

## Intact Gram-Negative *Helicobacter pylori*, *Helicobacter felis*, and *Helicobacter hepaticus* Bacteria Activate Innate Immunity via Toll-Like Receptor 2 but Not Toll-Like Receptor 4

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Received 3 March 2004/Returned for modification 5 April 2004/Accepted 20 May 2004

**Molecular and genetic studies have demonstrated that members of the Toll-like receptor (TLR) family are critical innate immune receptors. TLRs are recognition receptors for a diverse group of microbial ligands including bacteria, fungi, and viruses. This study demonstrates that distinct TLRs are responsible for the recognition of *Helicobacter* lipopolysaccharide (LPS) versus intact *Helicobacter* bacteria. We show that the cytokine-inducing activity of *Helicobacter* LPS was mediated by TLR4; i.e., TLR4-deficient macrophages were unresponsive to *Helicobacter pylori* LPS. Surprisingly, the cytokine response to whole *Helicobacter* bacteria (*H. pylori*, *H. hepaticus*, and *H. felis*) was mediated not by TLR4 but rather by TLR2. Studies of HEK293 transfectants revealed that expression of human TLR2 was sufficient to confer responsiveness to intact *Helicobacter* bacteria, but TLR4 transfection was not sufficient. Our studies further suggest that *cag* pathogenicity island genes may modulate the TLR2 agonist activity of *H. pylori* as *cagA*<sup>+</sup> bacteria were more active on a per-cell basis compared to *cagA* mutant bacteria for interleukin-8 (IL-8) cytokine secretion. Consistent with the transfection studies, analysis of knockout mice demonstrated that TLR2 was required for the cytokine response to intact *Helicobacter* bacteria. Macrophages from both wild-type and TLR4-deficient mice produced a robust cytokine secretion response (IL-6 and MCP-1) when stimulated with intact *Helicobacter* bacteria. In contrast, macrophages from TLR2-deficient mice were profoundly unresponsive to intact *Helicobacter* stimulation, failing to secrete cytokines even at high (100:1) bacterium-to-macrophage ratios. Our studies suggest that TLR2 may be the dominant innate immune receptor for recognition of gastrointestinal *Helicobacter* species.**

*Helicobacter pylori* is a gram-negative, spiral-shaped bacterium that infects half of the world's population (29). While infection with *H. pylori* invariably leads to a chronic inflammatory response (chronic active gastritis), most infected patients remain asymptomatic, with only minimal inflammation (62). However, a significant percentage of patients do progress to more serious outcomes, which include peptic ulcer disease, gastric lymphoma, and gastric cancer (12, 22, 42, 57). On the basis of its strong link to gastric cancer, *H. pylori* has been classified by the International Agency for Research on Cancer (a branch of the World Health Organization) as a class I carcinogen (29). In both animal models (40, 79) and human studies (14, 15), progression of *H. pylori* disease from superficial gastritis to gastric cancer appears to be related to the severity of the host inflammatory response. The identification of *H. pylori* components and host factors that contribute to the inflammatory response may lead to important insights into the mechanism of peptic ulcer disease and/or gastric malignancy.

Although *H. pylori* induces chronic mucosal inflammation to some degree in all infected patients, the organism does not appear to invade the gastric epithelium (reviewed in reference 27). Gastric epithelial cells and macrophages are considered to

be the main sources of proinflammatory cytokines and key components of innate immunity. With respect to *H. pylori*, the gastric epithelial cell layer is thought to represent the first line of defense and the initial trigger for host innate and inflammatory responses (8, 19, 65). *H. pylori* has been shown to activate intracellular signaling in gastric epithelial cells, leading to transcriptional responses. Epithelial cells release a variety of proinflammatory mediators including both cytokines and chemokines, leading to the subsequent attraction of monocytes/macrophages. In addition, both epithelial cells and macrophages appear to recognize microbial pathogens by sampling the environment with a family of receptors that discriminate between pathogens and self, pattern recognition receptors known as the Toll-like receptor (TLR) family (8, 19, 65).

Several animal models of *Helicobacter*-induced gastrointestinal inflammation and gastric cancer have been developed (40, 79, 80). *H. felis* is a mouse pathogen, and infection of C57BL/6 mice mimics many of the pathogenic changes commonly found in humans infected with *H. pylori* (40, 80). *H. pylori* from human isolates is not usually a mouse pathogen; however, a mouse-adapted strain, *H. pylori* SS1, has proven very useful in modeling gastric disease and cancer progression in mice (41, 75). *H. hepaticus* is an endogenous mouse pathogen. *H. hepaticus* infection produces a typhlocolitis in mice (16, 17). Recently, *H. hepaticus* infection has been shown to lead to the development of colon cancer in Rag-2-deficient mice (18). The receptors involved in the inflammatory response to *H. hepaticus*

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*cus* are largely unknown, although a prominent role for NF- $\kappa$ B has been demonstrated in studies by Erdman et al. (16).

In this study, we have investigated the role of TLRs in the recognition of *H. pylori*, *H. felis*, and *H. hepaticus*. With normal human monocytes and macrophages, transfected cell lines, and genetically deficient animals, we demonstrate that TLR2 is an important cytokine signaling receptor for *H. pylori*, *H. felis*, and *H. hepaticus*. Further, we demonstrate that although the lipopolysaccharide (LPS) of *H. pylori* is recognized by TLR4, the major TLR for intact *Helicobacter* bacteria is TLR2, not TLR4.

#### MATERIALS AND METHODS

**Human cells and cell lines.** Human embryonic kidney (HEK293) cells (American Type Culture Collection, Manassas, Va.) were grown in RPMI 1640 medium or Dulbecco modified Eagle medium (Gibco BRL, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Norcross, Ga.). HEK293 cells stably expressing human TLR2, TLR4, MD2, and/or CD14 were engineered as previously described (38). Peripheral blood mononuclear cells (PBMC) were isolated from normal human donors by Ficoll-Hypaque gradient centrifugation (Lymphocyte Separation Medium; Mediatech, Herndon, Va.). Monocytes were isolated by counter-current centrifugal elutriation of mononuclear leukocyte-enriched cell preparations from leukapheresis donors. Macrophages were differentiated from blood monocytes by culturing the cells in the presence of 1,000 U of macrophage colony-stimulating factor (R&D Systems, Minneapolis, Minn.) per ml for 2 days and in culture medium (Dulbecco modified Eagle medium plus 10% fetal bovine serum) alone for an additional 8 to 14 days (76).

