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Regulation of apoptosis by CD40 in B-cell lymphoma

Presented by

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ABSTRACT

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

MOLECULAR MECHANISMS IN CANCER

Doctor of Philosophy

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CD40 is a cell surface signalling molecule expressed on normal B-cells and B-cell malignancies. Activation of CD40 (via binding CD40 ligand, CD40L) in normal B-cells promotes B-cell survival and is vital for activation of the immune response. However, activation of CD40 can induce or prevent apoptosis in malignant B-cells. Furthermore, CD40-activating agents are entering clinical trails as cancer therapies.

B-cells were isolated from over twenty Non Hodgkin's lymphomas and non-malignant lymph nodes and the effect of CD40 ligation on apoptosis and expression of apoptosis regulators was assessed. CD40L suppressed apoptosis in all B-cell malignancies tested, accompanied by significant increases in Bcl-X_L protein expression and differential regulation of Bcl-X splicing and promoters. Mcl-1 protein expression was also regulated by CD40 stimulation. Although A20, survivin, Bfl-1 and BNIP3 (a novel CD40-target gene identified in this study) were regulated by CD40 in some samples, this was not consistent. Antisense oligonucleotides to Bcl-X_L and Mcl-1 demonstrated that these proteins were key for survival of lymphoma cells. NF-κB mediated both the pro-survival effects of CD40 and upregulation of Bcl-X_L and Mcl-1 expression. Microarrays were developed and used to characterise CD40-induced gene expression. This identified both novel CD40-target genes and also genes that were differentially regulated in cell lines with different survival outcomes to CD40 activation e.g. SLAM and SH2D1A.

Therefore CD40 acts as a potent survival signal for primary B-cell lymphomas leading to regulation of the key anti-apoptotic proteins Bcl-X_L and Mcl-1, via activation of NF-κB. This raises concerns over forthcoming clinical trails utilising CD40 activation as a treatment for cancer but suggests that targeting Bcl-X_L, Mcl-1 or NF-κB may be a useful approach for treating lymphoma.

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Dallman CL, Packham G. Purification of primary malignant B-cells and immunoblot analysis of Bcl-2 family proteins (in press). In: Molecular Methods in Cancer. Illidge T, Johnson PWM, eds. In press.

Michels M, O'Neill JW, **Dallman C**, Mouzakiti A, Habens F, Brimmell M, Zhang KYJ, Craig RW, Marcusson EG, Johnson PWM, Packham G. Mcl-1 is a key survival protein in B-lymphoma cells and is converted to a cell death molecule by caspase-mediated cleavage. *Oncogene*, in press.

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Abbreviations

ADP	Adenosine diphosphate
AID	Activation-induced cytidine deaminase
AIDS	Acquired immune deficiency syndrome
AIF	Apoptosis inducing factor
AML	Acute myeloid leukaemia
ANT	Adenine nucleotide translocator
ATP	Adenosine 5'-triphosphate
BD	Becton Dickinson
BH domain	Bcl-2 homology domain
BIR	Baculovirus IAP repeat
BL	Burkitt's lymphoma
BLIMP1	B-lymphocyte-induced maturation protein 1
bp	Base pair
BSA	Bovine serum albumin
C. elegans	*Caenorhabditis elegans*
CAD	Caspase-activated DNase
CAT	Chloramphenicol acetyltransferase
CD40L	CD40 ligand
cDNA	Complementary deoxyribonucleic acid
CGH	Comparative genomic hybridisation
CHOP	Cyclophosphamide, hydroxydoxorubicin (adriamycin), oncovin (vincristine), prednisone
CLL	Chronic lymphocytic leukaemia
CTL	Cytotoxic T-cell
CTP	Cytidine 5'-triphosphate
Cy	Cyanine
DD	Death domain
DEAE	Diethylaminoethyl
DED	Death effector domain
DISC	Death inducing signaling complex
DLBL	Diffuse-large B-cell lymphoma

DNA	Deoxyribonucleic acid
dNTP	Deoxyribose nucleotide 5'-triphosphate
DR	Death receptor
DTT	Dithiothreitol
EBV	Epstein Barr Virus
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(2-aminoethyl)-tetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-activated cell sorter
FADD	Fas associated death domain
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FL	Follicular lymphoma
FLICE	Fas-associated death domain-like interleukin 1β-converting enzyme
FLIP	FLICE-like inhibitory protein
GABA	Gamma-aminobutyric acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GBP-5	Guanylate binding protein 5
GCKR	Germinal centre kinase-related kinase
GSK3	Glycogen synthase kinase 3
GTP	Guanosine 5'-triphosphate
HEPES	N-2-Hydroxyethylpiperazine-N-2-ethanesulphonic acid
HGMP	Human Genome Mapping Project
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
hr	Hour
HRP	Horseradish peroxidase
IAP	Inhibitor of apoptosis protein
ICAD	Inhibitior of caspase-activated DNase
IFIT	Interferon-induced protein with tetratricopeptide repeats
Ig	Immunoglobulin

IKK	IκB kinase
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
IPTG	Isopropyl-1-thio-β-D-galactoside
IRES	Internal ribosome entry sequence
IRTA	Ig superfamily receptor translocation-associated gene
IκB	Inhibitor of nuclear factor-kappa B
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
KA	Kamebakaurin
kD	Dissociation constant
kDa	kilo Dalton
LAMP3	Lysosomal-associated membrane protein 3
LB	Luria-Bertani
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MAP2K	Mitogen activated protein kinase kinase
MAP3K	Mitogen activated protein kinase kinase kinase
MAP4K	Mitogen activated protein kinase kinase kinase kinase
MAPK	Mitogen activated protein kinase
MCL	Mantle cell lymphoma
MEKK	Mitogen activated protein kinase/extracellular signal regulatory kinase kinase
MIA	Melanoma inhibitory activity
M-MLV RT	Moloney Murine Leukaemia Virus Reverse Transcriptase
MOPS	3-morpholinopropanesulfonic acid
mRNA	Messenger ribonucleic acid
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NADH	Nicotinamide Adenine Dinucleotide
NF-κB	Nuclear factor-kappa B
NHL	Non-hodgkin's lymphoma
NIK	NF-κB inducing kinase

NK	Natural killer
ONPG	Orthonitrophenyl-β-D-galactoside
PAGE	Polyacrylamide gel electrophoresis
PAK	p21-activated kinase
PARP	Poly(ADP)ribose polymerase
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween 20
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDK	Phosphoinositide-dependent kinase
PE	Phycocoerythrein
PH	Pleckstrin homology
PI	Propidium iodide
PI3-K	Phosphatidylinositol 3-kinase
PIP_3	Phosphatidyl inositol 3,4,5 triphosphate
PRAME	Preferentially expressed antigen in melanoma
PT	Permeability transition
REAL	Revised European-American Classification of Lymphoid Neoplasms
RGS1	Regulator of G-protein signaling 1
RH	Rel homology
R-I	Ratio-intensity
RIP	Receptor interactive protein
RIPA	Radioimmunoprecipitation
RNA	Ribonucleic acid
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase – polymerase chain reaction
SAM domain	Sterile alpha motif
SAPK	Stress activated protein kinase
SCID	Severe combined immune deficiency
SDS	Sodium dodecylsulphate

SH2	Src homology 2
SHIP	SH2-containing inositol phosphatase
SHP2	SH2-containing protein tyrosine phosphatase 2
SLAM	Signalling lymphocytic activation molecule
SSC	Saline-Sodium Citrate
STAT	Signal transducer and activator of transcription
TAK	Transforming growth factor β activated kinase
TANK	TRAF-associated NF-κB activator
TBE	Tris-Borate-EDTA
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TNP	Trinitrophenol
TRAF	TNFR-associated factor
TRAIL	TNF-related apoptosis inducing ligand
TS	Tris-buffered saline
TST	Tris-buffered saline with Tween 20
UV	Ultraviolet
V	Volts
V_H	Ig heavy chain variable region
v/v	Volume for volume
VDAC	Voltage-dependent anion channel
VEGF	Vascular endothelial growth factor
w/v	Weight for volume
WHO	World health organisation
X-gal	5-Bromo-4-chloro-3-inodyl-β-D-galactoside
XIAP	X-linked inhibitor of apoptosis protein

Chapter 1: Introduction

Apoptosis, or programmed cell death, is an essential physiological process required for normal development, homeostasis and immune regulation in multicellular organisms. In particular, apoptosis is critical for the development and maturation of normal B-cells. B-cells are white blood cells responsible for making antibodies against bacterial, viral or tumour antigens. Deregulation of apoptosis can lead to pathogenesis: excessive apoptosis has been associated with cardiovascular diseases and neurodegenerative disorders e.g. Alzheimer's disease and AIDS, whereas suppression of apoptosis can lead to autoimmune diseases and tumorigenesis. Cancer formation can occur due to defects in apoptosis controlling genes leading to an extended cell life span, increasing the possibility that the cell will accumulate further genetic alterations that could contribute to malignancy. Moreover, successful cancer treatment using chemotherapy or radiotherapy, relies on the ability of malignant cells to undergo apoptosis, so often these treatments are unsuccessful and select for a population of cells that are resistant to apoptosis. Elucidation of the molecular mechanisms involved in the regulation of apoptosis is vitally important in order to understand tumorigenesis and to yield novel therapies to increase the propensity of malignant cells to commit suicide.

One of the key regulators of B-cell function and apoptosis is CD40, a member of the tumour necrosis factor (TNF) superfamily. The CD40 cell surface receptor is required for normal function of the immune system and is a positive regulator of cell survival for normal B-lymphocytes. However, there is evidence to support both pro- and anti-apoptotic functions for CD40 in malignant B-cells and epithelial cancers. There is increasing interest in the potential of CD40 activating agents as novel therapies for cancer and it is essential to understand the differential response of malignant cells, to inform the design of trials. The aim of this project was to investigate the effects of CD40 ligation on the regulation of apoptosis in B-cell lymphomas and to determine the molecular mechanisms involved. This introduction will discuss the mechanisms of apoptosis, the importance of apoptosis in B-cell development, the role and mechanisms of CD40 signalling in cell survival and apoptosis, and will also discuss the various B-cell lymphomas studied in this project.

1.1 Apoptosis

Although programmed cell death was first described in the 19th century, research did not begin until the early 1970's when Kerr, Wylie and Curry coined the term apoptosis to describe the morphology of programmed cell death (Lockshin & Zakeri 2001, Kerr *et al* 1972). Apoptosis is a Greek word describing leaves falling from trees or petals from flowers. Whereas cells that die from injury typically swell and burst (a process called necrosis), apoptosis involves the systematic dismantling of the cell. Cells shrink, detach from their neighbours and the nucleus becomes condensed. Condensed chromatin is cleaved into oligonucleosome-sized fragments of 180-200 bp (Wyllie 1980) and the nuclear envelope and nucleolus break apart. The plasma membrane retains its integrity and forms invaginations and protrusions called blebs which can pinch off forming apoptotic bodies, leading to cell fragmentation. Apoptotic bodies are rapidly phagocytosed by neighbouring cells (Collins *et al* 1994), preventing inflammatory and autoimmune responses.

Apoptosis is an active, tightly controlled process and is generally executed by a family of intracellular cysteine proteases called caspases. Caspases have aspartate specificity and exist as immature pro-enzymes that are activated upon apoptosis by cleavage. Firstly, initiator caspases (caspases 8 or 9) are autoactivated, whereby multiple procaspase molecules are brought close together in molecular complexes so they can cleave and activate each other. The activated initiator caspases then go on to cleave effector caspases (e.g. caspase 3, 7 and 6) which cleave cellular substrates and also activate other caspases, resulting in a caspase activation cascade. Caspases cleave a large number of substrates functioning to disrupt cellular protective mechanisms and switch on degradative pathways leading to destruction of the cell. For example, caspase-3 cleaves ICAD (inhibitor of caspase-activated DNase), which releases its repression on CAD (caspase-activated DNase), allowing CAD to cleave chromatin at nucleosomal boundaries, resulting in the characteristic DNA fragmentation of apoptosis (Enari *et al* 1998). DNA repair is also prevented by caspase cleavage of the DNA repair enzyme poly(ADP)ribose polymerase or PARP (Gu *et al* 1995). Caspases also cleave nuclear laminins, proteins involved in cytoskeletal regulation e.g. gelsolin, and other caspases themselves. However apoptosis can occur independently of caspases via activation of other proteases such as calpains, cathepsins and serine proteases (Mathiasen & Jaattela 2002).

Apoptosis can be triggered through two distinct pathways which converge on caspase activation (fig. 1.1). In the 'intrinsic' cell death pathway, insults such as growth factor withdrawal, DNA damage, hypoxia and cytotoxics lead to the release of proteins from the mitochondrial intermembrane space, such as cytochrome c (Kluck *et al* 1997). Bcl-2 family proteins regulate this pathway, as detailed in section 1.2. Released cytochrome c combines with Apaf-1 and procaspase-9, forming the apoptosome, the formation of which is dependent on ATP hydrolysis (Green & Reed 1998). The apoptosome is a wheel-like structure containing seven molecules of Apaf-1, cytochrome c, ATP and procaspase 9. Multiple procaspase 9 molecules are brought into close proximity in the apoptosome allowing autoactivation to occur producing active caspase 9 molecules. Caspase 9 dissociates from the complex and cleaves and activates effector caspases (e.g. caspases 3 and 7), which cleave cellular substrates and perpetuate the caspase cascade network resulting in cell death (Bleackley & Heibein 2001). In the 'extrinsic' pathway, the signal to die comes from outside the cell and occurs via the binding of death ligands e.g. Fas, to death receptors on the cell surface (see section 1.3). This directly leads to autoactivation of caspase-8, which then goes on to cleave and activate other effector caspases in a caspase cascade. There is also cross talk between the extrinsic and intrinsic pathways under some conditions. Caspase 8, activated by death receptor signalling, can cleave Bid, a pro-apoptotic Bcl-2 family protein, which can then target the mitochondria leading to cytochrome c release (Li *et al* 1998, Desagher *et al* 1999, Wei *et al* 2000).

Mitochondria play an important role in apoptosis, in addition to releasing cytochrome c other mediators of apoptosis are released into the cytosol and the mitochondrial membrane potential can be lost. Smac/DIABLO is released from the intermembrane space of the mitochondria during apoptosis and binds to XIAP (X-linked inhibitor of apoptosis protein), a protein which directly binds to caspases and inhibits their function. The binding of Smac/DIABLO to XIAP, and probably other members of the IAP family, prevents them from inhibiting caspases, therefore promoting apoptosis (Salvesen & Duckett 2002). Similar to Smac/DIABLO, HtrA2/Omi is released from the mitochondria during apoptosis and binds to XIAP inhibiting its function. HtrA2/Omi also has a serine protease dependent apoptosis-inducing activity when overexpressed extramitochondrially (Suzuki *et al* 2001). In some cell types

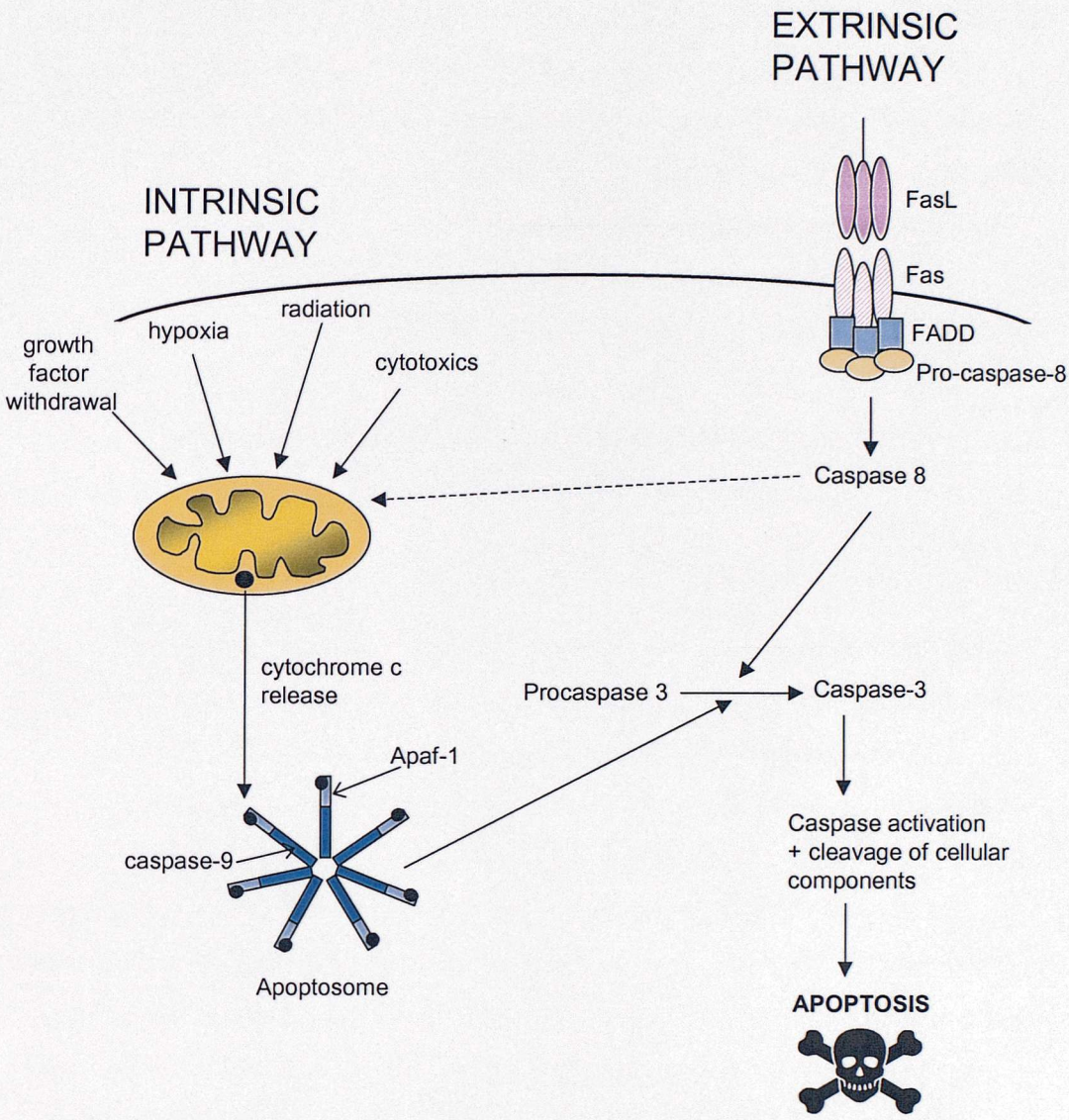


Figure 1.1: The two major apoptotic pathways. Apoptosis can be initiated by the extrinsic (death receptor pathway) or the intrinsic (mitochondrial) pathway. Activation of death receptors e.g. Fas, leads to processing of caspase 8 and activation of the caspase cascade. A wide range of apoptotic insults can activate the mitochondrial pathway, leading to release of proteins such as cytochrome c from the mitochondria, which promote apoptosis. Cytochrome c, in the presence of ATP, binds to Apaf-1 and procaspase 9 in a seven-pronged wheel structure called the apoptosome. This structure facilitates caspase 9 autoactivation. Caspase 9 can then activate effector caspases e.g. caspase 3, leading to activation of the caspase cascade and apoptosis. The extrinsic pathway can also activate the intrinsic pathway via caspase 8 activation of Bid, a pro-apoptotic Bcl-2 member, which can promote cytochrome c release.

procaspase-3 is released during apoptosis, although it is unclear whether it is activated prior to release (Green & Reed 1998). Apoptosis-inducing factor (AIF) and endonuclease G are also liberated from the mitochondrial intermembrane space and translocate to the nucleus upon an apoptotic signal, leading to chromatin condensation and large-scale DNA fragmentation, in a caspase independent manner (Susin *et al* 1999, Li *et al* 2001). Indeed, mutation or reduced expression of the C. elegans endonuclease G homolog cps-6 leads to decreased DNA degradation and delayed apoptosis, showing that DNA degradation is vital for the proper progression of apoptosis (Parrish *et al* 2001). Key regulators of mitochondrial function, cytochrome c release and caspase activation are members of the Bcl-2 family.

1.2 The Bcl-2 family

Bcl-2 is the founding member of a family of proteins that are important regulators of apoptosis. Bcl-2 was originally identified as a proto-oncogene in B-cell lymphoma. 85% of follicular lymphomas and 20% of diffuse B-cell lymphomas have a characteristic translocation between chromosome 18 and chromosome 14, which brings the Bcl-2 gene under control of the Ig heavy chain intron enhancer (reviewed in Yang & Korsmeyer 1996 and Antonsson & Martinou 2000). This results in transcriptional deregulation and overexpression of the Bcl-2 protein. Transgenic mice bearing a Bcl2-Ig minigene show a 4-fold expansion of the resting B-cell population, similar to human follicular lymphoma. These cells accumulate due to extended survival, consistent with an anti-apoptotic function of Bcl-2 and over time mice progress to diffuse large-cell lymphomas, similar to the transformation of follicular lymphoma in humans (reviewed in Korsmeyer 1999, MacCarthy-Morrogh *et al* 1999). The extended life span of B-cells afforded by Bcl-2 overexpression, allows cells to acquire additional genetic defects (e.g. c-myc mutation) without undergoing apoptosis, leading to tumorigenesis. Bcl-2 suppresses apoptosis induced by a wide range of stimuli both *in vitro* and *in vivo*, although not in all systems studied (Vaux *et al* 1988, Nunez *et al* 1990, Hockenbery *et al* 1990, Allsopp *et al* 1993) and provided the first example of oncogenesis mediated by decreased cell death.

Bcl-2 is now known to belong to a family of proteins that are all important regulators of apoptosis. The Bcl-2 family consists of at least 20 family members that all possess at least one of four conserved motifs known as Bcl-2 homology domains (BH1 to

BH4) (Adams & Cory 1998). The family comprises proteins that inhibit apoptosis (e.g. Bcl-2, Bcl-X_L, Bfl-1, Mcl-1 and Bcl-w) and proteins that promote apoptosis (e.g. Bax, Bak, Bcl-X_S, Bad, Bid, Bik and Hrk). Anti-apoptotic and pro-apoptotic members can heterodimerise and titrate one another's function, so the relative concentration of opposing members is important in determining if apoptosis occurs (Oltvai *et al* 1993). The Bcl-2 family can be divided into three subgroups (fig 1.2): (i) the anti-apoptotic proteins with 4 BH domains and a carboxy-terminal transmembrane domain (ii) the multidomain pro-apoptotic proteins which share sequence homology in BH1, BH2 and BH3 but lack BH4 and (iii) the BH3-only pro-apoptotic proteins that share homology only in BH3. The Bcl-2 family can localize to the outer mitochondrial membrane, the endoplasmic reticulum membrane and the nuclear envelope, via their C-terminal hydrophobic transmembrane domain. Localisation to the outer mitochondrial membrane is particularly important for their function and pro-apoptotic family members that do not express the transmembrane domain will translocate from the cytoplasm to the mitochondria during apoptosis (Wolter *et al* 1997, Hsu *et al* 1997).

High resolution, three-dimensional structures have been solved for Bcl-2, its closely related homologs Bcl-X_L and Bcl-w, the pro-apoptotic multidomain protein Bax and the pro-apoptotic BH3-only protein Bid (Muchmore *et al* 1996, Petros *et al* 2001, Suzuki *et al* 2000, Chou *et al* 1999, McDonnell *et al* 1999, Densiov *et al* 2003). Although primary sequence homology between these proteins is limited, their overall structures are remarkably similar. All comprise a central predominately hydrophobic α-helix packed against five amphipathic α-helices (fig. 1.3). A hydrophobic groove is present on the surface of Bcl-2 and Bcl-X_L, formed by the BH1, BH2 and BH3 domains, which is responsible for heterodimerisation with pro-apoptotic Bcl-2 family members. Heterodimerisation is mediated through insertion of the BH3 domain (helix 2) of the pro-apoptotic Bcl-2 family member into the hydrophobic groove of Bcl-2 or Bcl-X_L, via both electrostatic and hydrophobic interactions (fig. 1.3B) (Sattler *et al* 1997, Petros *et al* 2001). This protein-protein interaction neutralizes the effect of Bcl-2 or Bcl-X_L and promotes cell death, requiring only 9-16 residues of the BH3 domain. Therefore proteins containing the BH3 domain act as direct antagonists to Bcl-2. Synthetic BH3 domain peptides are also able to bind and inactivate Bcl-2, leading to

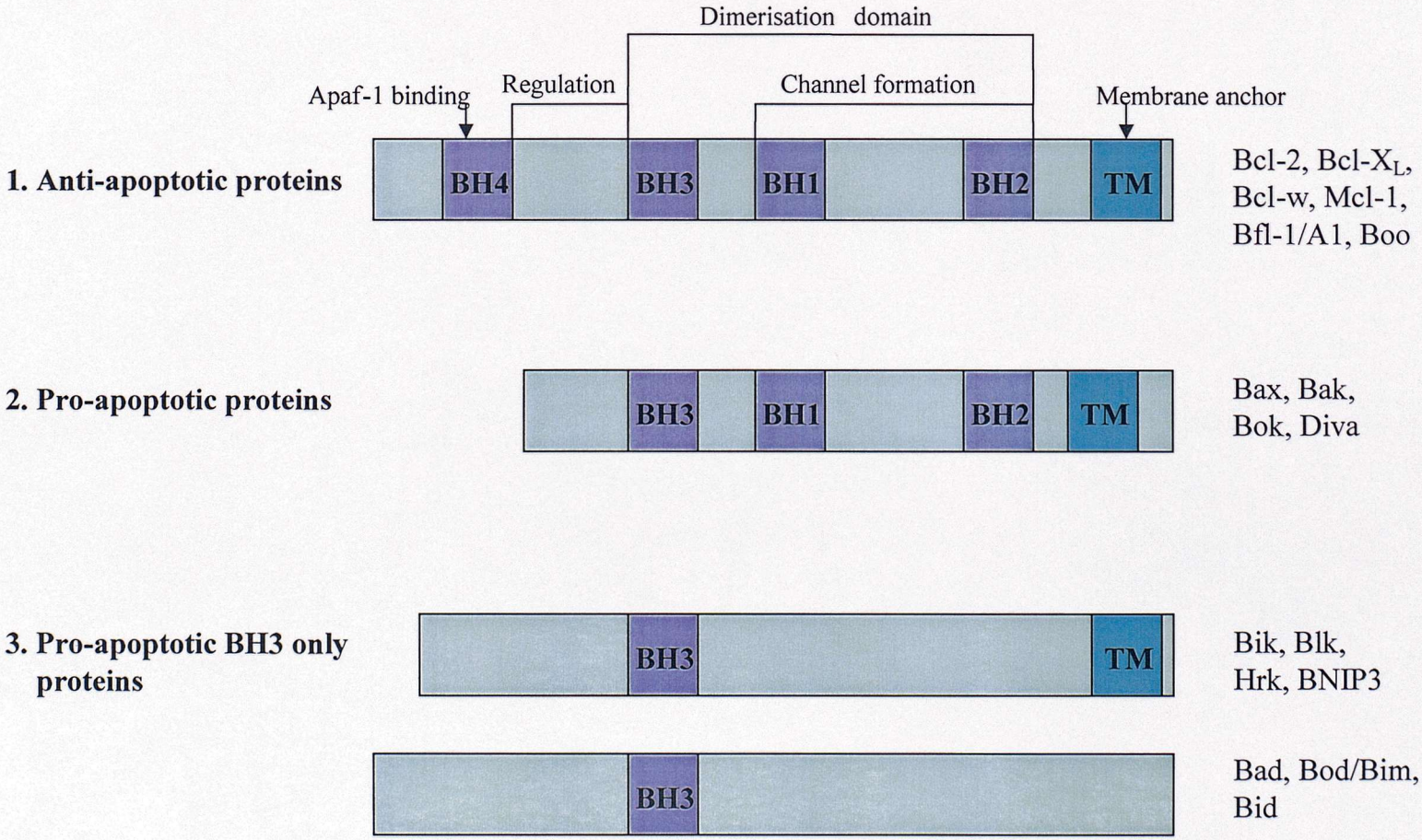
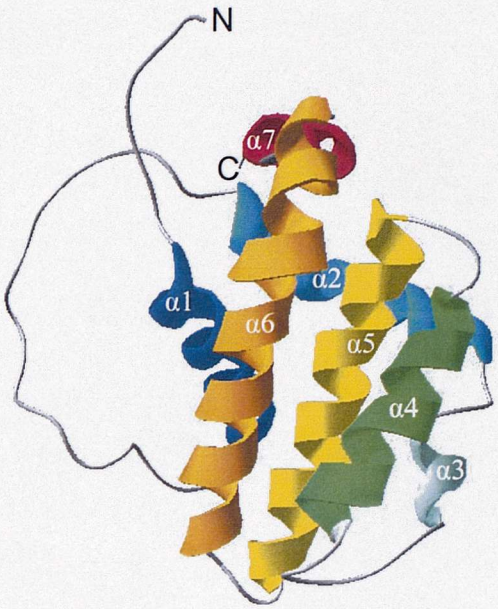


Figure 1.2: The Bcl-2 family. The family can be split into 3 subgroups based on structural and functional properties. BH = Bcl-2 homology domains, which are conserved regions. TM = transmembrane domain. Several functional domains have been identified in Bcl-2 family proteins and these are indicated. Proteins belonging to each subgroup are listed.

A Bcl-2



B Bcl-X_L-Bak

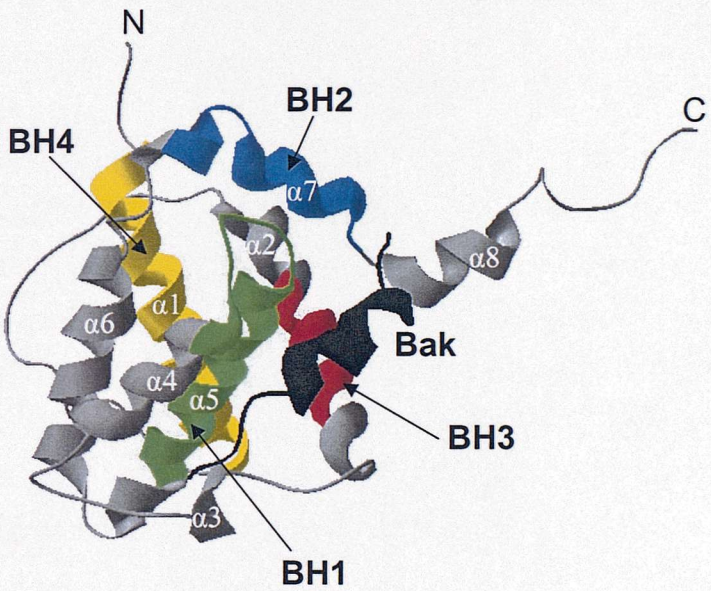


Figure 1.3: Crystal structures of Bcl-2 and Bcl-X_L/Bak. A - Bcl-2 structure (determined by Petros *et al* 2001). Here part of the unstructured loop region of Bcl-2 has been replaced by that of Bcl-X_L in order to improve solubility of the protein. The 7 alpha helices of Bcl-2 have been colour coded. **B -** Bcl-X_L structure showing insertion of the Bak BH3 domain (determined by Sattler *et al* 1997). The BH domains of Bcl-X_L are colour coded. A 16 amino acid peptide derived from the Bak BH3 domain is shown in black.

apoptosis and are attractive small molecule inhibitors for targeting Bcl-2 activity (Oxford *et al*, in press).

The structural topology and distribution of electrostatic potential in the hydrophobic groove differs between Bcl-2 family proteins such as Bcl-2 and Bcl-X_L. This may contribute to the specificity of interactions of BH3 domains with Bcl-2 or Bcl-X_L, for example Bad BH3 binds to Bcl-X_L and Bcl-2 with affinities (kD) of 0.6nM and 8nM respectively (Petros *et al* 2001). Interestingly, the C-terminal eighth α-helix of Bcl-w and the C-terminal transmembrane domain of Bax fold into their BH3 binding clefts (Densiov *et al* 2003, Suzuki *et al* 2000). This occurs in the soluble form of Bax and is thought to prevent hetrodimerisation with cytosolic proteins. When Bax translocates to the mitochondria, the transmembrane domain inserts into the outer mitochondrial membrane, leaving the cleft free to bind BH3 domains. The insertion of α-helix eight into the BH3 binding pocket of Bcl-w may restrict interactions of Bcl-w to partners that are capable of displacing this helix, thus exerting another level of control and specificity into Bcl-2 family interactions.

The N-terminal BH4 domain (helix 1), only possessed by the anti-apoptotic Bcl-2 family members (with the exception of Mcl-1), forms an amphipathic helix located on the opposite face of Bcl-2 to the hydrophobic groove. The BH4 domain is essential for the anti-apoptotic activity of Bcl-2 but is not involved in heterodimerisation with Bcl-2 family proteins. Instead, this region is involved in interactions with regulatory proteins such as CED-4 (a caspase activator in the nematode *Caenorhabditis elegans*, which is equivalent to Apaf-1), the protein phosphatase calcineurin and the protein kinase Raf-1 (Huang *et al* 1998, Shibasaki *et al* 1997, Wang *et al* 1996).

Bcl-2 and Bcl-X_L also possess a flexible loop region between the BH3 and BH4 domains consisting of 60 amino acids (Muchmore *et al* 1996). This is not essential for their anti-apoptotic activity but appears to have a negative regulatory role, as deletion mutations of this region have an enhanced ability to prevent apoptosis (Chang *et al* 1997). The loop domain has a number of serine and threonine phosphorylation sites and the outcome of phosphorylation is dependent on cellular context. Agents which induce cell cycle arrest e.g. Taxol, promote phosphorylation and inactivate Bcl-2,

whereas cytokine mediated phosphorylation of Bcl-2 prevents apoptosis in cytokine-dependent cell lines (Yamamoto *et al* 1999, Ito *et al* 1997, Blagosklonny 2001). Phospho-Bcl-2 was also shown to associate with Pin 1 (a peptidyl prolyl isomerase) leading to a transient conformational change in Bcl-2 which inhibits its function (Basu & Haldar 2003). The loop regions of Bcl-2 and Bcl-X_L can also be cleaved by caspase-3 during apoptosis and the carboxy-terminal cleavage products accelerate cell death, ensuring that apoptosis reaches completion (Cheng *et al* 1997, Clem *et al* 1998, Fujita *et al* 1998).

Bcl-2 family proteins regulate cell death through influencing mitochondrial events that are central to apoptosis. The precise mechanism/s by which Bcl-2 family proteins regulate apoptosis are not clear but may be explained by three properties of Bcl-2 proteins: they form membrane channels, they regulate cytochrome c release and they regulate caspase activation.

1. Membrane channels

Elucidation of the structure of Bcl-X_L revealed that it resembles the membrane insertion domain of pore forming bacterial toxins e.g. diphtheria toxin, particularly in the BH1 and BH2 domains. Indeed Bcl-X_L, Bcl-2, and Bax do form channels in lipid bilayers *in vitro*, involving the two core helices 5 and 6. These channels possess some ion selectivity and channel formation is favoured by changes in membrane potential or pH (Minn *et al* 1997, Schendel *et al* 1997). However there is no direct evidence for channel forming activity *in vivo* (Adams & Cory 1998, Zorning 2001). Therefore BH1 and BH2 domain containing Bcl-2 family proteins may form channels in organelle membranes, such as mitochondria, which may be voltage or pH regulated, and could influence mitochondrial function and apoptosis.

2. Cytochrome c release

Bcl-2 and Bcl-X_L prevent all mitochondrial changes occurring during apoptosis, including cytochrome c release (Kluck *et al* 1997), whereas addition of Bax or BH3 peptides to isolated mitochondria induce cytochrome c release and membrane potential loss (Rosse *et al* 1998, Cosulich *et al* 1997, Narita *et al* 1998). Recently Bcl-2 and Bcl-X_L have also been shown to prevent Smac/DIABLO release from mitochondria (Sun *et al* 2002). The mechanism by which cytochrome c is released

from mitochondria is not known but could occur through a specific channel in the outer mitochondrial membrane that is either formed or regulated by Bcl-2 proteins, or through non-specific swelling of the mitochondria and rupture of the mitochondrial outer membrane (fig 1.4). Bax oligomers and pro-apoptotic cleavage products of Bcl-X_L can form channels in lipid bilayers or outer mitochondrial membrane vesicles which are large enough for cytochrome c to pass through (Tsujimoto & Shimizu 2000, Basanez *et al* 2001, Kuwana *et al* 2002). There is also a conductance channel in the outer mitochondrial membrane termed VDAC (voltage-dependent anion channel), which is responsible for metabolite exchange across the mitochondrial membrane, but could also be the route of cytochrome c release. Endogenous Bcl-X_L can be coimmunoprecipitated with VDAC, whereas endogenous Bax and Bak can only be coimmunoprecipitated with VDAC in apoptotic, but not intact, cells (Shimizu *et al* 1999). In liposomes, Bax and Bak are able to interact with VDAC to release cytochrome c and this requires an intact BH3 domain, whereas Bcl-X_L interacts with VDAC preventing cytochrome c release. It was also demonstrated that the presence of VDAC in isolated mitochondria was essential for Bax or Bak induced membrane potential loss and cytochrome c release (Shimizu *et al* 1999). However a recent study has shown that oligomerised Bax can permeabilise membranes when VDAC was absent (Kuwana *et al* 2002). Although the diameter of VDAC is too small for cytochrome c to pass through, Bax or Bak may induce a conformational change in the protein or form a channel in combination with VDAC allowing cytochrome c to pass (fig. 1.4).

VDAC is also a component of the PT (permeability transition) pore, a large multi-protein channel consisting of VDAC, ANT (adenine nucleotide translocator), creatine kinase, hexokinase and cycophilin D, which spans both the inner and outer mitochondrial membrane. This channel is affected by changes in voltage, calcium, pH and redox. Opening of the channel is predicted to cause increased mitochondrial permeability, allowing water and solutes to enter leading to swelling of the matrix which could rupture the outer membrane and lead to cytochrome c release. However in many instances of apoptosis the mitochondria do not swell and burst, so this mechanism may not account for all types of apoptosis. The PT channel is proposed to be regulated by Bcl-2 family proteins. Indeed Bax can interact with both VDAC and

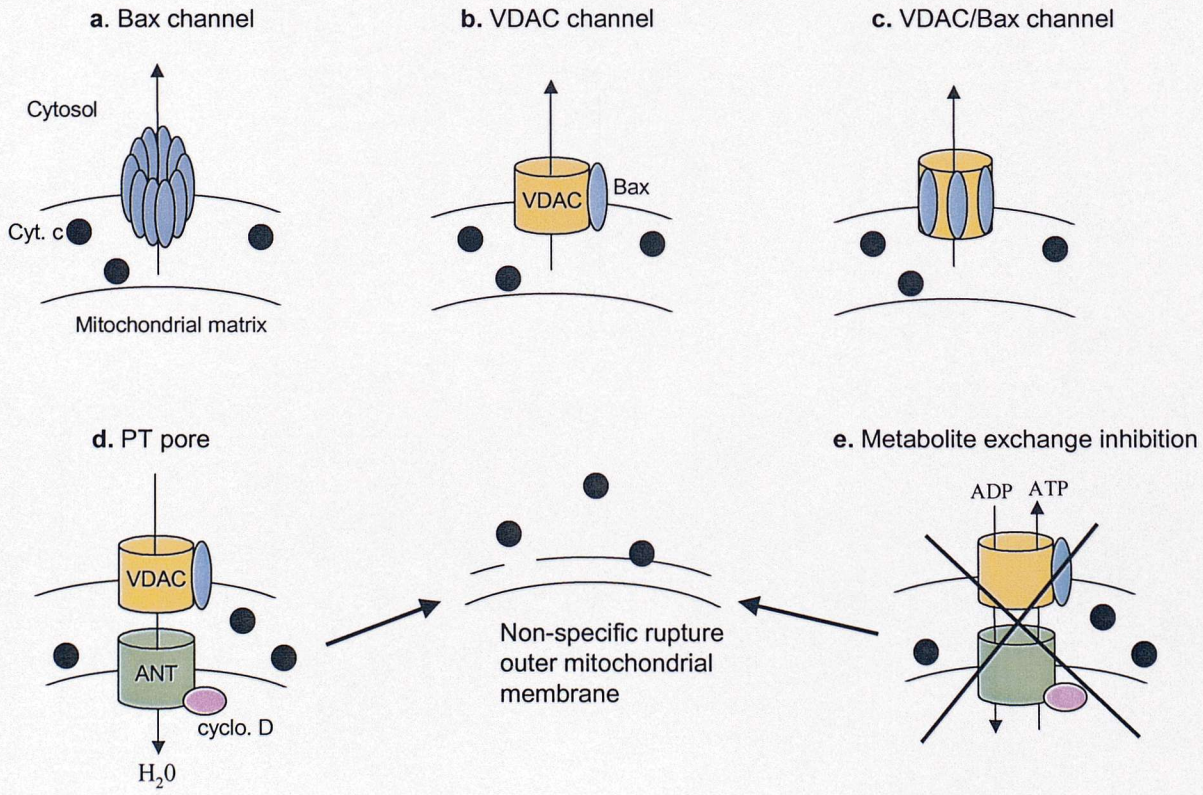


Figure 1.4: Possible mechanisms of cytochrome c release from mitochondria. A specific cytochrome c release channel could be formed by Bcl-2 family proteins or regulated by them. This channel could be composed of Bax molecules (a) or Bax could induce a conformational change in VDAC allowing cytochrome c to pass (b). Alternatively Bax and VDAC may associate to form a channel large enough for cytochrome c to pass through (c). Cytochrome c could also be released through non-specific rupture of the outer mitochondrial membrane. This could be achieved through opening of the PT pore (d) or through inhibition of metabolite exchange through VDAC/ANT (e), both of which can be regulated by Bcl-2 family proteins.

ANT to promote pore opening whereas Bcl-2 and Bcl-X_L may promote a closed conformation (Vander Heiden and Thompson 1999).

Another theory is that Bcl-2 family proteins may regulate mitochondrial homeostasis and outer membrane rupture through regulating metabolite exchange. Early in apoptosis prior to loss of membrane integrity and cytochrome c release, there is a defect in mitochondrial ATP/ADP exchange which could increase mitochondrial membrane potential and ultimately lead to matrix swelling and cytochrome c release (Vander Heiden *et al* 1999). The lack of metabolite exchange is due to closure of the VDAC channel and it has been shown that the addition of Bcl-X_L to isolated mitochondria prevents VDAC closure and maintains it in an open configuration thereby allowing normal mitochondrial metabolite exchange to take place and preventing loss of mitochondrial function and apoptosis (Vander Heiden *et al* 2001). This study contradicts the findings of Shimizu *et al* (1999) who found that Bcl-X_L closes the VDAC channel preventing cytochrome c release through VDAC. However this difference may reflect differences in *in vitro* and *in vivo* systems, and the stage of apoptosis studied. Clearly more studies are required to clarify this issue.

3. Caspase activation

Bcl-X_L has also been shown to bind Apaf-1 and caspase-9 in mammalian cells and prevents activation of caspase-9 mediated by Apaf-1 (Pan *et al* 1998, Hu *et al* 1998). A model has been proposed whereby pro-apoptotic Bcl-2 family members displace Bcl-X_L from the apoptosome complex, thereby facilitating caspase-9 activation (Adams & Cory 1998). Indeed, Diva an unusual pro-apoptotic Bcl-2 family protein which functions in a BH3-independent manner, coimmunoprecipitates with Apaf-1 and prevents binding of Bcl-X_L in an overexpression system (Inohara *et al* 1998). However recent data has failed to demonstrate a direct interaction of Apaf-1 with Bcl-2 family members (Conus *et al* 2000) but a number of adaptor molecules have been identified which link Bcl-2 family members to caspases. Aven binds both Bcl-X_L and Apaf-1, interfering with the ability of Apaf-1 to self-associate and activate caspases (Chau *et al* 2000). An anti-apoptotic protein BAR was identified which contains a Bcl-2 and Bcl-X_L-interacting SAM domain and a DED-like domain which interacts with the DED-containing procaspases 8 and 10 (Zhang *et al* 2000). BAR therefore bridges Bcl-2/Bcl-X_L to the death-receptor associated procaspases, linking the

extrinsic and intrinstic apoptotic pathways. Another adaptor molecule Bap31 was found to complex Bcl-2/Bcl-X_L to procaspase 8 in the ER (Ng *et al* 1997, Breckenridge *et al* 2002). In the absence of Bcl-2, Bap31 was degraded by caspases forming a product that promoted apoptosis. Spike, an ER-associated pro-apoptotic BH3-only protein, also interacted with Bap31 and inhibited complex formation between Bcl-X_L and Bap31 (Mund *et al* 2003). Therefore the displacement of Bcl-X_L/Bcl-2 from Bap31 by Spike could facilitate caspase activation and apoptosis (Mund *et al* 2003).

Recent studies have suggested that Bcl-2 family proteins may regulate caspase activation upstream of cytochrome c release and independently of the apoptosome. A study by Marsden *et al* (2002) showed that Apaf-1 and caspase-9 were dispensable for apoptosis in murine knockout cells, whereas cells overexpressing Bcl-2 were resistant to apoptosis. Caspases were clearly activated and cytochrome c was released in apoptotic cells lacking Apaf-1 or caspase 9, so another caspase activation pathway must be important. Also, Bcl-2 can protect heterozygous Apaf-1 knockout embryonic stem cells from apoptosis (Haraguchi *et al* 2000). However, in this study Apaf-1 null cells did not die through apoptosis, but through a caspase independent mechanism, although Bcl-2 could still protect these cells from cell death. Therefore Bcl-2 can protect cells from cell death independent of Apaf-1, in a caspase-dependent or caspase-independent manner.

A theory has been proposed whereby Bcl-2 family proteins regulate upstream initiator caspases, which may then directly or indirectly lead to cytochrome c release or may activate effector caspases directly. In this manner, Bcl-2 could act like its *C. elegans* homolog CED-9 which prevents activation of an initiator caspase. A number of potential upstream initiator caspases have been identified, including caspases 1, 11 or 12 (Marsden *et al* 2002) and caspase 2 (Guo *et al* 2002, Lassus *et al* 2002, Robertson *et al* 2002). In some cell lines activation of caspase-2 is clearly required for apoptosis and caspase-2 can directly and indirectly trigger the release of cytochrome c and Smac/DIABLO from mitochondria (Lassus *et al* 2002, Guo *et al* 2002, Robertson *et al* 2002). Caspase 2 activity was also required for Bax translocation and cytochrome c release (Lassus *et al* 2002) and caspase 2 induced cytochrome c release can be prevented by overexpressing Bcl-2 or Bcl-X_L (Guo *et al* 2002). However it is not

clear whether Bcl-2/Bcl-X_L directly inhibit caspase-2 activity or prevent caspase-2 induced apoptosis by inhibiting mitochondrial changes.

What is the specific role of individual Bcl-2 family proteins and how do interactions between classes of Bcl-2 family proteins determine apoptosis sensitivity? The BH3 only proteins seem to be activated by specific apoptotic signals and function as triggers of apoptosis by antagonising pro-survival molecules. BH3-only proteins are expressed at low levels or kept inactive in healthy cells but are unleashed in response to a death stimulus. Noxa, Puma, and BNIP3 are transcriptionally regulated, with Noxa and Puma being transcriptional targets of the tumour suppressor protein p53, that is activated in response to DNA damage (Oda *et al* 2000, Nakano *et al* 2001), whereas BNIP3 can be induced by hypoxia (Bruick 2000). Bim and Bmf are kept inactive by localization to cytoskeletal structures, Bim associates with the microtubule-associated dynein motor complex and the Bmf associates with the myosin V actin motor complex (Puthalakath *et al* 1999, Puthalakath *et al* 2001). Certain apoptotic stimuli disrupt these associations allowing Bim and Bmf to act. Bad is inactivated in cells stimulated with survival factors by phosphorylation. Phosphorylation on specific serine residues causes Bad to be sequestered by the 14-3-3 protein in the cytosol, preventing it from targeting to the mitochondria (Zha *et al* 1996). During apoptosis initiated by death receptors (e.g. Fas, TNFR-1) caspase-8 is activated which cleaves cytosolic Bid to form a truncated product, tBid. tBid translocates to the mitochondria and inserts into the mitochondrial membrane where it can activate Bax and Bak (Luo *et al* 1998, Li *et al* 1998).

Killing via BH3-only molecules is dependent on the multidomain pro-apoptotic molecules, Bak and Bax, as cells lacking both Bax and Bak do not die upon expression of BH3-only proteins, in contrast to cells expressing either Bax or Bak (Zong *et al* 2001, Wei *et al* 2001, Cheng *et al* 2001). There are two classes of BH3-only proteins: (i) proteins that can activate multidomain pro-apoptotic proteins Bax and Bak directly to trigger apoptosis, characterised by Bid or Bim. These proteins can also bind Bcl-2 and Bcl-2 may sequester these molecules when Bcl-2 is in excess (fig. 1.5A) (ii) proteins that cannot activate Bax/Bak directly but instead bind Bcl-2/Bcl-X_L, leading to displacement of pro-apoptotic proteins bound to Bcl-2/Bcl-X_L. The pro-apoptotic proteins bound to Bcl-2/Bcl-X_L may be Bax/Bak (fig. 1.5B) or could be

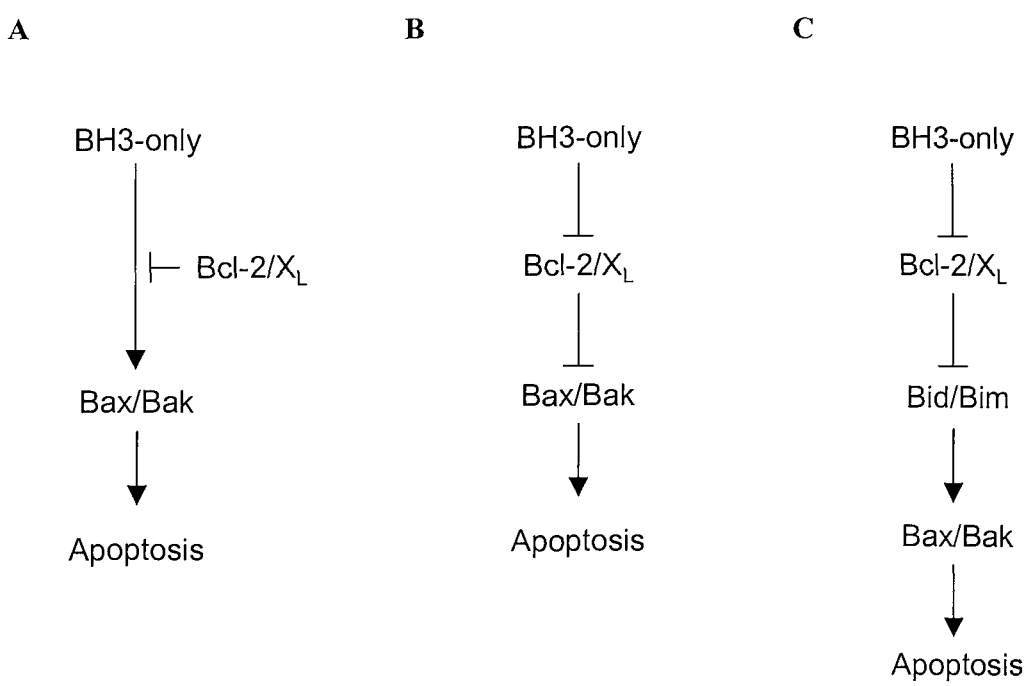


Figure 1.5: Mechanisms by which BH3-only proteins induce apoptosis. A – Some BH3 only proteins, e.g. Bid and Bim can activate and oligomerise the multidomain anti-apoptotics Bax and Bad directly, leading to cytochrome c release and apoptosis. In this model Bcl-2/Bcl-X_L prevent apoptosis by sequestering the BH3-only proteins. **B** – Other BH3-only proteins cannot activate Bax/Bak directly but can bind to Bcl-2/Bcl-X_L. This may displace any Bax/Bak molecules bound to Bcl-2/Bcl-X_L, facilitating apoptosis. **C** – This model is a combination of models A and B, whereby BH3-only proteins displace Bid/Bim sequestered by Bcl-2/Bcl-X_L, allowing Bid/Bim to activate Bax/Bak leading to apoptosis.

Bid/Bim type BH3-only proteins which then go on to activate Bax/Bak (fig. 1.5C) (Desagher *et al* 1999, Wei *et al* 2000, Eskes *et al* 2000,Yamaguchi *et al* 2002, Cheng *et al* 2001, Letai *et al* 2002, Moreau *et al* 2003). Current evidence favours a model whereby some BH3-only proteins act as antagonists of anti-apoptotic Bcl-2 family members and induce apoptosis by interfering with the interaction between pro and anti-apoptotics, thereby releasing pro-apoptotics to induce apoptosis. Elevated levels of Bcl-2/Bcl-X_L may therefore prevent apoptosis by sequestering all of the pro-apoptotic Bcl-2 family proteins. A recent paper has shown that, at least in UV irradiated HeLa cells, elimination of Mcl-1 is required for the events leading to apoptosis through the mitochondrial pathway, including Bax translocation and oligomerisation, and cytochrome c release (Nijhawan *et al* 2003). Following UV treatment in this system, Mcl-1 protein synthesis was blocked, and the exisiting pool of Mcl-1 protein was rapidly degraded by the proteasome. The elimination of Mcl-1 may allow the release of BH3-only proteins sequestered by Mcl-1, allowing the BH3-onlys to activate apoptosis. This puts Mcl-1 at the apical point in the Bcl-2 family pathway, upstream of BH3-only proteins. Our understanding of the mechanisms by which Bcl-2 family proteins control apoptosis is limited and so these models may be an oversimplification of the real events controlling cell death.

Expression and activity of Bcl-2 family members is regulated in a variety of ways. At the transcriptional level, anti-apoptotic members are induced by cytokines and growth factors e.g. interleukins 2,3 and 6 can induce Bcl-X_L expression, and Bax, Puma and Noxa transcription can be induced by p53. Both the Bcl-X and Mcl-1 RNAs can be alternatively spliced generating short forms of the proteins, called Bcl-X_S and Mcl-1S, that promote apoptosis. Bcl-X_S lacks a 63 amino acid region, which includes the BH1 and BH2 domains, whereas Mcl-1S contains only the BH3 domain (Bae *et al* 2000, Bingle *et al* 2000, Zorning *et al* 2001). Bcl-2 can be inactivated or activated by phosphorylation on different sites (Haldar *et al* 1995, Adams & Cory 1998) and Bad is also regulated by phosphorylation, as detailed above. Bcl-2 family members are also regulated by caspase cleavage. Caspase 8 can cleave the pro-apoptotic protein Bid to form tBid. tBid binds Bax or Bak resulting in their oligomerisation and cytochrome c release. Bcl-2, Bcl-X_L and Mcl-1 are also cleaved during apoptosis, producing proteins that promote apoptosis (Zorning *et al* 2001, J. Michels & G.Packham personal communication). Cleavage of Bax by calpains (calcium

dependent cysteine proteases which can be activated during apoptosis) has also been observed in response to interferon-gamma and chemotherapeutics (Toyota *et al* 2003). Cleaved Bax (p18) has increased cytotoxicity compared to wild type Bax, consistent with an inhibitory function for the cleaved N-terminus. These mechanisms amplify the apoptotic cascade, ensuring that once a cell embarks on the apoptotic pathway it cannot escape cell death.

1.3 Death receptors

Death receptors belong to the tumour necrosis factor receptor (TNFR) superfamily. There are at least 21 members of the TNFR family, including CD40 and the eight death receptors (TNF-R1, Fas, DR3, TRAIL-R1/DR4, TRAIL-R2/DR5, DR6, NGFR and EDAR). TNFRs are characterised by cysteine-rich sequences in their extracellular domain and are activated by binding ligands belonging to the TNF superfamily. At least 17 TNF family members have been identified and they all possess a trimeric structure, which can bind three receptors, leading to trimerisation or oligomerisation and activation of the receptors. In addition to the cysteine-rich extracellular domain, death receptors also contain a conserved cytoplasmic region of approximately 80 amino acids called the death domain (DD), which mediates protein-protein interactions and downstream signalling, generally leading to apoptosis. However, some death receptors can promote cell survival and some TNF family members can activate apoptosis even though they lack a DD e.g. CD40 (reviewed in Ozoren & El-Deiry 2003, Gupta 2001, Schneider & Tschopp 2000).

The best characterised death receptors are Fas, TNF and TRAIL. Activation of these molecules can also occur as a result of CD40 signalling (see section 1.7.3). Therefore, these molecules are discussed below.

1.3.1 Fas

Fas or CD95 is widely expressed with predominant expression on activated T-cells, whereas its ligand, FasL, is only expressed by cytotoxic T cells (CTLs) and natural killer (NK) cells and in sites of immune privilege (Ozoren & El-Deiry 2003). The Fas/FasL interaction is important for a number of immune functions including: (i) elimination of self-reactive T-cells (ii) deletion of activated T-cells at the end of an immune response (iii) killing target cells (e.g. virally infected cells or cancer cells) by

CTL and NK cells (iv) deletion of self-reactive B-cells (v) maintenance of immune privileged status of the retina and testis and (vi) homeostasis of the liver (Ozoren & El-Deiry 2003, Gupta 2001).

Ligation of Fas leads to aggregation of its death domains, which recruit an adaptor molecule Fas associated death domain (FADD), via its death domain. FADD also contains a death effector domain (DED), which binds the DED of procaspase 8. The complex of Fas, FADD and procaspase 8 molecules is called the death inducing signalling complex or DISC. The aggregation of procaspase 8 molecules in the DISC facilitates autoactivation, producing active caspase 8 molecules that dissociate from the DISC and cleave caspase 3 which perpetuates the caspase cascade leading to apoptosis. Cells can be grouped into two classes depending on the Fas signalling pathways utilised. In type I cells, strong activation of caspase 8 occurs at the DISC which is sufficient to activate caspase 3 and the caspase cascade. Activation of caspase 8 occurs before the loss of mitochondrial transmembrane potential, which is mediated by caspase 8 cleavage of Bid or caspase 3 mediated cleavage of pro-apoptotic Bcl-2 family members. However in type II cells, activation of caspase 8 mainly occurs downstream of the mitochondria and these cells are dependent on the mitochondrial pathway to undergo Fas-mediated apoptosis. In these cells only a small amount of caspase 8 may be activated at the DISC, which is sufficient to activate the mitochondrial pathway via Bid cleavage, but is not sufficient to activate caspase-3. Activation of caspases via the mitochondrial pathway then feedback onto the DISC, causing further caspase-8 activation, in an autoamplification loop that promotes apoptosis (Scaffidi *et al* 1998, Hennino *et al* 2000).

Other DISC components have also been identified which can modulate apoptosis. Procaspase 10 can also associate with FADD through homotypic association with its DED, where it is proteolytically activated. FLIP (FLICE-like inhibitory protein) occurs in two forms $FLIP_S$ and $FLIP_L$, which can associate with the DISC. $FLIP_S$ contains 2 tandem DEDs which are highly homologous to the N-terminus of caspase 8, and can block apoptosis by competing with procaspase 8 binding to FADD. $FLIP_L$ is a structural homolog of caspase-8, lacking protease activity because several of its active site residues are different. $FLIP_L$ is also a potent inhibitor of death receptor-induced apoptosis, but when overexpressed *in vitro* it has been shown to promote

apoptosis. FLIP deficient mice show enhanced sensitivity to apoptosis, promoting a death-suppressing role for FLIP *in vivo*. $FLIP_L$ is thought to inhibit apoptosis by formation of $FLIP_L$/procaspase 8 homodimers at the DISC. This association leads to limited caspase 8 processing, resulting in an activated caspase which remains tethered to the DISC complex. Here, it cleaves DISC components but cannot be released into the cytoplasm, therefore preventing apoptosis. In conditions of overexpression, $FLIP_L$ may accumulate in the cytoplasm and activate non-membrane procaspase 8, leading to apoptosis (Micheau *et al* 2002, Thome & Tschopp 2001, Peter & Krammer 2003).

1.3.2 TNF

TNF can signal through two receptors, TNF-R1 (has a DD) and TNF-R2 (does not have a DD), leading to either apoptosis or survival. Most of the effects of TNF are achieved through TNF-R1 engagement, although TNF-R2 does enhance TNF-R1 signalling. TNF is produced mainly by activated macrophages and T-cells in response to infection, whereas the receptors are widely expressed, leading to expression of proinflammatory and immunomodulatory genes, or apoptosis (Ozoren & El-Deiry 2003, Gupta 2001, Ashkenazi & Dixit 1998).

TNFR-1 trimerisation leads to association with the adaptor protein TRADD (TNFR-associated death domain) via their DDs. TRADD can recruit several other adaptor molecules including FADD (leading to caspase 8 activation and apoptosis, in a similar manner to Fas), TRAF 2 (TNFR-associated factor 2) and RIP (Receptor Interactive Protein, a DD-containing serine/threonine kinase). RIP is essential for activation of NF-κB, whereas TRAF 2 can activate the NF-κB and JNK pathways (Ozoren & El-Deiry 2003, Gupta 2001, Ashkenazi & Dixit 1998).

1.3.3 TRAIL

TRAIL (TNF-related apoptosis inducing ligand) or Apo2L can potently induce apoptosis in a broad range of cancer cell lines, but not in many normal cells. TRAIL can bind to five receptors, two of which carry a death domain, called DR4 and DR5. Two of the other receptors, DcR1 (TRAIL-R3 or TRID) and DcR2 (TRAIL-R4 or TRUNDD) are decoy receptors, capable of binding TRAIL but not transmitting a signal due to absent or incomplete death domains. A fifth TRAIL binding protein,

osteoprotegrin (OPG), has also been identified, which is a soluble TNFR that binds TRAIL relatively weakly (LeBlanc & Ashkenazi 2003).

The death domains of DR4 and DR5 recruit the DISC components FADD, procaspase 8 and procaspase 10 in a similar manner to Fas, leading to caspase activation and apoptosis. The physiological role of TRAIL is unclear but may be involved in NK cell, dendritic cell, T-cell and monocyte cytotoxicity, which is often augmented by interferon-regulated expression of TRAIL and its death receptors. Consistent with this, TRAIL may function in immune surveillance and defence against tumour initiation and metastasis (Ozoren & El-Deiry 2003, LeBlanc & Ashkenazi 2003).

The molecular mechanisms involved in the increased sensitivity of cancer cells versus normal cells to TRAIL-induced apoptosis are not clear cut but may depend on the following factors: (i) expression levels of DR4 or DR5 (ii) relative levels of either death receptor on the cell surface (iii) expression levels of decoy receptors (iv) high FLIP expression (v) release of Smac/DIABLO from the mitochondria and (vi) levels of NF-κB, protein kinase C, MAPK or AKT activity. Sensitivity to TRAIL can also be enhanced or induced by DNA damaging agents, ionising radiation, overexpression or activation of p53 (Ozoren & El-Deiry 2003, LeBlanc & Ashkenazi 2003).

Despite the apoptosis promoting effects of Fas and TNF, they have proved unsuitable for cancer therapy as systemic administration induces toxic side effects. However TRAIL remains promising as an anti-tumour agent since normal cells are generally TRAIL insensitive. *In vivo* studies have shown encouraging results with either TRAIL alone or in combination with chemotherapy or radiation. Various recombinant versions of TRAIL have been generated, as well as monoclonal antibodies that engage DR4 or DR5, which have varying toxicities e.g. some preparations show hepatocyte toxicity. However some preparations of TRAIL were well tolerated even at very high doses in mice and nonhuman primates (Ozoren & El-Deiry 2003, LeBlanc & Ashkenazi 2003). Although further research is necessary, some preparations of TRAIL may prove to be an effective cancer therapy for humans, either alone or in combination with traditional anti-cancer treatments.

1.4 Apoptosis and B-cell development

Apoptosis is critical to B-cell development. It is estimated that 95% of B-cell precursors die during development by apoptosis (Chao & Korsmeyer 1998) and widespread apoptosis occurs within lymph nodes during a humoral immune response.

B-cell generation occurs in the bone marrow independent of antigen (see fig. 1.6), beginning with the differentiation of lymphoid stem cells into progenitor B-cells (pro B-cells) (Goldsby *et al* 2000). Pro B-cells proliferate within the bone marrow and differentiate into precursor B-cells (pre B-cells), which requires interaction with bone marrow stromal cells. Pro B-cells undergo heavy chain immunoglobulin (Ig) gene rearrangements during their differentiation to pre B-cells. Development of a pre B-cell into an immature B-cell requires successful rearrangement of the Ig light chain genes. The vast majority of pre B-cells undergo apoptosis probably due to the faulty rearrangement of Ig genes (Cohen *et al* 1991). Those pre B-cells which possess functional Ig genes express IgM on the cell surface and are classified as immature B-cells. Immature B-cells that express self-reactive IgM die when they encounter self-antigens within the bone marrow. This negative selection deletes self-reactive B-cell clones, thus preventing autoimmunity. Further development of immature B-cells leads to the coexpression of surface IgM and IgD with a single antigenic specificity, which characterises mature B-cells.

Mature B-cells are exported to the blood and lymph, where they are termed naive B-cells as they have yet to encounter antigen, and are carried to the spleen and lymph nodes. In the absence of antigen, naive B-cells die by apoptosis within a few days. In the spleen and lymph nodes B-cell activation, selection and differentiation occur. Antigen is filtered from the blood and lymph by the spleen and lymph nodes, where it accumulates and is presented by antigen presenting cells e.g. follicular dendritic cells. Naive B-cells which encounter antigen specific to their membrane bound antibodies are activated and proliferate. Activated B-cell clones enter primary follicles within the lymph node, which consist of a network of follicular dendritic cells, naive B-cells and T helper cells, forming secondary follicles. Secondary follicles consist of a germinal center surrounded by the follicular mantle.

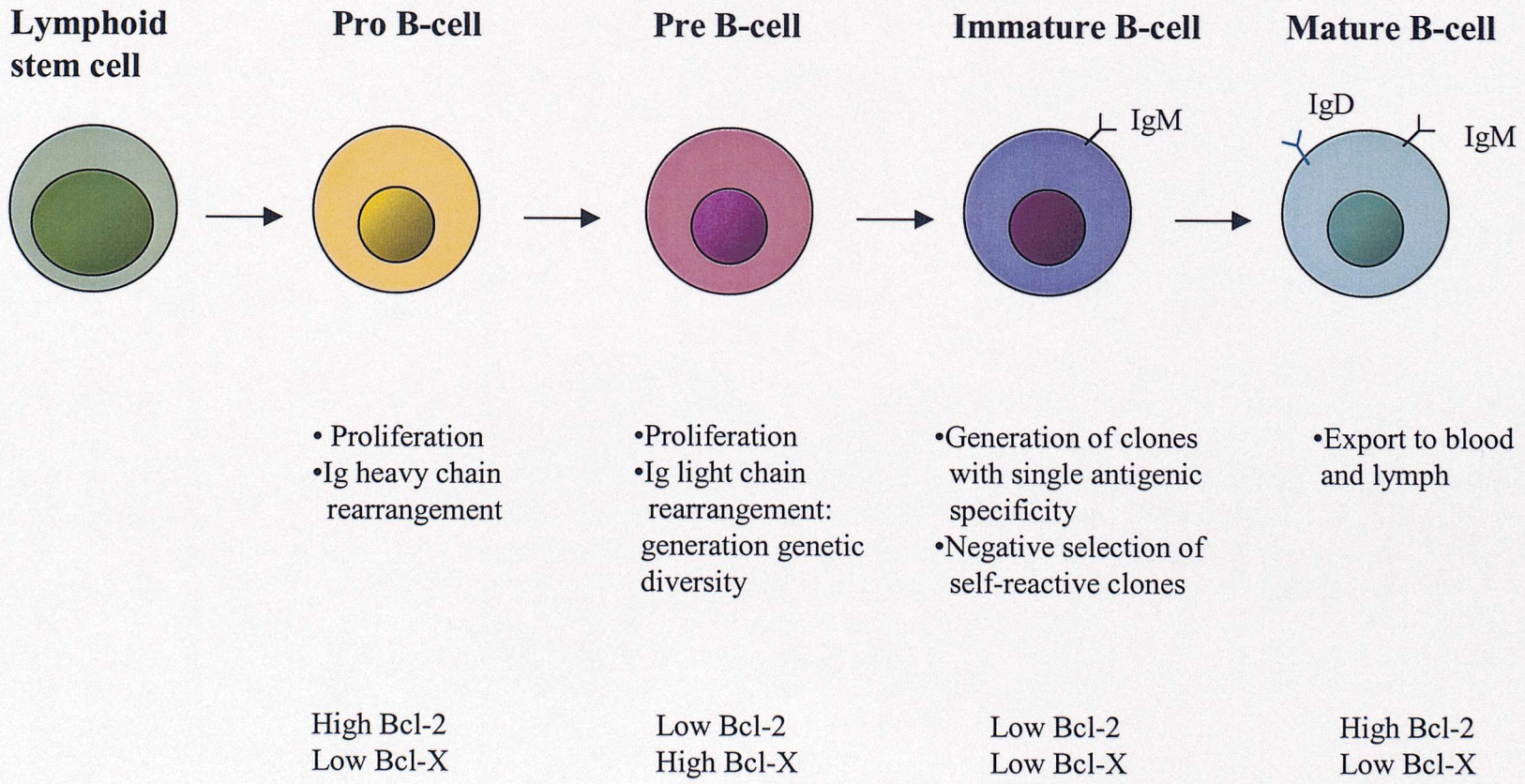


Figure 1.6: B-cell development in the bone marrow. Generation of mature B-cells, expressing membrane IgM and IgD, occurs through a specific sequence of events involving Ig gene rearrangements and strong selection for non self-reactive clones with a single antigenic specificity. Expression of the anti-apoptotic Bcl-2 and Bcl-X_L proteins is indicated at each developmental stage.

The first stage of germinal center formation involves intense proliferation of activated B-cells so they fill the spaces between the network of follicular dendritic cells. Polarisation of the germinal center develops when the activated B-cells move to one edge of the follicle, known as the dark zone (fig. 1.7). The activated B-cells are now termed centroblasts and have lost their membrane Ig. Centroblasts rapidly divide and undergo somatic mutation of heavy and light chain variable region genes, to increase the affinity of antibodies produced. The progeny of centroblasts are centrocytes which are small non-dividing cells that express membrane Ig. Centrocytes enter a region called the light zone at the center of the follicle where they interact with antigen presenting cells and T helper cells. In the light zone, centrocytes undergo selection to select only those B-cells that express antibodies with a high affinity for antigen. Those surviving centrocytes differentiate into memory cells and plasmablasts. Plasmablasts migrate into the center of the lymph node where they develop into antibody secreting plasma cells. During their differentiation to plasma cells, centrocytes undergo class switching, which changes the class of antibody that the B-cell produces e.g. from IgM to IgE, IgG or IgA (Goldsby *et al* 2000, Guzman-Rojas *et al* 2002).

The selection of high affinity B-cells in the germinal centre is regulated by membrane Ig, Fas and CD40. CD40 is a key molecule involved in the activation and proliferation of B-cells (see section 1.5). Antigen binding to membrane Ig is necessary for germinal centre B-cell survival but not sufficient. Germinal centre B-cells must also receive signals from CD4+ T helper cells, via, at least in part, interactions between CD40L on the T helper cell and CD40 on the B-cell. If the germinal centre B-cell fails to receive the correct signals it will undergo apoptosis, which may be mediated by Fas (Goldsby *et al* 2000, Krammer 2000).

During B-cell development the expression of Bcl-2 family proteins is tightly and reciprocally regulated. In the bone marrow, Bcl-2 expression is high in pro B-cells and mature B-cells, whereas it is low in pre B-cells and immature B-cells. Conversely, Bcl-X_L expression is low in pro B-cells and resting mature B-cells, but it is high in pre B-cells (Chao & Korsmeyer 1998) (fig 1.6). Within the lymph node, Bcl-2 expression is high in the mantle zone but absent in germinal center B-cells, however Mcl-1 expression is high in germinal center and activated B-cells and absent from mantle zones (Krajewski *et al* 1994). This reciprocal expression of Bcl-2 family proteins

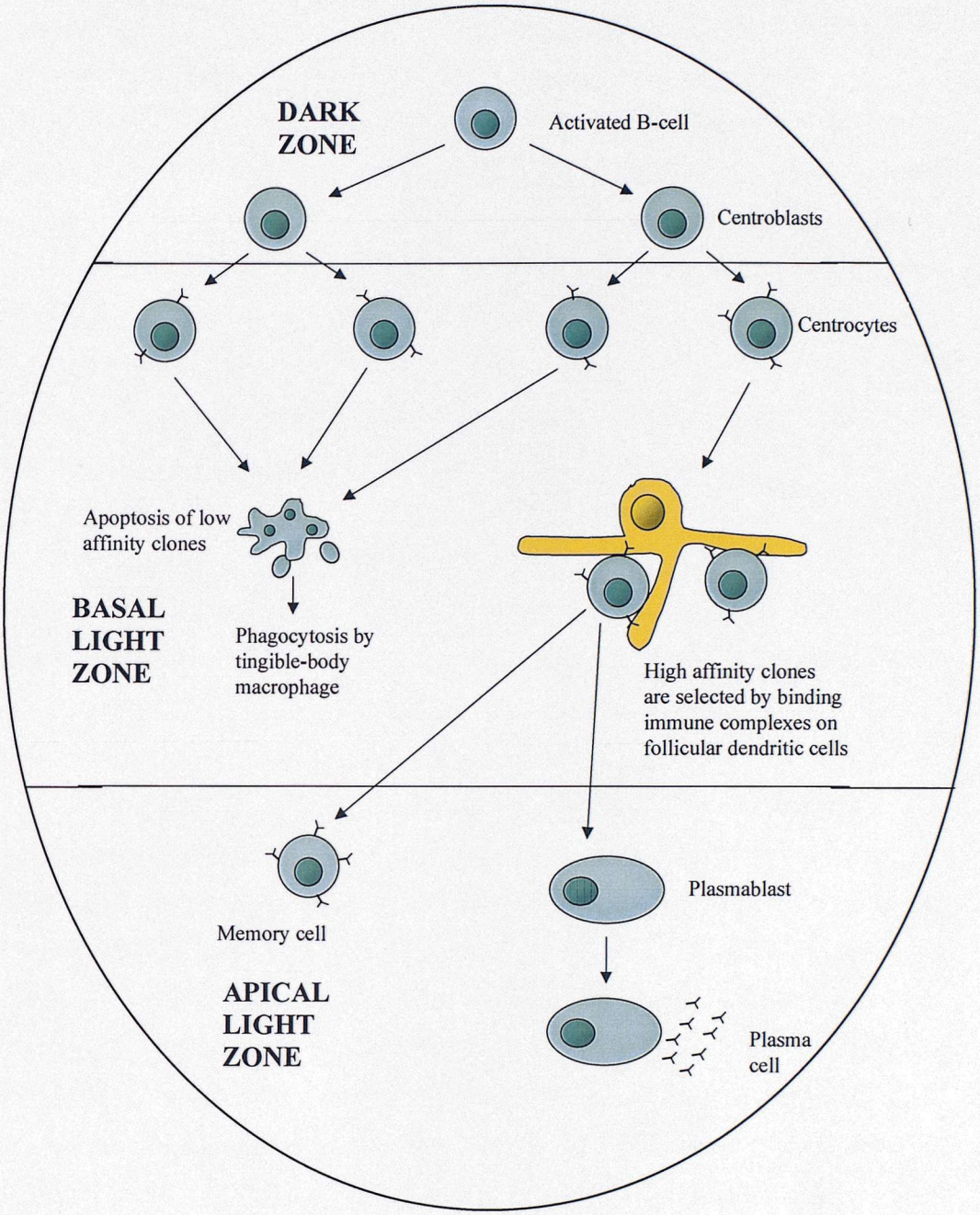


Figure 1.7: Events occurring within the germinal centres of lymph nodes. Activated B-cells proliferate within the dark zone of the germinal centre and undergo somatic mutation. In the light zone, clones with a high affinity for antigen are selected and differentiate into memory cells and plasma cells, which leave the germinal centre. Unselected centrocytes with low affinity membrane bound antibodies undergo apoptosis.

indicates tight regulation which is critical for lymphocyte development. Indeed, Bcl-X knockout mice show embryonic lethality with massive death of immature haematopoeitic cells. Chimeric mice containing Bcl-X deficient lymphocytes show a shortened lifespan and a decrease in the pre B-cell population (Motoyama *et al* 1995). Bcl-2 knockouts, although born normally, die within a few weeks after birth as they are unable to maintain B and T-cell homeostasis. Therefore Bcl-X_L is critical for the survival of immature B-cells, whereas Bcl-2 functions to maintain the mature B-cell population. The pro-apoptotic Bcl-2 family member Bim was shown to be involved in deleting autoreactive cells and reducing the population of antibody forming cells after the immune response (Marsden & Strasser 2003).

1.5 CD40 structure and function in normal B-cells

CD40 is a 48 kDa transmembrane glycoprotein cell surface receptor, which belongs to the tumour necrosis factor (TNF) receptor family (Kehry 1996). CD40 consists of a 193 amino acid residue extracellular domain, containing four cysteine-rich repeats, a 22 amino acid residue transmembrane domain and a 62 amino acid residue cytoplasmic tail, which can activate various cell signaling pathways through interaction with adaptor molecules (Fiumara & Younes 2001). CD40 was first identified and functionally characterised on mature B-cells but it is also expressed on immature B-cells, monocytes, dendritic cells, haematopoietic progenitors, endothelial cells and epithelial cells, as well as B-cell malignancies and some carcinomas (reviewed in van Kooten & Banchereau 1997, Younes & Carbone 1999). The ligand for CD40 (CD40L, CD154 or gp39) is a trimeric 33-39KDa type II transmembrane glycoprotein predominately expressed by activated CD4+ T-cells (Klaus *et al* 1997). However CD40L is also expressed on activated B-cells, natural killer cells, monocytes, eosinophils, basophils, dendritic cells, platlets, endothelial cells and smooth muscle cells (Fiumara & Younes 2001).

CD40-mediated stimulation of normal B-cells plays a key role in T-cell mediated B-cell activation and the humoral immune response. CD40 ligation has been shown to promote B-cell proliferation, immunoglobulin (Ig) production, Ig isotype switching, induction of B-cell memory and rescue from apoptosis. Blocking the CD40-CD40L interaction with soluble CD40 or monoclonal antibodies to CD40L, prevents B-cell proliferation or Ig production in response to T-cell signals (reviewed in Clark &

Ledbetter 1994). Mutations in the gene encoding CD40L causes a rare immunodeficiency disease called X-linked hyper-IgM syndrome (Korthauer *et al* 1993, DiSanto *et al* 1993). This is characterised by normal or elevated levels of IgM but no IgG, IgA or IgE production, and defects in germinal centre and memory B-cell formation. A similar phenotype is seen in CD40 or CD40L knockout mice, which cannot mount normal humoral immune responses (Klaus *et al* 1997).

At least some of the effects of CD40 on immune responses are thought to be dependent on its ability to promote B-cell proliferation and survival. Banchereau *et al* (1991) were the first group to show that CD40 ligation could induce B-cell proliferation by culturing human B-cells with anti-CD40 monoclonal antibodies (mAbs), presented by CDw32 Fc receptor-transfected fibroblasts. In combination with interleukin-4 (IL-4), long term normal human B-cell lines were generated. Recombinant soluble mouse CD40L also induces mouse and human B-cell proliferation, which can be enhanced with anti-Ig antibodies (Lane *et al* 1993).

The first indication that CD40 regulates apoptosis in normal B-cells resulted from experiments with purified germinal centre B-cells. These cells rapidly undergo apoptosis *in vitro,* however treatment of the cells with anti-CD40 mAbs induced long-term cell survival (Liu *et al* 1989, Liu *et al* 1991(i)). Subsequent studies have confirmed that CD40 ligation rescues germinal centre B-cells and peripheral blood B-cells from spontaneous apoptosis, using anti-CD40 mAbs or human soluble CD40L (Holder *et al* 1993, Lomo *et al* 1997). CD40 activation using CD40L transfected cells or anti-CD40 mAbs can also rescue germinal centre B-cells from apoptosis induced by Fas ligation (Cleary *et al* 1995, Koopman *et al* 1997).

1.6 CD40 responses in malignant cells

Although CD40 is considered a survival factor for normal B-cells, both suppression and induction of apoptosis have been observed in malignant cells (Dallman *et al* 2003).

1.6.1 Suppression of apoptosis by CD40 in B-cell malignancies

Many studies have shown that cross-linking of cell surface IgM with anti-IgM antibodies in the WEHI-231 cell line (immature murine B-cell lymphoma) and the Burkitt's Lymphoma cell line Ramos, leads to growth inhibition and apoptosis, which can be rescued by CD40 activation (Tsubata *et al* 1993, Kelly & Knox 1995, Siebelt *et al* 1997, Padmore *et al* 1997, Curnock *et al* 1998, Lin *et al* 1998, Schauer *et al* 1998, Kuss *et al* 1999, Craxton *et al* 2000). Ramos cells are also susceptible to Fas-mediated (Lee *et al* 1999) and calcium ionophore induced apoptosis (Kelly & Knox 1995), which can be rescued by CD40 ligation. CD40 activation was also shown to rescue five non-Hodgkin's lymphoma cell lines from apoptosis induced by the cytotoxic drug doxorubicin (Voorzanger-Rousselot *et al* 1998).

