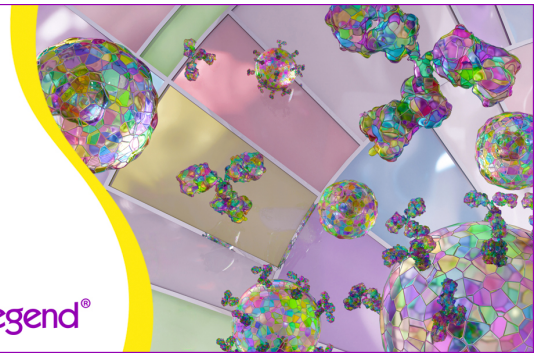


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### Lipopolysaccharide Activates Distinct Signaling Pathways in Intestinal Epithelial Cell Lines Expressing Toll-Like Receptors<sup>1</sup> ✓

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# Lipopolysaccharide Activates Distinct Signaling Pathways in Intestinal Epithelial Cell Lines Expressing Toll-Like Receptors<sup>1</sup>

Elke Cario, Ian M. Rosenberg, Steven L. Brandwein, Paul L. Beck, Hans-Christian Reinecker, and Daniel K. Podolsky<sup>2</sup>

LPS elicits several immediate proinflammatory responses in peripheral blood leukocytes via a recently described pathway including CD14, Toll-like receptors (TLR), serine-threonine kinases, and NF- $\kappa$ B transcription factor. However, the functional responses of intestinal epithelial cells (IEC) to stimulation with LPS are unknown. Expression of mRNA and protein for CD14 and TLRs were assessed by RT-PCR, immunoblotting, and immunohistochemistry in mouse and human IEC lines. LPS-induced activation of signaling pathways (p42/p44 mitogen-activated protein kinase (MAPK), c-Jun NH<sub>2</sub>-terminal kinase (JNK), p38, p65, NF- $\kappa$ B) were assessed by immunoblotting and gel shifts. CD14 mRNA and protein expression were not detectable in IEC. However, human TLR2, TLR3, and TLR4 mRNA were present in IEC. TLR4 protein was expressed in all cell lines; however, TLR2 protein was absent in HT29 cells. Immunofluorescent staining of T84 cells demonstrated the cell-surface presence of the TLRs. LPS-stimulation of IEC resulted in activation (>1.5-fold) of the three members of the MAPK family. In contrast, LPS did not significantly induce activation of JNK and p38 in CMT93 cells, p38 in T84 cells and MAPK and JNK in HT29 cells. Downstream, LPS activated NF- $\kappa$ B in IEC in a time-, dose-, and serum-dependent manner. IEC express TLRs that appear to mediate LPS stimulation of specific intracellular signal transduction pathways in IEC. Thus, IEC may play a frontline role in monitoring luminal bacteria. *The Journal of Immunology*, 2000, 164: 966–972.

The intestinal epithelium is the central component of the barrier between the myriad microbes and Ags of the lumen and inflammatory and immune cells of the lamina propria. The intestinal epithelial cell (IEC)<sup>3</sup> layer is constantly exposed to the resident microflora of obligate anaerobic bacteria, which play a crucial role in limiting direct contact of pathogenic threats with the mucosal epithelium. In response to invasive bacteria, the intestinal epithelial cell may produce a variety of cytokines and chemokines (1–3).

Potential pathogenic bacteria are able to directly deposit their toxic and proinflammatory constituents, such as LPS, a glycolipid derived from the outermost membrane of pathogenic Gram-negative bacteria, at the intestinal epithelial, apical surface. LPS may then be internalized, recycled, stored, or transcytosed from the apical to the basolateral pole of the intestinal epithelium (4, 5).

LPS is a potent toxin that elicits several immediate proinflammatory responses in mammalian cells (6). LPS-induced activation of monocytes, macrophages, or polymorphonuclear neutrophils is mainly mediated through CD14, a GPI-anchored membrane recep-

tor (7–9). Soluble CD14 is present in serum (10) and facilitates binding of LPS to cells that do not express membrane CD14 (11–13). Binding of LPS to CD14 is enhanced by the presence of the LPS binding protein (LBP) (12). LBP may also enhance binding of whole Gram-negative bacteria to cells via CD11/CD18 (14). However, because these integrins lack intracellular signaling domains, they probably function to transfer LPS to a second receptor that directly transduces the signal.

Recently, several Toll-like receptors (TLR) have been identified in blood macrophages and monocytes based on homology to the *Drosophila* protein (15, 16). Although initially detected as orphan receptors, recent studies have demonstrated that TLRs act as transmembrane coreceptors to CD14 in the cellular response to LPS (reviewed in Refs. 17 and 18). Recent studies have variably suggested that TLR2 or TLR4 serve as the main mediator of responses to LPS in vitro and in vivo (19–24). It appears that TLR4 plays the major role as LPS receptor (19, 21). However, the role of TLR2 still needs to be further defined. Heine et al. have shown that the presence of TLR2 is not essential for some cells to respond to LPS (20). However, Yang et al. have clearly demonstrated that TLR2 mediates LPS-induced intracellular signaling (22). LPS-LBP binding to CD14 can result in rapid phosphorylation of p42/p44 mitogen-activated protein kinase (MAPK), p38, and c-Jun NH<sub>2</sub>-terminal kinase (JNK) in monocytic cell lines (25–29). Downstream, LPS signaling through TLRs rapidly leads to NF- $\kappa$ B activation in monocytic cells (24, 30–32).

