Multiple TLRs Are Expressed in Human Cholangiocytes and Mediate Host Epithelial Defense Responses to Cryptosporidium parvum via Activation of NF-κB

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Multiple TLRs Are Expressed in Human Cholangiocytes and Mediate Host Epithelial Defense Responses to Cryptosporidium parvum via Activation of NF-κB

Xian-Ming Chen,* Steven P. O’Hara,* Jeremy B. Nelson,* Patrick L. Splinter,* Aaron J. Small,* Pamela S. Tietz,* Andrew H. Limper,† and Nicholas F. LaRusso‡*

Infection of epithelial cells by Cryptosporidium parvum triggers a variety of host-cell innate and adaptive immune responses including release of cytokines/chemokines and up-regulation of antimicrobial peptides. The mechanisms that trigger these host-cell responses are unclear. Thus, we evaluated the role of TLRs in host-cell responses during C. parvum infection of cultured human biliary epithelia (i.e., cholangiocytes). We found that normal human cholangiocytes express all known TLRs. C. parvum infection of cultured cholangiocytes induces the selective recruitment of TLR2 and TLR4 to the infection sites. Activation of several downstream effectors of TLRs including IL-1R-associated kinase, p-38, and NF-κB of cultured cholangiocytes induces the selective recruitment of TLR2 and TLR4 to the infection sites. Activation of several downstream effectors of TLRs including IL-1R-associated kinase, p-38, and NF-κB was detected in infected cells. Transfection of cholangiocytes with dominant-negative mutants of TLR2 and TLR4, as well as the adaptor molecule myeloid differentiation protein 88 (MyD88), inhibited C. parvum-induced activation of IL-1R-associated kinase, p-38, and NF-κB. Short-interfering RNA to TLR2, TLR4, and MyD88 also blocked C. parvum-induced NF-κB activation. Moreover, C. parvum selectively up-regulated human β-defensin-2 in directly infected cells, and inhibition of TLR2 and TLR4 signals or NF-κB activation were each associated with a reduction of C. parvum-induced human β-defensin-2 expression. A significantly higher number of parasites were detected in cells transfected with a MyD88 dominant-negative mutant than in the control cells at 48–96 h after initial exposure to parasites, suggesting MyD88-deficient cells were more susceptible to infection. These findings demonstrate that cholangiocytes express a variety of TLRs, and suggest that TLR2 and TLR4 mediate cholangiocyte defense responses to C. parvum via activation of NF-κB. The Journal of Immunology, 2005, 175: 7447–7456.

Cryptosporidium parvum, an intracellular parasite within the protist phylum Apicomplexa, is one of the most commonly reported enteric pathogens in both immunocompetent and immunocompromised individuals worldwide (1). The parasite infects gastrointestinal epithelia to produce diarrhea that is self-limited in immunocompetent subjects but potentially life-threatening in immunocompromised individuals, primarily those with AIDS (2). Infection by this parasite accounts for ~6% of all diarrheal disease in immunocompromised individuals and occurs in ~24% of AIDS patients with diarrhea worldwide (3). C. parvum is also the single most common identifiable pathogen in the biliary tract in patients with AIDS-cholangiopathy, an important biliary disorder caused by opportunistic infection of the biliary epithelium and resulting in significant morbidity and mortality in AIDS patients (4). Despite the magnitude and severity of C. parvum-induced infection, the pathogenesis is poorly understood, and there is currently no fully effective therapy (5–7).

The proportion of exposed individuals developing cryptosporidiosis reflects both parasite infectivity and host immune responses. Whereas both innate and adaptive immunity are involved in the resolution of cryptosporidiosis and resistance to infection (8), the mechanisms by which host epithelial cells elicit immune responses are not well understood. Specific attachment to the apical epithelial cell surface by C. parvum sporozoites, as well as parasite molecules inserted into epithelia after its attachment (9, 10), appear to activate host-cell secondary signal pathways and thereby alter cell function (10–12). The invasion of epithelial cells in vitro by C. parvum results in the rapid expression of anti-microbial peptides (e.g., β-defensins) and the inflammatory chemokines including IL-8, TNF-α, and prostaglandin E2, etc. (12–14). We previously reported that one intracellular signal pathway activated by C. parvum and associated with the cytokine/chemokine release in infected biliary epithelial cells (i.e., cholangiocytes) is the NF-κB signal pathway (12). Moreover, we showed that activation of NF-κB accounts for the antiapoptotic activation in C. parvum directly infected cholangiocytes and thus benefits parasite propagation (12). How C. parvum activates such host-cell intracellular signaling pathways such as NF-κB and mediates host-cell inflammatory and immune responses is still unclear.

TLRs are an evolutionarily conserved family of cell surface molecules, which are key to innate immunity by detecting invading pathogens (15). Most of the known TLRs, upon recognition of discrete pathogen-associated molecular patterns, activate a common set of adaptor proteins, such as myeloid differentiation protein 88 (MyD88), and protein kinases including IL-1 receptor-associated kinase (IRAK).3 IRAK, in turn, activates downstream effectors including p-38, ERK1/2, JNK, and IκB kinases (16). Activation of the kinases leads to the nuclear translocation of

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3 Abbreviations used in this paper: IRAK, IL-1R-associated kinase; HBD, human β-defensin; siRNA, short-interfering RNA; DN, dominant negative; DAPI, 4,6-diamidino-2-phenylindole; MBP, myelin basic protein.
corresponding nuclear transcription factors, such as AP-1 and NF-κB, and thus regulates host-cell responses to pathogens (15, 16). One important host epithelial defense response upon microbial infection is the production of a variety of small cationic antimicrobial peptides including β-defensins (17). Six human β-defensins (HBDS) have been identified and cloned in human epithelial cells. HBD-2 and -4 are identical and HBD-5 and -6 are primarily expressed in the epididymis (17, 18). The induction of HBD-2 transcription by IL-1 is dependent on a specific NF-κB binding site (~205 to −186) and its interaction with p-65-p50 NF-κB heterodimer (17, 19, 20). Whereas regulation of HBD expression has been well documented in epithelial cells upon microbial infection (17, 21−22), the role of TLRs in this process has not been fully explored. Moreover, expression and functions of TLRs in the liver, particularly in cholangiocytes, which are the target epithelial cells of a group of clinically important diseases, some of which are of infectious nature, have not been characterized.

