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Palmitoyl protein thioesterase 1 modulates tumor necrosis factor α-induced apoptosis

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ABSTRACT

Induction of apoptosis by TNF has recently been shown to implicate proteases from lysosomal origin, the cathepsins. Here, we investigated the role in apoptosis of palmitoyl protein thioesterase 1 (PPT1), another lysosomal enzyme that depalmitoylates proteins. We show that transformed fibroblasts derived from patients with the infantile form of neuronal ceroid lipofuscinosis (INCL), a neurodegenerative disease due to deficient activity of PPT1, are partially resistant to TNF-induced cell death (57–75% cell viability vs. 15–30% for control fibroblasts). TNF-initiated proteolytic cleavage of caspase-8, Bid and caspase-3, as well as cytochrome *c* release was strongly attenuated in INCL fibroblasts as compared to control cells. Noteworthy, activation of p42/p44 mitogen-activated protein kinase and of transcription factor NF-κB by TNF, and induction of cell death by staurosporine or chemotherapeutic drugs in INCL cells were unaffected by PPT1 deficiency. Resistance to TNF-induced apoptosis was also observed in embryonic fibroblasts derived from *Ppt1*/*Cln1*-deficient mice but not from mice with a targeted deletion of *Cln3* or *Cln5*. Finally, reconstitution of PPT1 activity in mutant cells was accompanied by resensitization to TNF-induced caspase activation and toxicity. These observations emphasize for the first time the role of PPT1 and, likely, protein depalmitoylation in the regulation of TNF-induced apoptosis.

1. Introduction

Considerable advances have been made in the characterization of the intracellular signaling events emanating from the tumor necrosis factor (TNF) receptors [1–4]. These receptors are expressed in almost all cell types and signal both cell death and survival [5,6]. TNFR1 signaling appears to require the sequential formation of two spatially distinct complexes [7]. The first complex, which comprises TNFR1, the adaptor TRADD, the kinase RIP1 and TRAF2, has been proposed to promote survival and inflammation via the activation of the transcription factor NF-κB. The second complex (complex II) involves TRADD, FADD and procaspase-8 and -10, forms in the cytoplasm and initiates apoptosis. The balance between apoptotic and proliferative outcomes could be regulated by TNFRI internalization [8], expression of anti-apoptotic genes such as the caspase-8 inhibitor FLICE-inhibitory protein (c-FLIP) or receptor localization to membrane microdomains [4,9,10].

Besides this two-step model of apoptotic signaling by TNFR1, there is mounting evidence that this receptor can trigger a lysosome-dependent death through the action of some lysosomal proteases (for a recent review, see [11]). Indeed, TNFR1 activation results in acidic sphingomyelinase-mediated generation of ceramide, which binds and activates the aspartyl protease cathepsin D by autocatalytic processing [12]. This would lead to apoptosis via the cleavage of Bid in the acidic environment of the endolysosomal compartment [13]. In U937 leukemic cells, TNF-induced cell death was associated with the release of cathepsin D and cytosol acidification [14]. In addition, involvement of the lysosomal cysteine protease cathepsin B in TNF-mediated apoptotic signaling has been shown in cathepsin B knockout hepatocytes that displayed resistance to TNF-induced mitochondrial release of cytochrome *c*, caspase activation, and

Abbreviations: Ac-DEVD-AMC, Ac-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin; INCL, Infantile Neuronal Ceroid Lipofuscinosis; MAPK, Mitogen-Activated Protein Kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NCL, Neuronal Ceroid Lipofuscinosis; PPT1, Palmitoyl protein thioesterase 1; TNF, Tumor Necrosis Factor-α; TNFR, TNF receptor

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apoptosis [15]. Similarly, immortalized murine embryonic fibroblasts from cathepsin B and L-deficient mice appear highly resistant to TNF [16]. The implication of cathepsin B in apoptosis has also been reported using TNF-treated tumor cells [17–20]. Finally, the well-known serine endopeptidase, chymotrypsin B, has been found to be released from the lysosomes in the cytosol of TNFα-treated rat hepatoma cells, an event that preceded mitochondrial alterations and apoptosis [21].

In addition to the implication of cathepsins in apoptosis, another lysosomal protease, palmitoyl protein thioesterase 1 (PPT1; EC 3.1.2.22) might also be involved in cell death signaling. Over-expression of PPT1 could protect human neuroblastoma cells against C_2-ceramide-induced caspase-3 activation and DNA fragmentation [22]. Conversely, PPT1 inhibition increased the susceptibility of these cells to apoptosis [23]. Moreover, PPT1-deficient lymphoblasts were more sensitive to apoptosis than control lymphoblasts [24]. PPT1 is targeted to the lysosome in a mannose 6-phosphate-dependent manner [25,26] and possesses, at least *in vitro*, thioesterase activity against fatty acid thioesters, including palmitoyl-CoA and fatty acid-modified proteins, such as H-Ras/p21 [27]. However, its role in the regulation of S-palmitoylated proteins within cells is not yet elucidated. Mutations in the *CLN1* gene encoding PPT1, located on chromosome 1p32, result in the infantile form of neuronal ceroid lipofuscinosis (INCL; MIM256730) [28], a human lysosomal storage disorder characterized clinically by early visual loss and progressive neurodegeneration due to a massive neuronal death [29–31]. In mice, targeted disruption of *Ppt1/Cln1* causes a severe neurological disorder closely resembling human NCL. Neuropathological findings include widespread neuronal loss accompanied by glial activation and apoptosis [32–34].

The present study aimed at investigating the specific role of PPT1 in TNF-induced apoptosis, by using fibroblasts isolated from patients affected with INCL. Here, we show that TNF-induced cell death signaling was strongly inhibited in INCL as compared to control cells. The same observation was made in Ppt1-deficient murine fibroblasts. Moreover, correction of the lysosomal enzyme defect in INCL cells restored their sensitivity to TNF, highlighting the importance of PPT1 in cell death triggered by the cytokine.

