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Necrosis-like death can engage multiple pro-apoptotic Bcl-2 protein family members

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Abstract

Necroptosis is a physiologically relevant mode of cell death with some well-described initiating events, but largely unknown executioners. Here we investigated necrostatin-1 (Nec-1) sensitive death elicited by different necroptosis stimuli in L929 mouse fibrosarcoma cells, mouse embryonic fibroblasts (MEF) and bone marrow-derived macrophages. We found that TNF α - or zVAD-induced necroptosis occurs independently of the recently implicated executioners Bmf or PARP-2, but can involve the Bcl-2 family proteins Bid and Bak. Furthermore, this type of necroptosis is associated with mitochondrial cytochrome *c* release and partly sensitive to cyclosporine A inhibition, suggesting a cross talk with the mitochondrial permeability transition pore. Necroptosis triggered by cadmium (Cd) exposure caused fully Nec-1-sensitive and caspase-independent death in L929 cells that was associated with autocrine TNF α -mediated feed-forward signalling. In MEF Cd-exposure elicited a mixed mode of cell death that was to some extent Nec-1-sensitive but also displayed features of apoptosis. It was partly dependent on Bmf and Bax/Bak, proteins typically considered to act pro-apoptotic, but ultimately insensitive to caspase inhibition. Overall, our study indicates that inducers of “extrinsic” and “intrinsic” necroptosis can both trigger TNF-receptor signalling. Further, necroptosis may depend on mitochondrial changes engaging proteins considered critical for MOMP during apoptosis that ultimately contribute to caspase-independent necrotic cell death.

Keywords

Bcl-2 proteins; Cadmium; Necroptosis; TNF α

Introduction

Over the past years, necroptosis, a form of programmed cell death displaying morphological features also seen in necrotic cells, has been firmly established as a cell death mode of physiological relevance distinct from classical “accidental” necrosis or genetically regulated

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apoptosis with key roles in host defence in the adult [1]. Necroptosis can be induced in a context- and cell-type specific manner and analogous to apoptotic cell death it may be triggered by extrinsic [2, 3] as well as intrinsic stimuli [4, 5]. As a hallmark for the distinction between necroptosis and other cell death modes serves the cytoprotective effect of necrostatin-1 (Nec-1), an allosteric inhibitor of the kinase activity of the receptor-interacting protein-1 (RIP-1) that prevents interaction with its binding partner RIP-3, thereby selectively blocking necroptosis, but not apoptosis [6]. Nec-1 was reported to provide at least partial protection against both extrinsic, death receptor-mediated necroptosis [6] as well as against intrinsic, DNA-damage elicited necroptosis [4, 7, 8], highlighting the central role for RIP-1 kinase in this mode of cell death. The interaction of RIP-1 with RIP-3 can be antagonized by caspase-8 dependent cleavage of the former, but possibly also other means, protecting cells from undergoing unwanted or accidental necroptosis [9]. The critical role of caspase-8, its optional dimerization partner FLIP and their common adapter FADD in preventing necroptosis has recently been documented in a series of studies demonstrating complete rescue of the embryonic lethality caused by caspase-8- or FADD-deficiency by simultaneous loss of RIP-3 or RIP-1, respectively [9–11]. Further specifics of the necroptotic pathway are under intense investigation but while the initiating events, particularly of extrinsic necroptosis, have been unravelled little is known about the molecules and events involved in the execution phase of necroptosis, downstream of the RIP-1/RIP-3 kinase module, assembled in the currently poorly defined “necroptosome” [1]. In an attempt to define novel components of the necroptosis network, a siRNA screen identified a number of molecules that impaired necroptosis in the mouse fibrosarcoma cell line L929. Induction of cell death by tumor necrosis factor- α (TNF α) as well as by the pan-caspase inhibitor zVAD-fmk is almost completely prevented by Nec-1 in L929 cells [6]. Interestingly, the necroptotic effect of zVAD-fmk, which has so far not been unequivocally related to its inhibition of caspase activity [12, 13], was shown to be most likely also mediated by TNF α , the autocrine production and liberation of which is triggered by zVAD-fmk, at least in this cellular system [13, 14]. However, in this screen additional candidates, next to TNF and TNF-receptor (TNF-R) were identified, including members of the PARP family, and the BH3-only protein Bcl-2 modifying factor (Bmf) [14], a member of the Bcl-2 family typically associated with apoptotic cell death [15]. Furthermore, it was recently shown that necroptosis induced by the DNA-damaging agent *N*-methyl-*N'*-nitro-*N'*-nitrosoguanidine (MNNG) is critically dependent on two other Bcl-2 family members, specifically on the activation of Bax by Bid [16]. Interestingly, a chemical compound screen and mass-spectrometric analysis recently identified additional mediators of necroptosis, i.e. mixed lineage kinase-like protein (MLKL) and the mitochondrial phosphatase PGAM5 as mediators of necroptosis-like cell death in HT29 colon carcinoma cells [17, 18]. Thereby, the latter protein apparently acts as an integration point for different necroptosis-inducing stimuli, besides RIP3-dependent TNF-mediated killing. Interestingly, PGAM5, initially identified as an interaction partner of anti-apoptotic Bcl-XL [19], seems to promote necroptosis via dephosphorylation and activation of the dynamin related protein 1 (DRP1), required for mitochondrial fission and previously also implicated in cytochrome *c* release during apoptosis induction [20]. Although the latter appears disputed, mitochondrial fission is clearly influenced by the interaction with Bcl-2 family proteins and hence we wondered if

pro-apoptotic Bcl-2 family proteins, besides promoting classical apoptosis, might also be required for an “intrinsic” necroptosis signalling pathway [21].

