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Abstract

Apoptosis is a fundamental process that contributes to tissue homeostasis, immune responses, and development. The receptor CD95, also called Fas, is a member of the tumor necrosis factor receptor (TNF-R) superfamily. Its cognate ligand, CD95L, is implicated in immune homeostasis and immune surveillance, and various lineages of malignant cells exhibit loss-of-function mutations in this pathway; therefore, CD95 was initially classified as a tumor suppressor gene. However, more recent data indicate that in different pathophysiological contexts, this receptor can transmit non-apoptotic signals, promote inflammation, and contribute to carcinogenesis. A comparison with the initial molecular events of the TNF-R signaling pathway leading to non-apoptotic, apoptotic, and necrotic pathways reveals that CD95 is probably using different molecular mechanisms to transmit its non-apoptotic signals (NF-κB, MAPK, and PI3K). As discussed in this review, the molecular process by which the receptor switches from an apoptotic function to an inflammatory role is unknown. More importantly, the biological functions of these signals remain elusive.

Keywords: Fas, apoptosis, cytokine, inflammation, carcinogenesis.

1. Introduction

The two main apoptotic signaling pathways are distinguished by the origins of the initiating signals. The intrinsic pathway is triggered by accumulation of DNA damage, deregulation of mitochondrial function, or viral infection, and induces the release of pro-apoptotic factors from the mitochondria. By contrast, the extrinsic pathway is activated by the binding of apoptotic ligands to death receptors on the cell surface. The pathways are interconnected, and both converge on activation of a family of cysteine proteases specific for aspartic acid residues, the caspases [1]. The apoptotic role of the mitochondrion is associated with a reduction in transmembrane potential and the loss of extracellular membrane integrity, leading to the release of various apoptogenic factors into the cytosol. One of these factors, cytochrome *c*, associates with the caspase-9/APAF1 complex to form the apoptosome and trigger apoptosis [2].

The intrinsic and extrinsic pathways share common features, and both require the aggregation of initiator caspases as an early event. During interactions with their respective ligands, members of the death-receptor superfamily recruit adaptor proteins such as Fas-associating protein with a death domain (FADD) [3, 4] or Tumor Necrosis Factor (TNF) Receptor 1-Associated Death Domain Protein (TRADD) [5], resulting in the aggregation and activation of initiator caspases (caspase-8 and -10) to form the death-inducing signaling complex (DISC) [6]. In a similar manner, release of cytochrome *c* and ATP from mitochondria promotes the formation of the apoptosome (along with cytosolic APAF-1), resulting in aggregation and activation of initiator caspase-9, which in turn cleaves caspase-3 [7].

It should be kept in mind that death receptors CD95 [8], TNFR1 [9], DR4 [10], DR5 [11], and DR6 [12] were cloned based on their ability to elicit apoptosis. Although the abilities of Fas/CD95, DR4, and DR5 to trigger non-apoptotic signaling pathways were observed immediately after the corresponding genes were cloned [13, 14], most if not all studies of these proteins have been focused on characterizing the molecular events leading to cell death. Accordingly, several agonistic molecules were developed in order to kill cancer cells, neglecting the impact of non-apoptotic signals in pathophysiological contexts. More recent data has altered this vision by highlighting the biological role of death receptor-mediated non-apoptotic signaling pathways in chronic inflammatory disorders and carcinogenesis.

2. TNF Receptor Family

Death receptors TNFR1, CD95, DR3, DR4, DR5, and DR6 belong to the tumor necrosis factor receptor (TNF-R) superfamily. These type I transmembrane proteins share common features: extracellular amino-terminal cysteine-rich domains (CRDs) [15, 16], which contribute to ligand specificity [17]; pre-association of the receptor at the plasma membrane [18-20]; and the death domain (DD), a conserved 80 amino-acid sequence located in the cytoplasmic tail, which is necessary for DISC formation and initiation of the apoptotic signal [21, 22].

2.1 TNFR1 signaling pathways

TNF-α exerts its effects by binding to two receptors, TNFR1 and TNFR2 [16]. Recently, progranulin was identified as a ligand of TNFR with a higher affinity than TNF-α. Progranulin antagonizes TNF-α signaling and plays a critical role in the pathogenesis of inflammatory arthritis in mice [23]. TNFR1, a 55 kDa protein with a DD in its intracellular

region, is expressed in almost all cell types, whereas, TNFR2, a 75 kDa protein, is mainly expressed in oligodendrocytes, astrocytes, T cells, myocytes, thymocytes, endothelial cells, and human mesenchymal stem cells [24]. Considerable uncertainty persists regarding the TNFR2 signaling pathway, which has been reviewed previously [24]. The CRD1 domains of CD95, TNFR1, and TNFR2 are involved in homotypic interactions, leading to pre-association of the receptor as a homotrimer in the absence of ligand [19, 20, 25]. Thus, this domain has been designated the pre-ligand binding assembly domain (PLAD) [25]. Receptors of the TNFR superfamily do not possess any enzymatic activity on their own, and therefore rely on the recruitment of adaptor proteins for signaling. Among these adaptor proteins, TRADD or FADD are instrumental in the implementation of cell death processes [3-6].

TNF-α is synthesized as a 26 kDa transmembrane type II protein (m-TNF-α) of 233 amino acids [26], which can be cleaved by the metalloprotease TACE [27, 28] to release the 17 kDa soluble form of the cytokine (cl-TNF-α). In contrast to cl-TNF-α, which only activates TNFR1, m-TNF-α can bind and activate both TNFR1 and TNFR2 [29].

Activation of TNFR1 induces cellular processes ranging from cell death (apoptosis or necroptosis) to cell proliferation, migration, and differentiation; the implementation of these cellular responses reflects the formation of different molecular complexes following receptor activation [24]. Binding of TNF to TNFR1 causes formation of two consecutive complexes, resulting in the divergence of their kinetic and spatial distributions. Whereas the plasma membrane complex (complex I) elicits a non-apoptotic signaling pathway, a second, internalized complex (complex II or DISC) triggers cell death [30]. In the presence of TNF-α, the adaptor protein TRADD interacts with TNFR1 and recruits other proteins involved in the signaling of the receptor, such as TRAF2, cIAP1, cIAP2, and RIP1, to form complex I. At the plasma membrane, this complex activates the NF-κB signaling pathway, which in turn promotes transcription of anti-apoptotic genes such as cIAP-1, cIAP-2, and c-FLIP [31]. The

linear ubiquitin chain assembly complex (LUBAC) is also recruited to complex I via cIAP-generated ubiquitin chains [32]. The LUBAC complex consists of HOIL-1, HOIP, and sharpin; HOIL-1 and HOIP add a linear ubiquitin chain by catalyzing the head-to-tail ligation of ubiquitin [33] to RIP1 and NEMO (IKKγ) in complex I [34], thereby activating NF-κB.

