



EPITHELIAL AND MESENCHYMAL CELL BIOLOGY

Lipopolysaccharide Causes an Increase in Intestinal Tight Junction Permeability *in Vitro* and *in Vivo* by Inducing Enterocyte Membrane Expression and Localization of TLR-4 and CD14

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Bacterial-derived lipopolysaccharides (LPS) play an essential role in the inflammatory process of inflammatory bowel disease. A defective intestinal tight junction (TJ) barrier is an important pathogenic factor of inflammatory bowel disease and other inflammatory conditions of the gut. Despite its importance in mediating intestinal inflammation, the physiological effects of LPS on the intestinal epithelial barrier remain unclear. The major aims of this study were to determine the effects of physiologically relevant concentrations of LPS (0 to 1 ng/mL) on intestinal barrier function using an *in vitro* (filter-grown Caco-2 monolayers) and an *in vivo* (mouse intestinal perfusion) intestinal epithelial model system. LPS, at physiologically relevant concentrations (0 to 1 ng/mL), in the basolateral compartment produced a time-dependent increase in Caco-2 TJ permeability without inducing cell death. Intraperitoneal injection of LPS (0.1 mg/kg), leading to clinically relevant plasma concentrations, also caused a time-dependent increase in intestinal permeability *in vivo*. The LPS-induced increase in intestinal TJ permeability was mediated by an increase in enterocyte membrane TLR-4 expression and a TLR-4-dependent increase in membrane colocalization of membrane-associated protein CD14. In conclusion, these studies show for the first time that LPS causes an increase in intestinal permeability via an intracellular mechanism involving TLR-4-dependent up-regulation of CD14 membrane expression. (*Am J Pathol* 2013, 182: 375–387; <http://dx.doi.org/10.1016/j.ajpath.2012.10.014>)

An integral function of intestinal epithelial cells is to act as a physical barrier, separating the noxious luminal environment from the underlying lamina propria and the deeper intestinal layers.^{1,2} The apically located tight junctions (TJs) form a paracellular seal between the lateral membranes of adjacent intestinal epithelial cells, and act as a structural and functional barrier against paracellular flux of luminal substances. Defective intestinal epithelial TJ barrier has been shown to be an important pathogenic factor of inflammatory bowel disease (IBD) and necrotizing enterocolitis (NEC) by allowing paracellular permeation of luminal antigens that elicit and promote inflammatory response.^{1,2} Both clinical and animal studies have shown the importance of a defective intestinal TJ barrier in the development and prolongation of intestinal inflammation in IBD and NEC.^{1–5} These studies have shown that normalization of intestinal barrier in patients with active Crohn's disease predicts prolonged clinical

remission, whereas a persistent increase in intestinal permeability portends poor clinical outcome with rapid recurrence of the disease.^{6,7} Additionally, animal studies have also shown that a primary defect in intestinal junctional complexes was sufficient to induce or aggravate intestinal inflammation in murine models of IBD,^{8,9} whereas therapeutic tightening or enhancement of the intestinal TJ barrier prevented the development of intestinal inflammation.^{3,10}

The terms *endotoxin* and *lipopolysaccharide* (LPS) are used interchangeably and refer to the major cell wall

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component of Gram-negative bacteria.^{11,12} LPS are complex amphiphilic molecules having a hydrophobic (consisting of lipid A) and a hydrophilic (consisting of carbohydrate core and polysaccharide O-antigen) component and are released from bacterial cell wall by shedding or through bacterial lysis.^{11–13} LPS concentrations are highest in the gut lumen, where many trillions of commensal bacteria reside. Normally, LPS in the gut lumen do not penetrate across the healthy intestinal epithelium^{14,15}; however, in intestinal permeability disorders, the defective TJ barrier allows paracellular flux of LPS and other luminal antigens.^{11–13,16–19} The intestinal tissue and circulating LPS levels are markedly elevated in IBD and NEC, and play an important role in mediating inflammatory response.^{11–13,16–18} The involvement of LPS in the initiation and propagation of intestinal inflammation in IBD and NEC has been well demonstrated.^{20–23} These studies have shown LPS to be an important contributing factor of intestinal inflammation, and removal of circulating LPS accelerated the clinical improvement of IBD and NEC.^{20,22,23} Despite the importance of a defective intestinal barrier in the accentuation and prolongation of intestinal inflammation in IBD and NEC,^{3,6,9,20,22} the effects of circulating levels of LPS on the intestinal epithelial barrier remain unknown. Because LPS levels are markedly elevated in these diseases and play an important role in the inflammatory process, understanding the effects of LPS on intestinal barrier function has important potential clinical significance.

In normal healthy individuals, plasma concentrations of LPS range from undetectable levels up to 0.2 ng/mL.^{11,12,20,22} A variety of physiological factors such as prolonged physical exertion, high-fat diet, physiological stresses, or heat can lead to elevated plasma LPS levels as high as 1 to 2 ng/mL.^{24–27} Patients with intestinal permeability disorders such as Crohn's disease, NEC, acute pancreatitis, alcoholic liver disease, and critical illnesses also have elevated plasma LPS levels ranging up to 2 to 10 ng/mL.^{11–13,20,22,28} Based on these reports, we consider LPS levels of 0 to 1.0 ng/mL to be physiologically relevant and 0 to 10 ng/mL to be clinically relevant. (For reference, the concentration of LPS in the gut lumen has been reported to be 1.8 µg/mL in the rat distal ileum.^{29,30}) Inexplicably, in most of the published studies, extreme pharmacological concentrations of LPS ranging between 50 and 1000 µg/mL, which exceed the physiologically achievable concentrations by 10⁴- to 10⁷-fold, have been used to assess various biological responses.^{30–34} At these extreme concentrations, LPS causes rapid cell death in various cell types studied, including in intestinal and immune cells,^{30,33–35} and does not provide accurate depiction of biological activity of LPS. Herein, we show that LPS, at physiologically and clinically relevant concentrations (0 to 10 ng/mL), does not cause intestinal epithelial cell death, but causes a selective increase in intestinal TJ permeability *in vitro* and *in vivo*. These studies also show for the first time that pattern recognition receptors Toll-like receptor 4 (TLR-4) and CD14 play

a central role in the modulation of the intestinal epithelial TJ barrier.

Materials and Methods

Reagents

Dulbecco's modified Eagle's medium, trypsin, fetal bovine serum, glutamine, penicillin, streptomycin, PBS, and horseradish peroxidase-conjugated secondary antibodies for Western blot analysis were purchased from Invitrogen/Life Technologies (Carlsbad, CA). Small-interfering RNA (siRNA) of TLR-4, CD14, and transfection reagents were from Dharmacon/Thermo Fisher Scientific (Lafayette, CO). LPS (O111:B4) was purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were of reagent grade and were purchased from Sigma-Aldrich, VWR (Aurora, CO), or Fisher Scientific (Pittsburgh, PA).

Cell Culture

Caco-2 cells (passage 20) were purchased from ATCC (Manassas, VA) and maintained at 37°C in a culture medium composed of Dulbecco's modified Eagle's medium with 4.5 mg/mL glucose, 50 U/mL penicillin, 50 U/mL streptomycin, 4 mmol/L glutamine, 25 mmol/L HEPES, and 10% fetal bovine serum as previously described.^{36,37} Caco-2 cells were used between passages 22 and 28 in this study. The human-derived, nontransformed colonic epithelial cell line NCM460 was maintained in M3:10 medium (INCELL, San Antonio, TX), but was shifted to Ham's F-12 culture medium supplemented with 20% fetal bovine serum and antibiotics as previously described.³⁸ NCM460 cells were used between passages 29 and 39 in this study. T84 intestinal epithelial cells (passage 53) were purchased from ATCC (Rockville, MD) and maintained at 37°C in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 16 mmol/L NaHCO₃, antibiotics, and 5% fetal bovine serum, as previously described.³⁹ Only T84 cells from passages 55 to 61 were used to maintain consistency. The cells were kept at 37°C in a 5% CO₂ environment. For growth on filters, high-density cells (1 × 10⁵ cells) were plated on Transwell filters with 0.4-µm pore size (Corning Incorporated, Corning, NY) and monitored regularly by visualization with an inverted microscope (Eclipse TS100/100-F; Nikon, Melville, NY) and by epithelial resistance measurements.

Determination of Epithelial Monolayer Resistance and Paracellular Permeability

Caco-2 transepithelial electrical resistance (TER) was measured by using an epithelial voltohmmeter (World Precision Instruments, Sarasota, FL) as previously reported.^{36,37} Both apical and basolateral sides of the epithelium were bathed with buffer solution. Electrical resistance was measured until similar values were recorded on three consecutive measurements.

