Toll-like receptor (TLR) 2 induced through TLR4 signaling initiated by Helicobacter pylori cooperatively amplifies iNOS induction in gastric epithelial cells

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The purpose of the present study is to elucidate the implications of TLR2 and TLR4 in gastric epithelial cells in the initiation of the innate immune response against the virulent factors of this organism.

The innate immune response is initiated by cell surface Toll-like receptors (TLRs) that recognize widely conserved molecular patterns on the microorganism’s surface (1, 2). Such pathogen-associated pattern recognition is essential for innate immune cells to discriminate between self and microbial non-self, and TLRs play an important role in host defense and tissue repair responses, thus maintaining mucosal homeostasis (6). To date, 11 mammalian TLRs have been isolated based on their divergence of molecular structure and their binding affinity for specific ligands. A variety of pathogen cell surface molecules, such as lipopolysaccharide (LPS), peptidoglycan (PGN), lipoteichoic acid, lipoolarabinomannan, lipoprotein, and unmethylated DNA with a CpG motif, are recognized as pathogen-associated patterns by their specific TLRs (31).

H. pylori is a gram-negative bacterium, and, therefore, it possesses LPS as a virulent factor in its outer membrane (20–22). Microbial LPS is specifically recognized by TLR4, which induces various innate immune responses (6, 31). The activation of TLR4, in cooperation with the adaptor molecule MyD88, signals the downstream mitogen-activating protein kinase (MAPK) pathway, which subsequently activates the nuclear factor-κB (NF-κB) system (6, 31). Inducible nitric oxide synthase (iNOS) is a target molecule for TLR4 signaling activated by microbial LPS (3, 7), and iNOS-derived nitric oxide (NO) is well-known to play important roles in various inflammatory responses (4, 5). Gastric mucosal cells express TLR4 on their cell surface membrane (11, 27), and the induction of iNOS is also reported in gastric epithelial cells following exposure to E. coli-LPS (32). Therefore, it is conceivable that H. pylori-LPS can activate the innate immune response of gastric epithelial cells via pattern recognition by the TLR4 system (13, 15, 17, 27). In contrast, recent studies also suggest that H. pylori-induced innate immune responses are (at least partially) mediated through TLR2 in vitro and in vivo (8, 17, 18, 28), although TLR2 is generally considered to respond to PGN and lipoproteins derived from gram-positive bacteria and mycobacteria (25).

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innate immune response against \textit{H. pylori} infection. Our in vitro study investigated the influence of \textit{H. pylori}-LPS on the expression of TLR2 and TLR4 and on the pathway of signal propagation using a mouse normal gastric epithelial GSM06 cell line (30). The innate immune response to \textit{H. pylori}-LPS in GSM06 was monitored with the induction of iNOS and its associated NO production. In vivo TLR2 expression was assessed in human gastric mucosa, with or without \textit{H. pylori} infection by immunohistochemistry.

**MATERIALS AND METHODS**

\textit{H. pylori}, \textit{H. pylori}, cytotoxin-associated gene-A-positive standard species ATCC no. 43504 (the American Type Culture Collection, Manassas, VA), was grown using a large-scale cultivation technique kindly provided by Dr. Suzuki (Keio University). Briefly, \textit{H. pylori} was inoculated and grown in Brucella broth (BD Diagnostic Systems, Franklin Lakes, NJ) containing 5% heat-inactivated horse serum (56°C for 30 min), under a microaerophilic condition at 37°C for 18–20 h, with rotary shaking at 60 rpm using the AnaeroPack system (Mitsubishi Gas Chemical, Tokyo, Japan), which contains oxygen-absorbing and carbon dioxide-producing agents to provide a microaerophilic environment of ~14% carbon dioxide and 6% oxygen, ideal for most Campylobacter species and \textit{H. pylori}. Thereafter, \textit{H. pylori} was harvested at a concentration of ~1 × 10^7 colony-forming units/ml.

