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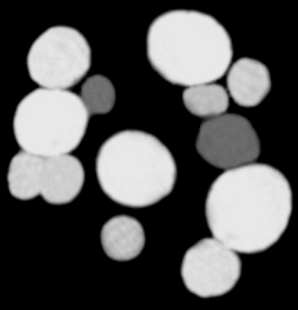

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Regulation of Death Receptor Responses by Hyperthermia

by

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ACADEMIC DISSERTATION

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Cover picture:
Graphic modification of a fluorescence microscopy image of a DAPI-labeled apoptotic nucleus

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Painosalama Oy – Turku, Finland, 2007

To my sisters

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ORIGINAL PUBLICATIONS

This thesis is based on the following original publications and manuscripts, which are referred to in the text by their Roman numbers. In addition, unpublished results are included.

I Elphick LM, Meinander A, Mikhailov A, Richard M, Toms NJ, Eriksson JE, and Kass GEN. (2006) Live cell detection of caspase-3 activation by a Discosoma-red-fluorescent-protein-based fluorescence resonance energy transfer construct. *Anal. Biochem.* **349:** 148-155.

II Tran SEF, Meinander A, Holmström TH, Riviero-Muller A, Heiskanen KM, Linnau EK, Courtney MJ, Mosser DD, Sistonen L, and Eriksson JE. (2003) Heat stress downregulates FLIP and sensitizes cells to Fas receptor-mediated apoptosis. *Cell Death Differ*. **10:** 1137-1147.

III Meinander A, Söderström TS, Kaunisto A, Poukkula M, Sistonen L, and Eriksson JE. (2007) Fever-like hyperthermia controls T lymphocyte persistence by inducing degradation of cellular FLIPshort. *J. Immunol.* **178:** 3944-3953.

ABBREVIATIONS

ActD	actinomycin D
AICD	activation-induced cell death
AIF	apoptosis-inducing factor
ALPS	autoimmune lymphoproliferative syndrome
Apaf-1	apoptotic protease-activating factor-1
Bad	Bcl-2-associated death protein
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma gene 2
BH	Bcl-2 homology
Bid	BH3-interacting domain death agonist
Bim	BH3-interacting mediator of cell death
BIR	baculoviral IAP repeat
BSA	bovine serum albumin
CAD	caspase-activated DNase
CaMKII	Ca^{2+}-calmodulin-dependent protein kinase II
CARD	caspase recruitment domain
CASH	caspase homologue
caspase	cysteinyl aspartate proteinase
Casper	caspase-eight-related protein
CD95L	CD95 ligand
CED	cell death abnormal
CFLAR	caspase-8 and FADD-like apoptosis regulator
CFP	cyan fluorescent protein
CHX	cycloheximide
CLARP	caspase-like apoptosis-regulatory protein
CNS	central nervous system
CrmA	cytokine response modifier gene A
COX	cytochrome *c* oxidase
CRD	cysteine-rich domain
CTL	cytotoxic T-lymphocyte
DEVD	Asp-Glu-Val-Asp
DcR	decoy receptor
DD	death domain
DED	death effector domain
DEDD	DED-containing DNA-binding protein
DIABLO	direct IAP-binding protein with low pI
DIC	differential interference contrast
DISC	death-inducing signaling complex
DMSO	dimethyl sulfoxide
DN	dominant negative
DR	death receptor
DsRed	discosoma red fluorescent protein
DTT	dithiothreitol
EAE	experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
EGL	egg lying defective
EHV	equine herpesvirus
ERK	extracellular-regulated kinase
FACS	fluorescence-activated cell sorter
FADD	Fas-associated death domain
FAF	Fas-associated protein factor
FAP	Fas-associated phosphatase
FBS/FCS	fetal bovine serum/fetal calf serum
FIST	Fas-interacting serine/threonine kinase
FITC	fluorescein isothiocyanate
FLAME	FADD-like anti-apoptotic molecule
FLICE	FADD-like ICE
FLIP	FLICE-inhibitory protein
fmk	fluoromethyl ketone
FRET	fluorescence resonance energy transfer
GLD	generalized lymphoproliferative disease
GFP	green fluorescent protein
HDAC	histone deacetylase
HGS	Human Genome Sciences
HHV	human herpesvirus
HIPK	homeodomain-interacting protein kinase
HRP	horseradish peroxidase
HS	heat shock
HSE	heat shock element
HSF	heat shock factor
Hsp	heat shock protein
HVS	herpesvirus saimiri
IAP	inhibitor of apoptosis
ICAD	inhibitor of CAD
ICE	interleukin-1β-processing enzyme

I-FLICE	inhibitor of FLICE
IFN-γ	interferon γ
I-κB	inhibitor κB
IKK	I-κB kinase
IL	interleukin
IRF	interferon regulatory factor
JNK	c-Jun N-terminal kinase
KSHV	Kaposi's sarcoma-associated herpesvirus
LDH	lactate dehydrogenase
LPR	lymhoproliferation
LPS	lipopolysaccharide
MALT	mucosa-associated lymphoid tissue lymphoma translocation protein
MAPK	mitogen-activated protein kinase
MCV	molluscum contagiosum virus
MEF	mouse embryonic fibroblast
MHC	major histocompatibility complex
MKK	MAP kinase kinase
MOMP	mitochondrial outer membrane permeabilization
MORT	mediator of receptor-induced toxicity
MRIT	MACH-related inducer of toxicity FLICE
MS	multiple sclerosis
MTT	methylthiazoletetrazolium
NEMO	NF-κB essential modulator
NFAT	nuclear factor in activated T-cells
NF-κB	nuclear factor κB
NGFR	nerve growth factor receptor
NIK	NF-κB-inducing kinase
NK cell	natural killer cell
OPG	osteoprotegerin
PAGE	polyacrylamide gel electrophoresis
Par-4	prostate apoptosis response factor 4
PARP	Poly (ADP-ribose) polymerase
PBS	phosphate-buffered saline
PE	phycoerythrin
PEA-15	phosphoprotein enriched in astrocytes of 15 kDa
PED	phosphoprotein enriched in diabetes
PHA	phytohemagglutinin
PLAD	pre-ligand association domain
PI3K	phosphatidylinositol 3-kinase
PIDD	p53-induced protein with a death domain
PKC	protein kinase C
PMSF	phenylmethylsulfonyl fluoride
PUMA	p53-upregulated modulator of apoptosis
RAIDD	RIP-associated ICH-1/CED-3-homologue with death domain
RING	really interesting new gene
RIP	receptor-interacting protein
RNAi	RNA interference
ROS	reactive oxygen species
RT	room temperature
rtTA	reverse tetracycline-controlled transactivator
SCID	severe combined immunodeficiency
SDS	sodium dodecyl sulfate
SEB	staphylococcal enterotoxin B
shRNA	small hairpin RNA
siRNA	small interfering RNA
Smac	second mitochondria-derived activator of caspases
SODD	silencer of death domains
STS	staurosporine
TET	tetracycline
TGF	transforming growth factor
THD	TNF homology domain
Th	T-helper
TMRM	tetramethyl rhodamine ester
TNF	tumor necrosis factor
TRADD	TNF-R-associated death domain
TRAF	TNF-R-associated factor
TRAIL	TNF-related apoptosis-inducing ligand
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
VEGF	vascular endothelial growth factor
YFP	yellow fluorescent protein
XIAP	X-linked IAP

ABSTRACT

Cells need a variety of responses to protect themselves against stresses caused by changes in the environment. Likewise, a complex apoptotic machinery is necessary for elimination of cells that are no longer needed in the organism or that have been terminally damaged. Hence, a carefully regulated balance between protecting and destructive signals is required. As T-lymphocytes are determinants of host defenses during infections, strict regulation of T-lymphocyte homeostasis is a prerequisite for a functional immune response. The T-lymphocyte population dramatically expands upon inflammatory responses and the excess cells need to be deleted to regain homeostasis after antigen clearance. The elimination of excess T-lymphocytes from the organism is conducted by activation-induced cell death, which involves death receptor signaling via CD95. The sensitivity to apoptosis mediated via CD95 is, therefore, a key determinant of T-lymphocyte persistence. Elevation of the body temperature during fever has been proposed to have a major impact on immune responses during infections. Fever influences the clonal expansion and proliferation of T-lymphocytes, and it may thereby modulate the survival and endurance of T-lymphocyte populations. In this study, we show that fever-like hyperthermia sensitizes activated human T-lymphocytes to CD95-mediated apoptosis. Interestingly, many of the proteins induced and/or activated during hyperthermia, such as the stress-induced MAP kinases and the molecular chaperone Hsp70, are not responsible for the hyperthermia-induced sensitization to CD95-mediated apoptosis, indicating that hyperthermia directly affects one or more components of the CD95-mediated apoptotic cascade. A modulator of death receptor signaling is the caspase-8 inhibitor c-FLIP, the expression of which is dynamically regulated. Hyperthermia triggers downregulation of both c-FLIP splicing variants, c-$FLIP_S$ and c-$FLIP_L$, with consequent sensitization to apoptosis mediated via CD95. Furthermore, c-FLIP downregulation and the subsequent sensitization are specific for hyperthermic stress. Finally, we demonstrate that the hyperthermia-mediated downregulation of c-FLIP as well as the sensitization to CD95-mediated apoptosis are due to increased ubiquitination and proteasomal degradation of c-$FLIP_S$. High quantities of c-FLIP and impaired lymphocyte apoptosis are associated with several autoimmune diseases. Our findings indicate that fever significantly influences the rate of lymphocyte elimination through c-$FLIP_S$ depletion, which might be beneficial for elimination of autoreactive lymphocytes otherwise able to induce autoimmune diseases or allergy. Such a general regulatory mechanism for lymphocyte removal has broad ramifications for the fever-mediated regulation of immune responses.

1. INTRODUCTION

Organisms are constantly exposed to different kinds of potentially harmful agents from the environment, such as viruses and microbes. To defend themselves against infections induced by such pathogens, organisms have developed a specialized immune system. The vertebrate immune system is comprised of both an innate immune response and a more sophisticated adaptive immune response. The innate immune response is mediated by physical barriers and phagocytosing cells that can directly destroy invading microorganisms. The adaptive immune system, on the other hand, functions specifically to recognize a particular antigen, either from an invading pathogen or a toxic molecule produced by it. Therefore, it is crucial that the adaptive immune system recognizes only harmful invaders, and does not react on molecules of the host itself. Occasionally the system fails to make a correct decision between foreign and self antigens and acts destructively against the host's own tissues. This may lead to autoimmune disease and be fatal for the organism. In the case of allergies, harmless molecules are recognized as harmful, inducing an inflammatory response.

The adaptive immune response is maintained by lymphocytes, which are specialists in recognizing antigens. While the B-lymphocytes function as antibody-producing cells, T-lymphocytes may be directly involved in killing infected host cells or assist other cells of the immune system. As lymphocytes are major regulators of the adaptive immune system, regulation of the lymphocyte number is of extreme importance to maintain a functional immune response. T-lymphocytes start to proliferate when they are introduced to an antigen by an antigen-presenting cell, leading to an increase in the T-lymphocyte population. When the antigen has been cleared out, it is crucial to eliminate excess activated T-lymphocytes not needed for the memory functions of the immune system. Active T-lymphocytes remaining in the organism may be autoreactive and impaired removal of activated T-lymphocytes has been associated with autoimmune diseases.

Activated T-lymphocytes are eliminated from the circulation by activation-induced cell death via death receptor-mediated apoptosis. The apoptotic cell death program is induced as a cause of both an increased expression of death ligands and an increased surface expression of the death receptors. Activation of death receptors engages the intracellular death program, which is mediated via a tightly regulated signaling machinery comprised of pro-apoptotic proteins involved in the execution of cell death as well as anti-apoptotic control proteins. The sensitivity towards death receptor-mediated apoptosis is determined by the balance between these death- and survival-promoting factors. The main apoptosis executioners are the proteolytical caspases, the enzymatic activity of which needs to be tightly regulated. The ability of the anti-apoptotic modulators to inhibit the death program is determined by their intracellular levels, which are regulated both by changes in gene expression and protein stability.

Elevated body temperatures associated with fever have been proposed to have a major impact on immune responses during infections, as fever influences the clonal expansion and proliferation of lymphocytes and enhances the lymphocyte-mediated cytotoxicity in target cells. In this thesis I have studied how fever-like hyperthermia influences the sensitivity of T-lymphocytes to death receptor-mediated apoptosis.

2. REVIEW OF THE LITERATURE

2.1. Programmed cell death determines tissue homeostasis

The adult human body consists of about 10^{13} cells that are organized into different tissues and that are differentiated for highly specific functions. The rate of cell division varies between cell types, but several millions of cells are produced in an adult body every second. To maintain homeostasis in tissues, the same amount of cells that are produced also needs to be removed to avoid cell accumulation or hyperplasia. On the other hand, too efficient removal of cells leads to atrophy or cell loss. Cells can die with different morphological patterns using different signaling pathways. Accidental cell death, also referred to as necrosis, is characterized by organelle swelling and rupture of the plasma membrane, leading to leakage of the intracellular components to the extracellular space. This kind of cell lysis is a pathological event that induces inflammation in the organism (reviewed by McConkey, 1998). To avoid inflammation, cells can be removed via so called programmed cell death.

Although dying cells were studied under the microscope by Walther Flemming in 1885 (reviewed by Lockshin and Zakeri, 2001), it was not until 1951 that cell death was described as a specific process (Glücksmann, 1951). The definition programmed cell death was used for the first time in 1964 (Lockshin and Williams, 1964). John Saunders described programmed cell death to be controlled by exogenous factors *in vivo* in 1966 (Saunders, 1966), although similar observations had already been made *in vitro* in 1934 (Fell and Canti, 1934). The morphological process of programmed cell death was first described as shrinkage necrosis in dying liver cells by John Kerr in 1971 (Kerr, 1971). The term apoptosis was introduced by John Kerr, Andrew Wyllie, and Sir Alastair Currie in 1972, who described a cell death process involving cell shrinkage, loss of contact with neighboring cells, formation of cytoplasmic vacuoles, plasma and nuclear membrane blebbing, and chromatin condensation (Kerr *et al*., 1972). Apoptosis is not the only type of programmed cell death, also other kinds of death pathways are strictly regulated and can thereby be described as programmed. Recommendations for classifying the different types of cell death have been made by the editors of the journal *Cell Death and Differentiation* (Kroemer *et al*., 2005). In this thesis I have concentrated on apoptotic cell death, other types of cell death will only be briefly mentioned.

To be able to digest all the components of the cell and induce the morphological changes that characterize apoptosis, an apoptotic breakdown machinery needs to be engaged. The caspases, a family of cysteine proteases, are involved in proteolytical processing of proteins in the dying cell. The caspases can activate different DNases such as CADs (caspase-activated DNases), which are involved in fragmenting DNA. Furthermore, the apoptotic cell needs to be engulfed by neighboring cells in order to avoid leakage of cytoplasmic components into surrounding tissues. In mammals, cell surface-exposed phosphatidylserine on dying cells serves as an “eat me” signal that surrounding phagocytosing cells recognize (reviewed by Danial and Korsmeyer, 2004). The apoptotic signaling pathways are summarized in a schematic figure on page 13 (Fig. I).

The genetic control of apoptotic cell death was determined in *C. elegans* by Sydney Brenner, Robert Horvitz, and John Sulston, who received the Nobel Prize in physiology or medicine for discovering cell death genes. During development of *C. elegans* from embryo to an adult organism the same 131 of the 1 090 cells produced undergo apoptosis. To understand the genetic control during development, *C. elegans* mutants were screened and specific genes required for apoptosis were identified. These genes were named *ced* (cell death abnormal) genes (reviewed by Metzstein *et al*., 1998). *ced-3* and its upstream activator *ced-4* were found to be required for execution of apoptosis, whereas the function of *ced-4* was shown to be blocked by the apoptosis inhibitor *ced-9*. Additionally, *egl-1* (egg lying defective) was shown to be able to inhibit the activity of *ced-9*. The mammalian homologues for *ced-3* and *ced-4* are the genes coding for caspases and Apaf-1, respectively (Yuan *et al*., 1993; Zou *et al*., 1997), and the gene products for *ced-9* and *egl-1* correspond to the mammalian anti-apoptotic Bcl-2 family-like proteins and pro-apoptotic BH3-only proteins (Hengartner and Horvitz, 1994; Conradt and Horvitz, 1998). The characterization of these genes provided evidence for the fact that the molecular mechanisms responsible for the cell death program have remained conserved during evolution.

The cell number during development is well controlled, as excess cells generated during development are removed by apoptosis. Also damaged and abnormal cells, such as T-lymphocytes that recognize self-antigens die from apoptosis. In addition, apoptosis is important when the structures in the adult body, such as the neural tube, fingers and toes are formed. Likewise, apoptosis is used for deletion of structures no longer needed, such as the tadpole of frogs (reviewed by Jacobson *et al*., 1997; Baehrecke, 2002). Since a balance between cell division and cell death is critical for maintaining tissue homeostasis, defects in the signaling pathways regulating apoptosis are associated with several types of human disease like cancer, autoimmune disease, and neurological disorders. One of the hallmarks of cancer is the capability of tumor cells to evade apoptosis (reviewed by Hanahan and Weinberg, 2000). Cancer cells have developed abilities to overcome death signals by both activation of mediators, such as the Bcl-2 family of anti-apoptotic proteins, the IAP family of apoptosis inhibitors, and the PI3K/Akt pathway, and by interfering with pro-apoptotic modulators such as p53 and the death receptor signaling pathway (reviewed by Schulze-Bergkamen and Krammer, 2004). Autoimmune disease can develop when autoreactive lymphocytes fail to undergo apoptosis, and death receptors and ligands have been suggested to be involved in tissue destruction during autoimmune disease (reviewed by O'Reilly and Strasser, 1999). Several neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and Huntington's disease are characterized by premature cell death of specific nerve cell populations. This premature death has been proposed to be a result of increased apoptotic signaling induced by the diseases (reviewed by Graeber and Moran, 2002). While defects in apoptotic signaling are responsible for development of several diseases, it is of great importance to understand the molecules involved in programmed cell death and the signaling mechanisms regulating this process.

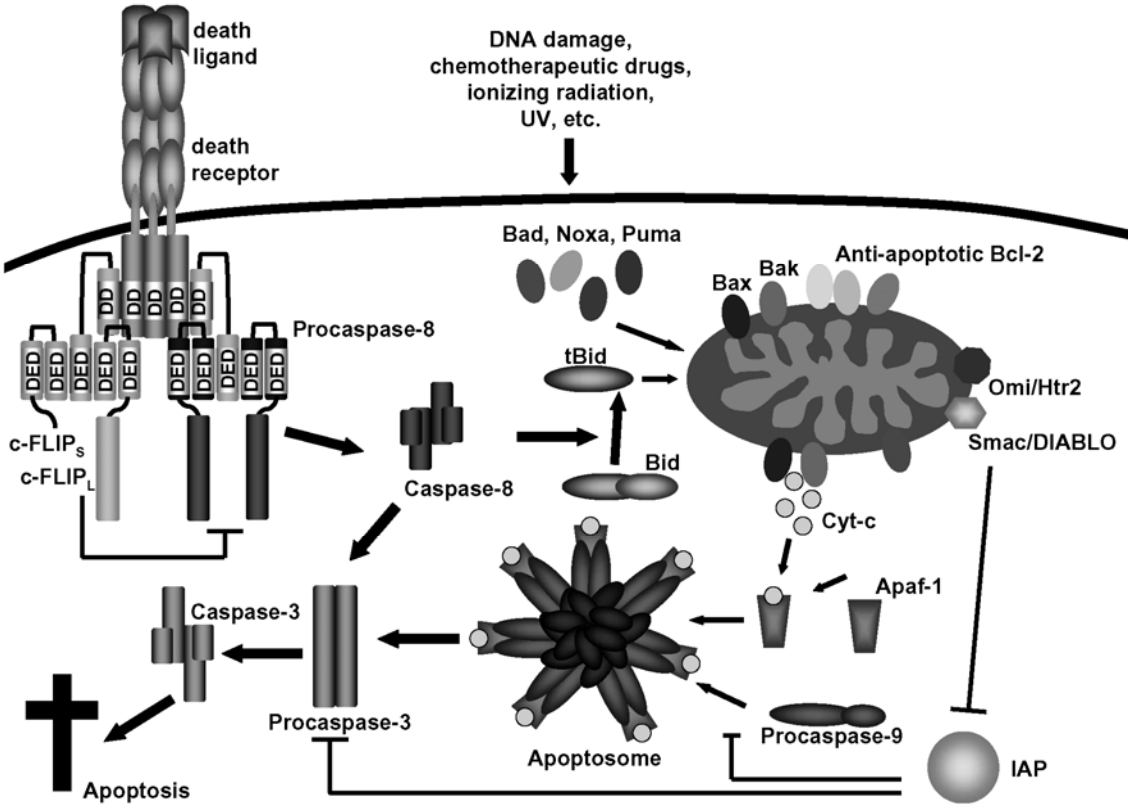


Figure I. Apoptotic signaling pathways. Cell death can be triggered either by the extrinsic apoptotic pathway mediated via death receptors or by the mitochondria-mediated intrinsic apoptotic pathway activated by several stresses lethal to the cell. Formation of the DISC (death-inducing signaling complex) is induced when a death ligand activates its corresponding receptor. Death effector domain-containing initiator caspases, such as caspase-8 are activated in the DISC. The active caspase-8 can further activate effector caspases such as caspase-3. Caspase-8 activation can be inhibited by c-FLIP, which competes with caspase-8 for binding to the DISC. Initiation of the intrinsic apoptotic pathway is regulated by the Bcl-2 family of anti- and pro-apoptotic proteins, the main guardians of the mitochondria. Mitochondrial outer membrane permeabilization leads to release of cytochrome *c*, Smac/DIABLO, and OMI/Htr. Cytochrome *c* is required for activation of Apaf-1 and apoptosome formation. Caspase-9, activated in the apoptosome, is a potent activator of downstream effector caspases. When released from the mitochondria, Smac/DIABLO and OMI/Htr are able to restrain the IAPs and are thereby important for efficient caspase activation. Active caspase-8 can also cleave Bid to the active tBid, which can further inactivate the anti-apoptotic Bcl-2 family members and activate the pro-apoptotic Bcl-2 family members. The death receptor-mediated extrinsic apoptotic pathway can thereby employ the intrinsic mitochondrial signaling machinery to induce more efficient caspase activation.

2.2. Caspases as regulators of death signaling

The caspases (cysteinyl aspartate proteinases) belong to an evolutionary conserved family of cysteine proteases that has been identified in all vertebrate species. The important role for caspases as apoptotic proteases was discovered in *C. elegans*, in which the death gene *ced-3*, the nematode homologue of ICE (interleukin-1β-processing enzyme), was shown to be indispensable for the execution of cell death (Yuan *et al*., 1993). ICE, later renamed to caspase-1, was the first member of the caspase family that was cloned and was shown to be

required for processing interleukin-1β (Cerretti *et al*., 1992; Thornberry *et al*., 1992). CED-3 is still the only caspase described in *C. elegans*, but eleven caspases have been described in humans, ten in mice, and seven in *Drosophila*. Depending on the size of the caspase prodomains, the caspases can be divided into initiator or apical caspases and effector or executioner caspases (Fig. II). The human initiator caspases, caspase-1, caspase-2, caspase-4, caspase-5, caspase-8, caspase-9, caspase-10, and caspase-12 possess long prodomains that contain motifs for interaction with upstream adaptor proteins. The prodomains of caspase-8 and caspase-10 consist of two death effector domains (DEDs), whereas the other initiator caspases contain a caspase activation and recruitment domain (CARD). The murine genome lacks caspase-4, caspase-5, and caspase-10. Instead, the murine caspase-11 is similar to human caspase-4 and caspase-5 (reviewed by Kumar, 2007). Among the initiator caspases, the human caspase-1, caspase-4, caspase-5, and caspase-12 are mainly involved in the control of inflammatory responses. The recruitment of these inflammatory caspases to a complex termed the inflammasome leads to processing and secretion of pro-inflammatory cytokines (reviewed by Petrilli *et al*., 2005; Martinon and Tschopp, 2007). Also the apoptotic caspases have been described to have apoptosis-independent functions regulating differentiation, cell proliferation, cell cycle progression, and cell migration (reviewed by Algeciras-Schimnich *et al*., 2002a; Lamkanfi *et al*., 2007).

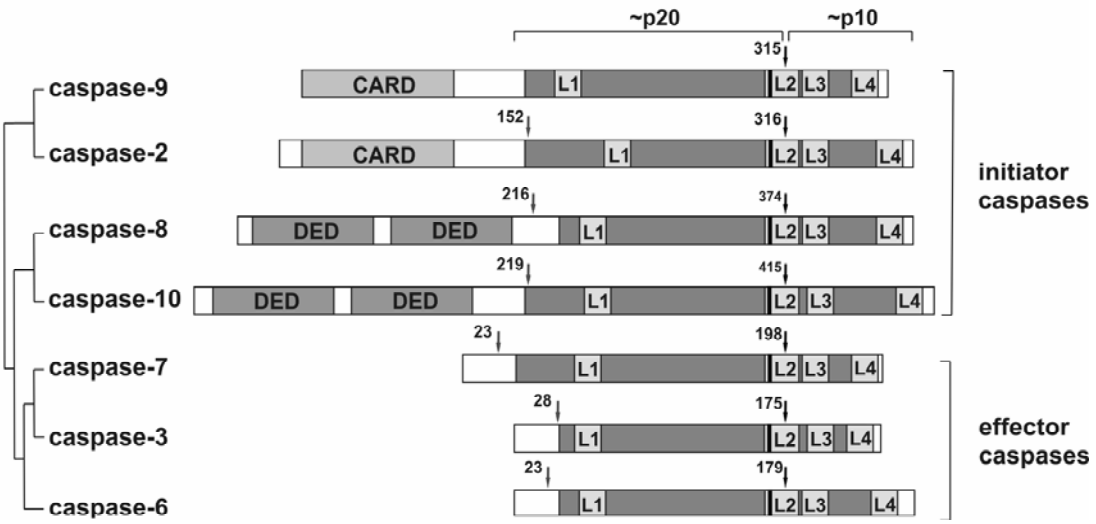


Figure II. Schematic presentation of the human apoptotic caspases. The initiator caspases have long N-terminal prodomains containing either two DEDs or one CARD, whereas the effector caspases have very short prodomains. The position of the first intra-chain cleavage between the large p20 and the small p10 subunits and the second intra-chain cleavage separating the large subunit form the prodomain are highlighted with arrows. The surface loops L1-L4 shaping the catalytic surface of the caspases are indicated as light grey boxes and the catalytically active cysteine is indicated by a black line in the N-terminal part of L2 (modified from Shi, 2002; Riedl and Shi, 2004).

Two major apoptotic pathways have been characterized in mammalian cells. The intrinsic apoptotic pathway is triggered upon different types of cellular stresses, such as DNA damage, chemotherapeutic drugs, and ionizing radiation, whereas the extrinsic apoptotic pathway, responsible for elimination of unwanted cells during development and education of the immune system is activated upon death receptor ligation (Fig. I). The DED-containing caspases function as initiator caspases for the extrinsic apoptotic pathway, whereas the

CARD-containing caspases mediate intrinsic apoptotic signaling. Since the effector caspases caspase-3, caspase-6, and caspase-7 are activated by upstream caspases, they do not need long prodomains with protein interaction motifs. The main responsibility for the initiator caspases is to cleave and activate effector caspases, whereas the effector caspases are responsible for proteolytical processing of other proteins. Caspase-9 is regarded as the canonical caspase for the intrinsic apoptotic pathway, caspase-8 as the key initiator for the extrinsic apoptotic pathway, and caspase-3 as the main effector caspase (reviewed by Kumar, 2007).

2.2.1. Initiation of death signaling by caspases

Caspase-8 and caspase-10 are the initiator caspases of the extrinsic apoptotic signaling pathway. These initiator caspases contain two DEDs for recruitment to the adaptor protein FADD (Fas-associated death domain), a component of the death-inducing signaling complex (DISC) formed at the intracellular part of activated death receptors. Caspase-8 (FLICE from FADD-like ICE/Mch5/Mach) was originally identified by yeast two-hybrid screens with the DED of FADD as bait and by sequencing components of immunoprecipitates of the CD95-DISC (Boldin *et al*., 1996; Muzio *et al*., 1996). Caspase-8, which is expressed as two splicing variants, caspase-8a and caspase-8b (Scaffidi *et al*., 1997), is recruited to the DISC upon death receptor triggering, where it is activated (Medema *et al*., 1997).

The role of caspase-10 (FLICE2/Mch4) in death receptor-mediated apoptosis was controversial in the initial studies. Some groups failed to detect interactions with caspase-10 and FADD and suggested that caspase-10 would not be part of the DISC (Bodmer *et al*., 2000; Sprick *et al*., 2000). However, certain commercially produced antibodies recognized Hsp60 instead of caspase-10, which might explain these results (Kischkel *et al*., 2001). Later it has been shown that caspase-10 is both recruited to and activated in the CD95 and TRAIL-R (TNF-related apoptosis-inducing ligand receptor) DISCs (Kischkel *et al*., 2001; Wang *et al*., 2001). At the protein level, caspase-10 exists as three different splicing variants, caspase-10a, caspase-10c, and caspase-10d, caspase-10b and caspase-10d are the same splicing product described by two different groups (Fernandes-Alnemri *et al*., 1996; Vincenz and Dixit, 1997; Ng *et al*., 1999). Caspase-10a and caspase-10d are the caspase-8 homologues extensively studied in the context of death receptor-mediated apoptosis. Caspase-10c, on the other hand, is a truncated form of caspase-10, lacking the caspase domain. No biological function of caspase-10c has been described in the literature, but due to its homology with c-FLIP$_S$, caspase-10c might have a similar apoptosis-inhibiting function. Isoform-specific downregulation of caspase-10c sensitizes Jurkat cells to both TRAIL- and CD95-mediated apoptosis, supporting the idea that caspase-10c would have anti-apoptotic properties (unpublished data by Anna Kreutzman and Annika Meinander). On the contrary, overexpression of caspase-10c has been shown to be cytotoxic (Ng *et al*., 1999).

Caspase-8 and caspase-10 seem to have both overlapping and distinct functions. Some reports suggest that caspase-10 is fully able to substitute for caspase-8 (Kischkel *et al*., 2001; Wang *et al*., 2001; Milhas *et al*., 2005). On the other hand, death receptor-mediated apoptosis is completely inhibited in caspase-8-deficient Jurkat cells, indicating that caspase-10 cannot

substitute for caspase-8 (Juo *et al*., 1998; Sprick *et al*., 2000 and 2002). While the mouse genome does not contain caspase-10, caspase-8 is the only initiator caspase in the murine CD95 and TRAIL-R DISCs, and is thereby required for death receptor-mediated apoptosis in mice (Reed *et al*., 2003). Caspase-8 and caspase-10 have been shown to be involved in activation of the NF-κB (nuclear factor κB) signaling pathway in a similar fashion (Shikama *et al*., 2003; Takahashi *et al*., 2006). In addition, both caspase-8 and caspase-10 are able to cleave the pro-apoptotic Bcl-2 family member Bid (Milhas *et al*., 2005) and RIP-1 (receptor-interacting protein-1), a protein involved in both apoptosis and NF-κB signaling induced via death receptors (Fischer *et al*., 2006). When comparing the ability of these initiator caspases to cleave peptides of a combinatory library, they seem to differ in substrate-specificity to some extent (Thornberry *et al*., 1997).

While *caspase-8*$^{-/-}$ mouse embryonic fibroblasts (MEFs) are resistant to CD95-mediated apoptosis, caspase-8 deficiency does not affect intrinsic apoptotic signaling (Varfolomeev *et al*., 1998). Caspase-8 deficiency is embryonic lethal around E12.5. The knock-out mice display impaired heart muscle development and accumulation of erythrocytes in the abdomen and in the blood vessels of the trunk (Table I). The severe proliferation defect of the lymphocytes in *caspase-8*$^{-/-}$ mice indicates important functions for caspase-8 outside regulation of cell death (Varfolomeev *et al*., 1998). Humans with mutations in caspase-8 or caspase-10 exhibit an autoimmune lymphoproliferative syndrome, ALPS, caused by defects in lymphocyte apoptosis. As caspase-8 mutations additionally lead to problems in lymphocyte activation and results in immunodeficiency, these mutations are more severe than caspase-10 mutations (Wang *et al*., 1999; Chun *et al*., 2002).

Caspase-9 is the only initiator caspase proposed to be involved in the mitochondrial apoptosis signaling and cells from *caspase-9*$^{-/-}$ mice are resistant to apoptosis mediated via the intrinsic apoptotic pathway (Hakem *et al*., 1998; Kuida *et al*., 1998). Caspase-9 contains an N-terminal CARD by which it can interact with Apaf-1 (apoptotic protease-activating factor-1). The interaction between caspase-9 and Apaf-1 in the apoptosome leads to caspase-9 activation (Zou *et al*., 1999).

Although caspase-2 contains a CARD, it is not associated with Apaf-1 in the apoptosome (Read *et al*., 2002). Caspase-2, which has been proposed to have a role in apoptosis induced by DNA damage (Lassus *et al*., 2002) and neurotrophic deprivation (Troy *et al*., 2001), is activated in another complex termed the PIDDosome (Tinel and Tschopp, 2004). The PIDDosome consists of the adaptor protein RAIDD (RIP-associated ICH-1/CED-3-homologue with death domain), PIDD (p53-induced protein with a death domain), and caspase-2. The adaptor protein RAIDD is associated with PIDD by death domain (DD) interactions. RAIDD contains a CARD in addition to its DD, and can recruit caspase-2 by CARD-CARD interactions (Tinel and Tschopp, 2004). Caspase-2 is suggested to work upstream of the mitochondria and to be required for mitochondrial outer membrane permeabilization (MOMP) in response to cytotoxic stress (Lassus *et al*., 2002). In addition, active caspase-2 has been suggested to contribute to caspase-8 activation in the DISC and thereby enhance CD95- and TRAIL-R-mediated apoptosis (Droin *et al*., 2001; Shin *et al*., 2005). Caspase-2 bound to the CD95-DISC was, however, recently shown not to be involved

in CD95-mediated apoptosis despite the receptor association-induced activation of caspase-2 (Lavrik *et al*., 2006).

Table I. Phenotypes of mutations and deficiencies in caspases and apoptotic signaling proteins.

Gene	Knock-out/mutant phenotypes	References
caspase-8	- deficiency is embryonic lethal - deficiency leads to defects in cardiac development - deficiency leads to defects in T-lymphocyte development - deficient MEFs are resistant to death receptor-mediated apoptosis - mutants are immunodeficient	Varfolomeev *et al*., 1998 Chun *et al*., 2002
caspase-10	- no caspase-10 homologue in mice - mutations are associated with ALPS II	Wang *et al*., 1999
caspase-9	- deficiency is perinatally lethal - MEFs are resistant to stress-induced apoptosis	Hakeem *et al*., 1998 Kuida *et al*., 1998
caspase-2	- deficient mice are viable and fertile	Kumar, 2007
caspase-3	- deficiency is perinatally lethal in mixed genetic background - deficient mice are viable with reduced fertility in pure genetic background	Kuida *et al*., 1996 Lakhani *et al*., 2006
caspase-7	- deficient mice are viable and fertile	Lakhani *et al*., 2006
caspase-3/7	- deficiency is perinatally lethal - deficiency leads to defects in cardiac development - deficient MEFs are resistant to apoptosis	Lakhani *et al*., 2006
caspase-6	- deficient mice are normal	Takahashi *et al*., 1996
FADD	- deficiency is embryonic lethal - deficiency leads to defects in cardiac development - deficiency leads to defects in T-lymphocyte development - deficient MEFs are resistant to death receptor-mediated apoptosis	Yeh *et al*., 1998 Zhang *et al*., 1998
cflar	- deficiency is embryonic lethal - deficiency leads to defects in cardiac development - deficiency leads to defects in T-lymphocyte development - deficient MEFs are sensitive to death receptor-mediated apoptosis	Yeh *et al*., 2000 Chau *et al*., 2005 Zhang and He, 2005
CD95/CD95L	- mutations are associated with ALPS I - mutations (lpr and gld) lead to lymphoproliferative disorders, lymphadenopathy, splenomegaly, defects in AICD, and elevated levels of autoantibodies	Nagata and Suda, 1995 Rieux-Laucat *et al*., 2003

2.2.2. Caspases as executioners of cell death

The initiator caspases activated either in the death receptor complex or in the apoptosome are mature to activate downstream effector caspases such as caspase-3 (CPP32), caspase-6, and caspase-7. The roles of the effector caspases in programmed cell death have been studied in knock-out mice. The phenotypes of the different *caspase-3* knock-out mice depend on their genetic background. In a genetically mixed 129/SvJ x C57BL/6 background, caspase-3 deficiency is prenatally lethal (Kuida *et al*., 1996), whereas the *caspase-3*$^{-/-}$ mice with a pure C57BL/6 background are viable. Also the *caspase-7*$^{-/-}$ mice are viable, but the *caspase-3/caspase-7* double knock-out mice die rapidly after birth displaying defects in heart development (Table I). MEFs derived form the double knock-out animals are resistant to apoptosis induced by UV, staurosporine, CD95L (CD95 ligand), and TNF-α (tumor necrosis factor α), proposing that these effector caspases are required for execution of cell death mediated via both the extrinsic and intrinsic apoptotic pathways (Lakhani *et al*., 2006). Caspase-6 is not as extensively studied as caspase-3 and caspase-7, and the exact role for

caspase-6 still remains enigmatic. Caspase-6 has been suggested to be involved in apoptotic cleavage of lamins (Takahashi *et al*., 1996), but does not seem to be indispensable for cell death signaling since *caspase-6*$^{-/-}$ mice develop normally (Zheng *et al*., 1999). As the phenotypes of mice deficient in a single effector caspase are not as severe as the phenotypes of the double knock-outs, the effector caspases might be able to compensate for each other.

