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REVIEW

CD95-mediated cell signaling in cancer: mutations and post-translational modulations

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Abstract Apoptosis has emerged as a fundamental process important in tissue homeostasis, immune response, and during development. CD95 (also known as Fas), a member of the tumor necrosis factor receptor (TNF-R) superfamily, has been initially cloned as a death receptor. Its cognate ligand, CD95L, is mainly found at the plasma membrane of activated T-lymphocytes and natural killer cells where it contributes to the elimination of transformed and infected cells. According to its implication in the immune homeostasis and immune surveillance, and since several malignant cells of various histological origins exhibit loss-of-function mutations, which cause resistance towards the CD95-mediated apoptotic signal, CD95 has been classified as a tumor suppressor gene. Nevertheless, this assumption has been recently challenged, as in certain pathophysiological contexts, CD95 engagement transmits non-apoptotic signals that promote inflammation, carcinogenesis or liver/peripheral nerve regeneration. The focus of this review is to discuss these apparent contradictions of the known function(s) of CD95.

Keywords Fas · Oncogene · Tumor suppressor · Lipid rafts · ALPS

Abbreviations

ASM	Acid sphingomyelinase
ADAM	A disintegrin and metalloproteinase domain
ALPS	Autoimmune lymphoproliferative syndrome
DD	Death domain
FADD	Fas-associating protein with a death domain
GC	Germinal center
LOH	Loss of heterozygosity
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinase
NF-κB	Nuclear factor Kappa B
TNF	Tumor necrosis factor

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Implementation of a CD95-mediated signaling pathway

CD95: the so-called death receptor

APT-1 (also known as CD95, Fas, or APO-1) gene spans approximately 25 kb on human chromosome 10 [1]. The gene consists of nine exons in which exon 6 encodes the transmembrane domain (Fig. 1a). CD95 is a type I transmembrane protein that belongs to the tumor necrosis factor (TNF) receptor family characterized by cysteine-rich extracellular domains. CD95 is resolved under denaturing conditions between 40 and 50 kDa in SDS-PAGE. This receptor has been initially cloned as a death receptor [2] bound and activated by the apoptotic-inducing mAb

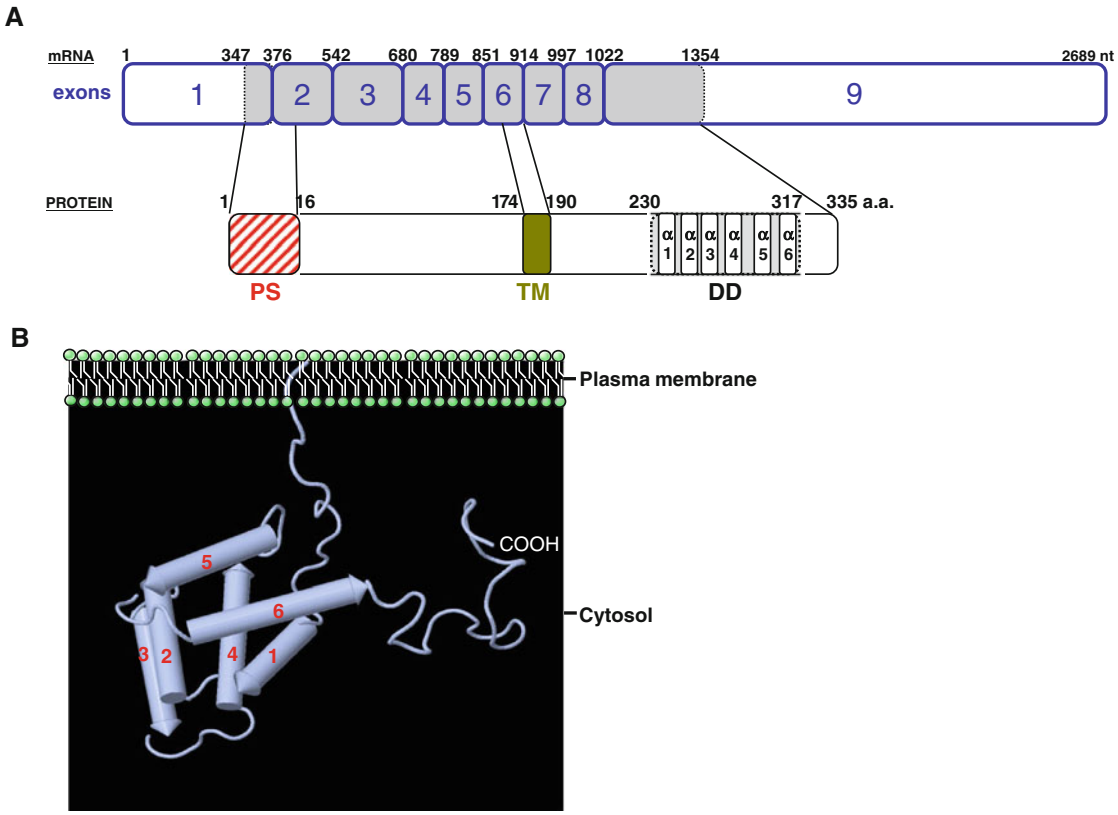

Fig. 1 CD95 structure. **a** CD95 mRNA consists of 9 exons as indicated in the *upper panel*. The CD95 mRNA (*upper panel*) and protein (*lower panel*) are depicted. *Numbers on the upper panel* stand for the position of nucleic acids in CD95 mRNA while on the *lower panel*, they represent the position of the amino acids in the protein sequence. The 6 α-helices constituting the CD95-death domain (DD) are indicated. *PS* peptide signal, *TM* transmembrane domain. Numbering is applied according to references [2, 59] (NM_000043). **b** Nuclear magnetic resonance (NMR) structure of the CD95 death domain according to 1DDF (Protein data bank) and analyzed using Jmol version 12.0.18. The rockets stand for the 6 α-helices and the plasma membrane has been added

designated APO-1 [3]. Consequently, the death-inducing activity of the antigen APO-1 has been extensively analyzed for the last 20 years and the pro-apoptotic role of CD95 is supported by the fact that the cognate ligand of CD95, the transmembrane CD95L (CD178/FasL) is present at the surface of activated lymphocytes [4] and natural killer cells [5] where it orchestrates the elimination of transformed and infected cells. In addition, CD95L is found expressed at the surface of neurons [6], corneal epithelia, and endothelia [7, 8] and Sertoli cells [9] to prevent the infiltration of immune cells and thus to prohibit the spread of inflammation in these sensitive organs (i.e., brain, eyes, testis, respectively), which are commonly called "immune-privileged" sites.