TNF-α–induced caspase activation is mediated by a second intracellular complex, known as complex II, which is formed when complex I dissociates from the receptor along with FADD and caspase-8 recruitment [30]. NF-κB activation leads to c-FLIP overexpression, preventing formation of complex II. Contrariwise, when NF-κB activation is blocked, the short-lived c-FLIP protein is depleted [35], and cells undergo programmed death [30]. In this context, RIP1 is deubiquitinated by enzymes such as Cezanne [36] and CYLD [37]. In addition, the complex composed of TRADD and RIP1 moves to the cytosol to form complex II. FADD is recruited to TRADD by the DD–DD interaction and binds caspase-8 [30]. Notably, when caspase-8 activity is inhibited or its expression is extinguished, DISC is unable to trigger the apoptotic signaling pathway, but TNFR1 or CD95 stimulation leads to the activation of another cell death signal, necroptosis [38, 39]. To prevent the induction of the necroptotic signal, caspase-8 cleaves and inactivates RIP1 and RIP3 [40]. The fine-tuned control of necroptosis by members of the apoptotic signaling pathway has been elegantly confirmed by experiments showing that the embryonic lethality of mice harboring single KO of caspase-8 or FADD can be rescued by an additional KO of the RIP3 gene [41-43].

2.2 TNF/TNFR: a gold mine for therapeutic tools

Many studies of TNF-α have demonstrated its pivotal role in fueling inflammation, a multistep process that promotes autoimmunity (*e.g.*, rheumatoid arthritis, ankylosing

spondylitis, Crohn's disease, psoriasis, and refractory asthma) and cancer. Many TNF inhibitors, such as neutralizing monoclonal antibodies (mAbs) (*e.g.*, infliximab, adalimumab, and golimumab) have been developed to treat these chronic inflammatory disorders, demonstrating that altering ligand/receptor interactions with neutralizing mAbs is an invaluable strategy for treating certain chronic inflammatory disorders. Other TNF-α antagonists, such as etanercept, a TNFR2-immunoglobulin Fc fusion protein, can improve the clinical course of rheumatoid arthritis [44].

A large and growing body of evidence has contributed to elucidation of the molecular mechanisms underlying induction of apoptotic and non-apoptotic signaling pathways by TNFR1, and also provided clues regarding how the receptor can switch from one signal to the other. However, the mechanistic links involved in implementation of non-apoptotic signaling pathways by CD95 remain elusive. However, several recent findings have revealed its pro-inflammatory effects [45-51].

3. CD95: a death receptor?

In 1989, identification of the mAb APO-1 by Peter Krammer et al. revealed the existence of a 52 kDa protein whose aggregation resulted in transmission of an apoptotic signal in cancer cells [52]. This receptor was cloned in 1991 by Nagata and colleagues, who named it Fas (CD95 or APO-1) [8]. Its ligand, CD95L, was cloned in 1993 by the same group, and was found to be primarily expressed at the surface of activated T lymphocytes [53] and natural killer (NK) cells [54]; however, its expression was also detected in tissues in which the presence of acute or chronic inflammation is highly undesirable, including the eyes [55] and testes [56].

3.1 Structure /function

The CD95 gene (*APT1)* consists of nine exons encoding a type I transmembrane protein harboring three CRDs, with exon 6 encoding the transmembrane domain [57] (Figure 1). Under denaturing conditions, CD95 migrates as a 40–50 kDa protein on SDS-PAGE. Similar to the TNF receptor [25], CD95 is pre-associated at the plasma membrane as a homotrimer, and this quaternary structure is mandatory for transmission of apoptotic signals in the presence of CD95L [19, 20]. Homotrimerization of CD95 occurs mainly through homotypic interactions involving the CD95-CRD1 domain [18-20]. Binding of CD95L or agonistic anti-CD95 mAbs to CD95 alters the receptor's conformation and the extent to which the receptor is multimerized at the plasma membrane [58]. The intracellular region of CD95 encompasses an 80 amino-acid stretch designated as the DD (Figure 1), which consists of six anti-parallel α-helices [59]. Upon addition of CD95L, CD95 undergoes conformational modification of the DD, inducing a shift of helix 6 and fusion with helix 5, promoting both oligomerization of the receptor and recruitment of the adaptor protein FADD [60]. One consequence of the opening of the globular structure of CD95 is that the receptor becomes connected through this bridge, which increases the extent of its homo-aggregation. This long helix allows stabilization of the complex by recruitment of FADD. The CD95-DD:FADD-DD crystal structure provides several insights into the formation of the large CD95 clusters observed by imaging or biochemical methods in cells stimulated with CD95L. In addition, the structure also confirms that alteration in the conformation of CD95 plays an instrumental role in signal induction [60]. However, the idea of an elongated C-terminal α-helix favoring the *cis*-dimerization of CD95-DD was challenged by Driscoll et al., who did not observe the fusion of the last two helices at a more neutral pH (pH 6.2), in contrast to the acidic condition (pH 4) used in the initial study in which Scott et al. resolved the CD95-DD:FADD-DD structure [60]. At pH 6.2, association of CD95 predominantly interacted with FADD in a 5:5 complex, which arose via a polymerization mechanism involving three types of asymmetric interactions, but without

major alteration of the DD globular structure [61, 62]. It is likely that the low-pH condition used by Scott et al. altered the conformation of CD95, resulting in the formation of non-physiological CD95:FADD oligomers [60]. Nonetheless, we cannot rule out the possibility that a local decrease in intracellular pH affects the initial steps of the CD95 signaling pathway *in vivo*, e.g., by promoting the opening of the CD95-DD and eventually contributing to formation of a complex that elicits a sequence of events distinct from that occurring at physiologic pH.

Once docked on CD95-DD, FADD self-associates [63], and binds procaspases-8 and -10, which are auto-processed and released in the cytosol as active caspases. Once activated, these caspases cleave many substrates, ultimately leading to the execution of the apoptotic program and cell death. The complex CD95/FADD/caspase-8/-10 is called DISC (Figure 2) [6]. Due to the importance of DISC formation to cell fate, it is not surprising that numerous cellular and viral proteins have evolved to hamper the formation of this structure: for example, both FLIP [64, 65] and PED/PEA-15 [66] interfere with the recruitment of caspase-8/-10 (Figure 2).

3.2 Type I /II signaling pathways

Following the discovery of CD95 and the elucidation of the initial steps in its signaling pathway, Peter and colleagues reported that cells can be divided in to two groups with regard to the kinetics with which they respond to CD95-mediated apoptotic signals, the magnitude of DISC formation, and the role played by the mitochondrion in this pathway [67]. DISC formation occurs rapidly and efficiently in type I cells, resulting in the release of a large amount of activated caspase-8 in the cytosol, whereas type II cells have difficulty forming this complex, and the amount of active caspase-8 is insufficient to directly activate the effector caspases-3 and -7 [67]. Nonetheless, type II cells experience cell death upon CD95

engagement and are even more sensitive to CD95-mediated apoptotic signals than type I cells [67-69]. This discrepancy can be partly explained by the fact that the low level of activated caspase-8 in type II cells is sufficient to cleave BID, a BH3-only protein, which constitutes the molecular link between caspase-8 activation and the apoptotic activity of mitochondria. Indeed, after cleavage by caspase-8, truncated BID (tBID) translocates to mitochondria, where it triggers the release of pro-apoptotic factors (Figure 2) [70, 71]. Although CD95 stimulation activates the mitochondrion-dependent apoptotic signal in type I and type II cells, it seems that only type II cells are addicted to this signal, because they contain higher levels of the caspase-3 inhibitor XIAP than type I cells [72]. Several members of the inhibitor of apoptosis (IAP) protein family, XIAP, c-IAP1, and c-IAP2 inhibit caspase-3, -7 [73, 74], and pro-caspase-9 [75] activity by direct binding, thereby preventing access to substrates. Furthermore, XIAP can function as an E3 ligase; this activity is involved in the ubiquitination of active caspase-3 and its subsequent degradation by the proteasome [76]. To detach XIAP from caspase-3 and restore the apoptotic signal, cells require the release of SMAC/DIABLO (second mitochondria-derived activator of caspase/direct IAP-binding protein with low PI) by the mitochondrion [77, 78], explaining why type II cells are more ‘addicted’ to this organelle than type I cells (Figure 2).

