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FLICE-Like Inhibitory Protein (FLIP) Protects Against Apoptosis and Suppresses NF-κB Activation Induced by Bacterial Lipopolysaccharide

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Bacterial lipopolysaccharide (LPS) via its activation of Toll-like receptor-4 contributes to much of the vascular injury/dysfunction associated with gram-negative sepsis. Inhibition of *de novo* gene expression has been shown to sensitize endothelial cells (EC) to LPS-induced apoptosis, the onset of which correlates with decreased expression of FLICE-like inhibitory protein (FLIP). We now have data that conclusively establish a role for FLIP in protecting EC against LPS-induced apoptosis. Overexpression of FLIP protected against LPS-induced apoptosis, whereas down-regulation of FLIP using antisense oligonucleotides sensitized EC to direct LPS killing. Interestingly, FLIP overexpression suppressed NF-κB activation induced by LPS, but not by phorbol ester, suggesting a specific role for FLIP in mediating LPS activation. Conversely, mouse embryo fibroblasts (MEF) obtained from FLIP −/− mice showed enhanced LPS-induced NF-κB activation relative to those obtained from wild-type mice. Reconstitution of FLIP−/− MEF with full-length FLIP reversed the enhanced NF-κB activity elicited by LPS in the FLIP −/− cells. Changes in the expression of FLIP had no demonstrable effect on other known LPS/Tlr-4-activated signaling pathways including the p38, Akt, and Jnk pathways. Together, these data support a dual role for FLIP in mediating LPS-induced apoptosis and NF-κB activation. *(Am J Pathol 2004, 165:1423–1431)*

Bacterial lipopolysaccharide (LPS) is a highly pro-inflammatory component of the outer membrane of gram-negative bacteria that has been implicated in the pathogenesis of gram-negative sepsis.[1] The difficulty in managing gram-negative sepsis is compounded by the development of such complications as systemic vascular collapse,[2] disseminated intravascular coagulation,[3] multiorgan failure,[4] and the development of vascular leak syndromes, including acute respiratory distress syndrome.[5] Injury to and/or dysfunction of the vascular endothelium has been implicated in the development of these sepsis-related complications.[6–9]

The vascular endothelium is a key host target of LPS and the first host tissue barrier to encounter circulating LPS shed from replicating or dying gram-negative bacteria.[10] Several lines of evidence exist for a direct effect of LPS on endothelial cells (EC) and this evidence has been reviewed extensively elsewhere.[11–13] EC express Toll-like receptor (Tlr)-4, an innate immune pattern recognition receptor that is activated by LPS.[14] Although the exact mechanism by which LPS is recognized by Tlr-4 remains unclear, EC activation is dependent on the cell surface assembly of a multi-protein recognition complex consisting of soluble CD-14, MD-2, and Tlr-4.[15] Following activation of the receptor complex, the adapter proteins myeloid differentiation factor 88 (MyD88) and MyD88 adapter-like protein (MAL) are recruited to the cytoplasmic domain of Tlr-4.[16,17] MyD88 contains an additional protein-binding domain, the death domain (DD), which facilitates its association with another DD-containing signaling molecule, IL-1 receptor-associated kinase-1 (IRAK-1).[18] MAL, via heterodimerization with MyD88, preferentially interacts with IRAK-2.[16,19] Subsequent interaction of IRAK family members with TNF receptor-associated factor-6 (TRAF-6) results in the activation of a downstream kinase cascade involving NF-κB-inducing kinase (NIK) and IκB kinase (IKK).[20,21] IKK-mediated

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phosphorylation of the NF-κB inhibitor, IκB, leads to IκB degradation, thus enabling NF-κB nuclear translocation and the induction of new gene expression.[18] Genes upregulated by NF-κB include pro-inflammatory cytokines, such as IL-1β, IL-6, IL-8, and TNF-α, as well as a variety of adhesion molecules.[22]

In addition to promoting NF-κB activation in EC, several of the intracellular Tlr-4 signaling molecules, including MyD88, IRAK, MAL, and TRAF-6, promote LPS pro-apoptotic signaling.[23–25] Although the mechanism by which these signaling molecules promote caspase activation and the induction of apoptosis remains unclear, a role for the Fas-associated death domain (FADD) protein in linking Toll-like receptor signaling to caspase activation has been reported.[26,27] Interestingly, inhibition of new gene expression, either at the mRNA or protein level is required to sensitize EC to LPS-induced caspase activation and apoptosis.[28] The mechanism by which inhibition of new gene expression sensitizes EC to LPS-induced apoptosis is presumed to be through inhibition of *de novo* synthesis of a constitutively expressed cytoprotective protein rather than blockade of an NF-κB-inducible protein.[29] We have shown that inhibition of protein synthesis with Shiga-like toxin sensitizes EC to LPS-induced apoptosis and that sensitization correlates with decreased expression of the anti-apoptotic protein, FLICE-like inhibitory protein (FLIP), a FADD binding protein.[30] Furthermore, we and others have shown a role for FADD in mediating NF-κB activation.[31–34] Based on initial data suggesting a role for FLIP in mediating apoptosis and the role of one of its binding partners, FADD, in mediating NF-κB activation, we investigated whether FLIP could definitely regulate one or both of these EC responses to LPS.

