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Decreased Expression of Toll-Like Receptor-4 and MD-2 Correlates with Intestinal Epithelial Cell Protection Against Dysregulated Proinflammatory Gene Expression in Response to Bacterial Lipopolysaccharide[1]

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The lumenal surface of the colonic epithelium is continually exposed to Gram-negative commensal bacteria and LPS. Recognition of LPS by Toll-like receptor (TLR)-4 results in proinflammatory gene expression in diverse cell types. Normally, however, commensal bacteria and their components do not elicit an inflammatory response from intestinal epithelial cells (IEC). The aim of this study is to understand the molecular mechanisms by which IEC limit chronic activation in the presence of LPS. Three IEC lines (Caco-2, T84, HT-29) were tested for their ability to activate an NF-κB reporter gene in response to purified, protein-free LPS. No IEC line responded to LPS, whereas human dermal microvessel endothelial cells (HMEC) did respond to LPS. IEC responded vigorously to IL-1β in this assay, demonstrating that the IL-1 receptor signaling pathway shared by TLRs was intact. To determine the reason for LPS hyporesponsiveness in IEC, we examined the expression of TLR4 and MD-2, a critical coreceptor for TLR4 signaling. IEC expressed low levels of TLR4 compared with HMEC and none expressed MD-2. To determine whether the low level of TLR4 expression or absent MD-2 was responsible for the LPS signaling defect in IEC, the TLR4 or MD-2 gene was transiently expressed in IEC lines. Transient transfection of either gene individually was not sufficient to restore LPS signaling, but cotransfection of TLR4 and MD-2 in IEC led to synergistic activation of NF-κB and IL-8 reporter genes in response to LPS. We conclude that IEC limit dysregulated LPS signaling by down-regulating expression of MD-2 and TLR4. The remainder of the intracellular LPS signaling pathway is functionally intact. *The Journal of Immunology,* **2001, 167: 1609–1617.**

The intestinal epithelium serves as a critical barrier to lumenal bacteria and food Ags. As well as constituting a barrier to the movement of lumenal contents, intestinal epithelial cells (IEC)[3] are active participants in the intestinal innate immune response, responding to signals in both the lumenal (apical) and lamina propria (basal) compartments (1, 2). Invasion by pathogenic bacteria or toxin-mediated injury to these cells elicits proinflammatory gene expression, secretion of cytokines and chemokines, and recruitment of inflammatory cells to the site of injury (1, 3). These innate immune responses likely have evolved to limit infection by pathogenic bacteria that use the gastrointestinal tract as the portal of entry. Even in the absence of pathogens, the colonic epithelium is exposed to $>10^{14}$ CFUs of commensal bacteria per milliliter consisting of both Gram-positive and Gram-negative organisms (4). Despite the density of these bacteria and their bacterial products, the intestinal epithelium does not activate proinflammatory signaling cascades in response to these commensal organisms.

Idiopathic inflammatory bowel disease is characterized by chronic intestinal inflammation in the absence of a recognized bacterial pathogen. Recent studies in animal models of inflammatory bowel disease have demonstrated that the presence of bacteria is required for the initiation of chronic inflammation (5–8). The bacteria required for the initiation of intestinal inflammation in these animal models are nonpathogenic, commensal organisms, suggesting that human inflammatory bowel disease may be the result of a perturbed host response to the microbial environment. For these reasons, several clinical studies have addressed the role of probiotic therapy, in which viable microorganisms are introduced to repopulate the intestine, as well as antimicrobial therapy, to treat patients with inflammatory bowel disease (9–14). A better understanding of intestinal innate immune responses and the molecular interactions between bacteria and the intestinal epithelium may lead to improved manipulation of host-microbial interactions.

In the past several years, much has been elucidated about the signal transduction pathways used by the innate immune system to fight invading microbial agents (15, 16). Vertebrates and invertebrates have evolved pattern-recognition receptors that sense the presence of pathogen-associated molecular patterns (PAMPs; Refs. 16 and 17). Examples of these PAMPs include mannans, peptidoglycans, bacterial DNA, and LPS. The outer cell wall of Gram-negative bacteria is characterized by LPS, and release of LPS results in septic shock. In mammalian cells, LPS and other bacterial products are recognized by a class of pattern-recognition

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[3] Abbreviations used in this paper: IEC, intestinal epithelial cells; PAMP, pathogen-associated molecular pattern; TLR, Toll-like receptor; HMEC, human dermal microvessel endothelial cells; ELAM, endothelial leukocyte adhesion molecule; IRAK, IL-1R-activated kinase.

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receptors known as Toll-like receptors (TLRs) (17). A total of ten TLRs have been cloned and are characterized by a leucine-rich extracellular domain and a cytoplasmic domain that is similar to the IL-1 receptor (TIR domain; Refs. 18–20). Only three TLRs, TLR2, TLR4, and TLR9, have been functionally characterized with respect to ligand recognition (16, 17, 21).

