ERK1/2 Has Divergent Roles in LPS-Induced Microvascular Endothelial Cell Cytokine Production and Permeability

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ERK1/2 has divergent roles in LPS-induced microvascular endothelial cell cytokine production and permeability

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Abstract

Endothelial cells play a major role in inflammatory responses to infection and sterile injury. Endothelial cells express Toll-like receptor 4 (TLR4) and are activated by LPS to express inflammatory cytokines/chemokines, and to undergo functional changes, including increased permeability. The extracellular signal-regulated kinase 1/2 (ERK1/2) mediates pro-inflammatory signaling in monocytes and macrophages, but the role of ERK1/2 in LPS-induced activation of microvascular endothelial cells has not been defined. We therefore studied the role of ERK1/2 in LPS-induced inflammatory activation and permeability of primary human lung microvascular endothelial cells (HMVEC). Inhibition of ERK1/2 augmented LPS-induced IL-6 and vascular cell adhesion protein (VCAM-1) production by HMVEC. ERK1/2 siRNA knockdown also augmented IL-6 production by LPS-treated HMVEC. Conversely, ERK1/2 inhibition abrogated permeability and restored cell-cell junctions of LPS-treated HMVEC. Consistent with the previously described pro-inflammatory role for ERK1/2 in leukocytes, inhibition of ERK1/2 reduced LPS-induced cytokine/chemokine production by primary human monocytes. Our study identifies a complex role for ERK1/2 in TLR4-activation of HMVEC, independent of myeloid differentiation primary response gene (MyD88) and TIR domain-containing adaptor inducing IFN-β (TRIF) signaling pathways. The activation of ERK1/2 limits LPS-induced IL-6 production by HMVEC, while at the same time promoting HMVEC permeability. Conversely, ERK1/2 activation promotes IL-6 production by human monocytes. Our results suggest that ERK1/2 may play an important role in the nuanced regulation of endothelial cell inflammation and vascular permeability in sepsis and injury.

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AUTHOR CONTRIBUTIONS

EW performed all *in* vitro and all *in vivo* experiments, analyzed data and wrote the first draft of the manuscript. JH conceptualized and helped to design the study and contributed to drafting, and critical revision of the manuscript. FX contributed to design of ECIS experiments. JJ and KW contributed to experimental design and edited the manuscript.

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Keywords

LPS; TLR4; acute inflammation; endothelial activation; NFκB

INTRODUCTION

The average human adult is estimated to contain in excess of 1 trillion endothelial cells (2, 7, 24, 53) as compared to 20–50 billion peripheral blood mononuclear cells and 1–4 billion circulating monocytes (40). Endothelial cells line the vast network of blood and lymphatic vessels of all tissues, and they dynamically regulate inflammation, leukocyte trafficking, coagulation, and the vascular barrier (4, 13, 22, 26, 38). Sepsis and tissue injury cause endothelial cell activation at the site of injury, as well as at remote sites. During these acute inflammatory processes, dysfunction of microvascular endothelial cells leads to vascular leak and coagulopathy, which contribute to shock, organ failure, and reduced survival (28, 31).

Endothelial cells have not traditionally been viewed as immune cells, but they are key players in the host's immune responses in sepsis (9, 31, 55, 57). Endothelial cells express innate immune receptors, including Toll-like receptors (TLRs), NOD-like receptors and RIG-I like receptors (28). Furthermore, the direct activation of endothelial TLRs by damage- and pathogen-associated molecular proteins (DAMPs and PAMPs, respectively) increases the expression of cytokines, chemokines and adhesion molecules, promotes neutrophil-endothelial adhesion, and induces endothelial permeability (28).

TLR-dependent signaling is mediated by nuclear factor κB (NFκB) and the family of mitogen-activated protein kinases (MAPKs), including p38-MAPK, JNK, ERK1/2 and ERK5 (12, 28, 50, 51). Notably, while endothelial cells and leukocytes express TLRs and share many of the same intracellular signaling intermediaries, there are differences in inflammatory signaling between endothelial cells and leukocytes. For example, direct activation of endothelial or monocyte TLR2 or TLR4 strongly upregulates IL-6 and IL-8 production. In contrast, activation of endothelial cell TLR2 and TLR4 does not upregulate IL-1β and TNFα production, whereas these cytokines are strongly upregulated in monocytes and macrophages activated with TLR2 and TLR4 agonists (23, 37, 52). While mapping out endothelial TLR2 signaling pathways, we observed that in human umbilical vein endothelial cells (HUVEC), inhibition of MEK1, the upstream kinase of ERK1/2, augmented TLR2-induced production of IL-6, but not IL-8 (51). This effect of MEK1 inhibition on TLR2-dependent activation of HUVEC was different from the pro-inflammatory role that MEK1 is known to play in leukocytes (51). Our results suggested to us that ERK1/2 activation plays an important role in regulating TLR-dependent activation of endothelial cells.

In the current report, we tested the hypothesis that ERK1/2 activation negatively regulates TLR4-dependent activation of primary human lung microvascular endothelial cells (HMVEC). We assessed the role of ERK1/2 in LPS-induced cytokine/chemokine production, permeability, and expression of endothelial tight junction proteins.

MATERIALS AND METHODS

Primary human endothelial cells and monocytes

HMVEC (Promocell) from male and female cadavers were cultured in endothelial growth media (EGM-2 Basal Medium, Lonza) supplemented with Microvascular Endothelial Cell Growth Medium supplements (EGM-2 MV, Lonza) as described (51). Human CD14+ monocytes from healthy male and female donors (Lonza) were thawed and cultured in RPMI supplemented with 10% heat-inactivated fetal calf serum (FCS), L-glutamine, and antibiotics and used immediately for experiments. Cells were incubated at 37°C under humidified 5% CO_2.