Materials and Methods

Materials

Highly purified LPS, phenol extracted from *Escherichia coli* serotype 0111:B4 and further purified by ion exchange chromatography, and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma Chemical Co., (St. Louis, MO). Cycloheximide (CHX) and staurosporine were obtained from Calbiochem-Novabiochem Corp., (San Diego, CA) and Kamiya Biochemical Co., (Seattle, WA), respectively.

Cell Culture

The human dermal microvascular endothelial cell line (HMEC-1) (developed and generously provided by F.J. Candal and Dr. E. Ades, Centers for Disease Control, and Dr. T. Lawley, Emory University, Atlanta, GA)[35] was cultured in RPMI medium (Cambrex Corp., Walkersville, MD) enriched with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), bovine brain extract (12 μg/ml), L-glutamine (2 mmol/L), sodium pyruvate (1 mmol/L), and nonessential amino acids, in the presence of penicillin (100 U/ml) and streptomycin (100 μg/ml) (all purchased from Cambrex). FLIP +/− and −/− mouse embryo fibroblasts (MEF) (generous gift of Dr. Wen-Chen Yeh, Amgen Institute, Toronto, Canada) were generated as previously described[36] and cultured in DMEM medium (Cambrex) enriched with 10% fetal bovine serum, L-glutamine (2 mmol/L), sodium pyruvate (1 mmol/L), and nonessential amino acids, in the presence of penicillin (100 U/ml) and streptomycin (100 μg/ml).

Caspase Assay

EC were seeded into 96-well plates at a density of 60,000 cells/well, cultured for 24 hours, treated, and caspase activity measured with a fluorimetric homogenous caspases assay according to the manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, IN). The plates were analyzed on a Cytofluor Series 4000 fluorescence plate reader (Perseptive Biosystems Inc., Framingham, MA) at 485 nm excitation and 530 nm emission, and caspase activity expressed relative to simultaneous medium control.

Cloning and Stable Expression of cDNA Constructs

cDNA encoding the long form of human FLIP, $FLIP_L$, (generous gift of Dr. Jurg Tschopp, Institute of Biochemistry of the University of Lausanne, Switzerland) was cloned into the bicistronic retroviral expression plasmid, pBMN-IRES-enhanced green fluorescent protein (EGFP) (kindly provided by Dr. Gary Nolan, Stanford University, Stanford, CA).[37] High-titer retrovirus was prepared from the Phoenix amphotropic packaging cell line (ATCC, Manassas, VA) transfected with 24 μg of the expression plasmid by calcium phosphate precipitation. Recombinant retroviral supernatants were collected 48 hours after transfection and filtered through a Millex-HV 0.45 μmol/L filter (Millipore Corp., Bedford, MA). For infection, 4×10^5 EC or MEF were seeded per well of a 6-well plate for 24 hours to achieve ~80% confluence. The growth medium was replaced with 2.5 ml of retroviral supernatant supplemented with 32 μg/ml polybrene and 10 mmol/L HEPES, and the plate centrifuged for 2 hours (1430 × *g*; 32°C). An additional 2.5 ml of growth medium were added to each well and the cells were then incubated for 8 hours (5% CO_2, 37°C) after which the retroviral-containing supernatant was replaced with normal growth medium. Cells were analyzed and sorted on the basis of EGFP expression using a FACVantage SE cell sorter (Becton Dickinson Corp., Franklin Lakes, NJ).

FLIP Antisense Oligonucleotide Design and Transfection

2′-O-methoxyethyl/2′-deoxynucleotide chimeric antisense oligonucleotides were used in all experiments (gift of Dr. C. F. Bennett, Isis Pharmaceuticals, Carlsbad, CA). Chimeric oligonucleotides were used to support an RNase H-dependent mechanism of action, which results

in a selective loss of target mRNA.[38] All oligonucleotides were synthesized and purified as previously described.[39] The c-FLIP antisense oligonucleotide (ACTTGTCCCTGCTCCTTGAA) was identified by screening a series of different antisense oligonucleotides designed to hybridize to their respective targets using quantitative RT-PCR and Northern blot assays. The control antisense oligonucleotide (TCTAGCCTCTCCTCGTAGTA) contained eight mismatches as compared to FLIP antisense oligonucleotide. For transfection, EC were seeded into 60-mm dishes and grown to ~80% confluence. EC were rinsed twice with Opti-MEM I medium (Life Technologies Inc., Grand Island, NY) followed by incubation with varying concentrations of oligonucleotides premixed with 10 μg/ml Lipofectin reagent (Life Technologies) in Opti-MEM I medium. After a 4-hour incubation at 37°C, the oligonulceotide solution was replaced with normal growth medium and EC allowed an 8-hour recovery period.

