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Krüppel-like factor 5 is an important mediator for lipopolysaccharide-induced proinflammatory response in intestinal epithelial cells

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ABSTRACT

Lipopolysaccharide (LPS) is a bacterially-derived endotoxin that elicits a strong proinflammatory response in intestinal epithelial cells. It is well established that LPS activates this response through NF-κB. In addition, LPS signals through the mitogen-activated protein kinase (MAPK) pathway. We previously demonstrated that the Krüppel-like factor 5 [KLF5; also known as intestine-enriched Krüppel-like factor (IKLF)] is activated by the MAPK. In the current study, we examined whether KLF5 mediates the signaling cascade elicited by LPS. Treatment of the intestinal epithelial cell line, IEC6, with LPS resulted in a dose- and time-dependent increase in KLF5 messenger RNA (mRNA) and protein levels. Concurrently, mRNA levels of the p50 and p65 subunits of NF-κB were increased by LPS treatment. Pretreatment with the MAPK inhibitor, U0126, or the LPS antagonist, polymyxin B, resulted in an attenuation of KLF5, p50 and p65 NF-κB subunit mRNA levels from LPS treatment. Importantly, suppression of KLF5 by small interfering RNA (siRNA) resulted in a reduction in p50 and p65 subunit mRNA levels and NF-κB DNA binding activity in response to LPS. LPS treatment also led to an increase in secretion of TNF-α and IL-6 from IEC6, both of which were reduced by siRNA inhibition of KLF5. In addition, intercellular adhesion molecule-1 (ICAM-1) levels were increased in LPS-treated IEC6 cells and this increase was associated with increased adhesion of Jurkat lymphocytes to IEC6. The induction of ICAM-1 expression and T cell adhesion to IEC6 by LPS were both abrogated by siRNA inhibition of KLF5. These results indicate that KLF5 is an important mediator for the proinflammatory response elicited by LPS in intestinal epithelial cells.

INTRODUCTION

The gastrointestinal tract is under constant assault by a wide variety of microbial pathogens. The body's first line of defense is the innate immune response. This requires the recognition of pattern-associated membrane proteins (PAMPs), which are bacterial components, by sensors called pattern recognition receptors (PRRs). Toll-like receptors (TLRs) are examples of these PRRs (1). A TLR is made up of single-spanning transmembrane domain, extracellular leucine-rich repeats and a cytosolic TIR (Toll/interleukin-1 receptor) domain. To date, 13 TLRs have been identified in mice and 11 in humans (2). TLR4, which is recognized by the bacterial endotoxin, lipopolysaccharide (LPS), utilizes a dual MyD88-dependent and -independent pathway (3–5). Both pathways lead to the activation of NF-κB and the subsequent induction of expression of proinflammatory genes (6–9).

Alternatively, LPS can trigger proinflammatory cytokine gene expression by activation of a number of mitogen-activated protein kinase (MAPK) pathways that include extracellular signal-regulated kinase1/2 (ERK1/2), p38 and c-jun-N-terminal kinase (JNK). The three MAPK pathways are regulated by different upstream components: ERK1/2 by MAP/extracellular-regulated kinase1/2 (MEK1/2); p38 by protein kinase R (PKR); and JNK by MEK1/4 (10,11). Previous reports indicate that endotoxin exposure results in the expression of tumor necrosis factor-α (TNF-α) through the Ras/MEK signaling pathway (10,12–14). Inhibition of the Ras/MEK pathway by U0126, a MEK1/2 inhibitor, results

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in a reduction of cytokine secretion (15). Furthermore, LPS also induces the expression of the early response gene, Egr-1, a downstream target of MEK1/2 (13,16). Collectively, these published results indicate that LPS can activate cellular response through the MAPK.

Krüppel-like factors (KLFs) are zinc finger-containing transcription factors that exhibit homology to Krüppel from *Drosophila* (17,18). Two KLFs are highly expressed in the intestinal epithelium: KLF4 (also known as gut-enriched Krüppel-like factor or GKLF), found in upper villus region, and KLF5 (also known as intestine-enriched Krüppel-like factor or IKLF), present in the lower crypt compartment (19,20). Expression of KLF4 is associated with epithelial differentiation and post-mitotic arrest (21). Recently, KLF4 has been shown to be regulated by LPS in macrophages (22). Our earlier studies indicate that KLF5 is a pro-proliferative regulator in cultured fibroblasts and intestinal epithelial cells (23–25). Studies also demonstrate that KLF5 is activated by the MAPK, ERK1/2, through Egr-1 (24,26). Because LPS also activates the MAPK pathway, we sought to determine whether KLF5 can be activated by LPS in cultured intestinal epithelial cells and to determine the consequence of such activation on signals elicited by LPS. We show that treatment of intestinal epithelial cells, IEC6, with LPS leads to the induction in KLF5 expression and subsequently KLF5-dependent induction in NF-κB expression. We also demonstrate that KLF5 is crucial in mediating LPS-elicited proinflammatory responses due to its critical involvement in the induction of expression of proinflammatory genes. These results indicate an important role played by KLF5 in innate immunity in response to LPS.

