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Human Intestinal Microvascular Endothelial Cells Express Toll-Like Receptor 5: A Binding Partner for Bacterial Flagellin

Christian Maaser,1,2* Jan Heidemann,1* Christof von Eiff, † Andreas Lugering,* Thomas W. Spahn,† David G. Binion,§ Wolfram Domschke,* Norbert Lugering,* and Torsten Kucharzik*

Bacterial flagellin has recently been identified as a ligand for Toll-like receptor 5 (TLR5). Human sites known to specifically express TLR5 include macrophages and gastric and intestinal epithelium. Because infection of intestinal epithelial cells with *Salmonella* leads to an active transport of flagellin to the subepithelial compartment in proximity to microvessels, we hypothesized that human intestinal endothelial cells functionally express TLR5, thus enabling an active inflammatory response upon binding of translocated flagellin. Endothelial expression of TLR5 in human macro- and microvascular endothelial cells was examined by RT-PCR, immunoblot analysis, and immunofluorescence. Endothelial expression of TLR5 in vivo was verified by immunohistochemistry. Endothelial modulation of ICAM-1 expression was quantitated using flow cytometry, and leukocyte transmigration in vitro was assessed by an endothelial transmigration assay. Epithelial-endothelial cellular interactions upon infection with viable *Salmonella* were investigated using a coculture system in vitro. We found that *Salmonella*-infected intestinal epithelial cells induce endothelial ICAM-1 expression in cocultured human endothelial cells. Both macro- (HUVEC) and microvascular endothelial cells derived from human skin (human dermal microvascular endothelial cell 1) and human colon (human intestinal microvascular endothelial cells) were found to express high constitutive amounts of TLR5 mRNA and protein. These findings were paralleled by strong immunoreactivity for TLR5 of normal human colonic microvessels in vivo. Furthermore, incubation of human dermal microvascular endothelial cells with flagellin from clinical isolates of *Escherichia* and *Salmonella* strains led to a marked up-regulation of ICAM-1, as well as to an enhanced leukocyte transendothelial cell migration. These results suggest that endothelially expressed TLR5 might play a previously unrecognized role in the innate immune response toward bacterial Ags. *The Journal of Immunology*, 2004, 172: 5056–5062.

The intestinal epithelium constitutes the major mechanical barrier between luminal pathogens and the mucosal immune system. An intact epithelial barrier function is thus critical to protect the host organism from invading luminal pathogens, restricting microbial entry and propagation. In turn, enteroinvasive bacteria, including *Salmonella*, are capable of traversing this barrier by infecting epithelial cells and ultimately translocating to the mucosal and submucosal layers. Eventually, enteroinvasive pathogens will enter the intestinal microcirculation, resulting in systemic infection and causing septic illness. Intestinal *Salmonella* infection in vivo is characterized by a marked inflammatory infiltration of the lamina propria (1), and infection of human endothelial cells with *Salmonella* has been shown to be a potent inducer of ICAM-1 and other endothelial adhesion molecules (2). In vitro studies with human endothelial cells have indicated that bacterial LPS are potent inducers of endothelial adhesion molecules by triggering the proinflammatory signal transduction response via the transcription factor NF-κB (3). However, it is not known whether organ-specific endothelial cells are also capable of recognizing distinct bacterial components apart from LPS and whether specific pathogens will selectively activate discrete mechanisms of innate immune defense in the microvasculature.

Recent studies have indicated that recognition of bacterial Ags is dependent on and mediated by members of the Toll-like receptor (TLR) superfamily, which are membrane expressed by various immune cells (4). TLRs represent a superfamily of highly conserved pattern recognition receptors that are activated upon binding and recognition of their bacterial ligands, playing an essential role in innate immunity and host defense (4). Originally identified as the human homologues of the *Drosophila* transmembrane receptor, Toll (5), the TLRs are members of the IL-1R family (6). The human TLR family is comprised of 10 highly conserved type I transmembrane receptors which are functionally expressed on APCs. TLRs have been shown to respond to various conserved motifs on pathogenic microorganisms, including polyacetylated lipopeptides (TLR1/2 and TLR2/6 heterodimers), dsRNA (TLR3), LPS (TLR4), imidazoquinolines (TLR7 and TLR8), and unmethylated CpG DNA (TLR9) (7). TLR5 has recently been identified as the specific receptor for the bacterial structural protein flagellin (8).