Immunoblotting

Cell monolayers were washed once with phosphate-buffered saline (PBS), lysed with ice-cold modified radioimmunoprecipitation assay lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L ethylenediaminetetra-acetic acid, protease inhibitor cocktail tablet (Roche), 1 mmol/L vanadate, 50 mmol/L NaF], scraped, transferred to microcentrifuge tubes, and centrifuged (16,000 $\times$ *g*, 10 minutes, 4°C). Total protein was determined using the BCA protein assay (Pierce Chemical Co., Rockford, IL). The supernatants were combined with 5X sample buffer (Genomic Solutions Inc., Chelmsford, MA), boiled for 3 minutes, and 20 μg of protein/lane were resolved by SDS-PAGE on a 4 to 20% Tris-Glycine gradient gel (Novex Inc., San Diego, CA). Protein was subsequently transferred for 1 hour at 100 v to polyvinylidene fluoride membrane (Millipore). Blots were blocked with 5% dry milk dissolved in Tris-buffered saline (TBS) and subsequently incubated with anti-FLIP (NF6; 1:80 dilution; generous gift of Dr. Peter H. Krammer of the German Cancer Research Center, Heidelberg, Germany)[40] for the detection of human FLIP, anti-FLIP (Dave-2; 1 μg/ml, Kamiya) for the detection of murine FLIP, or anti-IκB-α (1:1000 dilution; Cell Signaling Technology, Inc., Beverly, MA). All antibodies were prepared in TBS containing 0.1% Tween-20 and 5% dry milk and incubated with their respective blots for 1 hour at room temperature. The blots were incubated with 0.13 μg/ml of horseradish-peroxidase-conjugated to either anti-mouse or anti-rabbit IgG (both from BD Biosciences, San Diego, CA), developed with enhanced chemiluminescence (Amersham Life Sciences, Arlington Heights, IL), and exposed to Kodak X-Omat Blue film (NEN Life Sciences, Inc., Boston, MA).

Luciferase Assay

The recombinant adenovirus luciferase reporter construct (gift of Dr. James D. Kelly, Zymogenetics, Inc., Seattle, WA) containing NF-κB consensus binding sites was created as previously described.[31] For transfection of the luciferase reporter construct, HMEC-1 or MEF were seeded into 96-well black view plates (Corning Inc., Corning, NY) at a density of 40,000 or 20,000 cells/well, respectively, for 24 hours and subsequently incubated for 16 hours at a multiplicity of infection (m.o.i.) of 2000 in RPMI supplemented with 5% fetal bovine serum (FBS). Following infection, cells were exposed to experimental treatment in Ham's F12 medium supplemented with 2.5% FBS, 20 mmol/L HEPES, and 0.5% bovine serum albumin (BSA) for 4 hours at 37°C. Luciferase activity was determined using a commercially available assay kit and a TopCount NXT luminescence counter (both from Packard Instrument Co., Meriden, CT).

Phosphoprotein Enzyme-Linked Immunosorbent Assays (ELISA)

Commercially available ELISAs were used in accordance with the manufacturer's instructions to assay for both total and phosphorylated p38 mitogen-activated protein kinase (MAPK) ($pThr_{180}/pTyr_{182}$), Akt ($pSer_{473}$), and c-Jun N-terminal kinase (JNK) ($pThr_{183}/pTyr_{185}$) (Biosource International Inc., Camarillo, CA). For the quantitation of p38 and Akt, EC were grown to confluency in 60-mm dishes, treated, rinsed twice with ice-cold PBS, and lysed with 350 μl of commercially available cell extraction buffer (Biosource) supplemented with a protease inhibitor cocktail tablet (Roche). For the quantitation of JNK, cells were lysed in a buffer containing 6 mol/L urea, 0.5% Triton X-100, and protease inhibitor cocktail. Total protein was determined using the BCA protein assay (Pierce) and lysates were subsequently diluted in lysis buffer to equalize protein concentrations. Lysates assayed for p38 and Akt were further diluted 1:10 in dilution buffer, whereas those assayed for JNK were diluted 1:6. Lysates were then added to the plates and the manufacturer's protocols followed. Plates were read at an optical density of 450 nm on a microplate reader (Bio-Tec Instruments, Inc., Winooski, VT). The relative amounts of each phosphorylated protein assayed were standardized to the total (ie, phosphorylated and unphosphorylated) amount and expressed relative to time 0 levels.

Enzyme-Linked Immunosorbent Assay (ELISA)

MEF were seeded into 96-well plates at a density of 20,000 cells/well and cultured for 24 hours. Following treatment, plates were centrifuged (220 $\times$ *g*, 10 minutes) and the supernatants analyzed using commercially available kits for murine IL-6 (Pierce) or murine KC (R&D Systems, Minneapolis, MN). Supernatants derived from cells exposed to medium alone were diluted 1:2 before analysis for either cytokine, whereas supernatant derived from LPS-treated cells were diluted 1:4 or 1:8 before analysis for IL-6 or KC, respectively. The optical density at 450 nm and a correction wavelength of 550 nm were measured on a microplate reader (Bio-Tec Instruments). Values expressed in pg/ml were extrapolated from a standard curve using linear regression.

