THE ROLE OF TIS11 FAMILY IN THE PATHOPHYSIOLOGY OF CHRONIC LYMPHOCYTIC LEUKAEMIA

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ABSTRACT:

In this study the basal expression of Tis11 family and their regulation in B-CLL cells in response to stimuli that induce apoptosis (crosslinked Rituximab, XRituximab) or stimuli that inhibit spontaneous apoptosis (IL-4, CD40, CD40+IL-4, PMA) was tested. Tis11 family bind to AU Rich Elements (AREs) in the 3' Untranslated Region (3'UTR) and induce degradation of mRNAs bearing these target sequences. The Tis11 family consists of Tis11, Tis11b and Tis11d which induce apoptosis when overexpressed in a variety of human cell lines (epithelial, osteosarcoma and fibroblasts).

It was found that Tis11 mRNA is strongly expressed in unstimulated B-CLL cells, when compared to Tis11b mRNA levels, and was downregulated following stimulation with IL-4 or anti-CD40 at 3 hours post stimulation but remained unaffected by all other stimuli. Tis11b mRNA was found to be minimally expressed in unstimulated B-CLL cells and was strongly induced following XRituximab, PMA and anti-CD40 treatment in all patients tested but remained unchanged by IL-4 or anti-CD40+IL-4. Finally Tis11d was found to be strongly expressed in unstimulated B-CLL cells and was weakly induced by XRituximab and PMA in some but not all patients tested and showed no change after IL-4, anti-CD40 or anti-CD40+IL-4 stimulation.

Additionally it was found that when Tis11b is induced by XRituximab it is primarily regulated through p38 and to lesser extend through JNK pathway since inhibition of these pathways abrogated induction of the Tis11b mRNA and protein. On the contrary when Tis11b was induced by PMA or anti-CD40 it was found to be regulated through NF-κB pathway since inhibition of this pathway resulted in complete abrogation of Tis11b mRNA induction following PMA or anti-CD40 stimulation. In order to determine the function that Tis11b is involved in reponse to XRituximab and PMA or anti-CD40 treatment, Tis11b siRNA technology was utilised which revealed that inhibition of Tis11b significantly reduced (by 50-70%) the efficiency of XRituximab in inducing apoptosis in CLL cells while when Tis11b siRNA was applied in PMA or anti-CD40 stimulated CLL cells it significantly reduced their ability to induce plasma cell differentiation in these cells. Thus Tis11b is involved in induction of apoptosis following Rituximab treatment in CLL cells and also it is involved in induction of differentiation of CLL cells when such a stimulus (eg: anti-CD40) is present. Finally it was found that Tis11b/Berg36 is probably involved in B cell differentiation in general, since it was found that it has different basal expression and regulation at different stages

of B cell differentiation represented by Nalm6 (pre-B cells), Ramos (Germinal Centre B cells), AGLCL and WILCL (memory B cells) and RPMI8226 and MM1.S (plasma cells) cell lines. Indeed it was found that Multiple Myeloma cell lines have undetectable levels of Tis11b/Berg36 mRNA and neither PMA nor anti-CD40 could induce Tis11b in plasma cells even though these stimuli could modify expression of this gene in all other stages of differentiation.

Thus Tis11 family especially Tis11b and Tis11 may have an important role in the pathogenesis or progression of CLL (and possibly of B cell malignancies in general) and necessitate further investigation.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF PHOTOGRAPHS

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PAPERS AND ABSTRACTS SUBMITTED FOR PUBLICATION

PAPERS:

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ABSTRACTS:

1) Baou M., Murphy J., Yong K.L., Carr R., Jewell A.P. (2006) Involvement of the Tis11b/Berg36 Family in the Regulation of B-CLL Surivival and Differentiation. *Blood*; 108(11) Abstract: 4936

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3) Jewell A.P., Baou M., Yong K.L., Carr R., Murphy J. (2004) Regulation of Berg-36 Gene Expression by Survival and Apoptotic Stimuli in B-Chronic Lymphocytic Leukaemia Cells. *Blood*, 104(11), Abstract:4767

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LIST OF ABBREVIATIONS

ADCC: Antibody dependent cell cytotoxicity

AET: aminoethyl isothiouronium Bromide

AID: Activation induced cytidine deaminase

AIF: Activator of nuclear endonuclease

ALL: Acure Lymphocytic Leukaemia

AML: Acute Myeloid Leukaemia

APRIL: A proliferation-inducing ligand

AREs: AU Rich Elements

ATM: Ataxia Telangectasia mutated

AU: Airy Units

AUBPs: AREs Binding Proteins

BAFF: B-cell activating factor of the TNF family

Bcl-2: B cell CLL/lymphoma 2

B-CLL: B cell Chronic Lymphocytic Leukaemia

BCR: B cell Receptor

BH3: Bcl-2 homology 3

Bid: BH3 interacting domain death agonist

Bik: BCL2-interacting killer

Bmf: Bcl-2 modifying factor

BMMC: Bone Marrow Macrophages cells

B-PLL: B Pro Lymphocytic Leukaemia

CARD: caspase-activation and recruitment domain

Caspase: Cysteine-Aspartyl specific Proteases)

CD (eg: CD5,CD20,CD138, CD27 etc): Cluster of Differentiation

CDC: Complement dependent cytotoxicity

Cdk4: Cyclin dependent kinase 4

cDNA: complementary DNA

CLLD (eg:CLLD6 or 7):B-CLL deleted

CML: Chronic Myeloid Leukaemia

DED: Death Effector Domain

DEPC: Diethylpyrocarbonate

DISC: Death-Inducind signaling complex

DLBCL: Diffuse Large B-cell Lymphoma

DNA: deoxyribonucleic acid

DR (eg: DR4,5 etc): Death Receptor

DRAK-1: DAP(Death-associated protein) kinase-related apoptosis-inducing protein kinase

EDTA: ethylenediaminetetraacetic acid

ERK: Extracellular Signal Regulated Kinase

FACS: Fluorescnce –activated cell sorter

FADD: Fas associated death domain –containing protein

FCS: Foetal calf serum

FGFR1: Fibroblast growth factor receptor 1

FL: Follicular Lymphoma

FLIP or FLIP: FADD-like interleukin-1β-converting enzyme-like protease (FLICE/caspase 8) inhibitory proteins

GEF: Guanine Exchange Factor

GM-CSF: Granulocyte Macrophage colony stimulation factor

HCL: Hairy Cell Leukaemia

HEPES: 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid

HtrA2/Omi: High temperature requirement serine protease 2

IAPs: Inhibitors of apoptosis

ICAD: inhibitor of DNAase CAD

ICAM-1:Intercellular adhesion molecule-1

IFN (eg: IFN-γ): Interferon

IGFBP-4: Insulin growth factor binding protein 4

IGH: Immunoglobulins Heavy chains

IGL: Immunoglobulins Light chains

IL- (eg: IL-4, IL-2): Interleukin

iNOS: inducible Nitic-Oxide synthase

JNK: c-Jun N-terminal Kinase

LEF-1: Lymphocyte Enhancing Factor-1

LPS: Lipopolysaccharide

Mcl-1: myeloid cell leukemia sequence 1

MCL: Mantle Cell Lymphoma

MEFs: mouse embryonic fibroblasts

miRNA: micro-RNA

MLL: Mixed lineage leukaemia

MM: Multiple Myeloma

MOPS: 4-Morpholinepropanesulfonic acid

NES: Nuclear export signal

NF-κB: Nuclear Fcator κB

NK: Natural Killer

NLS: Nuclear localisation signal

PARN: PolyA Binding Protein

PCR: Polymerase Chain reaction

PB: Processing bodies

PBS: Phosphate buffered saline

PI3K: Phospho inositol 3 kinase

PKA: Protein kinase A

PKB: Protein kinase B

PKC: Protein kinase C

PLC-γ: Phospholipase C-γ

PMA: Phorbol myristate acetate

PI: Propidium Iodide

PI-3K: Phosphatidylinositol 3-kinase

RAIDD: receptor-interacting protein (RIP)-associated /CH3/CED3 homologous protein with a death domain/CARD

RISC: RNA-induced silencing complex

RNA: Ribonucleic acid

RNAi: RNA interference

mRNA: messenger RNA

rRNA: ribosomal RNA

RT: Reverse transcriptase

RT-PCR: Real Time PCR

SDS: Sodium dodecyl sulfate

SG: Stress granules

siRNA: short interfering RNA

SLL: Small Lymphocytic Lymphoma

Smac/DIABLO: second mitochondrial- derived activator of caspases/direct IAP binding protein with low Pi

SOCS-1: suppressor of cytokine signaling 1

STAT: Signal Transducer and Activator of Transcription

Tc: T cytotoxic cell

TCL-1: T cell leukaemia 1

TGF-β: Transforming growth factor β

TIA-1: T cell induced antigen-1

Th: T helper cells

TNF: Tumour Necrosis Factor

TNF-R: TNF receptor

TPA: 12-O-Tetradecanoylphorbol 13-acetate

TRADD: TNF-Receptor death domain protein

TRAF(reg: TRAF-1, 2 etc): TNF-R associated factor

TRAIL: TNF-related apoptosis-inducing ligand

UTR: Untranslated Region

UV: Ultraviolet

VDAC1: Voltage–dependent Anion-selective channel protein 1

VEGF: Vascular endothelial growth factor

XIAP: X-linked inhibitor of apoptosis

ZAP-70: zeta-chain (TCR) associated protein kinase 70kDa

ZF: Zinc Finger

ZNF36: Zinc Finger protein 36

ZNF36L1: Zinc Finger protein 36, C3H type-like 1

ZNF36L2: Zinc Finger protein 36, C3H type-like 2

CHAPTER 1

GENERAL INTRODUCTION

1.1.0 DEFINITION OF B CHRONIC LYMPHOCYTIC LEUKAEMIA

B Chronic Lymphocytic Leukaemia (B-CLL) is the commonest form of Leukaemia in the Western World accounting for almost 30% of all Leukaemias (Sellick *et al* 2005). It is characterised by a constitutive accumulation of monoclonal CD5+ B cells which are small mature lymphocytes that disrupt easily during the preparation of blood smears forming "smudge cells" (Caligaris–Cappio 2000, Caligaris-Cappio and Hamblin 1999). Interestingly first-degree relatives of CLL patients have a 7 fold higher risk to develop CLL (familial CLL) or another lymphoproliferative disorder than the general population (Sellick *et al* 2005). Thus there is an inherited susceptibility to CLL which probably functions as an autosomal recessive gene but this gene remains unknown even though a high-density Single Nucleotide Polymorphism (SNP) linkage scan (11,244 SNP studied) in several families with 2-5 affected individuals found as a possible locus for such a gene/susceptibility the 11p11 region (Sellick *et al* 2005). High expression of Bcl-2 in CLL is one of the hallmarks of the disease, however this has not been attributed to a certain chromosomal translocation involving chromosome 18q21.33. Instead hypomethylation of 5' end (promoter region) (Hanada *et al* 1993) has been proposed to be responsible for the high expression in CLL cells. Apart from direct regulation at the transcription level, post transcriptional modifications may explain this high expression in CLL cells. Two types of post transcriptional regulations have been described that affect either Bcl-2 function in memory B cells or Bcl-2 stability with the first one being phosphorylation (Torcia *et al* 2001) and the second one being the presence of AREs in the 3'UTR (Donnini *et al* 2001, Schiavone *et al* 2000) respectively.

1.1.1 DIAGNOSIS OF CLL

Diagnosis of B-CLL is usually made when a persistent lymphocytosis of at least $10x10^9/l$ and bone marrow infiltration of at least 40% are present. However CLL can be diagnosed when the WBC is between $5x10^9/$ and $10x10^9/l$ and a monoclonal population of B cells is detected (through light chain restriction) (Hoffbrand 2005).

B-CLL patients may present with non specific symptoms such as weight loss, malaise, night sweats and most of the cases are discovered by a routine blood test (Hoffbrand

2005). Symmetrical enlargement of the lymph nodes is the most frequent clinical symptom and can result in airway obstruction when it is very extensive. Patients also develop anaemia, thombocytopenia or pancytopenia as a result of extensive bone marrow involvement (Hoffbrand 2005). Splenomegaly and hepatomegaly appear at later stages of the disease as well as bacterial or fungal infections as a result of immunodeficiency and neutropaenia (Hoffbrand 2005).

Figure 1.1: CLL Blood smear stained with Wright-Gimsa
Typical CLL blood smear stained with Wright-Giemsa characterised by increased number of small lymphocytes with high nuclear to cytoplasmic ratio typical of CLL. This photo was obtained using a 40x objective. This photo was obtained from Postgraduate Haematology, Hoffbrand , 2005 p: 628

1.1.2 CLASSIFICATION OF CLL

Patients are currently classified according to Rai (0-IV stage) or Binet (A-C stage) when clinical findings are such as anaemia, thrombocytopenia etc are considered (Binet *et al* 1981, Rai *et al* 1975). The summary of the 2 classifications is shown in Table 1

Binet Classification	Lymphocytosis	Anaemia (Hb<10gr/L)	Thombocytopenia (PLT<100x10^9/L)	Enlarged areas (spleen,liver or lymph nodes)
Stage A	Yes	No	No	<3
Stage B	Yes, higher than in A	No	No	>3
Stage C	Yes, higher than B	Yes	Yes	Any
Rai Classification				
Stage 0	Yes	No	No	No
Stage I	Yes	No	No	1, lymph nodes
Stage II	Yes	No	No	At least 2
Stage III	Yes	Yes	No	At least 2
Stage IV	Yes	Yes	Yes	At least 2

Table 1.1: Summary of the two classifications (Binet and Rai) in CLL
Clinical characteristics and classification according toRai and Binet staging system.
Yes indicates presence of the mentioned clinical parameter for example anaemia or thrombocytopenia while no indicates the absence of the clinical parameter according to Binet and Rai classifications

1.1.4 CELL MARKERS IN CLL

Cell markers are essential for distinguishing B-from T-CLL and from Hairy Cell Leukaemia and Mantle Cell Lymphoma. B-CLL cells appear with weak to almost undetectable expression of surface Immunoglobulin (SmIg) and CD22 marker, positive expression for CD5, CD23, CD19/CD20/CD37 and MHC class II markers while they are negative for: CD10, CD79b, HC2/CD103 markers (Novak *et al* 2002, Hoffbrand 1999). In comparison with CD5+ normal B cells from age matched donors, B-CLL cells express similar levels of CD5, CD45RA, CD77, C86, HLA-DR, lower levels of CD22, CD32, CD38, CD40L, CD79b, CD95 but higher levels of CD23, CD25, CD27, CD39, CD69 and CD71 suggesting that they are antigen-experienced mature B cells and not naïve cells as their small size indicates (Damle et al 2002).

CD5 is normally found on the surface of T cells and in a small percentage of B cells called B1 cells (a self-renewing population of B cells). B1 cells will be analysed in terms of their origin in the introduction to Chapter 6. Expression of CD5 can be induced following BCR (B cell Receptor) and CD19 activation through Vav2 phosphorylation and NF-AT activation (Doody *et al* 2000) and thus the presence of this antigen in CLL can be the result of activation and not an indication of their origin (B1 cells). In terms of function, CD5 interacts with BCR signalling and either stimulates it or inhibits it (Gary-Gouy *et al* 2002, Lu et al 2002). Use of an anti-CD5 antibody both inhibited and induced apoptosis in B-CLL patients. Induction of apoptosis was seen only in patients with high expression of CD38, CD23, CD25 and other unfavourable clinical data such as trisomy 12 (Ciorca and Kitano 2002, Pers *et al* 2002) or ZAP-70 expression (Renaudineau *et al* 2005). In these patients there was co-localisation of CD5 with sIgM in lipid rafts followed by ZAP-70 recruitment and subsequent CD79a and Syk phosphorylation whereas in the resistant patients there was lack of one or more of the above events (Renaudineau *et al* 2005). Induction of apoptosis was accompanied by downregulation of Bcl-2, Mcl-1 and iNOS (inducible nitric Oxide synthetase) and upregulation of Bax (Ciorca and Kitano 2002, Pers *et al* 2002). On the contrary in the cases that anti-CD5 inhibited apoptosis, there was induction of Mcl-1 through activation of PKC (Perez-Chacon *et al* 2006).

Because BCR has a central role in determining B cell fate, the low levels of BCR (sIg and CD79b) may be involved in the pathogenesis of B-CLL (Alfarano *et al* 1999). This

defect has been recently associated with a defect in glycosylation of both μ and CD79a chains which results in their retention in the endoplasmic reticulum (as immature glycoforms) (Vuillier *et al* 2005). Association between CD79a-CD79b and CD79a and μ can occur only between mature glycoforms (Vuillier *et al* 2005). Reduced surface expression of CD79b has been correlated with reduced mRNA levels, presence of mutations in the coding region or a truncated transcript ΔCD79b (Gordon *et al* 2000, Alfarano *et al* 1999, Thompson *et al* 1997). Interestingly, the presence of the ΔCD79b transcript in Burkitt's Lymphoma cell lines resulted in resistance to apoptosis following anti-μ treatment (Cragg *et al* 2002) and may be another factor contributing to the apoptosis resistance of CLL cells.

1.1.4.1 CD38 Expression

CD38 is expressed in 27-30% of B-CLL patients (Chavallier *et al* 2002) with three patterns of expression: homogenous negative, homogenous positive and bimodal (Pittner *et al* 2005). CD38 positive patients also express higher levels of CD40, CD79b and CD69 (Damle *et al* 2002) and were induced to apoptosis by crosslinked anti-IgM treatment whereas CD38 negative cases were resistant (Zupo *et al* 2000, Zupo *et al* 1998). CD38 expression correlates with worse clinical prognosis and survival (His *et al* 2003) such as Binet stages B and C (Chevalier *et al* 2002), Unmutated cases (Kröber *et al* 2002, Lin *et al* 2002, Deaglio *et al* 1999) and 11q deletion (Chevalier *et al* 2002).

1.1.5 CHROMOSOMAL ABNORMALITIES IN CLL

Chromosomal abnormalities found in CLL include deletion of 13q14, trisomy 12 (25% of patients) (Buhl *et al* 2006, Winkler *et al* 2005), deletion of 11q23 (15% of patients) (Austen *et al* 2005, Sembries *et al* 1999), deletion or aberration of 17p (10% of patients) (Stankovic *et al* 2004, Dewald et al 2003, Cerretini et al 2003). Recently balanced or unbalanced translocations were found in some patients involving certain regions such as 13q14 (Mayr *et al* 2006) or 17p10 -17p12 region (Fink *et al* 2006) which correlated with worse prognosis. From these chromosomal abberations only the del13q14 will be further analysed because it may associate with the Tis11 family members.

Deletions of the 13q14 region are found in 40-64% of patients (Mayr *et al* 2006, Deawald *et al* 2003, van Everdink *et al* 2003) and can be monoallelic or biallelic. Deletion of 13q14 is the clonal event in B-CLL that precedes trisomy 12 (Jabbar et al 1995). Several genes have been isolated and were proposed to be the B-CLL tumour suppressor gene. These included Leu1, Leu2, Leu5, CLLD6, CLLD7, CLLD8, FAM104A4, and E4.5 a GEF protein (Guanine Exchange Factor) involved in Ran/Ras pathway (van Everdink *et al* 2003, Solomou *et al* 2003, Mabushi et al 2001, Kitamura *et al* 2000, Corcoran *et al* 1998) (Figure I2). Nevertheless with the exception of Leu2 and may be Leu5, none of them were found to be downregulated or mutated in B-CLL cells (van Everdink *et al* 2003, Mabushi et al 2001, Kitamura *et al* 2000, Corcoran *et al* 1998). More recent studies identified two miRNAs namely miR-15 and miR-16 (Figure 1.2) which were found to be reduced in 68% of B-CLL patients (Calin *et al* 2002). Initially low expression of miR-15/mi-R16 was correlated with low expression of arginyl-tRNA synthetase gene (SARS) but more recently it was reported that both miRNAs can target the 3'UTR of Bcl-2 mRNA *in vivo* and *in vitro* (Cimmino *et al* 2005). This may suggest a direct involvement of 13q deletion in the pathogenesis of CLL (Cimmino *et al* 2005). Interestingly during the timecourse of this project it was shown that miR-16 and miR-15 require Tis11 in order to mediate mRNA degradation of their target genes (Jing *et al* 2005).

1.1.6 MUTATED VERSUS UNMUTATED CLL

It was found by sequencing that the VH genes are hypermutated in almost 50% (38%-44%) of B-CLL cases which suggest that these cells are clonal expansions of postgerminal center memory B cells. On the contrary up to 63.5% of CLL cases appear with less than 2% mutations in their IgVH genes implying that they are clonal expansions of postgerminal centre naïve cells (Ferrer *et al* 2004, Hultdin *et al* 2003, Lin et al 2002, Tobin *et al* 2002). Patients with Unmutated IGH genes correlate with trisomy 12, p53 dysfunction, deletion of 11q22-23, expression of CD38, higher TCL1 expression, or shorter telomeres and overall worse prognosis and survival (Herling *et al* 2006, Damle *et al* 2004, Hultdin *et al* 2003, Lin et al 2002, Oscier *et al* 2002, Oscier *et al* 1997). Interestingly there is strong correlation between mutation status of IgH and mutation

status of IgL genes (κ or λ) with 90% of the CLL cases being mutated or unmutated for both IgH and IgL genes (Stamatopoulos *et al* 2005). These two subgroups will be further compared at the molecular level in the next section describing microarray analysis in CLL.

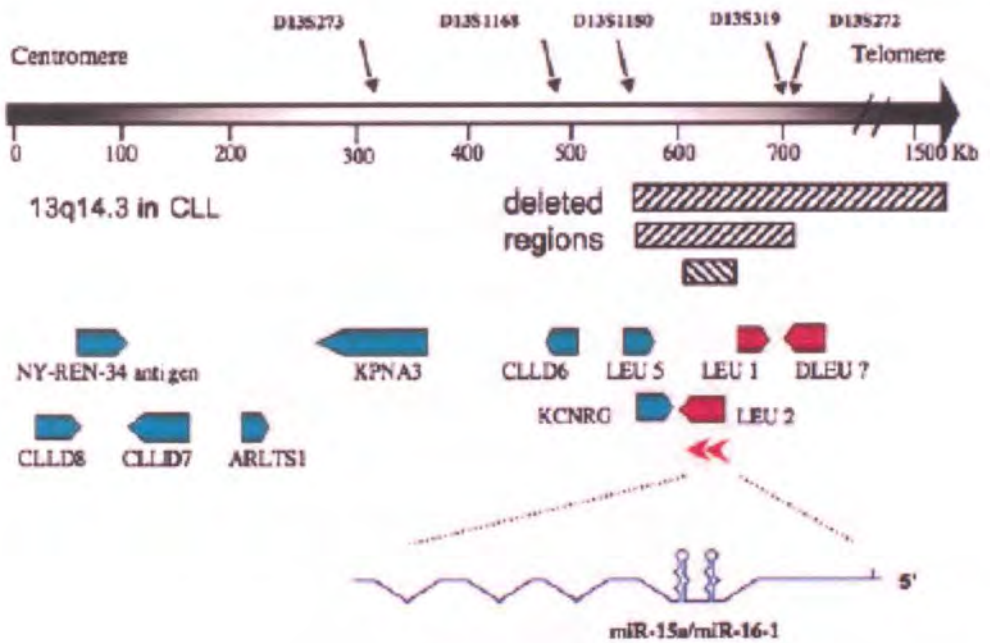


Figure 1.2: Diagrammatic representation of the 13q14.3 deletion in CLL (the most common deletion found, proposed to be the clonal event) with the recently intentified miR-15 and miR-16. The diagram was obtained from Calin and Groce 2006, *Oncogene,* **25,** pp: 6203

1.2.0 MICROARRAYS ANALYSIS IN CLL

Several studies using microarray analysis has been performed for the last 5 years in CLL comparing them to normal B cells or to different CLL subgroups such as Mutated versus Unmutated. This could identify candidate genes that could function as oncogenes or tumour suppressor genes in CLL and thus new therapeutic approaches or could explain the diversity seen between different subgroups at the clinical level.

1.2.1 CLL versus normal B cells

The two initial studies showed that B-CLL cells resemble memory B cells when B-CLL were compared to normal tonsillar B cells (Klein *et al* 2001) or cord blood B cells (>80% CD5+ B cells)) (Rosenwald *et al* 2001). Genes that were found to be different were primarily involved in proliferation, DNA repair, signal transduction pathways or apoptosis with some genes being upregulated in CLL in comparison to memory B cells (eg: Bcl-2, LEF-1, CDC25B, CD23, genes involved in IL-4, TGF-β or ERK signaling) and others being downregulated (eg: Cyclin B, Bid, DRAK1 and 2, SOCS-1, ICAM-1, CD18 etc) (Klein *et al* 2001). The second study that compared CLL with resting cord blood B cells or activated peripheral blood B cells also found that B-CLL resemble activated B cells and probably do not arise from CD5+ B1 cells (Rosenwald *et al* 2001). This study confirmed most of the findings by Klein *et al* and also reported that B-CLL cells overexpress the Wnt-3 signaling molecule while they underexpress Bcl-6 when compared to normal B cells (Rosenwald *et al* 2001). Another study has confirmed the above findings and has further shown that B-CLL when compared with normal tonsilar CD19+ B cells express higher levels of IL-24, TRAF-1, IGFBP4, Id3, TNFSF7 and NUMA mRNAs but lower levels of Lymphotoxin β, CD49d, IgM, IgG3, IGJ, IGL, IgV4-31, CD22, CD79α, CD81, CD20, CXCR4, JunB, MLL and ZFP36L2 (Jelinek *et al* 2003). A second study used purified CD5+ B cells from tonsils showed that CLL have higher levels of CD27, CD23, CCR7, Integrin β7, Dad-1 (apoptosis), Hsp27 as well as DAG kinase (Zheng *et* 2002) but lower levels of CD83, CD40, CD69, IL-8, IFN-γ Receptor α, CDC25B, JunB, c-myc, Cyclin B2 and Bfl-1 (or A1) genes (Zheng *et* 2002).

Even when CLL cells were compared to a mixture of mRNAs from several normal tissues such as bone marrow, spleen, thymus, placenta, liver, kidney, pancreas, skeletal muscle etc relatively few genes were found to be different. These included genes related with their B cell nature and certain receptors-signal transduction molecules-transcription factors such as PKC-βII, PKC-βI, IL-4Receptor, PLCγ2, TCL-1, Lyn, ERK1, ELF-1, JunB, spi-B and L-selectin which were found to be upregulated in CLL (Stratowa *et al* 2001) while serum albumin, human growth hormone and placental lactogen were found to be downregulated in CLL cells compared to the reference mRNA mixture (Stratowa *et al* 2001).

1.2.2 Microarrays in different B-CLL subgroups

Analysis was performed in Mutated versus Unmutated B-CLL, normal karyotype versus 13q deletion versus all other chromosomal abnormalities. When Mutated B-CLL were compared with Unmutated cases 23-30 genes were found to be different from a total of 12,000 or 18,000 tested (Klein *et al* 2001, Rosenwald *et al* 2001). Most of them were found to be overexpressed in Unmutated cases and included ZAP-70, BCL7A, CD30, Calcireticulin, IgV4-31, Lipoprotein Lipase, Dystrophin, FGFR1, Kalikrein (Klein *et al* 2001, Rosenwald *et al* 2001). The similarity between Unmutated and Mutated CLL at the genetic level was confirmed by another study where 24 genes involved in cell cycle regulation, signal transduction and apoptosis were tested (Kielne *et al* 2005). Again it was found that ZAP-70 was significantly higher expressed in Unmutated cases whereas PI3K, ATM, and Cyclin D1 and were significantly higher in Mutated cases (Kielne *et al* 2005). Finally a study using quantitative Real Time PCR showed increased expression of ZAP-70 in Unmutated cases but higher expression of CD44, RAF-1, MKK6, PAX5, NF-ATc1, TCF1, Nup214, CX3CR1 in mutated cases (Ferrer *et al* 2004). From all above mRNAs, ZAP-70 is the best studied one which is currently one of the most interesting fields of research in CLL.

Following the initial studies (Klein *et al* 2001, Rosenwald *et al* 2001) and several others have confirm higher expression of ZAP-70 in Unmutated cases which correlates with worse prognosis (Catherwood *et al* 2006, Richardson *et al* 2006, Kielne *et al* 2005, Crespo *et al* 2003). ZAP-70 expression has been correlated with better response of B-CLL cells to anti-IgM treatment due to activation of Syk, BLNK and PLCγ (Chen *et al*

2005) or increased homing of CLL to lymph nodes due to higher expression of CCR7 (Richardson *et al* 2006). The lymph node is a microenvironment where CLL cells may encounter TH cells that can provide survival or proliferation signals such as CD40L and IL-4 (Richardson *et al* 2006). B-CLL is not the only malignancy expressing ZAP-70, which was also detected in Small Lymphocytic Lymphoma (SLL), Mantle Cell Lymphoma (MCL), Diffuse Large B-cell Lymphoma (DLBCL), pre-B ALL (Sup *et al* 2004) primary cells as well as Nalm6, Daudi, Ramos cell lines (Scielzo *et al* 2006, Nolz *et al* 2005, Crespo *et al* 2005). Additionally normal B cells (tonsillar or splenic), naïve or memory express significant levels of ZAP-70 protein which can be further induced following stimulation with CD40L+IL-4+IL-6+IL-10, IL-15+CpG Oligonucleotides or anti-IgM even in peripheral blood B cells that bear very low basal ZAP-70 expression (Cutrona *et al* 2006, Scielzo *et al* 2006, Nolz *et al* 2005). Instead these findings suggest that ZAP-70 expression indicates B cell activation and indeed it has been correlated with higher expression of CD38 and CD27 (Cutrona *et al* 2006, Nolz *et al* 2005) but not CD5 cell markers (Scielzo *et al* 2006, Cutrona *et al* 2006, Crespo *et al* 2003). Additionally ZAP-70 is critical for the pro-B to the pre-B transition in early B cell development as shown by studies in knock out mice (Schweighoffer *et al* 2003).

When CLL cases were subdivided according to chromosomal abnormalities present (13q deletion, 11q deletion, trisomy 12 and 17p deletion) again few differences were identified. Patients with 13q deletions when compared with patients with normal karyotype had higher expression of PKB and TCL-1, while patients with trisomy 12 had higher levels of E2F, Bax, p27 and Cdk4 cell cycle proteins suggesting higher propensity to proliferation (Kielne *et al* 2005). In patients with 11q deletion there was significant increase of Mcl1 and NF-κB and significant decrease of Cyclin D3 and ATM while patients with 17p deletion had significantly higher levels of p21, c-myc but significantly lower levels of AID, Cyclin D1, PI3K, TCL1, ATM, p53, syk, Bcl-2, cyclin D3 and constituted the subgroup with the most genetic abnormalities (Kielne *et al* 2005).

1.2.3 MiRNAs and B-CLL

Despite the fact that microarray analysis and proteomics failed to show a significant difference between B-CLL and normal B cells or between B-CLL subgroups, miRNAs screening was found to be more informative. MiRNAs are 22 nucleotide long non coding RNAs that can bind and cause either mRNA degradation or translation arrest of coding mRNAs through the formation of RISC and the other components of RNA interference (Chen *et al* 2004). They represent 3% of human genes and their importance in cancer development has been highlighted by many studies which found that several miRNA are either upregulated or downregulated in most of the cancers tested (Chen *et al* 2004).

A total of 55 miRNAs were found to be different when CLL cells were compared with normal CD5+ B cells, or a total of 29 miRNAs were different when CLL cells were compared with PBMC (Calin *et al* 2004). Among the former ones were miR-181b pre, miR-19a, miR-123, miR-213, miR-33, miR-188, miR-141 while among the latter ones were: miR-181b per, miR-128, miR-123, miR-192 pre, miR-221, miR-223. let-7a-2 (Calin *et al* 2004). One miRNA (miR-181) which was found to be overexpressed in CLL has been shown to be involved in B cell differentiation and when overexpressed resulted in 3 fold increase in the percentage of CD19+ cells in the peripheral blood (Chen *et al* 2004) suggesting that it may form a candidate miRNA for the development of several leukeamias or lymphomas. When patients were classified according to their mutation status and ZAP-70 expression it was found that unmutated CLL patients with ZAP-70 expression have significantly higher levels of 9 miRNAs such as miR-15, miR-16-1 and -2, miR-195, miR-221, miR146 etc which primarily target Bcl-2, or CDKN1B for miR-221 (Calin *et al* 2005). Additionally 4 miRNAs were found to be lower expressed in Unmutated cases namely miR-223, miR-29a-2, miR29b-2 and miR29c. The significance of these findings in relation to the different behaviour of these two subgroups remains unknown (Calin *et al* 2005). A similar set of 9 miRNAs were identified when patients were subdivided based on their requirement for treatment among which were miR-221, miR-222, miR181-a, miR-24-2, miR-29c which were higher expressed in patients that required therapy sooner after diagnosis than patients that had longer interval to therapy (Calin *et al* 2005).

1.3.0 DEREGULATION OF NORMAL CELLULAR PROCESSES IN CLL

The following cellular functions have been found to be defective in CLL:

i) Apoptosis versus cell cycle

ii) Constitutive active signal transduction pathways

iii) Defective T cell development/differentiation

1.3.1 Apoptosis versus cell cycle

More than 95% of the CLL cells are in G0 phase and thus the disease arises as a result of defective induction of apoptosis rather than higher proliferation (Caligaris-Cappio 2000). This is well documented now since microarray analysis showed that B-CLL cells have downregulated most of the proteins involved in cell cycle progression such as CDC2, CDC25 etc while they overexpress anti-apoptotic proteins including Bcl-2, Mcl-1, Bfl-1, c-IAP2, XIAP, c-IAP1, TRAF-1, TRAF-2 and underexpress pro-apoptotic ones such as Bax or Bak (de Graff *et al* 2005, Munzert *et al* 2002, Granziero *et al* 2001, Klein *et al* 2001, Rosenwald *et al* 2001). Additionally other studies using Real Time PCR have shown dysregulation of BH3 only pro-apoptotic proteins such as downregulation of Bik and Bid (Mackus *et al* 2005) and upregulation of Noxa and Bmf in CLL cells compared with normal tonsillar or peripheral blood B cells (Mackus *et al* 2005, Morales *et al* 2004). This suggests that B-CLL cells probably fail to convert signals from the death receptor or from the cytoskeleton to apoptosis induction by the mitochondrial pathway (Mackus *et al* 2005). Indeed CLL cells are highly resistant to apoptosis induced by CD95 or TRAIL (TNF-related apoptosis-inducing ligand) (MacFarlane *et al* 2002, Roué *et al* 2001, Wang *et al* 1997). Their resistance to death receptor mediated apoptosis may be due to low or variable levels of CD95 (Damle *et al* 2002, Roué *et al* 2001, Sembries *et al* 1999, Tsuruda *et al* 1999, Wang *et al* 1997), high levels of soluble CD95 in their serum especially at advanced clinical stages (Kitada et al 2002), high expression of c-FLIP compared to normal B cells (Mathus *et al* 2005, MacFarlane *et al* 2002) or low expression of Bid (Mackus *et al* 2005) which connects the death receptor pathway to the mitochondrial one. Finally CLL cells have lower levels of FADD expression and DISC formation compared to transformed cell lines

highly sensitive to TRAIL induced apoptosis which may also contribute to their resistance to CD95 and TRAIL induced apoptosis (MacFarlane *et al* 2002).

Apart from CD95, other TNF family members are also dysregulated in B-CLL compared with normal CD19+ peripheral blood B cells. One study has shown that B-CLL cells express BAFF (or BlyS) and APRIL which are not found in the surface of normal B cells (Kern *et al* 2004, Novak *et al* 2002). BAFF and APRIL bind to BCMA and TAC1 members of the TNF-Receptors and can inhibit both spontaneous and drug induced apoptosis in B-CLL cells (Kern *et al* 2004, Novak *et al* 2002). Additionally BAFF and APRIL were found in the serum of B-CLL patients but not in the serum of normal individuals (Kern *et al* 2004, Planeles *et al* 2004). Because a study has shown that overexpression of APRIL in mice resulted in expansion of CD5+ B cells in the peritoneum of older mice with increased survival rather than proliferation (Planeles *et al* 2004), BAFF and APRIL may be directly involved in the development or maintenance and resistance to apoptosis phenotype of B-CLL.

In terms of cytoskeleton involvement in the resistance of B-CLL cells to apoptosis, it has been shown recently that a microtubule depolymerising drug called nocodazole, significantly induced apoptosis in B-CLL cells which was higher than in normal peripheral blood B cells (Beswick *et al* 2005).

Nevertheless despite the high resistance of CLL cells to apoptosis *in vivo*, they undergo spontaneous apoptosis *in vitro* such that at 72h more than half of the cells are dead. Spontaneous apoptosis was shown to be characterised by activation of caspases -3, -7, -8 suggesting that caspase activation is intact in CLL and thus their resistance to apoptosis *in vivo* results from defects that lie upstream and prevent caspase activation (King *et al* 1998). The phenomenon of spontaneous apoptosis implies strong dependence of CLL cells to environmental stimuli present *in vivo* such as in the bone marrow, spleen or lymph node microenvironments (Ghia *et al* 2002) or to soluble factors present in serum such as albumin as recently shown (Jones *et al* 2003, Moran *et al* 2002, Wickremasinghe *et al* 2001) or cytokines. All these phenomena, spontaneous apoptosis, effect of soluble factors such as cytokines and microenvironmental stimuli such as CD40 activation will be further discussed in the Introduction section of Chapter 4.

1.3.2 Constitutive active signal transduction pathways

Recently, certain pathways were found to be constitutively active in CLL and this correlated with their resistance to apoptosis. One such pathway was the PKC pathway whose inhibition by Bisindolylmaleimide (BisI) induced apoptosis and partially abrogated the protective effect of TPA or IL-4 on spontaneous apoptosis in CLL (Barragán *et al* 2002). Use of another PKC inhibitor, PKC412 also induced apoptosis in CLL (Ganeshaguru *et al* 2002). More recent studies have shown that PKCδ is constitutively phosphorylated at Serine and Threonine residues in CLL cells and inhibition of PKCδ by Rottlerin strongly induced apoptosis in CLL and had minimal effect on normal B cells (Ringshausen *et al* 2006, Ringshausen *et al* 2002). In further support for an involvement of PKC in the pathogenesis of CLL, it was found very recently that haematopoietic progenitor cells (HPC) when transformed with dominant negative PKCα, developed a disease very similar to B-CLL (high CD19, CD5, CD23 and Bcl-2 expression, cells mainly arrested at G0/G1 phase) both *in vitro* but also *in vivo,* in $Rag1^{-/-}$ mice (Nakagawa *et al* 2006). Apart from PKC, CLL cells have constitutively active p38 pathway (Sainz-Perez *et al* 2006, Ringshausen *et al* 2004) but there is controversy about whether it is involved in apoptosis. One report showed that p38 inhibition did not affect induction of apoptosis in CLL (Ringshausen *et al* 2004) while a more recent one showed the opposite when the inhibitor used by Ringshausen *et al* was used at a higher concentration (Sainz-Perez *et al* 2006). Activation of PI3K has been also correlated with the resistance of B-CLL cells to apoptosis (Barragán *et al* 2002). Finally CLL cells have constitutively expressed nuclear NF-ATp transcription factor in comparison to normal tonsillar B cells (Schuh *et al* 1996) and constitutively phosphorylated (active) STAT1 and STAT3 kinases at Ser727 which are absent in CD5+ or CD5- B cells from healthy individuals (Frank *et al* 1997). The importance of the latter defects in CLL pathogenesis remains currently unknown.

1.3.3 Defective T cell development/differentiation

There is oligoclonal expansion of T cells especially CD8+ cells that results in inverted CD4/CD8 ratio in peripheral blood of CLL patients, higher Th1/Th2 ratio and Tc1/Tc2 than in normal individuals primarily in Rai stage 0 and I (Podhorecka *et al* 2002). These imbalances may result in altered production of certain cytokines in CLL patients

compared with normal individuals and some of them such as IL-4, IL-2, IFN-γ or TNF-α have been shown to inhibit spontaneous apoptosis as described in the Introduction section of Chapter 3. Thus dysfunctional T cells may be involved in the increased survival of CLL cells (Scrivener *et al* 2003).

1.4.0 APOPTOSIS

Apoptosis is tightly regulated form of cell death different from necrosis. During necrosis the cell becomes swollen, then bursts releasing its cytoplasmic contents into the environment and causes inflammation. On the contrary, apoptosis is characterised by particular key features including: cell shrinkage, condensation of the nuclear membrane and nuclear fragmentation, DNA cleavage at internucleosomal sites, blebbing of the cell membrane resulting in the release of membrane–bound apoptotic bodies which are recognised by phagocytes due to the presence of phosphatidylserine on their surface (Degterev *et al* 2003, Hengartner *et al* 2000). Thus after apoptosis induction there is no inflammation because the cell membrane remains intact (Opferman and Korsmeyer *et al* 2003). However it should be noted that sometimes apoptosis converts to necrosis when, during apoptosis, the energy levels (ATP) are rapidly depleted because the multi step process of apoptosis requires sufficient amounts of energy (Nicotera and Melino *et al* 2004).

The effector molecules of apoptosis are called Caspases (Cysteine-Aspartyl specific Proteases) which are intracellular cysteine proteases able to cleave other proteins after aspartic acid residues. Fourteen mammalian caspases have been identified (Caspase1-14) but humans lack caspase-11 and -12 (Degterev *et al* 2003). Caspases are expressed as inactive proenzymes consisting of a N-terminal prodomain and two subunits p20 and p10 (Degterev *et al* 2003, Hengartner *et al* 2000). Their activation requires proteolytic cleavage between p20 and p10 subunits and removal of the pro-domain (Degterev *et al* 2003, Hengartner *et al* 2000). The cleavage of procaspases occurs at aspartic acid residues and thus caspases can be activated through autocatalysis (eg: caspase-1) or by other family members (eg: caspase-3 which is activated by caspase-9) resulting in a cascade of caspases able to amplify the apoptotic signal (Degterev *et al* 2003, Hengartner *et al* 2000). Accordingly caspases can be subdivided into initiator caspases (eg: caspases-1,-2,-8,-10) and executioner or effector caspases (caspases-3, -6, -7). Initiator caspases have additional domains such as DED (Death Effector Domain) present eg in caspase-8 and -10 or CARD (caspase-activation and recruitment domain) present in caspase-2, -4, -5, -9 which probably determine the proteins that can interact with them (Rield and Shi *et al* 2004, Degterev *et al* 2003) considering that DED is found also in FADD while CARD is also found in Apaf-1.

Activated effector caspases can cleave certain proteins such as nuclear lamins resulting in chromatin condensation and nuclear shrinkage, ICAD (inhibitor of DNAase CAD) allowing DNA fragmentation, DNA repair proteins such as Rad51, ATM and DNA-PK ensuring that no repair will occur after DNA fragmentation, cytoskeletal proteins such as actin, plextrin and gelsolin resulting in cell fragmentation and blebbing, all of these leading finally to the formation of apoptotic bodies and their removal by phagocytes (Degterev *et al* 2003). Caspases can cleave also proteins involved in apoptosis such as Bcl-2, Bcl-XL and cell survival such as p65/RelA and IKKβ involved in NF-κB activation as well as PKB (Degterev *et al* 2003) probably ensuring that no survival signal will reverse the initiation of apoptosis. They can also target cell cycle genes which are mainly inhibitors of cell cycle progression (eg: p21, p27) but this effect is more difficult to explain (Degterev *et al* 2003).

In B lyphocytes there are 4 main pathways involved in apoptosis:

1) B cell receptor signalling (BCR)
2) Death receptor pathway
3) Mitochondrial pathway
4) T-Cytotoxic Lymphocytes (CTL) and Natural Killer (NK) dependent release of granzymes

BCR can induce apoptosis in B cells but primarily in immature B cells rather than mature B cells and CTL and NK mediated cytotoxicity has not been reported to be dysfunctional in CLL. Thus only the Death Receptor and Mitochondrial pathway will be further analysed.

Figure 1.3: COMET assay in lymphocytes showing normal versus apoptotic versus necrotic lymphocytes. Note the significant larger size of necrotic cells compared with apoptotic and their tendency to leak their cellular contents to the surrounding environment causing inflammation. This photo was obtained from www.comet.itrcindia.org/Photos.PDF which is the website of COMET forum Assay

1.4.1 THE DEATH RECEPTOR PATHWAY :

The death receptors resemble the Tumour Necrosis Factor Receptors (TNF-R) superfamily, a heterogenous family consisting of several members. Among them are CD95 (Apo-1,Fas) ,TNF-R1, DR3, DR6, TRAIL-R1 or DR4 and TRAIL-R2 or DR5 (TNF-related apoptosis inducing ligand receptor 1 and 2) which are involved in induction of apoptosis whereas other family members such as CD40R, CD27, BAFFR are involved in proliferation or B cell differentiation (Aggarwal *et al* 2003). The best characterised TNF member involved in apoptosis is Fas (CD95). Binding of the Fas Ligand to CD95 causes trimerisation and recruitment of FADD (Fas associated death domain –containing protein) (Aggarwal *et al* 2003, Daniel *et.al* 2000, Hengartner *et al* 2000). FADD can then dimerise with other proteins through the DED domain (Death Effector Domain) such as procaspase-8 or -10 resulting in the formation of DISC (Death Inducing Signalling Complex) (Aggarwal *et al* 2003, Daniel *et al.* 2000, Hengartner *et al* 2000). This results in activation of caspase-8 or -10 by autocatalytic cleavage followed by either activation of caspase-3 in type I cells or cleavage of Bid in type II cells (Aggarwal *et al* 2003, Opferman and Korsmeyer *et al* 2003, Daniel *et al* 2000, Hengartner *et al* 2000). Cleavage of Bid (into p7 and p15 isoforms) precedes myristoylation of the p15 isoform which will translocate to the mitochondria to induce apoptosis through activation of Bax and Bak (Aggarwal *et al* 2003, Daniel *et al* 2000, Hengartner *et al* 2000). It should be mentioned that caspase-8 activation can be inhibited by $FLIP_L$ or FLIPs [FADD-like interleukin-1β-converting enzyme-like protease (FLICE/caspase 8) inhibitory proteins] suggesting that excess of these proteins may result in resistance to CD95/Fas induced apoptosis through direct inhibition of caspase-8 activation or through association with TRAFs or Raf-1 resulting in activation of NF-κB or ERK pathways respectively (Aggarwal *et al* 2003, Budd *et al* 2002, Daniel *et al* 2000, Hengartner *et al* 2000). Finally apart from FADD other similar proteins can be recruited to death receptors (mainly to DR4, 5 and 6) in order to form the DISC either alone or in combination with FADD and include TRADD (TNF-Receptor death domain protein), DAXX, RAIDD [receptor-interacting protein (RIP)-associated /CH3/CED3 homologous protein with a death domain/CARD] (Aggarwal *et al* 2003) with the subsequent events being the same as in FADD recruitment.

1.4.2 CD40 Receptor activation

Trimeric CD40L binds to CD40Receptor, and recruits TRAFs (TNF-receptor-associated factors) adaptor molecules namely TRAF1, 2, 3, 5, 6 (Aggarwal *et al* 2003). There are currently 6 TRAFs which apart from the death association domain contain another domain that binds to IAPs (Inhibitors of Apoptosis). Recruitment of TRAFs results in recruitment of cIAP1 and cIAP2 which inhibit apoptosis. Additionaly TRAFs (especially TRAF6 and TRAF2) can activate the NF-κB pathway resulting again in inhibition of apoptosis and cell proliferation (Aggarwal *et al* 2003, Bradley and Pober *et al* 2001). Apart from NF-κB activation, recruitment of TRAFs results in activation of several other pathways such as PI3K, Ras/ERK, p38, JNK, STAT3, STAT6 and NF-AT (van Kooten and Banchereau *et al* 2000) but with the exception of PI3K and ERK pathways the role of these pathways after CD40R activation are poorly understood. In conclusion, CD40 either through recruiting of IAPs or through activation of NF-κB, PI3K promotes cell survival. Nevertheless CD40 activation renders cells more sensitive to Fas and TNF-R1 cell killing which probably serves in the elimination of the B cell population at the end of an immune response.

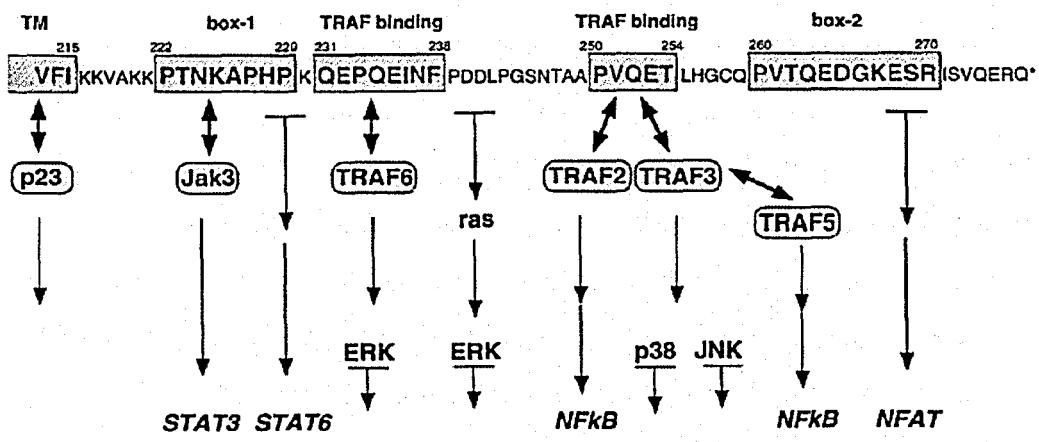


Figure 1.4: Signal transduction pathways activated following CD40Receptor activation. The intracellular domain of CD40R is shown in a one letter code for amino acids. Direct protein-protein interaction is shown by a double headed arrow while downstream activated pathways are shown by a single headed arrow. This diagram has been obtained from van Kooten C. and Banchereau J 2000, in Journal of Leukocyte Biology, Vol:67, pp:7

1.4.3 THE MITOCHONDRIAL PATHWAY:

The mitochondrial pathway is involved in the initiation of apoptosis following cytokine withdrawal, γ irradiation, DNA damage or death receptor activation accompanied by Bid cleavage. Key proteins in this pathway are the Bcl-2 family members which include: Bcl-2, Bcl-XL, Bcl-w, Mcl-1, NR-13, A1/Bfl-1 (anti-apoptotic), Bax, Bak, Bok (pro-apopoptic) and the BH3 only proteins Bad, Bid ,Bik, Hrk/DP5, Noxa, Bim, Bmf, Puma which are also pro-apoptotic (Cory *et al* 2003, Cory and Adams *et al* 2002). From the BH3 only proteins Puma and Bim can bind equally well to all anti-apoptotic members, Bad and Bmf preferentially bind to Bcl-2, Bcl-XL and Bcl-w, Noxa binds only to Mcl-1 and A1/Bfl-1 while Bik and Hrk/DP5 preferentially binds to Bcl-XL, Bcl-w and A1/Bfl-1 (Chen *et al* 2005).

Apoptosis or cell survival depends on the ratio of pro-apoptotic to anti-apoptotic proteins, on their cellular location, phospholrylation or other factors that results in their activation/inactivation (eg:cleavage) (Cory *et al* 2003, Cory and Adams *et al* 2002, Hengarther *et al* 2000). For example in healthy cells Bad protein resides in the cytosol, and is phosphorylated by Akt/PKB (at Ser136) resulting in binding to 14-3-3 proteins whereas in response to an apoptotic stimuli becomes dephosphorylated, is released from 14-3-3 and translocates to the mitochondria (Cory *et al* 2003, Opferman and Korsmeyer *et al* 2003, Cory and Adams *et al* 2002). On the contrary Bcl-2 in healthy cells resides in the mitochondrial, endoplasmic reticulum and nuclear membrane, is unphosphorylated but becomes phosphorylated at Ser70 following Taxol induced apoptosis (Blagosklonny *et al* 2001). In fact more than one sites can be phosphorylated and hyperphosphorylation of Bcl-2 decreases the anti-apoptotic activity which is a feature shared with Bcl-XL and Mcl-1 in response to certain drugs (Blagosklonny *et al* 2001). Finally Bim and Bmf members are associated with microtubules and actin cytoskeleton in healthy cells but also translocate to mitochondria to inhibit Bcl-2 in response to apoptotic stimuli whereas Puma and Noxa are transcriptionaly regulated by p53 and thus probably serve as sensors of DNA damage (Cory *et al* 2003, Cory and Adams *et al* 2002). From all the BH3 only proteins, Bim was found to be critical for leukemogenesis and deletion of autoreactive lymphocytes whereas the other family members have not been associated with leukemogenesis (Bouillet *et al* 2002, Cory and Adams *et al* 2002).

Following an apoptotic signal, Bax and Bak undergo conformational changes which allow the formation of homo-or hetero-dimers and association with the outer mitochondrial membrane resulting in increased fission of the mitochondria, fragmented appearance and release of cytochrome c (Conradt *et al* 2006, Cory *et al* 2003, Cory and Adams *et al* 2002, Hengarther *et al* 2000). Cytochrome c is accompanied by a drop in the mitochondrial transmembrane potential ($\Delta\Psi m$) which is considered as a downstream event following cytochrome c release (Aggarwal *et al* 2003). Cytochrome c binds to Apaf-1 which probably confers orientation to pro-caspase-9 (Chau *et al* 2000). Binding of cytochrome c to Apaf-1 and pro-caspase-9 form the apoptosome which in turn cleaves and activates caspase-9 which activates caspase-3 (Hengartner *et al* 2000). Caspase-3 is the main excecutioner/effector caspase resulting in cleavage of proteins already mentioned eg: lamins and thus formation of apoptotic bodies (Hengartner *et al* 2000).

Formation of the apoptosome is a carefully orchestrated process because mitochondria membrane permeabilisation results in the release of other proteins such as c-IAPs (Inhibitors of Apoptosis Proteins), Endonuclease G, AIF (Activator of nuclear endonuclease), Smac/DIABLO (second mitochondrial- derived activator of caspases/direct IAP binding protein with low pI), HtrA2/Omi (High temperature requirement serine protease 2) to prevent accidental induction of apoptosis (LeBlank *et al* 2003, Liston *et al* 2003, Hengartner *et al* 2000). Analytically endonuclease G probably co-operates with CAD to cause DNA fragmentation and AIF is also involved in DNA fragmentation while Smac/DIABLO and HtrA2/Omi inhibit the function of IAPs (LeBlank *et al* 2003, Liston *et al* 2003). There are 9 mammalian IAP family members: cIAP-1, cIAP-2, XIAP , NAIP, Apollon, Survivin, Livin, MLIAP, LP2 (Aggarwal *et al* 2003, LeBlank *et al* 2003, Liston *et al* 2003) and can inhibit caspase activation through direct association with caspases (eg: XIAP with caspase-9 or caspase-3) or through induction of proteasomal degradation in a ubiquitin dependent manner (eg: c-IAP2 and XIAP for caspase-3) (LeBlank *et al* 2003, Liston *et al* 2003). IAPs can induce degradation of Smac/DIABLO as well, making them powerful inhibitors of apoptosis (LeBlank *et al* 2003, Liston *et al* 2003). However when caspase-3 activation has irreversibly occured, it can cleave most of the IAPs ensuring that

they will not be able to inhibit its function (LeBlank *et al* 2003, Liston *et al* 2003). In conclusion the final outcome depends on the doses/ratios of all these factors released by mitochondria and probably ensure that apoptosis will not occur in the case of accidental release of cytochrome c after for example mitochondrial damage, or that apoptosis induction will not be prevented in the case that there is a strong apoptotic signal such as extensive DNA damage (Hengartner*et al* 2000). Until very recently it was considered that loss of $\Delta\Psi m$, cytochrome c and AIF release from mitochondria precede caspases activation however a very recent study showed that caspases 3 and 7 are necessary for the execution of these events following certain apoptotic stimuli such as UV irradiation (Lakhani *et al* 2006) possibly as a means of further amplifying the original apoptotic signal (Lakhani *et al* 2006)

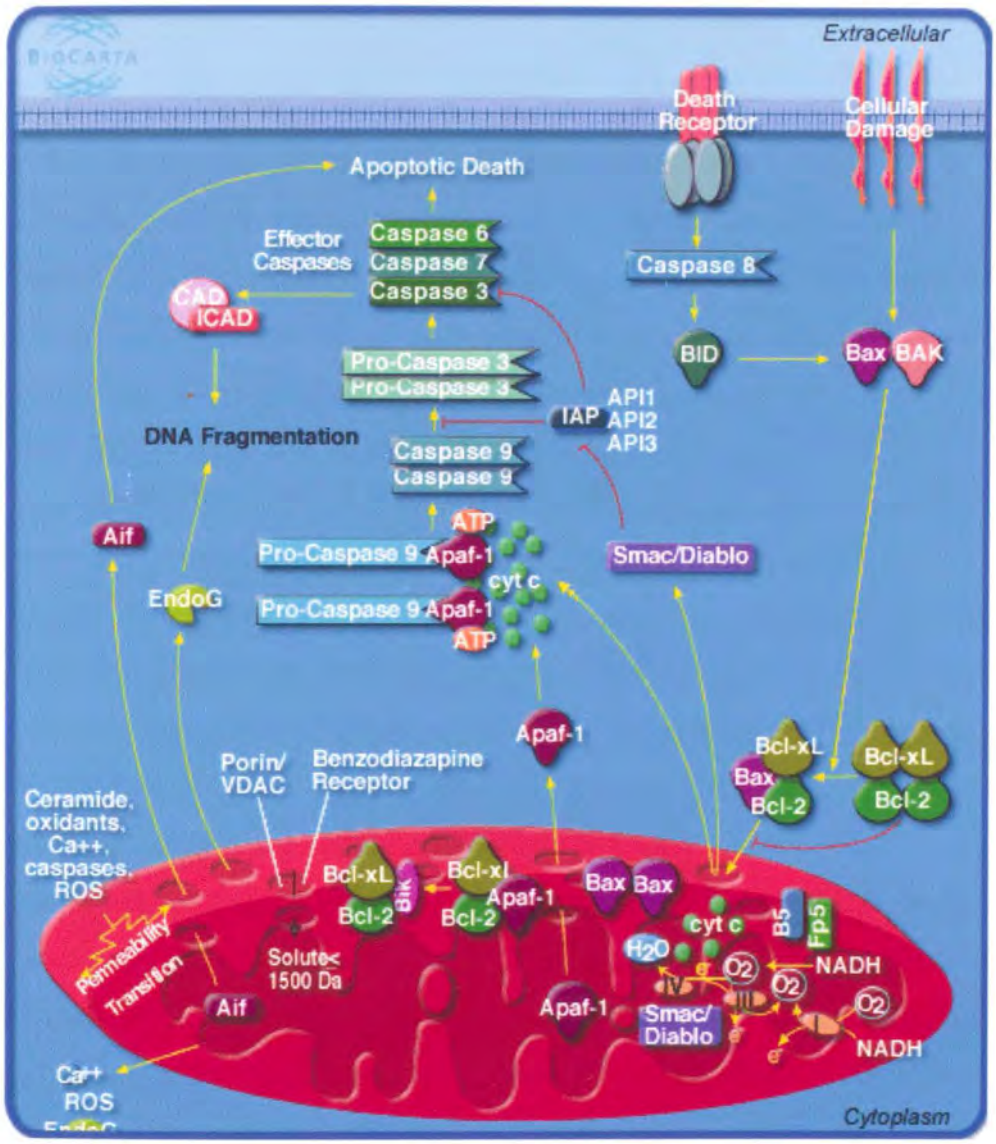


Figure 1.5: Diagrammatic presentation of Death Receptor and mitochondrial pathways of apoptosis

Brief summary of the genes activated following a death receptor signal or any extracellular signal resulting in induction of apoptosis through the mitochondrial pathway. All genes and abbreviations have been described in text apart from Reactive Oxygen Species (ROS) which is another signal involved in apoptosis. This pathway chart has been obtained from BioCarta website on http://www.biocarta.com/pathfiles/h_mitochondriaPathway.asp on the 30th of April 2006.

1.5.0 THE TIS11 FAMILY

1.5.1 Cloning and Isolation of Tis11 family members

The Tis11 (TPA inducible sequences 11) family consists of Tis11 (ZFP36, TTP, Nup475, GOS24), Tis11b (Berg36, ERF-1, ZFP36L1, BRF-1) and Tis11d (ZFP36L2, ERF-2, BRF-2). Recently a fourth family member was described in rodents, ZFP36L3, which was only expressed in mouse placenta but was not detected in human placenta or other human tissues (Blackshear *et al* 2005).

The human Tis11, located on chromosome 19q13.1, was isolated by several groups from cells stimulated with serum, PMA or insulin (Taylor *et al* 1991, Varnun *et al* 1991, DuBois *et al* 1990). Tis11b, located on chromosome 14q22-24, was isolated from CLL cells stimulated with PMA (Murphy and Norton 1990) or from a variety of human cDNA libraries probed with the rat cMG1 (homologue of Tis11b)(Bustin *et al* 1994, Varnum *et al* 1991) or as a gene that reverses the phenotype of a mutant cell line (H1080) lacking the ability to degrade IL-3 mRNA (Stoecklin *et al* 2002). Finally Tis11d, located on 2p22.3-p21, was isolated from human cDNA libraries probed with the mouse Tis11d mRNA (Taylor *et al* 1991, Nie *et al* 1995, Varnum *et al* 1991).

The sequence for each of the 3 human genes shows little variation within population and when polymorphisms are detected, these are mostly found in introns, promoter and 3' UTR regions (Carrick *et al* 2006). When polymorphisms are found in exons, they do not usually result in amino acid alteration (Carrick *et al* 2006, Blackshear *et al* 2003a). The total number of polymorphisms identified was 29 and heterozygosity for one particular in the protein coding region (base 1725 from gene M92844) was correlated with increased risk of developing Rheumatoid Arthritis (RA) in African–Americans and possibly in Caucasians as well (Carrick *et al* 2006). These data suggest that Tis11 family has an important function in cell physiology, and any significant change in their amino acid sequence may correlate with the development of a certain disease. This is further supported by the fact that the sequence of the two zinc finger motifs together with the linker sequence is highly conserved among organisms as diverse as human and zebrafish (Lai *et al* 2000). Even small differences in each Tis11 member are conserved between human, mouse and rat genes and are unique for that particular Tis11 member. For example at position 28 there is always Leucine for Tis11, Isoleucine for Tis11b and Phenylalanine for Tis11d in all 3 organisms mentioned above or at position 20 there is

Alanine for Tis11, Asparagine for Tis11b and Glutamic acid for Tis11d (Lai *et al* 2000). All 3 genes have been associated with induction of mRNA decay and even though redundancy in their function has been reported, studies with knock out mice suggest that they may operate in a cell specific manner. Tis11 knock out mice appeared normal at birth but later developed autoimmunity, arthritis, conjunctivitis and myeloid dysplasia (Taylor *et al* 1996b). This phenotype was reversed by administration of anti-TNF-α suggesting that it was due to the presence of large amounts of TNF-α (Taylor *et al* 1996b). On the contrary Tis11b knock out mice did not survive to birth but died *in utero* between embryonic day 8 and 13 due to either failure in the chorioallontoic fusion (Stumpo *et al* 2004) or defective extraembryonic vasculogenesis due to increased VEGF levels (Bell *et al* 2006). Finally Tis11d knock out mice appeared normal at birth but females were infertile due to defective ovulation and growth (Ramos *et al* 2004). In this study the RNA binding domain and the nuclear export signal were intact and thus the phenotype detected may not correspond to the phenotype caused by complete loss of Tis11d. In this case the defect in fertility may be due to a function that is mediated through the N-terminal domain of Tis11d (Ramos *et al* 2004).

Mouse Tis11 mRNA is highly expressed in spleen, thymus, lung, large intestine and liver, weakly expressed in brain and pancreas and is not expressed in testis and uterus (Cao *et al* 2004). Mouse Tis11b/Berg36 mRNA is highly expressed in kidneys, liver, lung, pancreas and heart and weaker in skeletal muscle, colon, thymus, spleen, small intestine, brain and peripheral blood leukocytes (Shimada *et al* 2000). Finally mouse Tis11d is highly expressed in lung, liver, skeletal muscle, kidneys, pancreas, placenta and less strongly in heart and brain (Ino *et al* 1995). Expression of the human Tis11 family members was tested using either GeneNote and Affymetrix analysis or Unigene and electronic northern blot analysis and is shown as a bar chart in Figure 1.6. Human Tis11 is highly expressed in all tissues tested with the lowest expression found in brain. In haematopoietic tissues shows invariably high expression in all Bone Marrow, Spleen and Thymus. Tis11b is expressed in all tissues tested with the lowest expression found in muscle cells. In haematopoietic tissues Tis11b is more highly expressed in Thymus than Bone Marrow. Finally Tis11d is also expressed in all tissues with the lowest levels found in brain. In haematopoietic tissues Tis11d is highly expressed in all 3 tissues.

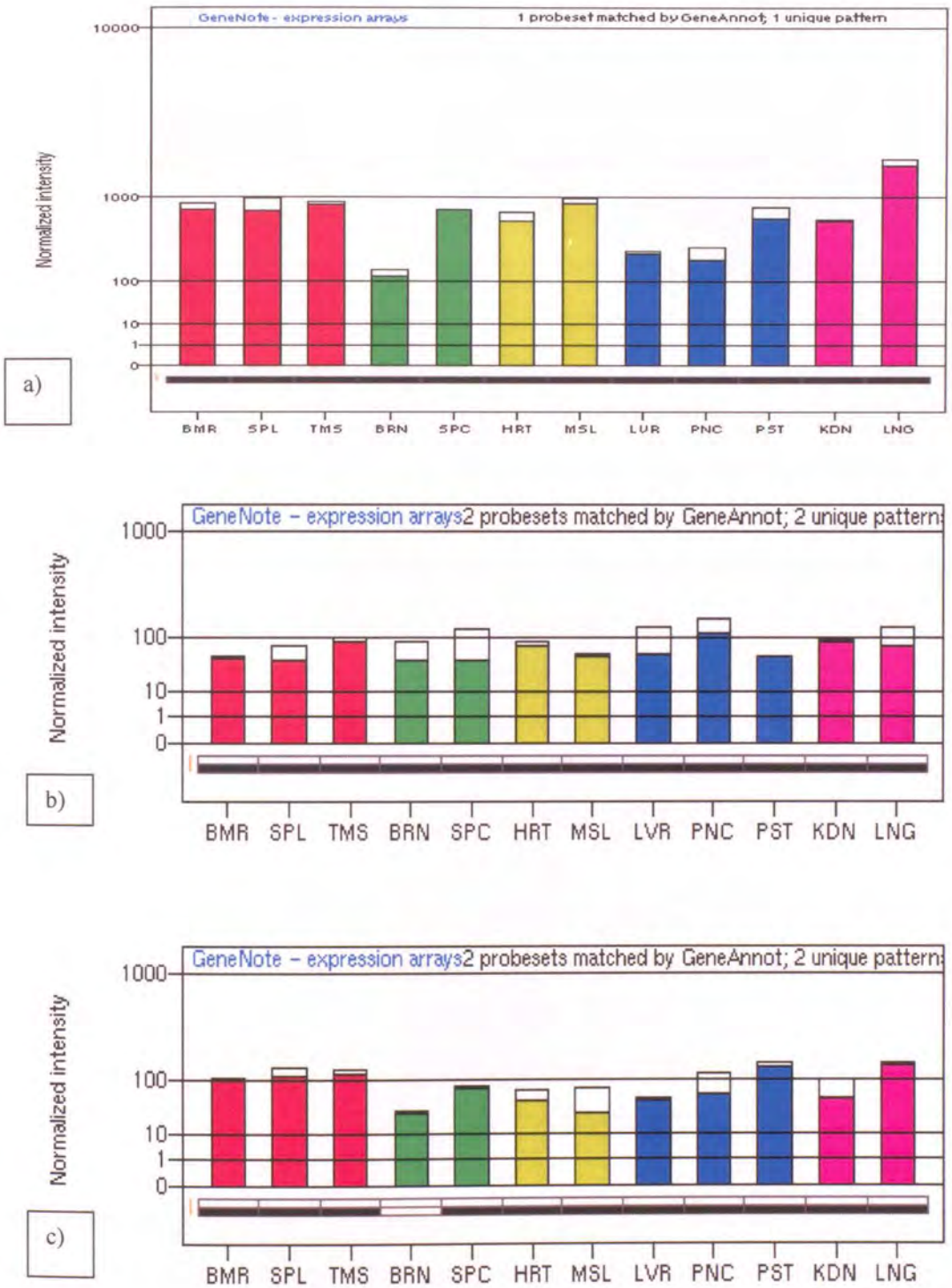


Figure 1.6: Expression arrays for Tis11 family expression in human tissues
GeneNote expression arrays detection of Tis11 family members in various human tissues, with a)Tis11 b)Tis11b and c)Tis11d expression. Tis11 is higher expressed than Tis11b and Tis11d in all tissues tested. In the haematopoietic tissues Bone Marrow (BMR), Spleen (SPL) and Thymus (TMS), Tis11 and Tis11d show no difference in expression between them while Tis11b expression is clearly lower in BMR compared to the other two haematopoietic tissues. The bar charts-data were obtained from Weizmann Gene cards Database on the 5th of April 2006. Abbreviations: BRN:Brain, SPC:Spinal cord, HRT:Heart, MSL: Skeletal Muscle, LVR:Liver, PNC: Pancreas, PST:Prostate, KDN:Kidney, LNG:Lung.

1.5.2 Tis11 family function

In terms of protein structure all 3 genes consist of 2 tandemly repeated zinc finger motifs (YKTEL) through which they bind to AU Rich elements (AREs) on the 3' UTR (untranslated region) of certain mRNAs and promote their deadenylation, decapping and degradation by either the exosome (3'-5' degradation) or XRN1 exonuclease (5'-3' degradation). Thus they are considered as destabilising AU Binding proteins (AUBPs). Apart from Tis11 family other proteins are classified as AUBPs and include HuR family which is considered as a stabilising agent and AUF1 family that is considered mainly as destabilising AUBPs (Wilusz *et al* 2001). AREs are found at the 3' end of several mRNAs that code for transcription factors, cell cycle regulators, apoptosis regulators, cytokines. They are subdivided into 3 classes namely Class I, Class II and Class III with Class II being further subdivided into Class IIA, IIB, IIC, IID, IIE (Wilusz *et al* 2001). Class I is characterised by the WAUUUAW motif and a U-rich region found for example in c-fos and c-myc onogenes, Class III is characterised by an U-rich sequence found in c-jun. Class IIA contains 5 repeats of the AUUUA sequence and is found for example in TNF-α and GM-CSF, ClassIIB contains 4 AUUUA repeats and is found for example in IFN-α, Class IIC contains 3 AUUUA repeats and is found in VEGF and IL-2, Class IID contains 2 AUUUA repeats and is found in FGF2 and finally Class IIE contains 1 AUUUA motif and is found in u-PA Receptor (Wilusz *et al* 2001).

One of the first indications that Tis11 functions as AUBPs came from Carballo *et al* who showed that Tis11 overexpression did not inhibit TNF-α transcription but instead reduced its half life by binding to AREs in the 3' end (Carballo *et al* 1996). Several others have confirmed this finding and showed that the optimal and minimally required sequence for Tis11 binding is UUAUUUAUU (Lai *et al* 2005, Brewer *et al* 2004, Blackshear *et al* 2003b, Wothington *et al* 2003) with the A residues and spacing between them being very critical in ensuring stable association between Tis11 and Mrna target sequence (Lai *et al* 2005, Brewer *et al* 2004). It should be noted that AREs are found at the 3' end of each of the three Tis11 genes suggesting that they may regulate their own stability-autocrine loop(negative feedback loop) (Bustin *et al* 1994, Ino *et al* 1995).

Overexpression of human Tis11 in HEK293 (embryonic kidney cells) caused a significant reduction in TNF-α mRNA which depended on the amount of Tis11 transfected (Lai *et al* 2000). The same was found for rat Tis11b or xenopus Tis11d even though Tis11d was less efficient in inducing TNF-α mRNA decay than Tis11 and Tis11b which were found to be equally efficient (Lai *et al* 2000). The two zinc finger motifs (especially Cys124, Cys147, His128, Cys162 and His166) were necessary and sufficient for binding to the AREs sequence and for inducing mRNA decay (Lai *et al* 2000). In the same study it was shown that the presence of a non binding mutant has a dominant negative effect on Tis11 induced destabilisation of TNF-α mRNA, possibly through interacting with proteins that inhibit Tis11 destabilising function (Lai *et al* 2002). Similarly another study showed that Tis11 family are involved in the mRNA decay of GM-CSF and IL-3 cytokines (Lai *et al* 2001).

Degradation of the mRNA requires deadenylation, which is induced by Tis11 family proteins themselves (Lai *et al* 2003). Deadenylation is strongly induced by Tis11 when at least two nonamers (UUAUUUAUU) are present (Lai *et al* 2005). Because the ability of Tis11 to promote deadenylation depended on the presence of Mg^{+2}, it was hypothesized and shown that PolyA Binding Protein (PARN) is required for deadenylation induced by Tis11 (Andersen *et al* 2005, Lai *et al* 2003). Association between Tis11 and PARN was not direct and thus other proteins may link them or Tis11 may displace an AREs stabilising factor that inhibits deadenylation caused by PARN (Lai *et al* 2003). Another study showed that Tis11 co-immunoprecipitates with hDcp1 and Dcp2 (decapping enzymes), hXrn1(5'-3' exonuclease), hCcr4(deadenylase), hRrp4 (component of exosome) or hEdc3 (enhances the activity of the decapping enzymes) through the N-terminal domain (Andersen *et al* 2005, Fenger-Grøn *et al* 2005). Because deletion of the N-terminal domain of Tis11 partially abrogated induction of mRNA decay, it was proposed that Tis11 is involved in deadenylation and degradation of mRNA by recruiting enzymes such as hDcp1, hDcp2, hCcr4 etc through it's N-terminal domain, a feature probably shared with Tis11b (Andersen *et al* 2005). Other *cis* acting elements should be involved in Tis11 function recruited through the C-terminal domain because deletion of this domain in both Tis11 and Tis11b resulted in stabilisation of an ARE containing β-globin construct (Andersen *et al* 2005). One such element may be the exosome (a multi-component complex with 3'-5' exonuclease activity) which is

recruited by Tis11 and is involved in degradation of mRNA (Chen *et al* 2001). The exosome consists of 14 different components such as hRrp4, hRrp41, hRrp42, hRrp43, hRrp46 hCsl4, hMtr3 etc that show similarity to yeast exosome components (Chen *et al* 2001). It was proposed that Tis11 and the rest of AUBP recruit a large multiprotein complex, that consists of PARN (deadenylase), decapping enzymes such as hDcp1, hDcp2, hEdc3, exonucleases among which the exosome and probably Xrn1 (5'-3' exonuclease) to preferentially degrade ARE containing mRNAs (Fenger-Grøn *et al* 2005, Chen *et al* 2001).

Targets of the Tis11 family include TNF-α in many cell types (Sauer *et al* 2006, Rigby *et al* 2005, Brooks *et al* 2004, Suzuki *et al* 2003, Twizere *et al* 2003, Lai *et al* 2000), GM-CSF (Lai *et al* 2002, Lai *et al* 2001), IL-3 (Schmidlin *et al* 2004, Lai *et al* 2002, Lai *et al* 2001), IL-2 (Ogilvie *et al* 2005), IL-6 (Sauer *et al* 2006), IL-1β (Chen *et al* 2006) while VEGF was shown to be specifically targeted by Tis11b (Ciais *et al* 2004). Most of these targets are cytokines and have been identified through overexpression of Tis11 family members in certain cell lines. A very recent study utilising Tis11 siRNA technology, revealed that it can target FOS (transcription factor), p21 (a cell cycle inhibitor) and possibly KLF6 (Kruppel like factor 6, transcription factor and cell cycle inhibitor) in THP-1 cell line and primary human monocytes (Patino *et al* 2006). Another very recent study has confirmed that Tis11 is involved in the regulation of c-myc and Cyclin D1 after treatment of a glioblastoma and a prostate cancer cell line with rapamycin (mTOR inhibitor) (Marderosian *et al* 2006). Finally use of knock out mice showed that Tis11 can target Ccl2 and Ccl3 chemokines in bone marrow derived macrophages (Sauer *et al* 2006).

1.5.3 Tis11 family members structure

The structure of the first zinc finger motif of Tis11 and Tis11d before and after binding to an ARE probe have been elucidated (Hudson *et al* 2004, Amann *et al* 2003). Because of the high similarity within the zinc finger motif between the 3 family members it is expected that the model of mRNA binding by Tis11d will apply to all family members. The first zinc finger (ZF1) motif of Tis11 lacks regular secondary structure (α helices or β-hairpins) and the whole structure is stabilised by zinc and NH or hydrophobic bonds (Amann *et al* 2003). Also there is a pocket formed by Tyr10, Lys11, Tyr26, Lys29 and

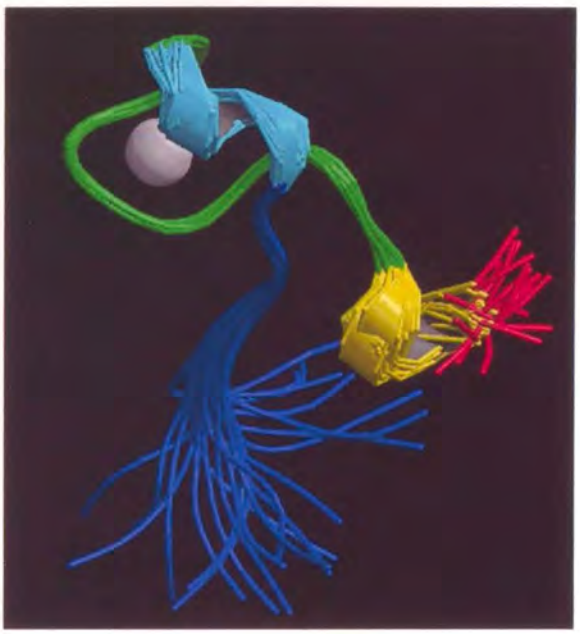
Phe32 with currently unknown function (Amann *et al* 2003). A 3D image of ZF1 from Tis11 as it appears in RCSB ProteinDataBank is shown in Figure 1.7.

