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Tribbles 3: A Novel Regulator of TLR2-Mediated Signaling in Response to *Helicobacter pylori* Lipopolysaccharide


*Helicobacter pylori* causes chronic gastritis, peptic ulcers, and gastric carcinoma. Gastric epithelial cells provide the first point of contact between *H. pylori* and the host. TLRs present on these cells recognize various microbial products, resulting in the initiation of innate immunity. Although previous reports investigated TLR signaling in response to intact *H. pylori*, the specific contribution of *H. pylori* LPS with regard to functional genomics and cell-signaling events has not been defined. This study set out to define downstream signaling components and altered gene expression triggered by *H. pylori* LPS and to investigate the role of the signaling protein tribbles 3 (TRIB3) during the TLR-mediated response to *H. pylori* LPS. Cotransfections using small interfering RNA and dominant-negative constructs demonstrated that *H. pylori* LPS functions as a classic TLR ligand by signaling through pathways involving the key TLR signaling components MyD88 adaptor-like, MyD88, IRAK1, IRAK4, TNFR-associated factor 6, IκB kinase β, and IκBα. Microarray analysis, real-time PCR, and ELISA revealed the induction of a discrete pattern of chemokines as a direct effect of LPS:TLR2 signaling. *H. pylori* infection was associated with decreased expression of TRIB3 in human gastric epithelial cell lines and tissue samples. Additionally, *H. pylori* decreased expression of C/EBP homologous protein and activating transcription factor 4, the transcription factors involved in the induction of TRIB3 expression. Furthermore, knockdown of TRIB3 and C/EBP homologous protein enhanced TLR2-mediated NF-κB activation and chemokine induction in response to *H. pylori* LPS. Thus, modulation of TRIB3 by *H. pylori* and/or its products may be an important mechanism during *H. pylori*-associated pathogenesis. *The Journal of Immunology*, 2011, 186: 2462–2471.

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The sequences presented in this article have been submitted to NCBI’s Gene Expression Omnibus under accession number GSE25515.

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The Journal of Immunology
thelial cells. Furthermore, we demonstrate a modulatory role for the TRIB3 protein during TLR-mediated signaling, in particular NF-κB activation and chemokine induction during the innate immune response to _H. pylori_ LPS.

**Materials and Methods**

**Cell culture and reagents**

HEK293, HEK-TLR2, and HEK-TLR4 cells were grown in MEM α medium (Life Technologies, NY). MNK45 cells (Health Science Research Resources Bank, Japan) were grown in RPMI 1640 medium (Life Technologies). A54 cells were grown in a 1:1 mixture of DMEM (Sigma) and nutrient F-12 Ham. Medium for all of the cell lines was supplemented with 10% FCS (Life Technologies), 2 mM l-glutamine (Sigma), 100 U/ml penicillin (Sigma), and 100 μg/ml streptomycin (Sigma). The media for HEK-TLR2 and HEK-TLR4 were further supplemented with 500 μg/ml Geneticin (Sigma). Escherichia coli LPS was purchased from Alexis (Lausen, Switzerland). Pam2CSK4 was from InvivoGen (Cayla, France), and polymyxin B sulfate (PMB) was from Sigma. mRNA was extracted with TRI reagent (Sigma) and further purified using the RNeasy kit. Total RNA extraction and real-time PCR were carried out as described below. 

**Growth of H. pylori**

Bacterial biomass was obtained by growth of the _H. pylori_ strains on blood agar under microaerophilic conditions (16). Before infection of cell cultures, bacteria were inoculated into _Brucella_ broth with 10% FCS and grown under microaerophilic conditions at 37°C overnight with shaking. Bacteria were washed in PBS (pH 7.4) and resuspended in antibiotic-free culture medium for the duration of infection. Bacteria were added to cell cultures at a multiplicity of infection of 100:1 for different time points.

**Preparation of LPS**

LPS was isolated from the reference strains of _H. pylori_ NCTC 11637, CCUG 17874, and NCTC 26695 and from four clinical isolates designated AM35, AM87, AM102, and J166. The LPS was obtained by phenol-water extraction and subsequent enzymatic purification with RNase A, DNase II, and proteinase K, as well as by ultracentrifugation, as described previously (17). The _H. pylori_ LPS obtained was essentially free of proteins (<0.1%) and nucleic acids (<0.1%), and it had an electrophoretic profile similar to that previously reported for the high-molecular-mass LPS of other _H. pylori_ strains (16). Moreover, in the _Limulus_ amebocyte lysate assay and for induction of TNF-α, the LPS exhibited bioactivities identical to those previously reported (18). Importantly, the _H. pylori_ LPS used in these studies was free of contaminating peptidoglycan, as determined in biochemical assays (9); furthermore, the bioactivities of these preparations were unaffected by treatment with lysozyme (data not shown) to remove trace peptidoglycan contamination.