A variety of recent studies have increasingly uncovered the important role of intestinal epithelial cells as a key component of the mucosal immune system (33). LPS is known to induce the proinflammatory cytokine IL-8 in HT29 and SW620 epithelial cells (12, 34). However, signaling pathways upstream of cytokine expression induced by LPS have not yet been fully established in IEC lines.

The effects of LPS on monocytes as one focal point of the host response to this key bacterial product have been well studied. We

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<sup>3</sup> Abbreviations used in this paper: IEC, intestinal epithelial cells; ERK, extracellular regulated kinase; p-ERK, phosphorylated ERK; JNK, c-Jun NH<sub>2</sub>-terminal kinase; p-JNK, phosphorylated JNK; MAPK, mitogen-activated protein kinase; p-MAPK, phosphorylated MAPK; TLR, Toll-like receptor; LBP, LPS binding protein; mCD14, membrane-bound CD14; sCD14, soluble CD14.

speculated that LPS may induce specific responses in IEC, the frontline of the mucosal immune system. To understand the functional role of the intestinal epithelium in mucosal host defense as part of the immune system, we characterized LPS-induced signal transduction pathways in IEC lines *in vitro*.

## Materials and Methods

### Materials and reagents

LPS (*Escherichia coli*, O26:B6) prepared by phenol extraction was purchased from Sigma (St. Louis, MO) and prepared as dispersed sonicate in endotoxin-free water (Life Technologies, Rockville, MD) before diluting to final concentration in supplemented media. IL-1 $\beta$  was obtained from R&D Systems (Minneapolis, MD). PMA was purchased from Sigma, and radiochemicals were obtained from NEN Life Science (Boston, MA).

### Cell culture

The human colon cancer cell lines HT29, Caco2, and T84 and the mouse rectal carcinoma cell line CMT93 were obtained from the American Type Culture Collection (Manassas, VA). Cells were grown in DMEM (Cellgro, Herndon, VA) containing 4.5g/L glucose and 10% (HT29, CMT93) or 20% (Caco2) FBS (Sigma). T84 cells were grown in a 1:1 mix of DMEM and Ham's F-12 medium (Cellgro) containing 10% FBS. All media were supplemented with 4 mmol/L L-glutamine (Life Technologies), 50 U/ml penicillin, and 50  $\mu$ g/ml streptomycin (Sigma). Cells were grown in 5% CO<sub>2</sub> at 37°C within a humidified incubator. T84 and Caco2 cells were grown on filters (0.4  $\mu$ m; Becton Dickinson, Franklin Lanes, NJ) to confluent monolayers to achieve a polarized and differentiated state within 14–21 days (35). Trans epithelial resistance was used to monitor changes in epithelial cell culture integrity and confluency and ranged from >1500  $\Omega$ cm<sup>2</sup> for confluent T84 and >500  $\Omega$ cm<sup>2</sup> for Caco2 cells (Millicell Electrical Resistance System; Millipore, Bedford, MA) before each experiment. THP1 cells were kindly provided by Dr. Ramnik Xavier and were seeded into culture flasks in DMEM supplemented with PMA (10 ng/ml) to induce differentiation and CD14 expression (26). U937 cells were grown in RPMI 1640 (Cellgro) containing 10% FBS and supplemented with PMA (10 ng/ml) to induce differentiation and CD14 expression and cell adherence. Whole-cell lysates from the monocytic cell line U937 were used as positive control for TLR protein expression.

### Assessment of LPS receptor mRNA expression: RT-PCR analysis

Expression of human TLR2, 3, and 4 mRNA (16) were assessed using total RNA by RT-PCR from HT29 and T84 cells (RNeasy Mini Kit; Qiagen, Valencia, CA). PCR primers for human TLR2 (346 bp) and human TLR4 (506 bp) have been previously described (31). PCR primers used for human TLR3 (320 bp) were 5'-AAA TFG GGC AAG AAC TCA CAG G and 5'-GTG TTT CCA GAG CCG TGC TAA. THP1 cDNA was used as positive source of human TLR expression to confirm the specificity of the primers and PCR, as recently shown (31). Human TLR2 cDNA from T84 cells and human TLR3 and TLR4 cDNAs from HT29 cells were inserted into pCR2.1 vector (TA Cloning Kit; Invitrogen, San Diego, CA), and subsequently sequenced (Massachusetts General Hospital DNA Sequencing Core Facility, Boston, MA).

Expression of human and murine CD14 mRNAs in HT29, T84, and CMT93 cells, respectively, were also assessed by RT-PCR (36). PCR primers used for human CD14 were 5'-GGT GCC GCT GTG TAG GAA AGA and 5'-GGT CCT CGA GCG TCA GTT CCT (450 bp). The PCR primers used for mouse CD14 (1326 bp) have previously been described (37). Species nonspecific GAPDH primers were 5'-CGG AGT CAA CGG ATT TGG TCG TAT and 5'-AGC CTT CTC CAT GGT GGT GAA GAC (306 bp). All PCR products were resolved by 1–2% agarose gel electrophoresis, and DNA bands were visualized by staining the gel with ethidium bromide.