Caco-2 paracellular permeability was determined by using an established paracellular marker inulin. Known concentrations of permeability marker (10 $\mu\text{mol/L}$) and its radioactive tracer were added to the apical solution.³⁶ Low concentrations of permeability markers were used to ensure that negligible osmotic or concentration gradients were introduced.

Assessment of Protein Expression by Western Blot Analysis

Protein expression from Caco-2 cells and mouse tissue was assessed by Western blot as previously described.^{36,37} Cells and mouse tissue were lysed with lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 500 $\mu\text{mol/L}$ NaF, 2 mmol/L EDTA, 100 $\mu\text{mol/L}$ vanadate, 100 $\mu\text{mol/L}$ phenylmethylsulfonyl fluoride, 1 $\mu\text{g/mL}$ leupeptin, 1 $\mu\text{g/mL}$ pepstatin A, 40 mmol/L para-nitrophenyl phosphate, 1 $\mu\text{g/mL}$ aprotinin, and 1% Triton X-100]. The lysates were centrifuged at 10,000 $\times g$ for 10 minutes in an Eppendorf Centrifuge (5417R; Hauppauge, NY) to obtain a clear lysate. The supernatant was collected and protein concentration was determined using the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA). Laemmli gel loading buffer (Bio-Rad Laboratories) was added to the lysate containing 10 to 20 μg of protein and boiled at 100°C for 7 minutes, after which proteins were separated on an SDS-PAGE gel. Proteins from the gel were transferred to the membrane (*Trans*-Blot Transfer Medium, nitrocellulose membrane; Bio-Rad Laboratories) overnight. The membrane was incubated for 2 hours in blocking solution (5% dry milk in TBS-Tween 20 buffer). The membrane was then incubated with antibody in blocking solution. After a wash in TBS-1% Tween buffer, the membrane was incubated in secondary antibody and developed using Santa Cruz Western Blotting Luminol Reagents (Santa Cruz Biotechnology, Santa Cruz, CA) on Kodak BioMax MS film (Fisher Scientific). The films were exposed between 5 seconds and 10 minutes.

Assessment of Apoptosis

Caco-2 apoptosis was assessed using the CytoGLO Annexin V-FITC Apoptosis Detection kit from Imgenex as previously described.³⁷ Following appropriate treatments, apoptosis was measured by flow cytometry using a FACScan flow cytometer (BD Biosciences, San Jose, CA). For each experimental sample, a total of 10,000 cells were counted for Annexin V-FITC and propidium iodide stain.

Proliferation Assay

Cell proliferation was measured using Quick Cell Proliferation Assay kit (ab65473; Abcam, Cambridge, MA) according to the manufacturer's instructions. This assay is based on cleavage of the tetrazolium salt WST-1 to formazan by cellular mitochondrial dehydrogenases. Cell proliferation is accompanied by an increase in the activity of the

mitochondrial dehydrogenases, which leads to an increase in conversion of tetrazolium salt, WST-1, to formazan. Briefly, Caco-2 cells (1×10^5 cells) were plated on Transwell filters. After LPS treatment, tetrazolium salt WST-1 solution was added to each filter, and the reaction was allowed to occur in standard culture condition for 4 hours. The optical density (formazan formation) was measured at 440 nm with a reference wavelength of 650 nm using a precision microplate reader (Molecular Devices, Sunnyvale, CA) as a measure of mitochondrial dehydrogenase activity.

Immunostaining

Colocalization of TLR-4 and CD14 was assessed by immunofluorescent antibody labeling.⁴⁰ Caco-2 monolayers or frozen mouse intestinal sections were washed and fixed with 2% paraformaldehyde. After being permeabilized with 0.1% Triton X-100 in PBS, Caco-2 monolayers or intestinal tissues were incubated in blocking solution composed of bovine serum albumin and normal donkey serum in PBS for 1 hour, then labeled with primary antibodies in blocking solution overnight at 4°C, followed by fluorescein isothiocyanate or Cy3-conjugated secondary antibodies for 1 hour at room temperature. ProLong Gold Antifade reagent (Invitrogen) was used to mount the filters onto coverslips. Immunolocalizations of TLR-4 and CD14 were visualized using a confocal fluorescence microscope (LSM 510 Meta Confocal; University of New Mexico Fluorescence Microscopy Shared Resource) equipped with a Hamamatsu Digital Camera 9 (Hamamatsu Photonics, Hamamatsu, Japan). Images were processed with LSM software (Carl Zeiss, Oberkochen, Germany).

siRNA Transfection

Caco-2 monolayers were transiently transfected with siRNA using DharmaFect transfection reagent (Dharmacon/Thermo Fisher Scientific) as previously described.⁴⁰ Briefly, cells (1×10^5 /filter) were seeded into a 12-well Transwell plate and grown to confluency. Caco-2 monolayers were then washed with PBS twice; 0.5 mL Accell medium (Thermo Fisher Scientific) was added to the apical compartment of each filter, and 1.5 mL were added to the basolateral compartment of each filter. siRNA (5 ng) of interest and DharmaFect reagent (2 μL) were preincubated in Accell medium. After 5 minutes of incubation, the two solutions were mixed, and the mixture was added to the apical compartment of each filter. For LPS experiments, Caco-2 cells were transfected with siRNA for 24 hours before the LPS treatment. The Caco-2 TJ barrier assessments were performed at the end of day 5 of LPS treatment.

In Vivo Determination of Mouse Intestinal Permeability and LPS Plasma Level

The Laboratory Animal Care and Use Committee at the University of New Mexico approved all experimental

protocols. Male C57BL/6 mice, *TLR-4* knockout mice, and *CD14* knockout mice (9 to 10 weeks) were purchased from the Jackson Laboratories (Bar Harbor, ME). The mice were kept two per cage in a temperature-controlled room at 25°C with a 12:12-hour light–dark cycle. Diet and drinking water were provided *ad libitum*.

LPS effect on intestinal permeability in an *in vivo* mouse model system was determined using a recycling intestinal perfusion method as previously described.^{36,40,41} Mice were injected with varying concentrations of LPS intraperitoneally (i.p.) every 24 hours for up to 5 days of the experimental period. A 6-cm segment of mouse small intestine was isolated and cannulated with a small-diameter plastic tube (in an anesthetized mouse maintained on 1% isoflurane in oxygen) and continuously perfused with 5 mL of Krebs-phosphate saline buffer for a 2-hour perfusion period.^{36,40} An external recirculating pump (Econo Pump; Bio-Rad Laboratories) was used to recirculate the perfusate at a constant flow rate (0.75 mL/min). The body temperature of the mouse was maintained at 37°C with a temperature-controlled warming blanket. The intestinal permeability was assessed by measuring the flux rate of the paracellular probe, Texas Red–labeled dextran (MW = 10,000 g/mol). Water absorption was determined by using a nonabsorbable marker, sodium ferrocyanide, or by measuring the difference between the initial and final volumes of the perfusate. The plasma LPS level was measured using Limulus Amebocyte Lysate Assay (Associates of Cape Cod, E. Falmouth, MA).

Statistical Analysis

Results are expressed as means \pm SE, and analyzed using Student's *t*-test for unpaired data (GraphPad Prism version 5.00 for Windows; GraphPad Software, La Jolla, CA). A *P* value of ≤ 0.05 was used to indicate statistical significance. All experiments were repeated at least three times to ensure reproducibility.

