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Lipopolysaccharide enhances transforming growth factor β 1-induced PDGF-B expression in bile duct epithelial cells

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Abstract

Background and Aim—Platelet-derived growth factor (PDGF)-B is a potent profibrogenic mediator expressed by bile duct epithelial cells (BDECs) that contributes to liver fibrosis after bile duct ligation. However, the mechanism of PDGF-B induction in BDECs during cholestasis is not known. Transforming growth factor β (TGF β) and lipopolysaccharide (LPS) also contribute to the profibrogenic response after bile duct ligation. We tested the hypothesis that LPS and TGF β 1 synergistically induce PDGF-B expression in BDECs.

Methods—Transformed human BDECs (MMNK-1 cells) and primary rat BDECs were stimulated with LPS and/or TGF β 1, and signaling pathways through which LPS potentiates TGF β 1-induced PDGF-B mRNA expression were investigated.

Results—Stimulation of MMNK-1 cells with LPS alone did not significantly induce PDGF-B mRNA expression. However, LPS cotreatment enhanced TGF β 1 induction of PDGF-B mRNA in MMNK-1 cells and also in primary rat BDECs. Importantly, cotreatment of MMNK-1 cells with LPS and TGF β 1 also significantly increased PDGF-BB protein expression. Interestingly, LPS did not affect TGF β 1 activation of a SMAD-dependent reporter construct. Rather, stimulation of MMNK-1 cells with LPS, but not TGF β 1, increased JNK1/2 phosphorylation. Expression of dominant negative JNK2, but not dominant negative JNK1, inhibited the LPS potentiation of TGF β 1-induced PDGF-B mRNA expression in MMNK-1 cells. In addition, LPS treatment caused I κ B α degradation and activation of a NF κ B-dependent reporter construct. Expression of an I κ B α super repressor inhibited activation of NF κ B and attenuated LPS potentiation of TGF β 1-induced PDGF-B mRNA.

Conclusions—The results indicate that LPS activation of NF κ B and JNK2 enhances TGF β 1-induced PDGF-B expression in BDECs.

Keywords

cholestasis; liver; gene expression; fibrosis; bile ducts

Introduction

Platelet derived growth factor-B (PDGF-B) plays an essential role in wound healing and fibrosis.^{1–3} PDGF isoforms homodimerize or heterodimerize and stimulate fibroblasts to produce extracellular matrix proteins (i.e., collagens).^{3,4} The PDGF-BB homodimer is a potent profibrogenic mediator in liver where it stimulates collagen production by portal

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fibroblasts and triggers hepatic stellate cell chemotaxis, which both contribute to peribiliary fibrosis.⁵ Inhibition of PDGF-B or its receptor reduced liver fibrosis in rodents after bile duct ligation (BDL),^{6,7} an established model of obstructive cholestasis and fibrosis. Interestingly, during cholestasis, the expression of PDGF-B increases in proliferating bile duct epithelial cells (BDECs).⁸ Of importance, the mechanism whereby PDGF-B expression increases in BDECs during chronic cholestatic liver injury is not known.

Potential inducers of PDGF-B expression in BDECs during cholestasis include transforming growth factor β (TGF β) and bacterial lipopolysaccharide (LPS), which both contribute to liver fibrosis during cholestasis.⁹⁻¹² The α V β 6 integrin expressed by BDECs during cholestasis localizes activation of latent TGF β near BDECs and portal fibroblasts.^{9,13} Of importance, previous studies indicate that PDGF-B expression is stimulated by TGF β .¹⁴ Additional studies found that translocation of LPS into the portal circulation and subsequent activation of toll-like receptor 4 (TLR4) signaling contributed to liver fibrosis.¹⁵ Because of their location, BDECs are poised to integrate profibrogenic signals from several mediators, including TGF β and LPS, and subsequently express factors that stimulate liver fibrosis. However, few studies have examined the synergistic effects of these mediators on intracellular signaling and profibrogenic gene induction in BDECs.

