The role of RIP-1 and cIAPs in apoptotic and non-apoptotic signalling via TLR3 and death receptors

Dissertation

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Summary

Skin cancers, like squamous cell carcinoma (SCC), which belongs to the most frequent tumors world wide, require novel strategies for treatment, since most malignant cancer cells resist current anti-tumour agents. In the recent years cytokines, such as TRAIL or CD95L, that activate apoptotic death receptor signalling pathways as well as synthetic compounds like poly (I:C) that may activate Toll-like receptor-3 (TLR3)-mediated cell death signalling pathways are discussed as potential targets for anti-tumour therapy. Overcoming apoptosis resistance of tumor cells is of paramount importance for the development of novel therapeutic strategies for specific elimination of cancer cells. However, many cancer cells resist apoptotic stimuli by up-regulation of anti-apoptotic proteins such as cFLIP, IAPs and Bcl-2. cIAPs were postulated as crucial inhibitors of TLR3- as well as CD95-induced apoptosis but the mechanisms as well as proof of this hypothesis are lacking to date. The goal of this thesis was to contribute to the understanding of the functional relevance of IAPs for the regulation of TLR3- and DR-mediated cell death and non-apoptotic signalling pathways in primary keratinocytes and SCC cells.

This work has demonstrated that cIAPs protect HaCaT keratinocytes from poly (I:C)- and CD95L-induced cell death by inhibition of both caspase and RIP-1-kinase activities. Interestingly, cIAPs inhibit the formation of an intracellular RIP-1 signalling complex, which consists of caspase-8, RIP-1 cFLIP, and FADD. This RIP-1 complex is necessary, but insufficient for induction of cell death. Upon TLR3 stimulation in the absence of cIAPs thus RIP-1 complex is binding to TRIF, and promotes enhanced apoptosis. However, inhibition of caspase-8 activity in this complex by chemical inhibitors (such as zVAD-fmk or QVD) or by overexpression of $cFLIP_S$ (a prominent caspase-8 interacting molecule and inhibitor) unmasks a necrotic form of cell death. In this pro-necrotic complex substantial accumulation of RIP-1 was observed, although this necrotic cell death was fully blocked by inhibition of RIP-1 kinase activity. Loss of RIP-1 in this complex mediated by specific shRNA protects cells from poly (I:C)/IAP antagonist and CD95L/IAP antagonist induced cell death.

However, cells expressing high levels of $cFLIP_L$ are resistant to poly (I:C) and CD95L-induced apoptosis in the absence of cIAPs. Interestingly the long isoform $cFLIP_L$, but not the short isoform $cFLIP_S$ conferred protection from IAP antagonist induced RIP-1-kinase-dependent necrotic cell death. Furthermore, degradation of cIAPs, induced by TWEAK signalling, duplicates findings with the IAP antagonist and shows the physiological relevance of cIAPs for the regulation of RIP-1 dependent cell death signalling pathways.

Studies of primary keratinocytes showed equal levels of sensitivity to TLR3-induced apoptosis of primary and immortalised keratinocytes (HaCaT). The IAPs protect these cells from TLR3-induced cell death by inhibition of both caspase and RIP-1-kinase activities. Intriguingly, the downregulation of RIP-1 in primary keratinocytes conferred no protection, but switches TLR3-induced cell death from apoptotic to necrotic cell death. Further inhibition of caspase activities by zVAD-fmk increases non-apoptotic cell death in RIP-1 knockdown primary keratinocytes and this cell death can only partially blocked by Necrostatin-1. Therefore poly (I:C) treated keratinocytes die in the absence of cIAPs in a caspase- and RIP-1-independent cell death and this cell death may be regulated by currently unknown factors.

TLR3 and DRs can also activate pro-survival NF-κB and MAPK dependent signalling pathways that promote resistance to cell death stimuli and therefore induce carcinogenesis. In this study it was also found that IAPs block spontaneous activation of both canonical and non-canonical NF-κB activation pathways in primary and SCC cell lines. TRAIL-induced signalling suppresses the spontaneous non-canonical NF-κB activation, induced by the absence of IAPs in primary and SCC keratinocytes. As well, TRAIL-induced canonical NF-κB activation was downregulated in the absence of IAPs. In contrast to NF-κB pathways, the MAPKs were not auto-activated upon IAP depletion, although TRAIL-induced p38 activation in the absence of IAPs was increased in cancer cells, unlike HaCaT and primary keratinocytes. TRAIL-induced JNK activation is not influenced by IAPs.

Taken together, the results of this study implicate cIAPs as crucial negative regulators of TLR3 and CD95 cell death signalling by modulation of RIP-1. Moreover, IAPs are critical inhibitors of both canonical and non-canonical NF-κB signalling. Furthermore, TWEAK signalling may modulate RIP-1-dependent necrosis, induced by different stimuli. Simultaneous treatment with IAP antagonists and pro-apoptotic stimuli, like TLR3- or DR-agonists, could thus be an interesting strategy for future anticancer therapy and may impact also anti-tumour immune response.

Zusammenfassung

Hautkrebs, wie das Plattenepithelkarzinom (SCC), gehört zu den weltweit am meist verbreiteten Tumorerkrankungen der Haut. Die Entwicklung von neuen Strategien zur Behandlung dieses Hautkrebses ist notwendig, da eine Vielzahl von Krebszellen Resistenzen gegenüber bekannten Anti-Tumor-Therapien zeigen. In den vergangenen Jahren wurden Zytokine, wie TRAIL und CD95L, die apoptotische Signalwege durch Aktivierung von Todesrezeptoren (TR) als auch synthetische Komponenten, wie Poly (I:C), die Toll-like-Rezeptor-3 (TLR3)-vermittelte Zelltodsignalwege initiieren können, als potentielle Zielstrukturen für Antitumortherapien diskutiert. Die Überwindung von Apoptoseresistenz von Tumorzellen ist dabei von höchster Bedeutung für die Entwicklung neuer therapeutischer Strategien zur spezifischen Eliminierung von Krebszellen. Viele Tumorzellen zeigen Resistenzen gegenüber apoptotischen Stimuli durch Aufregulierung zentraler anti-apoptotischer Proteine, wie cFLIP, IAPs und Bcl-2 Proteinen. cIAPs wurden als kritische Inhibitoren für die TLR3- und CD95-induzierte Apoptose postuliert, wobei sowohl diese Resistenzmechanismen bisher nicht genau bekannt sind, als auch die detaillierte Überprüfung dieser Hypothese zum jetzigen Zeitpunkt noch aussteht. Das Ziel dieser Arbeit bestand in der Identifizierung der funktionellen Relevanz von IAPs für die Regulierung des TLR3- und CD95-vermittelten Zelltods sowie der Bedeutung der cIAPs für die nicht-apoptotische Signalgebung in primären Keratinozyten und SCC Zellen.

In dieser Arbeit konnte demonstriert werden, dass cIAPs HaCaT Keratinozyten gegenüber Poly (I:C)- und CD95L-induzierte Zelltodinduktion durch Inhibition von Caspase- und RIP-1-Kinase-Aktivitäten schützen. Interessanterweise inhibieren cIAPs die Formierung eines intrazellulären RIP-1 Signalkomplexes, der Caspase-8, RIP-1, cFLIP und FADD Moleküle beinhaltet. Dieser RIP-1 Komplex ist nicht in der Lage Zelltod zu induzieren. Nach Stimulation des TLR3 in der Abwesenheit der cIAPs kommt es zur Bindung des RIP-1 Komplexes an TRIF, wodurch Apoptose gefördert und verstärkt wird. Die Inhibition der Caspase-Aktivität in diesem Komplex durch chemische Caspaseinhibitoren, wie zVAD-fmk oder QVD, oder durch Überexpression von $cFLIP_S$ (ein bekannter Caspase-8-Interaktionspartner und -Inhibitor) führte zum Nachweis eines nekrotischen Zelltods. In diesem Nekrose-fördernden Komplex wurde eine substantielle Akkumulation von RIP-1 beobachtet. Durch die Inhibition der RIP-1-Kinase Aktivität durch Necrostatin-1 in diesem Komplex kann die Poly (I:C)/IAP Antagonist geförderte Nekrose gehemmt werden. Auch der Verlust von RIP-1 in diesem Komplex, beispielsweise vermittelt durch spezifische shRNA, schützt die Zellen gegenüber Poly (I:C)/IAP Antagonist und gegenüber CD95L/IAP Antagonist induzierten Zelltods. Des Weiteren konnte gezeigt werden, dass die Expression von $cFLIP_L$ mit Resistenz gegenüber Poly (I:C)- und CD95L-induzierter Apoptose auch in Abwesenheit von cIAPs einhergeht. Interessanterweise schützt $cFLIP_L$, jedoch nicht $cFLIP_S$

vor dem IAP Antagonisten-induzierten RIP-1-Kinase-abhängigen nekrotischen Zelltod. Die physiologische Bedeutung der cIAPs für die Regulation von RIP-1-abhängigen Zelltodsignalwegen wird durch Experimente mit TWEAK deutlich. TWEAK bewirkt die Degradation von cIAPs und diese Experimente konnten die gefundenen Resultate mit dem IAP Antagonisten bezüglich der Mechanismen der Zelltodinduktion bestätigen.

Studien mit primären Keratinozyten konnten eine ähnliche Empfindlichkeit gegenüber TLR3-induzierte Apoptose von primären und immortalisierten Keratinozyten (HaCaT) zeigen. Auch hier schützen IAPs die Zellen gegenüber TLR3-induziertem Zelltod durch Inhibition von Caspase- und RIP-1-Kinase-Aktivitäten. Im Gegensatz zu HaCaT-Zellen führt eine Verminderung der RIP-1 Expression in primären Keratinozyten nicht zu einer Protektion des IAP Antagonist/Poly(I:C)-vermittelten Zelltods, sondern zu einem Wechsel von apoptotischem zum nekrotischem Zelltod. Ein weiterer Unterschied in der Todessignalgebung in primären Keratinozyten besteht darin, dass die Inhibition von Caspaseaktivitäten (mittels zVAD-fmk) in RIP-1-knockdown Zellen zu einer verstärkten Induktion des nicht-apoptotischen Zelltods führt, der aber nur teilweise durch Necrostatin-1 inhibiert werden kann. Demnach sterben Poly(I:C)-behandelte Keratinozyten in der Abwesenheit von cIAPs durch einen Caspase- und RIP-1-unabhängigen Zelltod und dieser Zelltod wird wahrscheinlich durch bisher noch nicht bekannte Faktoren reguliert.

TLR3 und TRs können auch überlebensfördernde NF-κB- und MAPK-abhängige Signalwege aktivieren, die Resistenzen gegenüber Zelltodstimuli und damit die Karzinogenese von Hautzellen fördern. In dieser Studie konnte weiterhin gezeigt werden, dass IAPs die spontane Aktivierung der kanonischen und nicht-kanonischen NF-κB Aktivierung in primären Keratinozyten und in SCC-Zellen hemmen. Des Weiteren wird die spontane nicht-kanonische NF-κB-Aktivierung der TRAIL-vermittelten Signalkaskade in der Abwesenheit von cIAPs in primären Keratinozyten und SCC Zellen unterdrückt. Die TRAIL-induzierte kanonische NF-κB-Aktivierung wird ebenfalls in der Abwesenheit von cIAPs vermindert. Im Gegensatz zum NF-κB Signalweg, werden die MAPK nach IAP Depletion nicht autoaktiviert. Eine Steigerung der p38 Aktivierung in der Abwesenheit der IAPs und nach TRAIL Stimulation wurde in MET1 und A5RT3 SCC Zellen, jedoch nicht in HaCaT Zellen und primären Keratinozyten beobachtet. Im Gegensatz dazu wird die TRAIL-induzierte JNK Aktivierung nicht von IAPs beeinflusst.

Zusammenfassend zeigen die Resultate dieser Studie zeigen somit, dass cIAPs durch Modulation der Funktion von RIP-1 TLR3- und CD95-Zelltodsignalwege negativ regulieren. Die cIAPs sind aber auch bedeutende Inhibitoren kanonischer und nicht-kanonischer NF-κB Signalgebung. Des Weiteren kann die kombinierte Signalgebung von TWEAK und anderen Stimuli die RIP-1-abhängige Nekrose modulieren. Die simultane Behandlung mit IAP Antagonisten und pro-apoptotischen Stimuli, wie zum Beispiel TLR3- oder TR-Agonisten,

könnte eine Strategie für Antitumortherapien darstellen, die auch für die antitumorale Immunantwort bedeutsam sein könnte.

Contents

Figures and Tables

1. Introduction

1.1.1 The role and function of the skin

The skin is the largest organ of the human body, which possesses multiple functions. The epidermis suffers direct, frequent, and damaging encounters with the external world than any other tissue of the body. Therefore, the skin plays the most important role in protecting against pathogens, as well as allows for temperature regulation, sensation and vitamin D and B synthesis (Schauber and Gallo, 2008). Skin cells are constantly renewed by their respective progenitor cells and keratinocytes are the major cell type of the epidermis (Lippens et al., 2009). Homeostasis in the skin is achieved when the rate of cell proliferation of the tissue is balanced by cell death. An insensitivity to apoptotic stimuli or an uncontrolled cell proliferation in the epidermis can result in skin tumour formation (Fesik, 2005), whereby massive keratinocyte apoptosis can result in Toxic epidermal necrolysis (TEN, Lyell's syndrome) (Trent et al., 2006) or Stevens Johnson syndrome (SJS). Both syndromes are rare acute dermatological diseases defined by epidermal cell death and mucosal erosions with extensive loss of contact between epidermis and dermis (LYELL, 1956). Further complicating the picture, keratinocytes also undergo a specialized form of programmed cell death, called cornification, which is different from classical apoptosis (Lippens et al., 2009).

1.1.2. Skin cancer

The three most common types of skin cancers are basal cell carcinoma (BCC), squamous cell carcinoma (SCC) and malignant melanoma.

BCC and SCC are the most frequent tumours of mankind and their number is still increasing worldwide (Boukamp, 2005). Often patients suffer from a second and third lesion and the severity of these tumours is linked to their number. SCCs in transplant recipients also appear to be more aggressive. They tend to grow rapidly, show a higher rate of local recurrences and metastasize in 5–8% of these patients (Euvrard et al., 2003). This largely differs from BCCs which are more frequent in the general population—at a ratio of 4:1 as compared with SCCs—but the number is only increased by a factor of 10 in transplant recipients (Boukamp, 2005).

Most skin SCCs are treated surgically, if surgery is not possible, radiotherapy may be used as a treatment. Other treatments used include curettage and cautery or cryotherapy. Sometimes combinations of treatment are used for more aggressive skin SCC that has a high risk of recurring and spreading. Occasionally SCC of the skin returns, even after apparently successful treatment and may spread to other parts of the body (Lansbury et al., 2010).

1.2. Cell death

Deregulation of apoptosis or other cell death forms in keratinocytes can trigger skin carcinogenesis.

1.2.1. Cornification in the skin

Cornification, is a slow coordinated process that takes about 2 weeks, occurs simultaneously in the different suprabasal layers of the epidermis (Figure 1) and requires the consecutive expression of typical differentiation-associated proteins (Lippens et al., 2009). A keratin filament network is formed and at the transition from the granular to cornified layer, other structural proteins are crosslinked to this protein network. The keratinocytes become

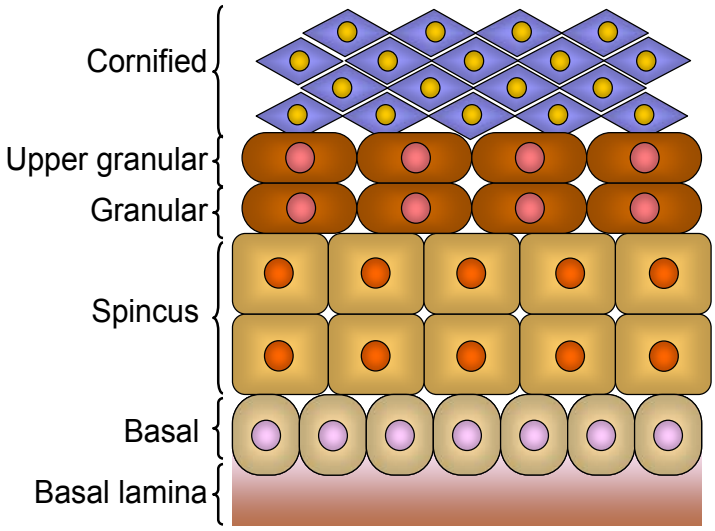


Figure 1. The scheme of epidermis layers. Apoptosis is restricted to the basal layer, whereas cornification occurs in the supra-basal layers.

metabolically inactive, the organelles are degraded and the resulting fully differentiated squames are flattened dead cells. During assembly of the epidermal cornified cell envelope, the plasma membrane fuses with the membranes of the lamellar bodies. The latter contain lipids that eventually replace the original plasma membrane and play a role in water maintenance. Ultimately, corneocytes are sloughed into the environment. In contrast to apoptotic cells, corneocytes are not phagocytozed by other cells (Lippens et al., 2005).

1.2.2. Caspase-independent cell death (CICD): Necrosis and Autophagy

Caspase-independent cell death (CICD) is a type of cell death that ensues when a signal that normally induces apoptosis fails to activate caspases. Even so, CICD often shares common characteristics with apoptotic cell death (Tait and Green, 2008) such as upstream signalling pathways that are critical for both forms of death like mitochondrial outer membrane permeabilization. Interestingly, caspase-dependent events such as phosphatidylserine

externalization and wide-scale chromatin condensation are absent during CICD. Cells undergoing CICD often display large-scale cytoplasmic vacuolization, autophagosome accumulation and peripheral nuclear condensation (Ekert et al., 2004). Furthermore, cells undergoing CICD may display very different characteristics depending on various factors like initial stimulus and cell type (Tait and Green, 2008).

Necrosis is one type of CICD. Necrotic cell death can be induced by injury, infection, inflammation and toxins. Necrosis is caused by special enzymes that are released by lysosomes, which are capable of digesting cellular components or the entire cell. Since the disorderly death generally does not send early cell signals it is more complicated for the immune system to locate and recycle dead cells that have died through necrosis. Consequently, the release of intracellular content after cellular membrane damage is one potential cause of inflammation in necrosis (Festjens et al., 2006b). Several histological features convey the gradual, irreversible transformation into necrosis from sub-lethal damage. Sequential structural changes like an eosinophilic cytoplasm, fragmentation of the nucleus, lysis of the nucleus can be observed.

Another type of CICD is autophagy. It is a process of isolation of cytoplasmic components into double membrane vesicles, followed by degradation upon fusion with lysosomal compartments (Reggiori, 2006). Depending upon the stimulus, autophagy can degrade cytoplasmic contents nonspecifically, or target the degradation of specific cellular components (Kundu and Thompson, 2008). In mammals, autophagy has been implicated in either the pathogenesis or response to a wide variety of diseases, including neurodegenerative disease, chronic bacterial and viral infections, atherosclerosis, and cancer, although the term “autophagic cell death” is controversial (Gozuacik and Kimchi, 2007).

1.2.3. Apoptosis

Apoptosis is highly important for the maintenance of tissue homeostasis and anti-cancer protection. In contrast to cornification and CICD, apoptosis is a fast process and occurs in the basal layer of the skin (Figure 1). During the course of apoptosis, the function of organelles ceases, but the organelles themselves are not degraded. The plasma membrane remains intact preventing leakage of the cellular content into the environment. Membrane blebbing results in formation of separate apoptotic bodies that are finally recognized and phagocytozed by macrophages or neighbouring cells (Kerr et al., 1972). A cell undergoing apoptosis also shows a characteristic biochemical changes e.g. like activation of caspases that relate to a family of cysteine proteases, required for the execution of the apoptotic process. Apoptosis can occur in case of cell damage or virus infection (Sinkovics, 1991) response to stress or DNA damage (such as exposure to UV or ionizing radiation) (Hanawalt,

1996) and is of paramount importance for development and immune cell regulation. There are two distinct ways of apoptosis activation: intrinsic or mitochondrial, and extrinsic, which is initiated via death receptors.

1.2.3.1. The intrinsic apoptotic signalling pathway

The intrinsic (mitochondrial) pathway of apoptosis is initiated by various factors, like growth factor withdrawal or DNA damage. The molecules that integrate the signalling of these different cell death stimuli converge to the level of the release of mitochondrial factors like cytochrome c, which leads to formation of the apoptosome (protein complex, consisting of caspase-9, AIF and cytochrome c) and activation of procaspase-9. Apoptosome formation leads to activation of the executioner caspases (like caspase-3), that targets and inhibits other downstream pro-survival molecules, and finally leads to chromatin condensation, and DNA fragmentation, ultimately to cell death (Beere, 2005).

The key players in the intrinsic pathway are the Bcl-2 family of proteins that are critical death regulators residing immediately upstream of mitochondria. The Bcl-2 family consists of both anti- and pro-apoptotic members. Detection of developmental death signals or intracellular damage is controlled by BH3-only members of the Bcl-2 family such as BID, BAD, NOXA, PUMA, BIM, and BMF, that act upstream in the pathway (Festjens et al., 2004; Sasi et al., 2009). Pro-apoptotic members of Bcl-2 family proteins such as Bax and Bak act downstream in the signalling pathway, in mitochondrial disruption (Cory et al., 2003; Sasi et al., 2009). Other members of Bcl-2 family like Bcl-2 or Bcl-X, are called anti-apoptotic. One of the critical functions of Bcl-2/Bcl-XL proteins is to maintain the integrity of the mitochondrial outer membrane, endoplasmatic reticulum and nucleus. These Bcl family protein may function as oncogenes not only by blocking apoptosis but also by blocking autophagy (Pattingre and Levine, 2006). The balance between anti-apoptotic and pro-apoptotic Bcl-2 family proteins is crucial for decision of the cell fate.

1.2.3.2. The extrinsic apoptotic signalling pathway

The extrinsic pathway is characterized by the ligation of cell surface death receptors via specific death ligands to generate catalytically active initiator caspase-8 or/and caspase-10 which depends on death receptor triggering. The extrinsic apoptotic pathway is also tightly linked to the mitochondrial pathway, which serves to amplify the apoptotic process. Bcl-2 family member Bid is cleaved by caspase-8 in response to death receptor mediated signalling and its cleavage product associates with mitochondria, which leads to cytochrome c release and apoptosis (Makin et al., 2001). As described for the intrinsic pathway, the extrinsic pathway also lastly results in activation of executioner caspases (like caspase-3) and cleavage of their target substrates to induce apoptosis (Beere, 2005).

1.2.3.4. Initiation of apoptosis by death receptors

The extrinsic apoptotic pathway is initiated by ligation of death receptors (TNF-R1/2, CD95, TRAIL-R1/2) and their respective ligands - TNF, CD95L, and TNF-related apoptosis-inducing

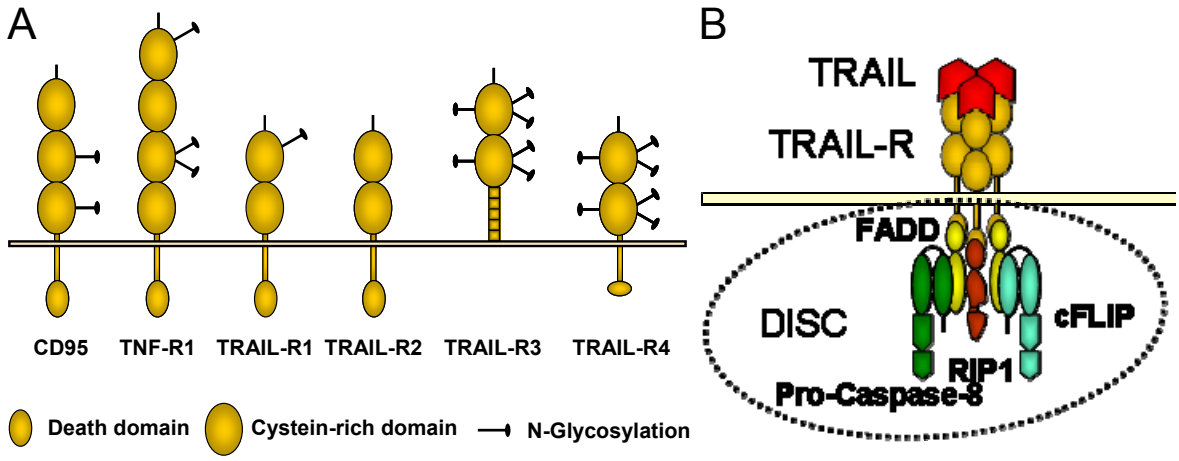


Figure 2. Death receptors and their signalling complex. A. Domain structure of the members of TNF superfamily. Most of the represented receptors have intracellular death domain, except TRAILR3, which lacks and TRAILR4 which has truncated death domain. **B.** Proteins associated with the TRAIL DISC. DISC consists of adaptor molecules (FADD), pro-apoptotic molecules (pro-caspase-8), anti-apoptotic molecules (cFLIP) and molecules with various functions (RIP-1)

ligand (TRAIL), which are members of the TNF superfamily (Wachter et al., 2004) (Figure 2 A). The TRAIL system, consisting of the ligand, two apoptosis promoting receptors (TRAIL-R1/2) and two decoy receptors, that are not able to induce apoptosis (TRAIL-R3 and 4), has attracted attention for its ability to preferentially kill tumour cells but not normal cells (Leverkus et al., 2000; Walczak et al., 1999). Binding of TRAIL to its receptors TRAIL receptor 1 (TRAIL-R1) and TRAIL-R2 on the cell surface leads to recruitment of adaptor proteins (e.g. FADD for TRAIL DISC) as well as the initiator caspase-8 and -10 to the death-inducing signalling complex (DISC) (Wehrli et al., 2000) (Figure 2 B). This subsequently results in activation of caspase-8 in these membrane-bound receptor complexes and finally leads to effector caspase activation (e.g. caspase-3) and ultimately apoptosis (Figure 3). There are several hypotheses about the functions of TRAIL-R3 and TRAIL-R4 “decoy” receptors (Figure 2 A). The current hypothesis are that these receptors either compete with the normal receptors for the ligand, or form mixed complexes of both decoy and normal receptors, therefore blocking apoptotic signal. In addition TRAIL-R4 is known to initiate antiapoptotic signalling via NF-κB (Kimberley and Screaton, 2004). In summary either way the signalling initiated by these receptors inhibits apoptosis.

Importantly, death receptors can also induce non-apoptotic signals, such as necrosis, inflammation, or proliferation via activation of MAPK or transcription factors (like AP-1 or NF-

κB) (Wachter et al., 2004) which may antagonise apoptotic pathways, induce inflammatory signals, or both (Figure 3) (Diessenbacher et al., 2008).

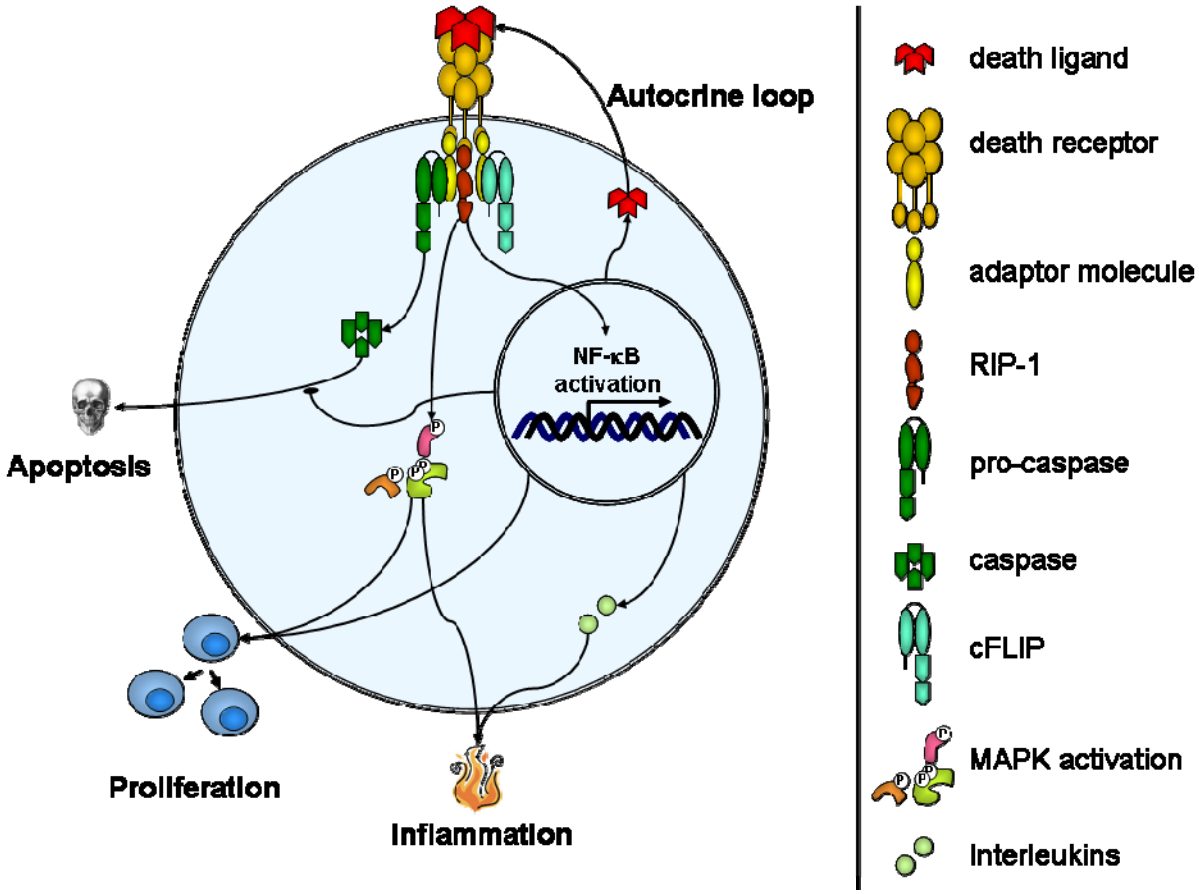


Figure 3. Death receptor signalling. Schematic picture of death receptor signalling pathways.

1.2.4. Inhibition of Apoptosis

Cell survival requires the active inhibition of apoptosis. This inhibition can be achieved by blocking the expression of pro-apoptotic factors, or by promoting the expression of antiapoptotic factors. These antiapoptotic factors can either directly block caspase activation like cFLIP or XIAP, or by blocking the release of pro-apoptotic factors and apoptosome formation, like antiapoptotic members of the Bcl-2 family such as Bcl-2 or Bcl-X.

1.2.4.1. cFLIP – inhibitor of caspases

The cellular FLICE-inhibitory protein (cFLIP) has 11 known isoforms, but only two are commonly detected in human cells: a long form ($cFLIP_L$) and a short form ($cFLIP_S$) (Figure 4). $cFLIP_L$, a 55 kDa protein, contains two DEDs and a caspase-like domain which however lacks some active site residues. Thus, $FLIP_L$ resembles caspase-8 in structure. In contrast, $cFLIP_S$, a 26 kDa protein, consists of only two DEDs and has high homology to the protein domains of viral FLIP isoforms (Scaffidi et al., 1999; Thurau et al., 2009).

Both isoforms of cFLIP form heterodimers with procaspase-8 and prevent its activation by blocking caspase cleavage in the DISC. Intriguingly, the mechanism of action differs for distinct cFLIP isoforms: while $cFLIP_S$ blocks caspase-8 cleavage by preventing the initial

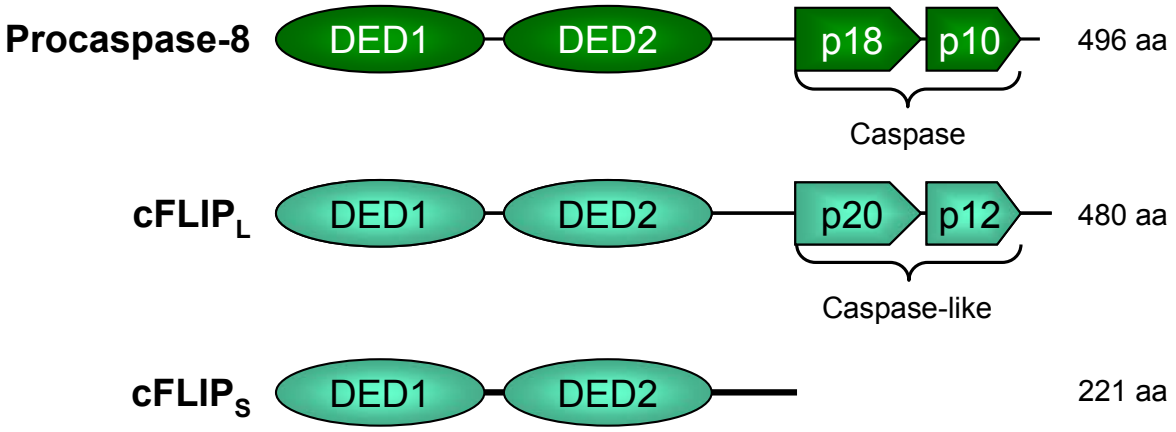


Figure 4. Domain structure of caspase8 and cFLIP isoforms. DED – death effector domain.

cleavage step of pro-caspase-8, $cFLIP_L$ allows this initial processing to a p43 fragment but inhibits the final cleavage step, thereby preventing the release of active caspase-8 into the cytosol and therefore blocks apoptosis (Geserick et al., 2008; Krueger et al., 2001). High expression of cFLIP is an important factor of melanoma resistance to pro-apoptotic stimuli (Geserick et al., 2008).

1.2.4.2. Inhibitor of apoptosis proteins (IAPs)

The IAP proteins are another family of caspase inhibitors. IAPs inhibit caspases by promoting the degradation of active caspases, or by sequestering the caspases away from their substrates (Tenev et al., 2005). There are eight members of IAP family found in humans: XIAP, cIAP1, cIAP2, MLIAP, ILP2, NAIP, BRUCE and Survivin (LaCasse et al., 2008)

IAP family members are characterized by the BIR (baculovirus inverted repeats) domain (Crook et al., 1993), which consist of approximately 70 amino acids containing the characteristic sequence CX2CX16HX6C. BIR has both hydrophobic and hydrophilic residues on its surface and therefore is theoretically capable of supporting protein-protein interactions. There are three subtypes of BIR domain, BIR1, BIR2, and BIR3, classified by their evolutionary relationship in phylogenesis (Wei et al., 2008). The protein domain structures of cIAP1 and 2 as well as XIAP, members of IAP family, are shown in Figure 5.

Some IAPs have RING (really interesting new gene) finger domain (C3HC4) at the C-terminus. It contains one zinc atom chelated to three cysteines and one histidine and another zinc atom bound to four cysteines. Some IAP family members also contain other structures, such as the caspase activation recruitment domain (CARD) found in cIAP1 and 2 (Wei et al.,

2008). The IAP protein sequences are highly conserved, some of baculoviral IAPs can even suppress apoptosis in mammalian cells (Huang et al., 2000).

Recently one group has described a novel ubiquitin-binding domain of IAPs (Figure 5) (Gyrd-Hansen et al., 2008), that was later conformed by Blankenship et al. (Blankenship et al., 2009) The UBA (ubiquitin-associated) domain of IAPs is located between the BIR domains and the CARD or the RING domain of cIAP1 and cIAP2, or XIAP, respectively. The cIAP1 UBA domain binds mono-ubiquitin and Lys48- and Lys63-linked polyubiquitin chains with low-micromolar affinities. The results suggested that ubiquitin binding may be an important mechanism for rapid turnover of auto-ubiquitinated cIAP1 and cIAP2 (Blankenship et al., 2009; Gyrd-Hansen et al., 2008).

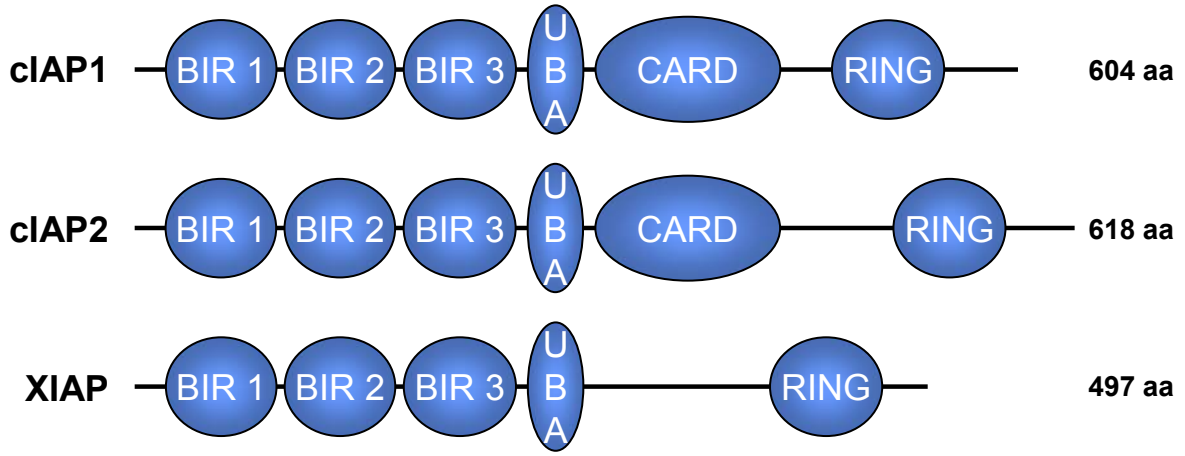


Figure 5. Domain structure of IAP family proteins. All three proteins have three BIR domains, UBA and RING. XIAP lacks CARD.