LPS-mediated activation of TLR4 culminates in NF-κB transcriptional activity and inflammatory cytokine production (17, 22, 23). LPS-dependent TLR4 signaling requires the presence of LPS-binding protein, which acts as an opsonin, and membrane or soluble CD14, which acts as an opsonic receptor (15, 16, 24–26). Another critical component of the TLR4 signaling complex is MD-2, a novel secreted protein (27), which binds to the extracellular domain of TLR4 where it facilitates LPS-mediated NF-κB activation and is required for LPS-dependent MAP kinase activation (28, 29). Whereas TLR4 is the sole sensor for LPS (17, 30), TLR2 is involved in recognition of peptidoglycans and lipoproteins present on Gram-positive bacteria and mycobacterial Ags (31–33). TLR expression is tissue specific, with abundant expression of TLR4 on macrophages, dendritic cells, and endothelial cells, but the expression and function of these receptors in other tissues is less clear (34, 35).

In the current study, we investigated the molecular mechanisms regulating the responses of IEC to purified LPS. Specifically, we investigated the mechanisms by which IEC are protected against continuous immune activation by commensal Gram-negative bacteria and LPS. Prior studies in well-characterized IEC lines have given variable results with respect to their ability to activate signal transduction pathways in response to LPS. For example, several studies have demonstrated that neither T84 cells nor Caco-2 cells respond to LPS stimulation with respect to proinflammatory cytokine expression or activation of the transcription factor NF-κB (36–41), whereas others have found modest activation of MAP kinase signaling pathways and NF-κB binding activity in response to LPS in T84 cells (42). By contrast, LPS resulted in secretion of IL-8 in HT-29 cells (36, 37, 41, 43). However, bacterial invasion reproducibly elicits a proinflammatory response by IEC characterized by activation of NF-κB and secretion of IL-8 (37, 40, 44, 45). Most of these studies predate the elucidation and characterization of the TLR family of molecules, the primary sensors of the innate immune response, and the MD-2 molecule. Furthermore, all of these studies used commercially available LPS preparations, which are frequently contaminated with lipoproteins (46). Because lipoproteins are recognized by and signal through TLR2 and not TLR4 (31–33), it is not clear whether these prior studies have measured TLR4- or TLR2-mediated signaling. Indeed, contaminating lipoprotein in commercial LPS preparations, and not purified protein-free LPS, was clearly shown to signal via TLR2 and explained the initial reports suggesting that LPS could signal through TLR2 (46).

We investigated the LPS responsiveness, expression pattern, and function of the TLR4/MD-2 signaling complex in various IEC lines. Here we report that IEC express no MD-2 and very weak TLR4 and are unresponsive to purified, protein-free LPS. We also demonstrate that transient expression of MD-2 and TLR4 restores the ability of IEC to respond to LPS, as measured by NF-κB and IL-8 reporter gene activity. These findings suggest that down-regulating the expression of the LPS signaling receptor TLR4 and the accessory molecule MD-2 may be a mechanism by which IEC protect against dysregulated immune signaling in response to Gram-negative commensal bacteria and their products. A better understanding of innate immunity in the gastrointestinal mucosa may improve our current knowledge of the pathogenesis of inflammatory bowel diseases.

Materials and Methods

Cells and reagents

IEC lines Caco-2, HT-29, and T84 were obtained from the American Type Culture Collection (Manassas, VA). Subconfluent monolayers of these cell lines were kept in a humidified incubator at 37°C with 5% CO_2. T84 were cultured on 12-mm Transwell, polycarbonate membranes (Costar 3401; Costar, Cambridge, MA) and maintained in DMEM/F12 (Life Technologies, Rockville, MD) with 5% Pen/Strep, 5% L-glutamine, supplemented with 5% FBS as described previously (47). T84 cells were used between passage number 16 and 35 (48). Caco-2 were maintained in MEM (Life Technologies) supplemented with 10% FBS, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and 5%Pen/Strep. HT-29 were maintained in McCoy's 5A medium supplemented with 10% FBS and 5% Pen/Strep. The immortalized human dermal microvessel endothelial cells (HMEC; Ref. 49; a generous gift of Dr. Candal of the Center for Disease Control and Prevention, Atlanta, GA) were cultured in MCDB-131 medium supplemented with 10% heat-inactivated FBS, 2 mM of glutamine, and 100 μg/ml of penicillin and streptomycin in 24-well plates, and used between passages 10–14, as described earlier (24, 34, 49).

Highly purified, phenol-water-extracted *Escherichia coli* K235 LPS (< 0.008% protein), which was prepared according to the method of McIntire et al. (50), was obtained from S. N. Vogel (Uniformed Services University of the Health Sciences, Bethesda, MD; Refs. 46, 51). The purity of this LPS preparation has been demonstrated previously (50, 52, 53), and this preparation of *E. coli* LPS is active on TLR4-transfected HEK 293 cells and not on TLR2 transfectants (S. N. Vogel, unpublished observations). Human IL-1β and TNF-α was purchased from R&D Systems (Minneapolis, MN).