**PECs.** Murine peritoneal exudate cells (PECs) were harvested from mice injected intraperitoneally with 1 ml of 4% thioglycolate after 4 days by peritoneal lavage with Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free sterile saline. TLR2-deficient mice were the gift of S. Akira (Osaka, Japan) (24). TLR4-deficient C57BL/10ScN mice were obtained from the National Cancer Institute. C57BL/6, C57BL/6  $\times$  129Sv F<sub>2</sub> (B6129F<sub>2</sub>), and C57BL/10SnJ wild-type control mice were purchased from The Jackson Laboratory (Bar Harbor, Maine).

Cells were cultured in 24-well culture dishes at densities of 10<sup>5</sup> to 10<sup>6</sup> cells per well in 1 ml of medium. HEK293 cells at 10<sup>5</sup>/ml, PBMC at 10<sup>6</sup>/ml, monocytes and macrophages at 10<sup>5</sup>/ml, and PECs at 10<sup>5</sup> to 10<sup>6</sup>/ml were used. Cells were stimulated with LPS (1 to 100 ng/ml), Pam<sub>3</sub>CSK<sub>4</sub> (10 to 100 ng/ml), or bacteria (10<sup>3</sup> to 10<sup>9</sup> CFU/ml). For analysis of cytokine secretion, culture supernatants were harvested 18 h after stimulation and cytokine levels were determined by enzyme-linked immunosorbent assay (ELISA) (OptEIA; BD-Pharmingen). For analysis of cytokine gene transcription, cells were stimulated with bacteria or medium alone for 2 h. The cells were harvested and lysed, and RNA was prepared with a commercial RNA extraction kit (RNeasy; QIAGEN, Valencia, Calif.). Total mRNA (5  $\mu$ g per blot) was reverse transcribed, labeled with biotin, and hybridized to a Mouse Inflammatory Cytokine SuperArray in accordance with the manufacturer's (SuperArray Inc., Frederick, Md.) instructions. Chemiluminescent images on film were transilluminated, scanned, and analyzed with GEArray software.

**LPS preparations.** LPS from *Escherichia coli* serotype O111:B4 was purchased from Sigma (St. Louis, Mo.). Phenol extraction of the LPS to remove contaminating lipopeptides was performed as described elsewhere (26). The following clinical isolates of *H. pylori* were also used as sources of LPS: AM1, isolated from a patient with chronic gastritis; AM2 and AM3, from patients with superficial gastritis; AM4, from a patient with chronic atrophic gastritis; AM5, from a patient with intestinal metaplasia; and AM6, from a patient with gastric adenocarcinoma. Similarly, LPS was obtained from clinical isolates of *E. coli* and *Salmonella enterica* (55).

Bacterial biomass was obtained by growth of *H. pylori* strains on blood agar under microaerobic conditions (54). *H. pylori* LPS was obtained from this biomass by phenol-water extraction and subsequent enzymatic purification with RNase A, DNase II, and proteinase K, as well as by ultracentrifugation as described previously (55). The *H. pylori* LPS obtained was essentially free of proteins (<0.1%) and nucleic acids (<0.1%) and had an electrophoretic profile similar to that reported previously for the high-molecular-mass LPS of other *H. pylori* strains (54). Moreover, in the *Limulus* amoebocyte lysate assay and for induction of tumor necrosis factor alpha, the LPS exhibited bioactivities identical to those reported previously (63). High-molecular-mass LPS was produced by these strains when they were grown either on solid medium or in liquid culture. Importantly, the *H. pylori* LPS used in these studies was free of contaminating peptidoglycan as determined in biochemical assays (A. P. Moran, unpublished

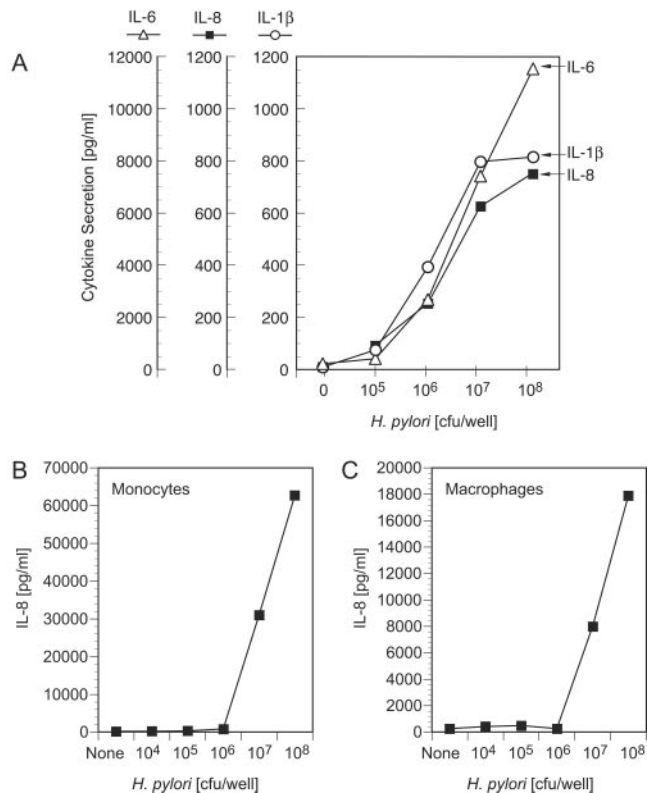


FIG. 1. Human monocytes and macrophages challenged with whole *H. pylori* bacteria secrete inflammatory cytokines. Human PBMC (A), purified monocytes (B), or monocyte-derived macrophages (C) were stimulated with various numbers of *H. pylori* SS1 bacteria or medium alone. Culture supernatants were harvested following an 18-h incubation, and IL-6, IL-8, and IL-1 $\beta$  levels were determined by cytokine-specific ELISA.

results), and furthermore, the bioactivities of these preparations was unaffected by treatment with lysozyme, which removes trace peptidoglycan contamination (data not shown).

**Helicobacter strains.** *H. pylori* SS1, *H. pylori* ATCC 43504 (*cagA*<sup>+</sup> *vacA*<sup>+</sup>), *H. pylori* Astra 244 (*cagA*<sup>-</sup> *vacA*<sup>+</sup>), *H. hepaticus* (ATCC 51449), and *H. felis* (ATCC 49179) were grown on tryptic soy broth-blood agar (Becton Dickinson, Cockeysville, Md.), harvested, and washed, and the optical density at 600 nm and CFU counts were determined (28). Bacteria were stored in aliquots at -70°C. On the day of assay, the bacteria were washed in sterile saline, recovery was checked by determining the optical density at 600 nm, and the bacteria were resuspended to 10<sup>9</sup>/ml in sterile saline. Serial dilutions of the bacteria were prepared in culture medium immediately prior to addition to the cell cultures. Cells and bacteria were incubated together in tissue culture medium for 18 h at 37°C, and culture supernatants were harvested for cytokine analysis. (Experiments with bacteria grown in liquid culture showed the same results. Data are representative of at least three independent experiments.)