The apoptotic caspases are crucial for both initiation and execution of cell death because of their capacity to cleave protein substrates. Some caspase targets are activated by processing, whereas cleavage of other substrates leads to degradation or inactivation. Several structural proteins such as the nuclear lamins and cytoskeletal proteins like actin and vimentin are targets for cleavage by caspases. Proteins involved in DNA repair such as Poly(ADP-ribose) polymerase (PARP) are cleaved and inactivated by caspases to ensure efficient fragmentation of the chromatin in an apoptotic cell. A strikingly big percentage of the caspase substrates are signaling proteins such as the kinases PKC (protein kinase C), Raf-1, and Akt. Finally, the DNase CAD is activated when caspases cleave ICAD, the inhibitor of CAD, leading to chromosome degradation (reviewed by Earnshaw *et al*., 1999). One of the main tasks of the caspases is to activate each other. The initiator caspases activate the effector caspases by cleavage; caspase-8, caspase-10, and caspase-9 can all cleave and activate caspase-3 and caspase-7 (Fernandes-Alnemri *et al*., 1996; Muzio *et al*., 1997; Srinivasula *et al*., 1998). Active caspase-3 can further cleave and activate caspase-6 (Srinivasula *et al*., 1996). Caspase-2, which is classified as an initiator caspase has, however, not been shown to be able to cleave and activate downstream caspases (reviewed by Kumar, 2007).

Since the caspases are very potent inducers of cell death, they have to be carefully regulated to avoid accidental caspase activation. Most of the caspases are constitutively expressed, and therefore they have to be regulated at the post-translational level. One mechanism of caspase inhibition can be provided by the IAPs (inhibitors of apoptosis). The effector caspases, caspase-3 and caspase-7, as well as the initiator caspase-9, are IAP targets (reviewed by Salvesen and Duckett, 2002; Ho and Hawkins, 2005). Both the viral and the cellular forms of FLIP (FLICE-inhibitory protein) are efficient inhibitors of the initiator caspases in the DISC. As apoptosis is a potent mechanism for elimination of virus-infected cells, it is not surprising that many apoptosis-inhibiting genes have evolved in viruses. In addition to the viral FLIPs, viral homologues of the IAPs and the anti-apoptotic Bcl-2 proteins have been found. The baculoviral protein p35 and the serpin CrmA (cytokine response modifier gene A), derived from a cowpox virus, can function as caspase pseudo-substrates and inhibit activation of several caspases (reviewed by Degterev *et al*., 2003; Ho and Hawkins, 2005).

2.2.3. The mechanism of caspase activation

The exact mechanisms of caspase activation are still controversial. While it seems clear that cleavage is not the only model for activation of caspases, other models involving allosteric interactions and induced proximity have been suggested. All caspases are synthesized as zymogens or procaspases with an N-terminal prodomain followed by a large subunit (p20) and a small subunit (p10). The mechanism of caspase activation is determined by the prodomain of each caspase. The effector caspases, which lack interaction motifs in their N-

terminal prodomains, are activated upon cleavage by upstream initiator caspases (Chai *et al*., 2001; Riedl *et al*., 2001; Kang *et al*., 2002). When caspases are activated by cleavage, the first step is cleavage after a specific internal aspartic acid residue between the small p10 subunit and the large p20 subunit (Fig. II). The p10 subunit is still associated with the p20 subunit after this cleavage. In the second step, the prodomain is cleaved off, separating the prodomain from the activated caspase-like domains. The initiator caspases are monomeric as procaspases, whereas the effector caspases exist as head-to-tail dimers both as inactive zymogens and as activated caspases (Fig. III) (Wei *et al*., 2000; Chai *et al*., 2001; Riedl *et al*., 2001).

The initiator caspases have been suggested to be able to self-activate upon oligomerization (Boatright *et al*., 2003). The DED-containing caspases are oligomerized by interactions between the N-terminal DED of the caspase and the DED-containing adaptor protein FADD, a component of the DISC, which is assembled upon death receptor ligation. The oligomerized zymogen has some enzymatic activity, but proteolytic cleavage seems to be necessary for full activation of the FADD-bound caspases. In this induced proximity model, the oligomerization of the zymogen is enough to induce the autoprocessing of caspase-8 to its active form (Muzio *et al*., 1998; Boatright *et al*., 2003; Donepudi *et al*., 2003). As caspase-10 and caspase-8 have similar DEDs, they are probably activated in a similar fashion. The auto-cleavage of caspase-8 proceeds in two steps, the first cleavage at aspartic acid 374 forms the p10 and the p43/41 subunit, which still contains the DEDs. The second cleavage at aspartic acid 216 separates the prodomain from the p18 large subunit, releasing the catalytically active p18-p10 caspase subunits that are mature to further process their target substrates in the cytosol (Medema *et al*., 1997; Chang *et al*., 2003).

Caspase-9 is activated upon MOMP and cytochrome *c* release. Activation of caspase-9 requires binding to the adaptor protein Apaf-1 in the apoptosome, inducing oligomerization of caspase-9. Caspase-9 seems to be unique among the caspases, since it does not require processing for activation. The active site of caspase-9 is hidden in the monomeric procaspase, but is exposed upon the conformational change induced by oligomerization in the apoptosome. The apoptosome thereby serves as an allosteric regulator for caspase-9 dimerization. As the other caspases form activated dimers with one active site per monomer, the caspase-9 dimer has been suggested to have only one active site (Renatus *et al*., 2001). Caspase-9 dimerization has, however, recently been shown to induce auto-cleavage of the active zymogen in an induced proximity fashion, similarly to caspase-8 (Pop *et al*., 2006). Nevertheless, caspase-9 processing may occur during apoptotic signaling regardless of whether it is required for its activation. The activated caspase-9 is a potent activator of downstream effector caspases (Li *et al*., 1997; Yin *et al*., 2006).

When the structure of the different types of caspases was resolved, valuable information for predicting mechanisms of caspase activation became available. To be recognized, a caspase substrate has to contain a tetrapeptide, P4-P3-P2-P1. The caspase substrates are cleaved after the P1 residue, which generally is an aspartic acid (Sleath *et al*., 1990; Thornberry *et al*., 1992). Most caspases prefer a glutamic acid residue at the P3 position, whereas the preferred residue at position P4 differs between the caspases (Talanian *et al*., 1997; Thornberry *et al*., 1997). The amino acid-specificity of the P2 residue allows for a greater degree of variation,

since the side chain of this residue does not face the active site of the caspase (Sleath *et al*., 1990; Talanian *et al*., 1997). The active site of the caspases is composed of a surface motif formed by four flexible surface loops (L1-L4) (Figs. II and III). Together the L1, L2, and L3 loops form the substrate-recognizing site that binds the aspartic acid P1 residue of the substrate. L2 harbors the catalytically active cysteine that is responsible for cleavage of the substrate (reviewed by Shi, 2002). The side chain of the P4 residue is recognized by L4, which differs between the caspases (Thornberry *et al*., 1997; Wei *et al*., 2000). Upon activation, caspases are cleaved after the P1 aspartic acid that is located between the large p20 subunit and the small p10 subunit in L2 (Fig. II). After cleavage, the catalytically active cysteine is exposed at the N-terminal segment of the cleaved L2 loop (Fig. III). In an active caspase dimer, the C-terminal part of the cleaved L2 stabilizes the neighboring activated monomer (reviewed by Shi, 2002). The multiple restrictions required for acceptance as a caspase substrate allows for a conserved substrate-specificity as well as for individuality between the different caspase family members. Thereby, the activity of caspases can be precisely regulated, making caspase activation a secure mechanism for execution of cell death.

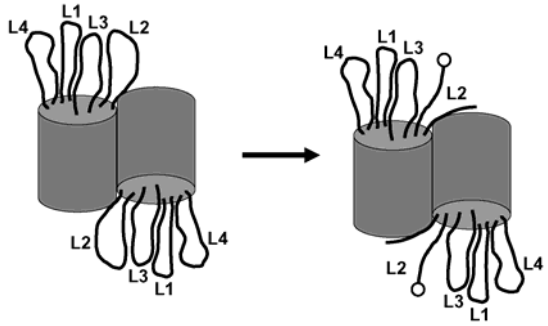


Figure III. Mechanisms of caspase activation. The inactive caspase is here illustrated as a dimer, since the model is based on the mechanism of effector caspase activation. The L1, L2, and L3 loops form the substrate-recognizing site. The structure of L4 varies between different caspases. Upon activation, the caspases are cleaved in L2, resulting in exposure of the catalytically active cysteine, indicated by a circle (modified from Shi, 2002; Riedl and Shi, 2004).

2.2.4. Detection of caspase activation and execution of apoptosis

Since caspases are potent mediators of apoptotic signaling, caspase activation is often used as a marker to detect apoptotic cell death. The activity of effector caspases can be measured as caspase cleavage, since the effector caspases are cleaved upon activation. Caspase activity can also be measured by detecting cleavage of caspase substrates such as PARP. Several assays for detection of the enzymatic activity of caspases have been developed (reviewed by Kohler *et al*., 2002). Other methods for apoptosis detection are assays that measure DNA fragmentation, cell membrane alterations, and nuclear morphology. DNA fragmentation can be measured by detection of DNA laddering by electrophoresis or by the TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) enzymatic labeling assay. In the TUNEL assay, the terminal deoxynucleotidyl transferase is able to label blunt ends of double-stranded DNA breaks with labeled dUTPs. Membrane alterations can be measured as changes in membrane composition or integrity. Upon induction of apoptosis cells lose their ability to transport phosphatidyl serine from the outer to the inner leaflet of the plasma membrane, leading to an accumulation of phosphatidyl serine on the outer membrane. This increase in surface phosphatidyl serine can be detected by staining with Annexin, a protein that labels phospholipids. Apoptosis is associated with chromatin condensation, which can be detected by chromatin staining both by microscopy and flow cytometry (reviewed by Saraste and

Pulkki, 2000). In addition, detection of modifications of other apoptotic signaling molecules can be used as markers for initiation and execution of apoptosis. Since apoptosis can be induced independently of caspases, measurement of caspase activity is not suitable for apoptosis detection in all systems. Cell death in general can be measured as LDH (lactate dehydrogenase) release. LDH is an enzyme that catalyzes the conversion of lactate to pyruvate and is released upon loss of membrane integrity in dying cells. LDH activity can be measured in cell suspensions, since LDH is able to reduce a tetrazolium salt to form a colored formazan, a product which may be quantified colorimetrically. Cell death can also be determined as loss of cell viability by measuring the activity of metabolic markers. The MTT (methylthiazoletetrazolium) assay is a colorimetric assay for detection of mitochondrial enzyme activity. MTT is reduced to a colorimetrically detectable formazan by mitochondrial reductases. Since these enzymes are active only in functional mitochondria, the conversion is directly related to the number of living cells (reviewed by Sgonc and Gruber, 1998).

2.3. Mitochondrial apoptotic signaling

2.3.1. The mitochondrion as a determinant of cell death

Apoptosis can be induced both by signals descending from within the cell, via the intrinsic apoptotic pathway, or via extrinsic signaling mediated by death receptors. Several apoptosis-inducing stresses such as DNA damage, cytokine deprivation, and cytotoxic drugs mediate apoptotic signaling via an intrinsic apoptotic pathway, involving the mitochondria. Activation of the caspases of the mitochondrial pathway requires mitochondrial outer membrane permeabilization, MOMP, leading to release of apoptotic modulator proteins otherwise residing in the intermembrane space (reviewed by Chipuk *et al*., 2006).

The Bcl-2 family of pro- and anti-apoptotic proteins

Members of the Bcl-2 (B-cell lymphoma) family of proteins are critical regulators of MOMP and the mitochondrial death pathways. The *bcl-2* proto-oncogene was discovered in human B-cell lymphomas and gave this protein family its name. Initially, Bcl-2 was determined to be anti-apoptotic as its homologue in *C. elegans*, CED-9, but later a whole family of Bcl-2 proteins, both anti- and pro-apoptotic, has been described (reviewed by Gross *et al*., 1999). The pro- and anti-apoptotic members of the Bcl-2 family can be distinguished by the number of Bcl-2 homology domains, termed BH1, BH2, BH3, and BH4 (Fig. IV). The anti-apoptotic Bcl-2 family members, Bcl-2, Bcl-XL, and Mcl-1, contain all four BH domains. The pro-apoptotic Bcl-2 members can be divided in two groups, multidomain members containing three BH domains (BH1, BH2, and BH3) such as Bax (Bcl-2-associated X protein) and Bak (Bcl-2 homologous antagonist/killer), and BH3-only proteins, such as Bid (BH3-interacting domain death agonist), Bad (Bcl-2-associated death protein), Bim (BH3-interacting mediator of cell death), Puma (p53-upregulated modulator of apoptosis), Noxa, and the homologue in *C. elegans* EGL-1. Characteristic for the Bcl-2 protein family is their ability to form homo- and heterodimers via their BH domains, either activating or neutralizing each other (reviewed by Gross *et al*., 1999; Chipuk *et al*., 2006). The pro-apoptotic multidomain proteins Bax and Bak are required for intrinsic apoptotic signaling and MOMP (Wei *et al*., 2001), whereas the

anti-apoptotic Bcl-2 proteins prevent MOMP. Activation of Bax and Bak results in formation of a pore in the outer mitochondrial membrane, allowing release of cytochrome *c* as well as other proteins from the intermembrane space. Permeabilization of the outer mitochondrial membrane simultaneously leads to a loss of mitochondrial transmembrane potential, resulting in disturbance of ATP synthesis. Cardiolipin has been suggested to be important for MOMP, but since Bax can induce MOMP also in yeast that lacks cardiolipin, it is questionable how important cardiolipin is for MOMP (reviewed by Chipuk *et al*., 2006).

The activity of the BH3-only pro-apoptotic Bcl-2 proteins is regulated both transcriptionally and by post-translational modifications. The transcription of Noxa and Puma is regulated by the tumor suppressor p53, which is able to induce Noxa and Puma expression in response to DNA damage (reviewed by Puthalakath and Strasser, 2002). Bim, which has been implied to be an important signal transducer in detachment-induced cell death, is kept sequestered in a microtubule-associated dynein motor complex in the cytoplasm in healthy cells (reviewed by Gross *et al*., 1999). The transcription of Bim has been suggested to be induced via MAPK (mitogen-activated protein kinase) and PI3K pathways. Several of the BH-only proteins are expressed constitutively and are thereby activated by different post-translational modifications. Phosphorylation of Bad at serines 112 and 136 by Akt keeps Bad sequestered in the cytoplasm by 14-3-3, a protein with the ability to bind and regulate a multitude of signaling proteins. Growth factor withdrawal results in dephosphorylation of Bad, a subsequent release from 14-3-3, and Bad relocalization to the mitochondria. Bid is activated when cleaved by the initiator caspases of the DISC, caspase-8 and caspase-10. The truncated Bid, tBid, can be N-myristoylated, which is required for targeting tBid to the mitochondria. tBid has a high affinity for both anti-apoptotic Bcl-2 and for pro-apoptotic Bax and Bak (reviewed by Puthalakath and Strasser, 2002).

The pro-apoptotic BH3-only proteins interfere with the functions of the anti-apoptotic Bcl-2 proteins and/or activate Bax and Bak. There are four models described, explaining how the Bcl-2 family of proteins interact to induce and inhibit MOMP. According to the first model (i), Bax and Bak are constitutively active, but are restrained by the anti-apoptotic Bcl-2 proteins. The BH3-only proteins neutralize the anti-apoptotic Bcl-2 proteins releasing Bax and Bak. However, different anti-apoptotic Bcl-2 proteins are neutralized by different BH3-only proteins, requiring the right combination of BH3-only proteins to inactivate the anti-apoptotic Bcl-2 proteins (Chen *et al*., 2005; Willis *et al*., 2005). The second model (ii) proposes that Bax and Bak are not constitutively active, but are directly activated by the BH3-only proteins Bid and Bim. Since these BH3-only proteins can be neutralized by anti-apoptotic Bcl-2 proteins, additional BH3-only proteins need to act as de-repressors on the anti-apoptotic Bcl-2 family members. In this model the different BH3-only proteins would not only have different targets, but also different roles, either as direct activators of Bax and Bak or as de-repressors, inactivating the anti-apoptotic Bcl-2 family members (Letai *et al*., 2002; Kuwana *et al*., 2005). In the third model (iii), it is suggested that cells can be primed for death by direct activator BH3-only proteins being sequestered by anti-apoptotic Bcl-2 family proteins. When the de-repressor BH3-only proteins are activated, they displace the direct activators from the anti-apoptotic Bcl-2 proteins, leading to activation of Bax and Bak (Certo *et al*., 2006). Finally, the fourth model (iiii) indicates that displacement of the direct activator BH3-only proteins by de-repressor BH3-only proteins does not only lead to release

of the direct activators, but might also release Bax and Bak from the anti-apoptotic Bcl-2 proteins, which induces MOMP (reviewed by Green, 2006).

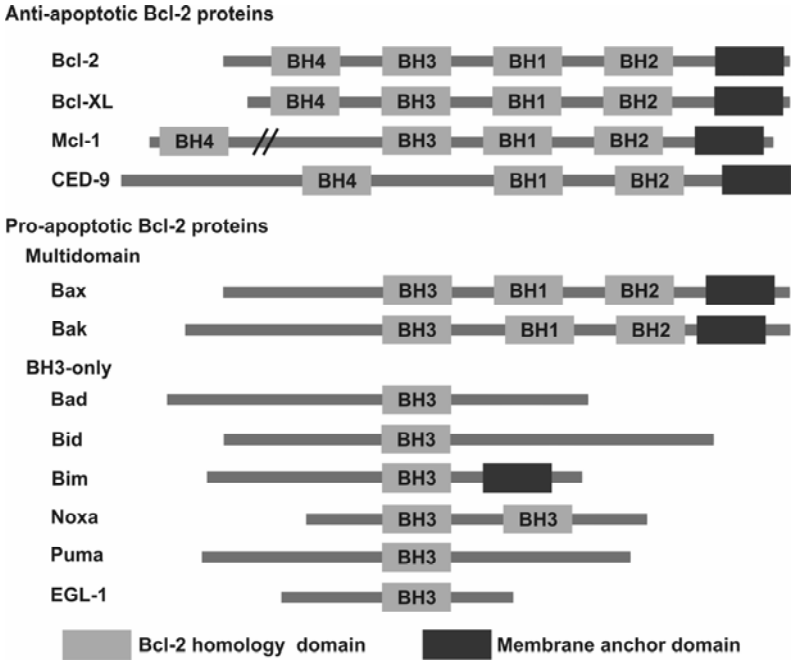


Figure IV. The Bcl-2 family of anti- and pro-apoptotic proteins. The proteins of the Bcl-2 family can be divided into three different subfamilies, the anti-apoptotic Bcl-2 subfamily, the pro-apoptotic multidomain subfamily, and the BH3-only subfamily. The Bcl-2 subfamily contains all four BH domains, the pro-apoptotic multidomain subfamily contains BH1, BH2 and BH3 domains, whereas the BH3-only subfamily contains only the BH3 domain as indicated by its name. Some of the Bcl-2 proteins contain an additional C-terminal membrane anchor domain (modified from Kuwana and Newmeyer, 2003).

Other proteins involved in mitochondrial death signaling

In addition to the Bcl-2 family of proteins, other apoptosis inducers and inhibitors are involved in the mitochondrial intrinsic signaling pathway. The IAPs are a family of proteins that function as potent caspase inhibitors. The IAPs were originally discovered in baculoviral genomes, but cellular orthologues in several species, such as yeasts, nematodes, flies, and mammals have subsequently been described. Eight human IAPs have been found, among them X-linked IAP (XIAP), c-IAP1, c-IAP2, and survivin. All IAPs contain one or more zinc-binding BIR (baculoviral IAP repeat) domains. The BIR domains bind to caspases and are responsible for the anti-apoptotic properties of the IAPs. In addition, most of the IAPs contain a C-terminal zinc-binding motif, a RING (really interesting new gene) domain. The RING domain can function as an E3 ligase targeting both the protein itself as well as other target proteins for ubiquitination and degradation (reviewed by Salvesen and Duckett, 2002). The IAPs do not participate in inhibition of caspase zymogen activation, but they bind to and inhibit activated caspases. The different IAPs target caspases at different levels of the apoptotic signaling cascade (reviewed by Salvesen and Duckett, 2002). Whereas c-IAP1 and c-IAP2 are involved in caspase-8 inhibition by binding TRAF1 and TRAF2 (TNF-R-associated factor), thereby switching TNF receptor (TNF-R) signaling towards NF-κB (nuclear factor κB) activation (Wang *et al*., 1998), XIAP binds most caspases and inhibits their activity (reviewed by Salvesen and Duckett, 2002).

Smac (second mitochondria-derived activator of caspases)/DIABLO (direct IAP-binding protein with low pI) is released from the mitochondria upon MOMP and promotes cytochrome *c*-dependent caspase activation by counteracting IAPs. Smac/DIABLO is N-terminally processed in the mitochondria, which is required for its pro-apoptotic properties (Du *et al*., 2000; Verhagen *et al*., 2000; reviewed by Danial and Korsmeyer, 2004). The serine protease Omi/HtrA2, which also is released from the mitochondria during MOMP, is able to inhibit IAP functions by binding to the BIR domain, inhibiting IAP-binding to caspases (Suzuki *et al*., 2001; Hegde *et al*., 2002; Danial and Korsmeyer, 2004). AIF (apoptosis-inducing factor), which also is released from the mitochondria upon MOMP, is implied to be important in caspase-independent cell death. When AIF is released from the mitochondria, it is translocated to the nucleus, where it causes chromatin condensation and DNA fragmentation. This translocation cannot be inhibited by caspase inhibitors (Susin *et al*., 1999). AIF-induced nuclear condensation and DNA fragmentation have been suggested to be important for apoptosis during embryoid body cavitation (Joza *et al*., 2001).

The apoptosome – a platform for caspase activation

The structure of the apoptosome reminds of a seven-spiked wheel, consisting of seven cytochrome *c*-bound Apaf-1 molecules surrounding seven caspase-9 molecules in the core (Acehan *et al*., 2002). The CARD of the scaffolding protein Apaf-1 is hidden between the WD40 domains in its quiescent state. Cytochrome *c* released upon MOMP interacts with the WD40 domains of Apaf-1. The interaction with cytochrome *c* induces an ATP-mediated conformational change in Apaf-1, leading to an assembly of the apoptosome (Li *et al*., 1997). Caspase-9 can now be recruited to Apaf-1 via the CARDs present in both caspase-9 and Apaf-1. The oligomerized caspase-9 is able to self-process when bound to Apaf-1 in the apoptosome. Active caspase-9 induces a subsequent activation of the executioner caspases, caspase-3, caspase-6, and caspase-7, leading to apoptosis (reviewed by Boatright and Salvesen, 2003; Spierings *et al*., 2005). Death receptor signaling can use the mitochondrial apoptotic pathway for amplification of caspase activation by caspase-8-induced cleavage of Bid. The effector caspases, caspase-3 and caspase-6, can also promote caspase-8 activation. Thus, the mitochondrial amplification loop can be re-activated by the effector caspases, allowing for efficient caspase activation (Engels *et al*., 2000; Cowling and Downward, 2002).

2.3.2. Stress-induced apoptosis

Genotoxic and proteotoxic stresses such as heat, radiation, hypoxia, and chemical agents can result in so called stress-induced cell death. These stresses also activate a protective stress response, but when the damage to the cells is beyond repair the cell death machinery is activated. The protective effects of the stress response will be described in more detail in a later chapter.

The tumor suppressor protein p53 is activated in response to DNA damage-inducing stresses, promoting either cell-cycle arrest to enable DNA repair or apoptosis to eliminate defective cells. p53 is a transcription factor capable of upregulating gene expression of several pro-apoptotic gene products, but p53 is also able to induce apoptosis independently of

transcription (reviewed by Yee and Vousden, 2005). In addition of upregulating the expression of some pro-apoptotic Bcl-2 family members, p53 interacts with the BH3-only pro-apoptotic Bcl-2 family members, enhancing their activity (reviewed by Fridman and Lowe, 2003). p53 can also disrupt the interaction between anti-apoptotic and pro-apoptotic Bcl-2 proteins enabling efficient activation of the pro-apoptotic proteins (reviewed by Yee and Vousden, 2005). Since CD95 is a target gene of p53, p53 has been suggested to activate the extrinsic apoptotic pathway (Müller *et al*., 1997). p53 can also induce apoptosis by ROS-(reactive oxygen species) mediated activation of caspases (reviewed by Yee and Vousden, 2005).

Heat stress has been shown to directly activate the pro-apoptotic Bax and Bak, inducing cytochrome *c* release and apoptosis. Interestingly, Bax/Bak-deficient cells were completely resistant to heat-induced apoptosis, indicating that the mitochondrial pathway is required for this type of stress-induced apoptotic signaling (Pagliari *et al*., 2005). Tu and co-workers suggested that caspase-2 is exclusively activated by heat stress, and that activation of caspase-2 is required for heat shock-induced apoptosis (Tu *et al*., 2006). Contradictory, another study showed that heat shock-induced apoptosis is independent of initiator caspase activation (Milleron and Bratton, 2006).

Oxidative stress involves generation of ROS, which can react chemically with DNA, proteins, lipids, and carbohydrates. The cells are protected from apoptosis induced by ROS by an anti-oxidant defense system, but cell death is induced if the protection is not sufficient. ROS can induce both apoptosis and necrosis. Since the thiol group in the active site of the caspases is sensitive to ROS, apoptosis is induced only by low levels of ROS and higher doses of ROS induce necrosis (reviewed by McConkey, 1998; Curtin *et al*., 2002). ROS generation occurs in TNF-R1- and CD95-mediated apoptosis, but also as a result of UV irradiation and chemotherapeutic drugs (reviewed by Curtin *et al*., 2002).

JNK (c-Jun N-terminal kinase), a MAPK that is activated upon stressful conditions, is able to activate apoptosis signaling pathways. JNK-induced apoptosis can involve c-Jun-mediated expression of *CD95L* (Faris *et al*., 1998a and 1998b) as well as activation of p53 and inhibition of the anti-apoptotic Bcl-2 family members Bcl-2 and Bcl-XL (Park *et al*., 1997). JNK activation alone is, however, not enough to induce apoptosis via death receptors (Herr *et al*., 1997 and 1999). Therefore, the stress-induced apoptosis signaling, although upregulating death receptors, induces apoptosis by signaling parallel to the death receptor pathways rather than by the same signaling cascades. This is supported by a study showing that the c-FLIP levels do not affect stress-induced apoptosis by chemotherapeutic drugs, such as doxorubicin, etoposide, and vincristine or by γ-irradiation (Kataoka *et al*., 1998). Other studies have also demonstrated that apoptosis induced by anti-cancer drugs, such as doxorubicin and cisplatin, indeed increases CD95L expression, but is still independent of caspases and the death receptor pathways although they can converge on common downstream effector molecules (Eischen *et al*., 1997; Villunger *et al*., 1997). However, contradictory results indicate that doxorubicin-induced apoptosis is mediated via CD95L upregulation and induces CD95 signaling (Friesen *et al*., 1996). Different stresses can induce cell death by several different mechanisms, not only restricted to apoptosis, but also via necrosis. The relationship between

protecting signals by molecular chaperones, such as the heat shock proteins (Hsps), and death signals induced by p53, ROS, and JNK, will determine whether the cell will survive or die.

2.3.3. Alternative death pathways

Although caspase activation is sufficient for activation of cell death, increasing evidence shows that cell death occurs also when caspases are inhibited and that programmed cell death can be achieved also independently of caspases. The morphology of cell death may, however, be shifted towards either necrosis or autophagic cell death when caspases are not involved (reviewed by Baehrecke, 2005; Kroemer and Martin, 2005). While necrosis is a pathological form of cell death, autophagic cell death has been observed under physiological conditions such as development. Autophagy is primarily associated with organelle turnover under physiological conditions and with cell survival in starving cells. Autophagy is characterized by the presence of autophagic vacuoles or autophagosomes that are surrounded by a double membrane sequestering organelles. The autophagosomes can fuse with lysosomes to form autolysosomes in which the sequestered material can be degraded. The lysosomes contain catabolic hydrolases that are able to digest the organelles sequestered in the autophagosome. Stressful conditions can induce lysosomal membrane permeabilization leading to release of cathepsins, which can result in both caspase-dependent classical apoptotic pathways and caspase-independent cell death (reviewed by Kroemer and Jäättelä, 2005). Autophagic cell death can occur also as a consequence of death receptor signaling in a RIP- and JNK-dependent manner (reviewed by Baehrecke, 2005).

Another pathway able to induce both caspase-independent and caspase-dependent apoptosis is the granzyme B/perforine pathway. T-lymphocytes and NK- (natural killer) cells utilize this granule-exocytosis pathway for elimination of their target cells. This is accomplished by delivery of perforine, a pore-forming protein, and granzymes, a family of serine proteases, via cytotoxic granules. Granzyme B can activate Bid and caspase-3 by cleavage, and induce DNA fragmentation (Heusel *et al*., 1994; Alimonti *et al*., 2001). Also calpains, which are calcium-dependent cysteine proteases, can promote death signaling by cleaving caspases as well as caspase substrates (Gil-Parrado *et al*., 2002).

2.4. Death receptor-mediated apoptosis

2.4.1. Death receptors and ligands

Death signaling via the extrinsic apoptotic pathway is mediated by death receptors. The death receptors are members of the TNF-R superfamily of type I transmembrane proteins, consisting of more than 20 proteins with a broad range of biological functions including cell death, survival, differentiation, and regulation of the immune response. The members of the TNF receptor superfamily are characterized by extracellular cysteine-rich domains (CRD), the number of which varies between the different family members (reviewed by Bodmer *et al*., 2002). Eight death receptors have been characterized; TNF-R1 (also known as DR1 from death receptor 1), CD95 (also known as Fas/APO-1/DR2), death receptor 3 (DR3, also

known as TRAMP/APO-3/LARD), TRAIL-R1 (also known as DR4/APO-2), TRAIL-R2 (also known as DR5/KILLER/TRICK2), death receptor 6 (DR6), ectodysplasin A receptor (EDAR), and nerve growth factor receptor (NGFR). Common for the death receptors is the cytoplasmic DD consisting of about 80 amino acids (Fig. V). The DD is required for transmission of death signals from the receptor to the intracellular signaling machinery (reviewed by Lavrik *et al*., 2005).

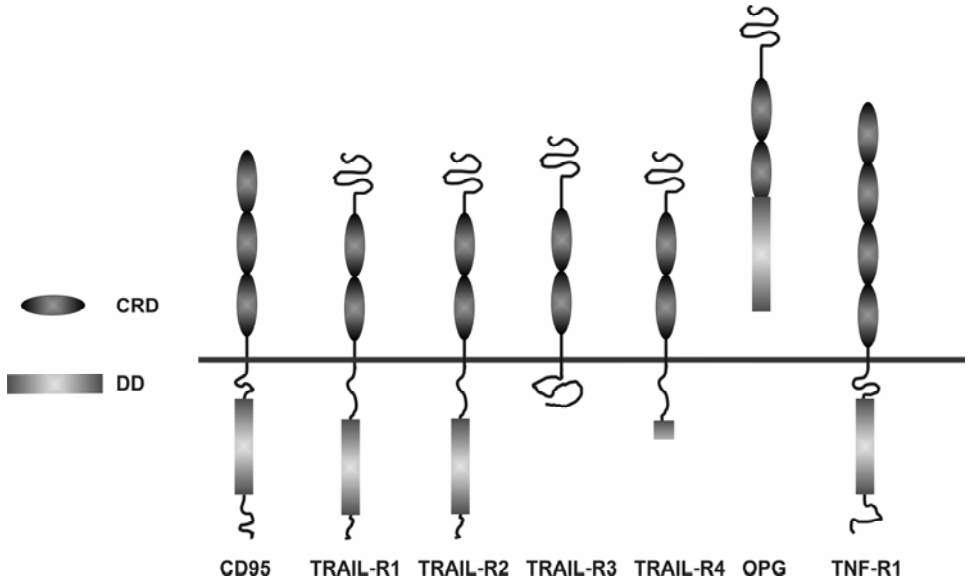


Figure V. The death receptors. The death receptors belong to the TNF receptor superfamily and are generally type I transmembrane proteins. CD95 has three, the TRAIL receptors have two and the TNF receptor 1 has four cysteine-rich domains (CRDs), indicated by an oval. The death receptors transmit apoptotic signals to the intracellular death machinery via their C-terminal death domains (DDs), presented as grey boxes. TRAIL-R3, which has a glycolipid anchor instead of a DD and TRAIL-R4, which has a truncated DD, are not able to transmit signals upon ligand binding and are termed decoy receptors. The soluble osteoprotegerin (OPG) can bind to TRAIL in the extracellular space, and can thereby compete with the death signal transmitting TRAIL-R1 and TRAIL-R2 for ligand binding.

The death receptors are activated by their corresponding death ligands. The best characterized death ligands are CD95L, which binds to CD95, TNF-α, which binds to TNF-R1, and TRAIL, which binds to TRAIL-R1 and TRAIL-R2 (reviewed by Bodmer *et al*., 2002; Fulda and Debatin, 2004). The TRAIL decoy receptors (DcR), which lack functional DDs can also interact with TRAIL (Fig. V). TRAIL-R3 (also known as DcR1 and TRID) is a GPI-anchored cell surface protein lacking a cytoplasmic tail (Degli-Esposti *et al*., 1997b; Pan *et al*., 1997a), TRAIL-R4 (also known as DcR2) has a cytoplasmic tail with a truncated DD (Degli-Esposti *et al*., 1997a), and osteoprotegerin (OPG) is a soluble decoy receptor that lacks the transmembrane domain and is thereby not bound to the cell membrane (Emery *et al*., 1998). The death ligands are members of the TNF ligand family of type II transmembrane proteins characterized by a conserved C-terminal extracellular TNF homology domain (THD) needed for ligand trimerization (Orlinick *et al*., 1997; Bodmer *et al*., 2002). The death ligands are synthesized as membrane-bound proteins, but they can be cleaved off the membrane by proteolytic enzymes such as matrix metalloproteases. Some of the TNF ligands require solubilization to be able to function, whereas shedding inhibits the physiological functions of others. Despite the broad range of biological functions, the members of the TNF receptor and

ligand family are structurally very similar and the receptor-ligand interactions are conserved. The interaction between the death receptor and its corresponding ligand requires the CRD of the death receptor. For the receptor to be activated, it has additionally been suggested that both the ligand and the receptor need to be at least trimerized. The receptor trimerization can be induced by ligand binding (reviewed by Bodmer *et al*., 2002), but the death receptors CD95 and TNF-R1 have been shown to exist as preformed oligomers at the membrane (Chan *et al*., 2000).

CD95-mediated apoptosis

The death receptor CD95 was discovered in 1989 by two research groups. Both groups used agonistic monoclonal antibodies that recognized a surface protein with the ability to induce apoptosis in the targeted cell (Trauth *et al*., 1989; Yonehara *et al*., 1989). When cloning the CD95 receptor, it was found to be a member of the TNF-R family of death receptors (Itoh *et al*., 1991; Oehm *et al*., 1992). Fc-conjugated soluble CD95 was used to find the ligand for CD95. CD95 was able to bind a ligand at the surface of a cytotoxic T-cell hybridoma, PC60-d10S, and CD95L was cloned from the cDNA of these cells (Suda *et al*., 1993). The CD95L is well conserved in the human and the mouse genomes, and cross-reaction between the species is possible (Takahashi *et al*., 1994; Peitsch and Tschopp, 1995). CD95 is expressed in liver, lung, heart, and ovary of adult mice, as well as in activated lymphocytes in lymphoid tissue (Watanabe-Fukunaga *et al*., 1992). CD95L is expressed in a more restricted way; only in activated lymphocytes in the spleen and the thymus and in immune privilege sites such as the eye and testis (Suda *et al*., 1993; Griffith *et al*., 1995). CD95L produced by activated T-lymphocytes triggers autocrine suicide or paracrine death of other lymphocytes and target cells. Furthermore, CD95L has been proposed to be involved in the immune escape of tumors. By expressing surface CD95L, tumor cells are able to delete attacking anti-tumor cytotoxic T-lymphocytes by activating their CD95 receptors (reviewed by Igney and Krammer, 2002).

