Serine protease inhibitor 2A inhibits caspase-independent cell death

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Abstract The release of cysteine cathepsins from the lysosome into the cytoplasm can trigger programs of cell death (PCD) that do not require caspase executioner proteases but instead are mediated by toxic reactive oxygen species (ROS). Here, we show that a cytoplasmic inhibitor of papain-like cathepsins – Serine protease inhibitor 2A (Spi2A) – is required for the protection of cells from caspase-independent PCD triggered by tumor necrosis factor-. In the absence of caspase activity, Spi2A suppressed PCD by inhibiting cathepsin B after it was released into the cytoplasm. Spi2A also directly protected against ROS-mediated PCD, which is consistent with a role in suppressing caspase-independent pathways of PCD. We conclude that inhibition of lysosomal executioner proteases by Spi2A is a physiological mechanism by which cells are protected from caspase-independent programmed cell death.

Keywords: Programmed cell death; Cathepsin B; Serine protease inhibitor 2A; Caspase-independent cell death; Lysosomal pathway of cell death

1. Introduction

Programmed cell death (PCD) is an active process by which signaling events induce the death of cells [1]. Classical apoptosis is the principle program of cell death in many developmental and physiological settings. Recent findings indicate that PCD can occur in the complete absence of caspase activity [2]. Like classical apoptosis, caspase-independent PCD can be triggered by death receptors, and results in the depolarization of mitochondria, generation of ROS and the release of apoptogenic factors like cytochrome *c* and apoptosis-inducing factor (AIF) [2]. This means that even in the absence of caspase activity the cell is still subjected to factors that still provoke PCD.

Lysosomal proteases called cathepsins can trigger the whole spectrum of morphologically distinct programs of cell death ranging from coagulative necrosis to classical apoptosis [3]. Depending on the cell type and the stimuli, cathepsins, most notably cathepsin B, released from the lysosome into the cytoplasm may function upstream [4,5] or downstream [6] of caspases. In tumor cells, following stimulation of tumor necrosis factor-α (TNF-α) receptor 1 (TNFR1), the release of cathepsin B from lysosomes and the initiation of PCD in the cytoplasm can occur in the absence of caspase activity through mechanisms which are not fully understood [6]. It has been suggested that the potent caspase-independent PCD, which is unmasked by mutations in caspases [7] or the use of pharmacological inhibitors such as Z-VAD.fmk [8–10], represents a back-up mechanism for cell death which may be especially important in pathological situations. It is believed that in the absence of caspase activity cathepsins play a dominant role as executioner proteases acting both upstream or downstream of toxic ROS generated in the mitochondria [2].

The program of gene expression induced by RelA/NF-κB transcription factors is critical to the control of cell survival [11]. Ligation of TNFR1 not only induces PCD, but also activates NF-κB, which counteracts this process by activating the transcription of genes encoding inhibitors of classical caspase-dependent apoptosis. However, whether NF-κB can protect against caspase-independent PCD is not known. We have recently reported that NF-κB protects murine fibroblasts from the lysosomal pathway of PCD by upregulating the expression of the mouse gene *Serine protease inhibitor 2A* (*Spi2A*) (Accession number: M64085) [12,13]. Spi2A is an unusual member of the anti-chymotrypsin family of serine protease inhibitors (serpins) because it is not secreted and resides in the cytoplasm. Furthermore, it is a potent inhibitor of papain-like cysteine proteases such as cathepsin B and L [14]. Induction of Spi2A expression by NF-κB extinguished cathepsin B activity in the cytosol and protected cells from classical caspase-dependent apoptosis [14].

We report that Spi2A was required for the protection of cells from death caused by the ligation of TNFR1 in the presence of Z-VAD.fmk. In the absence of caspase activity, protection by Spi2A was accompanied by the suppression of mitochondrial depolarization and ROS production. Experiments with the redox-cycling drug Naphthazarin indicate a direct role for Spi2A in protecting against cell injury from ROS. Thus, inhibition of lysosomal executioner proteases is a cyto-protective mechanism by which NF-κB can protect against programs of cell death not dependent on classical caspase-dependent apoptosis.