Microbial LPS was extracted using Moran’s hot phenol-water method (20). Briefly, the bacteria were dried with cold acetone and treated with pronase (Nacalai Tesque, Kyoto, Japan), and the LPS was extracted using 45% aqueous phenol at 68°C for 30 min. The crude LPS was purified by treating with RNase A, DNase II (Sigma-Aldrich, St. Louis, MO), and proteinase K (Nacalai Tesque), followed by centrifugation at 100,000 g for 18 h. The purity of the LPS was confirmed by SDS-PAGE, and the activity was determined colorimetrically using a standard endotoxin CSE-H kit, an Endospecy centrifugation at 100,000 M), and proteinase K (Nacalai Tesque), followed by centrifugation at 100,000 g for 18 h. The purity of the LPS was confirmed by SDS-PAGE, and the activity was determined colorimetrically using a standard endotoxin CSE-H kit, an Endospecy centrifugation at 100,000 g for 18 h.

When the cultures were 80–90% confluent, the cells were cultured in When the cultures were 80–90% confluent, the cells were cultured in Dulbecco’s modified Eagle’s medium F-12 supplemented with 10% low endotoxin fetal bovine serum, antibiotics, antimycotics, and insulin-transferrin selenium X (Invitrogen, Carlsbad, CA). When the cultures were 80–90% confluent, the cells were cultured in Dulbecco’s modified Eagle’s medium F-12 supplemented with 10% low endotoxin fetal bovine serum, antibiotics, antimycotics, and insulin-transferrin selenium X (Invitrogen, Carlsbad, CA). When the cultures were 80–90% confluent, the cells were cultured in Dulbecco’s modified Eagle’s medium F-12 supplemented with 10% low endotoxin fetal bovine serum, antibiotics, antimycotics, and insulin-transferrin selenium X (Invitrogen, Carlsbad, CA).

**Cell culture.** GSM06 cell line (Riken Cell Bank, Tsukuba, Japan) was cultured in Dulbecco’s modified Eagle’s medium F-12 supplemented with 10% low endotoxin fetal bovine serum, antibiotics, antimycotics, and insulin-transferrin selenium X (Invitrogen, Carlsbad, CA). When the cultures were 80–90% confluent, the cells were cultured in Dulbecco’s modified Eagle’s medium F-12 supplemented with 10% low endotoxin fetal bovine serum, antibiotics, antimycotics, and insulin-transferrin selenium X (Invitrogen, Carlsbad, CA). When the cultures were 80–90% confluent, the cells were cultured in Dulbecco’s modified Eagle’s medium F-12 supplemented with 10% low endotoxin fetal bovine serum, antibiotics, antimycotics, and insulin-transferrin selenium X (Invitrogen, Carlsbad, CA). When the cultures were 80–90% confluent, the cells were cultured in Dulbecco’s modified Eagle’s medium F-12 supplemented with 10% low endotoxin fetal bovine serum, antibiotics, antimycotics, and insulin-transferrin selenium X (Invitrogen, Carlsbad, CA). When the cultures were 80–90% confluent, the cells were cultured in Dulbecco’s modified Eagle’s medium F-12 supplemented with 10% low endotoxin fetal bovine serum, antibiotics, antimycotics, and insulin-transferrin selenium X (Invitrogen, Carlsbad, CA). When the cultures were 80–90% confluent, the cells were cultured in Dulbecco’s modified Eagle’s medium F-12 supplemented with 10% low endotoxin fetal bovine serum, antibiotics, antimycotics, and insulin-transferrin selenium X (Invitrogen, Carlsbad, CA).
protected least squares difference test. A minimum of three separate samples and analyzed by Fisher’s test to be statistically significant.

NO assay. The NO levels in the culture medium were measured indirectly as hydrolyzed NO derivatives (NOx: nitrates and nitrites) using the Griess reagent kit (Dojindo Molecular Technologies, Kumamoto, Japan), in accordance with the manufacturer’s instructions. The amount of culture medium per well was normalized to the total cell number in individual samples. The NOx concentration in the medium was determined by measuring absorbance at 540 nm and using a standard curve.

