Lipopolysaccharide Can Trigger a Cathepsin B-Dependent Programmed Death Response in Human Endothelial Cells

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In this study, we examined the mechanisms that contribute to lipopolysaccharide (LPS)-induced death responses in cultured human umbilical vein endothelial cells (HUVECs). In the presence of the protein synthesis inhibitor cycloheximide, LPS primarily induces caspase-dependent apoptotic cell death of HUVECs, which is blocked by siRNA-mediated knockdown of myeloid differentiation factor 88 adaptor protein but not of Toll-like receptor-associated interferon-inducing factor. Knockdown of Fas-associated death domain protein (FADD) by either siRNA or overexpression of a truncated version of FADD that lacks the N-terminal death effector domain (FADD_{DN}) increases the sensitivity of HUVECs to LPS plus cycloheximide-mediated death. However, based on the use of proteinase inhibitors, cell death changes from being principally caspase-dependent to being principally cathepsin B (Cat B)-dependent. Knockdown of cellular FLICE inhibitory protein potentiates the caspase-dependent pathway but does not activate the Cat B-dependent death response. Knockdown of either myeloid differentiation factor 88 or Toll-like receptor-associated interferon-inducing factor expression does not affect the LPS-triggered Cat B death response in FADD-deficient HUVECs. Finally, in the presence of either the phosphatidylinositol 3 kinase inhibitor LY294002 or the inflammatory cytokine interferon- γ , LPS activates both caspase- and Cat B-dependent death pathways. We conclude that LPS can activate a Cat-B-dependent programmed death response in human endothelial cells that is independent of both myeloid differentiation factor 88 and Toll-like receptor-associated interferon-inducing factor, is blocked by both FADD and phosphatidylinositol 3 kinase, and is potentiated

by interferon- γ . (Am J Pathol 2009, 175:1124–1135; DOI: 10.2353/ajpath.2009.090113)

Lipopolysaccharide (LPS) can mimic many of the features of bacterial sepsis including the release of inflammatory cytokines, fever, shock, coagulopathy, organ failure, and death. Vascular endothelial cells (ECs) are a significant target of LPS action, responding by expressing a variety of proinflammatory, procoagulant, and cytoprotective genes.¹ Circulating LPS forms a complex with soluble CD14, present in plasma, and the complex signals through a transmembrane receptor, Toll-like receptor (TLR) 4, which may then recruit either of two intracellular adaptor proteins, myeloid differentiation factor 88 (MyD88)² or TLR-associated interferon-inducing factor (TRIF).³ The MyD88 pathway involves additional adaptor proteins, including interleukin (IL)-1R-associated kinase 1 (IRAK1) and tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6), and results in activation of both nuclear factor kappa B (NF- κ B) and certain mitogen-activated protein kinases (MAPKs), notably c-Jun N-terminal kinase (JNK) and p38 MAPK.⁴ The TRIF pathway requires the adaptor protein TRIF-associated molecule (TRAM) and results in both NF- κ B activation and interferon regulatory factor (IRF)-7-dependent synthesis of type I interferon (IFN).⁴

In addition to activating new gene expression, LPS may directly cause EC injury, dysfunction, and death.⁵ The injurious response is most evident when the expression of inducible cytoprotective proteins is inhibited, for example when LPS is combined with agents that inhibit new protein synthesis such as cycloheximide (CHX) or Shiga-like toxins.⁶ In this respect, the EC response to LPS

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resembles that of TNF in which there is a competition between pathways that result in gene activation and those that result in cell death.⁷ TNF may activate death responses that involve caspase activation, dependent on Fas-associated death domain protein (FADD), or a caspase-independent death response initiated by cathepsin B (Cat B), whose activation is actually inhibited by FADD.⁸ The Cat B pathway is selectively activated in cultured human ECs when TNF is combined with the phosphatidylinositol 3 (PI3) kinase inhibitor LY294002 and both the caspase and Cat B pathways are activated when TNF is combined with IFN- γ . It is much less well established how LPS initiates cell death in ECs. Choi et al have reported that overexpression of a FADD dominant negative constructs (FADD_{DN}) protected an immortalized human dermal microvascular cell line (HMEC-1) from LPS-induced cell death in the presence of CHX.⁹ However, Bannerman et al, in a subsequent study using the same construct and the same cell line, failed to observe a protective effect, using a different assays for cell death.¹⁰ A more recent study has suggested that the intrinsic (mitochondrial) apoptotic pathway is required for LPS-induced lung endothelial cell death.¹¹

In the present report, we have further investigated the death response of ECs to LPS using untransformed human umbilical vein ECs (HUVECs). We find that in the presence of CHX, LPS primarily mediates caspase-dependent death that is initiated via MyD88 and FADD, but not TRIF. Reduced expression of cellular FLICE inhibitory protein (cFLIP), an inhibitor of FADD-mediated activation of caspase 8 (previously known as FLICE), further sensitizes EC to caspase-dependent death triggered by LPS. We also find that FADD serves as a negative regulator of an LPS-initiated Cat B-dependent death mechanism. Specifically, either siRNA knockdown of FADD or overexpression of FADD_{DN} blocks LPS-initiated caspase-dependent death while enhancing Cat B-dependent death in HUVECs. In HUVECs in which FADD has been knocked down, concomitant knockdown of either MyD88 or TRIF does not provide protection, suggesting that these adaptor proteins are not involved in Cat B activation. Like TNF, LPS efficiently activates the Cat B death pathway in ECs in presence of IFN- γ or LY294002. The ability of LPS to activate a Cat B-dependent death pathway as well as a caspase-dependent death pathway may explain some of the variable results previously reported for this mediator and has implications for how to protect ECs from injury in settings of sepsis.

Materials and Methods

Reagents

The protein synthesis inhibitor CHX, the Cat B-selective inhibitor CA074-methyl ester (CA074),^{12–15} LPS from *Escherichia coli* 0127:B8, digitonin, Pefabloc, 4'-6-diamidino-2-phenylindole (DAPI) and thiazolyl blue tetrazolium bromide (MTT) were all purchased from Sigma (St. Louis, MO). Recombinant human TNF, IFN- γ , and goat anti-human TNF IgG were purchased from R&D systems

(Minneapolis, MN). Anti-cytochrome *c* rabbit polyclonal antibody and mouse anti-human FADD monoclonal antibody were purchased from BD PharMingen (San Jose, CA). Anti-Fas and Fas ligand antibodies (DX-2 and NOK-1) were purchased from BioLegend (San Diego, CA). The broad-spectrum caspase inhibitor z-VAD-fmk (zVAD) and Cat B inhibitor zFA were purchased from Enzyme Systems Products (Livermore, CA). Rabbit anti-human Bid, anti-MyD88, and anti-TRIF polyclonal antibodies were purchased from Cell Signaling (Danvers, MA). Mouse anti-AU1 Tag (DTYRYI) antibody was purchased from Covance (Berkeley, CA). Horseradish peroxidase-conjugated secondary antibodies for immunoblotting were purchased from Jackson ImmunoResearch (West Grove, PA). Complete protease inhibitor mixture tablets were purchased from Roche Applied Science (Indianapolis, IN). Propidium iodide (P/I) was purchased from Invitrogen (Carlsbad, CA).

