Palmitoylation of human FasL modulates its cell death-inducing function

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Fas ligand (FasL) is a transmembrane protein that regulates cell death in Fas-bearing cells. FasL-mediated cell death is essential for immune system homeostasis and the elimination of viral or transformed cells. Because of its potent cytotoxic activity, FasL expression at the cell surface is tightly regulated, for example, via processing by ADAM10 and SPPL2a generating soluble FasL and the intracellular fragments APL (ADAM10-processed FasL form) and SPA (SPPL2a-processed APL). In this study, we report that FasL processing by ADAM10 counteracts Fas-mediated cell death and is strictly regulated by membrane localization, interactions and modifications of FasL. According to our observations, FasL processing occurs preferentially within cholesterol and sphingolipid-rich nanodomains (rafts) where efficient Fas–FasL contact occurs, Fas receptor and FasL interaction is also required for efficient FasL processing, and Fas palmitoylation, which occurs within its transmembrane domain, is critical for efficient FasL-mediated killing and FasL processing.

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Abbreviations: ADAM10, A disintegrin and metallopeptidase 10; APL, ADAM10-processed FasL; EGF, epidermal growth factor; FasL, Fas ligand; FCS, fluorescence correlation spectroscopy; GFP, green fluorescence protein; MMP, matrix metalloproteinase; NK, natural killer; PNS, post-nuclear supernatant; sFasL, soluble Fas ligand; SPA, SPPL2a-processed APL; SPPL2a, signal peptide peptidase-like 2A; TNF, tumor necrosis factor
tioning inside rafts, the interaction with Fas receptor and palmitoylation are all essential events for hFasL proteolysis and function.

Results

hFasL processing by metalloproteases, but not by SPPL2a, reduces hFasL-induced cell death. We first aimed at studying the importance of sequential hFasL shedding for the pro-apoptotic function of hFasL. For this purpose, hFasL cleavage in WSU B cells stably transfected with hFasL was prevented by using the SPPL2a inhibitor (Z-LL)2 or the MMP inhibitor TAPI-2, and cells were co-cultured with Fas-expressing JH6.2 Jurkat target cells. We found that the killing capacity of hFasL was unaffected upon inhibition of the intramembrane protease SPPL2a by (Z-LL)2 (Figure 1a), but was increased after inhibition of metalloproteases (Figure 1b). Sufficient inhibition of hFasL cleavage was confirmed by western blot analysis of lysates prepared from (Z-LL)2- and TAPI-2-treated WSU hFasL cells. These data indicate that Fas-mediated cell death is regulated by ADAM10-processing of hFasL, which may function to hinder the pro-apoptotic function of hFasL.

**Figure 1** Metalloprotease, but not SPPL2a-mediated. FasL processing decreases FasL-induced cell death. WSU cells were pre-treated with inhibitors of either MMP or SPPL2a before co-culture with the Fas-bearing JH6.2 cells (inhibitors remained in media during the co-culture period). Effective inhibition of ADAM10 or SPPL2 was confirmed by western blot. G247 antibody was used to detect the full-length FasL and the antibody Ab-3 was employed for the detection of the N-terminal APL and SPA fragments. Ezrin was used as loading control. Cell death was quantified by flow cytometric quantification of the subG1 population of propidium iodide-stained ethanol-fixed cells. (a) JH6.2 cells were co-cultured for 6 hours with WSU cells stably transfected with either hFasL or mock transfected, and were either pre-treated with the SPPL2 inhibitor (Z-LL)2 (2 μM, 2 h) or left untreated. The graph represents the average of two independent experiments, with error bars indicating the S.D. (b) JH6.2 cells were co-cultured for 6 h with WSU cells stably transfected with hFasL or mock transfected and either pre-treated with the MMP inhibitor TAPI-2 (50 μM, 2 h) or left untreated. The graph represents the average of four independent experiments, with error bars indicating the S.D.