Inflammatory agonist treatment

HMVEC were added to 48-well plates (3×10^5 cells/well) and grown to confluence. Human monocytes were added to 48-well plates (2×10^5 cells/well) and used immediately. HMVEC and monocytes were pre-incubated for 1 hour with vehicle (0.01% DMSO) or ERK1/2 inhibitor (SCH772984, 1μM, SelleckChem). Then ultrapure LPS (0.01 μg/ml, List Laboratories) was added to the wells in the continued presence or absence of ERK1/2 inhibitor (1μM). Cells were cultured for another 6 hours and supernatants were collected. Cytokines were quantified in culture supernatants of using Duoset ELISA kits (R&D systems).

Lipopolysaccharide (LPS)

We utilized ultrapure LPS from *E. coli* O111:B4 bacteria (Ultrapure LPS, List Laboratories, Lot 4219A1) for all experiments. The LPS was tested for purity by colloidal gold staining, according to manufacturer's instructions (Bio-Rad), and by immunoblotting for three outer membrane proteins (murein lipoprotein [MLP], peptidoglycan-associated lipoprotein [PAL], and outer membrane protein A [OmpA]), which are common contaminants of purified LPS (16, 17). The LPS contained no protein bands by gold staining, which can detect levels as low as 1 ng of protein (Supplemental Figure S1A). Similarly, none of the outer membrane proteins were detected in the LPS by immunoblotting (Figure S1B–D) (22, 23). Lysates of whole *E. coli* O111:B4 bacteria, which contain multiple proteins including MLP, PAL and OmpA, served as positive controls for gold stains and immunoblots.

Immunoblots

Immunoblots were performed as previously described (51). Primary antibodies used were p44/42-MAPK (ERK1/2; 1:1000; 4695; Cell Signaling), actin (0.1 mg/ml, A2066, Sigma), phospho-NF-κB p65 (1:1000; 3033; Cell Signaling), anti-murein lipoprotein (MLP), peptidoglycan-associated lipoprotein (PAL), and outer membrane protein A (OmpA). MLP, PAL, and OmpA antibodies were made as previously described and used at concentration of 1 μg/ml (17). Total protein concentrations of cell lysates were measured using the RCDC protein assay kit (Bio-Rad). Samples were separated by SDS-PAGE, transferred to PVDF membrane (Pall Corp), and blocked with 3% BSA in TBST (1 hour, room temperature). They were then incubated overnight at 4°C with primary antibodies, washed and incubated with suitable secondary antibodies conjugated to peroxidase (Jackson ImmunoResearch) and

developed with SuperSignal West Dura Extended Duration Substrate (Thermo Scientific). Signal was detected with ChemiDocMP Imaging System (BioRad). Target protein expression was normalized using densitometry software to quantify band intensity with Image Lab Software (BioRad).

Transfection of HMVEC with siRNAs

ERK1-specific (L-003592-00), ERK2-specific (L-003555-00), or non-targeting (D-001810-10) ON-TARGETplus SMARTpool siRNA (Dharmacon) were used following the manufacturer's instructions for transfecting HMVECs with DharmaFECT 4 Transfection Reagent (T-2004-01). We placed siRNA (5 μM) in serum-free medium, and diluted DharmaFECT transfection reagent in a separate tube, incubated both tubes for 5 minutes, and then combined the contents of both tubes and incubated the reagents together for another 20 minutes. HMVEC were grown to 80–90% confluence in a T-75 flask, and then the medium was replaced with antibiotic- and serum-free medium containing the transfection reaction. After 24 hours the transfection reagent media was removed, and cells were then treated with LPS (1 μg/ml) in complete media for 24 hours, and cytokines were quantified in culture supernatants.

Electric Cell-substrate Impedance Sensing (ECIS)

Transendothelial resistance (TER) was measured using the ECISZeta (Applied Biophysics) (44). HMVEC were placed in 96-well electrodes (40,000 cells/well) and allowed to adhere. Resistance (ohm), impedance (ohm), and capacitance (nanofarad) were measured at frequent intervals, using a frequency of 4000 Hz, which was determined to be the optimal frequency for endothelial cells. When cells reached a stable level of resistance, LPS (1 μg/ml) in the presence of ERK1/2 inhibitor (1μM), MyD88 inhibitor (10μM, Aobious), TRIF inhibitor (10μM, Novus), or vehicle was added to the wells, and TER was measured at frequent intervals through 15 hours. To account for differences in resistance of each well, data was normalized to the resistance of the individual well immediately before the addition of the agonist.

Immunofluorescence microscopy

HMVEC were grown to confluence on Lab Tek II chamber slides (Nunc) that had been pre-coated with collagen, and then treated for 2, 6, and 24 hours with LPS (1 μg/ml) in the presence of ERK1/2 inhibitor or vehicle. Cells were then fixed with 4% paraformaldehyde (15 minutes, 37°C) and permeabilized with 0.5% Triton X-100 in PBS (15 minutes, room temperature). After blocking (1% BSA in PBS, 30 minutes, room temperature), cells were incubated with either anti-ZO1-AlexaFluor594 (Invitrogen, 24 hours, 4°C), or anti-VE-cadherin (Santa Cruz Biotechnologies) (24 hours, 4°C) followed by AlexaFluor488-tagged secondary antibody (60 minutes, room temperature). Nuclei were counterstained with 4'6-diamidino-2-phenylindole (DAPI). Slides were visualized using fluorescent microscopy (Zeiss AxioImager D1), and images were obtained with 40X objective using AxioVision SE64 Rel 4.9.1 Software.

