
**Role of Bcl-2 family proteins in
mediating CD40-induced drug resistance
in
Chronic lymphocytic leukaemia
(CLL)**

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Philosophy

by

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Abstract

Despite the advent of novel therapeutic agents, fludarabine-based chemo-immunotherapy still forms the backbone of modern treatment regimens for fit patients with CLL. Even with the high efficacy of these regimens, a significant proportion of patients respond poorly to therapy. Although *TP53* deletion/mutation is strongly associated with fludarabine resistance, it does not account for all patients displaying this resistance phenotype. Understanding the mechanisms underlying fludarabine resistance in patients without *TP53* defects is therefore a major priority. In this study, I have investigated the role of the leukemic microenvironment in mediating such resistance. I focussed on the cytoprotective effect of CD40 stimulation as a result of engagement of CD40 on CLL cells by CD154 on activated T cells which is known to occur at sites of lymph node involvement. At a downstream level, I examined the changes in expression of and interaction between Bcl-2 family proteins given their role as master regulators of the mitochondrial death pathway. Using Western blotting, I showed that *in-vitro* treatment of resting CLL cells with fludarabine resulted in the consistent up-regulation of Puma and a concomitant increase in cell death. Knockdown of Puma expression by siRNA significantly reduced the amount of fludarabine-induced killing, suggesting that the killing of CLL cells by fludarabine requires Puma. Reciprocal immunoprecipitation experiments using antibodies to Puma or Bcl-2 followed by Western blotting

showed that these two Bcl-2 family members form a complex in resting CLL cells. In contrast, when CLL cells were co-cultured with CD154-expressing mouse fibroblasts to mimic interaction with T cells in the lymph node microenvironment, fludarabine-induced cell death was inhibited even though Puma was still induced in these cells. Immunoprecipitation experiments showed that Puma was bound to Bcl-XL and Mcl-1, two other members of anti-apoptotic Bcl-2 family proteins that are selectively up-regulated following CD40 stimulation. Taken together, the above findings suggest that CD40 stimulation up-regulates Bcl-XL and Mcl-1 which bind to Puma, preventing its activation of mitochondrial apoptosis and subsequently inhibiting fludarabine-induced killing. Therefore, my study revealed a potential mechanism responsible for CD40-mediated resistance to fludarabine in CLL.

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Declaration

All of the work presented in this thesis is my own.

List of abbreviations

AIF	Apoptosis-inducing factor
ATM	Ataxia telangiectasia-mutated gene
B cell	B lymphocyte
BCR	B cell receptor
BD	Becton Dickinson
BSA	Bovine serum albumin
cDNA	Complimentary deoxyribonucleic acid
CLL	Chronic lymphocytic leukaemia
CO ₂	Carbon dioxide
CpG-ODN	Deoxyribo(cytidine-phosphate-guanosine) motif containing oligodeoxynucleotide
CTD	C-terminal domain
CDK	cyclin-dependent kinase
CDR3	complementarity-determining region 3 (CDR3)
Dex	Dexamethasone
DIOC6	3,3-dihexyloxacarbocyanine iodide
PKC	Protein kinase C
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dCK	deoxycytidine kinase
DNTPs	Deoxyribonucleoside triphosphates
ECL	Electrochemiluminescence
ELISA	Enzyme Linked Immunosorbant Assay
EtBr	Ethidium bromide
Flu	fludarabine
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FSC	angles from the axis
FITC	Fluorescein isothiocyanate
FISH	fluorescence in situ hybridization
GR	Glucocorticoid receptor
GC	germinal centre (GC)
Hb	Haemoglobin
HRP	Horse radish peroxidase (HRP)-
Ig	Immunoglobulin
IGHV	Variable region of immunoglobulin heavy chain gene
IL	Interleukin
IAP	The inhibitor of apoptosis protein
IP	Immunoprecipitation
kDa	Kilo-Dalton
LREC	Liverpool Research Ethics Committee
M	Mutated
mAb	Monoclonal antibody
mRNA	Messenger RNA
NER	nucleotide excision repair

N/A	Non-applicable
NF- κ B	Nuclear factor κ B
ORR	Overall response rate
OS	Overall survival
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Propidium iodide
PI3-K	Phosphatidy inositol phosphatase 3
PKC	Protein kinase C
Poly-HEMA	Poly(2-hydroxyethyl methacrylate)
PR	Partial remission
PS	Phosphatidylserine
p53	Tumor protein p53
PVDF	Polyvinylidene fluoride
RCF	Relative centrifugal force
RIC	Reduced intensity conditioning
RNA	Ribonucleic acid
RNAi	RNA interference
RISC	RNA Induced Silencing Complex
RPMI	Roswell Park Memorial Institute Medium
s	Second
SD	Standard deviation
SD	standard error of mean
SDF-1	Stromal cell derived factor 1
SDS	Sodium dodecyl sulphate
SSC	scattered at right angle
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
TP53	p53 gene (human)
TBE	Tris/borate/EDTA
TBS	Tris-buffered saline
TBS-T	TBS-Tween
TEMED	Tetramethylethylenediamine
TFI	Treatment free interval
TLR	Toll-like receptor
TNF	Tumour necrosis factor
VDAC	The voltage-dependent anion channel
UM	Unmutated
WBC	White blood cells
WCL	Whole cell lysate
wt	Wild type

Chapter 1

Introduction to Chronic Lymphocytic Leukaemia

1.1 Introduction

Chronic lymphocytic leukaemia (CLL) is the most common blood cancer, accounting for approximately 30% of all leukaemia. Considerable morbidity and mortality is associated with this disease, and it remains incurable by conventional treatments. Patients with progressive forms of CLL will typically be subjected to several rounds of therapy and relapse prior to succumbing to the suppression of bone marrow and immune organ function that accompanies this disease. Development of therapy resistance by the malignant clone in CLL accounts for relapse of the disease and requirement for changed and more severe treatments. Recent studies of clonal evolution imply development of therapy resistance through selection of clones bearing an appropriate mutation (Woyach & Johnson et al. 2015). However, this is not the whole of story of treatment resistance. It is well known that microenvironment can provide protection signals to CLL cells exposed to cytotoxic stimuli, but the mechanism(s) involved have not been completely characterised. This thesis examines the role of CD40 ligation in providing CLL cells with protection against fludarabine (Kater et al. 2004).

According to current estimates, CLL is the most common leukaemia, with highest incidence rates in Europe and North America (Dores et al. 2007). Approximately 2,400 new cases of CLL are reported each year in the UK (Cramer & Hallek et al. 2011) and more than 15,000 in the USA (Siegel et al. 2014). CLL is considered as a disease of advanced age, with median age at diagnosis between 67 and 72 years, although sporadically there are CLL patients diagnosed in their 30s or 40s. Males are nearly twice more likely to develop CLL. Inherited genetic predisposition to CLL

has been documented, although is not yet fully understood (Neuland et al. 1983; Yuille et al. 2000).

1.2 Clinical presentation

CLL is a neoplastic lymphoproliferative disease characterised by clonal accumulation of B lymphocytes within the blood, bone marrow, lymph nodes, liver and spleen (Hallek & Pflug et al 2010; Schlette et al 2010). CLL is highly heterogeneous when it comes to the clinical course, with some patients presenting an indolent and asymptomatic disease and surviving for many years without any treatment, and others developing an aggressive disease that requires therapy relatively early (Dighiero 2005). At diagnosis, most patients are asymptomatic or present only minimal symptoms. Diagnosis is thus established by blood count (presence of $\geq 5 \times 10^3$ clonal B lymphocytes per μl of peripheral blood), microscopic evaluation of blood smear, and flow cytometric immunophenotyping of circulating lymphocytes (Hallek et al. 2008). The clinical manifestation of CLL may include lymph node enlargement, anaemia and/or thrombocytopenia, bone marrow failure, palpable hepatomegaly and/or splenomegaly, and may involve weight loss, shortness of breath, tiredness and repeated infections (Hallek & Pflug 2010). Despite recent advances in the management of CLL, it still remains an incurable disease.

1.3 Cellular origin of CLL

CLL cells exhibit phenotypic features of mature, activated B lymphocytes, and co-express the T-cell marker CD5, as well as B-cell surface markers CD19 and CD23. The levels of monoclonal surface immunoglobulins and CD20 are usually low (or even undetectable) compared to normal B lymphocytes (Chiorazzi, Rai & Ferrarini et al 2005; Ginaldi et al. 1998; Stevenson & Caligaris-Cappio et al 2004). Normal mature B cells undergo the process of activation, proliferation and differentiation upon encountering their antigen, a process that occurs either during the T-cell dependent germinal centre (GC) B-cell response or in a T-cell independent manner. Although the development of both memory B-cell types requires classical T-cell help, the generation of GC-dependent memory B cells requires TFH-cell help, while the generation of GC-independent memory cells does not need that (Takemori et al. 2014). Antigen stimulation induces the process of somatic hypermutation within the variable regions of immunoglobulin (Ig) genes, turning naive B cells with low-affinity surface Igs into long-lived memory B cells producing high-affinity antibodies. Of note, it is reported that somatic hypermutation can occur not only in the context of GCs, but also in a T-cell independent manner and outside classical GCs (Takemori et al. 2014). Thus, the presence of somatic mutations within the IGHV genes in at least 50% of CLL patients may suggest a clonal history of BCR stimulation, with mutated and un-mutated genes indicating the origin of tumour cells from an antigen-dependent or -independent developmental stage, respectively.

The two molecular subtypes of CLL are differentiated based on the presence or absence of somatic mutations within the immunoglobulin heavy-chain variable region (IGHV) genes (Tobin & Rosenquist et al 2005). In fact it has been observed

that the repertoire of the IGHV genes harboured by CLL cells is biased towards particular genes leading to observations of remarkable similarity in antigen receptor structure between unrelated (Messmer et al. 2004). The highly homologous regions include the sequences of complementarity-determining region 3 (CDR3) on heavy and light chains of B-cell receptors that are detected regardless of the IGHV genes mutational status (Lin 2010). This suggests that recognition of discrete antigens or structurally related epitopes may contribute to the selection of tumour clones. It also appears that cells with un-mutated IGHV genes carry more responsive B-cell receptors, while in other cases, mostly with mutated IGHV genes, cells are unresponsive to BCR stimulation. Thus, it is hypothesised that in cases with un-mutated IGHV genes low-affinity antigen stimulation contributes to the expansion of responsive leukaemic clones, whereas in cases with the mutated IGHV genes initial high-affinity stimulation (possibly with auto-antigen) selects a responsive clone which subsequently becomes desensitised and enters an anergic state (Melchers & Rolink et al 2006; Stevenson & Caligaris-Cappio et al 2004). In this context it has been observed that autoimmune diseases are quite frequent in CLL patients (Kipps & Carson 1993). Moreover, several reports indicate that disruption in the process of eliminating cellular debris and apoptotic cells, as well as pathogenic bacteria, may facilitate the development of CLL (Chiorazzi & Ferrarini 2003b; Rawstron et al. 2002). These observations clearly underscore the potential role of the extrinsic or auto-antigen encounter in the aetiology of CLL.

Nevertheless, the remarkable homogeneity of M and UM-CLL cells at the gene expression level suggests a common mechanism of oncogenic transformation (Caligaris-Cappio & Ghia 2007; Klein et al. 2001; Rosenwald et al. 2001). The cells

where this oncogenic transformation takes place are likely to be the small B cells that phenotypically resemble good-prognosis CLL cells (CD5+, CD38-, low level of CD20 and CD79b expression) that gradually accumulate in peripheral blood of healthy elderly people at the incidence rate varying between 3-6% (Ghia et al. 2004; Ghia & Hallek 2014; Rawstron et al. 2002). It is envisaged that further studies of these cells might provide important insights into the natural history of CLL.

1.4 Prognostic factors in CLL

1.4.1. IGHV mutational status

As described above immunoglobulin heavy chain variable region (IGHV) gene mutation status, defined as the presence or absence of somatic hypermutation in the IGHV gene of CLL cells as compared with the gene sequence of the nearest germline, is used to divide CLL patients into two prognostic groups. The presence of unmutated IGHV genes (i.e. <2% mutation) is considered to indicate significantly worse prognosis (Coscia et al. 2011).

1.4.2. Cytogenetic abnormalities

Molecular cytogenetic methods such as fluorescence in situ hybridization (FISH) have been used to detect genomic aberrations in over 80% of CLL cases, with high-risk aberrations more common in CLL cases with the un-mutated IGHV genes (Parikh & Shanafelt 2016). Based on cytogenetic analysis five different categories of CLL can be distinguished in terms of prognosis: i) 17p13 deletion in the TP53 gene; ii) 11q22-23 deletion in the ATM gene; iii) 12q trisomy (resulting in the presence of an extra copy of the MDM2 gene); iv) normal karyotype; and v) 13q14

deletion. Median treatment-free survival differs significantly between these groups, and was reported to reach 9, 13, 33, 49 and 92 months, respectively (Austen et al. 2005; Van Bockstaele, Verhasselt & Philippé 2009). The prognostic value of loss and/or mutation of ATM and p53 will be discussed below. Monoallelic and/or biallelic deletion of 13q14 is the most frequent chromosomal aberration, detected in over 50% of CLL cases (Austen et al. 2005). The deleted region associated with this chromosomal aberration includes the first exon of the DLEU1 gene and the long non-coding RNA (DLEU)-2. The latter affects the expression of two microRNAs, miR-15a and miR-16-1 (Aqeilan, Calin & Croce 2009). These two microRNAs target several proteins involved in regulation of cell cycle, such as cyclin D2, D3 and E, and cyclin-dependent kinases CDK4 and CDK6, as well as anti-apoptotic protein Bcl-2 (Decker et al. 2003). Thus, it is postulated that the absence or decreased levels of miR-15a and miR-16-1 following 13q14 deletion may contribute to lymphomagenesis (Aqeilan, Calin & Croce 2009).

1.4.3. Mutations within the DNA repair pathway

Two critical regulators of DNA damage, ATM and p53, are often mutated and/or lost in CLL cells. The ATM (ataxia telangiectasia mutated) gene acts upstream of p53 in the DNA damage pathway, by integrating cellular signalling caused by DNA double-strand breaks. Ataxia-telangiectasia patients that either lack ATM protein or express mutant ATM proteins often develop haematological malignancies, including mature B-cell leukaemia (Stankovic et al. 2002; Steele et al. 2008). Absent ATM expression or mutations in the ATM gene have been also reported in spontaneous CLL, including cases that harbour the un-mutated IGVH genes (Tobin & Rosenquist

2005). ATM mutations are scattered throughout the gene, and are often present in both alleles (Stankovic et al. 2002).

The acquisition of TP53 mutations leads to abnormal transcriptional activity of p53, manifesting for example by lack of p21 up-regulation in response to ionizing radiation (Zenz et al. 2010). While both p53 and ATM mutations may lead to impaired p21 up-regulation, constitutive p53 overexpression is observed only in cases with TP53 mutation (a phenomenon referred to as ‘type A’ p53 dysfunction) (Pettitt, Moran & Cawley 2001; Zenz et al. 2008). The presence of TP53 mutations is frequently associated with the transformation of CLL into an aggressive diffuse large B-cell lymphoma. Abrogation of p53 function may also occur as a result of gene deletion, and CLL patients with monoallelic deletion of the TP53 gene may also harbour mutations within the remaining TP53 allele (Zenz et al. 2008). The presence of TP53 mutation in the absence of 17p deletion is also indicative of poor prognosis. The mutations of ATM and p53 are not concurrent in CLL patients and lead to somehow different biological features in CLL cells, reflective of the fact that ATM and p53 pathway do not fully overlap (Stankovic et al. 2002; Steele et al. 2008).

1.4.4. CD38 and ZAP-70

The levels of CD38 and the T-cell associated tyrosine kinase ZAP-70 (zeta-associated protein of 70kDa) varies between CLL cases. Both the presence of CD38-positive cells and increased expression of ZAP-70 correlate with progressive disease and poor prognosis (Matrai 2005; Rassenti et al. 2004). Importantly, recent data indicate that the expression of ZAP-70, long considered as being stable during the course of the disease, can in fact change, particularly at the time of progression or

relapse (Wiestner et al. 2003). Furthermore, increased expression of the CLL up-regulated gene 1 (CLLU1) is observed in poor prognostic groups (as determined by IGVH mutational status, as well as the levels of ZAP-70 and CD38) and is indicative of high risk CLL with shorter time from diagnosis to therapy. Nevertheless, expression of CD38 or ZAP-70 may not be useful for predicting the outcome of fludarabine-based treatments (Matrai 2005; Wiestner et al. 2003).

Other than the prognostic factors discussed above, several other markers have been proposed to bear prognostic relevance in CLL patients. These have been summarised in (Table 1.1)

Table 1.1. Selected additional markers with suggested prognostic value in CLL.

Prognostic factor		Comments	References
Type	Name		
Serum makers	LDH (lactate dehydrogenase)	Increased LDL levels associated with shorter survival time and others	Van Bockstaele <i>et al.</i> , 2009
	TNF α (tumour necrosis factor alpha)	High plasma levels of TNF α predictive of shorter survival	Ferrajoli <i>et al.</i> , 2002
	β 2-microglobulin	Increased serum levels signify high-risk disease	Van Bockstaele <i>et al.</i> , 2009
	IL-6 (interleukin 6)	High IL-6 plasma levels correlate with shorter overall survival	Lai <i>et al.</i> , 2002
	MMP-9 (matrix metalloproteinase 9)	Increased serum levels correlate with shorter progression-free survival	Molica <i>et al.</i> , 2003
	TK1 (thymidine kinase 1)	High serum levels predict poorer overall survival	Konoplev <i>et al.</i> , 2010
Gene expression	LAG3 (lymphocyte-activation gene 3)	High LAG3 expression correlates with unmutated IGVH and reduced treatment-free survival	Kotaskova <i>et al.</i> , 2010; Zhang <i>et al.</i> , 2010
	p27(kip1)	High expression associated with poorer prognosis	Vrhovac <i>et al.</i> , 1998
Proliferation and apoptosis markers	Lymphocyte doubling time (LDT)	Short LDT is a predictor of shorter overall survival and treatment-free survival	Van Bockstaele <i>et al.</i> , 2009
	Inhibition of DPP2 (dipeptidyl peptidase 2)	Apoptotic response indicative of resting phenotype	Danilov <i>et al.</i> , 2010
	Telomere length and telomerase activity	Short telomeres and high activity predictive of shorter survival	Van Bockstaele <i>et al.</i> , 2009

1.5 The role of apoptosis in the pathogenesis of CLL

CLL is a disease characterized by defective apoptosis of the malignant cells. This next section of my thesis outlines the process of apoptosis, and how it is deregulated in CLL cells and related to disease pathogenesis.

1.5.1 Apoptosis--general mechanisms and regulation

The mammalian cell death network is comprised of many distinct functional modules, including apoptosis, autophagy and necrosis (Leist & Jäättelä 2001). Of these modules apoptosis (programmed cell death type I) has been studied most extensively, and is recognised to be of paramount importance for the health of multicellular organisms. It is widely known that disturbances in the apoptotic process within cells can lead to disease conditions such as cancer where it is recognised that evasion of cell death is a key hallmark (Hanahan & Weinberg 2000). Apoptosis is a highly complex process of removing damaged or superfluous cells, and is critical for tissue homeostasis. There are two main apoptotic pathways, the death receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway, both regulated by a concerted action of many pro- and anti-apoptotic proteins (Figure 1.1). The mitochondrial pathway of apoptosis is finely controlled up-stream of mitochondria by a large group of proteins that belong to the Bcl-2 family (Chipuk et al. 2010; Czabotar et al. 2014a; Youle & Strasser 2008) (Figure 1.1). The main event during the intrinsic pathway of apoptosis is the mitochondrial outer membrane permeabilisation (MOMP), which allows the release of proteins localised between the outer and inner mitochondrial membranes, such as cytochrome *c* or Smac, into the cytosol. Upon release, these proteins cooperate with cytosolic factors, leading to

the formation of a multiprotein complex called the apoptosome, resulting in activation of caspases and execution of apoptosis. Control over the integrity of the mitochondrial outer membrane is executed by proteins of the Bcl-2 family, which can be further divided into three subfamilies: anti-apoptotic (e.g. Bcl-2, Bcl-XL, Mcl-1 and Bcl-w), pro-apoptotic effectors (Bax and Bak), and BH3-only proteins (e.g. Bid, Puma, Bim and Bad), which form a complex network of interactions (Chipuk et al. 2010; Cory & Adams 2002; Czabotar et al. 2014a; Danial 2007; Skommer, Wlodkowic & Deptala 2007). Briefly, Bax and Bak become activated upon association with the BH3 activators, which induces a conformational change in Bax/Bak, leading to their oligomerisation and formation of pores within the outer mitochondrial membrane. The BH3 activator proteins are antagonised by the anti-apoptotic Bcl-2 family members, which are counteracted by the second group of BH3 proteins, referred to as sensitisers (e.g. Bad) (Figure 1.1). There is still controversy surrounding the process of direct activation of Bax and Bak, with reports showing that in some cases Bid, Bim and Puma can also interact with anti-apoptotic Bcl-2 proteins that have pre-formed complexes with Bax and Bak, resulting in the release of Bax and Bak which then induce MOMP (Willis et al. 2007). Irrespective of the exact mechanism of interaction between the members of Bcl-2 family of proteins, the function of anti-apoptotic members is to prevent MOMP. The inhibitor of apoptosis protein (IAP) family also consists of several members, including IAP1, IAP2, XIAP and surviving (Espinosa et al. 2006). These proteins play a role in regulating post-mitochondrial events in the pathway of apoptosis (Figure 1.1).

The extrinsic pathway of cell death is induced by ligation of plasma membrane-localised death receptors such as Fas (CD95) receptor or TRAIL receptors (e.g.

DR5), followed by activation of the initiator caspase 8 which can activate the executioner caspase-3 either directly, or via cleavage of Bid and the mitochondrial pathway of apoptosis (Figure 1.1).

1.5.2 Mitochondria and Apoptosis

As previously mentioned, the process of apoptosis is driven by a network of proteins connected to each other in an intricate manner, forming two main signalling pathways (intrinsic and extrinsic). The intrinsic (mitochondrial) pathway of apoptosis is heavily regulated by multiple proteins acting up-stream and down-stream of the mitochondria, determining the efficiency of apoptotic cell death. Mitochondria are double membrane intracellular organelles (Figure 1.1), whereby the intermembrane space houses a vast array of proteins, the highly convoluted inner membrane is a site of membrane-associated electron transport and ATP synthesis, while the matrix is a site of the citric acid cycle and fatty acid oxidation. The outer membrane of the mitochondria permits the passage of small molecules, but not proteins (Mohamad et al. 2005).

As described earlier, the main event during the mitochondrial pathway of apoptosis is the induction of MOMP, which allows the release of proteins localised between the outer and inner membranes, such as cytochrome c or Smac (mitochondrial protein that promotes cytochrome c), into the cytosol. Upon release these proteins cooperate with cytosolic factors, leading to the activation of caspases and execution of apoptosis. In particular, the release of cytochrome c allows its association with Apaf-1 and pro-caspase-9, leading to formation of a large protein scaffold called the apoptosome on which activation of pro-caspase-9 occurs. The initiator caspase-9 then

activates down-stream caspases, which then execute proteolytic disintegration of the cell (Mohamad et al. 2005) (Figure 1.1).

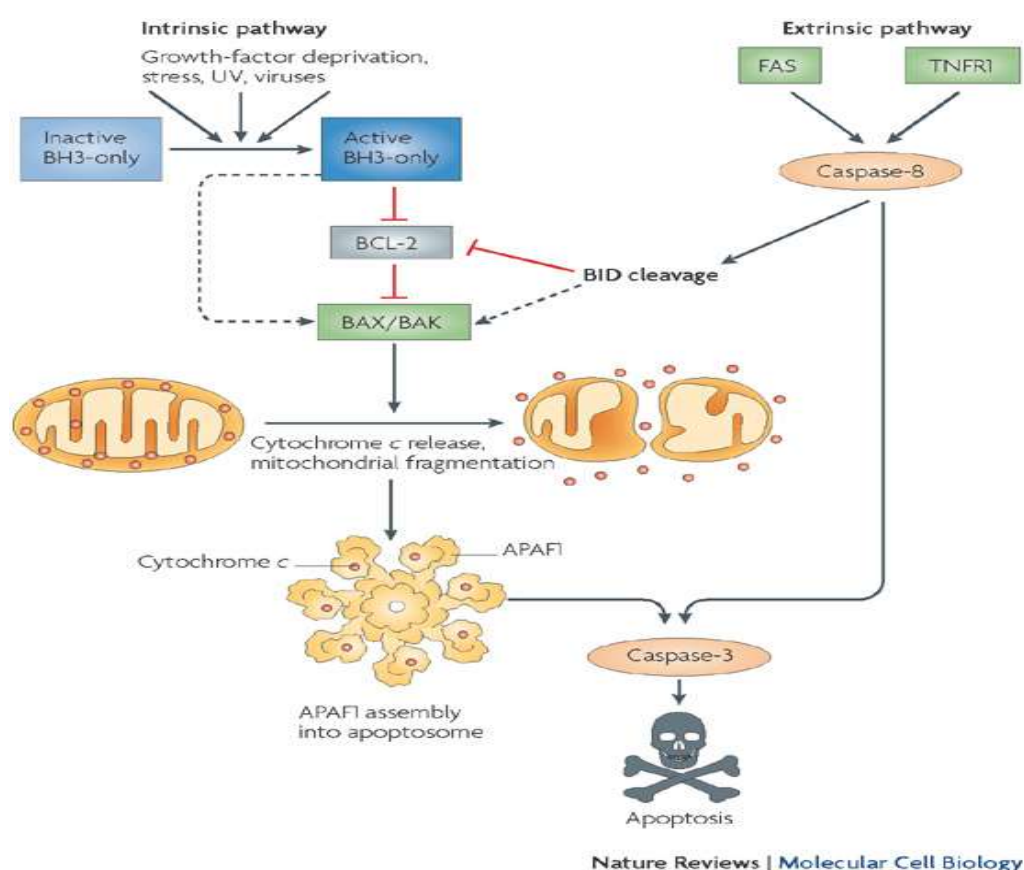


Figure 1.1. Pathway of apoptosis

Apoptosis can be induced by cell surface receptors, such as Fas and tumour necrosis factor receptor-1 (TNFR1) (extrinsic pathway, right), or by various genotoxic agents, metabolic insults or transcriptional cues (intrinsic pathway, left). The intrinsic pathway of apoptosis is regulated both up-stream and down-stream of mitochondria. Up-stream of the mitochondria the most important regulators are proteins from the Bcl-2 family, consisting of anti-apoptotic (e.g. Bcl-2 and Mcl-1), pro-apoptotic (e.g. Bax and Bak) and BH3-only proteins (Bid, Bad, Bim, Puma, or Noxa). Receptor ligation (the extrinsic pathway) or a wide variety of stress signals can also converge on mitochondria (the intrinsic pathway) (Czabotar et al. 2014b).

1.5.2.1 Bcl-2 family of proteins

As stated earlier, control over integrity of the mitochondrial outer membrane is executed by the different members of the Bcl-2 family of proteins, which form a complex network of interactions (Cory, Huang & Adams 2003; Shimizu et al. 1999). These interactions are facilitated by four conserved Bcl-2 homology (BH1-BH4) domains, with the anti-apoptotic members equipped with all four BH domains, while pro-apoptotic proteins containing either multiple BH domains (Bax and Bak) or a single BH3 domain. The BH3-only proteins can bind to the range of different anti-apoptotic Bcl-2 family proteins (Figure 1.2).

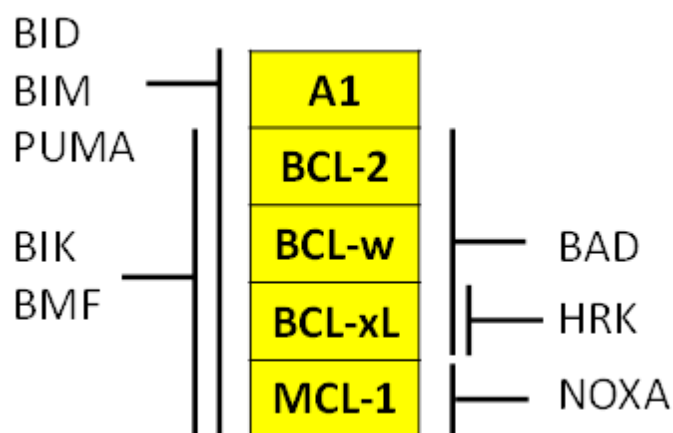


Figure 1.2. The binding profiles of selected BH3-only proteins.

The binding profiles of selected BH3-only proteins to the anti-apoptotic members of the Bcl-2 family (Chipuk et al. 2010).

It has been firmly established that the pro-apoptotic multi-domain Bcl-2 family members Bax and Bak mediate permeabilisation of the outer mitochondrial membrane although other proteins of the mitochondrial membrane, such as VDAC (the voltage-dependent anion channel), may also participate in this process (Kuwana et al. 2002). When cells encounter stress signals, either internal or external, Bax and Bak undergo conformational changes to form homo-oligomers, which then insert into the membrane to form pores. It has been shown that Bax has to first translocate from the cytosol to the mitochondrial membrane, while Bak is constitutively associated with the mitochondrial membrane (Chipuk et al. 2008; Kuwana et al. 2002). The observation that cells deficient in both Bax and Bak are extremely resistant to a wide range of apoptotic stimuli, while Bax or Bak single-deficient cells are still competent in undergoing apoptosis, confirmed that these two proteins are crucial within the mitochondrial pathway of apoptosis and exhibit mutual functional redundancy (Kang & Reynolds 2009).

Although it is widely accepted that the proteins Bax and Bak are essential for initiation of apoptosis at mitochondria, several mechanisms have been proposed to explain how these proteins are activated. The two predominant and non-mutually exclusive models describing the interactions between the Bcl-2 family members include the direct activation model and the indirect activation model (Adams & Cory 2007) (Figure 1.3).

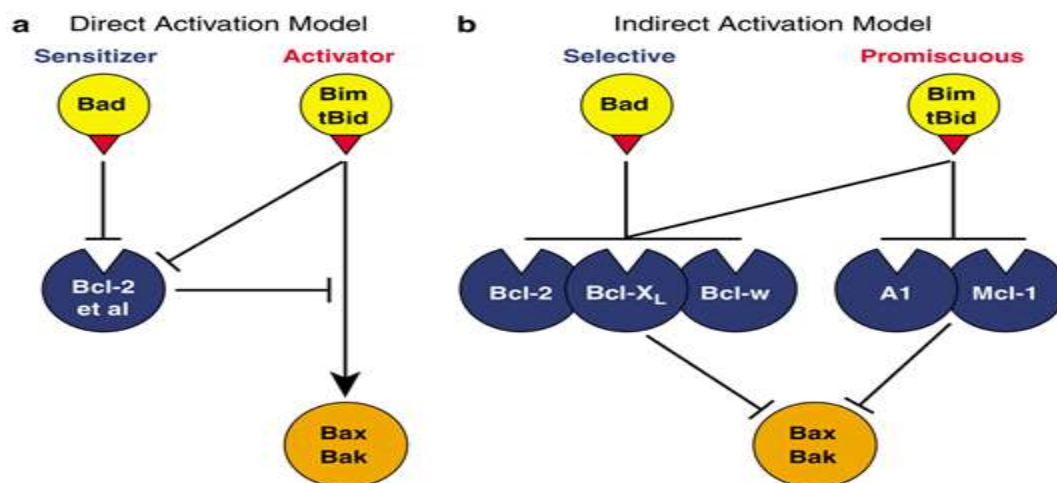


Figure 1.3. The models of Bax/Bak activation.

The binding profiles of selected BH3-only proteins activation during the initiation of the mitochondrial pathway of apoptosis (Adams & Cory 2007).

1.5.2.2 Activation of the mitochondrial pathway of apoptosis

In a direct activation model, when inactive cytosolic Bax is in contact with a group of BH3-only proteins (e.g. tBid, Bim, Puma), the latter directly induces conformational changes in Bax and prompts it to translocate to the outer mitochondrial membrane (Zhu et al. 2013). This is then followed by Bax oligomerisation driven by the bound BH3-only proteins (also known as activators). In an indirect activation model, in healthy cells the BH3-only proteins are antagonised by the anti-apoptotic Bcl-2 family members, which are counteracted by the second group of BH3-only proteins (sensitizers), such as Bad, Bik, Hrk, Noxa

and Bmf (Santidrián et al. 2010). Binding of the “sensitizers” liberates the “activators” that engage Bax and Bak. This model is strongly supported by a plethora of biochemical studies showing direct association of Bax/Bak with Bid, Bim and Puma (Adams & Cory 2007). Nevertheless, the extent of apoptosis resistance was somehow different from that observed in Bax/Bak double knock-out animals, with some lymphoid cells still dying in response to glucocorticoid treatment (Willis et al. 2007).

The indirect activation model is based on observations that apoptosis can proceed in the absence of direct activators Bim and tBid (Terrones et al. 2008), and supported by the observed apoptosis of Bim/Bid/Puma triple-deficient lymphoid cells in response to glucocorticoid treatment (Yu et al. 2001). In this model, activation of Bax and Bak can occur as a default event, with a small portion of these proteins constitutively “primed” in an apoptosis-inducing conformation and kept in check by the pro-survival Bcl-2 family proteins. As soon as all the anti-apoptotic Bcl-2 family proteins are neutralised by the BH-3 members, apoptosis will ensue.

The main difference between the direct and indirect models may be primarily the relative binding affinity of BH-3 proteins for their respective association partners (anti-apoptotic Bcl-2 family proteins or multi-domain pro-apoptotic Bcl-2 family proteins). Most of the results available in the literature, from both biochemical studies and animal models, can be reconciled by a model merging the direct and indirect activation scenario, as proposed (Chen et al. 2007) . In some situations, Bax/Bak may be activated by certain BH-3 protein, while in other situations other mechanisms of activation (e.g. phosphorylation, or spontaneous activation upon neutralisation of pro-survival Bcl-2 members) can occur (Hallaert et al. 2007).

1.5.3 p53 function in cell survival and apoptosis

p53 (known as Tumor protein and cellular Tumor antigen p53), it is an important tumour suppressor gene that acts to prevent the outgrowth and survival of malignant cells. These activities of p53 reflect its role as a transcription factor that regulates the expression of numerous genes in response to various stress stimuli. Classically, upon DNA damage, p53 induces cell cycle arrest allowing time for DNA repair to occur and promoting cell survival (Mohr et al. 2011). But p53 can also induce cell death programmes such as apoptosis or autophagy (Figure 1.4). The extent of DNA damage determines whether pro-survival or pro-apoptotic facets of p53 activity are induced (Bensaad et al. 2006). The apoptotic potential of p53 is also affected by MDM2-regulated ubiquitination, which leads to destruction of p53 protein. Some polymorphic variants of p53 having different binding affinity towards MDM2 (Dumont et al. 2003) and inhibitors of p53-MDM2 association are thus being considered as potential anti-cancer agents in CLL (Bixby et al. 2008).

1.5.4 Defective apoptotic signalling in CLL

For many years CLL was considered as a disease caused by unstoppable accumulation of long-lived lymphocytes in the G0/early G1 phase of the cell cycle that fail to undergo apoptotic cell death. Currently, it is well established that CLL is not a static disease and an on-going proliferation of CLL cells occurs in proliferation niches within the lymph nodes (Herishanu et al. 2011). How the balance between CLL cell proliferation and death is regulated, and how it correlates with the rates of disease progression, is still a matter of intense investigation. Of note, impaired

tumour surveillance also contributes to the increased survival of leukemic cells in CLL patients (Pytlik et al. 2008).

Aberrant apoptotic signalling has been widely reported in CLL. The importance of the Bcl-2 family of proteins in the pathogenesis of CLL has been firmly established, with over-expression of Bcl-2 considered as one of the hallmarks of the disease (Buggins et al. 2010). Another anti-apoptotic Bcl-2 protein, Mcl-1, as well as IAP proteins (survivin, CIAP1, CIAP2, and XIAP) are also up-regulated in CLL patients (Chiorazzi, Rai & Ferrarini 2005; Grzybowska-Izydorczyk et al. 2010). Moreover, decreased expression of pro-apoptotic molecules such as Smac has been observed in CLL (Grzybowska-Izydorczyk et al. 2010).

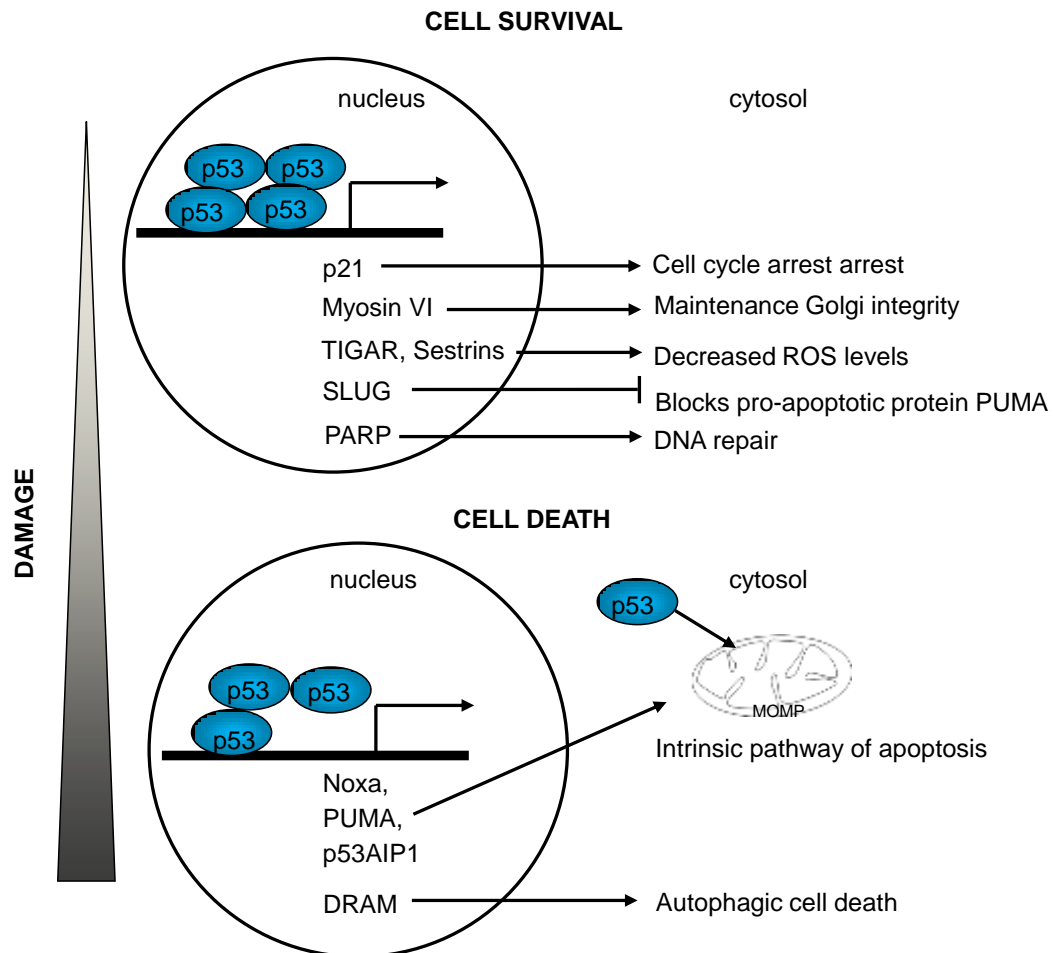


Figure 1.4. The role of p53 in cell survival and death.

Depending on the strength of damage, p53 may contribute to cell cycle arrest and activation of DNA repair processes (low or reparable damage), or to cell killing (more severe, irreparable or oncogenic stress). Mechanisms that contribute to induction of cell cycle arrest and survival include increased expression of p21WAF1/CIP1, as well as increased expression of genes that protect from oxidative stress, genes that promote the integrity of intracellular organelles, and most importantly genes facilitating DNA repair. Cell death processes induced by p53 include increased expression of pro-apoptotic genes such as Puma or p53AIP1, and, as recently discovered, genes involved in autophagy cell death (DRAM). Moreover, transcription-independent activity of p53 in the cytosol has been reported. Cytosolic

p53 function is comparable to the pro-apoptotic BH3-only protein (Bensaad et al. 2006).