Description of physiological "immune privilege" was later on followed by tumor-mediated "immune privileged area" since two groups reported that the ectopic expression of CD95L by malignant cells participated in the elimination of infiltrating T lymphocytes and thus could play a role in the establishment of a tumoral site whose access was denied to immune effector cells [10, 11]. However, these observations were rapidly challenged since both allogenic transplant of β-islets [12, 13] and tumor cells [14] expressing the membrane-bound CD95L led to a much rapid elimination of the cells as compared to control islets and malignant cells, respectively, due to an increase in infiltration of neutrophils and macrophages endowed with anti-tumoral activities. Confirming these latter findings, recent studies revealed that in certain pathophysiological contexts, engagement of the CD95 receptor promoted pro-inflammatory [15–17] or even pro-oncogenic processes [18–20]. In the following parts of this review, we will discuss how CD95 itself and its ligand organize the implementation of pro- or anti-apoptotic signals.

CD95-mediated apoptotic signaling pathway(s)

Similarly to the TNF-receptor [21], CD95 has been found expressed at the plasma membrane as a pre-associated homotrimer [22, 23]. Binding of CD95L or agonistic anti-CD95 mAbs to CD95 alters both the conformation and the extent to which the receptor aggregates at the plasma

membrane and mounts a signal occurring initially through protein–protein interactions. In this regard, the intracellular region of CD95 encompasses an 80-amino-acid-long stretch designated the death domain (DD) (Fig. 1a, b) whose structure consists of six amphipathic α-helixes arranged anti-parallel to one another (Fig. 1b) [24]. Upon addition of CD95L, CD95 undergoes conformational modifications of its DD, which elicit the shift of helix 6 and its fusion with helix 5 in order to promote both the oligomerization of the receptor and the recruitment of the adaptor protein *Fas-associating protein with a death domain* (FADD) [25]. A consequence of the opening of the globular structure of CD95 is that the receptor becomes connected through this bridge, which enables an increase in the magnitude of its homo-aggregation. In addition, this long helix also allows the stabilization of the complex by recruiting FADD. Overall, this CD95-DD:FADD-DD crystal structure not only revealed an attractive mechanism to explain the formation of large CD95 clusters observed using imaging or biochemical methods in cells exposed to CD95L, but also confirmed that alteration of the CD95 conformation was instrumental in the induction of the signal. However, this elongated C-terminal α-helix favoring the *cis*-dimerization of CD95-DD [25] was challenged by Driscoll and Wu's teams, which did not observe the fusion of the last two helices at a more neutral pH (pH 6.2) as compared to the acidic condition (pH 4) used in the initial study to resolve the CD95-DD:FADD-DD structure [25]. Consequently, at pH 6.2, association of CD95 with FADD consisted predominantly of a 5:5 complex that occurred via a polymerization mechanism involving three types of asymmetric interactions but without major alteration of the DD globular structure [26, 27]. It is likely that the low pH condition used in the study performed by Scott et al. [25] altered CD95 conformation and resulted in the formation of non-physiological CD95:FADD oligomers. Nonetheless, we cannot rule out that in vivo, a local decrease in the intracellular pH may affect the initial steps of the CD95 signal by promoting the opening of the CD95-DD, which in turn contributes to the formation of a complex eliciting a sequence of events different from the one occurring at physiologic pH.

Once docked on CD95-DD, FADD self-associates [28] and binds and aggregates procaspases 8 and -10, which are auto-processed and released in the cytosol as active caspases that cleave many substrates leading to the execution of the apoptotic program and the death of the cell. The complex CD95/FADD/Caspase-8/-10 is called DISC for "death-inducing signaling complex" [29]. Due to the importance of the DISC formation in the fate of the cells, it is not surprising that numerous cellular and viral proteins have been reported to negatively regulate the formation of this structure, such as FLIP [30, 31] and PED/PEA-15 [32] that interfere with the recruitment of caspase-8/-10.

CD95 mutations

Patients exhibiting germinal mutations in *APT-1,* the gene encoding the CD95 receptor, develop a syndrome termed autoimmune lymphoproliferative syndrome type Ia (ALPS, also called Canale–Smith syndrome) [33–35] (Table 1). ALPS patients show chronic lymphadenopathy and splenomegaly, expanded populations of double-negative α/β T lymphocytes ($CD3^{+}CD4^{-}CD8^{-}$) and often develop autoimmunity [33, 34, 36, 37]. In agreement with the notion that CD95 is a tumor suppressor gene, the ALPS patients display an increased risk to develop Hodgkin and non-Hodgkin lymphomas [38]. Predominance of post-germinal center (GC) lymphomas in patients exhibiting either germ line (Table 1) or somatic CD95 (Table 2) mutations can be explained by the fact that firstly, inside germinal centers of the secondary lymphoid follicles, the CD95 signal plays a pivotal role in the deletion of self-reactive maturating B lymphocytes [39] and secondly, *APT-1* belongs to a set of rare genes (i.e., PIM1, c-myc, PAX5, RhoH/TTF, Bcl-6), which are subjected to somatic hypermutation [40, 41] and thus accumulate mutations that at one point, may alter their biological function. In addition to post-GC lymphomas, significant amounts of mutations in CD95 gene have been found in tumors from various histological origins (summarized in Table 2). An extensive analysis of the CD95 mutations and their distribution in *APT-1* confirms that with some exceptions, most of them are gathered in exons 8 and 9 encoding the main part of the CD95 intracellular region (Fig. 2) [42]. From a biological standpoint, malignant and ALPS type Ia cells harboring a heterozygous mutation inside the CD95-DD exhibit resistance towards the CD95-mediated apoptotic signal. Indeed, in agreement with the notion that CD95 is expressed at the plasma membrane as a pre-associated oligomer (trimeric complex) [22, 23], the formation of heterocomplexes consisting of the association of wild-type with mutated CD95 will hamper FADD recruitment and thus, will alter the ignition of the apoptotic signal. Extensive analysis and positioning of various CD95 mutations described in literature seem to highlight "hot-spots" of mutation in the CD95 sequence (Tables 1, 2; Fig. 2a, b). Among these "hot-spots", it is worth noting that together arginine in position 234, aspartic acid in position 244 and valine in position 251, account for a significant amount of the documented CD95 mutations. Indeed, among the 189 mutations annotated in the 335-length CD95 amino acid sequence, 30 ($\sim$16%) are found localized on these three amino acids (Fig. 2a, b). Strikingly, the pivotal role played by these amino acids in the stabilization or formation of