#### RESULTS

***H. pylori* activates the innate immune response in human cells.** Normal human peripheral blood cells incubated with *H. pylori* SS1 bacteria secreted inflammatory cytokines, including interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-6, IL-8, and tumor necrosis factor alpha (Fig. 1). Cytokines were produced in a dose-dependent manner by PBMC upon incubation with *H. pylori* SS1 bacteria (Fig. 1A). Purified human monocytes and monocyte-derived human macrophages exhibited similar dose-dependent cytokine responses to *H. pylori* bacteria (Fig. 1B and C). Optimal cytokine

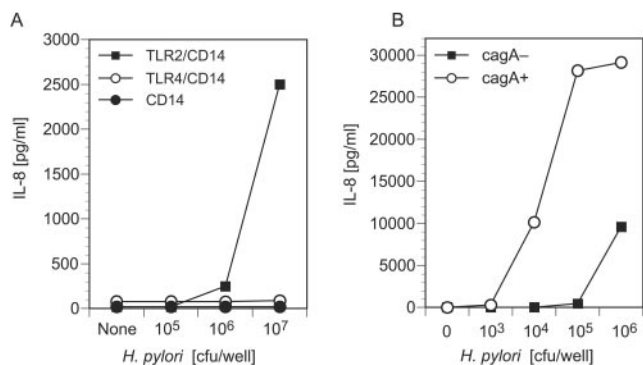


FIG. 2. Whole *H. pylori* bacteria are potent stimulators of HEK293 cells expressing TLR2 but not TLR4. (A) HEK293 cell clones stably expressing CD14, TLR2 and CD14, or TLR4 and CD14 were stimulated with *H. pylori* SS1 bacteria or medium alone. (B) TLR2- and CD14-expressing HEK293 cell clones were stimulated with various numbers of *cagA*<sup>+</sup> or *cagA* mutant *H. pylori* bacteria or medium alone. Culture supernatants were harvested after an 18-h incubation, and IL-8 levels were determined by ELISA.

secretion was observed at a dose of approximately 10<sup>8</sup> *H. pylori* SS1 CFU/10<sup>5</sup> monocytes (Fig. 1B) or macrophages (Fig. 1C).

Human monocytes and macrophages express innate immune receptors, including TLR2, TLR4, and the pattern recognition receptor CD14. These receptors participate in the inflammatory response to a variety of bacteria and bacterial products, including LPS from gram-negative bacteria, peptidoglycan from gram-positive bacteria, and yeast zymosan. Therefore, we determined if these receptors are also important for the response to *H. pylori*.

**Human TLR2 expression is sufficient to confer responsiveness to *H. pylori* bacteria.** We investigated the role of TLRs in the response to *H. pylori* in gain-of-function studies with stably transfected HEK293 cells. Transfection of HEK293 cells with human TLR4 confers responsiveness to the TLR4 ligand *E. coli* LPS (10, 38). Likewise, transfection with human TLR2 confers responsiveness to TLR2 ligands, such as yeast zymosan and *S.aureus* peptidoglycan (38, 44). CD14 expression enhances the response of HEK293 cells to both TLR2 and TLR4 ligands (10, 38, 44).

We examined the response of HEK293 cells stably transfected with CD14 alone or in combination with TLR2 or TLR4 to *H. pylori*. Intact *H. pylori* bacteria induced IL-8 secretion from cells expressing TLR2 and CD14 but not from cells expressing TLR4 and CD14 or CD14 alone (Fig. 2A). The IL-8 response of TLR2- and CD14-expressing cells to *H. pylori* SS1 bacteria was dose dependent (Fig. 2A and B). These results suggested that although *H. pylori* is a gram-negative bacterium, the cytokine secretion response to whole *H. pylori* bacteria occurs by TLR2, rather than TLR4, receptor stimulation.

***cagA*<sup>+</sup> *H. pylori* stimulation of TLR2-expressing cells.** Studies from several laboratories have shown that a set of *H. pylori* genes in the *cag* pathogenicity island influences the cytokine-stimulating activity of *H. pylori*. To address the role of the *cag* region genes in the TLR2 agonist activity of intact *H. pylori* bacteria, *cagA*<sup>+</sup> and *cagA* mutant *H. pylori* bacteria were used to stimulate TLR2- and CD14-expressing HEK293 cells (Fig. 2B). Both strains of bacteria stimulated IL-8 secretion from the TLR2- and CD14-expressing cells, indicating that *cagA* is not required for a TLR2-mediated cytokine response. Neverthe-

less, *cagA*<sup>+</sup> bacteria were 2 logs more potent stimulators of IL-8 secretion than *cagA* mutant bacteria and the response to *cagA*<sup>+</sup> bacteria was dependent on TLR2 expression, suggesting that *cagA* (or genes associated with *cagA* in the *cag* pathogenicity island) augments the TLR2 response either directly or by regulating the expression of other TLR2 target molecules on *H. pylori*.

***H. hepaticus* bacteria activate cytokine secretion via TLR2.** Similar to the response seen with *H. pylori*, whole *H. hepaticus* bacteria stimulated IL-8 secretion (Fig. 3) from HEK293 cells