HUVECs were isolated and serially cultured as previously described¹⁶ in accordance with protocols approved by the Yale University Human Investigation Committee. In brief, HUVEC cultures were pooled from three to five individual cords and were maintained in Medium 199 (M199) supplemented with 50 μ g/ml EC growth factor (Collaborative Biomedical Products, Bedford, MA), 100 μ g/ml porcine heparin (Sigma), 20% fetal calf serum, 200 μ mol/L L-glutamine, 100 units/ml penicillin, and 100 μ g/ml streptomycin (all from Invitrogen, Grand Island, NY) and serially propagated on gelatin-coated tissue culture plastic. By the second passage, HUVEC cultures uniformly express von Willebrand factor and CD31 and are free from detectable CD45⁺ contaminating leukocytes. Treatments of HUVECs were performed in complete growth medium using concentrations of reagents, listed in the figure legends, which had been determined to be optimal in preliminary experiments.

Construction of and Transduction with Recombinant Retroviral Vectors

Plasmid pcDNA3 AU1-FADDmt human FADD dominant negative (FADD_{DN}) with AU1 epitope tag,¹⁷ provided by Dr. V. Dixit (Genentech, South San Francisco, CA), was subcloned into the pFB retroviral vector (Stratagene, La Jolla, CA) using polymerase chain reaction (PCR) with primers 5'-CGACTCACTATAGGGAGACCCA-3' and 5'-GCGGCCGCTCAGGACGCTTCGGAGGTA-3', and topoisomerase cloning techniques (Invitrogen). The pFB vector was transfected into packaging cell line PA317 provided by Dr. G. Nolan (Stanford University, Palo Alto, CA) and G418 (Life Technologies)-resistant cells were derived as a source of retrovirus stock. Transduction and G418 selection of HUVECs was accomplished as described.¹⁸ In brief, this involved three serial transduction of primary cultures with retrovirus-containing supernatants followed by selection with G418. Approximately 50% of transduced HUVECs but no control HUVECs survived selection with 0.5 mg/ml G418. Multiple FADD_{DN}-transduced HUVEC clones were pooled to avoid clonal

variability. HUVECs transduced to overexpress FasL were prepared as described previously.¹⁸

Design and Transfection of cFLIP, FADD, MyD88, and TRIF siRNA Duplexes

Small interfering RNA duplex oligonucleotides against the coding sequence of human cFLIP, FADD, MyD88, or TRIF were designed by using software by Integrated DNA Technologies (Coralville, IA). We selected target sequences as follows: cFLIP-si1, 5'-CTACGAATAGCCACTGCATACCAAC-3' (sense 400); cFLIP-si2, 5'-CCATCAGGTTGAAGAAGCACTTGAT-3' (sense 2076); FADD-si1, 5'-ACACCAAGAU CGACAGCAUCGAGGA-3' (sense 368); FADD-si2, 5'-CCAUGCUGCUGGAGCAGAACGACC-3' (sense 269); FADD-si3 5'-AGAACACAGGAAGGAGAACGCA-3' (sense 446); MyD88-si1, 5'-GCAGCAGCAGGAGGAGGUGAGAA-3' (sense 417); MyD88-si2, 5'-CUGGAACAGACAAACUAUCGACUGA-3' (sense 604); TRIF-si1, 5'-CCACACGCACAGATGGTACAGCTG-3' (sense 2043); TRIF-si2, 5'-GGACAAGACGCAGGAGGCAGAATGA (sense 2115). Control siRNA duplexes were also purchased from Integrated DNA Technologies. Based on preliminary results to optimize responses, one round of transfection of HUVECs with 20 nmol/L siRNA was performed using Lipofectamine 2000 (0.1%, v/v) according to the manufacturer's instructions. Fresh medium was added 5 hours after transfection, and experiments were conducted 48 to 72 hours after transfection. The efficiency of knockdown was tested in each experiment both by qRT-PCR for mRNA expression and by immunoblotting for protein expression as described below.

Analysis of Cell Viability and Nuclear Morphology

The P/I method for loss of integrity of cell membranes was used to assess viability.¹⁸ In brief, cells were suspended in phosphate-buffered saline containing 25 μ g/ml P/I for 5 minutes at 37°C and then subjected to analytic flow cytometry on a FACSort (Becton Dickinson) immediately after labeling. A light-scatter gate was set up to eliminate cell debris from the analysis. The P/I fluorescence signal was recorded on the FL3 channel and analyzed by using Cellquest software. All assays were performed in triplicate and the data are reported as the mean $\pm$ SD of the percentage of cells that fail to exclude P/I. Nuclear morphology in HUVEC cultures was assessed by DAPI staining and fluorescence microscopy, as described previously.¹⁸

MTT viability assays were used to further evaluate the LPS-triggered Cat B-dependent cytotoxicity to HUVECs. Briefly, HUVECs were transfected with siRNAs targeting FADD or control siRNAs for 48 hours and subsequently stimulated with indicated treatments. At the end of treatment, MTT was added at a final concentration of 20 μ g/ml. After 2 hours of incubation, medium was removed and DMSO was added to dissolve the insoluble purple formazan generated in living cells. Optical density (OD₄₉₀,

subtracted background at 670 nm) indicated the cells survived from killing by LPS plus CHX.