2. Materials and methods

2.1. Reagents

Human and murine recombinant TNF and human recombinant TRAIL were purchased from Peprotech-Tebu (Le-Perray-en-Yvelines, France). Anti-CD95 was obtained from Immunotech (Marseille, France). Ac-Asp-Glu-Val-Asp-aminomethylcoumarin (Ac-DEVD-AMC) was from Bachem (Voisins-Le-Bretonneux, France). DMEM, trypsin-EDTA, fetal calf serum (FCS), penicillin and streptomycin were from Invitrogen (Cergy-Pontoise, France). Other reagents, including 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and cycloheximide, were from Sigma (Lisle d'Abeau, France).

2.2. Cell lines and transfections

Human SV40 large T-transformed skin fibroblasts derived from normal individuals or from three INCL patients were obtained as previously described [35]. Cells from these patients, who all carried the R151X mutation, were obtained from the laboratory of Dr. S. Hofmann (University of Texas Southwestern Medical Center, Dallas, TX) or Dr. O. van Diggelen (Erasmus University, Rotterdam, The Netherlands), These cells were grown in a humidified 5% CO_2 atmosphere at 37 °C in DMEM medium containing Glutamax (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% heat-inactivated FCS. Fibroblasts isolated from one INCL patient were transfected using Superfect (Quiagen, Courtaboeuf, France) with 5 μg of either pRcCMV or pRcCMV-hPPT1 plasmid (a kind gift from Dr. S. Hofmann). Transfected cells were then maintained in the presence of G418 (0.4 mg/ml).

Fibroblasts from *Cln1*−/−, *Cln3*−/− and *Cln5*−/− mice were obtained from the Department of Molecular Medicine, Helsinki (Finland). *Ppt1/Cln1*-deficient cells have an entire deletion of the *Cln1* exon 4 leading to a premature termination codon in exon 3 [33]. In *Cln3*−/− mice, a deletion of exons 1 to 6 reproduces a knockout model for juvenile NCL [36]. In *Cln5*-deficient animals, a model for Finnish variant late-infantile NCL, exon 3 was disrupted by a neomycin resistance cassette, inducing a frameshift and a premature stop codon in exon 4 [37]. All the mouse strains were inbred into the C57/BL genetic background and fibroblasts from C57/BL mice were used as controls.

2.3. Cytotoxicity assay

Subconfluent cells were incubated in DMEM medium containing 1% FCS and cycloheximide in the presence or absence of the indicated drug or cytokine. Then, cell viability was evaluated by using the tetrazolium-based MTT assay [38].

2.4. Fluorogenic DEVD cleavage enzyme assay

After incubation with TNF and cycloheximide, cells were sedimented and washed with PBS. Cell pellets were homogenized in 10 mM HEPES (pH 7.4), 42 mM KCl, 5 mM $MgCl_2$, 0.5% CHAPS, 1 mM dithiothreitol, 1 mM PMSF, and 2 μg/ml leupeptin. Reaction mixtures contained 100 μl of cell lysates and 100 μl of 40 μM Ac-DEVD-AMC. After 30 min incubation at room temperature, the amount of the released fluorescent product aminomethylcoumarin was determined at 351 and 430 nm for the excitation and emission wavelengths, respectively.

2.5. Release of cytochrome c

After incubation with TNF and cycloheximide, cells were sedimented and washed with PBS. Cell pellets were resuspended in 5 volumes of ice-cold homogenization buffer (20 mM HEPES/KOH (pH 7.4), 1 mM EDTA, 0.1% fatty acid-free BSA, 250 mM sucrose, 1 mM dithiothreitol, 0.1 mM PMSF, 20 μg/ml leupeptin, 10 μg/ml aprotinin and 10 μg/ml pepstatin A). After swelling for 10 min on ice, cells were homogenized by 15 strokes of a loose-fitting Dounce homogenizer. The suspension was then centrifuged at 750 *g* for 5 min at 4 °C, and post-nuclear supernatants were centrifuged at 10,000 *g* for 15 min at 4 °C. Equal amounts of protein were then analyzed by SDS-PAGE (15% gel) and Western blotting by using 1 μg/ml of anti-cytochrome *c* mAb (Becton-Dickinson, Le-Pont-de-Claix, France).

2.6. Western blot analyses

Equal amounts of proteins were separated in a 10 to 15% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). Proteins were detected using an ECL detection system (Pierce). Caspase-3 was detected by using a rabbit polyclonal antiserum given by Dr. D. Nicholson, Merk-Frosst Centre for Therapeutic Research (Pointe Claire-Dorval, Québec, Canada); caspase-8 with a rabbit polyclonal antiserum given by Dr. M. Peter, The Ben May Institute for Cancer Research (Chicago, IL); Bid and IκB-α by using rabbit polyclonal antibodies (Cell Signaling, Le-Perray-en-Yvelines, France and Santa Cruz Biotechnology, Santa Cruz, CA); p42/p44 phosphorylated MAPK and Ser32/36-phosphorylated IκB-α by using a mouse monoclonal antiserum (Cell Signaling). Goat anti-rabbit and anti-mouse secondary antibodies were from Cell Signaling. An anti-β-actin (Sigma) or an anti-ERK2 (Santa Cruz-Tebu) was used as controls for protein loading.

