The Cathepsin B Death Pathway Contributes to TNF Plus IFN-γ-Mediated Human Endothelial Injury

Jie Hui Li and Jordan S. Pober

*J Immunol* 2005; 175:1858-1866; doi: 10.4049/jimmunol.175.3.1858

http://www.jimmunol.org/content/175/3/1858

---

Why *The JI*?

- **Rapid Reviews!** 30 days* from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

---

**References**

This article **cites 41 articles**, 17 of which you can access for free at:

http://www.jimmunol.org/content/175/3/1858.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
The Cathepsin B Death Pathway Contributes to TNF Plus IFN-γ-Mediated Human Endothelial Injury

Jie Hui Li and Jordan S. Pober

Vascular endothelial cells (EC) perform a number of key functions essential for tissue survival, including regulation of perfusion, maintenance of blood fluidity and maintenance of perselectivity (1). EC injury, dysfunction, and death in response to cytokines, especially TNF acting in combination with IFN-γ, contributes to tissue injury and multiorgan failure observed in sepsis and other inflammatory conditions (2, 3). It has also been suggested that cytokine-mediated endothelial apoptosis contributes broadly to many aspects of cardiovascular disease (4). For these reasons, the basis of cytokine-mediated EC injury is a target of intense research.

Death receptors (DR), including TNFR1 (TNFR1/CD120a/DR-1), Fas (APO-1/CD95/DR-2), and DR-3, and DR-4/5 or TRAIL (R1/R2, respectively) can trigger apoptotic death upon ligand engagement (5, 6). Receptor-associated Fas-associated death domain protein (FADD) (7, 8), via its death effector domain, in turn mediates the recruitment and the autocatalytic activation of procaspase-8 (also known as FLICE) (9, 10). Activated caspase-8 is released from the death-inducing signaling complex (DISC) and proteolytically activates cytosolic effector caspase-3. Activated caspase-3, in turn, cleaves a variety of substrates, resulting in apoptotic cell death (11). In some cells, caspase-8 may proteolytically activate a cytosolic protein known as BID (12, 13). BID is a BH3 domain-only member of the Bcl-2 family, and BID cleavage by caspase-8 results in its translocation to the mitochondria where it combines with Bax to induce the release of proteins, such as cytochrome c or second mitochondria-derived activator of apoptosis (SMAC/Diablo). These proteins can initiate and amplify the apoptotic cascade (14).

Although the death pathway activated by Fas and TRAIL receptors is well understood, the molecular mechanisms involved in TNFR1-induced cell death are less well defined (15). Indeed, there is genetic evidence that FADD and caspase-8 are important for TNFR1-mediated apoptosis (6, 14, 16, 17). However, not all TNF-induced death responses depend on this pathway (6). For example, the initial TNFR-associated death domain protein/receptor-interacting protein 1 (TRADD/RIP-1) containing signaling complex has been proposed to bind the adaptor protein RACK1 (receptor-interacting protein-associated ICH-1/CED-3 homologous protein with a death domain), resulting in caspase-2-dependent cell death (18). Recently, a central role for caspase 8 (Caspase-8) in TNF-mediated cell death has been suggested by studies on fibrosarcoma cells (19) and hepatocytes from C57BL-deficient mice (20). Observations from our laboratory have shown that the compound LY294002, a selective inhibitor of PI3K, sensitizes HUVEC to Cat B-dependent programmed cell death in response to TNF or IL-1 (21). LY294002 contributes to this response by causing release of Cat B from lysosomes into the cytosol. Although LY294002 by itself results in some cell death, cell death is greatly amplified by TNF or IL-1. Activated Cat B, which is inhabitable by the selective protease inhibitor CA074-ME (CA074), causes mitochondrial membrane depolarization as well as release of death-inducing proteins from this organelle. The Cat B-dependent programmed death pathway is not intrinsically apoptotic, but may display features of apoptosis through incidental and delayed activation of caspases (21).

In the present study, we further investigate the biological significance of the Cat B death pathway in vascular EC. Our principal findings are that this pathway is activated by TNF plus IFN-γ, the...
combination of cytokines thought to underlie tissue injury in sep-
sis, but that this response is cell type restricted. Specifically, we
observe it to occur in HUVEC, and human dermal microvascular
EC (HDMEC), but not in HeLa cells or HEK293 cells. Cat B death
signaling in response to TNF plus IFN-γ involves mitochondria,
but is caspase- and Bid-independent. We also find that the Cat B
pathway is inhibited by FADD, the central adaptor protein for
DR-related caspase activation.

