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LPS-induced MCP-1 Expression in Human Microvascular Endothelial Cells is mediated by the tyrosine kinase, Pyk2 via the p38 MAPK/NF- κ B dependent pathway

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Abstract

Bacterial endotoxin (lipopolysaccharide or LPS) has potent pro-inflammatory properties and acts on many cell types including endothelial cells. Secretion of the CC chemokine, MCP-1 (CCL2) by LPS-activated endothelial cells contributes substantially to the pathogenesis of sepsis. However, the mechanism involved in LPS-induced MCP-1 production in endothelial cells is not well understood. Using human microvascular endothelial cells (HMVEC), we analyzed the involvement of the non-receptor tyrosine kinase, Pyk2, in LPS-mediated MCP-1 production. There was a marked activation of the non-receptor tyrosine kinase, Pyk2, in response to LPS. Inhibition of Pyk2 activity using a pharmacological inhibitor, Tyrphostin A9 significantly attenuated LPS-induced Pyk2 tyrosine phosphorylation, p38 MAP kinase (MAPK) activation, NF- κ B activation, and MCP-1 expression. Furthermore, specific inactivation of Pyk2 activity by transducing microvascular endothelial cells with catalytically inactive Pyk2 mutant (AAV-Pyk2MT) or Pyk2-specific siRNA significantly blocked LPS-induced MCP-1 production. The supernatants of these LPS-stimulated cells with attenuated Pyk2 activity demonstrated decreased trans-endothelial monocyte migration in comparison to LPS-treated controls, thus confirming the inhibition of functional MCP-1 production. In summary, our data suggest a critical role for the Pyk2 mediated pathway involving p38 MAP kinase and NF- κ B in LPS-induced MCP-1 production in human microvascular endothelial cells.

Keywords

Endothelial cells; Monocyte chemotactic protein MCP-1; Lipopolysaccharide LPS; Proline-rich kinase-2 Pyk2

1. Introduction

Gram-negative bacterial infection is a major cause of sepsis and septic shock, a syndrome characterized by a widespread inflammatory response that triggers organ damage and ultimately organ failure (Hack and Zeerleder, 2001). Endothelial cell (EC) injury and/or

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dysfunction, a commonality among several key complications associated with septic shock, is believed to be mediated at least in part by Lipopolysaccharide (LPS), a component of the outer envelope of all Gram-negative bacteria (Berman et al., 1993; Trepels et al., 2006). LPS directly activates the vascular endothelium and elicits an array of EC responses, including an increase in the expression of specific adhesion molecules and inflammatory cytokines such as IL-1, IL-8, and MCP-1 (monocyte chemotactic protein-1), which in turn results in the selective recruitment of leukocytes to inflammatory foci (Bierhaus et al., 2000). Among these cytokines, the chemokine that is the predominant attractant for neutrophils is IL-8, while the predominant attractant for monocytes is the MCP-1 (Rollins., 1997). Elucidation of how LPS signals through cell-surface receptors to induce chemokine production is of prime importance.

