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Review

CD95 ligand - death factor and costimulatory molecule?

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Abstract

The CD95 ligand is involved as a death factor in the regulation of activation-induced cell death, establishment of immune privilege and tumor cell survival. In addition, CD95L may serve as a costimulatory molecule for T-cell activation. Alterations in expression or shedding of membrane and soluble CD95L are associated with numerous diseases, and underscore the pathophysiological relevance of the CD95/CD95L system. In most cases, the causal link between altered CD95L expression and pathophysiology is unknown. Given the potency of the molecule to regulate death and survival of many different cell types, the control of CD95L production, transport, storage, shedding and inactivation is of tremendous biological and clinical interest. This review summarizes the current knowledge, hypotheses and controversies about CD95L as a multifunctional ligand and receptor. It considers the different roles of membrane and soluble forms of CD95L and the complex networks of intracellular dynamics of protein trafficking, as well as the potential bidirectional signal transduction capacity of CD95L, with a focus on molecular interactions that have been worked out over the past years.
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Tissue and Cellular Expression of CD95L

The CD95 ligand (CD95L, Apo-1L, FasL, CD178) is a 281-amino-acid-containing type II transmembrane protein of the TNF family of death factors (Figure 1).[1] Its death-inducing function is best documented in the context of activation-induced cell death (AICD) in T cells.[2] CD95L is expressed as a death factor in cytotoxic T lymphocytes (CTL) to kill virally infected or transformed target cells and in natural killer (NK) cells, where it is upregulated by CD16 engagement and cytokines including IL-2 and IL-12.[3] Similarly, high levels of intracellular CD95L have been detected in monocytic cells with an inducible release upon activation.[4] Under physiological conditions, CD95L is implicated in the control of erythroid differentiation,[5] angiogenesis in the eye[6] and skin homeostasis.[7] Furthermore, CD95L expression can be induced in a variety of cell types by stress including heat shock, exposure to chemotherapeutic agents or radiation, growth factor withdrawal and viral infection.[8]

CD95L expression has been associated with the establishment of immune privilege and tumor survival, although this is the matter of a still unresolved controversy.[9–11] In fact, constitutive or inducible membrane expression of CD95L was shown for numerous cell and tissue types including the eye, mature spermatozoa, uterus and placenta, lung, thyrocytes, keratinocytes (upregulated by UV-B light), CD68-positive histiocytes in the dermis,[12] neutrophils, mature erythroblasts, breast epithelial and vascular endothelial cells, astrocytes (upregulated by IL-1, IL-6, TNF-α and IFN-γ), microglia (downregulated by IFN-γ) and intervertebral discs. The assumption, however, that CD95L confers immune privilege and protection on organ transplants has been challenged by studies indicating a differential pro- or anti-inflammatory effect of CD95L depending on the microenvironmental context. Also, CD95L alone is not sufficient to prevent allograft rejection and other immunosuppressive factors such as TGF-β seem to be involved.

Many of the classical transcription factors have been identified to regulate CD95L expression under different conditions (see Kavurma and Khachigian[13] for review). Briefly, NFAT, NF-κB, c-myc, IRF-1 and Egr-3 mediate CD95L induction upon T-cell activation, for example, in the context of AICD. Moreover, Egr factors also contribute to CD95L expression in double-negative thymocytes. In mature T cells, Egr factors display different binding activities to Egr sites in the CD95L promoter of Th1 and Th2 cells, thereby potentially discriminating the two populations with regard to their AICD sensitivities. Upon cellular stress, the AP-1 complex, and upon growth factor withdrawal, the forkhead transcription factor, upregulate CD95L message and protein expression.

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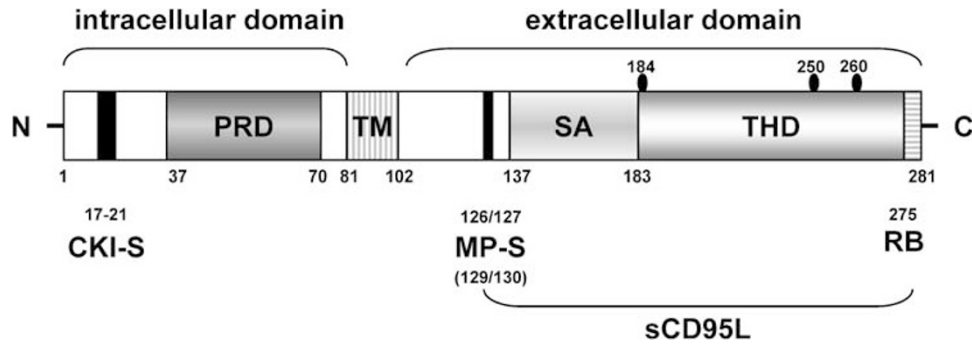
Figure 1 Structural features of a multifunctional death factor. Human CD95L is a type-II transmembrane molecule with 281 amino acids. The N-terminal cytoplasmic part (aa 1–81) contains a specific 'double' CKI substrate motif (CKI-S, aa 17–21). It also harbors an absolutely unique PRD (aa 37–70) which functions as a docking site for SH3 and WW domain proteins. The transmembrane region spans aa 81–102. Within the extracellular part, several metalloprotease substrate sites (MP-S) have been named to serve as cleavage sites for one or more metalloproteases (aa 109–115 (ELAELR), S126/127L and K129/130Q). These cleavage sites are located outside the region that is required for trimerization and self-assembly (SA). Thus, an sCD95L may also form trimers. The C-terminal THD (aa 183–281) contains several putative N-glycosylation sites (aa 184, 250, 260) and the very C-terminal receptor-binding site (RB) with a critical phenylalanin–residue at position 275.