As for Tis11d structure again it was found that the ZF are stabilised by hydrogen and van der Waals bonds, their orientation is relatively fixed because the linker region shows little flexibility (residues 186-189) (Hudson *et al* 2004).

When the minimal binding motif (UUAUUAUU) or the TNF-α ARE sequence was used:

```
                                  9,8,7,6
                                  | | | |
5'-UUAUUUAUUUAUUAUUUAUUUAUU-3'
   | |2|3|4,5                    | | | |
                                  16,17,18,19
```

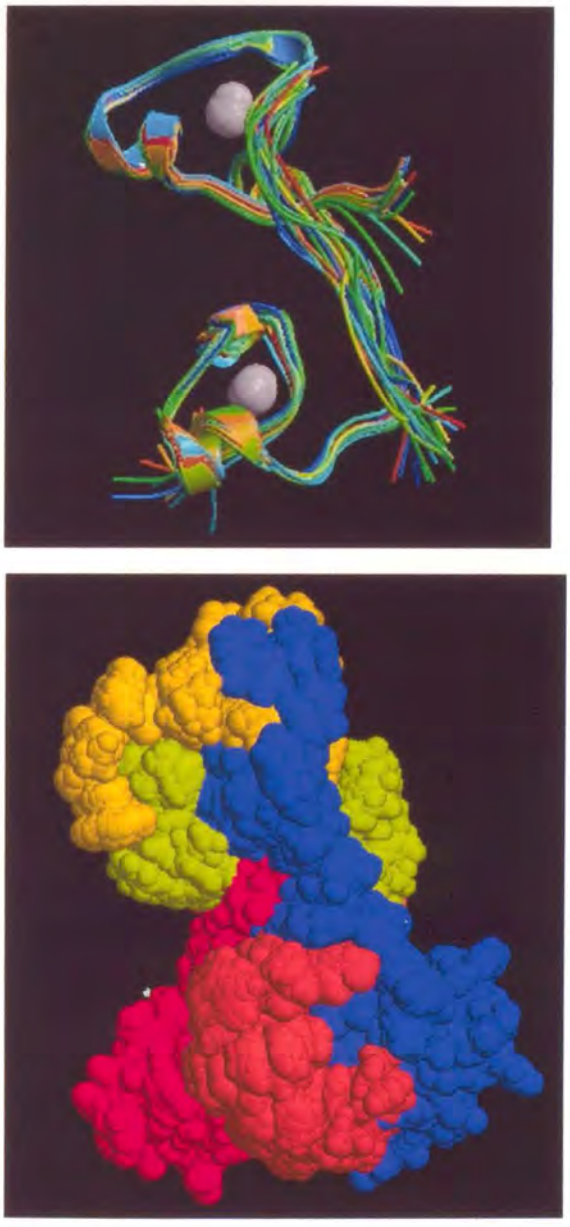
it was found that Tyr170 of ZF1 intercalated between U16 and U17 (or U8 and U9 counting from the 3' end) and Phe176 of the same ZF intercalated between U19 and A18 (or U6 and A7 counting from the 3' end) with the U8-Tyr170-U9 lying perpendicular to the U6-Phe176-A7 stack (Hudson *et al* 2004). Similarly Tyr208 from ZF2 intercalate between U4 and U5 and Phe214 intercalates between U2 and A3 (Hudson *et al* 2004). The binding of Tis11d to the mRNA is stabilised by hydrogen bonds. A photo of the predicted 3D structure from the RCSB Protein Databank is shown in Figure 1.8.



a)

b)

Figure 1.7: Structure of 1rts zinc finger motif of Tis11 protein
3D structure of the first zinc finger motif of Tis11 as it appears in a) RCSB Protein DataBank website, accession code 1M90 based on the study of Amann *et al* 2003 b) Spacefil model using the Rasmol programme of the Tis11 protein with orientation as in a) and group colour mode. The photo from the RCSB has been taken on the 31rst of March 2006 and analysed with the Rasmol programme the same day.



a)

b)

Figure 1.8: Structure of Tis11d protein after binding to mRNA target sequence
3D structure of Tis11d bound to mRNA as seen in a) RCSB Protein BataBank accession code 1RG0 and based on the study of Hudson *et al* 2004
b) Spacefil model after application of the Rasmol programme with orientation as seen in a) and group colour mode. The bound mRNA is indicated as blue colour while the ZF1 is represented in light green-yellow and ZF2 is represented in orange-red. The photo from the RCSB Protein DataBank has been taken on the 31^{rst} of March 2006 and analysed by Rasmol the same day.

1.5.4 Regulation of the Tis11 family at the mRNA level

All 3 Tis11 family members can be induced at the mRNA level by PMA in a wide variety of cells including CLL cells, mouse fibroblasts, human epithelial cells, and phaeochromocytoma cells (Taylor *et al* 1991, Varnum *et al* 1991, Murphy and Norton 1990). Insulin can induce Tis11 in NIH3T3 HIR 3.5 cells- mouse fibroblasts (Lai *et al* 1990) but in RIE-1 (rat intestinal epithelial) cells only Tis11b is induced (Corps and Brown 1995) suggesting cell type specific regulation. Another widely used stimulus is LPS in murine (RAW264.7 cell line) or human macrophages (THP-1 cell line) (Brooks *et al* 2004, Machtani *et al* 2001, Tchen *et al* 2004) which induced Tis11 as early as 30 min post stimulation. Primary human white blood cells including lymphocytes, monocytes and neutrophils were also treated with LPS which induced Tis11 primarily in macrophages and monocytes while had no effect in lymphocytes (Fairhurst et al 2003). Instead in human T lymphocytes, Tis11 was induced by GM-CSF (Varnum *et al* 1989), TGF-β (Ogawa *et al* 2003) or a combination of PMA plus Ionomycin (Raghavan *et al* 2001). Finally Tis11 was induced by anisomycin (a p38 activator) alone or synergistically with IFN-γ or IFN-β as early as 1h post-stimulation in MEFs (mouse embryonic fibroblasts) (Sauer *et al* 2006). In the same study it was shown that IFN-γ further increased Tis11 induction following stimulation of mouse Bone Marrow Macrophages cells (BMMC) with LPS, and comprises the first study showing that Interferons (IFNs) may also regulate Tis11 family (Sauer *et al* 2006).

Tis11b was also induced by PMA and Bryostatin in CLL cells (Murphy *et al* 1993, Murphy and Norton *et al* 1990), Calcium Ionophore, anti-IgM and anti-CD20 in Burkitt's lymphoma cell lines (Mathas *et al* 2000, Ning *et al* 1996b), Insulin in RIE-1 cells (Corps and Brown 1995), Parathyroid hormone in human primary osteoblasts (Reppe *et al* 2004) or ACTH hormone in adrenocortical cells (Chinn *et al* 2002).

Finally Tis11d is not as well studied as the other two family members but it has been shown to be induced by PMA in many cell types (Taylor *et al* 1991, Varnum *et al* 1991) and Dexamethasone in 697 pre-B cells (Yoshida *et al* 2002) or CD4+/CD8+ thymocytes (Bianchini *et al* 2006).

The kinetics of Tis11 family mRNA induction is stimulus and cell type specific. For example Tis11 mRNA is evident at 15 min after insulin or serum stimulation in mouse fibroblasts returning to baseline levels at 2h (Lai *et al* 1990, Taylor *et al* 1996a) but in

BMMC is induced by IL-4 at 30 min and is still evident at 3h post stimulation (Suzuki *et al* 2003) whereas in RAW264.7 cell line Tis11 mRNA induction by LPS is biphasic, with the first peak seen at 1h and the second one at 5h post stimulation (Sauer *et al* 2006, Tchen *et al* 2004). Similarly Tis11b peaks at 2h post stimulation and is clearly above basal levels at 4h in insulin treated RIE-1 cells (Corps and Brown 1995) or PMA treated CLL cells (Murphy *et al* 1993) but in ACTH treated human adrenocortical cells peak induction occurs at 3h post stimulation and is above basal levels for up to 24h (Chinn *et al* 2002). To further add to their complexity of regulation, different stimuli have different effects on the magnitude and kinetics of induction of Tis11b/Berg36 mRNA in the same cell type. For example PMA induced Tis11b/Berg36 stronger and earlier than Bryostatin in CLL cells (Murphy *et al* 1993) and the same was found for anti-IgM and anti-CD20 in BL60-2 Burkitt's Lymphoma cell line (Mathas *et al* 2000). Moreover the Tis11b mRNA is clearly above basal levels at 8h post stimulation with anti-IgM and PMA in the cells mentioned above but has returned to basal levels at 4h post stimulation with anti-CD20 in BL60-2 or PMA in normal tonsillar B cells (Murphy *et al* 1993). All above data suggest that Tis11 family members may be regulated through different signalling pathways in a stimulus and cell type dependent manner.

However little is known about the regulation of the Tis11 family mRNAs by signal transduction pathways. One study has shown that both phases of the biphasic Tis11 induction after LPS treatment, are regulated through the p38 pathway (Mahtani et al 2001, Tchen *et al* 2004) or p38 and ERK pathway at the protein level (Brook *et al* 2006) which was confirmed in THP-1 cell line as well (a human myelomonocytic cell line) (Brooks *et al* 2004). On the other hand JNK, PKC and PI3K pathways were not involved in the regulation of Tis11 by LPS in RAW264.7 (Brook *et al* 2006, Tchen *et al* 2004). In these studies it was shown that p38 regulates stability of Tis11 mRNA (half life) rather than transcription of the gene (Brook *et al* 2006, Tchen *et* al 2004). Another study in murine J774 macrophages confirmed that p38 inhibition abrogates Tis11 mRNA induction after LPS treatment and further showed that inhibition of NF-κB, calcineurin and Jak-2 pathways have no effect on the induction of the gene (Jalonen *et al* 2005). A more recent study, showed that p38 directly affects transcription of the Tis11 gene because induction of Tis11 mRNA by anisomycin plus IFNs was lower in $p38^{-/-}$ K.O. MEFs.

(Sauer *et al* 2006). In the same study it was also found that Tis11 transcription is regulated by STAT1 through a GAS element found on both mouse and human Tis11 promoters (Sauer *et al* 2006). STAT1 is also involved in Tis11 induction after LPS stimulation of BMMC which was partially attributed to secondary induction of IFN-β by LPS (Sauer *et al* 2006). Other factors directly regulating Tis11 transcription include Smad3 and Smad4 after treatment of HuT78 T cell lymphoma or primary T cells with TGF-β (Ogawa *et al* 2003).

Fewer studies have been conducted for Tis11b. One such study showed that H7 (a broad specificity inhibitor for PKC and PKA pathways) had no effect on Tis11b induction by PMA or Bryostatin in CLL cells (Murphy *et al* 1993). Another study showed that induction of Tis11b in RIE-1 cell line by insulin remained unaffected by PKC inhibition but PI3K inhibition (wortmannin) partially abrogated this induction (Corps *et al* 1995). However wortmannin had no effect on induction of Tis11b by PMA in RIE-1 cells (Corps *et al* 1995). Finally it was shown that Tis11b induction by Parathyroid hormone in OHS osteoblastic cell line was abrogated by inhibition of PKA pathway (Reppe *et al* 2004).

1.5.5 Regulation of the Tis11 family at the protein level

Apart from the transcriptional level, Tis11 genes family are regulated at the protein level through either phosphorylation or localisation in different cellular compartments. It is currently unclear whether phosphorylation controls localisation of Tis11 family members or their involvement in different functions.

1.5.5.1 Phosphorylation: The first indication of Tis11 phosphorylation came from Taylor *et al* 1995 which reported that stimulation of NIH3T3 cell line with various stimuli such as FCS, PMA, PDGF, FGF for 2h induced Tis11 protein detected as a 45kDa protein instead of 33kDa which is the predicted molecular mass. In this particular study phosphorylation of Tis11 was shown to be partially under p42 MAPK pathway (ERK1) which phosphorylates Ser220 in mouse Tis11 (Taylor *et al* 1995). In another study a timecourse experiment in LPS stimulated murine macrophages revealed that Tis11 protein is evident at 75 min post stimulation as a 36 kDa protein and increases in size over time up to 45 kDa at 4h post stimulation (Zhu *et al* 2001).

However a more recent study showed that Tis11 protein is phosphorylated in RAW264.7 cells as early as 5 min post stimulation with LPS and protects Tis11 from proteasome dependent degradation (Brook *et al* 2006). Phosphorylation occurred at Ser52 and Ser178 in a p38 and MK2 dependent manner (Brook *et al* 2006, Hitti *et al* 2006). Indeed it has been found that Tis11 protein is an excellent substrate for p38α and p38β isoforms which probably control multiple phosphorylation events (Zhu *et al* 2001) while another study showed that Tis11 can be phosphorylated by MAPKAPK2 (MK2) and MKK6 (Mahtani *et al* 2001). Ser52, Ser178, Thr249, Thr250, Ser 264, Ser 80/82 were suggested to be phosphorylated by MK2 and Ser264 and Thr250 was suggested to be regulated by the p38a pathway (Chrestensen *et al* 2004). From these sites, Ser52/58 found between Nuclear Localisation Signal (NLS) and Nuclear Export Signal (NES), Ser105 in NLS and Ser178 may determine the localisation of Tis11 protein in the nucleus or cytoplasm (Figure 4) (Brook *et al* 2006, Chrestensen *et al* 2004). Ser178 is also present in Tis11b and phosphorylation of this site creates a binding site for the 14-3-3 β isoform (Chrestensen *et al* 2004). Tis11 family members can bind equally well to 14-3-3 β and η isoforms (Johnson *et al* 2002). Tis11 could also bind strongly to the γ isoform but weakly to ζ, σ, τ (Chrestensen *et al* 2004) and binding of Tis11 to 14-3-3 proteins promoted localisation of Tis11 in the cytoplasm (Johnson *et al* 2002).

A very recent study using MudPIT or MALDI/MS methods showed that the major phosphorylation sites in TTP when purified from HEK293 cell line are: S66, S88, T92, S169, S186,S197, S218, S228, S276, S296 (Cao *et al* 2006). Phosphorylation of S197, S218 and S228 significantly affected the mobility of Tis11 protein in SDS-PAGE gels (Cao *et al* 2006) whereas all other sites alone or in combination did not significantly affect Tis11 mobility. Inspection for conserved motifs surrounding all potential phosphorylation sites found that S41, S88, S218 and S228 are potential targets of ERK1, S93 and T238 may be phosphorylated by p38 and S197 or T257 may be targets of GSK3 (Cao *et al* 2006). Common phosphorylation sites in all 3 family members include: T106, Y158, S184, S186, S217, S218, S273, S276, Y284, S294, S296, S323 whereas potential phosphorylation sites unique for Tis11 are: S66, S88, T92, S169, S186, S197 and S228 (Cao *et al* 2006). This study suggested that Tis11 family function may be altered through phosphorylation by several pathways that can be activated *in vivo*. It is still unclear whether phosphorylation of Tis11 affects Tis11 ability to promote

mRNA degradation. One study showed that transfection of cells with Tis11 and MKK6 (activates p38) resulted in phosphorylation of Tis11 protein but did not affect induction of mRNA decay (Tchen *et al* 2004). Two independent studies using a series of Serine or Threonine mutants alone or in combination showed that these phosphorylation events did not alter binding, deadenylation and mRNA degradation of TNF-a induced by Tis11 (Cao *et al* 2006, Rigby *et al* 2005). On the contrary one study showed that co-transfection of HEK293 cells with Tis11 and dominant active MKK6 (thus dominant active p38 pathway) resulted in moderate increase in the half life of a TNF-α construct suggesting some inhibition of Tis11 function (Zhu *et al* 2001). Another study showed that phosphorylation of Tis11 especially at Ser178 inhibited Tis11 binding ability to TNF-α mRNA and thus Tis11 function (Hitti *et al* 2006). In accordance with the previous two studies, another one found decreased binding of phosphoTis11 to IL-1β and abrogation of mRNA decay (Chen *et al* 2006). It was suggested that reduced binding activity of phosphorylated Tis11 could be due to competitive binding with 14-3-3 proteins since phosphorylation of Tis11 at Ser52 and Ser178 result in binding to 14-3-3 proteins (Hitti *et al* 2006). Finally a study involving Tis11b this time, showed that phosphorylation of Tis11b by Akt/PKB at Ser92 abrogated mRNA decay of an IL-3 ARE containing probe (Schmidlin *et al* 2004). Again in this study it was proposed that Akt/PKB phosphorylates Tis11b and results in inhibition of Tis11b function through binding to 14-3-3 proteins (Schmidlin *et al* 2004).

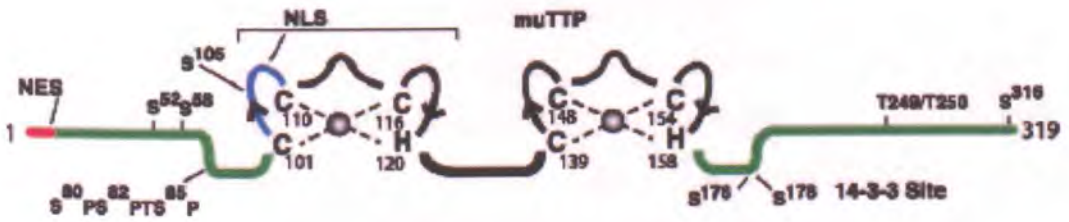


Figure 1.9: Nuclear Localisation(NLS) and Nuclear Export Signals (NES) in mouse Tis11 protein
Diagrammatic structure of mouse Tis11 (TTP) indicating the NES, NLS, a binding site for 14-3-3 proteins and other sites of phosphorylations regulated by either p38 or MK2. The two zinc fingers are shown as loops in the centre of which is zinc (grey circle). The diagram was obtained from Christensen *et al* 2004, pp:10183.

1.5.5.2 Localisation: Several studies in macrophages or fibroblasts showed that Tis11 protein is expressed at very low levels in the nucleus of unstimulated cells and shuttles to the cytoplasm after stimulation with LPS (Cao *et al* 2004, Phillips *et al* 2002, Carballo *et al* 1996, Taylor *et al* 1996a) even though one study reported that Tis11 is nuclear and remains nuclear after stimulation with serum (DuBois *et al* 1990). Most of these studies were carried out in transfected cells while a recent study on human leukocytes that tested localisation of endogenous Tis11 in neutrophils found that Tis11 is cytoplasmic in resting cells with little nuclear localisation and remains cytoplasmic following LPS stimulation (Fairhurst *et al* 2003). The only exception are HUVEC cells (Human Umbicullar Vein Endotehlial Cells) in which Tis11 is cytoplasmic in resting cells and becomes nuclear after stimulation with TNF-α (Gringhuis *et al* 2005). Nuclear to Cytoplasmic shuttling is regulated through phosphorylation at Ser52 and Ser178 primarily by the p38 pathway since inhibition of p38 resulted in nuclear accumulation of Tis11 protein after LPS treatment (Brook *et al* 2006). Interestingly hypophosphorylated forms of Tis11 were also found to be highly susceptible to proteasomal degradation (Brook *et al* 2006).

Nuclear localisation signals (NLS) have been mapped for all 3 genes initially in mouse Tis11 that has a NLS between amino acids 1-15 and a Nuclear Export Signal (NES) between 95-158 (Phillips *et al* 2002). In humans the NES has been mapped for Tis11 between amino acids 3-13 and 316-324, for Tis11b/Berg36 between 305-313 and for Tis11d between amino acids 471-479 (Phillips *et al* 2002). Neither RNA binding nor the zinc finger motifs are required for nuclear localisation of Tis11 (Murata *et al* 2002, Phillips *et al* 2002) but instead the linker region between the two zinc finger motifs, especially Arg127 and Arg131 are involved. On the contrary Leu3, Isol16 and Leu10 are critical for nuclear export (Murata *et al* 2002). Because it was shown by yeast two hybrid system that Tis11 associates with nucleoporin/Nup214 which is part of the nuclear pore on the cytoplasmic face of the pore complex (Carman *et al* 2004) probably this protein associates with the linker region to mediate nuclear localisation of Tis11. On the other hand nuclear export seems to be regulated through CRM1 (nuclear export receptor) since inhibition of CRM1 resulted in nuclear accumulation of Tis11 (Murata *et al* 2002, Phillips *et al* 2002).

When the cells are stimulated with a strong stress signal such as heat shock, FCCP (mitochondrial inhibitor), clotrimazole (mitochondrial stress) or oxidative stress, Tis11 localises in stress granules (SG) (Kedersha *et al* 2005, Murata *et al* 2005, Stoecklin *et al* 2004). In some cases a stress signal is not required and Tis11 co-localised with TIA-1 (a protein found specifically in SG) in a small percentage of cells overexpressing Tis11 (30-40% for COS-7, 5-12% for HepG2 hepatoma cell line) (Rigby *et al* 2005, Stoecklin *et al* 2004). Later on it was shown that localisation in SG, requires Tyr105, Tyr113, Phe119 and G109/G114 aromatic amino acids of Tis11 protein (Murata *et al* 2005). On the contrary it was shown that arsenite treatment excludes Tis11 from SG through MK2 mediated or p38 and JNK mediated phosphorylations which resulted in binding to 14-3-3 proteins (Rigby *et al* 2005, Stoecklin *et al* 2004). Apart from stress granules Tis11 and Tis11b when over expressed localise in Processing Bodies (PB), which have been proposed to be the sites of mRNA decay (Kedersha *et al* 2005, Fenger-Grøn *et al* 2005). Even more importantly it was shown that over expression of Tis11 and Tis11b resulted in stable association between SG and PB with SG engulfing PB suggesting that Tis11 family plays an active role in tethering SG to PB (Kedersha *et al* 2005). Stress granules are considered the sites where mRNA is stalled for storage, re-initiation of translation or degradation whereas Processing Bodies are the sites of mRNA degradation (Kedersha *et al* 2005, Cougot *et al* 2004). Additionally formation of PB is absolutely necessary for RNA interference (RNAi) activity and thus translation arrest or mRNA degradation through siRNA and miRNA (Jakymiw *et al* 2005) suggesting that Tis11 may be involved in these processes as well. This has been proved very recently in *Drosophila* cells in which a genome wide screen for genes involved in the RNAi pathway identified dTis11 as an important protein for RNAi activity (Dorner *et al* 2006). Further support for Tis11 involvement in RNAi in mammalian and human cells comes from Jing *et al* who showed that successful RNAi mediated by miR-16/miR-15 required Tis11 (Jing *et al* 2005).

1.5.6 Tis11 family in relation to malignancy

Because the Tis11 family can destabilise several mRNA whose inappropriate expression is related to malignancy, it was hypothesized that they may form a different class of tumour suppressor genes. In support of this hypothesis it was shown that injection of

mast cells transformed with v-H-ras resulted in tumour formation in mice as early as 6 weeks due to autocrine IL-3 overproduction. Transfection with v-H-ras clones over expressing Tis11 delayed tumour formation by at least 4 weeks suggesting that indeed Tis11 can be a tumour suppressor gene by destabilising certain mRNA bearing AREs at the 3' UTR (Stoecklin *et al* 2003). Another study also showed interference with Ras signalling/activation since Tis11b when bound to 14-3-3β and 14-3-3τ isoforms resulted in 80% abrogation of 14-3-3β binding to cRaf-1 which may have implications for constitutive Ras/ERK activation and malignant transformation (Bustin and McKay 1999). Another important molecule for malignant transformation is PKB/Akt (Schmidlin *et al* 2004). It was shown that Tis11b is phosphorylated at Ser92 by PKB or at Ser 90 when the first serine residue is absent (Schmidlin *et al* 2004). Because this phosphorylation resulted in inhibition of mRNA decay it was proposed that Akt/PKB, which is over expressed in most of the cancers, may cause malignant transformation through phosphorylation of Tis11b which will result in stabilisation of certain oncogenic proteins (Stoecklin *et al* 2003). Even though the Akt phosphorylation site is found only in Tis11b/Berg36, it was shown very recently that Tis11 function may be also affected by PKB. In the latter study, Tis11 induced mRNA decay of c-myc and Cyclin D1 following rapamycin treatment when Akt/PKB was present-over expressed but induced mRNA stabilisation of these genes when Akt/PKB was very low or absent (Marderosian *et al* 2006). From these two studies (Marderosian *et al* 2006, Stoecklin *et al* 2003) it seems that Tis11b and Tis11 have opposing effects in terms of inducing mRNA degradation in the case that Akt/PKB is present-over expressed but since the studies were performed in different cell types they may reflect intrinsic differences between these cell types.

In relation to Leukaemias it was shown that Tis11 (but not Tis11b) specifically interacts with Tax protein from BLV (Bovine Leukaemia Virus) or HTLV-1 (Human T lymphotropic Virus) (Twizere *et al* 2003). In the presence of Tis11, Tax was less oncogenic probably due to inhibition of Tax transactivation activity (Twizere *et al* 2003). Interestingly Tis11 was able to abrogate TNF-α induction by Tax in resting murine macrophages but the presence of Tax inhibited Tis11 induced TNF-α mRNA degradation in LPS stimulated RAW264.7 macrophages suggesting that Tis11 and Tax can inhibit each other-bipartite regulation (Twizere *et al* 2003). Tis11 was also found

by microarrays to be overexpressed in Marginal Zone Lymphoma but underexpressed in Mantle Cell Lymphoma (Thieblemont *et al* 2004).

On the other hand Tis11b was found to be over expressed in an IL-3 dependent murine myeloid precursor cell line in which it induced proliferation and delayed their differentiation to mature granulocytes in response to G-CSF (Shimada *et al* 2000). Additionally Tis11b was found to be one of the genes that are over expressed in cell lines and primary cells expressing the AML1-ETO [results from translocation t(8;21)(q22;q22)] fusion protein found in 40% of Acute Myeloid Leukaemia subtype M2 (Shimada *et al* 2000). High expression of Tis11b was also found in K562 cell line (CML/Erythroleukaemia) and HEL (Erythroleukaemia)(Shimada *et al* 2000). It is noteworthy that even though Tis11b was induced at least 3-fold in cells expressing AML-1-ETO, Tis11 and Tis11d were unaffected suggesting that only this family member is somehow involved in the malignant transformation caused by AML1-ETO fusion protein (Shimada *et al* 2000).

Finally Tis11d was found to be expressed in the vast majority of leukaemias including CML, ALL, AML, MM, T-ALL and T lymphoblastic lymphoma. Additionally it was found to be part of an insertion involving TCR β in a patient with acute T cell leukaemia (Ino *et al* 1995). In the case that the resulting fusion protein is expressed, it would have a longer and different C-terminal domain than wild type Tis11d and could result in either altered function of Tis11d protein or increased stability of it's own mRNA (Ino *et al* 1995).

In relation to CLL it was shown that miR-16 located on chromosome 13q14 requires Tis11 in order to mediate degradation of certain mRNAs (Jing *et al* 2005). Analytically it was shown that Dicer and miR-16 are required for degradation of ARE containing transcripts in HeLa cells and the presence of siRNA for Tis11 completely abrogated mRNA degradation caused by miR-16 (Jing *et al* 2005). It should be noted that miR-16 can target ARE containing mRNAs because it bears a sequence that is complementary to ARE (UAAAUAUU). Thus it was proposed that Tis11 promotes miR-16 recruitment/positioning to AREs which will then cause degradation by the RISC complex formed at the 3' end of a particular mRNA (Jing *et al* 2005). Because the siRNA used to target Tis11 could target all 3 family members it was suggested that all of them are required for miR16 mediated degradation. Even though Tis11 did not

directly bind to miR-16 it was proposed that Tis11 (or the other Tis11 members) and miR-16 participate in the formation of RISC which is absolutely necessary for mRNA degradation (Jing *et al* 2005). This finding is very important for CLL because miR-16 together with miR-15 are located on 13q14 the region that is deleted in 50% of CLL and were found to be reduced or absent in most of the CLL samples tested (Calin *et al* 2002). Very recently a study showed that miR-15/miR-16 have a short region that is complementary to Bcl-2 and thus miR-15/miR16 can directly interact with the 3' end of Bcl-2 causing degradation of Bcl-2 and induction of apoptosis (Cimmino *et al* 2005). The later finding suggests that Tis11 family may be involved in the regulation of Bcl-2 anti-apoptotic protein that is over expressed in CLL. Bcl-2 contains a Class II ARE motif in the 3'UTR with at least one UUAUUUAUU nonamer which is conserved between different species (humans, mice, chicken) and this sequence actively controls stability of Bcl-2 (Schiavone *et al* 2000). Interestingly following UVC irradiation the half life of Bcl-2 was reduced by at least 50%, accompanied by changes in the pattern of AUBP binding-increased binding of AUBP with low molecular weight further indicating that Tis11 family may be involved in the regulation of Bcl-2 (Donnini *et al* 2001) even though recently it was reported that Bcl-2 protein regulated the turnover of its own mRNA through the AREs present in the 3'UTR (Bevilacqua *et al* 2003).

1.5.7 Tis11 family and apoptosis

It has been shown that treatment of Ramos cell line with Calcium Ionophore induced Tis11b/Berg36 mRNA as early as 30 min post stimulation (Ning *et al* 1996b). Calcium Ionophore treatment induced apoptosis by more than 70% which could be reversed partially by co-treatment with IL-4 or anti-CD40 (Ning *et al* 1996b). Furthermore IL-4 stimulation abrogated induction of Tis11b/Berg36, whereas anti-CD40 did not significantly alter expression of this gene (Ning *et al* 1996b). In further support for a direct involvement of Tis11b/Berg36 in apoptosis, it was shown that transfection of Ramos with antisense Tis11b using a retrovirus system resulted in resistance to apoptosis after Calcium Ionophore treatment especially at 24 and 48 hours post stimulation (Ning *et al* 1996b). Later on it was shown that over expression of all 3 Tis11 family members induced apoptosis in a variety of cell lines such as HeLa (human epithelial cells), U20S and SAOS2 (human osteosarcoma) or NIH3T3 (mouse

fibroblasts) (Johnson *et al* 2000). This induction of apoptosis was completely abrogated in the presence of Bcl-2 or CrmA (inhibitor of death receptor activated caspases) (Johnson *et al* 2000). A unique feature of Tis11 in relation to apoptosis, was that it synergistically induced apoptosis with TNF-α in NIH3T3 (Johnson *et al* 2000) and over expression of Bcl-2 was unable to block this synergistic effect suggesting that the mitochondrial pathway is not involved in apoptosis induction by Tis11 and TNF-α (Johnson *et al* 2002). Interestingly when a mutant Tis11 lacking the zinc finger motifs was used, it localised in the nucleus and failed to induce apoptosis suggesting that for induction of apoptosis the zinc fingers should be intact and Tis11 should localise to the cytosol (Johnson *et a* 2002).

Another possible link of Tis11 with apoptosis may be through iNOS (inducing Nitric-Oxide synthase) and production of Nitric Oxide (NO) since it was shown that Tis11 results in induction of iNOS mRNA and protein in a human colon carcinoma cell line (Fechir *et al* 2005, Linker *et al* 2005). Production of iNOS and NO has been associated with induction of apoptosis in HL60 myeloid cells, CD4+/CD8+ early thymocytes and Jurkat cells (Krönche *et al* 2001). However in CLL cells production of iNOS mRNA and NO protein has been correlated with inhibition of apoptosis by IL-4 (Triscornia *et al* 2004) and thus if Tis11 can regulate iNOS and NO production in many different cell types, it is uncertain whether this will result in inhibition or induction of apoptosis.

Response to chemotheraupetic agents has been also correlated with levels of Tis11 family members. It was shown that head and neck squamous cell carcinoma cell lines expressing Tis11b were induced to apoptosis by cisplatin while cells not expressing Tis11b were resistant to apoptosis and required transfection with Tis11b in order to become sensitive (Lee *et al* 2005). In this study it was found that Tis11b down regulated cIAP2 that bears class IIA AREs at the 3' end but not Bcl-2 that contains exactly the same class of AREs (Lee *et al* 1 2005). When Dexamethasome (a synthetic glucocorticoid) was used to induce apoptosis in double positive thymocytes ($CD4^+/CD8^+$) it was found by microarrays that Tis11d was induced 2.9 or 6 fold (depending on the probe set) at 3h post stimulation confirming a pro-apoptotic role of this gene but Tis11 was 1.7 fold down regulated (Bianchini *et al* 2006). Use of Dexamethasone in 697 pre-B Acute Leukaemia cell line significantly induced apoptosis which was accompanied by downregulation of Tis11b at 6h, 24h or 48h tested (Yamada

et al 2003, Yoshida *et al* 2002) and induction of Tis11d (Yoshida *et al* 2002) pointing towards stimuli specific regulation of Tis11 family in relation to induction of apoptosis.

AIMS OF THIS STUDY :

According to the previous sections it was very appealing to assume that Tis11 family may be involved in the pathogenesis of CLL possible through the regulation of certain apoptosis regulating genes such as Bcl-2. Involvement of Tis11 family in the pathophysiology of CLL was further suggested by the following: a) TNF-α, a target of Tis11 family is overexpressed in serum of CLL patients when compared with healthy individuals and correlated with advanced clinical stage and unfavourable chromosomal abnormalities such as trisomy 12 or deletion 17p (Jablonska *et al* 2005, Ferrajoli *et al* 2002, Adami *et al* 1994, Guarini *et al* 1994) and additionally TNF-α is very stable in cultured B-CLL cells (Rambaldi *et al* 1993) which may suggest improper function of Tis11 family, b) it has emerged during the timecourse of this PhD thesis that VEGF is targeted by Tis11b, and VEGF is increased in serum of CLL patients when compared with healthy individuals and it associates with advanced clinical stage (Gora-Tybor *et al* 2005) while another study reported autocrine production of VEGF by CLL cells and secretion in the culture medium (Kay *et al* 2002)

Thus the primary aim of this project was to investigate the regulation of the Tis11 family in CLL and B-lymphocytes in response to stimuli that modulate apoptosis. Specifically to:

1) Investigate the regulation of Tis11 family in response to Rituximab treatment in CLL cells since there are no data available regarding such regulation

2) Provided that one or more Tis11 family member(s) would be significantly changed in response to Rituximab, aim of this project was to investigate the upstream pathways and possible downstream targets of Tis11 family

3) Investigate the regulation of Tis11 family in response to stimuli that inhibit spontaneous apoptosis in CLL cells since there are no such data available. Again provided that one or more members would be significantly changed, the upsteram pathways as well as possible downstream targets of Tis11 family would be investigated

4) Investigate the basal expression of Tis11 family at different stages of B cell development and their regulation primarily in response to stimuli that modify apoptosis.

The methodology as well as functional studies on Tis11 family in order to accomplish these aims would be decided according to the data obtained by each of these different directions.

CHAPTER 2

MATERIALS AND METHODS

Sheep Red cells treated with AET:

Sheep Blood Erythrocytes in alsevers were washed with sterile PBS and incubated at 37^0 C for 20 minutes with AET pH 9.0 (Aminoethylisothio-uronium bromide :1.97 gr in 50 ml dH2O). Then they were washed several times with sterile PBS before being resuspended in 50 ml Complete Culture Medium [88% RPMI 1640, 10% Foetal Calf Serum (FCS), 1% Penicillin-Streptomycin(10,000 units each), 1% L-Glutamine(29 mg)].

Separation of Mononuclear cells from whole blood of CLL patients:

Whole blood was collected from CLL patients attending the haematology clinics of University College Hospital and Guy's Hospital after written consent. Mononuclear cells were separated using density gradient centrifugation. T Lymphocytes were depleted using AET treated Sheep Blood Erythrocytes (E rosetting), and monocytes were depleted by adherence to plastic for 30 minutes at room temperature. The purity of isolated B-CLL cells was >90% as revealed by staining with anti-CD3 and anti-CD20 monoclonal antibodies and FACS analysis. A total of 56 patients participated in this study who ranged in age from 35 to 85 years old as calculated at the end of the experimental work (2005). From them 30 were males and 19 were females while for the rest 7 patients the sex is unknown. Mutation status could be retrieved from a atotal of 23 patients andfrom these patients 16 did not have more than 5% mutations in their immunoglobulins genes (unmutated cases)while the other 7 had more than 5% mutations in their immunoglobulins genes (mutated cases)

Separation of mononuclear cells from tonsils

Tonsils were collected from healthy individuals undergoing routine tonsillectomies at Guy's Hospital after written consent. Tonsils were placed in a sterile Petri dish containing Complete Culture Medium and were minced using a 20 ml syringe plunger. Mononuclear cells were separated by density gradient. T lymphocytes were depleted using AET treated Sheep Blood Erythrocytes, and monocytes were depleted by adherence to plastic for 30 minutes at room temperature. The purity of normal B cells isolated was >90% as revealed by staining with anti-CD3 and anti-CD20 monoclonal antibodies and FACS analysis. A total of 9 normal donors participated in this study.

Flow cytometry analysis of the purified B cells:

10^6 isolated B cells (B-CLL or normal tonsillar) were washed once in PBS and were stained with anti-CD3 FITC or anti-CD20 antibodies at the volume recommended by the manufacturer in FACS staining solution (PBS plus 0.2% Bovine Serum Albumin-BSA) for 30 minutes on ice. Then cells were washed twice in FACS staining solution and unstained (control) or anti-CD3 stained cells were prepared for FACScan acquisition. Cells stained with anti-CD20 were further incubated with the appropriate dilution of anti-mouse IgG FITC antibody for 30 minutes on ice, washed twice in FACS staining solution and prepared for FACScan acquisition. A total of 10,000 live cells were acquired each time and analysis was performed using the Cell Quest programme.

Cell Lines :

Two Multiple Myeloma cell lines namely RPM18226, MM1S, two Burkitt's Lymphoma cell lines: Ramos and Daudi, two Normal Lymphoblastoid cell lines: AGLCL and WILCL and a pre- B cell line called Nalm-6 were included in this study. All above cell lines were grown in Complete Culture medium changed every 2-3 days.
The mouse hybridoma cell line G28.5 producing the anti-CD40 antibody was grown in RPMI Dutch modification supplemented with 10% Foetal Calf Serum, 1% Penicillin /Streptomycin(10,000 units each), 1% L-Glutamine(29 mg) and 1mM Sodium Pyruvate.

Surface cell markers measurement by FACS analysis

10^6 cells from AGLCL, WILCL, Ramos, MM1.S, Nalm-6 cell lines were harvested, washed once in PBS and stained with the volume of antibody recommended by the manufacturer of anti-CD20, anti-19A or anti-CD138 FITC, anti-CD27 FITC, anti-CD19 FITC, anti-CD38 FITC, anti-CD80 FITC in FACS staining solution for 30 minutes on ice. Cells were washed twice with FACS staining solution and prepared for FACScan acquisition. Cells stained with anti-CD20 and anti-19A were further incubated with the volume recommended by the manufacturer of anti-mouse IgG FITC antibody in FACS staining solution for 30 minutes on ice, washed twice in FACS staining solution and prepared for FACScan acquisition. A total of 10,000 live cells were acquired each time and analysis was performed using the Cell Quest programme.
For staining with anti-CD95 PE, 10^6 Ramos cells were stimulated with the appropriate volume of G28-5 supernatant (containing anti-CD40) corresponding to 0.25, 0.5, 0.75 and 1 μg/ml of anti-CD40 for 48h. Cells were then harvested, washed with PBS and

incubated with the recommended by the manufacturer volume of anti-CD95 PE for 30 min on ice. At the end of the incubation period cells were washed twice in FACS staining solution and prepared for FACScan acquisition. A total of 10,000 live cells were acquired each time and analysis was performed using the Cell Quest programme.

ELISA to determine the concentration of anti-CD40 antibody produced by the G28-5 hybridoma cell line :

Elisa was performed in 96 well plates in order to measure the concentration of the anti-CD40 antibody present in the supernatant of the G28.5 hybridoma cell line. Each well was coated with 200 µl of coating antibody (rat anti-mouse IgG1 Heavy Chains at 5 µg/ml) and incubated overnight at 4^0 C. The next day the wells were washed twice in PBS /0.1% Tween20 and were blocked with PBS/2% FCS for 1hour at 37^0 C. The plate was washed 3 times with PBS/0.1% Tween20 and incubated with mouse IgG1 □ (control antibody) diluted 1/20, 1/40, 1/80, 1/160, 1/320 in Culture Medium or with undiluted, 1/20, 1/40, 1/80, 1/160 dilutions of G28-5 supernatant diluted in Culture Medium and further incubated 90 minutes at 37^0 C. Then the plate was washed 3 times with PBS/0.1% Tween20, anti-mouse IgG Peroxidase was added at the dilution recommended by the manufacturer and the plate was further incubated for 1 hour at 37^0 C. Finally the plate was washed 5 times with PBS/0.1% Tween20 before the addition of 100 µl 2,2'-Azino-Bis(3-Ethylbenzthiazoline-6-sulfonic acid) liquid substrate system for Elisa. This was further incubated for 20 minutes at room temperature and absorbance of the final reaction was measured at 405nm using a Beckman DU530 model UV/VIS spectrophotometer.

Cell culture conditions for inhibition of apoptosis :

10^6 B-CLL cells were left unstimulated or were stimulated with IL-4 (15 ng/ml,), CD40L trimeric (1µg/ml) or anti-CD40 produced by the G28-5 hybridoma cell line at the same concentration, PMA (30nM) and their combinations: CD40+IL-4, PMA+IL-4 for 24hours and 48hours. At the end of the culture period apoptosis was determined using Annexin/Propidium Iodide and Propidium Iodide methods

For the dose response experiment cells were stimulated with 5, 10, 15 ng/ml of IL-4 and 0.5 and 1 µg/ml CD40L for 24h and 48h and apoptosis was measured using the Annexin/PI method.

For RNA extraction 1-5x10^7 B-CLL, tonsillar B cells or Ramos, AGLCL, WILCL, Nalm6, RPMI8226, MM1.S cells were left unstimulated or were stimulated with 15 ng/ml IL-4, 1 μg/ml CD40, 30 nM PMA, and their combinations for 1hour up to 4hours. At the end of the culture period cells were harvested, washed once in PBS and processed for RNA extraction and Northern Blot Hybridisation.

Cell culture conditions for induction of Apoptosis

10^6 cells B-CLL cells were left unstimulated or were stimulated with Rituximab at 20 μg/ml alone or in the presence of 3x (60 μg/ml) F(ab)2 anti-human IgG specific for Fcγ for 24h and 48h and apoptosis was assayed by Annexin/Propidium Iodide and Propidium Iodide methods. When Rituximab is used together with 3x (ab)2 anti-human IgG specific for Fcγ will be referred as XRituximab.

For the dose response experiment cells were incubated with 4, 10, 20 μg/ml of Rituximab alone or in the presence of 3x (12, 30, 60 μg/ml respectively) F(ab)2 anti-human IgG specific for Fcγ for 24hours and 48hours and apoptosis was measured using Annexin/Ppopidium Iodide (Anenxin/PI) and Propidium Iodide methods.

Additionally 10^6 cells from Ramos, AGLCL and WILCL cells line were stimulated with 20 μg/ml Rituximab alone or XRituximab for 24hours and 48hours and apoptosis was measured using the Propidium Iodide method.

For RNA extraction 1-5x10^7 B-CLL, tonsillar B, Ramos, WILCL cells were left unstimulated or were stimulated with 20 μg/ml Rituximab alone or XRituximab for 3hours. At the end of the culture period cells were harvested, washed once in PBS and processed for RNA extraction and Northern Blot Hybridisation.

Additionally 1x10^7 Ramos cells were treated with 0.5 M Calcium Ionomycin or 15ng/ml anti-IgM for 3hours. At the end of the culture period cells were harvested, washed once in PBS and processed for RNA extraction and Northern Blot Hybridisation.

Flow cytometry analysis of apoptosis :

1)Annexin/PI method: 10^6 cells were harvested, washed once in PBS and resuspended in 200 μl of staining buffer provided by the manufacturer. Then the cells were treated with 10 μl of Annexin and 15 μl of Propidium Iodide (PI) for 15 minutes (ApoAlert Annexin staining kit) in the dark. At the end of the incubation period, cells were immediately acquired on a FACScan Flow cytometer and analysed using the Cell Quest

Programme. Live cells appeared as Annexin$^-$ /PI$^-$, early apoptotic cells appeared as Annexin$^+$/PI$^-$, late apoptotic or necrotic cells appeared as Annexin$^+$/PI$^+$.

2)Propidium Iodide method: 10^6 cells were harvested and washed once in PBS. Then they were fixed in 70% Ethanol for 30minutes at 4^0 C and washed twice in PBS. Finally the cells were stained with 200 µl of Propidium Iodide (50 µg/ml) for 30 minutes in the presence of 50 µl RNAase A(100 µg/ml). At the end of the incubation period, cells were immediately acquired on a FACScan Flow cytometer and analysed using the Cell Quest Programme. Apoptotic cells were considered cells with subdiploid DNA content.

Inhibition of signal transduction pathways:

The inhibitors used targeted NF-□B (BAY117280, Gliotoxin), p38(SB202190, SB203580), JNK(SP600125), ERK1/2(U0126) and PI3K (LY294002) pathways.

For the apoptosis experiment 10^6 cells were treated with 10µM SB203580, 10µM SP600125, 10µM U0126 inhibitors for 1hour prior to the addition of 20 µg/ml of XRituximab and further incubated for 24hour. At the end of the incubation time cells were harvested, washed once with PBS and apoptosis was monitored using Annexin/PI or Propidium Iodide methods.

For RNA extraction and Northern Blot analysis 1-3x10^7 B-CLL cells as well as Nalm6, Ramos, WILCL, AGLCL, were cultured in the presence of 5 µM Gliotoxin, 10µM BAY117280, 10µM SB203580, 10µM SB2022190, 10µM SP600125, 10µM U0126, 10µM LY294002 for 1hour prior to the addition of either PMA, anti-CD40, XRituximab or Calcium Ionophore, anti-IgM only in Ramos cells and cells were further incubated for 1hour or 3hours. At the end of the culture period cells were harvested, washed once in PBS and processed for RNA extraction and Northern Blot hybridisation

Transformation of XL-1 Blue cells with pBS(+) plasmid containing the human Tis11 cDNA :

Half of the membrane containing the pBluescript(+) plasmid and Tis11 cDNA (kindly provided by Dr. Perry Blackshear) was soaked in 10 µl TE for at least 30 minutes at room temperature. 2µl of the eluted pBluescript(+) plasmid were added to 50µl XL1-Blue competent cells, heat shocked at 42^0 C for 90 sec followed by chilling for 1-2 min on ice before the addition of 200 µl of LBroth medium. The cells were incubated for 45 minutes at 37^0 C and were spread onto LBroth agar plates containing 50µg/ml ampicillin and further incubated at 37^0 C overnight. The next day the plates were

checked for growth of colonies. A total of 30 colonies were formed from which 5 were selected, inoculated in LBroth medium containing 50 μg/ml ampicillin and further incubated at 37^0C for 18 hours before the plasmid was purified using the Wizard® Plus SV Minipreps DNA purification system

Similarly cells transformed with pOTB7 plasmid containing the Tis11d cDNA as obtained from I.M.A.G.E. clones were inoculated in LBroth medium containing 27 μg/ml Chloramphenicol and incubated at 37^0C for 18 hours before plasmid was extracted using the Wizard® Plus SV Minipreps DNA purification system

Plasmid Purification /Mini Prep :

The pBluescript(+) plasmid containing the Tis11 cDNA, pBluescript(+) plasmid containing the Tis11b/Berg36 cDNA, pOTB7 plasmid containing Tis11d, pUC9 plasmid containing 5L3 were extracted from bacterial cells using the Wizard® Plus SV Minipreps DNA purification system following the instructions of the manufacturer (Promega). Bacterial cells containing the plasmid were grown in LBroth medium containing the appropriate antibiotic for 18h at 37^0 C and were harvested by centrifugation at 10,000xg for 5 minutes at room temperature. The bacterial pellet was resuspended in 250 μl Wizard® Plus SV Minipreps Cell Resuspension solution and lysed in 250 μl Wizard® Plus SV Minipreps Cell lysis solution. Then 10 μl of alkaline protease solution was added and the mixture was incubated for 5 minutes at room temperature. The mixture was neutralised with the addition of 350 μl Wizard® Plus SV Minipreps Neutralisation solution and spun at 14,000xg for 10 minutes at room temperature. The clear lysate was placed in a Wizard® Plus SV Minipreps Spin column and was spun for 1min at 14,000xg to remove the bacterial lysates. The Spin column was washed twice with 250 μl Wizard® Plus SV Minipreps wash solution and the plasmid DNA was eluted using 100 μl Nuclease free Water. The purified plasmid was stored at -20^0 C until further use.

Preparation of cDNA probes for labbeling with ^{32}P

All cDNA probes were prepared after restriction digestion of the plasmids containing each cDNA as an insert. Restriction digestion were carried out for 3h at 37^0C, stained with 1μg/ml of Ethidium Bromide and run on a 0.8% low melting point agarose in 1xTBE buffer at 100V for 45 minutes-1 hour. At the end of electrophoresis the gel was

visualised under UV and each cDNA insert was removed from the gel using a sterile scalpel and placed in a sterile eppendorf until further use.

The restriction digestion reactions set were:

For Tis11b/Berg36 a reaction was set up containing 16µl of nuclease free water,1 µl of purified pBluescript plasmid containing the Berg36/Tis11b cDNA, 1µl of EcoRI and 2µl of digestion buffer H.

For Tis11 cDNA (kindly provided by Dr. P.Blackshear) a reaction was set up containing 16µl nuclease free water, 1 µl of purified pBluescript plasmid containing the Tis11 cDNA, 1µl HindIII and 2µl buffer.E

For Tis11 cDNA(kindly provided by Dr. S Brooks) the reaction contained 16µl nuclease free water, 1µl of purified pBluescript plasmid containing the Tis11 cDNA, 1 µl EcoRI and 2 µl Buffer H.

For Tis11d cDNA the reaction contained 15µl nuclease free water, 1µl of purified pOTB7 plasmid, 1µl ECORI, 1µl XhoI and 2 µl of buffer D.

For 5L3 cDNA the reaction contained 16µl nuclease free water, 1µl of purified pUC9 plasmid DNA, 1µl EcoRI, and 2 µl of buffer H.

RNA Extraction:

1-5x10^7 of unstimulated and stimulated cells were lysed in 1ml TRI Reagent and placed at -80^0 C until further use. Immediately after thawing 0.2 ml of chloroform was added to the lysate, vortexed and centrifuged at 13,000 rpm to separate the mixture into 3 phases: a colourless aqueous phase containing the RNA, an interphase containing the DNA and a red organic phase containing the proteins. The colourless aqueous phase was transferred into a clean sterile eppendorf and washed with 1ml isopropanol. The RNA was precipitated using 1 ml of 75% Ethanol and the precipitated RNA pellet was left to dry at room temperature for 1-2 hours. Finally the pellet was dissolved in 0.1% DEPC (Diethylpyrocarbonate) treated water. This stock RNA was stored at –80^0 C until further use.

Quality assessment and concentration of the isolated RNA

Both the purity and the quantity of the extracted RNA were checked using a Beckman DU530 model UV/VIS spectrophotometer. Purity of the isolated RNA was checked by measuring the ratio of the absorbance at 260nm versus 280nm (260/280 ratio) which for pure RNA should be ≥1.8. The quantity of RNA for each sample was calculated based

on the fact that absorbance of 1 O.D.at 260 nm equals with 40μg/ml of RNA in solution. Thus the concentration of RNA present in each sample would be Ax40 μg/ml where A is the Absorbance of that particular sample at 260 nm.

Quality of the extracted RNA, was checked also in a standard 1% agarose gel to identify the presence of the ribosomal RNA (rRNA) as 2 discrete bands, the top one representing 28S rRNA and the second one the 18S rRNA. When there was no degradation present both bands are clearly visible and the 28S rRNA band is twice the thickness of the 18S rRNA band.

Northern Blot Hybridisation

i)Transfer of RNA onto a GeneSreen plus nylon membrane:

2.5-5 μg of isolated RNA from B-CLL or 10 μg of RNA from the cell lines was denatured with equal volume of denaturing mix (10% 10xMOPS, 78% deionised Formamide, 12%Formaldehyde) for 5 min at 65^0 C and chilled on ice for another 5 min. Then 5 μl of loading mix (50%Glycerol, 10mM EDTA pH 8.0, 0.25% Bromophenol Blue and 0.25% Xylene Cyanol FF) was added before loading into a 1% agarose gel containing 6% Formaldehyde and 10% 10xMOPS buffer. The gel was run at 100V for 1h in 1xMOPS buffer. At the end of electrophoresis the gel was stained with 1μg/ml Ethidium Bromide in 1xMOPS buffer for 30min. The RNA was transferred from the gel onto a nylon membrane (GeneScreen Plus), using 10xSSC overnight. The next day both the gel and the membrane were tested under UV to check whether the transfer was complete. The membranes were well sealed in thick Wattman paper and stored at room temperature until further use.

ii)Labelling of the cDNA probes using of ^{32}P:

All cDNA used in this study were labelled with ALL-IN-ONE Random Prime Labelling mix (-dCTP) from Sigma following the instructions of the manufacturer. Initially the low melting point section containing each probe was melted at 50^0C and 1 μl of this linear probe was diluted in 44μl TE and denatured at 95^0 C for 3minutes followed by cooling on ice for 2 minutes. These 45 μl of denatured probe were added in the random primed tubes followed by the addition of 5μl of ^{32}P –dCTP. The mix was incubated at 37^0 C for 15 minutes and the reaction was stopped with 0.2M EDTA. The successfully labelled probe was purified from the unlabelled one using the ProbeQuant™ G-50 Micro Columns according to the instructions from the manufacturer. Briefly the

columns were vortexed to activate the resin and were centrifuged at 2.000 rpm for 1min. Then the labelled probe was added and further centrifuged at 2.000 rpm for 2 minutes. A liquid scintillation counter was used to measure the activity (counts/min) of the labelled cDNA probe.

iii)Hybridisation of the probes/Posthybridisation washes:

The membranes were pre-hybridised in annealing solution (1M NaCl, 1.0% SDS, 10% Dextran Sulfate, 10mM HEPES pH 7.0, 10mg/ml Salmon Sperm DNA) for 5 hours at 60^0 C. Then they were hybridised with 10^6 counts/ml of each cDNA probe at 60^0 C for >16 hours. At the end of hybridisation the membranes were rinsed extensively in 2xSSC (30 sec washes) followed by 4 washes in 2xSSC for 20 min, followed by a single wash in 2xSSC/1.0% SDS at 60^0 C, followed by a single wash in 0.1xSSC at 60^0C. Finally the membranes were placed in clingfilm and exposed on autoradiography film for 1 to 3 days at -80^0 C.

During development, the film was left for 2-3 minutes in Kodak developer, rinsed for 1-2 min in Kodak fixative and well rinsed in tap water.

Intracellular staining for Tis11b/Berg36 protein and FACS analysis

10^6 B-CLL cells were left unstimulated or were stimulated with 30 nM PMA, 1μg/ml anti-CD40 or 20 μg/ml XRituximab for 3 hours at 37^0 C. At the end of the culture period cells were harvested, washed once in PBS and fixed in 1% paraformaldehyde for 30 min at room temperature. Cells were washed twice in PBS and permeabilised with 0.5% Tritox-X for 30 minuts at room temperature followed by blocking with 3% human IgG for 30 min at room temperature. Then they were washed once in PBS and Tis11b/Berg36 antisera at 1/10 dilution was added in 0.25% Triton-X in PBS in the presence of 3% human IgG and incubated overnight at 4^0C. Then next day cells were washed twice in PBS and stained with anti-rabbit FITC at the recommended by the manufacturer dilution. Finally cells were washed twice in PBS and prepared for FACScan acquisition. In all cases 10,000 live cells were acquired and analysed using the CellQuest program.

When intracellular staining was performed after siRNA treatment, 10^6 B-CLL cells were transfected using 5 μl HyperFect reagent and either 3 μl Tis11b/Berg36 siRNA or 3 μl of control siRNA. These cells were incubated for 48h before stimulation with 20 μg/ml XRituximab and further incubated for 3 hours at 37^0C. The rest of the procedure is as described above.

Confocal Microscopy for Tis11b/Berg36 protein localisation

10^6 cells were left unstimulated or were stimulated with 30nM PMA or 1μg/ml anti-CD40 or 20μg/ml Rituximab in the presence of 3x secondary antibody F(ab)2 anti-human IgG specific for Fc□for 3 hours at 37^0 C. At the end of the culture period cells were harvested and washed once with PBS. Then they were fixed in 2% paraformaldehyde for 30 minutes at room temperature, washed once with PBS and permeabilised with 0.5% Triton-X in PBS for 30 minutes at room temperature. Cells were washed once in PBS and were stained either 1/10 dilution of normal rabbit serum or 1/10 dilution anti-Tis11b rabbit serum in 5% Bovine Serum albumin in PBS overnight at 4^0C. The following day cells were washed twice in PBS and further incubated with anti-rabbit FITC antibody at the recommended by the manufacturer dilution for 1 hour at room temperature. Finally cells were stained with 200 μl Propidium Iodide (50μg/ml) for 20 minutes at room temperature, spotted on a microscope slide and mounted with a fluorescence mounting solution before analysis under the LEICA confocal microscope using the Leica DM SDK software. All images were captured using a Retiga EXi CCD camera and a 63x objective on immersion. Fluorochromes were excised using the 488nm-line of a Ar/Ark laser for FITC and the 543/594 nm-line of a HeNe laser for Propidium Iodide. The pinhole was set at 1 Airy Unit (AU).

Intracellular staining for Bcl-2 protein and FACS analysis

10^6 cells were left unstimulated or were stimulated with 20μg/ml XRituximab for 24h at 37^0 C. At the end of the culture period cells were harvested, washed once in PBS and fixed in 1% paraformaldehyde for 30 minutes at room temperature. The cells were then washed twice in PBS and permeabilised with 0.5% Triton-X for 30 minutes at room temperature. Then anti-Bcl-2 PE or isotype control PE was added at the recommended by the manufacturer dilution in FACS staining solution and incubated for 45 minutes on ice. Finally cells were washed twice in PBS and prepared for FACScan acquitition. In all cases 10,000 live cells were acquired and analysed using the CellQuest program.

Transfection of CLL cells with Tis11b/Berg36 siRNA:

The sequence of Tis11b/Berg36 siRNA was:sense: CAAGAUGCUCAACUAUAGUdT dT, antisense: ACUAUAGUUGAGCAUCUUGdT dT. The sequence of control siRNA provided by QIAGEN or Invitrogen (as BLOCK-IT control siRNA) is unknown.

Tis11b/Berg36 siRNA was transfected into cells using Electroporation, Lipofectamine 2000 (Invitrogen) or HiPerFect (Qiagen) transfection reagents following the instructions of the manufacturer or modifying them appropriately.

For electroporation 1-3x10^7 B-CLL cells were placed in a cuvette with 1μg of either Tis11b or control siRNA FITC (provided by Invitrogen). The cuvette was electroporated at 980V, 720 □ for 1-2 sec. Cell were immediately washed once in Complete Culture medium and resuspended in a total volume of 5 ml Complete Culture medium. The cells were incubated at 37^0 C for 24h, 48h before they were harvested, washed once in PBS and prepared for FACScan acquisition and analysis.

For transfection with Lipofectamine, 5x10^5 cells were plated in 24 well plates containing 500 μl of complete culture medium. Initially 1-3μl of Lipofectamine plus 1-3μl of Tis11b or control siRNA were mixed in OPTI-MEM medium without serum and incubated for 5 min at room temperature. Then this mixture was added to cells and transfection efficiency was monitored at t24h, t48h and t72h post transfection.

For transfection with HiPerFect reagent, 5-10x10^5 cells were resuspended in 0.5 ml of complete culture medium in 24 well plates. Then 5μl of HiPerFect was mixed with 60nM of either negative control siRNA or Tis11b siRNA in 100 μl of serum free RPMI1640 medium and incubated for 10 min at room temperature. The mixture was was added to the cells which were further incubated for 24h or 48h to determine transfection efficiency or were stimulated with XRituximab.

In all cases that XRituximab was included (transfection with Lipofectamine or HiPerFect) cells were transfected 24h or 48h before stimulation with 20μg/ml and apoptosis was monitored at 24h or 48h post stimulation as indicated using Annexin PE or Propidium Iodide methods.

Quantitative Real Time PCR

A two step quantitative Real Time PCR was performed. For this experiment the RNA from stimulated and unstimulated CLL cells was extracted using the RNAeasy Mini Kit (Qiagen) according to the manufacturer instructions. Briefly cells were lysed in RLT buffer and homogenised using a 20 gauge sterile needle. The homogenate was mixed with equal volume of 70% Ethanol, applied to the spin column provided and after two washes the extracted RNA was eluted with appropriate volume of RNAase free waster.

cDNA synthesis: The extracted RNA was quantified using the Nanodrop and 0.5 μg of RNA was processed for cDNA synthesis using the QuantiTect Reverse Transcription kit

(Qiagen). Initially the RNA was treated with gDNA wipeout buffer for 2 min at 42^0 C before the addition of 6 µl reaction mix containing Quantiscript Reverse Transcriptase, Quantiscript RT Buffer and RT primer mix. This mixture was incubated for 15 minutes at 42^0 C and the reaction was stopped by incubation at 95^0 C for 3 minutes.

Real Time PCR: Real-Time PCR was performed using the QuantiTect® SYBR® Green PCR kit from QIAGEN following the manufacturer instructions. The primers for Tis11b and β-Actin were designed using the Roche software and were: 5'-GATGACCACCACCCTCGT-3', Tis11b Reverse:5'-TGGGAGCACTATAGTTGAGCATC-3', β-Actin Forward: 5'-CCAACCGCGAGAAGATGA-3',β-ActinReverse:5'-CAGAGGCGTACAGGGATAG-3. A reaction was set up containing 12.5 µl 2x QuantiTect SYBR Green PCR Master Mix provided by the manufacturer, 1µl of each set of primers corresponding to 1µM final concentration, the appropriate amount of cDNA corresponding to 500 ng, and the appropriate volume of RNAase free water to a final volume of 25 µl per reaction. The reaction mixtures were placed in a 96 well plate and run on the ABI7000 system. The programme consisted of 10 min at 95^0C initial activation, then 40 cycles of 15 sec at 95^0C, 15 sec at 60^0C and 30 sec at 72^0 C. The specificity of the Tis11b and β-Actin primers product was verified by their melting curve analysis. The fold change was calculated according to Livak Method as described analytically in Chapter 3

Statistical Analysis:

All statistical analysis shown in the bar graphs was perormed using a one tailed paired student *t* test, apart from the experiment involving induction of sIgM after PMA stimulation in the presence or absence of Tis11b siRNA in which a two tailed paired student *t test* was used.

Sequencing of the 5L3 clone

5 µl of the pUC9 plasmid purified with the Wizard® Plus SV Minipreps DNA purification system was transferred on ice at Dr. Marsh's lab where sequencing with M13 primers was set up. The results obtained were visualised using the CHROMAS program. The rest of the analysis involved use of certain databases such as BLASTN (from EBI or NCBI), ExPASy, or TRANSLATE as analysed in the chapter 7.

CHAPTER 3

RITUXIMAB AND REGULATION OF TIS11 FAMILY CLL

3.1 INTRODUCTION

Rituximab is a monoclonal chimeric mouse/human IgG □1 antibody raised against the CD20 Receptor which is expressed only in B lineage and specifically on mature B cells (stem cells, pre-B and plasma cells do not express CD20) forming tetramers or multiple dimers. CD20 protein exists as 3 different isoforms of 33, 35 and 37 KDa that result from multiple phosphorylations on serine and threonine residues (Janas *et al* 2005). The CD20 Receptor has emerged as an ideal target for immunotherapy because it is expressed on >90% of B cell malignancies such as Follicular Lymphoma (FL) and the rest of Non Hodgkin's Lymphoma. A diagram of the CD20 receptor as presented on the cell surface is shown in Figure I3.1. Rituximab (anti-CD20 antibody) binds between residues K142 and Y184 in the extracellular loop and this depends on the presence of a disulfide bond between C167-C183 (Ernst *et al* 2005).

Initially several antibodies were raised against the CD20 Receptor such as 1F5, B1, 11B8, AT80, KT20, 2F2, 7D8 as well as Rituximab (Chan *et al* 2003, Teeling *et al* 2004). In comparison with most of them, Rituximab has the advantage of bearing human constant regions (Fc□) instead of mouse ones making it a more efficient activator of complement and also less immunogenic. In general all the anti-CD20 antibodies are subdivided into Type I (Rituximab and most of the anti-CD20 antibodies) which are potent inducers of Complement Dependent Cytotoxicity (CDC) but weak inducers of apoptosis and Type II (B1 and 11B8) which are potent inducers of apoptosis but weak activators of complement (Teeling *et al* 2004). Both Type I and Type II antibodies are equally efficient in inducing Antibody Dependent Cell Cytotoxicity (ADCC) (Teeling *et al* 2004).

Rituximab has been used as a therapeutic agent in several patients including low grade Non Hogkin's Lymphoma (NHL) and CLL. Parameters measured following treatment were: the Overall Response Rate (ORR), Complete Remission (CR) and time to progression (TTP) which were better after multiple administrations compared to a single administration of Rituximab (Davis *et al* 2000, Ghielmini *et al* 2004). It should be noted that Rituximab treatment can induce cell death to all CD20 positive cells (normal or malignant) but normal B cells will re emerge after few months due to constant haematopoiesis from the bone marrow. Recovery of normal B cells has been shown to

correlate with the levels of SDF-1 (Stromal Derived Factor 1) in serum of previously treated patients (Dunleavy *et al* 2005).

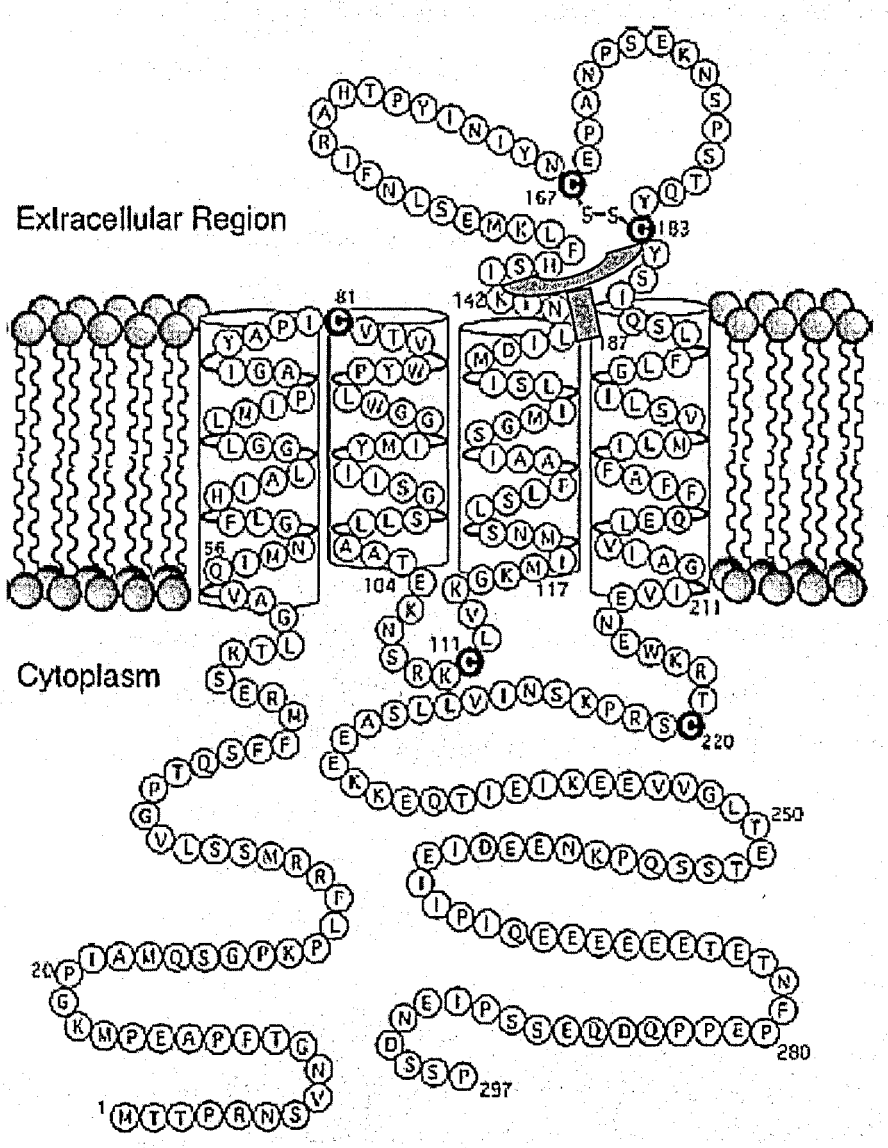


Figure I3.1: CD20 receptor on the B cell surface
This photo has been taken from Ernst *et al* 2005, *Bichemistry,* **44,** pp:15151 while Rituximab binding shown as  has been added by the author .

In CLL the standard dose of Rituximab (375 mg/m^2) and increased doses of 500, 650, 825, 1000, 1500, 2.250 mg/m^2 were tested. The same study included MCL (Mantle Cell Lymphoma) and B-PLL (B Pro Lymphocytic Leukaemia) patients (O'Brien et al 2001). The ORR achieved with the standard dose was 36% which increased to 75% by Rituximab at 2.250 mg/m^2 (O' Brien *et al* 2001). The response rates were better in Rai stage I and II than in Rai stages III and IV (O'Brien *et al* 2001). Later on Rituximab was combined with standard chemotherapeutic agents such as Fludarabine and CHOP which further increased the response rates. In combination with Fludarabine the CR was 70-83% in low grade NHL, (Cruczman *et al* 2005) while in CLL the CR achieved with this combination at 43 months was 33% (Byrd *et al* 2003b) or 70% primarily in ZAP-70 negative patients (Del Poeta *et al* 2005). When Byrd *et al* evaluated progression free survival for two clinical trials involving Fludarabine alone and Fludarabine combined with Rituximab, they found significantly better ORR, CR and progression free survival in combination therapy when compared with single Fludarabine therapy evaluated at 6 years post treatment (Byrd *et al* 2005). In combination with CHOP (Cyclophosphamide, Doxorubicin, Vincristine, Prednisone) the ORR was 100% with 58% being CR in low grade and follicular lymphoma (Cruczman *et al* 2004). Apart from B cell malignancies Rituximab has been used to treat autoimmune disorders such as Rheumatoid Arthritis (RA) and Systemic Lupus Erythomatosus (SLE) (Chambers and Isenberg 2005).

3.1.1 Mechanisms of apoptosis induction by Rituximab

Rituximab can induce apoptosis but requires cross-linking with a secondary antibody for *in vitro* studies (van der Kolk *et al* 2002, Pedersen *et al* 2002a, Shan *et al* 1998). Induction of apoptosis by Rituximab was evident as early as 4-8h in Ramos cells and was accompanied by activation of caspase 9, caspase 3 and PARP cleavage (van der Kolk *et al* 2002). Similarly another study showed apoptosis induction without changes in Bcl-2 and Bax protein levels (Shan *et al* 2000). In contrast to these studies Rituximab alone (no cross-linking) was able to downregulate Bcl-2 protein in two NHL cell lines derived from AIDS patients (Alas *et al* 2001) and this was correlated with inhibition of IL-10 secretion and downregulation of p38 and STAT3 pathways (Alas *et al* 2001, Vega *et al* 2004).

On the other hand in CLL it was shown that cross-linked Rituximab induced apoptosis at 24 hours and from the signalling pathways activated (ERK, p38 and JNK) only p38 MAPK pathway was involved in induction of apoptosis (Pedersen *et al* 2002a). Interestingly when a specific inhibitor of the p38 pathway was utilised the effect of Rituximab was only partially abrogated (Pedersen *et al* 2002a) suggesting that other signalling events/pathways may also be involved. Apart from these *in vitro* studies there is evidence of apoptosis induction by Rituximab *in vivo* because administration of Rituximab in CLL patients resulted in caspase 9 and caspase 3 activation as well as PARP cleavage 24 hours from the initial administration of Rituximab (Byrd *et al* 2002). This was accompanied by downregulation of Mcl1 and XIAP anti-apoptotic proteins (Byrd *et al* 2002).

3.1.2 Signal transduction pathways activated by Rituximab

Rituximab binding induces redistribution of CD20 into lipid raft microdomains (rich in cholesterol, sphingomyelin and other lipids), activation of Src kinases such as Lyn and subsequent activation of signal transduction pathways such as PLCγ, ERK, p38, JNK etc as well as calcium mobilisation (Hofmeister *et al* 2000, Shan *et al* 2000, Deans *et al* 1998, Deans *et al* 1993). Redistribution of CD20 into Triton-X insoluble lipid rafts in Ramos cells depended on the presence of cholesterol (Janas *et al* 2005). Caspase 3 and 7 activities were reduced to background levels when the cells were treated with Rituximab in the presence of a cholesterol inhibitor suggesting that induction of apoptosis by Rituximab is dependent on redistribution into lipid rafts. Because there was no direct association of caspase 3 with lipid rafts it was proposed that other proteins link redistribution of CD20 into lipid rafts and activation of caspase 3 (Javas *et al* 2005). Activation of Src kinases or further downstream events such as activation of p38 (Pedersen et al 2002) may mediate this link since inhibition of these events inhibit apoptosis induction by Rituximab.

AIM OF THE STUDY: was to investigate how Tis11 family members are modulated following Rituximab treatment in CLL cells. Provided that one or more members were changed aim of the study was to identify the mechanisms of this regulation and possible targets of this family following Rituximab treatment.

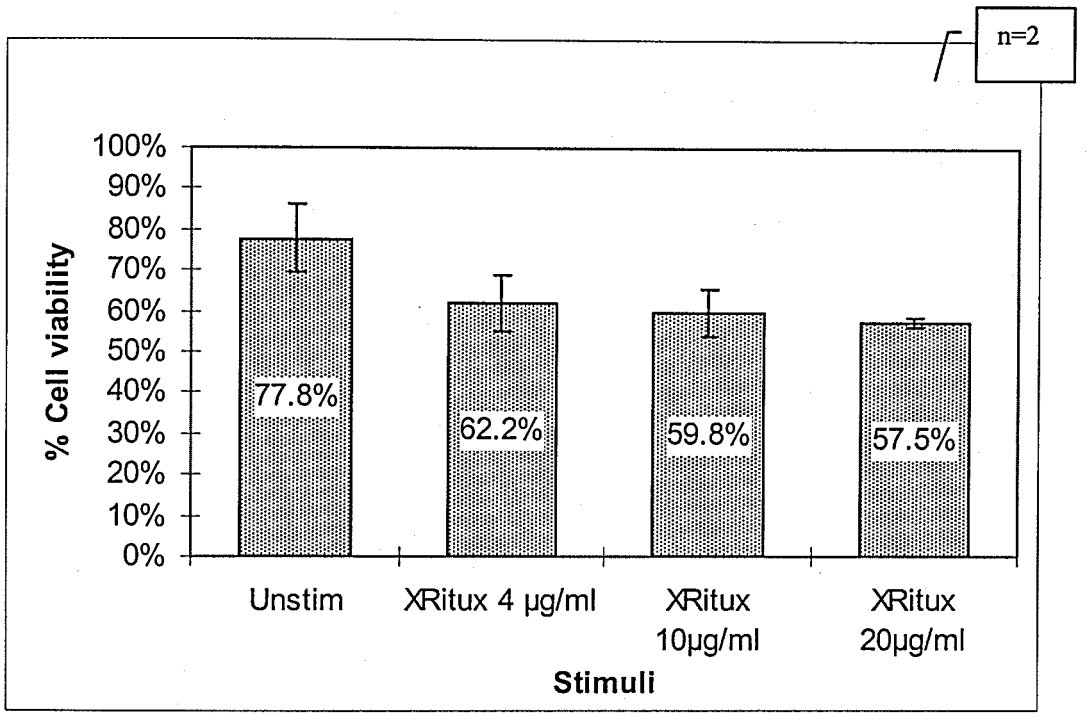
3.2 RESULTS

3.2.1 INDUCTION OF APOPTOSIS

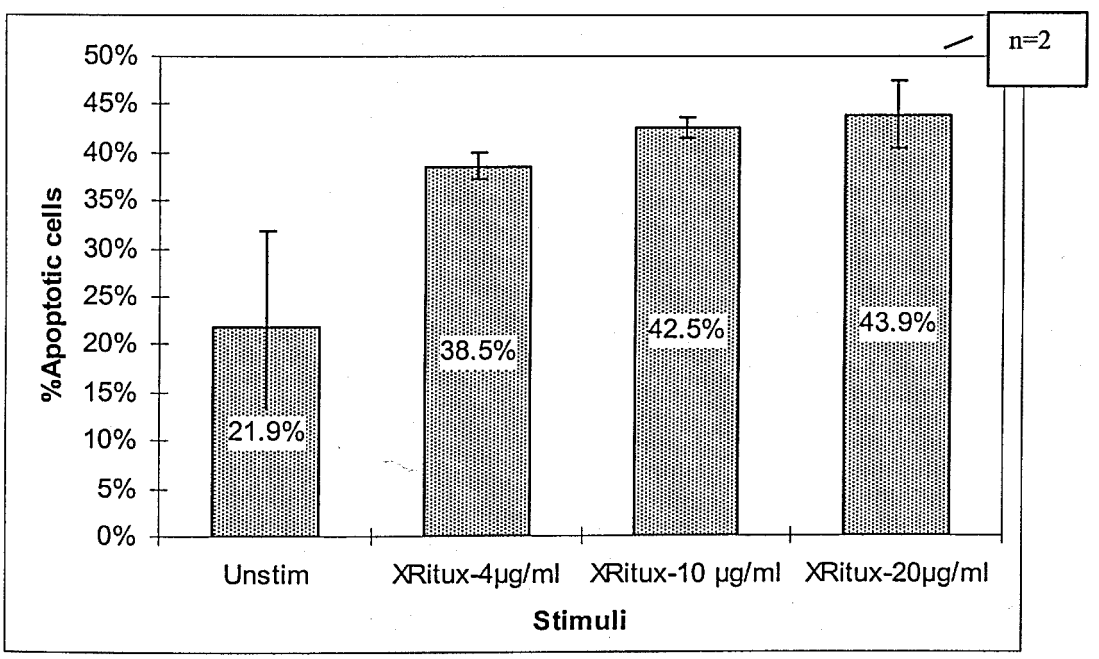
Rituximab, a chimeric anti-human CD20 used to treat NHLs, CLL or Hairy Cell Leukaemia patients, was utilised to induce apoptosis in B-CLL cells. For the first 2 patients 3 different doses of Rituximab were tested (4, 10, 20 μg/ml) either alone or in combination with a secondary antibody [F(ab)2 of anti-human IgG specific for Fcγ] at a dose 3x the dose of Rituximab (12, 30 and 60 μg/ml respectively) and apoptosis was monitored at 24 hours and 48 hours using the Annexin/PI and Propidium Iodide (PI) methods. When Rituximab is used in combination with the secondary antibody will be referred as XRituximab (cross-linked Rituximab).