Proline-rich kinase-2 (Pyk2) and Focal adhesion kinase (FAK) are closely related non-receptor protein tyrosine kinases that are activated by a variety of extracellular stimuli (Avraham et al., 2000). Unlike FAK which is ubiquitously expressed, Pyk2 has a more restricted tissue expression primarily in neuronal and hematopoietic tissues (Avraham et al., 2000). Pyk2 is rapidly activated and tyrosine phosphorylated by G-protein coupled receptor agonists, growth factors, cytokines, and stress signals that increase intracellular calcium (Astier et al., 1997; Avraham et al., 2000; Gismondi et al., 1997; Lev et al., 1995). Pyk2 functions by coupling several receptors (including integrin and chemokine receptors) with a variety of downstream effectors, thus regulating various functions such as cell adhesion, migration, proliferation and survival (Avraham et al., 2000; Di Cioccio et al., 2004; Liu et al., 1997; Zheng et al., 1998). We and others have shown that Pyk2 tyrosine phosphorylation could be closely associated with inflammatory processes in various cell types (Anand et al., 2008; Di Cioccio et al., 2004; Liu et al., 1997; Yamasaki et al., 2001). In the present study, we questioned whether Pyk2 was involved in the MCP-1 production that we observed upon LPS stimulation. Although umbilical vein endothelial cells are a well-characterized model for EC activation, much of the effect of LPS occurs at the level of microvasculature (Kirkpatrick et al., 1995). Hence we used human microvascular endothelial cells as a physiologically more relevant *in vitro* model to study the pathogenesis of LPS-induced microvascular changes. Our study demonstrates for the first time that activation of the non-receptor tyrosine kinase, Pyk2, is an important intermediate step in the pathway leading to MCP-1 secretion in LPS-stimulated microvascular endothelial cells. LPS induces marked Pyk2 phosphorylation in these cells. Inhibition of Pyk2 activation by the specific pharmacologic inhibitor Tyrphostin A9, over-expression of the kinase-dead mutant of Pyk2, and knock-down of Pyk2 using specific siRNA was paralleled by inhibition of both MCP-1 production and monocyte chemotaxis. Further investigation into LPS signaling revealed that Pyk2 regulates MCP-1 production through the p38 MAP kinase/NF- κ B pathway.

2. Materials and Methods

2.1 Reagents, cells and culture conditions

Lipopolysaccharide (LPS) was obtained from Sigma Chemical Co. (St. Louis, MO). The Pyk2 inhibitor (Tyrphostin A9) and the p38MAP kinase inhibitor (SB203580) were obtained from Calbiochem (San Diego, CA). Phospho-Pyk2 and p-FAK antibodies were obtained from Biosource (Carlsbad, CA), while Py99, p-ERK, ERK, p-p38, and p38 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Pyk2 antibodies were obtained from BD Transduction Laboratories (San Jose, CA). Human dermal microvascular endothelial cells (HMVEC) (Clonetics, San Diego, CA) were maintained in EGM-2MV growth medium containing growth factors, antimicrobials, cytokines and 5% FBS at 37°C in a humidified atmosphere containing 5% CO₂. To avoid phenotypic drift associated with decreasing expression of surface receptor molecules, HMVEC was not used beyond passage 4. Human umbilical vein endothelial cells (HUVEC) were also purchased from Clonetics (San Diego, CA) and grown in EGM growth medium containing supplements and 2% FBS.

2.2 Stimulation

In all experiments, HMVEC were grown to 80% confluence in 6-well assay plates. The cells were stimulated with LPS in the presence of 0.5% FBS. In the case of inhibitor treatments (Tyrphostin A9, SB203580), HMVEC were pretreated with the inhibitor for 1 hour after which they were stimulated with LPS for various time periods. The supernatant was used for the MCP-1 or trans-endothelial migration (TEM) assays and the cell lysates were used for the Western blotting analyses.

2.3 Recombinant adeno-associated virus transduction

High-efficiency gene delivery of the dominant-negative Pyk2 mutant, Pyk2K457A (Pyk2MT) or a control gene (β -galactosidase) was accomplished using a recombinant adeno-associated virus (rAAV)-based method. The AAV vectors were prepared as described previously (Madry et al., 2003). Before being exposed to the virus, the HMVEC were cultured overnight in complete medium. HMVEC were transduced by application of the AAV in a minimal amount of serum-free medium for 90 min at 37°C in a cell culture incubator. Equal volumes of complete EGM containing 10% serum were added to the cells to achieve a final serum concentration of 5%. The cells were cultured for 36 hours before being used for the experiments described later. After transduction, LPS was added to the medium and the cells were incubated for an additional 24 hours. The culture supernatant was removed and evaluated for MCP-1 content. Alternatively, the cells were lysed and subjected to Western blot analysis by using rabbit anti-human Pyk2 antibody or β -galactosidase staining in the case of the control.