Results

LPS Effect on Caco-2 Intestinal Epithelial TJ Permeability

The effects of physiologically and clinically achievable concentrations of LPS (0 to 10 ng/mL) on intestinal epithelial TJ permeability was determined *in vitro* by measuring TER and mucosal-to-serosal flux of paracellular marker inulin in filter-grown Caco-2 intestinal monolayers, over a 5-day experimental period. LPS (0, 0.1, 0.3, 0.5, 1, 5, and 20 ng/mL) caused a concentration-dependent drop in Caco-2 TER (Figure 1A) and an increase in inulin flux (Figure 1B). LPS caused a significant drop in TER and an increase in inulin flux at a concentration of 0.3 ng/mL. Increasing the LPS concentration above 0.3 ng/mL caused only a minimal additional drop in Caco-2 TER (Figure 1A) or increase in inulin flux (Figure 1B). These results

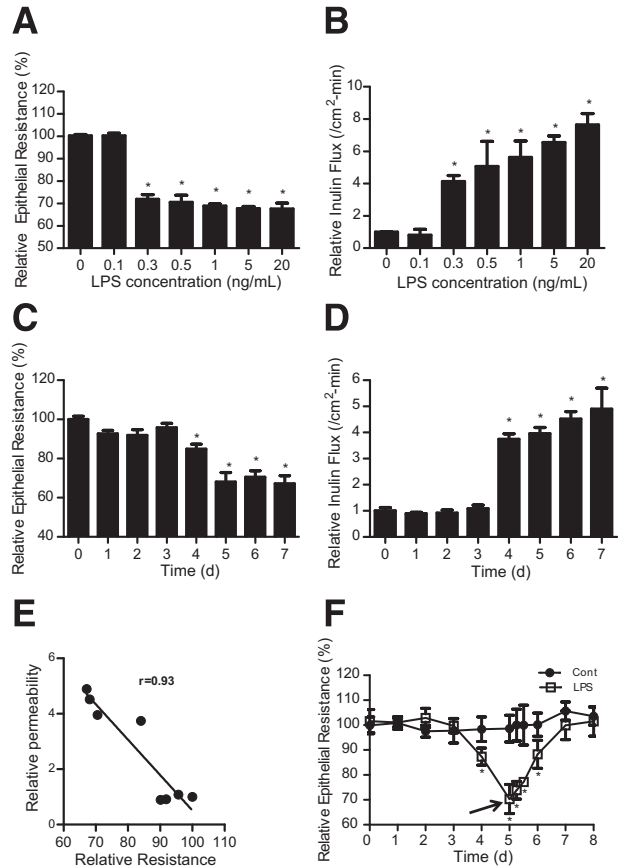


Figure 1 Effect of LPS on Caco-2 TJ permeability. **A:** Effect of increasing concentrations of LPS (0, 0.1, 0.3, 0.5, 1, 5, and 20 ng/mL) on Caco-2 TER. The mean TER for control (Cont) Caco-2 monolayers was $481 \pm 5 \Omega \cdot \text{cm}^2$. **B:** Effect of increasing concentrations of LPS (0, 0.1, 0.3, 0.5, 1, 5, and 20 ng/mL) on Caco-2 transepithelial flux of paracellular marker inulin. Filter-grown Caco-2 monolayers were treated with LPS for a 5-day experimental period. **C:** Time-course effect of LPS (0.3 ng/mL) on Caco-2 TER. The mean TER for day 0 was $450 \pm 14 \Omega \cdot \text{cm}^2$. **D:** Time-course effect of LPS (0.3 ng/mL) on Caco-2 inulin flux. The effect of LPS (0.3 ng/mL) on Caco-2 TER and inulin flux were measured over a 7-day experimental period. **E:** Graph of TER versus inulin flux ($r = 0.93$). **F:** Time course of Caco-2 TER recovery after LPS (0.3 ng/mL) removal. LPS was removed from the basolateral compartment after 5 days of treatment (arrow). The mean TER for control Caco-2 monolayers was $486 \pm 28 \Omega \cdot \text{cm}^2$. * $P < 0.0001$ versus control.

indicated that LPS at concentrations of 0.3 ng/mL or above cause an increase in Caco-2 TJ permeability. (In subsequent experiments, a LPS concentration of 0.3 ng/mL was used, unless stated otherwise.)

Next, the time-course effect of LPS (0.3 ng/mL) on Caco-2 TJ permeability was determined. LPS did not have significant effect on Caco-2 TER up to day 3, but caused a drop in TER between days 4 and 5. The drop in TER reached a trough level by day 5 and remained unchanged up to day 7 (Figure 1C). Conversely, LPS caused an increase in mucosal-to-serosal inulin flux by day 4, and the increase in inulin flux persisted up to day 7 (Figure 1D). A similar time-course effect was also observed for higher concentrations of LPS (1 and 20 ng/mL) (data not shown). The plot of the LPS-induced drop in TER and increase in paracellular flux

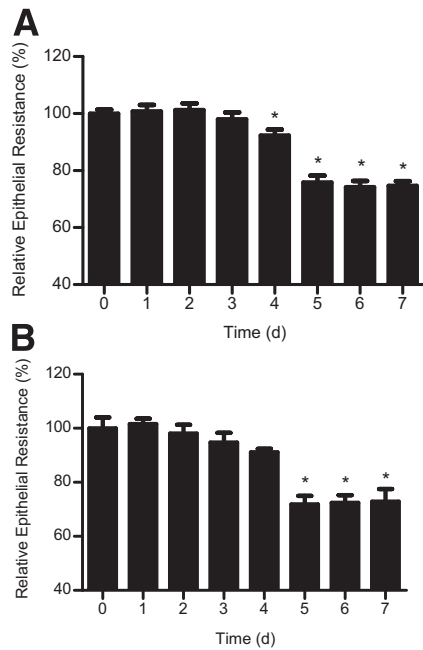


Figure 2 Time-course effect of LPS (0.3 ng/mL) on NCM460 TER (A) and T84 TER (B). The mean TER for NCM460 monolayers at day 0 was $418 \pm 14 \Omega \bullet \text{cm}^2$, and the mean TER for T84 monolayers at day 0 was $1570 \pm 108 \Omega \bullet \text{cm}^2$. * $P < 0.0001$ versus control.

demonstrates a linear relationship (relative correlation coefficient, $r = 0.93$) between the drop in Caco-2 TER and the increase in inulin flux (Figure 1E), indicating that the LPS-induced drop in Caco-2 TER correlates with an increase in paracellular permeability. The time course of Caco-2 TER recovery following LPS removal was also examined. The removal of LPS after 5 days of treatment resulted in full restoration of the Caco-2 TER within 2 days of removal (Figure 1F).

To confirm that the LPS effect extends to other intestinal epithelial cell lines, we also examined the effect of LPS (0.3 ng/mL) on NCM460 and T84 monolayer TER. NCM460 is a human-derived, nontransformed colonic epithelial cell line, and T84 is a colon cancer-derived cell line having high TER. LPS caused a similar time-dependent drop in NCM460 and T84 TER (Figure 2) as in the Caco-2 monolayers. There was an approximately 30% drop in TER by day 4 or 5 of LPS treatment. Because LPS preparation is known to contain traces of impurities, including cytokines and other lipoproteins, the effect of heat-inactivated LPS (exposure to 121°C for 30 minutes) on Caco-2, NCM460, and T84 TER was also examined. Heat-inactivated LPS did not affect the TER of intestinal monolayers (data not shown), suggesting that the impurities in LPS did not affect the intestinal monolayer TER and that active LPS was required for the drop in TER.

To assess the membrane specificity of LPS effect, LPS (0.3 ng/mL) was added to either the apical, basolateral, or combined apical and basolateral compartments. The addition of LPS to the apical solution did not have a significant effect

on Caco-2 TER (Figure 3A) or inulin flux (Figure 3B), whereas the addition of LPS to the basolateral solution caused a drop in Caco-2 TER and an increase in inulin flux. The addition of LPS to both apical and basolateral compartments caused a similar change in TJ permeability as the basolateral compartment alone (Figure 3, A and B). To investigate the possibility that the LPS effect on the apical membrane may be concentration related, higher concentrations of LPS were also added to the apical solution. The higher concentrations (20 ng/mL, 1 $\mu\text{g/mL}$, and 50 $\mu\text{g/mL}$) of LPS in the apical membrane surface also did not affect Caco-2 TER or inulin flux (Figure 3, C and D), suggesting that the presence of high concentrations of LPS on the mucosal surface do not affect intestinal TJ barrier function.