In addition to CD40 signalling suppressing apoptosis of normal B-cells and some B-cell lines, CD40 activation can induce positive effects in primary lymphoma cells. Isolated follicular lymphoma cells could be induced to proliferate and grow in prolonged culture using anti-CD40 mAbs presented by CDw32 Fc receptor-transfected fibroblasts, in the presence of IL-4 (Johnson *et al* 1993). Ghia *et al* (1998) demonstrated that primary follicular lymphoma cells treated with soluble CD40L showed increased survival compared to cells cultured in media alone or in the presence of various cytokines. Although soluble CD40L did not rescue primary mantle cell lymphoma cultures from spontaneous apoptosis in a study by Andersen *et al* (2000), it did induce DNA synthesis and cell cycle progression in malignant cells. A study using primary chronic lymphocytic leukaemia (CLL) B-cells found that culture with CD40L expressing fibroblasts rescued cells from spontaneous, fludarabine- and Fas-induced apoptosis (Kitada *et al* 1999), which confirmed the results of a previous study showing that anti-CD40 antibodies protected CLL cells from fludarabine-induced apoptosis (Romano *et al* 1998). Similarly, CD40 ligation of primary acute myeloid leukaemia (AML) samples induced proliferation, increased clonogenic potential and rescued cells from apoptosis induced by serum deprivation (Aldinucci *et al* 2002).

1.6.2 Growth inhibition or induction of apoptosis by CD40 in B-cell malignancies

A number of studies using malignant B-cell lines have found that CD40 activation can induce growth arrest and/or apoptosis. The first report showing CD40 stimulation is capable of inhibiting proliferation of a lymphoma cell line was reported by Heath *et al* (1993), who demonstrated that, in contrast to its effects on normal murine B-cells, a rat anti-mouse CD40 antibody inhibited the *in vitro* growth of A20 murine B lymphoma cells. Funakoshi *et al* (1994) showed that treatment of two human diffuse large B-cell lines and two Epstein-Barr Virus (EBV)-induced human B lymphoma lines with anti-CD40 mAbs or soluble CD40L produced significant growth inhibition. Cross-linking the mAbs resulted in greater inhibition of proliferation and also inhibited growth of the previously unresponsive Burkitt's Lymphoma cell line Raji.

A study treating seven EBV negative and seven EBV positive human Burkitt's lymphoma cell lines with anti-CD40 mAbs or soluble CD40L, found differential responses to CD40 ligation independent of their EBV status. Nine of the cell lines, including Ramos, were classified as high responders showing rapid aggregation and growth inhibition in response to CD40 ligation, whereas the remaining five lines, including L3055, showed no visible response to CD40 ligation (Henriquez *et al* 1999). However, in another study, CD40 ligation induced both growth arrest and rescue from anti-IgM induced apoptosis in L3055 cells (Baker *et al* 1998). Similarly, Henriquez *et al* (1999) showed CD40 ligation induced growth arrest in Ramos cells, but other studies have shown CD40 activation rescues Ramos cells from anti-IgM mediated apoptosis (Kelly & Knox 1995, Curnock *et al* 1998, Lin *et al* 1998, Craxton *et al* 2000). A recent report culturing B lymphoma cell lines with CD40L- transfected fibroblasts showed that CD40 activation not only decreased proliferation but also induced apoptosis in susceptible cell lines (Lefterova *et al* 2000). Similarly, anti-CD40 mAbs or soluble CD40L were both shown to induce apoptosis in Daudi cells (a Burkitt's lymphoma cell line) (Szocinski *et al* 2002).

CD40 activation can also produce negative effects on more differentiated malignant B-cells. CD40 ligation on XG2, a highly CD40 expressing multiple myeloma cell line, resulted in growth arrest (Pellat-Deceunynck *et al* 1996) and induced apoptosis (Bergamo *et al* 1997). However CD40 ligation in other multiple myeloma cell lines has been shown to induce proliferation (Teoh *et al* 2000).

To date, there has only been one report showing negative effects of CD40 ligation on human primary malignant B-cell cultures, but there have been no reports showing that CD40 activation alone can induce apoptosis in primary malignant B-cells. De Totero *et al* (2003) cultured CLL cells with murine L cells presenting CD40L for 72 hours and then cultured the CLL cells for an additional 24 hours with fludarabine. CD40L pretreatment increased fludarabine-induced apoptosis in eleven out of twenty CLL samples. In seven samples CD40 preactivation did not affect fludarabine-induced apoptosis and in two samples CD40-preactivation protected cells from the effects of fludarabine, showing CLL can have a heterogenous response to CD40 stimulation. However in all samples CD40L protected cells from spontaneous apoptosis. These results conflict those of Romano *et al* (1998) and Kitada *et al* (1999), who showed that CD40 protected CLL cells from fludarabine-induced apoptosis, but could be explained by the methods used to activate CD40 in each study and the fact that CD40 was preactivated in this study but cells were cultured with CD40 activating agents and fludarabine concurrently in the previous studies. Indeed, a recent study has shown that coculture of CLL cells with CD40L and fludarabine reduced fludarabine-induced apoptosis, whereas pretreatment with CD40L did not affect fludarabine-induced apoptosis (Grdisa 2003).

1.6.3 Carcinoma cells

Malignant non-B-cell lines generally undergo apoptosis as a result of CD40 ligation. SV80 (SV40-transformed human fibroblast cell line), HeLa (cervical carcinoma cell line) and A9 (murine L cell derivative) cells transfected with CD40 cDNA treated with membrane bound CD40L were efficiently killed in a dose-dependent manner, when *de novo* protein synthesis was inhibited. However, membrane bound CD40L had no effect on the viability of CD40 negative wild type cells or mock transfectants (Hess & Engelmann 1996). This phenomenon was not restricted to carcinoma cells expressing exogenous CD40, as MG75 and MG79 ovarian tumour cell lines, which constitutively express CD40, also responded to soluble CD40L by induction of apoptosis (Eliopoulos *et al* 2000). However, in the same study, CD40 positive A2780 ovarian carcinoma cells were resistant to CD40L induced apoptosis even when high concentrations of ligand were used. CD40 ligation also induces growth inhibition and apoptosis of CD40 expressing human malignant melanoma, multiple myeloma,

bladder carcinoma and pancreatic carcinoma cell lines and primary neuroblastoma cells (von Leoprechting *et al* 1999, Dotti *et al* 2001, Alexandroff *et al* 2000, Bugajska *et al* 2002, Airoldi *et al* 2003). CD40 engagement also directly inhibits growth of human breast carcinomas both *in vitro* and *in vivo* and ovarian tumour xenografts grown in mice (Wingett *et al* 1998, Hirano *et al* 1999, Tong *et al* 2001, Ghamande *et al* 2001).

However CD40 activation was shown to have positive effects on carcinoma cell survival in a limited number of studies. CD40L or anti-CD40 antibodies were shown to protect a urinary bladder carcinoma line from Fas-mediated apoptosis (Jakobson *et al* 1998) and CD40 activation stimulated proliferation of renal cell carcinoma cell lines and protected Kaposi's sarcoma cells from vincristine induced apoptosis (Bussolati *et al* 2002).

Despite CD40 activation having a generally negative effect on carcinoma cell survival, CD40 expression in lung cancer and melanoma has been correlated with poor prognosis and metastatic disease (van den Oord *et al* 1996, Sabel *et al* 2000). This could be because CD40 expressing carcinoma cells suppress T-cell activation, reducing the chance that malignant cells are eradicated by the immune system. A recent study has shown that incubation of primary renal cell carcinomas with primary healthy T-cells led to decreased CD40L expression on the T-cells. Furthermore, patients with CD40 positive breast or ovarian carcinomas possessed T-cells with decreased CD40L expression and impaired T-cell responses. When carcinoma cell lines were co-cultured with T-cells, the T-cells showed decreased interferon-gamma secretion, decreased IL-2 secretion and decreased proliferation and these responses were blocked with antibodies that inhibited the CD40-CD40L interaction (Batrla *et al* 2002). Therefore it is possible that the CD40-CD40L interaction between the carcinoma cells and T-cells leads to receptor-mediated endocytosis of CD40L on the T-cells, as carcinoma cells do not express costimulatory signals, like B-cells, thus inducing T-cell suppression.

CD40 may also have a role in invasion and angiogenesis of solid tumours. Upregulation of CD40 was observed in tumour blood vessels of renal carcinomas and Kaposi's sarcoma (Ottaiano *et al* 2002, Biancone *et al* 1999). CD40 activation on endothelial cells promotes *in vitro* tubule formation, proliferation and survival and *in vivo* angiogenesis, via production of vascular endothelial growth factor (VEGF) (Melter *et al* 2000, Deregibus *et al* 2003, Reinders *et al* 2003). CD40 also induces VEGF production in multiple myeloma cells (Tai *et al* 2002).

1.6.4 In vivo studies

Many *in vivo* studies have demonstrated that activating CD40, through a variety of methods, effectively reduces and/or prevents tumour growth in mice. Studies have either used mice with severe combined immune deficiency (SCID) or mice with normal immune responses. Funakoshi (1994) injected various human B-cell lymphomas into SCID mice and treatment with anti-CD40 mAbs resulted in a significant increase in survival. In a later paper by the same group (Funakoshi *et al* 1997) an *in vivo* model for human EBV induced lymphomagenesis was used. Here normal human peripheral blood lymphocytes from EBV positive donors were transferred into SCID mice and the mice spontaneously developed aggressive B-cell lymphomas. Treatment of the mice with anti-CD40 mAb every other day for 20 days significantly prevented the development of lymphoma compared to mice injected with an isotype matched control antibody. However, in Funakoshi's 1997 study, if human peripheral blood lymphocytes were incubated overnight with anti-CD40 mAb and subsequently injected into SCID mice, no increase in survival was seen compared to controls.

The eradication of tumours in SCID mice by anti-CD40 antibodies is probably largely due to direct effects of CD40 stimulation on the tumour, inducing cell death. However in many other studies using mice with normal immune responses, agents that activate CD40 can cure and protect mice from a range of tumours via activation of the immune system (Dilloo *et al* 1997, Tutt *et al* 1998, French *et al* 1999, Francisco *et al* 2000, Kikuchi *et al* 2000, Todryk *et al* 2001, van Mierlo *et al* 2002, Tutt *et al* 2002). Studies have shown that CD4+ T-helper cells, CD8+ cytotoxic T-cells (CTLs) and natural killer (NK) cells can be activated during CD40 immunotherapy, although the

role of NK cells and CD4+ T-cells is not consistent. Some studies have shown that activation of CD4+ cells or NK cells is required for optimal immunotherapy or primary rejection of the tumour, whereas other studies show that these cells are not required for CD40-induced anti-tumour immunity (Dilloo *et al* 1997, Francisco *et al* 2000, Todryk *et al* 2001, Liu *et al* 2002, Tutt *et al* 2002). However there is a consensus that CTLs are activated by CD40 immunotherapy and these cells can mediate anti-tumour immunity. CD40 negative tumours were also shown to be successfully treated by anti-CD40 antibodies (Todryk *et al* 2001, van Mierlo *et al* 2002). Therefore CD40 activation in mouse models elicits specific antitumour immunity, generally through activating CD8+ cytotoxic T-lymphocytes, which is effective against a number of tumours, independent of CD40 expression.

Tumour load also appears to be an important factor in CD40-based immunotherapy, as mice with the largest tumour burdens showed the most effective responses to anti-CD40 antibodies (Tutt *et al* 2002). This was also shown to be the case in combining anti-CD40 treatment with irradiation, which successfully treated lymphomas in mice over and above treatment with each agent alone (Honeychurch *et al* 2003). Large tumour loads may express more tumour antigens which are needed for CTL recognition and killing.

Activation of anti-tumour CTLs is believed to occur via activation of antigen presenting cells e.g. dendritic cells. Treatment with CD40L or anti-CD40 mAbs stimulates CD40 on antigen presenting cells leading to their maturation, which bypasses the need for CD40L expressing activated T helper cells (CD4+). Mature antigen presenting cells can activate cytotoxic T-lymphocytes through, at least in part, upregulation of costimulatory molecules, tumour antigen presentation and the production of inflammatory cytokines e.g. IL-12 (Bennet *et al* 1998, Ridge *et al* 1998). Indeed, transduction of human myeloma cells, primary renal cell carcinomas or bladder carcinoma cell lines with an adenovirus encoding CD40L and coculture with immature dendritic cells led to activation and maturation of the dendritic cells, which was inhibited with blocking CD40 antibodies (Bashey *et al* 2002, Loskog *et al* 2002). Also, CD40 signalling on the tumour itself can lead to upregulation of costimulatory molecules e.g. CD80 (B7.1), CD86 (B7.2) and HLA molecules, which increase

tumour antigen presentation and can directly activate CTLs, enhancing tumour immunogenicity (Younes & Kadin 2003, Airoldi *et al* 2003).

The anti-tumour therapeutic T-cell response initiated by CD40 ligation has recently been exploited in clinical studies. In a study trialing CD40L gene therapy for treatment of human CLL, the CD40L gene was transfected into CLL cells extracted from patients, using a replication-defective adenovirus vector, and the cells were then infused back into patients (Wierda *et al* 2000). This resulted in increased expression of immune accessory molecules, not only on the transfected cells but also on bystander leukaemia cells, and an increase in the number of T-cells and levels of Th1 cytokines produced. Clinically, patients showed significant reductions in blood lymphocyte counts and lymph node size, showing this may be a useful strategy for treating leukaemias. The anti-tumour T-cell response was suggested to kill CLL cells via activation of Fas-mediated apoptosis on CD40-activated CLL cells (Chu *et al* 2002). The effect of soluble recombinant CD40L on 32 patients with advanced solid tumours or non-Hodgkin lymphoma was tested in a phase I clinical trial. Limited anti-tumour activity was observed, with one patient with laryngeal cancer showing a long-term complete remission. Mild to moderate side effects were observed, including hepatic toxicity at large doses (Vonderheide *et al* 2001).

However, there is a concern that activation of CD40 in patients with B-cell malignancies will lead to further proliferation of malignant B-cells. The clinical effect of CD40 immunotherapy must depend on the balance between increased tumour growth and increased immunogenicity. Although the limited data so far show some therapeutic benefit of CD40 activation, this concern must be taken into account and CD40 immunotherapy may be a more suitable treatment for CD40 negative tumours.

1.7 CD40 target genes

Ligation of CD40 activates a range of signalling molecules leading to changes in gene expression. CD40 targets include both pro- and anti-apoptotic proteins impacting on both the intrinsic and extrinsic cell death pathways (figure 1.8). These molecules include the anti-apoptotic proteins Bcl-2, Bcl-X_L, Bfl-1, Mcl-1, A20 and survivin, and the pro-apoptotic proteins Bax and Fas. The molecular mechanisms for the differential regulation of cell survival by CD40 are not known (Dallman *et al* 2003). Here some

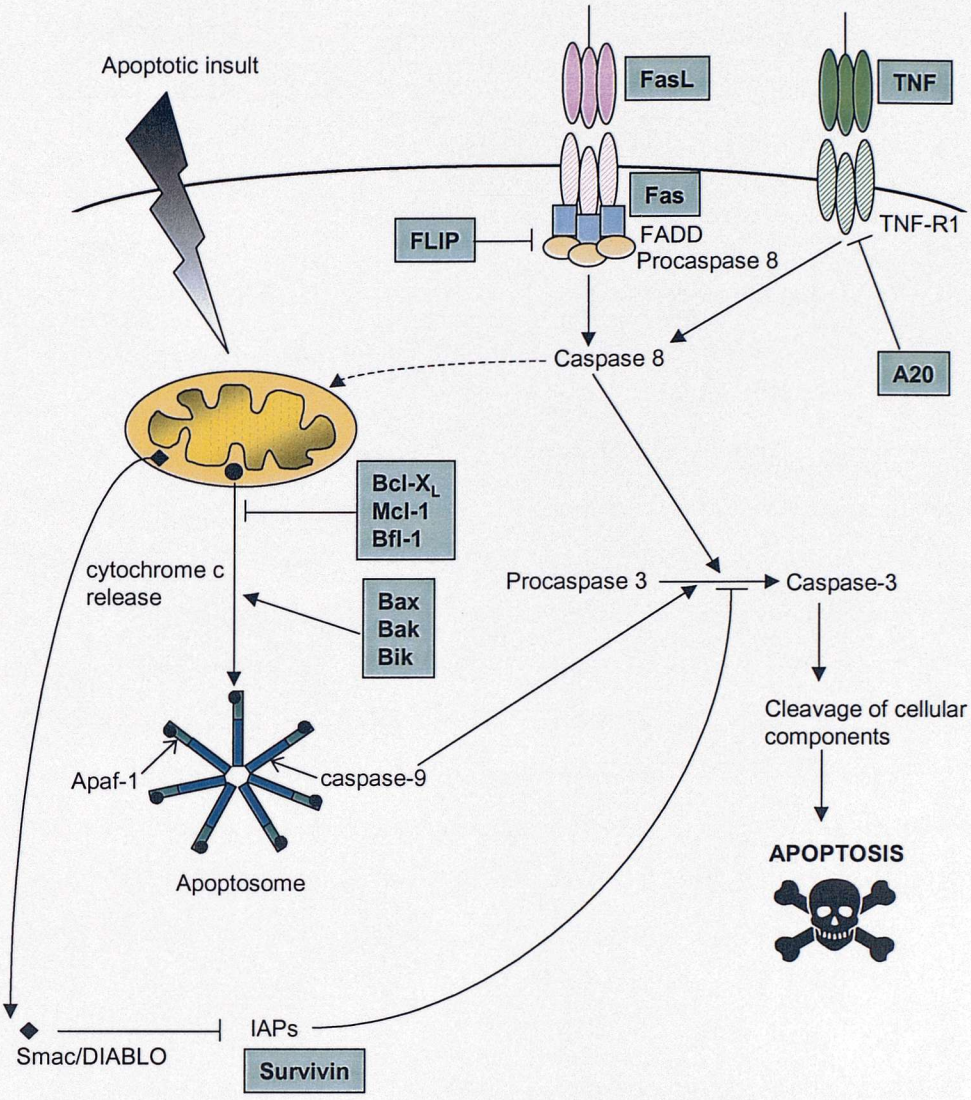


Figure 1.8: CD40 target genes and apoptosis. CD40 target genes, shown in blue boxes, impinge on both the intrinsic and extrinsic apoptotic pathways. The intrinsic pathway involves the release of factors from the mitochondria, such as cytochrome c and Smac/DIABLO. Cytochrome c combines with Apaf-1 and caspase-9 leading to caspase-3 activation and apoptosis. Cytochrome c release is prevented by Bcl-2 and CD40 induces expression of the Bcl-2 family proteins, Bcl-X_L, Mcl-1 and Bfl-1, which are thought to coordinately regulate mitochondrial function during apoptosis. Survivin is an IAP which prevents apoptosis by binding and directly blocking caspase 3 activation. The extrinsic pathway of apoptosis involves activation of death receptors such as Fas and TNF-R1. The anti-apoptotic CD40 target A20 prevents TNF-induced apoptosis but also prevents apoptosis induced by other factors e.g. p53 activation, although its mechanism of action is unknown. CD40 activation can also induce Fas, although this does not necessarily sensitise cells to Fas-mediated apoptosis. Induction of FLIP, which prevents signalling via Fas, may interfere with Fas-induced apoptosis. In cells in which CD40 signalling can induce apoptosis, induction of death receptor ligands (FasL, TNF) and pro-apoptotic Bcl-2 family proteins has been shown.

of the CD40 targets involved in control of apoptosis are described, whose regulation might play a prominent role in differential responses.

1.7.1 Anti-apoptotic Bcl-2 family proteins

(i) Bcl-2

CD40 ligation has been shown to induce expression of the Bcl-2 protein in germinal centre B-cells (Liu *et al* 1991(ii), Holder *et al* 1993) and in some Burkitt's Lymphoma cell lines (Ning *et al* 1996, Baker *et al* 1998). However Bcl-2 induction appears to be a long term phenomenon, requiring up to 72 hours of CD40 stimulation depending on the study, which does not correlate with the rapid rescue from apoptosis induced by CD40 ligation. Other studies have shown that CD40 ligation has no effect on Bcl-2 expression in MUTU-1 and L3055 Burkitt Lymphoma cells (Holder *et al* 1993), WEHI-231 cells (Choi MS *et al* 1995), naive human B-cells (Zhang *et al* 1996) and follicular lymphoma cells (Ghia *et al* 1998). Therefore the induction of Bcl-2 protein expression by CD40 ligation does not seem to account for the rapid ability of CD40 stimulation to rescue B-cells from apoptosis. However other anti-apoptotic Bcl-2 family members, namely Bcl-X_L, Bfl-1/A1 and Mcl-1, appear to play a more important role.

(ii) Bcl-X

Bcl-X_L shares a high degree of sequence homology with Bcl-2 (Boise *et al* 1993) and inhibits apoptosis in many assay systems. Bcl-X_L is overexpressed in many human malignancies including lymphoid and haematopoietic tumours, colorectal, stomach, prostate, breast and ovarian carcinomas (Krajewska *et al* 1996 (i) (ii) & (iii), Pallis *et al* 1997, Tu *et al* 1998, Olopade *et al* 1997, Xerri *et al* 1996, Lohmann *et al* 2000). Overexpression of Bcl-X_L can also promote tumour formation in mouse model systems (Naik *et al* 1996, Pena *et al* 1998). High Bcl-X_L expression has also been associated with progressive, chemoresistant cancers and poor prognosis (Tu *et al* 1998, Friess *et al* 1998, Deng *et al* 1998, Lohmann *et al* 2000, Biroccio *et al* 2001) and causes resistance to chemotherapeutics *in vitro* (Minn *et al* 1995).

The human Bcl-X gene contains at least 4 exons (fig. 1.9), the translation intiation codon is within exon II and the translation termination codon within exon III, whereas

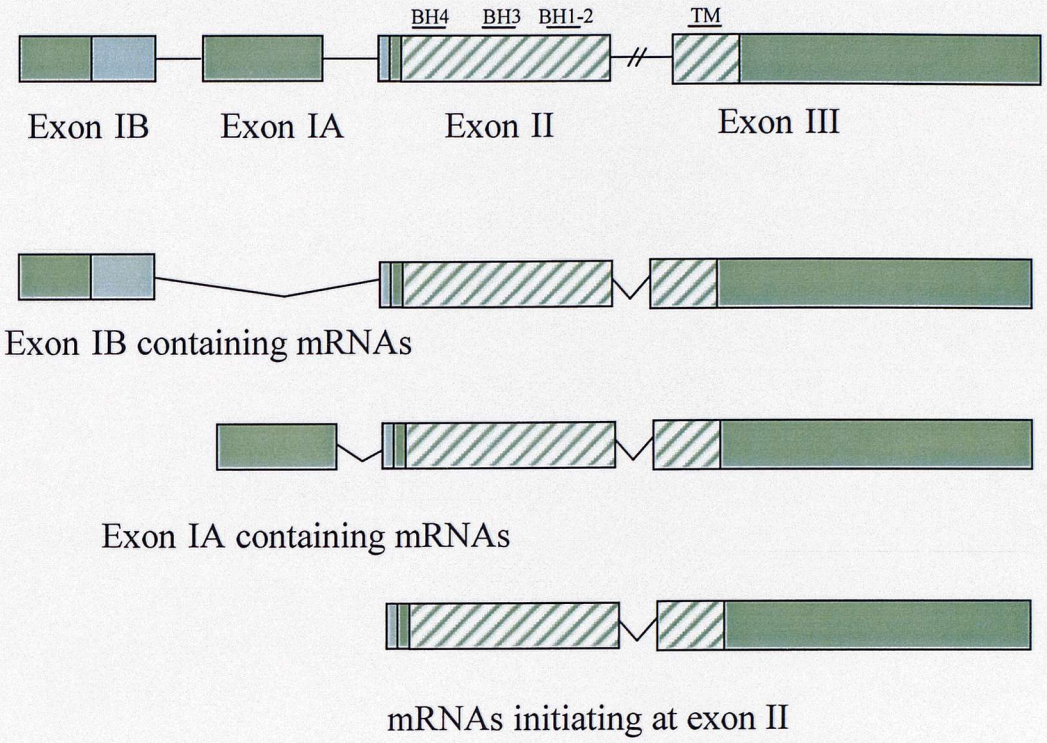


Figure 1.9: Human Bcl-X gene structure and mRNAs. The human Bcl-X gene contains 4 exons. The hashed region represents the coding sequence and the blue regions represent alternatively spliced non-coding regions. Exon IA and exon IB are non-coding and mRNAs can contain either exon IA or exon IB, not both. There is a 130bp alternatively spliced region at the 3' end of exon IB and an 18bp alternatively spliced region at the 5' end of exon II. Exon II encodes the BH1, BH2, BH3 and BH4 domains and contains the X_L/X_S splice site (see fig. 1.10). The first 138 base pairs of exon III encodes the transmembrane domain (TM) and carboxyl terminus of Bcl-X_L and Bcl-X_S. The intron separating exon II and III is over 9kb long. Multiple transcription initiation sites and promoters have been revealed, generating mRNAs containing either exon IA or exon IB or initiating at exon II.

exons IA and IB are non-coding. Exon II contains most of the coding sequence of Bcl-X, including the conserved BH1, BH2, BH3 and BH4 domains of Bcl-2 family proteins. The 5' end of exon III encodes the carboxyl terminus and transmembrane domain of both Bcl-X_L and Bcl-X_S (Grillot *et al* 1997). In human cells, Bcl-X RNAs initiate close to the start of exon II, or additionally contain either exon IA or exon IB, generated from three different promoters (Grillot *et al* 1997, MacCarthy-Morrogh *et al* 2000). The exon II promoter was identified in the mouse gene as a 57 base pair region located upstream of the translation initiation codon and is a TATA box less promoter (Grillot *et al* 1997). The promoter upstream of exon IA also lacks a TATA box, is GC rich and contains several Sp1-binding motifs (Grillot *et al* 1997). The exon IB promoter contains two TATA box sequences and has strong activity in lymphoid cells (MacCarthy-Morrogh *et al* 2000). In the mouse, three potential additional promoter regions have been identified. In addition to those identified by Grillot *et al* (exon II and exon IA promoter), P3 (-1886 bp from translation start, probably equivalent to the IB promoter), P4 (-2721 bp from translation start) and P5 (-3412 bp from translation start) were identified (Pecci *et al* 2001). However these promoters have not been studied in detail.

As indicated in fig. 1.9, there are alternative splicing events in non-coding exons of the Bcl-X gene. There is an alternatively spliced region of 130bp at the 3' end of exon IB (AS1) and an 18bp alternatively spliced region at the 5' end of exon II (AS2), which can result in four different exon IB containing splice forms, all of which can be detected in lymphoma cells (MacCarthy-Morrogh et al 2000). Therefore a variety of Bcl-X mRNAs can be generated from exon IB and exon IA with very different 5'-untranslated regions (5'-UTRs), the significance of which is unknown.

A number of different Bcl-X isoforms have been identified (5 in the mouse and 3 in humans) generated via alternative splicing of the Bcl-X gene (fig. 1.10). The three isoforms found in mouse, human and rat are Bcl-X_L, Bcl-X_S and Bcl-X_β. The coding sequences of Bcl-X_L and Bcl-X_S are formed by the juncture of exons II and III, but RNA encoding the pro-apoptotic Bcl-X_S protein lacks 189bp from the 3' end of exon II, causing deletion of the BH1 and BH2 domains (Boise *et al* 1993, Grillot *et al* 1997). Bcl-X_β lacks exon III and has a unique carboxyl terminus. The open reading

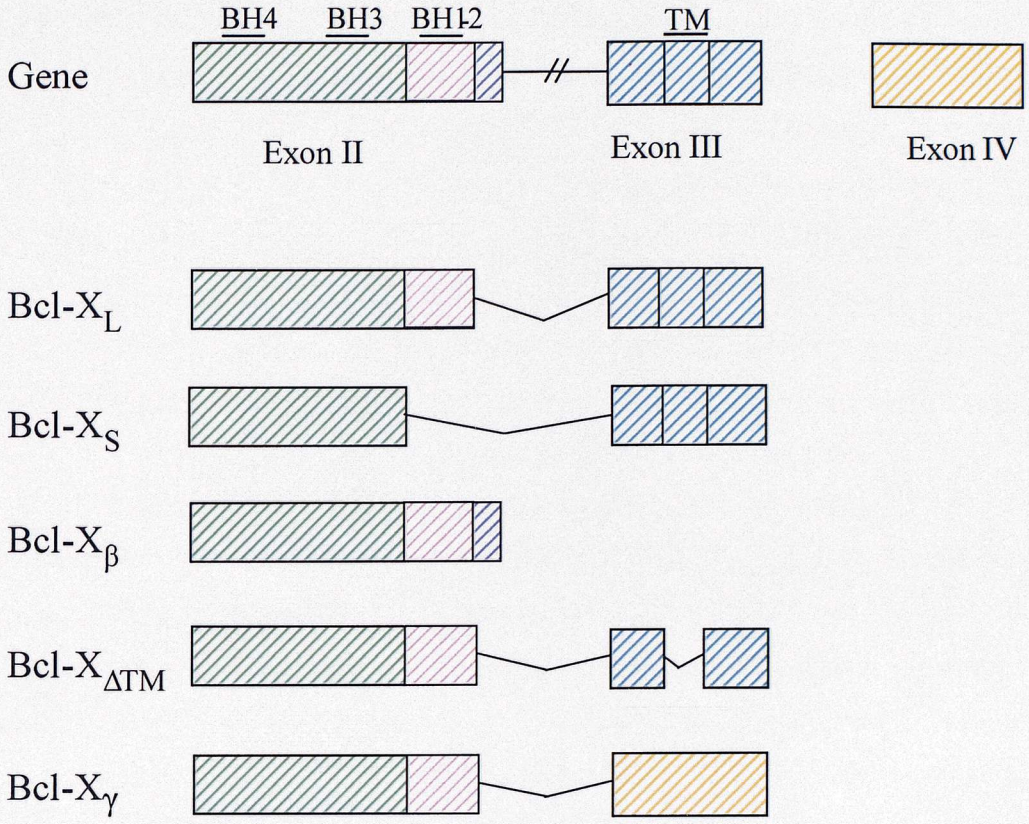


Figure 1.10: Human and murine Bcl-X isoforms. The coding sequence of the Bcl-X gene and Bcl-X isoforms are shown. Exon IV has also been identified in mouse cells. All isoforms have been identified in the mouse but only Bcl-X_L, Bcl-X_S and Bcl-X_β have been identified in human cells. The coding sequences of Bcl-X_L, Bcl-X_S and Bcl-$X_{\Delta TM}$ are formed by the juncture of exons II and exon III. Due to alternative splicing events, Bcl-X_S RNA lacks the region encoding the BH1-2 domains (pink region) in exon II and Bcl-$X_{\Delta TM}$ RNA lacks the region encoding the transmembrane (TM) domain in exon III. Bcl-X_β is generated from the continuous genomic sequence of exon II. Bcl-Xγ is generated from the inclusion of a distinct fourth exon, rather than exon III.

frame of Bcl-X_β arises from the continuous genomic sequence of exon II, extending over the splice donor sites utilized by the Bcl-X_L and Bcl-X_S transcripts (Gonzalez-Garcia *et al* 1994, Shiraiwa *et al* 1996, Ban *et al* 1998). Bcl-X_β expression correlates with that of Bcl-X_L but its function is unclear, as it was shown to act in an anti-apoptotic manner in murine neurons but promoted apoptosis in rat promyeloid cells (Gonzalez-Garcia *et al* 1994, Shiraiwa *et al* 1996, Ban *et al* 1998). Bcl-$X_{\Delta TM}$ has only been identified in the mouse and results from a 70 base pair deletion of the transmembrane domain in exon III, via alternative splicing. Thus it is a soluble form of Bcl-X with a diffuse localisation throughout the cytoplasm. Like Bcl-X_L, it prevents apoptosis (Fang *et al* 1994). The final murine Bcl-X isoform is Bcl-Xγ, which is primarily expressed in activated T-cells and prevents apoptosis. It contains a unique carboxyl-terminus generated from a distinct fourth exon, rather than exon III. Three different Bcl-Xγ RNAs were detected with different 3'-untranslated regions utilising 4 additional exons (exons V-VIII) (Yang *et al* 1997, Ye *et al* 2002, Yang *et al* 2002). However, despite the presence of these different Bcl-X isoforms at the RNA level, Bcl-X_L is the major protein detected in both human and mouse cells and in human B-cell lymphomas (Gonzalez-Garcia *et al* 1994, Grillot *et al* 1997, Olopade *et al* 1997, Xerri *et al* 1996).

Choi MS *et al* (1995) showed that treatment of WEHI-231 cells with anti-CD40 mAbs lead to a rapid induction of Bcl-X_L protein, which correlated with rescue of WEHI-231 cells from anti-μ and calcium ionophore induced apoptosis. Furthermore, WEHI-231 transfectants, expressing low or high levels of Bcl-X_L, were substantially protected from the effects of anti-μ or a calcium ionophore. Subsequent studies have shown that CD40 signalling increases Bcl-X_L protein expression in naive B-cells (Zhang *et al* 1996), peripheral blood B-cells in conjunction with IL-13 (Lomo *et al* 1997), Ramos and Daudi cells (Ning *et al* 1996, Lee *et al* 1999), and primary follicular lymphoma (Ghia *et al* 1998), CLL and AML cultures (Kitada *et al* 1999, Aldinucci *et al* 2003). A Ramos clone expressing a Bcl-X construct at levels similar to those induced in parental cells after CD40 stimulation was produced (Lee *et al* 1999). This clone was resistant to Fas-mediated apoptosis, thereby confirming that induction of Bcl-X_L by CD40 activation contributes to CD40-mediated apoptotic rescue.

(iii) Bfl-1/A1

The mouse A1 gene was identified as an early-response gene in haematopoietic cells treated with granulocyte-macrophage colony- stimulating factor, with sequence and functional similarity to Bcl-2 (Lin *et al* 1993, Lin *et al* 1996). The human homologue, Bfl-1, was isolated as a cDNA clone homologous to Bcl-2 from foetal liver, which is abundantly expressed in normal bone marrow and in several clinical samples of stomach cancer (Choi SS *et al* 1995). Bfl-1 suppresses apoptosis induced by p53 in a manner similar to Bcl-2 and Bcl-X_L but can also interfere with caspase-7 activation (D'Sa-Eipper *et al* 1996, Herold *et al* 2002). Bfl-1 can promote tumour formation by cooperating with a dominant nuclear oncogene, E1A, in transformation of primary epithelial cells *in vitro* and induction of Bfl-1 by the Bcr/Abl-STAT5 pathway was shown to be required for Bcr/Abl-induced transformation and leukaemogenesis (D'Sa-Eipper *et al* 1996, Nieborowska-Skorska *et al* 2002). However mice overexpressing A1 in lymphoid cells do not develop tumours but do show increased survival of thymocytes and pro B-cells but decreased numbers of pre B-cells, suggesting that A1 is important in the pro to pre B-cell transition (Chuang *et al* 2002). Unlike other Bcl-2 family proteins, expression of Bfl-1 permits limited cell proliferation over an extended period of time when cells are induced to undergo apoptosis (D'Sa-Eipper & Chinnadurai 1998).

Kuss *et al* (1999) demonstrated that treatment of primary murine B-cells and WEHI-231 cells with anti-CD40 mAbs increased A1 mRNA, more prominently than Bcl-X_L expression. WEHI-231 cells transfected with A1 cDNA showed a significantly higher survival rate after treatment with anti-IgM, however overexpression of A1 did not prevent anti-IgM induced cell cycle arrest. Similarly, Craxton *et al* (2000) found that cross-linking CD40 on WEHI-231, Ramos and germinal centre B-cells induced A1/Bfl-1 mRNA upregulation and that WEHI-231 clones overexpressing A1 were protected from anti-IgM and PI3-K inhibitor induced apoptosis, but not from calcium ionophore induced apoptosis. Daudi, Ramos and BJAB cells also upregulated Bfl-1 mRNA when CD40 was activated (Lee *et al* 1999). The kinetics of this response were investigated in Daudi cells and Bfl-1 mRNA was induced within 4 hours of CD40 stimulation and maintained for over 24 hours.

Induction of both Bcl-X_L and Bfl-1 by CD40 signalling is mediated by NF-κB activation. Lee *et al* (1999) showed that inhibition of the NF-κB pathway in Daudi and Ramos cells by overexpression of a dominant active IκB-α mutant which could not be phosphorylated, prevented CD40 induced upregulation of the Bcl-X_L and Bfl-1 genes and also abolished the ability of CD40 to rescue Fas-induced cell death.

(iv) Mcl-1

Mcl-1 was originally identified as a gene which is rapidly up-regulated early in the induced differentiation of a human myeloid leukaemia cell line (Kozopas *et al* 1993). Mcl-1 shows sequence similarity to Bcl-2 and transfection of Mcl-1 into Chinese hamster ovary (CHO) cells leads to inhibition of apoptosis induced by c-myc overexpression (Reynolds *et al* 1994). Furthermore, transfection of Mcl-1 into a haematopoietic stem cell line prolonged cell viability when cells were treated with cytotoxic agents (Zhou *et al* 1997). Transgenic mice expressing a Mcl-1 transgene in lymphoid tissues show enhanced viability of haematopoietic cell types and, in the long term, develop lymphomas which are predominately follicular and diffuse large B-cell in nature (Zhou *et al* 2001). The spontaneous apoptosis of normal peripheral blood B-cells cultured *in vitro* is associated with a marked decrease in Mcl-1 protein expression which precedes cell death (Lomo *et al* 1996). However Bcl-2, Bcl-X_L and Bax levels remain unaffected. The decrease in Mcl-1 protein expression was prevented with agents which rescue cells from spontaneous apoptosis (IL-4, anti-IgM mAbs and TPA). The same group later showed that CD40L in conjunction with IL-13 increased Mcl-1 protein expression in normal peripheral blood B-cells (Lomo *et al* 1997). Increased Mcl-1 protein expression by CD40 ligation has also been demonstrated in CLL cells (Kitada *et al* 1999), and germinal centre B-cells (Ghia *et al* 1998). However there is no direct evidence that the induction of Mcl-1 expression by CD40 signalling prevents B-cell apoptosis.

1.7.2 Other anti-apoptotic target genes

(i) A20

A20 was originally discovered as a TNF-α induced primary response gene, encoding a novel zinc finger protein, which protects cells from TNF-induced cell death (Opipari *et al* 1992, Heyninck *et al* 1999). A20 is also induced in human epithelial cell lines in

response to expression of the EBV oncogene LMP1 and inhibits apoptosis induced by p53, serum depletion and LPS, although its mechanism of action is unknown (Fries *et al* 1996, Beyaert *et al* 2000). Besides being an anti-apoptotic molecule, A20 is also a potent inhibitor of NF-κB. Indeed, A20 knockout mice die between 3 and 6 weeks after birth due to severe inflammation due to prolonged activation of NF-κB, and extensive apoptosis in the liver and kidney (Lee *et al* 2000).

CD40 activation in the Burkitt's Lymphoma cell lines Louckes and BJAB, and human peripheral blood B-cells was shown to induce A20 mRNA within one hour of stimulation. The presence of the protein synthesis inhibitor cycloheximide super-induced A20 mRNA, consistent with A20 being a primary response gene whose induction is independent of intervening protein synthesis (Sarma *et al* 1995). Transfection of the cell lines with A20, conferred resistance to apoptosis, therefore suggesting a role for A20 in CD40 mediated apoptotic rescue. A20 is also induced by CD40 signalling through the NF-κB pathway. Sarma *et al* (1995) showed that activation of CD40 on Louckes & BJAB-cells transfected with a wild type A20 promoter CAT reporter construct, led to a significant induction of CAT activity. However if the two NF-κB elements in the A20 promoter were mutated, then CD40 ligation did not induce CAT activity. They also showed by electrophoretic mobility shift assays that CD40 activation induced the binding of NF-κB proteins to NF-κB sites within the A20 promoter.

(ii) Survivin

Survivin is a member of the inhibitor of apoptosis protein (IAP) family. IAPs were originally identified in baculoviruses due to their ability to attenuate apoptosis induced by viral infection. IAPs are classified by the presence of a BIR (baculovirus IAP repeat) domain which directly binds caspases (reviewed in Jaattela 1999). All IAPs suppress apoptosis by directly inhibiting caspases and have ubiquitin ligase activity that may ubiquitinate caspases, targeting them for degradation (Guo & Hay 1999, Palaga & Osborne 2002). Survivin may also have a role in the cell cycle as it is upregulated in the G2/M phase of the cell cycle where it is associated with spindle microtubules. This association is essential for its anti-apoptotic activity (Guo & Hay 1999). A recent paper has shown upregulation of survivin expression after 48 to 96

hours of CD40 stimulation in primary CLL cultures that respond to CD40L by increasing cell viability and proliferation (Granziero *et al* 2001). This was seen at both the mRNA and protein level.

In addition to the full-length survivin protein, there are two survivin splice variants: survivin-ΔEx3 which lacks exon 3, and survivin-2B which retains part of intron 2. Survivin-ΔEx3 possesses similar anti-apoptotic activity to survivin whereas survivin-2B possesses decreased activity (Mahotka *et al* 1999). Survivin-ΔEx3 contains a putative BH2 domain and mitochondrial-targeting sequence. Studies with its viral homolog K7 have revealed that K7 binds to Bcl-2 via its BH2 domain and activates caspase-3 via its BIR domain. The BH2 domain of K7 is critical for its anti-apoptotic activity (Wang *et al* 2002). Therefore K7, and possibly its human homolog survivin-ΔEx3, act as adaptor molecules allowing Bcl-2 to prevent the actions of caspase-3.

Interestingly, survivin is expressed foetally but is undetectable in terminally differentiated adult tissues. However, survivin is expressed in cancer cell lines and in various primary cancers including breast, lung, colon and high-grade lymphomas (Ambrosini *et al* 1997). In fact survivin expression was shown to be an unfavourable prognostic factor in diffuse large B-cell lymphoma and acute myeloid leukaemia (Adida *et al* 2000 (i) & (ii)). In addition, survivin expression was shown to increase with tumour grade in endometrial adenocarcinoma and disease progression in melanoma patients (Lehner *et al* 2002, Gradilone *et al* 2003). The survivin splice forms have been detected in renal cell carcinomas and gastric carcinomas, with survivin-2B expression showing decreased expression with respect to the stage of disease (Krieg *et al* 2002). Therefore further upregulation of survivin by CD40 engagement in lymphoma, would further increase resistance to cell death and resistance to cancer therapies. As survivin is expressed solely in malignant cells, it could be an excellent candidate for apoptosis-based cancer therapy.

1.7.3 Pro-apoptotic genes

The molecular mechanisms by which CD40 induces apoptosis are not known and is a relatively new area of research. In a number of studies, CD40 ligation had no effect on expression of the pro-apoptotic Bcl-2 family members Bax, Bak and Bik (Zhang *et*

al 1996, Lomo *et al* 1997, Kitada *et al* 1999, Craxton *et al* 2000, Taylor *et al* 2000). However these studies used systems in which CD40 signalling prevents apoptosis. Two studies showing that CD40 can induce apoptosis have examined the expression of pro-apoptotic Bcl-2 members, finding that activation of CD40 on breast carcinoma cells leads to upregulation of Bax and activation of CD40 on lymphoma cell lines leads to upregulation of Bax, Bak and Bik mRNA and Bax protein (Szocinski *et al* 2002, Tong *et al* 2001). Indeed, antisense to Bax mRNA provided partial protection from CD40-mediated apoptosis, indicating that upregulation of Bax was part of the mechanism involved in CD40-mediated apoptosis (Szocinski *et al* 2002).

CD40 activation also affects expression and signalling of death receptor or TNF family members. CD40-induced apoptosis correlates with induction of cytotoxic ligands of the TNF family (Fas ligand, TRAIL and TNF) in ovarian carcinoma cell lines, HeLa cells stably transfected with CD40, biliary epithelial cells and CLL cells (Eliopoulos *et al* 2000, Afford *et al* 2001, Grell *et al* 1999, De Totero *et al* 2003). Furthermore, CD40-induced apoptosis in HeLa cells or biliary epithelial cells was dependent on the TNF-R1/TNF or Fas/FasL interaction respectively, as blocking these interactions with neutralising antibodies prevented CD40-induced apoptosis (Afford *et al* 2001, Grell *et al* 1999). Also, CD40-activated fludarabine-induced apoptosis of CLL cells was mediated through TNF-α and interferon-gamma production (De Totero *et al* 2003).

Many studies have shown that CD40 activation upregulates Fas expression in both normal and malignant B-cells and in carcinoma cell lines (Kitada *et al* 1999, Eliopoulos *et al* 2000, Alexandroff *et al* 2000, Garrone *et al* 1995, Schattner *et al* 1996, Wierda *et al* 2000, Wang *et al* 1997, Plumas *et al* 1998, Yamada *et al* 2001). However upregulation of Fas does not necessarily sensitise cells to Fas-mediated apoptosis. In both germinal centre B-cells and a variety of primary B-cell malignancies, ligation of Fas on CD40 activated cells only induces apoptosis in a subset of samples or studies (Garrone *et al* 1995, van Eijk *et al* 2001, Hennino *et al* 2000) whereas in other studies CD40 activation rescues germinal centre B-cells and Ramos cells from Fas-mediated apoptosis (Cleary *et al* 1995, Lee *et al* 1999, Hennino *et al* 2001). A possible reason for the resistance of CD40 activated cells to Fas

mediated apoptosis is that CD40 activation can lead to upregulation of FLIP in virgin and germinal centre B-cells (van Eijk *et al* 2001, Hennino *et al* 2000, Hennino *et al* 2001). FLIP associates with the Fas DISC and generally prevents complete processing of caspase-8, thereby preventing activation of the caspase cascade and apoptosis (see section 1.3.1). Therefore upregulation of FLIP by CD40 activation could prevent Fas-mediated apoptosis.

1.7.3 BNIP3, a potential CD40-target gene that promotes apoptosis

Data obtained from a microarray study by Alizadeh *et al* (2000), were analysed and showed that CD40 ligation of normal B-cells upregulated expression of BNIP3, a pro-apoptotic BH3-only Bcl-2 family member, in addition to previously identified anti-apoptotic genes e.g. Bcl-X_L.

BNIP3 was identified as a protein that interacts with both the anti-apoptotic E1B 19kDa protein and Bcl-2 (Boyd *et al* 1994). BNIP3 is a 60KDa homodimer, localised to mitochondria, which can induce apoptosis and antagonize the activity of Bcl-2 and Bcl-X_L (Chen *et al* 1997, Yasuda *et al* 1998, Ray *et al* 2000). BNIP3 requires the presence of its C-terminal transmembrane domain in order to promote apoptosis but the role of its BH3 domain is less clear, since studies have shown that the pro-apoptotic activity of BNIP3 is either dependent or independent of its BH3 domain. BNIP3 has also been shown to mediate a necrosis-like cell death when transiently transfected (Vande Velde *et al* 2000).

1.8 CD40 signalling

CD40 ligation influences gene expression via activation of a number of cell signalling pathways and transcription factors. The role of each signalling pathway in CD40-mediated survival is not well understood, nor are the exact pathways by which CD40 signalling occurs. CD40 activation has been shown to activate NF-κB, phospatidylinositol 3-kinase (PI3-K), the JNK, ERK and p38 mitogen activated protein kinase (MAPK) pathways and the transcription factors AP-1, NF-AT, STAT3 and STAT 6 (Berberich *et al* 1994, Ren *et al* 1994, Sakata *et al* 1995, Li *et al* 1996, Sutherland *et al* 1996, Francis *et al* 1995, Hannissian & Geha 1997, Karras *et al* 1997). The activation of each of these pathways by CD40 will be discussed. A

summary of the signalling pathways induced by CD40 ligation, resulting in cell survival, is shown in fig. 1.11.

CD40 itself possesses no intrinsic kinase activity and so adaptor molecules, associating with its short cytoplasmic domain (62 amino acids), mediate signal transduction following ligand-induced trimerisation of CD40. Thr^{255} in the cytoplasmic domain of CD40 is essential for signal transduction, NF-κB activation (Sarma *et al* 1995) and CD40 homodimer formation (Baker *et al* 1998). Yeast two-hybrid, *in vitro* binding assays and 293 cells transfected with soluble CD40 cytoplasmic domain have shown that TNFR-associated factor (TRAF) family proteins 1,2,3,5 and 6 can be associated with CD40 (Lee *et al* 1999, Wernburg *et al* 2001). TRAFs are recruited to the CD40 cytoplasmic domain following CD40 ligation and oligomerisation. There are two TRAF binding sites on the CD40 cytoplasmic domain: a membrane proximal site that binds TRAF6 (^{231}QEPQEINF) and a membrane distal site that binds TRAFs 1,2 and 3 (^{250}PVQET). The interaction of TRAF5 with CD40 is indirect, through TRAF3 binding (Pullen *et al* 1999). The membrane proximal TRAF 6 binding site was shown to be important in the activation of NF-κB, JNK and p38 MAPK in 293 cells but not in B-cell lines (Pullen *et al* 1999, Jalukar *et al* 2000, Wernburg *et al* 2001, Manning *et al* 2002). Disruption of the membrane proximal TRAF6 binding site was also shown to prevent affinity maturation and generation of plasma cells in mice (Ahonen *et al* 2002). The membrane distal TRAF 1,2,3 (and 5) binding site of CD40 has been shown to be essential for NF-κB, p38 MAPK and JNK activation (Lee *et al* 1999, Sutherland *et al* 1999, Werneburg *et al* 2001, Manning *et al* 2002). This region is also sufficient to rescue WEHI-231 cells from anti-IgM induced growth arrest (Hornung *et al* 1998, Sutherland *et al* 1999). TRAF3 was shown to be vital for p38 MAPK activation and Ig production in Ramos cells (Grammer *et al* 1998). Disruption of both TRAF binding sites in mice prevented germinal centre formation (Ahonen *et al* 2002).

There appears to be redundancy in the activation of signalling pathways by TRAFs, as neither TRAF2 nor TRAF3 is absolutely required for NF-κB induction or JNK activation (Leo *et al* 1999, Grammer *et al* 1998). Also, TRAF 2, 3 or 6 knockout mice show developmental problems, but do not show defects in NF-κB signalling (Ahonen

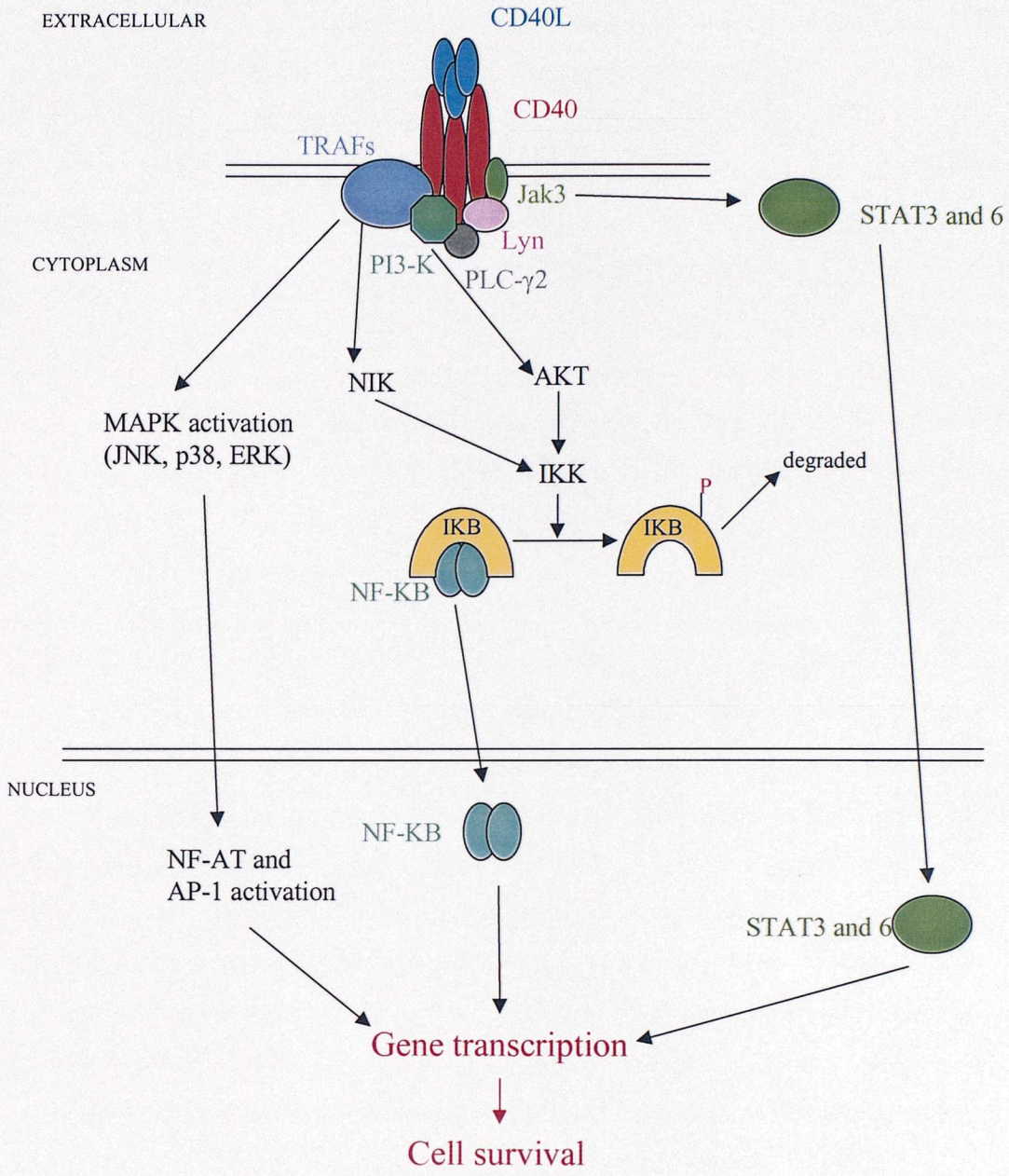


Figure 1.11: CD40 signalling pathways. Ligation of CD40 induces trimerisation and association, or phosphorylation, of adaptor molecules including TNFR-associated factors 1,2,3,5 and 6 (TRAFs), Lyn, phospholipase Cγ2, PI3-K and Jak3. These molecules activate NF-κB and the ERK, JNK and p38 MAPK cell signalling cascades, ultimately resulting in the transcription of genes that promote cell survival.

et al 2002). Furthermore, CD40 signalling and NF-κB activation can occur in the absence of TRAFs, since B-cell proliferation and early Ig production occured when all TRAF sites were mutated in mice (Ahonen *et al* 2002).

Therefore it is clear that CD40 recruits TRAF molecules to two sites on its cytoplasmic domain that are capable of activating the downstream signalling molecules NF-κB, JNK and p38 MAPK. However the role of each TRAF molecule in signalling is not clear and may be cell-type specific, require cooperation with other TRAFs or there may be functional redundancy in TRAF signalling. It is also apparent that TRAFs are not necessary for all CD40 signalling events and so other adaptor and signalling molecules associating with CD40 must be important.

The adaptor molecule JAK3 (Janus kinase 3) is constitutively associated with CD40, requiring a proline-rich sequence in the membrane proximal region of CD40, and is phosphorylated and activated upon CD40 ligation (Hanissian & Geha 1997). Activated JAKs phosphorylate receptors providing docking sites for STATs (signal transducer and activator of transcription). STAT3 and STAT6 are activated upon CD40 ligation (Hanissian & Geha 1997, Karras *et al* 1997) and so it is possible that these STATs are activated via JAK3, although this has not been proven. Once STATs have been activated they translocate to the nucleus where they can influence gene expression. The Src family tyrosine kinase Lyn, phospholipase Cγ2, and Vav (a proto-oncogene that can activate Ras and is a guanosine nucleotide exchange factor for Rho and Rac) are also phosphorylated and activated upon CD40 ligation (Ren *et al* 1994, Padmore *et al* 1997). CD40 ligation also leads to tyrosine phosphorylation and activation of PI3-K (Ren *et al* 1994, Padmore *et al* 1997).

1.8.1 PI3-K

There are three classes of PI3-Ks and multiple isoforms, which all catalyse the phosphorylation of phosphatidyl inositol lipids at the D-3 position of the inositol ring. The class involved in CD40 signalling is class IA, which catalyses the phosphorylation of phosphatidyl inositol 4,5 bisphosphate to phosphatidyl inositol 3,4,5 trisphosphate (PIP_3). Class IA PI3-Ks contain two subunits: a p110 catalytic subunit (of which there are 3 isoforms α, β, or δ) and a p85 adaptor or regulatory

subunit. At least seven different adaptors can be generated by alternative splicing from the p85α, p85β and p55γ genes. PI3-K is activated by cell surface receptors via p85 binding directly to phospho-tyrosine residues on activated receptor molecules or via p85 binding to tyrosine phosphorylated adaptor proteins bound to receptors. Ras can also stimulate PI3-K activation by directly binding to the p110 subunit (Cantley 2002).

The generation of PIP_3 by PI3-K mediates downstream signalling via activation of signalling proteins with pleckstrin-homology domains. Pleckstrin-homology (PH) domains directly bind PIP_3, leading to activation of the protein. Particularly important PH-domain containing proteins are AKT and phosphoinositide-dependent kinase 1 (PDK1). Binding of PIP_3 to AKT increases its activity, recruits it to the plasma membrane and alters the conformation of AKT exposing its phosphorylation sites. For full activation, AKT requires phosphorylation by PDK1 at threonine 308 and phosphorylation at serine 473 by a recently identified cytoskeleton-associated kinase termed PDK2 (Scheid *et al* 2002, Hresko *et al* 2003). AKT has a number of anti-apoptotic effects including phosphorylation and inactivation of both Bad and caspase-9, and also activation of the kinases that phosphorylate IκB therefore leading to NF-κB activation. It also suppresses the activity of the forkhead family of transcription factors, increases nitric oxide production, and inactivates glycogen synthase kinase 3 (GSK3). GSK3 is constitutively active in unstimulated cells and phosphorylates many proteins keeping them inactive or promoting their degradation e.g. c-myc, cyclin D. Inactivation of GSK3 by AKT phosphorylation therefore allows activation of pathways normally repressed by GSK3. Activated AKT can also translocate to the nucleus, where it may have nuclear targets such as transcription factors (Neri *et al* 2002). Other PH domain-containing proteins activated by PIP_3 include guanosine exchange factors for Rac, leading to Rac activation and actin polymerisation, and tyrosine kinases of the Bruton's tyrosine kinase and Tec family (Cantley 2002, Khwaja 1999). Therefore activation of PI3-K has many effects important for cell survival, proliferation and differentiation.

PI3-K knockout mice have been generated which show defects in CD40 signalling, illustrating the importance of this pathway in the response to CD40. p85α-p55α-p50α

knockout mice die within days of birth so chimeric mice were generated which showed decreased numbers of mature B-cells with diminished proliferative responses to CD40 (Fruman *et al* 1999). p100δ knockout mice were viable but showed decreased numbers of B-cells, decreased serum Ig and poor immune responses. Splenic B-cells did not proliferate in response to anti-CD40 and showed defective IκB-α phosphorylation and decreased Bcl-X_L levels compared to wild type mice (Clayton *et al* 2002). PI3-K activation was also shown to be critical for CD40-mediated rescue of Ramos cells from anti-IgM triggered growth inhibition and apoptosis (Curnock and Knox 1998). In primary mouse B-cells, inhibition of PI3-K did not prevent CD40 inducing cell survival but did decrease the proliferative response of CD40L treated cells. Microarray analysis revealed that the PI3-K pathway was important for CD40-mediated induction and inhibition of genes, particularly secondary response genes including metabolic enzymes and transporters (Dadgostar *et al* 2002).

1.8.2 NF-κB

CD40 ligation has been shown to induce NF-κB activity in many studies (Berberich *et al* 1994, Lee *et al* 1999, Sarma *et al* 1995, Francis *et al* 1995, Siebelt *et al* 1997, Schauer *et al* 1998). NF-κB activation is also required for CD40-induced upregulation of Bcl-X_L, Bfl-1 and A20 (Lee *et al* 1999, Sarma *et al* 1995, Dadgostar *et al* 2002), CD40-mediated rescue of WEHI-231 cells from anti-IgM mediated apoptosis (Schauer *et al* 1998), CD40-induced protection of CLL cells from fludarabine-induced apoptosis (Romano *et al* 1998) and CD40-mediated rescue of BJAB cells from Fas-mediated apoptosis (Zazzeroni *et al* 2003). In a microarray study in mouse B-cells, NF-κB activation was shown to be important for primary gene induction by CD40 (Dadgostar *et al* 2002).

NF-κB is a family of transcription factors for genes involved in cell survival, adhesion, inflammation, differentiation and growth. The NF-κB family includes 5 mammalian members p50 (NF-κB1), p52 (NF-κB2), c-Rel, RelA (p65) and RelB, which can form homo- and heterodimers. All of the members contain a 300 amino acid Rel homology domain (RH), which is involved in DNA-binding, nuclear transport and dimerisation. The family can be divided into two groups. RelA (p65), c-

Rel and RelB form one group, which in addition to the RH domain, possess one or more C-terminal transcriptional activation domains. The second group includes p50 and p52. These proteins are synthesised as p105 and p100 precursor proteins, respectively, which contain long C-terminal domains with multiple copies of ankyrin repeats, which inhibit these molecules. Limited proteolysis or arrested translation gives rise to the active, shorter p50 and p52 proteins. p50 and p52 bind DNA but can only act as transcriptional activators when they form heterodimers with members of the other group. NF-κB was the original name for the p50-p65 heterodimer (Li & Stark 2002, Bharti & Aggarwal 2002).

The activity of NF-κB factors is modulated by inhibitory IκB proteins (IκB-α, IκB-β, IκB-ε, p105, p100), which sequester the dimers in the cytoplasm, blocking their nuclear localisation sequence. Phosphorylation of IκB proteins on two serine residues targets them for ubiquitination and proteasomal degradation, and leads to partial degradation of p100 or p105 to p50 or p52. This releases free NF-κB complexes, exposing their nuclear localisation sequence, allowing the dimers to translocate to the nucleus where they activate transcription of various target genes. However liberation of NF-κB from IκB may not be sufficient to activate transcription, as phosphorylation of the first group of NF-κB proteins (p65, RelB, c-Rel) may also be required (Li & Stark 2002, Vermeulen *et al* 2002).

Phosphorylation of IκB proteins is achieved through activation of IκB kinase (IKK). IKK is composed of at least three subunits. Two catalytic subunits, IKK-α and IKK-β, have been identified plus a regulatory subunit IKK-γ (also called NEMO). Activation of the IKK complex involves phosphorylation of two serine residues within the kinase domains of IKK-α and IKK-β. Certain mitogen activated protein kinase kinase kinases (MAP3K) are involved in IKK activation *in vitro* (MEKK1, MEKK2, MEKK3 and NIK) but none are definitely proven to be an IKK kinase *in vivo* (Li & Stark 2002). Recently IKK-α was shown to move to the nucleus itself upon phosphorylation where it phosphorylates serine 10 of histone H3 at promoter regions of NF-κB regulated genes (Anest *et al* 2003, Yamamoto *et al* 2003). This histone modification has previously been shown to positively regulate transcription. This identifies another mechanism by which NF-κB-dependent transcription is regulated.

How does CD40 signalling lead to IKK activation? Some studies have linked TRAF2 and TRAF6 to NIK (NF-κB inducing kinase) activation, which then goes on to phosphorylate IKK-α and IKK-β (Malinin *et al* 1997, Brady *et al* 2000, Luftig *et al* 2001). The CD40 - TRAF6 – NIK – IKK - NK-κB pathway was shown to be involved in Cε promoter activation, which is needed for IgE class switching (Brady *et al* 2000). There is a naturally occurring mouse strain called alymphoplasia, caused by a mutation in NIK. These mice lack lymph nodes, have abnormal spleen and thymus development, low serum Ig and impaired B-cell proliferation in response to LPS or CD40L. Using this mouse, NIK was shown to be required for CD40-induced IκB-α phosphorylation, B-cell proliferation and Ig production in splenic B-cells (Garceau *et al* 2000). NIK activity was also essential for CD40 to trigger p100 processing to p52 (Coope *et al* 2002). However other studies have shown that NIK activity is not required for CD40-mediated NF-κB activation (Coope *et al* 2002, Yin *et al* 2001). Therefore other MAP3Ks, which may be redundant with NIK, may be important in linking CD40 to IKK activation. One of these MAP3Ks may be MEKK1 (mitogen-activated protein kinase/extracellular signal regulatory kinase kinase). MEKK1 is a central kinase in the JNK pathway and can activate IKK-α and IKK-β, leading to IκB-α phosphorylation (Lee *et al* 1997, Lee FS *et al* 1998). Although a link with MEKK1 and NF-κB has not been established in CD40 signalling, it is possible that activation of MEKK1 via CD40 leads to NF-κB activation and/or JNK activation. In epithelial cells, an NF-κB activator Act-1, which contains two TRAF binding sites, was recruited to CD40 after ligation. Act-1 activated TAK1 (transforming growth factor β activated kinase 1), a MAP3K, which then phosphorylated and activated IKK (Qian *et al* 2002).

NF-κB activity can also be induced by CD40 signalling via PI3-K activation. AKT (protein kinase B), a downstream target of PI3-K, can phosphorylate and activate IKK-α (Ozes *et al* 1999, Khwaja 1999). Andjelic *et al* (2000) showed that the PI3-K–AKT- NF-κB pathway was crucial for CD40-mediated proliferation and induction of Bcl-X expression in splenic B-cells. The PI3-K – AKT – NF-κB pathway was also responsible for CD40-induced multiple myeloma cell migration (Tai *et al* 2003) and

endothelial cell survival and angiogenesis (Deregibus *et al* 2003). However inhibition of PI3-K in murine B-cells did not interfere with the induction of NF-κB target genes in a microarray study (Dadgostar *et al* 2002). The PI3-K pathway has also been implicated in phosphorylation and activation of p65 and p50 in some cell types (Vermeulen *et al* 2002, Li and Stark 2002).

1.8.3 ERK

The extracellular signal-regulated kinases (ERKs) have been shown to be activated by CD40 in normal mouse B-cell systems, in a Hodgkin's disease cell line, in monocytes and a follicular dendritic cell line (Li *et al* 1996, Purkerson & Parker 1998, Dadgostar *et al* 2002, Eliopoulos *et al* 2003, Zheng *et al* 2003, Li & Nord 2002, Pearson *et al* 2001, Park *et al* 1999) However ERK was not activated by CD40 in primary human B-cells or B-cell lines (Sutherland *et al* 1995, Berberich *et al* 1996, Sakata *et al* 1995). A microarray study using normal mouse B-cells showed that although ERK was involved in CD40-induced proliferation it had little effect on gene expression patterns (Dadgostar *et al* 2002). In contrast, Eliopoulos *et al* (2003) found that ERK activation by CD40 was not involved in the proliferative response but did contribute to IgE production. Therefore the regulation of ERKs by CD40 is not consistent and may be cell-specific or play a minor role in CD40 signalling.

There are two ERKs, ERK1 and ERK2, which are mitogen-activated protein kinases (MAPKs). MAPKs are important signal transducing serine/threonine kinases which control a vast array of physiological processes. They have many downstream targets including protein kinases, phospholipases, transcription factors and cytoskeletal proteins. These enzymes are regulated by a phosphorylation cascade, comprising of at least three enzymes acting in series. MAPKs are activated directly by MAPK kinases (MAP2K), which themselves are phosphorylated and activated by MAPK kinase kinases (MAP3K). There are three well characterised MAPK cascades, the ERK, JNK (see section 1.8.4) and p38 (see section 1.8.5) pathways, which can all be regulated by CD40 (fig. 1.12).

ERKs are widely expressed and are involved in the control of cell division and proliferation. ERKs have many cellular substrates, although many physiologically

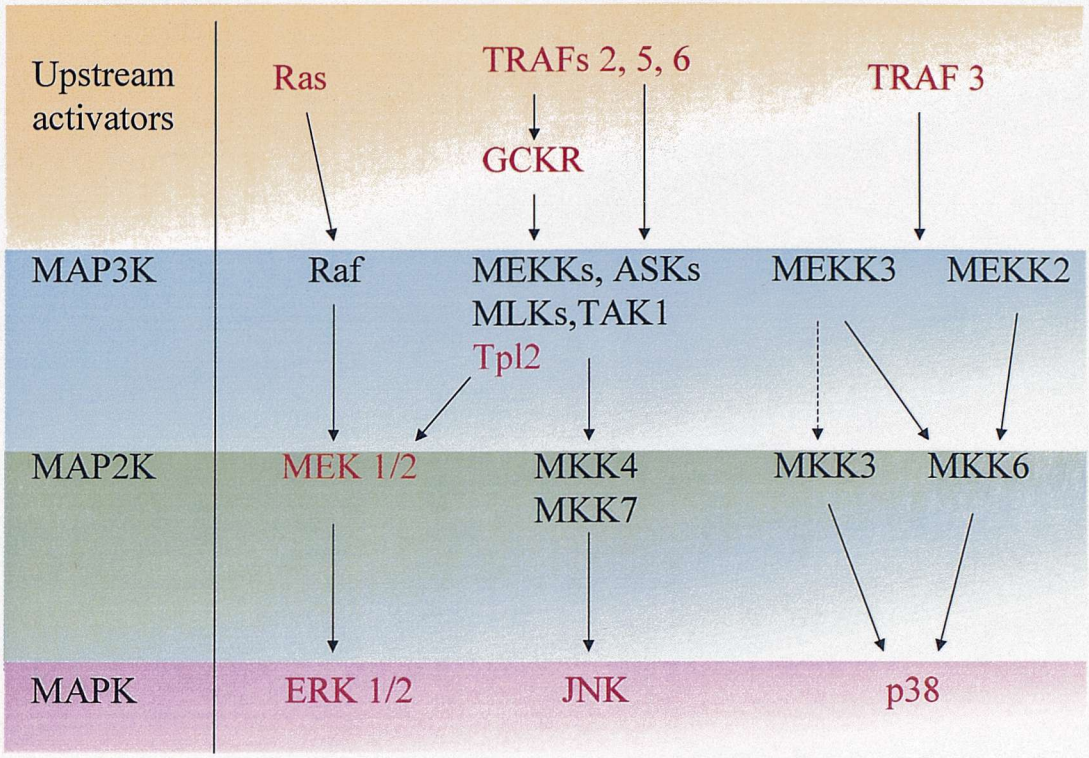


Figure 1.12: MAPK cascades. The ERK, JNK and p38 MAPK cascades can be activated by CD40. Proteins in red are those which have been linked to CD40 signalling. Proteins in black are classically involved in MAPK signalling, and so may link CD40 signalling to ERK, JNK or p38 activation. Activation of ERK can be Ras-dependent or Ras- independent. In a Ras-independent pathway, TRAF 6 activates the MAP3K Tpl2, which can then activate MEKs, leading to ERK activation. Activation of JNK by CD40 is generally mediated by TRAFs. During CD40 signalling, TRAF 2 can bind GCKR which can activate the JNK pathway. TRAFs 2 and 6 may also bind and activate MAP3Ks directly, or through association of adaptor molecules. p38 activation by CD40 has been shown to require TRAF 3.

relevant MAPK substrates have yet to be identified. ERKs can enhance activity of the transcription factors Elk-1, AP-1 and NF-AT, stimulate DNA synthesis through phosphorylation of carbamoyl phosphate synthetase II, a rate-limiting enzyme in pyrimidine nucleotide biosynthesis, and promote cell-cycle progression by inactivating MYT1, a cell-cycle inhibitory kinase (Park & Levitt 1993, Chang & Karin 2001). ERKs are activated by dual phosphorylation by the MAP2Ks MEK1 or MEK2. MEK1/2 are activated by the MAP3K Raf (a downstream target of Ras) in the case of growth factor signalling, but may be activated by other MAP3Ks in response to other stimuli.

CD40 signalling can activate ERK via Ras-dependent or Ras-independent pathways (Li *et al* 1996, Kashiwada *et al* 1998, Gulbins *et al* 1996, Eliopoulos *et al* 2003). The events leading from CD40 to Ras activation have not been well characterised but may involve tyrosine kinases and phospholipases (Gulbins *et al* 1996). The Ras-independent pathway can be mediated via TRAF6 activating the MAP3K Tpl2, in murine B-cells. Tpl2 then went on to phosphorylate MEK leading to ERK phosphorylation (Eliopoulos *et al* 2003).

1.8.4 JNK

CD40 can activate the JNK/SAPK (c-Jun N-terminal kinase/stress-activated protein kinase) pathway in normal B-cells (Sakata *et al* 1995, Li *et al* 1996). The JNK pathway is a critical regulator of transcription and apoptosis. There are three isoforms of JNK: p46 JNK1, p54 JNK2 and JNK3, plus may splice variants. JNK phosphorylates transcription factors such as c-Jun, ATF2, Elk-1, p53 and c-myc, modulating their activity. Phosphorylation of c-Jun on its N-terminal transactivation domain at serine 63 and serine 73 increases its transcriptional activity. Jun family proteins form the transcription factor AP-1, which has been shown to be activated by CD40 signalling, through homodimerisation, heterodimerisation with Fos proteins (synthesised due to promoter activation by Elk-1) or through heterodimerisation with ATF2. JNK can also phosphorylate Bcl-2 and Bcl-X_L, which may lead to their inactivation. JNK is activated by sequential protein phosphorylation through a MAPK cascade. JNK is activated via phosphorylation on threonine 183 and tyrosine 185 by the MAP2Ks MKK4 (also called JNKK1 or SEK1) and MKK7 (also called JNKK2). These protein kinases are expressed as a group of alternatively spliced isoforms. The

MKK7 protein kinase is primarily activated by cytokines whereas MKK4 is primarily activated by environmental stress. The MKK4 and MKK7 protein kinases are activated by dual phosphorylation by MAP3Ks. Several MAP3Ks have been reported to activate the JNK pathway including the MEKK group (MEKK1 to 4), the mixed-lineage protein kinase group (MLK1, MLK2, MLK3, DLK and LZK), the ASK group (ASK1, ASK2), TAK1 and Tpl-2. Most of these MAP3K have been identified as activators of MKK4 and MKK5 via *in vitro* experiments and it is unclear how they are regulated by physiological stimuli *in vivo*. Many of these MAP3Ks can activate more than one MAPK pathway and also the NF-κB pathway (reviewed in Lin & Dibling 2002, Davis 2000, Ichijo 1999).

MAP3Ks can be activated by a number of mechanisms, but these are not well understood. The Rho family GTPases, Cdc42 and Rac, mediate activation of JNK by some stimuli. This may be through a direct interaction with MEKKs or through activation of PAKs (p21-activated kinases), which may then phosphorylate and activate MEKK1. MEKKs are also activated by DNA damaging agents, microtubule damaging agents, c-Abl kinase and protein kinase C. Receptor mediated activation of JNK is achieved via adaptor proteins linking receptors to MEKKs. TRAFs 2 and 6 are required for JNK activation by TNF-α and IL-1 respectively (Davis 2002, Hagemann & Blank 2001). TRAF2 can bind the MAP3Ks MEKK1 and ASK1, leading to JNK activation (Nishitoh *et al* 1998, Chin *et al* 1999). TANK (TRAF-associated NF-κB activator) and GCKR (germinal centre kinase-related kinase) can also form a complex with TRAF 2 leading to JNK activation. GCK proteins are MAP4K and may activate MEKK1 (Chin *et al* 1999). TRAF6 can also bind MEKK1, although this interaction may be mediated by the adaptor protein ECSIT. ECSIT may increase MEKK1 activity via proteolytic processing. TRAF 6 also binds the MAP3K TAK1 via the adaptor protein TAB2 (Davis 2002).

The mechanism by which CD40 activates MAP3Ks and subsequent JNK activation has not been fully elucidated. However it is clear that both TRAF binding sites in the CD40 cytoplasmic tail can activate the JNK pathway in some cell types and TRAFs 2,5 and 6 induce JNK activation in cells when overexpressed. Dominant negative TRAF2 and dominant negative GCKR were shown to abrogate CD40-mediated JNK

activation in primary B-cells, suggesting that GCKR is a critical mediator of JNK activation by CD40 (Chin *et al* 1999). Since GCKR complexes with TRAF 2 and TANK and may activate MEKK1, CD40 ligation may lead to formation of a complex containing TRAF 2, TANK and GCKR associated with CD40, leading to MEKK1 activation and subsequent JNK activation. However, there may also be a TRAF-independent pathway for JNK activation, stimulated by a membrane-proximal region of CD40 (Leo *et al* 1999).

1.8.5 p38 MAPK

The p38 MAPK pathway is also activated by CD40 in normal B-cells (Sutherland *et al* 1996, Craxton *et al* 1998, Grammer *et al* 1998, Salmon *et al* 1997). p38 MAPK activation was shown to be necessary for CD40-mediated proliferation in human tonsillar B-cells and had an indirect role in NF-κB activation (Craxton *et al* 1998). In addition, p38 activation was required for Ig class switch recombination to IgE in normal B-cells (Zhang K *et al* 2002). In murine B-cells, activation of p38 by CD40 appeared to play a minor role in gene induction, requiring cooperation with other signalling pathways such as NF-κB and PI3-K for its effects, but p38 was shown to have a major role in CD40-mediated gene downregulation (Dadgostar *et al* 2002).

The p38 MAPK pathway is generally activated by inflammatory cytokines and environmental stresses. Activated p38 can phosphorylate the transcription factors ATF2, NF-AT, MEF2C and TCFs (Andrews *et al* 2003, Shaulin & Karin 2002). There are four p38 MAPKs, α, β, γ, δ. The MAP2Ks MKK3 and MKK6 activate p38. It is not clear which MAP3Ks activate MKK3 and MKK6. There are no known MAP3Ks that activate only the p38 pathway, although several of the JNK-activating MAP3Ks can activate p38 when overexpressed. MEKK2 and 3 can cause p38 activation and MKK6 is a direct substrate of these MEKKs. MEKK3 has also shown potential to activate MKK3. TAK1, ASK1 and MEKK4 have also been shown to strongly activate p38. (Hagemann & Blank 2001, Chang & Karin 2001, Ichijo 1999). It is not clear how CD40 activation is linked to p38 activation, but TRAF 3 was shown to be vital for CD40-induced p38 activation in Ramos cells (Grammer *et al* 1998).

1.8.6 Pro-apoptotic signalling pathways

The above signalling pathways were all activated by CD40 in systems where CD40 promotes cell survival. The signalling pathways linking CD40 to apoptosis are not well characterised and is a relatively new area of research. The ability of CD40 activation to induce apoptosis was shown to be dependent on the membrane proximal domain of CD40 (amino acids 216 to 239) but not the TRAF binding sites, in HeLa cells stably expressing CD40 (Eliopoulos *et al* 2000). CD40-mediated apoptosis was also caspase dependent, involving activation of caspase-3 and caspase–8 and lead to induction of cytotoxic ligands of the TNF family (Fas ligand, TRAIL and TNF – see section 1.7.5) (Eliopoulos *et al* 2000). In ovarian carcinoma cell lines that undergo apoptosis in response to CD40 ligation, CD40 activated the NF-κB and JNK pathways (Gallagher *et al* 2002). A comparison of two cell types with differing responses to CD40 revealed that in hepatocytes, CD40 activation led to transient NF-κB activation and sustained AP-1 activation, leading to apoptosis whereas in intrahepatic endothelial cells, CD40 led to prolonged NF-κB activation and no AP-1 activation, leading to proliferation (Ahmed-Choudhury *et al* 2003). In the mature murine B-cell line BAL-17, CD40 inhibited proliferation and this was dependent upon activation of the JNK and p38 MAPK pathways. CD45, a transmembrane tyrosine phosphatase that is thought to dephosphorylate Src family kinases, was also required for CD40 to inhibit proliferation but the role of this molecule was unclear (Arimura *et al* 2001). Therefore similar signalling pathways appear to be activated by CD40, in systems where CD40 can promote or inhibit cell survival. Cooperation between various signalling pathways and the kinetics of activation may play an important role in determining the outcome of CD40 signalling.

1.9 B-cell lymphoma

B-cell differentiation and activation is a hazardous process with many opportunities for mistakes to occur leading to the malignant transformation of B-cells generating lymphomas or leukaemias. The first stage during B-cell development with increased risk for oncogenesis is the rearrangement of Ig genes in the bone marrow to form the B-cell receptor. During this process, double-stranded DNA breaks are formed followed by non-homologous recombination. If this process is misdirected then

chromosomal translocations can occur which may bring a proto-oncogene under the control of a regulatory element which then drives inappropriate expression of that gene. Chromosomal translocations can also occur in the germinal centre as a result of misdirected somatic mutation or Ig class-switching, both of which involve DNA breaks. These chromosomal translocations may then prevent apoptosis, promote proliferation or block differentiation of the B-cell. Oncogenesis may also be facilitated by prolonged or unregulated antigenic stimulation, leading to the unchecked clonal expansion of B-cells (Shaffer *et al* 2002).

B-cell lymphomas were used in this study and the different diseases studied are discussed below. These lymphoproliferative diseases are often referred to as Non-Hodgkin's lymphoma (NHL), characterised by high concentrations of malignant B-cells in the lymph nodes with malignant cells also scattered throughout most tissues of the body (Magarath 2002). However the NHLs are a heterogeneous group of lymphoproliferative diseases with differing patterns of behaviour and responses to treatment. As there is no single factor which can distinguish between different B-cell malignancies, classification of these diseases is extremely complicated and is constantly being revised and updated. Currently, the World Health Organisation classification is in use which is based on the Revised European-American Classification of Lymphoid Neoplasms (REAL) (Harris *et al* 2000). This classifies each disease as a real entity defined by a combination of morphology, immunophenotype, genetic factors and clinical features. Therefore the descriptions used about each disease are based on this classification.

Lymphomas can be divided into indolent (low-grade, slow growing lymphomas) and aggressive (high-grade, fast growing lymphomas), as shown in table 1.1. Indolent lymphomas have a relatively good prognosis with a median survival of 10 years, even in the absence of therapy, however aggressive lymphomas have a much shorter natural survival (weeks or months). Paradoxically, aggressive lymphomas respond well to combination chemotherapy (with a 40-90% cure rate) whereas indolent lymphomas are not usually curable (Magarath 2002). NHL represent 2.4% of all cancers registered in England and Wales and 2.6% of all cancer deaths. Prevalence rises with age and is more common in men than women. Over the past 20 years

incidence of NHL has been rising steadily by 3-5% per year, for reasons which have yet to be identified (Evans and Hancock 2003).