Statistics

Data was analyzed using GraphPad Prism version 7.00 (GraphPad Software, Inc). Results are expressed as mean ± SD. With the exception of ECIS, results were analyzed using non-parametric-based biostatistics. Mann-Whitney tests were used to compare 2 groups. $P < 0.05$ was considered to be statistically significant. Data from ECIS experiments were analyzed by quantifying area under the curve from baseline = 1 with addition of negative peaks and graphed as means ± SD. One-way analysis of variance (ANOVA) followed by the Sidak's multiple comparison was then used to establish significance for multiple comparisons. $P < 0.05$ was considered to be statistically significant. Experiments were repeated at least twice.

RESULTS

ERK1/2 inhibitor augments LPS-induced IL-6 and VCAM-1 production, but not IL-8 or PAI-1 by HMVEC.

HMVEC were pre-incubated for 1 hour with vehicle or ERK1/2 inhibitor (SCH772984; 1 μM), and then LPS (10 ng/ml) was added in the continued presence of ERK1/2 inhibitor or vehicle. At 6 hours, levels of IL-6 and VCAM-1 were significantly higher in the supernatants of LPS-activated HMVEC treated with the ERK1/2 inhibitor (Figure 1A and 1B). In contrast, the inhibitor had no effect on the LPS-induced upregulation of IL-8 and plasminogen activator inhibitor-1 (PAI-1) in culture supernatants (Figure 1C and 1D).

Effects of siRNA knockdown of Erk1 and Erk2 in HMVEC on LPS-induced IL-6 production.

We performed siRNA knockdown to define the individual roles of the ERK1 and ERK2 in LPS-induced activation of HMVEC. Immunoblots confirmed successful knockdown of each protein in HMVEC (Figure 2A). We observed that HMVEC that had undergone siRNA knockdown of ERK2 had significantly augmented LPS-induced IL-6 production as compared with HMVEC transfected with non-targeted siRNA. In contrast, there were not significant differences in LPS-induced cytokine production between HMVEC with ERK1 siRNA knockdown and control cells. However, combined treatment with ERK1 and ERK2 siRNAs further augmented LPS-induced IL-6 production in HMVEC as compared with individual knockdown of either protein (Figure 2B).

ERK1/2 inhibition decreases LPS-induced NFκB activation.

We assessed for NFκB activation HMVEC by immunoblotting cell lysates for phosphorylated NFκB. Treatment with LPS increased phosphorylated NFκB within 30 minutes. The presence of ERK1/2 inhibitor suppressed the LPS-induced phosphorylation of NFκB (Figure 3A–3B).

Effects of ERK1/2 inhibitor on LPS-induced activation of primary human monocytes.

We tested the effects of ERK1/2 inhibition on primary human monocytes from healthy male and female donors in order to confirm that we were able to reproduce results of prior studies supporting a pro-inflammatory role for ERK1/2 in human monocytes. Monocytes were pre-treated with vehicle or with ERK1/2 inhibitor (1 μM), and then stimulated with LPS (0.01 μg/ml) for 6 hours. Consistent with previous studies showing that ERK1/2 plays a pro-

inflammatory role in monocytes, the inhibition of ERK1/2 led to decreased IL-6, TNFα, IL-8, and IL1β levels in culture supernatants of LPS-treated monocytes (Figure 4A–4D).

ERK1/2 inhibitor promotes stability of LPS-treated HMVEC monolayers.

Because ERK1/2 inhibition augments LPS-induced IL-6 production by HMVEC, we hypothesized that treatment with ERK1/2 inhibitor would exacerbate LPS-induced loosening of endothelial tight junctions and increase permeability. We treated HMVEC with LPS (1 μg/ml) in the presence and absence of ERK1/2 inhibitor, and quantified TER as a surrogate for permeability (44). Contrary to our hypothesis, treatment with ERK1/2 inhibitor abrogated LPS-induced TER (Figure 5A–5B). This suggests that ERK1/2 activation contributes to TLR4-dependent endothelial permeability. To further study the role of ERK1/2 in LPS-induced endothelial permeability, we performed fluorescence microscopy at 2, 6 and 24 hours to visualize cell-cell adhesion and tight junction proteins, including zonula occludens (ZO-1) and vascular endothelial (VE)-cadherin. Representative images following a 6-hour stimulation are shown in Figure 5B. At 6 hours, but not at 2 or 24 hours, we observed that ERK1/2 inhibitor reduced LPS-induced breakdown of cell-cell junctions and interendothelial gaps (white arrows; Figure 5C).

To begin to define the signaling pathways responsible for the disconnect in the apparent role of ERK1/2 in LPS-induced IL-6 production versus permeability, we investigated the roles of MyD88 and TRIF, two proximal adaptor proteins responsible for activation of a number of intermediary signaling molecules (10, 45). In the presence of the MyD88 inhibitor or TRIF inhibitor, TLR4-dependent IL-6 production and permeability were unaffected (S2A–S2F).

DISCUSSION

Our study identifies a novel and complex role for ERK1/2 in TLR4-dependent activation of HMVEC which differs substantially from the pro-inflammatory role of ERK1/2 in TLR4-dependent activation of leukocytes (14, 49). Furthermore, despite our finding the ERK1/2 inhibitor augments LPS-induced IL-6 production by HMVEC, we have found that the ERK1/2 inhibitor reduces LPS-induced permeability of HMVEC. This points to divergent roles of ERK1/2 in endothelial inflammatory activation and vascular leak.