Dose response experiment: According to the Annexin/PI method the cell viability of unstimulated cells at 24 hours was 77.8%±8.0% (mean, range: 69.8%-85.8%) and was reduced to 62.2%±7.0% (mean, range: 55.2%-69.2%, $p \leq 0.02$) by XRituximab at 4μg/ml. When the dose of XRituximab was increased to 10μg/ml, cell viability was further reduced to 59.8%±6.0 % (mean, range: 53.8%-65.8%, $p \leq 0.03$) and further escalation of XRituximab to 20 μg/ml reduced cell viability to 57.5%±1.5% (mean, range: 56%-59%, $p \leq 0.12$). Thus according to this method the most effective dose of Rituximab at 24 hours is 10 μg/ml (Figure 3.1a).

Similarly according to the PI method, the percentage of apoptotic cells in unstimulated cells at 24 hours was 21.9%±10.0% (mean, range 11.9%-31.9%) which was increased by 4μg/ml XRituximab to 38.50%±1.0% (mean, range: 37.5%-39.5%) and further increased to 42.5%±1.0% (mean, range: 41.5%-43.5%) by XRituximab at 10 μg/ml. Increasing the dose of XRituximab to 20 μg/ml increased apoptosis to 43.9% ±3.5% (mean, range: 40.4%-47.4%). According to this method the most efficient dose of XRituximab was the 20 μg/ml (Figure 3.1b).



a)



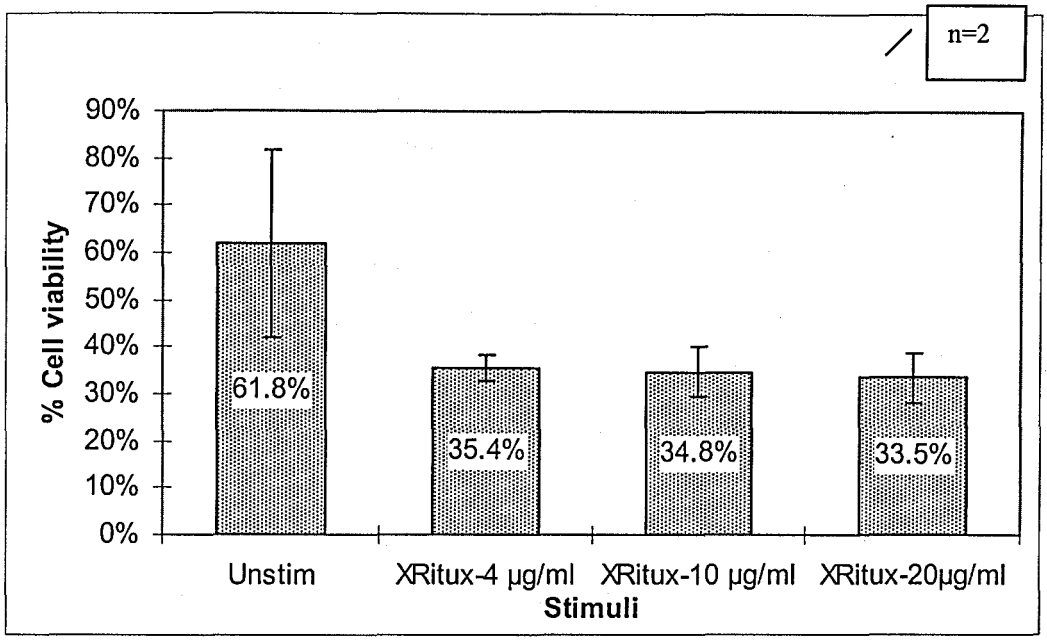
b)

Figure 3.1: Dose response experiment for XRituximab at 24 hours postimulation
Freshly isolated MNC from 2 CLL patients were left untreated (unstimulated) or were treated with Rituximab at 4 µg/ml,10 µg/ml and 20 µg/ml in the presence of 3x F(ab)2 goat anti-human IgG antibody (XRituximab) and apoptosis was monitored at 24 hours from the start of the culture period using **(a)** Annexin/PI and **(b)** and PI methods. When the Annexin /PI method is used the percentage of Live cells (Annexin$^-$/PI$^-$) are plotted whereas when the PI method is used the percentage of apoptotic cells (cells with subdiploid DNA content) is plotted. In each graph the mean±mean difference was plotted In the centre of each bar the mean percentage of a)live cells or b) apoptotic cells is shown.

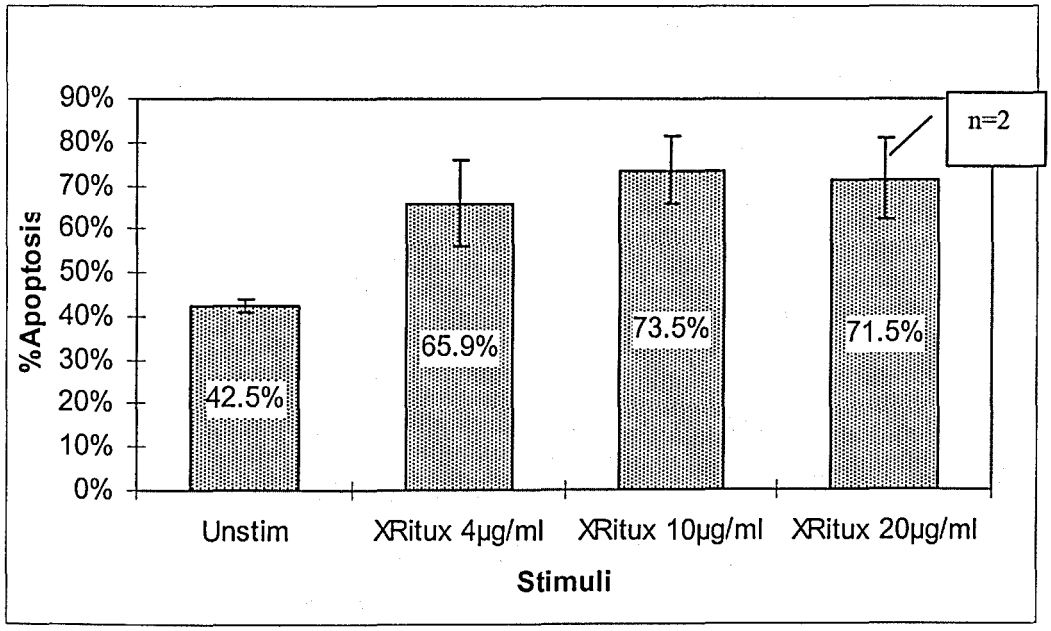
At 48 hours from the start of the culture period, according to the Annexin/PI method the cell viability of unstimulated cells was 61.84%±20.0%(mean, range: 41.84%-81.84%) which was reduced to 35.39%±3.0%(mean, range: 32.39%-38.39%) by XRituximab at 4μg/ml while XRituximab at 10μg/ml reduced cell viability to 34.75%±5.0% (mean, range: 29.75%-39.75%). Finally XRituximab at 20 μg/ml reduced viability to 33.5%±5.5%(mean, range: 28%-39%)(Figure 3.2a).

According to the PI method at the same timepoint the percentage of apoptotic cells in unstimulated cells was 42.5%±1.5%(mean, range: 41%-44%) which was increased to 65.9%±10% (mean, range: 55.9%-75.9%) by XRituximab at 4 μg/ml and further increased to 73.45%±8.0%(mean, range: 65.45%-81.45%) by XRituximab at 10 μg/ml. Increasing the dose of XRituximab at 20 μg/ml increased the percentage of apoptosis to 71.5%±9.0% (mean, range: 62.5%-80.5%)(Figure 3.2b).

Treatment of B-CLL cells from these 2 patients with secondary antibody only minimally induced apoptosis in B-CLL cells at an insignificant level XRituximab and was exactly the same for all 3 different doses tested (data not shown).



a)



b)

Figure 3.2: Dose response experiment for XRituximab at 48 hours postimulation
Freshly isolated MNC from 2 CLL patients were left untreated (unstimulated) or were treated with Rituximab at 4 µg/ml ,10 µg/ml and 20 µg/ml in the presence of 3x F(ab)2 goat anti-human IgG antibody (XRituximab) and apoptosis was monitored at 48 hours from the start of the culture period using **(a)** Annexin/PI and **(b)** and PI methods. When the Annexin /PI method is used the percentage of Live cells (Annexin$^-$/PI$^-$) are plotted whereas when the PI method is used the percentage of apoptotic cells (cells with subdiploid DNA content) is plotted. In each graph the mean ±mean difference was plotted In the centre of each bar the mean percentage of Live cells **(a)** or apoptotic cells **(b)** is shown.

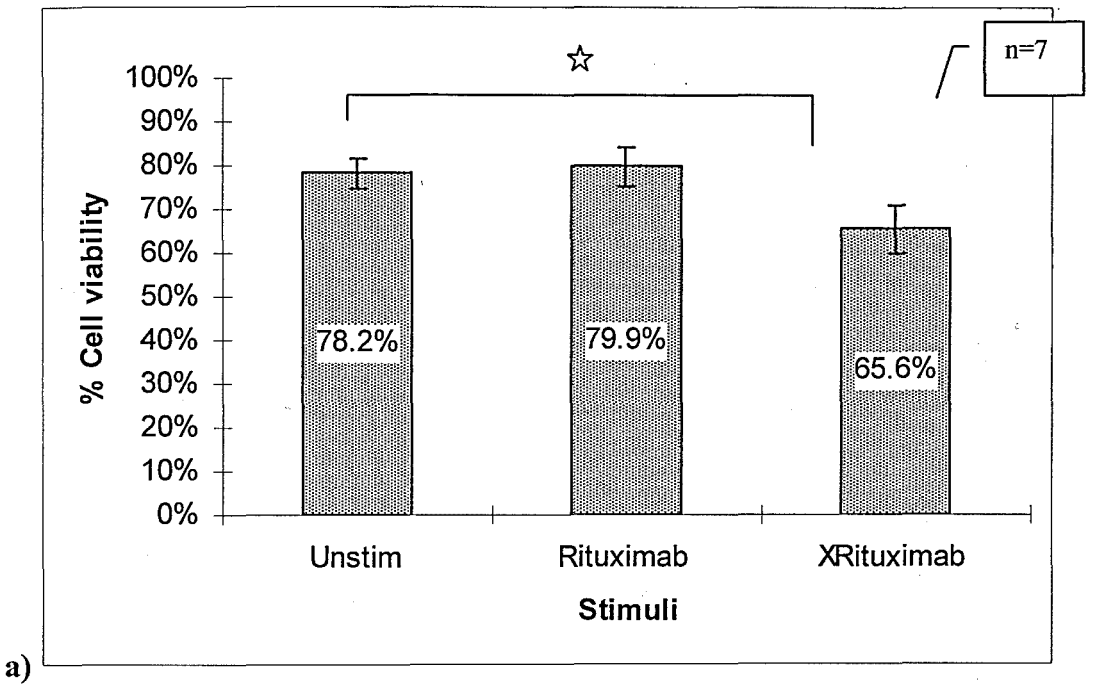
3.2.2 Induction of apoptosis by Rituximab at 20μg/ml:

In the subsequent experiments it was decided to use Rituximab at 20μg/ml since according to the dose response experiment is more effective in inducing apoptosis at 24 hours post stimulation and was also the dose utilised by Mathas *et al*, to study Tis11b/Berg36 (Mathas *et al* 2000). T lymphocytes were depleted using AET treated sheep blood erythrocytes while monocytes were depleted by adherence to plastic for 30 min. The purity of the B-CLL population was tested by staining for surface CD20 (specific for B cells) and CD3 (specific for T cells) expression. It was found that the purity of the isolated B-CLL cells was between 90% and 95% (Appendix 1).

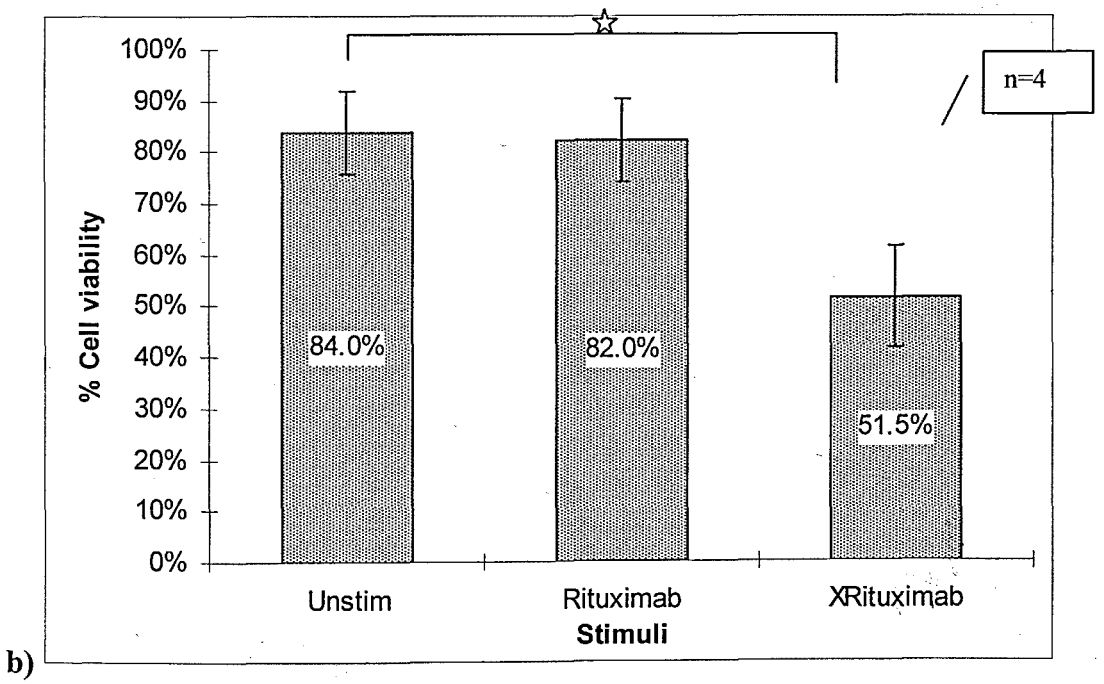
A total of 9 CLL patients were tested at this stage using 20μg/ml Rituximab alone or in the presence of 3x the secondary antibody (XRituximab) and apoptosis was monitored again at 24 and 48 hours post treatment using the Annexin/PI and PI methods.

According to the Annexin/PI method the cell viability of unstimulated B-CLL cells at 24 hours was 78.2%±3.5% (mean±sem) which was not significantly affected by Rituximab alone (79.9%±4.4, mean±sem $p \leq 0.25$) but was significantly reduced to 65.6%± 5.7%(mean±sem) ($p \leq 0.01$) by XRituximab (Figure 3.3a). Results from two representative patients are shown in Figure 3.4.

At 48 hours XRituximab was even more efficient in inducing apoptosis. According to the Annexin/PI method the percentage of cell viability in unstimulated cells was 84.0%±8.0% (mean±sem). Rituximab alone was unable to induce apoptosis (82.0%±8.0%, mean±sem, $p \leq 0.43$) but XRituximab significantly reduced cell viability to 51.5%±10.0% (mean±sem, $p \leq 0.04$) (Figure 3.3b). Data from two representative patients are shown in Figure 3.5.



a)



b)

Figure 3.3: Effect of XRituximab at 20 μg/ml at 24 or 48 hours post stimulation as assayed by the Annexin/PI method in B-CLL cells

Freshly isolated B-CLL patients were left unstimulated or were treated with Rituximab at 20 μg/ml in the presence of 3x F(ab)2 goat anti-human IgG antibody (XRituximab) and the percentage of cell viability ($Annexin^-/PI^-$) was measured by Annexin/PI at **a)**24hours and **b)**48hours after the initiation of the culture period. In each graph the mean ±sem of cell viability is plotted and error bars represent sem (sem=STDEV/√n where STDEV is Standard Deviation and n is the number of the patients tested). The presence of a star indicates statistical significance with $p \leq 0.05$.

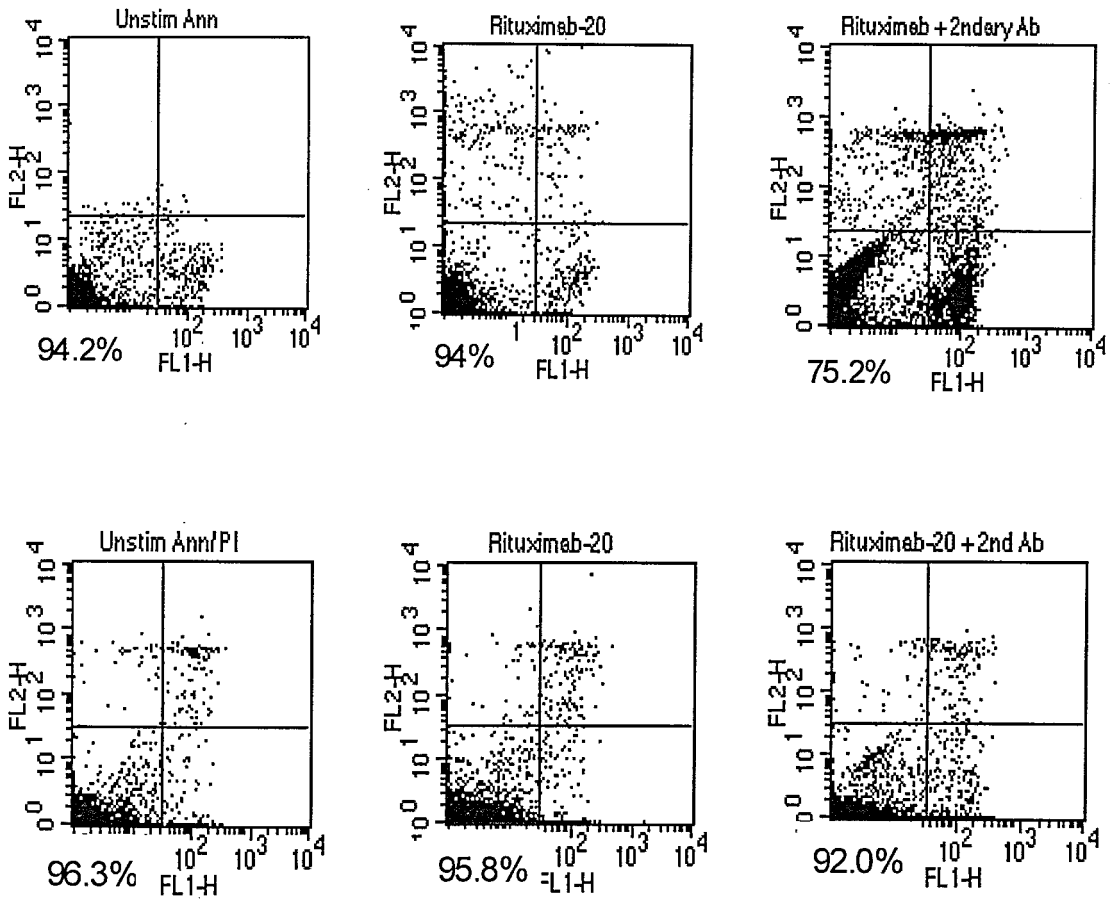


Figure 3.4: Primary data about the effect of XRituximab at 20 μg/ml at 24 hours post stimulation as assayed by the Annexin/PI method in B-CLL cells
B-CLL cells from CLL23 (top 3 quandrants) and from CLL25 (bottom 3 quandrants) were left unstimulated or were stimulated with 20 μg/ml Rituximab alone or crosslinked with 60 μg/ml of F(ab)2 goat anti-human IgG antibody and apoptosis was monitored at 24 hours post stimulation using the Anenxin/PI method. Note the significantly higher number of cells present in lower right (Annexin$^+$ cells) and upper right (Annexin$^+$/PI$^+$ cells) quadrants in XRituximab treated cells compared with unstimulated cells or Rituximab alone treated cells. Top 3 quandrants from the left to the right represent unstimulated cells, Rituximab treated cells and Rituximab+ F(ab)2 anti-human Fcγ specific secondary antibody (XRituximab) treated cells. Bottom 3 quandrants from the left to the right represent unstimulated cells, Rituximab treated cells and Rituximab+ Fab2 anti-human Fcγ specific secondary antibody (XRituximab) treated cells.

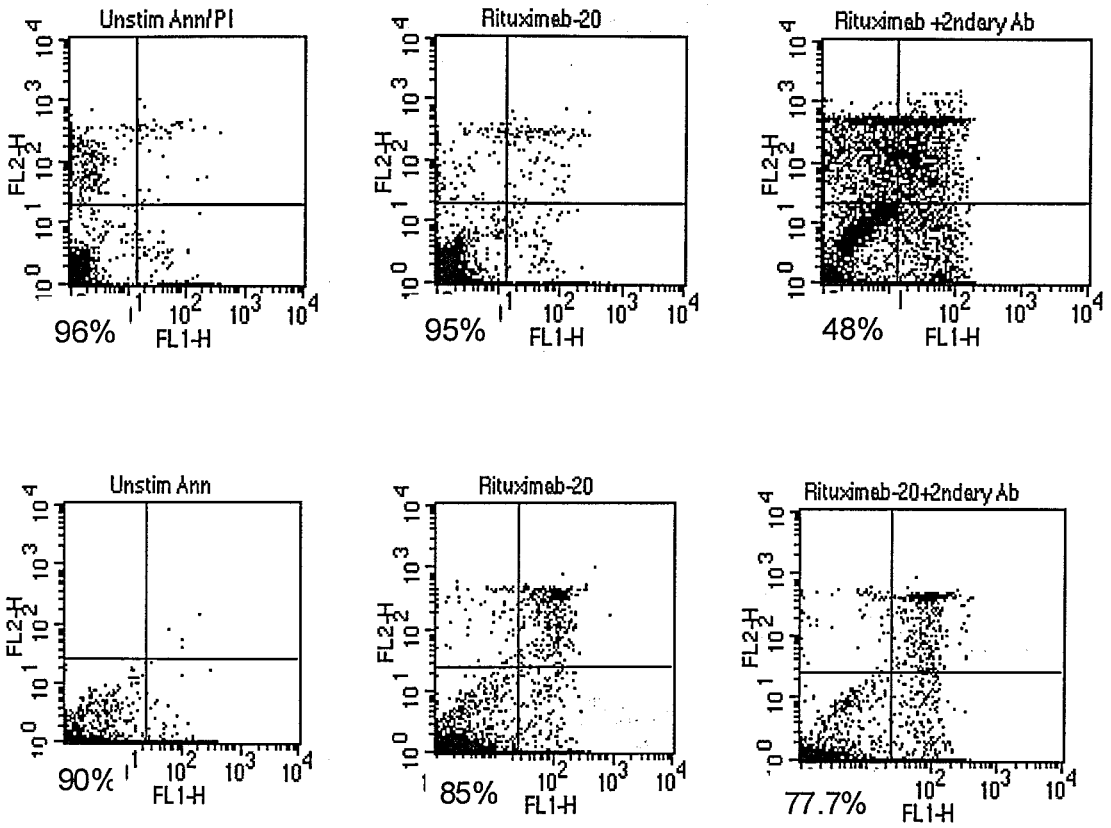


Figure 3.5: Primary data about the effect of XRituximab at 20 μg/ml at 48 hours post stimulation as assayed by the Annexin/PI method in B-CLL cells
B-CLL cells from CLL23 (top 3 quandrants) and from CLL25 (bottom 3 quandrants) were left unstimulated or were stimulated with 20 μg/ml Rituximab alone or crosslinked with 60 μg/ml of $F(ab)_2$ goat anti-human IgG antibody and apoptosis was monitored at 48 hours post stimulation using the Anenxin/PI method. Top 3 quandrants from the left to the right represent unstimulated cells, Rituximab treated cells and Rituximab+ $F(ab)_2$ anti-human Fcγ specific secondary antibody (XRituximab) treated cells. Bottom 3 quandrants from the left to the right represent unstimulated cells, Rituximab treated cells and Rituximab+ $F(ab)_2$ anti-human Fcγ specific secondary antibody (XRituximab) treated cells.

Similarly according to the PI method, at 24 hours from the initiation of the culture period, the percentage of apoptotic cells in unstimulated cells was 24.0%±5.0% (mean ± sem) and remained unchanged by Rituximab alone (18.0%±4.0%, mean±sem) but increased to 31.0%±4.0% (mean±sem, $p \leq 0.1$) by XRituximab (Figure 3.6a). Data from two representative patients are shown in Figure 3.7.

At 48 hours according to the same method, XRituximab increased the percentage of apoptotic cells from 35.0% ± 8.0% (mean±sem) in unstimulated cells to 63.0%±8.0% (mean±sem). This effect was found to be highly significant with $p \leq 0.001$. Rituximab alone was again ineffective (32.0%±8.2%, mean±sem) (Figure 3.6b). Data from two representative patients are shown in Figure 3.8.

An interesting finding revealed by the PI method was the fact that Rituximab alone and sometimes XRituximab as well affected the cell cycle progression of B-CLL cells causing a G2 arrest and sometimes an induction of S and G2 phases. This was seen by an increase in the presence of cells with tetraploid DNA content (M3 gate in the histograms shown in Figure 3.7 or 3.8). However it should be pointed that these cells with tetraploid DNA content may be doublets which may have arisen from two different cells on which Rituximab has bound crosslinked by the secondary antibody rather than actual proliferating cells.

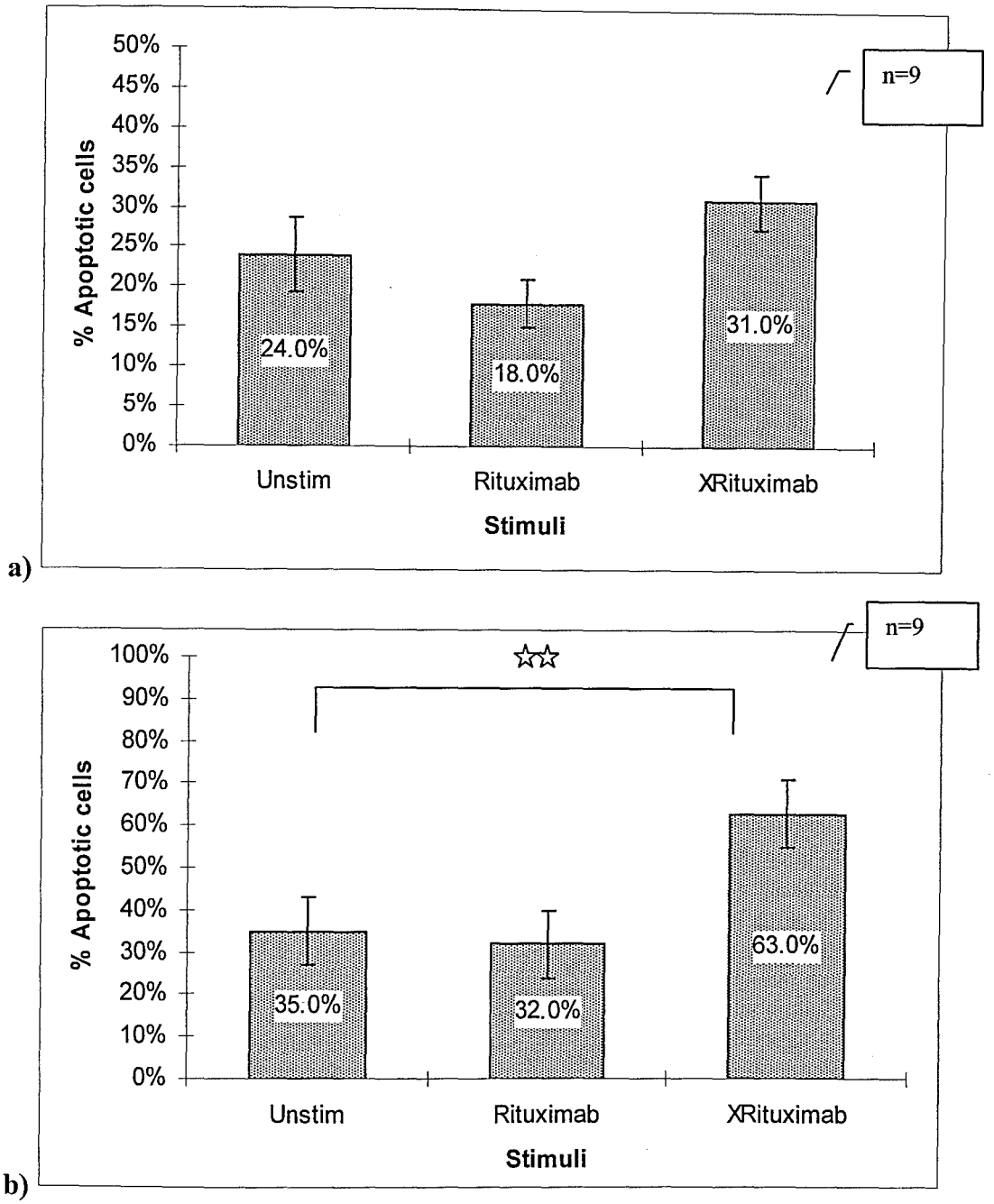


Figure 3.6: Effect of XRituximab at 20 μg/ml at 24 or 48 hours post stimulation as assayed by the Propidium Iodide (PI) method

Freshly isolated B-CLL from 9 CLL patients were left unstimulated or were stimulated with Rituximab at 20 μg/ml in the presence of 3x $F(ab)_2$ goat anti-human IgG antibody (XRituximab) and the percentage of apoptotic cells were measured by the PI method at **a)**24 hours and **b)**48 hours after the initiation of the culture period. In each graph the mean ±sem for each of the stimuli is plotted and error bars represent sem (sem=STDEV/√n where STDEV is Standard Deviation and n is the number of the patients tested). In the centre of each bar the mean percentage of apoptotic cells is shown and the presence of a star indicates statistical significance with $p \leq 0.05$ whereas the presence of two stars indicates statistical significance with $p \leq 0.001$.

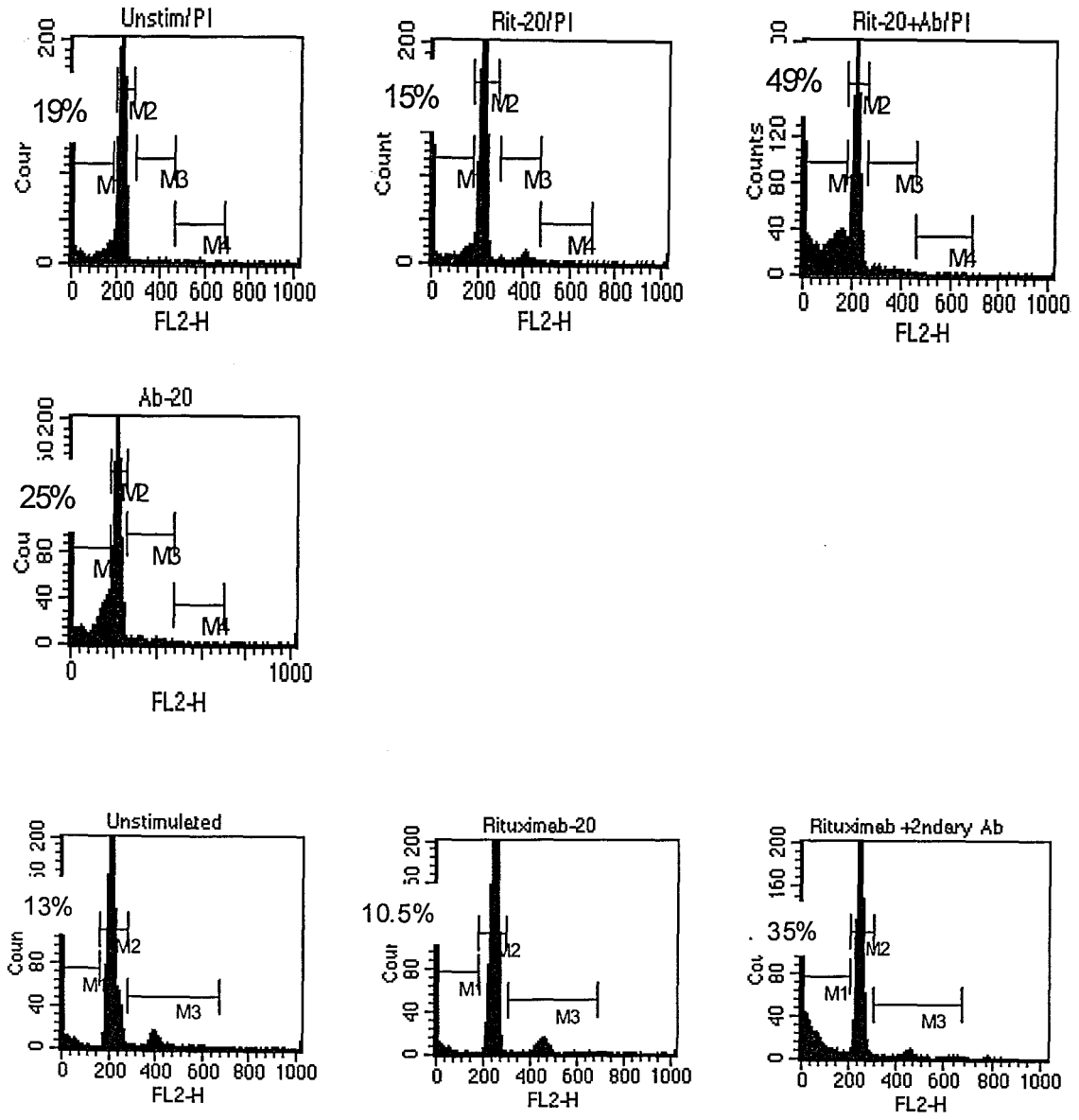


Figure 3.7: Primary data about the effect of XRituximab at 20 μg/ml at 24 hours post stimulation as assayed by the Propidium Iodide (PI) method in B-CLL cells
B-CLL cells from CLL18 (top 4 histograms) and from CLL24 (bottom 3 histograms) were left unstimulated or were stimulated with 20 μg/ml Rituximab alone or crosslinked with 60 μg/ml of F(ab)2 goat anti-human IgG antibody and apoptosis was monitored at 24 post stimulation using the PI method. The percentage of apoptotic cells is shown above the M1 gate. Top 4 histograms from left to the right represent unstimulated cells, Rituximab treated cells and Rituximab+ F(ab)2 anti-human Fcγ specific secondary antibody (XRituximab) treated cells. Single histogram in the middle of the page represents B-CLL cells from CLL18 treated with F(ab)2 goat anti-human IgG antibody at 60 μg/ml. Bottom 3 histograms from left to the right represent unstimulated cells, Rituximab treated cells and Rituximab+ F(ab)2 anti-human Fcγ specific secondary antibody (XRituximab) treated cells.

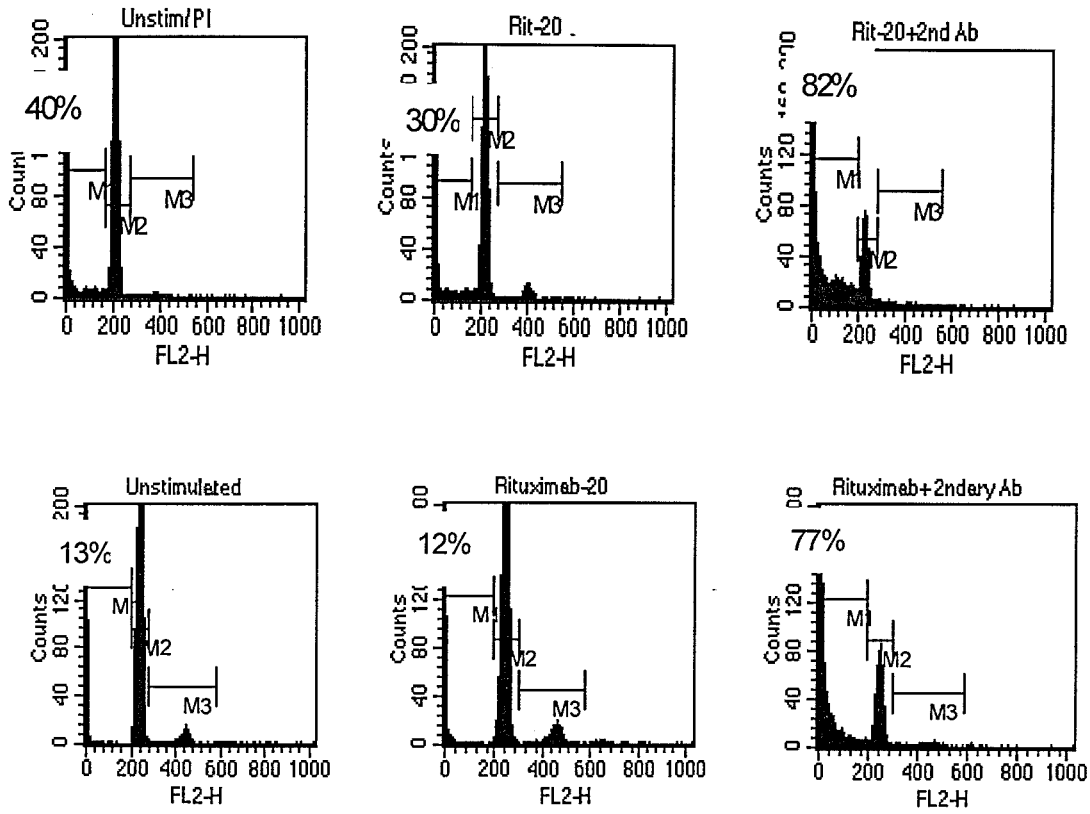


Figure 3.8: Primary data about the effect of XRituximab at 20 μg/ml at 48 hours post stimulation as assayed by the Propidium Iodide (PI) method in B-CLL cells
B-CLL cells from CLL18 (top 3 histograms) and from CLL24 (bottom 3 histograms) were left unstimulated or were stimulated with 20 μg/ml Rituximab alone or crosslinked with 60 μg/ml of $F(ab)_2$ goat anti-human IgG antibody and apoptosis was monitored at 48 hours post stimulation using the PI method. The percentage of apoptotic cells is shown above the M1 gate. These are data from 2 representative patients from a total of 9 patients tested. Top 3 histograms from left to the right represent unstimulated cells, Rituximab treated cells and Rituximab+ $F(ab)_2$ anti-human Fcγ specific secondary antibody (XRituximab) treated cells. Bottom 3 histograms from left to the right represent unstimulated cells, Rituximab treated cells and Rituximab+ $F(ab)_2$ anti-human Fcγ specific secondary antibody (XRituximab) treated cells.

Because a significant variation was found in response to XRituximab at 24 hours or 48 hours whatever the method used to determine apoptosis induction, it was decided to check whether this variation would correlate with surface CD20 expression of WBC as an indicator of the disease's aggressiveness. However there was no linear correlation between the levels of surface CD20 expression and efficiency of XRituximab (Figure 3.9a), (PEARSON correlation coefficiency was -0.14). When the total White Blood Count (WBC) was correlated with efficiency of XRituximab it was found that cases with WBC >90 $x10^9$/L responded better than cases with lower WBC. The correlation coefficient for the two parameters was +0.64 (according to PEARSON) but still this correlation coefficiency can not suggest linear correlation (Figure 3.9b)

Finally when the levels of spontaneous apoptosis were correlated with efficiency of XRituximab, an inverse correlation was found (the lower the levels of spontaneous apoptosis the better the response to Rituximab) (Figure 3.9c). The correlation coefficient calculated for these two parameters was -0.83 and according to PEARSON correlation of -1 indicates negative or inverse correlation between two parameters.

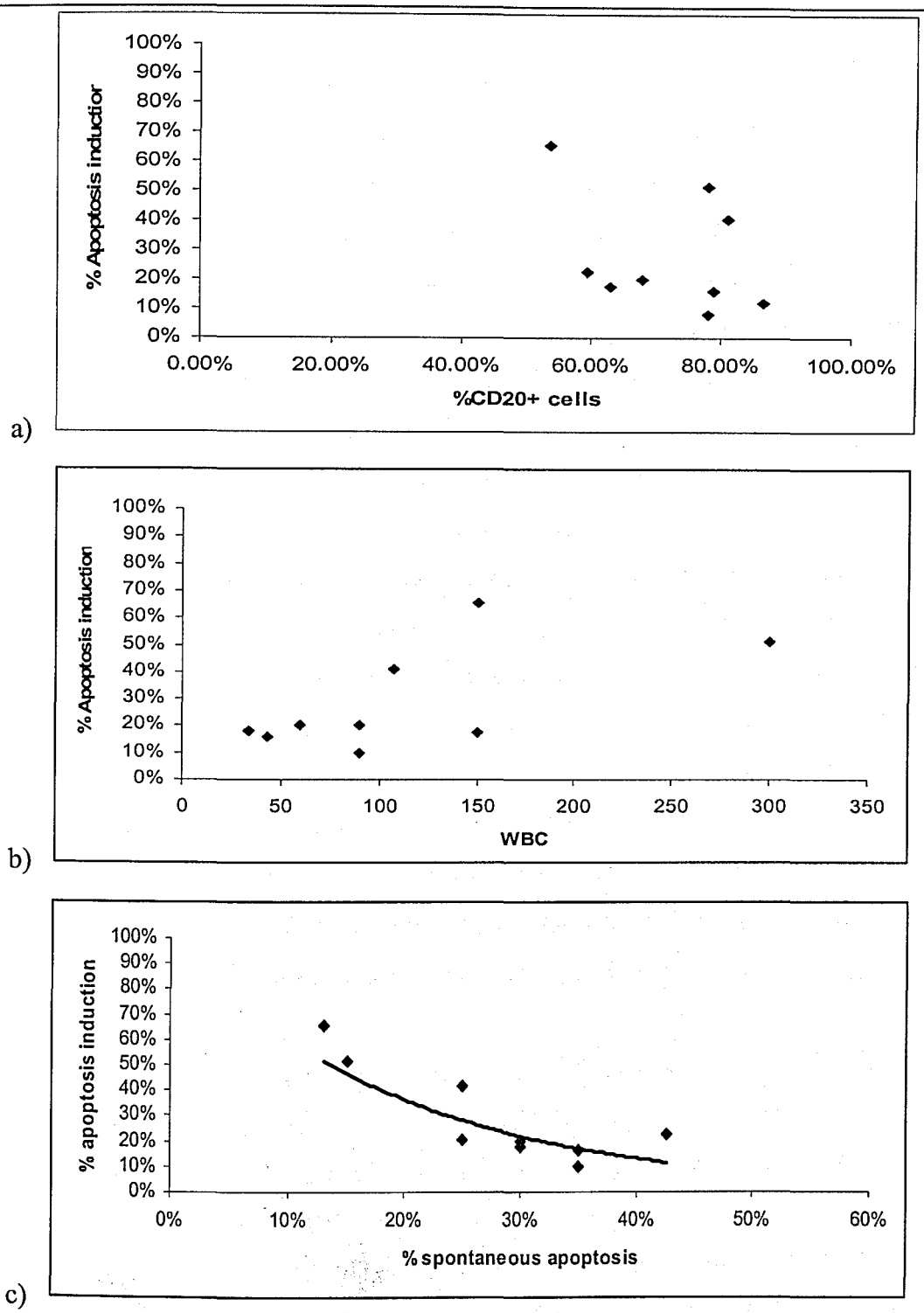


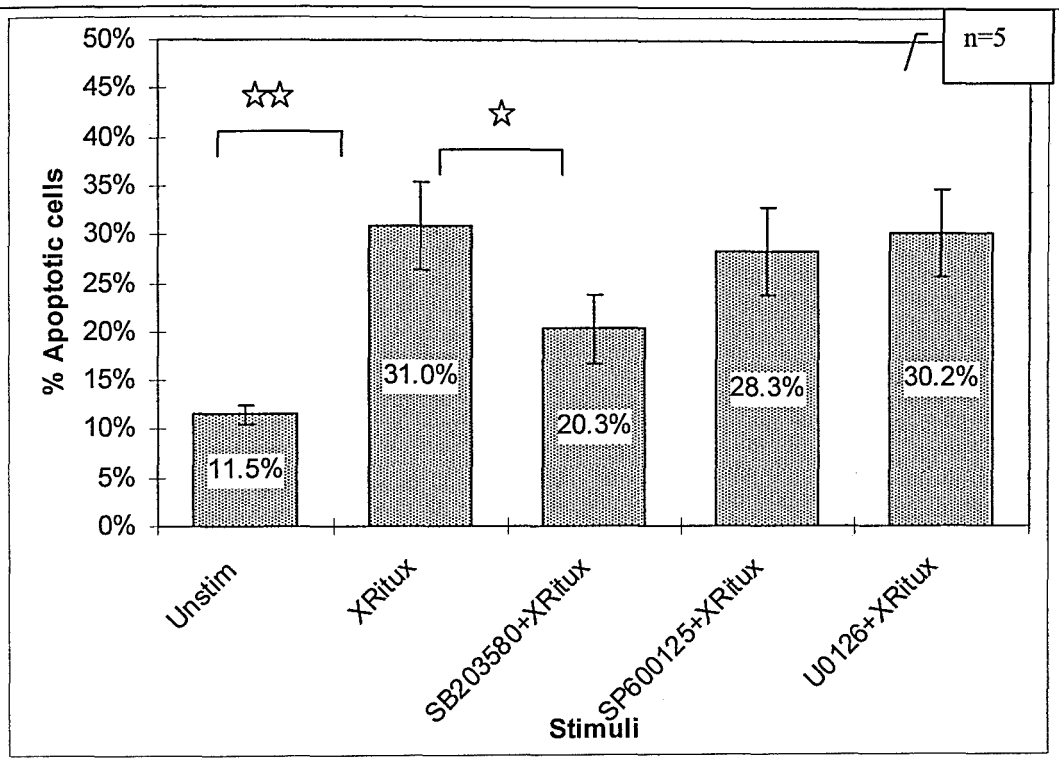
Figure 3.9: Correlation between surface CD20 expression, White Blood Count (WBC) and spontaneous apoptosis with response to XRituximab treatment in B-CLL cells. The a)Surface expression of CD20, b) WBC and c) Spontaneous apoptosis of CLL patients under test were correlated with the levels of apoptosis induction by XRituximab after 48 hours post stimulation. Apoptosis induction was calculated as percentage of apoptosis in XRituximab treated cells at 48 hours -percentage of apoptosis in unstimulated cells at 48 hours (spontaneous apoptosis). There is a inverse/ negative correlation between spontaneous apoptosis and effectiveness of XRituximab.

3.2.3 Inhibition of signalling pathways and effect on Rituximab induced apoptosis

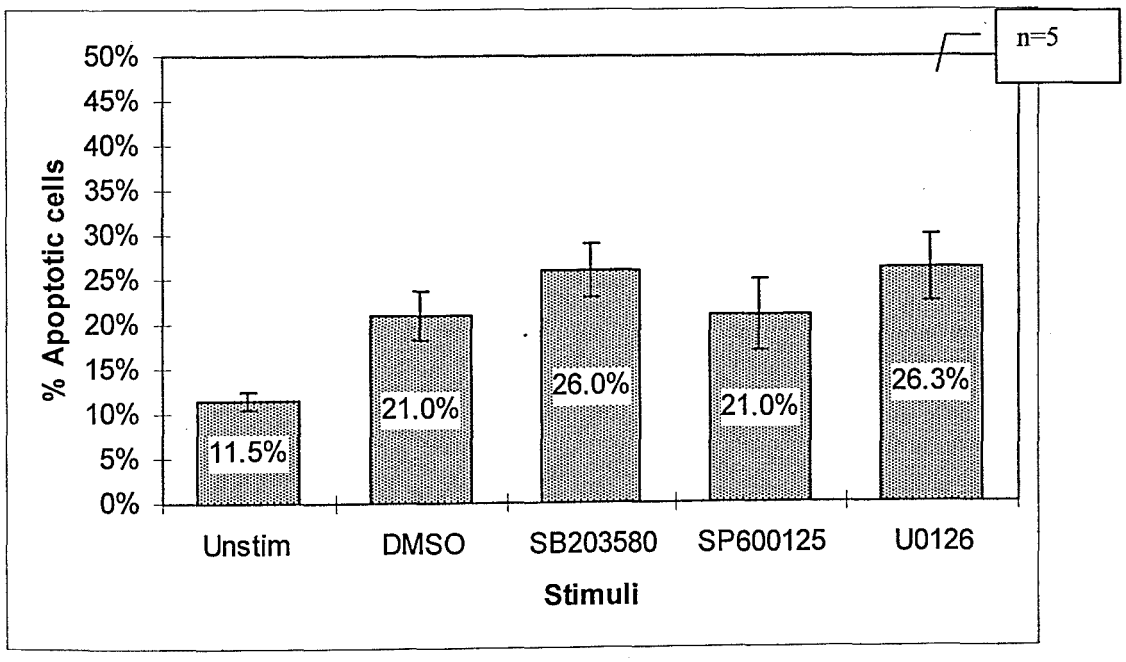
It has been previously shown (Pedersen et al 2002a) that treatment of B-CLL cells with XRituximab induced phosphorylation of ERK, JNK and p38 signalling pathways and that the p38 pathway was partially responsible for induction of apoptosis by Rituximab. Because in the above study a JNK inhibitor was not tested for possible involvement in Rituximab induced apoptosis, it was hypothesised that JNK could be the other pathway that mediates the pro-apoptotic effects of XRituximab in CLL patients. Thus B-CLL cells were treated with XRituximab for 24h in the presence of 3 inhibitors namely SP600125 (specific for JNK), SB203580 (specific for p38) and U0126 (specific for ERK1/2). Induction of apoptosis was monitored mainly using the PI method.

At 24 hours the mean percentage of apoptosis in unstimulated cells was 11.5%±1.0% (mean±sem) and was significantly increased to 31.0%±4.5% by XRituximab (mean±sem, $p \leq 0.003$). In the presence of SB203580 the mean percentage of apoptosis was 20.3%±3.50% (mean±sem, $p \leq 0.01$) while in the presence of SP600125 it was 28.3%±4.5% ($p \leq 0.26$). Finally in the presence of U0126 the mean percentage of apoptosis was 30.2%±4.50% (mean±sem, $p \leq 0.47$)(Figure 3.10a). These data strongly suggest that JNK even though it showed some effect, this did not reach statistical significance and that p38 is the only pathway known involved in induction of apoptosis by XRituximab. Data from two representative patients are shown in Figure 3.11a and 3.11b.

In order to exclude cytotoxicity caused by DMSO which is the dilutent for all the inhibitors used in this experiment, the same CLL patients were treated in parallel with DMSO alone at the maximal volume present in the inhibitors, SB203580 alone, SP600125 alone and U0126 alone at the concentration used in the XRituximab experiment (10 μM) for 24hours and apoptosis was monitored with the PI method. It was found indeed that DMSO was cytotoxic for the cells. Furthermore it was found that inhibition of the basal activity of p38 and ERK pathways further increased the mean percentage of spontaneous apoptosis in B-CLL cells while the cytotoxicity noticed for SP600125 is probably due to the presence of DMSO and not due to inhibition of the JNK pathway (Figure 3.10b).



a)



b)

Figure 3.10: Effect of signal transduction pathways inhibition on apoptosis induction by XRituximab in B-CLL cells

B-CLL cells were left unstimulated or were stimulated with:a) 20 μg/ml XRituximab in the presence or absence of inhibitors for p38 (SB203580), JNK (SP600125) and ERK1/2 (U0126) pathways or b)DMSO, SB203580, SP600125 and U0126 inhibitors for 24hours. Apoptosis was monitored with the PI method. In each graph the mean ±sem is plotted and error bars represent sem (sem=STDEV/$\sqrt{n}$ where STDEV is Standard Deviation and n is the number of the patients tested). In the centre of each bar the mean percentage of apoptotic cells is shown and the presence of a star indicates statistical significance with $p \leq 0.05$.

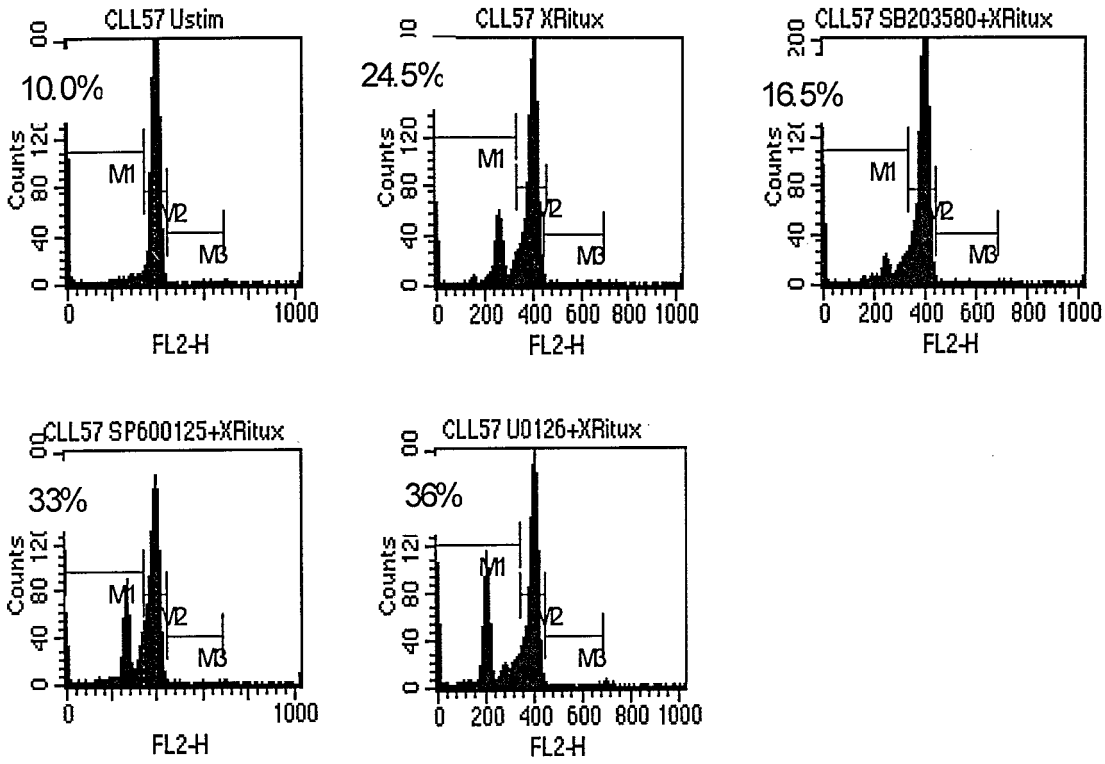


Figure 3.11a: Effect of inhibition of signal transduction pathways on apoptosis induction by XRituximab in CLL cells.
B-CLL cells from CLL57 were left unstimulated or were stimulated with XRituximab at 20 μg/ml in the presence or absence of 10 mM SB203580 (p38 specific inhibitor), SP600125 (JNK specific inhibitor) or U0126 (ERK1/2 specific inhibitor) as indicated above each histogram. At the end of the 24 hours culture period apoptosis was monitored using the PI method. The percentage of apoptotic cells is indicated in each histogram above the M1 gate. Top 3 histograms from left to the right represent unstimulated cells, XRituximab treated cells, and XRituximab+SB203580 while bottom 2 histograms from the left to the right represent XRituximab+SP600125 treated cells and XRituximab+U0126 treated cells.

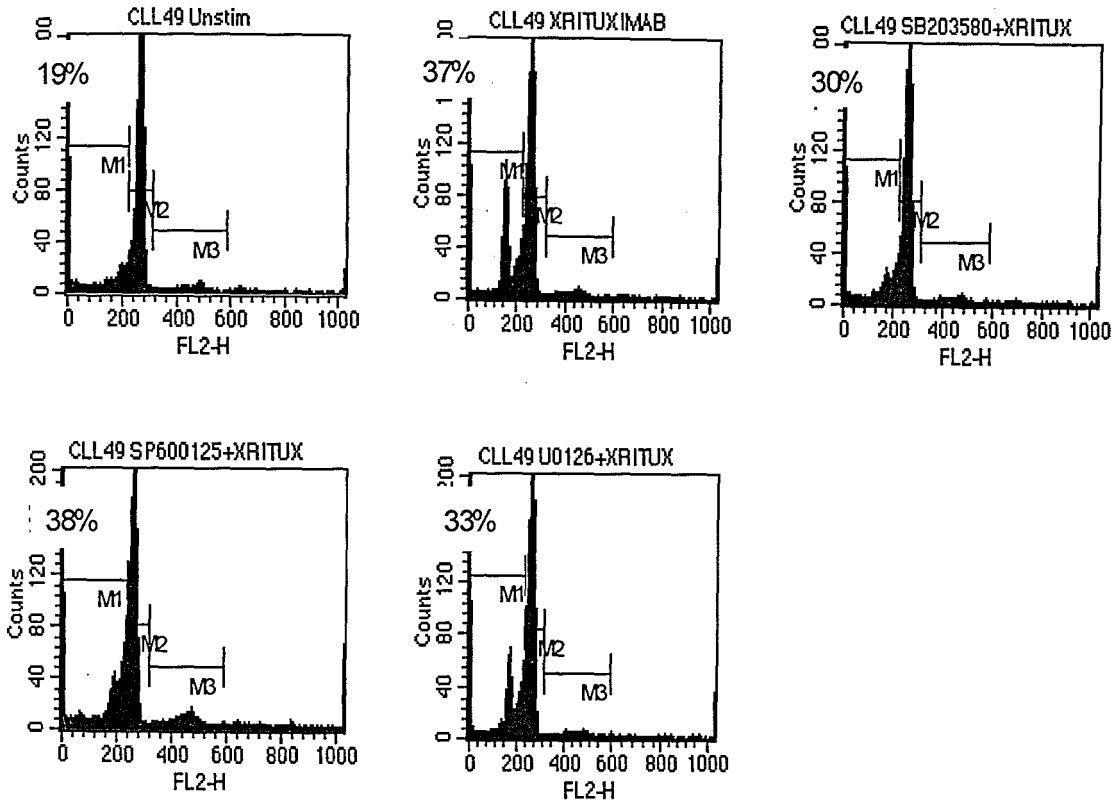


Figure 3.11b: Effect of inhibition of signal transduction pathways on apoptosis induction by XRituximab in CLL cells.
B-CLL cells CLL49 were left unstimulated or were stimulated with XRituximab at 20 μg/ml in the presence or absence of 10 mM SB203580 (p38 specific inhibitor), SP600125 (JNK specific inhibitor) or U0126 (ERK1/2 specific inhibitor) as indicated above each histogram. At the end of the 24 hours culture period apoptosis was monitored using the PI method. The percentage of apoptotic cells is indicated in each histogram above the M1 gate. Top 3 histograms from left to the right represent unstimulated cells, XRituximab treated cells, and XRituximab+SB203580 while bottom 2 histograms from the left to the right represent XRituximab+SP600125 treated cells and XRituximab+U0126 treated cells.

3.2.4 Rituximab treatment and induction of the Tis 11 family genes

Because previous work has shown that Tis11b/Berg36 was induced by immobilised Rituximab in BL60-2 (Mathas *et al* 2000) it was interesting to test whether XRituximab would induce the same gene in B-CLL cells. In order to do so, B-CLL cells were left untreated or were treated with XRituximab for 3h and expression of Tis11b/Berg36 mRNA was tested by Northern Blot. It was found that in unstimulated cells (To), Tis11b/Berg36 mRNA basal levels were low or undetectable and treatment with XRituximab strongly induced expression of this gene (Figure 3.12). Rituximab alone was also able to induce the gene but this induction was weaker in comparison with XRituximab treatment. Because it has been reported that Tis11 is induced by serum stimulation, B-CLL cells were also cultured for 3h in culture medium containing 10% FCS (Foetal Calf Serum) but no induction of Tis11b/Berg36 mRNA was seen at 3h. The latter was very critical for proper recovery of cells post separation.

In order to test whether Rituximab is able to induce all 3 Tis11 family genes, Northern Blot analysis was performed for Tis11 and Tis11d at 3h post stimulation with Rituximab alone or XRituximab. It was found that Tis11 was strongly expressed in unstimulated cells (basal expression) and remained unchanged in most of the patients tested or was weakly downregulated in some of them (Figure 3.13).

Finally Tis11d was also strongly expressed in unstimulated cells (basal expression) and was induced by XRituximab in some but not all patients tested (Figure 3.14). The magnitude (fold) of Tis11d mRNA induction was lower in comparison to Tis11b/Berg36 mRNA induction. Change in Tis11d was noticed in patients with relatively low basal expression of this gene.

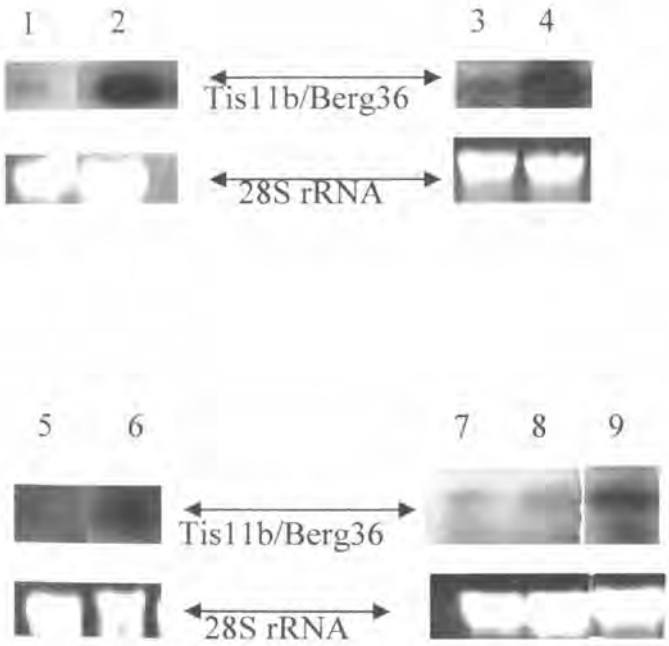


Figure 3.12: Northern Blot analysis of Tis11b/Berg36 expression in B-CLL cells: Freshly isolated B-CLL cells were processed for RNA extraction immediately after purification (To) or were stimulated with Rituximab alone or with XRituximab for 3h. At the end of the culture period RNA was extracted and processed for Northern Blot Hybridisation using a ^{32}P labelled cDNA probe for Tis11b/Berg36. Consistency of RNA loading as indicated by 28S rRNA staining is shown below each membrane. The arrows indicate either the Tis11b/Berg36 mRNA specific band detected by the radiolabelled probe or the 28S rRNA band present per lane.

Lanes:

1) CLL33 To(unstimulated cells)
2) CLL33 XRituximab 3h
3) CLL29 To(unstimulated cells)
4) CLL29 XRituximab treated cells
5) CLL38 To (unstimulated cells)
6) CLL38 XRituximab 3h
7) CLL40 To(unstimulated cells)
8) CLL40 serum 3h
9) CLL40 XRituximab 3h

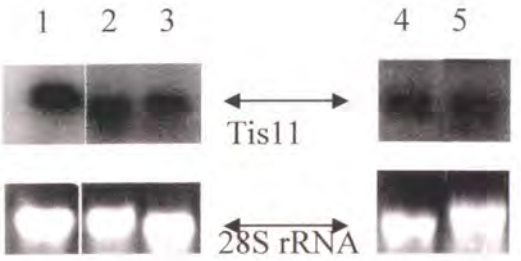


Figure 3.13: Northern Blot analysis of Tis11 expression in CLL :
Freshly isolated B-CLL cells were processed for RNA extraction immediately after purification (To) or were stimulated with Rituximab alone or with XRituximab for 3h. At the end of the culture period RNA was extracted and processed for Northern Blot Hybridisation using a ^{32}P labelled cDNA probe for Tis11. Consistency of RNA loading as indicated by 28S rRNA staining is shown below each membrane. The arrows indicate either the Tis11 mRNA specific band detected by the radiolabelled probe or the 28S rRNA band present in each lane.
Lanes:
1) CLL18 To(unstimulated cells)
2) CLL18 Rituximab 3h
3) CLL18 XRituximab 3h
4) CLL29 To(unstimulated cells)
5) CLL29 XRituximab 3h

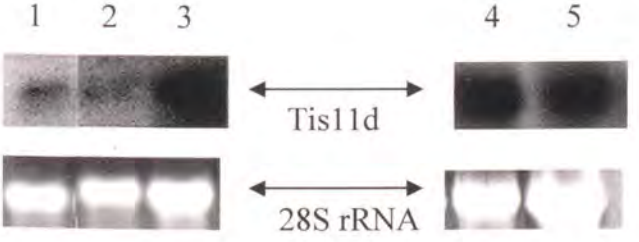


Figure 3.14: Northern Blot analysis of Tis11d expression in CLL :
Freshly isolated B-CLL cells were processed for RNA extraction immediately after purification (To) or were stimulated with Rituximab alone or with XRituximab for 3h. At the end of the culture period RNA was extracted and processed for Northern Blot Hybridisation using a ^{32}P labelled cDNA probe for Tis11d. Consistency of RNA loading as indicated by 28S rRNA staining is shown below each membrane. The arrows indicate either the Tis11 mRNA specific band detected by the radiolabelled probe or the 28S rRNA band present in each lane.
Lanes:
1) CLL20 To(unstimulated cells)
2) CLL20 Rituximab 3h
3) CLL20 XRituximab 3h
4) CLL23 To (unstimulated cells)
5) CLL23 XRituximab 3h

3.2.5 Regulation of Tis11b/Berg36 and Tis11d genes

In order to study the pathways involved in induction of Tis11b/Berg36 and Tis11d, B-CLL cells were cultured for 3h with XRituximab in the presence or absence of inhibitors specific for p38, JNK and ERK pathways at 10 μM.

It was found that p38 pathway is primarily involved in the regulation of Tis11b/Berg36 mRNA following XRituximab treatment since inhibition of this pathway resulted in partial or complete abrogation of Tis11b/Berg36 following XRituximab treatment. Inhibition ERK1/2 pathway did not affect induction of the gene (Figure 3.15) while inhibition of JNK was also found to be involved in the regulation of the gene but not as strongly as the p38 pathway.

Similarly Tis11d was also found to be regulated through the p38 pathway following XRituximab treatment in the patients that responded to treatment since inhibition of this pathway resulted in partial abrogation of Tis11d induction after XRituximab treatment (Figure 3.16). However in some patients inhibition of ERK pathway was also involved (data not shown). These findings suggest that Tis11b/Berg36 and Tis11d when induced by XRituximab are regulated primarily through the p38 pathway but they may differ in terms of function because Tis11b/Berg36 was found to be also partially regulated through JNK but Tis11d was also regulated through ERK1/2.

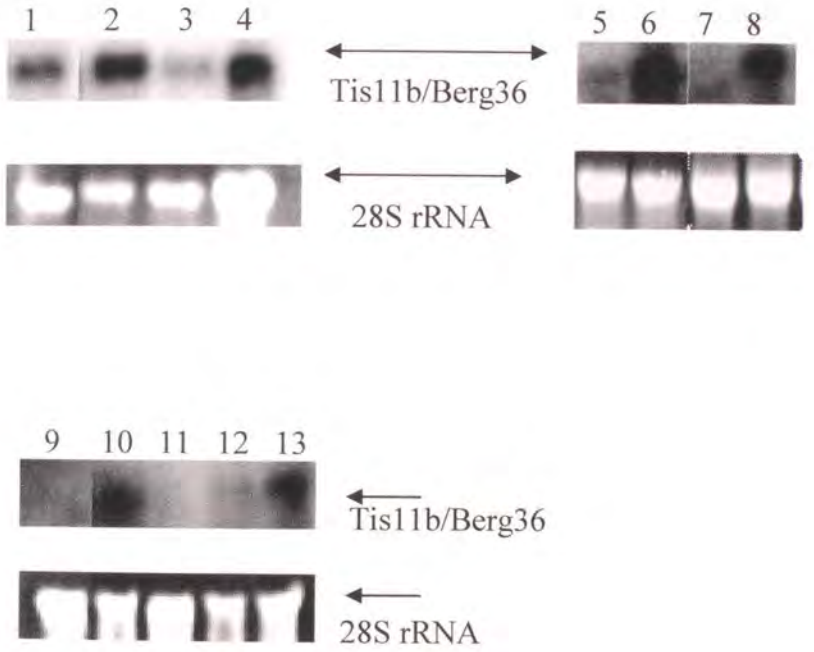


Figure 3.15: Northern Blot analysis of Tis11b expression in CLL :
Freshly isolated B-CLL cells were processed for RNA extraction immediately after purification (To) or were stimulated with XRituximab for 3h in the presence or absence of specific inhibitors for the p38 (SB203580 or SB202190), JNK (SP600125) and ERK1/2 (U0126) pathways. At the end of the culture period RNA was extracted and processed for Northern Blot Hybridisation using a ^{32}P labelled cDNA probe for Tis11b/Berg36. Consistency of RNA loading as indicated by 28S rRNA staining is shown below each membrane. The arrows indicate either the Tis11b/Berg36 mRNA specific band detected by the radiolabelled probe or the of 28S rRNA band present in each lane.
Lanes:
1) CLL32 To(unstimulated cells)
2) CLL32 XRituximab 3h
3) CLL32 SB202190+XRituximab 3h
4) CLL32 SP600125+XRituximab 3h
5) CLL29 To(unstimulated cells)
6) CLL29 XRituximab 3h
7) CLL29 SB202190+XRituximab 3h
8) CLL29 SP600125+XRituximab 3h
9) CLL38 To(unstimulated cells)
10) CLL38 XRituximab 3h
11) CLL38 SB203580+XRituximab 3h
12) CLL38 SP600125+XRituximab 3h
13) CLL38 U0126+XRituximab 3h

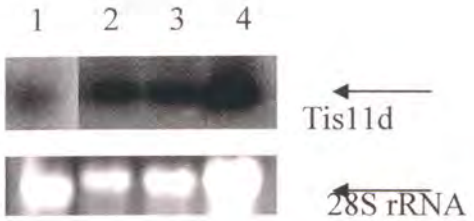


Figure 3.16: Northern Blot analysis of Tis11d expression in CLL
Freshly isolated B-CLL cells were processed for RNA extraction immediately after purification (To) or were stimulated with XRituximab for 3h in the presence or absence of specific inhibitors for the p38 (SB202190), JNK (SP600125) and ERK1/2 (U0126) pathways. At the end of the culture period RNA was extracted and processed for Northern Blot Hybridisation using a ^{32}P labelled cDNA probe for Tis11d. Consistency of RNA loading as indicated by 28S rRNA staining is shown below each membrane. The arrows indicate either the Tis11d mRNA specific band detected by the radiolabelled probe or the 28S rRNA band present in each lane.
Lanes:
1) CLL32 To(unstimulated cells)
2) CLL32 XRituximab 3h
3) CLL32 SB202190+XRituximab 3h
4) CLL32 SP600125+XRituximab

3.2.6. Involvement of p38 in the regulation of Tis11b/Berg36 protein

In order to confirm that p38 pathway is involved in the regulation of Tis11b/Berg36 protein, intracellular staining and FACS analysis was performed. Because at this stage we were interested only in the effects of each pathway on protein levels and not on the phosphorylation patterns it was decided to use FACS analysis. This method would allow rapid establishment of the appropriate protocol for Tis11b/Berg36 detection. It was found that the optimal dilution of Tis11b/Berg36 antisera was 1/10 in the presence of 3% human IgG as a blocking solution (Figure 3.17a).

After these conditions were established, B-CLL cells were left unstimulated or were stimulated with XRituximab in the absence or presence of 10 μM of SB203580 or SP600125. Analysis was performed over unstimulated cells and because there was high background staining of the secondary FITC antibody to the rabbit serum when compared with isotypic FITC antibody the Mean Fluorescence Intensity (MFI) data instead of percentage of cells will be presented here. It was found that the MFI of Tis11b/Berg36 protein in unstimulated cells was 60.3 and increased to 87.23 following XRituximab stimulation. In the presence of SB203580 the MFI of Tis11b/Berg36 protein following XRituximab treatment was MFI=54.5 while in the presence of SP600125 it was MFI=78.5 (Figure 3.17b). These data on the Tis11b protein regulation are consistent with the fact that p38 inhibition had stronger effect on induction of Tis11b/Berg36 mRNA following XRituximab treatment than inhibition of JNK pathway.

Thus the p38 pathway that is involved in the regulation of apoptosis following XRituximab treatment of B-CLL cells, appears to be involved in the regulation of Tis11b/Berg36 at the mRNA and protein level following this treatment. This suggests that Tis11b/Berg36 may be one of the downstream effectors in apoptosis induction following Rituximab treatment.

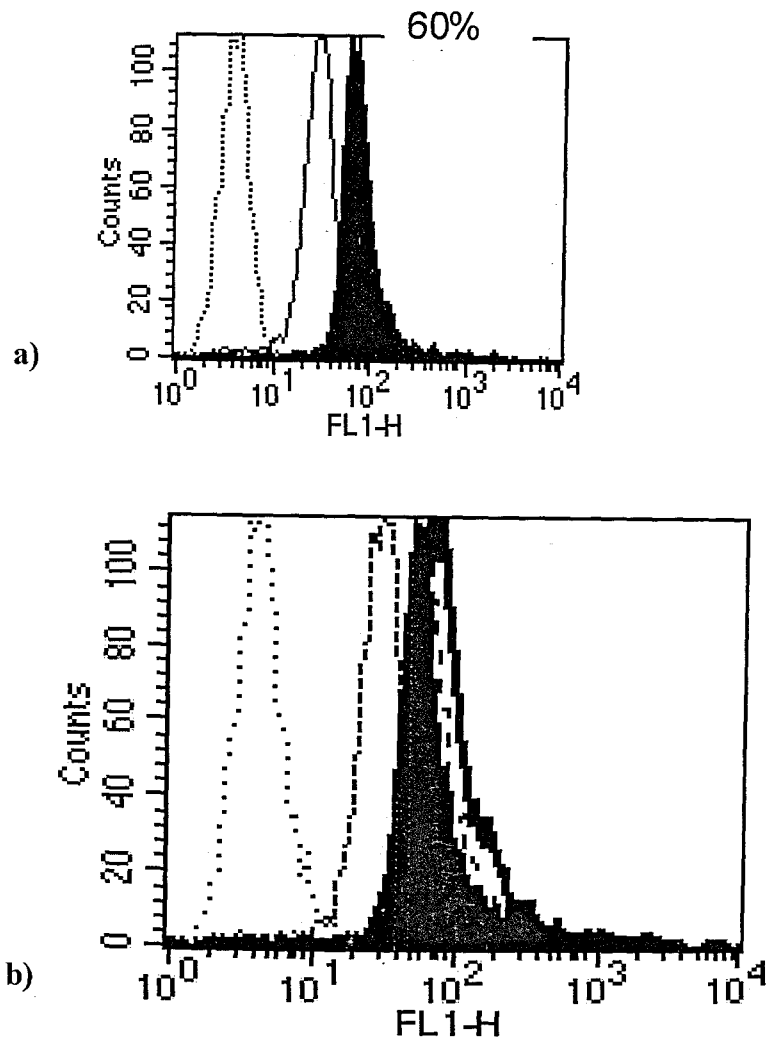


Figure 3.17: Effect of p38 and JNK inhibition of Tis11b/Berg36 protein following XRituximab treatment of B-CLL cells

a)B-CLL cells were left unstimulated (black open line) or were stimulated with 20 μg/ml XRituximab (black filled line-overlay) for 3h and were stained with Tis11b-Berg36 antisera overnight. Tis11b-Berg36 protein was detected by FACS analysis using an anti-rabbit FITC secondary antibody. Dotted line represents XRituximab treated cells stained with an isotype control (human IgG) antibody.

b) B-CLL cells were left unstimulated (black dashed open line) or were stimulated with 20 μg/ml XRituximab alone (thick black open line) or in the presence of 10 μM SB203580 inhibitor (filled black line) or 10 μM SP60015 inhibitor (dashed black line) for 3h and Tis11b/Berg36 protein was detected as in a)

3.2.7 Tis11b/Berg36 siRNA and effect on XRituximab induction of apoptosis

Since Tis11b/Berg36 was found to be strongly induced by XRituximab it was interesting to test whether inhibition of the gene would affect induction of apoptosis by this monoclonal antibody. For this purpose siRNA technology was utilised using a commercially available double stranded RNA oligo (siRNA) The sequence of the Tis11b/Berg36 siRNA is : CAA GAU GCU CAA CUA UAG UdT for the sense one and ACU AUA GUU GAG CAU CUU GdT for the antisense

Conditions of the transfection were improved as described in Appendix1 and set at 3 μl Lipofectamine 2000 plus 3 μl of siRNA (control or Tis11b/Berg36 siRNA). The first 3 patients were tested for apoptosis induction by Annexin/PI method. The transfection efficiency at 48 hours for the 3 patients tested were 76%, 55% and 59% (Table , Appendix1).