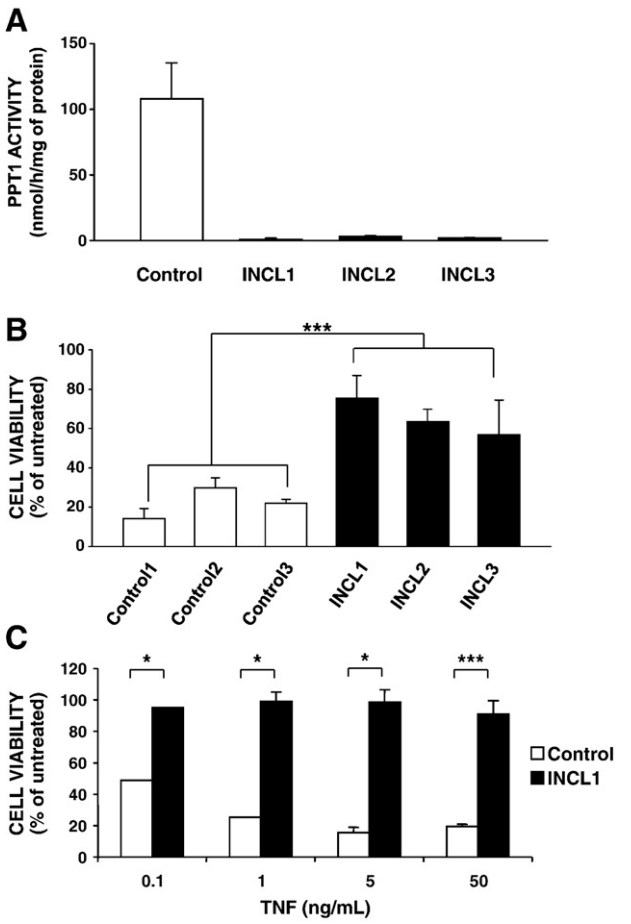

Fig. 1. INCL fibroblasts are partially resistant to TNF-induced cytotoxicity. (A) PPT1 activity in transformed fibroblasts from control subjects (mean of three different cell lines) or three different INCL patients (means ± S.E.M. of 3–9 independent determinations). (B) Control and INCL SV40-transformed fibroblasts were incubated for 16 h in DMEM medium containing 1% FCS and 50 μg/ml cycloheximide in the presence or absence of human TNF (50 ng/ml). Cell viability was assessed using the MTT assay, and is expressed as percentage of the viability of cells treated with cycloheximide only. Results are means ± S.E.M. of 3–7 independent determinations (all in triplicate). (C) Viability of control (mean of three different cell lines) and INCL1 transformed fibroblasts that were incubated for 16 h in 1% FCS in the presence of 50 μg/ml cycloheximide and the indicated concentration of TNF. Results are means ± S.E.M. of 3 independent experiments (for the 0.1 and 1 ng/ml TNF concentrations, the error bars in the control cells are too small to be seen).

2.7. Palmitoyl protein thioesterase 1 assay

PPT1 activity was assayed using 4-methylumbelliferyl-β-D-6-thiopalmitoyl-glucoside (Moscerdam, Rotterdam, The Netherlands), as reported [39]. Cell pellets were homogenized in 200 μl distilled water and briefly sonicated. Reaction mixtures contained 10 μl of cell lysate and 20 μl of substrate preparation (0.5 mM substrate, 1.5 mM dithiothreitol and 1 U β-glucosidase). After 1 hour incubation at 37 °C, the reaction was stopped by adding 2 ml of glycine/NaOH 0.2 M (pH 10.5) buffer. The amount of the released fluorescent product 4-methylumbelliferone was determined by fluorometry at 358 and 448 nm for the excitation and emission wavelengths, respectively. Protein concentration was measured with the Bio-Rad dye reagent using BSA as a standard.

2.8. TNFR1 expression analysis by flow cytometry

TNFR1 expression was analyzed on resting cells. Cells were detached using PBS containing 10 mM EDTA, sedimented at 4 °C and washed with PBS. Cells were incubated at 4 °C in the dark for 30 min with FITC-conjugated mouse anti-human TNFR1 (R&D Systems, Lille, France). Isotype control monoclonal antibody (Immunotech) was used as a negative control. Cytometric analyses were performed on a FACScan (Becton-Dickinson) flow cytometer.

2.9. Statistical analyses

Data are presented as means ± S.E.M. The Student's *t*-test was used for statistical analysis (*$p<0.05$; **$p<0.01$; ***$p<0.001$ versus control).

3. Results

3.1. TNF-induced apoptosis of transformed fibroblasts requires PPT1 activity

To evaluate the functional role of PPT1 in TNF-mediated cell death, we used a genetic model, i.e., SV40-transformed fibroblasts derived from patients affected with INCL. As compared to fibroblasts derived from control individuals, PPT1 enzymatic activity in INCL cells was severely impaired (Fig. 1A). In contrast, the activities of other lysosomal enzymes (e.g., β-galactosidase and β-hexosaminidase) in INCL cells were not different from those in controls (data not shown). The cytotoxic effect of TNF on control cells was dose-dependent (Fig. 1C), leading to a reduction in cell viability of ~70% after 16 h exposure to 50 ng/ml TNF (Fig. 1B and C). Under all concentrations tested, INCL fibroblasts were more resistant to the lethal effect of TNF than their normal counterparts. This finding was confirmed on several cell lines derived from different normal subjects or patients affected with INCL (Fig. 1B), demonstrating the involvement of PPT1 in TNF-induced cytotoxicity on human transformed fibroblasts.

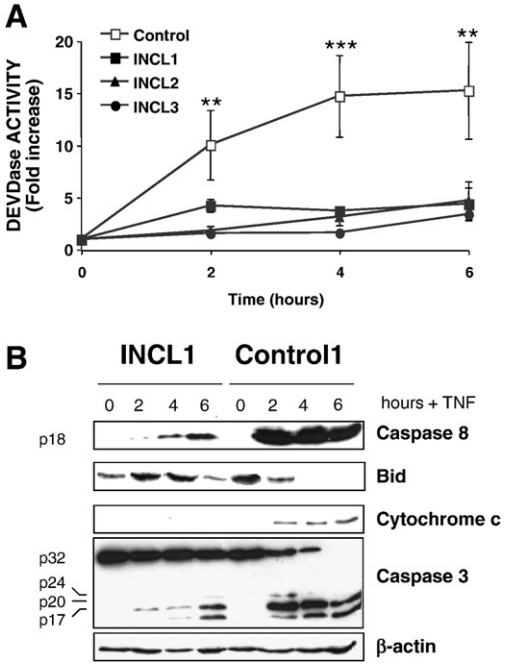

Fig. 2. The TNF-induced apoptotic cascade is impaired in INCL fibroblasts. (A) Control (mean of three different cell lines) and three different INCL transformed fibroblasts were incubated in medium containing 1% FCS with 50 ng/ml human TNF and 50 μg/ml cycloheximide for the indicated times. Cells were then harvested and DEVDase activity was determined. Data are means ± S.E.M. of 3–7 independent experiments. (B) Control1 and INCL1 fibroblasts were treated for the indicated periods with 50 μg/ml cycloheximide and 50 ng/ml TNF. Cell lysates and cytosolic extracts were prepared as described in Materials and methods, and analyzed by Western blotting for caspase-8 cleavage, Bid proform disappearance, cytochrome *c* release, and caspase-3 cleavage. An anti-β-actin was used as a control for protein loading. Results are representative of 3 independent experiments. Similar results were obtained using the INCL2 cell line.