2. Materials and methods

2.1. TNF-α death assays

NIH3T3 cells were transduced with MIGR1 retrovirus [15] encoding either GFP alone, or Spi2A in the forward (sense) or reverse (antisense) orientation and stable clones generated, as described previously [14]. Cells were treated with TNF-α (R&D) and after 16 h the number

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of live GFP-positive adherent cells were counted by flow cytometry [14]. Live cells were defined as those that excluded propidium iodide (PI-negative) and had the appropriate size, as defined by forward and side light scatter characteristics. Caspase activity was inhibited by pre-treatment of cells or extracts for 1 h with Z-VAD.fmk (ICN Biomedicals Inc; 50 μM). Complete inhibition of caspase activity was verified by enzyme assay [14]. The level of *Spi2A* mRNA was quantitated by real-time PCR using primers and probes specific for *Spi2A* [12] and *cyclofilin A* control mRNA [16], either before (Fig. 1B) or 4 h after (Fig. 2A) treatment with Z-VAD.fmk (50 μM) and TNF-α (10 ng ml^{-1}), as described previously [14].

2.2. ROS-mediated death assays

NIH3T3 cells were treated with the redox-cycling quinone Naphthazarin (5,8-dihydroxyl-1,4-naphthoquinone) (Sigma–Aldrich; 10 μM) and survival measured after 2 h at 37 °C, as described before [4]. Protection from ROS-mediated death was afforded by treatment with the anti-oxidant *N*,*N*′-diphenyl-*p*-phenylenediamine (DPPD) (Sigma–Aldrich; 1–2 μM), 30 min before addition of the quinone [17].

2.3. Death effector assays

Death effector pathways were induced by treatment of NIH3T3 cells with TNF-α (10 pg ml^{-1}) and Z-VAD.fmk (50 μM). Colorimetric assays for cathepsin B were performed on crude cytoplasmic extracts [18]. Briefly, NIH3T3 cells (10^6) were lysed in 10 mM Tris·Cl, pH 7.5, 100 mM NaCl, 1 mM EDTA, and 0.01% Triton X-100 (50 μl) for 30 min on ice, then centrifuged at $15\,000 \times g$ for 30 min at 4 °C and the supernatant recovered. Protein concentration was determined by Lowry assay (DC-protein assay kit, Biorad). Cathepsin B was assayed in reaction buffer using the *p*-Nitoaniline (*p*NA)-labeled substrate Z-RR-*p*NA (Calbiochem) [19]. Specific activity was determined by subtracting the apparent activity detected in the presence of the cathepsins B inhibitor, CA074-Me (30 μM) inhibitor (Peptide Institute) [14]. Mitochondrial membrane potential and ROS production was measured using the fluorescent dyes JC-1 (3 μg ml^{-1}) and Dihydroethidium (HE) (5 μM)(Molecular Probes), respectively, and flow cytometry according to the manufacturer's instructions.

3. Results

3.1. Spi2A protects from caspase-independent PCD

Complete inhibition of caspase activity by Z-VAD.fmk can sensitize normally resistant cells with wild-type levels of NF-κB to TNF-α-induced PCD [8,9]. In Fig. 1A, as expected [9], we show that complete inhibition of caspase activity by Z-VAD.fmk sensitized NIH3T3 fibroblasts to PCD by TNF-α.