**Patient samples**

Five _H. pylori_-positive patients and five _H. pylori_-negative patients undergoing upper esophagogastroduodenoscopy were enrolled in this study. The _H. pylori_ status was confirmed by the rapid urease test and histopathological examination of biopsy specimens taken from the corpus and antrum. Eight of the patients were Irish, one was Eastern European, and one patient was of Asian origin. Patients receiving antibiotics, proton pump inhibitors, steroids, or nonsteroidal anti-inflammatory drugs were excluded from the study. Ethical permission was granted by the St. James’s Hospital Research Ethics Committee, and informed written consent was obtained from all patients. Biopsies were stored in RNAlater (Ambion) at 4°C overnight to allow the solution to thoroughly penetrate the tissue and then at −80°C until processed for RNA. The tissue samples were homogenized in TRI reagent (Sigma) and further purified using the RNeasy MinElute cleanup kit (Qiagen, U.K.). Reverse transcription and real-time PCR were carried out as described below.

**Total RNA extraction and real-time PCR**

Total RNA was isolated from cell lines using a Nucleospin RNAII kit (Machery-Nagel GmbH, Düren, Germany); first-strand cDNA synthesis was performed using a RETROscript kit (Ambion), according to the manufacturer’s instructions. Gene-expression assays for IL-8, TLR2, TLR4, CXCL1, CXCL2, CXCL3, CCL20, ICAM1, TRIB3, CHOP, and ATF4 were performed using an Applied Biosystems 7900HT real-time PCR system (Applied Biosystems, Cheshire, U.K.). Relative gene expression of treated samples compared with untreated control samples was calculated using the comparative cycle threshold method (Applied Biosystems). The data were normalized to that of the housekeeping gene GAPDH.

**Plasmids**

The NF-κB luciferase reporter construct (NF-κB-LUC) contained three κB elements upstream of a minimal conalbumin promoter linked to the firefly luciferase gene (19). The IL-8 luciferase reporter construct (IL-8–LUC) contained the human IL-8 promoter sequence upstream of an SV40 promoter linked to the luciferase gene. The CCL20 promoter construct contained the promoter region (from −871 to +58) of the human CCL20 gene cloned into pGL2-basic (20). pCMV-LacZ (Clontech, Saint-Germain-en-Laye, France) expressed β-galactosidase from the human CMV immediate-early promoter. MyD88 adaptor-like (MAL)-DN encoded a mutant form in which the proline at position 125 had been substituted with histidine. MyD88-DN expressed a mutant version of MyD88 encoding the death domain (aa 1–151) (21). IRAK1-DN expressed only the N-terminal domain of the protein containing the death domain (aa 1–211) (22). IRAK4-DN expressed a kinase inactive form of the protein in which the lysine residues had been mutated to alanines at position 213 (KK213AA). TNFR-associated factor 6 (TRAF6)-DN (aa 289–522) lacked the N-terminal domain of the protein. IκB kinase (IKK) β-DN (IKK-β-DN) encoded a kinase inactive form of IKKβ in which the lysine residue at position 44 had been substituted with an alanine (23). The vector for IκB-αDN expressed a superpressor form of IκBα, in which the serine residues at positions 32 and 36 were replaced with alanines (24). The _TRIB3_ expression plasmid was generated using pCDNA3.1.

**Transfections and reporter assays**

Transfections using plasmid DNA and/or siRNA were performed using Lipofectamine (Life Technologies), according to the manufacturer’s instructions. Forty-eight hours posttransfection, cells were stimulated with 100 ng/ml Pam3CSK4 or 10 μg/ml _H. pylori_ LPS. Cells were harvested 8 h poststimulation using 1× lysis buffer (Promega, Mannheim, Germany). Luciferase activity was determined from cell extracts by means of the Luciferase Assay System (Promega). Luciferase levels were normalized after determining β-galactosidase activity expressed from a pCMV-LacZ vector, which was included in all transfections. β-galactosidase assays were carried out as described previously (25).

**ELISA**

Chemokine analysis was performed on supernatants from cell lines 24 h poststimulation with _H. pylori_ LPS, using DuoSet ELISA development kits for IL-8, CXCL1, and CCL20 (R&D Systems, Abingdon, U.K.), according to the manufacturer’s instructions.