Because TNFR1 is a well-known death receptor able to signal cell death, the implication of PPT1 in the apoptotic cascade activated by TNF in INCL transformed fibroblasts was examined. Determination of the global executioner caspase activity with the fluorogenic tetrapeptide substrate Ac-DEVD-AMC, which contains the cleavage site found in numerous caspase-3 and caspase-7 targets, showed that TNF-induced caspase processing was strongly inhibited in INCL cells (Fig. 2A). This effect on caspase-3-like activity was further demonstrated by Western blot analysis of individual caspases. As illustrated in Fig. 2B, the cleavage of caspase-3 (i.e., the decrease in the procaspase form and appearance of cleaved forms) triggered by TNF was considerably reduced and delayed in INCL cells. TNF treatment of control cells resulted in cleavage of the initiator caspase-8, which occurred quite concomitantly to that of effector caspases (Fig. 2B). This cleavage was accompanied by the complete disappearance of Bid, a member of the Bcl-2 family known as a substrate of caspase-8, and the progressive release of cytochrome *c* into the cytosol. Processing of both caspase-8 and Bid, as well as the release of cytochrome *c* were impaired in PPT1-deficient fibroblasts, further implicating this enzyme in the apoptotic cascade initiated by TNF with a site of action likely lying upstream of mitochondria.

3.2. PPT1 is involved in cell death triggered by death receptors but not receptor-independent cytotoxic agents

To explore further the site of action of PPT1 in the apoptotic cascade and to test whether PPT1 is involved only in TNF-induced apoptosis, control and mutant fibroblasts were exposed to TRAIL and a Fas/CD95 agonist (i.e., an anti-Fas antibody), two members of the TNF superfamily. These two molecules can induce apoptosis via the death receptors DR4 or DR5, and Fas/CD95, respectively. As observed upon TNF stimulation, INCL fibroblasts also resisted significantly to TRAIL and anti-Fas-mediated apoptosis (Fig. 3A). INCL fibroblasts were then challenged with the protein kinase inhibitor staurosporine and the anticancer drugs doxorubicin and etoposide, all stimuli that have been shown to promote apoptosis by activating the mitochondrial pathway. Fig. 3B demonstrates that the cytotoxic effects of these agents were similar in control and INCL cells. These observations suggest that whereas PPT1 can modulate the apoptotic pathway initiated by distinct death receptors, it does not affect receptor-independent cytotoxic signaling.

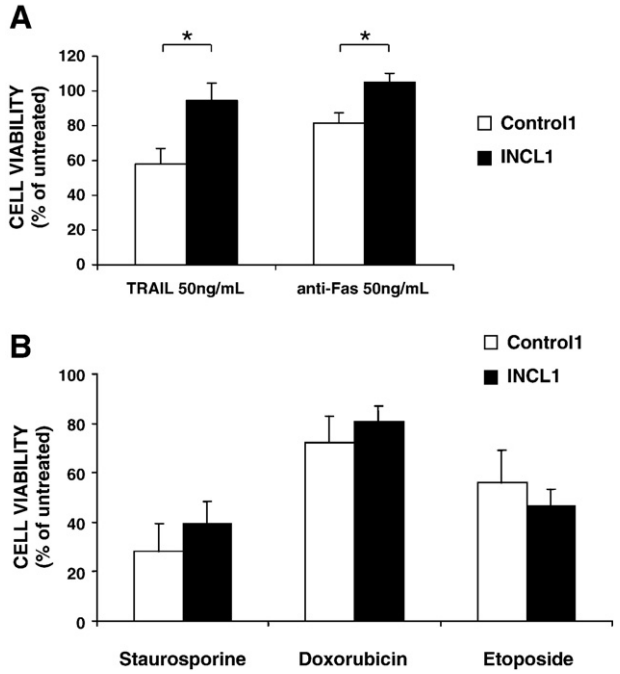

Fig. 3. INCL fibroblasts are partially resistant to TRAIL and anti-Fas but not to staurosporine and chemotherapeutic drugs. (A) Viability of control1 and INCL1 transformed fibroblasts that were incubated for 16 h in the presence of 50 μg/ml cycloheximide and the indicated concentration of TRAIL or anti-Fas. Cell viability was assessed using the MTT assay, and is expressed as percentage of the viability of cells treated with cycloheximide only. Results are means ± S.E.M. of 3 independent determinations (all in triplicate). (B) Control1 and INCL1 transformed fibroblasts were incubated for 24 h in DMEM medium containing 1% FCS with 100 nM staurosporine, 0.5 μM doxorubicin or 50 μM etoposide. Cell viability was assessed using the MTT assay. Results are means ± S.E.M. of 3–7 independent determinations (all in triplicate).

3.3. TNFR1 expression, and TNF-induced activation of p42/p44 MAPK and NF-κB do not require a functional PPT1

To test whether the impairment of TNF-induced toxicity in INCL fibroblasts might be due to an abnormal expression of TNFR1, the cell surface expression of this receptor was assessed by flow cytometry. Of note, INCL and normal cells expressed similar levels of TNFR1 at the plasma membrane (50% versus 41% in INCL and control cells, respectively) (Fig. 4A).

Ligation of TNFR1 not only signals cell death but also cell survival and can promote cell proliferation in some situations [2]. To investigate the role of PPT1 in TNF-induced cell survival signals, we compared the effects of TNF on p42/p44 MAPK and NF-κB activation in control and INCL fibroblasts (Fig. 4B). p42/p44 MAPK was found to be activated as soon as 15 min after exposure to TNF. Both kinetics and amplitude of p42/p44 MAPK activation were similar in control and mutant cells. Moreover, TNF elicited IκB phosphorylation and degradation in INCL as well as in control cells, suggesting that NF-κB was efficiently activated in PPT1-deficient cells. These findings indicate that PPT1 is preferentially implicated in the cytotoxic action of TNF but does not interfere with the cytokine-induced cell survival signaling.

