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TNF α -induced macrophage death via caspase-dependent and independent pathways

Tri M. Tran^{1,§}, Vladislav Temkin^{1,§}, Bo Shi¹, Lisa Pagliari¹, Soizic Daniel², Christiane Ferran², and Richard M. Pope^{1,†}

¹ Division of Rheumatology, Department of Medicine, Northwestern University Feinberg School of Medicine, Chicago, Illinois

² Division of Vascular Surgery and Nephrology, Departments of Surgery and Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA

Abstract

Macrophages are the principal source of TNF α , yet they are highly resistant to TNF α -mediated cell death. Previously, employing *in vitro* differentiated human macrophages, we showed that following the inhibition of NF- κ B, TNF α -induced caspase-8 activation contributes to DNA fragmentation but is not necessary for the loss of the inner mitochondrial transmembrane potential ($\Delta\Psi$ m) or cell death. We here extend these observations to demonstrate that, when NF- κ B is inhibited in macrophages, TNF α alters lysosomal membrane permeability (LMP). This results in the release of cathepsin B with subsequent loss of $\Delta\Psi$ m and caspase-8 independent cell death. Interestingly, the cytoprotective, NF- κ B-dependent protein A20 was rapidly induced in macrophages treated with TNF α . Ectopic expression of A20 in macrophages preserves LMP following treatment with TNF α , and as a result, mitochondrial integrity is safeguarded and macrophages are protected from cell death. These observations demonstrate that TNF α triggers both caspase 8-dependent and -independent cell death pathways in macrophages and identify a novel mechanism by which A20 protects these cells against both pathways.

Keywords

apoptosis; caspase-8; cathepsin B; A20

INTRODUCTION

Tumor necrosis factor- α (TNF α), which is primarily produced by macrophages, is a pivotal mediator in rheumatoid arthritis, inflammatory bowel disease, atherosclerosis, ischemia/reperfusion injury and viral hepatitis [1–4]. Macrophage resistance to TNF α -induced cell death may contribute to the persistence of these diseases. TNF α -induced apoptosis is signaled via TNF receptor 1 (TNF-R1) [5], which results in recruitment of adaptor proteins including TNF receptor associated protein with death domain (TRADD), receptor interacting protein (RIP), and TNF receptor associated factor 2 (TRAF2) into complex I at the plasma membrane [6]. Complex I then dissociates from TNFR1 and recruits Fas-associated death domain protein (FADD) and the initiator caspase 8, forming complex II. Activated caspase 8 may cleave either caspase 3 directly or activate the Bcl-2 family protein Bid [7] which targets the mitochondria,

[†]Corresponding author. Mailing address: Division of Rheumatology, Northwestern University Feinberg School of Medicine, 240 E. Huron St., Suite M300, Chicago, IL 60611. Phone: (312) 503-8003. Fax: (312) 503-0994. E-mail: E-mail: rmp158@northwestern.edu.

[§]T.M.T. and V.T. contributed equally to this manuscript.

activating the pro-apoptotic molecules Bax and Bak, resulting in mitochondrial outer membrane permeabilization (MOMP) and the release of intermembranous proteins such as cytochrome c which initiates an additional death signal through the activation of caspase 9 and 3. Both of these pathways lead to DNA fragmentation and the apoptotic phenotype [8]. These events ultimately result in the loss of inner mitochondrial transmembrane potential ($\Delta\Psi_m$) and cell death [9].

The formation of TNF-R1 complex I also transmits survival signals through activation of transcription factor NF- κ B [10,11]. Multiple cell types from mice deficient in mediators of TNF α -induced NF- κ B activation, including RelA (p65), I κ B kinase β , and I κ B kinase γ , are highly sensitive to TNF α -mediated apoptosis [12,13]. NF- κ B induces expression of several anti-apoptotic proteins, including FLIP, which interferes with caspase-8 activation in TNF-R1 complex II [6], Bcl-x_L that inhibits MOMP preventing the loss of mitochondrial intermembranous space proteins such as cytochrome c [14], and NF- κ B inhibitor and ubiquitin-editing enzyme protein A20 which targets RIP for proteasomal degradation [15–17].

Recent observations suggest that lysosomal membrane permeabilization (LMP), mediated through caspase-dependent or caspase-independent pathways, may also contribute to TNF α -mediated cell death [18–24]. In some cell types such as mouse embryonic fibroblasts (MEFs) and murine hepatocytes, LMP is mediated through caspase 8 activation [21,22,25]. TNF α may also induce LMP through activation of neutral sphingomyelinase (N-Smase), mediated by Factor Associated with N-Smase (FAN), ultimately generating ceramide and sphingosine [26,27]. Further, receptor-mediated cell death may occur independent of caspase activation, and it has been shown that RIP may contribute to caspase independent cell death mediated through TNFR1, Fas, TRAILR and TLR4 [28,29].

