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MOLECULAR CHARACTERIZATION OF APOPTOSIS IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA

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ABSTRACT

B-cell chronic lymphocytic leukemia (B-CLL), characterized by an accumulation of monclonal B cells, is the most common adult leukemia in the Western world. Defective apoptosis is considered to contribute to cell accumulation, disease progression and resistance to therapy in B-CLL. In this thesis regulation of apoptotic pathways in B-CLL cells were studied in relation to disease progression and chemotherapy responses.

TRAIL potently induces apoptosis in many tumor cells but exerts minimal cytotoxicity towards normal human cells. In the first study the TRAIL-apoptosis pathway was studied in B-CLL. B-CLL cells were relatively resistant to *in vitro* apoptosis-induction by recombinant TRAIL, although they expressed TRAIL-death receptors. Actinomycin D increased B-CLL susceptibility to TRAIL-induced apoptosis, which was not associated with the modulation of TRAIL-receptors. Down-regulation of the expression of $FLIP_L$ and $FLIP_S$ was correlated with the sensitization of B-CLL to TRAIL-induced apoptosis by actinomycin D. FLIP protein was also found to be expressed at higher levels in B-CLL cells as compared to normal tonsil B cells. Our results suggest the involvement of FLIP in the regulation of TRAIL resistance in B-CLL cells.

In the second study the proapoptotic BH3-only protein, Bmf, was studied in B-CLL cells. Two new splice variants, named bmf-II and bmf-III were described. They lacked the BH3 domain and, in agreement with this, also lacked the proapoptotic function of the previously described form of Bmf, but instead could promote survival, when overexpressed in HeLa cells. Expression of the isoforms was detected in B-CLL and normal B cells. In B-CLL undergoing serum deprivation-induced apoptosis, the pro-apoptotic form of Bmf was up-regulated while Bmf-III was down regulated. Taken together, we show that alternative splicing is used to switch between the apoptotic/non-apoptotic function of the Bmf protein and suggest that the relative levels of Bmf isoforms may have a role in regulating growth and survival in B cells and leukemic B-CLL cells.

In the third study the expression profile of apoptosis-regulating genes in B-CLL was investigated, in relation to chemoresistance and disease progression. We found higher expression of the anti-apoptotic Bcl-2-like proteins, Bfl-1, Mcl-1 and Bcl-2 in apoptosis-resistant B-CLL cells as compared to sensitive B-CLL. Bfl-1 was the most clearly discriminating gene between sensitive and resistant B-CLL cells. Investigation of the modulation of gene expression during serum deprivation-induced apoptosis was undertaken. A number of pro-apoptotic genes were induced and bfl-1 mRNA was down-regulated in B-CLL cells. In the fourth study the bfl-1 mRNA expression level was determined in a larger amount of patients and found to be significantly higher in patients failing to respond to chemotherapy compared to patients who responded to therapy and untreated patients. Bfl-1 expression was inversely correlated with *in vitro* fludarabine-induced apoptosis but its levels did not correlate with progression. RNA interference, that down-regulated bfl-1 expression in apoptosis-resistant B-CLL cells with high expression of bfl-1, led to induction of apoptosis in these cells. Taken together this indicates that bfl-1 might contribute to the development of apoptosis resistant phenotype in chemotherapy refractory B-CLL and could thus represent a future potential therapeutic target in B-CLL.

Key words: B-CLL, apoptosis, TRAIL, FLIP, Bmf, Bfl-1

LIST OF PUBLICATIONS

I. **Olsson A**, Diaz T, Celsing F, Österborg A, Jondal M and Osorio LM. Sensitization to TRAIL-induced apoptosis and modulation of FLICE-inhibitory protein in B chronic lymphocytic leukemia by actinomycin D. *Leukemia,* 15: 1868-1877, 2001

II. Morales AA, **Olsson A**, Celsing F, Österborg A, Jondal M and Osorio LM. Expression and transcriptional regulation of functionally distinct Bmf isoforms in B-chronic lymphocytic leukemia. *Leukemia*, 18: 41-47, 2004

III. Morales AA*, **Olsson A***, Celsing F, Österborg A, Jondal M and Osorio LM. High expression of Bfl-1 contributes to the apoptosis resistant phenotype in B-cell chronic lymphocytic leukemia. *International Journal of Cancer*, 113: 730-737, 2005

IV. **Olsson, A,** Ökvist, A, Choudhury A, Derkow K, Celsing F, Österborg A, Jondal M and Osorio LM. Up-regulation of Bfl-1 is a potential mechanism of chemoresistance in B-cell chronic lymphocytic leukemia. (Manuscript)

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CONTENTS

LIST OF ABBREVIATIONS

AIF	Apoptosis inducing factor
Apaf	Apoptotic protease activating factor
B-CLL	B-cell chronic lymphocytic leukemia
BCR	B cell receptor
BH	Bcl-2 homology
BIR	Baculovirus IAP repeat
CAD	Caspase activated DNase
CARD	Caspase activation recruitment domain
CMV	Cytomegalovirus
CR	Complete response
DD	Death domain
DED	Death effector domain
DISC	Death inducing signaling complex
DLC	Dynein light chain
DR	Death receptor
FADD	Fas associated protein with death domain
FDC	Follicular dendritic cells
FLICE	FADD-like ICE
FLIP	FLICE-inhibitory protein
IAP	Inhibitor of apoptosis protein
Ig	Immunoglobulin
IgV	Variable segment of Ig
IFN	Interferon
IL	Interleukin
NFκB	Nuclear factor κB
NK	Natural killer cell
NR	No response
OPG	Osteoprotegrin
PIDD	P53-inducible protein with a death domain
PR	Partial response
PTK	Protein tyrosine kinase
RAIDD	Receptor associated ICH-1 like protein with death domain
TCR	T cell receptor
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis inducing ligand

1 APOPTOSIS

Programmed cell death or apoptosis is an evolutionary conserved physiological process used by an organism to selectively eliminate cells that are no longer needed, have been damaged, infected or are dangerous. It is responsible for shaping organs during embryogenesis, maintaining tissue homeostasis and allowing controlled deletion of potentially harmful cells within the adult organism (Lockshin and Zakeri, 2001, Danial and Korsemeyer, 2004). In the immune system, apoptosis is involved in several aspects of immune function, including development of mature T and B cell populations, regulation of immune responses, and cell-mediated cytotoxicity (Strasser and Bouillet, 2003, Krammer, 2000).

The term apoptosis refers to a particular morphology of cell death in which the chromatin condenses in one or more masses in the nucleus, starting along the nuclear membrane forming a crescent or a ring-like structure (chromatin margination), followed by further shrinkage and fragmentation of the nucleus. The cell shrinks and becomes denser and often fragments into several pieces (Ziegler and Groscurth, 2004). The morphological changes are considered to be the result of caspase activity, which cleaves several vital proteins in the cell, and activates caspase-activated DNase (CAD), which is contributing to the degradation of DNA during apoptosis.

1.1 CASPASES

Demolition of cells during apoptosis requires a family of aspartate-specific cystein proteases, called caspases (Alnemri et al., 1996). Caspases are synthesized as procaspases, containing a prodomain and a large and a small caspase subunit (Figure 1). The active caspase consists of a homodimer containing two small and two large caspase subunits. Fourteen mammalian caspases are known, eleven so far in humans, seven of which have their major role in apoptosis (Riedl and Shi, 2004).

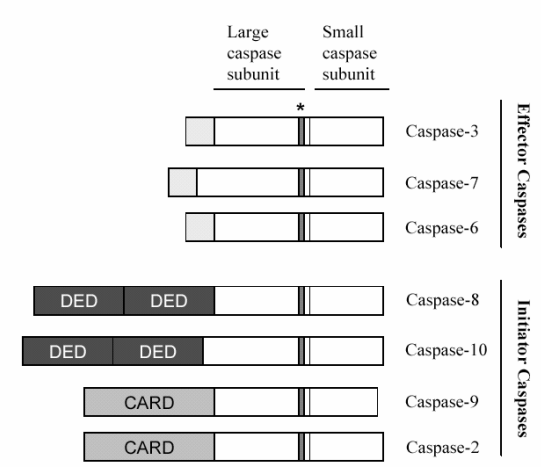

Figure 1. Schematic deptiction of caspase structure. Caspases are cystein proteases that all contain a small and a large caspase subunit including the active site. The asterisk indicates the catalytically active cystein residue. The initiator caspases in addition contain a long prodomain, which mediates their interaction with adaptor proteins upon apoptotic stimuli, leading to their activation.

These caspases can be divided in two groups based on their structure and role in apoptosis. One group, consisting of caspase-3, -6 and -7, has short prodomains and are the caspases performing the degradation of cellular substrates, and are thus referred to as effector caspases. Another group, consisting of caspase-2, -8, -9 and -10, has long prodomains and are referred to as initiator caspases since they are the first caspases to be activated in response to various apoptotic stimuli (Riedl and Shi, 2004) (Figure 1). The effector caspases exist as catalytically inactive homodimers in the cytoplasm and are activated by proteolytic cleavage, leading to a conformational change in the active site (Riedl and Shi, 2004). The initiator caspases have prodomains that are of two types: the caspase activation recruitment domain (CARD), found in caspase-2 and -9, and the death effector domain (DED) found in caspase-8 and -10. For activation the initiator caspase is recruited to a multiprotein complex, called the death inducing signaling complex (DISC), through homotypic interaction with adaptor proteins containing similar CARD or DED domains. Initiator caspases are also cleaved upon activation, similarly to the effector caspases. However, recent data indicate that cleavage is neither required nor sufficient for their activation. The zymogens of the initiator caspases exists as inactive monomers in the cytoplasm, and dimerization, occurring at the activating multiprotein complexes, is required for them to assume an active conformation, while the cleavage seem to have a stabilizing function in the active initiator caspase (Boatright and Salvesen, 2003).

Different types of apoptotic stimuli lead to the formation of different types of caspase-activating complexes. Caspase-8 and 10 are activated in the DISC complex formed by death receptor (DR) stimulation (Kischkel et al., 1995, Muzio et al., 1996, Medema et al., 1997, Kischkel et al., 2001, Sprick et al., 2002). Caspase-9 activation occurs in the apoptosome (Li et al., 1997, Zou et al., 1999) downstream of apoptotic signaling to the mitochondria. Caspase-2 is required for stress induced apoptosis and acts upstream of the mitochondria (Lassus et al., 2002, Robertson et al., 2002). A multiprotein complex initiating the activation of caspase-2 was recently described, and named the PIDDosome, since it contains the p53-inducible protein with a death domain DD (PIDD), together with the receptor associated ICH-1 like protein with death domain (RAIDD) (Tinel and Tschopp, 2004).

1.2 PATHWAYS FOR APOPTOSIS INDUCTION

Two major pathways for apoptosis induction exist, the death receptor pathway, often referred to as the extrinsic pathway, and the mitochondrial pathway, often called the intrinsic pathway (Figure 2).

1.2.1 Death receptor-mediated apoptosis

A subset of tumor necrosis factor receptor (TNF-R) family members, namely TNF-R1, Fas (CD95/APO-1), DR3/TRAMP, TRAIL-R1, TRAIL-R2 and DR6, can transmit cell death signals and are therefore referred to as the death receptors (Schneider and Tschopp, 2000). Members of this family contain one to five cystein-rich repeats in their extracellular domain, and a death domain (DD), which is essential for transduction of the apoptosis signal, in their intracellular domain (Schneider and Tschopp, 2000). Fas is the most studied death receptor (DR) and serves as the model DR. Fas oligomerizes

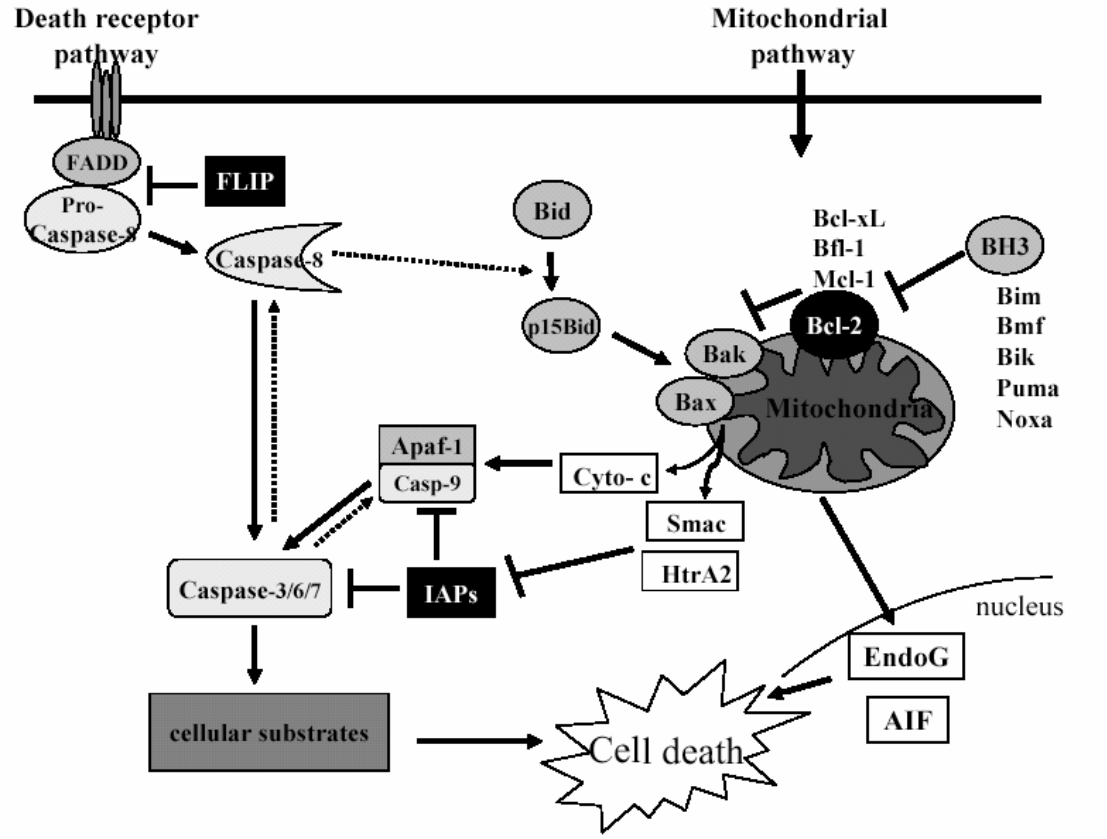

Figure 2. The two main pathways leading to apoptosis induction. The death receptor pathway is triggered by the ligation of cell surface receptors, such as Fas and TRAIL-receptors, leading to activation of caspase-8. The mitochondrial pathway is activated by several cytotoxic stimuli, such as cytokine deprivation, DNA damage and cell detachment, which leads to the release of apoptosis promoting factors from the mitochondria and results in the activation of caspase-9. This pathway is regulated by the Bcl-2 family of proteins. Activated caspase-8 and -9 in turn activate effector caspase-3, -6 and -7. Cross-talk between the pathways occurs through Bid, which is cleaved by caspase-8 and then can activate the mitochondrial pathway. Amplificatin loops where effector caspases can activate the initiator caspases also occur.

upon triggering by Fas ligand (FasL) binding, recruits the adaptor molecule Fas-associated death domain protein (FADD), through homotypic interaction of DDs found in both Fas and FADD, and caspase-8 to form the DISC (Kischkel et al., 1995, Boldin et al., 1996, Muzio et al., 1996). Caspase-10 is recruited to the DISC in a similar manner as caspase-8, although it cannot functionally substitute for caspase-8 (Kischkel et al., 2001, Sprick et al., 2002). Caspase-8 is believed to play an obligatory role in apoptosis initiated by death receptors, whereas the role of caspase-10 remains controversial (Sprick et al., 2001). Activated caspase-8 molecules are released into the cytosol where they can cleave and activate the effector caspases. Caspase-8 also processes the BH3-only Bcl-2 family member Bid, to generate a proapoptotic carboxy-terminal fragment, termed truncated Bid (tBid) (Li et al., 1998, Luo et al., 1998). tBid translocates to the mitochondria were it exerts its proapoptotic activity (Wei et al. 2000) (Figure 2).

Two types of Fas-mediated apoptosis pathways have been described (Scaffidi et al., 1998). In type I cells caspase-8 is recruited to the DISC and activated in sufficient

amount to activate caspase-3. In type II cells formation of DISC is so inefficient that only small quantities of caspase-8 are activated, which are not enough for caspase-3 activation, but sufficient to cleave Bid resulting in the activation of the mitochondrial apoptosis pathway (Kuwana et al., 1998, Li et al., 1998, Luo et al., 1998). As a consequence Fas-induced apoptosis is inhibited by over-expression of antiapoptotic Bcl-2 family members in type II cells but not in type I cells.

For the TNF-related apoptosis-inducing ligand (TRAIL) death receptors, TRAIL-R1 and TRAIL-R2, DISC formation occurs in the same way as for Fas, with the recruitment of FADD and caspase-8 and/or caspase-10 upon binding of the ligand TRAIL (Bodmer et al., 2000, Kischkel et al., 2000, Sprick et al., 2000, Sprick et al., 2002). Events downstream of TRAIL-induced activation of caspase-8 follow a similar pattern to Fas-mediated apoptosis signaling, which is independent or dependent on the mitochondrial activation in type I or type II cells, respectively. However, the regulation of events involved in cytochrome c release downstream of tBid is different in Fas and TRAIL apoptosis signaling (Werner et al., 2002b). In addition, ligation of TRAIL-Rs can also induce activation of caspase-2, which in turn induces cleavage of Bid in type II cells for initiation of apoptosis via the intrinsic pathway (Werner et al., 2004).

1.2.1.1 Special features of the TRAIL/TRAIL-Rs system

For the TRAIL/TRAIL-R system the ligand receptor interactions are more complicated than in the Fas/FasL system. TRAIL binds to five different receptors (Figure 3). Two of these can induce apoptosis, namely TRAIL-R1 (Pan et al., 1997) and TRAIL-R2 (Walczak et al., 1997, Sheridan et al., 1997). TRAIL-R3, on the other hand, lacks the intracellular part and is attached to the cell membrane by a glycophosphatidylinositol linker (Sheridan et al., 1997, Degli-Eposti et al., 1997a, MacFarlane et al., 1997) and TRAIL-R4 has a truncated intracellular domain with a non-functional DD (Degli-Eposti et al., 1997b, Marsters et al., 1997). The fifth receptor for TRAIL is the soluble TNF-R family member osteoprotegrin (OPG) that counteracts TRAIL-induced apoptosis (Emery et al., 1998). The non-apoptosis inducing receptors are often referred to as decoy receptors and their biological role is still unclear. Unlike TNF and FasL, TRAIL appears to have unique selectivity for triggering apoptosis in tumor cells while being non-toxic to normal tissues. TRAIL induces apoptosis in various tumor cell lines *in vitro*, but is less effective in inducing apoptosis in non-transformed cells (Griffith et al., 1998). TRAIL can trigger apoptosis independently through TRAIL-R1 or TRAIL-R2 (Kischkel et al., 2000, Sprick et al., 2000) and in cells where both receptors are present they can form heterocomplexes (Kischkel et al., 2000).