MATERIALS AND METHODS

Cell culture

The intestinal epithelial cells, IEC6, were maintained in DMEM containing 5% fetal bovine serum (FBS), 0.1 U/ml insulin and 1% streptomycin. Upon reaching confluency, cells were treated with varying concentrations of *E.coli* 0111:B4 LPS (List Biologicals; Campbell, CA) or vehicle only (water) for various periods of time. Where indicated, cells were also treated with 10 μg/ml polymyxin B (PMXB) (Sigma), a LPS antagonist (27); 1 μM U0126 (Promega), a MAPK inhibitor (15); or 10 μM Nα-*p*-Tosyl-L-lysine chloromethyl ketone (TLCK; Sigma), a NF-κB inhibitor (28). The human lymphocytic leukemic T cell line Jurkat E6.1 was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and maintained in RPMI medium 1640 (Invitrogen, Grand Island, NY) supplemented with 10% (v/v) heat-inactivated FBS (Invitrogen).

Northern blot analysis

Total RNA was extracted using the TRIzol reagent (Invitrogen). RNA was dissolved in deionized water and quantified using a spectrophotometer. Twenty micrograms of RNA were loaded into a denaturing agarose gel (1.2% agarose, 10× MOPS buffer, 37% formaldehyde and DEPC-treated water) and followed by transfer to nylon membrane (Hybond; Amersham). The full-length cDNA probe for mouse KLF5 was kindly provided by Dr J. Lingrel (19). Complementary DNA probes encoding the p50 and p65 subunits of NF-κB and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were generated by RT–PCR using the following primer pairs: p50, forward primer 5′-CACCTAGCTGCCAAAGAAGG-3′ and reverse primer 5′-CAGTGAGGGACTCCGAGAAG-3′; p65, forward primer 5′-GGCCTCATCCACATGAACTT-3′ and reverse primer 5′-GAGGTACCATGGCTGAGGAA-3′; GAPDH, forward primer 5′-ACCCAGAAGACTGTGGATGG-3′ and reverse primer 5′-GGATGCAGGGATGATGTTCT-3′. Probes were labeled with [α-^{32}P]dATP using a random-primed labeling kit (Stratagene). The membrane was hybridized in a QuikHyb solution (Stratagene), washed under high-stringency conditions and scanned with a phosphorimager (Amersham).

Western blot analysis

Cells were subjected to a 1× lysis buffer (Cell Signaling). Cell lysates were centrifuged at 10 000 *g* for 20 min and supernatants were collected. Protein concentrations were measured using BCA solution (Sigma) at 450 nm. Fifty micrograms of proteins per lane were loaded into a 10% Tris–HCl Criterion gel (Bio-Rad) and then transferred onto a nitrocellulose membrane (Schleicher & Schuell). A rabbit polyclonal KLF5 antibody (Biosource International/QCB; Hopkinton, MA) was generated against amino acids 106–122 of the mouse KLF5 (19). Antibodies against intercellular adhesion molecule-1 (ICAM-1) and actin were purchased from Santa Cruz and Calbiochem, respectively.

Electroporation

Cells cultured in 25 × 150 mm culture dishes (Corning) were washed with HANKS buffer, trypsinized, centrifuged and resuspended in DMEM (antibiotic- and serum-free). Ten micrograms of non-specific small interfering RNA (siRNA) or siRNA specific for KLF5 were introduced into 5×10^6 cells in a 0.5 mm cuvette (Bio-Rad), which was then subjected to electroporation using a Bio-Rad Gene Pulser Xcell (250 V and 500 Farads). The cells were then plated in 12-well dishes with standard media and incubated for an additional 24 h before treatment with LPS. The KLF5-specific siRNA sequence was 5′-AAC CCG GAU CUG GAG AAG CGA-3′ (Dharmacon). The non-specific siRNA sequence was 5′-GCG CGC UUU GUA GGA UUC G-3′.