Immunohistochemistry of TLR2. Gastric mucosal specimens, with or without H. pylori infection (6 cases each), obtained by endoscopic biopsy from the gastric antrum with patient consent were examined by immunohistochemistry. The specimens were fixed in 10% buffered formalin and embedded in paraffin. The sections were incubated with the primary antibody for TLR2 (1:100; H-175, Santa Cruz Biotechnology) overnight at 4°C, followed by reaction with the secondary antibody and detected using a modified streptoavidin-biotin method (Vectorstain ABC kit, Vector, Burlingame, CA). The antigen-antibody complexes were visualized by immersion in 3,3’-diaminobenizidine, 0.05 M Tris-diaminobenizidine complexed were visualized by immersion in 3,3’-diaminobenizidine solution (0.001 M 3,3’-diaminobenizidine, 0.05 M Tris/HCl buffer, pH 7.6, 0.01 M sodium azide, and 0.006% hydrogen peroxidase). The specificity of immunohistochemical staining was monitored in sections processed without the primary antibody (data not shown). This study protocol was approved by the ethical committee of Tohoku University School of Medicine.

Statistical analysis. The data are expressed as the means ± SD of a minimum of three separate samples and analyzed by Fisher’s protected least squares difference test. A P value <0.05 was defined to be statistically significant.

RESULTS

Expression of TLR2 and TLR4 in GSM06 cells and influence of treatment with H. pylori-LPS and/or IFN-γ. The levels of TLR2 and TLR4 mRNA in GSM06 cells were assessed in the presence or absence of H. pylori-LPS and IFN-γ by RT-PCR. Untreated cells exhibited very low expression of TLR2 mRNA, but H. pylori-LPS caused cells to increase TLR2 expression in a dose-dependent manner (Fig. 1A). IFN-γ (10–1,000 U/ml) alone did not affect the expression of TLR2 mRNA; nevertheless, this cytokine dose dependently induced expression with H. pylori-LPS to induce TLR2 mRNA (Fig. 1A). In contrast, TLR4 mRNA was constitutively expressed such that neither H. pylori-LPS nor IFN-γ influenced its expression level compared with control cells (Fig. 1A). Changes in protein levels were consistent with the mRNA data showing that IFN-γ alone did not affect the expression of TLR2 protein, but cotreatment with H. pylori-LPS synergistically induced the expression of TLR2 protein (Fig. 1B). The observation that the expression level of TLR2 protein significantly increased in the cells treated with H. pylori-LPS and IFN-γ but not with IFN-γ alone was confirmed by the densitometric analyses of blots (P < 0.0001, Fig. 1C). TLR4 protein was unchanged by these treatments, as was the mRNA (Fig. 1, B and C).

Participation of TLR4 signaling initiated by H. pylori-LPS in TLR2 induction. The expression levels of TLR2 and TLR4 diminished following transfection of GSM06 cells with siRNAs specific for mouse TLR2 and TLR4, respectively. Under unstimulated conditions, the cells transfected with TLR2- and TLR4-siRNAs exhibited declines in mRNA expression to 79.2 ± 2.3 and 76.4 ± 4.9% of the control values by 24 h posttransfection, and to 66.1 ± 4.2 and 62.9 ± 3.6% by 96 h, respectively (data not shown). The control siNC transfection did not affect the expression levels of TLR2 or TLR4 from 24 to 96 h posttransfection (data not shown).

TLR4 mRNA expression remained unchanged in TLR2-diminished cells vs. control siNC cells, but was significantly reduced in TLR4-diminished cells, regardless of treatment with H. pylori-LPS and IFN-γ (Fig. 2A). TLR2 mRNA expression was low in control siNC cells under the unstimulated condition
IFN-γ/H9253. Furthermore, TLR4-diminished cells also
significantly inhibited in TLR2-diminished cells as a conse-
sequence of TLR2 by Hp-LPS and IFN-γ. Changes in mRNA expression were assessed by RT-PCR for TLR4 and TLR2
and were quantified as densitometric ratios of TLR2 and TLR4/β-actin mRNA. A: TLR4 expression was unaltered in control and TLR2-diminished cells, but was significantly low in TLR4-diminished cells, regardless of treatment. B: TLR2 induction by the treatment was significantly inhibited in TLR2- and TLR4-diminished cells. Values are expressed as the means ± SD from three independent experiments. **P < 0.01: sINC with Hp-LPS + IFN-γ treatment vs. untreated control.