Soluble or Membrane CD95L

The 'default' receptor for CD95L is CD95 (Fas, APO-1). Human CD95L-expressing cells require direct cell-to-cell contact to generate a death signal via the CD95 receptor (Figure 2). The CD95L/CD95-initiated death pathway has been studied and elucidated in great detail over the past decade (for review, see Nagata,[14] Krammer[15] and Peter and Krammer[16]). In addition, CD95L binding to an alternative decoy receptor 3 (DcR3, TNFRSF6B) was reported to inhibit CD95L function in several malignancies. Thus, the DcR3 gene was amplified in about half of 35 primary lung and colon tumours studied, and DcR3 message was detected in malignant tissue.[17] Further, DcR3 is expressed by the majority of long-term and *ex vivo* malignant glioma cell lines, as well as in human glioblastoma *in vivo*. Chemotaxis assays revealed that in this context, DcR3 counteracts the chemotactic activity of CD95L against microglial cells *in vitro*.[18] These findings suggest that by blocking CD95L function, DcR3 may be involved in the progression and immune evasion of malignant gliomas and other tumors.

Like other members of the TNF family, CD95L also comes in two flavors, a membrane-bound CD95L form (mCD95L) with an approximal molecular weight of 40 kDa and a soluble CD95L variant (sCD95L) of 26–29 kDa. For both membrane and soluble CD95L, a spontaneous trimer formation has been suggested, which seems indispensable for triggering the death signal via CD95 oligomers.[19] In CD95L-transfected COS cells, mono-, di- and trimeric forms were detected under nonreducing conditions. The ability of CD95L to self-associate was localized to a 47-amino-acid region in its extracellular domain (SA in Figure 1), and seems independent of transmembrane or cytoplasmic sequences. As previously shown for proteins such as the IL-6 receptor, theoretically, sCD95L can be generated by several independent mechanisms including differential splicing (ds), post-translational modification (pm) or proteolytical cleavage (pc). Indeed, Ayroldi *et al.*[20] have been the first to describe a differentially spliced short variant of ds-sCD95L in unactivated spleen cells and hybridoma T cells, which protected cells from CD95L-induced apoptosis. This splice variant lacks both the intracellular and the transmembrane parts of CD95L, and

consists of a fraction of the extracellular domains encoded by exons 1 and 4 of the CD95L gene. To our knowledge, a pm-sCD95L has not been reported.

The existence of the proteolytically cleaved soluble variant (pc-sCD95L) is widely accepted. It was initially identified as a biologically active molecule in supernatants of CD95L COS transfectants and stimulated T cells. Metalloproteinase (MP) inhibitors decreased the soluble and increased the membrane-bound fraction of CD95L.[21] The putative MP cleavage site has been mapped to Lys129-Gln130 in murine cells and Ser126-Leu127 in human cells[22] (Figure 1). These findings suggest that murine and human cells utilize different enzymes to produce sCD95L, or that one MP uses different cleavage sites.[23] Since the MP which specifically cleaves TNF-α had been identified as a member of the 'a-disintegrin-and-metalloproteinase' (ADAM) MP family, and since the TNF-homologous portion of membrane-bound CD95L is similarly processed and shed, a closely related disintegrin has been suggested. Meanwhile, several candidate MPs have been analyzed in different cellular systems. Whereas CD95L cleavage could be achieved in some cells (i.e. glandular epithelial cells) by matrix metalloproteinase 7 (MMP-7=matrilysin), the MP(s) responsible for CD95L cleavage in other cell types remain to be identified.[23] In a specialized population of murine epithelial cells, MMP-7 colocalizes with CD95L and cleaves off a biologically active (death-inducing) sCD95L.[24] It was also reported that MMP-7 may play a role in the promotion of tumor survival and resistance to cytotoxic drugs (i.e. doxorubicin) by cleaving CD95L and reducing its effectiveness in triggering Fas-mediated apoptosis. Moreover, MMP-7 has been described to be highly expressed in pancreatic cancer precursors, regulating acinar-to-ductal metaplasia.[25] Obviously, MMP-7 does not only cleave CD95L but also the receptor CD95. The sCD95 then antagonizes the effects of cytotoxic CD95L to result in resistance to CD95L-induced apoptosis in tumor cells.[26]

Taken together, it is clear that sCD95 is generated in different cell types by different means. However, the exact function of sCD95L as a death inducer or death inhibitor or even as a chemoattractant is still a matter of debate, and obviously depends on a given cellular microenvironment. Thus, it has been shown that human sCD95L is functional in

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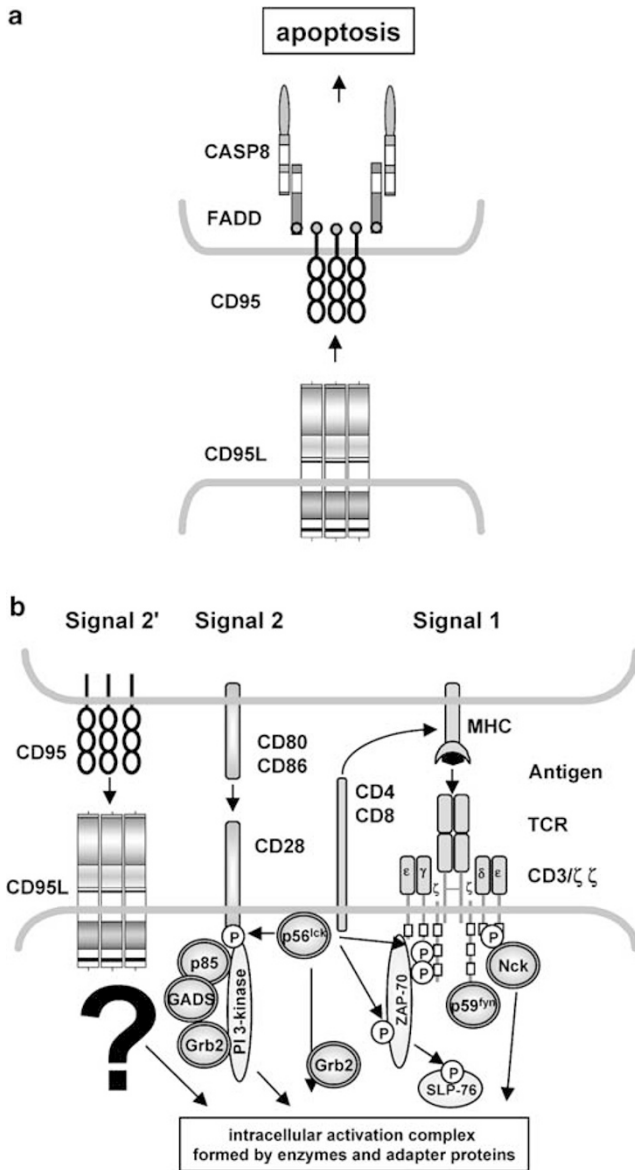
Figure 2 Dual function of CD95L. (**a**) CD95L trimers function as death factor to trigger CD95-induced apoptosis via FADD and caspase activation. This is the key event during activation-induced cell death. It plays a major role in target cell cytolysis, in the establishment of immune privilege and during tumor counter-attack. (**b**) Model for the costimulatory function of CD95L. By virtue of assembling with a number of cytosolic SH3 domain proteins required for TCR-dependent activation (double-circled), CD95L could alter the TCR/CD3 stimulus in different ways. In such a scenario, CD95L may serve as a costimulatory molecule to either directly interfere with signal 1 given by the TCR or by interfering with signal 2 given by classical costimulatory molecules such as CD28. In addition, CD95L could also create signals independent of other surface receptors.