IAPs act as endogenous regulators of caspases (Fan et al., 2005). Multiple BIR domains, even in the same IAP can use different functions to inhibit different caspases, therefore regulating caspase functions. XIAP is generally considered as the most potent endogenous caspase inhibitor (Wei et al., 2008). Interestingly, the larger body of research seems to prove that only a few IAP proteins, like DIAP1 in Drosophila and XIAP in mammals, possess the ability to inhibit caspase (Wilkinson et al., 2004). Eckelman and Salvesen suggested that cIAP1 and cIAP2, which can also bind caspases 3, 7, and 9 but with lesser affinity as XIAP, lost or never acquired the caspase inhibitory ability. Thus, cIAP1 and cIAP2 could only execute their binding function to caspase, rather than caspase inhibition (Eckelman and Salvesen, 2006).

The IAP family members are differentially overexpressed in many malignant tissues and not in their healthy counterparts, e.g. XIAP is highly expressed in melanomas (Nachmias et al., 2004). IAP family members are crucial in keeping the tumor cells alive and causing resistance to high doses of chemotherapy.

1.2.4.2.1. IAP and the ubiquitination process

IAPs can also inhibit the activity of caspases by modulation of the ubiquitination process. Ubiquitination is a post-translational protein modification procedure that plays important role in apoptosis and signal transduction. By operating the processes of ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2), and ubiquitin protein ligase (E3), target proteins are attached by ubiquitins. They are in turn recognized and degraded by proteasomes or represent modules able to modify binding or signalling properties (Broemer and Meier, 2009). The C-terminus RING domain of IAPs has been identified as the essential motif for the activity of ubiquitin ligase (E3) that is sufficient to cause ubiquitination and subsequent proteasome-mediated proteolysis (Vaux and Silke, 2005a). The RING domain in mammalian IAPs, including cIAP1, cIAP2, XIAP possesses E3 ligase activities (Vaux and Silke, 2005b), as well XIAP has been reported to ubiquitinate caspase-9 (Morizane et al., 2005).

Certain IAPs can negatively regulate their own activity by autoubiquitination. This process can functionally inactivate IAPs and allow the cell to undergo apoptosis. In cIAP2, either full-length protein or its RING domain alone could execute E3 ligase activity *in vitro* to promote autoubiquitination, as well as monoubiquitination of caspase-3 and caspase-7. The C-terminal RING domain of cIAP1 is required for binding to XIAP and promoting XIAP degradation in several cells (Silke et al., 2005). cIAP1 can heterodimerize with XIAP through a RING-RING interaction to regulate the endogenous XIAP abundance. These two mechanisms (ubiquitination and autoubiquitination) seem to work in counteraction to keep a fine balance, to regulate cell death and survival. Moreover IAPs appear to enhance the degradation of themselves or their targets in an unclear regulatory mechanism (Wei et al., 2008).

1.2.4.2.2. Regulation of IAP's function

IAPs, which are at the centre of virtually all apoptotic pathways, are also strictly regulated through feedback mechanisms. There are several kinds of endogenous pro-apoptotic proteins that function on almost every kind of IAP (Wei et al., 2008). Recently, neutralization between IAP and IAP antagonists has become a broadly discussed issue for the treatment of cancers.

These endogenous IAP binding proteins were first identified in Drosophila. They were defined as critical inhibitors of IAP activity. Later, mammalian counterparts of the IAP antagonists were identified, named second mitochondrial activator of caspases/direct IAP binding protein with low pI (Smac/DIABLO) (Du et al., 2000) and high-temperature-regulated A2/Omi (HtrA2/Omi) (Suzuki et al., 2001). These proteins are mitochondrial-derived proteins. X-linked IAP-associated factor 1 (XAF1) (Liston et al., 2001) is a nuclear protein that

operates as inactivator of XIAP. The IAP binding proteins in Drosophila and mammal all have a highly conserved homologous sequence, named the IAP-binding motif domain, at the N-terminus that can bind IAP BIR domains and compete for interaction with IAPs, thus displacing the bound caspases that are then free to amplify the caspase cascade continuously.

1.2.4.2.3 TWEAK-FN14 signalling regulates cIAPs level

The other way to alter the cellular levels of cIAP1 and 2 is the signalling via TNF-like weak inducer of apoptosis (TWEAK)-FN14 interaction. TWEAK is a cytokine of the TNF superfamily that activates the FN14 receptor (Chicheportiche et al., 1997). TWEAK is known to regulate cell proliferation, cell death, cell differentiation, angiogenesis and inflammation (Ortiz et al., 2009). The expression of TWEAK and FN14 is increased during vascular and renal injury (Sanz et al., 2008). Inflammatory cytokines increase FN14 receptor expression in tubular and vascular smooth muscle cells (Ortiz et al., 2009).

Unlike IAP antagonists that cause rapid proteasomal degradation of cIAP1 (Bertrand et al., 2008; Gaither et al., 2007; Petersen et al., 2007; Varfolomeev et al., 2007; Vince et al., 2007), signalling by FN14 promotes the lysosomal degradation of cIAP1–TRAF2 in a cIAP1-dependent manner (Vince et al., 2008). TWEAK-induced loss of the cIAP1–TRAF2 complex sensitizes immortalized and minimally passaged tumour cells to TNF-induced death, whereas primary cells remain resistant. Lysosomal degradation of cIAP1–TRAF2 by TWEAK/FN14 therefore critically alters the balance of life/death signals arising from TNF-R1 in immortalized cells (Vince et al., 2008).

1.2.4.2.4. Regulation of IAPs with synthetic antagonists

XIAP was shown to be highly expressed in many tumours, as well as induce the resistance of tumours to therapeutic agents (Nachmias et al., 2004). In order to restore the sensitivity of these tumours to pro-apoptotic stimuli synthetic IAP antagonists were developed. IAP antagonists are synthetic compounds that were modelled on the N-terminal IAP-binding motif of the mitochondrial protein Smac/DIABLO (Wright and Duckett, 2005). The XIAP-interfering function of Smac-mimetic compounds (IAP antagonists) is crucial for therapeutic efficiency of TNF-related apoptosis-inducing ligand (TRAIL) in xenograft tumour models (Li et al., 2004; Vogler et al., 2008). Recently, it has become apparent that compounds principally designed to target XIAP also target cIAPs by rapid autoubiquitination and proteasomal degradation of cIAP1 and -2 (Bertrand et al., 2008; Gaither et al., 2007; Petersen et al., 2007; Varfolomeev et al., 2007; Vince et al., 2007).

1.3. Death receptor induced non-apoptotic signalling pathways

The death receptor-mediated pathways that initiate the described variety of responses do not act independently in a parallel manner but are interconnected to a TRAIL-R as well as TNFR1-signalling network through various mechanisms. For example NF-κB activation is blocked by initiator as well as effector caspases that become activated during death receptor induced apoptosis (Wajant et al., 2003). In recent years, there is growing evidence that the balance between NF-κB activation and apoptosis induction in the context of TNFR1 signalling is critically regulated via MAPKs, specifically by the JNK pathway (Wicovsky et al., 2007) .

1.3.1. NF-κB activation

The NF-κB (Nuclear Factor kappa B) is a nuclear transcription factor found in all cell types and is involved in cellular responses to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, death receptor stimulation, and bacterial or viral antigens. Consistent with this role, incorrect regulation of NF-κB has been linked to cancer, inflammatory and autoimmune diseases, viral infection and improper immune development (Liang et al., 2006). The NF-κB family contains five members: p50, p52, RelA (also named p65), RelB, c-Rel. While all members of the NF-κB family share a Rel homology domain in their N-terminus, a subfamily including RelA, RelB and c-Rel also contain a trans-activation domain in their C-terminal protein domain (Karin and Ben-Neriah, 2000). NF-κB dimers bind to κB sites within the promoters/enhancers of target genes and regulate transcription through the recruitment of co-activators and co-repressors.

In its inactive state, NF-κB dimers are associated with one of three typical IκB proteins, IκBα, IκBβ, or IκBε, or the precursor proteins p100 and p105, which maintain NF-κB dimers in the cytoplasm and are crucial for signal responsiveness. The prototypical and most extensively studied member of the family is IκBα. During activation of canonical NF-κB pathways, IκBα is rapidly degraded leading to the release of multiple NF-κB dimers, although the p65/p50 heterodimer is likely the primary target of IκBα (Hayden and Ghosh, 2008).

Degradation of IκB is a rapidly induced signalling event that is initiated upon specific phosphorylation of these molecules by activated IKK. The IKK complex contains two highly homologous kinase subunits, IKKα/IKK1 and IKKβ/IKK2 and a regulatory subunit NEMO (NF-κB essential modulator)/IKKγ (Hacker and Karin, 2006).

The non-canonical or alternative NF-κB pathway, however, proceeds through proteasomal processing of p100 to p52, liberating p52 containing NF-κB dimers that drive a transcriptional response. The non-canonical NF-κB pathway is also characterized by its independence from IKKβ and NEMO. Instead the alternative pathway relies on the activation of IKKα by the NF-κB-inducing kinase (NIK) (Hacker and Karin, 2006; Scheidereit, 2006). In the resting state,

NIK is ubiquitinated and degraded, but upon stimulation it is accumulated, which leads to activation of the non-canonical pathway (Liao et al., 2004).

In part because IκBα degradation and p100 processing regulate different populations of NF-κB dimers, canonical and non-canonical NF-κB pathways regulate distinct sets of target genes.

NF-κB controls the expression of gene products linked with invasion, angiogenesis, and metastasis of cancer (Prasad et al., 2010). As well, constitutive NF-κB activation was detected in SCC (Tamatani et al., 2001) and melanoma (Uffort et al., 2009) cells.

1.3.2. NF-κB activation via Death receptors

NF-κB can be sufficiently activated by activation of death receptors (e.g. TNFR1/2). The TNF induced NF-κB activity may involve the five mammalian NF-κB/Rel proteins. The adaptor molecule TRADD interacts with TNFR1 and recruits additional adapter proteins like Receptor interacting protein 1 (RIP-1), TRAF2 and cIAP1. cIAP1 ubiquitinates several components of the formed complex, which causes ubiquitin-dependent recruitment of the linear ubiquitin chain assembly complex (LUBAC), ubiquitin receptor proteins TAB2 and TAB3, associated with kinase TAK1, which is activated by TAB2 and 3, and finally NEMO/IKKα/IKKβ complex, via their respective ubiquitin-binding domains (Haas et al., 2009). Once recruited LUBAC further ubiquitinates NEMO via linear ubiquitin chains, and most likely also other components of receptor complex. Several components of this complex harbour ubiquitin binding domains (UBDs) that bind to linear ubiquitin chains, thereby stabilizing complex and supporting further recruitment, retention, ubiquitination, and activation of NEMO/IKKα/IKKβ complex (Bianchi and Meier, 2009). The IKK complex induces phosphorylation and further degradation of IκB, which is associated with NF-κB. The free NF-κB translocates to the nucleus and induces transcriptional activation of certain genes (Hacker and Karin, 2006).

In most cells, TRAIL-induced NF-κB activation is most prominent when cell death pathways are caspase inhibitors (Wachter et al., 2004). In addition to its potent pro-inflammatory function, NF-κB has been demonstrated to regulate the transcription of numerous anti apoptotic target genes, for example cIAPs. Respectively, inhibition of NF-κB was shown to sensitize keratinocytes to death ligands such as TNF (Diessenbacher et al., 2008). In contrast, sensitization to TRAIL-induced apoptosis by NF-κB inhibition appears to be more cell type-restricted and does not play an important role in cell death of keratinocytes (Wachter et al., 2004).

1.3.3. MAPK

Mitogen-activated protein kinases (MAPKs) are one group of signal-transducing enzymes that have important functions in mediating responses to various extracellular stimuli.

Activation of MAPKs is one of the many cellular responses to the death ligands, such as TRAIL and TNF. Three subgroups of MAPKs have been identified: extracellular signal-regulated kinases (ERKs), c-Jun amino-terminal kinases (JNKs; also known as stress-activated protein kinases (SAPKs)) and p38 MAPKs (Pearson et al., 2001).

The JNKs are classic examples of stress-activated protein kinases. A number of stress stimuli, including UV radiation, heat shock, inflammatory cytokines, and chemotherapeutic drugs, induce potent and preferential activation of JNKs. Numerous reports show that JNK may regulate apoptosis (Lin et al., 2000). TNF was identified to activate all three types of MAPK, but this activation is highly cell-type-specific (Karin, 1998; Liu and Han, 2001; Whitmarsh and Davis, 1999; Wicovsky et al., 2007). However, in human epidermis MAPKs play important roles in cellular recovery. For example, inhibition of p38 completely abolishes the capacity of cells to recover from attack by α-toxin (Husmann et al., 2006), and the phosphorylated form of JNK, JunB is associated with protection from psoriasis-like skin disease and arthritis (Zenz et al., 2005). Furthermore TRAIL resistance in malignant cells was shown to be differentially mediated by p38, whereas in normal cells, resistance was mediated by NF-κB (Steele et al., 2006). Taken together, these studies point to a critical role of MAPK, in particular p38 and JNK as a potential resistance factor for apoptosis and a possible deviator of non-apoptotic signalling and prompted us to examine these signalling pathways in more detail.

1.4. Toll-like receptors

The first line of defence against pathogens is the initiation of innate immune responses induced by toll-like receptors (TLRs)-mediated recognition of pathogen-associated molecular pattern (PAMPs). These receptors are different from the above described DRs, but the signalling pathways share some similarities.

There are at least 10 TLRs identified so far in humans (Figure 6), and they recognize and specifically bind to a variety of pathogenic agonists such as lipopeptide (via TLR2), double-stranded RNA (via TLR3), lipopolysaccharide (LPS) (via TLR4), flagellin (via TLR5), and deoxycytidylate-phosphate-deoxyguanylate DNA (via TLR9) by molecular pattern recognition (Chao, 2009). TLRs usually form homo- or heterodimers (TLR1 and TLR2 or TLR2 and TLR6) and all, except TLR3, signal through the key adaptor myeloid differentiation primary-response gene 88 (MyD88). Some of TLRs, such as TLR2 and TLR4, require MyD88 adaptor-like protein (Mal) for recruiting MyD88. TLR3 signals through the adaptor TIR-domain-containing adaptor protein inducing interferon-β (IFN-β)-mediated transcription-factor (TRIF). In addition to Mal/MyD88-dependent pathway, TLR4 can also signal through TRIF. These signalling pathways lead to activation of transcription factors such as NF-κB and IFN

regulatory factor (IRFs), which result in production of various pro-inflammatory cytokines such as tumour necrosis factor (TNF), ILs, and IFNs (Figure 6).

Previous studies have demonstrated that human keratinocytes express TLRs 1–6 and 9 (Baker et al., 2003; Kawai et al., 2002; Kollisch et al., 2005; Mempel et al., 2003; Miller et al., 2005; Pivarcsi et al., 2003; Song et al., 2002). In addition, some of these studies have demonstrated that TLRs on keratinocytes are functional and respond to their respective ligands to produce cytokines and chemokines, and to activate NF-κB. For example TLR2 and TLR4 are expressed by human keratinocytes and can be activated by their ligands, bacterial lipopeptides and lipopolysaccharide, respectively (Kawai et al., 2002; Kollisch et al., 2005; Mempel et al., 2003; Pivarcsi et al., 2003; Song et al., 2002). Furthermore, additional studies have demonstrated that TLR3 and TLR5 are also expressed by human keratinocytes and can be activated by their ligands, double-stranded RNA (poly-I:C) and bacterial flagellin, respectively (Baker et al., 2003; Dai et al., 2006; Kollisch et al., 2005; Miller et al., 2005).

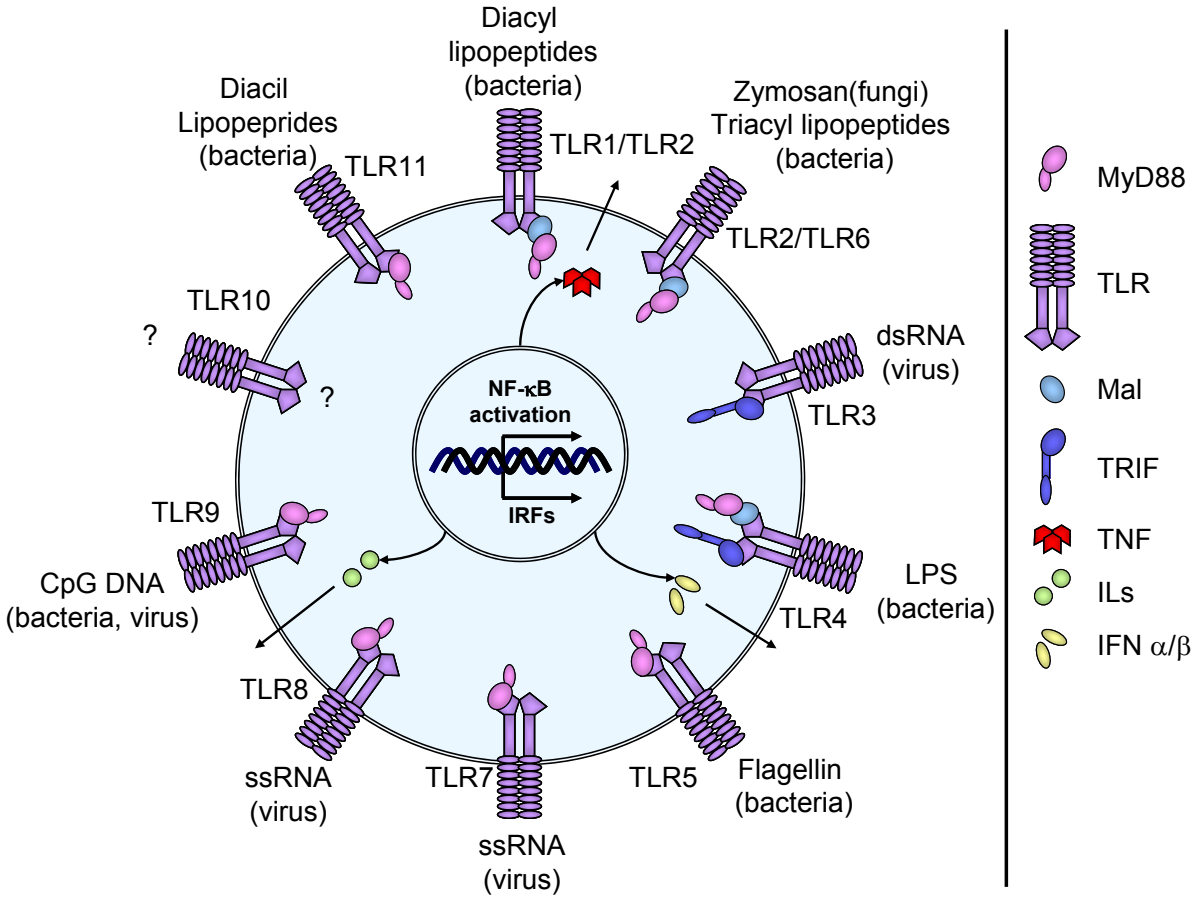


Figure 6. Toll-like receptor signalling. All TLRs are transmembrane proteins with a large extra-cellular domain containing leucine-rich repeats and a unique cytoplasmic Toll/IL-1 receptor (TIR) domain. TLR signalling pathways lead to activation of transcription factors such as NF-κB and IRFs, which result in production of various proinflammatory cytokines such as TNF, ILs, and IFNs.

Taken together, these studies highlight the importance of keratinocytes not only as a barrier to infectious microorganisms but also for detection of components of these organisms and activation of initiate immune responses via activation of TLRs (Miller and Modlin, 2007).

1.4.1. TLR-3 mediated signalling

TLR3 has been found in endosomal compartments or at the cell surface. The localization of TLR3 is cell type dependent, which may reflect the participation of cell type-specific pathways in antiviral IFN induction via TLR3. Human fibroblasts (e.g., the MRC-5 cell line) express TLR3 on the cell surface. However, in most cell types, including dendritic cells, macrophages, TLR3-transfected HEK293 cells and keratinocytes (Kollisch et al., 2005), TLR3 is detected predominantly in intracellular compartments (Vercammen et al., 2008)

TLR3 recognizes virus-derived double stranded RNA as well as its synthetic homologue poly (I:C) (Alexopoulou et al., 2001). TLR3 signals through the adaptor TIR-domain-containing adaptor protein inducing interferon-β (IFN- β)-mediated transcription-factor (TRIF) (Chao, 2009). TRIF knockout mice show defective responses to poly(I:C), indicating that TRIF is essential for TLR3-mediated signalling pathways (Yamamoto et al., 2003). The TIR domain of TLR3 binds the TIR domain of TRIF, which indirectly activates several transcription factors, including NF-κB, IRF3, and activating protein 1 (AP-1) (Vercammen et al., 2008).

Thus TLR3-mediated signalling is an important part of the inflammatory response upon injuries (Lai et al., 2009) and virus infection (Kalali et al., 2008).

1.4.2. TLR3-induced NF-κB and MAPK activation

TLR3-induced signals regulate inflammatory responses initiated by NF-κB activation (Santoro et al., 2003). The activation of NF-κB via TLR3 is achieved by two different signalling pathways bifurcating from TRIF. Different regions of TRIF can bind the ubiquitin ligase TRAF6 and the kinase RIP-1. In murine embryonic fibroblasts deficient in RIP-1, poly(I:C)-induced NF-κB is completely blocked, indicating that RIP-1 is an essential mediator of the TRIF pathway leading to NF-κB activation (Meylan et al., 2004). The interaction of TRIF and RIP-1 is mediated through the RIP homotypic interaction motif (RHIM) present in both proteins is mediated by the C-terminal part of TRIF and the intermediary domain of RIP-1 and RIP-3.

TRAF6 is recruited to the N-terminal domain of TRIF, but the role of TRAF6 is somewhat controversial and probably cell type specific (Gohda et al., 2004; Sato et al., 2003). At least in mouse embryonic fibroblasts, TRAF6 is recruited to TRIF along with RIP-1, followed by polyubiquitination of RIP-1 (Cusson-Hermance et al., 2005).

The activity of TRIF-associated TRAF6 might be responsible for the ubiquitination of RIP-1 in the TLR3 pathway. RIP-1 ubiquitination is recognized by the ubiquitin receptor proteins TAB2

and TAB3, leading to the activation of the kinase TAK1, which is part of the same complex. TAK1 phosphorylates and activates IKKα and IKKβ, which are part of a bigger IKK complex with the IKK adaptor protein NEMO. IKKβ is known to be the crucial IKK in TLR signalling and phosphorylates IκBα, which binds and keeps NF-κB (here depicted as a

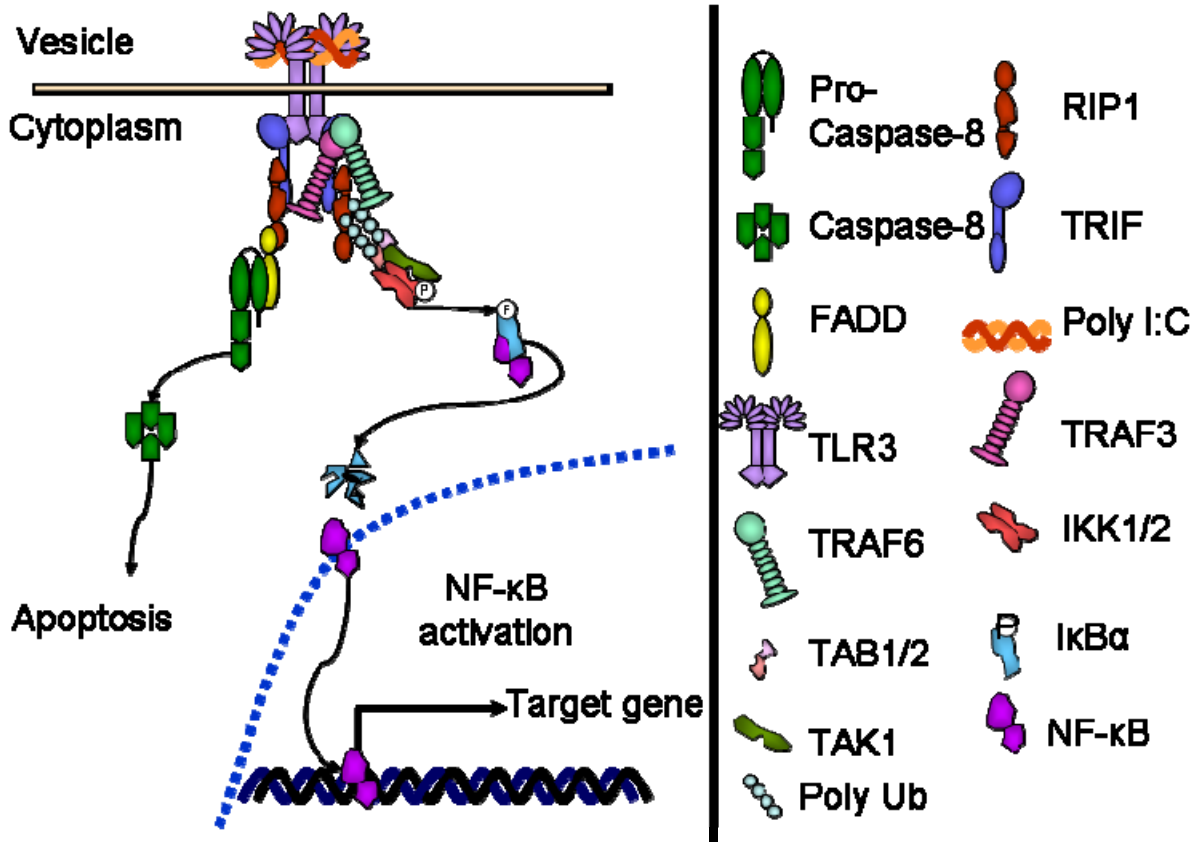


Figure 7. TLR3 signalling pathways. Ligation of the dsRNA to TLR3 can lead to NF-κB activation and apoptosis.

p65/p50 dimer) in an inactive state in the cytoplasm. IκBα phosphorylation leads to its recognition and degradation by the proteasome, thus allowing NF-κB to translocate to the nucleus, where it binds and activates specific gene promoters (e.g. cIAP1 and 2) (Figure 7) (Vercammen et al., 2008).

Poly(I:C)-induced NF-κB activation is decreased in TAK1-deficient mouse embryonic fibroblasts, showing that TAK1 is specifically needed for TLR3-induced NF-κB activation (Shim et al., 2005). TAK1 also activates the mitogen-activated protein kinases c-jun N-terminal kinase, p38, and extracellular signal-regulated kinase, leading to the phosphorylation and activation of members of the AP-1 family of transcription factors.

1.4.3. TLR3-induced apoptosis

TLRs have also been implicated in tumour cell proliferation and as well confer apoptosis resistance (for review see (Huang et al., 2008)). Contrasting the proinflammatory role of TLR3, it has also recently been suggested that this receptor may as well induce apoptosis in

a TRIF-dependent manner (Weber et al., 2010). The recruitment and binding of the kinase RIP-1 to TRIF by the RIP homotypic interaction motif (RHIM) domain is required for the transduction of apoptotic signals (Kaiser and Offermann, 2005; Weber et al., 2010). The binding of RIP-1 to TRIF not only activates NF-κB but also recruits the DD-containing adaptor protein FADD via a homotypic DD-DD interaction. FADD in turn interacts with procaspase-8 through the death effector domain (DED) present in both proteins (Figure 7). This signalling platform further initiates cell death induction (Vercammen et al., 2008).

Since TLR3 agonists induce apoptosis and the TLR3 is widely expressed on cancer cells (Rydberg et al., 2009), TLR agonists have been postulated as adjuvants for cancer vaccines (Salaun et al., 2007; Salem et al., 2005).

1.5. RIP-1 as the central signalling molecule

Protein kinases of the receptor interacting protein (RIP) family are important for death receptor signalling and regulation of cell death. RIP-1, the founding member of the RIP protein family was proposed to mediate the cell death as well as pro-survival signalling pathways, initiated by DRs as well as TLR3 (Kaiser and Offermann, 2005; Weber et al., 2010).

1.5.1 The role of RIP-1 for cell death signal transduction

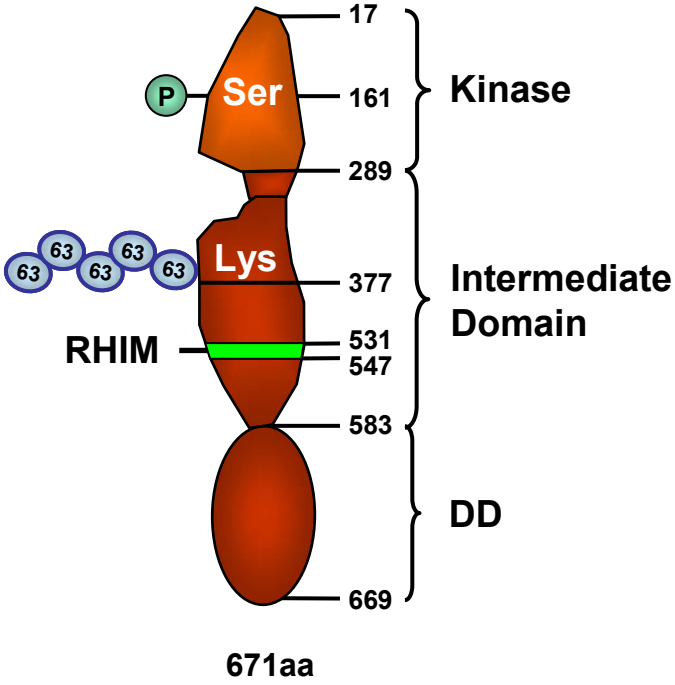


Figure 8. Domain organization of RIP-1. Abbreviations: DD - Death domain; RHIM - RIP homotypic interaction motif.

RIP-1 is constitutively expressed in many tissues such as the skin (Meylan and Tschopp, 2005).

RIP-1 consists of a C-terminal death domain (DD), intermediate domain (ID), which has a RIP homotypic interacting motive (RHIM) and a kinase domain (Figure 8). The DD of RIP-1 can be recruited to several death receptors (e.g. CD95, TRAIL, TNF) (Stanger et al., 1995).

TRADD and RIP-1 compete with each other for binding to TNFR1 through DD interactions and mediate separate events for programmed cell death. Recruitment of TRADD/FADD/caspase-8/10 triggers the execution of apoptosis, whereas

the receptor association of RIP-1 initiates a serine/threonine kinase-dependent necrosis-like death program that involves ROS accumulation (Zheng et al., 2006).

Dispensable for CD95L-induced apoptosis, RIP-1 is important for CD95L-, TRAIL- and TNF-induced necrosis (Festjens et al., 2006b). In line, reconstitution of a RIP-1-deficient Jurkat T-cell line with a kinase inactive form of RIP-1 fails to restore CD95L-induced necrosis. RIP-1, and specifically its DD, was reported to be critical for CD95-mediated necrosis independent of NF-κB–inducing activity or RIP-1 kinase (RIP-1K) activity (Degterev et al., 2005; Holler et al., 2000). The recent development of specific RIP-1K inhibitors has facilitated experiments examining the functional role of RIP-1K in necrosis (Degterev et al., 2008), but the potential targets of the kinase activity of RIP-1 are unknown (Degterev et al., 2008).

RIP-1 can interact with TRIF via its RHIM, and interact with FADD by the DD. This interaction is required for the transduction of apoptotic signals from TLR3 (Figure 7) (Kaiser and Offermann, 2005; Weber et al., 2010).

In summary, RIP-1 seem to play a role as an adaptor or scaffolding molecule during apoptosis and necrosis signalling in both DR and TLR3-induced signalling.

1.5.2. The role of RIP-1 for NF-κB and MAPK activation

The physiological function of RIP-1 in the TNF pathway was addressed with the generation of RIP-1-deficient mice and analysis of a RIP-1-deficient Jurkat T-cell line. Previous studies showed that cells devoid of RIP-1 do not mount strong NF-κB responses upon TNF stimulation, but still activate c-Jun N-terminal kinase (JNK) (Devin et al., 2003). Surprisingly, the recent studies, done in MEF cells have shown that RIP-1 in not essential for NF-κB activation (Wong et al., 2010).

CD95-induced apoptosis is normal in cells lacking RIP-1; these cells are not sensitized to TNF-induced apoptosis (Wong et al., 2010). In mice, loss of RIP-1 results in massive cell death of lymphoid and adipose tissues, reflecting one of the major roles of RIP-1 as a signalling intermediate of pro-survival pathways. RIP-1 does not require its kinase activity for the activation of NF-κB because reintroduction of a kinase-dead form of RIP-1 into the RIP-1-deficient T-cell line completely restores NF-κB activation. Thus, RIP-1 might function as a scaffolding protein instead (Devin et al., 2000). The intermediate region of RIP-1, which is important for downstream NF-κB signalling, not only binds to TRAF2 but also recruits IKKs to the activated TNF-R1 complex via interaction with NEMO (Hur et al., 2003).

In contrast, during TLR3 signalling RIP-1 is bound to TRIF and rapidly ubiquitinated, probably by TRAF6 resulting in canonical NF-κB activation (Vercammen et al., 2008).

Therefore, the intermediate domain of RIP-1 is crucial for NF-κB signal transduction in both DR and TLR signalling by interaction with a variety of signalling proteins. Although the importance of each interaction remains to be determined, certain observations have been

made, for example, ubiquitination of RIP-1 seems to modulate the duration of NF-κB-signalling activity and TNF-R1 stimulation results in massive RIP-1 ubiquitination, which enhances its proteasomal degradation.

1.5.3. Regulation of RIP-1 by ubiquitination

Protein ubiquitination has emerged as an important secondary protein modification that regulates various biological processes (Fang and Weissman, 2004). Following stimulation with TNF, RIP-1 binds to TNF-R1 and NEMO. TNFR1-associated RIP-1 undergoes polyubiquitination by A20 (an ubiquitin ligase that inhibits NF-κB signalling), leading to RIP-1 degradation (Wertz et al., 2004). A20 was shown to exert two opposing activities: sequential de-ubiquitination as well as ubiquitination of RIP-1, thereby targeting RIP-1 to proteasomal degradation (Heyninck and Beyaert, 2005). The stabilization of RIP-1 required interaction with NEMO through the polyubiquitin-binding region of the latter protein (Wu et al., 2006). Binding to NEMO may prevent conversion of Lys 63-linked RIP-1 to Lys 48-linked polyubiquitination, potentially by impairing interaction of RIP-1 with A20 (Wertz et al., 2004). More recently Bertrand et al. showed that that cIAP1 and cIAP2 directly ubiquitinate RIP-1 and induce constitutive RIP-1 ubiquitination in cancer cells. Constitutively ubiquitinated RIP-1 then associates with the pro-survival kinase TAK1 (Bertrand et al., 2008).

1.6. Aims

The skin is the first line defence against infections with pathogens. Numerous processes such as inflammation, activation and regulation of the innate and adaptive immune response are initiated by the skin cells, in particular keratinocytes. In contrast to this processes, infections of the skin with pathogens can result in initiation of autoimmune diseases and carcinogenesis of the skin (Wolska et al., 2009). However the mechanisms of bacterial and viral promotion of skin cancer progression are poorly understood to date. Virus-derived molecules such as vFLIP proteins are significant regulators of apoptotic processes (Thurau et al., 2009). The resistance to apoptotic stimuli is one way to evade host specific elimination by suppression of caspase activitiy of the tumour cells. Therefore overcoming apoptosis resistance by antagonism or downregulation of inhibitors of caspase activity has been advocated as a major improvement of tumour therapy (Fesik, 2005).

Agonists of TLR3 such as poly (I:C) can induce apoptosis in melanoma cells and were suggested for anti-cancer treatment (Salaun et al., 2007), whereas many other malignant cell lines are resistant to poly (I:C) induced cell death. These controversial findings raise the hypothesis that intracellular molecules regulate TLR3-induced cell death as well as other non-apoptotic signalling pathways. The role of cIAPs in regulation of TLR3 and DR-induced cell death and non-apoptotic signalling pathways was largely unclear at the beginning of this thesis.

Therefore the aim of this work was to contribute to the understanding of the mechanisms involved in TLR3- and DR-mediated cell death and non-apoptotic signalling pathways. The identification of the impact of cIAPs for the regulation of cell death processes in TLR3 and DR signalling is the object of this thesis and will be responded by following questions.

1. What is the relevance of cIAPs in TLR-3-mediated cell death in primary and transformed keratinocytes (HaCaT) and SCC cell lines?

   In order to achieve this aim, the responsiveness of primary keratinocytes as well as HaCaT and SCC cells to TLR3 agonist (poly (I:C)) will be analyzed. The inhibition of cIAPs and XIAP function will be performed with either specific shRNA-mediated knockdown or with a chemical IAP antagonist. The possible cell death induction will be characterized by biochemical and morphological analysis.