Lysosomal cathepsins, such as cathepsin B, can induce PCD in the absence of caspase activity [6]. This raises the possibility that because Spi2A is a potent inhibitor of cathepsin B, it may protect against a caspase-independent program of cell death [20]. To address this, NIH3T3 cells were transduced with retrovirus encoding *Spi2A* (Spi2A cells) on a polycistronic mRNA with green fluorescent protein (GFP) and stable clones which express high levels of Spi2A generated [14,15]. The expression of *Spi2A* in transduced clones was verified by real-time PCR (Fig. 1B). In the absence of caspase activity Spi2A cells exhibited markedly improved survival against TNF-α (0.1–30 ng ml^{-1}), compared to cloned cells transduced with GFP alone (GFP cells) (Fig. 1C). Therefore, we conclude that Spi2A can protect against caspase-independent PCD.

3.2. Spi2A is a physiological inhibitor of caspase-independent PCD

To verify that cyto-protection from caspase-independent PCD mediated by Spi2A was not due to overexpression, we generated clones of NIH3T3 cells expressing *Spi2A* in an anti-sense orientation (Spi2A-A cells) [14]. We have shown before that treatment with TNF-α induces the expression of Spi2A in an NF-κB-dependent manner [14]. As expected, real-time PCR revealed that in the presence of Z-VAD.fmk, treatment with TNF-α resulted in the upregulation of Spi2A mRNA in control NIH3T3 cells [16] (Fig. 2A). Importantly, after treatment with TNF-α, the upregulation of endogenous *Spi2A* mRNA was abrogated in stable clones of Spi2A-A cells (Fig. 2A).

In the absence of caspase activity, the inhibition of endogenous *Spi2A* mRNA expression by anti-sense message resulted in a marked increase in the susceptibility of cells to TNF-α-induced PCD, which was measured over the picomolar range of concentrations (0.03–10 pg ml^{-1}; Fig. 2B). Higher concentrations of TNF-α (nanomolar range) were required to demonstrate that overexpression of *Spi2A* decreased sensitivity to caspase-independent PCD (Fig. 1C). Importantly, in both experiments the survival of the GFP control cells with 10 pg ml^{-1}