Another significant feature of CLL cells is loss or mutations of tumour suppressor p53 protein. As described above, p53 plays a dual role. One role promotes survival of cells that are able to undergo DNA repair, while the second role promotes apoptosis in cells that are damaged beyond their capabilities to repair DNA. Loss or mutations in p53, as often detected in CLL patients, may thus promote tumourgenesis by leading to chromosomal instability, allowing acquisition of additional genetic abnormalities and survival of critically damaged cells (Steele et al. 2008).

Importantly, even though CLL cells exhibit prolonged survival *in vivo*, they undergo spontaneous apoptosis when cultured *ex vivo*. The spontaneous apoptosis *in vitro* can be inhibited upon increased cell density of homotypic cell cultures, or by co-culture with other cell types that play a role of nurse-like cells (Burger & Kipps 2002a; Martinez-Lostao et al. 2004; Pettitt et al. 2001). This indicates that the survival advantage is not entirely autonomous to CLL cells, and can potentially be reinforced by homotypic cell interactions and autocrine survival factors, as well as microenvironmental signals (Martinez-Lostao et al. 2004; Pettitt et al. 2001). The microenvironmental cues that have been recognised as factors promoting the survival of CLL cells include interactions with stromal and nurse-like cells, as well as cells expressing CD40 ligand (e.g. CD4⁺ T lymphocytes). In response to microenvironmental signals CLL may secrete chemokines (Burger et al. 2009) and support the survival of CLL cells by inducing anti-apoptotic signalling (Vauzour et al. 2007). Controversial data exist on the effect of triggering CD40 on CLL survival. Some data indicate that CD40 ligand stimulation increases sensitivity of CLL cells

towards apoptosis induced by CD95 ligand or fludarabine, potentially by decreasing the expression of Bcl-2 protein, and increasing the expression of pro-apoptotic BH3 protein Bid, death receptors CD95 and DR5, and cytokines TNF α and IFN γ (de Toter et al. 2003; Dicker et al. 2005). However, according to other reports, triggering of CD40 may induce pro-survival NF- κ B signalling and inhibit fludarabine-induced apoptosis in CLL cells (Romano et al. 1998).

1.5.5 CD40 signalling in CLL

1.5.5.1. Targeting tumour microenvironment

It is recognised that CLL microenvironment plays an important role in the survival of CLL cells and that it contributes to drug resistance. Understanding this role could lead to therapies targeting the microenvironment to result in efficient killing of CLL cells. For example (Burger & Gribben 2014), it is proposed that chemokine receptors on CLL cells are a therapeutic target because of the role they play in regulating trafficking of CLL cells between the blood, lymph nodes and bone marrow, and in facilitating interaction between CLL cells and accessory cells within the tumour microenvironment (Tsukada et al. 2002). In this respect, CXCR4 is arguably one of the most important chemokine receptors expressed by CLL cells as it mediates the response to stromal cell-derived factor 1 (SDF-1) secreted by blood-derived nurse-like cells as well as bone marrow and extramedullary stromal cells. Accordingly, CXCR4 antagonists are currently evaluated as a potential treatment strategy for CLL (Buchner et al. 2010; Burger & Kipps 2002b).

1.5.5.2. Role of T cells in development of CLL

Whilst antigenic stimulation via the B cell receptor (BCR) is generally considered to be one of the most important pro-survival factors that help CLL clones expand in vivo (Chiorazzi & Ferrarini 2003a; Chiorazzi, Rai & Ferrarini 2005), interaction of CLL cells with T cells in the bone marrow and lymph nodes also plays a critical role in the expansion and extended survival of the malignant cells (Ghia et al. 2002; Granziero et al. 2001; Schmid & Isaacson 1994; Trentin et al. 1997). Recent studies have shown that within lymphoid tissue proliferation centres a significant proportion of CD4⁺ T cells expressing CD40L (CD154) are interspersed with proliferating CLL cells that express CD40, thus providing in vivo evidence linking activated T cells to CLL clonal expansion (figure 1.5).. Further direct evidence for a role of CD4⁺ T cells in supporting CLL-cell proliferation is provided by an adoptive transfer model of CLL which demonstrated that activated autologous T cells were absolutely required for CLL cells to engraft, survive, and proliferate an in-vivo model of this disease (Oldreive et al. 2015; Pytlik et al. 2008).

Regarding the molecules involved in the interaction between T cells and CLL cells, one of the most important interactions is between CD40 - a member of the TNF receptor superfamily which is expressed on CLL cells - and its ligand CD154 which is expressed on T cells. Thus, it has been shown that in vitro activation of CLL cells with cross-linking antibodies to CD40 plus interleukin (IL)-4 not only promoted survival but also induced proliferation of CLL cells (Crawford & Catovsky 1993; Fluckiger et al. 1992). Further studies using primary CLL cells showed that engagement of CD40 by CD154 initiated a potent signalling cascade that led to activation of the pro-survival transcription factor NF- κ B (Dugas-Bourdages et al.

2014; Furman et al. 2000). Meanwhile, the role of CD40 in B cell development and function in vivo has been further reinforced by animal studies showing that B cells from CD40-deficient mice failed to proliferate and undergo immunoglobulin isotype switching in vitro in response to stimulation by CD40 ligand and IL-4 (Castigli et al. 1994; Kawabe et al. 1994). It is now firmly established that CD40-CD154 interaction is one of the most important mechanisms responsible for T cell-mediated CLL-cell survival and proliferation. Many of these effects can thus be recapitulated in vitro using a co-culture system where human or mouse epithelial cells (or fibroblasts) stably transfected with human CD40L are used as feeder layers, in combination with IL-4, to mimic interaction with T cells. Such in-vitro activation of CD40 has been shown to associate with prolonged survival and drug resistance in CLL cells (Dugas-Bourdages et al. 2014; Granziero et al. 2001; Kitada et al. 1999).

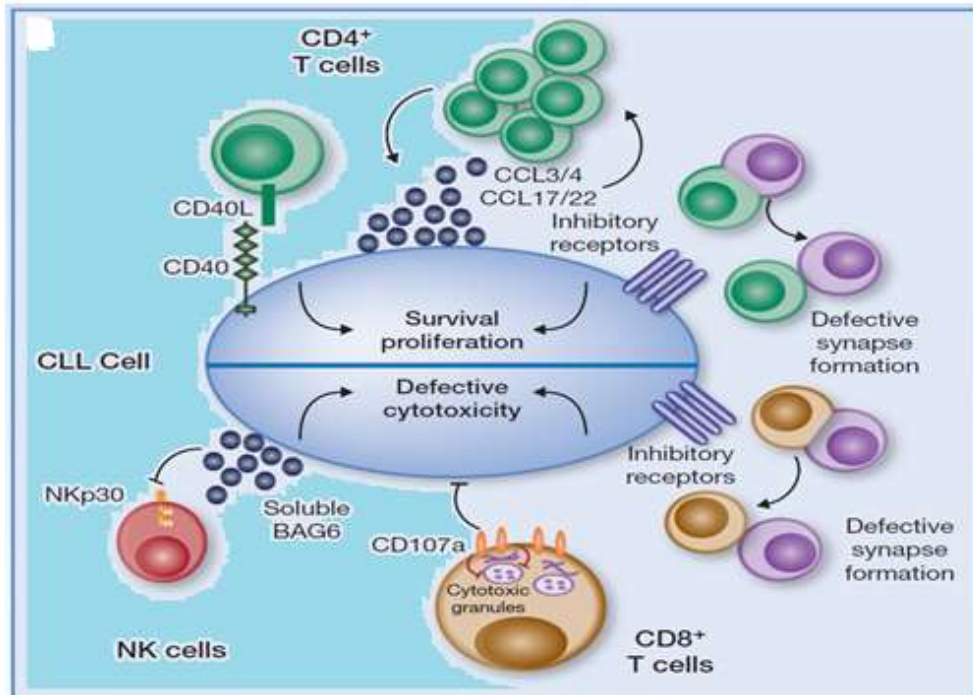


Figure 1.5. The CLL microenvironment

Microenvironment interactions for survival and proliferation of CLL cells, CD4⁺ T cells that express CD40 ligand along with immune and bone marrow stromal cells within CLL lymph nodes providing signals needed for activation of tumor clone (ten Hacken & Burger 2014).

1.6 Mechanisms of CD40L-mediated protection from drug-induced cell death in CLL

1.6.1 Multiple mechanisms involved in CD40L-mediated protection

CD40 ligation can rescue CLL cells from apoptosis, and constitutes an important survival cue for CLL cells within the lymph node microenvironment. The anti-apoptotic action is associated with enhanced expression of intracellular apoptosis-regulating proteins such as Bcl-2, Bcl-XL, Mcl-1 and A1/Bf1-1 (Ghia et al. 2001; Hallek et al. 2008) and the IAP family member survivin (Granziero et al. 2001). Moreover, CD40 stimulation was shown to reduce the level of pro-apoptotic proteins Bim-EL and Noxa (Hallek et al. 2008; Kater et al. 2004). These changes confer drug resistance by protecting mitochondria and thus preventing the release of cytochrome *c* into the cytosol (Chipuk et al. 2010; Youle & Strasser 2008). In addition, CD40 activation enhances secretion of chemoattractants, such as CCL22 and CCL17, as well as cytokines such as IL-6, IL-8, IL-10 and TNF-alpha, which promote tumour cell survival by enhancing the interaction of CLL cells with the microenvironment.

CLL cells are clonally heterogeneous, and not all respond to CD40L stimulation. The lack of *in vitro* CLL cell response to CD40L has been shown to correlate with shorter time to progression (Romano et al. 2000). It remains unknown what provides the subset of CD40-unresponsive CLL cells their strong proliferative and survival capability. The vital question remains also with regards to the strength of CD40 receptor stimulation encountered by CLL cells *in vivo*, particularly as clear heterogeneity in CLL responses can only be observed at relatively low doses of CD40L. CD40 activation is often studied together with additional stimuli, e.g.

cytokines, and in these cases it is unknown whether the observed response can be attributed solely to CD40 ligation (Schattner 2000).

1.6.2 Regulation of members of the Bcl-2 family of proteins by CD40 stimulation

The effect of CD40 activation on the expression of Bcl-2 family members appears to depend on the experimental design. Some authors have reported a decreased expression of Bcl-2 (Willimott et al. 2007) and an increased expression of pro-apoptotic BH3-only proteins (e.g. Bid) following CD40 activation (Kater et al. 2004). Although CD40 stimulation shifts the overall profile of the Bcl-2 family to support cell survival, single proteins may exhibit a counter-intuitive pattern of expression. The different results could also be accounted for by the different culture systems used, namely the administration of soluble recombinant CD40L (Schattner 2000) versus co-culture with fibroblasts expressing human CD40L with or without interleukins (Willimott et al. 2007)(e.g. IL-4, IL-2, IL-10 or IL-21).

1.7 Management of CLL

1.7.1 Current chemo-immunotherapy

1.7.1.1 Background

Chemotherapy is usually not advocated in early and stable disease, and is administered only to patients with more advanced or progressive CLL (Robak, Jamroziak & Robak 2009). For a long period, treatment with the alkylating agent chlorambucil was the standard regimen due to its low toxicity, low cost and convenience of oral delivery. Purine analogues such as fludarabine, pentostatin or cladribine are another widely used class of cytostatic drugs, with fludarabine established as a backbone improvement in the outcome of patient therapy for CLL. A series of clinical trials have also demonstrated the benefits of using fludarabine in combinational therapy for the treatment of CLL. The LRF CLL4 trial found that fludarabine was more effective when used in combination with the alkylating agent cyclophosphamide. Complete and overall response rates were better with fludarabine plus cyclophosphamide than with fludarabine alone (complete response rate 38% vs 15%, respectively; overall response rate 94% vs 80%, respectively) (Catovsky et al. 2007). Progression-free survival at 5 years was also found to be significantly better with fludarabine plus cyclophosphamide (36%) than with fludarabine or chlorambucil alone (10%). Furthermore, a more recent CLL8 trial demonstrated the benefits of using the CD20 monoclonal antibody rituximab in combination with fludarabine and cyclophosphamide (Hallek & Pflug 2011). The use of this chemoimmunotherapy was effective in prolonging progression-free survival and

overall survival of symptomatic CLL patients and helped establish a new standard of treatment for physically fit patients (Catovsky et al. 2007).

1.7.1.2 Fludarabine

Fludarabine (9-beta-D-arabinofuranosyl-2-fluoroadenine 5'-phosphate) is a water-soluble synthetic fluorinated purine nucleoside analogue of the antiviral agent vidarabine (ara-A) (Figure 1.6)(Ricci et al. 2009). On infusion into the bloodstream fludarabine phosphate undergoes rapid dephosphorylation to the respective nucleoside F-ara-A.

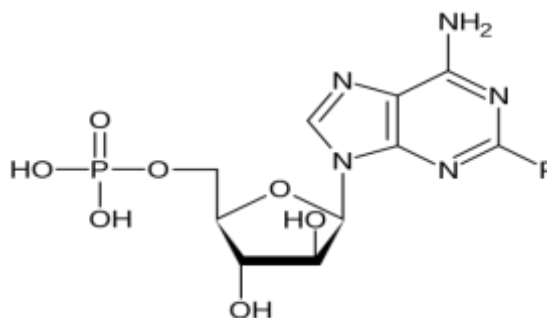


Figure 1.6. Structure of fludarabine.

Structure of fludarabine phosphate (prodrug), a fluorinated purine nucleoside analogue (Huang, Chubb & Plunkett 1990).

In this form it is actively taken up by the cells and then phosphorylated intracellularly by deoxycytidine kinase (dCK), forming the active triphosphate derivative 2-fluoro-ara-ATP. In this form, fludarabine is an active metabolite that is incorporated into DNA or RNA, blocking their further synthesis (Huang, Chubb & Plunkett 1990; Huang & Plunkett 1991). In addition, fludarabine exerts inhibitory activity against ribonucleotide reductase, leading to depletion of the deoxynucleotide

pool required for synthesis and repair of DNA, and potentially favouring incorporation of fludarabine into newly synthesised DNA strands (Tseng et al. 1982). Moreover, fludarabine is resistant to excision from DNA, potentially because it can inhibit DNA ligase and DNA primase (Catapano, Perrino & Fernandes 1993; Yang et al. 1992). Fludarabine has also been shown to inhibit several DNA repair processes, including excision and repair of inter-strand crosslinks and nucleotide excision repair (NER) (Li et al. 1997; Yang et al. 1995). The resulting DNA damage leads to histone H1.2 release and activation of p53-mediated transcription (Gine et al. 2008). A separate investigation has led to the finding that nuclear DNA damage can also be signalled to mitochondria which activates apoptosis (Zhivotovsky & Kroemer 2004). Both transcription-dependent and independent activation of apoptosis by p53 in CLL cells has been described (Steele et al. 2008). Despite the fact that fludarabine can induce apoptosis in p53-dependent and p53-independent manner (Pettitt, Sherrington & Cawley 2000), mutation of p53 signifies poor prognosis in CLL patients treated with fludarabine as TP53 mutations and 17p deletions are commonly found in fludarabine-refractory patients (Zenz et al. 2010; Zenz et al. 2008).

1.7.2 Development of chemoresistance

The strategies of drug resistance employed by CLL cells include intrinsic drug resistance, microenvironment-supported drug resistance, and acquired drug resistance (Figure 1.7). Following the course of chemotherapy, drug sensitive tumour cells may be completely eliminated, while populations of innately resistant peripheral blood CLL cells survive the treatment. This phenomenon was observed

following treatment with conventional as well as more recent chemotherapeutics, such as fludarabine, bendamustin, or rituximab (Gross et al. 2010). Such inherent drug resistance may be caused by abnormal apoptotic signalling, e.g. overexpression of anti-apoptotic proteins. In the absence of aberrant signalling, the survival support may be provided to CLL cells within the lymph nodes and bone marrow (Figure 1.7). Considering that CLL cells within the proliferation niches of the bone marrow and lymph nodes receive pro-survival cues from the microenvironment, it is not surprising that when cultured *ex vivo*, without the environmental support, they are more sensitive to fludarabine-induced apoptosis. (Podhorecka et al. 2010). Finally, the emergence of resistant CLL cells in the process of drug-driven evolution (acquired drug resistance) has been observed, for example following chemotherapy with fludarabine (Gross et al. 2010).

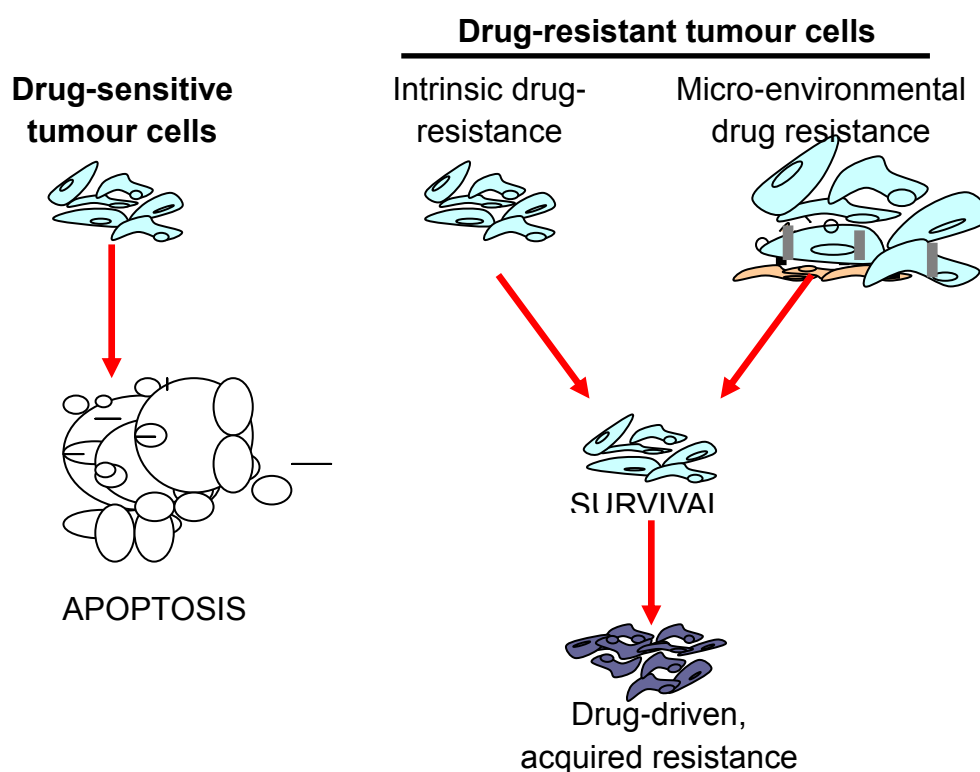


Figure 1.7. General mechanism of drug resistance in CLL

Following chemotherapy (red arrows) drug sensitive tumour cells undergo apoptosis, whereas cells with abnormal apoptotic signalling, e.g. Over-expression of Bcl-2, can survive the treatment (innate drug resistance). Additionally, the tumour microenvironment provides a rich source of survival and proliferative signals that can enhance the survival of drug sensitive CLL cells, allowing them to acquire new mutations and develop drug resistance (environmental drug resistance). Finally, new drug resistant clones can emerge following therapy (drug-driven resistance) (Burger et al. 2009).

1.8 New drugs – from bench to bedside

1.8.1 Targeting apoptosis

Current understanding of the molecular underpinnings that facilitate the development of CLL has a profound effect on the design of new targeted therapeutic approaches and management of CLL. For example, drugs that specifically target anti-apoptotic proteins and circumvent drug resistance are being tested as potential treatment strategies. Specific small molecule inhibitors of Bcl-2 and Bcl-XL, such as ABT-737 and ABT-263, have been extensively evaluated in preclinical models of CLL (Kang & Reynolds 2009; Lin 2010; Vogler et al. 2010). Latest clinical study using a highly potent, orally bioavailable and Bcl-2-selective inhibitor, ABT-199, demonstrated promising anti-leukemic activity (Souers et al. 2013).

1.8.2 Blocking BCR signalling

Of particular interest, Suljagic and co-workers (2010) reported that fostamatinib disodium (R788), the inhibitor of Syk, selectively decreases proliferation and survival of the malignant B-cell clones in Emu-TCL1 mice. Ibrutinib is a covalent Bruton's tyrosine kinase inhibitor that has demonstrated a 70% response rate, according to a recent clinical study (Cheng et al. 2014). In treatment-naïve patients, ibrutinib showed 90% progression-free survival (PFS) over two years and 75% over two years in relapsed patients. Idelalisib is an inhibitor of the delta isoform of PI3K and achieved a high response rate in relapsed refractory CLL (Davids & Brown 2013), ibrutinib and idelalisib have marketing authorisation, i.e. they are now used routinely. Other BTK inhibitors are also in development (Akinleye et al. 2013).

1.9 Questions that remain unanswered

Although multiple mechanisms are involved in and, in many cases, required for CD40-mediated protection of CLL cells from drug-induced cell death, it is likely that Bcl-2 family proteins play an important part in determining the sensitivity/resistance of CLL cells to therapeutic drugs. This is largely because of their essential role in regulating the mitochondrial death pathway. However, many questions still remain unanswered. In particular, the effect of CD40 stimulation on the expression of Bcl-2 family proteins in the presence or absence of cytotoxic drugs is unknown. Likewise, the effect of cytotoxic agents on the changes of expression of Bcl-2 family proteins induced by CD40-stimulation is unclear. Finally, CD40 ligation has sometimes opposite effects on the expression of Bcl-2 family proteins (e.g. Bcl-2), depending on the experimental conditions used in the studies, and thus better understanding on the interaction between the Bcl-2 family members specifically in CLL, and in response to various levels/scenarios of CD40 activation is needed (Buggins & Pepper 2010).

1.10 The hypothesis of my PhD thesis

Microenvironmental interactions, such as between CLL cell CD40 – and T cell CD40L interactions, facilitate cytotoxic drug resistance in CLL by changing the balance between anti-apoptotic and pro-apoptotic Bcl-2 family proteins within the affected malignant cells.

1.11 Aims of the PhD study

There is extreme variability in the therapeutic response to CLL treatment, and most patients eventually develop drug resistance. The microenvironment provides important survival and proliferative signals to CLL cells, and such signals can be provided by CD40L (CD154)-expressing T-cells engaging their corresponding receptor (CD40) on CLL cells. These signals are thought to contribute to drug resistance by inducing the expression of anti-apoptotic Bcl-2 family proteins, culminating in resistance to apoptosis. The fate of CLL cells following drug exposure is likely governed by specific interactions between pro-apoptotic Bcl-2 family proteins induced by drug treatment and anti-apoptotic Bcl-2 proteins induced by CD40L.

Therefore, my research aims to address the following research questions;

- 1) How do drugs that induce p53-dependent (fludarabine) or p53-independent (dexamethasone) apoptosis affect the levels of Bcl-2 family proteins in CLL cells?
- 2) How does stimulation of CLL cells with CD40L affect the levels of Bcl-2 family proteins?
- 3) How do pro- and anti-apoptotic Bcl-2 family proteins interact in CLL cells, and how are these interactions influenced by drug treatment and CD40L?
- 4) How do interactions between pro- and anti-apoptotic Bcl-2 family proteins influence the fate of CLL cells following drug treatment?
- 5) Can this knowledge be used to overcome drug resistance?

Chapter 2

Methodology and Materials

2.1 Materials

2.1.1 Flow cytometry

2.1.1.1 Principle

Flow cytometry measures several parameters of suspended cells according to their light scattering properties, which include fluorescence emission (Macey 1988), Where is FACS sorting cells based on flow cytometry data. The cells move in a stream of liquid through a light beam and past a sensing area which consists of several detectors (Figures 2.1) cells or particles between 0.2 – 50 μm ,also flow cytometry can be used in measure relative size,internal complexity and fluorescently-labelled cells (e.g. with antibodies conjugated to fluorescent dyes), the emission of fluorescence is also quantified using wavelength-specific detectors and filters (Ormerod,2006). Several measurements are recorded simultaneously for each cell, which combined with specific gating procedures allows identification of a homogenous cell population within a heterogeneous one (Macey 1988).

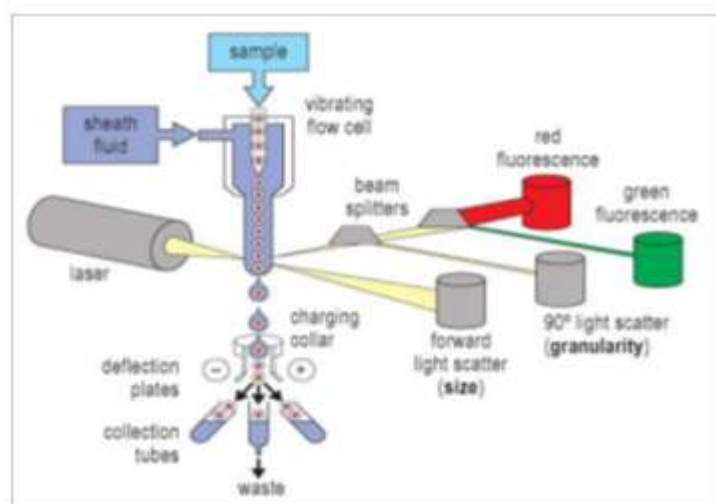


Figure 2.1 The principle of flow cytometry.

Hardware set-up in a typical flow cytometer includes fluidics, laser(s), a number of detectors and fluorescence detectors. Cells can be analysed and quantified, or, as depicted here, sorted using deflection plates and collection tubes.(Macey 1988).

2.1.1.2 Applications

Flow cytometry is routinely used to assess cell viability and to determine cellular immunophenotyping using fluorescence-labelled monoclonal antibodies against surface marker to identify subsets of cells of interest (Craig & Foon 2008). Cell viability assays are based on morphological changes (FSC/SSC), identification of plasma membrane permeability and phosphatidylserine exposure in dying cells with fluorescent probes such as propidium iodide (PI) and detection of caspase activation, loss of mitochondrial membrane potential, or fractional DNA content (sub-G1),

again using fluorescent probes (Wlodkowic, Skommer & Darzynkiewicz 2009). Immunophenotyping is performed with the use of fluorescently-labelled antibodies that recognise specific cell surface proteins (lineage markers), for example CD3 for T lymphocytes and CD19 for B lymphocytes or CD4 and CD8 for helper and cytotoxic T lymphocytes, respectively.

The BD fluorescence-activated cell sorting (FACS) Calibur™ was used for all experiments in my thesis.

2.1.1.3 Strength and limitation

The main advantages of FACS (fluorescence-activated cell sorting) are the speed of analysis, sensitivity and specificity, the ability to obtain multiple measurements for each cell within the cell population (Stacchini et al. 2012) and availability of a wide range of functional probes for staining of live, unfixed cells. The limitations include cost of equipment, a need to have single cells in suspension (problematic for adherent cells and cells that clump easily), inability to obtain microscopic images to correlate with light-scattering data (this has been overcome with the introduction of Image Stream technology) (Zuba-Surma & Ratajczak 2011) or to analyse tissue sections.

2.1.2 Cell Culture

2.1.2.1 Isolation of CLL cells

Peripheral blood (PB) samples were taken from patients previously diagnosed with CLL after giving informed consent and with the approval from the Liverpool Research Ethics Committee (LREC). Heparinised blood samples were slowly

layered on top of Lymphoprep TM (Axis-Shield, Kimbolton, U.K) and centrifuged at 800 g for 30 min at room temperature. The mononuclear layer of cells were carefully collected prior to washing and resuspending in ice-cold RPMI-1640 containing 10% v/v FCS, after which an equal volume of ice-cold RPMI-1640 plus 10% v/v FCS and 20% v/v DMSO was gradually added on ice. One mL aliquots of the final cell suspension were then placed in cryotubes housed in polystyrene holders and stored at -80°C for one week to freeze gradually before being transferred into liquid nitrogen for long-term storage in the University of Liverpool Leukaemia Biobank. For the majority of experiments, cells were not purified further and only CLL cases with white blood cell counts greater than $50 \times 10^9/L$ were employed to ensure there was minimal contamination from non-malignant cells.

2.1.2.2 Culture of CLL cells under standard conditions

Cryopreserved CLL cells were taken from the University of Liverpool Leukaemia Biobank and thawed rapidly at 37°C before transferring to a pre-chilled 25 mL universal. Ice-cold RPMI-1640 (supplemented with 10% v/v FCS, 2mM L-glutamine, 100units/mL penicillin and 100µg/mL streptomycin) was slowly added drop wise on ice to the 1mL CLL cell suspension until a final volume of 10 mL was reached. Cell suspensions were centrifuged at 500g for 5 min at 4°C and washed once with cold RPMI culture media to remove any remaining DMSO. Cells were resuspended in RPMI culture medium and recovered for 1 h in a 25 mL universal tube prior to culturing at 5% CO₂ at 37°C for 1 h to allow them to warm and 'recover' before any further treatment. Cellular viability was checked by trypan blue exclusion using a cellometer auto T4 slide (Peqlab Ltd, Hampshire, and U.K), and

only CLL samples with viability greater than 80% were used in subsequent experiments. Unless stated, CLL cells were seeded at 4×10^6 /mL in a 24-well plate for all experiments.

2.1.2.3 Fibroblasts maintained as feed layers to create co-culture conditions mimicking lymph node microenvironment

Parental or stably transfected NIH 3T3 mouse fibroblasts expressing human CD40L provided by Professor Gerry Cohen in University of Liverpool were cultured in high glucose DMEM (supplemented with 10% v/v heat inactivated FCS, 2mM L-glutamine, 100units/mL penicillin and 100 μ g/mL streptomycin) in a 37°C incubator with 5% CO², as described (Vogler et al. 2009). Both cell lines were maintained at a density of 2×10^5 /mL - 2×10^6 /mL. CD40L-expression was checked monthly by labelling the cells with a mouse anti-human CD154-FITC or mouse IgG1 κ control antibody prior to analysing FL1 fluorescence using a Becton Dickinson (BD) FACSCalibur machine. The data was analysed using BD CellQuest Pro software (BD Biosciences, Oxford, UK) (Figure 2.2).

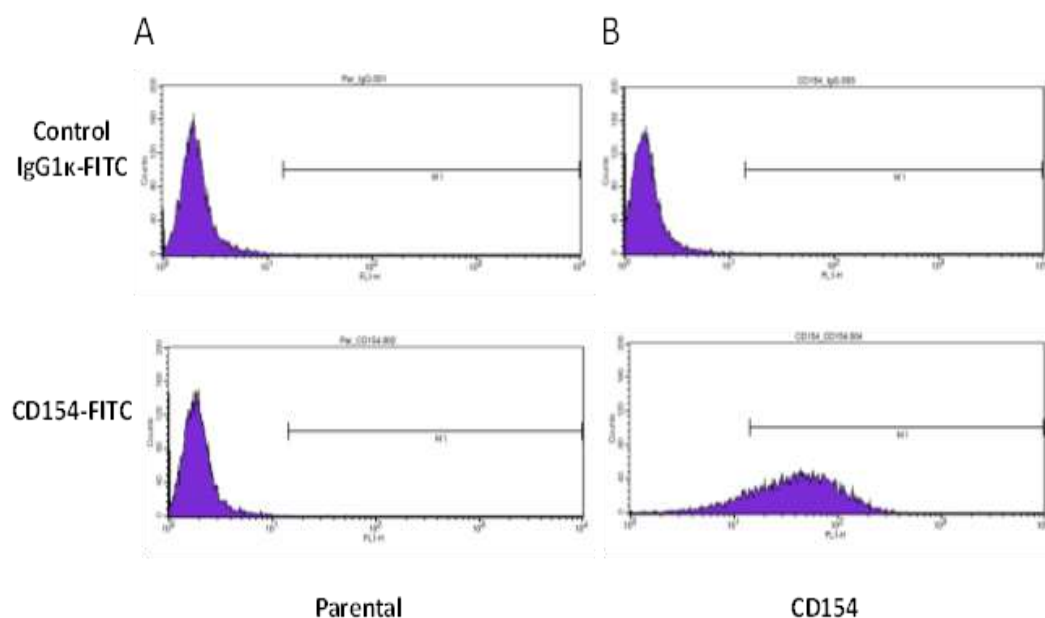


Figure 2.2 CD40L expression on mouse NIH 3T3 mouse fibroblasts.

To confirm that CD40L-expressing fibroblasts still expressed surface CD40L, every month parental (A) or CD40L-expressing (B) mouse fibroblast cells were labelled with either a FITC-conjugated control IgG1 κ antibody or CD40L-FITC antibody and surface expression was detected using FL1 fluorescence by FACS.

For co-culture studies, both parental and CD40L-expressing fibroblasts were γ -irradiated (to stop them dividing) and plated on the multi-well plate to form monolayers before co-culture of CLL cells.

2.1.2.4 CLL cells cultured under co-culture conditions

3×10^6 CLL cells were seeded on the respective monolayers prepared earlier and co-cultured with parental or CD40L (CD154)-expressing fibroblasts in RPMI-1640

(supplemented with 10% v/v FCS, 2mM L-glutamine, and 100units/mL penicillin and 100µg/mL streptomycin) in a 37°C incubator with 5% CO².

2.1.2.5 Measuring cell death by flow cytometry

6×10⁵ CLL cells were collected at the end of incubation and centrifuged at 550g for 5 min at 4°C and washed once with RPMI-1640 medium and again with phosphate buffered saline (PBS) comprised of 137mM NaCl, 2.7mM KCl, 4.3mM Na₂HPO₄, 1.47mM KH₂PO₄ (pH 7.4). CLL cells were suspended in 500µL of PBS and incubated with 1µg/mL propidium iodide (PI) in the dark for 10 min. Cellular viability was assessed by measuring the number of PI bright (dead) cells in a total of 10,000 gated events on the FACS. PI is a DNA-binding fluorochrome and commonly used for identifying dead cells whereas live cells with an intact plasma membrane exclude PI. Dead cells take up the fluorochrome as they have lost their membrane integrity and therefore fluoresce bright red (Figure 2.3).

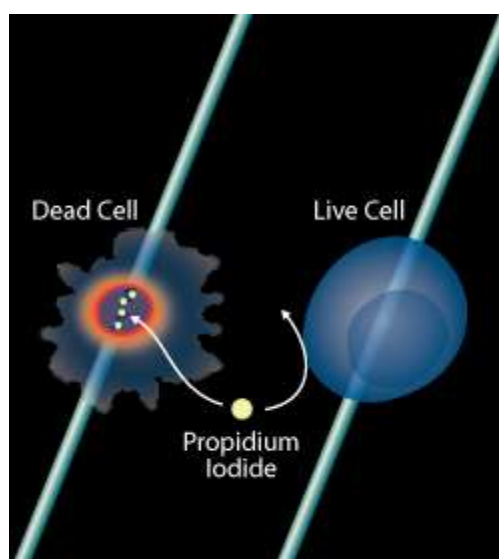


Figure 2.3. Propidium iodide staining mechanism.

Propidium iodide (PI) is standard viability dye in flow cytometry, PI can be used in combination with other fluorochromes excited at 488 nm such as fluorescein isothiocyanate (FITC) and phycoerythrin (PE). Figure and legend from (<https://www.rndsystems.com/resources/protocols/flow-cytometry-protocol-analysis-cell-viability-using-propidium-iodide>).

2.1.3 Expression of protein detected by Western blotting

2.1.3.1 Principle

In principle, the term Western blotting refers specifically to the immunological detection of proteins that have been electrophoretically-separated by molecular size and transferred to a membrane (Fulton & Twine 2013). Proteins are identified through the use of specific antibodies. In general, primary antibodies targeting the protein of interest are un-conjugated and require a second-layer conjugated detection antibody that recognises the source and type of primary antibody (e.g. mouse IgG).

The nature of the conjugation for the detection antibody is usually horse-radish peroxidase (HRP) where chemoluminescence reagents can be used to visualise the protein band of interest using either X-Ray film or imaging devices

2.1.3.2 Applications

Western blotting is used for detection of specific proteins, endogenous or ectopically expressed, in a mixture of proteins, in a qualitative and quantitative manner. For quantitative analysis, the expression level relative to a control sample, or a purified protein sample of known concentration, is used. Western blotting can be used to compare the level of expression of proteins of interest, monitor protein phosphorylation and changes in molecular weight of particular protein in cells with or without drug treatment.

2.1.3.3 Strength and limitation

The advantage of Western blotting over alternative techniques to detect protein expression is that it cannot only detect proteins of interest in a specific manner, but also measure the level of expression of proteins quantitatively. In addition, simultaneous determination of proteins of different sizes serves to increase the effectiveness of the technique. The limitations include the need to isolate protein samples from their native cellular environment (as compared to protein detection using fluorescent microscopy), inability to analyse protein expression on a single cell level, and thus inability to assess the heterogeneity of protein expression within a cell.

2.1.4 Protein-protein interaction detected by

Co-Immunoprecipitation

2.1.4.1 Principle

Co-Immunoprecipitation is a purification procedure to determine if two different molecules (usually proteins) interact with each other. An antibody specific to the protein of interest is added to a cell lysis. Then the antibody-protein complex is pelleted usually using protein-G sepharose which binds most antibodies. Generally, an antibody specific to that concerned antigen is used for this purpose. The antibody usually attached to an agarose resin which act as a supporting bead. The antigen may arise from various sources such as tissues or cells, translated proteins and metabolically labelled cells. After the pre-immobilization of the specific polyclonal or monoclonal antibody in the insoluble solid support, incubation is done with the cell lysate that contains the required antigen. Sometimes mild agitation is required for binding of the target antigen with the specific antibody. The immune complex thus formed is immobilized and collected followed by elution from the insoluble support for subsequent analysis.

In some special cases, immunoprecipitation can be performed using free antibody not bound to the insoluble support. Formation of immune complex using such technique is beneficial when the concentration of the target antigen is low, the antibody binding with the antigen has low binding affinity or the kinetics of the binding process are slow. Isolation of a single protein from a cell lysate is generally done by immunoprecipitation. The purpose of this technique is to provide a guide to

the analysis of such protein-protein interactions. This technique can also be used to evaluate how the specific protein molecule interacts with other molecules such as DNA (Masters 2004).

2.1.4.2 Application

Immunoprecipitation was used to detect protein-protein interactions in CLL cells cultured under standard or co-culture conditions. The two main uses were to identify whether two known Bcl-2 family proteins interact with one another, for example to investigate interactions between anti-apoptotic and pro-apoptotic proteins.

2.1.4.3 Strengths and limitations

The advantages of immunoprecipitation are native state of the proteins analysed, the relative ease of the protocol, and its cost-effectiveness (compared to e.g. bioluminescence resonance energy transfer). The limitations include the mixing of compartments during cell lysis, the need to stabilise low affinity or transient protein-protein interactions, and the risk of high background from non-specific interactions, particularly when gentle buffers are used. The presence of co-eluted antibody chains may interfere with sample analysis, particularly if several interacting proteins are co-precipitated with the target. Finally, immunoprecipitation does not allow analysis of native protein interactions in living cells in time course studies, or subcellular localisation of protein-protein interaction.

2.1.5 Knockdown of protein expression by siRNA using

nucleofection

2.1.5.1 Principle

Small interfering RNA or silencing RNA is generally termed as siRNA. These are double-stranded molecules of RNA comprising 20 to 25 base pairs. siRNAs have various biological functions but most importantly they act on the RNA interference (RNAi) pathway (Castanotto & Rossi 2009), siRNAs have a well-defined structure: a short (usually 20 to 24-bp) double-stranded RNA (dsRNA) with phosphorylated 5' ends and hydroxylated 3' ends with two overhanging nucleotides. Synthetic siRNAs can be introduced into cells by transfection. Since in principle any gene can be knocked down by a synthetic siRNA with a complementary sequence, siRNAs are an important tool for validating gene function and drug targeting in the post-genomic era (Bernstein et al. 2001).

The specific siRNA strand was guided by RNA Induced Silencing Complex (RISC) to bind to the targeted mRNA through complementary sequences (Elbashir 2001). At the 5' end of the siRNA strand there exist a 2 to 7 nucleotide stretch which conferred the mRNA specificity for siRNA which is also known as seed region. After annealing with the seed region of siRNA, the 10 nucleotides at the 5' end of the mRNA is subjected to degradation by Argonaute protein complex that contains a RNase H domain (Tomari & Zamore 2005). As a consequence the translation of the target mRNA is inhibited.

2.1.5.2 Applications

siRNA is used as a method to down-regulate the expression of target genes in order to establish the link between gene identity and function. It can be used to test function of newly discovered genes. In addition, siRNA is used in pathway analysis whereby disrupting one gene could affect the expression and/or activities of other genes in the same pathway. Finally, siRNA can be used to study gene redundancy (Katome et al. 2003).