Our previous studies demonstrated that, following inactivation of NF- κ B by the ectopic expression of a super repressor I κ B α , TNF α -induced caspase 8 activation contributed to DNA fragmentation but was not necessary for the loss of $\Delta\Psi_m$ or cell death [30]. In the present study, we demonstrate in macrophages, the principal source of TNF α , that following the inhibition of NF- κ B activation, TNF α also signals through a lysosomal cell death pathway, mediated by cathepsin B, which is independent of caspase-8. We also observed in macrophages, the principal source of TNF α , that both caspase 8-dependent and -independent pathways are triggered by this cytokine, and that the rapid induction of A20 protects against the caspase 8-independent, lysosomal pathway of cell death. These observations may be important in chronic inflammatory diseases, such as rheumatoid arthritis, which are mediated in large part through TNF α expressed by macrophages.

MATERIALS AND METHODS

Materials

Polymyxin B sulfate, cathepsin D inhibitor pepstatin A, Z-Arg-Arg-7-amido-4-methylcoumarin hydrochloride (Z-Arg-Arg-AMC), pyrrolidine dithiocarbamate (PDTC), digitonin, Histopaque, the serine protease inhibitor Pefabloc SC, HEPES, and the β -N-acetylglucosaminidase (NAG) fluorogenic substrate 4-Methylumbelliferyl N-acetyl- β -D-glucosaminide were obtained from Sigma-Aldrich (St. Louis, MO). RPMI, FBS, PBS, L-glutamine, penicillin and streptomycin were obtained from Gibco (Gaithersburg, MD). Propidium iodide (PI) was from Roche Molecular Biochemicals (Indianapolis, IN), Rhodamine 123 (Rh123) was from Molecular Probes (Eugene, OR). Caspase 8 (Ac-IETD-AFC) synthetic fluorogenic substrate and caspase 8 inhibitor (zIETD-fmk, IETD) were purchased from Enzyme Systems Products (Livermore, CA). Cathepsin B inhibitors CA and CA-074 Me, and cathepsin L inhibitor zFF-fmk were obtained from Calbiochem (San Diego, CA). Recombinant human TNF- α was from R&D Systems (Minneapolis, MN).

Cell isolation and culture

Buffy coats (Lifesource, Glenview, IL) were obtained from healthy donors. Mononuclear cells, isolated by Histopaque gradient centrifugation, were separated by countercurrent centrifugal elutriation (JE-6B, Beckman Coulter, Palo Alto, CA) in the presence of 10 µg/ml polymyxin B sulfate, as previously described [31,32]. Isolated monocytes were > 90% pure, as determined by morphology, non-specific esterase staining, and CD14 expression examined by flow cytometry (data not shown). Monocytes were adhered to plates (Costa, Cambridge, MA) for 1 hour in RPMI and 1 µg/ml polymyxin B sulfate. Following adherence, human blood-isolated monocytes were differentiated in vitro for 7 days in RPMI 1640 containing 20% heat-inactivated FBS, 1 µg/ml polymyxin B sulfate, 0.35 mg/ml L-glutamine, 120 Units/ml penicillin, and streptomycin (20% FBS/RPMI 1640) [33]. These studies have been approved by the Northwestern University Institutional Review Board.

Adenovirus infection of primary macrophages

Primary macrophages were infected at the concentrations identified in each experiment as a multiplicity of infection (moi) with adenoviral vectors expressing a super-repressor IκBα (Ad-IκBα) or a control vector (β-galactosidase or CMV-blank, Ad-Control) as previously described [31,33]. Where indicated, macrophages were co-infected with Ad-A20 (30 moi) or Ad-control at the same concentrations. Within each experiment, the total concentration of adenoviral vector was held constant. After infection, 20% FBS/RPMI was added, and the cells were incubated overnight. The macrophages were then washed twice with PBS and incubated in 20% FBS/RPMI for an additional 8 hours (total of 30 hours from the initiation of the infection). The infected cells were then treated with TNFα (10 ng/ml) or PBS for 16 hours, or as indicated in the figures.

Cell transfection

In vitro differentiated macrophages were transfected with either non specific, RIP or cathepsin B siRNA (final, 100 nM; Dharmacon, Inc, Lafayette, CO) employing Lipofectamine 2000 according to the manufacture's directions (Invitrogen). The cells were then incubated for 72 hours prior to analysis by immunoblot assay, or 48 hours before adenoviral infection.

Analysis of inner mitochondrial transmembrane potential ($\Delta\Psi_m$) and cell viability

Loss of $\Delta\Psi_m$ was assessed utilizing the cationic lipophilic green fluorochrome Rh123 as previously described [32,34]. Disruption of $\Delta\Psi_m$ is associated with the loss of Rh123 retention and the decrease of fluorescence. Cultures were incubated with Rh123 (20 ng/ml) for 30 minutes at 37°C, harvested and wash with PBS. To assess cell membrane integrity simultaneously, PI (3.3 µg/ml) was added to the cells just prior to analysis by flow cytometry.

