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CD95L concatemers highlight different stoichiometries of CD95-mediated apoptotic and non-apoptotic pathways.

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Running Title: novel soluble CD95L: structure and function

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Abstract

To better understand the stoichiometry of CD95L required to trigger apoptotic and non-apoptotic signals, we generated several CD95L concatemers from dimer to hexamer conjugated *via* a flexible link (GGGGS)₂. These ligands reveal that although the hexameric structure is the best stoichiometry to trigger cell death, a dimer is sufficient to induce the apoptotic response in CD95-sensitive Jurkat cells. Interestingly, only trimeric and hexameric forms can implement a potent Ca²⁺ response, suggesting that while CD95 aggregation controls the implementation of the apoptotic signal, both aggregation and conformation are required to implement the Ca²⁺ pathway.

Introduction.

CD95L (FasL/CD178), a member of the tumor necrosis factor (TNF) family, contributes to immune homeostasis and anti-tumor and anti-infectious responses [1]. CD95L is a type II transmembrane cytokine mainly expressed by activated T cells and NK cells to kill infected and transformed CD95-expressing cells. CD95L contains an extracellular stalk region (amino acid residues 103 to 143) cleaved by matrix metalloproteinases (MMP) and a disintegrin and metalloproteases (ADAMs) and a C-terminal TNF homology domain (THD) (amino acid residues 144 to 281) [2]. After cleavage by metalloproteases, the soluble extracellular region of CD95L (sCD95L) exhibits a homo-trimeric stoichiometry [3, 4]. On the other hand, the transmembrane CD95L (mCD95L) can be capped at the plasma membrane of activated T cells and NK cells to bind and aggregate its receptor, CD95 (also known as Fas). Binding of mCD95L to CD95 promotes the recruitment of the adaptor protein FADD (Fas Associated Death Domain) through homotypic interactions between their respective death domains (DDs) [5]. FADD in turn aggregates the initiator caspase-8 to form a CD95/FADD/caspase-8 complex, called death-inducing signaling complex (DISC), that can initiate the apoptotic program [6, 7]. sCD95L fails to trigger cell death but its binding to CD95 favors the recruitment of PLC γ 1 at the plasma membrane [4, 8], where the lipase cleaves phosphatidylinositol-4,5-bisphosphate into diacylglycerol and inositol triphosphate (IP3). IP3 in turn activates IP3

receptors (IP3Rs) on endoplasmic reticulum (ER) to release Ca^{2+} . This reduction of Ca^{2+} in ER lumen is sensed by STIM1 and STIM2, which traffic to the plasma membrane to activate Orai channels, allowing Ca^{2+} influx from the extracellular space [9], a molecular mechanism designated store-operated calcium entry (SOCE).

A potential explanation for the failure of sCD95L to induce cell death came from its stoichiometry, since the metalloprotease-cleaved ligand exhibits an homo-trimeric structure [4, 10, 11] and the minimal stoichiometry required to induce apoptosis has been suggested to be an hexamer [10]. Indeed, Holler and colleagues designed two chimeric CD95Ls in which the extracellular region of CD95L (*i.e.*, amino acid residues 139 to 281) devoid of its stalk region was fused to the collagen domain of muACRP30 or the human immunoglobulin G (IgG) region [10]. These recombinant CD95Ls indicated that the minimal stoichiometry required to trigger the CD95-mediated apoptotic signal corresponded to an hexamer [10].

The length of the metalloprotease-cleaved CD95L and thereby, probably its conformation seems also to affect the magnitude of the CD95-mediated apoptotic signal [12]. For instance, oxidants generated by neutrophil myeloperoxidase (MPO) from acute respiratory distress syndrome (ARDS) patients replace methionine in CD95L (*i.e.*, 8 methionine present in human CD95L extracellular region) by methionine sulfoxide in the THD and, increase in the aggregation level of sCD95L, which enhances its ability to kill small airway epithelial cells [13]. Oxidation of the unique methionine present in the CD95L stalk region at position 121 does not modify the magnitude of self-aggregation but it prevents the cleavage of mCD95L between $^{126}\text{SL}^{127}$ by MMP7 and promotes its processing closer to its plasma membrane [13]. The released soluble CD95L is longer, and it shows a stronger ability to kill epithelial cells as compared to the MMP7-cleaved ligand [13]. Overall, these results suggest that not only the stoichiometry of CD95L but also the conservation of its stalk region might account for the induction of robust apoptotic and non-apoptotic responses [14-17].