### Assessment of receptor and signaling proteins: immunoblotting

Cells, grown on 60 mm dishes or filters, were rinsed in cold PBS and placed on ice in 400  $\mu$ l lysis buffer per well (1% Triton X-100, 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, containing 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml aprotinin, 1 mM sodium fluoride, 1 mM sodium orthovanadate, and 2 mM PMSF). Lysates were centrifuged (12,000  $\times$  g, 15 min at 4°C), and protein concentration in each supernatant was determined by colorimetric Bradford protein assay (Bio-Rad, Hercules, CA). Proteins (per lane, 15–25  $\mu$ g) from the resulting supernatants were heated (85°C, 2 min), subjected to SDS-PAGE (4–12% Bis-Tris; Novex, San Diego, CA), and then transferred onto a polyvinylidene difluoride membrane (Millipore) followed by immunoblotting (according to the manufacturer's in-

structions) with specific polyclonal Abs to tyrosine-phosphorylated p42/p44 MAPK, JNK, or p38 (Promega, Madison, WI, and New England Biolabs, MA). Whole-cell lysates were used as positive controls after stimulation with PMA (10<sup>-7</sup> M for 30 min). The positive and negative control for phosphorylation of p38 (C6 glioma whole-cell lysates prepared with and without anisomycin treatment) were obtained from New England Biolabs. To confirm equal loading, immunoblots were stripped with 62.5 mM Tris-HCl, pH 6.8, 2% SDS containing 100 mM 2-ME at 50°C for 30 min and reprobed with polyclonal anti-extracellular signal-related kinase (ERK)-2 or anti-p38 Abs (Santa Cruz Biotechnology, Santa Cruz, CA). The membrane was developed with an enhanced chemiluminescence detection kit (NEN Life Science). Only results with significant increase of at least 1.5-fold phosphorylation of MAPK activation are presented. All experiments were repeated at least three times, and a representative result is shown for each experiment.

Anti-TLR2 and anti-TLR4 antisera (1:1000) were gifts from Tularik (San Francisco, CA) and have been previously described (31).

### Immunohistochemistry

T84 and U937 cells were seeded at different states of confluency onto plastic tissue culture slides. Slides were then washed with cold PBS and fixed in 4% paraformaldehyde/PBS containing 0.1% Triton X-100 for 60 min at 4°C (detergent was omitted for CD14 staining). After blocking with normal goat serum (Sigma), anti-TLR2, anti-TLR4, or normal anti-rabbit IgG (Santa Cruz Biotechnology) were added (1:100, 16 h), followed by FITC goat anti-rabbit (Vector Laboratories, Burlingame, CA). After blocking with horse serum (Life Technologies), anti-CD14 ("UCH-M1" from Santa Cruz Biotechnology and "MY4" from Coulter, Palo Alto) or normal anti-mouse IgG (Santa Cruz Biotechnology) were added (1:100, 16 h), followed by FITC horse anti-mouse (Vector Laboratories). Cells were then immediately viewed on an inverted immunofluorescence microscope ( $\times$ 40 objective, model IX70; Olympus, New Hyde Park, NY).

### Nuclear extracts and assessment of NF- $\kappa$ B activation by Western blotting and EMSA

Nuclear extracts were prepared according to the protocol described by Schreiber et al. (38). Nuclear content of the NF- $\kappa$ B subunit p65 was determined by Western blot analysis (anti-p65; Santa Cruz Biotechnology), as described above. NF- $\kappa$ B consensus (5'-AGT TGA GGC GAC TTT CCC AGG C) and mutant (5'-AGT TGA GGC GAC TTT CCC AGG C) oligonucleotides were obtained from Santa Cruz Biotechnology. Double-stranded oligonucleotides were 5'-end labeled with [ $\gamma$ -<sup>32</sup>P]ATP using T4 polynucleotide kinase (Promega). For competition, an 100-fold excess of cold oligonucleotide was added to the reaction. The reaction was conducted in a total volume of 20  $\mu$ l, using 0.5 ng of labeled oligonucleotide, 10–15  $\mu$ g of nuclear protein extract, and 1  $\mu$ g of poly(dI-dC) (Amersham Pharmacia, Piscataway, NJ) in 1 $\times$  buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM DTT, 1 mM EDTA, 5% glycerol). The samples were loaded onto a 6% polyacrylamide gel and run in 0.25 $\times$  TBE buffer. The resultant DNA-protein complexes were then detected by autoradiography.