Determination of Subdiploid DNA content

At the indicated time points, cultures were harvested, washed once with PBS, and fixed in 70% ethanol at -20°C overnight, followed by staining with PI (50 µg/ml). The apoptotic profile was determined by flow cytometry utilizing a Beckman-Coulter EpicsXL flow cytometer and system 2 software. The subdiploid DNA peak (<2N DNA) immediately adjacent to the G₀/G₁ peak (2N DNA) represented apoptotic cells was quantified by histogram analyses as previously described [29,32].

Analysis of loss of lysosomal integrity

The cultures were incubated with the fluorescent dye LysoTracker Green DND-26 (Invitrogen) (50 nM) for 30 min. at 37°C. Lack of lysosomal retention of LysoTracker detected as decreased

intensity of fluorescence which was determined by flow cytometry as previously described [35–37].

Caspase-8 activity assay

Cell lysates were prepared according to the manufacturer's directions (BioVision, Mountain View, CA). The lysates were incubated for 1 hour at 37°C with the fluorogenic caspase-8 substrate (Ac-IETD-AFC), and the samples read on a fluorometer at 400 nm excitation and 505 nm emission.

Measurement of cytosolic enzyme activities

To measure cytosolic enzyme activities, cells were washed twice with PBS, then treated with an extraction buffer (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, and 1 mM pefabloc, pH 7.5) containing 50 µg/ml digitonin for 15 minutes on ice. The digitonin concentration and treatment times were optimized to result in the total release of the cytosolic LDH activity without disruption of lysosomes as previously described [38]. The cytosolic extracts were collected and analyzed for cathepsin B activity by adding 50-µl volume of cytosolic extract to an equal volume of 20 µM Z-Arg-Arg-AMC in cathepsin reaction buffer (50 mM sodium acetate, 4 mM EDTA, 8 mM DTT, 1 mM pefabloc, pH 6.0) followed by incubation for 1 hour at 37°C. The liberated AMC was measured at 360 nm excitation and 460 nm emission on fluorescence reader. β-N-acetyl-glucosaminidase (NAG) activity was estimated by incubation with three volumes of 0.2M sodium citrate buffer, pH 4.5, containing 300 µg/ml 4-Methylumbelliferyl N-acetyl-β-D-glucosaminide for 30 minutes at 37°C. The liberated methylumbelliferyl (excitation, 356 nm; emission, 444 nm) was measured by fluorometer as previously described [39]. The amount of protein in each sample was used as an internal standard with which protease activities were normalized. Cytosolic LDH activity was determined employing the Cytotoxicity Detection Kit following manufacturer's instructions (Roche).

Cytosolic cytochrome c release assay

Cells were harvested and washed twice with PBS, then treated with an extraction buffer (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 8 mM DTT, and 1 mM pefabloc, pH 7.5) containing 50 µg/ml digitonin for 15 minutes on ice with constant rotation in order to extract the cytosol without disrupting the mitochondrial membrane as previously described [22]. The cells were then pelleted at 453×g, 4°C for 5 minutes. The supernatant was then collected, centrifuged at 18,000×g at 4°C for 20 minutes. The supernatants (cytosolic extracts, 15 µg) were subjected to 15% SDS-PAGE.

Microarray analysis

Affymetrix (Santa Clara, CA) GeneChip Human Genome Focus Array representing over 23,000 probe sets was performed as previously described [40].

Real time polymerase chain reaction (PCR)

Reverse transcription and real time PCR were performed as previously described [40]. Thermocycling was carried out in a final volume of 20 µl using a TaqMan universal PCR master mix and a FAM-labeled mixture of primers and probe for FAN.

Immunoblot analysis

Whole-cell or cytosolic extracts were prepared from in vitro differentiated macrophages and polyacrylamide gel electrophoresis was performed as previously described [32,41]. The antibodies used were: mouse monoclonal anti-caspase 8 (Cell Signaling Technology, Inc.

Beverly, MA), rabbit polyclonal anti-Bid and mouse monoclonal anti-cytochrome c antibody (BD PharMingen, San Diego, CA), rabbit anti- β -Actin (Calbiochem), mouse anti-RIP monoclonal antibody (BD Transduction Laboratories, Franklin Lakes, NJ), mouse anti-A20 monoclonal antibody (Imgenex, San Diego, CA), anti-FLAG monoclonal antibody (Sigma-Aldrich, St. Louis, MO). The secondary antibodies employed were donkey anti-rabbit or sheep anti-mouse secondary antibodies conjugated to horseradish peroxidase (Amersham Pharmacia Biotech, Piscataway, N.J.).

Statistical analysis

Significance was determined by Student's *t* test.