inducing apoptosis in presensitized cells at high doses even across species. In contrast, sCD95L has been shown to downregulate the apoptotic and inflammatory activity of its transmembrane counterpart in a number of cellular systems, suggesting that mCD95L is the functional molecule, and that shedding may counteract the cytotoxic activity of the membrane form. In terms of pathophysiology, sCD95L was found in the sera of patients with large granular lymphocytic leukemia (LGL) and NK cell lymphoma.[27] Later reports pointed to a correlation of sCD95L serum levels with various other diseases, for example, the asymptomatic stage of HIV infection, rheumatoid arthritis and osteoarthritis, ulcerative colitis, Sjögren's Syndrome, acute respiratory distress syndrome (ARDS) and a large number of malignancies (Table 1). Consequently, sCD95L found in the serum of HIV-positive patients has been discussed to reflect the antiviral immune defense.[28] Furthermore, CD95L shedding from the membrane might serve as a mechanism to adjust the killing activity of a T or NK cell. Interestingly, as soon as sCD95L interacts with fibronectin, cytotoxicity is restored,[29] indicating rapid internalization of sCD95L after binding to its receptor. Therefore, it appears likely that the connection of sCD95L to cell-membrane or intercellular matrix proteins prevents internalization and enhances the probability to generate an effective death signal. Finally, it should be mentioned that for some cell types, for example, human neutrophils, sCD95L functions as a chemotactic factor.[30,31]

The counterattack model

As mentioned, various tumors have been suggested to constitutively express relatively high levels of mCD95L or secrete sCD95L. This could be regarded as a mechanism to ensure tumor survival by blocking immune defense.[10,32–34] For example, Hahne *et al.*[35] reported that melanoma cells escape immune elimination, although tumor-specific cytolytic T cells directed against well-defined antigens are present in the population of tumor-infiltrating lymphocytes (TIL), or can even be detected in the peripheral blood of melanoma patients. When melanoma cells were analyzed for the expression of CD95L, both mCD95L and sCD95L could be detected, whereas normal melanocytes did not express CD95L. Since melanoma TIL mostly showed an activated phenotype expressing CD95, it was proposed that transformed melanocytes induce the apoptosis of CD95-sensitive TIL. As a proof of principle, CD95L-positive mouse melanoma cells led to rapid tumor formation when injected into normal mice, but to delayed tumor formation in CD95-deficient animals. In this regard, tumors might locally generate an immune-privileged microenvironment by attacking infiltrating T lymphocytes. Therfore, it was speculated that breaking this privilege or rendering TIL insensitive to CD95L-induced killing may provide a complementary approach in the therapy of malignant melanoma.

CD95L and Disease – Other Pathophysiological Aspects

The proof for a critical role of the CD95L molecule (and its receptor CD95) in the pathophysiology of autoimmune diseases was the analysis of generalized lymphoproliferative disease (*gld*) mice, which was shown to carry a spontaneous loss-of-function point mutation in the CD95L locus.[36] This mutation leads to the production of a nonfunctional protein and development of lymphadenopathy and splenomegaly with high titers of autoantibodies and defects in thymocyte maturation with a massive accumulation of CD4- and CD8-

double-negative T cells. However, no corresponding CD95L mutation has been found to be associated with autoimmune disease in humans. Since only one of 75 patients with systemic lupus erythematosus (now classified as autoimmune lymphoproliferative syndrome (ALPS) 1B) was found to carry an 84-bp deletion within exon 4 of the CD95L gene resulting in a 28-amino acid in-frame deletion, it was considered that CD95L mutations are an uncommon cause of the disease.[37] Interestingly, the study of peripheral blood mononuclear cells from this patient revealed decreased CD95L activity, decreased activation-induced cell death, and increased T-cell proliferation after activation. An involvement of a disturbed balance of CD95/CD95L interactions has also been postulated during organ-specific autoimmune diseases, such as Hashimoto's thyroiditis, insulin-dependent diabetes mellitus and multiple sclerosis. Furthermore, elevated levels of CD95L are thought to lead to the loss of virus-specific CTL observed during HIV infection, or to reduction of the antitumor activity of TIL in many tumors (see also Table 1).