and significantly increased in response to H. pylori-LPS and IFN-γ stimulation (Fig. 2B). Such increase in TLR2 mRNA expression induced by H. pylori-LPS and IFN-γ treatment was significantly inhibited in TLR2-diminished cells as a consequence of effective interference of TLR2 mRNA transcription by siTLR2 (Fig. 2B). Furthermore, TLR4-diminished cells also exhibited a significant reduction of TLR2 induction by H. pylori-LPS and IFN-γ treatment to nearly untreated control level (Fig. 2B), suggesting that TLR4 signaling may be essential for TLR2 induction by H. pylori-LPS and IFN-γ stimulation.

Possible signaling pathways of TLR2 induction by H. pylori-
LPS stimulation. The activation of ERK, p38, and JNK in GSM06 treated with H. pylori-LPS and IFN-γ was investigated by Western blotting using total and phosphorylation-specific antibodies. IFN-γ alone did not affect the phosphorylation levels of MAPKs compared with untreated cells (Fig. 3). In cells treated with H. pylori-LPS and IFN-γ, phosphorylated ERK protein, but no others, was clearly observed (Fig. 3).

Next, the activation of NF-κB was assessed by ELISA-based quantification of the DNA-binding p65 NF-κB molecule. The cells were pretreated with specific inhibitors for MAPKs and NF-κB, i.e., PD98059 (for ERK), SP600125 (for JNK), SB203580 (for p38), and PTDTC (for NF-κB) before treatment with H. pylori-LPS and IFN-γ. The cells treated with H. pylori-LPS and IFN-γ in the absence of inhibitor pretreatment showed a significant increase in the level of activated NF-κB compared with the control value (Fig. 4). On the one hand, when cells were pretreated with PTDTC and PD98059, NF-κB activation significantly diminished in a dose-dependent manner.

Fig. 2. Influences of declines in TLR2 and TLR4 expression on the induction of TLR2 by Hp-LPS and IFN-γ treatment. The negative control small interfering RNA (sINC) cells, small interfering TLR (siTLR) 2-, and siTLR4- diminished cells were treated with Hp-LPS (50 ng/ml) vs. TLR2- or TLR4-diminished cells. Values are expressed as the means ± SD from three independent experiments. **P < 0.01: sINC with Hp-LPS + IFN-γ treatment vs. TLR2- or TLR4-diminished cells.

Fig. 3. Activation of mitogen-activating protein (MAP) kinases by treatment with Hp-LPS and IFN-γ. The activation of MAP kinases in GSM06 cells treated with IFN-γ (1,000 U/ml) alone (left column) or Hp-LPS (50 ng/ml) + IFN-γ (1,000 U/ml) (right column) was analyzed by Western blotting using anti-phosphorylation-specific and total extracellular signal-regulated kinase (ERK) (top panels), p38 (middle panels), and c-Jun NH2-terminal kinase (JNK) (bottom panels) antibodies. Each pair of blots was run side by side. The marked phosphorylation of ERK, but neither p38 nor JNK, was identified in the cells treated with Hp-LPS + IFN-γ, while IFN-γ alone did not phosphorylate MAP kinases. The results represent three independent experiments.