Materials and Methods
Reagents and Abs
Recombinant human TNF, TRAIL, IFN-γ, the broad spectrum inhibitor of
caspases zVAD and DR-5 Fc fusion proteins were all purchased from R&D
Systems. A DNA fragment ELISA kit, RNase and complete protease in-
hibitor mixture tablets were purchased from Roche Applied Sciences.
The protein synthesis inhibitors cycloheximide (CHX) and emetine, the Cat B
selective inhibitor CA074, Triton X-100, ethidium bromide, 4’-6’-dia-
midino-2-phenylindole (DAPI), Z-Arg-Arg-amidomethylcoumarin (Z-
Arg-Arg-AMC), the cathepsin D selective inhibitor pepstatin A, Pefabloc,
and digitonin were all purchased from Sigma-Aldrich. CaspaTag fluores-
cein caspase-8 (DEVD) and caspase-3 (LETD) activity assay kits were
purchased from Serologicals. A FLICE/caspase-8 colorimetric assay kit
and the calfpan inhibitor Z-Leu-Leu-Tyr-FMK were purchased from Bio-
Vision. Propidium iodide (PI), calcine-AM, and LysoTracker red were pur-
bought from Molecular Probes. Mouse IgM agonistic anti-human Fas mAb
(IPO4) was purchased from Kamiya Biomedical. An annexin V staining
kit, antagonistic mouse IgG anti-human Fas mAb DX2, mouse anti-human
cytochrome c Epitope, and rabbit anti-human Bid and procaspase-8 polyclonal
Abs were purchased from BD Pharmingen. Rabbit anti-FADD polyclonal
Ab was purchased from Chemicon International. Mouse anti-Cat B Ab was
purchased from Oncogene Research Products. Mouse anti-AU1 Tag
(DTyrYR) Ab was purchased from Covance. Mouse anti-human poly-
(ADP-ribose) polymerase (PARP) Ab was purchased from The American
Type Culture Collection (ATCC). HRP-conjugated secondary Abs for im-
munoblotting were purchased from Jackson ImmunoResearch
Laboratories.

Cultured cells
HUVEC and HDMEC were isolated and serially cultured as previously
described (22) in accordance with protocols approved by the Yale Uni-
versity Human Investigation Committee. HUVEC cultures were pooled from
to three to five individual cords, were maintained in medium 199 (M199)
supplemented with 50 μg/ml EC growth factor (Collaborative Biomedical
Products), 100 μg/ml porcine heparin (Sigma-Aldrich), 20% FCS, 200 μM
t-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from
Invitrogen Life Technologies). Such cultures uniformly express von
Weberland factor and CD31 and are free from detectable CD45+ contamin-
ant leukocytes. HUVEC cultures were prepared from individual donors
and were used at passage levels 3–7. HDMEC were uniformly positive for
CD31, CD34, endoglin, and following TNF treatment, for E-selectin
-expression 24 h after transfection using puromycin (1
M Z-Arg-Arg-
Cys) and cytochrome
Measurement of Cat B release from lysosomes was performed as previ-
sely described (19, 21). EC were mock-treated or treated as indicated
with zVAD for 7 h. After 7 h, the medium was removed and cells were
washed twice in PBS before the addition of extraction buffer (50 μg/ml
digitonin, 250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl2,
1 mM EDTA, 1 mM EGTA, 1 mM Pefabloc, pH 7.5) and incubation on ice
for 20 min. These conditions allow for the selective permeabilization of
the plasma membrane by digitonin without perturbation of lysosomes as
determined in preliminary experiments using EC preloaded both with cal-
cein-AM and LysoTracker red. After incubation, the cytosolic extract
was collected. Samples were analyzed for Cat B either by immunoblotting (see
below) or with a Cat B activity assays. For the latter, a 50-μl volume of
cytosolic extract was added to an equal volume of cathespin reaction buffer
(50 mM sodium acetate, 4 mM EDTA, 8 mM DTT, 1 mM Pefabloc, pH
6.0). Cat B activity was measured by the addition of 20 μM Z-Arg-Arg-
Cys-Cleaved AMC was measured (ex 360 nm, em = 460 nm) using a
fluorescence plate reader both immediately following the addition of the
peptide substrate (t = 0) and following a 60 min incubation at 37°C (t =
60). Activity was determined by subtracting the background activity at t =
0 from activity at \( t = 60 \) and correcting for the amount of protein in each sample.

For the analysis of Bid cleavage and mitochondria cytochrome \( c \) release, HUVEC were treated for 14 h with or without treatments indicated. Floating and attached EC were separately harvested, pelleted, and incubated with the Cat B extraction buffer mentioned for 15 min on ice. The cytosolic extracts were spun in Eppendorf tubes at 14,000 rpm for 20 min and the resulting supernatants were analyzed for Bid and cytochrome \( c \) by immunoblotting.