To address this possibility and to evaluate previously documented findings implicating Bcl-2 family proteins in this cell death modality, we investigated the contribution of a series of BH3-only proteins as well as Bax and Bak to necroptosis induced by TNF α and zVAD-fmk triggered TNF-R stimulation. In addition, we studied the response to a more physiological trigger of necroptotic death, i.e. the metal and environmental pollutant, cadmium (Cd).

Materials and methods

Cells and reagents

Cells used throughout this study were either L929 mouse fibrosarcoma cells or mouse embryonic fibroblasts (MEF) immortalized with the SV40 large T antigen. Cells were maintained in DMEM with freshly added 2 mM L-glutamine (Invitrogen), 100 U/ml penicillin/streptomycin (Sigma-Aldrich) and 10 % fetal calf serum (PAA).

Macrophages from wt, Bmf^{-/-} [22] and Vav-Bcl-2 transgenic [23] mice were isolated from bone marrow. Cells (2×10^7), resuspended in 10 ml RPMI-medium containing 10 ng/ml M-CSF (Preprotech), 10 % FCS, 10 U/ml Pen/Strep, 2 mM L-glutamine, 50 μ M 2-mercaptoethanol, were seeded onto non coated Petri dishes. After 3 days of culture at 37 °C non-adherent cells were washed away and adherent cells, macrophages, were treated with Accutase™ for 5 min at 37 °C, washed and stained with the macrophage marker F4/80. The cell suspension with a purity of approximately 90 % of macrophages was then used for experiments.

Reagents and antibodies applied were as follows: fluorescence indicators dichlorofluorescein diacetate (DCF-DA), 5,5V,6,6V-tetrachloro-1,1V,3,3V-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1), and Hoechst 33342 from Molecular Probes (Leiden, The Netherlands); CellTiter-Glo (Promega Mannheim, Germany); MTT Cell Proliferation kit I (Roche Diagnostics Vienna, Austria); poly-(ADP-ribose) polymerase inhibitor 3-aminobenzamide, cycloheximide, staurosporine (STS), propidium iodide (PI), 3-methyl adenine (3-MA), 7-aminoactinomycin D (7AAD), 4',6-diamidino-2-phenylindole (DAPI), and α -GAPDH from Sigma (clone 71.1) (Deisenhofen, Germany); necrostatin-1 (Nec-1) and hsp90 inhibitor 17-(Dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG) from Eubio (Vienna, Austria); pan-caspase inhibitor Z-Val-Ala-DL-Asp(OMe)-fluoromethylketone (zVAD-fmk) (Bachem Weil am Rhein, Germany); cyclosporine A (CsA) (LC Laboratories Woburn, MA, USA); caspase-3 substrate Ac-DEVD-AMC, N-(2-quinolyl)valyl-aspartyl-(2,6-difluorophenoxy)methyl ketone) (QVD), etoposide, and rapamycin from Alexis Biochemicals (Lausen, Switzerland); histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA) from R. W. Johnstone, Peter MacCallum Cancer Centre, Melbourne, Australia; mTNF α (PeproTech), Vectashield antifade mounting medium (Vector Laboratories Burlingame, CA); α -Bmf (clone 17A9), α -tubulin (Santa Cruz Biotechnology sc-32293); α -PARP (#9542), α - γ H2AX (Ser139, clone 20E3), α -phospho-ATM (Ser1981, clone 10H11.E12) from Cell Signaling Biotechnology New England Biolabs (Frankfurt am Main, Germany); α -pADPr (clone 10H; SzaboScandic); α -53BP1 (Novus

Biologicals NB100-305); α -cytochrome *c* (clone 7H8.2C12) from BD Transduction Laboratories (Vienna, Austria); secondary antibodies Alexa Fluor 488 goat α -rabbit and Alexa Fluor 546 goat α -mouse from Invitrogen (Vienna, Austria); goat α -rabbit HRP and goat α -mouse HRP (Dako).

Cell survival and cell death

Cell viability was evaluated using a number of different methods. Cell proliferation and survival was initially evaluated by MTT assay following standard procedures. In addition, as an indirect readout of viability actually determining ATP levels of the cells the CellTiter-Glo assay was applied. Viability was also assessed by co-staining cells with AnnexinV/7AAD and subsequent FACS analysis. For assessing compromised membrane integrity as observed in necrosis-like cell death modes cells were either first trypsinized, PI-stained (5 μ g/ml) and then analyzed by FACS, or adhering cells were co-stained with the membrane impermeable PI and 2 μ g/ml of the membrane permeable DNA marker Hoechst 33342. After 5 min of incubation cells were imaged under a fluorescence microscope using appropriate filter settings and images then analyzed using Image J software. Furthermore, cells were FACS-analyzed for diminished DNA content as assessed by the sub-G1 assay [24].

Quantification of mitochondrial membrane potential, cytochrome c release, and acridine orange retention

Changes in mitochondrial membrane potential and cytochrome *c* release were determined with JC-1 loaded and α -cytochrome *c* stained cells, respectively, as described in detail before [25]. Acridine orange (AO) retention, reflecting lysosomal membrane integrity, was assayed according to Yuan et al. [26]. In brief, cells were harvested, incubated with 5 μ g/ml AO in D-MEM for 15 min, washed with PBS and FACS analyzed for red fluorescence.