RT-PCR Analysis

Total RNA extraction, reverse transcription, and PCR amplification were performed as described previously.^{18,19} Briefly, we performed real-time qRT-PCR using a Multicolor Real-Time PCR detection system (Bio-Rad, Hercules, CA) using the iQ SYBR Green Supermix (170-8882) according to the recommended protocol to determine the threshold cycle (Tc). The primer sequences used were as follows: FADD 5'-CCTAGACCTCTTCTCCATGCT-3' and 5'-AATGCTGCACACAGGTCTTCTT-3; MyD88, 5'-AATGAGGAGACTTGCTGCTGTA-3' and 5'-TATTGCATCTAGATTCTTGCTTT-3; cFLIP, 5'-ACCTTGTTTCGACTATAGAGTGCTGAT-3' and 5'-AGATTTAGTTTCTCCAACCAACCA-3'; IP-10, 5'-GAATGTGTAGAGACCATCATAATAAT-3' and 5'-AGGAAGAATTGRAGCTGAAGTTT-3'; TRIF 5'-ACCTCCTGCATGCCATGGTTCT-3' and 5'-TCAGCCAGCAGGTGGTACAA-3' β -actin, 5'-TGCACCACACCTTC-TACAATGA-3' and 5'-CAGCCTGGATAGCAACGTACAT-3'. In Poly IC-treated HUVECs, induction of IP-10 were expressed as fold induction (FI), calculated as follows: FI = 2^[(Poly IC (Tc target) - Tc β -actin) - Control(Tc target - Tc β -actin)]. In some experiments, the ratios of FI from experimental groups with different treatments were calculated. Error bars are used on graphs to indicate standard deviations.

Preparation of Cytosolic Extracts for the Analysis of Bid and Cytochrome c

HUVECs were mock treated or treated with LPS plus CHX and cotreatments as indicated in the text for 15 hours. Floating and attached ECs were separately harvested, pelleted, and incubated with the extraction buffer (250 mmol/L sucrose, 20 mmol/L Hepes, 10 mmol/L KCl, 1.5 mmol/L MgCl₂, 1 mmol/L EDTA, 1 mmol/L ethylene glycol bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid, 1 mmol/L Pefabloc, 50 μ g/ml digitonin, pH 7.5) for 15 minutes on ice to extract cytosolic contents as described previously.⁸ These conditions allow for the selective permeabilization of the plasma membrane by digitonin without perturbation of mitochondria. The cytosolic extracts were spun in Eppendorf tubes at 14,000 rpm for 20 minutes and the resulting supernatants were analyzed for Bid and cytochrome c by immunoblotting.

Immunoblotting

For immunoblotting, 4 $\times$ 10⁵ cells/sample were washed twice with cold phosphate-buffered saline before the addition of 100 μ 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 cocktail and 1 mmol/L phenylmethylsulfonyl fluoride (Boehringer Mannheim, Indianapolis, IN). 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 μ g of protein was separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, and transferred to a nitrocellulose membrane (Bio-Rad). After transfer, the membranes were blocked with 5% nonfat dry milk in Tris-buffered saline and then incubated overnight with primary antibody at 4°C. After washing, blots were further incubated with 1:5000 diluted secondary Ab (goat anti-mouse or anti-rabbit IgG horseradish peroxidase-conjugated) for 1 hour at room temperature. After additional washing, horseradish peroxidase activity was detected by chemiluminescence using SuperSignal West Dura (Pierce, Rockford, IL) according to the manufacturer's instructions.

Statistical Analysis

For cytotoxicity experiments, data are reported as means of triplicate samples from a single experiment $\pm$ SD (SD). Differences between treatment and control groups were analyzed using a two-sample, two tailed Student's *t*-test. A *P* value of <0.05 was considered as statistically significant. All experiments were repeated at least three times and showed similar outcomes.

Results

Death Induced by LPS Plus CHX in HUVECs Is Primarily Caspase-Mediated

Inhibition of new protein synthesis with agents such as CHX or Shiga-like toxin has been reported to sensitize cultured human ECs to LPS-induced programmed cell death.^{6,9} Consistent with these previous studies, we observed that as many as 40% of HUVECs lose membrane integrity after treatment with a combination of LPS plus CHX, whereas significantly fewer cells are killed by either agent used alone (Figure 1). A broad spectrum caspase inhibitor zVAD at 20 μ mol/L significantly inhibits the death of HUVECs triggered by LPS plus CHX (Figure 1A), although some level of cytotoxicity remains, and full inhibition is not achieved even at 150 μ mol/L concentrations of zVAD (data not shown). However, the combination of zVAD and a specific Cat B inhibitor, CA074, completely blocks the cytotoxicity induced by LPS plus CHX (Figure 1A).

We have previously reported that killing of HUVECs by TNF plus CHX causes caspase-8-mediated cleavage of Bid and its disappearance from the cytosol due to translocation to the mitochondria, and that Bid activation and translocation is associated with the release of cytochrome *c* from the mitochondria into the cytosol, indicating an amplification pathway for TNF-induced apoptosis.⁸ The appearance of cytochrome *c* in the cytosol of target cells and the simultaneous disappearance of Bid from the cytosol thus constitutes functional evidence of caspase-8 activation.⁸ The combined treatment of LPS and CHX induces cytochrome *c* release and Bid disap-

Figure 1. Analysis of HUVEC death triggered by LPS plus CHX. LPS (0.5 μ g/ml), CHX (3 μ g/ml), or caspase inhibitors (zVAD, 20 μ mol/L) and/or Cat B (CA074, 20 μ mol/L) were added to HUVEC cultures for a 15-hour treatment period, after which the cell viability was determined by P/I exclusion as described in *Materials and Methods*. **A:** Killing of HUVECs with LPS, CHX, or LPS plus CHX and inhibition of HUVEC death in responses to LPS plus CHX by zVAD, CA074, or combined treatment with zVAD plus CA074. In this and all subsequent cytotoxicity experiments, the bars represent the mean $\pm$ SD of triplicate determinations (ie, three replicate cultures from a single experiment) and significance was analyzed by two-sample, two-tailed Student's *t*-tests. Note that LPS plus CHX induced a significant degree of killing compared with controls and this was significantly reduced by either zVAD or zVAD plus CA074 but not by CA074 alone ($P < 0.05$). **B:** Bid disappearance from and cytochrome *c* release into the cytosol of HUVECs after treatment with LPS, CHX, or LPS plus CHX. After treatment, HUVECs were harvested and cytosolic extracts were prepared by digitonin extraction under conditions that cause selective permeabilization of the plasma membrane without perturbing lysosomes and mitochondria. Cytosolic extracts were analyzed for Bid and cytochrome *c* by immunoblotting as described in *Materials and Methods*. β -Actin was used as a loading control. Note that LPS plus CHX-triggered HUVEC death is accompanied by release of cytochrome *c* into and Bid disappearance from the cytosol. All experiments were repeated at least three times with similar results.

pearance in HUVECs (Figure 1B). Both Bid disappearance and cytochrome *c* release is largely prevented by the addition of zVAD to HUVEC cultures treated with LPS plus CHX (data not shown). These data are consistent with the finding that HUVEC death triggered by LPS plus CHX is mediated mainly (but not exclusively) through a caspase-dependent pathway with only a minor contribution of the Cat B-dependent pathway.