Table 1 Germinal mutations in the APT1 gene

	Mutations	Diseases	References	Observations
1	*Exon 3* Frameshift (−1) *Intron 3 and exon 7:* Splice variants *Exon 9* T225P Q257X	ALPS Ia	[34]	Five unrelated ALPS patients
2	*Exon 9* D253 (frameshift −2) L290 (deletion of the last 29 a.a. replaced by YKLHQE)	ALPS Ia	[35]	Case report of three patients (one sibling and unrelated patient) No surface expression of the L290 mutant
3	*Exon 9* K230X (+1 frameshift) D244Y (father and son) R234X	ALPS Ia	[33]	Case report of four patients
4	*Exon 1* T12A Second allele displayed deletion between aa 41 and 96 and exon 6 (no TM)	ALPS Ia in a patient with type 2 autoimmune hepatitis	[90]	Mutation inside exon 1 and deletion of a part of the PLAD domain
5	*Exon 4* R105 W *Exon 9* Y216C	ALPS Ia	[91]	Autosomal recessive mutations
6	*Exon 9* D244 V	ALPS Ia	[92]	
7	*Exon 3* C66R frameshift R1X C47X *Intron 7* S209X *Intron 8* S214X *Exon 9* R234P D244G D244Y T254I	ALPS Ia	[93]	Cohort of 11 families. Extracellular mutations (C66R/R1X/C47X) abolished the expression of the mutated CD95
8	*Exon 3* E63X *Intron 7* splice variant P201 frameshift *Exon 9* S214 frameshift (+2) S227 frameshift (+4) V233L R234P G237D G237S I243R T254K E256K W265 frameshift K280 frameshift (−1)	ALPS Ia	[37]	

Table 1 continued

	Mutations	Diseases	References	Observations
9	*Intron 3* P49 splice variant *Exon 3* C57X D62 frameshift (−1) *Intron 4* T131 splice variant *Intron 6* V174 splice variant *Exon 7* K181(del-11) *Intron 7* P201 splice variant *Exon 9* T225P T225K R234Q R234P A241D D244V Q257X Q260X L278X I294S	ALPS Ia	[46]	Cohort of 155 families 60 Individuals harbor a CD95 mutation. 17 different mutations. Penetrance of one or more ALPS features was 88% for individuals with intracellular mutations versus 18% for individuals with extracellular mutations. Exon 9 encompasses 59% of mutations causing ALPS
10	*Exon 7* K181 (del-11) P201 frameshift *Exon 9* T225P R234X D244V T254I E256G	Hodgkin and non-Hodgkin Lymphomas associated with ALPS type Ia patients	[38, 94]	Large-scale family case report. 223 members of 39 families, 130 individuals possessed heterozygous germline Fas mutations. ALPS patients displayed an increased risk of non-Hodgkin and Hodgkin lymphomas (14 and 51 times greater, respectively) as compared to general population
11	*Exon 9* R234Q	ALPS type Ia/Hodgkin Lymphomas	[95]	Family case report
12	Germline mutations (ALPS type Ia) K181X P201X S214X Somatic mutations (ALPS Type III) W173X P201X (3 cases) S214X D244V	ALPS Type Ia/Type III	[96]	Mutation originated in hematopoietic stem cells: 6/6 in double negative T cells from ALPS Type III 0/5 in healthy subjects
13	*Exon 9* I246S	ALPS Ia	[97]	
14	*Exon 3* H95R *Exon 7* E195X *Intron 7* (splice mutation/premature stop) E202X (5 patients) *Exon 8* N207X (del-8) L208X (del-2) *Exon 9* D244N V204 (del-2) D212X (del-5)	Autoimmune lymphoproliferative syndrome (ALPS type III)	[98]	12/31 of double-negative T cells displayed CD95 mutations

Table 2 Somatic mutations in the APT1 gene

	Mutations	Diseases	Mutation frequencies	References	Observations
1	*Exon 9* Y275S D253Y (2 patients) K235R N264H	Multiple myelomas	Among 54 patients, 6 did not express CD95 and 5–48 (10%) displayed mutation.	[99]	
2	*Exon 2* A(−1)T *Exon 4* C119 (+1) frameshift *Exon 6* L164F P167L *Exon 7* T182I L199X *Exon 8* L208X *Exon 9* D244V N248K (2 patients) E256K L262F K283N *Intron 7* splice variant *Intron 8* splice variant (2 patients)	Non-Hodgkin lymphomas	16/150 non-Hodgkin lymphomas (11%) 3/5 (60%) mucosa-associated lymphoid tissue (MALT)-type lymphoma 9/43 (21%) diffuse Large B-cell lymphomas 2/33 (6%) follicle center lymphomas 1/2 (50%) anaplastic large cell lymphoma 1/17 (6%) B-chronic lymphocytic leukemia 0/5 Burkitt lymphoma 0/9 mantle cell lymphoma 0/35 peripheral T-cell lymphoma	[100]	Missense mutations within exon 6 and 7 were associated with loss of heterozygosity (LOH)
3	*Exon 9* L213 (frameshift −20)	Adult T cell leukemia	Found in an ATL cell line (designated KOB)	[101]	
4	*Exon 9* A241T N250S V251I	Cutaneous malignant melanoma	3/44 (6.8%)	[102]	LOH for two patients
5	*Exon 6* C162R (2 patients) *Exon 9* V251I (8 patients) N237K D244 (+1) stop in 245	Bladder carcinomas	12/43 (28%)	[103]	11/12 mutations found in muscle-invasive transitional cell carcinomas Hotspot mutation in V251
6	*Exon 9* I300V[a] E323X[a]	Hodgkin and Reed–Sternberg lymphomas	1/10 patients (10%)	[104]	
7	*Exon 4* N102S *Exon 6* C162R *Exon 9* N239D	Squamous cell carcinoma	3/21 (14%) burn scar-related squamous cell carcinoma displayed mutated CD95. 2/6 (33%) with lymph node metastasis. 1/15 (7%) without metastasis. 0/50 conventional skin squamous carcinoma	[105]	Mutations are found in metastatic lesions

