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Cutting Edge: Fas Ligand (CD178) Cytoplasmic Tail Is a Positive Regulator of Fas Ligand-Mediated Cytotoxicity[1]

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CUTTING EDGE

Cutting Edge: Fas Ligand (CD178) Cytoplasmic Tail Is a Positive Regulator of Fas Ligand-Mediated Cytotoxicity[1]

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The cytotoxic function of CD178 (Fas ligand (FasL)) is critical to the maintenance of peripheral tolerance and immune-mediated tissue pathology. The active site of FasL resides at the FasL extracellular region ($FasL_{Ext}$) and it functions through binding/cross-linking Fas receptor on target cells. In this study, we report that $FasL_{Ext}$-mediated cytotoxicity is regulated by the FasL cytoplasmic tail ($FasL_{Cyt}$). Deleting the N-terminal 2–70 aa (Δ70) or N-terminal 2–33 aa (Δ33) reduced the cytotoxic strength as much as 30- to 100-fold. By contrast, change in the cytotoxic strength was not observed with FasL deleted of the proline-rich domains (45–74 aa, ΔPRD) in the $FasL_{Cyt}$. Our study identifies a novel function of $FasL_{Cyt}$ and demonstrates that $FasL_{2–33}$, a sequence unique to FasL, is critically required for the optimal expression of $FasL_{Ext}$-mediated cytotoxicity. The Journal of Immunology,* 2005, 174: 4470–4474.

Fas (CD95) is a type I transmembrane protein expressed by many nucleated cells (1). The physiological ligand for Fas (FasL,[4] or CD178) is a type II transmembrane protein expressed by activated T cells and non-T cells under a variety of conditions (2, 3). The extracellular domain of FasL ($FasL_{Ext}$) has the ability to bind Fas of target cells. Cross-linking of Fas induces target cells to undergo apoptosis (4). The FasL-mediated apoptosis pathway has been implicated in peripheral tolerance (1, 2), tissue pathology (5, 6), and maintenance of the immune privileged sites (7).

FasL expression is regulated at the transcriptional, translational, and posttranslational levels. An effective way to down-regulate FasL expression is by shedding that generates soluble FasL (sFasL). Shed sFasL exhibits weak cytotoxicity and excess sFasL inhibits FasL-based, cell-mediated cytotoxicity (8). FasL is also released from cells in the form of vesicles (FasL vesicle preparation (VP)). FasL VP display full-length FasL and express strong cytotoxicity (9, 10). The physiological significance of FasL VP remains unknown.

Among TNF family members, FasL possesses a distinctive cytoplasmic tail ($FasL_{Cyt}$) of 80 aa. The sequence of $FasL_{Cyt}$ is highly conserved among species, suggesting it may have specific functions (11–14). Here, we report a novel function of $FasL_{Cyt}$. We found that $FasL_{Cyt}$ is critically required for the full expression of FasL-mediated cytotoxicity, a function associated with $FasL_{Ext}$. Compared with $FasL_{Cyt}$ deletion mutants, $FasL_{Cyt}$ enhances cytotoxicity by as much as 30- to 100-fold. In addition, we identified $FasL_{2–33}$, a unique sequence not found in other proteins, as the positive regulator of FasL-mediated cytotoxicity. Our study demonstrates a novel regulatory function of $FasL_{2–33}$ for an effector mechanism that is critically involved in various important aspects of the immune system.

Materials and Methods

Cell lines and reagents

Neuro-2a (mouse neuroblastoma), NIH-3T3 (mouse fibroblast), and COS-7 (monkey kidney fibroblast) were obtained from American Type Culture Collection (ATCC). G247.4, NOK-1 mAb, and PE-conjugated streptavidin were obtained from BD Biosciences. All restriction endonucleases were obtained from New England Biolabs. The prokaryotic expression vector pBlueScript II KS was obtained from Stratagene. The human FasL cDNA construct and the mammalian expression vector BCMGSneo were kindly provided by Dr. S. Nagata of Osaka University Medical Center (Osaka, Japan) (11).