2. Which molecules are regulating the apoptotic and survival outcome of TLR3 signalling complexes?

   To target this question the formation and composition of intracellular protein complexes in HaCaT keratinocytes upon TLR3 stimulation in the presence and absence of cIAPs will be analyzed. The expression, recruitment, activation and modification of TLR3 complex-

related molecules such as RIP-1, TRIF, cFLIP and caspase-8 will be checked by co-immunoprecipitation studies with caspase-8 specific antibody. In addition, to verify the role of other cell death regulating molecules such as cFLIP and RIP-1 for TLR3 signalling, the protein expression levels of cFLIP isoforms and RIP-1 will be modified by generation of knockdown and overexpressing HaCaT cells.

3. What is the impact of cIAPs on regulation of CD95L-induced cell death in human keratinocytes?

Since CD95-induced cell death is tightly regulated by molecules that are also involved in TLR3 signalling, the role of cIAPs in controlling CD95-induced signalling pathways will be analyzed under the same condition as indicated in 1. This study could help in comparing if similar signalling platforms that regulate cell death signalling pathways exist.

4. What is the role of IAPs for the control of non-apoptotic signals such as NF-κB and MAPK activation in TRAIL stimulated keratinocytes and SCC cell lines?

To clarify this question the canonical and non-canonical NF-κB activation after TRAIL stimulation will be studied in the presence and absence of IAPs in primary human keratinocytes and SCC cell lines. MAPK activation will be studied by checking the phosphorylation status of respective MAPK in primary keratinocytes and SCC cell lines.

Taken together, this study will further the understanding of molecular mechanisms of resistance of skin tumours and possibly the use of IAP antagonist together with TLR3 agonists as a potential therapeutic strategy to overcome therapeutic resistance of skin tumour cells.

2. Material and Methods

2.1. Materials

The kits and chemicals that were used in this work were purchased from the described companies. The quality of the reagents was of analytical grade. If special reagents were used for experiments, it is described at the beginning of each section.

2.1.1. Enzymes and molecular biology reagents

Item	Company
Alkaline Phosphatase from calf intestine (CIAP)	Promega
Deoxynucleoside Triphosphate Set (dNTPs)	Promega, Fermentas
Endonucleases (Restriction enzymes)	Promega, Fermentas
NuPAGE® Novex 4-12% Bis-Tris Gels	Invitrogen
Oligonucleotides (Primer)	MWG-operon
Polyvinylidene fluoride (PVDF) membrane	GE Healthcare
Protein G beads	Roche
T4 DNA ligase	Promega
Taq DNA polymerase	Promega, Fermentas
NheI	Promega
XbaI	Promega
Bam HI	Promega

The buffers for the enzymes were purchased from the same companies.

Table 1: Molecular biology reagents

2.1.2. Kits

Item	Company
ECL Detection Reagents	GE Healthcare
ECL Plus™ Western Blotting Detection Reagents	GE Healthcare
EndoFree Plasmid Maxi Kit	Qiagen
Wizard Plus SV miniprep DNA Purification System	Promega
Wizard® SV Gel and PCR Clean-Up System	Promega
DC Protein assay	Bio-Rad

Table 2: Kits used in biochemical and molecular biological assays

2.1.3. Phoshpatase and protease inhibitors

Inhibitor	Company (Catalog number)
AEBSF Hydrochlorid	AppliChem (A1421)
Aprotinin	Roth (A162.3)

Benzamidine	Fluka (12072)
Complete (Protease Inhibitor Cocktail Tablets)	Roche (11836145001)
β-Glycerophosphate disodium salt hydrate	Sigma-Aldrich (G6376)
Leupeptin hemisulfate salt	Sigma-Aldrich (L2884)
Sodium orthovanadate	Sigma-Aldrich (S6508)
Sodium pyrophosphate tetrabasic decahydrate	Sigma-Aldrich (S6422)

Table 3: Phosphatase and protease inhibitors

2.1.4. Pharmacological stimulating substances

Substance	**Company (Catalog number)**
Compound A (CompA)	provided by TetraLogic Corp. (Vince et al., 2007)
LBW-242	Novartis (TRD 1223-139)
Necrostatin-1	Sigma-Aldrich (N9037)
4-Hydroxytamoxifen (4-HT)	Sigma-Aldrich (H7904)
z-Val-Ala-DL-Asp(OMe)-fluoromethylketone (zVAD-fmk)	Bachem (N-1560)
Q-VD-OPH (QVD)	Biovision (#1170)
Poly (I:C)	Sigma (p1530)

Table 4: Pharmacological stimulating substances

2.1.5. Stimulating Cytokine

Cytokine	**Source**
TNF-R2-Fc	M. Feoktistova (Geserick et al., 2009)
His-Flag-TRAIL (HF-TRAIL)	P. Diessenbacher (Diessenbacher et al., 2008)
CD95L-Fc	M. Feoktistova (Geserick et al., 2009)
TWEAK-Fc	The construct for producing of recombinant cytokines was provided by J. Silke, the TWEAK-Fc supernatant was produced and purified as described later.

Table 5: Stimulating Cytokine

2.1.6. Molecular weight markers

DNA molecular weight markers	**Company**
GeneRuler™ 1 kb DNA Ladder, ready-to-use	Fermentas
GeneRuler™ 100 bp Plus DNA Ladder, ready-to-use	Fermentas
Protein molecular weight markers	**Company**
SeeBlue® Pre-Stained Standard	Invitrogen

Table 6: Molecular weight markers for DNA and proteins

2.1.7. Buffers

Buffer	Composition
1 x PBS	2.7mM KCl, 1.5mM KH_2PO_4, 137mM NaCl, 8mM Na_2HPO_4, pH 7.4
1 x T-PBS	1xPBS, 0.1% Tween 20
5 x Laemmli sample buffer	60mM Tris-HCl (pH 6.8), 2% SDS, 10%l Glycerol, 5% β-Mercaptoethanol, 0.01% Bromophenol blue
AB-buffer	3% non-fat dry milk, 1.5% BSA in 1 x T-PBS
Blocking buffer	5% non-fat dry milk, 3% BSA in 1 x T-PBS
Crystal violet staining solution	0.5% crystal violet, 20% methanol
DNA sample buffer	30% (v/v) Glycerine, 50mM EDTA, 0.25% Bromophenol-blue, 0.25% Xylene Cyanol
Cell lysis buffer for isolation of total cell proteins	30mM TRIS-HCL (pH 7.5), 120mM NaCl, 10% Glycerol, 1% Triton X, 2 tablets Complete (Protease Inhibitor) per 100 ml
Triton lysis buffer for phospho-protein assays	20mM Tris (pH 7,4),137mM NaCl, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 2mM EDTA, 50mM Na β glycerophosphate, 1mM Na orthovanadate. Before us added: 1mM Pefabloc (ABSF), 5µg/ml aprotinin, 5µg/ml Leupeptin hemisulfate salt, 5mM benzamidine
SDS Running Buffer (MES)	50mM MES, 50mM Tris-base, 0.1% (w/v) SDS, 1mM EDTA, pH 7.3
SDS Running Buffer (MOPS)	50mM MOPS, 50mM Tris-base, 0.1% (w/v) SDS, 1mM EDTA, pH 7.7
TAE buffer	40mM Tris Base, 20mM Acetic acid, 10mM EDTA
HBS buffer	140mM NaCl, 50mM Hepes, 1.5mM Na_2PO_4, pH 7.05
Transfer buffer	25mM Tris, 192mM glycine, 10% methanol
EMSA Buffer-A	10mM Tris pH-7,9**,** 10mM KCl, 1,5mM $MgCl_2$**,** 10% Glycerol, 10mM K_2HPO_4, shortly before use added: 1mM Sodium vanadate, 10mM NaF, 0.5mM DTT, 1mM ABSF and protease inhibitors (Roche Diagnostik GmbH)
EMSA Buffer-C	20mM Tris pH-7.9, 0,42M NaCl, 1,5mM $MgCl_2$, 0,2mM EDTA, 10% Glycerol, 10mM K_2HPO_4, shortly before use added: 1mM Sodium vanadat, 10mM NaF, 0,5mM DTT, 1mM ABSF and protease inhibitors

Hypotonic fluorochrome solution	Sodium citrate 0,1% (w/v), Triton X 100 0,1% (v/v), Propidium Iodide (PI) 50µg/ml

Table 7: Buffers used in biochemical and molecular biological assays

2.1.8. Primary antibodies for western blot analysis

Antibody (clone)	Company (Catalog number)
Caspase-8 (C15)	provided by P.H. Krammer, (DKFZ, Heidelberg, Germany)
Caspase-8 (C20)	Santa Cruz (sc-6136)
Caspase-3	CPP32, provided by H. Mehmet, Merck Frost
FADD (1)	BD Transduction Laboratories (F36620)
cFLIP (NF-6)	Enzo Life Sciences, Inc
cIAP-1 (1E1)	provided by J. Silke (Silke et al., 2005)
cIAP-2 (207)	provided by J. Silke (Vince et al., 2009)
RIP-1 (38)	BD Transduction Laboratories (R41220)
RIP-3	provided by F. Chan (Cho et al., 2009)
TRADD (37)	BD Transduction Laboratories (T50320)
β-actin (A2103)	Sigma (A2103)
β-tubulin	Sigma (T4026)
IκBα	Sigma (sc-371)
p38	Sigma (sc-535)
XIAP (48)	BD Transduction Lab (H62120)
TRIF	Cell Signaling Technology (#4596)
p-IκBα	Cell Signaling Technology (#9246)
NIK	Cell Signaling Technology (#4994)
NF-κB p100/p52	Cell Signaling Technology (#4882)
p-HSP27	Cell Signaling Technology (#2401S)
p-p38	Cell Signaling Technology (#9215S)
p-JNK	Cell Signaling Technology (#9251S)
JNK	Cell Signaling Technology (#9252)
HMGB1	Abcam (ab18256)

Table 8: Primary antibodies for western blot analysis

2.1.9. HRP-coupled secondary antibodies

Antibody (clone)	Company (Catalog number)
Mouse IgG	Southern biotech (1031-05)
Mouse IgG1	Southern biotech (1070-05)
Mouse 1gG1	Southern biotech (1050-05)

Mouse IgG2a	Southern biotech (1080-05)
Mouse IgG2b	Southern biotech (1090-05)
Rabbit IgG	Southern biotech (4030-05)
Rat IgG	Southern biotech (3050-05)

Table 9: HRP-coupled secondary antibodies for western blot analysis

2.1.10. Vectors

Vector	Reference
Expression vector: PCR-3	Invitrogen
Lentiviral packaging vector: pMD2.G	(Rubinson et al., 2003)
Lentiviral packaging vector: pMDlg/pRRE	(Rubinson et al., 2003)
Lentiviral packaging vector: pRSV-Rev	(Rubinson et al., 2003)
Lentiviral vector: pFGEV16-Super-PGKHygro	(Vince et al., 2007)
Lentiviral vector: pF5xUAS-W-SV40-Puro	(Vince et al., 2007)
Lentiviral vector: pLKO.1	(Geserick et al., 2009)
Retroviral vector: pCF65-IEGZ	(Denk et al., 2001)
Retroviral vector for siRNA: pRS puro (pSuper.retro)	(Diessenbacher et al., 2008)
Retroviral vector for siRNA: pRS MIG (derived from pSuper.retro)	(Geserick et al., 2008)

Table 10: Vectors used for expression

2.1.11. siRNA-sequences for knockdown of:

Target	Sequence
cIAP2	64-mer oligomers containing cIAP2-targeting sequence nucleotide start position +316
cIAP1	64-mer oligomers containing cIAP1-targeting sequence nucleotide start position +3398
cIAP1	64-mer oligomers containing cIAP1-targeting sequence nucleotide start position +1685
RIP-1	64-mer oligomers containing RIP-1-targeting sequence nucleotide start position +193
HRS	64-mer oligomers containing a hyper random sequence not matched by any gene in the NCBI database

Table 11: siRNA-sequences for knockdown

2.1.12. Bacteria cells

Bacterial Cells	Company

E.coli DH5α	Clontech
E.coli M15 (pREP4)	Qiagen
E.coli TOP10F	Invitrogen
E.coli XL10 Gold	Stratagene

Table 12: Bacteria cells

2.1.13. Culture media and additives for bacterial cells

Item	**Company (Catalog number)**
Ampicillin sodium salt (100µg/ml)	Roth (K029.2)
Kanamycin sulfate (30µg/ml)	Roth (T832.1)
LB-Agar (Lennox)	Roth (X965.2)
LB-medium (Lennox)	Roth (X964.2)
SOC Medium	Sigma-Aldrich (S1797)

Table 13: Media and Reagents for bacterial cell culture. All media were autoclaved at 121°C for 15min. The additives were filtered with a 0.2µm filter-unit and stored at -20°C.

2.1.14. Human cells

Human cells	**Source**
A5RT3 parental (SCC cell line)	Mueller et al., 2001: provided by P. Boukamp (DKFZ, Heidelberg, Germany)
HaCaT parental (transformed keratinocytes)	Boukamp et al., 1988: were provided by P. Boukamp (DKFZ, Heidelberg, Germany)
HaCaT pCF65	M. Feoktistova (Geserick et al., 2009)
HaCaT $cFLIP_L$	M. Feoktistova (Geserick et al., 2009)
HaCaT $cFLIP_S$	M. Feoktistova (Geserick et al., 2009)
HaCaT pRS puro HRS	M. Feoktistova (Geserick et al., 2009)
HaCaT pRS puro shRNA cIAP2 (316)	M. Feoktistova (Geserick et al., 2009)
HaCaT pLKO.1	M. Feoktistova (Geserick et al., 2009)
HaCaT pLKO.1 shRNA cIAP1 (1685 and 3398)	M. Feoktistova (Geserick et al., 2009)
HaCaT pRS MIG shRNA HRS	M. Feoktistova (Geserick et al., 2009)
HaCaT pRS MIG shRNA RIP-1 (RIP-193)	M. Feoktistova (Geserick et al., 2009)
HaCaT GEV16 control	M. Feoktistova
HaCaT GEV16 RIP-1 WT	M. Feoktistova
HaCaT GEV16 RIP-1 K45A	M. Feoktistova
HaCaT GEV16 RIP-1 D324K	M. Feoktistova
HEK 293T (Human Embryonic Kidney 293 that stably express the large T-antigen of	American Type Culture Collection (ATCC)

SV40)	
MET1 parental (SCC cell line)	Poppet al., 2000: provided by I. Leigh (Skin Tumor Laboratory, London, UK)
ΦNX (amphotropic producer cell line derived from HEK 293T)	American Type Culture Collection (ATCC)
Primary keratinocytes. Donor 11	isolated from human skin
Primary keratinocytes. Donor I	isolated from human skin
Primary keratinocytes. Donor L	isolated from human skin
Primary keratinocytes. Donor E	isolated from human skin

Table 14: Human cells

2.1.15. Cell culture media and reagents for human cells

Item	**Company (Catalog number)**
Dulbecco's Modified Eagle Medium (D-MEM)	Invitrogen (41965-062)
Fetal Bovine Serum "Gold" (FBS)	PAA (A15-151)
Ultra Low IgG Fetal Bovine Serum	Invitrogen
HEPES	Sigma-Aldrich (H3375)
Hygromycin B	MERK (400050)
MEM Amino Acids	PAA (M11-003)
MEM Vitamins	PAA (N11-002)
Dulbecco's Phosphate-Buffered Salines (D-PBS)	Invitrogen (14190)
Puromycin dihydrochloride	Sigma-Aldrich (P9620)
Sodium Pyruvate	PAA (S11-003)
Trypsin EDTA	PAA (L11-004)
Zeocin™	Invitrogen (R250-01)
CnT-07	CellNTec (CnT-07.BM)

Table 15: Media and reagents for human cell culture

2.2. Methods

2.2.1. Molecular biological methods

All molecular biological work was carried out corresponding to standard protocols provided by the manufacturers. All methods are described in the literature in detail: Current Protocols in Molecular Biology (Ausubel et al., 1990) and Molecular Cloning (Sambrook et al., 1989). Therefore, only a brief description will be given and the modifications will be described in more detail if this applies.

2.2.1.1. Digestion of DNA with restriction endonucleases

The restriction reaction was performed in 20µl mix, containing 10-15 U of respective enzyme, compatible buffer and 1µg of analysed DNA solved in water. The reaction was performed for 1-1,5 h by 37°C.

2.2.1.2. DNA agarose gel electrophoresis

DNA fragments were separated according to their size by one-dimensional agarose (0,8-1,2%) gel electrophoresis. To visualize the DNA under UV light, 5-10µl Ethidium bromide solution (10 mg/ml in H_2O) was added before gel polymerization. The DNA samples were prepared in DNA sample buffer and were loaded onto the gel. Gels were run at 3-5V/cm, depending on the required quality, in TAE buffer. The DNA fragments were visualized under UV-light and photographed.

2.2.1.3. Cloning

DNA fragments of interest were purified (for kits look table 2) subjected to enzymatic digestion and ligated with T4 DNA ligase to the pre-digested and dephosphorylated vector (pF5xUAS-W-SV40-Puro). The ligations were performed at 16-20°C for 2-8 h. The used DNA fragment/vector ratio was 3:1 (sticky end cloning). The ligated fragment-vectors were transformed into competent bacterial cells and plated on antibiotic containing agar plates for bacteria colony formation. The DNA of formed bacteria colonies was isolated for restriction digestion, sequencing (by Agowa) and maxi plasmid preparations.

2.2.1.4. Heat shock transformation

The DNA ligation mixture was incubated with 100µl of heat shock competent bacteria cells for 30min on ice, followed by transformation, heat shock 45 sec by 42°C, followed by incubation on ice for 5min. Then 400µl of SOC Medium was added and the tube incubated at 37°C for 1 h shaking speeding bacterial shaker (80 rpm). Bacterial cells were pelleted by centrifugation at 1000 g for 1min and the supernatant was decanted. The pellet was resuspended by vortexing in the remaining drops of liquid. The entire suspension was plated on LB-Agar plates containing the respective antibiotics. Plates were incubated over night at 37°C.

2.2.1.5. Plasmid isolation

DNA plasmids were purified using Plasmid Kits (Table 2) and following manufactures protocols. The DNA concentration was determined by spectrophotometrical quantification at 260 nm by A_{260} * 50 = xµg/µl.

2.2.2. Cell culture techniques

Cells were cultured in their respective media in 5% CO_2 at 37°C and a humidity of 95%. All media were pre-incubated at 37°C before addition to cells.

Human cells	Medium
A5RT3/HEK 293T/HaCaT	D-MEM, 1% HEPES, 1% Sodium Pyruvate, 10% heat inactivated FBS
MET1	D-MEM, 1% HEPES, 1% Sodium Pyruvate, 1% MEM NEAA, 1%MEM Vitamins, 10% heat inactivated FBS
ΦNX ampho	D-MEM, 1% HEPES, 1% Sodium Pyruvate, 1% MEM Amino Acids, 10% heat inactivated FBS,
Primary keratinocytes	CnT-07 including the supplements

Table 16: Media and references for cell culture

2.2.2.1. Ca-phosphate mediated Transfection of vector-DNA

293T or ΦNX-ampho cells were grown in 10 cm plates $3x10^6$ per plate. Then the medium was changed with 6ml of respective medium per plate + 25μM Chloroquin followed by 40'-1h at 37°C in 5% CO_2. For generation of Ca-Phosphate-DNA precipitate 25μM $CaCl_2$, 20μg DNA were mixed with water up to final volume of 300μl (per plate), followed by addition dropwise into 300μl of 2x HBS buffer. Upon 30-40' incubation at RT the mix was added on the plate and incubated over night at 37°C in 5% CO_2 atmosphere before exchange of growth media.

2.2.2.2. Generation and purification of stimulating cytokines

For generation of CD95L-Fc (the construct was kindly provided by P. Schneider) and TWEAK-Fc (the construct was kindly provided by J. Silke) the corresponding constructs were transiently expressed in HEK 293T cells, cultured in IgG stripped medium. Supernatants were harvested and filtered (0.2μm, Millipore). The proteins from supernatant were precipitated with saturated solution of Ammonium sulfate, followed by dialysis with 20mM $NaPO_4$ pH-7,0 as dialysis buffer. Then the dialyzed solution was purified with protein-G agarose column. The activity of purified protein was tested by functional assays (e.g. Cristal violet staining upon stimulation of the cells).

2.2.2.3. Retroviral infection

The amphotrophic producer cell line ΦNX was transfected with 20µg of the retroviral vectors (pCF65 or pRS MIG) containing cDNA or shRNA of interest. The supernatants were harvested 48-72h post-transfection and filtered (0.45, Millipore). The virus containing supernatants were added to target cells with 5µg/ml Hexadimethrine bromide (Polybrene). Target cells were spin-infected (1,5 h at 30°C by 2100 rpm in a Hareaus centrifuge). Stable cell lines were selected with respective antibiotics: Zeocin 300µg/ml for 10-14 days for pCF65 and puromycine 3µg/ml for 3 days for pRS MIG. Western blot analyses were performed on polyclonal cells to confirm knockdown and ectopic expression of the respective molecules.

2.2.2.4. Lentiviral infection

To generate lentiviral supernatants, HEK 293T cells were transfected with pMD2.G, pMDlg/pRRE, and pRSV-Rev of the lentiviral packaging vectors together with the target vectors (e.g. pFGEV16-Super-PGKHygro, pF5xUAS-W-SV40-Puro and pLKO.1 vectors containing molecules of interest or respective shRNA). The supernatants were harvested 48-72h post-transfection and filtered (0.45µm, Millipore). The virus containing supernatants were added to target cells with 5µg/ml Hexadimethrine bromide (Polybrene). Target cells were spin-infected (as described before). Stable cell lines were selected either with hygromycin (100-150µg/ml for 1 week) or puromycin (3µg/ml for 3 days). Cells were subsequently tested for expression of the respective proteins. For the inducible protein expression the 24h transduced cells were stimulated with 10 to 100nM 4-HT (4-Hydroxytamoxifen) for 24h and total cell lysates were analysed by western blot.

2.2.2.5. Stable siRNA expression

RIP-1 siRNA as well as a hyper random sequence (HRS) not matched by any gene in the National Center for Biotechnology Information database (Vogler et al., 2007) were used. The random sequence construct was provided by S. Fulda (Ulm University, Ulm, Germany). For generation of the constructs, cDNA 64-mer oligomers containing RIP-1 targeting sequence (nucleotide start position +193) were cloned into the pRS-MIG retroviral vector using HindIII and BglII restriction sites. For generation of the cIAP2, cIAP1 and RIP-1 constructs, complementary DNA 64-mer oligomers containing either cIAP2 (nucleotide start position +316 or +466), cIAP1 (nucleotide start positions +3398 and +1685) or RIP-1 (nucleotide start position +193) targeting sequences were cloned either into the pRS-MIG retroviral vector using HindIII and BglII restriction sites (cIAP2 and RIP-1 shRNAs) or into pLKO.1 (cIAP1 shRNAs).

The resulting vectors were transfected either into the amphotrophic producer cell line ΦNX (pRS MIG) or into 293T cells together with the respective packaging plasminds (for pLKO.1, as was described before). The virus containing supernatants were used for spin infection of the target cells as described before (2.2.2.3 and 2.2.2.4.). Stable cell lines were selected in Puromycin (3µg/ml) for 3 days. Western blot analyses were performed on polyclonal cells to analyze expression of the downregulated proteins.

2.2.2.6. Cytotoxicity assay

Crystal violet staining of attached, living cells was performed 18-24 h after stimulation with the indicated concentrations of stimulating agents (DL or poly (I:C) and/or inhibitors (e.g. IAP antagonist zVAD-fmk, Necrostatin-1) in 96-well plates. Plates were washed two times with water. Subsequently, 50µl of Crystal violet staining solution (Table 7) were added per well. After incubation for 20min at room temperature, plates were washed several times with water to remove the non-intercalated dye. Plates were air dried, and 200µl methanol were added per well followed by 30min incubation at RT to solve the incorporated dye. The optical density of the wells was subsequently measured by a plate reader (Victor3; 1430 Multilabel counter, PerkinElmer). The optical density of control cultures was normalized to 100% and compared with stimulated cells. For statistical analysis, the Standard Error of Mean (SEM) was determined for at least of medium of three independent experiments of each cell line and stimulatory condition.

2.2.3. Cell lysate preparation

2.2.3.1. For Western blot analysis

For generation of total cell lysates parental and virally transduced cells were washed twice with ice-cold 1 x PBS and lysed for 30min on ice by the addition of Cell lysis buffer (Table 7). Cellular debris was removed by centrifugation at 20,000 g for 10min. The total amounts of isolated cell proteins were quantified by Bradford assay. 5µg of total cellular proteins was supplemented with 5 x Laemmli sample buffer and boiled at 95°C.

2.2.3.2. For phospho-protein analysis

For analysis of phospho-proteins the respective cells were starved in serum free medium for 6h to get rid of the basal phosphorylated MAPKs, activated by the FBS. Then the cells were scraped from the plate and resuspended in triton lysis buffer (see table 7). To quickly disrupt the cell membranes the lysates were pushed three times through a syringe with 0,4 mm needle, followed by 14 000rpm for 10min centrifugation and transfer of the supernatant to the

fresh tube for further experiments. The total amounts of isolated cell proteins were quantified by Bradford assay.

2.2.3.3. For EMSA

For generation of cytosolic and nuclear lysates for EMSA the respective cells were seeded in amount of $1,7x10^6$ cells in 10cm dishes. After desired stimulations on day 2, the cells were trypsinized and washed with PBS. Then the cells were resuspended in 300µl of EMSA Buffer-A (Table 7) followed by immediate addition of 0,125% NP-40 (Sigma NP-40). After incubation on ice for 5min, the cells were spun down for 10min at 1000 g at 4°C. The supernatant was transferred into fresh tube (represents cytosolic extracts). The pellet was washed with 300µl of EMSA Buffer-A. Then the pellet was resuspended in EMSA Buffer-C (Table 7). Then the lysates were centrifuged at 12,000g at 4°C for 10min. The supernatant represents the nuclear extracts which were used further for EMSA.

2.2.4. Electrophoretic mobility shift assay (EMSA)

The oligonucleotide probe used was a consensus NF-κB binding site derived from the κB element of the IL-2 promoter ("TCEdA>C"): 5′-CTAAATCCCCACTTTAGGGAGAACCAG-3′.
For the procedure: 4µg nuclear proteins were incubated with 10000cpm (equivalent of approximately 0.2ng) of a ^{32}P-labeled oligonucleotide probe and 2 pg poly (dI-dC) as nonspecific competitor. The samples were incubated for 30min on ice, separated on a non-denaturing 5% polyacrylamide (PAA) gel at 200 V/15 cm at room temperature followed by autoradiography.

2.2.5. Western Blot analysis

Proteins were separated by SDS-PAGE on 4-12% gradient gels (Invitrogen) using SDS Running Buffer and Invitrogene Power Ease 500 system. Proteins were transferred to PVDF membrane using Transfer buffer (Table 7). Membranes were incubated for 1 h in blocking buffer, washed with 1 x T-PBS, and incubated AB-buffer and primary antibodies overnight. After washing in 1 x T-PBS, blots were incubated with HRP-conjugated isotype-specific secondary antibody in 1 x T-PBS. After washing of the blots with 1 x T-PBS, bands were visualized with ECL detection kits by chemiluminescence and films (AGFA Curix HT and Amersham Hyperfilms).

2.2.6. Analysis of hypodiploid DNA content of the cells.

Around 150 000 cells per well were seeded in 6-well plate. The cells are treated with desired reagents for respective time. The cells and supernatants together were collected in 15 ml

falcon tubes, pelleted (200-400g for 5min) and washed 2 times with 1xPBS. Then 500 µl of cold hypotonic fluorochrome solution (Table 7) were gently added to the cells. Then the tubes were placed at 4°C in the dark for 36-48h before the flow cytometric analysis. The diploidy was measured by FACScan analysis (BD FACS Canto II).

2.2.7. Immunofluorescence microscopy

For detection of nuclear morphology and integrity of the cell membrane, $5x10^4$ cells of the respective cells were seeded per well in a 12-well plate. Following 24h of incubation for adherence, cells were stimulated as indicated in the figure legends for 24h. Subsequently, cells were incubated with Hoechst 33342 (5µg/ml; Polysciences Europe, Eppelheim, Germany) and SYTOX®Green (5pM; Invitrogen™, Molecular Probes™, Eugene, Oregon, USA) for 15min at 37°C immediately followed by phase contrast or fluorescence microscopy using a Zeiss HBO 50 microscope (Jena, Germany). Digital images were processed in an identical manner using the advanced SPOTSOFTWARE, Version 4.6 (Diagnostic Instruments Inc, MI, USA).

2.2.8. Co-immunoprecipitation of Caspase-8-bound complexes

For the precipitation of Caspase-8-bound proteins, $5x10^6$ cells were seeded and incubated for over night. Cells were washed once with medium at 37°C and subsequently pre-incubated for 1h with 100nM IAP antagonist at 37°C. Subsequently cells were treated with 20µg/ml of poly (I:C) for 2h. Stimulation was stopped by washing the monolayer four times with ice-cold PBS. Cells were lysed on ice by addition of 2 ml lysis buffer. After 30min lysis on ice, the lysates were centrifuged two times at 20,000xg for 5min and 30min, respectively, to remove cellular debris. A minor fraction of these clear lysates were used to control for the input of the respective proteins (TL- total lysates). Subsequently 1µg caspase-8 antibody (C-20) was added to all lysates. The caspase-8 containing complexes were precipitated from the lysates by co-incubation with 40µl protein G-beads (company) for 16 - 24h on an end-over-end shaker at 4°C. Ligand affinity precipitates were washed 4 times with lysis buffer before the protein complexes were eluted from dried beads by addition of standard reducing sample buffer and boiling at 95°C. Subsequently, proteins were separated by SDS-PAGE on 4-12% NuPAGE gradient gels, before detection of proteins by Western blot analysis.

3. Results

3.1. The role of cIAPs in TLR3-induced cell death

Recent studies show that TLRs are expressed in various types of tumours, such as multiple myeloma (Chiron et al., 2009) or melanoma (Salaun et al., 2007) and activation of tumour cell TLRs not only promotes tumour cell proliferation and resistance to apoptosis, but also enhances tumour cell invasion and metastasis (Huang et al., 2008). In contrast, active TLR3 signalling also promotes apoptosis induction (Salaun et al., 2006). Such dramatic differences indicate that the outcome of TLR3 signalling is controlled by intracellular downstream processes. Recently the probable role of TRL3 as a target for anti-cancer therapy has been proposed (Salaun et al., 2007). Previous studies (McEleny et al., 2004; Wang et al., 2005) have shown that cIAPs play a critical role in cell death signalling. Therefore it was proposed that cIAPs could be negative regulators of TLR3-induced cell death. The major goal of this study is to identify the functional relevance of cIAPs in regulating of apoptotic and non-apoptotic TLR3 signalling pathways.

3.1.1. Loss of cIAPs sensitizes to poly (I:C)-induced cell death

To identify the role of IAPs in regulation of TLR3 cell death signalling pathways the function of XIAP was inhibited and endogenous expression of cIAP1 and cIAP2 was downregulated by an IAP antagonist, compound A (Vince et al., 2007) in spontaneously transformed keratinocyte (HaCaT) and squamous cell carcinoma (SCC) cell lines. First the endogenous expression levels of cIAP1, cIAP2, XIAP, RIP-1 and RIP-3 proteins in HaCaT keratinocytes, MET1 SCC and metastatic A5RT3 SCC cell lines were compared to determine the basal IAP expression. These three cell lines show remarkable heterogeneous expression levels of IAPs. Both HaCaT and A5RT3 cells do not express XIAP at the protein level, whereas MET1 cells highly express XIAP (Figure 9 A). A5RT3 cells weakly express cIAP1, but high expression of cIAP2 was observed, compared to MET1 and HaCaT (Figure 9 A). RIP family members were also heterogeneously expressed in all three of described cell lines. RIP-1 was strongly expressed in HaCaT and MET1 cell lines, whereas in A5RT3 it was expressed at lower levels. RIP-3 was expressed in HaCaT, lower in A5RT3 and in MET1 cells RIP3 expression was absent. All three cell lines thus have different levels of IAP expression. To examine the effect of IAP antagonist on cIAP expression the HaCaT cells were incubated with IAP antagonist (Compound A) for the indicated time. This treatment induced a rapid degradation of cIAP1 and cIAP2 in all cell lines (Figure 9 B). In the next experiments the sensitivity to poly (I:C) was examined in IAP antagonist treated cells. HaCaT and MET1 cells, but not A5RT3 cells, show increased sensitivity to poly (I:C) induced cell death (Figure 9 C black bars). These results clearly show cIAPs as critical regulators of TLR3 induced cell

death in HaCaT cells independent of the anti-apoptotic function of XIAP. MET1 cells, in contrast to HaCaT and A5RT3, express high levels of XIAP. Therefore, it cannot be excluded that the observed increased sensitivity to poly (I:C) in the presence of IAP antagonist is due the inhibited function of XIAP (Figure 9 C black bars).

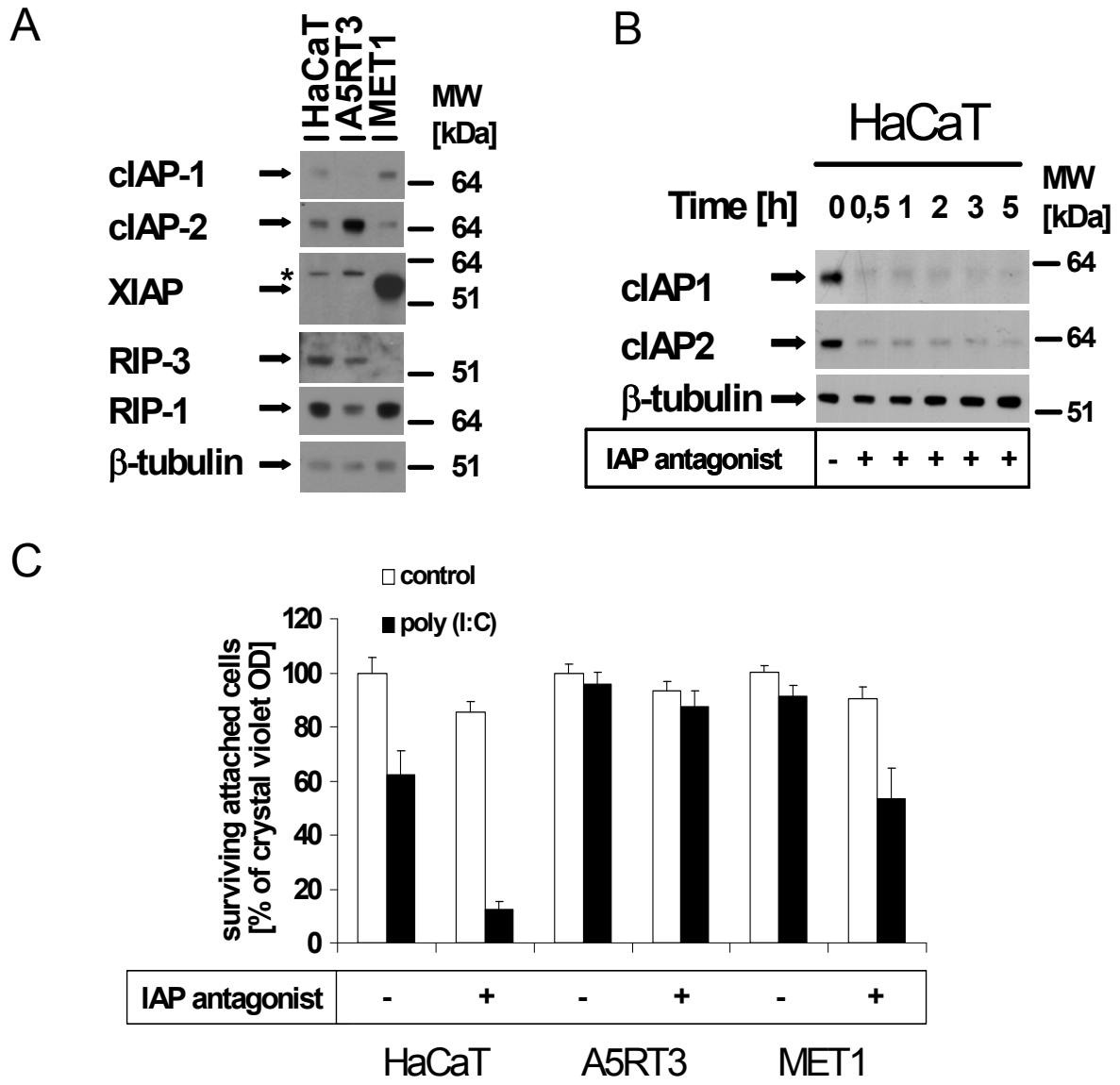


Figure 9. Loss of cIAPs sensitizes cells to poly (I:C)-mediated cell death. A. 5µg of total cell lysates from parental cells were analysed by Western-blot for the respective proteins. β-tubulin served as internal control for even protein loading. One of four representative experiments is shown (the lysates for these experiment were kindly provided by Dr. P. Geserick). The star indicates the non-specific band recognised by the antibody. **B.** HaCaT cells were treated for respective time with 100nM of IAP antagonist with further analysis of 5µg of the lysates by Western blot for the respective proteins, β-tubulin serves as a control. **C.** Parental HaCaT, MET1, or A5RT3 cells were pre-treated with 100nM IAP antagonist for 1h and subsequently stimulated with 50µg/ml of poly (I:C) in triplicate wells. Viability of cells was analyzed by crystal violet staining after 18-24h. Unstimulated cells served as control and were set as 100 % to allow comparison of poly (I:C)-independent sensitivity to IAP antagonist. The result of three independent experiments is shown, error bars describe standard error of mean (SEM).