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Abbreviations used in this paper: TLR, Toll-like receptor; HIMEC, human intestinal microvascular endothelial cells; C t, cycle threshold; MIF, median intensity fluorescence; FCF, flagellin-containing fraction; HIMEC-1, human dermal microvascular endothelial cell 1.
To date, TLR5 expression in humans has mainly been shown for intestinal epithelial cells, dendritic cells, and monocytes (7, 9, 10). Infection of intestinal epithelial cells with Salmonella thereby leads to an active transport of flagellin to the subepithelial compartment and subsequent binding to basolaterally expressed TLR5 in vitro, resulting in phosphorylation and nuclear translocation of the transcription factor NF-κB in intestinal epithelial cells (9, 11).

Recent work has demonstrated that gut-specific endothelial cells that exist in proximity to the enteric flora express TLR5 and are readily activated by its ligand, translocated bacterial cells and the intestinal mucosa, functionally express TLR5 and are expressed that human intestinal microvascular endothelial cells (HIMEC), representing the barrier between circulating immune cells and the intestinal mucosa, functionally express TLR5 and are readily activated by its ligand, translocated bacterial flagellin.

Materials and Methods

Cells and reagents

For HIMEC isolation, macroscopically normal colonic specimens were obtained from patients undergoing scheduled bowel resection for colorectal cancer and diverticulitis. The use of human tissues for immunohistochemistry and isolation of endothelial cells was approved by the ethical committee of the University of Muenster. HIMEC were isolated as previously described (13). In brief, mucosal strips from resected normal colon were washed, minced, and digested in collagenase type II solution (2 mg/ml; Worthington, Lakewood, NJ). Endothelial cells were extruded by mechanical compression and plated onto tissue culture dishes coated with collagen type I from rat tail (Upstate Biotechnology, Waltham, MA) in endothelial cell growth medium (PromoCell, Heidelberg, Germany) additionally supplemented with 10% (v/v) FBS. After 7–10 days of culture, microvascular endothelial cell clusters were physically isolated and a pure culture was obtained. HIMEC cultures were recognized by microscopic phenotype and expression of factor VIII-associated Ag. All experiments were conducted using HIMEC cultures between passages four and eight.

U937 cells (American Type Culture Collection, Manassas, VA), a human monocytic cell line derived from monocytic leukemia, were maintained in RPMI 1640 medium supplemented with 10% (v/v) FBS and 2 mM glutamine.

Bacterial infection and epithelial/endothelial coculture

Polarized intestinal epithelial cells grown on Transwell filter inserts were incubated with viable Salmonella for 1 h to allow for bacterial invasion. Cells were rinsed thoroughly, incubated with growth medium containing gentamicin (50 mg/ml) to kill remaining extracellular bacteria, and transferred to cell culture dishes containing confluent endothelial monolayers in the basolateral compartment.

Isolation of flagellin

Clinical isolates of two different strains of Salmonella typhimurium and one strain of Escherichia coli were used for flagellin isolation. Flagellin was isolated by acid depolymerization using a standard protocol (14, 15). Briefly, bacteria were grown with shaking overnight at 37°C, after which bacteria were pelleted and resuspended in 10 mM HCl, 150 mM NaCl, and incubated with rocking for 30 min at room temperature. After initial centrifugation to remove bacteria, supernatants were clarified by ultracentrifugation at 100,000 × g for 90 min and the pH was adjusted to 8.0 with 10 mM NaOH/50 mM Tris. LPS was removed from flagellin preparations by passage through a polymyxin B column according to the manufacturer’s instructions (Detoxi-Gel; Pierce, Rockford, IL) and as published recently by others (16). Supernatants were stored at −80°C until use. Purity of the preparations was confirmed by SDS-PAGE showing a single band with the expected size of flagellin on the Coomassie blue-stained gel. Furthermore, LPS content in the final preparation was <0.125 ng/ml as assessed by a Limulus amebocyte lysate-based assay (ICN Biomedicals; Eschwege, Germany).

