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Review

Regulation of CD95/Fas signaling at the DISC

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CD95 (APO-1/Fas) is a member of the death receptor (DR) family. Stimulation of CD95 leads to induction of apoptotic and non-apoptotic signaling pathways. The formation of the CD95 death-inducing signaling complex (DISC) is the initial step of CD95 signaling. Activation of procaspase-8 at the DISC leads to the induction of DR-mediated apoptosis. The activation of procaspase-8 is blocked by cellular FLICE-inhibitory proteins (c-FLIP). This review is focused on the role in the CD95-mediated signaling of the death effector domain-containing proteins procaspase-8 and c-FLIP. We discuss how dynamic cross-talk between procaspase-8 and c-FLIP at the DISC regulates life/death decisions at CD95.
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Facts

- CD95 (APO-1/Fas) is a member of the death receptor (DR) family
- CD95 stimulation leads to the formation of the CD95 death-inducing signaling complex (DISC).
- Procaspase-8 is activated at the DISC leading to the induction of both apoptotic and non-apoptotic pathways.
- Cellular FLICE-inhibitory proteins (c-FLIP) isoforms: c-FLIP$_L$, c-FLIP$_S$ and c-FLIP$_R$ control procaspase-8 activation at the DISC.

Open Questions

- The exact mechanism of CD95-mediated non-apoptotic signaling is not established.
- The stoichiometry of the CD95 DISC is a question of future studies.
- New molecules may be found to be associated with the DISC.

CD95 Signaling

CD95 (also called APO-1; Fas; fas antigen; tumor necrosis factor receptor superfamily member 6, TNFRSF6) is a member of the DR family, a subfamily of the tumor necrosis factor receptor superfamily.[1] All members of the DR family are characterized by a cytoplasmic region termed death domain (DD).[2,3] DD are 80–100 amino-acid long motifs involved in the transduction of the apoptotic signal. Crosslinking of CD95 with its natural ligand (L), CD95L (CD178)[4] or with agonistic antibodies such as anti-APO-1[5] induces apoptosis in sensitive cells.

Stimulation of CD95 has been also reported to trigger non-apoptotic pathways.[6–12] However, details of CD95-mediated non-apoptotic pathways remain largely unknown. Importantly, it has been shown that membrane-bound CD95L is essential for the cytotoxic activity, whereas soluble CD95L appears to promote autoimmunity and tumorigenesis via induction of non-apoptotic pathways, in particular NF-κB.[13] Future studies should elucidate more details on the mechanism of non-apoptotic action of CD95L.

Binding of CD95L or agonistic antibodies to CD95 leads to formation of a receptor complex at the cellular membrane, which was named DISC.[14] The DISC consists of oligomerized receptors, the DD-containing adaptor molecule FADD/MORT1 (Fas-associated DD), procaspase-8 (FLICE, MACHα, Mch5), procaspase-10 and the c-FLIP (Figure 1).[15–17] The interactions between the molecules at the DISC are based on homotypic contacts. The DD of the receptor interacts with the DD of FADD, whereas the death effector domain (DED) of FADD interacts with the N-terminal tandem DEDs of procaspases-8, -10 and c-FLIP. As a result of DISC formation procaspase-8 is activated at the DISC resulting in the formation of the active caspase-8, which leads to apoptosis.

The initial events of DISC formation have not been clarified yet. Pre-oligomerization of CD95 via the pre-ligand assembly domain has been reported to have an important role in apoptosis initiation.[18] Recently, there have been several new reports on the X-ray structure of the complex formed by isolated CD95 and FADD DDs.[19,20] Scott *et al.*[19] have suggested that binding of CD95L leads to an opening of the CD95 DD, which exposes the FADD-binding site and simultaneously generates a bridge between two CD95 molecules. They show that a basic unit of this oligomeric CD95 network is composed of a tetramer, comprising four FADD DDs and four CD95 DDs. In contrast, Wang *et al.*[20] have reported that a basic unit of the CD95 DISC comprises