RESULTS

Loss of lysosomal integrity and of $\Delta\Psi_m$ precedes DNA fragmentation or loss of cell viability

Employing *in vitro* matured macrophages infected with an adenoviral vector expressing a super repressor I κ B α (Ad-I κ B α), we previously demonstrated that incubation with the caspase 8 selective inhibitor zIETD.fmk (IETD) or CrmA (data not shown), or the broad specificity inhibitor zVAD.fmk, prevented DNA fragmentation, while cell survival, as defined by the ability to exclude propidium iodide (PI), and loss of inner mitochondrial transmembrane potential ($\Delta\Psi_m$) were not protected [30]. In order to identify a potential role of the lysosomal pathway in the induction of TNF α -induced cell death [21,42], experiments were performed employing the fluorescent plasma membrane-permeable dye Lysotracker, utilizing previously described methods [35–37], to determine the temporal relationship between the effects on lysosomal integrity and the relationship to the other events observed during the process of cell death. Macrophages infected with the Ad-I κ B α exhibited a significant ($p < 0.01$) decrease in lysosomal integrity as early as 2 hours after the addition of TNF α (Figure 1A), concomitant with the loss of $\Delta\Psi_m$ (Figure 1B). DNA fragmentation and loss of cell membrane integrity were first detected at 4 and 12 hours, respectively, after the addition of TNF α (Figure 1C, D). Additional experiments were performed to characterize the release of lysosomal contents into the cytoplasm. Cathepsin B and the lysosomal enzyme β -N-acetyl-glucosaminidase were detected in the cytosol 2 hours following the addition of TNF α (Figure 1E, F), confirming the results obtained with Lysotracker. These observations suggest that the LMP may contribute to macrophage death induced by TNF α .

Cathepsin B contributes to TNF α -induced macrophage death

Lysosomal membrane disruption results in the release of cathepsins into the cytosol, and cathepsin B, D and L have been reported to be execution proteases in TNF α -induced apoptotic and necrotic cell death in a variety of cell types [21,43–45]. To determine whether these lysosomal cathepsins were essential for macrophage cell death, we examined the effects of cell-permeable peptide inhibitors of these proteases. In contrast to the results obtained following the inhibition of caspase 8, the cathepsin B inhibitor CA-ME not only suppressed the apoptotic phenotype, but also reduced the loss of $\Delta\Psi_m$, and increased cell viability ($p < 0.01$, Figure 2A–C). The cell-impermeable cathepsin B inhibitor CA did not have any affect, nor did the cell-permeable inhibitors of cathepsin D (pepstatin A) or cathepsin L (zFF-FMK) (data not shown). To confirm the essential role of cathepsin B in mediating macrophage death, cathepsin B was reduced employing a specific siRNA (CatBi). Similar to the results obtained with CA-ME, the forced reduction of cathepsin B protected against the loss of $\Delta\Psi_m$ and apoptotic cell death (Figure 2D–F). Together, these observations demonstrate that the release of cathepsin B contributes to TNF α -induced cell death in macrophages.

The lysosomal pathway is independent of caspase 8

Since inhibition of caspase 8 only protected against DNA fragmentation [30] and the inhibition of cathepsin B only partially protected against apoptotic cell death (Figure 2), experiments were performed to determine the effects of the combined inhibition of both pathways. In contrast to the results observed by inhibition of either pathway individually, the combination of the caspase 8 inhibitor IETD plus CA-ME, almost completely prevented apoptotic cell death (Figure 3A, B). Further, with the combination, there was a marked suppression of the loss of $\Delta\Psi_m$ (Figure 3C) and protection of lysosomal integrity as determined by the retention of LysoTracker (Figure 3D). However, inhibition of caspase 8 alone resulted in no protection against the loss of lysosomal integrity, while inhibition of cathepsin B was effective (Figure 3D). Additionally, although the inhibition of caspase 8 had no effect on the loss of $\Delta\Psi_m$ (Figure 3C), it did reduce MOMP, as determined by the release of cytochrome c (Figure 3E). Further, while there was no significant reduction of MOMP by CA-ME alone, the combination of CA-ME plus IETD prevented the induction of MOMP (Figure 3E). Since both caspase 8 and cathepsin B have been shown to cleave Bid [9,23], the activation of Bid to tBid was examined by immunoblot analysis. In macrophages, inhibition of cathepsin B by CA-ME reduced Bid cleavage, although not as efficient as the caspase 8 inhibitor IETD. These observations suggest that the effects on the inner membrane ($\Delta\Psi_m$) and lysosomal integrity were mediated primarily by cathepsin B, and to a lesser degree by caspase 8, while caspase 8, and to a lesser degree cathepsin B, were responsible for MOMP and Bid cleavage.

Reactive Oxygen Species (ROS) mediate the loss of $\Delta\Psi_m$

ROS have been implicated in apoptosis and necrosis [46–48]. To determine if ROS contribute to TNF α -induced cell death, macrophages were incubated with TNF α plus the anti-oxidant BHA. BHA significantly reduced DNA fragmentation, cell death and the loss of $\Delta\Psi_m$, when examined 16 hours following the addition of TNF α (Figure 4A–C). In contrast, there was no protection against MOMP (Figure 4D) or LMP (Figure 4E), examined 6–7 hours following the addition of TNF α . These observations suggest that the effects of ROS were downstream of LMP. The data further indicate that, at the time points examined, ROS contributed to the loss of the $\Delta\Psi_m$ but not MOMP.

