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# Molecular Mechanisms in Lipopolysaccharide-Induced Interleukin 6 Release in Lymphatic Endothelial Cells

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## ABSTRACT

The lymphatic vasculature is involved in the transportation of tissue fluids, extravasated plasma proteins and cells back into blood circulation. Formation of lymphatic vessels by lymphatic endothelial cells (LECs) occurs both in normal tissues as well as in pathological processes including inflammation, lymphedema and tumor metastasis. Recent reports demonstrated that lymphatic vasculature is not just a major conduit for immune cell transport. It seems to be directly involved in both the induction and the resolution of inflammation. However, little is known about how lymphatic vessels themselves respond to inflammation. The purpose of this study was to investigate the molecular mechanism by which interleukin-6 release in LECs exposure to lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria. LPS was shown to cause an increase in IL-6 release in LECs. Pharmacological inhibitors of mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase (ERK), p38MAPK or c-jun N-terminal kinase (JNK), significantly abrogated the LPS-induced IL-6 release. In addition, LPS was shown to activate ERK, p38MAPK and JNK in LECs, suggesting functional cross-talk. The results of reporter assay further indicated that LPS increased the transcriptional activity of NF-κB, a critical transcription factor in inducing IL-6 expression. Our data suggest that MAPKs and NF-κB activation may contribute to LPS-induced IL-6 release in LECs. Interventions of MAPKs and NF-κB signaling may be beneficial in the treatment of lymphatic-associated inflammation.

Key words: lymphatic endothelial cells (LECs), lipopolysaccharide (LPS), mitogen-activated protein kinases (MAPKs), transcription factor, NF-κB

## INTRODUCTION

The lymphatic vascular system has an important role in the maintenance of tissue fluid homeostasis[1,2]. Tissues that frequently come into contact with foreign antigens, such as skin and mucous membranes, are particularly rich in lymphatic vessels, which are typically found in the vascularized tissues. The lymphatic vessels serve as the principle conduit for immune cells from peripheral tissues to the lymph nodes and also help to clear leukocytes from sites of resolving inflammation. The lymphatic vessels thus play a critical role in the regulation of inflammation[3]. In addition, lymphangiogenesis typically occurs at sites of tissue inflammation, such as bacterial infection[4]. Many lines of evidences demonstrated that lymphangiogenesis at sites of inflammatory insults induced by leukocytes, which produce VEGF-C and VEGF-D[1,4]. Baulk *et al.*[4] reported that blocking VEGF signaling suppresses lymphangiogenesis and exacerbates pulmonary edema in a Mycoplasma pulmonis infection mouse model. Lymphatic endothelial cells (LECs), which constitute lymphatic vessels, have been shown to express NF-κB. Activation of the NF-κB signaling in LECs may lead to upregulate VEGFR-3, which render LECs more sensitive to VEGF-C and VEGF-D produced by leukocytes[5]. On the other hand, inflammatory cytokines, such as TNF-α, released from leukocytes may also activate NF-κB signaling resulting in leukocyte adhesion molecules expression in LECs to regulate inflammatory responses[6]. However, little is known whether LECs are activated directly under bacterial infections independent of inflammatory cytokines.

There is increasing evidence from animal models that toll-like receptors (TLRs) mediate various vascular inflammatory diseases[7]. The TLR initiates a series of innate immune mechanisms against various microorganism infections by sensing the presence of pathogen-associated