In this experiment XRituximab at 24 hours post stimulation increased the number of Annexin positive(+) cells from 14.2%±3.0% (unstimulated cells mean ±sem) to 27.1%±5.5%(mean±sem). In the presence of Tis11b/Berg36 siRNA the percentage of Annexin+ cells was reduced to 7.3%±4.0% (p□0.02). It should be noted that use of the control siRNA reduced the percentage of Annexin+ cells to 19.0%±5.0% with p□0.16.When the cells were treated with Lipofectamine 2000 alone (no siRNA added) the percentage of Annexin+ cells was 11.1%±3.5% with p□0.03 (Figure 3.18a).

At 48 hours post stimulation the percentage of Annexin+ cells was 9.0%±3.0%(mean±sem) and was increased to 26.0%±5.0% (mean±sem) by XRituximab. In the presence of Tis11b/Berg36 siRNA the percentage of Annexin+ cells was 2.5%±1.0% and this effect was statistically significant (Figure 3.18b) while in the presence of the control siRNA the percentage of Annexin+cells was 6.0%±4.0% (mean±sem).

This experiment suggests that Lipofectamine affects exposure of Phosphatidylserine to the plasma membrane of cells and Annexin staining. Additionally it seems that the control siRNA interferes with apoptosis induction. For these reasons it was decided to repeat the above expreriment using another control siRNA and furthermore to monitor apoptosis induction using the Propidium Iodide (PI) method.

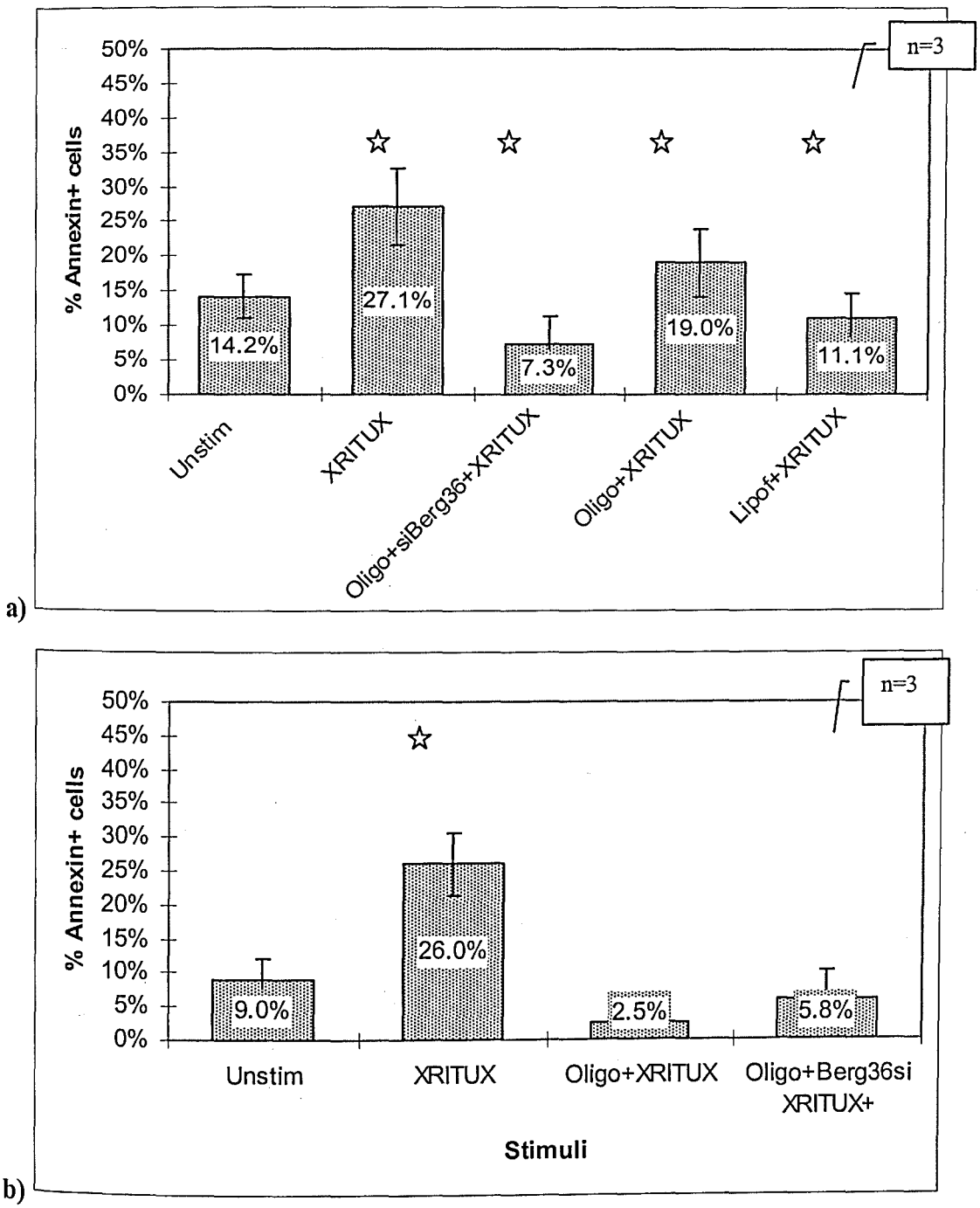


Figure 3.18: Effect of Tis11b/Berg36 siRNA on induction of apoptosis by XRituximab as assayed by the Anenxin/PI method in B-CLL cells

B-CLL cells were left unstimulated or were stimulated with XRituximab at 20 μg/ml in the presence or absence of Tis11b/ Berg36 siRNA or a negative control siRNA transfected using Lipofectamine 2000. The percentage of apoptotic cells (Annexin+) were measured by Annexin-PE at **a)**24 hours and **b)**48 hours after the addition of XRituximab. In each graph the mean ±sem is plotted and error bars represent sem (sem=STDEV/□n where STDEV is Standard Deviation and n is the number of the patients tested). In the centre of each bar the mean percentage of apoptotic (Annexin+) cells is shown and the presence of a star indicates statistical significance with p□0.05.

For the next 3 patients apoptosis induction was measured by the PI method and cells were transfected for 24 hours before addition of XRituximab and apoptosis was measured at 24 hours post stimulation. These 3 patients were all different from the 3 patients tested by Annexin/PI method.

It was found that addition of XRituximab increased the percentage of apoptosis from 19.0%±5.7% (unstimulated cells, mean±sem) to 38.0%±4.0%. In the precence of Tis11b/Berg36 siRNA, XRituximab increased apoptosis to 30%±4.20% while in the presence of control siRNA it increased apoptosis to 35%±1.0% (Figure 3.19a). Data from a representative patient is shown in Figure 3.19b.

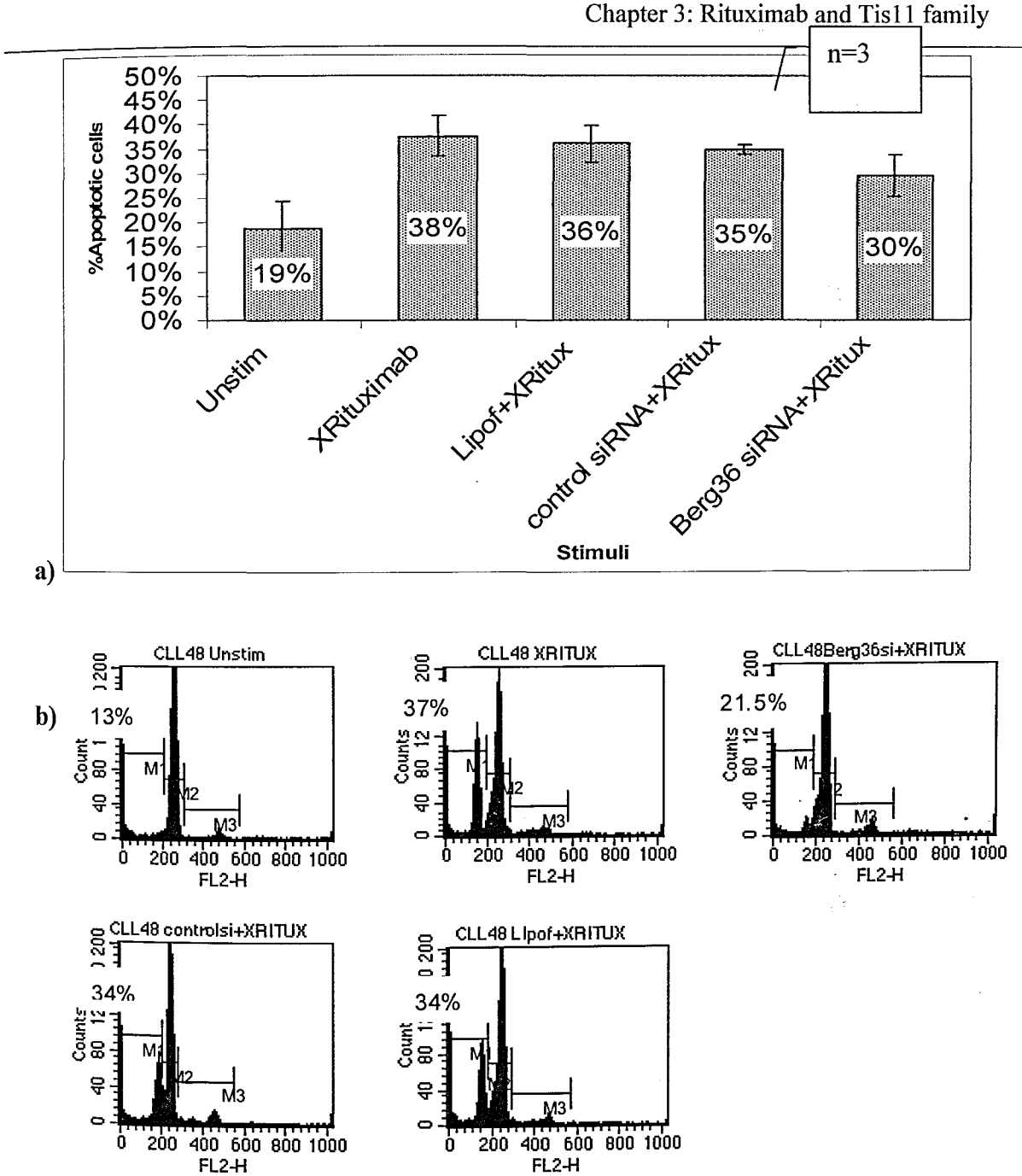


Figure 3.19: Effect of Tis11b/Berg36 siRNA on induction of apoptosis by XRituximab as assayed by the PI method in B-CLL cells

a)B-CLL cells were left unstimulated or were stimulated with XRituximab at 20 μg/ml in the presence or absence of Tis11b/Berg36 siRNA or a commercially available siRNA transfected using Lipofectamine 2000. Apoptosis was measured at 24 hours post stimulation using the PI method. In each graph the mean ±sem is plotted and error bars represent sem (sem=STDEV/√n where STDEV is Standard Deviation and n is the number of the patients tested). In the centre of each bar the mean percentage of apoptotic cells is shown. b) Primary data from one representative patient tested with the top 3 histograms from left to the right representing unstimulated cells, XRituximab treated cells and XRituximab+Tis11b/Berg36 siRNA treated cells and bottom 2 histograms from left to the right representing XRituximab+control siRNA treated cells and XRituximab +Lipofectamine 2000 reagent.

Because again there was interference of the control siRNA with apoptosis induction and from Lipofectamine (even though not so evident from bar graph because mean values are used), it was decided to change the control siRNA with one that shows no similarity with a known eukaryotic gene. This was provided by QIAGEN as the Tis11b/Berg36 siRNA and was dissolved at the same concentration and dilutent as the Tis11b/Berg36 siRNA. It is also labelled with Alexa 488 which is a green fluorochrome. Additionally the transfection reagent was changed from Lipofectamine to HyperFect which is more specific for siRNA transfection and claimed to be very efficient in delivering small amounts (like the ones used in this experiment) of siRNA into the cells. Finally XRituximab was added into CLL cells 48 hours post transfection, apoptosis was monitored at 24 hours post stimulation and thus at 72 hours post transfection and the PI method was used instead of Annexin/PI to measure apoptosis induction.

Before performing the actual experiment transfection efficiency was established for the new reagent (HyperFect) and control siRNA. For this purpose 3 different patients were transfected with 3 μl HyperFect and 3 μl control siRNA for 48 hours and transfection efficiency was monitored by FACS analysis. In all 3 cases only the live B-CLL cells were analysed and it was found that transfection efficiency was 63%, for CLL25, 34% for CLL54 and 41% for CLL24 (Figure 3.20) which was very similar and in the range obtained by Lipofectamine.

Immediately after establishing the transfection efficiency of the new reagent the actual experiment with XRituximab and Tis11b/Berg36 siRNA was performed. In this set of experiments it was found that the percentage of apoptotic cells at 24 hours after the addition of XRituximab was 29.7%±4.7% (mean ±sem) in unstimulated cells and increased to 44.7%±5.4% (mean±sem, $p \leq 0.001$) by XRituximab. In the presence of Tis11b/Berg36 siRNA the percentage of apoptotic cells was 33.5%±4.6%(mean±sem, $p \leq 0.05$) (Figure 3.20). The new transfection reagent and control siRNA did not affect the efficiency of XRituximab (46.1%±5.4, mean±sem and 43.7%±3.7%, mean±sem respectively) (Figure 3.21). Data from two representative paients are shown in Figure 3.22a and 3.22b.

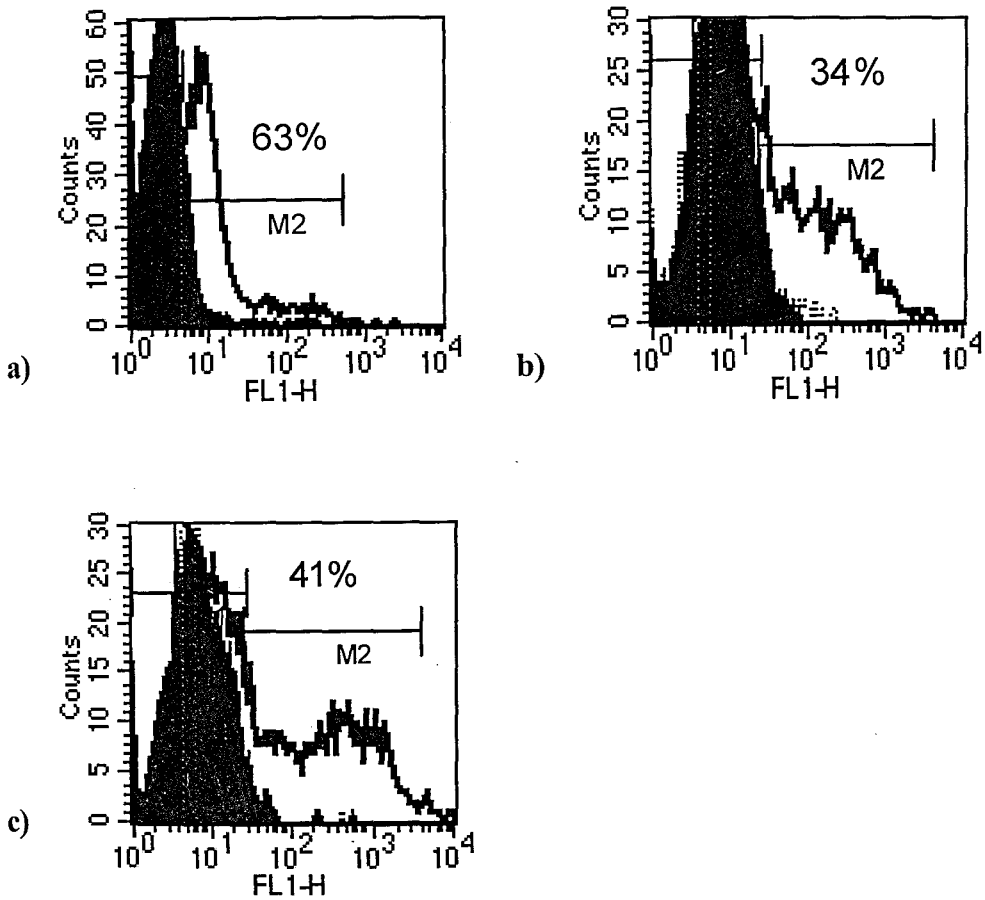


Figure 3.20: Transfection efficiency achieved with the HyperFect transfection reagent and a control siRNA in B-CLL cells
B-CLL from CLL25, CLL54, CLL24 were transfected using 3 µl HyperFect reagent and a control siRNA labelled with Alexa 488 provided by QIAGEN. Tranfection efficiency was monitored at 48 hours post transfection using FACS analysis. Analysis was performed on live gated B-CLL cells and the percentage of cells transfected with the control siRNA is shown for all 3 patients above gate M2. In all histograms the black filled line represents untransfected cells while the black open line represents cells transfected with control siRNA.

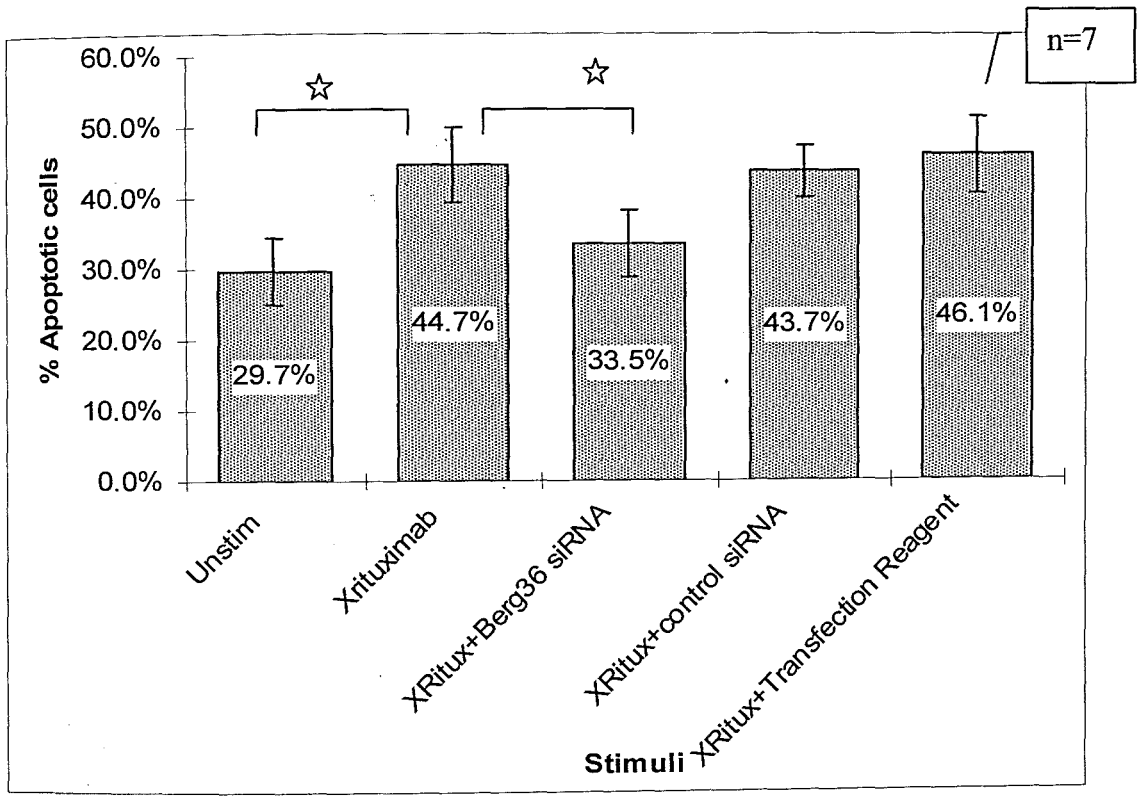


Figure 3.21: Effect of Tis11b/Berg36 siRNA on apoptosis induction by XRituximab in B-CLL cells

B-CLL cells were left unstimulated or were stimulated with XRituximab at 20 μg/ml in the presence or absence of Tis11b/Berg36 siRNA or a negative control siRNA transfected using HyperFect reagent .The percentage of apoptotic cells was measured by Propidium Iodide staining at 24hours post stimulation. In each graph the mean ±sem for each of the stimuli is plotted and error bars represent sem (sem=STDEV/√n where STDEV is Standard Deviation and n is the number of the patients tested). In the centre of each bar the mean percentage of apoptotic cells is shown. The presence of a star indicates statistical significance with $p \leq 0.05$

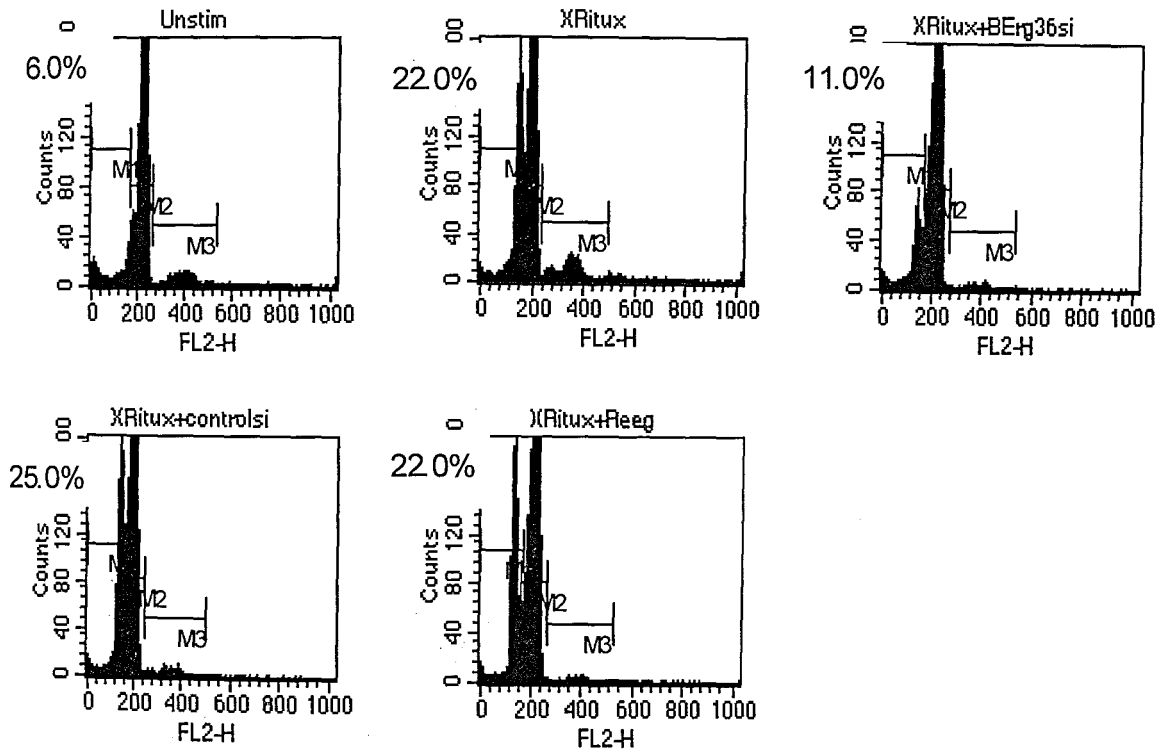


Figure 3.22a: Effect of Tis11b/Berg36 siRNA on apoptosis induction by XRituximab in CLL cells

B-CLL cells from CLL56 were left unstimulated (Unstim) or were stimulated with XRituximab (XRitux) for 24 hours in the presence of Tis11b/Berg36 siRNA (XRitux+Berg36 si) or control siRNA (XRitux+control si) which were transfected into the cells 48 hours prior stimulation. Apoptosis was measured by Propidium Iodide staining and FACS analysis. CLL56 is a representative of 3 patients tested in which 20,000 events were acquired. The percentage of apoptotic cells found in each case is shown above the M1 gate. Reagent refers to the transfection reagent used (HyperFect). Top 3 histograms from left to the right represent unstimulated cells, XRituximab treated cells and XRituximab+Tis11b/Berg36 siRNA treated cells while bottom 2 histograms from left to the right represent XRituximab+control siRNA treated cells and XRituximab +HyperFect reagent.

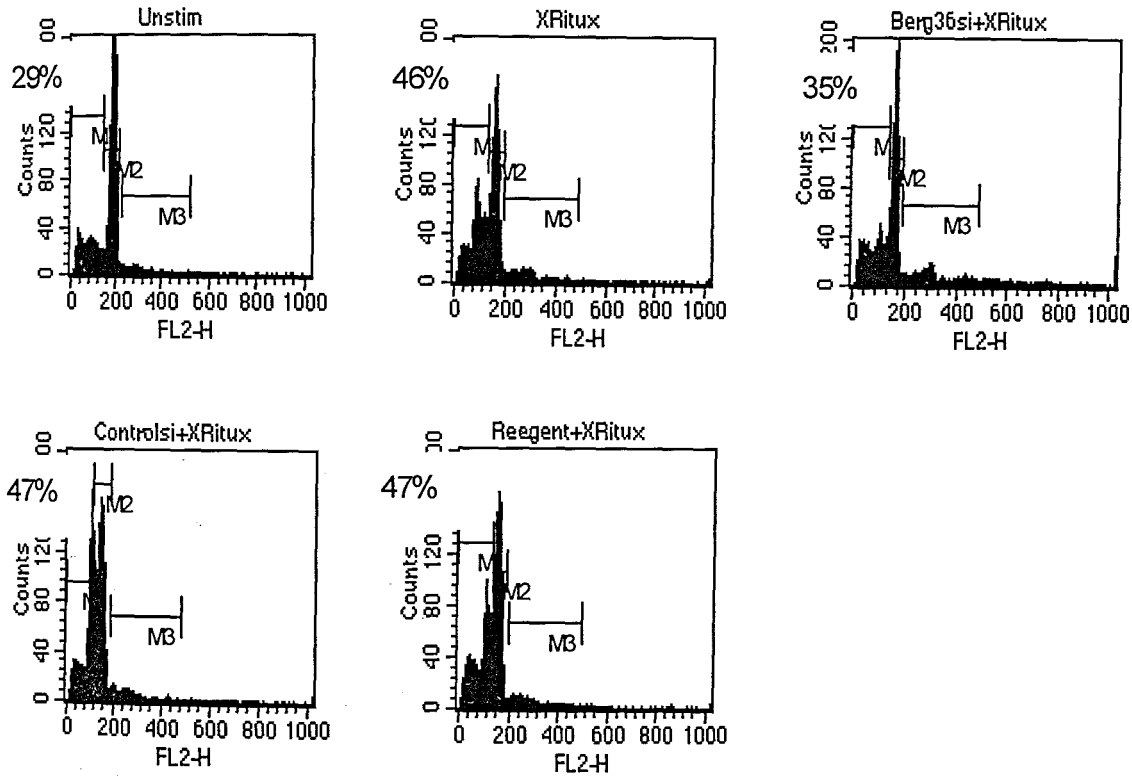


Figure 3.22b: Effect of Tis11b/Berg36 siRNA on apoptosis induction by XRituximab in CLL cells

B-CLL cells from CLL54 were left unstimulated (Unstim) or were stimulated with XRituximab (XRitux) for 24 hours in the presence of Tis11b/Berg36 siRNA (Berg36 si+XRitux) or control siRNA (control siRNA+XRitux) which were transfected into the cells 48h prior stimulation. Apoptosis was measured by Propidium Iodide staining and FACS analysis. CLL54 is a representative of 4 patients tested in which 10,000 events were acquired. The percentage of apoptotic cells found in each case is shown above the M1 gate. Reagent refers to the transfection reagent used (HyperFect). Top 3 histograms from left to the right represent unstimulated cells, XRituximab treated cells and XRituximab+Tis11b/Berg36 siRNA treated cells while bottom 2 histograms from left to the right represent XRituximab+control siRNA treated cells and XRituximab +HyperFect reagent.

3.2.8 Quantitative Real Time PCR

In order confirm downregulation by the Tis11b/Berg36 mRNA by the commercially used Tis11b/Berg36 siRNA, quantitative Real Time PCR (two step) was performed in a Roche LightCycler and in 96 well plate ABI 7000 system.

Efficiency of the primers was tested by a serial dilution of cDNA made from either unstimulated or XRituximab treated cells amplified by either Tis11b/Berg36 primers or β-Actin primers (control gene) (Appendix 1). Quantification of the results was attempted by a standard curve (in the Roche Light Cycler) and by a mathematical equation (Livak method) (Livak and Schmittgen 2001) which is $2^{-\Delta\Delta C_T}$ where $\Delta\Delta C_T = (C_{T\ Berg36} - C_{T\ \beta\text{-Actin}})_{Time\ 3h} - (C_{T\ Berg36} - C_{T\ \beta\text{-Actin}})_{Time\ 0h}$ and will be applied for XRituximab treated cells at 3h, XRituximab +Tis11b/Berg36 siRNA at 3h, XRituximab+control siRNA at 3h in comparison with unstimulated cells at time 0 hours. The above calculations were applied to all samples that was loaded in duplicate and run on the ABI 7000 system. The primary results obtained from this experiment together with the Ct value is shown in Table 3.1

Before applying the Livak method it is recommended for experiments run in duplicate or triplicate to calculate the mean C_T value for Tis11b and β-Actin at either the 3 hours timepoint or at 0 hours timepoint before performing all other calculations.

It was found that XRituximab treatment resulted in 1860 fold induction in Tis11b/Berg36 mRNA while in the presence of Tis11b/Berg36 siRNA it resulted in 190 fold increase. When XRituximab was used in the presence of the control siRNA it resulted in 2520 fold induction in Tis11b/Berg36 mRNA. The average C_T values, ΔC_T and $\Delta\Delta C_T$ calculations are shown in Table 4.2. These data suggest that there is at least 80% abrogation of Tis11b/Berg36 mRNA levels induced by XRituximab in the presence of Tis11b/Berg36 siRNA (Table 3.2). This efficiencyof downregulation is well above the 50% expected and required to perform any functional studies suggesting that indeed XRituximab is significantly less efficient in inducing apoptosis in the absence of Tis11b/Berg36 mRNA or protein. This experiment was loaded twice and the average change in fold induction and variation in relative expression are shown as a bar chart in Figure 3.23.

Well	Sample Name	Detector	Task	CT
C1	FZ Unstimulated Actin Primers	Bactin	Unknown	26.02
C2	FZ Unstimulated Berg36 Primers	Berg36	Unknown	34.81
C3	FZ Stimulated Actin Primers	Bactin	Unknown	25.21
C4	FZ Stimulated Berg36 Primers	Berg36	Unknown	22.94
C5	FZ Control siRNA Actin Primers	Bactin	Unknown	23.16
C6	FZ Control siRNA Berg36 Primers	Berg36	Unknown	20.05
C7	FZ Berg36 siRNA Actin Primers	Bactin	Unknown	25.38
C8	FZ Berg36 siRNA Berg36 Primers	Berg36	Unknown	26.37
D1	FZ Unstimulated Actin Primers	Bactin	Unknown	24.4
D2	FZ Unstimulated Berg36 Primers	Berg36	Unknown	32.43
D3	FZ Stimulated Actin Primers	Bactin	Unknown	23.02
D4	FZ Stimulated Berg36 Primers	Berg36	Unknown	20.39
D5	FZ Control siRNA Actin Primers	Bactin	Unknown	23.5
D6	FZ Control siRNA Berg36 Primers	Berg36	Unknown	20.81
D7	FZ Berg36 siRNA Actin Primers	Bactin	Unknown	25.58
D8	FZ Berg36 siRNA Berg36 Primers	Berg36	Unknown	26.67

Table 3.1: Primary data obtained from ABI7000 Real Time PCR system regarding Tis11b mRNA expression following XRituximab treatment in the presence or absence of Tis11b siRNA

These data were obtained from ABI7000 Real Time PCR system after using the QuantiTect SYBR Green PCR kit and cDNA obtained from CLL cells unstimulated, XRituximab stimulated and XRituximab+Tis11b/Berg36 siRNA or XRituximab +control siRNA. The primers used included Berg36 primers and β-Actin (as a loading control). CT value refers to the point at which each of the product Tis11b/Berg36 or β-Actin crossed the threshold of detection and becomes detected in each of the samples mentioned in the table. C1-C8 refers to the wells in a 96 well plate that the samples were loaded. This experiment was repeated twice in terms of setting (with fresh and frozen CLL cells from the same patient) and in terms of loading in the ABI7000 machine.

Average Ct	Tis11b/ Berg36	β-Actin	ΔCt	ΔΔCt	$2^{-\Delta\Delta C_T}$
Unstimulated	33.62	25.21	8.41	0	**1**
XRituximab	21.665	24.115	-2.45	-10.86	**1860**
XRituximab +Tis11b/Berg36 siRNA	26.52	25.68	0.84	-7.57	**190**
XRituximab +control siRNA	20.43	23.33	-2.9	-11.3	**2520**

Table 3.2; Summary of calculations for Real Time PCR experiment according to Livak method. The Real Time PCR data shown in Table 4.1 (above) were analysed according to Livak method in order to quantify the fold change in Tis11b/Berg36 mRNA level following stimulation with 20 μg/ml XRituximab in the absence of siRNA or in the presence of Tis11b/Berg36 siRNA or negative control siRNA. The mean fold induction (change) was calculated using the $2^{-\Delta\Delta C_T}$ equantion where $\Delta\Delta C_T = (C_{T\ Berg36} - C_{T\ \beta\text{-}Actin})_{Time\ 3h} - (C_{T\ Berg36} - C_{T\ \beta\text{-}Actin})_{Time\ 0h}$.

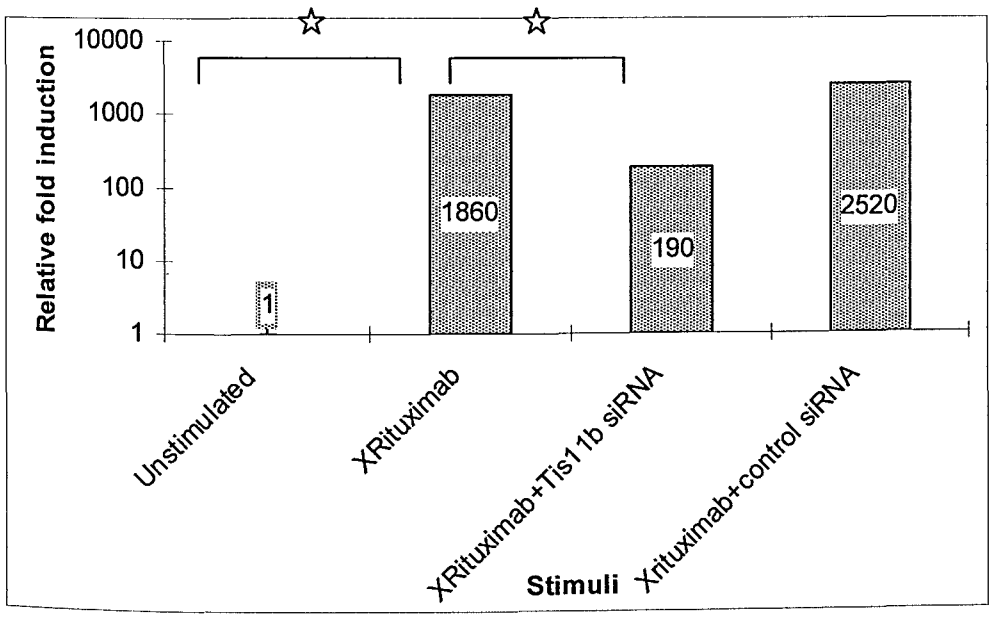


Figure 3.23: Effect of Tis11b/Berg36 siRNA on Tis11b induction following XRituximab treatment in CLL cells.
B-CLL were left stimulated or were stimulated with XRituximab in the presence or absence of either control siRNA or Tis11b/Berg36 siRNA for 3h and processed for RNA extraction and cDNA synthesis for quantitative Real Time PCR. This bar chart represents the average fold induction in Tis11b/Berg36 mRNA as calculated by the Livak method $2^{-\Delta\Delta C_T}$ equantion where $\Delta\Delta C_T = (C_{T\ Berg36} - C_{T\ \beta\text{-}Actin})_{Time\ 3h} - (C_{T\ Berg36} - C_{T\ \beta\text{-}Actin})_{Time\ 0h}$. described in Table 4.2 and error bars represent the mean difference in fold induction between the two experiments. Relative fold induction is shown in log scale and is relative to β-Actin changes In the centre of each bar chart the average fold induction is shown for each stimuli and the presence of a star indicates statistical significance with $p \leq 0.05$.

3.2.9 Effect of Tis11b/Berg36 siRNA on Tis11b/Berg36 protein

In order ensure downregulation by the siRNA at the protein level, intracellular staining and FACS analysis was performed. Again B-CLL were left unstimulated or were stimulated with XRituximab for 4h in the absence of siRNA or in the presence of either Tis11b/Berg36 siRNA or control siRNA. Tis11b/Berg36 protein was measured by FACS analysis after staining with 1/10 dilution of rabbit antisera. Transfection conditions were as in the apoptosis experiment.

In this CLL patients it was found that unstimulated cells express Tis11b/Berg36 in 20% of their cells (MFI:230). Treatment with XRituximab further induced the Tis11b/Berg36 protein in 32% of cells (thus total 52% of the cells being positive for Tis11b/Berg36 and MFI:240). In the presence of control siRNA, XRituximab further induced the Tis11b/Berg36 protein in 33% of cells (MFI:244) of cells while in the presence of Tis11b/Berg36 siRNA, XRituximab further induced the Tis11b/Berg36 protein in 7% of the cells (thus a total of 27% of cells being positive for Tis11b/Berg36 and MFI:222) (Figure 3.24). This represents a 45% reduction in the percentage of cells positive for Tis11b/Berg36 protein by XRituximab when the Tis11b/Berg36 is present.

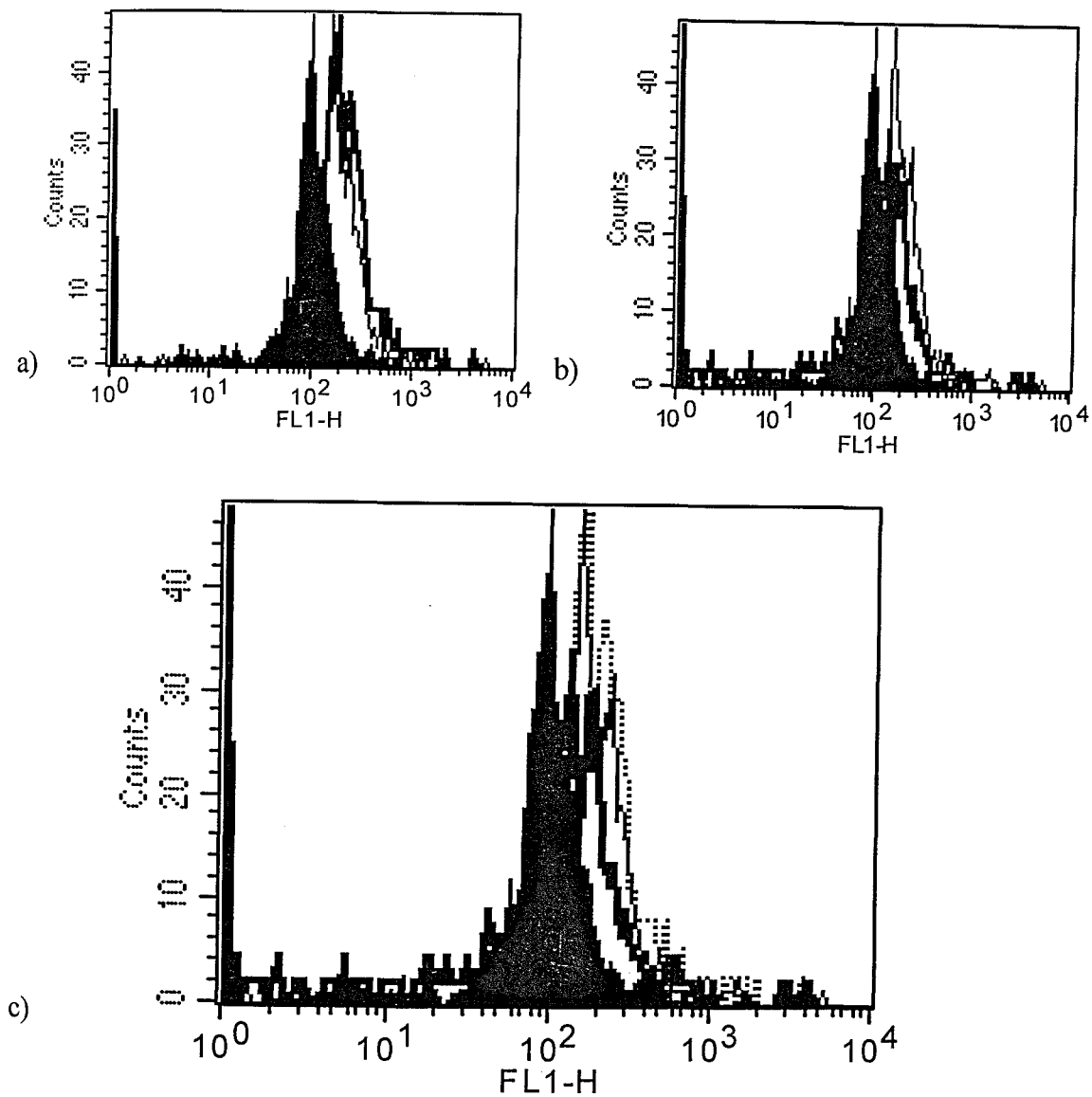


Figure 3.24: Effect of Tis11b/Berg36 siRNA on Tis11b/Berg36 protein
B-CLL cells were left unstimulated or were stimulated with XRituximab in the presence or absence of Tis11b/Berg36 siRNA or control siRNA for 3h. Tis11b/Berg36 protein was detected by FACS analysis after staining with Tis11b/Berg36 rabbit antisera at 1/10 dilution and a secondary anti-rabbit FITC antibody. a) The black filled line represents unstimulated cells, the black thin line-overlay represents XRituximab treated cells while the thick black line represents XRituximab treated cells in the presence of control siRNA b) The black filled line represents unstimulated cells, the black thin line-overlay represents XRituximab treated cells while the thick black line represents XRituximab treated cells in the presence of Tis11b/Berg36 siRNA c) The summary of a) and b) histograms. The black filled line represents unstimulated cells, the black thin line-overlay represents XRituximab treated cells, the dashed line represents XRituximab treated cells in the presence of control siRNA while the black thick line represents XRituximab treated cells in the presence of Tis11b/Berg36 siRNA.

3.2.10 Intracellular staining for Bcl-2 in B-CLL cells

After it was established that the Tis11b/Berg36 siRNA downregulated Tis11b/Berg36 mRNA and protein following XRituximab treatment, it was decided to test whether the presence of Tis11b/Berg36 siRNA treatment may affected Bcl-2 protein following XRituximab treatment. Bcl-2 as mentioned in the general introduction section, possesses AREs in the 3' UTR implying that it may be degraded by Tis11b/Berg36 or the rest of the Tis11 family. The levels of Bcl-2 protein were tested by intracellular staining and FACS analysis. It was found as expected that unstimulated fresh CLL express very high basal levels of Bcl-2 protein (Figure 3.25). A total of 5 different patients were tested, only live gated cells were analysed and 3 of them showed a weak downregulation of Bcl-2 levels. In one of these patients (CLL25) the percentage of Bcl-2 positive cells was 99.6% in unstimulated live gated cells and remained the same in XRituximab treated cells. However the MFI was 117.47 in unstimulated cells and was reduced to 107.8 in XRituximab treated cells (Figure 24a). In the other one (CLL24) the percentage of Bcl-2 positive cells in live gated cells was 98.71% and remained unaffected by XRituximab but there was a weak reduction in MFI from 116.31 to 105.09 (Figure 25b). In the last patients with some visible change again the change was noticed in MFI rather than in the percentage of positive cells and it was found that it was 102.24 in unstimulated cells and reduced to 98.93 in XRituximab treated cells. The results obtained in terms of percentage of positive cells for Bcl-2 or MFI for all 5 patients are shown in Table 3.3.

Both CLL24 and CLL25 were treated with XRituximab in the presence of Tis11b/Berg36 siRNA but no difference could be seen in terms of Bcl-2 protein levels when the Tis11b/Berg36 siRNA was present.

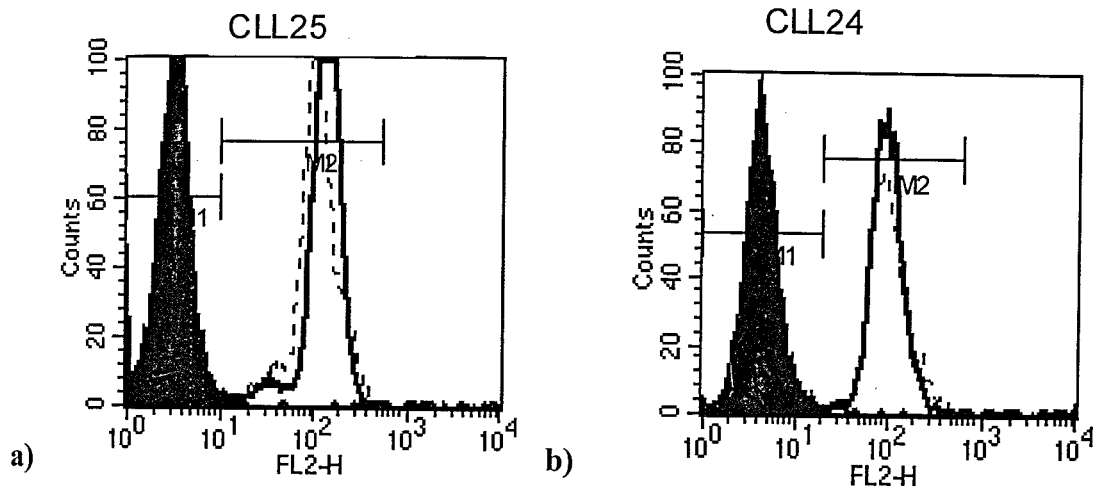


Figure 3.25: Intracellular staining for Bcl-2 protein in CLL cells.
10^6 CLL cells from a)CLL24 and b)CLL25 were fixed, permeabilised, stained with either an isotype control IgG PE antibody or with Bcl2 PE antibody and analysed in a FACScan Flow Cytometer. The black filled line represents unstimulated CLL cells stained with isotype control, the thick black open line-overlay represents unstimulated cells stained with Bcl-2 while dashed black open line represents XRituximab treated cells stained with Bcl-2 antibody. These are representative results from a total of 5 patients tested.

B-CLL cells	% of Bcl-2 positive cells	Bcl-2 MFI
B-CLL24	99.1% /**99.1%**	91/**93**
B-CLL25	99.5% /**99.6%**	117/**108**
B-CLL i	99.7% /**99.8%**	156/**176**
B-CLL ii	98.7%/ **98.6%**	106.3/**105.1**
B-CLL iii	95.6% / **97%**	102/**98**

Table 3.3: Summary of Bcl-2 protein staining before and after treatment of B-CLL cells with XRituximab
A total of 5 different CLL patients were left unstimulated or were stimulated with 20 µg/ml XRituximab for 24 hours. At the end of the culture period cells were fixed, permeabilised, stained with either an isotype control IgG PE antibody or with Bcl2 PE antibody and analysed in a FACScan Flow Cytometer. In all cases only live gated cells were analysed In both cases the first number indicates the percentage of Bcl-2 positive cells or the Bcl-2 MFI in unstimulated cells while the second number in bold indicates the percentage of Bcl-2 postive cells or Bcl-2 MFI in XRituximab treated CLL cells.

3.3 DISCUSSION

Previous work has shown that 2 strong inducers of apoptosis in Burkitt's Lymphoma cell lines, namely anti-IgM and immobilised Rituximab, induced Tis11b/Berg36 mRNA with similar kinetics (Mathas *et al* 2000) suggesting that Tis11b/Berg36 is involved in the regulation of apoptosis in Burkitt's Lymphoma B cells. It was very interesting to test whether Rituximab could induce Tis11b/Berg36 in CLL cells since it has been used *in vivo* to treat CLL patients. Additionally CLL cells resemble memory B cells (Klein *et al* 2001) while Burkitt's Lymphoma cell lines represent Germinal Centre B cells and thus different stages of B cell differentiation. For this reason the basal expression and regulation of Tis11b/Berg36 may differ. In support of this, anti-IgM treatment induces apoptosis in Burkitt's Lymphoma cell lines but inhibits apoptosis and promotes cell survival in CLL cells (Bernal *et al* 2001) .

Initially a dose response experiment in two patients revealed that the appropriate dose for efficient induction of apoptosis was 20μg/ml for Rituximab which was also the dose used by Mathas *et al* 2001. This was the dose used in all subsequent experiments and it was found that Rituximab alone was unable to induce apoptosis at 24 hours or 48 hours after the initiation of the culture period but was very efficient in the presence of 3x (60 μg/ml) F(ab)2 anti human IgG antibody (cross-linker) which is in accordance with published data (Pedersen *et al* 2002a, van der Kolk *et al* 2002). The antibody used as a crosslinker could induce apoptosis in the absence of Rituximab but this was always significantly lower than in the presence of Rituximab and was always the same whatever the dose of secondary antibody used also in accordance with published data (Pedersen *et al* 2002a). A possible explanation for the requirement of crosslinking is the fact that CD20 Receptor forms tetramers on the cell surface and thus Rituximab alone is insufficient for complete binding and activation of this receptor. It has been hypothesized that *in vivo* such crosslinking may be mediated by cells that bear Fcγ receptors (van der Kolk *et al* 2002, Shan *et al* 1998). The response of different patients to Rituximab varied significantly between individuals and did not depend on surface expression of CD20 or total White Blood Count/WBC but rather depended on the levels of spontaneous apoptosis (negative correlation) present at a certain time point for each patient. Even though this result was rather unexpected, a careful review of the literature

revealed surface CD20 expression was found not to correlate with response of CLL patients to Rituximab treatment *in vivo* as well (Pickartz *et al* 2001). Instead response to Rituximab may correlate directly with the levels of circulating CD20 (cCD20) which will neutralise the antibody. It has been shown that levels of cCD20 correlates with the stage of the disease and is an independent prognostic factor for CLL (Manshouri *et al* 2003). Other clinical data such as CD38 or ZAP-70 expression may determine response to Rituximab. It was shown CD38 positive undergo apoptosis more efficiently after treatment with crosslinked anti-IgM than CD38 negative patients (Zupo *et al* 1996). Similarly ZAP-70 expression was shown to determine apoptosis induction in response to both crosslinked anti-CD5 and anti- IgM in CLL patients (Nédellec *et al* 2005, Renaudineau *et al* 2005). Therefore Rituximab may be more efficient in patients that express ZAP-70. Furthermore chromosomal abnormalities and mutation status may affect the response of CLL patients to Rituximab. In support of the latter, it was shown that patients bearing the del(17)(p13.1) did not respond to Rituximab treatment at all, in contrast to the vast majority (86%) of patients bearing the 13q14.3 deletion who responded to this treatment (Byrd *et al* 2003).

Another interesting finding from the initial experiments was that treatment with Rituximab altered the cell cycle profile of CLL cells in some but not all patients tested in such way so that there was an increase in the percentage of cells in G2/M phase seen with the PI method even though these cells may represent duplets as explained in the results section (page 90). However if these are truly proliferating cells it is to our knowledge a novel finding for CLL cells. Effect on cell cycle was shown in Daudi mainly as a G1 arrest, while G2/M was not affected (Bezombes *et al* 2004). Use of another anti-CD20 antibody (1F5) in Ramos significantly reduced cell cycle progression (Shan *et al* 1998) while AT80 anti-CD20 also in Ramos increased proliferation (Chan *et al* 2003). Use of XRituximab in Ramos by the author showed anti-proliferative activity (data not shown). The differences noticed between these cells may be due to the nature of the cells since CLL cells are non cycling cells (G0/G1 arrested) while Daudi and Ramos are actively cycling cells or due to the type of antibody used. In any case these data suggest that anti-CD20 including Rituximab may affect cell cycle progression in B cells, with the outcome depending on the antibody used, presence of a crosslinker or the maturation state of the cells under test-treatment.

When the effects of Rituximab on apoptosis were established it was decided to check which signal transduction pathways may regulate induction of apoptosis. Previous work has shown that p38 is partially involved (Pedersen *et al* 2002a). Because another pathway activated by XRituximab with similar kinetics to p38 is JNK (Pedersen *et al* 2002a), it was hypothesised that JNK would be the other pathway involved in apoptosis induction since a JNK inhibitor was not used in the above study. Thus CLL were treated with XRituximab in the presence of a JNK specific inhibitor (SP600125) and surprisingly it was found that inhibition of JNK had no effect in induction of apoptosis at 24 hours and further induced apoptosis especially at 48 hours like inhibition of ERK1/2 pathway (U0126). This suggests that the two SAPK (Stress Activated MAP Kinases) pathways activated by XRituximab in B-CLL have distinct functions and also that there is another currently unknown pathway that together with p38 regulate induction of apoptosis by Rituximab in CLL cells.

Then Northern Blot analysis was performed initially for Tis11b/ Berg36 mRNA and then for the other 2 Tis11 family members (Tis11 and Tis11d) following XRituximab treatment for 3h. It was found that Tis11b/Berg36 mRNA is clearly induced at 3h by XRituximab and that this induction is regulated partially through the p38 pathway since inhibition of this pathway partially and in some cases completely abrogated induction of the gene. In some cases involvement of the JNK pathway was also noticed since inhibition of JNK by SP600125 abrogated by almost 30% induction of Tis11b/Berg36 following XRituximab treatment. Because there was no involvement of the JNK pathway in induction of apoptosis by XRituximab the latter result may suggest that Tis11b/Berg36 could be involved in another function following XRituximab treatment which may be regulated by JNK. Such function may be cell cycle regulation since Rituximab may affect cell cycle progression. The significant difference seen in the regulation of Tis11b/Berg36 mRNA after inhibition of p38 and JNK pathways was confirmed at the protein level as well further showing regulation of Tis11b/Berg36 by two independent pathways with the major one being the p38 pathway.

From the other 2 family members only Tis11d mRNA was induced by XRituximab in some but not all patients tested but the magnitude of induction was lower than Tis11b/Berg36. Induction of Tis11d was also under the p38 pathway in some cases. On the other hand Tis11 was found to be strongly expressed in unstimulated cells and

remained unaffected by XRituximab in most of the patients or was weakly downregulated in some of them. Variable induction of Tis11 in response to a stimuli (LPS) was shown in human Peripheral Blood Lymphocytes (Fairhurst et al 2003). In this study 4 normal donors were studied and in lymphocytes there was significant variation in response to LPS with 2 donors not responding at all, 1 donor showing a modest increase, and the remaining 1 donor showing a significant induction of Tis11 protein (Fairhurst et al 2003). The reasons for such variation are currently unknown. Another explanation for the results obtained for Tis11 could be the fact that Tis11 is induced with more rapid kinetics than Tis11b/Berg36 and thus returned to baseline levels at 3h post stimulation that was tested.

Then it was decided to test whether inhibition of Tis11b/Berg36 by a commercially available siRNA would affect apoptosis induction by XRituximab. Currently there is no commercially available siRNA for Tis11d which was also induced by XRituximab to test how it could affect apoptosis induction. B-CLL cells were transfected with a control siRNA labelled with FITC provided by the BLOCK-IT kit from Invitrogen which contains Lipofectamine. Several volumes of both Lipofectamine and siRNA was tested in order to improve transfection efficiency and in all cases it was found that transfection efficiency increased overtime (t72>t48>t24h) in all patients tested

It was found indeed that inhibition of Tis11b/Berg36 by siRNA significantly reduced by 30 up to 70% the efficiency of apoptosis induction by XRituximab using either Lipofectamine or a reagent called HyperFect claimed to be efficient in delivering small amounts of siRNA. In the latter case another control siRNA was included with no similarity to a known eukaryotic gene. Efficient downregulation of Tis11b/Berg36 mRNA and protein levels following Tis11b/Berg36 siRNA treatment (transfection by HyperFect) was shown by either quantitative Real Time PCR or intracellular staining and FACS analysis respectively.

Having shown downregulation of Tis11b/Berg36 by the siRNA and abrogation of apoptosis induction by XRituximab, it was decided to test whether Tis11b/Berg36 siRNA treatment would affect the levels of Bcl-2 since Bcl-2 has AREs in the 3' UTR and may be a target of Tis11b/Berg36. A total of 5 patients were tested and only 2 showed a weak downregulation of Bcl-2. This result even though disappointing, is in accordance with published data which showed that induction of apoptosis by Rituximab

treatment of CLL patients *in vivo* and *in vitro* was not accompanied by downregulation of Bcl-2 protein but instead there was downregulation of Mcl-1 and XIAP proteins (Byrd *et al* 2002). It was attempted to check what effect inclusion of Tis11b/Berg36 siRNA would have on Bcl-2 levels following XRituximab treatment in the two patients showing downregulation of Bcl-2 but no further change was noticed. This result may reflect limitations primarily due to minimal change in Bcl-2 protein levels following XRituximab treatment and transfection efficiency of CLL cells.

CHAPTER 4

IL-4, CD40 AND REGULATION OF TIS11 FAMILY IN CLL

4.1 INTRODUCTION

Even though B-CLL cells are highly resistant to apoptosis *in vivo,* they undergo spontaneous apoptosis when cultured *in vitro* in the absence of any stimuli which is evident 24 hours after the start of the culture period and increases over time. Spontaneous apoptosis was shown to be accompanied by activation of caspases -3,-7, -8 (King *et al* 1998) suggesting that caspase activation is intact in CLL cells and thus their resistance to apoptosis *in vivo* results from defects that lie upstream preventing caspase activation. The phenomenon of spontaneous apoptosis implies strong dependence of CLL cells on environmental stimuli found in the Bone Marrow, spleen or lymph node microenvironments such as cytokines or members of the Tumour Necrosis family (TNF) or on soluble factors present in serum such as albumin as recently shown (Jones *et al* 2003, Moran *et al* 2002).

4.1.1 Interleukins

Most of the Interleukins namely IL-1, IL-2, IL-4, IL-5, IL-6, IL-8, IL10, IL-13 have been studied for their effects on spontaneous apoptosis and among them IL-4 and IL-13 can strongly inhibit but IL-5 and IL-10 can further induce while IL-1 and IL-6 have no effect on spontaneous apoptosis in CLL cells. IL-8 was able to inhibit apoptosis when used up to 50ng/ml but induced apoptosis when used at a higher dose (di Celle *et al* 1996). IL-4 is the most efficient anti-apoptotic interleukin able to inhibit spontaneous apoptosis of CLL cells by up to 75% (Jewell *et al* 1995, Mainou-Fowler *et al* 1995, Panayiotidis *et al* 1993, Danescu *et al* 1992), followed by IL-13 which can inhibit spontaneous apoptosis by 50% (Chaouchi *et al* 1996). The effect of IL-4 was attributed to induction or stabilisation of Bcl-2 protein (Danescu *et al* 1992, Panayiotides *et al* 1993) or production of iNOS (inducible Nitric Oxide Synthase) which was shown to have anti-apoptotic effects in CLL cells (Levesque *et al* 2003). It is noteworthy that B-CLL cells have increased expression of IL-4 Receptor (Klein *et al* 2001) and thus it was appealing to speculate that there are increased levels of IL-4 in patients that may cause their resistance to apoptosis *in vivo.* Indeed one study showed that clonal B-CLL cells contain cytoplasmic IL-4 which they secrete in their culture medium and that the amount secreted is directly correlated with the number of the clonal B cells present (Kay *et al* 2001). It was also shown that CD4+ and CD8+ (Kay *et al* 2001) or CD2+

(Mainou-Fowler *et al* 2001) cells from CLL patients contain cytoplasmic IL-4 which they can also secrete (Kay *et al* 2001) in the culture medium. Both these studies suggest that IL-4 may act as an autocrine or paracrine growth factor for B-CLL cells. Among the other cytokines tested, IL-2 was able to inhibit spontaneous apoptosis and is the only cytokine that can also induce proliferation in CLL cells (Castejón *et al* 1999, Jewell *et al* 1995, Mainou-Fowler *et al* 1995). On the contrary IL-5 and IL-10 could further induce spontaneous apoptosis especially at 48 hours and 72 hours post stimulation (Castejón *et al* 1999, Mainou-Fowler *et al* 1994, Fluckiger *et al* 1994, Tangye *et al* 1998) accompanied by downregulation of Bcl-2 protein (Fluckiger *et al* 1994). However another study showed that IL-10 stimulation did not influence the levels of apoptosis or Bcl-2 protein in CLL cells (Jurlander 1997) or that inhibited spontaneous apoptosis without affecting the levels of Bcl-2 protein (Kitabayashi *et al* 1995). The reason for these discrepancies is unknown.

4.1.2 Interferons

IFN-□ and IFN-□ were shown to inhibit spontaneous and Hydrocortisone induced apoptosis in CLL (Zaki *et al* 2000, Jewell *et al* 1994, Panayiotides *et al* 1994, Buschle *et al* 1993).

4.1.3 Tumour Necrosis Factor Family (TNF family)

This heterogenous group consists of several members such as CD95 (Fas), TNF-□, CD40, BAFF, APRIL etc. Even though TNF-□was found to be increased in plasma of CLL patients compared to normal individuals it has been correlated with induction of cell proliferation rather than apoptosis (Jablonska *et al* 2005, Ferrajoli *et al* 2002, Guarini *et al* 1994, Foa *et al* 1990).

CD40 Receptor activation was also shown to increase proliferation of B-CLL cells *in vitro* either alone or in combination with IL-4 (Crawford and Catovsky *et al* 1993, Fluckiger *et al* 1992). Later on, other studies have clearly demonstrated that CD40 Receptor activation inhibits spontaneous (Bernal *et al* 2001, Cranziero *et al* 2001, Kitada *et al* 1999, Romano *et al* 1998, Wang *et al* 1997) as well as Fludarabine, Fas and Dexamethasone induced apoptosis in CLL cells (Kitada *et al* 1999, Romano *et al* 1998, Younes *et al* 1998). The anti-apoptotic effect of CD40 has been attributed to induction

of either NF-□B pathway (p65/RelA and p50/cRel complexes) (Romano *et al* 1998) or Bcl-XL, Mcl-1, Bfl-1, FLIPL and Survivin proteins (Bernal *et al* 2001, Granziero *et al* 2001, Kitada *et al* 1999). However not all patients respond to CD40 treatment (Granziero *et al* 2001, Wang *et al* 1997) and are thus subdivided into CD40 responders and non responders even though this response does not correlate with CD38 expression, stage of the disease, mutated or unmutated cases or previous treatments (Granziero *et al* 2001). CD40 has been shown to induce expression of CD40 Receptor on the surface of CLL cells suggesting that it may function as an autocrine growth factor especially in certain microenvironments such as the Bone Marrow or Lymph Nodes (Kitada *et al* 1999) affecting both proliferation and apoptosis. This is further supported by the fact that CLL patients have higher levels of CD40 Ligand in their serum in comparison with healthy individuals (Brugnoni *et al* 1995).

AIM:

To investigate the effect of CD40, IL-4, PMA and their combination on the regulation of Tis11 family in relation to inhibition of spontaneous apoptosis.

4.2 RESULTS

4.2.1 Inhibition of apoptosis

Because the molecular mechanisms underlying the phenomenon of spontaneous apoptosis and its reversion remain unresolved it was interesting to study the regulation of Tis11 family genes following inhibition of spontaneous apoptosis. This regulation will be compared with results obtained after treatment of CLL cells with Rituximab which is an inducer of apoptosis and have been discussed in the previous chapter (Chapter 3). Inhibition of spontaneous apoptosis in B-CLL cells was tested after culturing with IL-4, CD40L or their combination. In order to determine the optimal concentration of these agents, a dose response experiment was performed in the first patient (CLL1) using 5, 10, 15 μg/ml of IL-4 or 0.5 and 1 μg/ml of trimeric CD40L while apoptosis was determined using the Annexin/PI method. Due to poor compensation, analysis was performed for either Annexin or PI staining. According to Annexin staining the percentage of Annexin+ cells in unstimulated cells was 20% which was reduced to 10% by IL-4 at 5ng/ml, to 8.5% by IL-4 at 10 ng/ml and to 7.0% by IL-4 at 15 ng/ml (Figure 4.1). Similarly CD40L reduced Annexin$^+$ cells to 13% at 0.5 μg/ml and to 10% at 1μg/ml (Figure 4.1). Thus the most effective dose for IL-4 was 15 ng/ml and for CD40L was 1μg/ml and comprise the doses used in subsequent experiments. B-CLL were also treated with 30nM PMA alone or together with IL-4 because PMA will serve as a positive control for induction of Tis11b/Berg36 mRNA in CLL cells and it was previously shown to inhibit apoptosis in 4/12 patients tested (Barragán *et al* 2002). The cells were incubated with all the above stimuli for 24 hours and 48 hours at the end of which apoptosis was monitored using Annexin/PI and Propidium Iodide methods.

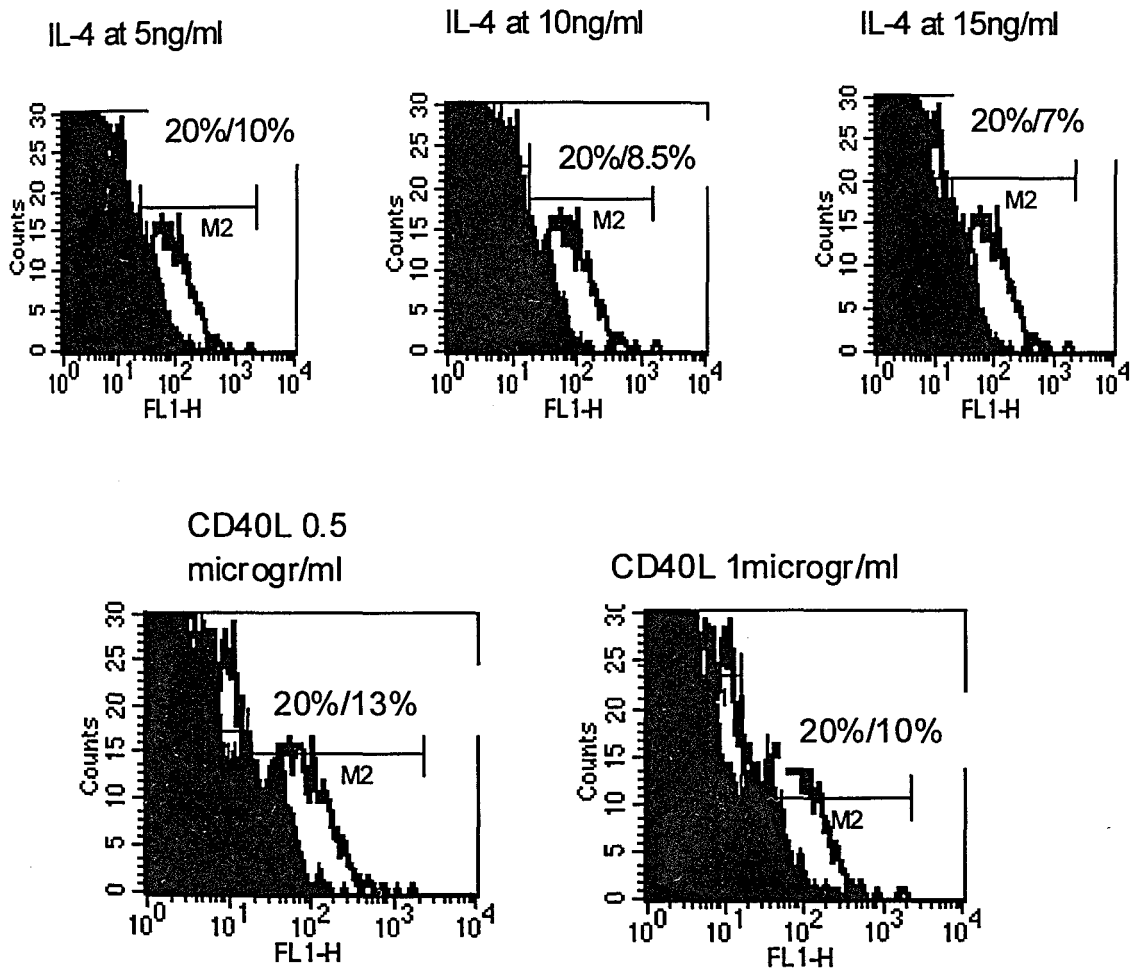


Figure 4.1: Dose response experiment for IL-4 and CD40L at 48 hours post stimulation.
CLL1 was left unstimulated or was stimulated with the indicated doses of IL-4 or CD40L for 48 hours and apoptosis was determined by Annexin staining. In all histograms the black filled line represents unstimulated cells and the open black line represents cells after stimulation. Gate M2 represents Annexin + cells and the first percentage above it, is Annexin+ cells in unstimulated cells while the second one is percentage of Annexin+ cells after stimulation with the indicated stimuli at the indicated dose.

According to the Annexin/PI method the percentage of cell viability (Annexin$^-$/ PI$^-$) at 24 hours from the start of the culture period, was 80.6%±4.0% (mean±sem) in unstimulated cells and was increased to 88.7%±2.3% (mean±sem) by IL-4 and to 85.4%±2.4% (mean ±sem) by CD40L (Figure 4.2a). When these two stimuli were combined the percentage of cell viability was further increased to 90.1%±2.00% (mean ±sem) (Figure 4.2a). The effect of all these stimuli was statistically significant with p□0.05. According to this method at 24 hours the most efficient inhibitor of spontaneous apoptosis is the CD40L+IL-4 combination followed by IL-4 followed by CD40L. PMA in combination with IL-4 did not affect spontaneous apoptosis at 24 hours even though PMA alone showed a tendency to inhibit spontaneous apoptosis (84.7%±4.6%) (Figure 4.2a) without reaching statistical significance. Data from two representative patients are shown in Figure 4.3a and 4.3b.

At 48 hours after the initiation of the culture period, the percentage of cell viability according to the Annexin/PI method in unstimulated cells was 77.7%±4.4% (mean±sem) and was increased to 90.0%±3.7% (mean±sem) by IL-4 and to 86.9%±2.5% by CD40L while their combination increased cell viability to 92.5%±3.0% (mean±sem) (Figure 4.2b). PMA alone did not have any significant effect on cell viability (78.2±5.6%) and surprisingly when combined with IL-4 showed a tendency to promote apoptosis (69.9%±7.0%)(Figure 4.2b). According to this method the most efficient inhibitor of spontaneous apoptosis at 48 hours is CD40L+IL-4 followed by IL-4, followed by CD40L. The effect of all 3 stimuli reached statistical significance with p□0.05. Data from two representative patients are shown in Figure 4.4a and 4.4b.

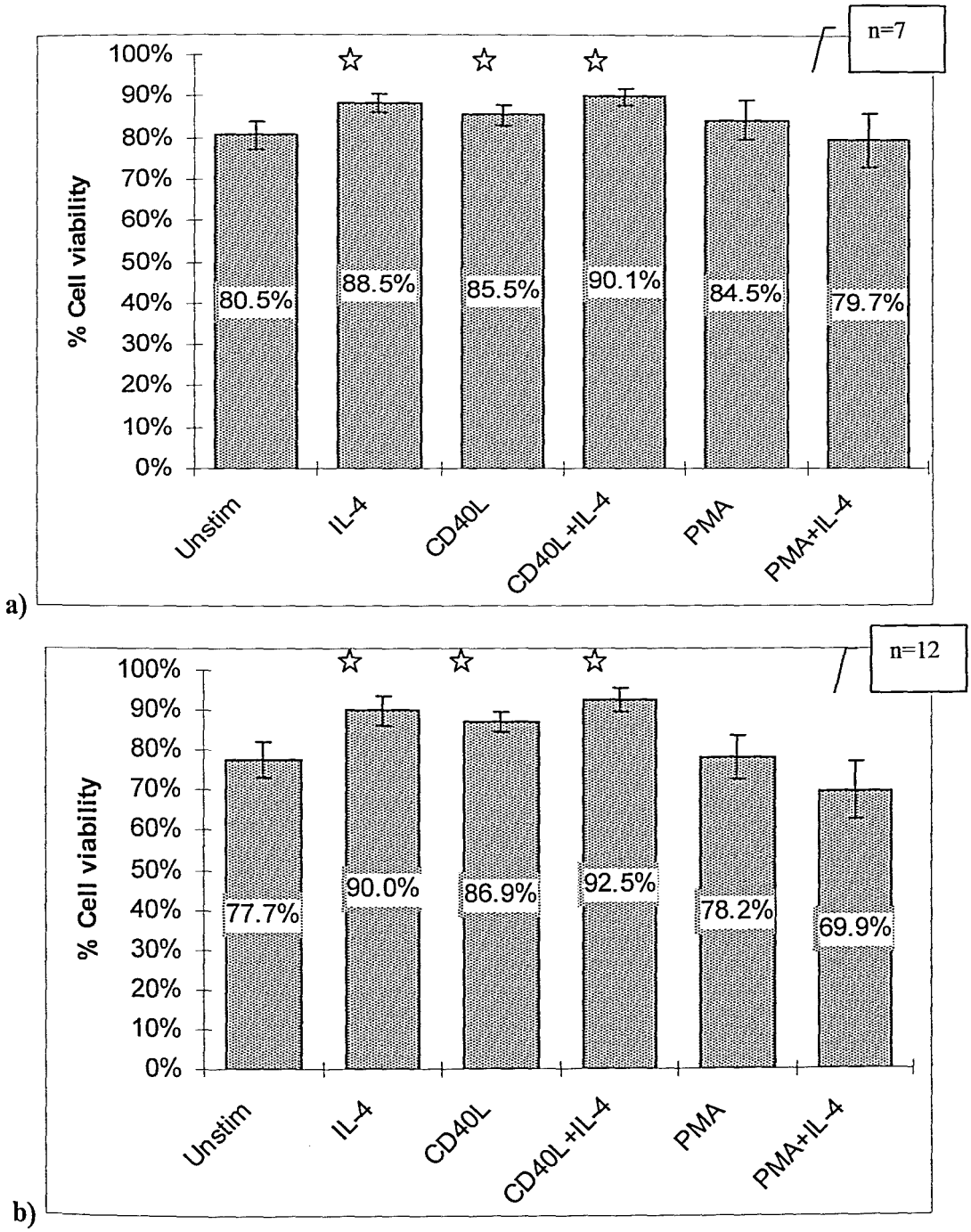


Figure 4.2: Inhibition of spontaneous apoptosis in B-CLL cells at 24 and 48 hours post stimulation as assayed by the Annexin/PI method

Freshly isolated B-CLL cells were left unstimulated or were stimulated with the indicated stimuli for **a)**24 hours or **b)**48 hours and apoptosis was measured using the Annexin/PI method which measures the percentage of cell viability ($Annexin^-/PI^-$) cells. In each bar graph the mean ±sem is plotted and error bars represent sem (sem=STDEV/$\sqrt{n}$ where STDEV is standard Deviation and n is the number of the patients tested). The presence of a star indicates statistical significance with $p \leq 0.05$.