Table 2 continued

	Mutations	Diseases	Mutation frequencies	References	Observations
8	*Exon 8* Splice variant (deletion codons 202–210) × 6 thyroid lymphoma (TL) and 2 chronic lymphocytic thyroiditis (CLTh) *Exon 9* Y216X E240V D244N D244H Frameshift exon 9 (+1 codon 285) (9 TL and 1 CLTh)	Thyroid lymphomas	17/26 thyroid lymphomas (65.4%) (consisting of 5/10 diffuse large B-cell lymphoma; 6/8 low-grade MALT; 6/8 follicle center cell lymphomas) 3/11 Chronic lymphocytic thyroiditis (27.3%)	[106]	Mutation hot-spots in thyroid lymphomas
9	*Exon 2* K23R *Exon 3* G50S *Exon 5* D152X *Exon 6* V172A *Exon 9* V229A 284 (Insertion +1) frameshift	Mucosa associated lymphoid tissue (MALT)-type lymphomas	4/5 mucosa associated lymphoid tissue (MALT)-type lymphomas 1/3 DLBLs	[107]	Patients display more than 1 mutation per gene
10	*Exon 9* T254A[b] Q260X Q260N N286S	High-grade prostatic intraepithelial neoplasia (HGPIN) and prostatic cancer	4/27 of HGPIN (14.8%)	[108]	
11	*Exon 9* N239D (2 patients) E240G D244V R263H	Gastric cancers	5/43 (11%)	[109]	
12	*Exon 9* D210G A221G N239S H266L K271M D276G I279V I302V	Testicular germ cell tumor	9/24 patients (37.5%) A total of 11 mutations were found in 10 lesions from the 9 patients (3 silent mutations)	[110]	All mutations in CD95 exon 9 were associated with a loss-of-function
13	*Exon 2* T24I *Exon 3* C69Y *Exon 4* E98G *Exon 7* Frameshift (−1) E195X *Exon 8* Frameshift (−1) I206X *Exon 9* T203P	Mycosis fungoide (MF) (cutaneous T-cell lymphoma)	6/44 (13%)	[111]	The mutation in CD95 could explain the common resistance of MF to chemotherapy

Table 2 continued

	Mutations	Diseases	Mutation frequencies	References	Observations
14	*Exon 4* C119 frameshift (−1) *Exon 6* S154 frameshift (−1) *Exon 9* A285 frameshift (+1) 2 cases I246V L287P	Nasal NK/T-cell lymphoma	7/14 (50%)	[112]	Insertion in polyA tract from nucleotide 1,088–1,094 (A285 frameshift): mutation hot spot
15	*Exon 3* G66D P84S *Exon 9* Q228X N250S	Non-small cell lung cancer (NSCLC)	4/80 in NSCLC (5%) 3/43 (7%) in NSCLC without metastasis. 1/37 (2%) in NSCLC with metastasis	[113]	All mutations found in NSCLC patients with metastasis were only detected in the metastatic lesion
16	*Exon 2* T27I S3F S16P *Intron 3* Splice variant *Intron 8* Splice variant *Exon 9* D244 N Q260X	Mucosa associated lymphoid tissue (MALT)-type lymphomas	7 mutations found in 5 patients 1/18 (5.6%) Mucosa associated lymphoid tissue (MALT) lymphomas 4/28 (14.3%) diffuse large B-cell lymphoma (DLBCL)	[114]	Mutations in exon 2 do not impair the transmission of the CD95-mediated apoptotic signal in T-47D (breast) and Jurkat (lymphocyte) cells
17	5′UTR	Diffuse large B cell lymphomas (DLBCL)	3 of 66	[115]	
18	RNA editing Frame shift (+1) K284 frameshift (+1)	Systemic lupus erythematosus (SLE)	Mutation accounted for 11% of *CD95* cDNA clones in SLE patients and the frequency of mutant clones was less than 1% in healthy subjects	[116]	Transcriptional mRNA editing. Not found in other human death receptor mRNAs such as DR5, DR6 or TNF receptor 1 (TNFR1) or in mouse CD95 mRNA

[a] Amino acid numbers have been modified according to the regular amino acid annotation

[b] Threonine 256 depicted in this study corresponded in fact to amino acid 254

intra and inter-bridges between CD95 and FADD may explain these "hot-spots" since for instance, both R234 and D244 contribute to the homotypic aggregation of the receptor and the FADD recruitment [24]. Nevertheless, the observation of death domain "hot-spots" is in contradiction with the study of Scott and colleagues [25] demonstrating that the region of the CD95-DD interacting with the FADD-DD spreads on a disperse surface through weak binding affinities. Most ALPS type Ia patients affected by malignancies do not undergo a loss of heterozygosity (LOH), which let us to hypothesize that this heterozygous configuration may promote carcinogenesis [43, 44]. Supporting this assumption, we demonstrated that although conservation of a wild-type allele failed to transmit the apoptotic signal, it was sufficient and mandatory to elicit non-apoptotic signals such as NF-κB, MAPK [43, 44], whose inductions promote invasiveness of tumor cells [42, 45]. Mutations found in the intracellular CD95-DD exhibit a higher penetrance of the ALPS phenotype features in mutation-bearing relatives than extracellular mutations, which suggest that these latter mutants require additional dysfunctions to efficiently abrogate the CD95-mediated apoptotic signal [46]. Alternatively, it could be tempting to speculate that in contrast to mutations inside the death domain, other CD95 mutations somehow prevent the apoptotic signal but fail to promote non-apoptotic ones, which may contribute to the disease progression.