Optimal processing by ADAM10 requires hFasL localization into rafts and binding to the Fas receptor. To test the consequences of raft localization on hFasL processing, we performed a cholesterol oxidase treatment, which partially inhibits lipid nanodomain formation by converting cholesterol to cholesterolone. The distribution of full-length FasL and APL in rafts were then analyzed using a biochemical approach. Post-nuclear supernatants (PNSs) were solubilized by polyoxyethylene ether Brij 98, and lysates were subjected to ultracentrifugation through a sucrose gradient. The various fractions were analyzed by western blotting. The membrane was probed with antibodies recognizing the raft protein marker Fyn and the non-raft marker Rab5α, as a control for proper separation. Our data show that whereas full-length FasL is only partially located in the raft fractions, the APL fragment was exclusively found there. In addition, the cholesterol oxidase treatment decreased the shedding of hFasL, thereby indicating that raft localization has a crucial role in ADAM10 processing of hFasL. (Figure 2a).

We also aimed at determining whether binding of hFasL to the Fas receptor is required for hFasL cleavage. The point mutation A247E in the Fas receptor-binding domain prevents interaction of hFasL with its receptor thereby preventing cell-mediated apoptosis (Supplementary Figure 1A and Del-Rey et al.24). Lysates prepared from hFasL247E-transfected WSU cells showed decreased levels of the APL fragment compared with cells transfected with wild-type hFasL (Figure 2b). Similarly, transient transfection of Fas receptor targeting siRNA in HEK293 cells dramatically decreased the amount of APL (Supplementary Figure 1B). Collectively, these data demonstrate that binding to the Fas receptor is required for the processing of hFasL by ADAM10.

ADAM10 processing of hFasL occurs within rafts. It has been shown that differential nanodomain localization of target protein and metalloprotease can regulate the proteolytic processing.18,19 Moreover, the above results indicate that hFasL processing mainly occurs within rafts. To obtain further evidence for this hypothesis, we treated hFasL-transfected WSU cells with the metalloprotease inhibitors TAPI-2, GI254023X or INCB-3619. Biochemical raft analysis showed that inhibition of ADAM10-mediated proteolysis of hFasL decreased APL production, while simultaneously increased the amount of full-length FasL (Figure 2c).

Palmitoylation of hFasL is required for efficient processing by ADAM10 and for FasL-induced cell death. Protein palmitoylation represents one of the raft targeting signals. Using acyl biotinyl-exchange chemistry, we show that hFasL is indeed palmitoylated. Incorporation of point-mutated hFasL revealed that this post-translational modification occurs at the cysteine position 82, which is located at the N-terminal portion of the transmembrane domain (Figures 3a and b).

To investigate whether binding to the receptor is necessary for hFasL palmitoylation, we tested the palmitoylation status of the hFasL247E mutant. Our results showed that this...
mutant, which is unable to bind to Fas, was also palmitoylated (Figure 3b). Therefore, palmitoylation of hFasL in the transmembrane domain at cysteine residue 82 does not require binding to Fas receptor.

We then assessed whether hFasL palmitoylation is important for Fas-mediated cell death. We generated WSU cells stably expressing comparable levels of wild-type hFasL or hFasLC82S at the cell surface (Figure 3c, inset). Co-culture experiments with Fas-expressing JH6.2 target cells revealed that the killing capacity of the hFasLC82S mutant was significantly lower than that of the wild-type hFasL (Figure 3c).

Next, we investigated the proteolysis of the palmitoylation-deficient mutant. Immunoblot analysis of WSU cells stably expressing wild-type hFasL or hFasLC82S revealed that less APL was produced by ADAM10 cleavage of the hFasLC82S mutant compared with wild-type hFasL (Figure 3d). In addition, WSU cells transfected with wild-type hFasL, and treated with the general palmitoylation inhibitor 2-bromopalmitate produced decreased amounts of APL compared with untreated cells (Supplementary Figure 1C). Similar to the decreased APL production, significantly lower level of soluble FasL was found in the supernatant of the hFasLC82S mutant cells compared with wild-type hFasL-expressing cells (Figure 3e). Taken together, our data indicate that palmitoylation of hFasL occurs upstream of Fas receptor binding and is a pre-requisite for efficient cleavage by ADAM10.

Neither FasL palmitoylation nor Fas receptor binding are required for the interaction of hFasL with ADAM10. The ADAM10 pro-form is processed and thereby converted to the mature form of the molecule by the pro-protein convertases furin or PC7. To investigate the nature of the ADAM10 forms interacting with hFasL and the location of this functional interaction, we analyzed the distribution of ADAM10 inside and outside the rafts. Biochemical analysis revealed that the pro-form of ADAM10 was excluded from rafts, whereas the mature form was localized within and outside the raft fractions (Figure 4a). Therefore, confinement of active ADAM10 does not restrict the interaction to any nanodomain while the interaction with the pro-form is restricted to the non-raft fraction.