To summarize, DISC formation and IAP amount are two cellular markers that allow a clear discrimination between type I and type II cells. Even though IAP overexpression can account for the mitochondrial dependency observed in type II cells, it remains unclear why DISC formation is hampered in type II cells and/or augmented in their type I counterparts. Recently, high activity of the lipid kinase phosphoinositide 3-kinase (PI3K) or down-regulation of its neutralizing phosphatase, phosphatase and tensin homologue on chromosome 10 (PTEN), were observed in type II cells, whereas this signal is blocked in type I cell lines [79, 80]. The PI3K signaling pathway prevents the aggregation of CD95 [81], probably by

retaining the receptor outside of lipid rafts [79, 82]. PEA-15, also known as PED, is a protein containing a death effector domain (DED) that inhibits the CD95 and TNFR1 apoptotic signals (Figure 2) [66]. Activation of PI3K and its downstream effector, serine-threonine kinase Akt, leads to phosphorylation of PEA-15 at serine 116 [79, 82]; this post-translational modification promotes its interaction with FADD, ultimately inhibiting DISC formation [83, 84].

Notably, the existence of type I and type II cells is not only an *in vitro* observation, but has been identified physiologically in the human body. CD95-mediated apoptotic signals cannot be altered in thymocytes or activated T cells expressing a Bcl-2 transgene, consistent with the type I nature of these cells [85], whereas hepatocytes expressing the same transgene resist CD95-induced apoptosis and thus behave as type II cells [86, 87].

3.3 What can we learn from CD95 mutations?

3.3.1 Human.

Germinal mutations in *APT1* have been reported in patients with autoimmune lymphoproliferative syndrome type Ia (ALPS, also called Canale-Smith syndrome) [88-90]. ALPS patients exhibit chronic lymphadenopathy and splenomegaly and expanded populations of double-negative α/β T lymphocytes (CD3$^+$CD4$^-$CD8$^-$), and often develop autoimmunity [88, 89, 91, 92]. In agreement with the notion that CD95 behaves as a tumor suppressor, ALPS patients display an increased risk of Hodgkin and non-Hodgkin lymphoma [93]. The predominance of post-germinal center (GC) lymphomas in patients with either germ line or somatic CD95 mutations can be explained by the fact that, inside germinal centers of the secondary lymphoid follicles, the CD95 signal plays a pivotal role in the deletion of self-reactive maturing B lymphocytes [94]; in addition, *APT1* belongs to a set of rare genes (*i.e.*,

PIM1, c-myc, PAX5, RhoH/TTF, and Bcl-6) subject to somatic hypermutation [95, 96], which may affect its biological function. In addition to post-GC lymphomas, tumors of various histological origins have been shown to exhibit significant numbers of mutations in the CD95 gene (reviewed in [51]). Extensive analysis of CD95 mutations and their distribution in *APT1* reveals that, with some exceptions, most are gathered in exons 8 and 9, which encode the CD95 intracellular region (Figure 3) [97]. Remarkably, most of these mutations are heterozygous, mainly localized in CD95-DD, and lead to inhibition of the CD95-mediated apoptotic signal. Indeed, in agreement with the notion that CD95 is expressed at the plasma membrane as a pre-associated homotrimer [19, 20], formation of heterocomplexes containing wild-type and mutated CD95 prevents FADD recruitment and dominantly abrogates the initiation of the apoptotic signal.

Extensive analysis of the positions of CD95 mutations described in the literature has revealed mutation "hot spots" in the CD95 sequence (Figure 3). Among these hot spots, arginine 234, aspartic acid 244, and valine 251 account for a considerable proportion of the documented CD95 mutations. Indeed, among the 189 mutations annotated in the 335 amino acids of CD95, 30 (~16%) are localized in one of these three amino acids (Figure 3). The pivotal roles played by these amino acids in stabilization or formation of intra- and inter-bridges between CD95 and FADD may explain the existence of these hot spots. For instance, both R234 and D244 contribute to homotypic aggregation of the receptor and FADD recruitment [59]. Nevertheless, the observation of death-domain hot spots contradicts the study of Scott and colleagues, who demonstrated that the region of CD95-DD that interacts with the FADD-DD extends over a dispersed surface and is mediated by a large number of low-affinity interactions [60].

Most ALPS type Ia patients affected by malignancies do not undergo loss of heterozygosity (LOH), leading some authors to hypothesize that preservation of a wild-type allele may

contribute to carcinogenesis [98, 99]. In the same vein, expression of a unique mutated CD95 allele blocks the induction of apoptotic signals, but fails to block non-apoptotic signals such as NF-κB and MAPK [98, 99], whose induction promotes invasiveness in tumor cells [97, 100]. In addition, mutations in the intracellular CD95-DD result in more highly penetrant ALPS phenotype features in mutation-bearing relatives than mutations in the extracellular domain. These results suggest that unlike DD mutations, CD95 mutations localized outside the DD somehow block apoptotic signaling but fail to promote non-apoptotic pathways that may contribute to disease aggressiveness.

3.3.2 Mouse models.

Three mouse models exist in which either CD95L affinity for CD95 is reduced (due to the germline mutation F273L in CD95L, called *generalized lymphoproliferative disease* [*gld*], which decreases CD95L binding to CD95) [101, 102]), the level of CD95 expression is down-regulated (due to an insertion of a retrotransposon in intron 2 of the receptor gene, these mice are called *lymphoproliferation* [Lpr] [103-105]), or DISC formation is hampered (due to a spontaneous mutation inside the CD95 DD at position 238, specifically, replacement of the valine 238 with asparagine; these mice are called lpr^{cg} for *lpr gene complementing gld* [106]). These mice have provided valuable insights into the pivotal role played by CD95 and CD95L in immune surveillance and immune tolerance [107]. In an attempt to simplify, some authors associated the phenotypes observed in these lpr, lpr^{cg} or gld mice with the complete loss of CD95 or CD95L [108]. However, conclusions must be drawn with caution, due to subtle differences between the phenotypes of spontaneous mouse models and genetically engineered mice. Indeed, in Lpr mice, insertion of an early transposon in intron 2 of CD95 causes premature termination of the CD95 transcript [104], which is leaky; consequently, CD95

mRNA and protein can be detected in mice homozygous for the spontaneous mutation [109, 110]. Also, the DD mutation in Lpr^{cg} mice reduces FADD recruitment but does not abrogate it [111]. Furthermore, CD95 can still interact with CD95L harboring the *gld* mutation, albeit somewhat more weakly than wild-type CD95L [112]. Finally, lpr, lpr^{cg}, and *gld* mice over-express CD95L relative to their wild-type counterparts [113]. Using T lymphocytes from ALPS type Ia patients or Lpr mice, we confirmed that far less intact CD95 is required to activate NF-κB than to induce apoptosis; therefore, although a single wild-type allele cannot restore cell death induction in these cells, it is sufficient to transduce NF-κB and MAPK cues [98, 99]. Overall, these observations support the idea that the biological roles ascribed to the CD95/CD95L pair, based on the analysis of these patients and mouse models, may correspond to the additive effects of the receptor's inability to induce cell death and its tendency to implement non-apoptotic signals.