RIP is an upstream initiator of caspase 8-induced apoptosis

RIP is critical for TNF α -induced NF- κ B activation and may also promote non-apoptotic cell death [28,29,49]. Therefore studies were performed to determine the role of RIP in the TNF α -induced cell death in macrophages when NF- κ B activation is suppressed. Employing RIP siRNA (RIPi), the forced reduction of RIP (Figure 5A) suppressed TNF α -induced DNA fragmentation (Figure 4B), but failed to protect against the loss of $\Delta\Psi_m$ (Figure 5C) or cell viability (Figure 5D). Consistent with its presence in TNFR1 complex II, the forced reduction of RIP suppressed caspase 8-like activity (Figure 5E). In contrast the forced reduction of RIP failed to protect against LMP determined by the release of cathepsin B (Figure 5F). These observations suggest that RIP is important for TNF α -induced caspase 8 activation, but is not essential for the caspase 8-independent, TNF α -mediated cell death in macrophages.

NF- κ B-regulated A20 is rapidly induced by TNF α

NF- κ B activation protects against apoptosis by inducing the expression of a number of genes whose products inhibit apoptosis [11,50]. In order to identify the mechanism responsible for preventing macrophage cell death when NF- κ B activation is suppressed, macrophages were pre-incubated with TNF α for 2 hours prior to the addition of PDTC, which we previously showed inhibits NF- κ B activation in macrophages [31]. Pre-incubation with TNF α prior to the inhibition of NF- κ B, significantly ($p < 0.005$) suppressed macrophage cell death compared to cells where PDTC was added at the same time as TNF α (Figure 6A). Pre-incubation with

TNF α also suppressed the loss of $\Delta\Psi_m$ and DNA fragmentation (data not shown). These observations suggest that pre-treatment with TNF α for 2 hours provides protection against both the caspase 8-independent and -dependent pathways of cell death.

Since many genes have been implicated in the protection against TNF α -mediated cell death, microarray analysis was performed to determine which protective genes might be up regulated by NF- κ B at 2 hours. Macrophages were infected with Ad-control or Ad-I κ B α vectors and then incubated with control medium or with TNF α for 2 hours. The cells were harvested and RNA was isolated and employed for microarray analysis. Of the 83 genes identified as up regulated by TNF α -induced NF- κ B activation at 2 hours, 10 were identified as related to apoptosis (Figure 6B). Among the genes with a potential role in protecting against apoptosis, the zinc-finger protein A20 was the most highly expressed at 2 hours and its induction was prevented by the super repressor I κ B α (Figure 6B). Employing immunoblot analysis, following the addition of TNF α , A20 was highly induced at 2 hours and it persisted through 24 hours (Figure 6C). These observations identify A20 as a candidate to protect against the caspase 8-independent cell death pathway in macrophages.

A20 prevents macrophages from TNF α -mediated cell death

To determine if A20 protects against TNF α -mediated cell death, macrophages were co-infected with a control adenoviral vector or one expressing A20 (Ad A20) plus Ad-I κ B α or Ad-control. The ectopic expression of A20 not only protected ($p < 0.01$) against the loss of lysosomal integrity, determined by Lysotracker retention and cathepsin B release (Figure 7A, B) and the loss of $\Delta\Psi_m$ (Figure 7C), but also against cell death and DNA fragmentation (Figure 7D, E). To further characterize the mechanism of action of A20, caspase 8-like activity was measured in macrophages. The expression of A20 also effectively ($p < 0.01$) suppressed the caspase 8-like activity observed in response to TNF α when NF- κ B activation was suppressed (Figure 7F). Therefore, A20 was effective at suppressing both the caspase 8-dependent and -independent pathways.

DISCUSSION

In macrophages, when NF- κ B activation is suppressed, TNF α -induced cell death is initiated by two pathways that converge on the mitochondria. One pathway is mediated by caspase 8, which is necessary for the DNA fragmentation which is characteristic of apoptosis. However, when caspase 8 activation is suppressed, even though DNA fragmentation is suppressed, there is no protection against the loss of $\Delta\Psi_m$ or cell death, which was necrotic, determined by electron microscopy (data not shown). The second, caspase 8-independent, pathway involves LMP and the release of cathepsin B. Inhibition of cathepsin B partially protects against TNF α -induced apoptotic cell death, but inhibition of both cathepsin B and caspase 8 results in nearly complete protection, demonstrating that both pathways are involved in TNF α -induced cell death. The NF- κ B mediated induction of A20 protects against both caspase 8-dependent and -independent pathways. These studies identify a novel mechanism, involving two pathways, for TNF α -induced cell death in macrophages and define a protective mechanism provided by the rapid NF- κ B-mediated induction of A20. These observations demonstrate that in macrophages, caspase 8 is critical for a characteristic feature of apoptosis, DNA fragmentation, while cathepsin B is dominant in the execution of cell death.