Table 1.1: Classification of B-cell lymphomas, showing examples of indolent and aggressive diseases, according to the REAL/WHO classification.

Indolent (low-grade)	**Aggressive (high-grade)**
Follicular lymphoma	Diffuse large B-cell lymphoma
CLL/small lymphocytic lymphoma	Mantle cell lymphoma
Lymphoplasmacytic lymphoma	Burkitt's lymphoma
Marginal zone B-cell lymphoma	Lymphoblastic lymphoma

1.9.1 Follicular lymphoma

The malignant cells in follicular lymphoma originate from germinal centre B-cells. Normal architecture of the lymph node is replaced by numerous, fairly uniform and closely apposed follicles (Alkan & Karcher 1996). The tumour cells generally express CD10, a membrane metalloendopeptidase, which is characteristic of germinal centre cells (Shaffer *et al* 2002). Most follicular lymphomas (60-90%) overexpress Bcl-2 due to a chromosomal translocation between chromosomes 14 and 18 (Alkan & Karcher 1996). This brings the Bcl-2 gene under control of the Ig heavy-chain enhancer, causing overproduction of Bcl-2. The structure of recombination breakpoints in this translocation is consistent with events that occur during V(D)J recombination (Shaffer *et al* 2002). Normal germinal centre B-cells express low levels of anti-apoptotic proteins, which prime the cells for apoptosis unless they are rescued by positive selection. Overexpression of Bcl-2 in follicular lymphomas therefore overcomes this propensity for apoptosis, contributing to cell survival.

Various NHLs, including follicular lymphomas and one third of diffuse large B-cell lymphomas, possess translocations or mutations in the BCL-6 gene. BCL-6 is a transcriptional repressor, present at high levels in germinal centre B-cells, which blocks terminal differentiation to plasma cells but also promotes proliferation. This is achieved through repression of genes such as B-lymphocyte-induced maturation protein 1 (BLIMP1) and p27KIP1 (cyclin-dependent kinase inhibitor). As cells are arrested at the germinal centre stage, this gives greater opportunity for the acquisition

of additional mutations, possibly mediated by somatic hypermutation or Ig class switching (Shaffer *et al* 2002).

Follicular lymphoma is the most common type of indolent lymphoma, comprising 20% of all NHLs and 70% of indolent lymphomas reported in American and European clinical trials (National Cancer Institute cancer database: www.nci.nih.gov/cancerinfo/pdq/ treatment/adult-non-hodgkins/healthprofessional). Most patients present with widespread disease at diagnosis and although they are not cured by conventional therapies (due to a very high rate of relapse), the median survival ranges from 8 to 12 years. Standard treatment is radiation for localised disease with a variety of treatment options for advanced stage disease. These include purine nucleoside analogs (fludarabine, 2-chlorodeoxyadenosine), oral alkaylating agents (cyclophosphamide, chlorambucil), interferon, or combinational chemotherapy. Combinational chemotherapy regimes involve treating with a number of drugs at the same time, for example CHOP therapy uses cyclophosphamide, doxorubicin, vincristine and predinisone. Other alternatives include treatment with the anti-CD20 monoclonal antibody Rituximab, use of radiolabeled monoclonal antibodies or treatment with an anti-idiotype vaccine (National Cancer Institute cancer database: www.nci.nih.gov/cancerinfo/pdq/treatment/adult-non-hodgkins/healthprofessional, Evans and Hancock 2003).

1.9.2 Chronic lymphocytic leukaemia (CLL)

CLL (molecularly and clinically related to small lymphocytic lymphoma) is the most common indolent lymphoproliferative disorder in Western Europe and North America and its incidence is rising (Alkan & Karcher 1996). It is characterised by clonal expansion of mature B-cells that express CD5 (a T-cell antigen) and express low levels of surface immunoglobulin (Shaffer *et al* 2002). These cells progressively accumulate in the blood, bone marrow and lymphatic tissues. Involved lymph nodes show a diffuse infiltrate of small, round lymphocytes with clumped chromatin and little cytoplasm (Alkan *et al* 1996). There is some confusion as to whether the disease should be classified as a leukaemia or lymphoma, as it presents with both massively inflated blood B-cell counts but also lymph node involvement.

There are two subtypes of CLL, as determined by the presence of somatic mutations in the Ig heavy chain variable region genes (V_H). One subtype has somatically mutated V_H genes and these patients have a good prognosis, often not requiring any treatment. However the second subtype has unmutated V_H genes and patients with this disease have a poor prognosis and need early treatment (Hamblin *et al* 1999, Damle *et al* 1999, Lanham *et al* 2003). The molecular mechanisms for this difference are currently under investigation, but recent data have shown that unmutated cases are able to signal through membrane IgM (causing Syk phosphorylation) whereas mutated cases can not (Lanham *et al* 2003). It is difficult to determine how this difference in signalling relates to tumour behaviour as B-cell receptor signalling can induce proliferation or apoptosis depending on the development stage of the cell and context of the signal.

Ig mutated CLL cells were believed to originate from post germinal-centre B-cells due to the presence of somatic hypermutation, whereas Ig unmutated CLL cells were believed to be pre germinal-centre B-cells. However microarray analysis has revealed that unmutated CLL cells have a closer profile to post germinal-centre B-cells than naive B-cells (Kipps 2002). Therefore unmutated CLL cells may have originated from post-germinal centre cells that failed to undergo somatic hypermutation.

CD38 expression is another prognostic factor for CLL. Patients with over 30% of CLL cells expressing CD38 (termed CD38 positive) show poor survival whereas CLL patients with less than 30% CD38 positive cells (CD38 negative) have a better prognosis (Hamblin *et al* 1999, Damle *et al* 1999, Del Poeta *et al* 2001, Ibrahim *et al* 2001). The relationship between CD38 positivity and V_H gene status is unclear, since some studies show that CD38 expression is unrelated to mutation status (Hamblin *et al* 1999, Lanham *et al* 2003) whereas others show that CD38 positive cells are those with unmutated V_H genes (Damle *et al* 1999). Also, the function of CD38 is not clear but its expression level varies with the stage of B-cell maturation and type of B-cell activation (Ibrahim *et al* 2001).

The overall 5 year survival of CLL patients is 60% and treatment with conventional chemotherapy is not curative (National Cancer Institute cancer database: www.nci.nih.gov/cancerinfo/pdq/treatment/adult-non-hodgkins/healthprofessional).

Patients with early stage CLL or who are minimally affected are generally not treated but carefully observed. If the disease progresses, the treatment options include oral alkylating agents, corticosteroids, purine nucleoside analogs, combination chemotherapy, interferon-alpha treatment, splenectomy or splenic irradiation and monoclonal antibodies (National Cancer Institute cancer database: www.nci.nih.gov/cancerinfo/pdq/treatment/adult-non-hodgkins/healthprofessional, Kalil and Cheson 1999).

1.9.3 Mantle Cell Lymphoma

Mantle cell lymphomas localise to the mantle zone surrounding germinal centres in lymph nodes and are believed to originate from pre-germinal centre B-cells. Involved lymph nodes show a diffuse lymphocytic infiltrate composed of small cells with little cytoplasm and irregular nuclei (Alkan *et al* 1996). Mantle cell lymphoma is associated with t(11;14) which brings the cyclin D1 gene under control of the Ig heavy-chain enhancer, causing overexpression of cyclin D1, which promotes proliferation. Like t(14;18), the t(11;14) is believed to occur early in B-cell differentiation.

Mantle cell lymphomas account for 6% of NHLs and although it is an aggressive lymphoma with a median survival of 3-5 years, it is refractory to most chemotherapeutics (Shaffer *et al* 2002). As it is unclear if current chemotherapeutics are of benefit in this disease other approaches are being tested. These include high-dose therapy with stem cell and bone marrow support, the use of interferon or treatment with anti-CD20 antibodies after CHOP chemotherapy (National Cancer Institute cancer database: www.nci.nih.gov/cancerinfo/pdq/treatment/adult-non-hodgkins/healthprofessional).

1.9.4 Diffuse large B-cell lymphoma (DLBL)

Diffuse large B-cell lymphomas are a heterogenous group of tumours and although they are treated as one disease in the clinic, at least three subtypes have been identified by microarray analysis with distinct gene-expression profiles. These groups are Germinal-centre B-cell like DLBL, Activated B-cell like DLBL and Type 3 DLBL (Alizadeh *et al* 2000, Rosenwald *et al* 2002).

Germinal centre B-cell like DLBL comprise about half of all DLBLs and, as the name suggests, originate from germinal centre B-cells. About 20% of these tumours carry the t(14;18) and some show amplification of the c-Rel gene leading to overexpression of this NF-κB subunit. Although these cells do not show increased expression of NF-κB target genes, overexpression of c-Rel could enhance anti-apoptotic and/or proliferative signals that operate via the NF-κB pathway, such as CD40 (Shaffer *et al* 2002). The t(14;18) and c-Rel amplification are unique to this DLBL subtype (Rosenwald *et al* 2002).

Activated B-cell like DLBL have a gene expression profile closely related to that of normal blood B-cells activated through the BCR. This DLBL subtype also has defects in the NF-κB pathway, and shows increased expression of NF-κB target genes compared to Germinal-centre B-cell like DLBLs. Many of these NF-κB target genes inhibit apoptosis (e.g. Bcl-2, FLIP) or promote proliferation (e.g. cyclin D2), so inappropriate activation of this pathway could have contributed to oncogenesis and could also render the tumours resistant to therapy. Analysis of two Activated B-cell like DLBL cell lines revealed that the cells possess constitutive activation of the IKK complex, leading to permanent activation of NF-κB (Davis RE *et al* 2001). Interestingly, treatment of these cells with a super-repressor form of IκBα or a dominant negative IKKβ, caused apoptosis and cell cycle arrest, but did not affect Germinal centre B-cell like DLBL. Therefore the NF-κB pathway could be an attractive molecular target for treatment of Activated B-cell like DLBL.

Type 3 DLBL is not a clearly defined subtype and includes samples which do not have gene expression profiles characteristic of the other two subtypes. This subtype may therefore consist of more than one type of DLBL (Rosenwald *et al* 2002).

DLBL is the commonest form of NHL (30-40% of cases), and as well as occurring spontaneously it can also occur through transformation of a pre-exisiting indolent lymphoma. Between 24% and 60% of follicular lymphomas transform to DLBL and this is associated with rapidly progressive disease, resistance to treatment and short survival, with a median survival time of 7 months (Bastion *et al* 1997, Martinez-Climent *et al* 2003). The molecular mechanisms responsible for transformation are

not well understood. Comparative genomic hybridisation (CGH) studies comparing genetic changes in FL biopsies and their transformed counterparts have revealed a wide range of alterations occurring upon transformation, including over 20 distinct regions of amplification or deletion (Martinez-Climent *et al* 2003, Hough *et al* 2001). Other reported changes include c-myc rearrangements, altered expression of c-myc and its target genes, and mutations in p53, Bcl-6 and the translocated Bcl-2 gene (Lossos *et al* 2002). As these changes are heterogeneous and occur in only a subset of transformed FL, there must be multiple genetic mechanisms responsible for transformation.

At present, 40% of DLBL patients can be cured with doxorubicin-based combination chemotherapy (National Cancer Institute cancer database: www.nci.nih.gov/ cancerinfo/pdq/treatment/adult-non-hodgkins/healthprofessional). Although all cases are treated similarly in the clinic, the three different DLBL subtypes show differing responses to chemotherapy. Patients with Germinal centre B-cell like DLBL have a 60% 5-year survival rate after doxorubicin-based chemotherapy, compared to 39% of patients with type 3 DLBL and 35% of patients with activated B-cell like DLBL (Rosenwald *et al* 2002). One of the reasons for this difference could be the constitutive activation of the NF-κB pathway in Activated B-cell like DLBL, so drugs targeting this pathway could be of real benefit.

1.9.5 Burkitt's Lymphoma

Burkitt's lymphoma (BL) is a highly aggressive B-cell malignancy, often presenting at extranodal sites such as the jaw, abdomen or breast or as an acute leukaemia (Diebold *et al* 2001). It is characterised by a diffuse infiltrate of medium sized B-cells, believed to be of germinal centre origin and an extremely high rate of both proliferation and apoptosis. This gives rise to a 'starry-sky' pattern observed in tumour sections, which is the histological hallmark of BL. It is caused by numerous benign macrophages that have ingested apoptotic tumour cells (Hecht and Aster 2000).

There are three clinical variants of BL. The endemic form of BL occurs in equatorial Africa and New Guinea and is the most frequent cancer in African children. It is the

form originally described by Dennis Burkitt in the 1950s and is associated with Epstein-Barr virus (EBV) infection and infection by the malarial parasite. The second form of BL is sporadic and seen worldwide mainly in children and young adults. It occurs a much lower frequency than the endemic form (20 to 100 fold lower incidence worldwide) but accounts for 30-50% of all childhood lymphomas (Farrell and Sinclair 1994, Diebold *et al* 2001). The third form of BL is immunodeficiency-associated BL and is seen primarily in HIV-infected individuals, often occuring as the initial manifestation of AIDS (Diebold *et al* 2001). Unlike endemic BL, where over 90% of tumours contain EBV, only a subset of sporadic BLs (20%) and immunodeficiency-associated BLs (40%) are EBV-associated (Hecht and Aster 2000).

The tumour cells of BLs are characterised by translocation of the c-myc gene on chromosome 8 to one of the Ig loci, forming t(8;14), t(2;8) or t(8;22) abnormalities. This results in transcription of inappropriately high levels of c-myc. c-myc is also frequently found to be mutated in BL in the region which normally binds the p107 repressor of myc function, and negative regulatory sequences in c-myc are often removed during the chromosomal translocation (Hecht and Aster 2000, Farrell *et al* 1997). These mechanisms further contribute to increased c-myc activity. c-myc is a sequence specific transcription factor and a well characterised oncogene. Upregulation of c-myc disrupts many aspects of cell function leading to cellular transformation, including promoting proliferation, preventing differentiation, increasing telomerase activity, decreasing cell adhesion, and can also increase apoptosis (Hecht and Aster 2000, Boxer and Dang 2001).

The role of EBV in the pathogenesis of BL is unclear. Although the majority of BL are EBV-associated, most humans are infected with EBV and maintain a few EBV positive B-cells throughout life so rare, random events must occur to produce BL. BL shows a monoclonal population of EBV genomes indicating that the tumour arose from a single cell that was infected with EBV before or at the time of transformation (Hecht and Aster 2000, Farell and Sinclair 1994). There are a number of different theories as to how EBV could contribute to malignancy including that acute EBV infection stimulates B-cell proliferation, which increases the chance of genetic alterations occurring, such as c-myc rearrangement. Infection in patients who are

immunosuppressed or who are infected with malaria may further increase B-cell proliferation and thus the risk of secondary genetic events. Decreased T-cell mediated immune responses are also observed in immunosuppression and malaria infection which may increase the likelihood that malignant cells will escape immune surveillance. Alternatively, EBV may infect after the initial oncogenic change and enhance proliferation of an early cancer cell causing it to grow into a tumour (Hecht and Aster 2000, Farrell *et al* 1997, Diebold *et al* 2001). Interestingly, a recent paper has shown that EBV infection induces CD40L gene expression in B-cells, and that the CD40/CD40L interaction inhibits apoptosis, facilitating persistent infection and host cell transformation (Imadome *et al* 2003).

Combined chemotherapy regimens e.g. CHOP, are the standard treatment for BL and are often very effective. Children with BL show over 80% long-term survival even with advanced staged disease (National Cancer Institute cancer database: www.nci.nih.gov/cancerinfo/pdq/treatment/adult-non-hodgkins/healthprofessional). However relapses are frequent and the prognosis of relapsed disease is poor with a median overall survival of 6 months (Maisey and Cunningham 2000).

1.10 Summary

Normal and malignant cells show differential responses to activation of CD40. Whereas CD40 stimulation induces proliferation and apoptotic rescue in normal B-cells, some malignant B-cell lines, follicular lymphoma, mantle cell lymphoma and CLL cultures; activation of CD40 induces growth arrest and apoptosis in other non-Hodgkin lymphoma cell lines, some multiple myeloma lines and carcinomas. The underlying mechanisms controlling differential control of cell survival by CD40 are not known and of great significance for understanding the function of CD40 in both normal and malignant cells. There is also considerable interest in using CD40 activating agents as novel cancer therapies. Impressive results have been obtained in mouse model systems, supported by some encouraging findings in early clinical trials. The anti-tumour effects of CD40 stimulation may derive from direct negative effects on CD40 positive tumour cells and/or stimulation of anti-tumour immune responses. It is clearly important to consider the likely response of tumour cells to CD40 ligation, to determine whether signalling will result in anti- or pro-apoptotic responses. Although increased apoptosis has been observed in some cell lines with CD40

expression (such as carcinomas and B-cell lines), it is important to note that no studies have been published showing induction of apoptosis by CD40 in primary malignant cells. The response of long term established cell lines may be very different to the situation in primary cells and it is essential that further studies are performed to determine CD40 responses in primary tumour material.

CD40 signalling can induce expression of anti-apoptotic (e.g. Bcl-X_L, Bfl-1, Mcl-1, survivin and A20) or pro-apoptotic proteins (e.g. Bax, Bak, Fas and TRAIL). However neither the significance of these targets nor their mechanism of regulation is well understood. These targets have been mainly studied in cell lines and so it is essential to determine the regulation of these molecules in primary cells. Detailed studies are required to determine whether the different outcomes observed following CD40 ligation are associated with differential regulation of pro- and anti- apoptotic targets, and whether these are mediated by distinct signalling pathways, particularly in primary cells. It will ultimately be important to determine if individual CD40 target genes can be used as surrogate markers to predict the outcome of response to CD40 activation and whether the survival molecules or pathways regulated by CD40 could be therapeutic targets for the treatment of cancer. Microarray analysis is a particularly useful approach to compare patterns of gene expression in different cellular settings and to identify novel CD40-target genes.

The aims of the project in each chapter were as follows:
Chapter 3: CD40 promotes survival of B-cell lymphomas and influences expression of apoptosis regulators. The aims of this chapter were (i) to determine the effect of CD40 ligation on cell survival in a range of primary B-cell lymphomas and non-malignant B-cells and (ii) to investigate the regulation and role of CD40-target genes in B-cell lymphomas. This chapter showed that CD40 ligation was a potent survival signal for B-cell lymphomas, accompanied by regulation of the key anti-apoptotic molecules Bcl-X_L and Mcl-1. The regulation of Bcl-X_L at the transcriptional and translational levels was investigated.

Chapter 4: Differential control of cell survival and cell signalling by CD40. The aims of this chapter were (i) to investigate the molecular mechanisms underlying the differential control of cell survival by CD40 and (ii) to investigate the activation and

role of CD40-induced cell signalling pathways in B-cell lymphomas. This chapter showed that the Fas or TRAIL pathways were not involved in the differential control of cell survival by CD40 but that survival outcome may be influenced by modulation of Bcl-2 family protein expression by CD40. NF-κB activity was shown to be essential for both the anti-apoptotic effects of CD40 and upregulation of Bcl-X_L and Mcl-1 expression by CD40.

Chapter 5: Characterisation of CD40-regulated genes in B-cell lymphoma using DNA microarrays. The aim of this chapter was to develop and implement DNA microarrays to characterise gene expression patterns induced by CD40. This chapter compared two different microarray platforms and identified many novel and differentially-regulated CD40 target genes.

Chapter 2: Materials and Methods

All chemicals were supplied by BDH Laboratory Supplies (Merck, UK) unless stated otherwise.

2.1 Solutions and Buffers

Listed in the order in which they appear in the text

PBS (phosphate buffered saline):	125mM NaCl
	16mM Na_2HPO_4
	10mM NaH_2PO_4
	pH 7.3
Cell preparation medium:	RPMI-1640 medium
	2mM L-glutamine
	100μg/ml penicillin G
	100μg/ml streptomycin
	1mM sodium pyruvate
	5ml non-essential amino acid solution
	0.5μg/ml fungizone
	(All supplied by Gibco, UK)
B-cell medium:	IMDM medium (Sigma, UK)
	10% (v/v) human serum (Sigma, UK).
	2mM L-glutamine
	20μg/ml gentamycin
	100μg/ml penicillin G
	100μg/ml streptomycin
Binding buffer:	1% (v/v) FCS (foetal calf serum)
(for flow cytometry)	0.1% (v/v) NaN_3
	in PBS
MOPS buffer:	From Sigma (UK), 1x contains:

	40mM MOPS 10mM sodium acetate 1mM EDTA pH 8.3
RNA loading buffer:	50% (v/v) deionised formamide 2.15M formaldehyde 5% (v/v) glycerol small amount of bromophenol blue to give blue colour in MOPS buffer (Sigma, UK)
TBE:	80mM Tris base 80mM orthoboric acid 1mM EDTA
Agarose gel loading buffer (6x):	0.25% (w/v) xylene cyanole 0.25% (w/v) bromophenol blue 15% (w/v) Ficoll 400
LB medium:	10g Tryptone 5g Yeast extract 10g NaCl in 1 litre water pH 7.0
LB/ampicillin plates:	15g agar to 1 litre LB medium, autoclave, cool to 50°C and add ampicillin to a final concentration of 100μg/ml. Pour into petri dishes, store at 4°C for up to 1 month.
Miniprep lysis buffer:	2g sucrose

	1ml 1M Tris pH8
	1ml 0.2M EDTA
	water to 10ml
	20mg lysozyme added on day of use
Maxiprep lysis buffer:	50mM glucose
	10mM EDTA
	25mM Tris-HCl pH8
Cytomix:	120mM KCl
(for electroporation)	0.15mM $CaCl_2$
	10mM K_2HPO_4
	10mM KH_2PO_4
	25mM HEPES
	2mM EGTA
	5mM $MgCl_2$
	2mM ATP
	5mM glutathione
	pH7.6, ATP and glutathione added on day of use
TS:	137mM NaCl
(for DEAE –dextran)	25mM Tris-HCl pH 7.5
	5mM KCl
	0.37mM Na_2HPO4
	0.68mM $CaCl_2$
	1mM $MgCl_2$
Lysis buffer:	10mM Tris-HCl
(for reporter gene asays)	1mM EDTA pH 8.0
	150mM NaCl
	0.65% (v/v) Nonident P40
ONPG substrate:	162mM Na_2HPO_4

	36mM NaH_2PO_4
	100mM β-mercaptoethanol
	2mM $MgCl_2$
	1.33 mg/ml ONPG
RIPA lysis buffer:	0.15M NaCl
	0.05M Tris-HCl pH 8.0
	1% (v/v) Nonident P40
	0.5% (w/v) deoxycholate
	0.1% (w/v) SDS
Gel fixing buffer:	10% (v/v) acetic acid
	45% (v/v) ethanol
Sample buffer:	50mM Tris-HCl pH 6.8
(for immunoblotting)	12% SDS
	0.1% (w/v) bromophenol blue
	10% (v/v) glycerol
Protein running buffer:	25mM Tris base
	200mM glycine
	0.1% (w/v) SDS
TS:	10mM Tris-HCl pH 8.0
	150mM NaCl.
TST:	TS + 0.05% (v/v) Tween 20
PBST:	PBS + 0.1% (v/v) Tween 20
BD buffer:	10mM Tris-HCl pH 7.5
	100mM NaCl
	0.1% (v/v) Tween 20

Microarray hybridization buffer:	6x SSC
	0.2% (w/v) SDS
	42% (v/v) deionised formamide (Sigma, UK)
20x SSC:	3M NaCl
	0.3M sodium citrate

2.2 Cell culture and treatments

2.2.1 Cell culture

All cell culture reagents were supplied by Gibco, UK. WEHI-231 cells (murine immature B-cell lymphoma cell line) were cultured in DMEM media supplemented with 5 x 10^{-5} M β- mercaptoethanol, plus 10% (v/v) FCS, 2mM L-glutamine, 100μg/ml penicillin G and 100μg/ml streptomycin. Akata 6 and Elijah (Group I EBV positive human Burkitt's lymphoma), DG75 (EBV negative human Burkitt's lymphoma cell line) and RL (human diffuse large B-cell lymphoma) cells were cultured in RPMI 1640 media supplemented with 10% (v/v) FCS, 2mM L-glutamine, 100μg/ml penicillin G and 100μg/ml streptomycin. 293 cells (human embryonic kidney) were cultured in DMEM media supplemented with 10% (v/v) FCS, 2mM L-glutamine, 100μg/ml penicillin G and 100μg/ml streptomycin. Cells were maintained in a humidified incubator at 37°C and 5% $C0_2$. All media was preheated to 37°C before addition to the cells.

2.2.2 Activating CD40

WEHI-231 cells were treated with IC10 mAb (5μg/ml) to activate CD40. IC10 is a mouse CD40-specific rat monoclonal antibody of isotype IgG2a. KT3 mAb (5μg/ml) was used as an isotype matched control (raised against mouse CD3). Alternatively soluble recombinant mouse CD40L (moCD40L) (Immunex Corporation, USA) was used at a concentration of 1μg/ml. Cells were left untreated as a control.

Human cells were treated with soluble recombinant human CD40L (Immunex Corporation, USA) at a concentration of 1μg/ml or 10μg/ml, or left untreated as a control. The recombinant CD40L molecules are trimeric leucine-zipper fusion

proteins. The incoroporation of a leucine zipper trimerisation motif enhances the biological activity over monomeric CD40L (Morris *et al* 1999).

Cells were diluted to a concentration of 0.3 x 10^6 cells/ml to ensure cells were growing exponentially, and cultured overnight in 75cm^3 flasks the day before treatment. CD40L was added to appropriate flasks and control flasks were left untreated. A control at the beginning of the experiment (before the addition of CD40L) was also included and this is referred to as the Time 0 control. Cells were harvested by centrifugation at 1,300rpm for 3 mins (in a Sorvall Legend RT centrifuge, used for all cell culture) and washed once in PBS, at various time points. Final traces of PBS were removed and the cell pellets were snap frozen in liquid nitrogen and then stored at -80°C.

2.2.3 Cell treatment

Cells were treated with various agents, alone or in combination with CD40L and the effect on apoptosis or cell viability was determined (see section 2.3). These agents are listed in table 2.1. Cells were treated at a cell density of 0.5 x 10^6 cells/ml typically for 48 hours.

2.3 Primary B-cell isolation and culture

2.3.1 Processing lymph node biopsies or spleens

Following ethical approval (Southampton & S.West Hants LREC submission 158/00), lymph node biopsies or spleens were homogenised in cell preparation medium. The tissue was placed in a sterile sieve and pushed through using a syringe plunger, to form a cell suspension. Samples were either frozen for later processing or used fresh. Cells were frozen in fresh freezing medium (40% preparation medium, 50% human serum, 10% dimethylsulphoxide) at 1 x 10^7 to 1 x 10^8 cells/ml.

Frozen samples were thawed in a 37°C water bath and washed in IMDM media (Sigma, UK) containing 10μg/ml DNase I (Sigma, UK). Cells were resuspended in B cell medium, at a concentration of 5 x 10^6 cells/ml. Cells were left to recover in a humidified incubator at 37°C and 10% CO_2, for approximately 3 hours.

Table 2.1: The function and final concentrations of various agents used to treat cells.

Agent	Supplier	Function	Final Concentration In Media
Etoposide	Bristol-Myers Pharmaceuticals Ltd.	Induce apoptosis	1μM to 20μM
Cycloheximide	Sigma	Inhibit protein synthesis/ induce cell death	10μg/ml
CH-11 mAb (IgM)	Upstate	Activate Fas	500ng/ml
TNP mAb (IgM)	Pharmingen	Isotype-matched control to CH-11	500ng/ml
ZB4 mAb (IgG)	Upstate	Neutralise Fas	500ng/ml
Human recombinant TRAIL (rTRAIL)	Alexis	Activate TRAIL	250ng/ml
Enhancer mAb	Alexis	Cross-links rTRAIL by binding to its FLAG-tag	1.25μg/ml
UO126	Promega	Inhibits MAPK pathway by inhibiting MEK	10μM
LY294002	Calbiochem	Inhibits PI3-K	10μM
SB 203580	Calbiochem	Inhibits p38 kinase	10μM
JNK Inhibitor II	Calbiochem	Inhibits JNK-1 and JNK-2	10μM
Sulfasalazine	Calbiochem	Prevents NF-κB activation by inhibiting IKK	500μM and 2mM
Kamebakaurin (KA)	Calbiochem	Inhibits NF-κB pathway by preventing DNA-binding activity of p50	0.1μM to 10μM
Gliotoxin	Sigma	Prevents NF-κB activation by inhibiting proteasome	0.1μM to 10μM

2.3.2 Isolation of B-cells

B-cell isolation was carried out as in Dallman & Packham (in press). Mononuclear cells were isolated from the cell suspension using Ficoll-Paque (Amersham Pharmacia Biotech, UK) which was warmed to room temperature. 15 ml cells were underlayed with 35 ml Ficoll and centrifuged at 500g for 25 mins. The cloudy interface was taken and placed in a fresh tube, which was filled with preparation medium and centrifuged at 300g for 10 mins. The supernatant was removed and the cells resuspended in 10ml preparation medium and counted.

Blood samples were also obtained. Mononuclear cells were isolated by layering 10ml blood over 12ml Ficoll-Paque. The tubes were centrifuged at 400g for 20 mins and the cloudy interface taken and placed in a fresh tube. The tube was filled with preparation medium and centrifuged at 800g for 5 mins.

B-cells were then immunomagnetically purified from the mononuclear cell suspension using a MACS B Cell Isolation Kit (Miltenyi Biotec, UK), according to the manufacturer's instructions. Briefly, non B-cells were indirectly magnetically labelled using a cocktail of hapten conjugated CD2, CD4, CD11b, CD16, CD36 and anti-IgE antibodies, and magnetic beads coupled to an anti-hapten monoclonal antibody. The magnetically labelled non B-cells were depleted by retention on a column in a magnetic field. The unlabelled B-cells pass through the column, resulting in a pure B-cell suspension.

2.3.3 Primary B-cell culture

Isolated B-cells were cultured in B-cell medium with and without CD40L (1μg/ml) at a cell density of 1 x 10^6 cells/ml. At various time points cells were harvested by centrifugation at 1,100rpm for 3 mins. Final traces of media were removed and cell pellets were snap frozen in liquid nitrogen and then stored at -80°C for later analysis.

2.4 Flow cytometry

Cell surface expression of various receptors was determined on human cell lines and primary B-cells by flow cytometry. This allowed determination of the purity and clonality of isolated primary B-cells. 4 x 10^5 to 1 x 10^6 cells were used per test. These

were resuspended in 100μl binding buffer plus 1μg antibody. The antibodies used are listed in table 2.2. The tubes were incubated for 30 mins in the dark on ice. Cells were then washed twice in binding buffer and resuspended in 0.5ml PBS for analysis by flow cytometry, using a FACSCalibur (BD, UK).

Table 2.2: Antibodies used for flow cytometry and their isotype-matched controls.
Unless otherwise stated all antibodies are directed against human epitopes.

Antibody	Supplier	Isotype-matched control	Supplier
CD40-FITC	Kind gift of MJ Glennie, Tenovus, UK	Mouse CD40-FITC	Kind gift of MJ Glennie, Tenovus, UK
CD20-PE	Pharmingen, UK	Mouse IgG1 kappa-PE	Sigma, UK
CD3-PE	Sigma, UK	Mouse IgG1 kappa-PE	Sigma, UK
Fas-FITC	Pharmingen, UK	Mouse IgG1 kappa-FITC	Pharmingen, UK

FITC-conjugated kappa specific antibodies and PE-conjugated lambda specific antibodies (Dako, UK) were also used to determine immunoglobulin light chain expression on isolated primary B-cells.

2.5 Cell viability and apoptosis assays

2.5.1 Trypan blue exclusion test

For determination of cell number and viability the trypan blue exclusion test was performed. An equal volume of trypan blue (0.4%, Sigma UK) was added to the cell suspension and the cells counted using a haemocytometer and light microscope. At least 100 cells were counted per sample. Dead cells were those which had lost membrane integrity and had taken up the trypan blue, thus appearing blue under the microscope.

2.5.2 Propidium iodide (PI) exclusion test

The PI exclusion test was used to determine viability of primary B-cells. 1 x 10^5 cells were plated per well in 50μl of B-cell media with or without CD40L (1μg/ml) in a 96 well plate. 50μl of B-cell media with or without varying concentrations of etoposide

was then added. The cells were left for 48 hrs in a humidified incubator at 37°C, 5% CO_2. After 48 hrs, cells were washed in PBS and resuspended in 0.5ml PBS containing 10mg/ml PI. The cells were incubated at 37°C for 5 mins and then analysed by flow cytometry.

Only dead cells, which have lost membrane integrity, take up PI. PI stains DNA, so dead cells will fluoresce when excited by lazer light and this fluorescence can be detected in the FL-2 channel. Viable cells, however, exclude PI and thus exhibit a lower fluorescence.

2.5.3 MTS assay

The MTS assay is an indirect method for measuring cell proliferation and viability. MTS is a tetrazolium dye, which is converted into a water-soluble formazan product by the action of NADH-generating dehydrogenases found in metabolically active cells. The conversion of MTS produces a shift in absorbance, which can be detected by spectrophotometry. Cells were plated in a 96 well plate at a density of 1×10^5 cells per well, in 100µl, with or without 1µg/ml CD40L. Following 48 hrs of culture, the MTS assay was performed by adding 20µl of the MTS reagent (Cell Titer-96 Aqueous Cell Proliferation Assay, Promega, UK) to the wells and incubating the plate for 3-4 hours at 37°C. The absorbance was then measured at 490nm with an ELISA plate reader.

2.5.4 Annexin V staining

One of the hallmarks of apoptotic cells is translocation of phosphatidylserine from the inner leaflet of the plasma membrane to the outer leaflet (Martin *et al* 1995). This can be detected using Annexin V. Annexin V binds to phosphatidylserine with high affinity, and when conjugated to fluorochromes can be used to identify apoptotic cells by flow cytometry. It is used in combination with PI to differentiate apoptotic cells (PI negative, Annexin V positive), from necrotic cells (PI positive, Annexin V positive).

To detect apoptosis by Annexin V and PI staining, an Annexin V-FITC kit (Immunotech, France) was used, following the manufacturer's instructions.

2.5.5 PARP cleavage

PARP is a DNA repair enzyme, which is cleaved rapidly and quantitatively by caspase-3 during the onset of apoptosis. Native PARP has a molecular weight of 116kDa and cleavage results in fragments of approximately 85kDa and 31kDa (Gu *et al* 1995). For detection of apoptosis by PARP cleavage, cells were treated with various agents for 48 hrs (see section 2.2). Following treatment, cells were washed in PBS and snap frozen for later analysis of PARP cleavage by Western blot (as in section 2.7).

2.5.6 Long-term clonogenic survival assays

To assess the effect of CD40L on long-term growth and survival, clonogenic assays were performed. Cells were cultured for 48 hrs with or without 1μg/ml CD40L, at a starting density of 1 x 10^5 cells/ml. After 48 hours, cells were counted and diluted to a concentration of 1 x 10^4 cells/ml with standard culture media. Eight serial dilutions were performed, each diluting the previous suspension by a factor of 3. 200μl of each dilution was aliquoted into 16 wells of a 96 well plate. This resulted in dilutions ranging from 2000 cells per well to <1 cell per well. The cells were cultured for 21 days. After this time, the number of wells containing healthy cells for each dilution, was determined by visual inspection under the light microscope. The cloning efficiency was determined as the dilution at which one third of the wells contained healthy cells (Godman 1983).

Clonogenic assays were also carried out with CD40L present at a concentration of 1μg/ml throughout the assay. This was achieved by using media containing CD40L for the initial dilutions and then changing half the media in the wells every week with fresh CD40L-containing media.

2.6 RNA extraction and manipulation

2.6.1 RNA isolation

Total RNA was extracted from cell pellets using Trizol reagent (Life Technologies, UK) following the manufacturer's instructions. Trizol is a solution of phenol and guanidine isothiocyanate that complexes with RNA, while disrupting cells and dissolving cell components. Chloroform is added which separates the solution into an

aqueous phase (containing RNA) and an organic phase (containing DNA and proteins). RNA is recovered from the aqueous phase by precipitation with isopropanol. RNA extracted from the cell lines was quantified using spectrophotometry in quartz cuvettes at 260nm. One absorbance unit at 260nm is equal to 40μg/ml RNA. The purity of RNA was estimated by measuring the ratio of absorbance at 260nm/280nm: a ratio above 1.8 indicates pure RNA. The RNA extracted from primary B-cells was quantified using a Ribogreen RNA Quantitation Kit (Molecular Probes, UK).

2.6.2 RNA Denaturing Gel Electrophoresis

The integrity of isolated RNA to be used for microarray analysis, was determined by RNA gel electrophoresis. All solutions and apparatus used for this procedure were RNAse free. This was achieved by rinsing apparatus with RNAse Away (BDH, UK) or baking glassware at 250°C for 4hrs. A 1% (w/v) agarose formaldehyde gel was prepared using MOPS buffer (Sigma, UK) and formaldehyde added to give a final conecentration of 0.66M. 2μg RNA was added to 9μl RNA loading buffer and ethidium bromide was added to give a final concentration of 0.1mg/ml. Samples were then heated to 65°C and cooled on ice. The samples were loaded into the gel and the gel run in MOPS buffer at 90 volts for 30-60 mins. RNA was visualized under UV illumination. Intact RNA should be present as a smear from 0.5-12kb with two bright sharp bands of 28S and 18S ribosomal RNA at approximately 5kb and 1.9kb respectively. The 28S ribosomal RNA band should be present at approximately twice the intensity of the 18S ribosomal RNA band. A band of transfer RNA may also be visualized between 0.1-0.3kb.

2.6.3 Removal of DNA from RNA preparations

For luciferase RT-PCRs, the RNA preparations were first treated to remove any contaminating transfected plasmid DNA. This was achieved using an RNeasy Mini kit (Qiagen, UK), following the manufacturer's instructions and incorporating the DNase digestion step. Briefly, a high-salt buffer allows RNA to bind to the RNeasy silica-gel membrane which is situated in a spin column. DNase is added to the membrane, to remove any contaminating DNA, the sample is washed and highly pure RNA is eluted with water.

2.6.4 RT-PCR

All reagents used were from Promega, UK unless stated otherwise. mRNA was converted to cDNA using M-MLV RT and oligo dT primers. For RNA extracted from cell lines, 1μg RNA was used. For RNA extracted from primary B-cells, equal amounts of RNA were used for each RT reaction. RNA + water up to 14μl + 1μl oligo-dT primer were placed in a tube. The samples were mixed and heated to 70°C for 5 mins. The tubes were then placed on ice and 5μl 5x Buffer, 1.25μl 10mM dNTP mix, 0.625μl RNAsin ribonuclease inhibitor (40u/μl), 1μl M-MLV RT and 2.125μl water were added to each tube. The samples were mixed and placed in a water bath at 42°C for 1 hour and then heated to 95°C for 5 mins. The samples were placed on ice and 75μl molecular biology grade water was added to the tubes and the cDNA stored at -20°C. As a control for DNA contamination, RNA samples were treated as above, except water was added instead of M-MLV RT.

The cDNA of interest was amplified using semi-quantitative PCR. The primers used are listed in table 2.3. Typically, PCR reaction mixes (25μl per reaction) were set up containing: 2.5μl 10x Reaction Buffer, 1.5μl 25mM $MgCl_2$, 0.5μl 10mM dNTP mix, 1μl (10pmol) forward primer, 1μl (10pmol) reverse primer, 5μl cDNA, 0.25μl HotGoldStar DNA Polymerase (Oswel, UK) and 13.25μl molecular biology grade water.

The cycling parameters for the PCR reactions were as follows (using a GeneAmp PCR System 9700):

10 mins 95°C (polymerase activation and initial denaturation)

30 cycles of: 1 min 94°C (denaturation)

1 min at annealing temperature (see table 2.3)

1 min 72°C (extension)

5 mins 72°C (final extension)

Table 2.3: Primers utilised for RT-PCR reactions. F = forward, sense primer. R = reverse, antisense primer. Primers were obtained from Invitrogen, UK, except for AID primers which were a kind gift of H. McCarthy, Southampton, UK.

cDNA	Primer sequences	Annealing temperature (°C)
A20	F: ccc gga gag gtg ttg gag ag R: ctg tgt cct gaa cgc ccc a	50
AID	F: agg caa gaa gac act ctg gac acc R: gtg aca ttc ctg gaa gtt gc	56
CCR7	F: cct tcc tgt gtg gtt tta ccg R: tgg aga gca ctg tgg cta gta	50
Bcl-X exon IA	F: ctg agc ttc gca att cc R: ggt ctc cat ctc cga ttc	45
Bcl-X exon IB	F: aag tga ctg agc ttg caa gt R: ggt ctc cat ctc cga ttc	46
Bfl-1	F: cca cca ggc aga aga tga cag ac R: tag gta tcc aca tcc ggg gc	55
BNIP3	F: gaa gca cat ccc gca gcc R: gcc acc cca gga tct aac agc	57
GAPDH	F: cca ccc atg gca aat tcc atg gca R: tct aga cgg cag gtc agg tcc acc	60
Luciferase	F: tgg tgc cct taa acg cct R: gaa gga aag tcc ttg ggg tc	50
Mcl-1	F: agg agg agg agg acg agt t R: ggt ggt ggt ggt tgg tt	46
RGC-32	F: aca gca agc ccc cga ata R: atg ctg caa gca ggt aaa ca	50
SH2D1A	F: cgc agt ggc tgt gta tca t R: act tct agc tga gga ctt ctt ctc	55
SLAM	F: atc act gga gaa cag tgt R: ccc agc ata cac tgc cc	55
36B4	F: ggc cga att ctg tct gtg gag acg gat tac acc R: ggc cgg atc cga ctc ttc ctt ggc ttc aac ctt ag	60

In order to obtain a semi-quantitative result, in which the PCR amplification was within a linear range, the number of cycles was decreased until a faint band was observed by agarose gel electrophoresis (see section 2.6.5).

All RT-PCR primers, except those for Bfl-1 and GAPDH, were either constructed at exon/intron boundries or constructed so the forward and reverse primers bound to sequences in different exons. This ensured that the primers specifically amplified cDNA rather than any contaminating genomic DNA. To ensure that bands visualised by the Bfl-1 and GAPDH PCRs were cDNA and not genomic DNA, RT-PCRs were also carried out using cDNA reactions in which the RT enzyme was not added. In most samples there was no contaminating DNA, but in those few instances where there was a small amount of genomic DNA present the band intensity was corrected for the presence of genomic DNA. To verify that the PCR products obtained by RT-PCR corresponded to the gene of interest, restriction digests were performed (see 2.7.3).

2.6.5 Agarose gel electrophoresis

PCR products were visualised by agarose gel electrophoresis. 1% (w/v) agarose (Life Technologies, UK) gels were made in TBE, and 0.1μg/ml ethidium bromide added. Samples were mixed with 6x agarose gel loading buffer and loaded into the gel. Agarose gels were run in TBE buffer at 100V. The DNA was visualized under UV illumination. Images were captured and bands were quantified using Kodak Digital Science 1D v2.0 software (Kodak, USA).

2.7 DNA manipulation

The following strategy was used to generate plasmid constructs (the details of which are listed in the following sections, 2.7.1 – 2.7.13): DNA fragments to be cloned were obtained by PCR amplification and the PCR products cloned into pGEM-Teasy vector (Promega, UK) by TA cloning. Following ligation, DNA was transformed into competent bacteria and clones analysed by restriction endonuclease digestion of small scale DNA preparations. Clones were sequenced and medium scale DNA preparations (midipreps) were made of plasmids with the correct sequence. DNA was subcloned by restriction digestion of the midipreps, followed by gel purification, and ligating the

resulting fragments into a linearised and dephosphorylated vector. DNA was transformed into competent bacteria and clones analysed by restriction endonuclease digestion of small scale DNA preparations. Large scale DNA preparations were produced of the correct plasmids and purified by centrifugation on caesium chloride gradients containing ethidium bromide. The construction of the plasmids used is detailed in section 2.7.14.

2.7.1 PCR amplification of DNA fragments

DNA to be cloned was amplified using the GC Advantage PCR kit (Clontech, UK), which contains a proofreading DNA polymerase. PCR reaction mixes (25µl per reaction) were set up as follows: 2.5µl 5x PCR buffer, 1.1µl $Mg(OAc)_2$, 50pg DNA, 1µl (10pmol) forward primer, 1µl (10pmol) reverse primer, 0.5µl 50x dNTP mix, 5µl GC Melt, 0.5µl 50x GC polymerase, water to 25µl.

The cycling parameters for the PCR reactions were as follows (using a GeneAmp PCR System 9700):

1 min 95°C (initial denaturation)

35 cycles of: 1 min 94°C (denaturation)

1 min annealing temperature

2 mins 68°C (extension)

5 mins 68°C (final extension)

The primers used were as follows (obtained from Invitrogen, UK):

Bcl-XIB cloning (sense primer): gcc aaa gct tag tga ctg agc ttg caa gtt c

(antisense primer): ggc ttt cga ata ggg atg ggc tca acc agt c

Annealing temperature = 55°C

Bcl-XIA cloning (sense primer): ggc aaa gct tca gta gga ggc gga gag cc

(antisense primer): ccg aaa gct tta ggg atg ggc tca acc agt c

Annealing temperature = 60°C

PCR products were visualized by agarose gel electrophoresis (see 2.6.5)

2.7.2 TA cloning of PCR products

All reagents were supplied by Promega, UK. PCR products were cloned using the pGEM-Teasy vector system, which contains a linearised vector with single 3'-T overhangs. PCR products were modified by A-Tailing, as they were generated using a proofreading polymerase. A-Tailing produces complementary 'sticky ends' on the vector and insert, facilitating ligation. The cloning site of the pGEM-Teasy vector interrupts the *lac*Z gene, which codes for β-galactosidase. Therefore recombinant clones can be detected by colour screening on IPTG/X-gal plates. IPTG is a lactose analog that induces transcription of the lacZ gene and X-gal is a β-galactosidase substrate. Clones that do not contain PCR products will form blue colonies, as X-gal is cleaved by β-galactosidase forming a blue product. Clones that contain PCR products, in most cases, produce white colonies as the coding sequence of β-galactosidase is interrupted, so X-gal cannot be cleaved. However recombinant clones can produce blue colonies if the PCR product is cloned in-frame with the *lacZ* gene.

A-Tailing was achieved by adding 1μl Taq DNA polymerase to 10μl PCR product. The mix was incubated at 72°C for 15 mins. A-tailed PCR products were ligated into pGEM-Teasy at 1:1 and 3:1 molar ratios of vector to insert, using 50ng vector. Ligation reactions (10μl per reaction) were set up containing 5μl 2x Rapid Ligation Buffer, 1μl (50ng) pGEM-Teasy vector, 1μl T4 DNA ligase, appropriate amount of insert DNA and molecular biology grade water to 10μl. The mixes were incubated for 1 hour at room temperature, and then stored at –20°C.

The ligation reactions were used to transform JM109 High Efficiency Competent Cells, via heat shock, following the manufacturer's instructions. IPTG/X-gal plates were made by spreading 25μl 40mg/ml X-gal and 33.3μl 300mM IPTG onto the surface of LB/ampicillin plates and allowed to absorb for 30 mins at 37°C.

Following incubation of the plates overnight at 37°C, white colonies were picked with a pipette tip and grown up overnight in 2ml LB medium supplemented with 100μg/ml ampicillin, in a shaking incubator at 200rpm and 37°C. Plasmid DNA was isolated from the cultures using a Wizard Plus SV Miniprep kit. Plasmid DNA was digested with restriction endonucleases at specific sites (see 2.7.3) to determine if the correct

insert was present. Plasmid DNA was then sequenced (MWG, Germany) to confirm that the sequence of the insert was correct.

2.7.3 Restriction Endonuclease Digestion

DNA digests with specific restriction endonucleases (New England Biolabs, UK), which cleave DNA at a specific sequence, were performed to determine if clones contain the correct insert, in the correct orientation. DNA digests were also performed to linearise vectors and prepare inserts. Typically, single enzyme digests were set up in a 15µl reaction volume, containing 1µl (approximately 10 units) restriction enzyme, 1.5µl enzyme buffer, 5µl DNA (1-2µg) and 7.5µl molecular biology grade water. Double enzyme digests were set up in a 25µl reaction volume, containing 1µl (10 units) each enzyme, 2.5µl appropriate buffer, 5µl (1-2µg) DNA and 15.5µl molecular biology grade water. Reactions were incubated overnight at the temperature for optimum activity of the restriction enzyme. Products resulting from DNA digests were visualised by agarose gel electrophoresis (see 2.6.5).

2.7.4 Phenol/Chloroform extraction and ethanol precipitation of DNA

An equal volume of phenol/chloroform was added to the DNA. The solution was vortexed and the phases separated by centrifugation for 2 mins at 13,000rpm in a microfuge (MSE Microcentaur, Sanyo). The top aqueous phase was removed (avoiding the interphase) and placed in a fresh tube. An equal volume of chloroform was added and the solution vortexed and centrifuged for 2 mins at 13,000rpm. The top phase was taken and the DNA precipitated by ethanol precipitation: 2.5 times the volume of ethanol and 10% of the volume of 3M sodium acetate, pH 5, were added. The sample was vortexed and placed at -20°C for 30 mins. The sample was then spun for 10 mins at 12,000rpm at 4°C. The supernatant was removed and the DNA pellet resuspended in 100µl 70% (v/v) ethanol. The tube was spun for 10 mins at 12,000rpm at 4°C and the supernatant removed. The pellet was left to air dry and then resuspended in an appropriate volume of molecular biology grade water.

2.7.5 Blunt ending DNA

Blunt ending was required in some instances to introduce an insert into a vector with incompatible 'sticky-ends'. All reagents used were supplied by Promega, UK. Blunt

ending DNA removes 5' protruding termini using the Klenow subunit of DNA polymerase I from E. coli. 25µl reaction mixes were set up containing: 20µl DNA (40µg DNA which has been previously purified by phenol/chloroform extraction and ethanol precipitation), 1µl Klenow subunit, 2.5µl 10x Klenow buffer, 0.1µl 10mM dNTPs, 0.2µl 10mg/ml acetylated BSA and 1.35µl molecular biology grade water. Mixes were incubated at room temperature for 10 mins and the reaction was terminated by incubating at 75°C for 10 mins.

2.7.6 Vector dephosphorylation

Vector DNA that was linearised by restriction endonuclease digestion was dephosphorylated to prevent vector religation. Digested vector DNA was ethanol precipitated (see *2.7.4*) and resuspended in 80µl molecular biology grade water. 10µl (10 units) shrimp alkaline phosphatase (Roche Molecular Biochemicals, UK) and 10µl 10x buffer were added. The reaction was incubated at 37°C for 20 mins and 65°C for 15 mins to denature the enzyme.

2.7.7 Gel purification of DNA fragments

DNA fragments, obtained by restriction digestion, were separated by agarose gel electrophoresis and then gel purified. DNA bands were visualized under UVB light and bands cut out of the gel using a scalpel. The mass of the gel fragment was recorded and a Qiaex II Gel Extraction kit (Qiagen, UK) was used, following the manufacturer's instructions.

2.7.8 DNA ligation

For ligations other than using the pGEM-Teasy system, DNA fragments were ligated into linearised and dephosphorylated vectors using T4 DNA ligase (Promega, UK). 1:1 and 3:1 molar ratios of vector to insert were used, using 50ng vector. Ligation reactions (10µl reaction volume) were set up containing 1µl T4 DNA ligase, 1µl 10x Ligase Buffer, 50ng vector, appropriate amount of insert DNA, molecular biology grade water to 10µl. Reactions were incubated in a 16°C waterbath overnight.

2.7.9 Transformation into competent bacteria

Ligation reactions were used to transform DH5α Subcloning Efficiency Competent Cells (Life Technonogies, UK). Competent cells were removed from the -80°C

freezer and thawed on ice. 50µl cells were aliquoted into chilled 10ml Falcon tubes and 1µl of the DNA ligation reaction was added to the cells. The cells were mixed by gently tapping the tube and were incubated on ice for 30 mins. The cells were then heat-shocked for 45 sec at 37°C and returned to the ice for 2 mins. 1ml LB medium was added to each tube and the samples placed in a shaking incubator at 37°C, 200rpm for 1 hour, for expression. 100µl of the bacterial broth was spread onto LB/ampicillin plates and incubated overnight at 37°C.

2.7.10 Small scale plasmid preparations (minipreps)

Individual colonies obtained from section 2.7.9 were picked with a pipette tip and cultured overnight in 2ml LB medium supplemented with 100µg/ml ampicillin, in a shaking incubator at 200rpm and 37°C. The culture was transferred to a microfuge tube and centrifuged at 13,000rpm for 5 mins in a microfuge. The supernatant was removed and the pellet resuspended in 100µl miniprep lysis buffer by vortexing. The pellet was incubated at room temperature for 5 mins. 200µl 0.2M NaOH, 1% SDS was added and the solution mixed. 150µl 3M sodium acetate pH5 was added and the tubes inverted to mix. The samples were incubated at -20°C for 8 mins and then cell debris was spun down at 13,000rpm for 15 mins in a microfuge. The supernatant was transferred to a fresh tube and 0.9ml 95% ethanol added. The tubes were inverted to mix and the plasmid DNA and RNA was centrifuged at 13,000rpm for 3 mins. The supernatant was removed and the pellet washed in 1ml ethanol. The pellet was air dried and resuspended in 60µl molecular biology grade water containing 0.6mg/ml Ribonuclease A (Sigma, UK).

2.7.11 Medium scale plasmid preparations (midipreps)

Clones of interest were picked with a pipette tip and grown up overnight in 100ml LB medium supplemented with 100µg/ml ampicillin, in a shaking incubator at 200rpm and 37°C. Plasmid DNA was isolated using a Wizard Plus SV Midiprep kit (Promega, UK), following the manufacturer's instructions.

2.7.12 Large scale, caesium chloride plasmid preparations (maxipreps)

Colonies containing the correct cloned plasmid were grown for 8 hours in 5ml LB medium supplemented with 100μg/ml ampicillin, in a shaking incubator at 200rpm and 37°C. This starter culture was added to 500ml LB medium supplemented with 100μg/ml ampicillin, and placed in a shaking incubator at 200rpm and 37°C overnight.

Cells were centrifuged at 6,200rpm (RC5C Sorvall centrifuge, F-16/250 rotor) for 10 mins, the supernatant was discarded and the pellet resuspended in 20ml maxiprep lysis buffer. A small spatula of lysozyme was added, the solution mixed and incubated at room temperature for 5 mins. 40ml 0.2M NaOH, 1% SDS was added and the solution swirled to mix and then centrifuged at 7,000rpm for 20 mins (RC5C Sorvall centrifuge, F-16/250 rotor). The supernatant was filtered through muslin into a clean centrifuge tube and 50ml propan-2-ol added. The solution was spun at 7,000rpm for 20 mins (RC5C Sorvall centrifuge, F-16/250 rotor) and the supernatant discarded. The pellet was left to air dry for approximately 1 hour. The pellet was resuspended in 20ml water and 25g caesium chloride added.

When the caesium chloride had dissolved, 0.7ml 10mg/ml ethidium bromide was added and the tubes spun at 10,000rpm for 10 mins (RC5C Sorvall centrifuge, SS34 rotor). The supernatant was transferred to an ultracentrifuge tube, the tubes were balanced, and spun in an ultracentrifuge at 42,000rpm (OTD55B Sorvall ultracentrifuge, TFT 50.38 rotor) for 65 hours. The ethidium bromide-stained plasmid DNA forms a band, which was collected with a peristaltic pump. An equal volume of water-saturated butan-1-ol was added to the plasmid DNA and the solution mixed. The sample was spun at 2,000 rpm for 5 mins (RC5C Sorvall centrifuge, SS34 rotor). The upper phase was discarded and the process repeated twice more. An equal volume of water was added to the DNA plus twice the total volume of ethanol. The tubes were incubated at -20°C for 1 hour and the DNA spun down at 10,000rpm for 10 mins (RC5C Sorvall centrifuge, SS34 rotor). 7ml 70% ethanol was added and the tubes spun again at 10,000rpm for 10 mins (RC5C Sorvall centrifuge, SS34 rotor). The supernatant was discarded and the pellet air-dried. The plasmid DNA was resuspended in molecular biology grade water at the desired concentration.

2.7.13 Site-directed mutagenesis

Specific mutations were made in plasmids using a Quikchange Site-Directed Mutagenesis Kit (Stratagene, UK), following the manufacturer's instructions. Briefly, two primers are synthesised containing the desired mutation, each complementary to opposite strands of the plasmid to be mutated. A PCR reaction is set up which denatures the plasmid and anneals the mutagenic primers. The mutagenic primers are incorporated into the plasmid using a high fidelity DNA polymerase which does not displace the primers, resulting in many copies of the mutated nicked plasmid. To remove the parental plasmid, a digestion with Dpn I is performed. Dpn I only digests methylated DNA, therefore it digests the parental DNA but leaves the newly synthesized, unmethylated, mutated plasmid intact. The mutated plasmid is used to transform competent bacteria, which repair nicks in the mutated plasmid. Clones were sequenced (MWG, Germany) to determine if they contain the correct mutation.

Primers used for mutagenesis are as follows:

Bclx1B MUT (sense primer): tac cgg gcc g**tt** gaa ggg gg**t** tgt ggc ccc

(antisense primer): ggg gcc aca **a**cc ccc ttc a**a**c ggc ccg gta

Bclx1A MUT1 (sense primer): ccc tcg atc cgg gcg **t**tg gag gag gaa gca agc

(antisense primer): gct tgc ttc ctc ctc ca**a** cgc ccg gat cga ggg

Bclx1A MUT2 (sense primer): gcc tgc cgg gtc gc**t** tga tcc ctc cgg cc

(antisense primer): ggc cgg agg gat ca**a** gcg acc cgg cag gc

Mutations are shown in bold.

2.7.14 Plasmid constructs

The pGL2 promoter, pGL2 basic plasmids and Renilla luciferase plasmid were obtained from Promega (UK). The human Bcl-X promoter luciferase reporter constructs (promoter 1B, promoter 1A, promoter 2 and promoter 1A-2) were generated by L. Wood and F.Habens, Southampton, UK (MacCarthy-Morrogh *et al* 2000). The mouse Bcl-X 3.2kb genomic region luciferase reporter construct was a kind gift of G. Nunez (Grillot *et al* 1997). The IκB-alpha promoter luciferase reporter

construct was a kind gift of D. Mann, Southampton, UK (Mann *et al* 2002). The β-galactosidase plasmid was a kind gift of J. Blaydes, Southampton, UK.

Plasmids TOPO AS1/2, TOPO AS1, TOPO AS2 and TOPO noAS were generated by L. Wood, Southampton, UK. cDNA from a T cell lymphoma cell line (HUT78) was amplified by PCR using primers complementary to a region in exon IB and a region in exon III of the Bcl-X gene. Multiple bands were visualized and these were TA cloned into pCRII-TOPO (Invitrogen, UK). TOPO AS1/2 contains a 130bp alternatively spliced region at the 3' end of exon IB (defined as alternatively spliced 1 or AS1) and an 18bp alternatively spliced region at the 5' end of exon II (AS2). TOPO AS1 contains AS1 but not AS2, TOPO AS2 contains AS2 only and TOPO noAS contains neither of the alternatively spliced regions.

Plasmids IB AS1/2, IB AS2 and IB noAS were derived from plasmids TOPO AS1/2, TOPO AS2 and TOPO noAS respectively, and contain the same Bcl-X sequences upstream of the luciferase gene. The Bcl-XIB cloning primers (see 2.7.1) were used to generate DNA fragments encompassing exon IB (missing 52bp at the 5' end) through to the non-coding region of exon II. The sense primer contained a HindIII site at the 5' end and the antisense primer a BstBI site at the 5' end. The PCR products were TA cloned into pGEM-Teasy. Midipreps of plasmids with the correct sequence were generated and the inserts subcloned into pGL2-promoter vector (Promega, UK) downstream of the SV40 promoter and upstream of the luciferase coding sequence in the HindIII site. This was achieved by digesting pGL2-promoter with HindIII and digesting the midipreps with HindIII and BstBI. 5' protruding termini were removed from the linearised vector and fragments by blunt ending, and the inserts were gel purified. The vector was dephosphorylated and the vector and inserts were ligated together and used to transform competent bacteria. Clones containing the correct plasmid, as determined by restriction endonuclease digestion, were grown up for large scale DNA preparation.

Plasmids Lucif AS1/2mut was generated by site-directed mutagenesis of plasmids Lucif AS1/2 using the BclxIB MUT mutagenesis primers.

Plasmid IA contains the non-coding exon IA region of the Bcl-X gene upstream of the luciferase gene. cDNA from Elijah cells treated with CD40L was amplified by PCR using the Bcl-X1A cloning primers (see 2.4.1) complementary to the 5' end of exon IA and the non-coding region of exon II of the Bcl-X gene. The primers contained a HindIII site at their respective 5' ends. The resulting PCR fragment was TA cloned into pGEM-Teasy. Midipreps of plasmids with the correct sequence were generated and the inserts subcloned into pGL2-promoter vector (Promega, UK) downstream of the SV40 promoter and upstream of the luciferase coding sequence in the HindIII site. This was achieved by digesting pGL2-promoter and the midipreps with HindIII. The inserts were gel purified and the vector was dephosphorylated. The vector and inserts were ligated together and and used to transform competent bacteria. Clones containing the correct plasmid, as determined by restriction endonuclease digestion, were cultured for large scale DNA preparation.

Plasmid IAmut was generated by site-directed mutagenesis of plasmid IA in two stages. Firstly site-directed mutagenesis was performed using the BclxIA MUT1 primers. Two clones from this procedure were then subjected to a second round of site-directed mutagenesis using the BclxIA MUT2 primers. Clones were then sequenced to verify the presence of both of the mutations.

2.8 DNA transfection and analysis of reporter gene expression

2.8.1 Electroporation

DNA was introduced into DG75, Akata 6 or RL cells by electroporation. 8 x 10^6 cells were used per test. The cells were washed in serum free media (retaining the original medium as conditioned media) and resuspended in 250µl serum free media per test. Construct DNA and 2µg β-galactosidase expression plasmid DNA were added to 0.4cm electroporation cuvettes (Equibio, UK) and the cells added. Cells were electroporated at 900µF, 250V using a Easyject Plus (Equibio, UK). Cells were immediately transferred to 8ml warmed media, comprising half fresh media and half conditioned media. Cells were placed in a humidified incubator at 37°C and 5% $C0_2$ for 24 hours.

Modifications to the above electroporation method were made in order to optimize transfection WEHI-231 cells. In particular, the number of cells and volume of serum free media per test and the capacitance and voltage of the pulse were varied. Tests were also carried out using 2 x 10^7 cells in 400µl cytomix per test.

2.8.2 Lipid-based transfection

293 cells were transfected using Fugene 6 Transfection Reagent (Roche Molecular Biochemicals, UK). Cells were plated at 2 x 10^5 cells per well, in a 6 well plate, overnight. The next day the media was changed 1 hour before the experiment and the cells transfected following the manufacturer's instructions. Cells were placed in a humidified incubator at 37°C and 5% $C0_2$ for 24 hours and the media was changed 4-5 hours following transfection.

Effectene Transfection Reagent (Qiagen, UK) was used to try to transfect WEHI-231 cells. Cells were cultured at 3.5 x 10^6 cells per well, in 1.6ml in a 6 well plate. The manufacturer's instructions were then followed. Cells were placed in a humidified incubator at 37°C and 5% $C0_2$ for 48 hours.

2.8.3 DEAE-dextran

RL cells were transfected using DEAE-dextran. 10 x 10^6 cells were used per test. Cells were washed twice in PBS-0.5mM EDTA and then washed once in TS. Cells were then resuspended in 1ml TS plus 0.25mg/ml DEAE-dextran (Amersham, UK). DNA was added (5-40µg) and the cells incubated at room temperature for 30 mins. Following incubation, 10ml cell culture media was added and the cells incubated at 37°C for a further 30 mins. Cells were then spun down and resuspended in 10ml warmed media. Cells were placed in a humidified incubator at 37°C and 5% $C0_2$ for 24 - 48 hours.

A DMSO shock was included in some experiments. After the 30 min incubation at 37°C, cells were spun down and resuspended in 15ml 10% (v/v) DMSO in PBS. Cells were incubated at room temperature for 2 mins, then washed in media and resuspended in 10ml warmed media. Cells were placed in a humidified incubator at 37°C and 5% $C0_2$ for 24 - 48 hours.

2.8.4 Reporter gene assays

Transfected cells were washed once in PBS and resuspended in 100µl lysis buffer. Cells were incubated for 2 mins at room temperature and cell debris was spun down by centrifugation at 12,000rpm for 5 mins in a microfuge. The luciferase assay was set up in a white 96 well OptiPlates (Packard BioScience, UK), with each well containing 30µl PBS (supplemented with 1mM $MgCl_2$ and 1mM $CaCl_2$), 20µl lysate and 100µl Luclite Plus substrate (Packard BioScience, UK). All samples were assayed in duplicate. Luminescence was detected using a TopCount microplate counter.

The β-galactosidase assay was set up in a 96 well plate, with each well containing 40µl lysis buffer, 10µl lysate and 50µl ONPG substrate. All samples were assayed in duplicate. The plate was incubated at 37°C until a yellow colour develops, which is the product of ONPG cleavage by β-galactosidase. The absorbance was then measured at 405nm using an ELISA plate reader. The β-galactosidase assay measures transfection efficiency so a value for the relative luciferase activity can be calculated by dividing the luciferase result by the β-galactosidase result.

2.8.5 Statistics

Transfection results were analysed using the SPSS statistics package. An independent samples t-test, with equal variances not assumed, was used as the data were unpaired and normally distributed (as determined by the Kolmogorov-Smirnov test for normality).

2.9 In vitro transcription and translation

In vitro transcription and translation reactions were performed using TNT Coupled Reticulocyte Lysate Systems (Promega, UK). Each reaction was set up (10µl reaction volume) containing 5µl rabbit reticulocyte lysate, 0.4µl TNT buffer, 0.2µl amino acid mixture minus methionine (1mM), 0.2µl RNAsin ribonuclease inhibitor (40u/µl), 400ng DNA template, 0.4µl [^{35}S] methionine (> 1000 Ci/mmol at 10mCi/ml, Amersham Pharmacia Biotech, UK), 0.2µl T7 RNA polymerase and molecular biology grade water to 10µl. Multiple reaction mixes were set up, minus the DNA, aliquoted and the template DNA added. Luciferase DNA was used as a positive control and pcDNA was used as a negative control. Reaction mixes were incubated at 30°C for 90 mins.

Following, incubation, 3x SDS sample buffer, supplemented with 0.1M DTT, (New England Biolabs, UK) was added to the sample. The samples were then heated to 95°C for 5 mins and 15µl separated by SDS-PAGE (see 2.7.2). The gel was then covered in gel fixing solution and slowly agitated for 3 x 15 min. The gel was then placed on a piece of Whatman 3MM chromatography paper (Merck, UK), covered with plastic wrap and dried at 80°C for 1 hour under a vacuum, using a gel dryer. The gel was exposed on a phosphorimaging screen (Fuji photo film, Japan) overnight and bands were detected and quantified using a molecular imager (BioRad, UK).

2.10 Immunoblotting (Western blotting)

2.10.1 Preparation of cell lysates

Total cellular protein was extracted from cell line pellets by detergent lysis in RIPA buffer supplemented with 1% (v/v) mammalian protease inhibitor cocktail (Sigma, UK) on the day of use. Cell lysates were incubated on ice for 30 mins and then clarified by centrifugation at 12,000rpm (Sigma 4K10 centrifuge) for 20 mins at 4°C. Protein Assay Reagent (BioRad, UK) was used to determine the protein content of the supernatant, using bovine serum albumin (Sigma, UK) as standard. The protein extracts were normalised so each sample contained 20µg protein/15µl sample, and 3x SDS sample buffer supplemented with 0.1M DTT (New England Biolabs, UK) was added. The samples were then heated to 95°C for 5 mins prior to loading on a SDS-polyacrylamide gel.

Primary B-cell pellets were solubilised in 1x sample buffer, with 0.1M DTT added on the day of use at a concentration of 5 x 10^5 cells per 15µl. Samples were sonicated and heated to 95°C for 5 mins prior to loading on a SDS-polyacrylamide gel.

2.10.2 SDS-PAGE and protein transfer

Pre-cast SDS-polyacrylamide gels (4-15% gradient ready gels, Bio-Rad, UK). were used. Cell lysates were separated by SDS-polyacrylamide gel electrophoresis using a Mini-Protean 3 Cell (BioRad, UK) in protein running buffer at 200V. Proteins were transferred onto nitrocellulose membranes (Protran, Schleicher & Schuell, Germany) in protein running buffer with 25% v/v ethanol in a Mini-Protean II transfer

unit (BioRad, UK) at 100V for 1 hour. For detection with phospho-specific antibodies, proteins were transferred onto Hybond C+ membranes (Amersham, UK).

2.10.3 Probing with antibodies

After transfer, the membranes were blocked by incubation with 5% (w/v) non-fat milk solution in TS for 1 hour at room temperature. Membranes were then incubated overnight at 4°C with one of the antibodies listed in table 2.4 in the appropriate buffer plus 5% non-fat milk powder. The membranes were then washed 3 x 5min in the appropriate buffer and incubated for 1 hour at room temperature with the appropriate HRP-conjugated secondary antibody (Amersham, UK) in the appropriate buffer plus 5% non-fat milk powder. In the case of the Bcl-2 antibody, a HRP-conjugated tertiary antibody was required. After incubation with the secondary antibody, the membrane was washed 3 x 5 min in TS and then incubated for 1 hour at room temperature with the tertiary antibody in TS plus 5% non-fat milk solution.

For detection with phospho-specific antibodies, 5% BSA was used instead of 5% non-fat milk in the blocking and incubation steps.

The membranes were then washed 3 x 5min in the appropriate buffer and bound immunocomplexes detected using Supersignal West Pico Chemiluminescent Substrate (Pierce, UK) according to the manufacturer's instructions. Bands were detected and quantified using a chemiluminescent imager (Fluor-S max, BioRad, UK) and Quantity One quantitation software (BioRad, UK).

2.11 Antisense oligonucleotides

20-mer 2'-O-methoxyethyl chimeric antisense oligonucleotides were obtained from Isis Pharmaceuticals, USA, to inhibit Bcl-X_L and Mcl-1 expression. The oligonucleotides contain a phosphorothioate-modified DNA backbone with methoxyethyl substitutions at the 2'-sugar positions in the first five and last five nucleotides (fig. 2.1). The phosphorathioate backbone brings about degradation of hybridized mRNA by RNase H cleavage (endonuclease which selectively cleaves the

Table 2.4: Antibodies used for Western Blotting.

Antibody:	Obtained from:	Dilution used	Secondary antibody (dilution)	Buffer used:	Approx. Mw (kDa)
β-tubulin	Sigma (clone 2.1)	1:500	Mouse-HRP (1:3000)	TST	55
A20	Active Motif (59A426)	1:200	Mouse-HRP (1:2000)	PBST	80
Actin	Sigma (20-33)	1: 500	Rabbit-HRP (1:5000)	TST	42
Bak	Santa Cruz (clone N-20)	1:400	Goat-HRP (1:2000)	TS	25
Bax	Santa Cruz (clone N-20)	1:400	Rabbit-HRP (1:5000)	TS	21
Bcl-2	Pharmingen (clone 6C8)	1:400	Rabbit anti-hamster (1:1000). Tertiary antibody = rabbit -HRP (1:5000)	TS	26
Bcl-X_L	R&D Systems (AF800)	1:1000	Rabbit-HRP (1:5000)	TS	30
BNIP3	Santa Cruz (clone C-18)	1:500	Goat-HRP (1:2000)	TS	60
Caspase-8	BD Pharmingen (clone 3-1-9)	1:1000	Mouse-HRP (1:2000)	BD buffer	50/55 – proform 40/36/23 – cleaved forms
Cathepsin L	AbCam (33/2)	1:200	Mouse-HRP (1:5000)	TS	40 25
Daxx	Santa Cruz (clone M-112)	1:200	Rabbit-HRP (1:5000)	TS	120
FADD	BD Transduction Labs (clone 1)	1:1000	Mouse-HRP (1:5000)	BD buffer	24
$FLIP_L$	Upstate (06-864)	1:1000	Rabbit-HRP (1:5000)	PBST	55
Mcl-1	Santa Cruz (clone S-19)	1:400	Rabbit-HRP (1:5000)	TS	42
PARP	R&D Systems (C2.10)	1:1000	Mouse-HRP (1:2000)	PBST	116 - uncleaved 84 - cleaved
PCNA	Xin Lu, Imperial College, London (PC10 ascites)	1:1000	Mouse-HRP (1:5000)	TS	36
Phospho-Akt (ser473)	Cell signaling (9271)	1:1000	Rabbit-HRP (1:5000)	TST	60
Phospho-p44/42 MAPK (Thr202/Tyr 204)	Cell signaling (910I)	1:1000	Rabbit-HRP (1:5000)	TST	42/44

Phospho-SAPK/JNK (Thr183/Tyr 185)	Cell signaling (9251)	1:1000	Rabbit-HRP (1:5000)	TST	54/46
Phospho-p38 MAPK (Thr180/Tyr 182)	Cell signaling (9211)	1:1000	Rabbit-HRP (1:5000)	TST	38
Survivin	Novus Biologicals (clone 60.11)	1:1000	Mouse-HRP (1:5000)	TST	16

RNA strand of a RNA:DNA duplex), whereas the methoxyethyl modification prevents nucleases cleaving the oligonucleotide itself (Dean & Giffey 1997). ISIS Bcl-X_L antisense oligonucleotides have previously been shown to specifically inhibit Bcl-X_L expression in normal keratinocytes, lung carcinoma and melanoma cells, sensitising these cells to apoptosis (Taylor *et al* 1999, Heere-Ress *et al* 2002). The Mcl-1 antisense oligonucleotide was also shown to specifically ablate Mcl-1 expression in melanoma and endothelial cells (Derenne *et al* 2002, Bannerman *et al* 2001). The various antisense oligonucleotides used and their sequences are shown in table 2.5

Table 2.5: Properties of antisense oligonucleotides obtained from ISIS Pharmaceuticals

ISIS ID	Target	Sequence
15999	Bcl-X_L	tcc cgg ttg ctc tga gac at
16011	Bcl-X_L	ctg gat cca agg ctc tag gt
129688	Orphan. Control for Bcl-X_L.	ttc gcg gct gga cga ttc ag
20408	Mcl-1	ttg gct ttg tgt cct tgg cg
105232	Mcl-1 8 base mismatch. Control for Mcl-1.	tgg gtc tgg ttt ctc tgt cg

Cells were electroporated with 50μM antisense, as in section 2.8.1, except 6 x 10^6 cells were used per test, in 360μl serum-free media. Cells were harvested at various time points, washed in PBS and snap frozen. Protein expression and PARP cleavage was detected by Western blot.

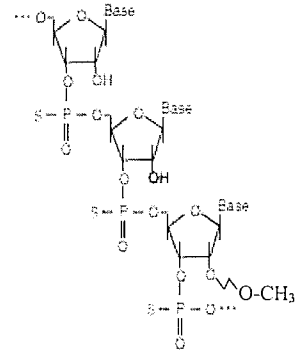


Fig 2.1: Structure of antisense oligonucleotides. Oligonucleotides used were phosphorothioate-modified, characterized by the introduction of sulphur into the DNA backbone. The first and last five nucleotides were modified by the addition of a methoxyethyl group on the 2' sugar position. This is illustrated on the last nucleotide in the figure. Figure modified from http://www.biognostik.com/ODN.

2.12 Microarrays

2.12.1 Reverse transcription

Total RNA was isolated using Trizol and assessed for integrity by denaturing gel elecrophoresis (as detailed in sections 2.6.1 and 2.6.2). 30µg RNA for each probe was added to 8µg oligo (dT_{15}) (Promega, UK) and the total volume made up to 48µl with molecular biology grade water. This was then heated to 70°C for 5 mins and cooled on ice. The following mix was then added to each sample: 20µl RT buffer (Roche, UK), 10µl 0.1M DTT (Roche, UK), 4µl 25mM dNTP mix (Amersham, UK), 2µl RNase inhibitor (Promega, UK) and 12µl molecular biology grade water. 2µl Superscript II (Invitrogen, UK) was then added and the samples incubated at 42°C for 4 hours, adding a further 2µl Superscript II after 1 hour and 1µl of Superscript II after 2 hours.

Following the incubation, 20 units of RNase A (Invitrogen, UK) were added and the samples incubated at 37°C for 20 mins. The samples were then passed through a Qiaquick PCR cleanup column (Qiagen, UK), following the manufacturer's instructions and eluting the product with 50µl water, and stored at -20°C. The cDNA obtained was then visualised by agarose gel electrophoresis (see section 2.6.5) to determine if the reverse transcription was successful.

2.12.2 Klenow labeling

Unless stated otherwise, all reagents were from the Bioprime labeling Kit (Invitrogen, UK). 20µl of Random primer/reaction buffer mix was added to 20µl of the cDNA obtained in section *2.12.1.* The samples were then incubated at 100°C for 5 mins and cooled on ice. While on ice the following were added to each sample: 5µl low dCTP mix, 3µl of either Cy5-dCTP or Cy3-dCTP (Amersham, UK), and 1.5µl Klenow fragment. The samples were then incubated at 37°C for 4 hours, in the dark. The reaction was stopped with the addition of 5µl 0.5M EDTA (pH8.0). The sample was then passed down a G50 sephadex column (Amersham, UK) to remove the unincorporated nucleotides, following the manufacturer's instructions. The contents of the Cy3 and Cy5 tubes were combined, 10µg Cot1 DNA (Invitrogen,UK) and 1µl 0.5M EDTA (pH 8.0) were added. The probe was then evaporated in a speedvac until

the volume was reduced to 10µl. The probe was resuspended in water to a final volume of 12.5µl and stored overnight at -20°C.

2.12.3 Hybridisation

37.5µl of microarray hybridisation solution (filtered prior to use) was added to the probe and incubated at 85°C for 5 minutes. The probe was then incubated at 42°C for 1 hour. At the same time, each microarray slide (obtained from Human Genome Mapping Project (HGMP), Harwell, UK) was placed in a slide cassette and incubated at 42°C for 30 minutes to 1 hour. The probe was spun at 13000rpm for 2 minutes in a microfuge (MSE Micrcentaur, Sanyo), to pellet any debris, and the probe was the applied to the microarray slide and a coverslip placed over the top. The microarray was then hybridized at 48°C in a waterbath overnight.

2.12.4 Post-hybridisation washes

Microarrays were firstly washed in a 2x SSC solution until the coverslip fell off and then were rapidly transferred to a 0.1x SSC, 0.1% SDS solution for 3 minutes with shaking. The slides were then rapidly transferred to a 0.1x SSC solution for 2 minutes with shaking and then dried by centrifugation at 800rpm for 5 minutes, in a Sorvall Legend RT centrifuge.

2.12.5 Scanning and analysis

Slides were scanned using a GenePix 4000B scanner and GenePix Pro 3.0 software (Axon, USA). Following scanning, spot registration was manually optimised in GenePix Pro 3.0, as suggested by the manufacturer. For each spot, the expression levels of the Cy3 and Cy5 probes was obtained by subtracting the median background intensity from the median spot intensity. The ratio of the median intensities for the Cy3 and Cy5 channels was then calculated for each spot using GenePix Pro 3.0 and imported into Acuity 3.0 (Axon, USA) for further analysis. Here, overall differences in the signal intensity of the Cy3 and Cy5 wavelengths were corrected for on a print-tip basis using Lowess (locally linear robust scatter plot smoother) normalization. This eliminates variations in Cy3 and Cy5 signal intensity due to different dye properties, differences in dye incorporation, differences in scanning of the two channels and spatial biases. Quality filtering of the spots was also undertaken removing values for spots which were flagged 'bad' or 'not found'.

2.12.6 Amersham CodeLink microarrays

10μg of Trizol-isolated RNA of good integrity (260/280 ratio = greater than 1.8) was sent to Amersham, UK, for hybridistion to their single-colour CodeLink microarrays. RNA samples were sent in duplicate and blinded. The raw data was sent back for analysis. Using Microsoft Excel, the mean of the duplicate normalised intensity values was calculated and a ratio of treated versus untreated intensity values were calculated. A one-tailed unpaired t-test (Microsoft Excel) was also performed on the data. Genes were selected according to the following criteria (i) plus or minus fold change of at least 4, (ii) $p< 0.05$, (iii) signal intensity more than 1% of the mean signal intensity value.

Chapter 3: CD40 promotes survival of B-cell lymphomas and influences expression of apoptosis regulators

3.1 Introduction

CD40 ligation can produce differential effects on cell survival and apoptosis in B-cell malignancies. In some settings activation of CD40 can rescue normal and malignant B-cells from apoptosis, however in other cellular settings CD40 activation can induce growth arrest and apoptosis (e.g. Kelly & Knox 1995, Tsubata *et al* 1993, Padmore *et al* 1997, Johnson *et al* 1993, Ghia *et al* 1998, Kitada *et al* 1999, Aldinucci *et al* 2003, Heath *et al* 1993, Funakoshi *et al* 1994). The mechanisms underlying this are unclear but may depend on the type of B-cell malignancy studied and the method or context of activating CD40. Most of the analyses of the effects of CD40 on cell survival have been carried out on cell lines and a systematic comparison of the outcome of CD40 signalling in various B-cell malignancies has not been performed. The first aim of this study was to address this problem by determining the effect of CD40 ligation on cell survival in freshly isolated B-cells from patients with various Non-Hodgkin's lymphomas, CLL or non-malignant lymph nodes. This is particularly important since there is considerable interest in utilizing agents that activate CD40 to treat lymphoma and other cancers, largely through stimulation of the immune system. This study could therefore inform the design of clinical trials, by indicating which B-cell malignancies might respond to CD40 therapy, and by determining if there is a danger of CD40 stimulation in the absence of immune killing.