Plasma membrane and CD95 signal

In addition to down-regulation of CD95 or accumulation of heterozygous mutations, the plasma membrane distribution

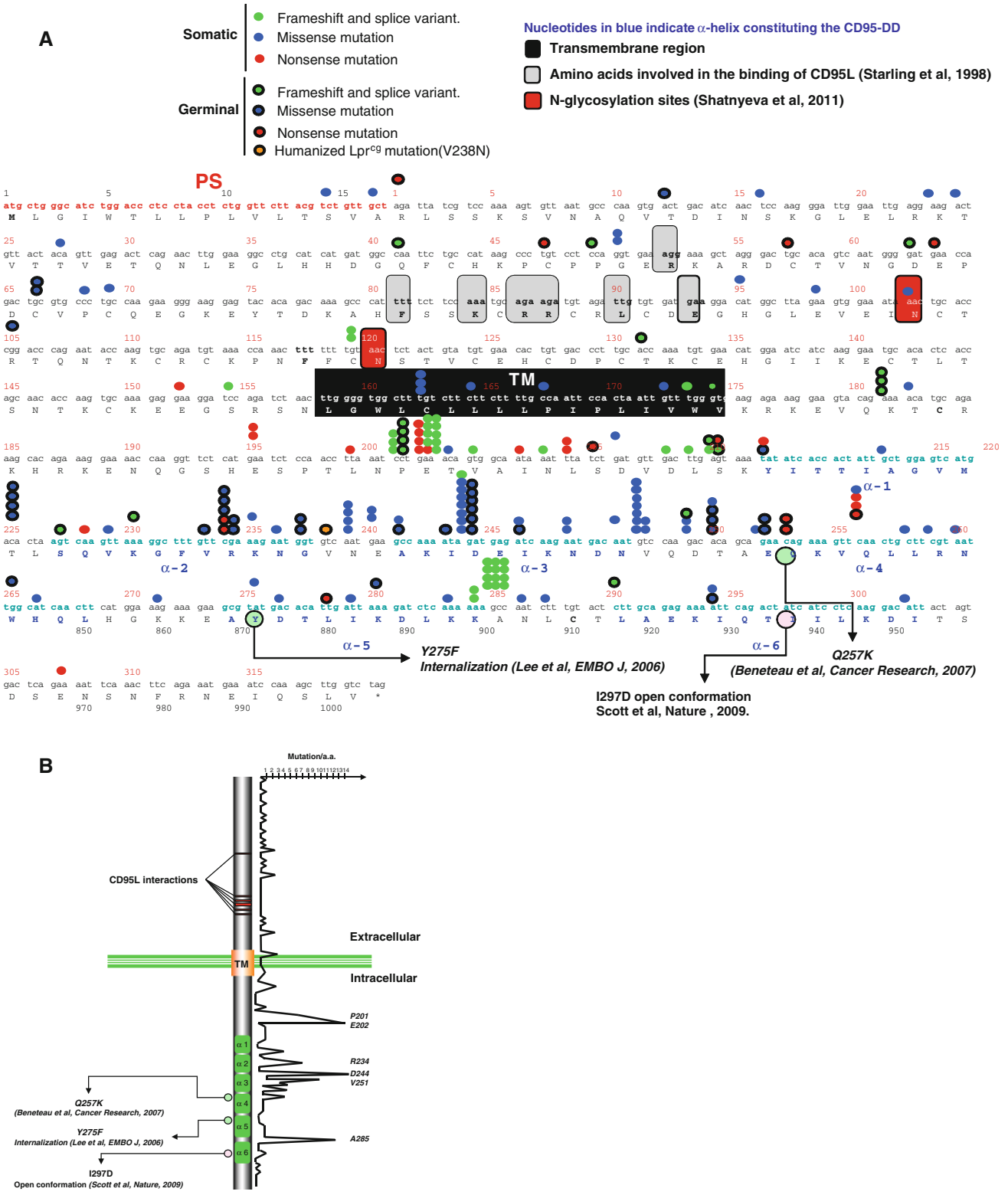

Fig. 2 Extensive analysis of the loss-of-function CD95 mutations. **a** Mutations reported in Tables 1 and 2 have been placed in the amino-acid sequence of CD95. The 6 α-helices constituting the death domain and amino acids implicated either in the CD95/CD95L interaction or the receptor glycosylation are depicted. **b** Schematic distribution of CD95 mutations in the CD95 protein. The number of mutations per amino acid is depicted on CD95 amino-acid sequence. The death domain and its 6 α-helices are reported

of CD95 may also represent an alternative way for tumor cells to circumvent the apoptotic signal. Indeed, plasma membrane is a heterogeneous lipid bilayer comprising compacted or "liquid-ordered" domains called microdomains, lipid rafts or detergent resistant microdomains (DRMs), which are described as floating in a more fluid or liquid-disordered 2-D lipid bilayer. Despite the fact that the composition and the kinetic of formation and disappearance of these plasma membrane structures remain poorly understood mostly due to limitation in the methodologies used to characterize them, the lipid rafts play a crucial role in the modulation of the initial steps induced by death receptor signaling. For instance, it has been elegantly reported that while CD95 is mostly excluded from lipid rafts in activated T lymphocytes, the TCR-dependent reactivation of these cells leads to the rapid distribution of the death receptor into lipid rafts [47]. This CD95 compartmentalization is crucial to decrease in the apoptotic threshold leading to the clonotypic elimination of activated T-lymphocytes through activation of the CD95-mediated apoptotic signal [47]. Similarly, the reorganization of CD95 into DRMs can occur independently of its ligand upon addition of certain chemotherapeutic drugs (e.g., rituximab [48], resveratrol [49, 50], edelfosine [51–53], aplidin [54], perifosine [53], cisplatin [55]). The intimate molecular cascades that underlie this process remain to be elucidated. Nevertheless, the current evidence lead us to postulate that alteration of intracellular signaling pathway(s) (e.g., the PI3K signal [51, 56]) may change biophysical properties of the plasma membrane such as the membrane fluidity, which may mimic the CD95L-induced initial steps in promoting the aggregation and clustering of CD95 into large lipid raft-enriched platforms, which in turn favor DISC formation and the induction of the apoptotic program [57]. On the other hand, these signaling pathways may exert post-translational modifications of the death receptor itself and thereby may promote or prevent its redistribution into lipid rafts.