Construction of FasL deletion mutants

The full-length human FasL cDNA cloned in pBlueScript II KS was used to generate deletion mutants by PCR using different 5′ primers and the same 3′ primer (Integrated DNA Technologies). All 5′ primers used contain the translation start sequence ATG that codes for methionine, therefore, deletion begins with amino acid residue 2 of FasL. The sequences of the 5′ primers are: 5′-ATGACCTCTGTGCCCAGAAGGCC-3′ (for Δ33 in which $FasL_{2–33}$ is deleted), 5′-ATGCTGAAGAAGAGAGGGAACCACAGC-3′ (for Δ70 in which $FasL_{2–70}$ is deleted), 5′-ATGCAGCTCTTCCACCTACAGAAG GAGC-3′ (for Δ102 in which $FasL_{2–102}$ is deleted) and 5′-GGCCTGGT CAAAGGAGGGGGAACCACAGCACAGGC-3′ (for ΔPRD (proline-rich domain deletion mutant) in which $FasL_{45–74}$ is deleted). We used Δ102 FasL together with BCMGSneo (vector control (Vc)) in every transfection experiment to control any unforeseen effect of our recombinant engineering process. The sequence of the 3′ primer is 5′-GTAAAACGACGGCCAGTGAGCG-3′ of the pBlueScript II KS. The PCR products were subcloned into pBlueScript II KS. The inserts were excised with *Not*I and *Xho*I and cloned into the BCMGSneo vector. The gene sequence of each construct was confirmed by DNA sequencing.

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[2] S.J. and V.J.P. contributed equally to this work.

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[4] Abbreviations used in this paper: FasL, Fas ligand; $FasL_{Ext}$, FasL extracellular domain; sFasL, soluble FasL; VP, vesicle preparation; $FasL_{Cyt}$, FasL cytoplasmic tail; PRD, proline-rich domain; Vc, vector control; WT, wild type.

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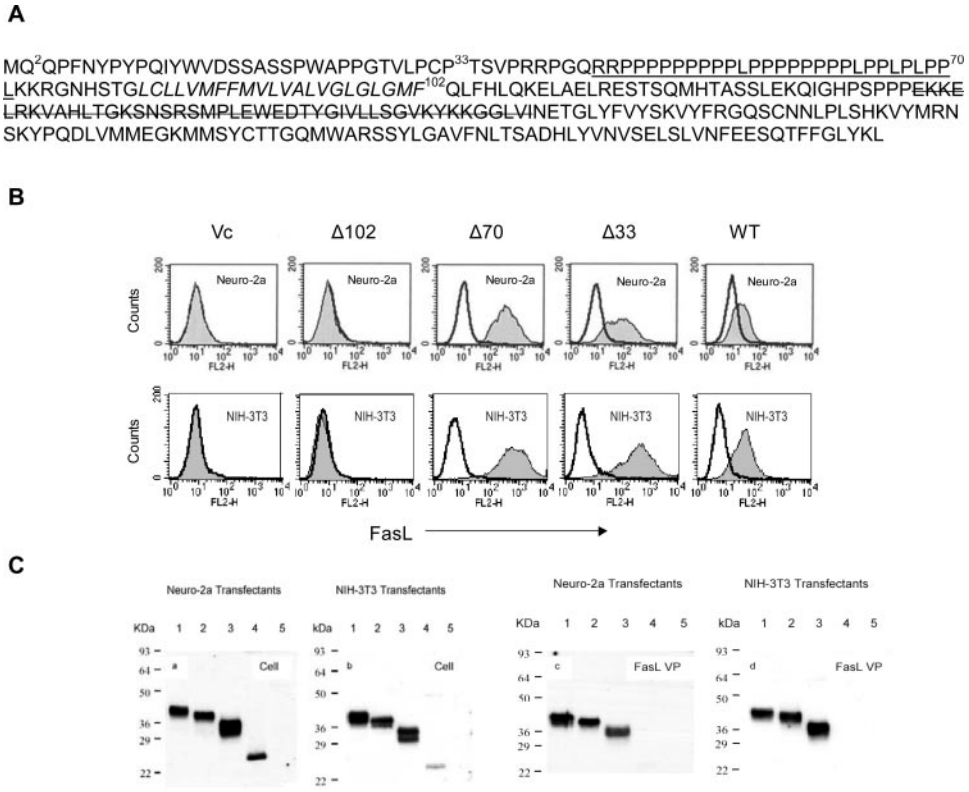