2.1.5.3 Strengths and limitations

The power of siRNA lies in its cost-effectiveness and ease of introduction into cells, the generation of double or triple knock downs, and an ability to perform loss-of-function studies in organisms or cells where classical genetic analysis is laborious and time consuming. The main disadvantage of this approach is that often there is an incomplete loss of gene function whereby residual protein synthesis could be sufficient to maintain function of the target gene. The effects are often transient, particularly in rapidly dividing cells. Finally, off-target silencing of genes that contain partial sequence identity can occur, necessitating the comparison of several siRNAs corresponding to different parts of the target RNA.

2.2 Materials

2.2.1 Reagents and cytotoxic agents

Cryotubes were from Nuncbrand (Fisher Scientific, Loughborough, U.K). Dimethyl sulfoxide (DMSO), trypsin, Dulbeccos Modified Eagles Medium (DMEM), trypan blue, phosphatase and protease inhibitor cocktails, propidium iodide (PI), 2-

mercaptoethanol and bovine serum albumin (BSA) were from Sigma-Aldrich (Exeter, U.K). Roswell Park Memorial Institute (RPMI) 1640 and fetal calf serum (FCS) were from Biosera (Ringmer, U.K). ECL Western blotting kit was from Millipore (Watford, U.K). NIH 3T3 mouse parental and CD40L-expressing fibroblasts were a kind gift from Professor Gerry Cohen (University of Liverpool, U.K). SDS-PAGE gel stacking buffer, resolving buffer, transfer buffer, and tetramethylethylenediamine (TEMED) were from Geneflow (Staffordshire, U.K). SDS-PAGE protein bench ladder was from Invitrogen, U.K. Human B cell Nucleofector kit was from (Lonza, U.K). Puma/BBC3 specific siRNA and non-specific siRNA were from Dharmacon/Thermo Scientific (via Abgene Ltd, Kent, UK). FITC Mouse anti-human CD40L and FITC Mouse IgG isotype control were from B.D. Pharmingen (Oxford, UK).

2.2.2 Antibodies

Below is a list of all the antibodies (Table 2.1), used for Western blotting and immunoprecipitation experiments. All secondary antibodies were purchased from Santa Cruz Biotechnology (Heidelberg, Germany)

Table 1.1 List of all antibodies used

Primary antibody	Source	Secondary antibody	Dilution
Mouse anti-Bcl2	Abcam (Cambridge,U.K)	Goat anti-mouse-HRP	1/2000
Rabbit anti-Bcl2 (IP)	New England Biolabs (Herts, U.K)		
Rabbit anti-Puma	New England Biolabs (Herts, U.K)	Goat anti-rabbit-HRP	1/2000
Rabbit anti-Bcl-xl	New England Biolabs (Herts, U.K)	Goat anti-rabbit-HRP	1/2000
Rabbit anti-Mcl-1	Santa Cruz (Heidelberg, Germany)	Goat anti-rabbit-HRP	1/2000
Rabbit anti-Bak	Santa Cruz (Heidelberg, Germany)	Goat anti-rabbit-HRP	1/2000
Mouse anti-Bax	New England Biolabs (Herts, U.K)	Goat anti-mouse-HRP	1/2000
Rabbit anti-Puma (IP)	Novusbio (Cambridge U.K)		
Mouse β -actin	Sigma (Exeter U.K)	Goat anti-mouse-HRP	1/10000
Rabbit anti-Bim	New England Biolabs (Herts, U.K)	Goat anti-rabbit-HRP	1/2000

2.2.3 Patients samples

All samples were obtained with informed consent and with the approval of the Liverpool Research Ethics Committee. The diagnosis of CLL was based on standard morphological, and immunophenotypic criteria, as described elsewhere (Melarangi et al 2012). The clinical details of the CLL patients are shown in (Table 2.2).

Table 1.2 Clinical features of the primary CLL samples used in the study.

No	Case number	Gender	Age at Dig	Patient status	WBC ($10^9/l$)	Deletion in 17p13	Deletion in 11q22	P53 mutation	P53 function	IGVH mutation
1	2866	M	80	A	229	N	N	N	Normal	0 U M
2	2911	M	80	D	223	N	N	N	Normal	0 U M
3	2968	M	81	D	87	N	N	N	Normal	3.47 M
4	2899	M	75	A	99	N	N	N	Normal	8.15 M
5	2929	M	59	A	93	N	N	N	Normal	1.01 UM
6	2746	M	81	D	306	N	N	N	Normal	2.43 M

IGVH status refers to somatic mutation in IGVH gene of CLL cells as compared with gene sequence of nearest germ-line using 2% as a cut-off.

M=mutated

UM=un-mutated

2.3 Statistical analysis

Statistical analysis was performed to compare the effects of fludarabine and dexamethasone on expression of Bcl-2 family proteins in CLL cells cultured under different conditions in Chapter 3, and the effects of Puma expression on fludarabine-induced CLL-cell death in Chapter 4. In these two chapters, all data analysed were of paired measurement and presented as mean \pm standard deviation (SD). To compare the effects between any two different conditions in each experiment, the

paired t-test was performed using the Graph Pad Prism 5 software (GraphPad Software, San Diego, CA, USA). The α level of < 0.05 (P-value) in 2-sided tests was set to accept any difference with the statistical significance. With no statistical analysis applied, results were descriptively presented in Chapter 5.

Finally in my thesis I have used paired t-test to compare the effects of multiple culture conditions/treatments on survival and protein expression of CLL cells in Chapters 3 and 4. Compared to anova test, it gave a bigger statistical power to find any difference between any two groups in this paired design for a limited sample size ($n=6$). In contrast, anova test is more suitable for comparison of means among multiple groups, but may not find the difference(s) existing between particular pair(s) of the multiple groups. Therefore, the multiple paired t-test might be more sensitive, but less stringent in identifying these statistical differences.

Chapter 3

Characterisation of the effects of fludarabine and dexamethasone on CLL cells cultured under standard and co- culture conditions

3.1 Background and aims

The fact that CLL cells are long-lived *in vivo* but rapidly undergo apoptosis *in vitro* (Collins et al. 1989; Coscia et al. 2011) clearly demonstrates that they retain the ability to execute apoptosis, and that their prolonged survival *in vivo* requires micro-environmental factors at sites of tissue involvement including bone marrow and lymph nodes. As described earlier, interaction of CLL cells with T cells in the bone marrow and lymph nodes of patients plays a critical role in the expansion and extended survival of the malignant cells. One of the most important interactions mediating these effects is stimulation of CD40 on CLL cells by CD40L on T cells. CD40 stimulation has been shown to protect CLL cells from spontaneous and drug-induced apoptosis *in vitro* (Kitada et al. 1999; Vogler et al. 2009; Zhuang et al. 2014), implicating its involvement in mediating drug resistance *in vivo*. Although multiple mechanisms are involved in CD40-mediated protection of CLL cells from drug-induced cell death, it is likely that Bcl-2 family proteins play a key role. With respect to drugs that induce p53-dependent (fludarabine) or p53-independent (dexamethasone) apoptosis in CLL, these treatments may affect the level of expression of Bcl-2 family proteins. Therefore, to understand how drug resistance might be overcome by CD40 stimulation, it is important to understand how this process affects drug-induced alteration in the expression of Bcl-2 family proteins.

The aim of this study was to investigate the effect of fludarabine or dexamethasone on CLL cells exposed to CD40 stimulation. In particular, I sought to establish the effects of these drugs on cell viability and expression of different anti-apoptotic and pro-apoptotic Bcl-2 family proteins in CLL cells that were cultured under standard

conditions or co-cultured with transfected fibroblasts that express human CD40L. The same sets of experiments were performed in parallel using parental fibroblasts that did not express CD40L as a control.

3.2 Methodology

3.2.1 Cell culture

Cryopreserved cells used in this chapter were thawed according to the method described in section 2.1.2.2, and were cultured under standard conditions as listed in section 2.1.2.2, or were co-cultured with either parental or CD40L-expressing fibroblasts as described in sections 2.1.2.3 and 2.1.2.4.

3.2.2 Flow cytometry protocol to detect apoptosis

CLL cells were stained with propidium iodide (PI) according to the procedure described in section 2.1.2.5. Flow cytometry was carried out following this staining using a BD FACS Calibur, and percentage cell apoptosis was calculated.

3.2.3 Sample preparation, SDS-PAGE and Western blotting

Western blotting was used to detect Bcl-2 proteins. Whole cell lysates were prepared by solubilising cell pellets from CLL cell samples with lysis buffer (10 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, 100 mM NaCl, 1% Triton X-100 and a protease inhibitor cocktail from Sigma-Aldrich (Exeter, U.K)). Cells lysates along with the BenchMaker™ Pre-stained protein ladder (life technologies catalogr no: 10748-010) were then sonicated using a tip sonicator set to maximum, and then centrifuged at 13,000rcf for 15min. A protein concentration of the supernatant was determined by Bio DC™ protein assay (Biorad) according to the manufacturer's instructions

(Lowry et al ,1951,Peterson.1979). A volume containing 10 μ g of protein was then mixed with an equal volume of double strength Laemmli sample buffer (10 mM Tris-HCl (pH 6.7), 1% sodium dodecyl sulphate (SDS), 2-mercaptoethanol and bromophenol blue), and then heated at 95°C for 5 min to fully denature the proteins.

Samples were then applied to a SDS-PAGE gel made up of a 5% acrylamide stacking gel and 15% acrylamide resolving gel to achieve good resolution of lower molecular weight proteins. Separated proteins were transferred to Immobilon-PVDF membranes (Millipore, Fisher Scientific UK Ltd, Loughborough, U.K.) by electrotransfer for 1h at 400mA in chilled transfer buffer (Figure 3.1). Membranes was washed in TBS-T (150mM NaCl, 25 mM Tris pH 7.5, 0.1% Tween 20) for 15 minutes to remove any residual transfer buffer, and then blocked in blocking buffer (TBS-T supplemented with 5% dry milk) for 45min. Membranes were briefly washed in TBS-T for 15 mins, and probed with primary antibodies targeting Bcl2 proteins diluted at 1:1000 in blocking buffer overnight at 4°C with gentle agitation. The membranes were washed 3 times for 15mins with TBS-T to remove unbound primary antibody, and then exposed to horse radish peroxidase (HRP)-conjugated secondary antibody diluted at 1:10,000 in blocking buffer for 1h at room temperature. Unbound secondary antibody was washed an additional 3 times for 15 mins with TBS-T. Specific proteins were detected using enhanced chemiluminescence (ECL) reagents (Millipore), and reactive bands were visualised using a Fujifilm LAS-1000 chemiluminescence imaging system (Fujifilm, Tokyo, Japan). Quantitative analysis of signals corresponding to the protein band of interest was carried out by densitometry for quantification of data, the images were further

analysed on the same instrument using 2D densitometry AIDA image analyser software package (Fujifilm)

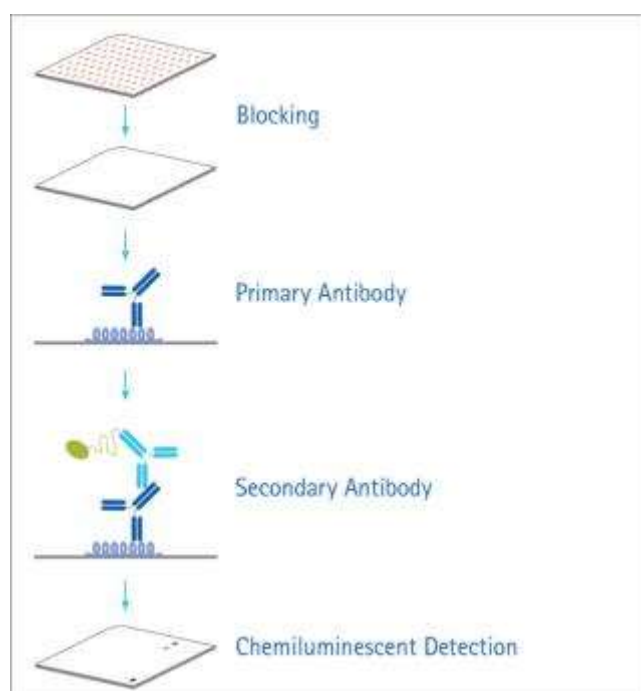


Figure 3.1. Western blotting

Western blotting Protein separation by SDS-PAGE

- A. Protein transfer to a membrane
- B. Blocking and probing with primary antibody specific to target protein
- C. Probing with a labeled secondary antibody specific to primary antibody
- D. ECL applied and signal is then detected using CCD camera.

Figure and legend taken from (GE Healthcare Bio-Sciences AB Björkgatan3075184 Uppsala Sweden www.gelifesciences.com, First published Feb 2015).

3.3 Effect of fludarabine or dexamethasone on the viability of

CLL cells cultured under standard and co-culture conditions

CLL cells cultured for 48h under standard conditions where no fibroblasts were present, or either with parental or CD40L-expressing fibroblasts exhibited different levels of spontaneous cell death as detected by PI (propidium iodide) uptake (Figure 3.2 A). As expected (Vogler et al. 2009), survival of CLL cells was greatly enhanced under co-culture conditions, and this was statistically significant when CLL cells were co-cultured with CD40L-expressing fibroblasts (Figure 3.2 B).

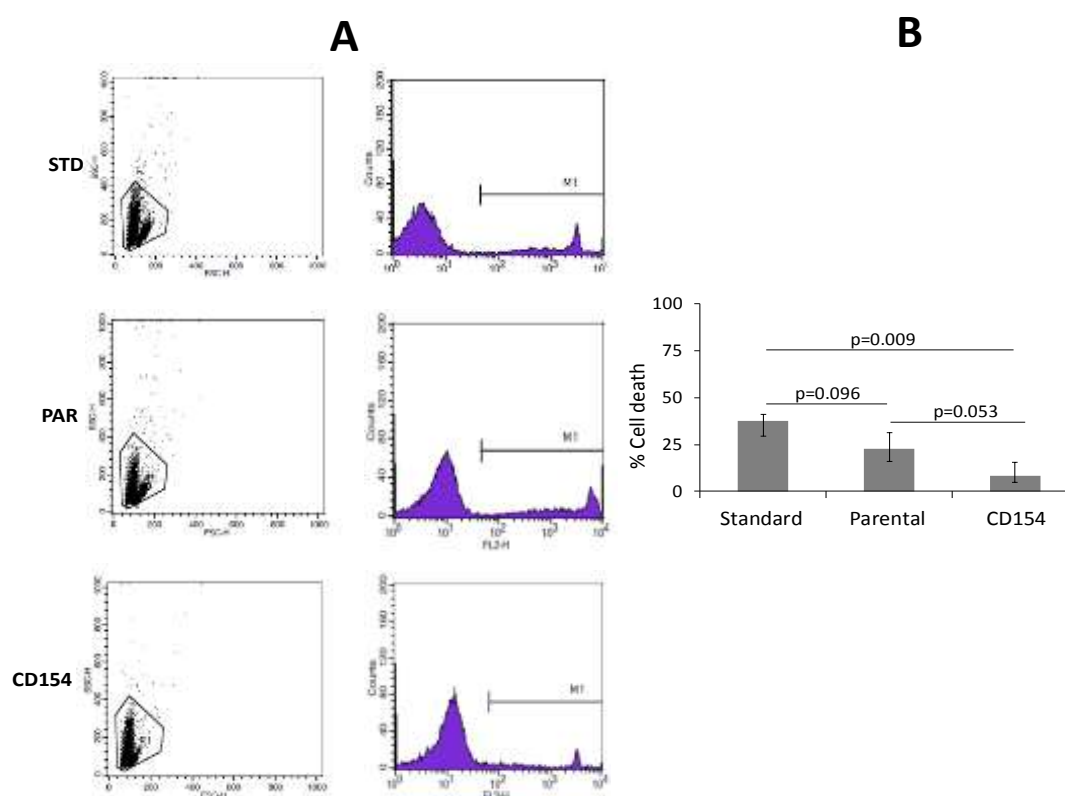


Figure 3.2. Effect of co-culture on spontaneous cell death measured by PI staining and flow cytometry.

A: Representative FSC/SSC dot-plots showing the gating used to select cells for fluorescence analysis and fluorescence histograms showing how PI positivity was defined. The population of cells in M1 region of the histogram are considered PI positive and therefore non-viable. **B:** Column charts showing difference in the % of spontaneous cell death (mean \pm SD) of CLL cells from 6 different patients under the three culture conditions.

Investigation of the effects of dexamethasone (100 nM) or fludarabine (10 μ M) were tested next. CLL cells cultured with these drugs for 48h under standard culture conditions showed a statistically significant increase in cell death compared to cells that were not treated with either compound (Figure 3.3). Both drugs were effective at killing CLL cells, with fludarabine having slightly greater cytotoxic effects compared to dexamethasone.

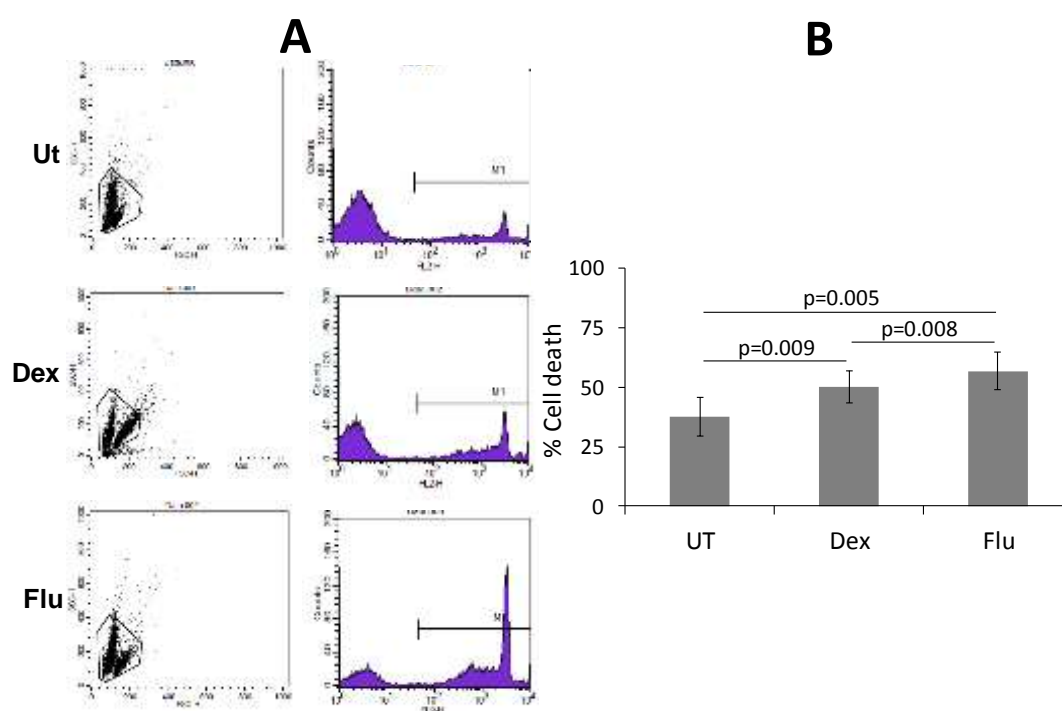


Figure 3.3 Effect of dexamethasone and fludarabine on cell death under standard conditions.

A: Representative FSC/SSC dot-plots and PI histograms. **B:** Column charts showing difference in the % of cell death (mean \pm SD) of CLL cells from 6 different patients in the presence or absence of the drugs. Statistical significance was determined using a Student's t-test for paired data.

Next, the effect of co-culture conditions on the cytotoxicity of dexamethasone and fludarabine was examined. CLL cells were cultured under standard conditions or co-cultured with either parental or CD154-expressing fibroblasts in the presence of either dexamethasone (100 nM) or fludarabine (10 μ M). The CLL cell death induced after treatment with dexamethasone under standard conditions was antagonized by co-culture with either parental or CD154-expressing fibroblasts (Figure 3.4). Co-culture of CLL cells with parental fibroblasts significantly reduced dexamethasone-

induced cell death ($p=0.045$) compared to standard conditions, suggesting that co-culture with fibroblasts alone has protected CLL cells from killing by this drug. This protective effect of co-culture was more pronounced when CLL cells were co-cultured with CD154-expressing fibroblasts ($p=0.002$), indicating that CD40 stimulation exerts specific and additional pro-survival effects to counteract the cytotoxicity of dexamethasone.

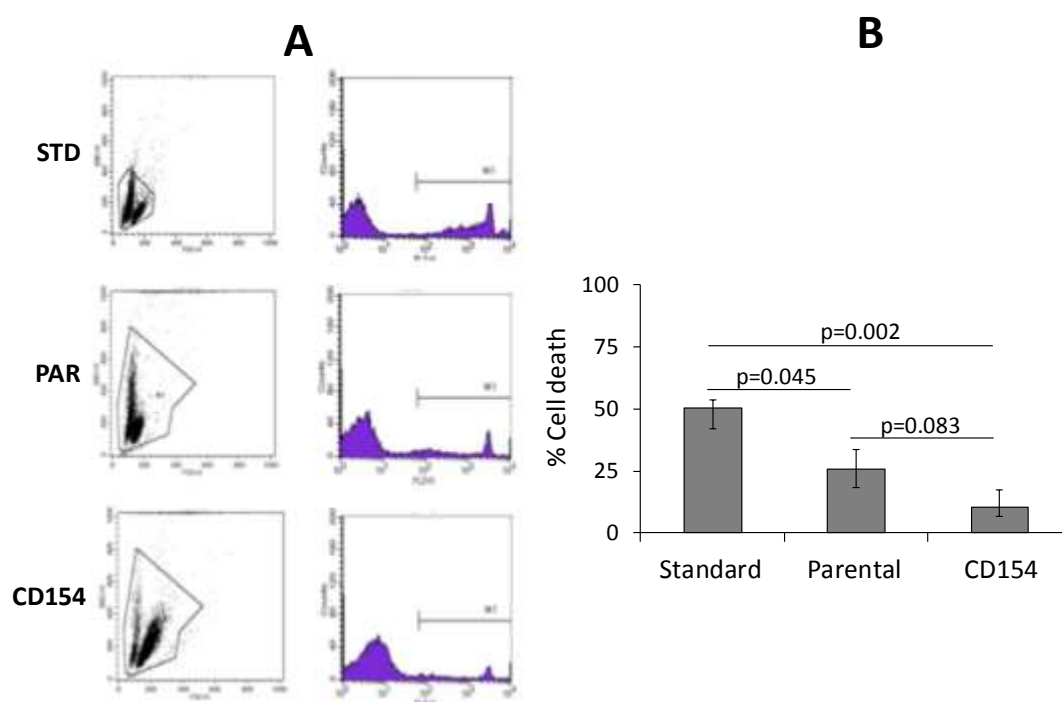


Figure 3.4. Effect of co-culture on dexamethasone-induced cell death.

A: Representative dot-plots and histograms. **B:** Column charts showing difference in the % of dexamethasone-induced killing (mean \pm SD) of CLL cells from 6 different patients under the three culture conditions. The % of drug-induced cell death was calculated as: $100 \times [(\% \text{ cell death of drug-treated cells} - \% \text{ cell death of untreated cells}) / (100 - \% \text{ cell death of untreated cells})]$.

Similarly, CLL cells were protected from the cytotoxic effects of fludarabine when they were co-cultured with either parental or CD154-expressing fibroblasts (Figure 3.5). Statistically significant reduction of drug-induced cell death was observed when fludarabine-treated CLL cells were co-cultured with parental cells ($p=0.023$) or with CD154-expressing fibroblasts ($p=0.007$). Again, the protective effect of co-culture with CD154-expressing fibroblasts was greatest, but did not show statistical significance compared to the co-culture with parental fibroblasts ($p=0.248$).

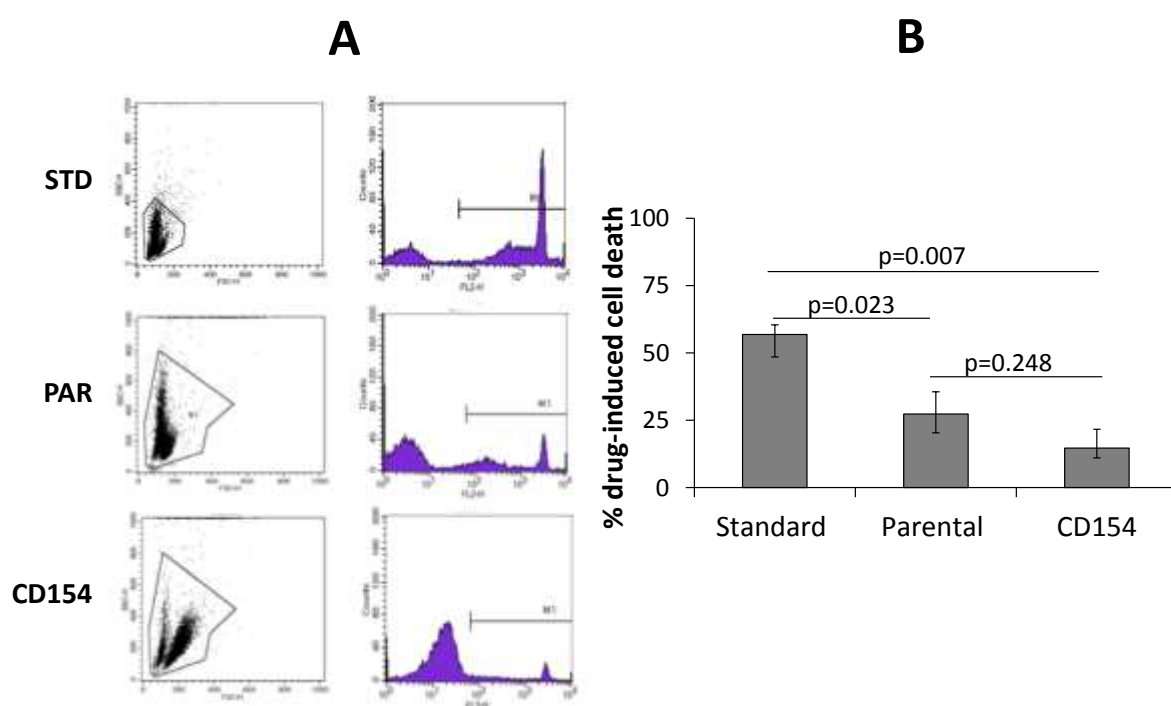


Figure 3.5. Effect of co-culture on fludarabine-induced cell death.

A: Representative dot-plots and histograms. **B:** Column charts showing difference in the % of fludarabine-induced killing (mean \pm SD) of CLL cells from 6 different patients under the three culture conditions. The % of drug-induced cell death was calculated as described in figure legend of Figure 3.3.

In summary, treatment of CLL cells with either dexamethasone or fludarabine induces cell death under standard culture conditions. In keeping with established findings (Melarangi et al. 2012), co-culture with either parental or CD154-expressing fibroblasts rescues CLL cells from the cytotoxic effects of these drugs (Figure 3.6, Table 3.1). The cytotoxic drugs used in this thesis operate through mechanisms that are p53-dependant fludarabine (Lin et al. 2013) and p53-independent dexamethasone (Melarangi et al. 2012). That CD154-expressing fibroblasts consistently offered CLL cells greater protection both fludarabine- and dexamethasone-induced cell death suggests that this protective effect supersedes these mechanisms.

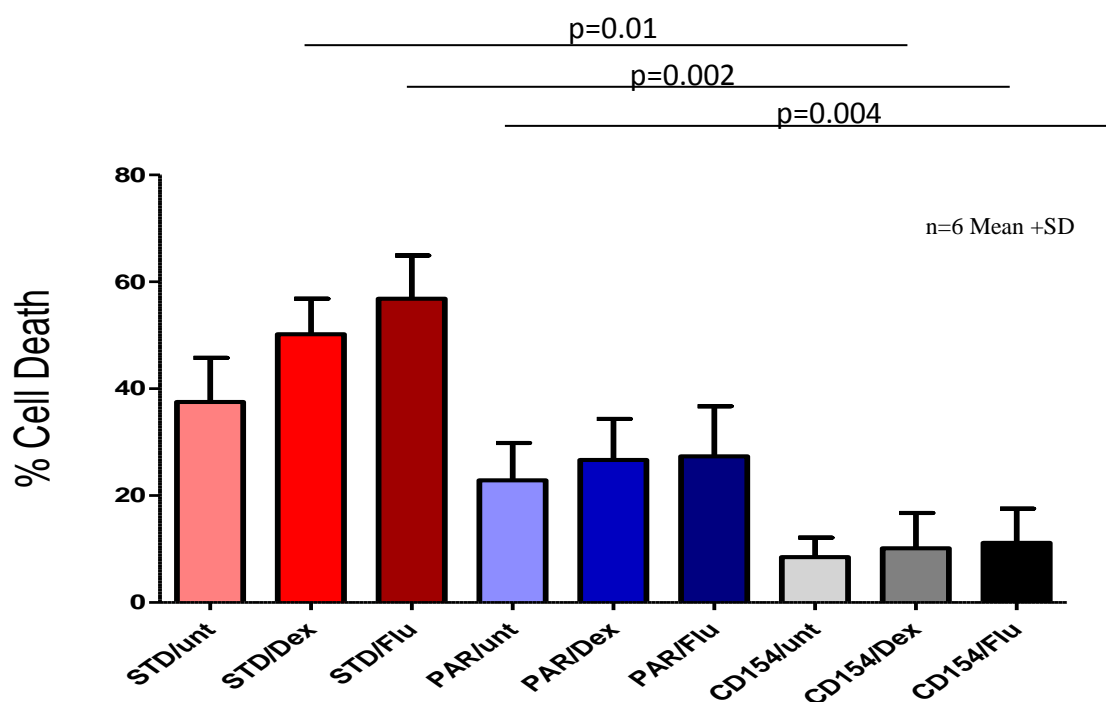


Figure 3.6. Induction of cell death in CLL cells.

% Cell death (mean + SD) under standard and co-culture conditions with parental cells or fibroblasts expressing CD40L for 6 CLL samples, in the presence or absence of dexamethasone or fludarabine.

Table 3.1. P values in the paired t test performed to compare cell death levels following drug treatment under different culture conditions outlined in (Figure 3.6).

	Treatment	STD			PAR			CD154		
Treatment		Ut	Dex	Flu	Ut	Dex	Flu	Ut	Dex	Flu
STD	Ut	X								
	Dex	0.01	X							
	Flu	0.004	0.2	X						
PAR	Ut	0.10	0.01	0.007	X					
	Dex	0.20	0.02	0.02	0.10	X				
	Flu	0.4	0.05	0.02	0.3	0.90	X			
CD154	Ut	0.01	0.001	0.002	0.03	0.02	0.09	X		
	Dex	0.01	0.002	0.003	0.04	0.03	0.11	0.67	X	
	Flu	0.02	0.002	0.004	0.05	0.04	0.15	0.44	0.45	X

3.4 Effect of fludarabine and dexamethasone on the expression of Bcl-2 family proteins in CLL cells cultured under standard and co-culture conditions

A principle mediator of the cytotoxic effects of many drugs is the process of apoptosis and this process is regulated by members of the Bcl-2 family of proteins. Previous studies have demonstrated that CD40 ligation coupled with IL-4 stimulation of CLL cells induces expression of Bcl2-A1, Bcl-XL and Mcl-1, and downregulates expression Bak, together resulting in protection from the pro-apoptotic effects of ABT-737 and staurosporine (Buggins & Pepper 2010; Butterworth et al. 2009). However, these studies only incompletely examined

expression of Bcl-2 family proteins. Moreover, previous work from this Department examined the mechanism of dexamethasone-induced apoptosis and found it to be dependent on Bim expression (Melarangi et al. 2012). Whereas others have found that CLL cell resistance to fludarabine is correlated with expression of Mcl-1 (Kitada et al. 1998). Therefore, to understand the interplay between expression of Bcl-2 family proteins and sensitivity of CLL cells to fludarabine- and dexamethasone-induced cytotoxicity within the CD40L co-culture system, protein expression of Puma, Bim-EL, Bak, Bax, Mcl-1, Bcl-XL and Bcl2 was examined by Western blot.

3.4.1 Comparison of Bcl-2 family protein expression in CLL cells exposed to fludarabine and dexamethasone cultured under standard conditions and co-culture conditions.

To examine Bcl-2 family proteins 6 CLL cell samples were cultured for 24h either under standard culture conditions, or co-cultured with parental or CD40L-expressing fibroblasts. These cultures were also incubated in the presence or absence of dexamethasone (100nM) or fludarabine (10 μ M). Following these cultures, CLL cells were analysed by flow cytometry to determine viability, and Bcl-2 family protein expression was determined by Western blot analysis. (Figures 3.7 - 3.18) show the results this analysis on each individual CLL cell sample. Variation in Bcl2 family protein expression was observed between each case. For example Bim-EL expression was induced by dexamethasone treatment regardless of culture conditions in 4 of the 6 CLL cases tested. In the two CLL cases were dexamethasone failed to induce Bim-EL expression, this was only observed when cells were cultured in the

presence of CD40L-expressing fibroblasts. The results of each individual case with respect to Puma, Bim-EL, Bak, Bax, Mcl-1, Bcl-XL and Bcl-2 are summarised in (Tables 3.2 – 3.7).

Table 3.2. Effect of culture condition and drug treatment on the expression of Bcl-2 family proteins in CLL sample 2746 (figures 3.7 and 3.8).

Bcl-2 family member	Effect of culture condition	Effect of drug treatment
Puma	↑ by CD40L	↑ by flu
Bim-EL	↓ by CD40L	↑ by dex > flu
Bak	No effect	↓ by dex and flu in PAR co-culture
Bax	No effect	No effect
Mcl-1	↑ by CD40L	↓ by dex and flu except in CD40L co-culture
Bcl-XL	↑ by CD40L > PAR	↓ by flu in PAR co-culture
Bcl-2	↓ by par and CD40L	↑ by dex in CD40L co-culture

PAR= parental

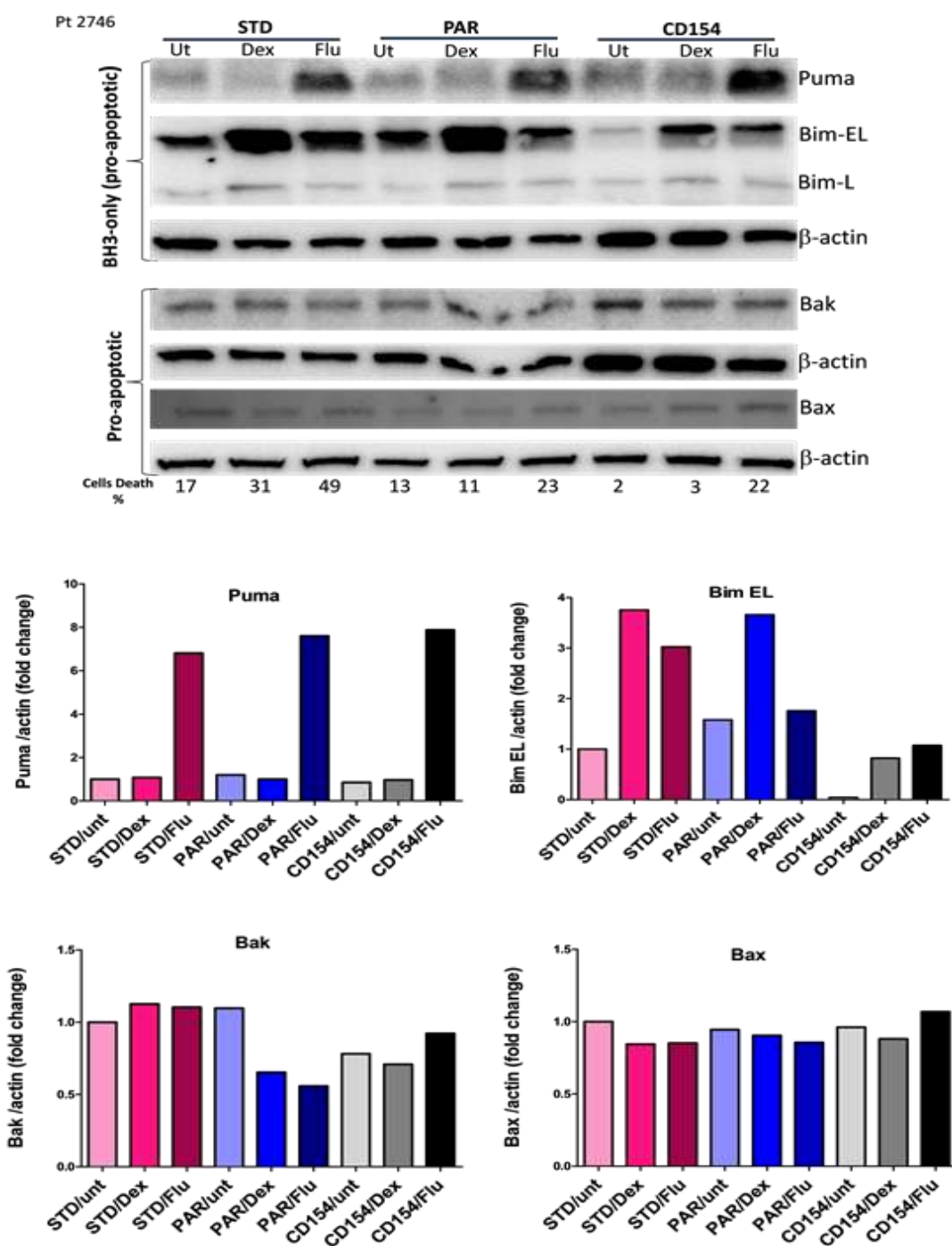


Figure 3.7. Protein expression analysis on pro-apoptotic proteins of the Bcl-2 family in CLL sample 2746.

The change in the level of Bcl-2 family proteins in dexamethasone or fludarabine under standard condition and co-culture with parental cells or fibroblasts expressing CD40L (treated or untreated) was relative to that in untreated cells under standard condition (fold change) also the blot was stripped and re-probed with anti-actin antibody.

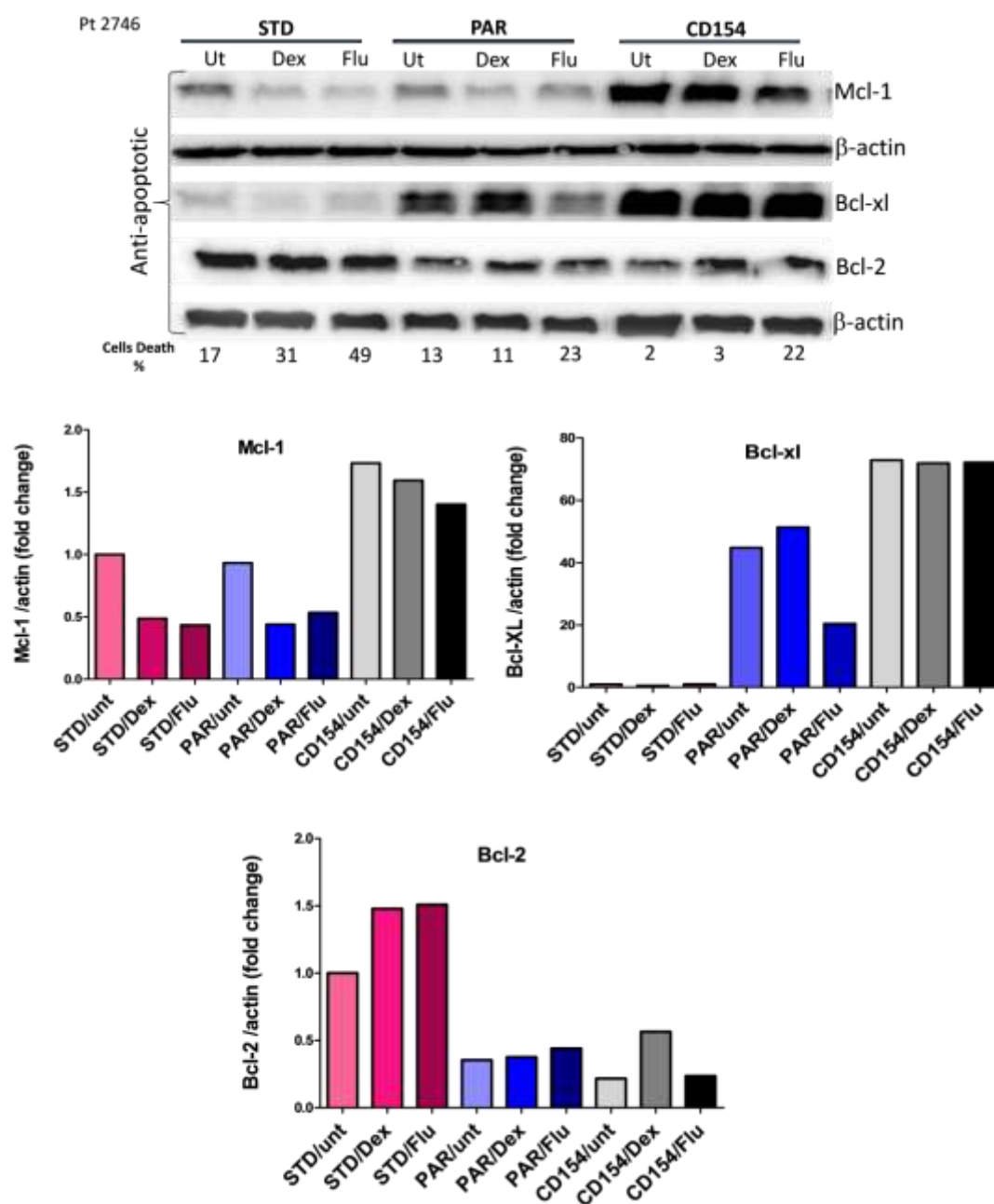


Figure 3.8. Protein expression analysis on anti-apoptotic proteins of the Bcl-2 family in CLL sample 2746.