2.4 MCP-1 ELISA

After various stimulations, the culture supernatants were collected, centrifuged, and processed for MCP-1 quantification by commercially available ELISA kits (Endogen), per the manufacturer's instructions.

2.5 Isolation of monocytes

The CD14⁺ monocytes were isolated from human peripheral blood mononuclear cells (PBMCs) by using the CD14 negative isolation kit (Dyna, Carlsbad, CA) according to the manufacturer's protocol. Briefly, human PBMCs were isolated by density-gradient centrifugation with Ficoll/Hypaque (GE Lifesciences, Piscataway, NJ) and then mixed with microbeads. The monocytes were isolated by magnetic bead separation. Flow cytometric analysis revealed a purity of the CD14⁺ cells to be more than 92%. (data not shown)

2.6 Trans-endothelial migration (TEM) assay of monocytes

Briefly, about 100,000 HMVEC cells were added to fibronectin-coated 24-well Transwell chambers with a pore size of 8 μ m (Costar Corp., Corning, NY) and grown for 3 days in 5% CO₂ at 37 °C. 0.6 ml of medium from the untreated or LPS/Tyrphostin A9- or AAV-Pyk2MT-treated HMVEC cells were added to the lower compartment. In the upper compartment, 1×10^6 monocytes in 0.1 ml of 0.5% FBS containing EBM medium were added onto the HMVEC monolayer. Supernatants pretreated with 20 μ g/ml MCP-1 antibody served as controls. The chambers were incubated for 4 hours at 37°C in 5% CO₂. The cells in the lower compartment were counted on a hemocytometer. The results are presented as the means $\pm$ S.D. of three separate experiments and are expressed as the number of cells migrating toward the lower compartment.

2.7 Western blotting and immunoprecipitation

Total cellular extracts from the LPS-treated cells were prepared by lysing the cells in radioimmunoprecipitation assay buffer (50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25%

sodium deoxycholate, 150 mM NaCl, 1 mM PMSF, 10 µg/ml aprotinin, leupeptin, and pepstatin, 10 mM sodium vanadate, 10 mM sodium fluoride, and 10 mM sodium pyrophosphate). Proteins (50 µg) were size-fractionated by 8% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked for 2–3 hours with 5% nonfat milk and then incubated with the respective primary and secondary Abs for 2–3 h each. The membranes were washed three to four times for 15 min each with TBS and 0.05% Tween 20, and later developed by chemiluminescence (ECL System; GE Healthcare, Piscataway, NJ).

2.8 siRNA-mediated knockdown of Pyk2

RNA interference-mediated knockdown of Pyk2 was performed using SMARTpool Pyk2 duplex RNA oligonucleotides obtained from Dharmacon (Lafayette, CO). A non-targeting siRNA was used as the control. HMVEC were transfected with the siRNA using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Pyk2 siRNA-mediated knockdown was estimated by detection of Pyk2 expression using Western blot analysis, 48 hours after the initial transfection.

2.9 NF-κB assay

At the end of the stimulation, cell and nuclear extracts were obtained by using a Nuclear Extract Kit (Active Motif, Carlsbad, CA). Briefly, cells were collected in the PBS/phosphatase inhibitors solution and lysed in a buffer (Active Motif, Carlsbad, CA) containing 10 mM DTT and a cocktail of protease inhibitors as per the manufacturer's recommendations. Solubilized proteins were then separated from cell debris by centrifugation (20 min at 14,000×g). The protein concentration in the cytoplasmic and nuclear fraction was measured by Bradford assay and the protein content was adjusted to have the same concentration in all the samples. NF-κB activation was measured by the NF-κB ELISA kit (Active Motif, CA, USA) according to the manufacturer's recommendations. The levels of NF-κB activation were measured by a Spectrophotometer at OD₄₅₀.

2.10 Statistical Analysis

Reported data are the means ± S.D. of at least three independent experiments performed in duplicate or triplicate. The statistical significance was determined by the Student's *t* test.