FIGURE 1. Cell surface expression of FasL by various transfectants. The WT human FasL sequence was shown in *A*. The amino acid positions 2, 33, 70, and 102 were marked to indicate the sequence deleted for Δ33 (2–33), Δ70 (2–70), and Δ102 (2–102) FasL mutants. PRD is underlined. The transmembrane domain is italicized. The strike-through sequence represents the trimerization motif. *B*, Various Neuro-2a transfectants (*upper panels*) and NIH-3T3 transfectants (*lower panels*) were stained with biotin-conjugated NOK-1 mAb (shaded area) or isotype control (open area), followed by FITC-conjugated streptavidin and analyzed with a flow cytometer. Data presented is representative of three separate experiments. *C*, Western blot analysis of FasL of various transfectants (*a* and *b*) and their FasL VP (*c* and *d*) of Neuro-2a and NIH-3T3 series. *Lanes 1–5* are samples from WT, Δ33, Δ70, Δ102, and Vc transfectants, respectively. Molecular mass markers are shown on the *left side* of each panel.

Transfection

The derivation, characterization, and culture condition for maintenance of transfectants of various cell lines have been described (15).

Flow cytometric analysis

Cells (0.5×10^6) were suspended in 0.1 ml of PBS containing 0.2% BSA and 1 μg of biotinylated NOK-1 or biotinylated control isotype. Binding reaction was conducted at 4°C for 30 min with gentle mixing periodically. Afterward, cells were washed twice with cold PBS. Bound Abs were measured by incubating with 0.5 μg of FITC-conjugated streptavidin for 30 min at 4°C. Cells were washed twice with cold PBS and then analyzed using FACScan (BD Biosciences) equipped with CellQuest software. At least 2×10^4 stained cells in the gated area were selected with each sample.

Preparation of sFasL and FasL VP

Cells at ~80% confluence were maintained in 150 mm × 25 mm petri dishes in 25 ml of culture medium for 48 h. FasL VP and sFasL were prepared as previously described (9, 10).

Table I. *FasL protein levels in various compartments of transfectant culture*[a]

	WT	Δ33	Δ70	Δ102[b]	Vc[b]
Neuro-2a[c]					
Cell lysate	3	19	89	<0.01	<0.01
FasL VP	17	14	50	<0.02	<0.02
sFasL	9	15	10	<0.2	<0.2
NIH-3T3[c]					
Cell lysate	8	65	72	<0.01	<0.01
FasL VP	31	44	54	<0.02	<0.02
sFasL	46	139	132	<0.2	<0.2

[a] FasL in samples was determined by ELISA. Data presented are representative of three separate experiments.

[b] The numbers indicate the limits of FasL detection, which depend on sample volumes used in the assay.

[c] The numbers indicate FasL total amounts in picomoles in samples. See *Materials and Methods* for sample preparation.

Quantification of FasL

The amounts of FasL in cell extract, FasL VP, and sFasL of all transfectants were determined using the $FasL_{Ext}$-specific ELISA kit (Oncogene) as previously described (10). A standard curve using recombinant sFasL provided with the kit is included in every individual assay.

Western blot analysis

Western blot analysis was conducted as previously described (9). Protein concentrations loaded were 0.1–5 μg. For samples lacking detectable FasL, 5 μg of total protein was loaded. FasL was detected using $FasL_{Ext}$-reactive G247.4 mAb followed by anti-mouse IgG-HRP (Sigma-Aldrich). Specific bands were developed using ECL (Amersham).

Cytotoxicity assay

A cytotoxicity assay was conducted as previously described using ^{51}Cr-labeled, A20 B lymphoma cells or Jurkat T lymphoma cells as targets (10). Various amounts of effector were incubated with 2×10^4 target cells for 4–8 h at 37°C in a 10% CO_2 incubator. At the end of incubation, cell-free supernatants were collected and counted with a gamma-counter (LKB). Cytotoxicity, expressed as percent-specific Cr release, was calculated by the formula: 100 × (experimental release − background release)/(total release − background release). Background release was determined by culturing target cells with medium. Total release was determined by lysing target cells with 2% Triton X-100. Experiments were conducted in duplicate and repeated at least twice.

Results and Discussion

$FasL_{Cyt}$ regulates FasL expression level

We prepared a series of FasL deletion mutant expression constructs and used them to transfect Neuro-2a and NIH-3T3 cells (Fig. 1*A*). G418-resistant transfectants were selected. We used flow cytometry to determine the cell surface expression of FasL (Fig. 1*B*). In both series of transfectants, wild-type (WT) FasL transfectants stained positive but a significantly stronger staining was observed with Δ33 and Δ70 FasL transfectants. Cell surface FasL expression was not observed with Δ102 FasL or Vc transfectants.