The change in the level of Bcl-2 family proteins in dexamethasone or fludarabine under standard condition and co-culture with parental cells or fibroblasts expressing CD40L (treated or untreated) was relative to that in untreated cells under standard condition (fold change) also the blot was stripped and re-probed with anti-actin antibody.

Table 3.3. Effect of culture condition and drug treatment on the expression of Bcl-2 family proteins in CLL sample 2866 (figures 3.9 and 3.10).

Bcl-2 family member	Effect of culture condition	Effect of drug treatment
Puma	No effect	↑ by flu
Bim-EL	↓ by CD40L	↑ by dex > flu
Bak	No effect	↓ by dex and flu in PAR co-culture
Bax	No effect	No effect
Mcl-1	↑ by CD40L	↓ by dex and flu except in CD40L co-culture
Bcl-XL	↑ by CD40L > PAR	↓ by flu in PAR co-culture
Bcl-2	↓ by par and CD40L	↓ by dex and flu in CD40L co-culture

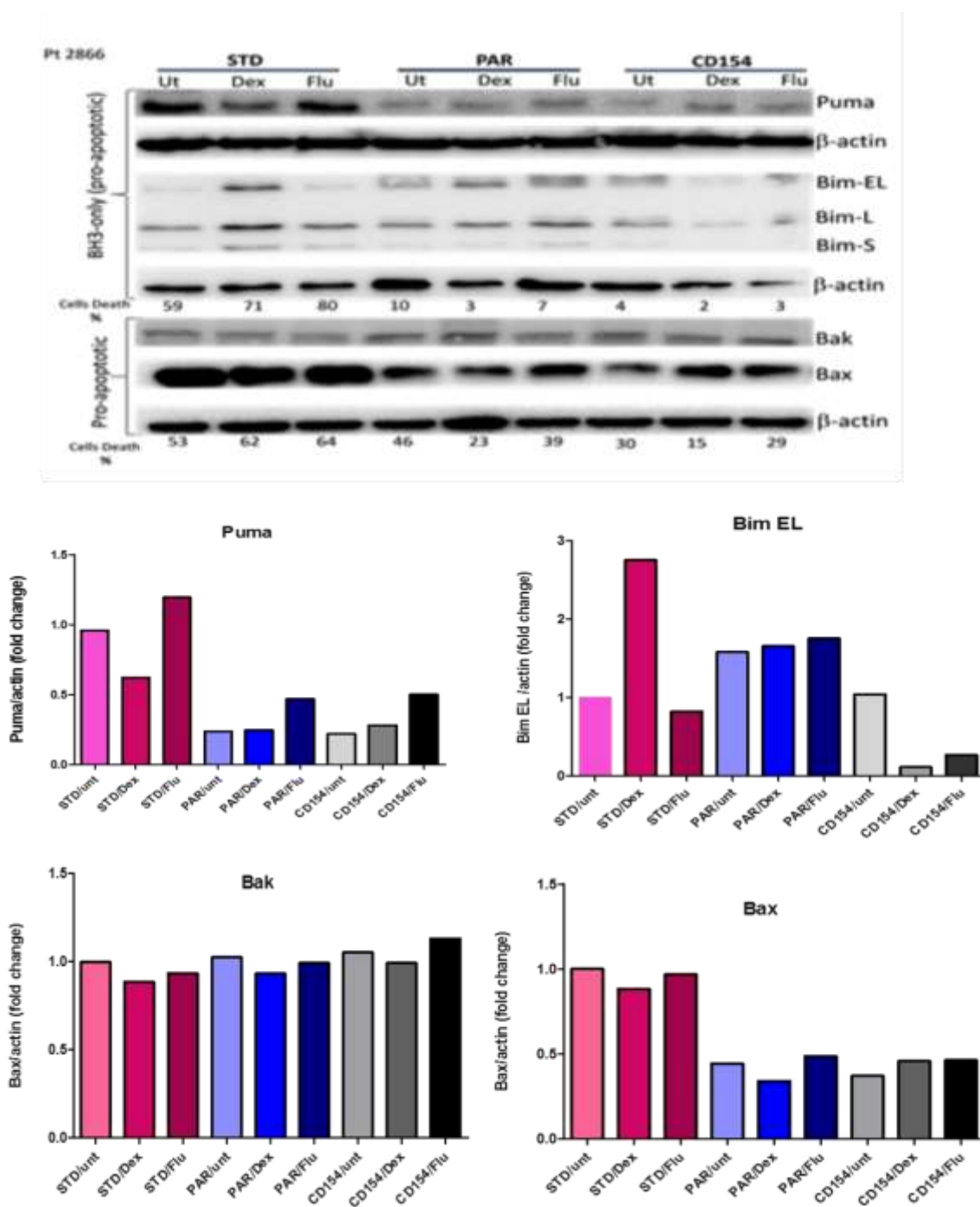


Figure 3.9. Protein expression analysis on pro-apoptotic proteins of the Bcl-2 family in CLL sample 2866.

The change in the level of Bcl-2 family proteins in dexamethasone or fludarabine under standard condition and co-culture with parental cells or fibroblasts expressing CD40L (treated or untreated) was relative to that in untreated cells under standard condition (fold change) also the blot was stripped and re-probed with anti-actin antibody.

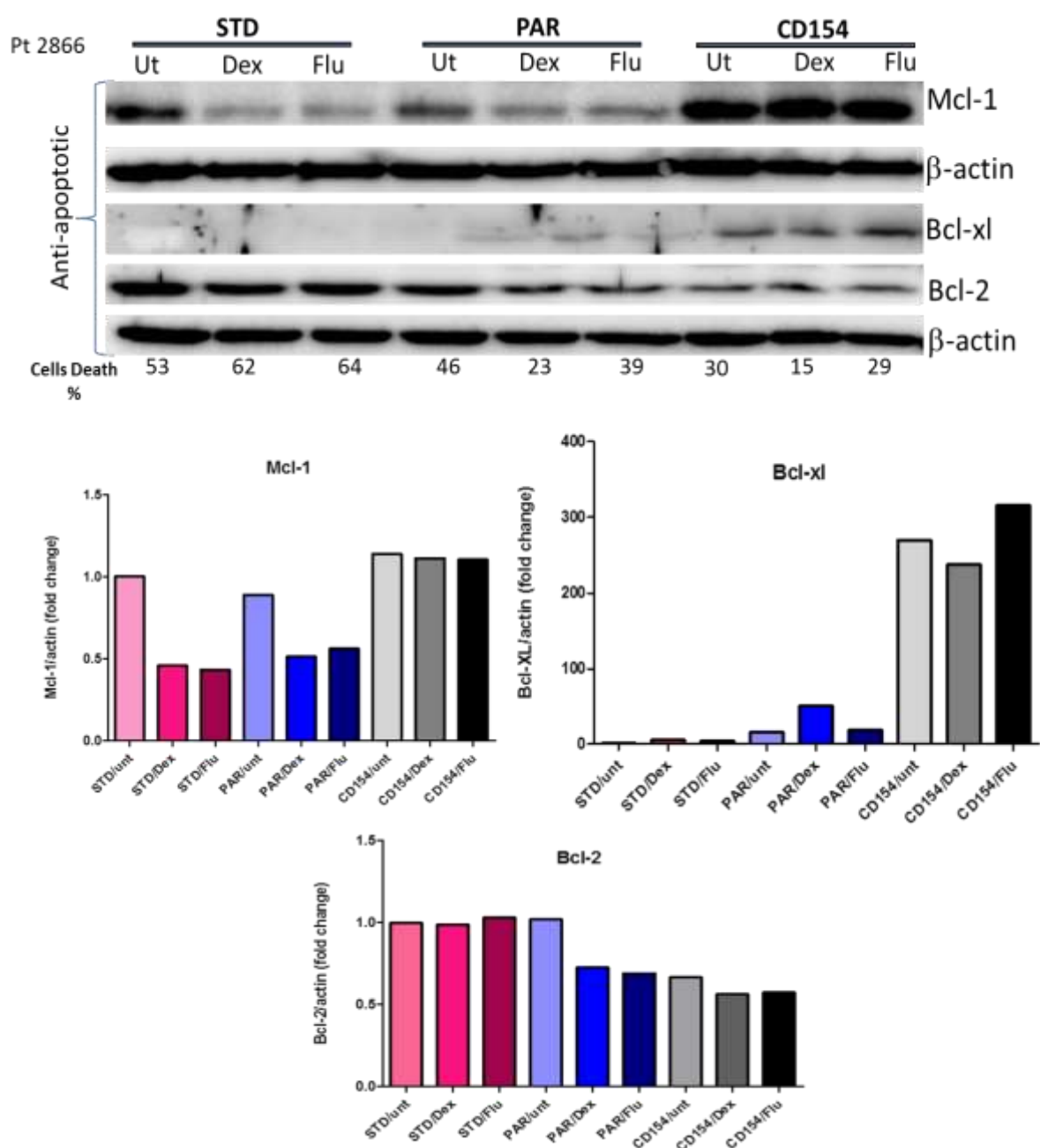


Figure 3.10. Protein expression analysis on anti-apoptotic proteins of the Bcl-2 family in CLL sample 2866.

The change in the level of Bcl-2 family proteins in dexamethasone or fludarabine under standard condition and co-culture with parental cells or fibroblasts expressing CD40L (treated or untreated) was relative to that in untreated cells under standard condition (fold change) the actin from the same patient, also the blot was stripped and re-probed with anti-actin antibody.

Table 3.4. Effect of culture condition and drug treatment on the expression of Bcl-2 family proteins in CLL sample 2899 (figures 3.11 and 3.12).

Bcl-2 family member	Effect of culture condition	Effect of drug treatment
Puma	No effect	↑ by flu
Bim-EL	↓ by CD40L	↑ by dex > flu
Bak	No effect	↓ by dex and flu in PAR co-culture
Bax	No effect	No effect
Mcl-1	↑ by CD40L	↓ by dex and flu except in CD40L co-culture
Bcl-XL	↑ by CD40L > PAR	↓ by flu in PAR co-culture
Bcl-2	↓ by par and CD40L	↓ by dex and flu in CD40L co-culture

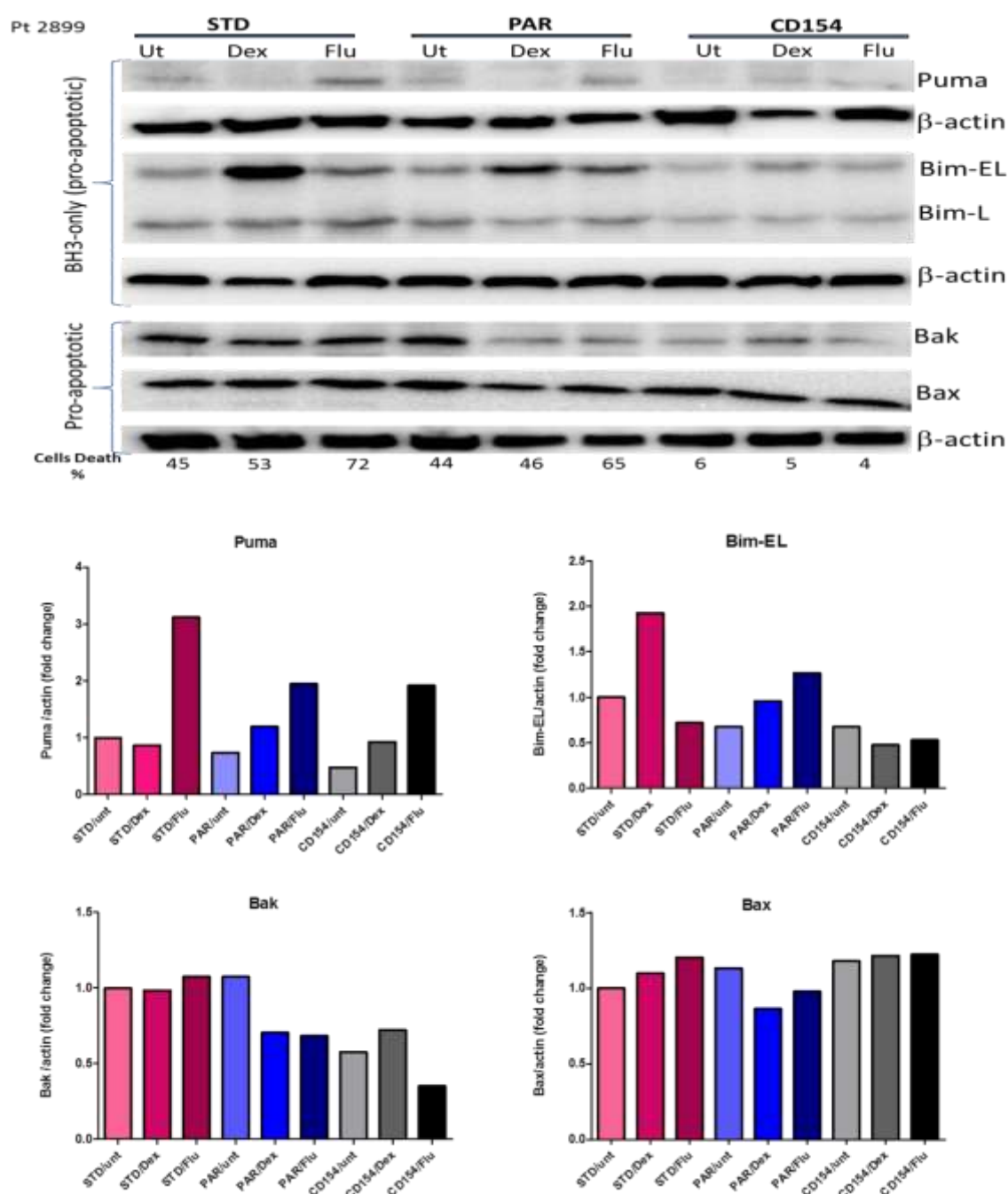


Figure 3.11. Protein expression analysis on pro-apoptotic proteins of the Bcl-2 family in CLL sample 2899.

The change in the level of Bcl-2 family proteins in dexamethasone or fludarabine under standard condition and co-culture with parental cells or fibroblasts expressing CD40L (treated or untreated) was relative to that in untreated cells under standard condition (fold change) also the blot was stripped and re-probed with anti-actin antibody.

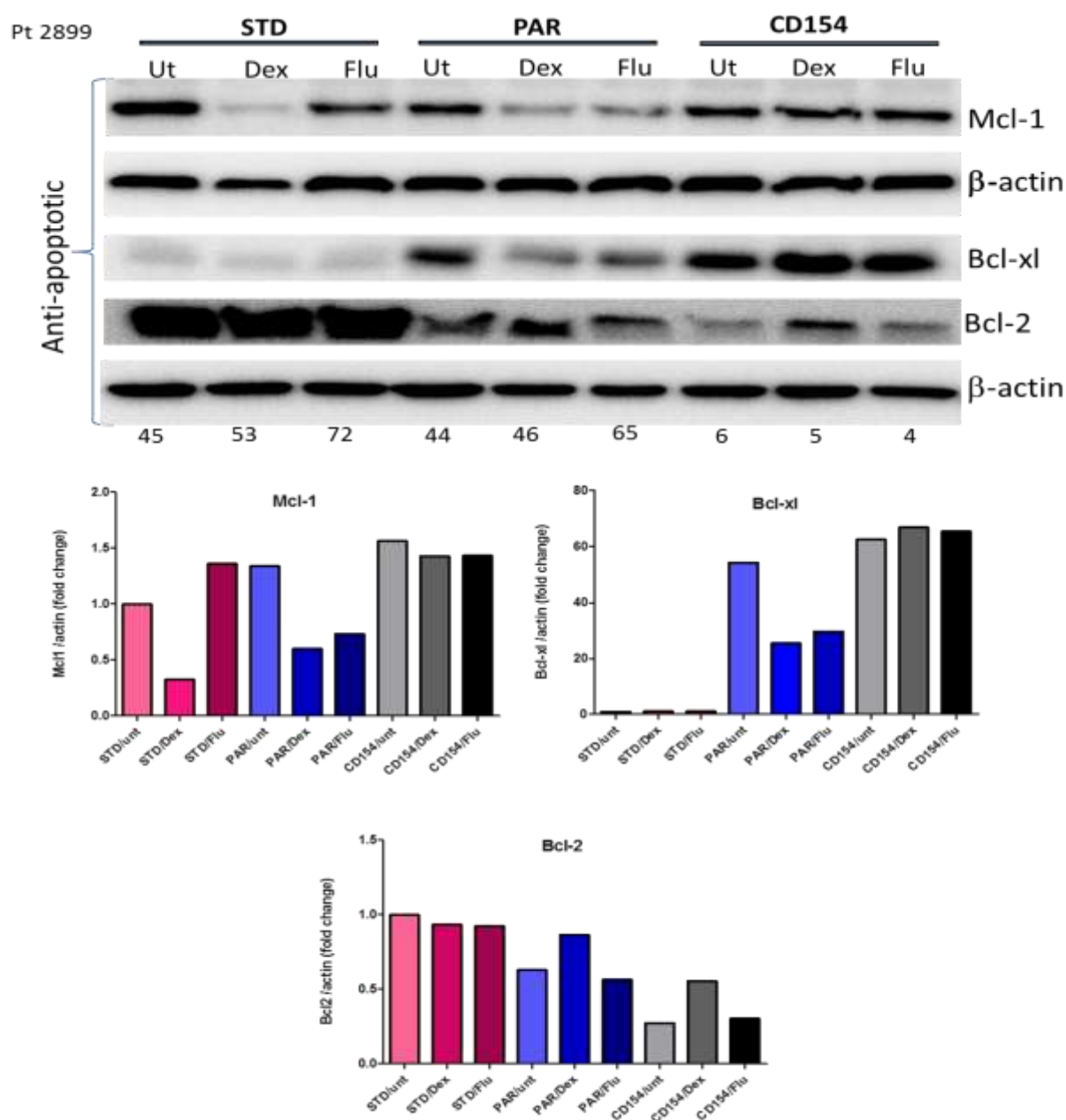


Figure 3.12. Protein expression analysis on anti-apoptotic proteins of the Bcl-2 family in CLL sample 2899.

The change in the level of Bcl-2 family proteins in dexamethasone or fludarabine under standard condition and co-culture with parental cells or fibroblasts expressing CD40L (treated or untreated) was relative to that in untreated cells under standard condition (fold change) also the blot was stripped and re-probed with anti-actin antibody.

Table 3.5. Effect of culture condition and drug treatment on the expression of Bcl-2 family proteins in CLL sample 2911 (figures 3.13 and 3.14).

Bcl-2 family member	Effect of culture condition	Effect of drug treatment
Puma	No effect	↑ by flu
Bim-EL	↓ by CD40L	↑ by dex > flu
Bak	No effect	↓ by dex and flu in PAR co-culture
Bax	No effect	↓ by dex and flu in PAR co-culture
Mcl-1	↑ by CD40L	↓ by dex and flu except in CD154 co-culture
Bcl-XL	↑ by CD40L > PAR	↓ by flu in PAR co-culture
Bcl-2	↓ by par and CD40L	↓ by dex and flu in CD40L co-culture

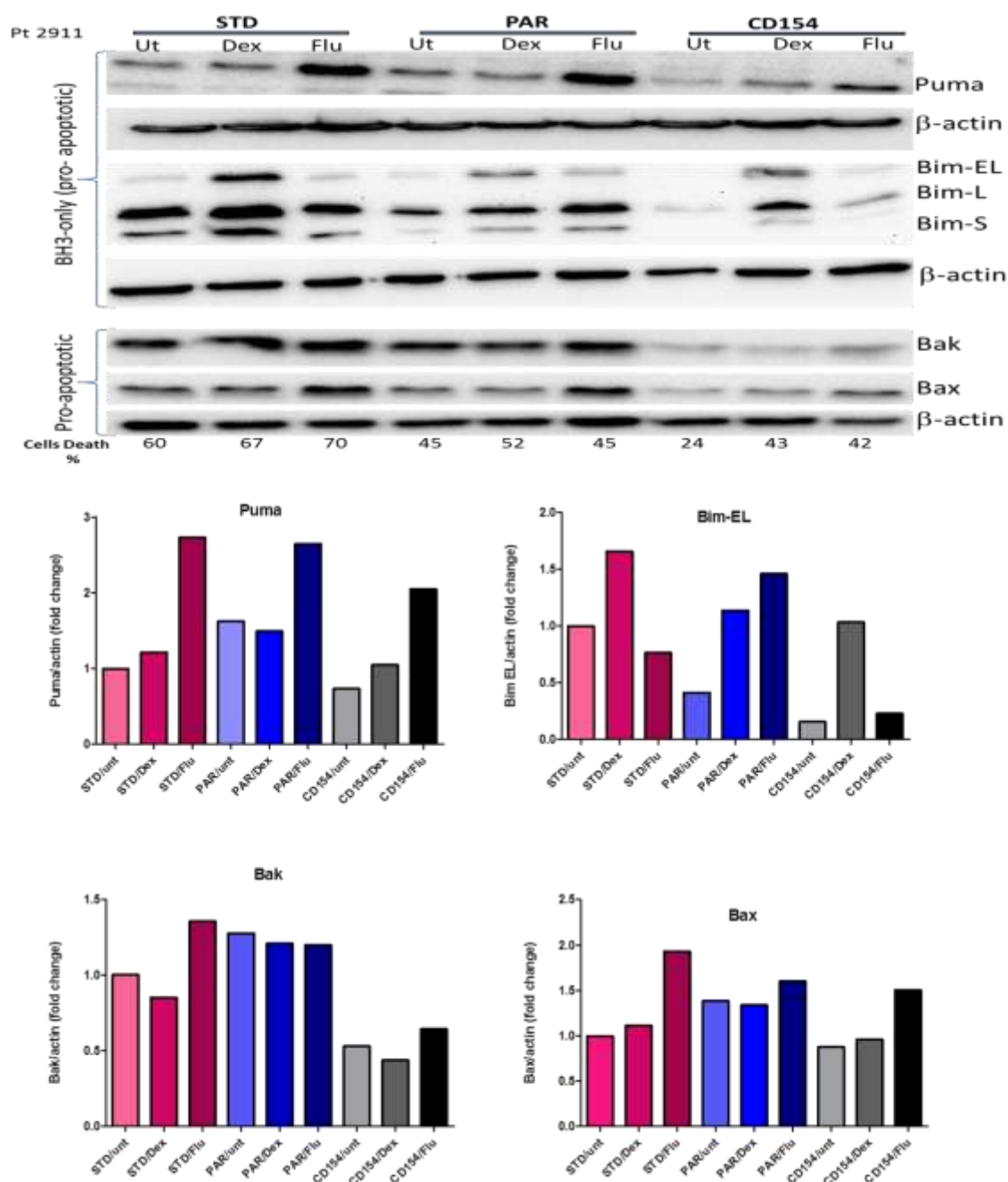


Figure 3.13. Protein expression analysis on pro-apoptotic proteins of the Bcl-2 family in CLL sample 2911.

The change in the level of Bcl-2 family proteins in dexamethasone or fludarabine under standard condition and co-culture with parental cells or fibroblasts expressing CD40L (treated or untreated) was relative to that in untreated cells under standard condition (fold change) also the actin blot was performed on a separate gel loaded with the same cell lysates.

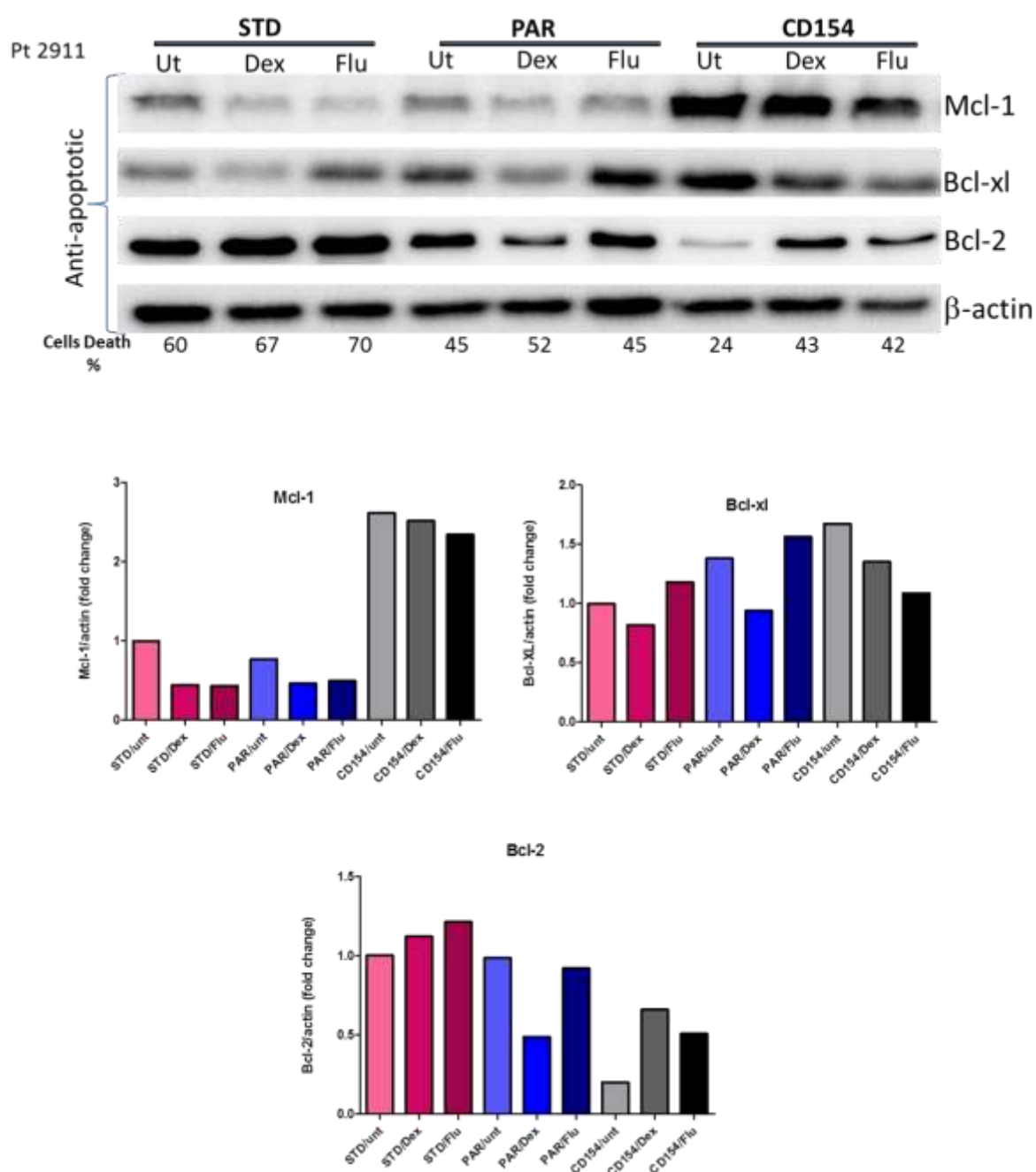


Figure 3.14. Protein expression analysis on anti-apoptotic proteins of the Bcl-2 family in CLL sample 2911.

The change in the level of Bcl-2 family proteins in dexamethasone or fludarabine under standard condition and co-culture with parental cells or fibroblasts expressing CD40L (treated or untreated) was relative to that in untreated cells under standard condition (fold change) also The actin blot was performed on a separate gel loaded with the same cell lysates

Table 3.6. Effect of culture condition and drug treatment on the expression of Bcl-2 family proteins in CLL sample 2968 (figures 3.15 and 3.16).

Bcl-2 family member	Effect of culture condition	Effect of drug treatment
Puma	No effect	↑ by flu
Bim-EL	↓ by CD40L	↑ by dex > flu
Bak	No effect	↓ by dex and flu in PAR co-culture
Bax	No effect	↑ on CD40L
Mcl-1	↑ by CD40L	↓ by dex and flu except in CD154 co-culture
Bcl-XL	↑ by CD40L > PAR	↓ by flu in PAR co-culture
Bcl-2	↓ by par and CD40L	↓ by dex and flu in CD40L co-culture

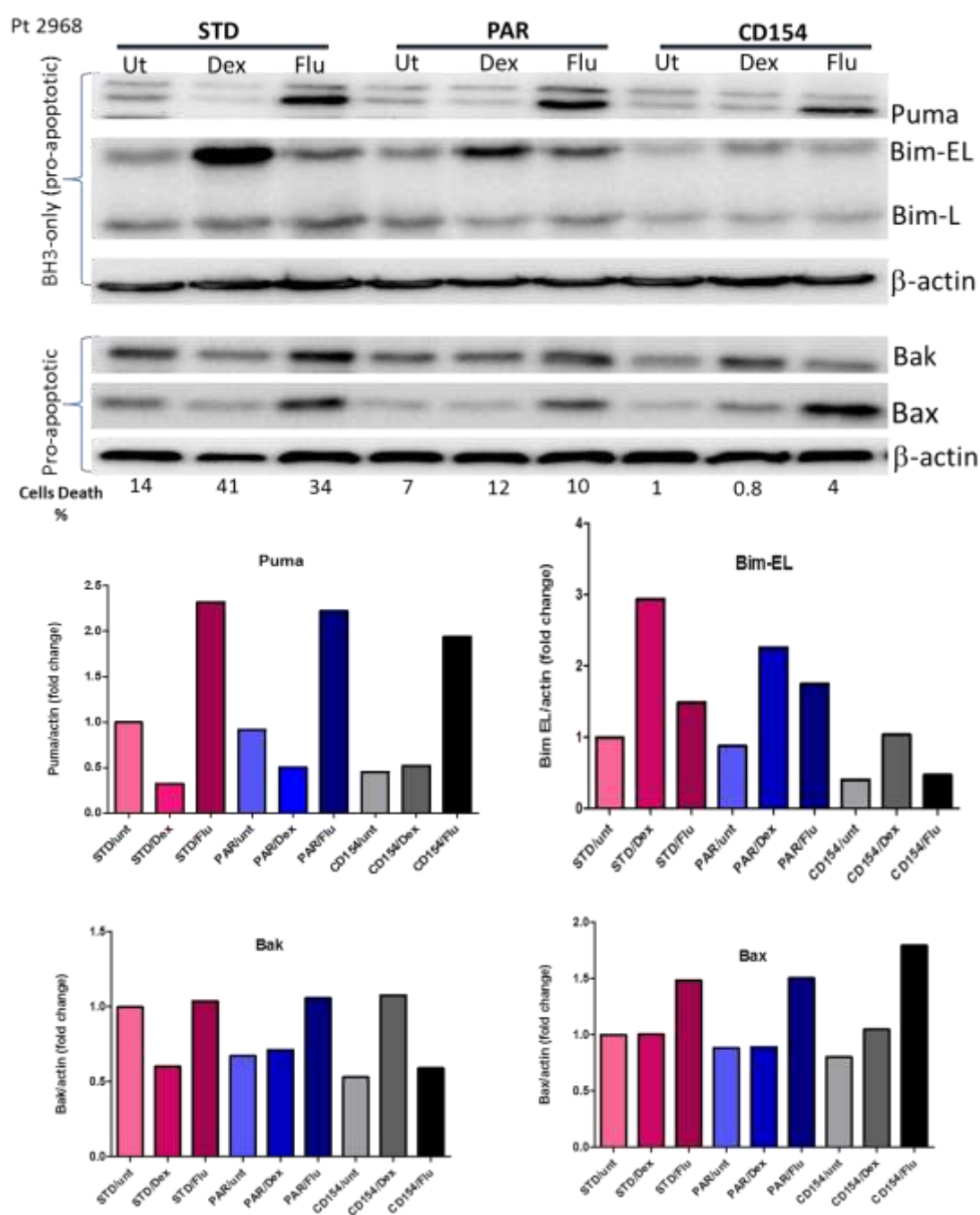


Figure 3.15. Protein expression analysis on pro-apoptotic proteins of the Bcl-2 family in CLL sample 2968.

The change in the level of Bcl-2 family proteins in dexamethasone or fludarabine under standard condition and co-culture with parental cells or fibroblasts expressing CD40L (treated or untreated) was relative to that in untreated cells under standard condition (fold change) also the blot was stripped and re-probed with anti-actin antibody.

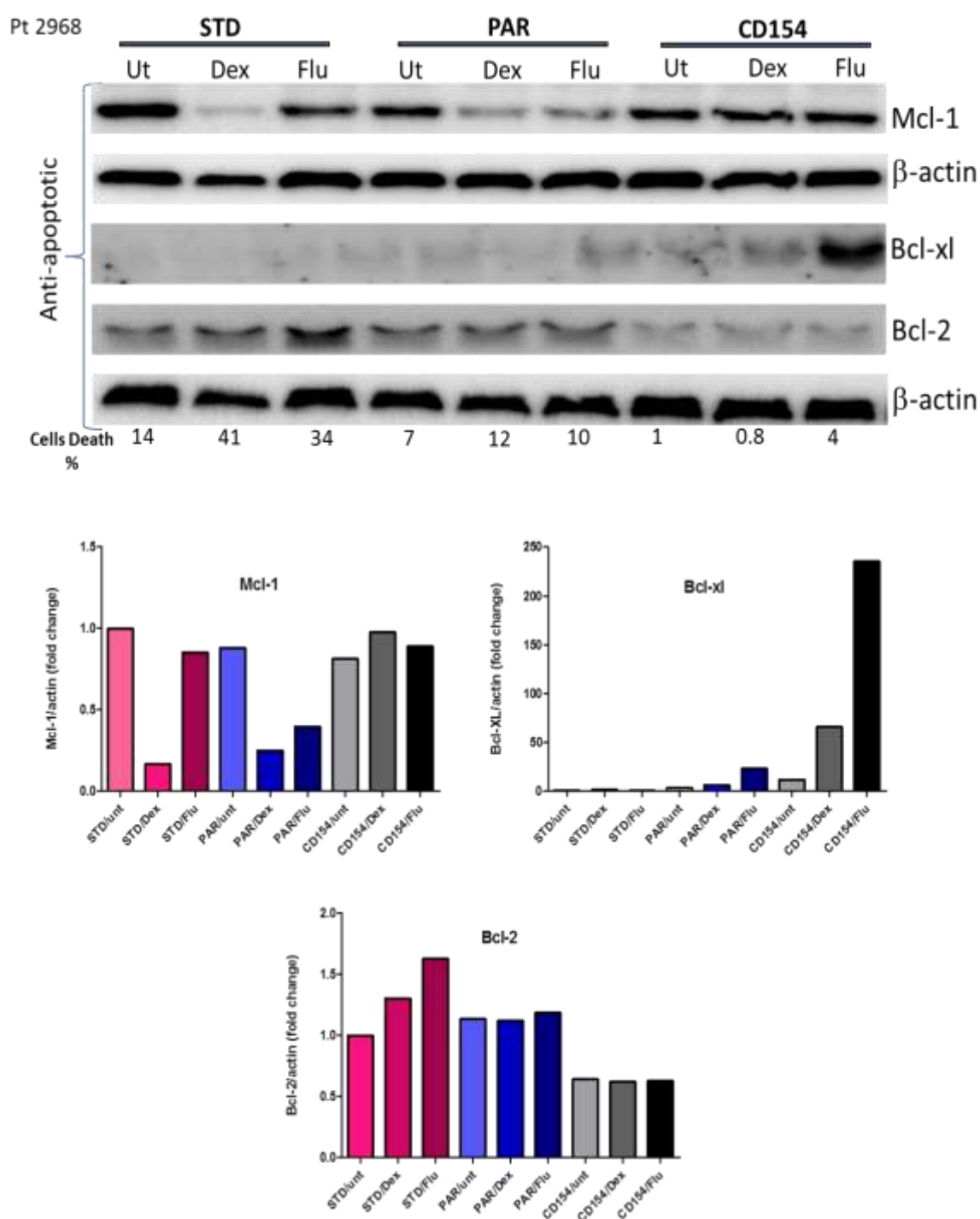


Figure 3.16. Protein expression analysis on anti-apoptotic proteins of the Bcl-2 family in CLL sample 2968.

The change in the level of Bcl-2 family proteins in dexamethasone or fludarabine under standard condition and co-culture with parental cells or fibroblasts expressing CD40L (treated or untreated) was relative to that in untreated cells under standard condition (fold change) also the blot was stripped and re-probed with anti-actin antibody.

Table 3.7. Effect of culture condition and drug treatment on the expression of Bcl-2 family proteins in CLL sample 2929 (figures 3.17 and 3.18).

Bcl-2 family member	Effect of culture condition	Effect of drug treatment
Puma	No effect	↑ by flu
Bim-EL	↓ by CD40L	↑ by dex > flu
Bak	No effect	No effect
Bax	No effect	No effect
Mcl-1	No effect	↓ by dex and flu except in CD154 co-culture
Bcl-XL	↑ by CD40L > PAR	↓ by flu in PAR co-culture
Bcl-2	↓ by PAR and CD40L	↓ by dex and flu in CD40L co-culture

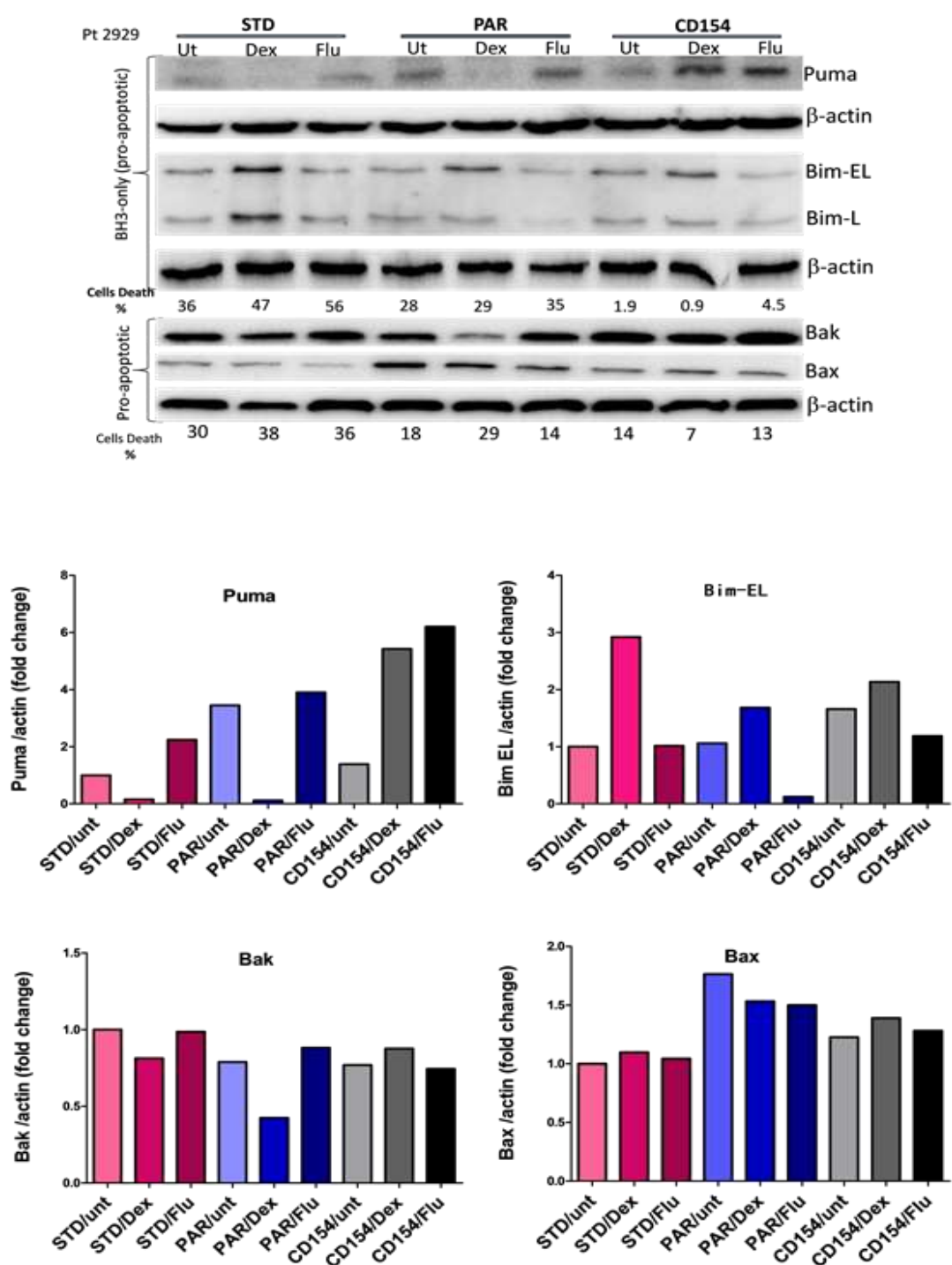


Figure 3.17. Protein expression analysis on pro-apoptotic proteins of the Bcl-2 family in CLL sample 2929.