3. Results

3.1 LPS induces MCP-1 production in human microvascular endothelial cells

To examine the modulation of MCP-1 secretion from endothelial cells on LPS treatment, HUVEC or HMVEC were grown to 80% confluence. These cells were stimulated with LPS for the indicated periods. MCP-1 concentrations in the clarified culture supernatants were then measured by ELISA. Exposure of microvascular endothelial cells (HMVEC) to LPS (100 ng/ml) resulted in a significantly greater increase in MCP-1 production compared to HUVEC (Figure 1A). Up-regulation of MCP-1 in both cell lines was time-dependent (Figure 1A) and dose-dependent (Figure 1B). MCP-1 was *de novo* synthesized upon stimulation with LPS, since minimal MCP-1 was detected in the supernatants of stimulated or unstimulated HMVEC that were cultured in the presence of cycloheximide (CX) (data not shown). Since microvascular endothelial cells have been reported to be a more relevant model in examining LPS-induced effects in comparison to macrovascular endothelial cells (Zachlederova and Jarolim, 2006), we further studied the signaling cascade leading to LPS-mediated secretion of MCP-1 using HMVECs.

3.2 LPS stimulates tyrosine phosphorylation of Pyk2 in HMVECs

To investigate the signaling processes involved in LPS-induced MCP-1 production, we initially examined the ability of LPS to activate the non-receptor tyrosine kinases Pyk2 and FAK. HMVEC stimulated with 100ng/ml LPS for different lengths of time did not show an increased FAK phosphorylation. Untreated HMVEC revealed a relatively high background of FAK tyrosine phosphorylation (Figure 2B). In contrast, there was consistent induction of Pyk2 activation as demonstrated by tyrosine phosphorylation of Pyk2 (Figure 2A). Tyrosine phosphorylation of Pyk2 was estimated by immunoblotting with anti-phospho-Pyk2 antibodies. Pyk2 phosphorylation was observed as early as 5 minutes after stimulation. Probing for total Pyk2 not only confirmed the abundance of expression of Pyk2 in HMVEC, but also equal loading. Since LPS induced activation of Pyk2, but not FAK, we focused our studies on the role of the tyrosine kinase, Pyk2.

3.3 Inhibition of Pyk2 kinase activity modulates LPS-induced MCP-1 production

Several lines of recent evidence suggest the involvement of Pyk2 in cytokine-induced signaling pathways as well as pathways leading to cytokine production (Anand et al., 2008; Di Cioccio et al., 2004; Katagiri et al., 2000; Miyazaki et al., 1998). In endothelial cells, LPS-induced production of inflammatory cytokines, specifically MCP-1, contributes significantly to the inflammatory process (Maus et al., 2002). Hence we tested the possible involvement of Pyk2 in LPS-induced MCP-1 production. We used various approaches to test the potential involvement of Pyk2 in LPS signaling in HMVEC.

3.3.1 Tyrphostin A9, a potent inhibitor of Pyk2 suppresses LPS-induced MCP-1 production and trans-endothelial migration of monocytes—Firstly, we used Tyrphostin A9 (AG17), a selective pharmacologic inhibitor blocking the Pyk2 signaling pathway (Fuortes et al., 1999). In the present study, we showed that Tyrphostin A9 pretreatment of HMVEC, at a concentration of 5 μ M, inhibited LPS-induced Pyk2 phosphorylation (Figure 3A). Furthermore, the inhibitor blocked LPS-induced MCP-1 expression in the endothelial cells in a concentration-dependent manner (Figure 3B).

MCP-1 has been shown to be a potent mediator of the extravasation and accumulation of monocytes at the sites of injury by their adhesion to and migration through the endothelial lining. Hence, we assessed the transendothelial migration (TEM) of monocytes towards the supernatants of LPS-stimulated endothelial cells pre-treated with Tyrphostin A9 to determine if the MCP-1 produced was functionally active. As shown in Figure 3C, pre-treatment with various concentrations of Tyrphostin A9 significantly inhibited monocyte migration across a HMVEC monolayer compared with supernatants of cells treated with LPS only. These results confirmed a physiological function of Pyk2 activity in regulating MCP-1 production.