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molecular patterns (PAMP) like lipopolysaccharide (LPS). LPS, a Gram-negative bacterial cell wall component, elicits most of the clinical manifestations of bacterial infections, including sepsis and septic shock[7]. LPS-induced cell activation is mediated by TLR4 in the presence of MD-2, CD14 and LPS-binding protein (LBP)[7]. LPS initially binds to LBP in the blood and is transferred to CD14[8]. The LPS/CD14 complex then interacts with the TLR4/MD-2 complex, leading to cell activation. Circulating LPS in blood directly promotes vascular inflammation via the activation of resident cells such as vascular endothelial cells and vascular smooth muscle cells[9,10]. Among the critical pathway mediating the inflammatory response is the mitogen-activated protein kinases (MAPKs) cascade, including extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK), and p38MAPK[8,11]. MAPKs have been shown to regulate the expression of inflammatory cytokines, including interleukin-6 (IL-6) in physiology and human diseases[12-14]. IL-6, a multifunctional cytokine was recognized as a regulator of immune and inflammatory responses[15]. IL-6 is also one of the important inducers of angiogenesis, which contributes to the process of many human diseases[16]. Many lines of evidence demonstrated that LPS induced cytokine release in vascular endothelial cells. However, little is known about the LPS actions in LECs. In this study, we elucidated whether the IL-6 level is altered in LECs exposure to LPS, which represents a lymphatic vascular inflammatory condition. The role of MAPKs in LPS-induced IL-6 release was also established. In this study, we demonstrated that LPS may activate MAPKs, leading to NF-κB activation and subsequent IL-6 release in LECs exposure to LPS.

## MATERIALS AND METHODS

### I. *Reagents*

Lipopolysaccharide (LPS) was purchased from Sigma (St. Louis, MO, USA). Endothelial cell growth medium MV2 was purchased from PromoCell (Heidelberg, Germany). Penicillin/streptomycin was purchased from Invitrogen (Carlsbad, CA, USA). PD98059, SB203580 and SP600125 were obtained from Calbiochem (San Diego, CA, USA). Turbofect$^{TM}$ *in vitro* transfection reagent was purchased from Upstate Biotechnology (Lake Placid, NY, USA). Antibodies specific for α-tubulin were purchased from Transduction Laboratories (Lexington, KY, USA). Anti-mouse and anti-rabbit immunoglobulin G (IgG)-conjugated alkaline phosphatase antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies specific for ERK1/2 phosphorylated at Thr202/Tyr204, p38MAPK phosphorylated at Thr180/Tyr182, and JNK1/2 phosphorylated at Thr183/Tyr185 were purchased from Cell Signaling (Beverly, MA, USA). The reporter plasmid, NFκB-Luc, Renilla-luc and Dual-Glo luciferase assay system were purchased from Promega (Madison, WI, USA). C/EBP reporter construct, p/T81 C/EBP-luc, was kindly provided by Dr. Kjetil Tasken (University of Oslo, Oslo, Norway). All materials for immunoblotting were purchased from Bio-Rad (Hercules, CA, USA) and GE Healthcare (Little Chalfont, UK). All other chemicals were obtained from Sigma (St. Louis, MO, USA).

### II. *Cell Culture*

Human lymphatic endothelial cells (HLEC, C-12217) were purchased from PromoCell (Heidelberg, Germany), and the cells were maintained in endothelial cell growth medium MV2 containing 100 U/mL of penicillin G, and 100 μg/mL of streptomycin in a humidified 37°C incubator.

### III. *Cell Viability Assay*

Cell viability was measured by a previously described colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay[17]. Briefly, cells ($2 \times 10^5$ cells/well) were cultured in 24-well plates and incubated with indicated pharmacological inhibitors for 30 min. The cells were then treated with vehicle or LPS for another 24 h. After various treatments, 1 mg/mL of MTT was added to the culture plates and incubated at 37°C for an additional 4 h. Then cells were lysed in 500 μL of dimethyl sulfoxide. The absorbance at 550 nm was measured on a microplate reader.

### IV. *Measurements of IL-6 Production*

Cells were cultured in 24-well culture plates. After reaching confluence, cells were treated with various stimulators or pretreated for 30 min with specific inhibitors as indicated, followed by LPS treatment in a humidified incubator at 37°C for another 24 h. After incubation, the medium was removed and stored at -80°C until assay. IL-6 in the medium was assayed using the IL-6 enzyme immunoassay kits (PeproTech), respectively, according to the procedure described by the manufacturer.