Caspase activity measurement

Activity of caspase-3 was determined in cell extracts using the artificial caspase-3 substrate Ac-DEVD-AMC as described before [25].

Immunocytochemistry

Cells cultured on sterilized glass cover slips were exposed to experimental conditions and then washed with PBS and fixed with 4 % PFA in PBS (15 min, RT). After three washes with PBS cells were incubated for 1 h in PBS with 0.1 % Triton X-100, 1 % bovine serum albumin and 10 % fetal calf serum for permeabilization and blocking. Next, cells were incubated with primary antibodies diluted in blocking solution at 1:25 (FITC-labeled α -cytochrome *c*), or 1:100 (all others) (o/n, 4 °C). Following another three washes with PBS, cells were labeled with secondary antibodies where appropriate (1:100 in blocking solution) (1 h, RT), again washed with PBS, incubated with DAPI (2 μ g/ml, in PBS) for nuclear staining (10 min, RT) and washed one more time. The samples were finally fixed on microscope slides with Vectashield and examined with a Leica SP5 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany) using LAS AF acquisition software Version 2.1.0. and Image J software for post-acquisition image processing.

Quantitative real-time PCR

Quantitative analysis of *Bmf* gene expression was conducted by use of real-time qPCR. To this end, experimentally exposed cells were lysed with Trizol (Invitrogen) and RNA isolated by chloroform-extraction. After DNase digestion RNA was reversely transcribed (Omniscript, Qiagen) using random hexamer primers and cDNA amplified with 5prime RealMasterMix SYBR ROX on a Eppendorf Mastercycler ep realplex² cyclor (40 cycles with annealing at 60 °C, elongation at 72 °C). Primers used for analysis of *Bmf* expression were: forward 5'-CCCA TAAGCCAGGAAGACAA-3'; reverse 5'-AGGGAGAGG AAGCCTGTAGC-3'); and for actin expression, determined for normalization: forward 5'-TTC GTT GCC GGT CCA CA-3'; reverse 5'-ACC AGC GCA GCG ATA TCG-3'). Relative induction compared to untreated cells was calculated by the delta-delta CT method.

Immuoblotting

For Western blot analyses, cells were harvested by trypsinization, washed with PBS and suspended in lysis buffer (10 mM HEPES, 300 mM sucrose, 10 mM KCl, 1.5 mM MgCl₂, 10 mM DTT, 0.5 % NP40; supplemented with complete protease inhibitor cocktail from Roche). After 30 min extraction on ice samples were spun down, protein content of supernatants determined with Bradford assay and 50–100 µg protein per sample separated on 4–20 % gradient gels (Lonza) and subsequently transferred to Hybond ECL nitrocellulose membranes (GE Healthcare). Following incubation with the desired antibodies, proteins were visualised by ECL (Pierce).

Statistics

All data shown are mean ± SE of at least three independent experiments, except where otherwise indicated. Statistical differences were evaluated by ANOVA followed by the appropriate post hoc tests, or by Mann–Whitney U-test, with a *p* value <0.05 considered as significant.

Results

TNFα- and zVAD-fmk induced necroptosis requires the RIP-1/RIP-3 necrosome while death is modulated by Bid and Bak, but not Bmf

In agreement with previous reports L929 cells were highly susceptible to cell death induction by TNFα and zVAD and in both cases addition of Nec-1 was fully cytoprotective (Fig. 1a). Further, we confirmed that only zVAD-fmk but not the chemically distinct pan-caspase inhibitor QVD had killing potency in this setting. TNFα and zVAD caused cell death associated with fully Nec-1-sensitive mitochondrial membrane rupture-triggered loss of cytochrome *c* (Fig. 1b). Consistent with non-apoptotic loss of mitochondrial membrane integrity, an inhibitor of the permeability transition pore complex (PTPC), CsA, was partly protective against these stimuli, in particular when TNFα was used as stimulus (Fig. 1c). Furthermore, L929 cells overexpressing Bcl-2 were not protected against either agent (Fig. S1a), but were significantly protected against DNA-damage induced cell death by etoposide. Relative survival, determined by MTT assay, was 38 ± 2 % in wt versus 50 ± 4 % in Bcl-2 overexpressing cells after 24 h of incubation with 50 µg/ml etoposide (*n* = 4, *p* < 0.05).