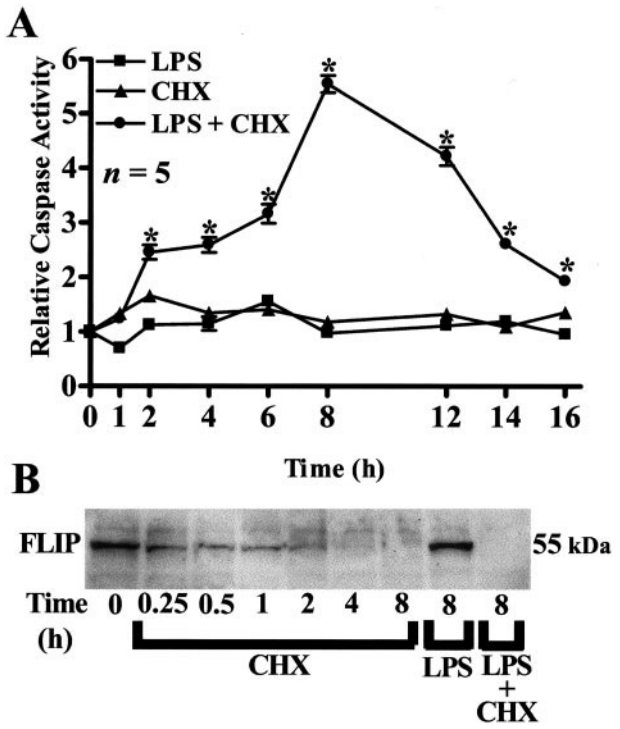

Figure 1. CHX sensitization of EC to LPS-induced apoptosis is temporally coincident with decreased FLIP expression. EC were incubated with medium or LPS (100 ng/ml) in the presence or absence of CHX (40 μg/ml) for increasing exposure times and assayed for caspase activity (**A**). Mean caspase activity (+SE) is reported relative to simultaneous media controls. *, Significantly increased compared to LPS or CHX alone. In other experiments, EC were exposed to CHX alone for increasing periods of time or exposed to LPS in the presence or absence of CHX for 8 hours, lysed and immunoblotted for FLIP expression (**B**). Molecular mass (in kd) is indicated.

Statistical Methods

A *t*-test or analysis of variance (analysis of variance) was used to compare the mean responses between a single experimental group or multiple experimental groups, respectively, and the control group. For experiments analyzed by analysis of variance, the Tukey post-hoc comparison test was used to determine between which groups, significant differences existed. All statistical analyses were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, Inc., San Diego, CA). A P value of < 0.05 was considered significant.

Results

CHX Sensitization of EC to LPS-Induced Apoptosis Is Preceded by CHX-Mediated Decrements in FLIP Expression

Consistent with previous reports,[24] CHX (40 μg/ml) sensitized EC to LPS (100 ng/ml)-induced apoptosis (Figure 1A). Increased caspase activity, a hallmark of apoptosis, initially increased within 2 hours of exposure to LPS+CHX and reached a maximum 6 hours later. There was no detectable increase in EC caspase activity following exposure to either LPS or CHX alone. CHX sensitization of EC to LPS-induced apoptosis was preceded by CHX-mediated decrements in the expression of FLIP (Figure 1B). Within 15 minutes of CHX treatment, decreased levels of FLIP expression were evident relative to untreated (time 0) EC. Within 2 hours of protein synthesis inhibition, a time at which initial increases were observed in caspase activity following LPS+CHX treatment, FLIP expression was almost completely ablated. EC exposed to LPS alone for 8 hours demonstrated no decrements in the expression of FLIP, whereas those exposed to LPS+CHX displayed a complete absence of FLIP expression similar to those EC exposed to CHX alone. Together, these data establish that CHX-mediated decrements in FLIP expression are temporally coincident with CHX-mediated sensitization to LPS-induced caspase activity.

Overexpression of FLIP Protects against CHX-Induced Sensitization of EC to LPS-Induced Apoptosis

Since CHX-mediated decreases in FLIP expression correlate with LPS+CHX-induced apoptosis, we hypothesized that increasing pre-existing EC levels of FLIP would protect against CHX sensitization to LPS-induced apoptosis. Using a retroviral infection system, the long form of FLIP, which is the predominant form expressed in EC,[41] was stably overexpressed in HMEC. Overexpression of FLIP was confirmed by Western blot analysis (data not shown). Relative to EC expressing vector alone, EC overexpressing FLIP demonstrated ~50% less caspase activity following LPS+CHX treatment (Figure 2A). To determine whether FLIP overexpression could non-specifically disrupt apoptosis evoked by other agonists, EC were exposed to staurosporine for 8 hours and assayed for caspase activity. In contrast to LPS, overexpression of FLIP had no effect on the ability of staurosporine to induce apoptosis.

Targeted Down-Regulation of FLIP Using Antisense Oligonucleotides Sensitizes EC to LPS-Induced Apoptosis

Since FLIP has a short half-life and its expression decreases rapidly in the presence of CHX, and that overexpression of FLIP protects against LPS+CHX-induced apoptosis, we postulated that decreasing the expression of FLIP would sensitize EC to LPS-induced apoptosis in the absence of global protein synthesis inhibition. To test this hypothesis, antisense oligonulceotides were designed to specifically reduce the expression of FLIP. Western blot analysis of EC transfected with FLIP antisense revealed a marked decrease in the expression of FLIP compared to EC transfected with a mismatch control oligonucleotide (Figure 2B). In subsequent experiments, EC transfected with either mismatch control or FLIP antisense were treated with media or LPS (100 ng/ml) for 8 hours and assayed for caspase activity (Figure 2C). EC transfected with FLIP antisense demonstrated significantly higher levels of caspase activity following expo-