In the present study, we explored the role of TLR signals in cholangiocyte responses to C. parvum infection. We found that normal human cholangiocytes express all known TLRs. Whereas C. parvum attachment to and invasion of cultured cholangiocytes appear not to be dependent on host-cell TLRs, C. parvum infection of cholangiocytes induces the recruitment of TLR2 and TLR4 to the infection sites, resulting in activation of IRAK and phosphorylation of p-38. Moreover, activation of NF-κB by C. parvum in directly infected cells is dependent on both TLR2 and TLR4 signals. In addition, a TLR2- and TLR4-dependent and NF-κB-associated induction of HBD-2, but not HBD-1 and HBD-3, occurs in cells infected with C. parvum, whereas MyD88-deficient cells are more susceptible to C. parvum infection in vitro. These findings demonstrate that cholangiocytes express a variety of TLRs, and TLR2 and TLR4 signals mediate cholangiocyte responses to C. parvum via activation of NF-κB, implicating an important role of TLRs in cholangiocyte defense responses to microbial infection in the biliary tree.

Materials and Methods

C. parvum and H69 cells

C. parvum oocysts of the Iowa strain were purchased from a commercial source (Bunch Grass Farms). Before infecting cells, oocysts were excysted to release infective sporozoites as described previously (23). H69 cells (a gift from Dr. D. Jefferson, Tufts University, Boston, MA) are SV40-transformed human bile duct epithelial cells originally derived from a normal liver harvested for transplant and have been extensively characterized (24). For experiments, H69 cells were used between passage 23 and 30 and maintained for three passages without coculture cells to ensure that the culture was free of 3T3 fibroblasts.

In vitro models and infection assay

Infection with C. parvum was done in a culture medium consisting of DMEM-F12, 10 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen Life Technologies), and freshly excysted C. parvum sporozoites (1 × 10^6 sporozoites/per slide well or culture plate). Inactivated organisms (treated at 65°C for 30 min) were used for sham infection controls. An attachment model and an attachment/invasion model were used to assay the attachment and invasion of C. parvum as described previously (23). Infection assays (attachment rate or attachment/invasion rate) were conducted after incubation for 2 h with the parasite using an indirect immunofluorescent technique as described previously (23). For the inhibitory experiments, two selective inhibitors of NF-κB, MG-132 and SN50 (25), were added in the medium at the same time as C. parvum. A concentration of 1 µM MG-132 or 50 µg/ml SN50, which showed no cytotoxic effects on H69 cells or on C. parvum sporozoites, was selected for the study. For the experiments to test whether TLRs and TLR-associated signals are involved in cholangiocyte defense responses against C. parvum infection, cells in T25 flasks were incubated with an equal number of C. parvum sporozoites (5 × 10^6) for 2 h, washed with culture medium to remove nonattached and noninternalized parasites, and were then further cultured for up to 96 h, followed by PCR analysis for C. parvum.

RT-PCR

Total cellular RNA was extracted from the cells using Tri-Reagent (Sigma-Aldrich). Total RNA (5 µg) was reverse transcribed to cDNA by using a Moloney Murine Leukemia Virus Reverse Transcriptase Kit (Invitrogen Life Technologies). After reverse transcription, cDNA was amplified using PCR with gene-specific primers designed to amplify a portion of the coding sequences (Table I). The PCR consisted of one cycle of 10-min denaturation at 94°C; 18−35 cycles of 1 min at 94°C, 1 min at 48−55°C, and 1 min at 72°C, and a final extension step at 72°C for 10 min. RNA (18s) was also amplified to confirm that an equal amount of total cDNA was used for each sample, and all experiments were done in duplicate. Template cDNA prepared from TIB-202, a normal human monocyte cell line that expresses all known TLRs except TLR3 (15, 26), was used as positive control for TLR mRNA detection in H69 cells. Sequencing was performed on all positive PCR products (Mayo Molecular Core Facility, Rochester, MN) to confirm the identity of amplified genes. To quantitatively measure C. parvum infection in H69 cells, a quantitative RT-PCR approach using a LightCycler (Roche Diagnostic Systems) was established by modification of a previous report (27). Briefly, total RNA was harvested from the cells after exposure to C. parvum and reverse transcribed to cDNA and amplified using AmpliTag Gold PCR Master Mix (Roche Diagnostic Systems). Primers specific for C. parvum 18s ribosomal RNA (Table I) were used to amplify the cDNA specific to the parasite. Primers specific for human plus C. parvum

