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Identification of TLR10 as a Key Mediator of the Inflammatory Response to Listeria monocytogenes in Intestinal Epithelial Cells and Macrophages

Tim Regan,*† Ken Nally,‡ Ruaidhri Carmody,‡ Aileen Houston,†§ Fergus Shanahan,†§ John MacSharry,†§,1 and Elizabeth Brint*,†,1

*Department of Pathology, University College Cork, National University of Ireland, Cork, Ireland; †Alimentary Pharmacobiotic Centre, University College Cork, National University of Ireland, Cork, Ireland; ‡University of Glasgow, Glasgow G61 1QH, United Kingdom; and §Department of Medicine, University College Cork, National University of Ireland, Cork, Ireland

J.M. and E.B. contributed equally to this work.

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Address correspondence and reprint requests to Dr. John MacSharry, Alimentary Pharmacobiotic Centre, School of Medicine, University College Cork, National University of Ireland, College Road, Cork, Ireland. E-mail address: j.macsharry@ucc.ie

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Abbreviations used in this article: EGR-1, early growth response protein-1; IEC, intestinal epithelial cell; ISRE, IFN-stimulated response element; MOI, multiplicity of infection; PRR, pattern recognition receptor; qRT-PCR, quantitative RT-PCR; siRNA, small interfering RNA.

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Listeria monocytogenes is a food-borne Gram-positive bacterium that can cause septicemia and meningitis. TLRs are central receptors of the innate immune system that drive inflammatory responses to invading microbes such as L. monocytogenes. Although intestinal epithelial cells (IECs) represent the initial point of entry used by L. monocytogenes for infection, the innate immune response to L. monocytogenes in these cells has been poorly characterized to date. The aim of this study was to determine which TLRs are involved in mediating the immune response to L. monocytogenes in IECs. We performed an RNA interference screen of TLRs 1–10 in the HT-29 IEC cell line and observed the most significant reduction in chemokine output following silencing of TLR10. This effect was also observed in the macrophage cell line THP-1. The chemokines CCL20, CCL1, and IL-8 were reduced following knockdown of TLR10. Silencing of TLR10 resulted in increased viability of L. monocytogenes in both HT-29 and THP-1 cells. TLR10 was found to be predominantly expressed intracellularly in epithelia, and activation required viable L. monocytogenes. NF-κB activation was seen to require TLR2 in addition to TLR10. Taken together, these data indicate novel roles for TLR10 in sensing pathogenic infection in both the epithelium and macrophages and have identified L. monocytogenes as a source of ligand for the orphan receptor TLR10. The Journal of Immunology, 2013; 191: 6084–6092.

L. monocytogenes is a Gram-positive bacterium that can cause septicemia and meningitis, particularly in immune-compromised individuals (1). Following ingestion, L. monocytogenes crosses the intestinal barrier by first invading intestinal epithelial cells. From there, infection of macrophages proceeds via phagocytosis followed by escape from the phagosome into the cytosol through the action of the pore-forming toxin listeriolysin O (2, 3). The infecting organisms reach their first target organs, the liver and the spleen, via the lymphoid system and the blood, where they are internalized by splenic and hepatic macrophages. They then actively multiply until the infection is controlled by a cell-mediated immune response.

Effective immune responses to L. monocytogenes infection relies on coordinated innate and adaptive immune responses, with the first line of innate defense being mediated by detection of the invading bacterium by pattern recognition receptors (PRRs). Several families of these receptors have been identified, including the transmembrane TLRs, the cytosolic NOD-like receptors and RIG-I–like receptors, and in more recent years C-type lectin receptors (4). The activation of innate immunity by PRRs in response to L. monocytogenes infection is still not fully understood. L. monocytogenes has been shown to be recognized by TLR2 (5, 6), NOD1 (7, 8), and NOD2 (9), resulting in NF-κB activation and proinflammatory gene expression. The role of TLR5 in detection of L. monocytogenes remains unclear because although flagellin from L. monocytogenes activates TLR5, bacterial mutants deficient in flagellin display an unaltered virulence (10, 11). More recent studies have identified several inflammasome components responsible for recognition of the bacterium such as NOD-like receptor C4 and AIM2. Recognition of L. monocytogenes through these receptors results in activation of caspase-1 and cleavage of IL-1β (12). L. monocytogenes infection has also been shown to induce a type I IFN response, potentially mediated by TLR2 and the adaptor protein TRIF in peritoneal macrophages (13). Most recently the IFN-β response has been shown to be triggered by nucleic acids released from L. monocytogenes acting through the intracellular sensor RIG-I (14). Previous in vitro mouse studies have predominantly used i.p. in vivo infection models where type I IFN responses were shown to be detrimental to bacterial clearance. Recently, however, using an in vivo mouse model of oral L. monocytogenes infection, Kernbauer et al. (15) proved that this is only the case when infection occurs through a route other than intragastric, highlighting the importance of study required in epithelial-mediated innate immunity.