The change in the level of Bcl-2 family proteins in dexamethasone or fludarabine under standard condition and co-culture with parental cells or fibroblasts expressing CD40L (treated or untreated) was relative to that in untreated cells under standard condition (fold change) also the blot was stripped and re-probed with anti-actin antibody.

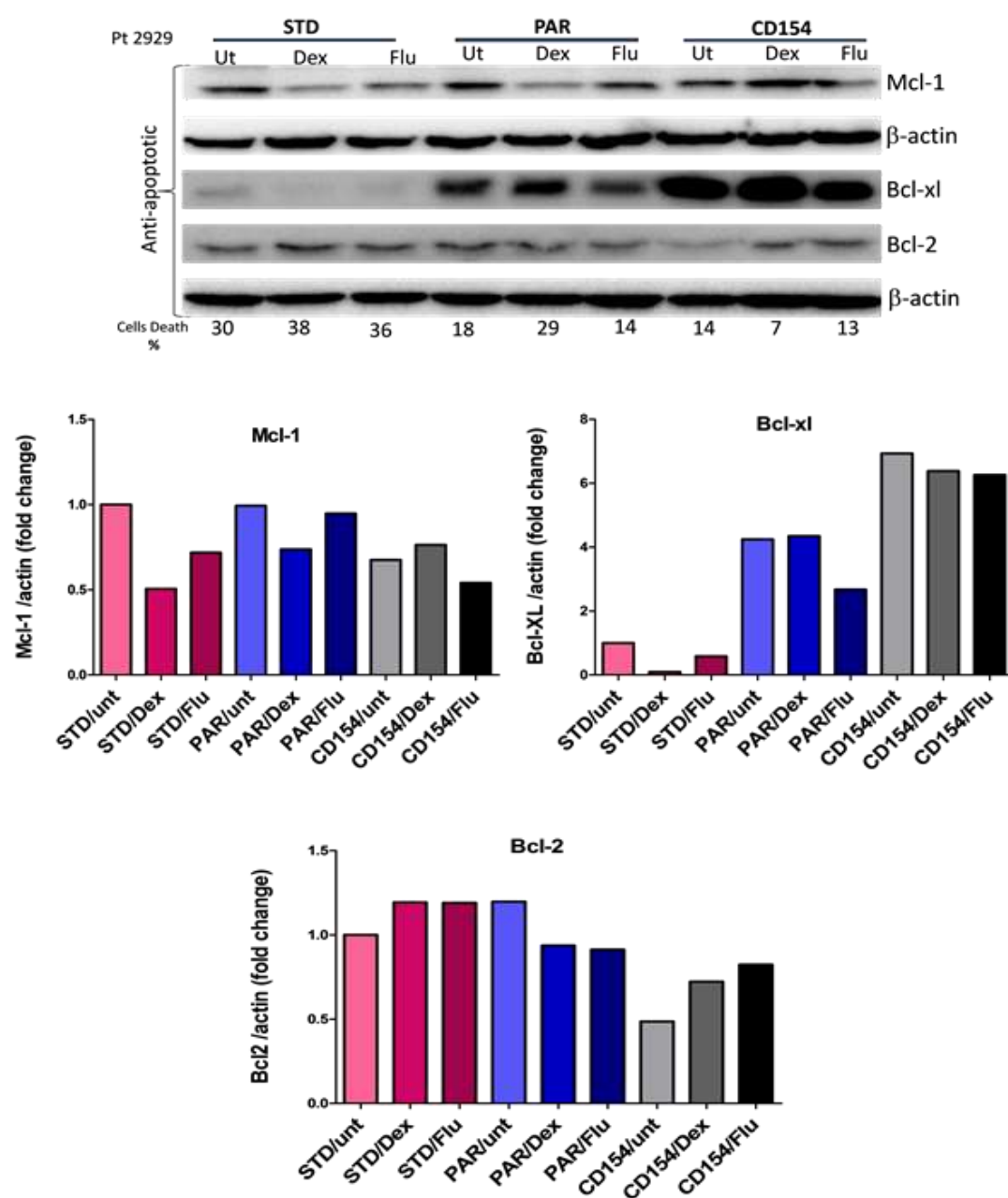


Figure 3.18. Protein expression analysis on anti-apoptotic proteins of the Bcl-2 family in CLL sample 2929.

The change in the level of Bcl-2 family proteins in dexamethasone or fludarabine under standard condition and co-culture with parental cells or fibroblasts expressing CD40L (treated or untreated) was relative to that in untreated cells under standard condition (fold change) also the blot was stripped and re-probed with anti-actin antibody.

3.5 Pooled analysis of the effect of fludarabine and dexamethasone on the expression of individual Bcl-2 family proteins in CLL cells cultured under standard and co-culture conditions

3.5.1 Puma

Overall in the 6 CLL samples tested, a consistent observation was an increase in levels of Puma following fludarabine treatment (Figure 3.19). It was noted that Puma levels were significantly elevated after fludarabine treatment regardless of whether CLL cells were cultured under standard conditions, or co-cultured with parental- or CD40L-expressing fibroblasts ($p < 0.05$, Table 3.8), and that the culture conditions had no effect on the ability of fludarabine to induce Puma expression. This latter observation is important because it suggests that the ability of CD40 ligation to rescue CLL cells from fludarabine cytotoxicity is not due to down regulation of Puma expression.

Levels of Puma were found to be unaffected by dexamethasone treatment.

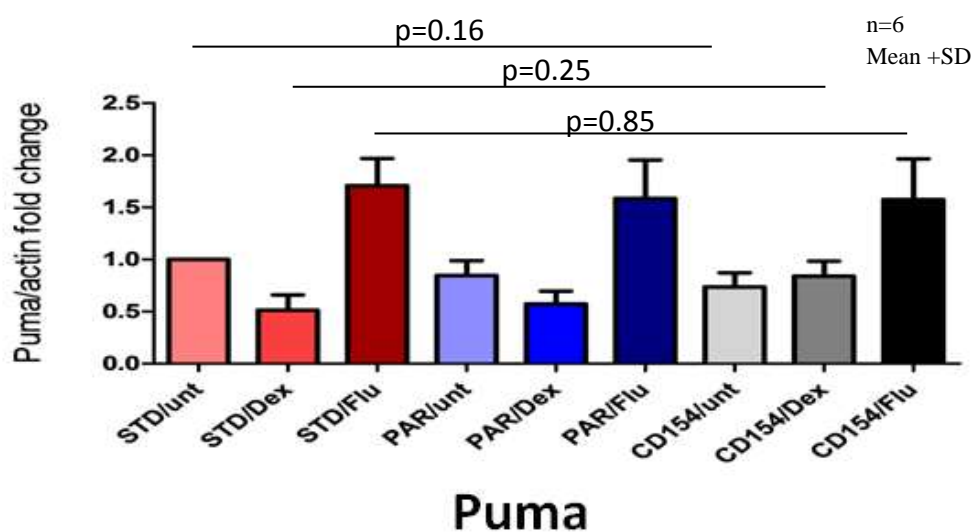


Figure 3.19. Puma levels in CLL cells from 6 samples under standard culture or co-culture condition.

Puma levels in CLL cells from 6 samples under standard culture condition, or co-culture with parental cells or fibroblasts expressing CD40L, in the presence or absence of dexamethasone or fludarabine were compared using paired t test for calculating P values as shown in (Table 3.8).

Table 3.8. P values in the paired t test performed to compare Puma levels under different treatment and culture conditions outlined in Figure 3.19.

Treatment	Treatment	STD			PAR			CD154		
		Ut	Dex	Flu	Ut	Dex	Flu	Ut	Dex	Flu
STD	Ut	X								
	Dex	0.05	X							
	Flu	0.03	0.01	X						
PAR	Ut	0.30	0.22	0.01	X					
	Dex	0.01	0.64	0.003	0.12	X				
	Flu	0.02	0.05	0.52	0.04	0.02	X			
CD154	Ut	0.16	0.38	0.003	0.43	0.30	0.01	X		
	Dex	0.30	0.25	0.01	0.92	0.15	0.04	0.17	X	
	Flu	0.22	0.09	0.85	0.07	0.06	0.96	0.04	0.08	X

3.5.2 Bim

In contrast to its induction of Puma expression in CLL cells, fludarabine did not affect the level of Bim (measured as Bim-EL) either under standard culture conditions, or after co-culture with parental or CD40L-expressing fibroblasts (Figure 3.19). Instead, elevation in the level of Bim-EL was observed after dexamethasone treatment (Figure 3.20). This increase was statistically significant compared to untreated cells in all three culture conditions used (Table 3.9), and, like fludarabine induction of Puma, was not affected by the culture condition. This finding agrees with previous work from this Department demonstrating that dexamethasone killing of CLL cells is dependent on the induction of Bim (Melarangi et al. 2012). However, findings from the current study show that CD40 ligation on CLL cells largely protects them from dexamethasone cytotoxicity. This, therefore, extends the findings of Melarangi et al to suggest that the microenvironment can negate the pro-apoptotic effects of induced Bim expression. Furthermore, that CLL cell treatment with fludarabine had no effect on the expression of Bim-EL, suggests that the induction of Bim expression in CLL cells treated with dexamethasone is a specific phenomenon.

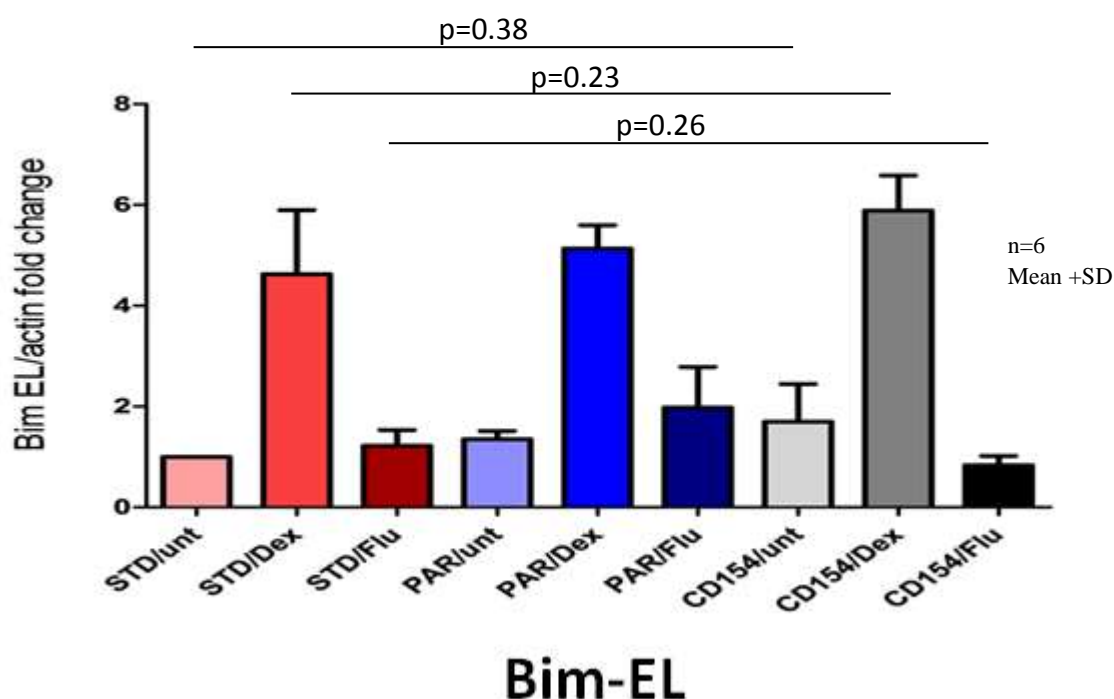


Figure 3.20. Bim-EL levels in CLL cells from 6 samples under standard culture or co-culture condition.

Bim-EL levels in CLL cells under standard condition, or co-culture with parental cells or fibroblasts expressing CD40L, in the presence or absence of dexamethasone or fludarabine were compared using paired t test for calculating P values as shown in (Table 3.9).

Table 3.9. P values of difference in the levels of expression of Bim-EL in CLL cells under all culture conditions, outlined in Figure 3.20.

	Treatment	STD			PAR			CD154		
Treatment		Ut	Dex	Flu	Ut	Dex	Flu	Ut	Dex	Flu
STD	Ut	X								
	Dex	0.03	X							
	Flu	0.49	0.04	X						
PAR	Ut	0.09	0.003	0.70	X					
	Dex	0.001	0.75	0.001	0.003	X				
	Flu	0.26	0.02	0.42	0.49	0.01	X			
CD154	Ut	0.38	0.001	0.62	0.68	0.006	0.49	X		
	Dex	0.004	0.23	0.006	0.009	0.47	0.005	0.006	X	
	Flu	0.40	0.01	0.26	0.11	0.002	0.12	0.23	0.002	X

3.5.3 Bak

Although there were differences in the expression of Bak in individual cases of CLL within the three culture systems, analysis of the pooled data showed Bak expression to remain constant across all culture conditions (standard or co-culture with parental cells or fibroblasts expressing CD40L) and that it was not significantly affected by treatment with either dexamethasone or fludarabine (Figure 3.21, Table 3.10).

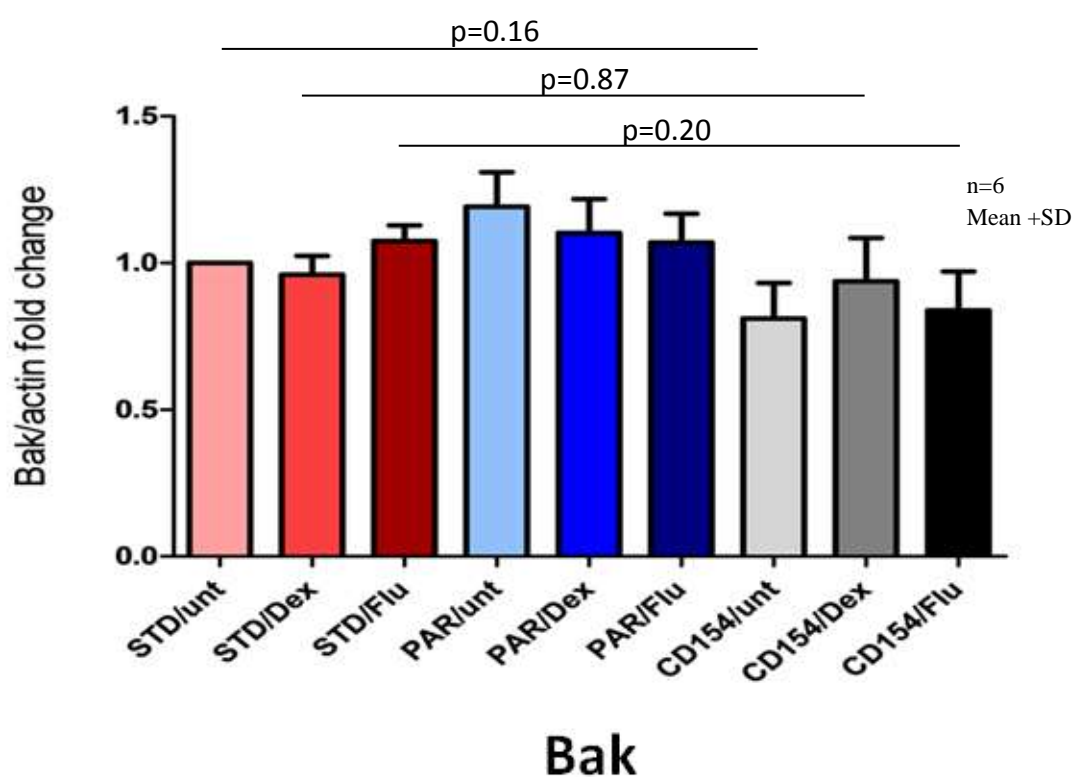


Figure 3.21. Bak levels in CLL cells from 6 samples under standard culture or co-culture condition.

Bak levels in CLL cells under standard condition, or co-culture with parental cells or fibroblasts expressing CD40L, in the presence or absence of dexamethasone or fludarabine were compared using paired t test for calculating P values as shown in Table 3.10.

Table 3.10. P values of difference in the levels of expression of Bak under all culture conditions, outlined in (Figure 3.21).

	Treatment	STD			PAR			CD154		
Treatment		Ut	Dex	Flu	Ut	Dex	Flu	Ut	Dex	Flu
STD	Ut	X								
	Dex	0.55	X							
	Flu	0.20	0.26	X						
PAR	Ut	0.15	0.03	0.41	X					
	Dex	0.41	0.16	0.84	0.17	X				
	Flu	0.50	0.30	0.96	0.13	0.53	X			
CD154	Ut	0.16	0.15	0.15	0.004	0.02	0.03	X		
	Dex	0.68	0.87	0.50	0.09	0.25	0.28	0.19	X	
	Flu	0.26	0.30	0.20	0.02	0.02	0.04	0.56	0.40	X

3.5.4 Bax

Similar to Bak, analysis of the pooled data showed that Bax expression in CLL cells remained unaffected by the culture condition used (standard, co-culture with parental or CD40L-expressing fibroblasts) (Figure 3.21). Treatment of CLL cells with dexamethasone did not affect Bax expression under any culture condition, whereas a slight increase in Bax expression was observed in fludarabine-treated CLL cells (Figure 3.22). This increase became statistically significant when fludarabine-treated CLL cells were co-cultured with parental or CD40L-expressing fibroblasts ($p < 0.05$, (Table 3.11).

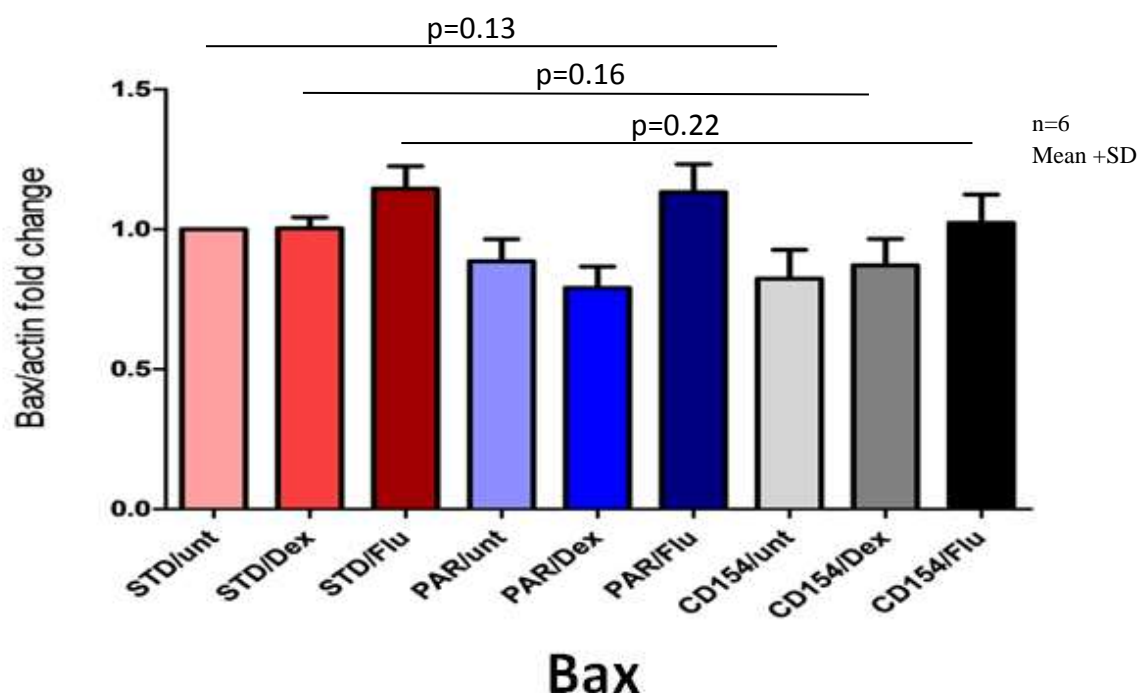


Figure 3.22. Bax levels in CLL cells from 6 samples under standard culture or co-culture condition.

Bax levels in CLL cells under standard culture condition co-culture with parental cells or fibroblasts expressing CD40L, in the presence or absence of dexamethasone or fludarabine were compared using paired t test for calculating P values as shown in Table 3.11.

Table 3.11. P values of difference in the levels of expression of Bax under all culture conditions with or without treatment of dexamethasone (Dex) or fludarabine (Flu), outlined in Figure 3.22.

	Treatment	STD			PAR			CD154		
Treatment		Ut	Dex	Flu	Ut	Dex	Flu	Ut	Dex	Flu
STD	Ut	X								
	Dex	0.93	X							
	Flu	0.12	0.08	X						
PAR	Ut	0.19	0.11	0.04	X					
	Dex	0.03	0.02	0.007	0.03	X				
	Flu	0.23	0.31	0.91	0.18	0.07	X			
CD154	Ut	0.13	0.10	0.05	0.28	0.68	0.13	X		
	Dex	0.22	0.16	0.04	0.79	0.29	0.14	0.27	X	
	Flu	0.83	0.83	0.22	0.03	0.002	0.53	0.03	0.28	X

3.5.5 Mcl-1

Treatment of CLL cells with either dexamethasone or fludarabine induced a reduction in Mcl-1 expression under standard culture condition (Figure 3.23). This reduction in Mcl-1 seemed in line with the induction of cell death by the drugs (Figure 3.3). Interestingly, Mcl-1 expression was also significantly reduced by co-culture of the cells with parental fibroblasts, but this decrease of Mcl-1 expression was not associated with induction of cell death. Furthermore, Mcl-1 expression in CLL cells co-cultured with parental fibroblasts was not largely affected by the presence of either dexamethasone or fludarabine (Table 3.12). These results suggest that the pro-survival effect of parental fibroblasts is not due to changes in Mcl-1 expression. In contrast, Mcl-1 expression was induced when CLL cells were co-cultured with fibroblasts expressing CD40L. Importantly, this increased expression was not affected by CLL cell treatment with dexamethasone or fludarabine under these conditions (Figure 3.23), consistent with the inability of these drugs to induce cytotoxicity in this system (Figure 3.6, Table 3.1).

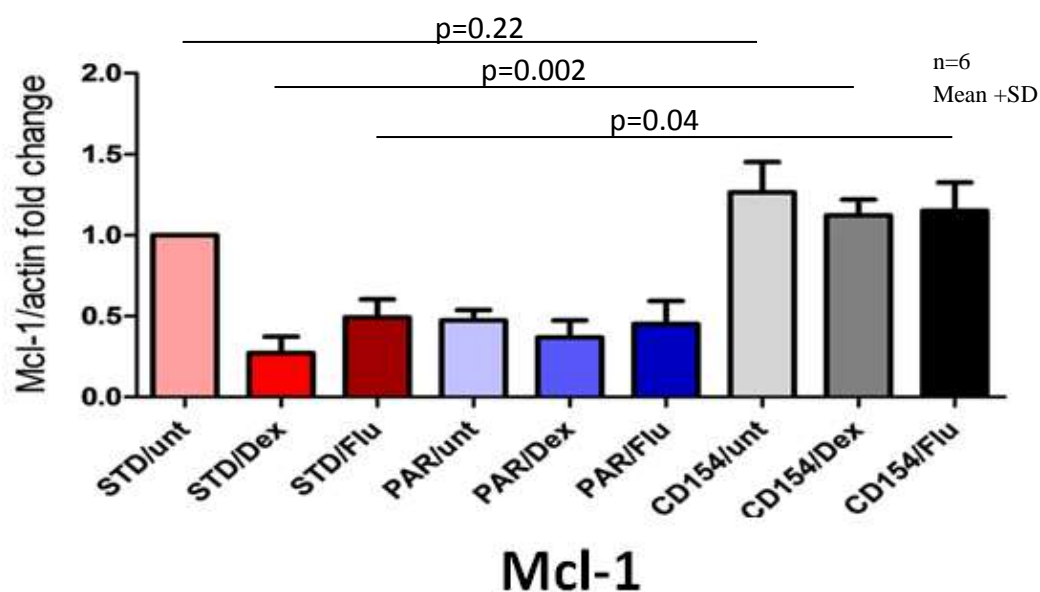


Figure 3.23. Mcl-1 levels in CLL cells from 6 samples under standard culture or co-culture condition.

Levels of Mcl-1 in CLL cells under standard culture condition or co-culture with parental cells or fibroblasts expressing CD40L, in the presence or absence of dexamethasone or fludarabine were compared using paired t test for calculating P values as shown in Table 3.12.

Table 3.12. P values of difference in the levels of expression of Mcl-1 under all culture conditions and in the presence or absence of dexamethasone or fludarabine, outlined in Figure 3.23.

	Treatment	STD			PAR			CD154		
Treatment		Ut	Dex	Flu	Ut	Dex	Flu	Ut	Dex	Flu
STD	Ut	X								
	Dex	0.008	X							
	Flu	0.006	0.21	X						
PAR	Ut	0.004	0.20	0.90	X					
	Dex	0.001	0.33	0.41	0.38	X				
	Flu	0.01	0.25	0.81	0.87	0.16	X			
CD154	Ut	0.22	0.001	0.02	0.02	0.007	0.02	X		
	Dex	0.27	0.002	0.01	0.004	0.003	0.01	0.41	X	
	Flu	0.43	0.005	0.04	0.02	0.006	0.02	0.25	0.86	X

3.5.6 Bcl-XL

Co-culture of CLL cells with either parental or CD40L-expressing fibroblasts both induced expression of Bcl-XL compared to culture of CLL cells under standard conditions (Figure 3.24). Because the co-culture system significantly reduces the level of CLL cell death (Figure 3.6, Table 3.1), this result suggest a role for Bcl-XL in protecting CLL cells from spontaneous apoptosis. Treatment with either dexamethasone or fludarabine did not prevent the up-regulation of Bcl-XL within the co-culture system. In fact, for unknown reasons, treatment with either drug appeared to further induce increased expression of this protein. In particular, this phenomenon was observed in CLL cells co-cultured with CD40L-expressing fibroblasts (Figure 3.24 and Table 3.13). Nevertheless, this increased expression of Bcl-XL by dexamethasone and fludarabine did not provide extra protection of CLL cells to that already provided by the co-culture system because there was essentially no change in the level of cell death (Figure 3.6, Table 3.1).

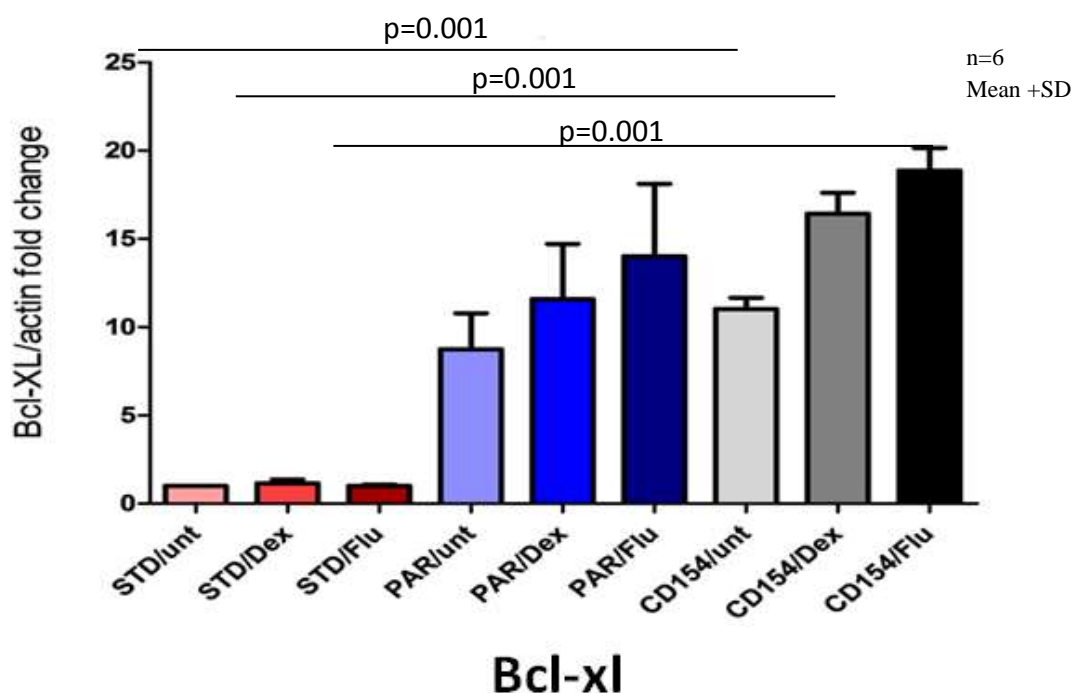


Figure 3.24. Bcl-XL levels in CLL cells from 6 samples under standard culture or co-culture condition.

The levels of Bcl-XL in CLL cells under standard condition, or co-culture with parental cells or fibroblasts expressing CD40L, in the presence or absence of dexamethasone or fludarabine were compared using paired t test for calculating P values as shown in (Table 3.13).

Table 3.13. P values of difference in the levels of expression of Bcl-xl under all culture conditions with or without dexamethasone or fludarabine, outlined in (Figure 3.24).

Treatment	Treatment	STD			PAR			CD154		
		Ut	Dex	Flu	Ut	Dex	Flu	Ut	Dex	Flu
STD	Ut	X								
	Dex	0.52	X							
	Flu	0.99	0.52	X						
PAR	Ut	0.01	0.01	0.001	X					
	Dex	0.02	0.02	0.01	0.10	X				
	Flu	0.02	0.02	0.02	0.10	0.23	X			
CD154	Ut	0.001	0.001	0.001	0.34	0.86	0.49	X		
	Dex	0.001	0.001	0.001	0.004	0.09	0.52	0.01	X	
	Flu	0.001	0.001	0.001	0.005	0.09	0.32	0.003	0.14	X

3.5.7 Bcl-2

Like Mcl-1 and Bcl-XL, expression of Bcl-2 in CLL cells was affected by the co-culture system compared culture under standard condition. Thus, culture of CLL cells with either parental or CD40L-expressing fibroblasts reduced the levels of Bcl-2 (Figure 3.25 and Table 3.14). Again, like Mcl-1, treatment of CLL cells with either dexamethasone or fludarabine had little or no effect on Bcl-2 levels within the co-culture system. However, when CLL cells were cultured under standard conditions, treatment with dexamethasone or fludarabine seemed to slightly induce Bcl-2 expression, but this did not achieve statistical significance within the current study (Figure 3.25, Table 3.14).

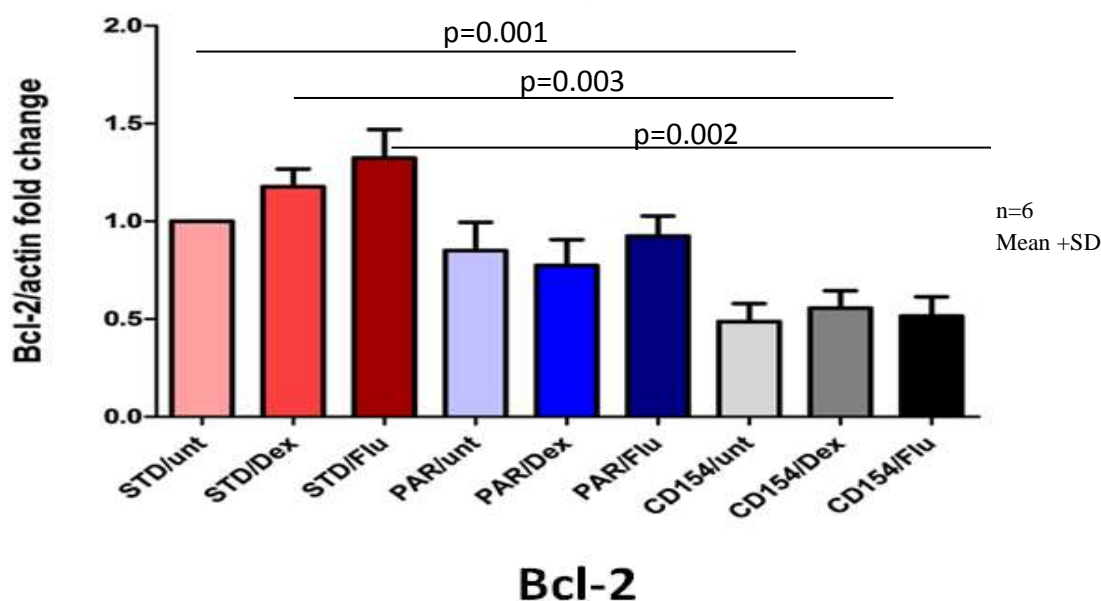


Figure 3.25. Bcl-2 levels in CLL cells from 6 samples under standard culture or co-culture condition.

The levels of Bcl-2 in CLL cells under standard culture condition or co-culture with parental cells or fibroblasts expressing CD40L, in the presence or absence of dexamethasone or fludarabine were compared using paired t test for calculating P values as shown in (Table 3.14).

Table 3.14. P values of difference in the levels of expression of Bcl-2 in CLL cells under all three culture conditions with or without dexamethasone or fludarabine, outlined in (Figure 3.25).

	Treatment	STD			PAR			CD154		
Treatment		Ut	Dex	Flu	Ut	Dex	Flu	Ut	Dex	Flu
STD	Ut	X								
	Dex	0.09	X							
	Flu	0.07	0.36	X						
PAR	Ut	0.34	0.04	0.03	X					
	Dex	0.14	0.009	0.003	0.27	X				
	Flu	0.49	0.001	0.06	0.63	0.27	X			
CD154	Ut	0.001	0.003	0.002	0.001	0.01	0.001	X		
	Dex	0.003	0.003	0.001	0.01	0.004	0.01	0.19	X	
	Flu	0.003	0.003	0.002	0.001	0.01	0.01	0.37	0.37	X

3.6 Summary of Results

- a. Spontaneous cell death is moderately reduced in CLL cells when co-cultured with parental fibroblasts and significantly reduced when co-cultured with CD40L (CD154)-expressing fibroblasts as compared with standard culture conditions ($p=0.01$).
- b. Compared to standard culture conditions, CLL cells are significantly protected from dexamethasone- or fludarabine-induced cell death when co-cultured with parental fibroblasts (p values of 0.02 and 0.05, respectively) and even more protected when co-cultured with CD40L-expressing fibroblasts ($P=0.002$ in both cases).
- c. The expression of different pro-apoptotic and anti-apoptotic Bcl-2 family of proteins followed a characteristic overall pattern when CLL cells were cultured under different conditions and exposed to different cytotoxic drugs, although there was some variation between individual cases.
- d. Fludarabine treatment consistently resulted in the increased expression of Puma in CLL cells cultured under all conditions.
- e. Dexamethasone treatment consistently resulted in the increased expression of Bim in CLL cells cultured under all conditions.
- f. Levels of Bak and Bax were largely unaffected by different culture conditions and treatment with dexamethasone or fludarabine.
- g. Levels of Mcl-1 were reduced by treatment with dexamethasone or fludarabine under standard culture conditions and increased by co-culturing CLL cells with fibroblasts expressing CD40L.

- h.** Bcl-xL levels were elevated after co-culture with both parental cells and fibroblasts expressing CD40L and further increased under these conditions by dexamethasone or fludarabine treatment.
- i.** Levels of Bcl-2 were reduced by co-culture of CLL cells with parental cells and even more so following by co-culture with CD40L-expressing fibroblasts. However, levels were unaffected by treatment with dexamethasone or fludarabine irrespective of the culture condition used.

The overall conclusions from this Chapter are summarised in the Table below (Table 3-15).

Table 3.15. Effect of culture condition and drug treatment on the expression of Bcl-2 family of proteins in 6 CLL samples studied.

Bcl-2 family member	Effect of culture condition	Effect of drug treatment
Puma	No effect	↑ by flu
Bim EL	No effect	↑ by dex
Bak	No effect	No effect
Bax	No effect	Slight ↑ by flu
Mcl-1	↑ by CD40L	↓ by dex and flu under standard conditions
Bcl-XL	↑ by CD40L > PAR	↑ by dex and flu in PAR and CD40L co-culture
Bcl-2	↓ by CD40L > PAR	No effect

3.7 Discussion

It is clear that microenvironment plays a key role in maintaining CLL cell survival and resistance to drug therapies (ten Hacken & Burger 2016). Within this thesis the role of CD40 ligation on CLL cells was studied because of its known role in providing cytoprotection (Elgueta et al. 2009). However, the mechanism of this cytoprotection is not well understood, particularly in relation to principal drug therapies used for CLL such as fludarabine and dexamethasone. This Chapter addressed the question how expression of Bcl2 family proteins are affected by co-culturing CLL cells with parental and CD40L-expressing fibroblasts, and then studied how fludarabine and dexamethasone influence this expression.

I confirmed that CD40 stimulation provides CLL cells with strong pro-survival signals, not only preventing their spontaneous apoptosis, but also inhibiting killing of CLL cells by drugs that induce cell death via p53-dependent (fludarabine) and p53-independent (dexamethasone) mechanisms.

Examination of this mechanism of cytoprotection was next investigated by measuring changes to protein expression of Bcl-2 family members. A consistent finding was that Puma was upregulated by treatment of CLL cells with fludarabine regardless of the culture conditions. Fludarabine is incorporated into DNA and mediates its cytotoxicity by blocking DNA synthesis. This would activate p53, which induce, among other p53 target genes, the expression of Puma in CLL cells (Mackus et al. 2005). The level of Puma induction by fludarabine was similar under each culture condition. However, this did not translate into similar levels of induced apoptosis, as there was a significant reduction in apoptosis when CLL cells were co-

cultured with parental or CD40L-expressing fibroblasts. This suggests that co-culture of CLL cells counteracts the pro-apoptotic effects of Puma.

Puma is a BH3-only protein that works by interacting with anti-apoptosis proteins such as Mcl-1, Bcl-XL, and Bcl-2. When Puma is induced, it binds to these anti-apoptosis proteins reducing their ability to bind Bax and Bak, subsequently resulting in activation of mitochondrial apoptosis. Conceivably, increased expression of Mcl-1 and Bcl-XL in CLL cells by the co-culture conditions should lead to rescue from Puma-induced apoptosis (Kater et al. 2004; Mackus et al. 2005; Romano et al. 1998). Indeed, experiments in this Chapter show that Bcl-XL levels are increased in CLL cells under both co-culture systems, whereas Mcl-1 expression is only induced when CLL cells are co-cultured with CD40L-expressing fibroblasts. However, although combined expression of Bcl-XL and Mcl-1 resulting from CLL-cell co-culture with CD40L-expressing fibroblasts provides protection from spontaneous apoptosis, it does not provide complete protection from fludarabine cytotoxicity. Clearly, this result suggests that the combined expression of Bcl-XL and Mcl-1 does not provide total cytoprotection against fludarabine in the co-culture system, challenging existing ideas that both of these anti-apoptosis proteins function in equivalent fashion. Puma is shown to bind all pro-survival Bcl-2-like proteins (Buggins & Pepper 2010; Chipuk et al. 2010), indicating that expression of equimolar levels of Mcl-1 and Bcl-XL should provide additive protection. That this is not the case suggests that Bcl-XL, because it is massively induced when CLL cells are co-cultured with parental and CD40L-expressing fibroblasts, must be the major protective influence from Puma-induced apoptosis when fludarabine is also present within these cultures.