Electrophoretic gel mobility shift assay

Nuclear proteins from mock-, non-specific siRNA- or KLF5 siRNA-transfected IEC6 cells treated with vehicle (water) or 5 μg/ml LPS were extracted according to the manufacturer's protocol (Panomics). A consensus NF-κB-binding sequence (5′-AGT TGA GGG GAC TTT CCC AGC C-3′) (29) was labeled with [γ-^{32}P]ATP (Amersham) using T4 polynucleotide kinase (Invitrogen). For binding assays, 5 μg of nuclear extracts were incubated at room temperature for 20 min with radiolabeled NF-κB-binding sequence in DNA binding buffer (20% glycerol, 5 mM $MgCl_2$, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris–HCl, pH 7.5 and 0.25 mg/ml poly(dI–dC). The protein–DNA complexes were resolved by 5% non-denaturing polyacrylamide gel electrophoresis. For competition experiments, 150-fold excess of unlabeled NF-κB probe

was pre-incubated with nuclear extracts prior to incubation with the labeled NF-κB probe.

Quantification of TNF-α and IL-6

Cells were treated with LPS or vehicle control for various periods of time. Supernatants were collected and assayed for the content of TNF-α and IL-6 by using enzyme-linked immunosorbent assay (ELISA) kits purchased from Biosource. Cytokines were measured using a microplate reader (Molecular Devices).

Cell adhesion assay

Cell adhesion assays were performed using published approach (30,31). Cultured human leukemic T lymphocyte, Jurkat E6.1, were labeled with 5 μM of the fluorescent dye, 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM; Molecular Probes, Eugene, OR) for 30 min at 37°C. Cells were washed twice and resuspended in RPMI (GIBCO). IEC6 cells were cultured in 12-well plates, pretreated with 5 μg/ml of LPS for 24 h and incubated with the fluorescently labeled Jurkat E6.1 cells at 37°C for 2 h. The co-cultured cells were washed twice with phosphate-buffered saline (PBS), and lysed in Tris/EDTA/NaCl with 1% Triton X-100. The lysed cells were then incubated in a revolver for 30 min, followed by centrifugation at 10 000 *g* for 30 min. The fluorescence intensity of the supernatant was measured at 520 nm using a Hitachi F4500 fluorescence spectrophotometer (Hitachi, Danbury, CT; excitation at 492 nm).

RESULTS

LPS activates KLF5 gene expression in IEC6 cells

We first determined whether LPS leads to an increase in KLF5 gene expression by treating IEC6 cells with 5 μg/ml of *E.coli* 0111:B4 LPS for various periods of time. As shown in Figure 1A, the addition of LPS to the cells led to a relatively acute but transient increase in the level of KLF5 messenger RNA (mRNA) after 2 h of treatment. In contrast, there was no such increase in cells treated with water control (Figure 1B). Figure 1C is a dose–response study in IEC6 cells treated with LPS for 2 h. As seen, there was a dose-dependent increase in the KLF5 mRNA levels. We used a concentration of 5 μg/ml LPS in all subsequent experiments.

We then measured the levels of KLF5 protein in IEC6 cells treated with LPS. Figure 1D shows that LPS treatment of IEC6 cells led to a sustained increase in the levels of KLF5 from 2 to 12 h. In contrast, no such increase was observed in cells treated with water control (Figure 1E). These results indicate that LPS leads to an increase in KLF5 transcript levels followed by an increase in KLF5 protein levels in IEC6 cells.

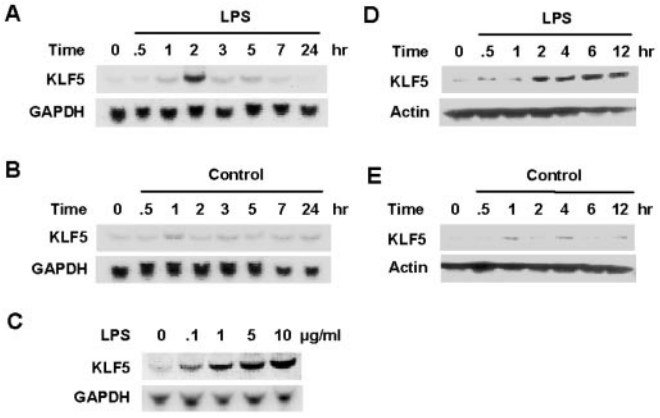

Figure 1. Northern and western blot analyses of KLF5 mRNA and protein in IEC6 cells in response to LPS. IEC6 cells were treated from 0 to 24 h with 5 μg/ml *E.coli* 0111:B4 LPS (**A**) or water control (**B**). Twenty micrograms of total RNA from each time point was analyzed by northern blot analysis using labeled cDNA probe for KLF5 or GAPDH (as a loading control.). (**C**) IEC6 cells were treated for 2 h with a range of LPS concentrations from 0 to 10 μg/ml. Blots were hybridized with cDNA probe for KLF5 or GAPDH. For protein measurement, IEC6 cells were treated from 0 to 12 h with 5 μg/ml LPS (**D**) or water control (**E**). Fifty micrograms of protein were loaded per lane and examined by western blot using antibodies against KLF5 or actin (as a loading control).