To prove whether cIAP1 or cIAP2 is more critical for TLR3 apoptosis resistance, a specific shRNA knock-down of cIAP1 or cIAP2 in XIAP deficient HaCaT cells was performed. The sensitivity to poly (I:C) of these cell lines (Figure 10 B) was analyzed. In line with our previous findings (Geserick et al., 2009), a robust up-regulation of cIAP2 expression in cIAP1 knockdown cells was found (Figure 10 A) that might be explained by posttranslational regulation of cIAPs (Conze et al., 2005) or by increased NF-κB activation (J. Vince and

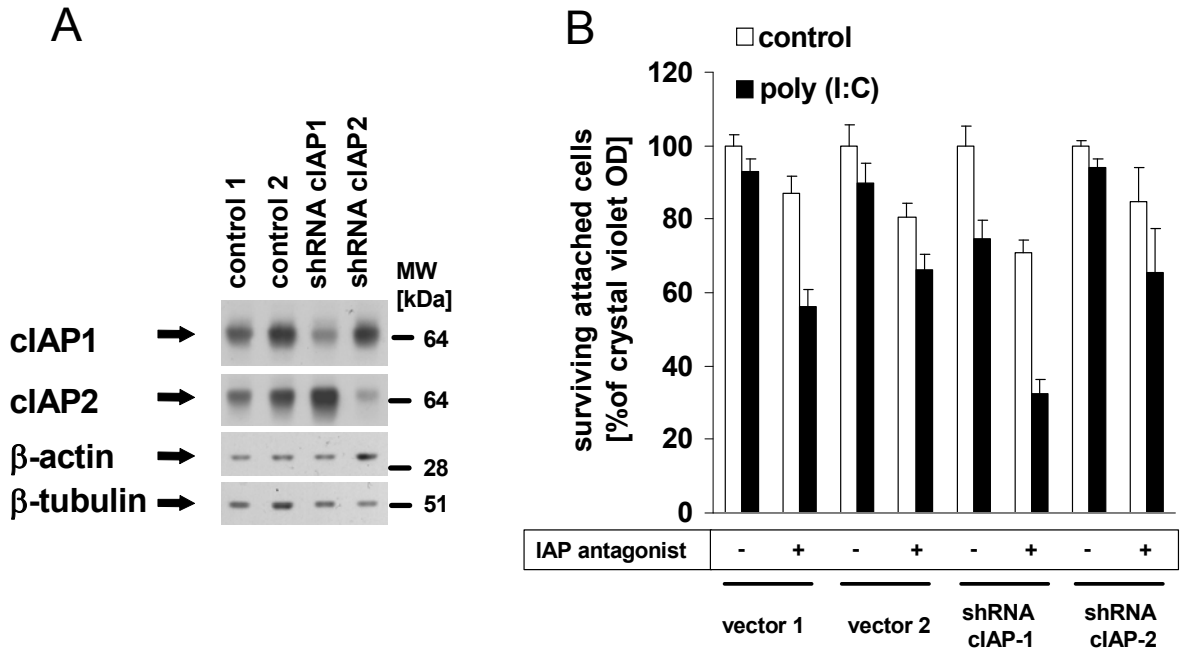


Figure 10. cIAPs regulate TLR3 induced cell death in XIAP independent manner. A. Parental HaCaT cells were either retrovirally transduced with specific shRNA for cIAP1 or cIAP2 or with the respective vector controls (control1 for cIAP1 and control2 for cIAP2). 5µg of total cell lysate from bulk infected cells were analysed for cIAP1 and cIAP2 expression by Western-blot analysis. β-tubulin and β-actin served as loading controls. **B.** For viability assays the transduced cells were either pre-treated IAP antagonist (100nM, 1h) and subsequently stimulated with 2µg/ml of poly (I:C) for 24h in triplicate wells. Viability of cells was analyzed by crystal violet assay after 18-24h. Unstimulated cells served as control and were set as 100 % to allow comparison of poly (I:C)-independent sensitivity. The summary of three independent experiments is shown and error bars show the standard error of mean (SEM).

J.Silke, unpublished observation). However knockdown of cIAP1 promoted poly (I:C)-induced cell death, providing further evidence that cIAP1 is a crucial negative regulator of TLR-induced cell death signalling (Figure 10 A white bars). Of note IAP antagonist further sensitized cIAP1 knockdown cells indicating that the additional downregulation of the upregulated cIAP2 level in this cell lines is relevant for the poly (I:C) induced cell death (Figure 10 B black bars). These results show that cIAP2 also controls the apoptotic TLR3 signalling pathway. In contrast, knockdown of cIAP2 did not affect cIAP1 expression, when compared to the respective control vector that substantially upregulated cIAP2. Despite this experimental limitation the marginal increase in cell death in these cells in cIAP2 knockdown cells suggests that cIAP2 is less important for the regulation of poly (I:C)-induced cell death

than cIAP1. Taken together these results demonstrate that cIAPs are important factors regulating TLR3-induced apoptosis resistance.

3.1.2. Loss of cIAPs sensitizes to poly (I:C)-induced cell death in a Caspase- and RIP-1-kinase dependent manner

Loss of cIAPs substantially increases the sensitivity of cells to TNF-induced cell death (Vince et al., 2007). In addition it was found that cIAPs also regulate TLR3-induced cell death (Figures 9-10). Furthermore, apoptotic cell death is strongly regulated by active caspases and necrotic cell death is critically controlled by the kinase activity of RIP-1. In addition, cIAPs are poor caspase inhibitors (Eckelman and Salvesen, 2006) but critical regulators of RIP-1 (Bertrand et al., 2008). Therefore, cIAPs could negatively regulate poly (I:C) induced cell death by controlling RIP-1 activity. To follow this hypothesis the experiments with the pan-caspase inhibitor zVAD-fmk and the recently described RIP-1-kinase inhibitor necrostatin-1 were performed (Degterev et al., 2008). zVAD-fmk completely blocked poly (I:C)-induced cell death in the presence of cIAPs (Figure 11 A panel 3). In the absence of cIAPs, both inhibitors were needed for protection against cell death implicating that a caspase-independent RIP-1 kinase-dependent form of cell death is operative whenever cIAPs are absent (Figure 11 A panel 10). To exclude the possibility that autocrine TNF-production induced by IAP antagonist (Vince et al., 2007) was responsible for the increased sensitivity to poly (I:C), a soluble TNF-R2-Fc was used to block autocrine TNF signalling. However TNF-R2-Fc treatment was ineffective to protect from poly (I:C), supporting a direct effect of cIAPs for TLR3-induced death signalling (Figure 11 A black bars). To further characterize the observed cell death the cell death morphology was analyzed by fluorescence microscopy studies in presence of caspase- and RIP-1-kinase inhibitors in poly (I:C)/IAP antagonist stimulated cells. The cells were stained with Hoechst-33342, to detect the chromatin condensation during the apoptosis and with SYTOX Green (high-affinity nucleic acid stain) which is able to stain nucleus only when the integrity of the membrane is disrupted. The SYTOX Green staining allows detection of late apoptotic events and necrosis. Increased numbers of typical apoptotic cells demonstrating membrane blebbing, DNA condensation and fragmentation were detectable after poly (I:C) stimulation even in the presence of RIP-1-kinase inhibitor whereas zVAD-fmk fully protected cell death and membrane integrity (Figure 11 B, left panel). In contrast, whenever IAPs were absent, zVAD-fmk only partially protected from poly (I:C) induced cell death. However a change in cell death morphology was observed as indicated by a rounded shape, a lack of DNA condensation and a retarded disruption of cell membranes (Figure 11 B, right panel). Combined addition of zVAD-fmk and Necrostatin-1 resulted in complete resistance to poly (I:C)/IAP antagonist mediated cell death (Figure 11 B lowest panel). These results

demonstrate that in the absence of cIAPs and caspase activity an alternative caspase-independent cell death signalling pathway was activated via TLR3. This TLR3 non-apoptotic cell death pathway critically depends on RIP-1 kinase activity. To further characterise the non-apoptotic cell death the release of high mobility group box 1 protein (HMGB-1) in the cellular supernatant was analysed as a characteristic of caspase-independent necrotic cell death (Scaffidi et al., 2002). HMGB-1 release, induced by TLR3-stimulation was unaffected by caspase inhibition, whereas Necrostatin-1 decreased HMGB-1 release to some extent. Most importantly, TLR3-induced HMGB-1 release was completely suppressed by the combination of zVAD-fmk and Necrostatin-1 (Figure 11 C).

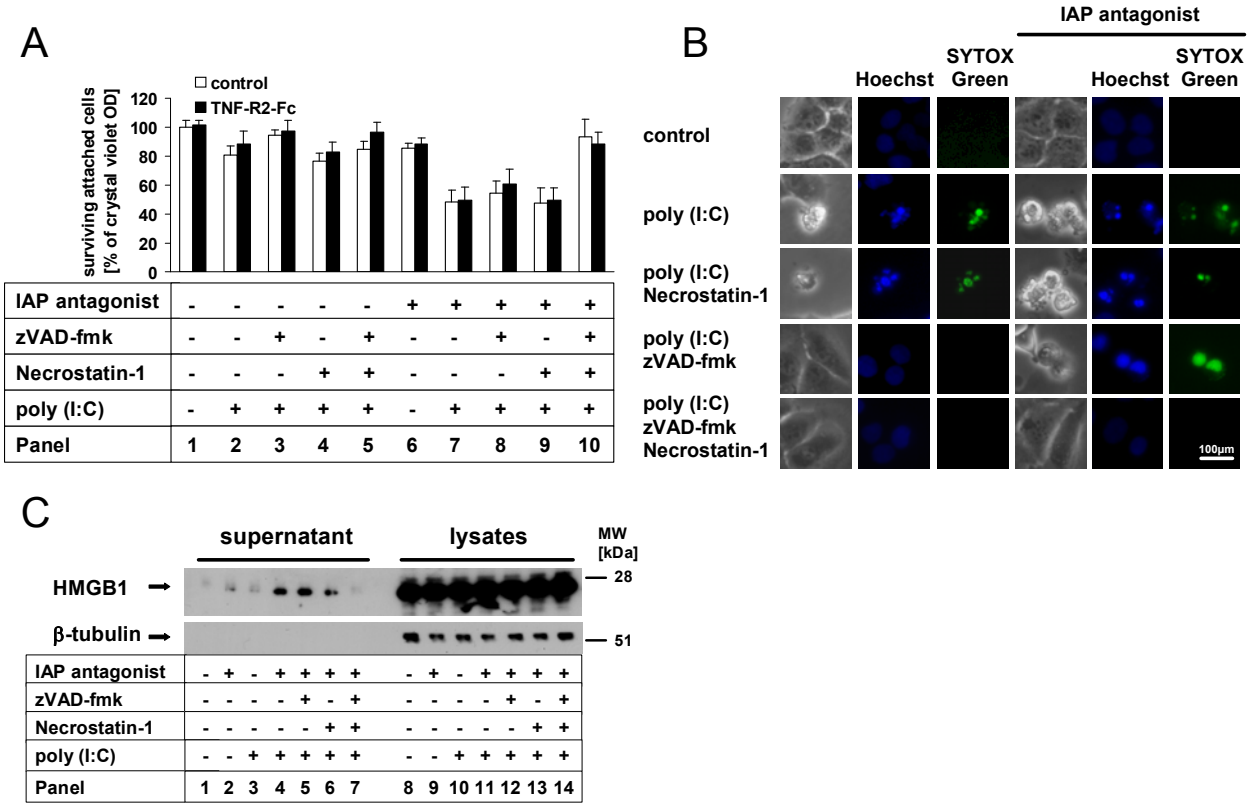


Figure 11. IAP antagonist sensitizes HaCaT cells to apoptotic and non-apoptotic cell death in caspase and RIP-1K dependent manner. A. Parental HaCaT cells were either separately or in combination pre-treated with 100nM IAP antagonist (1h), TNFR2-Fc (10µg/ml, 1h), zVAD-fmk (10µM; 1h), Necrostatin-1 (50µM, 1h), and subsequently stimulated with 2µg/ml of poly (I:C) in triplicate wells. Viability of cells was analysed and statistical analysis was performed as described before. The summary of three independent experiments is shown. **B.** For characterization of cell death morphologies, HaCaT cells were either pre-treated with zVAD-fmk (10µM, 1h), Necrostatin-1 (50µM, 1h) or IAP antagonist (100nM, 30min) and subsequently stimulated with poly (I:C) (2µg/ml) for 24h. Cells were stained with Hoechst-33342 (5µg/ml) and Sytox Green (5pM) for 15min at 37°C immediately followed by transmission and fluorescence microscopy. One representative of a total of three independent experiments is shown. **C.** HaCaT cells were stimulated as described in **D.** and the supernatants as well as the total cell lysates were analysed for HMGB-1 protein expression by Western blot analysis. β-tubulin served as loading control. One representative of three independent experiments is shown (This experiment was done together with Dr. P. Geserick).

These results suggest that RIP-1 kinase activity, in the absence of cIAPs, is critical for non-apoptotic TLR3-induced cell death signalling pathway. Furthermore, in the absence of caspase activity a necrotic cell death is unmasked in these cells. Taken together, our data demonstrate that cIAPs negatively regulate TLR3-mediated caspase-dependent as well as non-apoptotic (necrotic) cell death pathways. In the absence of cIAPs, RIP-1-kinase activity plays a critical role for the induction of non-apoptotic cell death.

3.1.3. Degradation of cIAPs by TWEAK sensitizes to poly (I:C)-induced cell death.

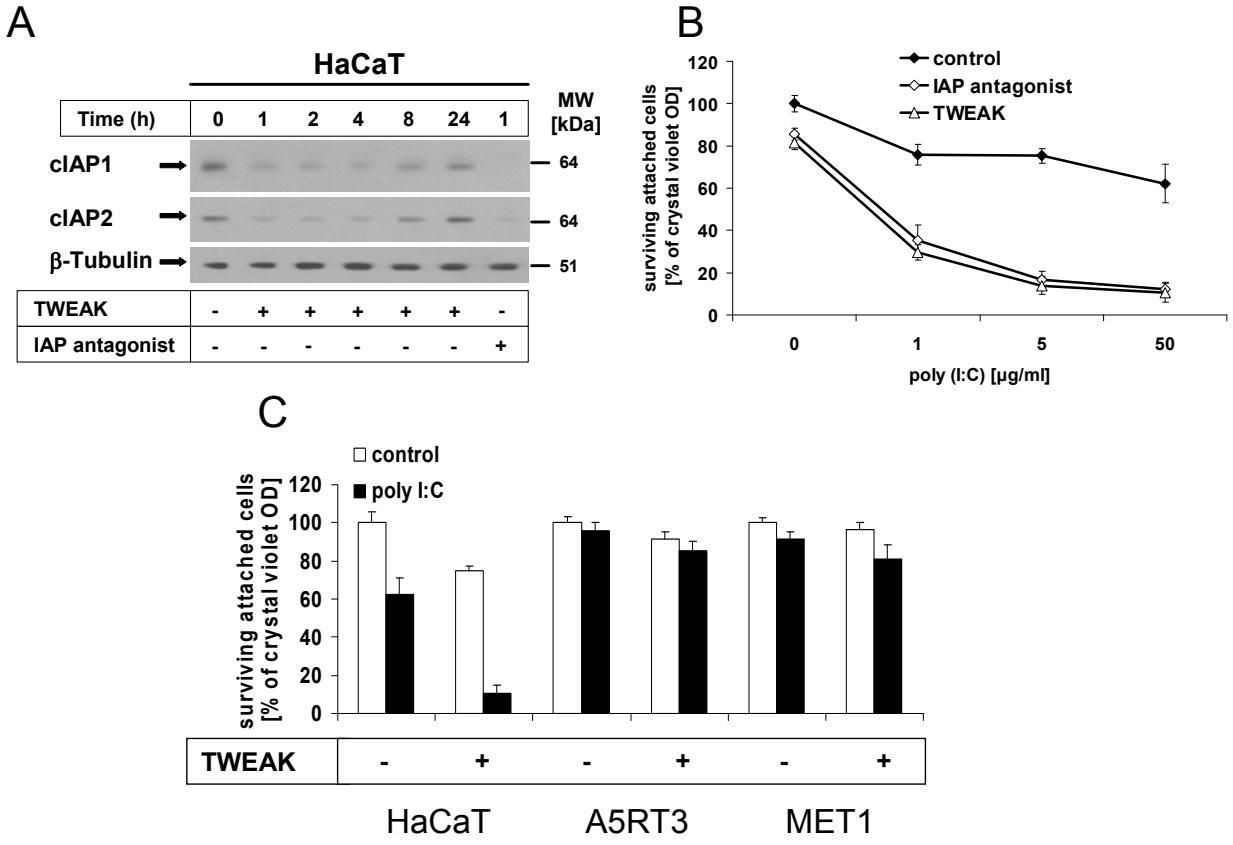


Figure 12. TWEAK sensitizes HaCaT, but not MET1 or A5RT3 to poly (I:C)-induced cell death. A. TWEAK leads to rapid downregulation of cIAP1 and -2 expression in HaCaT. Cells were stimulated with 0,5µg/ml TWEAK for the indicated time. Subsequently, total cellular lysates were analyzed for expression of cIAP1 or -2 by Western blotting. β-tubulin served as a loading control (this blot was kindly provided by Dr. P. Geserick). **B, C.** Viability of cells was analysed by crystal violet staining and statistical analysis was performed as described before. The summary of three independent experiments each condition is shown. Error bars represent SEM. **B.** For quantification of poly (I:C) induced cell death HaCaT cells were pre-incubated for 1h with either TWEAK or IAP antagonist alone and/or in combination with indicated concentrations of poly (I:C) for 24h in triplicate wells. **C.** For quantification of poly (I:C) induced cell death HaCaT, A5RT3 and MET1 cells were pre-incubated with 0,5µg/ml of TWEAK (1h) followed by poly (I:C) stimulation (50µg/ml for 24h). Error bars represent SEM.

To examine the role of cIAPs in a more physiologically relevant setting was employed. The

cytokine TWEAK (TNF-like weak inducer of apoptosis) was used. TWEAK binds to FN-14 receptor and the signal negatively regulates cIAP protein expression (Vince et al., 2008). Treatment with purified TWEAK substantially decreased the endogenous expression of cIAP proteins within one hour (Figure 12 A), but up-regulation of both cIAPs after 24h of TWEAK stimulation was observed. This could be due to the degradation of IAP antagonist of due to a possible increase in NF-κB activation and, therefore, increased transcription of NF-κB target genes (cIAPs).

The hypothesis was that TWEAK may also influence poly (I:C) induced cell death pathways similar to the IAP antagonist. TWEAK effectively enhanced poly (I:C) induced cell death in a similar manner as the IAP antagonist (Figure 12 B). The A5RT3 cells, which were not sensitized by IAP antagonist (Figure 9 C) were also not sensitized by TWEAK (Figure 12 C). Surprisingly the MET1 cells which could be sensitized by IAP antagonist (Figure 9 C) were not sensitized by TWEAK (Figure 12 C). This data indicate that the function of XIAP which is not affected by TWEAK, is also crucial for TLR3-induced cell death.

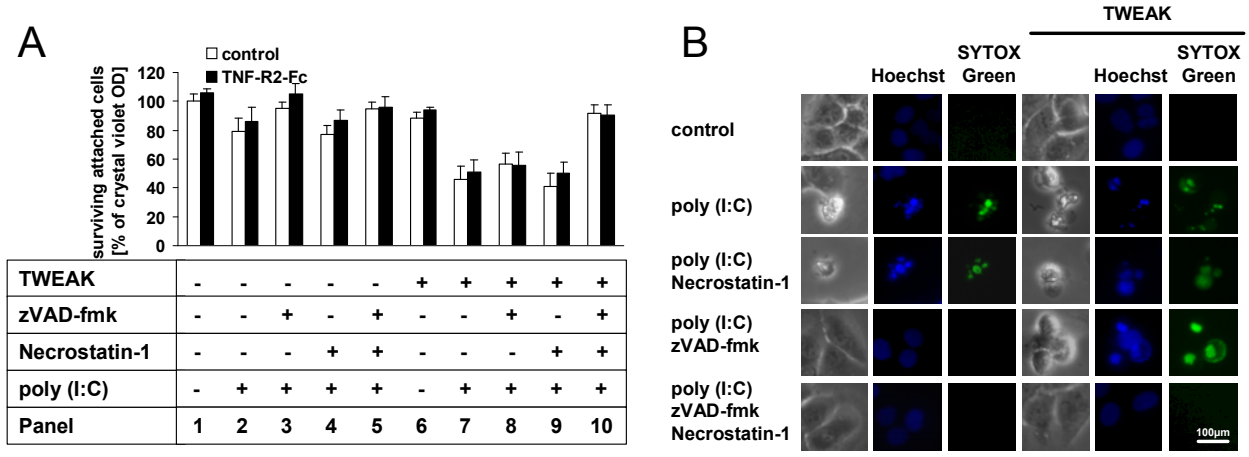


Figure 13. TWEAK sensitizes HaCaT cells to apoptotic and non-apoptotic cell death in caspase and RIP-1K dependent manner and independent of autocrine activated TNF cell death. A, B. For quantification and characterization of poly (I:C) induced cell death, parental HaCaT cells were either pre-treated or co-stimulated with zVAD-fmk (10µM, 1h), Necrostatin-1 (50µM, 1h), TNF-R2-Fc (10µg/ml, 1h, only in B.) or TWEAK (0,5µg/ml, 1 h) and subsequently stimulated with poly (I:C) (2µg/ml) for 24hrs in triplicate wells. **A.** For quantification of poly (I:C) induced cell death HaCaT cells were pre-incubated for 1h with either TWEAK or IAP antagonist alone and/or in combination with indicated concentrations of poly (I:C) for 24h in triplicate wells. Viability of cells was analysed by crystal violet staining and statistical analysis was performed as described before. The summary of three independent experiments each condition is shown. **B.** For characterization of cell death morphology, cells stimulated as described before were stained with Hoechst-33342 and Sytox Green dyes immediately followed by transmission and fluorescence microscopy.

This cell death, observed in HaCaT cells, upon treatment with TWEAK and poly (I:C) was independent of autocrine TNF signalling (Figure 13 A, black bars), which duplicated the results with IAP antagonist (Figure 11 A and B). At the same time this cell death was RIP-1 kinase- and caspase-dependent (Figure 13 A, white bars, and figure 13 B, right panel). Furthermore, similar to our findings with the IAP antagonist (Figure 11 B), apoptotic cell

death morphology of the cells upon poly (I:C) stimulation was detected (Figure 13 B left panel). In the presence of TWEAK the cells treated with poly (I:C) also showed apoptotic morphology during the cell death (Figure 13 B, right panel). This cell death was independent of RIP-1 kinase activity, since in presence of Necrostatin-1 the cell death had same apoptotic morphology (Figure 13 B right panel). However, when poly (I:C) induced apoptosis was suppressed by pan-caspase inhibitor zVAD-fmk, TWEAK promoted a necrotic cell death as shown by the markedly changed cell death morphology (Figure 13 B; right panel). This caspase-independent cell death was completely blocked by the combination of zVAD-fmk and Necrostatin-1 that highlights the essential role of RIP-1-kinase activity for necrotic cell death (Figure 13 B, right panel). TWEAK-FN14-mediated depletion of cIAPs thus reproduced our findings with IAP antagonist and highlighted the physiological relevance of cIAPs in TLR3 cell death pathways.

3.1.4. cFLIP isoforms negatively regulate poly (I:C) induced apoptosis but differentially control IAP antagonist/TWEAK-mediated caspase-dependent and independent cell death

Both members of the cFLIP protein family, $cFLIP_L$ and $cFLIP_S$, are competent inhibitors of death ligand (DL) induced cell death mediated by direct suppression of Caspase-8 activation in and release from the death inducing signalling complex (Geserick et al., 2008; Geserick et al., 2009). Caspase-8 has been suggested to be a crucial activator molecule for the execution of TLR-3 induced apoptosis (Kaiser and Offermann, 2005). To investigate the functional role of caspase-8 in the context of IAP antagonist-induced apoptotic and necrotic cell death, the caspase-8 inhibitors $cFLIP_L$ or $cFLIP_S$ were overexpressed in HaCaT cells (Geserick et al., 2008). The sensitivity to poly (I:C)/IAP antagonist were analysed in these cells (Figure 14 A). However, vector control transduced HaCaT cells are resistant to poly (I:C) mediated cell death (Figure 14 B, white bars, panel 2 and Figure 11 B, left panel). In line with the findings in parental HaCaT cells (Figure 11 A, white bars), loss of cIAPs promoted increased poly (I:C)-induced cell death (Figure 14 B; white bars; panel 7) in a RIP-1-kinase independent manner (Figure 14 B; white bars; panel 9 and Figure 14 C; left panel). This cell death can be partially inhibited by zVAD-fmk (Figure 13 B; white bars; panel 8) that promotes the switch to the necrotic cell death (Figure 14 C; left panel) and is completely suppressed by both inhibitors (Figure 14 B; white bars; panel 10) as also shown by microscopic analysis (Figure 14 C, left panel). Interestingly, a substantial basal sensitivity to the IAP antagonist was observed in cells overexpressing $cFLIP_S$ (Figure 14 B; gray bar, panel 6), but no increased sensitivity to poly (I:C) was detected (Figure 14 B; gray bar, panel 7). These data demonstrate that in the absence of cIAPs $cFLIP_S$ itself promotes a caspase-independent form of cell death (Figure 14 C, middle panel), but sufficiently protects against TLR-3

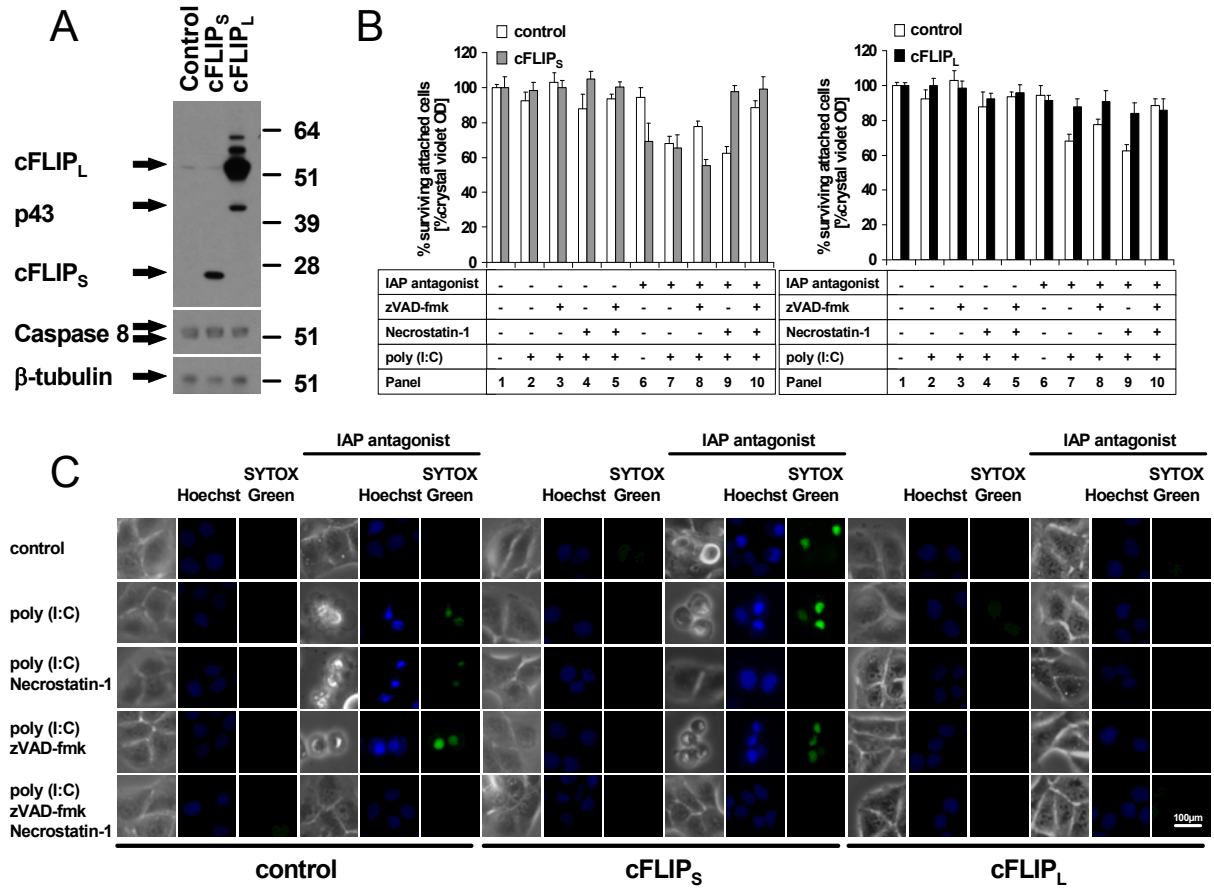


Figure 14. $cFLIP_L$ protects cells from poly (I:C)/IAP antagonist induced cell death, but $cFLIP_S$ induce spontaneous and poly (I:C) induced non-apoptotic cell death in the presence of IAP antagonist. A. For overexpression of cFLIP proteins, HaCaT cells were retrovirally transduced with vectors containing cDNA coding for $cFLIP_L$, $cFLIP_S$ or vector as control. The transduced cells were lysed with subsequent analysis of 5µg of protein by Western blot for indicated proteins. β-tubulin served as a loading control. One of three independent experiments is shown. **B.** The transduced cells were pre-treated or co-stimulated with zVAD-fmk (10µM; 1h), Necrostatin-1 (50µM, 1h), and IAP antagonist (100 nM, 1 h) and subsequently stimulated with 2µg/ml of poly (I:C) for 24h in triplicate wells. Viability was assayed by crystal violet staining. The summary of three independent experiments is shown and error bars describe SEM. **C.** For characterization of cell death morphology, bulk infected HaCaT cells were stimulated as described in **(B.)** followed by Hoechst-33342 and Sytox Green staining immediately followed by transmission and fluorescence microscopy.

mediated apoptosis. However, necrostatin-1 conferred complete protection against IAP antagonist induced cell death (Figure 14 B, gray bars, panel 9) and necrotic cell death morphology (Figure 14 C, middle panel), indicative of the important role of RIP-1-kinase activity in this cell death process. In contrast, cells expressing $cFLIP_L$ are fully resistant to any TLR-3-mediated cell death signalling (Figure 14 B, black bars and Figure 14 C; right panel). These results show a differential regulatory function of the cFLIP proteins in TLR-3 cell death signalling.

To further investigate the role of cFLIP and its isoforms in TLR3 signalling in the absence of IAPs TWEAK was used. In line with the data gained with IAP antagonist, shown in Figure 14,

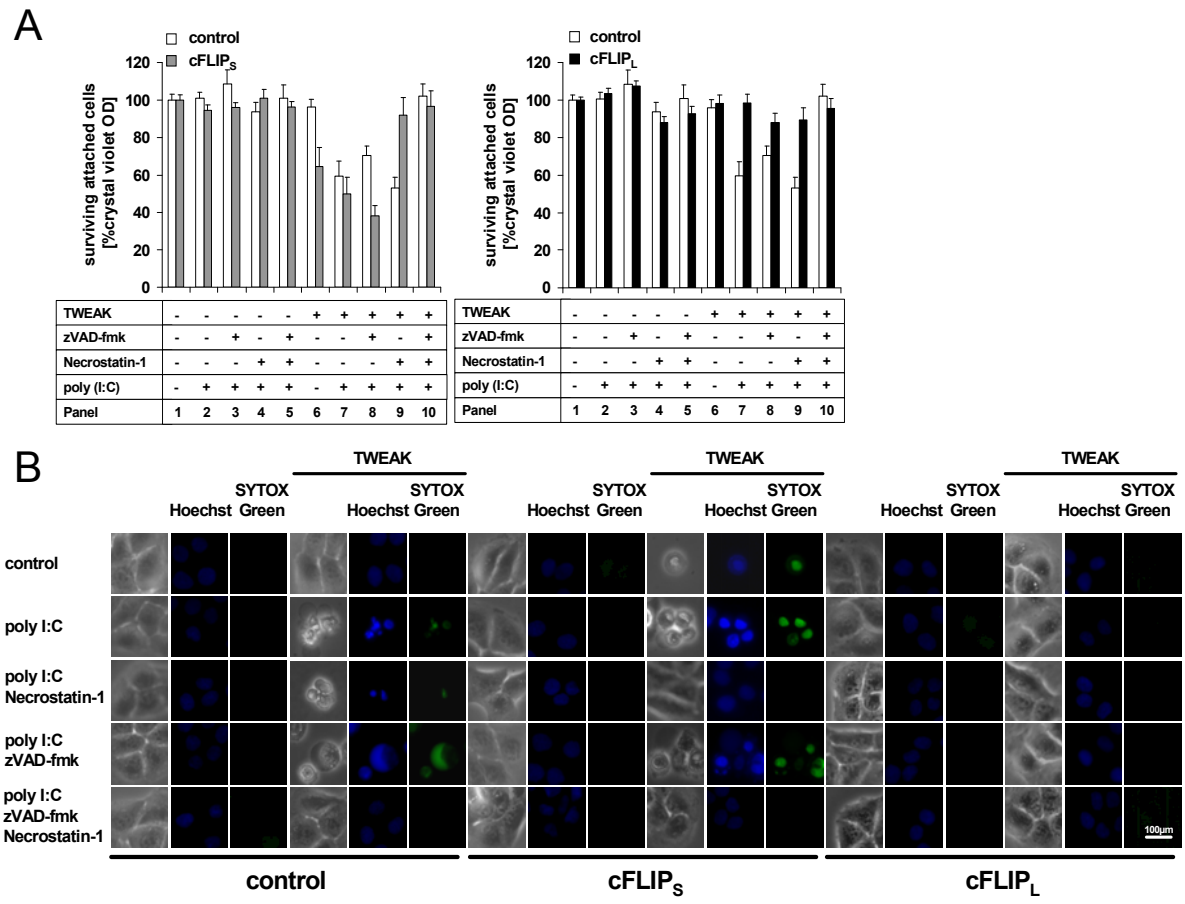


Figure 15. TWEAK duplicates findings obtained with IAP antagonist for cFLIP overexpressing HaCaT cells. A and B. For quantification and characterization of poly (I:C)-induced cell death, cells expressing $cFLIP_L$ and $cFLIP_S$ were either pre-treated or co-stimulated with zVAD-fmk (10µM, 1h), Necrostatin-1 (50µM, 1h), or TWEAK (0,5µg/ml, 1 h) and subsequently stimulated with poly (I:C) (2µg/ml) for 24h in triplicate wells. **A.** Viability of cells was analysed by crystal violet staining and statistical analysis was performed as described before. The summary of three independent experiments for each cell line under each condition is shown, the error bars represent SEM. **B.** For characterization of cell death morphology, cells stimulated as described before were stained with Hoechst-33342 and Sytox Green dyes immediately followed by transmission and fluorescence microscopy.

$cFLIP_L$ overexpression fully protected the cells from cell death induced by poly (I:C) as well as in the absence of IAP induced by TWEAK (Figure 15 A black bars and B, right panel). TWEAK, similar to IAP antagonist, induced necrosis in $cFLIP_S$ expressing cells even in the absence of TLR-3 stimulation (Figure 15 B; middle panel and Figure 15 A; gray bars; lane 6 and 9), a further increase of this cell death was achieved by adding poly (I:C) or zVAD/poly (I:C) (Figure 15 A, compare panels 6 , 7, and 8; gray bars and Figure 15 B; middle panel). Furthermore, this cell death was fully blocked by Necrostatin-1, indicating that $cFLIP_S$ promotes necrotic cell death whenever cIAPs are absent (Figure 15 A, gray bars; panels 9 and 10; and Figure 15 B, middle panel).

Taken together, these data suggest that cIAPs block TLR3-induced cell death signalling both in a caspase and RIP-1 kinase dependent manner. Thus downregulation of cIAPs, either by

synthetic IAP antagonists or by ligand-induced degradation as exemplified by TWEAK, is able to overcome apoptosis resistance in TLR3 apoptosis signalling. Moreover, these data suggest that the stoichiometry of $cFLIP_L$ and $cFLIP_S$ is important to determine the sensitivity to caspase-independent cell death once cIAPs are inactivated in a given cell irrespective of the mode of their degradation.