Outside the immune system, CD95L has also been implicated, for example, in the pathology of toxic epidermal necrolysis (TEN) by mediating keratinocyte apoptosis.[38] In the sera of patients suffering from TEN, high levels of soluble CD95L were detected, and CD95L produced by keratinocytes of TEN patients induced keratinocyte cell death. Incubating keratinocytes with intravenous immunoglobulin (IVIG) containing a naturally occurring anti-CD95 immunoglobulin completely inhibited CD95-mediated keratinocyte apoptosis. In TEN patients treated with IVIG, progression of skin disease was rapidly reversed.[38]

The CD95L/CD95 system also contributes to the control of infection. Grassme *et al.*[39] showed that *Pseudomonas aeruginosa* infection induces the apoptosis of lung epithelial cells by activation of the endogenous CD95/CD95L system only in CD95/CD95L wild type but not in deficient mice. Thus, inflammation and sepsis after *P. aeruginosa* infection was seen in mutant but not in normal mice – another example that controlled cell death and elimination by apoptosis is required to prevent inflammatory complications. One example for an obvious strategy of viruses to evade an immune response was reported by Raftery *et al.*[40] They observed that besides the downregulation of a number of accessory and costimulatory molecules (MHC I, CD40, CD86) on infected dendritic cells, some CMV strains further compromise the immune system by inducing the expression of death molecules such as TRAIL and CD95L. This upregulation of apoptosis-inducing ligands will not only suppress the T-cell response, but also delete activated T cells. However, although elevated (>50 pg/ml) levels of sCD95L have been observed also under various other pathological conditions in human sera, the clinical significance is still unclear.[14]

CD95L in T Lymphocytes

During T-cell development and maturation, CD95L was supposed to be involved in negative selection. In this context, CD95L is propagated by some investigators as an effector molecule leading to the deletion of autoreactive thymocytes.[41–43] However, the role of CD95L is discussed controversially also in this context, and from studies in transgenic mice the clear statement was that the CD95/CD95L system is involved in peripheral but not thymic deletion of T lymphocytes.[2,44] In mature T cells, CD95L is differentially expressed in individual T-cell subsets. On CD4$^+$ cells, CD95L appears as a membrane-bound receptor on T helper 1 (Th1) and only on some T helper 2 (Th2) cells following T-cell receptor (TCR) stimulation by antigen, anti-TCR/CD3 antibodies, mitogenic lectins, bacterial superantigens or other stimuli such as phorbol esters and calcium ionophore treatment. In such cells, CD95L is a major effector molecule in the course of AICD, thereby regulating the termination of T-cell responses, peripheral deletion and preventing autoimmunity. For Th2 cells, a reduced expression or absence of CD95L under similar conditions has been reported, which explains the resistance of such clones to AICD.[45] However, further mechanisms have been suggested to render these cells unresponsive to CD95-mediated cell death. Some reports indicate a role for FAP1, the Fas-associated phosphatase 1 and others a role of FLIP$_{short}$ or Bcl$_{xl}$ in the generation of Th2 resistance.[46] In cytotoxic T cells (and NK cells), CD95L is synthetized upon primary stimulation and then directed to and stored in secretory granules/lysosomes.[47] Besides CD95L, this type of lysosomes also contain perforins and granzymes. Upon contact, for example, with a virally infected target cell, the TCR-derived signal will lead to a redistribution of cytoskeletal elements, resulting in extrusion of the cytotoxic molecules into the immunological synapse,[48] accompanied by surface expression of mCD95L and binding to CD95 on the target cell or shedding of sCD95L.

CD95L as a Costimulator for T Cells

A series of reports employing mice defective for CD95L (*gld*) or CD95 (*lpr; lpr^{cg}*) suggest a costimulatory function for CD95L in both CD8$^+$ and CD4$^+$ T cells.[49–52] While CD4$^+$ cells showed a reduction in TCR/CD3-induced cell cycle progression and entered apoptosis, CD8$^+$ cells required the CD95L costimulation to rapidly progress through the cell cycle, resulting in proliferation and acquisition of cytolytic effector function. Although the molecular basis for the reverse costimulatory signalling of CD95L remains unknown, two motifs present in the cytosolic region of CD95L are likely to be involved in the regulation of receptor crosstalk and the reverse signalling: a casein kinase I (CKI) substrate motif and a proline-rich domain (PRD) (Figure 1).

CD95L and Other Bidirectional Signal Transducers with CKI Substrate Motifs

Most ligands of the tumor necrosis factor (TNF) superfamily are transmembrane proteins that share structurally related extracellular TNF homology domains (THD), which bind to cystein-rich domains (CRD) of their specific receptors (of the TNF-receptor superfamily). In general, ligand/receptor interactions of members of these families regulate key events in cellular activation, proliferation, differentiation, cell death and survival of immune cells and other tissues.[53] Since most of the TNF family members act as membrane-bound factors and

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require direct cell-to-cell contact, it seems obvious that signalling upon ligation triggers cellular responses in both directions. Substantial *in vivo* evidence for the physiological role of reverse signalling has been shown for CD40L, but bidirectional (i.e. costimulatory) functions have also been ascribed to several other members of the TNF superfamily in T cells, B cells and monocytes (for other individual references, see Locksley *et al.*[53] and Wenzel *et al.*[54]). Thus, the ligand/receptor complexes of OX40L/CD134 (TNFSF4/TNFRSF4), CD154/CD40 (TNFSF5/TNFRSF5), CD70/CD27 (TNFSF7/TNFRSF7), CD153/CD30 (TNFSF8/TNFRSF8), 4-1BB-L/CD137 (TNFSF9/TNFRSF9), TRANCE/RANK (TNFSF11/TNFRSF11), LIGHT/LIGHTR (TNFSF14/TNFRSF14)[55–57] and the death factors TNF, TRAIL (TNFSF10)[58] and CD95L (TNFSF6) were reported to convey retrograde signals. Of interest, so far the bidirectional signalling capacity has been mostly associated with those six of 16 TNF family members (CD27L, CD30L, CD40L, CD137L, TNFα and CD95L) that contain a putative casein kinase I (CKI) substrate motif (-SXXS-). Since the corresponding phosphorylation sites are not present in any of the remaining TNF family members, it seems likely that reverse signalling depends on this domain.[59] However, so far, CKI-dependent serine phosphorylation upon receptor activation has only been shown for TNF-α but none of the other ligands. In the case of CD95L, the membrane distal N-terminus in fact contains a unique 'double' CKI substrate site (amino acids 17–21 in man (-SSASS-) and 17–22 in mice (-SSATSS-)). The presence of such a motif (-S(P)XXS-) indicates a role for an additional kinase to regulate the sensitivity of this site itself for CKI by phosphorylation.[59] Nevertheless, no experimental data are available to prove the involvement of any kinase or phosphatase in the regulation of CD95L reverse signal transduction.