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This article is dedicated to Jürg Tschopp.
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Abbreviations: DISC, death-inducing signaling complex; c-FLIP, cellular FLICE-inhibitory proteins; cyt *C*, cytochrome *C*
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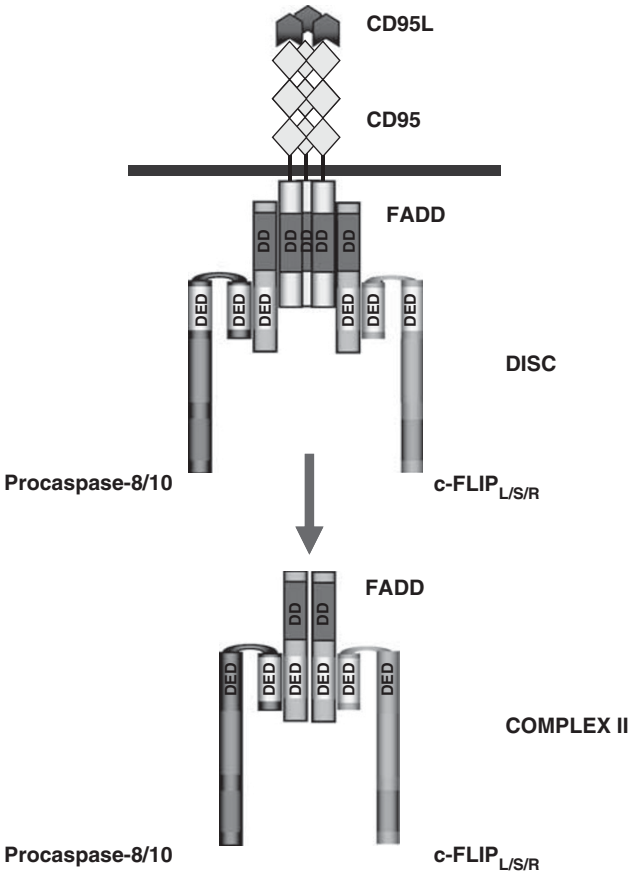

Figure 1 The CD95 DISC and complex II. The DISC consists of CD95, (depicted in yellow), FADD, (depicted in light blue), procaspase-8/procaspase-10, (depicted in green) and c-FLIP (depicted in violet). Complex II comprises FADD, procaspase-8/10 and c-FLIP. DD are shown in red; DED are shown in light yellow. The interactions between the molecules at the DISC and complex II are based on homotypic contacts. The DD of CD95 interacts with the DD of FADD while the DED of FADD interacts with the N-terminal tandem DEDs of procaspase-8, procaspase-10 and c-FLIP. The color reproduction of this figure is available at the *Cell Death and Differentiation* journal online

5–7 CD95 DDs and 5 FADD DD. The reported X-ray structures contradict each other with respect to the CD95/FADD complex; nevertheless, they provide a basis for a model of DD interactions at the DISC leading to procaspase-8 recruitment and activation. Certainly, the future challenge should involve obtaining the X-ray structure of the CD95 DISC assembled from the full-length CD95, FADD and procaspases, which would provide further insights into the structure of the complex.

In the CD95 apoptotic pathway two types of cells and signaling pathways have been established.[21] Type I cells are characterized by high levels of CD95 DISC formation and high amounts of active caspase-8. Activated caspase-8 directly leads to activation of downstream effectors caspases-3 and -7. In Type II cells, there are lower levels of CD95 DISC formation and, thus, lower levels of active caspase-8. In this case, signaling might require an amplification loop. This amplification loop involves the cleavage by caspase-8 of the Bcl-2-family protein Bid to generate truncated (t)Bid and subsequent bid-mediated release of cytochrome *C* (cyt *C*) from the mitochondria. The release of cyt *C* from the mitochondria results in apoptosome formation followed by the activation of procaspase-9, which in turn cleaves downstream effector caspases-3, -6 and -7. Importantly, it has been shown that Bid is essential for CD95-induced apoptosis in hepatocytes but dispensable in thymocytes.[22,23] This indicates that CD95-mediated signaling in thymocytes can be considered as Type I, while hepatocytes can be classified as Type II cells. In addition, an important role of XIAP in the amplification of Type I signal has been reported recently.[24] Jost *et al*.[24] have shown that the levels of XIAP expression can define Type I *versus* Type II signaling in a particular cell. They have demonstrated that BID/XIAP double-deficient mice are sensitive to CD95-induced apoptosis, whereas BID-deficient mice typically survive injection with CD95L or anti-CD95 antibodies.