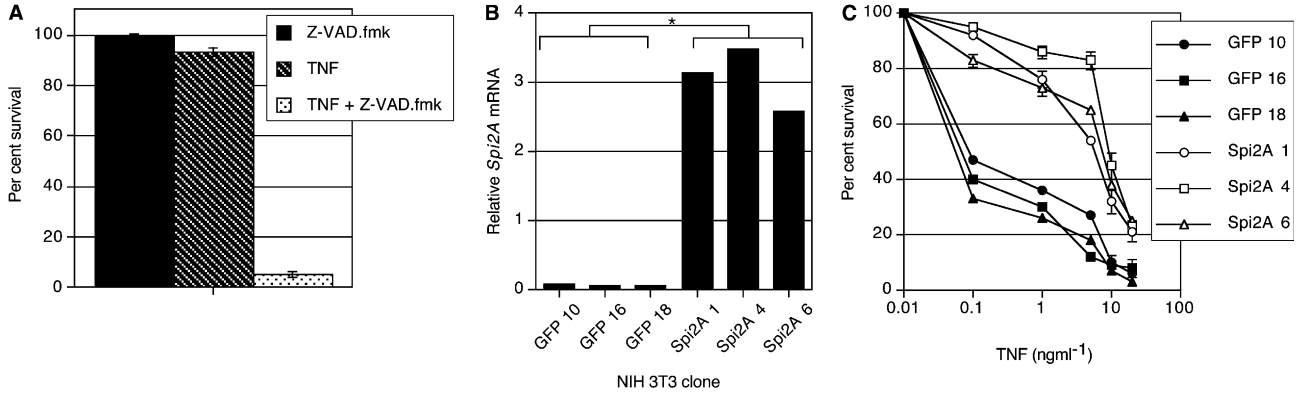

Fig. 1. Spi2A protects NIH3T3 cells from caspase-independent death induced by TNF-α. (A) Percentage survival of NIH3T3 cells after treatment with Z-VAD.fmk (50 μM) alone or TNF-α (10 ng ml^{-1}) alone or both. The recovery of cells after 16 h was compared with those incubated alone (100% recovery) to determine the percentage of recovery. Values are means (±S.E.M.) from four wells ($n = 4$). (B) Expression of *Spi2A* mRNA in three clones of NIH3T3 cells transduced with retrovirus encoding *GFP* alone (GFP) or *Spi2A* (Spi2A cells) as determined by real-time PCR. Significant differences between duplicate determinations on individual clones were determined by student's *t*-test ($P < 0.001^*$). The data are representatives of two independent experiments. (C) Percentage survival of cells after treatment with TNF-α + Z-VAD.fmk (50 μM). The recovery of cells incubated with TNF-α at 0.01 ng ml^{-1} (10 pg ml^{-1}) was nominally designated as 100% recovery and for GFP clones this was 70% of the recovery of cells incubated alone. Values are means (±S.E.M.) from four wells ($n = 4$).