Table I. Primer sequences

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<tr>
<th>Target mRNA</th>
<th>Forward</th>
<th>Reverse</th>
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<td>5′-GAGGAAATGATGACGACCTTAC-3′</td>
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<tr>
<td>TLR2</td>
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<td>5′-GGACCTGCTCACTTGTTACC-3′</td>
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<tr>
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<td>5′-TTTCGACCAAGATCGACATG-3′</td>
<td>5′-TTTCCAGGACGGCTGCTAATG-3′</td>
</tr>
<tr>
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<td>5′-GCCATGTTAAAGCAACTCTTG-3′</td>
</tr>
<tr>
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<td>5′-TGAGATACCGGGGAGATC-3′</td>
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<td>5′-GAGATACAGGGGAGATC-3′</td>
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<td>5′-AAGGTCGAACGACGACGTT-3′</td>
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<td>5′-GCTACACCTGAGCACCTTGGGCC-3′</td>
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<tr>
<td>HBD-2</td>
<td>5′-GACTGCACTCTGGTACGATC-3′</td>
<td>5′-GAGGCAACAGTGCCAAATTG-3′</td>
</tr>
<tr>
<td>HBD-3</td>
<td>5′-CCAGGTTGAGGCTAGATC-3′</td>
<td>5′-TCACGTTGCTCGAGGACATC-3′</td>
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<tr>
<td>C. parvum –18s</td>
<td>5′-TCTGTAAGAGGAGGAGGGAAATC-3′</td>
<td>5′-CTCCACAACTTGAAGACGGGCC-3′</td>
</tr>
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H69 cells stably transfected with constructs of TLR2-DN (dominant negative), TLR4-DN, and MyD88-DN were obtained by transfecting cells with the constructs followed by antibiotic selection. Cells were plated at 50–80% confluency and transfected with the plasmid DNA using the Lipofectamine Plus reagent kit (Invitrogen Life Technologies) according to the manufacturer’s instructions. TLR2-DN and TLR4-DN are kinase-inactive (DN) mutants of TLR2 and TLR4, respectively, and were kindly provided by Dr. M. F. Smith (University of Virginia, Charlottesville, VA). MyD88-DN is a DN mutant of MyD88 and was a gift from Prof. J. Tsopp (University of Lausanne, Lausanne, Switzerland). Stable-transfected clones were selected by adding Zeocin (600 μg/ml) (Invitrogen Life Technologies) to the culture medium. Selected transfected cells were cultured with Zeocin in the medium and then grown on 4-well chamber slides or T25 flasks for the experiments.

Specific siRNAs to TLR2, TLR4, and MyD88, which target human TLR2, TLR4, and MyD88 mRNA sequences were designed. For siRNA transfection, H69 cells were grown to 40–60% confluency on 10-mm dishes and were transfected with the siRNA using the siPORT lipid transfection agent (Ambion). siRNAs that showed the most inhibition of TLR2, TLR4, or MyD88 expression were selected with approaches we previously reported (28) and used in this study as follows: TLR2, AAATATCGTGTGCTGGATAACCCGATTC (sense) and AAATATCGTGTGCTGGATAACCCGATTC (antisense); TLR4, AACTGCGTGCCTCAAGATATACCC (sense) and AACTGCGTGCCTCAAGATATACCC (antisense); and MyD88, AACTTATCCAGCTCCTGTCTC (sense) and AACTTATCCAGCTCCTGTCTC (antisense). These siRNA oligonucleotides were determined to have no significant overlap with homologous gene sequences. Nonspecific siRNAs containing the same nucleotides but in irregular sequence (i.e., scrambled siRNAs) were used as the controls. The siRNAs were further labeled with Cy3 using the Silencer siRNA labeling kit (Ambion) for the identification of transfected cells by confocal microscopy.

Immunohistochemistry and immunofluorescent microscopy

For immunohistochemistry, paraffin-embedded normal human liver tissue samples (Institutional Review Board no. 725-00) were used for TLRs immunohistochemistry. Tissue sections of 5 μm were deparaffinized in xylen and rehydrated in ethanol followed by water and PBS. Endogenous peroxidase was blocked by immersion in 3% hydrogen peroxide. The tissue sections were then incubated with TLR Abs at a concentration of 5 μg/ml for 1 h at room temperature. Seven TLR Abs currently available and specific for TLR2–9 proteins were selected and used for immunohistochemistry (Imgenex, CA). The sections were incubated with PBS containing normal goat serum without a primary Ab. Immunostaining was then detected with the Vectastain peroxidase kit (Vector Laboratories) and developed with diaminobenzidine tetrahydrochloride. The sections were counterstained with hematoxylin followed by light microscopy.

For immunofluorescent microscopy, H69 cells were exposed to C. parvum sporozoites as described above. After 2 h of incubation, cells were fixed (0.1 mol/L, 1:4-piperazinediethanesulfonic acid (pH 6.95), 1 mmol/L EGTA, 3 mmol/L magnesium sulfate (Sigma-Aldrich), and 2% paraformaldehyde) at 37°C for 20 min and then permeabilized with 0.2% (v/v) Triton X-100 in PBS. For double-immunofluorescent labeling, fixed cells were incubated with TLR Abs at a concentration of 5 μg/ml for an additional 2 h at 4°C. The beads were then washed once with lysis buffer and twice with kinase inhibitor buffer (10 mM HEPES (pH 7.6), 20 mM MgCl₂, 20 mM β-glycerophosphate, 20 mM p-nitrophenyl phosphate, 1 mM EDTA, and 1 mM benzamidine). The beads were incubated for 30 min at 30°C in a final volume of 20 μl in the presence of 2 μg of myelin basic protein (MBP) (Sigma-Aldrich), 100 μM ATP, and 5 μCi of [γ³²P]ATP (DuPont NEN Research Products). SDS sample buffer was added to protein A beads and boiled for 5 min, and then subjected to SDS-PAGE analysis. The gels were dried, and the intensity of the radioactive signal was quantified using the Molecular Analyst software (Bio-Rad).

Western blotting

H69 cells were grown in T25 flasks to 95% confluence and exposed to C. parvum sporozoites. Cells were then lysed with the M-PER mammalian protein extraction reagent (Pierce), and protein concentrations were determined using Bradford reagent according to the instructions of the supplier (Sigma-Aldrich). Twenty micrograms of lysate protein per lane were separated on SDS-PAGE under reducing conditions, and then transferred onto nitrocellulose membranes. Membranes were incubated with the primary Abs to TLR2 and TLR4 (Imgenex, MyD88 (Imgenex), p-p-38 (Cell Signaling Technology), p-ERK1/2 (Santa Cruz Biotechnology), β-2-Microglobulin (Bio-Rad), and then with 0.2 μg/ml HRP-conjugated secondary Ab, and revealed with ECL light substrate (ECL, Amersham Biosciences).