The expression of CD95L is tightly regulated at the level of transcription. Activation of the T-cell receptor (TCR) induces upregulation of *CD95L* via NFAT (nuclear factor in activated T-cells), NF-κB, IRFs (interferon regulatory factors), and c-Myc. The *CD95L* expression induced during T-lymphocyte activation has been proposed to be regulated via several cytokines such as interleukin 2 (IL-2), TNF-α, and interferon-γ (IFN-γ). Other transcription factors controlling the transactivation of the *CD95L* gene are Sp1, Ap-1, and Egr (reviewed by Kavurma and Khachigian, 2003). *CD95L* expression is also induced in response to environmental stresses such as hyperthermia (Faris *et al*., 1998b; Cippitelli *et al*., 2005). The stress-induced expression of *CD95L* has been suggested to be mediated via JNK (Faris *et al*., 1998b).

When establishing mouse MRL strains, mutants developing lymphadenopathy and splenomegaly were discovered. The mutations were referred to as *lpr* for lymhoproliferation and *gld* for generalized lymphoproliferative disease. Later it has been established that CD95 expression is impaired in *lpr* mice and that *gld* mice have a mutation in their *CD95L* gene leading to expression of a CD95L with impaired ability to bind CD95. Studies in these mice have revealed that a malfunctioning CD95-CD95L system induces development of

lymphoproliferative disorders, suggesting that functional CD95L-CD95 signaling is important for the immune system. In addition to lymphadenopathy and splenomegaly, the *lpr* and *gld* mice have elevated levels of autoantibodies as a result of abnormal B-lymphocyte death. Also activation-induced cell death (AICD) is inhibited in the activated T-lymphocytes in these mice (reviewed by Nagata and Suda, 1995). Due to impaired lymphocyte apoptosis, defects in the CD95/CD95L signaling pathway are associated with autoimmune diseases such as ALPS in humans (see Table I). Three ALPS classifications have been defined; ALPS 0 that is caused by CD95 deficiency, ALPS I that is caused by mutations in *CD95* or *CD95L*, and ALPS II that is caused by mutations in the CD95 signaling mediators caspase-8 and caspase-10 (reviewed by Rieux-Laucat *et al*., 2003).

Even though CD95L can be cleaved from the membrane by metalloproteases, both the soluble and the membrane-bound CD95L have been suggested to be physiologically active (Tanaka *et al*., 1995). Knowledge-based protein modeling, subsequently supported by crystal structures, predicts that both the TNF ligands and receptors form trimers, implying that also the oligomerized form CD95 and CD95L consist of trimers (Banner *et al*., 1993; Peitsch and Tschopp, 1995). It has, however, been proposed that a CD95L trimer is not enough to activate CD95, but that a hexamer consisting of two trimers is required (Holler *et al*., 2003). The hexamer prerequisite might explain why some forms of soluble CD95L are not able to induce apoptosis (Suda *et al*., 1997). CD95 can already be assembled into trimer complexes prior to CD95L binding. This self-association is induced by the N-terminal domain termed pre-ligand association domain (PLAD) of CD95 (Papoff *et al*., 1999; Siegel *et al*., 2000). Upon CD95L binding the CD95 receptor is rapidly clustered forming a cap-like structure consisting of several CD95 trimers (Algeciras-Schimnich *et al*., 2002b). The clustered receptor is further internalized, concentrating the DISC components (Lee *et al*., 2006). CD95 clustering and internalization are, however, not required for apoptosis execution (Söderström *et al*., 2005).

TRAIL – the tumor-specific death ligand

TRAIL was identified and cloned based on its sequence homology to other members of the TNF superfamily (Wiley *et al*., 1995; Pitti *et al*., 1996). Like most of the other TNF family members, TRAIL forms homotrimers that bind and activate the trimerized receptor (Hymowitz *et al*., 1999; Mongkolsapaya *et al*., 1999). Unlike most TNF family members that show a specific expression pattern, TRAIL is expressed in a wide range of tissues (Wiley *et al*., 1995). Of the five TRAIL receptors identified only the TRAIL receptors with functional DDs, TRAIL-R1 (Pan *et al*., 1997b) and TRAIL-R2 (MacFarlane *et al*., 1997; Pan *et al*., 1997a; Screaton *et al*., 1997; Sheridan *et al*., 1997; Walczak *et al*., 1997), are able to transmit further apoptotic signals. Although ligation of both TRAIL-R1 and TRAIL-R2 can induce apoptosis, there might be differences in the signals these receptors transmit. It has been suggested that they have distinct cross-linking requirements; TRAIL-R2 would require a cross-linked ligand, whereas TRAIL-R2 would respond to both cross-linked and non-cross-linked TRAIL (Mühlenbeck *et al*., 2000). TRAIL-R1 and TRAIL-R2 can form both homo- and heterotrimers, but whether the heterotrimers are functional is still unknown (Schneider *et al*., 1997). The similarities and differences between the TRAIL receptors remain to be determined.

TRAIL differs from the other death ligands of the TNF family by its ability to specifically induce apoptosis in 40-60% of tumors without harming healthy cells (Wiley *et al*., 1995; Pitti *et al*., 1996), making TRAIL interesting as a potential anti-cancer treatment. Although cytotoxic side effects of TRAIL on normal tissues have been reported (Martin-Villalba *et al*., 1999; Jo *et al*., 2000), recombinant TRAIL has been shown to successfully suppress growth of human xenografts in SCID (severe combined immunodeficiency) mice (Walczak *et al*., 1999) and non-human primates (Ashkenazi *et al*., 1999) *in vivo*. Also the TRAIL-R monoclonal agonistic antibodies, HGS-ETR1 and HGS-ETR2 (Human Genome Sciences), can efficiently induce TRAIL-R-mediated apoptosis. These antibodies have been shown to be well tolerated in clinical experiments (reviewed by Melnikova and Golden, 2004). The mechanism by which TRAIL is able to selectively kill tumor cells is still unknown, but several hypotheses have been proposed. One suggestion has been that expression of decoy receptors is responsible for the resistance to TRAIL-induced apoptosis observed in normal cells. The results indicating protective effects of decoy receptors have, however, been obtained in overexpression studies, and studies with monoclonal antibodies targeting all the different TRAIL receptors implied that the resistance to TRAIL is mediated by intracellular events rather than the decoy receptors (Griffith *et al*., 1999).

Although several types of tumor cells are sensitive to TRAIL-induced apoptosis, a great number are resistant (Ganten *et al*., 2004 and 2005). Efforts to find methods to sensitize the resistant tumor cells to TRAIL have been made. Co-treatments with chemotherapeutic drugs and irradiation have successfully induced TRAIL sensitivity of several otherwise resistant tumors (Chinnaiyan *et al*., 2000; Kelly *et al*., 2002; Ganten *et al*., 2004; El-Zawahry *et al*., 2005; Galligan *et al*., 2005; Hamai *et al*., 2006). Both TRAIL and monoclonal agonistic TRAIL-R antibodies are in clinical trials for a variety of cancers (reviewed by Melnikova and Golden, 2004). Therefore, it is of great interest to further investigate and understand the TRAIL signaling pathways.

TNF-R1 signaling involves two signaling complexes

As both CD95 and TRAIL-R transmit the apoptotic program via the DISC, death receptor signaling via TNF-R1 utilizes two different signaling complexes mediating either survival or death. Activation of the TNF-R1 can induce NF-κB activation via complex I and apoptosis via complex II. Thereby, TNF-R1 signaling can be directed either towards survival or death (Liu *et al*., 1996; Micheau and Tschopp, 2003; Jin and El-Deiry, 2006). Complex I, which is assembled at the membrane at the intracellular part of the receptor, consists of the intracellular part of the TNF-R1, the adaptor protein TRADD (TNF-R-associated death domain), the kinase RIP-1, and the signal transducer TRAF2. In a similar way as FADD is recruited to the ligated CD95 and TRAIL-R, TRADD is recruited to the activated TNF-R1. TRADD has a DD in the N-terminus and is associated to the intracellular part of the TNF-R1 via DD interactions (Hsu *et al*., 1995). TRADD can further recruit RIP-1, a serine/threonine kinase domain-containing protein with a DD and TRAF2 (Hsu *et al*., 1996a and 1996b). The I-κB kinase (IKK) regulates I-κB phosphorylation and thereby also I-κB degradation and NF-κB activation. IKK is activated upon recruitment to complex I by RIP-1 and TRAF2. Thus, both TRAF2 and RIP-1 are required for and NF-κB activation induced via TNF-R1 (Hsu *et al*., 1996a and 1996b; reviewed by Chen and Goeddel, 2002).

Complex II is formed when complex I dissociates from the membrane to the cytoplasm (Micheau and Tschopp, 2003). In the TNF-R1 signaling cascade FADD is recruited to TRADD by DD interactions instead of binding directly to the DD of the activated receptor as is the case for CD95 and TRAIL-R (Hsu *et al*., 1996b; Harper *et al*., 2003). The inability of FADD to bind directly to the TNF-R1 has been proposed to be due to small structural differences in the DD surfaces. Based on studies on Pelle and Tube, two *Drosophila* proteins containing DDs, a model where FADD binds to CD95 and TRADD with Tube-like structures has been suggested. TRADD, on the other hand, binds FADD and TNF-R1 utilizing its Pelle-like surface (Sandu *et al*., 2005). Binding of caspase-8 to FADD in complex II leads to caspase-8 oligomerization and activation, similarly as caspase-8 activation is accomplished in the CD95 and TRAIL-R DISCs. FADD and caspase-8 are required for induction of apoptosis via TNF-R1, and since neither of them is recruited to complex I, formation of complex II is a prerequisite for the TNF-R1 death signaling (Harper *et al*., 2003). Modulation of caspase activation by proteins such as c-FLIP occurs in the same way in complex II and in the DISC (Micheau and Tschopp, 2003).

2.4.2. The death-inducing signaling complex – a platform for regulation of caspase activation

Death domain and death effector domain interactions as the backbone of the DISC

Ligation of the death receptors by their corresponding death ligands leads to recruitment of adaptor proteins to the intracellular DD of the activated receptor. Similarly as CARD interactions induce dimerization of CARD-containing proteins, also proteins with a DD can associate with each other by homotypic contact (Weber and Vincenz, 2001). Yeast two-hybrid screens with the cytoplasmic DD-containing part of CD95 as bait, revealed interactions between CD95 and FADD, also termed MORT, mediator of receptor-induced toxicity (Boldin *et al*., 1995; Chinnaiyan *et al*., 1995). Whereas the C-terminal DD of FADD interacts with the DD of CD95, the N-terminal part of FADD, containing a conserved DED, forms similar homotypic interactions with other DED-containing proteins (Fig. VI). Together with the activated receptor, the adaptor protein FADD, the DED-containing initiator caspases as well as other interacting proteins form the DISC. The proteins associated with the DISC can either further transmit the death signals to the intracellular apoptosis machinery, or inhibit the proceeding of death signals (Kischkel *et al*., 1995; Medema *et al*., 1997).

When FADD is bound to the activated CD95, it further recruits DED-containing initiator caspases via DED interactions (Muzio *et al*., 1998). Since the DED of FADD is necessary for caspase binding, CD95-mediated apoptosis is inhibited by FADD deletion mutants lacking the DED (Chinnaiyan *et al*., 1996). Likewise, CD95-mediated apoptosis is inhibited in *FADD*$^{-/-}$ MEFs and *FADD*$^{-/-}$ T-lymphocytes (see Table I) (Yeh *et al*., 1998; Zhang *et al*., 1998). Conversely, overexpression of full-length FADD induces apoptosis also in the absence of CD95 triggering. Accumulation of DED proteins leads to spontaneous oligomerization and formation of complexes with the ability to function like a DISC (Boldin *et al*., 1995; Chinnaiyan *et al*., 1995). These complexes have been described as caspase-recruiting filaments or death-effector filaments (Siegel *et al*., 1998). Although FADD was suggested to

be dispensable for TRAIL-induced apoptosis in early studies (Marsters *et al*., 1996; Yeh *et al*., 1998), later analyses of the TRAIL-R DISC have proven that FADD is recruited to the TRAIL-R and that it is required as an adaptor protein for the TRAIL receptors, making the TRAIL-R DISC similar to the CD95-DISC (Chinnaiyan *et al*., 1996; Chaudhary *et al*., 1997; Bodmer *et al*., 2000; Kischkel *et al*., 2000; Sprick *et al*., 2000).

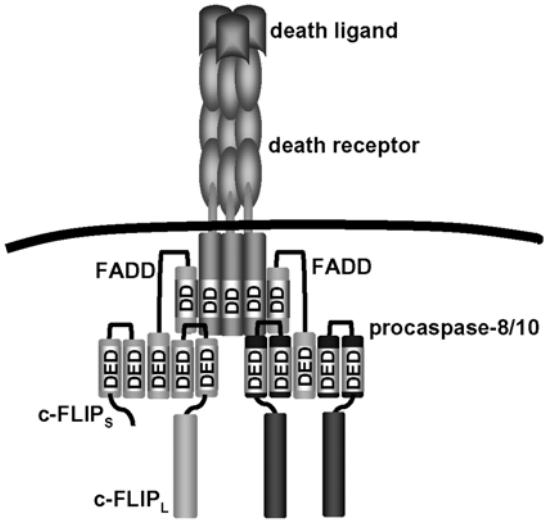


Figure VI. Schematic presentation of the DISC. Upon ligation of the death receptor, the adaptor protein FADD is bound to the intracellular part of the receptor by DD interactions. FADD, which in addition to its DD contains a DED, forms a platform for other DED-containing proteins to bind. Depending on the proteins recruited to the DISC, the apoptotic signaling is either inhibited or induced.

FADD is a phosphoprotein, but while the phosphorylation status of FADD seems not to affect its ability to mediate death signals, it regulates the subcellular localization of FADD. Phosphorylation of FADD leads to a nuclear localization of the protein (Alappat *et al*., 2005). The nuclear phosphorylated form of FADD has been suggested to have a role in cell cycle progression (Alappat *et al*., 2003) and FADD is predominantly found in its phosphorylated form during G2/M (Alappat *et al*., 2005). The non-apoptotic functions of FADD are not restricted to the cell cycle but also to regulation of lymphocyte homeostasis. FADD-deficient mice die *in utero* at E11.5, showing signs of cardiac failure and abdominal hemorrhage (Yeh *et al*., 1998), similarly to caspase-8- and c-FLIP-deficient embryos (Varfolomeev *et al*., 1998; Yeh *et al*., 2000). FADD-deficient T-lymphocytes derived from chimeric mice have a decreased capacity to proliferate in response to TCR stimulation (Zhang *et al*., 1998), indicating that FADD is important for proper activation of T-lymphocytes.

Regulation of DISC signaling

The caspases that have been activated upon binding to FADD and oligomerization in the DISC initiate further activation of the intracellular apoptotic program. Depending on the level of caspase-8 activation in the DISC, different effector molecules are required for execution of apoptosis, dividing cells into Type I or Type II cells. Type I cells are characterized by efficient DISC formation, leading to caspase-8 activation sufficient to directly activate downstream effector caspases such as caspase-3. In Type II cells, the DISC formation and thereby also FADD recruitment are weaker, leading to only a low level of caspase-8 activation, insufficient to directly activate downstream caspases (Scaffidi *et al*., 1998). In Type II cells, downstream caspase activation and execution of apoptosis can be accomplished by the help of the intrinsic apoptotic machinery. The mitochondrial amplification loop is

engaged by caspase-8-mediated cleavage of the BH3-only protein Bid, generating a truncated form of Bid, tBid (Li *et al*., 1998; Luo *et al*., 1998). tBid is a pro-apoptotic protein that participates in MOMP and cytochrome *c* release by activating the pro-apoptotic Bcl-2 family members Bax and Bak and inactivating the anti-apoptotic Bcl-2 family members Bcl-2 and Bcl-XL (reviewed by Puthalakath and Strasser, 2002). MOMP and cytochrome *c* release are also induced in Type I cells, but at a later stage, after caspase-3 activation. CD95-mediated apoptosis can be inhibited by overexpression of Bcl-2 and Bcl-XL in Type II cells, whereas caspase activation in Type I cells is strong enough regardless of the levels of the anti-apoptotic Bcl-2 family members and inhibition of the mitochondrial amplification loop (Scaffidi *et al*., 1998). Division of cells into Type I or Type II is not restricted to CD95 responses, as apoptosis induced via TRAIL-R1 and TRAIL-R2 can be separated depending on the efficiency of caspase activation at the level of the DISC (Fulda *et al*., 2001).

In addition to the initiator caspases, other regulatory proteins are associated with the DISC, either by direct binding to the receptor or via DED interactions with FADD. The proteolytically inactive caspase-8 homologue c-FLIP is a potent inhibitor of death receptor-mediated apoptosis due to its ability to compete with caspase-8 for binding to FADD (Scaffidi *et al*., 1999b). PEA-15 (phosphoprotein enriched in astrocytes of 15 kDa), also termed PED (phosphoprotein enriched in diabetes), is an anti-apoptotic DED protein able to compete for binding to FADD with the initiator caspases (Condorelli *et al*., 1999). DEDD (DED-containing DNA-binding protein) and DEDD2 are DED proteins with the capability to translocate to the nucleus, but they can additionally bind to caspase-8, caspase-10, and c-FLIP. The role of DEDD and DEDD2 in apoptotic signaling, however, remains unsolved (Barnhart *et al*., 2003). Proteins that are able to directly bind CD95, either promoting or inhibiting death signaling, include DAXX (Yang, X. *et al*., 1997), FAF1 (Fas-associated protein factor 1) (Chu *et al*., 1995), FAP-1 (Fas-associated phosphatase 1) (Sato *et al*., 1995), and FIST/HIPK3 (Fas-interacting serine/threonine kinase/homeodomain-interacting protein kinase 3) (Rochat-Steiner *et al*., 2000).

MAPK and PKC signaling has also been shown to modulate CD95 and TRAIL-R signaling at the level of initiator caspase activation. Activation of ERK or PKC during T-lymphocyte activation induces resistance to both CD95- and TRAIL-R-mediated apoptosis, and might thereby play a role in protection from death receptor signaling during clonal expansion of T-lymphocytes (Holmström *et al*., 1998, 1999, and 2000; Scaffidi *et al*., 1999b; Wilson *et al*., 1999; Söderström *et al*., 2002). Conversely, MAPK inhibition sensitizes cells to TRAIL-R and CD95-mediated apoptosis (Holmström *et al*., 1999 and 2000; Tran *et al*., 2001).

2.4.3. Modulation of death receptor responses by c-FLIP

Viral and cellular forms of FLIP

FLICE-inhibitory proteins, FLIPs, were first found in virus genomes when researchers searched for DED homologues. Viral FLIPs (v-FLIPs) have been described in several members of the γ-herpesvirus family; the v-FLIP E8 in equine herpesvirus-2 (EHV-2), the v-FLIP ORF71 in herpesvirus saimiri (HVS), the v-FLIP K13 in human herpesvirus-8 (HHV-8)

also called Kaposi's sarcoma-associated herpesvirus 2 (KSHV), and the v-FLIPs MC159 and MC160 in human molluscipoxvirus (molluscum contagiosum virus, MCV). Additionally, v-FLIP has been found in bovine herpesvirus-4 (Thome *et al*., 1997). The v-FLIPs consist of two DED-like structures, with C-terminal extensions of different length (Fig. VII). Most of the v-FLIPs interact with FADD in the DISC, where the v-FLIPs inhibit binding and activation of caspase-8 as well as apoptosis induced via death receptors (Bertin *et al*., 1997; Hu *et al*., 1997a; Thome *et al*., 1997). Thus, expression of v-FLIP provides protection of the host cell against apoptotic signaling (Thome *et al*., 1997; Glykofrydes *et al*., 2000; Godfrey *et al*., 2005).

Soon after the cloning of the v-FLIPs, a cellular version, c-FLIP (cellular FLICE-inhibitory protein), was cloned by Irmler and co-workers (Irmler *et al*., 1997). At the same time c-FLIP was cloned in several other laboratories and named as inhibitor of FLICE, I-FLICE (Hu *et al*., 1997b), caspase homologue, CASH (Goltsev *et al*., 1997), FADD-like anti-apoptotic molecule, FLAME-1 (Srinivasula *et al*., 1997), MACH-related inducer of toxicity, MRIT (Han *et al*., 1997), caspase-like apoptosis-regulatory protein, CLARP (Inohara *et al*., 1997), caspase-eight-related protein, casper (Shu *et al*., 1997), and Usurpin (Rasper *et al*., 1998). c-FLIP is expressed in a variety of human tissues (Han *et al*., 1997; Hu *et al*., 1997b; Inohara *et al*., 1997; Irmler *et al*., 1997; Shu *et al*., 1997; Srinivasula *et al*., 1997; Rasper *et al*., 1998), with a particularly high expression in the developing heart. c-FLIP-deficient mice do not survive past day 10.5 of embryogenesis. The day 10.5 c-FLIP-deficient embryos showed severe enlargements of the pericardial space and blood pooling in the head and abdominal regions. Except for the impaired heart development, the embryos developed normally until E9-9.5 (Yeh *et al*., 2000). This phenotype was reminiscent of those reported for *FADD*$^{-/-}$ and *caspase-8*$^{-/-}$ embryos (see Table I) (Varfolomeev *et al*., 1998; Yeh *et al*., 1998).

The gene coding for c-FLIP, *cflar* (caspase-8 and FADD-like apoptosis regulator), is localized in chromosome 2q33 together with caspase-8 and caspase-10. These three related genes have probably evolved by gene duplication (Goltsev *et al*., 1997; Irmler *et al*., 1997; Srinivasula *et al*., 1997; Rasper *et al*., 1998). *cflar* consists of at least 14 exons and combinations of these exons can give rise to at least 11 distinct isoforms (Djerbi *et al*., 2001). Yet, only three splicing variants of c-FLIP have been shown to be expressed as proteins (Fig. VII); a long form, c-FLIP-long (c-FLIP$_L$), and two short forms, c-FLIP-short (c-FLIP$_S$) and c-FLIP-Raji (c-FLIP$_R$). However, c-FLIP$_R$, which got its name from the Burkitt lymphoma Raji cell line where it was found, is expressed in a more tissue-restrictive fashion than the other c-FLIP isoforms (Djerbi *et al*., 2001; Golks *et al*., 2005). Like the v-FLIPs, the short forms of c-FLIP consist of two DEDs with short specific C-terminal extensions, whereas the DEDs in c-FLIP$_L$ are followed by a caspase-like domain (Irmler *et al*., 1997; Djerbi *et al*., 2001). All three splicing variants are similar up to amino acid 202, which corresponds to the termination of exon 6. c-FLIP$_S$ has a unique splicing tail that is encoded by exon 7, which is not included in c-FLIP$_L$ and c-FLIP$_R$. c-FLIP$_R$ is formed by an absence of splicing between exon 6 and 7, leading to a premature stop of translation in the beginning of intron 6 (see Fig. XIIa) (Djerbi *et al*., 2001).

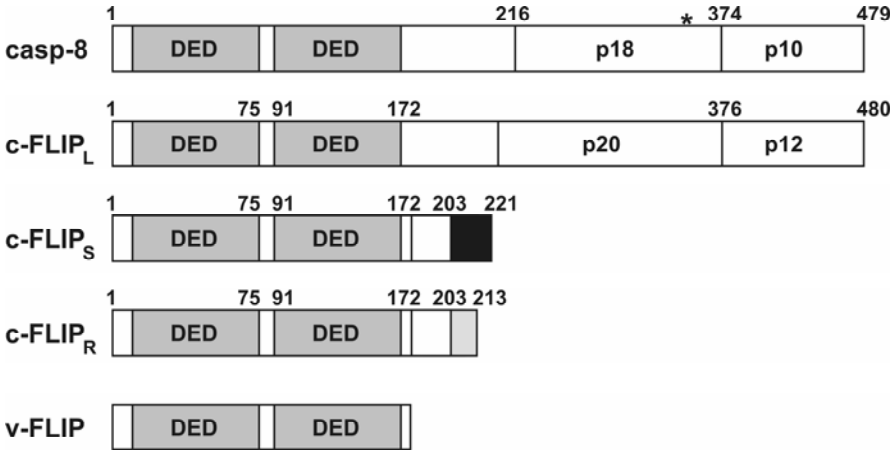


Figure VII. FLIP is an inactive homologue of caspase-8. As caspase-8, also the FLIPs possess two DEDs in their N-terminus, allowing for association to the DISC. c-FLIP$_L$ resembles caspase-8, but lacks the proteolytically active cysteine in its caspase homology domain, making c-FLIP$_L$ enzymatically inactive. v-FLIP and the short forms of c-FLIP, c-FLIP$_S$ and c-FLIP$_R$, lack the caspase homology domain, making them potent caspase inhibitors.

c-FLIP as a modulator of caspase activation

c-FLIP can form heterodimers with caspase-8 (Goltsev *et al*., 1997; Han *et al*., 1997; Hu *et al*., 1997b; Inohara *et al*., 1997; Irmler *et al*., 1997; Shu *et al*., 1997; Rasper *et al*., 1998), and caspase-10 (Goltsev *et al*., 1997; Hu *et al*., 1997b; Shu *et al*., 1997) in the DISC upon death receptor ligation. c-FLIP homodimers have not been found in the DISC *in vivo* (Irmler *et al*., 1997), but c-FLIP$_L$ has been shown be able to form homodimers *in vitro* (Rasper *et al*., 1998). The recruitment of c-FLIP to the DISC is mediated by direct interactions of the DEDs of c-FLIP and FADD (Goltsev *et al*., 1997; Han *et al*., 1997; Irmler *et al*., 1997; Shu *et al*., 1997; Srinivasula *et al*., 1997).

c-FLIP$_S$ functions as a physiological caspase inhibitor, interfering with initiator caspase activation on several levels (Fig. VIII). First, c-FLIP$_S$ competes with caspase-8 and caspase-10 for binding to the DISC due to the similar properties of their DEDs. Second, oligomerization-induced interactions between the caspase-like domains, and, thus, activation of caspase-8, is inhibited by c-FLIP$_S$, which consists of only two DEDs and lacks the caspase-like domain (Irmler *et al*., 1997; Krueger *et al*., 2001). When caspase-8 is activated and cleaved, it is released from the DISC and can therefore be replaced by a new procaspase-8 that in turn can be activated. Since c-FLIP$_S$ recruitment to the DISC inhibits processing of caspase-8, procaspase-8 is not released from the DISC and, thereby, recruitment of new procaspases is inhibited, providing for a third mechanism of caspase-8 inhibition (Scaffidi *et al*., 1999a). Therefore, most of caspase-8 in cells overexpressing c-FLIP$_S$ is localized in the cytoplasmic pool as procaspase-8 (Micheau *et al*., 2002). c-FLIP$_R$, which similarly to c-FLIP$_S$, lacks the caspase-like domain, is recruited to the DISC with comparable affinity and has the same anti-apoptotic properties as c-FLIP$_S$ (Golks *et al*., 2005).

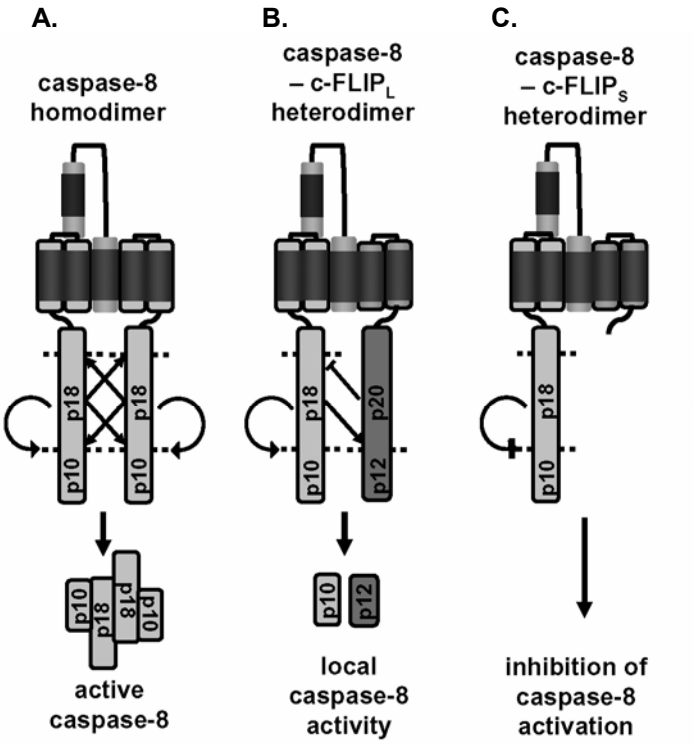


Figure VIII. Mechanism of c-FLIP-induced caspase inhibition in the DISC. (A) When a caspase-8 homodimer is formed in the DISC, the caspase-8 zymogen is activated, inducing autoproteolytic self-cleavage of the caspase-8 dimer. The small p10 subunit is cleaved by the first intra-chain cleavage, and the large p20 subunit is separated from the prodomain by the second intra-chain cleavage, releasing the active caspase-8 fragments from the DISC. Although caspase-8 is cleaved between the p10 and the p20 subunits, these two are never separated. **(B)** The caspase-8 zymogen is activated also when oligomerized with c-FLIP$_L$, but due to the lack of catalytic activity of c-FLIP$_L$, only the first cleavage between the small and the large subunits is accomplished. Therefore, local caspase-8 activity has been suggested to be accomplished upon formation of c-FLIP$_L$-caspase-8 heterodimers. **(C)** Since c-FLIP$_S$ lacks the whole caspase domain, association of c-FLIP$_S$ with caspase-8 inhibits all caspase-8 activation (modified from Budd *et al*., 2006).

Whereas c-FLIP$_S$ and c-FLIP$_R$ are established inhibitors of caspase-8 activation, the effect of c-FLIP$_L$ on caspase-8 activation is more complex (Fig. VIII). While c-FLIP$_L$ contains the P4-P1 consensus tetrapeptide for recognition and cleavage by caspases in its caspase-like domain, c-FLIP$_L$ lacks the proteolytically active cysteine required for being a functional protease (Hu *et al*., 1997b; Inohara *et al*., 1997; Irmler *et al*., 1997; Rasper *et al*., 1998). The oligomerized uncleaved proform as well as the partially cleaved form of caspase-8 possess local catalytic activity in the DISC. The active site of caspase-8 can be formed in a c-FLIP$_L$-caspase-8 heterodimer without processing of caspase-8 (Micheau *et al*., 2002), similarly as autoactivation without cleavage is proposed for caspase-9 (Renatus *et al*., 2001). The c-FLIP$_L$-induced activation of the procaspase-8 zymogen is enough to initiate autoprocessing of caspase-8. Nevertheless, c-FLIP$_L$ is not able to cleave caspase-8 and the autoprocessing is thereby restricted to the first cleavage of caspase-8 between the small p10 and the large p20 subunits (Krueger *et al*., 2001; Chang *et al*., 2002; Micheau *et al*., 2002; Boatright *et al*., 2004). Caspase-8 activated upon interaction with c-FLIP$_L$ has been shown to induce cleavage of caspase-8 substrates in vicinity, such as RIP (Micheau *et al*., 2002). Since the final activating cleavage of caspase-8 is inhibited by c-FLIP$_L$, caspase-8 is not released from the DISC. Therefore, recruitment of new procaspases is prohibited (Scaffidi *et al*., 1999a). c-

FLIP oligomerization with caspase-10 has similar effects on caspase-10 activation and processing as c-FLIP-caspase-8 oligomerization (Chang *et al*., 2002).

The expression levels of c-FLIP affect apoptosis sensitivity

The resistance to death receptor ligation has been shown to correlate directly with the expression levels of c-FLIP (Irisarri *et al*., 2000; Krueger *et al*., 2001). *cflar*$^{-/-}$ MEFs are highly sensitive to CD95- and TNF-R1-mediated apoptosis (Yeh *et al*., 2000) and knock-down of c-FLIP with RNAi or antisense oligonucleotides sensitizes a variety of cell types to TRAIL-, CD95L-, and TNF-α-induced apoptosis (Fulda *et al*., 2000; Kim, Y. *et al*., 2002; Siegmund *et al*., 2002; Chawla-Sarkar *et al*., 2004; Ganten *et al*., 2004; Ricci *et al*., 2004; Krueger *et al*., 2006; Mitsiades *et al*., 2006; Palacios *et al*., 2006; Saito *et al*., 2006). Due to its anti-apoptotic properties, manipulation of c-FLIP levels has been used to sensitize several types of cells otherwise resistant to death receptor-mediated apoptosis (Kataoka *et al*., 2002; Kim, Y. *et al*., 2002; Davidson *et al*., 2003; Hietakangas *et al*., 2003; Nitobe *et al*., 2003; Perez and White, 2003; Sayers *et al*., 2003; Dutton *et al*., 2004; Ganten *et al*., 2004; Ricci *et al*., 2004; El-Zawahry *et al*., 2005; Uriarte *et al*., 2005; Xiao *et al*., 2005). Caspase-8 is expressed at a 100 times higher level than c-FLIP (Scaffidi *et al*., 1999a), but c-FLIP$_L$ has 18 times better affinity to the DISC compared to caspase-8, resulting in a c-FLIP$_L$:caspase-8 ratio of 1:5. Additionally, the heterophilic c-FLIP$_L$-caspase-8 interaction is stronger than the homophilic caspase-8-caspase-8 interaction (Chang *et al*., 2002). c-FLIP$_R$, which has the same anti-apoptotic properties as c-FLIP$_S$, is also recruited to the DISC with comparable affinity (Golks *et al*., 2005). The high DISC-affinity of c-FLIP allows for small changes in the c-FLIP expression levels to affect the DISC composition and the outcome of apoptotic signaling.

Since c-FLIP$_L$ is able to induce partial caspase-8 activation, it cannot be classified solely as a caspase inhibitor like the short c-FLIP isoforms. While caspase-8 activation is more efficient in the absence of c-FLIP$_L$, c-FLIP$_L$ could instead be classified as a repressor of caspase activation. Isoform-specific downregulation of c-FLIP$_L$ (Flahaut *et al*., 2006; Hamai *et al*., 2006) or c-FLIP$_S$ (Flahaut *et al*., 2006; Salon *et al*., 2006) sensitizes to CD95L- and TRAIL-induced apoptosis, indicating that also c-FLIP$_L$ is an apoptosis inhibitor. Overexpression of c-FLIP$_S$ or c-FLIP$_L$ either separately or both isoforms simultaneously has been shown to rescue cells from death receptor-mediated apoptosis (Inohara *et al*., 1997; Irmler *et al*., 1997; Shu *et al*., 1997; Fulda *et al*., 2000; Chang *et al*., 2002; Kim, H. *et al*., 2002; Davidson *et al*., 2003). In contrast, others have not been able to detect any effects on apoptosis upon c-FLIP$_L$ overexpression (Panner *et al*., 2005). Reintroduction of c-FLIP$_S$ into *cflar*$^{-/-}$ MEFs rescues from TRAIL-induced apoptosis more efficiently than reintroduction of c-FLIP$_L$ (Bin *et al*., 2002), suggesting that c-FLIP$_S$ has stronger anti-apoptotic properties than c-FLIP$_L$. The effects of c-FLIP$_L$ on caspase activation in the DISC seem to depend on expression levels (Chang *et al*., 2002).

c-FLIP$_L$ is able to promote apoptosis, in addition to its apoptosis-repressing properties. High overexpression of c-FLIP$_L$, but not c-FLIP$_S$, leads to spontaneous apoptosis (Inohara *et al*., 1997; Irmler *et al*., 1997; Shu *et al*., 1997; Fulda *et al*., 2000; Chang *et al*., 2002). The cytotoxic effects of c-FLIP$_L$ overexpression were inhibited by caspase inhibitors, and were

mediated by caspase-8 and by the caspase-like domain of c-FLIP$_L$ (Inohara *et al*., 1997; Shu *et al*., 1997; Rasper *et al*., 1998; Fulda *et al*., 2000). The high c-FLIP$_L$ expression might induce interactions between caspase-8 and c-FLIP$_L$ outside the DISC, inducing cell death without death receptor ligation. Indeed, Micheau and co-workers showed that overexpressed c-FLIP$_L$ can bind to caspase-8 without death receptor ligation (Micheau *et al*., 2002). c-FLIP can, however, not interact with initiator caspases without death receptor stimulation *in vivo* unless overexpressed (Scaffidi *et al*., 1999a).