Epithelial cells line the enteric mucosal surface, providing barrier function against microbial invasion. Similar to immune cells, intestinal epithelial cells express many of these receptors of the innate immune system and are the first site of bacterial recognition in the intestine. Characterization of the innate immune responses to L. monocytogenes infection has been studied in several cell types, including endothelial cells, PBMCs, and macrophages (16). However, no extensive screen has been performed of innate mechanisms...
of detection of L. monocytogenes in the intestinal epithelia, and little focused attention has been paid to the role of specific PRRs in detecting L. monocytogenes infection at this barrier interface. To date only NOD2 has been directly shown to play a role in detecting L. monocytogenes in the intestinal epithelia (9). In this study, we examined the role of TLRs 1–10 in recognition of L. monocytogenes infection in epithelial cells. In doing so, we have uncovered a novel role for TLR10 in mediating the inflammatory response to infection by L. monocytogenes in IECs and also macrophages. Because ligand specificity for TLR10 has to date remained elusive, to our knowledge this is the first study to show an essential role for TLR10 in mediating the inflammatory response to infection by L. monocytogenes in IECs and also macrophages.

Materials and Methods

Cell culture

HCT-116, HCA-7, and HEK293T cells were maintained in DMEM (Thermo Scientific, Leicestershire, U.K.), HT-29 in McCoys 5A (Thermo Scientific), and THP-1 cells in RPMI 1640 (Thermo Scientific), supplemented with 10% FCS (Thermo Scientific) and 10 µg/ml penicillin and streptomycin (Sigma-Aldrich, Dorset, U.K.) and cultured in a humidified incubator at 37°C with 5% CO2. THP-1 cells were differentiated into macrophages by overnight stimulation with 5 ng/ml PMA (Sigma-Aldrich). The ligands Pam3Cys (50 µg/ml), peptidoglycan (10 µg/ml), lipoteichoic acid (10 µg/ml), FSL-1 (5 µg/ml), LPS (10 ng/ml), polyinosinic-polycytidylic acid (20 µg/ml), and TNF-α (10 ng/ml) (all from Invitrogen, Paisley, U.K.) were used for cell stimulation assays, as were bacteria (see below). Each of the ligands was obtained from Invitrogen.

Bacterial cell culture, infections, and survivability assays

The L. monocytogenes strain EGD (serotype 1/2a) was a gift from Prof. C. Hill (University College Cork, Cork, Ireland). Bacteria were grown to the logarithmic growth phase in brain–heart infusion broth (Sigma-Aldrich) at 37°C shaking at 200 rpm. Bacteria were subsequently washed with PBS by two steps of centrifugation (4000 × g for 5 min) and diluted in PBS for infection at multiplicity of infection (MOI) of 50:1. The cells were washed at 2 h postinfection with antibiotic-free cell culture medium and supplemented with cell culture medium containing gentamicin (Invitrogen) (50 µg/ml). Cells were then incubated for the times indicated in Results. For bacterial survival assays, the cells were washed at 8 h postinfection and treated with 0.2% Triton X-100 PBS. The lysates were then subjected to serial dilution and spotted on a brain–heart infusion agar plate. CFUs per lysate of internalized bacteria were then determined. The Salmonella typhimurium strain SJW1103 (wild-type) was a gift from Prof. P. O’Toole (University College Cork). Bacteria were grown to the logarithmic growth phase in Luria–Bertani broth (Sigma-Aldrich) at 37°C shaking at 200 rpm. Bacteria were subsequently washed with PBS by two steps of centrifugation (4000 × g for 5 min) and diluted in PBS for infection at an MOI of 10:1.