ELISA

To analyze the production of NF-κB-associated proinflammatory cytokines by H69 cells in response to C. parvum infection, H69 cells were grown to subconfluence in 4-well chamber slides. After incubation with assay medium containing freshly excreted sporozoites and various specific inhibitors for an additional 24 h at 37°C, supernatants were collected and analyzed for protein concentrations by Bradford reagent using the instructions of the supplier. Supernatants were then subjected to SDS-PAGE analysis. Immunoreactive bands were transferred to nitrocellulose membrane and detected using the Molecular Analyst software (Bio-Rad).

Immunoprecipitation and activity of IRAK

H69 cells were grown in 10-cm dishes to 95% confluence and exposed to C. parvum sporozoites at 37°C for 2 h. Cells were then rinsed with PBS and scraped into 1 ml of cold lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, and 0.1% SDS) supplemented with 1 mM PMSF, leupeptin and pepstatin at 20 μg/ml, and tyrocidine phosphatase inhibitors, sodium orthovanadate and sodium fluoride at 1 mM. The cell lysates were centrifuged at 13,000 × g for 10 min and were assayed for protein concentration using the Bradford reagent (Sigma-Aldrich). The cell lysates (1 mg of protein) were then incubated with 1 μg of anti-IRAK (Upstate Biotechnology) for 2 h at 4°C. Fifty microliters of 10% protein A-Sepharose (Sigma-Aldrich) was then added and incubated for an additional 2 h at 4°C. The beads were then washed once with lysis buffer and twice with kinase buffer (10 mM HEPES (pH 7.6), 20 mM MgCl₂, 20 mM β-glycerophosphate, 20 mM p-nitrophenyl phosphate, 1 mM EDTA, and 1 mM benzamidine). The beads were incubated for 30 min at 30°C in a final volume of 20 μl in the presence of 2 μg of myelin basic protein (MBP) (Sigma-Aldrich), 100 μM ATP, and 5 μCi of [γ³²P]ATP (DuPont NEN Research Products). SDS sample buffer was added to protein A beads and boiled for 5 min, and then subjected to SDS-PAGE analysis. The gels were dried, and the intensity of the radioactive signal was quantified using the Molecular Analyst software (Bio-Rad).

Statistical analysis

All values are given as mean ± SE. Means of groups were compared with the Student’s t test (unpaired) or ANOVA test when appropriate. p values <0.05 were considered statistically significant.

Results

Normal human cholangiocytes express multiple TLRs and accessory molecules

mRNA expression of TLRs and accessory molecules MD-2 and MyD88 was assessed by RT-PCR in a SV40-transformed normal human cholangiocyte cell line, H69. TIB-202 cells, a human monocyte cell line that expresses all known TLRs except TLR3 (25, 26), was used as the positive control. TLR1–10 mRNA was detected in H69 cells (Fig. 1, A and B). Consistent with previous reports, mRNA of all known TLRs except TLR3 was confirmed in TIB-202 cells (Fig. 1, A and B). Both MD-2 and MyD88 mRNA were also detected in H69 cells (Fig. 1B). Specificity of the PCR products was confirmed by sequencing. Expression of selected TLRs (i.e., TLR2 and TLR4) and MyD88 protein in H69 cells was also detected by Western blotting (Fig. 1C).

To further test expression of TLRs at the protein level in human cholangiocytes, we assessed TLR protein expression in cholangiocytes in normal human liver tissues with Abs currently available to TLR2–9 by immunohistochemistry. Although the negative controls, stained with normal goat serum and the secondary Ab, showed no specific staining in cholangiocytes (Fig. 2A), strong expression of TLR2–9 proteins was detected in cholangiocytes...
To test whether C. parvum recruits cholangiocyte TLR2 and TLR4, but not other TLRs, to the attachment sites in vitro

Previous studies by us and others (9, 10, 29, 30) demonstrated that TLRs, to the attachment sites in vitro, are involved in the infection process of C. parvum infection of cholangiocytes in vitro is not TLR2- and TLR4-dependent.

Expression of TLR2-9 proteins in normal human liver tissues. Expression of TLR2-9 proteins in normal human liver tissue sections was assessed by immunohistochemistry followed by light microscopy. Although the negative controls, stained with normal goat serum and the secondary Ab, showed no specific staining in cholangiocytes along the biliary duct (A), strong expression of TLR2-9 proteins was detected in cholangiocytes (B–I). Insets in B–I are high magnification of the boxed region in B–I. Arrowheads indicate staining at the apical surface. Bar, 10 μm.

Activation of downstream effectors of TLR2 and TLR4 signal pathways

To determine whether TLR signal pathways are activated during C. parvum infection of cholangiocytes, we measured the activation of various components of downstream effectors of TLRs. A significant increase of IRAK activity, an immediate downstream effector for both TLR2 and TLR4 (16), was detected in cholangiocytes upon C. parvum infection as seen by phosphorylation of MBP (31). A significant inhibition of IRAK activity was found in cells transfected with TLR2-DN, TLR4-DN, or MyD88-DN by quantitation of 32P labeling of the substrate MBP after SDS-PAGE (Fig. 5A). To further test which downstream effectors of IRAK are activated, cholangiocytes were exposed to C. parvum followed by Western blotting using Abs to the activated phosphorylated forms of various IRAK downstream kinases (16). A significant increase of phosphorylation of p-38, but not JNK and ERK1/2, was detected by immunoblotting using specific Abs against those phosphorylated kinases (Fig. 5, B and C). A significant inhibition of p-38 phosphorylation was found in cells transfected with TLR2-DN, TLR4-DN, or MyD88-DN (Fig. 5, B and C).