Expression vectors and cDNA constructs

Endothelial leukocyte adhesion molecule (ELAM)-NF-κB luciferase (34) and pCMV-EGFP (Clontech Laboratories**,** Palo Alto, CA; Ref. 54) were used as described previously. Human IL-8 promoter-luciferase construct was kindly provided by Dr. N. Mukaida (Kanazawa University, Kanazawa, Japan) (55). A flag-tagged human TLR4 construct was obtained from Tularik (San Francisco, CA). MD-2 cDNA construct was kindly provided by Dr. K. Miyake of Saga Medical School, Saga, Japan (27). The plasmids were prepared with endotoxin-free plasmid maxi-prep kit (Qiagen, Valencia, CA).

Transient gene expression assays

Caco-2 cells or T84 cells were plated at a density of 150,000 cells/well in 12-well plates 24 h before transfection. HMEC were plated at a concentration of 50,000 cells/well in 24-well plates. Cells were transfected the following day with Fugene 6 transfection reagent (Roche Biomedical Laboratories, Burlington, NC) as per manufacturer's instructions and as described earlier (24, 34). HT-29 cells were electroporated with an Electro Square Porator ECM 830 (BTX, San Diego, CA) set to the following parameters: voltage 600V, pulse length 500 μs, 9 pulses and a pulse interval of 100 ms. Reporter genes pCMV-β-galactosidase, pCMV-EGFP (0.1 μg), ELAM-NF-κB-luciferase (0.4 μg), or IL-8 luciferase (0.4 μg), and pCDNA3 empty vector (0.3–0.6 μg), Flag-tagged wild-type human TLR4 (0.3 μg) or human MD-2 cDNA (0.3 μg) constructs were cotransfected as indicated in the figure legends. After overnight transfection, cells were stimulated for 5 h with LPS (50 ng/ml), human IL-1β (10ng/ml), or TNF-α (20 ng/ml; R&D Systems). Cells then were lysed in 200 μl of reporter lysis buffer (Promega, Madison, WI), and luciferase activity was measured with a Promega firefly luciferase kit with a Wallac 1450 Microbeta liquid scintillation counter (PerkinElmer, Foster City, CA). Data shown are mean $\pm$ SD of three or more independent experiments, and are reported as fold-induction in relative luciferase activity over cells transfected with a control vector. Transfection efficiency was determined by counting green fluorescent protein (GFP)-positive cells with an Olympus (New Hyde Park, NY) BH2 RFCA microscope (54) or assaying for β-galactosidase activity with a colorimetric method (Promega) as previously described (34).

RT-PCR analysis

Total RNA was isolated from T84, Caco-2, HT-29, and HMEC with a Qiagen kit following manufacturer's instruction and treated with RNase free DNase I. For RT reaction, the MMLV Preamplification system (Life Technologies) was used. PCR amplification was performed with *Taq* polymerase (Perkin-Elmer) for 38 cycles at 95°C for 45 s, 54°C for 45 s, and 72°C for 1 min (for TLR2 and TLR4) as described earlier (24), and for 38 cycles at 94°C for 45 s, 55°C for 45 s, and 72°C for 45 s. (for MD-2). The TLR2 and TLR4 oligonucleotide primers used for RT-PCR were described

earlier (24). The oligonucleotide primers for MD-2 were as follows: forward, GAAGCTCAGAAGCAGTATTGGGTC; reverse, GGTTGGTG TAGGATGACAAACTCC (sequence kindly provided by Dr. J. C. Chow, Esai Research Institute, Wilmington, MA). GAPDH primers were obtained from Clontech and used as per manufacturer's instructions. The TLR2, TLR4, and MD-2 RT-PCR fragments were purified and sequenced to confirm the identity of the fragments.

ELISA and immunocytochemistry

For TLR4 immunocytochemistry, HMEC or T84 cells were grown on polycarbonate membranes. Cells were fixed in 4% paraformaldehyde and blocked in PBS-1% BSA, followed by incubation with 10 μg/ml of HTA125, a monoclonal Ab against TLR4, (a generous gift of Dr. K. Miyake, Saga Medical School; Ref. 27) or irrelevant isotype-matched Ab followed by incubation with FITC-conjugated anti-mouse Ab at 1/100 dilution (Vector Laboratories, Burlingame, CA). Cells were counterstained with 1 μg/ml propidium iodide after treatment with RNase A as described previously (47). Immunofluorescence was visualized with a Leica (Deerfield, IL) TCS SP laser scanning inverted confocal microscope and analyzed with the Leica TCS NT program.

For IL-8 ELISA, 10,000 cells were plated per well in 96-well plates. Cells were treated with LPS (50 ng/ml), IL-1β (10 ng/ml), or TNF-α (20 ng/ml) for 18 h and supernatants harvested for measurement of IL-8. IL-8 ELISA (BD PharMingen, San Diego, CA) were performed as per the manufacturer's instructions.