1.2.1.2 Physiological role of TRAIL

The biological role of TRAIL, which is widely expressed in many tissues, is not fully understood, but increasing evidence suggest that this apoptosis-inducing ligand may be an important player in immune surveillance against oncogenic transformation and virally infected cells (Kelley and Ashkenazi, 2004). The ability of TRAIL to trigger apoptosis in a variety of transformed cell lines suggests that it may be a physiological modulator of tumor cell apoptosis (Ashkenazi, 2001, Shankar and Srivastava, 2004).

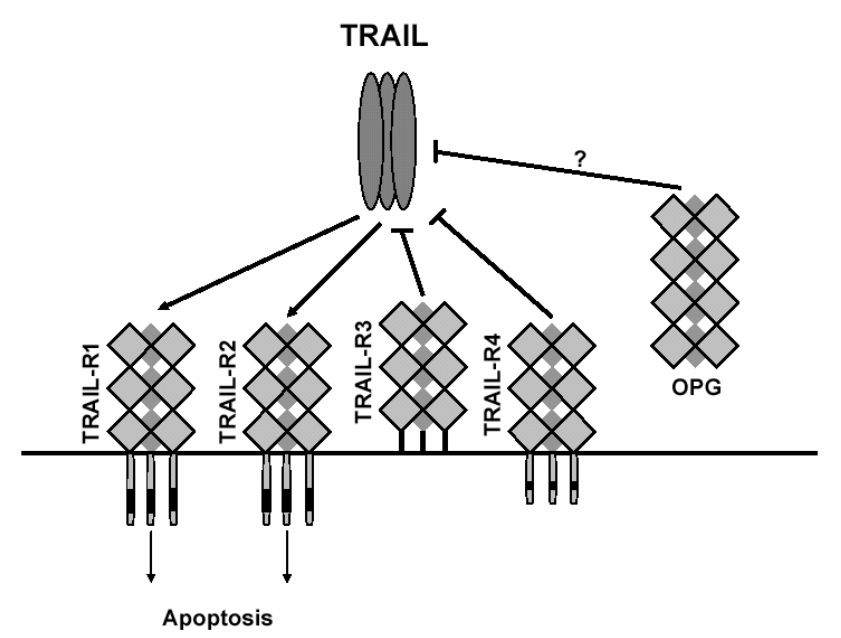

Figure 3. TRAIL and its receptors. TRAIL is a homotrimeric ligand that interacts with four closely related members of the TNF-receptor family. TRAIL-R1 and TRAIL-R2 contain a cytoplasmic death domain and signal apoptosis. TRAIL-R3 is linked to the plasma membrane by a glycophosphatidylinositol moiety and lacks signaling activity. TRAIL-R4 has a truncated, non-functional death domain. Osteoprotegrin (OPG) is a soluble, more distantly related receptor (adapted from Almasan and Ashkenazi, 2003).

There is evidence for involvement of TRAIL in target-killing by cytotoxic $CD4^+$ T cells and NK cells (Kayagaki et al., 1999a, 1999b). Studies with TRAIL gene knockout mice confirm a role for TRAIL in antitumor surveillance by NK cells, specifically in host defense against tumor initiation and metastasis (Takeda et al., 2001, Cretney et al., 2002, Smyth et al., 2003).

TRAIL might play a role in the early phases of IFN-dependent host defense against viral infection. For example, in response to IFNγ, CMV-infected human fibroblasts became sensitive to TRAIL killing, while uninfected neighboring cells up-regulate their TRAIL expression and down-regulate their TRAIL death receptors (Sedger et al., 1999).

1.2.2 The mitochondrial pathway

Many apoptosis inducing signals, including growth factor deprivation, oxidants, Ca^{2+}, oncogene activation, DNA-damage and microtubule attacking drugs, converge on the mitochondria, where proapoptotic members of the Bcl-2 family induce the release of several apoptosis promoting factors from the intermembrane space of the mitochondria, through poorly defined mechanisms.

Caspase activation downstream of the mitochondria is induced by the release of cytochrome c, which binds to Apaf-1, inducing a conformational change in Apaf-1 allowing it to bind caspase-9 through interaction of their respective CARD domains in

the presence of dATP/ATP (Figure 2). Binding of ATP/dATP is proposed to cause a conformational change facilitating heptamer assembly in the shape of a wheel known as the apoptosome (Hill et al., 2003).

Other apoptosis promoting factors released from the intermembrane space of the mitochondria are Smac/DIABLO, Omi/HtrA2, endonuclease G and apoptosis inducing factor (AIF). Smac/DIABLO and Omi/HtrA2 released from the mitochondria promote caspase activity by binding to the inhibitors of apoptosis protein (IAP) family, thereby antagonizing IAP inhibition of caspases (Du et al., 2000, Verhagen et al., 2000, Suzuki et al., 2001). Upon release of endonuclease G and AIF from the mitochondrial intermembrane space, they translocate into the nucleus where they perform DNA degradation in a caspase-independent manner (Li et al., 2001, Susin et al., 1999).

1.3 APOPTOSIS REGULATORS

1.3.1 FLIP

Although several isoforms of FLICE-inhibitory protein (FLIP) mRNA have been described, only two of them, $FLIP_S$ and $FLIP_L$ have been significantly studied at the protein level. $FLIP_L$, shares extensive amino acid sequence similarity with caspase-8 and -10 and features two DEDs at the N-terminus and one caspase-like domain at the C-terminus (see Figure 1). The caspase-like domain lacks enzymatic activity because it lacks the critical residues required for protease activity, including the catalytic cystein (Irmler et al., 1997). Similar to caspase-8, $FLIP_L$ can be cleaved at a conserved aspartic acid cleavage site between the large and small caspase subunits (Srinivasula et al., 1997, Irmler et al., 1997). $FLIP_S$ on the other hand lacks the caspase-like domains and is composed of the N-terminal DEDs and short C-terminal stretch of amino acids not found in $FLIP_L$ (Irmler et al., 1998). FLIP mRNA is widely expressed and high levels of $FLIP_L$ protein occur in heart, skeletal muscle, lymphoid tissues and kidney. $FLIP_S$ expression is more restricted and is characterized by high protein levels in lymphoid tissues (Rasper et al., 1998).

Both $FLIP_S$ and $FLIP_L$ can be recruited to the DISC, via homotypic DED interactions to FADD, where they block the procaspase-8 activation and protect cells from death receptor mediated apoptosis. However they function differently. $FLIP_S$ prevents the initial cleavage step of procaspase-8, while $FLIP_L$ allows the first cleavage step, releasing the small caspase subunit of caspase-8, but inhibits the second cleavage between the large caspase subunit and the DED domains. (Scaffidi et al., 1999, Kreuger et al., 2001). Whereas the antiapoptotic function of $FLIP_S$ is undisputed, there have been reports on proapoptotic as well as antiapoptotic effect of $FLIP_L$. The function of $FLIP_L$ depends on its concentration. At very low levels $FLIP_L$ can promote caspase-8 activation upon DR-ligation, whereas higher levels prevent apoptosis. At very high non-physiological levels $FLIP_L$ becomes cytotoxic on its own (Chang et al., 2002)

Over-expression of FLIP has been shown to activate the transcription factor NFκB and might therefore have a role in the regulation of NFκB-dependent gene expression, which could affect cellular proliferation in response to stimulation of death receptors (Chaudhary et al., 1999, 2000, Hu et al., 2000, Kataoka et al., 2000).

Several tumor types have inappropriately elevated levels of FLIP, e.g. melanoma, colon carcinoma and Hodgkin lymphoma (Roth and Reed, 2004). High expression of FLIP in tumor cells could lead to the resistance to apoptosis induction by death ligand-expressing cytotoxic lymphocytes and has been shown to promote tumor growth and facilitate immune escape of tumors (Medema et al., 1999, Djerbi et al., 1999). Resistance to TRAIL has been correlated with constitutive, increased FLIP expression in primary and transformed cells (Griffiths et al., 1998, Kim et al., 2000, Leverkus et al., 2000).

1.3.2 IAPs

The IAP family can suppress apoptosis by interacting with, and inhibiting the enzymatic activity of caspases (Deveraux and Reed, 1999). IAPs have also been implicated in cell division, cell cycle progression and signal transduction (Schimmer, 2004). So far eight human IAPs have been identified, including XIAP, cIAP1, cIAP2, and survivin (Schimmer, 2004). All IAP proteins contain so-called baculovirus IAP repeats (BIR), which are implicated in their function (Deveraux and Reed, 1999).

XIAP inhibits caspase-3, -7 and -9 but does not bind caspase-8 and is the most potent inhibitor of caspase-3 and -7 *in vitro*, while cIAP1 and cIAP2 have weaker activity (Deveraux et al., 1997, Roy et al., 1997). XIAP can bind to and inhibit the actions of activated effector caspases (Sun et al., 1999), and prevent the activation of caspase-9, which is not seen for cIAP1 and cIAP2 (Schimmer et al., 2004).

Survivin, which contain only a single BIR domain, is preferentially expressed in fetal tissues, suggesting that it plays a role in development (Ambrosini et al., 1997). Survivin is frequently expressed in a variety of malignancies including adenocarcinomas of the lung, pancreas, colon, breast and prostate (Ambrosini et al., 1999). There is conflicting data on the ability of survivin to inhibit caspase-activity and the mechanisms of its actions are not clear (Schimmer et al., 2004).

1.3.3 Bcl-2 family

Proteins of the Bcl-2 family are central regulators of apoptosis and are thought to act primarily on the mitochondria (Gross et al., 1999, Tsujimoto et al., 2003). The family comprises proteins with cell death inhibiting and cell death-promoting activity. They are characterized by the presence of conserved sequence motifs, known as Bcl-2 homology (BH) domains. Anti-apoptotic members share all four BH domains, designated as BH1-4. Proapoptotic members can be divided in two subgroups; the multidomain (Bax/Bak-like) pro-apoptotic members, which contain BH1-3 domains and closely resemble the antiapoptotic members in structure, and the BH3-only pro-apoptotic proteins, which are largely unrelated in sequence to Bcl-2 or each other except for the BH3 domain, which is essential for their proapoptotic activity. Most Bcl-2 family members contain a C-terminal transmembrane domain that targets them to intracellular membranes (Gross et al., 1999, Cory et al., 2003). The members of the different subgroups are shown in table 1.

Table 1. Bcl-2 family members

Antiapoptotic	**Multidomain proapoptotic**	**BH3-only proapoptotic**
Bcl-2	Bax	Bad
Bcl-x_L	Bak	Bik/Nbk
Bcl-w	Bok/Mtd	Blk
Mcl-1	Bcl-x_S	Bid
Bfl-1/A1		Hrk/DP5
Boo/Diva/Bcl-2-L10		Bim/Bod
		Bmf
		Noxa
		Puma/Bbc-3

How the Bcl-2 family regulates apoptosis is still controversial. An appropriate balance between the levels of prosurvival proteins and their BH3-only antagonist proteins is required for control of the balance between survival and cell death. Their activity on the mitochondria regulating the release of cytochrome c has been mostly studied, but regulation of caspase activation upstream of the mitochondria has also been suggested (Cory et al., 2003).

1.3.3.1 Bax/Bak

Genetic studies have demonstrated that Bax and Bak act in a redundant manner and are absolutely essential for intrinsic cell death signaling (Lindsten et al., 2000, Wei et al., 2001) and required for BH3-only proteins to induce apoptosis (Zong et al., 2001, Cheng et al., 2001). Inactive Bax resides in the cytosol or is loosely attached to the membranes and its pocket is occupied by its C-terminal helix (Wolter et al., 1997, Suzuki 2000). Upon receiving a death signal Bax changes conformation and inserts into the mitochondrial outer membrane as homooligomerized multimers (Wolter et al., 1997, Deshager et al., 1999). Inactive Bak, on the other hand, resides at the mitochondria and also undergoes an allosteric conformational activation and oligomerization in response to death signals (Griffiths et al., 1999).

Different models for how Bax/Bak mediate the release of apoptosis-promoting factors from the mitochondrial intermembrane space have been suggested, including direct pore formation, interaction with integral mitochondrial proteins and formation of large lipidic pores (Green and Kroemer, 2004).

1.3.3.2 Function of the antiapoptotic Bcl-2 family members.

How exactly Bcl-2 and its anti-apoptotic homologues promote survival is not clear (Cory et al., 2003), but mouse genetic studies indicate that the survival of every cell type requires protection by at least one Bcl-2 homolog (Cory et al., 2003, Ranger et al., 2001). The BH1-3 domains of the antiapoptotic Bcl-2 members form a hydrophobic groove through which they can bind the BH3 domain of the proapoptotic members (Muchmore et al., 1996, Petros et al., 2001). It is believed that heterodimerization between pro- and antiapoptotic Bcl-2 family members regulates their respective function. Bcl-2 appears to block the integration and aggregation of Bax/Bak in the outer mitochondrial membrane (Antonsson et al., 2001, Nechushtan et al., 2001). This might

be counteracted by BH3-only proteins that upon apoptotic stimuli will translocate to the mitochondria, bind to antiapoptotic members of the Bcl-2 family, which will in some way facilitate the aggregation of multidomain proapoptotic members (Bouillet and Strasser, 2002). An alternative model suggests that Bcl-2 proteins bind to BH3-only proteins at either the mitochondrial or the endoplasmatic reticulum, and inhibit them form activating Bax/Bak and inducing apoptosis, although only a few BH3-only proteins have been found to directly activate Bax or directly affect the mitochondria (Grinberg, et al., 2002, Sugiyama et al., 2002, Letai et al., 2003, Thomenius and Distelhorst, 2003). These two alternative models are shown in Figure 4.

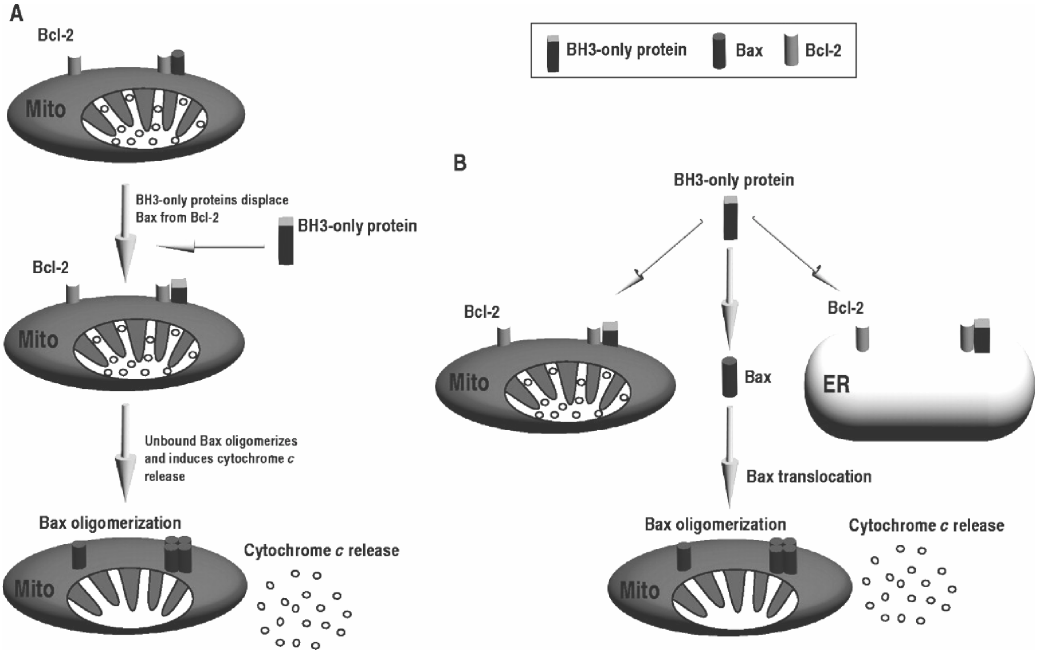

Figure 4. Alternative models of Bcl-2 family interactions. A: antiapoptotic Bcl-2 family members bind Bax/Bak-like proapoptotic proteins, preventing them from inducing cytochrome *c* release. BH3-only proteins relieve this inhibition, freeing the Bax/Bak-like proapoptotic family members. B: antiapoptotic Bcl-2 family members bind to BH3-only proteins, thus preventing them from inducing Bax activity and cytochrome *c* release (from Thomenius and Distelhorst, 2003).

1.3.3.3 Bfl-1

Bfl-1, also known as A1 and GRS, is an antiapoptotic member of the Bcl-2 family shown to protect from apoptosis induced by a variety of apoptotic stimuli, including death receptor ligation, DNA damage, cytokine or serum deprivation (Choi et al., 1995, Lin et al., 1993, Karsan et al., 1996, Kenny et al., 1997, D'Sa-Eipper et al., 1996, Lin et al., 1996, Wang et al., 1999, Zhang et al., 2000). Bfl-1 shares highest homology with Bcl-2 in the BH1 and BH2 domains, but also contains a BH3 and BH4 domain not found in the murine homolog A1, while a transmembrane domain is lacking (Choi et al., 1995, Karsan et al., 1996, Lin et al., 1993). Still, Bfl-1 localizes to intracellular membranes and is mainly localized to the mitochondria but also found in the cytoplasm (D'Sa-Eipper et al., 1996, Werner et al., 2002a, Ko et al., 2003).

An alternative splice variant of Bfl-1 has been identified and named Bfl-1S. Inclusion of an extra exon leads to a new C-terminal sequence and frame shift of the last exon

resulting in an early stop codon. Both isoforms protect against apoptotic stimuli, but the mechanism of action might be different since the new C-terminal of Bfl-1S contains a nuclear localization signal and Bfl-1S is mainly found in the nucleus (Ko et al., 2003).

Although the exact mechanisms of Bfl-1-mediated apoptosis inhibition are not clear Bfl-1 has been shown to be able to interact with the proapoptotic protein Bid (Werner et al., 2002a). Some studies have shown Bfl-1 mediated inhibition of the Bid cleavage (Duriez et al., 2000, Ko et al., 2003), while others found inhibition of Bid mediated cytochrome c release from the mitochondria but not of Bid cleavage (Werner et al., 2002a). Interaction with Bax and several BH3-only proteins, such as Bim, Puma and Noxa, has also been reported (Zhang et al., 2000, Chen et al., 2005). Bfl-1 also has functions that are different from those of other Bcl-2 type proteins. Unlike Bcl-2, Bfl-1 can cooperate with the oncogene E1A to provide a potent transforming activity *in vitro* (D'Sa-Eipper et al., 1996). Bfl-1 also lacks the anti-proliferative capacity that has been reported for Bcl-2 (D'Sa-Eipper et al., 1998).