RT-PCR

Total cellular RNA was extracted using an acid guanidinium-phenol-chloroform method (TRIzol Reagent, Life Technologies, Grand Island, NY) and treated with RNase-free deoxyribonuclease (Stratagene, La Jolla, CA). Reverse transcription and PCR were performed as described before (17). The following primers were used to amplify a 166-bp fragment of TLR5: sense primer, 5′-TCAAACTCCTTGAGAAGTCC-3′ and reverse primer, 5′-TTAGGATTTAGGCTTAGTCC-3′. Primers for the amplification of human β-actin mRNA were described before (17). The amplification time for TLR5 was 35 cycles of 45-s denaturation at 95°C and 2-min annealing and extension at 60°C. β-Actin was amplified for 26 cycles under the same conditions except the annealing and extension temperatures were 72 and 66°C, respectively.

Real-time RT-PCR

Real-time PCR was performed using an ABI Prism 5700 Sequence Detection System (PE Applied Biosystems, Foster City, CA). Each reaction contained 25 pmol of 2 × SYBR Green I/ROX, 400 mM dGTP, and dCTP, 2 mM MgCl2, 0.25 U uracil N-glycosylase, and 0.625 U Ampli-Taq Gold DNA polymerase, 25 pmol each of sense and antisense primers, and 2 μl of cDNA in a final volume of 50 μl. TLR5 primers and β-actin were as described above. Reactions were incubated at 50°C for 2 min followed by 95°C for 10 min. For TLR5 and β-actin, the amplification profile was 15-s denaturation at 95°C followed by 1-min annealing at 60°C for TLR5, and 1-min annealing at 62°C for β-actin for a total of 40 cycles. Amplification of the expected single products was confirmed on 1% agarose gels stained with ethidium bromide. Data analysis used sequence detection system software provided by the manufacturer where ΔCt was calculated using the equation ΔCt = (CtRn − CtRn), with Rn being the fluorescence signal of the product and Rn the fluorescence signal of the reference gene (Cyc). C is the cycle number at which the ΔRn crosses threshold. Fold changes in TLR5 mRNA expression were determined as fold change = 2ΔCt, where ΔCt = (CtTLR5 control − Ct actin control) − (CtTLR5 stimulated − Ct actin stimulated).

Immunohistochemistry

Resected specimens of normal colon were fixed in 4% (w/v) parafomaldehyde PBS, saturated in 20% (w/v) sucrose in PBS overnight, embedded in OCT compound (Sakura, Japan), and snap frozen in isopentane/liquid nitrogen. Five-micrometer sections were prepared, and endogenous peroxidase and biotin activities were blocked using 3% hydrogen peroxide and the avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA), respectively. Tissue sections were immunostained using a rabbit polyclonal anti-human TLR5 Ab (Santa Cruz Biotechnology, Santa Cruz, CA) and the DAKO LSAB+ tissue staining kit (DAKO, Carpinteria, CA). Sections were counterstained with hematoxylin, mounted with gelatin, and examined by light microscopy.