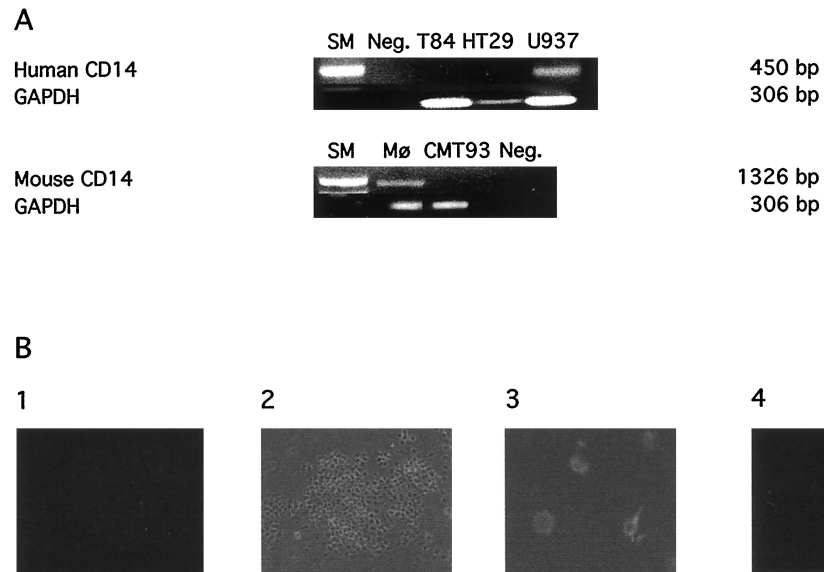
## Results

### Potential LPS receptors in IEC

Expression of CD14 mRNA in human (T84, HT29) and mouse (CMT93) IEC lines was initially assessed by RT-PCR. U937 (differentiated) and mouse macrophage cDNAs were used as known sources of human and mouse CD14 expression, respectively, to confirm the specificity of the primers and PCR (Fig. 1). No PCR product was detected in any of the intestinal epithelial-derived T84, HT29, or CMT93 cells using CD14-specific human or murine primers. However, signals of appropriate sizes were detected in U937 and macrophages. RT-PCR analysis of GAPDH expression confirmed the quality of all RNA preparations used for RT-PCR.

Human CD14 protein was not present in T84 cells, as assessed by immunohistochemistry (Fig. 1B, 1, immunofluorescence, 2, phase contrast). The monocytic differentiated cell line U937 (Fig. 1B, 3) was used as a positive control and demonstrated specific cell-surface CD14 staining (Fig. 1B, 4, mouse IgG as negative control). This result was confirmed using two different anti-CD14 Abs (results for anti-CD14 (My4) are shown).

**FIGURE 1.** Absence of CD14 mRNA and protein expression in IEC. *A*, RT-PCR human and murine CD14 mRNA was assessed using total RNA from T84, HT29, and CMT93 cells. U937 and macrophages were used as positive sources of human or murine CD14 mRNA. Species-unspecific GAPDH primers were used as a control for cDNAs. SM, Size marker; M $\phi$ , macrophage. *B*, Immunofluorescence histochemistry was used to assess the CD14 surface protein expression of T84 cells (1, immunofluorescence; 2, phase contrast; 3, U937 cells as positive control; 4, normal mouse IgG as negative control).



Primarily based on published cDNA sequences of human TLR2, TLR3, and TLR4 (15, 16) were used for RT-PCR to assess the presence of these TLRs in IEC. Primers specific for human TLRs were synthesized and used to assess expression of human TLR2, TLR3, and TLR4 mRNA in HT29 or T84 cells. Human THP1 cells served as positive sources of human Toll-like mRNA and confirmed the specificity of the primers and PCR. As shown in Fig. 2, TLR2, TLR3, and TLR4 mRNAs were present in both HT29 and T84 cells. The PCR products (346, 320, 506 bp) detected in T84 or HT29 cells were isolated, subcloned, and sequenced. The obtained sequences of these PCR products were >92% identical with the known nucleotide sequences of human TLRs (GenBank accession no. U88878, U88879, U88880).

Human TLR2 protein was present in T84 and Caco2 cells, as assessed by Western blotting (Fig. 3A). However, TLR2 protein was not detectable in HT29 cells or the monocytic cell line U937. Human TLR4 protein was expressed in all three human IEC lines. LPS stimulation did not modulate TLR4 protein expression in T84 cells. The presence of TLR2 (Fig. 3B, 1) and TLR4 (Fig. 3B, 2) on the cell surface of T84 cells (rabbit IgG as negative control; Fig 3B, 3) was confirmed by immunofluorescent histochemistry.

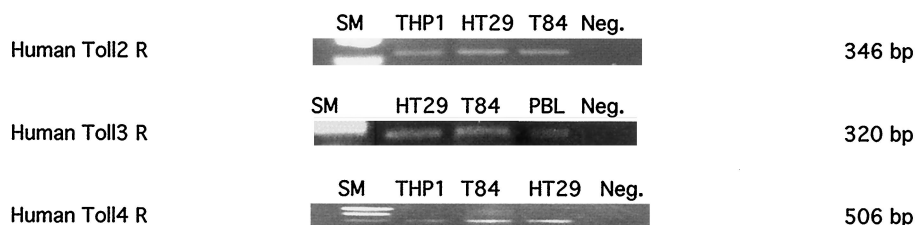
#### LPS-induced activation of signaling pathways in IEC

CMT93, T84, and HT29 cells were incubated with LPS for various time periods, and phosphorylation of MAPK, p38, and JNK was assessed by Western blotting. PMA ( $10^{-7}$  M for 30 min) was used as a positive control of MAPK phosphorylation in these cell lines. As demonstrated in Fig. 4A, LPS (10  $\mu$ g/ml) strongly up-regulated

p42/p44 Mapk in CMT93 cells within 5 min after stimulation. In contrast, LPS did not affect significantly activation of p38 and JNK (data not presented). In the absence of LPS stimulation (negative control), minimal baseline MAPK activity was measured, presumably reflecting primarily physical stress (change of the media). p42/p44 MAPK phosphorylation, as measured by immunoblotting, was proportional to increasing concentrations of LPS and required the presence of FBS (Fig. 4B). The absence of serum completely abolished the activation of MAPK in LPS-treated CMT93 cells.