LPS activation of TLR4 triggers intracellular signaling via mitogen-activated protein kinases (MAPKs) and NF κ B, among other pathways, which control LPS-induced gene expression.¹⁶ TGF β -induced gene expression is mediated by activation of the SMAD transcription factors and also via activation of non-canonical signaling through the MAPKs.¹⁷ Of importance, TGF β -induced PDGF-B expression is mediated by SMAD activation.¹⁴ Several studies have identified critical points of interaction between LPS and TGF β signaling.^{15,18,19} Suggesting a potential role for such an interaction in liver fibrosis, a previous study found that LPS-mediated downregulation of the TGF β pseudoreceptor Bambi increased TGF β -dependent SMAD activation in hepatic stellate cells.¹⁵ However, the interaction between TGF β and LPS signaling in BDECs has not been investigated.

The aim of the present study was to test the hypothesis that LPS stimulation enhances TGF β -induced PDGF-B mRNA expression in primary rat and transformed human BDECs. Moreover, we sought to determine the mechanism underlying the LPS-dependent enhancement of TGF β -induced PDGF-B mRNA expression in BDECs.

Methods

Cell culture and stimulation

Transformed human bile duct epithelial cells (MMNK-1) were kindly provided by Dr. Melissa Runge-Morris (Wayne State University, Detroit, MI) on behalf of Dr. Naoya Kobayashi (Okayama University, Okayama, Japan).²⁰ MMNK-1 cells were grown in low glucose Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 10 U/mL penicillin, and 10 μ g/mL streptomycin (Sigma, St. Louis, MO).²¹ Primary rat bile duct epithelial cells (rBDECs) were kindly provided by Dr. Vasanthi Bhaskaran, Dr. Monica Otieno, and Dr. Lois Lehman-McKeeman (Bristol-Myers Squibb, Princeton, NJ). rBDECs were maintained in LYD medium as described.⁹ All cells were maintained in a humidified 5% CO₂ incubator at 37°C. MMNK-1 cells were serum-starved for 1 hr then stimulated with 5 ng/mL TGF β 1 (Peprotech, Rocky Hill, NJ) or vehicle control (0.001% BSA in PBS) and/or 100 ng/mL LPS (*Escherichia coli*, serotype 0111:B4, Sigma) or vehicle control (PBS). rBDECs were provided fresh LYD medium then stimulated with 100 ng/mL LPS or vehicle control and one hour later stimulated with 5 ng/mL TGF β 1 or vehicle control.

DNA constructs

The NFκB-dependent and renilla luciferase reporter constructs were obtained from Promega (Madison, WI). The IκBα super repressor (IκBα-SR, Addgene plasmid 24143, donated by Dr. Warner Greene),²² dominant negative IκB kinase 2 (DN-IKK2) (Addgene plasmid 11104, donated by Dr. Anjana Rao),²³ DN-JNK1 (Addgene plasmid 13846, donated by Dr. Roger Davis),²⁴ DN-JNK2 (Addgene plasmid 13761, donated by Dr. Roger Davis),²⁵ and SMAD-dependent (SBE4-Luc) luciferase reporter construct (Addgene plasmid 16495, donated by Dr. Bert Vogelstein)²⁶ were purchased from Addgene (Cambridge, MA). Bacteria containing the DNA constructs were grown according to Addgene protocol for bacterial recovery. DNA was purified using an EndoFree Plasmid Maxi kit (Qiagen, Valencia, CA). pcDNA3.1 empty vector was kindly provided by Dr. Curtis Klaassen (University of Kansas Medical Center, Kansas City, KS).