A recent study elegantly showed that elimination of the remaining allele in cancer cells leads to the induction of an unconventional cell death program called "death induced by CD95R/L elimination" (DICE) [114].

These findings highlight the fact that distinct activation thresholds exist in the process of CD95 engagement. Although complete loss of CD95 expression in cancer cells leads to cell death, one wild-type allele (low activation threshold) is sufficient to elicit non-apoptotic signaling pathways, and the second allele (high activation threshold) is required to implement the canonical apoptotic signal [98, 115]. However, this rule suffers from an exception: metalloprotease-cleaved CD95L implements non-apoptotic signals in cells expressing two wild-type alleles of CD95 [48, 50, 116, 117] (further discussed in 3.6.2). In summary, because the characterization of CD95/CD95L biological roles has been carried out mainly by considering the default of apoptosis in ALPS type Ia patients and mouse models, we believe it is important to carefully reconsider these conclusions by integrating the notion that exposure

of these cells to CD95L will also lead to a chronic activation of non-apoptotic signaling pathways [99]. To better appreciate the complexity of the pathophysiological roles of CD95 and its ligand, it is therefore more appropriate to use conditional and tissue-specific CD95 and CD95L KO mice.

3.4 Regulation of the Initial Steps of CD95-mediated Signaling

3.4.1 Lipid rafts

In addition to CD95 down-regulation or expression of a mutated allele of the receptor, alteration of the plasma membrane distribution of CD95 represents an additional mechanism by which tumor cells could develop resistance to CD95L-expressing immune cells. The plasma membrane is a heterogeneous lipid bilayer comprising compacted or liquid-ordered domains, called microdomains, lipid rafts, or detergent-resistant microdomains (DRMs). These domains, which are enriched in ceramides, have been described as floating in a more fluid or liquid-disordered two-dimensional (2-D) lipid bilayer [118]. A series of elegant experiments showed that although CD95 is mostly excluded from lipid rafts in activated T lymphocytes, TCR-dependent re-activation of these cells leads to rapid distribution of the death receptor into lipid rafts [119]. This CD95 compartmentalization contributes to a reduction in the apoptotic threshold, leading to clonotypic elimination of activated T lymphocytes through activation of the CD95-mediated apoptotic signal [119]. Similarly, the reorganization of CD95 into DRMs can occur independently of ligand upon addition of certain chemotherapeutic drugs (*e.g.*, rituximab [120], resveratrol [121, 122], edelfosine [79, 123, 124], aplidin [125], perifosine [124], and cisplatin [126]). The molecular cascades underlying this process remain elusive. Nevertheless, a growing body of evidence leads us to postulate that alteration of intracellular signaling pathway(s), such as the aforementioned

PI3K signal [79, 82], may change biophysical properties of the plasma membrane, such as membrane fluidity, which in turn may facilitate CD95 clustering into large lipid raft-enriched platforms, favoring DISC formation and induction of the apoptotic program [82].

3.4.2 Post-translational modifications

Accumulation of CD95 mutations is not the only mechanism by which malignant cells inhibit the extrinsic signaling pathway. Post-translational modifications in the intracellular tail of CD95, such as reversible oxidation or covalent attachment of palmitic acid, alter the plasma membrane distribution of CD95 and thereby its downstream signaling. For instance, S-glutathionylation of mouse CD95 at cysteine 294 promotes clustering of CD95 and its distribution into lipid rafts [127]. This amino acid is conserved in the human CD95 sequence and corresponds to cysteine 304 (or C288 when the 16 amino-acid signal peptide is taken into consideration [8, 128]). Interestingly, Janssen-Heininger and colleagues emphasize that death receptor gluthationylation occurs downstream of activation of caspase-8 and -3; the catalytic activities of these caspases damage the thiol transferase glutaredoxin 1 (Grx1) [127]. One consequence of Grx1 inactivation is accumulation of glutathionylated CD95, which clusters into lipid rafts, thereby sensitizing cells to CD95-mediated apoptotic signals. Based on these findings, caspase-8 activation occurs prior to aggregation of CD95 and redistribution into lipid rafts, both of which are required to form the DISC and subsequently activate larger amounts of caspase-8. In agreement with these observations, activation of caspase-8 occurs in a two-step process. First, a small amount of activated caspase-8 (<1%) is generated immediately when CD95L interacts with CD95, resulting in acid sphingomyelinase (ASM) activation, ceramide production, and CD95 clustering; these in turn promote DISC formation

and the burst of caspase-8 processing that is essential for implementation of the apoptotic program [129].

S-glutathionylation consists of a bond between a reactive Cys-thiol and reduced glutathione (GSH), a tripeptide consisting of glycine, cysteine, and glutamate. Attachment of this group to a protein alters its structure and function in a manner similar to the addition of a phosphate [130]. S-glutathionylation is not the only post-translational modification of a cysteine in CD95: S-nitrosylation of cysteine 199 (corresponding to C183 after subtraction of signal peptide sequence) and 304 (C288) in colon and breast tumor cells also promotes the redistribution of CD95 into DRMs, formation of the DISC, and the transmission of the apoptotic signal [131].

Two reports have demonstrated that covalent coupling of a 16-carbon fatty acid (palmitic acid) to cysteine 199 (C183) elicits the redistribution of CD95 into DRMs, the formation of SDS-stable CD95 microaggregates resistant to denaturing and reducing treatments, and internalization of the receptor [132, 133]. Although the order of these events remains to be precisely determined, it is clear that these molecular steps play a critical role in the implementation of apoptotic signals.

As with S-nitrosylation, both the aforementioned S-glutathionylation at C304 (C288) and palmitoylation at C199 (C183) promote the partition of CD95 into lipid rafts and augment the subsequent apoptotic signal. Further investigation is required to determine whether these post-translational modifications are redundant, and occur simultaneously in dying cells, or instead are elicited in a cell-specific and/or in a microenvironment-specific manner. Understanding the molecular mechanisms controlling these post-translational modifications would be of great value in efforts to identify the mechanisms by which tumor cells block them, leading to resistance to the extrinsic signaling pathway.

Soon after CD95 was cloned, several groups investigated phosphorylation of this protein on serine/threonine and tyrosine and explored its biological role. Although serine/threonine phosphorylation may participate in the implementation of the CD95 signal, these authors mainly focused on the role of tyrosine phosphorylation in the cell death pathway. Phosphorylation can occur on two tyrosines located in the first (Y232, corresponding to Y216 starting from the first amino acid after the signal peptide) and fifth (Y291/Y275) α-helices of CD95-DD [134]. Y275 is located within a conserved YXXL motif reminiscent of the conserved 'I/VxYxxL' motif, termed the immunoreceptor tyrosine-based inhibitory motif (ITIM), which is responsible for the recruitment and activation of inhibitory phosphatases [135]. By recruiting the src homology domain 2 (SH2)-containing tyrosine phosphatase-1 (SHP-1), Y275 phosphorylation promotes CD95-mediated cell death in T cells [136] and counteracts the GM-CSF–driven pro-survival signals in neutrophils [135]. Notably, this Y^{275}DTL cytoplasmic domain is also a putative consensus YXXF sequence for AP-2 binding [137], which is instrumental in CD95 internalization (see below and [138]). Consistent with this, replacement of Y275 by a phenylalanine inhibits CD95 internalization and thereby blocks the induction of apoptosis, but does not affect non-apoptotic responses [138]. In addition, tyrosine phosphorylation of CD95 promotes the recruitment of the src kinases Fyn and Lyn through their SH2 domains, thereby promoting cell death [139, 140]. Accordingly, it is tempting to speculate that Y275 phosphorylation may guide the receptor through the induction of the apoptotic signal at the expense of non-apoptotic pathways. These data raise some questions about the identity of the tyrosine kinase involved in Y275 phosphorylation, the order of the molecular events leading to phosphatase and src kinase recruitment, and their respective roles in the CD95 signaling pathway.