Results

IEC lines fail to respond to purified, protein-free LPS

The lumenal aspect of IEC is continuously exposed to bacteria and bacterial products such as LPS. Previous studies have demonstrated a variable response to LPS in vitro as measured by the ability of IEC lines to transcribe proinflammatory genes (36), activate NF-κB-dependent reporter genes (40), or activate NF-κB binding to its consensus DNA sequence (42). Whereas HT-29 (36, 43) and T84 cells (42) are reported to be LPS responsive, Caco-2 cells are LPS unresponsive (36). Based on these published studies, we chose to characterize IEC lines that have been found to be responsive to LPS (T84 and HT-29) and one that is not (Caco-2). All of the published studies that have explored the response of IEC to LPS have used commercial LPS preparations, which are frequently contaminated with lipoproteins (46). Because lipoproteins signal through TLR2 whereas LPS signals through TLR4, we wished to determine whether IEC lines were responsive to purified, protein-free LPS, which has been shown to signal via TLR4 and not TLR2 (46). To answer this question, we used a functional assay measuring NF-κB-dependent gene transcription with an NF-κB-luciferase reporter gene construct derived from the ELAM promoter as well as transcriptional activation of the IL-8 gene with an IL-8 promoter-luciferase construct (24, 34, 55). We found that none of the three cell lines responded to LPS as measured by NF-κB reporter gene activity (Fig. 1*A*) or IL-8 gene activation (data shown in Fig. 2*B*). We have shown that HMEC respond vigorously to protein-free LPS through TLR4 as assessed by NF-κB transactivation (Fig. 1*A* and Refs. 24 and 34). IEC had robust responses to IL-1β and TNF-α (T84 cells) as assessed by NF-κB luciferase activity (Fig. 1*A*; Ref. 36). To confirm the findings of these transcriptional studies, we measured IEC production of IL-8 in response to LPS. Mucosal IL-8 levels correlate with the degree of inflammation in inflammatory bowel disease. IEC lines did not secrete significant IL-8 in response to LPS but were

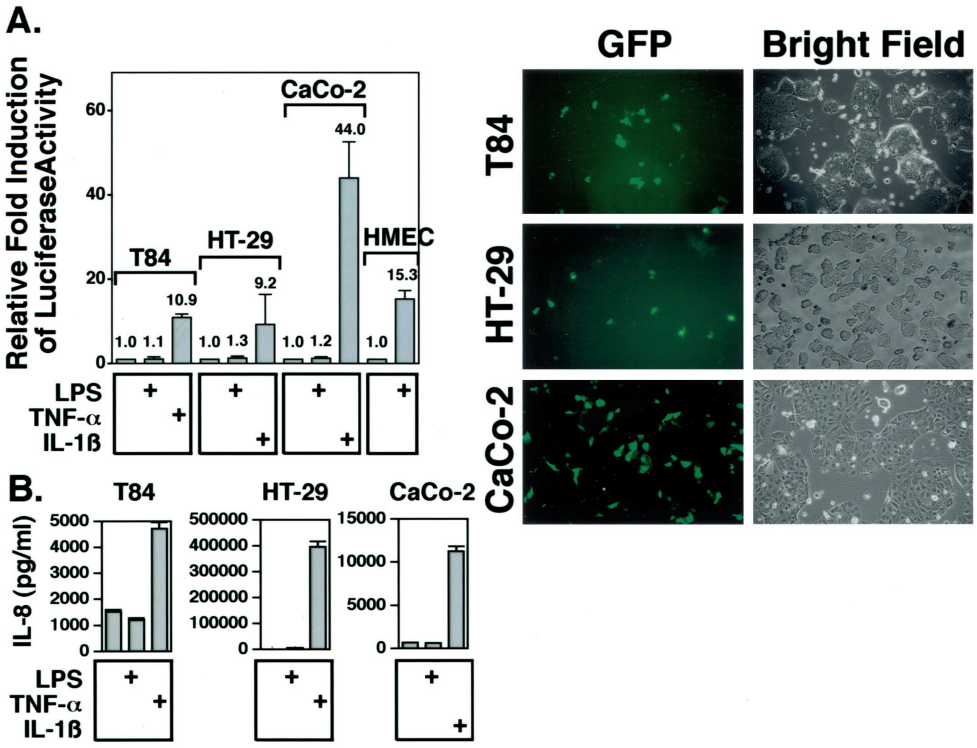

FIGURE 1. IEC lines do not respond to purified, protein-free LPS. *A*, IEC lines or HMEC as indicated were transfected with ELAM-NF-κB luciferase (0.4 μg) as described in *Materials and Methods*. The day after transfection, cells were exposed to purified, protein-free LPS (50 ng/ml), TNF-α (10 ng/ml), or IL-1β (10 ng/ml) for 5 h and cells lysed for luciferase activity. IEC lines transfected with an NF-κB reporter gene do not respond to LPS but do respond to IL-1β and TNF-α stimulation. *Right*, Transfection efficiency based on cotransfection of a CMV-driven green fluorescent protein-reporter gene and counting of cells with a fluorescent microscope (T84 and Caco-2: average efficiency, 10–20%; HT-29: average efficiency, 5%). These data are a representative experiment of three performed in triplicate, and y-error bars indicate the SD. *B*, IEC were stimulated with LPS (50 ng/ml), IL-1β (10 ng/ml), or TNF-α (20 ng/ml) as indicated for 18 h and supernatants harvested for measurement of IL-8 production by ELISA. These data are a representative experiment of three performed in triplicate, and y-error bars indicate the SD.