Regulation of the CD95 plasma membrane distribution

Plasma membrane distribution of CD95

Accumulation of CD95 mutations is not the only way by which malignant cells may impede the extrinsic signaling pathway elicited by immune cells or chemo- and radiotherapeutic regimens. Post-translational modifications in the intracellular tail of CD95 such as reversible oxidations or covalent attachment of a palmitic acid have been reported to alter the plasma membrane distribution of CD95 and thereby its subsequent signaling pathway (Fig. 3). For instance, S-glutathionylation of mouse CD95 at the cysteine residue 294 promotes the clustering of CD95 and its distribution into lipid rafts [58] (Fig. 3). This amino acid is conserved in the human CD95 sequence and corresponds to cysteine residue at position 304 (C288 in

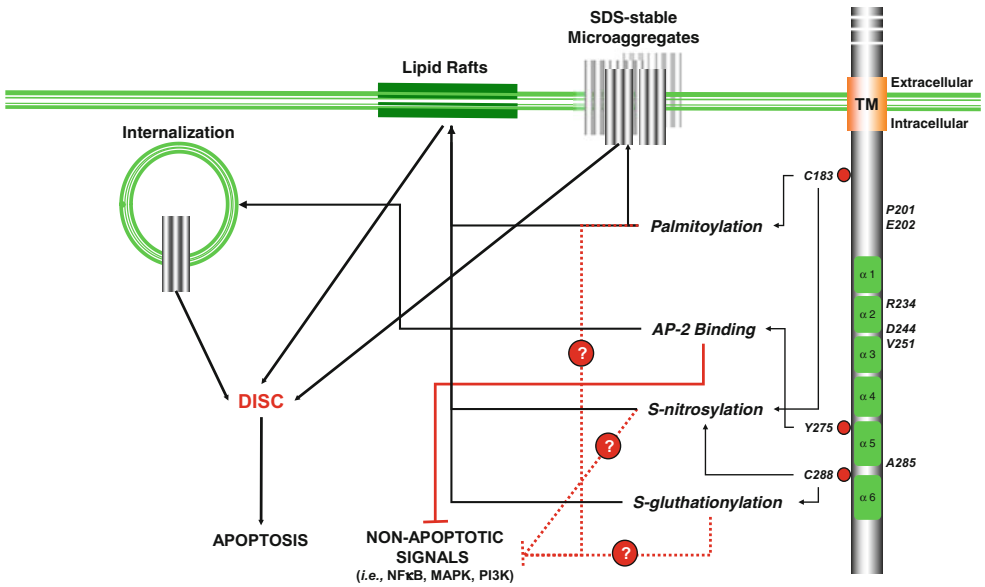

Fig. 3 Post-translational modifications and CD95 internalization. The main mutations described in Tables 1 and 2 have been placed in the amino-acid sequence of CD95. The 6 α-helices constituting the death domain are depicted. CD95 internalization (AP-2 binding) hampers the implementation of non-apoptotic signaling pathways while it elicits apoptosis. *Question marks* indicate experiments that remain to be conducted to decipher whether similarly to internalization, CD95 palmitoylation, S-nitrosylation and S-glutathionylation inhibit the CD95-mediated non-apoptotic outcomes. *Black arrows* indicate activations; *red lines* (*dashed* and *continuous*) stand for inhibitions

Figs. 2a, 3 whose numbering takes into consideration the subtraction of the 16-amino acid peptide signal [2, 59]). Interestingly, Janssen-Heininger Y.M. and colleagues [58] emphasize that the death receptor glutathionylation occurs downstream the activation of caspase-8 and -3 whose catalytic activity processes and inhibits the thiol transferases glutaredoxin 1 (Grx1), an enzyme implicated in the denitrosylation of proteins. The consequence of Grx1 inactivation is the accumulation of glutathionylated CD95, which cluster into lipid rafts sensitizing cells to the CD95-mediated apoptotic signal. Based on these findings and counterintuitively, we conclude that caspase-8 activation occurs prior to aggregation of CD95 and its redistribution into lipid rafts, which both are mandatory to form the DISC and subsequently to activate large amounts of caspase-8. To conciliate these observations, activation of caspase-8 has been reported to occur in a two-step process. First, an immediate and faint amount of activated caspase-8 (<1%) is generated when CD95L interacts with CD95 that orchestrates acid sphingomyelinase (ASM) activation, ceramide production and CD95 clustering that second, promote DISC formation and the outburst of caspase-8 processing essential to mount the apoptotic signal [60].

It is noteworthy that S-glutathionylation is not the only oxidation modulating the CD95 activity. In this regard, S-nitrosylation has also been reported to cause an augmentation of the plasma membrane expression and the activity of CD95 [61]. S-nitrosylation of cysteine residues 199 (corresponding to the residue C183 in Figs. 2a, 3) and 304 (C288) in colon and breast tumor cells leads to the redistribution of CD95 into DRMs, the formation of the DISC and the transmission of the apoptotic signal [61] (Fig. 3).

Two reports have brought to light that covalent coupling of a 16-carbon fatty acid (palmitic acid) to cysteine residue at position 199 (C183) elicits the redistribution of CD95 into DRMs, the formation of SDS-stable CD95 microaggregates, which resist to denaturing and reducing treatments and the internalization of the receptor [62, 63] (Fig. 3). These molecular steps remain to be more finely ordered but they play critical role in the implementation of the apoptotic signal (discussed below).

Of note, similarly to S-nitrosylation, both the aforementioned S-glutathionylation at C304 (C288) and palmitoylation at C199 (C183) promote the partition of CD95 into lipid rafts and enhance the subsequent apoptotic signal (Fig. 3). Further investigations addressing if these post-translational modifications are redundant and occur simultaneously in dying cells or if they are elicited in a cell specific and/or in a micro-environmental-specific manner would be of a great interest to better understand the molecular mechanisms used by tumor cells to overcome these post-translational alterations and thereby to resist to the extrinsic signaling pathway.

Receptor endocytosis

Using an elegant magnetic method to isolate receptor-containing endocytic vesicles, it has been shown that CD95 is promptly found associated with endosomal and lysosomal markers when incubated with an agonistic anti-CD95 mAb [64]. In addition, expression of a CD95 mutant in which the tyrosine 291 (Y275 in Figs. 2a, 3) is changed to phenylalanine does not impinge on FADD binding but compromises the CD95L-mediated CD95 internalization occurring through an AP-2/clathrin-driven endocytic pathway [64]. More strikingly, expression of the internalization defective CD95 mutant (Y291F) abrogates the transmission of the apoptotic signal while it fails to alter the non-apoptotic signaling pathways (*i.e.*, NF-κB and Erk) and even promotes them (Fig. 3). Overall, these findings provide insight into the presence in the death domain of a region interacting with AP2 and promoting a clathrin-dependent endocytic pathway in a FADD-independent manner. Regarding the role of palmitoylation in the receptor internalization, the interplay between lipid alteration and AP2/clathrin-driven internalization of CD95 remains to be elucidated.