### V. *Transfection and NF-κB-Luciferase or C/EBP-Luciferase Assays*

Cells ($2 \times 10^5$/well) were transfected with NFκB-luc plus renilla-luc or C/EBP-luc plus renilla-luc using Turbofect$^{TM}$ reagent (Upstate). Cells with or without treatment were then harvested, and the luciferase activity was determined using a Dual-Glo luciferase assay system kit (Promega), and normalized on the basis of Renilla luciferase activity. The level of induction of luciferase activity was compared as the ratio of cells with and without LPS stimulation.

### VI. *Immunoblot Analysis*

Immunoblot analyses were performed as described previously[18]. Briefly, cells were lysed in extraction buffer containing 10 mM of Tris (pH 7.0), 140 mM of NaCl, 2 mM of PMSF, 5 mM of DTT, 0.5% NP-40, 0.05 mM of

pepstatin A, and 0.2 mM of leupeptin. Samples of equal amounts of protein were subjected to SDS-PAGE and transferred onto a PVDF membrane, which was then incubated in TBST buffer (150 mM of NaCl, 20 mM of Tris-HCl, and 0.02% Tween 20; pH 7.4) containing 5% non-fat milk. Proteins were visualized by specific primary antibodies and then incubated with horseradish peroxidase- or alkaline phosphatase-conjugated secondary antibodies. Immunoreactivity was detected using NBT/BCIP following the manufacturer's instructions. Quantitative data were obtained using a computing densitometer with a scientific imaging system (Kodak, Rochester, NY, USA).

VII. *Statistical Analysis*

Results are presented as the mean ± SE from at least three independent experiments. One-way analysis of variance (ANOVA) followed by, when appropriate, the Newman-Keuls test was used to determine the statistical significance of the difference between means. A $p$ value of less than 0.05 was considered statistically significant.

## RESULTS

I. *Effects of PD98059, SB203580 and SP600125 on LPS-Induced IL-6 Release in LECs*

We first determined whether LPS induced IL-6 expression and release in LECs. As shown in Figure 1, treatment of LECs for 24 h with LPS (1 μg/mL) significantly induced IL-6 release from 185.0 ± 67.6 to 910.0 ± 143.9 pg/mL, respectively (n = 4) (Figure 1). Several lines of evidence demonstrated that MAPKs including ERK, JNK and p38MAPK contributed to IL-6 release in airway smooth muscle cells and macrophages[19,20]. We thus used PD98059 (a MEK1 inhibitor), SB203580 (a p38MAPK inhibitor) and SP600125 (a JNK inhibitor) to investigate whether ERK, JNK or p38MAPK contributed to LPS-induced IL-6 release in LECs. As shown in Figure 1, LPS-induced IL-6 release was suppressed significantly by the presence of these inhibitors while inhibitor alone was without effects. PD98059, SB203580 and SP600125 attenuated LPS-induced IL-6 release with the extent of IL-6 reduced from 910.0 ± 143.9 to 587.5 ± 67.9, 435.0 ± 93.1, and 565.0 ± 63.8 pg/mL, respectively (Figure 1). To further determine whether the cytotoxic effect was attributable to the actions of PD98059, SB203580 and SP600125 in LPS-treated LECs, an MTT assay was employed. As shown in Figure 2, treatment of cells with 30 μM of PD98059, 3 μM of SB203580 or 5 μM of SP600125 for 24 h did not alter cell viability. Together, these findings suggested that MAPK signaling cascades were involved in LPS-induced IL-6 release in LECs.

II. *LPS Induced ERK, JNK and p38MAPK Activation in LECs*

As PD98059, a MEK1 inhibitor, can eliminate the action of LPS in inducing IL-6 release, it was interesting to investigate if the downstream target of MEK1, ERK, is involved. Phosphorylation of ERK1 at Thr202/Tyr204 and ERK2 at Thr185/Tyr187 is required for ERK1 and ERK2 activation[21,22]. Thus, the phosphorylated form of the ERK1 and ERK2 was determined by immunoblotting with phosphorylated-specific antibody. As shown in Figure 3, the phosphorylated form of ERK1/2 increased time-dependently after LPS treatment. On the other hand, SB203580 and