Transfections

MMNK-1 cells were transfected using 6 μL FuGENE 6 (Roche, Indianapolis, IN) per well of a 6-well plate. For NFκB-related experiments, 0.5 μg pcDNA3.1, IκBα-SR, or DN-IKK2, 0.475 μg NFκB-dependent luciferase reporter, and 0.025 μg renilla were used per well of a 6-well plate. For SMAD-related experiments, 0.975 μg SMAD-dependent luciferase reporter and 0.025 μg renilla were used per well of a 6-well plate. For JNK-related experiments, 1 μg pcDNA3.1, DN-JNK1, or DN-JNK2 were used per well of a 6-well plate. Prior to transfection, FuGENE 6 and DNA were combined in serum- and antibiotic-free DMEM for 15 min before addition to MMNK-1 cell culture medium. Cells were then incubated for 16–24 hr with transfection reagent and DNA prior to treatment.

RNA isolation, cDNA synthesis, and real-time PCR

Total RNA was isolated from MMNK-1 cells and rBDECs using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH) per the manufacturer's protocol. One microgram of total RNA was utilized for the synthesis of cDNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) and C1000 thermal cycler (Bio-Rad, Hercules, CA). Levels of PDGF-B and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA were determined using either TaqMan gene expression assays (Applied Biosystems) or TaqMan Primetime qPCR assays (IDT, Coralville, IA), TaqMan gene expression master mix (Applied Biosystems), and a StepOnePlus thermal cycler (Applied Biosystems). Human PDGF-B (NM_002608) primer sequences were 5'-ATG ATC TCC AAC GCC TGC -3' (forward primer), 5'-TCA GCA ATG GTC AGG GAA C -3' (reverse primer) and 5'-/56-FAM/CAG AGT GGG/ZEN/AGC GGG TCA TGT T/3IABkFQ/-3' (probe). Human GAPDH (NM_002046) primer sequences were 5'-ACA TCG CTC AGA CAC CAT G -3' (forward primer), 5'-TGT AGT TGA GGT CAA TGA AGG G -3' (reverse primer) and 5'-/56-FAM/AAG GTC GGA/ZEN/GTC AAC GGA TTT GGT C/3IABkFQ/-3' (probe). Rat GAPDH (NM_017008) primer sequences were 5'- AAC CCA TCA CCA TCT TCC AG -3' (forward primer), 5'- CCA GTA GAC TCC ACG ACA TAC -3' (reverse primer) and 5'- /56-FAM/CAG CAC CAG/ZEN/CAT CAC CCC ATT TG/3IABkFQ/-3' (probe). Human PDGF-B and human and rat GAPDH were purchased from IDT. Rat PDGF-B primer/probe gene expression assays were purchased from Applied Biosystems (Assay ID Rn01502596.m1). The expression of PDGF-B was adjusted relative to GAPDH expression levels, and relative expression level was determined using the comparative Ct method.

Luciferase assay

MMNK-1 cells were transfected with SMAD-dependent or NFκB-dependent luciferase reporter constructs and a renilla luciferase reporter construct as described.²¹ Cells were scraped into PBS, subjected to centrifugation at 7500 × g for 5 min at 4°C, lysed in 100 μL

passive lysis buffer (Promega), and incubated on ice for 15 min. Firefly luciferase and renilla activities were determined using a Dual-Glo Luciferase Assay System (Promega) and a TECAN Infinite M200 plate reader (Tecan, Durham, NC). For each sample, luciferase relative light units were adjusted based on renilla activity as an estimation of transfection efficiency.

Western blotting

Cytosolic protein extracts (IkB blots) or RIPA extracts (JNK blots) were obtained as described previously,²¹ subjected to SDS-PAGE (Bio-Rad Criterion, 4–12% Bis-Tris gels, Hercules, CA), and transferred to Immobilon PVDF (Millipore, Billerica, MA) by semi-dry transfer. The membranes were blocked for 1 hr at room temperature in 3% BSA in TBST and incubated overnight at 4°C in either anti-IkB α (1:1000) antibody, anti-GAPDH (1:10000) antibody, anti-phospho-JNK1/2 (1:1000) antibody, or anti-JNK1/2 (1:2000) antibody (Cell Signaling Technology, Inc., Danvers, MA) in 1% BSA in TBST. Membranes were then washed with TBST and incubated 1 hr with anti-rabbit HRP-conjugated secondary antibody (1:1000) for IkB α and JNK1/2 blots (Pierce, Rockford, IL) or anti-mouse HRP-conjugated secondary antibody (1:1000) for GAPDH blots (Cell Signaling Technology). Membranes were incubated with West Pico ECL reagent (Pierce) before exposure to Blue Lite Autorad Film (ISC Bioexpress, Kaysville, UT). Developed films were scanned using an Epson Expression 1680 scanner (Epson America, Torrance, CA), and densitometry was performed using Quantity One 4.6.9 software (Bio-Rad).