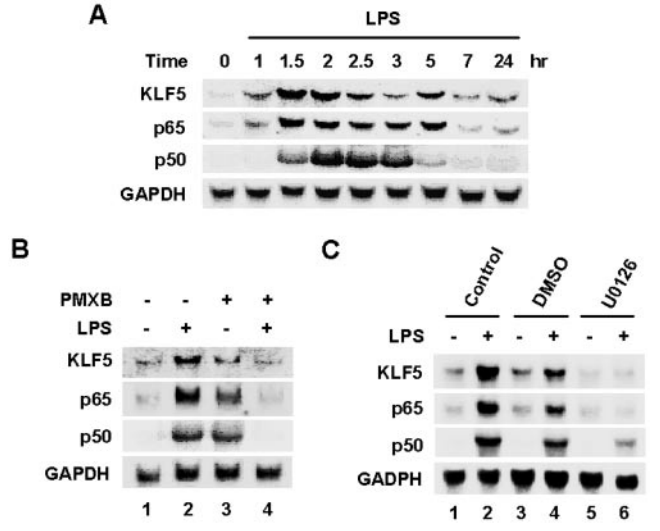

Figure 2. Northern blot analyses of the effects of LPS, PMXB and U0126 on expression of KLF5 and the p65 and p50 subunits of NF-κB in IEC6 cells. (**A**) IEC6 cells were treated with 5 μg/ml LPS for the time periods specified before extraction of RNA. Twenty micrograms of RNA were loaded in each lane and probed with cDNA encoding KLF5, and the p65 and p50 subunits of NF-κB. GAPDH was used as a loading control. (**B**) IEC6 cells were pretreated with 10 μg/ml PMXB for 30 min (lanes 3 and 4) and then treated with 5 μg/ml LPS (lanes 2 and 4) or water control (lanes 1 and 3) for 2 h before being analyzed for the mRNA levels of KLF5, and the p65 and p50 subunits of NF-κB. (**C**) IEC6 cells were pretreated with water control (lanes 1 and 2), the vehicle, DMSO (lanes 3 and 4), or the MAPK inhibitor, U0126 (lanes 5 and 6), for 30 min and followed by treatment with 5 μg/ml LPS (lanes 2, 4 and 6) or water control (lanes 1, 3 and 5) for 2 h before northern analyses for the mRNA stated. GAPDH was used as a loading control in both panels.

LPS activates NF-κB subunit gene expression in IEC6 cells

The transcription factor NF-κB plays an important role in mediating the proinflammatory response of cells to external stresses (32). Previous studies indicate that LPS activates NF-κB by a post-translational mechanism involving modification and degradation of the inhibitor of NF-κB, IκB, followed by nuclear translocation of NF-κB (33,34). We sought to determine whether LPS can regulate NF-κB activity at the level of transcription in IEC6 cells. Figure 2A shows

that LPS treatment of IEC6 cells led to an increase in the levels of mRNA for the p65 and p50 subunits of NF-κB, as well as those for KLF5. The induction of NF-κB subunit transcripts by LPS has not previously been reported.

We then determined the effect of PMXB, a LPS antagonist that interferes with the interaction of TLR4 and LPS (27), on expression of KLF5 and NF-κB subunits in IEC6 cells following LPS treatment. Figure 2B shows that cells treated with LPS alone had increased mRNA levels of KLF5, and p65 and p50 subunits of NF-κB (lane 2). The levels of these transcripts were also increased when cells were treated with PMXB alone (lane 3). This finding is consistent with a recent observation that PMXB had a direct effect on monocyte-derived human dendritic cells including activation of ERK1/2 (35). However, when cells were treated by both LPS and PMXB, there was a nearly complete abolishment of the three mRNAs (lane 4). This reveals that the induction in KLF5 expression is a response to LPS treatment, possibly through TLR signaling.