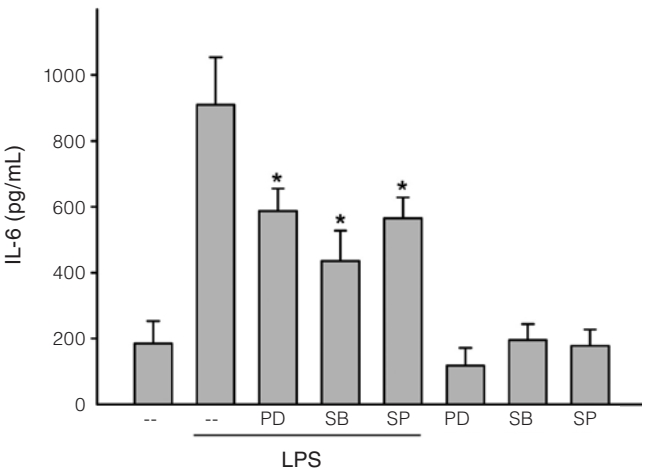


**Figure 1.** MAPKs were involved in LPS-induced IL-6 release in LECs. LECs ($10^5$ cells per well in 24-well plates) were pretreated for 30 min with vehicle, 30 μM of PD98059 (PD), 3 μM of SB203580 (SB) or 5 μM of SP600125 (SP), before being stimulated with 1 μg/mL of LPS for 24 h. IL-6 production was then measured as described in "Materials and Methods". Each column represents the mean ± SEM of four independent experiments performed in duplicate. * $p < 0.05$, compared to the group treated with LPS alone.

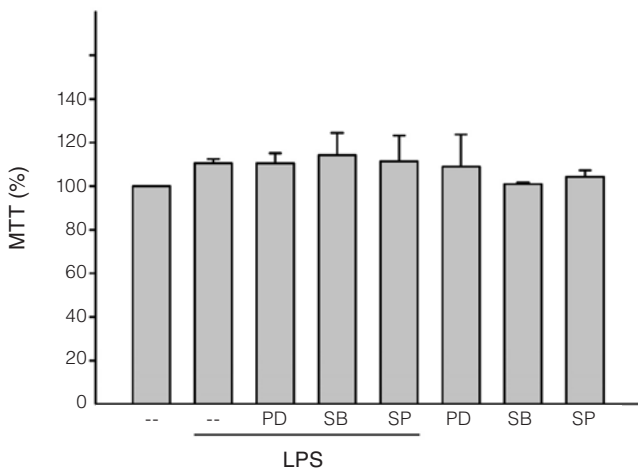


**Figure 2.** Effects of PD98059, SB203580 or SP600125 on cell viability in LECs. Cells were pretreated for 30 min with vehicle, 30 μM of PD98059 (PD), 3 μM of SB203580 (SB) or 5 μM of SP600125 (SP), before being stimulated with 1 μg/mL of LPS for 24 h. Cell viability was then determined by MTT assay as described in "Materials and Methods". Each column represents the mean ± SEM of four independent experiments performed in duplicate.

SP600125, which inhibited p38MAPK and JNK1/2, were also shown to suppress LPS-induced IL-6 release in LECs. Due to p38MAPK phosphorylation at Thr180/Tyr182 and JNK1/2 phosphorylation at Thr183/Tyr185 are correlated with p38MAPK and JNK activity[23,24]. We thus determined whether the extent of p38MAPK and JNK1/2 phosphorylation are altered by LPS in LECs. Treatment of cells with LPS increased p38MAPK phosphorylation significantly at 20 min and this was sustained to 60 min after LPS exposure (Figure 4). Results from Figure 5 also illustrate that LPS increased JNK phosphorylation significantly at 20 min after LPS exposure. These results suggested that LPS may activate ERK, JNK and p38MAPK, leading to IL-6 release in LECs.