**Immunoblotting**

For immunoblotting, \( 4 \times 10^5 \) cells/sample were washed twice with cold PBS, before the addition of 100 \( \mu \)l of lysis buffer (10 mmol/L Tris, pH 7.6, 150 mmol/L NaCl, 1% Triton X-100, 10 mmol/L EDTA) supplemented with a protease inhibitor mixture and 1 mmol/L PMSF (Boehringer Mannheim). Cells were maintained on ice and lysates were harvested by scraping. The protein concentration of each sample was determined using a Bio-Rad protein assay (based on the Bradford dye-binding procedure) as described by the manufacturer (Bio-Rad). For each lysate sample, 20 \( \mu \)g of protein was separated by SDS-PAGE, and transferred to a nitrocellulose membrane (Bio-Rad) as described (27). After transfer, the membranes were blocked with 5% nonfat dry milk in Tris-buffered saline and then incubated overnight with primary Ab at 4°C. After washing, blots were further incubated with 1/5000 diluted secondary Ab (goat anti-mouse or anti-rabbit IgG HRP-conjugated) for 1 h at room temperature. After additional washing, HRP activity was detected by chemiluminescence using SuperSignal West Dura (Pierce) according to the manufacturer’s instructions.

**Results**

**TNF or TRAIL can trigger Cat B-mediated cell death in human EC**

Previous studies from our laboratory had revealed that in the presence of LY294002, a pharmacological inhibitor of PI3K, TNF binding to TNFR1 on HUVEC could activate a cell death pathway that was independent of caspase activation but instead dependent on Cat B (21). The present study was conducted to further evaluate this pathway. We first examined whether other DR might similarly activate the Cat B-dependent death in HUVEC and other cell types. As we reported previously (27), human EC express TRAIL-R1 and TRAIL-R2 and ~30% of HUVEC lose membrane integrity, assessed by PI exclusion, after treatment with 20 ng/ml TRAIL for 17 h compared with 7.8% of cells in replicate control cultures not treated with TRAIL (Fig. 1A). The same condition of TRAIL treatment causes >50% of Jurkat, HEK293 or HeLa cells to undergo cell death, assessed by the same method (Fig. 1A). Death in response to TRAIL alone appears to be wholly caspase-dependent because it is completely inhibited by the presence of 20 \( \mu \)M zVAD (Fig. 1A). In contrast, treatment with TNF alone does not induce significant cell death in any of these cell types (data not shown). The addition of CHX, a protein synthesis inhibitor that reduces the expression level of cFLIP and other antiapoptotic genes, augments the cytotoxic effects of TRAIL and reveals those of TNF, triggering increased cell death in HUVEC, Jurkat, HEK293, and HeLa cells. In the presence of CHX, the efficacy of zVAD protection from cell death differs among these cell types (Fig. 1B). Specifically, when incubated with either TNF or TRAIL plus CHX, both HeLa and HEK293 cells are still completely protected by 20 \( \mu \)M zVAD. However, under the same conditions, ~40% of Jurkat cells still lose their membrane integrity (Fig. 1B). In HUVEC, zVAD successfully inhibits death by TNF plus CHX, but only partially inhibits cell death in response to TRAIL plus CHX (Fig. 1B), even at a concentration up to 100 \( \mu \)M (data not shown).
shown). CA074, a selective Cat B inhibitor, is not effective in protecting cell death when added alone in any of these settings, but as shown in Fig. 1C, combined addition of zVAD and CA074 effectively blocks the death of HUVEC or Jurkat cells triggered by TRAIL plus CHX. Moreover, zVAD and CA074 also completely protects Jurkat cells treated with TNF plus CHX (Fig. 1D). These data suggest that both HUVEC and Jurkat cells have a DR-triggered, Cat B-dependent death pathway, whereas HeLa and HEK293 cells do not.

Cat B-dependent death contributes to TNF plus IFN-γ-mediated EC injury

We have previously reported that IFN-γ can potentiate Fas-mediated cell death through a caspase-dependent pathway in HUVEC by increasing Fas and caspase-8 expression (25). IFN-γ is frequently present under the same conditions as those which lead to TNF production in vivo (e.g., in sepsis or immune reactions) and IFN-γ potentiates tissue injury and EC death caused by TNF (28).

We therefore examined whether IFN-γ potentiates the caspase and/or Cat B response of HUVEC to TNF. As shown in Fig. 2A, combined treatment with TNF and IFN-γ triggers death in ~40% of treated HUVEC or HDMEC. The TNF plus IFN-γ-treated HUVEC are not protected by antagonistic Fas Ab DX2 or by fusion protein DR-5 Fc, which are potent inhibitors of Fas and TRAIL death signaling respectively (25, 27) (Fig. 2A), ruling out secondary involvement of other DR. Most significantly, EC treated with TNF plus IFN-γ is not protected by zVAD or CA074 alone (Fig. 2A), but TNF- and IFN-γ-triggered death in HUVEC or HDMEC is effectively blocked by the combined treatment with zVAD and CA074 (Fig. 2A). These data suggest that both death pathways are activated in human EC by TNF plus IFN-γ. In contrast, death of HUVEC triggered by TNF plus IFN-γ was not reduced by inhibition of calpain (with z-Leu-Leu-Tyr-FMK) or cathepsin D (with pepstatin A), even in combination with the caspase inhibitor zVAD (data not shown). We then extended this analysis to other cell types. IFN-γ also enhances the TNF-induced killing of HeLa and HEK293 cells (Fig. 2B). However, in these cell types, TNF- and IFN-γ-induced cell death is completely inhibited by zVAD alone, without evidence of an effect of CA074 (Fig. 2B). Once again, the activation of the Cat B death pathway appears to be cell type-restricted.