3.3.2 A kinase inactive mutant of Pyk2 attenuates LPS-induced MCP-1 production and trans-endothelial migration (TEM) of monocytes—We next tested the inhibition of Pyk2 kinase activation using AAV vectors expressing a kinase-inactive Pyk2 mutant (Pyk2K457A). Over-expression of Pyk2MT was confirmed by Western blotting analysis of the cell lysates (Figure 4A). We observed that endothelial cells transduced with Pyk2MT exhibited decreased LPS-induced MCP-1 production as compared to the vector (β -galactosidase)-transduced cells (Figure 4B). In addition, the transduction of LPS-stimulated endothelial cells with Pyk2MT significantly inhibited monocyte migration across a HMVEC monolayer compared with supernatants of cells treated with LPS only (Figure 4C). This result provides direct evidence for the role of Pyk2 in LPS-induced MCP-1 production.

3.3.3 Knockdown of Pyk2 using siRNA suppresses LPS-induced MCP-1 production and trans-endothelial migration (TEM) of monocytes—Finally, we

knocked down Pyk2 expression in HMVEC using small interfering Pyk2 RNA (Pyk2siRNA). As shown in Figure 5A, transfection with Pyk2siRNA specifically reduced Pyk2 expression in comparison to the non-targeting siRNA duplex. The Pyk2siRNA-treated cells showed decreased MCP-1 production compared to cells treated with non-targeting control (Figure 5B). The supernatants from Pyk2-siRNA-treated cells demonstrated attenuated migration of monocytes in comparison to cells treated with non-targeting siRNA (Figure 5C).

Taken together, these data suggest that Pyk2-dependent activation is an important part of the LPS/MCP-1 signaling pathway.

3.4 Pyk2 regulates MCP-1 production through the p38/NF- κ B pathway

To investigate the signaling pathways by which Pyk2 mediates MCP-1 production, we focused on the MAP kinase pathways. The MAP kinase pathways, specifically p38 MAP kinase and the p44/42 MAP kinases, are known to be a major conduit for transmission of the signals downstream of Pyk2 that regulate cell proliferation, cell cycle control, and chemotaxis (Lev et al., 1995; Pandey et al., 1999). Preincubation of HMVEC with Pyk2 inhibitor strongly inhibited LPS-induced phosphorylation of p38 (Figure 6A), but not pERK (data not shown). We also investigated the effect of p38 MAP kinase inhibitor (SB203580) on MCP-1 production. Treatment of HMVEC with SB203580 significantly abrogated LPS-induced MCP-1 production (Figure 6B), thus confirming the role of p38 MAP kinase in the process.

NF- κ B is the most important factor that mediates transcription of the MCP-1 gene downstream of p38 MAP kinase in response to LPS in HMVEC (Goebeler et al., 2001). To decipher the role of NF- κ B in this pathway, we examined the effect of the Pyk2 inhibitor on LPS-induced NF- κ B activity. LPS induced a 4-fold increase in NF- κ B DNA binding activity (Figure 6C). Pretreatment of cells with 1 μ M, 5 μ M, or 10 μ M Tyrphostin A9 dose-dependently inhibited LPS-induced NF- κ B activity by $36.6 \pm 5.8\%$, $55 \pm 10\%$, and $57.5 \pm 4.1\%$, respectively ($P < 0.05$ compared with LPS only). This observation strongly suggests that the mechanism of action of Pyk2 in mediating MCP-1 production involves the NF- κ B pathway.