Herein, to decipher how the CD95L stoichiometry modulates the magnitude of apoptotic and non-apoptotic signaling pathways, we designed a set of CD95L concatemers and assessed their biological effects.

Results and discussion.

Generation of CD95L concatemers

Modeling and structural studies point out that soluble CD95L is a compact, non-covalently-linked homotrimer [18] whose binding to its receptor seems to occur by the insertion of the ligand into three individual CD95 receptors to initiate cell signaling pathways. Soluble CD95L exhibits a “jelly-roll” fold of β sheets [19], and its crystal structure was not complete since the stalk region encompassing amino acid residues 103 to 143 was missing. Because this stalk region has been suggested to modulate the aggregation level and/or conformation of the soluble CD95L (sCD95L)[12, 20], we reconstituted this region by performing molecular dynamics simulations with the CD95L homo-trimer (Fig.1A and 1B). The final state of the reconstituted extracellular CD95L trimeric model highlighted that the N- and C-terminal regions of sCD95L were at proximity and thereby, we wondered whether this feature could be used to generate concatemers by covalently linking CD95L monomers with a flexible linker (*i.e.*, GGGGS; Fig.1C).

Because molecular modeling of the CD95L homotrimer predicted that the length and flexibility of a GGGGS dimer (*i.e.*, (GGGGS)₂) would be sufficient to connect CD95L monomers without affecting their global conformation (Fig.1C), we generated concatemers using this linker (Fig.1D). All constructs were transfected in the mammalian cell line HEK/293T and produced for in a serum-low medium (*i.e.*, OPTI-MEM). Next, exosomes were eliminated by ultracentrifugation and the secreted ligands were analyzed in reducing and denaturing conditions by SDS-PAGE (Fig.1E) and in native condition using BN-PAGE (Fig.1F). Denaturing and reducing conditions (*i.e.*, SDS-PAGE) confirmed that both the secreted CD95L monomer and the metalloprotease-cleaved CD95L possessed similar molecular weights (MW) around 30 kDa (Fig.1E). Although this MW was superior to the theoretical molecular weight 20.4 kDa according to ExPASy website, this difference was in agreement with the N-glycosylation of CD95L in mammalian cells [18]. In native conditions, the recombinant soluble CD95L (a.a. 102 to 281) and its metalloprotease-cleaved counterpart exhibited a molecular weight below 148 kDa suggesting a homotrimeric or tetrameric stoichiometry (Fig.1F). Of note,

in native conditions, the dimeric concatemer migrated at 66 kDa, below its monomer counterpart indicating that dimers failed to self-associate to form tetramers or hexamers (Fig.1F). We also noticed that for trimeric, tetrameric, pentameric and hexameric concatemers, a faint band appeared at a higher molecular weight suggesting the formation of self-aggregate (Fig.1F).

CD95L concatemers and cell death

The ability of each construct to trigger cell death was first evaluated on primary T-cells. For this purpose, we exposed PHA-activated peripheral blood lymphocytes (PBLs) from healthy donors (Fig.2A) to different concentrations of the concatemers. Except the monomer, all concatemers killed activated T-lymphocytes (Fig.2A). Similarly, all concatemers but monomer induced the elimination of the CD95-sensitive Jurkat T-cell (Fig.S1A). Interestingly, while a hexamer of CD95L has been reported to be the minimal stoichiometry necessary to trigger cell death [10], we observed that the dimeric concatemer killed activated PBLs and Jurkat cells in a similar manner to trimer, tetramer and pentamer counterparts (EC_{50} measured in Jurkat cells corresponded to 169 ng/mL for the dimer, vs 152 for the trimer, 79 for the tetramer and 131 for the pentamer). Nonetheless, the most efficient stoichiometry to kill remained the hexamer [10], since this ligand killed 3 to 8 fold more efficiently activated PBLs and Jurkat cells as compared to the other concatemers (EC_{50} of 23 ng/mL in Jurkat cells). These findings pointed out that although a dimeric CD95L was sufficient to trigger cell death, the CD95-mediated apoptotic signal possess a threshold level that can be reached by the hexameric complex.