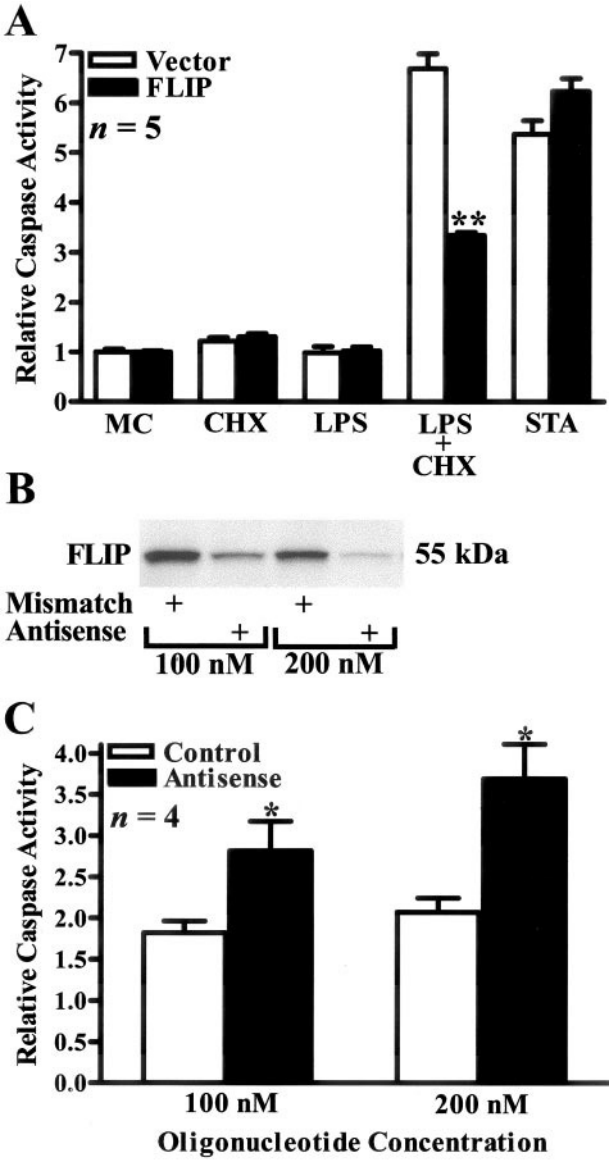

Figure 2. Changes in the expression of FLIP influence EC sensitivity to LPS-induced apoptosis. EC stably transfected with either vector alone or cDNA encoding FLIP were treated with medium (MC), CHX (40 μg/ml), LPS (100 ng/ml), LPS in combination with CHX, or staurosporine (STA) (50 nmol/L) for 8 hours and subsequently assayed for caspase activity (**A**). Mean (±SE) caspase activity is reported relative to simultaneous media controls. **, Significantly decreased relative to identically treated vector control EC. In other experiments, EC were exposed to 100 or 200 nmol/L of either FLIP antisense or mismatch control oligonucleotide, lysed, then immunoblotted with anti-FLIP antibody (**B**). In subsequent studies, EC transfected with oligonucleotides as above were exposed to medium or LPS (100 ng/ml) for 8 hours and assayed for caspase activity (**C**). *, Significantly increased compared to simultaneous mismatch oligonucleotide control-transfected EC.

sure to LPS than similarly treated EC transfected with mismatch control antisense.

Overexpression of FLIP Inhibits LPS-Induced NF-κB Activity in EC

FLIP has previously been shown to interact with other proteins that can influence NF-κB activation.[31,32] To determine whether FLIP could similarly mediate LPS-induced activation of NF-κB, EC stably transfected with the full-length long form of FLIP were assayed for inducible NF-κB dependent luciferase activity (Figure 3A). EC overexpressing FLIP displayed ~50% less NF-κB activity following LPS stimulation than EC transfected with vector alone. In contrast to LPS, overexpression of FLIP had no effect on the ability of PMA to induce NF-κB activation (Figure 3A).

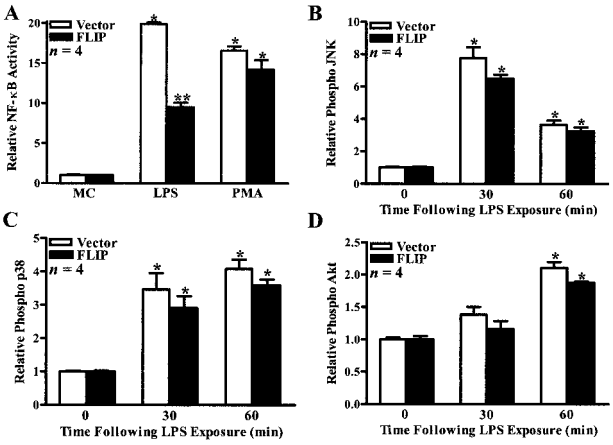

Figure 3. Overexpression of FLIP inhibits LPS-induced NF-κB-activation. EC stably transfected with either vector alone or FLIP were treated for 4.5 hours with medium (MC), LPS (100 ng/ml), or PMA (10 ng/ml), and assayed for NF-κB-dependent luciferase activity (**A**). **Vertical bars** represent mean (±SE) luciferase activity relative to simultaneous media controls. In other studies, transfected EC were treated with LPS (100 ng/ml) for varying time points and assayed by ELISA for cellular levels of phosphorylated JNK (**B**), p38 (**C**), and Akt (**D**). **Vertical bars** represent mean (±SE) phosphorylation relative to simultaneous media controls. *, Significantly increased relative to EC exposed to media alone. **, Significantly decreased compared to vector alone transfected EC exposed to identical treatment.