To find out whether FasL palmitoylation affects the interaction of hFasL with ADAM10, lysates from WSU cells stably transfected with hFasL, hFasLC82S or hFasLA247E were subjected to immunoprecipitation with an anti-ADAM10 antibody. Figure 4b shows that FasL interacts with the pro-form of ADAM10 regardless of its palmitoylation status.
Palmitoylation is required for optimal hFasL processing and cytotoxic activity. (a) Diagram of the hFasL transmembrane domain, highlighting the proposed palmitoylation site at aa 82, which we mutated to serine. (b) WSU cells stably transfected with constructs encoding for hFasL, hFasLC82S or hFasLA247E were subjected to an acyl-biotinyl exchange protocol as described in Materials and Methods. Specificity of the experiment was controlled by omitting the hydroxylamine (HA) treatment and Fyn was used as an internal control for reaction efficiency. (c) JH6.2 cells were co-cultured with WSU cells stably transfected with hFasL, hFasLC82S or mock. Cell death was then quantified by flow cytometric analysis of the subG1 population of propidium iodide-stained ethanol-fixed cells. The graph represents the average of four independent experiments with error bars indicating the S.D. (d) Cell lysates of WSU cells stably transfected with constructs encoding for hFasL and subjected to an acyl-biotinyl exchange protocol as described in Materials and Methods. Specificity of the experiment was controlled by omitting the hydroxylamine (HA) treatment and Fyn was used as an internal control for reaction efficiency. (e) Levels of soluble FasL were measured in the supernatant of WSU cells stably transfected with wild-type hFasL or hFasLC82S. The graph represents the average of two independent experiments with error bars indicating the S.D.

We also observed an increase in ADAM10 binding to hFasLA247E, indicating that this interaction occurs before binding of FasL to its receptor (Figure 4b).

To study the importance of the hFasL interaction with the ADAM10 pro-form in further detail, we treated the cells with the MMP inhibitor TAPI-2 or with the furin inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethylketone (Dec-CMK). Both treatments reduced the amount of cleaved APL fragment and the production of soluble FasL (Figures 4c and d), indicating that the interaction with the ADAM10 pro-form is important for the hFasL cleavage.

We also analyzed the interaction of ADAM10 with hFasL in the presence of these inhibitors. Both treatments led to increased amounts of the FasL co-immunoprecipitated with ADAM10 (Figure 4e). Our results indicate that the interaction with the ADAM10 pro-form is important for the production of APL.

Palmitoylation and receptor interaction alter the nanodomain localization of hFasL. We have previously demonstrated that the localization of hFasL within rafts is essential for maximum induction of cell death. In light of these findings, we investigated whether both palmitoylation and receptor binding are required for hFasL raft localization. To monitor hFasL localization of the wild-type FasL and its mutants, we investigated the in vivo lateral diffusion of hFasL-GFP, hFasLA247E-GFP and hFasLC82S-GFP using fluorescence correlation spectroscopy (FCS). FCS measures the apparent diffusion time of molecules, providing information on the diffusion mode of the molecule of interest. On the basis of diffusion law, the diffusion time of a protein (τd) can be plotted versus the square of the beam radius (w²), and this representation, the intercept to the y axis can be calculated (t0). The t0 value is indicative of the molecular confinement mode and is positive in the case of confinement to nanodomains, negative in the case of trapping in a meshwork and zero if the molecule is neither confined within discrete domains nor hindered by a network. We have previously reported that wild-type hFasL fused to GFP exhibited a diffusion behavior corresponding to a confinement inside rafts. For wild-type FasL, t0 was determined as 35.9 ± 8.5 ms, a diffusion behavior comparable to the raft marker BODIPY-C5-ganglioside-GM1 (25 ± 3 (Lenne et al.)). In contrast, the palmitoylation-deficient mutant hFasLC82S and the receptor-binding mutant hFasLA247E displayed a significantly different diffusion behavior, reflected by reduced t0 values (8.4 ± 2.9 and 6.2 ± 4.7, respectively), indicating a lower confinement of the proteins into nanodomains (Figure 5).