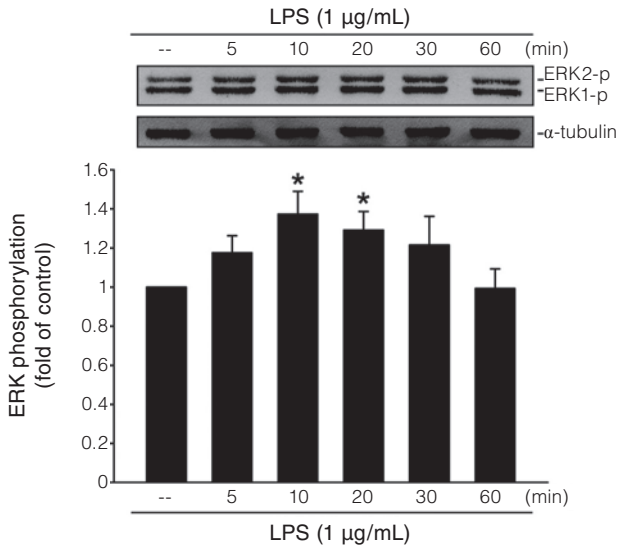


**Figure 3.** LPS induced ERK activation in LECs. Cells were treated with LPS (1 μg/mL) for the indicated time periods. The cells were then harvested and ERK phosphorylation was determined by immunoblotting. Data are presented as the means ± SEM $^{*}$ $p < 0.05$, compared to the control group.

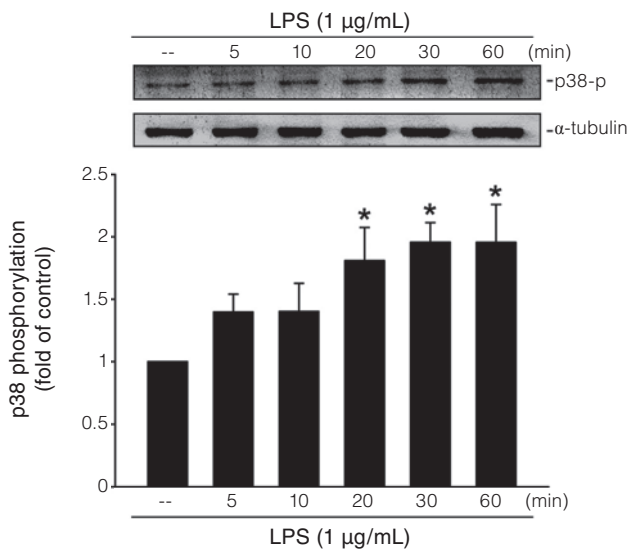


**Figure 4.** LPS induced p38MAPK activation in LECs. Cells were treated with LPS (1 μg/mL) for the indicated time periods. The cells were then harvested and the phosphorylation status of p38MAPK was determined by immunoblotting. Data are presented as the means ± SEM $^{*}$ $p < 0.05$, compared to the control group.

II. *ILPS Increased NF-kB Luciferase Activity in LECs*

It is conceivable that LPS may activate MAPKs, leading to the activation of transcription factor which transactivates IL-6 in LECs. The promoter region of the human IL-6 gene contains many transcription factor-binding sites. These transcription factors include the AP-1, C/EBP and NF-κB[25,26]. The NF-κB and C/EBP element is generally believed to play an important regulatory role in IL-6 expression in immune cells as well as in other cell types[25-27]. However, the role of NF-κB and C/EBP in regulating IL-6 expression following LPS stimulation in LECs is still unknown. A reporter assay was thus employed to determine whether LPS activated NF-κB in LECs. Cells were transiently transfected with a NFκB-Luc reporter construct. As shown in Figure 5, cells treated with LPS (1 μg/mL) for 24 h caused an increase in NFκB-Luciferase activity. The increase in NFκB-Luciferase activity was 1.9 ± 0.1 folds after 1 μg/mL LPS treatment (n = 5) (Figure 6). In addition, results from reporter assay also demonstrated that C/EBP is activated in LECs exposure to LPS. Treatment of cells with LPS (1 μg/mL) for 24 h also increased C/EBP-Luciferase activity by 1.5 ± 0.1 folds (n = 4) (Figure 7). Based on these results, we suggested that LPS activation of NFκB and C/EBP may result in IL-6 expression and subsequent release in LECs.