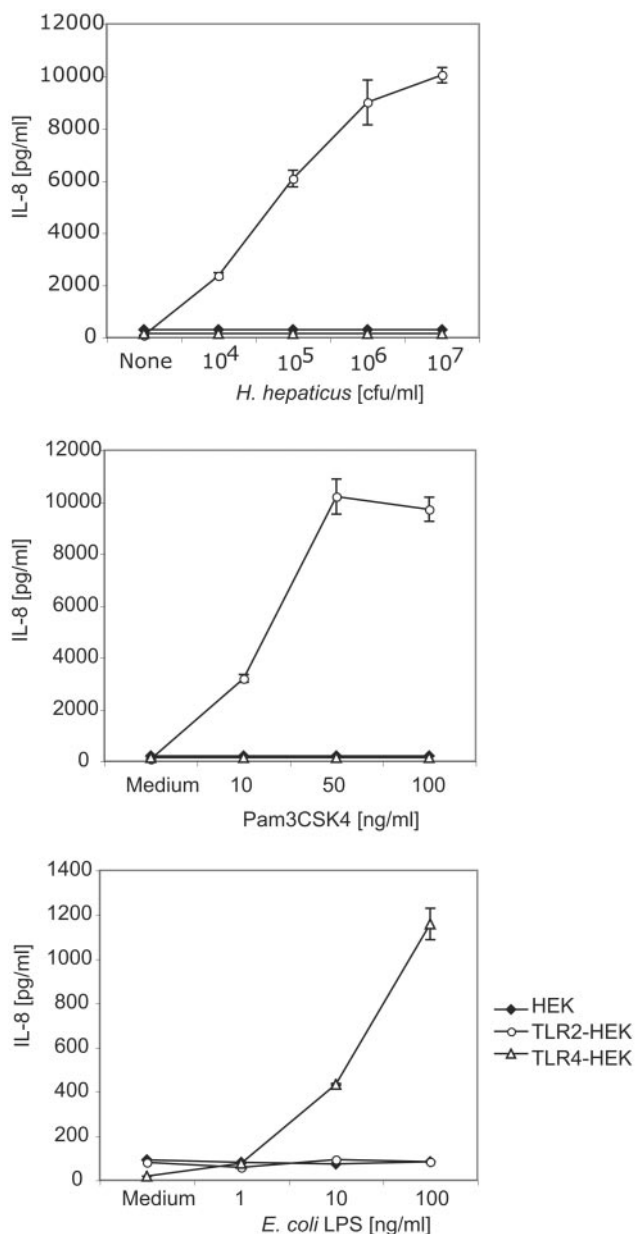


FIG. 3. Whole *H. hepaticus* bacteria activate TLR2-expressing but not TLR4-expressing HEK cells. HEK293 clones stably expressing TLR2 and CD14, TLR4 and MD2, or CD14 alone (control) were stimulated with *H. hepaticus* bacteria (upper panel), Pam<sub>3</sub>CSK<sub>4</sub> (TLR2 ligand, middle panel), *E. coli* LPS (TLR4 ligand, lower panel), or medium alone. Culture supernatants were harvested after an 18-h incubation, and IL-8 levels were determined by ELISA.

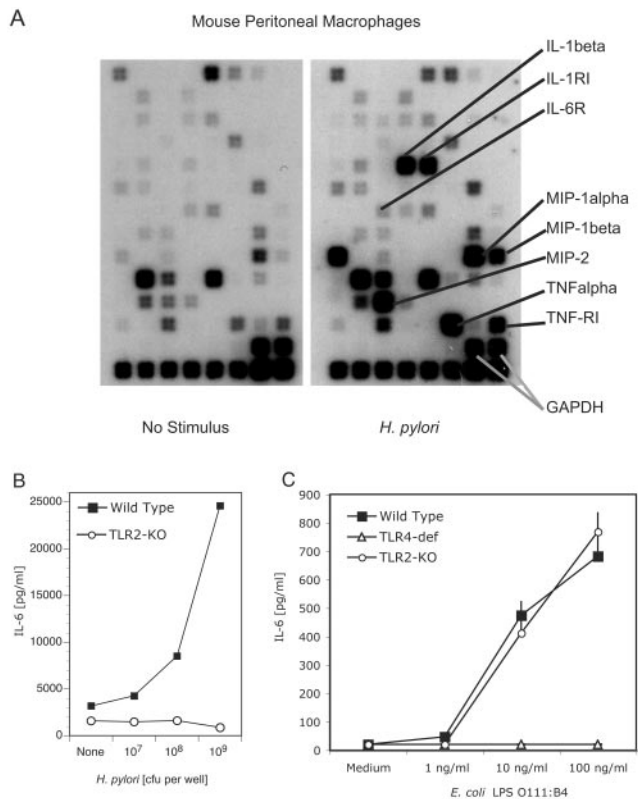


FIG. 4. Murine macrophages are activated by whole *H. pylori* SS1 bacteria in a TLR2-dependent manner. (A) Wild-type mouse peritoneal exudate macrophages were stimulated with medium (left panel) or *H. pylori* SS1 bacteria (right panel). Following a 2-h incubation, RNA was extracted, labeled, and hybridized to a mouse SuperArray blot. The positions of inflammatory cytokine and cytokine receptor spots are indicated. (Bottom two rows) Negative controls included blanks and pUC18. Positive controls included the gene for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and housekeeping genes (those for cyclophilin A and actin). (B) Wild-type and TLR2 knockout (KO) mouse peritoneal exudate macrophages were stimulated with medium (None) or *H. pylori* SS1 bacteria (10<sup>7</sup> to 10<sup>9</sup> per well). Supernatants were harvested 18 h later, and IL-6 levels were determined by ELISA. (C) Wild-type, TLR2 knockout, or TLR4-deficient (def) ScN mouse peritoneal exudate macrophages were stimulated with medium alone or *E. coli* LPS (TLR4 ligand). Supernatants were harvested 18 h later, and IL-6 levels were determined by ELISA.

transfected with human TLR2. The response was dose dependent, and IL-8 secretion was detectable at bacterium-to-cell ratios as low as 0.5:1 (Fig. 3). In contrast, neither *H. hepaticus* nor *H. pylori* bacteria activated TLR4 (and MD2)-expressing HEK293 cells, although the TLR4-expressing HEK293 cells responded to an LPS challenge (Fig. 2 and 3).

***H. pylori* bacteria activate inflammatory cytokine gene expression and secretion from murine macrophages.** Like human monocytes and macrophages, mouse peritoneal macrophages incubated with whole *H. pylori* bacteria exhibited a robust inflammatory cytokine response (Fig. 4). Gene array analysis of peritoneal macrophages demonstrated upregulation of a large number of inflammatory cytokine and chemokine genes within 2 h of stimulation of the macrophages with whole *H. pylori* bacteria (Fig. 4A). This increase in gene expression was reflected at the protein level; *H. pylori* induced a dose-

dependent increase in the synthesis and secretion of inflammatory cytokines, including IL-6 and MCP-1, from mouse macrophages (Fig. 4B).