Activated IRAK can further activate the NF-κB pathway (16). When cholangiocytes were exposed to C. parvum for 2 h, a significant decrease of IκBα protein was detected (Fig. 6A). In contrast, a partial but significant inhibition of IκBα decrease was found in cells transfected with TLR2-DN or TLR4-DN. A complete blockage of IκBα decrease upon C. parvum infection was
found in MyD88-DN-transfected cells (Fig. 6A). To further test the nuclear translocation of NF-κB in cells upon *C. parvum* infection, we used an Ab to p-65, a component of the NF-κB complex that displays nuclear translocation in directly infected cholangiocytes upon *C. parvum* infection (12), to measure NF-κB activation in *C. parvum*-infected cells by confocal immunofluorescent microscopy. Consistent with results of our previous studies, nuclear translocation of p-65 was found in directly infected cells, not in bystander noninfected cells (Fig. 6B). No nuclear translocation of p-65 was found in both directly infected and bystander noninfected cells in the TLR2-DN-, TLR4-DN-, and MyD88-DN-transfected cells (Fig. 6, C–E). Whereas scrambled siRNAs had no effect on *C. parvum*-induced NF-κB translocation (Fig. 6, F and G), no nuclear translocation of p-65 was found in infected cells transfected with siRNAs to TLR4 (Fig. 6, H and I), TLR2, and MyD88 (data not shown). In contrast, a control experiment using 100 ng/ml *Escherichia coli* LPS, a well-known ligand for TLR4, showed nuclear translocation of p-65 in most treated nontransfected and empty vector-transfected cells, but not in TLR2-DN- and MyD88-DN-transfected cells (data not shown), confirming the existence of the TLR4/MyD88/NF-κB pathway in H69 cells. Equally important, these results exclude LPS contamination in our

*FIGURE 3.* Accumulation of cholangiocyte TLR2 and TLR4, but not other TLRs, at the attachment sites during *C. parvum* infection. H69 cells were exposed to *C. parvum* for 2 h followed by immunofluorescent microscopy. A1–D3, Representative confocal micrographs by dual labeling of *C. parvum* (in green, left panel), TLRs (in red, middle panel), and the merged images (right panel). Accumulation of TLR2 (arrowhead in A2) and TLR4 (arrowhead in B2) was found at the host-cell-parasite interface. No obvious accumulation of TLR5 (arrowhead in C2), TLR9 (arrowhead in D2), and other TLRs (data not shown) was observed at the infection sites. E, Quantitative analysis of accumulation of TLR2 and TLR4 at the infection sites. No significant difference was found in the accumulation of TLR2 and TLR4 at the infection sites between cells transfected with DN mutants of TLR2 or TLR4 and cells transfected with empty vector controls. Bar, 2 μm.

C. *parvum* sporozoite preparation because NF-κB activation by *C. parvum* is limited only to directly infected cells, whereas in the control experiment, LPS activated NF-κB in virtually all cells. Taken together, the data suggest that various downstream effectors of TLR2 and TLR4 and associated intracellular signals such as NF-κB are activated in cholangiocytes upon *C. parvum* infection.

*FIGURE 4.* *C. parvum* attachment to and invasion of human cholangiocytes in culture is not dependent upon host-cell TLR2 and TLR4. H69 cells or cells stably transfected with functional inhibitory DN mutants of TLR2, TLR4, or MyD88 were exposed to *C. parvum* for 2 h followed by immunofluorescent confocal microscopy. A, Attachment assay in prefixed cells shows no significant difference of *C. parvum* attachment in all conditions. B, Attachment/invasion assay in nonfixed cells after exposure to *C. parvum* sporozoites. No significant change in attachment/invasion rate was found between nontransfected and transfected cells. Ctrl, control.

To further confirm the role of TLR2 and TLR4 in *C. parvum*-induced activation of the NF-κB pathway, expression and secretion of IL-8, a well-known gene product of NF-κB-dependent transcription (32), was assessed in cholangiocytes transfected with DN of TLR2, TLR4, and MyD88 in response to *C. parvum* infection. Cells were incubated with *C. parvum* for 12 h, and expression of IL-8 mRNA was measured by RT-PCR. An increase of IL-8 mRNA expression was detected in H69 cells after exposure to *C. parvum*. *C. parvum*-induced IL-8 mRNA expression was significantly reduced in cells transfected with TLR2-DN and TLR4-DN (Fig. 7A), revealing only a slight increase over the sham-infected control H69 cells, whereas IL-8 mRNA expression in response to *C. parvum* was completely inhibited in MyD88-DN-transfected cells (Fig. 7A). Inhibition of NF-κB by two selective functional inhibitors, SN50 and MG-132 (25), also diminished *C. parvum*-induced IL-8 mRNA expression in cholangiocytes. Consequently, when cells were incubated with *C. parvum* for 12 h, a significant increase of IL-8 secretion was found in the supernatants from cells incubated with *C. parvum* as measured by ELISA.
A significant decrease of IL-8 release was detected in the supernatants from cells transfected with functional inhibitory DN mutants of TLR2, TLR4, or MyD88 or cells treated with NF-κB inhibitors (Fig. 7B).