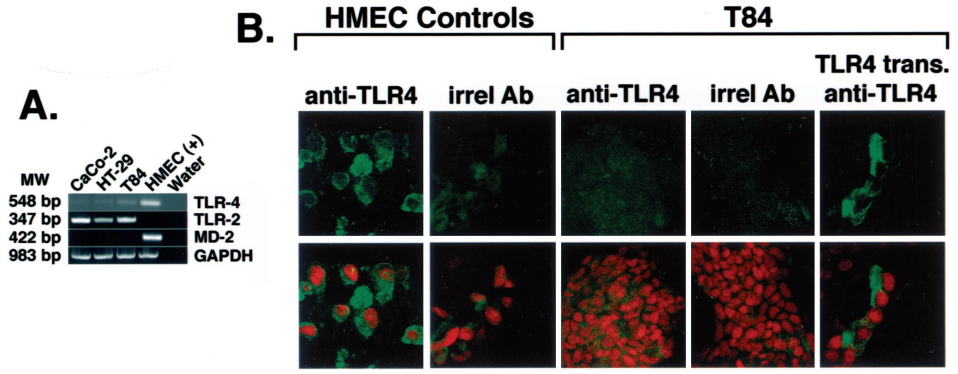

FIGURE 2. TLR4, TLR2, and MD-2 expression pattern in human IEC lines. *A*, Expression of TLR4 (548 bp), TLR2 (347 bp), and MD-2 (422 bp) were analyzed by PCR after reverse transcription of total RNA from IEC lines and HMEC cells as indicated. GAPDH was analyzed to verify similar cDNA loading. All cell lines tested express TLR4 and TLR2. The IEC lines tested do not express MD-2. *B*, Immunofluorescent analysis of TLR4 expression in T84 IEC. T84 cells and HMEC cells were cultured on polycarbonate membranes, fixed, and stained with a monoclonal Ab specific for human TLR4 (74) or an irrelevant isotype-matched Ab as indicated. *Bottom*, propidium-iodide-stained nuclei in red and TLR4 in green as merged images. HMEC demonstrate specific, plasma membrane and cytoplasmic staining for TLR4 (labeled HMEC/anti-TLR4), whereas T84 cells have weak TLR4 staining (labeled T84/anti-TLR4) that is similar to that seen with an irrelevant Ab (labeled Irrel Ab). Transfection of T84 cells with a human TLR4-expressing plasmid (0.4 μg; labeled TLR4Trans/anti-TLR4) results in TLR4-positive cells that are similar in intensity to TLR4-positive HMEC cells.

responsive to TNF-α and IL-1β (Fig. 1*B*). We and others also have shown that the LPS-TLR4 signaling pathway shares the IL-1 receptor signaling molecules, including MyD88, IL-1R-activated kinase (IRAK), IRAK2, and TRAF-6 to mediate NF-κB activation (34, 56). Because IEC responded to IL-1β as measured by NF-κB transcriptional activation and IL-8 production, these data suggest the presence of an intact intracellular signaling pathway leading from the IL-1 receptor to NF-κB. The results of our studies suggest that IEC are unresponsive to purified protein-free LPS as measured by NF-κB transcriptional activation despite an intact IL-1 receptor signaling pathway.

IEC lines express low levels of TLR4 and the coreceptor molecule MD-2

For IEC to respond to LPS, the TLR4/MD-2 signaling complex must be expressed and functional. We hypothesized that IEC lines fail to respond to LPS because they do not express critical components of the TLR4/MD-2 signaling complex (57). We investigated the mRNA expression profile of TLR4 and MD-2 by RT-PCR in these representative intestinal cell lines and used HMEC, an LPS-responsive cell line (24) as a control. All three IEC expressed very low levels of TLR4 mRNA by RT-PCR when compared with LPS-responsive HMEC (Fig. 3*A*). All three cell lines tested express TLR2 (Fig. 2*A*, *second panel*). Of interest, none of the three IEC lines tested expressed detectable MD-2, whereas the LPS-responsive HMEC strongly expressed MD-2 (Fig. 2*A*, *third panel*). The housekeeping gene GAPDH was expressed equally in all cell lines tested (Fig. 2*A*, *bottom panel*).

Our data demonstrate that IEC express low levels of TLR4 mRNA and undetectable MD-2 mRNA when compared with LPS-responsive HMEC cells. Because low levels of mRNA expression may result in variable levels of protein expression, we wished to