In contrast to L929 cells, neither TNF α nor zVAD caused cell death in SV40 large T-transformed MEF (SV40-MEF) or in NIH3T3 fibroblasts when administered alone (not shown). However, concurrent addition of both agents combined with the protein synthesis inhibitor cycloheximide (CHX) to either wild type (wt) or Casp-3/7 double-deficient SV40-MEF induced cell death that was significantly reduced in the presence of Nec-1 (Fig. 1d, Fig. S1b). This observation allowed us to assess whether various Bcl-2 family proteins typically involved in apoptosis might contribute to necroptosis by using SV40-transformed MEF from gene-modified mice. We found that the lack of RIP-1 and RIP-3 as well as that of Bid or of Bak, but not that of any other pro-apoptotic Bcl-2 family member tested (Bmf, Bad, Bim, Puma, Noxa, Bax) protected cells against the CHX + TNF + zVAD treatment (Fig. 1e; Fig. S1c). At the same time, only SV40-MEF deficient in Bax/Bak were protected against the apoptosis inducer STS, while cells lacking RIP-1, Bid, or Bmf were just as sensitive as wt cells, supporting that the protection afforded was stimulus-specific (Fig. S1d). Overexpression of Bcl-2 also failed to prevent necroptosis (Fig. 1e). In addition, MEF derived from Bim/Bmf double-deficient mice responded like wt cells to the treatment, although the mice from which these cells were derived showed persisting interdigital webbing [27], possibly even suggestive of impaired necrotic cell death [28]. Finally, we also tested the response of primary macrophages, reported to be susceptible to zVAD alone [29], and observed Nec-1 sensitive killing (Fig. S1e). Of note, the extent of zVAD-induced killing was unaltered in primary macrophages derived from Bcl-2 overexpressing or Bmf-deficient bone marrow, further indicating that neither Bmf nor Bcl-2 play critical roles in this scenario (Fig. S1e). To explore a role for Bmf in necroptosis restricted to L929 cells, we also generated a number of clones in which we ablated Bmf expression by RNA interference (Fig. S1f). Cells lacking Bmf protein were just as sensitive towards TNF α or zVAD as parental L929 cells (Fig. 1f). Ultimately, quantification of Bmf mRNA levels in L929 cells before and after treatment with inducers of necroptosis failed to reveal transcriptional activation of the Bmf gene and we could also not detect any changes in protein abundance (Fig. S1g, h).

Cd-exposure triggers a mixed cell death response in mouse fibroblasts

To extend our observations beyond the rather non-physiological treatment of cells with zVAD, TNF plus CHX, we next investigated necroptosis in response to Cd, an environmental pollutant that can trigger cell death with necrotic features in the absence of caspase activation in different cell types [30]. Based on experiments determining dose-dependent cell death induction (Fig. 2a, b and Fig. S2) we chose 5 μ M and 20 μ M Cd, causing intermediate and high levels of cell death, respectively, for subsequent more detailed analyses. At both concentrations L929 cells were almost fully protected against cell death by Nec-1 but not by QVD (Fig. 2c, d and not shown). Similar results were obtained when cell death was determined by PI uptake using flow cytometric analysis (Fig. 2e), but surprisingly also when analysing the percentage of sub-G1 cells, generally considered a measure of pure apoptosis (Fig. 2f and not shown). This was also true for cytochrome *c* release from mitochondria, which was not detected at 5 μ M but at 20 μ M Cd and proved to be sensitive to Nec-1 but not QVD. In fact, QVD actually enhanced cell death in this setting (Fig. S3a, b). To test whether these findings indeed reflected a contribution of classical apoptosis we thus determined caspase-3/7 activity but found no activation of these proteases over 24 h by Cd,

whereas STS caused an approximately 8-fold elevation of caspase-3/7 activity within 6 h of exposure (Fig. S3c). Caspase-3/7 activity was also not induced by co-incubation with Cd plus Nec-1 (Fig. S3d), supporting complete effector caspase-independence in this cell death model. In line, we also found no processing of caspase-3 in Cd-treated cells by Western analysis (Fig. S3e). Altogether this suggests that the sub-G1 population did in this case not reflect apoptotic cells but possibly chromatinolysis associated with programmed necrosis [31]. Mitochondrial cytochrome c release may thus also be observed in the necroptotic death, possibly as a consequence of DRP1-mediated mitochondrial fragmentation.

Of note, the situation was completely different in SV40-MEF, in which Nec-1 conferred only slight protection to the cells (Fig. 3a). However, at 5 μ M Cd significant and additive protection was seen for Nec-1 or QVD when quantifying PI exclusion while in the sub-G1 analysis protection was only significant for the combination of both compounds (Fig. 3b). This suggested mixed apoptotic/necroptotic death and indeed we found significantly elevated caspase-3/7 activity levels after 18–24 h (5 μ M) and 12–18 h (20 μ M) of metal exposure (Fig. 3c). Co-incubation of cells with 5 μ M Cd and Nec-1 enhanced caspase activity even further, possibly indicating a shift to apoptosis, whereas QVD completely suppressed the protease (Fig. 3d). Nec-1 alone had no impact on caspase-3 activity, ruling out an off-target effect of the compound as reported by Cho et al. [32]. In addition, Cd also induced cytochrome c release in MEF (Fig. S4a, b), but differently from L929 cells, this was insensitive to Nec-1 (Fig. S4c). This suggests that the release observed may have been due to RIP-1/3 independent fragmentation of mitochondria.

Necroptosis-like cell death induced by Cd can occur in the absence of RIP-1/-3 or PARP-1/-2 and does not engage LMP or autophagy

Next we examined if Cd-induced death of mouse fibro-blasts involved other specifics previously associated with necroptosis. We found that inhibition of the RIP-1 molecular chaperone hsp-90 using 17-DMAG, indirectly blocking RIP-1 by allowing for its degradation, conferred similar protection as Nec-1 to L929 cells at 5 μ M Cd (Fig. 4a, b). In comparison, it did not protect SV40-MEF (Fig. 4c, d) and we also observed that MEF deficient for RIP-1 or RIP-3 were just as sensitive against Cd-toxicity as their wt counterparts (Fig. 4e).