Adaptor Function of MyD88 and TRIF for the LPS-Mediated Death Signaling in ECs

Both MyD88 and TRIF are adaptor proteins of LPS-TLR4 signaling pathways that result in new gene transcription.^{20,21} We further tested if LPS-triggered death signaling involved either of these adaptor proteins in HUVECs. We first established conditions under which MyD88 expression in HUVECs can be effectively reduced by siRNA transfection (Figure 2, A and B). To examine the effects of MyD88 knockdown on the LPS-triggered death signaling in HUVECs, we then measured the cell viability of the siRNA-transfected HUVECs when treated by LPS plus CHX. MyD88 siRNA-transfected HUVECs become markedly resistant to death triggered by LPS plus CHX (Figure 2C), whereas control siRNA-transfected HUVECs remained sensitive to this treatment. We similarly tested if TRIF contributes to the death response of HUVECs to LPS by means of TRIF siRNA knockdown (Figure 3, A and B). In contrast to MyD88 siRNA-transfected HUVECs, TRIF siRNA-transfected HUVECs remain fully sensitive to

Figure 2. Sensitivity of MyD88 siRNA-transfected HUVECs to LPS plus CHX-induced killing. **A** and **B**: HUVECs were transfected with siRNA targeting MyD88 for 48 hours and subjected to real-time qRT-PCR (**A**) and immunoblotting analysis (**B**). Note that two different MyD88 siRNAs, but not the control siRNA, knocked down MyD88 expression. **C**: HUVECs were transfected with siRNA (control, MyD88 si1 or MyD88 si2 for 48 hours, subsequently stimulated with indicated treatment CHX, LPS, or both for 15 hours and viability was determined by P/I exclusion as described as in *Materials and Methods*. After treatment of LPS plus CHX, differences of P/I-stained cells between the control siRNA-transfected group and MyD88 siRNA-transfected groups were analyzed. Note that killing by LPS plus CHX is significant ($P < 0.05$) compared with LPS or CHX alone in untransduced or control-transduced HUVECs but is not significant following knockdown of MyD88 with either of two siRNAs. All experiments were repeated at least three times with similar results.

caspase-dependent killing in response to LPS plus CHX (Figure 3C). TRIF knockdown in replicate wells is functionally effective since IP-10 induction by poly IC, a TLR3 ligand that exclusively signals via TRIF, is inhibited (Figure 3D). We conclude that LPS-triggered caspase acti-

vation in HUVECs is largely, if not wholly, MyD88-mediated and TRIF-independent.

FADD Inhibits the LPS-Triggered Cat B Death Signaling in HUVECs

It is unclear how MyD88 is connected to caspase activation. It has been shown that in cells which overexpress both molecules, MyD88 may interact with FADD,^{22,23} a known activator of caspase 8. However, the role of FADD in death signaling triggered by LPS plus CHX has yielded conflicting results in experiments using the HMEC-1 endothelial-derived immortalized cell line.^{9,10} To explore this issue in untransformed HUVECs, we first reduced FADD expression in HUVECs by knockdown with three different FADD siRNAs; FADD siRNA3 showed the highest efficiency and a minimum of 80% knockdown was achieved, as assessed by real-time qRT-PCR for mRNA levels (Figure 4A) and immunoblotting for protein expression (Figure 4B). Transfection with a control siRNAs did not reduce FADD expression levels (Figure 4A). We then measured the cell viability of FADD siRNA-transfected HUVECs treated with LPS plus CHX. In contrast to our results with MyD88 knockdown, FADD siRNA-transfected HUVECs showed comparable or even increased levels of cell death triggered by LPS plus CHX (Figure 4C). More than 40% of FADD siRNA1-transfected HUVECs lost membrane integrity, and 60% of the FADD siRNA3-transfected HUVECs did so (Figure 4B). This result is reminiscent of our previous findings that JURKAT T cells lacking FADD or HUVECs transduced with a dominant negative FADD construct (FADD_{DN}) showed increased sensitivity to TNF plus CHX.⁸ In these instances, death became Cat B-dependent and caspase-independent.⁸ In the FADD siRNA-transfected HUVECs, the caspase inhibitor zVAD alone provides very limited protection against death induced by LPS plus CHX (Figure 4C). However, the death signaling in the FADD siRNA-transfected HUVECs is sig-

Figure 3. Sensitivity of TRIF siRNA-transfected HUVECs to LPS plus CHX-induced killing. **A** and **B**: HUVECs were transfected with siRNA targeting TRIF for 48 hours and subjected to real-time qRT-PCR (**A**) and immunoblotting analysis (**B**). Note that two different TRIF siRNAs, but not the control siRNA, reduced TRIF expression. **C**: HUVECs were transfected with siRNA (control, TRIF si1, or TRIF si2) for 48 hours, subsequently stimulated with indicated treatment CHX, LPS, or both for 15 hours, and viability was determined by P/I exclusion as described as in *Materials and Methods*. LPS plus CHX killing of HUVECs is not significantly changed by TRIF knockdown. **D**: HUVECs were transfected with siRNA-targeted TRIF or controls for 48 hours, subsequently stimulated with or without Poly IC (2 μ g/ml) for 3 hours, and their RNA was isolated and subjected to real time qRT-PCR analysis using primers for IP-10. Note that the IP-10 induction by Poly IC was significantly decreased ($P < 0.05$) in HUVECs transfected with either of the two different TRIF siRNAs but not with the control siRNA. All experiments were repeated at least three times with similar results.

Figure 4. Sensitivity of FADD siRNA-transfected HUVECs to LPS plus CHX-induced killing. **A** and **B:** HUVECs were transfected with siRNAs targeting FADD or control siRNAs for 48 hours and subjected to real-time qRT-PCR (**A**) and Western blotting analysis (**B**) to measure FADD expression. **C:** HUVECs were transfected with siRNA (controls, FADD si1, si2, or si3) for 48 hours and subsequently stimulated with indicated treatment, CHX, LPS, or both, for 15 hours. Caspase inhibitor zVAD, Cat B inhibitor CA074, or both combined were added to cultures treated with LPS plus CHX. Cell viability was determined by P/I exclusion as described in *Materials and Methods*. Differences of P/I-stained cells between the cells with CHX plus LPS treatment and cells with other treatments in each group (with control, FADD si1, or FADD si3 siRNA transfection) were analyzed. Note that LPS plus CHX killing of HUVECs appears to be potentiated by the most efficient knockdown of FADD, produced by transfection with FADD si3, and that killing is significantly reduced by inhibiting Cat B following FADD knockdown with either FADD si1 or si3 but not in control cells ($P < 0.05$). Knockdown of FADD also results in loss of significant protection by caspase inhibition alone. All experiments were repeated at least three times with similar results.

nificantly inhibited by the Cat B inhibitor CA074 and is only completely blocked by the combination of zVAD and CA074 (Figure 4C). In contrast, in control siRNA-transfected HUVECs, the death signaling triggered by LPS plus CHX remains largely sensitive to zVAD (Figure 4C). Similar results were observed when FADD siRNA-transduced cells were subjected to LPS plus CHX, and killing was assessed by an MTT assay of mitochondrial function (Table 1).