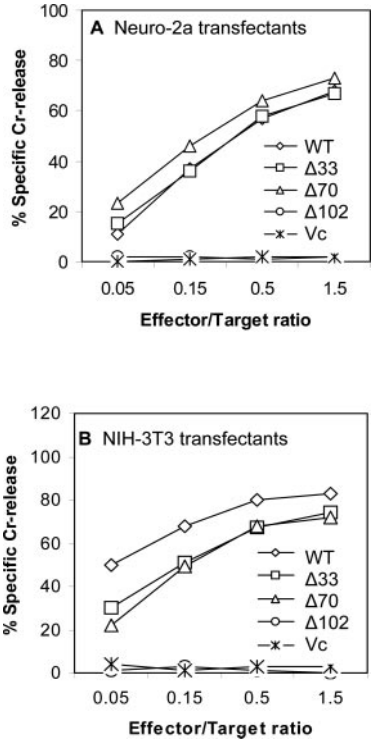

FIGURE 2. FasL-mediated cytotoxicity does not correlate with FasL membrane expression levels of transfectants. Cell-mediated cytotoxicity was conducted with various transfectants of Neuro-2a (*A*) and NIH-3T3 (*B*) series. Transfectants were cultured with ^{51}Cr-labeled Jurkat cells at various E:T ratios. The percent-specific Cr release was determined after 4 h of incubation.

We used FasL-specific ELISA to determine the total FasL levels in transfectants. Under the specific condition, WT transfectants of NIH-3T3 and Neuro-2a cell lines expressed 8 and 3 picomoles of FasL, respectively. A 6- to 30-fold increase in FasL level was observed for Δ33 FasL and Δ70 FasL transfectants. No FasL was detected in Δ102 FasL or Vc transfectants. Thus, the total FasL levels in transfectants correlated with their cell surface expression. In contrast, FasL levels in FasL VP and sFasL preparations did not correlate with the total FasL levels of transfectants (Table I). We have recently reported that the increase in FasL expression in Δ33 and Δ70 FasL transfectants is the result of an increase in the FasL translation rate (15).

We validated the size of FasL deletion mutants by Western blot analysis (Fig. 1*C*). WT, Δ33, and Δ70 FasL transfectants expressed the recombinant proteins of the predicted sizes (Fig. 1, *A* and *B*). A small size and faintly stained band was observed with the Δ102 FasL transfectant. No band was observed with Vc transfectants. FasL of predicted sizes were also observed with FasL VP prepared from the corresponding WT, Δ33, and Δ70 FasL transfectants. No band was observed with vesicles prepared from Δ102 FasL and Vc transfectants (Fig. 1*C*).

Both WT and $FasL_{Cyt}$ deletion mutants express FasL-mediated cytotoxicity

We tested these transfectants for cell-mediated cytotoxicity against the ^{51}Cr-labeled Jurkat target (Fig. 2). Cytotoxicity was not detected with Δ102 FasL and Vc transfectants. Transfectants expressing cell surface FasL displayed a dose-dependent killing based on various E:T ratios. Interestingly, the cytotoxic strength of WT FasL transfectants was comparable to that of Δ33 or Δ70 FasL transfectants despite the fact that the latter transfectants expressed significantly more FasL.

We also determined the cytotoxic strength based on the total FasL amount of transfectant using ^{51}Cr-labeled A20 B lymphoma cells as target (Fig. 3, *A* and *B*). For both series of transfectants, the cytotoxic strength of WT FasL was 10- to 30-fold stronger than that of Δ33 or Δ70 FasL transfectants. This dramatic difference is surprising because the cytotoxicity is dependent on cross-linking Fas receptors on target cells by $FasL_{Ext}$. The data therefore strongly suggest that $FasL_{Cyt}$ regulates $FasL_{Ext}$-mediated cytotoxicity across a membrane barrier. This difference in the strength of cell-mediated killing could be intrinsic to $FasL_{Cyt}$ or due to the cellular environment of FasL transfectants, or both.