Fig. 4. Activation of nuclear factor-κB (NF-κB) by treatment with Hp-LPS and IFN-γ and effects of inhibitors for NF-κB and MAP kinases. NF-κB activation in GSM06 cells, with or without pretreatments with pyrrolidine dithiocarbamate (PDTC) (10, 100 μM), PD98059 (1, 10 μM), SB203580 (1, 10 μM), and SP600125 (1, 10 μM) before Hp-LPS + IFN-γ stimulation, was quantitatively assessed as free p65 NF-κB binding activity. Treatment with Hp-LPS + IFN-γ significantly increased free p65 NF-κB binding activity. Pretreatment with either PTDTC or PD98059 inhibited NF-κB activation in a dose-dependent fashion. Neither SB203580 nor SP600125 elicited an inhibitory effect on NF-κB activation. A450, absorbance at 450 nm. Values are expressed as means ± SD from four independent experiments. *P < 0.05 and **P < 0.01 vs. untreated control. ¥P < 0.01: untreated control vs. Hp-LPS + IFN-γ. ¥¥P < 0.05: PTDTC 10 vs. 100 μM. SP < 0.05: PD98059 10 vs. 100 μM.
Roles of TLR2 and TLR4 in iNOS induction as an innate immune response against H. pylori-LPS. The innate immune response in GSM06 cells treated with H. pylori-LPS and/or INF-γ was monitored by changes in iNOS mRNA level and its associated NO production. The treatment with H. pylori-LPS alone was very weak in iNOS induction and NO production, whereas INF-γ alone significantly induced these responses in GSM06 cells (P < 0.0001, Fig. 6A). Furthermore, treatment with H. pylori-LPS together with INF-γ significantly multiplied the increment of iNOS mRNA and NO production compared with their individual treatments (P < 0.0001, Fig. 6A). This induction of iNOS mRNA was inhibited by PDTC in a dose-dependent manner (Fig. 6B).

Next, the influence of declines in TLR2 and TLR4 on iNOS induction by H. pylori-LPS and INF-γ stimulation was investigated. siNC cells responded to treatment with H. pylori-LPS and INF-γ with a significant increase in iNOS expression (Fig. 7). As anticipated, such iNOS induction by H. pylori-LPS and INF-γ stimulation was significantly inhibited in TLR4-diminished cells (P < 0.01, Fig. 7). Surprisingly, TLR2-diminished cells, which expressed TLR4 at the same level as siNC cells, as shown in Fig. 2A, also exhibited a significant reduction of iNOS induction by H. pylori-LPS and INF-γ stimulation (P < 0.01, Fig. 7). In other words, declines in not only TLR4 but also TLR2 attenuated responsiveness to treatment with H. pylori-LPS and INF-γ. These data were corroborated by changes in NO production. Following treatment with H. pylori-LPS and INF-γ, NOx content as well as iNOS mRNA markedly increased in siNC cells, but such increase in NO production was significantly inhibited in both TLR2- and TLR4-diminished cells (P < 0.0001, Fig. 8).

To investigate whether TLR2 responsiveness to H. pylori-LPS was particular to this pathogen, the ligand-specific reaction for individual receptors was also assessed in TLR2- and TLR4-diminished cells by treatment with PAM2CSK4 (for TLR2) and E. coli-LPS (for TLR4) in the presence of INF-γ. On the one hand, an increase in NO production (NOx content) following E. coli-LPS stimulation was observed in both siNC cells and TLR2-diminished cells, but was significantly inhibited in TLR4-diminished cells (P < 0.0001, Fig. 8). On the other hand, there was no significant inhibition following pretreatment with SB203580 or SP600125 (Fig. 4).

In addition, the influence of pretreatment with the inhibitors on H. pylori-LPS- and INF-γ-induced TLR2 mRNA was investigated by RT-PCR. The induction of TLR2 was blocked to nearly control levels by PDTC and was reduced in a dose-dependent fashion by PD98059 (Fig. 5). However, neither SB203580 nor SP600125 attenuated TLR2 induction in the treated cells (Fig. 5).