3.4.3 CD95 internalization

A powerful magnetic method for isolating receptor-containing endocytic vesicles was used to show that CD95 promptly associates with endosomal and lysosomal markers upon incubation of cells with agonistic anti-CD95 mAb [138]. In addition, expression of a CD95 mutant in which the DD-located tyrosine 291 (Y275) is changed to phenylalanine does not seem to alter the capacity to bind FADD, but instead compromises CD95L-mediated CD95 internalization occurring through an AP-2/clathrin-driven endocytic pathway [138]. More strikingly, expression of the internalization-defective CD95 mutant Y291F abrogates the transmission of apoptotic signals, but fails to block the non-apoptotic signaling pathways (*i.e.*, NF-κB and ERK); indeed, the mutant even promotes these pathways (Figure 3). These findings highlight the presence of a region in the DD, which interacts with AP2 and promotes a clathrin-dependent endocytic pathway in a FADD-independent manner. The role of palmitoylation in the AP2/clathrin-driven internalization of CD95 remains to be elucidated.

3.4.4 Ca^{2+} response

A recent study demonstrated that CD95 engagement evokes rapid and transient Ca^{2+} signaling, which stimulates the recruitment of protein kinase C-β2 (PKC-β2) from the cytosol to the DISC[141]. This kinase transiently halts DISC formation, providing a checkpoint before the irreversible commitment to cell death [142]. These findings raised two important questions: what are the Ca^{2+}-dependent molecular mechanisms transiently inhibiting DISC formation, and do tumor cells use this signal to escape the immune response and/or resist chemotherapy?

3.5 Programmed necrosis, also known as necroptosis

In 1998, inhibition of caspase activity was shown to sensitize the fibroblastic L929 cell line to TNF-mediated necrotic cell death [39]. With respect to the CD95 signal, Tschopp et al. showed that FADD and RIP1 participate in the implementation of a non-apoptotic signaling pathway that leads to a necrotic morphology associated with loss of plasma membrane integrity but not with chromatin condensation [38]. Of note, BID cleavage was not observed in the context of this necrotic signal. Whereas FADD plays a crucial role in both apoptotic and necrotic pathways, RIP1 recruitment to CD95 occurs independently of this adaptor protein. Indeed, yeast two-hybrid experiments showed that RIP1 can bind directly to the CD95 DD, whereas this interaction is lost when a bait corresponding to mutated CD95-DD (replacement of Val 238 to Asn) is used [143]. In addition, RIP3 (RIPK3, a member of the RIP kinase family) is an indispensable factor for the induction of the necrotic signaling pathway [70-72]. Identification of necrostatin, a chemical inhibitor of necroptosis [144] that specifically inhibits RIP1 kinase activity [145], has accelerated the pace of discovery in this field of cell death. The apoptosis and necroptosis pathways interact: for instance, caspase-8 exerts a potent inhibitory effect on CD95 and TNFR1-mediated necroptosis [146] through its ability to process and inactivate RIP1 and RIP3 [147, 148]. At least in the case of TNF signaling, the necrotic signal relies on the activity of CYLD, a deubiquitinating enzyme that is also cleaved and inactivated by caspase-8 [149].

Overall, these findings suggest that the apoptotic machinery controls the necrotic pathway. This concept was recently confirmed by the results of *in vivo* double-KO experiments [41-43, 150]. Indeed, FADD and caspase-8 can be considered to be a pro-survival factor, mainly because both of these two “apoptotic” molecules inhibit the RIP1/RIP3-dependent necrotic signal; consequently, their loss unleashes the necroptotic program and leads to embryonic lethality. However, most studies on necroptosis have focused on the TNF signaling pathway, whereas the mechanism by which CD95 elicits this cell death

pathway, as well as how this receptor switches between non-apoptotic, apoptotic, and necroptotic signals, remains to be elucidated. Importantly, the impact of each type of cell death on antigen presentation, and on the efficiency of immune response after elimination of infected or transformed cells, also remains unclear.

3.6 CD95L, an Inflammatory/Oncogenic Cytokine?

3.6.1 A ligand that creates immune privilege

The transmembrane CD95L (CD178/FasL) is present on the surface of activated lymphocytes [102] and NK cells [151], where it orchestrates the elimination of transformed and infected cells. In addition, CD95L is expressed on the surface of neurons [152], corneal epithelia and endothelia [55, 153], and Sertoli cells [56], where it acts to prevent the infiltration of immune cells and thereby inhibit the spread of inflammation in these sensitive organs (*i.e.*, brain, eyes, and testis, respectively), commonly referred to as "immune-privileged" sites. The initial description of physiological immune privilege was followed by an understanding of tumor-mediated immune privilege: two groups independently reported that the ectopic expression of CD95L by malignant cells participated in the elimination of infiltrating T lymphocytes, and could thus play a role in the establishment of a tumor site to which immune cells are denied access [154, 155]. However, these observations are controversial, because ectopic expression of CD95L in allogenic transplant of β-islets [156, 157] and in tumor cell lines [158] led to a more rapid elimination of these cells relative to control cells, at least in part due to increased infiltration of neutrophils and macrophages endowed with antitumor activity.

3.6.2 (At least) two different ligands and two different signals

Among the weapons at the disposal of immune cells is transmembrane CD95L, which contributes to the elimination of pre-tumor cells. Therefore, pre-tumor cells that escape immune surveillance will tend to have developed resistance to CD95, a process termed immunoediting [159]. In other words, the influence of the immune system on pre-tumor cells will select for malignant cells with elevated resistance to the CD95L-induced signal. As previously mentioned, these alterations to the CD95 signal not only block the CD95-mediated apoptosis, but also promote the transmission of non-apoptotic signals by CD95L, which may also play a critical role in carcinogenesis [98-100, 160]. In support of this hypothesis, complete loss of CD95 expression is rarely observed in malignant cells [161].