**TLR2 knockout macrophages are unresponsive to *H. pylori* bacteria, while TLR4-deficient macrophages respond normally.** The gain-of-function studies of transfected cell lines indicated that TLR2 plays an important role in the response to whole *H. pylori* bacteria. We next determined if the targeted deletion of TLR2 in knockout mice would affect the inflammatory response to *H. pylori* bacteria. Peritoneal macrophages from wild-type and TLR2<sup>-/-</sup> mice were cultured with *H. pylori* bacteria, and the release of cytokines was measured. Wild-type macrophages secreted IL-6 in response to *H. pylori* bacteria, while TLR2<sup>-/-</sup> macrophages failed to respond to *H. pylori* (Fig. 4B), even at very high doses of bacteria (10<sup>9</sup> CFU/10<sup>6</sup> macrophages). Control cultures demonstrated a robust cytokine response of wild-type and TLR2<sup>-/-</sup> macrophages to *E. coli* LPS (Fig. 4C) and to IL-1β (data not shown). As expected, TLR2<sup>-/-</sup> macrophages were unresponsive when challenged with the TLR2 ligands (i.e., zymosan and peptidoglycan) (11, 70; data not shown), whereas TLR4-deficient macrophages were unresponsive to LPS (Fig. 4C).

In a similar experiment, we compared wild-type and TLR2- and TLR4-deficient macrophage responses to *H. pylori* SS1 and *H. felis* (Fig. 5). We wanted to determine whether (i) the failure of TLR2<sup>-/-</sup> mice to respond to *H. pylori* SS1 was peculiar to this species of *Helicobacter* or whether responses to other gastric *Helicobacter* species were also deficient in these animals and (ii) whether TLR4 is necessary for the cytokine response to *Helicobacter* bacteria. These studies demonstrate that TLR2<sup>-/-</sup> macrophages failed to respond to either *Helicobacter* species (Fig. 5A and B). In contrast, TLR4-deficient mice had an enhanced response to both *Helicobacter* species compared to the response of control (wild-type) animals (Fig. 5A and B). (The basis for the hyperresponsiveness of TLR4-deficient macrophages to *Helicobacter* bacteria is not clear, but it may reflect higher levels of TLR2 expression on these macrophages or increased availability of intracellular adaptor or signaling components in the absence of TLR4 expression.) Thus, TLR2 but not TLR4 was necessary and sufficient for the cytokine response to intact *Helicobacter* bacteria.

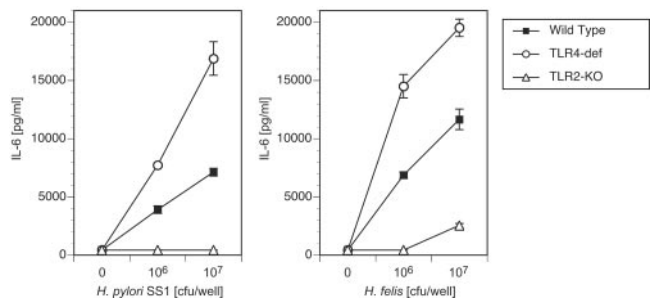


FIG. 5. *H. pylori* SS1 and *H. felis* both activate murine macrophages in a TLR2-dependent, TLR4-independent manner. Wild-type, TLR2-knockout (KO), and TLR4-deficient (def) mouse peritoneal exudate macrophages were stimulated with medium alone or with *H. pylori* SS1 (left panel) or *H. felis* (right panel) bacteria. Supernatants were harvested 18 h later, and IL-6 levels were determined by ELISA.

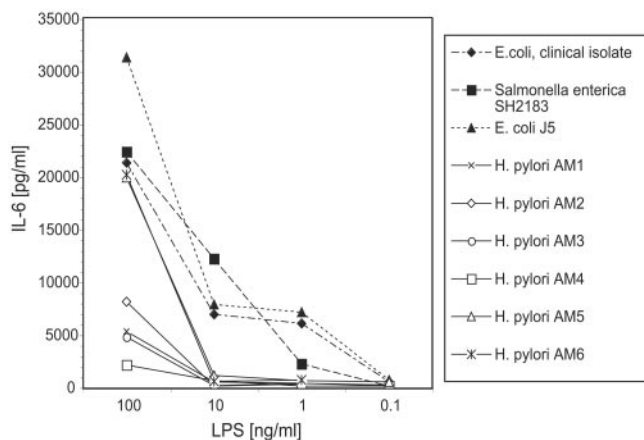


FIG. 6. Dose-dependent activation of cytokine secretion and comparison of LPS preparations purified from *E. coli* with those from clinical isolates of *H. pylori*. Wild-type peritoneal exudate macrophages were stimulated with medium alone or with various doses of LPS isolated from *E. coli*, *S. enterica*, or clinical isolates of *H. pylori* (designated AM1 to -6). Following an 18-h incubation, supernatants were harvested and IL-6 levels were determined by ELISA.

***H. pylori* LPS stimulates cytokine secretion via TLR4.** We next determined if the LPS component of *H. pylori* could stimulate an inflammatory response similar to that seen with whole bacteria. LPS from gram-negative bacteria, such as *E. coli*, stimulates the innate immune response through TLR4. This response is independent of TLR2 expression (23, 26, 77). In contrast to *E. coli*, the innate immune response to *H. pylori* was dependent on TLR2 but not TLR4. To determine if differences in the structure of the LPS of *E. coli* compared to that of *H. pylori* LPS could account for differences in TLR activation, we compared the *E. coli* and *H. pylori* LPSs as stimulants of wild-type, TLR2<sup>-/-</sup>, and TLR4-deficient macrophages. The LPSs of both *E. coli* and *H. pylori* activated macrophages in a dose-dependent (Fig. 6) and TLR4-dependent (Fig. 7) manner. Thus, *H. pylori* LPS preparations triggered cytokine secretion from wild-type and TLR2<sup>-/-</sup> macrophages, as well as from TLR4-transfected HEK293 cells (Fig. 7A, B, and D). In contrast, *H. pylori* LPS failed to activate TLR4<sup>-/-</sup> macrophages or control HEK293 cells (Fig. 7C and data not shown).

As noted in other studies, LPS prepared from clinical isolates of *H. pylori* varies substantially in its cytokine-stimulating activity (5, 31, 35). We observed that LPS from isolates AM5 and AM6 induced a robust IL-6 cytokine response (Fig. 6). LPS from isolates AM1, -2, -3, and -4 induced lesser levels of IL-6 (Fig. 6). These differences in activity may reflect variations in the degree of acylation and/or phosphorylation of the LPS in these different clinical isolates (Moran, unpublished). Differences in the core and O chains of the LPS from the individual isolates are also observed that could perhaps modulate the cytokine-inducing activity of the lipid A component of LPS (Moran, unpublished).