Analysis of the cellular events involved in CD40 signalling have revealed that molecules which control apoptosis are CD40 targets and so regulation of these molecules by CD40 may be responsible for the effects of CD40 activation on cell survival. Previously identified CD40 targets include the Bcl-2 family molecules Bcl-X_L, Mcl-1 and Bfl-1, the anti-apoptotic zinc finger protein A20 and the caspase-inhibitor survivin (Lee *et al* 1999, Kitada *et al* 1999, Kuss *et al* 1999, Sarma *et al* 1999, Granziero *et al* 2001). However, analysis of these molecules has been restricted to a limited number of lymphoma cell lines and few studies have been carried out using primary material. The role of these molecules in CD40-mediated survival is not well understood, nor is their regulation by CD40 ligation in primary human

lymphoma cultures. Therefore the second aim of this study was to systematically determine the effect of CD40 ligation on the expression of these apoptosis regulators in a variety of primary B-cell malignancies. This will help to elucidate which CD40 target genes are key to the regulation of cell survival in real human tumours and may reveal suitable targets which can be exploited for cancer therapy.

Potential CD40 target genes have been identified from data generated from a study using DNA microarrays to characterise gene expression in diffuse large B-cell lymphomas (Alizadeh *et al* 2000). This included the pro-apoptotic Bcl-2 interacting protein BNIP3. Therefore, in addition to the previously reported CD40-target genes, the effect of CD40 activation on the expression of BNIP3 was also determined in primary B-cells.

Bcl-X_L was identified as a key CD40-target gene in both cell lines and primary lymphoma cells in this study, at both the transcriptional and post-transcriptional levels. Bcl-X_L is an important regulator of apoptosis that is critical for B-cell development and normal lymphoid cell survival (Motoyama *et al* 1995, Chao & Korsmeyer 1998). Overexpression of Bcl-X_L is associated with many malignancies, particularly those which are chemoresistant and have poor prognosis, and can promote tumour formation in mouse model systems (Naik *et al* 1996, Pena *et al* 1998). Therefore Bcl-X_L is an attractive target for cancer therapy and interfering with its function or regulation may induce or sensitise tumour cells to apoptosis. To further understand the mechanisms that control Bcl-X_L expression, the transcriptional and translational control of Bcl-X were investigated.

3.2 CD40 activation induces expression of apoptosis regulators in lymphoma cell lines

Initial experiments were performed using two Burkitt's lymphoma cell lines Akata 6 and Elijah, prior to experiments using primary material, in order to develop techniques to detect protein and mRNA expression of apoptosis regulators. Cells were treated with CD40L (1μg/ml) or left untreated as a control, for up to 24 hours. Protein expression of Bcl-X_L, Mcl-1 and BNIP3 were determined by western blot. mRNA expression of Bfl-1 and A20 were determined by semi-quantitative RT-PCR analysis,

as antibodies were not available for these proteins. In the semi-quantative RT-PCR experiments, care was taken to ensure the PCR reaction was in its linear phase, by modifying the number of PCR cycles until a band just above the level of detection was observed by agarose gel electrophoresis. mRNA expression of different Bcl-X transcripts were also determined. The results of these experiments are shown in fig. 3.1.

In Akata 6 cells CD40L treatment increased Bcl-X_L protein levels after 4 hours of stimulation and this was sustained over the course of the experiment, whereas there was no change in Bcl-X_L levels in control cells (fig. 3.1A). The increase in Bcl-X_L protein with CD40 ligation was fairly modest, since basal levels of Bcl-X_L in these cells was high. RT-PCRs specific for mRNAs generated from the Bcl-X IA and IB promoters were undertaken (see fig. 1.9 for Bcl-X gene and RNA structure) and revealed that exon IA-containing transcripts were rapidly increased by stimulation with CD40L (within 2 hours), whereas there was no change in the expression of Bcl-X exon IB-containing transcripts (fig, 3.1B). The two bands in the exon IB RT-PCRs correspond to alternatively spliced forms of exon IB, whereby a 130bp region in the 3' end of exon IB is spliced out in the smaller form. Mcl-1 protein levels were modestly downregulated in control Akata 6 cells over time whereas levels were sustained in CD40L treated cells during the first 8 hours of CD40 stimulation (fig. 3.1A). Similar to Bcl-X_L, Akata 6 cells also express constitutively high levels of Mcl-1. The levels of BNIP3 were unaffected by CD40L treatment. Bfl-1 and A20 mRNAs were rapidly induced in Akata 6 cells treated with CD40L within 2 hours of stimulation and the induction of Bfl-1 by CD40 was extremely strong (fig. 3.1B).

In Elijah cells, Bcl-X_L protein levels were also upregulated by CD40 ligation within 4-8 hours of stimulation (fig. 3.1C), and this may be due, at least in part, to increased transcription of exon IA containing mRNAs (fig. 3.1D). There was no consistent change in the expression of exon IB containing transcripts. Mcl-1 protein levels did not increase in CD40 stimulated cells and there was also no change in BNIP3 protein levels (fig. 3.1C). Interestingly, there was strong induction of A20 mRNA and a modest induction of Bfl-1 mRNA (fig. 3.1D), which is converse to the Akata 6 results.

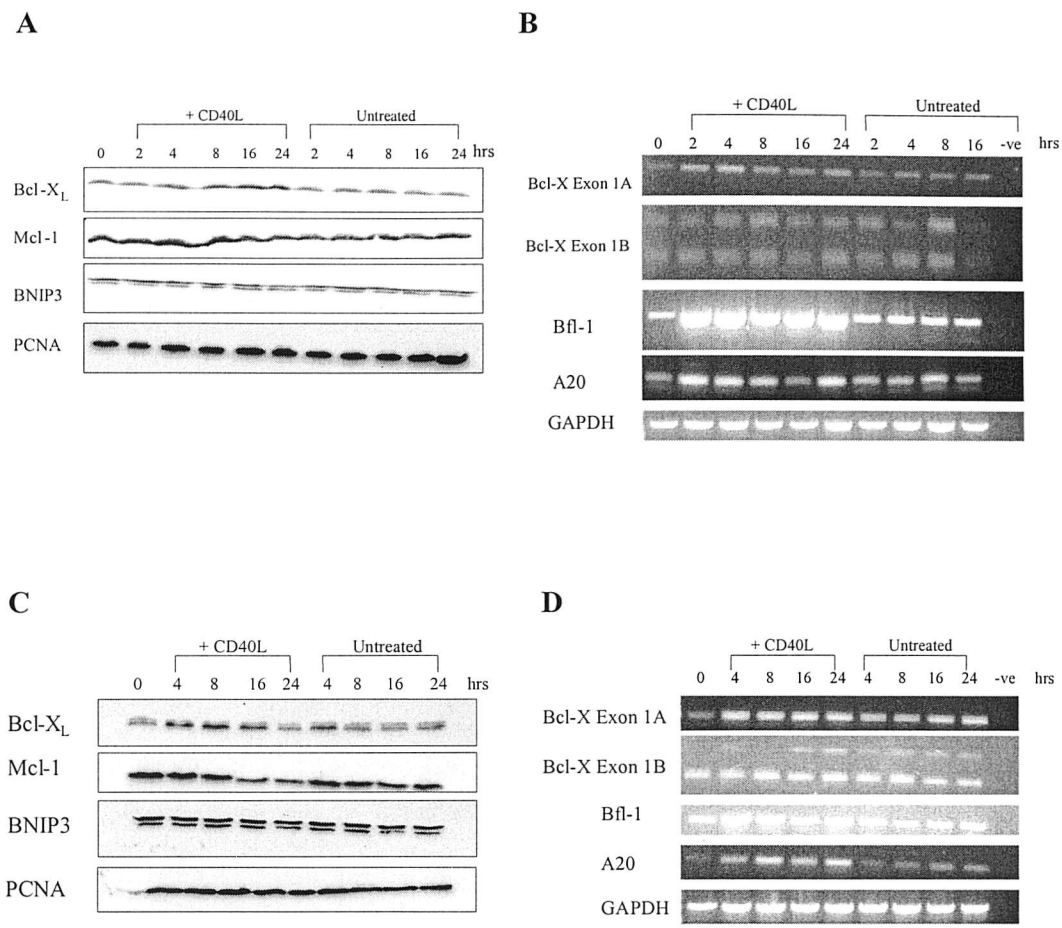


Figure 3.1: The effect of CD40L on expression of apoptosis regulators in Akata 6 (A and B) and Elijah (C and D) cells. The day before treatment, cells were diluted to a density of 0.3 x 10^6 cells/ml and cultured overnight. 24 hours later CD40L (1μg/ml) was added or cells were left untreated as a control. Pellets were taken at the time points indicated and snap frozen. **A and C** - For Western blot analysis, cells were lysed and 20μg of total protein was loaded per well. Proteins were transferred onto nitrocellulose membranes and probed with appropriate antibodies. Equal loading was demonstrated by re-probing one of the blots with an anti-PCNA antibody. **B and D** – Semi-quantitative RT-PCR analysis. Total RNA was isolated and reverse transcribed to cDNA using oligo dT primers. Specific cDNAs were amplified by PCR and visualised by agarose gel electrophoresis. GAPDH is shown to demonstrate equal amounts of cDNA were added to each PCR reaction. Water was added instead of cDNA as a control in the –ve lane.

3.3 CD40L rescues Akata 6 and Elijah cells from apoptosis

To determine the effect of CD40L treatment on apoptosis in Akata 6 and Elijah cells, cells were treated with combinations of CD40L and the apoptosis inducing agent etoposide. Apoptosis was assessed by detection of poly(ADP)ribose polymerase (PARP) cleavage by Western blot (fig. 3.2). PARP is a DNA repair enzyme, which is cleaved rapidly and quantitatively by caspase-3 during the onset of apoptosis. Native PARP has a molecular weight of 116kDa and cleavage results in fragments of approximately 85kDa and 31kDa (Gu *et al* 1995). In both Akata 6 and Elijah cells, treatment with 1μM or 2μM etoposide resulted in cleavage of most of the PARP detected in control cells. The addition of CD40L prevented the majority of PARP cleavage, indicating that CD40 activation could prevent most cells from undergoing etoposide-induced apoptosis. However one would not expect to observe complete rescue from apoptosis as the cells were treated concurrently with etoposide and CD40L. The survival response signalled by CD40 ligation would not be immediate as it involves the transcription and translation of genes. In Elijah cells there was a small amount of spontaneous apoptosis indicated by the presence of a weak band corresponding to 85kDa PARP in untreated cells. This band was not seen in cells treated with CD40L, showing that CD40L can rescue cells from spontaneous apoptosis.

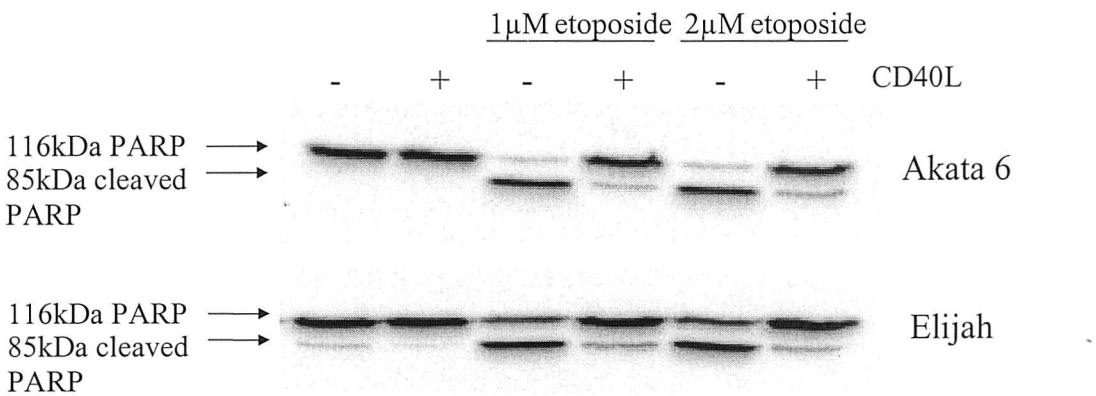


Figure 3.2: The effect of etoposide and CD40L on PARP cleavage. Cells were cultured for 48 hours alone, with 1μg/ml CD40L, with 1μM or 2μM etoposide, or with a combination of 1μM or 2μM etoposide and CD40L (1μg/ml). After 48 hours cells were pelleted and snap frozen. Cell pellets were lysed and and 20μg of total protein was analysed by Western blot.

Apoptosis was also assessed by Annexin V and PI staining. One of the earliest features of apoptosis is the movement of phosphatidylserine from the inner leaflet of the plasma membrane to the outer leaflet (Martin *et al* 1995). This triggers recognition and phagocytosis of the apoptotic cell. The mechanism for the movement of phosphatidylserine is not well understood but is associated with loss of aminophospholipid translocase activity, which moves phosphatidylserine from the outer to the inner leaflet, and is calcium-dependent (Bratton *et al* 1997, Bratton *et al* 1999). Annexin V binds to phosphatidylserine with high affinity, and when conjugated to fluorochromes can be used to identify apoptotic cells by flow cytometry. Cells are also stained with PI to assess membrane integrity. Cells negative for both stains are viable (lower left quadrant on fig 3.3), cells positive for annexin V and negative for PI are considered apoptotic (lower right quadrant) and cells positive for both annexin V and PI are considered necrotic or late apoptotic (upper right quadrant). Therefore cells dying by apoptosis will move from the lower left to the lower right and onto the upper right quadrant.

As shown in fig. 3.3, when both Akata 6 and Elijah cells were treated with etoposide a large amount of cell death occurred, although Akata 6 cells were more sensitive to the effects of etoposide than Elijah cells. Total cell death in etoposide-treated cells, calculated by adding together the percentage of cells in the upper right and lower right quadrants, was 74% in Akata 6 cells and 53% in Elijah cells. Treatment with both etoposide and CD40L improved cell survival (55% total cell death in Akata 6 and 22% total cell death in Elijah). Elijah cells showed a clear reduction in the percentage of both apoptotic and late apoptotic/necrotic cells when treated with CD40L (22% apoptotic in etoposide-treated cells compared to 5% apoptotic in etoposide plus CD40L-treated cells; 29% late apoptotic/necrotic in etoposide-treated cells compared to 17% in etoposide plus CD40L-treated cells). This clearly demonstrates that CD40 ligation reduces etoposide-mediated apoptosis of Elijah cells. Although a clear decrease in the percentage of late apoptotic/necrotic cells was observed in CD40L-treated Akata 6 cells (60% late apoptotic/necrotic etoposide-treated cells compared to 35% in etoposide plus CD40L-treated cells), there was actually a slight increase in cells present in the apoptotic quadrant (14% in etoposide-treated cells compared to 20% in etoposide plus CD40L-treated cells). This suggests that CD40L slows the rate of etoposide-induced apoptosis in Akata 6 cells, rather than completely preventing it.

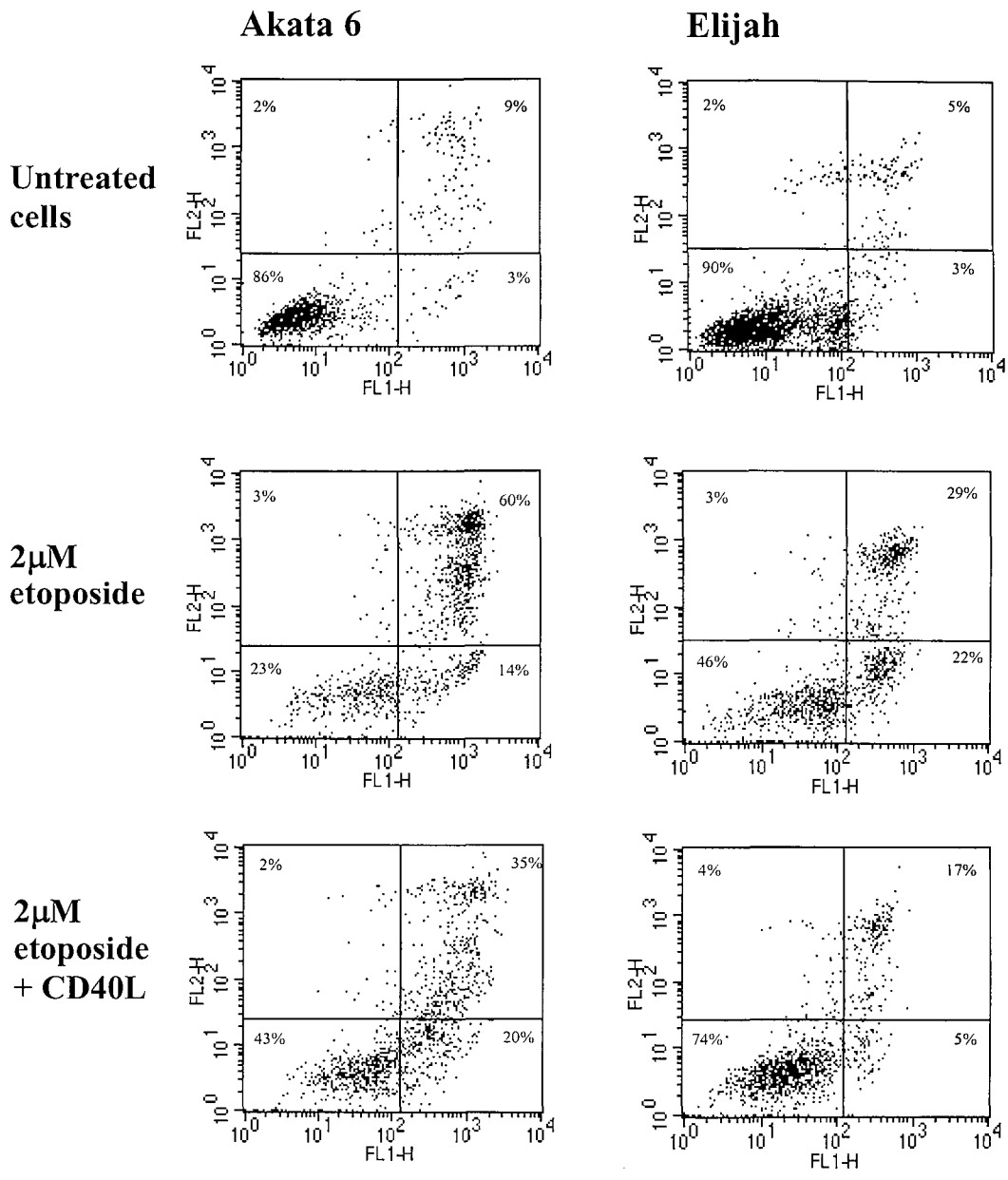


Figure 3.3: The effect of etoposide and CD40L treatment on Annexin V/PI staining in Akata 6 and Elijah cells. Cells were cultured for 48 hours alone, with 2μM etoposide or 2μM etoposide and 1μg/ml CD40L. After 48 hours cells were stained using Annexin V-FITC (x axis) and propidium iodide (y axis). Percentages indicate the percentage of cells in each quadrant. These results were representative of two experiments.

3.4 Isolation of B-cells from primary material

In order to study the effects of CD40 signalling on primary B-cells, a method was developed to isolate B-cells from lymph node biopsies, spleens or blood samples from patients with B-cell lymphomas or non-malignant lymph nodes (reactive nodes) (Dallman & Packham, in press). Lymph nodes or spleens, from Cancer Sciences Divison tumour bank, were homogenised to obtain a cell suspension, and then viable mononuclear cells were isolated from cell suspensions, or blood samples, using density centrifugation. Various methods were tested to purify B-cells from the mononuclear cell suspension. Positive cell selection was tested, whereby B-cells were labelled with anti-CD19 magnetic beads and passed through a column in a magnetic field. B-cells were then recovered from the column. However this procedure could only process small numbers of B-cells and the anti-CD19 magnetic beads attached to the B-cell surface may have interfered with subsequent signalling experiments. Due to the limitations of positive selection, a negative selection method was tested which gave much improved results. Negative selection was achieved by labelling the mononuclear cell suspension with a cocktail of antibodies against non B-cell markers e.g. CD4 (T helper cell marker) and CD16 (expressed on macrophages, neutrophils and natural killer cells), which were then coupled to magnetic beads. The sample was run down a column in a magnetic field. The non-B cells, which were labelled with the magnetic beads, remained in the column, whereas the B-cells passed untouched through the column.

To verify that a pure B-cell population was isolated, cell surface staining for the B-cell markers CD20 and CD40, and the T-cell marker CD3 was undertaken and the cells analysed by flow cytometry. Fig 3.4A shows the results from a patient with follicular lymphoma. All contaminating CD40/CD20 negative and CD3 positive T cells were removed by the purification procedure. The resulting pure B-cell population was representative of most of the other samples purified, as shown by the CD40 expression in table 3.1. Although this purification method resulted in a pure, untouched population of B-cells, the yields were fairly low and variable. Yields of pure B-cells ranged from approximately 5% to 50% of the starting cell suspension, which limited subsequent experiments that could be performed.

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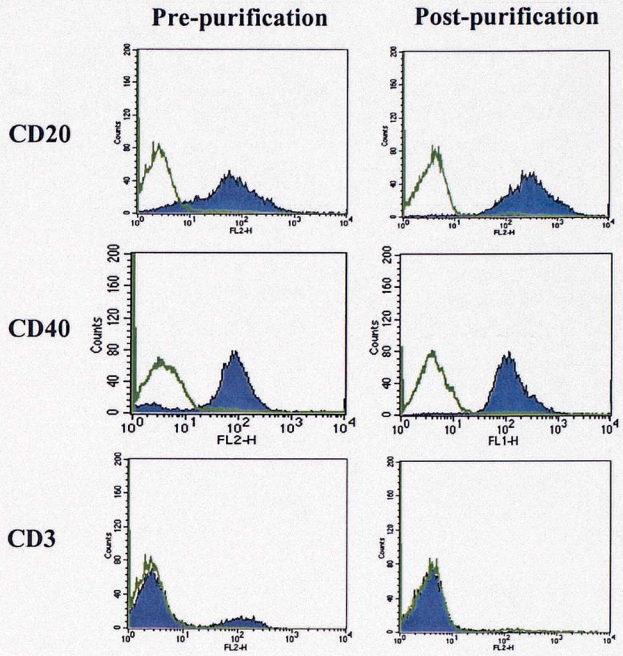

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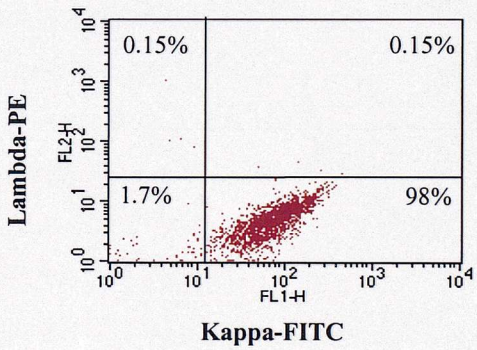


Figure 3.4: Representative flow cytometric analysis of cell surface markers on purified follicular lymphoma cells (FL-b). B-cells were purified from a lymph node biopsy of a follicular lymphoma (FL-b) by immunomagnetic depletion. **A** - Assessment of purity. Open histogram: immunostaining with a fluorescently conjugated isotype matched control antibody. Closed histogram: immunostaining with a fluorescently conjugated antibody against either CD20, CD40 (B-cell markers) or CD3 (T-cell marker). Cell counts are shown on the y-axis and fluorescence intensity is shown on the x-axis. CD3 positive, CD40/CD20 negative T-cells were completely removed by the selection protocol leaving pure B-cells. **B** - Assessment of clonality. Immunoglobulin light chain expression was analysed by double staining using FITC-conjugated anti-kappa (x-axis) and PE-conjugated anti-lambda (y-axis) antibodies. Percentages of cells in each quadrant are indicated. See table 3.1 for quantification of other primary samples.

In total, twenty two primary B-cell samples were used throughout this study, comprising seven follicular lymphomas, two mantle cell lymphomas, one diffuse large B-cell lymphoma, eight chronic lymphocytic leukaemias (CLL) and four reactive lymph nodes (table 3.1). Fresh samples were obtained or samples were used which had been stored as viable cell suspensions under liquid nitrogen (frozen samples). Wherever possible fresh samples were used since more viable cells were present, resulting in larger yields. However in some instances only frozen material was available. The majority of samples used were diagnostic samples, however in some instances samples were obtained from patients with previously diagnosed lymphoma who had previously been treated with chemotherapy (table 3.1).

Most samples were also stained for kappa and lambda immunoglobulin light chains. A single B-cell expresses either kappa or lambda light chains. Expression of the two immunoglobulin light chains kappa and lambda normally occurs at a ratio of 60:40 in a non-clonal population of B-cells, whereas clonal B-cell malignancies show clonal restriction and express a single light chain. Therefore, clonal restriction is a simple measure of the proportion of malignant cells in a B-cell preparation. Fig. 3.4B shows light chain staining of purified follicular lymphoma cells, 98% of cells are B-cells (express light chains) and are almost all clonal (express kappa) with very few contaminating normal lambda-positive B-cells (0.15%). As shown in table 3.1, all the lymphoma samples were light chain restricted, with very few normal B-cells present. In most samples every B-cell in the sample expressed the same light chain. This shows that a pure malignant B-cell population, which is clonal in origin, existed in the sample and that the production of normal B-cells was suppressed. In contrast, the reactive node samples showed two distinct populations of cells, with approximately 60% of cells expressing kappa light chains and approximately 40% of cells expressing lambda light chains.

Cytogenetic results were obtained for a number of the primary samples (table 3.1), carried out by the Cytogenetics Department at Southampton General Hospital. All the follicular lymphomas tested carried the characteristic translocation between chromosome 18 and chromosome 14, which brings the Bcl-2 gene under control of the Ig heavy chain intron enhancer, leading to overexpression of the Bcl-2 protein. The mantle cell lymphomas contained the translocation between chromosome 11 and

Table 3.1 Primary B-cell samples. Reactive node samples are shown overleaf. Pretreated indicates whether patients had previously been treated with chemotherapy. Ig light chain expression as determined by flow cytometry. The percentage of B-cells expressing the respective light chain is indicated in brackets. CD40 expression is stated as % of purified cells that were CD40 positive by FACS analysis. Aggregation with CD40L was determined by visually comparing treated and untreated cells under the light microscope after 48 hours of culture. n.a. = information not available.

Sample	Diagnosis	Tissue	Fresh or frozen	Cytogenetics	Pretreated	Ig light chain (% restriction)	CD40 expression	Aggregation with CD40L
FL-a	Grade 1 Follicular Lymphoma	Lymph node	Fresh	t(14;18)	No	Kappa (97%)	100%	Yes
FL-b	Grade 1 Follicular Lymphoma	Lymph node	Frozen	t(14;18)	Yes	Kappa (100%)	98%	Yes
FL-c	Grade 2 Follicular Lymphoma	Lymph node	Fresh	t(14;18)	No	Lambda (96%)	89%	Yes
FL-d	Low grade Follicular Lymphoma	Lymph node	Frozen	t(14;18), +7	No	Lambda (93%)	99%	Yes
FL-e	Grade 1 Follicular Lymphoma	Lymph node	Fresh	n.a.	No	Kappa (94%)	100%	Yes
FL-f	Grade 3A Follicular Lymphoma	Lymph node	Fresh	n.a	No	Lambda (96%)	99%	Yes
FL-g	Follicular Lymphoma (Grade unknown)	Blood	Fresh	t(14;18), +2, abnormal 1 & 3	Yes	Lambda (100%)	70%	No
MCL-a	Mantle Cell Lymphoma	Lymph node	Frozen	t(11;14)	n.a.	Kappa (100%)	99%	Yes
MCL-b	Mantle Cell Lymphoma	Lymph node	Fresh	t(11;14)	No	Kappa (100%)	99%	Yes
DLBL	Diffuse Large B-cell Lymphoma (transformed from low-grade FL)	Lymph node	Frozen	n.a.	Yes	n.a.	99%	Yes
CLL-a	Chronic Lymphocytic Leukaemia	Blood	Fresh	n.a.	n.a.	Kappa (100%)	72%	Yes
CLL-b	Chronic Lymphocytic Leukaemia	Spleen	Fresh	abnormal 8	Yes	Lambda (100%)	98%	No
CLL-c	Chronic Lymphocytic leukaemia	Spleen	Fresh	n.a.	Yes	Lambda (100%)	98%	No
CLL-d	Chronic Lymphocytic Leukaemia (mutated VH gene)	Blood	Frozen	n.a	No	Lambda (100%)	98%	No
CLL-e	Chronic Lymphocytic Leukaemia (unmutated VH gene)	Blood	Frozen	n.a.	No	Kappa (100%)	98%	No
CLL-f	Chronic Lymphocytic Leukaemia (aggressive disease)	Lymph node	Fresh	del 11q, -10 abnormal 7 & 13	No	Lambda (98%)	97%	Yes
CLL-g	Chronic Lymphocytic Leukaemia	Spleen	Fresh	n.a.	Yes	Lambda (96%)	83%	Yes
CLL-h	Chronic Lymphocytic Leukaemia	Lymph node	Fresh	t(10;14), abnormal 8	No	Kappa (100%)	97%	No

Sample	Diagnosis	Tissue	Fresh or frozen	Cytogenetics	Pretreated	IgM light chain	CD40 expression	Aggregation with CD40L
Reactive -a	No lymphoma	Spleen	Fresh	n.a.	No	Kappa (58%) + lambda (42%)	86%	Yes
Reactive -b	No lymphoma	Lymph node	Fresh	n.a.	No	Kappa (59%) + lambda (41%)	98%	Yes
Reactive -c	No lymphoma	Lymph node	Fresh	n.a.	No	Kappa (63%) + lambda (37%)	99%	Yes
Reactive -d	No lymphoma	Lymph node	Fresh	n.a.	No	kappa (57%) + lambda (43%)	95%	Yes

chromosome 14. This is characteristic of mantle cell lymphoma and causes rearrangement of the Bcl-1 gene, leading to overexpression of cyclin D1 protein, which promotes cell proliferation. Other abnormalities were also detected such as the addition or deletion of whole chromosomes and abnormalites in individual chromosomes.

3.5 CD40 rescues primary malignant B-cells from apoptosis

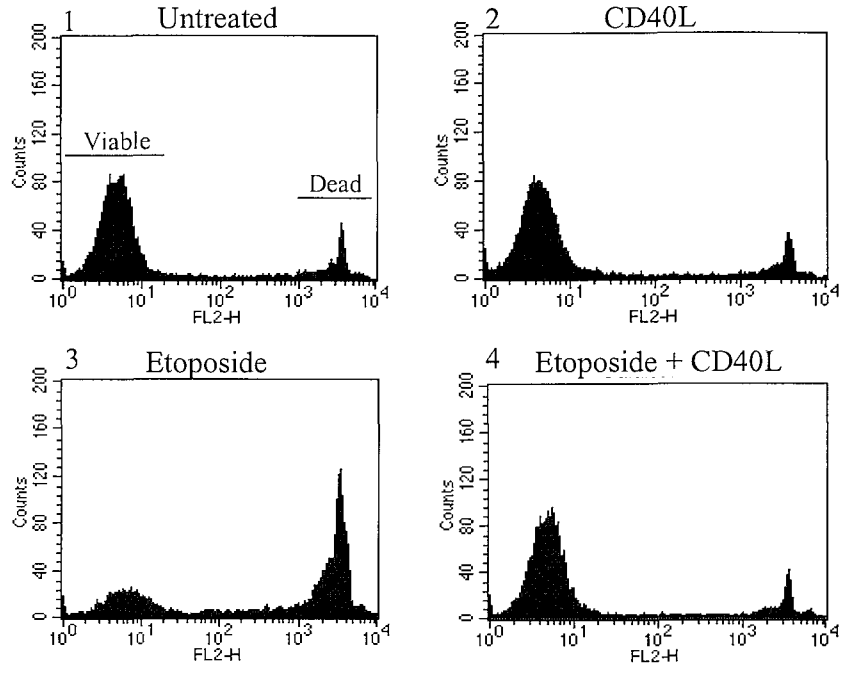
The survival response of the purified primary B-cells to CD40L was assessed by the PI exclusion test (figs. 3.5 and 3.6), the MTS assay (fig. 3.7) and PARP cleavage (fig. 3.8).

For the PI exclusion test, cells were cultured for 48 hours in the presence or absence of CD40L and with or without etoposide. PI is a dye that can enter dead cells through their leaky membranes and stain DNA. These cells will fluoresce when excited by lazer light and this fluorescence can be detected in the FL-2 channel of the flow cytometer. Viable cells, however, will exclude PI and thus exhibit a lower fluorescence.

Figure 3.5 shows representative examples of primary samples where CD40L treatment prevented etoposide-induced cell death or spontaneous cell death. Figure 3.5A demonstrates that B-cells from a patient with follicular lymphoma (FL-a) did not undergo a great deal of spontaneous cell death (graphs 1 and 2), but were sensitive to etoposide treatment (73% of cells die when treated with 2μM etoposide – graph 3). CD40L acted as a potent survival signal, completely rescuing cells from etoposide induced cell-death (graph 4). In contrast, the mantle cell lymphoma (MCL-a) (fig. 3.5B) sample showed a large degree of spontaneous cell death (graph 5), which was decreased by CD40L (graph 6).

Fig. 3.6 summarises the PI data from all the primary samples. It shows that samples show varying degrees of spontaneous apoptosis and have differing sensitivities to etoposide. There is no clear distinction in the amount of spontaneous and etoposide induced cell killing between different types of malignancy. It has been demonstrated that higher grade NHLs (e.g. diffuse large B-cell lymphomas and mantle cell

A Follicular lymphoma (FL-a)



B Mantle cell lymphoma (MCL-a)

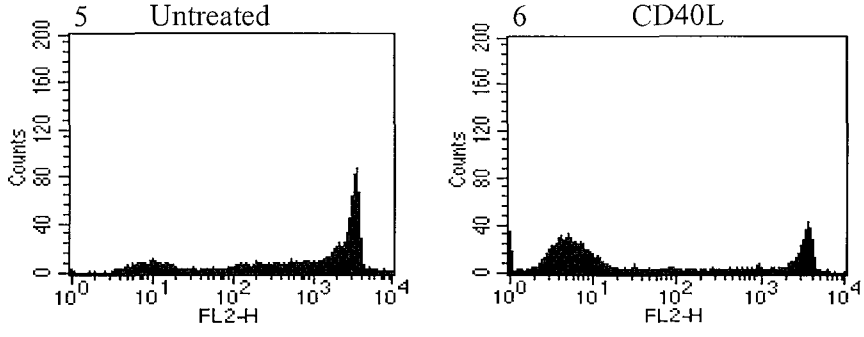


Figure 3.5: CD40L rescues primary lymphoma cells from spontaneous and etoposide-induced cell death. B-cells were purified by immunomagnetic depletion and cultured at 1 x 10^6 cells/ml for 48 hours in the presence or absence of CD40L (1μg/ml) and/or etoposide (2μM). PI was added to the cells and samples analysed by flow cytometry. Viable and dead cells are indicated. These samples were representative of other primary samples (see fig. 3.6).

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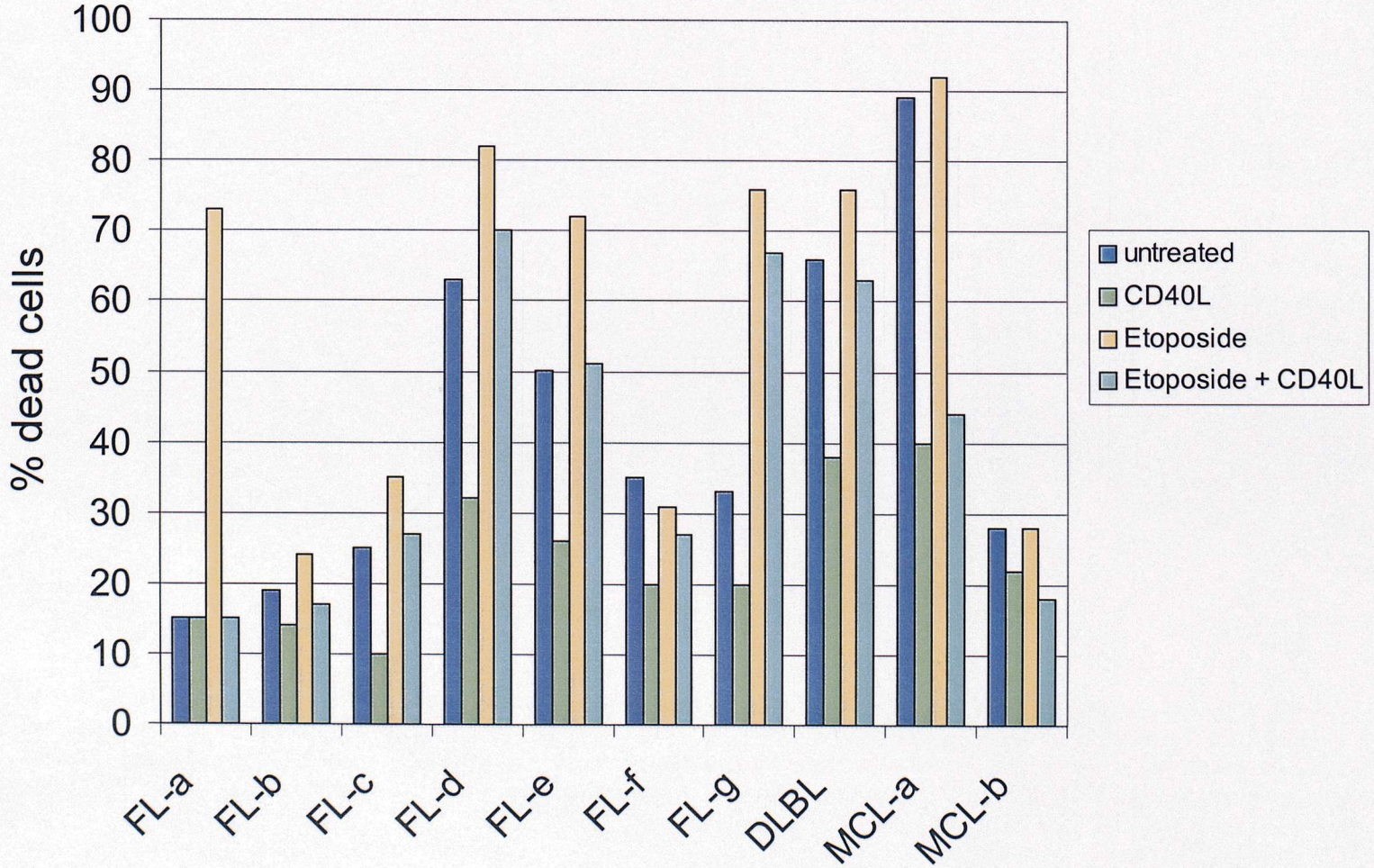
100
90
80
70
60
50
40
30
20
10
0
% dead cells
FL-a
FL-b
FL-c
FL-d
FL-e
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untreated
CD40L
Etoposide
Etoposide + CD40L

B

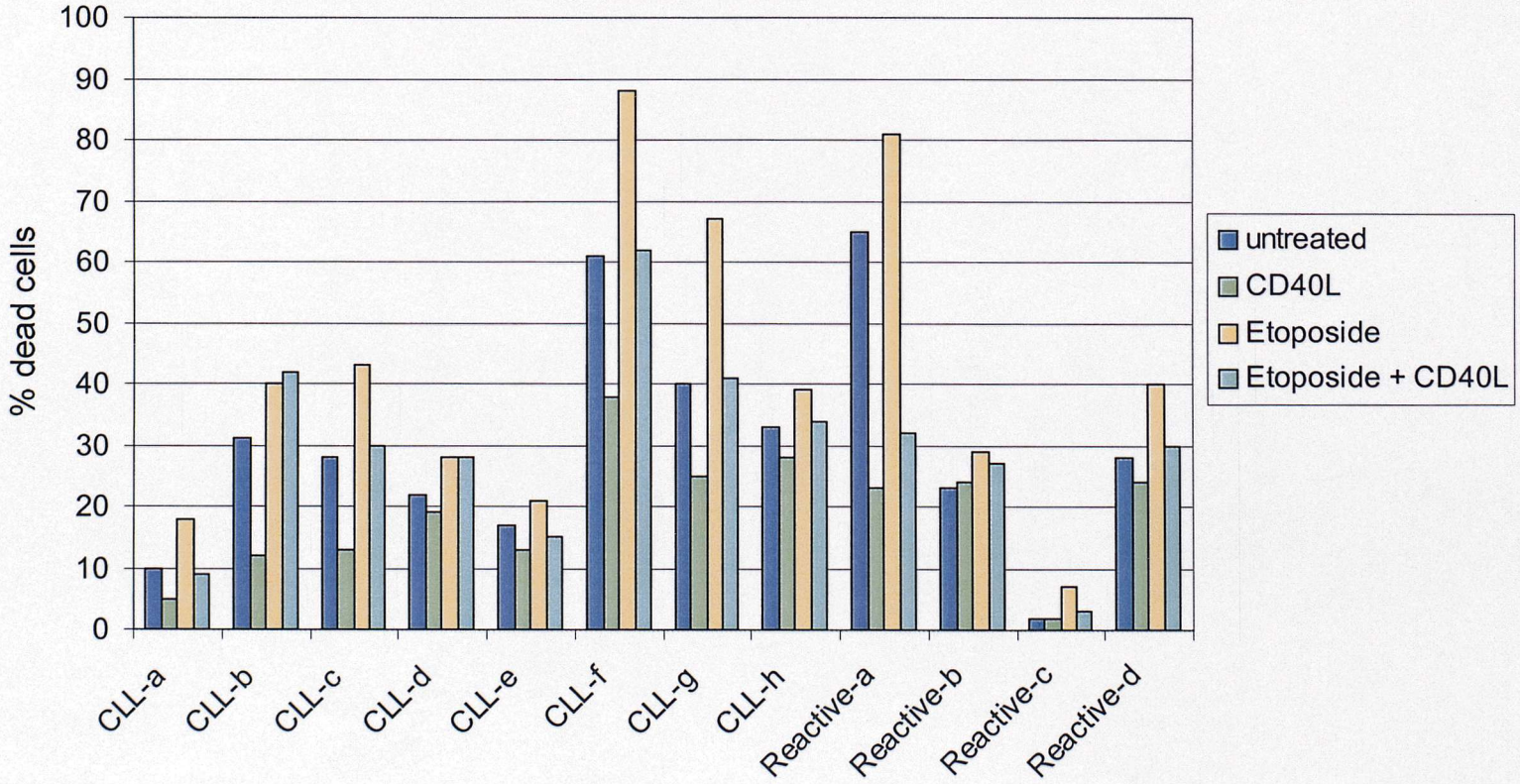

Figure 3.6: CD40L rescues primary malignant B-cells from spontaneous and etoposide-induced apoptosis. B-cells were purified from lymph node biopsies, spleens or blood by immunomagnetic depletion. Cells were cultured at 1x 10^6 cells/ml in a 96 well plate (100 000 cells per well) and treated with or without CD40L (1μg/ml) and with or without 2μM etoposide. After 48 hrs PI was added to the cells, the samples analysed by flow cytometry and the percentage of dead (PI positive) cells determined. **A**- Follicular lymphoma, mantle cell lymphoma and diffuse-large B-cell lymphoma samples. **B**- CLL and reactive nodes.

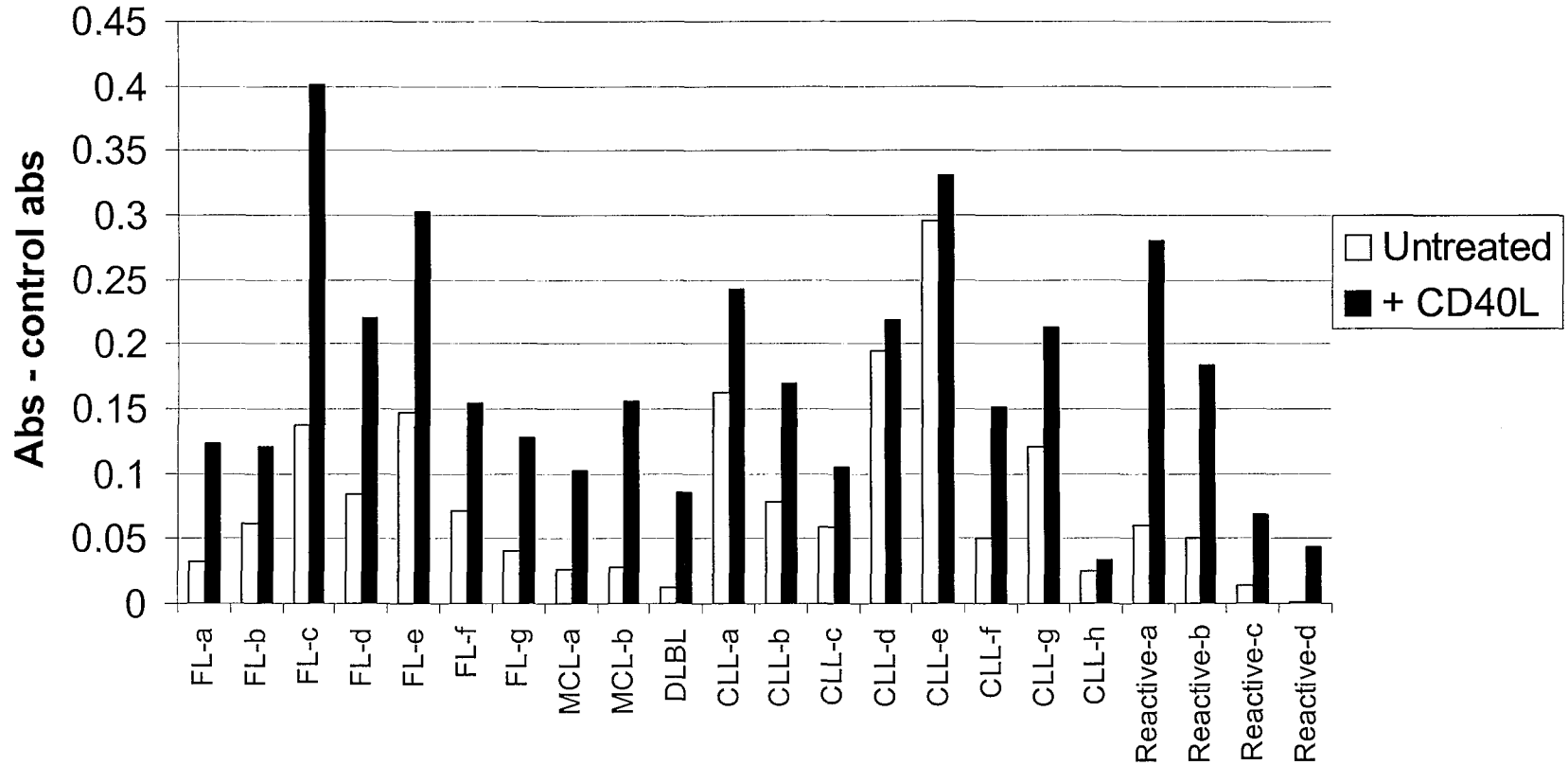

Figure 3.7: The effect of CD40L on MTS conversion in primary B-cells. Purified B-cells were cultured at 1x 10^6 cells/ml in a 96 well plate (100, 000 cells per well) and treated with or without CD40L (1μg/ml). After 48 hours the MTS reagent was added and the plate was incubated for 4 hrs, the absorbance was then read using an ELISA plate reader. The data are represented as absorbance minus control absorbance (absorbance of media alone + MTS reagent).

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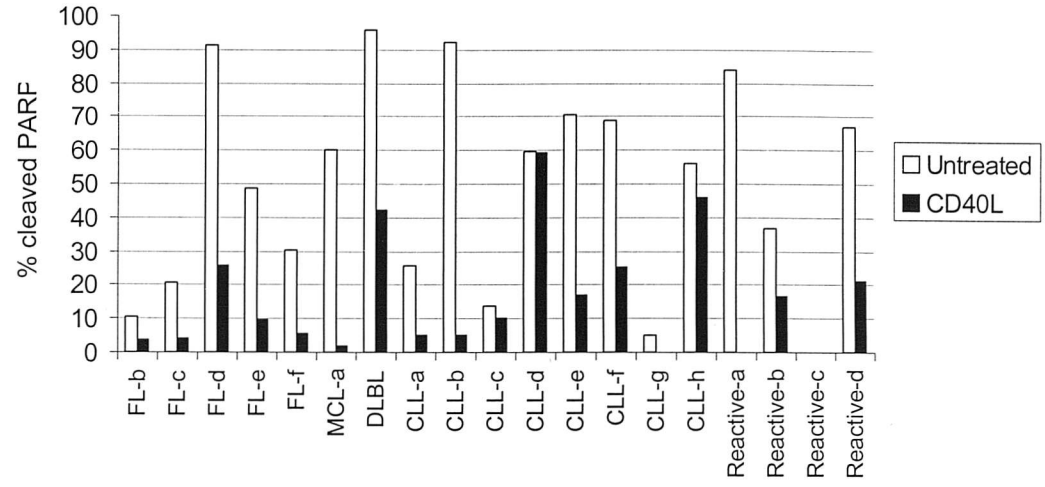

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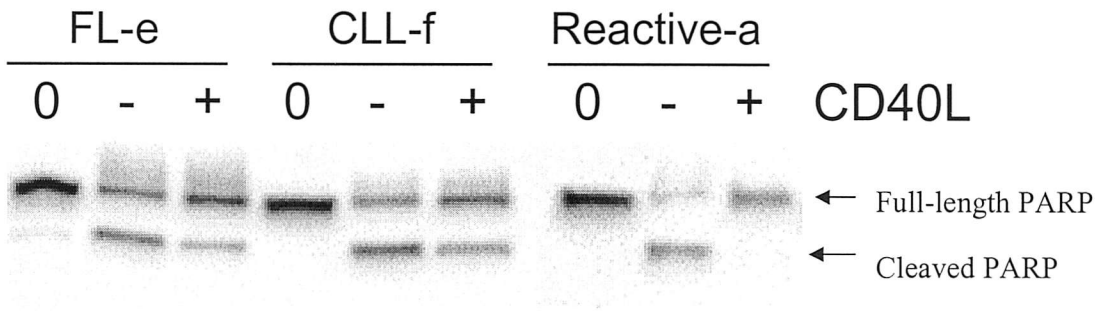


Figure 3.8: The effect of CD40L on PARP cleavage in primary B-cells. Cells were cultured for 24 hours with CD40L (1μg/ml) or left untreated as a control. PARP cleavage was detected by Western blot. **A** – Summary of PARP cleavage in primary samples. The full-length and cleaved PARP bands were quantified and the percentage of cleaved PARP calculated from the total intensity of the PARP bands. **B** – Examples of western blots showing PARP cleavage and rescue by CD40L.

lymphomas) show a greater degree of apoptosis than lower grade malignancies (e.g. follicular lymphomas) (Soini *et al* 1998). Although the DLBL sample and MCL-a sample showed 65 to 90% spontaneous apoptosis, the MCL-b sample showed a much reduced level of spontaneous cell death (28%). Also the FL-d, CLL-f and Reactive-a samples showed similar levels of spontaneous apoptosis to the DLBL sample. However it must be noted that numbers of high grade lymphomas in this study were small. There is also considerable heterogenerity in the amount of apoptosis occurring in samples of the same malignancy.

In 19/22 samples CD40L treatment greatly improved cell survival, reducing the amount of spontaneous death and/or etoposide induced-death detected. The three samples where CD40L treatment had little effect on cell survival were CLL-d, CLL-h and Reactive-b. In the FL-a and Reactive-c samples where CD40L did not affect spontaneous apoptosis, CD40 ligation reduced the amount of etoposide-induced cell death (fig. 3.6). There was variability in the sensitivity of samples to CD40L, particulary in the CLL and reactive nodes. Therefore these data demonstrate that CD40 signalling prevents both spontaneous and etoposide-induced cell death, in both normal and malignant B-cells, regardless of histological type, although there is variation in the sensitivity of samples to CD40 ligation.

Isolated primary B-cells were also treated with CD40L for 48 hours, or left untreated as a control, and cell proliferation and viability determined by MTS assay. The MTS assay is an indirect method for measuring cell proliferation and viability. MTS is a tetrazolium dye, which is converted into a water-soluble formazan product by the action of NADH-generating dehydrogenases found in metabolically active cells. The conversion of MTS produces a shift in absorbance, which can be detected by spectrophotometry. The MTS assay (fig. 3.7) showed that CD40L had a positive effect on all purified B-cells, although some samples were more sensitive to the effects of CD40L than others. This data suggests that CD40 signalling increases proliferation and/or viability of both normal and malignant B-cells. However since no difference in cell viability of CD40L treated and untreated FL-a, Reactive-b and Reactive-c samples was observed in the PI exclusion assay, the positive effect in the MTS assay was unlikely to be due to CD40L improving cell viability. Instead, this

suggests that CD40L improved cell proliferation or had a direct effect on mitochondrial function enabling cells to convert more MTS molecules.

Apoptosis of primary B-cells was assessed by detection of PARP cleavage by Western blot. The results are shown in fig. 3.8. Fig. 3.8A summarises the PARP cleavage data from all the samples tested and fig. 3.8B shows examples of Western blots obtained. In all samples tested, except Reactive-c, PARP cleavage occurred after 24 hours of culture (fig. 3.8A). Treatment with CD40L reduced or prevented PARP cleavage in all samples, except in sample CLL-d. Therefore CD40 signalling decreased the amount of apoptosis occurring in both normal and malignant primary B-cell cultures. Fig 3.8 also confirms the findings from the PI data (fig. 3.6), showing that different primary samples showed different amounts of spontaneous apoptosis e.g. a large amount of spontaneous apoptosis (over 60% of PARP cleaved) occurred in FL-d, MCL-a, DLBL, CLL-b, CLL-e, CLL-f, Reactive-a and Reactive-d. Samples also had varying sensitivities to the effects of CD40, for example CLL-h showed only a slight improvement in cell survival with CD40L treatment whereas CD40L prevented almost all PARP cleavage and thus apoptosis in the MCL-a and Reactive-a samples.

CD40 signalling can cause cell adhesion in culture, mediated by intercellular adhesion molecule-1 (ICAM-1 or CD54) and lymphocyte functional antigen –1 (LFA-1 or CD11a/CD18) (Barrett *et al* 1991, Katada *et al* 1996). Aggregate formation was assessed in the primary samples by visual inspection under the light microscope after 48 hours in culture in the presence or absence of CD40L. As shown in table 3.1, all of the follicular lymphoma, mantle cell lymphoma, diffuse large B-cell lymphoma samples and reactive nodes showed aggregate formation, with the exception of FL-g, although FL-g did respond to CD40 ligation in the cell survival assays (figs. 3.6-3.8). Five out of the eight CLL samples did not show aggregate formation, although CLL-b and CLL-c showed a fairly large degree of rescue from apoptosis by CD40L, whereas the other samples showed minor effects of CD40 ligation on cell survival (figs. 3.6-3.8). Therefore in general, CD40 ligation induces aggregate formation of primary lymphoma cells, but has variable effects on the aggregation of CLL cells, which does not appear to correlate with the ability of CD40L to protect cells from apoptosis.

In summary, all of the primary samples tested, showed an improvement in cell survival or decreased apoptosis when treated with CD40L, in at least one assay and for the majority of samples this was confirmed in all three assays (PI exclusion, MTS and PARP cleavage). However the sensitivity of cells to CD40 ligation was variable, particularly between CLL samples. Taken together, these data clearly show that CD40 ligation delivers a potent survival signal to both normal and malignant B-cells, regardless of histological type, reducing or preventing spontaneous apoptosis and also preventing etoposide-induced cell death.

3.6 Expression of apoptosis regulators in CD40L treated primary malignant B-cells

Isolated and purified B-cells were cultured with CD40L, or left untreated as a control, for up to 72 hours and samples taken at various time points for protein and mRNA analysis of apoptosis regulators. Figures 3.9 to 3.11 show examples of the western blots and RT-PCR results obtained. All bands on western blots were quantified and normalised to either actin or β-tubulin. All RT-PCR bands were quantified and normalised to GAPDH (codes for a constitutive enzyme in the glycolytic pathway) and 36B4 (codes for a constitutive acidic ribosomal phosphoprotein) mRNA levels.

Initially samples were cultured for up to 72 hours but in the majority of these samples a large amount of cell death occurred in the untreated samples from 44 to 72 hours. The decreased GAPDH and 36B4 mRNA levels in untreated cells after 44 hours in fig. 3.10, indicate this problem. Therefore all analyses from these samples were performed using the 24 hour time point data and subsequent samples were cultured for 16 to 24 hours. Figure 3.11 shows some semi-quantitative RT-PCR results from samples cultured for 16 hours with or without CD40L. The results from all the primary samples are summarised in table 3.2. CD40 ligation induced various patterns of protein and RNA expression in the primary samples. CD40L increased expression of many apoptotic regulators to varying degrees, as indicated by the number of arrows in table 3.1, calculated by comparing expression of molecules with untreated cells. It was also important to distinguish between increased and sustained patterns of expression. Increased expression was defined as when CD40L treatment increased expression above that of both untreated cells and cells at the start of the experiment

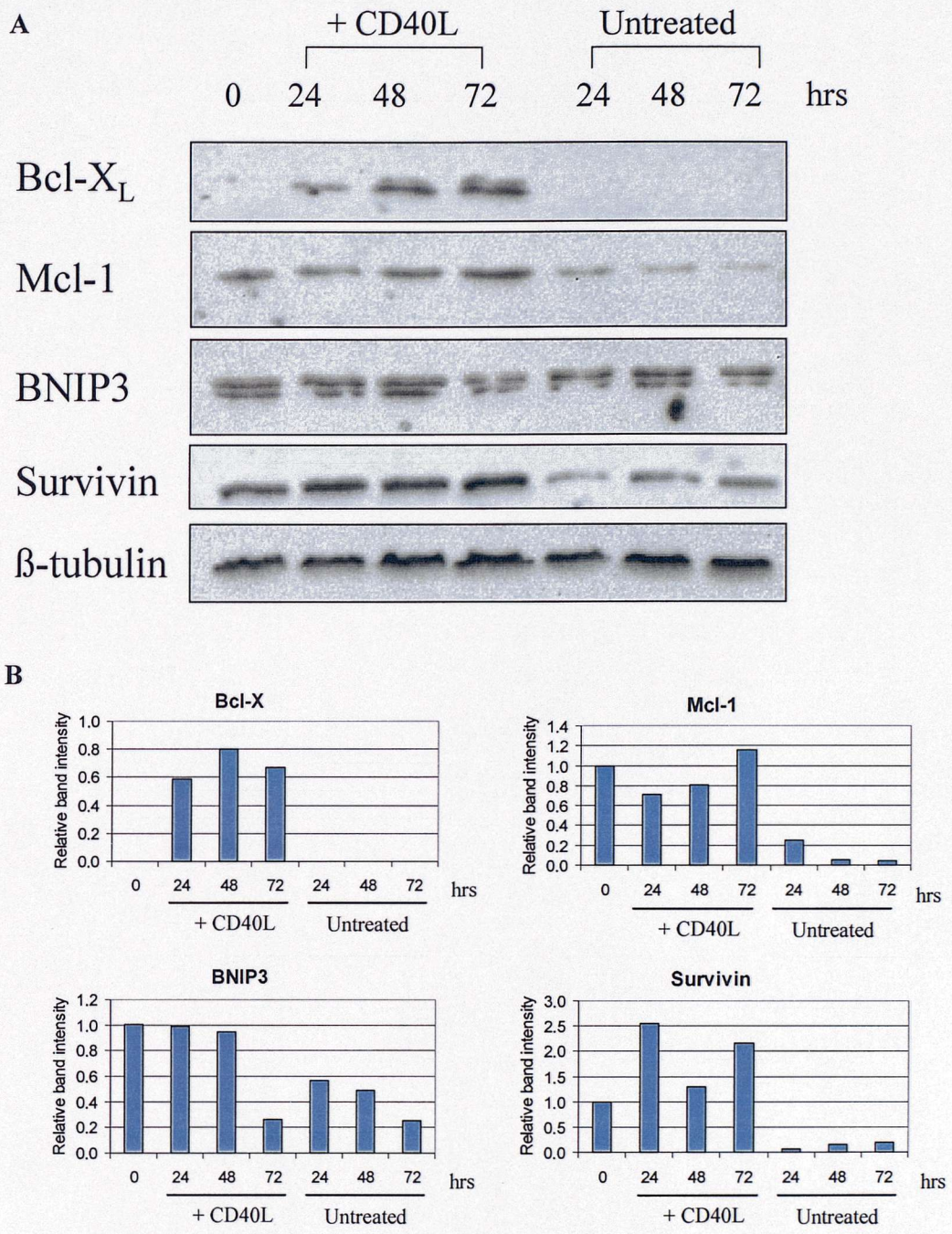


Figure 3.9: The effect of CD40L on protein expression of apoptosis regulators in a follicular lymphoma (FL-c). Immunomagnetically purified B-cells were cultured for 48 hours either with CD40L (1μg/ml) or left untreated as a control. Samples were taken at the time points indicated and snap frozen. **A** – Western blots: samples were lysed in SDS sample buffer and approximately 5 x 10^5 cells were loaded per well on SDS-PAGE gels. Proteins were transferred onto nitrocellulose membranes and probed with appropriate antibodies. **B** – Blots were quantified and normalised by dividing the band intensity by that of β-tubulin. The relative band intensity of the time 0 band was set to 1.

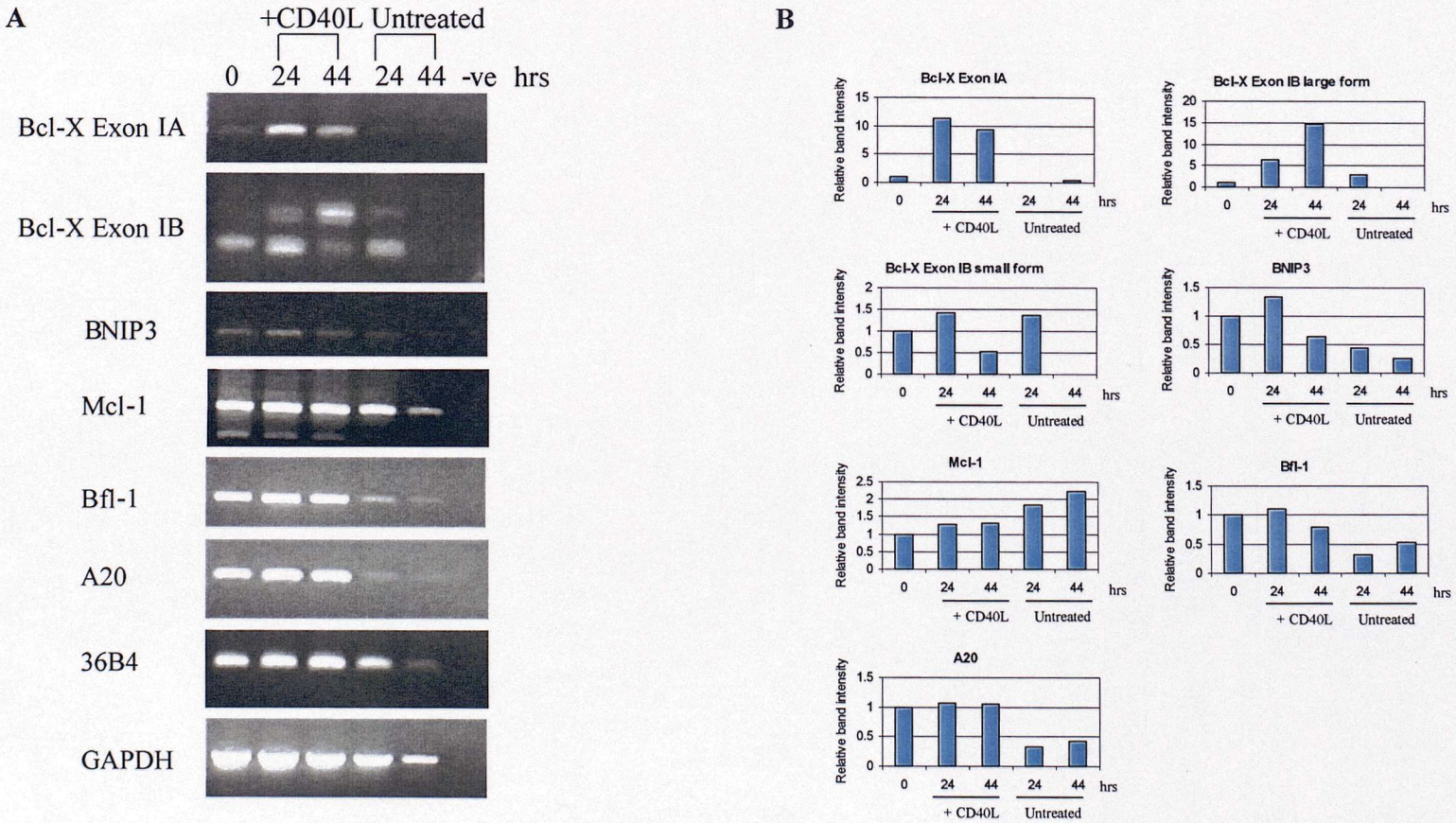


Figure 3.10: The effect of CD40L on mRNA expression of apoptosis regulators in a diffuse large B-cell lymphoma (DLBL). Immunomagnetically purified B-cells were cultured with CD40L (1μg/ml) or left untreated as a control. Samples were taken at the time points indicated. **A** – Semi-quantitative RT-PCR analysis. –ve = water added instead of cDNA. **B** – Quantification of bands, band intensity was quantified and normalised by dividing by the mean of the GAPDH and 36B4 band intensities. The normalised band intensity of the time 0 band was set to 1.

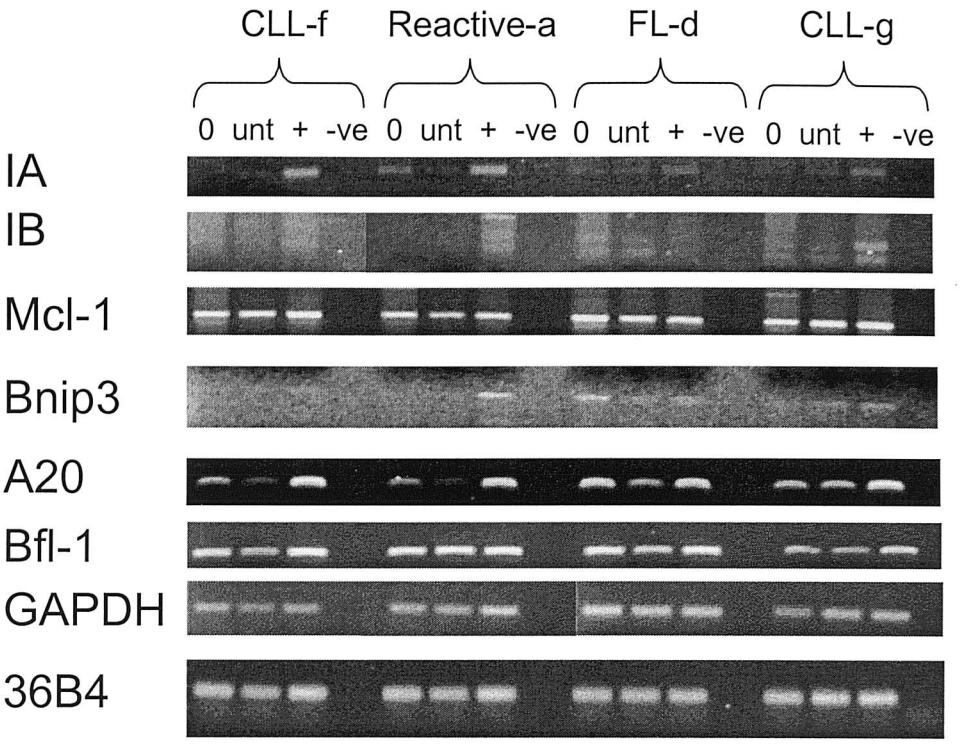


Figure 3.11: The effect of CD40L on mRNA expression of apoptosis regulators in primary B-cells. Immunomagnetically purified B-cells were cultured with 1μg/ml CD40L (+) or left untreated as a control (unt) for 16 hours, RNA was isolated, reverse transcribed and semi-quantitative RT-PCR analysis carried out. 0 = Time 0, pellet taken at the start of the experiment –ve = water added instead of cDNA.

Table 3.2: The effect of CD40L on the expression of apoptosis regulators in primary malignant B-cells. Cells were treated with CD40L (1μg/ml), or left untreated as a control, for 16 or 24 hours. Protein and mRNA expression were determined by Western blot and semi-quantitative PCR, respectively. Bands were quantified and normalized to actin or β-tubulin protein levels or the mean values of GAPDH and 36B4 mRNA levels. The expression of apoptosis regulators in untreated and CD40L treated cells was compared at 16 or 24 hours. Key is shown overleaf.

Sample	*Protein Expression*				*RNA Expression*						
	Bcl-X	Mcl-1	BNIP3	Survivin	Bcl-X 1A	Bcl-X 1B lg	Bcl-X 1B sml	Mcl-1	BNIP3	A20	Bfl-1
FL-a	na	na	na	na	↑↑↑	n.d	—	—	↑	↑↑↑	>
FL-b	↑↑↑	>	>	—	↑	n.d	—	—	↑↑	↑	↑
FL-c	↑↑↑	>	>	↑	—	↑	↑	—	>	—	↓
FL-d	↑↑↑	↑↑	n.d.	n.d.	↑↑	↓	↓	—	—	>	—
FL-e	↑↑↑	↑↑	>	n.d.	↑	↑↑↑	↑↑↑	—	↑	>	↑↑
FL-f	↑↑↑	↑↑	↑↑	↑↑	↑	—	—	—	—	>	↑
MCL-a	↑↑	↑	>	↑↑↑	—	↑	↑	—	↑	>	>
MCL-b	na	na	na	na	—	—	—	—	—	>	↑↑
DLBL	↑↑↑	>	>	n.d.	↑↑↑	↑↑	↑	—	↑↑	>	>
CLL-a	↑↑	↑↑	—	>	—	>	—	—	↑	—	—
CLL-b	↑	↑↑↑	>	↑	↑	↑	↑	—	↑	↑	>
CLL-c	↑↑↑	↑↑↑	↑↑	—	↑↑	↑	—	—	—	—	↑
CLL-d	↑↑↑	>	—	—	—	n.d	n.d	—	↓	>	—
CLL-e	↑↑↑	>	—	—	↑↑	n.d	n.d	>	↑	>	>
CLL-f	↑↑↑	—	>	—	↑↑	↑↑	↑↑	—	↑	↑↑	↑
CLL-g	↑↑↑	↑↑	n.d.	↑↑	↑↑↑	↑	↑	—	↑	↑	↑
Reactive-a	↑↑↑	↑↑	n.d.	>	↑↑	↑↑↑	↑↑↑	—	↑↑↑	↑↑	—
Reactive-b	↑↑↑	>	n.d.	↓	↑	—	—	—	↑	>	—
Reactive-c	↑↑↑	>	n.d.	n.d.	↑↑	n.d	n.d	—	↑	↑	>

KEY:

↑	1.5 to 3 fold increase in expression with CD40L
↑↑	3 to10 fold increase in expression with CD40L
↑↑↑	Above 10 fold increase in expression with CD40L or CD40L induces expression
↓	1.5 to 3 fold decrease in expression with CD40L
>	Sustained (CD40L maintained expression at similar levels to Time 0, whereas expression decreased in untreated samples)
—	No effect of CD40L
n.d	No protein or RNA detected
na	Not appropriate, as not enough sample for analysis

(T0) e.g. Bcl-X exon IA expression in fig. 3.10. Sustained expression was defined as when expression decreased in untreated samples, but CD40L maintained expression at similar levels to Time 0 e.g. A20 and Bfl-1 expression in fig. 3.10. Sustained expression by CD40L could either be a direct effect of CD40 signalling or could be a consequence of increased cell death in the control samples compared to CD40L treated cells.

From table 3.2 it is clear that Bcl-X_L protein levels are consistently regulated by CD40. In all samples, Bcl-X_L expression was either induced or increased by CD40 ligation and in most cases this was extremely strong e.g. a 137 times increase in Bcl-X_L expression was observed after 48 hours of treatment with CD40L in FL-b. At the mRNA level, both the exon IA and exon IB transcripts responded to CD40L. Exon IA containing transcripts were increased by CD40 ligation in the majority of samples (14/19 samples) whereas the exon IB transcripts were increased or sustained by CD40 in approximately half of the samples compared to untreated cells (IB large 10/19 samples, IB small 8/19 samples). There was no clear pattern of Bcl-X promoter usage, since some samples responded to CD40L by increasing expression of both IA and IB transcripts, whereas other samples just utilised either IA or IB. Differential regulation of the exon IB splice variants by CD40L was also observed in some samples (figs. 3.10 & 3.12). For example, only the large exon IB splice form was regulated by CD40 in CLL-c, whereas the small and large splice variants were regulated by CD40 with different kinetics in the DLBL and FL-c sample. In other samples exon IB containing transcripts were not expressed or only the small splice form could be detected.

Mcl-1 protein levels were also regulated by CD40, as all samples, with the exception of CLL-f, either showed an increase in Mcl-1 expression when treated with CD40L or Mcl-1 expression was sustained by CD40 ligation (table 3.2). However, Mcl-1 mRNA levels were only influenced by CD40L treatment in one sample (CLL-e). Fig. 3.13A shows examples of samples where Mcl-1 protein was regulated by CD40 but mRNA levels were not. This suggests that CD40 regulates Mcl-1 levels post-transcriptionally. One mechanism by which CD40 signalling may sustain Mcl-1 levels post-transcriptionally is through preventing cleavage of Mcl-1. Mcl-1 was shown to be downregulated and cleaved by caspases during apoptosis in B-cells, forming a 28kDa cleavage product (J. Michels & G. Packham, personal communication). The 28kDa

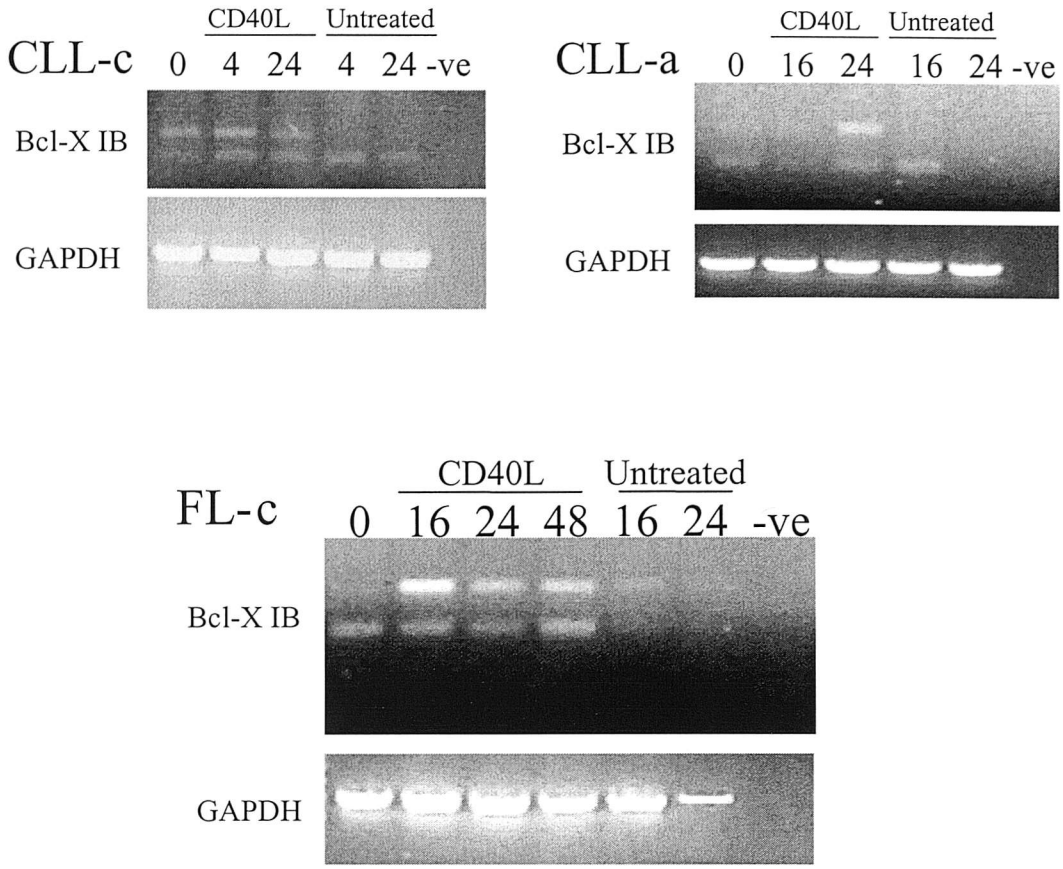


Figure 3.12: Examples of differential regulation of Bcl-X exon IB splice forms by CD40L. Immunomagnetically purified B-cells were cultured with 1μg/ml CD40L (+) or left untreated as a control for various time points up to 48 hours, RNA was isolated, reverse transcribed and semi-quantitative RT-PCR analysis carried out. Two splice variants for exon IB were detected and GAPDH is shown as a loading control. 0 = Time 0, pellet taken at the start of the experiment, –ve = water added instead of cDNA. Numbers indicate the time (in hours) cells were cultured for. See also fig. 3.10.

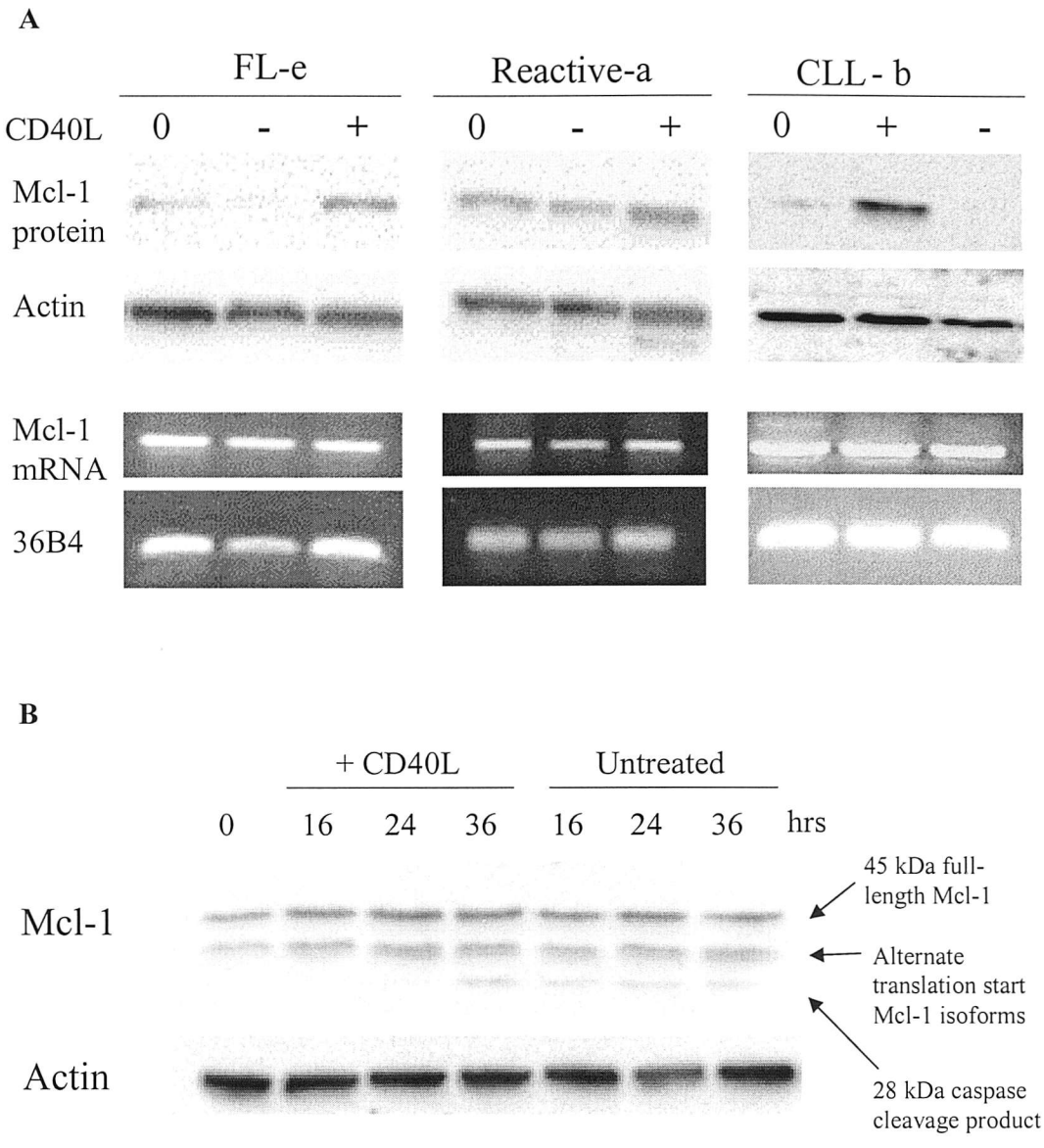


Figure 3.13: Regulation of Mcl-1 expression by CD40 in primary malignant B-cells.
A – Examples of samples showing post-transcriptional regulation of Mcl-1 by CD40. Samples were treated with CD40L (1μg/ml) (+) or left untreated as a control (-) for 16 hours and protein and RNA analysed by western blot and semi-quantitative RT-PCR. 0 = sample taken at the start of the experiment. Actin and 36B4 are shown as loading controls for protein and RNA respectively. **B** – CD40L prevents caspase-mediated cleavage of Mcl-1 in the CLL-a sample. Cells were treated with CD40L (1μg/ml) or left untreated for the time points indicated and protein expression analysed by Western blot.