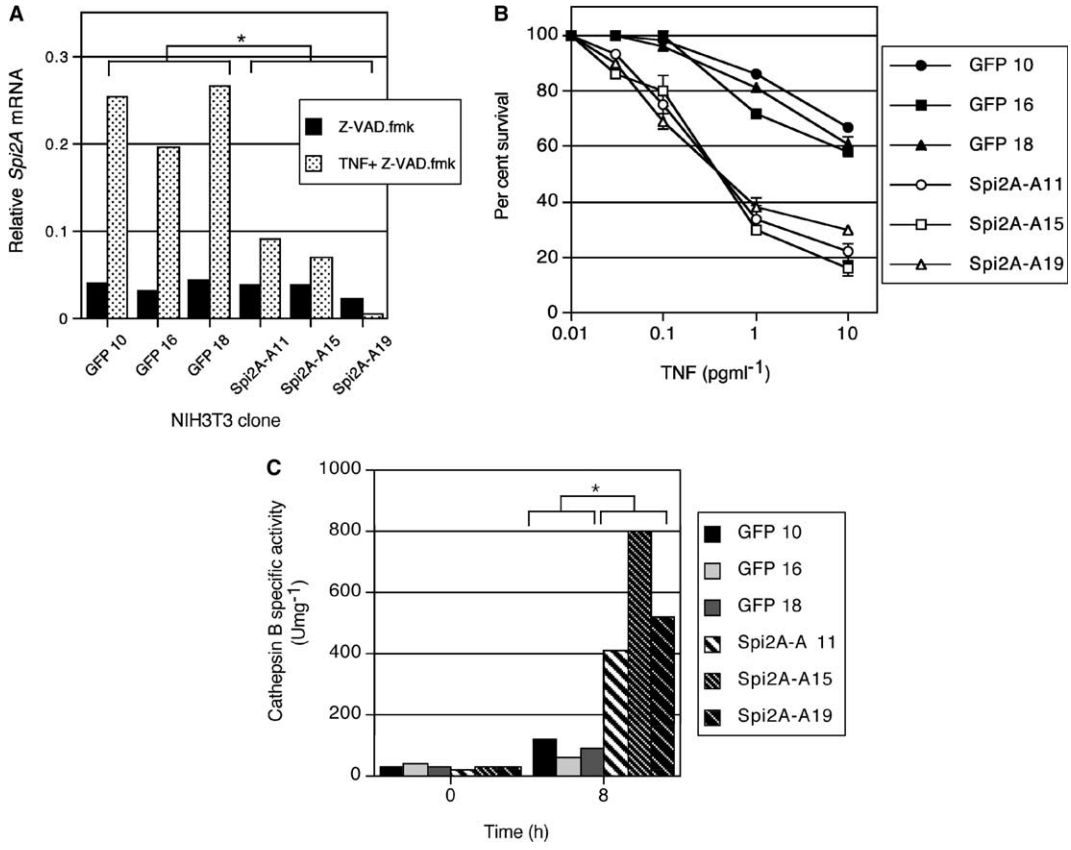

Fig. 2. Spi2A is a physiological inhibitor of lysosomal pathway of death in the absence of caspase activity. (A) Endogenous *Spi2A* mRNA levels measured by real-time PCR in three clones of NIH3T3 cells transduced by retrovirus encoding *GFP* alone (GFP clones) or anti-sense *Spi2A* (Spi2A-A clones) 4 h after treatment with either Z-VAD.fmk (50 μM) or Z-VAD.fmk + TNF-α (10 ng ml^{-1}). There was a significant difference in Spi2A expression ($P < 0.001^*$) between GFP and Spi2A-A clones after treatment with VAD.fmk + TNF-α. The data are representatives of two independent experiments. (B) Percentage survival of three clones of GFP or Spi2A-A NIH3T3 cells after treatment with TNF-α + Z-VAD.fmk (50 μM). The recovery of cells after 16 h was compared with those incubated with TNF-α at 0.01 pg ml^{-1} (100% recovery). Values are the means (±S.E.M.) from four wells ($n = 4$). (C) Cathepsin B activity in crude cytoplasmic extracts from three clones of GFP or Spi2A-A NIH3T3 cells before (time = 0 h) or after (time = 8 h) treatment with TNF-α + Z-VAD.fmk (50 μM). There was a significant difference in cathepsin B specific activity between GFP and Spi2A-A clones ($P < 0.001^*$). The data are representatives of two independent experiments.

(0.01 ng ml^{-1}) TNF-α was the same (70% survival compared to GFP-cells with Z-VAD.fmk alone; legend to Figs. 1 and 2B). We have shown previously that Spi2A has no direct effect on NF-κB activation, therefore it is unlikely that the knock-down in Spi2A expression increased PCD by impairing NF-κB function [14]. Thus, Spi2A is required to antagonize TNF-α-induced PCD in the absence of caspase activity.

3.3. *Spi2A is a physiological inhibitor of the lysosomal pathway of death in the absence of caspase activity*

Spi2A is located in the cytoplasm and so can protect from classical caspase-dependent apoptosis by suppressing cytoplasmic cathepsin B activity after it is released from the lysosome [14]. We wanted to determine if this mechanism of cyto-protection by Spi2A extends to the inhibition of caspase-independent PCD. In the absence of caspase activity, the inhibition of endogenous *Spi2A* mRNA expression by anti-sense *Spi2A* resulted in the induction of cytoplasmic cathepsin B activity after treatment of NIH3T3 cells with TNF-α (Fig. 2C). Thus, the inhibition of cytosolic cathepsin B by Spi2A is a physiologically relevant mechanism by which Spi2A blocks the lysosomal pathway of cell death in the absence of caspase activity.

3.4. *Spi2A suppresses mitochondrial pathways of PCD in the absence of caspase activity*

The permeabilization of the outer membrane of the mitochondrion is central to most caspase-independent death programs [2]. One important consequence of damaged mitochondria is the release of ROS, which are thought to be particularly important in mediating TNF-α cytotoxicity [21]. Given the ability of Spi2A to protect from caspase-independent PCD, we determined whether Spi2A could protect cells from mitochondrial depolarization and ROS production. The knock-down in *Spi2A* expression resulted in the onset of mitochondrial depolarization (Fig. 3A) and ROS production (Fig. 3B) after treatment of Spi2A-A cells with Z-VAD.fmk and TNF-α. Therefore, we conclude that Spi2A is a physiological inhibitor of ROS production in the absence of caspase-activity.