Recently, we and others have shown that there is a second CD95 signaling complex formed upon CD95 stimulation, which was termed as complex II. Complex II comprises DED-proteins FADD, procaspase-8 and c-FLIP, and does not contain CD95 (Figure 1).[25,26] Complex II is formed within minutes after receptor stimulation. Apparently, complex II contributes to the activation of caspase-8. However, the amount of procaspase-8, which is activated in complex II *versus* in the DISC, and the mechanism of CD95 complex II formation remain unclear.[25] Notably, the formation of a second complex was first discovered in TNF signaling for TNFR1 in Jürg Tschopp laboratory in 2003.[27] Complex II in TNFR1 signaling does also not contain the receptor itself but comprises all the other components of the TNFR1 complex, for example TRADD, receptor-interacting protein 1 (RIP1), TRAF2 as well as the apoptosis-inducing components FADD, caspase-8 and caspase-10. In TNFR1 signaling, the generation of complex II has an essential role in caspase-8 activation and apoptosis induction. The formation of complex II was reported to be a characteristic feature of a number of TNFR family members including TRAIL-R1/R2 and CD95.[25,28] Therefore, the formation of complex II can be considered as a universal paradigm of TNFR signaling.

CD95 pro-and anti-apoptotic signaling is regulated at multiple levels: at the DISC, at complex II and at the mitochondria. Procaspase-8 and c-FLIP are two DED proteins, which are essential for the initiation of the apoptotic and non-apoptotic signals at the CD95 DISC. Below we describe in detail the DED-proteins procaspase-8 and c-FLIP, and the mechanism of their pro- and anti-apoptotic action.

Procaspase-8 and its Activation at the DISC

Procaspase-8 belongs to the family of caspases. Procaspase-8 is activated at the DISC.[29] Two isoforms of procaspase-8 (procaspase-8a and procaspase-8b) were reported to be bound to the DISC.[30] Procaspase-8a/b possess two tandem DED domains as well as the catalytic subunits p18 and p10 (Figure 2). Procaspase-8a contains an additional 2 kDa (15 aa) fragment, which results from translation of exon 9. This small fragment is located between the second DED and the large catalytic subunit resulting in the different length of procaspase-8a (p55) and procaspase-8b (p53).

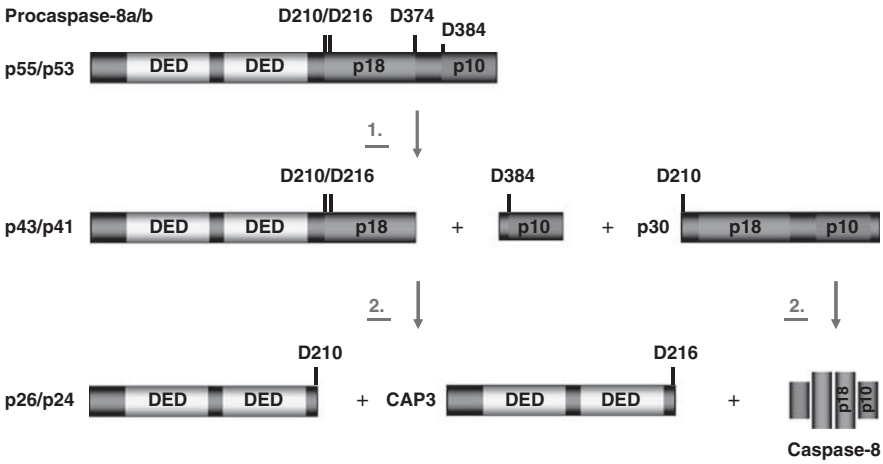

Figure 2 Scheme of procaspase-8 and the two-step mechanism of procaspase-8 activation. Procaspase-8a/b (p55/p53) is shown in green; DEDs are presented in light yellow. The N-terminal cleavage products: p43/p41, prodomains p26/p24, CAP3; C-terminal cleavage product p30 as well as caspase-active domains p18 and p10 are indicated. Two cleavage steps with the resulting products are presented. The color reproduction of this figure is available at the *Cell Death and Differentiation* journal online

Activation of procaspase-8a/b is believed to occur via oligomerization at the DISC.[31–33] Procaspase-8a/b was shown to form homodimers at the CD95 DISC, which is a prerequisite for its activation. Furthermore, according to the interdimer model of procaspase-8 activation, each procaspase-8 homodimer activates another procaspase-8 homodimer.[33] Hence, the DISC structure provides a platform for the oligomerization of procaspase-8 that allows two procaspase-8 homodimers to be in the close proximity leading to the initial activation of procaspase-8.