Fig. 5. Effects of NF-κB and MAP kinase inhibitors on TLR2 induction upon treatment with H. pylori-LPS and INF-γ. TLR2 expression in GSM06 cells pretreated with PDTC (10, 100 μM), PD98059 (10, 100 μM), SB203580 (1, 10 μM), and SP600125 (1, 10 μM) before H. pylori-LPS (50 ng/ml) + INF-γ (1,000 U/ml) treatment was assessed. PDTC blocked TLR2 induction to nearly control levels, and PD98059 also reduced it in a dose-dependent fashion. Neither SB203580 nor SP600125 attenuated TLR2 induction. The results represent three independent studies.
resulted in the loss of functions specific to these receptors. NO cated that declines in TLR2 or TLR4 by siRNA treatments /H11021 diminished cells (P<0.0001, Fig. 8). PAM₃CSK₄ was also weak in NO induction (P<0.0001, Fig. 8), reflecting low TLR2 expression in the cells (Fig. 2B). Nevertheless, NO production was significantly high upon every treatment compared with the untreated control (P<0.0001, Fig. 8), as cotreatment with IFN-γ may synergistically enhance NO production in addition to ligand-specific reaction, as shown in Fig. 6A.

Increased expression of TLR2 in human gastric mucosa infected with _H. pylori_. An immunohistochemical analysis of TLR2 expression was performed using biopsy specimens obtained from human gastric mucosa, with or without _H. pylori_ infection. _H. pylori_-infected gastric mucosa showed an intense reaction on the apical surface of the epithelial cells and on invading inflammatory cells (Fig. 9, A, B, and C). However, gastric mucosa without _H. pylori_ infection exhibited very little immune reaction against TLR2 antibody (Fig. 9D).

**DISCUSSION**

TLRs are thought to play important roles in the first line of defense against _H. pylori_ infection in gastric epithelial cells; however, their pathophysiological roles have not been completely resolved. In the present study, we investigated innate immune responses to _H. pylori_-LPS through the action of TLR2 and TLR4 using the mouse normal gastric epithelial GSM06 cell line (30). This cell line is derived from transgenic mice harboring the temperature-sensitive SV40 large T-antigen gene and exhibits 1) nontransformed cell characteristics similar to gastric mucosal cells, and 2) significant homologous sequences in TLR2 and TLR4 proteins with humans (NIH Blast2). IFN-γ was used in the present study, since this Th1 cytokine is known to play a crucial role in _H. pylori_-associated gastritis, i.e., IFN-γ mediates the induction of iNOS by _H. pylori_ in mouse gastric epithelial cells (24).

![Fig. 8](https://example.com/image8.png)  
**Fig. 8.** iNOS-associated NO production induced by _H. pylori_-, _E. coli_ (Ec)-LPS, and PAM₃CSK₄ treatment with Ec-LPS (50 ng/ml), IFN-γ (1,000 U/ml) were assessed in siNC cells and TLR2- and TLR4-diminished cells. Every treatment significantly increased NO production compared with the untreated control (P<0.0001). Values are expressed as means±SD from three independent experiments. **P<0.0001 vs. siNC cells stimulated with Ec-LPS + IFN-γ, and §§P<0.0001 vs. siNC cells stimulated with PAM₃CSK₄ + IFN-γ.

![Fig. 9](https://example.com/image9.png)  
**Fig. 9.** Immunohistochemistry of TLR2 in human gastric mucosa, with or without Hp infection. A: immunohistochemistry of TLR2 with formalin-fixed and paraffin-embedded specimens from human gastric antral mucosa with Hp infection. A positive reaction of TLR2 staining was identified at the apical surface of the epithelial cells and on invading inflammatory cells. Black squares in A indicate enlarged images of TLR2 expression corresponding to the apical surface of gastric epithelial cells (B) and invading inflammatory cells (C). D: noninflamed gastric mucosa without Hp infection exhibited little expression of TLR2. Original magnifications of A and D are at x40.
GSM06 cells exhibited constitutive expression of TLR4 such that neither *H. pylori*-LPS nor IFN-γ influenced the level of TLR4 mRNA or protein compared with control cells. While IFN-γ alone had no effect on TLR2 levels, there was a small but significant increase in TLR2 expression upon treatment with *H. pylori*-LPS. Furthermore, IFN-γ, together with *H. pylori*-LPS, synergistically enhanced TLR2 induction, suggesting that this cytokine may play a role in the posttranscriptional stabilization of TLR2 mRNA induced by *H. pylori*-LPS. In comparison, the transcriptional activation of iNOS by IFN-γ results from the IFN-γ-respecting element located in the promoter region of the iNOS gene (12).