An important trigger of necroptosis induced by DNA damage or, as recently shown by TNF-related apoptosis inducing ligand (TRAIL) [33], is the DNA repair enzyme PARP-1, the excessive activation of which may lead to cellular ATP depletion [4, 33, 34]. Further, also the homologue PARP-2 has been implicated in necroptosis in L929 cells [14]. Inhibition of both homologues using the nonselective competitive inhibitor 3-AB was indeed cytoprotective in L929 cells incubated with 5 μ M Cd (Fig. 4a, b), while in MEF it did preserve ATP levels but did not prevent cellular PI uptake (Fig. 4c, d), consistent with apoptotic cell death being activated. Western analysis and immunofluorescence imaging suggested that PARP activity was in fact enhanced in both cell types (Fig. 4f and Fig. S5a), a response obviously elicited by the DNA damage induced during Cd exposure (Fig. S6). Cd mediated DNA damage seems to be due to its ability to induce oxidative stress and to non-selectively inhibit DNA damage repair mechanisms [32]. However, at the same time we

detected the occurrence of diverse fragments of PARP-1 over time (Fig. 4f and Fig. S5b), presumably induced by processing through caspases or, particularly in L929 cells, through other proteases such as those released from lysosomes, e.g. cathepsins or calpain [35–37]. Regardless, analysis of SV40-MEF deficient for either PARP-1 or PARP-2 indicated that neither of them was significantly protected against Cd (Fig. S5c). Further, we examined the potential involvement of lysosomal membrane permeabilisation (LMP), prompted by previous observations supporting a role of the lysosomes in necroptosis [23, 38]. Our results indicated that in L929 cells LMP occurred only at 20 μ M Cd and was then sensitive to inhibition with Nec-1 but not with QVD (Fig. S7a-c). In contrast, LMP occurred at both 5 μ M and 20 μ M Cd in MEF and at the lower concentration it was in part—although not significantly- inhibited by both Nec-1 and QVD (Fig. S7d, e). This suggests that necroptosis and apoptosis are activated simultaneously under these conditions and that LMP can occur secondary to the activation of caspases or the RIP1/3 necrosome. Finally, prompted by studies on zVAD induced killing indicating a role for autophagy in the process [39, 40], we exposed fibroblasts to Cd concurrently with either the autophagy inhibitor 3-MA or with the autophagy inducer rapamycin, but could in neither case observe an aggravation of or rescue from cell death, irrespective of cell type and Cd concentration used (not shown).

Cd-induced cell death engages TNF-receptor stimulation as well as multiple BH3-only proteins

Since even in zVAD induced necroptosis TNF-R stimulation appears to be important [13], we next evaluated if this was also the case in Cd-exposed cells. First we tested the combined action of Cd and TNF α on cell viability but could not detect significant additive effects in L929 cells or SV40-MEF (Fig. 5a, b). However, this did not allow us to rule out that Cd and TNF α may trigger one and the same linear signalling pathway in a type of feed-forward loop (Cd $\rightarrow$ TNF $\rightarrow$ TNF-R $\rightarrow$ RIP-1/3 $\rightarrow$ death). We thus examined the effect of blocking TNF-R by adding neutralizing α -TNF α antibody before Cd exposure. We observed that at 5 μ M Cd this conferred partial protection to both L929 cells and SV40-MEF (Fig. 5c-f). At 20 μ M Cd cytoprotection was no longer observed, indicating that either autocrine TNF production superseded the effect of the neutralizing antibody or that TNF-R stimulation was of little importance at this higher dose. It appears possible that under these conditions other additional cell death mediators, e.g. members of the Bcl-2 family, take command.

Hence, the next series of experiments addressed the role of BH3-only proteins in Cd-induced cell death, focusing initially on Bmf, due to its proposed role in necroptosis. Interestingly, Bmf-knockout MEF were significantly less sensitive to Cd than wt MEF, as assessed by PI exclusion (Fig. 6a, Fig. S8a, b) or MTT assay (not shown), as well as in terms of a smaller sub-G1 population (Fig. 6b). Bmf-deficient MEF also displayed a lower number of cells releasing mitochondrial cytochrome *c* (Fig. 6c) or showing diminished mitochondrial membrane potential (Fig. S8c). Of note, Bmf-knockout MEF were no longer significantly protected by Nec-1 (Fig. 6d), as opposed to wt MEF, indicating that Bmf is involved in mediating RIP-1/-3-dependent cell death. Surprisingly, despite of these observations we could not detect any induction of Bmf in MEFs at either the RNA- or the protein level (Fig. 6e, f), in contrast to enhanced transcription and translation induced by stimuli causing Bmf-dependent cell death, i.e. the histone deacetylase inhibitor SAHA or serum withdrawal,

respectively. Noteworthy, we even detected a slight decrease of Bmf abundance during the first 2–6 h before recovery to approximate baseline levels (Fig. 6f). Similar observations were made with L929 cells (Fig. S8d, e), but these cells were not at all protected against Cd when Bmf expression was silenced by RNAi or when Bcl-2 was overexpressed (not shown).

Most strikingly, we observed that Bax/Bak double knockout MEF were almost completely protected against Cd as indicated by PI exclusion and sub-G1 assay (Fig. 6a, b). In contrast, despite of a slight reduction in cell death we saw no significant cytoprotection in Bax and Bak single knockout MEF, or in MEF lacking Bid or the closest Bmf relative Bim (not shown). Together this suggests that activation of Bax and/or Bak, possibly by a subset of BH3-only proteins, contributes to Cd toxicity in MEF.