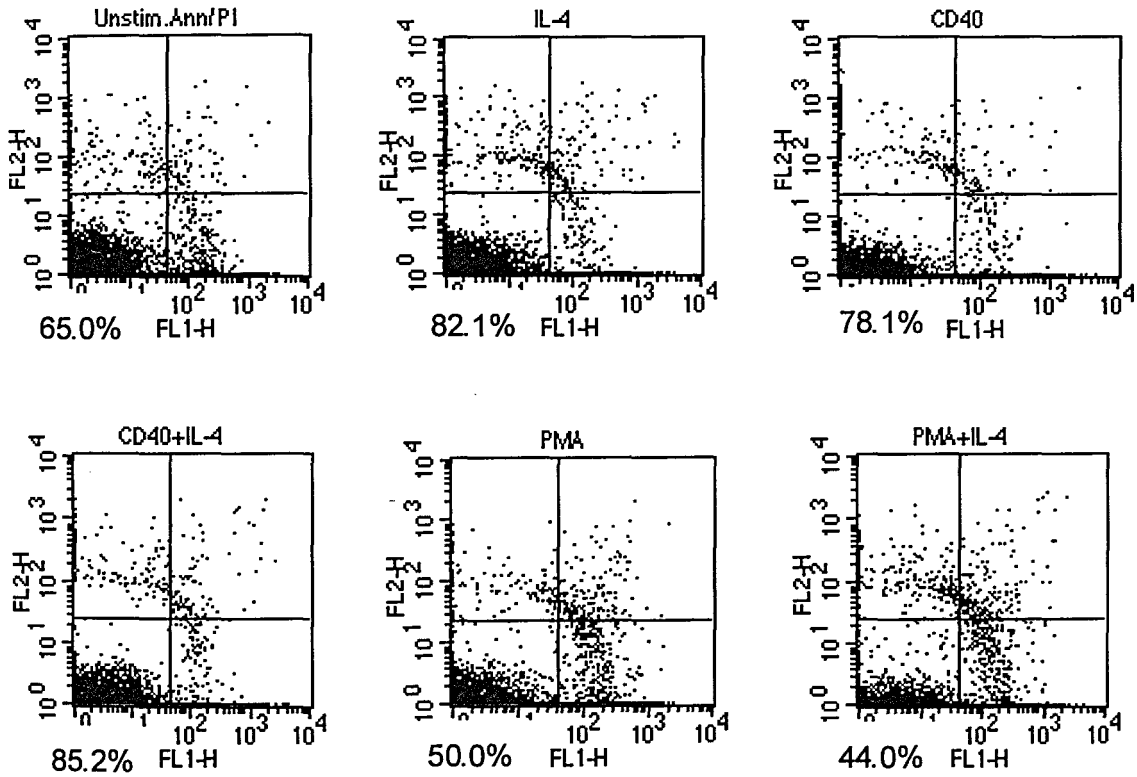


Figure 4.3a: Inhibition of spontaneous apoptosis in CLL cells assayed by the Annexin/PI method at 24 hours post stimulation.
B-CLL cells from CLL9 were left unstimulated or were stimulated with the indicated stimuli for 24 hours. Apoptosis was measured using the Annexin/PI method. The percentage of live cells is indicated in each case below the lower left quadrant. This is a representative patient from a total of 7 CLL patients tested. Top 3 quandrants from left to the right represent unstimulated cells, IL-4 stimulated cells and CD40L stimulated cells while bottom 3 quandrants from the left to the right represent CD40L+IL-4 stimulated cells, PMA stimulated cells and PMA+IL-4 stimulated cells.

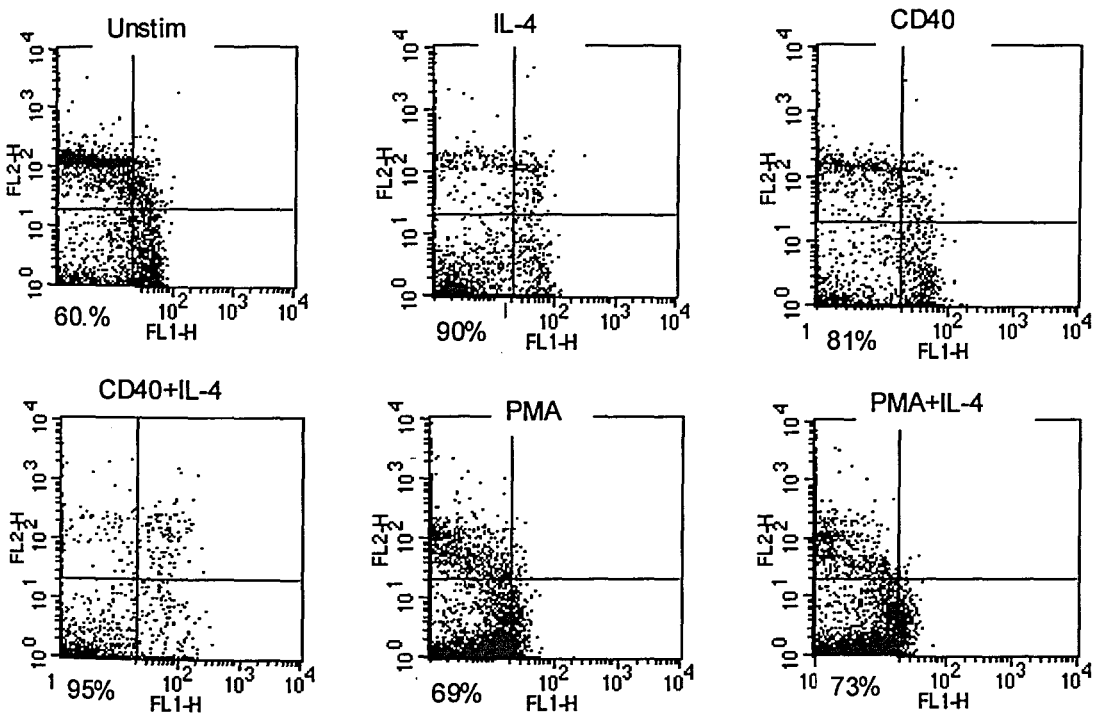


Figure 4.3b: Inhibition of spontaneous apoptosis in CLL cells assayed by the Annexin/PI method at 24 hours post stimulation.
B-CLL cells from CLL6 were left unstimulated or were stimulated with the indicated stimuli for 24 hours. Apoptosis was measured using the Annexin/PI method. The percentage of live cells is indicated in each case below the lower left quadrant. This is another representative patient from a total of 7 CLL patients tested. Top 3 quandrants from left to the right represent unstimulated cells, IL-4 stimulated cells and CD40L stimulated cells while bottom 3 quandrants from the left to the right represent CD40L+IL-4 stimulated cells, PMA stimulated cells and PMA+IL-4 stimulated cells.

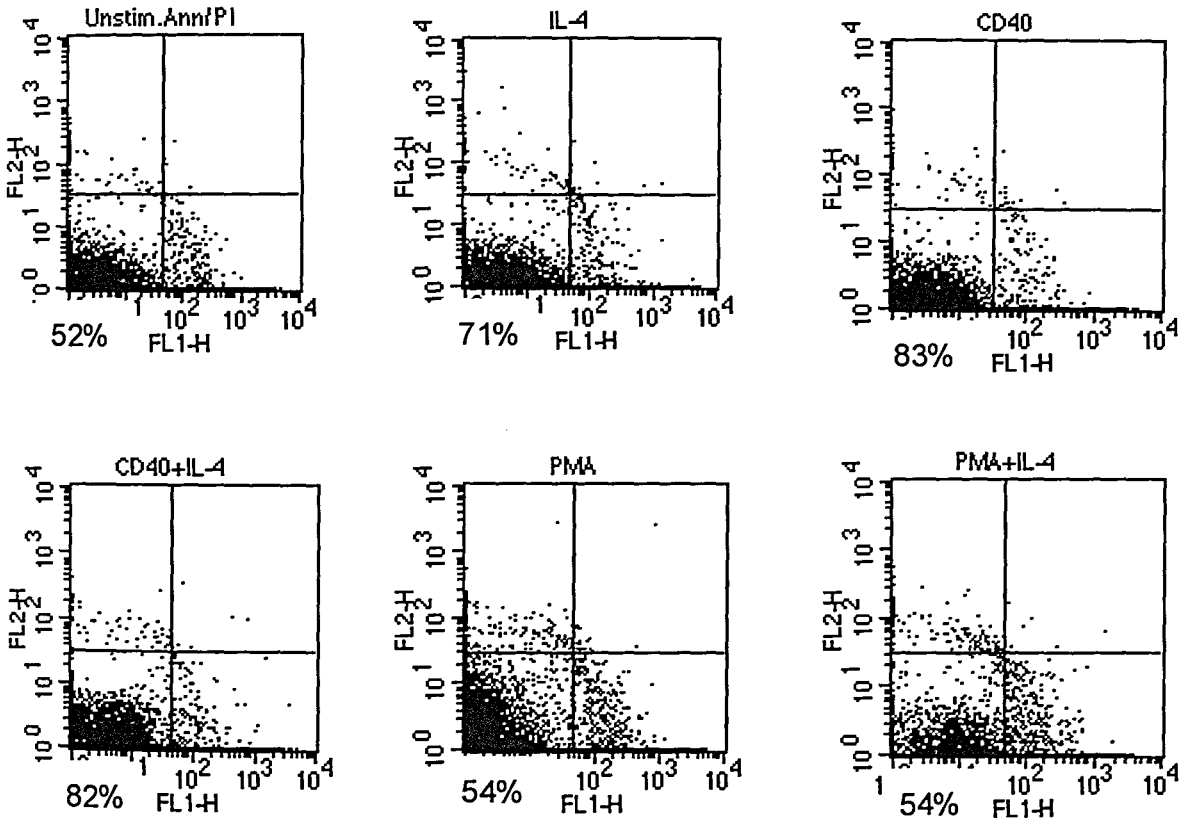


Figure 4.4a: Inhibition of spontaneous apoptosis in CLL cells assayed by the Annexin/PI method at 48 hours post stimulation.
B-CLL cells from CLL9 were left unstimulated or were stimulated with the indicated stimuli above each quandrant for 48 hours. Apoptosis was measured using the Annexin/PI method. The percentage of live cells is indicated in each case below the lower left quadrant. This is one representative patient from a total of 7 CLL patients tested. Top 3 quandrants from left to the right represent unstimulated cells, IL-4 stimulated cells and CD40L stimulated cells while bottom 3 quandrants from left to the right represent CD40L+IL-4 stimulated cells, PMA stimulated cells and PMA+IL-4 stimulated cells.

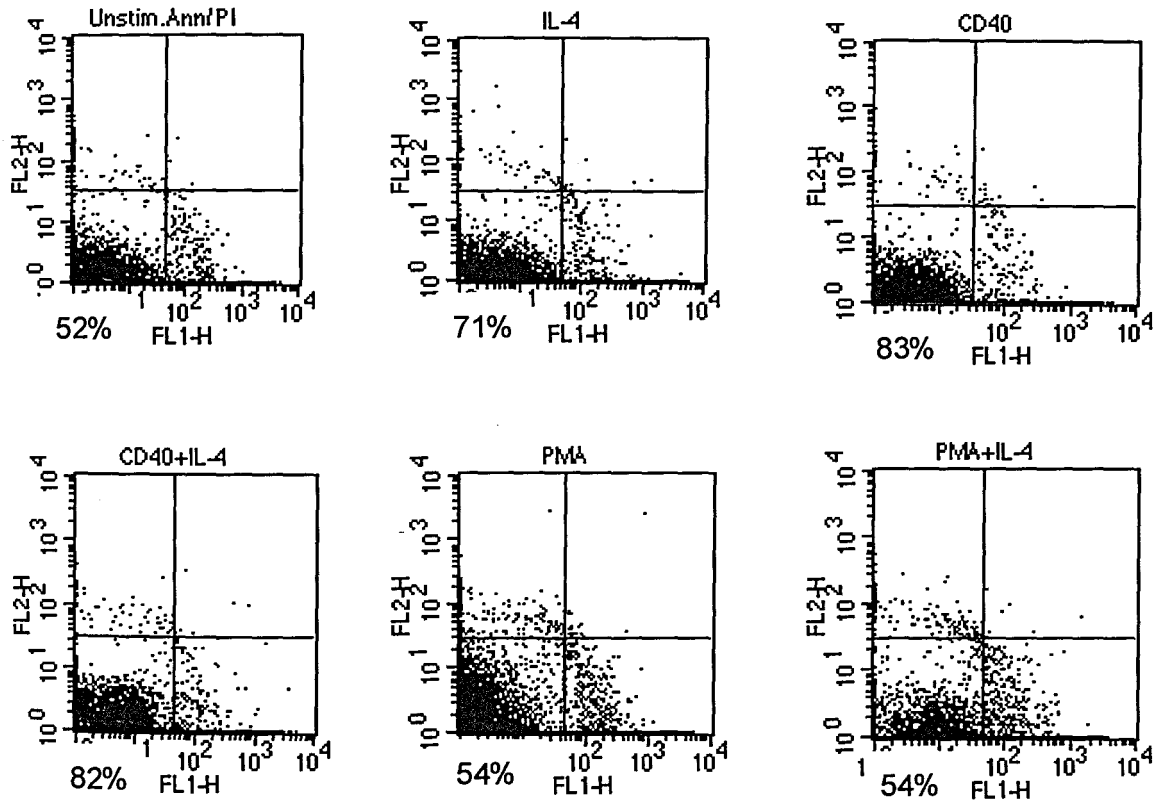


Figure 4.4b: Inhibition of spontaneous apoptosis in CLL cells assayed by the Annexin/PI method at 48 hours post stimulation.
B-CLL cells from CLL6 were left unstimulated or were stimulated with the indicated stimuli above each quandrant for 48 hours. Apoptosis was measured using the Annexin/PI method. The percentage of live cells is indicated in each case below the lower left quadrant. This is another representative patient from a total of 7 CLL patients tested. Top 3 quandrants from left to the right represent unstimulated cells, IL-4 stimulated cells and anti-CD40 stimulated cells while bottom 3 quandrants from left to the right represent anti-CD40+IL-4 stimulated cells, PMA stimulated cells and PMA+IL-4 stimulated cells.

According to the PI method the percentage of apoptotic cells at 24 hours was 21.0%±4.9% (mean ±sem) and was reduced to 6.3%±1.2% (mean±sem) by IL-4 and to 14.2%±4.2 (mean±sem) by CD40L while their combination reduced apoptosis to 9.80%±5.80% (mean±sem) (Figure 4.5a). PMA alone reduced apoptosis to 12.3%±3.4% (mean±sem) and when combined with IL-4 further reduced apoptosis to 11.1±3.20% (mean±sem) (Figure 4.5a) suggesting that IL-4 can enhance the effect not only of CD40 but of PMA as well towards inhibition of apoptosis. Based on the PI data the most efficient inhibitor of apoptosis at 24 hours post stimulation was IL-4 followed by CD40L+IL-4 followed by PMA+IL-4. The effect of all stimuli used reached statistical significance with $p \leq 0.05$ apart from PMA ($p \leq 0.06$). Data from two representative patients are shown in Figure 4.6a and 4.6b.

According to the PI method at the same timepoint the percentage of apoptotic cells was 27.0%±5.0% (mean ±sem) in unstimulated cells and was reduced to 11.0%±2.6% (mean±sem) by IL-4 and to 15.8%±4.7% (mean±sem) by CD40L while their combination reduced apoptosis to 12.4%±5.3% (mean±sem). PMA alone reduced apoptosis to 17.8%±3.7% (mean±sem) and when combined with IL-4 reduced apoptosis to 18.8%±4.1% (mean±sem) and suggests that at this timepoint IL-4 does not further enhance the anti-apoptotic effect of PMA (Figure 4.5b). According to the PI method the most efficient inhibitor of spontaneous apoptosis at 48 hours post stimulation is IL-4 followed by CD40+IL-4 followed by CD40L (Figure 4.5b). The effect of all stimuli including PMA and PMA+IL-4 is statistically significant with $p \leq 0.05$. Results obtained from two repressenative patients are shown in Figure 4.7a and 4.7b.

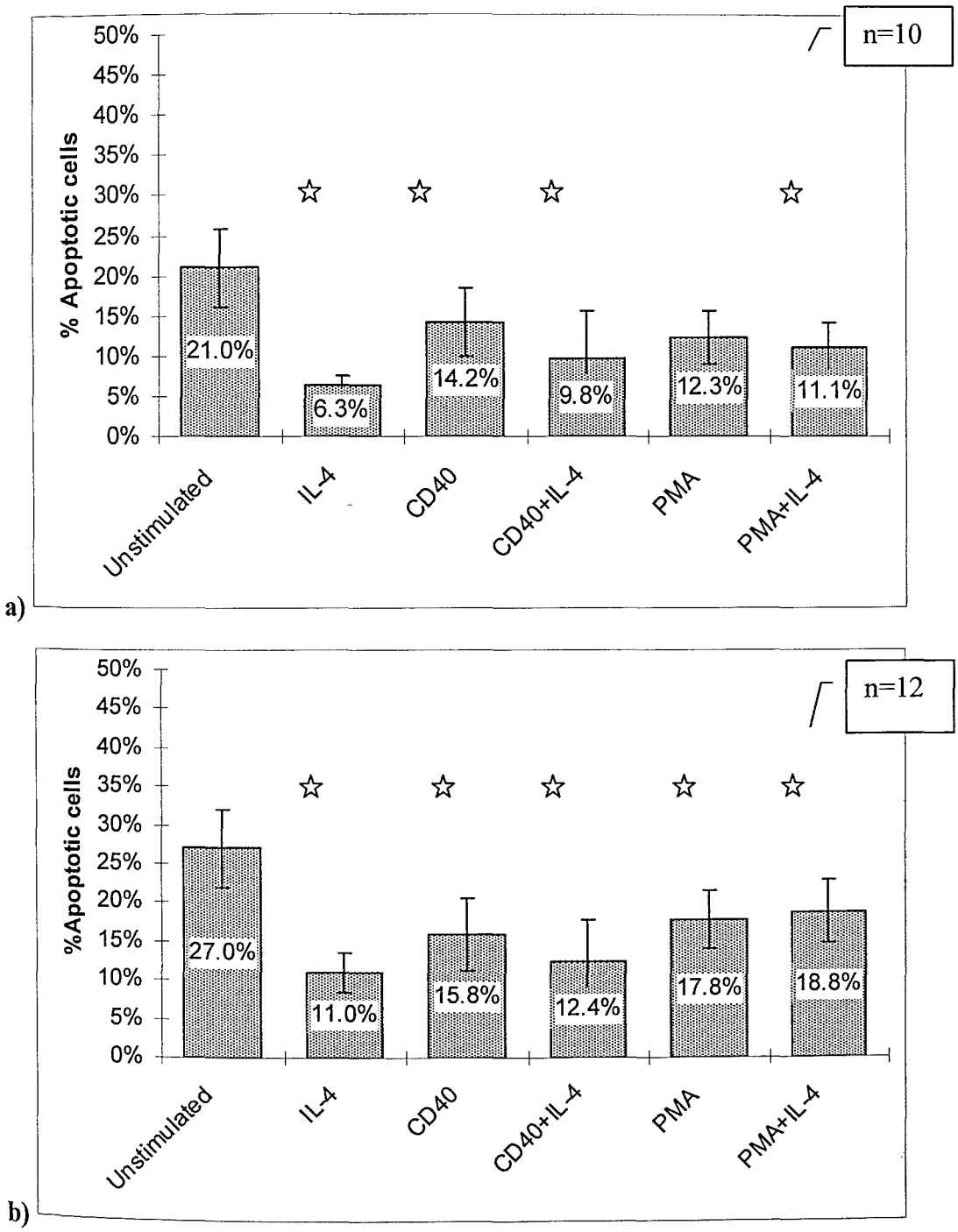


Figure 4.5: Inhibition of spontaneous apoptosis in B-CLL cells at 24 and 48 hours post stimulation as assayed by the Propidium Iodide (PI) method.
Freshly isolated B-CLL cells were left unstimulated or were stimulated with the indicated stimuli for **a)**24 hours or **b)**48 hours and apoptosis was measured using the PI method. In each bar chart the mean ±sem for each of the stimuli is plotted and error bars represent sem (sem=STDEV/$\sqrt{n}$ where STDEV is standard Deviation and n is the number of the patients tested). The presence of a star indicates statistical significance with $p \leq 0.05$

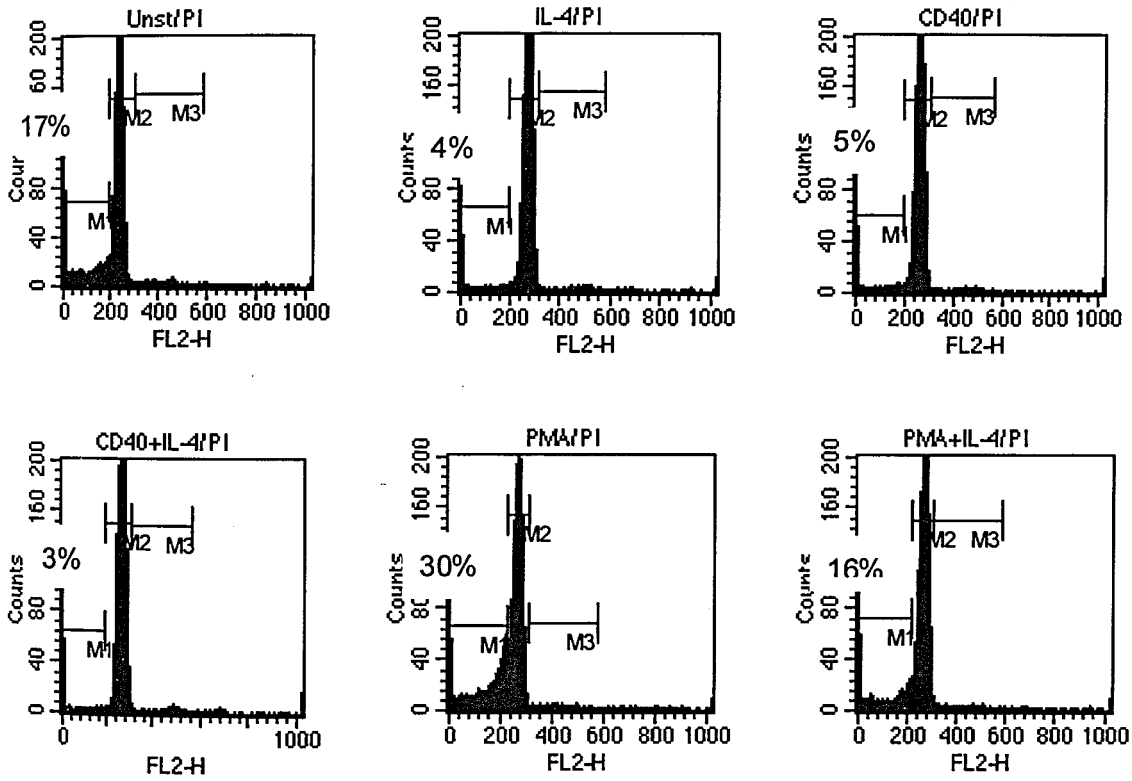


Figure 4.6a: Inhibition of spontaneous apoptosis in CLL cells assayed by the PI method at 24 hours post stimulation.
B-CLL cells from CLL9 were left unstimulated or were stimulated with the indicated stimuli for 24 hours and apoptosis was measured using the PI method. The percentage of apoptotic cells is indicated in each case above the M1 gate. This is one representative patient from a total of 10 CLL patients tested. Top 3 histograms from left to the right represent unstimulated cells, IL-4 stimulated cells and CD40L stimulated cells while bottom 3 histograms from left to right represent CD40L+IL-4 stimulated cells, PMA stimulated cells and PMA+IL-4 stimulated cells.

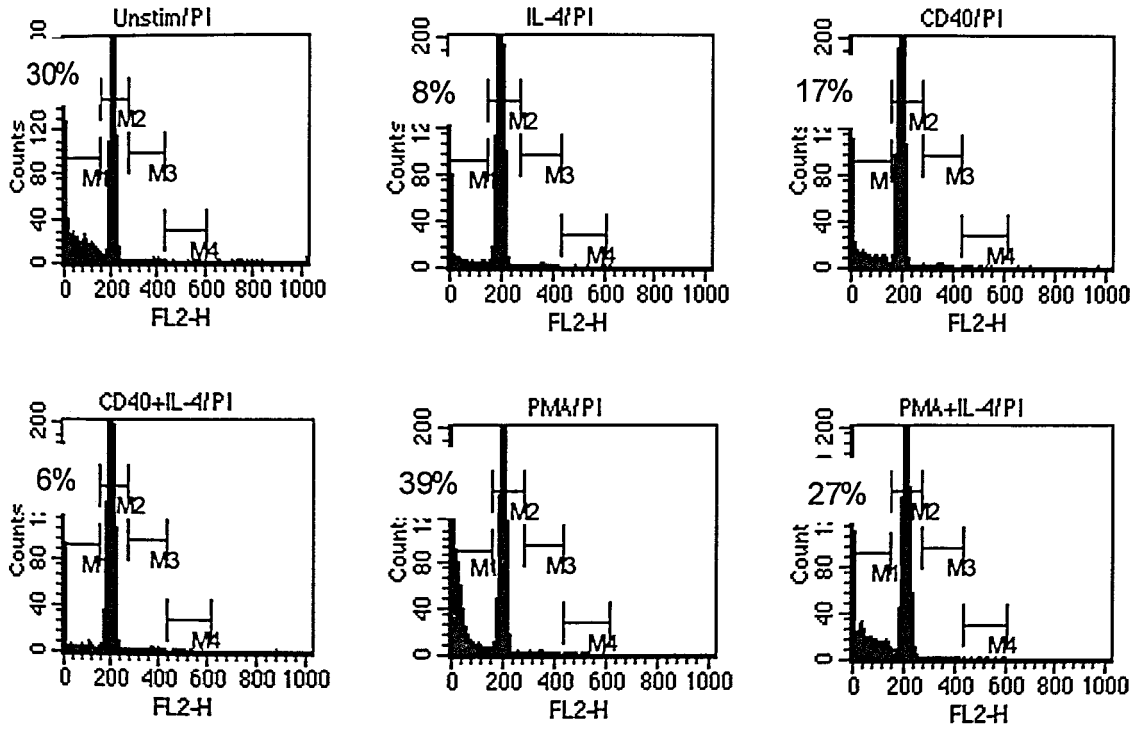


Figure 4.6b: Inhibition of spontaneous apoptosis in CLL cells assayed by the PI method at 24 hours post stimulation.

Purified B-CLL cells from CLL14 were left unstimulated or were stimulated with the indicated stimuli for 24 hours and apoptosis was measured using the PI method. The percentage of apoptotic cells is indicated in each case above the M1 gate. This is another representative patient from a total of 10 CLL patients tested. Top 3 histograms from left to the right represent unstimulated cells, IL-4 stimulated cells and CD40L stimulated cells while bottom 3 histograms from left to right represent CD40L+IL-4 stimulated cells, PMA stimulated cells and PMA+IL-4 stimulated cells.

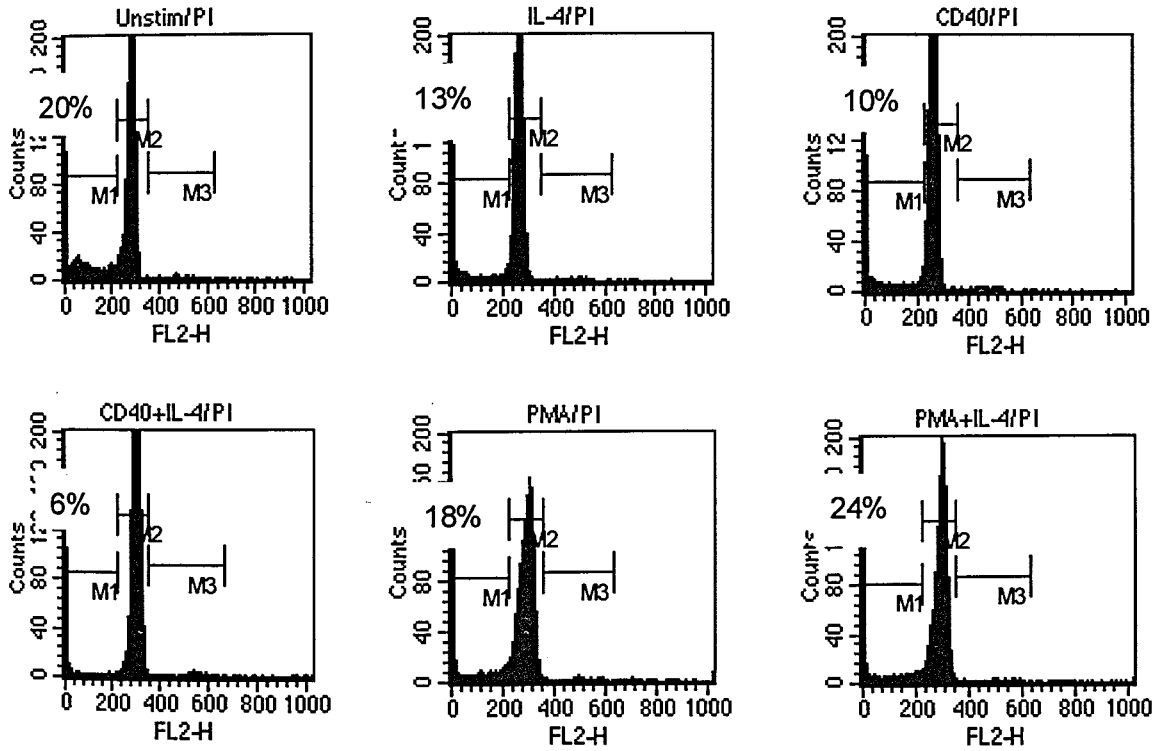


Figure 4.7a: Inhibition of spontaneous apoptosis in CLL cells assayed by the PI method at 48 hours post stimulation.
B-CLL cells from CLL9 were left unstimulated or were stimulated with the indicated stimuli for 48 hours and apoptosis was measured using the PI method. The percentage of apoptotic cells is indicated in each case above the M1 gate. This is one representative patient from a total of 10 CLL patients tested. Top 3 histograms from left to the right represent unstimulated cells, IL-4 stimulated cells and CD40L stimulated cells while bottom 3 histograms from left to the right represent CD40L+IL-4 stimulated cells, PMA stimulated cells and PMA+IL-4 stimulated cells.

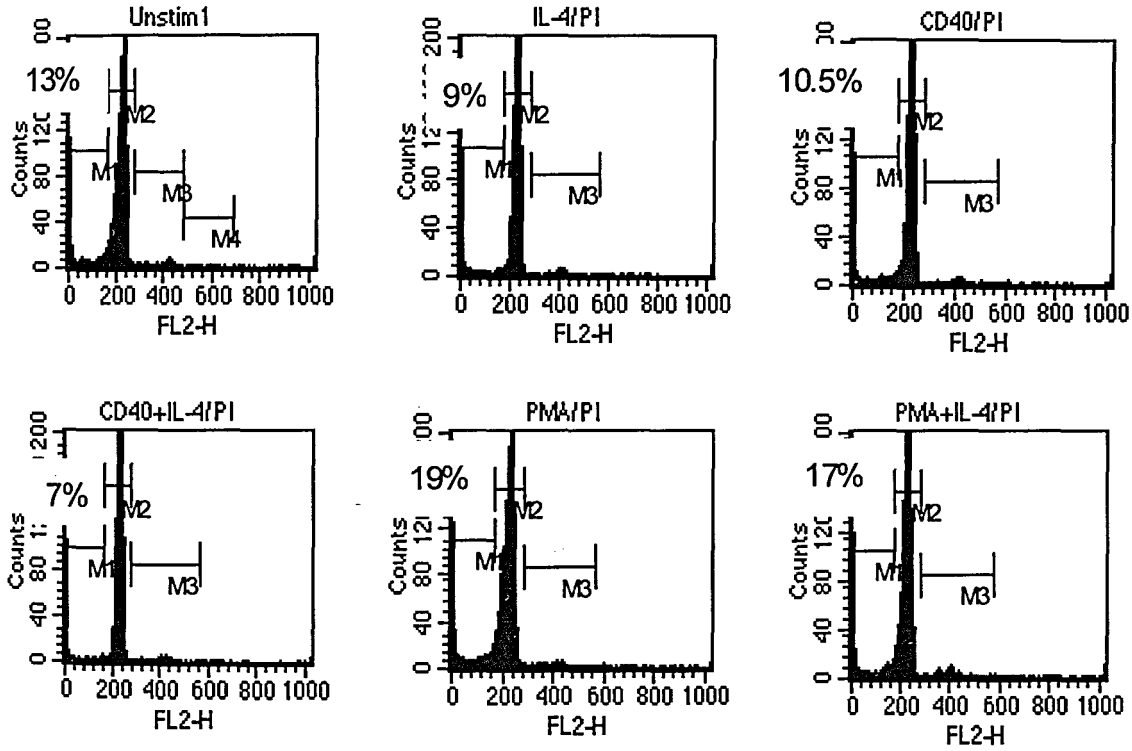


Figure 4.7: Inhibition of spontaneous apoptosis in CLL cells assayed by the PI method at 48 hours post stimulation.
B-CLL cells from CLL14 were left unstimulated or were stimulated with the indicated stimuli for 48 hours and apoptosis was measured using the PI method. The percentage of apoptotic cells is indicated in each case above the M1 gate. This is another representative patient from a total of 10 CLL patients tested. Top 3 histograms from left to the right represent unstimulated cells, IL-4 stimulated cells and CD40L stimulated cells while bottom 3 histograms from left to the right represent CD40L+IL-4 stimulated cells, PMA stimulated cells and PMA+IL-4 stimulated cells.

4.2.2 Regulation of the Tis11 family genes

After the effect on apoptosis was established, B-CLL cells were cultured in the presence of all these stimuli for 3h and the regulation of Tis11b/Berg36, Tis11 and Tis11d mRNA levels were tested by Northern Blot.

It was found that B-CLL cells do not express Tis11b/Berg36 mRNA in unstimulated cells (To-basal expression) or express it very weakly and this expression was strongly induced by PMA (as expected/positive control) and CD40L or anti-CD40. Even though in some cases CD40Receptor activation induced the gene as strongly as PMA, in most of the patients this activation was found to be weaker than PMA (Figure 4.8).

On the other hand IL-4 had no effect on basal or CD40 induced Tis11b/Berg36 mRNA levels. However when IL-4 was combined with PMA it further induced expression of the gene suggesting that this interleukin may be involved in the positive regulation of the gene but only under certain circumstances (Figure 4.8). Tis11b/Berg36 mRNA was also unaffected by serum since cultivation of the B-CLL cells for 3h in culture medium without any stimuli did not affect the basal expression of the gene (Figure 4.8).

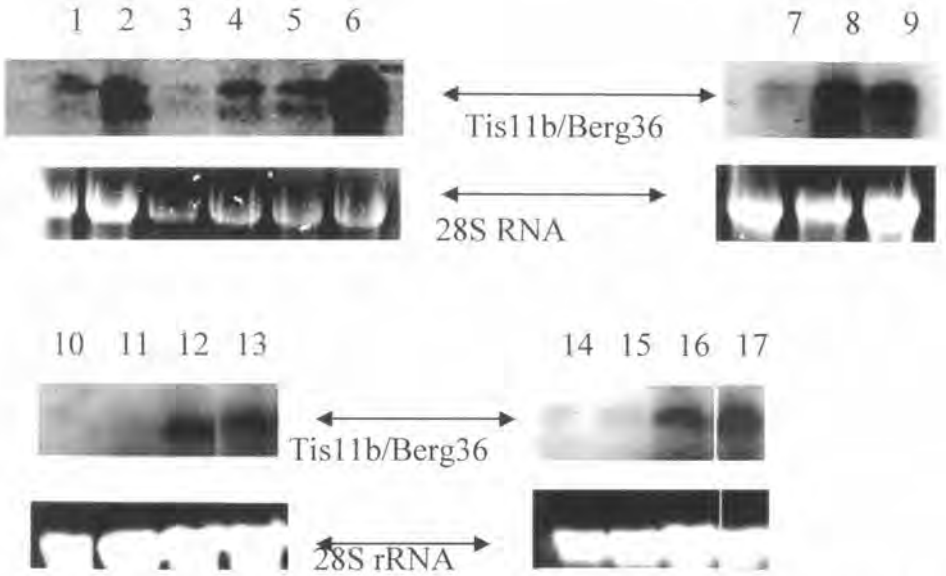


Figure 4.8: Northern Blot analysis of the Tis11b/ Berg36 expression in B-CLL cells
Freshly isolated B-CLL cells were processed for RNA extraction immediately after purification (To) or were cultured with the indicated stimuli for 3h. At the end of the culture period, the cells were processed for RNA extraction and Northern Blot hybridisation using a ^{32}P labelled cDNA probe for Tis11b/Berg36. Consistency of RNA loading as indicated by 28S rRNA staining is shown below each membrane. The double headed arrows indicate either the Tis11b/Berg36 mRNA specific band detected by the radiolabelled probe or the 28S rRNA band present in each lane.
Lanes:

1) CLL15 To(unstimulated cells),
2) CLL15 PMA 3h
3) CLL15 IL-4 3h
4) CLL15 CD40L 3h
5) CLL15 CD40L+IL-4
6) CLL15 PMA+IL-4 3h
7) CLL25 To (unstimulated cells)
8) CLL25 PMA 3h
9) CLL25 anti-CD40 3h
10) CLL38 To (unstimulated cells)
11) CLL38 Serum 3h
12) CLL38 PMA 3h
13) CLL38 anti-CD40 3h
14)CLL40 To (unstimulated cells)
15) CLL40 Serum 3h
16) CLL40 PMA 3h
17) CLL40 anti-CD40 3h

When Tis11 mRNA levels were tested in the same set of patients, it was found that in contrast to Tis11b/Berg36, Tis11 is strongly expressed at To (basal expression). This expression was not induced but instead it was found to be downregulated by CD40 alone or in combination with IL-4, in which case the basal expression of Tis11 mRNA was completely abrogated (Figure 4.9). The gene was weakly induced by PMA but this response was variable between different patients with some patients showing weak but clear induction and some others showing no response (Figure 4.9). Interestingly IL-4 when combined with PMA significantly induced Tis11 mRNA and formed the only combination that clearly induced expression of the gene at 3h (Figure 4.9) further supporting that IL-4 has different effects in gene regulation when combined with different stimuli. Also in contrast to Tis11b/Berg36, the basal expression of Tis11 was induced by serum at 3h and suggests that the induction noticed by PMA may be due to the presence of serum in the culture medium (Figure 4.9).

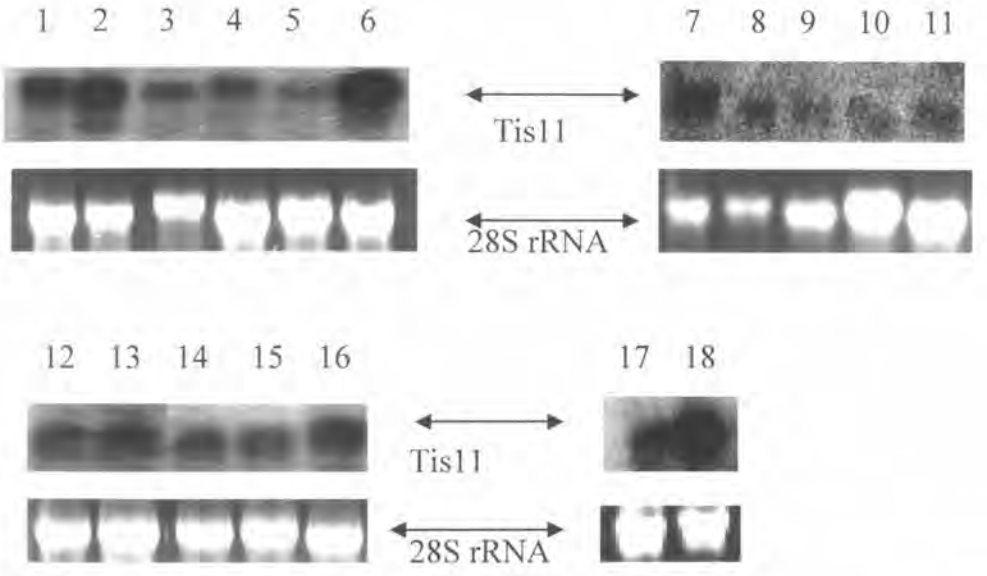


Figure 4.9: Northern Blot analysis of the Tis11 gene in B-CLL cells.
Freshly isolated B-CLL cells were processed for RNA extraction immediately after purification (To) or were cultured with the indicated stimuli for 3h. At the end of the culture period the cells were processed for RNA extraction and Northern Blot hybridisation using a ^{32}P labelled cDNA probe for Tis11 kindly provided by Dr. Brooks. Consistency of RNA loading as indicated by 28S rRNA staining is shown below each membrane. The arrows indicate either the Tis11 mRNA specific band detected by the radiolabelled probe or the 28S rRNA band present in each lane.
Lanes:
1) CLL15 To(unstimulated cells)
2) CLL15 PMA 3h
3) CLL15 IL-4 3h
4) CLL15 CD40L 3h
5) CLL15 CD40L+IL-4
6) CLL15 PMA+IL-4 3h
7) CLL33 To(unstimulated cells)
8) CLL33 PMA 3h, 9) CLL33 IL-4 3h
10) CLL33 anti-CD40 3h
11) CLL33 anti-CD40+IL-4
12) CLL23 To(unstimulated cells)
13) CLL23 PMA 3h
14) CLL23 anti-CD40 3h
15) CLL23 anti-CD40+IL-4 3h
16) CLL23 PMA+IL-4 3h
17) CLL39 To(unstimulated cells)
18) CLL39 Serum 3h

Finally Tis11d mRNA was found to be strongly expressed at To (basal expression) in B-CLL cells and this expression was not further induced by any of the stimuli used apart from PMA that clearly induced the gene in some but not all patients tested (Figure 4.8). In contrast to Tis11 the basal expression of Tis11d was not found to be downregulated by the CD40+IL-4 combination or anti-CD40 nor was affected by the PMA+IL-4 combination (Figure 4.10). Finally Tis11d mRNA was also induced by serum at 3h suggesting different regulation of Tis11 and Tis11d in comparison with Tis11b/Berg36 gene.

Overall these results suggest that Tis11 family members are regulated by distinct stimuli and probably pathways in B-CLL and thus probably there is no redundancy in their functions. Among the 3 genes Tis11 and Tis11d seem to be similar in terms of basal expression and PMA regulation. The major difference found between the 3 family members involved their regulation in response to CD40 Receptor activation (by either CD40L or anti-CD40) since it was found that Tis11b/Berg36 was induced by this stimuli, Tis11 was downregulated and Tis11d remained unaffected.

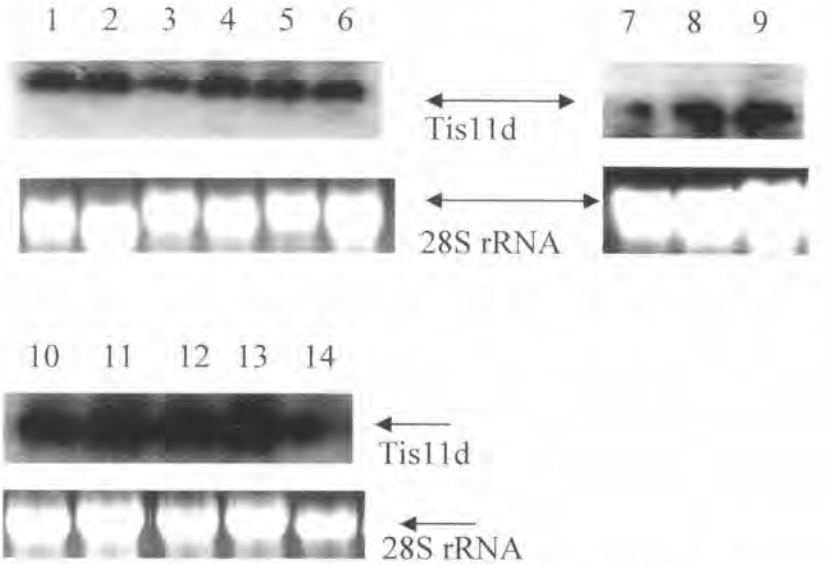


Figure 4.10: Northern Blot analysis of the Tis11d gene in B-CLL cells
Freshly isolated B-CLL cells were processed for RNA extraction immediately after purification (To) or were cultured with the indicated stimuli for 3h. At the end of the culture period the cells were processed for RNA extraction and Northern Blot hybridisation using a ^{32}P labelled cDNA probe for Tis11d. Consistency of RNA loading as indicated by 28S rRNA staining is shown below each membrane. The arrows indicate either the Tis11d mRNA specific band detected by the radiolabelled probe or the 28S rRNA band present in each lane.
Lanes:
1) CLL15 To (unstimulated cells)
2) CLL15 PMA 3h
3) CLL15 IL-4 3h
4) CLL15 CD40L 3h
5) CLL15 CD40L+IL-4
6) CLL15 PMA+IL-4 3h
7) CLL40 To(unstimulated cells)
8) CLL40 Serum 3h
9) CLL40 PMA 3h
10) CLL23 To(unstimulated cells)
11) CLL23 PMA 3h
12) CLL23 anti-CD40 3h
13) CLL23 anti-CD40+IL-4 3h
14) CLL23 PMA+IL-4 3h

4.2.3 Time course experiment for Tis11

The fact that Tis11 was weakly induced by PMA in some but not all patients tested was rather unexpected, because this agent is expected to induce all Tis11 family members in almost all cell types. One possible explanation could be that Tis11 is induced in B-CLL cells by PMA with kinetics more rapid than Tis11b/Berg36 mRNA, and since it has AREs at the 3' end could return to baseline by the 3h timepoint tested in the previous experiments.

In order to test this hypothesis, B-CLL cells were stimulated with the same agents apart from the PMA+ IL-4 combination for 1h, 2h and 3h and expression of the Tis11 mRNA was tested by Northern Blot. For this experiment the cDNA used was kindly provided by Dr. Perry Blackshear. According to the Blackshear group the Tis11 cDNA hybridisation will result in the appearance of two bands with the lower one being the Tis11 specific band while a higher migrating band represents non specific binding (Lai *et al* 1990). It was found that IL-4 or anti-CD40 stimulation clearly downregulated the basal expression of Tis11 mRNA as early as 1h post stimulation which was complete by 2h post stimulation (Figure 4.11). The effects of PMA or the CD40+IL-4 combination are difficult to interpet due to unequal loading (Figure 4.11). Thus downregulation of Tis11 mRNA following CD40 or IL-4 treatment was found using two different cDNA probes and is a novel finding of this study.

To further confirm the differences in regulation of Tis11 and Tis11b/Berg36 mRNAs in B-CLL cells, the same timecourse experiment was performed for Tis11b/Berg36 mRNA. It was found that PMA induced the gene peaking at 2h and at 3h while anti-CD40 also strongly induced the gene but peaking at 1h post stimulation and remaining above basal levels at 3h post stimulation (Figure 4.12). The effects of IL-4 on Tis11b/Berg36 shown in Figure 4.12 are probably due to unequal loading.

These results clearly show that Tis11 and Tis11b/Berg36 are regulated by distinct stimuli and probably pathways in CLL in response to stimuli that inhibit spontaneous apoptosis. CD40 stimulation induces Tis11b/Berg36 but downregulates Tis11 mRNA, IL-4 has no effect on Tis11b/Berg36 mRNA but downregulates Tis11 and finally PMA induces Tis11b/Berg36 mRNA but is very difficult to say from the above experiments whether it has an effect on Tis11 mRNA.

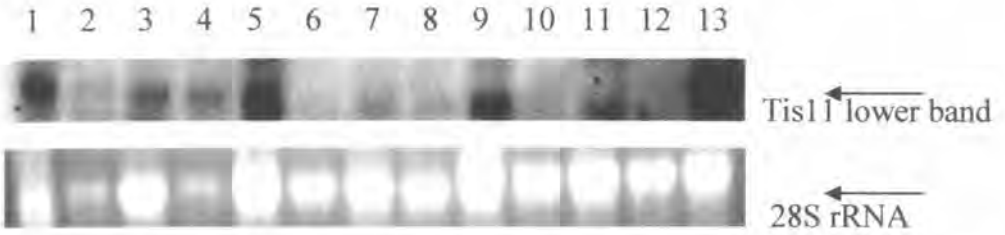


Figure 4.11: Timecourse experiment for Tis11 regulation in B-CLL cells
Freshly isolated B-CLL cells were processed for RNA extraction immediately after purification (To) or were stimulated with PMA, IL-4, CD40 and CD40+IL-4 for 1h, 2h, 3h as indicated and RNA was extracted and processed for Northern Blot analysis using a ^{32}P labelled cDNA for Tis11 (kindly provided by Dr. Blackshear). Consistency of RNA loading as indicated by 28S rRNA staining is shown below each membrane. The arrows indicate either the Tis11 mRNA specific band detected by the radiolabelled probe or the 28S rRNA band present in each lane.
Lanes:
1) CLL52 To(unstimulated cells),
2) CLL52 PMA 1h,
3) CLL52 IL-4 1h,
4) CLL52 anti-CD40 1h,
5) CLL52 anti-CD40+IL-4 1h,
6) CLL52 PMA 2h,
7) CLL52 IL-4 2h,
8) CLL52 anti-CD40 2h,
9) CLL52 anti-CD40+IL-4 2h,
10) CLL52 PMA 3h,
11) CLL52 IL-4 3h,
12) anti-CD40 3h,
13) CLL52 anti-CD40+IL-4 3h

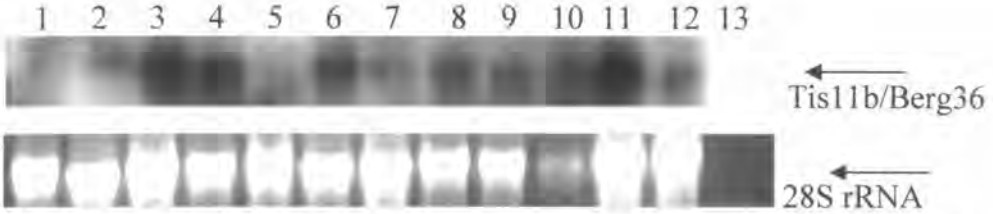


Figure 4.12: Timecourse experiment for Tis11b/Berg36 induction in B-CLL cells
Freshly isolated B-CLL cells were processed for RNA extraction immediately after purification (To) or were stimulated with PMA, IL-4, CD40, CD40+IL-4 for 1h, 2h or 3h as indicated and RNA was extracted and processed for Northern Blot analysis with a ^{32}P labelled cDNA probe for Tis11b/Berg36. Consistency of RNA loading as indicated by 28S rRNA staining is shown below each membrane. The arrows indicate either the Tis11b/Berg36 mRNA specific band detected by the radiolabelled probe or the 28S rRNA band present in each lane.
Lanes:
1) CLL52 To(unstimulated cells),
2) CLL52 PMA 1h,
3) CLL52 IL-4 1h,
4) CLL52 anti-CD40 1h,
5) CLL52 anti-CD40+IL-4 1h,
6) CLL52 PMA 2h,
7) CLL52 IL-4 2h,
8) CLL52 anti-CD40 2h,
9) CLL52 anti-CD40+IL-4 2h,
10) CLL52 PMA 3h,
11) CLL52 IL-4 3h,
12) anti-CD40 3h,
13) CLL52 anti-CD40+IL-4 3h

4.2.4 Regulation of Tis11 gene in normal tonsillar B cells

Then induction of the Tis11 family was tested in tonsillar B cells as a source of normal B lymphocytes and compared with their regulation in B-CLL cells in case the malignant phenotype of B-CLL cells may affect regulation of the Tis11 family genes.

It was found that Tis11b/Berg36 mRNA is strongly expressed at To (unstimulated cells/basal expression) and is not affected by any of the PMA, IL-4 or anti-CD40 stimuli at 1h but is induced by PMA and CD40 at 3h and 4h post stimulation in most of the samples tested. Because of the high basal expression, the response varied between samples with some samples showing no response. IL-4 alone was unable to induce the gene at 3 or 4h and did not affect induction of the gene when combined with anti-CD40 at 3 or 4h post stimulation. In contrast to CLL cells Tis11b/Berg36 mRNA, was induced by serum in tonsillar B cells (Figure 4.13).

On the other hand Tis11 was strongly expressed at To (unstimulated cells /basal expression) and remained unaffected by PMA at 1h or 3h. Stimulation with anti-CD40 clearly downregulated the basal expression of the gene at 1h and 3h and the same was noticed when anti-CD40 was combined with IL-4 (Figure 4.14). Thus the malignant phenotype of CLL cells can not explain downregulation of Tis11 following anti-CD40 treatment.

Finally Tis11d was also expressed strongly at To (unstimulated /basal expression) in tonsillar B cells and remained unaffected by all stimuli used at 1h post stimulation. However there was clear induction by PMA at 3h (Figure 4.14).

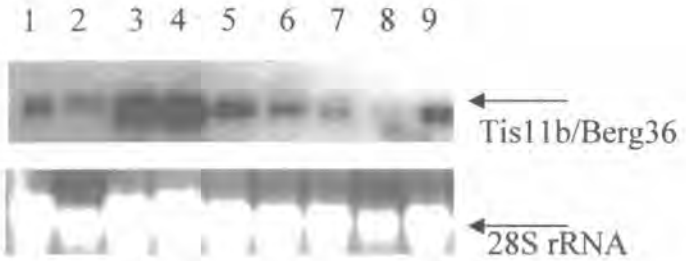


Figure 4.13: Regulation of Tis11b/Berg36 in tonsillar B cells.
Freshly isolated tonsillar B cells were processed for RNA extraction immediately after purification (To) or were cultured with the indicated stimuli for 1h to 4h. At the end of the culture period the cells were processed for RNA extraction and Northern Blot hybridisation using a ^{32}P labelled cDNA probe for Tis11b/Berg36. Consistency of RNA loading as indicated by 28S rRNA staining is shown below the membrane. The arrows indicate either the Tis11b/Berg36 mRNA specific band detected by the radiolabelled probe or the 28S rRNA band present in each lane.
Lanes:

1) TBC3 To(unstimulated cells) Tis11b,
2) TBC3 PMA 1h Tis11b,
3) TBC3 anti-CD40 1h Tis11b,
4) TBC3 TGF-β 1h Tis11b,
5) TBC3 ant-CD40+IL-4 1h Tis11b,
6) TBC3 PMA 3h Tis11b,
7) TBC3 IL-4 3h Tis11b,
8) TBC3 anti-CD40 3h Tis11b,
9) TBC3 anti-CD40+IL-4 3h

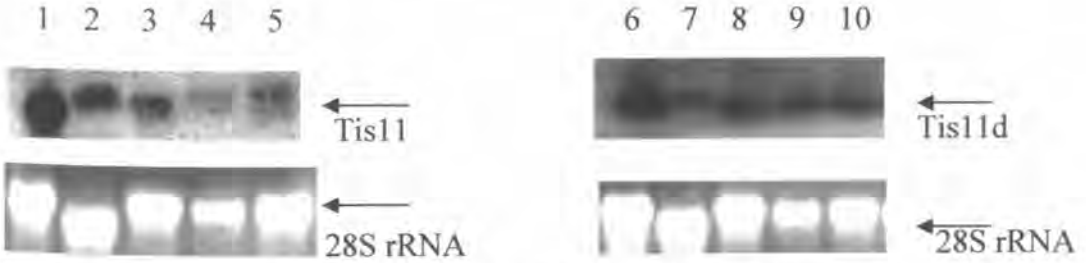


Figure 4.14: Regulation of Tis11 and Tis11d genes in tonsillar B cells.
Freshly isolated tonsillar B cells were procssed for RNA extraction immediately after purification (To) or were cultured with the indicated stimuli for 1h to 4h. At the end of the culture period the cells were processed for RNA extraction and Northern Blot hybridisation using a ^{32}P labelled cDNA probe for Tis11 (kindly provided by Dr. S. Brooks) or Tis11d. Consistency of RNA loading as indicated by 28S rRNA staining is shown below each membrane. The arrows indicate either the Tis11 or Tis11d mRNA specific bands detected by the radiolabelled probes or the 28S rRNA band present in each lane.
Lanes:

1) TBC4 To(unstimulated cells) Tis11,
2) TBC4 PMA 1h Tis11,
3) TBC4 IL-4 1h Tis11,
4) TBC4 anti-CD40 1h Tis11,
5) TBC4 anti-CD40+IL-4 1h Tis11,
6) TBC4 To(unstimulated cells) Tis11d,
7) TBC4 PMA 1h Tis11d,
8) TBC4 IL-4 1h Tis11d,
9) TBC4 anti-CD40 1h Tis11d,
10) TBC4 anti-CD40+IL-4 1h Tis11d

4.2.5 Induction of Tis11b/Berg36 protein by anti-CD40

Because according to the above experiments there was a clear induction of Tis11b/Berg36 mRNA by either CD40L or anti-CD40 in malignant (B-CLL) or normal B lymphocytes (tonsillar B cells), it was decided to test whether this change reflected change at the protein level. This was a significant finding since Tis11b/Berg36 mRNA was also induced by XRituximab a stimulus that induced apoptosis in both CLL and normal tonsillar B cells. Several hypotheses were made in order to explain this finding. The first one was that Tis11b/Berg36 mRNA regulation may not reflect regulation at the protein level. This becomes much clearer especially since Tis11b/Berg36 possesses AREs in its 3'UTR (just like Tis11) and it is induced by anti-CD40 earlier than when it is induced by PMA (Figure 4.12) or Rituximab (Mathas *et al* 2000). Thus under this scenario protein would be present at a certain timepoint for one stimulus but not for the other. A second possible explanation could be the fact that XRituximab and anti-CD40 may induce the gene through different pathways which would imply possible involvement of Tis11b/Berg36 protein in different functions. Another explanation that somehow links the first two, could be the fact that Tis11b/Berg36 protein is detected at a certain timepoint for both stimulus but possibly due to different regulation by different signal transduction pathways may be differentially phosphorylated and thus active or inactive since it was reported that Tis11 protein function is affected by phosphorylation (Chen *et al* 2006, Hitti *et al* 2006 Zhu *et al* 2001). Finally because mRNA degradation-decay occurs in the cytoplasm another possible explanation could be that Tis11b/Berg36 is localised in different cellular compartments following XRituximab or anti-CD40 treatment possibly and thus could be involved in mRNA decay in one case but not in the other.

The first priority was to test whether induction of Tis11b/Berg36 mRNA reflects induction of Tis11b/Berg36 protein as well. For this reason B-CLL cells were left untreated or were stimulated with anti-CD40, XRituximab or PMA for 3h and Tis11b/Berg36 protein was detected by FACS analysis after staining with anti-Tis11b/Berg36 antisera.

It was found that the change noticed at the mRNA level reflected the changes found at the protein level since all three stimuli used were able to induce the Tis11b/Berg36 protein at 3h post stimulation. PMA further induced the Tis11b/Berg36 protein in 64%

of cells, XRituximab further induced the protein in 54% of the cells while anti-CD40 treatment further induced the protein in 35% of cells (Figure 4.15) while the basal expression of the protein was detected in almost 15% of the cells. When the MFI values were analysed it was found that in unstimulated cells the MFI of Tis11b/Berg36 protein was 28.92 which was increased to 42.01 by PMA and to 33.85 by XRituximab (Figure 4.15).When anti-CD40 cells were analysed it was found that anti-CD40 increased the MFI of Tis11b/Berg36 to 21.1 from 19.1 found in unstimulated cells (Figure 4.15).
Thus, at the protein level Tis11b/Berg36 regulation mirrors changes found at the mRNA level, at which PMA was the strongest stimulus followed by XRituximab followed by anti-CD40 or CD40L. In conclusion Tis11b/Berg36 protein is induced by both stimuli that can induce (XRituximab) and inhibit (anti-CD40 or PMA) spontaneous apoptosis in B-CLL cells

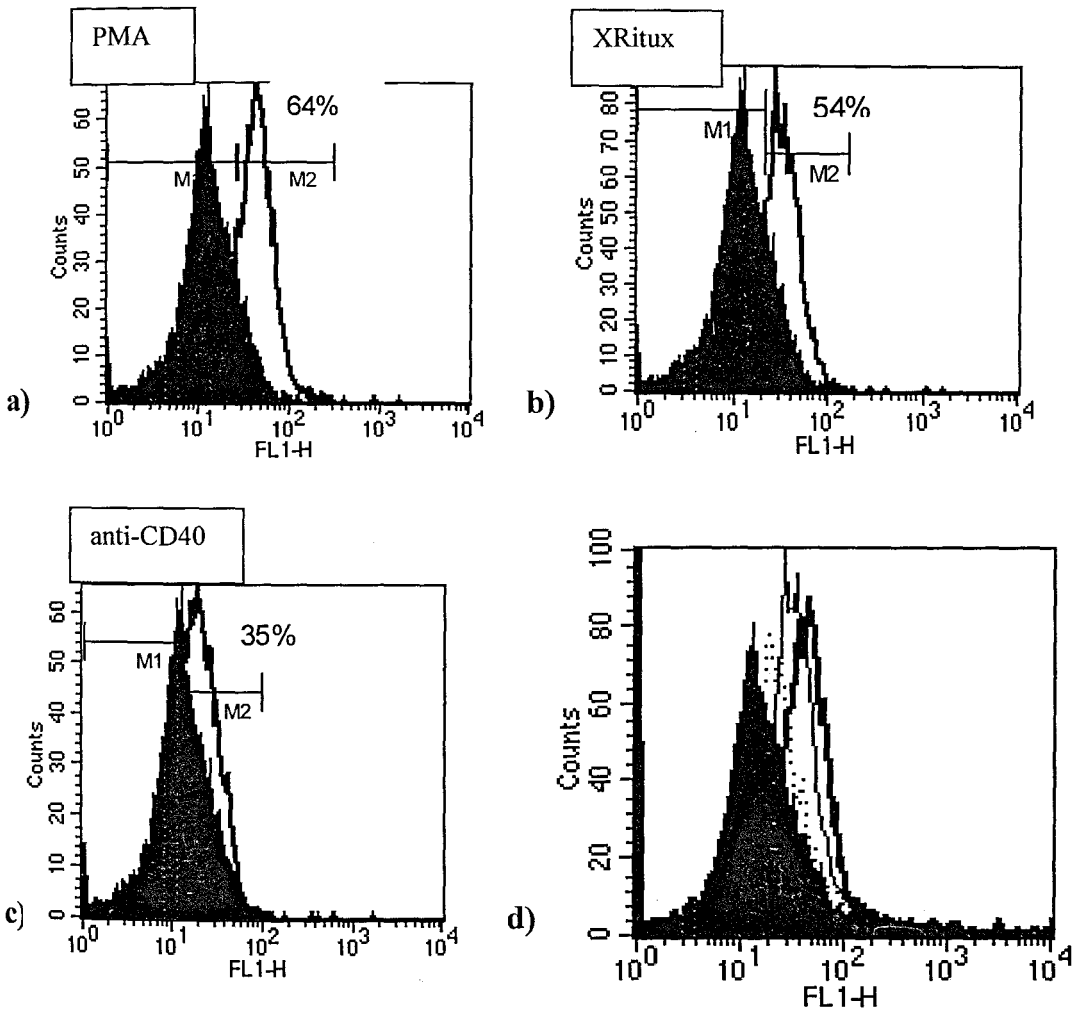


Figure 4.15: Induction of Tis11b/Berg36 protein by PMA, XRituximab, anti-CD40 in B-CLL cells as assayed by FACS analysis.
B-CLL cells from the same patient were left unstimulated (black filled line) or were stimulated with PMA, XRituximab or anti-CD40 for 3h and Tis11b/Berg36 protein was detected using Tis11b/Berg36 antisera and FACS analysis. a) Cells were stimulated with 30 nM PMA (black open line-overlay), b) Cells were stimulated with 20 μM XRituximab (black open line-overlay), c)Cells were stimulated with 1μg/ml anti-CD40 (black open line-overlay). The percentage above M2 gate represents the percentage of cells positive for Tis11b/Berg36 expression following stimulation-the percentage of cells positive for Tis11b/Berg36 protein in unstimulated cells (basal expression).
d) The collective histogram of a),b) and c) showing unstimulated B-CLL cells (black filled line-peak), PMA stimulated cells (thick black open line-overlay), XRituximab stimulated cells (thin black open line-overlay) and anti-CD40 stimulated cells (dotted black open line-overlay).

4.2.6 Inhibition of signal transduction pathways:

Because it was shown in the previous section that all 3 stimuli are capable of inducing the Tis11b/Berg36 protein it was decided to test the second hypothesis that Tis11b/Berg36 induction could be regulated through different pathways following XRituximab (involvement of p38 mainly) and anti-CD40 or PMA stimulation. In order to test this hypothesis B- CLL cells were treated with PMA, anti-CD40 for 3h in the presence or absence of inhibitors specific for NF-□B (Gliotoxin, BAY117082), p38 (SB203580, SB202190), JNK (SP600125), ERK (U0126) and PI3K (LY294002) pathways. From the two NF-□B inhibitors used Gliotoxin is a proteasome inhibitor and has been reported to strongly inhibit NF-□B in lymphocytes while BAY117082 is an inhibitor of IKKa phosphorylation and thus a more selective inhibitor of the classical NF-□B pathway. Previous work has shown that Tis11b/Berg36 induction by PMA is PKC independent (Murphy *et al* 1993) and thus such an inhibitor was not included in this study.

It was found that p38 pathway is not involved in the induction of Tis11b/Berg36 mRNA by PMA or CD40L/anti-CD40. Instead it was found that the gene is regulated through NF-□B pathway following PMA or anti-CD40 treatment since inhibition of this pathway abrogated almost completely induction of the gene at 3h (Figure 4.16). Furthermore inhibition of NF-□B resulted in downregulation of the basal expression of Tis11b/Berg36 in the cases that the gene was clearly present. Surprisingly inhibition of ERK pathway did not affect the induction of the gene by PMA or anti-CD40.

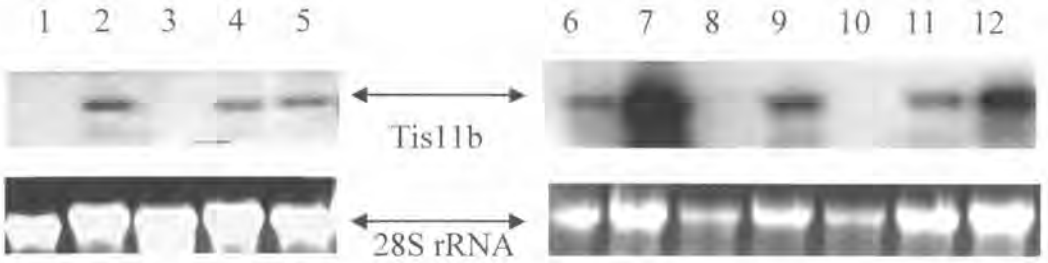


Figure 4.16: Inhibition of signal transduction pathways and their effects on Tis11b/Berg36 mRNA induction following PMA or anti-CD40 stimulation in B-CLL cells:

Freshly isolated B-CLL cells have been lysed for RNA extraction immediately after purification (To) or were stimulated for 3h with of PMA or a-CD40 alone or in the presence of specific inhibitors for the p38 (SB203580 or SB202190), JNK (SP600125) and ERK1/2 (U0126) pathways. At the end of the culture period the cells were lysed and RNA was extracted which and processed for Northern Blot hybridisation using a ^{32}P labelled cDNA probe for Tis11b/Berg36. Consistency of RNA loading as indicated by 28S rRNA staining is shown below each membrane. The arrows indicate either the Tis11b/Berg36 mRNA specific band detected by the radiolabelled probe or the 28S rRNA band present in each lane.

Lanes:

1) CLL30 To(unstimulated cells) Tis11b,
2) CLL30 PMA 3h Tis11b
3) CLL30 Glt+PMA 3h Tis11b
4) CLL30 SB202190+PMA 3h Tis11b
5) CLL30 SP600125+PMA 3h Tis11b
6) CLL24 To(unstimulated cells) Tis11b
7) CLL24 PMA 3h Tis11b
8) CLL24 Glt+PMA 3h Tis11b
9) CLL24 anti-CD40 3h Tis11b
10) CLL24 Glt+anti-CD40 3h Tis11b
11) CLL24 SB202190+anti-CD40 3h Tis11b
12) CLL24 SP600125+ anti-CD40 3h Tis11b

Because this experiment was done before the timecourse experiment (Figure 4.12) revealed that peak induction of Tis11b/Berg36 mRNA by anti-CD40 was at 1h post stimulation, the above experiment was repeated. This time B-CLL cells were stimulated for 1h with anti-CD40 in the presence or absence of the inhibitors to ensure that the same pathway regulates the gene at 1h post stimulation just like at 3h and exclude effect on the stability of the Tis11b/Berg36 mRNA rather than transcription. Indeed it was found that inhibition of NF-□B pathway completely abrogated induction of Tis11b/Berg36 even at 1h post stimulation (Figure 4.17). Stimulation with PMA in the presence of inhibitors was not repeated at an earlier timepoint because the Tis11b/Berg36 mRNA levels were very similar at 3h and 2h following this stimulation.
In conclusion it was found indeed that XRituximab and PMA or anti-CD40 regulate Tis11b/Berg36 through different pathways (p38 and NF-□B respectively) and since protein was detected following treatment with 3 stimuli it suggests that Tis11b/Berg36 protein may have a function different from apoptosis at least in B-CLL cells.

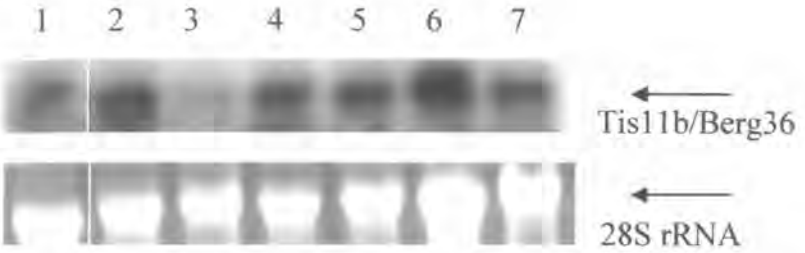


Figure 4.15: Regulation of Tis11b/Berg36 mRNA by anti-CD40 at 1h post stimulation in B-CLL cells

Freshly isolated B-CLL cells have been lysed for RNA extraction immediately after purification (To) or were stimulated for 1h with a-CD40 alone or in the presence of specific inhibitors for the NF-κB (BAY117280), p38 (SB203580), JNK (SP600125) and ERK1/2 (U0126) pathways. At the end of the culture period the cells were lysed and RNA was extracted which and processed for Northern Blot hybridisation using a ^{32}P labelled cDNA probe for Tis11b/Berg36. Consistency of RNA loading as indicated by 28S rRNA staining is shown below each membrane. The arrows indicate either the Tis11b/Berg36 mRNA specific band detected by the radiolabelled probe or the 28S rRNA band present in each lane.

Lanes:

1) CLL44 To(unstimulated cells)
2) CLL44 anti-CD40 1h
3) CLL44 BAY117280+anti-CD40 1h
4) CLL44 SB203580+anti-CD40 1h
5) CLL44 SP600125+ anti-CD40 1h,
6) CLL44 LY294005+anti-CD40 1h
7) CLL44 U0126+anti-CD40 1h

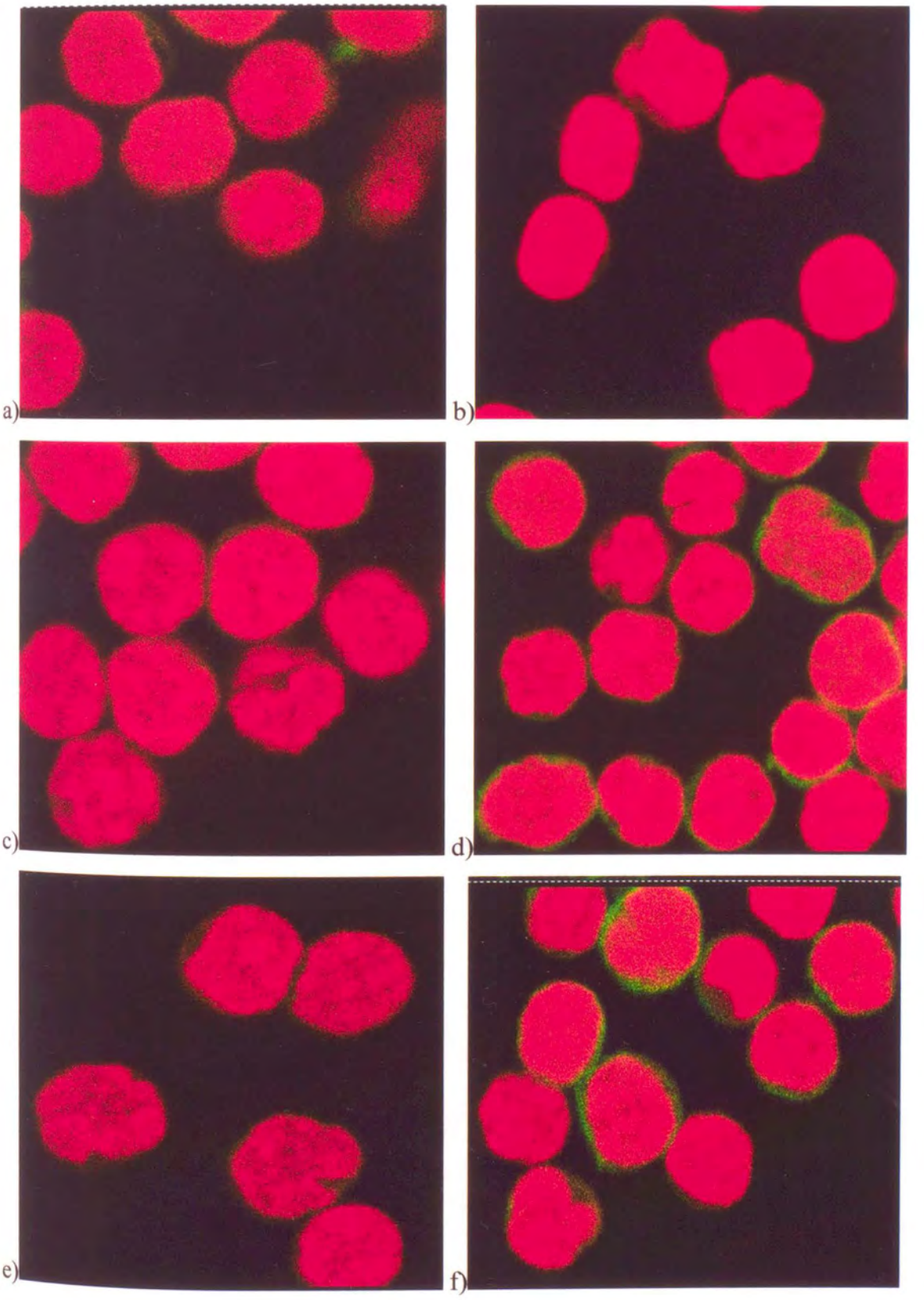
4.2.7 Confocal Microscopy and localisation of Tis11b/Berg36 protein

The next hypothesis to be tested in order to establish involvement of Tis11b/Berg36 in another function, was proper localisation of the protein for all 3 stimuli or in other words following regulation through at least 2 different pathways namely p38 and NF-□B.

It has been reported that Tis11 is located in the nucleus in unstimulated cells but it associates with Nup214/nucleoporin and shuttles in the cytoplasm after stimulation (Carman *et al* 2004, Taylor *et al* 1996a). The cytoplasm is considered the site where Tis11 induces degradation of the genes that contain AREs in the 3'UTR (Kedersha *et al* 2005, Fenger-Grøn *et al* 2005)

Thus B-CLL cells were left unstimulated or were stimulated with PMA, anti-CD40 and XRituximab for 3h and the localisation of the Tis11b/Berg36 protein was monitored using a LEICA confocal microscope. It was found that Tis11b/Berg36 protein is barely detectable in unstimulated B-CLL cells and when detectable is seen as a thin ring around the nucleus in roughly 8-12% of cells. Following stimulation with PMA, anti-CD40 or XRituximab the protein is clearly visible and it localises predominantly in the cytoplasm. It is seen as a diffuse staining resembling a thick ring around the nucleus. However in some cells stimulated with PMA, the Tis11b/Berg36 protein is visible in both the cytoplasm and the nucleus (Figure 4.16 and 4.17). Again even with this method the protein changes reflected changes at the mRNA level with PMA being the stronger inducer of the gene (higher percentage of cells with detectable Tis11b/Berg36 protein) followed by XRituximab followed by anti-CD40.