To test the role of TLRs in C. parvum-induced HBD production, expression of HBD-1–3 was measured in cells after exposure to C. parvum for 12 h. A constitutive expression of HBD-1 and HBD-3 mRNA was found in the sham-infected, C. parvum-infected nontransfected cells, cells treated with NF-κB inhibitors, and cells transfected with TLR2-DN, TLR4-DN, and MyD88-DN (Fig. 8A). In contrast, a low expression of HBD-2 was found in the sham-infected cells, and a significant increase of HBD-2 mRNA was

FIGURE 5. C. parvum activates TLR2- and TLR4-associated signals in human cholangiocytes in culture. H69 cells or cells stably transfected with functional inhibitory DN mutants of TLR2, TLR4, or MyD88 were exposed to C. parvum for 2 h. A, Activation of IRAK as measured by immunoprecipitation followed by incubation with [γ-32P]ATP using MBP as a substrate. IRAK was also blotted by Western blot to confirm an equal amount of total IRAK used for the assay. The intensity of the radioactive signals was quantified and expressed as densitometric arbitrary values, representative of three distinct experiments. B, Phosphorylation of p-38, JNK, and ERK1/2 as assessed by immunoblotting using specific Abs. To confirm equal loading, membranes were reprobed with an actin Ab. C, Quantitative analysis of phosphorylation of p-38, JNK, and ERK1/2. The intensity of the signals was quantified using a PhosphorImager and expressed as densitometric arbitrary values. Data are representative of three distinct experiments. * p < 0.05, compared with sham-infection control; #, p < 0.05, compared C. parvum-infected cells.

FIGURE 6. C. parvum activates the NF-κB pathway in directly infected cholangiocytes via TLR2 and TLR4 signals. A, Degradation of IκBα as assessed by quantitative Western blot. H69 cells were exposed to C. parvum for 2 h, and IκBα in the cell lysates was determined by Western blot using an Ab against IκBα. Actin was also blotted to confirm an equal loading. The intensity of the signals was quantified using a PhosphorImager and expressed as densitometric arbitrary values. Data are representative of three distinct experiments. B–F and H, Nuclear translocation of p-65 of the NF-κB complex. Cells were exposed to C. parvum for 2 h followed by immunofluorescent staining using a mAb against p-65 and a polyclonal Ab to C. parvum. Nuclear translocation of p-65 was found in directly infected cells (p-65 in green by arrowheads in B), but not in bystander noninfected cells. No nuclear translocation of p-65 was found in both directly infected (asterisks in C–E) and nondirectly infected cells in the TLR2-DN-, TLR4-DN-, and MyD88-DN-transfected cells. Whereas nuclear translocation of p-65 was found in infected cells transfected with a scrambled siRNA (F), it was absent in directly infected cells (G) transfected with Cy3-tagged TLR4-siRNA. G and I show merged images of the DAPI and Cy3 channels of the same field in F and H, respectively. C. parvum was identified with DAPI staining of its nucleus in blue (arrowheads in G and I). Nuclear translocation of p-65 was confirmed by positive Cy3 signal in red (asterisks in G and I). * p < 0.05, compared with sham-infection control; #, p < 0.05, compared C. parvum-infected cells. Bar, 2 μm.
No obvious HBD-2 expression was found in both directly infected and noninfected cells (Fig. 8). Consistent with nuclear translocation of NF-κB, we measured HBD-2 expression in cells after exposure to *C. parvum*. Inhibition of NF-κB by SN50 and MG-132 also diminished *C. parvum*-induced HBD-2 mRNA expression in cholangiocytes (Fig. 8, A and B). Consistent with mRNA expression of HBD-2, expression of HBD-2 peptide was detected in infected cholangiocyte culture by Western blotting (Fig. 8C). A low expression of HBD-2 peptide was detected in the sham-infected cells, and strong expression was found in cells exposed to the parasite. A decreased expression of HBD-2 was found in cells transfected with TLR2-DN, TLR4-DN, and MyD88-DN after exposure to *C. parvum*. To further clarify the relationship between NF-κB activation and HBD-2 expression induced by *C. parvum*, we measured HBD-2 expression in *C. parvum*-infected cells by immunofluorescent microscopy. Consistent with nuclear translocation of NF-κB in directly infected cells, expression of HBD-2 was found only in cells directly infected by the parasite not in bystander noninfected cells (Fig. 8D). No obvious HBD-2 expression was found in both directly infected

**FIGURE 7.** *C. parvum* induces IL-8 release in cholangiocytes via TLR2- and TLR4-associated activation of NF-κB. A, Expression of IL-8 mRNA. H69 cells were exposed to *C. parvum* for 2 h followed by RT-PCR. 18s rRNA was amplified to confirm an equal amount of total mRNA used for each sample. The intensity of the signals was quantified using a PhosphorImager and expressed as densitometric arbitrary values. Data are representative of three distinct experiments. B, IL-8 concentration in the supernatants of the cell cultures. H69 cells were exposed to *C. parvum* for 12 h, and supernatants were collected. IL-8 concentration was determined by ELISA. Data are expressed as picograms of IL-8 per milligram of total protein and representative of three distinct experiments. +, *p < 0.05*, compared with sham-infection control; #, *p < 0.05*, compared *C. parvum*-infected cells.

**FIGURE 8.** *C. parvum* induces HBD2 expression in cholangiocytes via TLR2- and TLR4-associated activation of NF-κB. H69 cells were exposed to *C. parvum* for 12 h, and expression of HBDs was determined by RT-PCR, Western blot, and immunofluorescent microscopy. A, Expression of mRNA of HBD-1–3 as assessed by RT-PCR. 18s rRNA was amplified to confirm an equal amount of total mRNA used for each sample. B, Quantitative analysis of HBD mRNA signals using a PhosphorImager. C, Expression of HBD-2 as determined by Western blot using an Ab against HBD-2. The intensity of the signals was quantified and expressed as densitometric arbitrary values. D, Expression of HBD-2 in *C. parvum* directly infected cells. H69 cells were exposed to *C. parvum* for 12 h followed by triple immunofluorescent staining using a polyclonal Ab to label HBD-2 (in green), a mAb to *C. parvum* (in red as indicated by arrowheads), and DAPI to label cell nucleus. +, *p < 0.05*, compared with sham-infection control; #, *p < 0.05*, compared *C. parvum*-infected cells. Data are representative of three distinct experiments. Bar, 5 μm.

and uninfected cells transfected with TLR2-DN, TLR4-DN, and MyD88-DN, or cells treated with NF-κB inhibitors (data not shown).