Implementation of a CD95-mediated non-apoptotic signaling pathway

CD95L promotes carcinogenesis

Increase rate in malignancies after organ transplantation represents the major cause of cancer-related mortality in immunomodulated transplant recipients [65] and provides the basis for the crucial role played by the immune system in tumor surveillance. Since among the weapons set at the disposal of immune cells, CD95L contributes to the elimination of pre-tumoral cells, it is envisioned that pre-tumoral cells escaping the immune surveillance will be shaped to develop resistance to the CD95, a process that has been termed immunoediting [66]. In other words, the imprinting of the immune system on pre-tumoral cells could ultimately select malignant cells with increased resistance towards the CD95L-induced signal (e.g., down modulating CD95, expressing dominant negative mutants of CD95). As heterozygous "dominant negative" mutations of CD95 prevent the CD95-mediated apoptotic signal triggered by "weak agonistic" molecules such as agonistic antibodies but do not abrogate the non-apoptotic signals induced by highly aggregated CD95 ligand (e.g., membrane-embedded CD95L) [67], it is conceivable that these mutations may increase the apoptotic threshold enough to switch the signal from apoptotic to non-apoptotic pathways [44]. Apart from the classical inhibitory effect on apoptosis

exerted by the expression of a dominant-negative mutant of CD95, which may be sufficient to account for carcinogenesis, we propose that the switch in the CD95 signal (i.e., from apoptotic to non-apoptotic signaling pathway) could account for malignancy progression. In agreement with this hypothesis, complete loss of CD95 expression [68] and LOH are rarely observed in malignant cells [42], which thereby, are still capable to transmit the CD95-mediated non-apoptotic signals when exposed to CD95L [44]. In this regard, recent reports confirmed a role of the couple CD95/CD95L in carcinogenesis through either the activation of JNK (C-Jun kinase also called stress-activated protein kinase) [19], NF-κB (nuclear factor-kappa B) [16] or PI3K (phosphatidylinositol 3-kinase) [20]. Overall, these recent studies do not rule out the pro-apoptotic function of the "death receptor" CD95 but they emphasize that in certain pathophysiological contexts, CD95 engagement enables the transmission of non-apoptotic and even pro-oncogenic signaling pathways.

From a molecular standpoint, binding of the membrane-bound CD95L or homemade generated crosslinked soluble CD95L to CD95 evokes DISC formation building the platform for the ignition of the apoptotic signal. On the other hand, we and others recently observed that once cleaved by metalloprotease, the soluble version of CD95L (cl-CD95L) fails to induce DISC formation [17, 20] but it orchestrates the infiltration and accumulation of activated T lymphocytes in damaged organs of SLE patients through the formation of a complex that we called the MISC for motility inducing signaling complex [17]. This complex is devoid of FADD and caspase-8/-10 but it encompasses the c-yes src kinase, whose activity contributes to the activation of the PI3K signaling pathway [17, 20, 69]. These studies do not only reveal a molecular link between the "death receptor" CD95 and the class I PI3Ks—p110γ (also known as PIK3CG) and δ (PIK3CD) isoforms—in T cells but they also revisit the biological role of CD95L whose shedding by metalloproteases, frequently over-expressed in the inflammatory areas, affects the initial events of the CD95 signaling pathway.

Likewise, decrease in the plasma membrane level of CD95 or expression of a mutated CD95 allele as observed in ALPS patients (Table 1) and various malignant cells (Table 2) impedes the implementation of the apoptotic signal but does not affect the transmission of non-apoptotic signals such as NF-κB, MAPK and PI3K [19, 43, 44] suggesting that these signals stem from different domains of CD95 or rely on different thresholds to be elicited. One important question that remains to be addressed is how the magnitude of the CD95 aggregation controls the formation of "death"- and/or "motility"-ISCs. In other words, although it is well-accepted that the couple CD95/CD95L can eliminate malignant cells by the implementation of the DISC or can promote carcinogenesis by fueling inflammation and/or by inducing metastatic dissemination [15–20, 45], the molecular mechanism(s) underlying the switch between these different signaling pathways remains enigmatic. Addressing this question will bring to light new therapeutical agents able to contain the spreading of inflammation or to impede carcinogenesis mechanisms at least in pathologies in which increase in soluble CD95L amounts has been reported such as cancers (e.g., pancreatic cancers [70], large granular lymphocytic leukemia and natural killer cell lymphoma [71]) or autoimmune disorders (e.g., rheumatoid arthritis and osteoarthritis [72], graft versus host disease (GVDH) [73, 74] or SLE patients [17, 75]).

CD95L stoichiometries elicit different cell signaling pathways

CD95L is a type II transmembrane protein (carboxy-terminal extracellular region), which belongs to the TNF (tumor necrosis factor) family. As aforementioned, CD95L is mainly found at the surface of immune cells where it contributes to the elimination of infected or transformed cells through cell-to-cell contact. CD95L is also detected on macrophages and dendritic cells upon HIV infection [76] and on surface of epithelial cells in inflammatory disorders [77]. It is noteworthy that CD95L can be cleaved by metalloproteases such as MMP3 [78], MMP7 [79], MMP9 [80] and ADAM-10 (A disintegrin and metalloproteinase 10) [81, 82] and shed from the plasma membrane to be released in the connective tissue and the bloodstream as a soluble and homotrimeric ligand. Seminal studies on the metalloprotease-cleaved CD95L have revealed that this ligand exhibits a homotrimeric stoichiometry [83, 84]. Considering that hexameric CD95L represents the minimal stoichiometry required to signal apoptosis [83], the cleaved form of CD95L (cl-CD95L) has long been considered as an inert ligand competing with its membrane-bound counterpart (m-CD95L) to antagonize the transmission of the apoptotic signal [84, 85].