Finally and more importantly there was no difference in protein localisation between stimuli that inhibit (PMA, anti-CD40) or induce apoptosis (XRituximab) or p38 regulation and NF-□B regulation in B-CLL cells.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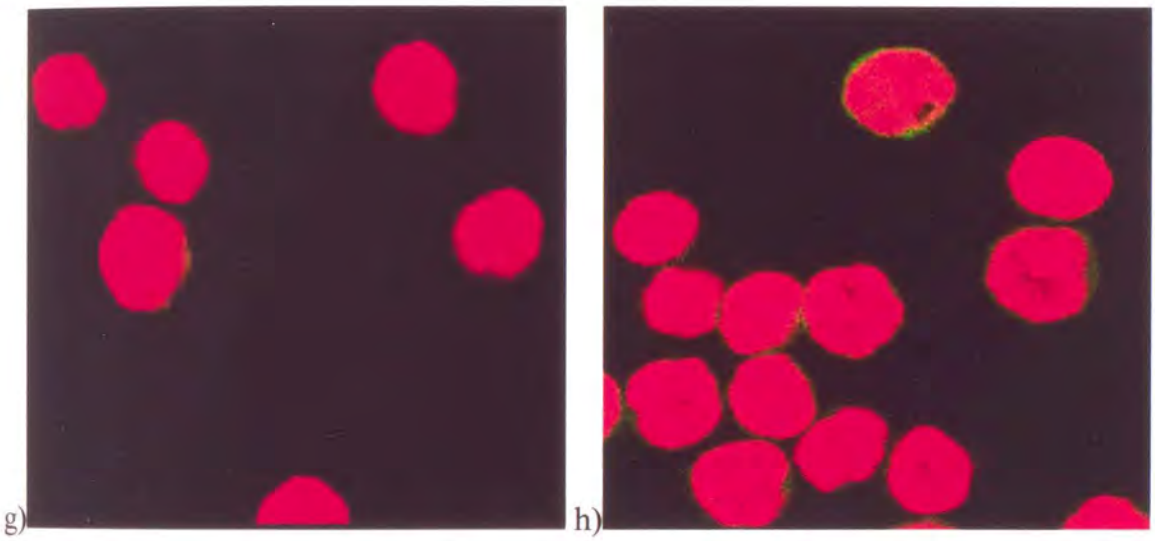
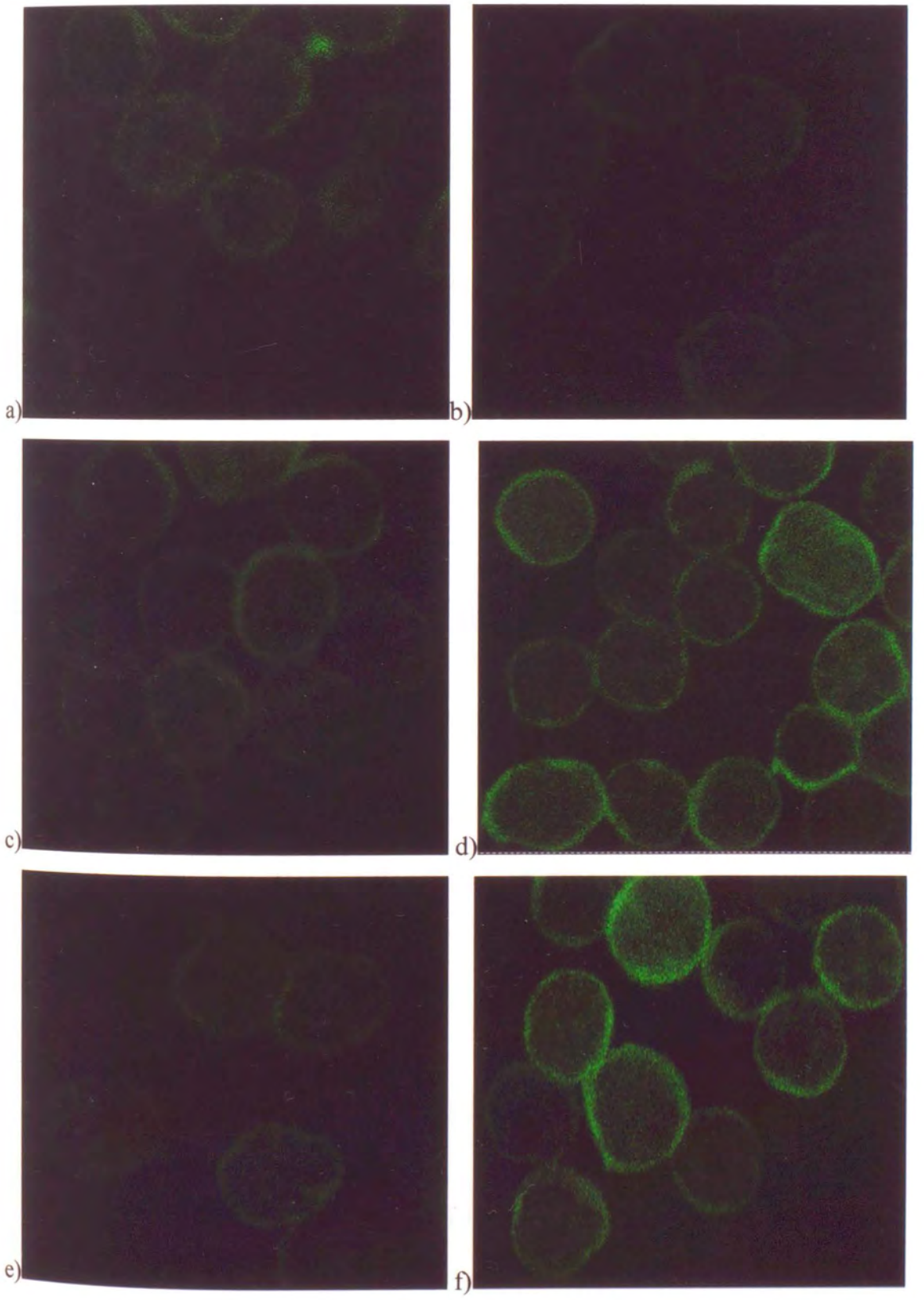


Figure 4.16: Induction and localisation of Tis11b/Berg36 protein following stimulation of B-CLL cells with PMA, XRituximab and anti-CD40 as assayed by Confocal microscope analysis.

B-CLL cells were left unstimulated or were stimulated with PMA, anti-CD40 or XRituximab for 3h and were stained with Tis11b/Berg36 antisera or normal rabbit serum plus anti-rabbit FITC, counterstained with Propidium Iodide to visualise integrity of the nucleus and analysed by LEICA confocal microscopy. A) unstimulated cells stained with normal rabbit serum, b)Unstimulated cells stained with Tis11b/Berg36 antisera, c) XRituximab treated cells stained with normal rabbit serum, d)XRituximab treated cells stained with Tis11b/Berg36 antisera, e) PMA treated cells stained with normal serum, f)PMA treated cells stained with Tis11b/Berg36 antisera, g) anti-CD40 treated cells stained with normal serum, h)anti-CD40 treated cells stained with Tis11b/Berg36 antisera. All images have been captured using a 60x lenses and represent the combined image obtained from P1 lazer (green for FITC) and the P2 lazer (red for Propidium Iodide).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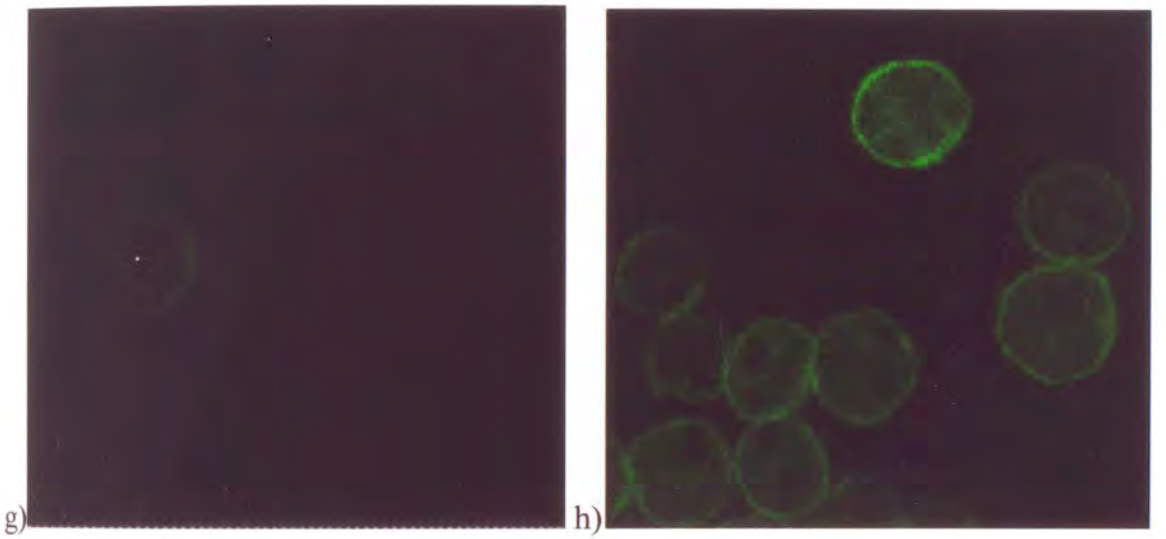


Figure 4.17: Induction and localisation of Tis11b/Berg36 protein following stimulation of B-CLL cells with PMA, XRituximab and anti-CD40 as assayed by Confocal microscope analysis.

B-CLL cells were left unstimulated or were stimulated with PMA, anti-CD40 or XRituximab for 3h and were stained with Tis11b/Berg36 antisera or normal rabbit serum plus anti-rabbit FITC, counterstained with Propidium Iodide to visualise integrity of the nucleus and analysed by LEICA confocal microscopy. a) Unstimulated cells stained with normal rabbit serum, b)Unstimulated cells stained with Tis11b/Berg36 antisera, c) XRituximab treated cells stained with normal rabbit serum, d)XRituximab treated cells stained with Tis11b/Berg36 antisera, e) PMA treated cells stained with normal serum, f)PMA treated cells stained with Tis11b/Berg36 antisera, g) anti-CD40 treated cells stained with normal serum, h)anti-CD40 treated cells stained with Tis11b/Berg36 antisera. All images have been captured using a 60x lenses and represent the image obtained from the P1 lazer (green for FITC).

4.2.8 Effect of Tis11b/Berg36 siRNA on apoptosis or differentiation after anti-CD40 or PMA treatment.

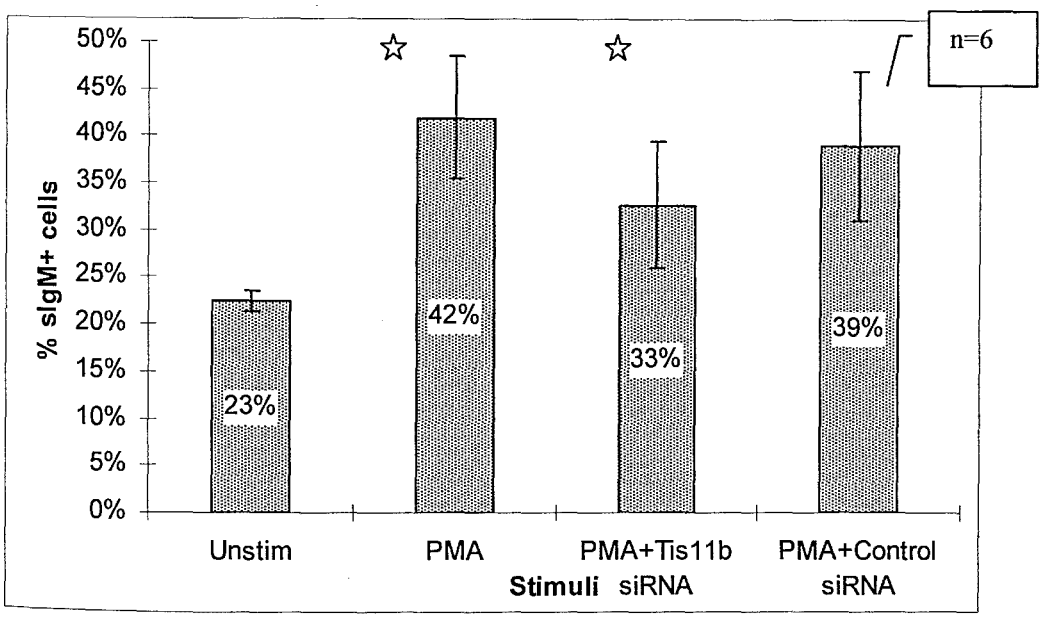
It was suggested by the previous experiments (regulation of Tis11b/Berg36 by NF-□B, proper localisation in the cytoplasm) that Tis11b/Berg36 may have a function other than apoptosis. The possible functions were cell cycle and differentiation since PMA and CD40Receptor activation can induce both functions in CLL cells (Minuzzo *et al* 2005, Takeuchi *et al* 2000, Tangye *et al* 1995, Fluckiger *et al* 1992).

In order to test possible involvement of Tis11b/Berg36 in differentiation siRNA technology was utilised which was applied primarily in PMA stimulated CLL cells even though 2 patients were stimulated with anti-CD40 as well. An involvement of Tis11b/Berg36 in differentiation is supported by the results in Chapter 6 in which is shown that CD40 ligation downregulated the basal expression of Tis11b/Berg36 mRNA in terminally differentiated plasma cells (Multiple Myeloma cell line) and by the studies in knock out mice that are embryonic lethal (Bell *et al* 2006, Stumpo *et al* 2004).

Induction of differentiation following PMA treatment was studied by induction of surface expression of IgM (sIgM) since unstimulated CLL cells have low or negative expression of sIgM. Thus B-CLL cells were stimulated with PMA and anti-CD40 for 72h in the presence or absence of Tis11b/Berg36 siRNA or control siRNA from QIAGEN and sIgM was measured by FACS analysis using a directly labelled anti-IgM-PE antibody. It was found that PMA strongly induced expression of sIgM in all patients tested while anti-CD40 ligation induced expression of the same cell marker in some but not all patients tested similar to what it has been noticed for inhibition of apoptosis. Induction of sIgM was stronger after PMA stimulation than after CD40 stimulation.

Analytically it was found that unstimulated CLL cells are 23.0%±1.1% (mean±sem) positive for sIgM which increased to 42.0%±6.5% (mean±sem, p□0.02) after stimulation with 30 nM PMA for 72h. In the presence of Tis11b/Berg36 siRNA, PMA increased the percentage of sIgM+ cells to 33.0%±6.7% (mean±sem, p□0.05) while in the presence of control siRNA it increased it to 39%±8.0% (mean±sem, p□0.2) (Figure 4.21). Data from a representative patient are shown in Figure 4.22.

This suggests that Tis11b/Berg36 is involved in differentiation of B-CLL cells to plasmablasts and probably it induces differentiation since inhibition of Tis11b/Berg36 by siRNA treatment reduced the efficiency of PMA to induce expression of sIgM as an indicator of B-CLL differentiation.



a)

Figure 4.21: Effect of Tis11b/Berg36 siRNA on sIgM induction following PMA treatment in B-CLL cells.
B-CLL cells were left unstimulated or were stimulated with 30 nM PMA for 72 hours in the absence or presence of either Tis11b/Berg36 siRNA or control siRNA. At the end of the culture period cells were stained with a mouse anti-human IgM PE antibody at the recommended by the manufacturer dilution. In the centre of each bar the mean percentage of cells positive for sIgM is shown and error bars represent sem (sem=STDEV/$\sqrt{n}$ where STDEV is standard Deviation and n is the number of the patients tested). The presence of a star indicates statistical significance with $p \leq 0.05$

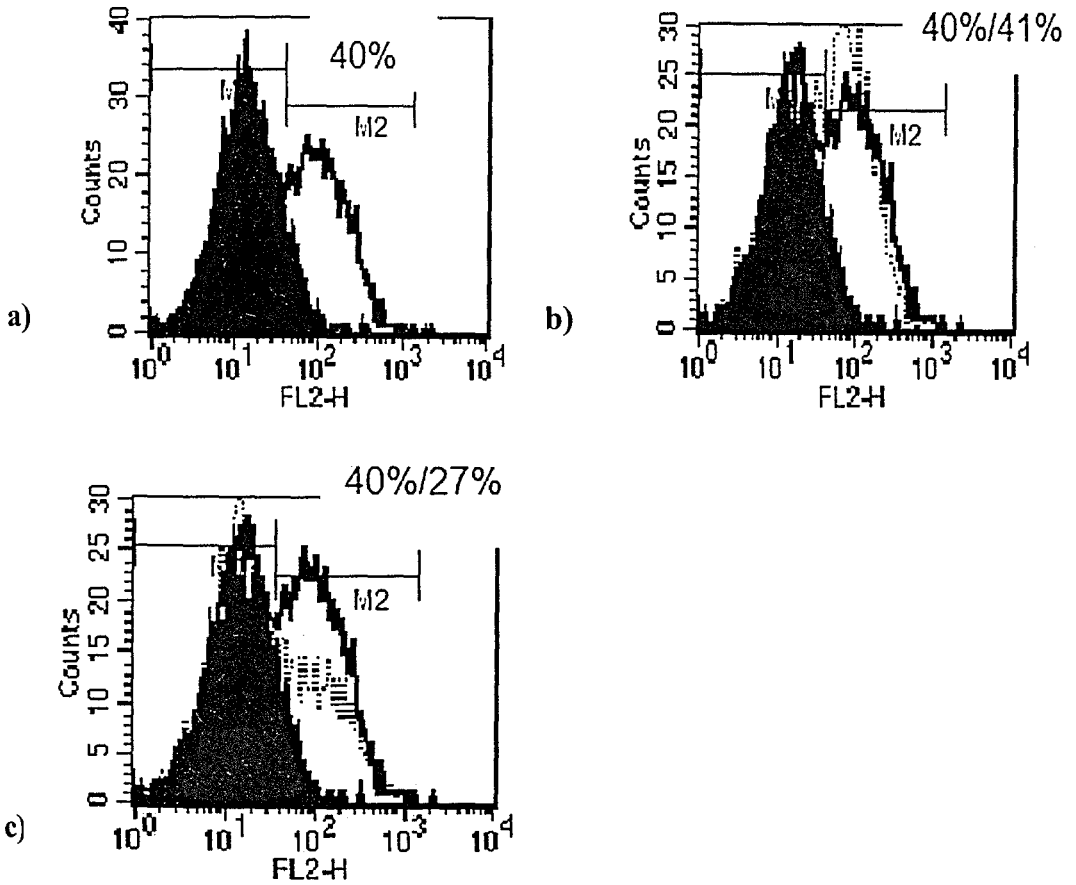


Figure 4.22: Effect of Tis11b/Berg36 siRNA on sIgM induction after PMA treatment in B-CLL cells

B-CLL cells from CLL30 were left unstimulated or were stimulated with 30 nM PMA for 72 hours in the absence or presence of either a control siRNA or Tis11b/Berg36. At the end of culture period cells were stained with sIgM PE and analysed by FACS analysis. a) The black filled overlay represents unstimulated cells while the thick black line-overlay represents PMA stimulated cells, b) The black filled overlay represents unstimulated cells while the thick black line-overlay indicates PMA stimulated cells and the dashed black line-overlay indicates PMA+control siRNA stimulated cells, c) The black filled overlay represents unstimulated cells while the thick black line-overlay indicates PMA stimulated cells and the dashed black line-overlay indicates PMA+Tis11b/Berg36 siRNA stimulated cells. In all cases 10,000 events were analysed and the percentage above M2 indicates the percentage of cells positive for sIgM. In b) and c) the first percentage is the percentage of sIgM positive cells after PMA and the second one is after PMA+control siRNA or Tis11b/Berg36 siRNA respectively

4.3 DISCUSSION

It was shown in Chapter 3 that Tis11b/Berg36 is strongly induced by XRitruximab and that inhibition of p38 pathway (that is partially responsible for induction of apoptosis by XRituximab) partially abrogated induction of Tis11b/Berg36 as well. Finally inhibition of Tis11b/Berg36 induction by siRNA technology significantly reduced apoptosis induction by XRituximab. Also it is well known that CLL cells when cultured *in vitro* in the absence of any stimuli undergo apoptosis defined as spontaneous apoptosis which can be inhibited by the presence of certain cytokines (eg:IL-4, IFN-□ IFN-□) or members of the TNF family (CD40) (Bernal *et al* 2001, Granziero *et al* 2001, Kitada *et al* 1999, Romano *et al* 1998, Wang *et al* 1997, Jewell *et al* 1995, Mainou-Fowler *et al* 1995, Panayiotidis *et al* 1993, Dancescu *et al* 1992). Because there are no data available regarding the regulation of the Tis11 family by any of the stimuli that inhibit spontaneous apoptosis it was interesting to check what effect such stimuli would have on their expression especially since Tis11 and Tis11d were found to be highly expressed in unstimulated B-CLL cells (Chapter 3). This basal expression may be downregulated by stimuli that inhibit spontaneous apoptosis. The stimuli that were chosen to inhibit apoptosis included IL-4, CD40 ligation and their combination, PMA alone or combined with IL-4.

IL-4 was chosen because it is a potent inhibitor of spontaneous apoptosis in CLL cells and also because previous work has shown that it abrogates induction of Tis11b/Berg36 by calcium ionophore in Ramos cells (Ning et al 1996b). CD40 was chosen because during the last 5-7 years has been correlated with inhibition of apoptosis (Bernal *et al* 2001, Cranziero *et al* 2001, Kitada *et al* 1999, Romano *et al* 1998, Wang *et al* 1997) and also it is involved in differentiation and cell proliferation of CLL cells (Minuzzo *et al* 2005, Fluckiger *et al* 1992). The effects of the CD40 when combined with IL-4 on spontaneous apoptosis are unknown. PMA or TPA was correlated with inhibition of apoptosis in some but not all patients tested (Barragán *et al* 2002, Bellosillo *et al* 1997) but is the agent by which Tis11b/Berg36 was isolated from B-CLL cells and would serve as a positive control for the Northern Blot experiments. Finally the effects of PMA in combination with IL-4 on spontaneous apoptosis of B-CLL cells are unknown.

All experiments were performed in purified B-CLL cells, and it was found that at 24 hours post stimulation, CD40L+IL-4, IL-4, and CD40L all significantly inhibited spontaneous apoptosis (p□0.05) whatever the method used to measure their effect. The effect of CD40L or anti-CD40 combined with IL-4 which was able to inhibit spontaneous apoptosis by more than 60% in most of the patients tested, is a novel finding of this study. This effect may be due to the synergistic effect of these two stimuli on the activation of NF-□B (Furman *et al* 2000). IL-4 was found to inhibit spontaneous apoptosis by at least 50% which is in accordance with published data (Jewell *et al* 1995, Mainou-Fowler *et al* 1995, Panayiotidis *et al* 1993, Danescu *et al* 1992). Recently it was shown that IL-4 requires PKC or PI3K pathway to elicit the anti-apoptotic effect whereas other studies suggested that this effect may be due to induction of NF- □B pathway in CLL cells (Zaninoni *et al* 2003, Barragán *et al* 2002). When CD40L or anti-CD40 was used alone, it inhibited spontaneous apoptosis by 30%-40% even though some patients did not respond to stimulation which is also in accordance with published data (Bernal *et al* 2001, Cranziero *et al* 2001, Kitada *et al* 1999, Romano *et al* 1998, Wang *et al* 1997). The effect of CD40 on spontaneous apoptosis has been attributed to activation of PI3K pathway, followed by Akt phosphorylation/activation, followed by NF-□B activation (Cuni *et al* 2004). Finally PMA showed significant variation between patients at 24 hours and in the cases that there was inhibition of apoptosis this effect did not reach statistical significance (p□0.05). Nevertheless when PMA was combined with IL-4, it significantly inhibited spontaneous apoptosis at this timepoint and this is another novel finding of this study.

At 48 hours from the initiation of the culture period CD40L+IL-4, IL-4 and CD40 were more efficient in inhibiting spontaneous apoptosis than at 24 hours and additionally PMA was a significant inhibitor of apoptosis when assayed by the PI method. The anti-apoptotic effect of PMA in CLL cells that responded has been associated with activation of PKC pathway and PI3K pathways but not ERK pathway (Barragán *et al* 2002). An important difference found involved the PMA+IL-4 combination which did not significantly inhibit spontaneous apoptosis at 48 hours as at 24 hours suggesting that the effect of this combination is rapid rather than sustained and probably due to alterations in early signalling events.

Since IL-4, CD40 and PMA inhibit apoptosis and since their effect is due to activation of different signalling pathways (NF-□B for IL-4, PI3K and NF-□B for CD40 and PI3K and PKC for PMA) it was very interesting to test how each of these stimuli alone or in combination could regulate the Tis11 family genes. The regulation of these genes at the mRNA level was tested by Northern Blot analysis after stimulating the cells with the above stimuli for 3h.

Tis11b/Berg36 mRNA was induced by PMA as expected (Murphy and Norton 1990) and surprisingly by CD40 (either CD40L or anti-CD40) even though induction by CD40 was weaker than PMA induction. Between CD40L and anti-CD40 the latter one induced the gene stronger than CD40L. A timecourse experiment revealed that the kinetics of Tis11b/Berg36 induction by PMA and anti-CD40 were different since peak induction by PMA was noticed at 2h post stimulation while peak induction by anti-CD40 was noticed at 1h post stimulation. IL-4 either alone or combined with CD40 did not have any effect on the regulation of Tis11b/Berg36 and thus there was no down regulation of the basal expression of this gene as expected. On the contrary when combined with PMA it further induced the gene in some but not all patients tested. The reason for this difference in Tis11b/Berg36 regulation by IL-4 when combined with CD40 or PMA is unknown.

Tis11 on the other hand was not induced by CD40 but instead it was down regulated at 1h post stimulation and even more clearly at 3h post stimulation and suggests that Tis11 may be a pro-apoptotic gene in B-CLL cells similar to what has been shown in a variety of cell lines such as fibroblasts, epithelial cells, osteosarcoma etc in which over expression of Tis11 induced apoptosis (Johnson *et al* 2000). When anti-CD40 was combined with IL-4, Tis11 was down regulated at 3h post stimulation and this regulation was delayed in comparison with anti-CD40 alone since there was no down regulation of the gene at 1h post stimulation. Again the effect of anti-CD40+IL-4 on Tis11 is different from the effect of Tis11b/Berg36 at the same timepoint suggesting that probably there is no redundancy in function between the two genes even though both of them seem to be pro-apoptotic.

On the other hand expression of Tis11 was induced weakly by PMA especially when combined with IL-4. Again the reason for the difference noticed when IL-4 was combined with CD40 or PMA in terms of Tis11 regulation remains unknown. IL-4 alone did not affect the expression of Tis11 and it may even down regulated the gene at 3h post stimulation in CLL cells. Because Tis11 was induced by insulin as early as 15 min (Lai *et al* 1990), a timecourse experiment was performed to test Tis11 regulation at earlier timepoints. This experiment showed a clear downregulation of Tis11 mRNA following anti-CD40 at 2h post stimulation and by IL-4 at the same timepoint. The latter finding is in contrast with what has been reported for mouse Bone Marrow Mononuclear cells in which IL-4 induced Tis11 expression in a STAT6 dependent manner at 1h post-stimulation and remained above basal levels for up to 3h (Suzuki et al 2003). Since CLL cells have highly active IL-4 signalling pathway (Klein *et al* 2001) it is unexpected the fact that Tis11 shows such regulation in this cell type.

Finally Tis11d which was also expressed at high levels in unstimulated cells but remained unchanged by most of the stimuli used. It was neither induced nor down regulated by IL-4, CD40 alone or in combination and surprisingly it was only weakly induced by PMA in some but not all patients tested.

The regulation of the TIs11 family by PMA in CLL cells is exactly the opposite from what has been reported in Swiss 3T3 cells in which stimulation with PMA resulted in very strong induction of Tis11 but very weak or no induction of Tis11b/Berg36 and Tis11d, suggesting again cell type dependent regulation of Tis11 family and that Tis11b/Berg36 is the predominant Tis11 family member in B lymphocytes.

B-CLL cells have several constitutively active several pathways including PKC, PI3K, NF-□B and p38 (Ringshausen *et al* 2004, Zaninoni *et a*l 2003, Barragán *et al* 2002, Ringshausen *et al* 2002). Since p38 was the pathway that was partially responsible for induction of Tis11b/Berg36 by XRituximab in CLL cells (Chapter 3) it was hypothesised that the pre-activated state of B-CLL cells may lower the threshold required for induction of Tis11b/Berg36. In order to test this hypothesis normal tonsillar B cells were stimulated with PMA, IL-4, anti-CD40 for 1 up to 4h and tested for regulation of the Tis11 family genes.

In comparison to CLL cells, tonsillar B cells were found to express higher amounts of Tis11b/Berg36 mRNA at To (basal expression) but similar amounts of Tis11 and Tis11d mRNA. Tis11b/Berg36 was again induced by anti-CD40 at 3h and even though there is weak induction by serum in tonsillar B cells this was lower than induction by anti-CD40. This suggests that anti-CD40 can induce the gene at the mRNA level not only in malignant but also in normal B cells and further strengthen involvement of Tis11b/Berg36 in a function different from apoptosis. Interestingly induction of Tis11d/Berg36 was not evident at 1h but only at 3 and 4h suggesting delayed kinetics of induction compared with B-CLL and probably that indeed the pre-activated/malignant state of B-CLL cells alters the kinetics of Tis11b/Berg36 induction by anti-CD40.

On the other hand Tis11 was down regulated by anti-CD40 at 3h post stimulation similar to what was found in B-CLL cells and IL-4 also had a negative effect evident at 1h post stimulation suggesting same regulation of Tis11 by anti-CD40 and IL-4 in B-CLL and normal tonsillar B cells. Finally Tis11d showed no change by any of the stimuli further supporting that Tis11d is not affected by the stimuli used in this study in B cells.

In order to explain induction of Tis11b/Berg36 by anti-CD40 several hypotheses were made. One of them was that induction of this gene by CD40L or anti-CD40 may be due to the activation of a different signalling pathway in comparison with the pathway activated (p38 as shown in Chapter 3) in the case that the gene is involved in apoptosis. In order to test this hypothesis B-CLL cells were cultured in the presence or absence of the inhibitors used for XRituximab and additionally two other inhibitors were included one for NF-□B (Gliotoxin or BAY117082) since anti-CD40 is a very strong activator of this pathway and one for PI3K (LY294002) for the same reason. Gliotoxin is an inhibitor of the proteasome thus inhibits I□B-a proteolysis while BAY117082 inhibits phosphorylation of I□B□ and thus is more selective inhibitor for the classical NF-□B pathway. It is well known that CD40Receptor activation induces PI3K, NF-□B, ERK, p38 and JNK activation in normal B and CLL cells (Cuni *et al* 2004, van Kooten and Banchereau *et al* 2000) and also PMA induces these pathways apart from PI3K in CLL cells (Petlikovski *et al* 2005).

It was found that inhibition of NF-□B pathway abrogated Tis11b/Berg36 mRNA induction by PMA or anti-CD40 which is different from what was found for XRituximab. Interestingly it was found that the strong basal expression of Tis11b/Berg36 in tonsillar B cells was also under NF-□B regulation. All these results indicate that Tis11b/Berg36 is involved in another function such as proliferation or differentiation in B cells, since NF-□B pathway has been correlated with both these functions in B cells. Involvement of Tis11b/Berg36 in differentiation was supported by the fact that PMA can induce proliferation and differentiation in CLL cells (Tangye *et al* 1995) and the same has been shown for anti-CD40 stimulation of CLL cells (Minuzzo *et al* 2005, Takeuchi *et al* 2000, Fluckiger *et al* 1992). Involvement of Tis11b/Berg36 in B cell differentiation towards plasma cells is further supported by the finding that this gene was induced by Bryostatin in CLL at 3h (data not shown and Murphy *et al* 1993) whereas the other two remained unaffected and it was reported that Bryostatin induces expression of CD11c and CD22 cell markers in CLL cells suggesting induction of differentiation (Varterasian *et al* 2000). Finally Tis11b/Berg36 knock out mice were found to be embryonic lethal at day E9-E12 by two independent studies (Bell *et al* 2006, Stumpo *et al* 2004) which is a period that differentiation of many organs occurs.

Another explanation for induction of Tis11b/Berg36 by PMA, anti-CD40 and XRituximab is that the protein may localise in different cellular compartments after stimulation by each of the stimuli. When B-CLL were stimulated with all 3 stimuli for 3h it was found that Tis11b/Berg36 localises in the cytoplasm with a diffuse pattern of staining whatever the stimuli used. The only difference noticed was in the number of cells being positive for Tis11b/Berg36 staining reflecting the differences in the fold of induction found at the mRNA level. Finally because Tis11b/Berg36 can be regulated by phosphorylation a Western Blot experiment was performed with cell lysates from unstimulated and PMA, anti-CD40 or XRituximab stimulated B-CLL cells for 3h. However no difference in phosphorylation was seen (data not shown) suggesting that Tis11b/Berg36 is not differentially phosphorylated by the above 3 stimuli

Thus all the data support the hypothesis that Tis11b/Berg36 is probably involved in the cell cycle or differentiation of B cells In order to test involvement of Tis11b/Berg36 in

differentiation two approaches were utilised. One involved the application of siRNA technology in PMA stimulated B-CLL cells in relation to induction of sIgM as a marker of CLL differentiation. The other approach involved utilisation of several B cell lines arrested at different stages of differentiation in which the basal expression and regulation of Tis11b/Berg36 was studied and will be presented-analysed in the next chapter (Chapter 5).

According to the first approach it was found that PMA stimulation and to lesser extent anti-CD40 stimulation significantly induced the expression of sIgM whose basal expression was found to be low in B-CLL cells However in the presence of Tis11b/Berg36 siRNA both stimuli and especially PMA was significantly less efficient in inducing sIgM expression. This finding suggests that Tis11b/Berg36 is a positive regulator of B-CLL differentiation at least in response to PMA. This consists a novel finding of this study and it is to our knowledge the first one implying involvement of Tis11b/Berg36 or Tis11 family in general in differentiation of a certain cell type.

CHAPTER 5

Tis11 family at different stages of B cell differentiation

5.1 INTRODUCTION

5.1.1 B cell differentiation and regulation

B lymphocytes arise in the Bone Marrow from the common lymphoid progenitor cell (CLP) (Lin^-, KIT^{low}, $AA4.1^+$, $SCA1^{low}$, $IL\text{-}7R^+$) (Nagasawa *et al* 2006). CLP cells arise from lymphoid-primed multipotent progenitor cells (LPMP) (Lin^-, $B220^-$, KIT^+, $SCA1^+$, $FLT3^+$, $CD34^+$) which in turn arise from haematopoietic multipotential progenitors (MPP) (Lin^-, KIT^+, $SCA1^+$, $FLT3^-$, $CD34^+$) which arise from haematopoietic stem cells (HSC) (Lin^-, KIT^+, $SCA1^+$, $FLT3^-$, $CD34^-$) (Nagasawa *et al* 2006). The earliest detectable progenitor of B cells is called pre pro-B cell ($B220^+$, KIT^-, $CD19^-$, $FLT3^+$, $CD24^{-/low}$, $CD43^-$, IgM^-) which will differentiate to an early pro-B cell ($B220^+$, KIT^+, $CD19^+$, $FLT3^-$, $CD24^+$, $CD43^+$, IgM^-) (Nagasawa *et al* 2006). The early pro-B cell ($B220^+$, $CD43^+$, AA 4.1^+) is characterised by the D_HJ_H recombination which will be followed by the joining of V_H to the D_HJ_H at the late pro-B cell (Montenico-Rodriguez *et al* 2006). $V_HD_HJ_H$ recombination occurs only in cells committed to the B lineage probably due to the absence of H3K9 methylation in V_H promoter (Johnson *et al* 2004) which is present in T cells, myeloid and progenitor haematopoietic cells (Johnson *et al* 2004). A productive (in-frame) $V_HD_HJ_H$ recombination defines pre-B cells ($B220^-$, KIT^-, $CD19^+$, $FLT3^-$, $CD24^+$, $CD43^-$, IgM^-) which express a pre-BCR receptor consisting of μH chain, surrogate □5, VpreB light chains and intracellular Ig□ and Ig□ (Nagasawa *et al* 2006). At the next stage (large pre-B or pre-B I) cells proliferate probably through CD19 mediated signals (Engel *et al* 1999) and form the small pre-B or pre B II stage where the light chain rearrange (V_LJ_L). Following proper rearrangement the light chains interact with the heavy chains to form surface IgM (sIgM) a feature that defines the immature B cell ($B220^-$, KIT^-, $CD19^+$, $FLT3^-$, $CD24^+$, $CD43^-$, IgM^+) (Nagasawa *et al* 2006). The three possible outcomes in the case that an immature B cell encounters an autoantigen are clonal deletion, receptor editing or anergy depending on the affinity of the autoantigen for the B cell receptor (BCR). (Chung *et al* 2003). Immature B cells that have escaped clonal deletion or anergic B cells migrate to the periphery (spleen or the lymph nodes) (Chung *et al* 2003) to further differentiate to Follicular B cells (IgM^{low}, IgD^{high},

CD21^{med}, CD23^{high}, CD1^{neg}), or Marginal Zone B cells (IgM^{high}, IgD^{low}, CD21^{high}, CD23$^{low/neg}$, CD1^{high}) or B1 cells (CD5^{+}, B220^{low}, CD23^{-}) (Samardzic *et al* 2002). Immature B cells migrating to the spleen are called transitional B cells or T1 cells (sIgM^{high}, sIgD^{low}, CD21 and CD23 low or negative) and are found in the bone marrow (15-20% of B lymphocytes), peripheral blood (15-20% of B cells) and in the spleen (10-15% of all B cell) (Loder *et al* 1999). T1 are non proliferating cells and can differentiate into T2 (sIgM^{high}, sIgD^{high}, CD21^{+}, CD23^{+}) or mature B cells (Loder *et al* 1999). T2 represent 10-15% of splenic B cells, they are proliferating cells and form an intermediate stage of differentiation between T1 and mature B cells (Follicular B cells) even though mature B cells may develop directly from T1 through an unknown mechanism (Loder *et al* 1999). This was elegantly shown by transferring T1 cells into RAG 2$^{-/-}$ mice (lack B and T cells) which differentiated into T2 and Follicular B cells after 48h, whereas injection of T2 cells resulted in differentiation to Follicular B cells after 24h (Loder *et al* 1999).

Marginal zone B cells represent 3-5% of splenic B cells (Loder *et al* 1999) and are non proliferating B cells found only at the borders of red and white pulp (Srivastava *et al* 2005). They may arise from T2 either directly or indirectly from Follicular B cells under certain circumstances such as lymphopenic environment (Srivastava *et al* 2005). The strength of B Cell Receptor (BCR) signalling determines probably differentiation to follicular or marginal zone B cells. A strong signal is necessary for development of follicular B cells whereas a weaker signal is sufficient for the generation of marginal zone B cells as well as T2 cells from T1 (Loder *et al* 1999).

Finally B1 (IgM^{high}, IgD^{low}, CD11b^{+}) cells are primarily found in the peritoneum and pleural cavities and are further subdivided into B1a and B1b depending on whether there is expression of CD5 marker (Montenico-Rodriguez *et al* 2006). There was a strong controversy for years about the ancestor of B1 cells with one theory suggesting that they arise from T2 and another one suggesting that they arise separately. Very recently it was shown that the latter theory is probably right. It was found that injection of SCID mice with an early B cell progenitor: Lin^{-}, B220^{-}(or CD45R^{-}), CD19^{+} derived from either adult or foetal bone marrow resulted in the appearance of B1 cells in the peritoneum (almost 90% of the cells) and absence of B2 B cells in the bone marrow and spleen whereas injection with adult bone marrow Lin^{-}, B220^{+}(or CD45R^{-}), CD19^{+},

AA4.1$^+$ pro-B cells gave rise to B2 cells in the bone marrow and spleen, and to both B1 (60%) and B2(40%) B cells in the peritoneum (Montenico-Rodriguez *et al* 2006). Collectively these data suggest that B1 and B2 B cells derive from different pro-B progenitors that share expression of CD19 but differ in the expression of B220$^-$, with B220$^-$ progenitors giving rise to B1 cells and B220$^+$ giving rise mainly to B2 cells (Montenico-Rodriguez *et al* 2006). Additionally proper development of B1 B cells depends on CD19, Vav, Btk, Bcl10 and Oct-2 expression since disruption of either of them resulted in reduced numbers of B1 cells (Xue *et al* 2003, Samardzic *et al* 2002, Humbert P.O. and Corcoran *et al* 1997, Engel *et al* 1995) while CD22 disruption resulted in increased B1 numbers (Sato *et al* 1996).

Terminal differentiation into antibody secreting cells plasma cells occurs when B cells interact with antigen specific T cells in the T zones of the spleen or lymph nodes. For certain types of antigen such as blood borne bacteria, Marginal Zone B cells are activated resulting in the production of short-lived plasma cells that produce antibodies with low affinity to the antigen (Oliver *et al* 1999). However for most of the antigens Follicular B cells are activated, resulting in the formation of Germinal centres (GC). In GC cells B cells will differentiate into either memory B cells or antibody secreting plasma cells (Huntington *et al* 2006, McHeyzer –Williams and Ahmed *et al* 1999, Song and Cerny *et al* 2003). In GC centroblasts proliferate rapidly and undergo somatic hypemutation in the V_H or J□regions of their immunoglobulins (affinity maturation of B cells) (Song and Cerny *et al* 2003, Shapiro *et al* 1999). Depending on the affinity of the newly formed receptor to the antigen (McHeyzen-Williams and Ahmed *et al* 1999) B cells will either re-enter the GC and start a new cycle of somatic hypermutation-affinity maturation or exit the GC as memory B cells or as an antibody forming cell (plasma cell) (McHeyzen-Williams and Ahmed *et al* 1999).

Memory B cells have a lower threshold of activation, can present antigen to T cells better than naïve B cells, and can be distinguished from naïve cells by the surface expression of CD27 (positive for memory, negative for naïve) (Weller *et al* 2003).

AFC (Plasma cells) are long lived cells that reside in the bone marrow, secrete antibodies with high affinity to antigen and express CD138 (Syndecan) (Song and Cerny 2003). They can develop from memory B cells when they encounter the same antigen for the second time. In the latter case, one distinction between memory B cell

and memory-plasma B cell or plasmablastic B cell is the surface expression of B220 which is present in memory B cells but absent in memory-pre plasma B cells (Driver *et al* 2001). Plasmablastic cells are proliferating cells and secrete antibody but at lower levels while plasma cells are non proliferating cells and secrete higher amounts of antibody. The summary of the B cell stages of differentiation is shown in Figure 6.1.

AIM OF THIS STUDY: Aim of this study is to test whether the Tis11 family is differentially expressed and regulated at different stages of B cell differentiation which would indicate involvement in B cell maturation process.

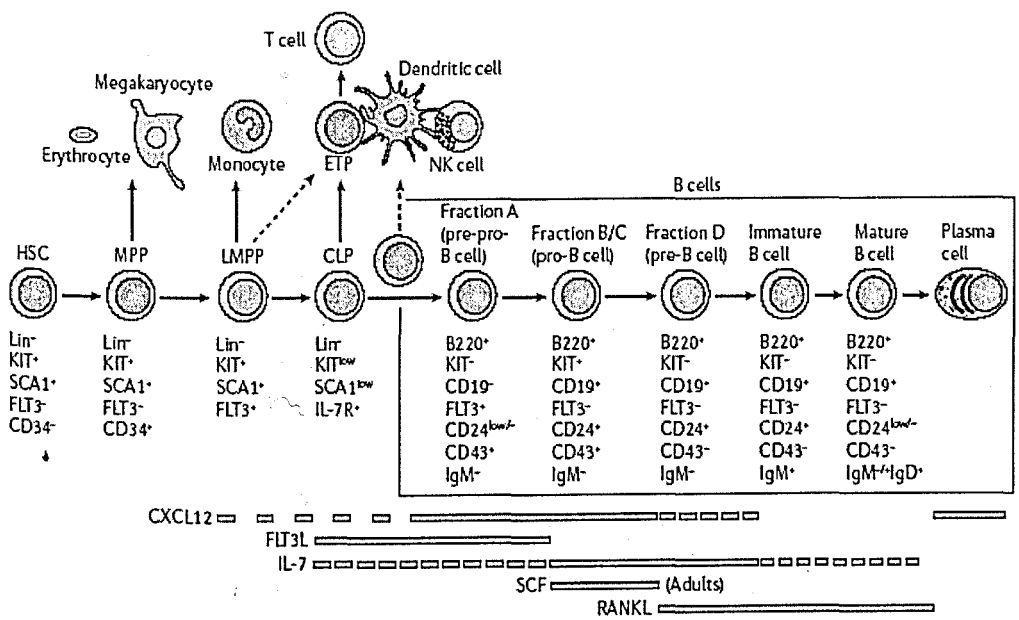


Figure I 5.1: Summary of B cell development/differentiation
Diagrammatic presentation of the stages of B cell differentiation from Haematopoietic stem cells (HSC) to antibody secreting Plasma cells. The cell markers that characterise each stage of B cell differentiation and some growth factors required for the proper growth at certain stages are indicated. HSC: Haematopoietic Stem Cells, MPP: haematopoietic Multipotential Progenitors, LMPP: Lymphoid primed Multipotential Progenitors, CLP: Common Lymphoid Progenitor cell, NK: Natural Killer T cell, SCF: Stem Cell Factor, RANKL: Receptor Activator of nuclear factor-□B Ligand. This photo was obtained from Nature Reviews in Immunology.2006; 6, pp:109

5.2. RESULTS

Tis11b/Berg36 was induced by stimuli that can induce differentiation of CLL cells (PMA and CD40Receptor activation) (Chapter 4) and this induction was found to be partially regulated through NF-□B pathway (Chapter 4).

For these reasons it was decided to utilise B cell lines arrested at different stages of differentiation and examine what are the basal levels of Tis11b/Berg36 expression at different stages of differentiation and how this may be altered by the stimuli used in the previous chapters. Apart from Tis11b/Berg36, Tis11 and Tis11d would be also tested because there are currently no data about their basal expression and regulation at different stages of B cell differentiation.

The cell lines used represent most stages of B cell differentiation and include NALM6 which is an Acute Lymphoblastic Leukaemia cell line representing pre-B cells, RAMOS which are Burkitt's Lymphoma cell lines representing Germinal Center B cells (CG), AGLCL and WILCL which are normal lymphoblastoid cell lines representing mature B cells and finally RPMI8226 and MM1.S which are Multiple Myeloma cell lines representing Plasma cells.

5.2.1 Characterisation of AGLCL and WILCL normal lymphoblastoid cell lines.

Because there are no data available regarding the immunophenotype of AGLCL and WILCL cell lines even after personal communication with Dr. Rabbitts who established these cell lines in 1983, it was decided to characterise them in terms of surface cell markers. The cell markers tested were CD19, CD138, CD86, CD27 and CD38. Additionally CD20 staining was performed in all cell lines in order to determine their eligibility for Rituximab treatment. It was found that AGLCL is highly positive for CD19 (79% of live cells express CD19 on their surface), clearly positive for CD27 (54%), and weakly positive for CD86 (25%), CD38 (21%) and CD138 (20%) (Figure 5.1). This phenotype suggests that they are primarily memory B cells. Weak expression of CD138 suggests either that they may contain a percentage of plasmablasts (memory B/ pre-plasma cells) or that they were induced to express CD138 following *in vitro* cultivation.

On the other hand WILCL were found to be highly positive for CD19 (76% of live cells express CD19), clearly positive for CD27 (40%) and weakly positive for CD86 (17 %) and CD38 (25%) (Figure 5.2). Interestingly CD138 was expressed only in 3% and thus considered negative for CD138 expression while AGLCL are weakly but clearly positive.

In summary AGLCL express higher levels of CD27 and CD86 than WILCL, suggesting that WILCL are memory B cells but less activated than AGLCL and probably represent an earlier point in B cell differentiation. In support of this it was found that AGLCL express CRACC or 19A (a CD2 like receptor present only in activated B cells) in 60% of their cells whereas WILCL express the same receptor in 40% of their cells (Figure 5.2 and 5.3). The difference in expression of CD27, CD86 and CD138 between AGLCL and WILCL is shown in Figure 5.3.

Finally in terms of CD20 expression, it was found that NALM6, RPMI8226 and MM1.S are negative for CD20 expression, Ramos, AGLCL and WILCL cells were strongly positive for CD20 (95-98%, 95% and 90% respectively) (Figure 5.1)

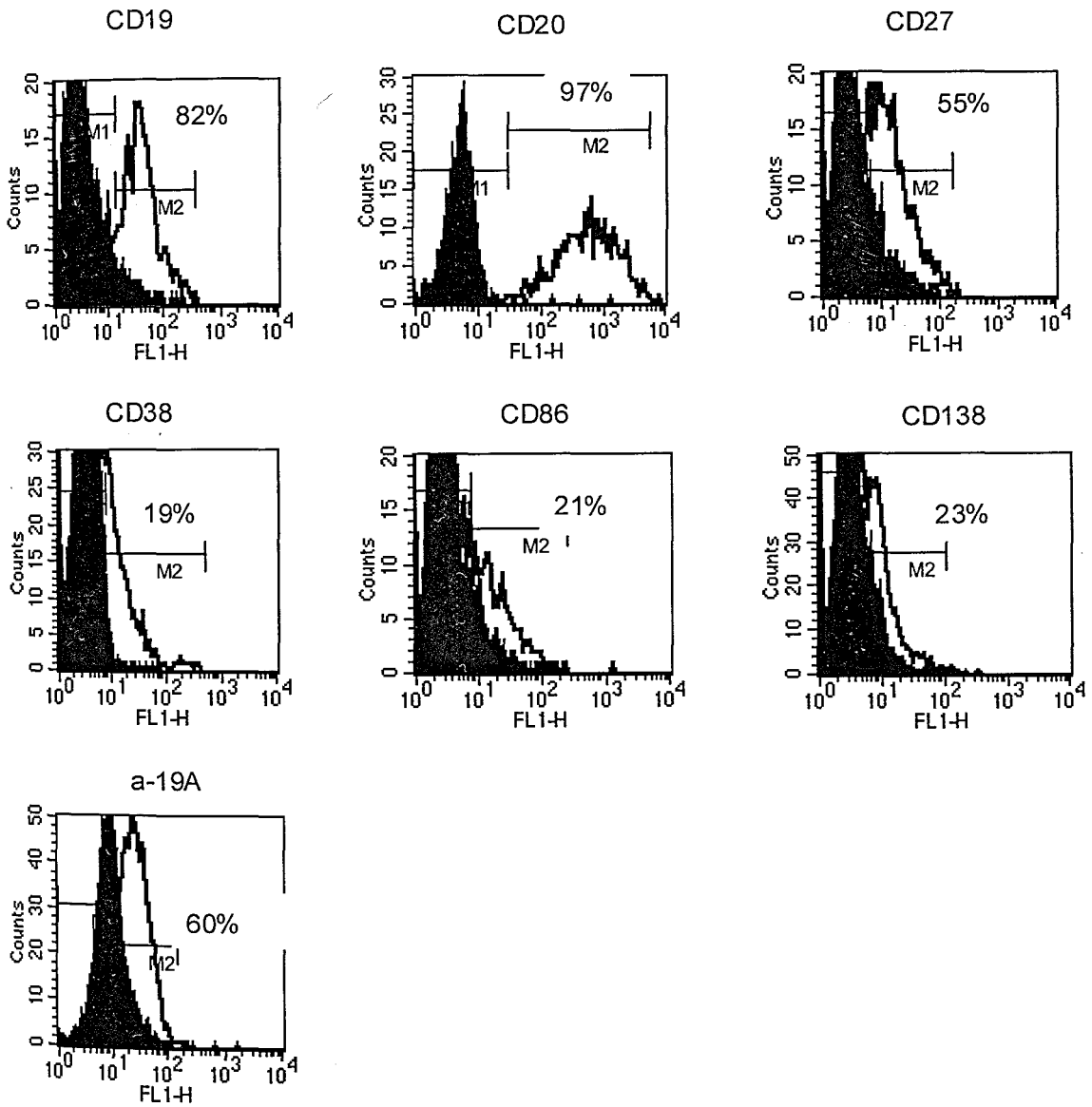


Figure 5.1: Cell markers expression in AGLCL cells.
AGLCL cells were stained with CD19, CD38, CD86, CD138 directly labelled with FITC, or CD20 and 19A (CRACC) together with a secondary anti-mouse FITC. In all cases 10,000 live cells were acquired and analysis was performed on live gated cells. Black filled line represents background staining with secondary FITC antibody whereas black open line-overlay represents cells stained with the indicated surface marker. The percentage of positive cells for each cell marker is shown above M2 gate. This experiment was repeated twice and very similar results were obtained.

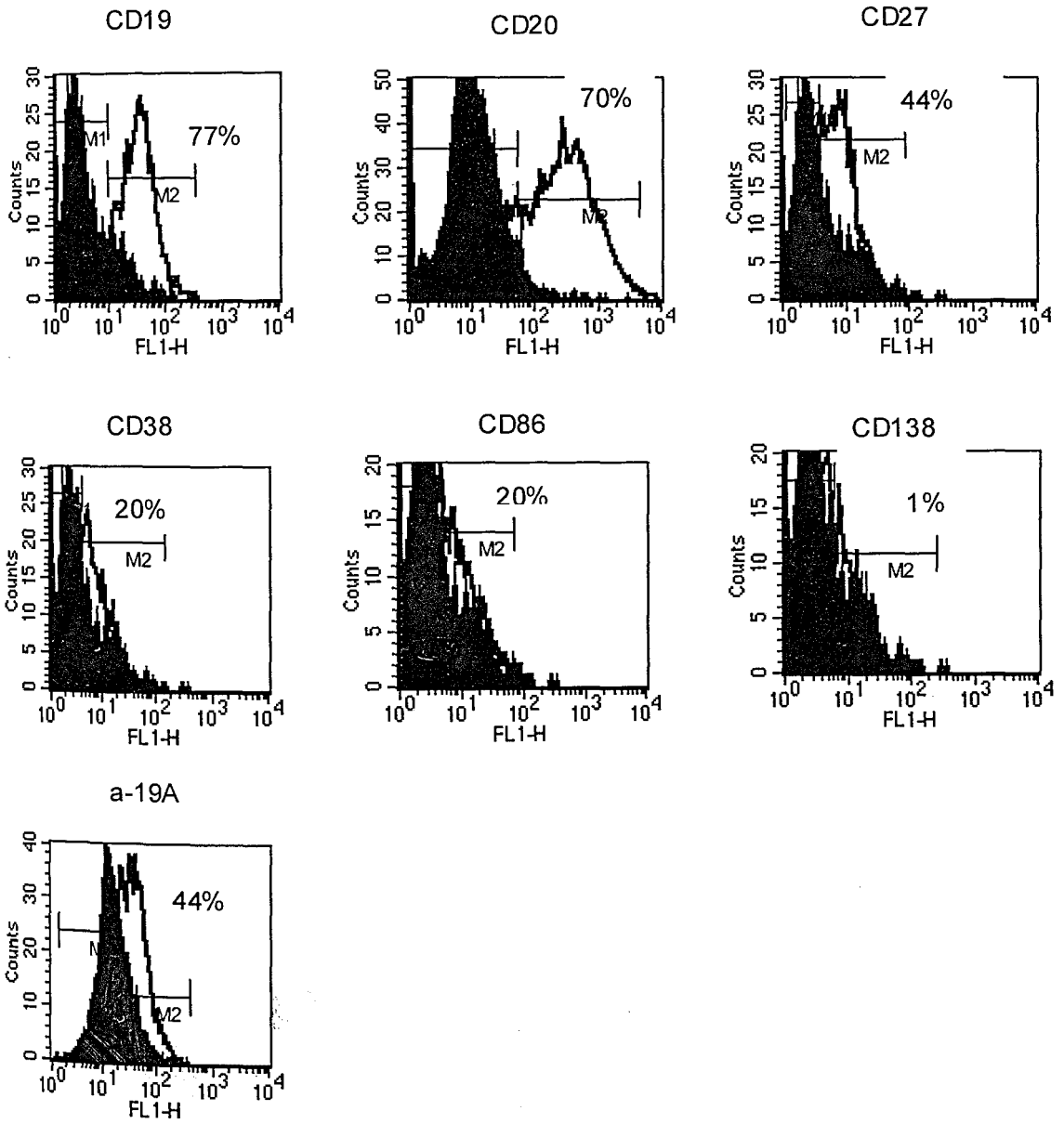


Figure 5.2: Cell markers expression in WILCL cells.
WILCL cells were stained with CD19, CD38, CD86, CD138 directly labelled with FITC, or CD20 and 19A (CRACC) together with a secondary anti-mouse FITC. In all cases 10,000 live cells were acquired and analysis was performed on live gated cells. Black filled line represents background staining with secondary FITC antibody whereas black open line-overlay represents cells stained with the indicated surface marker. The percentage of positive cells positive for each cell marker is shown above M2 gate. This experiment was repeated twice and very similar results were obtained

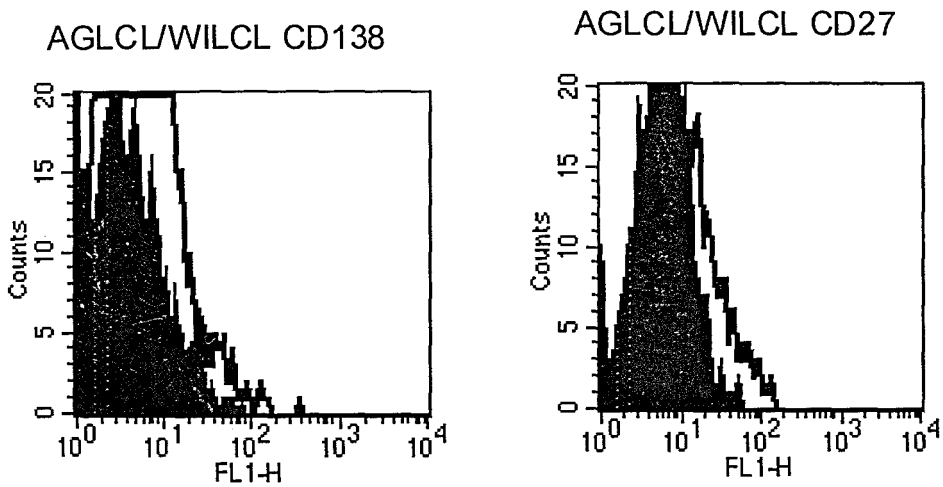


Figure 5.3: Differential expression of cell markers in AGLCL and WILCL cell lines.

Cells were stained as mentioned in Figures 5.1 and 5.2. Analysis was performed in order to check for difference in expression of CD138 and CD27 in AGLCL and WILCL cells. Again analysis was performed in live gated cells and the black filled line represents WILCL cells stained with CD27 or CD138 whereas the black open lien-overlay represents AGLCL cells stained witheither of them. It was found that CD27 and CD138 is higher expressed in AGLCL than in WILCL cells.

5.2.2 Basal expression of Tis11 family at different stages of B cell differentiation

Initially the basal expression of Tis11b/Berg36 mRNA in all cell lines was determined and it was found that RPMI8226 and MM1.S have undetectable levels of Tis11b/Berg36 mRNA, Nalm6 and Ramos express low levels, whereas AGLCL and WILCL showed high basal expression of this gene. From the malignant cell lines used (Nalm6, Ramos, RPMI8226 and MM1.S), Nalm6 showed the highest level of expression whereas RPMI8226 and MM1.S showed the lowest. This may imply that Tis11b/Berg36 basal expression is down regulated as B cells become more differentiated towards plasma cells (Figure 5.4).

Then the basal Tis11 expression was determined in a similar manner (Figure 5.5). It was found that Ramos, RPMI8226, MM1.S have undetectable levels of Tis11 mRNA, Nalm6 have very low basal levels whereas AGLCL and WILCL showed strong basal expression. Compared to Tis11b/Berg36 basal levels, Tis11 is also absent in the two multiple Myeloma cell lines, and is expressed at lower levels than Tis11b/Berg36 in Nalm6 and Ramos cell lines since for both genes 10 μg of RNA were loaded per lane.

Finally the basal expression of Tis11d was assayed by Northern Blot and it was found to be high in all cell lines tested whatever their stage of differentiation (Figure 5.6).

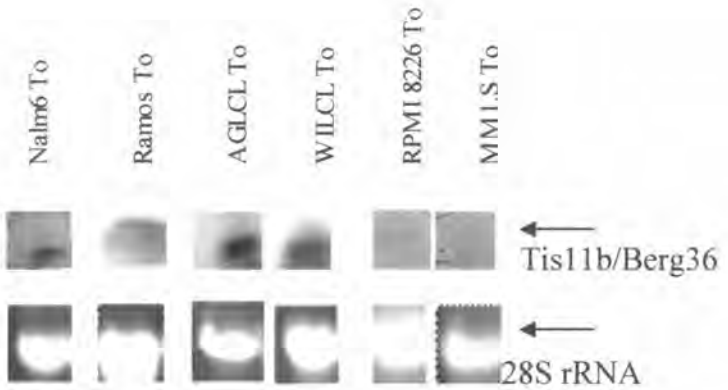


Figure 5.4: Basal Tis11b/Berg36 mRNA expression in different cell lines.
10^7 cells (To) from Nalm6, Ramos, AGLCL, WILCL, MM1.S cell lines were processed for RNA extraction and Northern Blot hybridisation using a ^{32}P labelled Tis11b/Berg36 cDNA probe. Consistency of RNA loading as indicated by 28S rRNA staining is shown below each membrane. The arrows indicate either the Tis11b/Berg36 mRNA specific band detected by the radiolabelled probe or the 28S rRNA band present in each lane. All the membranes have been exposed to a radiography film for 3 days.

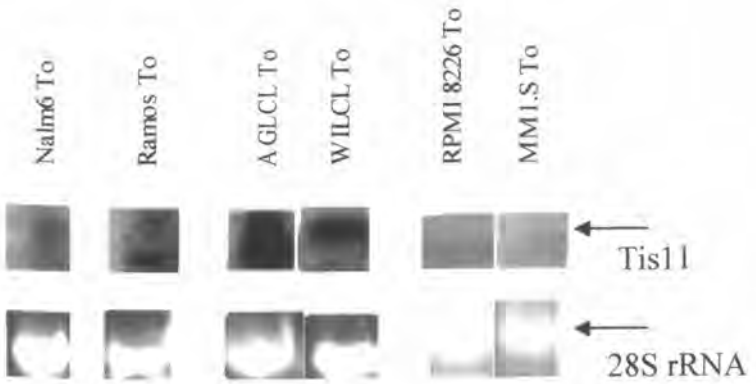


Figure 5.5: Basal Tis11 mRNA expression in different cell lines.
10^7 cells (To) from Nalm6, Ramos, AGLCL, WILCL, MM1.S cell lines were processed for RNA extraction and Northern Blot hybridisation with a ^{32}P labelled Tis11 cDNA probe kindly provided by Dr. Brooks. Consistency of RNA loading as indicated by 28S rRNA staining is shown below each membrane. The arrows indicate either the Tis11 mRNA specific band detected by the radiolabelled probe or the 28S rRNA band present in each lane. All the membranes have been exposed to a radiography film for 5 days.

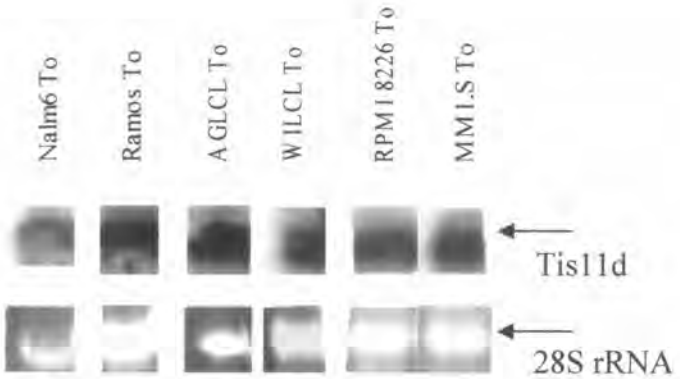


Figure 5.6: Basal Tis11d mRNA expression in different cell lines.
10^7 cells (To) from Nalm6, Ramos, AGLCL, WILCL, MM1.S cell lines were processed for RNA extraction and Northern Blot hybridisation with a ^{32}P labelled Tis11d cDNA probe. Consistency of RNA loading as indicated by 28S rRNA staining is shown below each membrane. The arrows indicate either the Tis11d mRNA specific band detected by the radiolabelled probe or the 28S rRNA band present in each lane. All the membranes have been exposed to a radiography film for 3 days.

5.2.3 Regulation of the Tis11 family gene at different stages of B cell differentiation

After the basal expression of all 3 genes was determined, it was decided to test whether the same patterns of regulation for Tis11 family genes found in CLL cells apply throughout B cell differentiation. Therefore all the above cell lines were stimulated with PMA, IL-4, anti-CD40 etc for 1 or 3h as indicated and induction of the Tis11 family genes was tested at the mRNA level by Northern Blot. Rituximab either alone or XRituximab was used Ramos, AGLCL, WILCL. Additionally in Ramos, two new stimuli were included anti-IgM and Ionomycin since they can induce Tis11b/Berg36 mRNA (Mathas *et al* 2000, Ning *et al* 1996b) but it is not known how these stimuli may affect Tis11 or Tis11d.

5.2.3.1 Tis11b/Berg36 regulation

Tis11b/Berg36 was found to be induced by PMA in Ramos at 1h and 3h post stimulation and WILCL at 3h post stimulation tested, while stimulation of AGLCL, MM1.S and RPMI8226 with PMA resulted in no significant change at 3h post stimulation. Finally in Nalm6, PMA stimulation resulted in no change or weak down regulation of the basal Tis11b/Berg36 mRNA levels at 3h and 4h post stimulation (Figure 5.7).

Anti-CD40 stimulation resulted in clear induction of Tis11b/Berg36 mRNA in Nalm6 and Ramos cell lines but in weak or no change in WILCL cell line (Figure 5.8). On the contrary the same stimuli clearly down regulated the gene in AGLCL and MM1.S cell lines (Figure 5.8). It should be noted that the MM1.S membrane was exposed for 2 weeks rather than 3 days in order to visualise this difference due to the very low basal expression of Tis11b/Berg36. These results suggest that as B cells differentiate towards plasma cells lose their ability to induce Tis11b/Berg36 mRNA in response to anti-CD40 stimulation.

Stimulation with anti-IgM resulted in strong induction of Tis11b/Berg36 mRNA at 3h post stimulation in Ramos cells but in clear down regulation of the gene in AGLCL which reflects probably the fact that they represent different stages of differentiation and thus respond differently to BCR signalling (data not shown).

Rituximab alone or crosslinked (XRituximab) strongly induced Tis11b/Berg36 at 3h post stimulation in all cell lines tested (Figure 5.9). In Ramos cells XRituximab was found to be more efficient in inducing apoptosis (personal data not shown, van der Kolk *et al* 2002) and in inducing Tis11b/Berg36 mRNA than Rituximab alone, suggesting a quantitative and qualitative difference in terms of apoptosis induction and Tis11b/Berg36 mRNA levels at least in Ramos cells.

Finally IL-4 did not significantly affect the basal expression of the gene in all the cell lines tested at 3h post stimulation.

All these stimuli and their effects on different cell lines are summarised in Table 5.1

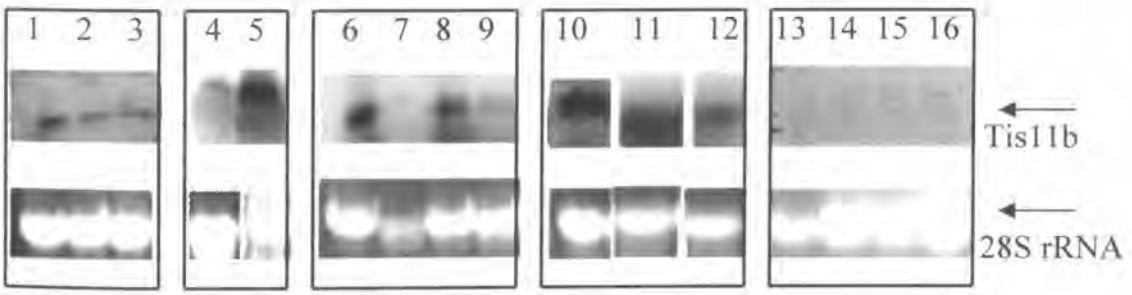


Figure 5.7: Regulation of Tis11b/Berg36 mRNA by PMA in different cell lines: The indicated cell lines were stimulated with 30 nM PMA for 3h or 4h and RNA was extracted and processed for Northern Blot hybridisation using as a probe the ^{32}P labelled Tis11b/Berg36 cDNA. Consistency of RNA loading as indicated by 28S rRNA staining is shown below each membrane. The arrows indicate either the Tis11b/Berg36 mRNA specific band detected by the radiolabelled probe or the 28S rRNA band present in each lane:

Lanes:

1) Nalm6 To(unstimulated cells), 2) Nalm6 PMA 3h,
3) Nalm6 PMA 4h, 4) Ramos To(unstimulated cells),
5) Ramos PMA 3h, 6) AGLCL To(unstimulated cells),
7) AGLCL PMA 1h, 8) AGLCL PMA 3h,
9) AGLCL PMA 4h, 10) WILCL To(unstimulated cells),
11) WILCL PMA 1h, 12) WILCL PMA 3h,
13) MM1.S To(unstimulated cells), 14) MM1.S PMA 3h,
15) RPMI8226 To(unstimulated cells), 16) RPMI8226 PMA 3h

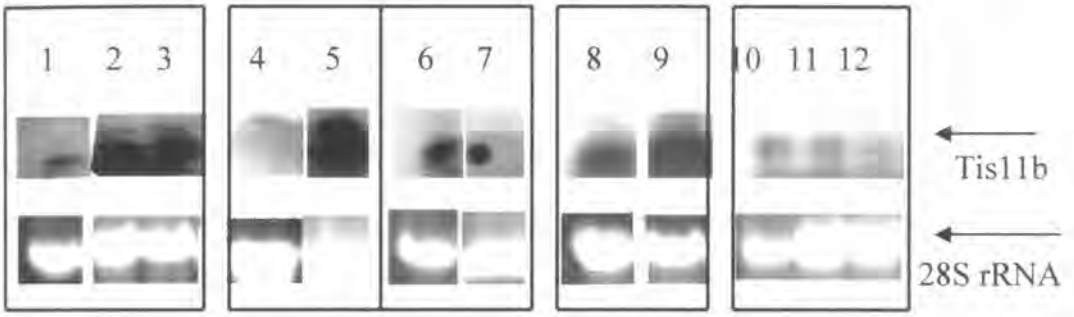


Figure5.8: Regulation of Tis11b/Berg36 mRNA by anti-CD40 in different cell lines
The indicated cell lines were stimulated with 1 μg/ml anti-CD40 for 3h or 1h and RNA was extracted and processed for Northern Blot hybridisation using a ^{32}P labelled Tis11b/Berg36 cDNA probe. Consistency of RNA loading as indicated by 28S rRNA staining is shown below each membrane. The arrows indicate either the Tis11b/Berg36 mRNA specific band detected by the radiolabelled probe or the 28S rRNA band present in each lane.
Lanes:
1) Nalm6 To(unstimulated cells), 2) Nalm6 anti-CD40 1h,
3) Nalm6 anti-CD40 3h, 4) Ramos To(unstimulated cells),
5) Ramos anti-CD40 3h, 6) AGLCL To(unstimulated cells),
7) AGLCL anti-CD40 3h, 8) WILCL To(unstimulated cells),
9) WILCL anti-CD40 3h, 10) MM1.S To(unstimulated cells),
11) MM1.S PMA 3h, 12) MM1.S anti-CD40 3h.

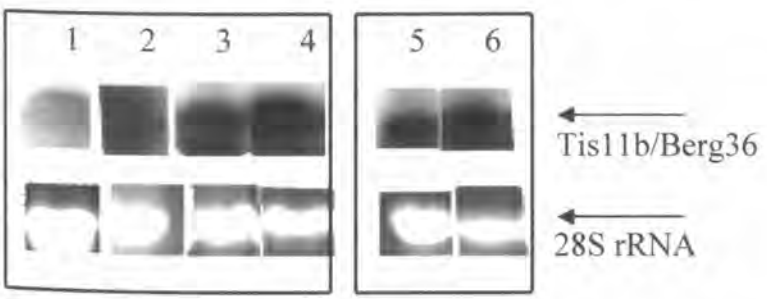


Figure 5.9: Regulation of Tis11b/Berg36 mRNA by stimuli that induce in Ramos and WILCL cell lines
The indicated cell lines were stimulated with 15 ng/ml anti-IgM, 20 μg/ml Rituximab or 20 μg/ml XRituximab for 3h and RNA was extracted and processed for Northern Blot hybridisation using a ^{32}P labelled Tis11b/Berg36 cDNA probe. Consistency of RNA loading as indicated by 28S rRNA staining is shown below each membrane. The arrows indicate either the Tis11b/Berg36 mRNA specific band detected by the radiolabelled probe or the 28S rRNA band present in each lane.
Lanes:
1) Ramos To(unstimulated cells), 2) Ramos anti-IgM 3h,
3) Ramos Rituximab 3h, 4) Ramos XRituximab 3h,
5) WILCL To(usntimulated cells), 6) WILCL XRituximab 3h

Tis11b/Berg36 regulation at 1h or 3h					
	PMA	Anti-CD40	Anti-IgM	XRituximab	IL-4
NALM6	No change	+	NA	NA	No change
RAMOS	++	+	++ at 3h	++	-
WILCL	+	+/No change	ND	+	No change
AGLCL	-	-	-	ND	No change
MM1.S	No change	- at 3h	ND	NA	ND
RPMI822	No change	ND	ND	NA	ND

Table 5.1: Summary of Tis11b/Berg36 mRNA regulation in different B cell lines. The indicated cell lines stimulated for 1h or 3h with the indicated stimuli and tested for the regulation of Tis11b/Berg36 mRNA. NA=not applicable, ND=not done, + weak induction, ++ strong induction, - downregulation. When no timepoint is mentioned, it refers to the effect seen at both timepoints tested but when is mentioned it refers to the timepoint tested and the effect seen.

5.2.3.2 Tis11 regulation

Then Tis11 was tested and was found not to be altered by PMA in Nalm6, Ramos, RPMI8226 and MM1.S cell lines but was down regulated in AGLCL and WILCL cell lines (Figure 5.10 and data not shown).

Surprisingly anti-CD40 stimulation induced the gene at 1h and 3h post stimulation in Ramos cells, had no effect in WILCL cells and down regulated the gene in AGLCL cells at 1h and 3h post stimulation (Figure 5.10 and 5.11). The induction of Tis11 in Ramos was rather unexpected because in CLL cells anti-CD40 clearly down regulated the gene and probably reflects different regulation at different stages of B cell differentiation (memory B and GC cells respectively).

XRituximab also clearly induced Tis11 mRNA at 3h post stimulation in Ramos cells, stronger than Rituximab alone (Figure 5.11). However in WILCL there was no induction of Tis11 mRNA following XRituximab treatment (not shown) just like in CLL cells suggesting different regulation by Rituximab treatment at different stages of B cell differentiation. Finally IL-4 resulted in no significant change at 1h in Ramos or WILCL cell lines tested. The summary of results obtained for Tis11 regulation is shown in Table 6.2.

5.2.3.3 Tis11d regulation

Finally Tis11d mRNA regulation was tested, and it was found to be weakly induced by PMA at 3h post stimulation in Ramos and at 1h post stimulation in AGLCL cells but showed no change in the rest of the cell lines tested. Most of the stimuli used such as IL-4, anti-CD40, Ionomycin, Rituximab or anti-IgM did not significantly affect the basal expression of the gene in all cell lines tested (Figure 5.12). There was no change of Tis11d in MM1.S cell line in response to anti-CD40 stimulation which is in contrast to what was found for Tis11b/Berg36 (Figure 5.12). The summary of results obtained for Tis11d regulation is shown in Table 5.3

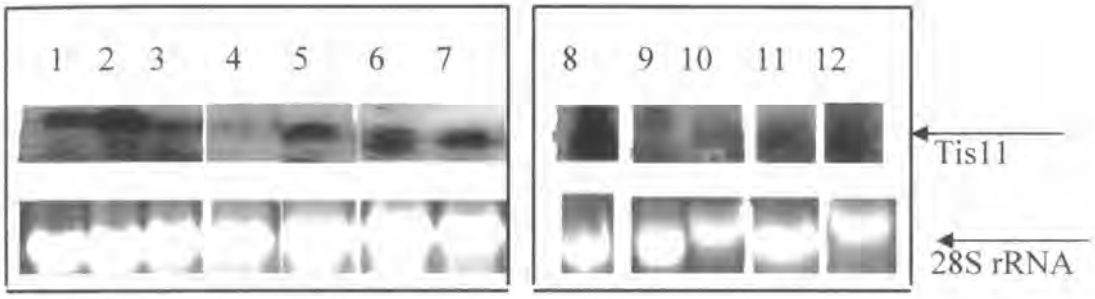


Figure 5.10: Regulation of Tis11 mRNA in different cell lines
The indicated cell lines were stimulated with the indicated stimuli for the indicated times and RNA was extracted and processes for Northern Blot hybridisation using a ^{32}P labelled Tis11 cDNA probe kindly provided by Dr. Brooks. Consistency of RNA loading as indicated by 28S rRNA staining is shown below each membrane. The arrows indicate either the Tis11 mRNA specific band detected by the radiolabelled probe or the 28S rRNA band present in each lane.
Lanes:
1)WILCL To(unstimulated cells), 2) WILCL IL-4 1h,
3) WILCL PMA 1h, 4) WILCL PMA 3h,
5) WILCL anti-CD40 3h, 6) WILCL Rituximab 3h,
7) WILCL XRituximab 3h, 8) AGLCL To(unstimulated cells),
9) AGLCL IL-4 3h, 10) AGLCL PMA 3h,
11) AGLCL anti-CD40 1h, 12) AGLCL anti-CD40 3h

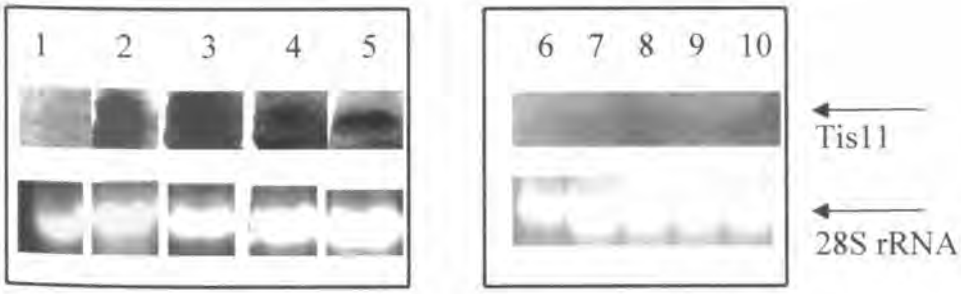


Figure 5.11: Regulation of Tis11 mRNA in different cell lines
Ramos and MM1.S and RPMI8226 cell lines were stimulated with the indicated stimuli for the indicated times and RNA was extracted and processes for Northern Blot hybridisation using a ^{32}P labelled Tis11 cDNA probe kindly provided by Dr. Brooks. Consistency of RNA loading as indicated by 28S rRNA staining is shown below each membrane. The arrows indicate either the Tis11 mRNA specific band detected by the radiolabelled probe or the 28S rRNA band present in each lane.
Lanes:
1) Ramos To(unstimulated cells), 2) Ramos anti-CD40 1h,
3) Ramos anti-CD40 3h, 4) Ramos Rituximab 3h,
5) Ramos XRituximab 3h, 6) MM1.S To(unstimulated cells)
7) MM1.S PMA 3h, 8) MM1.S anti-CD40 3h,
9)RPMI8226 To(unstimulated cells), 10) RPMI8226 PMA 3h

Tis11 regulation at 1h or 3h					
	PMA	Anti-CD40	Anti-IgM	XRituximab	IL-4
NALM6	No change	No change	NA	NA	No change
RAMOS	No change	+	No change at 3h	++ at 3h	No change
WILCL	-	No change at 3h	ND	No change	No change
AGLCL	-	-	ND	ND	-
MM1.S	No change	No change	ND	NA	ND
RPMI8226	No change	ND	ND	NA	ND

Table 5.2: Summary of Tis11 mRNA regulation in different B cell lines.
The indicated cell lines were stimulated with the indicated stimuli for 1h or 3h and tested for the regulation of Tis11 mRNA. NA=not applicable, ND=not done, + weak induction, ++ strong induction, - downregulation. When no timepoint is mentioned, it refers to the effect seen at both timepoints tested but when is mentioned it refers to the timepoint tested and the effect seen.