Accumulating evidence indicates that the apoptotic ligand CD95L behaves as a chemoattractant for neutrophils, macrophages [47, 157, 158], T lymphocytes [50], and malignant cells in which the CD95-mediated apoptotic signal is non-productive [100, 116]. Nonetheless, the biological role of CD95L must be further clarified, given that in pathophysiological settings, the ligand is present in at least two forms with different stoichiometries. CD95L is a transmembrane cytokine whose ectodomain can be cleaved by metalloproteases such as MMP3[162], MMP7[163], MMP9[164], and ADAM-10 (A disintegrin and metalloproteinase 10) [165, 166], and then released into the bloodstream as a soluble ligand. Based on data showing that hexameric CD95L represents the minimal level of self-association required to signal apoptosis [167], and that cleavage by metalloproteases releases an homotrimeric ligand [167, 168], this soluble ligand has long been considered to be an inert molecule that competes with its membrane-bound counterpart for CD95 binding, thereby antagonizing the death signal [168, 169]. However, recent work has demonstrated that this metalloprotease-cleaved CD95L (cl-CD95L) actively aggravates inflammation and autoimmunity in patients affected by systemic lupus erythematosus (SLE) by inducing the non-apoptotic NF-κB and PI3K [48, 50] signaling pathways (Figure 4). In contrast to the case

of transmembrane CD95L, induction of the PI3K signaling pathway by its metalloprotease-cleaved counterpart occurs through the formation of a molecular complex devoid of FADD and caspase-8 that instead recruits the src kinase c-yes [50, 116]; this unconventional receptosome was designated the motility-inducing signaling complex (MISC) [50, 117](Figure 4). Even though we did not detect any trace of caspase-8 in MISC, this enzyme has been shown to participate in cell migration. The protease activity of caspase-8 can be abolished by phosphorylation at tyrosine 380 by src kinase [170]. This post-translational modification was observed in cells stimulated with EGF and in colon cancer cells with constitutive activation of src; from a molecular standpoint, the modification does not alter caspase homodimerization or recruitment in DISC [170]. Moreover, the epidermal growth factor receptor (EGFR)-driven phosphorylation of caspase-8 at Y380 turns out to potently induce the PI3K signaling pathway by recruiting the PI3K adaptor p85 alpha subunit [171]. Ultimately, caspase-8 phosphorylation triggers cell migration. Nonetheless, it is noteworthy that CD95-induced migration and invasion does not appear to require an intact DD (reviewed in [172]), suggesting either that the caspase-8–dependent mode of cell migration reflects the action of an alternative signal mediated by death receptors or that it only participates in non-death receptor-induced cell motility. It would be interesting to address this question in the future. At present, we can only surmise that phosphorylation of caspase-8 at Y380 following EGFR stimulation primes certain cancer cells to become unresponsive to the apoptotic signal triggered by cytotoxic CD95L, and meanwhile promotes cell migration, an essential event in cancer cell metastasis (Figure 4). We recently showed that CD95 implements the PI3K signaling pathway by recruiting EGFR. This CD95-dependent EGFR activation relies on the recruitment of the NADPH oxidase 3 (Nox3), the production of reactive oxygen species, which in turn activate the src kinase c-yes [117]. In triple-negative breast cancer cells exposed to metalloprotease-cleaved CD95L, c-yes activation is instrumental in forming an EGFR-

containing MISC, and this receptor tyrosine kinase (RTK) orchestrates the activation of PI3K in an EGF-independent manner (Figure 4). These data are in accordance with a recent study showing that another RTK, platelet-derived growth factor receptor-β, is recruited in colon cancer cells exposed to CD95L and thereby triggers cell migration [173]. Accordingly, we postulate that RTK recruitment is a common process in CD95 stimulation that can simultaneously inhibit the apoptotic signal by phosphorylating DISC-recruited caspase-8, and promote cell motility by activating the PI3K signaling pathway. This hypothesis will be investigated further in future work.

In a similar manner, a reduction in the plasma membrane level of CD95 or expression of a mutated CD95 allele, as observed in ALPS patients and malignant cells, inhibits the implementation of the apoptotic signal but does not affect the transmission of non-apoptotic signals, e.g., via NF-κB, MAPK, and PI3K [98, 99, 161], suggesting that these signals may stem from a domain other than CD95-DD, or are elicited at different thresholds. In summary, although the CD95/CD95L interaction can eliminate malignant cells by promoting formation of the DISC, or contribute to carcinogenesis by sustaining inflammation and/or inducing metastatic dissemination [47, 48, 50, 100, 116, 161, 174], the molecular mechanisms underlying the switch between these different signaling pathways remain enigmatic. An important question to be addressed is how the magnitude of CD95 aggregation regulates the formation of "Death"- vs. "Motility"-ISCs. Answering these questions will lead to the development of new therapeutic agents with the ability to contain the spread of inflammation or impede carcinogenesis, at least in pathologies associated with increased soluble CD95L, such as cancers (e.g., pancreatic cancer [175], large granular lymphocytic leukemia, breast cancer [176], and NK-cell lymphoma, [177]) or autoimmune disorders (e.g., rheumatoid arthritis and osteoarthritis [178], and graft-versus-host-disease (GVDH) [179, 180] or SLE[50, 181]). Together, these studies support the notion that the death function of CD95 corresponds

to its “day job” while the receptor may act as “a night killer” by fueling inflammation in certain pathophysiological contexts.

Strikingly, although the soluble form of CD95L generated by MMP7 (cleavage site inside the ^{113}ELR^{115} sequence, Figure 5) induces apoptosis [163], its counterpart processed between serine 126 and leucine 127 does not [48, 50, 168]. To explain this discrepancy, we speculate that the different quaternary structures of naturally processed CD95L molecules underlie the implementation of death-inducing vs. non-death-inducing signaling complexes. Consistent with this notion, soluble CD95L incubated in the bronchoalveolar lavage (BAL) fluid of patients suffering from acute respiratory distress syndrome (ARDS) undergoes oxidation at methionines 224 and 225 (Figure 5), promoting aggregation of the soluble ligand and boosting its cytotoxic activity [182]. The same authors observed that the stalk region of CD95L, corresponding to amino acids 103–136 and encompassing the metalloprotease cleavage sites (Figure 5), participates in the multimerization of CD95L, accounting for the damage to the lung epithelium that occurs in ARDS [182]. Of note, in ARDS BAL fluid, additional oxidation occurs at methionine 121 (Figure 5), which prevents the processing of CD95L by MMP7, potentially explaining why this cytotoxic ligand retains its stalk region [182]. Nonetheless, preservation of this region in soluble CD95L raises the possibility that an unidentified MMP7-independent cleavage site exists in the juxtamembrane region of CD95L, near the plasma membrane, or alternatively that the ligand detected in ARDS patients corresponds to full-length CD95L embedded in exosomes [183, 184]. Indeed, this peculiar exosome-bound CD95L is sometimes expressed by human prostate cancer cells (i.e., LNCaP), and it evokes apoptosis in activated T lymphocytes [185].

Overall, these findings emphasize the importance of finely characterizing the quaternary structure of naturally processed CD95L from the sera of patients affected by cancers or chronic/acute inflammatory disorders. Such investigations will improve our understanding of

the molecular mechanisms set in motion by this ligand, and thus our appreciation of its downstream biological functions.