Evidence from FasL VP

To firmly establish that $FasL_{Cyt}$ regulates FasL-mediated cytotoxicity, we determined the cytotoxic strength of FasL VP prepared from transfectants (Fig. 3, *C* and *D*). FasL VP is presumably a minimum subcellular component capable of expressing functional FasL transmembrane protein. It is free from sFasL. Its cytotoxicity, unlike transfectants, does not depend on protein synthesis (10). Using the same amount of FasL, FasL VP derived from WT FasL transfectants of Neuro-2a or NIH-3T3 delivered 10- to 30-fold stronger cytotoxicity than the FasL VP

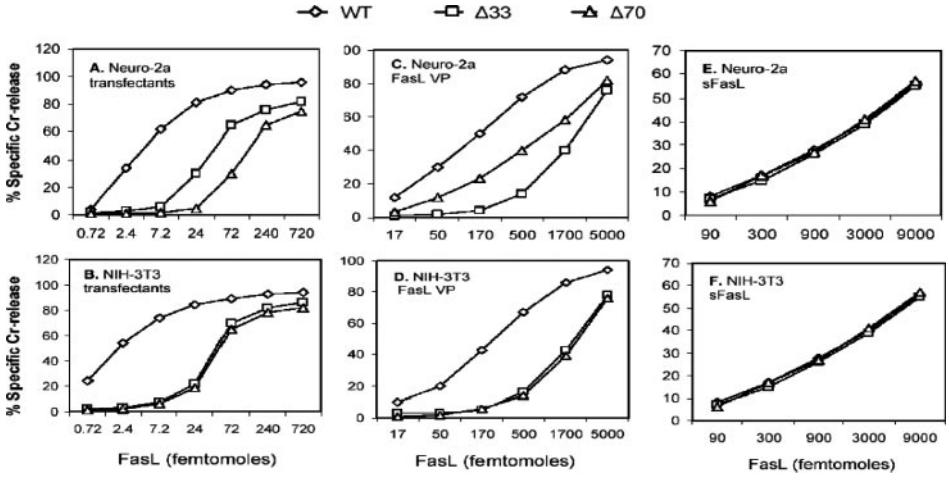

FIGURE 3. Comparison of cytotoxicity mediated by cells, FasL VP, and sFasL of various transfectants. Cells (*A* and *B*), FasL VP (*C* and *D*), and sFasL (*E* and *F*) of Neuro-2a (*A*, *C*, and *E*) and NIH-3T3 series (*B*, *D*, and *F*) were incubated with ^{51}Cr-labeled A20 target cells at various molar concentrations of FasL for 4 (cells and FasL VP) or 8 h (sFasL). Afterward, supernatants were removed and counted. The cytotoxicity data presented is representative of three experiments.

derived from Δ33 or Δ70 FasL transfectants. By contrast, the sFasL derived from these transfectants, either from Neuro-2a series (Fig. 3*E*) or from NIH-3T3 series (Fig. 3*F*), displayed nearly identical cytotoxicity. The data suggest that $FasL_{2–33}$ is important for the optimal expression of FasL-mediated cytotoxicity.

$FasL_{2–33}$ but not $FasL_{PRD}$ is required for the optimal expression of FasL-mediated cytotoxicity

$FasL_{Cyt}$ contains PRD that may interact with certain cellular proteins (12). To determine whether $FasL_{PRD}$ plays a role in FasL-mediated cytotoxicity, we generated PRD-deleted (ΔPRD) FasL transfectants from Neuro-2a and COS-7 cell lines. In contrast to Δ33 and Δ70 FasL transfectants, FasL expression was not increased in ΔPRD transfectants (data not shown). We prepared FasL VP from these transfectants and determined their cytotoxic strength (Fig. 4). For both Neuro-2a and COS-7 transfectants, the cytotoxic strength of ΔPRD FasL VP was comparable to WT FasL VP. As controls, FasL VP prepared from Δ33 FasL Neuro-2a transfectant and Δ70 FasL COS-7 transfectant displayed cytotoxicity 30- to 100-fold less than WT FasL VP. The data indicate that $FasL_{PRD}$ is not required for the optimal expression of FasL-mediated cytotoxicity. Taken together, the critical role of $FasL_{2–33}$ is demonstrated both by its deletion (as in Δ33 FasL and Δ70 FasL) that resulted in losing the $FasL_{Ext}$ cytotoxic strength and by its presence (as in ΔPRD FasL and WT FasL) that resulted in optimal display of $FasL_{Ext}$ cytotoxicity.