3.4. Enzymatic correction of INCL fibroblasts restores their sensitivity to TNF

To establish the role of PPT1 in TNF-mediated cell death, we used human PPT1-deficient fibroblasts that were stably transfected with a cDNA encoding human PPT1 (INCL2 PPT) and compared their response to the lethal effect of TNF with those of parental cells (INCL2) or mutant cells transfected with the corresponding empty vector (INCL2 neo). In PPT1-transfected cells, the activity of PPT1 was increased as compared to that found in mutant or even control fibroblasts (Fig. 5A). Importantly, expression of PPT1 cDNA in INCL cells restored the TNF sensitivity (Fig. 5B).

The sensitivity of INCL2 PPT cells was further confirmed by examining caspase activity. As shown in Fig. 5C, the cleavage of Ac-DEVD-AMC was significantly increased in PPT1-transfected cells within 2 h, peaking at 6 h post-treatment. No progressive increase was seen in mutant cells transfected with an empty vector. Moreover, Western blot analysis demonstrated that executioner caspase-3 processing was enhanced in INCL2 PPT cells, resembling that observed in normal fibroblasts (see Fig. 2B). Altogether, these data indicate that in human transformed fibroblasts PPT1 plays a critical role in TNF apoptotic signaling by regulating effector caspase activation.

3.5. Ppt1-deficient murine fibroblasts also resist TNF-induced killing

In order to consolidate our observations on human cells, we analyzed the responses to TNF in Ppt1-deficient cells obtained from transgenic mice with a targeted disruption of the *Ppt1/Cln1* gene [33]. Embryonic fibroblasts from *Cln1*−/− mice exhibited a deficient activity of PPT1 (Fig. 6A) and were resistant both to human and murine TNF-induced cell death (Fig. 6B), indicating that the impaired pathway is mediated by TNFR1 but not TNFR2 [40]. As observed in INCL human fibroblasts, the resistance to TNF was

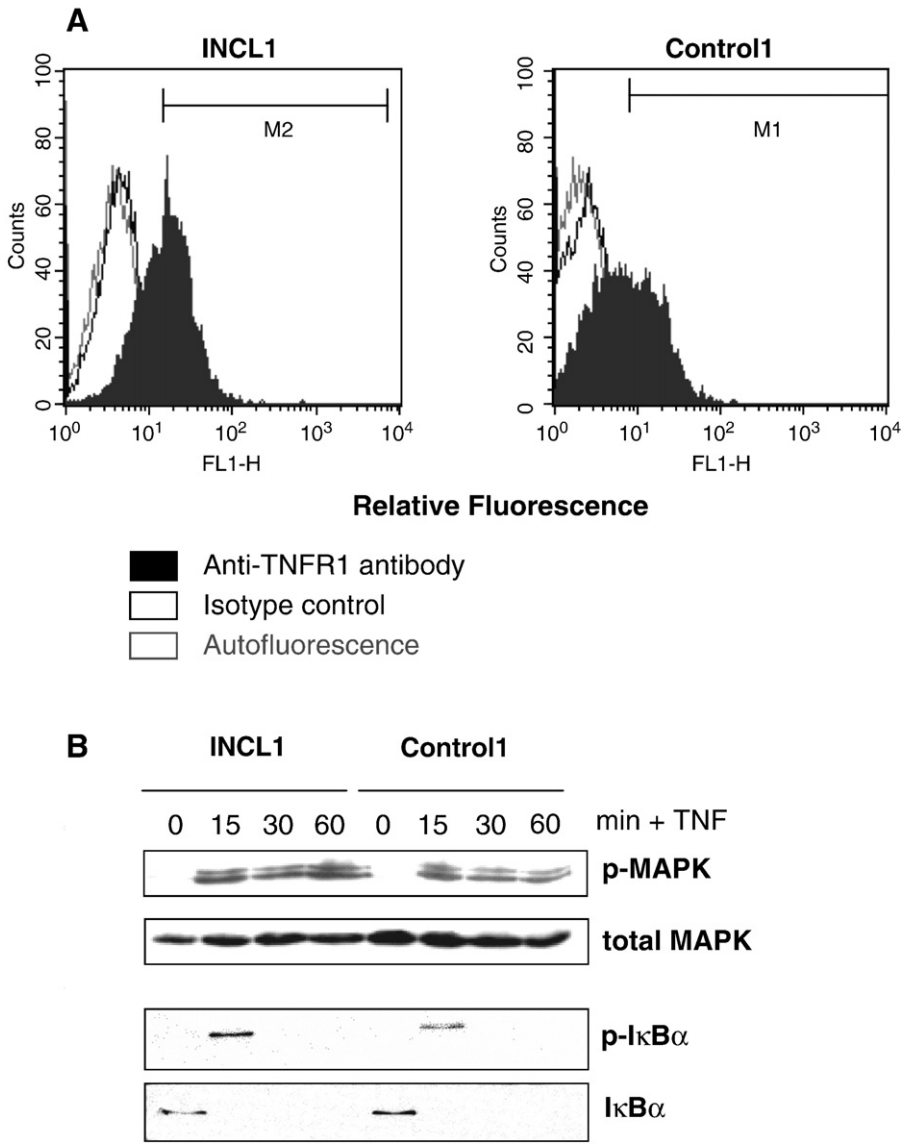

Fig. 4. TNF triggers activation of p42/p44 MAPK and NF-κB equally well in control and INCL fibroblasts. (A) Cell surface expression of TNFR1 in control1 and INCL1 fibroblasts, as assessed by flow cytometry. The mean fluorescence intensity of gated control and INCL cells was 17.2 and 26.1, respectively; 41% and 50% of the gated cells were positive for TNFR1. (B) TNF-induced activation of p42/p44 MAPK and NF-κB. Cells were incubated in 1% FCS with or without 50 ng/ml human TNF and 50 μg/ml cycloheximide for the indicated times, and then harvested. Expression of activated (dually phosphorylated; p-MAPK) or total MAPK (as a loading control), as well as phosphorylated (p-IκBα) and IκBα, was evaluated by Western blot. Results are representative of 3 independent experiments.

associated with a defective cleavage of caspase 3 and Bid (Fig. 6C). However, as observed in human cells, TNF-induced activation of p42/p44 MAPK (Fig. 6D) and NF-κB (data not shown) remained unaffected in *Cln1*−/− cells.