Small interfering RNA transfection

All small interfering RNA (siRNA) transfections were performed with SMARTpool siRNAs (Thermo Scientific). Sequences are delineated in Supplemental Table 1A. For the TLR siRNA screen, cells were seeded at 1 × 10^4/well in a 96-well plate the day before transfection. DharmaFECT 4 (Thermo Scientific) was then used to transfect the cells with siRNA (50 nM) according to the manufacturer’s instructions 48 h prior to infection. THP-1 siRNA transfections were performed using the Amaxa system (Lonza, Basel, Switzerland) according to the manufacturer’s protocol. Cells were cultured for a further 48 h prior to infection. The “control siRNA” used in all experiments indicates the nontargeting scrambled siRNA. All analyses on siRNA treatment are of independent triplicate biological replicates. Technical replicates were included in each plate screen to address any intra-assay variation.

Gene expression analysis using quantitative RT-PCR

Total RNA was isolated using an RNeasy kit (Qiagen, West Sussex, U.K.). cDNA synthesis was performed using Transcriptor reverse transcriptase (Roche, West Sussex, U.K.) according to the manufacturer’s instructions. Total RNA (1 µg) was incubated with 50 pmol random hexamers at 25°C for 5 min, 42°C for 50 min, and 4°C for 5 min. Individual PCR primer pairs and probes were designed using the Roche Universal ProbeLibrary Assay Design Centre (https://www.roche-applied-science.com/sis/rtpcr/upl/adc.jsp). Primer sequences and probe combinations are provided in Supplemental Table 1B. PCR reactions were performed using SensiMix II (Bioline, London, UK). 900 nM each primer, and 250 nM probe mix in the LightCycler480 System (Roche). Thermal cycling conditions were as recommended by the manufacturer (Roche). β–Actin was used as a housekeeping gene, and the 2^ΔΔCt method (17) was used to calculate relative changes in gene expression. Relative expression in each figure refers to induction levels of the gene of interest relative to a housekeeping control, β–actin, and then compared to an untreated control transfection assay.

Flow cytometry

Abs against TLR1, TLR2, and TLR6 (IMG-5012, IMG-6720A, IMG-304A) were from Imagegen (San Diego, CA), and the TLR10 Ab was from Santa Cruz Biotechnology (TLR10 H-165). As secondary Abs, we used Alexa Fluor 488 goat anti-mouse IgG (Invitrogen) and PE-conjugated goat anti–rabbit IgG H+L (Imagegen). The respective isotype controls for each Ab were also from Imagegen. On the day of analysis, 1 × 10^6 cells were harvested from the 0.2% EDTA/PBS and washed twice in running buffer (0.5% BSA PBS, 1% FBS, 0.1% sodium azide). For intracellular staining, cells were fixed in BD Cytofix/Cytoper (Becton Dickinson, Franklin Lakes, NJ) for 30 min on ice. For extracellular staining, cells were not fixed. Each primary Ab was used at 0.5 µg/ml for 30 min on ice. Each wash step for intracellular staining was performed using BD Perm/Wash buffer (Becton Dickinson) whereas the running buffer was used for extracellular staining washes. The secondary Abs were used at a 1:1000 dilution for 20 min in the dark on ice, and cells were analyzed using the Accuri C6 flow cytometer (Becton Dickinson). The gating method employed was designed to include the live cell population only. Population gates were set using negative controls and IgG isotype controls. The results were expressed as the percent or mean fluorescence intensity of the total live cell population after subtracting the mean fluorescence intensity of control cells stained with the appropriate isotype control Abs.

Western blot analysis

Cells were lysed in RIPA lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 1 mM PMSF, and 10% protease inhibitor cocktail; Sigma-Aldrich). The following Abs were used: anti–TLR10 (H-165) (sc-30198, Santa Cruz Biotechnology, Heidelberg, Germany) and β–actin Ab (Sigma-Aldrich). Proteins were detected using the Fujifilm LAS-3000 imager (R&D Systems, Abingdon, U.K.).

ELISA analysis of supernatants for cytokine production

CCL20 levels were quantified using the anti-CCL20 kit (Meso Scale Discovery, Rockville, MD) according to the manufacturer’s instructions. Concentrations were determined using the Meso Scale Discovery Imager C4000. IL-8 levels were quantified using the Ready-Set-Go! human IL-8 ELISA kit (eBioscience, San Diego, CA) according to the manufacturer’s instructions.