**Microarrays**

Five micrograms total RNA was used to generate cDNA using the One Cycle cDNA synthesis Kit (Affymetrix, High Wycombe, U.K.), according to the manufacturer’s instructions. In vitro transcription was carried on the entire cDNA sample to generate biotin-labeled cRNA using the IVT Labeling Kit (Affymetrix), followed by fragmentation using the Sample Cleanup Module (Affymetrix). The fragmented-biotin-labeled cRNA was then hybridized against Affymetrix U133 plus 2.0 GeneChips followed by washing, staining, and scanning, as outlined in the Affymetrix Expression Analysis Technical Manual. Scanned images, obtained using Affymetrix Software (MASS), were normalized using robust multichip average (RMA) analysis (26). For each time-point, an average RMA value was computed for samples from two individual experiments. To ensure that the average was statistically representative, a Student _t_ test was performed, and a _p_ value was generated. Only those genes with a value of _p_ ≤ 0.05 were included in the subsequent bioinformatics analysis. Expression data for each time point were compared with the corresponding untreated control data and a signal log ratio of 0.585 or greater (equivalent to a ≥ 1.5-fold change in expression) was taken to identify significant differential regulation. Lists of differentially regulated genes were annotated using NetAffx software (www.affymetrix.com). The data discussed in this publication have been deposited in the NCBI’s Gene Expression Omnibus and are accessible through the accession number GSE25515 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE25515).
**Results**

**H. pylori LPS induces IL-8 expression via TLR2**

There have been conflicting findings with regard to the TLR responsible for the recognition of *H. pylori* LPS. In this study, the ability of *H. pylori* LPS to engage specific TLRs was assessed by monitoring IL-8 expression in HEK293 cells stably transfected with TLR2 or TLR4. Nontransfected HEK293 cells acted as a suitable negative control because they do not express TLR2 or TLR4 endogenously (12, 27). The TLR-expression status of the HEK cell lines used in this study was confirmed by RT-PCR (data not shown). Similarly to the synthetic TLR2 ligand Pam3CSK4, LPS from reference stains (NCTC 11637, NCTC 26695, and CCUG 17874) and clinical isolates of *H. pylori* (AM35, AM87, AM102, and J166) significantly upregulated IL-8 mRNA expression in HEK-TLR2 cells but not HEK293 or HEK-TLR4 cells (Fig. 1A) following treatment for 8 h. Treatment of the HEK cell lines with the TLR4 ligand *E. coli* LPS (Alexis) upregulated IL-8 only in HEK-TLR4 cells (Fig. 1A). In stimulated HEK-TLR2 cells, there was a degree of variation in the IL-8–inducing ability of LPS prepared from different strains of *H. pylori*. These differences in activity were reported previously and may be due to variations in the degree of acylation and/or phosphorylation of the LPS from different isolates (9, 28). Differences in the core and O-chains of the LPS from individual isolates could also modulate the IL-8–inducing activity of the lipid A component (9). IL-8 induction in HEK-TLR2 cells in response to *H. pylori* LPS was found to be dose dependent (Fig. 1B). Infection with *H. pylori* (NCTC 11637) resulted in a significant increase in IL-8 induction in HEK293, HEK-TLR2, and HEK-TLR4 cells (Fig. 1C). HEK293 cells were shown to respond to *H. pylori* via nucleotide-binding oligomerization domain–containing protein 1-mediated recognition of peptidoglycan (29), and certain components of the bacterium have also been suggested as TLR4 ligands (30). Notably, TLR2-expressing HEK cells demonstrated the highest level of IL-8 expression in response to the intact bacterium, with the level of induction following treatment for 8 h (Fig. 1C), similar to the level of induction following treatment with 10 μg/ml LPS from the same strain at the same time point (Fig. 1A, 1B). Based on the similarity in response to the intact bacterium, 10 μg/ml LPS was used in subsequent experiments.

To demonstrate that the observed TLR-mediated effects were LPS specific, LPS was preincubated with the antibiotic PMB, a well-known inhibitor of the activating properties of LPS (10). Preincubation of *E. coli* LPS and *H. pylori* LPS with PMB resulted in a dose-dependent decrease in IL-8 expression (Fig. 1D, 1E). It was observed that higher concentrations of PMB were required to inhibit *H. pylori* LPS-mediated IL-8 induction compared with the quantities used to inhibit the *E. coli* LPS-induced effect. This is a reflection of the lower extent of phosphorylation in the lipid A of *H. pylori* LPS, which is required for binding PMB (28).