LPS Effect on Caco-2 Apoptosis and Cell Death

In previous studies, the effects of high pharmacological concentrations (50 $\mu\text{g/mL}$ to 1 mg/mL) of LPS on intestinal epithelial barrier integrity were evaluated.^{30–32} These studies indicated that at these extreme concentrations (which exceed the physiological levels by 10^4 - to 10^7 -fold), LPS causes a rapid apoptosis and death of intestinal epithelial cells and apoptosis-dependent loss of epithelial integrity.^{30–32} However, the effects of physiologically relevant concentrations of LPS (0 to 1 ng/mL) on intestinal epithelial cell

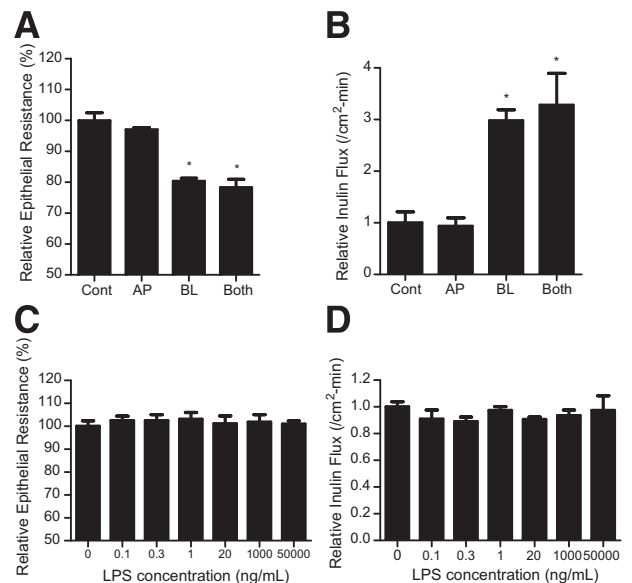


Figure 3 Membrane specificity of the LPS effect on Caco-2 permeability. **A:** Effect of LPS (0.3 ng/mL) on Caco-2 TER. The mean TER for control (Cont) Caco-2 monolayers was $475 \pm 12 \Omega \bullet \text{cm}^2$. **B:** Effect of LPS (0.3 ng/mL) on Caco-2 inulin flux. LPS (0.3 ng/mL) was added to either apical (AP), basolateral (BL), or combined apical and basolateral compartments (Both) for the 5-day experimental period. **C:** Effect of increasing concentrations of LPS (0, 0.1, 0.3, 1, 20, 1000, and 50,000 ng/mL) on Caco-2 TER. The mean TER for control Caco-2 monolayers was $454 \pm 19 \Omega \bullet \text{cm}^2$. **D:** Effect of increasing concentrations of LPS (0, 0.1, 0.3, 1, 20, 1000, and 50,000 ng/mL) on Caco-2 inulin flux. LPS was added to the apical compartment at each 24 hours treatment period for 5 days. * $P < 0.0001$ versus control.

apoptosis or death remain unclear. The LPS effect on Caco-2 apoptosis and necrosis was determined over a 5-day experimental period by Annexin V-FITC labeling of phosphatidylserine on plasma membrane of apoptotic cells and by propidium iodide labeling of DNA in necrotic cells using flow cytometry³⁷ and by caspase-3 expression in filter-grown Caco-2 monolayers.³⁰ Increasing the concentration of LPS up to 100 ng/mL did not induce Caco-2 cell apoptosis (Figure 4A) or necrosis (data not shown). As a positive control, Caco-2 monolayers were also treated with high LPS concentration (1 mg/mL) that had been previously shown to induce Caco-2 cell apoptosis (Figure 4A). In separate experiments, the time-course effect of LPS (0.3 ng/mL) on caspase-3 expression in Caco-2 monolayers was also examined. LPS did not cause an increase in caspase-3 expression (Figure 4B). Additionally, the LPS effect on morphology of Caco-2 monolayers was examined using a Nikon inverted microscope ($\times 400$ magnification). LPS (0 to 100 ng/mL) did not cause morphological changes to the epithelial monolayer surface or detachment of epithelial cells (data not shown). To examine the effect on cell turnover, we also measured the effect of LPS (0.3 ng/mL) on cell proliferation over a 5-day experimental period by assessing mitochondrial dehydrogenase activity. LPS did not have a significant effect on Caco-2 cell proliferation (Figure 4C). Together, these results indicate that LPS at concentrations up to 100 ng/mL does not induce Caco-2 cell death and that the LPS effect on Caco-2 TJ permeability is not due to cell death leading to a breach in the epithelial integrity.

LPS-Induced Increase in Caco-2 TJ Permeability Requires an Increase in TLR-4 Expression

In the following studies, the possible involvement of TLR-2, TLR-4, or TLR-5 in the LPS-induced increase in Caco-2

TJ permeability was determined. The basal expressions of TLR-2 and TLR-4 were low in unstimulated Caco-2 monolayers, whereas TLR-5 levels were higher (Figure 5A). LPS (0.3 ng/mL) treatment resulted in a time-dependent increase in TLR-4 expression (Figure 5A), but did not significantly affect TLR-2 or TLR-5 levels. LPS did not cause an increase in TLR-4 expression until day 4 of the treatment. The time course of the LPS-induced increase in Caco-2 TLR-4 expression paralleled the drop in Caco-2 TER and the increase in paracellular permeability (Figure 1, C and D), suggesting a possible cause-and-effect relationship. To determine the possible causal role of TLR-4 in the LPS-induced increase in Caco-2 TJ permeability, the effect of siRNA-induced silencing of TLR-4 expression was examined. The TLR-4 siRNA transfection resulted in an inhibition of the LPS-induced increase in Caco-2 TLR-4 expression (Figure 5B), drop in Caco-2 TER (Figure 5C), and increase in inulin flux (Figure 5D), confirming the specific requirement of TLR-4 in LPS-induced increase in Caco-2 TJ permeability. In a separate set of experiments, the requirement of TLR-2 or TLR-5 in the LPS-induced increase in Caco-2 TJ permeability was also examined. The siRNA-induced knockdown of *TLR-2* or *TLR-5* did not affect the LPS-induced drop in Caco-2 TER or the increase in inulin flux (Figure 6). To further validate the involvement of TLR-4 signal transduction pathway, the effect of a pharmacological inhibitor of the TLR-4 pathway, TAK-242,⁴² on the LPS-induced increase in Caco-2 TJ permeability was examined. TAK-242 (3 $\mu\text{mol/L}$) prevented the LPS-induced drop in Caco-2 TER (Figure 5E) and the increase in inulin flux (Figure 5F). Together, these findings suggest that the LPS effect on Caco-2 TJ permeability is mediated by an increase in TLR-4 expression and activation of the TLR-4 signal transduction pathway.

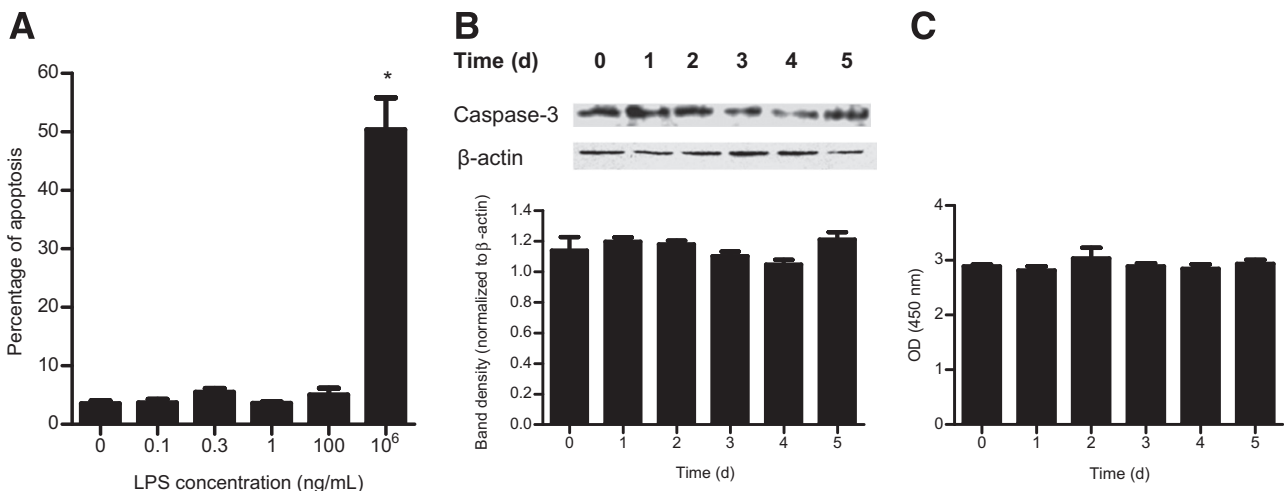


Figure 4 Effect of LPS on Caco-2 cell apoptosis and proliferation. **A:** Effect of increasing concentrations of LPS on Caco-2 cell apoptosis was assessed by flow cytometry. LPS concentration of 1 mg/mL was used as a positive control of apoptosis.²³ There were a total of 10,000 events for each sample. **B:** Effect of LPS on caspase-3 expression assessed by Western blot analysis. **C:** Effect of LPS on Caco-2 cell proliferation as assessed by mitochondrial dehydrogenase conversion of tetrazolium salt WST-1 to formazan. * $P < 0.0001$ versus control.