Discussion

The pathophysiological relevance of necroptosis in developmental and immunological processes is now firmly established [9–11], whereas mechanistic aspects remain to be fully elucidated. Thus, although Nec-1 sensitive cell death, which we here use to define necroptosis, may be triggered by both extrinsic and intrinsic stimuli, the involvement of common proteins other than RIP-1 and RIP-3 in a yet to be defined canonical necroptotic pathway is just beginning to emerge. In this regard, several studies indicated that activation of TNF-R is a key event for extrinsic necroptosis, as blocking its stimulation protected not only against TNF α but also against zVAD-induced cell death [13, 14].

Here we show that this may also hold for Cd-induced cell death, which, consistent with previous studies [7, 8] was almost completely (L929 cells) or partially (MEF) sensitive to Nec-1. Accordingly, cell death was also reduced in mouse fibroblasts by adding a neutralizing antibody directed against TNF α . A potential trigger for the presumptive release of the cytokine may be the DNA damage caused by Cd, in line with a release of TNF α induced by DNA damaging conditions [41–43]. Indeed, a Nec-1 sensitive key role of TNF-R feed-forward signalling in cell killing has been reported for HeLa cells exposed to high-dose etoposide [43], whereas Nec-1 sensitive but TNF-R independent cell death was seen in a number of other cancer cell lines [44]. Our own experiments indicate that L929 cells and SV40-MEF are also partly protected against etoposide by Nec-1 (unpublished observation). Furthermore, despite of dose-dependent caspase-activation the cells were not rescued from death by QVD (unpublished observation). However, we have not yet addressed the role of TNF-R signalling in these conditions.

Different from the importance of TNF-R signalling, several other aspects of the Hitomi study could be neither confirmed nor expanded to the scenarios investigated here. For example, while the knockdown of PARP-2 reportedly protected L929 cells against zVAD or TNF α [14], PARP-2 deficient MEF were neither resistant to TNF α /CHX + zVAD nor to Cd in our study. Surprisingly, even PARP-1 knockout MEF were not Cd-resistant, despite of the fact that PARP was indeed activated and that chemical inhibition of PARP activity in wt MEF helped preserving cellular ATP levels. Unlike the importance of PARP-1 in RIP-dependent necrosis induced by the DNA damaging agent MNNG [4, 5, 34], its rather late activation in response to Cd exposure may thus be merely a collateral event. This would

agree with the relatively rapid onset of its degradation, presumably mediated by caspases and, in L929 cells, other proteases [35–37].

We could also not corroborate the suggested involvement of the BH3-only protein Bmf in cell death of L929 cells exposed to TNF or zVAD. Importantly, this was not restricted to L929 cells, but the lack of protection against TNF α /CHX + zVAD also held for MEF and macrophages derived from Bmf knockout mice.

Out of numerous other MEF deficient in Bcl-2 family proteins only those derived from Bid and Bak knockout mice were significantly less sensitive against TNF α /CHX + zVAD than wt MEF. Furthermore, in contrast to previous reports [12] we also found that both TNF α and zVAD-induced death of L929 cells were accompanied by cytochrome *c* release, and that cell death was sensitive to CsA, an inhibitor of the PTPC, while Bcl-2 overexpression was not cytoprotective. These findings largely confirm a recent study by Irrinki et al. [45], except for a reported protective effect of Bcl-XL, but this difference might be related to different protein expression levels and/or antagonist binding characteristics as compared to Bcl-2 [46]. In particular Bcl-XL's ability to interact with PGAM5, a recently identified mediator of intrinsic and extrinsic necroptosis may be critical here [29, 47]. Regardless, both studies can be interpreted to indicate that mitochondria play a critical role in necroptosis. However, rather than indicating a contribution of apoptotic MOMP, in conjunction with the observed protection afforded by CsA they might underscore previously suggested interactions of components of the outer and the inner mitochondrial pore in some cell death models [48] or deregulated impaired mitochondrial fission/fusion events in the absence of Bax/Bak ultimately contributing to DRP1-mediated fragmentation.

An important role for Bid has been recently reported for SV40-MEF exposed to MNNG [16]. Interestingly, it appeared that Bid activated Bax, while Bak was not involved [49]. In contrast, here we found that MEF deficient for Bid or Bak, but not Bax, were protected against TNF α /CHX + zVAD, adding to the relatively few reported cases where Bax and Bak are not redundant in promoting cell death [50]. However, we have not investigated if both Bcl-2 family proteins are functionally related in this context, an aspect worthwhile pursuing in future studies.

In light of the above-discussed finding that the absence of Bmf conferred no protection against TNF α -induced necroptosis, we were surprised that SV40-MEF deficient for Bmf were to a significant extent protected against Cd-toxicity, as reflected by a number of viability parameters. In these cells, Cd-exposure was accompanied by elevated caspase activity and only partial inhibition by Nec-1, indicating a mixed apoptotic/necroptotic cell fate. Of note, addition of Nec-1 stimulated caspase-3 activity even further, suggesting a shift from necroptosis to apoptosis in this situation. Furthermore, Nec-1 remained without any effect on viability in Bmf-deficient MEF. This seems to confirm that Bmf was a core component of necroptosis in this cell death model, leaving little to be inhibited in its absence. Alternatively, it might indicate that Cd-induced death was in fact primarily apoptosis that is not inhibited by Nec-1. In either case, the importance of Bmf appears surprising, as the protein was neither transcriptionally nor translationally induced in the Cd-exposed fibroblasts. In line with previous reports [22, 51] Bmf was strongly induced by

SAHA or serum deprivation and accordingly Bmf-deficient MEF were significantly less sensitive to these stimuli than wt cells (Fig. S8f and not shown). The importance of Bmf in Cd-induced death will thus not be primarily based on altered protein abundance but rather on a change in sub-cellular localization [15] and/or some other post-translational modification [52].