PDGF-BB ELISA

MMNK-1 cells were serum-starved for 1 hr then stimulated with 5 ng/mL TGF β 1 (Peprotech, Rocky Hill, NJ) or vehicle control (0.001% BSA in PBS) and/or 100 ng/mL LPS (*Escherichia coli*, serotype 0111:B4, Sigma) or vehicle control (PBS) for 4 hr. Media was collected, and the concentration of PDGF-BB in the media was determined using a commercially available DuoSet ELISA (R&D Systems, Minneapolis, MN).

Statistics

Comparison of two groups was performed using a paired t-test. Comparison of three or more groups was performed using two-way analysis of variance followed by the Student-Newman-Keuls post-hoc test for multiple comparisons. For data that was resistant to transformation, comparison of three or more groups was performed using a Kruskal-Wallis one way analysis of variance by ranks followed by a pairwise comparison with Dunn's method. The criterion for statistical significance was $p < 0.05$.

Results

LPS potentiates TGF β 1-stimulated PDGF-B mRNA in MMNK-1 cells and primary rat BDECs

MMNK-1 cells and rBDECs were stimulated with 5 ng/mL TGF β 1 in the presence or absence of 100 ng/mL LPS. LPS stimulation alone had no effect on PDGF-B mRNA or protein expression in MMNK-1 cells (Figure 1A,B) but did slightly increase PDGF-B mRNA expression at 2 hr in rBDECs (Figure 1C). TGF β 1 stimulation alone increased the expression of PDGF-B mRNA in both MMNK-1 cells (Figure 1A) and in rBDECs (Figure 1C) but did not increase PDGF-BB protein expression in MMNK-1 cells at 4 hr (Figure 1B). Interestingly, LPS cotreatment significantly enhanced TGF β 1-stimulated PDGF-B mRNA in both cell types, with peak mRNA induction evident at 2 hr (Figure 1A,C). In addition, cotreatment of MMNK-1 cells with LPS and TGF β 1 significantly increased PDGF-BB protein expression at 4 hr (Figure 1B).

LPS does not affect TGF β 1-induced activation of SMAD in MMNK-1 cells

TGF β 1 induces PDGF-B expression by activating the SMAD family of transcription factors.¹⁴ Therefore, one mechanism whereby LPS could enhance TGF β 1-induced PDGF-B mRNA expression is through regulating SMAD activation. Stimulation of MMNK-1 cells with TGF β 1 alone, but not LPS alone, activated a SMAD-dependent reporter construct after 4 hr (Figure 2). Moreover, LPS cotreatment did not affect TGF β 1-induced SMAD-dependent luciferase reporter activity (Figure 2). The results indicate that LPS cotreatment does not affect TGF β 1 activation of SMADs in MMNK-1 cells.

LPS activation of JNK2 enhances TGF β 1-induced PDGF-B mRNA expression in MMNK-1 cells