LPS Causes an Increase in Mouse Intestinal Permeability *in Vivo*

In the following studies, an *in vivo* model of the LPS-induced increase in mouse intestinal permeability was established, using a modification of a previously described method of recycling mouse intestinal perfusion.^{36,40,41} To determine the appropriate LPS concentration to use *in vivo*, the effects of increasing i.p. doses of LPS (0, 0.05, 0.1, 1.0, or 10 mg/kg body weight) on mouse plasma LPS level were determined. The basal plasma LPS level in control mice (C57BL/6, the Jackson Laboratory, Bar Harbor, ME) was 0.017 ng/mL; increasing the i.p. LPS concentration resulted in a dose-dependent increase in mouse plasma LPS levels (Table 1). The LPS concentration of 0.1 mg/kg body weight produced a 1-hour peak level of 2.230 ng/mL and a 24-hour steady-state plasma level of 0.405 ng/mL (Table 1), which were within physiologically and clinically relevant plasma

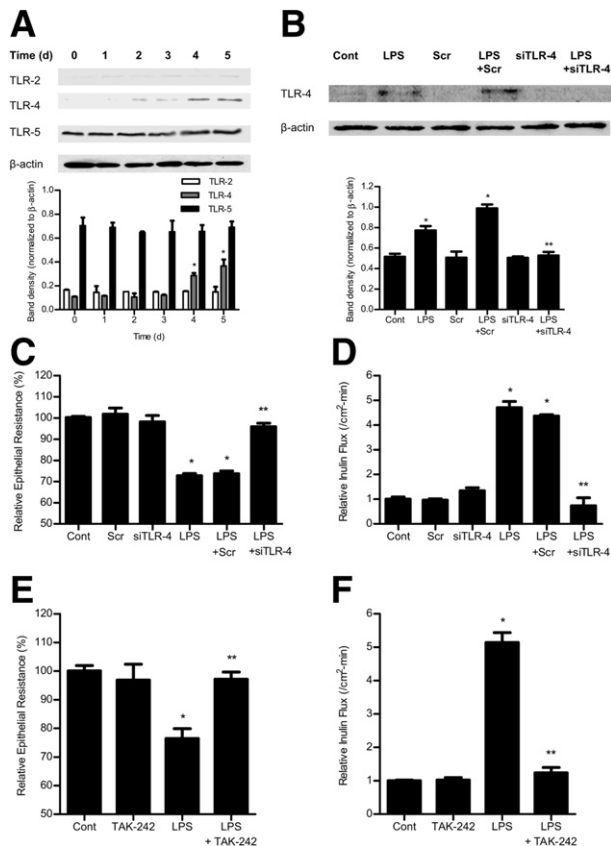


Figure 5 Effect of LPS on Toll-like receptors. **A:** Time-course effect of LPS on TLR-2, TLR-4, and TLR-5 expression in Caco-2 monolayers. **B:** Effect of TLR-4 siRNA transfection on TLR-4 expression in Caco-2 monolayers. Effect of inhibition of TLR-4 by siRNA silencing on Caco-2 TER (**C**) and inulin flux (**D**). The Caco-2 cells were transfected with TLR-4 siRNA, 24 hours before the LPS treatment. The mean TER for control (Cont) Caco-2 monolayers was $441 \pm 4 \Omega \cdot \text{cm}^2$. Effect of TLR-4 inhibitor, TAK-242 (3 $\mu\text{mol/L}$), on Caco-2 TER (**E**) and inulin flux (**F**). TAK-242 was added to the apical compartment 1 hour before the LPS treatment. The mean TER for control Caco-2 monolayers was $463 \pm 15 \Omega \cdot \text{cm}^2$. Scr, scrambled. * $P < 0.001$ versus control; ** $P < 0.001$ versus LPS treatment.

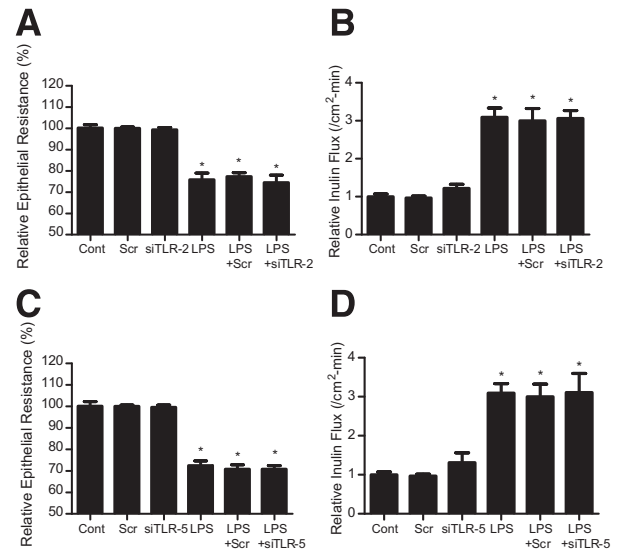


Figure 6 Effect of siRNA silencing of TLR-2 or TLR-5 on LPS-induced alteration in Caco-2 TER and inulin flux. Caco-2 cells were transfected with TLR-2 siRNA or TLR-5 siRNA for 24 hours before the LPS treatment. siRNA-induced silencing of TLR-2 did not affect Caco-2 TER (**A**) or inulin flux (**B**). The mean TER for control Caco-2 monolayers was $468 \pm 17 \Omega \cdot \text{cm}^2$. siRNA-induced silencing of TLR-5 also did not affect Caco-2 TER (**C**) or inulin flux (**D**). The mean TER for control Caco-2 monolayers was $492 \pm 24 \Omega \cdot \text{cm}^2$. Scr, scrambled. * $P < 0.001$ versus control.

concentrations.^{11–13,20,22} By contrast, the lethal LPS dose (10 mg/kg) used in previous animal studies³² resulted in very high peak (13.49 $\mu\text{g/mL}$) and trough (252.4 ng/mL) plasma LPS levels, which produced a rapid intestinal mucosal damage (Table 1). On the basis of the above data, the LPS dose of 0.1 mg/kg was selected for the mouse intestinal permeability studies. Over a 5-day treatment period (LPS injection every 24 hours), the steady-state plasma LPS level remained between 0.4 and 0.5 ng/mL (Figure 7A). Similar to the *in vitro* studies, intraperitoneal LPS (0.1 mg/kg) injection did not have a significant effect on mouse intestinal permeability up to day 3, as assessed by mucosal-to-serosal flux of Texas Red–dextran 10 kDa, but caused an increase in intestinal permeability at day 5 (Figure 7B). LPS (0.1 mg/kg) treatment did not cause intestinal mucosal damage (Figure 7C) or changes in mouse behavior or body weight (data not shown). These data

Table 1 Effect of Increasing Concentration of i.p. LPS (0, 0.05, 0.1, 1.0, 10.0 mg/kg Body Weight) on Mouse Plasma LPS Level *in Vivo*

LPS (mg/kg)	0 hours (ng/mL)	1 hour (ng/mL)	24 hours (ng/mL)
0.05	0.017	0.366	0.020
0.1	0.017	2.230	0.405
1	0.018	1327.1	20.732
10	0.018	13,491.0	252.427

Plasma LPS level was measured prior to i.p. LPS injection and 1 hour and 24 hours post-LPS injection ($n = 3$ to 4).

indicate that LPS, at clinically relevant plasma concentrations, causes an increase in mouse intestinal permeability without causing acute intestinal mucosal damage.