Previous studies demonstrate that LPS induces cellular response through the MAPK pathway (13). Our recent studies indicate that KLF5 is regulated by MAPK (24). To determine whether LPS can stimulate KLF5 through the MAPK pathway, we pretreated IEC6 cells with U0126, a MAPK inhibitor. In both water control- and vehicle- [dimethyl sulfoxide (DMSO)] treated cells, the levels of KLF5, p50 and p65 transcripts were significantly elevated in the presence of LPS (Figure 2C; lanes 2 and 4, respectively). However, when cells were treated with U0126, the increase in KLF5 and NF-κB subunit mRNAs in response to LPS was abrogated (Figure 2C; lane 6). These results indicate that LPS-stimulated KLF5 expression is mediated by the MAPK pathway, as is expression of the two NF-κB subunits.

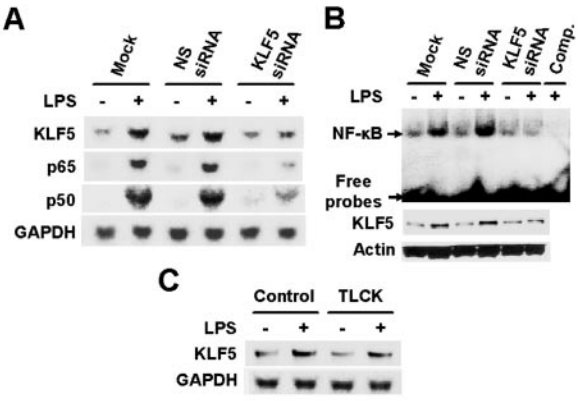

Figure 3. Inhibition of KLF5 expression by siRNA abrogates induction of NF-κB subunit levels and binding activity in response to LPS. (**A**) IEC6 cells were transfected by electroporation with non-specific (NS) siRNA or KLF5-specific siRNA. Mock-transfected cells were used as control. Twenty-four hours following transfection, cells were treated with 5 μg/ml LPS or water control for 2 h, followed by northern blot analysis for the various mRNAs indicated. (**B**) Nuclear extracts were prepared from mock-transfected IEC6 cells or cells transfected with non-specific (NS) siRNA or KLF5-specific siRNA that have been treated with 5 μg/ml LPS or water control for 2 h. Electrophoretic mobility shift assay (EMSA) was then performed with a labeled consensus NF-κB binding sequence using 5 μg nuclear extracts per lane. In the last lane (Comp.), 150-fold excess of unlabeled NF-κB probe was included in the reaction containing nuclear extracts from mock-transfected and LPS-treated cells. The same nuclear extracts were also analyzed for the content of KLF5 or actin by western blotting, as shown below the EMSA. (**C**) IEC6 cells were pretreated with 10 μM of the NF-κB inhibitor, TLCK, or water control for 1 h and then treated with 5 μg/ml LPS or water control for 2 h before being analyzed for the mRNA levels for KLF5 or GAPDH by northern hybridization.

Inhibition of KLF5 by siRNA results in abrogation of LPS-stimulated expression of NF-κB subunits in IEC6 cells

We next determined whether the LPS-stimulated KLF5 expression in IEC6 cells is responsible for the increased expression of NF-κB subunits. This was accomplished by siRNA. Figure 3A shows that the increased levels of KLF5 mRNA in response to LPS were significantly attenuated in cells transfected with KLF5-specific siRNA as compared to mock-transfected cells or cells transfected with non-specific (NS) siRNA. Importantly, increased mRNA levels of the p65 and p50 NF-κB subunits in response to LPS were also reduced in cells transfected with KLF5-specific siRNA as compared to mock-transfected cells or cells transfected with non-specific siRNA (Figure 3A). Moreover, NF-κB binding activity in response to LPS was significantly reduced in cells transfected with KLF5-specific siRNA when compared to mock-transfected cells or cells transfected with non-specific (NS) siRNA (Figure 3B). This reduction correlated with blocked induction of KLF5 protein in the presence of LPS in cells transfected with KLF5-specific siRNA (Figure 3B). Lastly, treatment of cells with TLCK, an NF-κB inhibitor, did not result in a reduction in the levels of KLF5 mRNA in response to LPS (Figure 3C). These results indicate that KLF5 is an upstream mediator of NF-κB subunit expression following LPS treatment.