Immunofluorescence

HIMEC were seeded on collagen-coated glass chamber slides (LabTek; Nalgen Nunc International, Naperville, IL), cultured to subconfluence in growth medium, and stimulated as indicated in Results. After brief fixation with 2% (w/v) paraformaldehyde in PBS, formaldehyde-fixed cultures were permeabilized with Triton X-100 (0.1% (v/v) in PBS) for 5 min, and blocked in blocking buffer (10% (v/v) normal donkey serum in PBS) for 1 h at room temperature. Normal goat IgG (Sigma-Aldrich, St. Louis, MO) or polyclonal goat anti-human TLR5 Ab (Santa Cruz Biotechnology) were diluted in blocking buffer and were incubated at 4°C overnight. After extensive washing, immunodetection was performed using a polyclonal Cy3-labeled donkey anti-goat Ab (Jackson ImmunoResearch, West Grove, PA), and nuclei were visualized by counterstaining with 4',6-diamidino-2-phenylindole (Sigma-Aldrich). In some experiments, specific fluorescence for TLR5 was quenched by prior preincubation of the TLR5 Ab with the corresponding blocking peptide (Santa Cruz Biotechnology).

Immunoblotting

HIMEC were stimulated for 16 h with TNF-α (20 ng/ml), IFN-γ (40 ng/ml), E. coli, or S. typhimurium-flagellin containing fractions. Control cells remained without any stimulation. HIMEC monolayers were lysed in modified RIPA buffer (50 mM Tris–HCl (pH 7.6), 1 mM EDTA, 150 mM NaCl, 0.25% (w/v) sodium deoxycholate, 1% (v/v) Igepal CA-630, and 0.1% (w/v) SDS; all Sigma-Aldrich) containing protease inhibitor mixture III (Calbiochem, San Diego, CA) on ice. Lysates were cleared by centrifugation, and total protein concentration was determined by Bradford assay (Bio-Rad, Hercules, CA). Thirty micrograms of total cellular protein were size separated on a 7.5% SDS-PAGE gel, blotted onto nitrocellulose membranes (Amersham Pharmacia Biotech, Arlington Heights, IL), and blocked with blocking buffer (PBS containing 0.1% (v/v) Tween 20, 10% (w/v) nonfat dry milk, and 1% (w/v) BSA). Blots were incubated with polyclonal goat anti-human TLR5 Ab (1/100; Abcam, Cambridge, UK).
overnight at 4°C. Immunodetection was performed using HRP-conjugated rabbit anti-goat Ab (Sigma-Aldrich) and ECL (Amersham Pharmacia Biotech). Goat IgG (Sigma-Aldrich) used at the same concentration served as isotype control (data not shown).

**Endothelial leukocyte transmigration assay**

Endothelial transmigration assays were performed as described before (18). In brief, HIMEC were seeded onto collagen-coated Transwell polycarbonate filter inserts (24-well format, pore size 5 μm) in growth medium and grown to confluence. Monolayer integrity was assessed in parallel inserts by crystal violet staining followed by light microscopy. Forty-eight hours after reaching confluence, endothelial monolayers were stimulated with growth medium containing flagellin-containing fractions (FCF) from S. typhimurium H737. U937 cells were suspended in RPMI 1640 medium containing 10% (v/v) of FBS and 1% (w/v) of glucose and 2 × 10⁴ cells were added on top of the endothelial monolayers. U937 cells were allowed to migrate for 3 h at 37°C/5% (v/v) CO₂, and transmigrated cells were counted in the lower chamber by flow cytometry using equal volumes and counting intervals. Each condition was assessed in triplicates.

**Statistical analysis**

Values are expressed as mean ± SEM. Student’s unpaired t test was used to determine statistical significance of values between groups; p values <0.05 were considered significant.

All studies involving human subjects were approved by the ethical committee of the University of Munster.