The susceptibility towards death receptor-mediated apoptosis during development, differentiation, and disease can be altered by changing the expression levels of c-FLIP. When monocytes differentiate to macrophages, they gain resistance to CD95-mediated apoptosis by an increased expression of both c-FLIP$_L$ and c-FLIP$_S$ (Perlman *et al*., 1999). Hemin-induced differentiation results in downregulation of c-FLIP, thereby sensitizing K562 cells to TRAIL-induced apoptosis (Hietakangas *et al*., 2003). Human T-cell leukemia virus type 1 is able to increase the expression of c-FLIP via activation of the transactivator protein Tax, which protects infected cells from AICD (Krueger *et al*., 2006). Also host cells infected by *Trypanosoma cruzi* have elevated expression levels of c-FLIP$_L$ and are therefore resistant to CD95 signaling (Hashimoto *et al*., 2005). Tumor cells have been shown to express elevated levels of c-FLIP rendering these cells resistant to death receptor-mediated apoptosis (Irmler *et al*., 1997; reviewed by Thome and Tschopp, 2001; Debatin and Krammer, 2004; Schulze-Bergkamen and Krammer, 2004). Different approaches to downregulate the high c-FLIP levels and to sensitize tumor cells to death receptor-mediated apoptosis have been explored.

c-FLIP protects cells from apoptosis induced by CD95L, TRAIL, and TNF-α, but not from intrinsic apoptotic signaling induced by staurosporine (Krueger *et al*., 2001), γ-irradiation or chemotherapeutic drugs such as doxorubicin, vincristine or etoposide (Kataoka *et al*., 1998). On the other hand, DNA-damaging drugs can downregulate c-FLIP via increased p53 activation leading to an increased sensitivity to death receptor-mediated apoptosis (Fukazawa *et al*., 2001). Cytotoxic lymphocytes can induce apoptosis in target cells via both CD95 and granzyme B/perforin. c-FLIP cannot, however, protect from granzyme B/perforin-induced apoptosis (Kataoka *et al*., 1998). The *cflar*$^{-/-}$ cells were, as expected, similarly sensitized to death signaling facilitated by the mitochondrial pathway as wild-type cells (Yeh *et al*., 2000). In all cases where downregulation of c-FLIP has been shown to sensitize cells to death receptor-mediated apoptosis, it could be inhibited by caspase inhibitors.

Regulation of the c-FLIP expression at the level of protein synthesis

The c-FLIP expression can be upregulated and downregulated depending on the conditions when the cells need to be protected or sensitized to death receptor-mediated apoptosis. The exact mechanisms regulating the expression of c-FLIP are not solved, but several regulators of c-FLIP transcription and translation have been suggested. The transcription factor NF-κB regulates the expression of several survival promoting genes, among them *cflar*. NF-κB may, thus, protect cells from death receptor-mediated apoptosis by increasing the cellular c-FLIP levels (Kreuz *et al*., 2001; Micheau *et al*., 2001; Wang *et al*., 2006). Anti-proliferative and anti-metastatic drugs that inhibit activation of NF-κB have been used to sensitize cells to

apoptosis. Among them, evodamine efficiently blocks the expression of c-FLIP as well as of other NF-κB-induced anti-apoptotic proteins (Takada *et al*., 2005). The levels of c-FLIP are transiently upregulated during B-cell activation, and the B-cell receptor CD40 increases an NF-κB-dependent c-FLIP synthesis correlating with a reduced sensitivity to CD95-mediated apoptosis (Hennino *et al*., 2000; Eeva *et al*., 2007).

When the mechanisms of cell death mediated via p53 were investigated in a human colon carcinoma cell line, it was found that although p53 upregulates the death receptors CD95, TRAIL-R1, and TRAIL-R2, apoptosis induced via p53 does not involve caspase-8 activation. p53 enhanced the expression of several pro-apoptotic genes and downregulated several anti-apoptotic genes. Therefore, it was a surprise that the effect of p53 on the expression of c-FLIP was stimulation rather than inhibition. *cflar* promoter reporter assays revealed that the transcriptional activity of the *cflar* gene is regulated by p53. The upregulation of c-FLIP explains why caspase-8 is not activated in p53-induced apoptosis, although death receptor expression is enhanced. This suggests that the pro-apoptotic signaling mediated via p53 employs the intrinsic apoptotic pathway (Bartke *et al*., 2001).

Several other transcription factors have been shown to bind to the promoter of *cflar* either inhibiting or activating transcription. NFAT (nuclear factor of activated T-cells) binds to the *cflar* promoter and enhances the expression of c-FLIP in a VEGF-dependent (vascular endothelial growth factor) manner during angiogenesis (Zaichuk *et al*., 2004). The androgen receptor, a ligand-binding transcription factor essential for growth and differentiation of the cells in the prostate gland, binds to the *cflar* promoter upon androgen stimulation, enhancing the transcriptional activity of the *cflar* gene (Gao *et al*., 2005). Par-4 (prostate apoptosis response factor-4), a co-activator of the androgen receptor, can further enhance the expression of c-FLIP (Gao *et al*., 2006). c-Myc sensitizes to TRAIL-induced apoptosis by binding to the promoter of *cflar*, repressing its transcription (Ricci *et al*., 2004). Also the transcription factor E2F has been suggested to inhibit the synthesis of c-FLIP$_S$ (Salon *et al*., 2006). Inhibition of histone deacetylases (HDACs) is a major determinant of transcriptional activity and differential acetylation of histone tails regulates initiation of transcription. HDAC inhibition induces downregulation of both c-FLIP mRNA and protein (Watanabe *et al*., 2005; Pathil *et al*., 2006; Schuchmann *et al*., 2006). The HDAC inhibition-induced loss of c-FLIP sensitizes tumor cells to TRAIL ligation while primary cells are not affected (Pathil *et al*., 2006; Schuchmann *et al*., 2006). The inhibitory effect on *cflar* transcription mediated by HDAC inhibitors is not dependent on protein synthesis, implying that HDAC inhibition affects the c-FLIP expression directly (Watanabe *et al*., 2005).

The expression levels of c-FLIP are also regulated by the PI3K/Akt signaling pathway. Inhibition of the PI3K/Akt pathway sensitizes primary as well as tumor cells to apoptosis induced by TRAIL and CD95L. The sensitization has been shown to be a consequence of a decrease in the expression levels of c-FLIP (Panka *et al*., 2001; Suhara *et al*., 2001; Nam *et al*., 2002 and 2003; Bortul *et al*., 2003; Davies *et al*., 2004; Alladina *et al*., 2005; Uriarte *et al*., 2005; Kondo *et al*., 2006; Szegezdi *et al*., 2006). Conversely, activation of the PI3K/Akt pathway increases c-FLIP expression, thereby protecting cells from death receptor-mediated apoptosis (Panka *et al*., 2001; Suhara *et al*., 2001; Bortul *et al*., 2003; Nam *et al*., 2003; Kang *et al*., 2004; Starck *et al*., 2005). Since the PI3K/Akt signaling pathway stimulates growth

and protects from apoptosis, and is constitutively active in several cancer cells, it is a good target for anti-tumor therapies. However, also primary cells have been shown to be sensitized to death receptor-mediated apoptosis upon Akt inactivation due to decreased expression levels of c-FLIP. Luciferase assays suggested that the promoter region from -414 to -133 of the *cflar* promoter is needed for the PI3K/Akt-mediated transcriptional induction of *cflar* gene (Chiou *et al*., 2006). The forkhead transcription factor FOXO3a is negatively regulated by Akt. While FOXO3a has been shown to repress the transcription of *cflar*, Akt may release the repression and thereby enhance *cflar* transcription (Skurk *et al*., 2004). PI3K/Akt can directly target the translational initiation factor mTOR, which increases the levels of translated c-FLIP$_S$. The translation of c-FLIP$_L$ is not, however, affected by mTOR, indicating that mTOR recruits splicing factors that favor c-FLIP$_S$ translation instead of c-FLIP$_L$ (Panner *et al*., 2005). Both c-FLIP$_L$ and c-FLIP$_S$ are expressed from the same gene and are thereby controlled by the same promoter. Isoform-specific changes in the levels of c-FLIP mRNA may be explained either by different recruitment of splicing factors or by different regulation of mRNA stability.

Regulation of c-FLIP stability by post-translational modifications

Both c-FLIP$_L$ and c-FLIP$_S$ are targeted by ubiquitination and subjected to proteasomal degradation, providing an efficient mechanism of regulation of the intracellular levels of c-FLIP (reviewed by Tran *et al*., 2004). The ubiquitination-mediated degradation of c-FLIP, inducing sensitization to death receptor-mediated apoptosis has been shown to be enhanced upon hemin-induced differentiation (Poukkula *et al*., 2005), inhibition of cyclooxygenase-2 (Liu *et al*., 2006), infection with the adenovirus E1A (Perez and White, 2003), activation of p53 (Fukazawa *et al*., 2001; Chandrasekaran *et al*., 2006), and in response to PPARγ modulators (Kim, H. *et al*., 2002). Interestingly, both CD95L alone (Chanvorachote *et al*., 2005), and TRAIL in combination with 3,3'-diindolylmethane (Zhang *et al*., 2005) have been proposed to increase ubiquitination and proteasomal degradation of c-FLIP. In addition, TNF-α treatment has been shown to induce c-FLIP$_L$ ubiquitination mediated via JNK-induced phosphorylation and activation of the ubiquitin ligase Itch. The activated Itch interacts with the caspase-like domain of c-FLIP$_L$, and induces proteasomal degradation of c-FLIP$_L$. This provides a mechanism where JNK and Itch mediate increased sensitivity to TNF-α-induced apoptosis. However, TNF-α-induced apoptosis mediated by JNK requires inhibition of the NF-κB pathway (Chang *et al*., 2006).

The ubiquitination of c-FLIP$_L$ and c-FLIP$_S$ seems to be regulated differently, which concurrently explains the shorter turn-over of c-FLIP$_S$ compared to that of c-FLIP$_L$. The ubiquitination of c-FLIP$_S$ is regulated by its isoform-specific splicing tail. Lysines 192 and 195 are the primary target lysines during hemin-induced ubiquitination of c-FLIP$_S$. Although these target lysines are located before the splicing tail and are present also in c-FLIP$_L$, they are not targets for c-FLIP$_L$ ubiquitination. Therefore, the structure of the C-terminal tail regulates the ubiquitination of c-FLIP$_S$ (Poukkula *et al*., 2005).

Proteasome inhibitors are in clinical trials for their ability to sensitize cancer cells to death receptor-mediated apoptosis, especially to TRAIL. As c-FLIP is both constitutively and inducibly ubiquitinated and degraded via the proteasome, proteasome inhibition by

bortezomib, MG132, lactacystin, or epoxomicin has been shown to induce accumulation of c-FLIP (Fukazawa *et al*., 2001; Kreuz *et al*., 2001; Kim, H. *et al*., 2002; Perez and White, 2003; Chanvorachote *et al*., 2005; Poukkula *et al*., 2005; Zhang *et al*., 2005; Anan *et al*., 2006; Chang *et al*., 2006). However, proteasome inhibition has been shown not to affect (Johnson *et al*., 2003) and even to reduce (Sayers *et al*., 2003) the c-FLIP levels. The contradicting results could be explained by the proteasome inhibitor dose, exposure times, or the cell types used. Since proteasome inhibitors also inhibit NF-κB-mediated new synthesis of c-FLIP (Kreuz *et al*., 2001), the levels of c-FLIP might be depleted during longer treatments. By this means, no c-FLIP accumulation would be induced and the cells could be sensitized to death receptor-mediated apoptosis.

In addition to ubiquitination, c-FLIP is subjected to other post-translational modifications. Phosphorylation at serine 193 inhibits the ubiquitination of c-$FLIP_S$ and stabilizes the protein (Kaunisto *et al*., unpublished data). Interestingly, this phosphorylation site is located between the target lysines 192 and 195 for ubiquitination of c-$FLIP_S$ during hemin-induced differentiation. Also others have reported a stabilizing effect of phosphorylation on c-FLIP. CaMKII-mediated phosphorylation stabilizes c-$FLIP_L$ and protects against TRAIL- and CD95L-induced apoptosis (Yang *et al*., 2003; Xiao *et al*., 2005). Conversely, CaMKII inhibitors induce downregulation of c-$FLIP_L$, sensitizing resistant cells to death receptor signaling (Yang *et al*., 2003; Xiao *et al*., 2005; Song *et al*., 2006). The stabilized phosphorylated c-$FLIP_L$ remains in the DISC, where it is accumulated and inhibits caspase-8 activation (Yang *et al*., 2003). Both PI3K/Akt and PKC induced phosphorylation of c-$FLIP_L$ with the same consequences as CaMKII (Xiao *et al*., 2005). In contrast, PKC has been shown to phosphorylate c-FLIP in response to bile acids, leading to a loss of the phosphorylated c-FLIP from the DISC. The loss of c-FLIP allows for more efficient caspase-8 and caspase-10 activation and thereby sensitization to TRAIL-induced apoptosis (Higuchi *et al*., 2003). The contradictory outcome of phosphorylation on c-FLIP stability might be due to targeting of different phosphorylation sites, which might provide additional regulation of c-FLIP.

2.4.4. Alternative pathways regulated via death receptors

The NF-κB transcription factor consists of five subunits; RelA/p65, c-Rel, Rel-B, p50/NF-κB1, and p52/NF-κB2. Most of the NF-κB subunits are retained in the cytoplasm by specific inhibitors of NF-κB, the I-κBs. The IKK (I-κB kinase) complex is composed of two catalytic subunits IKK-α and IKK-β and a regulatory subunit IKK-γ/NEMO (NF-κB essential modulator). Upon phosphorylation by the IKK complex, the I-κBs are ubiquitinated and degraded, releasing NF-κB to the nucleus where it can function as an active transcription factor (Fig. IX) (Liu *et al*., 1996; reviewed by Karin, 2006).

TNF-α is a well known inducer of both NF-κB activation and apoptosis. In addition to inducing apoptosis, ligation of CD95 (Ponton *et al*., 1996), TRAIL-R1 and TRAIL-R2 (Schneider *et al*., 1997; Jeremias *et al*., 1998), as well as the decoy receptor TRAIL-R4 (Degli-Esposti *et al*., 1997a) can activate NF-κB-dependent survival signaling (Fig. IX). Thus, death receptor ligation may promote survival signaling in cells in which the apoptotic pathway for some reason is blocked. While TNF-α utilizes two complexes for induction of

either NF-κB or apoptosis signaling cascades, CD95 and TRAIL-R ligation leads to formation of only one signaling complex, the DISC, responsible for both the death and the survival signaling (reviewed by MacFarlane, 2003). Similarly to TNF-R1, TRAIL-R1 and TRAIL-R2 employ TRADD for NF-κB signaling and FADD for apoptosis signaling (Chaudhary *et al*., 1997; Schneider *et al*., 1997). In contrast, the NF-κB activation induced via CD95 is mediated via FADD instead of TRADD (Imamura *et al*., 2004). Overexpression of FADD, c-FLIP, caspase-8, caspase-10, TRADD, and TRAF2 have been shown to activate NF-κB without death receptor ligation (Fig. IX) (Chaudhary *et al*., 2000; Hu *et al*., 2000; Shikama *et al*., 2003; Kreuz *et al*., 2004). In order to induce degradation of IκBα and NF-κB activation, these proteins need to interact with and activate TRAF2, NIK (NF-κB-inducing kinase), IKKα, and IKKβ (Chaudhary *et al*., 2000; Kataoka *et al*., 2000; Shikama *et al*., 2003).

Caspase-8 is able to cleave RIP-1, and the cleaved form of RIP-1 loses its ability to activate NF-κB in response to TNF-α ligation. The caspase-8-cleaved form of RIP-1 enhances the association of FADD to TRADD and thereby the formation of the apoptosis-inducing TNF-R1 signaling complex II (Lin *et al*., 1999). TNF-α-induced apoptosis is inhibited by a mutant of RIP-1 that is resistant to cleavage by caspase-8 (Lin *et al*., 1999), indicating that the cleavage of RIP-1 is required for the formation of complex II. RIP-1 is cleaved also during CD95L- and TRAIL-induced apoptosis, and thereby RIP-1 cleavage seems to determine whether death receptor signaling via TNF-R1 as well as TRAIL-R and CD95 is directed towards death or NF-κB activation (Liu *et al*., 1996; Chaudhary *et al*., 1997; Lin *et al*., 1999; Martinon *et al*., 2000). TRAF2 has been shown to bind to both the activated TNF-R1 and to TRADD (Hsu *et al*., 1996b) and to be associated with the CD95-DISC (Wajant *et al*., 2003). TRAF2 is crucial for CD95- and TNF-R1-mediated NF-κB activation, but is, surprisingly, dispensable for TRAIL-induced NF-κB activation (Lin *et al*., 2000).

While caspase-8 seems to turn death receptor signaling towards the apoptotic pathways via RIP-1 cleavage, cleavage of c-FLIP by caspase-8 has been shown to enhance NF-κB survival signaling. The cleaved p43-c-FLIP$_L$ is more potent in recruiting TRAF2 and RIP1 to the c-FLIP$_L$-caspase-8 complex than the uncleaved c-FLIP$_L$ (Kataoka and Tschopp, 2004; Dohrman *et al*., 2005a). Golks and co-workers have shown that caspase-8 can cleave both c-FLIP$_L$ and c-FLIP$_S$ downstream of their DED to form p22-c-FLIP. p22-c-FLIP is able to bind IKKγ/NEMO and efficiently activate NF-κB (Golks *et al*., 2006). This result indicates that NF-κB signaling mediated via c-FLIP requires caspase-8. Also FADD-induced NF-κB activation has been suggested depend on caspase-8 (Hu *et al*., 2000). Therefore, it seems that processing of c-FLIP is sufficient for induction of NF-κB, and that a possible RIP-1 cleavage cannot interfere with this.

Surprisingly, overexpression of c-FLIP$_L$ has been shown to inhibit NF-κB signaling induced by both CD95L (Imamura *et al*., 2004) and TRAIL (Wachter *et al*., 2004). This effect might be caused by inhibition of caspase-8 activation induced by the overexpression of c-FLIP. Additionally, inhibition of caspase activity prevented NF-κB activation through c-FLIP, caspase-8 (Chaudhary *et al*., 2000; Hu *et al*., 2000; Kataoka *et al*., 2000; Shikama *et al*., 2003), and CD95 (Imamura *et al*., 2004; Kreuz *et al*., 2004). Interestingly, both c-FLIP and caspase-8 have been shown to be dispensable for induction of NF-κB in some studies. NF-κB

activation was normal in T-lymphocytes lacking c-FLIP (Zhang and He, 2005), in *cflar*$^{-/-}$ MEFs (Yeh *et al*., 2000), as well as in *caspase-8*$^{-/-}$ MEFs (Varfolomeev *et al*., 1998). According to these observations, neither c-FLIP nor caspase-8 is required for the NF-κB signaling pathway.

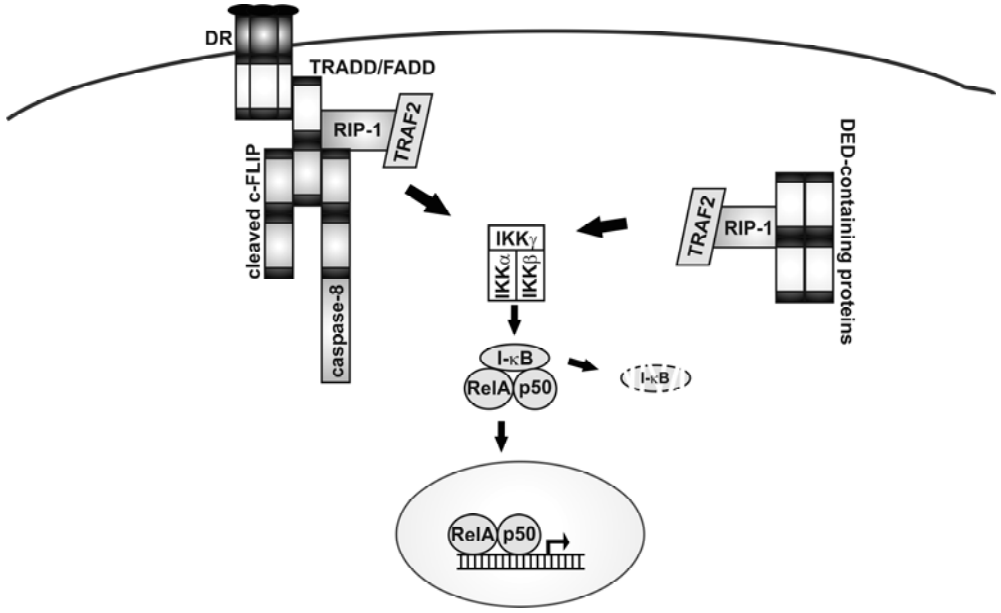


Figure IX. DED proteins as mediators of NF-κB activation. RIP-1 and TRAF2 are recruited to the receptor-bound TRADD or FADD, inducing activation of the IKK complex. The active IKKs phosphorylate I-κB, which results in I-κB ubiquitination and proteasomal degradation. When the NF-κB subunits are released from I-κB, they translocate into the nucleus, where NF-κB can promote transcription of its target genes. Caspase-8-mediated cleavage of c-FLIP is additionally promoting NF-κB activation. DED-containing proteins can oligomerize also in the absence of death receptor ligation when they are abundantly expressed. The oligomerized DED proteins can recruit RIP-1 and TRAF2 and activate the IKK complex, which further activates the NF-κB signaling cascades.

TCR stimulation induces NF-κB-mediated expression of several genes coding for proteins needed for T-lymphocyte proliferation. NF-κB signaling induced in response to TCR ligation requires caspase-8. Caspase-8 is able to recruit Bcl-10 and MALT (mucosa-associated lymphoid tissue lymphoma translocation protein), forming a complex necessary for the engagement of IKK (Su *et al*., 2005). Also c-FLIP$_L$ has been shown to be involved in NF-κB signaling induced by TCR stimulation, and the hyperproliferative features of T-lymphocytes in c-FLIP$_L$ transgenic mice have been proposed to be a consequence of increased NF-κB transcriptional activity (Dohrman *et al*., 2005a). Both CD8+ T-lymphocytes from c-FLIP$_L$ transgenic mice and Jurkat cells overexpressing c-FLIP produced more IL-2 following TCR stimulation than wild-type cells (Kataoka *et al*., 2000; Dohrman *et al*., 2005a). Reduced NF-κB activation upon T-lymphocyte stimulation in c-FLIP transgenic mice has been reported (Tai *et al*., 2004), but the inhibitory effect might be due to an overexpression high enough to inhibit caspase-8.

In addition to the activation of initiator caspases and NF-κB, death receptor ligation can affect death- and survival-inducing kinases such as JNK (Liu *et al*., 1996; Lin *et al*., 2000;

Wajant *et al*., 2003), ERK (Kataoka *et al*., 2000), and PI3K/Akt (Zauli *et al*., 2005). TRAF2 and RIP-1 are both involved in TNF-α-induced JNK activation, whereas FADD is not, suggesting that the signaling complex I is employed for TNF-R1-mediated JNK signaling (Liu *et al*., 1996). Interestingly, TRAF2, which is dispensable for TRAIL-induced NF-κB activation, can mediate TRAIL-induced JNK activation (Lin *et al*., 2000). c-FLIP is not required for JNK activation, since no defects in this signaling pathway is observed in *cflar*$^{-/-}$ MEFs (Yeh *et al*., 2000). c-FLIP$_L$ has even been suggested to inhibit TNF-α-induced JNK activation by binding the upstream JNK activator MKK7 (Nakajima *et al*., 2006). c-FLIP can recruit the ERK upstream MAP kinase kinase kinase Raf-1 to the DISC. While both c-FLIP$_L$ and c-FLIP$_S$ have been shown to enhance ERK activation, c-FLIP$_S$ is a more potent activator than c-FLIP$_L$ (Kataoka *et al*., 2000). On the other hand, c-FLIP is not required for activation of ERK during T-lymphocyte proliferation, since the activation induced by TCR stimulation is normal in T-lymphocytes from Rag chimeric mutant mice deficient in c-FLIP (Chau *et al*., 2005; Zhang and He, 2005). Even reduced ERK activation in response to TCR stimulation in c-FLIP transgenic mice has been shown (Tai *et al*., 2004). Taken together, the survival-promoting properties of the anti-apoptotic proteins of the DISC are extended beyond inhibition of the initiator caspases and the pro-apoptotic proteins are evidently involved in proliferative signaling in addition to their death-promoting functions.

2.4.5. DISC proteins in the regulation of lymphocyte proliferation and cell death

Regulation of T-lymphocyte homeostasis

In addition to being involved in lymphocyte apoptosis, the proteins of the death-inducing signaling complex are important for activation and proliferation of T-lymphocytes. T-progenitor cells migrate from the bone marrow to the thymus, where their TCRs are exposed to self-antigens. Depending on the affinity of the self-antigens to their TCRs the T-lymphocytes are either positively or negatively selected (reviewed by Krammer, 2000). The T-lymphocytes not accepted during selection are removed, and CD95 might be involved in the elimination of these cells (reviewed by Nagata and Suda, 1995; Cui *et al*., 1996). T-lymphocytes exposed to a peptide/MHC (major histocompatibility complex) class I molecules will turn into CD8+ T-lymphocytes, whereas T-lymphocytes exposed to peptide/MHC class II molecules will develop to CD4+ T-lymphocytes. The CD4+ cells differentiate to T-helper (Th) cells involved in cytokine secretion regulating cellular immune responses (Th1 cells) or in regulation of antibody responses (Th2 cells). The CD8+ cells are cytotoxic T-lymphocytes involved in killing targeted cells (reviewed by Krammer, 2000). The resting T-lymphocytes leave the thymus generating the splenic, lymphatic, and peripheral T-lymphocyte pools. Antigen-presenting cells, such as dendritic cells, introduce antigens to the T-lymphocytes. The antigen presentation is mediated via the MHC on the antigen-presenting cell. In addition, co-stimulatory molecules are required for full activation of T-lymphocytes. Activation of T-lymphocytes by their specific antigen leads to sequestration of the T-lymphocytes to lymphoid tissues where they proliferate rapidly. After differentiation into effector cells they are again released to the circulation (reviewed by Sprent and Tough, 2001).

The apoptotic signaling in lymphocytes is subjected to a stringent but dynamic regulation. Following T-lymphocyte activation, the death receptors of proliferating lymphocytes need to be carefully secured against apoptotic signaling. On the other hand, the T-lymphocytes produced during clonal expansion need to regain their sensitivity towards death receptor-mediated apoptosis to be eliminated when the antigens have been cleared out (Fig. X). Resting T-lymphocytes are resistant to CD95-mediated apoptosis due to the lack of surface-expressed CD95 (Klas *et al*., 1993). Although the expression of CD95 is increased during activation, the T-lymphocytes remain resistant to CD95-mediated apoptosis early upon activation (Schmitz *et al*., 2004). Caspase-8 is inhibited from binding to the CD95-DISC during this early activation, probably by high expression of c-FLIP$_S$ (Peter *et al*., 1997; Inaba *et al*., 1999). The increased expression of c-FLIP$_S$ during activation of T-lymphocytes has been suggested to be due to an increased NF-κB activation (Schmitz *et al*., 2004; Bosque *et al*., 2005). The sensitivity towards CD95 signaling is regained in the fully activated lymphocytes by a subsequent decrease in the c-FLIP$_S$ levels (Algeciras-Schimnich *et al*., 1999; Kirchhoff *et al*., 2000; Schmitz *et al*., 2004; Bosque *et al*., 2005), allowing for efficient binding of caspase-8 to the DISC. The resistance to CD95-mediated apoptosis during T-lymphocyte activation corresponds to the levels of c-FLIP$_S$ (Irmler *et al*., 1997), but not the levels of c-FLIP$_L$, the expression of which remain constant (Scaffidi *et al*., 1999a).

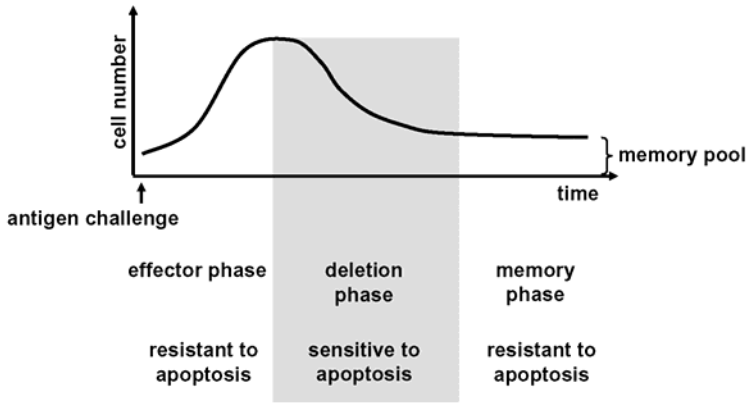


Figure X. Time course of apoptosis sensitivity during T-lymphocyte activation. Naïve T-lymphocytes undergo clonal expansion upon antigen challenge and differentiate into effector cells. During proliferation in this effector phase T-lymphocytes are resistant to apoptotic signals. The T-lymphocytes regain sensitivity after an immune response and are eliminated via AICD. Also the T-lymphocytes that survive AICD and enter the memory T-lymphocyte pool are resistant to apoptosis (modified from Krueger *et al*., 2003).

The excess of activated T-lymphocytes needs to be efficiently eliminated at the end of an immune response in order to avoid autoreactivity. When the TCR of an activated lymphocyte is repeatedly stimulated with its specific antigen, it is removed via AICD. AICD is induced within a population of activated T-lymphocytes via an increased expression and release of CD95L from fully activated T-lymphocytes (Peter *et al*., 1997; Norian *et al*., 2000; Li-Weber and Krammer, 2002; Schmitz *et al*., 2004). To form a memory cell pool, some cells survive the restimulation-induced AICD. These cells are resistant to CD95 ligation due to increased expression of c-FLIP$_S$ (Kirchhoff *et al*., 2000; Bosque *et al*., 2005; Oehme *et al*., 2005) and decreased expression of caspase-8, caspase-10, and caspase-3 (Strauss *et al*., 2003). The T-lymphocytes of *lpr* and CD95-deficient mice are not sufficiently eliminated after activation,

provoking unrestrained T-lymphocyte proliferation, which causes lymphadenopathy and lymphocytic infiltration in lungs and liver (Mogil *et al*., 1995; Adachi *et al*., 1996; Fortner and Budd, 2005). The CD95 pathway is, however, not the only pathway involved in the elimination of activated T-lymphocytes (Mogil *et al*., 1995). Another mechanism is "death by neglect" in response to growth factor and cytokine deprivation (reviewed by Krammer, 2000).

Cytotoxic T-lymphocytes (CTLs) eliminate autoreactive B-lymphocytes via AICD when they present autoantigens/self-antigens to the CTLs. Retrovirus-induced expression of c-FLIP blocks CD95-mediated apoptosis and AICD in activated B- and T-lymphocytes. In experimental conditions, self-antigens can be mimicked by superantigens such as staphylococcal enterotoxin B (SEB). c-FLIP-overexpressing cells do not respond to superantigens, indicating that cell death induced by presentation of self-antigens is impaired. Overexpression of c-FLIP *in vivo* results in accumulation of mature lymphocytes in the spleen and lymph nodes. It also increases the pool of autoreactive B-lymphocytes and causes autoimmune tissue damage. Interestingly, retroviral overexpression of c-FLIP does not affect lymphocyte development, activation, or immune responses to foreign antigens. Due to the disturbance in the elimination of autoreactive T-lymphocytes and B-lymphocytes, high quantities of c-FLIP and impaired lymphocyte apoptosis are associated with several cases of autoimmune diseases (van Parijs *et al*., 1999).

c-FLIP in T-lymphocyte survival and proliferation

c-FLIP$_L$ overexpression protects from CD95-mediated apoptosis in *in vitro* activated T-lymphocytes isolated from c-FLIP$_L$ transgenic mice (Lens *et al*., 2002; Tai *et al*., 2004; Tseveleki *et al*., 2004) and in activated T-lymphocytes overexpressing c-FLIP$_L$ (Refaeli *et al*., 1998; Charo and Robbins, 2007). When studying the effects of c-FLIP$_L$ overexpression on AICD, different research groups show different results. As Tai and co-workers detected a resistance to AICD in c-FLIP$_L$ transgenic T-lymphocytes activated *in vitro* (Tai *et al*., 2004), neither Tseveleki and co-workers nor Lens and co-workers could observe such resistance (Lens *et al*., 2002; Tseveleki *et al*., 2004). The controversy might be due to different c-FLIP expression levels in the different transgenes. Like the T-lymphocytes from c-FLIP$_L$ transgenic mice, also the cells from c-FLIP$_S$ transgenic mice were resistant to CD95-mediated apoptosis, whereas AICD was not affected in T-lymphocytes from c-FLIP$_S$-overexpressing mice (Oehme *et al*., 2005). Interestingly, the memory cell pool formed after an immune response was increased in the c-FLIP$_S$ transgenic mice (Oehme *et al*., 2005). Caspase-8-deficient T-lymphocytes were also protected from CD95-mediated apoptosis, but not from AICD, indicating that AICD may be induced without caspase-8 activation (Salmena *et al*., 2003).

Several studies have shown that c-FLIP$_L$ overexpression in transgenic mice increases T-lymphocyte proliferation (Lens *et al*., 2002; Dohrman *et al*., 2005b). While overexpression of c-FLIP$_L$ also augments caspase-8 activation, increasing cell death, c-FLIP$_L$-transgenic mice have a normal number of T-lymphocytes (Lens *et al*., 2002; Dohrman *et al*., 2005b). The role of c-FLIP in T-lymphocyte proliferation was investigated in c-FLIP-deficient T-lymphocytes that were developed by reconstituting Rag$^{-/-}$ blastocysts with c-FLIP-deficient embryonic

stem cells as well as in conditional c-FLIP knock-outs. Like FADD- and caspase-8-deficient cells, the c-FLIP-deficient T-lymphocytes did not proliferate properly in response to TCR stimulation (Chau *et al*., 2005; Zhang and He, 2005). The c-FLIP-deficient T-lymphocytes were, like FADD- and caspase-8-deficient T-lymphocytes, also defective in responding to IL-2 (reviewed by Budd *et al*., 2006). Interestingly, T-lymphocytes deficient in c-FLIP show a reduced IL-2 production, indicating that c-FLIP is involved in the control of IL-2 expression (Zhang and He, 2005). The c-$FLIP_L$-induced IL-2 production has been proposed to depend on NF-κB and ERK signaling (Kataoka *et al*., 2000; Lens *et al*., 2002).

Transgenic mice with targeted caspase-8 mutations restricted to T-lymphocytes have a decreased number of peripheral T-lymphocytes and show impaired T-lymphocyte activation (Salmena *et al*., 2003). These mice do not respond properly to viral infections, indicating immunodeficiency (Salmena *et al*., 2003). T-lymphocyte-specific deletion of *caspase-8* is associated with lethal lymphoproliferative immune disorders characterized by lymphadenopathy, splenomegaly, and accumulation of T-lymphocytes in lungs, liver, and kidney (Salmena and Hakem, 2005). A mutation in the *caspase-8* gene, giving rise to a caspase-8 protein without enzymatic function and capability of binding to the DISC has been found. In addition to the defective lymphocyte apoptosis that is also described in ALPS patients, individuals homozygous for this mutation have defects in the activation of T-lymphocytes, B-lymphocytes, and NK cells, leading to immunodeficiency (Chun *et al*., 2002). Studies with caspase inhibitors have similarly indicated that T-lymphocyte activation requires caspase-8 activation (Alam *et al*., 1999; Kennedy *et al*., 1999; Gilot *et al*., 2005). In contrast, both *lpr* and *CrmA* mice showed normal proliferation (Newton *et al*., 1998). Contradictory to the reported proliferation promoting effects of c-$FLIP_L$, T-lymphocyte activation and IL-2 production have also been shown to be impaired both in c-$FLIP_L$ transgenic mice (Tai *et al*., 2004; Charo and Robbins, 2007) and in T-lymphocytes overexpressing c-$FLIP_S$ (Oehme *et al*., 2005). Since caspase activation is required for TCR-mediated proliferation and IL-2 production, it is possible that the impaired T-lymphocyte proliferation observed in some c-FLIP transgenic models is due to inhibited caspase activation (Kennedy *et al*., 1999). Therefore, the effects of c-FLIP overexpression on T-lymphocyte proliferation may depend on the different levels of expression in the transgenic mice.