Bfl-1 is a direct transcriptional target of NFκB (Zong et al., 1999), and is inducible by inflammatory stimuli, such as TNFα and IL-1β, in various cell types (Karsan et al. 1996, Moreb and Schweder, 1997). In humans bfl-1 expression is found in various types of hematopoietic cells in the bone marrow, in germinal centers of peripheral lymphoid organs, hematopoietic cells of fetal liver (Jung-Ha et al., 1998), endothelial cells and in smooth muscle cells (Karsan et al., 1996).

Some DNA damaging agents have been shown to induce Bfl-1 in a NFκB dependent manner (Cheng et al., 2000) and increase expression of Bfl-1 has been found in an *in vivo* established vinflunine resistant cell line as well as in *in vitro* established cisplatin resistant cell lines compared to their respective sensitive parental cell lines (Kruczynski et al., 2002, Kim et al, 2004), indicating that elevated expression of Bfl-1 could contribute to development of chemoresistance in tumor cells.

Whereas human Bfl-1 has a more widespread expression pattern the mouse homolog, known as A1, is specifically expressed in hematopoietic cells (Lin et al., 1993). In mouse, four genes exist for A1, three of which (A1-a, -b and -d) encode for highly conserved full length proteins with antiapoptotic activity while a frame shift in A1-c gives rise to a truncated protein (Hatakeyama et al., 1998). Mice deficient for A1-a develop without any apparent abnormalities, but neutrophiles from these mice exhibit accelerated spontaneous apoptosis *in vitro* (Hamazaki et al., 1998) and the mice exhibit a dampened acute inflammatory response (Orlofsky et al., 2002). No effect on apoptotic response of T lymphocytes was observed (Hamazaki et al., 1998), but it was previously reported that peripheral blood mononuclear cells express only A1-b and -d but not A1-a (Hatakeyama et al., 1998). Differential regulation of the various A1 gene has also been observed in macrophages that constitutively express only A1-b and A1-d, but strongly up-regulate A1-a upon inflammatory stimuli (Orlofsky et al., 2002).

In B cells A1 expression is low throughout bone marrow development. Induction of A1 expression correlates with the acquisition of longevity in mature spleenic B cells (Tomayko et al., 1998). Enforced A1 expression protects immature B cells from BCR-

crosslinking induced apoptosis, and CD40 ligation induces A1 in these cells (Kuss et al., 1999, Craxton et al., 2000).

1.3.3.4 BH3-only proteins

The BH3-only proteins connect proximal death signals to the core apoptotic pathway (Huang and Strasser, 2002). The BH3-domain of these proteins is essential for heterodimerization with the antiapoptotic Bcl-2 relatives and for proapoptotic function. Individual BH3-only proteins are expressed only in certain cell types, and some appear to monitor particular subcellular compartments for stress or damage, and/or to respond to specific sets of cytotoxic signals (Cory et al., 2003) (Figure 5).

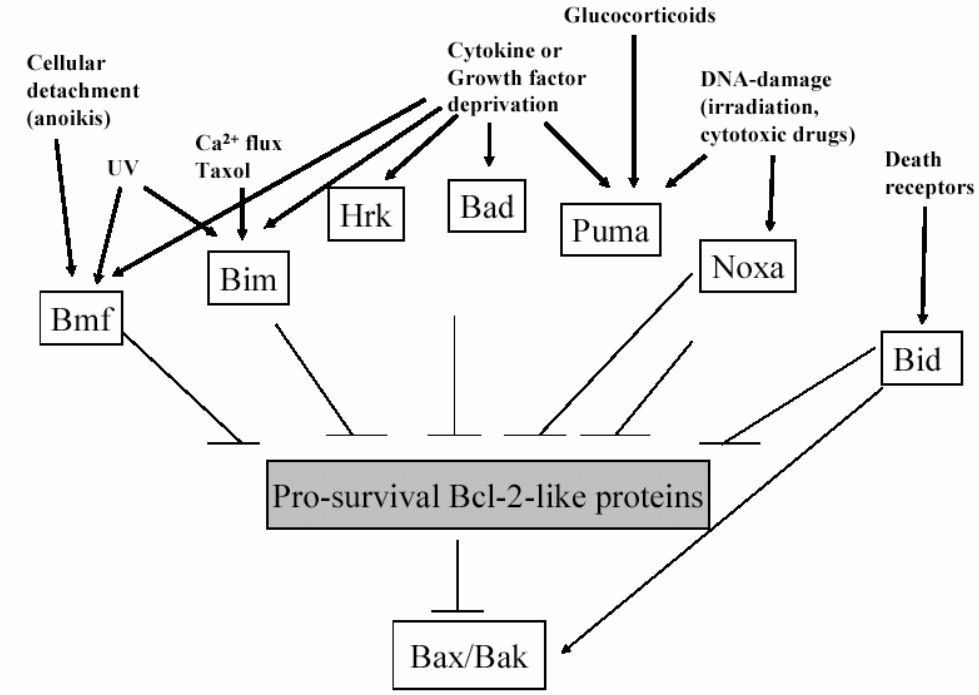

Figure 5. BH-3 only proteins act as damage censors in the cell. Inidvidual BH3-only proteins are activated in response to different types of intracellular damage, in a partly overlapping way. Upon activation they translocate, bind to and inactivate Bcl-2 antiapoptotic proteins or alternatively, as is the case for Bid, activate the Bax/Bak-like proapoptotic proteins.

Individual BH3-only proteins are also activated in different ways. In healthy cells, the proapoptotic activity of BH3-only proteins is kept in check by transcriptional and post-translational mechanisms to prevent inappropriate cell death. Several BH3-only proteins are regulated through transcriptional control. Puma and Noxa are p53 inducible genes (Nakano et al., 2000, Han et al., 2001, Oda et al., 2000), Hrk/dp5 and Bim expression are induced by growth factor deprivation in neurons in a JNK-dependendent way (Harris and Johnson, 2001), while the forkhead transcription factor FKHR-L1 induces Bim in hematopoietic cells upon cytokine withdrawal (Dijkers et al., 2000). At the posttranslational level BH3-protiens can be prevented from performing their apoptosis inducing activities in several ways. Phosphorylation mediates the sequestration of Bad by binding to 14-3-3 proteins (Zha et al., 1996). Upon cytokine stimulation Bad is dephosphorylated and released from 14-3-3 proteins allowing it to

bind to antiapoptotic Bcl-2 proteins. For Bik, on the other hand, phosphorylation seems to have an activating function (Verma et al., 2001). Bim and Bmf activity is controlled by sequestering to cytoskeletal components. Bim binds to the dynein light chain 1 (DLC-1, also known as LC8) of the microtubular dynein motor complex (Puthalakath et al., 1999) (Figure 6) and Bmf is sequestered to the myosin V motor complex by binding to DLC-2 (Puthalakath et al., 2001). Both proteins are released upon specific apoptotic stimuli (Puthalakath et al., 1999, 2001). Full-length Bid is inactive in the cytosol, but upon cleavage by caspase-8 the truncated Bid can be myristoilated, which mediates its translocation to the mitochondria where it can directly activate Bax and Bak (Wei et al., 2000).

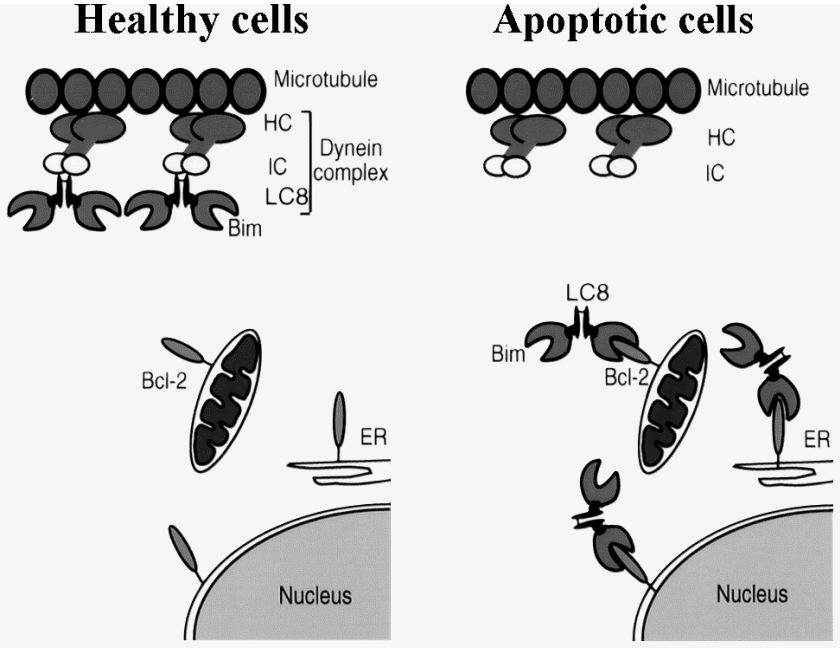

Figure 6. Model for the regulation of Bim. In healthy cells, Bim is bound to LC8 (also known as DLC-1) and thereby sequestered to the microtubule-associated dynein motor complex, which also contains dynein intermediate chains (IC) and dynein heavy chains (HC). Certain apoptotic stimuli cause release of a complex of Bim and LC8. Free Bim, still complexed with LC8, binds to Bcl-2-like antapoptotic proteins, found on intracellular membranes, and thereby neutralizes their ability to counteract apoptosis promotion (adapted from Puthalakath et al., 1999).

There are two types of BH3 domains: Bad-like "sensitizing" and Bid-like "activating". The activating BH3 domain s (e.g. in Bid and Bim) induces oligomerization of Bax and Bak, while the "sensitizing" (e.g. in Bad and Bik) occupy the pocket of antiapoptotic Bcl-2 family members, Bcl-2 and Bcl-xL (Letai et al 2002). Differences among BH3-only proteins in the affinity to different antiapoptotic Bcl-2 family members exist. Some BH3-only proteins, like Bim and Puma, have high affinity for all antiapoptotic members whereas others had selective affinity to some specific antiapoptotic members (Chen et al., 2005). Broad-spectrum binding to antiapoptotic proteins correlates with strong potency to induce apoptosis, whereas those BH3-only proteins that interact with just a few antiapoptotic proteins are weaker inducers of apoptosis. (Chen et al., 2005).

1.3.3.5 Bmf

The BH3-only protein Bmf is normally sequestered to the actin cytoskeleton-based myosinV motor complex, through its interaction with DLC-2. Bmf is released, together with its partner DLC-2 from the myosinV motor complex in response to ultraviolet radiation and loss of adhesion signals usually preceding a distinct form of cell death only observed in fibroblasts, epithelial or endothelial cells, i.e. anoikis, that prevents detached cells from colonizing elsewhere (Puthalakath, et al. 2001). Phosphorylation of Bmf in the DLC-2-binding domain by JNK in response to stress signals has been shown to mediate the release of Bmf (Lei et al., 2003) After release Bmf translocates and binds to the antiapoptotic Bcl-2 proteins, Mcl-1, Bcl-2, Bcl-xL and Bcl-w to counteract their function (Puthalakath et al., 2001).

Bmf mRNA has been found in several cell lines of B- and T-lymphoid, myeloid, or fibroblast origin. Bmf proteins has been detected in many mouse organs, with abundant expression in pancreas, liver, kidney and hematopoietic tissues (Puthalakath et al., 2001) In hematopoietic cells, Bmf has been implicated in cytokine withdrawal-induced cell death of granulocytes where the protein was reported to accumulate during *in vitro* culture (Villunger et al, 2003). Studies of Bmf deficient mice indicate a role for Bmf in blood vessel regression in the vitreus of the eye in new-born animals, as well as in controlling the homeostasis in the hematopoietic system (Villunger, unpublished observations). After 12 month, Bmf-deficient mice display enlarged spleens with predominantly increased B, but also elevated T cell numbers (Villunger, unpublished observations).

2 APOPTOSIS IN CANCER

Deregulation of apoptosis can lead to severe pathological conditions. Excessive apoptotic elimination of cells may result in degenerative disease and immuno-deficiency, while abnormal survival of cells that should be killed may lead to autoimmunity, tumorigenesis, tumor progression and failure of treatment of human cancers. Defective apoptosis in tumor cells contributes to the survival of cells beyond intended lifespan, allowing time for accumulation of genetic alterations that deregulate cell proliferation, interfere with differentiation, promote angiogenesis and increase cell motility and invasiveness during tumor progression. Defective apoptosis also leads to resistance to the immune system since CTL and NK killing of tumor cells depend on functional apoptotic machinery (Reed, 2003, Johnston et al., 2002).

Disruption in the mitochondrial pathway is extremely common in cancer cells. The p53 tumor suppressor gene is the most frequently mutated gene in human tumors and antiapoptotic Bcl-2 members are over-expressed in a variety of tumors, while proapoptotic Bcl-2 family members are inactivated in certain cancers. Defects of downstream apoptosis regulators are also observed in human cancers, such as silencing of Apaf-1 and IAP over-expression. Tumor cells are often also resistant to death receptor-mediated apoptosis, which could contribute to tumor immune evasion (Reed 2003, Johnston et al., 2002).

Altered expression or mutations of genes encoding key apoptotic proteins can provide cancer cells with both an intrinsic survival advantage and inherent resistance to chemotherapeutic drugs. Since apoptosis defects can promote resistance downstream of the drug-target interaction it is possible that genotoxic agents may induce further genetic mutations owing to damage without death (Johnston et al., 2002).

Many strategies for directly targeting apoptosis regulation as anti-tumor treatment are being pursued preclinically or clinically, including targeting of death receptor pathway, Bcl-2 family members, IAPs, NFκB, Akt/PKB activity and reactivation of p53 (Reed, 2003).

2.1 TRAIL AS A THERAPEUTIC STRATEGY IN CANCER

Chemotherapeutic drugs and radiation used for anti-cancer treatment usually require functional p53, which engages primarily the mitochondrial apoptosis pathway. However many tumors have or develop inactivating mutations of p53 which leads to resistance of tumors to therapy. Most death ligands induce apoptosis independent of p53 status and thus have a potential as complement to conventional chemotherapy and radiation therapy in treating cancer (Almasan and Ashkenazi, 2003).

Administration of TNFα in mice causes a lethal inflammatory response (Lehmann et al., 1987) and FasL causes liver damage and rapid death, when administered systemically in mice (Ogasawara et al., 1993). TRAIL and agonistic antibodies that bind TRAIL receptors, on the other hand, appear to be well tolerated. Several studies have shown anti-tumor effects without toxic side effects of TRAIL in mice. (Walczak et al., 1999, Ashkenazi et al., 1999, Chinnaiyan et al., 2000). TRAIL has also been

shown to be non-toxic in nonhuman primates (Ashkenazi et al., 1999). Some concern about the clinical use of recombinant TRAIL was raised when a study revealed induction of apoptosis in primary human hepatocytes (Jo et al., 2000). However, others have not been able to see the same effect, and different preparations of recombinant TRAIL have been proposed have different effects (Lawrence et al., 2001). Currently a monoclonal agonistic anti-TRAIL-R1 antibody is in clinical trials. Initial reports show no apparent toxicity and some biological activity (Tolcher et al., 2004, Hotte et al., 2004).

Although TRAIL is capable of inducing apoptosis in tumor cells of diverse origin, many tumor cells display intrinsic resistance to TRAIL, despite expressing its receptors, suggesting that TRAIL alone might not be effective for treatment of these cancers. It has been shown *in vitro* and *in vivo* that TRAIL acts in synergy with other chemotherapeutic agents. In this regard, chemotherapeutical drugs, such as, doxorubicine, adriamycine, cisplatin or etoposide, are capable of sensitizing TRAIL-resistant human tumor cells, including breast carcinoma, multiple myeloma and bladder tumor cells (Shankar and Srivastava, 2004).

2.2 MODULATION OF THE ACTIVITY OF BCL-2 FAMILY AS CANCER THERAPY

Several strategies to target the Bcl-2 are being investigated. Antisenese oligo-nucleotides targeting Bcl-2, leading to the degradation of Bcl-2 transcripts are being evaluated in clinical trials. Clinical responses as single agent therapy and/or a chemosensitizing effect have been observed in several hematological malignancies, including B-CLL (Chanan-Khan et al., 2004, Rai et al., 2002). The use of apoptosis inducing BH3-domain peptides and small molecules binding to the BH3-binding pocket of antiapoptotic Bcl-2 members, thereby mimicking the function of BH3-only proteins are also being explored (Shangary and Johnson, 2003).

3 B-CLL

3.1 CLINICAL CHARACTERISTICS AND CLASSIFICATION

3.1.1 Epidemiology and diagnosis

In most western countries B-CLL is the predominant type of leukemia among people aged 50 and older. Median age at diagnosis is 65 years with a male:female ratio of approximately 2:1 (Oscier et al., 2004). Most B-CLL patients are asymptomatic at diagnosis and the disease is detected incidentally by routine full blood count, but it may also present with lymphadenopathy, infections, hemolysis or non-specific symptoms such as weight loss, fatigue due to anemia and night sweats (Oscier et al., 2004). The criteria for diagnosis is lymphocytosis of $>5x10^9$/l, with a light chain restricted monoclonal B cell population with dim immunoglobulin expression and co-expression of CD5, CD19 and CD23 (Zent et al., 2004). Most common sites of involvement are the bone marrow, peripheral blood and lymph nodes (Ferrarini and Chiorazzi, 2004).

3.1.2 Progressive disease

Patients with B-CLL follow a highly variable course. Some remain stable for a long time, without requiring therapy, while others progress rapidly to a more advanced disease and die despite aggressive treatment. Disease progression is defined according to a modification of the criteria by the National Cancer Institute Committee in 1978 (Silver et al., 1978). Patients are considered to have a progressive disease if there is progression during 3 months in disease-related anemia (and Hb < 100g/L), in thrombocytopenia (and platelet count < 100 x 10^9/L) and/or in spleen/liver/lymph node size (evaluated by both clinical examination and computer tomography of the abdomen) and/or in more than a doubling of the blood lymphocyte counts and/or appearance of constitutional symptoms.

3.1.3 Staging systems for prognosis evaluation

Two major systems for staging of the disease, in order to correlate clinical findings with survival times, are used throughout the world, the Rai system (Rai et al., 1975) and the Binet system (Binet et al., 1981). The criteria for both systems are shown in Table 2. One of the major problems with the staging systems is their failure to predict progression of the disease and hence there has been continual effort to identify other prognostic factors in B-CLL.

Table 2. Staging systems for B-CLL.

Rai staging system	
Stage	**Criteria**
Rai 0	lymphocytosis in the blood and bone marrow
Rai I	lymphocytosis and enlarged lymph nodes
Rai II	lymphocytosis and hepatomegaly and/or splenomegaly (with or without engaged nodes)
Rai III	lymphocytosis and anemia (with or without enlarged nodes and organ involvement)
Rai IV	lymphocytosis and thrombocytopenia (with or without enlarged nodes, anemia or organomegaly)

Binet staging system	
Stage	**Criteria**
Binet A	lymphocytosis in the blood and bone marrow and less than tree areas of palpable lymphoid involvement
Binet B	lymphocytosis in the blood and bone marrow and three or more areas of palpable lymphoid involvement
Binet C	lymphocytosis in the blood and bone marrow and anemia (< 10 g/dL Hb) or thrombocytopenia (<100 x 10^9/L platelets)

The Rai staging system has later been simplified to include only three categories of risk: low (stage 0), intermediate (stage I and II) and high (stage III and IV) (Rai, 1987).