**Results**

**Salmonella-infected intestinal epithelial cells induce endothelial ICAM-1 expression**

To define the specific interactions between the two barrier-forming cell types of the human intestinal mucosa in response to epithelial Salmonella infection, we used an epithelial/endothelial coculture system using Transwell tissue culture inserts. Caco-2 human intestinal epithelial cells were grown to confluence as polarized monolayers on cell culture inserts and infected with viable Salmonella as indicated in Materials and Methods. Polarization was verified by measuring the transepithelial resistance. Extracellular bacteria were killed by a non cell-permeable antibiotic, and cell culture inserts were transferred to cell culture dishes containing human endothelial cells and cocultured for 16 h. Endothelial ICAM-1 expression was quantitated by flow cytometry. As indicated in Fig. 1, coculture with intestinal epithelial cells infected with Salmonella led to a significant increase in endothelial ICAM-1 expression (Fig. 1, 5), comparable to that achieved by endothelial stimulation with recombinant human TNF-α (Fig. 1, 4). To ensure that the detected effect on endothelial ICAM-1 expression was not simply caused by translocated LPS, epithelial cells were incubated with LPS from the same Salmonella strain as the control (Fig. 1, 2). LPS was thereby added exclusively to the apical side of the polarized epithelial monolayer, while endothelial cells were brought in coculture with the basolateral side of the epithelial monolayer. However, addition of LPS to the apical side of epithelial cells did not enhance endothelial ICAM-1 expression (Fig. 1, 2). Isotype IgG served as a negative control (Fig. 1, 1), while Fig. 1, 3, shows constitutive ICAM-1 expression. Consistent with a previous report in which proinflammatory actions of flagellin from enteropathogenic Salmonella have been demonstrated (19), these data suggest that bacterial products other than LPS, in conjunction with possible epithelially produced mediators as suggested by work from our own group (20), elicit activation of microvascular endothelial cells in this model of epithelial/endothelial coculture.

**Human macro- and microvascular endothelial cells constitutively express TLR5 in vitro**

As Salmonella-infected human intestinal epithelial cells transduced a proinflammatory response in cocultured microvascular en-
cells with proinflammatory mediators such as IL-1β, TNF-α, and IFN-γ yielded no further up-regulation of endothelially expressed TLR5 mRNA as assessed by quantitative real-time RT-PCR (data not shown).

In accordance with constitutive mRNA levels of TLR5, human endothelial cells constitutively express TLR5 protein as depicted in Fig. 3A, showing whole cell lysates of HIMEC size separated by SDS-PAGE and immunoblotted with pAbs to TLR5. In accordance with our real-time RT-PCR data, endothelial TLR5 protein expression remained unaltered upon incubation with combined IFN-γ (40 ng/ml) and IL-1β (20 ng/ml) as well as FCF from E. coli and S. typhimurium (Fig. 3A). To reveal the subcellular localization of TLR5, indirect immunofluorescence on HIMEC was performed. As shown in Fig. 3B, unstimulated HIMEC display a speckled immunofluorescence pattern (I) characteristic of the membrane localization of TLR5 protein. Specificity of binding was verified by preincubation of the primary Ab with the corresponding blocking peptide (Fig. 3BII), leading to an almost complete abolishment of staining as well as preimmune IgG as primary Ab (Fig. 3BIII).

**Normal human intestinal microvessels express TLR5 in vivo**

Since both constitutive TLR5 mRNA and protein expression were detected in vitro, we next sought to define TLR5 expression in normal human intestinal microvessels in vivo. Using frozen tissue sections from normal human colon for performing immunohistochemistry, strong immunoreactivity for TLR5 was detected. Interestingly, some lamina propria mononuclear cells (arrows) also showed specific immunostaining for TLR5 (Fig. 4A). Control IgG served as a negative control, lacking any specific signal (Fig. 4B). These results demonstrate constitutive expression of TLR5 in colonic microvascular endothelium in vivo, pointing to its potential role as a flagellin receptor, mediating innate immune responses toward mucosal invading bacteria and/or bacterial products.