Surprisingly, although monomer and trimer were resolved as homo-trimers in native conditions (see BN-PAGE shown in Fig.1F), the former did not induce cell death, while the latter did (Fig.2A and Fig.S1A). These data suggested that either the minor self-association of trimer concatemer observed in BN-PAGE (Fig.1F) or a different trimeric conformation exhibited by these trimers accounted for their difference in the induction of the apoptotic signal. Like the monomer, the metalloprotease-cleaved CD95L produced in HEK cells, mainly corresponding

to a ligand devoid of a large part of the stalk region because cleaved between amino acid residues ¹²⁶SL¹²⁷ [4], self-associated as an homotrimer (Fig. 1F) and failed to trigger cell death in both Jurkat and H9 T-cells (Fig.S1A) indicating that the presence of the stalk region in the soluble CD95Ls cannot by itself promote the induction of the apoptotic signal.

To establish that the newly generated recombinant CD95Ls triggered a caspase-dependent apoptotic response, we evaluated whether FADD and caspase-8 were necessary to implement the cell death signal. Elimination of these DISC components in Jurkat cells (Fig.2B) abrogated the cell death signal induced by concatemers (Fig.2C-D). Next, Jurkat and H9 T-cell lines were incubated for a short incubation time (2 hours) with the different concatemers and the metalloprotease-cleaved CD95L (sCD95L) and we quantified the protease activity of initiator caspase-8 (Fig.S1B) and of the executioner caspases -3 and/or -7 (Fig.S1C). Except monomers and sCD95L, all concatemers increased the caspase-8 activity in these T-cells, even if the intensity levels for dimer and trimer were not significant (Fig.S1B). All concatemers but sCD95L, stimulated the caspase-3/7 activity (Fig.S1C). Interestingly, these data indicated that although dimer, trimer and pentamer concatemers exhibited similar efficiency to kill activated PBLs or Jurkat cells when cell death was measured after 24 hours (Fig.2A and S1A), the dimer and trimer ligands induced lower caspase-8 and caspase-3/7 activities compared to that of pentamer (Fig.S1B-C). These data suggested that either the lower caspase activity observed for the dimer/trimer treatments could be compensated by a more sustained receptor activation compared to that of the pentamer or that the different aggregation and/or conformation modification exerted on CD95 by dimer and trimer might promote the recruitment of additional factors enhancing the apoptotic signal downstream the caspase-3/7 activations. Finally, pre-incubation of Jurkat (Fig.S1D) and H9 T-cells (Fig.S1E) with zVAD-fmk, a broad-spectrum caspase inhibitor, totally abrogated the cell death signal induced by concatemers (Fig.S1C-D) supporting that the CD95L concatemers implemented a classical apoptotic signaling pathway.

CD95L concatemers and Ca²⁺ response

The CD95-mediated calcium response exerts a pleiotropic role, but we established that this signal is essential for cell migration of Th17 cells in autoimmune diseases such as lupus [11] and thereby, for the implementation of non-apoptotic functions [4, 21]. A dose effect performed with all concatemers revealed that cells exposed to trimeric and hexameric ligands underwent stronger CD95-mediated Ca²⁺ response as compared to those measured with the other ligands (Fig.3A). In agreement with our previous data [11, 22], the metalloprotease-cleaved CD95L, which exhibited a homotrimeric structure (Fig.1F) also induced a strong Ca²⁺ response. Interestingly, although monomer, trimer and the metalloprotease-cleaved CD95L exhibited a homotrimeric structure (Fig.1F), the robust calcium signal observed with the metalloprotease-cleaved ligand and trimer concatemer (Fig.3B and 3C) was not phenocopied with the monomer (Fig.3A and 3C). CD95L can be cleaved by different metalloproteases and at different positions in its stalk region [13] such as ¹¹³EL¹¹⁴ [23], ¹²⁶SL¹²⁷ [3, 4] or ¹²⁹KQ¹³⁰ [24, 25] according to the literature. We previously observed that the metalloprotease-driven cleavage of CD95L in HEK/293T cells mainly occurs between amino acid residues ¹²⁶SL¹²⁷ [4] and thereby leads to the elimination of a large part of its stalk region. Although the role of this stalk region remains to be elucidated, a long form of soluble CD95L containing the stalk region has been reported to induce cell death [20] and be responsible for the pathology severity in acute respiratory distress syndrome [12]. Unlike the metalloprotease-cleaved CD95L, the monomer encompassed the stalk region and failed to trigger apoptosis (Fig.2 and S1) and calcium response (Fig.3A and 3C), suggesting that although the stalk region did not affect the CD95L stoichiometry, it could change the ligand conformation, and thereby alter CD95 activation. Nonetheless, it is noteworthy that the trimeric concatemer also possessed the stalk region and triggered both cell death (Fig.2 and S1) and calcium response (Fig.3A & 3C) rendering difficult to apprehend the exact role of this juxtamembrane region.