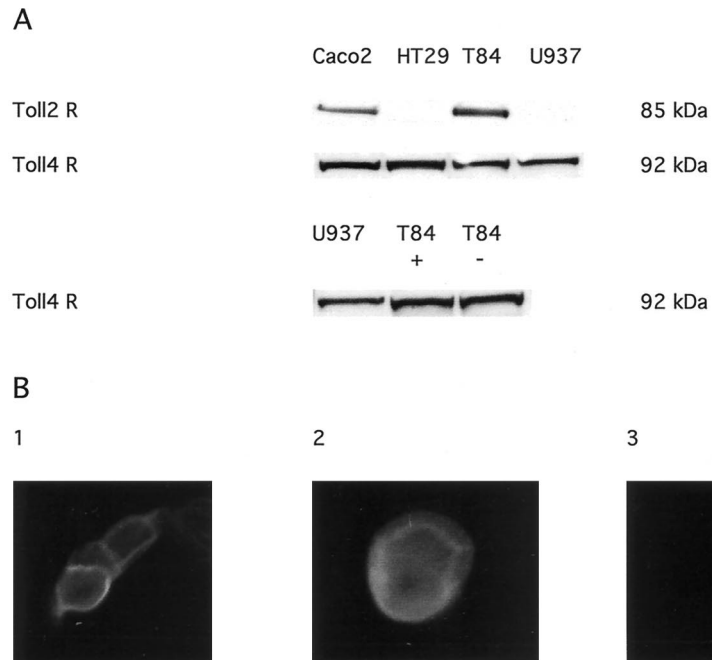
In contrast, phosphorylation of p42/p44 MAPK following stimulation of T84 cells with LPS was delayed with maximal stimulation at 30 min. Activation returned to resting levels after 1 h (Fig. 5). JNK was minimally activated by LPS in a time-dependent fashion in differentiated T84 cells. However, we failed to detect activation of p38 in T84 cells in response to LPS (data not presented). Phosphorylation of p38 was assessed in lysates of HT29 cells prepared at various times after the addition of LPS. As shown in Fig. 6, a major peak of p38 activity was observed at 15 min. However, cell lysates of HT29 stimulated with 5  $\mu$ g/ml LPS did not exhibit specific induction of JNK or p42/p44 MAPK activity at any time (data not presented). As PMA did not induce significant phosphorylation of p38 in HT29 cells (data not shown), the positive and negative controls provided from the manufacturer are presented.

HT29 cells were incubated with LPS (0.5 or 5  $\mu$ g/ml) for various time periods, and EMSAs were performed to determine whether LPS activates NF- $\kappa$ B consensus sequence binding in IEC. As shown in Fig. 7A, LPS up-regulated the binding of proteins to the NF- $\kappa$ B consensus sequence in a time- and dose-dependent



**FIGURE 2.** TLR2, 3, and 4 mRNA expression in IEC. Expression of human TLR2, TLR3, and TLR4 mRNA in T84 and HT29 cells were analyzed by RT-PCR as detailed in *Materials and Methods*. THP1 cells were used as positive sources of human TLR mRNA expression to confirm the specificity of the primers and PCR. As representatives, cDNA products of TLR2 in T84 and TLR3 and TLR4 in HT29 cells were confirmed by sequencing after subcloning into the TA cloning vector pCR2.1. SM, Size marker.

**FIGURE 3.** TLR2 and 4 protein expression in human IEC. *A*, Western blotting, as described in *Materials and Methods*, was used to assess protein expression of TLR2 and TLR4 in T84, HT29, or Caco2 cells. The monocytic cell line U937 was used as presumed positive control for TLR protein expression. Additionally, T84 cells were stimulated with LPS (5  $\mu\text{g/ml}$ ) for 15 min to assess the effect on TLR4 protein expression. *B*, Immunofluorescence histochemistry was used to visualize the TLR's surface expression of T84 cells (1, staining with anti-TLR2; 2, anti-TLR4; 3, normal anti-rabbit IgG as negative control).



manner. Activation was specifically inhibited by addition of 100-fold excess of unlabeled NF- $\kappa$ B oligonucleotides containing a wild-type NF- $\kappa$ B binding sequence. This result was confirmed (Fig. 7*B*) by Western blot analysis of the expression of the NF- $\kappa$ B subunit p65 in nuclear extracts of HT29 cells after stimulation for 10 min with LPS (0.5 or 5  $\mu\text{g/ml}$ ). As demonstrated in Fig. 7*C*, LPS-induced nuclear translocation of p65 required the presence of serum in T84 cells.