Although CA074 is reasonably specific for Cat B, we wished to directly confirm that Cat B is activated following TNF plus IFN-γ treatment. Following 7 h of treatment with IFN-γ, with or without TNF, we observed an increase in immunoreactive Cat B in the cytosol assessed by immunoblotting (Fig. 2C). The appearance of immunoreactive Cat B is paralleled by an increase in the cytosolic

**FIGURE 2.** Cat B contributes to cell death of the human EC treated with TNF plus IFN-γ. TNF (10 ng/ml), IFN-γ (80 ng/ml), caspase inhibitor zVAD (20 μM), and/or Cat B inhibitor CA074 (20 μM) were included during the entire treatment period as indicated, the cell viability was determined by PI exclusion staining as described in Materials and Methods. A. Both HUVEC and HDMEC can be protected from cell death induced by TNF plus IFN-γ by CA074 in combination with zVAD, but not by either agent alone or by antagonistic anti-Fas Ab (DX-2, 2 μg/ml) and DR5 Fc (10 μg/ml) during the entire 17 h treatment period. B. HeLa and HEK293 cells treated by TNF and IFN-γ, are fully protected by zVAD alone, during the entire 17-h treatment period. All experiments (A and B) were repeated at least three times with similar results. C. IFN-γ results in lysosomal release of Cat B into the cytosol of HUVEC analyzed by digitonin extraction and immunoblotting as described in Materials and Methods. D. IFN-γ results in Cat B activation in cytosolic extracts of HUVEC assayed at pH 6.0 as described in Materials and Methods. Experiments (C and D) were performed three times with similar results. For the analysis of Cat B activity, EC were treated as in C, with or without zVAD and CA074. T, TNF; IFN, IFN-γ.

**FIGURE 3.** Caspase-8 and caspase-3 activation during TNF- and IFN-γ-induced HUVEC cell death. HUVEC were either untreated or treated with TNF, IFN-γ, or both, in the presence or absence of zVAD (20 μM). Fluorescent probe substrates of caspase-3 (20 μM) and caspase-8 (20 μM) were included during the 7 h entire treatment period. HUVEC were harvested and the activities of caspase-8 (A) and caspase-3 (B) were determined by FACS analysis as described in Materials and Methods. C. In vitro analysis of caspase-8 activity. HUVEC were treated as in A and B, in the absence of fluorescent probe substrates of caspase-3 and caspase-8. Caspase-8 activation in cytosolic extracts of HUVEC was assayed using FLICE/caspase-8 colorimetric assay kit following the manufacturer’s instructions. OD 405 indicates the caspase-8 activity of HUVEC after the different treatments. All experiments were repeated three times with similar results.
activity of the enzyme, measured at pH 6.0, that is completely blocked in the presence of CA074 (20 μM) (Fig. 2D). To determine the efficiency of caspase inhibitor zVAD in intact HUVEC, we directly measured caspase-8 and caspase-3 activation in HUVEC after TNF and IFN-γ treatment. Combined treatment of TNF and IFN-γ, effectively activates caspase-8 (Fig. 3A) and caspase-3 (Fig. 3B) in HUVEC (Fig. 3). However, in presence of the broad-spectrum caspase inhibitor zVAD, TNF- and IFN-γ-treated HUVEC do not show caspase-8 and caspase-3 activation (Fig. 3). Both activation and inhibition of caspase-8 was confirmed in lysates prepared from cytokine-treated HUVEC in the absence or presence of specific caspase inhibitor (Fig. 3C). Because zVAD alone effectively inhibits caspase-8 and caspase-3 activation, but does not prevent cell death, it is reasonable to conclude that caspase activation is dispensable for Cat B-mediated cell death.

**Mitochondria are involved in Cat B-mediated cell death**

Previous studies using TNF plus LY294002 suggested that Cat B-triggered death depended on changes in mitochondria (21). We wished to test whether this is also true of Cat B-dependent death mechanism triggered by TNF and IFN-γ. To demonstrate a causal role of mitochondria in death pathways evoked by TNF and IFN-γ, HUVEC were subjected to retroviral transduction with a caspase-resistant form of Bcl-2. Four rounds of infection with either LZRS-EGFP or LZRS-Bcl-2 results in over 95% expression of the transgenes in HUVEC (26). Transduction with Bcl-2 protects EC from TNF- and IFN-γ-initiated cell death and detachment, compared with cells transduced with EGFP or untransduced cells (Fig. 4C). These data are wholly consistent with previous results using TNF plus LY294002 and indicate that the Cat B death response depends on mitochondria in HUVEC treated with TNF plus IFN-γ.