Concluding remarks

This new set of ligands confirmed that the apoptotic signal relies on an aggregation threshold to be efficiently activated and hexamer is the best ligand to do it. On the other hand, the CD95-mediated calcium response relies on both conformation and stoichiometry because only trimer and hexamer (Fig.3A-C) can efficiently induce this response. Although the maintain of the stalk domain might explain why monomer, which self-associates as a trimer (Fig.1F), does not phenocopy the metalloprotease-cleaved ligand for the calcium response, this hypothesis must be further investigated since the trimeric concatemer also encompasses this domain and efficiently implements apoptotic and non-apoptotic signals.

Material and Methods.

Antibodies and reagents

Mouse monoclonal anti-FLAG M2 (#F3165), anti-CD95 mAb CH-11 (#05-201), anti- β -actin (#A5316), Phytohemagglutinin-L (PHA-L; #11249738001) and human interleukin-2 (IL2; #11011456001) were from sigma-Aldrich (Sigma Aldrich Chimie, Saint-Quentin-Fallavier, France). Anti-CD95L antibody (G247-4, #556387) and anti-FADD (#556402) were acquired from BD Bioscience. Anti-caspase-8 antibody (clone 1C12, #9746), was from cell signaling (Beverly, MA, USA). MTS ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (#G3581) was purchased from Promega (Charbonnières-les-Bains, France).

Production of the soluble CD95Ls

Concatemers and full length CD95L-encoding vectors were transfected using calcium/phosphate method in HEK/293T cells. After 7 days of culture in serum-low Opti-MEM (Life technologies, Saint Aubin, France), secreted constructs or metalloprotease-cleaved CD95L were collected. Supernatants were ultracentrifuged for 1 h (100 000 g) to eliminate exosome contamination. Supernatant were dialyzed using spectra/por®1 dialysis membrane (6-8 kDa) (Sigma Aldrich) against PBS (Sigma Aldrich) and next concentrated using

centrifugal filters (Amicon® Ultra-15, Ultracel® -10K, Millipore, Molsheim, France). Soluble CD95L concentration was quantified by ELISA.

CD95L ELISA

Soluble CD95L was dosed by ELISA (#ab45892) following the manufacturer's instructions (Abcam, MA, USA).

Caspase-8 and caspase-3/7-glo assays

Jurkat cells (2×10^4 cells) were incubated with the different concatemers at 100 ng/mL for 2 hours and then, the caspase-8 (#G8200, Promega) and caspase-3/7 (#G8090, Promega) activities were assessed following the manufacturer's recommendation. Reading was performed in white 96 well plates.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflict of interest disclosure

MJ, PV and PL have filled patents to protect the use of CD95/CD95L inhibitors.

Ethics approval statement for human and/or animal studies

No human or animal studies

Author contributions

EL, CO, KK, NL, RJ, PV conceived, performed, and analyzed experiments. MJ analyzed data and reviewed manuscript. PL supervised, analyzed data, and wrote the manuscript.

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Figure legends.