LPS is a well-known activator of other EC signaling pathways including those involving JNK,[42] p38,[43] and Akt.[44] To determine whether FLIP could similarly influence the ability of LPS to activate these pathways, the phosphorylation state of these proteins was assessed by ELISA. Within 30 minutes of and for up to 60 minutes following LPS (100 ng/ml) treatment, increases in the phosphorylation of JNK (Figure 3B) and p38 (Figure 3C) relative to time 0 were observed in both control and FLIP overexpressing EC. Within 60 minutes of exposure to LPS, increases in the phosphorylation state of Akt were detected (Figure 3D). EC overexpressing FLIP or vector alone demonstrated equivalent changes in the levels of phosphorylation of all three signaling proteins following LPS exposure.

Absence of FLIP Enhances LPS-Induced NF-κB Activity and NF-κB-Dependent Cytokine Production in MEF

Since FLIP overexpression suppresses LPS-induced NF-κB activation, it was predicted that decreased expression of FLIP would enhance NF-κB signaling. To test this prediction, FLIP +/− or −/− MEF were obtained,[36] and their phenotype confirmed by Western blotting with an anti-FLIP antibody that cross-reacts with both human and mouse FLIP (Figure 4A). FLIP +/− or −/− MEF were

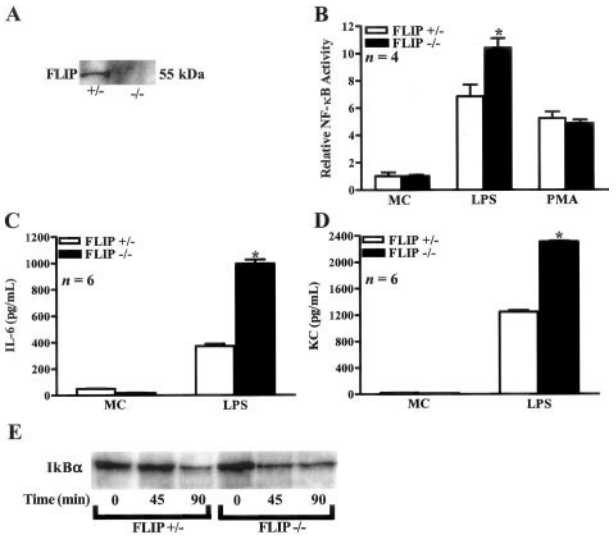

Figure 4. Loss of FLIP enhances LPS-induced NF-κB activity. FLIP +/− and −/− MEF lysates were immunoblotted with anti-FLIP antibody to confirm the genetic phenotype of these cells (**A**). Molecular mass (in kd) is indicated. In other experiments, FLIP +/− and −/− MEF were treated for 4.5 hours with either medium (MC), LPS (100 ng/ml), or PMA (10 ng/ml), lysed, and assayed for NF-κB-dependent luciferase activity (**B**). **Vertical bars** represent mean (±SE) luciferase activity in arbitrary units relative to simultaneous media controls. Alternatively, MEF were treated for 16 hours and the culture supernatants analyzed for IL-6 (**C**) or KC (**D**). **Vertical bars** represent mean (±SE) cytokine production in pg/ml (**C** and **D**). *, Significantly increased compared to FLIP +/− MEF exposed to the same treatment. In subsequent studies, FLIP +/− and −/− MEF were treated for increasing exposures to LPS (100 ng/ml), lysed, and immunoblotted for IκBα (**E**).

subsequently exposed to LPS or PMA for 4 hours and assayed for NF-κB-dependent luciferase activity (Figure 4B). FLIP −/− MEF displayed significantly enhanced NF-κB activation relative to FLIP +/− MEF following LPS exposure. That loss of FLIP enhances NF-κB activation is consistent with the finding that overexpression of FLIP represses NF-κB activation induced by LPS (Figure 3A). In contrast to stimulation with LPS, FLIP +/− and −/− MEF demonstrated equivalent NF-κB activation following exposure to PMA (Figure 4B).

To determine whether FLIP could similarly down-regulate expression of naturally expressed endogenous NF-κB dependent gene products, FLIP +/− and −/− MEF were assayed for IL-6 and KC production. Similar to luciferase activity, IL-6 (Figure 4C) and KC (Figure 4D) production were both enhanced in LPS-treated FLIP −/− MEF compared with similarly treated FLIP +/− MEF. Together, these data demonstrate that NF-κB-dependent gene expression is enhanced in the absence of FLIP.

Absence of FLIP Enhances LPS-Induced IκB Degradation

IκB degradation is a prerequisite step in the LPS-induced activation of NF-κB and the expression of NF-κB-regulated gene products.[29] To determine whether FLIP can exert its down-regulatory effect on NF-κB activation upstream or downstream of IκB degradation, FLIP +/− and −/− MEF were stimulated with LPS and assayed for IκB degradation (Figure 4E). Relative to the FLIP +/− MEF, the FLIP −/− MEF demonstrated enhanced degradation of IκB following a 45-minute exposure to LPS. Within 90 minutes of treatment, equivalent degradation was evident in both cell types. These data suggest that FLIP negatively regulates NF-κB upstream of IκB degradation.