3.5. *Spi2A protects from death caused by ROS*

A mechanism by which ROS can cause cellular injury is through the release of digestive cathepsins into the cytoplasm after damaging lysosomal membranes [4,17]. Therefore, we determined whether Spi2A can protect cells from death caused directly by ROS. In Fig. 4A, as expected we show that the

redox cycling quinone Naphthazarin induces the PCD of NIH3T3 cells and that this can be reversed by the addition of the anti-oxidant DPPD. As has been shown for cathepsin D [4], we show that oxidative damage induced by Naphthazarin results in the release of cathepsin B from the lysosome into the cytoplasm (Fig. 4B). In Fig. 2C and in another study [14], we show that Spi2A is a physiologically relevant inhibitor of cathepsin B in the cytoplasm. Importantly, the expression of *Spi2A* protected cells from PCD caused by Naphthazarin (Fig. 4C). We conclude that Spi2A can protect from ROS-mediated PCD, most likely through the suppression of cathepsin B in the cytoplasm.

4. Discussion

There is an increasing realization that programs of cell death that do not require caspases may be of consequence in a variety of physiological settings [2]. Our present study is the first description of an endogenous serpin protecting cells from caspase-independent PCD. Caspase-independent PCD is an important component of the TNF-α-induced toxicity. Therefore, the finding that the *Spi2A* gene maps to the "TNF-protection locus" on mouse chromosome 12 suggests that the suppression of caspase-independent PCD by Spi2A may be of wide physiological relevance [22].

We have reported previously that Spi2A can abrogate classical caspase-dependent apoptosis not by direct inhibition of caspases, but rather through the inhibition of mitochondrial damage through suppression of cathepsin B activity in the cytoplasm [14]. This conclusion was based on the inability of Spi2A to inhibit caspase 3, 8 or 9 activity in vitro. However, we could not exclude the possibility that the suppression of PCD by Spi2A was due not only to the inhibition of lysosomal cathepsins but also the inhibition of caspases other than the ones we have tested. In this study, we observed as have others [8–10] that even when caspase activation was suppressed by Z-VAD.fmk, TNF-α still triggered the release of cathepsin B from the lysosomes, induced mitochondrial permeabilization and induced ROS production. Importantly, the present study clearly indicates for the first time that the abrogation of PCD by Spi2A does not require caspases as targets of inhibition.

In the absence of caspase-activity the cell is still susceptible to PCD from a range of factors. For example, AIF and the pro-apoptotic member of the Bcl-2 family – Bax – can induce apoptosis by dissipating mitochondrial potential in a Z-VAD.fmk-insentitive manner [20]. The damage of mitochondria that occurs in the absence of caspase activity leads to the

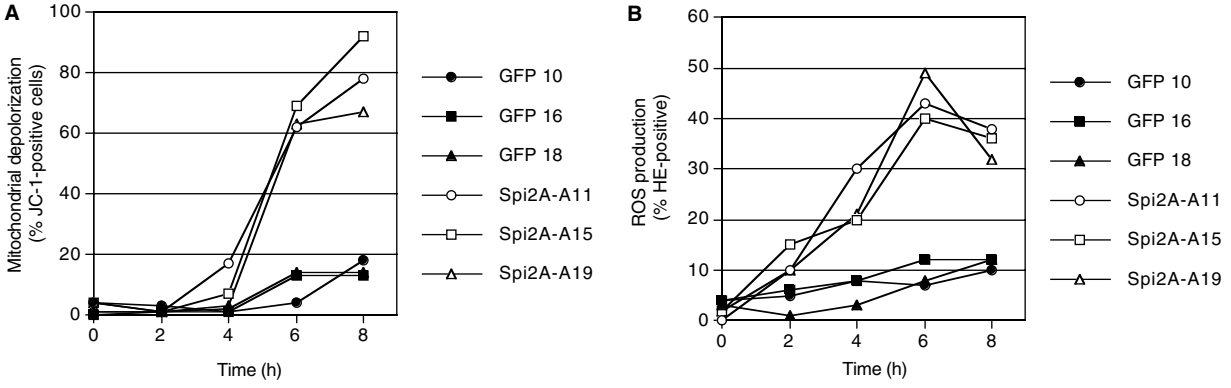

Fig. 3. Spi2A inhibits mitochondrial PCD in the absence of caspase activity. Cloned GFP ($n = 3$ clones) or Spi2A-A NIH3T3 cells ($n = 3$ clones) were treated with TNF-α (10 pg ml^{-1}) + Z-VAD.fmk (50 μM), then (A) mitochondrial depolarization, and (B) ROS production measured over time. The values are the means of duplicate determinations ($n = 2$). The data are representatives of two independent experiments.