Inhibition of KLF5 results in diminished secretion of TNF-α and IL-6 from IEC6 cells following LPS treatment

Studies showed that LPS activates expression of proinflammatory genes, such as those encoding cytokines and cell adhesion molecules, in a pathway that is dependent on NF-κB (36). We thus treated IEC6 cells with LPS for various periods of time and measured the quantities of two cytokines, TNF-α and IL-6, in the media. As shown in Figure 4A and B, LPS significantly increased the secretion of both cytokines over that of the control from 8 to 72 h. To determine whether KLF5 plays a role in LPS-stimulated secretion of TNF-α and IL-6, we inhibited expression of KLF5 by siRNA followed by LPS treatment. Figure 4C and D show that secretion of both cytokines was increased in mock-transfected or cells transfected with non-specific (NS) siRNA following 48 h of LPS treatment. In contrast, LPS treatment failed to increase the secretion of either TNF-α or IL-6 in cells transfected with KLF5-specific siRNA (Figure 4C and D). Combining the results of Figures 3 and 4, our studies demonstrate that KLF5 is necessary for the induction of NF-κB gene expression and subsequent TNF-α and IL-6 production in IEC6 cells treated with LPS.

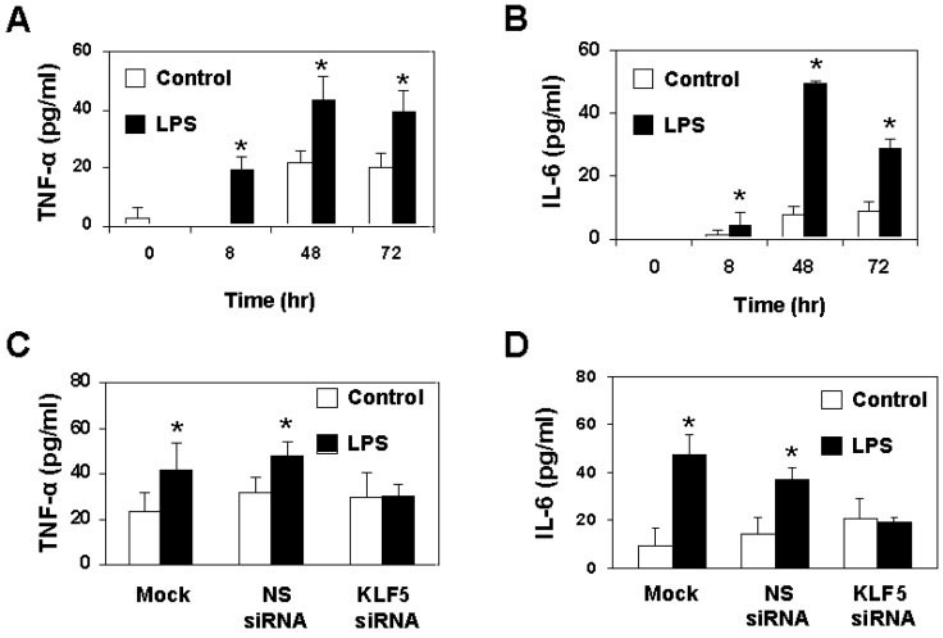

Figure 4. Increased secretion of TNF-α and IL-6 in IEC6 cells in response to LPS requires KLF5. IEC6 cells were treated with water control (open bars) or 5 μg/ml LPS (filled bars) for the periods of time specified. The amounts of TNF-α (**A**) and IL-6 (**B**) in the supernatants were determined by ELISA. $N = 4$ in all experiments. $^*P < 0.05$ compared to control. Mock-transfected IEC6 cells or IEC6 cells transfected with non-specific (NS) siRNA or KLF5-specific siRNA were treated with water control (open bars) or 5 μg/ml LPS (filled bars) for 48 h. The amounts of TNF-α (**C**) and IL-6 (**D**) in the supernatants were determined by ELISA. $N = 4$ in all experiments. $^*P < 0.05$ compared to control.

KLF5 inhibition in IEC6 cells results in diminished induction of ICAM-1 expression and heterotopic cell adhesion in response to LPS

Previous studies showed that expression of ICAM-1 is increased in LPS-treated endothelial cells and that this increase mediates subsequent neutrophil–endothelial interactions (37,38). We sought to determine whether ICAM-1 expression is also increased in IEC6 cells treated with LPS and whether such increase mediates increased cell–cell adhesion. Figure 5A shows that LPS treatment of IEC6 cells exhibited a time-dependent increase in the level of ICAM-1 protein up to 24 h and that this increase was preceded by the increase in KLF5 protein level. Transfection of cells with KLF5-specific siRNA resulted in a diminished induction of KLF5 and ICAM-1 protein levels in response to LPS as compared to mock-transfected cells or cells transfected with non-specific (NS) siRNA (Figure 5C). Importantly, using a co-culture system, we showed that adhesion of the Jurkat T lymphocytes to IEC6 cells in response to LPS treatment was abolished in IEC6 cells transfected with KLF5-specific siRNA (Figure 5B). In contrast, adhesion of Jurkat to IEC6 cells was increased after LPS treatment in mock-transfected or cells transfected with non-specific siRNA (Figure 5B). These results demonstrate that KLF5 is crucial in mediating increased cell adhesion between lymphocytes and IEC6 cells from LPS treatment by activating expression of ICAM-1.