The effective diffusion coefficient (Deff), defined by the inverse of the diffusion law slope, represents the long-range diffusion of the proteins of interest. Deff was dramatically decreased for the hFasLA247E receptor-binding mutant (0.29 ± 0.02 versus 0.54 ± 0.17 μm²/s for wt hFasL), indicating a greatly reduced long-range diffusion of hFasLA247E.
Discussion

Our results demonstrate that shedding of hFasL by ADAM10 counteracts the cytotoxic activity of hFasL. In this study, we show that ADAM10 processing of hFasL depends on the integrity of the cholesterol- and sphingolipid-enriched nanodomains (rafts) and on the interaction with the Fas receptor.

Finally, we have reported the first description of hFasL palmitoylation. This post-translational modification is required for appropriate partitioning of hFasL into rafts, for efficient Fas-mediated cell death and hFasL processing by ADAM10 (Figure 6).

Data from other groups substantiate our observations that metalloprotease inhibition decreases hFasL processing and increases its killing activity.11 The increased cytotoxicity of hFasL may depend either on the accumulation of the full-length FasL form or on the decrease in the proteolysis products, namely sFasL, APL and SPA. Our results clarify that neither APL nor SPA has an effect on hFasL-induced cell death or on hFasL processing. The decrease in hFasL cytotoxicity is likely due to the reduced amount of full-length FasL and/or the production of soluble FasL. It is possible that sFasL may compete with the full-length form for binding to the Fas receptor. It also remains unclear whether sFasL proteins are exclusively produced by processing of full-length hFasL at the plasma membrane. Overall, the role of hFasL processing by ADAM10 might be a down-modulation or termination of the death signal, as has been described for shedding of receptors, such as TNFR1 following its engagement,22 protection of the neighboring cells by soluble FasL23 or reverse signaling into the ligand-bearing cells, mediated by the liberated SPA fragment.

We demonstrated that FasL interaction with its receptor Fas is necessary for FasL processing. The A247E mutation located within the receptor-binding site, which causes reduced killing of target cells (Supplementary Figure S1A and Del-Rey et al.24), displays reduced FasL processing.
hFasL processing following its interaction with Fas would restrict hFasL cytotoxicity to the target cell and avoid excessive cell death. Several ligands and receptors are processed upon binding by their partner, such as the Notch receptor which is cleaved after activation by its ligand Delta (reviewed by Kopan and Weinmaster) and the Ephrin2 ligand, which is processed once it binds to its receptor Eph3A. Proteolysis can also be promoted by cross-talk with other receptors. Examples include shedding of the Ephrin ligand which is stimulated by recruitment of G proteins and shedding of CD44 which can occur after binding to small hyaluronan oligosaccharides or by EGF stimulation through activation of Rac GTPases. It remains an open question whether and how other receptors might affect the processing of hFasL. Using the hFasLA247E mutant, which is excluded from rafts, we demonstrated that hFasL recruitment by the Fas receptor is important for its localization into these nanodomains. This hypothesis is further corroborated by our previously published data showing that interaction of hFasL with Fas increases FasL localization inside rafts.

We then demonstrate that although hFasL and ADAM10 display a similar distribution inside and outside rafts, FasL processing depends on the integrity of the raft domains. Indeed, this result also explains the decreased processing capacity of the receptor-binding mutant hFasLA247E. Individual rafts are relatively small and display heterogeneous protein content. This heterogeneity allows regulation of protein activity owing to differential raft localization. In contrast to FasL/ADAM10, differential nanodomain localization of the target protein and the metalloprotease responsible for its cleavage has been reported for other molecules. In these cases, this differential protein localization might control molecule processing. This strategy has been observed for the CD30 protein which is located in rafts, whereas the metalloprotease responsible for its shedding, ADAM17, is excluded. In the case of the amyloid precursor protein, the presence of two distinct pools distributed inside and outside of rafts results in differential processing.

FasL is capable of binding to ADAM10 before interaction with the Fas receptor, and according to our results, this early binding event occurs outside rafts and is important for the proteolysis of hFasL. In this FasL population, hFasL proteolysis is activated by a cleavage of the ADAM10 pro-form by Furin. We cannot exclude the possibility that the FasL cleavage might also occur by direct interaction with the active form of ADAM10. We have yet to determine whether binding to ADAM10 outside rafts is important for FasL processing at the plasma membrane or for intracellular processing. In general, recruitment of ADAM10 to a receptor–ligand system following ligand binding of the receptor EphA3 has also been described. ADAM10 then cleaves the ligand Ephrin 5 in trans. Interaction with additional unidentified proteins might also be essential for processing of hFasL by ADAM10 as, for example, recruitment of tetraspanins by ADAM10 has been reported to increase cleavage of ADAM10 substrates and/or proteolytic maturation of ADAM10.