Luciferase assays

HEK293 cells were seeded at 2.5 × 10^4/well in a 96-well plate 24 h prior to transfection. Cells were transfected using TurboFect (Fermentas) according to the manufacturer’s protocol using 50 ng Ifn-stimulated response element (ISRE) luciferase plasmid and 5 ng thymidine kinase Renilla luciferase along with varying amounts of either TLR1, TLR2, TLR6, or TLR10 (gift from Prof. L. O’Neill, Dublin, Ireland). Twenty-four hours after transfection cells were infected with L. monocytogenes as described above for 8 h. Extracts were prepared by lysing the cells for 15 min in 50 µl passive lysis buffer (Promega, Madison, WI) and measured for firefly luciferase and Renilla luciferase activity. Firefly luminescence readings were corrected for Renilla activity and expressed as fold stimulation over unstimulated empty vector control.

NF-κB activity

Secreted embryonic alkaline phosphatase reporter HEK-Blue MD2-CD14 cells (InvivoGen, Toulouse, France) were used according to the manufacturer’s instructions. Briefly, cells were maintained in selective antibiotics and seeded at 2 × 10^5 cells/well on a 96-well plate the day before transfection. Cells were then transfected and infected with L. monocytogenes as described above. Supernatants were collected 8 h later and activity measured using QUANTI-Blue (InvivoGen).

Statistics

Results were evaluated using a Student t test. A value of p < 0.05 was considered statistically significant.
Results

Role of different TLRs in L. monocytogenes–induced gene expression

We initially investigated the involvement of all human TLRs in the response of HT-29 IECs to L. monocytogenes infection by use of a siRNA screening approach (Supplemental Fig. 1). Inflammatory readouts selected were IL-8 and early growth response protein-1 (EGR-1), as these demonstrate consistently robust expression patterns in response to infection. EGR-1 mRNA was induced 6-fold (p ≤ 0.001) by L. monocytogenes infection. Knockdown of TLRs 1–10 did not affect EGR-1 induction by L. monocytogenes, indicating that activation of EGR-1 expression is via a TLR-independent mechanism (Fig. 1A). IL-8 mRNA expression was increased 4.5-fold following L. monocytogenes infection, and this effect was significantly abrogated following silencing of TLRs 1 and 10 (Fig. 1B). Knockdown of TLR2 resulted in reduced IL-8 expression following infection; however, this did not reach statistical significance. Of the three genes where any effect was observed following siRNA-mediated knockdown (TLRs 1, 2, and 10), TLR10 silencing had the most significant effect on reduction of IL-8 expression. Additionally, silencing of TLRs 1, 2, and 10 were found to be specific and have no off-target effects on the other members of the closely related TLR1, 2, 6, or 10 gene expression by quantitative RT-PCR (qRT-PCR) (Fig. 1C–F) and by flow cytometry (Supplemental Fig. 2).

Role of TLR10 in mediating chemokine production in response to L. monocytogenes infection

This novel identification of TLR10 as a potential sensor of L. monocytogenes infection in an IEC cell line was intriguing given the fact that TLR10 expression is more commonly associated with immune cell subtypes (18). We confirmed both expression and effective silencing by siRNA of TLR10 in the HT-29 cell line by qRT-PCR (Fig. 2A) and Western blotting (Fig. 2B). To fully characterize which cytokines and chemokines were induced by L. monocytogenes in a TLR10-dependent manner, we initially examined mRNA expression of an extensive number of TLR-induced genes (EGR-1, IL-1β, IL-2, IL-6, IL-8, IL-9, IL-12, IL-18, IL-23, caspase-1, CCL1, CCL2, CCL5, CCL20, CXCL1, CXCL10) in HT-29 cells following infection. Of these genes only caspase-1, IL-1β, IL-8, IL-18, IL-23, CCL1, CCL20, and EGR-1 demonstrated detectable levels of expression by HT-29 cells, and of these only EGR-1, CCL1, CCL20, and IL-8 were significantly induced following infection (Supplemental Fig. 3B). Because results from the original screen implicated possible roles for TLRs 1 and 2 as well as TLR10 in the L. monocytogenes–induced immune response, we performed single knockdown with siRNA against TLRs 1, 2, and 10 and measured CCL20 and IL-8 mRNA expression. TLR10 silencing resulted in the most significant decreases in CCL20 and IL-8 mRNA levels in response to L. monocytogenes infection (Fig. 2C and 2D, respectively). TLR2 silencing reduced IL-8 expression, whereas TLR1 silencing reduced CCL20 expression. Because CCL20 was the most strongly induced chemokine gene in response to L. monocytogenes, protein production was measured during a longer period of time, and a consistent reduction in CCL20 was observed following knockdown of TLR10 by siRNA at all time points (Fig. 2E). Given that L. monocytogenes is an invasive bacterium, we hypothesized that TLR10 might be detecting the pathogen intracellularly. In Fig. 2F intracellular versus extracellular staining for TLR10 by flow cytometric analysis revealed much greater expression levels of intracellular TLR10 than extracellular TLR10. Furthermore, heat-killed L. monocytogenes failed to induce CCL20 or IL-8 to levels comparable to the induction seen when invasive live bacteria were administered to HT-29 cells (Fig. 2G, 2H). This implies that bacterial invasion must be responsible for such high levels of CCL20 and IL-8 induction. Together with the data from Fig. 2F, it implies that this induction could be mediated by intracellular TLR10.