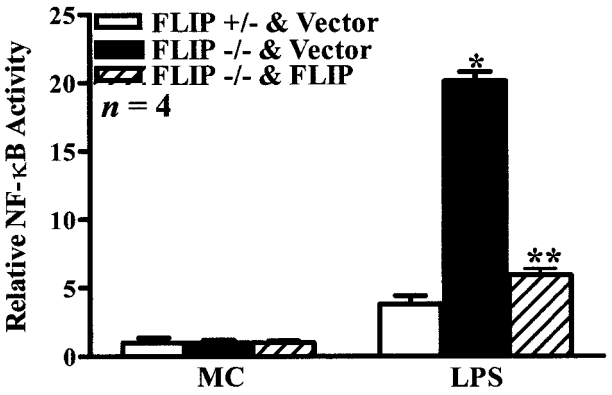

Figure 5. Reconstitution of FLIP reverses the enhanced NF-κB activation in FLIP −/− MEF exposed to LPS. FLIP +/− or −/− MEF stably transfected with either vector alone or FLIP were treated for 4.5 hours with medium (MC) or LPS (100 ng/ml), lysed, and assayed for NF-κB-dependent luciferase activity. **Vertical bars** represent mean (±SE) luciferase activity in arbitrary units. *, Significantly increased compared to vector alone transfected FLIP +/− MEF exposed to the same treatment. **, Significantly decreased compared to vector alone transfected FLIP −/− MEF cells exposed to the same treatment.

Reconstitution of FLIP Reverses the Enhanced NF-κB Activation in FLIP −/− MEF Stimulated with LPS

To confirm that the enhanced NF-κB activity in LPS-treated FLIP −/− MEF could be ascribed to the loss of FLIP and not to a developmental anomaly unique to the mice from which the MEF were derived, vector alone or vector encoding the full-length FLIP cDNA was retrovirally-transduced into FLIP +/− or −/− MEF, and NF-κB activation assayed. Western blot analysis confirmed expression of FLIP in the FLIP −/− MEF transfected with FLIP cDNA (data not shown). Similar to the non-transduced MEF, FLIP −/− MEF expressing vector alone demonstrated higher NF-κB activity following exposure to LPS than similarly treated FLIP +/− MEF expressing vector alone (Figure 5). Reconstitution of FLIP in the FLIP −/− MEF completely reversed the enhancement in NF-κB activation following LPS exposure.

Discussion

In the present report, we have shown that changes in the expression of FLIP alter EC sensitivity to LPS-induced apoptosis. In the absence of *de novo* protein synthesis, EC were sensitized to LPS-induced apoptosis in a time-dependent manner (Figure 1). The sensitization to LPS-induced apoptosis by CHX-mediated inhibition of new gene expression is not unique to CHX, as others have reported that actinomycin D, an mRNA synthesis inhibitor, confers sensitization.[28] Of greater patho-physiologi-

cal relevance, protein synthesis inhibition by Shiga-like toxins has been similarly shown to sensitize EC to LPS-induced apoptosis.[30] Thus, a common denominator to sensitization of EC to LPS-induced apoptosis is the inhibition of new gene expression.

CHX-mediated sensitization of EC to LPS-induced apoptosis was temporally coincident with decreases in the anti-apoptotic protein, FLIP (Figure 1B). FLIP is an anti-apoptotic protein with significant homology to caspase-8, however, a substitution of two amino acids in the region of FLIP that corresponds to the catalytic active site of caspase-8 renders it incapable of proteolysis.[45] The role of FLIP in mediating apoptotic signaling by the classic death receptor Fas has been well described.[46,47] On ligand binding to its cognate death receptor, the adapter protein FADD is recruited to the Fas receptor via homophilic interactions of the death domains (DD) contained within each protein. FADD, is an adapter protein that can recruit FLIP and pro-caspase-8 to the death receptor complex via protein-protein interactions of death effector domains (DED) contained within all three proteins. Pro-caspase-8, which has intrinsically low levels of proteolytic activity, is only activated when other pro-caspase-8 molecules are brought into close proximity, whereby neighboring pro-caspase-8 molecules may transactivate one another leading to the formation of fully active caspase-8 molecules and the onset of apoptosis.[48] FLIP, which lacks intrinsic proteolytic activity, functions as an endogenous dominant-negative protein to block pro-caspase-8 activation.[46] Thus, the relative ratios of pro-caspase-8 and FLIP can determine whether a cell undergoes apoptosis in response to a caspase-8-dependent pro-apoptotic stimulus.

In the present study, we provide evidence demonstrating a definitive role for FLIP in suppressing LPS-induced apoptosis. Overexpression of FLIP protected against CHX-mediated sensitization to LPS-induced apoptosis (Figure 2A). This protection was not the result of non-specific inhibition, as FLIP overexpressing EC remained sensitive to staurosporine-induced apoptosis. Further, specific down-regulation of FLIP with antisense oligonucleotides sensitized EC to direct LPS killing (Figure 2C). The present data are the first to demonstrate that decreasing the basal levels of FLIP directly sensitizes EC to the direct apoptotic effects of LPS in the absence of other non-host-derived mediators.