The vascular endothelium plays a substantial role in the innate immune response, and regulates systemic inflammation, coagulation and vascular permeability. During sepsis, the upregulation of a number of biomarkers correlate with worse prognosis and increased organ dysfunction, including, but not limited to IL-6, IL-8, VCAM-1 and PAI-1 (5, 18, 33, 34, 41). Our data show that ERK1/2 inhibition augments upregulation of IL-6 and VCAM-1, but not IL-8 or the anti-fibrinolytic coagulation intermediary, PAI-1. This suggests that despite having a role in reducing TLR4-dependent IL-6 and adhesion molecule expression of VCAM-1 by LPS-activated endothelial cells, ERK1/2 may not play a predominant role in TLR4-dependent coagulopathy in HMVEC. This is contrary to the pro-inflammatory role of ERK1/2 in TLR4-dependent activation of leukocytes that we confirmed in LPS-activated primary human monocytes with reduced production of IL-6, TNFα, IL-8 and IL1β in the presence of the ERK1/2 inhibitor.

Because NF-κB has previously been shown to mediate LPS-induced cytokine production and adhesion molecule expression by endothelial cells (6, 11, 42, 47), we hypothesized that ERK1/2 might restrain inflammatory activation of HMVEC by modulating NF-κB activation. However, we observed that ERK1/2 inhibition reduced LPS-induced activation of NF-κB in HMVEC, despite the augmented production of IL-6 and VCAM-1. This finding has led us to speculate that in endothelial cells, non-NF-κB pathways may mediate LPS-induced upregulation of IL-6 and VCAM-1.

The differences in the role of ERK1/2 in inflammatory activation of endothelial cells and leukocytes could potentially be exploited therapeutically in patients with sepsis and other inflammatory processes that are driven by TLR4 activation, such as ischemia reperfusion injury (36). As endothelial activation and dysfunction are believed to contribute to the development of organ injury and failure in sepsis (5, 21, 34, 43), it is conceivable that augmentation of the ERK1/2 activity specifically in endothelial cells might reduce organ injury by limiting endothelial inflammatory responses while still preserving the ability of leukocyte populations to combat infection. This concept is supported by reports that ERK1/2 is critical for monocyte and macrophage development (1, 29, 39).

A perplexing aspect of our data is that ERK1/2 inhibition augmented IL-6 and VCAM-1 production but reduced permeability of HMVEC treated with LPS. Our permeability results are consistent with other reports that ERK1/2 activation increases permeability and alters cell-cell junctions in HUVEC and bovine lung artery endothelial cells (27, 46). The effects of ERK1/2 inhibition on reducing LPS-induced permeability were paralleled by preservation of adherens junction and tight junction proteins, specifically ZO-1 and VE-Cadherin, in the presence of the ERK1/2 inhibitor. Disruption of these same endothelial tight junction and adhesion proteins has been shown to cause endothelial hyperpermeability in many other inflammatory conditions, including burn injury and acute lung injury too (19, 20, 32). Thus our results on the role of ERK1/2 in TLR4-dependent endothelial permeability, and on tight junction and adhesion proteins, may have mechanistic and therapeutic relevance not only in sepsis, but also to these other inflammatory processes.

Upon LPS recognition, TLR4 intracellular signaling is divided into two signaling pathways based on the adapter proteins, MyD88 and TRIF (30, 48). We thought that it was possible that TRIF-dependent versus MyD88-dependent signaling might account for differences in the apparent role of ERK1/2 in TLR4-dependent induction of IL-6 *versus* permeability. However, our data using TRIF and MyD88 inhibitors suggest that neither of these proximal adapter proteins are critical for TLR4-dependent IL-6 production in HMVEC, and that ERK1/2's effects on permeability appear to be independent of both of these adaptor proteins. Future studies will address potential alternative TLR4-dependent signaling mechanisms in human endothelial cells and further delineate proximal and distal ERK1/2 signaling pathways. It is also plausible that the downregulation of NF-κB in presence of ERK1/2 inhibitor we demonstrated can explain this preservation of the endothelial monolayer with ERK1/2 inhibition as it has been shown that inhibition of NF-κB reverses endothelial leakage in septic mice (56).

While the details of the mechanism remain to be elucidated, this disconnect between the effects of ERK1/2 in endothelial inflammation and permeability challenges the paradigm that LPS-induced inflammation per se leads to increased vascular permeability. Other studies support this notion in which signaling pathways that modulate cytokine production are separate from those that affect permeability. Interleukin signaling is an example and while an NF-κB-dependent pathway leads to transcriptional inflammatory activation in endothelial cells, a more proximal pathway separate from that is responsible for vascular barrier disruption (58). The differential effects of ERK1/2 on cytokine production and permeability may facilitate leukocyte migration and movement across the endothelial barrier by loosening cell-cell junctions, while also limiting generalized endothelial cell production of pro-inflammatory mediators. Finally, it is possible that ERK1 and ERK2 each have different functions. Our ERK1/2 inhibitor, while specific, inhibits both ERK1 and ERK2. While these proteins are generally described together and are approximately 80% homologous, there appears to be significant differences between ERK1 and ERK2 as ERK2 deficiency is embryonically lethal in mice (15). Consistent with the possibility that ERK1 and ERK2 play different roles, we found that siRNA knockdown of ERK2, but not ERK1, significantly augmented LPS-induced IL-6 production by HMVEC.