Overall, the *H. pylori* LPS preparations were 1 to 2 logs less stimulatory than either *E. coli* or *Salmonella* LPS similarly prepared from clinical isolates (Fig. 6). Nevertheless, all of the *H. pylori* LPS preparations tested that were active in assays with wild-type cells (i.e., AM1, AM4, AM5, and AM6) displayed the same TLR4-dependent, TLR2-independent pattern

of response (Fig. 7). Similarly, differences in the structure of the LPS derived from *H. pylori* compared to that of the LPS from *E. coli* cannot account for the TLR2 dependence of the response to intact *H. pylori* bacteria (53, 56). Taken together, our studies suggest that TLR4 expression was both necessary and sufficient for a robust cytokine secretion response to *H. pylori* LPS, whereas TLR2 expression was required for responsiveness to intact *Helicobacter* bacteria.

**Intact *E. coli* bacteria stimulate both TLR2- and TLR4-expressing cells.** We directly compared the TLR2 and TLR4 dependence of cytokine secretion induced by intact *E. coli* and *H. pylori* bacteria (Fig. 8). *E. coli* bacteria induced IL-6 secretion from both wild-type and TLR2 knockout peritoneal macrophages but only weakly activated TLR4 knockout cells (Fig. 8). In contrast, *H. pylori* bacteria activated wild-type and TLR4 knockout cells but not TLR2 knockout cells. *H. pylori* bacteria were weak inducers of cytokine secretion compared to *E. coli* bacteria on a per-cell basis (Fig. 8 and data not shown). Although the response of peritoneal macrophages to *E. coli* bacteria was largely TLR4 dependent, both TLR2- and TLR4-expressing HEK293 cells secreted cytokines in response to *E. coli* bacteria, indicating that both receptors potentially contributed to the response to intact *E. coli* (Fig. 8). In contrast, *H. pylori* bacteria activated TLR2-expressing, but not TLR4-expressing, HEK293 cells (Fig. 8). Thus, intact *H. pylori* activated TLR2, while intact *E. coli* activated both TLR2 and TLR4, although TLR4 was the predominant activating receptor for *E. coli* on normal macrophages.

## DISCUSSION

TLRs are transmembrane proteins that function as pattern recognition receptors for the detection and response to microbial ligands (reviewed in references 30, 32, and 37). To date, 10 TLRs have been identified in humans and natural or synthetic ligands for at least 9 TLRs have been identified (9, 30, 32, 33, 37, 52, 67). The extracellular regions of TLRs are diverse but contain variable numbers of leucine-rich repeat regions and conserved cysteine domains that are thought to contribute to receptor structure and function. All of the TLRs have a signature intracellular signaling motif in common with the IL-1 receptor, called the TIR (Toll-IL-1-R) domain (6, 9, 52, 67). Activation of TLRs results in the recruitment of adaptor proteins, including MyD88 and Mal/TIRAP, to the TIR domain. A series of phosphorylation-recruitment-activation events leads to the activation and translocation of NF- $\kappa$ B to the nucleus and the transcription of inflammatory cytokine genes (1, 30, 50, 51, 59).

Ligands recognized by TLRs can be found among many genera of bacteria, viruses, and fungi (3, 7, 44, 49, 77, 78, 82). These include LPS from gram-negative bacteria (TLR4 ligand), peptidoglycan and lipoteichoic acid from gram-positive bacteria (TLR2 ligands), and zymosan from yeast (TLR2 ligand). Recent data also indicate that TLRs are important in the innate immune response to bacterial DNA (TLR9 [24]) and viral RNA (TLR3 [2]), as well as intact bacteria and viruses (TLR2 and TLR4 [11, 39]).

Our data demonstrate that TLR2 is a critical receptor for the recognition of intact *H. pylori* bacteria. This is remarkable since most gram-negative bacteria preferentially activate TLR4

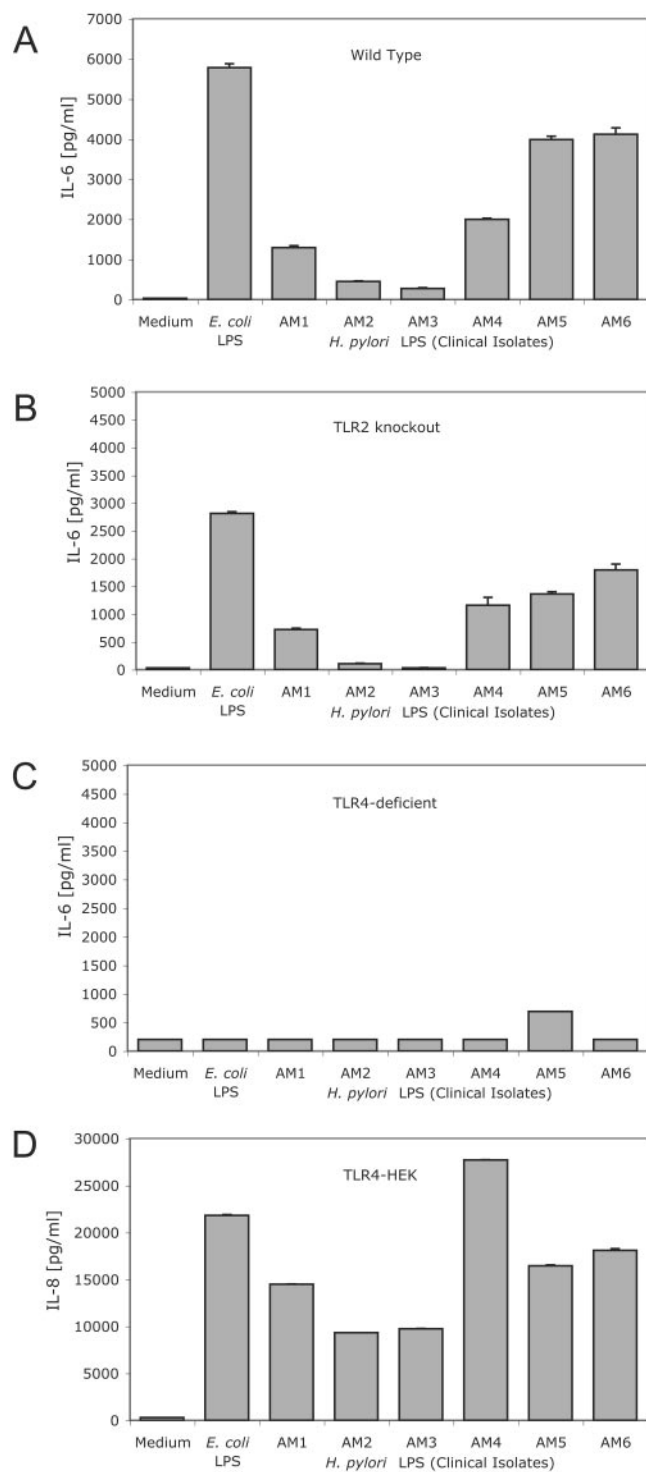


FIG. 7. LPS preparations from clinical isolates of *H. pylori* are TLR4 ligands. Wild-type peritoneal exudate macrophages (A), TLR2 knockout macrophages (B), TLR4-deficient macrophages (C), or TLR4-expressing HEK293 cells (D) were stimulated with medium alone, LPS isolated from *E. coli* O11B4, or LPS from clinical isolates of *H. pylori*. All LPS stimulations were done with protein-free LPS re-extracted with 10 ng of phenol/ml. *H. pylori* LPS was prepared from six different clinical isolates (designated AM1 to -6) as detailed in Materials and Methods. Following an 18-h incubation, supernatants were harvested and IL-6 (macrophages) or IL-8 (TLR4-expressing HEK293 cells) levels were determined by ELISA.