Consistent with our observations, it has become increasingly clear that caspase activation is often not obligatory for the loss of mitochondrial integrity or sufficient for the induction of cell death [51]. In some cases, caspase inhibition actually increases TNF α -induced cell death [42, 48]. Nonetheless, following the inhibition of NF- κ B, TNF α does not uniformly induce caspase-independent cell death in all cell types. In human synovial fibroblasts, when NF- κ B activation was suppressed, TNF α -induced apoptotic cell death was prevented by the inhibition of caspase

8 [41]. In contrast, cell death induced through the intrinsic pathway, for example by cellular stress or DNA damage, does not require caspase activation, although caspase activation is observed following the induction of MOMP and the release of cytochrome c, which results in the activation of caspases 9 and 3 [51]. However, in macrophages, caspase-dependent and -independent pathways are concurrent and converge on the mitochondria.

TNF α -induced activation of caspase 8 may lead to MOMP, mediated through Bid and Bax/Bak, which results in the release of cytochrome c and the activation of caspases 9 and 3 [9, 52–54]. Release of cathepsin B into the cytosol may also result in Bid cleavage [23]. In macrophages, caspase 8, and to a lesser degree cathepsin B, contributed to the activation of Bid. However the activation of Bid and the induction of MOMP did not appear critical for TNF α -induced cell death in macrophages since inhibition of caspase 8 suppressed Bid cleavage and the release of cytochrome c but had no effect of cell death. Supporting this interpretation, the ectopic expression of Bcl-x_L in macrophages, which protects against Bax/Bak-mediated cell death, prevented the TNF α -mediated release of cytochrome c but did not suppress the cell death or the loss of $\Delta\Psi_m$ (data not shown). The role of Bid is likely cell type specific since Bid $-/-$ hepatocytes demonstrated decreased TNF α -induced LMP and cell death, while Bid $-/-$ MEFs did not [22,25].

The hallmark of a cathepsin-mediated death pathway is LMP, which results in the release of active cathepsins into the cytosol. Our observations demonstrate that the induction of LMP and the release of cathepsin B were independent of caspase 8 activation. This contrasts with earlier studies showing that LMP may occur secondary to caspase 8 activation [21,22,42]. These differences may be due to cell type- and species differences. Indeed, in murine hepatocytes and mouse embryonic fibroblasts (MEFs), caspase 8 is required for LMP [21, 22]. It is well documented that in MEFs caspase 8 directly cleaves caspase 9 at Asp349, independent of the apoptosome [22]. Cleaved caspase 9 results in LMP, through a mechanism that has not been fully characterized [22]. Although we did not directly examine the role of caspase 9, inhibition with IETD or CrmA (data not shown) or the pan-caspase inhibitor zVAD.fmk [30] failed to protect macrophages from cell death, even though DNA fragmentation was markedly reduced. Further, the Asp349 caspase 8 cleavage site is not conserved in human caspase 9, suggesting that this process may not be operative in humans [22]. In fact, reports in the literature identify differences between the human fibrosarcoma cell line WEHI-S, where TNF α -induced cathepsin B mediated cell death is independent of caspase 8, and ME180 cervical cancer cells, MCF-7 breast cancer cells, and Jurkat T cells where cathepsin B-mediated cell death does depend on caspase 8 activation [37,42,55]. Together, these observations demonstrate that the caspase 8 dependence of cathepsin B-mediated cell death may be cell type-, disease- and species-specific.

Our data demonstrate that the cathepsin B mediates LMP in macrophages. LMP was up stream of the loss of $\Delta\Psi_m$ and cell death. It remains to be determined whether cathepsin B directly affects $\Delta\Psi_m$ or if this is mediated through another mechanism. Potential candidates contributing to the release of cathepsin B include the FAN-mediated activation of N-Smase, RIP or reactive oxygen species [20,24]. In macrophages, we were not able to detect any increase of N-Smase activity following the addition of TNF α (data not shown), suggesting that this was not the mechanism responsible for LMP. Neither the forced reduction of RIP nor the anti-oxidant BHA protected against lysosome rupture, excluding their role in LMP. RIP has been implicated in necrotic, caspase-independent, cell death mediated by the accumulation of cellular ROS which causes the loss of mitochondrial integrity [47]. We previously demonstrated that the addition of the broad specificity caspase inhibitor zVAD.fmk to TNF α in macrophages resulted in necrotic cell death by a mechanism implicating RIP [49]. Also, in macrophages, we demonstrated that the forced reduction of RIP protected macrophages from TLR4-mediated cell death when NF- κ B activation was suppressed [29]. However, in the

current study, RIP was not responsible for the loss of lysosomal integrity or of $\Delta\Psi_m$, although RIP was necessary for TNF α -induced caspase 8 activation [6,56].

Although our data do not suggest that ROS contributed to LMP, they do suggest that ROS contributed to the loss of the $\Delta\Psi_m$. Although the mechanisms remain to be clarified, our observations suggest that the accumulation of ROS, mediated by cathepsin B, may contribute to the necrotic phenotype by promoting the loss of the $\Delta\Psi_m$. It is possible that this leads to the formation of permeability transition pores in the inner mitochondrial membrane, which may result in necrosis [57]. Supporting this interpretation, when caspase activation was suppressed, the loss of the $\Delta\Psi_m$ was unabated even though MOMP was greatly reduced, at the time points examined. These observations suggest that effects of LMP on the inner mitochondrial membrane, mediated at least in part through ROS, contributed to necrosis when caspase activation was suppressed.