During sepsis, widespread microvascular endotheliopathy leads to vascular leak, inflammation, and coagulopathy, and promotes sepsis-induced organ failure (25). However, the precise mechanisms of sepsis-induced endotheliopathy, and the role of endothelial inflammatory pathways in driving organ injury and failure in sepsis and injury remain elusive. ERK1/2 plays key roles in numerous cellular processes including cell adhesion, differentiation, and proliferation, and in pro-inflammatory signaling in diseases such as asthma, vascular disease, ischemia-reperfusion and rheumatoid arthritis (3, 8, 35, 54). Our study has uncovered a substantial difference in the role of ERK1/2 activation in TLR4-dependent cytokine responses of HMVEC *versus* leukocytes. Our data suggest that ERK1/2 activity restrains LPS-induced production of IL-6 in HMVEC, in contrast to the known pro-inflammatory role of ERK1/2 in monocytes and macrophages. Paradoxically, however, ERK1/2 activity also promotes LPS-induced HMVEC permeability. Our study focused on lung microvascular endothelial cells because they are centrally involved in lung injury and respiratory failure in sepsis. Further studies are needed to determine the role of ERK1/2 in LPS-induced activation and permeability of microvascular endothelial cell from other organs, to determine the functional relevance of endothelial ERK1/2 activation in sepsis and organ injury, and to define the differences in ERK1/2-dependent signaling in endothelial cells *versus* leukocytes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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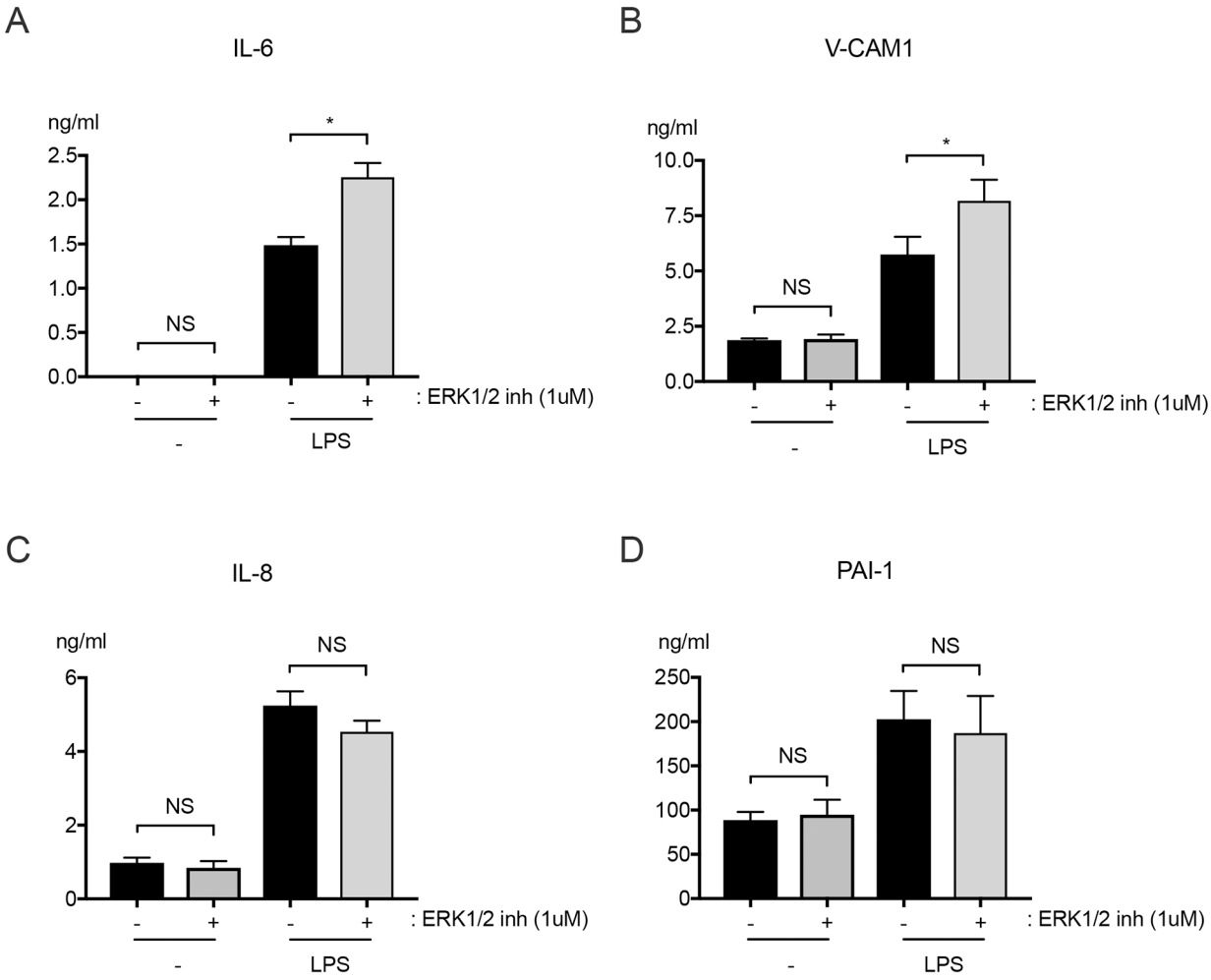

Figure 1: ERK1/2 inhibitor augments LPS-induced production of IL-6 and VCAM-1, but not IL-8 or PAI-1 by HMVEC.