A puzzling observation regarding Cd-induced death of MEF was that, unlike the partial protection afforded by Nec-1, there was absolutely no protection detectably in MEF lacking RIP-1 or RIP-3. This seems to indicate that in SV40-MEF complete inhibition of RIP-1 function due to genetic ablation does not adequately mimic inhibition by Nec-1. On the one hand, this would be in line with observations by Hitomi et al. [14] who suggested that the lack of cytoprotection by siRNA-mediated knock-down of RIP-1 in L929 cells against TNF α -induced death, as opposed to protection by Nec-1, was due to concurrent inhibition of RIP-1 kinase activity and pro-survival NF κ B activation in the former as compared to the latter treatment. On the other hand, this is not easily reconciled with the persistent protection against TNF/zVAD induced death seen in RIP knockout MEF. It remains formally possible that under conditions of Cd-exposure Nec-1 may block other components that contribute to death, next to RIP-1, that may be responsible for the death of RIP-1/-3 knockout cells. Alternatively, in the absence of RIP-1/-3, unrestrained (TNF-dependent) apoptotic cell death may proceed, as e.g. seen in cells that lack FLIP and RIP-3 (i.e. failure of RIP-3 deficiency to rescue FLIP lethality) [9].

In contrast to MEF, in L929 cells Cd-induced death occurred in the absence of caspase-activation and was fully sensitive to Nec-1 but not QVD, supporting a primarily necroptotic mode of cell death. At the same time, however, short-hairpin mediated knockdown of Bmf did not protect these cells against Cd, suggesting that this Bcl-2 family protein was not important here. Surprisingly, these cells were nonetheless similarly responsive as MEF in terms of Bmf transcriptional and translational activation upon exposure to SAHA or serum deprivation, respectively. Altogether, these findings point to important cell-type specific differences and response patterns, which seems to prohibit too far-reaching generalizations. As to the underlying reasons, we can only speculate here. However, previous studies have suggested that a correlation exists between the extent of ripoptosome formation and, for example, the expression level of cFLIP_L and/or the stoichiometry of the molecular components of the ripoptosome [53]. Given that L929 cells are remarkably sensitive to Nec-1 inhibition towards a number of different death stimuli, this may well just reflect highly favorably absolute and/or relative expression levels of ripoptosome components in these cells, resulting in their large dependence on RIP-1 kinase activity mediated cell death.

A final noteworthy characteristic of Cd-induced cell death observed here is the apparent lack of an autophagic component, as neither inhibition nor stimulation of autophagy altered the death rate elicited by the metal. Degterev et al. [6] described induction of autophagy as a downstream event of necroptotic stimuli, but could also not see cytoprotection by 3-MA or in autophagy-deficient MEF. Further, Wu et al. [39] suggested a pro-survival role of autophagy in zVAD-exposed L929 cells, with zVAD supposedly blocking lysosomal function and thereby suppressing maturation of autophagosomes. Here, we observed Cd-induced destabilization of lysosomes that was Nec-1 sensitive in L929 cells, but rather

Nec-1 insensitive in MEF, which would position it downstream or upstream of RIP-1, respectively. Of note, a recent study suggested a death-promoting role of autophagy in zVAD-exposed L929 cells [40], adding further controversy to this issue.

Overall, our study indicates that extrinsic and intrinsic necroptosis share TNF-receptor signalling components as well as the involvement of mitochondrial changes similar to those reported for apoptotic cell death warranting re-examination of cell death paradigms previously considered strictly apoptotic. Further, we observed that the role of cell death executioners such as Bmf can be strictly cell-type and/or stimulus-dependent, making the definition of a canonical necroptosis pathway a challenging task for further research.

Electronic supplementary material

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Acknowledgments

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Fig. 1.

TNF α - and zVAD-induced necroptosis in mouse fibroblasts. **a** L929 cells were cultured either alone or in the presence of 10 ng/ml TNF- α , 20 μ M zVAD-fmk, 20 μ M QVD, 30 μ M Nec-1 or a combination of either TNF- α and Nec-1 or zVAD-fmk and Nec-1 for 18 h. Cell survival was assessed by AnnexinV/7AAD staining and only cells negative for both AnnexinV and 7AAD were considered as living cells. Data are expressed as relative survival normalized to medium culture. **b** L929 cells were cultured either alone, in the presence of 10 ng/ml TNF- α , 20 μ M zVAD-fmk with or without 30 μ M Nec-1 for 12 h. Cytochrome c

release was analyzed by flow cytometry (c) L929 cells were cultured either alone, in the presence of 10 ng/ml TNF- α , 20 μ M zVAD-fmk with or without 10 μ g/ml Cyclosporin A for 12 h. Cell survival was assessed by AnnexinV/7AAD staining and data expressed as relative survival normalized to medium culture. **d** Casp-3/7 deficient SV40-MEF were cultured in the presence of 1 μ g/ml cycloheximide (CHX) with or without 10 ng/ml TNF- α and 20 μ M zVAD-fmk as indicated and after 18 h viability was estimated from changes in JC-1 staining. **e** MEF of the indicated genotype were cultured in the presence of 10 ng/ml TNF- α , 1 μ g/ml cycloheximide and 20 μ M zVAD-fmk for 18 h. Cell survival was estimated from changes in JC-1 staining and normalized to CHX-treated cells. **f** L929 stably expressing short hairpin RNA directed against Bmf expression and their wt counterparts were exposed to 10 ng/ml TNF- α or to 20 μ M zVAD-fmk and after 24 h cell viability was assessed by MTT assay. Data in **a–f** are mean $\pm$ SE of at least three experiments. * $p < 0.05$ compared to indicated treatments or to wild type cells **f**. *n.s.* Non-significant. Statistical analysis was performed using Mann–Whitney U-test, ANOVA followed by Student–Newman–Keuls test, or Dunn’s test as appropriate

Fig. 2.