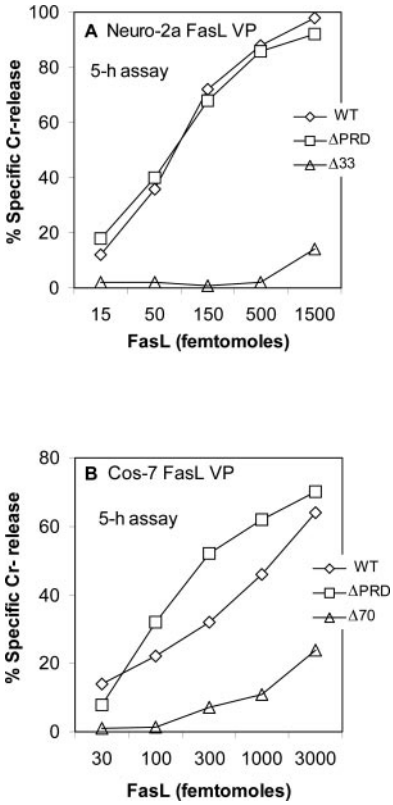

FIGURE 4. $FasL_{PRD}$ is not required for the optimal expression of FasL-mediated cytotoxicity. Samples of FasL VP were prepared from WT and ΔPRD FasL transfectants of Neuro-2a and COS-7 cell lines. FasL contents were determined using ELISA. Various amounts of FasL were compared for cytotoxic strength against ^{51}Cr-labeled A20 target. As controls, FasL VP prepared from Δ33 FasL Neuro-2a transfectant and Δ70 FasL COS-7 transfectant were analyzed in parallel. The data presented is representative of three experiments.

Our study used an artificial expression system to determine the structure and function relationship between $FasL_{Cyt}$ and $FasL_{Ext}$-mediated cytotoxicity. Deletion of $FasL_{Cyt}$ could potentially result in different localization of cell membrane FasL, change in sFasL production, faster FasL membrane movement, loss of interaction with $FasL_{Cyt}$-interacting proteins, loss of the ability to form oligomers, changes in $FasL_{Ext}$ conformation, change in phosphorylation state, etc. Additional studies are needed to determine the precise mechanism(s) and FasL-bearing vesicles offer a simple and novel system for this purpose and perhaps for other bioactive transmembrane proteins. Among these possibilities, we have ruled out the overproduction of sFasL as a mechanism for the down-regulation of cell-mediated cytotoxicity. Moreover, under the assay conditions, sFasL released were insufficient to influence the cell-mediated cytotoxicity (10). Our data further indicate that the weakened cytotoxic potential of sFasL is in part due to loss of $FasL_{Cyt}$, in addition to loss of multivalency as previously described (8). The observation that $FasL_{PRD}$ did not play a role in FasL-mediated cytotoxicity rules out the participation of potential $FasL_{PRD}$-interacting proteins such as Grb2, Grap, $p47^{phox}$, and Nck in this process (12). Moreover, no interacting protein was detected using GST-$FasL_{2–29}$ (12). These data suggest the ability to optimize FasL cytotoxicity is intrinsic to $FasL_{2–33}$. $FasL_{2–33}$ contains a DSSASSP motif that is a potential substrate for CK-I, CK-II, and GSK-3 kinases. Among them, the CK-I site (SXXS) is shared with several TNF superfamily members. Otherwise, $FasL_{2–33}$, including the Cys_{32}, a potential site for disulfide bonding and acetylation/palmitoylation, is unique among members of the TNF superfamily (⟨www.ncbi.nlm.nih.gov⟩). These properties should be helpful in determining the molecular mechanism by which the FasL-based cytotoxicity is optimized. The dramatic enhancement of cytotoxicity by $FasL_{2–33}$ may explain why FasL plays a critical role in peripheral tolerance and immune-mediated tissue damage that is dependent on cytotoxicity strength.

A regulatory role of $FasL_{Cyt}$ on $FasL_{Ext}$-mediated cytotoxicity had not been previously envisaged and our study provides the first definitive evidence supporting this novel function. Our study has significant implications with respect to regulation of membrane protein function in general and we have demonstrated this significant point in one effector function that is critically involved in peripheral tolerance, lymphocyte homeostasis, and immune-mediated tissue pathology. Our results also point out a potential complication in studies in which the cytoplasmic tail of a transmembrane protein is deleted by recombinant engineering as well as the potential use of $FasL_{Cyt}$ and $FasL_{2–33}$ to control the expression levels and biochemical properties of transmembrane proteins.

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Disclosures

The authors have no financial conflict of interest.

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