Mcl-1 cleavage product was observed in three CLL samples (CLL-a, CLL-g and CLL-f) and CD40L treatment was associated with the absence of the cleavage product (fig. 3.13B). Therefore maintenance of Mcl-1 levels by CD40 signalling, may, at least in part, be due to an indirect mechanism through CD40 signalling preventing apoptosis and thereby preventing caspase-mediated Mcl-1 cleavage. Mcl-1 cleavage may have occurred in other primary samples but this might not have been detected as the first batch of Mcl-1 antibody used did not detect the cleavage product and in other samples the cleavage product may have been degraded or there may not have been enough spontaneous apoptosis to detect the cleavage product.

CD40 ligation sustained or increased BNIP3 protein expression in nine of the nineteen primary samples tested, suggesting that BNIP3 may be a novel CD40-target gene for at least some B-cell malignancies. In three samples there was no effect of CD40L on BNIP3 protein expression and in five samples, BNIP3 protein was not detected despite a positive control producing a strong signal. However, at the mRNA level BNIP3 transcripts were regulated by CD40 ligation in most samples (14/19 samples). In most samples CD40L treatment strongly increased BNIP3 mRNA expression, even though in some of those cases BNIP3 protein was not detected or was not influenced by CD40 treatment. There is therefore, not a clear correlation between BNIP3 mRNA and protein expression, although this study is the first to show that both can be regulated by CD40 in primary B-cell malignancies.

Survivin, A20 and Bfl-1 all showed regulation by CD40 ligation in some patients but did not appear to be consistent CD40 target genes. Survivin expression was increased or sustained by CD40L in 7 out of the 19 samples and actually decreased in expression with CD40L in one sample (Reactive-a). It was reported that survivin was not expressed in normal adult tissues (Ambrosini *et al* 1997), however survivin was detected in two of the three reactive nodes. A20 mRNA expression was generally sustained by CD40L (9/19 samples) but was increased in some samples (6/19) and CD40 signalling had no effect on A20 expression in three samples. Bfl-1 expression was increased or sustained by CD40L treatment in 13/19 samples, decreased in one sample (FL-c) and was unaffected by CD40L in 5 samples.

No clear differences in the expression and regulation of apoptosis regulators by CD40 were observed between different B-cell malignancies or between normal and malignant cells. CD40 signalling was able to regulate expression of each apoptosis regulator in samples from each disease or normal subgroup. Even samples which showed little effect of CD40 ligation on cell survival e.g. CLL-d, showed regulation of apoptosis regulators by CD40L e.g. increased Bcl-X_L expression.

3.7 Antisense oligonucleotides to Bcl-X_L and Mcl-1 induce apoptosis in Akata 6 cells

Since Bcl-X_L and Mcl-1 were the most consistently and strongly CD40 regulated genes in the primary B-cell malignancies, antisense oligonucleotides to these molecules were obtained to define the role of these molecules in CD40-mediated cell survival. In particular, the importance of Mcl-1 for cell survival has been relatively poorly studied. Many different methods were used to try and introduce the antisense molecules into Akata 6 cells including lipid-based transfection reagents, passive uptake and electroporation. However electroporation was the only method that produced any satisfactory transfection efficiency. It would have been interesting to introduce antisense molecules into primary cells, however electroporation is not a suitable method for transfecting primary material, since it induces an extremely large degree of cell death and requires a large number of cells per transfection.

Two different Bcl-X antisense oligonucleotides were obtained from Isis Pharmaceuticals (San Diego, USA), Bcl-X1 or 15999 and Bcl-X2 or 16011, which target different regions of the Bcl-X mRNA. The control for these oligonucleotides was an orphan oligonucleotide whose sequence does not correspond to any mRNAs in the cell. These antisense oligonucleotides were previously shown to specifically inhibit Bcl-X_L expression in normal keratinocytes, lung carcinoma and melanoma cells, sensitising these cells to apoptosis (Taylor *et al* 1999, Heere-Ress *et al* 2002). A Mcl-1 antisense oligonucleotide was obtained along with its mismatch control oligonucleotide, which contains the same bases as the Mcl-1 oligonucleotide but in a different order. The Mcl-1 antisense oligonucleotide was shown to specifically ablate Mcl-1 expression in melanoma and endothelial cells (Derenne *et al* 2002, Bannerman *et al* 2001).

Akata 6 cells were chosen as a model lymphoma system to study the effects of antisense since they constitutively express high levels of Bcl-X and Mcl-1 and are relatively easy to transfect. Akata 6 cells were electroporated with these antisense molecules and protein expression detected after 4 and 24 hours. As shown in fig. 3.14, the Bcl-X antisense molecules began to downregulate Bcl-X_L expression after 4 hours of treatment and at 24 hours Bcl-X_L levels were approximately 15% of the level of orphan oligonucleotide treated cells. One would not expect complete removal of Bcl-X protein expression in a population of cells via this method, since it takes time to turnover the existing Bcl-X_L protein in the cells and electroporation is not 100% efficient at transfecting cells. The Bcl-X antisense molecules increased PARP cleavage approximately 4-fold relative to the control oligonucleotides (fig. 3.14C), indicating that Bcl-X_L is a key survival molecule for Akata 6 cells. It was also noted that the control oligonucleotides increased apoptosis relative to mock transfected cells (cells which were electroporated in the absence of DNA), but treatment with the antisense molecules promoted a much larger amount of cell death. Mcl-1 levels also decreased with the Bcl-X antisense molecules but this could be attributed to the increased apoptosis of the cells, rather than a non-specific action of the Bcl-X oligonucleotides. Mcl-1 is known to be downregulated during apoptosis which can be mediated by Mcl-1 cleavage and/or decreased Mcl-1 transcription and translation, as Mcl-1 has a short half-life (Iglesias-Serret *et al* 2003, J. Michels & G. Packham, personal communication).

The Mcl-1 antisense oligonucleotide reduced Mcl-1 expression 4 and 24 hours after administration, showing a decrease in Mcl-1 expression to one third of its mismatch control after 24 hours (fig. 3.14). The Mcl-1 antisense molecule increased PARP cleavage, relative to its mismatch control, and did not significantly affect Bcl-X_L expression (fig. 3.14). Therefore Mcl-1 is also a key survival protein for Akata 6 cells.

It would have been interesting to treat cells with the antisense molecules and CD40L in combination to determine if Bcl-X_L and Mcl-1 were important mediators of CD40-induced cell survival. However data presented in section 3.8 suggested that electroporation may disrupt or reduce CD40 signalling and so electroporation of antisense molecules could not be used. DEAE-dextran is a gentle transfection method which should still allow extracellular signalling and so DEAE-dextran was used to try

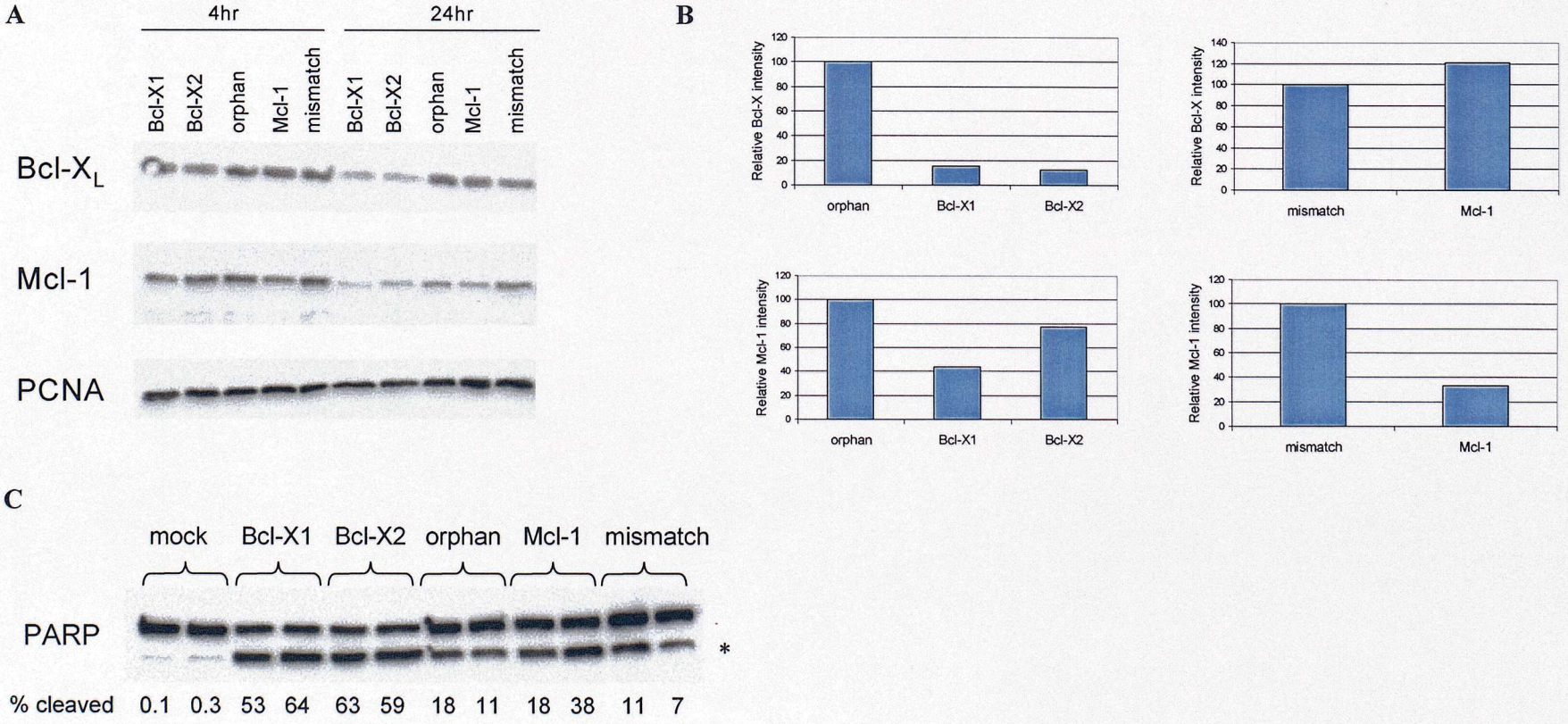


Figure 3.14: Antisense oligonucleotides against Bcl-X and Mcl-1 promote apoptosis. Akata 6 cells were electroporated with 50μM of each antisense oligonucleotide or were electroporated without any DNA (mock). See text for descriptions of each antisense molecule. Cells were cultured for up to 24 hours and pellets taken at 4 and 24 hours for protein analysis of Bcl-X_L and Mcl-1 by Western blot (**A**). **B** - Bcl-X_L and Mcl-1 bands, at the 24 hour time point, were quantified, normalised to PCNA levels and expressed as a percentage of the control antisense intensities (set to 100%). **C** - PARP cleavage is shown at 24 hours, as a measure of apoptosis, cleaved PARP is annotated by *. These results are representative of two independent experiments.

and introduce the antisense oligonucleotides. However this did not produce any reduction in Bcl-X_L or Mcl-1 protein levels (data not shown). Therefore although, the role of Bcl-X_L and Mcl-1 in CD40-mediated cell survival could not be determined, these data demonstrate that Bcl-X_L and Mcl-1 are key survival proteins for lymphoma cells.

3.8 Transcriptional control of Bcl-X_L

This study has shown that Bcl-X_L protein was consistently induced or upregulated by CD40 in both B-cell lines and primary B-cell malignancies, regardless of histological type. Analysis of Bcl-X mRNAs revealed that both exon IA and exon IB containing mRNAs were regulated by CD40 in primary samples. Therefore Bcl-X_L is a key CD40 target gene that appears to be regulated by CD40, at least in part, at the level of transcription in B-cell lymphomas. In order to explore this further, this study sought to determine how CD40 regulates Bcl-X promoter activity.

WEHI-231 cells (mouse immature B-cells) were chosen as a model system to investigate the regulation of Bcl-X transcription by CD40. This was due to a number of factors. The WEHI-231 cell line has been extensively studied as cross-linking surface IgM on WEHI-231 cells leads to apoptosis, which is rescued by CD40 activation (Tsubata *et al* 1993, Choi MS *et al* 1995, Siebelt *et al* 1997, Schauer *et al* 1998, Kuss *et al* 1999, Craxton *et al* 2000). This rescue is associated with induction of Bcl-X_L (Choi MS *et al* 1995, Kuss *et al* 1999, Craxton *et al* 2000). A number of published studies have also used WEHI-231 cells as a model system to study promoter regulation of various genes (Lee *et al* 1995, Lam *et al* 1998, Lee JR *et al* 1998, Wu *et al* 1996, Yang *et al* 2001). As WEHI-231 cells are mouse cells, human CD40 constructs with various deletions or mutations in specific domains or signalling molecule binding sites could be transfected into the cells and specifically stimulated to investigate the function of CD40 receptor domains in Bcl-X regulation.

To confirm that CD40 activation regulated Bcl-X_L expression in WEHI-231 cells, cells were treated with a either a mouse CD40-activating antibody (IC10) or its isotype-matched control antibody (KT3); or mouse CD40L (moCD40L) and left untreated as a control, and protein expression analysed by western blot. As shown in fig. 3.15, stimulation of CD40 through IC10 or moCD40L led to induction of Bcl-X_L

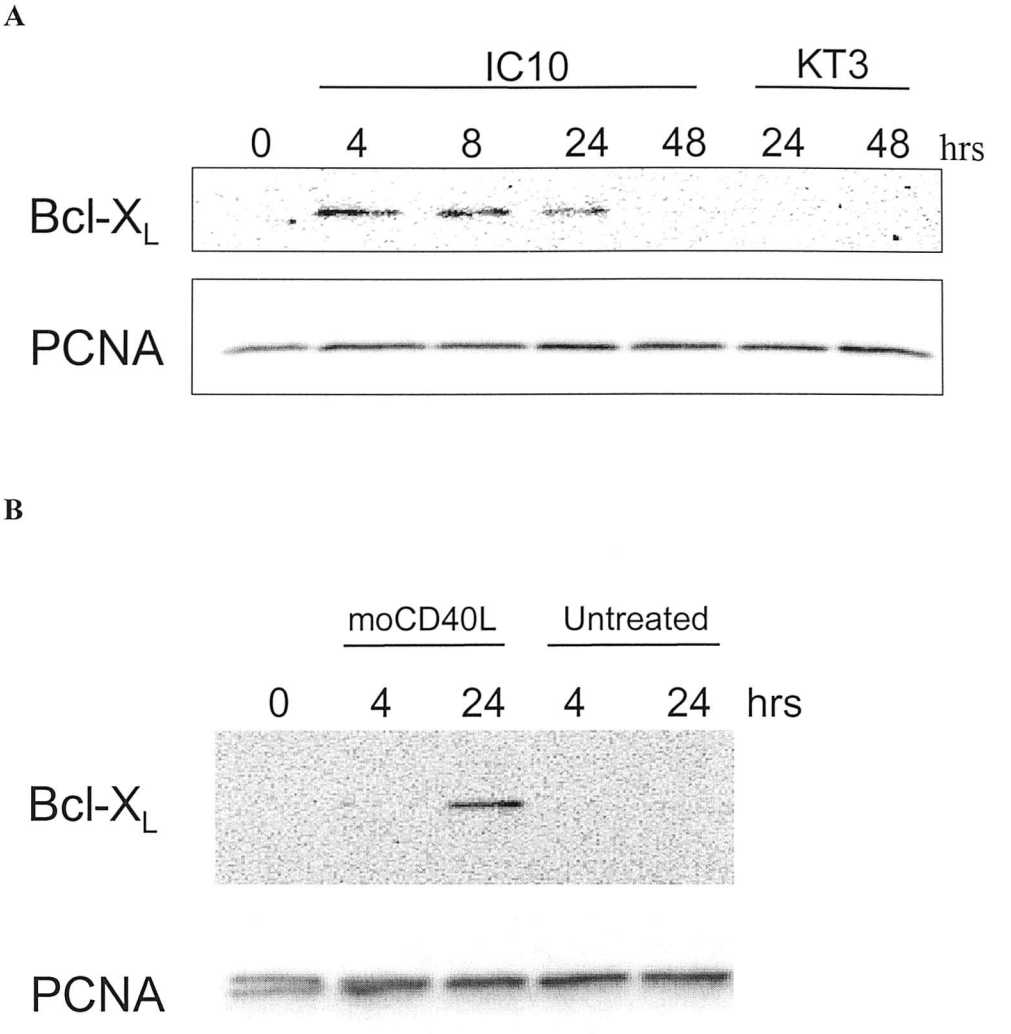


Figure 3.15: CD40 activation induces Bcl-X_L expression in WEHI-231 cells. A- WEHI-231 cells were treated with 5μg/ml IC10, a CD40-activating antibody, or 5μg/ml KT3, an isotype-matched control antibody, for 4 to 48 hours. A pellet was also taken at the start of the experiment (0). **B** -WEHI-231 cells were treated with either 1μg/ml fresh mouse CD40L (moCD40L) or left untreated as a control. Pellets were taken at the start of the experiment (0) and at 4 and 24 hours. In both experiments, total protein was isolated and analysed by Western blot with antibodies for Bcl-X_L and PCNA. PCNA expression was used as a loading control.

protein over 4 to 24 hours. Bcl-X_L induction showed different kinetics when treated with IC10 or moCD40L. Bcl-X_L induction by IC10 was maximal at 4-8 hours, declined at 24 hours and was no longer detected at 48 hours, whereas moCD40L lead to optimal expression after 24 hours of treatment.

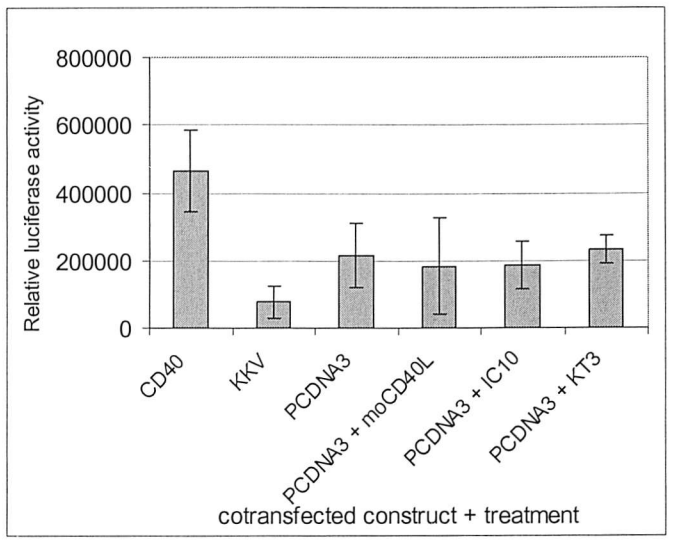
Although many published studies have successfully transfected WEHI-231 cells, mainly through electroporation (Wu *et al* 1995, Lam *et al* 1998, Lee JR *et al* 1998), transfection of WEHI-231 cells proved extremely difficult. Many attempts and methods were used to try and determine suitable transfection conditions, using luciferase and β-galactosidase reporter constructs. The luciferase reporter constructs used included the Bcl-X IB promoter and the IκB-alpha promoter. These constructs were chosen since they were previously shown to drive strong luciferase activity in other studies performed in the laboratory. The IκB-alpha promoter was also shown to be CD40-responsive (Mann *et al* 2002). The following electroporation conditions were tested: number of cells transfected (8×10^6 cells or 2×10^7 cells), electroporation buffer used (serum-free media or cytomix), volume of electroporation buffer used (250μl, 400μl or 800μl), amount of DNA transfected (20μg to 40μg) and electrical parameters (900μF, 960μF or 1050μF and from 250mV to 340mV). These conditions were used based on published conditions used in the literature (Wu *et al* 1995, Lam *et al* 1998, Lee JR *et al* 1998). The only electroporation conditions that resulted in measurable luciferase and β-galactosidase activity were using 8×10^6 cells in 250μl serum-free media, transfecting with 40μg total DNA at 960μF and 270mV. Using these conditions, luciferase activity values of approximately 140,000 were obtained with the IκB-alpha promoter, and values of approximately 500 were obtained with the Bcl-X IB promoter construct. The lipid-based transfection reagents Fugene 6 and Effectene were also tried but did not result in any detectable luciferase or β-galactosidase activity.

Having identified reasonably successful transfection conditions, the IκB-alpha promoter plasmid was used to test which method of activating CD40 increased promoter activity in WEHI-231 cells. The IκB-alpha promoter was used as it had high activity in WEHI-231 cells and was previously shown to be activated by CD40 in dendritic cell lines (Mann *et al* 2002). IκB-alpha was also confirmed as a CD40 target

in B-cells through microarray experiments (see chapter 5). Previous studies have shown that overexpressing CD40 is sufficient to activate a range of different promoter constructs (Luftig *et al* 2001, Mann *et al* 2002), so WEHI-231 cells were cotransfected with 20μg IκB-alpha promoter plasmid and 20μg human CD40 plasmid to overexpress human CD40 on the cell surface. As a control, cells were cotransfected with 20μg IκB-alpha promoter plasmid and either 20μg pcDNA3 (parental plasmid from which the CD40 plasmid was derived) or 20μg KKV plasmid. KKV encodes a CD40 molecule that is missing the intracellular domain, with only KKV residues of the intracellular domain present to anchor the receptor in the cell membrane. This CD40 molecule is incapable of intracellular signalling and was therefore used as a control for CD40 transfected cells (Mann *et al* 2002). The mouse CD40-activating antibody IC10 and soluble moCD40L were also tested to determine if they could activate signalling through endogenous mouse CD40 to the IκB-alpha promoter. pcDNA3 cotransfected cells were either left untreated for 48 hours or after 24 hours they were treated with moCD40L or IC10 to activate mouse CD40 signalling, or KT3 as an isotype-matched control for the IC10 antibody. As shown in fig. 3.16A, the only treatment that increased IκB-alpha promoter activity was cotransfecting with human CD40, although this was fairly modest. Interestingly, cotransfection with KKV decreased luciferase activity relative to pcDNA3 levels, suggesting that KKV expression may negatively regulate IκB-alpha promoter activity, perhaps by acting in a dominant negative manner.

Cotransfection with CD40 was the only method that increased IκB-alpha promoter activity in WEHI-231 cells (fig. 3.16A). However, induction of IκB-alpha promoter activity was only approximately 2-fold. It was therefore determined whether cotransfecting with human CD40 and then treating with human CD40L could increase IκB-alpha promoter activity further. However, treatment with human CD40L did not specifically increase activity of the IκB-alpha promoter (fig. 3.16B). Non-specific increases in IκB-alpha promoter activity were obtained when treating KKV or pcDNA3 cotransfected cells with CD40. Therefore, although human CD40L is documented to be specific for human CD40 and should not affect mouse cells, it was having a non-specific effect on transcription. However these results confirm the

A



B

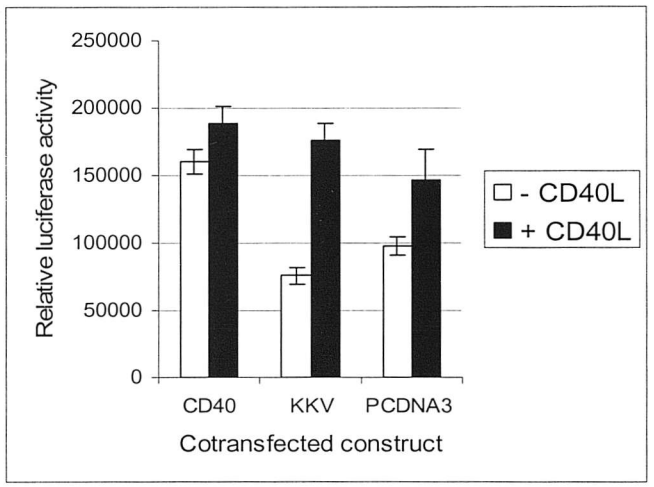


Figure 3.16: Optimising methods to activate CD40 in WEHI-231 cells transfected with the CD40-responsive IKB-alpha promoter. A – 20μg of IKB-alpha promoter plasmid plus 20μg of either pcDNA3, CD40 or KKV plasmids were transfected into WEHI-231 cells by electroporation. pcDNA3 cotransfected cells were split after 24 hours and cultured for a further 24 hours with 1μg/ml moCD40L or 5μg/ml IC10 antibody to activate CD40, or 5μg/ml KT3 antibody as an isotype-matched control for IC10. **B** - 20μg of IKB-alpha promoter plasmid plus 20μg of either pcDNA3, CD40 or KKV plasmids were transfected into WEHI-231 cells by electroporation. Each transfection was then split in two after 24 hours, one half was left untreated and the other half was treated with 1μg/ml human CD40L for 24 hours. In both experiments, luciferase activity was measured and normalised to β-galactosidase activity, to account for differences in transfection efficiency. Data are mean of duplicate transfections from the same experiment and error bars show plus and minus one standard deviation. These results are representative of two independent experiments.

findings of fig. 3.16A, showing that overexpression of CD40 was sufficient to increase activity of the IκB-alpha promoter.

Since cotransfection with human CD40 was the best method for inducing CD40 signalling in transfected WEHI-231 cells, cells were cotransfected with Bcl-X promoter constructs and CD40, or pcDNA3 as the empty vector control. The different Bcl-X promoter luciferase reporter constructs used are shown in fig. 3.17A. The Bcl-X gene contains at least three promoters, one upstream of exon IB (promoter 1B) (MacCarthy-Morrogh *et al* 2000), one upstream of exon IA (promoter 1A) and one upstream of exon II (promoter 2) (Grillot *et al* 1997). Constructs were used with these sequences cloned upstream of the luciferase coding sequence in pGL2 basic. The entire region between and including the 1A and 2 promoters was also cloned into pGL2 basic resulting in the promoter 1A-2 construct. Cells were also cotransfected with CD40 or pcDNA3 and the entire mouse Bcl-X genomic region (3.2kb), as a positive control for CD40 signalling. This construct contains multiple promoters and should respond to CD40 signalling (Grillot *et al* 1997). Cells were also cotransfected with CD40 or pcDNA3 and the IκB-alpha promoter, as a positive control. As shown in fig. 3.17B, the Bcl-X IB promoter was far more active (approximately 20-fold) than the IA, 2 or IA-2 constructs. The activity of the 1A, 2 or 1A-2 promoters was similar to that of the empty pGL2 basic plasmid alone. However, even activity of the Bcl-X IB promoter and mouse 3.2kb region was very modest compared to the activity of the IκB-alpha promoter. This is consistent with the fact that WEHI-231 cells do not express any Bcl-X protein when unstimulated. Cotransfection of CD40 with either the human or mouse Bcl-X promoter constructs did not specifically enhance promoter activity, despite increasing activity of the IκB-alpha promoter. To confirm that transfection of the CD40 expression construct induced expression of CD40 on the cell surface, 293 cells, which do not express CD40, were transfected with the CD40 expression plasmid and CD40 expression determined by flow cytometry (data not shown).

Since WEHI-231 cells were not suitable to study regulation of Bcl-X transcription by CD40, it was decided to try a different system. RL cells (human diffuse large B-cell lymphoma line) were chosen since they upregulate Bcl-X_L in response to CD40L (see

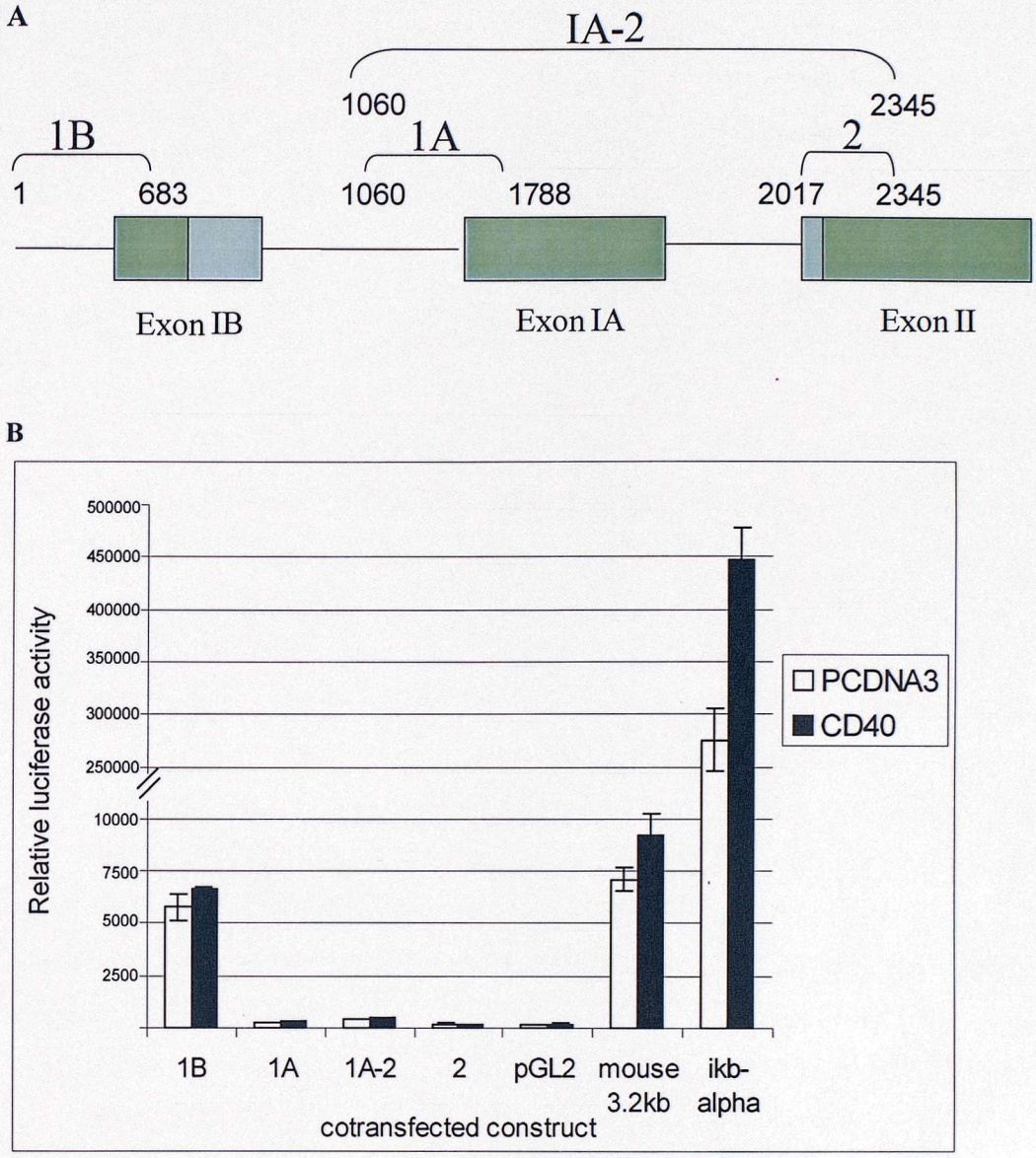


Figure 3.17: A - Bcl-X promoter reporter constructs. Constructs were obtained with the promoter regions indicated (1B, 1A, 1A-2 and 2) cloned upstream of luciferase in pGL2 basic. The values indicate the position in bases of each promoter, relative to the start of the 1B promoter region. Blue regions indicate alternatively spliced regions. The translation start site is within exon II. **B - The effect of cotransfecting CD40 on activity of Bcl-X promoters and IκB-alpha promoter.** 20μg of each promoter construct were cotransfected with either 20μg PCDNA3 or 20μg CD40 plasmid, plus 2μg β-gal plasmid, into WEHI-231 cells by electroporation. The constructs used were Bcl-X promoter constructs (1B, 1A, 1A-2, 2) or pGL2 basic as a control, the entire mouse Bcl-X genomic region (3.2kb) and IκB-alpha promoter as a positive control. Luciferase activity was measured and normalised to β-galactosidase activity, to account for differences in transfection efficiency. Data are mean of duplicate transfections from the same experiment and error bars show plus and minus one standard deviation. These results were representative of two independent experiments.

chapter 4) and proved easy to transfect by electroporation. RL cells also constitutively express Bcl-X_L and should therefore have a higher basal level of Bcl-X promoter activity than WEHI-231 cells. RL cells were transfected with the Bcl-X promoter constructs and each transfection was then split after 24 hours. Half of the cells were left untreated and the other half was treated with 1μg/ml CD40L for 24 hours. As shown in fig. 3.18, promoter 1B has the highest activity, followed by promoter 2 and promoter 1A-2, with promoter IA having the least activity. All promoters showed increased activity relative to pGL2 basic, consistent with basal expression of Bcl-X in these cells. Treatment with CD40L did not enhance activity of any of the Bcl-X promoters. In fact treatment actually appeared to modestly decrease activity of promoter 1B and promoter 2 (fig. 3.18A).

Since the addition of CD40L to transfected RL cells did not increase Bcl-X promoter activity, cells were cotransfected with the plasmid encoding human CD40 as a means to induce CD40 signalling. This was because electroporation disrupts cell membranes and so the native CD40 expressed by RL cells may no longer be in the correct context for signalling following electroporation. Overexpression of CD40 may overcome this problem and induce trimerisation of the receptor and receptor activation due to expression of large amounts of the receptor. However cotransfecting CD40 with the Bcl-X promoter constructs also did not enhance promoter activity (fig. 3.18B). A last attempt was to try cotransfecting CD40 and also treating cells with CD40L. Perhaps overexpressed CD40 is not sufficient to activate signalling in this system and so CD40L may be required to activate signalling. As shown in fig 3.18C, treatment of CD40 transfected cells did not enhance Bcl-X promoter activity.

Therefore electroporation of RL cells was not a sufficient model system to study the regulation of Bcl-X transcription by CD40. Electroporation may be too harsh a transfection method to allow CD40 signalling to the Bcl-X promoters since it kills a high proportion of cells and disrupts cell membranes and so may interfere with cell surface signalling. It was therefore decided to try DEAE-dextran to transfect RL cells. DEAE-dextran is a gentle transfection method, which causes little cell death but at the expense of decreased transfection efficiency. This system has previously been used to study promoter regulation (ornithine decarboxylase) via extracellular stimuli (IL-3) in

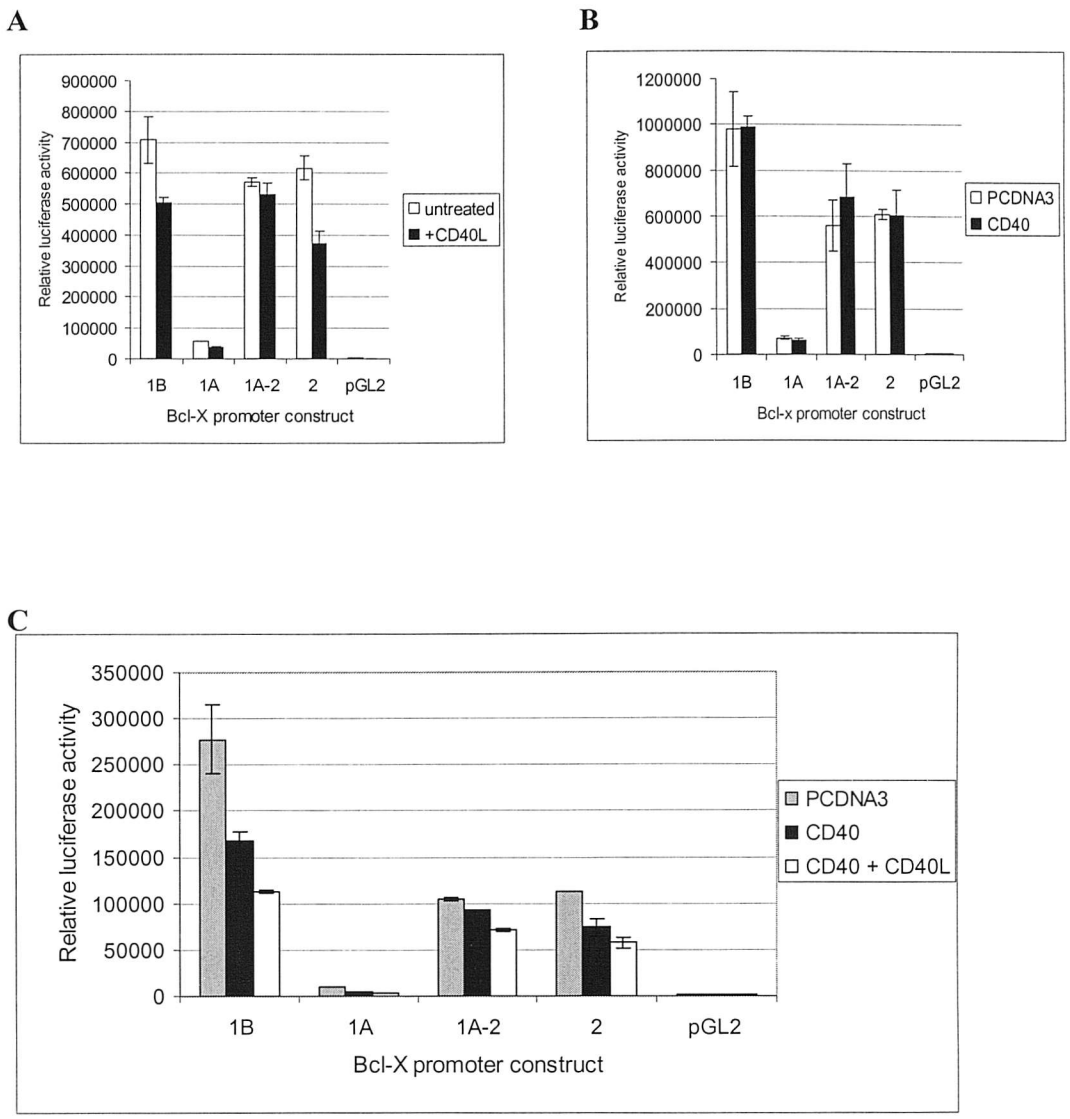


Figure 3.18: The effect of activating CD40 on Bcl-X promoter constructs in RL cells. A – RL cells were transfected by electroporation with 20μg of each Bcl-X promoter construct or pGL2 basic, plus 2μg β-gal plasmid. After 24 hours, the cells were split in two, half were left untreated and half were treated with 1μg/ml CD40L for 24 hours. **B** - RL cells were transfected by electroporation with 20μg of each Bcl-X promoter construct or pGL2 basic, plus 20μg of either pcDNA3 or CD40 plasmid, plus 2μg β-gal plasmid. Cells were harvested after 48 hours. **C** – Experiment was performed as in B except after 24 hours each transfection was split in two and half of the CD40 transfected cells were treated with 1μg/ml CD40L for 24 hours. The remaining cells were left untreated. In each experiment, luciferase activity was measured and normalised to β-galactosidase activity, to account for differences in transfection efficiency. Data are mean of duplicate transfections from the same experiment and error bars show plus and minus one standard deviation.

a myeloid cell system (Packham & Cleveland 1997) and has been used to study promoter regulation by CD40 in B-cells (Agresti & Vercelli 2002, Dunn *et al* 2000, Worm *et al* 1998). RL cells were transfectable at a very low efficiency with DEAE dextran. Addition of CD40L to the transfected cells did not influence Bcl-X promoter activity (data not shown). However DEAE-dextran transfected cells were still able to increase Bcl-X_L protein expression in response to CD40L treatment (data not shown).

As trying to determine how CD40 regulates the Bcl-X promoters proved difficult in B-cell lines, non-lymphoid cell systems were tried. The mouse dendritic cell lines DC2.4 and FSDC were used since they have been used as a model system to study CD40 regulation of the IL-6 promoter (Mann *et al* 2002). Cells were cultured with 1μg/ml and 2μg/ml moCD40L for up to 24 hours, or left untreated as a control, and Bcl-X_L protein expression analysed by western blot. Both of the cell lines showed high basal levels of Bcl-X_L and neither of the lines upregulated Bcl-X_L expression in response to CD40 activation (data not shown) and were therefore of no use to study Bcl-X regulation by CD40. 293 cells were also tried as a model system. These cells are easy to transfect using Fugene-6 and many studies have used this line to study CD40 signalling events by transfecting a CD40 expression vector (Tsukamoto *et al* 1999, Pullen *et al* 1999, Luftig *et al* 2001, Werneburg *et al* 2001, Ye *et al* 2002). Overexpressing CD40 on the cell surface of 293 cells did not affect activity of the Bcl-X promoters (data not shown).

Therefore despite the use of many different systems no induction of Bcl-X promoter activity by CD40 was observed. Overexpression of CD40 or activation of endogenous CD40 did not influence Bcl-X promoter activity in either WEHI-231, RL or 293 cells, despite the use of a variety of transfection methods. These results suggest that the Bcl-X promoter constructs used may not be the elements that respond to CD40 signalling or they could in the wrong context for transcriptional activation e.g. they may need the presence of additional enhancer elements for their function or they may need to be modified by histones. Alternatively, Bcl-X induction by CD40 may not occur through a transcriptional mechanism e.g. through stabilisation of RNAs.

3.9 Translational control of Bcl-X_L

This study also investigated translational control of Bcl-X expression. Many genes involved in the regulation of proliferation and apoptosis have long (over 200 bases) and highly structured 5'-untranslated regions (5'-UTRs) which regulate downstream translation, e.g. Bcl-2, TGF-β family, c-myc, p53 (Willis *et al* 1999, Clemens & Bommer 1999). A variety of Bcl-X mRNAs can be generated with very different 5'-UTRs, through utilisation of different promoters and alternative splicing events (see fig. 1. 9) and the significance of these different leader sequences is unknown. Bcl-X mRNAs can either contain exon IA or exon IB as an upstream non-coding exon. There is an alternatively spliced region of 130bp at the 3' end of exon IB (Alterantively Spliced region 1 or AS1) and an 18bp alternatively spliced region at the 5' end of exon II (Alternatively Spliced region 2 or AS2). These alternative splicing events can result in four different exon IB containing splice forms, all of which can be detected in lymphoma cells (MacCarthy-Morrogh *et al* 2000).

One of the mechanisms by which translation is influenced by 5' UTRs is through the presence of upstream open reading frames (uORFs) in the 5'-UTR which can inhibit translation of the downstream coding region. In the Bcl-X sequence, both exon IA and AS1 of exon IB contain two uORFs, which have an unknown function (figs. 3.19-3.20). The Bcl-2 gene also contains a single uORF which represses Bcl-2 translation (Harigai *et al* 1996) and as the Bcl-X gene is highly homologous to the Bcl-2 gene in both sequence and structure (Boise *et al* 1993), it is possible that translation of Bcl-X is regulated by a similar mechanism.

Translational control of Bcl-X_L protein expression by CD40 may also be important in primary B-cell lymphomas. Differential regulation of exon IB containing splice forms was observed, in primary malignant B-cells, by CD40 (fig 3.12). This suggests that alternative splicing of exon IB containing transcripts could generate an extra level of control on Bcl-X expression. Furthermore, increases in Bcl-X_L protein expression in CD40L treated lymphoma cells could not be completely accounted for by changes in mRNA expression, indicating translational control maybe important.

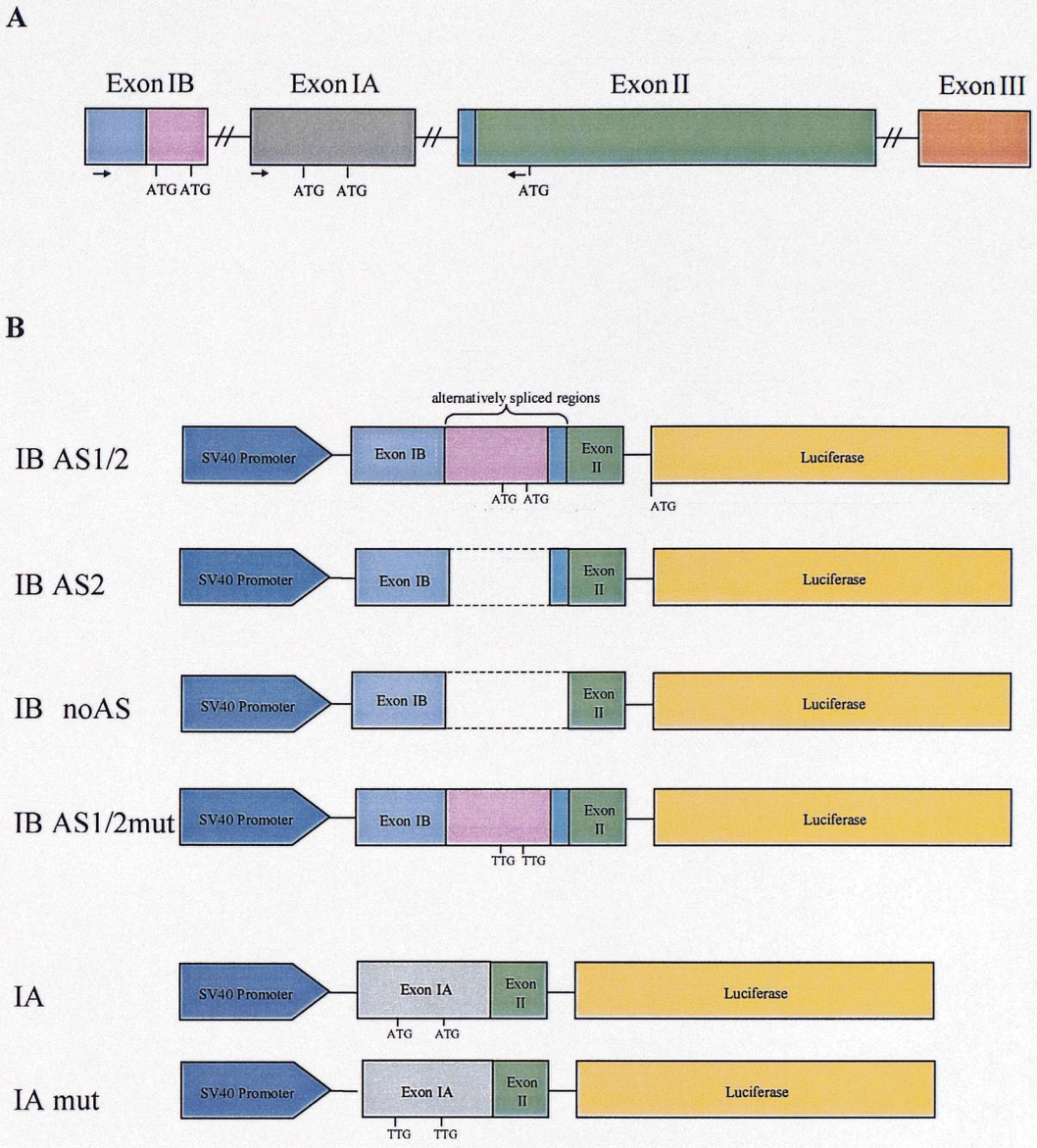


Figure 3.19: Bcl-X 5' UTR pGL2 promoter constructs. A - The Bcl-X gene. The Bcl-X gene contains 4 exons. Exons IA and IB are non-coding and mRNAs can contain either of these exons, not both. The pink region of exon IB (AS1) and the blue region of exon II (AS2) can be alternatively spliced. The translation start site (ATG) is in exon II, but there are also upstream open reading frames (uORFs) in AS1of exon IB and in exon IA (as indicated by ATG). The Bcl-X_L/X_S splice site is located 189bp from the 3' end of exon II. The translation termination site is within exon III. Arrows indicate the position of PCR primers used to generate the clones shown in section B. A common reverse primer was used in combination with primers specific for either exon IB or exon IA. **B** – Bcl-X 5' UTRs, including different splice forms, were cloned into pGL2 promoter vector upstream of luciferase and downstream of the SV40 promoter. The two ATGs in exon IB AS1 and in exon IA were mutated to TTG, generating constructs IB AS1/2mut and IA mut.

A

aag tga ctg agc ttg caa gtt ccc ctg tct ctt
cag ggg aaa ctg agg ccg gct tgt tcg gga gag
acg gcg cga gca gtc agc cag gta ggc cgg cag
cca ggt agg ccg gcc cgg gtc cgc ggc gcg gaa
ctc ggc cgc gaa gag ctc ttg cgt ctg gaa gct
acc ggg ccg atg aag ggg gat gtg gcc ccc cac
ggc tcg cgg ggc tcg cag ctc ttt ctc tcc ctt
cag aat ctt atc ttg gct ttg gat ctt aga aga
gaa tca cta acc aga gac gag act cag tga gtg
agc agg tgt ttt tgg gac aat gga ctg gtt gag
ccc atc cct a

B

cag tag gag agc caa ggg gcg tgc aag aga gag
ggg gct ggg ctc ccg ggt ggc agg agg ccg cgg
ctg cgg agc ggc cgc cct cga tcc ggg cga tgg
agg agg aag caa gcg agg ggg ctg gtt cct gag
ctt cgc aat tcc tgt gtc gcc ttc tgg gct ccc
agc ctg ccg ggt cgc atg atc cct ccg gcc gga
gct ggt ttt ttt gcc agc cac cgc gag gcc ggc
tga gtt acc ggc atc ccc gca gcc acc tcc tct
ccc gac ctg tga tac aaa aga tct tcc ggg ggc
tgc acc tgc ctg cct ttg cct aag gcg gat ttg
aat ctc ttt ctc tcc ctt cag aat ctt atc ttg
gct ttg gat ctt aga aga gaa tca cta acc aga
gac gag act cag tga gtg agc agg tgt ttt tgg

C

Kozak:	gcc	gcc	**a**cc	aug	**g**
IB uORF1:	acc	ggg	ccg	aug	**a**
IB uORF2:	uga	agg	**g**gg	aug	u
IA uORF1:	atc	cgg	**g**cg	aug	**g**
IA uORF2:	ccg	ggu	cgc	aug	**a**

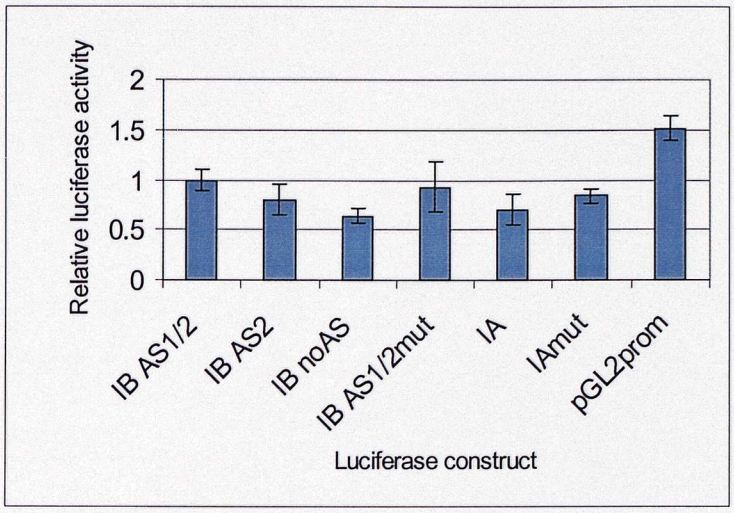
Figure 3.20: Bcl-X 5' untranslated regions cloned into pGL2 promoter A – Bcl-X exon IB and exon II sequence 5' to the translation start site, showing alternatively spliced regions and uORFs. AS1 is highlighted in pink and AS2 is highlighted in blue. The ATGs of the uORFs in exon IB are highlighted in green and the stop codons in red. The first uORF is 40 or 34 codons in length and the second uORF is 29 or 33 codons in length, depending if AS2 is present. **B** - Bcl-X exon IA and exon II sequence 5' to the translation start site, showing alternatively spliced regions and uORFs. AS2 is highlighted in blue. The ATGs of the uORFs in exon IA are highlighted in green and the stop codons in red. The first uORF is 12 codons in length and the second uORF is 18 codons long. **C** – Comparison of the uORF initiator codons to the Kozak consensus sequence for translation initiation. Strong initiator codons have purines at positions –3 and +4, relative to the translation initiation codon (aug), which are highlighted in bold.

Therefore the aim of this study was to determine whether different 5'-UTRs of Bcl-X transcripts were important in the control of Bcl-X_L expression. To determine if these leader sequences altered expression of a downstream ORF, the various Bcl-X 5'-UTRs were cloned downstream of the SV40 promoter and upstream of the luciferase ORF, in the pGL2 promoter vector, thereby substituting the Bcl-X coding sequence for that of luciferase (fig. 3.19 and 3.20). The plasmids contained either exon IB-exon II with AS1 and AS2 (IB AS1/2), exon IB-exon II without AS1 but containing AS2 (IB AS2), exon IB-exon II without any alternatively spliced regions (IB noAS), or exon IA-exon II without any alternatively spliced regions (IA). Unfortunately it was not possible to successfully clone exon IB-exon II containing AS1 but not AS2, although this mRNA can be detected in lymphoma cells (MacCarthy-Morrogh *et al* 2000). In theory, two versions of exon IA-exon II were possible, via alternative splicing of AS2. However, only the version missing AS2 was cloned from Elijah Burkitt's lymphoma cells.

The translation start codons (ATG) of the uORFs in exon IA and AS1 of exon IB are conserved in the mouse Bcl-X gene and are in a reasonable context for translation initiation, as determined by the Kozak consensus sequence, as they possess purines at either of the critical –3 and +4 positions (fig. 3.20C) (Kozak 1989). Site-directed mutagenesis was undertaken to mutate the ATGs in exon IA and AS1 of exon IB to TTG, in order to determine the effect of these uORFs on expression of the downstream luciferase ORF. This resulted in constructs IB AS1/2mut and IA mut (fig. 3.19).

The resulting reporter constructs were transfected into the Burkitt's lymphoma line DG75 and 293 cells, and luciferase activity detected (fig. 3.21). All luciferase constructs in DG75 cells showed decreased expression compared to the parental pGL2 promoter vector (fig. 3.21A) and this was highly statistically significant for the IA and IB AS1/2 constructs (table 3.3). This is probably due to the fact that longer mRNAs are produced from the constructs that are translated less efficiently. There was no significant difference in expression between the exon IB or exon IA containing constructs nor was there any influence of the alternatively spliced regions on luciferase expression.

A DG75



B 293

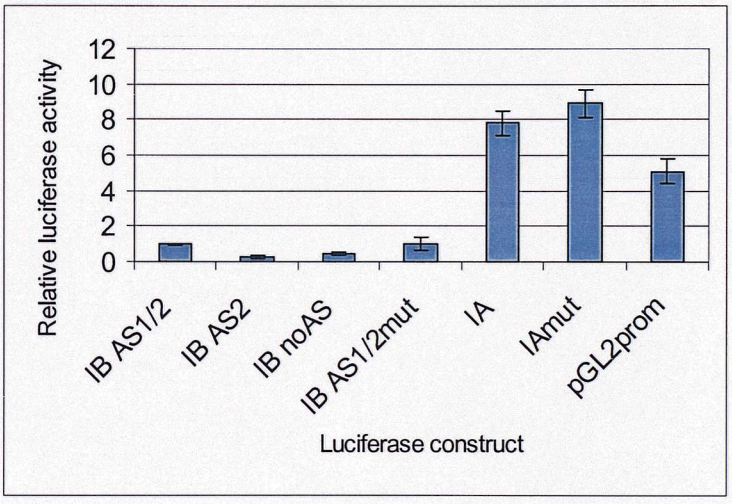


Figure 3.21: Expression of Bcl-X IB and IA constructs in DG75 (A) and 293 (B) cells. A – 10μg of each construct was transfected into DG75 cells by electroporation, along with 2μg β-galactosidase plasmid. **B** – 0.6μg of each construct was transfected into 293 cells, using Fugene, along with 0.4μg β-galactosidase plasmid. In both cell lines studied, luciferase activity was measured and normalised to β-galactosidase activity. All transfections were carried out in duplicate. The bars represent the mean of two independent experiments, each performed in duplicate, and are normalised to the mean value obtained for the first bar (set to 1). Error bars show plus and minus one standard deviation of the mean.

Table 3.3: Independent samples t-test results for data from fig. 3.21. Independent samples t-tests were performed using SPSS, equal variances not assumed. n.s. = not significant, p > 0.05. * = p less than or equal to 0.05. ** = p less than or equal to 0.01. ***= p less than or equal to 0.001. If a difference was significant than the relationship is indicated in the significance column.

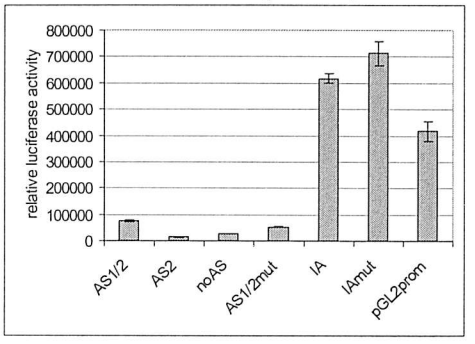
	DG75			**293**		
	t value	p value	significance	t value	p value	significance
AS1/2 v AS2	2.07	0.09	n.s	14.1	<0.001	*** AS1/2>AS2
noAS v AS2	1.88	0.13	n.s	2.45	0.06	n.s.
AS1/2 v AS1/2 mut	0.51	0.64	n.s.	0.61	0.58	n.s.
AS1/2 vs IA	2.40	0.06	n.s.	5.59	0.011	** IA>AS1/2
IA v IAmut	1.56	0.19	n.s	1.56	0.19	n.s.
IA v pGL2prom	8.17	<0.001	*** pGL2>IA	8.17	<0.001	*** IA>pGL2
AS1/2 v pGL2 prom	6.45	0.001	*** pGL2>IB	6.45	0.001	*** pGL2>IB

In contrast, the Bcl-X 5' UTRs showed dramatic changes in luciferase expression when transfected into 293 cells. IA constructs showed 8 fold more activity than the IB AS1/2 construct, in 293 cells, which was highly significant (fig. 3.21B, table 3.3). Indeed, the presence of exon IA in the 5'UTR of the reporter construct, significantly enhanced luciferase activity relative to the parental pGL2 promoter construct (fig. 3.21B, table 3.3). Interestingly, the exon IB construct containing AS1 showed 4 fold more activity than exon IB constructs lacking this region, which was also highly statistically significant (fig. 3.21B, table 3.3).

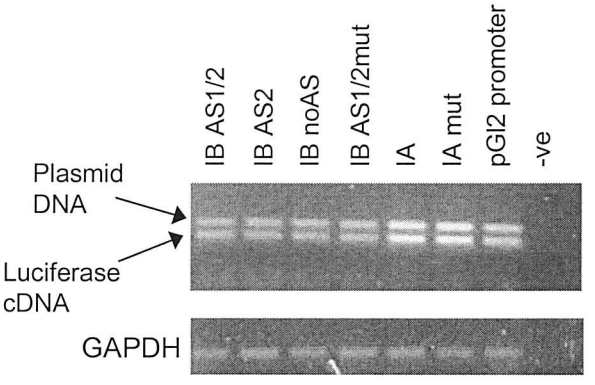
Mutating the uORFs in exon IA or AS1 of exon IB did not significantly alter luciferase activity relative to the unmutated construct in either cell line, indicating that the uORFs in exon IA or exon IB do not act as repressors of translation (fig 3.21, table 3.3).

The differences in luciferase activity observed in figure 3.21 could be due to a number of factors, such as increased translation of exon IA and AS1 containing RNAs or the inserted IA or IB sequences could influence transcription of the luciferase gene, mRNA processing or mRNA transport. In order to account for these factors and to determine if the IA and IB exons were influencing translation, the levels of luciferase RNA were studied directly. 293 or DG75 cells were transfected with the IB and IA constructs and half of the transfected cells were used for a luciferase assay and RNA was isolated from the remainder. The RNA was treated with DNase to remove as much plasmid DNA as possible and then reverse transcribed. It was impossible to remove all the plasmid DNA from the RNA preparations, so luciferase PCR primers were designed which spanned the SV40 intron (65 base pairs) present in the luciferase ORF, to distinguish between luciferase cDNA and plasmid DNA (fig. 3.22B). The number of PCR cycles performed was carefully optimised to gain a semi-quantative result. The number of PCR cycles was decreased until faint bands just at the level of detection were observed. These bands were therefore generated while the PCR reaction was in its linear phase. Luciferase expression was quantified and normalised to GAPDH levels, to account for any variability in the efficiency of the reverse transcription reaction. The values from the luciferase assay were then normalised to the RT-PCR values, to give an indirect value for translation.

A Luciferase Activity Assay



B Luciferase RT-PCR



C 'Translational' effect

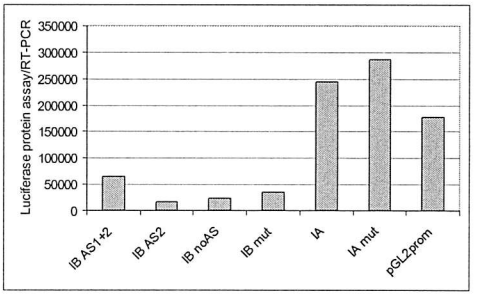


Figure 3.22: 293 cells were transfected with 0.6μg of the various constructs + 0.4μg β-gal plasmid, using Fugene. **A - Luciferase assay**. As previously described in fig. 3.22. **B – Semi-quantitative RT-PCR**. RNA was isolated from transfected cells, treated with DNase and reverse transcribed. Primers specific for luciferase or GAPDH were used to amplify fragments by PCR. Luciferase primers were designed across the SV40 intron to discriminate any plasmid DNA from luciferase cDNA. PCR cycle number was decreased until faint bands were observed to give a semi-quantitative result. **C – Effect of translation.** RT-PCR values were quantified and normalised to GAPDH to account for any variability in cDNA synthesis. These values were then used to exclude variations in RNA expression from the luciferase activity assay values by dividing the luciferase assay values by the RT-PCR values. This experiment was representative of two independent experiments.

As shown in fig. 3.22, there were relatively equal levels of luciferase RNA generated from the IB constructs in 293 cells, demonstrating that the positive effect of AS1 was probably due to increased translation. Although there was slightly increased IA luciferase RNA expression (approximately 2-fold), relative to the IB constructs, this was not as great as the increase observed in the luciferase activity assay (approximately 12-fold in this experiment). Therefore most of the activity of the IA region in 293 cells probably results from increased translation of the luciferase gene. Consistent with the lack of effect of the Bcl-X 5' UTRs on luciferase activity in DG75 cells, luciferase RNA expression was equal from each construct (data not shown).

In summary, the 5' UTRs of Bcl-X mRNAs can influence gene expression in a cell-type specific manner. In DG75 cells the presence of different Bcl-X 5' UTRs did not influence gene expression. However, in 293 cells, the presence of exon IA promoted translation and exon IA containing Bcl-X mRNAs were translated with much greater efficiency than exon IB containing mRNAs. Alternative splicing events in exon IB modified the efficiency of translation of the downstream gene, since mRNAs containing AS1 of exon IB were translated with greater efficiency than exon IB containing mRNAs lacking this region. Mutating the uORFs in exon IA or exon IB did not influence gene expression in either cell line.

3.10 Discussion

The aims of this study were to determine the effect of CD40 ligation on both cell survival and the expression of apoptosis regulators in primary B-cell lymphomas. The role and regulation of the key CD40-target genes Bcl-X_L and Mcl-1 was also explored. This study demonstrated that CD40L is a potent survival signal for human lymphoma cells, regardless of histological type. CD40L can rescue the Burkitt's lymphoma cell lines, Akata 6 and Elijah, from etoposide-induced apoptosis, as assessed by quantification of PARP cleavage and Annexin-V staining. In over twenty primary samples, CD40L rescued B-cell malignancies and normal B-cells from both spontaneous apoptosis occurring in the culture and etoposide-induced cell death.

Primary samples showed varying degrees of spontaneous apoptosis and different sensitivities to the effects of both etoposide and CD40L, but in all cases a positive effect of CD40 signalling on cell survival was observed. Differences in sensitivities

did not appear related to the type of malignancy, although CLL samples showed the greatest variation in sensitivity to CD40 signalling. Differences in sensitivity to CD40 signalling could be a function of the heterogenous nature of the samples, for example many differences in cytogenetics were observed, fresh or frozen samples were used and some patients had been treated with chemotherapy prior to analysis. It is difficult to make meaningful comparisons between these small groups but despite these differences CD40 signalling consistently improved cell survival. It is well documented that CD40 signalling protects normal B-cells, lymphoma cell lines, follicular lymphoma, CLL, and AML cells from apoptosis (Kelly & Knox 1995, Tsubata *et al* 1993, Padmore *et al* 1997, Johnson *et al* 1993, Ghia *et al* 1998, Kitada *et al* 1999, Aldinucci *et al* 2003, Dallman *et al* 2003). However a number of studies using malignant B-cell lines have shown that CD40 activation can induce growth arrest and apoptosis (Heath *et al* 1993, Funakoshi *et al* 1994, Lefterova *et al* 2000, Bergamo *et al* 1997). CD40-induced cell death has not, however, been observed in any primary B-cell malignancies cultured *ex vivo.*

Agents that activate CD40, including anti-CD40 antibodies, soluble CD40L or cells modified to express CD40L, have been used to successfully treat both CD40 positive and CD40 negative tumours in mouse model systems (Funakoshi *et al* 1997, Tutt *et al* 1998, French *et al* 1999, Kikuchi *et al* 2000, Todryk *et al* 2001). This anti-tumour effect of CD40-based therapy was largely due to destruction of the tumour by cytotoxic T-cells, which were activated by CD40-stimulated antigen presenting cells e.g. dendritic cells, and occured independent of the CD40 status of the tumour (French *et al*1999, Todryk *et al* 2001, Bennet *et al* 1998). However anti-tumour effects were also observed in SCID mice, which have a severe deficiency in both B and T-cells (Funaksohi *et al* 1997, Szocinski *et al* 2002, Francisco *et al* 2000). Therefore CD40-therapy may also be achieved via other mechanisms e.g. through direct signalling effects of CD40 on tumour cells leading to apoptosis, antibody-mediated cell cytotoxicity, activation of complement by the antibody, or through activation of natural killer cells. These studies led to interest in using CD40-based therapy to treat human malignancy. A CD40L-gene therapy trial in CLL and a phase I clinical trial using CD40L have shown some encouraging results (Wierda *et al* 2000, Vonderheide *et al* 2001). However data presented here raises concerns over the safety of these clinical trials. According to this data, activation of CD40 on primary B-cell

malignancies will promote tumour cell survival and even protect cells from the cytotoxic effects of chemotherapy. Unless the immune modulatory or other anti-tumour effects of CD40-therapy are strong enough to outweigh the survival advantage of CD40 signalling to the tumour, CD40-based therapy could prove detrimental to the patient. Future trials may be better restricted to CD40 negative tumours, or CD40 positive tumours where a clear apoptotic effect of CD40 ligation has been demonstrated.

However this study was performed on isolated primary B-cell malignancies cultured *ex vivo* and this may not reflect the situation *in vivo*. The presence of other cellular signals and the cell microenvironment may influence the outcome of CD40 signalling *in vivo*. Spontaneous apoptosis observed in the primary culture *in vitro* may reflect the absence of a survival signal in culture that may be present in body. CD40 may be one such signal, as addition of CD40L rescued cells from spontaneous apoptosis. Infiltrating T-cells in the tumour could provide the CD40 stimulus but coexpression of CD40 and CD40L in CLL and Hodgkin's disease has also been observed (Clodi *et al* 1998, Schattner *et al* 1998, Clodi *et al* 2002). The serum of CLL patients was also shown to contain elevated cells of biologically active soluble CD40L (Clodi *et al* 1998, Younes *et al* 1998). Interestingly, transcription of CD40 and CD40L can be coordinately regulated by the AT-hook transcription factor AKNA (Siddiqa *et al* 2001). Therefore, in lymphoma constitutive activation of the CD40-CD40L pathway may be present due to autocrine or paracrine stimulation and this may promote the survival of the tumour. Blocking the CD40/CD40L interaction in lymphoma could represent a target for therapy.

The effect of CD40L on the expression of apoptosis regulators in lymphoma cell lines and primary malignant B-cell samples was investigated in this study. CD40 ligation is known to induce expression of the anti-apoptotic proteins Bcl-X_L, Mcl-1, Bfl-1 and A20 in B-cell lines but very few studies have been performed using primary human lymphoma samples (Lee *et al* 1999, Kuss *et al* 1999, Sarma *et al* 1995). This is the first study to systematically compare expression of these CD40 targets in a range of different primary B-cell malignancies. The results show that CD40 activation leads to an increase in protein expression of the anti-apoptotic protein Bcl-X_L in all cells studied. In the cell lines, this occurred after 4-8 hours of CD40 stimulation and peaked

at 16-24 hours, whereas in the primary samples Bcl-X_L protein expression was maximal at 48 hours of CD40 stimulation. The extent to which CD40 influenced Bcl-X_L protein expression in the primary samples was dramatic, with every single sample upregulating Bcl-X_L expression in response to CD40 ligation. This is consistent with the findings of studies in cell lines, normal B-cells, CLL, AML and follicular lymphoma (Zhang *et al* 1996, Ning *et al* 1996, Lee *et al* 1999, Kitada *et al* 1999, Ghia *et al* 1998, Aldinucci *et al* 2003).

Bcl-X_L is a key anti-apoptotic protein, shown to protect cells from a wide range of apoptotic stimuli, including chemotherapeutics (Boise *et al* 1993, Minn *et al* 1995, Fang *et al* 1995, Schott *et al* 1995). In normal B-cells, it is expressed at the pro B-cell stage or upon B-cell activation (Chao & Korsmeyer 1998) and in the lymphoma samples tested in this study basal Bcl-X_L expression was very low. Therefore upregulation of Bcl-X_L by CD40 in lymphoma cells should increase tumour cell survival and resistance to chemotherapy, especially if the CD40 pathway is constitutively activated. However the role of Bcl-X_L in CD40-mediated cell survival has not been determined. Although Bcl-X_L was clearly upregulated in all primary samples tested, these samples had differing sensitivities to CD40 ligation e.g. CD40 ligation of CLL-d resulted in a very modest improvement in cell survival but also greatly upregulated Bcl-X_L expression. Clearly expression of other apoptosis controlling proteins also determines the extent to which CD40 promotes survival. Antisense oligonucleotides to Bcl-X were used in this study in a Burkitt's lymphoma cell line which overexpresses Bcl-X_L (Akata 6) and demonstrated that Bcl-X_L is indeed a critical protein for lymphoma cell survival.

As Bcl-X_L is a key CD40 target gene in lymphoma cells and promotes tumour cell survival it could be an attractive target for cancer therapy, either alone or in combination with CD40-based therapies. A variety of approaches have been undertaken to target anti-apoptotic Bcl-2 family proteins in cancer, including antisense therapy and design of small molecule inhibitors that bind the BH3-domain binding pocket (BH3 mimetics) (Oxford *et al*, in press).

RT-PCR analysis was carried out to determine which Bcl-X transcripts were regulated by CD40 signalling. Bcl-X transcription can occur from at least three different

promoters located upstream of exon II, exon IA or exon IB. Bcl-X mRNAs can therefore contain either exon IA or exon IB upstream of the coding sequence (Grillot *et al* 1997, MacCarthy-Morrogh *et al* 2000). There is alternative splicing of the exon IB transcripts, generating a large and small splice variant. In Akata 6 and Elijah cells, only exon IA-containing transcripts were induced during CD40 signalling. In the primary samples, CD40 signalling was capable of regulating both exon IA and exon IB containing Bcl-X transcripts but there was differential regulation of these transcripts between samples.

To elucidate the mechanisms by which CD40 leads to induction of Bcl-X transcription, the different Bcl-X promoter regions (promoter 1A, promoter 1B, promoter 2 and the region spanning promoter 1A to promoter 2) were transfected into various cell lines and different methods were used to try and activate CD40 in these systems, including overexpression of CD40 or activation of endogenous CD40. However none of these treatments activated the Bcl-X promoter constructs despite the use of appropriate controls. This could be due to a number of factors. Firstly, electroporation was used to transfect some of the cell lines and this is a harsh method of transfection leading to a high proportion of cell death and disruption of cell membranes, which may affect the ability of cells to signal through CD40. Activation of the IκB-alpha promoter by CD40 was observed using electroporation but this was fairly modest (2-fold induction) compared with previous studies (at least 10-fold induction, Mann *et al* 2002) suggesting that electroporation may have diminished the ability of cells to signal through CD40. A gentler method of transfection, DEAE-dextran was used to transfect RL cells, which still allows CD40 signalling (Agresti & Vercelli 2001, Dunn *et al* 2000, Worm *et al* 1998), but regulation of Bcl-X promoter activity was not observed. Secondly, the Bcl-X promoter constructs cloned may not be the CD40-responsive regions, although this seems unlikely since the IA-2 promoter contains the CD40-responsive NF-κB sites described by Lee *et al* (1999) and the exon IA promoter is also likely to be regulated by CD40 as suggested by the RT-PCR results. Thirdly, the Bcl-X promoter regions may not be in the correct context for transcriptional activation by CD40. They may require the presence of additional enhancer elements or may be modified by histones *in vivo* that contribute to their regulation. Indeed, histone deacetylase inhibitors have been shown to decrease Bcl-X_L

mRNA in mesothelioma cell lines, leading to apoptosis (Cao *et al* 2001). Fourthly, in the cell lines used CD40 may upregulate Bcl-X through a post-transcriptional mechanism or, in the case of 293 cells, may not possess the necessary factors for CD40 signalling to the Bcl-X promoter. A post-transcriptional mechanism of Bcl-X activation by CD40 is unlikely in RL cells, since CD40 upregulates exon IA mRNAs in this cell line and this is mediated through the DNA-binding activity of the NF-κB subunit p50 (chapter 4).

Data were obtained from these experiments regarding the relative activities of the Bcl-X promoters in B-cell lines. In WEHI-231 cells and RL cells, the exon IB promoter had the highest activity. In WEHI-231 cells the 1A, 1A-2 and 2 promoters had activities similar to the parental pGL2 basic construct, which is consistent with the fact that these cells do not express Bcl-X protein. In RL cells, the 1A-2 and 2 promoter constructs had similar activities, whereas the 1A promoter was relatively inactive. Therefore most of the promoter activity in the 1A-2 construct must be derived from the promoter 2 region.

RT-PCR analysis of Bcl-X expression in primary lymphoma cells also revealed differential regulation of the alternatively spliced exon IB-containing transcripts by CD40 in some samples (fig. 3.12). In addition, the massive increases in Bcl-X_L protein expression could not completely be explained by increased Bcl-X mRNA expression, therefore post-transcriptional control mechanisms such as translation must have a role in the regulation of Bcl-X expression. The functions of the upstream non-coding exons IA and IB are not known, nor is the effect of alternative splicing of exon IB, so the effect of these leader sequences on translation of the Bcl-X gene was investigated.

Bcl-X mRNAs can possess different 5' UTRs due to usage of different promoters and alternative splicing. The effect of these leader sequences on gene expression was determined by cloning these sequences upstream of the luciferase ORF and determining luciferase activity and RNA expression. Cell-type specific effects of the leader sequences were observed. All constructs were translated with similar efficiency in DG75 cells and repressed gene expression relative to the parental pGL2 promoter

construct. However, in 293 cells, the exon IA 5' UTR dramatically improved translation of the downstream sequence, compared to both the exon IB constructs and the parental pGL2 promoter construct. Also in 293 cells, the presence of the alternatively spliced region in exon IB (AS1) improved translation relative to IB constructs lacking this region. Both exon IA and AS1 of exon IB contain uORFs. Although the presence of uORFs is inhibitory for translation of the downstream for a number of genes e.g. Bcl-2, c-mos, Lck, TGFβ-3; mutating the translation start codons of the uORFs in the Bcl-X gene did not influence translation (Geballe & Morris 1994, Harigai *et al* 1996, Willis 1999).

Despite the positive effect of AS1 on translation in 293 cells, *in vitro* translation of the IB constructs, using a rabbit reticulolysate system, revealed that the presence of AS1 was actually inhibitory for translation *in vitro* (data not shown).

Therefore, exon IA and the AS1 region of exon IB must contain regions that are capable of increasing translation of the downstream gene, in a cell-type specific manner. Also, translation inhibition mechanisms, other than the presence of uORFs, must be present in AS1 that are responsible for the decreased translation efficiency *in vitro* but that are overcome *in vivo*. Perhaps the AS1 region is involved in forming secondary structures within the mRNA, such as hairpin loop structures, which are inhibitory for translation *in vitro* but promote translation *in vivo*. Secondary structures can inhibit translation by providing an obstacle to cap recognition and scanning of the mRNA. Cap recognition and scanning is dependent on binding of eIF4E to the cap, binding of eIF4E to the ribosome complex via eIF4G and unwinding of secondary structure by the helicase activity of eIF4A and eIF4B. Perhaps these factors are limiting *in vitro* therefore preventing translation, whereas *in vivo* these factors are present in excess allowing the effect of secondary structure to be overcome. Indeed eIF4E overexpressed in cells facilitates translation of mRNAs with extensive secondary structures (Clemens & Bommer 1999) but a study by Rau *et al* disputed previous *in vitro* data showing that eIF4E was not limiting in rabbit reticulocyte lysates (Rau *et al* 1996). However the activity of eIFs could be modulated *in vivo* through cell signalling pathways, leading to increased translation. Indeed eIFs, such as eIF4E and eIF2, can be regulated by phosphorylation. eIF4E can be phosphorylated

through protein kinase C and the p38 and ERK MAPK pathways (Kleijn *et al* 1998). eIF4E activity is also regulated by binding its binding proteins (4E-BPs), which are in turn regulated by phosphorylation through PI3-K (Clemens & Bommer 1999). 4E-BP1 sequesters eIF4E imparing its ability to form active complexes in association with eIF4G and may have an important regulatory role in reticulocyte lysates (Rau *et al* 1996).

Another possibility for translational regulation is that the AS1 or IA regions contain internal ribosome entry segments (IRESes). These sequences are highly structured and bind the ribosomal pre-initiation complex and direct the complex to the translation start codon, bypassing the need for cap recognition and scanning. IRESes were initially discovered in picornaviruses and have since been found in a number of cellular mRNAs including Bip, IGF-II, eIF4G, c-myc, FGF-2 and PDGF (Jackson *et al* 1990, Nanbru *et al* 1997, Bernstein *et al* 1997). Interestingly these mRNAs have 5' UTRs that repress translation of downstream reporters *in vitro* but promote translation *in vivo* (Willis 1999). This is thought to be due to the fact that the internal entry process requires cellular trans-acting factors. Indeed, IRESes can be considered as translation enhancers, allowing activation of genes in response to specific stimuli e.g. the PDGF-2 mRNA contains an IRES that gains activity upon differentiation of K562 cells. Also, the profile of proteins that bind to the PDGF-2 5' UTR dramatically changes during the differentiation of K562 cells (Bernstein *et al* 1997). Therefore an IRES in AS1 or exon IA could account for the differences in translation observed *in vitro* and *in vivo*. The presence of an IRES in AS1 of exon IB was tested by Anne Willis' labroratory (Leicester, UK), using a dicistronic reporter construct, with the AS1 region inserted between the two coding sequences. In the parental construct, the downstream cistron is translated much less efficiently than the upstream one, but if a sequence containing an IRES is inserted between the two cistrons, the protein product of the downstream cistron is detected earlier and in higher yield than the upstream one (Jackson *et al* 1990). However no IRES activity was found in the AS1 region in a variety of cell types. It remains to be determined if there is an IRES in the exon 1A region.

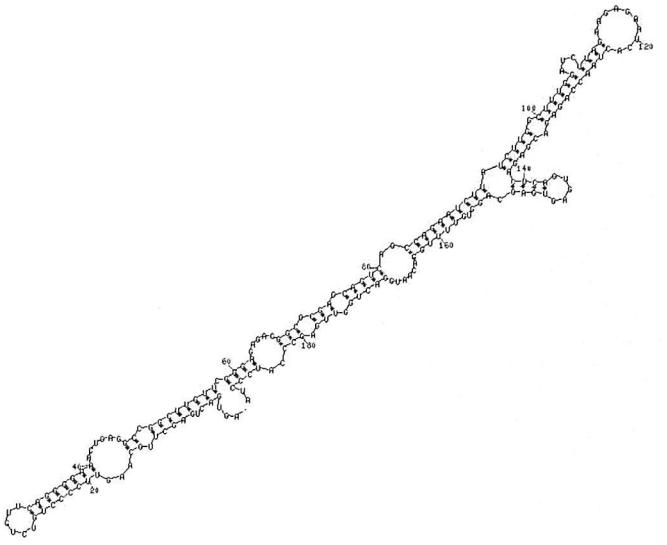
Since it is possible that the exon IA or AS1 regions modify translation through their secondary structure, the structures of the exon IB and exon IA regions were predicted

using Zuker's online RNA folding program mfold (http://ibc.wustl.edu/~zuker/rna). The most stable structures predicted with the lowest free energy (delta G) are shown in figure 3.23. The RNAs containing AS1 or exon IA are the most highly structured, which is not just related to their size, as if the delta G value is divided by the number of bases, both AS1 and exon IA containing RNAs have the lowest free energies per base (table 3.4). Since the uORFS of AS1 and exon IA have little impact on translation, it was hypothesised that the initiation codons of the uORFs may be inaccessible to the ribosome complex if they are incorporated within secondary structure. However, as shown in figure 3.24C & D, the initiation codons are not within regions of secondary structure. Both the AS1 and exon IA regions contain extensive hairpin loop structures that could influence translation. The region that actually promotes translation may not be in the AS1 or exon IA regions but the addition of these regions could influence the secondary structure of other parts of the mRNA molecule.