**FCF enhance endothelial ICAM-1 expression**

Having demonstrated constitutive TLR5 expression in human microvascular endothelial cells in vitro and in vivo, we next sought to determine the effects of FCF from Enterobacteriaceae species on microvascular cell adhesion molecule expression. Stimulation of HIMEC with FCF from clinical isolates of E. coli and Salmonella (data not shown) strains for 16 h resulted in a marked up-regulation of the endothelial leukocyte adhesion molecule, ICAM-1, as determined by flow cytometry (Fig. 5, 3; median intensity fluorescence (MIF) = 147.2). As a positive control for ICAM-1 up-regulation, the proinflammatory cytokine TNF-α (20 ng/ml) was used (Fig. 5, 4; MIF = 484.6). Unstimulated cells were used to quantify constitutive endothelial ICAM-1 expression (Fig. 5, 2; MIF = 45.7), and isotype control IgG was used as a negative control (Fig. 5, 1; MIF = 29.1). Taken together, our data suggest that endothelial cells expressing TLR5 can be directly activated by flagellin to express ICAM-1.

**FCF enhance transendothelial leukocyte migration**

As a functional readout for intestinal endothelial function in response to flagellin, we next examined whether FCF were able to enhance leukocyte transendothelial migration. HIMEC were grown on Transwell cell culture inserts to confluence, stimulated with flagellin-containing Salmonella fractions, and subjected to a transmigration assay by adding a defined number of U937 cells, a human monocytic cell line, to the upper wells. After 3 h, cells that had migrated to the lower wells were enumerated by flow cytometry using fixed acquisition settings. As shown in Fig. 6, Salmonella-derived FCF led to a marked increase in U937 transendothelial migration (relative cell number, 450 ± 30), as compared with unstimulated cells (90 ± 30). This finding ascribes a potential role to flagellin as a potential proinflammatory mediator in the human intestinal microvasculature.

**Discussion**

In this study, we have shown constitutive expression of TLR5 in human endothelial cells, including primary cultures of HIMEC and...
in human colonic tissue. This finding provides important insight into the mechanisms underlying the innate immune response to pathogenic enteroinvasive bacteria, including Salmonella and Escherichia strains, which are known to traverse the intestinal epithelial barrier and gain proximity to mucosal and submucosal microvessels. Our data suggest that HIMEC might serve an important sentinel function in the rapid detection of translocated flagellin in the mucosal layers. This finding is directly relevant to the pathophysiology of intestinal infections, but also implicates a role in the propagation and continuation of chronic inflammation, which characterizes inflammatory bowel diseases. Disruption of an intact intestinal epithelial barrier, with an associated disturbance of epithelial tight junctions, has been suggested to play an important pathophysiological role in both Crohn’s disease (21, 22) and ulcerative colitis (23). Specifically, the expression of the epithelial intercellular tight junction compound protein occludin was found to be substantially diminished in active inflammatory bowel disease at sites of high polymorphonuclear cell transmigration into the colonic epithelium (24). As a result of decreased barrier integrity, luminal Ags, including bacterial flagellin, are more likely to traverse the damaged epithelium at higher concentrations, which may result in activation of TLR5 molecules expressed throughout mucosal sites.

Mucosal cells expressing TLR5 include dendritic cells in the lamina propria, which respond with maturation and chemokine production upon binding of bacterial flagellin (7). In addition, dendritic cells are regarded as key players in the procession and presentation of luminal Ags. HIMEC, however, have also recently been shown to be potent APCs in vitro (25), pointing to a previously unrecognized role of endothelial cells in the detection of and response to bacterial flagellin. Upon encounter by bacterial flagellin, intestinal endothelial cells might thus be capable of serving multiple rapid effector functions, including up-regulation of endothelial leukocyte adhesion molecules, enhanced expression of chemotactrant cytokines, and Ag presentation to circulating lymphocytes leading to enhanced leukocyte infiltration into the lamina propria at sites of inflammation, thereby initiating the host’s defense. These possible effector functions are paralleled and substantiated by a recent study from Andonegui et al. (26), in which endothelially expressed TLR4 has been identified as the key molecule in LPS-induced leukocyte sequestration into lungs using an endothelium-specific TLR4−/− knockout mouse model (26).