To ensure that the effect seen was not restricted to one cell line (HT-29), an additional IEC line, HCA-7, was assessed. TLR10 silencing was confirmed by qRT-PCR in HCA-7 cells (Fig. 3A). mRNA expression of TLR10 in HCA-7 cells was observed to be much higher than in HT-29 cells. Silencing of TLR10 resulted in reduced CCL20 and IL-8 mRNA expression in the HCA-7 cell line (Fig. 3B, 3C), as seen in HT-29 cells. CCL1 induction was, however, not significantly affected, unlike TLR10-silenced HT-29 cells in response to L. monocytogenes infection (Supplemental Fig. 3C, 3D). A consistent reduction in CCL20 protein production was observed following knockdown of TLR10 by siRNA in HCT-116 cells at all time points (Fig. 3D).

Because both L. monocytogenes infection and TLR10 have been predominantly studied in immune cell subtypes, we next chose to examine PMA-differentiated THP-1 macrophage-like cells to see whether this was an IEC-specific effect. A consistent reduction in CCL20 protein production was observed following knockdown of TLR10 by siRNA after 48 h (Fig. 3E). Confirmation of ~52% TLR10 silencing in THP-1 cells was confirmed by flow cytometry (Fig. 3F, 3G).

TLR10 affects L. monocytogenes survival in IECs and macrophages

Altering expression of the PRRs NALP3 and Aim2 has been previously shown to influence the ability of L. monocytogenes to survive and replicate within cells (12). We wanted to identify whether altering expression of TLR10 would have a similar effect. Survival of L. monocytogenes was analyzed by calculating the intracellular bacterial burden in both HT-29 and THP-1 cells following TLR10 silencing during an 8-h period. Increased survival of the bacteria is seen in both cell lines, indicating a critical role for the receptor in antagonizing bacterial intracellular viability (Fig. 4A and 4B, respectively).

TLR10 requires TLR2 to mediate optimal NF-κB activation in response to L. monocytogenes

Recognition of L. monocytogenes by different PRRs has been shown to lead to NF-κB–dependent proinflammatory gene expression, inflammasome activation, and caspase-1 cleavage and IFN-β responses (19). Because both NF-κB and IFN-β are major downstream signaling outputs of TLRs, we investigated activation of these in response to overexpression of TLR constructs with or without stimulation by L. monocytogenes. We overexpressed a TLR10 construct in either an NF-κB reporter cell line or in conjunction with an ISRE-luciferase reporter construct. Because TLR10 has been shown to dimerize with TLR2 (20), we overexpressed a variety of combinations of TLRs 1, 2, and 10 in conjunction with the reporter constructs. Fig. 5A shows that overexpression of TLRs 1 or 10 alone did not activate NF-κB significantly above control level. Stimulation of the overexpressed constructs with L. monocytogenes, however, results in a 2-fold level of activation above control, with TLR2 and significantly higher NF-κB activation following cotransfection of TLR2 and TLR10 (5-fold above control), which was comparable to cotransfection of TLR1 and TLR2. These data indicate that TLR10 alone is not able to respond to L. monocytogenes but instead requires TLR2. Endogenous TLR10 levels may explain the response to L. monocytogenes when TLR2 is transfected alone (see Supplemental Fig 3A). ISRE activation was also measured by a luciferase assay in response to L. monocytogenes following overexpression of combinations of TLR constructs. Unlike
NF-κB, no increase in the ability of any of the TLR combinations to drive an ISRE-luciferase construct was seen following *L. monocytogenes* stimulation (Fig. 5B).