4. Discussion

The bacterial endotoxin, LPS stimulates endothelial cells to express chemokines that initiate the activation and recruitment of circulating leukocytes at inflammatory tissue sites. MCP-1 is a member of the beta (C-C) subfamily of chemokines that attracts blood monocytes and T-cells and facilitates their trans-endothelial migration (Uguccioni et al., 1995). In particular, during septicemia MCP-1, along with other chemokines, participates in a series of cellular events that severely damage the endothelium and surrounding tissues (Baggiolini et al., 1997). Though the effects of LPS on cytokine production in endothelial cells are well elucidated, the mechanism involved in this process has not been characterized in detail. In this regard, we have recently demonstrated the involvement of Pyk2 in LPS-mediated IL-8 expression in macrovascular endothelial cells (Anand et al., 2008). Despite the differences in the pathways governing the inducible expression of IL-8 and MCP-1 in endothelial cells, they share molecules that modulate their expression. Hence we questioned whether Pyk2 also has a role in LPS-induced production of MCP-1, attempting to elucidate the molecular mechanism involved in this pathway. Here, we demonstrate a signaling pathway involving the Pyk2, p38 MAP kinase and NF- κ B pathway, that mediates LPS-induced MCP-1 production

The non-receptor tyrosine kinase Pyk2 is rapidly becoming recognized as a tyrosine kinase of central importance (Avraham et al., 2000). It has been implicated in the regulation of key signaling intermediates and thus its role in cellular functions is only beginning to be defined. Diverse stimuli such as G-protein-coupled receptor (GPCR) agonists, vascular endothelial growth factor (VEGF) and IL-1 α have been shown to activate Pyk2 in endothelial cells (Keogh

et al., 2002). Our experiments demonstrate that LPS rapidly increases tyrosine phosphorylation of Pyk2 in microvascular endothelial cells. The increase in Pyk2 phosphorylation was observed in as little as 5 minutes. The involvement of Pyk2 in LPS-induced MCP-1 production was confirmed using multiple strategies, namely AAV-expressing Pyk2 kinase-inactive mutant, Tyrphostin A9, and Pyk2-specific siRNA. Tyrphostin A9, a specific pharmacologic inhibitor has been identified as an effective tool for investigating the role of Pyk2 in cellular signaling (Fuortes et al., 1999). AAV vectors have been used successfully to deliver genes into endothelial cells both *in vitro* and *in vivo* (Fan et al., 2008; Stachler and Bartlett, 2006). Our standardization experiments revealed greater than 85% HMVEC transduced with the control AAV- β -gal vector to be β -galactosidase positive (data not shown), indicating an extremely high efficiency of the rAAV-based approach.

Culture supernatants from LPS-treated cells displayed significant chemotactic activity for monocytes. We observed a slightly higher proportion of monocyte migration using supernatants of LPS-treated cells (containing 0.8 ng/ml MCP-1 as measured by ELISA) compared with the positive control (1 ng/ml MCP-1). These data imply that the culture supernatants of LPS-treated HMVEC contain other chemoattractants including MCP-1 for the TEM of monocytes. Inhibition of monocyte migration towards supernatants of LPS-treated HMVEC that were transduced with AAV-Pyk2MT, pre-treated with Tyrphostin A9, or transfected with Pyk2 specific siRNA suggested that inhibition of Pyk2 activity blocked monocyte infiltration across microvascular endothelial cells by predominantly down-regulating MCP-1.

A significant contributor to endothelial pathobiology is the heterogeneity of endothelial cells: in particular, the difference between cells derived from large vessels and from the microvasculature (Zachlederova and Jarolim, 2006). Multiple differences in between HUVEC and HMVEC have been reported, such as expression of chemokine receptors and other cell-surface markers (Beck et al., 1999; Salcedo et al., 2000). Although the majority of the previous *in vitro* studies on EC activation use HUVEC, the HMVEC is the primary site at which extravasation of leukocytes occurs (Zachlederova and Jarolim, 2006). We therefore used HMVEC to evaluate LPS-induced MCP-1 expression in the present study. A comparison of LPS-induced MCP-1 production between HMVEC and HUVEC revealed an enhanced response in the microvascular endothelial cells (HMVEC). Similar findings have been reported in a previous study which showed that lung microvascular endothelial cells (LMVEC) are more sensitive to LPS-induced MCP-1 production than HUVEC (Beck et al., 1999).