**The role of FADD in the TNF-triggered Cat B-mediated death mechanism**

FADD is the central adaptor protein of the DISC (30). We examined whether FADD also plays a role in the Cat B-dependent death

---

**FIGURE 4.** Mitochondrial changes in the Cat B death pathway activated by TNF plus IFN-γ. A. Loss of mitochondrial membrane potential (Δψ). HUVEC were either untreated or treated for 14 h with TNF (10 ng/ml), IFN-γ (80 ng/ml), or both, in the presence or absence of zVAD (20 μM). Floating and attached EC were harvested, pooled, washed, and resuspended in PBS containing JC-1 (10 μg/ml). After 15 min at 37°C HUVEC were washed again, and fluorescence was measured by FACS. The numbers in the figure indicate the percent of HUVEC, which have lost their mitochondria membrane potential in response to these cytokines (Fig. 4A). To identify the mechanism through which an increase in Cat B activity might affect mitochondria, we examined both Bid cleavage and cytochrome c release in response to TNF and IFN-γ. In the absence of zVAD, combined treatment with TNF and IFN-γ results in cytochrome c release from mitochondria and disappearance of Bid from the cytosol of dying cells (Fig. 4B). However, in the presence of zVAD, TNF plus IFN-γ still induces cytochrome c release from mitochondria but no longer result in loss of Bid (Fig 4B). In other words, mitochondria appear to be involved in the Cat B-dependent death mechanism triggered by TNF and IFN-γ, but this pathway is independent of Bid cleavage.

Bcl-2 blocks release of death-inducing proteins from mitochondria, largely by antagonizing the activation of Bax (29). To demonstrate a causal role of mitochondria in death pathways evoked by TNF and IFN-γ, HUVEC were subjected to retroviral transduction with a caspase-resistant form of Bcl-2. Four rounds of infection with either LZRS-EGFP or LZRS-Bcl-2 results in over 95% expression of the transgenes in HUVEC (26). Transduction with Bcl-2 protects EC from TNF- and IFN-γ-initiated cell death and detachment, compared with cells transduced with EGFP or untransduced cells (Fig. 4C). These data are wholly consistent with previous results using TNF plus LY294002 and indicate that the Cat B death response depends on mitochondria in HUVEC treated with TNF plus IFN-γ.
mechanism in HUVEC. AU1-tagged FADD.DN mutant, which lacks the FADD death effector domain, was transduced into HUVEC using a retroviral vector pFB. Expression in the transduced cells (HUVECFADD.DN) was confirmed by immunoblotting, compared with an empty vector control (HUVECpFB). Moreover, HUVECFADD.DN are resistant to TRAIL-triggered death signal compared with the HUVECpFB.control demonstrating than that of HUVECFADD.DN (Fig. 5A). TNF-triggered death signaling in HUVECFADD.DN is blocked by 20 μM CA074, but not by zVAD (Fig. 5C). In other words, FADD blocks TNF-induced killing through the caspase-dependent pathway as expected, but simultaneously potentiates killing through the Cat B-dependent pathway. Consistent with this interpretation, HUVECFADD.DN are also sensitive to the combined treatment of TNF and IFN-γ, and the death mechanism in HUVECFADD.DN is completely B2-dependent (Fig. 5D). In contrast, TNF- and IFN-γ-mediated killing of HUVECFADD.DN appears to involve both caspase- and B2-dependent death mechanisms, similar to our findings with untransduced cells (data not shown).

To confirm that FADD can inhibit the B2 pathway, we examined the effects of TNF on genetically mutated Jurkat cells, which lack FADD (Jurkat2572) or caspase-8 (Jurkat2571). We first confirmed that these cell lines do not express FADD (Jurkat2572) or caspase-8 by immunoblotting (Jurkat2571) (Fig. 6A). Importantly, both mutated cell types resist death signaling triggered by TRAIL or agonistic anti-Fas Ab IPO-4 (Fig. 6B). However, upon incubation with TNF for 7 h, up to 50% of FADD-deficient Jurkat2572 lose their membrane integrity in the presence or absence of CHX (2.5 μg/ml) (Fig. 6C). The death signaling is significantly inhibited by 20 μM CA074, but not by zVAD (Fig. 6C). TNF also kills caspase-8-deficient Jurkat2571 by the caspase-independent, B2-dependent death mechanism, but only in presence of CHX (2.5 μg/ml) (Fig. 6C). Because FADD-deficient cells are killed by TNF in the absence of CHX but caspase-8-deficient cells are not, caspase-8 activation cannot fully explain the role of FADD in blocking the B2 pathway.

Nonapoptotic features of B2-mediated cell death

In a final set of experiments, we characterized features of B2-dependent HUVEC death in cells treated with TNF plus IFN-γ. TNF plus IFN-γ-treated HUVEC show several apoptotic features. Specifically, TNF plus IFN-γ-treated HUVEC display early (7 h) PS translocation assessed by binding annexin V before their loss of membrane integrity (Fig. 7A); and supernatants from TNF plus IFN-γ-treated HUVEC contain nucleosomal DNA fragments as evidenced by DNA fragment ELISA (Fig. 7B). However, in the presence of zVAD, TNF and IFN-γ-treated HUVEC no longer released nucleosomal DNA fragments (Fig. 7B), and annexin V binding no longer precedes loss of membrane integrity (Fig. 7A), yet >30% of HUVEC with 14 h treatment still lose their membrane integrity as evidenced by PI staining (Fig. 7C). The mode of death signaling in Jurkat2571 by the caspase-independent, B2-dependent death mechanism, but only in presence of CHX (2.5 μg/ml) (Fig. 6C). Because FADD-deficient cells are killed by TNF in the absence of CHX but caspase-8-deficient cells are not, caspase-8 activation cannot fully explain the role of FADD in blocking the B2 pathway.