Expression of MyD88-DN in cultured cholangiocytes hampers epithelial defense against *C. parvum* infection

To test whether TLRs and TLR-associated signals are involved in cholangiocyte defense responses against *C. parvum* infection, we assessed the number of parasites detected over time in H69 cells stably transfected with a control empty vector or MyD88-DN. After incubation with an equal number of *C. parvum* sporozoites for 2 h, cells were washed with culture medium to remove nonattached and noninternalized parasites and were then further cultured for up to 96 h. We then isolated RNA from the cells to measure *C. parvum* infection in cells by quantitative RT-PCR using *C. parvum*-specific primers. We found that the number of *C. parvum* detected in cells transfected with the control vector gradually but consistently declined over time (Fig. 9, □), consistent with previous studies (23). We also detected a significantly higher number of parasites in MyD88-deficient cells than in cells transfected with the control vector at 48, 72, and 96 h after initial infection (Fig. 9, ▪). Although other interpretations are possible, these data suggest that cholangiocytes exhibit innate epithelial defense responses to inhibit *C. parvum* infection, and that MyD88 functional inhibition in cholangiocytes hampers this epithelial defense against *C. parvum*.

**Discussion**

In the work described in this study, we demonstrated that normal human cholangiocytes in vivo and in culture express all known
TLRs. Moreover, C. parvum infection of human cholangiocytes in culture induces the recruitment of TLR2 and TLR4, but not other TLRs, to the infection sites. However, whereas neither TLR2 nor TLR4 appears necessary for C. parvum attachment to or invasion of cholangiocytes in vitro, the parasite activates several downstream effectors of TLR2 and TLR4. More specifically, TLR2- and TLR4-induced signaling is required for the activation of NF-κB induced by C. parvum in directly infected cells. More importantly, a TLR2- and TLR4-dependent and NF-κB-associated interaction of HBD-2, but not HBD-1 and HBD-3, was found in C. parvum directly infected cells. MyD88-deficient cells are more susceptible to C. parvum infection in vitro. These findings demonstrate that cholangiocytes express a variety of TLRs, and TLR2 and TLR4 mediate cholangiocyte defense responses to C. parvum via activation of NF-κB.

The family of TLRs plays a key role in controlling innate immune responses. Recent studies revealed a broad and regulated expression of TLRs in human tissues. Most tissues express at least one TLR, whereas phagocytes and B cells in particular show abundant and constitutive expression of all known TLRs (33–36). Intestinal epithelial cells normally express very low levels of TLRs such as TLR2 and TLR4 (37, 38). In contrast, TLR5 is expressed exclusively on the basolateral surface of the intestinal epithelial cells (39). The characteristic of TLR expression in intestinal epithelial cells, which are consistently exposed to microbiota including commensal bacteria in the intestine, may explain why the commensal bacteria usually do not induce inflammatory responses (38, 40). Although human bile is sterile under physiological conditions, duodenal microorganisms are believed to be a major source of bacterial infection in several biliary diseases. In particular, enteric bacteria, demonstrable in bile, may be responsible for chronic proliferative cholangitis associated with hepatolithiasis (41). Nevertheless, whereas expression of TLR2–5 was recently shown in normal murine cholangiocytes and in several human cholangiocarcinoma cell lines (42), expression of TLRs in the normal human liver, particularly in cholangiocytes, has never been systemically examined. Extensive expression and cellular distribution of multiple TLRs in human cholangiocytes, as suggested by our results, indicated that TLRs may play a key role in cholangiocyte innate immune responses. These TLRs participate in a complex pattern-recognition system (15) and, therefore, provide cholangiocytes the capacity to quickly recognize and respond to microorganisms in the bile, initiating the first defense machinery in the biliary tree to keep the bile sterile and maintain the integrity of the biliary epithelial barrier.

TLRs participate in epithelial immune responses to a variety of invading pathogens including parasites, such as Trypanosoma cruzi, malaria parasites, and Leishmania (43–46). Whether TLRs also play a role in C. parvum infection of any epithelia is unclear. C. parvum sporozoites attack and invade host cells, resulting in the formation of a parasitophorous vacuole via a host-cell actin-dependent mechanism (23, 47–49). We previously showed that a variety of host-cell membrane-associated kinases, e.g., c-Src and PI3K, and actin-associated proteins, e.g., cortactin, and Arp2/3 complex, are recruited to the infection sites and induce actin remodeling and facilitate parasite invasion of host epithelial cells (10, 23, 29, 30). TLR4 has been reported as a coreceptor for E. coli P fimbriae attachment to host epithelial cells (50). In this study, we found that TLR2 and TLR4, but not other TLRs, are recruited to the infection sites. However, C. parvum attachment to and invasion of cholangiocytes seems independent of TLRs, because DN mutation of TLR2 and TLR4 did not affect parasite attachment and invasion. Instead, we identified that a set of downstream effectors of TLR2 and TLR4 are activated in cholangiocytes during C. parvum infection, suggesting that parasite host-cell interactions may trigger cholangiocyte reactivity in response to C. parvum via activation of TLR2- and TLR4-mediated intracellular signaling pathways.