We have previously shown that the death receptors Fas and DR4 are palmitoylated. This modification is necessary for receptor partitioning into rafts and for receptor engagement. We demonstrated in this study that FasL palmitoylation...
also occurs. Indeed, palmitoylation of both ligand and receptor might represent an extended modification for efficient interaction and recruitment. However, palmitoylation of both components is not always necessary, as exemplified by TNF-α, where TNF-α is palmitoylated, but its receptor TNFR1 remains unmodified.

Our data imply that palmitoylation of hFasL is important for its biological function, as mutation of the palmitoylation site decreases the level of hFasL-induced cell death. As with the receptor-binding mutant hFasLA247E, the hFasL palmitoylation mutant also exhibits decreased proteolysis by ADAM10. Fluorescence correlation spectroscopy suggests that localization of both the palmitoylation and the receptor-binding mutants is not confined to raft nanodomains, in contrast to the wild-type hFasL. This delocalization would explain the observed decrease in FasL-mediated cell death and proteolytic cleavage.

In summary, hFasL proteolysis by ADAM10 depends on the interaction of the ligand with its receptor Fas, and the integrity of the raft nanodomains. Post-translational modifications, such as palmitoylation, which change the partitioning of hFasL into rafts, might enable the cell to fine-tune the processing of hFasL by ADAM10 and, consequently, hFasL-mediated cell death.

Materials and Methods

Antibodies and reagents. The antibodies used for immunoblotting were as follows: anti-ADAM10 (Chemicon/Millipore, Billerica, MA, USA), anti-Ezrin (Zymed laboratories, San Francisco, CA, USA), anti-FasL clone G247-4 (BD PharMingen, San Diego, CA, USA), referred to as G247 in the manuscript), anti-FasL clone Ab-3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Rab5 (Santa Cruz Biotechnology) and horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies (Jackson ImmunoResearch, West Grove, PA, USA). The anti-FasL antibody clone BR-17 (Diacone, Besançon, France) was used for the expression analysis by flow cytometry. The matrix metalloproteinase inhibitor TAPI-2 (N-(R)-(2-(hydroxyamino-arynoyl)methyl)-4-methylpentanoyl-L-t-butyl-alanyl-L-alanine, 2-aminoethyl amide) was purchased from Merck (Nottingham, UK). The ADAM10 inhibitor GI24023X ((2R,3S)-3-(formyl-hydroxyamino)-2-(3-phenyl-1-propyl) butanoic acid ((1S)-2,2-dimethyl-1-(methyl-carbamoyl-1-propyl) amide) has been described elsewhere.

The small molecule protease inhibitor INCB-3619 obtained from P. Scherle (Incyte Corporation, Wilmington, DE, USA) was previously described. Furin convertase inhibitor Dec-CMK was purchased from Alexis (Lausen, Switzerland).

Cell culture and transfection procedures. WSU and JH6.2 cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum at 37°C in a 5% CO2 atmosphere. HEK293 and HEK293 T cells (ATCC no. CRL-1653) were cultured in Dulbecco’s Modified Eagle’s Medium (Invitrogen), supplemented with 10% FBS at 37°C in a 5% CO2 atmosphere. COS-7 cells (ATCC no. CRL-1657) were grown in DMEM supplemented with 10% FBS, 10% fetal bovine serum at 37°C and 10% glycerol for 1 h at room temperature. Biotinylated proteins were purified with neutravidin beads (Pierce/Thermo Scientific), separated by SDS-PAGE and submitted to immunoblotting using an antibody-recognizing FasL. Specificity of the experiment was controlled by omitting the hydrolymtime reaction.

Cell death assays. The killing potential of FasL-expressing cells was determined using co-culture assays with Fas-expressing target cells (JH6.2) at a killer-to-target ratio of 1:1. Target cells were pre-stained with 5 μM diacetyl succinimidy ester (CFSE, Molecular Probes/Invitrogen, Carlsbad, CA, USA) before co-culturing to distinguish them from killer cells during FACS analysis. The quantification of apoptosis in the target cells was performed by flow cytometry. Briefly, cells were fixed with ice-cold 70% ethanol, washed with 38 mM sodium citrate (pH 7.0) and stained for 20 min at 37°C with 69 μM propidium iodide (Sigma)/38 mM sodium citrate/5 μg per ml RNaseA (Sigma). Cells were subsequently analyzed by flow cytometry (FACS Calibur; Becton Dickinson, Franklin Lakes, NJ, USA). The proportion of apoptotic cells was determined as represented by the subG1 peak.