3.1.4 New prognostic factors

Due to the heterogeneity in clinical behavior of the disease it is of great importance to identify new prognostic factors that are better in predicting the outcome in B-CLL. Two subgroups of B-CLL with clear difference in survival have been identified based on the presence or absence of somatic hypermutation in the variable region of the rearranged immunoglobulin (IgV) genes (Hamblin et al., 1999, Damle et al., 1999). Cases with somatically mutated IgV genes were found to have a better prognosis than those without mutations in IgV genes, which has also been confirmed in several multivariate analysis studies (Oscier et al., 2002, Kröber et al., 2002). Recent data suggests that the use of particular IgV segments such as VH 3.21 may confer a poor prognosis regardless of mutational status (Tobin et al., 2002).

It has been argued that performing IgV sequencing in routine diagnostic laboratories is difficult, very costly and time consuming. Surrogate markers for IgV mutation status has thus been sought for. Microarray studies have shown that all B-CLL cells share a characteristic gene expression pattern and only the expression of a few genes differ between IgV mutated and unmutated cases (Klein et al., 2001, Rosenwald et al., 2001). One gene found to be differently expressed was ZAP70, which was found in cases with unmutated IgV genes but was absent in cases with mutated IgV genes (Klein et al., 2001). ZAP70 can be easily detected by flow cytometry and concordance with mutational status was high in two studies, 93% and 92% respectively (Crespo et al., 2003, Orchard et al., 2004). However, this correlation was only 77% in another study, but ZAP70 was a better predictor for requirement of therapy than was IgV mutation status (Rassenti et al., 2004).

CD38 has also been suggested as a surrogate marker for the IgV mutation status in B-CLL, with high CD38 expression correlating with unmutated IgV (Damle et al., 1999). However, poor correlation to mutation status has been found in other studies (Hamblin et al., 2000, Hamblin et al., 2002, Kröber et al., 2002), although CD38 may serve as an independent prognostic marker (Ibrahim et al., 2001, Hamblin et al., 2002, Lin et al., 2002). Some concern that the CD38 expression levels can vary over time has been raised (Hamblin et al., 2002, Kröber et al., 2002).

Genomic aberrations have also been shown to have prognostic significance. Chromosome 11q deletions and structural abnormalities in chromosome 17p are associated with short survival and poor response to therapy (Dohner et al 1995, 1997).

3.1.5 Therapy in B-CLL

The disease course is heterogeneous among B-CLL patients. Some patients remain indolent, without requirement for therapy for many years while others rapidly progress to fatal disease. A diagnosis of CLL does not imply the need for therapy. Patients with stable disease are not treated unless symptoms develop or disease progresses (Cheson et al., 1996). There is currently no curative treatment for B-CLL. First-line therapy in B-CLL is the alkylating agent chlorambucil or the purine analog fludarabine (Yee et al., 2004, Redaelli et al., 2004). Overall response rates were reported to be higher for fludarabine than for chlorambucil, although no difference in survival has been noted (Rai et al., 2001). Fludarabine response rates after alkylating agent failure have been reported to be as high as 45% (Redaelli et al., 2004). Other agents used include the alkylating agent cyclophosphamide, the purine analogue cladribine as well as combinations therapies such as CVP (cyclophosphamide, vincristine and prednisone), CHOP (cyclophosphamide, doxorubicine vincristine and prednisone) and CAP (cyclo-phosphamide, adriamycin and prednisone). Combination therapies are typically used in patients who fail to respond to single agent therapy (Redaelli et al., 2004).

Despite high initial response rates to conventional therapy, relapse and subsequent resistance to chemotherapy frequently occur. Hence, new treatment modalities with different mechanisms of action are needed and include monoclonal antibodies and stem cell transplantation. Rituximab is a chimeric monoclonal antibody targeting CD20, which is expressed on both malignant and normal B cells (Liu and O'Brien, 2004). As monotherapy rituximab results in higher response rates in previously untreated than in relapsed patients but CR rates are low (Liu and O'Brien, 2004). The most efficacious use of rituximab is in combination with chemotherapy. The addition of rituximab to fludarabine resulted in higher CR rates than the use of fludarabine alone. Rituximab has also been used to treat complications of B-CLL, including autoimmune hemolytic anemia, cold agglutinin disease, autoimmune thrombocytopenia and pure red cell aplasia (Liu and O'Brien, 2004). Alemtuzumab (Campath-1H) is a humanized rat antibody targeting CD52, which is a small glycoprotein with unknown function expressed on almost all lymphocytes, monocytes, macrophages and eosinophils, but not on erythrocytes, platelets and hematopoietic stem cells (Lundin and Österborg, 2004). Clinical trials have shown that alemtuzumab has important clinical activity in patients with chemotherapy-refractory B-CLL, and is even effective in patients with p53 mutations or deletions. Activity may be further enhanced by the combination with fludarabine. Alemtuzumab is most effective in reducing leukemia counts, bone marrow disease and spleen size and less effective at shrinking bulky lymphadenopathy. In young patients with poor prognosis autologous and allogeneic stem cell transplantations is pursued with the intent to cure (Rizouli and Gribben, 2004).

3.2 BIOLOGY OF B-CLL CELLS

3.2.1 Immunophenotype

B-CLL is characterized by the progressive accumulation of monoclonal B cells. Distinctive phenotypic features are CD5 expression, low levels of surface IgM and absent or low expression of CD79b and CD22, which are involved in signaling from the B cell receptor (BCR) (Oscier et al., 2004). B-CLL cells share with normal B cells the expression of several surface markers, including CD19, CD20, CD24 and CD40 (Oscier et al., 2004). Several activation markers, such as CD23, CD25, CD69 and CD71 and CD27 are also expressed in B-CLL (Damle et al., 2002).

3.2.2 Immunological dysfunction

Dysfunction of the immune system is commonly seen in B-CLL patients with prevalence of both immunosuppression and autoimmunity. Ineffective production of antigen-specific antibodies and antibody mediated autoimmune phenomena are associated with deregulated humoral immunity. Hypogammaglobulinemia is a common and progressive immune defect in patients with B-CLL. Reduced production of Ig by non-leukemic B cells and a reduced number of normal B cells available to produce Ig are observed (Weirda, 2003). T cell defects include overall increase in numbers of both $CD4^+$ and $CD8^+$ T cells (although circulating T cells are far outnumbered by the leukemic B cells), with a disproportionate increase in $CD8^+$ T cells in blood and disproportionate increase in $CD4^+$ T cells in lymph nodes in chemotherapy naïve patients. Reduced responsiveness of T cells is also seen (Weirda, 2003).

3.2.3 The normal counterpart of B-CLL

Previously, B-CLL was believed to derive from naïve $CD5^+$ B cells, but several recent findings have changed the view of the normal counterpart of B-CLL to that of an activated, antigen-experience B cell. First, the finding of somatic hypermutations in the V regions of Ig in approximately half of B-CLL cases (Fais et al., 1998, Damle et al., 1999, Hamblin et al., 1999) indicates that in these cases the malignant cells must have arisen from B cells that have encountered antigen in a germinal center. The lack of mutations in other cases could indicate that malignant cells are descendants of naïve B cells or alternatively from antigen experienced B cells that failed to undergo somatic hypermutations upon antigen encounter, for example as a result of stimulation with a T-independent antigen (Chiorazzi and Ferrarini 2003). Several lines of evidence support that also unmutated B-CLL derive from antigen-experienced cells. There is a biased usage of V genes in both mutational subgroups indicating a selection by antigen (Messmer et al., 2004, Tobin et al., 2004). The gene expression profiles of mutated and unmutated cases are very similar and both subsets have an expression profile most similar to memory B cells (Klein et al., 2001). In both mutation subgroups the B-CLL cells express activation markers, such as CD23, CD25, CD69 and CD71 as well as CD27, which is believed to be a marker for memory B cells, while there is low expression of markers that are generally down-regulated upon cell activation such as CD22, FcγRIIb, CD79b and IgD (Damle et al., 2002). Short telomere lengths and high telomerase activity, specifically in unmutated cases, indicates a considerable replicative history, and more cell divisions in the past of unmutated than in mutated cases (Damle

et al., 2004). Thus the current view is that B-CLL cells, irrespective of IgV mutation status, derive from antigen-experienced cells.

3.2.4 Signaling through the B cell receptor in B-CLL

Stimulation through the BCR may be important for survival and proliferation of B-CLL cells and may contribute to inferior clinical outcome of some patients (Stevenson and Caligaris-Cappio, 2004). Although gene expression profiles are very similar in all B-CLL cases, B-CLL cells that express unmutated IgV genes have a gene expression profile that differs from patients whose IgV genes have undergone somatic hypermutation in that they express higher levels of genes that are induced during activation of blood B cells. This suggests that the unmutated B-CLL subpopulation may have ongoing BCR signaling (Rosenwald et al., 2001). A majority of unmutated B-CLL cells respond to IgM ligation, as measured by phosphorylation of Syk, whereas only approximately one third of mutated cases do (Lanham et al., 2004, Chen et al., 2002). The response to IgM ligation correlates with CD38 expression status in the mutated, but not unmutated, cases (Lanham et al., 2004). Strong correlation also exists between ZAP70 expression and response to IgM ligation (Chen et al., 2002). ZAP70 is a protein tyrosine kinase normally expressed in T cells, contributing to TCR signaling. In B-CLL cells ZAP70 participates in BCR signaling (Chen et al., 2002). Introduction of ZAP70 in $ZAP70^-$ B-CLL cells render them responsive to IgM-ligation (Chen et al., 2004).

3.2.5 The role of the microenvironment in the maintenance of the malignant clone

B-CLL is currently interpreted as an accumulative disorder. The malignant cells relentlessly increase in peripheral organs, bone marrow and peripheral blood presumably because defective apoptosis causes their extended survival. More than 99% of circulating B-CLL cells are in the G0/early G1 phase of the cell cycle and are unresponsive to the exogenous stimuli that favor cell cycle progression of normal B cells (Caligaris-Cappio, 2002). B-CLL cells undergo apoptosis *in vitro* when cultured without supporting stromal cells or combinations of cytokines, indicating that the accumulation of apoptosis resistant cells *in vivo* is supported by the microenvironment. Data indicate that $CD4^+$ T lymphocytes (Granziero et al., 2000, Ghia et al., 2001) and accessory cells, such as bone marrow stromal cells (Lagneaux et al., 1998) and follicular dendritic cells (Pedersen et al., 2002), may be involved in cross-talk between malignant cells and the microenvironment. (Caligaris-Cappio, 2002). A number of cytokines, including IL-4, IFNα, TNFα, IL-8 and IL-13 have been shown to promote the survival of B-CLL cells (Dancescu et al., 1992, Tangy et al., 1997, Francia de Celle et al., 1996).

The proliferation compartment that conceivably feeds the downstream accumulation compartment is represented by focal aggregates of proliferating cells that form so-called pseudo-follicles in lymph nodes and bone marrow. These pseudo-follicles or nodules represent the histopathological hallmark of B-CLL (Caligaris-Cappio, 2001). Pseudo-follicles have scattered aggregates of B-CLL cells, co-expressing Bcl-2 and proliferation markers, and bystander cells such as $CD4^+$, CD40L expressing T cells and

some follicular dendritic cells, which might promote the survival and proliferation of the B-CLL cells.

3.2.6 Apoptosis regulation in B-CLL

Several mechanisms, including various apoptosis-regulating proteins have been described to contribute to the resistance towards apoptosis in B-CLL.

3.2.6.1 Bcl-2 family

Bcl-2 is over-expressed in most B-CLL patients (Schena et al., 1992, Hanada et al., 1994). Translocations involving the Bcl-2 gene are rare in B-CLL and the mechanism for the high Bcl-2 expression has been proposed to be hypomethylation of the Bcl-2 promotor (Hanada et al., 1994). Higher levels of Bcl-2 protein or higher Bcl-2/Bax ratios have been associated with *in vitro* resistance to drug-induced apoptosis, a more aggressive disease, refractoriness to chemotherapy and shorter overall survival (McConkey et al., 1996, Thomas et al., 1996, Aguilar-Santelises et al., 1996, Pepper et al., 1998). Up-regulation or delay in down-regulation of Bcl-2 has also been proposed as mechanisms for cytokine-mediated prevention of B-CLL apoptosis (Tangye and Raison, 1997). However, not all studies have identified an association between Bcl-2 or Bax expression and chemoresistance and/or outcome in B-CLL (Johnston et al., 1997, Robertson et al., 1996, Kitada et al., 1998). Antisense oligonucleotides targeting Bcl-2 transcripts for degradation, induce apoptosis in B-CLL cells, and sensitize them to chlorambucil and fludarabine *in vitro* (Pepper et al., 2001, Auer et al., 2001). Clinical responses were reported from initial clinical trials with Bcl-2 antisense as single agent therapy in relapsed/refractory B-CLL (Rai et al., 2002).

A polymorphism in Bax promoter causing reduced protein expression was found in B-CLL and was associated with disease progression (Saxena et al., 2002). The *in vitro* response to many chemotherapeutic agents has been correlated with the levels of Bax (Bosanquet et al., 2002).

The antiapoptotic Bcl-2 family member Mcl-1 is frequently over-expressed in B-CLL and higher levels of Mcl-1 are associated with a failure to achieve complete remission following chemotherapy (Kitada et al., 1998). Mcl-1 expression levels have been correlated with *in vitro* chlorambucil-induced apoptosis (Johnston 2004). Mcl-1 can be induced by survival promoting CD40-ligation (Kitada et al., 1999). Rituximab-induced apoptosis *in vitro* is associated with down-modulation of Mcl-1 (Byrd et al., 2002).

3.2.6.2 IAPs

XIAP, cIAP1 and cIAP2 are abundantly expressed in B-CLL (Granziero et al., 2001, Munzert et al., 2002), but their importance in regulation of B-CLL apoptosis has been questioned since B-CLL also express high levels of Smac/DIABLO, which is released form the mitochondria upon apoptotic stimuli, counteracting the IAPs (Schliep et al., 2004). Survivin is generally not expressed in peripheral blood B-CLL cells but is found in proliferating cells of the pseudo-follicles, and can be induced by CD40-ligation in a majority of B-CLL cases (Granziero et al., 2001).

3.2.6.3 Fas/FasL system

B-CLL cells express none or low levels of Fas on their surface, although transcripts for both Fas and FasL are commonly detected. Several stimuli have been shown to up-regulate Fas expression, for example IFN and CD40-ligation, but B-CLL cells remained resistant to Fas-mediated apoptosis (Osorio et al., 1998). However, it was found that after an initial resistance to Fas-induced apoptosis, sensitivity to Fas-ligation was achieved upon prolonged CD40-stimulation, concomitant to FLIP down-regulation and up-regulation of FADD (Chu et al., 2002).

Activation of B-CLL cells leads to expression of FasL and to the release of soluble Fas and FasL. Increased levels of soluble Fas are found in B-CLL patients and higher levels are correlated with disease progression and late clinical stage. Soluble Fas has been shown to be able to inhibit FasL-induced apoptosis, which may represent a mechanism to avoid induction of apoptosis by FasL-expressing T cells (Osorio et al., 1998).

3.2.6.4 p53

Mutations of the p53 gene occur in about 10-15% of B-CLL (el Rouby et al., 1993, Wattel et al., 1994, Döhner et al., 1995). Aberrations of the p53 gene is one of the most predictive molecular markers for resistance to first-line therapy and short overall survival in B-CLL. Mutations become more frequent as the disease progresses and predict aggressive disease that will be unresponsive to chemotherapy (Fenaux et al., 1992, Wattel et al., 1994, Döhner et al., 1995, Cordone et al., 1998). Treated patients have an increased frequency of p53 mutations, especially patients treated with alkylating agents (Sturm et al., 2003). Mutated p53 is correlated with reduced *in vitro* sensitivity to γ-irradiation, chlorambucil and fludarabine (Sturm et al., 2003).

3.2.6.5 NFκB

NFκB is a transcription factor that can promote survival through induction of several antiapoptotic proteins. Higher levels of constitutively active NFκB are seen in unstimulated B-CLL cells compared to healthy peripheral blood B cells and are further induced by CD40 stimulation (Romano et al., 1999, Furman et al., 2000). The chemotherapeutic agents, fludarabine, dexamethasone and proteasome inhibitors, which all induce apoptosis in B-CLL cells, also down-regulate NFκB activity (Romano et al., 1999, Furman et al., 2000, Chandra et al., 1998).

4 AIMS OF THE PRESENT STUDY

The aim of this thesis was to characterize apoptosis regulation in B-CLL, with a special focus on characterization of the mechanisms that contribute to apoptosis resistance, and their involvement in disease progression and resistance to chemotherapy. In particular the objectives were:

- To explore the TRAIL death pathway in B-CLL, in terms of TRAIL-receptor expression and response to TRAIL-induced apoptosis.
- To characterize Bmf and its alternative splice variants, and their potential involvement in the regulation of apoptosis in B-CLL.
- To explore the expression profile of apoptosis-regulating genes in B-CLL in relation to disease progression, chemoresistance and apoptosis induction by deprivation of survival signals from the microenvironment.
- To study the role of Bfl-1 in B-CLL apoptosis, disease progression and chemoresistance.

5 PATIENT CRITERIA USED IN THE STUDIES

5.1 B-CLL DIAGNOSIS CRITERIA

The criteria for diagnosis of B-CLL is lymphocytosis of $>5x10^9$/l, with a light chain restricted monoclonal B cell population with dim immunoglobulin expression and co-expression of CD5, CD19 and CD23 (Zent et al., 2004).

5.2 DISEASE PROGRESSION

Patients were considered to have a progressive disease, according to a modification of the criteria by the National Cancer Institute Committee (Silver et al., 1978), if there was progression during the preceding 3 months in disease-related anemia (and Hb < 100g/L), in thrombocytopenia (and platelet count < 100 x 10^9/L) and/or in spleen/liver/lymph node size (evaluated by both clinical examination and computer tomography of the abdomen) and/or in more than a doubling of the blood lymphocyte counts and/or appearance of constitutional symptoms.

5.3 RESPONSE TO CHEMOTHERAPY

Criteria for assessing the response to therapy was defined by the NCI sponsored working group in 1996 as follows (Cheson et al., 1996).
Complete remission (CR), requires all of the following for at least 2 months:

- Absence of lymphadenopathy
- No hepatomegaly or splenomegaly
- Absence of constitutional symptoms.
- Normal complete blood cell count (CBC) as exhibited by: polymorphonuclear leukocytes ≥ 1500/μL, platelets >100000/μL and hemoglobin > 11.0 g/dL.
- <30%lymphocytes and no nodules in the bone marrow.