Nonapoptotic features of B2-mediated cell death

In a final set of experiments, we characterized features of B2-dependent HUVEC death in cells treated with TNF plus IFN-γ. TNF plus IFN-γ-treated HUVEC show several apoptotic features. Specifically, TNF plus IFN-γ-treated HUVEC display early (7 h) PS translocation assessed by binding annexin V before their loss of membrane integrity (Fig. 7A). However, in the presence of zVAD, TNF and IFN-γ-treated HUVEC no longer released nucleosomal DNA fragments (Fig. 7B), and annexin V binding no longer precedes loss of membrane integrity (Fig. 7A), yet >30% of HUVEC with 14 h treatment still lose their membrane integrity as evidenced by PI staining (Fig. 7C). The mode of death signaling in Jurkat2571 by the caspase-independent, B2-dependent death mechanism, but only in presence of CHX (2.5 μg/ml) (Fig. 6C). Because FADD-deficient cells are killed by TNF in the absence of CHX but caspase-8-deficient cells are not, caspase-8 activation cannot fully explain the role of FADD in blocking the B2 pathway.

Nonapoptotic features of B2-mediated cell death

In a final set of experiments, we characterized features of B2-dependent HUVEC death in cells treated with TNF plus IFN-γ. TNF plus IFN-γ-treated HUVEC show several apoptotic features. Specifically, TNF plus IFN-γ-treated HUVEC display early (7 h) PS translocation assessed by binding annexin V before their loss of membrane integrity (Fig. 7A). However, in the presence of zVAD, TNF and IFN-γ-treated HUVEC no longer released nucleosomal DNA fragments (Fig. 7B), and annexin V binding no longer precedes loss of membrane integrity (Fig. 7A), yet >30% of HUVEC with 14 h treatment still lose their membrane integrity as evidenced by PI staining (Fig. 7C). The mode of death signaling in Jurkat2571 by the caspase-independent, B2-dependent death mechanism, but only in presence of CHX (2.5 μg/ml) (Fig. 6C). Because FADD-deficient cells are killed by TNF in the absence of CHX but caspase-8-deficient cells are not, caspase-8 activation cannot fully explain the role of FADD in blocking the B2 pathway.

Nonapoptotic features of B2-mediated cell death

In a final set of experiments, we characterized features of B2-dependent HUVEC death in cells treated with TNF plus IFN-γ. TNF plus IFN-γ-treated HUVEC show several apoptotic features. Specifically, TNF plus IFN-γ-treated HUVEC display early (7 h) PS translocation assessed by binding annexin V before their loss of membrane integrity (Fig. 7A). However, in the presence of zVAD, TNF and IFN-γ-treated HUVEC no longer released nucleosomal DNA fragments (Fig. 7B), and annexin V binding no longer precedes loss of membrane integrity (Fig. 7A), yet >30% of HUVEC with 14 h treatment still lose their membrane integrity as evidenced by PI staining (Fig. 7C). The mode of death signaling in Jurkat2571 by the caspase-independent, B2-dependent death mechanism, but only in presence of CHX (2.5 μg/ml) (Fig. 6C). Because FADD-deficient cells are killed by TNF in the absence of CHX but caspase-8-deficient cells are not, caspase-8 activation cannot fully explain the role of FADD in blocking the B2 pathway.
cell death was further assessed by DAPI staining. Treatment of HUVEC with TNF plus IFN-γ causes nuclei to shrink and fragment, a hallmark of apoptosis. Addition of zVAD prevents nuclear fragmentation but the nuclei still underwent condensation (Fig. 7D). This is the same result we reported previously for HUVEC treated with TNF and PI3K inhibitor LY294002 (21), and it implies that caspases contribute to apoptotic features but are not required for Cat B-dependent cell death. Similarly, apoptotic features are not observed in TNF-treated FADD-deficient Jurkat2572 (Fig. 8) in which the death mechanism is wholly Cat B-dependent. Specifically, although TNF induced Jurkat2572 cell death (assessed by loss of membrane integrity) is observed at 3.5 or 7 h, Jurkat2572