by the interaction of the potent TLR4 ligand with LPS in their outer membrane (reviewed in reference 43). Although the LPS produced by most gram-negative bacteria activates via TLR4, a reported notable exception is the LPS of *Porphyromonas gingivalis*, which is a ligand for TLR2 but not TLR4 (48). Our studies of the LPS derived from different *H. pylori* isolates indicate that *H. pylori* LPS is a TLR4 ligand similar to the LPS of other gram-negative strains, such as *E. coli* and *Salmonella* spp. Thus, the TLR2 dependence of the response to intact *H. pylori* cannot be explained by the expression of an unusual LPS.

Consistent with our finding, the previously published studies of Kawahara et al. (34, 36) demonstrated that the response to *H. pylori* LPS occurs via TLR4 in guinea pig gastric pit cells. In contrast, Smith et al. (74) recently reported that *H. pylori* LPS activated via TLR2, not TLR4. The reason for the discrepancy between their study and the present report (as well as the studies of Kawahara et al. [34, 36]) is not clear. However, by using the same experimental approach as Smith et al. (74), i.e., transfected HEK293 cells, we have found that *H. pylori* LPS from clinical isolates signals preferentially via TLR4. Furthermore, with knockout macrophages we found that *H. pylori* LPS does not elicit a cytokine response from TLR4-deficient macrophages; the response of TLR4-deficient macrophage to TLR2 ligands such as zymosan, peptidoglycan, and lipopeptides was indistinguishable from the response of wild-type macrophages, indicating that the TLR4-deficient macrophages had an intact TLR2-signaling capacity. One possible explanation for the discrepancy between our results (as well as the studies of Kawahara et al. [36]) and those of Smith et al. (74) is the strain of bacteria from which the LPS was isolated. Smith et al. (74) used only one *H. pylori* strain (26695), a heavily passaged laboratory strain, to prepare their LPS. In the present studies, we used a number of clinical isolates of *H. pylori* for LPS purification. It has been previously established that clinical isolates and laboratory strains of *H. pylori* have very different LPS molecules and that passage of *H. pylori* and culture conditions can induce variation in the LPS structures expressed (54, 55, 71).

It is also noteworthy that the dosages of *H. pylori* LPS used for cytokine stimulation in the present study were much lower than the dosages at which Smith et al. detect TLR2 agonist activity in their *H. pylori* LPS (i.e., nanograms per milliliter compared to micrograms per milliliter) (74). A potential difference between the *H. pylori* LPS studied by Smith et al. (74) and the LPS preparations in the present study may be the level of trace contaminants such as peptidoglycan or lipopeptides, both potent TLR2 agonists (26, 38, 72).

Despite the capacity of *H. pylori* LPS to activate via TLR4, the response to intact *H. pylori* bacteria was dependent on TLR2, and not TLR4, expression. This suggests that a non-LPS component of the bacterium is the major cytokine-activating molecule. It also raises the question of why it is difficult to detect a TLR4-dependent response to *H. pylori* LPS on the intact bacterium. One reason may be that *H. pylori* LPS is only weakly active as a cytokine inducer, even under ideal conditions, i.e., when it is added in a purified form to cultured cells. We and others (Fig. 7 and references 5, 58, 63, and 64) have noted that *H. pylori* LPS is 100- to 10,000-fold less active than the LPS of other gram-negative bacteria, such as *E. coli*. It is estimated that *E. coli* bacteria yield 10 ng of LPS per 10<sup>6</sup> CFU

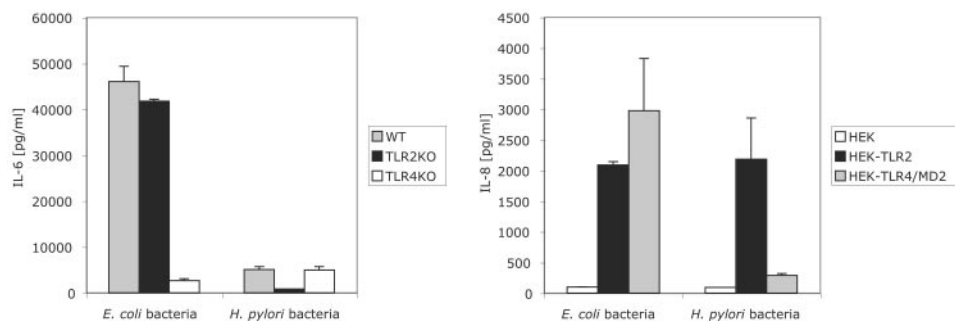


FIG. 8. *E. coli* bacteria activate cytokine secretion via both TLR2 and TLR4. Wild-type (WT), TLR2 knockout (KO), and TLR4 knockout mouse peritoneal exudate macrophages were stimulated with *E. coli* or *H. pylori* bacteria ( $2.5 \times 10^5$  CFU/well, left panel). Supernatants were harvested 18 h later, and IL-6 levels were determined by ELISA. (IL-6 levels were less than 200 pg/ml in medium controls.) HEK293 cells transfected with TLR2 or TLR4 were stimulated with *E. coli* or *H. pylori* bacteria ( $10^6$  CFU/well, right panel). Supernatants were harvested 18 h later, and IL-8 levels were determined by ELISA. (IL-8 levels in medium controls were less than 200 pg/ml in HEK and HEK-TLR2 cells and 350 pg/ml in HEK-TLR4 cells.)

of bacteria (66). Given its weak intrinsic activity (1,000-fold lower than the activity of *E. coli* LPS), a response to *H. pylori* LPS on intact bacteria would be difficult to detect with challenge doses of less than  $10^8$  to  $10^9$  CFU even if all of the *H. pylori* LPS were exposed and accessible for interaction with TLR4.