PRD as a Protein-Docking Site of CD95L

The second prominent signalling motif within the cytosolic region of CD95L is a PRD spanning amino acids 37–70 of the human CD95L (Figure 1). This PRD is unique among the members of the TNF family, and certainly plays a major role in the biology of CD95L. When CD95L was cloned and sequenced, the PRD was thought to function as a docking site for proteins that regulate signalling, trafficking and expression of CD95L. After 1 year, it was found that peptides corresponding to the proline-rich region of the murine CD95L (aa 40–77) were able to selectively interact with the SH3 domains of the Src-related kinase p59[fyn(T)].[60] From this it was suggested that CD95L membrane expression is primarily regulated through an activation-independent SH3-mediated association with p59[fyn(T)]. In 1996, Hachiya and co-workers deposited several protein fragments in the NCBI database, which were found to interact with CD95L in a yeast two-hybrid screen. Although these 'Fas-ligand associated factors' (FLAF1-3-AAB93495, AAB93496, AAB93497) contained either a WW domain (characterized by two tryptophane (W) residues spaced in a certain distance) or one or more SH3 (Src homology 3) domains as the PRD-interacting region, and were supposed to regulate CD95L stability, no further results were published. Sequence comparison reveals that FLAF-1

forms part of the formin-binding protein 11 (FBP11, also called the huntingtin-interacting protein HYPA), FLAF2 is part of the c-Cbl-associated protein SH3P12='sorbin and SH3 domain containing 1' (containing 3 SH3 domains), and FLAF3 represents a portion of the BAI1-associated protein 2 (BAP2)-beta (with a single SH3 domain).

Association of Proteins with Proline Recognition Domains

In order to identify proteins that interact with the PRD of CD95L cytoplasmic tail, we used two different protein-based approaches. First, we screened a large panel of SH3 and WW domains expressed as GST fusion proteins for their ability to precipitate CD95L from stable transfectants and T lymphoblasts.[54] Second, we constructed fusion proteins that contain the cytosolic part of CD95L with or without proline stretch, and used these for pull-down assays from T-cell lysates.[61] Our analyses revealed that in addition to Fyn SH3 domains, a number of other SH3 or WW modules are also capable of effectively and specifically interacting with the full-length CD95L overexpressed in transfectants and inducibly expressed in stimulated human T lymphocytes. The identification of various other Src-related kinases and different adapter proteins of the growth factor receptor-binding protein 2 (Grb2) family, phosphoinositol 3 kinase (PI 3-kinase), Nck and Phox-47 and the WW domain proteins FE65, FBP11 and dystrophin as putative CD95L-interacting molecules, opens new insights into the potential of CD95L to regulate and modulate activation processes in T cells and other CD95L-expressing cells. It should be stressed, however, that not all SH3 or WW domains bind to CD95L, and if there are more than one SH3 domains in a given protein, binding is not seen for all domains. Moreover, many SH3 domains that we tested in our screening assay did not show any binding to CD95L, and not all SH3 domain-containing proteins that we found to interact with CD95L *in vitro* are present, for example, in T cells indicating a cell-type-specific set of interactors and interactions regulating CD95L biology in a given cell type.

Speculations about Reverse Signalling in T Cells

For T cells, the role of Src-related kinases (i.e. Lck, Fyn and Yes), PI 3-kinase, adapter proteins including Grb2, Gads and Nck-1 in activation has been extensively investigated over the past two decades. Most of these molecules mediate critical steps in the assembly of activation complexes associated with antigen receptors, adhesion molecules and costimulatory downstream signalling mediators. Since we found Grb2 in precipitates with CD95L fusion proteins, this interaction may serve as a model scenario for CD95L-to-TCR crosstalk (Figure 2b). Grb2 is a well-defined adapter protein consisting of two SH3 domains flanking a single SH2 domain that plays a pivotal role in signalling of the insulin receptor, growth factor receptors, the TCR and others. TCR ligation rapidly results in recruitment of the Grb2-Sos complex to the phosphorylated transmembrane adapter LAT (linker for activated T cells), thereby triggering the Ras/MAPK pathway, leading to

transcription of a number of proteins required for T-cell activation and differentiation.[62] An interaction of Grb2 and CD95L could therefore point to a competitive or synergistic crosstalk between CD95L and the TCR, and explain the increased proliferation described for CD8^{+} cells *in vivo*.[50,52] Since the Grb2-Sos complex is well known to recruit the small G-protein Ras, and since spatial proximity between Ras and the active p110 subunit is crucial for PI-3K activation, Grb2 might not only directly provide a stimulus for proliferation (as has been shown for the EGF-R-pathway), but also faciliate PI-3K activation. The PI-3K product PIP_3 was shown to activate both the PIP_3-dependent kinase 1 (PDK-1) and protein kinase B (PKB)/AKT, resulting in antiapoptotic signals in skin tumor cells via phosphorylation of Bad or the transcription factor FOXO3a. Taken together, Grb2 and the p85 adapter subunit of PI-3K, both binding to the PRD via their SH3 domains, might generate both a proliferative (via Ras and MAP-kinase) and antiapoptotic (via PKB) signal at the same time, regardless of whether the CD95L extracellular domain is cleaved from the surface or not.