In addition to establishing a role for FLIP in conferring resistance to LPS-induced apoptosis, the data presented here suggest an additional role for FLIP in down-regulating LPS-induced NF-κB activation. Overexpression of FLIP significantly impaired the ability of LPS to induce NF-κB (Figure 3A). Conversely, in cells deficient for FLIP, LPS-induced NF-κB activity was enhanced relative to FLIP expressing cells (Figure 4B). In addition, LPS-elicited production of IL-6 and KC, two endogenously expressed LPS-inducible gene products whose expression is dependent on NF-κB activation,[49,50] was enhanced in the FLIP $-/-$ MEF relative to the FLIP $+/-$ MEF (Figure 4, C and D). This latter finding rules out that the observations with the NF-κB luciferase reporter assay could be attributed to FLIP interference with either the exogenous gene product used to assay for NF-κB activity, luciferase, or the adenoviral infection process used to transfect the reporter construct.

NF-κB activation is often associated with a predominantly anti-apoptotic role through its ability to up-regulate cytoprotective gene products such as cellular inhibitors of apoptosis,[51] however, NF-κB has been reported to promote apoptosis as well.[52] Since overexpression of FLIP protects against LPS-induced apoptosis and inhibits NF-κB activation, one may postulate that the protection conferred against LPS-induced apoptosis is mediated by FLIP's ability to down-regulate NF-κB activation. However, we have previously shown that EC overexpressing an IκBα construct containing mutations that prevent its degradation and that functions as a super-repressor of NF-κB, are equally sensitive to LPS+CHX-induced apoptosis as those EC with normal NF-κB activity.[24] Further, since the presumed mechanism of the pro-apoptotic properties of NF-κB is through the promotion of new gene expression and that LPS evokes EC apoptosis in the absence of new gene expression, it is unlikely that the down-regulation of NF-κB by FLIP is responsible for its cytoprotective properties against LPS.

Because NF-κB promotes the expression of gene products that are anti-apoptotic, the finding that FLIP both directly protects against LPS-induced apoptosis and down-regulates NF-κB may appear contradictory. However, there is evidence to suggest that the anti-apoptotic effect conferred by NF-κB is limited to certain agonists such as TNF-α and that NF-κB has no influence on LPS-induced EC apoptosis. In a previous study, specific blockade of NF-κB activation was reported to sensitize EC to TNF-α-, but not to LPS-induced apoptosis, suggesting the lack of an anti-apoptotic role for NF-κB in moderating EC responses to LPS.[29] Thus, FLIP appears to play distinct and non-conflicting roles in mediating EC responses to LPS that include both the conferring of resistance to LPS-induced apoptosis and the down-regulating of LPS-induced NF-κB activation.

The mechanism by which FLIP inhibits NF-κB activation remains to be elucidated. There are reports that FLIP, via its death effector domains, interacts with NIK and IKK.[32,33,53,54] Since these molecules are involved in the LPS/Tlr-4 induction of NF-κB activation,[55,56] FLIP binding and/or sequestering of NIK and IKK could restrict their ability to promote NF-κB signaling. Another possible mechanism by which FLIP may down-regulate inducible NF-κB activity is via its interaction with FADD. We have previously shown that similar to FLIP, FADD inhibits LPS-induced NF-κB activation.[31] The interaction of FLIP and FADD is mediated by homophilic interactions of the death effector domains (DED) contained within each molecule.[47] FADD, in turn, has been shown to bind to MyD88, an upstream adapter protein involved in the LPS activation of NF-κB, via homophilic interactions of the death domains (DD) contained within each molecule.[26,57] Thus FLIP, via recruitment of FADD, may indirectly sequester MyD88. In addition, members of the IRAK family which also contain DD's and interact with MyD88 via DD-DD interactions may also possibly bind FADD and, thus, be indirectly sequestered by FLIP. The finding that IκB deg-

radation is enhanced in the FLIP −/− MEF (Figure 4E) suggests that FLIP exerts its inhibitory effect upstream of IκB and is consistent with these proposed mechanisms of FLIP inhibition. Future experiments will be needed to address the exact mechanism by which FLIP can down-regulate LPS-induced NF-κB activation.

In summary, using overexpression and anti-sense strategies to modulate the cellular levels of FLIP, we have established a role for FLIP in protecting against LPS-induced apoptosis. In addition, we have established that FLIP down-regulates LPS-induced NF-κB activation. Whereas stable overexpression of FLIP inhibits LPS-induced activation of NF-κB, the absence of FLIP enhances LPS-induced NF-κB activation. The increased NF-κB activity in FLIP-deficient MEF corresponded with enhanced IκB degradation, suggesting that FLIP negatively regulates NF-κB activation upstream of IκB. Although FLIP could down-regulate LPS-induced NF-κB activation, FLIP overexpression had no effect on other LPS-inducible signaling pathways, including those involving JNK, Akt, and p38. The dual role of FLIP in protecting against LPS-induced apoptosis and down-regulating NF-κB activation and the accompanying up-regulation of pro-inflammatory gene products may be of particular significance in EC during gram-negative sepsis. By lining the vascular surface, EC are one of the first host cell types exposed to circulating LPS.[11] Thus, a mechanism for protecting against EC injury induced by LPS, such as by sustained expression of high levels of FLIP, would be clearly advantageous. LPS is also a potent activator of EC and several of the pro-inflammatory EC responses to LPS, including cytokine production and adhesion molecule expression, are mediated by NF-κB.[12] Thus, expression of FLIP and its corresponding ability to down-regulate NF-κB activation and moderate the exuberant and deleterious inflammatory response that accompanies sepsis could be beneficial as well.

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