Because *H. pylori* is a gastric pathogen, the ability of *H. pylori* LPS to induce IL-8 expression in gastrointestinal cell lines was also investigated. Treatment of the gastric adenocarcinoma-derived cell line AGS with *H. pylori* LPS did not result in an upregulation of IL-8 mRNA expression (Fig. 1F). These cells do not express TLR2 endogenously (31, 32). In contrast, the stomach cancer-derived cell line MKN45 and T84 colorectal carcinoma cells were shown to express TLR2 (14, 33, 34) and, notably, *H. pylori* LPS was found to induce IL-8 expression in MKN45 and T84 cells (Figs. 1D, 1E, 1F). Intact *H. pylori* LPS-mediated induction of IL-8 mRNA expression in HEK-TLR4 cells (D) and *H. pylori* LPS–induced IL-8 expression in HEK-TLR2 cells (E). F, IL-8 expression was upregulated in the TLR2+ gastrointestinal cell lines MKN45 and T84, but not in TLR2– gastric AGS cells, in response to *H. pylori* LPS. Error bars represent the SD of values obtained from three experiments. Statistical difference between treated and control samples were analyzed using the Student *t* test. *p* < 0.05.
T84 cells (Fig. 1F). The TLR-expression status of the cell lines used was confirmed by real-time PCR. Although other investigators demonstrated increased cell surface expression of TLR4 following *H. pylori* infection (8), neither *H. pylori* infection nor treatment with *H. pylori* LPS significantly altered TLR2 or TLR4 mRNA expression in AGS or MKN45 cells (data not shown). Taken together, these findings supported existing evidence implicating TLR2 in the recognition of *H. pylori* LPS.

*H. pylori* LPS activates NF-κB and the IL-8 promoter through pathways involving key TLR signaling components

A complete understanding of the role of TLRs in response to *H. pylori* LPS requires elucidation of downstream cell-signaling events. Having confirmed a role for TLR2 in the innate recognition of *H. pylori* LPS, we next investigated the role of downstream TLR-signaling proteins during this response. To this end, cotransfections were carried out using NF-κB–LUC and either an empty expression vector (EV) or expression vectors for dominant-negative mutant forms of known TLR-signaling proteins. Forty-eight hours posttransfection, HEK-TLR2 cells were stimulated with 100 ng/ml Pam3CSK4 (Fig. 2A, C, E) or 10 μg/ml of *H. pylori* LPS (B, D, F). Cells were harvested at 8 h poststimulation, and luciferase values were normalized for transfection efficiency based on β-galactosidase activity. Error bars represent the SD of values obtained from three experiments. Statistical differences between normalized luciferase readings from cells transfected with expression vectors for dominant negative proteins/siMyD88 and the EV/siCTRL were analyzed using the Student *t* test. *p* < 0.05.

**FIGURE 2.** *H. pylori* LPS activates NF-κB and the IL-8 promoter through pathways involving key TLR signaling components. HEK-TLR2 cells were cotransfected with NF-κB–LUC or IL-8–LUC, together with an EV or expression vectors for dominant negative forms of TLR-signaling molecules. Cotransfections were also performed using the luciferase constructs and an siCTRL or siRNA for MyD88. Forty-eight hours posttransfection, cells were stimulated with 100 ng/ml of Pam3CSK4 (A, C, E) or 10 μg/ml of *H. pylori* LPS (B, D, F). Cells were harvested at 8 h poststimulation, and luciferase values were normalized for transfection efficiency based on β-galactosidase activity. Error bars represent the SD of values obtained from three experiments. Statistical differences between normalized luciferase readings from cells transfected with expression vectors for dominant negative proteins/siMyD88 and the EV/siCTRL were analyzed using the Student *t* test. *p* < 0.05.

**FIGURE 3.** Global changes in gene expression in HEK-TLR2, AGS, and MKN45 cells treated with *H. pylori* LPS. A, HEK-TLR2 cells were treated with 10 μg/ml of *H. pylori* LPS, and total RNA was isolated at intervals over 48 h. B, AGS and MKN45 cells were treated with *H. pylori* LPS, and total RNA was isolated following 8 h of treatment. RNA analysis was performed to identify genes whose expression was up- or downregulated in response to LPS treatment from two independent experiments.
Global changes in gene expression in response to \textit{H. pylori} LPS

Many studies have investigated the global gene-expression profile in epithelial cells in response to infection with live \textit{H. pylori} (31, 33, 35–40); however, the specific contribution of \textit{H. pylori} LPS has not been demonstrated. In this study, DNA microarray-based analysis was performed to monitor global gene-expression changes in HEK-TLR2 cells in response to \textit{H. pylori} LPS over a 48-h time course. A range of genes was identified as being consistently expressed in LPS-treated cells compared with resting cells at corresponding time points. Fig. 3A displays the number of differentially expressed transcripts over time. The greatest number of differentially expressed transcripts occurred at 8 h poststimulation. A complete list of differentially expressed transcripts in HEK-TLR2 cells treated with \textit{H. pylori} LPS for 8 h is provided in Supplemental Table I. The transcripts displaying the greatest increase in expression are listed in Table I. Of particular note, IL-8 was identified as the most significantly upregulated gene, with an \sim{} 10-fold increase in expression following an 8-h stimulation with \textit{H. pylori} LPS. This finding was consistent with observations obtained using real-time PCR (Fig. 1A).