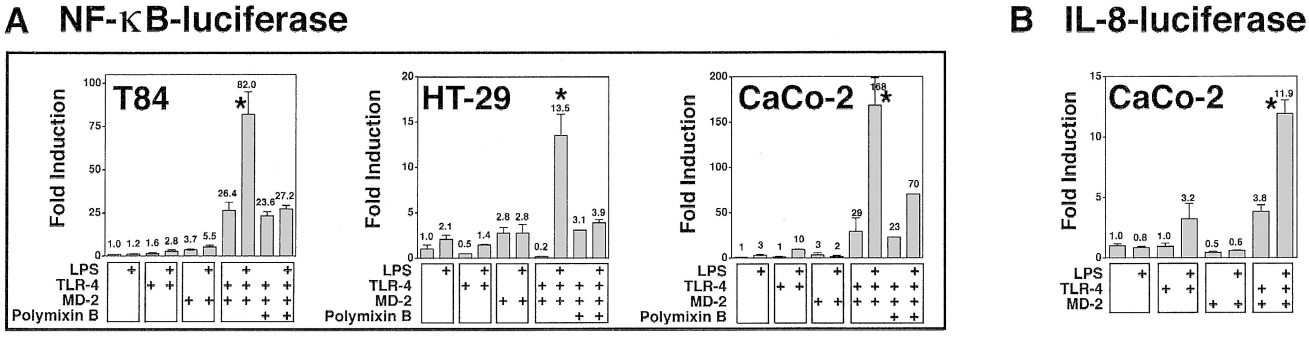

FIGURE 3. Expression of TLR4 and MD-2 restores LPS responsiveness to human IEC lines. *A*, T84, HT-29, and Caco-2 cells were transfected with ELAM-NF-κB-luciferase (0.4 μg) and cotransfected with 0.3 μg of MD-2, TLR4, or both as indicated. Amount of transfected DNA was kept constant with a pCDNA3 vector control. The day after the transfection, indicated cells were exposed to LPS (50 ng/ml) for 5 h and lysed for luciferase activity. Polymixin B (10 μg/ml) was added after the transfection to indicated conditions and demonstrated inhibition of LPS-mediated activation. The data are expressed as fold-induction of relative light units when compared with transfection of the vector control. These data are one representative experiment of three independent experiments performed in triplicate, and y-error bars indicate SD. Values of p for control compared with TLR4 + MD-2 + LPS are T84 = 0.006, HT-29 = 0.043, and Caco-2 = 0.0002. Values of p values TLR4 + MD-2 (no LPS) compared with TLR4 + MD-2 + LPS are T84 = 0.018, HT-29 = 0.028, and Caco-2 = 0.001. *B*, IL-8-luciferase (0.4 μg) was transfected into Caco-2 cells and cotransfected with 0.3 μg of MD-2, TLR4, or both as indicated. The amount of transfected DNA was kept constant with a pCDNA3 vector control. The day after the transfection, indicated cells were exposed to LPS (50 ng/ml) for 5 h and lysed for luciferase activity. Polymixin B (10 μg/ml) was added after the transfection to indicated conditions and demonstrated inhibition of LPS-mediated activation. The data are expressed as fold-induction of relative light units when compared with transfection of the vector control. These data are one representative experiment of three independent experiments performed in triplicate, and y-error bars indicate SD. Value of p for control compared with TLR4 + MD-2 + LPS is <0.0001 and for TLR4 + MD-2 (no LPS) compared with TLR4 + MD-2 + LPS is <0.0001.

investigate whether the level of mRNA expression correlated with protein expression of TLR4 in our system. To address this question, we performed immunofluorescent staining for the presence of TLR4 in a representative IEC line, T84, and the LPS-responsive HMEC cells (Fig. 2*B*). HMEC demonstrate specific plasma membrane and cytoplasmic staining for TLR4, whereas T84 cells have weak TLR4 staining, which was similar to that seen with secondary Ab alone (Fig. 2*B*, as indicated). Transient transfection of T84 cells with a TLR4-expressing plasmid resulted in TLR4-positive T84 cells with immunofluorescence that was of similar intensity to that observed in LPS-responsive HMEC cells. These data suggest that the LPS unresponsiveness of these IEC lines may be attributable to the absence of MD-2 expression and/or the low levels of TLR4 expression.

Expression of both TLR4 and MD-2 confers LPS responsiveness to IEC

Our data above demonstrate that IEC lines fail to respond to LPS and that these cells express low levels of endogenous TLR4 and MD-2. In macrophages, the level of TLR4 expression correlates with the degree of LPS responsiveness (58). We hypothesized that transgenic expression of TLR4 could restore LPS responsiveness in IEC if the reason for LPS hyporesponsiveness is the relatively low expression of TLR4. To test this hypothesis, we transiently transfected IEC with cDNA for human TLR4 and stimulated transfected cells with LPS. An example of TLR4 transfection of T84 cells is seen in Fig. 2*B*. Despite TLR4 expression in transfected cells, IEC did not become responsive to LPS as measured by transcriptional activation of an NF-κB reporter gene (Fig. 3*A*) or an IL-8 reporter gene (Caco-2; Fig. 3*B*). Expression of TLR4 alone led to a small degree of NF-κB and IL-8 activation in Caco-2 cells in the presence of LPS but not in the other cell lines (Fig. 3*A*). We conclude from these experiments that increased expression of TLR4 in IEC is not sufficient to restore LPS responsiveness, suggesting that another critical signaling molecule in the receptor complex may be missing.

In addition to the requirement for TLR4 to mediate LPS-dependent signaling (16, 17), MD-2 is required for activation of NF-κB and MAP kinases in cellular responses to LPS (29, 57). Because IEC express low levels of TLR4, we hypothesized that expression of MD-2 could restore LPS responsiveness in IEC if the reason for LPS hyporesponsiveness is the absence of MD-2 expression. To test this hypothesis, we transiently transfected IEC with cDNA for MD-2 and stimulated transfected cells with LPS. Despite low levels of TLR4 expression in IEC, expression of MD-2 was not sufficient to restore LPS-dependent signaling as measured by transcriptional activation of an NF-κB reporter gene (Fig. 3*A*) or IL-8 reporter gene (Caco-2; Fig. 3*B*). We conclude from these experiments that restoration of MD-2 expression alone also is not sufficient to permit LPS-dependent signaling in IEC.