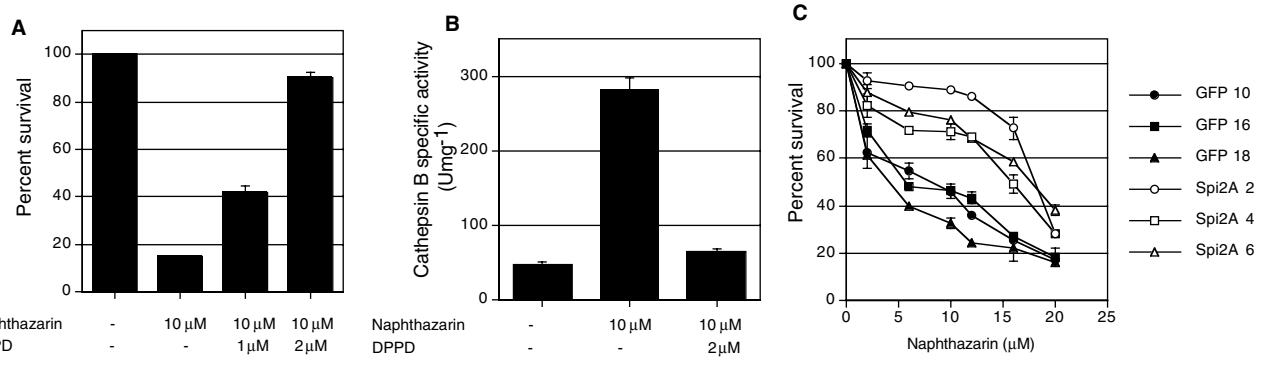

Fig. 4. Spi2A protects from death caused by ROS. (A) Percentage survival of NIH3T3 cells after treatment with the ROS-initiator Naphthazarin alone or with varying concentrations of the anti-oxidant DPPD. (B) Cathepsin B activity in crude cytoplasmic extracts from NIH3T3 cells after treatment with drugs. (C) Percentage survival of cells after treatment with drug. The recovery of cells after 2 h was compared with those incubated alone (100% recovery) to determine the percentage of recovery. In all parts, measurements were made 2 h after the addition of drugs and are the means (±S.E.M.) of determinations from four wells ($n = 4$).

leakage of ROS [10]. In the absence of caspases it has been suggested that other proteases, such as calpains, serine proteases and cathepsins substitute as dominant executioner proteases [20]. We show that in the absence of caspase activity TNFR1-ligation induces mitochondrial depolarization and ROS production, which damages lysosomes and releases cathespin B into the cytoplasm. Spi2A is required to protect against death caused by TNFR1 by inhibiting cytoplasmic cathepsin B released after lysosomal damage. Therefore, we propose that this activity of Spi2A mediates protection against ROS-mediated PCD.

Cyto-protection from PCD that does not require caspase activity may be afforded by other serpins that inhibit cathepsins. For example, the inhibition of cathepsins has been described by the human serpins SCCA 1 and 2 and the mouse serpin SQN-5 [23,24]. Furthermore, overexpression of SCCA2 protected HeLa cells from TNF-α-mediated PCD [25].

Lysosomal cathepsins play a role in both apoptosis and necrosis [3]. The importance of necrotic cell death in pathological processes in fact predates the discovery of apoptosis [1,3]. However, more recent findings indicate that necrotic, caspase-independent PCD is of physiological relevance in limb development in mice [26] and cell specification in Drosophila and *C. elegans* [20]. Studies with in vivo models will hopefully shed light on the role of *Spi2A* in both development and disease.

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