Flagellin, a protein of 40–60 kDa, is known as the principle constituent of flagella in motile bacteria (27) and has recently been identified as the bacterial component responsible for TLR5-stimulating activity. Bacterial species known to express TLR5 comprise all flagellated bacteria, including Salmonella, Listeria, Pseudomonas, and Escherichia strains, whereas nonflagellated bacteria, such as Haemophilus influenzae, are therefore devoid of TLR5-stimulating activity (8). Consequently, TLR5 is capable of recognizing flagellin derived from pathogenic and nonpathogenic bacteria. As reported by Gewirtz and coworkers (9), TLR5 is expressed constitutively at high levels on the basolateral side of the intestinal epithelium, a position normally devoid of bacterial flagellin. This observation seems logical, as abundant luminal flagellin is thus unable to evoke any inflammatory response, whereas in the setting of an injured intestinal epithelial barrier TLR5 receptors will be challenged, resulting in the up-regulation of proinflammatory response genes.

Signaling pathways involved in the propagation of the TLR5 stimulus include activation of the IL-1R-associated kinase (28), the adaptor protein myeloid differentiation factor 88 (29) and ultimately, nuclear translocation of the proinflammation transcription factor NF-κB (30), although NF-κB-independent TLR5-mediated activation of epithelial IL-8 expression via p38 mitogen-activated protein kinase activation has been demonstrated recently (31). Interestingly, tolerance toward the TLR5 stimulus has been demonstrated in human monocyes and THP-1 cells, and this tolerance mechanism was shown to be independent of the TLR5 surface expression levels (28). In homology, tolerance toward bacterial LPS, the stimulus for TLR4, was similarly shown for HIMEC in vitro (12), further suggesting that human microvascular endothelial cells, a “nonimmune” cell population, possess features characteristic of immune cells involved in the pathogenesis of intestinal inflammatory disease. Defining mechanisms of endotoxin tolerance in various gut cell populations is presently an area of intense investigation in mucosal immunity and inflammatory bowel disease research. The concept of an impaired ability to develop programmed hyporesponsiveness to bacterial products has gained momentum as a central mechanism driving chronic inflammation in
inflammatory bowel disease. The interplay of the various TLRs has demonstrated further complexity in the interaction of these molecules in innate immunity. A recent publication has shown that TLR5 is able to congregate with TLR4, forming TLR5/TLR4 heterodimers, being involved in the induction of NO production in macrophages (32).

We have shown herein that infection of polarized Caco-2 intestinal epithelial cells with viable *Salmonella*, but not incubation of these cells with LPS derived from the same *Salmonella* strain, led to a marked increase in ICAM-1 expression in cocultured endothelial cells. These data reflect the presence of a proinflammatory stimulus or stimuli other than LPS propagated by infected epithelial cells. As shown by RT-PCR and immunoblot/immunofluorescence analysis, TLR5 is constitutively expressed in human microvascular endothelial cells (HMEC and HIMEC-1) and macrovascular (HUVEC) endothelial cells. Moreover, TLR5 protein expression in the normal human colonic microvasculature was confirmed in vivo by immunohistochemistry. Of interest, no up-regulation of TLR5 could be detected after incubation with different proinflammatory stimuli. These findings potentially reflect a homeostatic/constitutive rather than an inducible/reactive function of endothelial TLR5 expression in vivo. This is well in accordance with the observations reported by Mizel and Snipes (28), who did not detect any regulation of TLR5 expression levels in Jurkat T cells rendered tolerant to bacterial flagellin. Because endothelially expressed TLR5 might serve an important sentinel function, constitutively high levels appear reasonable to allow for a rapid response to an acute flagellin challenge. Incubation of human microvascular endothelial cells with FCF led to a significant enhancement of endothelial ICAM-1 expression, suggesting functional relevance of this pathogen receptor being expressed on human endothelial cells.

As summarized in the schematic in Fig. 7, HIMEC are therefore potential targets for flagellin translocated across the intestinal barrier. Detection of mucosal flagellin by endothelial cells may represent a novel mechanism of intestinal innate immune response, aiding in the resolution of intestinal infections by enhancing the expression of endothelial leukocyte adhesion molecules.

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**References**