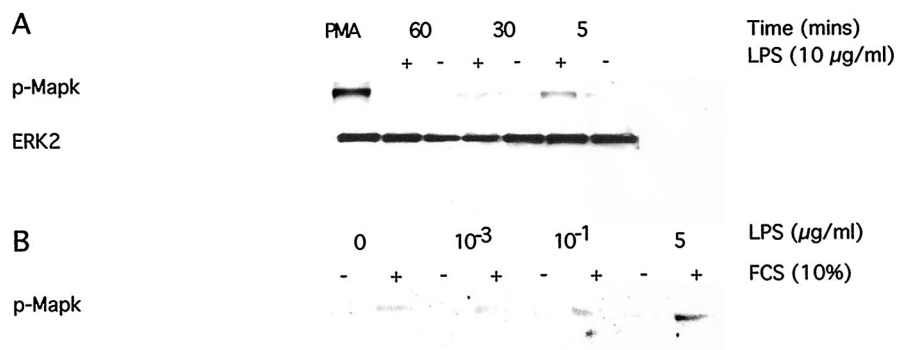
**Discussion**

The intestinal mucosal barrier is regulated by a complex network of cytokine mediating epithelial cell-immune cell interactions. A broad spectrum of several peptides has been demonstrated to play an essential role in regulating the intestinal immune system to balance host mucosal defense, tolerance of resident colonic microorganisms, and repair of the intestinal epithelial surface barrier following intestinal epithelial injury (39). The intestinal epithelium

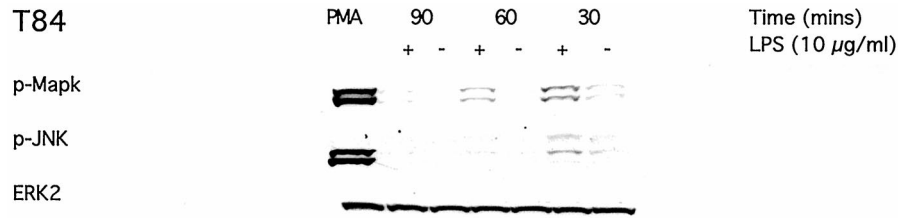
itself appears to play a key role in up-regulation of the host immune defense by recognizing and subsequently responding to invading pathogenic threats by secretion of proinflammatory cytokines (2).

We hypothesized that LPS, a key product of pathogenic Gram-negative bacteria, might stimulate different signal transduction pathways in IEC. LPS activates myeloid lineage cells by binding to membrane-bound CD14 (mCD14) (9). To determine whether the receptor mCD14 could be involved in LPS-induced IEC activation, we examined CD14 mRNA and protein expression in IEC lines. However, none of the three tumor IEC lines used in this study expressed constitutive mRNA for CD14. Using immunohistochemistry, CD14 cell-surface protein expression was not detectable in our *in vitro* studies of intestinal tumor cell lines. However, mCD14 expression has been shown to be significantly up-regulated in response to LPS in various systems *in vivo* (40). Despite the inability to detect mCD14 in these cell lines *in vitro*, possibly

**CMT93**



**FIGURE 4.** Effects of LPS on activity of p42/p44 MAPK in mouse colonic epithelial cells. *A*, Time course of activation of p42/p44 MAPK by LPS (10  $\mu\text{g/ml}$ ). In parallel studies, CMT93 monolayers were exposed to fresh DMEM containing 10% FBS, without LPS (negative control). PMA ( $10^{-7}$  M) has been used as a positive control (30 min of stimulation). To confirm equal loading, the blot was reprobbed with anti-ERK2 (total). *B*, Serum-dependent effects of different doses of LPS on MAPK activation. Cells were washed twice with serum-free DMEM and then preincubated with fresh DMEM containing 0 or 10% FBS for 3 h. Cells were stimulated for 7 min with LPS added directly to the media. Whole-cell lysates from confluent CMT93 monolayers were harvested after stimulation with or without LPS. Phosphorylation of MAPK was determined by Western blotting as described in *Materials and Methods*.



**FIGURE 5.** Time-dependent effects of apical LPS-stimulation on activity of MAPK in differentiated T84 cells. Time course of activation of p42/p44 MAPK and JNK by LPS (10 µg/ml), applied to the apical chamber, in T84 cells. In parallel studies, cell monolayers were exposed to fresh media containing full serum, without LPS (negative control). T84 cells formed confluent monolayers within 14–21 days from seeding on filters, with transepithelial resistance (TER) values >1500 Ωcm<sup>2</sup>. Phosphorylation of MAPK was determined as described in *Materials and Methods*. PMA (10<sup>-7</sup> M) has been used as a positive control (30 min of stimulation). To confirm equal protein loading, the blot was reprobed with anti-ERK2 (total).

exposure to abnormal high concentrations of luminal LPS during acute bacterial infection *in vivo* might produce mCD14 gene expression by IEC. Interestingly, IL-2-deficient (-/-) mice overexpress intestinal epithelial mCD14 RNA, which is not detectable in the wild type (41).

CD14 also exists as a soluble plasma protein (sCD14), which, together with another serum protein designated LBP, can facilitate binding of LPS to epithelial and endothelial cells (12). Our results demonstrate that to activate distinct intracellular signal transduction pathways in IEC by LPS, serum is required, presumably as a source of soluble CD14 and LBP (10). These proteins may function as opsonins that capture pathogenic microbes facilitating recognition of luminal bacterial pathogens by the mCD14-negative intestinal epithelium. These serum proteins may be released from the vascular space when the intestinal epithelial monolayer is disrupted and invaded by pathogens. Injury of the intestinal mucosa may also lead to recruitment and migration of acute inflammatory cells into the mucosa (42), which could release sCD14 by cleavage of mCD14 (43), inducing a defensive response of the intestinal epithelium to bacterial toxins.