Additionally, global changes in gene expression were compared between AGS and MKN45 cells treated with 10 $\mu$g/ml \textit{H. pylori} LPS for 8 h (Fig. 3B). A complete list of the differentially expressed genes is provided in Supplemental Tables II and III. A greater number of differentially expressed transcripts was observed in the TLR2+ cell line MKN45, with 36 differentially regulated transcripts, compared with 7 transcripts in the TLR2 cell line AGS, suggesting that expression of TLR2 enhances the responsiveness of gastric epithelial cells to \textit{H. pylori} LPS. The

Table I. Transcripts displaying the greatest upregulation following \textit{H. pylori} LPS stimulation for 8 h

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<th>Accession No.</th>
<th>Gene Title</th>
<th>Gene Symbol</th>
<th>Fold Change HEK-TLR2</th>
<th>Fold Change MKN45</th>
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<td>ICAM 1 (CD54)</td>
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</table>

\textit{--}, No gene symbol is available for this transcript.

FIGURE 4. CXCL1, CXCL2, CXCL3, CCL20, and ICAM1 are upregulated in HEK-TLR2 cells in response to \textit{H. pylori} LPS. HEK293, HEK-TLR2, and HEK-TLR4 cells were treated with 10 $\mu$g/ml \textit{H. pylori} LPS, and total RNA was isolated at intervals over a 48-h period. Real-time PCR revealed that \textit{H. pylori} LPS upregulated CXCL1 (A), CXCL2 (B), CXCL3 (C), CCL20 (D), and ICAM1 (E) expression in HEK-TLR2 cells but not in HEK293 or HEK-TLR4 cells. In addition, ELISA was performed on supernatants from HEK293, HEK-TLR2, and HEK-TLR4 cells treated with \textit{H. pylori} LPS for 24 h. F. IL-8, CXCL1, and CCL20 protein was only detected in supernatants from LPS-treated HEK-TLR2 cells. Error bars represent the SD of values obtained from three experiments. Statistical differences between treated and control samples were analyzed using the Student $t$ test. *$p < 0.05$. 

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transcripts displaying the greatest increase in expression in MKN45 cells are listed in Table I. Similar to results obtained using real-time PCR (Fig. 1F), IL-8 was upregulated in MKN45 cells but not in AGS cells.

Certain genes whose expression was identified by microarray analysis as being upregulated in response to *H. pylori* LPS were chosen for validation. Of these, real-time PCR analysis confirmed an increase in expression of the genes encoding ICAM1 and the chemokines CXCL1, CXCL2, CXCL3, and CCL20 in *H. pylori* LPS-treated HEK-TLR2 cells (Fig. 4). These chemokines were not differentially regulated in HEK293 or HEK-TLR4 cells (Fig. 4), suggesting that the effect is TLR2 dependent. Furthermore, these transcripts were consistently upregulated in MKN45 cells but not in AGS cells (Fig. 5). ELISAs were carried out to confirm

**FIGURE 5.** *H. pylori* LPS induces CXCL1 (A), CXCL2 (B), CXCL3 (C), CCL20 (D), and ICAM1 (E) expression in MKN45 cells. MKN45 and AGS cells were treated with 10 µg/ml *H. pylori* LPS, and total RNA was isolated at intervals over a 48-h period. Real-time PCR indicated that *H. pylori* LPS upregulated all of these transcripts in MKN45 cells but not in AGS cells. F, IL-8, CXCL1, and CCL20 protein was detected by ELISA in supernatants from LPS-treated MKN45 cells but not from AGS cells. Error bars represent the SD of values obtained from three experiments. Statistical differences between treated and control samples were analyzed using the Student *t* test. *p < 0.05.