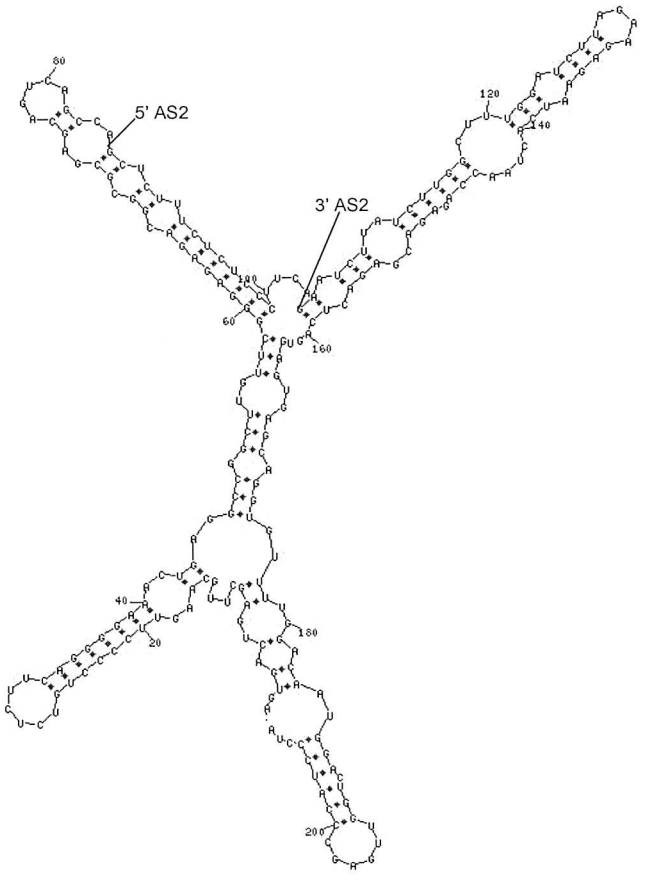
The cell-type dependent effects of both exon IA and AS1 of exon IB could be due to differences in the expression of factors that regulate translation in the two cell types. Perhaps 293 cells express regulatory factors that promote translation of exon IA containing mRNAs, for example by binding to an IRES, but DG75 cells may lack these factors. Perhaps the ability of DG75 cells to translate all exon IB splice forms equally is reflected in the fact that these cells express a high level of Bcl-X_L.

Therefore this study has shown that Bcl-X is a key CD40-target gene for primary B-cell lymphomas. Bcl-X expression is regulated through usage of different Bcl-X promoters and through modification of the 5'-UTR by alternative splicing. The utilisation of different promoters and alternative splicing therefore exerts additional levels of control on Bcl-X expression. This may allow cells to integrate information from different signalling pathways at both the transcriptional and post-transcriptional levels, facilitating a rapid and cohesive response. It is also imperative that Bcl-X expression is tightly controlled as underexpression would lead to inappropriate cell death and overexpression could contribute to cell survival and tumour formation. Further understanding of the regulation of Bcl-X at the transcriptional and translational levels, including elucidating the mechanisms by which CD40 regulates Bcl-X expression, may therefore lead to ways in which Bcl-X expression can be

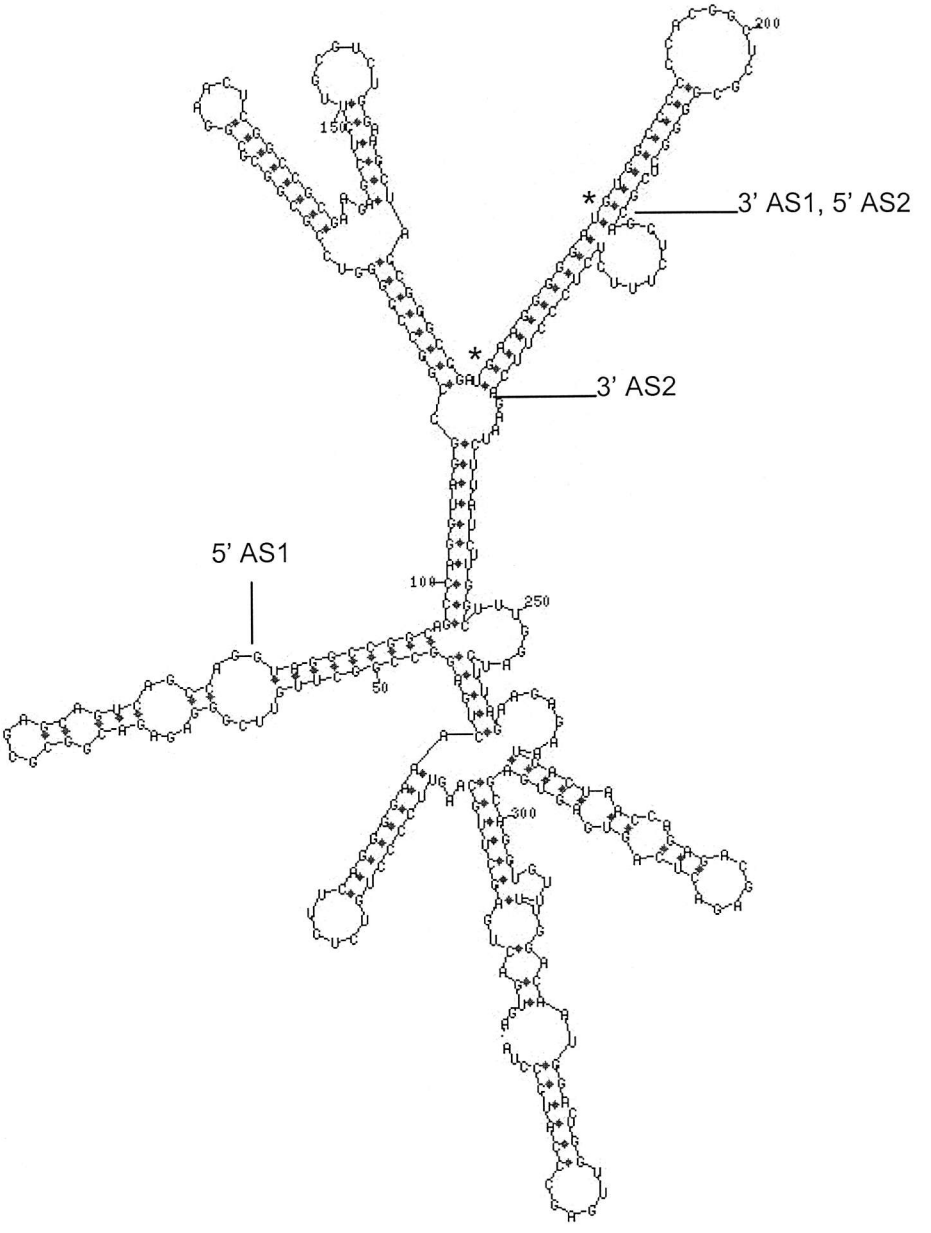
A Exon IB noAS structure. Delta G = -64.1



B Exon IB AS2 structure. Delta G = -65.45



C Exon IB AS1/2 structure. Delta G = -134.18



D Exon IA structure. Delta G = -183.19

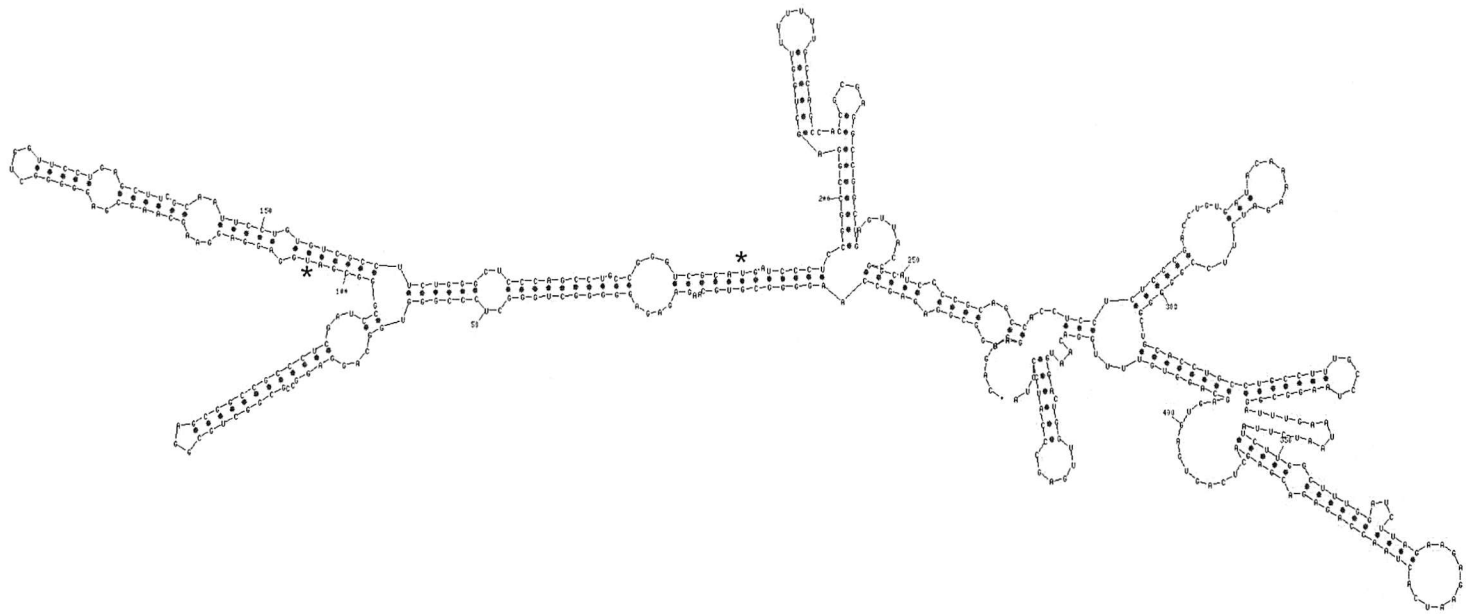


Figure 3.23: Predicted RNA structures of exon IB and exon IA regions. RNA structures were predicted using M. Zuker's and D. Stewart's mfold program (http://ibc.wustl.edu/~zuker/rna), Washington University. The alternatively spliced regions of exon IB are indicated on the structures and the AUGs of the uORFs in AS1 and exon IA are indicated with a *.

Table 3.4: Properties of predicted RNA structures, as determined by Zuker's mfold program. The number of predicted structures is the number of structures which have delta G values within 5% of the minimum delta G value.

	IB AS1/2	**IB AS2**	**IB noAS**	**IA**
No. bases	337	208	190	443
No. predicted structures	28	13	4	27
Minimum Delta G value	-134.18	-65.45	-64.1	-183.19
Delta G value per base	-0.398	-0.315	-0.337	-0.414

manipulated for therapeutic advantage in cancer and other diseases associated with defects in apoptosis.

Mcl-1, another anti-apoptotic Bcl-2 family member, was also identified as a key CD40 target gene in this study. In the primary samples, CD40L increased or sustained Mcl-1 protein expression, in all samples tested except one CLL sample (CLL-f). This is consistent with the findings of Kitada *et al* (1999) and Ghia *et al* (1998) who showed that CD40 ligation increased Mcl-1 expression in CLL cells and germinal centre B-cells, respectively. The maintenance of Mcl-1 levels in CD40L treated cells, when Mcl-1 levels decreased in untreated cells, observed in this study may not, however, be a direct consequence of CD40 signalling. Mcl-1 expression is known to decline during the spontaneous apoptosis of B-cells (Lomo *et* al 1996), which may, at least in part, be mediated by caspase cleavage of Mcl-1 (J. Michels & G.Packham, personal communication). Mcl-1 was cleaved at evolutionary conserved aspartic acid residues, forming a 28kDa cleavage product that could be detected by Western blot, during apoptosis of B-cell lines and primary material. One of the cleavage products was also shown to promote apoptosis (J. Michels & G.Packham, personal communication). The 28kDa Mcl-1 cleavage product was observed in three CLL samples in this study and less cleavage was observed when cells were treated with CD40L, consistent with the cell survival promoting function of CD40 in these cells. Mcl-1 cleavage was not observed in all samples where Mcl-1 was downregulated so other mechanisms may also account for the downregulation of Mcl-1 during apoptosis. Mcl-1 has a short half-life and a decrease in the both the transcription and translation of Mcl-1 have been observed during apoptosis (Iglesias-Serret *et al* 2003, J. Michels & G.Packham, personal communication). The downregulation of Mcl-1 observed in primary samples may therefore reflect the viability of the cells, with CD40L treated cells showing less spontaneous apoptosis over time than untreated cells. In contrast to Mcl-1 protein expression, Mcl-1 mRNA levels remained unchanged with CD40 treatment even in samples where CD40 ligation greatly induced Mcl-1 protein expression. This suggests that Mcl-1 is regulated post-transcriptionally by CD40.

Like Bcl-X_L, Mcl-1 is an important anti-apoptotic protein shown to promote tumour cell survival. Transgenic mice overexpressing Mcl-1 develop lymphomas with a high

frequency (approximately 80%) and expression of Mcl-1 is critical for multiple myeloma cell survival and in the response of CLL cells to survival signals (Zhou *et al* 2001, Derenne *et al* 2002, Zhang B *et al* 2002, Pedersen *et al* 2002). B-cell lymphomas constitutively express Mcl-1 and further enhancement by CD40 signalling could contribute to tumour cell survival. Indeed, antisense oligonucleotides to Mcl-1 used in this study demonstrated that Mcl-1 is a key survival protein for lymphoma cells. Therefore targetting Mcl-1, for example with antisense or BH3 mimetics, may be a useful strategy for treating B-cell lymphomas.

Both Bfl-1 and A20 have previously been described as NF-κB induced CD40 target-genes in lymphoma cell lines and normal B-cells (Kuss *et al* 1999, Craxton *et al* 2000, Lee *et al* 1999, Sarma *et al* 1995). Bfl-1 is an anti-apoptotic Bcl-2 family member whereas A20 is an anti-apoptotic zinc finger protein with an unknown mechanism of action (D'Sa-Eipper *et al* 1996, Opipari *et al* 1992, Fries *et al* 1996). This is the first study to determine their regulation by CD40 in primary B-cell lymphoma samples. Both Bfl-1 and A20 mRNAs were induced by CD40L in the cell lines studied, although Bfl-1 was more strongly induced in Akata 6 cells, whereas A20 was more strongly induced in Elijah cells. However, there was differential regulation of these molecules in primary material. CD40L increased or sustained expression of Bfl-1 or A20 in the majority of samples, but expression was unaffected by CD40L in some samples and CD40L treatment even decreased expression of Bfl-1 mRNAs in one sample (FL-c). The ability of CD40L to signal to Bfl-1 or A20 was not related and there was also no correlation with the type of malignancy. Therefore regulation of Bfl-1 or A20 expression by CD40 in lymphoma cells may contribute to the cell survival promoting ability of CD40 in some cases. However, these analyses were performed at the mRNA level as antibodies are not available to these proteins, so it remains to be elucidated if these changes persist at the protein level.

The regulation of survivin expression was also determined in primary lymphoma samples. Survivin inhibits apoptosis by directly inhibiting caspases (Guo & Hay 1999) and showed differential regulation by CD40 ligation in different primary samples. However, samples of each disease type showed CD40L-mediated increases in survivin expression. The inconsistent regulation of survivin by CD40 agrees with

the original research paper describing survivin as a CD40 target, which showed that in some CLL cultures CD40L upregulated survivin expression, whereas in others survivin was not expressed or expression was not influenced by CD40 ligation (Granziero *et al* 2001). Interestingly, two of the reactive nodes expressed survivin when it has been previously reported that survivin is expressed foetally and in cancer cells but not in terminally differentiated adult tissues (Ambrosini *et al* 1997).

BNIP3 expression was also studied in primary material and demonstrated, for the first time, that BNIP3 can be regulated by CD40. BNIP3 is a pro-apoptotic Bcl-2 family protein, identified as being upregulated by CD40 from analysis of DNA microarray data (Alizadeh *et al* 2000). BNIP3 protein levels were upregulated or sustained by CD40 ligation in approximately half of the samples, however CD40L treatment resulted in increased or sustained BNIP3 mRNA levels in the majority of samples, even when BNIP3 protein was not detected. This result confirms the finding of the DNA microarray study (Alizadeh *et al* 2000), demonstrating that BNIP3 is a novel CD40-target gene. Although BNIP3 is a CD40 target at the transcriptional level, this does not translate into protein in all cases. CD40 often sustained BNIP3 protein levels, suggesting that, like Mcl-1, BNIP3 is downregulated during apoptosis. In the literature, the only factor described regulating BNIP3 expression is hypoxia but the significance of this is unclear. BNIP3 is induced at the transcriptional level by hypoxia, mediated by hypoxia-inducible factor-1 (HIF-1) (Bruick 2000). Although there have been no published studies linking CD40 to hypoxia, CD40 activation can promote angiogenesis and VEGF production, which can also occur under hypoxic conditions (Melter *et al* 2000, Deregibus *et al* 2003, Reinders *et al* 2003, Tai *et al* 2002).

In summary, CD40 ligation delivers a strong anti-apoptotic signal to primary B-cell malignancies, which is consistently accompanied by increased expression of the anti-apoptotic proteins Bcl-X_L and Mcl-1. Bcl-X_L expression can be influenced at many levels through usage of different promoters and alternative splicing events, which can affect translation of Bcl-X_L. CD40 signalling also influenced expression of the apoptosis regulators A20, Bfl-1, survivin and BNIP3, but this was not consistent between samples. The cell survival promoting effect of CD40 raises concerns over the safety and design of clinical trials for CD40-based therapy of lymphoma. Both Bcl-X_L

and Mcl-1 are key survival proteins for lymphoma cells and disruption of their expression or function may be of therapeutic benefit for lymphoma.

Chapter 4: Differential control of cell survival and cell signalling by CD40

4.1 Introduction

CD40 is an important cell survival signalling molecule for normal B-cells, but CD40 ligation can produce differential effects on cell survival in malignant cells. In many B-cell lymphoma lines and primary lymphoma cultures, CD40 activation promotes cell survival and suppresses apoptosis (e.g. Kelly & Knox 1995, Tsubata *et al* 1993, Padmore *et al* 1997, Johnson *et al* 1993, Ghia *et al* 1998, Kitada *et al* 1999). Indeed, data presented in chapter 3 demonstrated that CD40 acts as a survival signalling molecule for isolated primary lymphoma cells. However there is also considerable evidence showing that CD40 signalling can cause growth inhibition or induce apoptosis, in some cellular settings. These include some B-cell lymphoma lines (Heath *et al* 1993, Funakoshi *et al* 1994) and carcinoma cells (e.g. Hess *et al* 1996, Eliopoulos *et al* 2000, Hirano *et al* 1999).

The molecular mechanisms for this differential control of cell survival by CD40 are not well understood. Understanding why CD40 activation can produce differential effects is of great importance considering the large degree of interest in utilising CD40 therapy as a treatment for cancer. Impressive results have been obtained in mouse model systems, supported by some encouraging findings in early clinical trials (Funakoshi *et al* 1994, Tutt *et al* 1998, Vonderheide *et al* 2001). The molecular mechanisms underlying the differential control of cell survival could reveal markers to predict the outcome of tumours to CD40 activation. This would then ensure that CD40 therapy would be given to patients likely to respond to the treatment. In addition, molecules regulated by CD40 could be therapeutic targets for the treatment of cancer. CD40 regulated pathways could be targeted to switch cells from a CD40 survival-promoting to a CD40 death inducing-phenotype.

This study therefore sought to characterise and compare the responses of two B-cell lymphoma lines, Akata 6 and RL, to CD40 ligation. These cell lines were chosen as model systems as they have very different responses to CD40 ligation. CD40

suppresses apoptosis of Akata 6 cells, a Burkitt's lymphoma line, (as shown in chapter 3) whereas CD40 inhibits proliferation and promotes death of RL cells, a diffuse large B-cell line, both *in vitro* and *in vivo* (Funakoshi *et al* 1994).

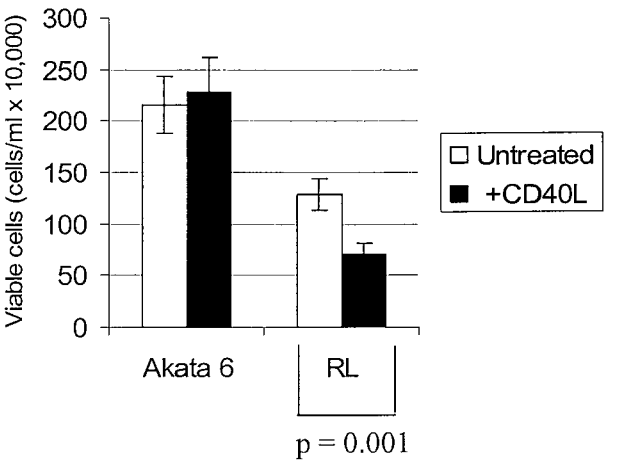
Ligation of CD40 activates a range of signalling molecules leading to changes in gene expression. CD40 targets include both pro- and anti-apoptotic genes that impinge on both the intrinsic (mitochondrial) and extrinsic (death receptor) apoptosis pathways (Dallman *et al* 2003). It is likely that some of these CD40 targets play a prominent role in differential control of cell survival. This chapter therefore sought to determine the effect of CD40L on the expression and function of Bcl-2 family members and components of the Fas and TRAIL pathways, in RL and Akata 6 cells.

Signalling molecules previously shown to be activated by CD40 include PI3-K, NF-κB and three MAPK cascades: the ERK pathway, JNK pathway and the p38 pathway (Ren *et al* 1994, Padmore *et al* 1997, Lee *et al* 1999, Sakata *et al* 1995, Sutherland *et al* 1996, Li *et al* 1996). Differential regulation of cell signalling pathways could play a role in determining the outcome of CD40 signalling. A comparison of the effects of these signalling pathways on cell survival and expression of apoptosis regulators has not been undertaken in lymphoma cell lines or primary B-cell malignancies. Therefore this study performed this comparison to elucidate which signalling pathways were important in the regulation of cell survival and regulation of the key apoptosis regulators Bcl-X_L and Mcl-1 by CD40 in RL, Akata 6 cells and in primary B-cell lymphomas.

4.2 Short-term survival

The short-term survival responses of Akata 6 and RL cells to CD40L were determined over 48 hours using a variety of techniques. Untreated and CD40L treated cells were stained with trypan blue and counted to assess the effect of CD40 ligation on cell viability (fig. 4.1). CD40L treatment significantly decreased the number of viable RL cells compared to untreated controls (independent samples t-test: t=6.3, n=8, p=0.001) (fig. 4.1A). This could be achieved through CD40L decreasing cell proliferation or increasing cell death. As shown in fig. 4.1B, CD40L treatment significantly increased cell death in RL cells (independent samples t-test: t=7.1, n=8, p=0.002), causing approximately 30% cell death after 48 hours of treatment. In contrast, there was no

A



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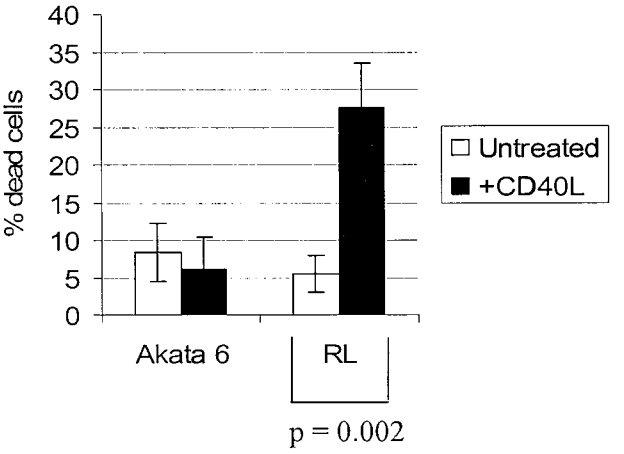


Figure 4.1: The effect of CD40L on short-term cell survival in Akata 6 and RL cells. Cells were cultured at 5 x 10^5 cells/ml and treated with 1μg/ml CD40L or left untreated as a control for 48 hours. After 48 hrs of treatment the number of viable cells (trypan blue negative) and dead cells (trypan blue positive) were counted. **A** - Number of viable cells. Data show number of viable cells per ml of culture x 10,000. **B** - Percentage of dead cells. Data show percentage of dead cells relative to total cell number. For both graphs, data are represented as the mean of single counts from four independent experiments. Error bars show plus and minus one standard deviation. For all experiments, an independent sample t-test (SPSS) was used to derive p values for untreated vs CD40L treated RL cells.

difference in cell number or viability of CD40L treated Akata 6 cells compared to untreated cells. This finding is probably due to the fact that Akata 6 cells are proliferating at a maximal rate, so CD40 signalling cannot improve proliferation. However CD40 signalling can rescue Akata 6 cells from etoposide-induced apoptosis, as assessed by PARP cleavage (fig 4.2A) and Annexin V assay (fig 4.3A, see also chapter 3). Therefore CD40 acts as a positive survival signal for Akata 6 cells, whereas it acts as a negative survival signal for RL cells, inducing cell death.

To determine if CD40L-induced death was via apoptosis in RL cells, PARP cleavage and Annexin V/PI staining were assessed. As shown in fig. 4.2A + B, CD40L did not enhance PARP cleavage in RL cells. This could be because RL cells have a deficiency in cleaving PARP or CD40L does not kill RL cells through apoptosis. However, this was not entirely consistent and later experiments showed that CD40 ligation did induce PARP cleavage in RL cells (see section 4.6) and so this was not pursued.

The Annexin V assay showed that CD40L treatment induced RL cell death, as CD40L treatment gave 23.1% total dead cells (upper right plus lower right quadrant) compared to 14.4% for untreated cells (fig. 4.3B). Most of the dead cells were present in the upper right quadrant and are therefore classed as secondary apoptotic or necrotic cells. There was not a clear apoptotic population of RL cells. Therefore either the assay missed apoptosis as it occurred quickly and so any apoptotic cells were already secondary apoptotic by the time of the assay, or CD40L did not induce phosphatidylserine exposure in RL cells.

RL cells were also treated with etoposide and CD40L in combination to determine if CD40 could rescue RL cells from etoposide-induced apoptosis, as is the case for Akata 6 cells (fig. 4.2 and 4.3). At a concentration of etoposide (2μM) which induced apoptosis and concomitant PARP cleavage in Akata 6 cells, no enhanced PARP cleavage was observed in RL cells (fig. 4.2A). The Annexin V assay revealed that etoposide was slightly enhancing RL cell death (18.8% total cell death vs 14.4%), but that RL cells were not as sensitive to etoposide as Akata 6 cells (18.8% total cell death vs 38.7%) (fig. 4.3). The addition of both CD40L and etoposide to RL cells did

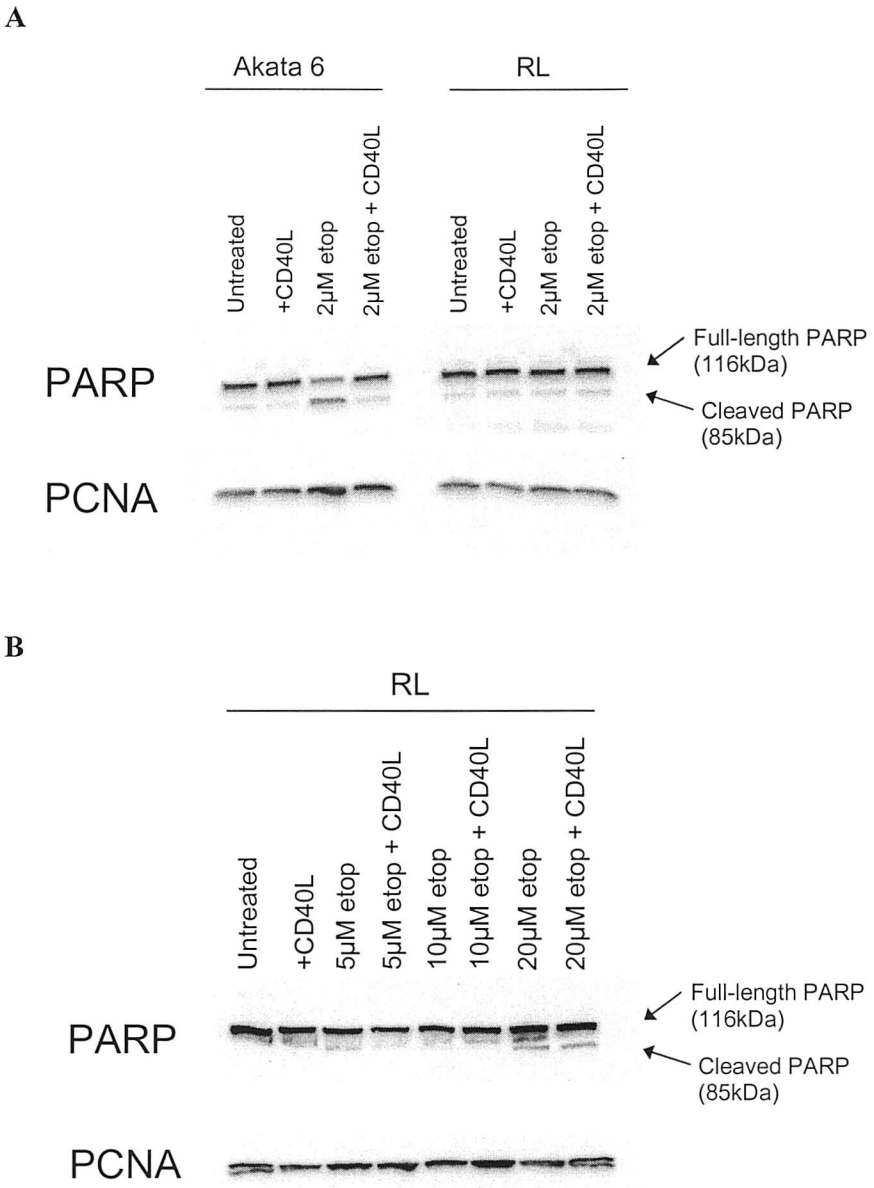
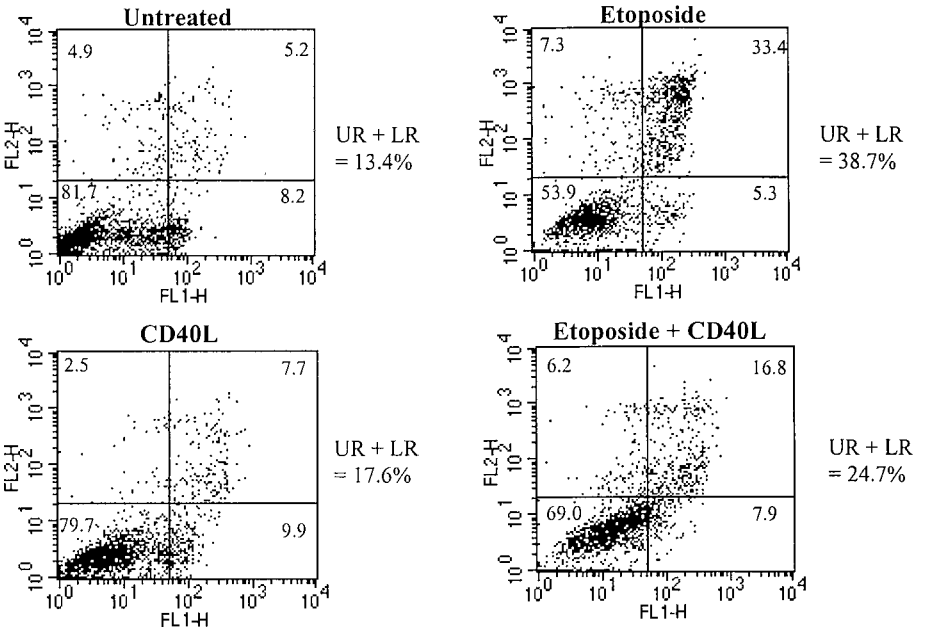


Figure 4.2: The effect of CD40L and etoposide on PARP cleavage in Akata 6 and RL cells. For both experiments, cells were cultured for 48 hrs alone, with 1μg/ml CD40L, with 2μM to 20μM etoposide or with both agents in combination. Total protein was isolated and analysed by Western blot. PCNA is shown as a loading control.

A Akata 6



B RL

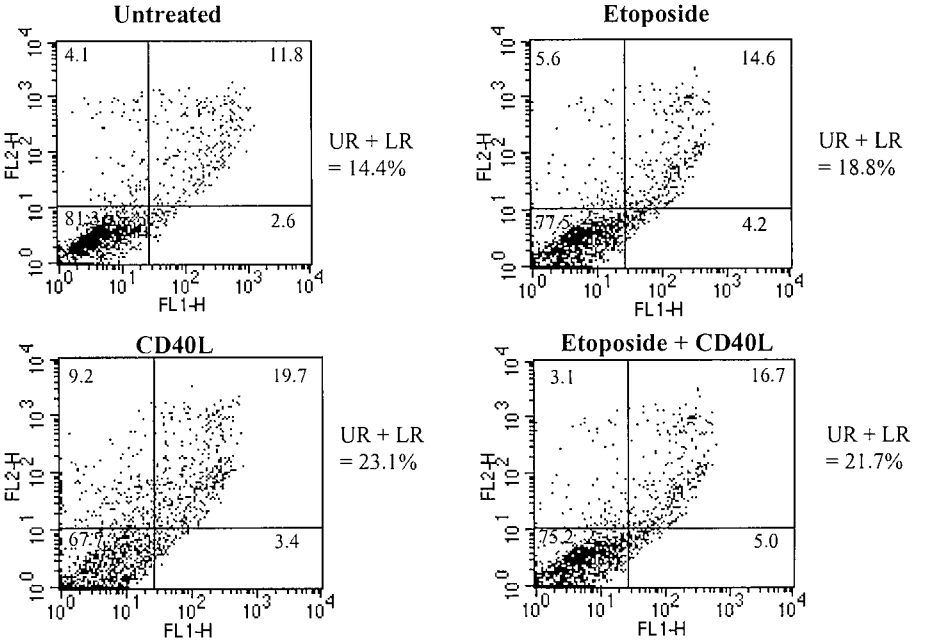


Figure 4.3: The effect of etoposide and CD40L treatment on AnnexinV/PI staining in Akata 6 (A) and RL (B) cells. Cells were cultured for 48 hrs, untreated, with 1μg/ml CD40L, with 2μM etoposide, or both agents in combination. Cells were then stained with Annexin V-FITC (x axis) and propidium iodide (y axis), and analysed using flow cytometry. Values indicate the percentage of cells in each quadrant. UR + UL value indicates the percentage of cells in the upper right plus the lower right quadrant. This is the total number of dead cells. Data are representative of two experiments.

not have any significant effect over treating the cells with either agent alone (21.7% total dead cells for the combination, compared to 23.1% for CD40L treated cells and 18.8% for etoposide treated cells).

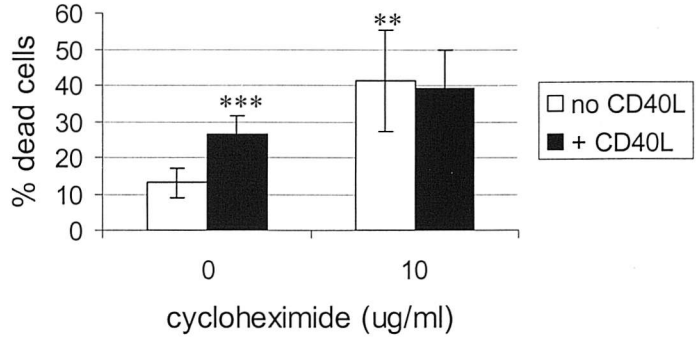
As RL cells were less sensitive to etoposide than Akata 6 cells, further investigations were carried out treating RL cells with a range of etoposide concentrations, in order to obtain a level of etoposide-induced PARP cleavage similar to that of Akata 6 cells. As shown in fig. 4.2B, 20μM etoposide caused some PARP cleavage and this was not influenced by CD40L treatment. Therefore CD40L does not influence etoposide-induced apoptosis of RL cells, confirming that CD40L acts as a negative survival signal for RL cells.

Previous studies of CD40 signalling in epithelial tumours have shown that blocking protein synthesis via cycloheximide treatment either increases sensitivity to CD40L-induced death or actually leads to the induction of apoptosis via CD40 (Hess & Engelmann 1996, Eliopoulos *et al* 2000, Gallagher *et al* 2002). To determine if this was the case for RL cells, they were treated with CD40L, or cycloheximide, or both agents in combination and cell death assessed by trypan blue assay (fig. 4.4A) and PARP cleavage (fig. 4.4B). As shown in fig. 4.4A, treatment with CD40L or cycloheximide increased cell death, at statistically significant levels (independent samples t-test for CD40L: $t=5.2$, $n=12$, $p<0.001$; independent samples t-test for cycloheximide: $t=4.8$, $n=12$, $p=0.003$). However, cycloheximide treatment induced PARP cleavage whereas CD40L treatment did not. The combination of CD40L and cycloheximide did not increase cell death or PARP cleavage over that observed with cycloheximide treatment alone (fig. 4.4). Therefore cycloheximide does not influence CD40L-induced RL cell death.

4.3 Long-term survival

The previous section showed that CD40L acts as a positive survival signal for Akata 6 cells but a negative survival signal for RL cells, at least in the short-term. However the effect of CD40 activation on long-term cell survival has not been previously studied. Experiments were therefore carried out to determine if the effects of CD40L persist in long-term clonogenic survival of Akata 6 and RL cells. Cells were initially

A



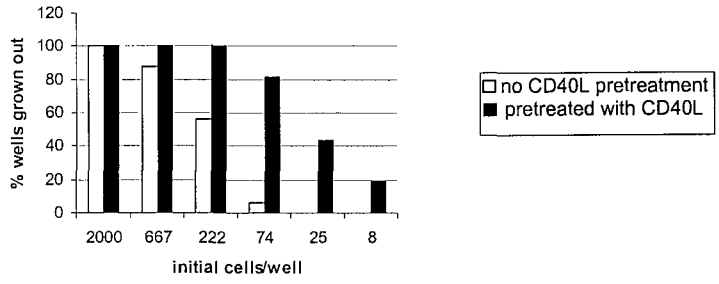
B

Figure 4.4. The effect of cycloheximide on CD40L-mediated death in RL cells. Cells were treated for 48 hrs with CD40L (1μg/ml) or cycloheximide (CHX) at 10μg/ml, or both agents in combination. **A – Trypan blue assay**. Data are represented as the mean percentage of trypan blue positive cells (dead cells) for three independent experiments, each performed in duplicate. Error bars show plus and minus one standard deviation. Independent sample t-tests (SPSS) were performed, comparing untreated to CD40L treated cells (***, $p = < 0.001$) and comparing untreated to CHX treated cells (**, $p = 0.003$). **B – PARP cleavage**. Following treatment, total protein was extracted and analysed by Western blot. This blot is representative of three independent experiments.

cultured for 48 hrs in the presence or absence of CD40L and then serial dilutions performed, using standard culture media. Cells were then plated out and cultured for 21 days. After this time the percentage of wells containing healthy, growing cells was assessed for each dilution. This assay was therefore assessing the effect of short-term CD40L exposure on long-term cell survival. As shown in fig. 4.5A, CD40L pretreatment significantly improved survival and clonogenic potential of Akata 6 cells, improving cloning efficiency 8 fold (table 4.1). Akata 6 cells have a low clonogenic potential, requiring on average 149 cells per well for a single cell to grow out. The cloning procedure may act as a cellular insult that signalling through CD40 can help to overcome. Surprisingly, CD40L pretreatment also improved long-term survival of RL cells (fig. 4.5B), improving RL cloning efficiency by an average of 5.75 fold (table 4.1). This result was unexpected, considering the negative survival role of CD40 in RL cells in the short term.

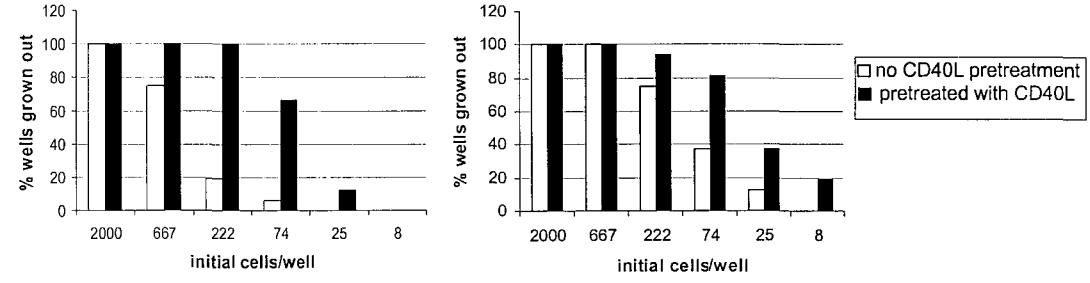
A possible reason for the positive effect of CD40L pretreatment on RL long-term cell survival could be that CD40 signalling was killing sensitive cells in the short-term, leaving more robust cells which were better able to form clones in the long-term assay. Therefore the clonogenic assay was repeated with CD40L present in the media throughout the experiment. In this experiment, CD40L treated cells still showed improved survival, compared to untreated cells (fig. 4.5C) but to a lesser extent, with CD40L improving cloning efficiency 1.3 fold (table 4.1). Therefore although CD40L induces RL approximately 30% cell death in the short-term, this is overcome in long-term assays and CD40 signalling actually confers a net survival advantage. These data suggest that CD40L could improve long-term RL cell survival in two ways. Firstly a short-term death phase occurs, killing sensitive cells and acting as a selection pressure for a more robust phenotype. However this short-term death phase is then countered by a long-term survival advantage.

A Akata 6



B RL

(i) **(ii)**



C RL

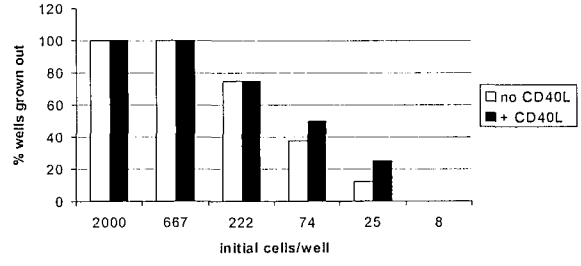


Figure 4.5: Long-term clonogenic assays. A and B – Akata 6 (A) and RL (B) cells were cultured for 48 hrs with or without 1μg/ml CD40L, at a starting density of 1 x 10^5 cells/ml. After 48 hours, cells were diluted to a concentration of 1 x 10^4 cells/ml with standard culture media. Eight serial dilutions were performed down to the equivalent of <1 cell per well. 16 wells of a 96-well plate were plated out for each dilution. Data are represented as the percentage of wells grown out i.e. wells containing healthy cells, for each dilution after 21 days. Two independent experiments were performed for RL cells (B(i) and (ii)). **C** – RL clonogenic assay with CD40L present throughout. This was performed as A and B, except CD40L (1μg/ml) containing media was used for the dilutions and each week half of the media was replaced with CD40L-containing media.

Table 4.1 The effect of CD40L on cloning efficiencies of Akata 6 and RL cells. This table shows the cloning efficiencies from the experiments shown in fig. 5.5. To obtain a high probability that wells with growth contain single clones, cloning efficiency was defined as the dilution at which one third of the wells contained healthy cells after 21 days (Godman 1983). Cloning efficiency was calculated by plotting a scatter plot using the data from fig. 5.5 and deriving the equation from the trend line produced. % cloning efficiency is calculated as (1/cloning efficiency)x 100. 100% cloning efficiency indicates that if on average each well contained 1 cell, then one third of the wells would grow out.

Cell line	Experiment	Cloning efficiency untreated cells	Cloning efficiency CD40L treated	% cloning efficiency untreated cells	% cloning efficiency CD40L treated	Fold increase in cloning efficiency with CD40L
Akata 6	CD40L pretreatment	149	18.5	0.67	5.40	8
RL	CD40L pretreatment – expt 1	318	42	0.31	2.38	7.5
RL	CD40L pretreatment – expt 2	88.6	22	1.12	4.54	4
RL	CD40L present throughout expt	88.6	68.6	1.12	1.45	1.3

4.4 The role of Fas in the differential control of cell survival by CD40

Previous studies have shown that CD40L-induced death in epithelial cells can be mediated via death receptor signalling, through upregulation of cytotoxic ligands (Fas ligand, TRAIL and TNF) and through the TNF-R1 and Fas pathways (Eliopoulos *et al* 2000, Afford *et al* 2001, Grell *et al* 1999). CD40 activation can also upregulate Fas expression in both normal and malignant B-cells and in carcinoma cell lines, but this does not necessarily sensitise cells to Fas-mediated apoptosis (Kitada *et al* 1999, Alexandroff *et al* 2000, Garrone *et al* 1995, Schattner *et al* 1996, Wang *et al* 1997, Plumas *et al* 1998, Yamada *et al* 2001). It was decided to determine if differential regulation of Fas in Akata 6 and RL cells was responsible for the different outcome of CD40 signalling in these cells.

Firstly, experiments were undertaken to determine if CD40 signalling upregulates Fas expression in RL and Akata 6 cells. Cells were treated with CD40L for 48 hours, or left untreated as a control and then stained with either a FITC-conjugated anti-Fas antibody or a FITC-conjugated isotype matched control antibody, and analysed by flow cytometry. As shown in fig. 4.6, both cell lines express Fas at low levels and upregulate Fas expression following CD40L treatment. Akata 6 cells upregulate Fas expression 6.5 fold (mean fluorescence intensity of Fas stained CD40L treated cells/ mean fluorescence intensity of Fas stained untreated cells) whereas RL cells upregulate Fas expression 4.2 fold.

The effect of CD40L on the expression of Fas death-inducing signalling complex (DISC) components in RL and Akata 6 cells was also determined, to see if they could give any insight into the differential control of cell survival by CD40. Fas, FADD, caspase-8 and FLIP comprise the DISC. FADD transduces signals from the death receptors to caspase-8, leading to caspase-8 cleavage and activation. FLIP can prevent processing of caspase-8, thus negatively regulating death receptor signalling. Alterations in expression of any of these DISC components by CD40 could therefore affect death receptor signalling and apoptosis induction. Indeed, FLIP was previously shown to be upregulated by CD40 in normal B-cells (van Eijk *et al* 2001, Hennino *et al* 2000, Hennino *et al* 2001).

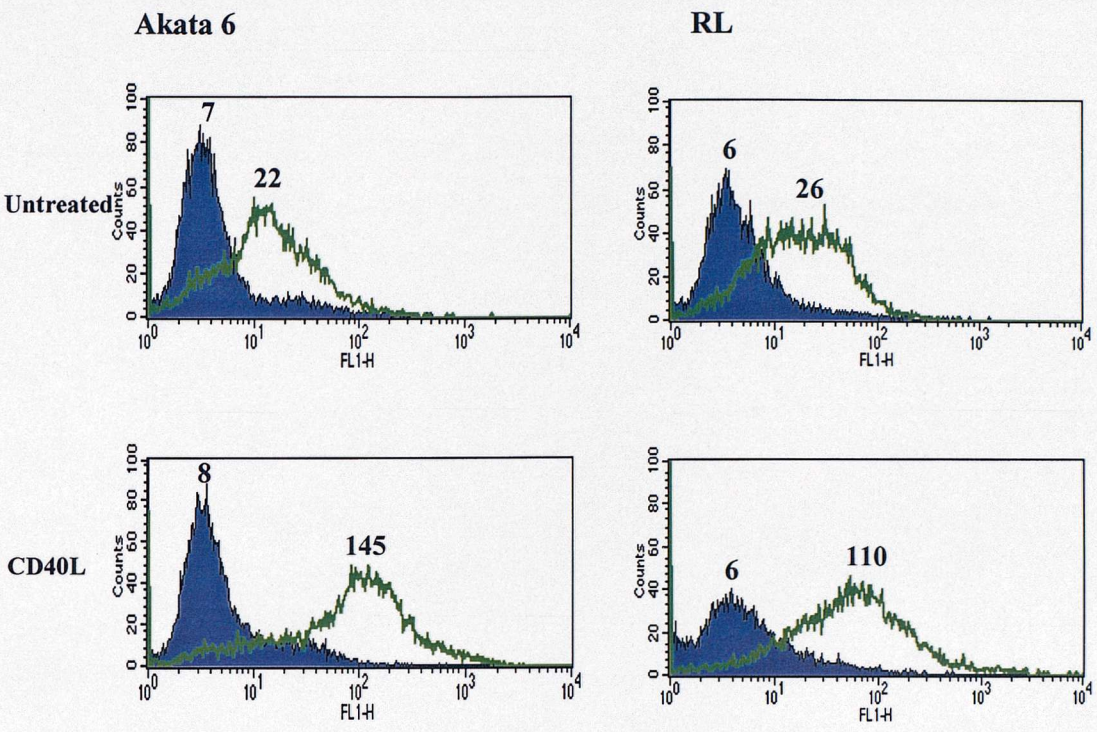


Figure 4.6: CD40L upregulates Fas expression in Akata 6 and RL cells. Cells were treated plus or minus CD40L (1μg/ml) for 48 hrs and then stained with either a FITC-conjugated isotype matched control antibody (blue closed peaks) or a FITC-conjugated anti-Fas antibody (green open peaks). Cells were then analysed by flow cytometry. Cell counts are shown on the y-axis and fluoresence intensity on the x axis. Values shown are mean fluorescence intensity for each peak. Data are representative of two independent experiments.

As shown in fig. 4.7, CD40L treatment did not affect FADD or $FLIP_L$ expression, but it increased caspase-8 expression in both RL and Akata 6 cells. There was no evidence of caspase-8 cleavage in either cell line, which would cleave the 55/50 kDa observed doublet into a 40/36 kDa doublet and a 23 kDa band. This suggests that caspase-8 was not being activated or was cleaved at a level that was below detection. Levels of DISC components also appeared similar in both cell lines. It appears that CD40 signalling was attempting to sensitise both cell lines to death receptor-mediated apoptosis by upregulating Fas and caspase-8 expression

To determine if Akata 6 or RL cells were differentially sensitive to Fas signalling, cells were treated with a Fas-activating antibody CH-11, (Mouzakiti & Packham 2003), or an isotype-matched control antibody (IgM), in the presence or absence of CD40L and cell death assessed by trypan blue assay. RL cells were Fas sensitive (independent samples t-test: $t=5.1$, $n=8$, $p=0.01$) but did not show any enhanced Fas-mediated killing in the presence of CD40L (fig. 4.8A(i)), whereas Akata 6 cells were insensitive to Fas and CD40L treatment did not affect this (fig, 4.8A(ii)). Next, RL cells were treated with a Fas-neutralising antibody ZB4 (Mouzakiti & Packham 2003), to determine if CD40L-induced death was Fas-mediated. As shown in fig. 4.8B, ZB4 treatment did not affect the ability of CD40 to kill RL cells. The ZB4 used in this experiment was fully functional as the same preparation of ZB4 blocked Fas-mediated apoptosis in Jurkat cells (data not shown). Therefore CD40-induced RL cell death is not mediated via Fas.

As Fas signalling was not involved in CD40-induced death, it was decided to determine if TRAIL signalling had a role. TRAIL signalling utilises similar DISC components to Fas leading to caspase 8 activation. Cells were treated with recombinant TRAIL and an enhancer antibody which oligomerises TRAIL, in the presence or absence of CD40L, and cell death assessed by trypan blue assay. Treatment with enhancer antibody alone was used as a control. Both RL and Akata 6 cells were insensitive to TRAIL, and CD40L did not sensitise cells to TRAIL-mediated apoptosis (fig. 4.8C). This preparation of recombinant TRAIL and enhancer was shown to induce apoptosis in Jurkat cells and sensitive B-cell lines (Mouzakiti & Packham 2003).

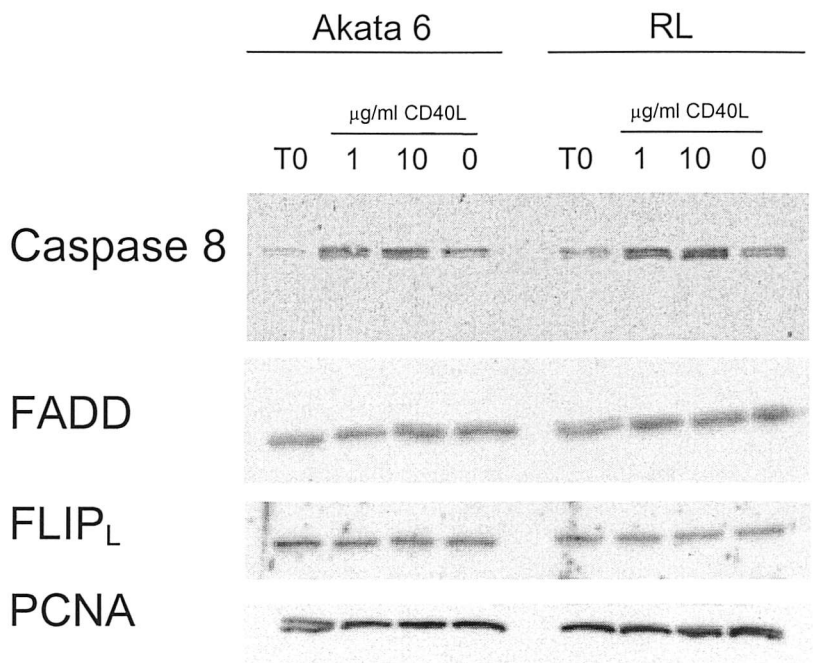


Figure 4.7: The effect of CD40L on expression of DISC components. Cells were either left untreated or treated for 48 hours with either 1µg/ml or 10µg/ml CD40L. A cell pellet was also taken at the start of the experiment (T0). Cells were snap frozen and then lysed. 20µg total protein was analysed by Western blot. PCNA is shown as a loading control.

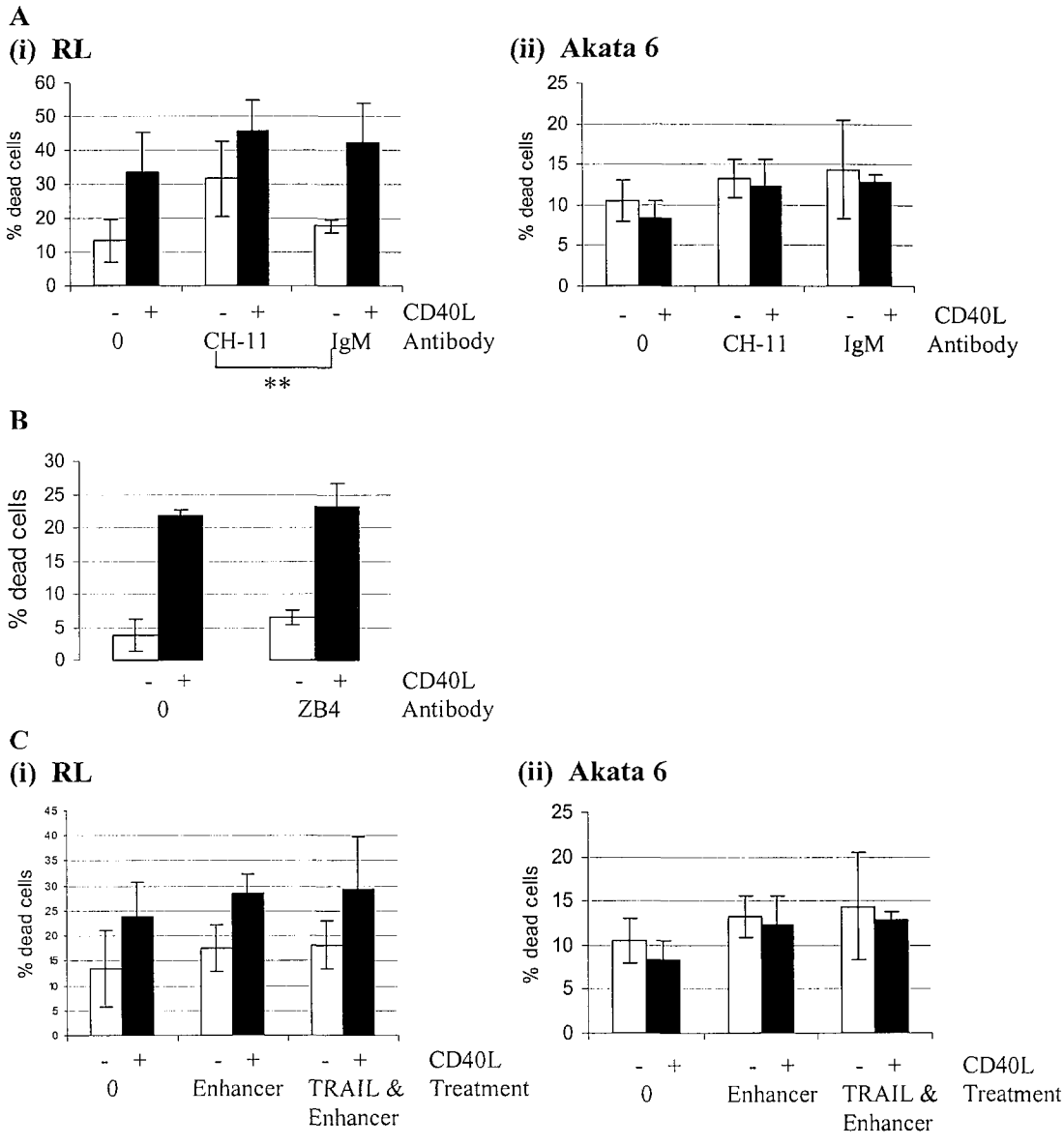


Figure 4.8: The effect of activating Fas or TRAIL and CD40L on RL and Akata 6 cell death. In all experiments cells were cultured for 48 hrs with the various agents, CD40L was used at 1μg/ml and cell death measured by counting the number of trypan blue positive cells. Error bars are plus and minus one standard deviation. **A – Activating Fas.** RL (i) or Akata 6 (ii) were treated with Fas activating antibody (CH-11 500ng/ml) or IgM antibody as an isotype-matched control. Data are mean of two or three independent experiments, each performed in duplicate. An independent samples t-test (SPSS) was used to compare CH-11 with IgM treated RL cells (**, p = 0.01). **B – Blocking Fas.** RL cells were treated with ZB4 (500ng/ml), a Fas neutralising antibody (ref) or left untreated as a control. Data are mean of two independent experiments, each performed in duplicate. **C –Activating TRAIL.** RL (i) or Akata 6 (ii) were treated with recombinant TRAIL (250ng/ml) in combination with Enhancer antibody (1.25μg/ml), which oligomerizes TRAIL. Cells were also treated with Enhancer alone as a control. Data are mean of two independent experiments, each performed in duplicate.

4.5 The role of Bcl-2 family proteins in the differential control of cell survival by CD40

Differences in death receptor signalling do not appear to account for the different effects of CD40 on cell survival in RL and Akata 6 cells, or CD40-induced RL cell death. This study also looked at the expression of Bcl-2 family members in untreated and CD40L treated cells, as they are key regulators of cell death. Firstly, a time-course experiment was performed with CD40L and expression of apoptosis regulators detected by western blot (fig. 4.9). This showed that CD40L treatment increased Bcl-X_L expression strongly in RL cells and more modestly in Akata 6 cells. Mcl-1 expression was modestly increased in Akata 6 cells but remained unchanged in RL cells. This confirms previous Akata 6 results presented in chapter 3 (see fig. 3.1). The lower molecular weight bands on the Mcl-1 blot are isoforms of Mcl-1 generated by alternate translation initiation. Mcl-1 is cleaved to a 28kDa form during apoptosis of B-cell lines and some primary samples (fig. 3.13, J. Michels & G.Packham personal communication), however no Mcl-1 cleavage was observed in RL cells treated with CD40L. Levels of Bcl-2, Bax and BNIP3 remained unchanged.

As a recent paper showed that CD40L increased Bax expression in RL cells at 10μg/ml after 48 hrs of treatment (Szocinski *et al* 2002), it was decided to treat RL and Akata 6 cells with this higher concentration of CD40L and determine the expression of Bcl-2 family members. As shown in fig. 4.10, 10μg/ml CD40L but not 1μg/ml CD40L slightly increased Bax expression in RL cells. There was no change in Bax expression in Akata 6 cells. 10μg/ml CD40L increased Bcl-2 expression two fold in Akata 6 cells, but this did not occur in RL cells. This experiment also confirms results of the previous experiment, showing that CD40 signalling increased Bcl-X_L expression in both cell lines but modestly increased Mcl-1 expression in only Akata 6 cells. Although the overall level of induction of Bcl-X_L by CD40L was reduced in this experiment, Bcl-X_L expression was still clearly increased by CD40 engagement in Akata 6 and RL cells. An A20 antibody became available at the time of this experiment, and as this was previously shown to be a CD40 target at the mRNA level (see chapter 3, Sarma *et al* 1995), it was decided to determine if the A20 protein behaved in a similar fashion. In contrast to the induction of A20 mRNA by CD40 in Akata 6 cells (see chapter 3), there was no change in A20 protein expression with

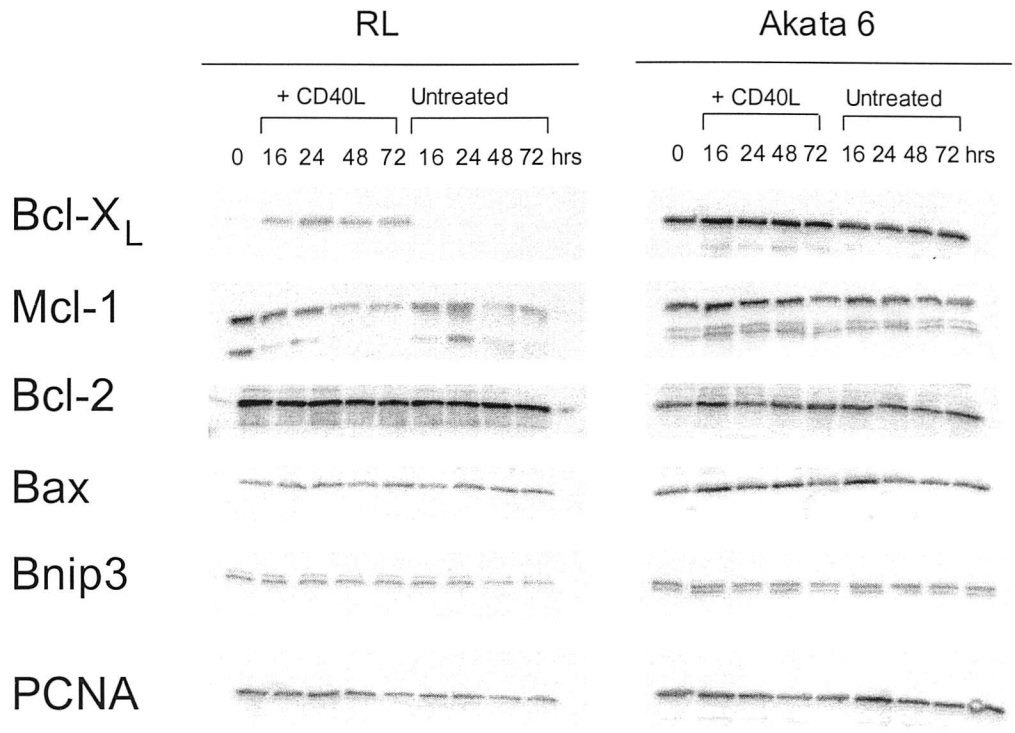


Figure 4.9: The effect of CD40L on the expression of apoptosis regulators in RL and Akata 6 cells. Cells were cultured plus or minus CD40L (1μg/ml) for the time points indicated and pellets snap frozen. A cell pellet was also taken at the start of the experiment (Time 0).Pellets were lysed and 20μg total protein analysed by Western blot.

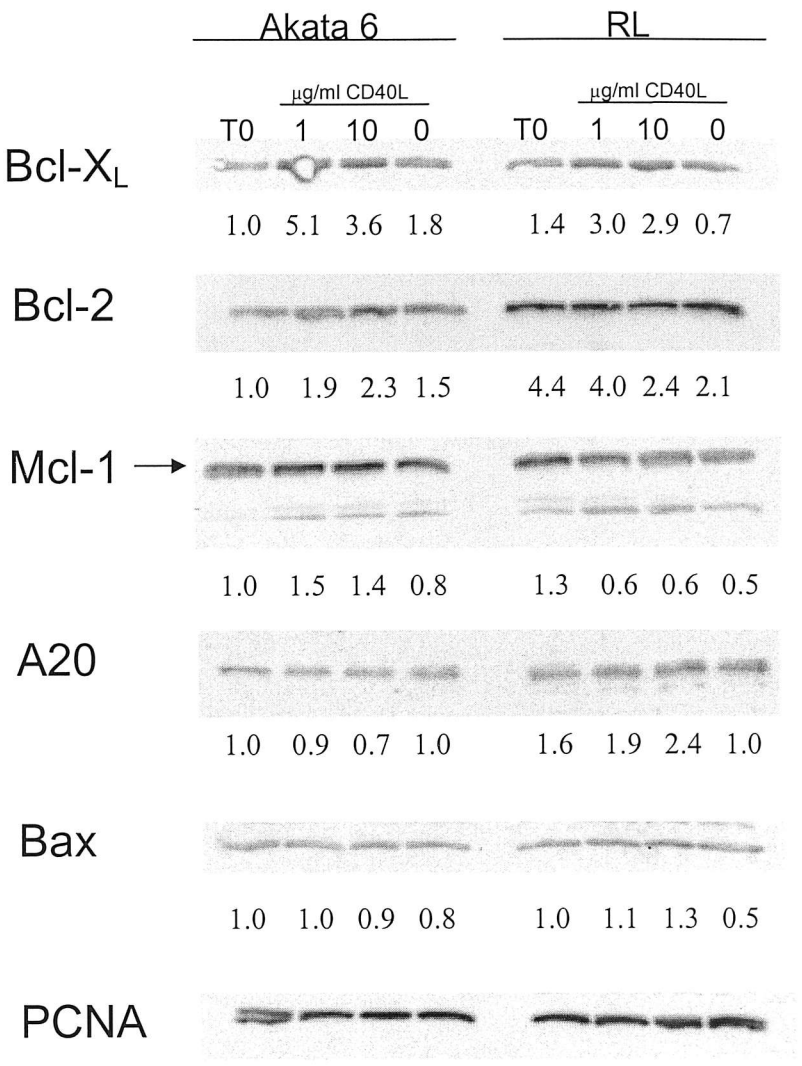


Figure 4.10: The effect of CD40L at 10μg/ml on the expression of apoptosis regulators in RL and Akata 6 cells. Cells were cultured with CD40L (1μg/ml or 10μg/ml) or left untreated for 48 hours and pellets snap frozen. A cell pellet was also taken at the start of the experiment (T0). Pellets were lysed and 20μg total protein analysed by Western blot. Values beneath each blot represent the band intensity normalised to PCNA.

CD40L treatment. However in RL cells there was a two-fold increase in A20 expression following CD40L treatment.

4.6 The role of signalling pathways in regulation of cell survival, Bcl-X_L and Mcl-1 expression by CD40 in RL and Akata 6 cells

Differences in the regulation of cell signalling pathways by CD40 may influence the outcome of CD40 signalling. Therefore a comparison of the activation of various signalling pathways by CD40 and their effects on cell survival was undertaken in Akata 6 and RL cells. Bcl-X_L and Mcl-1 were key CD40 regulated genes in primary B-cell malignancies (chapter 3) and so the signalling pathways responsible for upregulation of these molecules were also investigated. This was achieved through treating cells with various specific inhibitors to signalling molecules, in the presence or absence of CD40L, and assessing PARP cleavage, Mcl-1 and Bcl-X_L expression by Western blot. In addition, the expression of phosphorylated forms of signalling intermediates were determined to investigate if CD40 signalling activated these molecules and if the inhibitors were specifically preventing signalling. The inhibitors used were against pathways that were previously shown to be activated by CD40 (Ren *et al* 1994, Padmore *et al* 1997, Lee *et al* 1999, Sakata *et al* 1995, Sutherland *et al* 1996, Dadgostar *et al* 2002). These were the NF-κB inhibitors sulfasalazine, gliotoxin and kamebakaurin (KA), the MAPK inhibitor UO126, the PI3-K inhibitor LY294002, the p38 kinase inhibitor SB203580 and JNK inhibitor II.

Sulfasalazine is used to treat inflammatory bowel disease and rheumatoid arthritis. It is a potent and specific inhibitor of NF-κB through inhibiting the IκB kinases IKK-α and IKK-β, by antagonizing ATP binding (Wahl *et al* 1998, Weber *et al* 2000). Gliotoxin is a fungal metabolite which inhibits NF-κB activation through inhibition of the 20S proteasome (Kroll *et al* 1999). This therefore prevents proteasome-mediated degradation of IκB proteins, keeping NF-κB sequestered by IκB proteins in the cytoplasm. KA specifically inhibits NF-κB activity by directly targeting the DNA-binding activity of p50. Its exact mechanism of action is not clear but KA may directly bind to and modify a key cysteine residue (cys-62) in the DNA-binding domain of p50 (Lee *et al* 2002).

Three MAPK signalling cascades have been well characterised (the ERK pathway, p38 pathway and JNK pathway) and each has been shown to be activated by CD40. UO126 blocks the ERK-MAPK cascade through specifically inhibiting MEK. Activated MEK proteins are responsible for phosphorylating and activating ERK 1 and 2. SB203580 specifically inhibits p38 kinase by binding the ATP-binding pocket of p38 and JNK inhibitor II is a competitive inhibitor of JNK-1, 2 and 3, thereby preventing phosphorylation of c-Jun (English & Cobb 2002). LY294002 was also used as an inhibitor of the PI3-K pathway. This molecule functions by specifically inhibiting PI3-kinase through preventing ATP binding.

The inhibitors were used in pilot experiments using Akata 6 cells and RL cells in order to ascertain whether the inhibitors were functional and which pathways were important in CD40 signalling (data not shown). In these experiments, CD40L treatment increased Bcl-X_L and Mcl-1 expression in Akata 6 cells and reduced the level of spontaneous apoptosis in Akata 6 cells, which was consistent with previous results presented in this chapter and also in chapter 3. CD40 ligation also increased Bcl-X_L expression in RL cells (as in figs. 4.9 and 4.10) and promoted apoptosis of RL cells, as indicated by increased PARP cleavage in CD40L treated cells. Previous experiments in section 4.1 could not detect any enhanced PARP cleavage with CD40 ligation in RL cells although CD40L clearly promoted cell death. However the experiments presented in this section were performed at a later date and so RL cells may have acquired the ability to cleave PARP in response to CD40L due to a change in culture conditions.

In the pilot experiments, treatment with LY294002 or JNK inhibitor II enhanced PARP cleavage in Akata 6 cells, but not RL cells, and this was diminished by CD40 ligation. This suggests that PI3-K and JNK activity are required for survival of Akata 6 cells, but not the survival promoting effects of CD40 ligand. By contrast, inhibition of the ERK or p38 MAPK pathways did not influence PARP cleavage in either cell line. Inhibition of PI3-K, ERK, JNK or p38 MAPK did not influence the upregulation of Bcl-X_L or Mcl-1 by CD40L in either cell line. Despite the fact that UO126, LY294002 and JNK inhibitor II did not influence Bcl-X_L or Mcl-1 regulation, it was clear that UO126 was functional since it clearly decreased phosphorylation of ERK in

both cell lines, and both LY294002 and JNK inhibitor II promoted apoptosis. It was not clear, however, if SB203580 was fully functional since activation of downstream p38 targets were not assessed. In contrast to the other inhibitors, the NF-κB inhibitors gliotoxin and KA, prevented the CD40L-mediated increase in Bcl-X_L expression and Mcl-1 expression, indicating a major role for NF-κB in the induction of these molecules by CD40. Further experiments were therefore performed using NF-κB inhibitors.

Akata 6 and RL cells were treated with CD40L in the presence or absence of NF-κB inhibitors (figs. 4.11 and 4.12). A range of gliotoxin and KA concentrations were used (from 10μM to 0.1μM) and sulfasalazine was used at 500 μM and 2mM. Although this concentration of sulfasalazine appears high it has been used at this concentration and higher in other studies (Wahl *et al* 1998, Munzert *et al* 2002).

In Akata 6 cells, western analysis demonstrated increased Bcl-X_L and Mcl-1 expression in CD40 ligand treated cells and PARP analysis demonstrated a small amount of spontaneous apoptosis and this was decreased by CD40L (fig. 4.11). Sulfasalazine (2mM) prevented induction of Mcl-1 and Bcl-X_L, and prevented CD40 ligand from suppressing spontaneous apoptosis. These effects were not observed with 0.5mM sulfasalazine and were therefore dose dependent. Similarly, gliotoxin and KA also prevented induction of Bcl-X_L and Mcl-1, and suppression of spontaneous apoptosis in a dose dependant manner. Mcl-1 expression was actually lower in cells treated with gliotoxin or sulfasalazine (2 mM). However, it is difficult to determine whether this is a direct effect on Mcl-1 transcription, or reflects somewhat higher levels of apoptosis in cells treated with these inhibitors.

Interestingly, in Akata 6 cells, gliotoxin altered the migration of the Bcl-X_L band, producing bands of a higher molecular weight (fig. 4.11). The Bcl-X shift was similar to deamidated Bcl-X, observed by Deverman *et al* (2002). Deamidation of Bcl-X occurs at two asparagines (52 and 66) in the loop region, converting them to asparate residues. This introduces negative charges and may influence the structure and function of Bcl-X. Indeed, deaminated Bcl-X was unable to bind Bim and lost its anti-apoptotic activity (Deverman *et al* 2002).

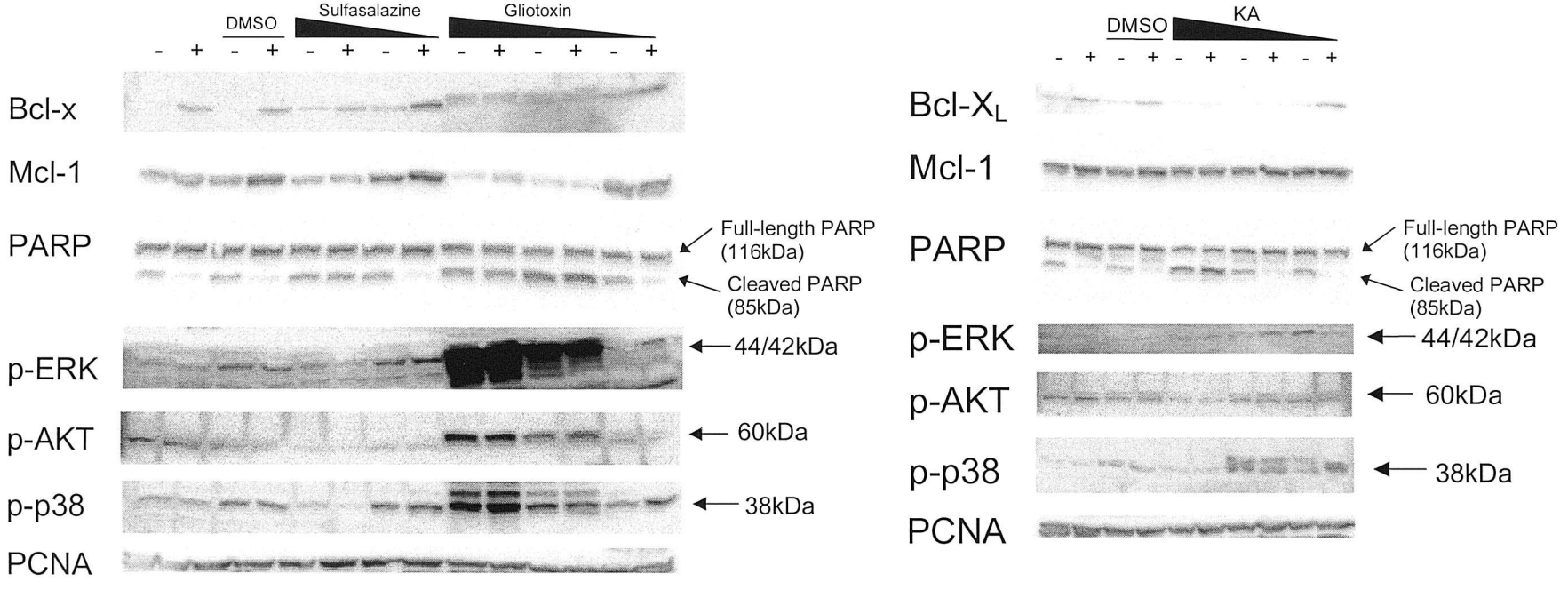


Figure 4.11: Effect of inhibiting NF-κB on CD40 signalling in Akata 6 cells. Cells were cultured for 16 hours in the presence or absence of CD40L (1μg/ml) and the presence or absence of various NF-κB signalling inhibitors at various concentartions. Sulfasalazine was used at 2mM or 500μM final concentration and Gliotoxin was used at 10μM, 1μM and 0.1μM final concentartion. Kamebakaurin (KA) was used at 10μM, 1μM and 0.1μM final concentration. The inhibitors were dissolved in DMSO and so a volume of DMSO equivalent to the maximum volume of inhibitor solution added was used as a control. Following treatment, protein expression and detection of phosphorylated signalling intermediates was detected by Western blot. PCNA is shown as a loading control. Results are representative of two independent experiments.

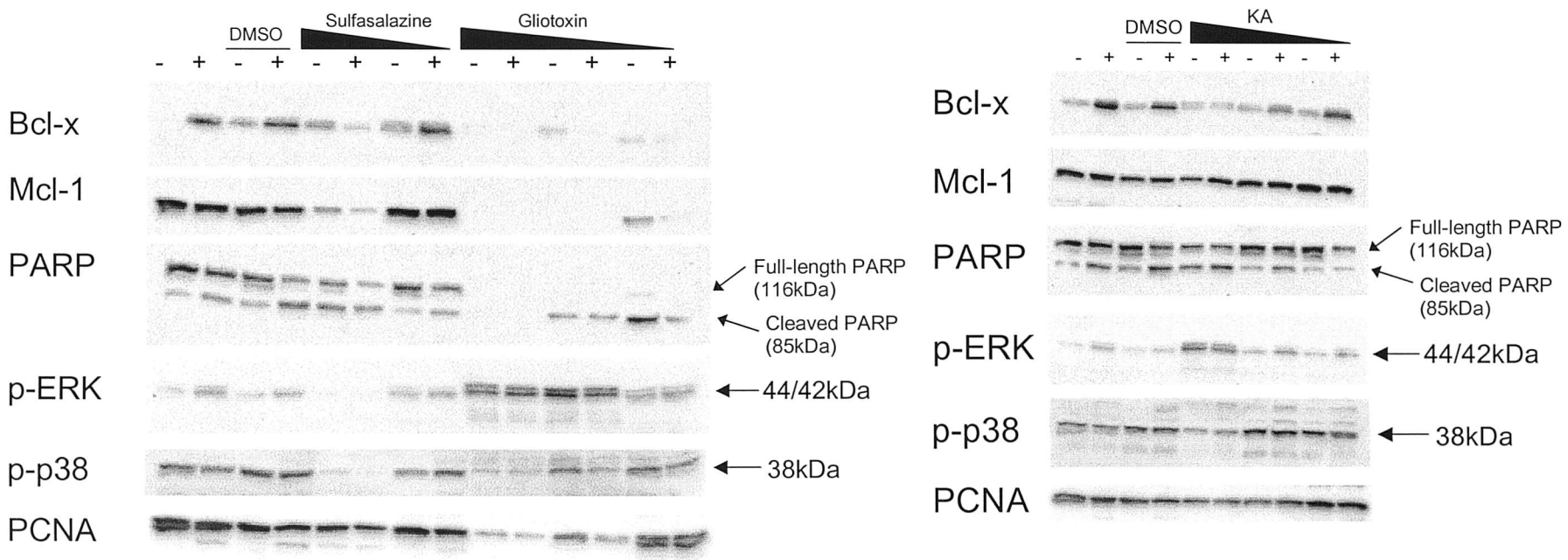


Figure 4.12: Effect of inhibiting NF-κB on CD40 signalling in RL cells. Cells were cultured for 16 hours in the presence or absence of CD40L (1μg/ml) and the presence or absence of various NF-κB signalling inhibitors at various concentartions. Sulfasalazine was used at 2mM or 500μM final concentration and Gliotoxin was used at 10μM, 1μM and 0.1μM final concentartion. Kamebakaurin (KA) was used at 10μM, 1μM and 0.1μM final concentration. The inhibitors were dissolved in DMSO and so a volume of DMSO equivalent to the maximum volume of inhibitor solution added was used as a control. Following treatment, protein expression and detection of phosphorylated signalling intermediates was detected by Western blot. PCNA is shown as a loading control. Results are representative of two independent experiments.

In RL cells, CD40 ligand induced Bcl-X_L expression, but not Mcl-1, and induced PARP cleavage (fig. 4.12). Similar to Akata 6 cells, sulfasalazine (2mM) prevented induction of Bcl-X_L expression and decreased basal levels of Mcl-1. However sulfasalazine did not alter the effects of CD40 ligand on PARP cleavage. Gliotoxin induced massive apoptosis, even in the absence of CD40 ligand, suggesting that RL cells are exquisitely sensitive to proteasome inhibition. Similar to the effects of sulfasalazine, KA prevented induction of Bcl-X_L expression but did not alter the effects of CD40 ligand on PARP cleavage. The effects of KA and sulfasalazine were dose dependent.

Therefore, NF-κB appears to play a major role in induction of Bcl-X_L (and Mcl-1) in both Akata 6 and RL cells, and is important for promoting cell survival by CD40 ligand in Akata 6 cells. Despite the profound difference in survival outcome following stimulation of CD40 in Akata 6 and RL cells, there were no major differences in the signalling pathways regulating Bcl-X_L or Mcl-1 expression.

Figures 4.11 and 4.12 also demonstrate that CD40 ligation differentially influenced ERK phosphorylation in Akata 6 and RL cells. In Akata 6 cells, CD40 ligation did not influence ERK phosphorylation, whereas it increased ERK phosphorylation in RL cells. However, in pilot experiments inhibition of ERK did not effect gene expression or apoptosis and this was therefore not pursued.

Interestingly, gliotoxin massively increased phosphorylation of ERK, AKT and p38 in a dose-dependent manner in Akata 6 cells (fig. 4.11). Gliotoxin also increased phosphorylation of ERK and influenced the pattern of phosphorylation of p38 in RL cells (fig. 4.12). Gliotoxin inhibits the proteasome and so perhaps activators of the ERK and PI3-K pathways, which would normally be degraded by the proteasome, were not degraded, leading to hyper-phosphorylation of signalling molecules. A recent paper showed that the proteasome inhibitors MG132 and lactacystin increased ERK, JNK and p38 phosphorylation and it was suggested that this was due to reactive oxygen species targeting upstream signalling molecules (Wu *et al* 2002).