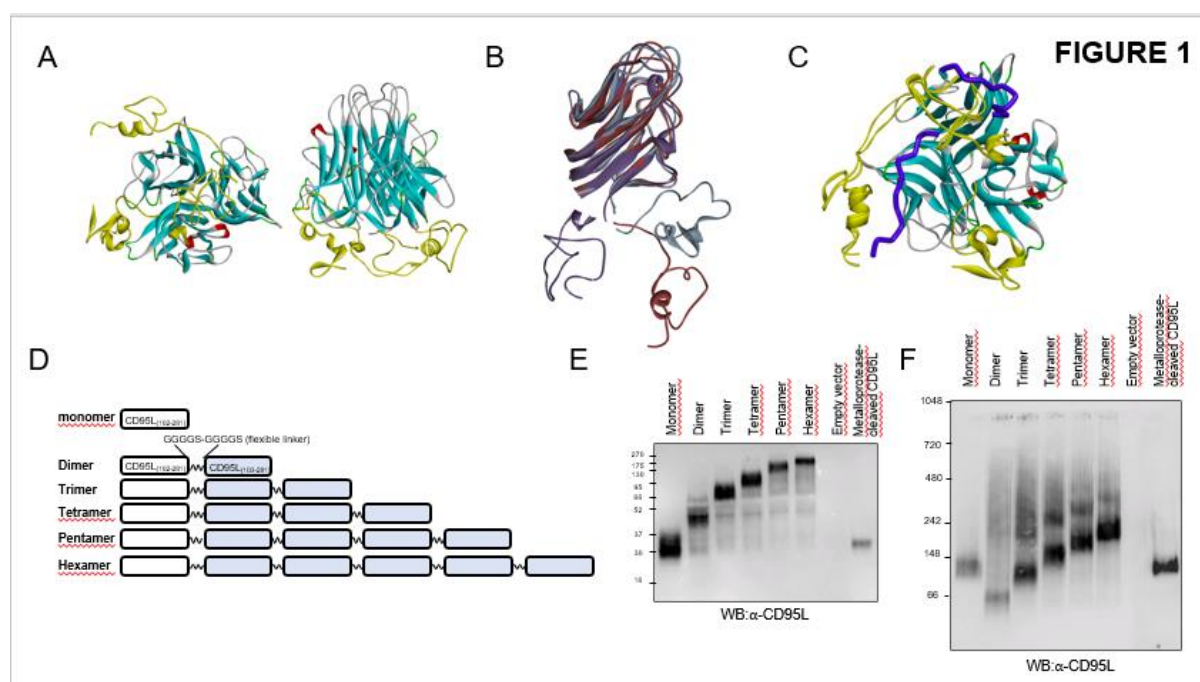


Figure 1. Generation of CD95L concatemers

A. The amino acid residues 103 to 143 were missing in the crystal structure. Their structure was built through extensive molecular dynamic simulations. The final state of the reconstituted CD95L trimeric model with Nt region in yellow is depicted.

B. Addition of the amino acid residues 103 to 143 to the crystallized CD95L did not affect the experimental 3D structure of the protomer.

C. Based on the reconstituted CD95L trimeric model showed in Figure A, a molecular modeling approach was applied to predict the minimal repetition of the flexible linker GGGGS (in blue) to connect covalently three CD95L protomers without affecting the global structure of the crystallized homotrimeric CD95L.

D. Schematic representation of the indicated concatemers.

E. The CD95L concatemer-encoding pcDNA3 vectors and empty or human full length CD95L-encoding pLVX-IRES-tdTomato vectors were transfected in HEK/293T cells and supernatants were harvested after 7 days. After ultracentrifugation to eliminate exosomes, supernatants were resolved in reducing and denaturing conditions (SDS-PAGE). Indicated immunoblotting was performed.