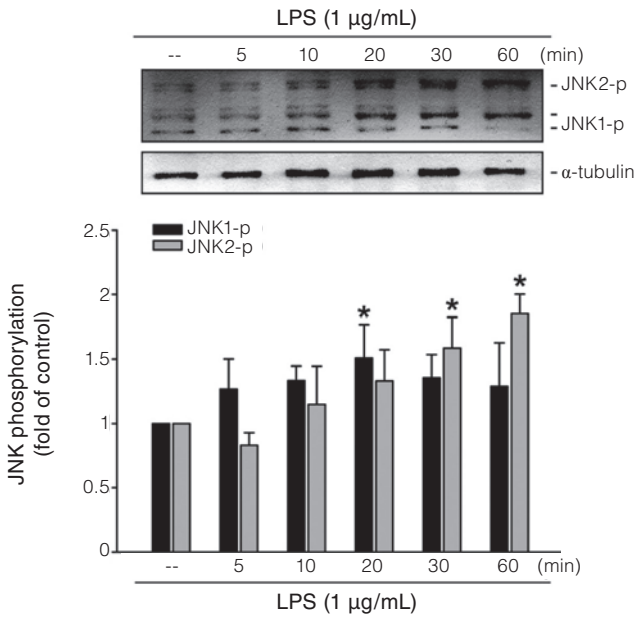


**Figure 5.** LPS induced JNK activation in LECs. Cells were treated with LPS (1 μg/mL) for the indicated time periods. The cells were then harvested and the phosphorylation status of JNK was determined by immunoblotting. Data are presented as the means ± SEM $^{*}$ $p < 0.05$, compared to the control group.

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## DISCUSSION

Lymphatic vascular inflammatory responses seem to be the result of interactions between LECs and exogenous stimuli. LPS has been proven to play a causal role in inducing vascular endothelial cell injury and subsequent tissue damage during Gram negative infection[28]. Many lines of evidence demonstrated that LPS exposure can lead to the activation of vascular endothelial cells, secretion of inflammatory cytokines and complicated immune responses. However, whether LPS activates LECs resulting in inflammatory cytokine release remains unknown. In this study, we demonstrated that LPS induced IL-6 expression in LECs. The underlying mechanism of LPS shown in this study may involve the activation of ERK, JNK and p38MAPK.

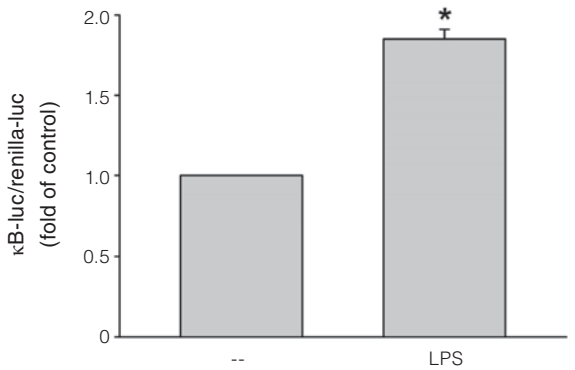


**Figure 6.** LPS induced increase in NF-κB-luciferase in LECs. Cells were transiently transfected with NF-κB-luc and renilla-luc for 48 h and treated with LPS (1 μg/mL) for another 24 h. NF-κB-luciferase assay was then determined as described in "Materials and Methods". Data are presented as the mean ± SEM of four independent experiments performed in duplicate. * $p < 0.05$, as compared with the control group.

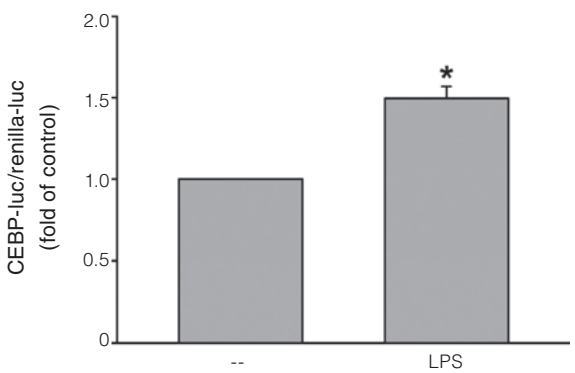


**Figure 7.** LPS induced increase in C/EBP-luciferase in LECs. Cells were transiently transfected with C/EBP-luc and renilla-luc for 48 h and treated with LPS (1 μg/mL) for another 24 h. NF-κB-luciferase assay was then determined as described in "Materials and Methods". Data are presented as the mean ± SEM of four independent experiments performed in duplicate. * $p < 0.05$, as compared with the control group.