Tis11d regulation at 1h or 3h					
	PMA	Anti-CD40	Anti-IgM	XRituximab	IL-4
NALM6	No change	No change	NA	NA	No change
RAMOS	+	No change	No change at 3h	No change	No change
WILCL	No change	No change at	ND	No change	No change
AGLCL	No change	No change	ND	ND	No change
MM1.S	No change	No change	ND	NA	ND
RPMI8226	No change	ND	ND	NA	ND

Table 5..3: Summary of Tis11d mRNA regulation in different B cell lines.
The indicated cell lines were stimulated with the indicated stimuli for 1h or 3h and tested for the regulation of Tis11d mRNA. NA=not applicable, ND=not done, + weak induction, ++ strong induction, - downregulation. When no timepoint is mentioned, it refers to the effect seen at both timepoints tested but when is mentioned it refers to the timepoint tested and the effect seen.

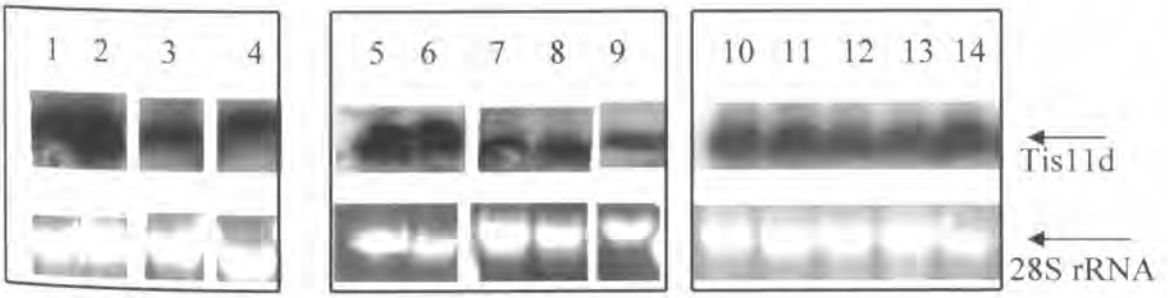


Figure 5.12: Regulation of Tis11d mRNA in different cell lines:
The indicated cell lines were stimulated with the indicated stimuli for the indicated times and RNA was extracted and processed for Northern Blot hybridisation using as a ^{32}P labelled Tis11d cDNA probe. Consistency of RNA loading as indicated by 28S rRNA staining is shown below each membrane. The arrows indicate either the Tis11d mRNA specific band detected by the radiolabelled probe or the 28S rRNA band present in each lane.
Lanes:
1) Ramos To(unstimulated cells), 2) Ramos PMA 3h,
3) Ramos anti-IgM 3h, 4) Ramos XRituximab 3h,
5) AGLCL To(unstimulated cells), 6) AGLCL PMA 1h,
7) AGLCL anti-CD40 3h, 8) AGLCL anti-CD40 4h,
9) AGLCL anti-IgM 3h, 10) MM1.S To(unstimulated cells),
11) MM1.S PMA 3h, 12) MM1.S anti-CD40 3h,
13) RPMI8226 To(unstimulated cells), 14) RPMI8226 PMA 3h

5.2.4 Regulation of Tis11b/Berg36 by different signal transduction pathways at different stages of B cell differentiation.

As shown above Tis11b/Berg36 was the only gene that was induced at different stages of B cell differentiation by a certain stimuli such as PMA or anti-CD40 in CLL, Ramos, WILCL cells. According to the data from previous chapters it was interesting to check whether induction of Tis11b/Berg36 by PMA and anti-CD40 in these cell lines was under NF-□B regulation as in CLL cells which would suggest same patterns of regulation at all stages of B cells differentiation. Accordingly it was interesting to test whether Ionomycin, anti-IgM and XRituximab that induce apoptosis and Tis11b/Berg36 mRNA in Ramos regulate the gene through p38 as was shown for XRituximab in CLL (see Chapter 3). These hypotheses could clarify whether Tis11b/Berg36 regulation is stimuli dependent, B cell stage dependent or function specific. To address these matters specific inhibitors of signal transduction pathways already discussed were included. The inhibitors used were: Gliotoxin (Glt) and BAY117280 for NF-□B, SB203580 and SB202190 for p38 pathway, SP600125 for JNK, U0126 for ERK and LY294005 for PI3K pathway at the same concentrations as in Chapters 4 and 5. It was found that in the case of anti-CD40 stimulation inhibition of NF-□B, p38 and JNK in Nalm6 cells partially or completely abrogated induction of the Tis11b/Berg36 gene while in Ramos cells inhibition of NF-□B, p38 and ERK pathways resulted in abrogation of Tis11b/Berg36 induction. In both cell lines the stronger effect was seen by the p38 inhibitor which completely abrogated Tis11b/Berg36 mRNA induction (Figure 5.13). In the case of Ionomycin stimulation, inhibition of NF-□B and JNK pathways resulted in complete abrogation of Tis11b/Berg36 mRNA induction at 3h post stimulation in both Ramos (Figure 14) and Nalm6 cells(data not shown). When anti-IgM was used, inhibition of p38 and PI3K pathways partially abrogated Tis11b/Berg36 induction in Ramos (Figure 14) even though not very clear in the photo due to unequal loading and scanning efficiency/clarity. Finally XRituximab mediated induction of Tis11b/Berg36 was abrogated by inhibition of NF-□B in Ramos cells and not p38 (Figure 5.14).

Collectively these data suggest that Tis11b/Berg36 is regulated in a stimulus-dependent and stage-dependent manner in B lymphocytes. Additionally stimuli that have a certain effect-function at the certain stage of differentiation eg: XRituximab, anti-IgM and

Ionomycin in Ramos did not regulate the gene through the same pathway suggesting that its regulation is stimuli dependent rather than function dependent.

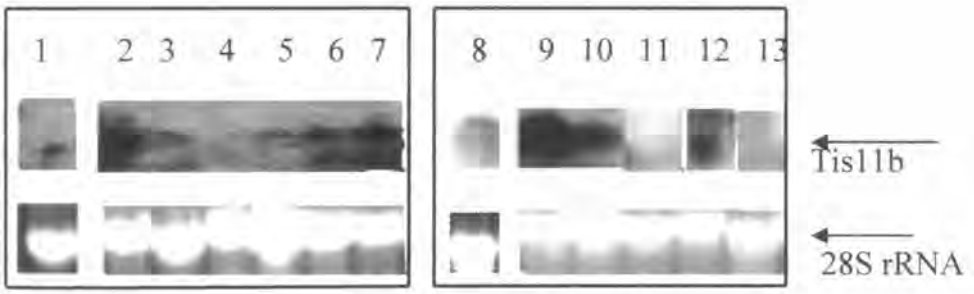


Figure 5.13: Inhibition of signal transduction pathways in relation to Tis11b/Berg36 mRNA induction by anti-CD40 in different cell lines
Nalm6 and Ramos cell lines were treated with anti-CD40 for 3h in the presence or absence of specific inhibitors for NF-κB (Glt), p38 (SB202190, SB203580), JNK (SP600125), ERK1/2 (U0126) and PI3K (LY294002) pathways. At the end of the incubation time RNA was extracted for Northern Blot analysis using a ^{32}P labelled Tis11b/Berg36 cDNA probe. Consistency of RNA loading as indicated by 28S rRNA staining is shown below each membrane. The arrows indicate either the Tis11b/Berg36 mRNA specific band detected by the radiolabelled probe or the 28S rRNA band present in each lane.
Lanes:
1) Nalm6 To(usntimulated cells)
2) Nalm6 anti-CD40 3h
3) Nalm6 Glt+anti-CD40 3h
4) Nalm6 SB202190+anti-CD40 3h
5) Nalm6 SP600125+anti-CD40 3h
6) Nalm6 LY294005+anti-CD40 3h
7) Nalm6 U0126+anti-CD40 3h
8) Ramos To(unstimulated cells)
9) Ramos anti-CD40 3h
10) Ramos Glt+anti-CD40 3h
11) Ramos SB203580+anti-CD40 3h
12) Ramos SP600125+anti-CD40 3h
13) Ramos U0126+anti-CD40 3h

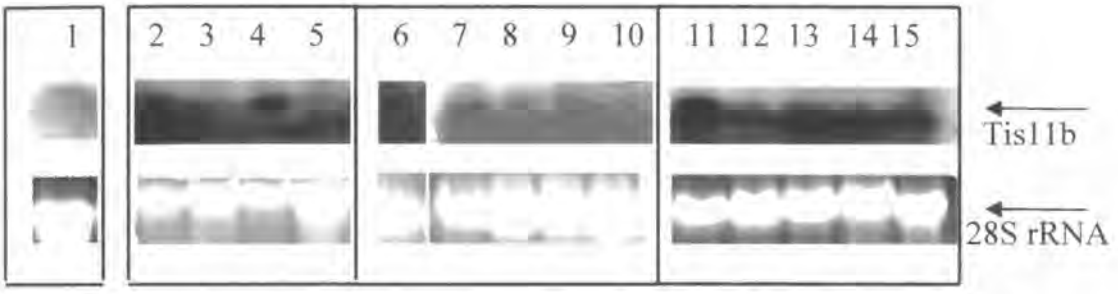


Figure 5.14: Inhibition of signal transduction pathways in relation to Tis11b/Berg36 mRNA induction by Ionomycin and anti-IgM in Ramos cells
Ramos cells were treated with Ionomycin or anti-IgM for 3h in the presence or absence of specific inhibitors for NF-κB (Glt), p38 (SB203580), JNK (SP600125), ERK1/2 (U0126) and PI3K (LY294002) pathways. At the end of the incubation time RNA was extracted for Northern Blot analysis using a ^{32}P labelled Tis11b/Berg36 cDNA probe. Consistency of RNA loading as indicated by 28S rRNA staining is shown below each membrane. The arrows indicate either the Tis11b/Berg36 mRNA specific band detected by the radiolabelled probe or the f 28S rRNA band persent ine ach lane.
Lanes:
1) Ramos To(unstimulated cells)
2) Ramos Ionomycin 3h
3) Ramos Glt+Ionomycin 3h
4)Ramos SB203580+Ionomycin 3h
5) Ramos SP600125+Ionomycin 3h
6) Ramos anti-IgM 3h
7) Ramos BAY117280+anti-IgM 3h
8) Ramos SB203580+anti-IgM 3h
9) Ramos SP600125+anti-IgM 3h
10) Ramos LY294005+anti-IgM 3h
11)Ramos XRituximab 3h
12) Ramos Glt+XRituximab
13) Ramos SB203580+XRituximab
14) Ramos SP600125+XRituximab
15) Ramos U0126+XRituximab 3h

5.3 DISCUSSION

In the previous chapter (Chapter 4) it was found that Tis11b/Berg36 was induced by stimuli involved in B cell differentiation namely PMA and anti-CD40. More importantly inhibition of Tis11b/Berg36 using a commercially available siRNA reduced the efficiency of PMA in inducing differentiation of B-CLL cells. On the contrary it was shown that Tis11 is down regulated following anti-CD40 treatment of CLL cells as early as 1h post stimulation. For these reasons it was decided to test the basal expression and regulation of the Tis11 family members at different stages of B cell differentiation. Thus cell lines arrested at different stages of differentiation were included namely Nalm6 (pre-B cells), Ramos (GC cells), AGLCL and WILCL (peripheral blood mature B cells) and finally RPMI8226 and MM1.S (plasma cells). With the exception of AGLCL and WILCL all the cell lines are malignant/leukaemic cell lines.

AGLCL and WILCL are not well characterised in terms of their immunophenotype and before using them it was necessary to characterise them. These cell lines were established from peripheral blood B cells of two different individuals and as such they are expected to contain naïve B cells, memory B cells and T1 cells. In order to identify these populations the cell lines were stained with CD19 and CD20 to confirm their B lymphocyte identity, CD86, CD38 and CRACC (19A) to check whether they are activated cells or not, CD27 to discriminate between naïve and memory B cells (Nagumo *et al* 2002) and finally CD138 to exclude the presence of plasma cells. Additionally they were tested for expression of Bcl-2 (data not shown), since Bcl-2 protein is developmentally regulated in B lymphocytes (Vigorito *et al* 2005, Tarte *et al* 2003, Merino *et al* 1994, Li *et al* 1993). It was found that AGLCL and WILCL highly express CD19 and CD20 as expected, and consist mainly of memory B cells with almost half of their cells expressing CD27. The cells have been previously activated because they express CD38, CD86 and CRACC/19A (a CD2 like receptor present only in activated/stimulated B cells and not in resting B cells) (Murphy *et al,* personal communication). Between the two cell lines, AGLCL express higher CD27 and CD86 than WILCL but the opposite was found for CD38. Since CD38 is a receptor highly expressed in

GC cells and declines thereafter (Lin *et al* 2002) this finding suggests that AGLCL represent a later stage in differentiation than WILCL. In further support of the latter, AGLCL were found to be weakly positive for CD138 (25%) which is characteristic of plasma cell differentiation, whereas WILCL were negative (3%). The CD138 expression in AGLCL is in accordance with their larger size under the light microscope and their slower proliferation rate –doubling time noticed in comparison to WILCL cells.

This suggests that AGLCL may contain some Antibody Forming Cells (AFC) or plasmablastic cells (thus positive for CD138). Because plasma cells reside in the bone marrow, these can be only memory-plasmablastic cells. It should be noted though that one study reported generation of peripheral blood plasma cells *in vitro* after cultivation of peripheral blood B cells with CD40L transfectants plus IL-2, plus IL-4, plus IL-10, plus IL12 which were also CD138+ cells but lower than CD138+ plasma cells (Tarte *et al* 2003). After the nature of the two normal lymphoblastoid cell lines was established the basal expression and regulation of Tis11 family was studied in all cell lines participating in this study.

It was found that Tis11b/Berg36 is highly expressed in WILCL and AGLCL normal lymphoblastoid cell lines but was low or undetectable in all malignant cell lines including Nalm6, Ramos and MM1.S or RPMI8226. Among malignant cell lines Nalm6 expressed the higher amounts of Tis11b/Berg36 whereas MM1.S and RPMI8226 had undetectable levels of this gene suggesting a decline in basal Tis11b/Berg36 expression as cells progress to plasma cell development.

Similarly the basal expression of Tis11 was found to be high in AGLCL and WILCL cells but very low or even undetectable in all the malignant cell lines especially the 2 Multiple Myeloma ones (MM1.S and RPMI8226). Between the two normal cell lines, the basal expression of Tis11 was higher in AGLCL than in WILCL and among the malignant cell lines Nalm6 was the only cell line with low but detectable expression of Tis11.

Finally Tis11d was very strongly expressed in all the cell lines tested with almost no difference in the levels of expression between normal and malignant cell lines. In the two normal lymphoblastoid Tis11d was found to be higher in AGLCL than in WILCL cells, and in the two multiple myeloma Tis11d was found to be higher in MM1.S than

RPMI8226. In conclusion Tis11d was always highly expressed at To (basal expression) in all the cell lines tested and showed no difference between normal and malignant cell lines whereas Tis11b/Berg36 and Tis11 were expressed at lower levels than Tis11d in all cell lines tested and their basal expression was higher in normal than in malignant cell lines and undetectable in multiple myeloma cell lines.

After the basal expression of the Tis11 family was elucidated, cells were stimulated with various stimuli to determine the regulation of Tis11 family at different stages of B cell maturation. The stimuli used included PMA, IL-4, CD40, Rituximab and XRituximab similarly to B-CLL cells at the same concentrations. Additionally Ionomycin and anti-IgM were included in Ramos cells because they strongly induce apoptosis in this cell line and it was very interesting to test whether all stimuli inducing apoptosis in Ramos (Ionomycin, anti-IgM, Rituximab, XRituximab) regulate Tis11b/Berg36 and Tis11 in a similar manner.

It was found that Tis11b/Berg36 mRNA was induced by PMA in Ramos at all timepoints tested but remained unaffected in AGLCL, MM1.S and RPMI8226. Interestingly the same stimuli (PMA) weakly down regulated the basal expression of Tis11b/Berg36 in Nalm6 suggesting that the same stimuli has different effects on Tis11b/Berg36 expression at different stages of differentiation. In Ramos cells it was found that inhibition of NF-□B almost completely abrogated induction of Tsi11b/Berg36 mRNA while at 3h post stimulation more than one pathways were involved.

Ionomycin induced Tis11b/Berg36 at 3h post stimulation in Ramos as expected (Ning *et al* 1996b) and in Nalm6 cells. In both cell lines inhibition of NF-□B and JNK pathways resulted in abrogation of Tis11b/Berg36 mRNA induction at 3h post stimulation. An ERK Inhibitor was not used in Ramos because Ionomycin did not activate ERK in another Burkitt's Lymphoma cell line (Graves *et al* 1996) and was assumed that the same would apply in Ramos cells as well.

Anti-CD40 stimulation induced Tis11b/Berg36 mRNA at 1h or 3h post stimulation in Nalm6, Ramos and WILCL but down regulated the gene in AGLCL and MM1.S at 3h post stimulation. Induction of Tis11b/Berg36 by this stimuli in Ramos cells is in accordance with published data that utilised microarray analysis (Basso *et al* 2004)

while all the other findings are novel findings of this study. The effect on MM1.S cell line was evident only after long exposure (15 days) because of the very low basal levels detected with the standard 3 days exposure. Because treatment of MM1.S with a human anti-CD40 antibody induced cell death (assayed by ^{51}Cr-release) (Tai *et al* 2004, Hayashi *et al* 2003) without affecting cell proliferation (Tai *et al* 2004) the above finding may suggest that Tis11b/Berg36 has another function than induction of apoptosis as cells differentiate to plasma cells. Notably inhibition of NF-□B and JNK pathways partially abrogated induction of Tis11b/Berg36 by anti-CD40 at 3h in Ramos cells while inhibition of p38 and ERK pathways completely abrogated induction of the gene at 3h post stimulation. It has been reported that anti-CD40 rapidly induces JNK (within 5 min) in a Burkitt's Lymphoma cell line and that this induction precedes activation of NF-□B activation suggesting that JNK is upstream of NF-□B activation after CD40 signalling (Berberich *et al* 1996). This finding is confirmed here, because their inhibition had an identical effect on Tis11b/Berg36 mRNA regulation following anti-CD40 treatment and suggest that NF-□B is the upstream regulator of Tis11b/Berg36. Additionally because anti-CD40 activated p38 pathway (within 5 min post stimulation) in human B cells and use of SB203580 (the same inhibitor used in this study) inhibited anti-CD40 induced proliferation by almost 70% (Craxton *et al* 1998), complete abrogation of Tis11b/Berg36 by p38 inhibition after anti-CD40 may suggest that this gene may be involved in proliferation as well.

Anti-IgM treatment strongly induced Tis11b/Berg36 mRNA in Ramos and inhibition of p38 and PI3K pathways.partially abrogated induction of the gene It has been reported that PI3K is upstream of p38 and ERK1/2 activation after anti-IgM treatment (Sakata *et al* 1999) suggesting that actually p38 is the upstream regulator of Tis11b/Berg36 in this case. It was also shown that p38 and JNK pathways are involved in induction of apoptosis by anti-IgM and Ionomycin in B104 Burkitt's Lymphoma cell line (Graves *et al* 1998, Graves *et al* 1996), suggesting that Tis11b/Berg36 is involved in induction of apoptosis following BCR stimulation (anti-IgM) in GC cells.

Finally XRituximab strongly induced Tis11b/Berg36 in Ramos and WILCL cell lines tested and in WILCL cells inhibition of p38 abrogated by 50% induction of Tis11b/Berg36. It is noteworthy that induction of Tis11b/Berg36 following XRituximab

in both WILCL (memory B cells) and CLL cells (resemble memory B cells as shown by microarrays) was at least partially under the control of p38 pathway and suggests probably that in memory cells p38 is the main regulator of Tis11b/Berg36 mRNA in response to CD20Receptor activation. However in Ramos cells induction of Tis11b/Berg36 by XRituximab was not affected by inhibition of p38 pathway suggesting possibly that at the GC stage another than p38 pathway regulates induction of Tis11b/Berg36 in response to CD20Receptor activation.

In conclusion Tis11b/Berg36 can be: i)Induced or down regulated by the same stimuli at different stages of B cell differentiation , ii) when induced by a certain stimuli at different stages of differentiation is regulated through a different set of pathways in each of these stages suggesting stimuli dependent and developmental stage dependent regulation of the gene.

As for Tis11 regulation, it was induced by a certain stimuli at one stage and downregulated by the same stimuli at another stage (eg: by IL-4 in WILCL or Ramos and Nalm6 respectively). XRituximab weakly induced the gene at 3h post stimulation in Ramos and WILCL cells which is completely different from what was found in B-CLL cells suggesting again developmental stage dependent regulation.

Finally Tis11d mRNA remained unchanged by all the stimuli used at all stages of differentiation. The only exception was XRituximab which weakly induced the gene in WILCL and probably Ramos cells but in the latter cell type there was poor quality of RNA and hybridisation signal. Interestingly Tis11d was down regulated by IL-4 in AGLCL cells and by anti-CD40 in WILCL and AGLCL normal lymphoblastoid cells which is in contrast to what was found in B-CLL cells. All findings regarding regulation of Tis11 and Tis11d in all stages of B cell differentiation constitute novel findings of this study.

In summary, all 3 Tis11 family members showed a B cell stage dependent regulation in response to the same stimuli implying that they may be involved in B cell differentiation.

CHAPTER 6

5L3 Sequencing and analysis

6.1 INTRODUCTION

In early 1990s stimulation of CLL cells led to the identification of a set of 12 Early Response Genes (ERGs) with unknown function at the time (Murphy and Norton 1990). Among them was Tis11b/Berg36 which has been described and studied in the previous sections of this PhD thesis and another gene called 5L3 (Murphy and Norton 1990). Interestingly 5L3 was induced by PMA only in CLL cells and not in normal tonsillar B cells (tested up to 24h) (Murphy and Norton 1993) or in other B cell Leukaemias-Lymphomas such as Multiple Myeloma (RPMI8226 cell line), Burkitt's Lymphoma (Daudi and Raji cell lines) and primary Hairy Cell Leukaemia (HCL) and Non Hodgkin's Lymphoma (NHL) cells (Green *et al* 1991). Thus it was interesting to further investigate the regulation of this gene in CLL in case a novel regulator of apoptosis could be identified. In order to test this hypothesis two different approaches were utilised. The first one involved sequencing and extensive Bioinformatics analysis is order to identify similarity with a known gene or protein while the second one involved Northern Blot analysis after stimulation of CLL cells with stimuli that inhibit (IL-4, PMA, CD40) and induce apoptosis (XRItuximab) in CLL. None of these stimuli has been tested before in association with 5L3 expression in CLL or other B cell malignancies.

6.2 RESULTS

6.2.1 SEQUENCING RESULTS

Because at the start of this project, no known genes were found to have similarity with 5L3, it was decided to sequence the clone and perform Bioinformatics analysis. Initially the pUC9 plasmid containing the 1.1 Kb 5L3 clone insert was grown into LBroth medium containing ampicillin, plasmid was extracted and sent to the Molecular Biology Service Unit, King's College London for sequencing using the M13 primer (forward and reverse).

The sequence data from M13 primers is shown in Figure 6.1 and 6.2 and their summary in Figure 6.3.

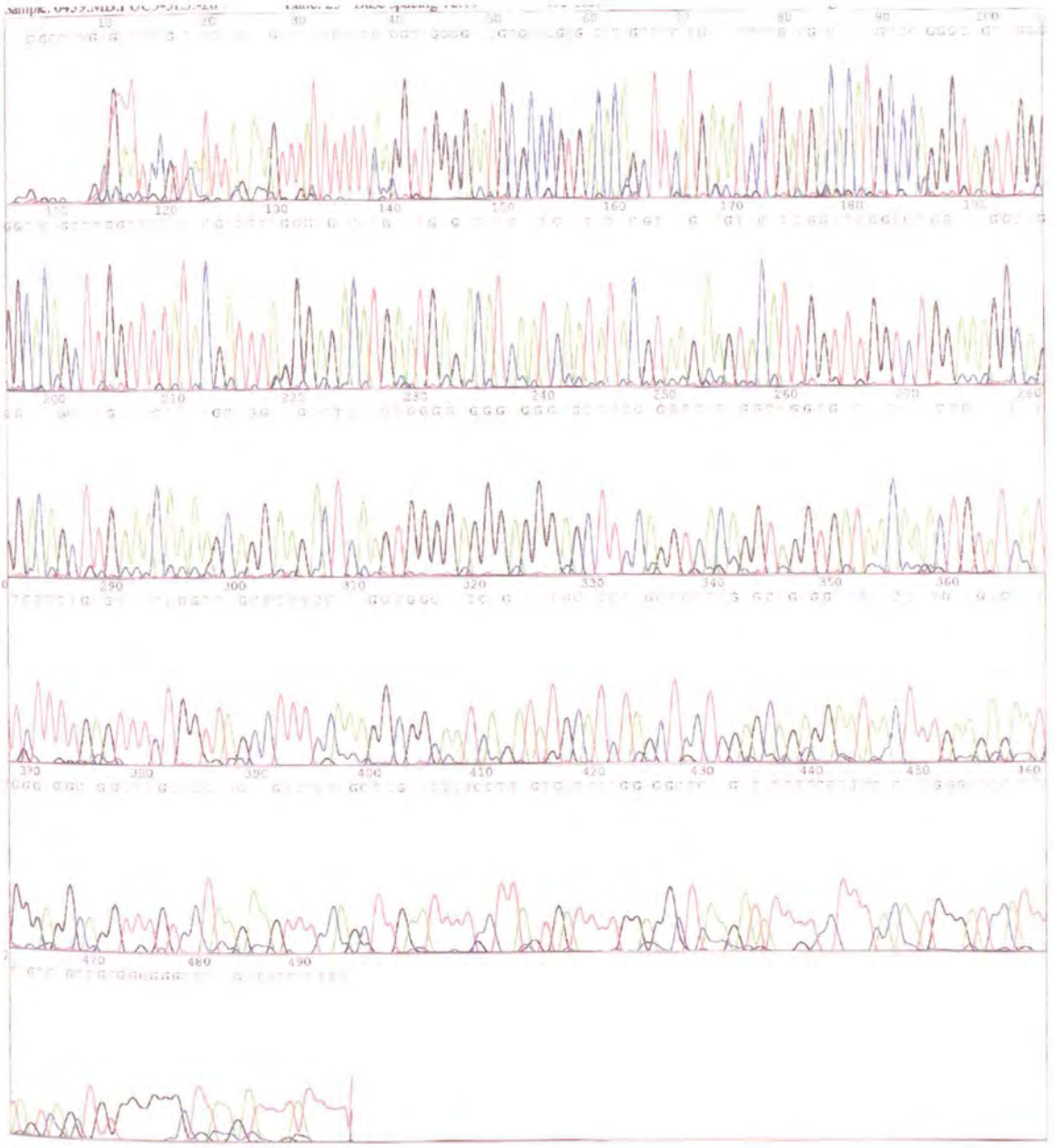


Figure 6.1:Results from sequencing analysis of the 5L3 clone using the M13 Forward Primer

Sequencing results from the M13 Forward Primer for 5L3 clone after processing with the CHROMAS programme. Each base is represented by a single colour peak :with red representing Thymine, green representing Adenine, blue representing Cytidine and black representing Guanine and the number below the peaks indicate the position for each of the nucleotides from the start of the sequence.

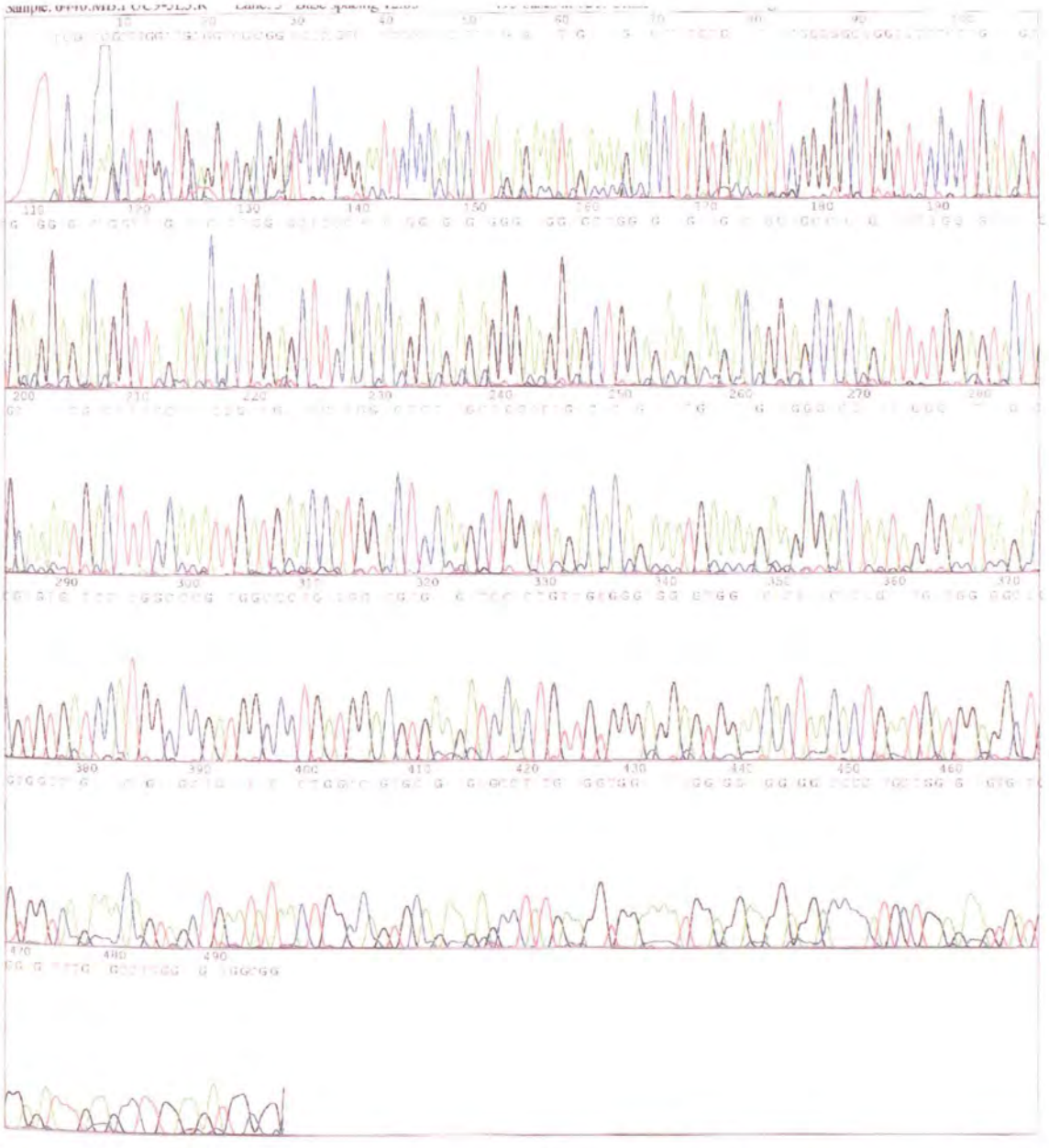


Figure 6.2 :Results from sequencing analysis of the 5L3 clone using the M13 Reverse Primer

Sequencing results from the M13 Reverse Primer for 5L3 clone after processing with the CHROMAS programme. Each base is represented by a single colour peak :with red representing Thymine, green representing Adenine, blue representing Cytidine and black representing Guanine and the number below the peaks indicate the position for each of the nucleotides from the start of the sequence.

Sequence from Forward `Primer

1 GGCCNNNGNGTTTCCGTATTTAGAAGTTTATTTTTTNAGGTTGGGGAATG

51 CGCCCGTGACACAGCTTCATGAAATCCTGATGACACATGCCCAGGGTAGT

101 TGGGGCACAGCTTGGTTTTATACGATTTAGGGAGACATGAATGAGACATC

151 AATCAATATACGTAAGAAGTACATTGGTTTGGTCTGGAAAGGCAGGACAG

201 CTTGAAACAAAAGCAGGAAGACTCAAGTGGGGAGGGAGGGAGCTTCCAGG

251 TCACAGGTAGGTGATAACAAACTGTATCATTCTTTTGAGTTTCTGGTTAG

301 CCTTTCCAAAGGCGGCAATCAGATATGCATCTAGCTCCTCGAGCAGAGGA

351 ATAACTTTGAAGAGAATGGGAGGCAGGTTTGTCCTAAGAAGTTTCCAGCT

401 TGAATTTTCCTTAGTGATTTTGGAGGCTCAAGATATTTTCCTTTCACAAT

451 GGGATCCATTTAGTCAGCTGTGGGGGGCTTAAGATTTTATTT

Sequence from Reverse primer

1 TTCGCCCGCTTGGCTGCAGGTCGACGGANCCCCGGGAATTCCCCAACACT

51 TAGAGAAATAGAAAAGAACCTATGTGAAATATCGGGGGCTGGTTTCCCCT

101 GATAGTGAAGGAGACAGGTTAGATACACTGGAGCTTCCACACAGGAGAGA

151 GGGAAGGAGCTGGAGAAGAAGACAGGAGCCACAGATTTTGGAGTACTGCA

201 AAATGACTTTCCAAATTGATGAACCATGGACTCCAAGCTGGATAGACACA

251 GAAATGAAAAGAAGGGACTAATAGGGAATAAGACGTGTGATCCATGGCCC

301 GATGGCCCTGATGGACGTGAAGATCCACTGTTGTGGGAGGAGTGGAACAC

351 TAACACTGAATGATGGAGGCTGTGGTCAGAAACAGTAGCTGAATATAAC

401 TGGCCAGTGCAGAAGAGCCTATGAAGGTGGAAAATGGAGGAAGGAGGACC

451 CCATGCTGGA GAAGTGATGG AGATTTGAAG CCTTGGAAGA TGGCGG

Figure 6.3: Summary of the sequencing results obtained by the Forward and Reverse M13 primers.
The sequences above represent the summary of the sequencing results obtained by the two M13 primers (forward and reverse) and were presented in the previous two figures (Figure 6.1 and 6.2 respectively) in a CHROMAS format.

Reverse and complementary sequence to the sequence derived from the forward primer

AAAATAAAATCTTAAGCCCCCCACAGCTGACTAAATGGATCCCATTGTGAAAGGAAAATA
TCTTGAGCCTCCAAAATCACTAAGGAAAATTCAAGCTGGAAACTTCTTAGGACAAACCTG
CCTCCCATTCTCTTCAAAGTTATTCCTCTGCTCGAGGAGCTAGATGCATATCTGATTGCC
GCCTTTGGAAAGGCTAACCAGAAACTCAAAAGAATGATACAGTTTGTTATCACCTACCTG
TGACCTGGAAGCTCCCTCCCTCCCCACTTGAGTCTTCCTGCTTTTGTTTCAAGCTGTCCT
GCCTTTCCAGACCAAACCAATGTACTTCTTACGTATATTGATTGATGTCTCATTCATGTC
TCCCTAAATCGTATAAAACCAAGCTGTGCCCCAACTACCCTGGGCATGTGTCATCAGGAT
TTCATGAAGCTGTGTCACGGGCGCATTCCCCAACCTNAAAAAATAAACTTCTAAATACGG

Reverse and complementary sequence to the sequence derived from the reverse primer

CCGCCATCTTCCAAGGCTTCAAATCTCCATCACTTCTCCAGCATGGGGTCCTCCTTCCTC
CATTTTCCACCTTCATAGGCTCTTCTGCACTGGCCAGTTATATTCAGCTACTGTTTCTGA
CCACAGCCTCCATCATTCAGTGTTAGTGTTCCACTCCTCCCACAACAGTGGATCTTCACG
TCCATCAGGGCCATCGGGCCATGGATCACACGTCTTATTCCCTATTAGTCCCTTCTTTTC
ATTTCTGTGTCTATCCAGCTTGGAGTCCATGGTTCATCAATTTGGAAAGTCATTTTGCAG
TACTCCAAAATCTGTGGCTCCTGTCTTCTTCTCCAGCTCCTTCCCTCTCTCCTGTGTGGA
AGCTCCAGTGTATCTAACCTGTCTCCTTCACTATCAGGGGAAACCAGCCCCCGATATTTC
ACATAGGTTCTTTTCTATTTCTCTAAGTGTTGGGGAATTCCCGGGGNTCCGTCGACCTGC

Figure 6.4: Reverse and complementary sequences to the sequencing data obtained by the Forward and Reverse M13 primers.
The sequences shown in Figure 6.3 were processed through CHROMAS programm to obtain their reverse and complementary sequence in order to ensure that both 5'-3' and 3'-5' directions and analysed by BLAST searches to identify similarity with known transcripts.

6.2.2 BIOINFORMATICS ANALYSIS

6.2.2.1 BLASTN

The sequencing data have been compared with known sequences available on NCBI or EBI (Ensembl) databases using BLASTN search. The latter was carried out in comparison to genomic DNA sequences or cDNA (Ensembl) and RefSeq- RNA (NCBI) sequences since this clone is a cDNA and thus represents mRNA sequence rather than genomic DNA sequence.

BLASTN analysis, non restricted using the NCBI database, for the sequence derived from the Forward primer identified a BAC clone called RP11-242J7 located on chromosome 4 which showed the highest similarity with 5L3 sequence (455 bases matching from 24-492 with 99% similarity) (Figure 6.5b). The same match was also identified by the Ensembl database but as a contig this time (Figure 6.5a). The same type of analysis (non restricted by NCBI) for the sequence derived from the reverse primer identified the same BAC clone in chromosome 4 with a score of 430 bits (217 bases matching from 169-385 with 99% similarity) (Figure 6.6)

When BLASTN search was performed for the reverse and complementary sequences for both the forward and reverse primer over genomic DNA, the highest score of similarity for both of them was the BAC clone RP11-242J7 on Chromosome 4 (data not shown because similar to figures 5 and 6).

When the BLASTN analysis was restricted to RefSeq-RNA (NCBI database) only the sequence from the Forward primer showed a significant similarity to a known transcript which was the product of KIAA0565 gene transcript variant 3 located on chromosome 17p11.2 to which 5L3 showed a 244 score with 83% similarity for bases 54-450 (Figure 7). The reverse and complementary sequence identified the same match. This KIAA0565 gene has 5 different transcription variants that arise through alternative splicing but its function is currently unknown.

When BLASTN was performed through Ensembl (EBI) database in comparison to cDNAs including novel, non coding cDNAs, and pseudogenes no match was found for the sequence derived from the Forward primer in both orientations. The sequence from

the reverse primer showed low score of similarity for the matches detected (data not shown). The reason for the different results obtained through NCBI and EBI databases is unknown.

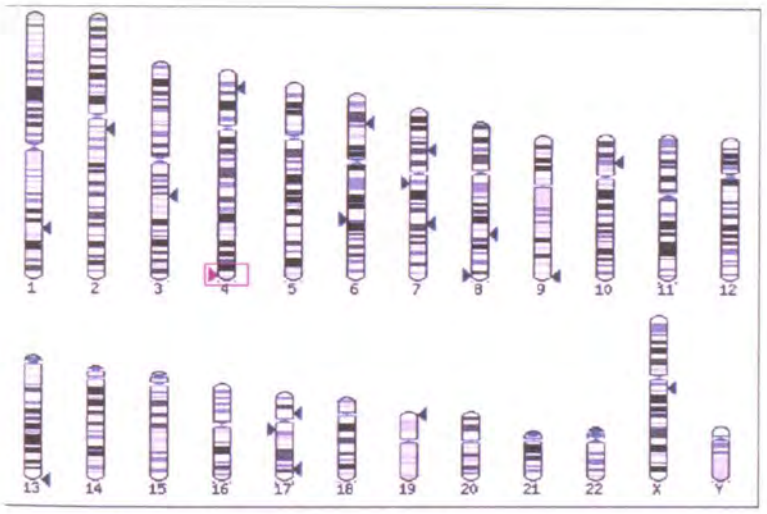


Figure 6.5a: BLASTN analysis of the sequenceof 5L3 obtained from the M13 Forward primer using Ensembl (EBI) database

Chromosomal distribution of BLASTN analysis for the sequence obtained by the Forward Primer over genomic DNA using the Ensembl (EBI) database. The arrows indicate all matches detected while the red arrow and box indicate the highest score-similarity found which corresponds to BAC clone RP11-242J7 on chromosome 4. Note that the same region was identified as the highest score match for the sequence derived from the Reverse primer using the same search engine. The same results were also obtained using non restricted (nr) BLASTN from NCBI database for both primers.

```
gi|18042387|gb|AC073932.4|

Query  24     AAGTTTATTTTTTNA-GGTTGGGGAATGCGCCCGTGACACAGCTTCATGAAATCCTGATG  82
              |||||||||||||| | ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  38646  AAGTTTATTTTTTCAAGGTTGGGGAATGCGCCCGTGACACAGCTTCATGAAATCCTGATG  38705

Query  83     ACACATGCCCAGGGTAGTTGGGGCACAGCTTGGTTTTATACGATTTAGGGAGACATGAAT  142
              |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |
Sbjct  38706  ACACATGCCCAGGGTAGTTGGGGCACAGCTTGGTTTTATACGATTTAGGGAGACATGAGT  38765

Query  143    GAGACATCAATCAATATACGTAAGAAGTACATTGGTTTGGTCTGGAAAGGCAGGACAGCT  202
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  38766  GAGACATCAATCAATATACGTAAGAAGTACATTGGTTTGGTCTGGAAAGGCAGGACAGCT  38825

Query  203    TGAAACAAAAGCAGGAAGACTCAAGTGGGGAGGGAGGGAGCTTCCAGGTCACAGGTAGGT  262
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  38826  TGAAACAAAAGCAGGAAGACTCAAGTGGGGAGGGAGGGAGCTTCCAGGTCACAGGTAGGT  38885

Query  263    GATAACAAACTGTATCATTCTTTTGAGTTTCTGGTTAGCCTTTCCAAAGGCGGCAATCAG  322
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  38886  GATAACAAACTGTATCATTCTTTTGAGTTTCTGGTTAGCCTTTCCAAAGGCGGCAATCAG  38945

Query  323    ATATGCATCTAGCTCCTCGAGCAGAGGAATAACTTTGAAGAGAATGGGAGGCAGGTTTGT  382
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  38946  ATATGCATCTAGCTCCTCGAGCAGAGGAATAACTTTGAAGAGAATGGGAGGCAGGTTTGT  39005

Query  383    CCTAAGAAGTTTCCAGCTTGAATTTTCCTTAGTGATTTTGGAGGCTCAAGATATTTTCCT  442
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  39006  CCTAAGAAGTTTCCAGCTTGAATTTTCCTTAGTGATTTTGGAGGCTCAAGATATTTTCCT  39065

Query  443    TTCACAATGGGATCCATTTAGTCAGCTGTGGGGGGCTTAAGATTTTATTT  492
              ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  39066  TTCACAATGGGATCCATTTAGTCAGCTGTGGGGGGCTTAAGATTTTATTT  39115
```

Figure 6.5b: Alignment of sequence of 5L3 obtained from Reverse primer to BAC clone RP11-242J7. Alignment of the sequencing results from the Forward primer to the RP11-242J7 BAC clone. This was obtained using a BLASTN analysis (non restricted) from NCBI database. The score and degree of similarity between the 5L3 sequencing data and the target sequence is shown. The results were recently confirmed on the 14/04/06

```
gi|18042387|gb|AC073932.4|

Query  169    AAGACAGGAGCCACAGATTTTGGAGTACTGCAAAATGACTTTCCAAATTGATGAACCATG  228
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  44971  AAGACAGGAGCCACAGATTTTGGAGTACTGCAAAATGACTTTCCAAATTGATGAACCATG  44912

Query  229    GACTCCAAGCTGGATAGACACAGAAATGAAAAGAAGGGACTAATAGGGAATAAGACGTGT  288
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  44911  GACTCCAAGCTGGATAGACACAGAAATGAAAAGAAGGGACTAATAGGGAATAAGACGTGT  44852

Query  289    GATCCATGGCCCGATGGCCCTGATGGACGTGAAGATCCACTGTTGTGGGAGGAGTGGAAC  348
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  44851  GATCCATGGCCCGATGGCCCTGATGGACGTGAAGATCCACTGTTGTGGGAGGAGTGGAAC  44792

Query  349    ACTAACACTGAATGATGGAGGCTGTGGTCAGAAACAG  385
              |||||||||||||||||||||||||||||||||||||
Sbjct  44791  ACTAACACTGAATGATGGAGGCTGTGGTCAGAAACAG  44755
```

Figure 6.6: Alignment of sequence of 5L3 obtained from the M13 Reverse primer to BAC clone RP11-242J7. Alignment of the sequencing results from the Reverse primer to the RP11-242J7 BAC clone. This was obtained using a BLASTN analysis (nr, non restricted) from NCBI database. This BAC clone showed the higher degree of similarity with the 5L3 sequence. The score and degree of similarity between the 5L3 sequnecind data and the target sequence is shown. The results were recently confirmed on the 14/04/06.

```
>gi|89041256|ref|XM_934727.1| G PREDICTED: Homo sapiens KIAA0565 gene product, t
3 (KIAA0565), mRNA
Length=5115

 Score =  244 bits (123),  Expect = 1e-62
 Identities = 337/405 (83%), Gaps = 15/405 (3%)
 Strand=Plus/Plus

Query  54    CCGTGACACAGCTTCATGAAATCCTGATGACACATGCCCAGGGTAGTTGGGGCACAGCTT  113
             ||||||||||||| ||| ||| ||||||||||||  || ||| ||| ||||||||||| |||
Sbjct  4372  CCGTGACACAGCCTCAGGAAGTCCTGATGACATGTGTCCAAGGTGGTTGGGGCACAACTT  4431

Query  114   GGTTTTATACGATTTAGGGAGACATGAATGAGACATCAATCAATATACGTAAGAAGTACA  173
             ||||||||||| ||||||||||||    ||||||||||||||||||||  ||||||||||||
Sbjct  4432  GGTTTTATACATTTTAGGGAGAC----ATGAGACATCAATCAATATATTTAAGAAGTACA  4487

Query  174   TTGGTTTGGTCTGGAAAGGCAGGACAGCTTGAAACAAAAGCAGGAAGACTCAAGTGGGGA  233
             ||||||   ||||||||||| |||||  ||||| |||| | |||||||||   || | |
Sbjct  4488  TTGGTTCACTCTGGAAAGGCGGGACAATTTGAAGCAAAGGGAGGAAGACT--GGTAGCG-  4544

Query  234   GGGAGGGAGCTTCCAGGTCACAGGTAGGTGATA-ACAAACTGTATCATTCTTTTGAGTTT  292
             ||||||| |||||||||||||||| ||| ||| | |||||| ||  || ||||||||||||
Sbjct  4545  GGGAGGGGGCTTCCAGGTCACAGATAGATGAGAGACAAACAGTTGCACTCTTTTGAGTTT  4604

Query  293   CTGGTTAGCCTTTCCAAAGGCGGCAATCAGATATGCATCTAGCTCCTCGAGCAGAGGAAT  352
             ||| |||||||||||||||| | || |||||| ||||| || ||    ||||||||   |
Sbjct  4605  CTGATTAGCCTTTCCAAAGGAGACAGTCAGATCTGCATTTATCTTAGTGAGCAGAGAGGT  4664

Query  353   AACTTTGAAGAGAATGGGAGGCAGGTTTGTCCTAAGAAGTTTCCAGCTTGAATTTTC---  409
             ||||||||| |||||||||||||||||||| |||||| |||||||||| ||| |||||
Sbjct  4665  AACTTTGAATAGAATGGGAGGCAGGTTTGCCCTAAGCAGTTTCCAGCCTGAGTTTTCCTT  4724

Query  410   ----CTTAGTGATTTTGGAGGCTCAAGATATTTTCCTTTCACAAT  450
                 |||||||||||||||||| |||||||||||||||||||||||
Sbjct  4725  TTAGCTTAGTGATTTTGGAGGCCCAAGATATTTTCCTTTCACAAT  4769
```

Figure 6.7: Alignment of 5L3 sequence to KIAA0565 transcript variant 3.
Alignment of the sequence of 5L3 obtained from the Forward primer to KIAA0565 transcript variant 3 after BLASTN analysis over RefSeq-RNA sequences in the NCBI database. This transcript gave the higher score of similarity to the 5L3 sequence. This score of similarity is indicated as well as the gaps found between the two sequences. These data were not obtained when the sequence was initially analysed on June 2003 but were identified recently on the 14/04/06

6.2.2.2 TRANSLATE and BLASTP

Finally the sequencing data were translated to a protein sequence using TRANSLATE from ExPaSy database or EMBL-EBI translation tool from the same database. The former one identified 6 Open Reading Frames (ORFs) for the sequence derived from the forward primer but all of them (either in the 5'-3' or in the 3'-5' direction) had many stop codons sometimes immediately after Methionine thus probably do not code for a protein (Figures 6.8 and 6.9). When these ORFs were compared with protein sequences through BLASTP no similarity was found (data not shown)

Results from the TRANSLATE database for the Reverse primer sequence revealed only one ORF at the 3'-5' direction (3'-5' Frame 2) that could be the sequence of a protein. From all 3 frames only the 3'-5' frame was checked through BALSTP for similarity with a known protein but no such match was identified. Translation of the sequencing results from both primers and their reverse and complementary sequence was done later on using the CHROMAS programme as well but no difference was obtained for any of the 3 ORFs.

5'3' Frame 1

G X X V S V F R S L F F X V G E C A R D T A S **Stop** N P D D T C P G **Stop** L G H S L V L Y D L G R H E **Stop** D I N Q Y T **Stop** E V H W F G L E R Q D S L K Q K Q E D S S G E G G S F Q V T G R **Stop Stop** Q T V S F F **Stop** V S G **Stop** P F Q R R Q S D **Met** H L A P R A E E **Stop** L **Stop** R E W E A G L S **Stop** E V S S L N F P **Stop Stop** F W R L K I F S F H N G I H L V S C G G L K I L F

5'3' Frame 2

A X X F P Y L E V Y F X R L G N A P V T Q L H E I L **Met** T H A Q G S W G T A W F Y T I **Stop** G D **Met** N E T S I N I R K K Y I G L V W K G R T A **Stop** N K S R K T Q V G R E G A S R S Q V G D N K L Y H S F E F L V S L S K G G N Q I C I **Stop** L L E Q R N N F E E N G R Q V C P K K F P A **Stop** I F L S D F G G S R Y F P F T **Met** G S I **Stop** S A V G G L R F Y

5'3' Frame 3

X X X F R I **Stop** K F I F X G W G **Met** R P **Stop** H S F **Met** K S **Stop Stop** H **Met** P R V V G A Q L G F I R F R E T **Stop Met** R H Q S I Y V R S T L V W S G K A G Q L E T K A G R L K W G G R E L P G H R **Stop** V I T N C I I L L S F W L A F P K A A I R Y A S S S S S R G I T L K R **Met** G G R F V L R S F Q L E F S L V I L E A Q D I F L S Q W D P F S Q L W G A **Stop** D F I

3'5' Frame 1

K **Stop** N L K P P T A D **Stop Met** D P I V K G K Y L E P P K S L R K I Q A G N F L G Q T C L P F S S K L F L C S R S **Stop Met** H I **Stop** L P P L E R L T R N S K E **Stop** Y S L L S P T C D L E A P S L P T **Stop** V F L L L F Q A V L P F Q T K P **Met** Y F L R I L I D V S F **Met** S P **Stop** I V **Stop** N Q A V P Q L P W A C V I R I S **Stop** S C V T G A F P N X K K **Stop** T S K Y G N X X A

3'5' Frame 2

N K I L S P P Q L T K W I P L **Stop** K E N I L S L Q N H **Stop** G K F K L E T S **Stop** D K P A S H S L Q S Y S S A R G A R C I S D C R L W K G **Stop** P E T Q K N D T V C Y H L P V T W K L P P S P L E S S C F C F K L S C L S R P N Q C T S Y V Y **Stop** L **Met** S H S C L P K S Y K T K L C P N Y P G H V S S G F H E A V S R A H S P T X K N K L L N T E X X X

3'5' Frame 3

I K S **Stop** A P H S **Stop** L N G S H C E R K I S **Stop** A S K I T K E N S S W K L L R T N L P P I L F K V I P L L E E L D A Y L I A A F G K A N Q K L K R **Met** I Q F V I T Y L **Stop** P G S S L P P H L S L P A F V S S C P A F P D Q T N V L L T Y I D **Stop** C L I H V S L N R I K P S C A P T T L G

Figure 6.8: TRANSLATE results for the sequence of 5L3 obtained from the M13 Forward Primer.

The sequence obtained using the M13 Forward primer was translated using the TRANSLATE programm from ExPAsy database which identified 3 Open Reading Frames (ORFs) in 5'-3' or 3'-5' direction. The sequence of the possible proteins is shown in the single letter code with the start codon (Methionine) and Stop Codons shown in bold. These data were initially obtained on June 2003 and were recently confirmed on the 14/04/06.

5'3' Frame 1

F A R L A A G R R X P G N S P T L R E I E K N L C E I S G A G F P **Stop Stop Stop** R R Q V R Y T
G A S T Q E R G K E L E K K T G A T D F G V L Q N D F P N **Stop Stop** T **Met** D S K L D R H R N
E K K G L I G N K T C D P W P D G P D G R E D P L L W E E W N T N T E **Stop** W R L W S E T V
A E Y N W P V Q K S L **Stop** R W K **Met** E E G G P H A G E V **Met** E I **Stop** S L G R W R

5'3' Frame 2

S P A W L Q V D G X P G I P Q H L E K **Stop** K R T Y V K Y R G L V S P D S E G D R L D T L E L
P H R R E G R S W R R R Q E P Q I L E Y C K **Met** T F Q I D E P W T P S W I D T E **Met** K R R D
Stop Stop G I R R V I H G P **Met** A L **Met** D V K I H C C G R S G T L T L N D G G C G Q K Q **Stop**
L N I T G Q C R R A Y E G G K W R K E D P **Met** L E K **Stop** W R F E A L E D G

5'3' Frame 3

R P L G C R S T X P R E F P N T **Stop** R N R K E P **Met Stop** N I G G W F P L I V K E T G **Stop** I H
W S F H T G E R E G A G E E D R S H R F W S T A K **Stop** L S K L **Met** N H G L Q A G **Stop** T Q K
Stop K E G T N R E **Stop** D V **Stop** S **Met** A R W P **Stop** W T **Stop** R S T V V G G V E H **Stop** H **Stop**
Met Met E A V V R N S S **Stop** I **Stop** L A S A E E P **Met** K V E N G G R R T P C W R S D G D L K
P W K **Met** A

3'5' Frame 1

P P S S K A S N L H H F S S **Met** G S S F L H F P P S **Stop** A L L H W P V I F S Y C F **Stop** P Q P P S
F S V S V P L L P Q Q W I F T S I R A I G P W I T R L I P Y **Stop** S L L F I S V S I Q L G V H G S S I
W K V I L Q Y S K I C G S C L L L Q L L P S L L C G S S S V S N L S P S L S G E T S P R Y F T
Stop V L F Y F S K C W G I P G X P S T C S Q A G E

3'5' Frame 2

R H L P R L Q I S I T S P A W G P P S S I F H L H R L F C T G Q L Y S A T V S D H S L H H S
V L V F H S S H N S G S S R P S G P S G H G S H V L F P I S P F F S F L C L S S L E S Met V H
Q F G K S F C S T P K S V A P V F F S S S F P L S C V E A P V Y L T C L L H Y Q G K P A P D
I S H R F F S I S L S V G E F P G X R R P A A K R A

3'5' Frame 3

A I F Q G F K S P S L L Q H G V L L P P F S T F I G S S A L A S Y I Q L L F L T T A S I I Q C **Stop**
C S T P P T T V D L H V H Q G H R A **Met** D H T S Y S L L V P S F H F C V Y P A W S P W F I N L
E S H F A V L Q N L W L L S S S P A P S L S P V W K L Q C I **Stop** P V S F T I R G N Q P P I F H I
G S F L F L **Stop** V L G N S R X S V D L Q P S G R

Figure 6.9: TRANSLATE results for the sequence of 5L3 obtained from the M13 Forward Primer

The sequence obtained using the M13 Reverse primer was translated using the TRANSLATE programm from the ExPAsy database which identified 3 Open Reading Frames (ORFs) in 5'-3' or 3'-5' direction. The sequence of the possible proteins is shown in the single letter code with the start codon (Methionine) and Stop Codons shown in bold. These data were initially obtained on June 2003 and were recently confirmed on the 14/04/06.

6.2.3 NORTHERN BLOT ANALYSIS

Despite the fact that no known protein was identified for 5L3 clone, it was decided to check the regulation of this gene in CLL in response to PMA (positive control), IL-4, CD40, CD40+IL-4, PMA+IL-4, Rituximab or XRituximab. CLL cells were stimulated with the above stimuli for 3h and Northern Blot analysis revealed that 5L3 was strongly induced by PMA as expected and addition of IL-4 to PMA did not further changed this induction. Stimulation with IL-4 alone, CD40L, CD40L+IL-4, Rituximab and XRituximab did not change the basal expression of the gene (Figure 6.11). In one patient, CLL15 there was strong induction of 5L3 by CD40L but because it was not seen in the rest of the patients tested (a total of 5) it was considered as a unique feature of that particular patient. Inclusion of two different Multiple Myeloma cell lines namely RPMI826 and MM1.S confirmed the fact that 5L3 is specifically induced by PMA in CLL cells and showed that RPMI8226 had higher basal levels of 5L3 than MM1.S and than most of the CLL tested (Figure 6.11) .

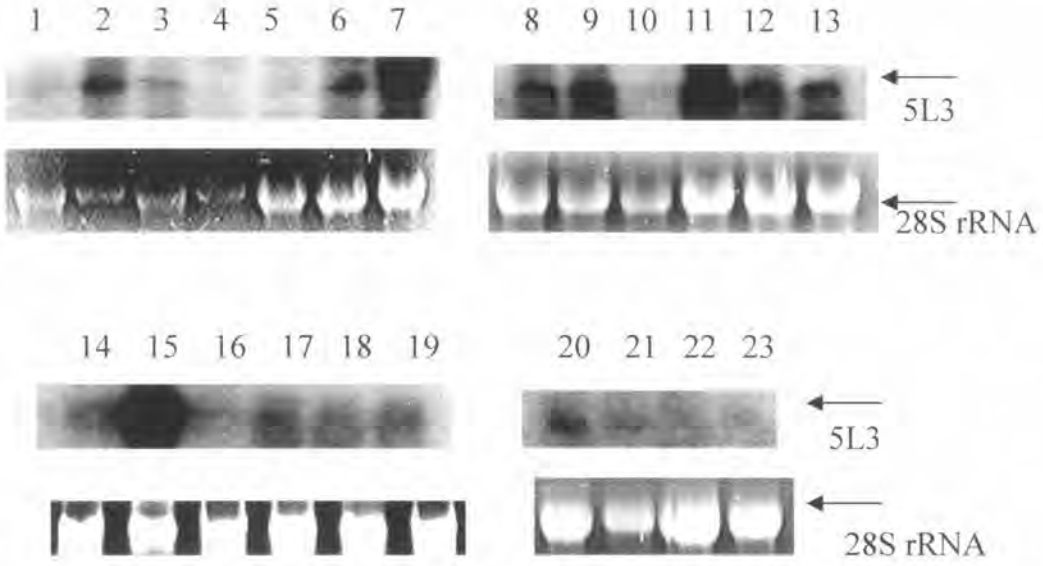


Figure 6.11: Northern Blot analysis of 5L3 mRNA expression in B-CLL cells and Multiple Myeloma cell lines .

B-CLL cells were processed for RNA extraction immediately after purification (To) or were stimulated with the indicated stimuli for 3h and RNA was extracted and processed for Northern Blot Analysis using a ^{32}P labelled 5L3 cDNA. Two Multiple Myeloma cell lines RPMI8226 and MM1.S were included as well mainly as negative controls. Consistency of RNA loading as indicated by 28S rRNA staining is shown below each membrane. The double headed arrows indicate either the 5L3 mRNA specific band detected by the radiolabelled probe or the 28S rRNA band present in each lane.

Lanes:

1) CLL14 To(unstimulated cells), 2) CLL14 PMA 3h,
3) CLL14 IL-4 3h, 4) CLL14 CD40L 3h,
5) CLL14 CD40L+IL-4 3h, 6) CLL14 anti-IgM 3h,
7) CLL17 PMA+IL-4 3h, 8) CLL15 To(unstimulated cells),
9) CLL15 PMA 3h, 10) CLL15 IL-4 3h,
11) CLL15 CD40L 3h, 12) CLL15 CD40+IL-4 3h,
13) CLL15 PMA+IL-4 3h, 14) CLL18 To(usntimulated cells),
15) CLL18 PMA 3h, 16) CLL18 IL-4 3h,
17) CLL18 CD40L 3h, 18) CLL18 Rituximab 3h,
19) CLL18 XRituximab 3h, 20) RPMI 8226 To(unstimulated cells),
21) RPMI8226 PMA 3h, 22) MM1.S To (unstimulated cells),
23) MM1.S PMA 3h

5.3 DISCUSSION

Previous work suggested that 5L3 could be a CLL specific gene which may be involved in the regulation of apoptosis (Green *et al* 1991). This cell type specific regulation of 5L3 in association with the fact that PMA can inhibit apoptosis in CLL cells (Barragán *et al* 2002), led to the hypothesis that it may be a novel gene involved in the regulation of apoptosis in CLL. Because there was no similarity identified to a known gene at the start of this PhD sequencing and extensive BLAST searches were necessary in order to further characterise the 5L3 transcript.

Initially a BLASTN analysis was performed which revealed very strong similarity to a BAC clone (RP11-242J7) which is located telomeric to Chromosome 4. This similarity was identified using two different search engines, one from NCBI and one from Ensembl (EBI) databases. Since 5L3 represents cDNA rather than genomic DNA, extensive search was carried out using these two search engines over known cDNAs, novel cDNAs, non coding cDNAs and cDNAs from pseudogenes (Ensembl), or RefSeq RNA sequences (NCBI) in order to identify a better sequence match. However only the latter database and search criteria revealed a significant match with a gene called KIAA0565, located on Chromosome 17p11.2 which function remains unknown. This KIAA0565 is a gene with 5 transcription variants. Transcription variant 3 (XM_934727) has a predicted size of 5.1 Kb which is quite larger than the predicted size of 5L3 clone. However there are other two transcription variants namely transcription variant 2 (XM_934726) which is 2.2 Kb long and is close to the predicted 1.8 Kb size of 5L3 (Murphy and Norton 1990) , and transcription variant 5 (XM_934729) which is 4.2 Kb long and is close to the predicted 4Kb minor transcript of 5L3 (Murphy and Norton 1990). It is currently unknown if indeed 5L3 is part of this KIAA0565 transcript and why BLASTN identified as a match of 5L3 the transcript variant 3 and not 2 or 5 variants.

Then the sequencing data were translated to a protein sequence using TRANSLATE tool from ExPaSy database and the translation tool from CHROMAS programme and both identified 6 different ORFs (3 in the 5'-3' direction and 3 in the 3'-5' direction) for both primers. Unfortunately most of them were characterised by many stop codons and

thus probably they do not code for a protein. The only exception was ORF2 in the 3'-5' direction from the reverse primer which was compared through BLASTP with known proteins but no similarity was identified. Finally in terms of Bioinformatics analysis, PSIpred a tool for prediction of secondary structure revealed that the possible coded protein from OFR2 has 3 helixes and 7 β strands. When this structure was compared with the secondary structure for KIAA0565 no significant similarity was found since KIAA0565 was very rich in helixes (a total of 24) which were much longer than the ones found for the ORF2 and only 3 β strands.

After the end of Bioinformatics analysis, expression and regulation of 5L3 was tested in CLL cells after stimulation with CD40, IL-4, PMA and XRituximab but only PMA was found to induce the gene as expected (Green *et al* 1991). Rituximab alone or XRituximab had no effect on the basal expression of 5L3. Thus it was concluded that probably it is not involved in the regulation of apoptosis in CLL cells but instead it may be involved in other functions that arise following PMA stimulation of CLL cells. At this stage further work with 5L3 was discontinued.

CHAPTER 7

GENERAL DISCUSSION

This study was conducted in order to test possible involvement of the Tis11 family in the regulation of apoptosis and pathogenesis of Chronic Lymphocytic Leukaemia (CLL). This was hypothesised for several reasons already explained in the General Introduction and AIMS sections of this project. Thus initially B-CLL cells were treated with stimuli that induce apoptosis (anti-CD20, Rituximab monoclonal antibody) and stimuli that inhibit apoptosis (IL-4, CD40L or anti-CD40, anti-CD40+IL-4) and monitored for changes in Tis11, Tis11b, Tis11d mRNA expression and Tis11b protein expression.

In summary it was noticed that spontaneous apoptosis varied significantly between patients at both 24 hours and 48 hours timepoints tested from a minimum of 20% to a maximum of 32% at 48 hours from the start of the culture period (Annexin/PI and PI methods). Variation in the levels of spontaneous apoptosis has been correlated with chromosomal abnormalities present (Jahrsdörfer *et al* 2005), patients with the 13q14.3 deletion showed the lower levels of spontaneous apoptosis when compared with patients having other chromosomal abnormalities or even with normal karyotype patients, while patients with trisomy 12 showed the highest ones (Jahrsdörfer *et al* 2005). Additionally the levels of spontaneous apoptosis have been positively correlated with the levels of serum lactic dehydrogenase (LDH) and negatively with Bcl-2 levels (Jahrsdörfer *et al* 2005). These findings may explain the variation in the levels of spontaneous apoptosis found in this study, even though there are no data available regarding the chromosomal abnormalities present in the patients that participated to attempt a correlation. When Rituximab was used to induce apoptosis, it significantly induced apoptosis in CLL cells only in the presence of a secondary-crosslinker antibody (XRituximab) especially at 48 hours post stimulation. Induction of apoptosis by XRituximab was accompanied by induction of Tis11b/Berg36 mRNA and protein. Interestingly Tis11b was the only gene from the Tis11 family that was induced following this treatment while Tis11 and Tis11d remained unaffected or were weakly induced in some patients. Additionally this study has found that Tis11b/Berg36 is one of the downstream genes activated by the p38 pathway following XRituximab treatment since inhibition of p38 resulted in partial abrogation of Ti11b mRNA and protein following XRituximab. This indicated a direct role of Tis11b in induction of apoptosis by XRituximab which was indeed shown through siRNA technology because inhibition

of Tis11b/Berg36 using a commercially available siRNA severely affected the efficiency of XRituximab to induce apoptosis in CLL. All above data regarding Tis11b induction, regulation and direct involvement in induction of apoptosis by Rituximab constitute novel findings of this study. These findings are of major importance since Tis11b may regulate Bcl-2 or Mcl-1 and XIAP that were shown to be modified in response to Rituximab treatment in Lymphoma cell lines or CLL respectively (Byrd *et al* 2003b, Alas *et al* 2001).

When spontaneous apoptosis was inhibited by CD40+IL-4, IL-4 or CD40L or anti-CD40 it was found that the basal expression of Tis11 was downregulated by CD40+IL-4 and CD40L or anti-CD40. Since inhibition of spontaneous apoptosis by IL-4 has been correlated with increase in the protein levels of Bcl-2 (Jewell *et al* 1994, Danescu *et al* 1992, Panayiotides *et al* 1992) the above data may suggest that Tis11 can actually target Bcl-2 mRNA in B-CLL cells. Tis11 may be involved in inhibition of spontaneous apoptosis by IL-4 through iNOS and NO production since it was shown that IL-4 utilised this pathway to inhibit apoptosis in CLL cells (Levesque *et al* 2003) and Tis11 has been shown to modify iNOS (Fechir *et al* 2005, Linker *et al* 2005). In the concept that Tis11 is pro-apoptotic (Johnson *et al* 2002, Johnson *et al* 2000) its basal expression should be down regulated following IL-4 stimulation and CD40Receptor activation since both of them significantly inhibited spontaneous apoptosis. Down regulation of Tis11 by IL-4 or anti-CD40 and no significant change by PMA constitute novel findings of this study.

Surprisingly though Tis11b/Berg36 was not downregulated by CD40+IL-4 or anti-CD40 but instead it was induced by CD40L or anti-CD40 (another novel finding of this study) and remained unchanged by the CD40+IL-4 combination. It should be noted that PMA that strongly induced Tis11b/Berg36 in all patients tested was found to inhibit apoptosis in some patients especially at 48 hours post stimulation. Finally Tis11d basal expression remained unaffcted by all stimuli used to inhibit spontaneous apoptosis.

A major hypothesis made to explain the finding that Tis11b/Berg36 mRNA or protein was induced by anti-CD40 was that it may have another function different and possibly

independent from induction of apoptosis. This was further supported by the fact that PMA is well known inducer of proliferation and differentiation in CLL cells (Tangye *et al* 1995) rather than being a regulator of apoptosis. Indeed it was shown in this study that inhibition of NF-κB abrogated induction of Tis11b mRNA following anti-CD40 (even at 1 hour post stimulation) or PMA stimulation. Interestingly inhibition of p38 had no effect on induction of Tis11b following stimulation with these two stimuli thus XRituximab and anti-CD40 or PMA do not share a common pathway in inducing Tis11b. The latter finding further indicates that Tis11b may target a completely different set of genes in reponse to these signals (CD20 Receptor activation or CD40Receptor activation) *in vivo* which may further depend on the overall set stimuli present in a certain microenvironment. This becomes more evident especially since CD20Receptor participates in BCR signalling while CD40Receptor activation is one of the main T cell derived signal during immune activation that usually is activated in parallel to IL-4 Receptor.

Furthermore utilisation of Tis11b siRNA showed that downregulation of Tis11b mRNA following PMA or anti-CD40 treatment reduced their efficiency to induce sIgM expression which is considered as a marker of B-CLL differentiation to plasmablasts (Minuzzo *et al* 2005, Takeuchi *et al* 2000, Malisan *et al* 1996, Tangye *et al* 1995, Fluckiger *et al* 1992). Thus Tis11b promotes differentiation of B-CLL to plasma cells in response to PMA. This is another novel finding of this study which requires further investigation especially since the target(s) that Tis11b may have in relation to induction of CLL differentiation are completely unknown.

Finally because it was found in this study that the basal expression of Tis11b and Tis11 is different at different stages of B cell differentiation (from pre-B to plasma cells) and this basal expression was found to be regulated in a B cell stage specific manner by certain stimuli, this study is the first to suggest that Tis11 family and especially Tis11b may have an important role in B cell maturation and terminal differentiation. At the early stages of B cells differentiation (pre-B cells) that target(s) of Tis11b are unknown while during the last stages of B cell differentiation Tis11b may regulate Bcl-6 or Blimp-1 in order to mediate terminal differentiation to plasma cells.

Other possible targets of Tis11 family and Tis11b that may justify a very important role in the pathophysiology of the disease are TNF-α (Jablonska *et al* 2005) and VEGF at least when produced in an autocrine manner. These factors can affect proliferation or survival in CLL cells but the stimuli utilised in this study were not the appropriate ones to suggest possible regulation of these mRNAs by Tis11b in CLL cells. Provided that this is true then overexpression of Tis11 family primarily Tis11b may delay progression of CLL patients to more advanced clinical stages.

In conclusion Tis11b and Tis11 are both involved in apoptosis in CLL cells, Tis11b is probably involved in B-CLL differentiation and both of them may be involved in B cell differentiation in general. Both of them may have a direct role in the pathophysiology and progression of the disease, however because of their differences in regulation in CLL in response to a certain stimuli they probably target for different mRNAs with AREs in the 3'UTR.

FUTURE WORK:

Further work in required for the completion of this project especially towards identification of mRNAs that are targeted by either Tis11b in response to Rituximab or by Tis11b and Tis11 in response to CD40Receptor activation or by Tis11 in response to IL-4 stimulation. This may include overexpression of Tis11 and Tis11b in CLL cells in relation to changes in mRNA and protein level of several possible candidate genes. Another approach would be expanding the siRNA technology already utilised in this project in order to determine the targets of Tis11b and Tis11 in CLL in response to stimulation. Further methods involving REMSA or RNP (ribonucleoprotein complexes) under certain conditions may be another way to identify targets of this family in CLL cells. Certain candidate targets such as Bcl-2 may require cell free systems because of the intrinsic resistance of CLL cells to downregulate Bcl-2 in response to many stimuli. Even more extensive work is required to determine the targets of Tis11b in relation to B cell differentiation, since no candidate genes can be suggested at the moment. In this case probably studies with the cell lines already included in this study namely Nalm6, Ramos and Daudi, RPMI8226 and MM1.S should be further developed and possibly utilisation of mouse cell lines such as BCL-1 should be of great interest and advantage. Methods involved have been already described in Chapter 5 (dealing with the cell lines) and many others as necessary. Additionally development of transgenic mice or conditional knock out (K.O.) or B cell specific knock out mice for Tis11b should provide valuable insights towards understanding Tis11b involvement in B cell development since embryonic lethality of Tis11b knock out mice between day 9 and 13 precludes studying of the function of this gene in lymphocyte development and maturation.

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APPENDIX 1

A1.1 Purification of B-CLL cells from mononuclear cells isolated from CLL patients

In order to test the basal expression and regulation of Tis11 family in B-CLL cells, B cells were purified from mononuclear cells from patients with WBC<$90x10^9$. B cells as described in Materials and Methods. After the last wash cells were processed for cultivation or staining with anti-CD3 and anti-CD20 to check the purity of the isolated B cell population.

In most of the patients tested the purity of B cells was >90% as revealed by staining with anti-CD3 reaching 95% in some cases. The percentage of CD20+ cells varied significantly between different patients. The summary of the data is shown in Table 3.1 and one representative example of CD3, CD20 staining and FACS analysis is shown in Figure 1.

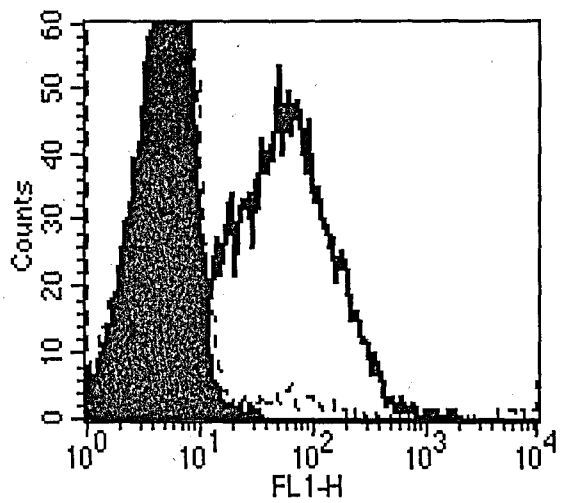


Figure A1.1: CD3, CD20 staining of purified B-CLL cells
B-CLL were purified as described above from CLL2 and were stained with anti-CD3 FITC and anti-CD20 followed by anti-mouse FITC at the dilution recommended by the manufacturer. Cells were immediately analysed by FACScan and CellQuest programm. The black filled line represents background fluorescence, the thick open black line-overlay represents cells stained with anti-CD20 while the black dotted line-overlay represents cells stained with anti-CD3. In all cases the analysis was performed on live gated cells and the percentage above M2 gate represent the percentage of cells positive for CD20(89%) and CD3 (4%) respectively.