IL-2 is required for T-lymphocyte activation, and the increased expression of IL-2 during activation might be mediated via FADD-, caspase-8-, and c-FLIP-induced NF-κB and ERK activation. IL-2 has been suggested to be responsible for the downregulation of the c-$FLIP_S$ levels at the end of immune responses. In addition to the downregulation of c-$FLIP_S$, the enhanced expression of CD95 and CD95L in activated T-lymphocytes is implied to be mediated via IL-2 (Refaeli *et al*., 1998; Algeciras-Schimnich *et al*., 1999). Inhibition of IL-2 production and function with cyclosporine A or IL-2 neutralizing antibodies, inhibit both T-lymphocyte activation and the downregulation of c-$FLIP_S$ (Algeciras-Schimnich *et al*., 1999). Resting as well as recently activated T-lymphocytes are Type II cells, and can be protected from apoptotic signaling via high expression of Bcl-2 or Bcl-XL, in addition to the protective effect of high c-$FLIP_S$ levels. During activation, T-lymphocytes switch their Type II cell properties to those of Type I cells. In addition to downregulation of c-$FLIP_S$, IL-2 has been proposed to induce Type I cell properties in activated T-lymphocytes, allowing for efficient

apoptotic signaling without requirement for the mitochondrial amplification loop (Schmitz *et al*., 2003).

2.4.6. Diseases involving abnormal death receptor responses – the role of c-FLIP

High c-FLIP levels are associated with cancer

Several tumor cells have an enhanced resistance to death receptor-mediated apoptosis. One of the common mechanisms tumor cells use to protect themselves from cell death is an increased expression of c-FLIP (Irmler *et al*., 1997; reviewed by Thome and Tschopp, 2001; Debatin and Krammer, 2004; Schulze-Bergkamen and Krammer, 2004). Most Epstein-Barr virus (EBV) positive Burkitt's lymphoma cells express high levels of c-FLIP$_L$ and are resistant to CD95-mediated apoptosis. Changing the balance between caspase-8 and c-FLIP in the DISC in EBV-positive cells by overexpressing caspase-8 sensitizes to CD95-mediated apoptosis, whereas overexpression of c-FLIP in EBV-negative cells protects from it (Tepper and Seldin, 1999). Also malignant mesothelial cells are resistant to death receptor-mediated caspase-8 activation and killing, while normal mesothelial cells are not. The levels of c-FLIP are upregulated in malignant mesothelial cells compared to normal mesothelial cells, and downregulation of c-FLIP$_L$ with siRNA sensitizes the malignant mesothelial cells to apoptosis induced by TRAIL or CD95L (Rippo *et al*., 2004). In addition, TRAIL-resistant prostate cancer cells have high expression levels of c-FLIP$_L$ but can be sensitized to TRAIL-ligation by downregulation of c-FLIP$_L$ by siRNA. Similarly, overexpression of c-FLIP$_L$ in TRAIL-sensitive cells renders resistance, suggesting that the levels of c-FLIP determine the sensitivity to TRAIL (Zhang *et al*., 2004). Isoform-specific knock-down of either c-FLIP$_L$ or c-FLIP$_S$ sensitizes several cancer cell lines to apoptosis induced by TRAIL or CD95L. The sensitization depends on the initial expression level of the isoforms and the efficiency of the siRNA-mediated knock-down (Sharp *et al*., 2005).

The increased c-FLIP levels in tumor cells can be explained either by induced gene expression or by stabilization of the c-FLIP protein. The c-FLIP levels have been shown to maintain high during cycloheximide treatments in certain tumor cells (Ahmad and Shi, 2000; Youn *et al*., 2001; MacFarlane *et al*., 2002; Nesterov *et al*., 2002; Thomas *et al*., 2002), indicating that the c-FLIP degradation is inhibited. Hodgkin and Reed-Sternberg cells, the malignant cells of classical Hodgkin's disease, constitutively overexpress c-FLIP and are resistant to CD95-mediated apoptosis (Thomas *et al*., 2002; Mathas *et al*., 2004). One group demonstrated that the increased c-FLIP levels in these cells were a result of increased stabilization of the c-FLIP protein (Thomas *et al*., 2002), whereas other groups state that the levels of c-FLIP are reduced in these cells upon cycloheximide treatments, suggesting that enhanced protein synthesis would be responsible for the high c-FLIP levels (Dutton *et al*., 2004; Mathas *et al*., 2004). These results indicate that tumor cells can both increase the expression of c-FLIP and stabilize the already synthesized c-FLIP protein, and that the mechanism applied can vary.

Some chemotherapeutic drugs, such as Actinomycin D and 3,3'-diindolylmethane induce downregulation of c-FLIP and sensitizes to apoptosis induced by TRAIL (Griffith *et al*.,

1998; Leverkus *et al*., 2000; Zhang *et al*., 2005). Also proteasome inhibitors, such as PS-341 (also known as Velcade® or bortezomib), have been shown to efficiently sensitize tumor cells to TRAIL-induced apoptosis (Sayers *et al*., 2003; Ganten *et al*., 2005). Various mechanisms behind the proteasome inhibitor-induced sensitization have been proposed. While some studies show stabilization of c-FLIP in response to proteasome inhibition (Fukazawa *et al*., 2001; Poukkula *et al*., 2005), other studies demonstrate that proteasome inhibition can induce c-FLIP downregulation (Sayers *et al*., 2003; Ganten *et al*., 2005; Kabore *et al*., 2006). The reduction in the c-FLIP levels caused by proteasome inhibitors may be due to inhibition of I-κB degradation and thereby inhibition of NF-κB activation. Since *cflar* is an NF-κB target gene, this might lead to reduced levels of c-FLIP. The role of NF-κB inhibition in the TRAIL sensitization mediated by proteasome inhibition has been both supported (Rajkumar *et al*., 2005) and opposed (Sayers *et al*., 2003; Ganten *et al*., 2005). Although it has been reported that proteasome inhibitors specifically sensitize tumor cells to TRAIL (Ganten *et al*., 2005), also primary cells have been shown to be sensitized (Leverkus *et al*., 2003), which might complicate the clinical applicability of a combination of TRAIL and proteasome inhibitors. A functional proteasome has even been proposed to be required for death receptor-mediated apoptosis in proliferating tumor cells (Sohn *et al*., 2006).

Cytotoxic T-lymphocytes induce apoptosis via both CD95L and granzyme B/perforin, which is critical in immunity against tumors (Lowin *et al*., 1994). This CTL response is inhibited in murine tumor cells overexpressing c-$FLIP_L$. c-$FLIP_L$ overexpression does not affect the granzyme B/perforin pathway, but can still sufficiently inhibit death induced by cytotoxic T-lymphocytes *in vivo*, suggesting that the CD95 pathway is indispensable for tumor clearance (Medema *et al*., 1999). Also viral FLIP is able to protect tumor cells from apoptosis induced by cytotoxic T-lymphocytes and thereby promote tumor progression and growth (Djerbi *et al*., 1999). Overexpression of c-FLIP protects tumor cells from NK cell-mediated killing both *in vitro* and *in vivo*. In addition, overexpression of c-FLIP inhibited graft rejection and induced engraftment of bone marrow stem cells in the absence of perforin (Taylor *et al*., 2001).

Since high expression levels of c-FLIP are associated with resistance to death receptor-mediated apoptosis in cancer cells, efforts have been made to develop anti-cancer agents targeting c-FLIP. Doxorubicin is an anti-cancer drug that induces apoptosis via DNA damage-induced activation of p53 and by induction of ROS. Doxorubicin was shown to promote downregulation of c-$FLIP_L$ in cardiac myocytes, sensitizing them to CD95L-induced apoptosis (Nitobe *et al*., 2003). The downregulation of c-FLIP may have been mediated via p53, which is able to induce ubiquitination and degradation of c-FLIP (Fukazawa *et al*., 2001; Kataoka *et al*., 2002). Doxorubicin can sensitize to TRAIL-induced apoptosis in several cancer cell lines otherwise resistant to TRAIL (Kelly *et al*., 2002; El-Zawahry *et al*., 2005). Other anti-cancer drugs, such as the cyclin-dependent kinase inhibitor flavopiridol (Palacios *et al*., 2006), the anti-metabolite 5-fluorouracil (Ganten *et al*., 2004), the tyrosine kinase inhibitor STI-571, more commonly known as imatinib mesylate (Hamai *et al*., 2006), the DNA-damaging agent oxalipatin, and the topoisomerase-1 inhibitors irinotecan (Galligan *et al*., 2005) and rubitecan (9-nitrocampothecin) (Chatterjee *et al*., 2001), are able to downregulate c-FLIP and thereby sensitize tumor cells to TRAIL- or CD95L-induced

apoptosis. In addition to c-FLIP, other signaling molecules both upstream and downstream of c-FLIP may contribute to the apoptosis-resistance in tumor cells.

c-FLIP as a determinant of lymphocyte survival in autoimmune diseases

Naïve T-lymphocytes are activated and become effector cells when they encounter their specific antigen. The antigen is presented to the T-lymphocytes by antigen-presenting cells. Autoimmune diseases are caused by T-lymphocytes and B-lymphocytes that have lost their tolerance towards autoantigens, and are thereby activated in the absence of an ongoing infection or pathogen stimulation. The cause of this breakdown of immune tolerance to self-antigens is unknown, but it probably involves impairment of AICD of autoreactive T-lymphocytes. Due to the increased activation of B-lymphocytes, elevated levels of autoantibodies and serum immunoglobulins are associated with autoimmune diseases (reviewed by Aktas *et al*., 2006).

Multiple sclerosis (MS) is believed to involve potentially pathogenic, autoimmune, or autoreactive T-lymphocytes that react with myelin antigens, such as myelin basic protein. When comparing the expression of c-FLIP in peripheral activated T-lymphocytes from patients with clinically active, clinically stable, and healthy donors, the levels of both c-$FLIP_L$ and c-$FLIP_S$ were downregulated at the end of lymphocyte activation in the cells from clinically stable and healthy donors, whereas the c-$FLIP_L$ and c-$FLIP_S$ levels maintained high in the activated T-lymphocytes of patients with active MS. Accordingly, the T-lymphocytes of the patients with active state MS were less sensitive to CD95-mediated apoptosis than the lymphocytes from patients with stable MS (Semra *et al*., 2001). Little apoptosis and high levels of c-FLIP have also been reported in macrophages of patients with longstanding rheumatoid arthritis (Catrina *et al*., 2002).

Naïve CD4+ T-lymphocytes can differentiate to Th1 or Th2 cells depending on the cytokines they are exposed to. Th1 cells activate macrophages, neutrophils, and CD8+ cytotoxic T-lymphocytes, whereas Th2 cells promote B-lymphocyte proliferation, maturation, and antibody production. Loss of the balance in Th1 and Th2 differentiation is linked with several diseases. Increased Th2 responses can lead to asthma and allergic disorders, whereas increased Th1 responses are associated with autoimmune diseases (Wu *et al*., 2004). Studies in transgenic mice overexpressing c-$FLIP_L$ in the T-lymphocytes have shown that c-$FLIP_L$ enhances Th2 type responses and thereby immunoglobulin production and hypersensitivity to allergens in addition to protecting from CD95-mediated apoptosis. The high amount of Th2 cells may also increase allergic airway inflammation (Tseveleki *et al*., 2004; Wu *et al*., 2004). Th1 responses, on the other hand, are decreased in transgenic mice overexpressing c-$FLIP_L$ in their T-lymphocytes. Since Th1 cells are more sensitive to caspase-8 activation, the overexpression of c-$FLIP_L$ may increase cell death in Th1 cells due to the ability of c-$FLIP_L$ to augment caspase-8 activation (Wu *et al*., 2004).

Experimental autoimmune encephalomyelitis (EAE) is a Th1-driven autoimmune disease. c-$FLIP_L$ overexpression, enhancing Th2 responses in favor of Th1 responses, has been shown to protect mice from this autoimmune disease. When c-$FLIP_L$-transgenic mice were exposed to EAE peptides and T-lymphocytes from these mice were introduced to wild-type mice by

adoptive transfer, the recipient mice were protected from EAE. This indicates that the c-$FLIP_L$-overexpressing T-lymphocytes alter cytokine production and differentiate into EAE-suppressive T-lymphocytes (Tseveleki *et al*., 2004). On the other hand, overexpression of c-$FLIP_L$ by retroviral transduction of hematopoietic stem cells boosted the already induced EAE, by strengthening and prolonging the inflammatory response in the CNS (central nervous system) (Djerbi *et al*., 2003). The differences between these studies are that Tseveleki and co-workers constitutively overexpressed c-$FLIP_L$, enhancing Th2 responses, which protect from the induction of Th1-driven disease EAE, whereas Djerbi and co-workers used a system where EAE is induced before the c-$FLIP_L$ levels are increased. Thus, the retrovirally transduced c-$FLIP_L$ might suppress cell death of the T-lymphocytes already produced before the transduction, enhancing the pool of autoreactive, damaging cells. Also the high levels of c-FLIP found in T-lymphocytes of MS patients may have been induced to protect the autoreactive cells after the Th1 and Th2 differentiation and the onset of disease (Sharief, 2000).

2.5. Effects of hyperthermia on cell survival

2.5.1. The heat shock response

The cells in an organism are constantly exposed to changes in their environment that they have to cope with in order to survive. In addition to physiological processes, such as development and differentiation, cells can be exposed to potentially harmful chemical and physical environments. The heat shock response is activated upon different stimuli stressful for the cell and is found in all species (reviewed by Lindquist, 1986). The heat shock response is characterized by an induced expression of molecular chaperones, Hsps in particular (reviewed by Lindquist, 1986; Morimoto and Santoro, 1998). The increased gene expression was first detected as chromosomal puffs in the salivary gland of *Drosophila melanogaster* exposed to elevated temperatures (Ritossa, 1962). To express Hsps as efficiently as possible, transcription and translation of most other proteins are inhibited during the heat shock response (Tissieres *et al*., 1974; McKenzie *et al*., 1975; Duncan and Hershey, 1989). As molecular chaperones are responsible for the folding of both newly synthesized and damaged proteins, they protect cells from proteotoxic stress. The expression of Hsps is regulated by heat shock factors (HSFs), which bind to the specific heat shock elements on the promoter regions of heat shock-inducible genes. Vertebrates express several different HSFs, but HSF1, which corresponds to the only HSF found in invertebrates, is the main stress-responsive HSF, required for a proper heat shock response (reviewed by Wu, 1995; Morimoto, 1998; Pirkkala *et al*., 2001; Anckar and Sistonen, 2007).

2.5.2. The role of fever and inflammation in cell survival

All endothermic organisms have a core body temperature, defined by a thermoregulatory set point. Hyperthermia is characterized by an increase in the body temperature, without requirement of changes in the thermoregulatory set point. During fever on the other hand, the thermoregulatory set point is elevated, which subsequently increases the body temperature.

Due to an elevated set point, a fever patient feels cold until the actual body temperature corresponds to the unusually high thermoregulatory set point. The thermoregulatory mechanism is thereby intact during fever and functions similarly as when maintaining the body temperature in a cold environment. The physical raise in temperature is suggested to be mediated by activation of the sympathetic nervous system and by heat production in brown fat (reviewed by Rothwell, 1994). Important for obtaining an increased body temperature is the redistribution of blood flow to deep vascular beds to minimize heat loss through the skin, decreased vasopressin secretion reducing the volume of body fluids to be heated as well as shivering and decreased sweating (reviewed by Saper and Breder, 1994).

Fever can be induced by exogenous and endogenous pyrogens. The exogenous pyrogens such as viruses, fungi, bacterial products like endotoxins, or other infectious agents are able to produce and release endogenous pyrogens. The main thermoregulatory center of the brain is the preoptic area in the anterior hypothalamus. Endogenous pyrogens affect the central nervous system by setting the cellular thermostat at an elevated level. This is accomplished by depressing the activity of the warm-sensitive neurons and by stimulating the cold-sensitive neurons. An increase in body temperature is thereby required to maintain the level of neuronal activity of the warm-sensitive neurons. In addition, the endogenous pyrogens stimulate thermogenesis via synthesis of prostaglandins and corticotrophin-releasing factor (reviewed by Rothwell, 1994). Several cytokines such as IL-1α, IL-1β, IL-6, TNF-α, IFN-α, and IFN-γ can function as endogenous pyrogens (reviewed by Kluger, 1991; Luheshi, 1998).

Fever has been shown to be beneficial for the infected host. Elevated temperatures can inhibit growth and even directly kill pathogens. Fever can also activate the heat shock response, which, in addition to protecting the host cell, activates host defenses. Besides the Hsps, several pivotal cytokines and genes involved in early immune responses are targets for HSF1. In addition, the elevated temperature is able to induce a heat shock response in the pathogen itself, and bacterial Hsps may activate innate immune response reactions followed by expression of endogenous pyrogens in the host (reviewed by Hasday and Singh, 2000). Fever has been reported to affect adhesion molecules and increase lymphocyte trafficking across endothelial venules (Chen *et al*., 2006). Although elevated temperature induced during fever can exert stimulation of the immune response, very high temperatures inhibit inflammatory responses. T-lymphocyte proliferation is increased upon mild hyperthermia, but upon temperatures over 41ºC, proliferation is reduced. Likewise, the activities of neutrophils, macrophages, and other lymphocytes are enhanced at temperatures between 38ºC and 41ºC, but attenuated upon exposure to higher temperatures. If the temperature rises too high, many immunological reactions and cellular functions are completely blocked. The expression of IL-1, TNF-α, and the interferons, which are required for an optimal host defense, is regulated differently depending on the body temperature. LPS (lipopolysaccharide), which is a component of the cell wall of Gram-negative bacteria, functions as an endotoxin and induces a strong immune response reaction. IL-1, TNF-α, and interferons are induced upon LPS stimulation only during mild hyperthermia, whereas their expression is suppressed at higher temperatures. The threshold for effects of temperature changes varies between species, for example mice are more sensitive to elevated body temperatures than humans (reviewed by Hasday and Singh, 2000).

The higher the rise in temperature is, the stronger is the activation of HSF1 and the heat shock response. Thus, the heat shock response may protect against too severe pro-inflammatory responses that otherwise could induce sepsis. Indeed, high Hsp expression levels have been shown to protect from LPS-induced sepsis (reviewed by Hasday and Singh, 2000). Moreover, the TNF-α production is triggered in *Hsf1*$^{-/-}$ mice, rendering these mice hyperresponsive to LPS (Xiao *et al*., 1999). This may be due to the ability of HSF1 to repress the transcription of IL-1β and TNF-α, and thereby attenuate immune responses (Cahill *et al*., 1996; Xiao *et al*., 1999). Fever has also been shown to enhance host defenses and protect mice from sepsis by decreasing the expression of the pro-inflammatory cytokines TNF-α, IFN-γ, IL-6 and IL-1β in a bacterial peritonitis model (Jiang *et al*., 2000). The activation of HSF1 is not only directly induced by the elevated temperature during fever, but HSF1 can also be activated by prostaglandins, which are synthesized in response to endogenous pyrogens (Rossi *et al*., 1997; reviewed by Morimoto and Santoro, 1998). Many of the pro-inflammatory cytokines are target genes for NF-κB (Lee *et al*., 2003), and activation of the heat shock response is suggested to inhibit NF-κB activation making the heat shock response a potent regulator of inflammation (Rossi *et al*., 1998; Heneka *et al*., 2000 and 2001). HSF1 is, however not required for the inhibition of NF-κB activation induced by the heat shock response (Malhotra *et al*., 2002). The ability of HSF1 to counteract pro-inflammatory responses also implies that HSF1 may prohibit autoimmune characteristics. Indeed, it has been shown that the heat shock response reduces experimental autoimmune encephalomyelitis in mice by reducing NF-κB activation (Heneka *et al*., 2001). The temperatures required for activation of the heat shock response varies between different types of tissues, for example T-lymphocytes have a lowered set point for activation of the heat shock response than B-lymphocytes (Gothard *et al*., 2003).

2.5.3. The effects of hyperthermia and the heat shock response on apoptotic signaling

Thermotolerant cells are protected from apoptotic signaling

Hyperthermia is able to protect cells from otherwise lethal stimuli. The protective effect is mediated by activation of the heat shock response leading to an increased expression of molecular chaperones such as Hsp70, Hsp90, and Hsp27. These different Hsps inhibit pro-apoptotic proteins at different levels of the cell death signaling pathways (Fig. XI). Cells that express elevated levels of Hsps are referred to as thermotolerant. Preconditioning cells with mild hyperthermia to induce Hsp70 expression, other stresses activating the heat shock response as well as overexpression of Hsp70 have been used to study the effect of thermotolerance and Hsp expression on cell survival. The chaperone activity of Hsp70 has been suggested to be the mechanism of protection in thermotolerant cells (Nollen *et al*., 1999; Mosser *et al*., 2000). Since thermotolerance cannot be induced in MEFs from *Hsf1*$^{-/-}$ mice, HSF1 is required for the elevated Hsp production and induction of thermotolerance (McMillan *et al*., 1998). Thermotolerant cells have been shown to be protected from apoptosis induced by a lethal heat shock (Mosser and Martin, 1992; Mosser *et al*., 1997 and 2000; Buzzard *et al*., 1998; Nollen *et al*., 1999), lethal concentrations of hydrogen peroxide (Creagh and Cotter, 1999), UV irradiation (Park *et al*., 2000), ceramide (Mosser *et al*., 1997; Buzzard *et al*., 1998), cytotoxic drugs such as camphothecin and actinomycin D (Creagh and

Cotter, 1999), and staurosporine (Mehlen *et al*., 1996). Heat shock-induced expression of Hsp70 can protect also from death receptor-mediated apoptosis induced by TNF-α (Jäättelä *et al*., 1989; Buzzard *et al*., 1998; Gabai *et al*., 2002) and TRAIL (Özören and El-Deiry, 2002). Interestingly, the chaperone function of Hsp70 has been suggested to be dispensable for protection against TNF-α-induced apoptosis (Buzzard *et al*., 1998; Gabai *et al*., 2002). Elevated expression of Hsp27 has been shown to protect from apoptosis mediated via CD95 (Mehlen *et al*., 1996), but not to affect TNF-α-induced apoptosis (Jäättelä, 1993).

The effect of Hsps upstream of the mitochondria

The protecting effect of elevated Hsp expression has been shown on several levels of the apoptotic signaling cascades, both upstream and downstream of the mitochondria (Fig. XI). The effects of Hsp expression on apoptosis induced via death receptors have been suggested to be mediated by molecules involved in the mitochondrial amplification loop. Hsp70 has been demonstrated not to affect death receptor-mediated apoptosis in Type I cells, the cell death of which can be induced without involvement of intrinsic apoptotic signaling (Özören and El-Deiry, 2002; Clemons *et al*., 2005). Hsp70, on the other hand, has been shown to protect Type II cells from TRAIL- (Özören and El-Deiry, 2002) and CD95L-induced apoptosis (Clemons *et al*., 2005), indicating that Hsp70 could affect mediators of the intrinsic apoptotic pathway. Indeed, Hsp70 overexpression does not protected fibroblasts of *bid*$^{-/-}$ mice from TNF-α-induced apoptosis similarly as wild-type fibroblasts, implying that the Hsp70-mediated protection affects only Bid-dependent apoptotic signaling (Gabai *et al*., 2002). Additionally, downregulation of Hsp27 with anti-sense oligonucleotides decreases the cytosolic fraction of Bid, indicating that Hsp27 is able to prevent the mitochondrial translocation of Bid. This is proposed partly to be due to Hsp27-induced stabilization of the F-actin network, and not due to inhibition of Bid cleavage (Paul *et al*., 2002). Hsp70 together with its co-chaperone Hsp40 have also been shown to interact with Bax and thereby inhibit translocation of Bax from the cytosol to the mitochondria (Gotoh *et al*., 2004). Overexpression of Hsp70 has also been demonstrated to increase the surface expression of TRAIL-R specifically in Type II cells, sensitizing them to TRAIL-induced apoptosis (Clemons and Anderson, 2006). In addition, overexpression of Hsp70 promoted TCR-mediated apoptosis by stabilizing CAD (caspase-activated DNase), which increased DNA fragmentation during AICD (Liu *et al*., 2003).

Hsp70 is a potent inhibitor of stress-induced apoptosis due to its ability to interfere with the JNK signaling pathway. In addition of inhibiting MKK4, an upstream kinase of JNK (Mosser *et al*., 1997 and 2000), Hsp70 can prevent apoptosis downstream of JNK (Mosser *et al*., 1997; Buzzard *et al*., 1998). Hsp27 has also been shown to be able to interfere with death receptor-mediated apoptosis. Hsp27 can directly interact with Daxx, preventing Daxx-mediated activation of Ask1 and its downstream target JNK, as well as inhibiting translocation of Daxx from the nucleus to the cytoplasm (Charette *et al*., 2000). Although the role of Daxx in CD95-mediated apoptosis is controversial, Hsp27 may function as an inhibitor of such signaling (reviewed by Beere, 2004 and 2005). In addition, Hsp70 is able to block TNF-α-induced JNK activation (Gabai *et al*., 2002).

Hsp90 can prevent apoptosis by supporting several survival pathways (Fig. XI). The PI3K/Akt survival pathway is controlled by cytokine signaling. Hsp90 is able to protect cells from cytokine withdrawal-induced death by binding Akt and maintaining its activity also in the absence of cytokines. The active Akt keeps Bad phosphorylated and sequestered to 14-3-3, preventing dimerization between Bad and the anti-apoptotic Bcl-2 family members and thereby induction of mitochondrial membrane permeabilization. In addition, Hsp90 has been shown to stabilize several mediators of the NF-κB signaling pathway. Hsp90 can directly associate with and stabilize RIP1 as well as the IKK complex (reviewed by Beere, 2004 and 2005).

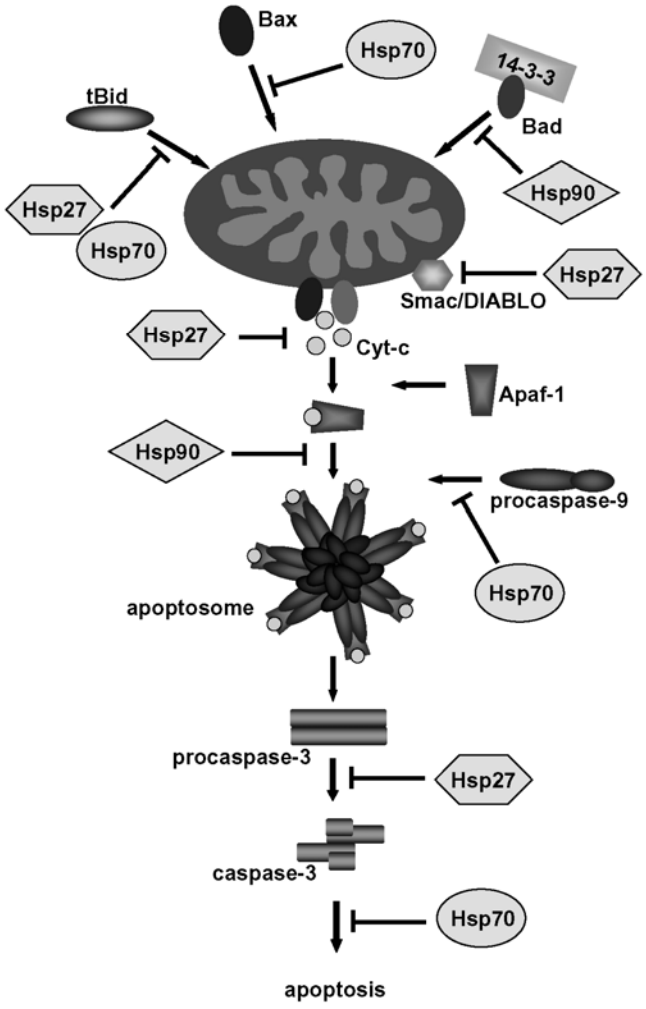


Figure XI. Inhibition of apoptotic signaling pathways by Hsps. Hsp70, Hsp90, and Hsp27 target signaling molecules at different levels of the apoptotic signaling pathways. Hsp70 and Hsp27 inhibit translocation of tBid and Bax from the cytoplasm to the mitochondria. Bad translocation to the mitochondria is inhibited by Hsp90, which stabilizes the interaction between 14-3-3 and Bad. Hsp27 can inhibit release of cytochrome *c* and Smac/DIABLO from the mitochondria. Caspase-9 activation in the apoptosome is inhibited by both Hsp70 and Hsp90. Further downstream in the apoptotic signaling pathway, Hsp27 has been shown to inhibit activation of caspase-3. Hsp70 can even inhibit execution of apoptosis downstream of caspase-3 activation (modified form Beere, 2004 and 2005).

The effect of Hsps downstream of the mitochondria

Although some studies have suggested that Hsps confer protection against apoptosis only by inhibiting events upstream of the mitochondria (Creagh *et al*., 2000; Steel *et al*., 2004), many studies have shown that Hsps mediate protective effects downstream of cytochrome *c* release from the mitochondria (Fig. XI) (Garrido *et al*., 1999; Pandey *et al*., 2000a). The protective effects of the Hsps on the apoptosome complex have been extensively studied. Direct binding of Hsp70 to Apaf-1 has been proposed to prevent recruitment of procaspase-9 to the apoptosome, either by inhibiting oligomerization of Apaf-1 (Saleh *et al*., 2000) or by affecting the conformation of Apaf-1, thereby inhibiting binding of caspase-9 (Beere *et al*., 2000). However, the direct binding of Hsp70 to Apaf-1 in these studies was demonstrated

only in a cell-free system and has later been challenged by *in vivo* data (Steel *et al*., 2004). On the other hand, Hsp90 interactions with Apaf-1 inhibiting Apaf-1-oligomerization and caspase-9 activation have been detected also in *in vivo* studies (Pandey *et al*., 2000b). Hsp27 has been shown to prevent activation of caspase-9 by binding to cytochrome *c* when released from the mitochondria. Therefore, Hsp27 can prevent cytochrome *c*-mediated interaction of caspase-9 and Apaf-1 (Garrido *et al*., 1999; Bruey *et al*., 2000). In contrast, Pandey and co-workers did not detect interactions between Hsp27 and Apaf-1 or caspase-9, but they showed an interaction between Hsp27 and caspase-3 instead. They conclude that Hsp27 inhibits the caspase-9-mediated proteolysis of caspase-3 without interfering with caspase-9 activation (Pandey *et al*., 2000a). Hsp70 is also involved in protection from caspase-independent apoptosis by interacting with AIF and preventing nuclear localization of AIF, thus, interfering with AIF-mediated apoptosis (Ravagnan *et al*., 2001; Gurbuxani *et al*., 2003). In addition, release of Smac/DIABLO from the mitochondria is inhibited by Hsp27 (reviewed by Beere, 2004 and 2005).

Hsp70 has been suggested to protect form apoptotic signaling also downstream of caspase-3 (Fig. XI), and thereby interfere with apoptosis induced by extrinsic as well as by intrinsic apoptotic signaling (Jäättelä *et al*., 1998). Both heat shock and overexpression of Hsp70 were shown to protect against TNF-α-induced apoptosis by inhibiting phospholipase A_2. Activation of phospholipase A_2 by late effector caspases, induces cytotoxicity by increasing the release of arachidonic acid from membrane phospholipids (Jäättelä, 1993; Jäättelä *et al*., 1998). In addition, Hsp70 can directly bind to and inactivate both caspase-3 and caspase-7 (Komarova *et al*., 2004). Also caspase-independent cell death can be inhibited by Hsp70. In cancer cells, depletion of Hsp70 results in loss of lysosomal membrane potential followed by cysteine cathepsin release from the lysosomes and subsequent cell death (Nylandsted *et al*., 2004). Since Hsps can target death signaling at multiple steps of the apoptotic signaling cascades, they are able to protect differently depending on the physiological conditions and the apoptotic stimuli.

High expression levels of Hsps protect cancer cells from cell death

The expression levels of Hsps are elevated in several types of cancer (reviewed by Jäättelä, 1999; Calderwood *et al*., 2006). Because tumor cells are growing under stressful conditions, the chaperone functions of the Hsps are very important for both repair and degradation of damaged proteins (reviewed by Jolly and Morimoto, 2000). The different Hsp70 family members promote cancer cell growth by distinct mechanisms (Rohde *et al*., 2005). Overexpression of Hsp70 or Hsp27 can increase the tumor potential of rodent cells and the metastatic potential of human breast cancer cells in nude mice (Jäättelä, 1993). It has also been shown that overexpression of Hsp70 in primary cells can induce malignant transformation, and that the transformed phenotype can be reversed by a subsequent withdrawal of the overexpression (reviewed by Jolly and Morimoto, 2000). Depletion of Hsp70 by anti-sense oligonucleotides sensitizes tumor cells to apoptosis induced by TNF-α (Jäättelä *et al*., 1992). Interestingly, depletion of Hsp70 also activates a death program, independent of both caspases and Bcl-2, specific for tumor cells (Nylandsted *et al*., 2000).

Inhibition of HSF1 with a dominant negative mutant decreased the Hsp70 levels and sensitized otherwise thermotolerant breast cancer cells to hyperthermia-induced cell death, indicating that the high expression levels of Hsp70 and tolerance against apoptotic stimuli is regulated via HSF1 (Wang *et al*., 2002). HSF1 was upregulated also in prostate cancer cells, inducing increased expression of Hsp27 (Hoang *et al*., 2000). Interestingly, Kim and co-workers showed that the Hsp70 expression was decreased upon treatment with the anti-cancer drug vincristine, although the treatment induced DNA-binding of HSF1. The authors speculated that the outcome of HSF1 activation in response to anti-cancer agents would be different from that induced by other stresses and that JNK signaling would be involved in this differential regulation (Kim *et al*., 1999).

Given the capacity of Hsps to protect against cell death at several levels of apoptotic signaling, and since the expression levels of the Hsps are high in many tumor cells, which in certain cancer types correlates with poor prognosis, they are a tempting target for tumor therapies and prognostics. To date, Hsp90 is the only member of the Hsp family that can be targeted by a specific inhibitor. The Hsp90 inhibitor geldanamycin and its derivates have been extensively studied and are currently used in clinical trials with promising results. The main drawback with Hsp90 inhibitors as therapeutical tools is their ability to induce HSF1 activation, which may protect instead of sensitize to death signals (reviewed by Jäättelä, 1999; Jolly and Morimoto, 2000; Calderwood *et al*., 2006).

2.5.4. Hyperthermia and disease

Hyperthermia in cancer therapy

Hyperthermia and fever have been regarded as mechanisms for curing diseases such as cancer for many centuries. There are several reports from the eighteenth and nineteenth century about improved disease course in cancer patients with fever caused by bacterial infections (reviewed by Hobohm, 2001; Hildebrandt *et al*., 2002; Wust *et al*., 2002). Fever in response to infectious agents can induce cancer cell death at several levels. Pro-inflammatory cytokines are released upon infection, leading to activation of dendritic cells and proliferation of cytotoxic T-lymphocytes. Cancer cells have also been suggested to be less heat-resistant than normal cells, and thereby the physical heat can induce damage of cancer cells, releasing cancer antigens. Activated dendritic cells can subsequently induce T-lymphocyte responses against the cancer cells (reviewed by Hobohm, 2001). Several phase III trials have shown benefits of hyperthermia-treatments combined with radiotherapy or chemotherapy. The beneficial effects may be due to increased drug delivery and reoxygenation due to a hyperthermia-induced increase in perfusion. The increased perfusion followed by oxygenation induced by hyperthermia may on the other hand also benefit tumor growth (reviewed by Wust *et al*., 2002). Severe hyperthermia has, however, been implied to decrease blood flow into tumors, impairing oxygen and nutrient supply (reviewed by Hildebrandt *et al*., 2002).

Hyperthermia in the absence of inflammation leads to activation of the heat shock response if the temperature elevation is severe enough, and is thereby different from hyperthermia

induced during fever. There are, however, studies showing an increase in several pro-inflammatory Th1-cytokines such as IL-1β, IL-6, and TNF-α in response to whole body hyperthermia at 41.8 degrees in humans (Robins *et al*., 1995). Whole body hyperthermia induces apoptosis in CD4+ T-lymphocytes, with a subsequent increase in the CD8+ T-lymphocyte population and NK cells (Hammami *et al*., 1998; Dieing *et al*., 2003; Ahlers *et al*., 2005; Kida *et al*., 2006). If cytotoxic T-lymphocyte populations targeting tumor cells are increased while T-helper cells are eliminated, killing tumor cells by hyperthermia might not induce a severe inflammatory response. Indeed, it has been shown that whole body hyperthermia combined with chemotherapy induces a strong but reversible anti-inflammatory response in cancer patients during therapy (Ahlers *et al*., 2005). Interestingly, Hsps have been shown to be expressed on the surface of several cancer cells. The Hsps form complexes with tumor-specific peptides, and the complex is internalized into antigen-presenting cells. Presentation of parts of the Hsp/tumor peptide by dendritic cells may activate specific immune responses against the tumor, and might thereby be a delicate strategy for anti-cancer vaccination (reviewed by Hobohm, 2001; Hildebrandt *et al*., 2002). However, it has to be taken into account that hyperthermia itself may be cytotoxic to some tissues, especially to the nervous system (reviewed by Haveman *et al*., 2004). In addition, elevated body temperatures during pregnancy may induce severe birth defects or spontaneous abortions (reviewed by Edwards, 1998).