Partial response (PR) requires for at least 2 months:

- ≥50% decrease in peripheral blood lymphocyte count from pre-treatment baseline value

And

- ≥50% reduction in lymphadenopathy

and/or

- ≥50% reduction in the size of the liver and/or spleen

plus at least one of the following:

- polymorphonuclear leukocytes ≥ 1500/μL or 50% improvement over baseline
- platelets >100000/μL or 50% improvement over baseline
- hemoglobin > 11.0 g/dL or 50% improvement over baseline.

6 RESULTS AND DISCUSSION

6.1 TRAIL-MEDIATED APOPTOSIS IN B-CLL (PAPER I)

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a potent activator of the cell death pathway and exerts tumoricidal activity *in vivo* with minimal toxicity. To investigate the therapeutic potential use of TRAIL in B-CLL, we studied the expression levels of TRAIL-receptors and the *in vitro* response to TRAIL-induced apoptosis in B-CLL cells. mRNA for all TRAIL-receptors were expressed, but on the cell surface TRAIL-R1 and TRAIL-R2 proteins were mainly expressed with only low levels of TRAIL-R3 and TRAIL-R4. TRAIL-R3 protein was highly expressed intracellularly. The significance of this intracellular expression is unclear, but it has been shown that TRAIL-R3 and TRAIL-R4 translocate to the cell surface upon ligation of TRAIL-R1 of TRAIL-R2 (Zhang et al., 2002). Most B-CLL cells were completely resistant to TRAIL induced apoptosis but, a few cases showed a small increase in apoptotic cells after TRAIL treatment, which was weakly correlated with the expression levels of TRAIL-R2.

To further explore the TRAIL death pathway and the regulation of the resistance to TRAIL in B-CLL cells, the transcription inhibitor actinomycin D was used in an attempt to sensitize B-CLL cells to TRAIL-induced apoptosis. Concomitant treatment with actinomycin D and TRAIL synergistically induced apoptosis in B-CLL cells. This was achieved without significant modulation of TRAIL-receptor levels. Instead we found that $FLIP_L$ and $FLIP_S$ were down-regulated by actinomycin D treatment, in correlation with the gain in TRAIL susceptibility. In addition $FLIP_L$ was constitutively expressed at higher levels in B-CLL cells than in normal B cells. These results together suggest that FLIP contributes to the resistance towards TRAIL in B-CLL cells. Others have found $FLIP_L$ but not $FLIP_S$ in the TRAIL-DISC in B-CLL, although only low levels of DISC formation was observed (MacFarlane et al., 2002). The same study found sensitization of B-CLL cells to TRAIL by the protein synthesis inhibitor cyclohexamide, without any apparent effect on the protein levels of FLIP, while the triterpenoid CDDO sensitized B-CLL cell to TRAIL-induced apoptosis, with a concomitant down-modulation of FLIP (Pedersen et al., 2002). However, specific down-modulation of FLIP using antisense treatment failed to induce apoptosis (Pedersen et al., 2002). Taken together, FLIP seems to contribute to the resistance of B-CLL to TRAIL-induced apoptosis but it is not the only factor involved.

Since chemotherapeutic agents have been shown in several cellular systems to sensitize resistant tumor cells to TRAIL (Shankar and Srivastava, 2004), combination therapy with TRAIL and conventional therapy could be an attractive strategy in B-CLL. This potential was explored by Johnston et al., who found that the chemotherapeutic agents, chlorambucil and fludarabine induced TRAIL-R1 and TRAIL-R2, and sensitized B-CLL cells to TRAIL-induced apoptosis. However this only applied to B-CLL cells that were sensitive to apoptosis induced by the drugs (Johnston et al., 2003). In our study, B-CLL samples that were either resistant or sensitive to chlorambucil and fludarabine were included (data not shown). Actinomycin D treatment could sensitize to TRAIL-induced apoptosis irrespective of their response to fludarabine or chlorambucil. Understanding of the mechanism behind actinomycin D sensitization to

TRAIL-induced apoptosis could thus help in finding therapeutic targets for treatment of chemorefractory B-CLL patients, which are in greatest need of new therapeutic modalities.

6.2 BMF SPLICE VARIANTS IN B-CLL (PAPER II)

Bmf is a relatively new proapoptotic protein belonging to the BH3-only group in the Bcl-2 family (Puthalakath et al., 2001). Since relatively little is known about this protein we wanted to investigate its expression levels in B-CLL cells, and its involvement in the regulation of apoptosis in B-CLL.

RT-PCR using bmf specific primers on B-CLL cells amplified several transcripts. After cloning and sequencing of them we found two new splice variants of *bmf*. These novel variants of *bmf*, termed *bmf-II* and *bmf-III*, lacked the functional BH3-domain and bmf-III also had a different C-terminal sequence. Both splice variants retained the DLC-2 binding motif, mediating the sequestration of Bmf to the myosin V actin motor complex. As expected, Bmf-II and Bmf-III lacking the apoptosis inducing BH3-domain, unlike the originally described Bmf protein, could not induce apoptosis when over-expressed *in vitro*. Surprisingly, they provided a survival advantage to these cells, as illustrated by an increase colony formation.

Since Bmf is sequestered to the actin cytoskeleton in non-apoptotic cells, until certain apoptotic stimuli induces its release (Puthalakath et al., 2001), one might envision that enforced expression of the alternative splice variants could promote Bmf-induced apoptosis by competing for binding to the DLC-2 of the myosin V motors. However, instead we observed promotion of survival by these new isoforms. How this is achieved is currently unknown. A hypothetical model could be based on observations of the related BH3-only protein Bim, which is similarly sequestered to cytoskeletal components through the binding to DLC-1 in the dynein motor complex of the microtubules. DLC-1 forms dimers, which can bind two Bim molecules. Upon apoptotic stimuli the complex of two DLC-1 and two Bim molecules is released (Puthatlakath et al., 1999) (see Figure 6). Bmf is similarly released together with DLC-2 (Puthalakath et al., 2001). If DLC-2, which is highly homologous to DLC-1, also forms dimers, then the release of dimers of DCL-2 bound to mixed isoforms of Bmf could have less activating potential. Further studies on the function and interactions of these isoforms on cells expressing physiological levels of individual isoforms and combinations are required to understand the role of these new splice variants.

Bmf and its splice variants were found to be highly expressed in B-CLL and normal tonsil-derived B cells compared to peripheral blood T cells and a number of cell lines of various cellular origin. This could indicate a specific role of Bmf in B cells, which is also supported by the observation that mouse deficient for Bmf develop lymph-adenopathy, with predominantly increased B cells with age (Villunger, unpublished observation). However, an extended study of the expression pattern of Bmf and its splice variant would shed light on this issue.

mRNA expression of the full-length form of bmf was enhanced in B-CLL cells during serum deprivation-induced apoptosis, while bmf-III levels were decreased. Induction of bmf-I was also seen in tonsil-derived B cells and several cell lines during serum deprivation-induced apoptosis, although the concomitant down-modulation of bmf-III was not observed. It might be possible that in B-CLL cells up-regulation of the proapoptotic form of Bmf is not enough to induce apoptosis, but requires the concomitant down-regulation of Bmf-III. These observations implicate that the balance between the individual bmf-isoforms may critically influence the susceptibility of the B-CLL cells to undergo apoptosis. Differences in apoptosis sensitivity of B-CLL cells did not correlate with differences in bmf expression or with the modulation of expression levels during induction of apoptosis. Although our results indicate that Bmf might act as critical initiator of apoptosis, at least in the absence of survival factors, the cell survival decision may be under additional control of other Bcl-2 family proteins, or other apoptosis regulating proteins.

6.3 EXPRESSION PROFILE OF APOPTOSIS-REGULATING GENES IN B-CLL (PAPER III)

Resistance to apoptosis is one mechanism contributing to chemotherapy resistance. In order to identify regulatory genes involved in the development of an apoptosis resistant phenotype in patients with chemotherapy refractory B-CLL expression of apoptosis-regulating genes in B-CLL cells was quantified using cDNA arrays and RT-PCR. Expression profiles were compared between leukemic cells from a patient group with non-progressive, indolent, previously untreated disease, and leukemic cells sensitive to *in vitro* fludarabine-induced apoptosis (sB-CLL), and a group with progressive, chemotherapy refractory disease and leukemic cells resistant to *in vitro* fludarabine-induced apoptosis (rB-CLL).

Supervised hierarchical clustering analysis of apoptosis-regulating gene expression using 8 genes, with more than 1.5 fold difference in median expression between the two B-CLL subgroups resulted in one cluster with 7 of 8 sB-CLL cases and one cluster with all the rB-CLL cases and one sB-CLL case. The bcl-2 family genes, bfl-1, bcl-2 and mcl-1, in accordance with their anti-apoptotic function, appeared in the same cluster, with higher expression in the rB-CLL as compared to the sB-CLL group. Within the rB-CLL group, although the number of patients in our study is small, we observed that patients with lower bfl-1 expression had high bcl-2 expression, so that bfl-1 and bcl-2 expression seems to be complementary to each other. Both bcl-2 and mcl-1 has been reported to contribute to disease progression and/or chemotherapy response (Aguilar-Santelises et al., 1996, McConkey et al., 1996, Pepper et al., 1998, Kitada et al., 1998), but all studies have not been able to find such a correlation (Johnston et al., 1997, Roberson et al., 1996). The results of our study indicate that combined expression levels of bfl-1, bcl-2 and mcl-1 should be considered, which is not surprising since they are functional homologues and could perform the same protective function.

Caspase-1, caspase-4 and caspase-5 were also expressed at higher levels in the rB-CLL group. The primary role of these caspases is not in apoptosis transduction, but in the maturation of some pro-inflammatory cytokines such as IL-1β and IL-18 (Martinon F and Tschopp J, 2004), and their role in B-CLL is currently unknown. Lower expression

of TRAF3 and hus1 were found in rB-CLL. TRAF3 has been implicated in apoptosis signaling from the lymphotoxin-β-receptor (VanArsdale et al., 1997). hus1, in complex with rad9 and rad1, is involved in the DNA damage cell cycle check point and has been suggested as a tumor suppressor gene (Weiss et al., 2000). Low expression hus1 could thus be involved in the chemoresistance in the rB-CLL group.

As the products of the genes identified here correlate with the development of a highly apoptosis resistant phenotype in B-CLL they may represent potential targets for future drug development.

Despite their prolonged survival *in vivo*, most B-CLL cells undergo spontaneous apoptosis when cultured *in vitro* under suboptimal conditions suggesting a dependence of the leukemic cells on the microenvironment (Caligaris-Cappio et al., 2002), where humoral factors and/or cellular interactions provide survival signals to the tumor cells. Therefore we also investigated how apoptosis-regulating genes are modulated during deprivation of survival factors by *in vitro* culture. A homogeneous pattern of gene expression modulation was seen irrespective of B-CLL subgroup. This modulation included several members of the Bcl-2 family. These changes involved the down-modulation of the antiapoptotic gene bfl-1 and induction of the proapoptotic members bax, bmf and bid, while, on the contrary, proapoptotic bim was reduced. Caspase-2, which has been implicated in stress-induced apoptosis (Lassos et al., 2002, Robertson et al., 2002) was also induced. Similar to the observations in **paper I**, expression levels of FLIP, which confers protection against death-receptor mediated apoptosis (Irmler et al., 1997), were increased. The relevance of FLIP up-regulation during serum deprivation-induced apoptosis is unclear since FLIP has been found not to protect from apoptosis induced by growth factor deprivation or DNA damage. Bfl-1 was the only gene differently modulated between sB-CLL and rB-CLL, being more strongly down-regulated in the sB-CLL group.

Taken together these changes shift the balance between pro- and antiapoptotic Bcl-2 family members towards apoptosis promotion, which might be potentiated by the increased levels of caspase-2, suggesting that these genes are controlled in B-CLL cells by survival factors *in vivo*. Understanding of how interactions within the microenvironment control the expression of these genes could identify new targets for therapeutic intervention.

6.4 ROLE OF BFL-1 IN B-CLL APOPTOSIS AND CHEMORESISTANCE (PAPERS III AND IV)

Since the function of bfl-1 in B-CLL has not previously been studied in detail and we found it, in **paper III,** to be the gene most differently expressed between apoptosis sensitive and resistant B-CLL samples in the gene expression profiling, we wanted to further characterize its role in B-CLL.

In **paper III** bfl-1 was found as the most discriminating gene between a group with non-progressive, indolent, previously untreated disease and a group with progressive, chemotherapy refractory disease. To determine if increased bfl-1 expression is related to the natural disease course or the effect of chemotherapy, in **paper IV**, bfl-1

expression was determined by competitive PCR in a group of 38 patients, including all stages of disease, progressive and non-progressive patients, treated and untreated and responding or not to chemotherapy. Bfl-1 expression was significantly correlated with failure to respond to chemotherapy, whereas no significant difference was seen between progressive and non-progressive patients, indicating a role of bfl-1 in chemoresistance in B-CLL.

The groups in **paper III** also included as criteria for patient selection, the *in vitro* response to fludarabine-induced apoptosis. The expression of bfl-1 was higher in the resistant group, than in the sensitive group. This was confirmed in the larger group of patients studied in **paper IV**. In both papers a few samples (from 3 patients) were resistant to fludarabine-induced apoptosis without expressing high levels of bfl-1, indicating that the resistance might be due to other factors, such as bcl-2 or defective p53, both of which have been associated with resistance to drug-induced apoptosis and poor response to therapy in B-CLL (McConkey et al., 1996, Pepper et al., 1998, Wattel et al., 1994, Döhner et al., 1995, Cordone et al., 1998). Conversely, a few cases (3 patients) were found to be sensitive to fludarabine-induced apoptosis in spite of high levels of bfl-1. The one fludarabine-sensitive patient in **paper III** that expressed high levels of bfl-1, lost this expression during *in vitro* culture, while resistant cells did not.

Bfl-1 expression levels did not correlate with *in vitro* chlorambucil-induced apoptosis. This was somewhat surprising since most of the treated patients had received chlorambucil, and we found a correlation between no response to chemotherapy and high expression levels of bfl-1. One explanation might be that most of the patients were given chlorambucil together with glucocorticoids, while the *in vitro* induction of apoptosis was explored with chlorambucil alone.

Several studies have shown modulation of gene expression by fludarabine treatment in B-CLL. (Kitada et al., 1998, Plate et al., 2000, Rosenwald et al., 2004) and bfl-1 has been found to be up-regulated by DNA damaging agents in several cell lines (Cheng et al., 2000, Kim et al., 2004). We could not, however, see any particular modulation of bfl-1 expression by fludarabine treatment of B-CLL cells *in vitro*. This does not exclude the possibility of long-term effects on the bfl-1 expression of chemotherapy treatment of B-CLL patients, for example through selective killing of malignant cells with lower bfl-1 expression. Prospective studies are required to determine if bfl-1 is a predictive factor for the outcome of chemotherapy or if modulation of its expression is part of the development of chemoresistance.

In **paper III** we found that bfl-1 expression levels are decreased in apoptosis sensitive cells during spontaneous apoptosis *in vitro*. In **paper IV,** we selectively down-modulated bfl-1, using specific siRNA, in fludarabine resistant, bfl-1 high-expressing B-CLL cells, which resulted in induction of apoptosis, showing that bfl-1 has a protective role against apoptosis in these cells. CD40L, known to promote survival of B-CLL cells *in vivo*, has been shown to induce the expression of bfl-1 in B-CLL cells, protecting them from spontaneous and fludarabine-induced apoptosis *in vitro* (Kater et al., 2004). This, together with our results suggests that bfl-1 may be important for the extended survival of the leukemic cells *in vivo*.

7 CONCLUSIONS AND FUTURE PERSPECTIVES

The studies in this thesis have been focused on characterization on apoptosis regulation in B-CLL. We have studied mechanisms that contribute to apoptosis resistance, and their involvement in disease progression and resistance to chemotherapy.

TRAIL-induced apoptosis in B-CLL

B-CLL cells are relatively resistant to *in vitro* induction of apoptosis by TRAIL, despite expressing the TRAIL death receptors. Treatment with actinomycin D sensitizes the B-CLL cells to TRAIL-induced apoptosis, which is not due to modulation of TRAIL receptors. Down-regulation of $FLIP_L$ and $FLIP_S$ explains, at least in part, the actinomycin D-mediated sensitization of B-CLL cells to TRAIL-induced apoptosis. Constitutive $FLIP_L$ expression levels in B-CLL cells are significantly elevated compared to expression levels in normal B cells. Our findings suggest the involvement of FLIP and possibly other pro- or anti-apoptotic proteins in regulating TRAIL-mediated apoptosis in B-CLL, which could have implications for effective combined treatment regimes that enhance the apoptotic response of B-CLL cells.

Bmf splice variants in B-CLL

Two new splice variants of bmf are described here. These novel variants of bmf, termed bmf-II and bmf-III, lack a functional BH3-domain and, unlike the originally described Bmf protein, cannot induce apoptosis when over-expressed *in vitro* but can instead provide a survival advantage. During growth factor withdrawal-induced apoptosis in B-CLL cells, transcription of the bmf-isoforms are differently regulated, with a selective increase of the proapoptotic variant, while the non-apoptotic promoting bmf-III variant is decreased. These data suggest that the balance between the individual bmf-isoforms may critically influence the susceptibility of B-CLL cells to undergo apoptosis and that survival signals *in vivo* could promote survival of B-CLL cells by controlling the transcription of bmf.

Further characterization of the function of bmf and its splice variants is required to understand the biological role of this protein and the relevance for B-CLL. This should include detailed analysis of the expression pattern of bmf splice variants in normal tissues and studies on the function and interaction of these isoforms in cells expressing physiological levels of individual isoforms and combinations thereof. The role of bmf isoforms in tumorigenesis models might also be important to study.

Role of bfl-1 in chemoresistance in B-CLL

Expression analysis of apoptosis-regulating genes revealed that the antiapoptotic Bcl-2 family members, bfl-1, bcl-2 and mcl-1, are implicated in the regulation of B-CLL apoptosis and chemoresistance. Bfl-1 is the strongest discriminating gene between resistant and sensitive cells. High bfl-1 mRNA expression is correlated with chemotherapy refractoriness of B-CLL and resistance to *in vitro* fludarabine-induced apoptosis, but is not correlated with progression of the disease. Specific down-modulation of bfl-1 expression by RNA interference induces apoptosis of resistant B-CLL cells. Taken together these data indicate that up-regulation of bfl-1 expression

might be a mechanism of chemoresistance in B-CLL and that bfl-1 could serve as one potential therapeutic target in B-CLL.

Prospective studies should be performed to clarify if bfl-1 could serve as a predictor of therapy outcome. In addition, the potential of synergistic killing of fludarabine and bfl-1 targeting siRNA should be further explored *in vitro*. The function of bfl-1 on the protein level in B-CLL, should be defined.