HMVEC were pre-treated with ERK1/2 inhibitor (SCH772984; 1μM), or vehicle for 1 hour, and then with LPS (10 ng/ml) for another 6 hours in the continued presence of ERK1/2 inhibitor or vehicle. Levels of **(A)** IL-6, **(B)** VCAM-1, **(C)** IL-8, and **(D)** PAI-1 were quantified in culture supernatants. *n = 4; *p≤0.05; NS, not significant.*

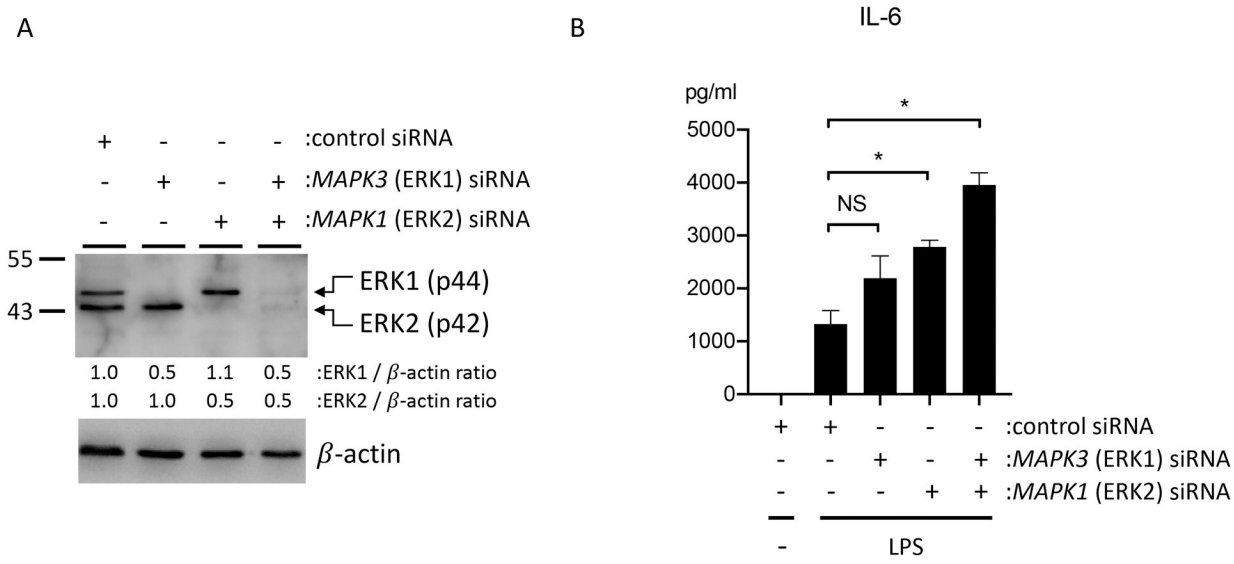

Figure 2: siRNA knockdown of ERK1/2 in HMVEC augments LPS-induced IL-6 production.

HMVEC were transfected with siRNAs for ERK1, ERK2, or both ERK1 and ERK2 for 24 hours, and then treated with LPS (1 μg/ml). **(A)** Expression of total ERK1/2 after siRNA transfection by western blotting. Relative density of ERK1/2 was compared to that of β-actin. **(B)** IL-6 levels in supernatants of LPS-treated HMVEC that had undergone siRNA knockdown of ERK1, ERK2, or both ERK1 and ERK2. *n = 4; *p≤0.05; NS, not significant.*