The initial activation of procaspase-8 at the DISC upon dimerization is followed by the subsequent cleavage of procaspase-8a/b. This processing occurs at Asp residues located between the prodomain and the large and the small catalytic subunits (Figure 2).[30,34] We have shown recently that cleavage between the prodomain and the large and the small catalytic subunits occurs simultaneously, though there is a preference towards a cleavage between the large and small catalytic subunits.[35] This processing results in the generation of a number of the cleavage products, including p43/p41, p26/p24, CAP3, p30, p18 and p10 (Figure 2).[34–37] At the first cleavage step, the N-terminal p43/p41 and the C-terminal p30 cleavage products are generated. Importantly, these cleavage products already possess catalytic activity. At the second cleavage step, p43/p41 and p30 are processed to p10 and p18, respectively, which leads to the generation of the active caspase-8 heterotetramer $(p18/p10)_2$.[38] Hence, activation of procaspase-8 at the DISC is a complex process that involves generation of several catalytically active cleavage products leading to the initiation of apoptosis.

Furthermore, in the course of procaspase-8 activation at the DISC procaspase-8 undergoes a substrate specificity switch as has been recently shown by Mac Farlane *et al.*[37] using *in vitro* reconstituted DISC. Upon initial dimerization procaspase-8 at the DISC has a very restricted substrate range, which is limited to itself and c-FLIP. Following cleavage, caspase-8 is fully activated and can cleave apoptotic substrates, for example caspase-3 and Bid. The differences in the substrate specificity of procaspase-8 *versus* caspase-8 have also been demonstrated by other authors using recombinant proteins.[33] Thus, there are two DISC-mediated caspase-8 activities: procaspase-8 activity and 'active caspase-8' activity. The procaspase-8 activity is required for the initiation of procaspase-8 processing whereas active caspase-8 induces apoptosis. Furthermore, procaspase-8 activity has been suggested to be indispensible for the initiation of non-apoptotic pathways.[37] In line with this, it has been recently reported using transgenic mice that the perturbation of the caspase-8 cleavage site abrogates its pro-apoptotic function without influencing its non-apoptotic function.[39]

Another important mechanism of the regulation of procaspase-8 activity, which has been discovered recently, is the protein modification of procaspase-8. Phosphorylation of procaspase-8 at the catalytic subunits has been shown to have an important role in the activity of procaspase-8, likely by interfering with processing of procaspase-8.[40] Phosphorylation at Y380 of procaspase-8 has been shown to inhibit CD95-mediated apoptosis.[41] In addition, polyubiquitination of procaspase-8 at the DISC has been reported recently to stabilize the active caspase-8 heterotetramer and, in this way, to have a pro-apoptotic role.[42] The mechanistic role of protein modifications of procaspase-8 has to be investigated in more detail in future studies.

In addition to its role in apoptosis there is emerging evidence of the role of procaspase-8 in non-apoptotic signaling pathways and cell survival. Caspase-8 knockout mice are characterized by prenatal lethality and by deficiencies in heart muscle development.[43] Interestingly, there is a recent report showing that RIP3 knockout can compensate for this effect, providing the evidence for a common signaling pathway mediated by procaspase-8 and RIP3.[44] In addition, the activity of procaspase-8 has been shown to be essential for the activation of the NF-κB signaling pathway during lymphocyte activation.[45]

Cellular FLICE-inhibitory Proteins

c-FLIP, also known as FLAME-1/I-FLICE/CASPER/CASH/MRIT/CLARP/Usurpin, is a well-described inhibitor of DR-mediated apoptosis.[46] c-FLIP was first described in 1997,

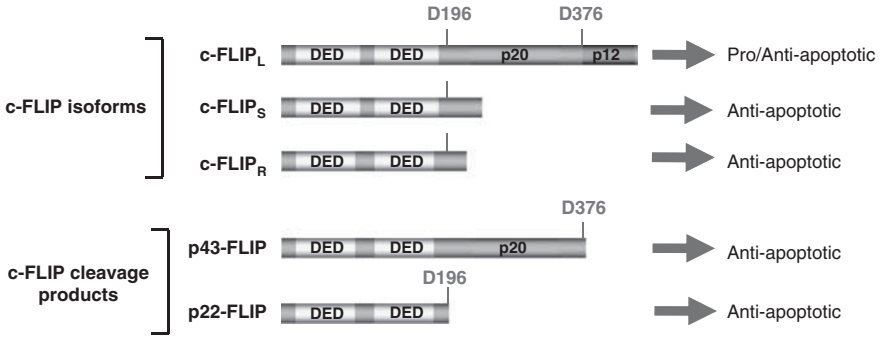

Figure 3 The scheme of c-FLIP proteins. c-FLIP isoforms and c-FLIP cleavage products are shown. DEDs and caspase-like domains (p20 and p12) are indicated. D376 and D196 leading to the generation of p43-FLIP and p22-FLIP, respectively, are presented in red. The color reproduction of this figure is available at the *Cell Death and Differentiation* journal online