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9 REFERENCES

Aguilar-Santelises M, Rottenberg ME, Lewin N, Mellstedt H and Jondal M. 1996. Bcl-2, Bax and p53 expression in B-CLL in relation to *in vitro* survival and clinical progression. Int J Cancer. 69: 114-9.

Almasan A, Ashkenazi A. 2003. Apo2L/TRAIL: apoptosis signaling, biology, and potential for cancer therapy.Cytokine Growth Factor Rev. 14: 337-48.

Alnemri ES, Livingston DJ, Nicholson DW, Salvesen G, Thornberry NA, Wong WW, Yuan J. 1996. Human ICE/CED-3 protease nomenclature. Cell. 87: 171.

Ambrosini G, Adida C, Altieri DC. 1997. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. Nat Med. 3: 917-21.

Antonsson B, Montessuit S, Sanchez B, Martinou JC. 2001. Bax is present as a high molecular weight oligomer/complex in the mitochondrial membrane of apoptotic cells. J Biol Chem. 276: 11615-23.

Ashkenazi A, Pai RC, Fong S, Leung S, Lawrence DA, Marsters SA, Blackie C, Chang L, McMurtrey AE, Hebert A, DeForge L, Koumenis IL, Lewis D, Harris L, Bussiere J, Koeppen H, Shahrokh Z, Schwall RH. 1999. Safety and antitumor activity of recombinant soluble Apo2 ligand. J Clin Invest. 104: 155-62.

Auer R, Corbo M: C F: 2001. Bcl-2 antisense (Genasense) induces apoptosis and potentiates activity of both cytotoxic chemotherapy and rituximab in primary CLL cells. Blood, 98:808a.

Binet JL, Auquier A, Dighiero G, Chastang C, Piguet H, Goasguen J, Vaugier G, Potron G, Colona P, Oberling F, Thomas M, Tchernia G, Jacquillat C, Boivin P, Lesty C, Duault MT, Monconduit M, Belabbes S, Gremy F. 1981. A new prognostic classification of chronic lymphocytic leukemia derived from a multivariate survival analysis. Cancer. 48: 198-206.

Boatright KM, Salvesen GS. 2003. Mechanisms of caspase activation. Curr Opin Cell Biol. 15: 725-31.

Bodmer JL, Holler N, Reynard S, Vinciguerra P, Schneider P, Juo P, Blenis J, Tschopp J. 2000. TRAIL receptor-2 signals apoptosis through FADD and caspase-8. Nat Cell Biol. 2: 241-3.

Boldin MP, Goncharov TM, Goltsev YV and Wallach D. 1996. Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. Cell, 85: 803–815.

Bosanquet AG, Sturm I, Wieder T, Essmann F, Bosanquet MI, Head DJ, Dorken B, Daniel PT. 2002. Bax expression correlates with cellular drug sensitivity to doxorubicin, cyclophosphamide and chlorambucil but not fludarabine, cladribine or corticosteroids in B cell chronic lymphocytic leukemia. Leukemia. 16: 1035-44.

Bouillet P, Purton JF, Godfrey DI, Zhang LC, Coultas L, Puthalakath H, Pellegrini M, Cory S, Adams JM, Strasser A. 2002. BH3-only Bcl-2 family member Bim is required for apoptosis of autoreactive thymocytes. Nature. 415: 922-6.

Bouillet P, Strasser A. 2002. BH3-only proteins - evolutionarily conserved proapoptotic Bcl-2 family members essential for initiating programmed cell death. J Cell Sci. : 115: 1567-74

Byrd JC, Kitada S, Flinn IW, Aron JL, Pearson M, Lucas D, Reed JC. 2002. The mechanism of tumor cell clearance by rituximab *in vivo* in patients with B-cell chronic lymphocytic leukemia: evidence of caspase activation and apoptosis induction. Blood. 99: 1038-43.

Caligaris-Cappio F, Cignetti A, Granziero L, Ghia P. 2002. Chronic lymphocytic leukaemia: a model for investigating potential new targets for the therapy of indolent lymphomas. Best Pract Res Clin Haematol. 15: 563-75.

Chanan-Khan A. 2004. Bcl-2 antisense therapy in hematologic malignancies. Curr Opin Oncol. 16: 581-5.

Chandra J, Niemer I, Gilbreath J, Kliche KO, Andreeff M, Freireich EJ, Keating M, McConkey DJ. 1998. Proteasome inhibitors induce apoptosis in glucocorticoid-resistant chronic lymphocytic leukemic lymphocytes. Blood. 92: 4220-9.

Chang DW, Xing Z, Pan Y, Algeciras-Schimnich A, Barnhart BC, Yaish-Ohad S, Peter ME, Yang X. 2002. c-FLIP(L) is a dual function regulator for caspase-8 activation and CD95-mediated apoptosis. EMBO J. 21: 3704-14.

Chaudhary PM, Jasmin A, Eby MT, Hood L. 1999. Modulation of the NF-kappa B pathway by virally encoded death effector domains-containing proteins. Oncogene. 18: 5738-46.

Chen L, Apgar J, Huynh L, Dicker F, Giago-McGahan T, Rassenti L, Weiss A, Kipps TJ. 2005. ZAP-70 directly enhances IgM signaling in chronic lymphocytic leukemia. Blood. 105: 2036-41.

Chen L, Widhopf G, Huynh L, Rassenti L, Rai KR, Weiss A, Kipps TJ. 2002. Expression of ZAP-70 is associated with increased B-cell receptor signaling in chronic lymphocytic leukemia. Blood. 100: 4609-14.

Chen L, Willis SN, Wei A, Smith BJ, Fletcher JI, Hinds MG, Colman PM, Day CL, Adams JM, Huang DC. 2005. Differential Targeting of Prosurvival Bcl-2 Proteins by Their BH3-Only Ligands Allows Complementary Apoptotic Function. Mol Cell. 17: 393-403.

Cheng EH, Wei MC, Weiler S, Flavell RA, Mak TW, Lindsten T, Korsmeyer SJ. 2001. BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. Mol Cell. 8: 705-11.

Cheng Q, Lee HH, Ying L Parks TP and Cheng G. 2000. Upregulation of Bcl-x and Bfl-1 as a potential mechanism of chemoresistance, which can be overcome by NFκB inhibition. Oncogene. 19: 4936-40.

Cheson BD, Bennet JM, Grevre M, Kay N, Keating MJ, O'Brien S and Rai KR. 1996. National Cancer Institute-sponsored working group guildlines for chronic lymphocytic leukemia: revised guidlines for diagnosis and treatment. Blood. 87: 4990-7.

Chinnaiyan AM, Prasad U, Shankar S, Hamstra DA, Shanaiah M, Chenevert TL, Ross BD, Rehemtulla A. 2000. Combined effect of tumor necrosis factor-related apoptosis-inducing ligand and ionizing radiation in breast cancer therapy. Proc Natl Acad Sci U S A. 97: 1754-9.

Choi SS, Park IC, Yun JW, Sung YC, Hong SI, Shin HS. 1995. A novel Bcl-2 related gene, Bfl-1, is overexpressed in stomach cancer and preferentially expressed in bone marrow. Oncogene. 11: 1693-8.

Chu P, Deforce D, Pedersen IM, Kim Y, Kitada S, Reed JC, Kipps TJ. 2002. Latent sensitivity to Fas-mediated apoptosis after CD40 ligation may explain activity of CD154 gene therapy in chronic lymphocytic leukemia. Proc Natl Acad Sci U S A. 99: 3854-9.

Cordone I, Masi S, Mauro FR, Soddu S, Morsilli O, Valentini T, Vegna ML, Guglielmi C, Mancini F, Giuliacci S, Sacchi A, Mandelli F, Foa R. 1998. p53 expression in B-cell chronic lymphocytic leukemia: a marker of disease progression and poor prognosis. Blood. 91: 4342-9.

Cory S, Huang DCS, Adams JL. 2003. The Bcl-2 family: roles in cell survival and oncogenesis. Oncogene. 22: 8590-607.

Craxton A, Chuang PI, Shu G, Harlan JM and Clark EA. 2000. The CD40-inducible Bcl-2 family member A1 protects B cells form antigen receptor-mediated apoptosis. Cell Immunol. 200: 56-62

Crespo M, Bosch F, Villamor N, Bellosillo B, Colomer D, Rozman M, Marce S, Lopez-Guillermo A, Campo E, Montserrat E. 2003. ZAP-70 expression as a surrogate for immunoglobulin-variable-region mutations in chronic lymphocytic leukemia. N Engl J Med. 348: 1764-75.

Cretney E, Takeda K, Yagita H, Glaccum M, Peschon JJ, Smyth MJ. 2002. Increased susceptibility to tumor initiation and metastasis in TNF-related apoptosis-inducing ligand-deficient mice. J Immunol. 168: 1356-61

Damle RN, Batliwalla FM, Ghiotto F, Valetto A, Albesiano E, Sison C, Allen SL, Kolitz J, Vinciguerra VP, Kudalkar P, Wasil T, Rai KR, Ferrarini M, Gregersen PK, Chiorazzi N. 2004. Telomere length and telomerase activity delineate distinctive replicative features of the B-CLL subgroups defined by immunoglobulin V gene mutations. Blood. 103: 375-82.

Damle RN, Ghiotto F, Valetto A, Albesiano E, Fais F, Yan XJ, Sison CP, Allen SL, Kolitz J, Schulman P, Vinciguerra VP, Budde P, Frey J, Rai KR, Ferrarini M, Chiorazzi N. 2002. B-cell chronic lymphocytic leukemia cells express a surface membrane phenotype of activated, antigen-experienced B lymphocytes. Blood. 99: 4087-93.

Damle RN, Wasil T, Fais F, Ghiotto F, Valetto A, Allen SL, Buchbinder A, Budman D, Dittmar K, Kolitz J, Lichtman SM, Schulman P, et al. 1999. IgVH gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. Blood. 94: 1840-7.

Dancescu M, Rubio-Trujillo M, Biron G, Bron D, Delespesse G and Sarfati M. Interleukin 4 protects chronic lymphocytic leukemic B cells from death by apoptosis and upregulates Bcl-2 expression. J Exp Med 1992; 176: 1119-26.

Degli-Esposti MA, Dougall WC, Smolak PJ, Waugh JY, Smith CA, Goodwin RG. 1997. The novel receptor TRAIL-R4 induces NF-_B and protects against TRAIL-mediated apoptosis, yet retains an incomplete death domain. Immunity 7: 813–820.

Degli-Esposti MA, Smolak PJ, Walczak H, Waugh J, Huang C-P, DuBose RF, Goodwin RG, Smith CA. 1997. Cloning and characterization of TRAIL-R3, a novel member of the emerging TRAIL receptor family. J ExpMed; 186: 1165–1170.

Desagher S, Osen-Sand A, Nichols A, Eskes R, Montessuit S, Lauper S, Maundrell K, Antonsson B, Martinou JC. . Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome c release during apoptosis. J Cell Biol. 144: 891-901.

Deveraux QL, Reed JC. 1999. IAP family proteins--suppressors of apoptosis. Genes Dev. 13: 239-52.

Deveraux QL, Takahashi R, Salvesen GS, Reed JC. 1997. X-linked IAP is a direct inhibitor of cell-death proteases. Nature. 388: 300-4.

Dijkers PF, Medema RH, Lammers JW, Koenderman L, Coffer PJ. 2000. Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. Curr Biol. 10: 1201-4.

Djerbi M, Screpanti V, Catrina AI, Bogen B, Biberfeld P, Grandien A. 1999. The inhibitor of death receptor signaling, FLICE-inhibitory protein defines a new class of tumor progression factors. J Exp Med. 190: 1025-32.

Dohner H, Fischer K, Bentz M, Hansen K, Benner A, Cabot G, Diehl D, Schlenk R, Coy J, Stilgenbauer S, et al. 1995. p53 gene deletion predicts for poor survival and non-response to therapy with purine analogs in chronic B-cell leukemias. Blood. 85: 1580-9.

Dohner H, Stilgenbauer S, Fischer K, Schroder M, Bentz M, Lichter P. 1995. Diagnosis and monitoring of chromosome aberrations in hematological malignancies by fluorescence in situ hybridization. Stem Cells. 13: 76-82.

Dohner H, Stilgenbauer S, James MR, Benner A, Weilguni T, Bentz M, Fischer K, Hunstein W, Lichter P. 1997. 11q deletions identify a new subset of B-cell chronic lymphocytic leukemia characterized by extensive nodal involvement and inferior prognosis. Blood. 89: 2516-22.

D'Sa-Eipper C, Chinnadurai G. 1998. Functional dissection of Bfl-1, a Bcl-2 homolog: anti-apoptosis, oncogene-cooperation and cell proliferation activities. Oncogene. 16: 3105-14.

D'Sa-Eipper C, Subramanian T and Chinnadurai G. 1996. bfl-1, a bcl-2 homologue, suppresses p53-induced apoptosis and exhibits potent cooperative transforming activity. Cancer Res. 56: 3879-82.

Du C, Fang M, Li Y, Li L, Wang X. 2000. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell. 102: 33-42.

Duriez PJ, Wong F, Dorovini-Zis K, Shahidi R, Karsan A. 2000. A1 functions at the mitochondria to delay endothelial apoptosis in response to tumor necrosis factor. J Biol Chem. 275: 18099-107.

el Rouby S, Thomas A, Costin D, Rosenberg CR, Potmesil M, Silber R, Newcomb EW. 1993. p53 gene mutation in B-cell chronic lymphocytic leukemia is associated with drug resistance and is independent of MDR1/MDR3 gene expression. Blood. 82: 3452-9.

Emery JG, McDonnell P, Burke MB, Deen KC, Lyn S, Silverman C, Dul E, Appelbaum ER, Eichman C, DiPrinzio R, Dodds RA, James IE, Rosenberg M, Lee JC, Young PR. 1998. Osteoprotegerin is a receptor for the cytotoxic ligand TRAIL. J Biol Chem. 273 14363-7.

Eskes R, Desagher S, Antonsson B, Martinou JC. 2000. Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane. Mol Cell Biol. 20: 929-35.

Fais F, Ghiotto F, Hashimoto S, Sellars B, Valetto A, Allen SL, Schulman P, Vinciguerra VP, Rai K, Rassenti LZ, Kipps TJ, Dighiero G, Schroeder HW Jr, Ferrarini M, Chiorazzi N. 1998. Chronic lymphocytic leukemia B cells express restricted sets of mutated and unmutated antigen receptors. J Clin Invest. 102: 1515-25.

Fenaux P, Preudhomme C, Lai JL, Quiquandon I, Jonveaux P, Vanrumbeke M, Sartiaux C, Morel P, Loucheux-Lefebvre MH, Bauters F, et al. 1992. Mutations of the p53 gene in B-cell chronic lymphocytic leukemia: a report on 39 cases with cytogenetic analysis. Leukemia. 6: 246-50.

Ferrarini M, Chiorazzi N. 2004. Recent advances in the molecular biology and immunobiology of chronic lymphocytic leukemia. Semin Hematol, 41: 207-23.

Francia di Celle P, Mariani S, Riera L, Stacchini A, Reato G and Foa R. Interleukin-8 induces the accumulation of B-cell chronic lymphocytic leukemia cells by prolonging survival in an autocrine fashion. Blood 1996; 87: 4382-9.

Furman RR, Asgary Z, Mascarenhas JO, Liou HC, Schattner EJ. 2000. Modulation of NF-kappa B activity and apoptosis in chronic lymphocytic leukemia B cells. J Immunol. 164: 2200-6.

Ghia P, Strola G, Granziero L, Geuna M, Guida G, Sallusto F, Ruffing N, Montagna L, Piccoli P, Chilosi M, Caligaris-Cappio F. 2002. Chronic lymphocytic leukemia B cells are endowed with the capacity to attract CD4+, CD40L+ T cells by producing CCL22. Eur J Immunol. 32: 1403-13.

Granziero L, Ghia P, Circosta P, Gottardi D, Strola G, Geuna M, Montagna L, Piccoli P, Chilosi M, Caligaris-Cappio F. 2001. Survivin is expressed on CD40 stimulation and interfaces proliferation and apoptosis in B-cell chronic lymphocytic leukemia.Blood. 97: 2777-83.

Green DR, Kroemer G. 2004. The pathophysiology of mitochondrial cell death. Science. 305: 626-9.

Griffith TS, Chin WA, Jackson GC, Lynch DH, Kubin MZ. 1998. Intracellular regulation of TRAIL-induced apoptosis in human melanoma cells. J Immunol. 161: 2833-40.

Griffiths GJ, Dubrez L, Morgan CP, Jones NA, Whitehouse J, Corfe BM, Dive C, Hickman JA. 1999. Cell damage-induced conformational changes of the pro-apoptotic protein Bak *in vivo* precede the onset of apoptosis. J Cell Biol. 144: 903-14.

Grinberg M, Sarig R, Zaltsman Y, Frumkin D, Grammatikakis N, Reuveny E, Gross A. 2002. tBID Homooligomerizes in the mitochondrial membrane to induce apoptosis. J Biol Chem. 277: 12237-45.

Gross A, McDonnell JM, Korsmeyer SJ. 1999. BCL-2 family members and the mitochondria in apoptosis. Genes Dev. 13: 1899-911.

Hamasaki A, Sendo F, Nakayama K, Ishida N, Negishi I, Nakayama K, Hatakeyama S. 1998. Accelerated neutrophil apoptosis in mice lacking A1-a, a subtype of the bcl-2-related A1 gene. J Exp Med. 188: 1985-92.

Hamblin TJ, Davis Z, Gardiner A, Oscier DG and Stevenson FK. 1999. Unmutated Ig VH genes are associated with a more aggressive form of chronic lymphocytic leukemia. Blood. 94: 1848-54.

Hamblin TJ, Orchard JA, Gardiner A, Oscier DG, Davis Z, Stevenson FK. 2000. Immunoglobulin V genes and CD38 expression in CLL. Blood. 95: 2455-7.

Hamblin TJ, Orchard JA, Ibbotson RE, Davis Z, Thomas PW, Stevenson FK, Oscier DG. 2002. CD38 expression and immunoglobulin variable region mutations are independent prognostic variables in chronic lymphocytic leukemia, but CD38 expression may vary during the course of the disease.Blood. 99: 1023-9.

Han J, Flemington C, Houghton AB, Gu Z, Zambetti GP, Lutz RJ, Zhu L, Chittenden T. 2001. Expression of bbc3, a pro-apoptotic BH3-only gene, is regulated by diverse cell death and survival signals. Proc Natl Acad Sci U S A. 98: 11318-23.

Hanada M, Delia D, Aiello A, Stadtmauer E, Reed JC. 1993. bcl-2 gene hypomethylation and high-level expression in B-cell chronic lymphocytic leukemia. Blood. 82: 1820-8.