3.1.5. cFLIP isoforms differentially influence the modification and composition of the intracellular TLR3 signalling complexes

The observed switch from the apoptotic to the necrotic cell death in cells stimulated with IAP antagonist/poly (I:C) in the absence of caspase activity led to the hypothesis that molecules involved in the TLR3 cell death signalling cascade are either differentially modified or recruited in potentially different apoptotic or necrotic intracellular signalling platforms. To identify the differences in the function of $cFLIP_L$ and $cFLIP_S$ for the TLR3 cell death signalling platform caspase-8 immunoprecipitation was performed (Figure 16 A). Caspase-8 was chosen since it was proposed to be the key molecule on the apoptotic pathway in TLR3 signalling (Kaiser and Offermann, 2005). Therefore caspase-8 could be present in the receptor signalling complex and molecules associated with apoptotic and necrotic cell death could be identified. Since $cFLIP_S$ but not $cFLIP_L$ spontaneously induced caspase-independent cell death in the absence of cIAPs, the hypothesis was that resistance to and alterations of the poly (I:C) induced cell death characteristics might be explained by modification, activation and recruitment of molecules involved in the TLR-3 cell death signalling platform. It was found that upon TLR3 stimulation an intracellular complex, consisting of Caspase-8, TRIF, RIP-1 FADD and cFLIP isoforms is formed (Figure 16 B panel 3). Interestingly in the absence of cIAPs this complex formation was also detected at a lower level, although this complex lacked TRIF (Figure 16 B panel 2). Both cIAPs were absent in the complex, indicating that modification of molecules by cIAPs is probably taking place outside of this complex. In the absence of cIAPs, but in the presence of TLR3 stimulation the complex was formed more robustly than in presence of IAPs (Figure 16 B panel 4). This indicates that cIAPs are blocking the formation of this complex and therefore the TLR3-induced signalling.

When compared to control cells, the absence of cIAPs in $cFLIP_S$ overexpressing cells led to a robustly increased Caspase-8 binding complex consisting of TRIF, RIP-1 and FADD (Figure 16 B, compare panels 4 and 8). Interestingly, IAP antagonist alone in $cFLIP_S$ expressing cells also let to strongly increased spontaneous complex formation that contained caspase-8, FADD, $cFLIP_S$ and RIP-1, and lacked TRIF (Figure 16 B; panel 6). These results indicate that TRIF is not critical for the induction of IAP antagonist mediated spontaneous necrotic cell death and propose a TLR-3 stimulation independent complex formation

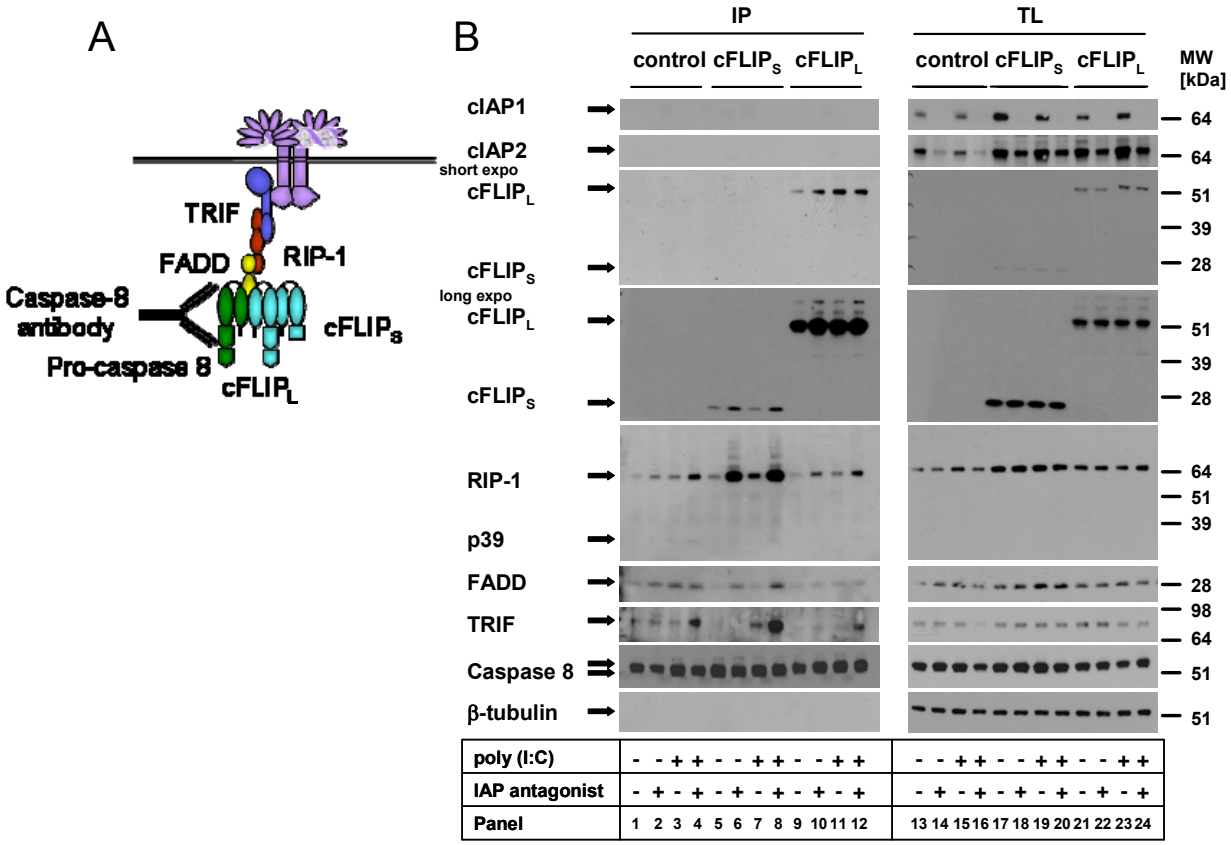


Figure 16. cIAPs block formation of intracellular TLR3 signalling complex. $cFLIP_S$ enhances formation of the complex in the absence of IAPs. A. The schematic picture shows the intracellular complex, precipitated with Caspase-8 antibody. **B.** For caspase-8-containing protein complexes analysis, the complex was immune precipitated (IP) from bulk infected HaCaT cells expressing $cFLIP_L$, $cFLIP_S$ or vector after stimulation with poly (I:C) (40µg/ml for 2 h). Equal amounts of IP and total cell lysates (TL) were subsequently analyzed by Western blot for the indicated molecules.

whenever $cFLIP_S$ is dominantly expressed. However, in $cFLIP_L$ expressing cells a partial suppression of complex formation upon IAP antagonist/poly (I:C) stimulation was observed. This correlated with decreased recruitment of FADD, TRIF, $cFLIP_L$, caspase-8 and decreased RIP-1 modification in the absence of cIAPs when compared to control cells (Figure 16 B, compare panels 4 and 12). These results indicate that $cFLIP_L$ inhibit TLR-3 mediated cell death by limiting the formation of caspase-8 interaction with RIP-1 and TRIF.

the data suggest diverse impacts of different cFLIP isoforms for TLR3 signalling complex formation and RIP-1 recruitment to this complex. The enhanced RIP-1 recruitment to the signalling complex in $cFLIP_S$ could be responsible for increased necrotic cell death.

3.1.6. cIAPs are crucial negative regulators of RIP-1 recruitment in an apoptotic TRIF containing Caspase-8-interacting complex

To further investigate the relevance of RIP-1 molecule for TLR 3 signalling HaCaT cells with manipulated RIP-1 expression were analysed for sensitivity to poly (I:C) in presence or absence of cIAPs. Since cIAPs regulate RIP-1 function and block the recruitment of RIP-1 to

the TLR3 complex the hypothesis arose that cIAPs promote resistance to poly (I:C) induced cell death by blocking a pro-death function of RIP-1 in TLR-3 signalling. To test this hypothesis HaCaT cells with decreased levels of RIP-1 were generated (Figure 17 A) and

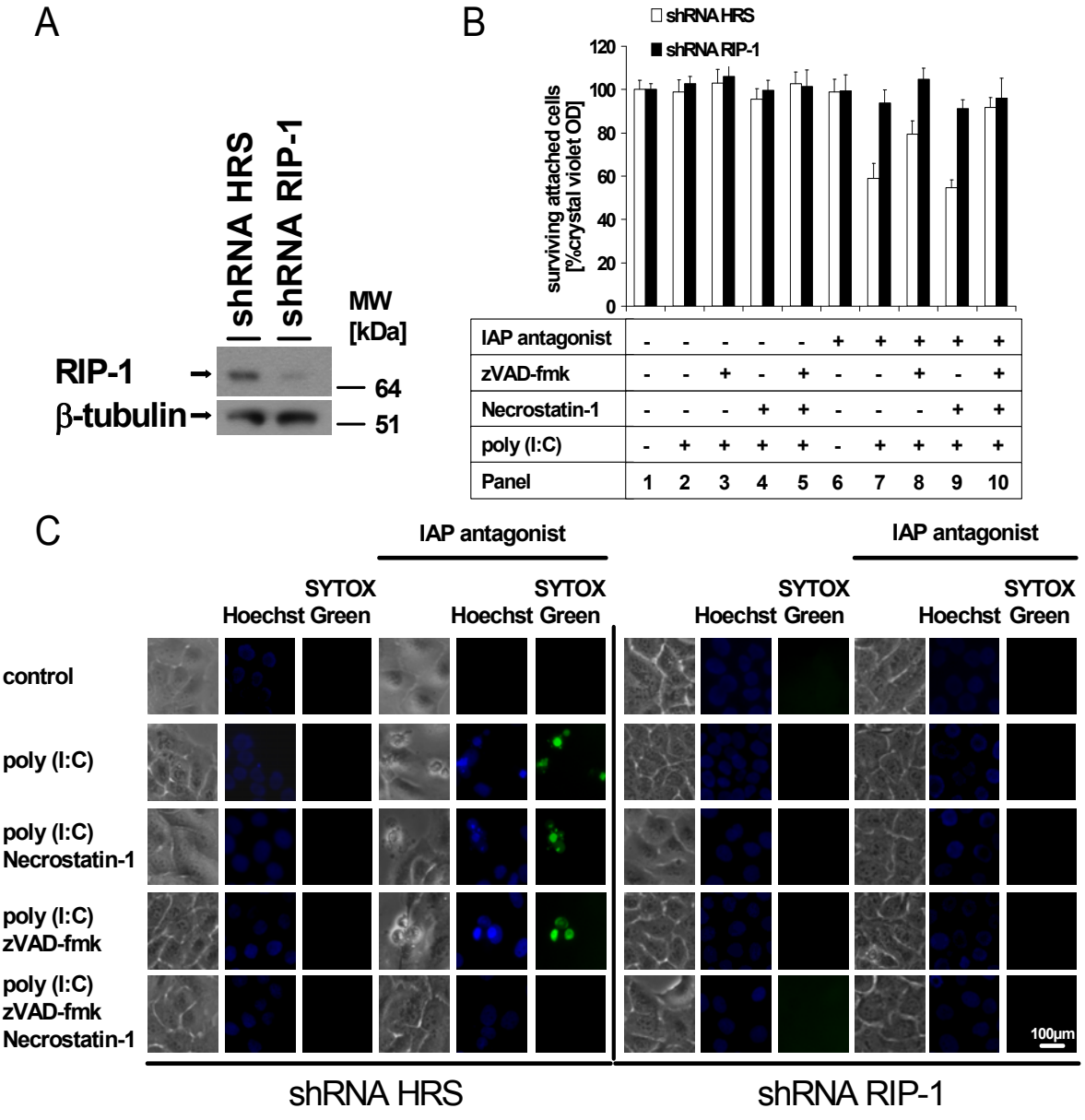
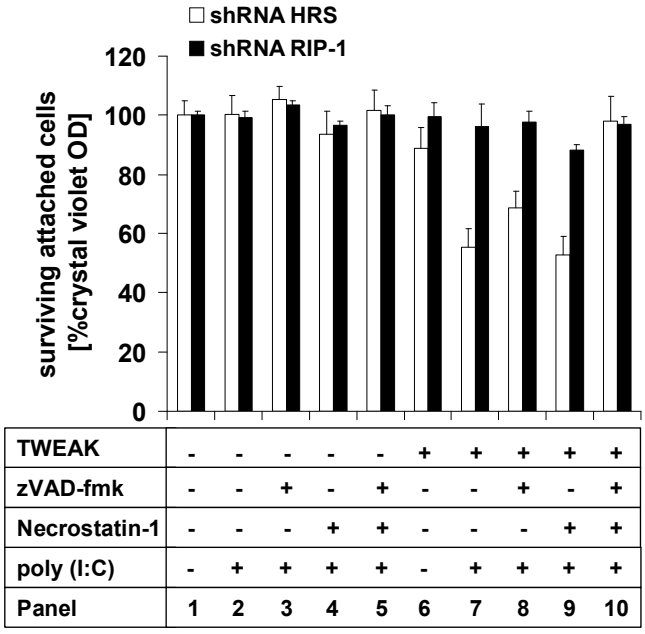


Figure 17. RIP-1 is required for poly (I:C)/IAP antagonist-induced cell death.
A. HaCaT cells were retrovirally transduced with HRS and RIP-1 specific shRNA. Knockdown of RIP-1 protein expression was analysed by Western-Blot analysis. β-tubulin expression served as an internal loading control. One representative of three independent experiments is shown. **B.** For viability analysis, bulk infected HaCaT cells were either pre-treated with zVAD-fmk (10µM, 1h), Necrostatin-1 (50µM, 1h) or IAP antagonist (100 nM, 30min) and subsequently stimulated with poly (I:C) (2µg/ml) for 24h followed by crystal violet staining. The summary of three independent experiments is shown, the error bars represent SEM. **C.** For characterization of cell death morphology, bulk infected HaCaT cells were stimulated as described in **(B.)** followed by Hoechst-33342 and Sytox Green staining immediately followed by transmission and fluorescence microscopy.

their sensitivity to poly (I:C)/IAP antagonist induced cell death (Figure 17 B) was quantified.

Interestingly, infection with retroviral vectors substantially protected cells from poly (I:C)-induced cell death, when compared to parental cells (Figure 17 B white bars, C left panel). This induced resistance to cell death might be explained by the retroviral shRNA transfer into the cells, as plausible by the fact that TLR3 recognize viral derived dsRNA, which in turn could induce NF-κB activation and therefore increased protection form apoptosis. However, in the absence of cIAPs control-infected cells were still substantially sensitized to poly (I:C) induced apoptotic cell death (Figure 17 B white bars, C left panel). In contrast, the knockdown of RIP-1 fully protected cells from all types of TLR3-induced cell death in presence and absence of cIAPs (Figure 17 B black bars; C right panel). These data provide evidence that in the absence of cIAPs RIP-1 is a crucial regulator of non-apoptotic TLR-3-mediated cell death.



TWEAK	-	-	-	-	-	+	+	+	+	+
zVAD-fmk	-	-	+	-	+	-	-	+	-	+
Necrostatin-1	-	-	-	+	+	-	-	-	+	+
poly (I:C)	-	+	+	+	+	-	+	+	+	+
Panel	1	2	3	4	5	6	7	8	9	10

Figure 18. RIP-1 is required for poly (I:C)/TWEAK-induced cell death. For viability analysis, bulk infected HaCaT cells were either pre-treated with zVAD-fmk (10μM, 1h), Necrostatin-1 (50μM, 1h) or TWEAK (0,5μg/ml, 1 h) and subsequently stimulated with poly (I:C) (2μg/ml) for 24h followed by crystal violet staining. The summary of three independent experiments is shown, the error bars represent SEM.

In addition, the stimulation of RIP-1 knockdown cells with TWEAK duplicated the findings with the IAP antagonist. These results indicate that during execution of other signalling pathways cIAP levels could be altered and this could trigger RIP-1 dependent TLR3 death signals. TWEAK was able to sensitize control cells to poly (I:C)-induced cell death (Figure 18, white bars, panel 7), which could be fully blocked by inhibiting both caspases and RIP-1 kinase activity (Figure 18, white bars, panel 10). In the absence of RIP-1 full resistance to all types of cell death induced by poly (I:C) as well as TWEAK/poly (I:C) was observed (Figure 18 black bars). Thus, RIP-1 is not only a crucial regulator of necrosis, as shown in previous experiments, but also a critical pro-apoptotic regulator of TLR3 signalling that is negatively regulated by cIAPs.

To further determine the intracellular regulation of poly (I:C)-induced cell death by cIAPs and more specifically the impact of RIP-1 in this signalling pathway, the caspase-8-interacting protein complexes in both control and RIP-1 knockdown cells were analyzed (Figure 19 A). TLR3 ligation induced the interaction of RIP-1, FADD and marginal amounts of TRIF with endogenous caspase-8 (Figure 19 B panel 3) in control cells, similar to control

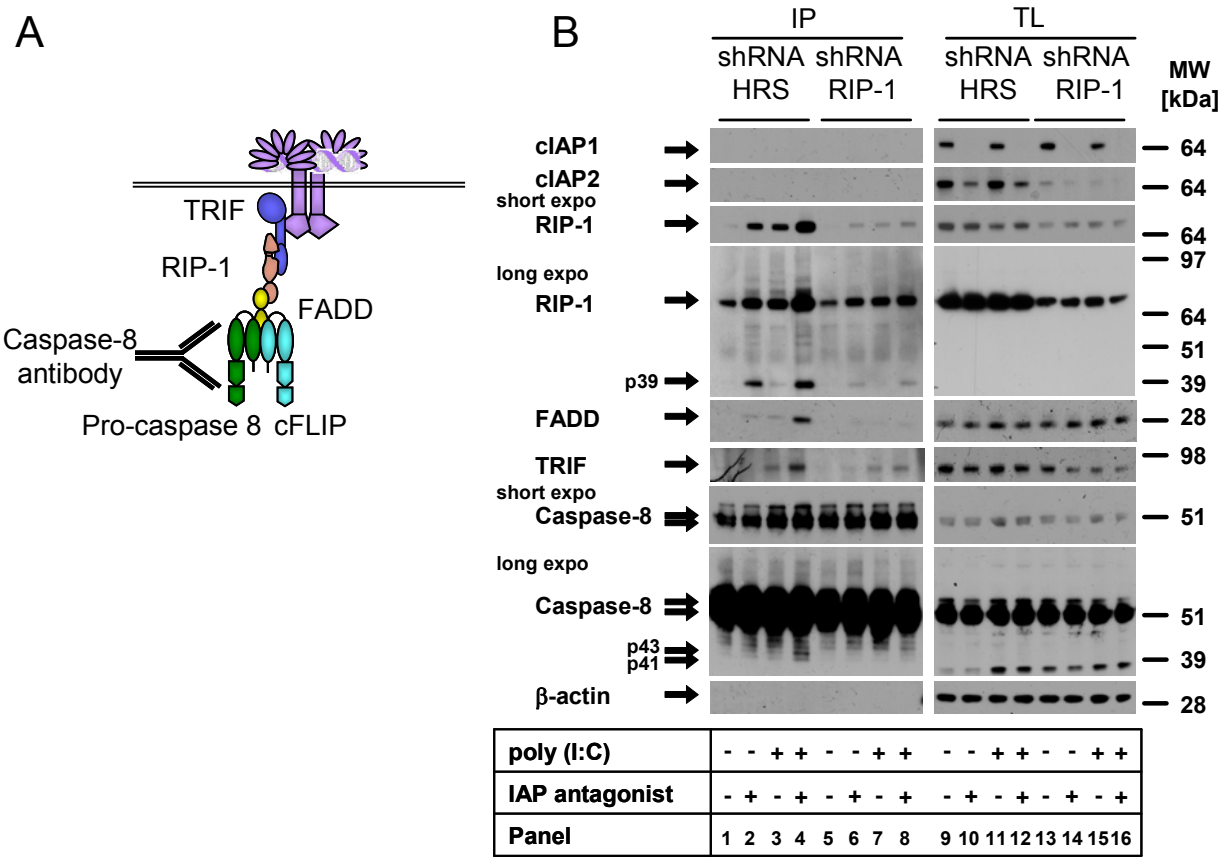


Figure 19. RIP-1 is required for intracellular TLR3 complex formation. A. The schematic picture showing the composition of the intracellular complex in RIP-1 downregulated cells. **B.** For complex analysis, the caspase-8-containing complex was precipitated from bulk infected HaCaT cells upon stimulation with poly (I:C) (40µg/ml, 2 h) using caspase-8 co-immuneprecipitation (IP). Equal amounts of immunoprecipitates were subsequently analyzed by Western blotting for the indicated molecules. Equal amounts of total cellular lysates (TL) were loaded on the same gels to allow comparison of signal strength between IP and TL.

cells in the cFLIP experiments (compare Figure 16 B). Interestingly, in the presence of IAP antagonist alone, which did not led to cell death, (compare figure 8 B panel 6), a robust caspase-8 interaction with RIP-1, but not TRIF and to a lesser extent FADD was noted (Figure 19 B; panel 2), comparable to the cFLIP experiments. In contrast TLR3-induced interaction of RIP-1, FADD and TRIF to the intracellular caspase-8 interacting complex in the absence of cIAPs was substantially increased (Figure 19 B; panel 4). These results demonstrate a cIAP-dependent negative regulation of TLR-3 induced cell death by limiting TRIF and RIP-1 recruitment to the caspase-8 containing complex. In line with our functional data (compare figure 8 B, black bars), knockdown of RIP-1 blocked the formation of this signalling platform from caspase-8 complex, as indicated by the blocked ligand/IAP

antagonist-induced recruitment of TRIF into this complex (Figure 19 B, panel 8). Interestingly, the cIAP2 expression level in RIP-1 downregulated cells was significantly decreased (Figure 19 B), indicating that RIP-1 is promoting the expression of cIAP2, probably by participating in the NF-κB activation.

These data show that RIP-1 expression is required for TLR-3 cell death signals. Furthermore, these experiments demonstrate that RIP-1 is critical adapter molecule for activation and formation of the cell death-inducing TLR-3 signalling complex. Loss of RIP-1 protects from all death signalling and interferes with formation of the death promoting signalling platform.

3.1.7. RIP-1 overexpression induces cell death

In previous experiments the crucial role of RIP-1 for the TLR3 cell death signalling was identified (Figure 17-19). Since downregulation of RIP-1 in HaCaT cells significantly reduced sensitivity to TLR3 induced cell death, the hypothesis arose that increased RIP-1 levels could sensitize cells to pro-cell death stimuli.

To further investigate the impact of RIP-1 and its enzymatic activities for TLR3 cell death signalling pathways overexpression of wild type RIP-1 (WT) as well as the kinase dead mutant (K45A) or the cleavage site mutant (D324K) was performed (Figure 20 A). Therefore the respective cDNAs for inducible expression of wild type RIP-1 and it mutants were subcloned from the pcDNA 3 vector into the PF 5x UAS MCS W SV40 lentiviral vector by using NheI and XbaI (Figure 20 B). The successfully subcloned constructs were analyzed by specific restriction with Bam HI (Figure 20 C), and subsequently verified by sequencing. The vectors were used for generation of lentiviral supernatants and subsequent transduction of HaCaT cells.

The overexpression of RIP-1 and its mutants was analyzed after treatment with different concentrations of H-Tamoxifen for 24h. In all three cell lines RIP-1 was overexpressed in comparable amounts upon treatment with 10nM and higher concentrations of H-Tamoxifen (Figure 21 A). Surprisingly the induced RIP-1 protein was slightly bigger then the endogenous one. Since 10nM of H-Tamoxifen was the lowest concentration sufficient for induction of RIP-1 expression at physiological level, and considering that high overexpression might induce non-specific effects, 10nM of H-Tamoxifen was used for subsequent experiments.

Induction of wild type as well as the mutant variants of RIP-1 decreased the cell viability when compared to control cells (Figure 21 B panel 2), indicating that RIP-1 is a crucial regulator of cell death. To analyze the caspase- and RIP-1 kinase dependency of RIP-1 induced cell death the pan-caspase inhibitor zVAD-fmk and the RIP-1 kinase inhibitor Necrostatin-1 were used. The inhibition of caspase activity partially blocked RIP-1 WT- and

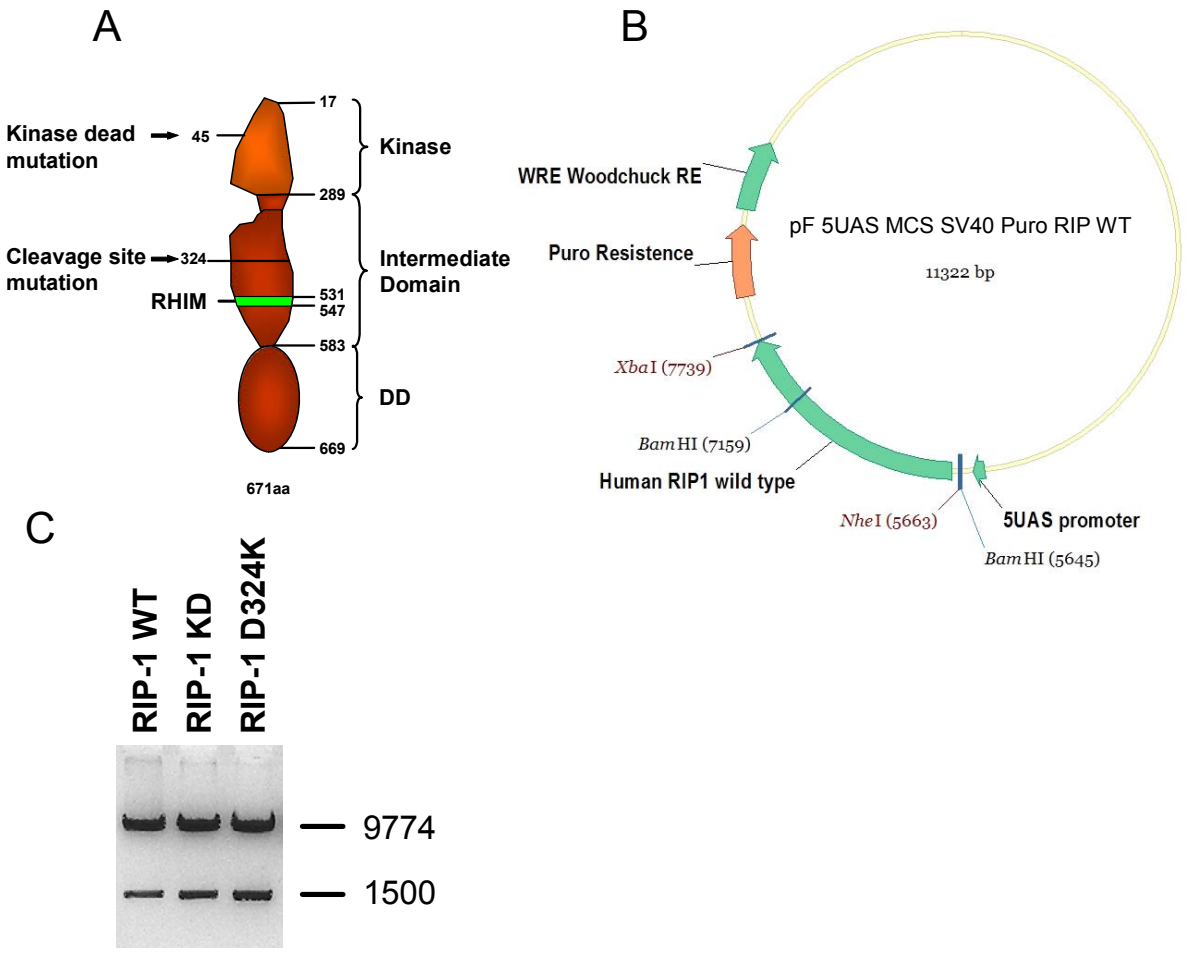
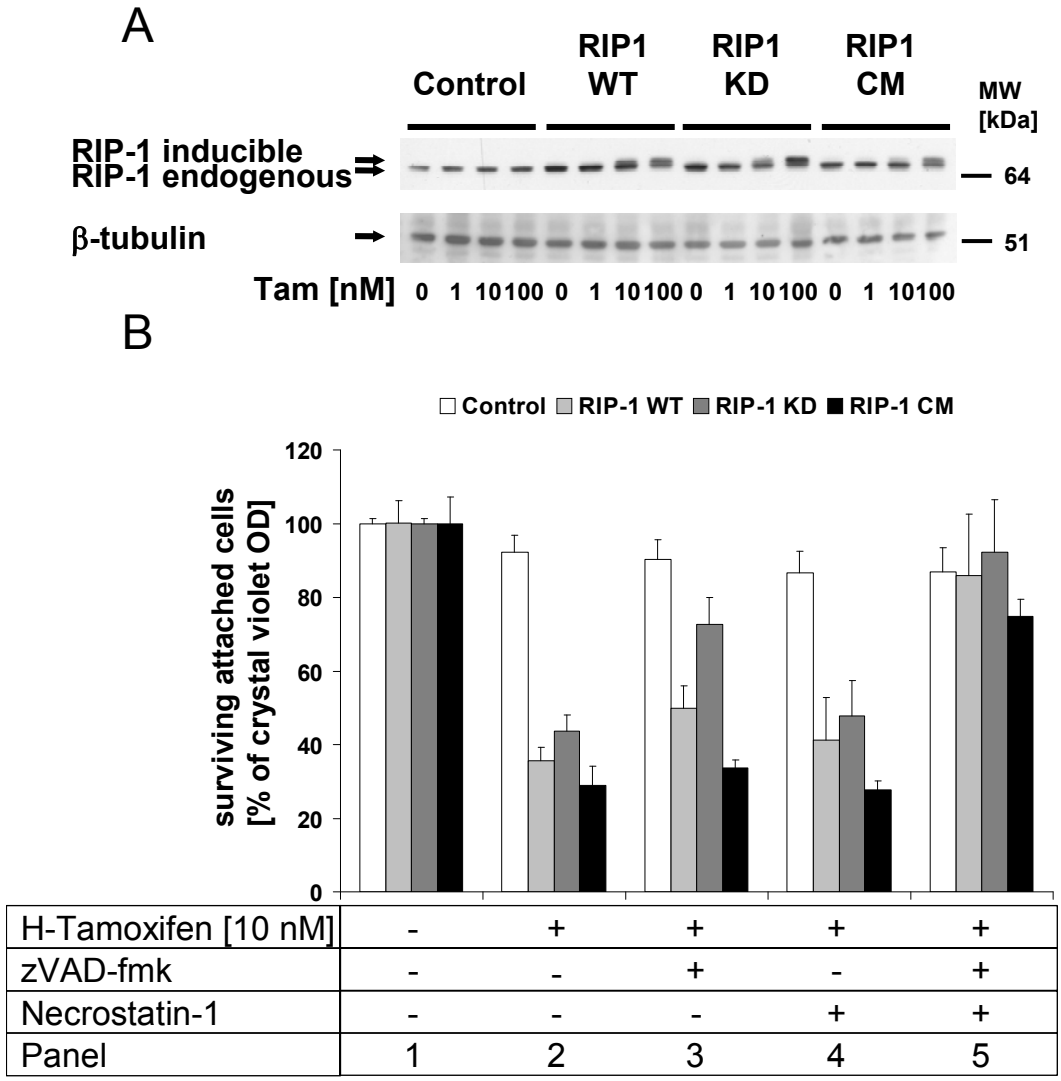


Figure 20. Cloning of wild type and mutant variants of RIP-1 into the inducible expression vector system. A. Schematic domain structure of RIP-1. The sites of kinase and cleavage site mutations are marked. **B.** The map of the inducible lentiviral RIP-1 wild type construct as an example. **C.** 1µg of the final constructs were digested with *Bam* HI followed by separation on the agarose gel.

CM-induced cell death, whereas in RIP-1 KD cells more than 70% of the cells were protected (Figure 21 B panel 3). This result demonstrates that RIP-1 kinase dead mutant-induced cell death is caspase-dependent, whereas when the kinase domain is active (RIP-1 WT and RIP-1 CD) the observed cell death is not completely caspase dependent. The blocking of RIP-1 kinase activity did not show any effect in all four cell lines (Figure 21 B panel 4). Whereas using of both caspase and RIP-1 kinase activity inhibitors fully protect all RIP-1 overexpressing cell lines to cell death (Figure 21 B panel 5).

These results demonstrate that RIP-1 and its enzymatic activities itself are substantial regulators of cell death. Furthermore overexpression of RIP-1 in general and RIP-1 with active kinase domain specifically activates caspase and RIP-1 kinase dependent types of cell death. In addition, RIP-1 KD overexpressing cells do not show complete protection in presence of caspase inhibitors, indicating that either other molecules are involved in the non-apoptotic cell death or the endogenous RIP-1 expression in HaCaT cells promotes caspase independent cell death. Since even the lowest overexpression of RIP-1 as well as its mutants



H-Tamoxifen [10 nM]	-	+	+	+	+
zVAD-fmk	-	-	+	-	+
Necrostatin-1	-	-	-	+	+
Panel	1	2	3	4	5

Figure 21. RIP-1 overexpression induces cell death in both caspase dependent and independent manner. A. The transduced cells were treated with indicated amounts of H-Tamoxifen for 24h, followed by lysis and western blot analysis for RIP-1 expression, β-tubulin served as a loading control. **B.** The control, RIP-1 wild type as well as mutant RIP-1 overexpressing cells were either left unstimulated, or pre-treated with 10nM of H-Tamoxifen for 12-18h followed by either separate or in combined treatment with 100 nM IAP antagonist (1h), zVAD-fmk (10μM; 1h), Necrostatin-1 (50μM, 1h) in triplicate wells for 24h. Viability of cells was analysed and statistical analysis was performed as described before. The summary of three independent experiments is shown, the error bars represent SEM.

induced massive cell death further experiments with TLR3 ligand or IAP antagonist couldn't be performed. Despite these experimental limitations, these gained results once again show that RIP-1 itself is a key molecule in cell death signalling and even can induce cell death by itself.

3.2. The role of cIAPs in CD95L-induced cell death

Previous studies have shown that cIAPs can inhibit CD95L- and TRAIL-induced apoptosis (McEleny et al., 2004; Wang et al., 2005). Since cIAPs regulate RIP-1 modifications induced by TNF-R1 and RIP-1 is one of the key players in CD95 signalling, the mechanism of DR cell

death in the context of IAP inhibition was investigated. In the previous part it was shown that cIAPs block TLR3-mediated cell death, whereas their absence enhances cell death and thus cell death proceeds in a caspase and RIP-1 kinase-dependent manner. In this part of the studies the impact of cIAPs on CD95L induced cell death will be examined. The aim of this part of the studies is thus to compare the role of IAPs in pro-death signalling initiated by membrane-bound receptors of different protein families (TLR3 and CD95).

3.2.1. Loss of IAPs sensitizes to CD95L-induced cell death in both caspase-dependent and independent manner

Death ligands, such as CD95L, are known to form apoptosis-competent signalling clusters, followed by recruitment to the cytoplasmic death domain–containing adaptor protein FADD, which in turn recruits procaspase-8. In context of this signalling complex, procaspase-8 is activated by dimerization, resulting in processing and release of the mature and active enzyme (Boatright et al., 2003). Previous studies have shown that cIAPs can inhibit CD95-induced apoptosis (McEleny et al., 2004; Wang et al., 2005). Since the previous experiments, described above, have shown the relevance of IAPs for protection against apoptotic as well as non-apoptotic cell death in the absence of caspase activity during TLR3-induced signalling, it was proposed that cIAPs could also negatively regulate CD95-induced cell death.

Our experiments have shown that CD95L is inducing cell death in HaCaT cells, and this effect can be strongly enhanced by downregulation of cIAPs (Figure 22 A). To determine the morphology of the cell death, fluorescent microscopy studies were performed. Caspase inhibitor zVAD-fmk was used in order to check the caspase dependency of observed cell death. Upon stimulation with CD95L the first early apoptosis events (judging by morphological features, like membrane blebbing, and chromatin condensation) were observed after 4h, both in presence and in absence of IAP antagonist (Figure 22 B, upper panel). Although the cells could be fully protected from this cell death by blocking of caspases after 4h stimulation, the picture changed after 24h, indicating that non-apoptotic cell death proceeds with a prolonged kinetic. Thus a number of some late apoptotic events (blebbed membrane, condensed chromatin, although the membrane is already ruptured) upon CD95L stimulation in presence and absence of IAPs have been detected (Figure 22 B lower panel). Surprisingly CD95L-induced cell death could not be blocked by caspase inhibitor zVAD-fmk in the absence of IAPs (Figure 22 B lowest panel). However a change in cell death morphology was observed as indicated by a rounded shape, a lack of DNA condensation and a retarded disruption of cell membranes (Figure 22 B lowest panel).