Of the signaling pathways activated by LPS, the MAP kinase pathways have both been shown to be directly involved in the production of cytokines (Adams et al., 2001). In particular, the central role of p38 MAP kinase-dependent signaling in LPS-stimulated endothelium has been highlighted in recent studies (Hashimoto et al., 2000; Hippenstiel et al., 2000; Kotlyarov et al., 1999). Since Pyk2 has been implicated in the regulation of MAP kinases (Della Rocca et al., 1997; Rocic et al., 2001), we examined the role of p38 MAP kinase and p44/42 MAP kinase downstream of Pyk2 in MCP-1 production. We demonstrate that LPS-induced MCP-1 production in HMVEC is mediated through the p38 MAP kinase pathway. Inhibition of Pyk2 activity blocked LPS-induced p38 MAP kinase activation, indicating that p38 MAP kinase functions downstream to Pyk2.

NF- κ B is a ubiquitous and pleiotropic regulator of many genes involved in LPS-induced inflammatory responses in endothelial cells. A previous study has demonstrated that MCP-1 expression is tightly controlled by the transcription factor NF- κ B downstream of p38 MAP kinase in endothelial cells (Nemeth et al., 2002). Therefore, we investigated whether inhibition of Pyk2 blocked NF- κ B activation in response to LPS. We demonstrate that tyrosine

phosphorylation of Pyk2 may be critical in activating NF- κ B, further leading to transcription of genes that regulate cytokine expression.

5. Conclusions

In summary, our results indicate a signal transduction pathway, where LPS activates Pyk2, which in turn activates p38 MAP kinase. The p38 MAP kinase then activates the transcription factor, NF- κ B, driving the transcriptional activation of MCP-1 and possibly other cytokines. Our results reiterate an active role for microvascular endothelial cells in the induction of MCP-1 in response to LPS. Additional studies to characterize the molecules upstream of Pyk2, as well as its binding partners, should provide an insight into the complete mechanism by which LPS modulates MCP-1 production in endothelial cells. Consistent with the pathophysiological role played by MCP-1 in several inflammatory diseases, our results indicate that Pyk2 may represent a suitable target for the development of new anti-inflammatory drugs.

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Abbreviations

LPS	Lipopolysaccharide
rAAV	recombinant adeno-associated virus
MCP-1	Monocyte chemotactic protein-1
HMVEC	Human microvascular vein endothelial cells
HUVEC	Human umbilical vein endothelial cells
siRNA	small inhibitory RNA
FAK	Focal adhesion kinase
IL	interleukin
EGM	Endothelial Growth Medium
PBMCs	peripheral blood mononuclear cells

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Figure 1. LPS-induces time and dose-dependent MCP-1 production in both human umbilical vein endothelial cells (HUVEC) and human microvascular endothelial cells (HMVEC)

HUVEC (open) and HMVEC (filled) were treated with LPS (100 ng/ml) for various periods of time (A) or were treated with various doses of LPS (100 ng/ml) for 16 hours (B). The supernatants were collected and assayed for MCP-1 production by ELISA. * $P < 0.05$ compared to the HUVEC cells. Data represent the mean $\pm$ S.D of three independent experiments.

Figure 2. LPS induces tyrosine phosphorylation of Pyk2 in HMVEC

(A) Human microvascular endothelial cells were stimulated with LPS (100 ng/ml) for the indicated periods of time. The lysates were analyzed by Western blotting with antibodies to phospho-Pyk2 (Tyr402). The same blot was probed with anti-Pyk2 antibody. (B) Lysates obtained from HMVEC stimulated with LPS (100 ng) for various periods of time were also analyzed by Western blotting with antibodies to phospho-FAK (Tyr397). Data show one representative experiment out of three independent experiments.