The data above demonstrate that neither expression of TLR4 nor MD-2 individually was sufficient to confer LPS responsiveness in IEC. We hypothesized that LPS unresponsiveness in IEC was attributable to both the low levels of TLR4 and the absence of MD-2. To test this hypothesis, we cotransfected MD-2 and TLR4 in IEC and measured their response to LPS. Cotransfection of TLR4 and MD-2 in IEC led to synergistic activation of NF-κB (T84, 82-fold; HT-29, 13-fold; Caco-2, 168-fold) and IL-8 (Caco-2, 12-fold) reporter genes in response to LPS when compared with cells transfected with the empty-vector control or cells transfected with TLR4 and MD-2 but not stimulated with LPS (Fig. 3, *A* and *B*). Expression of MD-2 and TLR4 in the absence of LPS led to moderate transcriptional activation of the reporter gene over basal levels in T84 (NF-κB, 26-fold) and Caco-2 (NF-κB, 29-fold; IL-8, 3.8-fold) consistent with prior reports in other in vitro systems (29). However, LPS stimulation in MD-2- and TLR4-transfected cells showed significantly increased response over that observed in cells transfected with TLR4 and MD-2 in the absence of LPS. Furthermore preincubation with polymixin B inhibited LPS-mediated reporter gene activation, supporting the specificity of LPS-induced responses (Fig. 3, *A* and *B*). These results demonstrate that the absence of MD-2 and low levels of TLR4 expression in IEC lines are responsible for the unresponsiveness of these cells to protein-free LPS. The data further support the presence of an intact intracellular signal transduction pathway leading to NF-κB activation in IEC.

Discussion

Evidence from human and animal models of inflammatory bowel disease supports a model of perturbed host-microbial interactions in the pathogenesis of chronic intestinal inflammation (5–8). Inflammation of the ileal reservoir, i.e., pouchitis, in patients following a total proctocolectomy for ulcerative colitis is treated effectively with antibiotics or probiotic therapy (9, 11). Animal models of inflammatory bowel disease secondary to aberrant T cell function or toxin-mediated damage do not develop intestinal inflammation in the absence of bacteria (59, 60). In murine models of colitis, administration of dextran-sulfate sodium is associated with milder colitis in animals with mutations in their TLR4 gene (i.e., C3H/HeJ and C57BL/10Sc) than in the matched strain without this TLR4 mutation, suggesting that TLR4-mediated signaling plays a role in the development of murine colitis (61). IEC are the first point of contact between lumenal bacteria and the mucosal immune system; therefore, their response to the commensal flora may provide an important link to understanding the diathesis that occurs during dysregulated inflammation. The pathologic and microscopic similarity between infectious colitides and idiopathic inflammatory bowel disease suggests that the intestinal immune system is participating in a stereotypic reaction to pathogenic bacteria (62). We investigated the potential mechanism(s) by which IEC protect themselves against massive amounts of bacteria and bacterial LPS present in the intestinal lumen and do not initiate acute inflammatory responses as would be elicited along other mucosal surfaces such as the bronchial or uroepithelium in response to bacterial LPS (63, 64).

Although the signaling pathways leading from LPS to TLR4 and NF-κB activation have been described previously, relatively little is known about mechanisms to limit ubiquitous activation of these pathways in the presence of LPS. The closest in vitro model to explore these mechanisms is successive exposure of cells to LPS which results in a state of LPS tolerance. In monocytic cells and peritoneal macrophages, previous challenge with LPS results in diminished expression of proinflammatory cytokines in response to LPS. The molecular mechanisms for this hyporesponsiveness include down-regulation of IRAK and diminished recruitment of the MyD88 adapter molecule in monocytes (65) and down-regulation of the TLR4 receptor in peritoneal macrophages, suggesting that different cell types have evolved distinct strategies to limit LPS responsiveness (28). In addition to intracellular pathways that are altered in LPS-tolerized cells, polymorphisms in the extracellular domain of the TLR4 are associated with hyporesponsiveness to inhaled LPS in humans (66). The mutation in TLR4 present in the C3H/HeJ mouse acts as a dominant negative mutation and inhibits the function of wild-type TLR4 (30, 67). Recent studies have identified viral products that can interfere with TLR signaling and may represent a mechanism by which viruses inhibit the immune response and predispose to subsequent bacterial infection (68).