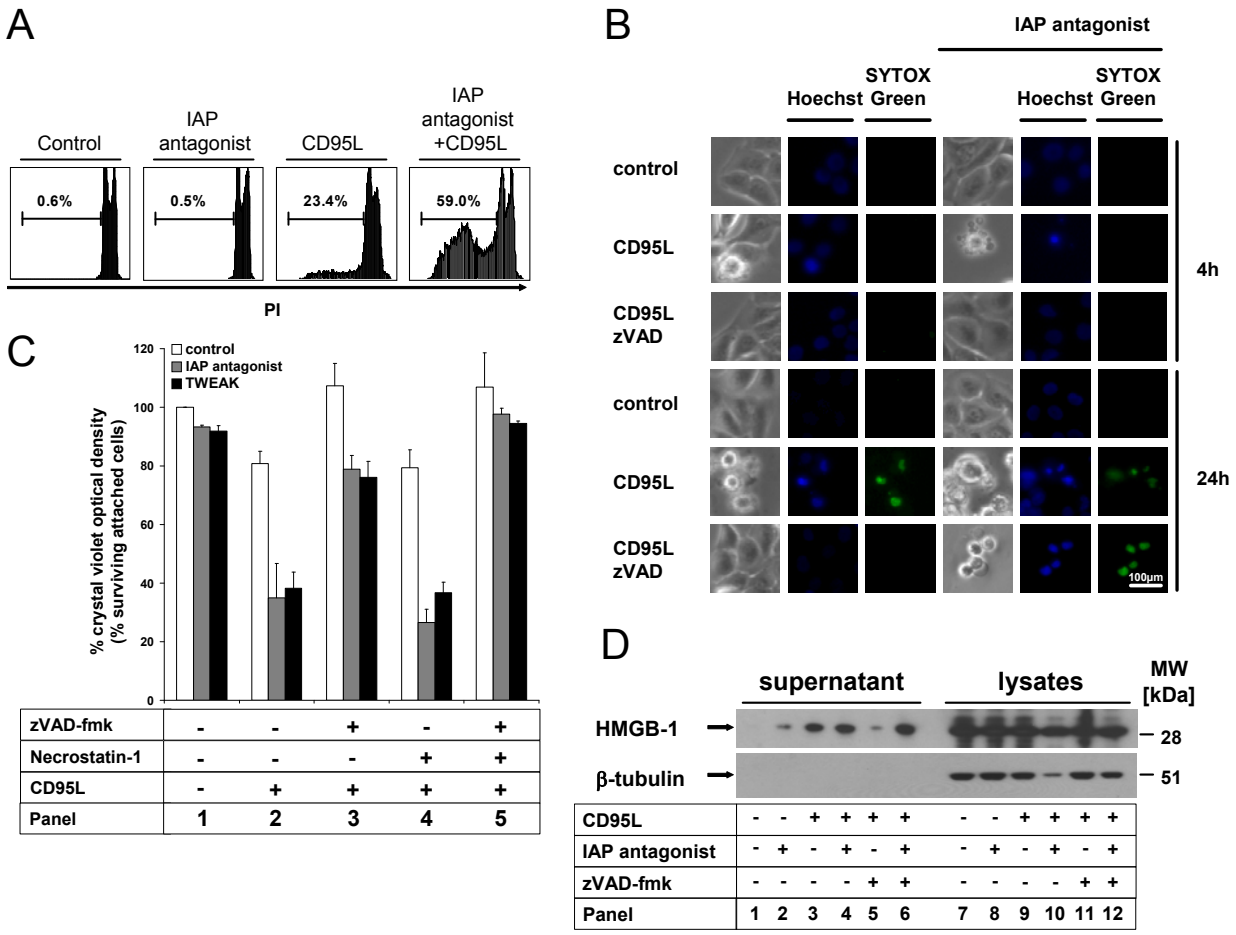


Figure 22. IAP antagonist sensitizes HaCaTs to CD95L induced cell death in both caspase dependent and independent manner. A. Cells were incubated for 8 h and subsequently analyzed for hypodiploidy by FACS analysis (see Materials and methods). **B.** HaCaT cells were either pretreated with 10µM zVAD-fmk for 1h or 100 nM of the IAP antagonist for 30min. Cells were subsequently stimulated with 5U/ml CD95L for 4 or 24 h. 5µg/ml Hoechst 33342 and 5pM SYTOX green were added for 15min at 37°C, immediately followed by transmission (left) or fluorescence (right) microscopy. One of two independent experiments is representatively shown. **C.** HaCaT cells were either pretreated or co stimulated with zVAD-fmk (10µM, 1h), Necrostatin-1 (50µM, 1h) IAP antagonist (100nM, 1h) or TWEAK (0,5µg/ml, 1 h) and subsequently stimulated with 5 U/ml CD95L in triplicate wells for 24h. Viability of cells was analysed and statistical analysis was performed as described before. The summary of three independent experiments is shown, the error bars represent SEM. **D.** For analysis of the release of HMGB-1 protein into the supernatant, HaCaT cells were pre- or co-stimulated with 10µM zVAD-fmk for 1h and 100nM of the IAP antagonist for 30min and subsequently stimulated with 5U/ml CD95L for 24h. Cell-free supernatants as well as total cellular lysates were analyzed for HMGB-1 protein expression. β-tubulin was used as a loading control. One of two representative experiments is shown. Experiments **A**, **C** and **D** were kindly provided by Dr. P. Geserick, experiment **B** was done together with Dr. P. Geserick.

To examine the role of cIAPs in CD95 death signalling in a more physiological setting the cytokine TWEAK was used. The results obtained with TWEAK for CD95 signalling were similar to the results obtained with IAP antagonist (Figure 22 C). The cells were sensitized to the CD95L cell death by both TWEAK and IAP antagonist at to a similar extent (Figure 22 C panel 2), this effect could be partially blocked by zVAD-fmk (Figure 14 C panel 3), whereas

Necrostatin-1 did not show much effect when used alone (Figure 22 C panel 4), and complete protection was achieved by both zVAD-fmk and Necrostatin-1 together (Figure 22 C panel 5). These results indicate mechanistic similarities between TLR3 signalling pathways and CD95 induced cell death pathways and an important role of both caspase- and RIP-1 kinase functions for cell death induced by CD95L in the absence of cIAPs.

However, HMGB-1 was released upon CD95L stimulation in the presence and absence of IAP antagonist (Figure 22 D panel 3 and 4). Although in the presence of IAPs this release could be blocked by zVAD-fmk (Figure 22 D panel 5), in its absence HMGB-1 release was not inhibited by zVAD-fmk (Figure 22 D panel 6). These results demonstrate that cIAPs are not only critical players in TLR3 death signalling but also in CD95 cell death signalling pathways. The CD95L-induced cell death also requires both caspase and RIP-1 kinase activity in the absence of cIAPs.

3.2.2. The role of RIP-1 and cFLIP isoforms in CD95-mediated cell death in presence of TWEAK

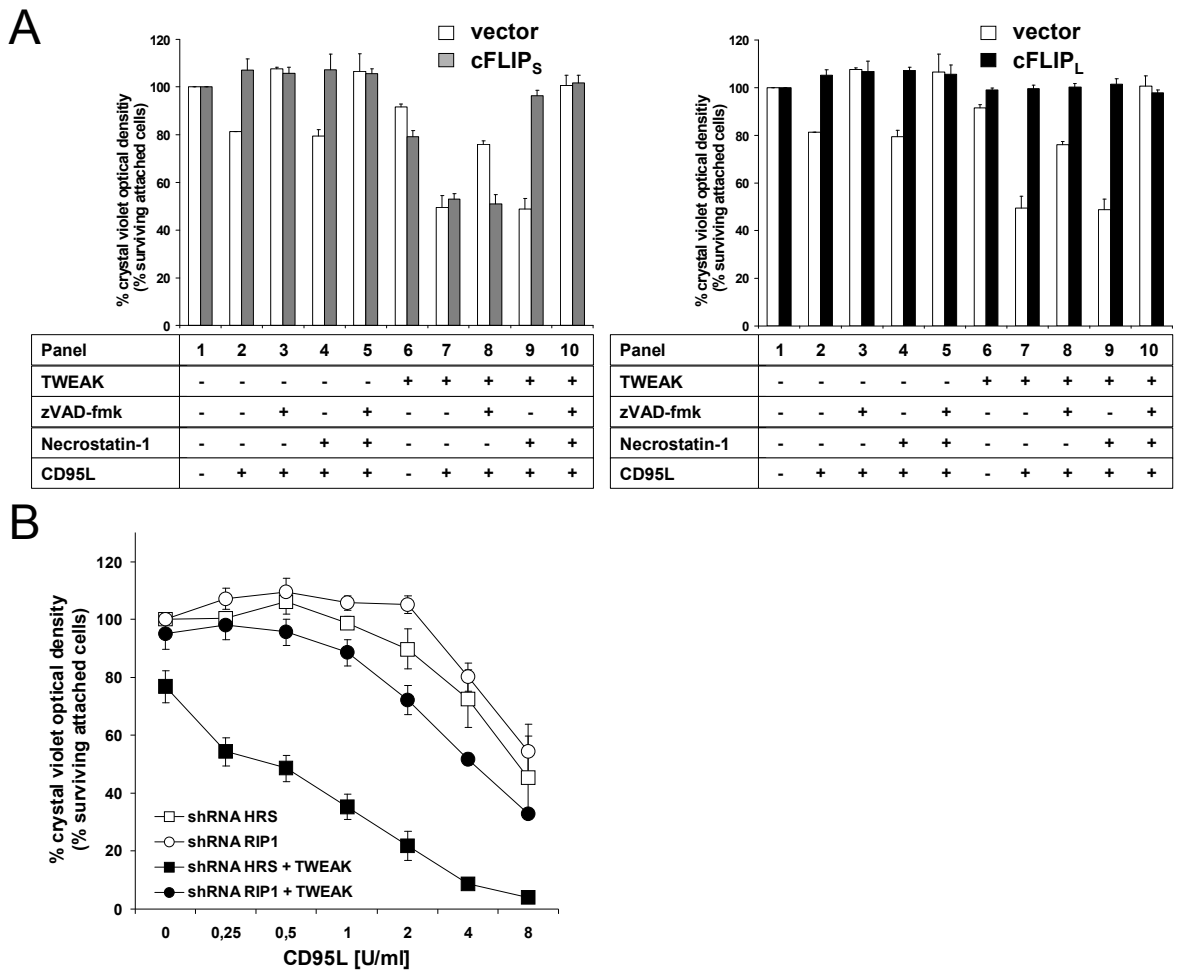
To further investigate the CD95 signalling in the absence of cIAPs, induced by TWEAK, the long and short isoforms of caspase inhibitor cFLIP were overexpressed in HaCaT cells.

The vector control transduced cells (Figure 23 A white bars) were sensitive to CD95L-induced cell death (Figure 23 A, white bars, panel 2); which was inhibited by zVAD-fmk (Figure 23 A, white bars, panel 3), whereas addition of Necrostatin-1 was ineffective (Figure 23 A white bars panel 4). In the presence of TWEAK the cells were more sensitive to CD95L (Figure 23 A white bars panel 7) and inhibition of caspases did not allow for full protection (Figure 23 A white bars panel 8). Similarly, inhibition of RIP-1 kinase activity (Figure 23 A white bars panel 9) alone was ineffective. In marked contrast, inhibition of both caspase and RIP-1 kinase activities could fully protect the cells (Figure 23 A white bars panel 10). In line with the data shown for TLR3 signalling in Figure 15, $cFLIP_L$ fully protected cells against cell death induced by CD95L, (Figure 23 A black bars) whereas TWEAK also induced necrosis in $cFLIP_S$ expressing cells in the absence of TLR-3 stimulation, which could be blocked by Necrostatin-1 (Figure 23 A; gray bars; lane 6 and 9). In contrast, a further increase of this cell death was observed in the presence of CD95L/TWEAK and zVAD/CD95L/TWEAK (Figure 23 A compare panels 6, 7, and 8; gray bars). Furthermore, CD95L-induced cell death was fully blocked by necrostatin-1, indicating that $cFLIP_S$ promotes necrotic cell death whenever cIAPs are absent (Figure 23 A, gray bars; panels 9 and 10). These results again show that cFLIP isoforms contribute to antiapoptotic protection of the cells; whereas $cFLIP_S$ is a crucial regulator of the necrotic pathway.

To further investigate the relevance of RIP-1 for CD95 death signalling, RIP-1 was downregulated in HaCaT cells. Interestingly, there was not much difference detected in

sensitivity to CD95L between control and RIP-1 downregulated cells. Although in the absence of IAPs, induced by TWEAK, a sufficient sensitization to CD95L in control cells, but not in RIP-1 knockdown cells was detected (Figure 23 B).

Taken together, these data suggest that cIAPs block TLR3- and CD95L-induced cell death signalling in a RIP-1 dependent manner. Thus downregulation of cIAPs, either by synthetic



Panel	1	2	3	4	5	6	7	8	9	10
TWEAK	-	-	-	-	-	+	+	+	+	+
zVAD-fmk	-	-	+	-	+	-	-	+	-	+
Necrostatin-1	-	-	-	+	+	-	-	-	+	+
CD95L	-	+	+	+	+	-	+	+	+	+

Panel	1	2	3	4	5	6	7	8	9	10
TWEAK	-	-	-	-	-	+	+	+	+	+
zVAD-fmk	-	-	+	-	+	-	-	+	-	+
Necrostatin-1	-	-	-	+	+	-	-	-	+	+
CD95L	-	+	+	+	+	-	+	+	+	+

Figure 23. CD95L duplicates findings obtained with poly (I:C) A. For quantification and characterization of CD95L-induced cell death, HaCaT expressing cFLIP$_L$ and cFLIP$_S$ were either pre-treated or co-stimulated with zVAD-fmk (10µM, 1h), Necrostatin-1 (50µM, 1h), or TWEAK (0,5µg/ml, 1h) and subsequently stimulated with 2,5U/ml of CD95L respectively for 24h in triplicate wells. Viability of cells was analysed by crystal violet staining and statistical analysis was performed as described before. The summary of three independent experiments is shown, error bars represent SEM. **B.** Cells expressing shRNA HRS, shRNA RIP-1 were pre-treated with TWEAK (0,5µg/ml, 1h) and subsequently stimulated with indicated concentrations of CD95L for 24h in triplicate wells. Viability of cells was analysed by crystal violet staining and statistical analysis was performed as described before. The summary of three independent experiments is shown, error bars represent SEM. These experiments were kindly provided by Dr. P. Geserick.

IAP antagonists or by ligand induced degradation as exemplified by TWEAK, is able to overcome apoptosis resistance in CD95 apoptosis signalling. Moreover the observed similarities between CD95- and TLR3-mediated signalling led us to the conclusion that cIAPs are at the cross road of cell death signalling pathways induced by diverse and different

stimuli. The hypothesis arose, that there is a conserved cell death signalling platform, triggered by different stimuli.

3.3. Role of cIAPs in TLR3 cell death in primary keratinocytes

Our studies in A5RT3 and MET1 skin cancer cell lines, as well as HaCaT, which have mutation in both copies of the p53 gene, have shown the relevance of cIAPs for TLR3 induced cell death signalling. The next goal of this study was to investigate the relevance of these findings for non-transformed and non-malignant primary human keratinocytes.

3.3.1. Loss of cIAPs sensitizes primary keratinocytes to poly (I:C)-induced cell death in a Caspase- and RIP-1-kinase dependent manner

Primary keratinocytes significantly differ from transformed HaCaT cells. HaCaT cells for example have two copies of a mutated p53 gene, whereas in primary keratinocytes p53 is wild type. Moreover, HaCaT and primary keratinocytes differ by the pattern of expressed proteins: of main interest is that primary keratinocytes express high protein levels of XIAP in contrast to HaCaTs that lack XIAP (Figure 24 A). In addition, primary keratinocytes have high levels of $cFLIP_L$, but not $cFLIP_S$ expression, and different donors of primary cells express different levels of cIAP1 and cIAP2. For our studies primary keratinocytes obtained from three different donors (donor E, donor I and donor L) were used.

The first experiments showed that poly (I:C) can decrease cellular viability in primary keratinocytes of all three donors, although Donors I (Figures 24 B, panel 2) and L (data not shown, since it duplicated data form donor I) proved to be less sensitive than Donor E (Figure 25 A, panel 2). Interestingly, although an increase of cellular viability was detected by caspase inhibition (Figure 24 B for Donor I and Figure 25 A for Donor E, panels 3), the inhibition of RIP-1 kinase activity decreased viability (Figure 24 B for Donor I and Figure 25 A for Donor E, panels 4). Blocking of both caspase- and RIP-1 kinase, however, could not confer complete resistance to TLR3 induced cell death in all three donors (Figure 24 B for Donor I, and Figure 25 A for Donor E, panels 5). Downregulation of IAPs decreased cellular viability (Figure 24 B for donor I and Figure 25 B for Donor E, panels 6) in all three donors. TLR3 stimulation in the absence of IAPs further decreased cellular viability, with lesser amount of cell death in donors I and L (Figure 24 B panel 7 and Figure 25 A, panel 7). Inhibition of caspases in the absence of IAPs did not show any effect in donors I and L (Figure 23 B panel 8), but increased cellular viability in Donor E (Figure 25 A, panel 8). RIP-1 kinase inhibition, in the absence of IAPs, did not show any effect on cellular viability in all donors (Figure 24 B and 25 A, panels 9). Inhibition of both caspase- and RIP-1 kinase

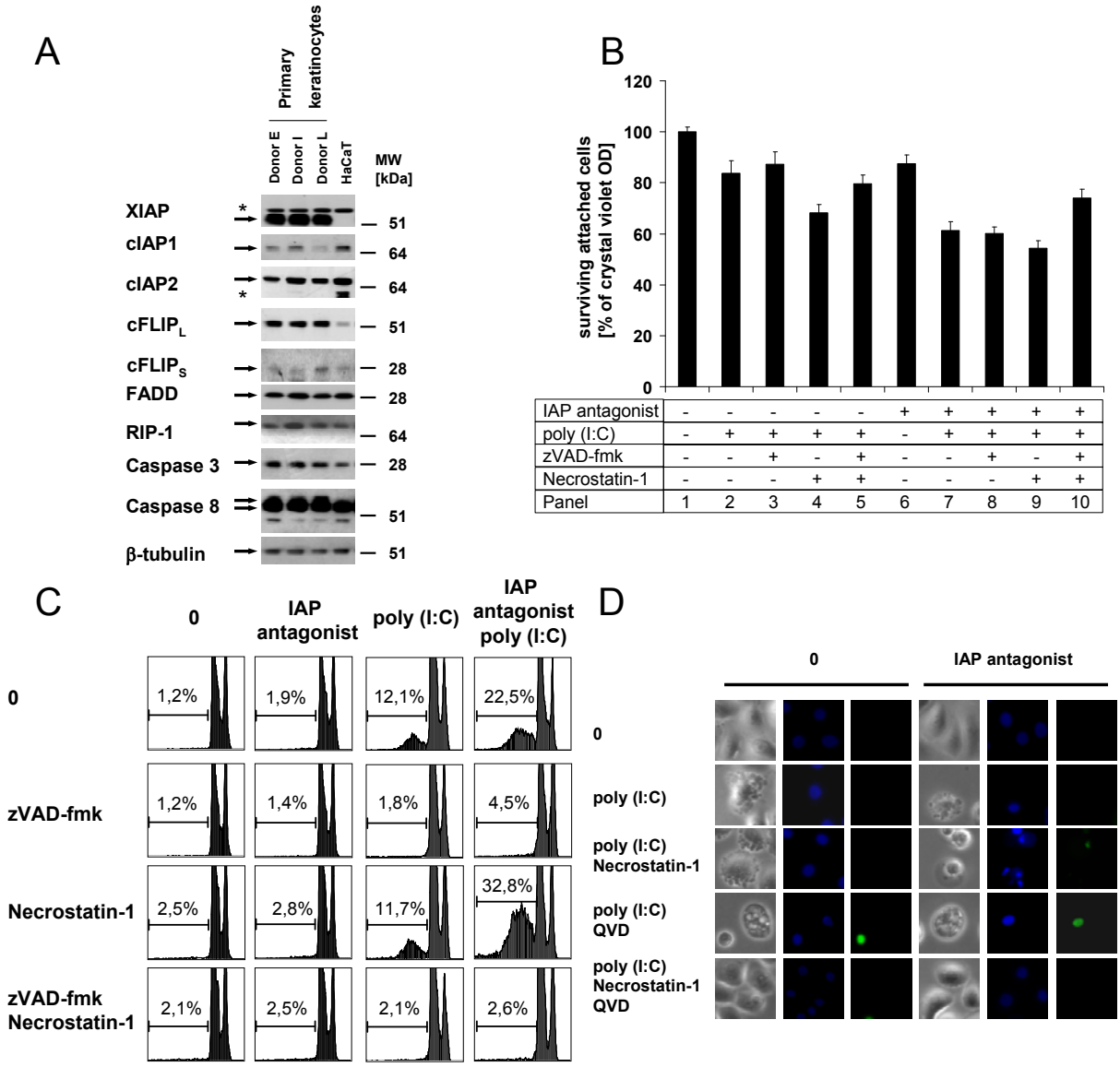


Figure 24. Loss of cIAPs sensitizes primary keratinocytes to poly (I:C)-mediated apoptotic and non-apoptotic cell death is caspase and RIP-1K dependent manner. A. The analysis of 5µg of total cell lysates from HaCaT and primary keratinocytes (donors E, I and L) by Western-blot analysis for the respective proteins. β-tubulin served as internal control for even loading. One of two representative experiments is shown. The stars indicate the nonspecific binding of the antibody. These blots were kindly provided by Dr. P. Geserick. **B.** Primary keratinocytes (donor I) were either separately or in combination pre-treated with 100nM IAP antagonist (1h), zVAD-fmk (10µM; 1h), Necrostatin-1 (50µM, 1h), and subsequently stimulated with 2µg/ml of poly (I:C) in triplicate wells. The summary of five independent experiments is shown, error bars represent SEM. **C.** Primary keratinocytes (donors I and L) were either separately or in combination pre-treated with 100 nM IAP antagonist (1h), zVAD-fmk (10µM; 1h), Necrostatin-1 (50µM, 1h), and subsequently stimulated with 2µg/ml of poly (I:C) for 18h and subsequently analysed for hypodiploid DNA content by FACS. One representative experiment (donor I) of 2, performed for each donor is shown. **D.** For characterization of cell death morphologies, primary keratinocytes (donor I) were either pre-treated with QVD (10µM, 1h), Necrostatin-1 (50µM, 1h) or IAP antagonist (100nM, 30min) and subsequently stimulated with poly (I:C) (2µg/ml) for 24h. Cells were stained with Hoechst-33342 and SYTOX Green immediately followed by transmission and fluorescence microscopy. One representative (donor I) of a total of two independent experiments performed for each of the donors is shown.

activities in the absence of IAPs protected viability in all three donors (Figures 24 B and 25 A,

panel 10). These data indicate that the observed effects are donor-specific. IAPs critically regulate TLR3-induced cell death in primary keratinocytes, although signalling molecules such as caspases and RIP-1 kinase are involved in cell death regulation of TLR3-induced signalling. In contrast to the data in HaCaT, RIP-1 kinase activity is required for primary

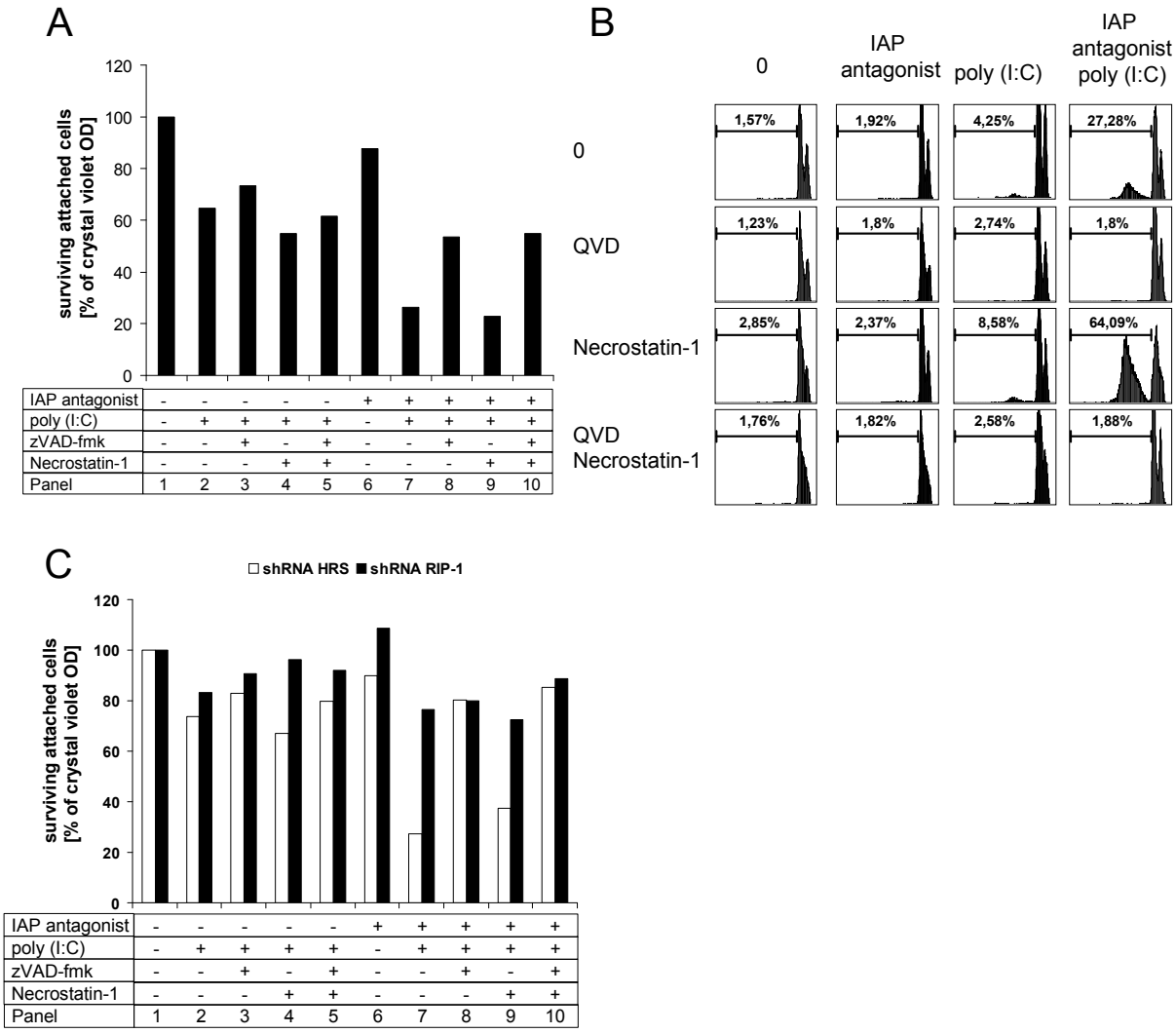


Figure 25. Primary keratinocytes (donor E) are more sensitive to TLR3-induced cell death, than donors I and L and RIP-1 is essential for its execution. A. Primary keratinocytes from Donor E were either separately or in combination pre-treated with 100 nM IAP antagonist (1h), zVAD-fmk (10μM; 1h), Necrostatin-1 (50μM, 1h), and subsequently stimulated with 2μg/ml of poly (I:C) in triplicate wells. Viability of cells was analysed as described before. **B.** Primary keratinocytes (donor E) were either separately or in combination pre-treated with 100 nM IAP antagonist (1h), QVD (10μM; 1h), Necrostatin-1 (50μM, 1h), and subsequently stimulated with 2μg/ml of poly (I:C) for 18h and subsequently analysed for hypodiploid content by FACS. **C.** For viability analysis, bulk infected primary keratinocytes (donor E) were either pre-treated with zVAD-fmk (10μM, 1h), Necrostatin-1 (50μM, 1h) or IAP antagonist (100 nM, 30 min) and subsequently stimulated with poly (I:C) (2μg/ml) for 24h followed by crystal violet

keratinocyte survival.

To check if the observed decrease in cellular viability is due to increased cell death, hypodiploidy analysis (Figure 24 C) and live imaging using Hoechst and SYTOX-Green dyes (Figure 24 D) were performed. Upon treatment with poly (I:C), as well as in the absence of

RIP-1 kinase activity, the cells were dying with prominent apoptotic features: membrane blebbing, chromatin condensation and increased amounts of hypodiploid nuclei in donors I and L (Figure 24 C and 24 D, left panel), as well as in Donor E (Figure 25 B). Blocking of caspase activity by respective inhibitors repressed hypodiploidy (Figure 24 C and 25 B) and morphological change of the dying cells (Figure 24 D). The cells first became rounded and swollen followed by membrane rupture (Figure 24 D). In the absence of IAPs the effects of TLR3 stimulation were further intensified, which increased cell death as determined by hypodiploidy analysis (Figure 24 C and 25 B). Moreover morphologically apoptotic cells were noted under those conditions (Figure 24 D, right panel). Surprisingly, blocking of RIP-1 kinase function by Necrostatin-1 in the absence of IAPs increased the amount of TLR3-induced apoptosis (Figure 24 D, right panel and Figure 24 C and 25 B). Inhibition of caspase activity by zVAD-fmk or QVD led to complete loss of hypodiploid nuclei also in the absence of IAPs (Figure 24 C and 25 D), whereas the dead cells acquired necrotic morphology. Absence of both caspase and RIP-1 kinase activity led to complete protection against both types of cell death, indicating the relevance of both enzymes for apoptosis and necrosis, respectively. Furthermore these results suggest that the observed loss of viable cells upon treatment with caspase and RIP-1 kinase inhibitors in the presence and absence of IAPs (Figures 24 B and 25 A) is not related to a cell death phenotype, but rather reflect an anti-proliferative effect of the combination of inhibitors.

The results gained for primary keratinocytes (donors I, L and E) clearly differ from analogous results in HaCaT cells. The HaCaTs did not show increased apoptosis in response to blocking of RIP-1 kinase activity in the absence of IAPs, in contrast to primary keratinocytes. These results indicate that RIP-1 kinase function seem to play an important role in protection against cell death in primary keratinocytes.

3.3.2. The function of RIP-1 in TLR3-induced cell death in primary keratinocytes

To further investigate the role of RIP-1 and the difference in cell death signalling between primary keratinocytes and HaCaT cells RIP-1 shRNA downregulated primary keratinocytes were generated. As described in Figure 17, downregulation of RIP-1 in HaCaTs led to complete protection from both types of TLR3-induced cell death in the absence of IAPs. To check if cell death protection in primary keratinocytes is controlled by the same mechanisms, RIP-1 was downregulated in primary keratinocytes (Donors I, L and E). The level of RIP-1 downregulation in primary keratinocytes was comparable to the level of downregulation in HaCaTs (Figure 26 A). In contrast to HaCaT cells, the primary keratinocytes donors I and L (For L data is not shown, since it duplicates the data from donor I) were unable to protect from TLR3-induced cell death (Figure 26 B, white bar, panel 2). Moreover, surprisingly, RIP-1

downregulation did not protect from poly (I:C) induced cell death, when compared to control cells (Figure 26 B, panel 2). This indicates significant differences between TLR3-induced death signalling in primary keratinocytes and HaCaT cells. Furthermore RIP-1 proved to be not critical for TLR3-induced cell death in primary keratinocytes.

Unexpectedly in the absence of caspase activity further sensitization of RIP-1 downregulated cells was noted, although partial protection of the control cells against TLR3-induced cell death (Figure 26 B, panel 3) was observed. This indicates that cell death in primary keratinocytes is partially dependent of caspases, although the RIP-1 is crucial for protection from caspase-independent cell death. The further decrease of RIP-1 kinase activity did not alter the cell death sensitivity in both control and RIP-1 knockdown cells (Figure 26 B, panel 4). Inhibition of both caspases and RIP-1 kinase activity did fully protect, in difference to parental primary keratinocytes (compare Figure 26 B, panel 5 and Figure 24 B, panel 5). Inhibition of IAP activity did not influence the cell survival, indicating that IAP's function is not required for survival of primary keratinocytes (Figure 26 B panel 6). In the absence of IAPs both control and RIP-1 downregulated cells were sensitized to TLR3-induced cell death (Figure 26 B, panel 7), in contrast to the results from HaCaTs (compare Figure 17 B). Therefore IAPs are critical factors for TLR3-induced cell death, whereas in primary keratinocytes further enzymatic analysis of the mode of all cell death is required to dissect this pathway. In the absence of both IAP and caspase activities the viability of RIP-1 knockdown cells was sufficiently decreased, compared to the control cells, indicating the relevance of RIP-1 for protection from caspase-independent cell death (Figure 26 B, panel 8). The decrease of RIP-1 kinase activity protected RIP-1 knockdown cells, whereas was ineffective in control cells (Figure 26 B, panel 9). This might indicate that Necrostatin-1 is not only inhibiting RIP-1 kinase, but may also target other, currently unknown, proteins, which are necessary for survival of primary keratinocytes.

The combination of caspase and RIP-1 kinase inhibitors conferred the best protection in both control and RIP-1 knockdown cells from TLR3-induced cell death in the absence of IAPs (Figure 26 B, panel 10).

Interestingly, in contrast to donors I and L, but in line with the data in HaCaT cells, RIP-1 downregulation in Donor E protected these cells from TLR3-induced cell death in the absence of IAPs (compare Figures 26 B, 8 B and 16 C, black bars, panel 7).

The analysis of the hypodiploid nuclear content in the control cell line have revealed the same effects as in the parental cells of donors I and L. Poly (I:C) induced apoptotic cell death, which was increased in presence of IAP antagonist and further increased in the absence of RIP-1 kinase activity, whereas the blocking of caspases led to nearly complete absence of hypodoploid DNA content (Figure 26 C, left panel). In contrast the RIP-1 downregulated cell line has shown less apoptosis upon TLR3 stimulation in presence and

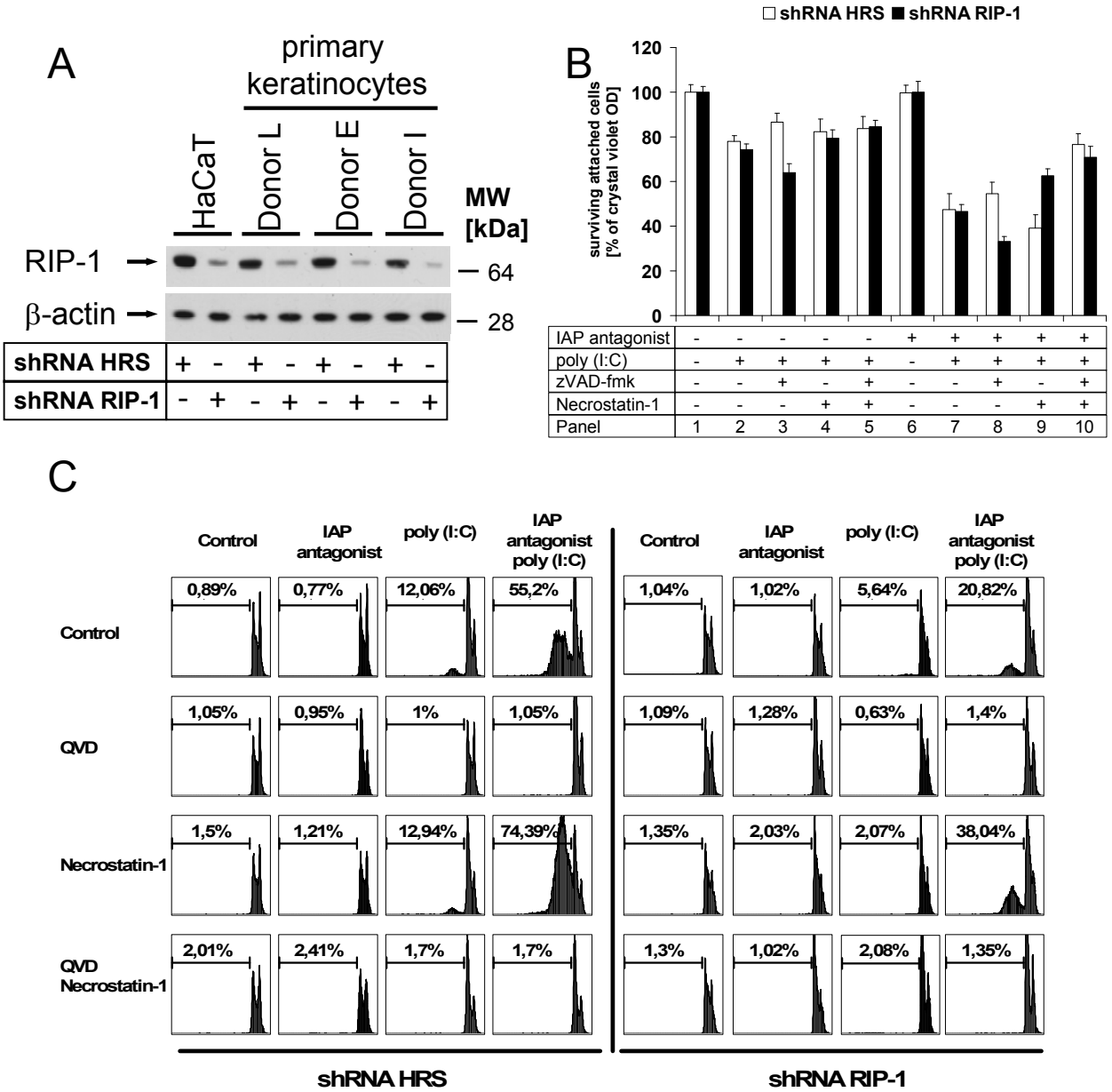


Figure 26. RIP-1 protects primary keratinocytes (donors I and L) from poly (I:C) induced apoptosis in absence of IAPs. A. Primary keratinocytes donors L, E and I and HaCaT cells as a control were retrovirally transduced with HRS and RIP-1 specific shRNA. Knockdown of RIP-1 was analysed by Western-blot analysis. β-actin expression served as an internal loading control. One representative of two independent experiments is shown. **B.** For viability analysis, bulk infected primary keratinocytes (donors I and L) were either pre-treated with zVAD-fmk (10μM, 1h), Necrostatin-1 (50μM, 1h) or IAP antagonist (100 nM, 30 min) and subsequently stimulated with poly (I:C) (2μg/ml) for 24h followed by crystal violet staining. The summary of five independent experiments is shown, as described in materials and methods. **C.** Transduced primary keratinocytes (donors I and L) were either separately or in combination pre-treated with 100 nM IAP antagonist (1h), QVD (10μM; 1h), Necrostatin-1 (50μM, 1h), and subsequently stimulated with 2μg/ml of poly (I:C) for 18h and subsequently analysed for hypodiploid DNA content by FACS. One representative experiment (donor I) of 2, performed for each donor is shown.

absence of IAPs, compared to the control cell line (Figure 26 C, right panel). These data suggest that RIP-1 as a whole protein is required for apoptosis execution in some donors of

primary keratinocytes (see Figure 26 C, right panel); moreover RIP-1 is also required for protection from caspase-independent cell death. Contrasting data in HaCaT cells, RIP-1 kinase function, in primary keratinocytes, is an important component of anti-apoptotic protection (Figure 26 C, left panel). Another hypothesis is that necrostatin-1 is targeting other molecules, which are responsible for anti-apoptotic protection. Donor E is different form the other two donors and much more resembles HaCaT, indicating that there are a least two different types of primary keratinocytes with different types of cell death signalling.