**TLR10 is not involved in recognition of other known TLR2 ligands**

As TLR10 is most closely related to TLR1 (20), we wanted to compare TLR1- versus TLR10-mediated responses to some known TLR2 ligands. Using THP-1-derived macrophages, IL-8 production was measured in response to a range of TLR2 ligands, including two strains of intracellular bacteria, following siRNA treatment against TLR1 or TLR10 (Fig. 6). Whereas a reduction in IL-8 production was seen following both TLR1 and TLR10 siRNA treatment in response to *Salmonella* infection, it was not as significant as the reduction seen during *L. monocytogenes* infection in TLR10 siRNA-treated cells. Besides the significant reduction of IL-8 production seen in response to Pam3Cys following TLR1 siRNA treatment, no further statistically significant changes in IL-8 secretion were observed in response to the other TLR2 ligands or the positive control, LPS.

**Discussion**

TLRs have been clearly established as the major sensors of the innate immune system. Currently a total of 10 human TLRs (1–10) and 12 mouse TLRs (1–9 and 11–13) have been identified. Each TLR recognizes a limited repertoire of broadly conserved molecules of microbial origin, with ligand specificity having been elucidated for many of the TLRs thus far (4). Importantly, despite being identified >10 y ago as a member of this family, TLR10 has remained the only orphan TLR, having no known ligand (18). The absence of TLR10 in mice has, to date, precluded the identification of synthetic or natural ligands for this receptor. To our knowledge, this study is...
FIGURE 2. TLR10 silencing affects *L. monocytogenes*–induced chemokine gene expression. HT-29 cells were treated with TLR10 siRNA and silencing was confirmed by qRT-PCR (A) and Western blots (B), which contain the lysates from HT-29 cells, which were transfected as follows: untreated (lane A), TLR10-expressing plasmid (0.5 μg) (lane B), TLR10-expressing plasmid (1 μg) (lane C), TLR10-expressing plasmid (2 μg) (lane D), TLR10 siRNA (lane E), and control siRNA (lane F). Upper panel, TLR10 Ab; lower panel, β-actin Ab. The effects of siRNA for TLR1 and TLR2 on CCL20 and IL-8 induction were determined following infection with *L. monocytogenes* (C, D). Secretion of CCL20 was determined by ELISA 8, 24, and 48 h following infection in cells transfected with control or TLR10 siRNA (E). Extracellular versus intracellular expression of TLR10 in HT-29 cells was measured by flow cytometry (F). TLR10 siRNA-treated cells were infected with heat-killed *L. monocytogenes* (HKLM) or live *L. monocytogenes* (MOI of 50:1) as described previously, and the expression of the chemokines IL-8 and CCL20 were measured by qRT-PCR (G and H, respectively). Values are shown as means ± SEM; n = 3. *p < 0.05, **p < 0.01, ***p < 0.001.
The first identification of an essential role for TLR10 in mediating the inflammatory response to a specific microorganism, *L. monocytogenes*, in both intestinal epithelial cells and macrophages.

The initial aim of this study was to identify which TLRs play a key role in detection of *L. monocytogenes* in the intestinal epithelia, as most prior work has focused predominantly on systemic infections.
in doing so, we have identified TLR10 as a key mediator of the innate immune response to infection in intestinal epithelial cells. Many human microbial infections are acquired via an oral transmission route and as such the first opportunity the immune system has to encounter and counter the infection occurs in the gastrointestinal tract. Despite this, very few studies have addressed which PRRs are directly involved in detecting *L. monocytogenes* at this interface. Although TLR2 (5) and MyD88 (21) have been shown to detect *L. monocytogenes* infection in other cell types, only NOD2 has been directly shown to mediate the inflammatory response to *L. monocytogenes* in the intestine (9). Our screen investigating the role of TLRs in *L. monocytogenes* infection has yielded several interesting observations. TLR2 alone does not seem to play as critical a role in response to infection in epithelia as has been observed in immune cell types as shown by the slight but not significant reduction in chemokine expression following knockdown of TLR2. Knockdown of TLR1 seems to have a more significant effect on IL-8 production than does knockdown of TLR2, and finally knockdown of TLR10 has the greatest effect on the ability of these cells to mount an immune response to *L. monocytogenes*.