**FIGURE 6.** Decreased TRIB3 expression enhances the TLR2-mediated activating properties of *H. pylori* LPS. A, Treatment of HEK-TLR2, AGS, and MKN45 cells with *H. pylori* NCTC 11637 significantly decreased TRIB3 mRNA expression. B, TRIB3 expression was decreased in gastric biopsies from *H. pylori*-positive patients compared with *H. pylori*-negative patients. C, *H. pylori* LPS decreased TRIB3 expression in HEK-TLR2 and MKN45 cells. HEK-TLR2 cells were cotransfected with NF-κB–LUC (D), IL-8–LUC (E), or CCL20 luciferase construct (F), together with a scrambled siCTRL or siTRIB3. Transfected cells were stimulated with 100 ng/ml of Pam3CSK4 or 10 µg/ml of *H. pylori* LPS 48 h later and were harvested at 8 h poststimulation. TRIB3 knockdown enhanced the TLR2-mediated activating properties of Pam3CSK4 and *H. pylori* LPS. Statistical differences between treated and control samples were analyzed using the Student *t* test. *p < 0.05.
such changes at the protein level. IL-8, CXCL1, and CCL20 protein was detected in supernatants from HEK-TLR2 (Fig. 4F) and MKN45 cells (Fig. 5F) treated with H. pylori LPS but not in supernatants from LPS-treated HEK293, HEK-TLR4 (Fig. 4F), or AGS cells (Fig. 5F). CXCL2 and CXCL3 chemokine secretion was not assayed, because ELISA kits for these proteins were unavailable. Similarly to previous reports (38, 39, 41–46), we detected increased levels of IL-8, CXCL1, CXCL2, CXCL3, and CCL20 in gastric antrum biopsies from H. pylori-positive patients compared with H. pylori-negative patients (Supplemental Fig. 1). Although ICAM1 expression was detected in all biopsy samples, no statistically significant difference was observed between the H. pylori-positive and -negative groups (Supplemental Fig. 1). Taken together, these data indicated that TLR2-mediated recognition of the LPS component of H. pylori contributes to the induction of the chemokines IL-8, CXCL1, CXCL2, CXCL3, and CCL20 during H. pylori infection.

Modulation of TRIB3 by H. pylori enhances TLR2-mediated NF-κB activation and chemokine induction in response to H. pylori LPS

Having established that H. pylori LPS activates NF-κB through cell-signaling events using known TLR-signaling proteins, we next set out to identify the role of the recently identified TRIB3 protein during the TLR2-mediated response. TRIB3 is a homolog of Drosophila tribbles and modulates phosphorylation of the p65 subunit of NF-κB (15). Initially, the effect of H. pylori infection on TRIB3 mRNA expression was investigated. Treatment of HEK-TLR2, AGS, and MKN45 cells with H. pylori NCTC 11637 (Fig. 6A) resulted in a significant decrease in TRIB3 mRNA (up to 60% in MKN45 cells). Because AGS cells do not express TLR2 (31, 32), H. pylori has the ability to repress TRIB3 by a TLR2-independent mechanism and by bacterial components other than LPS. In addition, TRIB3 mRNA expression was decreased in antrum biopsies from H. pylori-positive patients compared with H. pylori-negative individuals (Fig. 6B).

Stimulation of HEK-TLR2 and MKN45 cells with H. pylori LPS resulted in a significant decrease in TRIB3 mRNA expression (Fig. 6C), albeit to a lesser extent than with whole bacteria (Fig. 6A). This change was not detected in the microarray experiments using LPS-treated cells, possibly as a result of the low level of decreased expression resulting in a false-negative result during the analysis of the large data sets. LPS stimulation had no effect on TRIB3 mRNA expression in AGS cells (data not shown). No statistically significant difference in the level of TRIB3 inhibition was observed among LPS isolated from different clinical and reference strains of H. pylori (data not shown). To investigate the effect of decreased TRIB3 mRNA expression on the activating properties of H. pylori LPS, cotransfection experiments were carried out in HEK-TLR2 cells using siRNA for TRIB3 (siTRIB3) and NF-κB–LUC, IL-8–LUC, and CCL20 luciferase reporter constructs. Real-time PCR revealed >80% decrease in TRIB3 mRNA following transfection with siTRIB3 (data not shown). Knockdown of TRIB3 significantly enhanced basal and TLR2-mediated activation of NF-κB (Fig. 6D), the IL-8 promoter (Fig. 6E), and the CCL20 promoter (Fig. 6F) in HEK-TLR2 cells in response to Pam3CSK4 and H. pylori LPS compared with cells transfected with a scrambled siCTRL. In similar experiments, TRIB3 knockdown in HEK-TLR4 cells significantly enhanced NF-κB activation in response to E. coli LPS (data not shown).

Gain-of-function experiments involving transfection of increasing quantities of an expression vector for TRIB3 (pcDNA3-TRIB3) resulted in dose-dependent inhibition of basal and TLR2-mediated activation of NF-κB (Fig. 7A), the IL-8 promoter (Fig. 7B), and the CCL20 promoter (Fig. 7C) in response to Pam3CSK4 and H. pylori LPS. These results demonstrated a negative regulatory role for TRIB3 during TLR-mediated NF-κB activation. Collectively, these data showed that decreased TRIB3 expression in response to H. pylori significantly enhances the TLR2-mediated activating properties of H. pylori LPS.