The co-culture system also provided protection to p53-independent cytotoxic drugs. Dexamethasone primarily up-regulates Bim in CLL cells and does not affect the expression of Puma or Bax, which are induced by fludarabine. This result is in keeping with previous studies (Melarangi et al. 2012; Ren et al. 2010), and reflects the differential action of dexamethasone and fludarabine at a molecular level. Dexamethasone binds to and activates the intracellular glucocorticoid receptor which regulates the expression of a number of target genes including Bim (Melarangi et al. 2012), and explains the consistent finding of Bim upregulation in CLL cells within all three culture conditions. Similar to the rescue effects of the co-culture system on fludarabine-induced cytotoxicity, co-culture of CLL cells with either parental or CD40L-fibroblasts also provided rescue from dexamethasone-induced apoptosis. The mechanism of this rescue is likely provided by increased expression of Bcl-XL and not Mcl-1. This is because although, like Puma, Bim also binds Bcl-2-like prosurvival proteins comparably (Mol Cell. 2005 Feb 4;17(3):393-403), there was no difference between the level of cytoprotection provided by co-culture with parental fibroblasts or with co-culture with CD40L-expressing fibroblasts.

To my knowledge, this is the first study to directly compare dexamethasone and fludarabine for their effect on the expression of Bcl-2 family proteins and associated cytotoxicity. It also provides insight into how microenvironment provides rescue from this cytotoxicity. It would seem that increased Bcl-XL expression induced in CLL cells by co-culture with fibroblasts provides the major protective influence against fludarabine- and dexamethasone-induced apoptosis. This observation has impact on our understanding of the potential use of drugs targeting Bcl-2 family proteins, particularly with respect to anatomical location of the malignant cells. The

observations from this Chapter predict that the selective Bcl-2 inhibitor ABT-199 should be more effective at killing CLL cells in the blood than in the tissues because Bcl-2 expression in CLL cells is downregulated within the co-culture system despite increased survival. In the same way, inhibitors that target Bcl-XL (e.g. ABT-263) should be able to kill CLL cells within tissues but not necessarily in the blood because Bcl-XL is expressed at very low levels in circulating CLL cells, but is induced in CLL cells within the co-culture system. When considering combination therapy, it therefore makes sense to combine fludarabine or dexamethasone with Bcl-XL inhibitors in order to target CLL cells within tissues.

In addition, the effect on the expression of Bcl-2 family proteins from drug treatment and stimulation by fibroblasts with or without CD40L was independent from each other. Drug treatment of CLL cells with fludarabine or dexamethasone resulted in up-regulation of pro-apoptotic BH3-only proteins Puma and Bim, respectively, and co-culture conditions did not alter drug-induced up-regulation of Puma and Bim in CLL cells. Vice versa, co-culture of CLL cells with CD154-expressing fibroblasts led to strong induction of anti-apoptotic Bcl-2 family proteins Bcl-xL and Mcl-1, which was not affected by the addition of either fludarabine or dexamethasone. However, there was one exception. The expression of Bcl-XL was increased following treatment with dexamethasone or fludarabine in CLL cells co-cultured with CD40L-expressing or parental fibroblasts and this effect was not observed in CLL cells cultured under standard conditions. Presumably, the two drugs activate certain signalling pathways that further complement those activated following CD40-stimulation to enhance the expression of Bcl-XL by the transcriptional or post-transcriptional mechanisms. This observation provides an example of how

cytotoxic drugs can activate both pro- and anti-apoptotic signalling pathways and suggests that their effectiveness might be enhanced if the drug-induced anti-apoptotic pathways could be inhibited, in this case by blocking Bcl-XL or the upstream signalling pathways that result in Bcl- XL up-regulation.

Chapter 4

Functional study of Puma in fludarabine-induced death of CLL cells under standard conditions

4.1. Background and aims

A consistent finding within the previous chapter was that Puma is upregulated by fludarabine treatment of CLL cells regardless of culture condition. So far it is assumed that induced expression of Puma results in CLL cell apoptosis because of established studies on the mechanism of fludarabine-induced cytotoxicity in other cell systems) (Zhang, Li & Xu 2013). In order to understand the way in which co-culture of CLL cells with parental and CD40L-expressing fibroblasts provides cytoprotection, it was important to establish whether Puma is required for fludarabine-induced cytotoxicity of CLL cells cultured under standard conditions. The aim of this chapter was to employ Puma-specific siRNA to knock down Puma expression in primary CLL cells and investigate the effect of such knock down on both spontaneous and fludarabine-induced apoptosis.

4.2. Methodology

Pooled Puma siRNA (cat#L-004380-00-0005, Dharmacon/Thermo Scientific /GE Healthcare, Little Chalfont, Bucks, UK,) was transfected into CLL cells using nucleofection. 1×10^7 CLL cells were resuspended in 100 μ l transfection solution V from the human B cell nucleofector kit (Amaza AG/Lonza). 0.5nmol of either Puma siRNA duplexes or non-specific control siRNA (cat#D-001810-02-05, Dharmacon/Thermo Scientific/GE Healthcare,) were added, and CLL cells were electroporated using a Nucleofector apparatus (Amaza AG/Lonza, Cologne, Germany) set to program X-01. Following electroporation, cells were mixed with 0.9 ml of pre-warmed medium, and then cultured overnight at 37°C. The cells were subsequently incubated at a density of 5×10^6 cells/ml, and cultured with or without

fludarabine (10 μ g/ml) for a further 48h before harvesting for analysis of cell death by flow cytometry and Puma protein expression by Western blot.

4.3. Results

4.3.1 Screening for transfection efficiency

Previous work in this laboratory has shown that the transfection efficiency using the nucleofection method varies considerably between individual CLL samples (Melarangi et al. 2012). To address this variability I initially screened six patient samples (2911, 2929, 2746, 2866, 2899, and 2968) to determine which was suitable for the siRNA knockdown experiments. This screen was performed by flow cytometry where CLL cells 24h after being transfected with 2 μ g pMaxGFP (a GFP plasmid supplied in the Nucleofector Kit from Amaxa AG) were analysed. As expected, the transfection efficiency varied among individual CLL samples (Table 4.1), with a mean of 29.16% and standard deviation (SD) of 5.636% (n=6). Those samples with a transfection efficiency of $\geq 30\%$ were chosen for the knock-down experiments. The cases selected were 2911, 2929, and 2746 which had transfection efficiencies of 36%, 35%, and 31%, respectively (Table 4.1).

Table 4.1. Transfection efficiency of six screened CLL samples

	Patients	Transfection efficiency %
1	2911	36
2	2929	35
3	2746	31
4	2866	23
5	2899	24
6	2968	26

4.3.1 siRNA-mediated knockdown of Puma rescues CLL cells from Fludarabine-induced killing

After establishing which patient samples responded within the nucleofection technique, we then proceeded to knock down Puma expression in CLL cells with siRNA. We first used Western blot to examine the efficiency of the Puma knockdown. Within these experiments it is important to note that Puma is not normally expressed, but must be induced by cell damage that results in p53 activation (Bender & Martinou 2013; Ren et al. 2010). A control was prepared by irradiating CLL cells in order to induce p53 and Puma (Figures 4.1 – 4.3, the IR lane). Figures 4.1 – 4.3 also show that Puma expression is up-regulated in untransfected CLL cells from patient samples 2746, 2929 and 2911 following exposure

to fludarabine. When CLL cells were transfected with siRNA targeting Puma, exposure to Fludarabine failed to upregulate Puma. In contrast, CLL cells transfected with non-specific control siRNA acted like un-transfected CLL cells and up-regulated Puma upon exposure to Fludarabine. When Puma expression is normalised to β -actin in the Western blots and the data pooled together it is clear that treatment of CLL cells with Puma-specific siRNA significantly inhibits (control siRNA untreated and puma siRNA untreated by 40% whereas control siRNA + flu and puma siRNA + flu by 26 %) the induction Puma expression by Fludarabine (Figure 4.4).

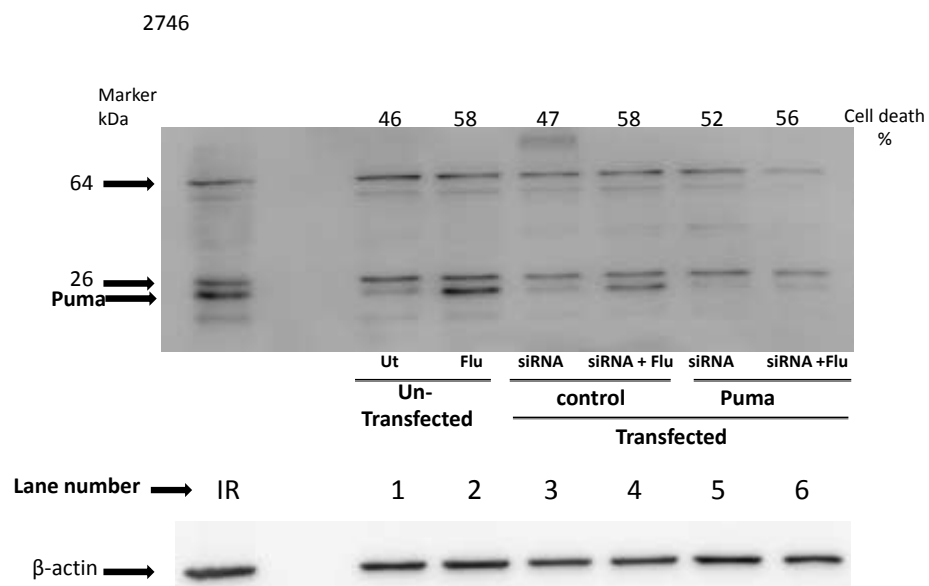


Figure 4.1. Effect of Puma-specific siRNA on Puma protein levels in CLL cells treated (sample 2746).

CLL cells (un-transfected, transfected with control siRNA or Puma specific siRNA) were cultured in the standard condition for 48 hours in the presence or absence of 10 μ M fludarabine (Flu) and cell death was measured by the PI/FACS method as described previously. Western blot was employed to examine the levels of Puma protein expression in the same samples with β -actin probed as loading controls. Lysate prepared from γ -irradiated CLL cells was loaded as a positive control for Puma (lane IR) also the blot was stripped and re-probed with anti-actin antibody.

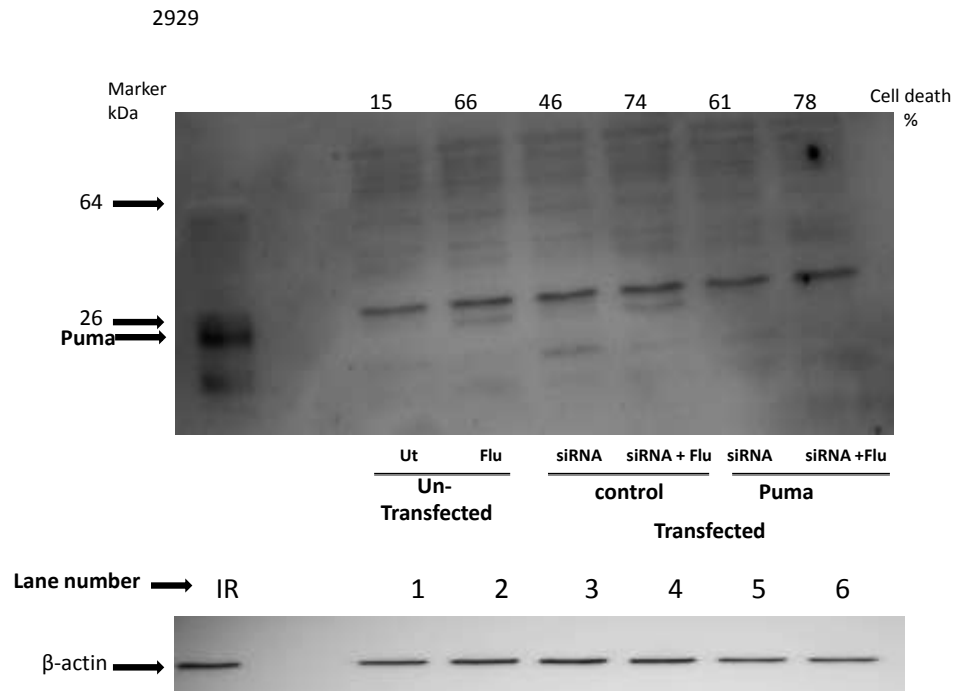


Figure 4.2. Effect of Puma-specific siRNA on levels of Puma protein in CLL cells treated with fludarabine (sample 2929).

CLL cells (un-transfected, transfected with control siRNA or Puma specific siRNA) were cultured in the standard condition for 48 hours in the presence or absence of 10 μ M fludarabine (Flu) and cell death was measured by the PI/FACS method as described previously. Western blot was employed to examine the levels of Puma protein expression in the same samples with β -actin probed as loading control. Lysate prepared from γ -irradiated CLL cells was loaded as a positive control for Puma (lane IR) also the actin blot was performed on a separate gel loaded with the same cell lysates/actin from the same patient, also the blot was stripped and re-probed with anti-actin antibody.

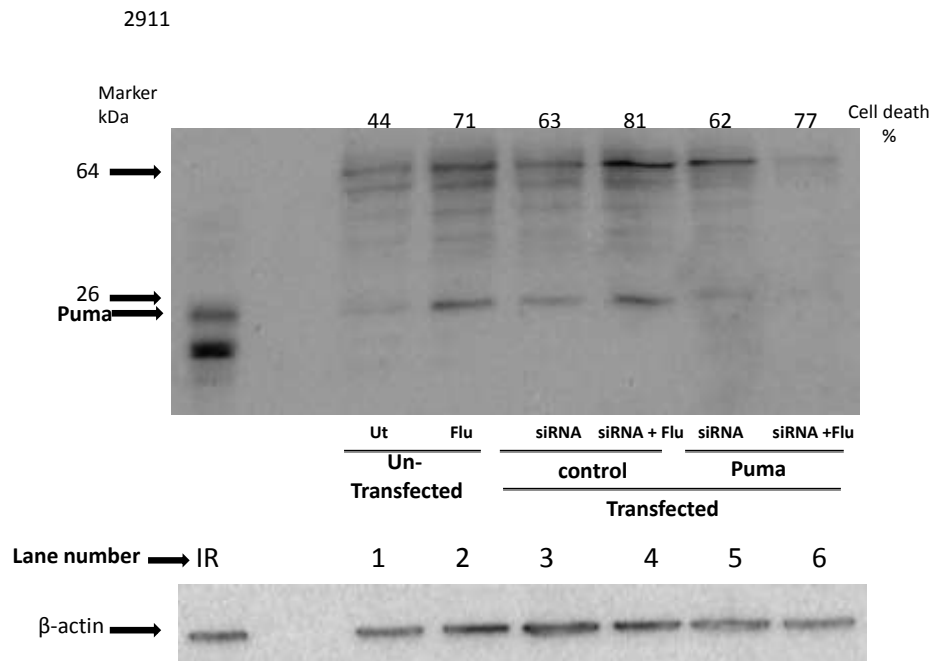


Figure 4.3. Effect of Puma -specific siRNA on levels of Puma protein in CLL cells treated with fludarabine (sample 2911).

CLL cells (un-transfected, transfected with control siRNA or Puma specific siRNA) were cultured in the standard condition for 48 hours in the presence or absence of 10 μ M fludarabine (Flu) and cell death was measured by the PI/FACS method as described previously. Western blot was employed to examine the levels of Puma protein expression in the same samples with β -actin probed as loading control. Lysate prepared from γ -irradiated CLL cells was loaded as a positive control for Puma (lane IR) also the actin blot was performed on a separate gel loaded with the same cell lysates actin from the same patient, also the blot was stripped and re-probed with anti-actin antibody.

n=3

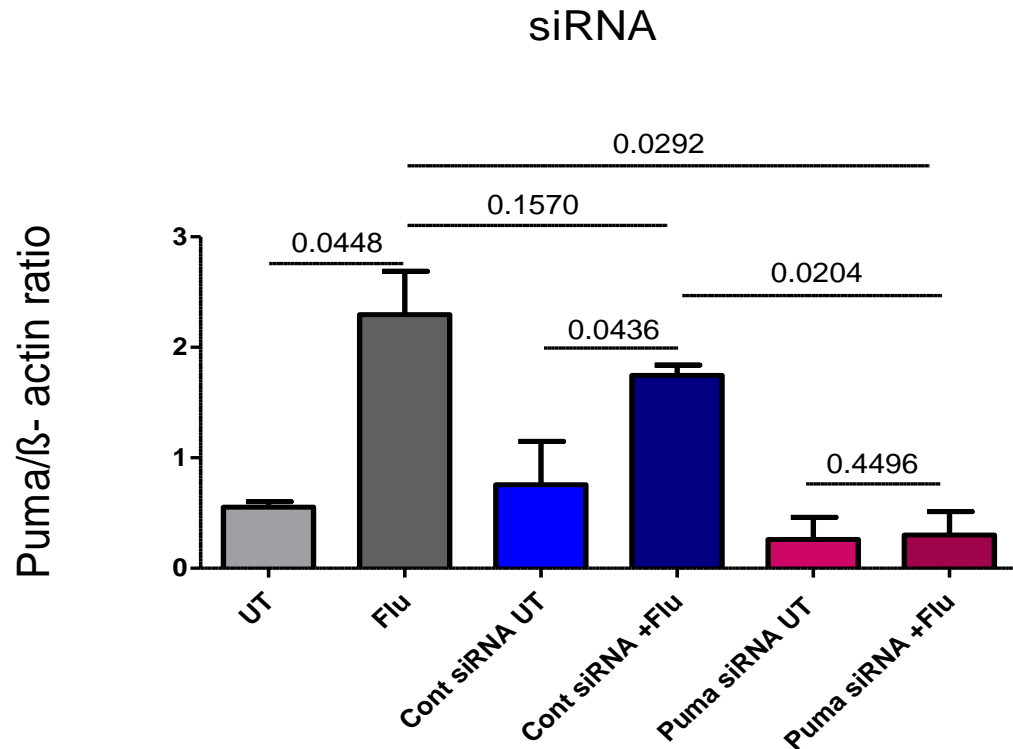


Figure 4.4. Pooled analysis of the effect of siRNA on Puma expression (n=3).

The ratio of Puma to actin was measured by densitometry in untreated (UT) or fludarabine-treated (Flu) CLL cells that had or had not been transfected with control siRNA or Puma-specific siRNA. Each data point in the graph represents the mean + SD of independent experiments using primary CLL cells from three patients. A two-tailed, paired T-test was performed to determine the statistical significance of the difference between the two groups of data. The respective *P* values have been shown.

I next examined the effect of Puma knockdown on Fludarabine-induced killing of CLL cells. To do this CLL cells (un-transfected and transfected with either control or Puma-specific siRNA) were cultured under standard conditions for 48 hours in the presence or absence of 10 μ M fludarabine. Cell death was measured using the PI/FACS method as described previously, and is shown for each CLL case in Figures 4.5 – 4.7, and summarized in Figure 4.8. As expected, fludarabine treatment

increased cell death in both un-transfected CLL cells and in CLL cells transfected with nonspecific siRNA. This is in line with previous results generated in this thesis (which figures 3.3 in chapter 3), and corresponds to induction of Puma expression in fludarabine treated cells (Figures 4.1 – 4.4). No significant difference in drug-induced cell death was observed between un-transfected CLL cells and CLL cells transfected with nonspecific siRNA ($p=0.121$), and this is consistent with the induction of Puma; the levels of Puma in fludarabine-treated cells was no different between un-transfected and control siRNA-transfected CLL cells ($p=0.157$). However, there was a reduction in fludarabine-induced cell death in cells that had previously been transfected with Puma-specific siRNA, only 30% of drug-specific cell death was observed in these cells. This reduction was statistically significant when compared with fludarabine-induced cell death in un-transfected ($p=0.0384$) and control siRNA-transfected cells ($p=0.0065$), and is consistent with the ability of fludarabine to induce Puma expression in cells transfected with Puma-specific siRNA which was significantly lower than in un-transfected ($p=0.0292$) and control siRNA-transfected cells ($p=0.0204$). Taken together, these results demonstrate that Fludarabine-induced upregulation of Puma is responsible for the induction of death in treated CLL cells.

n=3

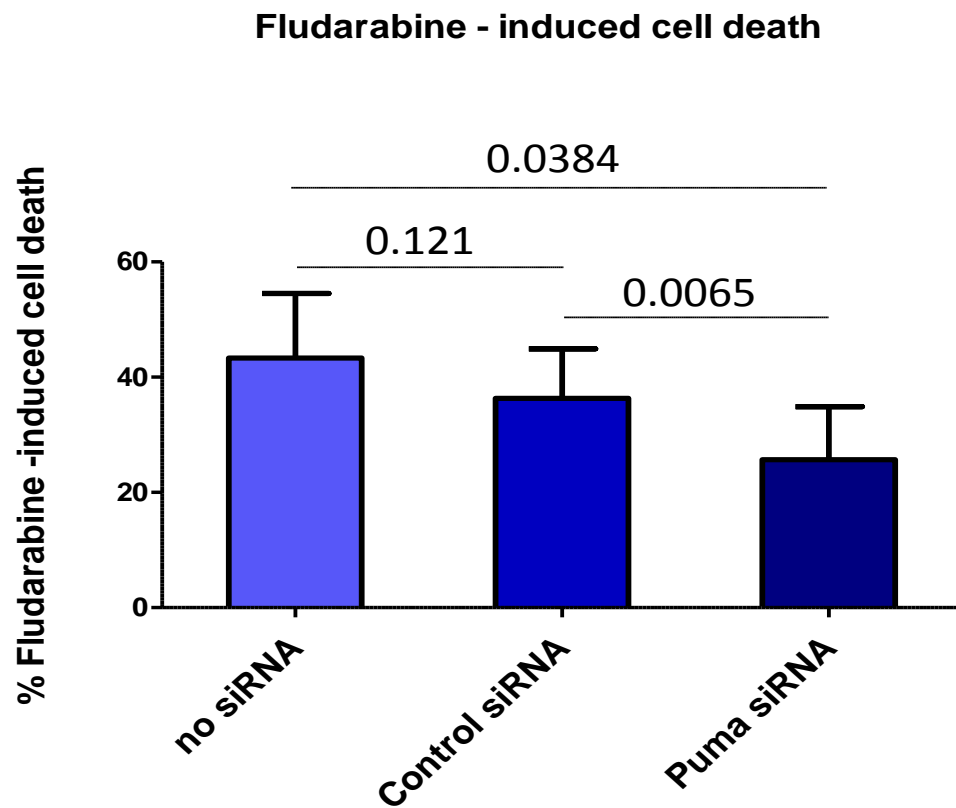


Figure 4.5. Pooled analysis of the effect of siRNA on fludarabine-induced killing as measured by the PI/FACS method (n=3).

The percentage of fludarabine-induced cell death was calculated as: $100 \times [(\% \text{ cell death of treated cells} - \% \text{ cell death of untreated cells}) / (100 - \% \text{ cell death of untreated cells})]$. Each data point in the graph represents the mean + SD of independent experiments using primary CLL cells from three patients. A two-tailed, paired t-test was performed to determine the statistical significance of the difference between the two groups of data.

4.4 Summary of results

In this Chapter I presented data using siRNA technology investigating the functional importance of Puma up-regulation within the mechanism of fludarabine-induced killing of CLL cells. Three CLL samples with high transfection efficiency by Nucleofector (2746, 2929, and 2911) were selected. Western blotting analysis of untreated and fludarabine-treated CLL cells showed that Puma-specific siRNA but not control siRNA prevented the up-regulation of Puma protein by fludarabine. Drug-specific killing was significantly reduced in the Puma siRNA-transfected cells compared with un-transfected cells or control siRNA-transfected cells.

4.5 Discussion

The aim of this Chapter was to clarify the functional importance of Puma concerning the mechanism of fludarabine-induced killing of CLL cells. Although it has been known for some time that fludarabine increases Puma expression in CLL cells at both the mRNA and protein level in a p53-dependent fashion (Mackus et al. 2005), the contribution of Puma to fludarabine-induced cytotoxicity has not been directly investigated. The data presented in this Chapter clearly show that knockdown of Puma by siRNA prevented fludarabine-induced Puma up-regulation and reduced fludarabine-induced cell death, thus demonstrating that the cytotoxicity of fludarabine in CLL cells is at least partly dependent on Puma. To my knowledge, this result provides the first direct demonstration that Puma contributes to the killing of CLL cells by fludarabine.

I attempted to measure the expression of Noxa and Bmf proteins by Western blotting, but for reasons yet unknown I could not detect the expression of either protein under the experimental conditions used. Time constraints prevented me from pursuing this further. However, future experiments could be designed to dissect the functional importance of these other pro-apoptotic Bcl-2 family proteins in the killing of CLL cells by fludarabine.

The experiments described in this Chapter do not include the co-culture system. This is because co-culture with either parental or CD40L-expressing fibroblasts rescued CLL cells from fludarabine-induced cell death. It is interesting, however, that Puma was similarly upregulated in CLL cells cultured under all the conditions described in this thesis when fludarabine was present. This suggests that the co-culture system must induce the expression of some factor which mitigates the pro-apoptotic role of Puma. Potentially, this rescue could be mediated by changes in expression of anti-apoptotic Bcl-2 family proteins in co-cultured cells as has been suggested in previous studies (Vogler et al. 2011; Willimott et al. 2007). Chapter 5 therefore investigates the interaction between Puma and anti-apoptotic Bcl-2 family proteins with the aim of clarifying the mechanism of the rescue effects provided by the co-culture system on fludarabine-induced CLL cell death.

Chapter 5

Characterisation of Puma-interacting proteins in CLL cells treated with fludarabine

5.1 Background and aims

Data in the previous Chapter show that Puma is a pivotal mediator of killing induced by fludarabine in CLL cells cultured under standard conditions. The data in Chapter 3 show that Puma is up-regulated by fludarabine in CLL cells cultured under standard and co-culture conditions. However, data in Chapter 3 also show that co-culture conditions, and particularly co-culture with CD40L-expressing fibroblasts, rescue CLL cells from drug-induced killing. Potentially, this rescue could be mediated by changes in expression of Bcl-2 family proteins in co-cultured cells as has been suggested in previous studies (Vogler et al. 2011; Willimott et al. 2007). Indeed, Figures 3.23 and 3.24 show that the anti-apoptotic Bcl-2 family proteins Mcl-1 and Bcl-XL are up-regulated in CLL cells co-cultured with CD40L-expressing fibroblasts. However, it is not clear from these observations how up-regulation of these proteins rescue CLL cells from Puma-induced apoptosis. The aim of this Chapter is, therefore, to investigate interactions between Puma and predominant anti-apoptotic Bcl-2 family proteins that are expressed in primary CLL cells, comparing associations observed in cells cultured under standard conditions with those co-cultured with CD40L-expressing and parental fibroblasts.

5.2 Methodology

5.2.1 Immunoprecipitation

Principle

Immunoprecipitation is a technique that is used for the purification of an antigen from a mixture of antigens by using the basic precipitation principle. Generally, an antibody specific to that concerned antigen is used for this purpose. The antibody usually attached to an agarose resin which act as a supporting bead. The antigen may arise from various sources such as tissues or cells, translated proteins and metabolically labelled cells. After the pre-immobilization of the specific polyclonal or monoclonal antibody in the insoluble solid support, incubation is done with the cell lysate that contains the required antigen. Sometimes mild agitation is required for binding of the target antigen with the specific antibody. The immune complex thus formed is immobilized and collected followed by elution from the insoluble support for subsequent analysis.

CLL cells were first washed in ice-cold PBS. The cell pellet from this washing step was lysed with 200µl IP lysis buffer (10mM HEPES (pH7.4), 150mM NaCl, 2mM EDTA, 1% CHAPS (Sigma, UK) and 1/100 dilutions of protease and phosphatase inhibitor cocktails from Sigma-Aldrich (Exeter, U.K). Cells suspended in this lysis were then agitated on a rotor mixer at 4°C for 1-2h. Samples were centrifuged at 13,000rcf for 15secs at 4°C, and pellets were discarded. 30µl of both protein A sepharose and protein G sepharose beads that were pre-rinsed in lysis buffer were

added to each sample, which were then rotor-mixed for 1h at 4°C (preclearing). Samples were pulse centrifuged at 10,000rcf, and a protein concentration of the pre-cleared supernatant was measured by DC protein assay (Biorad) according to the manufacturer's instructions. 100µg of protein/sample was used for immunoprecipitation with 2µg/sample of antibody targeting either Puma, Bcl-2, Mcl-1, or Bcl-XL. The antibody/cell lysate mixture was incubated overnight at 4°C, and then with 30µl of protein A sepharose beads that were pre-rinsed in lysis buffer. The samples were further mixed for 1h at 4°C on a rotor mixer, and then pulse centrifuged. Beads were washed in IP wash buffer (10mM HEPES (pH7.4), 150mM NaCl, 2mM EDTA, 0.2% CHAPS) 3 times, and the final pellet resuspended in 30µl total Laemmli sample buffer and prepared for PAGE.

SDS-PAGE and Western blotting were performed as described in section 3.2.3, with the exception that Exactacruz™ (Santa Cruz) secondary antibodies were used rather than standard secondary antibodies to reduce heavy and light-chain background.

5.3 Identification of Puma binding proteins in fludarabine-treated CLL cells cultured under standard conditions

Three CLL samples (2746, 2911 and 2929) were analysed using immunoprecipitation and Western blotting to assess interactions between Puma and the anti-apoptotic Bcl-2 family members. These particular samples were chosen because treatment with fludarabine increased the percentage of apoptotic CLL cells compared to untreated (control) cells under standard culture conditions (Figure 3.3),

and because fludarabine induced expression of Puma regardless of the conditions the CLL cells were cultured under (Figure 3.19).

Immunoprecipitation experiments were performed using CLL cells that were untreated or treated with fludarabine. In some experiments CLL cells were treated with dexamethasone as a control since this drug induces a similar level of killing but does not increase Puma expression.

CLL cells from sample 2746 were treated under standard conditions with fludarabine or dexamethasone for 48 h and then analysed for cell death using the PI/FACS assay. As expected, both dexamethasone and fludarabine increased the percentage cell death (Figure 5.1). Cell lysates were prepared for immunoprecipitation and analysed by Western blot. (Figure 5.1) (lanes 1 – 3) shows that Puma was detectable in whole cell lysates prepared from CLL cells that had been treated with fludarabine, but was not detectable in cell lysates prepared from untreated or dexamethasone-treated CLL cells. Puma was also detected in the pull-down fraction prepared from fludarabine-treated cells, but not from that prepared from untreated or dexamethasone-treated cells (Figure 5.1, lanes 4 – 6). Importantly, the post-IP fraction corresponding to fludarabine-treated cells showed little or no detectable Puma (Figure 5.1, lane 9), confirming that a high proportion of Puma had been immunoprecipitated.

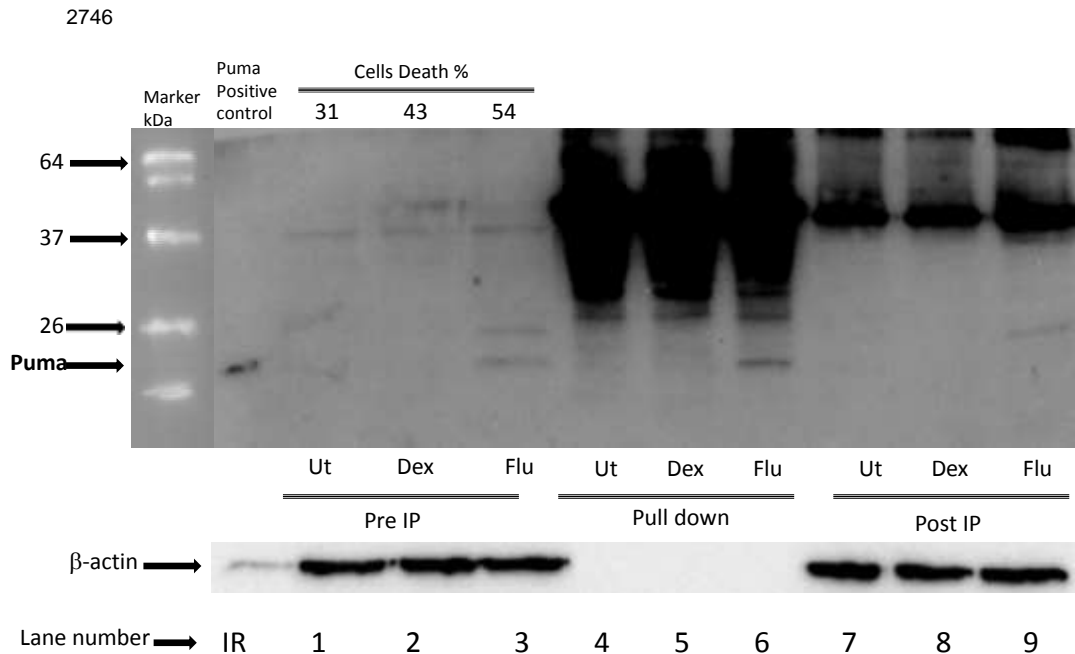


Figure 5.1. Immunoprecipitation of Puma from CLL cell lysates (sample 2746).

CLL cells were incubated with fludarabine (Flu, 10 μ M) or dexamethasone (Dex, 100 nM) for 48h. At the end of incubation, cells were harvested for analysis of cell death by FACS. Cell lysates were immunoprecipitated using an anti-Puma antibody (from Novus). The presence of Puma was then analysed by Western blot in un-manipulated whole cell lysates (Pre-IP fractions, lanes 1-3), in the pull-down protein complexes (lanes 4-6) and in post-immunoprecipitated lysates (Post-IP fractions, lanes 7-9). The anti-Puma antibody used for Western blotting was from Cell Signalling. Western blotting for the presence of β -actin was used as a protein loading control (bottom panel). Whole cell lysates of irradiated CLL cells (IR lane) was used as a positive control for Puma. A pre-stained protein mass marker was used to guide determination of the molecular weight associated with Puma also the blot was stripped and re-probed with anti-actin antibody

Having validated Puma immunoprecipitation I next investigated the proteins that co-immunoprecipitate with Puma. First, Bcl-2 was chosen because of its known interaction with Puma in other cell types (Edwards et al. 2013). Puma pull-down fractions, together with pre- and post-IP cell lysates, were subjected to Western blotting using an anti-Bcl-2 antibody (Figure 5.2). As expected, Bcl-2 was clearly detected in pre-IP lysates from untreated and drug-treated cells. Consistent with data presented in Chapter 3, Bcl-2 expression levels seemed to be higher in lysates from CLL cells treated with fludarabine. Analysis of the Puma pull down complexes (Figure 5.2, lanes 5 – 7) showed that Bcl-2 was present in the immunoprecipitate derived from fludarabine-treated CLL cells but not from untreated or dexamethasone-treated cells. Analysis of post-IP lysates showed that Bcl-2 was absent from fludarabine-treated CLL cells (Figure 5.2, lane 10), suggesting that it had been quantitatively removed with the immunoprecipitation of Puma. No such absence was observed in post-IP lysates derived from untreated or dexamethasone-treated CLL cells. These findings indicate that all available Bcl-2 was bound to up-regulated Puma.

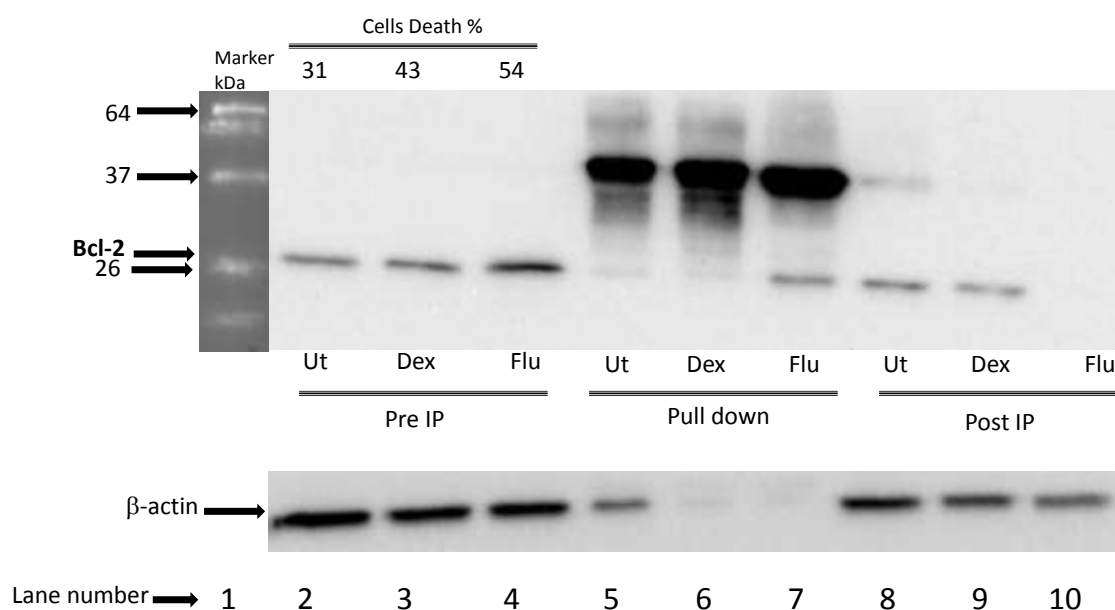


Figure 5.2. Puma interacts with Bcl-2 in fludarabine-treated CLL cells (sample 2746).

Un-manipulated whole cell lysates (lanes 2-4), the pull-down protein complexes associated with Puma immunoprecipitation (lanes 5-7) and post-IP protein lysates (lanes 8-10) from CLL cells prepared in Figure 5.1 were analysed by Western blot using an anti-Bcl-2 antibody (from Cell Signalling). Western blotting for β -actin was used as a protein loading control (bottom panel). Pre-stained protein mass marker was used as a guide for determining molecular weight of the protein of interest (lane 1) also the actin blot was performed on a separate gel loaded with the same cell lysates.

To confirm the co-association of Puma and Bcl-2 I performed a reciprocal immunoprecipitation experiment where lysates were immunoprecipitated using an anti-Bcl-2 antibody and pull-down samples analysed by immunoblotting with an anti-Puma antibody (Figure 5.3). Analysis of whole cell lysates showed, as expected, that Puma was up-regulated following fludarabine treatment in the pre-IP lysates (Figure 5.3, lanes 1 – 3). Examination of the Bcl-2 pull-down fractions

showed co-immunoprecipitation of Puma only in fludarabine-treated samples (Figure 5.3, lanes 4 – 6), confirming that up-regulated Puma binds to Bcl-2 in CLL cells. Importantly, Puma could be detected – albeit weakly - in the post-IP samples (Figure 5.3, lane 9), indicating up-regulated Puma was not completely bound to Bcl-2.

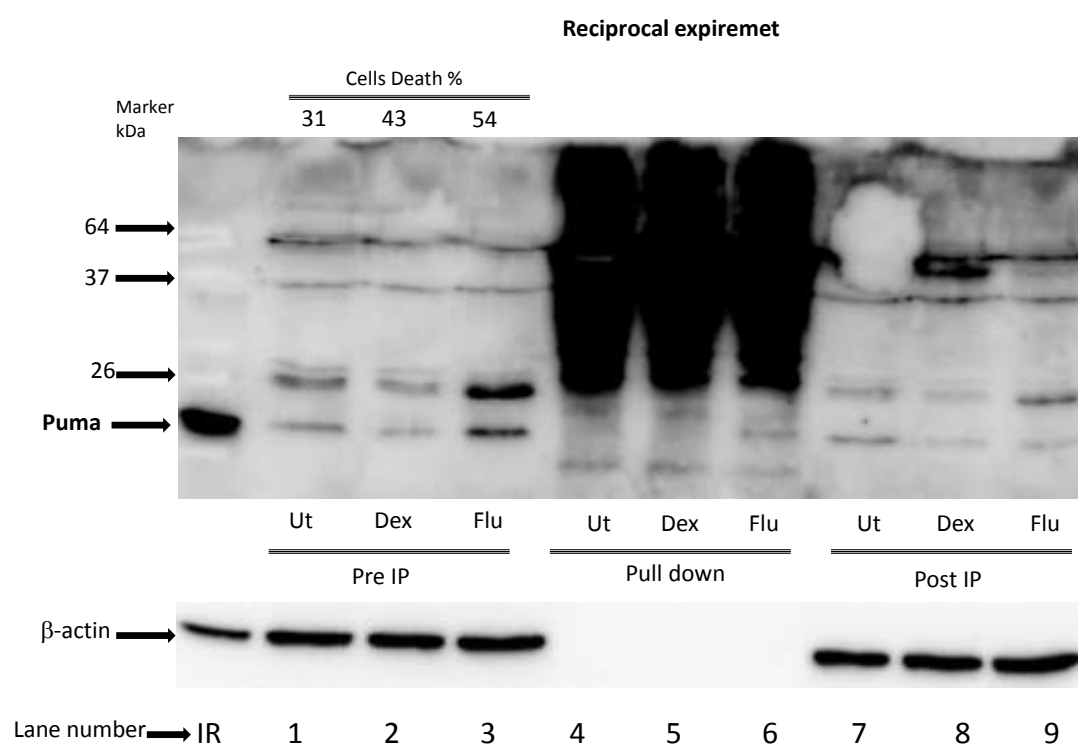


Figure 5.3. Reciprocal experiments confirmation of Bcl-2-Puma interaction in fludarabine-treated CLL cells (sample 2746). .)