Figure 3. Inhibition of Pyk2 activity with Tyrphostin A9 inhibits LPS-induced MCP-1 production and trans-endothelial migration of human monocytes

(A) HMVEC were pretreated with vehicle or Tyrphostin A9 (5 μM) for 1 hour at 37°C. The cells were then stimulated with LPS(100ng/ml) for 15 minutes in Endothelial Basal Medium (EBM) with 0.5% FBS. The cells were lysed and analyzed by Western blotting with anti-phospho-Pyk2 (Tyr 402) antibodies. The same blots were probed with anti-Pyk2 antibodies. (B) HMVEC pre-incubated with vehicle or various concentrations of Tyrphostin A9 for 1 hour were cultured with or without LPS (100 ng/ml) and the concentration of MCP-1 in the culture supernatants was determined 16 hours after stimulation. * P< 0.05 compared to the vehicle control. Data represent the mean ± S.D of three independent experiments. (C) HMVEC cells

pre-treated with Tyrphostin A9 were stimulated with LPS (100ng/ml) for 16 hours. The supernatants were then tested for their ability to induce monocyte migration using a trans-endothelial migration assay (TEM) *P<0.05 versus vehicle control.

A. **B.** **C.**

Figure 4. Transduction of HMVECs with AAV-expressing kinase-inactive Pyk2 inhibits LPS-induced MCP-1 production and trans-endothelial migration of human monocytes
 HMVEC cells were transduced with rAAV-expressing Pyk2 mutant (Pyk2MT) or AAV- β galactosidase (Vector Control - VC). (A) Overexpression of the mutant was demonstrated by Western blot analysis with anti-Pyk2 antibodies 48 hours after transduction (left panel). (B) Transduced cells were tested for their ability to produce MCP-1 upon LPS stimulation (100 ng/ml) using a commercial ELISA kit (Endogen) (right panel). * P<0.05 versus the AAV-Vector control. (C) Supernatants from the Pyk2 mutant-overexpressing HMVEC stimulated with LPS (100 ng/ml) were also tested for their ability to induce monocyte migration using a

trans-endothelial membrane assay (TEM). *P<0.05 compared to the AAV-Vector control. Data represent the mean $\pm$ S.D of three independent experiments.

Figure 5. Knockdown of Pyk2 expression using specific Pyk2 siRNA attenuates LPS-induced MCP-1 production and decreases the trans-endothelial migration of human monocytes
 (A) HMVEC were transfected with 100 nM Pyk2 specific siRNA and non-targeting siRNA (NT siRNA) using lipofectamine (Invitrogen). The knock-down of Pyk2 expression was analyzed by Western blot analysis with Pyk2 antibodies. Anti-actin antibody was used as an internal control. * $P < 0.05$ compared to the non-targeting siRNA control. (B) The supernatants of the siRNA-treated cells were analyzed for MCP-1 expression after stimulation with LPS (100 ng/ml). * $P < 0.05$ compared to the non-targeting siRNA control. Data represent the mean $\pm$ S.D of three independent experiments.

Figure 6. Pyk2 mediates MCP-1 production through the p-38 MAP kinase/NF- κ B pathway
 (A) HMVEC pre-treated with vehicle or Tyrphostin A9 (5 μ M) for 1 hour at 37°C were stimulated with LPS for 15 minutes. The cells were lysed and analyzed by Western blotting with anti-phospho-p38 antibodies. The same blots were probed with anti-p38 antibodies. Data show one representative experiment out of three independent experiments. (B) HMVEC pre-treated with vehicle or the p38 MAP kinase inhibitor (SB203580) for 1 hour were stimulated with LPS (100 ng/ml), and the concentrations of MCP-1 in the supernatants was measured 16 hours after stimulation. *P<0.05 compared to the vehicle control. (C) HMVEC cells were pre-incubated with Tyrphostin A9 (5 μ M) for 1 hour before stimulation with LPS (100 ng/ml) for the indicated periods of time. After treatment, nuclear extracts were prepared and NF- κ B (p65)

DNA binding activity was quantified by ELISA. Data shown are means $\pm$ SEM of three independent experiments. *P < 0.05 compared with cells treated with LPS only.