Cd-induced cell death in mouse fibroblasts. **a, b** Dose-dependent effect of incubation with Cd for 24 h on viability of L929 cells (**a**) and SV40-MEF (**b**) as assessed by MTT assay and ATP-determination, respectively. **c–f** Impact of Nec-1 and QVD on cell viability determined by MTT assay (**c**), by PI exclusion (**e**), or by sub-G1 assay (**f**) of L929 cells exposed to 5 μM Cd, and on cell morphology of L929 cells exposed to 20 μM Cd (**d**). Data are mean ± SE of at least three independent experiments. * $p < 0.05$ compared to controls; § $p < 0.05$ compared

to cells treated with Cd only. Statistical analysis was performed using ANOVA followed by Student–Newman–Keuls test or Dunn’s test as appropriate

Fig. 3.

Cd causes Nec-1- and QVD-sensitive cell death in SV40-MEF. **a, b** Impact of Nec-1 and QVD on cell viability of SV40-MEF exposed to Cd for 24 h as determined by MTT assay (**a**), and by measuring PI-exclusion or the sub-G1 population (**b**). **c** Caspase-3 activity, expressed relative to initial values, of SV40-MEF exposed to 5 μ M or 20 μ M Cd over a time-course of 24 h. **d** Effect of Nec-1 and QVD on caspase-3 activity of cells exposed to 5 μ M Cd for 18 h. Data are mean $\pm$ SE of at least 3 independent experiments. * $p < 0.05$ compared to controls; § $p < 0.05$ compared to cells treated with Cd only. Statistical analysis was performed using ANOVA followed by Student–Newman–Keuls test or Dunn’s test as appropriate

Fig. 4.

The impact of inhibitors of proteins implicated in necroptosis on Cd-induced cell death. **a–d** Effect of 0.1 μM 17-DMAG (hsp-90 inhibitor), 1 mM 3-aminobenzamide (3-AB, PARP inhibitor), or 0.5 μM cyclosporine A (CsA, PTPC inhibitor) on cell viability after 24 h determined by measuring ATP levels and PI-exclusion of L929 cells (**a, b**) and SV40-MEF (**c, d**) exposed to 5 μM or 20 μM Cd as indicated. **e** Effect of exposure to 5 μM or 20 μM Cd for 24 h on viability of wt and RIP-1 deficient or RIP-3 deficient SV40-MEF as assessed by MTT assay. **f** Western blot analysis of PARP expression and processing and of poly(ADP-

ribose) formation of L929 cells exposed to 5 μM or 20 μM Cd over 2–24 h. Asterisks mark bands of unknown origin or unspecific bands. GAPDH and tubulin were used as a loading control. All data are from at least three independent experiments. *n.s.* Non-significant. * $p < 0.05$ compared to controls; § $p < 0.05$ compared to cells treated with Cd only. Statistical analysis was performed using ANOVA followed by Student–Newman–Keuls test or Dunn’s test as appropriate

Fig. 5.

Autocrine TNF α production elicited by Cd contributes to cell death (a), (b) Effect of TNF α , added at 10 ng/ml (a) and 100 ng/ml (b) on cell viability estimated by MTT assay of L929 cells and SV40-MEF in the absence or presence of 5 μ M Cd. c–f Effect of TNF α neutralizing antibody, added at 2 μ g/ml or 10 μ g/ml 1 h before treatment, on Cd-induced cell death of L929 cells and SV40-MEF as assessed by MTT assay (c, e), determination of ATP levels (d), and estimated from PI-exclusion (f). Data are from at least three independent

experiments. Statistical analysis was performed using ANOVA followed by Student–Newman–Keuls test or Dunn’s test as appropriate

Fig. 6.

Bmf-deficient cells are partially protected against Cd toxicity. **a, b** Cell viability estimated from PI-exclusion (**a**) and from sub-G1 assay (**b**) of S40-MEF from the indicated genotype. **c** Mitochondrial cytochrome *c* release measured after 24 h of exposure to 5 μM or 20 μM Cd or to 0.1 μM staurosporine (STS) in S40-MEF derived from wt or Bmf-deficient mice. **d** Impact of Nec-1 and QVD on cell viability of Bmf-deficient SV40-MEF exposed to 5 μM Cd for 24 h as determined by measuring PI-exclusion or the sub-G1 population. **e** Relative transcription of Bmf of S40-MEF determined after 6 h of exposure to Cd or to 4 μM histone

deacetylase inhibitor SAHA. **f** Western blot analysis of Bmf expression of S40-MEF exposed to 5 μ M or 20 μ M Cd over 12–24 h. Serum deprivation (SD) served as a positive control. Tubulin was used as a loading control. All data are from at least three independent experiments. * $p < 0.05$ compared to wt cells or untreated cells (**d**); § $p < 0.05$ compared to controls of the same genotype