Separation of detergent-resistant membranes. PNS from 20 x 106 WSU cells was solubilized in 1 ml buffer A (25 mM HEPES, 150 mM NaCl, 1 mM EGTA, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 5 mM orthovanadate, protease inhibitor cocktail) containing 1% Brij 98 (Aldrich, St Louis, MO, USA) and 10% glycerol for 1 h on ice, followed by the addition of 2 ml of 2 M sucrose in buffer A, before being placed at the bottom of a step sucrose gradient (1.33-0.9-0.8-0.75-0.7 M) in buffer A. The gradient was centrifuged at 250,000 x g for 16 h in an SW41 Beckman rotor (Beckman Instruments, Gagny, France) at 4°C. One-milliliter fractions were harvested from the top, except for the last fraction that contained 3 ml.

Fluorescence correlation spectroscopy experiments. Fluorescence correlation spectroscopy (FCS) measurements were performed with a custom apparatus as previously described. Briefly, FCS data were collected using a confocal-based microscope (Axiovert 200M; Zeiss, Oberhochen, Germany) equipped with an Aprochromat 40 x 1 /2 numeric aperture water-immersion objective and excitation from a 488-nm line of an Ar+ ion laser. The laser waist was set by selecting the lateral extension of laser beam falling into the back aperture of the objective with a diaphragm. Fluorescence was collected through a 325 to 565-nm bandpass filter. A confocal pinhole reduced the out-of-focus fluorescence. FCS measurements were carried out in Hanks balanced salt solution with 10 mM HEPES, pH 7.4, by illuminating the sample with an excitation power of 3 J/w at the back aperture of the objective lens. Autocorrelations were processed by a hardware correlator (http://www.correlator.com, Hong Kong) and data were analyzed with built-in functions of IGOR Pro (WaveMetrics, Lake Oswego, OR, USA).

Immunoprecipitation. WSU cells (20 x 10^6) were sonicated in buffer A (25 mM HEPES, 150 mM NaCl, 1 mM EGTA, 10 mM NaF-P-P, 10 mM NaF, 5 mM Na3VO4, 25 mM HEPES, 10 mM [γ-glycerophosphate, 5 μg/ml leupeptin, 0.125 μg/ml pepstatin A, 10 μg/ml chymostatin) containing 0.5% Nonidet P-40 (NP-40) and 10% glycerol at 43°C and then centrifuged to discard cell debris. The supernatant was incubated overnight at 4°C with 30 μl Protein G sepharose beads (Invitrogen) pre-incubated with an anti-ADAM10 antibody (Millipore). The beads were washed four times with buffer A containing 0.5% NP-40 and 10% glycerol and the immunoprecipitates were eluted from the beads with Laemmli buffer at 95°C for 5 min. Samples were separated by SDS-PAGE and analyzed by immunoblotting.
FasL-specific ELISA. The culture medium of WSU cells incubated at 10 × 10^6 cells per ml for 4 h was harvested to analyze soluble proteins. sFasL present in the supernatant was measured by ELISA using the human sFasL module set (Bender Medsystems, Vienna, Austria). Briefly, a 96-well microplate was coated overnight with 2.5 μg/ml mouse anti-FasL capture antibody at 4°C, washed with phosphate-buffered saline (PBS)/0.05% Tween (PBS-T) and then blocked with 3% bovine serum albumin in PBS for 2 h at room temperature. After washing with PBS-T, samples and standard were added. Next, biotinylated anti-FasL detection antibody was applied to each well and the plate was incubated for 2 h at room temperature, washed and incubated for 1 h at room temperature with a streptavidin-peroxidase conjugate. The TMB super-sensitive microwell one component peroxidase substrate (Tebu-bio, Le-Perray-en-Yvelines, France) was added and the reaction was stopped after 20 min incubation at room temperature by the addition of 4 N H2SO4 and the microwell plate was read at 450 nm.

Conflict of interest

The authors declare no conflict of interest.

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