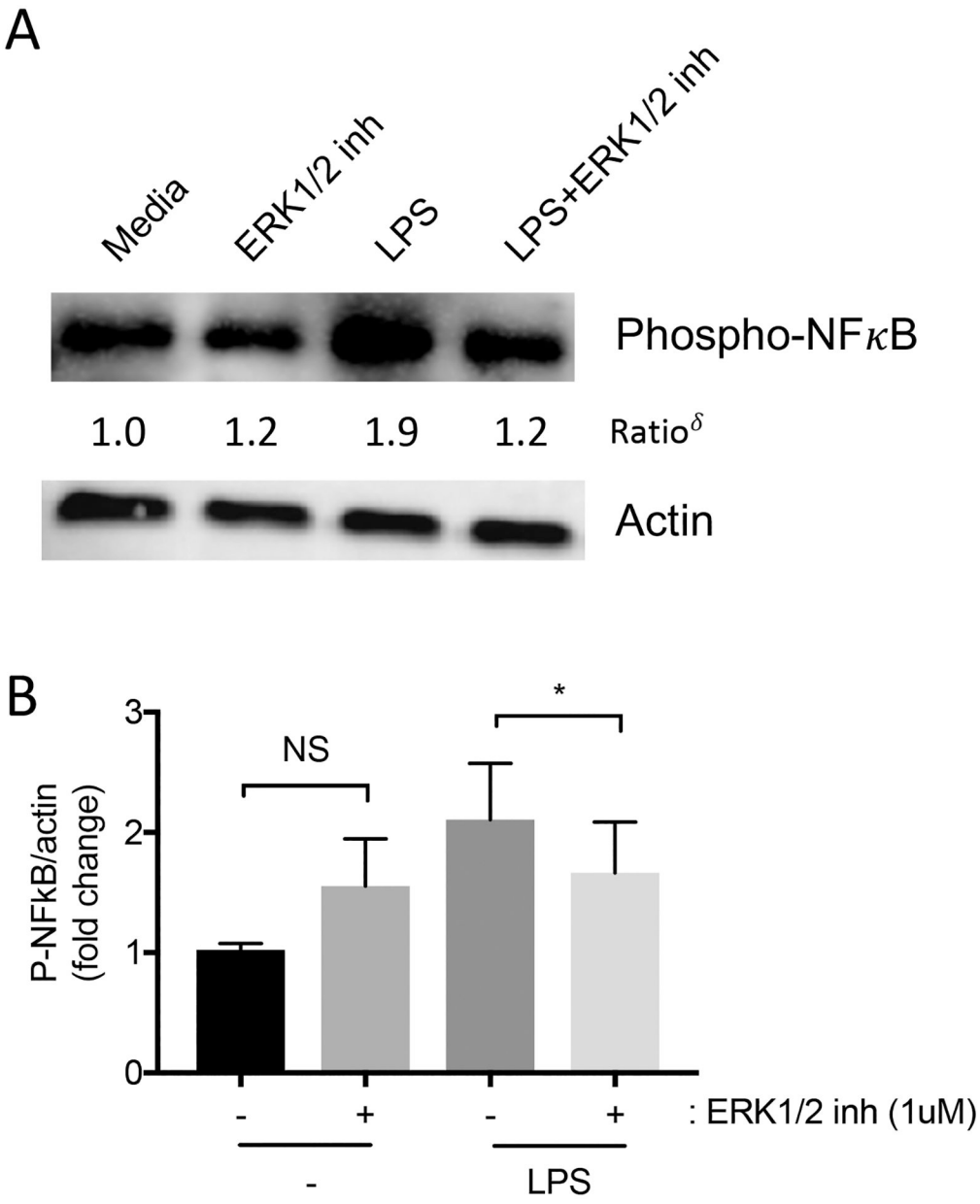

Figure 3: ERK1/2 inhibitor suppresses LPS-induced NFκB activation in HMVEC.

HMVEC were pre-treated for 1 hour with ERK1/2 inhibitor (1μM) or vehicle, and then with LPS (10 ng/ml) in the continued presence of ERK1/2 inhibitor for 30 minutes. (A) Representative images of phospho-NF-κB and actin were detected by immunoblots (δ: normalized density of phospho-NF-$\kappa B_{treatment}$/normalized density of phospho-NF-κB_{media}). (B) Bar graphs showing the relative abundance of phospho-NF-κB after normalization to actin. *n=4; *p ≤ 0.05; NS, not significant.*

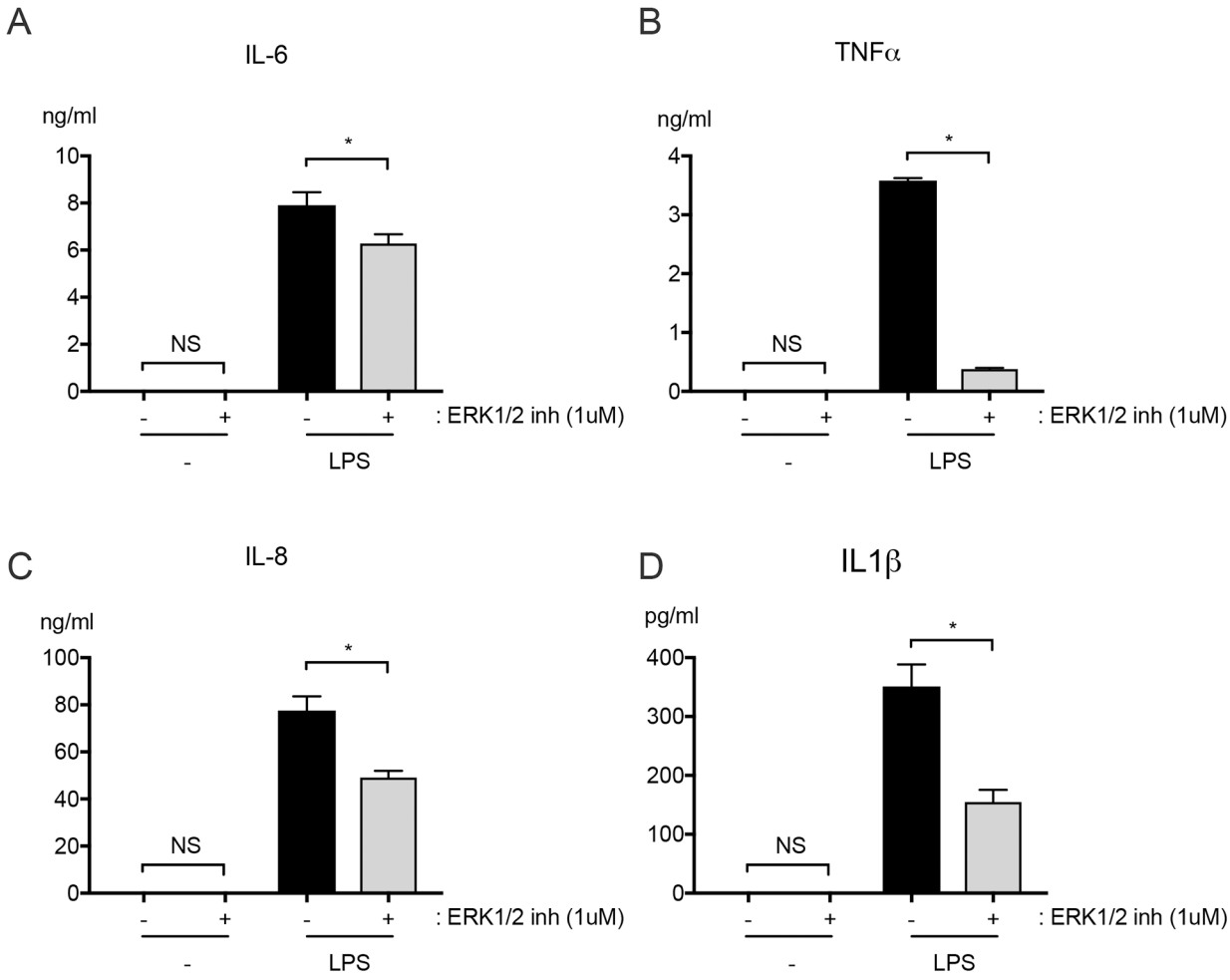

Figure 4: ERK1/2 inhibitor decreased LPS-induced IL-6, TNFα, IL-8, and IL1β production by human monocytes.