In this study, we have reported TLR10 to play a functional role in intestinal epithelial cells. In humans, TLR10 expression has been reported to be somewhat restricted. It was initially characterized to be primarily expressed on immune cell subtypes, with a predominance found in spleen, lymph nodes, thymus, and tonsils (18). Note that in the study by Chuang and Ulevitch (18) expression of TLR10 in colon tissue was not investigated. Within immune cell populations the highest levels of TLR10 expression have been reported on T regulatory cells (22), B lymphocytes (23), and plasmacytoid DCs (24), with expression also reported on THP-1 monocytes (25). The initial siRNA screen performed in this study, which identified this novel role for TLR10, was performed on HT-29 intestinal epithelial cells, not on an immune cell type. Expression of TLR10 mRNA has previously been reported in the human SW480 intestinal
epithelial cell line (26). In previous studies, we have detected TLR10 mRNA in human colon biopsy samples, which comprise ~90% intestinal epithelial cells (27). Because we have coexposed expression of TLR10 in two intestinal epithelial cell lines, HT-29 and HCA-7, by both mRNA and protein, our data indicate a previously unappreciated role for TLR10 in nonimmune cell types in the gut. Moreover, we have confirmed our findings in the macrophage cell line THP-1, indicating that the role of TRL10 in the detection and immune response to L. monocytogenes is not limited to intestinal epithelial cells.

We have investigated signaling from TLR10 in response to L. monocytogenes and have shown that TLR10 alone is not sufficient to mediate L. monocytogenes–induced NF-κB activation but that it requires TLR2 to be present. We have also demonstrated coexpression TLR2 and TLR10 drives NF-κB activation significantly more than does TLR2 alone. However, a caveat to this observation is that endogenous TLR10 levels may be present at low levels in HEK cells. Many studies involving TLR2 have shown that it can combine with either TLR1 or TLR6 and that this interaction is essential for effective ligand binding by TLR2 and also for discrimination of triacycl and diacyl lipopeptides from bacteria (4). Phylogenetic analysis has shown that TLR10 is most closely related to TLR1 and TLR6 (18). The level of sequence homology among these three suggests the idea of a common TLR1/6/10 ancestor that evolved to elaborate this question.

Although we have not yet fully investigated the role of TLR10 in detection of other pathogens, it seems unlikely that it will be limited to L. monocytogenes detection. In a screen of TLR2 ligands to compare TLR1–against TLR10-induced IL-8 production, we have shown that TLR10 is also involved in detection of the intracellular pathogen Salmonella typhimurium. Indeed, the intracellular role of TLR10 detection in epithelia appears to be crucial, as heat-killed L. monocytogenes did not result in TLR10 activation and subsequent cytokine expression. Further work is required to identify other pathogens detected by TLR10.

We have also shown that depletion of TLR10 increases Listeria survival in both HT-29 epithelial and THP-1 macrophage cells. Previous studies have linked TLR10 to ROS activation in THP-1 cells (25), and, together with our data, suggests that TLR10 may play a role in ROS-mediated clearance of Listeria. How it acts to reduce bacterial survivability in intestinal epithelial cells such as HT-29 cells is unclear, although reduced expression of antimicrobial factors such as CCL20 in the absence of TLR10 signaling may be a key factor. Indeed, observations by Yang et al. (30) and Starner et al. (31) have demonstrated the antimicrobial effect of chemokines and cytokines; however, specific mutagenesis studies of both TLR10 and bacterial strains may also further elaborate this question.

The role of TLRs in defense against many pathogenic infections in the intestine remains unclear. Whereas the primary role of TLRs in the intestine is the same as in other tissues (i.e., defense against infection), other homeostatic functions have been described. TLR2, for example, is found to be expressed on both apical and basolateral surfaces of intestinal epithelial cells, and activation of TLR2 from the apical surface has been reported to result in cell homeostasis, not inflammation (32). Similarly, MyD88 has been shown to have a homeostatic role in the epithelium, as mice lacking MyD88 are hyperresponsive to dextran sulfate sodium–induced colitis (33). Conversely, however, another recent study has shown an essential role for MyD88 in reducing the bacterial lectin RegIIIγ in response to L. monocytogenes infection in epithelial cells (34). Indeed, a recent study has found that the circadian rhythm of intestinal epithelial cells governs the signaling of TLR-mediated homeostasis (35). Our data showing TLR10 as the dominant TLR involved in mediating the immune response to L. monocytogenes in the intestine open up intriguing possibilities concerning the balance of proinflammatory versus homeostatic TLR responses to infection in epithelial cells. It is possible that a TLR2/10 dimer may be proinflammatory in epithelial responses whereas TLR2 on its own or in

FIGURE 6. Effects of TLR1 versus TLR10 siRNA treatment on IL-8 production in THP-1 macrophages following 24 h stimulation with TLR2 ligands. THP-1 cells were treated with nontargeting control, TLR1, or TLR10 siRNA with 5 ng/ml PMA for 48 h. The cells were then subject to stimulation with the ligands indicated. Bacterial infections were performed as described in Materials and Methods. Twenty-four hours after stimulation, supernatants were analyzed by ELISA for IL-8 production. *p < 0.05, **p < 0.01.
combination with TLRs 1 and 6 may mediate a more homeostatic effect.