3.6. TNF-induced apoptosis is not altered in the CLN3 and CLN5 murine models of ceroid lipofuscinosis

INCL belongs to the group of neuronal ceroid lipofuscinoses (NCLs) which comprise different inherited diseases, all characterized by lysosomal storage of autofluorescent lipopigments and by a neurodegenerative process [41]. In order to test whether the resistance to TNF-induced apoptosis was a common feature to all ceroid lipofuscinoses, we analyzed the response to TNF of murine fibroblasts carrying a deletion in the *Cln3* and *Cln5* genes responsible for the juvenile and variant late-infantile forms of NCL, respectively. As illustrated in Fig. 7A, control, *Cln3*−/− and *Cln5*−/− cells were equally sensitive to the cytotoxic effect of TNF. Moreover, TNF triggered a similar cleavage of caspase-3 in embryonic fibroblasts from *Cln3*−/− and *Cln5*−/− mice (Fig. 7B). Thus, the resistance of PPT1-deficient cells to TNF-induced apoptosis is not a common feature to all forms of NCL.

4. Discussion

Accumulating evidence suggests that in addition to caspases, lysosomal proteases serve as important mediators in programmed cell death [11]. This lysosomal pathway of cell death might even be exacerbated in transformed and immortalized cells [16]. By using transformed fibroblasts, we show for the first time that PPT1, an enzyme that removes long-chain fatty acids from lipid-modified cysteine residues in proteins [42,43], represents one of the lysosomal regulators of TNF-induced apoptosis. This conclusion is supported by two lines of evidence. First, both human and murine PPT1-deficient fibroblasts were more resistant to TNF-induced cytotoxicity than their normal counterparts. Secondly, reconstitution of PPT1 activity in INCL cells restored susceptibility to killing. In addition, in other models of NCL where PPT1 integrity is preserved, such as in *Cln3*−/− and *Cln5*−/− cells, TNF apoptotic signaling was not affected. The participation

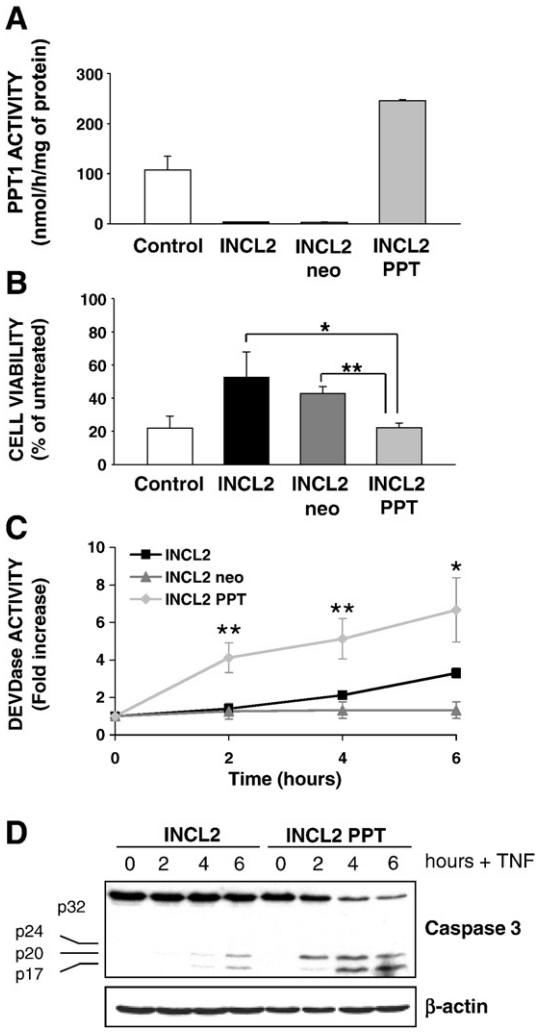

Fig. 5. Enzymatic correction of PPT1 activity restores TNF-induced apoptosis in INCL fibroblasts. (A) PPT1 enzyme activity of control and INCL2 fibroblasts that were untreated, transfected with the empty vector (INCL2 neo) or the pRcCMV-hPPT1 vector (INCL2 PPT). Results are representative of 3–9 independent experiments. (B) Cell viability of control, INCL2, INCL2 neo and INCL2 PPT fibroblasts that were incubated for 16 h in DMEM medium containing 1% FCS and 50 μg/ml cycloheximide in the presence or absence of human TNF (50 ng/ml). Results are means ± S.E.M. of 3–8 independent determinations (all in triplicate). (C) DEVDase activity in control, INCL2, INCL2 neo and INCL2 PPT fibroblasts that were incubated in medium containing 1% FCS with 50 ng/ml TNF and 50 μg/ml cycloheximide for the indicated times. Data are means ± S.E.M. of 3–4 independent experiments. (D) Analysis of caspase-3 cleavage in INCL2 and INCL2 PPT fibroblasts treated with 50 ng/ml TNF and 50 μg/ml cycloheximide for the indicated times. An anti-β-actin was used as a control for protein loading.

of PPT1 in TNF-induced apoptosis was also demonstrated by the fact that caspase activation, cleavage of Bid, and the subsequent release of mitochondrial cytochrome *c*, were all significantly reduced in PPT1-deficient fibroblasts. However, other TNF activities than cell death induction, such as p42/p44 MAPK and NF-κB activation, remained unaffected in mutant cells, suggesting that PPT1 is not involved in TNF-induced cell survival signaling.