Although the LPS of *H. pylori* is apparently not the major determinant of the response to intact *H. pylori* bacteria, the LPS-TLR4 interaction may, nevertheless, play a role in disease pathogenesis. This may be particularly important in the stomach, where we and others have failed to detect TLR2 expression (60; unpublished observations). In contrast, TLR4 has been detected on gastric pit cells, where it may sample the stomach environment for pathogens (34). In the absence of TLR2, the interaction of *H. pylori* LPS with TLR4 may be critical to the early detection of *H. pylori* infection or colonization. Several previous studies have suggested that *H. pylori* may activate the innate immune response through interactions between its LPS and TLR4. Initial work by Sakagami et al. (68) demonstrated that C3H/He mice (with an intact TLR4-encoding gene) show severe atrophic gastritis in response to *H. felis* infection, whereas C3H/HeJ mice (which have a mutated TLR4-encoding gene and are LPS nonresponders) show heavy colonization but minimal atrophic gastritis with a much reduced macrophage infiltration of the lamina propria. Recently, Panthel et al. (61) showed that, in short-term colonization studies, TLR2 knockout mice had reduced colonization compared to C57BL/6J mice, but the degree of gastric inflammation was not reported. In addition, strain differences play an important role in colonization, as C57BL/6 mice are reported to have lower levels of colonization than either C3H/HeN or C3H/HeJ mice (46). The importance of TLR4 in the *H. pylori* response has been supported by work by another group that has reported that guinea pig gastric pit cells express TLR4 and show significant responses to *H. pylori* LPS (34, 36). Another group has suggested that *H. pylori* NF- $\kappa$ B activation in macrophages also involves TLR4 and CD14 (45).

Nevertheless, *H. pylori* LPS has been shown to be a weak inducer of TLR4 activation with a potency that is 1,000- to 10,000-fold less than that of LPS from *E. coli* or *S. enterica* (5, 58, 63, 64). The lower bioactivity of *H. pylori* LPS compared to that of *E. coli* LPS may reflect a lower interaction with TLR4 and/or MD2. The biochemical basis of the lower activity of

*H. pylori* LPS compared to that of *E. coli* LPS is the underphosphorylation of lipid A, as well as the presence of longer-chain fatty acids than normally encountered with *E. coli* LPS (53, 56). In addition, *H. pylori* LPS generally contains only four fatty acids (56) rather than the six-fatty-acid architecture seen in *E. coli* and other bioactive LPS molecules (53). Given its relatively weak bioactivity, *H. pylori* LPS may not contribute to the overall profile of bacterial stimulation or signaling seen with intact *H. pylori* bacteria. Furthermore, it has also been shown that *H. pylori* can activate cells by an LPS-independent and TLR4-independent mechanism (4, 47). The importance of the latter pathway relative to the LPS-dependent pathway in macrophages has not been examined.

Our studies reveal a role for genes in the *cag* pathogenicity island. In humans the induction of cytokine gene expression in infected stomach tissue has been linked to the level of colonization with *cagA*<sup>+</sup> *H. pylori* bacteria (73, 81). *cagA*<sup>+</sup> *H. pylori* bacteria were more potent activators of TLR2-expressing cells than *cagA* mutant *H. pylori*, suggesting that *cagA* or other associated *cag* pathogenicity island genes may themselves stimulate cytokine secretion by activating TLR2 and CD14 receptors. Alternatively, *cag* pathogenicity island-encoded genes may regulate the expression levels of *H. pylori* components that signal through TLR2 receptors. (Although *cagA* is a useful marker for the *cag* pathogenicity island, *cagA*<sup>+</sup> strains of *H. pylori* SS1 may have an incomplete *cag* region [13, 69].) In previous studies (reviewed in 21), *cagA* has been linked to IL-8 secretion from epithelial cells and is proposed to involve the translocation of the CagA protein into epithelial cells and its subsequent intracellular phosphorylation and activation of IL-8-encoding gene expression (20, 25). Our studies suggest that *cagA*-induced IL-8 secretion from transfected HEK cells is a TLR2-dependent process, since HEK cells lacking TLR2 failed to secrete IL-8 when challenged with *cagA*<sup>+</sup> or *cagA* mutant *H. pylori* bacteria. Nevertheless, *cagA* expression was not required for TLR2-induced cytokine secretion, since *cagA* mutant strains of *H. pylori*, as well as *H. felis* and *H. hepaticus*, which do not express *cagA*, all induced cytokine secretion via TLR2.

In conclusion, our studies demonstrate that TLR2 is both necessary and sufficient for responses to whole bacteria of several *Helicobacter* species. A major, nonredundant role for

TLR2 in the recognition of intact *Helicobacter* bacteria was seen both with cell lines stably transfected with TLRs and with normal cells from wild-type, TLR2 knockout, and TLR4 knockout animals. Moreover, TLR4 was neither necessary nor sufficient for responses to intact *Helicobacter* bacteria. In contrast to the TLR2-dependent response to intact *Helicobacter* bacteria, LPS as a pure lipid product extracted from the outer membrane of *Helicobacter* bacteria and depleted of lipoproteins and glycan cell wall components was a ligand for TLR4 but its activity was variable. We demonstrated this with both knockout animals and transfected cell lines. Thus, TLR4 (but not TLR2) was both necessary and sufficient for response to the isolated bacterial lipid, while TLR2 was required for response to intact *Helicobacter* bacteria. (Control cultures demonstrated that intact *E. coli* bacteria activated cytokine secretion via both TLR2 and TLR4 and that TLR4 was the dominant receptor on macrophages, consistent with the potent agonist activity of *E. coli* LPS.)

We hypothesize that, upon initial infection in the stomach, intact *H. pylori* bacteria may be weakly recognized by interaction of its LPS with TLR4. However, a substantial inflammatory cytokine response to *H. pylori* may only develop after the recruitment and accumulation of TLR2-expressing cells, such as infiltrating blood leukocytes (polymorphonuclear granulocytes and monocytes) in the stomach (38). Thus, *H. pylori* may escape detection and elimination by the immune system because it colonizes a TLR2-deficient environment (i.e., the stomach) and at the same time expresses an LPS with very weak TLR4 agonist activity.

#### ACKNOWLEDGMENTS

This work was supported by grants from the National Institutes of Health (RO1 AI51415 [to E.K.J.], RO1 CA93405 [to T.C.W.], RO1 AI37750, and RO1 CA67529 [to J.G.F.]) and the Irish Health Research Board (to A.P.M.).

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