The signaling events following ligation of different TLRs involve the activation of a common set of adaptor proteins (e.g., MyD88) and protein kinases (15, 16). Activation of IRAK, an immediate downstream kinase to MyD88, was detected in cholangiocytes upon C. parvum infection. Downstream effectors of IRAK in epithelia include p-38, JNK, ERK1/2, and IκB kinase (15, 16). Phosphorylation of p-38, but not JNK and ERK1/2, and activation of the NF-κB signaling pathway were found in cells exposed to C. parvum, suggesting a differential activation of downstream effectors of IRAK in cholangiocytes during C. parvum infection, similar to results of previous studies in respiratory epithelial cells during Pneumocystis infection (51) or Pseudomonas aeruginosa flagella activation (52). We showed previously that C. parvum activates the NF-κB system via degradation of IκBα in directly infected epithelial cells resulting in host-cell secretion of associated gene products (e.g., IL-8) (12). In this study, we found that C. parvum-induced degradation of IκBα was blocked in cells transfected with TLR2-DN, TLR4-DN, and MyD88-DN. Nuclear translocation of p-65 was inhibited by transfection of cells with TLR2-DN, TLR4-DN, and MyD88-DN. Activation of NF-κB by C. parvum was also inhibited by specific siRNAs to TLR2, TLR4, and MyD88. Consequently, IL-8 release from cholangiocytes upon C. parvum infection was inhibited. Moreover, our data suggest that both TLR2 and TLR4 contribute to C. parvum-induced NF-κB activation, because neither TLR2-DN nor TLR4-DN alone could completely block induced NF-κB activation and IL-8 release, whereas transfection with a DN mutant of their common adaptor protein MyD88 completely inhibited NF-κB activation. Taken together, these data indicate the following: 1) that C. parvum activates TLR2- and TLR4-dependent signal pathways in infected cholangiocytes; and 2) that both TLR2 and TLR4 are involved in activation of the NF-κB signaling pathway in directly infected cholangiocytes in which the adaptor protein MyD88 plays an essential role. Whether host-cell endosomes and intracellular pattern recognition receptors, such as nucleotide-binding oligomerization domain 1 (NOD1) and NOD2 (53, 54), also play a role in host-cell activation by C. parvum is unclear. Whether C. parvum expresses
TLRs such as LPS and peptidoglycans (55) and why direct parasite-host-cell interactions are required for TLR activation need further investigation.

HBDs are key elements of the innate immune system against invading pathogens in both respiratory and gastrointestinal epithelial cells (17). Whereas HBD-1 is extensively expressed in these epithelia under physiological conditions, HBD-2 expression is induced by exposure to microbes or cytokines, such as TNF-α (17–20). A constitutive expression of HBD-1 and LPS-induced expression of HBD-2 has also been reported in human cholangiocytes (41). The NF-κB signaling pathway has been implicated in the expression of HBD-2 in response to various stimuli (56, 57). Whereas TLR signaling-associated HBD-2 expression has been demonstrated in tracheobronchial and intestinal epithelial cells (21, 22), the role of TLRs in microbial-induced HBD expression in cholangiocytes has not been fully characterized. In this study, we provide substantial evidence supporting the notion that C. parvum induces expression of HBD-2 in cholangiocytes via TLR-mediated NF-κB activation. First, TLR2 and TLR4 are recruited to the parasite attachment sites. Secondly, TLR2 and TLR4 are associated with activation of the NF-κB signaling pathway induced by C. parvum, and both nuclear translocation of NF-κB and expression of HBD-2 are limited to directly infected cells. More importantly, DN mutants of TLR2, TLR4, and MyD88, or inhibition of NF-κB by specific inhibitors block C. parvum-induced HBD-2 expression. Both HBD-1 and HBD-2 have anti-C. parvum activity, as evidenced by a decrease of C. parvum sporozoite viability after incubation with recombinant HBD-1 or HBD-2 (14). Indeed, we detected a significantly higher number of parasites in cells transfected with MyD88-DN than in the control cells at 48–96 h after initial exposure to an equal number of parasites. Besides HBD-1, a constitutive expression of HBD-3 was also detected in both C. parvum-infected and sham-infected cultured cholangiocytes, suggesting that both HBD-1 and HBD-3 may play a general antimicrobial role in the defense of the biliary system, similar to that documented in other epithelia (58). In contrast, TLR-induced expression of HBD-2, as demonstrated by our results, may provide epithelial cells (e.g., cholangiocytes) a critical local defense response against microbial infection.

In conclusion, our study demonstrated that human cholangiocytes normally express TLR1–10. Using an in vitro model of biliary epithelial response against microbial infection, we provide substantial evidence supporting the notion that C. parvum induces expression of HBD-2 in cholangiocytes via TLR-mediated NF-κB activation. First, TLR2 and TLR4 are recruited to the parasite attachment sites. Secondly, TLR2 and TLR4 are associated with activation of the NF-κB signaling pathway induced by C. parvum, and both nuclear translocation of NF-κB and expression of HBD-2 are limited to directly infected cells. More importantly, DN mutants of TLR2, TLR4, and MyD88, or inhibition of NF-κB by specific inhibitors block C. parvum-induced HBD-2 expression. Both HBD-1 and HBD-2 have anti-C. parvum activity, as evidenced by a decrease of C. parvum sporozoite viability after incubation with recombinant HBD-1 or HBD-2 (14). Indeed, we detected a significantly higher number of parasites in cells transfected with MyD88-DN than in the control cells at 48–96 h after initial exposure to an equal number of parasites. Besides HBD-1, a constitutive expression of HBD-3 was also detected in both C. parvum-infected and sham-infected cultured cholangiocytes, suggesting that both HBD-1 and HBD-3 may play a general antimicrobial role in the defense of the biliary system, similar to that documented in other epithelia (58). In contrast, TLR-induced expression of HBD-2, as demonstrated by our results, may provide epithelial cells (e.g., cholangiocytes) a critical local defense response against microbial infection.

In conclusion, our study demonstrated that human cholangiocytes normally express TLR1–10. Using an in vitro model of biliary cryptosporidiosis, we found that C. parvum recruits TLR2 and TLR4, but not other analyzed TLRs, to the host-cell-parasite interface, an event that results in activation of NF-κB thus triggering host-cell responses such as cytokine/chemokine release and HBD expression. Future studies should define the molecular mechanisms by which C. parvum activates host-cell TLRs and investigate the potential synergistic effects of C. parvum and other pathogens (e.g., HIV-1) infection in the pathogenesis of biliary disease via the TLR pathways.

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Disclosures
The authors have no financial conflict of interest.

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