Next, the involvement of TLR-4 in the LPS-induced increase in mouse intestinal permeability was examined. Intraperitoneal LPS (0.1 mg/kg) caused a time-dependent increase in intestinal tissue TLR-4 expression (Figure 7D). In the control mice, there was only minimal expression of TLR-4 in the intestinal tissue. LPS treatment did not affect TLR-4 expression up to day 3; however, by day 5, there was a marked increase in TLR-4 expression (Figure 7D). The time course of TLR-4 expression paralleled the increase in mouse intestinal permeability (Figure 7B). To determine the requirement of TLR-4 in the LPS-induced increase in mouse intestinal permeability, the LPS effect was examined in

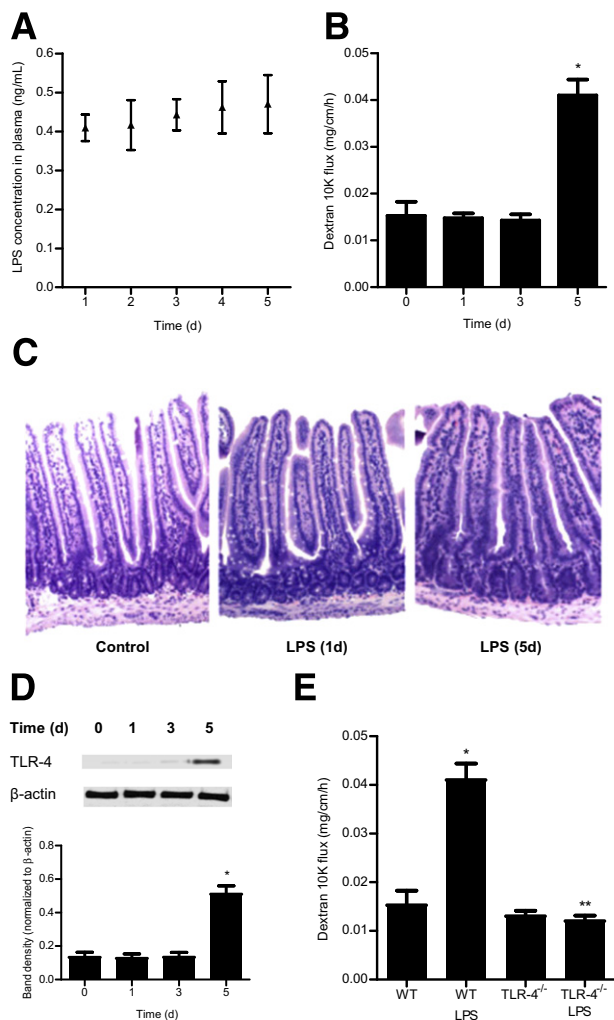


Figure 7 Effect of i.p. LPS (0.1 mg/kg body weight) on mouse intestinal permeability and plasma TLR-4 level. **A:** Time-course effect of i.p. LPS on plasma LPS level in mice. **B:** Time-course effect of i.p. LPS on intestinal permeability. **C:** Effect of LPS administration on intestinal tissue: wild-type (WT); LPS 1 day treatment; LPS 5 days treatment. H&E stain, original magnification, $\times 200$. **D:** Time-course effect of LPS on TLR-4 expression in mice. **E:** Effect of LPS on intestinal permeability in $TLR-4^{-/-}$ mice. * $P < 0.001$ versus WT control; ** $P < 0.001$ versus WT LPS treatment.

$TLR-4$ deficient mice (C57BL/6 $Tlr4^{lps-d}$, the Jackson Laboratory). Intraperitoneal LPS did not cause an increase in intestinal permeability in $TLR-4^{-/-}$ mice (Figure 7E), indicating that TLR-4 was also required for the LPS-induced increase in mouse intestinal permeability.

LPS-Induced Increase in Mouse Intestinal TJ Permeability Is Mediated by a TLR-4–Dependent Up-Regulation and Membrane Recruitment of CD14

TLR-4 forms an aggregate receptor complex with membrane-associated proteins CD14 and myeloid differentiation protein 2 (MD2) and other adaptor proteins.^{43,44} CD14 appears to be the receptor protein involved in the binding of LPS/LPS binding protein complex.^{43,44} In the following studies, the LPS effect on expression and localization of TLR-4 and CD14 in Caco-2 monolayers was examined by immunostaining and imaging by confocal microscopy. In the control Caco-2 monolayers, there was only minimal TLR-4 and CD14 expression (Figure 8). LPS (0.3 ng/mL) treatment caused a time-dependent increase in TLR-4 expression, both in the cytoplasm and at the level of the plasma membrane; and by day 5, a marked increase in plasma membrane localization of TLR-4 was seen (Figure 8). Corresponding to the increase in TLR-4 expression and membrane localization, there was also an increase in cellular expression and membrane colocalization of CD14 by day 5 (Figure 8). The time course of the LPS-induced increase in CD14 expression was also confirmed by immunoblot analysis (Figure 9A). Next, the involvement of CD14 in the LPS-induced increase in Caco-2 TJ permeability was examined. The siRNA-induced knockdown of *CD14* prevented the LPS-induced drop in Caco-2 TER (Figure 9B) and increase in paracellular permeability (Figure 9C), indicating that CD14 expression was also required for the LPS-induced increase in Caco-2 TJ permeability. In the following studies, the possible dependence of CD14 expression and membrane localization on TLR-4 expression was examined. The siRNA-induced knockdown of TLR-4 inhibited the LPS-induced increase in Caco-2 CD14 expression (Figure 9D) and membrane localization (Figure 8), suggesting that the increase in TLR-4 was required for the increase in CD14 expression.

Next, the role of TLR-4/CD14 receptor complex in the LPS-induced increase in intestinal permeability was also determined *in vivo*. In the control mice, there was only a minimal expression of TLR-4 or CD14 in mouse enterocytes (Figure 10A). LPS administration (0.1 mg/kg i.p.) resulted in a marked increase in mouse enterocyte TLR-4 and CD14 expression, both in the cytoplasm and at the level of the plasma membrane (Figure 10A). LPS treatment did not cause an increase in enterocyte CD14 expression in $TLR-4^{-/-}$ mice. Additionally, LPS did not cause an increase in intestinal permeability in $CD14^{-/-}$ mice (C57BL/6 $CD14^{-/-}$; the Jackson Laboratory) (Figure 10B). Together, these data suggest that the LPS-induced increase in

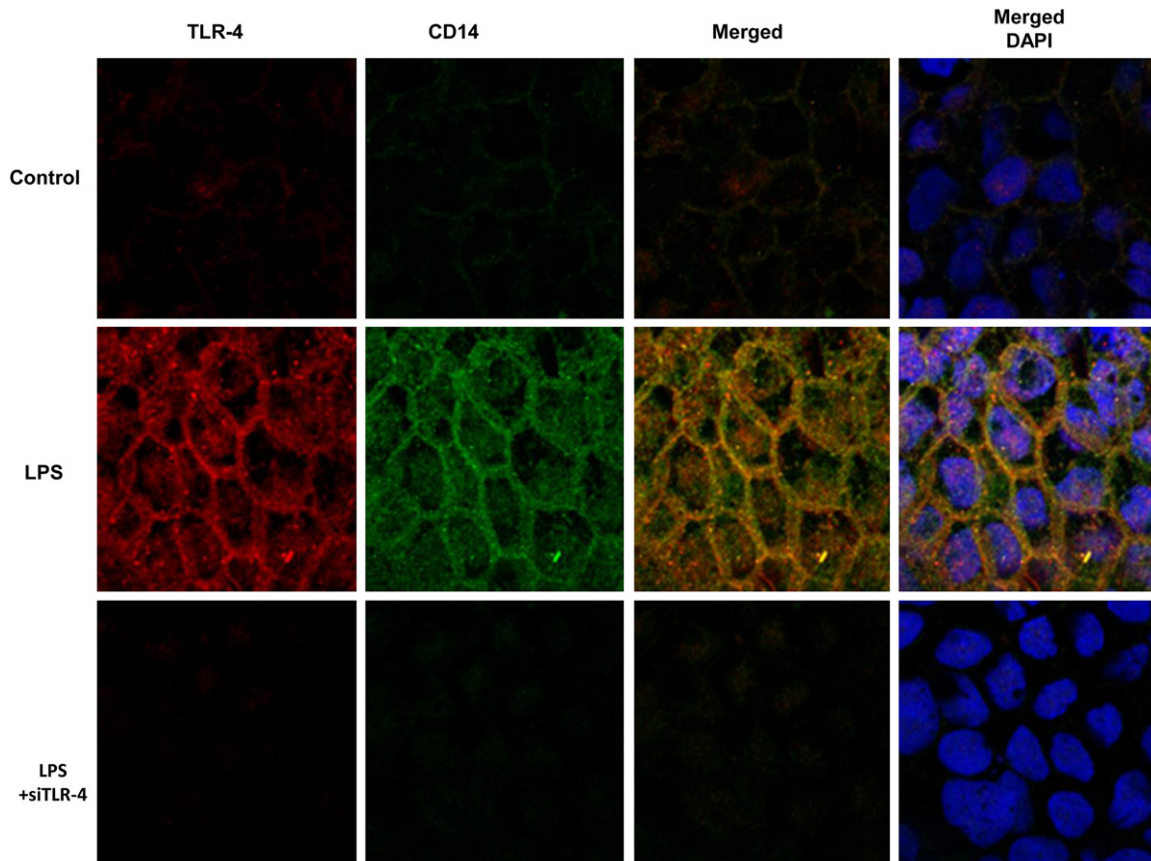


Figure 8 Effect of LPS on TLR-4 and CD14 localization in filter-grown Caco-2 monolayers as assessed by immunostaining. Original magnification, $\times 600$.

enterocyte CD14 expression and membrane localization is dependent on TLR-4 expression and that CD14 expression is necessary for the LPS-induced increase in mouse intestinal permeability.