F. The molecular weights of the different CD95L were assessed in native condition using BN-PAGE method.

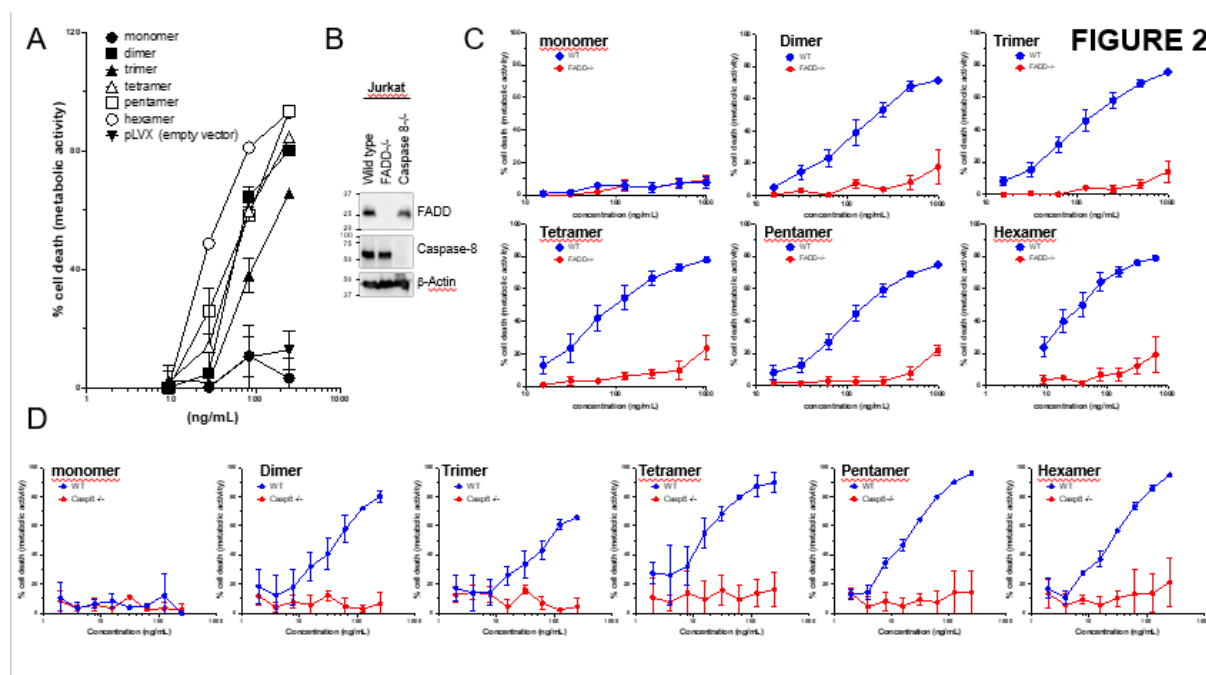


Figure 2. Concatemers of CD95L and induction of apoptosis

A. CD95L constructs secreted by HEK/293T cells were dosed by ELISA and their cytotoxic activity was evaluated by MTS assay using PHA-activated PBLs from health donor. Data represent means and SD of three independently performed experiments.

B. Wild type Jurkat (A3) and its FADD-deficient (FADD^{-/-}; clone I2.1) and caspase-8 deficient (Casp8^{-/-}; clone I9.2) were lysed. Lysates (10 μ g of protein) were resolved in a 12% SDS-PAGE and indicated immunoblots were performed. β -actin serves as loading control.

C. The cytotoxic activity of CD95L constructs was evaluated by MTS assay using the CD95 sensitive cell line Jurkat and its FADD-deficient counterpart, namely I2.1 cell line. Data represent means and SD of three independently performed experiments.

D. The cytotoxic activity of CD95L constructs was evaluated by MTS assay using the CD95 sensitive cell line Jurkat and its Caspase-8-deficient counterpart, namely I9.2 cell line. Data represent means and SD of three independently performed experiments.