NF- κ B regulated A20, which in turn is capable of suppressing TNF α -induced NF- κ B activation.[58], was important in the protection of macrophages against TNF α -induced cell death. Pre-treatment of macrophages with TNF α for 2 hours prior to the inhibition of NF- κ B activation resulted in the induction of A20, which was associated with protection against the TNF α -induced loss of $\Delta\Psi_m$ (data not shown) and cell death. Although many anti-apoptotic proteins are regulated by NF- κ B, at 2 hours, there was no major effect on other anti-apoptotic proteins including FLIP, GADD45 β or XBP1, suggesting that they were not contributing to the protection noted at this time point. Supporting the importance of A20 in macrophages, the ectopic expression of A20 protected against all aspects of TNF α -induced cell death including LMP. Prior studies in endothelial cells demonstrated that A20 suppressed the TNF apoptotic pathway by inhibiting activation of the apical caspases 8 and 2, the executioner caspases 3 and 6, Bid cleavage, and the release of cytochrome *c*, which maintained mitochondrial integrity [59]. Studies in Jurkat T-cells demonstrated that A20 inhibits TNF α -induced apoptosis by disrupting the recruitment of TRADD and RIP to TNFR-1 complex [60]. Therefore because RIP contributes to TNF α -induced caspase 8 activation through its role in complex II [6], A20 may suppress caspase 8 through the reduction of RIP by ubiquitination, targeting it for proteasomal degradation [15], and/or the disruption of complex I [60] and subsequently complex II. Nonetheless, the forced reduction of A20 by a specific siRNA, in the absence of inhibition of NF- κ B, failed to sensitize macrophages to TNF α -mediated cell death (data not shown), suggesting that the absence of A20 alone is not sufficient to permit TNF α -induced LMP.

Taken together, the current study identifies a novel mechanism of TNF α -induced cell death in macrophages, which involves caspase-dependent and -independent pathways concurrently. Our data demonstrates that the caspase 8-dependent pathway is mediated through RIP, while the caspase-independent pathway is mediated through LMP and cathepsin B, and that A20, a protein up regulated by TNF α as part of the NF- κ B-dependent response, is important for protecting against both pathways. Further investigation is needed to define the mechanism by which A20 blocks these pathways. The importance of understanding the function of A20 is highlighted by the recent discovery that a susceptibility allele in this gene has been identified in patients with rheumatoid arthritis [61]. The expression of A20 may be important for preventing TNF α -mediated cell death of macrophages in chronic inflammatory conditions such as rheumatoid arthritis and inflammatory bowel disease, suggesting that A20 may be a potential therapeutic target in chronic inflammatory conditions.

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Figure 1. Loss of lysosomal and mitochondrial integrity precedes DNA fragmentation or loss of cell membrane integrity

Differentiated macrophages were infected with Ad-IκBα (200 moi) or control vector and then incubated with TNFα (10 ng/ml) for the indicated time periods. Lysosomal integrity (defined by Lysotracker retention, **A**), loss of $\Delta\Psi_m$ (% of control Rh123, **B**), apoptosis (<2N DNA, **C**), loss of cell membrane integrity (% PI+ cells, **D**), cytosolic cathepsin B activity (**E**) and cytosolic NAG activity (**F**) were analyzed. **A–D**, Results are the mean $\pm$ S.E. of 3 to 6 independent experiments, performed in duplicate. *, $p < 0.01$ or **, $p < 0.005$ versus control treatment. **E–F**, the values represent mean $\pm$ S.E. of an experiment performed in duplicate, which was representative of 2 independent experiments.

Figure 2. Cathepsin B contributes to TNF α -induced cell death

Differentiated macrophages were infected with Ad-IkB (200 moi) or a control adenoviral vector, followed by incubation with TNF α (10 ng/ml) for 24 hours. **A–C**, The cell permeable cathepsin B inhibitor CA-ME (2.5, 5 and 10 μ M) was added to the cells 1 hour before TNF α . Loss of cell membrane integrity (**A**), < 2N DNA (**B**) and loss of mitochondrial membrane potential (**C**) were analyzed. **D–F**, Macrophages were transfected with cathepsin B siRNA (100nM, CatBi) or non-specific siRNA (NS). The effect of the siRNA on cathepsin B expression was examined by western blotting (**D**, panel on right). Following the addition of siRNA, the macrophages were infected with Ad-IkB α (200 moi) or the control vector, followed by incubation with TNF α (10 ng/ml) for 16 hours. The cells were then harvested and examined for the loss of cell membrane integrity (**D**), apoptosis (**E**) and $\Delta\Psi$ m (**F**). The results represent the mean $\pm$ S.E. of 3 independent experiments, performed in duplicate. * represents $p < 0.01$ or **, $p < 0.005$ versus control treatment.