Discussion

A defective intestinal epithelial TJ barrier plays an important pathogenic role in intestinal inflammation by allowing increased intestinal permeation and systemic circulation of gut-derived bacterial antigens.^{1,2} Previous clinical studies have demonstrated a correlation between an increase in intestinal permeability and plasma LPS levels and an increase in disease activity in IBD.^{20–23} Although the cause-and-effect relationship between a defective intestinal TJ barrier and intestinal penetration of LPS and systemic endotoxemia is well established,^{11,13,16–20,22} the effects of clinically relevant concentrations of LPS on the intestinal barrier remain unknown. Because LPS levels are significantly elevated during physiological stresses and in intestinal permeability disorders,^{20,22,24–28} and because circulating LPS are an important determinant of inflammatory response and multiorgan failure,^{11–13,16–19,22} understanding the effects of clinically relevant concentrations of LPS on intestinal barrier function has important potential

clinical significance. Herein, we show for the first time that physiologically and clinically relevant concentrations (0 to 10 ng/mL) of LPS cause an increase in intestinal epithelial TJ permeability *in vitro* and *in vivo*, when present in the interstitial fluid or basolateral membrane compartment. Consistent with the presence of high concentrations of bacteria and LPS in the gut lumen, LPS in the apical membrane surface did not affect the intestinal barrier function. These data suggest that the high concentrations of LPS present in the normal gut lumen do not affect intestinal epithelial barrier function,^{14,15,25} but that relatively low levels of LPS in the interstitial fluid, which are readily achievable during physiological stresses or in intestinal permeability disorders, lead to an increase in intestinal permeability.

In the healthy intestine, luminal LPS do not permeate the intact intestinal epithelial barrier to any significant extent.^{14,15} However, in intestinal permeability disorders, the defective TJ barrier allows paracellular permeation of LPS, leading to elevated levels of LPS in the intestinal tissue and in systemic circulation.^{11,16,17,20,22} LPS is a prototypical pathogen-associated molecular pattern (PAMP) and binds to a pattern recognition receptor (PRR).^{43,44} In the enterocytes, TLRs play an integral role in the recognition of PAMPs. Among the TLRs, TLR-4 is known to be the PRR for LPS.^{43,45} Our data indicate that the TLR-4 levels are low in

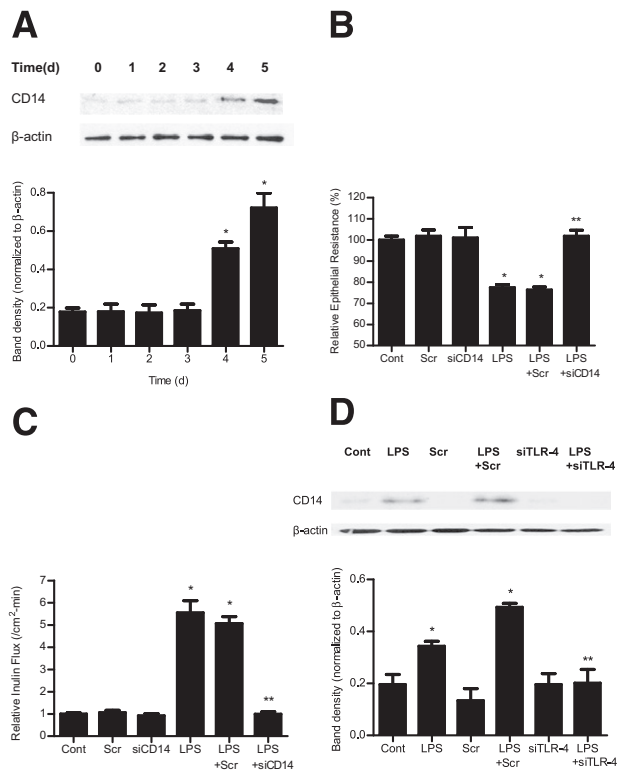


Figure 9 Effect of LPS on CD14 expression in filter-grown Caco-2 monolayers. **A:** Time-course effect of LPS on CD14 expression. **B:** Effect of siRNA CD14 transfection on Caco-2 TER. The mean TER for control Caco-2 monolayers was $482 \pm 15 \Omega \cdot \text{cm}^2$. **C:** Effect of siRNA CD14 transfection on Caco-2 inulin flux. **D:** Effect of siRNA TLR-4 transfection on expression of CD14 in Caco-2 cells. Scr, scrambled. * $P < 0.001$ versus control; ** $P < 0.001$ versus LPS treatment.

quiescent Caco-2 cells and healthy mouse enterocytes. LPS produced a time-dependent increase in TLR-4 expression in Caco-2 cells, and by day 4 to 5, a marked increase in TLR-4 expression was observed. The time course of TLR-4 expression paralleled the increase in Caco-2 TJ permeability; targeted knockdown of *TLR-4* expression or pharmacological inhibition of the TLR-4 signal transduction pathway prevented the LPS-induced increase in Caco-2 TJ permeability, demonstrating the requirement of TLR-4 and the activation of the signal transduction pathway in mediating the LPS-induced increase in Caco-2 TJ permeability. The possibility of TLR-2 or TLR-5 involvement was also considered, but LPS did not affect the expression of TLR-2 or TLR-5, and siRNA-induced silencing of TLR-2 or TLR-5 did not prevent the LPS-induced increase in intestinal TJ permeability, confirming that the LPS effect is regulated specifically by the TLR-4 signal transduction pathway. Consistent with our data, previous studies have also shown low or undetectable levels of TLR-4 in healthy human intestine and markedly elevated TLR-4 expression in patients with IBD or NEC.^{43,45} An important potential implication of these findings is that the increase in TLR-4 expression could be an important pathogenic mechanism contributing to the persistent increase in intestinal permeability and prolongation of intestinal inflammation in IBD and NEC.²²

Sheth et al⁴⁶ previously reported that a LPS concentration of 500 ng/mL causes an acute drop in NRC-1 cholangiocyte monolayer TER, with associated changes in junctional localization of TJ proteins [occludin and Zona occludens protein 1 (ZO-1)]. The LPS effect on NRC-1 TJ permeability also required a rapid increase (within minutes) in tyrosine phosphorylation of TJ proteins and myosin light chain kinase activation, suggesting that the LPS-induced increase in NRC-1 TJ permeability was mediated by an acute signaling process leading to an activation of tyrosine kinase and myosin light chain kinase.⁴⁶ By contrast, in our studies, the lower concentration of LPS (0 to 20 ng/mL) did not cause an acute change in TJ permeability or TJ protein localization in intestinal monolayers, but did cause a delayed increase in intestinal TJ permeability (4 to 5 days), which was dependent on TLR-4 expression. In our preliminary studies, we also examined the possibility that NF- κ B activation may play a role in LPS modulation of Caco-2 TJ permeability. These studies suggest that the LPS-induced increase in TLR-4 expression leads to an activation of NF- κ B. Our initial studies also suggests that siRNA-induced knockdown of *NF- κ B* and/or *p65* prevents the LPS-induced increase in Caco-2 TJ permeability. These studies suggest the possibility that NF- κ B may be an important down-stream mediator of LPS. An important focus of our future studies will be to validate the role of NF- κ B and to delineate the molecular mechanisms by which LPS modulates the intestinal TJ barrier.