4.1. Place of PPT1 in TNF-induced apoptosis

Consistent with previous observations that cleavage of Bid, a pro-apoptotic member of the Bcl-2 family which controls the mitochondrial checkpoint of apoptosis, is the most likely route for lysosome-directed apoptosis [44], the present study suggests that the site of action of PPT1 lies upstream of mitochondria. The observation that drugs such as staurosporine induced apoptosis equally well in control and PPT1-deficient cells reinforces the notion that the apoptotic signaling components at and downstream the mitochondrion are intact in INCL cells. How PPT1 regulates the apoptotic machinery that operates downstream of TNFR1 and upstream of mitochondria is still unclear. PPT1 is a depalmitoylating enzyme that has been reported to release, *in vitro*, palmitate from the small GTP-binding proteins, including p21Ras [43]. Its intracellular physiological substrates, however, are still unknown. Moreover, despite the lysosomal targeting of PPT1 [26], its enzymatic activity has a neutral pH optimum, and extralysosomal localization of PPT1 protein has been reported in neurons [43,45], suggesting that PPT1 functions might not be limited to lysosomes [27]. By controlling S-palmitoylation of signaling proteins, a dynamic process regulating protein function [46–48], PPT1 could influence protein distribution between membrane compartments or subdomains within the same membrane or could be implicated in protein–protein interactions. For example, some early steps of TNFR1-mediated signaling, including initiation of apoptosis, have been reported to take place in caveolae-like domains [9,49–51],

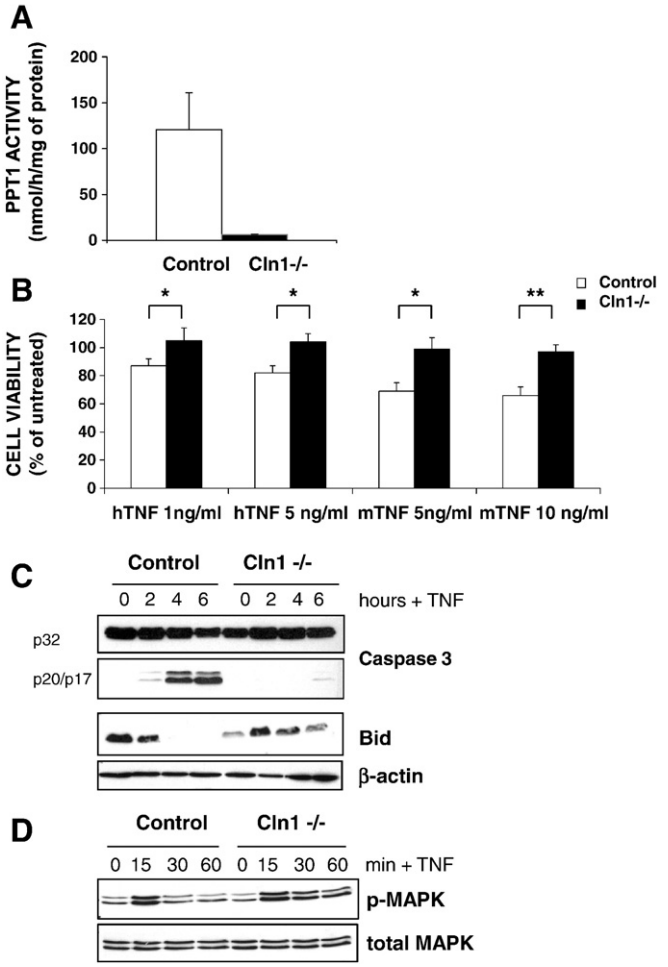

Fig. 6. *Ppt1/Cln1*-deficient murine fibroblasts behave as INCL human fibroblasts with regard to TNF-induced cytotoxicity. (A) Ppt1 activity in control or *Cln1*−/− murine transformed fibroblasts (means ± S.E.M. of 3 independent determinations). (B) Control and *Cln1*−/− murine fibroblasts were incubated for 16 h in DMEM medium containing 1% FCS and 10 μg/ml cycloheximide in the presence of the indicated concentration of human or murine TNF. Cell viability was assessed using the MTT assay, and is expressed as percentage of the viability of cells treated with cycloheximide only. Results are means ± S.E.M. of 5–7 independent determinations (all in triplicate). (C) Control and *Cln1*−/− murine fibroblasts were treated for the indicated periods with 10 μg/ml cycloheximide and 10 ng/ml of murine TNF. Cell lysates were prepared and analyzed by Western blotting for caspase-3 cleavage and Bid proform disappearance; β-actin was used as a loading control. (D) Control and *Cln1*−/− murine fibroblasts were treated for the indicated periods with 50 ng/ml of human TNF. Cell lysates were analyzed by Western blotting for MAPK phosphorylation as in Fig. 4B.

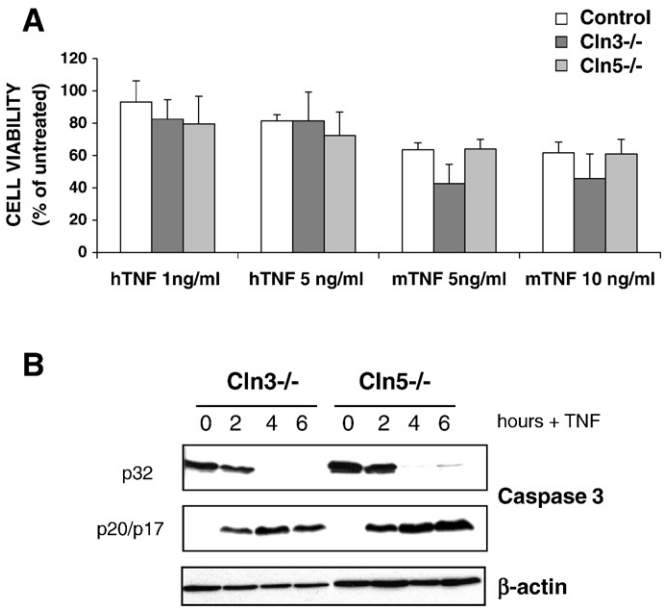

Fig. 7. TNF-induced apoptosis is not altered in other murine models of ceroid lipofuscinosis. (A) Control, *Cln3*−/− and *Cln5*−/− murine transformed fibroblasts were incubated for 16 h in DMEM medium containing 1% FCS and 10 μg/ml cycloheximide in the presence of the indicated concentration of human or murine TNF. Cell viability was assessed using the MTT assay, and is expressed as percentage of the viability of cells treated with cycloheximide only. Results are means ± S.E.M. of 3 independent determinations (all in triplicate). (B) *Cln3*−/− and *Cln5*−/− murine fibroblasts were treated for the indicated periods with 10 μg/ml cycloheximide and 10 ng/ml of murine TNF. Cell lysates were prepared and analyzed by Western blotting for caspase-3 cleavage; β-actin was used as a loading control. For control cells, see Fig. 6C.

which represent specialized microdomains of the plasma membrane enriched in sphingolipids, cholesterol [52,53] and several palmitoylated proteins [46]. It is therefore possible that PPT1 regulates cytokine-induced cell death by affecting protein association within lipid rafts.