In this study, we have begun to characterize the response of IEC to LPS in vitro and found that IEC lines do not respond to purified

protein-free LPS. Our data demonstrate that the IEC lines tested express low levels of TLR4 mRNA and do not constitutively express the coreceptor molecule MD-2 mRNA. We chose these IEC lines because they are well-characterized and their response to LPS has been studied previously. Although we used IEC lines derived from colon cancers, it is unlikely that all three cell lines derived from distinct sources would contain a deletion in the region of chromosome 8 containing the MD-2 gene. A recent study corroborates our TLR4 findings in primary human IEC and found very low levels of TLR4 expression in healthy intestine but increased expression in inflammatory bowel disease by immunohistochemistry (69). This study did not address the functional aspect of increased TLR4 expression in inflamed mucosa. Our findings suggest that both TLR4 and MD-2 are required by IEC to activate proinflammatory cytokine genes in response to LPS; therefore, increased TLR4 expression by itself would not result in LPS reactivity without concomitant MD-2 expression. Thus, an additional mechanism by which cells that are normally chronically exposed to LPS may down-regulate their response to LPS is to down-regulate the expression of TLR4 and MD-2. Recently, a CHO cell subline has been described that fails to respond to LPS and expresses TLR4 but no MD-2 (57). Expression of MD-2 restores LPS responsiveness in these CHO cells, supporting the concept that MD-2 is required for LPS signaling. The recent findings by Cario and Podolsky (69) of increased TLR4 expression in the mucosa of patients with inflammatory bowel disease suggests that aberrant TLR expression may play an important role in the loss of tolerance to enteric bacteria. Preliminary studies in our laboratory with laser-capture microscopy to dissect IEC and lamina propria mononuclear cells from intestinal biopsies reveal that IEC do not express MD-2, whereas lamina propria cells do express MD-2 (unpublished observations). Further studies will address the expression of MD-2 in normal mucosa and in the mucosa of patients with inflammatory bowel disease once Abs specific for human MD-2 become available.

LPS is only one of many PAMPs found in the gastrointestinal lumen. To maintain tolerance to Gram-positive and Gram-negative commensal bacteria, the normal intestinal epithelium should be unresponsiveness or hyporesponsive to diverse bacterial products. We have tested IEC responses to various TLR2 ligands and found that, despite TLR2 and TLR6 mRNA expression, these cells are unresponsive to these ligands (unpublished observations). These data suggest that other mechanisms may contribute to IEC unresponsiveness to PAMPs.

In this study, we used NF-κB reporter gene activity and IL-8 promoter activity as functional measures of the IEC response to LPS. NF-κB is a central regulator of the mucosal immune response and modulates proinflammatory cytokine secretion and antiapoptotic mechanisms in IEC (70). Unlike previously published studies, we did not find significant NF-κB or IL-8 transcriptional activation in response to LPS in IEC lines (36, 37, 41, 42). One reason for this discrepancy may be the amount and source of LPS used in our study. LPS is active at concentrations as low as 1 ng/ml. We used LPS at a concentration of 50 ng/ml for our studies compared with concentrations ranging from 5–100 μg/ml in other studies (40, 42, 43). Additionally, we used purified, lipoprotein-free LPS rather than commercially available LPS, which frequently contains contaminants including lipoproteins (46). These lipoprotein contaminants stimulate TLR2 receptors, especially at concentrations above 1 μg/ml. Indeed, we found that these IEC lines express TLR2, which is required for responses to lipoprotein (Fig. 3*A*; Refs. 16, 31, and 71–73). Future studies will address the role of TLR2-mediated responses in IEC. Another reason for the discrepancy could be the relative sensitivity of the assays used in other studies. We did not find significant NF-κB or IL-8 transcriptional activation in IEC stimulated with LPS alone, whereas IEC expressing both TLR4 and MD-2 are highly responsive to LPS (Fig. 2*A*). Thus, the responses measured in other systems in response to LPS such as NF-κB DNA binding activity or IL-8 secretion may be measurable but relatively small when compared with cells expressing TLR4 and MD-2. HT-29 cells have been found to be responsive to *E. Coli* O26:B6 LPS (36, 42). In addition to the possibility of lipoprotein contamination, the response to LPS as measured by IL-8 secretion is ~10-fold less when compared with the same concentration of IL-1β (36), suggesting that the ability of these cells to respond to LPS is reduced when compared with IL-1β, which uses the same signaling machinery. In this context, our results are similar to that in the literature when comparing the relative response to LPS vs IL-1β in IEC.

Based on our model, IEC would be expected to have a muted response to LPS normally present in the intestinal lumen. The presence of an intact IL-1R/TLR intracellular signaling pathway suggests that a pathogenic stimulus may lead to a specific increase in TLR4 and MD-2 expression and thus provide a rapid mechanism of response while avoiding chronic activation. In idiopathic inflammatory bowel disease, Cario and Podolsky (69) have demonstrated increased TLR4 expression by IEC. Increased TLR4 expression as a primary defect or secondary to the inflammatory milieu might initiate or perpetuate chronic inflammation in the presence of commensal bacteria. We suggest that MD-2 expression may be another critical point of regulation in the intestinal innate immune response to commensal organisms. Absent or low MD-2 expression may protect against dysregulated innate immune responses, whereas increased expression of MD-2 in idiopathic inflammatory bowel disease may contribute to the inflammatory cascade. Thus, careful regulation of both TLR4 and MD-2 is necessary to maintain homeostasis in an organ that is continuously exposed to high concentrations of bacteria. An understanding of the mechanisms used in health to limit deleterious activation of TLR pathways in the presence of bacteria may help in understanding the pathogenesis of inflammatory bowel disease and guiding therapy.

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