FIGURE 7. Apoptotic features of HUVEC death triggered by TNF plus IFN-γ. A, At the indicated time points, HUVEC cells treated with TNF and IFN-γ were stained with annexin V-FITC and -PI and analyzed by FACS. The value indicated in the lower right quadrant represent the percentage of annexin V-positive and PI-negative cells in the population, which represents an early stage of apoptosis. In contrast, the upper right quadrant contains double-stained cells, which can represent either necrotic or apoptotic cells. B, Assessment of apoptosis by DNA fragment ELISA. HUVEC were incubated with TNF and IFN-γ for 17 h, in the presence or absence of zVAD (20 μM) or CA074 (20 μM). All of the treated HUVEC were harvested and their fragmented DNA was quantified using a DNA fragment ELISA kit. Absorbance at 450 nm indicates the amounts of fragmented DNA released from apoptotic HUVEC. C, With the similar treatment as indicated in A, in the presence or absence of zVAD (20 μM), TNF- and IFN-γ-treated cells were stained with PI. Note that zVAD reduces apoptosis but does not prevent cell death. D, Examination of nuclear changes. HUVEC were treated with TNF and IFN-γ for 14 h in the presence or absence of zVAD (20 μM), and were centrifuged onto slides by cytospin. Nuclear morphology was examined by DAPI staining as described in Materials and Methods. Arrows indicate the marked nuclear changes. Note: In the TNF- and IFN-γ-treated group, the nuclear condensation and fragmentation indicative of apoptosis, incubation of zVAD prevents nuclear fragmentation but the nuclei still condense, which is consistent with the data of DNA fragment ELISA shown in B. All experiments were repeated at least three times with similar results. T, TNF; I, IFN-γ.

FIGURE 8. Apoptotic features of FADD-deficient Jurkat cells after TNF treatment. A, At the indicated time points, FADD-deficient Jurkat cells (Jurkat2572) treated by TNF (10 ng/ml) or by emetine (15 μM), were stained with annexin V-FITC and PI as analyzed by FACS. B, As described in Materials and Methods, Jurkat2572 were treated by TNF (10 ng/ml) or by emetine (15 μM) for 14 h and their DNA was isolated, separated, and visualized. C, Jurkat2572 and Jurkat (wild type) cells were treated for 14 h using TNF (10 ng/ml), TRAIL (20 ng/ml), or emetine (15 μM), respectively and subjected to immunoblot with anti-PARP Ab as described in Materials and Methods. Note that TNF cell death is non-apoptotic in FADD-deficient Jurkat T cells. All experiments were repeated at least three times with similar results.
do not show signs of PS translocation, assessed by annexin V binding (Fig. 8A), nor do TNF-treated Jurkat<sub>372</sub> cells show nucleosomal DNA fragmentation assessed by gel electrophoresis (Fig. 8B) or PARP cleavage assessed by immunoblotting (Fig. 8C).

In contrast, etamine-treated Jurkat<sub>372</sub>, which undergo apoptosis independently of caspase-8, do show early PS translocation (Fig. 8A), DNA fragmentation (Fig. 8B), and PARP cleavage (Fig. 8C).

**Discussion**

Programmed cell death in response to DR signaling is generally considered to be apoptotic and dependent upon caspases (6). However, DR-initiated caspase-independent programmed cell death has previously been observed in a few of other cell systems (31), such as TNF plus CHX-mediated death of NIH 3T3 cells (32) or TNF-mediated cell death of WEHI-S fibrosarcoma cells (19). The activation of noncaspase proteases, such as cathepsins (33) or calpains (34), has emerged as an alternative means to induce cell death (31).

We have previously reported that in the presence of the pharmacological agent LY294002, TNF- or IL-1-mediated HUVEC death is induced by Cat B (21). In this study, we show that TNF plus IFN-γ, a pathophysiologically relevant combination of cytokines, also activates Cat B-dependent death mechanisms in HUVEC (Fig. 2). However, the Cat B pathway could not be elicited in HeLa cells or HEK293 cells (Fig. 1B) and this cell type restriction may explain why it was not observed in many previous investigations.

The Cat B pathway involves at least two steps, release of Cat B from lysosomes and subsequent activation of Cat B in the neutral pH environment of the cytosol. Guicciardi et al. (20) reported that in hepatocytes lysosome release of Cat B by TNF plus actinomycin D was likely to be caspase-dependent because recombinant caspase-8 could cause the release of Cat B from purified lysosomes. In the present study, we show that Cat B-mediated HUVEC death triggered by TNF plus IFN-γ cannot be blocked by dominant negative FADD (Fig. 5D) or by effective inhibition of caspases with zVAD (Fig. 2A). Also we did not observe cFLIP down-regulation in response to TNF- and IFN-γ-activated HUVEC (our unpublished observation).

Thus, in HUVEC, Cat B release appears to be independent of caspases; Cat B is liberated from the lysosomes by the actions of IFN-γ alone (Fig. 2, C and D). Although IFN-γ treatment killed only a small number of HUVEC (Fig. 2A), the presence of TNF markedly enhanced this effect (Fig. 2A). This effect of IFN-γ is similar to that previously described for LY294002 which also releases Cat B from the lysosomes (21). Under normal circumstance, the neutral pH of the cytosol should limit Cat B activity. We speculate that TNF somehow alters the conditions under which Cat B may act perhaps by recruitment of the enzyme into a multiprotein complex. This possibility is under active investigation.