Our previous studies of TNF-mediated activation of the Cat B death pathway had further shown that while zVAD no longer prevented cell death or mitochondrial release of cytochrome c, it did prevent disappearance of Bid from the cytosol and it prevented nuclear fragmentation but

not nuclear condensation in dying cells.⁸ Using DAPI staining and immunoblotting, we analyzed these responses in cell death triggered by LPS plus CHX in HUVECs following FADD knockdown. In the absence of zVAD, the combined treatment of LPS and CHX caused nuclei to condense and fragment (Figure 5A). Addition of zVAD prevented nuclear fragmentation but the nuclei still underwent condensation (Figure 5A). In the absence of zVAD, LPS plus CHX induced both Bid disappearance from and cytochrome c release into the cytosol of FADD siRNA3-transfected HUVECs; in the presence of zVAD, cytochrome c was still released into the cytosol by LPS plus CHX, but was no longer accompanied by disappearance of Bid (Figure 5B). Thus in HUVECs lacking FADD, LPS plus CHX still activates caspases, and caspases contribute to the appearance of cell death, but Cat B-dependent cell death still occurs even when caspases are inhibited, albeit with distinct morphological features. Moreover, the fact that CA074 provides strong protection as a single agent implies that Cat B is upstream of caspase activation, presumably through Bid-independent mitochondrial release of cytochrome c.

To supplement our findings with FADD siRNA, we also transduced HUVECs to overexpress an AU1 tagged human FADD_{DN}¹⁷ using retroviral vector pFB.¹⁹ The expression of FADD_{DN} was confirmed by immunoblotting (Figure 6A). In the presence of CHX, LPS killed FADD_{DN}-transduced HUVECs (HUVEC_{FADD-DN}) as well as empty vector-transduced HUVECs (HUVEC_{VEC}). The Cat B inhibitor CA074, but not the caspase inhibitor zVAD, significantly protected HUVEC_{FADD-DN} from the cytotoxicity triggered by LPS plus CHX (Figure 6B). In contrast, with the same treatment, HUVEC_{VEC} are significantly protected by the caspase inhibitor zVAD but not by the Cat B inhibitor CA074 (Figure 6B). These findings further support the interpretation that death signaling triggered by LPS plus CHX, like that triggered by TNF plus CHX,⁸ is regulated by FADD. Specifically, in the presence of intact

Table 1. MTT Viability Assay for the Cat B-Mediated Death Response to LPS

siRNA	Treatments	OD ₄₉₀
Control	CHX	0.61
	CHX+LPS	0.50
	CHX+LPS+zVAD	0.70
	CHX+LPS+CA074	0.75
FADD1	CHX	0.55
	CHX+LPS	0.30
	CHX+LPS+zVAD	0.31
	CHX+LPS+CA074	0.67
FADD3	CHX	0.48
	CHX+LPS	0.24
	CHX+LPS+zVAD	0.22
	CHX+LPS+CA074	0.56

HUVECs were transfected with siRNAs targeting FADD or control siRNAs for 48 hours and subsequently stimulated with indicated treatment, CHX, LPS, or both for 15 hours. Caspase inhibitor zVAD or Cat B inhibitor CA074 were added to cultures treated with LPS plus CHX. Cell viability was determined by MTT viability assay. Note that LPS plus CHX killing of HUVECs is potentiated by knockdown of FADD and that killing becomes more dependent upon Cat B. Statistical analyses showed that CA074, but not zVAD, significantly protected FADD siRNA transfected HUVECs from cytotoxicity induced by LPS plus CHX. All experiments were repeated three times with similar results.

Figure 5. zVAD effects on the appearance of HUVEC death triggered by LPS plus CHX in the absence of FADD. HUVECs were transfected with FADD siRNA (pool of siRNA 1, 2, 3) for 48 hours, subsequently stimulated with indicated treatment with CHX, LPS, or both, in the presence or absence of zVAD at the indicated time points. **A:** Examination of nuclear changes. Following 7 hours of treatment, HUVECs were harvested and centrifuged onto slides by cytospinning. Nuclear morphology was examined by DAPI staining as described in *Materials and Methods*. **Arrows** indicate the marked nuclear changes. Note that in the LPS plus CHX-treated group, HUVECs lacking FADD undergo both nuclear condensation and fragmentation indicative of apoptosis and that addition of zVAD prevents nuclear fragmentation but the nuclei still undergo condensation. **B:** Bid cleavage and cytochrome *c* release in the FADD-independent HUVEC cell death triggered by LPS plus CHX. Following 15 hours of treatment, HUVECs were harvested, and cytosolic extracts were prepared by digitonin extraction under conditions that cause selective permeabilization of the plasma membrane without perturbing lysosomes and mitochondria. Cytosolic extracts were analyzed for Bid and cytochrome *c* (Cyto C) by immunoblotting as described in *Materials and Methods*. Note that in the presence of zVAD, LPS plus CHX induced significant cytochrome *c* release without Bid disappearance from the cytosol, indicative of a Bid-independent mitochondrial release reaction. β-Actin was used as a loading control. All experiments were repeated at least three times with similar results.