Taken together, our results show that L. monocytogenes–infected intestinal epithelial cells produce chemokines in a TLR10-dependent manner. Whereas mucosal and systemic immunity to pathogenic infection can be very distinct, our finding of a role for TLR10 in the inflammatory response in macrophages as well as IECs indicates a general role for TLR10 in response to L. monocytogenes infection. Although the precise ligand remains to be identified, to our knowledge this study shows the first definitive role for TLR10 in pathogen detection.

Disclosures
The authors have no financial conflicts of interest.

References
SUPPLEMENTAL FIGURE 1. EGR-1 induction in HT-29 epithelial cells in response to *L. monocytogenes* following silencing of PRR expression. Cells were treated with siRNA to PRRs as indicated for TLRs, NLRs or CLRs. Following siRNA transfection, cells were then infected with *L. monocytogenes* (MOI 50:1) for 3 hours and gene expression of EGR1 (A) or IL-8 (B) was determined by qRT-PCR. * P<0.05, ** P<0.01 and *** P<0.001, Values are shown as Mean ± SEM, n=3.

SUPPLEMENTAL FIGURE 2. Specificity of silencing of siRNA used against TLR1, TLR2 and TLR10. THP-1 cells were treated with control (scrambled) siRNA and TLR1 siRNA (A), TLR2 siRNA (B) or TLR10 siRNA (C) with 5ng/ml PMA for 48 hours. Flow cytometry was used as described in the methods to monitor expression levels of TLR1, TLR2 and TLR10. Gating was performed against the isotype control and all control siRNA samples were measured against the corresponding TLR siRNA at the time of analysis to avoid intra-assay variation in staining efficiency. Representative scatter plots of flow data are shown with relative knockdown (KD) of target TLR.

SUPPLEMENTAL FIGURE 3. Western Blot of TLR10 expression levels in HEK and immune response to *L. monocytogenes* in IEC. Lysates of HEK cells were prepared for Western blotting analysis 48 hours after transfection as described in the methods section. TLR10 detection was confirmed using transfected TLR10 as a positive control. β-actin expression was also examined to ensure equal levels of protein were loaded on the gel (A). HT-29 cells were infected with *L. monocytogenes*, (MOI 50:1), for 3 hours. qRT-PCR was used to measure induction of the cytokine and chemokines indicated (B). Cells were treated with control siRNA or TLR10 siRNA as indicated and infected with *L. monocytogenes* (MOI 50:1) for 3 hours. Gene expression of CCL-1 was determined by qRT-PCR in HT-29 (C) or HCA-7 cells (D). * P<0.05, ** P<0.01 and *** P<0.001, Values are shown as Mean ± SEM, n=3.
A  TLR1 siRNA
Control siRNA
* ~85% KD

Target Controls

B  TLR2 siRNA
Control siRNA
* ~33% KD

C  TLR10 siRNA
Control siRNA
* ~52% KD

A: HEK 293 T

- Untreated
- Transfection Reagent only
- Empty Vector
- TLR10 (0.5 μg)
- TLR10 (2 μg)
- TLR10 (3 μg)

B: HT-29

Relative Expression (ΔCt) for different genes:
- Caspase1
- IL-1β
- IL-8
- IL-6
- IL-23
- CCL-1
- CCL-20
- EGR-1

C: HT-29

Relative Expression (ΔCt) for CCL-1:
- Control siRNA
- TLR10 siRNA

D: HCA-7

Relative Expression (ΔCt) for CCL-1:
- Control siRNA
- TLR10 siRNA

Gene expression levels are compared under different conditions, including control and TLR10 siRNA treatments.
**Table 1A**

Sequences of siRNA pools used:

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**Table 1B**

qRT-PCR primers and corresponding Universal Probe Library (UPL) probe numbers

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