LPS stimulation of MMNK-1 cells increased JNK1/2 phosphorylation within 60 min (Figure 3A). Densitometry indicated a slight reduction in JNK1/2 phosphorylation in cells co-stimulated with LPS and TGF β 1, but this did not achieve statistical significance (Figure 3A, LPS = 1.0 ± 0.19 vs. LPS+TGF β 1 = 0.76 ± 0.10 , $p > 0.05$, $n=3$). To determine if the activation of either JNK1 or JNK2 contributed to PDGF-B expression, MMNK-1 cells were transfected with DN-JNK1, DN-JNK2, or control expression vectors, and PDGF-B mRNA expression was evaluated 2 hr and 4 hr after stimulation with LPS and TGF β 1. Neither DN-JNK1 nor DN-JNK2 affected induction of PDGF-B mRNA in cells treated with TGF β 1 alone (Figure 3B,C). Interestingly, expression of DN-JNK2, but not DN-JNK1, significantly reduced LPS potentiation of TGF β 1-induced PDGF-B mRNA expression in MMNK-1 cells at 2 hr (52.3% decrease, Figure 3B) but not at 4 hr (Figure 3C).

LPS activation of NF κ B enhances TGF β 1-induced PDGF-B mRNA expression in MMNK-1 cells

Stimulation of MMNK-1 cells with TGF β 1 did not affect cytosolic I κ B α levels (Figure 4A). In contrast, LPS stimulation triggered I κ B α degradation after 1 hr in MMNK-1 cells and this was not impacted by TGF β 1 cotreatment (Figure 4A). LPS also stimulated I κ B α degradation in rBDECs after 1 hr (data not shown). In agreement with the inability of TGF β 1 to initiate I κ B α degradation, stimulation of MMNK-1 cells with TGF β 1 did not activate a NF κ B-dependent reporter construct (Figure 4B). In contrast, LPS stimulation significantly increased NF κ B-dependent luciferase activity in MMNK-1 cells, and this was unaffected by TGF β 1 cotreatment (Figure 4B).

To determine if LPS activation of NF κ B contributed to potentiation of PDGF-B mRNA expression in this model, we transfected MMNK-1 cells with a DN-IKK2 expression vector. Expression of DN-IKK2 significantly inhibited LPS-induced NF κ B-dependent luciferase reporter activity, although the inhibition was not complete (Figure 5A). In MMNK-1 cells transfected with control vector, LPS enhanced TGF β 1 induction of PDGF-B mRNA at both 2 hr and 4 hr (Figure 5B–C). DN-IKK2 did not affect PDGF-B mRNA expression in MMNK-1 cells stimulated with TGF β 1 alone (Figure 5B–C). However, DN-IKK2 expression decreased LPS potentiation of PDGF-B mRNA expression at 2 hr (30.8% decrease) and at 4 hr (32.5% decrease), although the decrease only achieved statistical significance at 4 hr (Figure 5B–C).

Next, we transfected MMNK-1 cells with an I κ B α super repressor (I κ B α -SR), which is resistant to IKK β phosphorylation and subsequent degradation, thus inhibiting NF κ B activation.²² Expression of I κ B α -SR completely inhibited LPS-stimulated NF κ B-dependent luciferase reporter activity in MMNK-1 cells after 4 hr (Figure 5D). Expression of I κ B α -SR had no effect on PDGF-B mRNA induction in MMNK-1 cells stimulated with TGF β 1 alone (Figure 5E–F). In agreement with the ability of I κ B α -SR to inhibit NF κ B activation to a greater extent than DN-IKK2, the inhibition of PDGF-B expression with I κ B α -SR after

TGF β 1 and LPS cotreatment at 2 hr was much greater than the inhibition with DN-IKK2 (63.8% decrease). In the presence of the I κ B α -SR, LPS no longer significantly potentiated TGF β 1-induced PDGF-B mRNA expression at 2 hr (Figure 5E), and LPS potentiation of TGF β 1-induced PDGF-B mRNA expression was significantly decreased at 4 hr (38.6% decrease, Figure 5F).

Discussion

BDECs actively participate in biliary fibrosis by expressing profibrogenic mediators.^{9,27,28} BDEC expression of the heterodimeric α V β 6 integrin during chronic cholestasis results in activation of latent TGF β ¹³ and subsequent stimulation of portal fibroblasts to produce extracellular matrix.²⁹ Activated TGF β further amplifies profibrogenic responses by inducing profibrogenic gene expression in BDECs. The studies presented here indicate that TGF β 1 increases PDGF-B expression in BDECs. Importantly, neutralizing PDGF-BB antibodies or PDGF receptor deficiency reduces liver fibrosis in models of cholestasis.^{6,7} Accordingly, induction of PDGF-B expression in BDECs is one potential mechanism whereby TGF β contributes to the development of liver fibrosis during cholestasis.