CHOP and ATF4 expression is decreased during H. pylori infection

Previous studies of the promoter region of the TRIB3 gene identified a segment consisting of tandemly arranged 33-bp repeats that contain a C/EBP-ATF element that is essential for TRIB3 induction in response to anoxia (47) and endoplasmic reticulum stress (48, 49). The transcription factor ATF4 interacts with this 33-bp element and has the ability to activate the TRIB3 promoter (47–49). In addition, overexpression of the C/EBP transcription factor CHOP activates TRIB3 promoter activity, and induction is further enhanced upon cotransfection with ATF4, suggesting a cooperative effect (48). Based on the evidence that CHOP and ATF4 are involved in the induction of TRIB3, we set out to investigate whether these transcription factors play a role during H. pylori-mediated TRIB3 repression. Expression of CHOP (Fig. 8A) and ATF4 (Fig. 8B) was significantly decreased in HEK-TLR2,
AGS, and MKN45 cells following *H. pylori* infection. LPS isolated from *H. pylori* did not significantly alter the expression of either of these genes (data not shown), indicating that other components of the bacterium are responsible. To investigate the effect of CHOP and ATF4 inhibition on TLR2-mediated signaling in response to *H. pylori* LPS, loss-of-function experiments were performed using siRNA for CHOP or ATF4 together with the NF-kB and chemokine reporter constructs. Transfection of HEK-TLR2 cells with siRNA for CHOP or ATF4 resulted in mRNA expression decreases of 66 and 68%, respectively, compared with mock-transfected cells (Fig. 8C). Knockdown of both of these genes resulted in ~30% reduction in TRIB3 mRNA expression (Fig. 8C). Knockdown of CHOP expression decreased TRIB3-mediated NF-κB (Fig. 8D), the IL-8 promoter (Fig. 8E), and the CCL20 promoter (Fig. 8F) activation in response to Pam3CSK4 and *H. pylori* LPS. However, decreased ATF4 expression did not significantly enhance the signaling activity (Fig. 8D–F), implying that additional factors may be required. Because CHOP and ATF4 are decreased in response to *H. pylori* and knockdown of both of these genes decreases TRIB3 expression, these data strongly suggested that inhibition of CHOP and ATF4 by *H. pylori* decreases TRIB3 expression, albeit through an LPS-independent mechanism. Hence, it is likely that there are multiple synergistic mechanisms through which *H. pylori* may modulate TRIB3 expression.

**Discussion**

Cytokines play a key role in *H. pylori*-associated gastroduodenal disease, but the exact mechanism of cytokine induction triggered by individual *H. pylori* virulence factors is not fully understood. In this article, we present findings implicating *H. pylori* LPS in the induction of a discrete set of chemokines in a TLR2-dependent fashion. Interestingly, TLR2 has been detected in epithelial cells from human gastric biopsy samples, with increased TLR2 expression reported in samples from *H. pylori*-infected patients (3, 32). Moreover, the cytokine response to live *H. pylori* is significantly impaired in TLR2−/− cells (9, 50). In recent years, there have been conflicting reports with regard to the TLR responsible for the recognition of *H. pylori* LPS. Consistent with the findings presented in this article, there is accumulating evidence implicating TLR2 in the recognition of *H. pylori* LPS. Smith et al. (14), Triantafilou et al. (13), and Lepper et al. (12) also showed that LPS from *H. pylori* activates NF-κB via TLR2. Furthermore, Triantafilou et al. (13) showed that TLR2 knockdown in vascular endothelial cells inhibited *H. pylori* LPS-mediated cytokine induction (13). Yokota et al. (11) showed that IL-8 induction in T24 uroepithelial cells in response to *H. pylori* LPS was suppressed by expression of a dominant-negative TLR2 but not a dominant-negative TLR4. In contrast with the current study, other investigators have implicated TLR4 in the recognition of *H. pylori* LPS (3, 7–10). The reason for the discrepancies among the various reports outlined above is not clear. However, the TLR2-dependent activity observed in the current study was common to all LPS preparations tested, including clinical and reference strains of *H. pylori*. Moreover, experiments using PMB suggested that the response was LPS specific and not due to the presence of other contaminating TLR ligands.