4.7 Role of NF-κB in the control of alternative Bcl-X promoters

To determine whether NF-κB inhibition affected the regulation of Bcl-X RNAs by CD40, RL and Akata 6 cells were treated with CD40 ligand for 4 hours and 8 hours in the presence or absence of 10μM or 5μM KA and RT-PCRs were performed specific for the Bcl-X exon IA and exon IB transcripts. 5μM KA was used in addition to 10μM KA, as the data in figures 4.11 and 4.12 suggested that the optimal dose of KA was between 10μM and 1μM.

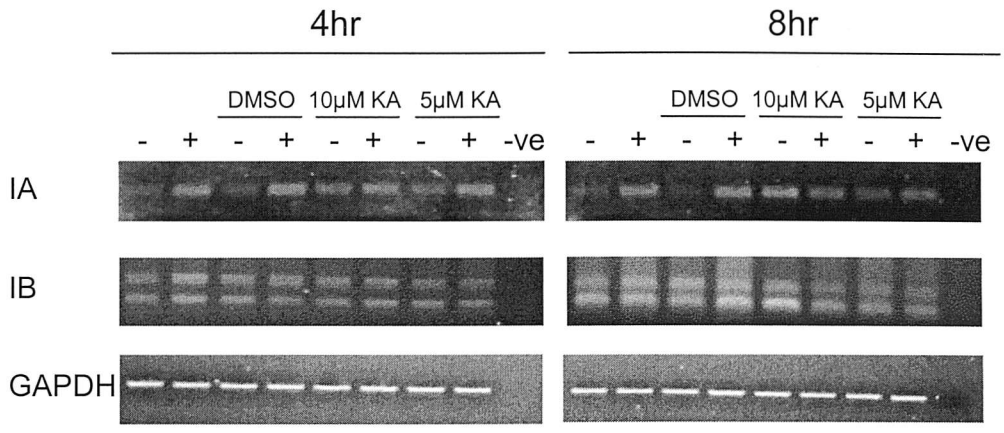
As shown in fig. 4.13, CD40L or KA did not dramatically influence expression of exon IB containing transcripts in either Akata 6 or RL cells. However expression of the exon IA-containing mRNAs was increased by CD40L treatment, suggesting that CD40 signalling targets the Bcl-X exon IA promoter. This is consistent with results presented in chapter 3 but extends these results to include RL cells.

In RL cells (fig. 4.13B), upregulation of exon IA mRNAs by CD40 was clearly prevented by KA treatment. This shows that the NF-κB pathway, and indeed the DNA-binding activity of the p50 subunit, was essential for CD40-mediated Bcl-X mRNA upregulation. In Akata 6 cells (fig. 4.13A), the effects of KA were less clear. KA increased basal levels of exon IA containing mRNAs, bringing them to a similar level to that observed with CD40L treatment. Therefore p50 activity may have a role in suppressing normal levels of exon IA-containing mRNAs in Akata 6 cells. KA did not affect expression of exon IA transcripts in CD40L treated cells, showing that p50 activity is not involved in the upregulation of exon IA transcripts by CD40 in this cell line. Perhaps other NF-κB subunits e.g. p65, are involved in upregulating Bcl-X exon IA transcript expression in Akata 6 cells.

4.8 The role of signalling pathways in regulation of cell survival, Bcl-X_L and Mcl-1 expression by CD40 in primary malignant B-cells

Inhibitors to cell signalling pathways were also used to treat freshly isolated primary malignant B-cells, in conjugation with CD40L. The use of primary cells is important since cell signalling may be abnormal in established cell lines. An experiment was performed using follicular lymphoma cells (sample FL-f) and treating these cells with

A Akata 6



B RL

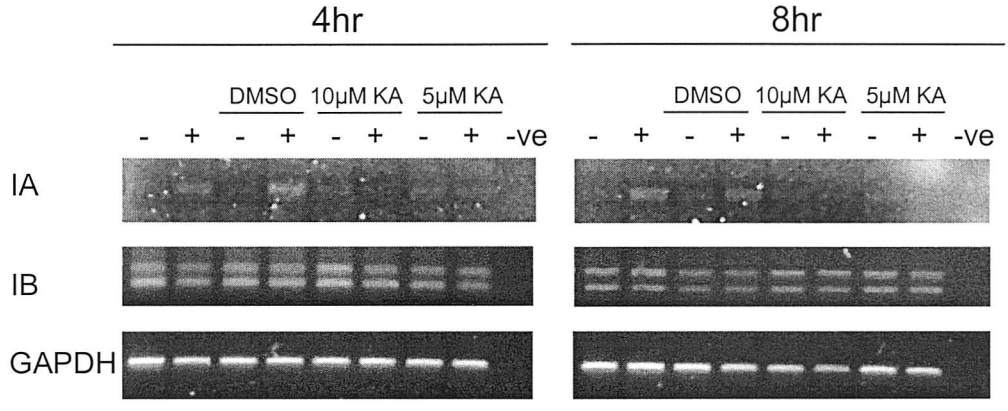


Figure 4.13: Kamebakaurin (KA) prevents the CD40L-mediated increase in Bcl-X exon IA mRNAs. Akata 6 (**A**) or RL cells (**B**) were treated in the presence or absence of CD40L (1μg/ml) and the presence or absence of 10μM or 5μM KA for 4 or 8 hours. The equivalent volume of DMSO was used as a control. RNA was isolated and expression of Bcl-X exon IA and exon IB containing mRNAs detected by semi-quantitative RT-PCR. GAPDH is shown as a loading control.

either UO126 or LY294002, in the presence or absence of CD40L and protein expression analysed by Western blot. Only these inhibitors were used since the other inhibitors were unavailable at the time of the experiment. As shown in fig. 4.14, Bcl-X_L and Mcl-1 were clearly upregulated by CD40. CD40 ligation also reduced PARP cleavage, indicating that CD40 signalling rescued these cells from spontaneous apoptosis. CD40 signalling led to increased ERK and AKT phosphorylation, suggesting that the ERK-MAPK cascade and the PI3-K pathway were activated by CD40 in these cells.

Despite increased phosphorylation of ERK with CD40L, UO126 did not affect CD40 signalling to Bcl-X_L or Mcl-1. UO126 also did not affect PARP cleavage. However UO126 was functional since it clearly reduced ERK phosphorylation (fig. 4.14). Treatment with LY294002 prevented the CD40L-mediated induction of Bcl-X_L, suggesting that PI3-K has a major role in regulating Bcl-X_L expression, which was not observed in the cell lines. LY294002 also decreased Mcl-1 levels but probably still allowed CD40 to signal to Mcl-1 (fig. 4.14). The decrease in Mcl-1 expression may be due to a direct role of PI3-K in regulating Mcl-1 levels or may be a function of the increased apoptosis observed with LY294002 treatment, as indicated by increased PARP cleavage and the presence of Mcl-1 cleavage products. The increased PARP cleavage associated with LY294002 treatment was not influenced by CD40 ligation, suggesting that PI3-K activity is required for the cell-survival promoting effects of CD40 in these cells. LY294002 clearly blocked the PI3-K pathway as phosphorylation of AKT (a downstream target of PI3-K) was prevented with the inhibitor. Treatment with LY294002 also decreased ERK phosphorylation suggesting that the inhibitor may have non-specific effects on cell signalling or that there is cross-talk between the PI3-K and ERK pathways. Indeed, stimulation of PI3-K/AKT by integrins was required for activation of the ERK MAPK cascade in a fibroblast cell line (King *et al* 1997).

As results from the experiments treating the cell lines with signalling inhibitors showed that the NF-κB pathway was instrumental in CD40 signalling and the data presented with FL-f, suggested that the PI3-K pathway may be important in primary samples, further primary samples were treated with KA, gliotoxin or LY294002.

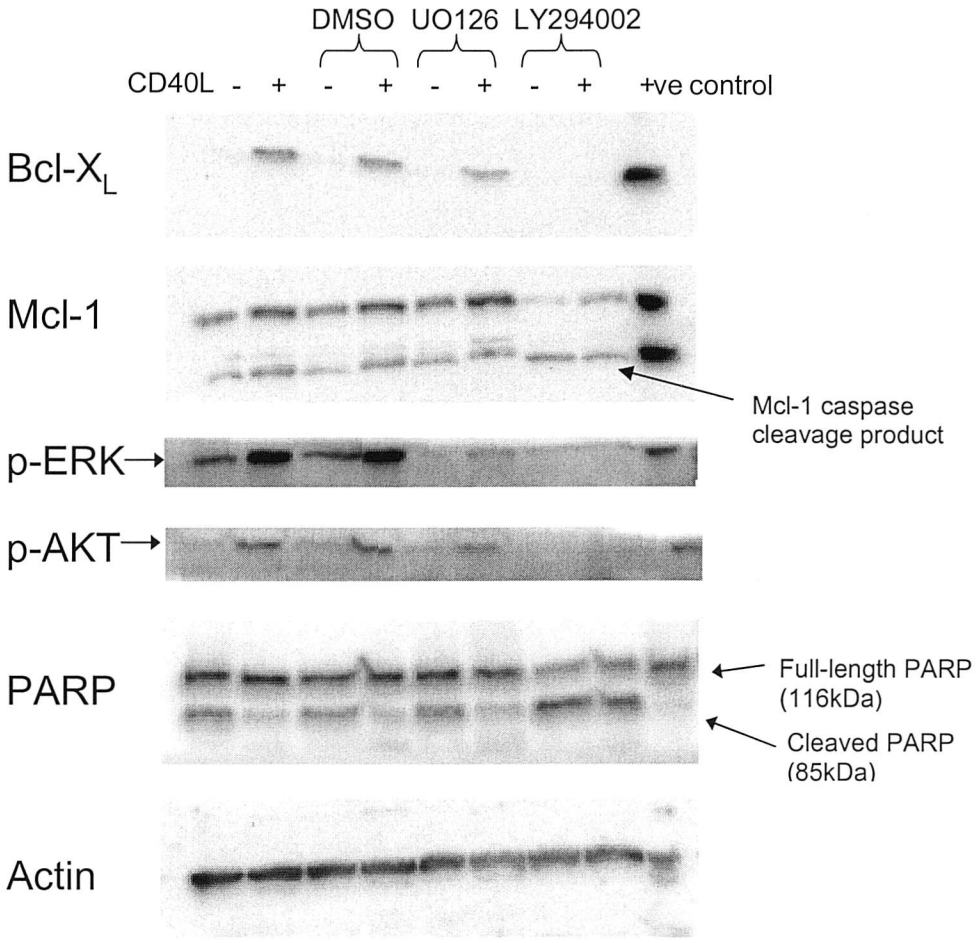


Figure 4.14: Effect of inhibiting the ERK-MAPK and PI3-K pathways on CD40 signalling in a follicular lymphoma (FL-f). B-cells were immunomagnetically purified from a fresh lymph node and cultured for 16 hours in the presence or absence of CD40L (1μg/ml) and the presence or absence of UO126 (10μM) or LY294002 (10μM). The equivalent volume of DMSO was used as a control. Following treatment, protein expression and expression of phosphorylated signalling proteins was detected by Western blot. Actin is shown as a loading control.

These samples were CLL-h and Reactive-d. Figure 4.15 shows the Western blots obtained.

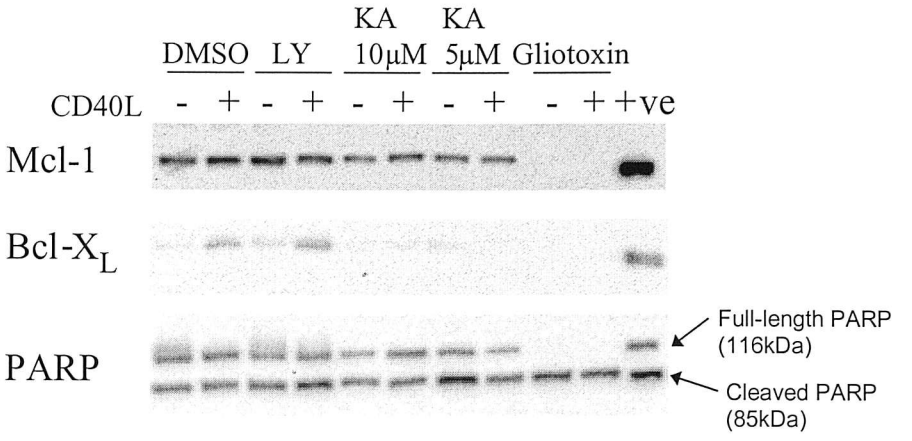
In the CLL-h sample (fig. 4.15A), CD40L treatment did not influence Mcl-1 expression or greatly affect PARP cleavage. However treatment with CD40L increased Bcl-X_L expression. Treatment with LY294002 did not affect CD40 signalling to Bcl-X_L. However, both KA and gliotoxin prevented the CD40-mediated increase in Bcl-X_L expression. Even at a very low dose (0.1μM), gliotoxin caused complete PARP cleavage and disappearance of both Bcl-X_L and Mcl-1.

In the Reactive-d sample, (fig. 4.15B), CD40 ligation induced Bcl-X_L expression and increased Mcl-1 expression. LY294002 reduced induction of Bcl-X_L by CD40 but it did not affect signalling to Mcl-1. KA, however, prevented the CD40-mediated increase in both Mcl-1 and Bcl-X_L expression. KA also reduced basal Mcl-1 levels.

Therefore these data show that in both normal and malignant primary B-cells, the activity of the NF-κB subunit p50 is essential for the regulation of Bcl-X_L (and Mcl-1) by CD40. The activity of PI3-K is also necessary for the regulation of Bcl-X_L in some primary samples.

RT-PCRs for the different Bcl-X transcripts were undertaken on the CLL-h sample (fig. 4.16). Unfortunately this could not be carried out on the other primary samples as there were too few cells for analysis. As shown in figure 4.16, CLL-h did not express any Bcl-X exon IB-containing mRNAs but CD40L treatment clearly led to the induction of the exon IA transcript. This increase was slightly reduced with LY294002, but was completely inhibited by 10μM or 5μM KA treatment. Gliotoxin treatment completely prevented any expression of exon IA mRNAs. Therefore, this data suggests that the activity of p50 is necessary for the upregulation of Bcl-X expression by CD40, via inducing expression of Bcl-X exon IA mRNA.

A CLL-h



B Reactive-d

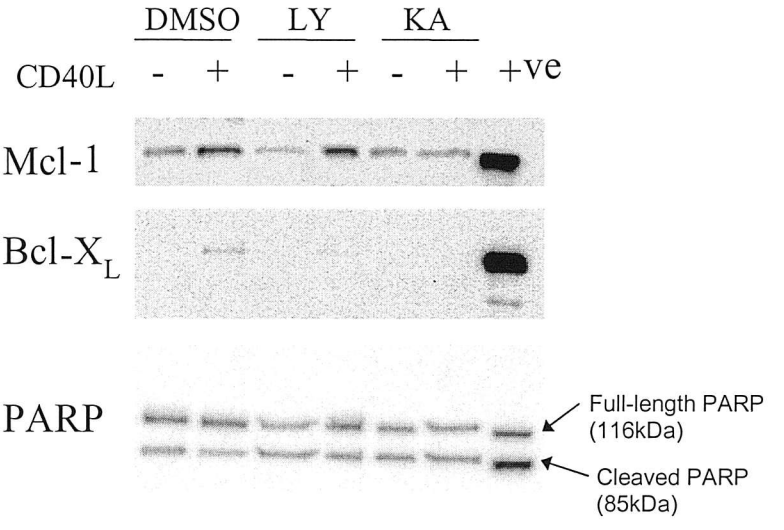


Figure 4.15: Effect of inhibiting the PI3-K and NF-κB pathways on CD40 signalling in a CLL (CLL-h) and reactive node (Reactive-d). B-cells were immunomagnetically purified from fresh lymph nodes and cultured for 12 hours in the presence or absence of CD40L (1μg/ml) and the presence or absence of LY294002 (10μM), kamebakaurin (KA) (10μM or 5μM) or gliotoxin (0.1μM). The highest equivalent volume of DMSO was used as a control. Following treatment, protein expression and PARP cleavage was detected by Western blot.

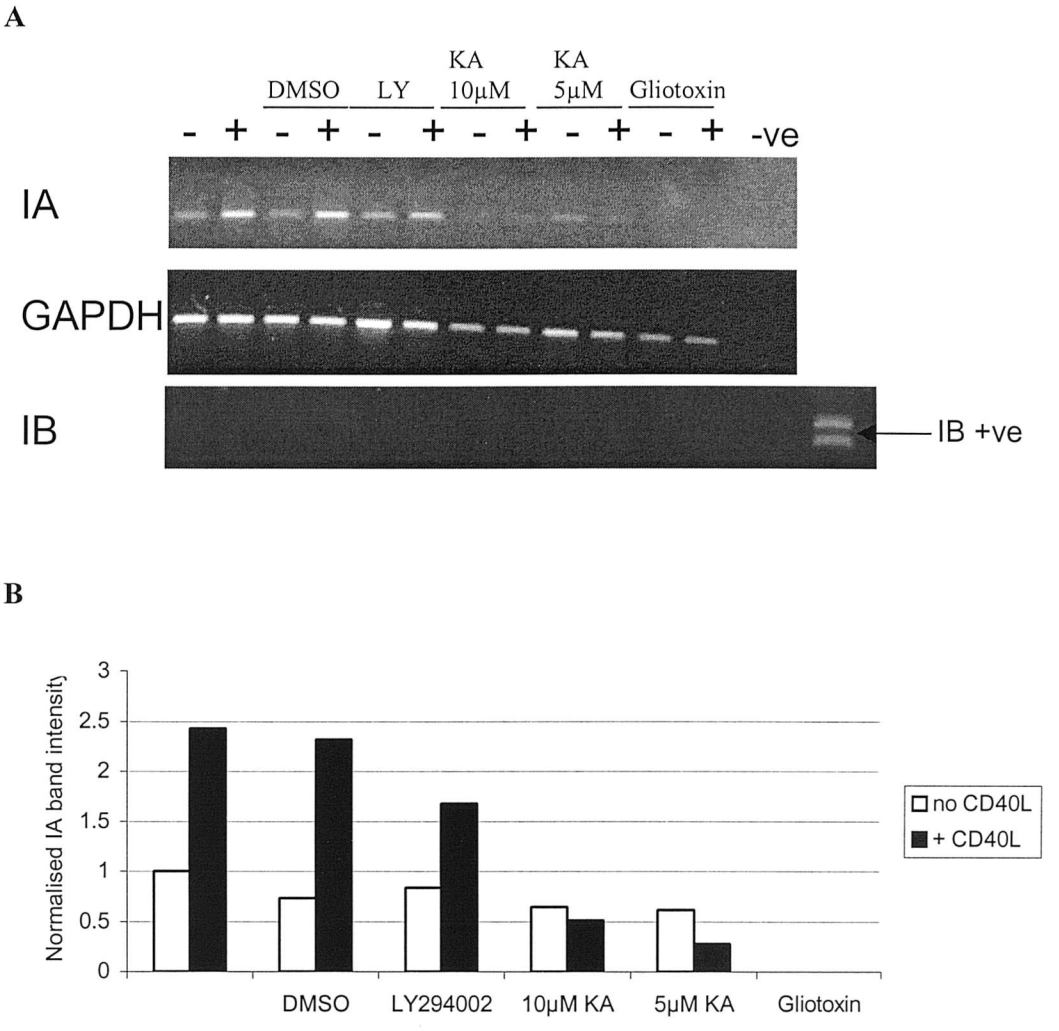


Figure 4.16: Kamebakaurin (KA) prevents the CD40L-mediated increase in Bcl-X exon IA mRNAs in a CLL sample. Immunomagnetically purified CLL cells were treated in the presence or absence of CD40L (1μg/ml) and the presence or absence of LY294002 (10μM) or kamebakaurin (KA) (5μM or 10μM) or gliotoxin (0.1μM) 12 hours. The equivalent volume of DMSO was used as a control. RNA was isolated and expression of Bcl-X exon IA and exon IB containing mRNAs detected by semi-quantitative RT-PCR. GAPDH is shown as a loading control. **A** – shows the gels obtained. RL cDNA was used as the Bcl-X exon IB positive control. **B** - quantification of the Bcl-X exon IA transcript. IA and GAPDH bands were quantified and data is expressed as IA band intensity divided by GAPDH band intensity.

4.9 Discussion

CD40 can act either as a pro-survival or death-inducing signalling molecule for malignant B-cell lines, depending on the cell type or system used. This study confirmed this observation, clearly demonstrating that CD40 ligation rescues Akata 6 cells from etoposide-induced apoptosis but induces cell death in RL cells, in the short-term. It is not clear if CD40L-induced RL cell death is via classical apoptosis or another mechanism, since RL cells did not consistently cleave PARP in response to CD40. RL cells also did not show a clear apoptotic population of cells in the Annexin V assay. However it is clear that CD40L treatment does induce approximately 30% RL cell death in the short-term (48 hours). Therefore CD40L-induced death in RL cells could occur, at least in part, through necrosis or alternative forms of programmed cell death such as autophagic degeneration.

Previous studies using carcinoma cells have shown that blocking protein synthesis with cycloheximide allows CD40 ligation to induce apoptosis (Hess & Engelmann 1996, Eliopoulos *et al* 2000, Gallagher *et al* 2002), suggesting that anti-apoptotic proteins are synthesised either via activation of the CD40 signalling pathway or through another active survival pathway in the cell e.g. constitutive expression of the PI3K-AKT pathway or Bcl-2 overexpression. In this study cycloheximide treatment of RL cells did not influence the ability of CD40 to induce cell death or apoptosis. This may suggest that CD40L-induced RL cell death occurs via a different mechanism to that in epithelial cells.

CD40 signalling was also suggested to sensitise epithelial tumours to drug-induced apoptosis (Eliopoulos *et al* 1996, Young *et al* 1998), suggesting that CD40 therapy in combination with chemotherapy may be a valid option for treatment of CD40 positive carcinomas. However, in primary B-cell lymphomas and in B-cell lymphoma lines, including Akata 6, CD40 ligation suppresses apoptosis by cytotoxic drugs (see chapter 3, Kitada *et al* 1998, Voorzanger-Rousselot *et al* 1998). In RL cells, CD40 ligation did not significantly sensitise or suppress etoposide-induced death as assessed by PARP cleavage or Annexin V assay. The data from chapter 3 and this study therefore suggest that CD40 therapy in combination with chemotherapy is probably not a viable treatment for B-cell lymphomas, as CD40 signalling would prevent

chemotherapeutics from inducing tumour cell apoptosis and could actually confer a survival advantage on the tumour, proving detrimental for patients.

Long-term clonogenic assays were performed to assess the effect of CD40L on RL and Akata 6 cell survival over 21 days. This is the first study to assess the effect of CD40 ligation on long-term cell survival. Surprisingly, CD40L pretreatment improved the long-term survival and clonogenic ability of both Akata 6 and RL cells. In RL cells, this was due in part to selection of a more robust phenotype by CD40L. CD40 signalling kills sensitive cells in the short-term, selecting for more robust cells to grow and form clones. This short-term death phase was then overcome in RL cells, and was followed by a long-term growth advantage. These findings have key consequences for CD40 based cancer therapy. There is much interest in treating lymphoma, and other cancers, with CD40L or antibodies to CD40, or CD40L gene therapy. This is based on encouraging findings in animal models (Funakoshi *et al* 1994, Tutt *et al* 1998, Todryk *et al* 2001) and a phase I clinical trial treating a variety of tumours with CD40L (Vonderheide *et al* 2001). In these systems a therapeutic effect is probably largely achieved through CD40 stimulating an anti-tumour immune response. However these agents will also directly stimulate CD40 signalling on the tumour itself. Although many studies have shown CD40 signalling induces growth inhibition or cell death in some malignant cells, these assays have always been performed in the short-term (up to 96 hours) (Hirano *et al* 1999, Eliopoulos *et al* 2000, Gallagher *et al* 2002). These data show that despite a short-term death phase, CD40 promotes long-term survival of RL cells. CD40 also confers a massive survival advantage to Akata 6 cells in the long-term. These findings, in addition to the results obtained in chapter 3 showing that CD40 ligation delivers a potent survival signal to primary B-cell lymphomas, have important implications for the future of CD40-based therapies. Treating B-cell lymphomas with CD40 activating agents in the clinic could actually increase tumour cell survival and lead to progression of the disease, if a sufficient anti-tumour immune response is not mounted. Even in tumours where a short-term killing effect of CD40 occurs, this may be overcome by a survival advantage in the long-term. Therefore a safer option may be to restrict CD40 therapy to CD40 negative tumours, or to patients in which a sufficient immune response is expected to occur.

It is also important to determine the mechanisms by which CD40 differentially controls cell survival. If we can understand why CD40 causes a particular cell to die, perhaps we can use that information to target the appropriate pathway in cells where CD40 causes cell survival, switching them to a CD40 death inducing-phenotype. In addition, markers of the response may be discovered which could predict if cancer cells will live or die following CD40 treatment.

Death receptor signalling has been shown to be an important mediator of CD40-induced apoptosis in epithelial cells. Upregulation of death receptors and their ligands occurs in epithelial cells, suggesting an autocrine or paracrine mechanism of action for apoptosis. CD40-induced apoptosis correlates with induction of Fas ligand, TRAIL or TNF in ovarian carcinoma cell lines, HeLa cells stably expressing CD40 and biliary epithelial cells (Eliopoulos *et al* 2000, Afford *et al* 2001, Grell *et al* 1999). Blocking the TNF-R1/TNF or Fas/FasL interactions with neutralising antibodies prevented CD40-induced apoptosis in HeLa cells or biliary epithelial cells respectively (Eliopoulos *et al* 2000, Grell *et al* 1999). Many studies have also shown that CD40 activation leads to Fas upregulation, in both normal and malignant B-cells and carcinoma cell lines, although this does not necessarily sensitise cells to Fas-mediated apoptosis.

In this study, although CD40 ligation upregulated expression of Fas on both RL and Akata 6 cells, only RL cells were Fas sensitive. Akata 6 cells were previously shown to be insensitive to Fas (Mouzakiti & Packham, personal communication) and EBV-positive Burkitt's lymphoma lines, like Akata 6 cells, were also shown to be Fas-resistant (Falk *et al* 1992). Fas sensitivity was not enhanced by CD40L in RL cells and a Fas-blocking antibody did not prevent RL CD40L-induced cell death. RL and Akata 6 were also insensitive to TRAIL and CD40 did not sensitise cells to TRAIL-mediated apoptosis. A recent study showed that Akata 6 cells were TRAIL insensitive, despite expressing the DR4 and DR5 receptors for TRAIL (Mouzakiti & Packham 2003). Therefore, in contrast to epithelial cells, the Fas and TRAIL pathways are not involved in CD40L induced cell death in RL cells and do not appear to be involved in the differential regulation of cell survival by CD40.

A possible reason why Fas and TRAIL signalling are not influenced by CD40 could be due to alterations in expression of signalling components downstream of the receptor, for example in the DISC. FLIP can negatively regulate Fas and TRAIL signalling by interfering with caspase-8 activation (Thome & Tschopp 2001). FLIP was also shown to be upregulated by CD40 activation in normal B-cells (van Eijk *et al* 2001, Hennino *et al* 2000, Hennino *et al* 2001) and CLL (Kitada *et al* 1999), providing a possible explanation as to why CD40 signalling does not sensitise some cells to Fas-mediated apoptosis, even though it upregulates Fas expression. In RL and Akata 6 cells, $FLIP_L$ expression was not influenced by CD40L treatment and neither was FADD expression. Levels of FADD and FLIP appeared similar between the two cell lines. Caspase-8 expression was increased by CD40L in both cell lines, suggesting that caspase-8 may be a CD40 target gene. There was no evidence of caspase-8 cleavage and activation in either Akata 6 or RL cells. Taken together, these results suggest that CD40 signalling is attempting to sensitise cells to Fas-mediated apoptosis by upregulating Fas and caspase-8 expression, but this does not affect Fas sensitivity, possibly due to the presence of FLIP and as yet unidentified inhibitors of the death receptor signalling pathway.

This study also determined the effect of CD40 on the expression of Bcl-2 family members in Akata 6 and RL cells. Bcl-X_L is a consistent CD40 target and was upregulated by CD40 both in Akata 6 and RL cells, in primary lymphoma cells (see chapter 3) and in a number of published studies (Choi MS *et al* 1995, Ghia *et al* 1998, Lee *et al* 1999, Kitada *et al* 1999). It is intriguing that such a potent cell survival molecule should be upregulated by CD40 in RL cells, when CD40 induces short-term cell death in these cells. Perhaps in the short-term, the balance between death-inducing proteins and death-suppressing proteins is tipped towards death, but in the long-term death-suppressing proteins, such as Bcl-X_L predominate, leading to a long-term survival advantage.

Possible molecules that could account for short-term CD40L-induced cell death in RL cells are pro-apoptotic Bcl-2 family members. A recent study showed that Bax, Bak and Bik mRNAs were upregulated after CD40 stimulation on Daudi cells and that Bax protein increased in RL cells following treatment with 10μg/ml CD40L for 48 hrs

compared to untreated cells (Szocinski *et al* 2002). The same conditions were used in this study and confirmed that CD40L treatment increased Bax expression, relative to untreated cells. However this increase was largely due to a decrease in Bax expression in untreated cells over 48 hours. Therefore Bax levels were merely sustained at a slightly higher level than basal levels. It is unlikely, therefore, that regulation of Bax by CD40 is entirely responsible for CD40-induced death. There was also no evidence of increased Bax expression following 1μg/ml CD40L treatment in the initial time course experiment, even though this concentration of CD40L clearly induces RL cell death. This study therefore questions the importance of Bax as a mediator of CD40L-induced death in RL cells.

Differences in the expression of Bcl-2 family members following CD40L treatment in Akata 6 and RL cells, might contribute to the differential regulation of cell survival by CD40. CD40L treatment upregulated Mcl-1 expression in Akata 6 cells but not in RL cells, whereas upregulation of Bax expression was only observed in RL cells. Data presented in chapter 3 demonstrated that Mcl-1 was a key survival protein for Akata 6 cells using antisense oligonucleotides and so upregulation of Mcl-1 by CD40 should contribute to cell survival. At 10μg/ml CD40L, Bcl-2 expression increased in Akata 6 cells but not in RL cells. Even though these differences appear slight, they could be enough to tip the balance of apoptosis controlling proteins, towards cell death for RL cells and cell survival for Akata 6 cells.

CD40 signalling has been shown to activate a number of cell signalling pathways, including the ERK, JNK and p38 MAPK cascades, and the NF-κB and PI3-K pathways, although their role in CD40 signalling is not clear (Li *et al* 1996, Sakata *et al* 1995, Craxton & Sutherland 1996, Berberich 1994, Padmore *et al* 1997). The role of these pathways in CD40 signalling in Akata 6 and RL cells was investigated to determine if they had a function in the control of cell survival by CD40. In Akata 6 cells, and in a CLL sample and reactive node, inhibition of the NF-κB pathway prevented the anti-apoptotic activity of CD40 ligation. However the signalling pathways responsible for the death-promoting effect of CD40 ligation in RL cells were unclear.

The only discernable difference in signalling between the two cell lines was that ERK phosphorylation was increased with CD40L treatment in RL cells but not Akata 6 cells. However inhibition of the ERK pathway with UO126 did not influence the survival outcome of CD40 signalling in either cell line. This is the first study to report activation of ERK by CD40 in human NHL cells. ERK has been shown to be activated by CD40 in normal mouse B-cell systems (Li *et al* 1996, Purkerson & Parker 1998, Dadgostar *et al* 2002, Eliopoulos *et al* 2003), in a Hodgkin's disease cell line (Zheng *et al* 2003), in non B-cell systems (Li & Nord 2002, Pearson *et al* 2001, Park *et al* 1999) but ERK was not activated by CD40 in human B-cells or some cell lines (Sutherland *et al* 1995, Berberich *et al* 1996, Sakata *et al* 1995). The role of ERK in CD40-mediated responses appears minor. A microarray study using normal mouse B-cells showed that although ERK is involved in CD40-induced proliferation it has little effect on gene expression patterns (Dadgostar *et al* 2002). In contrast, Eliopoulos *et al* (2003) found that ERK activation by CD40 was not involved in the proliferative response or expression of activation markers but did contribute to IgE production.

The mechanism by which CD40 upregulates Bcl-X_L expression in both Akata 6 and RL cells and primary material was investigated using specific inhibitors to the NF-κB, PI3-K, ERK, JNK and p38 MAPK pathways. In all cells studied the NF-κB inhibitors prevented the CD40-mediated increase in Bcl-X_L expression. This was most specific with the NF-κB inhibitor kamebakaurin (KA), which specifically targets the DNA-binding activity of the p50 NF-κB subunit (Lee *et al* 2002). KA also prevented the increase in exon IA mRNA with CD40L, suggesting that activation of p50 via CD40 signalling is involved in increasing activity of the exon IA promoter.

Previous studies have identified Bcl-X_L as a NF-κB regulated gene. Two functional NF-κB sites have been identified upstream of exon II (-77 and –62 from the exon II transcription start site or –362 and –339 from the translation initiation codon) which bind c-Rel, p65 and p50 in a fibrosarcoma cell line (HT1080) and a Burkitt's lymphoma line (Daudi) (Lee *et al* 1999, Chen *et al* 2000). Lee *et al* (1999) demonstrated that these sites were CD40-responsive and that blocking NF-κB signalling by stably transfecting cells with a form of IκB that resists degradation,

prevented upregulation of Bcl-X in response to anti-CD40. Studies with the murine Bcl-X promoter region have revealed two additional functional NF-κB sites (at –847 and –967 from the translation initiation codon) that are also conserved in the human sequence and are located in the exon IA promoter region. In the mouse, these sites were shown to be bound by or activated by p50/p50 homodimers, p49/p65 heterodimers and p50/p65 heterodimers (Glasgow *et al* 2000). The subunits that bind these sites were also tissue-specific and showed hypoxia-induced regulation in the rat brain (Qiu *et al* 2001). Data presented here suggested that the activity of the exon IA promoter was also increased by CD40 signalling in lymphoma cells, requiring the DNA-binding activity of the NF-κB subunit p50. It is therefore feasible that this is mediated through p50 binding the conserved NF-κB binding sites in the exon IA promoter.

The PI3-K pathway was implicated in the regulation of Bcl-X_L by CD40 in a follicular lymphoma and reactive node sample in this study, but not in a CLL sample, Akata 6 or RL cells. The variability of the role of PI3-K in these samples could be related to the stage of B-cell development or activation. PI3-K has many downstream targets, which can activate transcription factors e.g. ets-2, which could possibly influence Bcl-X expression (Smith *et al* 2000). Bcl-X_L was also shown to be under control of the PI3-K pathway in other cellular systems. In Baf-3 cells (IL-3 dependent bone marrow derived cell line), the PI3-K pathway was responsible for increasing Bcl-X mRNA expression in response to IL-3 and IGF-1 (Leverrier *et al* 1999) and in megakaryocytic cells thrombopoietin increases Bcl-X_L expression partly through the PI3-K pathway (Kirito *et al* 2002). PI3-K can also activate the NF-κB pathway through phosphorylation of AKT (protein kinase B) which can then activate IκB-kinase (IKK) leading to NF-κB activation (Khwaja *et al* 1999, Ozes *et al* 1999, Beraud *et al* 1999). This pathway to NF-κB activation has been shown to be activated by CD40 in multiple myeloma cells, where it is responsible for cell migration and in normal B-cells where it is necessary for proliferation but not cell survival (Tai *et al* 2003, Andjelic *et al* 2000). Therefore activation of PI3-K by CD40 could influence Bcl-X_L expression through as yet unidentified downstream mediators and/or through activation of NF-κB.

The upregulation of Mcl-1 levels by CD40 was shown to be regulated by the NF-κB pathway in Akata 6 cells and a reactive node sample in this study, using specific inhibitors. The inhibitor of p50 DNA-binding kamebakaurin (KA) prevented CD40-induced Mcl-1 upregulation suggesting a potential mechanism of action. Although no evidence of control of Mcl-1 at the mRNA level by CD40 was observed in the primary samples, this was not actually tested in the above samples and so it is possible that p50 could target the Mcl-1 promoter in response to CD40 in these samples. A putative NF-κB site was identified in both the human and murine Mcl-1 promoters but mutation of the site did not decrease reporter gene activity, so it may not be a functional NF-κB site (Akgul *et al* 2000).

The proteasome inhibitor gliotoxin showed some interesting effects on cellular signalling in this study. Treatment with gliotoxin massively increased phosphorylation of ERK, AKT, JNK and p38 in a dose-dependent manner. This suggests that the proteasome system may participate in degradation of short-lived upstream molecules crucial for phosphorylation of these molecules or inhibitors of these pathways could be downregulated by proteasome inhibition. Previous studies have also noted that various proteasome inhibitors can cause activation of the ERK, JNK or p38 MAPK pathways in non-B cell systems (Giasson *et al* 1999, Meriin *et al* 1998, Nakayama *et al* 2001, Wu *et al* 2002). Reactive oxygen species (ROS) have been implicated in the activation of signalling cascades by proteasome inhibitors (Wu *et al* 2002) and gliotoxin does indeed, increase ROS production, at least in macrophages and hepatic stellate cells (Suen *et al* 2001, Kweon *et al* 2003).

Gliotoxin also shifted the migration of both Bcl-X_L and Mcl-1 bands on Western blots which is consistent with deamidated Bcl-X, observed by Deverman *et al* 2002. Deamidation of Bcl-X occurs at two asparagines (52 and 66) in the loop region, converting them to asparate residues. This introduces negative charges and may influence the structure and function of Bcl-X. Indeed, deaminated Bcl-X was unable to bind Bim and lost its anti-apoptotic activity (Deverman *et al* 2002). This data suggests that deamidated Bcl-X may normally be degraded by the proteasome or that, as gliotoxin induced a large amount of apoptosis, deamidation could occur during

apoptosis converting anti-apoptotic Bcl-X to a pro-apoptotic form. Mcl-1 may also be deamidated or another modification may cause the observed shift in molecular weight.

Table 4.2 summarises the effects of CD40 signalling on RL and Akata 6 cells in this study. Although it is clear that CD40 signalling produces differential effects on cell survival in RL and Akata 6 cells, the reasons for this remain unclear. Survival outcome does not correlate with Fas or TRAIL signalling but may be influenced by alterations in expression of Bcl-2 family proteins. NF-κB activity was found to be essential for both the anti-apoptotic effects of CD40 signalling and upregulation of Bcl-X_L and Mcl-1 by CD40 in cell lines and in primary material. In some primary samples PI3-K activity was also necessary for upregulation of Bcl-X_L by CD40. Despite short-term differences in survival outcome through CD40, in the long-term CD40 ligation improved cell survival for both Akata 6 and RL cells which raises concerns over forthcoming trials for CD40-based cancer therapy of CD40 positive tumour cells.

Table 4.2 Comparison of the effects of CD40 on RL and Akata 6 cells.
Nd = not determined.

Effect of CD40L on:	RL	Akata 6
Cell viability	Cell death	No effect
Etoposide mediated death	No effect	Rescues from apoptosis
Cycloheximide mediated death	No effect	Nd
Clonogenic survival	Increases 6 fold with CD40L pre-treatment. Increases by 30% with CD40L present throughout expt.	Increases 8 fold with CD40L pre-treatment.
Fas expression	Upregulates	Upregulates
Fas sensitivity	Fas sensitive, no enhancement with CD40L	Fas insensitive, no effect of CD40L on sensitivity
TRAIL sensitivity	TRAIL insensitive, no effect of CD40L on sensitivity	TRAIL insensitive, no effect of CD40L on sensitivity
Blocking Fas (ZB4)	CD40L mediated death not through Fas pathway	Nd
Caspase 8 expression	Increases	Increases
Bcl-X_L expression	Increases	Increases
Mcl-1 expression	No effect	Slight increase
Bcl-2 expression	No effect	Slight increase at higher CD40L conc.
Bax expression	Slight increase at higher CD40L conc.	No effect
A20 expression	Increases	No effect
ERK phosphorylation	Increases	No effect

Chapter 5: Characterisation of CD40-regulated genes in B-cell lymphoma using DNA microarrays

5.1 Introduction

DNA microarrays are a relatively new and rapidly expanding technology, used to simultaneously measure changes in expression of thousands of genes. Since their conception in the mid-1990s (Schena *et al* 1995), the use of microarray technology has exploded so now there are at least 4,000 studies concerning microarrays in the literature. A single microarray contains thousands of cDNA molecules or oligonucleotides, each specific for a certain gene, spotted onto a glass slide. RNA of interest is then typically reverse transcribed and fluorescently labelled and then applied to the slide. If the cell expresses a certain gene, then the fluorescently labelled probe will bind to the appropriate spot on the array, causing it to fluoresce. The amount of fluorescence should be proportional to the starting amount of RNA and so one can quantify levels of gene expression. Microarray technology has a wide range of applications, including classifying tumours, identifying potential targets for treatment of disease, identifying microbes, comparative genomics and determining the effects of drug treatments on gene expression.

The use of DNA microarrays to define gene expression changes upon CD40 signalling is an important approach to further understand CD40 biology. Although many CD40 target genes have been identified (e.g. Bcl-X_L, Mcl-1, Bfl-1, A20), it is also possible that key CD40 target genes, or networks of genes, important for the functions of CD40 have yet to be identified (Dallman *et al* 2003). DNA microarrays comparing gene expression in untreated versus CD40-activated cells may therefore help to identify novel CD40-targets. The mechanisms controlling the differential control of cell survival in tumour cells by CD40 are not known (see chapter 4). Therefore the use of microarrays to compare gene expression profiles in cells that have different responses to CD40 activation could reveal important genes involved in determining the cell survival response to CD40.

DNA microarrays were not being used in the University of Southampton at the time of this project and so the first aim of this study was to identify and develop a suitable microarray technology. Different microarray platforms were tested and two were compared in this project. These were a dual-colour system (HGMP microarrays), where two RNA samples labelled with different fluorescent dyes were hybridised to a single slide, and a novel single-colour microarray system (Amersham CodeLink microarrays), where a single probe was hybridised to each slide and then fluorescently labelled post-hybridisation. These systems were used to characterise CD40-regulated genes in Akata 6 cells, a primary follicular lymphoma sample, and RL cells. Akata 6 and RL cells were chosen in order to study differential responses to CD40 ligation. CD40 ligation promotes cell survival in Akata 6 cells but cell death in RL cells (see chapter 4) and so differential changes in gene expression by CD40 may reveal key genes responsible for these effects. Primary follicular lymphoma cells were also used to determine if the technology was applicable to primary material and to further understand the effects of CD40 signalling in a real human tumour. A single microarray study has been published regarding the effects of CD40L on primary mouse B-cells (Dadgostar *et al* 2002), but this is the first study to address this question in human malignant B-cells and to study differential responses to CD40.

5.2 Dual-colour microarrays

Different dual-colour microarray systems were tested and optimised to find a suitable technology. In dual-colour systems, two RNA samples are reverse transcribed and labelled with two different fluorescent dyes (typically Cy3, which fluoresces green, and Cy5, which fluoresces red). These fluorescently labelled cDNA probes are then hybridised to a single array and the ratio of fluorescence from the two samples is calculated to compare gene expression in the two samples. A gene that is upregulated in the Cy3 sample versus the Cy5 sample will appear green, whereas a gene that is upregulated in the Cy5 sample versus the Cy3 sample will appear red and if the two samples have equal gene expression the spot will appear yellow.

Microarrays were obtained from Cancer Research UK and various protocols were tested and modified to try and obtain suitable results. These included: (i) a direct-incorporation method, whereby RNA was reverse transcribed into cDNA in the

presence of fluorescent dyes (Cy3 or Cy5) linked to dCTP molecules, but this resulted in very poor incorporation of the fluorescent nucleotides, (ii) an indirect-incorporation method, whereby aminoallyl-dUTP was incorporated during reverse transcription of RNA and then monofunctional forms of Cy3 or Cy5 dyes were reacted with the amine-modified cDNA, (iii) use of a specific reverse transcriptase enzyme which has improved incorporation rates of fluorescently labelled nucleotides (FluoroScript, Invitrogen, UK), and (iv) modifying hybridisation and washing conditions. However, none of these methods were very successful and so an alternative system was sought from HGMP (Human Genome Mapping Project, Medical Research Council, UK).

cDNA microarrays were obtained from HGMP, that represent nearly 20,000 genes, spotted over two arrays. Each array was composed of 48 blocks of spots, and each block contained two replicate spots for a gene (fig. 5.1). RNA was labelled by reverse transcription using oligo(dT) primers and then Cy3-dCTP or Cy5-dCTP were incorporated into the cDNA by second strand synthesis using Klenow polymerase and random primers. The use of Klenow polymerase and random priming of first-strand cDNA results in greater incorporation of fluorescently labelled nucleotides than conventional direct incorporation methods (Smith *et al* 2003). The probes were then purified and a Cy3 and a Cy5 probe hybridised to each microarray.

To determine the limitations of the HGMP system, a self-self hybridisation was performed, whereby RNA from the same sample was reverse transcribed and labelled with either Cy3 or Cy5 and applied to a single array. The resulting image is shown in figure 5.1A. In the self-self hybridisation, all of the spots should be yellow with a ratio of fluorescence of 1, since the same RNA sample was used for each probe. However in practice this is often not the case due to a number of factors: (i) differences in labelling efficiency between the two dyes, (ii) differences in the power of the two lasers used to detect fluorescence of the probes, (iii) differing amounts of RNA labelled between the two channels and (iv) spatial biases in ratios across the surface of the microarray. This variation can clearly be seen in fig. 5.1A, as one side of the array has mainly yellow and green spots, whereas the other side contains mostly red and yellow spots. The median fluorescence intensities (minus background) for each spot were plotted against each other, resulting in fig. 5.2A. In this scatter plot, the spots with a ratio close to 1 are coloured black, whereas the genes detected

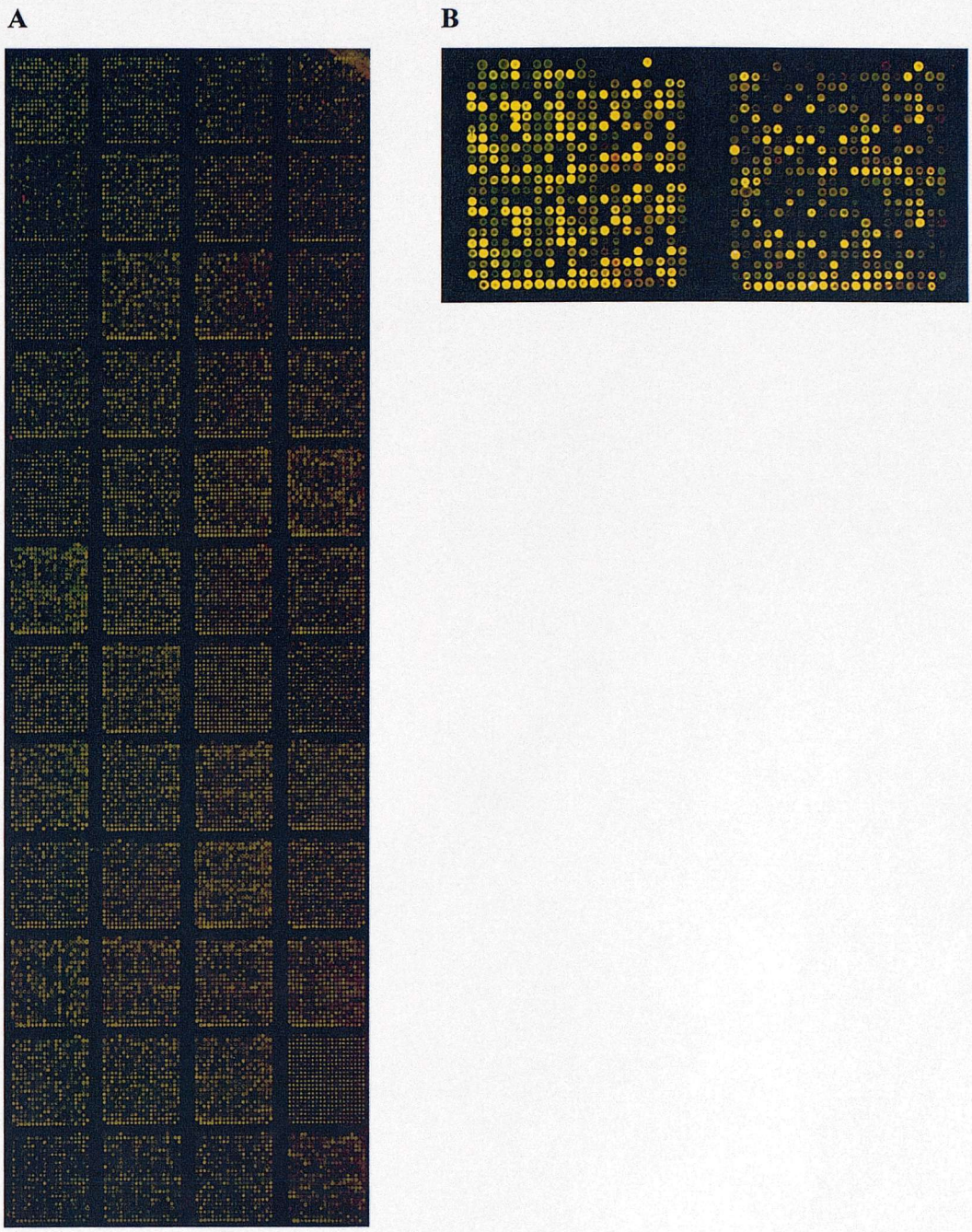
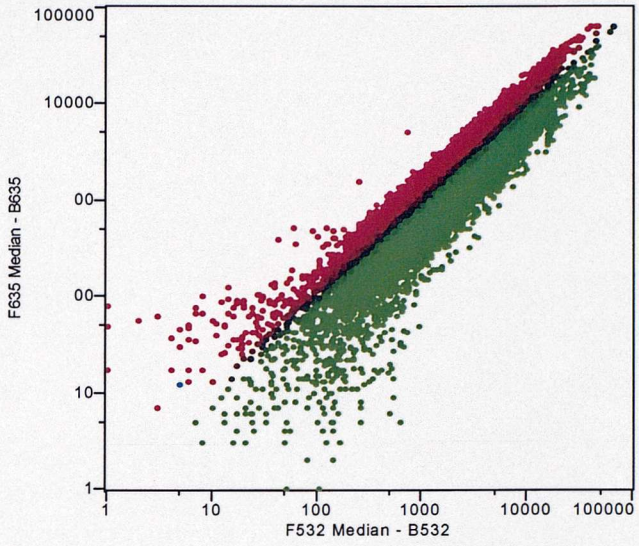


Figure 5.1: HGMP dual-colour microarrays. A – Self-self hybridisation. Image shows the whole microarray slide consisting of 48 blocks of genes, totalling approximately 10,000 genes, each in duplicate. Universal reference RNA was reverse transcribed and then labelled with either Cy3 (green) or Cy5 (red). The probes were then hybridised to the microarray and scanned. **B** – Close-up of two blocks in an array where untreated RL RNA was labelled with Cy3 and CD40L-treated (1μg/ml, 16 hours) RL RNA was labelled with Cy5. Replicate spots can be observed in the top and bottom halves of the block.

A Unnormalised



B Global normalisation

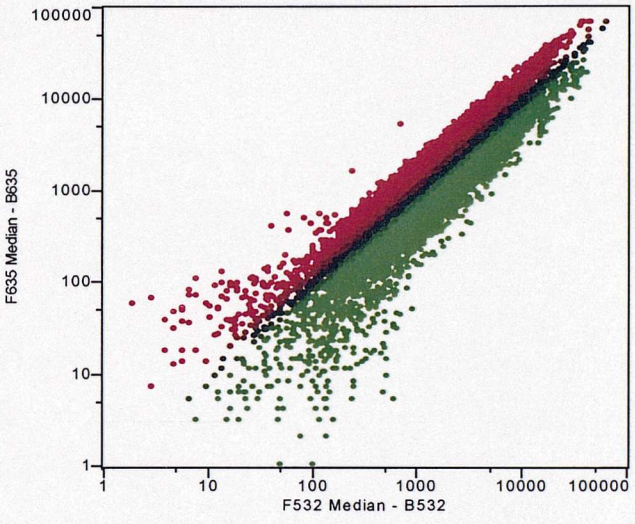


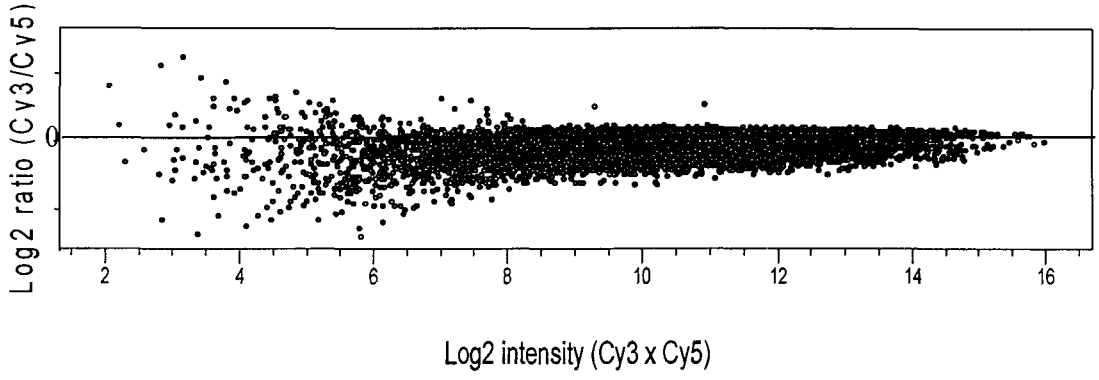
Figure 5.2: The effect of global normalisation. The median Cy3 fluoresecence intensities (F532, x axis) were plotted against the median Cy5 fluorescence intensities (F635, y axis) for each spot from the self-self hybridisation (array in fig. 5.1A). Black coloured spots have an intensity ratio close to 1, whereas green and red spots show upregulation or downregulation of gene expression. **A** – unnormalised data. **B** - data that has undergone global normalisation.

with the Cy5 or Cy3 probes to different extents are coloured red or green. Therefore, there is a lot of systematic variation in the self-self hybridisation that needs to be corrected for.

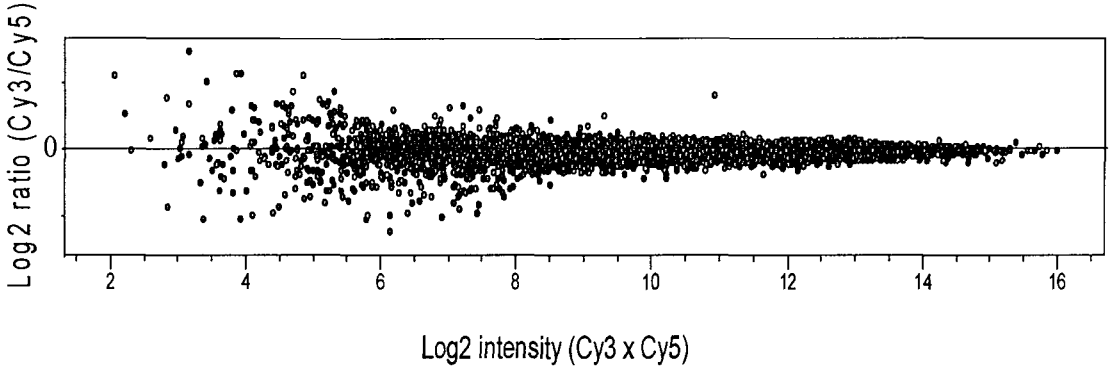
In order to correct for systematic variation normalisation has to be employed. Global normalisation and lowess normalisation were undertaken, to determine which method resulted in the majority of ratios being closest to 1. Global normalisation is achieved by using all the spots on the array to generate a normalisation factor which will scale the mean of the intensity ratios to one. However, global normalisation does not take into account spatial or intensity dependent dye biases. As shown in figure 5.2B, global normalisation does increase the number of spots with a ratio close to 1 (black points on the scatter plot), but there are still many outlying spots (red or green points on the scatter plot). Intensity dependent effects were also observed (fig. 5.2), as at low fluorescence intensities there was much more variation in fluorescence than at higher fluorescence intensities.

Intensity dependent effects can be more easily visualised by plotting the log2 Cy3/Cy5 ratio (y axis) against the log2 intensity (Cy3 x Cy5, x axis) for each spot, in an R-I (ratio-intensity) plot (fig. 5.3A). Log2 ratios are often used for analysis since this produces a continuous spectrum of values which treats upregulated and downregulated genes in a similar fashion, e.g. log2(1) = 0, log2(2) = 1, log2(0.5) = -1, log2(4) = 2, log2(0.25) = -2. In the R-I plot all the data points should lay on a straight line around zero but the unnormalised self-self data is skewed (fig. 5.3A), with the majority of data points lying below zero. A large variation in the ratios was also observed at lower signal intensities. Lowess normalisation (locally weighted linear regression) removes intensity-dependent dye effects by carrying out a local weighted linear regression as a function of the log2 intensity and subtracting the calculated best-fit average log2 ratio from the experimentally observed ratio for each data point. Divergent spots do not influence lowess normalisation since lowess uses a weight function that decreases the contribution of data from divergent spots (Quackenbush 2002). Lowess normalisation can either be applied globally or locally to each block (print-tip), which can then correct for spatial differences across the slide. Since significant spatial differences were observed on the microarray, print-tip lowess was

A Unnormalised



B Lowess normalisation



C

	Range of ratios	**% spots with a ratio >1.5**	**% spots with a ratio <0.66**	**% spots with a ratio >2**	**% spots with a ratio <0.5**
Global	0.234 to 2.70	24.82%	22.21%	2.60%	8.19%
Lowess	0.412 to 2.31	2.06%	2.92%	0.07%	0.24%

Figure 5.3: Lowess normalisation. A & B – show ratio versus intensity (R-I) plots of unnormalised and lowess normalised self-self hybridisation data (from array in fig. 5.1A), on a log2 scale. Each point represents a single spot on the array and they are colour coded according to which block they were part of. **C** – table comparing global normalisation (fig. 5.2) to lowess normalisation of the self-self hybridisation. Ratio = cy3/cy5 intensity for each spot.

used. As shown in fig. 5.3B, print-tip lowess normalisation centred the data well and reduced intensity-dependent effects.

In order to determine which normalisation method was the most appropriate and to ascertain a meaningful limit to assess fold changes in later experiments, the percentage of spots falling into various limits was calculated (fig. 5.3C). These results clearly demonstrate that lowess normalisation is most effective at reducing systematic variation in the experiment and that a 2-fold change should be a sufficient cut-off to obtain meaningful results in later experiments.

Microarray experiments were then performed with RNA isolated from untreated and CD40L treated RL and Akata 6 cells. To account for any dye bias, reciprocal labelling was performed whereby on one array RNA from untreated cells was labelled with Cy3 and RNA from CD40L treated cells was labelled with Cy5 and on a second array the dyes were swapped so that RNA from untreated cells was labelled with Cy5 and RNA from CD40L treated cells was labelled with Cy3. Ratios on each array were calculated as CD40L treated/untreated, to ascertain which genes were upregulated or downregulated by CD40 ligation. Replicate spots on each array were averaged to give a mean ratio. As shown in fig. 5.4A, ratios from replicate spots on a single array were very consistent, with an R^2 value of 0.911. However, comparison of replicate spots between the two reciprocally labelled slides revealed that there were major inconsistencies and no meaningful correlation between the two data sets (fig. 5.4B). Plotting the ratios from the replicate arrays should result in a straight line (see fig. 5.6A), however this was not observed (fig. 5.4B). Therefore it seems that the results obtained from dual-colour microarray experiments largely depend on the allocation of each dye to a particular sample.

Analysis of the relative intensity of individual spots hybridised with RNA isolated from control or CD40L treated cells, revealed that very few genes were more than 2-fold upregulated or downregulated by CD40 ligation in either Akata 6 or RL cells, ranging from 0.01% to 4.3% of the total genes on the array (table 5.1). Also the maximum fold-changes on each array were highly variable ranging from only 2.3 fold to over 53 fold (table 5.1). There was a clear pattern of dye bias on the reciprocally labelled arrays. Both a greater percentage of upregulated genes than downregulated genes and a greater maximum fold increase rather than fold decrease were observed

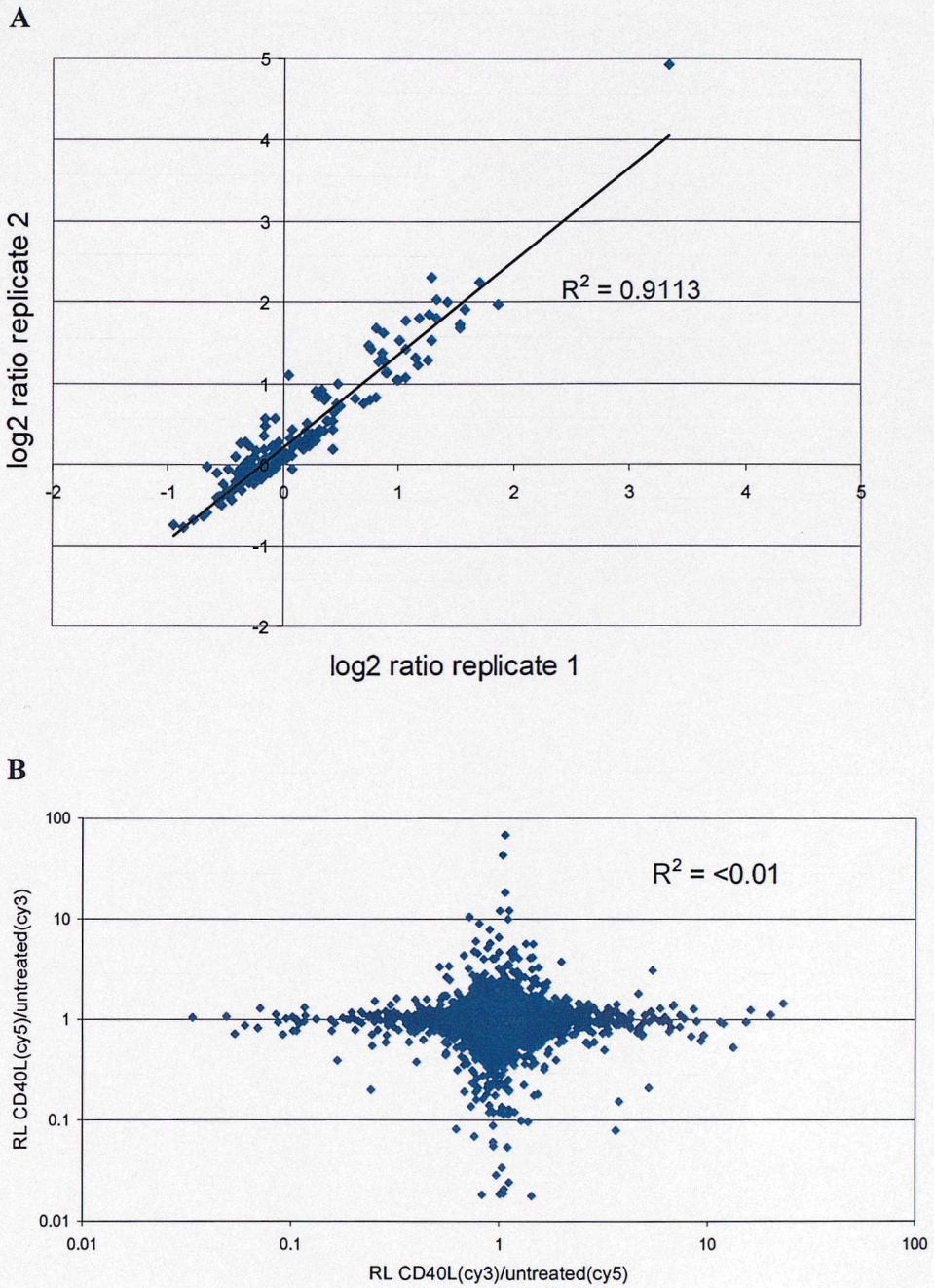


Figure 5.4: Dual-colour microarray replicates. A – Comparison of fluorescence ratios of replicate spots on the same array, where RNA from CD40L treated RL cells was labelled with cy3 and RNA from untreated RL cells was labelled with cy5, showing good reproducibility of values. 250 representative points were plotted on this graph. **B** - comparison of mean fluorescence ratios for each gene on reciprocally labelled microarrays comparing gene expression in untreated and CD40L treated RL cells, showing little reproducibility between dye-swap replicates.

Table 5.1: Gene expression changes with CD40L treatment using HGMP microarrays. The percentage of genes more than 2-fold upregulated or downregulated with CD40L treatment are shown, as are the maximum fold increases and decreases. For each cell line 4 arrays were used. These comprised two different gene sets (1 and 2) that were reciprocally labelled. The ratio of gene expression is calculated in each case as CD40L-treated intensity/untreated intensity.

Cell line	Array	% genes > 2 fold upregulated	% genes >2 fold downregulated	Maximum fold increase	Maximum fold decrease
Akata 6	1 cy5/cy3	0.50	1.48	3.26	7.34
	1 cy3/cy5	2.20	1.11	28.4	6.51
	2 cy5/cy3	0.41	1.16	3.05	24.3
	2 cy3/cy5	4.30	1.50	29.2	7.17
RL	1 cy5/cy3	0.23	0.51	5.51	25.1
	1 cy3/cy5	0.14	0.01	5.91	2.29
	2 cy5/cy3	0.40	1.30	5.69	53.8
	2 cy3/cy5	1.51	0.33	22.9	6.87

when CD40L treated RNA was labelled with Cy3 (i.e. Cy3/Cy5 ratio). Similarly, a greater number of downregulated genes than upregulated genes and a greater maximum fold decrease rather than increase were observed when untreated RNA was labelled with Cy3 (i.e. Cy5/Cy3 ratio). Therefore Cy3 labelling gives greater predominance to the RNA than Cy5 labelling.

Due to the limitation of dye bias, genes were only considered significantly upregulated or downregulated by CD40 ligation if their mean ratio was more than 2-fold upregulated or downregulated in both reciprocally labelled microarrays. These results are shown in tables 5.2-5.5. Despite the limited number of genes regulated on both arrays, many known CD40 target genes were identified e.g. FLIP, A20, SLAM, lymphotoxin-alpha, c-IAP2 (Punnonen *et al* 1997, Bleharski *et al* 2001, Worm & Geha 1995, Hong *et al* 2000), showing that this system can correctly identify CD40-induced gene changes. The microarrays also identified some novel potential CD40 target genes e.g. lymphocyte cytosolic protein 1 (L-plastin), STAT1, the IL-13 receptor, RGC-32, plexin A3 and many genes with unknown functions. Many of the genes were regulated in both Akata 6 and RL cells e.g. sorting nexin 11, chemokine (C-C) motif receptor 7 (CCR7) and lymphotoxin-alpha.

Since Akata 6 and RL cells show differing responses to CD40 ligation, it was determined if any genes were differentially regulated by CD40 between the two cell lines. These results are shown in tables 5.6-5.8 and only included genes where information was present and consistent between the reciprocally labelled microarrays. Expression of genes such as the IL-13 receptor and STAT1 were upregulated by CD40 ligation in Akata 6 cells, but expression of these genes was not significantly altered by CD40 ligation in RL cells. Conversely, SLAM, CD83 and TRAF1 were upregulated by CD40 ligation in RL cells but were unaffected by CD40 signalling in Akata 6 cells. Interestingly, one gene KIAA1268, a putative transcription factor, was upregulated by CD40 ligation more than 2-fold in RL cells but was downregulated more than 2-fold in Akata 6 cells.

Only judging genes as significant if they were regulated by CD40 in both reciprocally labelled microarrays was a very strict analysis and probably excluded many significant genes, due to missing data on one array or due to the dye bias. In particular, a large number of genes were co-regulated by CD40 on one Akata 6 array

Tables 5.2-5.5: CD40L regulated genes from HGMP dual-colour microarrays. Genes that were more than 2 fold upregulated or downregulated following CD40L treatment (1μg/ml, 16 hours) in both reciprocally labelled microarrays are listed in the following tables. The mean fold change and standard devaition (SD) are shown for each gene.

Table 5.2: Akata 6 CD40L upregulated genes. * = also upregulated in RL cells (in at least one array), raw data for these genes are shown in table A.1

Gene	Function	Mean fold upregulated	SD
Cholinergic receptor, neuronal nicotinic, alpha polypeptide 6 (CHRNA6)	Ligand gated ion channel	3.50	0.75
Chemokine (C-C) motif receptor 7 (CCR7)*	Extracellular signalling	2.94	0.53
IL-13 receptor	Extracellular signalling	2.67	0.75
STAT1	Transcription factor	2.53	0.62
Sorting nexin 11*	Intracellular trafficking	2.47	0.45
clone DKFZp434M0927	Strong similarity cysteine string protein, which is involved in neurotransmitter release	2.30	0.22
Major histocompatibility complex, class I, F (HLA-F)*	Antigen presentation	2.28	0.31
Farnesyl-diphosphate farnesyltransferase 1 (FDFT1)	Metabolism	2.26	0.60
Clone 24761*	Unknown	2.21	0.42

Table 5.3: Akata 6 CD40L downregulated genes.

Gene	Function	Mean fold downregulated	SD
FLJ11170	Unknown	6.00	2.2
DnaJ-like heat shock protein 40	Chaperone	4.68	2.44
SH2 domain protein 1A (SH2D1A)	Intracellular signalling, associates with SLAM	3.51	1.11
Hypothetical protein LOC55580	Unknown	2.62	0.51
Hypothetical protein PRO2037	Unknown	2.40	0.43
KIAA1268	Putative transcription factor	2.27	0.05
ED70-2A	Unknown	2.25	0.40

Table 5.4: RL CD40L upregulated genes. Genes with a * were also upregulated in Akata 6 cells (in at least one array), raw data for these genes are shown in table A.1.

Gene	Function	Mean fold upregulated	SD
Lymphotoxin alpha*	Extracellular signalling	7.83	5.23
FLIP*	Apoptosis	6.06	0.74
CD83	Extracellular signalling	4.56	1.50
Signalling lymphocytic activation molecule (SLAM)	Extracellular signalling	4.14	2.15
A20*	Apoptosis	3.14	0.77
HSPC049*	Unknown	3.01	1.11
Sorting nexin 11*	Intracellular trafficking	2.87	0.38
Histone family 2A, member X	Transcription	2.85	0.26
G protein-coupled receptor kinase 7	Intracellular signalling	2.77	0.24
DKFZp434I0535*	Putative DNA binding protein	2.76	0.50
IL-12 receptor related* (Epstein-Barr virus induced gene 3)	Extracellular signalling	2.75	0.77
Transmembrane mucin 12	Signalling	2.75	0.80
TRAF1	Intracellular signalling	2.61	0.20
Apoptosis inhibitor 2 (cIAP 2)	Apoptosis	2.60	0.71
Guanylate cyclase 1, soluble, alpha 3*	Intracellular signalling	2.50	0.25
KIAA1268	Putative transcription factor	2.42	0.52
Mitogen-activated protein kinase phosphatase x (MKPX)	Intracellular signalling	2.36	0.25
Plexin A3 (plexin 4, SEX gene)*	Extracellular signalling	2.35	0.57
CD82	Extracellular signalling	2.30	0.35
Transcription elongation factor B (SIII), polypeptide 3 (elongin A)	Transcription	2.21	0.48
Lymphocyte cytosolic protein 1 (L-plastin)	Actin bundling	2.15	0.07

Table 5.5: RL CD40L downregulated genes. Genes with a * were also downregulated in Akata 6 cells (in at least one array), raw data for these genes are shown in table A.2.

Gene	Function	Mean fold downregulated	SD
Lymphoid-restricted membrane protein*	Vesicle targetting	2.70	0.44
FLJ22612*	ATP dependent peptidase	2.75	0.58
RGC-32*	Cell cycle	2.20	0.31
FLJ12185	Unknown	2.14	0.30
TBC1 domain family, member 4*	Intracellular signalling	2.14	0.36

Tables 5.6-5.8: Differentially regulated genes by CD40 ligation in Akata 6 and RL cells. Genes that were more than 2-fold upregulated or downregulated following CD40L treatment in one cell line (in both reciprocally labelled microarrays) that were unaffected or differently regulated by CD40L treatment in the other cell line (in both reciprocally labelled microarrays) are listed in the following tables. The mean fold change and standard deviation (SD) are shown for each gene. The functions of each gene are listed in tables 5.2-5.5. There were no genes that were consistently downregulated in RL cells that were unaffected by CD40L in Akata 6 cells.

Table 5.6: Akata 6 CD40L upregulated genes that were unaffected by CD40L treatment in RL cells.

Gene	Akata 6 mean fold upregulated	SD	RL mean ratio	SD
Cholinergic receptor, neuronal nicotinic, alpha polypeptide 6 (CHRNA6)	3.50	0.75	1.42	0.11
IL-13 receptor	2.67	0.75	0.93	0.16
STAT1	2.53	0.62	1.26	0.08
clone DKFZp434M0927	2.30	0.22	1.39	0.35
Farnesyl-diphosphate farnesyltransferase 1 (FDFT1)	2.26	0.60	1.19	0.16

Table 5.7: Akata 6 CD40L downregulated genes that were unaffected by CD40L treatment in RL cells.

Gene	Akata 6 mean fold downregulated	SD	RL mean ratio	SD
FLJ11170	6.00	2.2	0.87	0.17
DnaJ-like heat shock protein 40	4.68	2.44	1.09	0.15
SH2 domain protein 1A (SH2D1A)	3.51	1.11	1.13	0.11
Hypothetical protein LOC55580	2.62	0.51	0.76	0.03
Hypothetical protein PRO2037	2.40	0.43	0.97	0.13
ED70-2A	2.25	0.40	0.77	0.05

Table 5.8: RL CD40L upregulated genes that were unaffected by CD40L treatment in Akata 6 cells.

Gene	RL mean fold upregulated	SD	Akata 6 mean ratio	SD
CD83	4.56	1.50	1.15	0.33
Signalling lymphocytic activation molecule (SLAM)	4.14	2.15	0.90	0.31
Histone family 2A, member X	2.85	0.26	0.92	0.42
Transmembrane mucin 12	2.75	0.80	1.22	0.33
TRAF1	2.61	0.20	1.17	0.17
KIAA1268	2.42	0.52	0.44	0.05
Mitogen-activated protein kinase phosphatase x (MKPX)	2.36	0.25	0.86	0.07
CD82	2.30	0.35	1.23	0.38
Transcription elongation factor B (SIII), polypeptide 3 (elongin A)	2.21	0.48	0.80	0.08

and one RL array (see appendix tables A.1-A.2) e.g. cytokine receptor CRL2 precursor. These common genes were always identified from arrays in which the dye allocation was the same e.g. RNA from CD40L-treated cells was labelled with Cy3 and RNA from untreated cells was labelled with Cy5 on both RL and Akata 6 arrays. In fact there were more similarities between Akata 6 and RL arrays labelled with the same dye allocation, than between reciprocally labelled arrays from the same sample!

In order to verify some of the potential CD40-target genes identified by the HGMP microarray system, semi-quantitative RT-PCRs were undertaken. RT-PCRs were performed for SLAM, SH2D1A, CCR7 and RGC-32 (fig. 5.5). SLAM and SH2D1A showed differential regulation by CD40 ligation in the microarray experiments, whereas CCR7 and RGC-32 showed co-regulation.

SLAM is a cell surface signalling molecule, which was shown to be upregulated by CD40 ligation in both normal B-cells and dendritic cells (Punnonen *et al* 1997, Bleharski *et al* 2001). The microarrays showed that SLAM was upregulated by CD40 ligation over 4-fold in both RL arrays but was unaffected by CD40L in Akata 6 cells (table 5.8). This was confirmed in the RT-PCR results, clearly showing that RL cells upregulated SLAM expression 2.5-fold in response to CD40 signalling (fig. 5.5). Interestingly, Akata 6 cells did not express detectable levels of SLAM and CD40 ligation did not modulate expression levels.

SH2D1A binds to SLAM and can both inhibit and recruit binding of adaptor molecules to SLAM (Li *et al* 2003). A previous study showed that SH2D1A expression was induced by CD40 signalling in a B lymphoblastoid cell line (Shlapatska *et al* 2001), however the microarray results suggested that SH2D1A was downregulated by CD40 ligation (3.5-fold), with no change in expression in RL cells (table 5.7). The RT-PCR results confirmed this, showing that expression of SH2D1A was decreased almost 2-fold upon CD40 ligation in Akata 6 cells. SH2D1A was not expressed in RL cells and CD40L did not influence its expression (fig. 5.5). Therefore the expression of both SLAM and SH2D1A is differentially regulated in Akata 6 and RL cells. These results suggest that RL cells may be able to signal through SLAM, which may be further potentiated by CD40 signalling, whereas Akata 6 cells cannot signal through SLAM due to low or absent levels of SLAM and high levels of its inhibitor SH2D1A.

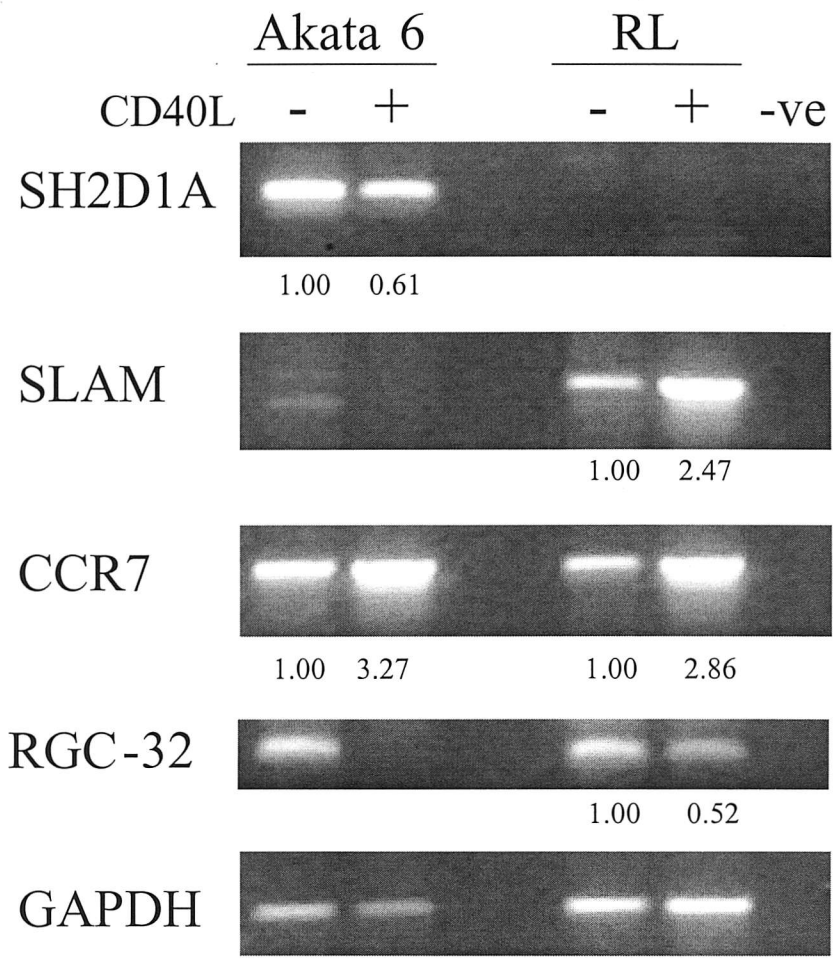


Figure 5.5: CD40L regulates SH2D1A, SLAM, CCR7 and RGC-32 RNA expression. These molecules were identified as possible CD40 target genes from HGMP microarray results, and their regulation by CD40 was confirmed by RT-PCR. Akata 6 and RL cells were treated with CD40L (1μg/ml) (+) or left untreated as a control (-) for 16 hours and RNA isolated. These RNA samples were the same samples used for the HGMP microarrays. RNA was reverse transcribed to cDNA. Specific primers were used to amplify specific cDNAs, in a semi-quantitative manner. GAPDH is shown as an internal control. –ve = water used instead of cDNA in PCR reaction. Values beneath each blot represent the relative band intensity normalised to GAPDH. The values for untreated cells were set to 1.

CCR7 is a chemokine receptor that was more than 2 fold upregulated with CD40L treatment on one RL array and both Akata 6 arrays (table A.1). RT-PCRs for CCR7 mRNA indeed showed that this was the case (fig. 5.5), showing that CCR7 mRNA was upregulated approximately 3-fold in both cell lines, identifying CCR7 as a novel CD40 target gene. RGC-32 is a cell cycle regulatory protein that was more than 2 fold downregulated by CD40 ligation in both RL arrays and one Akata 6 array (table A.2). RT-PCR results, again verified the microarray results, showing that CD40 ligation completely abrogated RGC-32 expression in Akata 6 cells and decreased expression of RGC-32 approximately 2-fold in both Akata 6 and RL cells (fig. 5.5).

Therefore, although the HGMP system gave disappointing results due to the large amount of variation in reciprocally labelled microarray replicates, it was able to correctly identify known and novel CD40 target genes, and genes that were either co-regulated or differentially regulated in Akata 6 and RL cells.