The effect of fever and infections on allergy and autoimmunity

According to the hygiene hypothesis, the increased incidence of allergy and autoimmune diseases in developed countries is due to a decreased exposure to pathogens inducing infections. Fever, a common denominator for most childhood infections, is also associated with lower odds of allergic sensitization later in life (Williams *et al*., 2004). Since anti-infectious immune responses and responses directed towards allergens and autoantigens have been suggested not to be able to be active simultaneously, the loss of immune responses against infectious agents induces an increase in responses to autoantigens and allergens (reviewed by Bach, 2005). As mentioned previously, increased Th2 responses may lead to asthma and allergic disorders, whereas increased Th1 responses are associated with autoimmune diseases (Wu *et al*., 2004). Contact to pathogens that induce inflammation has been shown to evoke anti-inflammatory responses mediated via mucosa-driven T-lymphocytes referred to as T-regulatory cells or Th3 cells. Th3 responses induce release of both TGF-β and IL-10 that have suppressive effects on Th1 and Th2 responses (reviewed by Yazdanbakhsh *et al*., 2002; Rautava *et al*., 2004). Therefore, exposure to pathogens activates Th3 responses, able to inhibit later inappropriate Th1 and Th2 responses that have the ability of inducing allergy and autoimmune diseases.

3. OUTLINE OF THE STUDY

The aim this thesis was to determine how hyperthermia affects death receptor-mediated apoptosis. Death receptors, CD95 in particular, are important regulators of homeostasis within cell populations of the immune system. As physiological model systems for detection of CD95-mediated apoptosis, we used primary human T-lymphocytes and a human Jurkat T-lymphocyte cell line. Fever is a physiological hyperthermia that is induced upon immune responses against infections. Hence, it was of particular interest to investigate the effects of fever-like hyperthermia in T-lymphocytes.

In the beginning of this thesis project we found that hyperthermia sensitizes T-lymphocytes to death receptor-mediated apoptosis. To determine the molecular mechanisms responsible for the hyperthermia-induced sensitization, we studied different pathways known to be modulated during hyperthermic stress. As hyperthermia is known to activate of MAPK signaling pathways (reviewed by Dorion and Landry, 2002), we investigated the effects of JNK, p38, and ERK on the hyperthermia-induced sensitization. We also investigated the role of Hsp70 expression in the induced sensitivity, as hyperthermia also activates the heat shock response (reviewed by Lindquist, 1986). We further studied the level at which hyperthermia affects death signaling, and found that the target was the death-inducing signaling complex. Since hyperthermia induced a rapid downregulation of both c-FLIP$_L$ and c-FLIP$_S$, we examined the impact of c-FLIP downregulation on the sensitization to CD95 signaling. Additionally, we characterized the mechanisms that regulate the decrease in intracellular c-FLIP during hyperthermia. In addition to c-FLIP$_L$ and c-FLIP$_S$, a third form of c-FLIP has been reported to exist in certain human cell types (Djerbi *et al*., 2001; Golks *et al*., 2005). To elucidate a possible existence of three c-FLIP isoforms also in other species, a comparative study of the c-FLIP genomes was performed.

Additionally, this study includes development of new tools for detection of apoptosis. Since no satisfactory quantitative methods for detecting apoptosis in living cells at a single-cell level as well as in cell populations and in tissues *in vivo* were available, we developed FRET-based tools for *in vivo* detection of caspase-3 activity. This apoptosis detection tool was validated in a cell model.

4. MATERIALS AND METHODS

4.1. Cell culture

Primary human peripheral T-lymphocytes were collected from several healthy volunteers by venipuncture. The blood samples were diluted 1:2 in PBS, overlaid onto Ficoll-Paque™PLUS (Amersham), and centrifuged at 1 500 rpm for 30 min. Monocytes from the Ficoll-Paque separation were depleted by plastic adherence for 2 h. Non-adhering cells were further separated in a nylon wool column to exclude B-lymphocytes. Resting T-lymphocytes (day 0) were stimulated with 1 μg/ml PHA (phytohemagglutinin, Sigma) for 22 h. After one day of activation cells were washed and T-lymphocyte proliferation was supported by addition of 20 U/ml IL-2 (interleukin-2, Sigma). The same dose of IL-2 was added at day 3 of activation. Human Jurkat T-lymphoma cells (clone E6-1, ATCC), modified cell lines originating from Jurkat cells, and primary human peripheral T-lymphocytes were cultured in RPMI-1640 medium (Sigma-Aldrich), murine hippocampal HT-22 neuronal cells and human HeLa cervical carcinoma cells (ATCC) in DMEM (Dulbecco modified Eagle's medium, Sigma-Aldrich) in a humidified 5% CO_2 atmosphere at 37°C. Both media were supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin, and 2 mM L-glutamine. In contrast to the Jurkat cells, which have a normal chromosome number, the HeLa cell genome contains 82 chromosomes. The increased chromosome number is partly due to gene transfer from the human papillomavirus 18 to the cervical carcinoma cell genome (Macville *et al*., 1999).

4.2. Plasmids and constructed cell lines

The sequence for a 19-amino-acid peptide chain including the caspase-3 cleavage site, GSGGSGSG**DEVD**NGSGSGS, was added in front of the start codon of pDsRed2-N1 (Clontech) by PCR cloning. The cDNA for the peptide sequence along with that of pDsRed2-N1 was then digested with *Bsp*EI and *Eco*RI and subcloned into the multiple cloning site of pEYFP-C1 (Clontech) to generate pFRET-casp3. The pFRET-casp3 construct was introduced into HT-22 cells using the FuGENE 5 transfection reagent (Roche) according to the manufacturer's instructions.

GFP-tagged kinase-dead mouse MKK4a (DN-MKK4) was prepared by releasing the kinase-dead fragment from the pEBG-SEK1(K129R) plasmid (a kind gift from John Kyriakis, Diabetes Research Laboratory, Massachusetts General Hospital, Charlestown, MA, USA, Sanchez *et al*., 1994) with BamHI, and ligating it in-frame with the C-terminus of GFP into EGFP-C1 cut with BglII and BamHI and treated with Shrimp Alkaline Phosphatase (USB, Amersham Pharmacia). Jurkat cells were transfected with DN-MKK4 by electroporation 48 h before performing the experiment.

For transfections of FLAG-c-$FLIP_L$ and FLAG-c-$FLIP_S$ (a kind gift from Jürg Tschopp, Institute of Biochemistry, University of Lausanne, Switzerland), as well as FLAG-c-$FLIP_S$Δ203-221 (described by Poukkula *et al*., 2005), Jurkat cells were subjected to a single electric pulse (220 V, 975 μF). The stably overexpressing cells were selected by geneticin (G418, 1.5 mg/ml, Calbiochem).

A Jurkat cell line expressing the reverse tetracycline-controlled transactivator (rtTA) was generated by electroporation-mediated transfection with the plasmid pUHD17201neo. A Jurkat-rtTA clone was then transfected with the tetracycline-regulated dicistronic Hsp70/GFP expression plasmid pTR5-DC/Hsp70-GFP*tk/hygro. Stably transfected clones were selected and screened as previously described (Caron *et al*., 2000; Mosser *et al*., 2000).

For shRNA-mediated downregulation of c-FLIP, oligonucleotides were cloned into the pSuperior vector (Oligoengine) according to the manufacturer's instructions. The following sequences were used for downregulation of c-FLIP: c-$FLIP_{L+S}$ (5'-GAT CCC CGC TGT GGA GAC CCA CCT GCT TCA AGA GAG CAG GTG GGT CTC CAC AGC TTT TTA-3'), c-$FLIP_L$ (5'-GAT CCC CTT CAA GGC TCA GAA GCG AGT TCA AGA GAC TCG CTT CTG AGC CTT GAATTT TTA-3'), and c-$FLIP_S$ (5'-GAT CCC CCA CCC TAT GCC CAT TGT CCT TCA AGA GAG GAC AAT GGG CAT AGG GTG TTT TTA-3'). A scrambled sequence not coding for any gene found in the data bases was used as a control: scram (5'-GAT CCC CGC GCG CTT TGT AGG ATT CGT TCA AGA GAC GAA TCC TAC AAA GCG CGC TTT TTA-3'). The constructed plasmids were controlled to be correct by sequencing. To induce shRNA expression, HeLa cells were electroporated (220 V, 975 μF) 72 h before performing the experiment.

4.3. Reagents and treatments

Apoptosis was induced with agonistic α-CD95 antibody CH-11 (Biosite), 200 ng/ml for 2 h in Jurkat cells if not otherwise indicated, and 1 μg/ml for 2 or 12 h in primary human T-lymphocytes. To induce apoptosis in HT-22 cells another agonistic α-CD95 antibody, Jo2 (BD Pharmingen), was used at a concentration of 200 ng/ml for indicated times with or without co-treatment with the protein synthesis inhibitor cycloheximide (CHX, Sigma) at a concentration of 1 μg/ml Apoptosis was induced in HeLa cells with the indicated concentrations of recombinant CD95L (a kind gift from Jürg Tschopp, Institute of Biochemistry, University of Lausanne, Switzerland) for 2 h. Recombinant FLAG-tagged TRAIL (Alexis) was used at indicated concentrations together with 2 μg/ml crosslinking M2 anti-FLAG antibody (Sigma). Both Jurkat cells and HeLa cells are sensitive to death receptor triggering with recombinant CD95L. Primary T-lymphocytes and Jurkat cells respond also to death receptor ligation with agonistic α-CD95 antibody, whereas HeLa cells do not unless sensitized with cycloheximide or other protein synthesis inhibitors.

For inhibition of caspase activation cells were incubated with the following peptide inhibitors 1 h prior to other treatments; the general caspase inhibitor z-VAD-fmk (20 μM), the caspase-8 inhibitor z-IETD-fmk (50 μM), and the caspase-3, caspase-6, caspase-7, caspase-8, and caspase-10 inhibitor z-DEVD-fmk (50 μM) (Sigma). These are competitive irreversible fluoromethyl ketone- (fmk) derivatized peptide inhibitors. CD95-mediated apoptosis was measured in samples treated with a range of concentrations (1–100 μM) of caspase inhibitors to determine the concentration needed to inhibit caspase activation. The proteasome inhibitor epoxomicin (epoxo, Calbiochem) was used at 200 nM. CHX was used at 5 μM to determine the c-FLIP half-lives. The CD95L blocking antibody NOK-1 (BD Pharmingen) was used at 1 μg/ml for 15 min prior to apoptosis induction. Triton X-100, DMSO, Hoechst 33258, and

saponin were purchased from Sigma, digitonin was obtained from VWR, and bovine serum albumin (BSA) was obtained from Sigma or Roche.

To induce hyperthermia, heat shock treatments (HS) were performed in a water bath at 40ºC or 42ºC for 30 min if not otherwise indicated. Controls were left in the incubator at 37ºC. As the heat shock treatments were done using cell culture flasks or cell culture plates sealed with parafilm, tests with sealed and unsealed control plates and flasks were made to exclude effects of the sealing itself. After exposure to hyperthermia, cells were either harvested or returned to 37ºC for recovery and/or additional treatments.

4.4. Detection of apoptosis

Apoptosis in all Jurkat cell lines and primary peripheral blood T-lymphocytes was detected by Annexin V analysis. The cells were incubated for 10 min on ice with 0.2 mg/ml human recombinant FITC-conjugated Annexin V (Sigma) in Annexin V binding buffer (2.5 mM HEPES pH 7.4, 35 mM NaCl, and 0.6 mM $CaCl_2$) and analyzed on a FACScan flow cytometer (BD Biosciences). Annexin-V-PE (phycoerythrin) was used for apoptosis detection in GFP-expressing Jurkat cell lines.

Caspase-3 activity was measured in cell extracts from Jurkat cells lysed with RIPA lysis buffer (PBS pH 7.4, 1% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate, 1 mM Na_3VO_4, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 1 mM PMSF, 0.5 mM DTT) without protease inhibitors, using the homogeneous time-resolved fluorescence quenching assay LANCE kit for caspase-3 (Perkin-Elmer Life Science), as described by the manufacturer. Results were measured on VICTOR (Perkin-Elmer Life Science).

HeLa cell survival was quantified either by detection of active caspase-3 or by MTT viability assay. For detection of active caspase-3, cells were harvested by trypsin-EDTA dissociation, washed twice in ice-cold PBS, fixed, and stained with a phycoerythrin-conjugated α-caspase-3 antibody (1:20) according to manufacturer's instructions (BD Pharmingen). The cells were analyzed by flow cytometry using a FACScan flow cytometer (BD Biosciences). For MTT viability assays, HeLa cells were seeded on 96-well plates. After treatments the medium was replaced with fresh medium containing 1 mM MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide, Sigma-Aldrich) and incubated for 2–4 h at 37ºC before washing and solubilizing the MTT-formazan with 50 µl DMSO. Results were measured at 540 nm on a plate reader.

In HT-22 cells, the levels of apoptosis were determined either by detection of lactate dehydrogenase (LDH) release or by detection of active caspase-3. For detection of apoptosis by LDH release, the cells were treated in DMEM containing 2% FBS with 200 ng/ml agonistic α-CD95 monoclonal antibody (Jo2) and 1 µg/ml CHX. Aliquots of the media were then removed at the indicated time points and assayed for LDH activity according to the manufacturer's instructions (Roche). The release of LDH from cells into the culture medium was used as an indicator of cell death and was expressed as percentage of the total (Triton X-100-releasable) LDH from the culture. Morphological evidence for chromatin condensation

was obtained using the fluorescent chromatin dye Hoechst 33258 (2 μg/ml) as previously described (Jones *et al*., 1998).

4.5. Western blotting

For Western blot analysis, cells were lysed in either a lysis buffer containing 20mM NaCl, 100 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 1 μM cyclosporine A, 25 mM HEPES, pH 7.2, and 700 μg/ml digitonin, in a RIPA lysis buffer (PBS pH 7.4, 1% NP-40, 0.5% sodium deoxycholate, 1 mM Na_3VO_4, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 1 mM PMSF, 0.5 mM DTT) with addition of complete protease inhibitor cocktail (Roche) or directly in 3x Laemmli sample buffer. Protein lysates were subjected to SDS-PAGE, transferred to nitrocellulose membrane (Schleicher and Schuell), and the membrane was blocked in PBS containing milk or BSA. Western blotting was performed using antibodies against c-FLIP (clone NF6, either purchased from Alexis or received as a kind gift from Peter Krammer, German Cancer Research Center, Heidelberg, Germany), CD95L (clone G247-4, BD Pharmingen), caspase-8 (clone C15, either purchased from Alexis or received as a kind gift from Peter Krammer), caspase-9 (Cell Signaling Technology Inc.), caspase-3 (BD Pharmingen), Bcl-XL (H-62, Santa Cruz Biotechnology), Bcl-2 (clone 7, BD Transduction Laboratories), CD95 (C-20, Santa Cruz Biotechnology), phospho-p44/42-MAPK (New England Biolabs), Hsp70 (SPA-810, StressGen and 4g4, Affinity Bioreagents), ERK2 (Santa Cruz Biotechnology), Hsc70 (SPA-815, StressGen), ubiquitin (clone FK-1, BioMol), subunit IV of cytochrome *c* oxidase (20E8-C12, Molecular Probes), and Bax (clone B9, Santa Cruz Biotechnology). HRP- (horseradish peroxidase) conjugated secondary antibodies were from Southern Biotechnology, Promega, DAKO, and Amersham. The results were visualized using the enhanced chemiluminescence method (ECL, Amersham) on X-ray film.

4.6. Mitochondrial fractionation

To obtain cytosolic and membrane-bound fractions, respectively, the permeabilized cells were centrifuged (15,000 x g, 15 min, 4°C) and the supernatant (cytosolic fraction) was removed. The pellet was resuspended in lysis buffer (0.15 M NaCl, 50 mM Tris–HCl (pH 7.5), 0.002% SDS, 1% NP-40, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1μg/ml aprotinin, and 1 μg/ml pepstatin) and centrifuged again (15,000 x g, 15 min, 4°C), and the supernatant (fraction comprising membrane-bound and organelle-bound proteins including mitochondrial proteins) was harvested. Protein concentrations of both fractions were determined and a total of 20-50 μg of proteins was subjected to SDS-PAGE and immunoblotting using antibodies against Bax and cytochrome *c* oxidase (COX). Cytosolic fractions were found free of mitochondrial contamination as confirmed by the lack of COX.

4.7. Electrophoretic mobility shift assay

Cells were washed in cold PBS, lysed by freeze–thaw in buffer C (25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20 mM HEPES pH 7.9) containing PMSF and DTT (0.5 mM each), and supernatant was recovered by centrifugation at 4ºC. The whole-cell extracts (15 μg proteins) were incubated with a ^{32}P-labeled oligonucleotide probe corresponding to the consensus heat shock element (Mosser *et al*., 1988). The protein–DNA

complexes were then resolved on 4% polyacrylamide native gel electrophoresis and detected by autoradiography using BAS Imaging plates (FujiFilm) and a BAS-1800 Imaging plate reader (FujiFilm).

4.8. Kinase assays

ERK2 and JNK were immunoprecipitated from 400 µl RIPA lysates ($6x10^6$ cells/sample) by incubation with α-ERK2 and α-JNK antibody coupled to protein-A-Sepharose. Immunoprecipitates were washed three times in RIPA buffer. ERK2 immunoprecipitates were then washed three times in ERK assay buffer (10 mM Tris pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 0.5 mM DTT). JNK immunoprecipitates were additionally washed three times with LiCl buffer (500 mM LiCl, 100 mM Tris pH 7.6, 0.1% Triton X-100, 1 mM DTT) and three times with JNK assay buffer (20 mM MOPS pH 7.2, 2 mM EGTA, 10 mM $MgCl_2$, 0.1% Triton X-100, 1 mM DTT). The kinase reactions were carried out in 120 µl kinase assay buffer containing 25 µM ATP, 2.5 µCi ^{32}P γ-ATP and 1 mg/ml myelin basic protein or GST-c-Jun, respectively, as substrates, for 15 min at 37ºC. Reactions were stopped by addition of 3x Laemmli sample buffer and the samples were resolved by SDS-PAGE, analyzed on a phosphor imager (BioRad), and autoradiographed.

4.9. Microscopy

Jurkat cells transfected with vector-GFP or dominant negative MKK4-GFP were fixed with 3% paraformaldehyde in PBS for 30 min, printed on cover slips using a cytospin, and DNA-stained with Hoechst 33342 (Molecular Probes). After three washes, cells were mounted in Mowiol (Sigma) and visualized with a fluorescence microscope (Leica). Hsp70-GFP-Tet-ON cell lines were treated with 1 µg/ml doxycycline (Sigma), resulting in a broad range of GFP induction, as detected by fluorescence microcopy. Hsp70 expression, assessed by cell staining with α-Hsp70 antibody, correlated well with GFP-fluorescence intensity. To visualize cells retaining mitochondrial membrane potential, tetramethyl rhodamine ester (TMRM, 200 nM, Molecular Probes) was added to the medium, together with Hoechst 33342 (Molecular Probes). Images were taken by confocal microscopy (Leica TCS40) on the live cells at different time points after treatment with 200 ng/ml CH11 agonistic α-CD95 antibody. For differential interference contrast (DIC) imaging, Jurkat cells were printed on cover slips using a cytospin.

For detection of cytochrome *c* release HT-22 cells were treated with agonistic Jo2 α-CD95 antibody with or without co-treatment with CHX. Following fixation in 4% paraformaldehyde, cells were washed once in blocking buffer (3% BSA, 0.05% saponin, pH 7.2) and incubated (o/n, 4°C) in 1:100 monoclonal α-cytochrome *c* antibody (clone 6H2.B4, BD Pharmingen). Following further washing, cells were then incubated in 100 µl of 1:100-diluted FITC-conjugated α-mouse secondary antibody (DAKO) for 1 h at RT. After final washes, cells were mounted in VectaShield (Vector Laboratories) prior to imaging.

In situ caspase-3 activity in HT-22 was analyzed using the pFRET-casp3 construct. Following 72 h incubation, the pFRET-casp3 transfected cells were treated with Jo2 (+ CHX) or carrier solvent and examined using a Zeiss Axiovert 135 fluorescence microscope using

the following filter sets to assess FRET: excitation, 450–490 nm for the donor (EYFP); emission, 515 nm long-pass filter. This choice of filter settings was derived from initial experiments where transfected cells showed a distinct red fluorescence when the expressed DsRed was mature (at 72 h) as compared to immature (green-fluorescent DsRed) at 24 h (Baird *et al*., 2000; Jakobs *et al*., 2000). Caspase-3 activation following CD95 ligation was quantified by differential counting of green versus red fluorescent cells. Analysis of FRET by confocal laser scanning microscopy was carried out with a Zeiss LSM510 META. Donor (EYFP) was excited at 488 nm using an argon ion laser, and EYFP and FRET were detected at bandwidths of 500-550 nm (EYFP channel) and 565-615 nm (FRET channel), respectively. For this purpose, the cells were grown on glass cover slips, transfected with pFRET-casp3 as described above, and following the initiation of apoptosis analyzed by confocal laser scanning microscopy. Bleaching of the probes as a result of illumination by the xenon lamp or argon ion laser was negligible.

4.10. Detection of activated Bax

Harvested HT-22 cells were washed twice in PBS, followed by permeabilization, fixation, and resuspension with the N-terminal-specific N-20 antibody (1:100, o/n, 4°C), previously shown to be specific to the activated form of Bax (Santa Cruz Biotechnology). Following washing, the cells were exposed to FITC-conjugated α-rabbit secondary antibody (DAKO), 1:100, 1 h at RT, washed, and then analyzed using a Beckman Coulter Epics XC flow cytometer.

4.11. Surface expression analysis of CD95

Cells were washed twice with PBS. After washing, cells were blocked for 30 min with 1% BSA in PBS. Cells were then incubated with CH-11 agonistic α-CD95 antibody (Biosite), 1:500 in 1% BSA in PBS for 30 min followed by washing with PBS. Finally, cells were incubated with Alexa 488-conjugated goat α-mouse IgG (Molecular Probes) for 30 min. After washes, cells were analyzed by flow cytometry on a FACScan flow cytometer (BD Biosciences). Samples without primary antibody were used as negative controls (2nd ab control).

4.12. DISC analysis

$5x10^7$ Jurkat cells were resuspended in 1 ml of pre-warmed RPMI-1640 medium. To ligate CD95 and induce DISC formation, 1 μg Fc-CD95 ligand fusion protein (CD95L:Fc, a kind gift from Pascal Schneider, Institute of Biochemistry, University of Lausanne, Switzerland), was added to the cell suspension. The cells were incubated at 37°C for 12 min, and the reaction was stopped by adding 10 ml of ice-cold PBS. After washing, the cells were lysed in 1 ml of lysis buffer [20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 0.2% NP-40, and Complete protease inhibitor cocktail (Roche)] for 20 min on ice. The cell debris was removed by centrifugation at 15 000 x g for 12 min at 4°C. 1 μg of CD95L:Fc was added to the lysates from control cells that were not treated with CD95 with the purpose to trigger the death receptor. Samples were immunoprecipitated with 15 μl protein G beads (Amersham) for 2.5 h at 4°C. The beads were washed five times with lysis buffer, resuspended in 3x

Laemmli sample buffer and finally boiled for 5 min. The immunoprecipitates and corresponding cell lysates were analyzed by Western blotting. A control sample without CD95L:Fc was used to exclude unspecific binding to the protein G beads (ab control).

4.13. RNase protection assay

Hyperthermia was induced in Jurkat cells at 42ºC for indicated times and RNA was prepared with the QIAGEN kit for RNA isolation according to the manufacturer's protocol. The RNase protection assay was done with the RiboQuant™ Multi-Probe RNase Protection Assay System (BD Pharmingen) according to the manufacturer's standard RPA protocol using the hApo-3b probe (cat. # 45611P). The results were visualized by autoradiography using BAS Imaging plates (FujiFilm) and a BAS-1800 Imaging plate reader (FujiFilm).

4.14. Ubiquitination assays

For immunoprecipitation of c-FLIP, the cell pellet from $3x10^7$ stably c-FLIP-overexpressing Jurkat cells was resuspended in 75 µl of boiling 1% SDS in PBS, and the resulting lysate was heated at 100°C for 5 min. The lysates were then suspended in 1 ml 0.5% Triton X-100 in PBS. DNA was sheared by sonication, and the particulate material was centrifuged at 15 000 x g for 10 min. Samples were taken from the cleared lysates for input control. The lysates were precleared for 30 min without antibody at RT, and then incubated with α-FLIP antibody 1:10 (NF6 supernatant, a kind gift from Peter Krammer) and 20 µl of a 50% slurry of protein A-Sepharose beads under rotation for 2 h at 4°C. After incubation, the Sepharose beads were washed four times with 0.5% Triton X-100 in PBS, and the immunoprecipitated proteins were run on SDS-PAGE, and immunoblotted with α-ubiquitin antibody (FK-1, BioMol).

4.15. Detection of caspase-3 activation by FRET microfluorimetry

HT-22 cells were transfected with pFRET-casp3. Following 72 h incubation, the transfected cells were treated with the apoptosis-inducing drug staurosporine (STS, 1 µM) or vehicle control (dimethyl sulfoxide) for 18 h. After trypsinization, cells were washed and resuspended in PBS and transferred to a black 96-well plate (Costar) before FRET measurement using a Spectra Max Gemini XS microfluorometer (Molecular Devices) (excitation, 488 nm; emission 525–550 nm for EYFP and 570–595 nm for DsRed). The FRET ratios were obtained by dividing the integrated DsRed signal (γ_{em} 570-595 nm) by the integrated EYFP signal (γ_{em} 525–550 nm).

4.16. Image analysis and statistics

For densitometric analysis of Western blots the X-ray films were scanned and the analysis was done with the ScionImage GelPlot2 software. The values were normalized to the untreated control sample, which was given the value 1. Radioactive autoradiographs were quantified with the AIS-Analytical Imaging Software. The bar graphs represent mean values (± SD) from at two or more independent experiments. For statistical analysis, comparisons of treatments were made using GraphPad Prism software. Unpaired *t* tests were performed on data from different individuals, i.e. primary T-lymphocytes, whereas paired *t* tests were

performed on data from HeLa cells, Jurkat cells and Jurkat-based cell lines. Unpaired *t* tests or one-way ANOVA followed by Tukey-Kramer post tests were made to compare data from HT-22 cells. Statistical significance is marked with asterisks (***: $p < 0.001$, **: $p < 0.005$, and *: $p < 0.05$), and ns ($p > 0.05$) stands for not statistically significant.

5. RESULTS AND DISCUSSION

5.1. Development of tools for *in vivo* detection of apoptosis (I)

A part of this thesis study included development of tools for detection of apoptosis *in vivo*, and validation of these tools for analytical purposes. We chose caspase-3 activity as a marker for apoptosis, because execution of apoptosis seldom is inhibited downstream of this late effector caspase. In general, caspase activity is detected in cell lysates with tetrapeptide substrates linked to cleavable fluorescent tags. Caspase-specific substrates are available for live cell detection of caspase activity, but their peptide-based nature restricts their permeability into cells. More recently, efforts have been made to design GFP-based tandem constructs where caspase activity can be detected in living cells at a single-cell level as well as in cell populations and in tissues. In these constructs, the recognition substrate peptide of the caspase of interest links a donor cyan fluorescent protein (CFP) to an acceptor GFP or yellow fluorescent protein (YFP) (Xu *et al*., 1998; Mahajan *et al*., 1999; Tyas *et al*., 2000; Donahue *et al*., 2001; Luo *et al*., 2001; Morgan and Thorburn, 2001; Tawa *et al*., 2001). Since the distance between the donor and the acceptor fluorophore is very short in these fusion proteins, the excitation of the donor fluorophore leads also to the excitation of the acceptor fluorophore by fluorescence resonance energy transfer (FRET). Upon caspase activation the substrate linker peptide is cleaved, leading to an increase in the distance between the donor and acceptor resulting in loss of FRET. The excitation of CFP requires a special excitation filter when using conventional fluorescence microscopes and CFP excitation is only poorly achieved with lasers found in most flow cytometers and confocal laser microscopes. Therefore, we developed a new tandem construct called pFRET-casp3, consisting of Discosoma red fluorescent protein (DsRed) and enhanced yellow fluorescent protein (EYFP) linked by a 19-amino-acid chain (GSGGSGSG**DEVD**NGSGSGS) that contains the caspase-3 substrate recognition sequence and cleavage site aspartic acid/glutamic acid/valine/aspartic acid (DEVD) (Fig. 1A, I). The regions flanking the caspase-3 substrate recognition site consist of eight and six small, uncharged amino acids to make the linker as linear and unreactive as possible. In addition, the DEVD is immediately followed by an aspargine residue, which is a polar uncharged residue that has been suggested to be well tolerated as an amino acid following the P1 position of a caspase substrate recognition site (reviewed by Degterev *et al*., 2003). If the linker region between the donor and acceptor fluorophore is too short, the activated caspases may not reach the cleavage site as efficiently. An earlier version of pFRET-casp3 encoding a shorter linker of only twelve amino acids (SGGS**DEVD**GSGS) taught us that a small difference in the length of these two linkers was already significant for the sensitivity to detect caspase-3-mediated cleavage. In addition, the fusion protein with the short linker was aggregated in the cells, appearing as distinct globular inclusions (unpublished observations). Others have also reported similar problems with aggregation when using very short linker regions in their FRET constructs (Mahajan *et al*., 1999).

Validation of the use of pFRET-casp3 was performed by exposing pFRET-casp3-transfected murine HT-22 hippocampal neuronal cells to staurosporine or agonistic α-CD95 antibody Jo2 in combination with CHX. The expression of pFRET-casp3 yields a strong red fluorescence

when excited with 450 to 490 nm light or with a 488 nm argon ion laser line under control conditions due to FRET from donor EYFP to acceptor DsRed. Upon caspase-3 activation, the active caspase-3 cleaves the YFP-DsRed fusion protein at the caspase cleavage site, separating the two fluorescent proteins. Thus, no energy transfer from YFP to DsRed is possible, resulting in enhanced yellow fluorescent and decreased red fluorescent emission (Fig. 1B, I). Indeed, when testing p-FRET-casp3, the induction of apoptosis correlated with a loss of FRET ratio (DsRed signal/EYFP signal) (Fig. 1C and 2A, I). The time course of detection of caspase-3 activity correlated with the increase in pro-caspase-3 processing (Fig. 2B, I).

To ensure that the cells also showed other apoptotic features in response to treatment with the agonistic α-CD95 antibody Jo2 and CHX, additional methods for apoptosis detection were used. Increases in LDH release (Fig. 3A, I), chromatin condensation (Fig. 3B, I), Bax translocation to the mitochondria (Fig. 4B, I), Bax activity (Fig. 4C and D, I), and cytochrome *c* release (Fig. 4A, I) were observed in response to CD95 ligation with Jo2, confirming that the classical apoptotic signaling pathways were activated and that the caspase-3 activation observed with pFRET-casp3 was specific. The substrate-specificity of caspases is not absolute, and also other caspases might contribute to the cleavage of pFRET-casp3 during apoptosis. It has, however, been reported that DEVD-based tandem constructs are predominantly cleaved by caspase-3 and to a lesser degree by caspase-7 (Rehm *et al*., 2002; Takemoto *et al*., 2003; Kawai *et al*., 2004). Furthermore, mutation of the P1 aspartic acid cleavage site abrogates the loss of FRET in similar constructs (Tyas *et al*., 2000; Rehm *et al*., 2002).

Our tandem construct has several advantages over the CFP-EYFP constructs. The expressed, mature YFP-DEVD-DsRed fusion protein is expected to form a 220 kDa complex due to the obligate tetrameric nature of mature DsRed. The tetrameric nature of DsRed may thereby help to prevent EYFP dimerization (Zacharias *et al*., 2002), which otherwise might interfere with the FRET signal (Nagai and Miyawaki, 2004). Moreover, the channel separation of the EYFP-DsRed pair is better than that of the CFP-GFP pair (Jakobs *et al*., 2000). Since the excitation obtained with argon ion lasers found in most flow cytometers and confocal laser microscopes is higher than the 430 to 440 nm excitation maxima of CFP, special excitation filter blocks are required for efficient excitation of donor CFP. In contrast, the EYFP donor is easily excited with the 488 nm line generated by an argon ion laser and can therefore be detected with any flow cytometer or conventional fluorescence microscope. Thus, our construct can be used when excitation wavelengths lower than 488 nm are not available. Indeed, the loss of FRET can be detected in apoptotic cells transfected with pFRET-casp3 both by microscopy and flow cytometry using a 488 nm laser line (unpublished data). Another advantage of DsRed over EYFP as the acceptor fluorescent protein is that its absorbance and fluorescence is insensitive to pH changes over a range of pH 5–12 (Baird *et al*., 2000). Additionally, the relative autofluorescence intensity of the red-shifting DsRed is low in the transfected cells (Nagy *et al*., 1998). Due to these properties, the pFRET-casp3 construct is a valuable tool for detection of *in vivo* caspase-3 activity.

5.2. c-FLIP$_S$ is absent in rodent genomes

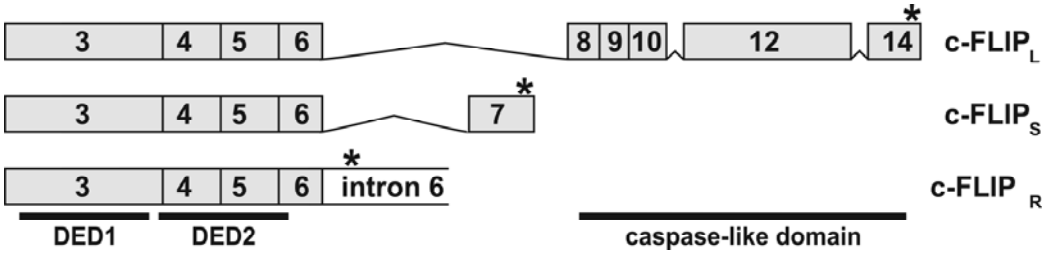
The human c-FLIP gene, termed *cflar* (caspase-8 and FADD-like apoptosis regulator), is located at the chromosome locus 2q33-q34 in front of the *caspase-10* and the *caspase-8* genes. Three different splicing variants of c-FLIP have been reported to be expressed at the protein level, c-FLIP$_L$, c-FLIP$_S$, and c-FLIP$_R$ (Irmler *et al*., 1997; Golks *et al*., 2005). While c-FLIP$_L$ is composed of nine exons, the short splicing variants c-FLIP$_S$ and c-FLIP$_R$ consist of five and four exons, respectively (Golks *et al*., 2005). The unique splicing tail of c-FLIP$_S$, which is formed from exon 7, is absent from both c-FLIP$_L$ and c-FLIP$_R$. c-FLIP$_R$ is a run-through from exon 6 into intron 6, and it is thereby also terminated by a unique tail (Fig. XIIa). Interestingly, we noticed that the amino acid sequence of the murine c-FLIP$_S$ was similar to that of human c-FLIP$_R$ and not to that of human c-FLIP$_S$. In addition to the human and murine short forms of c-FLIP, short splicing variants of c-FLIP have been reported to exist in rats (Xiao *et al*., 2002) and in pigs (Goto *et al*., 2004), as well as in the predicted canine genome. We performed multiple sequence alignments with Clustal W (1.83) on all reported short forms of c-FLIP, and noticed that all their C-termini were homologues of human c-FLIP$_R$ rather than human c-FLIP$_S$ (Fig. XIIb).

To analyze whether there are more than one short form of c-FLIP also in species other than *Homo sapiens*, we studied the region between exons 6 and 8 of the human, bovine, murine, rat, macaque, chimpanzee, as well as the predicted canine *cflar* gene. Multiple sequence alignments showed that intron 6, exon 7, and intron 7 in human and chimpanzee *cflar* are almost identical (unpublished data). Murine *cflar* is very similar to human and chimpanzee *cflar* immediately downstream of exon 6 (Fig. XIIIa) and immediately upstream of exon 8 (Fig. XIIIb). Otherwise, the area between exon 6 and exon 8 in the murine *cflar* gene differs surprisingly much from the human and chimpanzee *cflar* sequences (Fig. XIIIa/b). The splicing tails of both the murine c-FLIP$_S$ and human c-FLIP$_R$ reside immediately downstream of exon 6 and are very well conserved (Fig. XIIIa).