Harris CA, Johnson EM Jr. 2001. BH3-only Bcl-2 family members are coordinately regulated by the JNK pathway and require Bax to induce apoptosis in neurons. J Biol Chem. 276: 37754-60.

Hatakeyama S, Hamasaki A, Negishi I, Loh DY, Sendo F, Nakayama K, Nakayama K. 1998. Multiple gene duplication and expression of mouse bcl-2-related genes, A1. Int Immunol. 10: 631-7.

Hill MM, Adrain C, Martin SJ. 2003. Portrait of a killer: the mitochondrial apoptosome emerges from the shadows. Mol Interv. 3: 19-26

Hotte SJ, Oza AM, Lê LH, MacLean M, Iacobucci A, Corey A, Fox NL, Hirte HW. 2004. Phase 1 Study of a Fully Human Monoclonal Antibody to the Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand Receptor 1 (TRAIL-R1) in Subjects with Advanced Solid Malignancies or Non-Hodgkin's Lymphoma (NHL). 16th EORTC-NCI-AACR Symposium on Molecular Targets and Cancer Therapeutics. Abstract #208.

Hu WH, Johnson H, Shu HB. 2000. Activation of NF-kappaB by FADD, Casper, and caspase-8. J Biol Chem. 275: 10838-44.

Huang DC, Strasser A. 2000. BH3-Only proteins-essential initiators of apoptotic cell death. Cell. 103: 839-42.

Ibrahim S, Keating M, Do KA, O'Brien S, Huh YO, Jilani I, Lerner S, Kantarjian HM, Albitar M. 2001. CD38 expression as an important prognostic factor in B-cell chronic lymphocytic leukemia. Blood. 98: 181-6.

Irmler M, Thome M, Hahne M, Schneider P, Hofmann K, Steiner V, Bodmer JL, Schroter M, Burns K, Mattmann C, Rimoldi D, French LE, Tschopp J. 1997. Inhibition of death receptor signals by cellular FLIP. Nature. 388: 190-5.

Jo M, Kim TH, Seol DW, Esplen JE, Dorko K, Billiar TR, Strom SC. 2000. Apoptosis induced in normal human hepatocytes by tumor necrosis factor-related apoptosis-inducing ligand. Nat Med. 6: 564-7.

Johnston JB, Daeninck P, Verburg L, Lee K, Williams G, Israels LG, Mowat MR. and Begleiter A. 1997. P53, MDM-2, BAX and BCL-2 and drug resistance in chronic lymphocytic leukemia. Leuk. Lymphoma. 26: 435-49.

Johnston JB, Kabore AF, Strutinsky J, Hu X, Paul JT, Kropp DM, Kuschak B, Begleiter A, Gibson SB. 2003. Role of the TRAIL/APO2-L death receptors in chlorambucil- and fludarabine-induced apoptosis in chronic lymphocytic leukemia. Oncogene. 22: 8356-69.

Johnston JB, Paul JT, Neufeld NJ, Haney N, Kropp DM, Hu X, Cheang M, Gibson SB. 2004. Role of myeloid cell factor-1 (Mcl-1) in chronic lymphocytic leukemia. Leuk Lymphoma. 45: 2017-27.

Johnston RW, Ruefli AA, Lowe SW. 2002. Apoptosis; A link between cancer genetics and chemotherapy. Cell. 108: 153-164

Jung-Ha H, Kim D, Lee SB, Hong SI, Park SY, Huh J, Kim CW, Kim SS, Lee Y, Choi SS, Shin HS. 1998. Expression of Bfl-1 in normal and tumor tissues: Bfl-1 overexpression in cancer is attributable to its preferential expression in infiltrating inflammatory cells. Hum Pathol. 29: 723-8.

Karsan A, Yee E, Kaushansky K, Harlan JM. 1996. Cloning of human Bcl-2 homologue: inflammatory cytokines induce human A1 in cultured endothelial cells. Blood. 87: 3089-96.

Kataoka T, Budd RC, Holler N, Thome M, Martinon F, Irmler M, Burns K, Hahne M, Kennedy N, Kovacsovics M, Tschopp J. 2000. The caspase-8 inhibitor FLIP promotes activation of NF-kappaB and Erk signaling pathways. Curr Biol. 10: 640-8.

Kayagaki N, Yamaguchi N, Nakayama M, Kawasaki A, Akiba H, Okumura K, Yagita H. 1999. Involvement of TNF-related apoptosis-inducing ligand in human CD4+ T cell-mediated cytotoxicity. J Immunol.162: 2639-47.

Kayagaki N, Yamaguchi N, Nakayama M, Takeda K, Akiba H, Tsutsui H, Okamura H, Nakanishi K, Okumura K, Yagita H. 1999. Expression and function of TNF-related apoptosis-inducing ligand on murine activated NK cells. J Immunol. 163: 1906-13.

Kelley SK, Ashkenazi A. Targeting death receptors in cancer with Apo2L/TRAIL. 2004. Curr Opin Pharmacol. 4: 333-9.

Kenny JJ, Knobloch TJ, Augustus M, Carter KC, Rosen CA, Lang JC. 1997. GRS, a novel member of the Bcl-2 gene family, is highly expressed in multiple cancer cell lines and in normal leukocytes. Oncogene. 14: 997-1001.

Kim JK, Kim KD, Lee E, Lim JS, Cho HJ, Yoon HK, Cho MY, Baek KE, Park YP, Paik SG, Choe YK, Lee HG. 2004. Up-regulation of Bfl-1/A1 via NF-kappaB activation in cisplatin-resistant human bladder cancer cell line. Cancer Lett. 212: 61-70.

Kim K, Fisher MJ, Xu SQ, el-Deiry WS. 2000. Molecular determinants of response to TRAIL in killing of normal and cancer cells. Clin Cancer Res. 6: 335-46.

Kischkel FC, Hellbardt S, Behrmann I, Germer M, Pawlita M, Krammer PH and Peter ME. 1995. Cytotoxicity-dependent APO-1(Fas/CD95)-associated proteins form a death-inducing signalling complex (DISC) with the receptor. EMBO J, 14: 5579–5588.

Kischkel FC, Lawrence DA, Chuntharapai A, Schow P, Kim KJ, Ashkenazi A. 2000. Apo2L/TRAIL-dependent recruitment of endogenous FADD and caspase-8 to death receptors 4 and 5. Immunity. 12: 611-20.

Kischkel FC, Lawrence DA, Tinel A, LeBlanc H, Virmani A, Schow P, Gazdar A, Blenis J, Arnott D, Ashkenazi A. 2001. Death receptor recruitment of endogenous caspase-10 and apoptosis initiation in the absence of caspase-8. J Biol Chem. 276: 46639-46.

Kitada S, Zapata JM, Andreeff M, Reed JC. 1999. Bryostatin and CD40-ligand enhance apoptosis resistance and induce expression of cell survival genes in B-cell chronic lymphocytic leukaemia. Br J Haematol. 106: 995-1004.

Kitada, S., Andersen, J., Akar, S., Zapata, J. M., Takayama, S., Krajewski, S., Wang, H. G., Zhang, X., Bullrich, F., Croce, C. M., Rai, K., Hines, J. and Reed, J. C. 1998. Expression of apoptosis-regulating proteins in chronic lymphocytic leukemia: correlations with *in vivo* and *in vitro* chemoresponses. Blood. 91: 3379-89.

Ko JK, Lee MJ, Cho SH, Cho JA, Lee BY, Koh JS, Lee SS, Shim YH, Kim CW. 2003. Bfl-1S, a novel alternative splice variant of Bfl-1, localizes in the nucleus via its C-terminus and prevents cell death. Oncogene. 22: 2457-65.

Krammer PH. 2000. CD95's deadly mission in the immune system. Nature 407: 789-95.

Krober A, Seiler T, Benner A, Bullinger L, Bruckle E, Lichter P, Dohner H, Stilgenbauer S. 2002. V(H) mutation status, CD38 expression level, genomic aberrations, and survival in chronic lymphocytic leukemia. Blood. 100: 1410-6.

Kruczynski A, Hill BT. 2001. Vinflunine, the latest Vinca alkaloid in clinical development. A review of its preclinical anticancer properties. Crit Rev Oncol Hematol. 40: 159-73.

Krueger A, Schmitz I, Baumann S, Krammer PH, Kirchhoff S. 2001. Cellular FLICE-inhibitory protein splice variants inhibit different steps of caspase-8 activation at the CD95 death-inducing signaling complex. J Biol Chem. 276: 20633-40.

Kuss AW, Knodel M, Berberich-Siebelt F, Lindemann D, Schimpl A, Berberich I. 1999. A1 expression is stimulated by CD40 in B cells and rescues WEHI 231 cells from anti-IgM-induced cell death. Eur J Immunol. 29: 3077-88.

Kuwana T, Smith JJ, Muzio M, Dixit V, Newmeyer DD, Kornbluth S. 1998. Apoptosis induction by caspase-8 is amplified through the mitochondrial release of cytochrome c. J Biol Chem. 273: 16589-94.

Lagneaux L, Delforge A, Bron D, De Bruyn C, Stryckmans P. 1998. Chronic lymphocytic leukemic B cells but not normal B cells are rescued from apoptosis by contact with normal bone marrow stromal cells. Blood. 91: 2387-96.

Lanham S, Hamblin T, Oscier D, Ibbotson R, Stevenson F, Packham G. 2003. Differential signaling via surface IgM is associated with VH gene mutational status and CD38 expression in chronic lymphocytic leukemia. Blood. 101: 1087-93.

Lassus P, Opitz-Araya X, Lazebnik Y. 2002. Requirement for caspase-2 in stress-induced apoptosis before mitochondrial permeabilization. Science. 297: 1352-4.

Lawrence D, Shahrokh Z, Marsters S, Achilles K, Shih D, Mounho B, Hillan K, Totpal K, DeForge L, Schow P, Hooley J, Sherwood S, Pai R, Leung S, Khan L, Gliniak B, Bussiere J, Smith CA, Strom SS, Kelley S, Fox JA, Thomas D, Ashkenazi A. 2001. Differential hepatocyte toxicity of recombinant Apo2L/TRAIL versions. Nat Med. 7: 383-5.

Lehmann V, Freudenberg MA, Galanos C. 1987. Lethal toxicity of lipopolysaccharide and tumor necrosis factor in normal and D-galactosamine-treated mice. J Exp Med. 165: 657-63.

Lei K, Davis RJ. 2003. JNK phosphorylation of Bim-related members of the Bcl2 family induces Bax-dependent apoptosis. Proc Natl Acad Sci U S A. 100: 2432-7.

Letai A, Bassik MC, Walensky LD, Sorcinelli MD, Weiler S, Korsmeyer SJ. 2002. Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. Cancer Cell. 2: 183-92.

Leverkus M, Neumann M, Mengling T, Rauch CT, Brocker EB, Krammer PH, Walczak H. 2000. Regulation of tumor necrosis factor-related apoptosis-inducing ligand sensitivity in primary and transformed human keratinocytes. Cancer Res. 60: 553-9.

Li H, Zhu H, Xu CJ, Yuan J. 1998. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell. 94: 491-501.

Li LY, Luo X, Wang X. 2001. Endonuclease G is an apoptotic DNase when released from mitochondria. Nature. 412: 95-9.

Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X. 1997. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell. 91: 479-89.

Lin EY, Orlofsky A, Berger MS, Prystowsky MB. 1993. Characterization of A1, a novel hemopoietic-specific early-response gene with sequence similarity to bcl-2. J Immunol. 151: 1979-88.

Lin EY, Orlofsky A, Wang HG, Reed JC, Prystowsky MB. 1996. A1, a Bcl-2 family member, prolongs cell survival and permits myeloid differentiation. Blood. 87: 983-92.

Lin K, Sherrington PD, Dennis M, Matrai Z, Cawley JC, Pettitt AR. 2002. Relationship between p53 dysfunction, CD38 expression, and IgV(H) mutation in chronic lymphocytic leukemia. Blood. 100: 1404-9.

Lindsten T, Ross AJ, King A, Zong WX, Rathmell JC, Shiels HA, Ulrich E, Waymire KG, Mahar P, Frauwirth K, Chen Y, Wei M, Eng VM, Adelman DM, Simon MC, Ma A, Golden JA, Evan G, Korsmeyer SJ, MacGregor GR, Thompson CB. 2000. The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. Mol Cell. 6: 1389-99.

Liu NS, O'Brien S. 2004. Monoclonal antibodies in the treatment of chronic lymphocytic leukemia.Med Oncol. 21: 297-304.

Lockshin RA, Zakeri Z. 2001. Programmed cell death and apoptosis: origins of the theory. Nat Rev Mol Cell Biol. 2: 545-50.

Lundin J, Osterborg A. 2004. Advances in the use of monoclonal antibodies in the therapy of chronic lymphocytic leukemia. Semin Hematol. 41: 234-45.

Luo X, Budihardjo I, Zou H, Slaughter C, Wang X. 1998. Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cell. 94: 481-90.

MacFarlane M, Ahmad M, Srinivasula SM, Fernandes-Alnemri T, Cohen GM, Alnemri ES. 1997. Identification and molecular cloning of two novel receptors for the cytotoxic ligand TRAIL. J Biol Chem 272: 25417–25420.

MacFarlane M, Harper N, Snowden RT, Dyer MJ, Barnett GA, Pringle JH, Cohen GM. 2002. Mechanisms of resistance to TRAIL-induced apoptosis in primary B cell chronic lymphocytic leukaemia. Oncogene. 21: 6809-18.

Marsters SA, Sheridan JP, Pitti RM, Huang A, Skubatch M, Baldwin D, Yuan J, Gurney A, Goddard AD, Godowski P, Ashkenazi A. 1997. A novel receptor for Apo2L/TRAIL contains a truncated death domain. Curr Biol 7: 1003–1006.

Martinon F and Tschopp J. Inflammatory caspases: linking an intracellular innate immune system to autoinflammatory diseases. Cell 2004; 117: 561-74 VanArsdale, T. L., VanArsdale, S. L., Force, W. R., Walter, B. N., Mosialos, G., Kieff, E., Reed, J. C. and Ware, C. F. Lymphotoxin-beta receptor signaling complex: role of tumor necrosis factor receptor-associated factor 3 recruitment in cell death and activation of nuclear factor kappaB. Proc. Natl. Acad. Sci. U S A, 94: 2460-2465, 1997.

McConkey DJ, Chandra J, Wright S, Plunkett W, McDonnell TJ, Reed JC and Keating M. 1996. Apoptosis sensitivity in chronic lymphocytic leukemia is determined by endogenous endonuclease content and relative expression of BCL-2 and BAX. J Immunol. 156: 2624-30.

Medema JP, de Jong J, van Hall T, Melief CJ, Offringa R. 1999. Immune escape of tumors *in vivo* by expression of cellular FLICE-inhibitory protein. J Exp Med. 190: 1033-8.

Medema JP, Scaffidi C, Kischkel FC, Shevchenko A, Mann M, Krammer PH, Peter ME. 1997. FLICE is activated by association with the CD95 death-inducing signaling complex (DISC). EMBO J. 16: 2794-804.

Messmer BT, Albesiano E, Efremov DG, Ghiotto F, Allen SL, Kolitz J, Foa R, Damle RN, Fais F, Messmer D, Rai KR, Ferrarini M, Chiorazzi N. 2004. Multiple distinct sets of stereotyped antigen receptors indicate a role for antigen in promoting chronic lymphocytic leukemia. J Exp Med. 200: 519-25.

Micheau O, Tschopp J. 2003. Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. Cell. 114: 181-90.

Moreb JS and Schweder M. 1997. Human A1, a bcl-2-related gene, is induced in leukemic cells by cytokines as well as differentiating factors. Leukemia. 11: 998-1004.

Muchmore SW, Sattler M, Liang H, Meadows RP, Harlan JE, Yoon HS, Nettesheim D, Chang BS, Thompson CB, Wong SL, Ng SL, Fesik SW. 1996. X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death. Nature. 381: 335-41.

Munzert G, Kirchner D, Stobbe H, Bergmann L, Schmid RM, Dohner H, Heimpel H. 2002. Tumor necrosis factor receptor-associated factor 1 gene overexpression in B-cell chronic lymphocytic leukemia: analysis of NF-kappa B/Rel-regulated inhibitors of apoptosis. Blood. 100: 3749-56.

Muzio M, Chinnaiyan AM, Kischkel FC, O'Rourke K, Shevchenko A, Ni J, Scaffidi C, Bretz JD, Zhang M, Gentz R, Mann M, Krammer PH, Peter ME, Dixit VM. 1996. FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death--inducing signaling complex. Cell. 85: 817-27.

Nakano K, Vousden KH. 2001. PUMA, a novel proapoptotic gene, is induced by p53. Mol Cell. 7: 683-94.

Nechushtan A, Smith CL, Lamensdorf I, Yoon SH, Youle RJ. 2001. Bax and Bak coalesce into novel mitochondria-associated clusters during apoptosis. J Cell Biol. 153: 1265-76.

Oda E, Ohki R, Murasawa H, Nemoto J, Shibue T, Yamashita T, Tokino T, Taniguchi T, Tanaka N. 2000. Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. Science. 288: 1053-8.

Ogasawara J, Watanabe-Fukunaga R, Adachi M, Matsuzawa A, Kasugai T, Kitamura Y, Itoh N, Suda T, Nagata S. 1993. Lethal effect of the anti-Fas antibody in mice. Nature. 364: 806-9.

Orchard JA, Ibbotson RE, Davis Z, Wiestner A, Rosenwald A, Thomas PW, Hamblin TJ, Staudt LM, Oscier DG. 2004. ZAP-70 expression and prognosis in chronic lymphocytic leukaemia. Lancet. 363: 105-11.

Orlofsky A, Weiss LM, Kawachi N, Prystowsky MB. 2002. Deficiency in the anti-apoptotic protein A1-a results in a diminished acute inflammatory response. J Immunol. 168: 1840-6.

Oscier D, Fegan C, Hillmen P, Illidge T, Johnson S, Maguire P, Matutes E, Milligan D; Guidelines Working Group of the UK CLL Forum. British Committee for Standards in Haematology. 2004.Guidelines on the diagnosis and management of chronic lymphocytic leukaemia. Br J Haematol,125:294-317.

Oscier DG, Gardiner AC, Mould SJ, Glide S, Davis ZA, Ibbotson RE, Corcoran MM, Chapman RM, Thomas PW, Copplestone JA, Orchard JA, Hamblin TJ. 2002. Multivariate analysis of prognostic factors in CLL: clinical stage, IGVH gene mutational status, and loss or mutation of the p53 gene are independent prognostic factors. Blood. 100: 1177-84.

Osorio LM, Aguilar-Santelises M. 1998. Apoptosis in B-chronic lymphocytic leukaemia. Med Oncol. 15: 234-40.

Pan G, O'Rourke K, Chinnaiyan AM, Gentz R, Ebner R, Ni J, Dixit VM. 1997. The receptor for the cytotoxic ligand TRAIL. Science 276: 111–113.