Our own and other laboratories' experiments to prove a direct involvement of CD95L in the regulation of TCR/CD3 responses as a costimulatory molecule turned out to become puzzling. We failed to detect changes in tyrosine phosphorylation, Erk activation and AKT activation as a direct action of CD95L ligation. We also failed to detect CD95L-dependent changes in TCR/CD3 or phorbolester/ionophore stimulation of activated T cells. However, the antibodies and the CD95Fc fusion protein used in our studies are functional, for example, in blocking AICD in the very same clones and lines. In addition, we reproducibly observe an inhibitory effect of CD95L crosslinking on the proliferative capacity of freshly isolated peripheral blood T cells upon stimulation with anti-CD3mAb or superantigen. Nonetheless, the exact experimental conditions to biochemically identify the pathways of CD95L reverse signalling still need to be worked out. Such analyses are further hampered by the fact that the regulation of CD95L expression is far more complex and dynamic than expected, and that CD8^{+} and CD4^{+} or Th1 and Th2 cells differ in their expression patterns. Therefore, upcoming experiments will focus on the use of stably transfected CD95L T-cell lines established in our laboratory, on the mechanisms of inhibition of primary T-cell stimulation and on the role of oligomerization induced by crosslinking of CD95L.

Nck-linking CD95L, the Cytoskeleton and the TCR/CD3 Complex

We recently also described the interaction of two of three SH3 domains of Nck-1 with the PRD of CD95L.[54] These data were now confirmed by cotransfection, co-precipitation and colocalization studies with wild type and mutants of Nck-1 in different cell lines (Qian *et al.*, manuscript in preparation). Nck-1 is a classical adapter protein of 377 amino acids, which is built of one c-terminal SH2 and three SH3 domains. It was shown to constitutively associate with the active $\gamma 2$ isoform of the serine/threonine casein kinase I (CKI-$\gamma 2$),[63] and could therefore be involved in sequestering the kinase to the substrate CD95L. As mentioned, Watts *et al.*[59] described

the CKI-dependent phosphorylation of a corresponding motif in the TNF-R. Similarly, serine phosphorylation by a casein kinase recruited via Nck-1 could be responsible for reverse signalling through CD95L. Nck-1 is also implicated in regulation of the cytoskeleton through interaction with the Wiskott–Aldrich syndrom–protein (WASP), the WASP interacting protein (WIP), WASP-family verprolin homologous protein (WAVE1), the Centaurin-α family of PIP3-binding proteins, the p21-activated kinase (PAK1), the ε-chain of CD3 and others. The cytoskeletal association of Nck-1 may also play a role in CD95L transport to the cell membrane (Figure 3, see below).

PACSIN and FBP17 as Interactors for CD95L Models and Hypotheses

We used variants of the intracellular region of human CD95L with or without PRD, expressed as GST fusion proteins to precipitate binding partners from T-cell lysates. The precipitates were separated by two-dimensional gel electrophoresis and sequence information obtained by MALDI-TOF-based peptide finger-print analyses. As already mentioned, we verified the Grb2 association reported earlier. Besides, with PACSIN2 and FBP17, we identified two structurally related proteins that are likely to be involved in protein trafficking and cytoskeletal organization, and may also be responsible for the presence of β-tubulin and actin in the same precipitates.[61]

PACSIN2 is a 53 kDa human splice variant of the rat Syndapin II, and a member of a family of cytosolic adapter proteins that link clathrin-dependent endocytosis to the actin cytoskeleton, and are involved in the regulation of vesicular traffic.[61] To date, three PACSINs are known, all of which contain phosphorylation sites for casein kinase II and protein kinase C (the name PACSIN refers to 'protein kinase C and casein kinase substrate in neurons'). In contrast to the ubiquitously expressed PACSIN2, PACSIN1 was not detected in cells other than neurons. In terms of function, it was recently reported that PACSIN isoforms specifically interact with Sos, thereby regulating actin dynamics via MAP kinase signalling. In the context of CD95L, PACSIN2 could be involved in the reorganization of the cytoskeleton and in trafficking of cytoplasmic vesicles, leading to storage and regulation of extrusion of CD95L or to endocytosis, recycling or degradation. The potential association of PACSINs with the GEF Sos could also suggest a role of this interaction in downstream signalling to MAP kinases. Most interestingly, the activity of the sheddase ADAM13 that contains three proline-rich repeats in its long cytoplasmic tail was shown to be efficiently inhibited by the SH3 domain of PACSIN2 *in vivo*. However, due to the presence of only one SH3 domain per PACSIN2 molecule, CD95L and ADAM13 should either compete for this docking site, or are connected via multimerization of PACSIN2, probably involving the coiled-coil domain.

Formin-binding protein 17 (FBP17), a 72 kDa protein, belongs to the heterogeneous family of formin-binding proteins, which, in association with the various formins, also regulate the organization and assembly of the actin cytoskeleton, and are involved in orchestrating cell motility, adhesion