Primary human $CD14^+$ monocytes were pretreated for 1 hour with ERK1/2 inhibitor (1 μM) and then stimulated with LPS (1 μg/ml) for 6 hours in the continued presence of ERK1/2 inhibitor or vehicle. Levels of **(A)** IL-6, **(B)** TNFα, **(C)** IL-8 and **(D)** IL1β were quantified in culture supernatants. *n = 6; *p≤0.05; NS, not significant.*

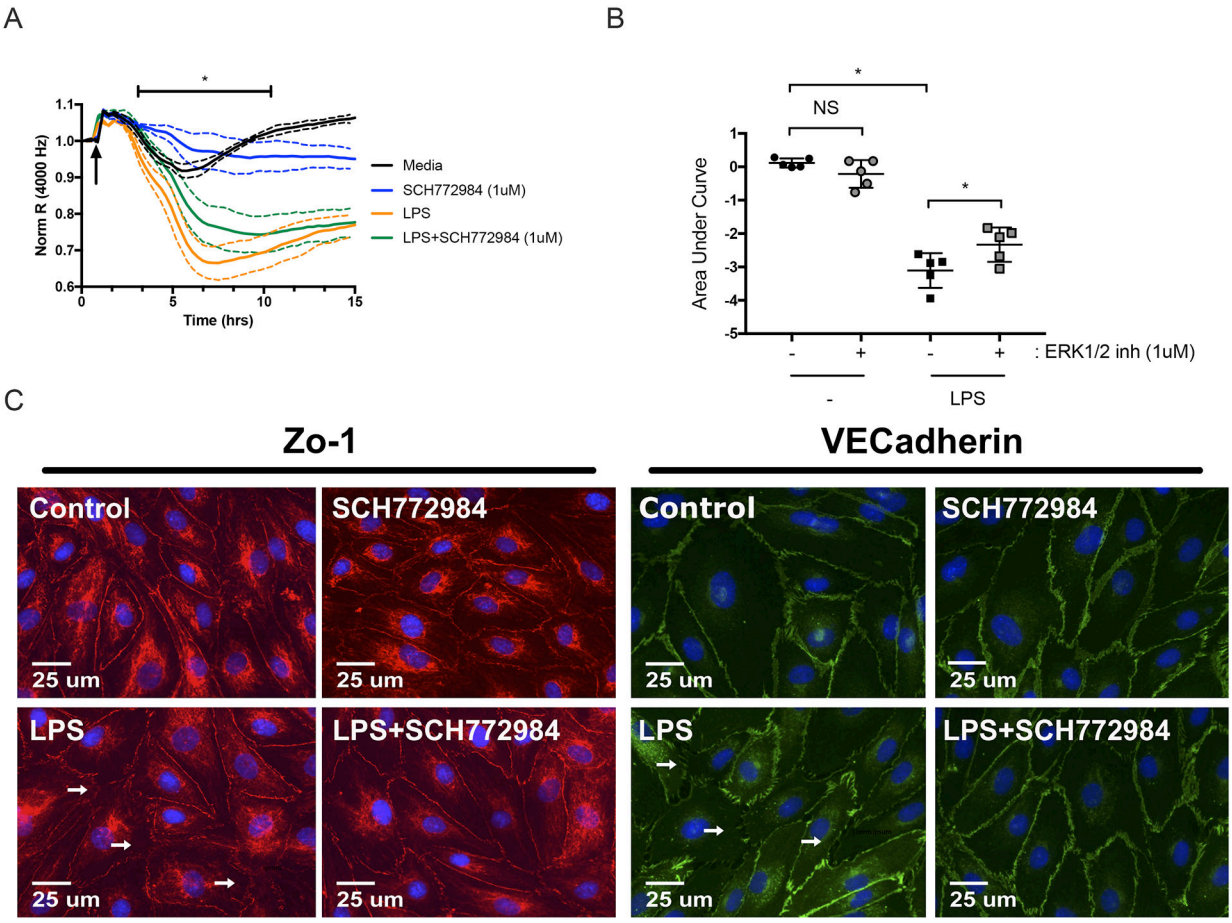

Figure 5: ERK1/2 inhibitor promotes stability of LPS-activated HMVEC monolayers.

(A) ECIS was used to assess the effects of ERK1/2 inhibitor on LPS-induced HMVEC permeability. Cells were treated with LPS (1 μg/ml) in the continued presence of ERK1/2 inhibitor (1 μM) or vehicle. Data was normalized to the resistance immediately before the addition of LPS (arrow). **(B)** Area under the curve was then quantified from baseline = 1 with addition of negative peaks and graphed as means ± SD. Data was analyzed by one-way analysis of variance (ANOVA). *n = 5; *p≤0.05; NS, not significant*. **(C)** Representative fluorescence microscopy images of HMVEC treated with LPS (1 μg/ml) in the presence and absence of ERK1/2 inhibitor. Images were taken at 40x magnification. ZO-1(red), VEcadherin (green), or nuclei (blue). White arrows indicate examples of disrupted cellular junctions. Scale bar = 25 μm.