CLL patient	% of CD20+ cells	% of CD3+ cells
CLL1	90%	5.0%
CLL2	89%	4.0%
CLL3	Not available	Not available
CLL4	97%	1-2%
CLL5	60%	1%
CLL6	93%	2%
CLL7	88%	2%
CLL8	98%	1%
CLL9	60%	2.5%
CLL10	91%	2.5%
CLL11	61%	2.5%
CLL12	95%	2.5%
CLL13	97%	1.5%
CLL14	97%	1%
CLL15	87%	5%
CLL16	59%	2%
CLL17	70%	15%
CLL18	82%	1.5%
CLL19	86.5%	3.7%
CLL20	74%	1%
CLL21	63%	8%
CLL22	71%	9%
CLL23	95%	1%
CLL24	83%	2.5%
CLL25	82.5%	8%
CLL30	91.5%	5%

Table A1.1: Summary of purity of isolated B-CLL cells as assayed by anti-CD3 and anti-CD20 staining. 10^5 cells were stained using anti-human CD3 FITC or anti-human CD20 followed by anti-mouse FITC antibody in the presence of 2% BSA (Bovine Serum Albumin) in PBS. Cells were aquired using a FACScan flow cytometer and analysed using the CellQuest programme. Analysis was performed on gated live cells

A1.2 Quantification and quality assessment of ant-CD40 antibody produced by the G28-5 cell line

Because the anti-CD40 antibody was produced by the author by growing the G28-5 hybridoma cell line, it was necessary to determine the concentration of the antibody in the culture supernatant and also whether this antibody was fully functional before further use.

The concentration of the anti-CD40 in the supernatant was measured by sandwich ELISA after the culture supernatant containing the anti-CD40 was diluted 1/20, 1/40, 1/80, 1/160. Undiluted supernatant and the diluted as above were loaded in 96 well plates containing the appropriate coating antibody.

The standard curve was made by a serial dilution (1/20, 1/40, 1/80, 1/160, 1/320) of a mouse IgG1, κ (MOPC-21) antibody in culture medium. The starting concentration of the antibody as provided by the manufacturer (Sigma-Aldrich) was 0.2mg/ml or 0.2μg/μl. Detection was made using an anti-mouse peroxidase conjugated antibody. Each of the samples (mouse IgG1 or anti-CD40) was loaded twice and the mean absorbance of each loading was calculated before further calculations One example of such an experiment is shown in Table 1 and the standard curve produced is shown in Figure 2. According to this standard curve and measurements the concentration of the undiluted G28-5 supernatant containing the anti-CD40 antibody was 8.6×10^{-3} μg/μl. Thus a final concentration of 1μg/ml anti-CD40 which is the concentration used in the experiments, would required 116 μl of G28-5 supernatant which would be used per ml of cultured cells. The same procedure was repeated for a total of 3 times and very little variation was obtained in the concentration of anti-CD40 between different passages. For example the second time the anti-CD40 concentration was found to be 8.8×10^{-3} μg/μl and the volume that should be used in this case was 114 μl.

Mouse IgG (μg/μl	Absorbance 405nm(mean)	G28-5 Supernatant dilutions	Absorbance 405nm(mean)
0.01	0.73	Undiluted	0.640
0.005	0.44	1/20	0.390
0.0025	0.37	1/40	0.280
0.00125	0.34	1/80	0.230
0.000625	0.25	1/160	0.190

Table A1..2: ELISA results for dtermination of anti-CD40 concntration in G28-5 culture supernatant

Serial dilution of mouse IgG of known concentration (1mg/ml) and of G28-5 supernatant with unknown concentration for anti-CD40. The serial dilutions of mouse IgG were made in culture medium just like the G28-5 supernatant. The absorbance was read at 405nm following addition of anti-mouse peroxidase (HRP) antibody and ready to use ELISA substrate.

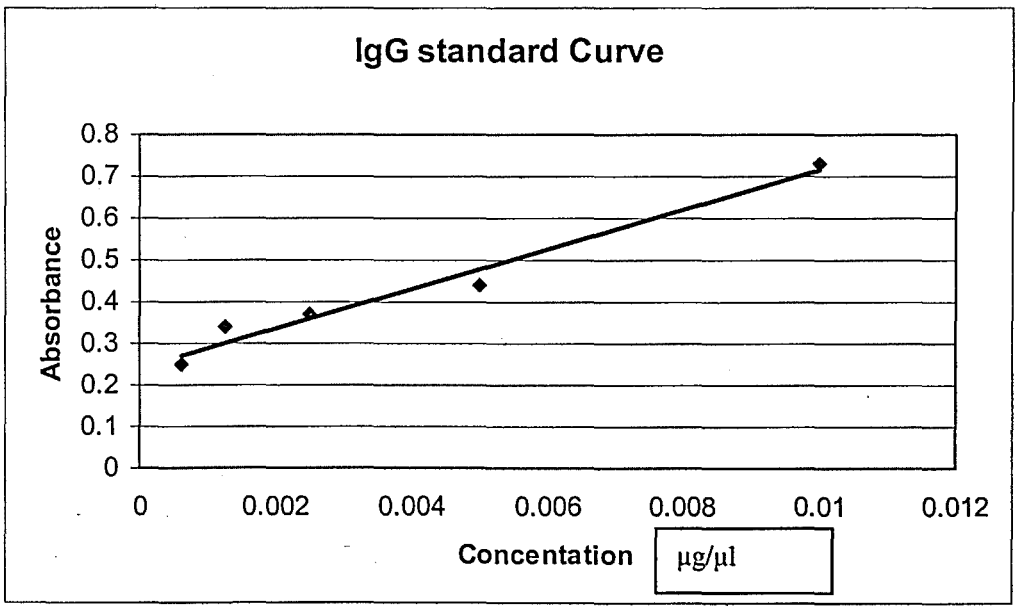


Figure A1.2: Standard curve for the IgG ELISA results shown in Table 1 after they have been plotted in a XY scatter using Excel programme. According to this the concentration of anti-CD40 present in G28-5 supernatant is 8.6×10^{-3} μg/μl or 8.6 μg/ml.

In order to test whether the anti-CD40 produced was fully functional, Ramos cells were stimulated with 4 different volumes of G28-5 supernatant corresponding to 0.25, 0.5, 0.75 and 1 μg of anti-CD40 for 48h and cells were then stained with anti-CD95 because stimulation of B lymphocytes with anti-CD40 or CD40L is known to induce surface expression of CD95 (Craxton *et al* 1998, Wang *et al* 1997). It was found that Ramos cells have some basal expression of CD95 (45% of the cells) which was increased to 70% by 0.25 μg/ml anti-CD40, to 78% by 0.5 μg/ml anti-CD40, to 80% by 0.75 μg/ml anti-CD40 and finally to 88% by 1 μg/ml anti-CD40 present in the G28-5 supernatant (Figure 3). When the analysis was carried out using the MFI as an indicator of CD95 expression it was found that the basal expression of CD95 was 40.0 which was increased to 104.6 (thus 64.6 change in MFI) by anti-CD40 at 0.25 μg/ml, further increased to 108.33 (thus 68.33 change in MFI) by anti-CD40 at 0.5 μg/ml, and was further increased to 123.78 (thus change of 83.78 in MFI) by anti-CD40 at 1 μg/ml.
Thus the G28-5 supernatant contains fully functional anti-CD40 antibody which is able to mediate its effect in a dose dependent manner.

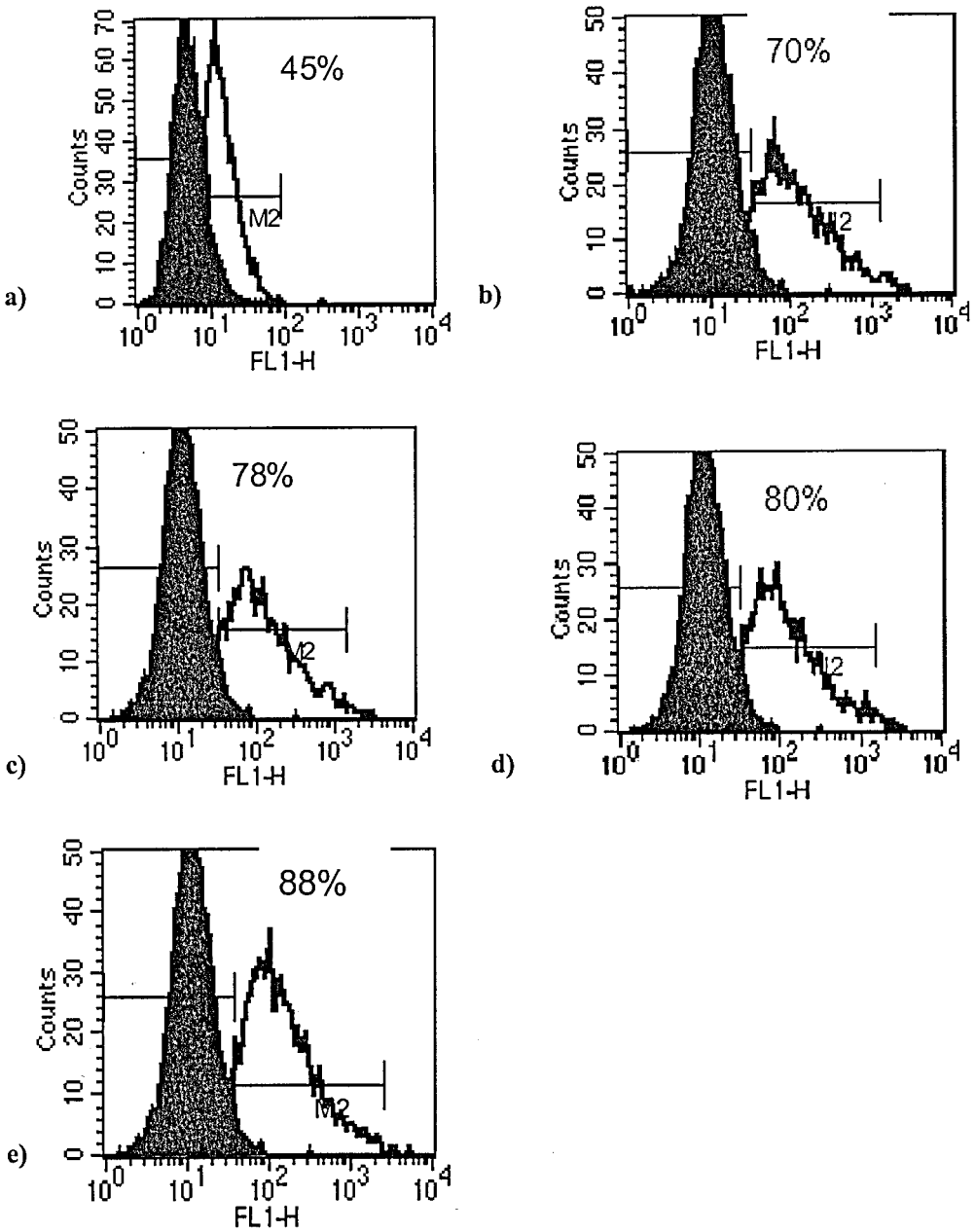


Figure A1.3: Quality assesment of G28-5 supernatant containing the anti-CD40 antibody. Ramos cells were left unstimulated or were stimulated with 4 different doses of G28-5 supernatant corresponding to 0.25, 0.5, 0.75 and 1 μg/ml of anti-CD40 for 48h at the end of which the cells were stained with anti-CD95. a) The black filled histogram represents background staining in unstimulated cells while the black open line-overlay represents unstimulated Ramos stained with anti-CD95 b)-e) The black filled line represents unstimulated cells stained with anti-CD95 while open black line-overlay represents stimulated with the indicated concentration of anti-CD40 stained with anti-CD95. was performed in live cells. In all histograms the percentage of CD95 positive cells after stimulation – the percentage of CD95 positive cells in unstimulated cells in gate M2. Analysis was performed in live gated cells.

A1.3 Preparation of cDNA probes for Tis11b/Berg36, Tis11, Tis11d and 5L3 used for Northern Blot Hybridisations

i) Tis11b/Berg36 cDNA probe:

The Tis11b/Berg36 cDNA which is 1.5 Kb long has been ligated to EcoRI restriction enzyme recognition sequence and cloned into a pBluescript plasmid. Sufficient amount of this recombinant plasmid has been digested with EcoRI in the presence of the appropriate buffer and DNAase free water for 3h at 37^0 C and the rest as described in Materials and Methods. A diagrammatic description of the plasmid into which the Tis11b/Berg36 has been cloned is shown in Figure 4 and a representative photo of a restriction digestion experiment is shown in Figure 5

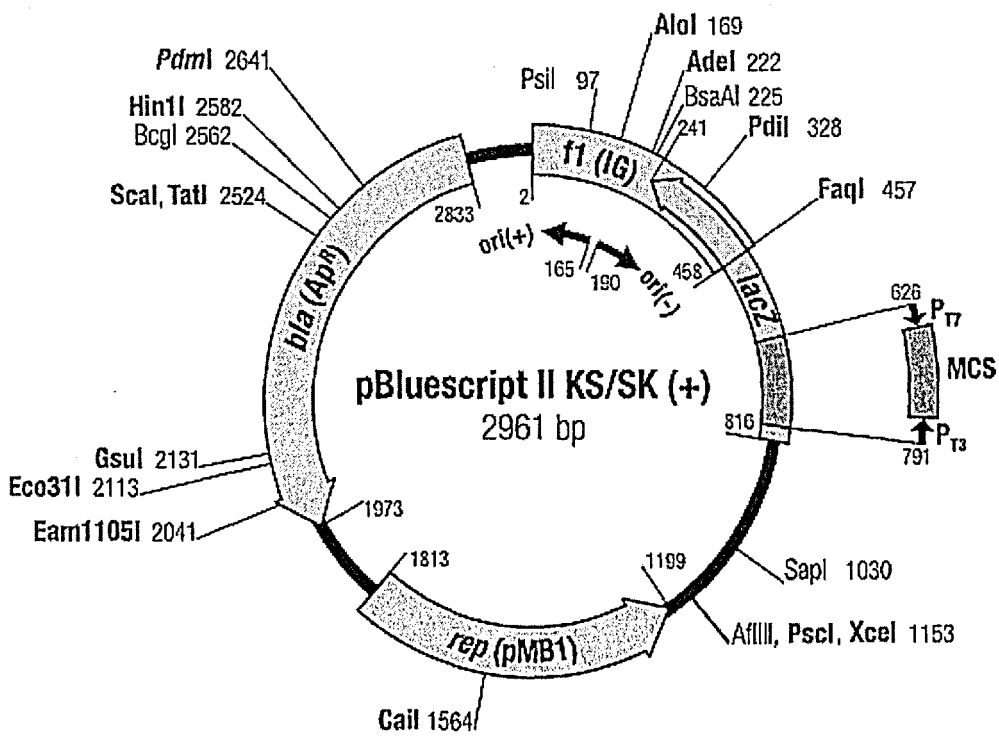


Figure A1.4: Diagrammatic presentation of pBluescipt plasmid that contains the Tis11b/Berg36 cDNA inserted within the ECORI restriction site and Tis11 cDNA kindly provided by Dr. Blackshear P. inserted within HindIII restriction site

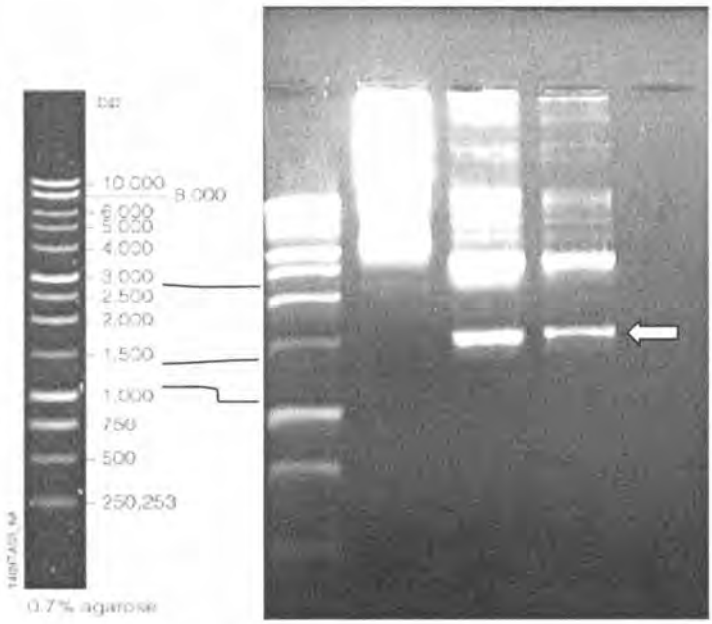


Figure A1.5: Restriction digestion of pBluescript plasmid containing the Tis11b/Berg36 cDNA

Undigested and digested with ECORI for 2h at 37^0C pBluescript plasmid to reveal the Tis11b/Berg36 cDNA was run in a 0.8% Low melting point agarose gel

Lane 1: 1Kb ladder, Lane 2: Undigested pBluescript Lane 3: Digested with ECORI pBluescript plasmid (4 µl) to reveal the Tis11b/Berg36 cDNA (1.5 Kb, lower DNA band), Lane 4: Digested with ECORI pBluescript plasmid (2µl) to reveal the Tis11b/Berg36 cDNA (1.5 kb, lower DNA band). The white arrow indicates the 1.5 Tis11b/Berg36 cDNA insert- clone. On the left hand side the 1kb ladder run in 0.7% agarose as supplied by the manufacturer (Promega) is shown.

ii) Tis11 and Tis11d cDNA probes

The cDNA clones of Tis11 and Tis11d have been obtained from I.M.A.G.E. clones and they have been cloned in CMV-SPORT and p0TB7 plasmids between restriction sites for Tis11 and EcoRI and XhoI restriction sites for Tis11d. Each of these plasmids have been grown in LBroth Agar medium containing the appropriate antibiotic(Ampicillin for the Tis11 clone and Chloramphenicol for Tis11d clone) for 24h at 37^0 C to form distinct colonies. Inspection of the plates revealed sufficient number of colonies grown for each plasmid (data not shown) and 10 different colonies have been randomly selected from each plate and further grown in 5 ml of LBroth medium containing the appropriate antibiotic for further 24h at 37^0 C. Then the two different plasmids have been isolated using the Wizard Plus Minipreps DNA purification system and digested with the mentioned above restriction enzymes for 3h at 37^0. The Tis11 containing plasmid failed to reveal a cDNA insert after several digestions or even after isolation of the plasmid with a QIAGEN Midi prep kit which allows extraction from 25 ml of bacterial culture instead of 5 ml used for Wizard Mini prep kit. On the contrary the Tis11d cDNA has been successfully isolated and all the 10 selected colonies contained the appropriate insert as revealed by the restriction digestion. A diagrammatic description of the plasmic containing Tis11d is shown (Figure 6) while pCMV-SPORT6 will not be shown because it was not used.

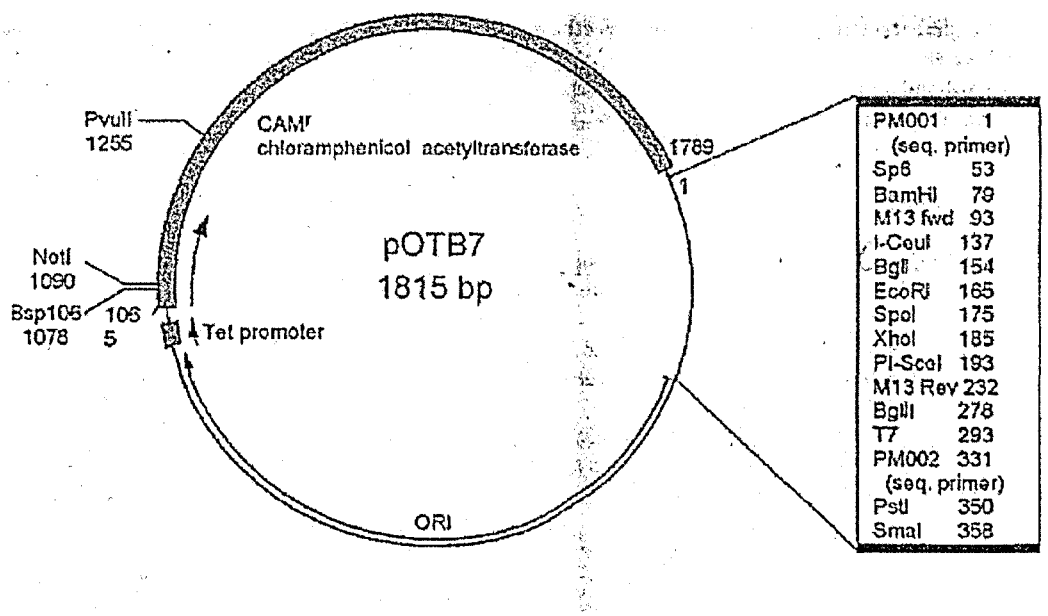


Figure A1.6: Diagrammatic description of the pOTB7 plasmid containing the Tis11d cDNA clone which has been inserted between ECORI and HindIII restriction sites. This picture was supplied together with the Tis11d clones by the manufacturer (MRC clones).

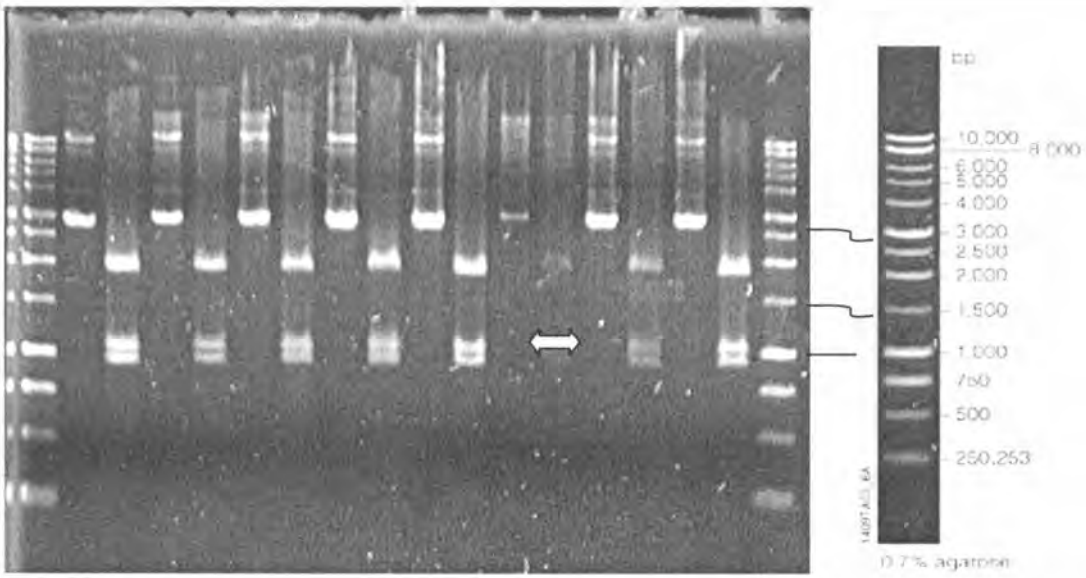


Figure A1.7: Restriction Digestion of the Tis11d containing plasmid from I.M.A.G.E
(AU24-D9 clone) with EcoRI, XhoI enzymes in 0.8% low melting point agarose
Lane 1: 1Kb ladder , Lane 2: Colony 1 Undigested, Lane 3: Colony 1 Digested
Lane 4: Colony 2 Undigested, Lane 5: Colony 2 Digested, Lane 6: Colony 4 Undigested, Lane 7: Colony 4 Digested, Lane 8: Colony 6 Undigested, Lane 9: Colony 6 Digested, Lane 10 : Colony 7 Undigested,Lane 11: Colony 7 Digested
Lane 12:Colony 8 Undigested, Lane 13:Colony 8 Digested, Lane 14:Colony 9 Undigested, Lane 15:Colony 9 Digested, Lane 16:Colony 10 Undigested
Lane 17:Colony 10 Digested, Lane 18: 1Kb ladder. The double headed arrow indicates the Tis11d cDNA insert- clone. On the right hand side the 1Kb ladder run in 0.7% agarose as supplied by the manufacturer (Promega) is shown.

The I.M.A.G.E. clone technical support has been conducted and they suggested that it is probably a faulty clone which led us to contact two independent researchers in USA namely Dr. Perry Blackshear and Dr. Seth Brooks to provide us with a human Tis11 cDNA. The plasmid provided by Dr. Brooks has been digested with EcoRI and used immediately for Northern hybridisation (Figure 8). The plasmid provided by Dr. Blackshear was sent on a transferring membrane, thus half of the membrane was soaked into TE for 15 min at room temperature and then used to transform XL1-Blue competent cells. After transformation these cells were plated onto LBroth agar plates containing ampicillin. The competent cells have been successfully transfected with pBluescript-Tis11 plasmid and at least 15-20 different colonies were clearly formed (Figure 9c). Two negative controls were also included, one plate contained only the LBroth agar and ampicillin and a second plate containing the LBroth Agar and ampicillin spread with XL1-Blue competent cells that have not been transformed with the pBS plasmid containing Tis11. Colonies were grown only in the plate containing XL-1 Blue cells transformed with pBluescript-Tis11 plasmid. Five different colonies have been isolated and were grown in LBroth medium containing ampicillin for 24h at 37^0 C before the plasmid has been isolated using the Wizard Mini prep kit and digested with HindIII for 2h at 37^0 C to reveal the 1Kb Tis11 human cDNA clone (Figure 10).

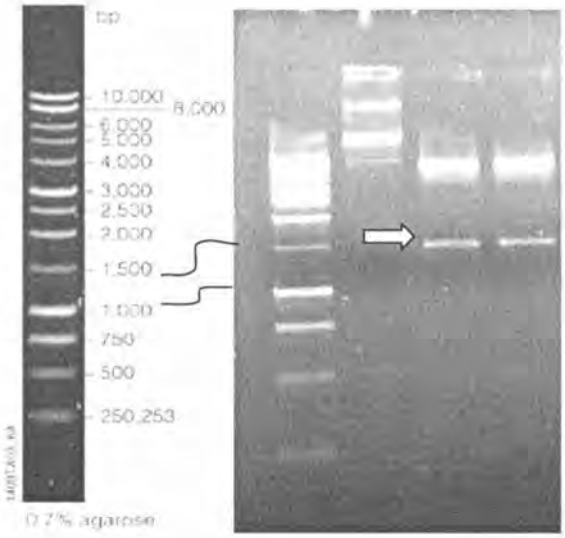


Figure A1.8: Restriction digestion of plasmid containing human Tis11 cDNA kindly provided by Dr. Brooks S.

Undigested and digested with EcoRI for 2h at 37^0C plasmid containing the Tis11 cDNA were run into a 0.8% Low melting point agarose. The Tis11 cDNA insert was excided using a sterile scalper and was used after labelling with ^{32}P as a probe for Northern Blot Hybridisation.

Lane 1: 1Kb DNA ladder, Lane 2: Undigested plasmid, Lane 2: Digested plasmid Lane 3: Digested plasmid . The human Tis11 cDNA insert is approximately 1.5 Kb long and is shown by a arrow. On the left hand side the 1kb ladder run in 0.7% agarose as supplied by the manufacturer (Promega) is shown.

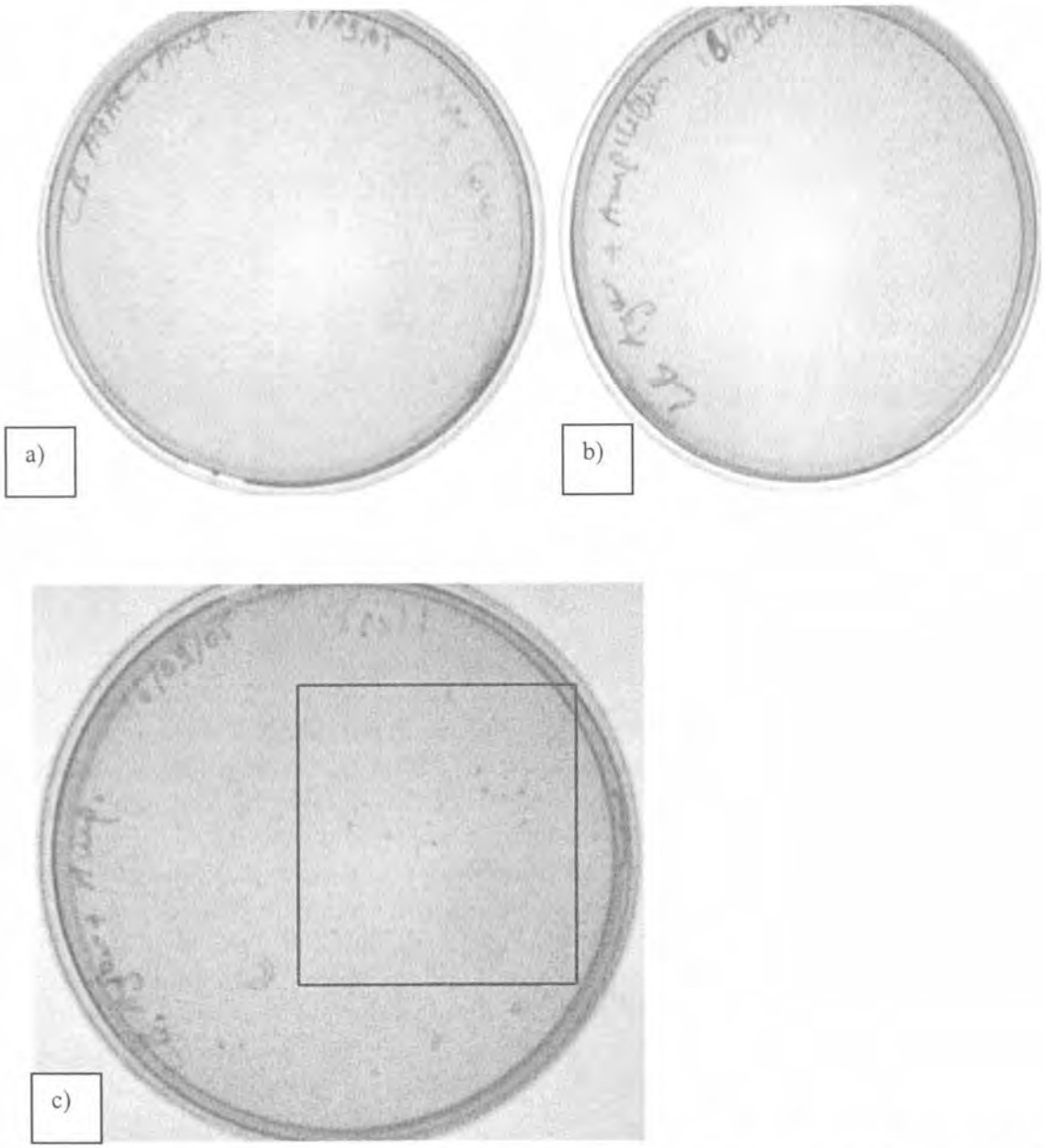


Figure A1.9: Transformation of XL-1 Blue cells with pBluescript+ plasmid containing the Tis11 cDNA kindly provided by Dr. Blackshear P.
a) LBroth Agar containing the appropriate amount of Ampicillin was incubated at 37^0 C overnight to test the sterility of the agar plates and general preparation during setting up the plates, b) XL1-Blue Competent cells that have not been transformed with the pBluescript plasmid were spread on LBroth Agar containing the appropriate amount of ampicillin in order to check that the competent cells do not contain another plasmid than could grow on ampicillin and thus interfere with Tis11 plasmid growth.
c): Tranfection of XL1-Blue competent cells with pBluescript plasmid containing the Tis11 cDNA were spread on LBroth Agar containing the appropriate amount of ampicillin. A total of almost 30 different colonies were grown, and this photo has been taken after 5 different colonies were processed for plasmid purification using the Promega Mini kit. The main area where the colonies were seen is marked by a red box while the are other 5 colonies below that box

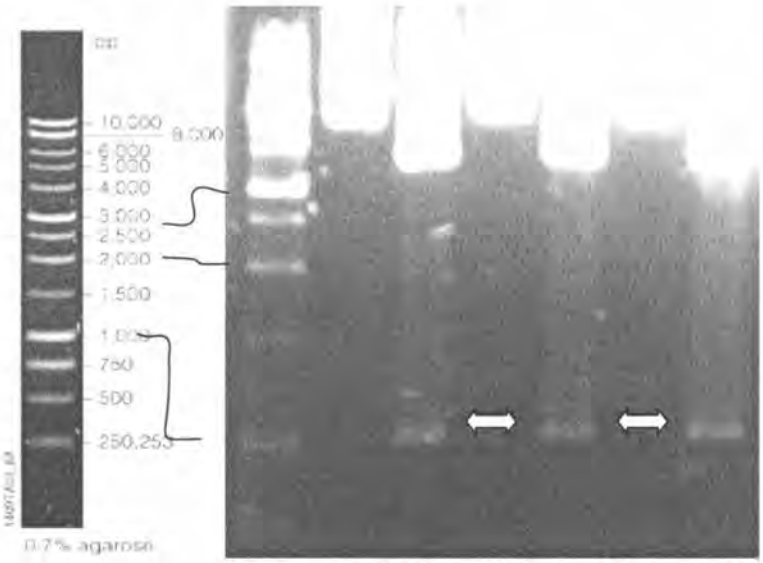


Figure A1.10: Restriction digestion of pBluescript plasmid containing the Tis11 cDNA clone kindly provided by Dr. Blackshear Perry.

Udigested and digested pBluescript plasmid were run into a 0.8% Low melting point agarose and the Tis11 cDNA was excised using a sterile scalper and served after labelling with ^{32}P as the probe for Northern Blot hybridisations.

Lane 1: 1 Kb DNA ladder, Lane 2: Undigested plasmid from colony 1, Lane 3: Digested plasmid with HindIII from Colony 1, Lane 4: Undigested plasmid from Colony 2, Lane 5: Digested plasmid with HindIII from colony 2, Lane 6: Undigested plasmid from colony 3, Lane 7: Digested plasmid with HindIII from colony 3.

The human Tis11 cDNA insert is shown by a double headed arrow. On the left hand side the 1kb ladder run in 0.7% agarose as supplied by the manufacturer (Promega) is shown.

iii)5L3 cDNA probe

In order to prepare the 5L3 cDNA clone, the pUC9 plasmid containing this cDNA was digested with ECORI to reveal the 1.1 Kb insert of 5L3 clone (Figure 11) that was labelled with ^{32}P before hybridisation of the membranes

Figure A1.11: 5L3 Digestion in 0.8% Low melting point agarose
The pUC9 plasmid was left undigested or was digested for 2h with ECORI at 37^0 C to reveal the 1.1 Kb insert corresponding to 5L3 cDNA clone.
Lane 1: 1 Kb DNA ladder, Lane 2: Digested pUC9 plasmid with ECORI , Lane 3: Undigested pUC9 plasmid. The white arrow indicates the 1.1 Kb 5L3 insert –clone
On the right hand side the 1Kb DNA ladder in 0.7% Agarose as provided by the manufacturer (Promega) is shown.

A1.4 Transfection of CLL cells with control siRNA and improvement of transfection efficiency

Initial experiments to establish the transfection efficiency of B-CLL cells with siRNA, were carried out using the BLOCK-IT kit from Invitrogen providing Lipofectamine and a control siRNA labelled with FITC. This control siRNA was transfected into B-CLL cells using Lipofectamine and Electroporation and the transfection efficiency was monitored with either a FACScan Flow Cytometer or a Fluorescence microscope. In general it was found that when the analysis was performed under the fluorescence microscope the transfection efficiency was roughly 10% higher than the one obtain for the same patient using the FACS analysis.

For the Lipofectamine method the volumes of Lipofectamine and siRNA had to be adjusted because the ones recommended by the manufacturer resulted in poor transfection efficiency which ranged from 7-17% as determined by fluorescence microscope analysis even though transfection efficiency was monitored at t18h instead of t24h and probably would be higher at this timepoint. Analytically for CLL29 the transfection efficiency was 6.4% (when $1x10^6$ cells were transfected) , for CLL41 it was 16.8% (for $1x10^6$ cell transfected) and for CLL42 it was 10% (for $1x10^6$) (data not shown) .

For the next 3 patients transfected: CLL33,CLL36,CLL38 the volume of Lipofectamine was increased to 2μl and the volume of the control siRNA was increased to 2μl while the cells were grown in OPTIMEM (the medium recommended to reconstitute Lipofectamine and siRNA) instead of RPMI 1640 medium .The transfection efficiencies at 24h were 32%, 45% and 18.5% respectively by flow cytometry analysis (Figure 12) but treatment with Lipofectamine resulted in induction of at least 30-40% cell death as assayed by Region analysis in a FSC/SSC dot plot analysis.

Between the two different methods used to transfect cells electroporation resulted in slightly better transfection efficiency whatever the method used to check this efficiency but also resulted in more cell death (at least 20% more than Lipofectamine) (Figure 12). However transfection efficiency increased significantly over time since the % of FITC+ cells was always higher at t48h than t24h whatever the method used to transfect the

cells (Figure 12). Because electroporation resulted in more cytotoxicity (Figure 13) it was decided to use Lipofectamine to transfect B-CLL cells with the Tis11b/Berg36 siRNA especially since the scope of this experiment was to test the effect of Tis11b/Berg36 in apoptosis following XRituximab treatment .

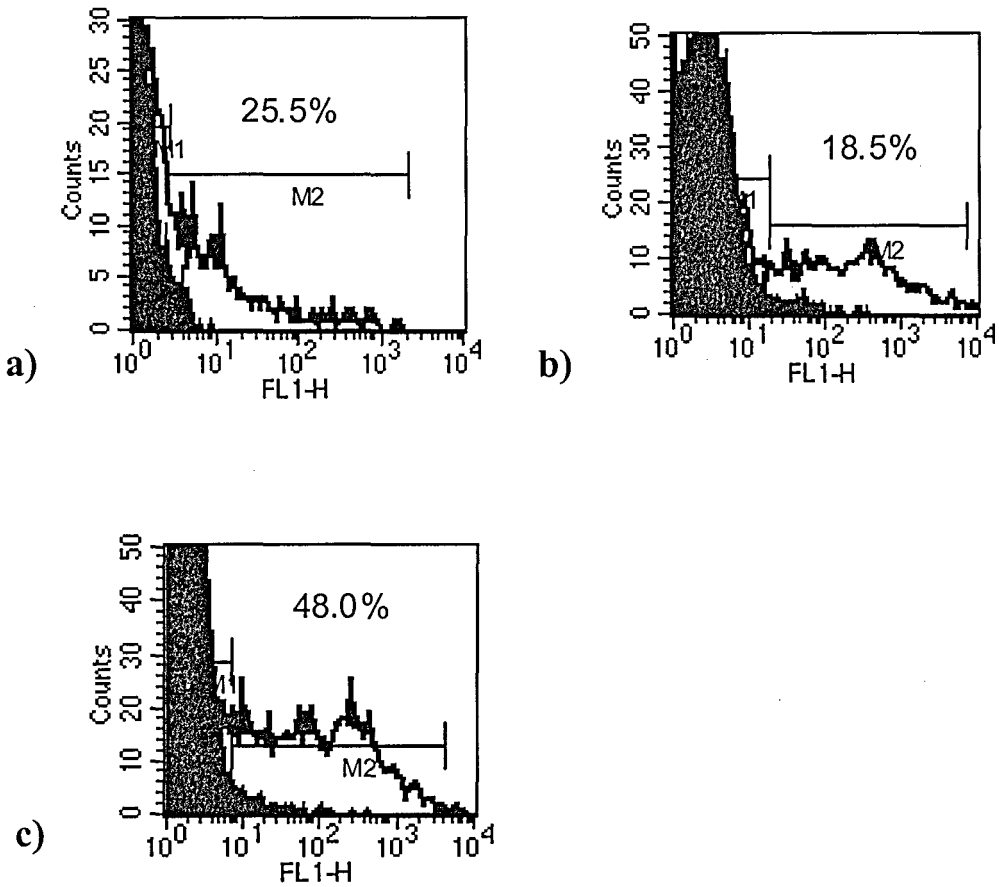


Figure A1.12.:Transfection efficiency in CLL using Lipofectamine or Electroporation.
CLL38 has been transfected with the same amount of control siRNA labelled with FITC using **a)**electroporation at t24h **b)**Lipofectamine at t24h and **c)** Lipofectamine at t48h and transfection efficiency was monitored using FACS analysis. In all a), b), c) the black filled line represents untrasfecetd cells while the black open line-overlay represents transfected cells with control siRNA using either Electroporation or Lipofectamine as indicated. The percentage of positive/ transfected cells is indicated in each plot above the M2 gate.

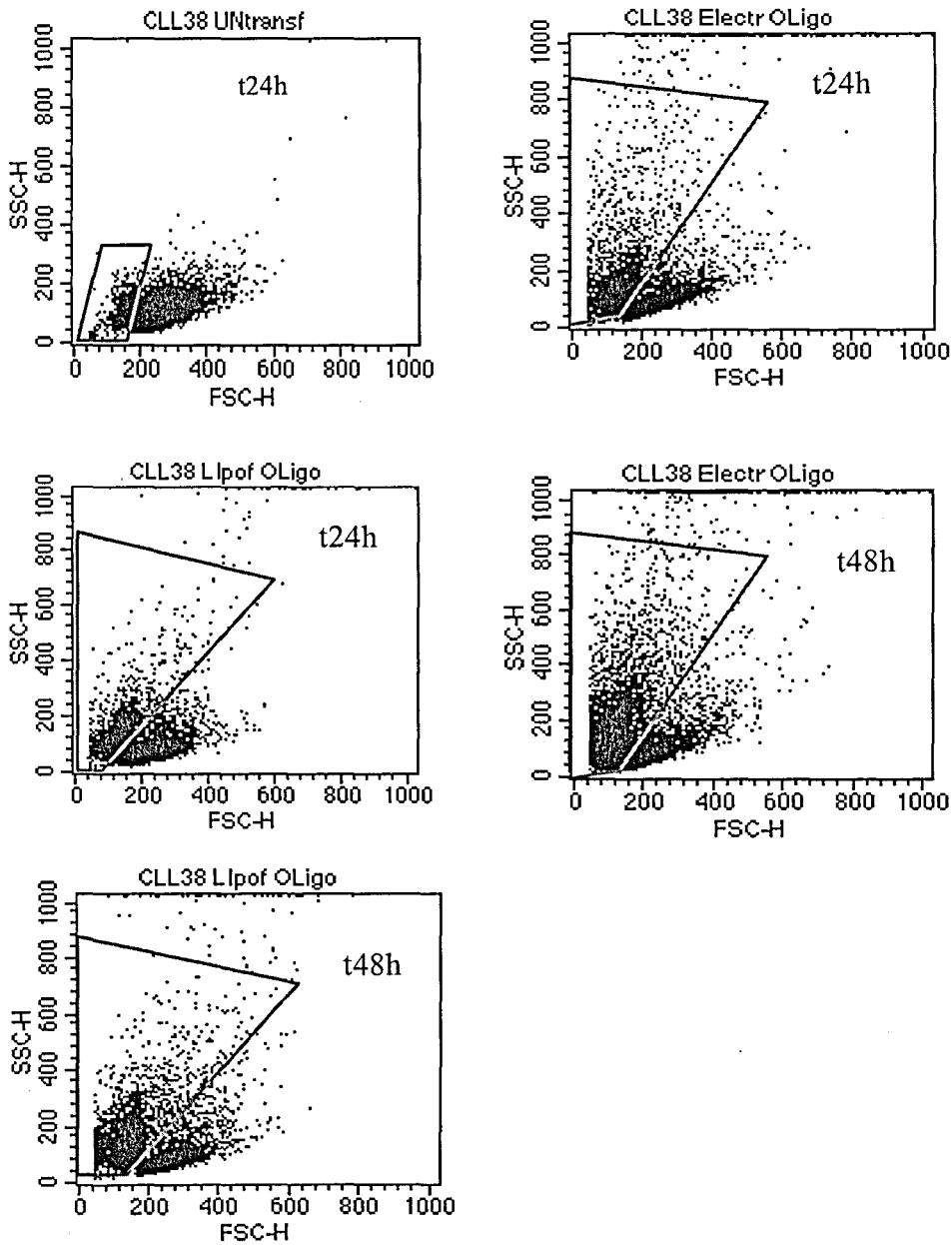


Figure A1.13: Effect on cell death by Lipofectamine or Electroporation mediated transfection of CLL cells.
CLL38 was transfected with 2 µl Tis11b/Berg36 siRNA using the Lipofectamine (2µl) or electroporation and the effect on cell viability was checked by FSC/SSC analysis on a FACScan Flow Cytometer 24h or 48h post transfection. Apoptotic /dead cells in comparison with healthy/live cells are characterised by smaller size and higher granularity thus appear as a population of cells with lower FCS and higher SSC always to the left of live/healthy cells. Here dead cells are shown in a red gate. Note that CLL38 has been when transfected with Lipofectamine were grown in OPTIMEM instead of RPMI1680 medium which was proved to be cytotoxic and still electroporation is more cytotoxic than Lipofectamine.

For the next experiment CLL36 ,CLL38 were grown in RPMI and transfected with 3 μl of Lipofectamine and 2 μl of control siRNA whereas in the presence of 15% FCS CLL33 and a new patient CLL43 were transfected with 3 μl Lipofectamine and 3 μl of control siRNA in the presence of 10% FCS. Transfection efficiency was assayed at t24h by Flow cytometry analysis and it was found to be 56% for CLL36 and 32% for CLL38 , 41% for CLL33 and 34% for CLL43 (Figure 14). The most important finding of the last experiment was that further increase of Lipofectamine did not further induce cell death as revealed by Region analysis on a FSC/SSC dot plot and that induction of cell death in the previous experiment was due to the fact that cells were grown not in RPMI 1640 but in OPTIMEM medium which contains glycine. Because in CLL36 there was a decrease in transfection efficiency (from 56% in the first experiment to 50% in this experiment) it was decided to keep the concentration of serum at 10% since it is considered generally as an agent that reduces transfection efficiency of cells. Finally since increase of Lipofectamine and control siRNA to 3μl increased transfection efficiency of CLL33 from 32% to 49% (Figure 13) it was decided to use this volumes in subsequent experiments in the presence of 10% FCS.

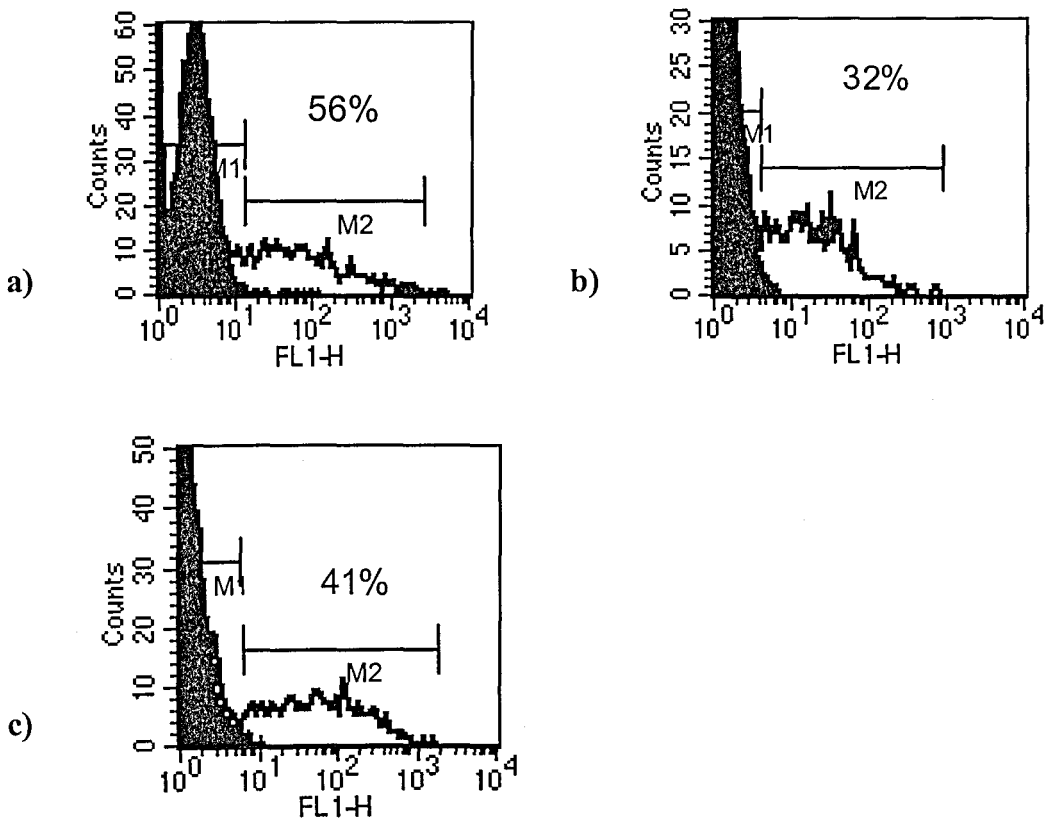


Figure A1.14: Transfection efficiency in CLL cells
a) and b)B-CLL from CLL36 and CLL38 were transfected with 3µl of Lipofectamine plus 2µl of control siRNA in the presence of 10% FCS and transfection efficiency was monitored at t24h with the use of FACS analysis c) CLL33 was transfected with 3µl Lipofectamine plus 3 µl control siRNA in the presence of 15% FCS and tranfection efficiency was monitored by FACS analysis.
a), b) and c) The black filled line represents unstimulated cells and the black open line-overlay represents cells transfected with Lipofectamine +control siRNA. In all 3 histograms the percentage FITC positive/ transfected cells is indicated above the M2 gate.

Patients	FITC+ cells at t24h	FITC + cells at t48h	FITC+ cells at t72h
CLL33	41%	ND	ND
CLL36	56%	76%	ND
CLL38	30%	50%	ND
CLL41	57%	80%	85%
CLL42	27.85%	62%	68.7%
CLL43	35%	Analysis	ND
ICR1	44.70%	63%	69.0%

Table A1.3: Transfection efficiency in B-CLL cells at different timepoints using Lipofectamine 2000

Transfection efficiency of B-CLL cells transfected with 3µl of Lipofectamine and either 2 µl(CLL36,CLL38) or 3µl (all other CLL patients tested) of control siRNA labelled with FITC. The cells were analysed on a FACS Scan flow cytometer for fluorescence on FL1 channel over background fluorescence of untransfected cells.A total of 10,000 cells was acquired and % of FITC cells reveal % MFI of siRNA transfected cells-% MFI of untransfected cells

A1.5 Quantitative Real Time PCR set up

i) PCR to confirm cDNA synthesis and specificity of primers specificity used in quantitative Real Time PCR

Because it was necessary to establish successful downregulation of Tis11b/Berg36 mRNA by the Tis11b/Berg36 siRNA used, it was decided to perform quantitative Real Time PCR. This was a two step reaction with the first one being the cDNA synthesis and the second one being the actual Real Time PCR experiment using SYBR Green to quantify the product of each reaction. Before starting the Real Time PCR experiment it was necessary to check the successful construction of cDNA from the mRNA isolated and the specificity of the primers used namely Tis11b/Berg36 and β-Actin (control).
In order to do so after the cDNA was constructed using following the manufacturers instruction, 2 μl of the cDNA made from unstimulated cells, XRituximab treated cells, XRituximab+control siRNA and XRituximab +Tis11b/Berg36 siRNA treated cells was amplified by standard PCR using either the Tis11b/Berg36 primers or β-Actin primers. Amplification was carried out at 62^0C for 30 cycles and the product was run in 2% Agarose gel (Figure 15)

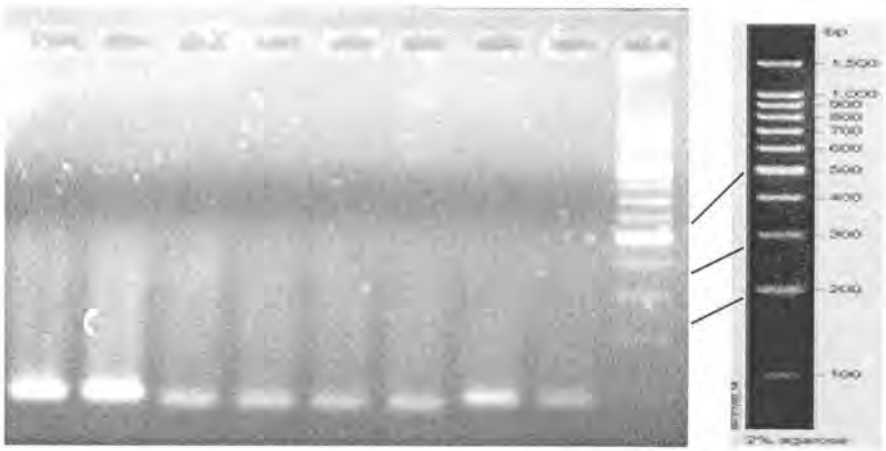


Figure A1.15:PCR with β-Actin and Tis11b/Berg36 primers following cDNA synthesis from unstimulated and XRituximab treated B-CLL cells
Lane 1: Unstimulated β-Actin primers, Lane 2: XRituximab β-Actin primers, Lane 3: Unstimulated Tis11b/Berg36 primers, Lane 4: XRituximab Tis11/Berg36 primers, Lane 5: XRituximab+Tis1b/Berg36 siRNA Tis11b/Berg36 primers, Lane 6: XRituximab +control siRNA-Tis11b/Berg36 primers, Lane 7: XRituximab +control siRNA β-Actin primers, Lane 8: XRituximab+Tis11b siRNA β-Actin primers, Lane 9: 100 bp DNA ladder. Note that the expected size of a product from the β-Actin primers is 100 bp while the expected size of a product from the Tis11b/Berg36 primers is 89 bp.

ii) Melting curve analysis and quantification

Another way to assess specificity of primers, exclude presence of primers dimmers is to perform a melting curve analysis during the actual Real Time PCR experiment. Additionally this analysis indicates the maximum temperature that the reaction product is stable and thus it's fluorescence by the incorporated SYBR Green dye can be measured. This is quite valuable information during the set up of the machine when the temperature at which the fluorescence is measured has to be inserted in the programme set up.

In order to perform a melting curve analysis a 1/10, 1/100, 1/1000 dilution from cDNA made from XRituximab treated cells or unstimulated cells was processed for Real Time PCR using the manufacturer instructions using β-Actin or Tis11b/Berg36 primers. This experiment was repeated in total of 3 times in a ABI 7000 cycler (96 well plate format) and a total of 3 times in a Roche LightCycler (Capillaries format) and always a single product was obtained by either the β-Actin or the Tis11b/Berg36 primers and there was no evidence of primers dimmers in both cases.

Additionally even more importantly it was found from these experiments that the melting temperature is well above 80^0 C for β-Actin but at around 78^0 C for Tis11b/Berg36 and thus the melting temperature was set at 77^0 C for both.

Here one experiment is shown from the ABI 7000 cycler (Figure 16).

Then the Real Time PCR results were quantified using a plot of Delta Rn versus cycle (Delta Rn is the point where the product passes the threshold of detection) which indicates the cycles at which the product is at the linear phase of amplification and thus is measured. This analysis should also provide information about the difference in cycle between the different dilutions for each set of primers. This analysis was performed in 3 different experiment and it was found that for every 1/10 dilution there is a reduction of 3 cycles in detection and this was consistent in all 3 experiments and for both primers suggesting that the efficiency of β-Actin and Tis11b/Berg36 primers is the same and reaches 100% (Figure 17). Thus log of 100 is 2 and this will be used in the Pfafll equation when quantifying the results from the actual experiment (Chapter 4)

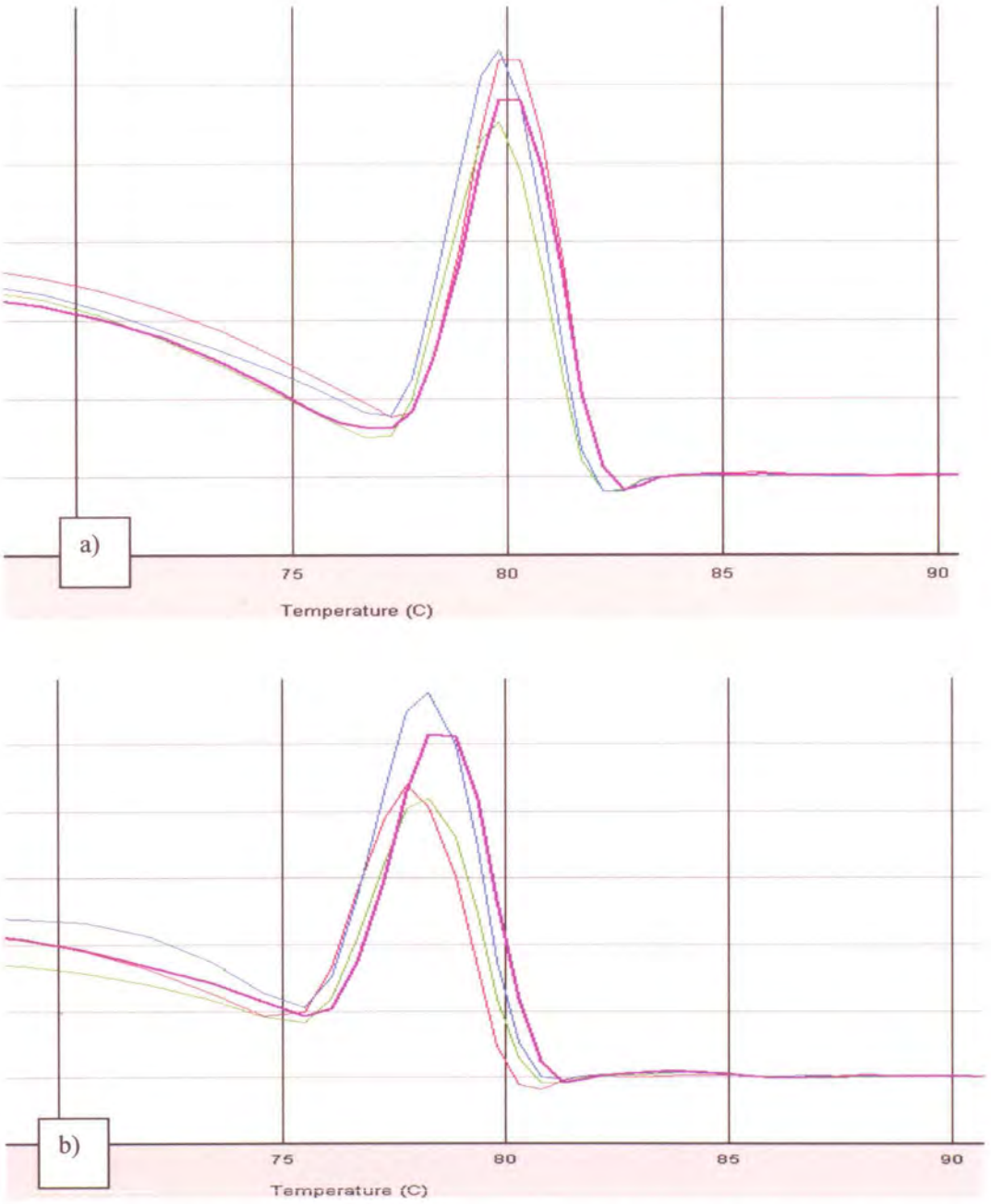
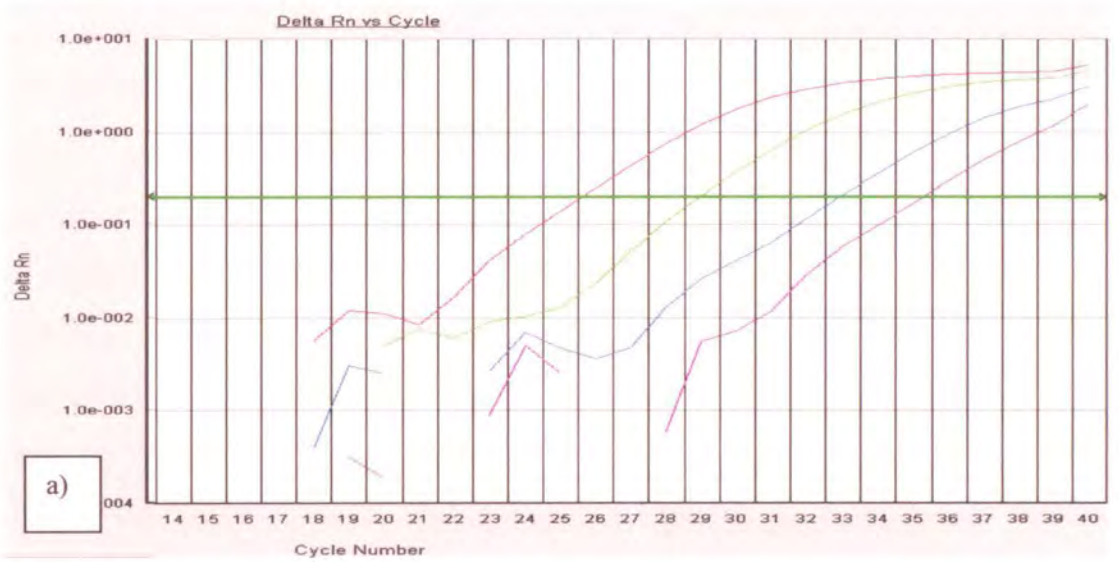


Figure A1.13: Melting curve analysis for a) β-Actin primers or b) Tis11b/Berg36 primers

cDNA constructed from XRituximab treated cells was diluted 1/10 (light blue curve), 1/100 (dark blue-purple curve), 1/1000(green curve) and was processed for Real Time PCR using SYBR Green following the manufacturer instruction with β-Actin primers (a) or Tis11b/Berg36 primers (b). In this particular experiment an undiluted sample from unstimulated cells B-CLL was loaded as well (red peak). Clearly there are no primer dimmers -would appear at the right hand side of the peak seen above as a distinct peak lower that the actual product) and also the primers are highly specific since there is no other product detected – would appear as a distinct peak probably at the left hand side of peak seen above with the same height as this one. The melting temperature for β-Actin is at around 80^0C and for Tis11b/Berg36 at around 77^0C.



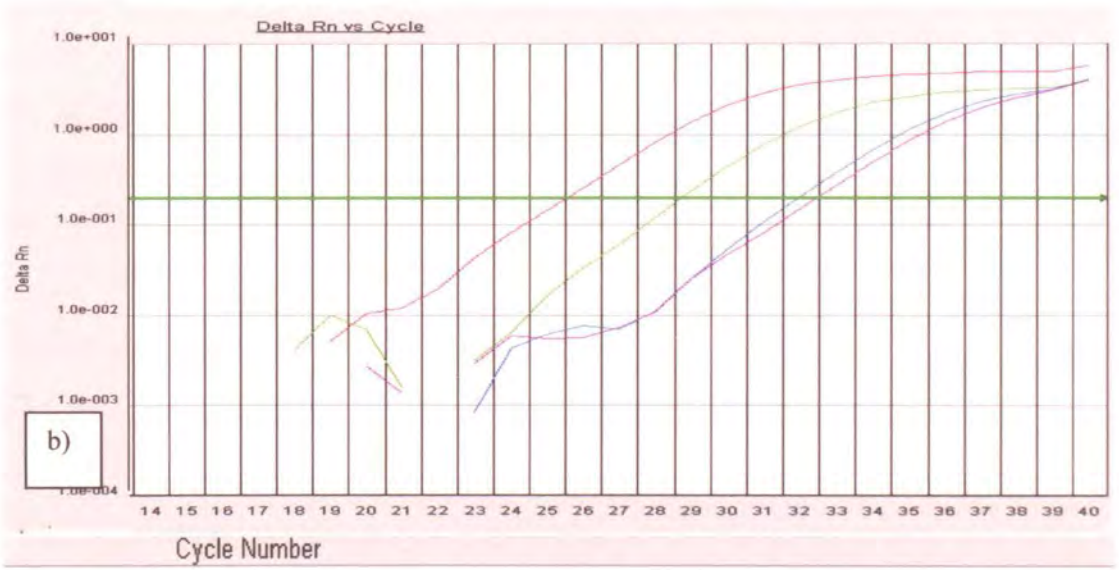


Figure A1.17: Quantification curve for a) β-Actin primers, and b) Tis11b/Berg36 primers

XRituximab+ Control siRNA treated cells were diluted 1/10, 1/100, 1/1000 or 1/10000 and were processed for Real Time PCR using SYBR Green and following the manufacturer instructions. The primers used were β-Actin (a) and Tis11b/Berg36 (b). Note that there is a reduction of 3 cycles between 1/10, 1/100, $1/10^3$ dilution whatever the primer set used apart from the $1/10^4$ dilution which has not worked properly for both primer sets probably due to error during making the dilution. This indicates equal efficiency of amplification for both primer sets and also since the experiment was repeated 3 times with the 1/10, 1/10 and $1/10^3$ dilution and the result was the same in terms of cycle difference between the dilutions this suggests that the primers efficiency is 100% and thus log of 100 is 2 which will be used in the Pfafll equation

APPENDIX 2: BUFFERS AND SOLUTIONS

Agarose-Formaldehyde gel (1%) : was made by dissolving 0.5 gr of agarose in 42 ml of DEPC treated water and adjusting the final volume to 50 ml by addition of 5 ml of 10x MOPS buffer and 3 ml of Formaldehyde (36%).

DEPC treated water: was made by addition of 1ml of DEPC (Diethylpyrocarbonate) to 1lt of deionised, was incubated overnight and autoclaved the next day to deactivate the DEPC

MOPS Buffer (10x): was made by dissolving 41.8 gr of MOPS (0.2M) in DEPC treated water and after the pH was adjusted to 7.0, 20 ml of 1M sodium acetate (20mM) and 20 ml of 0.5M EDTA (pH: 8.0) (10mM) were added in a final solution of 1lt. EDTA and Sodium Acetate were also made in DEPC treated water.

PBS Buffer: was made by dissolving 1 PBS tablet (Oxoid) per 100 ml of deionized water

Resolving gel (12%) was made by addition of 2.0 ml of 30% Acrylamide, 1.3 ml of 1.5M Trizma Base (pH: 8.8) , 0.05 ml of 10% SDS, 0.05 ml of 10% Ammunium Persulfate and 0.002 ml of TEMED adjusting the final volume to 5ml with deionised water.

RNA gel loading Buffer: constituted of 95% (v/v) deionised formamide, 0.025 (w/v) bromophenol blue, 0.025% (w/v) xylene cyanole FF, 5mM EDTA (pH: 8.0) and 0.025 (w/v) SDS

20xSSC: was made by dissolving 175.3 gr of Nacl (3.0M) and 88.2 of Sodium Citrate (0.3M) in a final volume of 1lt.

Stacking gel : was made by addition of 0.5 ml of 30% Acrylamide, 0.38 ml of 1.0M Trizma Base (pH: 6.8), 0.03 ml of 10% SDS, 0.03 ml of 10% Ammonium Persulfate, 0.003 TEMED and adjusting the final volume to 3ml with deionised water

Transfer Buffer (1x) consisted of 24Mm Trizma Base, 192 mM glycine, and 20% methanol. The buffer was made as a 5x stock solution without methanol and was diluted before use and before addition of 20% of ethanol

Tris-Glycine electrophoresis buffer (1x): constituted of 25mM Trizma Base, 250mM glycine electrophoresis grade, 0.1% (w/v) SDS. The buffer was made as a 10X stock solution and was diluted appropriately before use

APPENDIX 3

REAGENTS AND SUPPLIERS

2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt liquid substrate system for Eliza was from Sigma-Aldrich, Cat No: A3219

Acrylamide/bis-Acrylamide 30% solution electrophoresis reagent was from Sigma-Aldrich, Cat No: A-3574

Acetic acid glacial was from BDH AnalaR, Cat No: 10001CU

AET (2-Amonoethylisothiouronium Bromide hydrobromide) was from Sigma-Aldrich, Cat No: A5879

Affinity purified anti-human IgM F(ab')2 fragment from goat was from DOCKLAND, Cat No: 709-1131

Affinity isolated anti-human IgG (Fc specific), F(ab')2 fragment was from Sigma-Aldrich, Cat No: I 3391

Affinity purified anti-human IgG Fcγ specific, F(ab')2 fragment was from DAKO Cytomation, Cat No: A

AffiniPure goat anti-human IgG, Fcγ specific, F(ab')2 fragment was from Jackson Immunoresearch, Cat No: 109-006-008

Agarose for routine use (Molecular Biology tested) was from Sigma-Aldrich, Cat No: A9556

Agarose low melting point was from Sigma-Aldrich , Cat No: A9414

Aminoethylisothio-uronium bromide was from Sigma-Aldrich, Cat No:

Ammonium Persulfate electrophoresis reagent was from Sigma-Aldrich, Cat No: A3678

Albumin, bovine serum fraction V was from Sigma-Aldrich,
Cat No: A3059

ALL-IN-ONE Random Prime Labeling mix (-dCTP) was from Sigma-Aldrich, Cat No: R7522

Ampicillin sodium salt was Sigma-Aldrich, Cat No: A9518

Anti-Berg36 rabbit antisera was produced by Genosys to the peptide sequence : CHSGSDSPTLDNSRR

Anti-CD40 was produced by growing the G28-5 hybridoma cell line

Anti-Mouse IgG1 isotype control was from Sigma-Aldrich, Cat No: M5284

ApoAlert Annexin V-FITC Apoptosis kit was from BD Clontech, Cat No: K2025-1

BAY117082 (NF-κB inhibitor) was from Calbiochem, Cat No: 196870

Berg-36 (Tis11b) primers for Real Time PCR was designed using the ROCHE software and constructed by MWG-Biotech AG. Their sequence was Forward: 5'-GAT GAC CAC CAC CCT CGT-3'and Reverse: 5'-TGG GAG CAC TAT AGT TGA GCA TC-3'

Bicinchroninic Acid protein Assay kit was from Sigma-Aldrich, Cat No: BCA-1 and B9643

BLOCK-IT transfection kit containing Lipofectamine and control siRNA FITC labelled was from Invitrogen, Cat No: 13750-070

BRF-1 (Tis11b) siRNA provided with the appropriate siRNA dilution buffer was from QIAGEN, Cat No: 1024645

Bryostatin was from Sigma-Aldrich, Cat No: B7431

CD3 (IgG1)-FITC was from Coulter, Cat No: 6604625

CD19-FITC was from Becton Dickinson

CD20 (Leu-16) was from BD (Beckton Dickinson), Cat No: 347670

CD27-FITC was from Beckton Dickinson. Cat No:

CD38 (clone AT13/5)-FITC was from DAKO Cytomation, Cat No:

CD86-FITC was from Serotec, Cat No: MCA1118F

CD138-FITC was from Becton Dickinson, Cat No:

Coomassie Blue Stain solution 1x was from BioRAD, Cat No: 74355A

Deoxycytidine 5' [a- 32P] triphosphate triethylammonium salt (3000 Ci/mmol) was from Amersham Biosciences, Cat No: PB10205

Detection Reagent for Western Blot was from Amersham Biosciences, Cat No: RPN2109

Dextran Sulfate sodium salt was from Sigma-Aldrich , Cat No: D8906

DNA Ladder 100bp was from Promega, Cat No: G2101

DNA Ladder 1kb was from Promega, Cat No: G6941

ECORI restriction enzyme provided with the appropriate dilution buffer was from Promega, Cat No: R601

EDTA (Ethylenediamine Tetraacetic Acid) was from Sigma, Cat No: E4884

Ethanol 99.7-100% v/v was from BDH AnalaR, Cat No: 10107

Ethidium Bromide was from Sigma-Aldrich, Cat No: E-7637

Extra Thick Blot paper was from BioRad, Cat No: 11U1503

Foetal Bovine Serum (FCS) EU approved origin was from GIBCO BRL, Cat No: 10106-151

Formaldehyde solution for molecular biology 36.5% was from Sigma-Aldrich, Cat No: F8775

GBX Developer and replenisher was from Sigma-Aldrich, Cat No: P7042

GBX Fixer and replenisher was Sigma-Aldrich, Cat No: P7167

GeneScreen Plus® Hybridisation transfer membranes were from NEN Life Sciences Products, Cat No: NEF97630

Gliotoxin (NF-κB inhibitor) from *Gladiocladium fimbriatum* was from Calbiochem, Cat No: 371715

Glycine for electrophoresis (mimimum 99%) was from Sigma-Aldrich, Cat No: G8898

HEPES (N-[2-Hydroxyethyl] piperazine-N'[2-ethanesulfonic acid) was from Sigma-Aldrich, Cat No: H-3375

HindIII restriction enzyme provided with the appropriate dilution buffer was from Promega, Cat No: R6041

HiPerFect Transfection Reagent was from QIAGEN, Cat No: 301704

Histopaque 1077 was from Sigma-Aldrich, Cat No: 10771

Hydrochloric acid was from BDH AnalaR, Cat No: 101254H

Hyperfilm ECL was from Amersham Biosciences, Cat No: RPN2103K

Hyperfilm for ^{32}P was from Amersham Biosciences, Cat No: RPN6L

IL-4 (human recombinant) was from R&D Systems, Cat No: 204-IL

Ionomycin (Calcium Ionophore) was from Sigma-Aldrich, Cat No: C7522

LBroth Agar was Sigma-Aldrich , Cat No: L-2897

LBroth was from Sigma-Aldrich, Cat No: L-3022

LightCycler® FastStart DNA master SYBR Green I kit was from Roche, Cat No: 03003230

Loading Dye Blue-Orange 6x was from Promega, Cat No: G1090A

LY294005 (PI3K Inhibitor) was from Calbiochem, Cat No: 440204

Methanol was from BDH AnalaR, Cat No: 101586B

MOPS (minimum 99% titration) was from Sigma-Aldrich, Cat No: M1254

Mouse IgG1 , κ (MOPC-21) was from Sigma-Aldrich, Cat No: M5284

Mouse Immunoglobulins HRP were from

Needles 21G2'' were from BD Microlance, Cat No: 301155

Negative Control siRNA (with no similarity to eukaryotic gene) labelled with Alexa Fluorochrome provided with the appropriate siRNA diluion buffer was from QIAGEN, Cat No: 1022563

NotI restriction enzyme provided with the appropriate dilution buffer was from Promega, Cat No: R643A

OptiMEM medium was from GIBCO BRL, Cat No: 31985-047

PBS (Phosphate Buffered Saline) Tablets were from Oxoid, Cat No: BR00146

PCR Master Mix was from Promega, Cat No:

PE-conjugated Bcl-2 antibody reagent test was from BD Pharmingen, Cat No: 556536

Penicillin-Streptomycin-Glutamin (100x) liquid (contains 10,000 units of Penicillin and 10,000 units of Streptomycin) was from Invitrogen, Cat No: 10378-016

Phenol: Chloroform: Isoamyl Alcohol 25:24:1 saturated with 10 mM Tris, Ph:8.0 , 1mM EDTA was from Sigma-Aldrich, Cat No: P3803

PMA (Phorbol12-Myristate 13-Acetate) was from Sigma-Aldrich, Cat No: P1585

ProbeQuant™ G-50 Microcolumns were from Amersham Biosciences, Cat No: 275335-01

Polyclonal Goat anti-Mouse Immunoglobulins F(ab)2 was from DAKO Cytomation, Cat No: F0479

Polyclonal Swine anti-Rabbit Immunoglobulins was from DAKO Cytomation, Cat No: F0054

Polyvinylpyrrolidone for molecular biology was from Sigma-Aldrich, Cat No: P5288

Propidium Iodide was from Sigma-Aldrich, Cat No: P4170

QuantiTect Reverse Transcription kit was from QIAGEN, Cat No: 205311

Rabbit Immunoglobulins HPR(Peroxidase) was from DAKO Cytomation, Cat No: P0399

Rabbit Immunoglobulins HPR(Peroxidase) was from DAKO Cytomation, Cat No: P0217

Rabbit Serum (Normal) was DAKO Cytomation, Cat No: X0902

Rainbow™ Colour protein molecular weight markers was from Amersham Biosciences, Cat No: RPN756

Rat anti-mouse IgG1 heavy chain was from Serotec, Cat No: MCA336 OR LO-MG1-2

Ribonuclease A (RNAase) was from Sigma-Aldrich, Cat No: R-4875

Rituximab (anti-CD20) was kindly provided by

RNeasy Mini Kit was from QIAGEN, Cat No: 74104

RPMI1840 medium with L-Glutamine was from GIBCO BRL,

Cat No: 21875-034

SalI restriction enzyme provided with the appropriate dilution buffer was from Promega, Cat No: R605A

Salmon Sperm DNA was from Sigma-Aldrich, Cat No:

SB202190 (p38 inhibitor) was from Calbiochem, Cat No:

SB203580 (p38 inhibitor) was from Calbiochem, Cat No: 559398

Sheep Blood Erythrocytes in Alsevers were from TCS Biosciences, Cat No: SB068

Sodium Acetate was from Sigma-Aldrich, Cat No: S-8625

Sodium Azide was from Sigma-Aldrich, Cat No: S2002

Sodium Chloride for molecular Biology was from Sigma-Aldrich, Cat No: S3014

Sodium Citrate was from BDH AnalaR, Cat No: 300627B

SDS (Sodium Dodecyl Sulfate) was from BDH AnalaR, Cat No: 30175

Sodium Hydroxide pellets were from BDH AnalaR, Cat No: 102524P

SP600125 (JNK inhibitor II) was from Calbiochem, Cat No: 420119

Syringes of 20 ml were from BD, Cat No: 300613

TEMED (N,N,N',N'-Tetramethylethylenediamine) electrophoresis reagent was from Sigma-Aldrich , Cat No: T9281

TRI REAGENT was from Sigma-Aldrich, Cat No: T9424

Tris-Glycine SDS Sample Buffer (2x) was from Invitrogen, Cat No: LC2676 or 46-5010

TRITON X was from Sigma-Aldrich, Cat No: X-100

TRIZMA BASE was from Sigma-Aldrich, Cat No: T6066

Tween 20 (Polyoxyethylene-Sorbitan Monolaurate) was from Sigma-Aldrich , Cat No:

U0126 (ERK1/2 inhibitor) was from Calbiochem, Cat No: 662005

Wizard® Plus Minipreps DNA purification System was from Promega, Cat No: A1330