Un-manipulated whole cell lysates (lanes 2-4), the pull-down protein complexes associated with Bcl2 immunoprecipitation using a Bcl-2 antibody from Cell Signalling (lanes 5-7) and post-IP protein lysates (lanes 8-10) from CLL cells prepared in Figure 5.1 were analysed by Western blot using an anti-Puma antibody (from Cell Signalling). Western blotting for β -actin was used as a protein loading control. Whole cell lysates of irradiated CLL cells (IR lane) was used as a positive control for Puma also the actin blot was performed on a separate gel loaded with the same cell lysates.

Taken together, the above results using reciprocal immunoprecipitation assays clearly demonstrate that Puma interacts with Bcl-2 in fludarabine-treated CLL cells. Importantly, whereas all of the Bcl-2 co-immunoprecipitated with Puma, not all of the Puma co-immunoprecipitated with Bcl-2. This observation is consistent with the idea that fludarabine-induced killing is triggered when levels of Puma exceed the neutralising capacity of Bcl-2. However, it is also important to understand the role of other anti-apoptotic Bcl-2 proteins as “Puma neutralisers”. Within this context I therefore investigated whether Puma interacted with Mcl-1 due to my own observation of Mcl-1 expression in CLL cells culture under standard conditions (Figure 3.23) and in reports of the abundant expression of this protein in unstimulated CLL cells (Clohessy, Zhuang & Brady 2004; Houlston & Catovsky 2008).

Analysis of Mcl-1 presence in Puma immunoprecipitates and in pre-IP and post-IP lysates derived from untreated, dexamethasone-treated and fludarabine-treated CLL cells cultured under standard conditions showed that Mcl-1 was up-regulated in pre-IP lysates (Figure 5.4, lane 4) and co-immunoprecipitated with Puma (Figure 5.4, lane 7) of CLL cells that were exposed to fludarabine. Importantly, Mcl-1 was completely depleted from post-IP lysates derived from fludarabine-treated cells (Figure 5.4., lane 10). This suggests that all of the available Mcl-1 was bound to up-regulated Puma. However, I was unable to confirm interaction between Puma and Mcl-1 in a reciprocal IP experiment involving IP for Mcl-1 and WB for Puma as the IP experiment did not work.

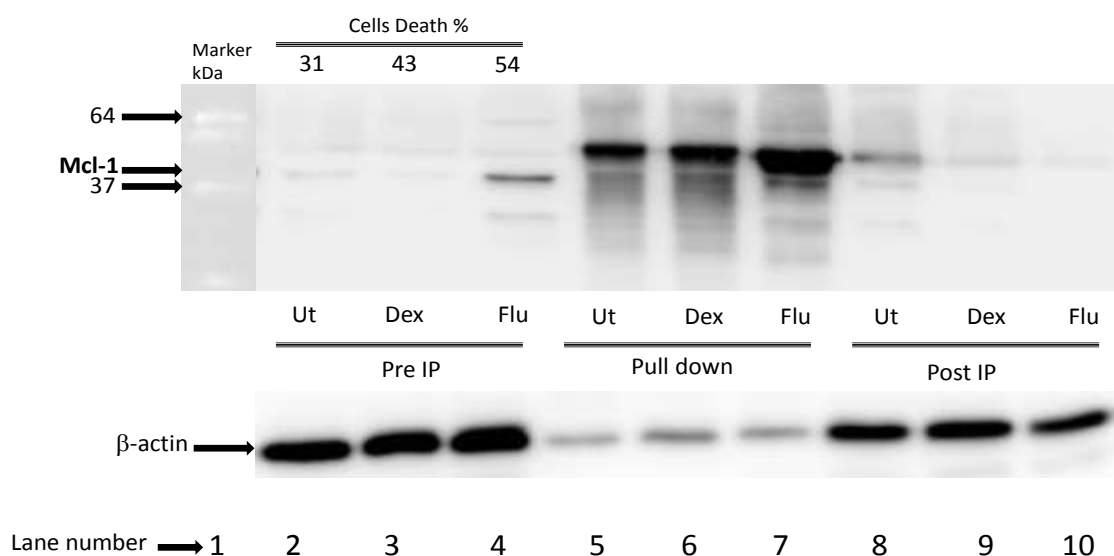


Figure 5.4. Detection of Puma-Mcl-1 binding in fludarabine-treated CLL cells (sample 2746).

Pre-IP whole cell lysates (lanes 2-4), the pull-down protein complexes associated with Puma immunoprecipitation (lanes 5-7) and post-IP protein lysates (lanes 8-10) from CLL cells prepared in Figure 5.1 were analysed by Western blot using an anti-Mcl-1 antibody (from Santa Cruz). Western blotting for β -actin was used as a protein loading control (bottom panel). Pre-stained protein mass marker was used as a guide for determining molecular weight of protein of interest (lane 1) also the actin blot was performed on a separate gel loaded with the same cell lysates.

These immunoprecipitation experiments were repeated twice more using CLL cases 2929 and 2911, and the Western blots are shown in supplementary data (figures 1- 8) of this thesis. A summary of all the results generated is shown in (Table 5.1).

Table 5.1. Overall summary of the immunoprecipitation data obtained from three fludarabine-treated CLL cells.

IP	WB	Case	WB target detected in pre-IP lysate	WB target detected in pull-down fraction	WB target detected in post-IP lysate
Puma	Bcl-2	2476	+	+	-
		2929	+	+	-
		2911	+	+	±
Bcl-2	Puma	2476	+	+	±
		2929	+	+	±
		2911	n/a	n/a	n/a
Puma	Mcl-1	2476	+	+	-
		2929	+	+	+
		2911	n/a	n/a	n/a
Mcl-1	Puma	2476	n/a	n/a	n/a
		2929	n/a	n/a	n/a
		2911	n/a	n/a	n/a

n/a indicates where data is unavailable.

In all three patient samples, Puma was consistently and selectively up-regulated by fludarabine, and Bcl-2 co-immunoprecipitated with up-regulated Puma and was not detectable in the corresponding post-IP lysates in two of the fludarabine-treated samples (2476 and 2929). Bcl-2 also co-immunoprecipitated with up-regulated Puma in the third sample (2911). However, the signal was weaker than in the previous two cases, and Bcl-2 was still detectable in the post-IP lysate of this case indicating incomplete depletion. Reciprocal immunoprecipitation experiments involving cases

2476 and 2929 showed that Puma co-immunoprecipitated with Bcl-2, but that residual levels of Puma was still detectable in the post-IP lysates, indicating that depletion was incomplete. Like Bcl-2, Mcl-1 co-immunoprecipitated with up-regulated Puma in cases 2476 and 2929. It was undetectable in post-IP lysates from case 2476, indicating complete depletion, whereas it was still detectable in post-IP lysates from case 2911, indicating partial depletion. Similar experiments involving case 2911 were not informative due to technical problems. A reciprocal immunoprecipitation of Mcl-1 and examination for co-associated Puma also did not yield information data due to technical problems. Overall, the data presented here show that Puma clearly interacts with Bcl-2 in fludarabine-treated CLL cells under standard culture conditions. Puma may also interact with Mcl-1 in these cells, but the evidence is not as strong.

5.4 Identification of Puma binding proteins in fludarabine-treated CLL cells cultured with parental and CD40L-expressing fibroblasts.

The previous section identified Bcl-2 and Mcl-1 as proteins which co-associate with Puma in fludarabine-treated CLL cells which have been cultured under standard conditions. It is also noted that up-regulated Puma appears to sequester most, if not all of Bcl-2 and Mcl-1 in these cells. This would account for the induction of cell death because Puma levels are likely to be in excess of Bcl-2 and Mcl-1. In contrast, co-culture with either parental or CD40L-expressing fibroblasts rescues CLL cells from fludarabine-induced killing. Considering that expression of Bcl-2 decreases in

CLL cells co-cultured with parental or CD40L-expressing fibroblasts (Figure 3.25), it seemed therefore necessary to examine expression of other Bcl-2-family proteins associated with Puma under these conditions.

(Figure 5.5) shows that fludarabine treatment results in up-regulation of Puma in CLL cells co-cultured with parental cells (PAR) or CD40L-expressing fibroblasts. The level of induction appears to be equivalent in each case because β -actin expression is constant in untreated and fludarabine-treated samples cultured with parental cells (PAR) or CD40L-expressing fibroblasts.

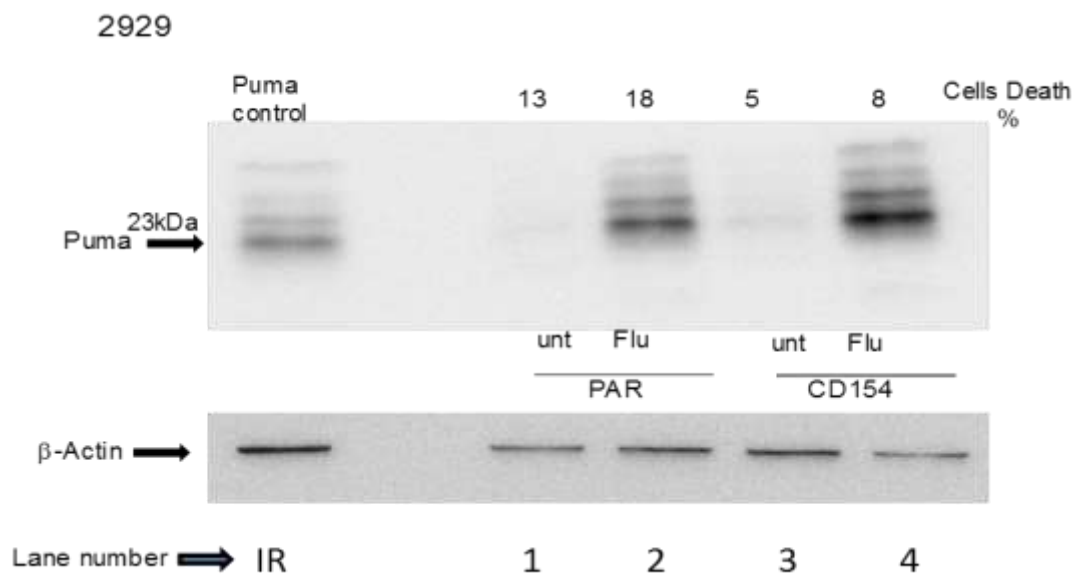


Figure 5.5. Detection of Puma in co-cultured CLL cells treated with fludarabine (sample 2929).

CLL cells were co-cultured with parental (PAR) or CD40L-expressing (CD154) fibroblasts. Co-cultured CLL cells were incubated with or without fludarabine (10 μ M) for 48 hours. At the end of incubation, cells were harvested for analysis of cell death by FACS and percent cell death is reported as indicated. Cell lysates were also prepared for Western blotting using anti-Puma antibody for the detection of Puma expression. Western blotting for β -actin was used as a protein loading control (bottom panel). Lysate prepared from γ -irradiated CLL cells was used as a positive control for Puma (lane IR), also the blot was stripped and re-probed with anti-actin antibody

After ascertaining that Puma was up-regulated in co-cultured CLL cells that were treated with fludarabine, I next performed immunoprecipitation experiments using an anti-Puma antibody and initially examined for the presence of co-associated Bcl-XL. Western blotting showed that Bcl-XL was present in pre-IP lysates prepared from untreated and fludarabine-treated CLL cells co-cultured with parental or CD40L-expressing fibroblasts (Figure 5.6, lanes 2 – 5). When Puma was immunoprecipitated, it was found that Bcl-xl associated with the pull down protein complex from the lysate sample prepared from fludarabine-treated CLL cells that had been co-cultured with CD40L-expressing fibroblasts (Figure 5.6, lanes 6 – 9). However, immunoprecipitation with Puma was unable to remove all Bcl-xl from CLL cell lysates (Figure 5.6, lanes 10 –13), indicating that Bcl- XL is either in excess of Puma or that it has a lower affinity for Puma than do proteins such as Bcl-2 and Mcl-1. Nevertheless, this experiment indicates that Puma and Bcl- XL are able to interact with each other in CLL cells.

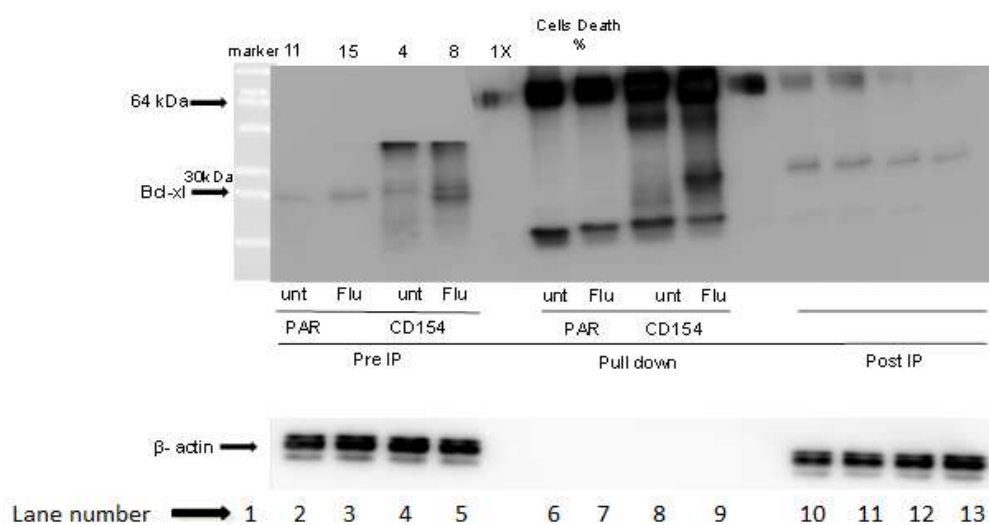


Figure 5.6. Puma interacts with Bcl-XL in fludarabine-treated CLL cells co-cultured with CD40L -expressing fibroblasts (CLL sample 2929).

Pre-IP whole cell lysates (lanes 2-5), the pull-down protein complexes associated with Puma immunoprecipitation (lanes 6-9) and post-IP protein lysates (lanes 10-12) from co-cultured CLL cells prepared in Figure 5.6 were analysed by Western blot using an anti-Bcl-XL antibody (from Cell Signalling). Western blotting for β -actin was used as a protein loading control (bottom panel). Pre-stained protein mass marker was used as a guide for determining molecular weight of protein of interest (lane 1) also the actin blot was performed on a separate gel loaded with the same cell lysates.

To confirm the interaction of Puma with Bcl-XL a reciprocal immunoprecipitation experiment was performed. (Figure 5.7), shows that Puma protein is detectable in pre-IP lysates (Figure 5.7, lanes 1-4) and in samples pulled-down with Bcl-XL (Figure 5.7, lanes 5-8) in those CLL samples treated with fludarabine. Puma was not detected within the post-IP lysates (Figure 5.7 lanes 9 – 12). Taken together with the results presented in (Figure 5.6), these results confirm co-association of Bcl-XL with Puma. Moreover, because residual Bcl-XL can be detected following Puma IP but residual Puma cannot be detected following Bcl-XL IP, these results further

suggest that Bcl-XL is in excess of Puma and is able to provide cytoprotection. This provides a framework for the pro-survival effect of co-culture.

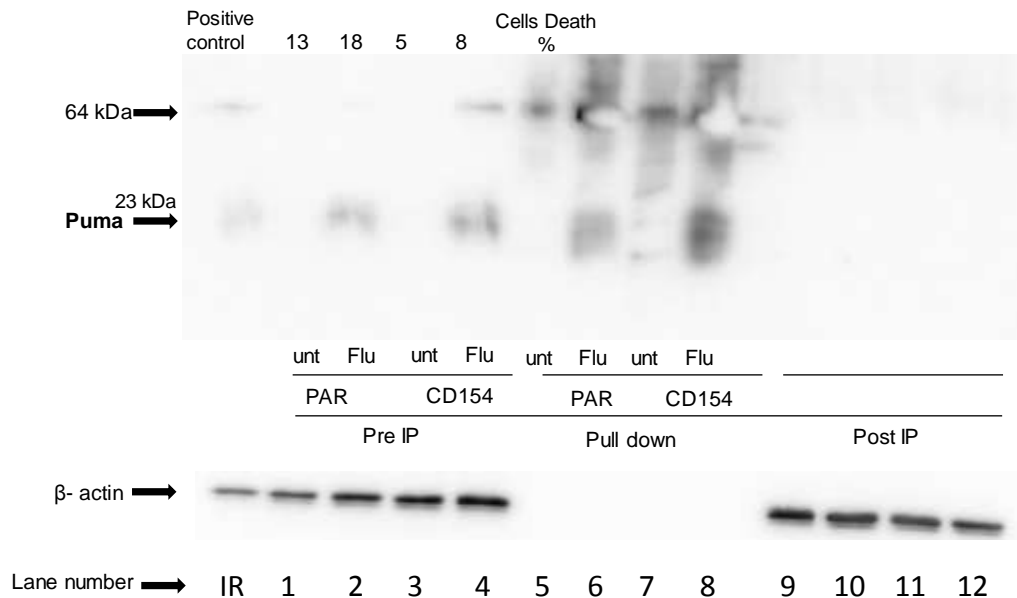


Figure 5.7. Detection of interaction of Bcl-XL with Puma in fludarabine-treated CLL cells on co-cultures (CLL sample 2929).

CLL cells co-cultured with parental (PAR) or CD40L-expressing fibroblasts (CD154) were incubated with fludarabine (Flu, 10 μ M) for 48h. At the end of incubation, cells were harvested for analysis of cell death by FACS as described earlier. Cell lysates were also prepared for immunoprecipitation assay using a Bcl-XL antibody (from Cell Signalling). Un-manipulated whole cell lysates (lanes 1-4), the pull-down protein complexes (lanes 5-8) and post-IP protein lysates (lanes 9-12) from the control cells (Ut) and fludarabine (Flu)-treated cells were analysed using SDS-PAGE, followed by Western blotting using anti-Puma antibody (from Cell Signalling). β -actin was probed for as protein loading controls. Lysate prepared from γ -irradiated CLL cells was used as a positive control for Puma (lane IR) also the blot was stripped and re-probed with anti-actin antibody.

Next, interaction of Puma with Mcl-1 was examined by performing similar immunoprecipitation experiments with the same set of lysates. As shown in (Figure 5.7) and supplementary data (Figures 9-17) equivocal results were produced. The Western blots produced by the anti-Mcl-1 antibody showed many reactive bands, and the band corresponding in Western blots to Mcl-1 did not change between parental- and CD40L-expressing fibroblasts (Figure 5.8).

Reciprocal immunoprecipitation experiments using anti Mcl-1 antibody were also performed on these lysates, and the results shown in (Figure 5.9). Western blotting using anti-Puma antibodies detected Puma protein in pre-IP lysates from fludarabine-treated CLL cells cultured either with parental or CD40L-expressing fibroblasts (Figure 5.9, lanes 1 - 4). Puma also seemed to co-immunoprecipitate with Mcl-1 in the pull-down fractions (Figure 5.9, lanes 5 - 8), whereas no Puma was observed in post-IP lysates (Figure 5.9, lanes 9 – 12). These results therefore suggest that Mcl-1 associates with Puma in co-cultured CLL cells.

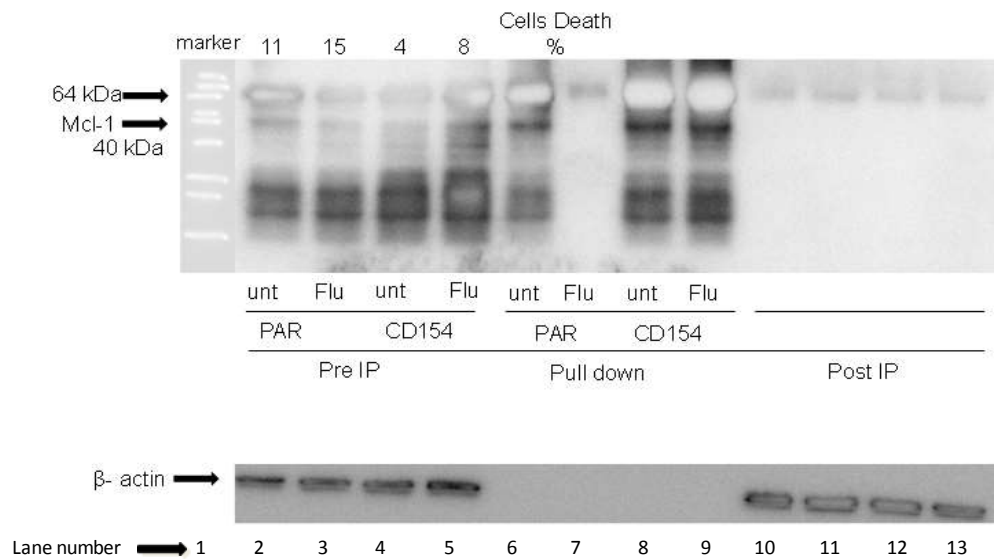


Figure 5.8. Detection of interaction of Puma with Mcl-1 in fludarabine-treated CLL cells co-cultured with CD154-expressing fibroblasts (CLL sample 2929).

CLL cells were incubated with fludarabine (Flu, 10 μ M) for 48h. At the end of incubation, cells were harvested for analysis of cell death by FACS as described earlier. Cell lysates were also prepared for immunoprecipitation assay using a Puma antibody (from Novus). Un-manipulated whole cell lysates (lanes 2-5), the pull-down protein complexes (lanes 6-9) and post-IP protein lysates (lanes 10-13) from the control cells (Ut) and fludarabine (Flu)-treated cells were analysed using SDS-PAGE, followed by Western blotting using anti- Mcl-1 antibody (from Santa Cruz). β -actin was probed for as protein loading controls. Pre-stained protein mass marker was used as a guide for determining molecular weight of protein of interest (lane 1) also the blot was stripped and re-probed with anti-actin antibody.

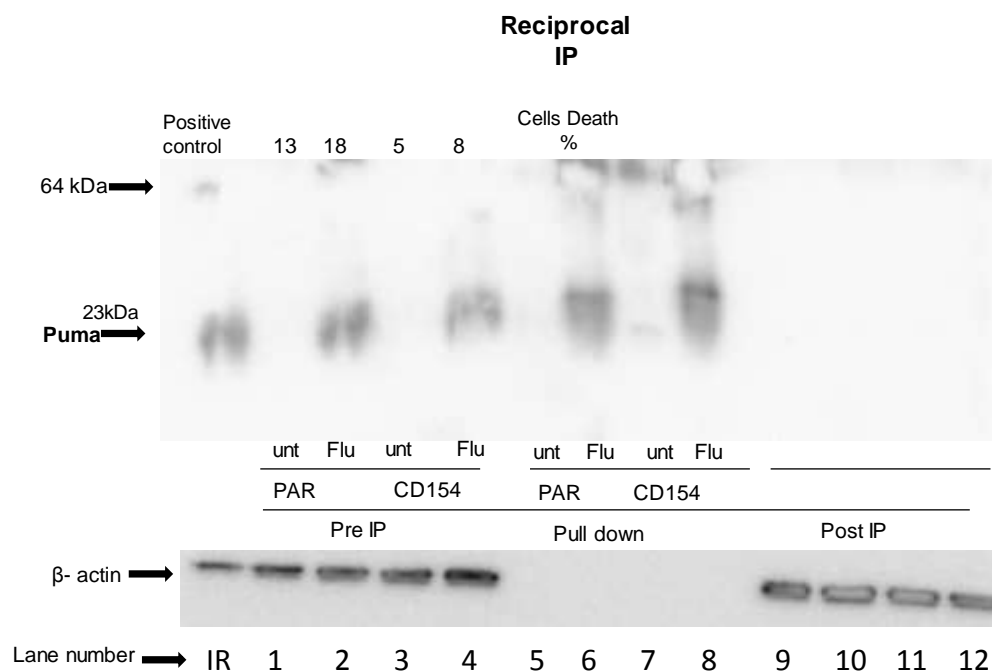


Figure 5.9. Detection of interaction of Mcl-1 with Puma in fludarabine-treated CLL cells on co-cultures (CLL sample 2929).

CLL cells co-cultured with parental (PAR) or CD40L -expressing fibroblasts (CD154) were incubated with fludarabine (Flu, 10 μ M) for 48h. At the end of incubation, cells were harvested for analysis of cell death by FACS as described earlier. Cell lysates were also prepared for immunoprecipitation assay using a Mcl-1 antibody (from Santa Cruz). Un-manipulated whole cell lysates (lanes 2-5), the pull-down protein complexes (lanes 6-9) and post-IP protein lysates (lanes 10-13) from the control cells (Ut) and fludarabine (Flu)-treated cells were analysed using SDS-PAGE, followed by Western blotting using anti-Puma antibody (from cell signalling). β -actin was probed for as protein loading controls. Pre-stained protein mass marker was used as a guide for determining molecular weight of protein of interest (lane 1) also the actin blot was performed on a separate gel loaded with the same cell lysates

5.5 Summary of results

Having shown that fludarabine treatment up-regulates Puma in CLL cells (Chapter 3), and that Puma contributes to fludarabine-induced killing of these cells under standard culture conditions (Chapter 4), the present Chapter sought to identify interactions between up-regulated Puma and specific anti-apoptotic Bcl-2 proteins. I focussed my attention on Bcl-2, Bcl-XL and Mcl-1 as these are thought to be the most abundant anti-apoptotic Bcl-2 proteins in CLL cells. I show that Puma associates with Bcl-2 and Mcl-1 in fludarabine-treated CLL cells cultured under standard conditions. When CLL cells are subjected to co-culture conditions Puma co-associated also with Bcl- XL. It seemed that when CLL cells were cultured under standard conditions, the level of induced Puma exceeded the levels of Bcl-2 and Mcl-1. Conversely, when CLL cells were co-cultured with parental or CD40L-expressing fibroblasts then the levels of Bcl- XL and perhaps also Mcl-1 exceeded the level of Puma. These results therefore suggest that the excess of Bcl- XL and Mcl-1 induced under co-culture conditions was sufficient to provide fludarabine-treated CLL cells with protection against apoptosis.

5.6. Discussion

Puma is a pro-apoptotic BH3-only protein which can be activated by cellular stresses including DNA damage, endoplasmic reticulum stress and growth factor deprivation (Wang & Kaufman 2014). Understanding how this protein functions on a molecular level within CLL cells gives understanding of the effectiveness of therapy using agents, such as fludarabine, which stimulate its expression. This understanding is particularly important with respect to mechanisms of drug resistance induced by microenvironment signals.

In this chapter I examine proteins which co-immunoprecipitate with Puma in CLL cells. Under standard culture conditions Puma primarily co-associates with Bcl-2 and Mcl-1. However, I find that the level of expression of these proteins is insufficient to overcome apoptotic effect of Puma induced by fludarabine. Thus, my results show that immunoprecipitation of Puma results in depletion of Bcl-2 and Mcl-1 from cell lysates of CLL cells cultured under standard conditions (Figure 5.4). However, immunoprecipitation of Bcl-2 does not completely remove Puma (Figure 5.3). Therefore, the residual Puma present in these cells likely accounts for the induction of apoptosis. If Puma expression can be reduced, such as through using targeted siRNA as I demonstrated in the previous chapter, CLL cell viability in the presence of fludarabine could be maintained. Similarly, if expression of an anti-apoptotic Bcl-2 family protein, such as Bcl-XL, can be induced so that Puma is fully sequestered, then rescue from fludarabine-induced death can also be achieved. This latter case is observed when CLL cells are co-cultured with parental and CD40L-expressing fibroblasts.

My experiments also show that Mcl-1 associates with Puma in co-cultured CLL cells. However, technical problems prevented me from making quantitative assessment of the data. The quality of Western blots of Mcl-1 was poor and bands corresponding to Mcl-1 in molecular weight seemed to migrate close to the Ig heavy chain in immunoprecipitated samples. This made interpretation difficult, particularly because upregulated Mcl-1 expression was not clearly observed in the CD40L-stimulated CLL cells.

Nevertheless, my experiments present a model of drug resistance mechanism using the co-culture conditions. Thus, co-culture induces the expression of Bcl-XL and Mcl-1 in sufficient quantity to overcome the pro-apoptotic effect of Puma induced by fludarabine. This then rescues the cells from fludarabine-induced apoptosis. This model therefore presents a potential avenue of therapeutic intervention. Specific targeting of either Bcl-XL or Mcl-1 in CLL cells on co-culture should release sequestered Puma and cause increased cell death. This notion is supported by studies showing that compounds such as (Bcl-XL inhibitor) and (Mcl-1 inhibitor) can effectively restore cell death in co-culture models (Choudhary et al. 2015; Pan et al. 2015). How Puma activates Bax and/or Bak is still a subject of debate; some have suggesting a direct activating role where activation of Bax/Bak occurs through direct interaction with Puma, while others have suggesting a sensitisation role where Puma binds to anti-apoptotic Bcl-2 family proteins resulting in the release of Bax and/or Bak (Bender & Martinou 2013; Ren et al. 2010).

According to the indirect model of Bax/Bak activation, even a relatively small increase in Puma may be sufficient to displace Bax and/or Bak from Bcl-2 and Mcl-1 to activate the mitochondrial death pathway. In contrast, the direct model of Bak/Bak

activation requires that the amount of Puma exceeds that of Bcl-2 and Mcl-1 to neutralise the latter in order for apoptosis to occur (Ren et al. 2010). This criterion appears to have been reached in case 2476 since lysates that had been depleted of Puma were also completely depleted of Bcl-2 and Mcl-1, whereas lysates that had been depleted of Bcl-2 and Mcl-1 still contained detectable amounts of Puma. This suggests that the amount of Puma exceeded the that of Bcl-2 and Mcl-1 to neutralise it. The situation in case 2911 was quite different in that Bcl-2 was readily detectable in lysates that has been depleted of Puma. It therefore seems likely that the fludarabine-induced killing that occurred in this sample was triggered before the maximum Puma-neutralising capacity of Bcl-2 had been reached; only the indirect model of Bax/Bak activation can explain the induction of apoptosis under these circumstances. Finally, interaction of Puma with Bcl-XL and Mcl-1 was shown by pull down experiments using anti-Puma antibodies, which were also confirmed by reciprocal immunoprecipitation experiments with anti-Bcl-XL and anti-Mcl-1 antibodies, respectively. With the exception of reciprocal immunoprecipitation experiments with CLL sample 2746, in which there were technical problems, results of interactions between Puma with Bcl-XL and Mcl-1 were consistent in the CLL samples studied. This suggests that the Puma protein upregulated by fludarabine in co-cultured CLL cells was bound to Bcl-XL and Mcl-1, two molecules specifically up-regulated by CD40 stimulation. Although, due to time constraints, I did not perform knockdown experiments in CD40-stimulated CLL cells to test if knockdown of Bcl-XL or Mcl-1 by their respective siRNAs will restore the sensitivity to fludarabine, it is most likely that the fludarabine-upregulated Puma was sequestered

by Bcl-XL and Mcl-1 in CD40-stimulated CLL cells, resulting in inhibition of Puma to activate downstream apoptotic effector molecules such as Bax and/or Bak.

Chapter 6

General Discussion

6.1 General Discussion

The aim of this thesis was to gain further insight into the mechanisms of drug resistance in CLL. The heterogeneous response of CLL patients to therapy suggest that there are multiple mechanisms involved, yet it is likely that the Bcl-2 family of proteins play an essential role in determining the sensitivity of CLL cells to therapeutic agents because of the instrumental role this family of proteins plays in regulating the mitochondrial death pathway. CLL cells rapidly undergo apoptosis *in vitro* and this strongly suggests that these cells receive important pro-survival signals from their microenvironment. Within the microenvironment pro-survival and proliferative signals can be provided by accessory cells such as stromal cells, dendritic cells, nurse-like cells and T-cells. The latter express CD40L (also known as CD154) on their surface that engages its corresponding receptor on the surface of CLL cells. This stimulation has been shown to protect CLL cells from spontaneous and drug-induced apoptosis. Others have also shown that such stimulation can cause increased expression of anti-apoptotic members Bcl-2 family proteins (Hussein et al. 2009; Vogler et al. 2011; Willimott et al. 2007).

However, several questions still remain un-answered in this area of research. Therefore, this thesis sought to expand on this knowledge by addressing the specific research questions articulated in section 1.11, i.e.:

- 1). How do drugs that induce p53-dependent (fludarabine) or p53-independent (dexamethasone) apoptosis affect the levels of Bcl-2 family proteins in CLL cells cultured in the presence or absence of CD40 stimulation?
- 2). How does CD40 stimulation affect the levels of Bcl-2 family proteins in CLL cells cultured in the presence or absence of cytotoxic drugs?
- 3). How do pro- and anti-apoptotic Bcl-2 family proteins interact in CLL cells, and how are these interactions influenced by drug treatment and CD40 stimulation?
- 4). How do interactions between pro- and anti-apoptotic Bcl-2 family proteins influence the fate of CLL cells following drug treatment?

The purine analogue fludarabine is a widely used class of cytotoxic drugs that has been established as a backbone of chemotherapy for CLL. However, most patients eventually become resistant to the chemotherapy, a situation that is frequently associated with deletion/mutation of the TP53 tumour suppressor gene. In keeping with their p53-independent mechanism of action, glucocorticoids (GCs) such as dexamethasone, either alone or in combination with other agents, have emerged as a useful and important treatment option for patients with fludarabine-refractory or TP53-defective CLL (Pettitt et al. 2012; Steele et al. 2008; Zenz et al. 2010). However, as with chemotherapy, response to glucocorticoids is variable.

In Chapter 3, I evaluated the effect of fludarabine and dexamethasone on CLL cells cultured alone or with mouse fibroblasts expressing human CD40L. Both parental

and CD40L-expressing fibroblasts inhibited the spontaneous apoptosis of CLL cells as previously reported. Under standard conditions, dexamethasone and fludarabine caused a significant increase in cell death following 48h incubation in primary CLL cells from 6 different CLL patients. However, co-culturing of CLL cells on parental, and to a greater extent CD40L-expressing fibroblasts markedly attenuated spontaneous, fludarabine- and dexamethasone-induced apoptosis, confirming previous reports showing that CLL cells become resistant to drug-induced apoptosis when they are stimulated by the microenvironmental factors (de Toter et al. 2003; Kater et al. 2004).

It has already been shown that CLL cells isolated from the peripheral blood differ in levels of expression of Bcl-2 family of proteins to those extracted from the lymph node, but the effect of therapeutic agents on these levels of expression was still unknown. The data presented in this study demonstrated that culturing of CLL cells on CD40L-expressing fibroblasts increased the expression of Mcl-1 and Bcl-XL and this increase was associated with insensitiveness to fludarabine or dexamethasone treatment. Culturing CLL cells on parental fibroblasts was sufficient enough to cause an increase in Bcl-XL expression suggesting its expression can also be upregulated by a CD40-independent mechanism. Treatment with fludarabine induced the expression of Puma in CLL cells under standard and co-culture conditions but failed to cause apoptosis in cells co-cultured with CD40L-expressing fibroblasts. This demonstrated that regulation of apoptosis is far more complex than at the expression levels of Bcl-2 family of proteins. Each anti-apoptotic member has a preference as to which pro-apoptotic member it binds and this can be cell-type specific (Willimott et al. 2007; Youle & Strasser 2008). The increase in other anti-apoptotic proteins such

as Mcl-1, and Bcl-XL induced by CD40 stimulation may shift the balance in favour of survival by changing protein-protein interaction pattern with corresponding binding partners (Edwards et al. 2013).

In Chapter 4, I have established the role of Puma in fludarabine-induced cell death in CLL cells. Although it has been known for a while that Puma was induced at both messenger and protein levels by fludarabine via p53-dependent mechanism in CLL (Mackus et al. 2005), the exact role of Puma in fludarabine-induced cell death was not clearly established. The data presented in this study clearly showed that fludarabine up-regulated the expression of Puma and that knockdown of Puma by siRNA resulted in a reduction in cell death induced by fludarabine, thus demonstrating that Puma is required for fludarabine-induced cell death in CLL cells. This result is, to my knowledge, the first demonstration that Puma is critically involved in mediating cell killing by fludarabine in CLL cells.

In Chapter 5, I have investigated binding partners of BH3-only protein Puma up-regulated by fludarabine, as how it activates Bax and/or Bak is still a subject of debate. Thus, some studies provided experimental evidence suggesting that a direct activating mechanism was involved while others reported findings supporting an indirect activation model (Bender & Martinou 2013; Moldoveanu et al. 2014; Ren et al. 2010). The undisputable importance of Puma in the regulation of cell death is underscored by its ability to interact with all anti-apoptotic Bcl-2 family proteins. I have detected two interacting partners of Puma, Bcl-2 and Mcl-1, in un-stimulated CLL cells. I have also detected additional Puma-interacting protein Bcl-XL in CLL cells co-cultured with CD40L-expressing fibroblasts. Given that the overexpression

of Bcl-2 is one of the hallmarks of CLL (Buggins & Pepper 2010; Kitada et al. 1998) and that high Mcl-1 expression is associated with a poor disease prognosis understanding the characteristics of these interactions is important as it may provide a rational basis in selecting targets for intervention in cancer. Puma expression is p53-inducible (Jeffers et al. 2003; Mackus et al. 2005; Ren et al. 2010), and increases rapidly upon treatment with chemotherapeutic agents such as fludarabine that are used in the first-line treatment of CLL. In the present study I confirmed that Puma expression increases upon fludarabine treatment in primary CLL cells. I also detected the interactions of Puma with both Bcl-2 and Mcl-1 in fludarabine-treated CLL cells, indicating that Puma may bind to these two anti-apoptotic members of Bcl-2 family of proteins to release Bax and/or Bak activating mitochondrial apoptosis pathway. Considering that fludarabine induced a similar level of CLL-cell death in all samples, and taking into account previous reports on the additive effect of Bcl-2 inhibitors to cytotoxicity by fludarabine in CLL cells, it is likely that Bcl-2 inhibitors will increase cytotoxic efficacy of fludarabine (Campas et al. 2006; Kang & Reynolds 2009).

Although I established that Puma is required for fludarabine-induced cell death, other proteins such as Noxa and Bmf may also be involved because even in the absence of Puma as a result of siRNA knockdown, fludarabine still induced cell death, albeit to a lesser degree.

Also I analysed in this chapter the binding partners of Puma in fludarabine-treated CLL cells co-cultured with CD40L-expressing fibroblasts and found that Bcl-XL and Mcl-1 were bound to Puma. There was some heterogeneity as I could not detect binding of Puma to Bcl-XL or Mcl-1 in one sample (2911).

I have shown that co-cultured CLL cells displayed increased Bcl-XL and Mcl-1 and that Puma binds to these two proteins. Therefore, it is possible that fludarabine resistance is partly due to increased expression of Bcl-XL and Mcl-1 which bind and inhibit Puma preventing apoptosis induction.

Puma is a pro-apoptotic BH3-only protein which can be activated by cellular stresses including DNA damage, endoplasmic reticulum stress and growth factor deprivation (Wang & Kaufman 2014). Understanding how this protein functions on a molecular level within CLL cells could provide insight into the effectiveness of therapeutic agents, such as fludarabine, which stimulate its expression. This understanding is particularly important with respect to mechanisms of drug resistance mediated by microenvironment-derived pro-survival signals.

In this chapter I examined proteins which interact with Puma in CLL cells. Under standard culture conditions Puma primarily co-associates with Bcl-2 and Mcl-1. However, I find that the level of expression of these proteins is insufficient to overcome the induction of apoptosis by Puma induced by fludarabine. If Puma expression can be reduced, such as through using targeted siRNA as I demonstrated in the previous chapter, CLL-cell viability in the presence of fludarabine is maintained. Similarly, if expression of an anti-apoptotic Bcl-2 family protein, such as Bcl-XL, can be induced so that Puma is fully sequestered, then rescue from fludarabine-induced death can also be achieved. This latter scenario is observed when CLL cells are co-cultured with parental and CD40L-expressing fibroblasts. Such co-culture induces expression of Bcl-XL which interacts with Puma. This is demonstrated by my experiments showing that immunoprecipitation of Bcl-XL

quantitatively removes Puma (Figure 5.7), but the converse experiment leaves residual Bcl-XL within lysates of fludarabine-treated CLL cells (Figure 5.6).