To find a sequence in the *cflar* genes that would correspond to the human c-FLIP$_S$-specific splicing tail, we performed sequence alignments using Clustal W (1.83). We searched for sequences homologous to the unique human c-FLIP$_S$ splicing tail within chimpanzee, macaque, bovine, murine, and rat as well as the predicted canine *cflar* gene. Corresponding sequences were found downstream of exon 6 in all species, and these sequences were aligned to analyze whether they were conserved. While the sequence of the c-FLIP$_S$ splicing tail was very well conserved in the human, chimpanzee, macaque, bovine and the predicted canine *cflar*, it was less conserved in mouse and rat *cflar* (Fig. XIVa). The translated amino acid sequence from these gene sequences predicted even larger differences on the protein level comparing the rodents to the other species (Fig. XIVb). This suggests that the two short isoforms of c-FLIP expressed in humans may be expressed in chimpanzee, macaque, cow, and dog. The predicted macaque c-FLIP$_S$ splicing tail contains a premature stop codon and it is thereby shorter than the other c-FLIP$_S$ splicing tails. The rodent *cflar* gene, on the other hand, seems to include only the short c-FLIP isoform homologous to the human c-FLIP$_R$. However, it cannot be excluded that rodent c-FLIP isoforms including the splicing tails corresponding to the human c-FLIP$_S$, would be expressed and have similar functions as the human c-FLIP$_S$ although the predicted sequences are very different. The nucleotide sequence for long and short splicing variants of porcine and orangutan c-FLIP can also be found in the

data bases, but neither the chromosomal locus of *cflar* nor the sequence between exon 6 and 8 are known in these species, and therefore, they were not included in the analysis.

A.



B.

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CLUSTAL W (1.83) multiple sequence alignment

Human c-FLIP-S       -----MSAEVIHQVEEALDTDEKEMLLFLCRDVAIDVVPPNVRDLLDILRERGKLSVGDL
Human c-FLIP-R       -----MSAEVIHQVEEALDTDEKEMLLFLCRDVAIDVVPPNVRDLLDILRERGKLSVGDL
Murine c-FLIP-S      MAQSPVSAEVIHQVEECLDEDEKEMMLFLCRDVTENLAAPNVRDLLDSLSERGQLSFATL
Rat c-FLIP-S         MALSTVSAEVIHQVEESLDEDEKEMMLFLCRDVTENLAPPNVRDLLDCLSERGQLSFAAL
Canine c-FLIP-S      ----------------------------------------------LSERGELSAMGL
Porcine c-FLIP-S     MTLYRMSAEVIHQVEEALDEDEKEILLFLCRDIAADVVPLNVRDLLDILRERGKLSLVSL

Human c-FLIP-S       AELLYRVRRFDLLKRILKMDRKAVETHLLRNPHLVSDYRVLMAEIGEDLDKSDVSSLIFL
Human c-FLIP-R       AELLYRVRRFDLLKRILKMDRKAVETHLLRNPHLVSDYRVLMAEIGEDLDKSDVSSLIFL
Murine c-FLIP-S      AELLYRVRRFDLLKRILKTDKATVEDHLRRNPHLVSDYRVLLMEIGESLDQNDVSSLVFL
Rat c-FLIP-S         AELLYRVRRFDLLKRILKTDKAAVEDHLCRSPHLVSDYRVLLMEIGENLNQSDVSSLIFL
Canine c-FLIP-S      AELLYRVRRFDLLKRIFKMDRRAVEAHLLRHPRLISDYRVLMTEIGDDLDKSDVSSLIFL
Porcine c-FLIP-S     AELLYRVRRFDLLKRILKMDRRTVEAQLLRHPHLISDYRVLMMEIGEGLDKSDVSSLIFL

Human c-FLIP-S       MKDYMGRGKISKEKSFLDLVVELEKLNLVAPDQLDLLEKCLKNIHRIDLKTKIQKYKQSV
Human c-FLIP-R       MKDYMGRGKISKEKSFLDLVVELEKLNLVAPDQLDLLEKCLKNIHRIDLKTKIQKYKQSV
Murine c-FLIP-S      TRDYTGRGKIAKDKSFLDLVIELEKLNLIASDQLNLLEKCLKNIHRIDLNTKIQKYTQSS
Rat c-FLIP-S         TKDYTGRGKVAKDKSFLDLVIELEKLNLIGSDQLNLLEKCLKSIHRIDLKTKIQKYTQSS
Canine c-FLIP-S      MRDHMGRNKVAKDKSFLDLVIELEKLNLIAPDQLDLLEKCLKNIHRIDLKTKIQKYKQSA
Porcine c-FLIP-S     MRDHISRSKMAKDKSFLDLVIELEKLNLVAPDHLDLLEKCLRNIHRIDLKTKIQKYKQSA

Human c-FLIP-S       QGAGTSYRNVLQAAIQ-KSLKDPSNNFRMITPYAHCPDLKILGNCSM
Human c-FLIP-R       QGAGTSYRNVLQAAIQ-KSLKDPSNNFRVSLEKTYG----IPA----
Murine c-FLIP-S      QGARSN-MNTLQASLPKLSIK---YNSRVSLEPVYG----VPA----
Rat c-FLIP-S         QEARSN-MNALQASLPKLSIKEHLYNSRVSLEPVCG----ISA----
Canine c-FLIP-S      QGAGTNYTNALQASFPNLSLKDPSCNLRVSLEQMFG----IPA----
Porcine c-FLIP-S     QGAETNYVNALQASLPNLSIKDPSYNLRVSLEQVCG----IPA----
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Figure XII. Murine c-FLIP$_S$ corresponds to human c-FLIP$_R$. (A) The c-FLIP isoforms are built up by the following exons; all three c-FLIP isoforms contain exons 3, 4, 5, and 6 coding for the two DEDs. Due to alternative splicing, the c-FLIP$_L$ mRNA additionally contains the exons 8, 9, 10, 12, and 14, c-FLIP$_S$ mRNA contains exon 7, whereas c-FLIP$_R$ mRNA is a run-through intron 6. The stop codons in the different isoforms are marked by an asterisk (modified from Golks *et al*., 2005). **(B)** Multiple sequence alignments of the short c-FLIP isoforms of the species reported, human c-FLIP$_S$ (ID: AAC51623, GI: 2253681) and c-FLIP$_R$ (Djerbi *et al*., 2001), murine c-FLIP$_S$ (ID: BAE25546, GI: 74180622), rat c-FLIP$_S$ (ID: AAK28358, GI: 13506834), canine c-FLIP$_S$ (ID: XP_858329, GI: 74005137), and porcine c-FLIP$_S$ (ID: AAS22336, GI: 42652429) were done with Clustal W (1.83).

A.

```
CLUSTAL W (1.83) multiple sequence alignment

human  cflar downstream of exon 6 GTGAGTCTGGAGAAAACATATGGAATCCCAGCATGAAACCGTTTCAGAGTTCTAATAAAA
monkey cflar downstream of exon 6 GTGAGTCTGGAGAAAACATATGGAATCCCAGCCTGAAACCGTTTCAGAGTTCTAATAAAA
murine cflar downstream of exon 6 GTGAGTCTGGAGCCAGTGTATGGAGTACCAGCATGAACCAGTCTCAGAGATGTAATAAAA
                                  ************  *   ****** * ***** **** * ** ****** * ********

human  cflar downstream of exon 6 ATATGCATATTCTCACAGCATGTACTTTATTTAATATCTGAAAAAATTGTGATAGAAATG
monkey cflar downstream of exon 6 ATATGCATATTCTCACAGCATGTGCTTTATTTAATATCTGAAAAAATTGTGATAGAAATG
murine cflar downstream of exon 6 ATAAACAT---CTCATTTCATATGCT----GTAATAGCTAAACAAATTCTGATAGATATG
                                  ***  ***   ****   *** * **     ***** ** ** ***** ******* ***

human  cflar downstream of exon 6 TGTATTTGTTTTAAAAACGTGTAACTTCTTAT-ATTTCAAAGCTAATACATGTTCATTGA
monkey cflar downstream of exon 6 TGTATTTGTTTTAAAAACGTGTAACTTCTTAT-ATTTCAAAGCTAATACATGTTCATTGA
murine cflar downstream of exon 6 TGT-TTGATTAAGAATGTGTATAATTTCTTATGATTATAAACCTTAGTAGTGTTCAAAAA
                                  *** **  **   **   ** *** ******* ***  *** ** *    ******   *

human  cflar downstream of exon 6 GATATTTGGAGACTATAGAGAAAGATAAAAGAAAATAAATCACCTATAT-TCCACTATCC
monkey cflar downstream of exon 6 GATATTTGGAGACTATAGAGAAAGATAAAAGAAAATAAATCACCTATAT-TCCACTACCC
murine cflar downstream of exon 6 TATATTTGGAAAAATTTATGAAATATATAACAAGA-AAATAATTTTTGTGCCCATTATCT
                                   ********* *   *   **** *** ** ** * **** *  * * *  *** ** *

human  cflar downstream of exon 6 AAAGACAACCACTGTTAGTATTTTGGTATATTTCCTTCTAGACTCTTTTTTATGTGGGTT
monkey cflar downstream of exon 6 AAAGACAACCACTGTTAGCATTTTGGTATATTTCCTTCTAGACTCTTTTTTATGTGGGTT
murine cflar downstream of exon 6 GGGCATGACTACTG---------TGGAAAGCTTTCTTTTAGTCTCTGTCCTATGTGCATT
                                      *  ** ****         *** *   ** *** *** **** *  ******  **

human  cflar downstream of exon 6 TGCATCTGTATCTGTATGTATGTATATATATATA--------------------------
monkey cflar downstream of exon 6 TGCATCTGTATCTTTATATATATATATATATATACGTATATATATATATATATACGTATATA
murine cflar downstream of exon 6 AGCAAATGTGTCTATTTATACAGTTGAATATCTTTTTCATTCTTTGTTTCTTTGAAGAGT
                                   ***  *** *** * * **    *  **** *

human  cflar downstream of exon 6 ----------------TATATACATACATACATACATACAGTTGGACAATTGGACATTTT
monkey cflar downstream of exon 6 TATATATACATATATATATATATACACACACATACATACAGTTGGACAATTGGACGTTTT
murine cflar downstream of exon 6 CAATTTTAAAAATTAAAGTAGGTAGA-ATGTACCCATAGAAAGAAAAAGTTAAATGTCCC
                                              **   * * *   *  **** *     * * **  *  *

human  cflar downstream of exon 6 TTTCACTGTTCATTTATTCACTTGAAATTGTAACTTAAAAAAAGAAAATGTAACTTTAAA
monkey cflar downstream of exon 6 TTTCACTGTTCATTTATTCACTTGAAATTGTAACTTAAAAAAAGAAAATGTAACTTTAAA
murine cflar downstream of exon 6 CAA-AGAGATTTTAAAGTTGTTTC--CTTCTACCTCACGGAACTCATGTCCTACCTCCTT
                                      *  * *  *  * *   **    ** ** ** *   **   *  *   ** *

human  cflar downstream of exon 6 AATAA-AAATAGATGCACCATGCCTATGGAAAAAAATTTAAATGTACTACAAGAGGTTTT
monkey cflar downstream of exon 6 AATAA-AAATAGATGCACCATGCCTATGGAAAAAAATTTAAATGTACTACAAGAGGTTTT
murine cflar downstream of exon 6 CCTGTTAAGGAGACTAATCTAGACCAGTTTCTTCTATAACCATGCACAGAGAATCTACCC
                                    *   **  ***   * *  * * *         **    *** **    *

human  cflar downstream of exon 6 A-AAAGTATTTTTTTGCCCGGGTGCGGTGGCTCATGCGTGTAATCCCAGCACTTTGGGAG
monkey cflar downstream of exon 6 A-AAAGTATTTTTTTGCCCGGGTGCGGTGGCTCATGCGTGTAATCCCAGCACTTTGGGAG
murine cflar downstream of exon 6 ACAGAGTGTCTACTTTTATACAAGTGGTAGCATAT-CATGTCTGCTC----TTCTGAACA
                                  * * *** * *  **        * *** **  ** * ***   * *     * **

human  cflar downstream of exon 6 GCCGAGCCGGAAGGATCACCTGAGGTCAGGAGTTCGAGACTGGCCTGGCCAACATGGTGA
monkey cflar downstream of exon 6 GCCGAGCCGGAAGGATCACCTGAGGTCAGGAGTTCGAGACTGGCCTGGCCAACATGGTGA
murine cflar downstream of exon 6 GAGACTCCTTAGATATTGTTCCATATAGTTAATAGGAGATTGTTTCGACTTAATTATT--
                                  *     **  *   **      *  *    * *  **** **    * *  *  *  *

human  cflar downstream of exon 6 AACTCCATCTCTACTAAAAAATACAAAAATTAGCCAGGTATGGTGGCAGGTGCCTATAAT
monkey cflar downstream of exon 6 AACTCCATCTCTACTAAAAAATACAAAAATTAGCCAGGTATGGTGGCAGGTGCCTATAAT
murine cflar downstream of exon 6 -ATTTGTATTATTTTGAATGATACCC---CTACCCTTTTATCTTCTTTTGAGACAAGAA-
                                   * *     * *  * **  ****      ** **   ***  *     * * * * **

human  cflar downstream of exon 6 CCCAGCTACTTGGGAGGCTGAGGCAGGAGAATCATATG-AACCCAGGAAG---TGGAGGT
monkey cflar downstream of exon 6 CCCAGCTACTTGGGAGGCTGAGGCAGGAGAATCATATG-AACCCAGGAAG---TGGAGGT
murine cflar downstream of exon 6 CTTACCTGTAATCCAGCCTG-GCCTGGAATCCATTATGTAACCTAGGCTGGCCTTGAACT
                                  *  * **       ** *** * * ***      **** **** ***  *   * **  *

human  cflar downstream of exon 6 TGCAGT-AGCCAAATCGCGCCATTGTACTCCAGCCTGGGTGACAAGAGCAAAACTCTGTC
monkey cflar downstream of exon 6 TGCAGT-AGCCAGATCGCGCCATCGTACTCCAGCCTGGGTGACAAGAGCAAAACTCTGTC
murine cflar downstream of exon 6 TGCAATGAGCCTCCTCTTGCT-TCAGCCTCC--TCGGGCTCATGGCTTCCATTTTCTGCA
                                  **** * ****   **  **  *    ****   * ** * *      * *   ****
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```
human  cflar downstream of exon 6 TCAAAAAAAATGT------TTTCTTCCTACCCCTATAATACTGACCTCCAATTATTT-TT
monkey cflar downstream of exon 6 TCAAAAAAAATGT------TTTCTTCCTACCCCTATAATACTGACCTCCAATTATTT-TT
murine cflar downstream of exon 6 TGTACTAAAATGTATTTAGTTCTTTCTTGCTGATGTATAAATTGCCTCCTTTCCTTTGTT
                                   *  *  *******      **  *** * *   * **  * *  *****  *  *** **

human  cflar downstream of exon 6 CCTAAAGGCA--GCTGTTG--TGAACTAGTTCATTCTGTAACTATCCAGAGATA-GTCTA
monkey cflar downstream of exon 6 CCTAAAGGCA--GCTGTTG--TGAACTAGTTCATTCTGTAACTATCCAGAGATA-GTCTA
murine cflar downstream of exon 6 ACTAGAAACAATGCTGCAAAATAAACTTCCTGATTCT-TATCTCTTTGTGCATTTGCTTT
                                    *** *  **  ****     * ****   * ***** ** ** *      **  *  *

human  cflar downstream of exon 6 CACATATATTTCATTTTTGCTTTTTTCTCTTCTACAGATGATAACACCC-TATGCCCATT
monkey cflar downstream of exon 6 CACATATGTTTCATTTTTGCTTTTTTCTCTTATACAAATGATAGCACCC-TATGCCCATT
murine cflar downstream of exon 6 TAAAAAGACTATTTAGAAACTATTTTTACTTATTTATTTTAATTTGCATATATGCTTGT-
                                    * * *   *   *     ** ****  *** *  *  * *     *   *****   *

human  cflar downstream of exon 6 GTCCTGATCTGAAAATTCTTGGA--AATTGT---TCCATGTG-ATTAACATGGAACTGCC
monkey cflar downstream of exon 6 GTCCTGATCTGAAAATTCTTGGA--AATTGT---TCCATGTG-ATTAACATGGAACTGCC
murine cflar downstream of exon 6 GTGCCTGCATGAATTTTTGTGAACCATTTGTGTATGCATGTGTGCCTGCATGAATTTATG
                                   ** *     ****  **  ** *  * ****   * ******      **** *  *
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B.

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CLUSTAL W (1.83) multiple sequence alignment

human  cflar upstream of exon 8  --CAGCAAATTTTTGTATTTTTAGTAGAGATGGGGTTTTACCATGTTGGCCAGGCCGGTC
monkey cflar upstream of exon 8  --CAGCAAATTTTTGTATTTTTAGTAGAGATGGGGTTTTACCATGTTGGCCAGGCCGGTC
murine cflar upstream of exon 8  TGCAGTCAAGCTGTGCTTTGCCCGGTGTTAAAGGAATAGATTA-GATGGCAGGCTCAGTC
                                   ***  **  * **  **    *  *  *  **  *  *  * * ****  *  * ***

human  cflar upstream of exon 8  T--GGAACTCCTGGCCTCAGATGATCCACCCACCTTGGCAT-CCCAAGGTGTTGGGATTA
monkey cflar upstream of exon 8  T--GGAACTCCTGGCCTCAGATGATCCACCCACCTTGGCAT-CCCAAGGTGTTGGGATTA
murine cflar upstream of exon 8  CCAGCATATTCCAAGTTGGGACCTTCTAACACCCATGCTGTACTTGTAAGACCTGGTTTA
                                    * *  * *     *  **   ** * *  ** **   * *           ** ***

human  cflar upstream of exon 8  CAGGTGTC----AGCCAT------CGCTCCCGGCC-------TATTTTCATCTTTTAAAA
monkey cflar upstream of exon 8  CAGGTGTC----AGCCAT------CGCTCCCGGCC-------TATTTTCGTCTTTTAAAA
murine cflar upstream of exon 8  GAGCTGTTCTGAAGCAATATTCTATGCATCCCACCCTCCTAATGTTTTCATTTCTTAATG
                                  ** ***     *** **       **  **  **       * ***** * * ****

human  cflar upstream of exon 8  GCAGAATTTCCCTGTTCTTTGGTAATATGATACGGGATTTATGGAGTTAACAGCTCTTGC
monkey cflar upstream of exon 8  GCAGAATTTCCCTGTTCTTTGGTAATATGATATGGGATTTATGGAGTTAACAGCTCTTGC
murine cflar upstream of exon 8  G-GAAACTGATGTGATCTTTGACAATATAGTCTGGAATTTATGGAGTTAACAGTCCCTGC
                                 *   ** *    ** ******  *****  *  ** ****************  * ***

human  cflar upstream of exon 8  TATTCTTTAGATAATTTAACTGTTGGGACGATATTTTTATTTTTTTCCTGAGTTAAGAAT
monkey cflar upstream of exon 8  TATTCTTTAGATAATTTAACTGTCGGGACAATATTTTTATTTTTTTCCTGAGTTAAGAAT
murine cflar upstream of exon 8  ACTTCATT--TTTAGGTAACTGTGAAGGTGATATTTTTATTCCCCCCCTCCCCGTGTAAA
                                   *** **   * *  *******   *   ***********     ***        **

human  cflar upstream of exon 8  CCTATTGAGACATTTAAAGAGGTGTAATGTATAGTGTACCTCTGAAATAACTAACAGGAA
monkey cflar upstream of exon 8  CCTATTGAGACATTTAAAGAGGTGTAATGTACAGTGTACCTCTGAAATAACTAACAGGAA
murine cflar upstream of exon 8  AATATCAAGCTATTTAAAGAGGTGTAAC--ATAGTGAGTCTCTGAAGAAACTAACAC--A
                                   ***  **  ****************   * ****   *******  ********   *

human  cflar upstream of exon 8  GTATGACCTTATTCTTTGTATTTGAAG
monkey cflar upstream of exon 8  GTATGACCTTATTCTTTGTATTTGAAG
murine cflar upstream of exon 8  GTGTGACCTTCTGTTTTATATTTGAAG
                                 ** ******* *  *** *********
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Figure XIII. The sequence of the *cflar* gene between exons 6 and 8 is well conserved in human and chimpanzee, but less conserved in rodents. Multiple alignments on the human, chimpanzee (monkey) and the murine *cflar* sequence from the beginning of intron 6 to the end of intron 7 were done with Clustal W (1.83). Intron 6 **(A)** and the end of intron 7, upstream of exon 8 **(B)** are shown. The sequences encoding the splicing tails that are homologous with human c-FLIP$_R$ are highlighted with black and the sequences corresponding to human the c-FLIP$_S$-specific splicing tail are highlighted with grey color. The murine sequence homologous to the human c-FLIP$_S$ splicing tail is located higher upstream than the corresponding human and chimpanzee sequences. Nucleotides conserved between the human, chimpanzee, and murine genomes are indicated with an asterisk.

A.

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CLUSTAL W (1.83) multiple sequence alignment

Human           ATGATAACACCCTATGCCCATTGTCCTGATCTGAAAATTCTTGGAAATTGTTCCATGTGA
Chimpanzee      ATGATAGCACCCTATGCCCATTGTCCTGATCTGAAAATTCTTGGAAATTGTTCCATGTGA
Macaque         ATGATAGCACCCTATGTCCATTTTCCTGACTTGTAAATTCTTGGAAATTGTTCCATATGA
Bovine          ATGGTAGCATGCTATGCCCACTGTCCTGACCTGAGAATTCTTAGAAATTGTTCCATGTGA
Canine          ATGGTAGAACACTGTACCCATTGTCCTGAGCTGAGAATTCTTAGAAATTGTTCTGTATGA
Murine          GTGGTAGCATATCATGTCTGCTCTTCTGAACAGAGACTCCTTAGATATTGTTCCATATAG
Rat             GTGGTAGCATATCATGTCTGCTCCTCTGAACAGAGAAGTCTTAGACATTGTTCTATATGG
```

B.

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CLUSTAL W (1.83) multiple sequence alignment