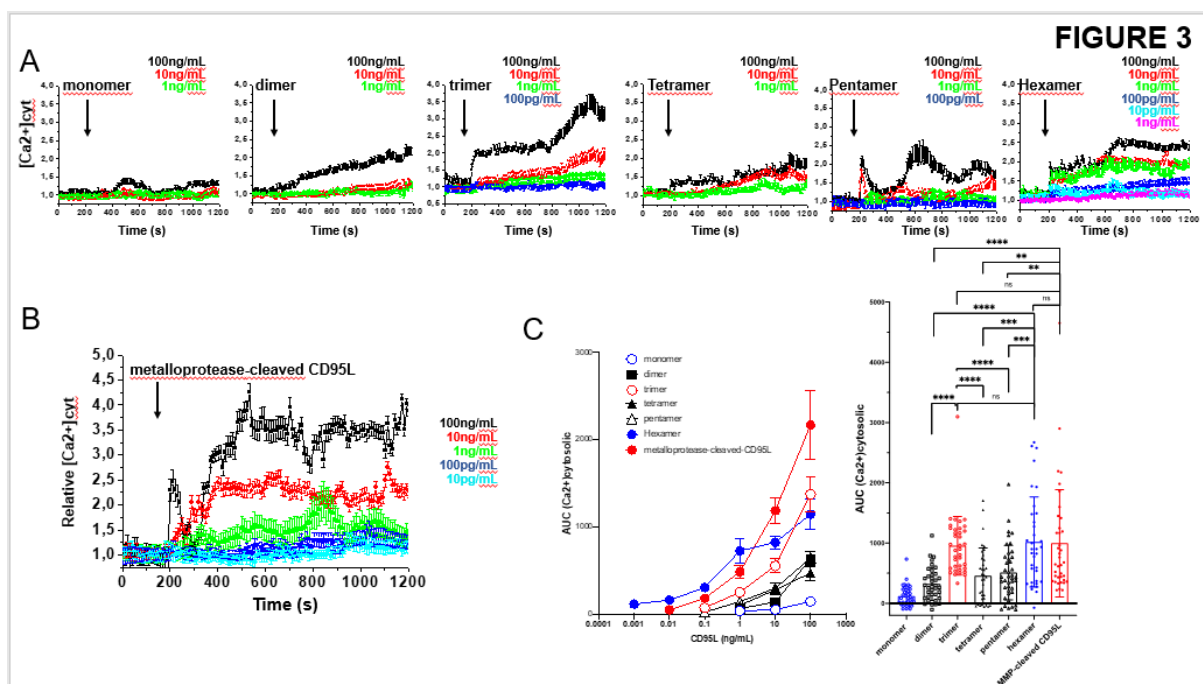
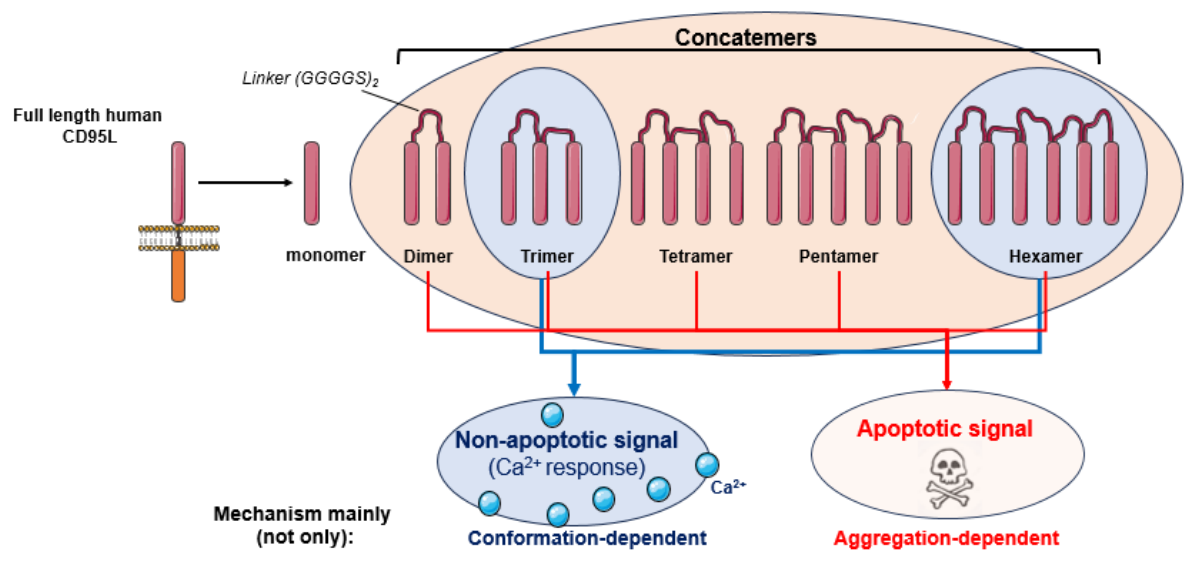


Figure 3. Concatemers of CD95L and induction of calcium response

A. The cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) in Jurkat cells exposed to the indicated concentration of CD95L was assessed using the calcium fluorescent probe Cali-520. Ratio values (R) were normalized to pre-stimulated values (R_0) to yield R/R_0 values (relative $[Ca^{2+}]$). Data represent the mean \pm SD of three independently performed experiments.

B. Same experiment as A with the metalloprotease-cleaved CD95L (sCD95L).

C. Left panel: The histogram represents the area under the curve (AUC) of the Ca^{2+} response for the different CD95Ls at the indicated concentrations obtained from experiments depicted in A and B. For each condition, the AUC was calculated for a stimulation of 1200 s. **Right panel:** AUC (1200 s) of the Ca^{2+} response obtained in Jurkat cells exposed to the higher concentration of indicated ligands (100 ng/mL). Data represent mean \pm SD. The statistical analyses of the AUC values were performed for the indicated ligands using non-parametric two-tailed Mann-Whitney tests. **, *** and **** indicate p values=0.01, 0.001 and 0.0001, respectively.



Graphical abstract: Using the extracellular region of CD95L (FasL), concatemers were designed, synthesized and expressed. The biological effects of these molecules on the apoptotic and non-apoptotic (intracellular Ca²⁺ response) signals were analyzed. The findings highlight the major role of conformation (non-apoptotic) and aggregation (apoptotic) in the CD95-mediated signals.