3.4. The role of cIAPs in MAPK and NF-κB activation

In the previous parts of the thesis the role of cIAPs in cell death pathways induced by TLR3 and CD95 pathways in different cellular models was described. These receptors can also induce non-cell death pathways such as activation of NF-κB and MAPK (Stone et al., 2009). Activation of NF-κB is tightly connected to up-regulation of anti-apoptotic proteins such as cFLIP and cIAPs. In contrast cFLIP and cIAPs can also regulate NF-κB activation (Diessenbacher et al., 2008; Dohrman et al., 2005; Wachter et al., 2004). These processes are therefore tightly interconnected and can confer protection against apoptosis and potentially carcinogenesis (Stone et al., 2009). Therefore it is important to understand the influence of cIAPs on non-cell death signalling pathways for future therapeutic applications of IAP antagonist.

3.4.1. Inhibition of cIAPs led to both canonical and non-canonical NF-κB activation

To characterise the relevance of IAPs for modulation of non-cell death signalling pathways the canonical and non-canonical NF-κB activation pathways were analysed in HaCaT and SCC cells in presence of IAP antagonist and DL or poly (I:C). Therefore in initial experiments with another inhibitor of IAPs – IAP antagonist 2 (LBW242) was used at different concentrations and time points to detect time and dose dependency of NF-κB activation. The NIK stabilisation and p100 degradation were detected upon IAP inhibition (Figure 27 A) in HaCaT cells. These results are an indication for non-canonical NF-κB activation. This finding indicates that cIAPs are blocking non-canonical NF-κB activation in HaCaT cells. The stabilisation of NIK was detected at all concentrations used within 1h of treatment. Interestingly cIAP1 was degraded after 30min of treatment at all concentration, whereas cIAP2 was not affected by the lowest concentration of IAP antagonist 2. Interestingly after 24h of treatment with IAP antagonist 2 NIK stabilisation was decreased, whereas cIAP2 protein levels were increased in IAP antagonist 2-dependent manner (Figure 27 A). These results were repeated with IAP antagonist with HaCaT, A5RT3 and MET1 cells. The cells were treated with IAP antagonist and the levels of NIK and p100 were evaluated (Figure 27

B). In all three cell lines NIK stabilisation as well as p100 degradation upon IAP downregulation was observed. Taken together these results indicate that cIAPs are blocking non-canonical NF-κB activation in keratinocyte cell lines.

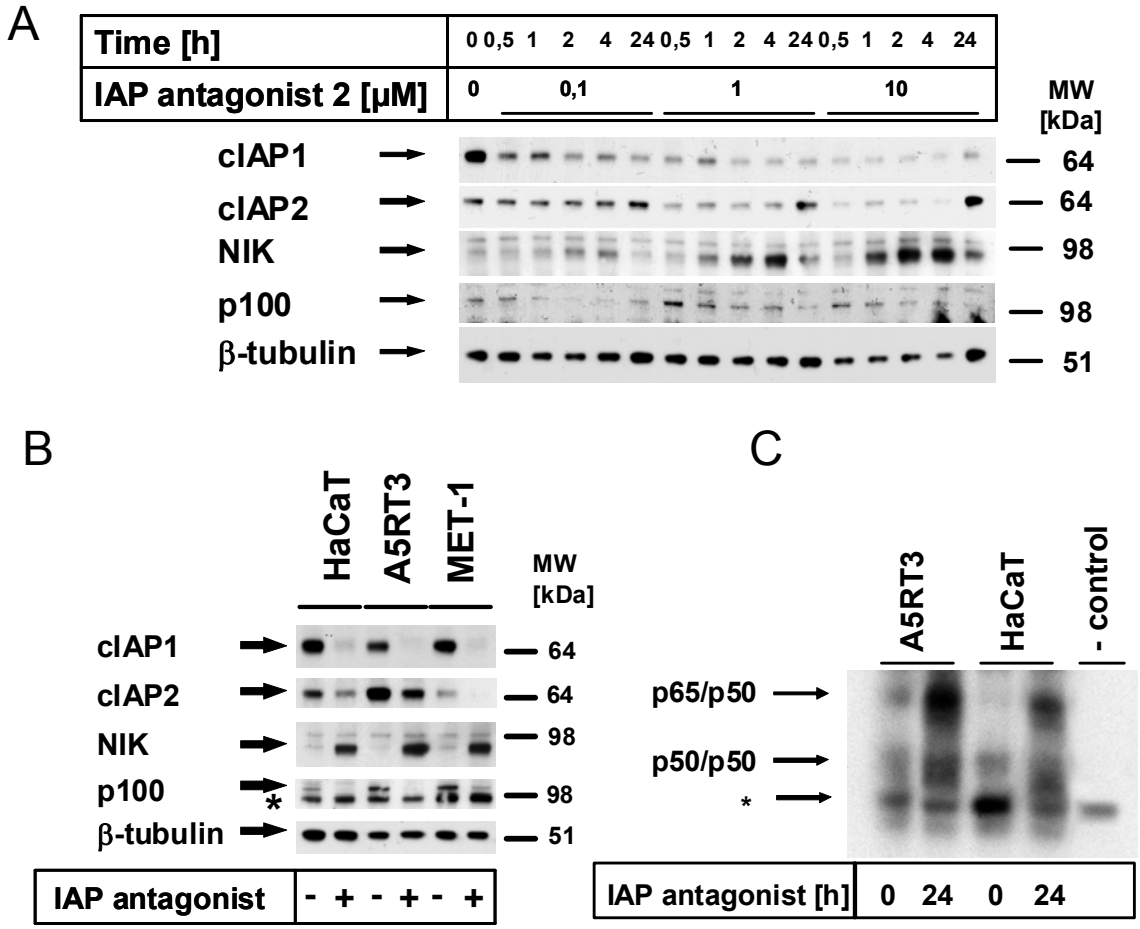


Figure 27. IAPs are blocking canonical and non-canonical NF-κB activation in HaCaTs. A. HaCaT cells were treated with indicated concentrations of LBW242 for respective time. The lysates were loaded in amount of 5μg and analysed by Western blot for respective proteins. **B.** HaCaT, A5RT3 and MET1 cells were treated with 100nM of IAP antagonist for 4h, followed by Western-blot analysis of 5μg total cell lysates form respective proteins. β-tubulin served as internal control for even protein loading. One of three representative experiments is shown. **C.** A5RT3 and HaCaT cells were treated with 100nM of IAP antagonist for 24h and the nuclear lysates were used for NF-κB EMSA one of 2 experiments is shown. Stars indicate nonspecific binding of the antibody and probe.

To further characterise the impact of cIAPs on canonical NF-κB activation, HaCaT and A5RT3 cell lines were used and checked for DNA binding of p65/p50 heterodimers by EMSA (Figure 27 C). Increased amounts of p65/p50 heterodimer-DNA binding was detected in both cell lines after 24h of treatment with IAP antagonist. The results indicate that IAPs are also blocking the canonical NF-κB pathway.

Taken together, the results suggest that IAPs do not only interfere with the cell death pathways but also control both canonical and non-canonical NF-κB activation pathways.

3.4.2. The role of IAPs for NF-κB and MAPK activation in HaCaTs, SCC and primary keratinocytes

MAPK signalling is important for the control of normal skin development and homeostasis. Its deregulation promotes epidermal carcinogenesis (Khavari and Rinn, 2007). TRAIL is known to induce apoptosis in variety of tumour and transformed cells without toxicity to normal cells (Mahmood and Shukla, 2010), and the absence of IAPs sensitizes various cell lines to TRAIL induced cell death (Geserick et al., 2009). TRAIL is a known inducer of NF-κB (Falschlehner et al., 2007) and MAPK, such as JNK (Lin et al., 2000) and p38 (Morel et al., 2005). Thus TRAIL and IAP antagonist could be potentially used for cancer treatment. Therefore NF-κB as well as MAPK activation, induced by TRAIL in the absence of IAPs should be studied in order to predict the possible and potentially deleterial pro-carcinogenic effects.

Since downregulation of IAPs led to increased canonical and non-canonical NF-κB activation, the influence of IAPs in DL-induced NF-κB as well as MAPK activation was studied in different cell lines. The first comparison was performed between HaCaT and primary keratinocytes (donor 11) (Figure 28), to check the difference between parental and spontaneously transformed keratinocytes. In both cell lines cIAP1 and cIAP2 were lost in time dependent manner upon TRAIL stimulation. In both cell types, cIAP1 was completely absent within 30min of IAP antagonist stimulation, whereas the level of cIAP2 was only reduced, but not completely degraded (which is consistent with the previous findings in HaCaT cells, compare Figure 9 B).

The IκBα phosphorylation and degradation served as an indicator of canonical NF-κB activation. In both cell types TRAIL treatment led to increased p-IκBα level, whereas total IκBα was lowered, in line with the previously published kinetics (Leverkus et al., 2003). Interestingly the level of p-IκBα was also significantly increased upon IAP antagonist treatment alone, whereas the total level of IκBα remained largely unchanged. In the presence of IAP antagonist the TRAIL-induced IκBα phosphorylation and degradation was decreased in primary keratinocytes, whereas in HaCaT cells the level of IκBα activation remained the same. These data suggest that the activation of the canonical pathway of NF-κB is constantly suppressed by IAPs, whereas canonical TRAIL-induced NF-κB activation is partially suppressed in primary keratinocytes in the absence of IAPs.

NIK stabilisation is one of the features of activation of the non-canonical NF-κB pathway. NIK stabilisation was not detected upon TRAIL stimulation, whereas NIK was rapidly stabilised in the absence of IAPs within 1 h of IAPs degradation. Although the absence of IAPs stabilizes NIK, further stimulation with TRAIL significantly repressed NIK stabilisation in both cell types. Thus cIAPs are important negative regulators of non-canonical NF-κB pathway, whereas TRAIL-induced signalling suppresses the spontaneous non-canonical NF-κB activation in absence of IAPs.

Other important non-cell death signalling pathways are the activation of MAPK, which can promote processes such as inflammation and carcinogenesis. The activity of MAPK in response to IAP downregulation and TRAIL stimulation were detected by phosphorylation of p38, JNK and HSP27, an important target protein of p38. Phosphorylation of HSP27, JNK and p38 activation were first detected after 1-2h of TRAIL stimulation and, in contrast to NF-κB pathways, both MAPK were not activated in the absence of IAPs in both cell types. TRAIL-induced p38 and JNK activation, in the absence of IAPs, were unchanged in HaCaT and, interestingly, decreased in primary keratinocytes for both p38 and JNK.

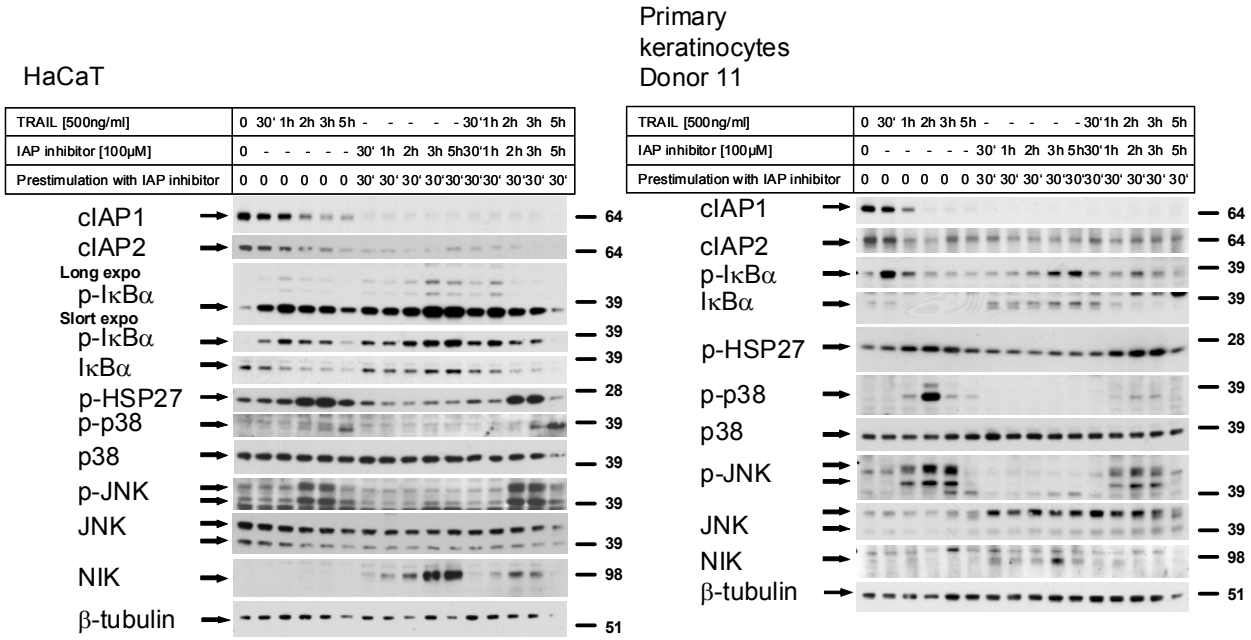


Figure 28. TRAIL induced MAPK and NF-κB activation in presence and absence of IAPs in HaCaT cells and primary keratinocytes. HaCaT and primary keratinocytes (donor 11) were starved for 6h and either pre-treated with IAP antagonist for 30min or left untreated followed by stimulation with 500ng/ml of TRAIL for the indicated time periods. The cells were lysed and 5μg of total cellular proteins were analysed by Western blot for the respective proteins. β-tubulin served as a control.

These data suggest that the presence of IAPs, in contrast to NF-κB pathways, does not affect MAPK activation. The TRAIL-induced MAPK activation is partially suppressed in the absence of IAPs in primary keratinocytes. Together these data indicate that IAPs are not crucial regulators of MAPK in primary keratinocytes and HaCaT cells.

Next NF-κB and MAPK activation were compared in MET1 and A5RT3 cells (Figure 29) in order to check the difference between tumour cells (A5RT3 and MET1) and normal (primary keratinocytes), or immortalised (HaCaT) keratinocytes. A5RT3 is a part of the HaCaT tumour progression model, derived from HaCaT by introduction of the Harvey-ras oncogene (codon 12 mutation); this cell line was proved to form metastatic tumours (Mueller et al., 2001). MET1 cell line is part of the MET model system; this cell line is derived from a primary skin

tumour. Furthermore, MET1 carry a number of aberrations typically seen in SCCs, although these cells are devoid of p53 mutations (Popp et al., 2000).

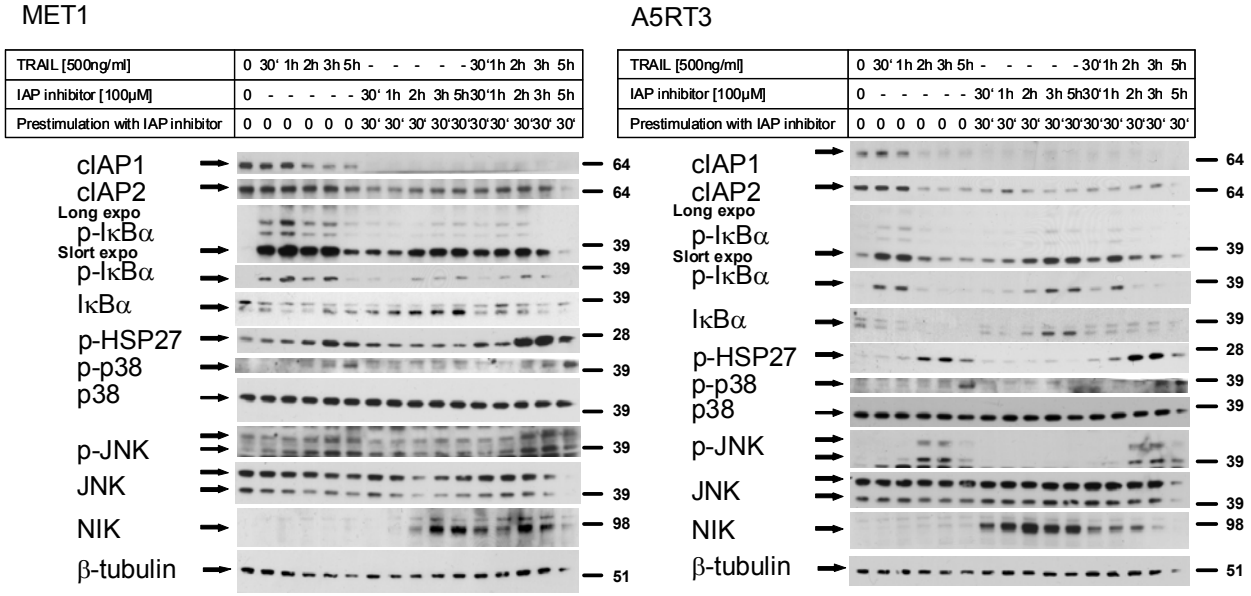


Figure 29. TRAIL induced MAPK and NF-κB activation in presence and absence of IAPs in MET1 and A5RT3 cell lines. MET1 and A5RT3 were starved for 6h and either pretreated with IAP antagonist for 30min. or left untreated followed by stimulation with 500ng/ml of TRAIL for indicated time periods. The cells were lysed and 5μg were analysed by Western blot for respective proteins. β-tubulin served as a control.

In both cell lines cIAP1 protein was lost upon TRAIL stimulation, whereas cIAP2 was only lost by TRAIL stimulation in A5RT3, whereas in MET1 cells the cIAP2 level did not change significantly upon TRAIL stimulation. In both cases cIAP1 was rapidly degraded after 30min of IAP antagonist treatment, whereas cIAP2 was degraded only partially.

In both cell lines the p-IκBα level was significantly enhanced and total IκBα level was lowered within 1h of TRAIL treatment, in line with data from HaCaT and primary keratinocytes. The level of p-IκBα was also significantly increased upon IAP antagonist treatment whereas the total level of IκBα was largely unchanged. In the presence of IAP antagonist the TRAIL-induced IκBα phosphorylation and degradation was decreased in both cell lines (Figure 29), in line with the data in primary keratinocytes, but different from HaCaT (Figure 28). Thus, the activation of the canonical pathway of NF-κB is constantly suppressed by IAPs also in tumour cell lines. In contrast, whereas the canonical TRAIL induced NF-κB activation is partially suppressed in the absence of IAPs.

Interestingly, NIK stabilisation was not detected upon TRAIL stimulation (Figure 29), similar to the data in HaCaT and primary keratinocytes (Figure 28), whereas the absence of IAPs stabilized NIK within 1 h (Figure 29). Although the absence of IAPs stabilised NIK, further stimulation with TRAIL significantly repressed NIK stabilisation in both cell lines, in line with data in HaCaT and primary keratinocytes. The non-canonical NF-κB pathway is also significantly suppressed by IAPs, although TRAIL-induced signalling suppresses the non-canonical NF-κB activation.

In both cell lines JNK and p38 as well as HSP27 were activated within 1-2h of TRAIL stimulation. In line with the data in HaCaT and primary keratinocytes, downregulation of IAPs did not influence p38 and JNK activation. In both cell lines TRAIL-induced p38 activation was unchanged or slightly increased in the absence of IAPs, in line with data from HaCaT cells. In the absence of IAPs, the TRAIL-induced JNK activation was slightly decreased in A5RT3, in line with data from primary keratinocytes, whereas in MET1 it was slightly increased.

In general these data suggest that IAPs negatively regulate both canonical and non-canonical NF-κB activation. TRAIL-induced signalling induces canonical, but not non-canonical NF-κB activation. Although in the absence of IAPs TRAIL-induced signalling is decreased for both canonical and non-canonical NF-κB pathways, primary as well as tumour cells exhibit these features. On the opposite, p38 as well as JNK activation were induced only by TRAIL, but not by IAP degradation, indicating that MAPK pathway is independent of IAPs. Interestingly in primary keratinocytes activation of both MAPK pathways was decreased in the absence of IAPs, whereas in both tumour cell lines activation of these pathways was enhanced. This might indicate that simultaneous usage of both TRAIL and IAP antagonist on the skin tumours may cause increased inflammation in tumour but not in normal cells. The significance of these findings is to be evaluated *in vivo* settings.

4. Discussion

4.1. cIAPs are critical regulators of TLR3-induced cell death in transformed keratinocytes and SCC cells

TLR3 signalling is tightly connected to cellular proliferation, apoptosis resistance and tumour progression (for review see (Li et al., 2010)). Controversially, TLR3 was also shown to induce apoptosis in cancer cells (Salaun et al., 2006) indicating that TLR3 agonists (such as poly (I:C)) could be potentially used as anti-tumour agents. However, several melanomas are resistant to TLR3 agonists (Salaun et al., 2007), leading to the hypothesis that a number of intracellular molecules regulate TLR3-induced cell death pathways.

For further understanding of these mechanisms, which determine TLR3 signalling outcome the role of IAPs was studied, since these proteins regulate cell death processes (Geserick et al., 2009; Varfolomeev et al., 2007). The goal of this study was to investigate the role of cIAPs in regulation of TLR3-induced cell death. While using a synthetic compound (IAP antagonist), siRNA targeting cIAPs or activation of signalling pathways (TWEAK) that antagonize the function of cIAPs, a dramatic increase in sensitivity of primary as well as immortalized keratinocytes (HaCaT) to poly (I:C)-induced cell death was observed. In line with a recent report (Weber et al., 2010), the results of this study now also show that cIAPs profoundly regulate TLR3-triggered cell death. IAP antagonist, which was used in this study, originally was established to disrupt the function of XIAP. In this study it was found that XIAP deficient HaCaT cells are substantially sensitized to poly (I:C) induced cell death. The data clearly show that cIAPs confer resistance to TLR3-induced cell death. Since HaCaT cells lack XIAP at the protein level, and TWEAK-FN14 signalling leaves XIAP levels unaffected (Wicovsky et al., 2009), the inhibition of cIAP function leads to the suggestion that cIAPs regulate TLR3-mediated cell death in a XIAP independent manner. In contrast the metastatic A5RT3 cells, that are more resistant to cell death induced by poly (I:C) treatment were not sensitized by TWEAK in a similar way. This result indicates the existence of other signalling pathways which regulate the TLR3 signalling, independent of IAPs.

Furthermore, MET1 SCC cancer cells, which have high XIAP expression at the protein level and show less sensitivity to TLR3-induced cell death, can be further sensitized by IAP antagonist. In contrast, TWEAK failed to sensitize MET1 to poly (I:C) treatment, indicating the relevance of XIAP for protection against TLR3-induced cell death in this cell line.

When discussing which molecules have greater impact for the cell death signalling pathways (cIAPs or XIAP) it has to be noted that the majority of the cell lines in the National Cancer Institute panel have overexpressed level of XIAP, when compared with matched normal tissue (Tamm et al., 2000). As well, XIAP overexpression has been correlated with poor prognosis in a variety of cancer types: adult and childhood acute myelogenous leukemia

(Tamm et al., 2000; Tamm et al., 2004), clear-cell renal carcinoma (Ramp et al., 2004), multiple myeloma (Nakagawa et al., 2006) and bladder cancer (Li et al., 2007). However, XIAP expression and the relative levels of other IAP family members vary between tumour lines. This suggests different regulation of these antiapoptotic genes between distinct types of cancer (Tamm et al., 2000). Moreover, mRNA levels of XIAP do not correlate with protein levels in the tumours, indicating post-transcriptional regulation of expressed proteins (IAP) (Dean et al., 2007). As well, evidence gained in this and other studies of our group in TLR3 and DR signalling suggest that cIAP1 has a stronger impact to the antiapoptotic protection of the cells than cIAP2 (Diessenbacher et al., 2008; Geserick et al., 2009). Summing up – all three members of IAP family grant significant protection from cell death, and the quantitative impact of each molecule in these signalling is highly cell type dependent.

Nonetheless, the observed sensitization of HaCaT as well as MET1 cells to TLR3-induced cell death by IAP depletion signifies that IAP downregulation could be used as a potential strategy to overcome the resistance of tumour cells to TLR3-induced cell death in a therapeutic setting.

4.2. cIAPs negatively regulate apoptotic and necrotic TLR3-induced signalling by limiting caspase and RIP-1 kinase activity in HaCaT keratinocytes

This study has shown the high relevance of cIAPs for regulation of TLR3-induced cell death signalling pathways. It has to be kept in mind for further possible usage of IAP antagonist and TLR3 ligand in anti-cancer therapy that the type of cell death is of critical importance: since apoptosis and necrosis utilize different ways of cleaning up the dead cells (Krysko et al., 2006), different consequences for the affected organ and organism might well be of critical importance (Festjens et al., 2006b; Greenhalgh, 1998). In transformed keratinocytes (HaCaT cells) the cell death induced by TLR3 in the absence of cIAPs had pronounced apoptotic features. In contrast, blocking of caspases dramatically changed the phenotype of the cell death giving it necrotic features (in the absence of cIAPs). This necrotic cell death is dependent on RIP-1 kinase activity, indicating RIP-1 as a key player in the necrotic cell death. This finding is in line with the landmark study that showed that necrotic cell death can be inhibited by RIP-1 kinase inhibitor Necrostatin-1 (Degterev et al., 2008). This observed switch to the necrotic cell death highlights cIAPs as negative regulators of RIP-1 kinase function and thus cell death resistance from TLR3 signals. RIP-1 in general plays significant role in the cell signalling e.g. RIP-1 knockout mice are born but die rapidly because of an increased sensitivity to TNF (Kelliher et al., 1998). RIP-1, and specifically its DD, was reported to be critical for CD95-mediated necrosis independent of NF-κB-inducing activity or RIP-1 kinase activity (Degterev et al., 2005; Holler et al., 2000). The precise role or potential

targets of the kinase activity of RIP-1 remain largely unknown (Hitomi et al., 2008), although a recent study claims a formation of RIP-1-RIP-3 complex, which regulates programmed necrosis and virus-induced inflammation (Cho et al., 2009).

Furthermore the downregulation of RIP-1 on the protein level in this study led to complete protection form both types of cell death induce by TLR3 in the absence of cIAPs. This indicates RIP-1 as a critical regulator of apoptosis signalling at the crossroad of all cell death pathways, induced by TLR3.

4.3. $cFLIP_L$ and $cFLIP_S$ differentially regulate apoptotic and necrotic TLR3 signalling pathways in the absence of cIAPs

The previous studies showed that cIAPs control apoptotic and necrotic TLR3-induced cell death signalling pathways. Furthermore, the inhibition of caspase activity in the absence of cIAPs induces a switch from apoptotic to necrotic type of cell death. The cFLIP proteins, $cFLIP_L$ and $cFLIP_S$, are both inhibitors of caspase-8 (Kataoka, 2005; Scaffidi et al., 1999) in cell death signalling complexes (Geserick et al., 2008) and therefore both cFLIP isoforms were proposed as potent cellular factors providing tumour protection from apoptosis (Zhang and Fang, 2005). In this study the role of caspase-8 activation for TLR3 induced apoptotic and necrotic cell death was further investigated by means of overexpression of cFLIP isoforms. Expression of both cFLIP isoforms induced resistance to TLR3-induced apoptosis in the absence of cIAPs. Interestingly, the overexpression of $cFLIP_S$, but not $cFLIP_L$ in the absence of cIAPs induced necrosis even in the absence of TLR3 stimulation. This indicates the critical relevance of cIAPs for protection of the cells against spontaneous necrotic cell death induced by $cFLIP_S$. Interestingly, blocking of RIP-1 kinase function with chemical inhibitor completely protected these cells from $cFLIP_S$ induced necrosis in the absence of cIAPs. This again confirms RIP-1 kinase function as crucial component of necrotic signalling in the cell.

The physiological relevance of this spontaneous necrosis is not clear to date, although recent studies have shown that viral variants of FLIP proteins (vFLIP), expressed by Human herpesvirus 8 (HHV-8), HVS8 (Nicholas, 2007), and $cFLIP_S$ isoform share high homology in the protein sequence as well as in their function (Thurau et al., 2009). For example the vFLIP isoform expressed by HHV-8 virus can inhibit caspase-8 activation (Glykofrydes et al., 2000) and protects from CD95L-induced cell death signalling pathways (Wu et al., 2004). Furthermore it was proposed that vFLIP from HHV-8 virus could be a molecule that promotes carcinogenesis of endothelial cells and leads to the formation of Kaposi's sarcomas (KS) (Thurau et al., 2009). In the context of cIAPs downregulation, vFLIP proteins could be critical factors of anti-tumour protection. The role of vFLIP proteins in the cell signalling and the possible role of IAPs in these signalling pathways will be the issue of future studies.

$cFLIP_L$ overexpression led to complete protection from apoptotic type of cell death, which was proposed before, since $cFLIP_L$ is known to block the cellular caspase activity (Kataoka, 2005). Surprisingly $cFLIP_L$ overexpression also fully blocked the necrotic cell death pathway, which could be due to increased interaction between RIP-1 and $cFLIP_L$ (Dohrman et al., 2005), or due to a changed stoichiometric ratio between RIP-1 and $cFLIP_L$. This may result in the blockade of the RIP-1 kinase function. Moreover, inhibition of active caspase-8 mediated cleavage of RIP-1, by $cFLIP_L$ could influence the RIP-1 kinase function and therefore necrotic signalling. The further hypothesis is described in chapter 4.4 of this thesis.
The further insight to the mechanism of TLR3 signalling was in the understanding of receptor complex composition and the function of its elements.

4.4. cIAPs control TLR3-induced cell death signalling by regulating the formation of intracellular complexes

The concept of the formation of intracellular signalling complexes that triggers either cell death or other cellular responses was firstly studied in death ligand signalling pathways (Festjens et al., 2006a). The recruitment and the activation of pro- and anti-apoptotic proteins to membrane bound death receptor complexes (such as the DISC) (Peter and Krammer, 2003) or to cytoplasmic intracellular complexes (complex II) are crucial for the cell signalling outcome (Dohrman et al., 2005). Growing evidence rose that the enzymatic activities of RIP-1, cFLIP and caspase-8 play an important role either for cell death induction or cell survival.
When TLR3-dependent signalling events were examined, it was found that cIAPs control the composition of TLR3 signalling complexes as well as the modification of molecules within that complex that ultimately regulates the outcome of cell near responses (Figure 30). Activated TLR3 leads to formation of an intracellular complex that contains RIP-1, caspase-8, cFLIP isoforms, FADD and TRIF. The formation of this signalling platform results in caspase-dependent apoptosis induction (Figure 30, gray area). This cell death can be blocked by caspase inhibitors such as zVAD-fmk, $cFLIP_L$ and $cFLIP_S$, which indicates the crucial role of caspase-8 activation in such pro-apoptotic complexes.
The presence of IAPs blocks formation of intracytoplasmic signalling platform, which in this study is called “RIP-1 complex”, containing RIP-1, Caspase-8, FADD and cFLIP (Figure 30 compare white and pink areas). This spontaneously formed complex is necessary but insufficient for cell death induction. Since the adaptor molecule TRIF is lacking in this complex, we propose that upstream signalling via TLR3 receptor is required to trigger the formation of the pro-apoptotic and necrotic signalling platform (Figure 30, green area).
At endogenous protein levels TRIF was found in the caspase-8-interacting complex in this study. Previous findings in TRIF knockout mice (Kaiser and Offermann, 2005; Yamamoto et al., 2003) or siRNA-mediated TRIF knockdown cells (Weber et al., 2010) showed that the

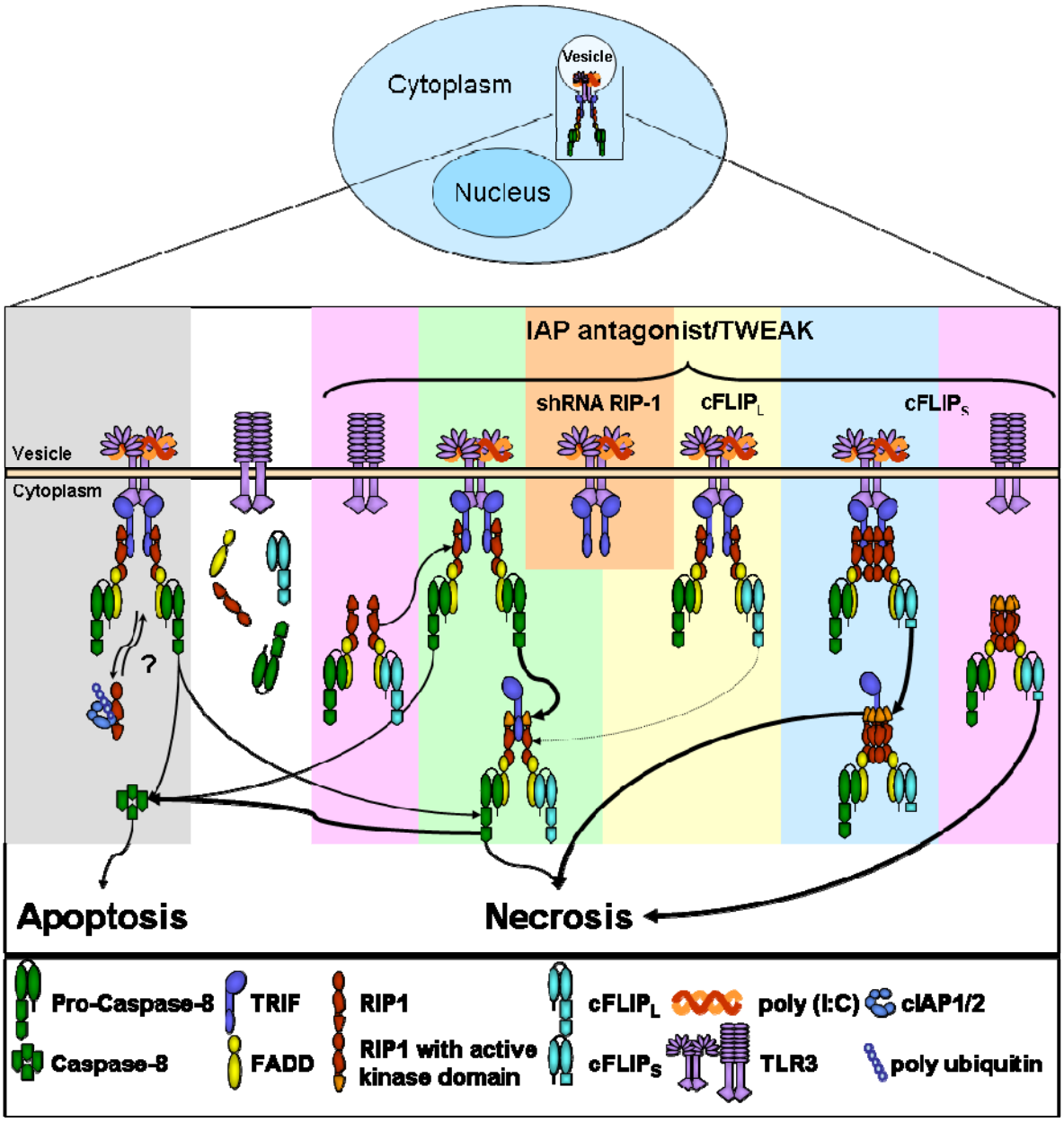


Figure 30. The role of cIAPs during TLR3-mediated cell death in HaCaTs.
cIAPs block formation of a cytoplasmic Caspase-8-bound complex. The complex contains the initiator Caspase-8, RIP-1, cFLIP, and FADD and is able to bind to the cytoplasmic domain of TLR3 via TRIF. This signalling platform induces cell death in a caspase-dependent as well as caspase-independent manner. In the absence of RIP-1, the formation of this complex is suppressed, and therefore further signalling is blocked. cFLIP$_L$ also suppresses caspase-8 complex formation by competition with Caspase-8 for binding to FADD. cFLIP$_L$ protects from apoptotic as well as non-apoptotic cell death. cFLIP$_S$ leads to massive formation of a cytoplasmic caspase-8 bound complex (without TRIF) with active RIP-1 in the absence of cIAPs independent of TLR3 stimulation. RIP-1 kinase activity in this complex is the reason for a constitutive sensitivity to treatment with IAP antagonist. cFLIP$_S$ overexpression protects from TLR3 induced apoptosis, but not from necrotic-like cell death.

adaptor function of TRIF in apoptotic TLR3 signalling is required. The findings of this study now further extend these data and show that in poly (I:C)/IAP antagonist-stimulated cells the interaction of FADD and TRIF as well as RIP-1 that are bound to caspase-8 are dramatically

increased. This is ultimately sufficient to promote apoptotic and necrotic cell death (Figure 30, green area).