FADD, death is initiated by caspase activation and amplified by caspase-induced cleavage of Bid while in the absence of FADD or in the presence of a form of FADD lacking the death effector domain, death results from a caspase-independent pathway involving Cat B activation and a Bid-independent mitochondrial release reaction.

cFLIP Inhibits the Caspase-Dependent Death Response of HUVECs to LPS

cFLIP may block death receptor-induced apoptosis by preventing FADD-mediated caspase 8 activation, and it has been reported that reduction of cFLIP expression by an antisense oligonucleotide sensitized HUVEC to LPS-triggered death signaling.²⁴ Since cFLIP interacts with FADD and since FADD appears to function as a switch between the caspase 8-dependent death pathway and the Cat B death pathway, we wished to examine whether cFLIP played a similar role. We first reduced cFLIP expression by siRNA transfection in HUVECs by greater than 85%, as measured by real-time qRT-PCR for mRNA (Figure 7A) or immunoblotting for protein (Figure 7B). In the absence of CHX, LPS does not induce death in the cFLIP siRNA-transfected HUVECs. However, with a low concentration of CHX (1 μg/ml), LPS effectively killed cFLIP siRNA-transfected HUVECs (Figure 7C), while control siRNA-transfected HUVECs remained relatively resistant to this combined treatment (Figure 7C). Under these conditions, death is blocked by caspase inhibitor zVAD (Figure 7C). In other words, reducing cFLIP levels sensitizes HUVECs to an LPS-induced caspase-dependent death mechanism in HUVECs, but unlike knockdown of FADD, knockdown of cFLIP does not switch the LPS-induced death response of HUVEC to the Cat B death pathway.

The Role of MyD88 and TRIF in LPS-Triggered Cat B Death Signaling in HUVEC

To test if LPS-triggered Cat B death signaling in HUVEC requires MyD88, we simultaneously knocked down MyD88 and FADD expression by siRNA transfection. More than 90% of knockdown efficiency of mRNA encoding FADD and MyD88 is achieved, as measured by real-time qRT-PCR, while transfection with control siRNAs does not reduce either FADD or MyD88 expression levels (Figure 8A). About 50% of FADD and MyD88 siRNA-transfected HUVECs are killed by LPS plus CHX, and cell death was significantly inhibited by Cat B inhibitor CA074 but not by caspase inhibitor zVAD (Figure 8B). HUVECs cotransfected with FADD siRNA and control siRNA showed a similar level of sensitivity to LPS and CHX treatment and were also protected by Cat B inhibitor CA074 (Figure 8B). We then examined the effect of simultaneous knockdown of FADD and TRIF (Figure 8B). HUVECs with reduced FADD and TRIF expression were not protected from killing triggered by LPS plus CHX (Figure 8B). These experiments suggest that neither MyD88 nor TRIF is involved in LPS activation of the Cat B death pathway.

Figure 6. Cell death in FADD-dominant negative transduced HUVECs killed by LPS plus CHX. **A:** Dominant negative FADD (FADD_{DN}) expression by retroviral transduced HUVECs or by pFB (mock-vector transduced HUVEC) was determined by immunoblotting using mouse anti-AU1(Flag) antibody. **B:** The sensitivity of these two cell cultures to LPS plus CHX was determined by P/I exclusion after incubation for 15 hours in the presence or absence of zVAD, CA074, or both. Differences of P/I-stained cells between the cells with CHX+LPS treatment and cells with other treatments in each group (HUVEC_{FADD-DN} and HUVEC_{Vector}) were analyzed. Note that FADD_{DN} both significantly reduces caspase-mediated killing and significantly potentiates the Cat B-mediated pathway triggered by LPS plus CHX ($P < 0.05$).

LPS Cotreatment with LY294002 or IFN- γ Activates both Caspase and Cat B Death Pathways

We had previously shown that TNF killing of HUVECs is potentiated by addition of the PI3 kinase inhibitor, LY294002.²⁵ This response is largely resistant to the presence of zVAD and mainly involves the activation of the Cat B pathway as judged by inhibition with CA074.²⁵ We therefore examined the effects of combining LPS with LY294002. These conditions also resulted in HUVEC death, but protection was only conferred by combining zVAD and CA074, implying that both death pathways are being activated (Figure 9A).

IFN- γ , like LY294002, can also potentiate TNF-induced killing of HUVECs and can promote activation of the Cat B-mediated death pathway even in the presence of FADD.⁹ We therefore tested if the combination of LPS with IFN- γ could induce human EC injury and if so, by which pathway(s). This seemed particularly relevant as Gram-negative sepsis represents a situation in which ECs may be exposed both to LPS and to IFN- γ .²⁶ As shown in Figure 9B, about 40% of HUVECs treated with LPS plus IFN- γ for 15 hours die as assessed by loss of membrane integrity. The death signaling triggered by the combination of LPS and IFN- γ was only inhibited by the combination of zVAD and CA074 and not by either agent alone.

Confirmation of the Specificity of Cat B Inhibition

Although CA074 is described as a specific inhibitor of Cat B¹²⁻¹⁵ and we have previously shown that it does

Figure 7. Sensitivity of cFLIP siRNA-transfected HUVECs to LPS plus CHX-induced killing. **A** and **B:** HUVECs were transfected with siRNAs targeting cFLIP or control siRNAs for 36 hours and subjected to real-time qRT-PCR (**A**) and immunoblotting analysis (**B**) to measure cFLIP expression. **C:** HUVECs were transfected with siRNA (controls, cFLIP si1, or si2) for 36 hours, subsequently stimulated with indicated treatment, CHX (1 μ g/ml), LPS, or both for 15 hours. Caspase inhibitor zVAD, Cat B inhibitor CA074, or both combined were added to cultures treated with LPS plus CHX. Cell viability was determined by P/I exclusion as described in *Materials and Methods*. After treatment of LPS plus CHX, differences of P/I-stained cells between the control cells (with or without control siRNA transfection) and cFLIP siRNA-transfected cells were analyzed using a two-sample, two-tailed Student's *t*-test. Note that LPS plus CHX killing of HUVECs is significantly potentiated by knockdown of cFLIP ($P < 0.05$) and that killing was inhibited by zVAD. All experiments were repeated at least three times with similar results.

Figure 8. Sensitivity of MyD88 or TRIF siRNA-transfected HUVECs to Cat B-dependent death signal triggered by LPS plus CHX. **A:** HUVECs were transfected with control siRNAs or cotransfected with control and FADD siRNAs, MyD88, and FADD siRNAs or TRIF and FADD siRNAs for 48 hours and subjected to real-time qRT-PCR to monitor FADD, MyD88 and TRIF knockdown efficiency. **B:** HUVECs with the same siRNA transfections as described above (**A**), subsequently stimulated with indicated treatment, CHX (2 μ g/ml), or LPS (0.5 μ g/ml) plus CHX for 15 hours. Caspase inhibitor zVAD (20 μ mol/L) or Cat B inhibitor CA074 (20 μ mol/L) was added to cultures treated with LPS plus CHX. After treatments indicated, differences of P/I-stained cells between the control siRNA-transfected group and FADD siRNA-transfected groups were analyzed using a two-sample, two-tailed Student's *t*-test. Note that HUVECs following FADD plus MyD88 or FADD plus TRIF knockdown retain their sensitivity to LPS plus CHX and that cell death is significantly inhibited by CA074 ($P < 0.05$) but not by zVAD. One of two experiments with similar results.