Utilizing BDL, a model of obstructive cholestasis, previous studies indicated that LPS derived from the portal circulation contributes to liver fibrosis by activating TLR4 signaling on nonhematopoietic cells.¹⁵ Potential nonhematopoietic cells activated by LPS during cholestasis are hepatic stellate cells or portal fibroblasts. LPS-induced activation of TLR4 expressed on stellate cells contributes to fibrosis by increasing chemokine secretion and increasing chemotaxis of Kupffer cells.^{15,30} Of interest, BDECs also express TLR4 and have been shown to produce cytokines in response to LPS stimulation.^{31,32} In agreement with a previous study in primary BDECs,³³ we found that LPS stimulation alone did not affect PDGF-B mRNA expression in MMNK-1 cells. However, LPS did slightly increase PDGF-B mRNA in primary rat BDECs in our studies. Importantly, LPS cotreatment significantly enhanced TGF β 1-dependent PDGF-B mRNA expression in both MMNK-1 cells and primary rat BDECs. In addition, LPS and TGF β 1 cotreatment significantly increased PDGF-BB protein expression. These results suggest that a novel pathway through which LPS could contribute to the development of fibrosis during cholestasis is by sensitizing BDECs to increase TGF β -induced PDGF-B expression.

Our studies focused on determining the mechanism whereby cotreatment of BDECs with TGF β and LPS increases PDGF-B mRNA expression. Cotreatment of MMNK-1 cells or primary rat BDECs with these mediators caused a rapid induction of PDGF-B mRNA, allowing us to identify signaling pathways regulating PDGF-B in this model. Although LPS and TGF β are both increased in cholestasis, it is difficult to determine the exact timing and duration of BDEC exposure to these mediators *in vivo*. Cholestatic liver injury is a chronic condition and is not fully replicated by the acute exposure of BDECs to various mediators in culture. Nonetheless, our studies suggest that coexposure of BDECs to LPS activates signaling pathways capable of enhancing TGF β -induced PDGF-B mRNA expression.

One potential mechanism whereby LPS could enhance TGF β 1-induced PDGF-B expression is by increasing activation of SMAD transcription factors. Previous studies have shown that SMADs participate in TGF β induction of PDGF-B expression.¹⁴ SMADs are a critical hub for crosstalk between LPS and TGF β signaling. In some cell culture models, prolonged LPS treatment enhanced expression of inhibitory SMAD7 to inhibit SMAD2/3 signaling, thus reducing TGF β -induced gene induction.^{18,19} In other models, TLR4-dependent downregulation of the TGF β pseudoreceptor Bambi sensitized hepatic stellate cells to TGF β 1 stimulation and enhanced activation of SMADs.¹⁵ Importantly, exposure of MMNK-1 cells to LPS for up to 24 hours did not affect Bambi mRNA expression (data not

shown). In agreement, we found that LPS costimulation did not affect basal or TGF β 1 activation of a SMAD-dependent reporter construct in MMNK-1 cells. Although SMADs are essential for TGF β 1 induction of PDGF-B mRNA expression,¹⁴ these results indicate that LPS does not increase SMAD activation in this model, suggesting that enhanced SMAD activation is not a critical mechanism whereby LPS potentiates TGF β 1-induced PDGF-B mRNA expression in BDECs.