In addition, activation of transcription factors, NF-κB and C/EBP, may also contribute to LPS actions in inducing IL-6 expression in LECs.

IL-6 was reported to have various biological properties in endothelial cell dysfunctions[29]. Many inflammatory stimuli including tumor necrosis factor-α, IL-1 and LPS enhanced IL-6 expression in vascular endothelial cells[30,31]. Although LECs are terminally differentiated cells distinct from blood vascular endothelial cells[32,33], we noted in this study that LPS also induced IL-6 expression in LECs. The molecular mechanism involved in IL-6 expression by LPS in LECs remains unresolved. MAPK signaling cascades has been shown to participate in a variety of cellular functions in physiology and diseases[12]. Activation of MAPKs is thus postulated to be required for LPS induction of IL-6 in LECs. Recent studies demonstrated that ERK and p38MAPK played a critical role in inflammatory gene expression in human vascular endothelial cells exposed to LPS[30]. Whether ERK and p38MAPK contribute to LPS induction of IL-6 in LECs has not been previously demonstrated. In agreement with these observations, we noted that MAPKs signaling blockade by specific inhibitors markedly suppressed LPS-induced IL-6 release in LECs. LPS was also shown to activate MAPKs, including ERK, JNK and p38MAPK. We thus suggested that MAPKs may be causally related to LPS-induced IL-6 expression in LECs.

Transcription factor NF-κB is mainly involved in regulating inflammatory and immune responses to extracellular stimuli. Upon activation, NF-κB dissociates from its inhibitory protein IκB-α and translocates from cytosol to the nucleus[34], where NF-κB binds to related DNA elements (NF-κB response element) and activates its target genes such as IL-6[30]. Previous studies have demonstrated that ERK and p38MAPK are upstream regulators of NF-κB in activated vascular endothelial cells[35]. We noted that LPS significantly increased NF-κB activity as determined by reporter assay. A reporter assay showed further that C/EBP activity was also elicited by LPS in LECs. As the promoter region of the IL-6 gene contains AP-1 binding sites[25,26], activation of AP-1 is also likely to be responsible for the increase in IL-6 expression. This is supported by the observation that LPS induced the activation of JNK, a critical upstream regulator of AP-1 and by the blockade of LPS-induced IL-6 expression by the JNK inhibitor (SP600125) in LECs. In addition, our recent study revealed that JNK signaling may also regulate C/EBP activity in the induction of inflammatory genes[36]. Together, these findings raise the possibility that LPS activation of MAPKs may regulate at least three separate pathways in LECs: one on the JNK-AP-1 signaling cascade, one on the JNK-C/EBP signaling cascade and another on the ERK or p38MAPK-NF-κB signaling cascade. The differential mechanisms of LPS actions in driving these signaling pathways remain to be elucidated. It is likely that these pathways may culminate in inducing IL-6 expression in LECs. Furthermore, several reports have demonstrated the physical and functional interactions between C/EBP and NF-κB[37]. However, whether C/EBP

cooperates with other transcription factors, such as NF-κB or AP-1 in the induction of IL-6 in LPS-stimulated LECs, need to be investigated further. Our findings revealed, at least, that MAPKs and transcription factors C/EBP and NF-κB may attribute to LPS-induced IL-6 expression in LECs. It thus abrogates the invasion of exogenous stimuli or pharmacological approaches to diminish the effects of pathogens by the modulation of MAPKs-NF-κB or C/EBP cascade may provide new strategies for managing gram-negative infection-associated lymphatic vascular diseases.

## ACKNOWLEDGMENTS

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