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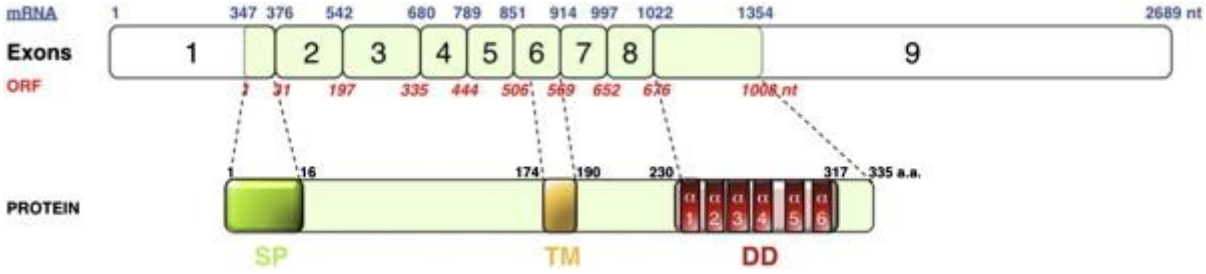
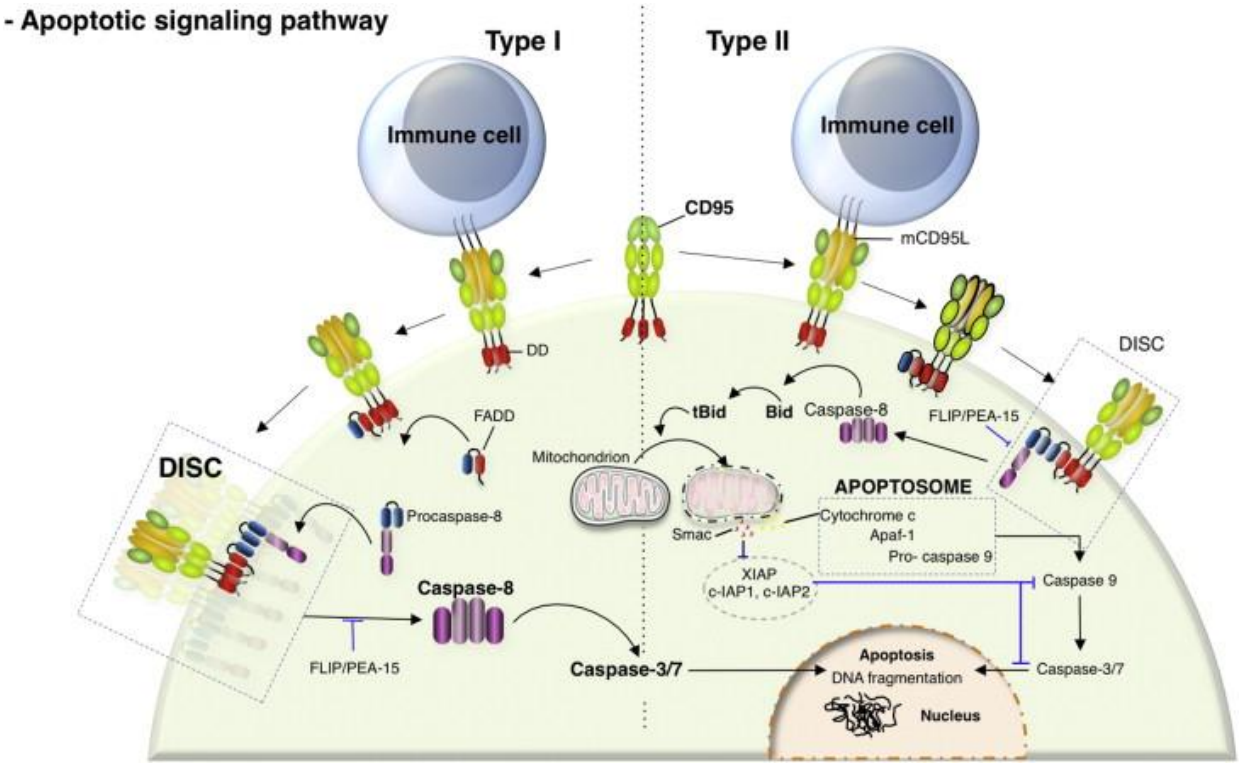
Figure Legends

Figure 1. CD95: mRNA to protein.



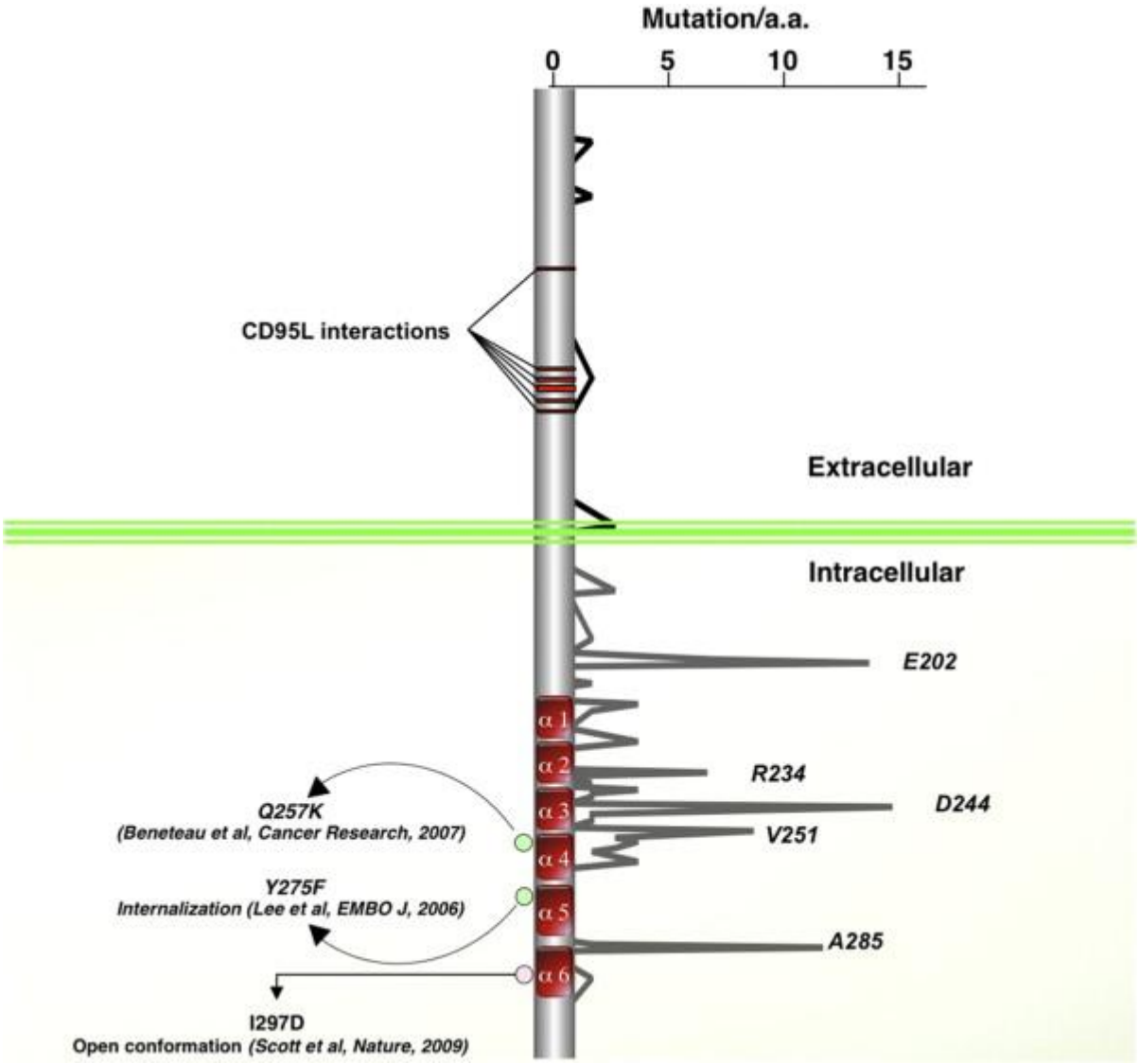
Upper panel: The CD95 mRNA consists of nine exons. The open reading frame (ORF) is indicated. **Lower panel:** Three main domains of the protein are depicted: signal peptide (SP), transmembrane domain (TM), and death domain (DD). DD is a protein module composed of a bundle of six alpha-helices

Figure 2. Type I / II cells.