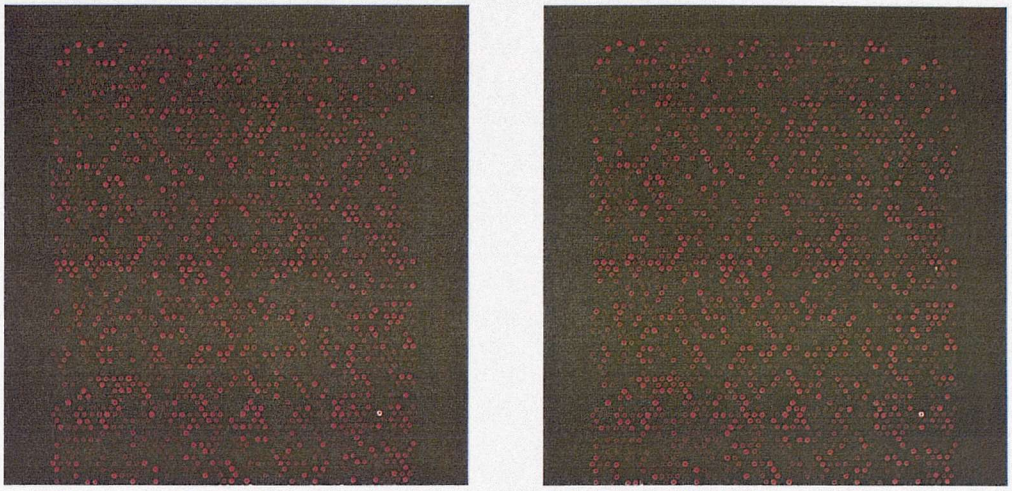
5.3 Amersham CodeLink Expression Bioarrays

Since fairly disappointing results were obtained using the dual-colour HGMP system, CodeLink Expression Bioarrays (Amersham, UK), a novel single-colour system, were evaluated. Each CodeLink Bioarray contains 30-base oligonucleotides from 20,000 genes that are attached to a polyacrylamide aqueous gel slide surface by their amine-terminated ends. This results in a three-dimensional gel matrix that holds oligonucleotides away from the cell surface, allowing greater target access to the oligonucleotides. In this system, mRNA is reverse transcribed by a DNA oligonucleotide containing a T7 RNA polymerase promoter 5' to a $d(T)_{24}$ sequence. Following second-strand cDNA synthesis, the cDNA serves as a template for an *in vitro* transcription reaction to produce the target cRNA. The *in vitro* transcription reaction is performed in the presence of biotinylated nucleotides to label the target cRNA. This procedure produces a 50 to 200 fold linear amplification of the input RNA. The biotinylated cRNA is then hybridised to the array and is detected using Cy5-streptavidin, which fluoresces when excited by laser light.

RNA was isolated from untreated and CD40L treated (16 hours) Akata 6 cells and a primary follicular lymphoma sample (FL-e, see table 3.1) and hybridisations were performed by Amersham. Each RNA sample was assayed in duplicate and each sample was hybridised to a separate array and normalised for overall differences in signal intensity. Examples of the array images produced are shown in figure 5.6A. The raw data was subjected to extensive analysis. The data obtained were extremely reproducible between replicate arrays (fig. 5.6B), with an R^2 value of 0.9885, showing excellent correlation, linearity across signal intensities and reproducibility. The mean of the replicate values was calculated and a ratio of untreated versus CD40L-treated signal intensity was calculated for each spot on the array. The data were quality controlled by removing any spots that had intensity close to background (spots had to have a signal intensity which was at least 1% of the mean signal intensity) and by performing a Student's t-test on the replicate values. Genes were considered significantly upregulated or downregulated by CD40L if a significant difference in signal intensity between untreated and CD40L-treated cells ($p<0.05$) was obtained. In contrast to the HGMP microarrays, many genes were significantly upregulated or downregulated by CD40 ligation more than 2-fold, e.g. 7.4% of genes in the FL-e sample were more than 2-fold downregulated (table 5.9). Since a 2-fold cut-off gave a very large number of genes to identify (almost 3500 in total), a cut-off of 4-fold was used as this gave more manageable numbers of genes to deal with. The maximum fold changes were also very large e.g. a maximum fold increase of 77.4 ($p=0.01$) was observed in FL-e cells (table 5.9).

Tables 5.10 to 5.22 show genes that were significantly upregulated or downregulated by CD40 ligation by a factor of 4 or greater in Akata 6 cells. Tables 5.23 to 5.40 show genes that were significantly upregulated or downregulated by CD40 ligation by a factor of 4 or greater in the FL-e sample. Genes with an asterix were upregulated or downregulated by CD40L over 4 fold in both Akata 6 and FL-e cells (21 genes in total). The fold changes observed in this system were extremely large, with over 60-fold changes in some instances e.g. upregulation of the chemokine CXCL9, and amino oxidase by CD40 ligation.

Many genes involved in signalling, apoptosis, transcription, cytoskeletal regulation and metabolism were significantly upregulated or downregulated by CD40 ligation in



A Akata replicate 1 array **Akata replicate 2 array**

B

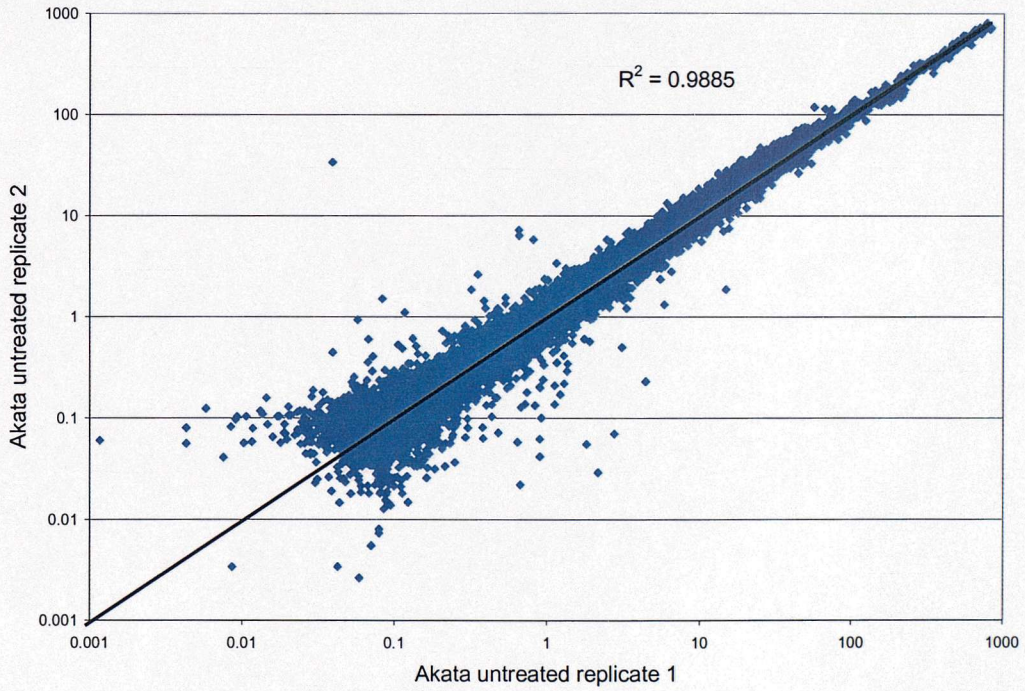


Figure 5.6: Reproducibility of Codelink arrays. Duplicate samples of untreated Akata 6 RNA were converted to cRNA, biotinylated, hydridised and Cy5 labelled to separate Codelink microarrays. **A** – shows a portion of each duplicate microarray. **B** – normalised signal intensities for every gene of each replicate array were plotted against each other, showing extremely good reproducibility.

Table 5.9: Gene expression changes with CD40L treatment using CodeLink microarrays. The percentage of genes which had a mean ratio of more than 2-fold or 4-fold upregulated or downregulated with CD40L treatment are shown, as are the maximum fold increases and decreases. p values were calculated using a one-tailed, unpaired Student's t-test (Microsoft Excel) on replicate values (n=4) and are shown in brackets. Each array was performed in duplicate.

	Akata 6	**FL-e**
% genes > 4 fold upregulated	0.50	0.81
% genes >4 fold downregulated	0.20	1.50
% genes > 2 fold upregulated	2.85	5.22
% genes >2 fold downregulated	1.66	7.40
Maximum fold increase	63.8 (p=0.004)	77.4 (p=0.01)
Maximum fold decrease	41.6 (p<0.001)	30.0 (p=0.002)

Tables 5.10 to 5.17: CD40L upregulated genes in Akata 6 cells. Genes listed were identified on Codelink arrays as being significantly upregulated ($p<0.05$) more than 4 fold with CD40L treatment in Akata 6 cells. Genes are grouped according to their functions. Genes with a * were also upregulated by CD40 ligation in the FL-e sample. Genes that were also identified as CD40-targets using the HGMP system, in at least one of the reciprocally labelled arrays, are colour-coded. Genes in red were identified as regulated by CD40 in both Akata 6 and RL cells in the HGMP arrays, genes in green were identified as regulated by CD40 in Akata 6 cells only in the HGMP arrays and genes in blue were identified as regulated by CD40 in RL cells only in the HGMP arrays (see table A.3). In some instances two values are present (separated by a + sign), which relate to two independent spots on the array.

Table 5.10: Extracellular signalling (receptors + ligands)

Gene	Fold upregulated with CD40L
Chemokine CXCL9	63.9
IL-12 receptor-related (EBV induced gene 3)*	48.9
Chemokine CXCL10	35.3
EBI2 (lymphocyte-specific G-protein coupled receptor)	29.9
TNF-α-induced protein 6	10.7
IL-10*	10.0
Gamma-aminobutyric acid (GABA) A receptor	8.6
Chemokine CCL17*	7.8
Immunoglobulin superfamily receptor translocation associated 4 (IRTA4)	7.7
CD58*	6.5
Immunoglobulin superfamily receptor translocation associated 1 (IRTA1)	6.5
IL-1 receptor 1	5.3
CD70 (CD27-ligand)	4.9
CD83	4.8
Chemokine CXCL11	4.7
Leukocyte-associated Ig-like receptor 2 (LAIR2)	4.6
CD20 like protein 4 (CD20L4)	4.3 + 4.1

Table 5.11: Intracellular signalling

Gene	Fold upregulated with CD40L
Guanylate binding protein 5 (GBP5)	37.3
TRAF1*	11.7
Ras and Rab interactor 2	10.8
Regulator of G-protein signalling 1 (RGS1)	9.1 + 8.2
Guanylate binding protein 1 (GBP1)	6.6
Rhophilin 2	5.0
Interferon-induced protein with tetratricopeptide repeats 1 (IFIT1)	4.8
Mitogen-activated protein kinase kinase kinase 8 (MAP3K8)	4.8
Interferon-induced protein with tetratricopeptide repeats 1 (IFIT4)	4.7
Guanylate binding protein 2 (GBP2)	4.3
Tyrosine kinase 2 (TYK2)	4.2

Table 5.12: Apoptosis

Gene	Fold upregulated with CD40L
Bfl-1*	28.3
FLIP*	10.8
Fas*	8.1
A20*	7.8
Serglycin	5.6

Table 5.13: Transcription

Gene	Fold upregulated with CD40L
Interferon regulatory factor 4 (IRF4)	35
IκB-zeta	6.9
IκB-alpha*	6.6 + 6.4
STAT5A	6.3
NF-κB (p49/p100)*	5.4
IκB-epsilon*	5.0

Table 5.14: Cytoskeleton

Gene	Fold upregulated with CD40L
Capping protein (actin filament), gelsolin-like (CAPG)	4.7
Tymosin beta-4	4.4

Table 5.15: Metabolism

Gene	Fold upregulated with CD40L
Serine hydrolase-like	18.0
Amino oxidase*	11.3
Cytochrome B5 reductase	6.9
Ureidopropionase beta	5.4
dCMP deaminase	4.5
N-acetylneuraminate pyruvate lyase	4.1
Phythanoyl-CoA hydroxylase	4.1

Table 5.16: Other functions

Gene	Function	Fold upregulated with CD40L
Calgizzarin	Calcium binding	20.6
Potassium large conductance calcium-activated channel, subfamily M, beta member 1	Membrane channel	14.1
Visinin-like 1	Neuronal calcium sensor	9.8 + 7.3
Mucosal vascular addressin cell adhesion molecule 1 (MADCAM1)	Adhesion	9.4
Collagen, type 1, alpha 1	Extracellular matrix	9.2
Neutrophil oxidase*	Defence	8.1
Testican-2	Extracellular matrix	7.7
Preferentially expressed antigen in	Tumour antigen	7.4

melanoma (PRAME)		
Retinal protein 4	Neurotransmitter release	6.8
Melanoma inhibitory activity (MIA)	Tumour marker	5.6
Mitochondrial ribosomal protein L56	Translation	5.6
Crumbs 3	Adhesion?	5.2
HRas-like suppressor 2	Putative tumour suppressor	5.2
MGC15619	Cation channel	4.3

Table 5.17: Genes with unknown functions

Gene	Fold upregulated with CD40L
Transmembrane protein induced by TNF-α (TMPIT)	9.8
Hypothetical protein FLJ10901	8.6
SAM domain, SH3 domain and nuclear localisation signals 1 (SAMSN1)	7.9
FLJ20376	7.0
Deme-6	7.0
Beta-actin pseudogene	5.8
Hypothetical protein GS3686	5.4
Hypothetical protein FLJ10157	4.9
Hypothetical protein MGC1203*	4.6
Similar to common salivary protein 1 (LOC124220)	4.1

Tables 5.18 to 5.22: CD40L downregulated genes in Akata 6 cells. Genes listed were identified on Codelink arrays as being significantly downregulated ($p<0.05$) more than 4 fold with CD40L treatment in Akata 6 cells. Genes are grouped according to their functions. Genes with a * were also upregulated by CD40 ligation in the FL-e sample. One gene, RGC-32, was also identified as being downregulated with CD40L treatment using the HGMP system in Akata 6 and RL cells, and is coloured red (see table A.4).

Table 5.18: Extracellular signalling

Gene	Fold downregulated with CD40L
Killer cell lectin-like receptor subfamily C, member 3 (KLRC3 or NKG2-E)	6.2
IL-7 receptor	4.6
Epidermal growth factor receptor	4.3
G-protein coupled receptor 30	4.2

Table 5.19: Intracellular signalling

Gene	Fold downregulated with CD40L
BMX non-receptor tyrosine kinase	6.2
Src-2	5.1

Table 5.20: Cytoskeleton

Gene	Fold downregulated with CD40L
Syntrophin, alpha 1	5.5
Tubulin beta 4	4.1
Wave 3	4.0

Table 5.21: Other functions

Gene	Function	Fold downregulated with CD40L
Activation-induced cytidine deaminase (AID)*	Germinal centre reaction	41.7
Oxysterol binding protein-like 1A (ORP-1)	Phospholipid binding	10.0
Slug*	Transcription	8.5
Immunoglobulin J chain*	Immune response	7.3
RGC-32*	Cell cycle activation	6.5
Spanx C	Tumour antigen	5.6
Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3 (KCNN3)*	Membrane channel	4.6
Hypothetical protein BC015003	Lipid metabolism?	4.4
Potassium voltage-gated channel, KQT-like subfamily, member 1 (KCNQ1)	Membrane channel	4.3
Ankyrin repeat domain 2	Muscle protein	4.0

Table 5.22: Unknown functions

Gene	Fold downregulated with CD40L
BTB (POZ) domain containing 3 (BTBD3)	8.5
Hypothetical protein FLJ22297	7.1
KIAA1446	5.6
Clone 25028	5.1
FLJ23124	5.1
Hypothetical protein FLJ20356	5.0
cDNA clone ZB42D04*	5.0
KIAA0633 (COBL)	4.2

Tables 5.23 to 5.31: CD40L upregulated genes in a follicular lymphoma sample (FL-e). Genes listed were identified on Codelink arrays as being significantly upregulated ($p<0.05$) more than 4 fold with CD40L treatment in FL-e cells. Genes are grouped according to their functions. Genes with a * were also upregulated by CD40 ligation in Akata 6 cells. Genes that were also identified as CD40-targets using the HGMP system, in at least one of the reciprocally labelled arrays, are colour-coded. Genes in red were identified as regulated by CD40 in both Akata 6 and RL cells in the HGMP arrays, genes in green were identified as regulated by CD40 in Akata 6 cells only in the HGMP arrays and genes in blue were identified as regulated by CD40 in RL cells only in the HGMP arrays (see table A.3). In some instances two values are present (separated by a + sign), which relate to two independent spots on the array.

Table 5.23: Extracellular signalling

Gene	Fold upregulated with CD40L
IL-12 receptor related (EBV induced gene 3)*	63.2
Urokinase plasminogen activator	32.5
Chemokine CCL17*	24.2
IL-10*	15.5
IL-13 receptor alpha 1	15.3
IL-17B receptor	14.8 + 6.4
Vascular endothelial growth factor (VEGF)	8.9
Somatostatin receptor 2	7.7
IL-2 receptor alpha	7.0
CD80	6.9
Orphan G protein-coupled receptor (RDC1)	6.5
CD58*	6.5
Chemokine CCL3	5.5
Nectin 1	5.4
IL-21 receptor	5.1
Neurexin 4	5.0
OX40 ligand	5.0
Chemokine CCL5	4.8
Chemokine CCL4	4.7

Table 5.24: Intracellular signalling

Gene	Fold upregulated with CD40L
TRAF1*	12.0
TRAF4	11.1 + 7.1
Insulin receptor tyrosine kinase substrate	9.2 + 7.7
Dual specificity phosphatase 2	7.4
PIM-1 oncogene	5.7
Cdc-42 GAP	5.4
KIAA0856	5.28
Thyroid hormone receptor interactor 10	4.8
Protein tyrosine phosphatase, non-receptor type substrate 1 (PTPNS1)	4.1
Phospholipase A2, Group IVC	4.0

Table 5.25: Apoptosis

Gene	Fold upregulated with CD40L
A20*	14.3
Fas*	8.2
FLIP*	7.0
Bid	4.9

Table 5.26: Transcription

Gene	Fold upregulated with CD40L
Transcription factor BMAL2	14.8
SMAP31	14.1
IκB-alpha*	10.0 + 9.9
NF-κB (p49/p100)*	8.5
Transcription factor 7	7.6
Activating transcription factor 5 (ATF5)	7.4
Jun dimerisation protein p21SNFT	7.4
p63	7.1
Interferon regulatory factor 4 (IRF4)*	6.5
c-myc	6.0
HXBP-1 transcription factor	5.1
Activating transcription 3 (ATF3)	4.6
IκB-epsilon*	4.5

Table 5.27: Cytoskeleton

Gene	Fold upregulated with CD40L
Myristolyated alanine-rich protein kinase C substrate (MARCKS)	15.7
Lamin A/C	7.2
Ras homolog gene family, member F (ARHF)	6.5 + 4.5
Synaptopodin	6.0
Myosin IC	5.7
Filamin A, alpha (actin binding protein 280)	5.3
Beta tubulin	4.9
Lamin B2	4.4

Table 5.28: Metabolism and biosynthesis

Gene	Fold upregulated with CD40L
Amino oxidase*	77.4
Pyrroline-5-carboxylate reductase 1 (PYCR1)	11.8
Cystathionine beta synthase	11.6
Phosphoserine aminotransferase	11.4
Tryptophanyl-tRNA-synthetase (WARS)	8.4 + 7.4
Thioredoxin	8.3
Branched chain aminotransferase 1	6.6
Argininosuccinate synthetase	6.3
Phosphoenolpyruvate carboxykinase 2	5.4
Fatty acid synthase	5.3
Adenylate kinase 3	5.1
Non-metastatic cells 1 (NME 1)	5.1
Methylene tetrahydrofolate dehydrogenase	4.8
Hexokinase 2	4.6
Tyrosyl-tRNA-synthetase	4.4

Beta 1,4-galactosyl transferase, polypeptide 5	4.3
Multifunctional polypeptide similar to SAICAR synthetase and AIR carboxylase (ADE2H1)	4.3
Kringle containing transmembrane proein 2	4.1
Pyruvate kinase	4.1

Table 5.29: Membrane channels/transporters

Gene	Fold upregulated with CD40L
Very low density lipoprotein receptor (VLDLR)	11.2 + 8.3
MGC4504 (cation transporter)	10.6
Solute carrier family 1 (neutral amino acid transporter), member 5	7.2
Organic anion transporter polypeptide-related protein 4	6.3
FXYD domain-containing ion transport regulator 2	5.1
Chloride intracellular channel 2	4.5
Solute carrier family 7 (cationic amino acid transporter, Y+ system), member 5	4.5
Folate receptor 3	4.3
Hypothetical protein DKFZP434K0427 (cation transporter)	4.2

Table 5.30: Other functions

Gene	Function	Fold upregulated with CD40L
Tescalin	calcium binding	13.6
Adam-like, decysin 1	proteinase	12.4
Ninjurin 1	adhesion	11.1
Neutrophil oxidase*	defence	9.5
Natural killer ecll transcript 4	Lymphocyte activation	8.8
Niban	Renal tumour marker	8.4
Bystin	adhesion	8.4
Galectin 3	IgE binding	8.0
Reticulocalbin 1	Calcium binding	7.4
Lysosomal-associated membrane protein 3 (LAMP3)	Tumour marker	6.6
Synaptoporin	Component of synaptic vesicles	6.3
Chromosome 20 open reading frame 97	Putative kinase	6.3
Hypothetical protein FLJ32009	Calcium binding	5.4
TNF-α induced protein 2	Inflammation and angiogenesis	5.0
P311	Myelofibroblast transformation	4.7
Sorting nexin 11	Intracellular trafficking	4.3
Semaphorin 4C	Axon guidance	4.3
Cyclin D2	Cell cycle regulation	4.2
Statherin	Salivary protein	4.2
p21 (Cyclin-dependent kinase inhibitor 1A)	Cell cycle regulation	4.1
Separin	Chromosome segregation during mitosis	4.1

Table 5.31: Unknown functions

Gene	Fold upregulated by CD40L
FLJ32301	32.2
DKFZP434H1235	10.8
Glycoprotein A repetitions predominant (GARP)	10.4
MGC1203*	7.6
FLJ10116	7.3 + 7.2
FLJ23313	7.2
Pleckstrin	6.6
Hypothetical protein PRO2714	6.3
Hypothetical protein DKFZP564K0822	5.3
KIAA0540	4.7
YY51E04	4.5
HNC31-1-D12.R	4.5
Phosphoserine-phosphatase-like	4.3
FLJ22505	4.1
Clone 24877	4.0

Tables 5.32 to 5.40: CD40L downregulated genes in a follicular lymphoma sample (FL-e). Genes listed were identified on Codelink arrays as being significantly downregulated ($p<0.05$) more than 4 fold with CD40L treatment in FL-e cells. Genes are grouped according to their functions. Genes with a * were also upregulated by CD40 ligation in Akata 6 cells. One gene, RGC-32, was also identified as being downregulated with CD40L treatment using the HGMP system in Akata 6 and RL cells, and is coloured red (see table A.4). In some instances two values are present (separated by a + sign), which relate to two independent spots on the array.

Table 5.32: Extracellular signalling

Gene	Fold downregulated with CD40L
Chemokine (C-C) motif receptor 6 (CCR6)	12.7
Interferon gamma	9.0
CD9	7.6
Fc fragment of IgG, low affinity IIB, receptor for CD32	7.6
Cysteine-rich motor neuron	7.5
Endothelial differentiation, sphingolipid G-protein coupled receptor 1	7.1
Connective tissue growth factor	5.9
Inhibin, beta A	5.9
G protein coupled receptor 34	5.8
Fibroblast growth factor 14 (FGF-14)	5.7
48kDa FKBP-associated protein	5.2
Chorionic somatomammotrophin hormone 1	4.7
Tetraspan-1	4.5
CD24	4.2
Serotonin receptor 3A	4.2
Glucocoricoid receptor alpha	4.1

Table 5.33: Intracellular signalling

Gene	Fold downregulated with CD40L
Protein kinase A anchoring protein 9	7.1
Lax	6.4
Calcyon (D1 dopamine receptor-interacting protein)	5.7
guanine nucleotide exchange factor DBS	5.6
Beta-adrenergic receptor kinase	5.2
SNF-1 related kinase	5.1
Rho-GTPase activating protein, ARHGAP9	4.4
Insulin receptor substrate	4.1

Table 5.34: Apoptosis

Gene	Fold downregulated with CD40L
Cathepsin B	5.3
TOSO (regulator of Fas-induced apoptosis)	5.2

Table 5.35: Transcription

Gene	Fold downregulated with CD40L
SET binding protein 1	14.5
Kruppel-like factor	10.5
Histone H2BA	9.7
FLJ33887	9.5
Histone H4G	7.9
KIAA0780	7.6
Histone H4J	6.8
Core binding factor beta isoform PEBP2B	6.6
Ring finger protein 24	6.4
Histone H2A	6.2
ETV6/AML1 translocation breakpoint region	5.7
Lymphoid transcription factor 1	5.5
Zinc finger protein 288	5.1
TAF9-like RNA polymerase II, TATA box binding protein (TBP)-associated factor, 31 kD	4.8
Aldosterone receptor (nuclear receptor subfamily 3, group C, member 2)	4.7
Histone H3B	4.7
Slug*	4.4
Human displacement protein (CCAAT)	4.3
FLJ21616	4.1

Table 5.36: Cytoskeleton

Gene	Fold downregulated with CD40L
Ectoderm-neural cortex-1 protein (ENC-1)	9.8 + 16.4
WIRE	4.5

Table 5.37: Metabolism and biosynthesis

Gene	Fold downregulated with CD40L
FLJ20581 (lipid metabolism)	16.2
FLJ20330 (oxidoreductase)	12.4
Carbohydrate sulfotransferase 2	12.1
5' nucleotidase (CD73)	11.2
Sialyltransferase 4A	8.5
Chromosome 7 ORF 10	7.9
Fructose-1,6-bisphosphate	7.5
N-myristoyl transferase 2	6.4
Nuclear-encoded mitochondrial NADH-ubiquinone reductase 24kDa subunit	5.8
Serine hydrolase-like	5.8
2',5'-oligoadenylate synthetase 1	5.4
Acetyl-coenzyme A synthetase 2 (ADP forming)	5.4
Gamma-glutamyltransferase-like 3	5.3
Phosphodiesterase 3B	5.2
Cat eye syndrome chromosome region, candidate 1 (CECR1) = adenosine deaminase	5.0
Fucosyltransferase 7	4.5
Serine palmitoyl transferase, subunit II	4.5
pyruvate dehydrogense complex, component X	4.1

Table 5.38: Membrane channels/transporters

Gene	Fold downregulated with CD40L
Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 1*	30.0
Potassium voltage-gated channel, shaker related subfamily, member 5	25.8
Potassium voltage-gated channel, subfamily H, member 8	5.8
Potassium voltage-gated channel, KQT-like subfamily, member 1 (KCNQ1)*	5.6
Hereditary haemochromatosis region	5.3
Sortilin-related receptor L	5.1
Apolipoprotein C-III (APOC3)	5.1
ATP binding cassette, subfamily A, member 7 (macrophage ABC transporter)	5.0
Potassium voltage-gated channel, ISK-related family, member 4 (KCNE4)	4.7
Purigenic receptor P2X, ligand-gated ion channel, 1	4.7

Table 5.39: Other functions

Gene	Function	Fold downregulated with CD40L
KIAA0882	Calcium binding	25.7
Cyclin-dependent kinase inhibitor 1C (Cdk-1C, p57, Kip2)	Cell cycle regulation	13.2
Hypothetical protein FLJ12363	Putative intracellular signalling protein. Linked to GTPases.	12.0
Pre-B lymphocyte gene 3	Immunoglobulin	11.4
Retrotransposon	Transposase	5.5 + 5.7
MEGF6 protein (KIAA0815)	Putative calcium binding protein	10.6
Ribonuclease A family 5	RNA degradation	10.0
Putative dipeptidase (LOC64174)	Membrane dipeptidase	9.9
splicing factor, arginine/serine-rich 2, interacting protein	RNA splicing	7.4
DKFZP564O2423	Putative lectin	7.3
Prickle 1	Tissue polarity	7.3
Ribonuclease A family 4	RNA degradation	7.1
MAGE-E1 protein	Tumour antigen	6.4
Hypothetical protein FLJ11838	May be involved in DNA synthesis and/or pantothenate metabolism	6.4
Replication protein A complex 34kDa subunit homolog RPA4	DNA binding	5.8
RGC-32*	Cell cycle activation	5.7
Niemann-Pick C1 protein (NCP1)	Intracellular trafficking	5.5
Human interferon induced protein p78	Dynamin family member. GTP-binding.	5.2
Activation-induced cytidine	Germinal centre reaction	5.2

deaminase (AID)*		
Prepronociceptin	Neuropeptide ligand precursor	5.1
Hypothetical protein DKFZP761G058	Putative serine/threonine phosphatase	4.9
RNA binding motif, single stranded interacting protein 1 (RBMS1)	DNA replication	4.9
Mariner transposon HSMAR1	Transposase	4.8
FLJ38991	Putative membrane signalling protein	4.8
Lymphocyte antigen 9	adhesion	4.8
T-cell leukaemia/lymphoma 6 (TCL6)	Tumour marker	4.7
Immunoglobulin J chain*	Immune response	4.7
Heme binding protein 1	Iron binding	4.6
EF-1a	Translation elongation factor	4.5
F-box only protein 32	Protein degradation	4.3
MXRA1	Extracellular matrix remodelling	4.3
Beta haemoglobin	Oxygen binding	4.2
FLJ21006	Putative G protein	4.1
Melanoma adhesion molecule (MCAM) (CD146)	adhesion	4.1

Table 5.40: Unknown functions

Gene	Fold downregulated with CD40L
YI29A01	13.2
FLJ13843	12.3
Transmembrane protein 2	12.1
PAC 106H8, similar to dynamin	11.9
Chromosome 6 ORF 37	11.4
Chromosome 20 clones 97 and 127	10.7
FLJ21130	9.8
UI-E-EJ1-AJY-N-20-0-UI.R1	9.3
FLJ12030	7.9
AV734838	7.8
FLJ13268	7.6
Hypothetical protein FLJ10213	6.9
Hypothetical protein FLJ13480	6.9
Clone 24694	6.8
15kDa selenoprotein	6.7
FLJ21208	6.4
WT3F701.X1	6.3
DKFZP434K1111	6.3
FLJ14081	6.2
FLJ23475	6.0
CpG island DNA genomic MSE1 fragment, clone 161E9	6.0
KIAA1164	5.8
DKFZP667A2417	5.7
Basement-membrane induced gene 1	5.6
UI-E-EO1-AID-M-20-0-UI.R1	5.6

FLJ90113	5.6
FLJ11906	5.3
Hypothetical protein PRO2893	5.3
YI30F01	5.2
ZB42D04*	5.1
KIAA0907	5.1
Chromosome 1 specific transcript KIAA0500	5.0
Placenta-specific 8	4.9
FLJ11890	4.9
KIAA0794	4.9
TCCCIA00427	4.9
601649390F1	4.9
FLJ13741	4.9
DKFZP241L157	4.8
DKFZP566O134	4.8
IMAGE:4642270	4.7
Hypothetical protein FLJ10707	4.7
602154358F1	4.6
Hypothetical protein FLJ14001	4.6
FLJ20861	4.6
IMAGE:4243767	4.5
FLJ23247	4.5
FLJ14280	4.5
FLJ11691	4.4
QO19F11.X1	4.3
CD52	4.3
QV3-CT0656-120201-543-C09	4.2
FLJ13279	4.2
FLJ14085	4.2
DKFZP586F071	4.1
601490158F1	4.1
FLJ10232	4.1
FLJ21006	4.1
AGENCOURT_6480263	4.0
FLJ13027	4.0

both Akata 6 and FL-e cells, showing that CD40 signalling impinges on a wide range of important processes occurring in the cell, that can determine cell fate. More changes were observed in the FL-e sample than Akata 6 cells, particularly involving genes concerned with metabolism and membrane transport (tables 5.28, 5.29, 5.37, 5.38), perhaps reflecting that the untreated primary cells were not proliferating, whereas Akata 6 cells were actively proliferating.

Many known CD40-target genes were upregulated by CD40L in both the cell systems, including A20, FLIP, Fas, Bfl-1 and IκB-alpha (tables 5.12, 5.13, 5.25, 5.26). Bfl-1 was upregulated over 28 fold in Akata 6 cells and 3.7 fold in FL-e cells. Indeed, both Bfl-1 and A20 were shown to be induced by CD40 using RT-PCR in both Akata 6 and FL-e cells in this study (see chapter 3). Fas protein expression was also shown to be upregulated by CD40L in Akata 6 cells in this study (see chapter 4). Bcl-X_L, a key CD40-target gene, shown to be modestly upregulated in Akata 6 and also upregulated in FL-e cells in this study (see chapter 3) was upregulated 1.8 fold on the Akata 6 array and 2.1 fold on the FL-e array. Taken together, these results show that the CodeLink microarray system can produce reliable results, is a highly sensitive system and can correctly identify CD40 targets.

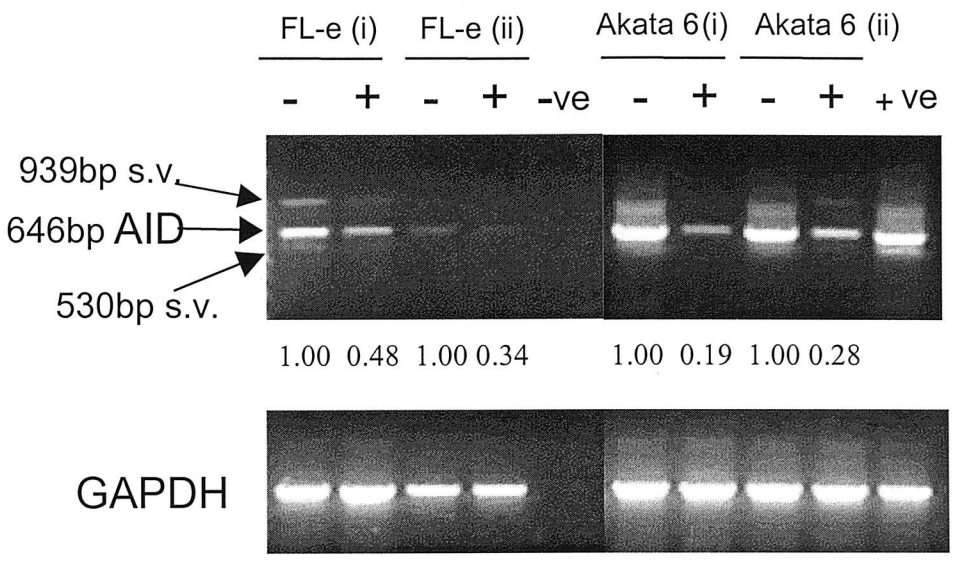
In addition to known CD40-target genes, this microarray system potentially identified many novel CD40-target genes that could play important roles in many aspects of CD40 biology. In both Akata 6 and FL-e cells, a large number of genes involved in extracellular signalling were significantly upregulated by CD40 ligation (tables 5.1 and 5.14). These included many chemokines (e.g. CXCL9 and CCL17, which influence migration of leukocytes) and interleukins and their receptors (e.g. IL-10, which influence leukocyte proliferation and differentiation). Interestingly, some of the genes upregulated by CD40 ligation are also interferon-inducible genes e.g. CXCL9, CXCL10, CXCL11, guanylate binding protein 2, WARS, IFIT 1 and IFIT 4 (Hiroi & Ohmori 2003, Rebhan *et al* 1997). However interferon-gamma itself, was downregulated by CD40 ligation in the FL-e sample (table 5.32). Many NF-κB-regulated genes and genes involved in NF-κB signalling were also upregulated by CD40 ligation including three different IκB isoforms (IκB-zeta, IκB-alpha and IκB-epsilon), p49/p100, A20 and Bfl-1.

Interestingly, some tumour antigens and tumour markers were shown to be significantly regulated by CD40 ligation on the arrays, including preferentially expressed antigen in melanoma (PRAME), melanoma inhibitory activity (MIA) (table 5.16) and lysosomal-associated membrane protein 3 (LAMP3) (table 5.30). Also some of the genes, particularly those downregulated by CD40 ligation in FL-e, have functions that have yet to be defined (tables 5.17, 5.22, 5.31, 5.40).

Many of the genes identified using the CodeLink system were also identified as CD40-regulated genes in at least one of the HGMP arrays (as shown by colour-coding on tables 5.10 to 5.40, appendix tables A.3 & A.4). These included FLIP, A20, IκB-epsilon, CXCL10 and RGC-32. The fold changes detected with the dual-colour HGMP microarray system were generally much reduced compared to the CodeLink system e.g. A20 was upregulated 2.43 fold in the Akata dual-colour arrays whereas it was upregulated 7.8 fold in the CodeLink arrays and the Epstein-Barr virus induced gene 3 was upregulated 2.12 fold in the Akata dual-colour arrays whereas it was upregulated almost 50 fold in the CodeLink arrays.

One gene shown to be significantly downregulated by CD40 in both Akata 6 and FL-e cells was activation-induced cytidine deaminase (AID). AID was downregulated over 40 fold in Akata 6 cells (the most downregulated gene) and over 5 fold in FL-e (tables 5.21 and 5.39). AID is expressed in germinal centre cells and is essential for somatic hypermutation and class switch recombination. It also has prognostic significance in CLL and was previously shown to be *upregulated* by CD40 signalling alone, or in combination with interleukins, in normal B-cells (MacCarthy *et al* 2003, Zhou *et al* 2003, Zan *et al* 2003, Greeve *et al* 2003, Nagumo *et al* 2002). To verify that the microarray result was correct, RT-PCRs were undertaken for AID on Akata 6 and FL-e samples treated with CD40L, or left untreated as a control. As shown in fig. 5.7A, the RT-PCR results confirmed the microarray findings, showing that CD40 ligation downregulated AID expression over 2-fold in FL-e cells and approximately 5-fold in Akata 6 cells. The microarray results suggested that AID was downregulated to a greater degree in Akata 6 than FL-e cells and the RT-PCR results showed this to be

A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FL-e (ii)
Akata 6(i)
Akata 6 (ii)
- + - + -ve - + - + +ve
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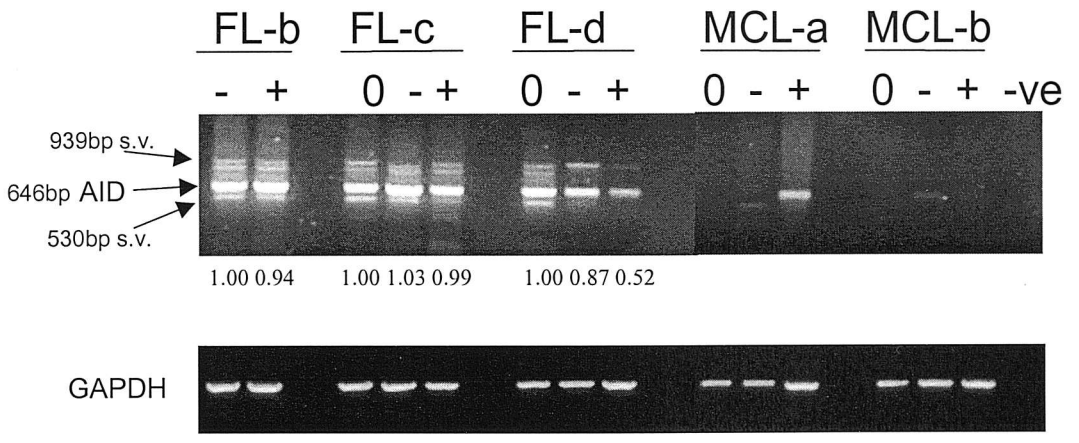
646bp AID
530bp s.v.
1.00 0.48 1.00 0.34
1.00 0.19 1.00 0.28
GAPDH

B


FL-b
FL-c
FL-d
MCL-a
MCL-b
- + 0 - + 0 - + 0 - + 0 - + -ve
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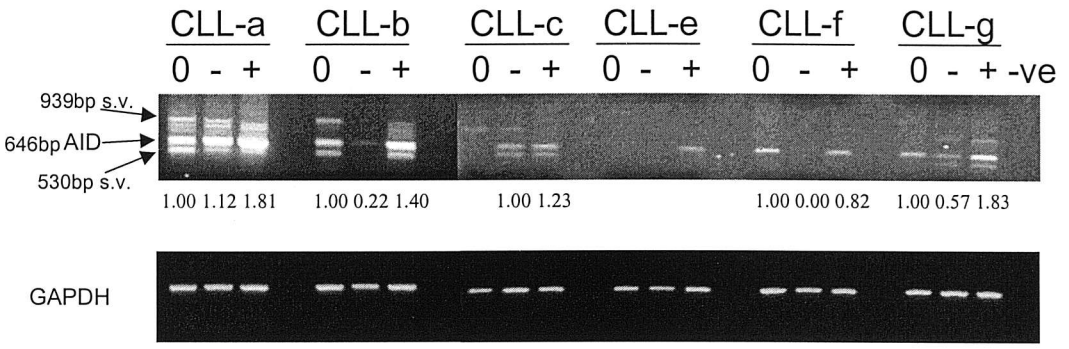
1.00 0.87 0.52
GAPDH

C



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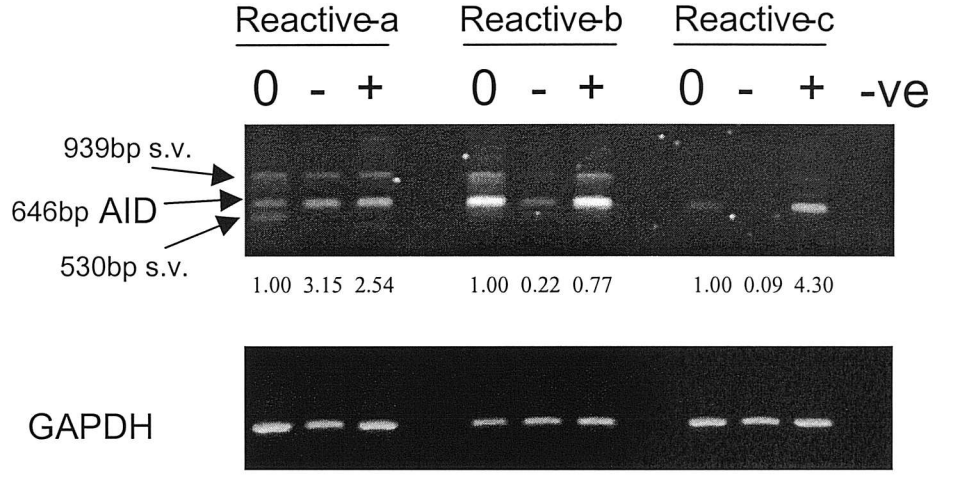

Figure 5.7: CD40L regulates AID expression in normal and malignant B-cells. In all experiments cells were treated with CD40L (1μg/ml) (+) or left untreated as a control (-) for 16 hours. 0 = Time 0, at the start of the experiment. RNA was isolated and reverse transcribed to cDNA. Primers specific for AID were used to amplify AID cDNA, in a semi-quantitative manner. AID is shown as a band at 646bp. There are two AID splice variants (s.v.), one which retains intron 4 (939bp band) and one which omits exon 4 (530bp band). GAPDH is shown as an internal control. –ve = water used instead of cDNA in PCR reaction. See table 3.1 for characteristics of primary samples. Values beneath each blot represent the relative band intensity normalised to GAPDH. The first band of each sample was set to 1. **A** - Akata 6 and FL-e AID RT-PCRs. RNA samples from two different experiments were used (i) and (ii). (i) = RNA used in the Codelink microarray, (ii) = RNA from an additional experiment. +ve = AID positive control (GA10 Burkitt's lymphoma cell line). **B** – Primary follicular lymphoma (FL) and mantle cell lymphoma (MCL) AID RT-PCRs. **C** – Primary CLL AID RT-PCRs. **D** – Primary reactive node RT-PCRs.

the case, although the fold changes observed using RT-PCR were not as great as those detected using microarrays, perhaps as the RT-PCR was only semi-quantitative.

Since these results showed that CD40L downregulated AID expression in malignant B-cells, whereas reports in the literature have demonstrated that CD40 signalling upregulates AID expression in normal B-cells, the effect of CD40L on AID expression in more primary samples was determined. As shown in figures 5.7B-5.7D, CD40 ligation either upregulated, downregulated, or did not influence AID expression in a variety of B-cell malignancies and reactive nodes. AID was highly expressed in follicular lymphoma cells, which was expected as these are derived from germinal-centre B-cells. CD40L decreased AID expression in two follicular lymphoma samples (FL-d and FL-e) but did not influence AID expression in two other follicular lymphoma samples (FL-b and FL-c). AID was not expressed in the two mantle cell lymphoma samples, and CD40L induced AID expression in the MCL-a sample but not the MCL-b sample (fig. 5.7B). The CLL samples had variable constitutive levels of AID e.g. CLL-a and CLL-b expressed high levels of AID whereas CLL-c and CLL-e did not express AID (fig. 5.7C). This could be related to the V_H gene status of the CLL samples, since CLL with unmutated V_H genes were shown to express AID, whereas CLL with mutated V_H genes did not express AID (McCarthy *et al* 2003). Unfortunately the V_H genes status for the majority of these CLL samples was not available, except for CLL-e which is, infact, unmutated. In the majority of CLL samples (CLL-a, CLL-b, CLL-e and CLL-g), CD40L upregulated AID expression, in one sample (CLL-f) CD40L sustained AID expression and in one sample (CLL-c) CD40L did not influence AID expression (fig. 5.7C). In the reactive node samples, AID was constitutively expressed in all three samples, although relatively weakly in Reactive-a and Reactive-c. CD40 ligation did not influence AID expression in Reactive-a, it sustained AID expression in Reactive-b and increased AID expression in Reactive-c (fig. 5.7D). Therefore, although CD40 signalling was previously associated with increasing AID expression, this study has shown that CD40 can induce diverse responses in various primary B-cell malignancies and non-malignant B-cells.

Two AID splice variants have been described, one retaining intron 4 and one omitting exon 4 (McCarthy *et al* 2003). These splice variants were detected in Akata 6 cells

and the majority of primary samples. Interestingly, extra bands were also detected in the follicular lymphoma, CLL-a and CLL-b samples, which were intermediate in size between AID and the intron 4-containing splice variant. It is possible that these bands could be additional AID splice variants that have yet to be identified.

5.4 Discussion

The aim of this study was to develop a microarray system and to characterise gene expression changes induced by CD40 ligation in malignant B-cells. Two microarray platforms were compared: CodeLink microarrays, a single-colour system, and HGMP microarrays, a dual-colour system. Both systems were able to correctly identify both known CD40-target genes e.g. FLIP, Bfl-1 and A20, and novel CD40 target genes, which were verified by RT-PCR e.g. AID, RGC-32 and CCR7. However results from the CodeLink microarray system were far more reproducible, and accurate than the HGMP dual-colour system (compare figs. 5.1B and 5.6B). The CodeLink system was also more sensitive requiring less than a tenth of the RNA needed for the HGMP system. Fold changes in gene expression were also much reduced in the dual-colour system than the single-colour system. The benefits of the single-colour system are probably due to a number of factors. Firstly, the single-colour system employs a 3-dimensional arrangement of nucleotides on the microarray surface, whereas the dual-colour system uses a two-dimensional arrangement. The 3-D arrangement gives greater access and specificity to the probe, facilitating more efficient probe binding. Secondly, labelling of the probe with fluorescent dyes is performed after hybridisation to the microarray in the single-colour system, whereas probes are fluorescently labelled before hybridisation in the single-colour system. Pre-hybridisation labelling may cause the fluorescent label to interfere with hybridisation of the probe, decreasing binding efficiency. Thirdly, the two differently labelled probes will compete for binding to the array in the dual-colour system, which may reduce hybridisation efficiency. Fourthly, there is a large amount of dye bias in the dual-colour system, causing Cy3-labelled spots to show more fold changes than Cy5-labelled spots. Reciprocally-labelled replicates were performed to account for this but very few genes were regulated in common on the reciprocally labelled arrays. Notably, many of the genes identified by the CodeLink system were identified as

regulated by CD40 on the HGMP arrays on only one of the reciprocally-labelled replicates (table A.3), confirming that dye bias limits detection in the HGMP system. Dye bias may be due to different incorporation rates of the Cy3 and Cy5-labelled nucleotides; differences in the power or sensitivity of the two lasers used to detect the probes; or differences in hybridisation efficiency of Cy3 and Cy5 labelled probes. Obviously, the use of a single-colour system prevents all of these concerns. Fifthly, the quality of the single-colour CodeLink arrays was far superior to the HGMP microarrays. However, despite the short-comings of the HGMP system, it must be noted that these are freely available microarrays (whereas CodeLink is a commercial system) and can be successfully used to identify gene expression changes if the appropriate reciprocally-labelled replicates are employed.

Both microarray systems correctly identified known CD40-target and/or NF-κB-target genes, such as Fas, FLIP, A20, Bfl-1, IκB-α, c-IAP2, CD83 and lymphotoxin-α (see chapter 3 and 4, Worm & Geha 1995, Hong *et al* 2000). Comparison of the results of this study with that of Dadgostar *et al* (2002), who characterised CD40 gene-expression changes in primary murine B-cells, revealed many common CD40-regulated genes. These included upregulation of A20, Bfl-1, TRAF1, Fas, STAT5a, IRF4, lymphotoxin-α and IκB-α by CD40 ligation in both Akata 6 cells and primary murine B-cells. Upregulation of c-myc, Pim-1 kinase, fatty acid synthase, OX40 ligand and cyclin D2 by CD40 ligation was observed in both FL-e cells in this study and primary murine B-cells in Dadgostar *et al* (2002). This therefore shows that the microarray platforms employed in this study were able to correctly identify known CD40-targets and that there are some similarities in CD40-induced gene expression between malignant human B-cells and normal murine B-cells.

This study also potentially identified many novel CD40-target genes. These included many genes involved in regulation of the immune response, lymphocyte migration and adhesion. For example, many chemokines and their receptors were upregulated by CD40 ligation, such as the interferon-induced chemokines CXCL9, CXCL10 and CXCL11, and the chemokine receptor CCR7. Upregulation of CCR7 mRNA by CD40 ligation in RL and Akata 6 cells was confirmed by RT-PCR. CD40 ligation was previously shown to alter the response of normal tonsillar B-cells to certain

chemokines (Casamayor-Palleja *et al* 2002). Chemokines are secreted cell signalling molecules which bind G-protein coupled receptors, influencing migration of leukocytes. They are important in inflammatory and immune responses and also in the control of normal lymphocyte homeostasis, including lymphoid development and homing into and out of lymph nodes. CCR7 binds two B-cell chemoattractant chemokines CCL21 and CCL19, and CCR7 knockout mice display defective B-cell entry into nodes (Till *et al* 2002). Chemokines have also been implicated in tumour growth, angiogenesis and metastasis (Vicari & Caux 2002). Indeed, overexpression of CCR7 was correlated with tumour stage and lymph node metastasis in nonsmall cell lung cancer (Takanami 2003), and CCR7 was found to be upregulated in classical Hodgkin's disease (Hopken *et al* 2002). In addition, the expression of CCR7 correlated with clinical lymphadenpathy in CLL, and blocking CCR7 prevented transendothelial cell migration of CLL cells (Till *et al* 2002). Therefore upregulation of chemokines and their receptors, particularly CCR7, by CD40 signalling, may be important for the normal functions of CD40 in B-cell development and regulation of the immune response, but could also promote tumour spread in malignant cells.

However CD40 ligation also upregulated expression of the B-cell specific molecule RGS1 (Regulator of G-protein signalling 1) in Akata 6 cells, which inhibits signalling through G-protein coupled receptors such as certain chemokine receptors (Moratz *et al* 2000, Reif & Cyster 2000). RGS1 is highly expressed in germinal centre B-cells and its expression is also induced by antigen. Therefore, upregulation of RGS1, in addition to chemokines and their receptors, by CD40 ligation may further modulate chemokine signalling.

Tumour antigens and tumour markers were also identified as CD40-regulated genes using microarrays. These included PRAME (preferentially expressed antigen in melanoma), MIA (melanoma inhibitory activity), GBP-5 (guanylate binding protein 5), LAMP3 (lysosomal-associated membrane protein 3) and NY-REN-34 (Rebhan *et al* 1997, Bosserhoff & Buettner 2002, Scanlan *et al* 1999). PRAME is highly expressed in various solid tumour cells, multiple myeloma, AML, ALL, and CML and is recognised by cytotoxic T-cells (Matsushita *et al* 2003). Therefore upregulation of these molecules on CD40-activated tumour cells could help tumour recognition and killing by CTLs during CD40-based immunotherapy.

Interestingly, CD40 ligation also upregulated genes which were previously thought to be neuronal specific genes e.g. GABA (Gamma-aminobutyric acid) A receptor and plexin A3, in Akata 6 and RL cells, respectively. GABA is an inhibitory neurotransmitter but it has also been found at sites of immune and inflammatory reactions. For example, GABA treatment has been shown to modulate T-cell mediated cytotoxicity (Bergeret *et al* 1998). Plexin A3 is a transmembrane protein which regulates neuronal development and axon guidance through binding semaphorins (Rebhan *et al* 1997). However semaphorins and plexins have been detected in cells of the immune system where they may regulate lymphocyte activation. For example, CD100 (semaphorin 4D) interacts with plexin-B1 on CD5+ B-cells leading to cell proliferation and survival (Granziero *et al* 2003). Interestingly, CD40 ligation increased CD100 expression in CLL cells (Granziero *et al* 2003). Therefore it is conceivable that increased expression of the GABA A receptor and Plexin A3 through CD40 signalling could modulate B-cell function.

IRTA1 and IRTA4 were also identified as novel CD40-target genes in Akata 6 cells from the microarray experiments. IRTAs (Ig superfamily receptor translocation-associated gene, also called Fc receptor homologs) are transmembrane receptors, expressed predominately in B-cells, with different members showing restricted expression to certain B-cell compartments. For example, IRTA1 is specific to the marginal zone, whereas IRTA4 is expressed predominately in the mantle zone of lymph nodes (Miller *et al* 2002, Davis RS *et al* 2001). The IRTA locus (1q21) is also often involved in translocations in B-cell malignancies. The function of the IRTA family is unknown but they are thought to play a role in normal and neoplastic B-cell development.

CD40 signalling also downregulated many novel genes in Akata 6, RL and FL-e cells, such as RGC-32 and AID. Downregulation of RGC-32 in Akata 6 and RL cells, and downregulation of AID in Akata 6 and FL-e cells was confirmed by RT-PCR. RGC-32 is a recently identified gene, shown to increase activity of the cyclin dependent kinase $p34^{CDC2}$ and induce quiescent aortic smooth muscle cells to enter S-phase (Badea *et al* 2002). AID (Activation-induced cytidine deaminase) is essential for somatic hypermutation and class switch recombination in the germinal centre.

Previous studies have shown that CD40 signalling, either alone or in combination with interleukins, led to induction of AID expression in normal B-cells (Zhou *et al* 2003, Zan *et al* 2003, Greeve *et al* 2003, Nagumo *et al* 2002). In this study AID expression was also determined in a variety of primary samples, showing that CD40 ligation can induce diverse patterns of AID expression in various primary B-cell malignancies and non-malignant B-cells. The reasons for this differential regulation of AID expression by CD40 are unclear and analysis of more primary samples may be useful. It would also be interesting to determine if the mutational status of the V_H genes of CLL cells influences the outcome of CD40 signalling to AID, since CLL with unmutated V_H genes were shown to express AID, whereas CLL with mutated V_H genes did not express AID (McCarthy *et al* 2003).

One of the aims of this study was to identify differentially regulated genes by CD40 in Akata 6 and RL cells, since these cell lines have differing responses to CD40 ligation. This proved difficult using the HGMP microarray system due to the lack of reproducibility and confidence between replicates, however a number of potential differentially regulated genes were identified including KIAA1268, a putative transcription factor characterised from high-risk diffuse large B-cell lymphoma (Aguiar *et al* 2000), the IL-13 receptor, STAT1, SLAM and SH2D1A. The HGMP data suggested that CD83 and TRAF1 were upregulated by CD40 ligation in RL cells but were unaffected by CD40 signalling in Akata 6 cells, however CD83 and TRAF1 were significantly upregulated in Akata 6 cells using the CodeLink microarrays. As the CodeLink system is much more reliable, this system will be used in the future to determine CD40-induced gene expression changes in RL cells and to compare them with the Akata 6 data.

Differential regulation of SLAM (signalling lymphocytic activation molecule) and SH2D1A (or SAP, SLAM-associated protein) by CD40 was confirmed by RT-PCR. SLAM was constitutively expressed in RL cells, where it was upregulated by CD40 ligation, whereas SLAM expression was not detected in Akata 6 cells and was not influenced by CD40 signalling. Conversely, SH2D1A was expressed in Akata 6 cells, but not in RL cells, and CD40 ligation decreased SH2D1A expression in Akata 6 cells. SLAM, or CDw150, is a cell-surface receptor that can also be secreted in a soluble form. SLAM functions as a self-ligand and engagement of SLAM induces T-

cell proliferation and interferon-gamma production, B-cell proliferation, differentiation and Ig synthesis. CD40 signalling was previously shown to upregulate SLAM expression in both normal B-cells and dendritic cells (Punnonen *et al* 1997, Bleharski *et al* 2001). In contrast with the ability of SLAM to induce B-cell proliferation, SLAM can enhance Fas-mediated apoptosis and can prevent rescue of Fas-mediated apoptosis by CD40 (Mikhalap *et al* 1999). Therefore activation of SLAM can result in positive or negative B-cell survival signals. SLAM associates with a number of adaptor molecules including Fgr (Src family kinase), SHIP (SH2-containing inositol phosphatase), SH2D1A and SHP2 (SH2 domain containing protein tyrosine phosphatase 2) via interaction with phospho-tyrosines on the cytoplasmic tail of SLAM (Engel *et al* 2003). Ligation of SLAM induces rapid dephosphorylation of Fgr and SHIP, and association of Lyn and Fgr with SHIP (Mikhalap *et al* 1999). SH2D1A has a dual role in SLAM signalling. It can act as an adaptor molecule that recruits Src family kinases such as Fyn to SLAM. SH2D1A can also act as an inhibitor of SLAM signalling, blocking the binding of other SH2-domain containing molecules to SLAM e.g. SHP2 and SH2D1A bind to the same sites in SLAM and SHP2 only binds to SLAM in the absence of SH2D1A (Li *et al* 2003, Engel *et al* 2003). One study showed that CD40 signalling upregulated SH2D1A in a lymphoblastoid line (Schlapatska *et al* 2001), whereas SH2D1A was shown to be downregulated by CD40 activation in Burkitt's lymphoma lines (Nagy *et al* 2002), which agrees with the results obtained with Akata 6 cells, a Burkitt's lymphoma line, in this study. Therefore the results obtained in this study suggest that RL cells should be able to signal through SLAM, which is potentiated by CD40 ligation, whereas Akata 6 cells should not signal through SLAM due to low or absent levels of SLAM and high levels of its inhibitor SH2D1A. Although further work is necessary, it is tempting to speculate that this could impact on the differential control of cell survival signalling by CD40, since SLAM activation can produce negative effects on B-cell survival.

Chapter 6: Summary and further work

The aim of this study was to investigate the regulation of apoptosis by CD40 in B-cell malignancies. This is of particular importance for a number of reasons. Firstly, insights into the function and mechanisms of action of CD40 are key to understanding normal B-cell biology, since CD40 is an important signalling molecule in B-cell development and regulation of the immune response. Secondly, CD40 activation can either promote survival or induce apoptosis in malignant cells but the mechanisms responsible for this differential control of cell survival are not known (Dallman *et al* 2003). Understanding why CD40 activation can produce such contrasting effects on cell survival is important not only for CD40 and cancer cell biology, but also for forthcoming clinical trails using CD40-based immunotherapy to treat cancer. Little research has been undertaken on the response of primary B-cell malignancies to CD40 and so use of primary samples is of vital importance to determine whether CD40 promotes apoptosis or cell survival in real human tumours, and how this differs to normal B-cells. Furthermore, determining which genes are regulated by CD40 in malignant cells and investigating their role, regulation and function may give insight into the differential control of cell survival and may also identify suitable novel targets for cancer therapy.

This study demonstrated that CD40 is a potent survival signalling molecule for primary B-cell malignancies, regardless of histological type, and also non-malignant B-cells. CD40 also promoted long term clonogenic survival of the lymphoma cell lines Akata 6 and RL, despite inducing short-term cell death in RL cells. This has important implications for CD40-based immunotherapy. There is considerable interest in using agents that activate CD40 e.g. soluble CD40L, anti-CD40 mAb, or gene therapy with CD40L, to treat cancer based on the findings that CD40 can induce apoptosis in some malignant cell lines and encouraging results in animal models and early clinical trials (Funakoshi *et al* 1994, French *et al* 1999, Wierda *et al* 2000, Vonderheide *et al* 2001). The efficacy of CD40-based therapies is largely due to activation of cytotoxic T-cells, which destroy the tumour. However if induction of the immune response is insufficient than this study has revealed that direct activation of CD40 on the tumour could actually promote tumour cell survival, even in cells where

a short-term killing effect of CD40 occurs. This study also demonstrated that CD40 signalling could reduce the apoptotic effects of the cytotoxic drug etoposide, suggesting that CD40-based therapies in combination with chemotherapy may not be a useful approach to treat cancers. Therefore future clinical trials using CD40-based therapies may be better restricted to CD40 negative tumours; tumours in which a clear long-term apoptotic response to CD40 has been defined; or patients in which a strong anti-tumour immune response is expected to occur. The signalling pathways responsible for the anti-apoptotic effect of CD40 were investigated and showed that activity of NF-κB was important for both the normal survival and CD40-induced survival of lymphoma cell lines and primary samples, suggesting that NF-κB inhibitors may have potential use in the treatment of B-cell lymphoma. Various small molecule inhibitors of NF-κB activity are being developed or have been approved for use in the clinic, and function by inhibiting the NF-κB activating kinase IKK (Haefner 2002) or inhibiting the proteasome e.g. Velcade.

CD40 activation is likely to regulate cell survival through altering expression of pro- or anti-apoptotic genes. CD40 ligation is known to induce expression of the anti-apoptotic proteins Bcl-X_L, Mcl-1, Bfl-1 and A20 in B-cell lines (Lee *et al* 1999, Kuss *et al* 1999, Sarma *et al* 1995) but this was the first study to systematically compare expression of these CD40 targets in a range of different primary B-cell malignancies. In this study, Bcl-X_L and Mcl-1 were identified as the key CD40-upregulated genes in primary B-cell lymphomas and non-malignant B-cells. Regulation of A20, Bfl-1 and the anti-apoptotic protein survivin by CD40, was not consistent between primary samples but did occur in some samples. BNIP3, a pro-apoptotic Bcl-2 family protein, was identified as a novel CD40-regulated gene in approximately half of the primary samples tested. Further investigations into the role and regulation of Bcl-X_L and Mcl-1 in lymphoma cells revealed that these proteins were vital for the survival of lymphoma cells and that their upregulation by CD40 was dependent on NF-κB activity. Therefore disruption of expression or function of Bcl-X_L and/or Mcl-1 may be of therapeutic benefit for lymphoma.

Regulation of Bcl-X_L expression can occur at many levels, for example through usage of different promoters and alternative splicing events. mRNAs from both the Bcl-X

exon IA and exon IB promoters were upregulated by CD40 ligation in primary samples and the upregulation of mRNAs derived from the Bcl-X exon IA promoter was mediated via the DNA-binding activity of the p50 NF-κB subunit. Various Bcl-X mRNAs can be generated with different 5'-UTRs, due to alternative promoter use and alternative splicing events, and this study showed that these 5'UTRs can influence translation of the downstream open reading frame, thereby exerting an additional level of control on Bcl-X expression.

Akata 6 and RL cells were used as model systems to investigate the differential control of cell survival by CD40, since CD40 has an anti-apoptotic role in Akata 6 cells but a pro-apoptotic role in RL cells in the short-term. Survival outcome to CD40 activation was not influenced by signalling through the death receptors Fas and TRAIL, but may be modulated by alterations in expression of Bcl-2 family proteins. DNA microarrays were developed and used to characterise CD40-induced gene expression changes in these cells and also in a primary follicular lymphoma sample, and this identified many novel CD40-regulated genes e.g. CCR7, RGC-32 and also differentially regulated genes in RL and Akata 6 cells e.g. SH2D1A and SLAM. The microarray study also revealed that CD40 ligation can differentially regulate AID expression in primary B-cell malignancies. Two microarray platforms were compared: traditional dual-colour cDNA microarrays (HGMP) and a novel single-colour microarray system (Amersham CodeLink arrays). The CodeLink system was far superior to the dual-colour system in terms of both sensitivity and reproducibility.

Further work that could be undertaken based on this project is listed below.

(i) The effect of CD40 activation on cell survival.

This study demonstrated that activation of CD40 using soluble trimeric CD40L improved cell survival for a wide-range of different B-cell lymphomas. However differing sensitivities to CD40 ligation were observed, particularly between CLL samples. It would be interesting to determine if this was related to the mutational status or CD38 status of CLL samples. CLL patients with unmutated V_H genes have a poor prognosis whereas those with mutated V_H genes have a good prognosis and often do not require any treatment. CD38 also has prognostic significance in CLL as those patients with CLL cells that express CD38 show

poor survival (Hamblin *et al* 1999, Damle *et al* 1999). The molecular mechanisms underlying these differences are not well understood and perhaps the response of CLL cells from different subgroups to CD40 activation could give valuable insight into the mechanisms behind these differences. If these subgroups of CLL show differing responses to CD40 ligation, then this could have important implications for use of CD40-based therapies, since novel treatments for CLL patients with unmutated V_H genes are necessary.

Different sensitivities to CD40 ligation in primary samples could be related to differences in the developmental stage or activation of B-cells. Differences in CD40-target gene expression were also observed in different B-cell malignancies using microarrays. Investigations into the effect of B-cell development stage on CD40 signalling could be carried out using normal B-cells at various stages of development and assessing their response to CD40 ligation in terms of cell survival and regulation of CD40-target genes.

This study used soluble trimeric CD40L to activate CD40 signalling, however other methods to activate CD40, such as anti-CD40 monoclonal antibodies are additionally being developed for use in clinical trials. It would therefore be important to determine if the survival responses to CD40 activation were similar to both CD40L and anti-CD40 mAbs. This work could also be extended to investigate the survival responses to CD40 in primary carcinoma cells, since very few studies have analysed the effect of CD40 activation in primary carcinoma cells. It will also be important to determine long-term survival responses to CD40 in carcinoma cells.

(ii) The role of CD40-target genes in CD40-mediated survival.
This study identified key and novel CD40-target genes in B-cell lymphomas. This could be extended by using quantitative-PCR analysis e.g. Taqman, to further validate CD40-targets and investigate their regulation by CD40. The role of CD40-target genes in CD40-mediated responses is not well understood and could be tested by using RNA interference approaches (RNAi) to ablate gene expression. In this study, antisense oligonucleotides were used to ablate Bcl-X_L and Mcl-1 expression, showing that these molecules were key for the survival of

lymphoma cells, but their exact role in CD40-induced survival could not be determined due to difficulties with delivery of the antisense molecules into cells. This could be overcome using RNAi, since an inducible plasmid encoding a specific RNAi molecule could be stably transfected into cells and RNAi production, and hence target ablation, induced when required.

(iii) The role of NF-κB in CD40 signalling.

NF-κB activity was shown to be required for both the cell-survival promoting effect of CD40 and the induction of Bcl-X_L and Mcl-1 expression by CD40 in primary B-cell lymphomas and cell lines. To further investigate NF-κB activity, electrophoretic mobility shift assays (EMSA) could be performed on nuclear extracts from untreated cells or cells treated with CD40L to determine which NF-κB subunits bind DNA upon CD40 ligation. Probes spanning the NF-κB sites in the Bcl-X and Mcl-1 promoters could be used to investigate whether these promoters may be activated via NF-κB. Chromatin immunoprecipitation (CHIP) assays could be used to verify NF-κB binding to distinct promoter regions in intact cells.

It would also be interesting to determine if the promoter regions of novel CD40-target genes identified in this study e.g. BNIP3, contain NF-κB consensus sites, using a bioinformatics approach. If NF-κB consensus sites were found, regulation by NF-κB could be determined by EMSA, CHIP assays and transfection experiments using promoter reporter constructs containing mutated NF-κB sites.

To determine the role of NF-κB activation in CD40-mediated survival signalling or in the regulation of certain CD40-target genes, cells could be transfected with plasmids encoding various NF-κB subunits to overexpress NF-κB or cells could be transfected with a plasmid encoding a non-phosphorylatable IκB-alpha protein, to inhibit NF-κB activity. It would also be useful to treat cells with NF-κB inhibitors and CD40L and characterise gene expression using DNA microarrays. This may identify novel NF-κB regulated genes that are important in controlling the cellular response to CD40 activation.

(iv)Translational control of Bcl-X_L

The 5' UTRs of Bcl-X mRNAs were shown to influence translation of the downstream gene in this study. In particular the presence of exon IA in the 5' UTR greatly enhanced expression of this mRNA compared to mRNAs lacking this region. It is possible that this region could contain an IRES and this could be tested by cloning the exon IA sequence into a dicistronic reporter plasmid. In a dicistronic reporter plasmid lacking an IRES, the upstream gene will be translated with much greater efficiency than the downstream gene. However if an IRES is placed in between the two genes, then this will promote translation of the downstream gene.

(v) Differential responses to CD40 activation.

Differential regulation of cell survival by CD40 was studied using Akata 6 and RL cells. Microarray analysis using HGMP dual-colour arrays identified genes that were differentially regulated by CD40 in these samples. However the CodeLink microarray system was subsequently shown to be a greatly improved microarray platform in terms of sensitivity and reproducibility. Therefore this system will be used to characterise CD40-induced gene expression changes in RL cells and to compare them with the Akata 6 data.

SLAM and SH2D1A were identified as differentially regulated genes in Akata 6 and RL using microarrays, which may impact on the survival response to CD40 activation. This was verified by semi-quantitative RT-PCR but further work should involve verification of these CD40-targets at the protein level using western blots for SH2D1A and SLAM or flow cytometry for cell surface expression of SLAM. SLAM is a self-ligand and could be activated using anti-SLAM activating antibodies or recombinant soluble SLAM to determine its function in RL cells. Overexpression or RNAi approaches could be used to determine the importance of these molecules in CD40-mediated survival responses.

Appendix

Tables A.1 – A.2: CD40 Co-regulated genes in Akata 6 and RL cells. The following tables show genes that were more than 2 fold up- or downregulated in at least one Akata 6 array and at least one RL array. Values shown are log2 ratio of CD40L/untreated fluorescence intensity. N.d. = not determined i.e. spot was not flagged as bad or not found on the array., n.a. = standard deviation not applicable, as there was only one replicate value.

Table A.1: CD40L upregulated genes in both Akata 6 and RL cells.

Gene	Function	Akata array 1		Akata array 2		Mean	SD	RL array 1		RL array2		Mean	SD
FLIP	Apoptosis	1.89	3.50	n.d.	n.d.	2.70	1.14	2.41	2.61	2.76	n.d.	2.59	0.18
Beta-actin	Cytoskeleton	2.23	n.d.	n.d.	n.d.	2.23	n.a.	1.65	1.84	0.33	0.80	1.16	0.71
CD56	Cell adhesion	-0.10	3.92	n.d.	n.d.	1.91	2.84	0.74	1.39	-1.62	0.00	0.13	1.29
Programmed cell death 1 (PCD1)	Apoptosis	1.93	n.d.	n.d.	n.d.	1.93	n.a.	0.79	1.45	-0.12	n.d.	0.50	0.76
Annexin 7	Exocytosis	1.93	n.d.	n.d.	n.d.	1.93	n.a.	1.19	1.25	0.53	0.81	0.95	0.34
Heterogeneous nuclear ribonucleoprotein K	RNA binding	1.76	n.d.	n.d.	n.d.	1.76	n.a.	1.22	n.d.	-0.04	n.d.	0.59	0.72
IκB-epsilon	Transcription	0.98	2.25	n.d.	n.d.	1.62	0.90	1.42	1.67	0.34	0.53	0.99	0.65
mitochondrial ribosomal protein L39	Translation	1.63	n.d.	n.d.	n.d.	1.63	n.a.	1.25	2.24	0.77	n.d.	1.26	0.69
Hyaluronan synthase 1	Biosynthesis	1.51	1.59	n.d.	n.d.	1.55	0.06	1.30	1.36	-1.05	-0.81	0.20	1.31
Chemokine (C-C) motif receptor 7 (CCR7)	Extracellular signalling	1.22	1.43	1.66	1.83	1.54	0.27	1.31	1.48	0.74	0.78	1.08	0.37
Clone 24658	unknown	1.91	3.17	0.52	0.53	1.53	1.27	1.66	1.80	0.17	0.69	1.08	0.78
Sorting nexin 11	Intracellular trafficking	0.92	1.40	1.25	1.57	1.28	0.28	1.65	1.71	1.31	1.40	1.52	0.19
DKFZp434I0535	Putative DNA binding protein	1.25	1.29	n.d.	n.d.	1.27	0.02	1.20	1.25	1.66	1.69	1.45	0.26

HSPC049	Unknown	1.15	1.34	n.d.	n.d.	1.24	0.14	1.09	1.30	1.52	2.21	1.53	0.49
Vasodilator-stimulated phosphoprotein (VASP)	Cytoskeletal regulation	1.20	2.06	0.74	0.93	1.23	0.58	1.19	1.46	0.42	0.86	0.98	0.45
Lymphotoxin alpha	Extracellular signalling	1.49	2.26	0.64	0.47	1.22	0.83	1.62	2.81	2.58	3.93	2.73	0.95
A20	Apoptosis	1.49	1.84	0.49	0.94	1.19	0.59	1.20	1.45	1.82	2.01	1.62	0.36
Major histocompatibility complex, class I, F (HLA-F)	Antigen presentation	1.34	1.36	1.00	1.04	1.18	0.19	1.72	1.78	0.31	0.35	1.04	0.82
Nuclear matrix protein p84	RNA export and processing	0.71	1.56	n.d.	n.d.	1.13	0.60	0.79	1.54	-0.12	0.16	0.60	0.73
Clone 24761	Unknown	0.71	1.30	1.11	1.36	1.12	0.29	1.08	1.13	0.73	n.d.	0.92	0.21
Plexin A3 (plexin 4, SEX gene)	Extracellular signalling	0.79	1.39	n.d.	n.d.	1.08	0.43	1.01	n.d.	1.59	n.d.	1.21	0.33
Cytokine receptor CRL2 precursor	Extracellular signalling	0.35	1.65	n.d.	n.d.	1.00	0.92	1.07	1.78	-0.23	-0.03	0.65	0.95
Guanylate cyclase 1, soluble, alpha 3	Intracellular signalling	0.51	1.71	0.74	1.12	1.02	0.52	1.27	1.49	1.14	1.35	1.31	0.15
IL-12 receptor related (Epstein-Barr virus induced gene 3)	Extracellular signalling	1.23	1.80	0.38	0.43	0.96	0.68	1.06	1.17	1.54	1.91	1.42	0.39
Zinc finger protein RINZF	Protein binding	0.95	1.83	0.48	0.56	0.96	0.62	1.15	1.43	0.43	0.67	0.92	0.45
Aldolase C, fructose-bisphosphate	Metabolism	1.51	1.54	0.29	0.41	0.94	0.68	1.17	1.24	0.49	0.55	0.86	0.40
DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 15 (DDX15)	RNA splicing	0.19	2.57	0.25	n.d.	0.81	1.17	1.25	1.43	-0.41	-0.21	0.52	0.96
Visual system homeobox 1 homolog (VSX1)	Transcription factor	1.15	n.d.	0.29	0.38	0.74	0.47	1.74	n.d.	-0.53	0.12	0.76	1.15
TLS-associated serine-arginine protein (TASR)	RNA splicing	0.38	2.62	-0.22	0.15	0.73	1.28	1.16	n.d.	0.06	0.13	0.63	0.61
Nuclear distribution gene C homolog (NUDC)	Cytokinesis during mitosis	0.68	1.72	-0.30	0.46	0.64	0.83	0.95	1.05	0.40	0.43	0.71	0.34

Hypothetical protein FLJ20244	Probable N(2),N(2)-dimethylguanosine tRNA methyltransferase	1.05	1.12	-0.47	-0.20	0.37	0.83	0.90	1.27	-0.34	-0.29	0.38	0.82
Splicing factor, arginine/serine-rich 4	RNA splicing	0.89	1.59	-0.67	-0.37	0.36	1.06	0.69	1.39	-1.09	-0.69	0.07	1.16
Splicing factor 45kD	RNA splicing	0.20	1.84	-0.42	-0.30	0.33	1.04	0.78	1.56	-0.28	0.03	0.52	0.82
Zinc finger protein NY-REN-34	Transcription	1.46	1.93	-2.41	-1.89	-0.23	2.23	1.24	1.54	0.73	0.84	1.09	0.37

Table A.2: CD40L downregulated genes in both Akata 6 and RL cells

Gene	**Function**	**Akata array 1**		**Akata array 2**		**Mean**	**SD**	**RL array 1**		**RL array2**		**Mean**	**SD**
Clone SEL 125	Unknown	-1.95	-1.86	n.d.	n.d.	-1.90	0.06	-1.52	-1.29	-0.25	-0.23	-0.82	0.68
Scaffold attachment factor B	Transcription	-1.74	n.d.	n.d.	n.d.	-1.74	n.a.	-1.49	-0.77	0.02	0.49	-0.44	0.87
TBC1 domain family 4	Signalling	-1.72	-1.30	-1.72	-1.30	-1.51	0.24	-1.34	-1.19	-1.04	-0.75	-1.08	0.25
Thrombospondin 1	Cell-cell, cell-matrix interactions	-1.10	n.d.	n.d.	n.d.	-1.10	n.a.	-1.49	n.d.	-0.36	0.64	-0.68	1.03
RGC-32	Cell cycle	-1.24	-1.09	n.d.	n.d.	-1.17	0.11	-1.30	-0.89	-1.19	n.d.	-1.13	0.21
Clone 25023	Unknown	-0.95	n.d.	n.d.	n.d.	-0.95	n.d.	-1.10	-1.02	-0.31	-0.16	-0.65	0.48
Clone 24923	Unknown	-1.86	-1.38	-0.53	0.006	-0.94	0.84	-1.2	-1.00	-0.56	-0.24	-0.75	0.43

Lymphoid-restricted membrane protein (LRMP)	Vesicle targetting	-1.29	-0.98	-0.67	-0.54	-0.87	0.33	-1.31	-1.13	-1.65	-1.58	-1.40	0.24
General transcription factor IIE, polypeptide 1	Transcription	-1.08	-0.95	-0.70	-0.53	-0.81	0.24	-5.56	0.63	n.d.	n.d.	-2.46	4.38
FLJ22612	ATP dependent peptidase	-1.34	n.d.	-0.48	-0.11	-0.82	0.62	-1.64	n.d.	-1.52	-0.93	-1.43	0.34
NGFI-A binding protein 1 (ERG1 binding protein 1)	Transcriptional repressor	-1.41	-0.81	-0.23	n.d.	-0.67	0.56	-0.21	-0.01	-1.09	-0.96	-0.57	0.53
FLJ10080	unknown	-2.09	-2.02	0.81	1.01	-0.57	1.71	-1.20	n.d.	0.63	0.82	-0.24	1.11

Tables A.3 – A.4: CD40 regulated genes in both HGMP and CodeLink microarrays. The following tables show genes that were more than 2 fold upregulated or downregulated following CD40L treatment in one HGMP array and were significantly regulated over 4-fold in the CodeLink microarrays. Values shown are the mean ratio or standard deviation (SD). n.a. = not applicable, as data was not available.

Table A.3: CD40 upregulated genes in both HGMP and Codelink microarray systems

Gene	Function	Akata 6 HGMP		Mean ratio Akata 6 Codelink	RL HGMP		Mean ratio FL-e Codelink
		Mean	SD		Mean	SD	
FLIP	Apoptosis	7.51	5.30	10.8	6.06	0.74	7.0
Thymosin beta-4	Cytoskeletal regulation	3.39	0.10	4.4	0.98	0.11	1.17
IκB-epsilon	Transcription	3.37	1.98	5.0	2.14	0.93	4.50
A20	Apoptosis	2.43	0.96	7.8	3.14	0.77	14.3
CXCL10	Extracellular signalling	2.17	0.10	35.3	n.a.	n.a.	n.a.
Testican-2	ECM component	2.16	0.52	7.7	1.02	0.20	0.29
IL-12 receptor related (Epstein-Barr virus induced gene 3)	Extracellular signalling	2.12	1.03	48.9	2.75	0.77	63.2
Regulator of G-protein signalling 1 (RGS1)	Intracellular signalling	1.94	0.59	9.1 + 8.2	1.37	0.28	3.43
Mitogen activated protein kinase kinase kinase 8 (MAP3K8)	Intracellular signalling	1.93	0.60	4.8	1.39	0.08	2.09
Activating transcription factor 5 (ATF5)	Transcription factor	1.92	0.76	n.a.	0.89	0.06	7.4

Interferon-induced protein with tetratricopeptide repeats 4 (IFIT4)	Unknown	1.70	0.36	4.7	1.20	0.17	0.54
CD83	Extracellular signalling	1.44	0.62	4.8	4.56	1.50	2.79
NF-κB p49/p100	Transcription	1.85	0.17	5.4	3.13	1.79	8.5
TRAF1	Intracellular signalling	1.16	0.17	11.7	2.61	0.20	12.0
Retinal protein 4	Neurotransmitter release	1.94	n.a.	6.8	1.64	0.81	1.38
Tryptophanyl-tRNA synthetase (WARS)	Metabolism	1.13	0.25	1.24	1.60	0.72	8.4 + 7.4

Table A.4: CD40 downregulated genes in both HGMP and Codelink microarray systems.

Gene	**Function**	**Akata 6 HGMP**		**Mean fold-decrease Akata 6 Codelink**	**RL HGMP**		**Mean fold-decrease FL-e Codelink**
		Mean	**SD**		**Mean**	**SD**	
RGC-32	Cell cycle	7.51	5.30	6.5	6.06	0.74	5.7

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