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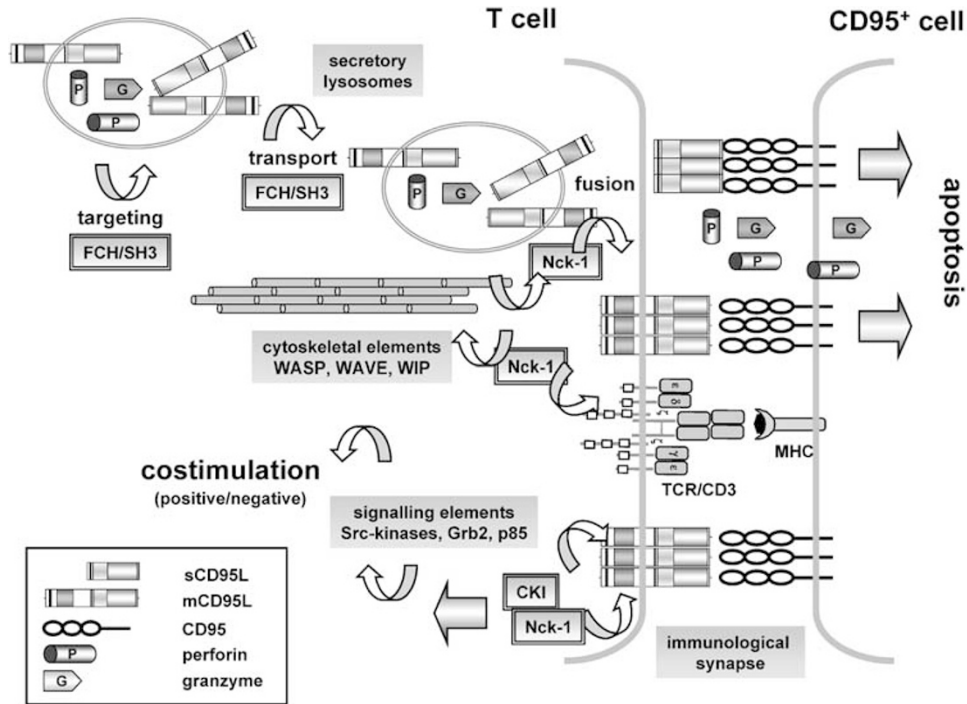
Figure 3 CD95L interacting proteins – facts and hypotheses. Recent work by Griffiths *et al.*[47,48,71] showed that in cytotoxic cells, CD95L is targeted to secretory granules/lysosomes. Besides CD95L, these lysosomes contain other cytotoxic molecules such as granzymes and perforins, which in concert contribute to an effective target cell lysis. Targeting of CD95 to these lysosomes strictly depends on the PRD within the cytoplasmic tail of the molecule. In this context, members of the FCH/SH3 family (including FBP17, PACSINs, CD2BP1 and others) are good candidates for directing the localization and transport of CD95L (and/or secretory lysosomes in general). All proteins of this family are involved in actin cytoskeleton reorganization and protein/organelle trafficking. Other interactors for CD95L are the small adapter proteins Grb2 and Nck-1. Therefore, a working model for CD95L function would be that the PRD is not only required for lysosomal targeting, but also for transport and fusion of these lysosomes to the area where the immunological synapse is formed. This could be followed by the release of the various cytolytic factors and in the presence of a MMP (such as matrilysin, MMP-7) by CD95L cleavage. Whether the sCD95L induces or inhibits apoptosis then depends on the potential to form active multimers. The fact that CD95L has been found to act as a 'negative' or 'positive' costimulatory molecule for T cells underscores the complex biology of the molecule. Whether the same (FCH/SH3-family, Grb2 or Nck-1) or other (e.g. CKI, PI 3-kinases or Src kinases) CD95L interacting molecules are involved in this aspect, remains open.

and cytokinesis.[64] Interestingly, PACSINs and FBP17 are very similar with regard to their overall modular composition. Both contain a C-terminal SH3 domain (that interacts with CD95L) and an N-terminal FCH (Fes/CIP homology) domain that shares homology, for example, to regulators of the actin cytoskeleton in yeast. Fuchs *et al.*[65] showed that FBP17 is widely expressed in a variety of human tissues. They demonstrated the interaction of FBP17 with sorting nexin (SNX2), and thus provided a link between MLL, a gene associated with myelogenous leukemia, and the epidermal growth factor receptor (EGFR) pathway. In general, SNX-proteins are molecules crucially involved in directed protein trafficking and lysosomal targeting.[66] Therefore, association of CD95L with FBP17 could represent an alternative mode of lysosomal transport of CD95L, and also point to a signalling crosstalk between the death factor and the EGFR pathway. As mentioned, we also found actin and β-tubulin in pulldown precipitates. Although we did not yet formally prove the interactions, this could provide more evidence for the association of CD95L with lysosomes and/or cytoskeletal elements, since β-tubulin is a major component of microtubules, arranging the mitotic spindle during cell division and

regulating axonal transport and organelle positioning. In addition, it was reported that tubulin plays an important role in G-protein-mediated signal transduction. Obviously, even though tubulins are structural proteins, they participate in cellular signalling albeit through physical forces. In this scenario, it is discussed whether tubulins modulate the conformation of receptors or G -proteins, either leading to a change in receptor–ligand interaction and/or influencing the G-protein-mediated signal.

PACSIN2 and FBP17 belong to a larger family of structurally related proteins, which, as mentioned, are in most cases associated with lysosomal transport processes, cytoskeletal organization and signal transduction. At least nine members of this family are known in humans. They all share an overall modular composition with an N-terminal FCH (Fes/CIP4 homology) domain, a central coiled-coil region and a C-terminal SH3 domain. Interestingly, SH3 domains of all other members of this protein family (PACSIN1 and PACSIN3, CD2-binding protein 1 (CD2BP1=PSTPIP), RhoGAP C1 and Rho GAP C1-related protein, FLJ00007 protein and CDC42-interacting protein CIP4), bind to CD95L *in vitro* (Qian *et al.*, unpublished; Baum and Zörnig, unpublished).

Targeting of CD95L to secretory lysosomes

The phenomena associated with CD95L, namely surface expression on CD4$^+$ T cells and lysosomal expression and transport associated with cytotoxic (CD4$^+$ or CD8$^+$) T cells and NK cells, may be regulated differentially, and thus require distinct sets of CD95L-binding proteins. In both cases, the association with regulatory elements seems to depend on the PRD of the cytosolic portion of the molecule.[67] It has been suggested that sorting of CD95L from the Golgi network to secretory lysosomes may provide a general mechanism for controlling the cell surface appearance of proteins involved in immune regulation. The association of membrane-bound or