and has been shown to be a major inhibitor of procaspase-8 activation at the DISC.[47] This finding has been followed by many studies discovering the complexity of DR signaling regulation by c-FLIP proteins and also elucidating the role of c-FLIP proteins in NF-κB activation.[17,48–52] Jürg Tschopp *et al*. have significantly contributed to the discovery of c-FLIP proteins as well as their role in NF-κB activation. The current view on c-FLIP proteins is shown in Figure 3. Five c-FLIP proteins have been characterized so far: three c-FLIP isoforms and two cleavage products.[17,46,53–56]

The three c-FLIP isoforms comprise: Long (L), Short (S) and Raji (R), for example c-FLIP$_L$, c-FLIP$_S$ and c-FLIP$_R$, respectively (Figure 3). All three isoforms possess two DED domains and thereby bind to the DISC. The short FLIP isoforms, c-FLIP$_S$ and c-FLIP$_R$, block procaspase-8 activation and apoptosis.[52,55] c-FLIP$_L$ can act as an anti-apoptotic molecule, functioning in a way similar to c-FLIP$_S$, when it is present at high concentrations at the DISC.[52,57] c-FLIP$_L$ can also act as a pro-apoptotic molecule.[52] This occurs upon strong receptor stimulation or in the presence of high amounts of one of the short c-FLIP isoforms, c-FLIP$_S$ or c-FLIP$_R$. Under these conditions c-FLIP$_L$ promotes the activation of procaspase-8 at the DISC.[52]

Two cleavage products of c-FLIP have been reported namely p43-FLIP and p22-FLIP.[17,56] p43-FLIP is generated from c-FLIP$_L$ at the CD95 DISC as a result of procaspase-8 cleavage at D376. p22-FLIP is the N-terminal cleavage product of c-FLIP resulting from procaspase-8 cleavage at D196. In contrast to p43-FLIP, p22-FLIP is formed in the cytosol independently of DR stimulation. In addition, both cleavage products of c-FLIP turned out to be inducers of NF-κB activity by binding to the IKK complex.[8,56]

Dynamics of Procaspase-8 and c-FLIP Interactions

Procaspase-8 and c-FLIP regulate CD95 signaling directly at the DISC (Figure 4). As pointed out above procaspase-8 is activated at the CD95 DISC inducing the apoptotic process. This activation can be inhibited by all reported c-FLIP proteins. The only exception is the c-FLIP$_L$ isoform that might also induce procaspase-8 activation, and might block procaspase-8 activation when expressed at high concentrations. Therefore, procaspase-8 at the DISC has a pro-apoptotic role and

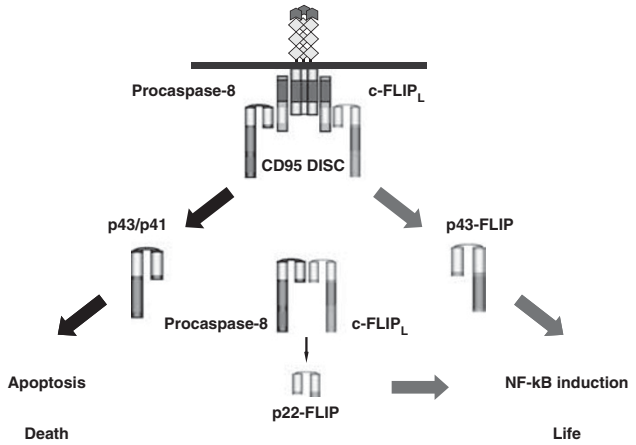

Figure 4 DED proteins: procaspase-8 and c-FLIP at the DISC and in the cytosol. Upon CD95 stimulation the CD95 DISC is formed. Cleavage products of procaspase-8 and c-FLIP$_L$, p43/p41 and p43-FLIP, respectively, are generated at the DISC. p43/p41 leads to the induction of apoptosis, while p43-FLIP binds to the IKK complex, which results in the induction of NF-κB. Procaspase-8 and c-FLIP form dimers producing p22-FLIP. p22-FLIP binds to the IKK complex via IKKγ, which leads to the induction of NF-κB. The color reproduction of this figure is available at the *Cell Death and Differentiation* journal online

c-FLIP proteins, with the exception of the c-FLIP$_L$ isoform, possess an anti-apoptotic function.