Consistent with this hypothesis, recent findings demonstrated that palmitoylation of the death receptor Fas, which belongs to the TNF superfamily, was essential for its localization into lipid rafts and its endocytosis [54,55]. Fas palmitoylation appears as a prerequisite to the apoptotic complex (DISC) assembly into endosomes and the correct initiation of apoptosis [56]. As reported for Fas, TNF-induced apoptosis was shown to require also TNF receptor internalization whereas TNF-triggered survival pathways did not [57]. It is thus conceivable that, as described for Fas, palmitoylation of TNFR1 influences its internalization and the subsequent TNF-induced apoptosis. This hypothesis would be in accordance with our findings that PPT1 deficiency affects only the TNF-initiated cell death pathway but not the survival one. Two observations would support this hypothesis. First, a defect in receptor-mediated endocytosis was reported in PPT1-deficient fibroblasts [58]. Second, although palmitoylation of TNFR1 has not yet been documented, analysis of its protein sequence reveals the presence of a cysteine residue (Cys248) that resides just downstream the transmembrane domain, i.e., in an equivalent site where the palmitoylated cysteine in Fas is found (data not shown). However, this exciting hypothesis needs detailed examination to be confirmed.

PPT1 could also influence lipidic plasma membrane composition. Recent findings revealed that PPT1-deficient neurons express an increased amount of ATP synthase at the plasma membrane and show an increased uptake of apolipoprotein A1 [59]. This is associated with a dysregulated metabolism of cholesterol [60], a major component of plasma membrane and particularly of lipid rafts. Furthermore, PPT1 was shown to localize into lipid rafts and to regulate their lipid content in CHO cells [61]. Such modifications in lipid raft composition may perturb receptor signaling.

Another possible mode of action of PPT1 in modulating the TNFR1 death signal would be the promotion of complex II formation or the activation of a factor controlling initiator caspase or Bid cleavage. Conversely, PPT1 may modulate TNFR1-induced apoptosis by triggering the depalmitoylation and degradation of an anti-apoptotic molecule (e.g., cFLIP) or more indirectly that of a factor that regulates the expression levels of anti-apoptotic molecules. Again, these hypotheses await further characterization of the PPT1 substrates to be tested. Alternatively, depalmitoylation of p21Ras by PPT1 may alter its function [46] and subsequently influence TNFR1 signaling since Ras activation has been reported to modulate TNF-induced apoptosis [62]. A further possibility is that the palmitate released by PPT1 is used for generating a pro-apoptotic messenger, such as ceramide [63]. However, the quantity of palmitate produced by PPT1 is likely to be insufficient to account for a substantial ceramide generation upon TNF stimulation, which is considered to occur mostly through sphingomyelin hydrolysis [64]. Finally, the possibility that the lysosomal ceroid storage present in INCL cells somehow affects TNFR1 apoptotic signal transduction, for instance by impairing release of cathepsins, cannot be totally excluded.

4.2. Pathophysiology of apoptosis in PPT1 deficiency

The major pathological findings in patients affected with INCL, as well as *Ppt1*-deficient mice include accumulation of autofluorescent lipopigments (GRODs), neuronal loss and glial activation [30,65] but apoptosis in the cortex and retina have also been reported [29,32]. In line with the link to apoptotic cell death, overexpression of PPT1 has been described to protect human neuroblastoma cells from apoptosis induced by short-chain ceramide or a phosphatidylinositol 3-kinase inhibitor [22]. Reduction of PPT1 expression in the same cell line by antisense oligonucleotides enhanced the susceptibility to cell death [23]. The data of the present study on TNF-induced apoptosis of transformed fibroblasts and the above findings may seem at odds. However, some discrepancies have already been reported regarding PPT1 processing in neurons and fibroblasts [66]. Furthermore, whereas PPT1 deficiency increased the amount of the F1-ATP synthase at the plasma membrane of neurons, no similar modifications were observed in fibroblasts [59]. On the other hand, the nature of PPT1 substrates in neurons and fibroblasts may differ; alternatively, other acyl-protein thioesterases might be differentially expressed in these cell types. In this respect, the possibility that the cytosolic acyl-protein thioesterase 1 [67] modulates TNF-induced apoptosis cannot be ruled out. In addition, PPT1 is present in the synaptosomes and synaptic vesicles of neurons, and in presynaptic terminals of axons [45]. It is therefore possible that PPT1 also acts extracellularly in the nervous system, and that a defect in this activity accounts for a toxicity on neuronal cells. Finally, in contrast to the rather selective defect of TNF-induced apoptosis we observed, the endoplasmic reticulum and oxidative stresses seen in PPT1-deficient neurons [68–70] appear to be common manifestations of NCLs and lysosomal storage disorders [71].

NCLs are diseases due to mutations in at least eight distinct genes. Despite this genetic heterogeneity, they share common clinical features that could suggest common pathogenic mechanisms. This is why we assessed whether TNF-induced apoptosis was also altered in CLN3 and CLN5-deficient fibroblasts. Emerging evidence indicates that there exists some functional relationship between the different CLN gene products [72,73]. However, in our study, murine *Cln3*−/− and *Cln5*−/− fibroblasts did not behave as their *Cln1*−/− counterparts, as they did not resist TNF-induced cell death. As to the susceptibility to TNF of fibroblasts with cathepsin D deficiency (a defect underlying CLN10), discordant results have been reported [13,74]. Thus, resistance to TNF-induced apoptosis may not be a

common pathogenic mechanism to all forms of NCL. Whereas the mechanism of dysregulation of apoptosis in cells harboring a defect in PPT1 activity might be indirect and still remains to be elucidated, it appears that this protein thioesterase plays a key role in the regulation of mammalian cell death. Interestingly, further indication that PPT1 has a prominent role in the regulation of cell death has been provided in *Drosophila* where its increased expression dose-dependently led to apoptosis of photoreceptors [75].

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