Figure 9. The death response of HUVECs to LPS cotreatment with LY294002 or IFN- γ . LPS (0.5 μ g/ml), LY294002 (20 μ mol/L), IFN- γ (150 ng/ml), or the inhibitors of caspases (zVAD, 20 μ mol/L) and/or Cat B (CA074 or zFA) were added to HUVEC cultures for a 15-hour treatment period, after which the cell viability was determined by P/I exclusion as described as indicated in *Materials and Methods*. Differences of P/I-stained cells between cells with LPS + LY or LPS + IFN- γ and cells with other treatment as indicated, were analyzed. **A:** Killing of HUVECs with LPS, LY294002, or LPS plus LY294002 and inhibition of HUVEC cell death in response to LPS plus LY294002 by zVAD, CA074, or combined treatment with zVAD plus CA074. Note that significant levels of killing require both LPS and LY294002 and are only significantly inhibited by the combined treatment with zVAD plus CA074. **B:** Killing of HUVECs with LPS, IFN- γ , or LPS plus IFN- γ and inhibition of HUVEC death. Note that significant levels of killing require both LPS and IFN- γ and are only significantly inhibited by the combined treatment of zVAD and CA074. **C:** Killing of HUVECs with LPS, LY294002, or LPS plus LY294002 and inhibition of HUVEC death in responses to LPS plus LY294002 by zVAD, zFA or combined treatment with zVAD plus zFA. All experiments were repeated at least three times with similar results.

inhibit Cat B in extracts of HUVECs,⁸ the role of Cat B in the present set of experiments has been based solely on this single agent. Attempts to knock down Cat B expression with siRNA in HUVECs have been unsuccessful. An alternative approach is to examine the role of additional pharmacological inhibitors. Therefore, in a final set of experiments we tested the ability of a structurally distinct Cat B inhibitor, namely zFA,^{27,28} to protect HUVECs from cell death triggered by LPS plus LY294002. As shown in Figure 9C, zFA or zVAD alone did not inhibit the death signaling triggered by LPS plus LY294002, but the death signal was completely blocked by the combination of zFA and zVAD. In other words, two different Cat B inhibitors provided a similar degree of protection, supporting the identification of Cat B as the relevant initiator of the alternative death pathway.

Discussion

Many of the pathological responses associated with LPS, such as Schwartzman reactions, depend on responses of vascular ECs.²⁹ Human ECs express TLR4,³⁰ although not CD14, but can respond to LPS by using soluble CD14 present in plasma.³¹ The responses of ECs to LPS may be divided into the activation of new gene expression, encoding proinflammatory, procoagulant and antiapoptotic proteins, and activation of cell death pathways. In the present report, we find that the apoptotic death response of HUVECs to LPS is dependent on the MyD88 adaptor protein (Figure 2) but not on TRIF (Figure 3). We also demonstrate that FADD, an adaptor protein originally described as coupling Fas (CD95) to the activation of caspase-8 and subsequently of TNFR1 via TRADD to activation of caspase-8, is a modulator of EC responses to LPS (Figure 4). In the case of cell death responses, which are most evident when antiapoptotic gene product expression is inhibited, eg, by CHX, FADD appears to couple MyD88 to caspase activation (Figure 4). However, the absence of FADD, caused by knockdown of FADD expression by siRNA, actually enhances death by enabling LPS to activate a caspase-independent, Cat

B-dependent cell death pathway (Figure 4). Overexpression of a truncated FADD mutant lacking the N-terminal death effector domain (FADD_{DN}), also enhances activation of the Cat B pathway (Figure 6). These findings are similar to our previous results in HUVECs treated with TNF.⁸ We interpret our findings to suggest that FADD is a regulatory switch for LPS-induced death responses in human ECs. Knockdown of cFLIP, an inhibitor of the interaction of FADD with caspase-8, can potentiate the caspase-dependent killing of HUVECs by LPS plus CHX but does not appear to influence the Cat B pathway (Figure 7). Thus the molecular targets with which FADD interacts to inhibit the Cat B pathway are unknown. Furthermore, neither concomitant MyD88 nor TRIF knockdown by siRNA transfection blocks the Cat B-mediated death response of FADD knockdown HUVECs to LPS plus CHX (Figure 8), implying that an as yet unidentified adaptor protein(s) must be involved in activation of this pathway. Finally, we have shown that the combined treatment of LPS plus IFN- γ or the PI3 kinase inhibitor LY294002 activates both caspase and Cat B death pathways in HUVECs (Figure 9). We have summarized our findings in this study in Figure 10.

Choi et al were the first to suggest that FADD might contribute to LPS plus CHX killing of human ECs.⁹ These workers used the SV40 large T immortalized line HMEC-1 and transfected a dominant negative FADD construct (FADD_{DN}) in their studies, reporting inhibition of killing. Bannerman et al subsequently expressed the same construct in the same cell line and found no diminution of killing.¹⁰ The latter authors have suggested that the discrepant results may relate to different assays used to assess cell death. Our study advances this prior work in three ways. First, we have used untransformed HUVECs, avoiding potential alterations in cell death mechanisms induced by transformation. Second, we have used siRNA to complement overexpression approaches, avoiding potential pitfalls of non-physiological protein-protein interactions. And third, we have applied findings from our studies of TNF responses in which we had first established that FADD may participate in more than one death

Figure 10. Schematic summary of LPS-triggered programmed cell death in human endothelial cells. LPS bound to CD14 triggers responses through TLR4. In the presence of FADD, LPS-activated TLR4 recruits MyD88 which, in turn, engages FADD. FADD recruits pro-caspase 8, which undergoes auto-catalytic activation. This response is normally inhibited by cFLIP, the level of which rapidly decays in the absence of new protein synthesis (eg, when CHX is present). Activated caspase 8 may directly activate effector caspases, causing apoptotic cell death. This response may also be amplified by caspase 8-mediated cleavage of cytosolic Bid, forming active tBid. tBid translocates to the mitochondria, initiating release of cytochrome *c* into the cytosol where, in combination with other proteins, activates caspase 9. Active caspase 9, like caspase 8, activates effector caspases and also leads to apoptotic cell death. In the absence of FADD or when phosphatidylinositol 3 kinase is inhibited by LY294002 or when IFN- γ is present, LPS activates a MyD88-independent release of cathepsin B (cat B) from the lysosome into the cytosol. Cytosolic Cat B initiates mitochondrial release of cytochrome *c* and other death-inducing proteins from the mitochondria through a mechanism that is independent of Bid. Some of these released proteins, such as apoptosis inducing factor, can cause cell death through a pathway that does not involve caspases. Caspase inhibitors like zVAD can block the actions of caspase 8, caspase 9 and effector caspases, preventing death triggered via MyD88 and FADD. However, zVAD does not inhibit cat B and cannot prevent mitochondrial release of proteins like apoptosis inducing factor and thus cannot prevent cell death when Cat B is activated, although it may prevent the appearance of certain features of death caused by caspases such as nuclear fragmentation. Programmed cell death initiated by Cat B can be prevented by Cat B inhibitors such as CA074 or zFA.