The role of JNK in PDGF-B expression has not been extensively examined, although a DN-JNK construct reduced PDGF-B induction by angiotensin II.³⁴ Our results would suggest that JNK2 deficiency could reduce fibrosis after BDL by decreasing PDGF-B expression by BDECs. However, a previous study found that liver fibrosis after BDL is significantly reduced by JNK1 deficiency, whereas JNK2 deficiency increased fibrosis.³⁵ Of importance, the mice in this *in vivo* study lacked each JNK isoform in all cells, making conclusions about the relative contribution of JNK in BDECs challenging. Moreover, it is noteworthy to mention that JNK2 appears to be the dominant isoform in MMNK-1 cells. Indeed, JNK1 and JNK2 may play differing roles in hepatic stellate cells, portal fibroblasts, and BDECs to determine the overall outcome of fibrosis caused by chronic cholestasis.

Although activation of NF κ B alone is not sufficient to stimulate PDGF-B mRNA expression in MMNK-1 cells, we found that LPS activation of NF κ B enhances TGF β 1-induced PDGF-B mRNA expression. Of importance, NF κ B-dependent PDGF-B expression has been demonstrated in other models, including endothelial cells exposed to shear stress³⁶ and fibroblasts treated with TNF α .³⁷ However, in the context of BDECs, NF κ B may require the presence of an additional transcription factor such as SMADs to increase transcription of the PDGF-B gene. These results indicate that the ability of NF κ B to stimulate PDGF-B mRNA expression on its own is cell-type dependent.

Inhibition of NF κ B activation attenuated PDGF-B expression in MMNK-1 cells cotreated with LPS and TGF β 1, but not with TGF β 1 alone, suggesting that nuclear translocation of NF κ B contributes to the potentiation of TGF β 1-induced PDGF-B expression by LPS. Indeed, our studies suggest that in MMNK-1 cells, TGF β 1 does not stimulate NF κ B activation on its own, nor does it modulate the activation of NF κ B by LPS. Rather, TGF β 1 and LPS regulate parallel signaling pathways that both contribute to the maximal induction of PDGF-B mRNA expression. Interestingly, other studies have shown that TGF β 1-induced signaling inhibits LPS activation of MAPK and NF κ B and the induction of pro-inflammatory genes.^{38,39} Indeed, we found that LPS induction of the pro-inflammatory gene interleukin-8 was significantly reduced by TGF β 1 (data not shown), suggesting that although LPS can promote TGF β 1-induced profibrogenic gene induction in MMNK-1 cells, TGF β 1 retains anti-inflammatory activity in this *in vitro* model. These results suggest that the ultimate effect of LPS and TGF β 1 co-stimulation on gene expression in BDECs is highly dependent on the target gene. The inhibition of LPS-induced IL-8 expression by TGF β 1 is consistent with the known anti-inflammatory properties of this cytokine. Moreover, the finding that LPS enhances TGF β 1 induction of PDGF-B suggests a novel mechanism whereby LPS could contribute to liver fibrosis.

In conclusion, we found that TGF β 1 induction of PDGF-B mRNA expression in both MMNK-1 cells and in primary rat BDECs was enhanced by cotreatment with LPS. In MMNK-1 cells, this enhancement required activation of JNK2 and NF κ B. Each of these critical profibrogenic mediators are expressed during the progression of chronic cholestasis, suggesting possible coexposure of BDECs to these mediators. Accordingly, the synergistic induction of PDGF-B by LPS and TGF β 1 could represent an important mechanism of amplification of fibrosis during cholestasis.

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Figure 1. Effect of TGFβ1 and LPS on PDGF-B mRNA and protein induction in MMNK-1 cells and rat BDECs

(A,B) MMNK-1 cells were treated with 100 ng/mL LPS and/or 5 ng/mL TGFβ1 for the indicated times, and (A) PDGF-B mRNA and (B) PDGF-BB protein levels were determined. Data are expressed as mean ± SEM and as fold change vs. control-treated cells (mRNA) or as pg/ml (protein). (C) Primary rat BDECs were stimulated with 100 ng/mL LPS for one hour then treated with 5 ng/mL TGFβ1 for the indicated times, and PDGF-B mRNA levels were determined. Data are expressed as mean ± SEM and as fold change vs. control-treated cells. n=3–5 independent experiments *Significantly different than TGFβ1 alone, p<0.05.