This study also indicated that *H. pylori* LPS functions as a classic TLR2 ligand with regard to cell-signaling events. Although *H. pylori* LPS is known to activate NF-κB and the IL-8 promoter (8, 13, 14), this study demonstrated that this process involves the TLR adaptor proteins MAL and MyD88, as well as the signaling components IRAK1, IRAK4, TRAF6, IKKβ, and IκBα. To our knowledge, this is the first study to delineate cell-signaling components triggered by *H. pylori* LPS. Because NF-κB is a key transcription factor involved in the regulation of genes involved in inflammation, cell proliferation, and apoptosis, understanding how NF-κB is activated by the LPS component of *H. pylori* may be useful in the development of novel therapeutic strategies for inhibiting inflammation during *H. pylori*-associated disease.
Although many studies have investigated the global gene-expression profile in epithelial cells in response to H. pylori infection, the expression profile in response to H. pylori LPS has not been demonstrated. Using a combination of microarray analysis, real-time PCR, and ELISA, this study demonstrated increased expression of ICAM1 and the chemokines CXCL1, CXCL2, CXCL3, and CCL20 in H. pylori LPS-treated HEK-TLR2 and MKN45 cells. Similar to IL-8, the chemokines CXCL1, CXCL2, and CXCL3 are involved in neutrophil attraction (51), whereas CCL20 attracts immature dendritic cells and memory T cells and plays a role in inflammation on mucosal surfaces (45). In support of previous reports (38, 39, 41–46), we detected increased levels of IL-8, CXCL1, CXCL2, CXCL3, and CCL20 in gastric antrum biopsies from patients infected with H. pylori compared with H. pylori-negative patients. Although ICAM1 expression was detected in all biopsy samples, no statistically significant difference was observed between the two patient groups. The LPS-mediated effect was only observed in TLR2-expressing cell lines, indicating that the specific contribution of the LPS component of H. pylori to the induction of a discrete set of chemokines during H. pylori infection is mediated via LPS–TLR2 interactions.

Notably, to our knowledge, the current study presents the novel finding that H. pylori modulates expression of the signaling protein TRIB3. A previous study showed that TRIB3 interacts with the p65 subunit of NF-kB and inhibits its phosphorylation by PKAc, thereby inhibiting NF-kB transcriptional competence (15). In this article, we showed that endogenous TRIB3 mRNA expression is downregulated in HEK-TLR2, AGS, and MKN45 gastric cells in response to H. pylori infection, as well as gastric biopsies from H. pylori-infected individuals. AGS cells do not express TLR2, implying that H. pylori-mediated TRIB3 inhibition can occur independently of TLR2. A key role for the transcription factors ATF4 and CHOP in the induction of TRIB3 expression in response to cellular stress was demonstrated previously (47–49).

In this study, we showed that in addition to TRIB3, ATF4 and CHOP expression is decreased in H. pylori-infected HEK-TLR2, AGS, and MKN45 cells. Furthermore, knockdown of ATF4 and CHOP expression using siRNA resulted in decreased TRIB3 expression. These data strongly suggested that inhibition of TRIB3 expression by intact H. pylori occurs via repression of ATF4 and CHOP.

Inhibition of TRIB3 was also observed in HEK-TLR2 and MKN45 cells in response to H. pylori LPS, albeit to a lesser extent than with the intact bacterium. Because H. pylori LPS was not found to inhibit expression of ATF4 or CHOP, it is possible that other mechanisms are responsible for the LPS-mediated inhibition of TRIB3. Indeed, regulation of TRIB3 expression was suggested to involve a complex set of pathways, with evidence to suggest transcriptional regulation by NF-kB, as well as stabilization of the TRIB3 transcript by the RNA-binding protein HuR (47). Further studies are required to investigate whether LPS modulates these pathways to inhibit TRIB3 expression. In any case, in cells in which CHOP and TRIB3 mRNA expression is decreased, the TLR2-mediated NF-kB and chemokine promoter activation by Pam3CSK4 and H. pylori LPS is enhanced. Overexpression experiments showed that increased TRIB3 expression inhibits TLR-mediated NF-kB activation and chemokine induction in response to H. pylori LPS. Although ATF4 knockdown inhibited TRIB3 expression, this was not sufficient to amplify TLR2-mediated signaling in response to H. pylori LPS. It may be that although ATF4 and CHOP are involved in the repression of TRIB3 by H. pylori, CHOP inhibition modulates NF-kB activation through an additional mechanism. Indeed, members of the C/EBP family have been suggested to interact with NF-kB subunits and may regulate NF-kB activity in some cell types (52, 53), and overexpression of CHOP decreases NF-kB activation in response to TNF-α (54). Because NF-kB promotes inflammation through transcriptional induction of proinflammatory genes, enhanced NF-kB activation and chemokine induction via repression of CHOP and TRIB3 signaling by H. pylori and/or its products may represent an important mechanism during H. pylori-associated pathogenesis.

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Disclosures

The authors have no financial conflicts of interest.

References