Furthermore, other reports suggested that TRIF-RIP-1 interaction is critical for TLR3-induced pro-inflammatory signalling (Meylan et al., 2004) and cell death (Kaiser and Offermann, 2005). Results of this study now indicate that cIAPs suppress TLR3 mediated cell death induction by limiting RIP-1 recruitment. In addition, this RIP-1/caspase-8 complex favours apoptotic cell death, but inhibition of caspase activity unmasks the alternative RIP-1 kinase activity dependent form of cell death. The role of RIP-1 in TLR3 signalling is controversially discussed, since RIP-1 has proved to be an essential molecule for execution of cell death as well as for many other signalling pathways such as NF-κB activation (for review see (Declercq et al., 2009)). However, when RIP-1 expression was reduced, a decrease in cytoplasmic complex formation was observed, and such cells were fully resistant to both apoptotic and necrotic cell death (Figure 30, orange area). Thus results of this study identified a number of RIP-1 functions in TLR3 signalling. RIP-1 acts not only as an indispensible adapter molecule, but is also critical for cell death induction, whereas the kinase function of RIP-1 is solely required for necrotic cell death.

To further understand the role of caspase activation in TLR3 signalling, the impact of the endogenous caspase inhibitor cFLIP and its isoforms on caspase-8 inhibition within these cytoplasmic complexes was studied. Results of this study support the concept that cFLIP isoforms differentially regulate TLR3-induced cell death signalling. Noteworthy both cFLIP isoforms suppressed TLR3-triggered apoptotic cell death, and upon overexpression of both isoforms the amount of cFLIP in caspase-8 complex was increased. $cFLIP_S$ can promote necrosis in the absence of cIAPs upon TLR3 stimulation (Figure 30 blue area), as well as without any TLR3 stimulation (Figure 30 right pink area). This necrotic cell death is critically dependent on RIP-1-kinase activity and RIP-1 recruitment to the caspase as well as RIP-1 complexes is increased. In addition, high $cFLIP_L$ levels not only correlate with resistance of cells to apoptotic but also to caspase-independent cell death correlating with reduced RIP-1 recruitment to the cytoplasmic complex (Figure 30, yellow area). The hypothesis is that, $cFLIP_L$ may interfere in caspase-8-mediated RIP-1 cleavage in the cellular signalling platform, which contains cFLIP isoforms and caspase-8 as shown for CD95 signalling pathway (Geserick et al., 2009). Future mechanistic studies are required to elucidate this point in more detail.

The composition of the cytoplasmic complexes, formed upon TLR3 stimulation, has crucial relevance for the cell fate. The increased amount of cFLIP isoforms in the complex leads to anti-apoptotic protection, whereas the increased amount of RIP-1 promotes necrosis. Future studies will now aim to discover other components of these complexes, relevant for the cell signalling.

4.5. cIAPs control apoptotic and necrotic cell death induced by CD95L

In recent years the death receptors in general (Mahmood and Shukla, 2010; Russo et al., 2010) and CD95 specifically (Gerspach et al., 2009; Wicovsky et al., 2009) were studied as potential targets for tumour treatment. Growing evidence indicates that cIAPs regulate death ligand induced cell death and non-apoptotic signalling pathways (Geserick et al., 2009; Varfolomeev et al., 2007). Data gained in this study with IAP antagonist and TWEAK-FN14 supports these findings. Upon CD95 stimulation HaCaT cells activate the apoptotic pathway, whereas the reduction of cIAPs level in the cell leads to significant increase of apoptotic cell death, indicating the essential relevance of cIAPs for the regulation of CD95-induced apoptosis. The blocking of caspases by chemical inhibitors or $cFLIP_S$ overexpression also unmasked the necrotic cell death pathway, which was controlled by RIP-1 kinase activity, further supporting the position of RIP-1 as a key necrotic regulator. The downregulation of RIP-1 by shRNA did not influence CD95-induced apoptosis, but significantly decreased the CD95-induced cell death in the absence of IAPs. Furthermore $cFLIP_L$ isoform blocked both apoptotic and necrotic cell death induced by CD95L. Taken together these results nearly duplicate the data gained in the study of TLR3 signalling, indicating that the role of cIAPs is broader and involves for both TLR3 and CD95 signalling pathways.

Recent studies have shown that the formation of cytoplasmic complexes upon TNF (Varfolomeev and Vucic, 2008), CD95L or TRAIL (Geserick et al., 2009) stimulation is also enhanced by loss of IAPs. These studies have proposed that cIAPs inhibit the recruitment of RIP-1 to these complexes and therefore protect the cells from DR-induced apoptosis as well as necrosis mediated by RIP-1 kinase activity (Geserick et al., 2009). Intriguingly, $cFLIP_L$, but not the $cFLIP_S$ isoform inhibited the formation of the cytoplasmic complex (Geserick et al., 2009). Taken together these findings indicate common activation mechanism of apoptosis as well as necrosis (in the absence of caspase activity) which are suppressed by cIAPs under physiological conditions.

4.6. cIAPs are suppressors of RIP-1 complex formation

This study together with other works of our group (Geserick et al., 2009) show, that two distinct groups of receptors (DR and TLR) share similar mechanisms of the molecular machinery of cell death pathways. This indicates that signalling platform could prove important for a larger number of cell death pathways. The current hypothesis is that cIAPs are constantly ubiquitinating RIP-1, which may either lead to subsequent proteasomal degradation of RIP-1 (Figure 31 left part). Alternatively cIAP-mediated ubiquitination, most

likely by Lys63, may provide critical for inhibition of formation of such a signalling platform. This RIP-1 complex, which contains RIP-1, caspase-8, FADD and cFLIP, but is unable to induce cell death by itself (Figure 31 middle part) is a major finding of this thesis. When $cFLIP_S$ isoform is recruited to the RIP-1 complex it can promote activation of RIP-1 kinase and therefore induce necrotic cell death. In the absence of IAPs, which can also be mediated by TWEAK (Vince et al., 2008), the RIP-1 complex, is spontaneously formed (Figure 31 middle part). Although the RIP-1 complex is unable induce cell death by itself, its formation simplifies the cell death signalling via other cellular signals. Upon stimulation of the respective receptor (e.g. DR or TLR3) the RIP-1 complex is able to bind to these receptors, followed by posttranslational modification of the molecular components and further dissociation of the complex from the receptor (Figure 31 right part). This dissociation of the c

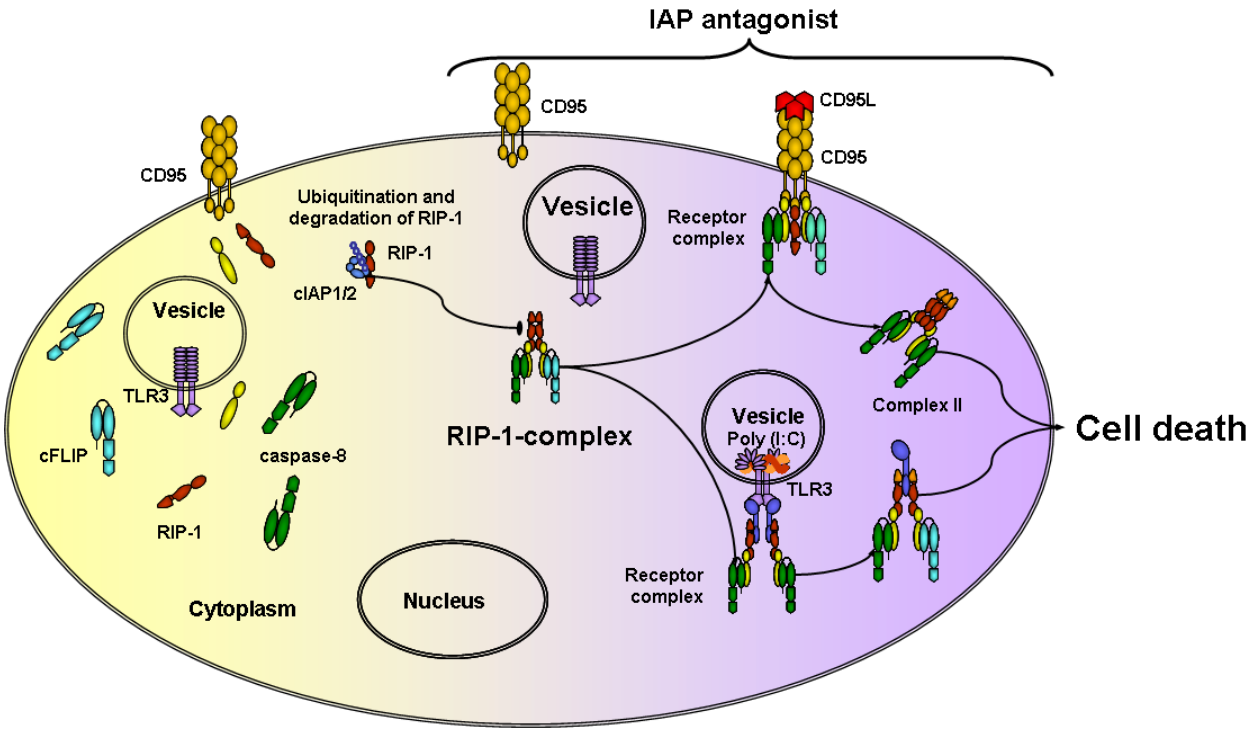


Figure 31. Cell death signalling in the absence of IAPs. IAPs block formation of a RIP-1 complex, which does not induce cell death by itself, but simplifies formation of receptor complexes (TLR3 and TRAIL), which leads to intensive formation of "second complexes" which leads to cell death.

omplex from the receptor was shown for DR signalling (Geserick et al., 2009) and will be a subject of future studies in the TLR3 signalling pathway. These dissociated complexes are called "complex II" (Geserick et al., 2009) (Figure 31 right part). It can trigger cell death in both caspase and RIP-1 kinase dependent manner. Understanding of the regulation and composition of such RIP-1 complexes might prove highly useful in the attempt to design novel therapeutic strategies that are needed to overcome cell death resistance, such as for tumour therapy or virus elimination from an infected host.

4.7. cIAPs and XIAP protect primary keratinocytes from TLR3-induced apoptotic and non-apoptotic cell death

The probable use of IAP antagonists, combined with TLR3 or death receptor agonists as treatment for tumours, should always relate to studied in primary keratinocytes in this thesis. Beside the obvious differences between primary keratinocytes and the model SCC cell lines, such as wild type p53 and the likely absence of chromosomal aberrations in primary keratinocytes (Boukamp, 2005), there are also several other differences. For example, primary keratinocytes have high levels of cFLIP expression and therefore are resistant to TRAIL-induced cell death (Leverkus et al., 2000). In contrast, the sensitivity of primary keratinocytes to TLR3 in this study was on the same level as in HaCaT (immortalised keratinocytes). An additional important difference of primary keratinocytes is that they express high levels of XIAP, which is also expressed by MET1 cells, but is absent in HaCaT and the derived cell line A5RT3. Similar to the data in the SCC models, primary keratinocytes were further sensitized to TLR3-induced cell death by the absence of IAPs, and cell death showed apoptotic features. Whereas blocking of caspases also revealed the cryptic necrotic pathway (Figure 32, blue field). This necrotic cell death was also dependent on RIP-1 kinase activity, duplicating the findings in the immortalised keratinocytes. Intriguingly and in contrast to the SCC cell lines, blocking of RIP-1 kinase functions in the presence and absence of IAPs led to an increase of apoptotic cell death, indicating the crucial relevance of RIP-1 kinase function for antiapoptotic protection of primary keratinocytes (Figure 32, blue field). Intriguingly the combined inhibition of RIP-1 and caspase activity could not grant full protection against TLR3-iduced cell death in the presence as well as in the absence of IAPs. This indicates that there are other TLR3 pro-death signalling pathways, which are not mediated by caspases or RIP-1 kinase function. One of the possibilities could be the activation of IFN regulatory factors (IRF) 3, 5 and 7 upon stimulation of TLR3 (Boo and Yang, 2010). Activation of these factors can be induced by binding of TRAF3 to TRIF and further association of TRAF3 into a complex with IKKε and TBK1 (Hacker and Karin, 2006). IRF5 is a strong transcription activator for IFN-α production, and IRF7 can induce IFN-α and also IFN-β preferentially (Boo and Yang, 2010). The IFNs in turn can induce apoptosis in various cell types (Chawla-Sarkar et al., 2003).

Furthermore, RIP-1 downregulation did not induce protection from TLR3-induced cell death both in presence and absence of IAPs in two of three donors, although morphologically the induced cell death had more necrotic than apoptotic features. Interestingly blocking of RIP-1 kinase function in RIP-1 knockdown primary keratinocytes induced strong protection against TLR-3-induced cell death (Figure 32, green field). The complete protection as found in HaCaT was not achieved in the absence of combined caspase and RIP-1 kinase activity.

These results suggest that in HaCaT immortalised keratinocytes the signalling is performed

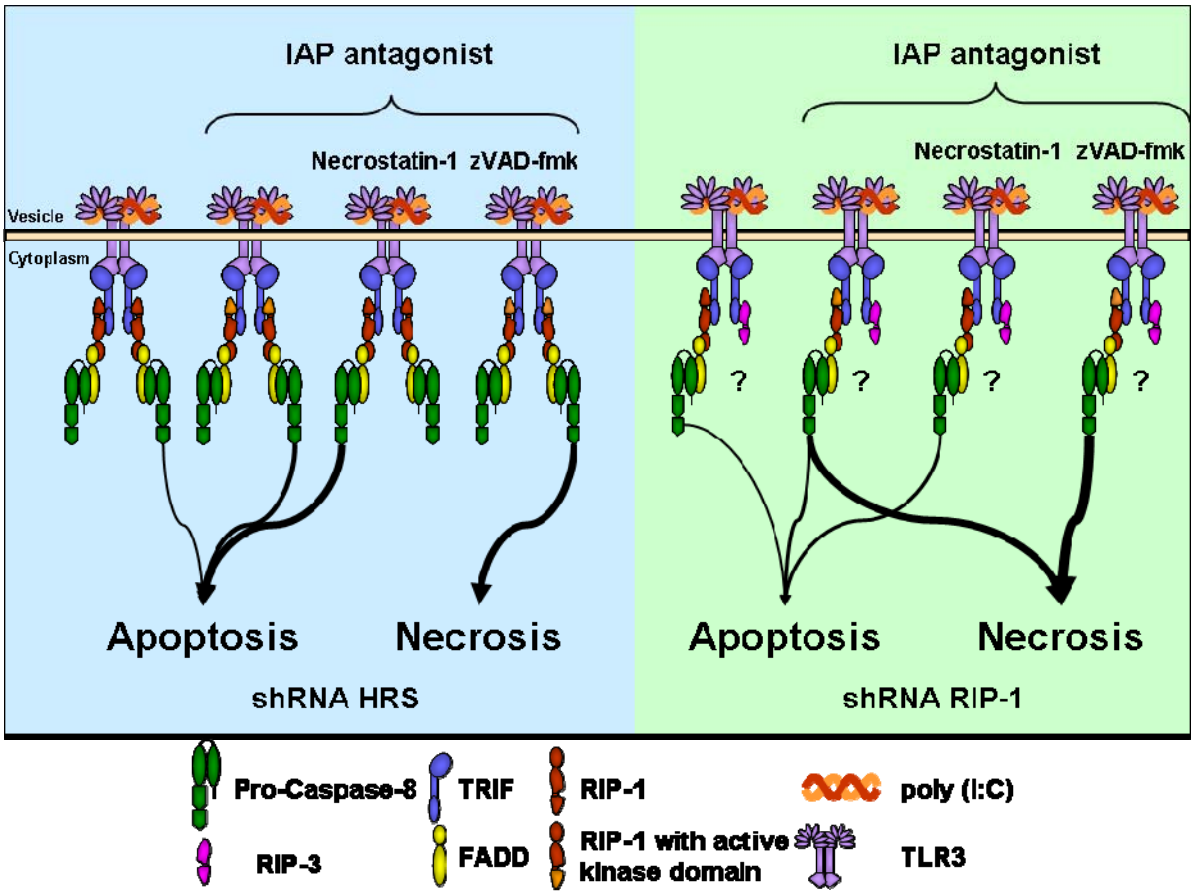


Figure 32.The role of IAPs in TLR3-mediated cell death in primary keratinocytes. Blue field: TLR3 induces apoptosis in the primary keratinocytes, independent of RIP-1 expression level. The absence of IAPs sensitizes the cells to TLR3-induced apoptosis and blocking of RIP-1 kinase function is increasing the effect, whereas the blocking of caspases changed the cell death type to necrosis. **Green field:** RIP-1 downregulated cells are sensitive to TLR3 induced cell death in the presence and absence of IAPs. Although in the absence of IAPs these cells suffer necrosis upon TLR3 stimulation, which is partially reduced by blocking of RIP-1 kinase function and is promoted by blocking of caspase functions.

otherwise, compared to primary keratinocytes. The possible explanation could be the high XIAP expression in primary keratinocytes, which is absent in HaCaTs, and XIAP function is also targeted by IAP antagonist. The role of XIAP in keratinocyte cell death processes is aimed to dissect in the future by using TWEAK. Moreover, the other RIP family members, like RIP-3 could influence these signalling pathways. RIP-3 can interact with TRIF and RIP-1 via its RHIM (Declercq et al., 2009; Kaiser and Offermann, 2005) and is known to induce necroptosis (Cho et al., 2009; Feng et al., 2007; He et al., 2009; Zhang et al., 2009). Therefore in RIP-1 knockdown cells RIP-3 could be recruited to the TLR3 cytoplasmic signalling complex and promote the necrosis shift (Figure 32, green area). The role of RIP-3 in the cell death signalling in SCC as well as in primary keratinocytes will be a topic of future separate studies.

Summing up the data in primary keratinocytes, the combined use of TLR3 stimulation and IAP antagonist as a therapeutic agent is debatable, since primary keratinocytes were shown to be as sensitive as immortalised cell lines, whereas the tumour and metastatic cells proved to be insensitive, or at least less sensitized by IAPs downregulation. Although, more extended studies are needed to solve these perplexing issues, why primary keratinocytes react differently, as compared to tumour cells in respect to inhibition of RIP-1 kinase activity.

4.8. The impact of IAPs for the control of non-apoptotic signals activation in TRAIL stimulated primary keratinocytes and SCC cell lines

TRAIL is proposed to be a probable anticancer therapeutic agent, since the primary keratinocytes proved to be more resistant to TRAIL-induced apoptosis, when compared to immortalised keratinocytes and cancer cells (Leverkus et al., 2000). IAPs proved to play an important role for TRAIL-induced cell death (Geserick et al., 2009). Therefore the combined use of TRAIL and IAP antagonist appears possible as anti-tumour strategy. Since NF-κB activation is tightly connected to up-regulation of anti-apoptotic proteins, such as cFLIP and cIAPs (Chiron et al., 2009; Wang et al., 1998), NF-κB induced signalling can activate apoptosis resistance and therefore carcinogenesis (Stone et al., 2009).

TLR3 as well as DR signals affect a wide range of physiological and pathophysiological processes in human patients such as autoimmune diseases and cancer progression (Huang et al., 2008; Suhir and Etzioni, 2010). NF-κB dependent suppression of apoptosis or the creation of an inflammatory microenvironment that promotes growth, survival and potentially metastasis of tumour cells has been proposed (Li et al., 2010).

In this study it was shown that TRIAL, in presence of IAPs, is inducing only canonical NF-κB activation (Figure 33, yellow area). IAPs are regulating non-canonical NF-κB activation (Figure 33, compare yellow and violet areas) (Varfolomeev et al., 2007), probably by promoting NIK degradation. Also, IAPs block spontaneous activation of the canonical NF-κB pathway. Some studies propose that NIK, which is a part of the non-canonical NF-κB pathway, may as well regulate the canonical NF-κB signalling by yet unknown mechanism (Varfolomeev and Vucic, 2008) (Figure 33, violet are). Interestingly TRAIL-induced signalling in the absence of cIAPs showed partially blocked canonical and completely blocked non-canonical NF-κB pathways. Therefore the combined usage of TRAIL and IAP antagonist on keratinocytes should have repressed NF-κB induction and, consequently should not lead to following inflammation or expression of antiapoptotic proteins. Thus our data support the combined use of TRAIL and IAP antagonist as an anti-tumour therapy, although our data about RIP-1 kinase function in primary keratinocytes clearly merit cautious testing in this context

In contrast to NF-κB pathways, MAPK signalling is not auto-activated upon IAP depletion, although TRAIL treatment leads to p38 and JNK MAPK activation (Song and Lee, 2008) in primary as well as in SCC cell lines. Interestingly, TRAIL resistance in malignant cells was shown to be differentially mediated by p38, whereas in normal cells, resistance was mediated by NF-κB (Steele et al., 2006). In this study it was shown that cancer MET1 and

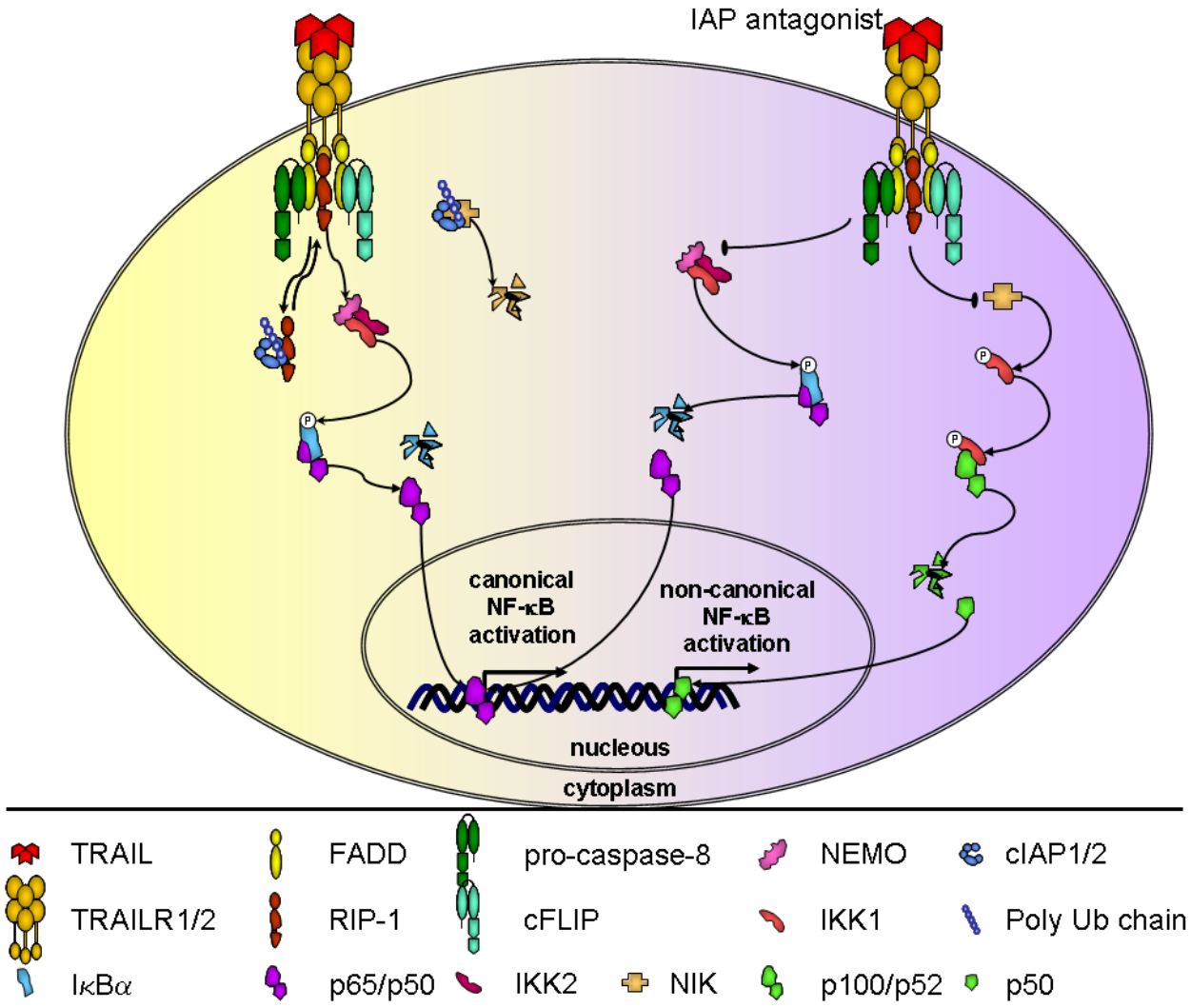


Figure 33. Canonical and non-canonical NF-κB activation in presence and absence of IAPs. IAPs block non-canonical NF-κB activation by promotion of NIK degradation. IAPs block spontaneous NF-κB activation. TRAIL signalling blocks spontaneous activation of both pathways

A5RT3 cells display increased p38 activation upon TRAIL stimulation in the absence of IAPs. In contrast, p38 activation was unchanged in HaCaT cells and decreased in primary keratinocytes. The hypothesis is that this could be one of the reasons for reduced sensitivity of MET1 and A5RT3 to TRAIL in the absence of IAPs, compared to HaCaT cells data, which was shown in (Geserick et al., 2009). In contrast, TRAIL-induced activation of JNK seems to be not influenced by the absence of IAPs in SCC cell lines, whereas in primary keratinocytes the absence of IAPs reduces the TRAIL-induced JNK activation.

Taken together these results suggest that in order to increase the sensitivity of tumour cell to TRAIL, inhibition of IAPs could be combined with p38 chemical inhibitors, like SB203580. However the detailed study of TRAIL-induced p38 activation and the interplay of MAPK and IAPs and its role in anti-tumour therapy must be the object of future studies.

4.9. Perspectives

The current study has clearly demonstrated the crucial role of IAPs, RIP-1, and cFLIP variants in the regulation of TLR3 as well as DR signalling. Based on studies of this thesis, a number of intriguing questions arise:

- The RIP-1 complex formation and modification of its components

This study has shown cIAPs as negative regulators of intracellular RIP-1 complex formation. The regulation and composition of such RIP-1 complexes will be the topic of further studies. These future studies might prove useful to come to novel therapeutic strategies to overcome cell death resistance, such as tumour therapy or virus elimination from an infected host.

- RIP-3 and its role in cell death signalling

RIP-3 is another RIP family member, which also has a kinase and intermediate domain with a RHIM, although RIP-3 is lacking a death domain. Furthermore, RIP-3 can interact with TRIF and RIP-1 via its RHIM (Declercq et al., 2009; Kaiser and Offermann, 2005). RIP-3 is also known to induce necroptosis (Cho et al., 2009; Feng et al., 2007; He et al., 2009; Zhang et al., 2009). Consequently, in this study RIP-3 was proposed as a potential candidate for recruitment to TLR3 signalling complexes in the absence of RIP-1 and promotion of necrosis shift of the cell death signalling pathway. Clarifying the role of RIP-3 will be important for the understanding of cell death signalling in general, and specifically for necrotic signalling, induced by different membrane-bound receptors.

- Role of cIAPs in DR and TLR3 signalling in primary keratinocytes

This study has identified the IAP proteins as crucial regulators of cell death signalling in primary keratinocytes, although the exact role of cIAPs and XIAP requires further elaboration. Using TWEAK as alternative for IAP inactivation is one of the ways to address this question.

- The role of p38 activation in protection of tumour cells from apoptosis.

This study has also revealed that IAPs inhibit TRAIL-induced p38 activation in tumour cells, which were not, or were only partially sensitive to TLR3 and DR stimulation in presence and absence of IAPs. Since TRAIL resistance in malignant cells was shown to be differentially mediated by p38 (Steele et al., 2006), a potential role of p38 in cell death protection of the tumour cells as well as the role of IAPs in this process will be compelling future tasks to understand the interplay of cell death and inflammatory signalling pathways.

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6. Appendix

6.1 Abbreviations

% (v/v)	percent by volume
% (w/v)	percent by mass
4-HT	4-Hydroxytamoxifen
aa	amino acids
AIF	Apoptosis-Inducing Factor
AP-1	Activating Protein 1
BAD	Bcl-2-associated death promoter
BAK	Bcl-2 homologous antagonist/killer
BCC	Basal Cell Carcinoma
Bcl-2	B-cell lymphoma 2
Bcl-X	Bcl-2-like protein 1
BIM	Bcl-2-like protein 11
BIR	Baculoviral IAP Repeat
BMF	Bcl-2-modifying factor
CARD	Caspase Activation and Recruitment Domain
Caspase	Cysteinyl-Aspartate Specific Protease
CD95	=FasL (FS7-associated cell surface antigen)
CD95L	=FasL (FS7-associated cell surface antigen Ligand)
cFLIP	cellular FLICE-Inhibitory Protein
cIAP	cellular Inhibitor of Apoptosis Protein
CIAP	Alkaline Phosphatase from calf intestine
CICD	Caspase-independent cell death
CM	Cleavage mutant
DD	Death Domain
DED	Death Effector Domain
DISC	Death Inducing Signalling Complex
D-MEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
dNTPs	Deoxynucleoside Triphosphate Set
DR	death receptor
E1	ubiquitin activating enzyme
E2	ubiquitin conjugating enzyme
E3	ubiquitin ligase enzyme
E.coli	Escherichia coli
EDTA	ethylenediaminetetraacetic acid
ERK	extracellular-signal-regulated kinase
Expo	exposition
F-	Flag (tag)
FADD	Fas-Associated Death Domain protein
FBS	Fetal Bovine Serum
Fc	Fragment crystallizable
FLICE	FADD-like interleukin-1 beta-converting enzyme
GFP	Green fluorescent protein
HaCaT	human adult low calcium temperature (keratinocytes)
HEK 293T	Human Embryonic Kidney 293 large T transformed
HF	His-Flag (tag)
HHV-8	Human herpesvirus 8
HRP	Horseradish peroxidase
HRS	Hyper random sequence
HVS8	herpesvirus saimiri 8
IFN	Interferon
IgG	Immunoglobulin G
IκB	Inhibitor of NF-κB
IKK	Inhibitor of κB Kinase
IL	Interleukin
IP	Immunoprecipitation
IRF	IFN regulatory factor

JNK	c-Jun N-terminal kinase
KD	Kinase Death
kDa	kilo Dalton
KS	Kaposi sarcoma
LUBAC	The linear ubiquitin chain assembly complex
Mal	MyD88-adapter-like
MAPK	Mitogen-Activated Protein Kinase
MW	Molecular Weight
MyD88-	Myeloid differentiation primary response gene (88)
NOXA	Phorbol-12-myristate-13-acetate-induced protein 1
NEMO	NF-κB Essential Modulator
NF-κB	Nuclear Factor 'kappa-light-chain-enhancer' of activated B-cells
NIK	NF-κB-Inducing Kinase
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate-Buffered Salines
PCR	Polymerase chain reaction
PI	Propidium Iodide
PUMA	BH3-only protein, p53 upregulated modulator of apoptosis
PVDF	Polyvinylidene fluoride
QVD	(Q-VD-OPH) Q-Val-Asp(non-omethylated)-OPh
R	Receptor
RHIM	RIP homotypic interaction motif
RING	Really Interesting New Gene
RIP	Receptor-Interacting Protein
SAPK	stress-activated protein kinase
SCC	Squamous cell carcinoma
SDS	Sodium Dodecyl Sulfate
SEM	Standard Error of Mean
shRNA	Small hairpin RNA
SiRNA	Small interfering RNA
SMAC/DIABLO	Second Mitochondrial Activator of Caspases/Direct IAP Binding protein with Low pI
TAE	Tris-acetate-EDTA
TAK1	Transforming Growth Factor-β (TGF-β)-Activated Kinase 1
TBK1	TANK-binding kinase
TIR	Toll/IL-1 receptor domain
TL	Total Lysate
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRADD	TNF Receptor Associated Death Domain
TRAF	TNF Receptor Associated Factor
TRAIL	TNF-related apoptosis-inducing ligand
TRIF	TIR-domain-containing adapter-inducing interferon-β
TWEAK	Tumor necrosis factor-like weak inducer of apoptosis
U	Unit
UBA	Ubiquitin-Associated (domain)
UBD	Ubiquitin binding domain
UV	Ultra Violet
vFLIP	viral FLICE-Inhibitory Protein
WT	Wild type
XAF1	X-linked IAP-associated factor 1
XIAP	X-linked IAP
ZVAD-fmk	z-Val-Ala-DL-Asp(OMe)-fluoromethylketone

6.2. Curriculum vitae

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1.10.2005 – 30.09.2008	Scholarship from GRK 1167, Otto-von-Guericke University, Magdeburg Department of Experimental Dermatology, Prof. M. Leverkus PhD thesis work: “The role of RIP-1 and cIAPs in apoptotic and non-apoptotic signalling via TLR3 and death receptors”
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23.08.2010	Submission of the PhD Thesis

Magdeburg, 23.08.2010

Feoktistova M.

6.3. Publications

Kotnova A.P., Karpova N.N., **Feoktistova M.A.**, Liubomirskaya N.V., Kim A.I. and Il'yin Y.V. Retrotransposon *gtwin*: structural analysis and distribution in *Drosophila melanogaster* strains. *Genetika* 2005, V. 41 (1), p. 23-29. Russian. (Article)

Kotnova A.P., **Feoktistova M.A**., Glukhov I.A., Salenko V.B., Lyubomirskaya N.V., Kim A.I., Ilyin Y.V.. Retrotransposon gtwin specific for the *Drosophila melanogaster* subgroup. *Dokl Biochem Biophys*. 2006 Jul-Aug;409:233-5. (Article)

Wachter T, Felcht M, **Feoktistova M**, Kavuri S, Geserick P, Siegmund D, Brocker EB, Leverkus M. – TRAIL-mediated activation of mitogenactivated protein kinases in human keratinocytes: p38 activation is necessary for TRAIL-induced IL-8 secretion. – *Journal of Investigative Dermatology*, OCT 2007, V.127, p.S95-S95 (Meeting Abstract).

Geserick P, Hupe M, Moulin M, Wong WWL, **Feoktistova M**, Kellert B, Gollnick H, Silke J, Leverkus M – Cellular IAPs inhibit a cryptic CD95-induced cell death by limiting RIP1 kinase recruitment. – *Journal of Cell Biology*, 2009, V.187, №7, p. 1037-1054 (Article)

Gnad T, **Feoktistova M**, Leverkus M, Lendeckel U, Naumann M – *Helicobacter pylori*-induced activation of β-catenin involves low density lipoprotein receptor-related protein 6 and Dishevelled. – *Molecular Cancer*, 2010, **9**:31 (Article)

Kavuri S, Berg D, Geserick P, Panayotova Dimitrova D, **Feoktistova M**, Siegmund D, Gollnick H, Neumann M, Wajant H, Leverkus M. – cFLIP isoforms block death receptor-induced NF-kappa B activation irrespective of caspase-8 or cFLIP processing, – *Journal of Biological Chemistry* 2010 (Article in revision)

Feoktistova M, Geserick P, Hupe M, Häcker G, Leverkus M. – cIAPs block TLR3-mediated cell death by interference with Ripoptosome formation, a RIP1/caspase-8 containing intracellular complex. – *Molecular Cell*, 2010 (Article submitted)

6.4. Erklärung

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Erklärung
Hiermit erkläre ich, dass die von mir eingereichte Dissertation zum Thema:

The role of RIP-1 and cIAPs in apoptotic and non-apoptotic signalling via TLR3 and death receptors

selbständig verfasst, nicht schon als Dissertation verwendet habe und die benutzten Hilfsmittel und Quellen vollständig angegeben wurden.

Weiterhin erkläre ich, dass ich weder diese noch eine andere Arbeit zur Erlangung des akademischen Grades doctor rerum naturalium (Dr. rer. nat.) an anderen Einrichtungen eingereicht habe.

Magdeburg, der 23.08.2010

Diplom Genetikerin Maria Feoktistova