Table 1 Examples for disease-association of sCD95L expression

Disease	sCD95L in patient's sera	Reference
(a) Cancers		
Colon adenocarcinoma	Significantly increased (mCD95L also increased)	Song E *et al*. (2001) Br J Cancer 85: 1047
LGL leukemia	Significantly increased (mCD95L also increased)	Tanaka M *et al*. (1995) EMBO J. 14: 1129
NK cell lymphoma	Significantly increased (mCD95L also increased)	Tanaka M *et al*. (1995) EMBO J. 14: 1129
Melanoma	Unchanged	Melzani G *et al*. (2002) Dermatology 205: 111
Multiple myeloma	Unchanged	Kanda Y *et al*. (2001) Leuk. Lymphoma 40: 351
Bladder cancer	Significantly increased	Mizutani Y *et al*. (2001) Cancer 92: 287
(b) Immunologic diseases		
Graves' hyperthyroidism	Significantly increased	Wang CY *et al*. (2002) Metabolism 51: 769
Multiple sclerosis	Significantly decreased	Macchi B *et al*. (2001) Neuroreport 12: 4113
Graft-versus-host-disease	Significantly increased	Kanda Y *et al*. (1998) Bone Marrow Transplant. 22: 751
Sjögren's syndrome	Significantly increased	Ishimaru N *et al*. (2001) J. Immunol. 167: 6031
Churg–Strauss vasculitis	Significantly increased (mCD95L decreased)	Muschen M *et al*. (1999) Am. J. Pathol. 155: 915
(c) Infectious diseases		
Viral liver cirrhosis	Significantly increased	Wang PS *et al*. (2002) Lung 180: 25
Pulmonary sarcoidosis	Significantly increased	Shikuwa C *et al*. (2002) Respiration 69: 242
Human immunodeficiency virus infection	Significantly increased in asymptomatic stage	Bahr GM *et al*. (1997) Blood 90: 896
Helicobacter-pylori-positive gastric ulcer	Significantly increased	Shimada M *et al*. (2002) Scand. J. Gastroenterol. 37: 501
Acute pancreatitis	Significantly decreased	Endo S *et al*. (2000) Res. Commun. Mol. Pathol. Pharmacol. 108: 179
(d) Other diseases		
Atherosclerosis in hypertensive patients	Significantly increased	Okura T *et al*. (2002) J. Hypertens. 20: 895
Acute myocardial infarction	Significantly increased	Shimizu M *et al*. (2002) J. Am. Coll. Cardiol. 39: 585
Acute respiratory distress Syndrome (ARDS)	Significantly increased	Matute-Bello G *et al*. (1999) J. Immunol. 163: 2217
Fibrosing lung disease	Significantly increased	Kuwano K *et al*. (2002) Respirology 7: 15
Severe traumatic brain injury	Prolonged intrathecal release	Lenzlinger PM *et al*. (2002) J. Neuroimmunol. 122: 167
Rheumatoid arthritis/ osteoarthritis	Increased in synovial fluid	Hashimoto H *et al*. (1998) Arthritis Rheum. 41: 657
Histiocytic necrotizing lymphadenitis	Significantly increased	Kato K *et al*. (2001) Int. J. Hematol. 73: 84
Alzheimer's disease	Not investigated	Tourneur L *et al*. (2001) J. Immunol. 167: 1338
Severe acute alcoholic hepatitis and alcoholic cirrhosis	Significantly increased	Taieb J *et al*. (1998) Lancet 351: 1930
Hepatic failure	Significantly decreased	Nakae H *et al*. (2001) J. Crit. Care 16: 59

intracellular CD95L to proline-interacting adapter proteins or enzymes may therefore not only explain the crosstalk between the death factor and other surface receptors. Of note, also in this context, the CD95L-associated SH3 adapters of the Grb2 family and especially Nck-1 have crucial roles for the formation of the immunological synapse upon TCR ligation, and the link to cytoskeletal reorganization.[68]

Secretory lysosomes and the 'kiss of death'

The specialized lysosomal compartment where CD95L is sorted to contains granzymes A, B, H, K and M, pore-forming perforin monomers and, as a transmembrane component, CD95L[69] (Figure 3). Upon interaction with a target cell, the lipid bilayer of the secretory lysosomes fuses with the plasma membrane, thereby releasing the soluble lysosome components and presenting mCD95L on the cell surface. This trigger of target cell apoptosis has been referred to as the 'kiss of death'.[70] Unlike other proteins that are sorted to the lysosomal compartment by di-leucine or tyrosine-based sorting motifs, Griffiths et al.[67] convincingly showed that CD95L is targeted to secretory lysosomes via the PRD. This study proposed a new model for sorting pathways in cells with secretory lysosomes compared to conventional lysosomes. Upon transfection, the presence of the PRD was sufficient to sort CD95L to secretory lysosomes, whereas deletion of the PRD, but not of tyrosine or di-leucine motifs in the tail of CD95L (7Y-9Y-13Y and 29V-30L), resulted in surface expression of the protein.[67] As detailed earlier, various tumors have been suggested to constitutively express high levels of CD95L on their cell membrane, as a mechanism to effectively kill TILs by a mechanism termed the 'Fas counterattack'.[10,32,34] One example that also highlights the targeting of CD95L to specific subcellular lysosomal compartments is the identification of CD95L-loaded melanosomes.[33] In these structures, CD95L colocalizes with both melanosomal and lysosomal antigens. Isolated melanosomes express CD95L and exert CD95-mediated apoptosis in Jurkat cells. The mechanisms leading to expression and storage or secretion of CD95L in such tumor cells have not yet been investigated. It will be interesting to find out whether alterations or mutations occuring in those malignancies might be related to mutations in the PRD of CD95L, or any of the transport proteins involved. But, although a number of possible interaction partners of CD95L have been identified, the exact regulatory molecules guiding CD95L either to the secretory lysosomes or the plasma membrane remain unknown.

Conclusions

Although the CD95L has been identified 10 years back, we are still far from understanding the complex biology of the molecule. CD95L is found in both immunologically active and immune-privileged tissues and in immune cells as well as in tumor cells. The regulation of expression and the function seem to be cell-type-specific and microenvironment-dependent. This concerns the cytotoxic function and the potential costimulatory signalling capacity. To monitor a reverse

signalling in vitro proved to be a frustrating experience. However, as has been demonstrated for TNF, the upcoming analyses of the CKI substrate motif may shed light on this function that has been clearly demonstrated in vivo. In addition, the role of the soluble form is unclear, and it is not known under which (pathological) conditions it is produced. In terms of CD95L transport and storage, the identification of the PRD interacting proteins will help to position the molecule in the complex cytoskeleton and transport network.

Acknowledgements

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