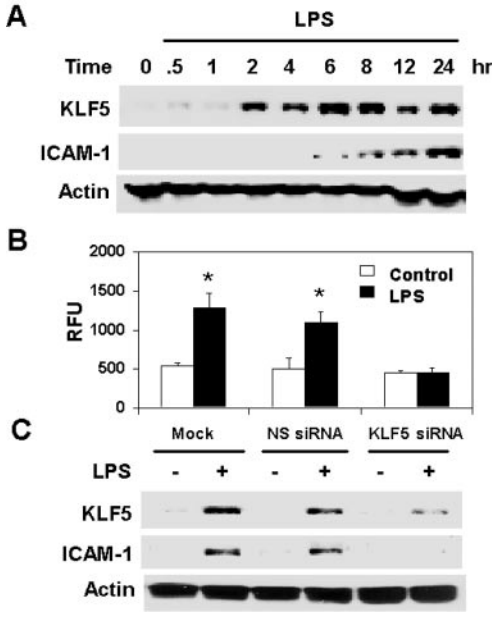

Figure 5. Increased expression of ICAM-1 and adhesion of Jurkat T cells to IEC6 cells in response to LPS requires KLF5. (**A**) IEC6 cells were treated with 5 μg/ml LPS for the time periods specified before protein extraction. Fifty micrograms of protein were loaded in each lane and analyzed for the content of KLF5, ICAM-1 or actin by western blotting. (**B**) Fluorescently labeled Jurkat E6.1 lymphocytes were co-cultured with mock-, non-specific (NS) siRNA or KLF5-specific siRNA-transfected IEC6 cells that were pretreated with either water control (open bars) or 5 μg/ml LPS (filled bars) for 24 h. After 2 h of co-culturing, cells were washed, lysed and attachment of Jurkat cells to IEC6 cells determined by a spectrophotometer. Results are expressed as relative fluorescence units (RFU). $N = 6$ in all experiments. $^*P < 0.01$ compared to control. (**C**) Western blot analysis was performed using the same set of cells that were used in (B) for the protein content of KLF5, ICAM-1 or actin.

DISCUSSION

TLRs are pathogen recognition molecules that activate the immune system as part of the innate immune response. Microbial recognition by TLRs plays a crucial role in the host

immune system's decision to respond or not to a particular microbial infection. LPS, a membrane glycolipid of Gram-negative bacteria, exhibits strong immunostimulating activity among TLR ligands and has been studied in great detail. Recent studies have shown that cell surface TLR4-MD2 physically interacts with LPS and triggers the release of an LPS signal, revealing a host–pathogen interaction mediated by TLR (39). It is now well established that the nuclear transcription factor, NF-κB, plays a crucial role in mediating the intracellular signaling generated by LPS that eventually leads to the induction of expression of proinflammatory genes (4). Abundant literature has documented that the activation of NF-κB by LPS is mediated by post-translational modification and proteosomal degradation of IκB, an inhibitor of NF-κB (40).

The intestinal epithelium is an integral part of the innate immunity in defense of bacterial infection (41). Previous studies indicate that TLR4 is present in several intestinal epithelial cell lines, including IEC6, rendering the cells responsive to LPS (9,42–45). The rationale behind our study was based on the previous findings that LPS activates the MAPK, ERK1/2 (46–48) and that ERK1/2 stimulates KLF5 expression (24). Furthermore, recently published data showed a member of the Krüppel-like factor family, KLF4, is induced in macrophages in response to LPS (22). However, in IEC6 cells, expression of KLF4 is not significantly induced by LPS (results not shown). Instead, we demonstrated an alternative and independent mechanism by which NF-κB becomes activated by LPS that requires the induction of expression of the gene encoding KLF5 in IEC6 cells. We showed that the levels of KLF5 mRNA and protein are increased in IEC6 cells treated with LPS (Figure 1A and D, respectively). This increase is inhibited by treatment of cells with the MAPK inhibitor, U0126 (Figure 2C), confirming that the induction of KLF5 in response to LPS is mediated by MAPK. Importantly, LPS treatment of IEC6 cells also increases the mRNA levels of the two NF-κB subunits, p65 and p50 (Figure 2A), and that this increase is attenuated by the transfection of cells with siRNA that inhibits KLF5 expression (Figure 3A). Furthermore, the increase in NF-κB DNA binding activity in response to LPS is also abrogated in cells transfected with KLF5-specific siRNA (Figure 3B). These results indicate that KLF5 is necessary and sufficient for the induction of NF-κB subunit expression secondary to LPS. This finding is further confirmed by the observation that TLCK, an NF-κB inhibitor, fails to inhibit the increase in KLF5 mRNA level in response to LPS, therefore placing KLF5 upstream of NF-κB in the LPS-induced signaling pathway.