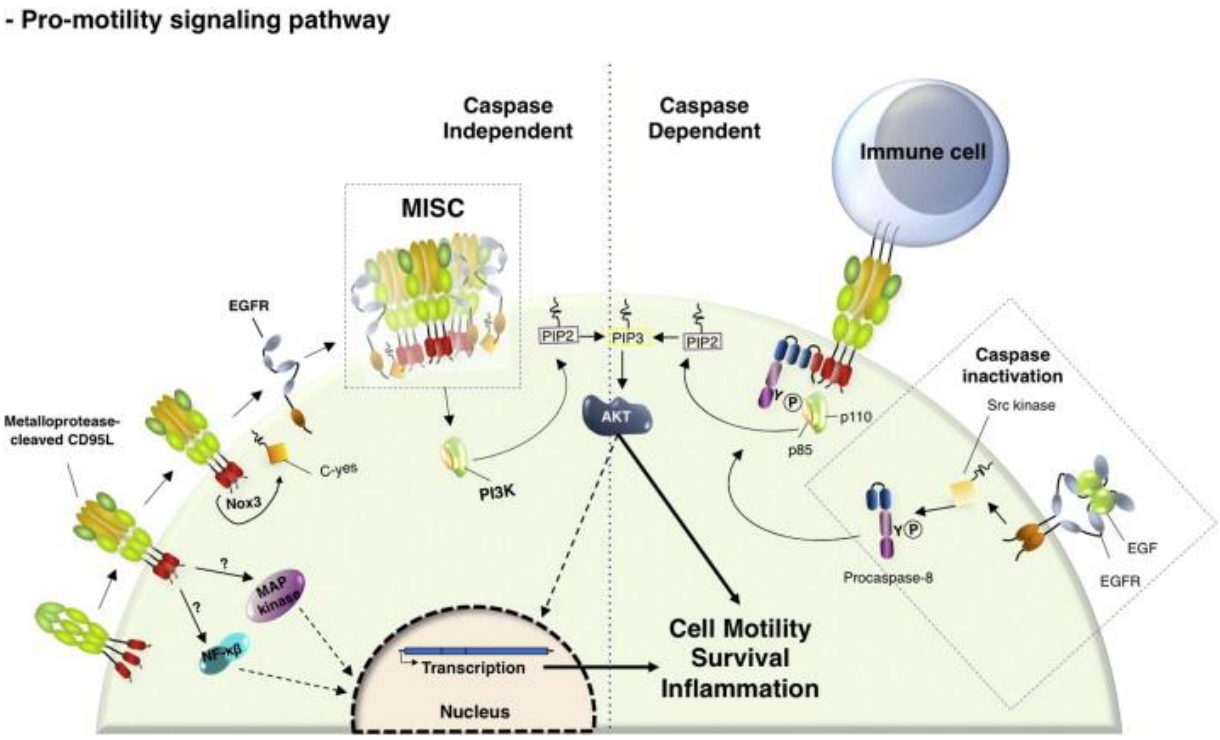
Binding of transmembrane CD95L to CD95 leads to formation of the DISC, which consists of FADD and procaspase-8. C-FLIP and PEA-15 bind to FADD, thereby preventing caspase-8 recruitment. At the level of the DISC, aggregation of procaspase-8 promotes its auto-cleavage and activation. Cleaved caspase-8 is then released to the cytosol, where it promotes the cascade of caspase activation leading to apoptosis. Type I cells are characterized by an efficient DISC formation, which releases sufficient caspase-8 to directly activate caspase-3. By contrast, type II cells form low levels of the DISC, and the resultant weak amount of released caspase-8 activates the mitochondrion-dependent apoptotic pathway to amplify the death signal.

Figure 3. Distribution of somatic and germinal mutations within the CD95 protein sequence.



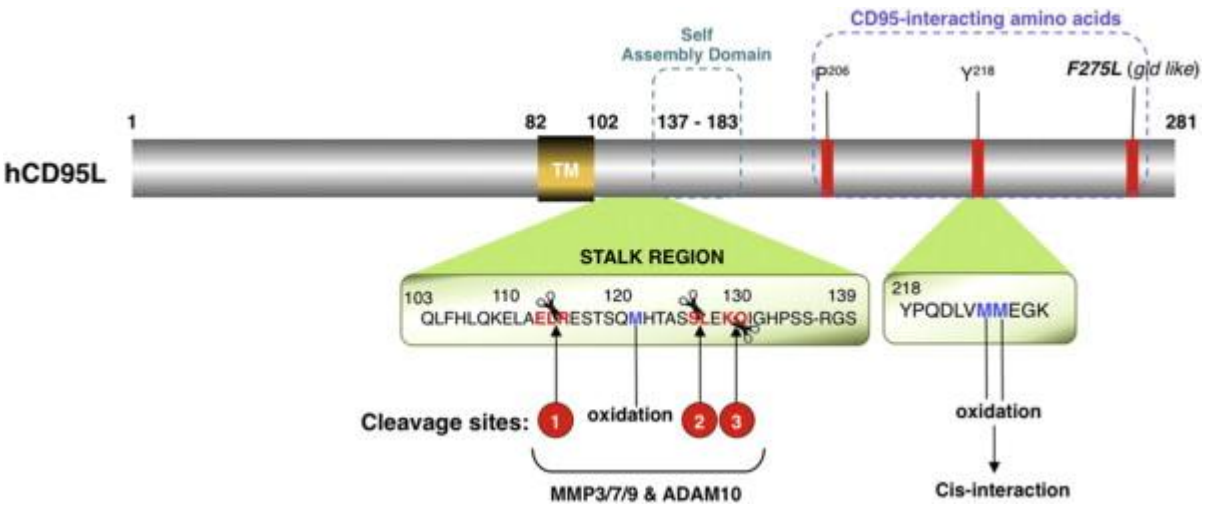
A comprehensive analysis of CD95 mutations is shown. Most of the mutations are distributed within the CD95-DD[51]. Whereas Q257K perturbs FADD binding, replacement of tyrosine at position 275 by phenylalanine prevents CD95 internalization without altering FADD recruitment [138], and I297D maintains the CD95-DD in an open conformation and promotes apoptosis[60].

Figure 4. CD95 triggers an unconventional PI3K signaling pathway.



Left panel: In the presence of cl-CD95L, CD95 triggers MISC formation. The MISC complex is devoid of FADD and caspase-8, but instead recruits the src kinase c-yes, which implements the PI3K signaling pathway. CD95 engagement is also capable of activating NF-κB and MAPK through an as-yet-unknown mechanism. ***Right Panel:*** Procaspase-8 can be phosphorylated by the tyrosine kinase src upon EGFR stimulation. This post-translational modification not only blocks the catalytic activity of caspase-8, but also promotes the recruitment of the p85 subunit of PI3K. We surmise that caspase-8 phosphorylation promotes the non-apoptotic signals induced by CD95.

Figure 5. CD95L: metalloprotease cleavage sites and domains.



Schematic of the human CD95L protein. The juxtamembrane region, also called the stalk region, encompasses three different cleavage sites. Amino acids involved in its interaction with CD95 are indicated [186]. Oxidation of methionine residues in position 224 and 225 increases the aggregation level of the soluble ligand, whereas oxidation at methionine 121 prevents the processing of CD95L by metalloproteases [182]. TM: transmembrane domain.