Figure 2. Effect of TGFβ1 and LPS on SMAD reporter activity in MMNK-1 cells

MMNK-1 cells transfected overnight with a SMAD-dependent luciferase reporter construct were treated with 100 ng/mL LPS and/or 5 ng/mL TGFβ1 for 4 hr. Firefly luciferase relative light units were determined and normalized to renilla luciferase expression. Data are expressed as mean ± SEM and as fold change vs. control-treated cells, n=3–4 independent experiments *Significantly different than the same treatment in the absence of TGFβ1, p<0.05.

Figure 3. Role of the JNK1/2 MAPK pathway in LPS potentiation of TGFβ1-induced PDGF-B mRNA expression in MMNK-1 cells

(A) MMNK-1 cells were treated with 100 ng/mL LPS and/or 5 ng/mL TGFβ1 for 1 hr. Levels of phosphorylated and total JNK1/2 were determined by western blotting. A western blot representative of three independent experiments is shown. (B–C) MMNK-1 cells were transfected overnight with pcDNA3.1 (CTL), DN-JNK1, or DN-JNK2 expression vectors. Cells were treated with 100 ng/mL LPS and/or 5 ng/mL TGFβ1, and PDGF-B mRNA levels were determined (B) 2 hr or (C) 4 hr later. Data are expressed as mean ± SEM and as fold change vs. control-treated cells. n=3–4 independent experiments *Significantly different

than the same treatment in the absence of LPS, $p < 0.05$. #Significantly different from cells transfected with pcDNA3.1 and given the same treatment, $p < 0.05$.

Figure 4. NF κ B activation in MMNK-1 cells stimulated with LPS and TGF β 1

(A) MMNK-1 cells were treated with 100 ng/mL LPS and/or 5 ng/mL TGF β 1 for 1 hr. Cytosolic levels of IkB α and GAPDH were determined by western blotting. A western blot representative of four independent experiments is shown. (B) MMNK-1 cells were transfected overnight with a NF κ B-dependent luciferase reporter construct then treated with 100 ng/mL LPS and/or 5 ng/mL TGF β 1 for 4 hr. Firefly luciferase relative light units were determined and normalized to renilla luciferase expression. Data are expressed as mean $\pm$ SEM and as fold change vs. control-treated cells. n=10 independent experiments

*Significantly different than the same treatment in the absence of LPS, $p < 0.05$.

Figure 5. Role of NFκB in LPS potentiation of TGFβ1-induced PDGF-B mRNA expression in MMNK-1 cells
 (A,D) MMNK-1 cells were transfected overnight with a NFκB-dependent luciferase reporter construct and pcDNA3.1 (CTL) or (A) a DN-IKK2 construct or (D) an IκBα super repressor (IκBα-SR). Cells were treated with 100 ng/mL LPS and/or 5 ng/mL TGFβ1 for 4 hr. Firefly luciferase relative light units were determined and normalized to renilla luciferase expression. Data are expressed as mean ± SEM and as fold change vs. control-treated cells. (B–C) MMNK-1 cells were transfected overnight with CTL or DN-IKK2 then treated with 100 ng/mL LPS and/or 5 ng/mL TGFβ1. PDGF-B mRNA expression was determined (B) 2 hr or (C) 4 hr later. Data are expressed as mean ± SEM and as fold change vs. control-treated cells. (E–F) MMNK-1 cells were transfected overnight with CTL or IκBα-SR then treated with 100 ng/mL LPS and/or 5 ng/mL TGFβ1. PDGF-B mRNA expression was determined (E) 2 hr or (F) 4 hr later. Data are expressed as mean ± SEM and as fold change vs. control-treated cells. n=3–6 independent experiments *Significantly different than the same treatment in the absence of LPS, p<0.05. #Significantly different from cells transfected with pcDNA3.1 and given the same treatment, p<0.05.