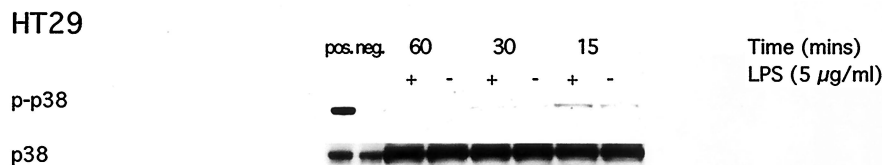
Recently, CD14-deficient mice have been shown to be highly resistant to shock induced by purified LPS or bacteremia (44). In active inflammatory bowel disease, CD14 is highly up-regulated in recruited monocytes of the intestinal mucosa (45) and could play an important role promoting hyperresponsiveness of the intestinal epithelium to LPS. TLR appear to act cooperatively with CD14 in LPS-induced cellular signal transduction in peripheral blood leukocytes. Yang et al. have shown that TLR2 expression is activated by LPS in a response that depends on LBP and is enhanced by CD14 (22). The present study demonstrates that IEC express at least two TLRs. Recently, it has been shown that TLR mRNA is absent in macrophages from normal mucosa but is expressed in macrophages in inflamed mucosa of patients with inflammatory bowel disease (46). *In vivo* studies are needed to clarify the role of CD14 and TLR proteins in the intestinal epithelium as well as inflammatory cells in the pathogenesis of inflammatory bowel dis-

eases. Immune imbalance in these patients could result from undue activation by LPS and bacteria through these epithelial receptors. Conceptually, neutralizing Abs against CD14 and/or LBP could ameliorate colitis.

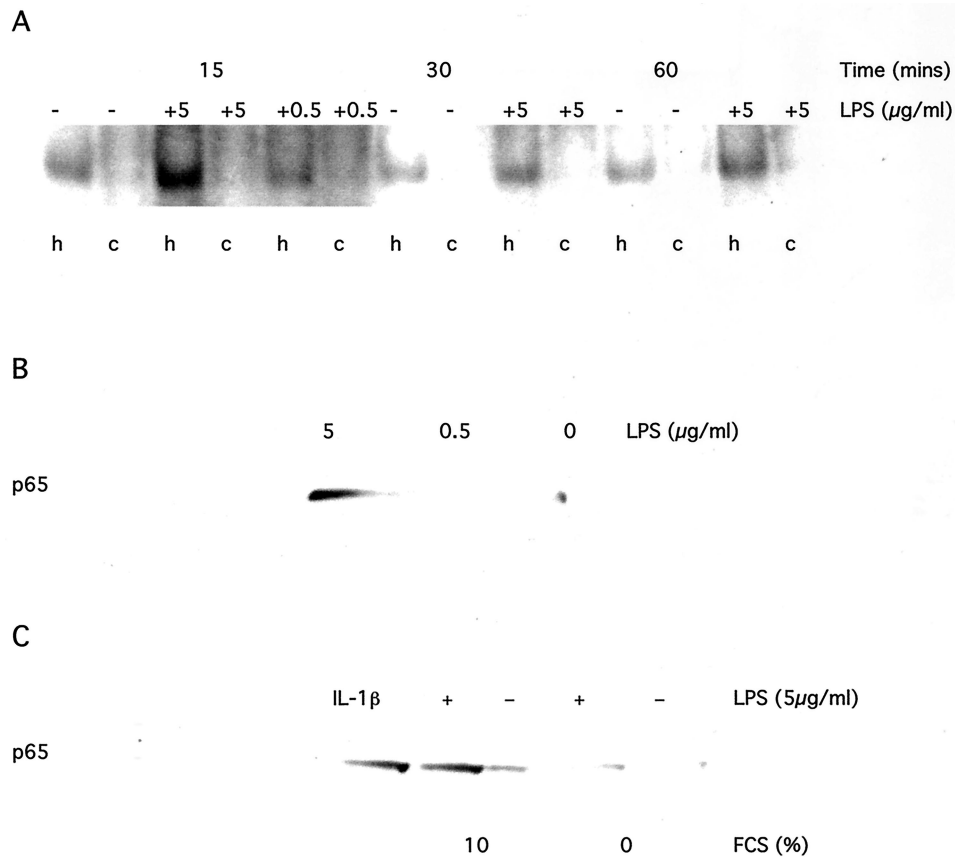
MAPK appears to be an important mediator of LPS activation in IEC. We show that p42/p44 MAPK is selectively activated in a concentration-dependent manner by LPS. Of interest, stimulation of p42/p44 MAPK was less strong than that observed after PMA. This suggests that IEC may be partially desensitized or tolerant of LPS, limiting activation of the underlying immune cells in the face of constant exposure to LPS at the apical surface of the epithelium. Hyporesponsiveness may primarily result from the absence of mCD14. The time courses of activation of these kinases in IEC lines following stimulation with LPS was maximal for p42/p44 at 5–10 min and for p38 and JNK at later time points (10–30 min), which is consistent with observations in other cell lines (25, 26, 47). LPS activation of p42/p44 in differentiated T84 cells was delayed and peaked later at 30 min.