In addition to the regulation of apoptosis, it was shown that the main DED-containing proteins of the CD95 DISC, procaspase-8 and c-FLIP, have a prominent role in NF-κB activation.[14] c-FLIP N-terminal cleavage products p43-FLIP and p22-FLIP strongly induce NF-κB.[56,58] Procaspase-8 activity is required for generation of these cleavage products.

p43-FLIP is generated by procaspase-8 at the DISC upon CD95 stimulation. p43-FLIP interacts with components of the TNFR-mediated NF-κB activation pathway, TNFR-associated factor 1 (TRAF1), TRAF2 and RIP, which together promote NF-κB activation.[58] In addition, we have shown that p43-FLIP generated at the DISC might directly activate the IKK complex leading to NF-κB induction (Figure 4).[8]

p22-FLIP also induces NF-κB by direct binding to the IKK complex.[56] p22-FLIP is generated by procaspase-8 activity in non-apoptotic cells without DR stimulation (Figure 4).[56] Under these conditions procaspase-8 does not undergo processing leading to apoptosis induction but rather utilizes

its procaspase activity, which leads to the processing of c-FLIP to the p22-FLIP cleavage product. Procaspase-8 can induce NF-κB not only via generation of p22-FLIP or p43-FLIP, but also by direct binding to the CBM adapter complex formed upon TCR stimulation.[45] Thus, several studies indicate that the most prominent DED-proteins of the CD95 DISC, procaspase-8 and c-FLIP, have an important role both in CD95-induced apoptotic and non-apoptotic pathways.

This complex interplay between procaspase-8 and c-FLIP leading to the induction of non-apoptotic *versus* apoptotic pathways has encouraged us to apply a systems biology approach to understand life/death decisions at CD95.[9,59–61] By using mathematical modeling and experimental data, we have shown that the induction of the apoptotic pathway can be directly connected to the amount of the generated cleavage product p43/p41 of procaspase-8, whereas the induction of the non-apoptotic pathway depends upon the amount of the generated cleavage product p43-FLIP of c-FLIP$_L$.[8] The generation of both cleavage products occurs at the CD95 DISC. Furthermore, procaspase-8 and c-FLIP$_L$ compete for the same binding sites at the CD95 DISC. Therefore, the generation of p43-FLIP is non-linear. Upon low concentrations of c-FLIP$_L$ at the DISC, there will be almost no p43-FLIP generated. Upon increase of c-FLIP$_L$ amounts, the amount of p43-FLIP produced will be increased. Upon high concentrations of c-FLIP$_L$ all binding sites at the DISC will be occupied by c-FLIP$_L$ proteins, and this would also block procaspase-8 recruitment and subsequent activation. Consequently, under high c-FLIP overexpression, c-FLIP$_L$ could not be processed to p43-FLIP at the DISC as procaspase-8 activity at the DISC will be missing. Therefore, a high concentration of c-FLIP$_L$ would block both apoptotic and non-apoptotic pathways at the DISC. Thus, c-FLIP$_L$, depending whether its concentration at the DISC is medium or high, can promote or inhibit CD95-mediated NF-κB activation.[8] Finally, this analysis show that the regulation of the apoptotic *versus* non-apoptotic signaling pathways has non-linear dynamics depending on the amount of the generated cleavage products of procaspase-8 or c-FLIP$_L$.

These studies demonstrate that the ratio between DED-containing proteins procaspase-8 and c-FLIP$_L$ at the DISC has a major role in the regulation of apoptotic *versus* non-apoptotic signaling pathways in CD95 signaling. The initiation of CD95-mediated apoptotic and non-apoptotic signals occurs at the DISC and, therefore, the DISC can be considered as the central regulation node of CD95 signaling. Future work should involve a detailed analysis of the stoichiometry of DED proteins at the DISC. The quantitative analysis of the DISC stoichiometry should certainly provide a novel view on the regulation of life and death decisions at the DISC and in DR signaling pathways.

Conflict of Interest

The authors declare no conflict of interest.

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