Human           MITPYAHCP-DLKILGNCSM-
Chimpanzee      MIAPYAHCP-DLKILGNCSM-
Macaque         MIAPYVHFP-DL---------
Bovine          MVACYAHCP-DLRILRNCSM-
Canine          MVEHCTHCP-ELRILRNCSV-
Murine          -VVAYHVCSSEQRLLRYCSI-
Rat             -VVAYHVCSSEQRSLRHCSIW
```

Figure XIV. The sequence for the c-FLIP$_S$-specific splicing tail is absent in the rodent *cflar* gene. The nucleotide **(A)** and the predicted amino acid **(B)** sequences most homologous to the human c-FLIP$_S$-specific splicing tail were compared between species using Clustal W (1.83).

No dramatic differences in the function of c-FLIP$_S$ and c-FLIP$_R$ have been observed so far. Both proteins have similar half-lives and the same effects on both death receptor signaling and NF-κB activation (Golks *et al*., 2005). However, we have reported that the turn-over of c-FLIP$_S$ is regulated by its C-terminal splicing tail (Poukkula *et al*., 2005). The folding of the splicing tails of the short c-FLIP isoforms might mediate ligation of ubiquitin chains to c-FLIP as well as interactions between c-FLIP and proteasomes. Therefore, it will be interesting to investigate whether the splicing tails of human c-FLIP$_R$ and murine c-FLIP$_S$ also are capable of mediating ubiquitination and degradation. If this is not the case, the human c-FLIP$_S$ seems to be a unique protein, the turn-over of which is specifically regulated.

5.3. The effects of hyperthermia on CD95-mediated apoptosis (II, III)

5.3.1. Hyperthermia sensitizes T-lymphocytes to CD95-mediated apoptosis (II, III)

Fever is a physiological hyperthermia that is induced upon immune responses against infections. Hyperthermia has been shown to induce thermotolerance in cells and thereby to protect them from different otherwise lethal treatments such as cytotoxic drugs (Creagh and Cotter, 1999). Homeostasis in immune cell populations is partly regulated by the death receptor CD95. To investigate the effects of hyperthermia on CD95 signaling, we used Jurkat T-lymphocytes. The Jurkat T-lymphocyte cell line was established from the peripheral blood of a 14-year old boy with acute T cell leukemia in the late 1970's (Schneider *et al*., 1977; Weiss *et al*., 1984; reviewed by Abraham and Weiss, 2004). While subjecting Jurkat T-lymphocytes to a hyperthermia treatment of 30 min at 42ºC prior to the addition of an agonistic α-CD95 antibody, the level of apoptosis was increased compared to the level of apoptosis induced with CD95 ligation alone (Fig. 1A and B, II). To investigate the effect of hyperthermia on CD95 responses in a more physiological system, we used primary human T-lymphocytes. The primary human peripheral T-lymphocytes were isolated from blood and

activated by stimulation with phytohemagglutinin (PHA) and interleukin-2 (IL-2). The sensitivity to CD95-mediated apoptosis in primary T-lymphocytes was tested by treating them with an agonistic α-CD95 antibody for twelve hours. Consistent with earlier reports (Kirchhoff *et al*., 2000), the primary T-lymphocytes were resistant to CD95-mediated apoptosis during early activation (day 1 and day 3 of activation), but responded to a twelve-hour treatment with an agonistic α-CD95 antibody after six days of activation (Fig. XVa). CD95 ligation was unable to induce apoptosis during the first three days of activation also when cells were subjected to a pretreatment with hyperthermia at 42ºC (Fig. 1A, III). However, CD95 ligation in cells that were activated for six days induced apoptosis in most of the cells pretreated at 42ºC already after two hours, whereas the cells treated with agonistic α-CD95 antibody alone were barely affected at this time point (Fig. 1A, III). To elucidate whether this observation could have relevance under fever-like conditions, we studied the effects of hyperthermia at lower temperatures on human primary peripheral T-lymphocytes. Indeed, when the temperature was lowered to 40ºC, a similar sensitization to CD95-mediated apoptosis was observed (Fig. 1B, III).

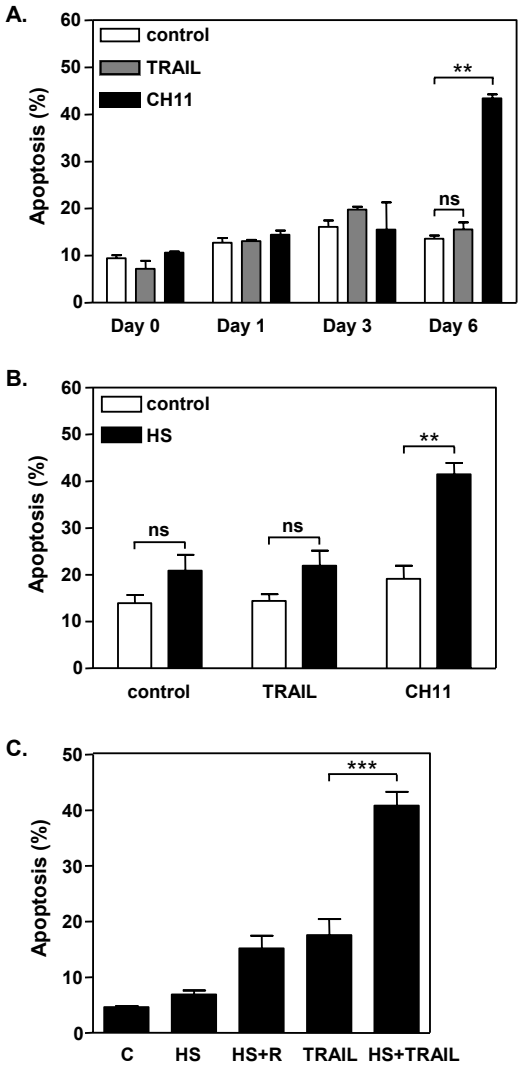

Figure XV. Hyperthermia does not sensitize primary human T-lymphocytes to TRAIL-induced apoptosis. (A) Apoptosis was quantified by Annexin V analysis after resting, day 1-, day 3-, and day 6-activated primary human T-lymphocytes were treated with 1 μg/ml recombinant TRAIL in combination with M2 cross-linking antibody or 1 μg/ml agonistic α-CD95 antibody CH11 for 12 h. The bar graphs represent mean values (± SD) of samples from two individuals. Statistical significance was determined with unpaired *t* tests and marked with asterisks (**: $p < 0.005$), ns ($p > 0.05$) stands for not statistically significant. **(B)** Apoptosis was quantified as in panel (A) after day 6-activated primary human T-lymphocytes were treated with 1 μg/ml recombinant TRAIL in combination with M2 cross-linking antibody or 1 μg/ml agonistic α-CD95 antibody CH11 for 2 h with or without pretreatment with hyperthermia at 42ºC (HS) for 30 min. The bar graphs represent mean values (± SD) of samples from two individuals. Statistical significance was determined with unpaired *t* tests and marked with asterisks (**: $p < 0.005$), ns ($p > 0.05$) stands for not statistically significant. **(C)** Apoptosis was quantified as in panel (A) after Jurkat cells were treated with 200 ng/ml recombinant TRAIL in combination with M2 cross-linking antibody for 2 h with or without pretreatment with hyperthermia at 42ºC (HS) for 30 min. The bar graphs represent mean values (± SD) of samples from six independent experiments. Statistical significance was determined with paired *t* tests and marked with asterisks (***: $p < 0.001$).

In our model system, the primary cells were treated with PHA and IL-2 for six days to completely activate T-lymphocytes. In general, the highest fever temperatures are obtained during the first days of infection. However, during a response to infections, the T-lymphocytes in the body are not simultaneously activated. Therefore, in the animal, T-lymphocyte populations are comprised of T-cells at different activation states. In the intact organism, one could suspect that part of the T-lymphocytes are fully activated already during the first days of infection, when the increase in temperature is at maximum. Fever temperatures would thereby sensitize the already activated cells to CD95L-induced AICD, which protects the organism from the damaging effects of a possible accumulation of autoreactive T-lymphocytes.

Primary cells have been suggested not to be sensitive to TRAIL (Wiley *et al*., 1995; Pitti *et al*., 1996). Therefore, we further analyzed if the hyperthermia-induced sensitization was restricted to CD95 signaling or if hyperthermia was able to sensitize also primary T-lymphocytes to TRAIL-induced apoptosis. As expected, T-lymphocytes were resistant to TRAIL ligation during early activation (Fig. XVa). TRAIL ligation failed to induce apoptosis also during twelve-hour incubations in day 6-activated cells, regardless whether the cells were pretreated with hyperthermia or not (Fig. XVa/b). However, Jurkat cells, which are sensitive to TRAIL-induced apoptosis, were further sensitized by hyperthermia (Fig. XVc). Hyperthermia has been shown to sensitize Jurkat cells but not primary T-lymphocytes to TRAIL-induced apoptosis even twelve hours after the hyperthermia-treatment. This long-lasting sensitization was suggested to increase TRAIL-TRAIL-R interactions. However, the increased interactions were demonstrated as a decreased affinity of a α-TRAIL-R antibody to the TRAIL-R, which does not provide solid evidence for the ligand-receptor interactions to be increased (Moulin and Arrigo, 2006; Moulin *et al*., 2006).

HeLa cells are cervical adenocarcinoma epithelial cells obtained from a tumor derived from Henrietta Lacks, who died of cancer in 1951 (reviewed by Masters, 2002). The hyperthermia-induced sensitization to CD95-mediated apoptosis was not restricted to Jurkat cells or the use of an agonistic α-CD95 antibody, because HeLa cells subjected to hyperthermia were also sensitized to treatment with recombinant CD95L (Fig. 1C, II). Similarly, malignant glioma cells have been shown to be sensitized to CD95-mediated apoptosis by hyperthermia (Hermisson *et al*., 2000).

5.3.2. Hyperthermia enhances CD95-mediated caspase activation (II)

To elucidate the mechanism behind the hyperthermia-induced sensitization to death receptor-mediated apoptosis, we investigated the signaling pathways activated upon death receptor triggering. While the pancaspase inhibitor zVAD-fmk completely inhibited apoptosis induced by CD95 ligation also when the cells were pre-treated with hyperthermia, we concluded that the hyperthermia-mediated sensitization was caspase dependent (Fig. 6A, II). Hyperthermia increased the caspase-8 processing induced by CD95 ligation, as can be seen from the cleavage of caspase-8 both to its intermediate p41/p43 form and to its active p18 form (Fig. 6B, II). Interestingly, we observed that hyperthermia alone activated caspase-8 without inducing apoptosis (Fig. 6B, II). To assess whether hyperthermia was able to increase caspase

activation in general, we examined the cleavage of caspase-9 and caspase-3. Although caspase-9 cleavage has been suggested not to be required for its activation, it is an indication of caspase-9 activation, since caspase-9 can be cleaved upon binding to Apaf-1 in the apoptosome (Pop *et al*., 2006). Hyperthermia itself did not, however, induce any cleavage of caspase-9 or caspase-3 (Fig. 6C, II), indicating that hyperthermia was not able to induce universal caspase activation on its own. Caspase-3 activity detected as DEVDase activity was not either increased upon hyperthermia treatment alone (Fig. 6D, II). On the other hand, hyperthermia did increase CD95-mediated cleavage of both caspase-9 and caspase-3 (Fig. 6C, II), as well as CD95-mediated caspase-3 activity (Fig. 6D, II). In addition, hyperthermia increased both CD95-mediated cleavage of Bid and loss of cytochrome *c* from the mitochondria (unpublished data). These results show that hyperthermia promotes the activation of mediators involved in apoptotic signaling induced via CD95. The caspase-8 inhibitor zIETD-fmk completely blocked the hyperthermia-induced caspase-8 cleavage, the increase in CD95-mediated caspase-8 and caspase-3 activation, as well as apoptosis (Fig. 7A, II). Moreover, we observed the sensitizing effect both in Type I (H9 cells) and Type II (Jurkat and HeLa cells) cells, indicating that the hyperthermia-induced sensitization does not require the mitochondrial amplification loop (unpublished data). Thus, it can be concluded that hyperthermia affects CD95 signaling at the level of caspase-8 activation.

5.3.3. Hyperthermia does not affect the expression of either CD95 ligand or receptor (II, III)

Cell stress has been proposed to increase the expression of *CD95L* in a JNK-dependent manner (Faris *et al*., 1998a and 1998b), and HSF1 has been shown to induce *CD95L* expression in response to hyperthermia (Cippitelli *et al*., 2005). Studies in *lpr* mice revealed that apoptosis induced by both γ-irradiation and hyperthermia are dependent on CD95 signaling (Reap *et al*., 1997). As stress signaling was suggested to engage death receptors for execution of apoptosis, we further examined the effects of hyperthermia on the CD95 receptor and ligand expression. CD95 was similarly expressed on the surface of control and hyperthermia-exposed primary T-lymphocytes and Jurkat cells (Fig. 5A, II and 2A, III). SDS-stable high molecular weight forms of CD95 that appear in Western blots after CD95 ligation were observed in samples regardless of whether the cells were treated with hyperthermia or not. These high molecular weight forms might be aggregates of oligomerized CD95, indicating that hyperthermia does not affect the oligomerization of CD95. Likewise, there were no changes in the overall expression of CD95 (Fig. 5B, II). Furthermore, we could not detect any changes in the levels of CD95L during hyperthermia followed by a two-hour recovery (Fig. 2B, III). In addition, the CD95L-blocking antibody (NOK-1) did not affect the hyperthermia-induced sensitization to CD95 ligation with agonistic α-CD95 antibody (Fig. 2C, III). Taken together, these results exclude elevated CD95 surface expression and CD95L upregulation as causes for the observed sensitization. Moreover, these results suggest that hyperthermia affects CD95 signaling downstream of death receptor ligation. The results by Cippitelli and co-workers showing a hyperthermia-induced increase in the CD95L expression were obtained under different experimental conditions than ours. While they detected an increased expression of CD95L after a 24-hour hyperthermia treatment or after a five-hour hyperthermia in the presence of PMA and ionomycin (Cippitelli *et al*., 2005), we used only

30-min hyperthermia treatments followed by a two-hour recovery. As the *CD95L* is a target gene for NF-κB and PMA is a strong inducer of NF-κB activation, the increased CD95L expression that Cippitelli and co-workers detected could be caused by the PMA treatment and not by hyperthermia. Prolonged exposure to elevated temperatures might, however, additionally increase CD95L expression, enhancing the sensitizing effect we observed.

5.3.4. The hyperthermia-induced sensitization to CD95-mediated apoptosis does not involve MAPK signaling (II)

Hyperthermia activates several members of the MAPK family, among them ERK, p38, and JNK (reviewed by Dorion and Landry, 2002). Thus, a stress-induced activation of JNK during pretreatment with hyperthermia might result in additional activation of apoptotic signaling pathways. In Jurkat cells, JNK was activated both by hyperthermia and CD95 stimulation (Fig. 4A, II). MKK4 has been shown to be necessary for hyperthermia-induced JNK activation (Yang, D. *et al*., 1997). To disrupt the JNK pathway, we used a dominant negative mutant of the JNK activator MKK4 (DN-MKK4) tagged to GFP (green fluorescent protein). However, expression of DN-MKK4 did not prevent the hyperthermia-induced increase in CD95-mediated apoptosis in Jurkat cells (Fig. 4B and C, II), indicating that JNK is not responsible for the sensitization. Furthermore, rather than inducing protection, inhibition of the MAPK family members p38 and ERK resulted in further increased sensitization to CD95-mediated apoptosis in response to hyperthermia (unpublished data). We have previously shown in HeLa cells that CD95 stimulation results in a rapid activation of ERK protecting from death receptor-mediated apoptosis (Holmström *et al*., 1999; Tran *et al*., 2001). Accordingly, the hyperthermia-induced sensitization might be caused by abrogation of ERK-mediated survival signaling. However, hyperthermia did not reduce CD95-mediated activation of ERK. Instead, hyperthermia strongly increased ERK phosphorylation, an activation state that disappeared rapidly when the cells were left to recover at 37ºC (Fig. 4D, II). In Jurkat cells, on the contrary, no significant ERK activity was detected after either hyperthermia or CD95 ligation (unpublished data). Taken together, these results exclude the modulation of MAPK activity as a determinant of the hyperthermia-induced sensitization to CD95-mediated apoptosis. Additionally, these data strongly suggests that the observed sensitization is not a combination of stress-specific apoptotic signaling and death receptor signaling, but rather a direct effect of hyperthermia on one or more components of the apoptotic cascade induced upon CD95 ligation.

5.4. The hyperthermia-induced sensitization to CD95-mediated apoptosis is caused by c-FLIP$_S$ downregulation (II, III)

5.4.1. Hyperthermia downregulates c-FLIP and sensitizes to CD95-mediated apoptosis (II, III)

The finding that caspase-8 could be the target for the hyperthermia-induced sensitization to CD95-mediated apoptosis, prompted us to investigate whether the modulators of death signaling in the DISC were responsible for the induced sensitivity. We observed that the

levels of both c-FLIP$_S$ and c-FLIP$_L$, which are able to interfere with caspase-8 activation in the DISC, were downregulated rapidly during hyperthermia treatment (Fig. 8A, II). As c-FLIP$_R$ is not found in Jurkat cells and is expressed at very low levels in primary T-lymphocytes (Golks *et al*., 2005), c-FLIP$_R$ was not included in this study. The levels of both c-FLIP$_L$ and c-FLIP$_S$ decreased markedly upon hyperthermia also in primary T-lymphocytes, at temperatures of 40ºC as well as 42ºC (Fig. 1C and D, III). The resistance to CD95 signaling during early T-lymphocyte activation has been suggested to be regulated by increased expression of c-FLIP$_S$ (Kirchhoff *et al*., 2000; Schmitz *et al*., 2004). As the c-FLIP amounts decrease upon T-lymphocyte activation, we showed that elevated temperatures further deplete c-FLIP, allowing for efficient CD95-dependent elimination of the activated T-lymphocytes (Fig. 1A and B, III). The levels of c-FLIP were reduced upon hyperthermia also earlier during T-lymphocyte activation (day 1 - day 3). Nevertheless, the c-FLIP$_S$ quantities were much higher at this time. Hence, hyperthermia did not downregulate c-FLIP$_S$ to the extent that would sensitize these recently activated cells to apoptosis (unpublished data).

We and others have shown that treatments that downregulate c-FLIP, sensitize cells to death receptor-mediated apoptosis (Kataoka *et al*., 2002; Kim, H. *et al*., 2002; Davidson *et al*., 2003; Hietakangas *et al*., 2003; Nitobe *et al*., 2003; Perez and White, 2003; Sayers *et al*., 2003; Dutton *et al*., 2004; Ganten *et al*., 2004; Ricci *et al*., 2004; El-Zawahry *et al*., 2005; Uriarte *et al*., 2005; Xiao *et al*., 2005). To be able to determine the role of c-FLIP in death receptor-mediated apoptosis, and to be able to analyze the distinct functions of the different c-FLIP isoforms, we designed isoform-specific shRNA pSuperior constructs for c-FLIP. HeLa cells were transfected with shRNA constructs that downregulate either both c-FLIP$_L$ and c-FLIP$_S$ or each isoform separately (Fig. XVIa). Downregulation of both c-FLIP isoforms sensitized HeLa cells efficiently to CD95L-induced apoptosis, whereas downregulation of only one of the isoforms at a time was less potent in sensitizing to CD95L (Fig. XVIb). However, the shRNA construct targeting c-FLIP$_L$ was less efficient than that targeting c-FLIP$_S$ (Fig. XVIa). Therefore, the level of apoptosis induced in the cells in which c-FLIP$_S$ has been downregulated cannot be compared to the level of apoptosis induced in the cells with downregulated c-FLIP$_L$. However, these results indicate that both c-FLIP isoforms are anti-apoptotic, and that downregulation of both isoforms is required for efficient sensitization to CD95-mediated apoptosis in HeLa cells.

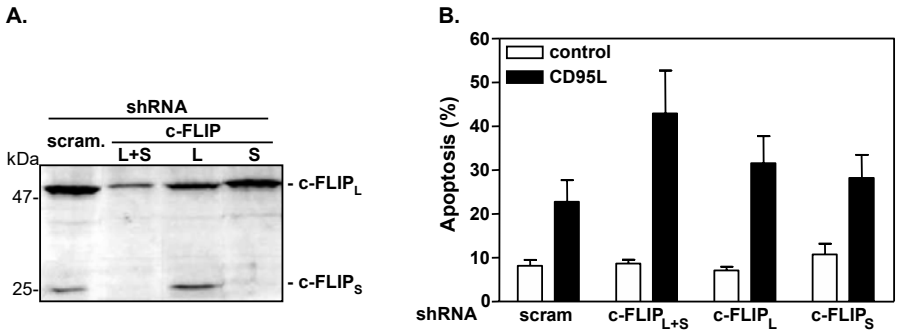

Figure XVI. Downregulation of c-FLIP sensitizes HeLa cells to CD95-mediated apoptosis. HeLa cells were transfected with pSuperior scrambled and c-FLIP shRNA vectors downregulating both c-FLIP$_L$ and c-FLIP$_S$ or the two isoforms specifically. **(A)** The shRNA-mediated downregulation of c-FLIP was controlled by Western blotting. **(B)** HeLa cells were treated with 200 ng/ml recombinant CD95L in combination with M2 cross-linking antibody for 3 h. Apoptosis was analyzed by flow cytometry using a PE-conjugated antibody recognizing activated caspase-3. The bar graphs represent mean values (± SD) of samples from three independent experiments.

We also analyzed whether other stresses, such as oxidative stress, osmotic shock or heavy metals, could downregulate c-FLIP and sensitize Jurkat cells to death receptor-mediated apoptosis. However, no downregulation of c-FLIP could be detected during these stresses (unpublished data). Likewise, these stresses did not have a sensitizing effect on CD95-mediated apoptosis (unpublished data), indicating that the downregulation of c-FLIP and its sensitizing effect were specific for hyperthermia and is not a phenomenon induced by cell stress in general. However, none of the treatments used to induce these stresses lasted more than one hour and longer treatments might affect the apoptosis sensitivity differently. Extended treatments (four to six hours) with oxidative stress have been shown to upregulate both CD95L and CD95 in murine intestinal epithelial cells, sensitizing these cells to apoptosis (Denning *et al*., 2002).

5.4.2. Downregulation of c-FLIP$_S$ is required for the hyperthermia-induced sensitization to CD95-mediated apoptosis (III)

The hyperthermia-induced decrease in c-FLIP levels was rapidly restored when cells were left to recover at 37ºC (Fig. 3A, III). To study whether the intracellular levels of c-FLIP correlated with the hyperthermia-induced sensitization to CD95-mediated apoptosis, cells were treated with the agonistic α-CD95 antibody either immediately after exposure to hyperthermia, or were left to recover for up to six hours before ligation of CD95. The kinetics of c-FLIP depletion and reappearance corresponded perfectly to the kinetics of hyperthermia-mediated sensitization and desensitization to CD95-mediated apoptosis (Fig. 3B, III). A similar inverse correlation between c-FLIP levels and CD95 sensitivity could be observed in primary human T-lymphocytes (Figs. 3C, D, III), indicating that downregulation of c-FLIP at the time of CD95 ligation is the mechanism responsible for the sensitization in both Jurkat cells and primary human T-lymphocytes. The hyperthermia-induced increase in apoptosis could still be detected after 48 h of treatment with agonistic α-CD95 antibody (Fig. XVIIa) and the cells were not sensitized if hyperthermia was initiated after CD95 ligation (Fig. XVIIb). These results provide further evidence that the sensitizing effect is determined by the levels of c-FLIP at the time of CD95 ligation.

To determine the c-FLIP isoform-specific roles in the observed sensitization to CD95-mediated apoptosis, we used Jurkat cell lines stably overexpressing either c-FLIP$_S$ or c-FLIP$_L$ (Fig. 4A, III). The overexpressed c-FLIP was downregulated upon hyperthermia, but with slower kinetics than the endogenous c-FLIP. Hence, high c-FLIP levels were maintained in the overexpressing cells also after exposure to hyperthermia (Fig. 7, III and unpublished data). Overexpression of c-FLIP$_S$ was able to rescue the cells from the hyperthermia-induced sensitization to CD95-mediated apoptosis, whereas cells overexpressing c-FLIP$_L$ were sensitized equally well as wild-type Jurkat cells (Fig. 4B, III). These results indicate that while both isoforms of c-FLIP are downregulated upon hyperthermia (Figs. 1C, 3A, III), the sensitization to apoptosis induced via CD95 is primarily mediated by downregulation of c-FLIP$_S$. It is important to note that despite downregulation of c-FLIP$_S$ was both necessary and sufficient for the hyperthermia-induced sensitization to CD95-mediated apoptosis in T-

lymphocytes, the efficient shRNA-mediated c-FLIP$_S$ depletion did not have more prominent effects on apoptosis in HeLa cells (Fig. XVI).

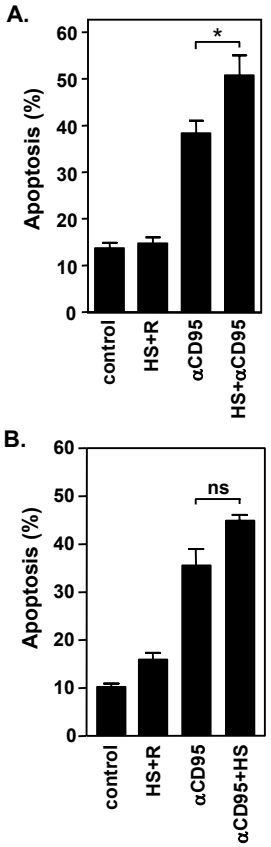

Figure XVII. The hyperthermia-induced sensitization depends on the c-FLIP levels at the time of CD95 ligation. (A) Apoptosis was quantified by Annexin V analysis after Jurkat cells were treated with 0.5 ng/ml agonistic α-CD95 CH11 antibody for 48 h, with or without a 30-min pretreatment with hyperthermia at 42ºC (HS). The bar graphs represent mean values (± SD) of samples from three independent experiments. Statistical significance was determined with paired *t* tests and marked with asterisks (*: $p < 0.05$). **(B)** Jurkat cells were either treated with hyperthermia at 42ºC for 30 min followed by a 1 h 15-min recovery (HS+R), with 200 ng/ml agonistic α-CD95 antibody CH11 for 2 h, or with α-CD95 antibody for 2 h combined with the 30-min hyperthermia treatment at 42ºC starting 15 min after addition of the α-CD95 antibody. Apoptosis was quantified by Annexin V analysis. Notice that some apoptosis was induced upon hyperthermia treatment also in the absence of CD95 ligation. The bar graphs represent mean values (± SD) of samples from two independent experiments. Statistical significance was determined with unpaired *t* tests and marked with asterisks, ns ($p > 0.05$) stands for not statistically significant.

Several studies have shown that overexpression of c-FLIP$_L$ (Kataoka *et al*., 2000; Kataoka and Tschopp, 2004; Kreuz *et al*., 2004; Dohrman *et al*., 2005a) and c-FLIP$_S$ (Golks *et al*., 2006) efficiently activates NF-κB. To detect whether the protective effect of the c-FLIP$_S$ overexpression was due to increased NF-κB activation, we analyzed the ability of the overexpressed c-FLIP to activate NF-κB. Interestingly, the level of NF-κB activity was higher in the parental Jurkat cells than in the overexpressing cell lines (Fig. XVIII), which suggests that overexpression of c-FLIP$_L$ or c-FLIP$_S$ decreases the NF-κB activity. The c-FLIP-overexpressing cell lines were, however, grown in the presence of geneticin selection, whereas the parental cell line was not. Thus, the NF-κB response might have been altered in the manipulated cell lines. To exclude the possibility that NF-κB activity is affected in the c-FLIP-overexpressing cell lines, they should be compared to a mock-transfected line that would have undergone a similar antibiotics-mediated selection.

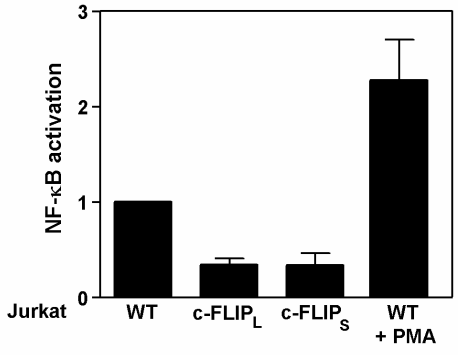

Figure XVIII. Stable overexpression of c-FLIP in cell lines does not enhance NF-κB activation. The level of NF-κB activation in Jurkat parental cells and Jurkat stable transfectants overexpressing c-FLIP$_S$ or c-FLIP$_L$ was determined by detecting NF-κB binding activity *in vitro* by electrophoretic mobility shift assay. Parental Jurkat cells treated with PMA at a concentration of 100 nM for 1 h were used as a positive control for the induction of NF-κB activity. The bar graph represents mean values (± SD) from autoradiographs from three independent experiments quantified with the AIS-Analytical Imaging Software.

5.4.3. The loss of c-FLIP from the DISC allows for efficient activation of caspase-8 (III)

To analyze whether hyperthermia affects the DISC composition, we used Fc-conjugated CD95L for immunoprecipitation of the CD95-DISC. The levels of both c-FLIP$_S$ and c-FLIP$_L$ were significantly decreased in the activated DISC of cells exposed to hyperthermia prior to receptor triggering (Fig. 5, III). However, induction of hyperthermia did not markedly affect the recruitment of caspase-8 to the DISC (Fig. 5, III). The absence of c-FLIP in the DISC is most likely caused by downregulation of expression and not by impaired recruitment, as reduced c-FLIP levels were detected both in the cell lysates and DISC-immunoprecipitates. The loss of c-FLIP from the activated DISC changes the ratio of caspase-8 and c-FLIP, allowing for efficient activation of caspase-8 in the absence of its inhibitor (Fig. 6B and 7A, II). Similarly to our studies, a change in the ratio of caspase-8 and c-FLIP within the DISC, resulting in enhanced caspase activation and more efficient apoptotic signaling, has been shown with 5-fluorouracil (5-FU) treatment (Ganten *et al*., 2004). Interestingly, the change in the balance of caspase-8 and c-FLIP in the DISC was obtained by increased recruitment of caspase-8, since 5-FU did not affect recruitment of c-FLIP. As c-FLIP has been suggested to have a stronger affinity for binding in the DISC than caspase-8 (Chang *et al*., 2002), a more dramatic downregulation of c-FLIP might be required to also deplete c-FLIP from the DISC.

Hyperthermia alone was able to induce caspase-8 cleavage without inducing apoptosis (Fig. 6B, II). While caspase-8 activation requires oligomerization, hyperthermia has to induce the formation of either a DISC or another complex where caspase-8 can be activated. We have not been able to detect any DISC assembly in the absence of CD95 ligation (Fig. 5, III and unpublished data). Caspase-8 can be activated upon treatment with anti-cancer agents also in the absence of death receptor ligation (Engels *et al*., 2000). However, this DISC-independent caspase-8 activation is induced via the mitochondrial apoptotic pathway (Engels *et al*., 2000), which is not activated upon hyperthermia alone (Fig. 6C and D, II, and unpublished data). Caspase-8 has been reported to be activated also in death-effector filaments that are formed upon overexpression of DED proteins (Siegel *et al*., 1998). Hyperthermia might induce assembly of such kind of aggregates in which caspase-8 activation could occur. Recently, it was shown that caspase-2 can be activated upon hyperthermia (Tu *et al*., 2006). In addition, active caspase-2 has been suggested to contribute to caspase-8 activation in the DISC, thereby enhancing CD95- and TRAIL-R-mediated apoptosis (Droin *et al*., 2001; Shin *et al*., 2005). Hence, caspase-2 may be involved also in the hyperthermia-induced caspase-8 activation and sensitization to CD95-mediated apoptosis.

5.5. Regulation of c-FLIP ubiquitination and degradation during hyperthermia (III)

5.5.1. Hyperthermia does not affect transcription or translation of c-FLIP (III)

Hyperthermia is known to silence general gene expression in order to maximize production of chaperones and other proteins required for survival under stress conditions (Tissieres *et al*., 1974; McKenzie *et al*., 1975; Duncan and Hershey, 1989). Therefore, we wanted to investigate whether the hyperthermia-induced downregulation of c-FLIP was caused by silencing of c-FLIP transcription or translation. The hyperthermia-induced downregulation of c-FLIP occurred very rapidly at the protein level; c-FLIP$_S$ was downregulated to 50% already

after 15 min and c-FLIP$_L$ after 30 min (Fig. 6A, III). As the c-FLIPs have short half-lives, silencing of the *cflar* gene expression would rapidly downregulate c-FLIP protein levels. To study if the hyperthermia-induced downregulation was due to inhibition of *de novo* synthesis of c-FLIP mRNA, we performed RNase protection assays. No inhibition of c-FLIP expression at the mRNA level was evident at the early time points (Fig. 6B, III), when hyperthermia downregulated the c-FLIP protein (Fig. 6A, III), indicating that the transcription of c-FLIP was constant also during hyperthermia. Determination of the turnover of c-FLIP with CHX chases revealed that the half-life of c-FLIP$_L$ in Jurkat cells was approximately 2 h, whereas the half-life of c-FLIP$_S$ was between 30 min and 1 h (Fig. 6C, III). If the downregulation of c-FLIP would be due to a hyperthermia-induced general shutdown in translation, the kinetics of protein loss would be the same as the half-life of the protein. Since c-FLIP was downregulated significantly faster than indicated by its half-life, inhibition of protein synthesis as a mechanism for c-FLIP elimination during hyperthermia can be excluded. It is, however, plausible that, over a longer period of time, the hyperthermia-induced shutdown of protein synthesis may contribute to the loss of c-FLIP. Additionally, it has been suggested that a 42ºC hyperthermia is required for a general shut down of protein synthesis (Duncan and Hershey, 1989). Since, c-FLIP is downregulated already upon a 40ºC hyperthermia treatment, stress-induced silencing of gene expression can be excluded as an explanation for the c-FLIP depletion.

5.5.2. The ubiquitination and degradation of c-FLIP are increased upon hyperthermia (III)

Abundant evidence for c-FLIP being degraded via the ubiquitin-proteasome pathway (Fukazawa *et al*., 2001; Perez and White, 2003; Poukkula *et al*., 2005; Chandrasekaran *et al*., 2006; Liu *et al*., 2006), prompted us to examine whether proteasomal degradation of c-FLIP was enhanced upon hyperthermia. Indeed, pretreatment with the specific proteasome inhibitor epoxomicin inhibited the hyperthermia-induced downregulation of c-FLIP in Jurkat cells (Fig. 7A, III). As the half-life of c-FLIP$_S$ is short, the protein was markedly stabilized by epoxomicin treatment (Fig. 7A, III). The hyperthermia-induced downregulation of c-FLIP$_L$ was also inhibited by epoxomicin, although the effect were less obvious than that of c-FLIP$_S$ (Fig. 7A, III), due to the slower turnover of c-FLIP$_L$ (Fig. 6C, III). Ubiquitination assays further proved that the ubiquitination of both c-FLIP$_L$ and c-FLIP$_S$ is increased upon hyperthermia (Fig. 7B, III). Enhanced ubiquitination of endogenous c-FLIP could be seen in all hyperthermia-treated samples, as the NF6 antibody used for immunoprecipitation also recognizes endogenous c-FLIP (Fig. 7B, D, III).

We have previously shown that the rapid turnover of c-FLIP$_S$ is determined by its C-terminal region, since the mutated c-FLIP$_S$, lacking this part (c-FLIP$_S$Δ203-221), is unable to be ubiquitinated and degraded via the proteasome (Poukkula *et al*., 2005). To investigate the mechanism of the hyperthermia-induced ubiquitination of c-FLIP$_S$, we used the same c-FLIP$_S$Δ203-221 mutant. Hyperthermia did not affect the protein levels or ubiquitination of this c-FLIP$_S$ mutant, whereas hyperthermia induced both downregulation and ubiquitination of the exogenously expressed wild-type c-FLIP$_S$ (Fig. 7C, D, III). Taken together, these results indicate that the C-terminal splicing tail is a general determinant of c-FLIP$_S$ stability,

regulating also the hyperthermia-induced ubiquitination and degradation of c-FLIP$_S$. The reduction in hyperthermia-induced ubiquitination (Fig. 7D, III), the loss of hyperthermia-induced downregulation (Fig. 7C, III) as well as the resistance to hyperthermia-induced sensitization to CD95-mediated apoptosis of the ubiquitination-resistant c-FLIP$_S$ mutant (Fig. 7E, III), verified the involvement of the ubiquitin-proteasome pathway in the hyperthermia-induced downregulation of c-FLIP and sensitization to CD95-mediated to apoptosis. Mutation of the lysines 192 and 195, previously shown to be the primary targets for c-FLIP$_S$ ubiquitination during hemin-induced differentiation of K562 cells (Poukkula *et al*., 2005), did not decrease the hyperthermia-induced ubiquitination of c-FLIP$_S$ (unpublished data). Therefore, the hyperthermia-induced ubiquitination of c-FLIP$_S$ seems to targ*et al*so lysines other than 192 and 195, and accordingly, these lysines are not completely indispensable for c-FLIP$_S$ ubiquitination. Replacement of a target lysine with an arginine has been reported to transfer ubiquitination to another lysine also in other substrates. Even mutation of all lysines has been shown to be required to inhibit ubiquitination of some substrates, indicating that under certain circumstances any lysine may be able to function as a target for ubiquitination (reviewed by Pickart, 2001).

The hyperthermia-induced proteasomal degradation of c-FLIP implicates that the ubiquitin-proteasome pathway is an efficient route to dynamically regulate the c-FLIP levels to sensitize cells to death receptor ligation. c-FLIP$_L$ and c-FLIP$_S$ are similar until the beginning of the c-FLIP$_L$ caspase-like domain and the c-FLIP$_S$ splicing tail (Fig. VII). Since deletion of the splicing tail completely inhibits ubiquitination of c-FLIP$_S$ (Poukkula *et al*., 2005), the caspase-like domain has to regulate the ubiquitination of c-FLIP$_L$. Due to the longer caspase-like domain of c-FLIP$_L$, there are also more possibilities for regulation of c-FLIP$_L$ ubiquitination. Recently, Chang and co-workers showed that the ubiquitination of c-FLIP$_L$ is regulated by JNK1-mediated phosphorylation and activation of the ubiquitin ligase Itch. The interaction between Itch and c-FLIP$_L$ required the caspase-like domain of c-FLIP$_L$, explaining why Itch was not able to mediate ubiquitination of c-FLIP$_S$ (Chang *et al*., 2006). No ubiquitin ligase for c-FLIP$_S$ has yet been found. Hyperthermia is known to trigger several signaling pathways, and since the c-FLIP depletion upon hyperthermia treatment is very rapid, it will be important to examine whether c-FLIP$_S$ is regulated by similar kinds of signaling mechanisms as the one suggested for c-FLIP$_L$.

5.6. The hyperthermia-induced sensitization to CD95-mediated apoptosis is independent of Hsp70 and the heat shock response (II, III)

When cells are subjected to stressful conditions, such as elevated temperatures, the heat shock response is activated, leading to enhanced expression of Hsp70 (Lindquist, 1986; Morimoto, 1998). Such cells, referred to as thermotolerant, are less sensitive to several stimuli otherwise lethal for the cell (Mosser and Martin, 1992; Nollen *et al*., 1999; Mosser *et al*., 2000). The same protective effect is obtained by overexpression of Hsp70 (Mosser *et al*., 1997 and 2000; Buzzard *et al*., 1998; Nollen *et al*., 1999). Thermotolerant cells have also been shown to be protected from death receptor-mediated apoptosis (Jäättelä *et al*., 1989; Buzzard *et al*., 1998; Özören and El-Deiry, 2002). To examine the protective effect of Hsp70 on CD95-mediated apoptosis, we used a Jurkat-based cell line inducibly overexpressing an

Hsp70-GFP fusion protein upon induction with doxycycline (Fig. 3A, II). Both mitochondrial polarization and nuclear fragmentation were similar in the cells expressing increased levels of Hsp70-GFP and in GFP-negative cells (Fig. 3B, II). The effect of CD95 ligation on the level of apoptosis was also similar in cells expressing high levels of Hsp70 and cells without elevated Hsp70 expression (Fig. 3C, II). These results indicate that the overexpression of Hsp70 cannot protect Jurkat cells from CD95-mediated apoptosis. Another study also suggests that elevated Hsp70 expression does not affect CD95-mediated apoptosis in Jurkat cells (Creagh and Cotter, 1999).

Although death receptor signaling is capable of bypassing the protective effects of Hsp70 overexpression, we wanted to further examine whether thermotolerance plays a role in CD95-mediated apoptosis. To acquire thermotolerance, we induced hyperthermia in Jurkat cells and left the cells to recover. After twelve hours of recovery, no sensitizing effect of hyperthermia to CD95 ligation was detected (Fig. 8A, III). At this point, the c-FLIP expression levels were restored to normal, but the levels of Hsp70 were increased during the recovery from hyperthermia (Fig. 8B, III). To downregulate the c-FLIP levels in the thermotolerant cells, we exposed the thermotolerant cells to a second hyperthermia for 30 min. The second hyperthermia exposure downregulated the c-FLIP levels that had been restored during the recovery from the first treatment (Fig. 8B, III), and despite the high levels of Hsp70, the cells were again sensitized to CD95 ligation similarly to the cells treated with agonistic α-CD95 antibody immediately after the first hyperthermia induction (Fig. 8A, III). Based on these results, we conclude that the sensitizing effect of c-FLIP downregulation overrides the possible protective effect of thermotolerance.

We also studied the effect of CD95 ligation on the induction of the heat shock response. Our results clearly showed that CD95 ligation does not affect the DNA-binding capacity of HSF1 or the hyperthermia-induced expression of Hsp70 (Fig. 2A and B, II), suggesting that the hyperthermia-induced sensitization to CD95-mediated apoptosis is not a consequence of CD95-induced changes in the heat shock response.

An increased expression of c-FLIP has been reported to protect tumor cells from death receptor-mediated apoptosis (Djerbi *et al*., 1999; Medema *et al*., 1999; reviewed by Thome and Tschopp, 2001; Thomas *et al*., 2002; reviewed by Debatin and Krammer, 2004; reviewed by Schulze-Bergkamen and Krammer, 2004). Several tumor cells have also been shown to express high levels of the molecular chaperone Hsp70, which can protect them from stressful conditions such as insufficiency of oxygen and growth factors induced by the tumor state (Jäättelä *et al*., 1992; reviewed by Jäättelä, 1999; Calderwood *et al*., 2006). Interestingly, we show that also thermotolerant cells, with high Hsp70 expression levels, can be sensitized to death receptor-mediated apoptosis by hyperthermia-induced downregulation of c-FLIP. Thus, the elevated Hsp70 levels were not able to help the cells escaping the sensitizing effects of c-FLIP downregulation. It has been shown that treatment with the protein synthesis inhibitor CHX or the RNA synthesis inhibitor ActD (actinomycine D) abolishes the protective effect of hyperthermia on TNF-α-induced apoptosis (Jäättelä *et al*., 1989). Since c-FLIP is rapidly depleted upon protein synthesis inhibition, the lost protection to TNF-α-induced apoptosis might have been due to depletion of c-FLIP. Therefore, this study further supports our results,

according to which c-FLIP downregulation during hyperthermia is a stronger sensitizer to death receptor-mediated apoptosis than Hsp70 is a protector.

Hyperthermia has been used as a clinical approach to enhance the effects of different cancer therapies such as radiotherapy or chemotherapy (reviewed by Hobohm, 2001; Hildebrandt *et al*., 2002; van der Zee, 2002; Wust *et al*., 2002). We have shown that the hyperthermia-induced downregulation of c-FLIP is a powerful method to sensitize cells to death receptor-mediated apoptosis. Thus, the rapid depletion of the intracellular pool of c-FLIP induced upon hyperthermia treatment could be a mechanism underlying the observed sensitization of tumor cells to anti-cancer agents. Increased drug delivery and reoxygenation due to a hyperthermia-induced increase in perfusion may also contribute to the enhanced tumor cell death upon elevation of the body temperature (reviewed by Wust *et al*., 2002). Cancer cells have also been suggested to be less heat-resistant than normal cells, thus, the physical heat can damage cancer cells. Activated dendritic cells can subsequently induce T-lymphocyte responses against cancer antigens released from the cancer cells (reviewed by Hobohm, 2001).

5.7. Hyperthermia and apoptosis during fever and immune responses (III)

The persistence of lymphocyte populations in an organism is determined by the balance of death- and survival-promoting factors in these cells. At normal temperatures, this balance is primarily determined by growth factors and cytokines (reviewed by Krammer, 2000). In fever, however, stress and stress-specific cytokine signaling are important for the fate-determining balance (reviewed by Hammami *et al*., 1998; Hasday and Singh, 2000; Jiang *et al*., 2000; Hildebrandt *et al*., 2002; Ahlers *et al*., 2005; Kida *et al*., 2006). The regulation of lymphocyte survival and persistence is critical for immune responses during infections. Interestingly, several studies done on fever patients and individuals exposed to local or whole body hyperthermia treatment have shown a decrease in the T-lymphocyte number (Hammami *et al*., 1998; Hildebrandt *et al*., 2002; Ahlers *et al*., 2005; Kida *et al*., 2006). The elevation of the body temperature during fever has been proposed to have a major impact on immune responses during infections, as fever influences the clonal expansion and proliferation of lymphocytes and enhances the lymphocyte-mediated cytotoxicity in target cells (reviewed by Kluger, 1991; Roberts, 1991; Hasday and Singh, 2000).

A murine model system would be suitable to elucidate the *in vivo* effects of fever on T-lymphocyte homeostasis. However, the bioinformatics study shown in figures XII-XIV suggests that the c-FLIP isoform corresponding to human c-FLIP$_S$ is absent in rodent genomes. We have demonstrated that downregulation of particularly c-FLIP$_S$ is responsible for the hyperthermia-induced sensitization to CD95-mediated apoptosis, and that the c-FLIP$_S$ degradation is regulated by its isoform-specific splicing tail. Thus, it needs to be examined whether the ubiquitination and degradation of the murine short c-FLIP isoform, corresponding to the human c-FLIP$_R$, are regulated in a similar manner as the human c-FLIP$_S$ during fever. If the stability of all the human and murine short c-FLIP isoforms is regulated similarly, it would be interesting to investigate if fever induces downregulation of c-FLIP and sensitizes activated T-lymphocytes to AICD *in vivo* in mice.

The sensitivity towards apoptotic signaling in T-lymphocytes has been shown to be modulated by the intracellular c-FLIP levels (Peter *et al*., 1997; Kirchhoff *et al*., 2000; Schmitz *et al*., 2004). It is critical that excess activated T-lymphocytes are eliminated to avoid formation of unwanted autoreactive or allergy-promoting lymphocyte populations (Sharief, 2000; Semra *et al*., 2001; Catrina *et al*., 2002). Our results indicate that hyperthermia is an important determinant for appropriate apoptotic signaling in activated T-lymphocytes. Therefore, fever may constitute a clearly beneficial effect, relevant for elimination of unwanted lymphocytes (Fig. XIX). Related to this, a further subject for speculation is whether the declining incidence of infections and febrile illnesses in industrialized countries due to decreased infection rates could be linked to the steadily increasing prevalence of allergies and autoimmune diseases (Yazdanbakhsh *et al*., 2002; Williams *et al*., 2004; Bach, 2005), as a reflection of unspecific lymphocyte activation.

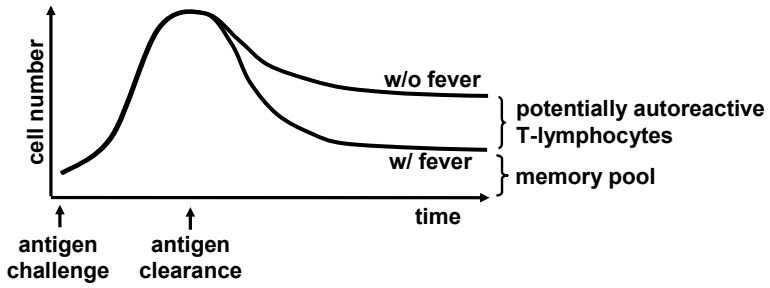

Figure XIX. Fever facilitates efficient deletion of excess activated T-lymphocytes. T-lymphocytes start to proliferate upon exposure to their specific antigen. After antigen clearance, the excess activated T-lymphocytes need to be removed from the body in order to avoid formation of an autoreactive T-lymphocyte population. Exposure to fever temperatures provides for efficient deletion of activated T-lymphocytes.

6. CONCLUSIONS

It is critical that excess activated T-lymphocytes are eliminated from the body to avoid persistence of autoreactive or allergy-promoting lymphocyte populations. Therefore, the sensitivity to CD95 signaling has to be carefully regulated in T-lymphocytes. The focus of this study was to determine the effects of hyperthermia on CD95-mediated apoptosis in T-lymphocytes, and the results revealed that fever-like hyperthermia sensitizes fully activated, yet not resting or recently activated primary human T-lymphocytes to CD95-mediated apoptosis. To investigate the molecular mechanisms behind the hyperthermia-induced sensitization we used a Jurkat T-lymphocyte cell line in addition to the primary human T-lymphocytes. Our results show that hyperthermia enhances the death receptor-mediated activation of both initiator and effector caspases. We were also able to identify the DISC as a target for the hyperthermia-induced effects. A modulator of death receptor signaling in the DISC is the caspase-8 inhibitor c-FLIP. We showed that hyperthermia corresponding to fever triggers downregulation of both c-FLIP$_L$ and c-FLIP$_S$, with consequent sensitization to apoptosis induced via CD95. Additionally, we demonstrated that the hyperthermia-mediated downregulation is due to increased ubiquitination and proteasomal degradation of c-FLIP$_S$.

Hyperthermia itself has been shown to activate MAPK signaling pathways as well as the heat shock response. In the light of our study, MAPK signaling was, however, not involved in the hyperthermia-induced sensitization to CD95-mediated apoptosis. Even the substantially elevated levels of Hsp70 failed to rescue thermotolerant cells from the hyperthermia-induced sensitization caused by c-FLIP downregulation. These findings demonstrate that apart from regulating death receptor signaling during normal conditions, c-FLIP acts as a highly dynamic determinant of death receptor signaling during stress.

Our results implicate that fever plays a major role in regulating the termination of T-lymphocyte populations and that hyperthermia may be critical for the removal of unwanted T-lymphocytes that could become autoreactive or allergy-promoting. Therefore, it will be interesting to investigate *in vivo* how fever conditions affect the elimination of activated T-lymphocytes, and to study whether fever is able to decrease the population of autoreactive T-lymphocytes in autoimmune disease or allergy animal models. Our study may explain the molecular mechanism behind the clinically observed reduced number of T-lymphocytes during fever and in hyperthermia-treated patients. However, the link between fever and lymphocyte elimination warrants further studies on the hyperthermia-mediated effects on c-FLIP levels in T-lymphocytes under normal and pathological conditions.

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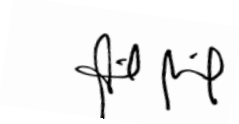

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