Interestingly, LPS-induced stimulation of different IEC lines involves selected activation of MAPK pathways. Thus, we did not observe significant LPS-induced activation (>1.5 fold) of JNK or p38 in CMT93 cells, p38 in T84 cells or MAPK, and JNK in HT29 cells. These results may reflect cell-specific features, including the state of differentiation or idiosyncratic alterations of signal transduction pathways in these colon cancer cell lines. It is known that LPS stimulation does not always result in the activation of p42/p44 MAPK, JNK, or p38 in other cell lines, including subgroups of peripheral blood leukocytes. Nick et al. have shown that neither p42/p44 MAPK nor JNK are activated by LPS stimulation of neutrophils (47).

NF-κB is an abundantly expressed transcription factor that is central to several immune and inflammatory responses, leading to rapid induction of cytokine secretion (48). Awane et al. have recently shown that NF-κB-inducing kinase serves as the common mediator in the NF-κB signaling cascades triggered by IL-17, TNF-α, and IL-1β in IEC (49). In immune effector cells like



**FIGURE 6.** Effects of LPS on activity of p38 in HT29 cells. Time course of activation of p38 by LPS (5 µg/ml) in HT29 cells. In parallel studies, HT29 monolayers were exposed to fresh DMEM containing 10% FBS, without LPS (negative control). Whole-cell lysates from 90% confluent HT29 monolayers were harvested after stimulation with or without LPS for the indicated time periods. Phosphorylation of p38 was determined by Western blotting, as described in *Materials and Methods*. The positive and negative control (C6 glioma whole-cell lysates prepared with and without anisomycin treatment) were obtained from the manufacturer. To confirm equal protein loading, each blot was reprobed with anti-p38 (total). p-p38, Phosphorylated p38 protein.



**FIGURE 7.** Effects of LPS on NF- $\kappa$ B activation in human IEC. **A**, Time course of NF- $\kappa$ B activation by LPS in HT29 cells. EMSA was conducted using labeled double-stranded oligonucleotide encoding the NF- $\kappa$ B binding consensus sequence (*h*) and cold competitor (*c*). HT29 cells were stimulated with LPS (0.5 or 5  $\mu$ g/ml) in fresh media for the indicated time periods. In parallel studies, cell monolayers were changed to fresh media without LPS and also incubated for the same time periods. **B**, Dose-dependent nuclear translocation of p65 by LPS in HT29 cells. The expression of the NF- $\kappa$ B subunit p65 in HT29 cells was determined by Western blot analysis in nuclear extracts with LPS (0.5 or 5  $\mu$ g/ml) in fresh media (10% FBS) after stimulation for 10 min. As negative control, HT29 cells were changed to fresh media (10% FBS) without LPS and also incubated for 10 min. **C**, Serum-dependent nuclear translocation of p65 by LPS in T84 cells. The expression of the NF- $\kappa$ B subunit p65 in T84 cells was determined by Western blot analysis in nuclear extracts with or without LPS (5  $\mu$ g/ml) in the presence or absence of serum after incubation for 10 min. Cells were washed twice with serum-free media and then preincubated for 12 h with fresh media containing 0% FBS. LPS, with or without serum, was then directly added to the media. T84 cells were stimulated for 10 min, under serum-free conditions, with IL-1 $\beta$  (10 ng/ml) as positive control.

monocytes, bacterial LPS has been demonstrated to be a potent stimulus of NF- $\kappa$ B (50). After stimulation with LPS, TLR4 can activate NF- $\kappa$ B transcription and induces expression of inflammatory cytokines and costimulatory molecules, suggesting that human TLRs participate in the innate immune response and signal the activation of adaptive immunity (15). Enteropathogenic *Escherichia coli* are known to activate NF- $\kappa$ B in IEC, which is causally linked to IL-8 production (51). Naumann et al. have suggested that the activation of NF- $\kappa$ B in epithelial cells is independent of penetration and invasion of pathogenic bacteria (52). We demonstrate by gel shift assays and Western blotting that, in the absence of bacteria, the pathogenic toxin LPS itself is an effective inducer of NF- $\kappa$ B expression in the IEC lines HT29 and T84. LPS induction of NF- $\kappa$ B activation is time- (maximal at 15 min) and concentration-dependent in these cell lines. LPS-induced activation of NF- $\kappa$ B factor in IEC depends on the presence of serum. Whether LPS directly transduces activation of NF- $\kappa$ B via TLR in IEC needs to be further investigated. Held et al. have shown that IFN- $\gamma$  may trigger LPS activity in macrophages by LPS-induced NF- $\kappa$ B transcription (53). Synergistic induction of NF- $\kappa$ B activation might also play an important role in host defense of the intestinal epithelium, enabling quiescent cells to respond quicker to bacterial Ags.

Finally, we conclude that the lack of constitutive mCD14 may make IEC hyporesponsive and tolerant to the constant luminal exposure of resident microflora and nondangerous amounts of pathogenic bacterial toxins. However, our results also suggest that any release or expression of specific serum mediator proteins may turn quiescent IEC into defensive immune cells with the capability to immediately recognize serious infectious challenges. IEC constitutively express TLRs that might be a critical link to readily up-regulate distinct intracellular signal transduction pathways as stress response—analogue to primary effector cells of the immune system.

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