Strikingly, while the soluble form of CD95L generated by MMP7 (cleavage site inside the ^{113}ELR^{115} sequence, Fig. 4) induces apoptosis [79], its counterpart processed between the serine^{126} and the leucine^{127} does not [16, 17, 84]. To explain this discrepancy, one may postulate that the different quaternary structures of the naturally processed CD95L underlie the implementation of "death" or "non-death"-inducing signaling complex and their downstream signals. In agreement with this notion, it has been reported that soluble CD95L bathed in bronchoalveolar lavages (BALs) of patients suffering from acute respiratory distress syndrome (ARDS) undergoes oxidation of two methionine residues in position 224 and 225 of CD95L (Fig. 4) that

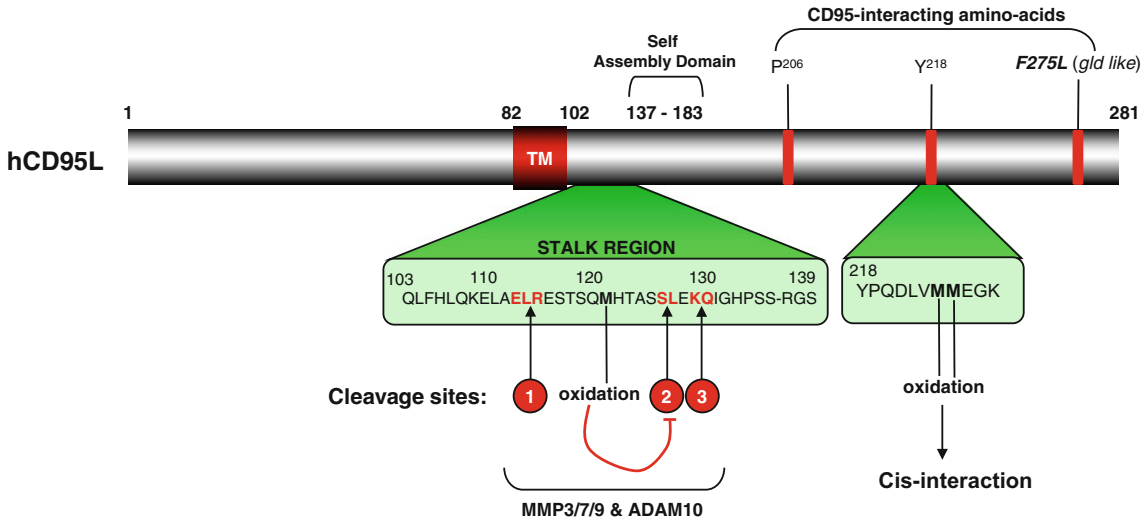

Fig. 4 Human CD95L protein structure. Different domains (CD95L self-assembly/CD95 binding sites/Stalk region) of the type II protein CD95L are depicted in this scheme. In the stalk region, the amino acids corresponding to the metalloprotease cleavage sites are depicted in *red*. Three targets of oxidation (i.e., methionine) are reported and their role on the CD95L structure is indicated

enhances the aggregation level of the soluble ligand and thus its cytotoxic activity [86]. The same authors observed that the stalk region of CD95L corresponding to amino acids 103–136 and encompassing the metalloprotease cleavage sites (Fig. 4) is also instrumental in the multimerization of CD95L, which accounts for the damage of lung epithelium in ARDS patients [86]. Of note, in the ARDS BALs an additional oxidation occurs at methionine 121 (Fig. 4), which in turn prevents the processing of CD95L by MMP7 and explains why this cytotoxic ligand keeps its stalk region [86]. Nonetheless, preservation of this region in soluble CD95L raises the question if a yet unidentified MMP7-independent cleavage site exists in the juxtamembrane region of CD95L, near the plasma membrane, or if the ligand detected in ARDS patients corresponds in fact to a full length CD95L embedded in exosomes [87, 88]. Indeed, this peculiar exosome-bound CD95L can be expressed by human prostate cancer cells (i.e., LNCaP) and evokes apoptosis in activated T lymphocytes [89].

Although the soluble CD95L cleaved between its serine^{126} and leucine^{127} exhibits no apoptotic activity, this cytokine fueled inflammation and auto-immunity both in a lupus-prone mouse model [16] and in systemic lupus erythematosus (SLE) patients [17]. Recent findings on CD95L emphasize that it may be of a great interest in the future to finely characterize the quaternary structure of the naturally processed CD95L found increased in sera of patients affected by cancers or chronic/acute inflammatory disorders to better comprehend the molecular mechanism engaged by the ligand and its subsequent biological functions.

Overall, these results indicate that instead of a monolithic and apoptotic function for the couple CD95/CD95L, the ratio of transmembrane versus cleaved CD95L or the plasma membrane amounts of functional CD95 may account for the induction of apoptotic or non-apoptotic signals and that way, may favor elimination of transformed cells or promote carcinogenesis, respectively.

Conclusions

The cellular response to CD95 relies on its expression level, its alteration by post-translational modifications, the presence or absence of heterozygous mutations and/or the stoichiometry of the exposed ligand. However, altogether these features are not sufficient to foresee the main signal transmitted by CD95. Fifteen years ago, Stuart and colleagues already reported that "immune privilege" does not only result from the expression of CD95L on epithelial and endothelial corneal cells but also relies on the site itself in which the CD95L-expressing cells are transplanted. Indeed, although transplanted corneas display high rate of acceptance in the eye, allogeneic corneas grafted heterotopically to the skin are rapidly rejected [8]. Likewise, the group of Nabel shed light on the role of TGF-β in the CD95-mediated recruitment of neutrophils and thus strengthened the biological effect of the microenvironment in the modulation of the CD95 signal [14]. We recently ascertained that the epithelial-mesenchymal transition (EMT) causes resistance of tumor cells towards the CD95-mediated apoptotic signal [68]. Indeed, while tumor cells displaying an epithelial-like signature were sensitive to the

CD95 signal, the mesenchymal malignant cells resisted to the transmission of the apoptotic-signaling pathway through a yet totally unknown process. As TGF-β is the prototype cytokine contributing to EMT process, it may correspond to the principal micro-environmental factor, whose tissue concentration accounts for the CD95 response both in malignant cells and in innate immune cells. Hitherto, it remains to decipher whether the presence of TGF-β will elicit the expression of factors that hinders the transmission of the CD95 apoptotic signal and promotes the non-apoptotic signal and/or whether TGF-β will circumvent the apoptotic signal and promotes pro-oncogenic signaling pathways by directly acting on the CD95 expression level and/or the mechanisms of CD95L cleavage.

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