Previous studies have indicated that the membrane-associated protein CD14 forms a receptor complex with TLR-4 and MD2, and plays an important role in the recognition of LPS/LPS binding protein (LBP) complex and in facilitating the interaction with TLR-4.^{43,44} LBP is a lipid transfer protein that catalyzes the transfer of LPS monomer to a binding site on CD14 via a ternary complex of LBP-LPS-CD14, to form a monomeric LPS-CD14 complex. CD14 then transfers LPS to TLR-4/MD2.⁴⁷⁻⁵² The LPS effect on biological activity may be CD14 dependent or independent.^{44,53} Our data suggest that CD14 also plays an integral role in the modulation of intestinal TJ barrier. Immunostaining studies have shown that the LPS-induced increase in plasma membrane TLR-4 expression and aggregation is associated with an increase in CD14 membrane expression and colocalization, consistent with the formation of the TLR-4 receptor cluster.^{43,44} The requirement of CD14 membrane localization in LPS modulation of the Caco-2 TJ barrier was confirmed by siRNA silencing studies, which showed that *CD14* knockdown inhibited the LPS-induced increase in Caco-2 TJ permeability. These findings indicate that the plasma membrane expression and colocalization of CD14 and TLR-4 are prerequisites for the LPS modulation of the Caco-2 TJ barrier. Another novel finding related to the CD14/TLR-4 interaction in the modulation of Caco-2 TJ barrier is the dependence of enterocyte CD14 expression and membrane localization on TLR-4 expression. Our results show that CD14 expression and membrane localization are

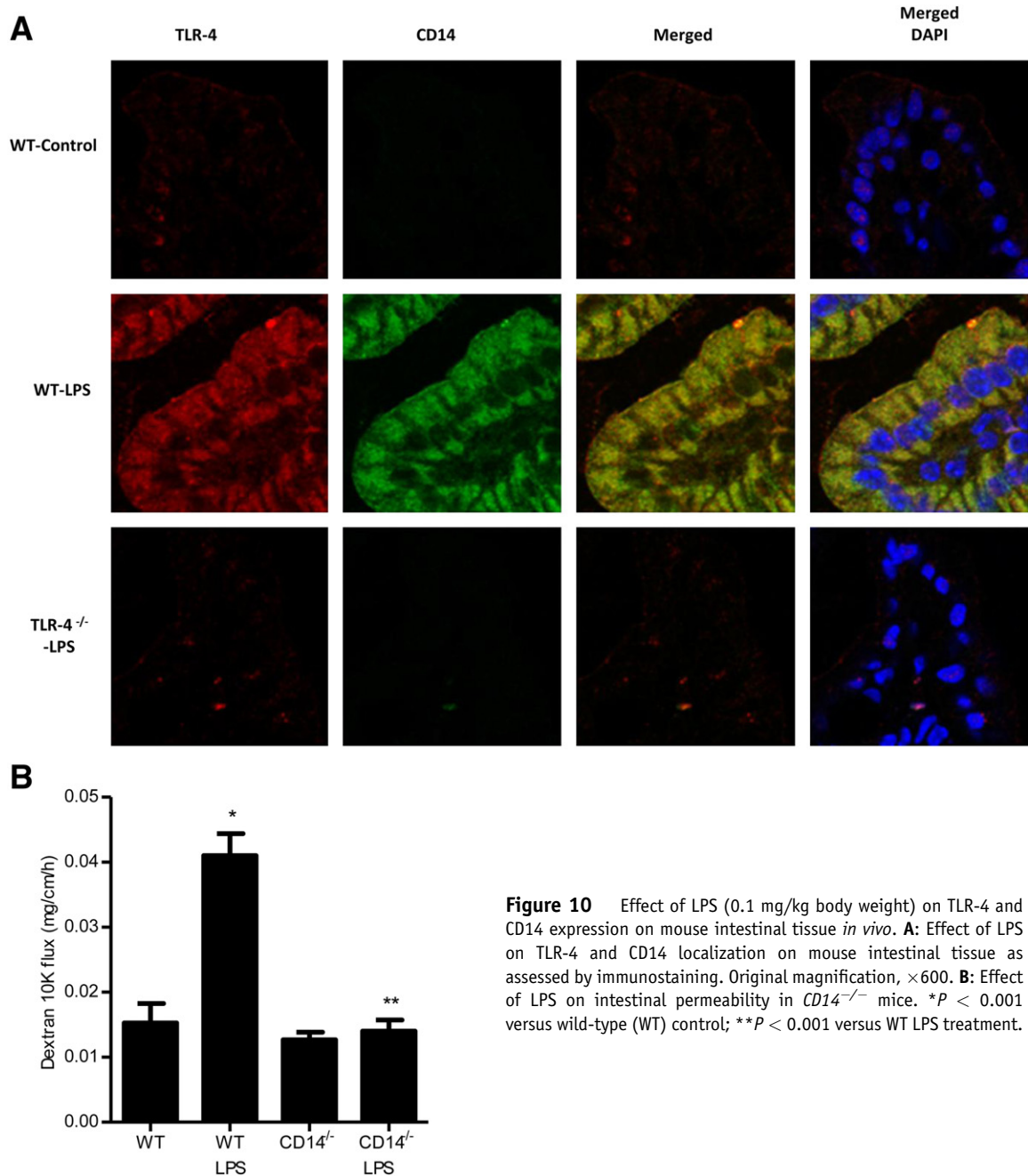


Figure 10 Effect of LPS (0.1 mg/kg body weight) on TLR-4 and CD14 expression on mouse intestinal tissue *in vivo*. **A:** Effect of LPS on TLR-4 and CD14 localization on mouse intestinal tissue as assessed by immunostaining. Original magnification, $\times 600$. **B:** Effect of LPS on intestinal permeability in *CD14*^{-/-} mice. * $P < 0.001$ versus wild-type (WT) control; ** $P < 0.001$ versus WT LPS treatment.

dependent on TLR-4 expression; and that targeted knock-down of *TLR-4* prevents the LPS-induced increase in CD14 expression and membrane localization in Caco-2 cells and mouse enterocytes. Thus, our data suggest for the first time that TLR-4 is an important regulator of CD14 expression in enterocytes.

Previous studies have shown that in Caco-2 monolayers, high pharmacological concentrations of LPS (50 $\mu\text{g}/\text{mL}$ to 1 mg/mL) cause rapid intestinal epithelial cell death (within 1 to 2 hours of LPS treatment), leading to a loss of epithelial integrity.^{30,31} Similarly, intraperitoneal injection of a high lethal dose (10 mg/kg) of LPS in mice and rats also resulted in rapid (within 2 to 3 hours) intestinal mucosal damage,

with epithelial cell apoptosis and rapid loss of intestinal barrier function.³² However, these high LPS concentrations are not clinically achievable.^{33,34} At these high pharmacological concentrations, LPS-treated mice were very sick, and most died shortly after the treatment.^{32,54,55} Our *in vitro* studies indicate that LPS, at physiologically or clinically relevant concentrations (0 to 10 ng/mL), does not cause cell death, but leads to a selective increase in Caco-2 paracellular permeability. To assess the *in vivo* effects of LPS, we also developed an *in vivo* mouse model of LPS modulation of intestinal barrier function, which closely recapitulates the plasma LPS levels reported in the clinical studies.^{11–13,24–27} To establish the appropriate LPS dose to

use in the *in vivo* studies, mice were injected i.p. with increasing concentrations of LPS. These studies indicate that a LPS dose of 0.1 mg/kg produces LPS plasma levels (peak level of 2.23 ng/mL and trough level of 0.41 ng/mL) that are achievable in clinical studies (Table 1). The LPS dose of 10 mg/kg, which had been used in previous animal studies,³² resulted in a very high plasma LPS level (peak level of 13.49 µg/mL and trough level of 252.4 ng/mL) that was not achievable clinically and led to rapid intestinal mucosal damage and apoptosis. LPS at the dose used (0.1 mg/kg) in this study did not cause any apparent illness or intestinal mucosal damage in the LPS-treated mice (data not shown), and produced a similar time-course effect on mouse intestinal permeability as the *in vitro* studies with Caco-2 monolayers. The increase in mouse intestinal permeability was associated with an increase in TLR-4 expression in mouse enterocytes; and the increase in intestinal permeability was inhibited in *TLR-4*^{-/-} mice, indicating the requirement of TLR-4 in the LPS-induced increase in mouse intestinal permeability. Our *in vivo* studies also suggest that the increase in mouse intestinal permeability is due to a TLR-4-dependent increase in enterocyte CD14 expression and membrane colocalization. Together, these *in vivo* studies show that the LPS-induced increase in mouse intestinal permeability is mediated by an increase in enterocyte membrane expression of TLR-4, and TLR-4-dependent expression and colocalization of CD14.

In conclusion, LPS at physiologically relevant concentrations cause an increase in intestinal TJ permeability *in vitro* and *in vivo*, without inducing epithelial cell death or intestinal mucosal damage. The LPS effect on intestinal TJ permeability *in vitro* and *in vivo* is mediated by an increase in enterocyte expression and membrane localization of TLR-4, and TLR-4-dependent expression and colocalization of CD14. An important potential clinical implication of these studies is that LPS, at physiologically relevant concentrations, causes an increase in intestinal permeability and could play an important role in further deterioration and prolongation of intestinal TJ barrier defect in intestinal permeability disorders and inflammatory diseases of the gut. These data also show for the first time that TLR-4 signal transduction pathway plays an important role in intestinal barrier regulation and may be targeted therapeutically.

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