Figure 3. Inhibition of both caspase-8 and cathepsin B protects against TNF α -induced cell death Differentiated macrophages were infected with Ad-I κ B α (50 moi, **A–D**; or 200 moi **E**). The cell membrane permeable caspase 8 selective inhibitor (IETD, 20 μ M) or the cathepsin B inhibitor (CA-ME, 20 μ M) were added to the cells 1 hour before the addition TNF α (10 ng/ml) for 16hours (**AD**) or 7 hours (**E**). The loss of cell membrane integrity (**A**), apoptosis (**B**), $\Delta\Psi$ m (**C**) and lysosomal integrity (**D**) were analyzed. The results represent the mean $\pm$ S.E. of 3 independent experiments, performed in triplicate. * represents $p < 0.05$ and ** < 0.01 versus control treatment. **E**, Cytosolic extracts were examined by cytochrome c released from the mitochondria by immunoblot analysis employing an anti-cytochrome c antibody. β -actin served as the loading control. The release of cytochrome c was compared with that observed following treatment with the control (DMSO), normalized to β -actin. The upper panel is representative of 2 independent experiments and the lower panel represents the mean $\pm$ S.E. of 2 independent experiments. * represents $p < 0.05$. **F**, Bid cleavage was examined by immunoblot analysis employing anti-Bid antibody. The cells were harvested 4 hours following the addition of TNF α . β -actin was used as the loading control. The experiment is representative of 2 independent experiments.

Figure 4. ROS is downstream regulator of lysosome pathway

Differentiated macrophages were infected with Ad IkBα (50 moi (A, B, C) or 200 moi (D, E)) followed by the addition of BHA (100 μM) and TNFα (10 ng/ml) for 16 hours (A, B, C) or 6–7 hours (D, E). The cells were then examined for loss of membrane integrity (A), DNA fragmentation (B), and mitochondrial inner membrane potential (C). The results are the mean ± S.E. of 2 independent experiments, performed in duplicate. D. Cytosolic cytochrome c was determined by western blot with anti-cytochrome c antibody. β-actin was used as control. EtOH was the vehicle for the BHA. The panel on the left is representative of 2 independent experiments. The panel on the right presents the mean ± S.E. of 2 independent experiments. The density of the cytochrome c bands was normalized with those for β-actin. E, Cytosolic cathepsin B activity. The results are the mean ± SE of 3 independent experiments, performed in duplicate. *, $p < 0.05$ versus control treatment.

Figure 5. The forced reduction of RIP only protects macrophages from DNA fragmentation

Differentiated macrophages were transfected with RIP siRNA (100nM, RIPi) or non-specific siRNA (NSi), and the expression of RIP repression was examined by western blotting (A). Cells were then infected with Ad-IkBα (50 moi) (A–D) or 200 moi (E,F) followed by incubation with TNFα (10 ng/ml) for 16 hours (B–D) or 4 hours (E, F), respectively. Loss of cell membrane integrity (D), DNA fragmentation (B), $\Delta\Psi_m$ (C), caspase 8-like activity (E), and cytosolic cathepsin B (F) were determined. B–E. The results are the mean $\pm$ S.E. of 2 (F) or 3 (B–E) independent experiments, performed in triplicate. * represents $p < 0.05$ and ** $p < 0.01$ versus control treatment.

Figure 6. NF-κB-regulated A20 is rapidly induced by TNFα in macrophages

A. Differentiated macrophages were pretreated with TNFα (10 ng/ml) for 2 hours; the medium was then replaced with a new medium supplemented with PDTC (200 μM) plus TNFα (10 ng/ml), followed by incubation for 6 to 24 hours. PBS and DMSO were used as the controls for TNFα and PDTC, respectively. Loss of cell viability was determined. The experiment was repeated twice with similar results. * represents $p < 0.01$ and ** $p < 0.005$ versus control treatment. **B.** differentiated macrophages were infected with Ad-IκBα (200 moi) or control adenoviral vector, followed by incubation with TNFα (10 ng/ml) or PBS for 2 hours. mRNA of TNFα-induced NF-κB-regulated genes were determined by microarray analysis. **C.** Macrophages were treated with TNFα (10 ng/ml) for the indicated times, after which the cells were harvested and the lysates examined by immunoblot analysis using an anti-A20 antibody.

Figure 7. A20 protects macrophages from TNF α -induced death

Differentiated macrophages were co-infected with Ad-IκBα or Ad-control vectors (50 moi) together with Ad-A20 (or the control) vectors (30 moi), followed by TNF α (10 ng/ml) for 16 hours (A, C–E) or for 4 hours (B, F). Lysosomal integrity (A), cytosolic cathepsin B activity (B), $\Delta\Psi_m$ (C), cell viability (D), DNA fragmentation (E), and caspase-8-like activity (F) were determined. The results represent the mean $\pm$ S.E. of 3 independent experiments, performed in triplicate. **, $p < 0.01$ versus control treatment.