The protein synthesis inhibitor CHX has been widely used to enhance (or reveal) apoptotic death responses by DR, presumably by reducing expression of cFLIP and other short-lived proteins that antagonize caspase-8 or other proteases of apoptosis. We used CHX to determine whether short-lived proteins similarly regulate apoptosis signaling. TNF effects on mitochondria can be triggered via Bid cleavage by caspase-8 or via a protease activated by JNK signaling (35). In both cases, cleaved Bid fragment then causes mitochondrial cristae reorganization by its interactions with either Bax or, in some cases, Bak, releasing mitochondrial apoptotic factors, such as cytochrome c and SMAC/Diablo or apoptosis inducing factor (36).

In our study, Bid cleavage is detectable in apoptotic (detached) HUVEC in response to TNF and IFN-γ as assessed by loss of intact Bid on immunoblot. However, in the presence of zVAD, TNF and IFN-γ treated HUVEC lose mitochondrial membrane potential and release cytochrome c (Fig. 4, A and B), yet caspase-8 activation is no longer detectable (Fig. 3, A and C) and Bid remains intact, even in the detached cells. We do not know yet how Cat B death signaling induces cytochrome c release from mitochondria independent of caspase-8 activation and Bid cleavage.

An additional implication of our studies relates to the role of FADD in Cat B-mediated cell death. Apoptosis signaling of DR appears to be transduced by the adaptor FADD protein and the protease caspase-8 (6, 30, 37), but several studies suggest that FADD may have additional roles in alternative signaling pathways (14, 17, 38, 39). For example, FADD deficiency causes defective proliferation in T lymphocytes (14, 40). In addition, FADD deficiency leads to early embryonic lethality in mice (14, 17). The mechanism by which FADD gene disruption causes organismal death is unexplained. We find that dominant negative FADD-transfected HUVEC grow and proliferate as well as their control cells (our unpublished observations), but become sensitive to TNF-triggered Cat B-dependent death signaling (Figs. 5B and 6C). In contrast, dominant negative FADD transfected HeLa and HEK293 cells completely resist TRAIL and TNF-triggered death signaling (our unpublished observations). These data suggest that FADD inhibits TNF-triggered Cat B-dependent signaling in those cell types in which this pathway exists. Because hepatocytes appear to have Cat B death pathway, we speculate that FADD deficiency also sensitizes hepatocytes to TNF-mediated cell death.

Finally, there are important implications of our findings that relate to protection of tissues from injury. Vascular EC are found in every organ and EC death or dysfunction can affect every organ system. Cytokines are important effectors of tissue injury in addition to serving as mediators of inflammatory responses (1). Combined treatment of TNF and IFN-γ induces significant EC injury in zVAD prevents nuclear fragmentation but the nuclei still condense. This is the same result we reported previously for HUVEC treated with TNF plus P38 kinase inhibitor LY294002 (21). It differs from results observed with TNF plus CHX in which zVAD prevents cell death and prevents nuclear condensation as well as fragmentation (21). In the presence of zVAD, TNF- and IFN-γ-treated HUVEC no longer released nucleosomal DNA fragments (Fig. 7B), and annexin V binding no longer precedes loss of membrane integrity (Fig. 7A), yet >30% of HUVEC with 14 h treatment still lose their membrane integrity as evidenced by PI staining (Fig. 7C). Thus TNF plus IFN-γ can, in the presence of zVAD, initiate nonapoptotic cell death. Nonapoptotic cell death is frequently described as “necrosis.” It is important to note that cellular necrosis, more accurately described by the term “oncosis,” is characterized by nuclear swelling, not condensation. Thus Cat B-dependent death is neither apoptotic nor oncotic and appears to belong to a different category of orderly or programmed cell death.
vivo, even when both TNF and IFN-γ are present at low concentrations (41). TNF and IFN-γ are often present in combination during pathophysiologic conditions such as sepsis (2). In the present study, we have demonstrated that TNF and IFN-γ-triggered human EC injury involves two different death mechanisms: caspase-dependent and Cat B-dependent. Both pathways appear to depend on TNF signaling through TNFR1 as revealed by Ab blocking experiments (our unpublished observations).

In summary, we have found that Cat B-mediated death mechanism contributes to human endothelial injury triggered by a combination of the inflammatory cytokines TNF and IFN-γ. This cytokine-triggered death response in human EC and Jurkat cells is not apoptotic and is normally inhibited by FADD. These observations may help develop strategies to prevent inflammatory cytokine-mediated human endothelial injury in various diseases settings.

Acknowledgments
We thank colleagues Alessio D’Alessio, Jae Choi, David Enis, and Stephen L. Shiao for suggestions for this manuscript. We are also grateful to Gwendolyn Davis, Louise Benson, and Lisa Gras for excellent technical assistance with cell culture.

Disclosures
The authors have no financial conflict of interest.

References