pathway.^{8,32} A major conclusion of this part of the present study is that FADD plays a remarkably similar role in LPS-initiated death responses as it plays in TNF-initiated death responses (Figure 10). It is likely to act similarly in IL-1-initiated death responses of HUVECs as well, since IL-1 signaling also depends on MyD88.³³

We think that the involvement of FADD in LPS-triggered caspase activation is most easily interpreted as there being an interaction between MyD88 and FADD. This has proven difficult to confirm. Others have reported that overexpressed MyD88 and FADD do interact,^{22,23} but non-physiological levels of expression of DD-containing proteins may lead to artifactual interactions. We have made repeated attempts to coimmunoprecipitate endogenous FADD and MyD88 from HUVECs and have had inconsistent results, positive in some experiments but

negative in others. Addition of LPS sometimes appeared to transiently increase the interaction but in other experiments did not. Therefore, we cannot firmly conclude that there is a direct interaction between these two proteins, probably because such interactions are of low affinity, even though our siRNA experiments are consistent with such an interpretation. Moreover, reduction of FADD expression appears to potentiate HUVECs to Cat B-mediated death (Figure 4) in a pathway that does not appear to involve MyD88. Consequently, if siRNA or antisense therapies are effectively translated to the clinic,³⁴ our data suggest that FADD is not an appropriate target for reducing tissue injury.

The involvement of FADD in LPS-induced programmed cell death raised the possibility that killing might be due to activation of a death receptor, such as Fas or TNFR1, by LPS-mediated induction or activation of a death receptor ligand. It is highly unlikely that such a ligand would be synthesized *de novo*, since LPS-triggered killing required the presence of a protein synthesis inhibitor. We examined this possibility by conducting treatments of HUVECs with LPS plus CHX in the presence of an anti-TNF, anti-Fas, or anti-Fas ligand neutralizing antibody. None of these antibodies reduced killing by LPS plus CHX (data not shown) but were effective in protecting replicate wells from killing, ie, using TNF plus CHX treatment to test anti-TNF antibody or using IFN- γ treatment of FasL-transduced HUVECs to test anti-Fas or anti-Fas ligand antibodies as previously described.¹⁸

The adaptor protein(s) that activate Cat B-mediated death in response to LPS in HUVECs is an open question (Figure 10). A prior study with a dominant negative construct of MyD88 suggested that LPS-mediated death signaling in human ECs is strictly MyD88-dependent.¹⁰ With a different experimental approach, namely RNA interference, we confirmed that LPS-triggered apoptosis in HUVECs is strictly MyD88-dependent (Figure 2). However, MyD88 siRNA-transfected HUVECs do not alter their Cat B-mediated death response to LPS (Figure 8). These observations suggest that LPS-triggered caspase- and Cat B-dependent death pathways in HUVEC use different adaptor proteins. TRIF has also been reported to act as an adaptor protein for LPS-triggered caspase-independent cell death signaling,³⁵ although a specific link to Cat B was not examined. We have found that knockdown of TRIF in HUVECs does not alter either caspase-dependent (Figure 3C) or Cat B-dependent death responses to LPS (Figure 8B). We believe that our knockdown of TRIF is adequate to draw this conclusion since more than 80% knockdown efficiency is achieved in the TRIF siRNA-transfected HUVECs, and IP-10 induction by poly IC, a TRIF-dependent response, is significantly reduced following TRIF knockdown (Figure 3D).

There is precedent for the role of Cat B in programmed cell death from other systems. Cat B knockout mice are resistant to tumor necrosis factor-mediated hepatocyte apoptosis and liver injury.³⁶ In previous studies with human ECs and tumor cells, Cat B was implicated as a mediator of TNF-triggered cell death.^{8,14,25} However, the interpretation of the human EC experiments have depended on the specificity of CA074 as an inhibitor of

intracellular Cat B.^{12–14} In the present study, we have extensively used CA074 to determine the role of Cat B death mechanism in the LPS-triggered HUVEC cell death. Although we were unable to achieve knockdown of Cat B by siRNA transfection, a second Cat B inhibitor, zFA, shows similar protection of HUVECs against death signaling triggered by LPS plus LY294002, supporting our identification of Cat B as the relevant target. It is important to note that the Cat B response leads to caspase activation and that death in this pathway normally appears to be apoptotic. The relevant point here is that inhibition of caspases eliminates the features of apoptosis but does not protect cells from death. In our studies, we use loss of plasma membrane integrity as our primary method of quantifying cell death. This response occurs at later times than early markers of apoptosis, such as annexin V binding, but in our experience is more reliable and occurs in both apoptotic and nonapoptotic pathways. We have also confirmed our P/I exclusion assays with morphological assessment of nuclear changes and by an MTT assay of mitochondrial function. All three approaches yield similar answers.

Many of the experiments described in this report make use of pharmacological or RNAi manipulations of endogenous EC molecules that may not commonly occur *in vivo*. A notable exception to this is the combination of LPS plus IFN- γ , which has been shown to occur in sepsis.^{37,38} The neutralization of LPS by antibody does not ameliorate clinical sepsis, most likely because LPS leads to the release of additional endogenous mediators, such as TNF and IL-1, that produce many of the same responses as LPS itself. The ability of all of these agents to kill ECs depends on a cofactor, and like LPS, the endogenous cofactor for this effect is possibly IFN- γ . Our data therefore suggest that neutralization of this cytokine may have broadly protective effects for EC that are exposed to LPS or its induced effector cytokines. They further suggest that protection of ECs from injury by inhibition of caspases alone is unlikely to have a significant impact, since the combination of LPS and IFN- γ is likely to involve a caspase-independent, Cat B-dependent cell death response.

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