My experiments also showed that Mcl-1 associates with Puma in co-cultured CLL cells. It is likely that induced Mcl-1 also interacts with Puma because immunoprecipitation of the former quantitatively removed the latter from lysates of fludarabine-treated CLL cells (Figure 5.9). However, technical problems render this conclusion inconclusive. The quality of Western blots of Mcl-1 was poor and bands corresponding to Mcl-1 in molecular weight seemed to migrate close to the Ig heavy chain in immunoprecipitated samples. This made interpretation difficult, particularly because upregulated Mcl-1 expression was not clearly observed in the CD40-stimulated CLL cells.

Nevertheless, my experiments potentially provide an explanation for CD40-mediated drug resistance as mimicked by the co-culture conditions. Co-culture induces the expression of Bcl-XL and Mcl-1 in sufficient quantities to bind and inhibit the apoptotic function of the upregulated Puma. This then rescues the cells from fludarabine-induced apoptosis. This in turn presents a potential avenue of therapeutic intervention. Specific targeting of either Bcl-XL or Mcl-1 in co-cultured CLL cells should release sequestered Puma and cause cell death. This notion is supported by studies showing that compounds such as (Bcl-XL inhibitor) and (Mcl-1 inhibitor) can effectively restore cell death in co-culture models (Choudhary et al. 2015).

6.2 Conclusion

In conclusion, this work sought to explore in greater detail the resistance mechanism in CD40-stimulated CLL cells in response to drug-induced, p53-dependent apoptosis. Microenvironmental stimuli such as CD40 stimulation shift the balance of cellular fate in favour of survival by up-regulating anti-apoptotic members of Bcl-2 family of proteins including Bcl-XL and Mcl-1. It is shown that these proteins can bind to pro-apoptotic members of the Bcl-2 family of proteins and prevent apoptosis induction. This study is the first to demonstrate a possible mechanism of fludarabine resistance in CLL cells where CD40 stimulation up-regulated expression of Mcl-1 and Bcl-XL which sequestered and inhibited apoptotic activity of fludarabine-induced Puma in CLL cells. This inhibition might be relevant in the survival and expansion of the malignant clone in these cells. Future studies would be to clarify the importance of the interacting partners of Puma in mediating fludarabine resistance using siRNAs specific to Bcl-XL and/or Mcl-1 in CD40-stimulated CLL cells. Furthermore, it would be very interesting to examine if fludarabine-resistant CLL cells can be induced to undergo apoptosis when treated in combination with selective Bcl-XL or Mcl-1 inhibitors. The findings from these studies would be very important in helping design less toxic individualised therapies in CLL.

Chapter 7

References

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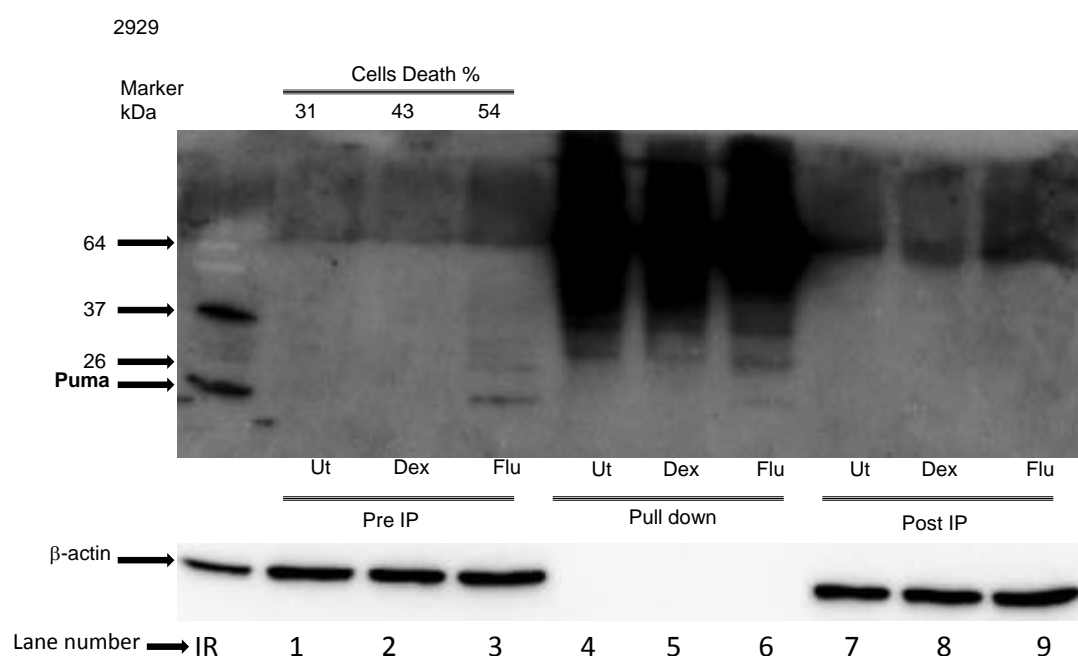
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Supplementary data

1. Figures associated with repeated Puma co-association experiments using different CLL cases.

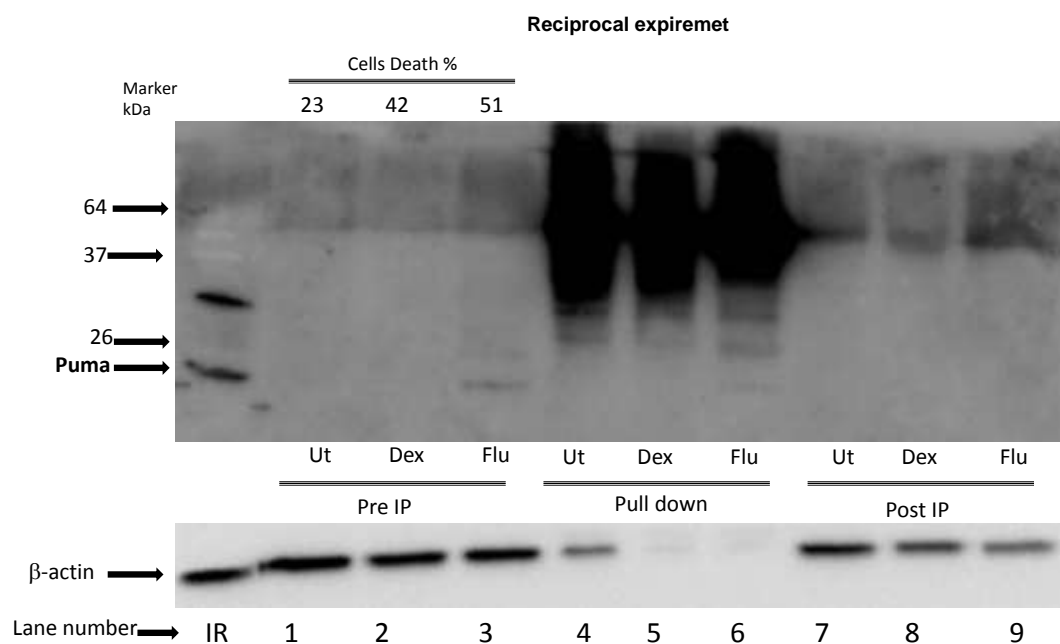
1.1. Identification of Puma binding proteins in fludarabine-treated CLL cells cultured under standard conditions

- Case no 2929



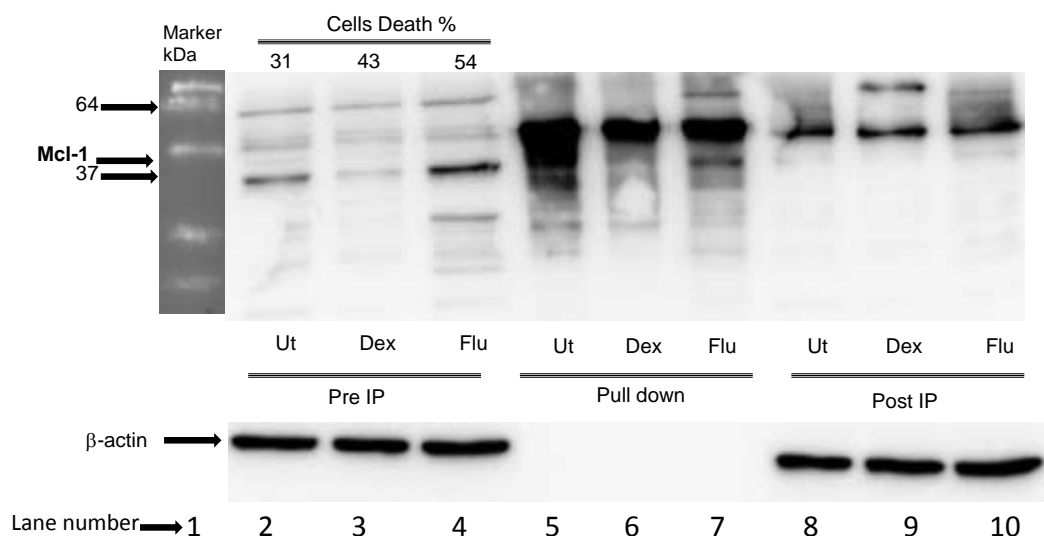
(Figure 1) Immunoprecipitation of Puma in CLL cells (sample 2929).

CLL cells were incubated with fludarabine (Flu, 10 μ M) or dexamethasone (Dex, 100 nM) for 48h. At the end of incubation, cells were harvested for analysis of cell death by FACS as described earlier. Cell lysates were also prepared for immunoprecipitation assay using an anti-Puma antibody (from Novus) as described in Methodology. The presence of Puma in the pull-down protein complexes (lanes 4-6), together with un-manipulated whole cell lysate (Pre-IP fractions, lanes 1-3) and post-immunoprecipitated lysates (Post-IP fractions, lanes 7-9) was analysed using SDS-PAGE, followed by Western blotting using a second anti-Puma antibody (from Cell Signalling). β -actin was probed for as protein loading controls. Pre-stained protein mass marker and a positive control for Puma (IR) were also used. actin blot was performed on a separate gel loaded with the same cell lysates



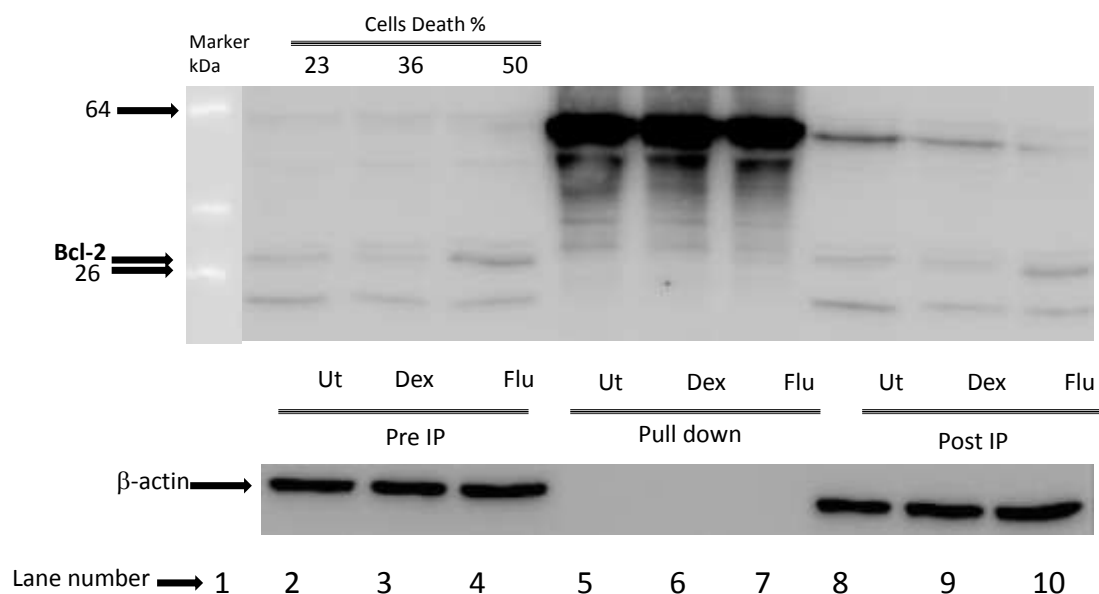
(Figure 3) Confirmation of Bcl-2-Puma interaction in primary CLL cells treated with fludarabine (sample 2929).

CLL cells were incubated with fludarabine (Flu, 10 μ M) or dexamethasone (Dex, 100 nM) for 48h. At the end of incubation, cells were harvested for analysis of cell death by FACS as described earlier. Cell lysates were also prepared for immunoprecipitation assay using a Bcl-2 antibody (from Cell Signalling). Un-manipulated whole cell lysates (Pre IP samples, lanes 1-3), the pull-down protein complexes (lanes 4-6) and post IP protein lysates (lanes 7-9) from the control cells (Ut), dexamethasone (Dex)- and fludarabine (Flu)-treated cells were analysed using SDS-PAGE, followed by Western blotting using anti-Puma antibody (from Cell Signalling). β -actin was probed for as protein loading controls. Pre-stained protein mass marker and a positive sample for Puma (IR) also the actin blot was performed on a separate gel loaded with the same cell lysates.



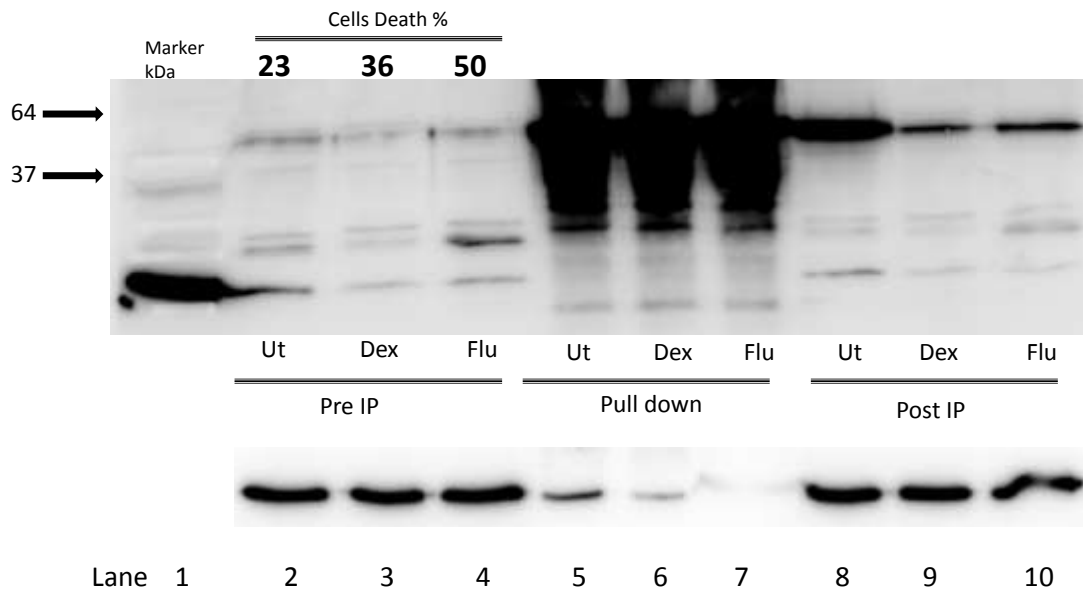
(Figure 4) Detection of Puma-Mcl-1 binding in primary CLL cells treated with fludarabine (sample 2929).

CLL cells were incubated with fludarabine (Flu, 10 μ M) or dexamethasone (Dex, 100 nM) for 48h. At the end of incubation, cells were harvested for analysis of cell death by FACS as described earlier. Cell lysates were also prepared for immunoprecipitation assay using a Puma antibody (from Novus). Un-manipulated whole cell lysates (Pre IP samples, lanes 2-4), the pull-down protein complexes (lanes 5-7) and post IP protein lysates (lanes 8-10) from the control cells (Ut), dexamethasone (Dex)- and fludarabine (Flu)-treated cells were analysed using SDS-PAGE, followed by Western blotting using anti-Mcl-1 antibody (from Santa Cruz). β -actin was probed for as protein loading controls. Pre-stained protein mass marker was used as a guide for determining molecular weight of protein of interest (lane 1) also he actin blot was performed on a separate gel loaded with the same cell lysates.



(Figure 6) Interaction of Puma with Bcl-2 in fludarabine-treated CLL cells (sample 2911).

CLL cells were incubated with fludarabine (Flu, 10 μ M) or dexamethasone (Dex, 100 nM) for 48h. At the end of incubation, cells were harvested for analysis of cell death by FACS as described earlier. Cell lysates were also prepared for immunoprecipitation assay using a Puma antibody (from Novus). Un-manipulated whole cell lysates (lanes 2-4), the pull-down protein complexes (lanes 5-7) and post-IP protein lysates (lanes 8-10) from the control cells (Ut), dexamethasone (Dex)- and fludarabine (Flu)-treated cells were analysed using SDS-PAGE, followed by Western blotting using anti-Bcl-2 antibody (from Cell Signalling). β -actin was probed for as protein loading controls. Pre-stained protein mass marker was used as a guide for determining molecular weight of protein of interest (lane 1) also the actin blot was performed on a separate gel loaded with the same cell lysates.

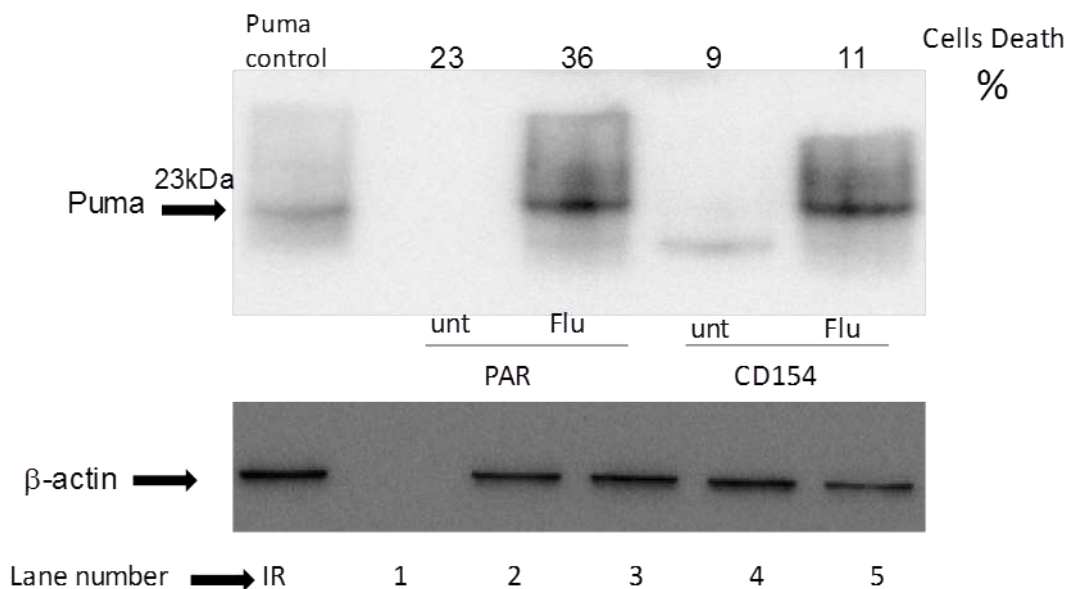


(Figure 8) Detection of Puma-Mcl-1 binding in primary CLL cells treated with fludarabine (sample 2911).

CLL cells were incubated with fludarabine (Flu, 10 μ M) or dexamethasone (Dex, 100 nM) for 48h. At the end of incubation, cells were harvested for analysis of cell death by FACS as described earlier. Cell lysates were also prepared for immunoprecipitation assay using a Puma antibody (from Novus). Un-manipulated whole cell lysates (Pre IP samples, lanes 2-4), the pull-down protein complexes (lanes 5-7) and post IP protein lysates (lanes 8-10) from the control cells (Ut), dexamethasone (Dex)- and fludarabine (Flu)-treated cells were analysed using SDS-PAGE, followed by Western blotting using anti-Mcl-1 antibody (from Santa Cruz). β -actin was probed for as protein loading controls. Pre-stained protein mass marker was used as a guide for determining molecular weight of protein of interest (lane 1) also the actin blot was performed on a separate gel loaded with the same cell lysates.

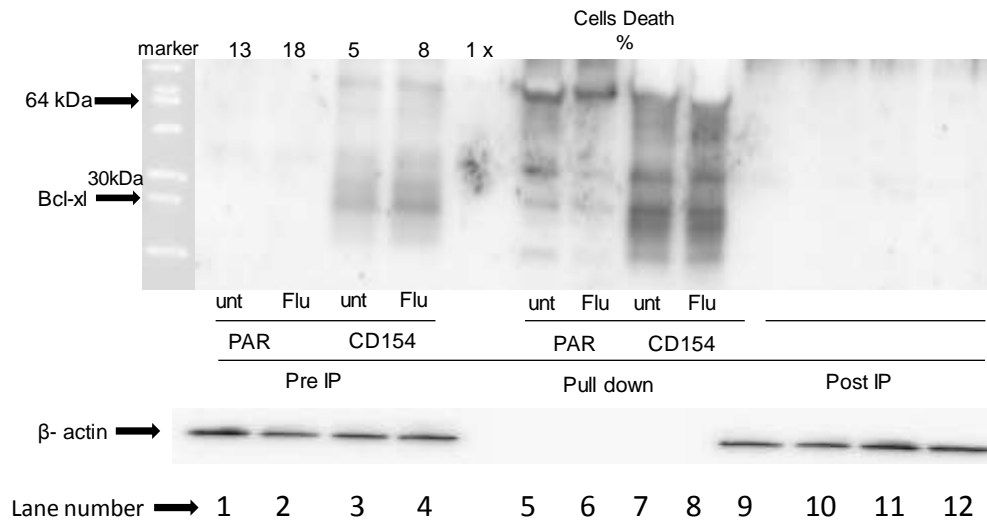
2. Identification of Puma binding proteins in fludarabine-treated CLL cells cultured with parental and CD40L-expressing fibroblasts.

- Case no 2746



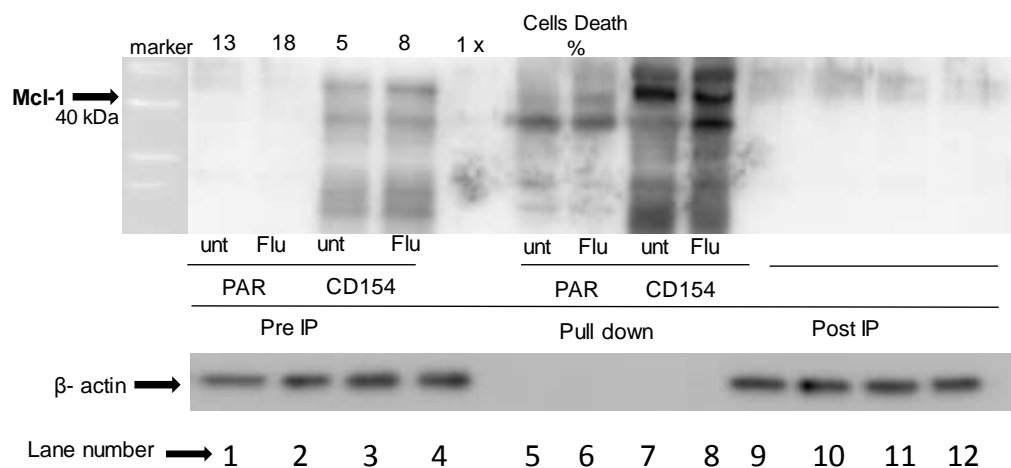
(Figure 9) Detection of expression of Puma with or with out fludarabine (sample 2746).

Co-cultured with parental (PAR) or CD40L-expressing (CD154) fibroblasts. Co-cultured CLL cells were incubated with or without fludarabine (10 μ M) for 48 hours. At the end of incubation, cells were harvested for analysis of cell death by FACS as described earlier. For the detection of expression of Puma, lysates were prepared from the above cells for Western blotting using anti-Puma antibody. Lysate prepared from γ -irradiated CLL cells was used as a positive control for Puma (lane IR) also the actin blot was performed on a separate gel loaded with the same cell lysates.



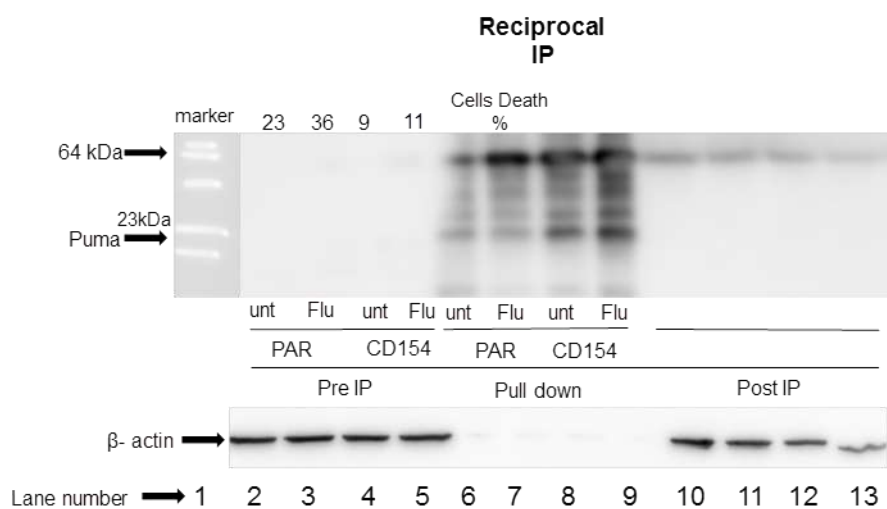
(Figure 10) Puma interacts with Bcl-XL in fludarabine-treated CLL cells co-cultured with CD40L-expressing fibroblasts (CLL sample 2746).

CLL cells were incubated with fludarabine (Flu, 10 μ M) for 48h. At the end of incubation, cells were harvested for analysis of cell death by FACS as described earlier. Cell lysates were also prepared for immunoprecipitation assay using a Puma antibody (from Novus). Un-manipulated whole cell lysates (pre-IP, lanes 1-4), the pull-down protein complexes (lanes 5-8) and post-IP protein lysates (lanes 9-12) from the untreated cells (Ut) or fludarabine - treated cells (Flu) were analysed by Western blotting for expression of Bcl-XL using anti-Bcl-XL antibody (from Cell Signalling). β -actin was probed for as protein loading controls also the actin blot was performed on a separate gel loaded with the same cell lysates.



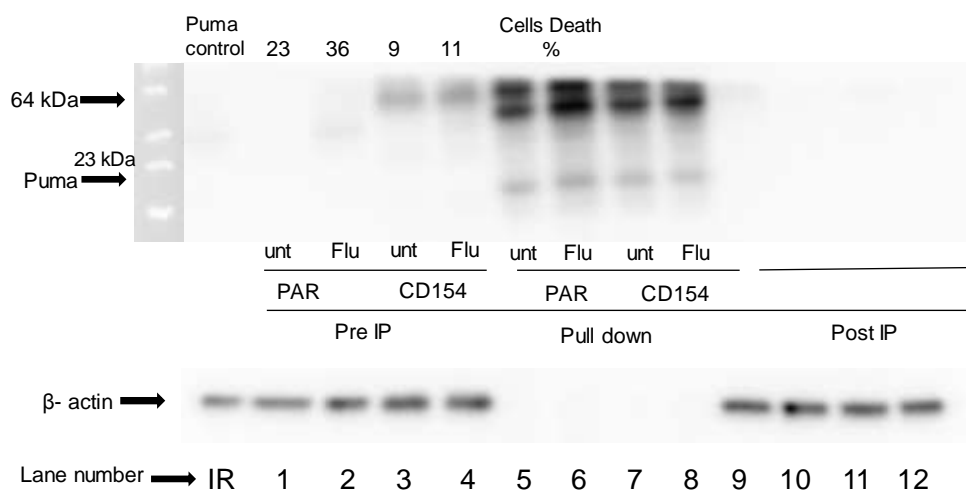
(Figure 11) Detection of interaction of Mcl-1 with Puma in fludarabine-treated CLL cells on co-cultures (CLL sample 2746).

Co-cultured CLL cells were incubated with fludarabine (Flu, 10 μ M) for 48h. At the end of incubation, cells were harvested for analysis of cell death by FACS as described earlier. Cell lysates were also prepared for immunoprecipitation assay using a Puma antibody (from Novus). Un-manipulated whole cell lysates (lanes 1-4), the pull-down protein complexes (lanes 5-8) and post-IP protein lysates (lanes 9-12) from the control cells (Ut) and fludarabine (Flu)-treated cells were analysed by Western blotting for expression of Mcl-1 using anti-Mcl-1 antibody (from Santa Cruz). β -actin was probed for as protein loading controls also the blot was stripped and re-probed with anti-actin antibody.



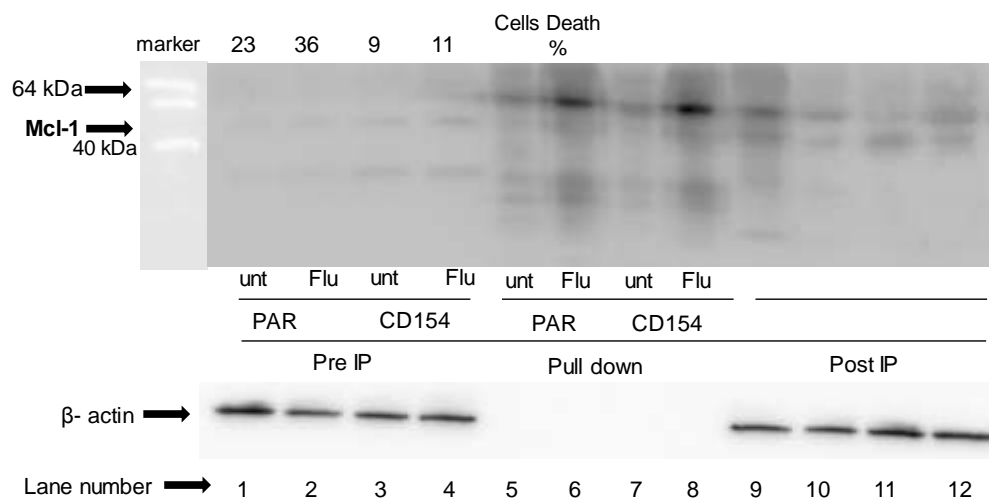
(Figure 12) Detection of interaction of Puma with Mcl-1 in fludarabine-treated CLL cells on co-cultures (sample 2746).

Co-cultured CLL cells were incubated with fludarabine (Flu, 10 μ M) for 48h. At the end of incubation, cells were harvested for analysis of cell death by FACS as described earlier. Cell lysates were also prepared for immunoprecipitation assay using a Mcl-1 antibody (from Santa Cruz). Un-manipulated whole cell lysates (lanes 1-4), the pull-down protein complexes (lanes 5-8) and post-IP protein lysates (lanes 9-12) from the control cells (Ut) and fludarabine (Flu)-treated cells were analysed by Western blotting for expression of Puma using anti-Puma antibody (from Cell Signalling). β -actin was probed for as protein loading controls. Lysate prepared from γ -irradiated CLL cells was used as a positive control for Puma also the blot was stripped and re-probed with anti-actin antibody.



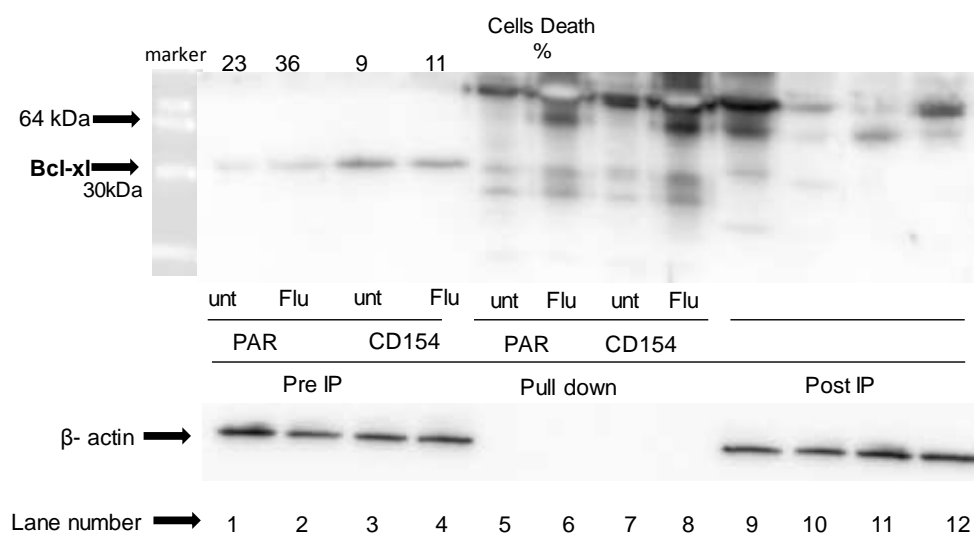
(Figure 13) Detection of interaction of Bcl-XL with Puma in fludarabine-treated CLL cells (sample 2746) on co-cultures.

Co-cultured CLL cells (sample 2746) were incubated with fludarabine (Flu, 10 μ M) for 48h. At the end of incubation, cells were harvested for analysis of cell death by FACS as described earlier. Cell lysates were also prepared for immunoprecipitation assay using a Bcl-XL antibody (Cell Signalling). Un-manipulated whole cell lysates (lanes 1-4), the pull-down protein complexes (lanes 5-8) and post-IP protein lysates (lanes 9-12) from the control cells (Ut) and fludarabine (Flu)-treated cells were analysed by Western blotting for expression of Puma using an anti-Puma antibody (from Cell Signalling). β -actin was probed for as protein loading controls. Lysate prepared from γ -irradiated CLL cells was used as a positive control for Puma (lane IR) also the actin blot was performed on a separate gel loaded with the same cell lysates.

- Case no 2911

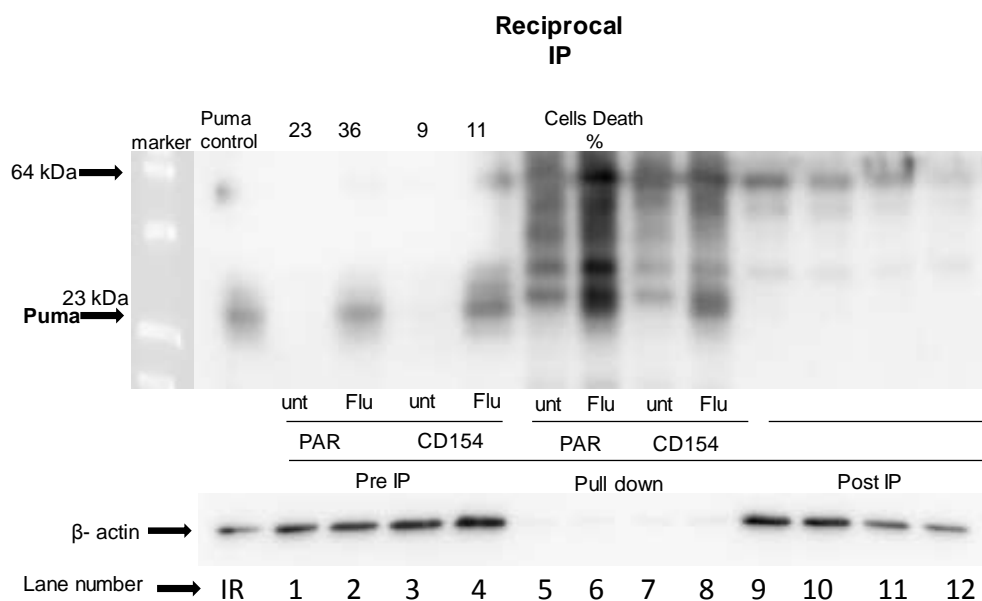
(Figure 14) Detection of interaction of Puma with Bcl-XL in fludarabine-treated CLL cells on co-cultures (sample 2911).

Co-cultured CLL cells were incubated with fludarabine (Flu, 10 μ M) for 48h. At the end of incubation, cells were harvested for analysis of cell death by FACS as described earlier. Cell lysates were also prepared for immunoprecipitation assay using a Puma antibody (from Novus). Un-manipulated whole cell lysates (lanes 1-4), the pull-down protein complexes (lanes 5-8) and post-IP protein lysates (lanes 9-12) from the control cells (Ut) and fludarabine (Flu)-treated cells were analysed by Western blotting for the expression of Bcl-XL using an anti-Bcl-XL antibody (from Cell Signalling). β -actin was probed for as protein loading controls also the actin blot was performed on a separate gel loaded with the same cell lysates.



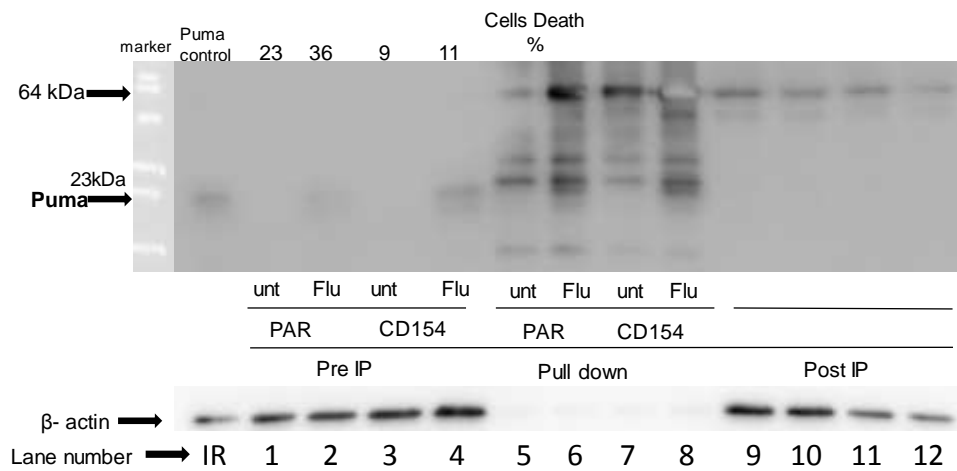
(Figure 15) Detection of interaction of Mcl-1 with Puma in fludarabine-treated CLL cells on co-cultures (CLL sample 2911).

Co-cultured CLL cells were incubated with fludarabine (Flu, 10 μ M) for 48h. At the end of incubation, cells were harvested for analysis of cell death by FACS as described earlier. Cell lysates were also prepared for immunoprecipitation assay using a Puma antibody (from Novus). Un-manipulated whole cell lysates (lanes 1-4), the pull-down protein complexes (lanes 5-8) and post-IP protein lysates (lanes 9-12) from the control cells (Ut) and fludarabine (Flu)-treated cells were analysed by Western blotting for the expression of Mcl-1 using an anti-Mcl-1 antibody (from Santa Cruz). β -actin was probed for as protein loading controls also the actin blot was performed on a separate gel loaded with the same cell lysates.



(Figure 16) Detection of interaction of Mcl-1 with Puma in fludarabine-treated CLL cells on co-cultures (from sample 2911).

Co-cultured CLL cells were incubated with fludarabine (Flu, 10 μ M) for 48h. At the end of incubation, cells were harvested for analysis of cell death by FACS as described earlier. Cell lysates were also prepared for immunoprecipitation assay using a Mcl-1 antibody (from Santa Cruz). Un-manipulated whole cell lysates (lanes 1-4), the pull-down protein complexes (lanes 5-8) and post-IP protein lysates (lanes 9-12) from the control cells (Ut) and fludarabine (Flu)-treated cells were analysed by Western blotting for expression of Puma using an anti-Puma antibody (from Cell Signalling). β -actin was probed for as protein loading controls. Lysate prepared from γ -irradiated CLL cells was used as a positive control for Puma (lane IR) also The actin blot was performed on a separate gel loaded with the same cell lysates.



(Figure 17) Detection of interaction of Bcl-XL with Puma in fludarabine-treated CLL cells on co-cultures (sample 2911).

Co-cultured CLL cells were incubated with fludarabine (Flu, 10 μ M) for 48h. At the end of incubation, cells were harvested for analysis of cell death by FACS as described earlier. Cell lysates were also prepared for immunoprecipitation assay using a Bcl-XL antibody (Cell Signalling). Un-manipulated whole cell lysates (lanes 1-4), the pull-down protein complexes (lanes 5-8) and post-IP protein lysates (lanes 9-12) from the control cells (Ut) and fludarabine (Flu)-treated cells were analysed by Western blotting for expression of Puma using an anti-Puma antibody (from Cell Signalling). β -actin was probed for as protein loading controls. Lysate prepared from γ -irradiated CLL cells was used as a positive control for Puma (lane IR) also the actin blot was performed on a separate gel loaded with the same cell lysates