Pedersen IM, Kitada S, Leoni LM, Zapata JM, Karras JG, Tsukada N, Kipps TJ, Choi YS, Bennett F, Reed JC. 2002. Protection of CLL B cells by a follicular dendritic cell line is dependent on induction of Mcl-1. Blood. 100: 1795-801.

Pedersen IM, Kitada S, Schimmer A, Kim Y, Zapata JM, Charboneau L, Rassenti L, Andreeff M, Bennett F, Sporn MB, Liotta LD, Kipps TJ, Reed JC. 2002. The triterpenoid CDDO induces apoptosis in refractory CLL B cells. Blood. 100: 2965-72.

Pepper C, Hooper K, Thomas A, Hoy T, Bentley P. 2001. Bcl-2 antisense oligonucleotides enhance the cytotoxicity of chlorambucil in B-cell chronic lymphocytic leukaemia cells. Leuk Lymphoma. 42: 491-8.

Pepper C, Hoy T and Bentley P. 1998. Elevated Bcl-2/Bax are consistent feature of apoptosis resistance in B-cell chronic lymphocytic leukemia and are correlated with *in vivo* chemoresistance. Leuk. Lymphoma. 28: 355-61.

Pepper C, Thomas A, Hoy T, Bentley P. 2002. Antisense oligonucleotides complementary to Bax transcripts reduce the susceptibility of B-cell chronic lymphocytic leukaemia cells to apoptosis in a bcl-2 independent manner. Leuk Lymphoma. 43: 2003-9.

Petros AM, Medek A, Nettesheim DG, Kim DH, Yoon HS, Swift K, Matayoshi ED, Oltersdorf T, Fesik SW. Solution structure of the antiapoptotic protein bcl-2. Proc Natl Acad Sci U S A. 2001 98: 3012-7.

Pettitt AR, Sherrington PD, Cawley JC. 1999. The effect of p53 dysfunction on purine analogue cytotoxicity in chronic lymphocytic leukaemia. Br J Haematol. 106: 1049-51.

Pickeral OK, Rassenti LZ, Powell J, Botstein D, Byrd JC, Grever MR, Cheson BD, Chiorazzi N, Wilson WH, Kipps TJ, Brown PO, Staudt LM. 2001. Relation of gene expression phenotype to immunoglobulin mutation genotype in B cell chronic lymphocytic leukemia. J Exp Med. 194: 1639-47.

Puthalakath H, Huang DC, O'Reilly LA, King SM, Strasser A. 1999. The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. Mol Cell.;3: 287-96.

Puthalakath H, Strasser A. 2002. Keeping killers on a tight leash: transcriptional and post-translational control of the pro-apoptotic activity of BH3-only proteins. Cell Death Differ. 9: 505-12.

Rai KR, Dohner H, Keating MJ, Montserrat E. 2001. Chronic lymphocytic leukemia: case-based session. Hematology (Am Soc Hematol Educ Program): 140-56. 44. Rizouli V, Gribben Jg J. 2004. The role of stem cell transplantation in chronic lymphocytic leukemia. Semin Hematol. 41: 246-53.

Rai KR, O'Brien S, Cunningham C, Turkina AG, Ochoa L, Frankel SR, Golenkov AK. 2002. Genasense (Bcl-2 Antisense) Monotherapy in Patients with Relapsed or Refractory Chronic Lymphocytic Leukemia: Phase 1 and 2 Results. Blood. 100

Rai KR, Sawitsky A, Cronkite EP, Chanana AD, Levy RN and Pasternack BS. 1975. Clinical staging of chronic lymphocytic leukemia. Blood. 46: 219-34.

Rai KR, Sawitsky A. 1987. A review of the prognostic role of cytogenetic, phenotypic, morphologic, and immune function characteristics in chronic lymphocytic leukemia. Blood Cells. 12: 327-38.

Ranger AM, Malynn BA, Korsmeyer SJ. 2001. Mouse models of cell death. Nat Genet. 28: 113-8.

Rassenti LZ, Huynh L, Toy TL, Chen L, Keating MJ, Gribben JG, Neuberg DS, Flinn IW, Rai KR, Byrd JC, Kay NE, Greaves A, Weiss A, Kipps TJ. 2004. ZAP-70 compared with immunoglobulin heavy-chain gene mutation status as a predictor of disease progression in chronic lymphocytic leukemia. N Engl J Med. 351: 893-901.

Redaelli A, Laskin BL, Stephens JM, Botteman MF, Pashos CL. 2004. The clinical and epidemiological burden of chronic lymphocytic leukaemia. Eur J Cancer Care 13: 279-87.

Reed JC. 2003. Apoptosis-targeted therapies for cancer. Cancer Cell. 3: 17-22.

Riedl SJ, Shi Y. 2004. Molecular mechanisms of caspase regulation during apoptosis. Nat Rev Mol Cell Biol. 5: 897-907.

Robertson JD, Enoksson M, Suomela M, Zhivotovsky B, Orrenius S. 2002. Caspase-2 acts upstream of mitochondria to promote cytochrome c release during etoposide-induced apoptosis. J Biol Chem. 277: 29803-9

Robertson LE, Plunkett W, McConnell K, Keating MJ, McDonnell TJ. 1996. Bcl-2 expression in chronic lymphocytic leukemia and its correlation with the induction of apoptosis and clinical outcome. Leukemia. 10: 456-9.

Romano MF, Lamberti A, Tassone P, Alfinito F, Costantini S, Chiurazzi F, Defrance T, Bonelli P, Tuccillo F, Turco MC, Venuta S. 1998. Triggering of CD40 antigen inhibits fludarabine-induced apoptosis in B chronic lymphocytic leukemia cells. Blood. 92: 990-5.

Roth W, Reed JC. 2004. FLIP protein and TRAIL-induced apoptosis.Vitam Horm. 67: 189-206.

Roy N, Deveraux QL, Takahashi R, Salvesen GS, Reed JC. 1997. The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. EMBO J. 16: 6914-25.

Saxena A, Moshynska O, Sankaran K, Viswanathan S, Sheridan DP. 2002. Association of a novel single nucleotide polymorphism, G(-248)A, in the 5'-UTR of BAX gene in chronic lymphocytic leukemia with disease progression and treatment resistance. Cancer Lett. 187: 199-205.

Scaffidi C, Krammer PH, Peter ME. 1999. Isolation and analysis of components of CD95 (APO-1/Fas) death-inducing signaling complex. Methods. 17: 287-91.

Scaffidi, S. Fulda, A. Srinivasan, L. Feng, C. Friesen, K.J. Tomasseli et al. 1998. Two CD95 (APO-1/Fas) signaling pathways. EMBO J. 17: 1675–1687.

Schena M, Larsson LG, Gottardi D, Gaidano G, Carlsson M, Nilsson K, Caligaris-Cappio F. 1992. Growth- and differentiation-associated expression of bcl-2 in B-chronic lymphocytic leukemia cells. Blood. 79: 2981-9.

Schimmer AD. 2004. Inhibitor of apoptosis proteins: translating basic knowledge into clinical practice. Cancer Res. 64: 7183-90.

Schliep S, Decker T, Schneller F, Wagner H, Hacker G. 2004. Functional evaluation of the role of inhibitor of apoptosis proteins in chronic lymphocytic leukemia. Exp Hematol. 32: 556-62.

Schneider P, Tschopp J. 2000. Modulation of death receptor signalling. Symp Soc Exp Biol. 52: 31-42.

Sedger LM, Shows DM, Blanton RA, Peschon JJ, Goodwin RG, Cosman D, Wiley SR. 1999. IFN-gamma mediates a novel antiviral activity through dynamic modulation of TRAIL and TRAIL receptor expression. J Immunol. 163: 920-6.

Shangary S, Johnson DE. 2003. Recent advances in the development of anticancer agents targeting cell death inhibitors in the Bcl-2 protein family. Leukemia. 171470-81.

Shankar S, Srivastava RK. 2004. Enhancement of therapeutic potential of TRAIL by cancer chemotherapy and irradiation: mechanisms and clinical implications. Drug Resist Updat. 7: 139-56.

Sheridan JP, Marsters SA, Pitti RM, Gurney A, Skubatch M, Baldwin D, Ramakrishnan L, Gray CL, Baker K, Wood WI, Goddard AD, Godowski P, Ashkenazi A. 1997. Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. Science 277: 818–821.

Silver RT, Sawitsky A, Rai K, Holland JF and Glidewell O. 1978. Guidelines for protocol studies in chronic lymphocytic leukemia. Am J Hematol. 4: 343-58.

Smyth MJ, Takeda K, Hayakawa Y, Peschon JJ, van den Brink MR, Yagita H. 2003. Nature's TRAIL--on a path to cancer immunotherapy. Immunity. 18: 1-6.

Sprick MR, Rieser E, Stahl H, Grosse-Wilde A, Weigand MA, Walczak H. 2002. Caspase-10 is recruited to and activated at the native TRAIL and CD95 death-inducing signalling complexes in a FADD-dependent manner but can not functionally substitute caspase-8. EMBO J. 21: 4520-30.

Sprick MR, Weigand MA, Rieser E, Rauch CT, Juo P, Blenis J, Krammer PH, Walczak H. 2000. FADD/MORT1 and caspase-8 are recruited to TRAIL receptors 1 and 2 and are essential for apoptosis mediated by TRAIL receptor 2. Immunity. 12: 599-609.

Stevenson FK, Caligaris-Cappio F. 2004. Chronic lymphocytic leukemia: revelations from the B-cell receptor. Blood. 103: 4389-95.

Strasser A, Bouillet P. 2003. The control of apoptosis in lymphocyte selection. Immunol Rev.82-92.

Sturm I, Bosanquet AG, Hermann S, Guner D, Dorken B, Daniel PT. 2003. Mutation of p53 and consecutive selective drug resistance in B-CLL occurs as a consequence of prior DNA-damaging chemotherapy. Cell Death Differ. 10: 477-84.

Sugiyama T, Shimizu S, Matsuoka Y, Yoneda Y, Tsujimoto Y. 2002. Activation of mitochondrial voltage-dependent anion channel by apro-apoptotic BH3-only protein Bim. Oncogene. 21: 4944-56.

Susin SA, Lorenzo HK, Zamzami N, Marzo I, Brenner C, Larochette N, Prevost MC, Alzari PM, Kroemer G. 1999. Mitochondrial release of caspase-2 and -9 during the apoptotic process. J Exp Med. 189: 381-94.

Suzuki M, Youle RJ, Tjandra N. 2000. Structure of Bax: coregulation of dimer formation and intracellular localization. Cell. 103: 645-54.

Takeda K, Hayakawa Y, Smyth MJ, Kayagaki N, Yamaguchi N, Kakuta S, Iwakura Y, Yagita H, Okumura K. 2001. Involvement of tumor necrosis factor-related apoptosis-inducing ligand in surveillance of tumor metastasis by liver natural killer cells. Nat Med. 7: 94-100.

Tangye SG and Raison RL. Human cytokines suppress apoptosis of leukaemic CD5+ B cells and preserve expression of bcl-2. Immunol Cell Biol 1997; 75: 107-15.

Tangye SG, Raison RL. 1997. Human cytokines suppress apoptosis of leukaemic CD5+ B cells and preserve expression of bcl-2. Immunol Cell Biol. 75: 127-35.

Thomas A, El Rouby S, Reed JC, Krajewski S, Silber R, Potmesil M, Newcomb EW. 1996. Drug-induced apoptosis in B-cell chronic lymphocytic leukemia: relationship between p53 gene mutation and bcl-2/bax proteins in drug resistance. Oncogene 12: 1055-62.

Thomenius MJ, Distelhorst CW. 2003. Bcl-2 on the endoplasmic reticulum: protecting the mitochondria from a distance. J Cell Sci. Nov 116: 4493-9.

Tinel A, Tschopp J. 2004. The PIDDosome, a protein complex implicated in activation of caspase-2 in response to genotoxic stress. Science. 304: 843-6.

Tobin G, Thunberg U, Johnson A, Eriksson I, Soderberg O, Karlsson K, Merup M, Juliusson G, Vilpo J, Enblad G, Sundstrom C, Roos G, Rosenquist R. 2003. Chronic lymphocytic leukemias utilizing the VH3-21 gene display highly restricted Vlambda2-14 gene use and homologous CDR3s: implicating recognition of a common antigen epitope. Blood. 101: 4952-7. Rosenwald A, Alizadeh AA, Widhopf G, Simon R, Davis RE, Yu X, Yang L,

Tobin G, Thunberg U, Karlsson K, Murray F, Laurell A, Willander K, Enblad G, Merup M, Vilpo J, Juliusson G, Sundstrom C, Soderberg O, Roos G, Rosenquist R. 2004. Subsets with restricted immunoglobulin gene rearrangement features indicate a role for antigen selection in the development of chronic lymphocytic leukemia. Blood. 104: 2879-85.

Tolcher AW, Mita M, Patnaik A, Rowinsky EK, Corey3A, Fleming M, Fox NL, Weiner LM, Meropol NJ, Padavic K, Cohen RB. 2004. A Phase 1 and Pharmacokinetic Study of HGS-ETR1, A Fully Human Monoclonal Antibody to TRAIL-R1 (TRM-1), in Patients with Advanced Solid Tumors. American Society of Clinical Oncology Annual Meeting, 2004: Abstract #3060.

Tomayko MM, Cancro MP. 1998. Long-lived B cells are distinguished by elevated expression of A1. J Immunol. 160: 107-11.

Tsujimoto Y. 2003. Cell death regulation by the Bcl-2 protein family in the mitochondria. J Cell Physiol. 195: 158-67.

Wagner KW, Engels IH, Deveraux QL. 2004. Caspase-2 can function upstream of bid cleavage in the TRAIL apoptosis pathway. J Biol Chem. 279: 35047-52

Walczak H, Degli-Esposti MA, Johnson RS, Smolak PJ, Waugh JY, Boiani N, Timour MS, Gerhart MJ, Schooley KA, Smith CA, Goodwin RG, Rauch CT. 1997. TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL. EMBO J 16: 5386–5397.

Walczak H, Miller RE, Ariail K, Gliniak B, Griffith TS, Kubin M, Chin W, Jones J, Woodward A, Le T, Smith C, Smolak P, Goodwin RG, Rauch CT, Schuh JC, Lynch DH. 1999. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand *in vivo*.Nat Med 5: 157-63.

Wang CY, Guttridge DC, Mayo MW and Baldwin AS Jr. 1999. NF-κB induces expression of the Bcl-2 homologue A1/Bfl-1 to preferentially suppress chemotherapy-induced apoptosis. Mol Cell Biol. 19: 5923-9.

Wattel E, Preudhomme C, Hecquet B, Vanrumbeke M, Quesnel B, Dervite I, Morel P, Fenaux P. 1994. p53 mutations are associated with resistance to chemotherapy and short survival in hematologic malignancies. Blood. 84: 3148-57.

Wei MC, Lindsten T, Mootha VK, Weiler S, Gross A, Ashiya M, Thompson CB, Korsmeyer SJ. 2000. tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. Genes Dev. 14: 2060-71.

Wei MC, Zong WX, Cheng EH, Lindsten T, Panoutsakopoulou V, Ross AJ, Roth KA, MacGregor GR, Thompson CB, Korsmeyer SJ. 2001. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science. 292: 727-30.

Weiss, R.S., Enoch, T. and Leder, P. Inactivation of mouse Hus1 results in genomic instability and impaired responses to genotoxic stress. Genes Dev, 14: 1886-1898, 2000.

Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE, Moritz RL,Simpson RJ, Vaux DL. 2000. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. Cell. 102: 43-53.

Verma S, Zhao LJ, Chinnadurai G. 2001. Phosphorylation of the pro-apoptotic protein BIK: mapping of phosphorylation sites and effect on apoptosis. J Biol Chem. 276: 4671-6.

Werner AB, de Vries E, Tait SW, Bontjer I, Borst J. 2002a. Bcl-2 family member Bfl-1/A1 sequesters truncated bid to inhibit is collaboration with pro-apoptotic Bak or Bax. J Biol Chem. 277: 22781-8

Werner AB, de Vries E, Tait SW, Bontjer I, Borst J. 2002b. TRAIL receptor and CD95 signal to mitochondria via FADD, caspase-8/10, Bid, and Bax but differentially regulate events downstream from truncated Bid. J Biol Chem. 277: 40760-7.

Werner AB, Tait SW, de Vries E, Eldering E, Borst J. 2004. Requirement for aspartate-cleaved bid in apoptosis signalling by DNA-damaging anti-cancer regimens. J Biol Chem. 279: 28771-80.

Wierda WG. 2003. Immunologic monitoring in chronic lymphocytic leukemia. Curr Oncol Rep. 5: 419-25.

Villunger A, Scott C, Bouillet P, Strasser A. 2003. Essential role for the BH3-only protein Bim but redundant roles for Bax, Bcl-2, and Bcl-w in the control of granulocyte survival. Blood. 101: 2393-400.

Wolter KG, Hsu YT, Smith CL, Nechushtan A, Xi XG, Youle RJ. 1997. Movement of Bax from the cytosol to mitochondria during apoptosis. J Cell Biol. 139: 1281-92.

Yee KW, O'Brien SM, Giles FJ. 2004. An update on the management of chronic lymphocytic leukaemia. Expert Opin Pharmacother. 5: 1535-54.

Zent CS, Kay NE. 2004. Advances in the understanding of biology and prognosis in chronic lymphocytic leukemia. Curr Oncol Rep, 6:348-54.

Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ. 1996. Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L) Cell. 87: 619-28.

Zhang H, Cowan-Jacob SW, Simonen M, Greenhalf W, Heim J and Meyhack B. 2000. Structural basis of BFL-1 for its interaction with BAX and its anti-apoptotic action in mammalian and yeast cells. J Biol Chem. 275: 11092-9.

Zhang XD, Franco AV, Nguyen T, Gray CP, Hersey P. 2000. Differential localization and regulation of death and decoy receptors for TNF-related apoptosis-inducing ligand (TRAIL) in human melanoma cells. J Immunol. 164: 3961-70.

Ziegler U, Groscurth P. 2004. Morphological features of cell death. News Physiol Sci. 19: 124-8.

Zong WX, Edelstein LC, Chen C, Bash J and Gélinas C. 1999. The prosurvival Bcl-2 homolog Bfl-1/A1 is a direct transcriptional target of NFκB that blocks TNFα-induced apoptosis. Genes Dev. 13: 382-7.

Zong WX, Lindsten T, Ross AJ, MacGregor GR, Thompson CB. 2001. BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak. Genes Dev. 15: 1481-6.

Zou H, Li Y, Liu X, Wang X. 1999. An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9.J Biol Chem. 274: 11549-56.

Zupo S, Isnardi L, Megna M, Massara R, Malavasi F, Dono M, Cosulich E, Ferrarini M. 1996. CD38 expression distinguishes two groups of B-cell chronic lymphocytic leukemias with different responses to anti-IgM antibodies and propensity to apoptosis. Blood. 88: 1365-74.