NF-κB has been shown to be critically involved in the transcriptional activation of numerous downstream genes that encode mediators of inflammatory and immune responses (36). Among the cytokines activated by NF-κB are TNF-α and IL-6 (49). We showed that secretion of TNF-α and IL-6 is significantly increased in IEC6 cells treated with LPS (Figure 4A and B), a response likely mediated by the activation of NF-κB. Similar findings have been documented in previously published studies (9,50). Importantly, inhibition of KLF5 by siRNA abolishes LPS-stimulated secretion of both TNF-α and IL-6 (Figure 4C and D), indicating a critical role for KLF5 in mediating the production of proinflammatory cytokines as a result of LPS treatment.

In addition to secretion of proinflammatory cytokines, an occurrence during innate immune response is the recruitment of leukocytes to the target of infection. ICAM-1, a cell adhesion molecule expressed in endothelial and epithelial cells mediates interactions with immune cells expressing CD11a/CD18 and CD11b/CD18 (51). LPS stimulation results in an increase in ICAM-1 expression in intestinal epithelial cells and subsequent adhesion of monocytic cells (52). Here, we demonstrated that LPS stimulates ICAM-1 production in IEC6 cells (Figure 5A) and increases adhesion of the Jurkat T lymphocytes (Figure 5B). Notably, the induction of KLF-5 production in response to LPS precedes that of ICAM-1 (Figure 5A). Furthermore, inhibition of KLF5 expression abolishes LPS-stimulated ICAM-1 production and adhesion of lymphocytes (Figure 5B and C, respectively). Thus, KLF5 also plays an important physiological role in recruiting leukocytes by LPS-activated IEC6 cells.

Based on the findings of this and previous studies, we present a model in which KLF5 plays an important role in mediating the proinflammatory response elicited by LPS in intestinal epithelial cells (Figure 6). Here, KLF5 functions primarily as an upstream activator of expression of the two NF-κB subunits, p65 and p50, which subsequently stimulates expression of the three proinflammatory genes studies, TNF-α, IL-6 and ICAM-1. This mechanism may represent a relatively 'late' response as compared to the classical mechanism of NF-κB activation through post-translational modification. Additionally, although NF-κB has been shown to activate the expression of each of the three downstream proinflammatory genes (49,52), our results do not rule out a role for the direct involvement of KLF5 in the transcriptional activation of any or all of the three target genes. Moreover, results of previous studies demonstrate a synergistic effect of KLF5 and NF-κB on transcriptional activation of target genes through a mechanism that involves physical interaction between KLF5 and NF-κB subunits (53,54). It is possible that all of these mechanisms exist as a way to ensure the establishment of a

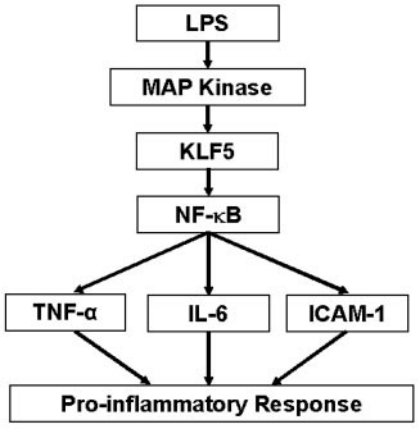

Figure 6. A model for the role of KLF5 in mediating the proinflammatory response in IEC6 cells elicited by LPS. Treatment of IEC6 cells by LPS activates MAP kinase activity, which leads to induction of KLF5 mRNA and protein levels. The increased KLF5 then transcriptionally activates expression of the p65 and p50 subunits of NF-κB with a subsequent increase in NF-κB binding activity, leading to increased production of TNF-α, IL-6 and ICAM-1, and subsequent proinflammatory response.

robust proinflammatory response to a harmful bacterial product. Be that as it may, the results of our studies clearly establish a physiologically significant role played by KLF5 in mediating signaling elicited by LPS. Whether KLF5 may mediate signaling by other TLR ligands is currently being investigated.

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