Participation of TLR4 signaling in the induction of TLR2 by *H. pylori*-LPS stimulation was corroborated in TLR2- and TLR4-diminished cells using the siRNA technique. TLR2 induction was significantly inhibited in TLR4-diminished cells compared with control siNC cells, although TLR4 siRNA did not interfere with the expression of TLR2 mRNA directly. In contrast, TLR4 expression was unchanged in TLR2-diminished cells. The facts suggest that TLR2 induction in GSM06 cells possibly results from the interaction between *H. pylori*-LPS and TLR4. Previous studies reported TLR2 induction via the interaction of a microbial LPS with TLR4 in mouse macrophages (18) and endothelial cells (9), an observation that is consistent with the present data. Furthermore, TLR2 induction by LPS stimulation is reported to be severely impaired in splenic macrophages isolated from TLR4-deficient C3H/HeJ mice (18), suggesting that TLR4 plays an essential role in the process. Controversially, TLR4-independent TLR2 induction by exposure to high concentrations of LPS is also reported in peritoneal macrophages from C3H/HeJ mice (16).

In general, the activation of TLR4, in cooperation with the adaptor molecule MyD88, signals the downstream MAPK pathway, which subsequently activates the NF-κB system (6, 31). TLR4 signaling for TLR2 induction initiated by *H. pylori*-LPS stimulation is possibly propagated through phosphorylation of the ERK pathway and, subsequently, NF-κB activation. Several lines of evidence support this notion: 1) phosphorylation of ERK but not p38 or JNK was identified in cells treated with *H. pylori*-LPS and INF-γ; 2) the level of activated p65 NF-κB was significantly increased in treated cells; 3) the activated NF-κB level significantly declined by pretreatment with the ERK inhibitor PD98059; and 4) TLR2 induction by *H. pylori*-LPS and INF-γ stimulation was inhibited by pretreatment with PD98059 and the NF-κB inhibitor PDTC. Neither p38 (SB203580) nor JNK (SP600125) inhibitors elicited such inhibitory effects. Thus TLR4 signaling through ERK-mediated NF-κB activation is crucial for TLR2 induction by *H. pylori*-LPS in gastric epithelial cells. Musikacharoen et al. (23) reported that the DNA element that binds activated NF-κB molecules is located in the promoter region of the mouse TLR2 gene, which is in agreement with our data.

In the present study, the innate immune response to *H. pylori*-LPS in GSM06 was monitored with the induction of iNOS and its associated NO production. iNOS is a target molecule for TLR4 signaling initiated by microbial LPS, not only in inflammatory cells (3, 7), but also in gastric epithelial cells (32). The induced iNOS in gastric epithelial cells exerts antimicrobial activity by NO attacking microorganisms (5). While iNOS-derived NO is possibly causative of gastric mucosal damages, it also works for exclusion of injured gastric epithelial cells by promoting apoptosis and eventually contributes to maintenance of gastric mucosa; therefore, it seems presumable that persistent inflammation without apoptosis in iNOS-deficient mice with *H. pylori* infection may be rather linked to pre-neoplastic transformation (19). Thus iNOS induction and its associated NO production play crucial roles in the formation of various pathological features in *H. pylori*-infected gastric mucosa.

*H. pylori*-LPS, with the synergistic assistance of IFN-γ and NF-κB activation, induced iNOS expression and increased NO production in GSM06 cells. In sharp contrast to TLR2 induction by *H. pylori*-LPS and/or INF-γ stimulation, iNOS induction and its associated NO production were also facilitated by treatment with IFN-γ alone. Such difference is possibly accounted for by the fact that transcriptional activation of the iNOS gene may occur via direct interaction of IFN-γ with the promoter region of this gene (12). TLR4-diminished cells significantly deteriorated the responsiveness to *H. pylori*-LPS stimulation, suggesting that TLR4 signaling initiated by *H. pylori*-LPS causes this response as well as TLR2 induction. Furthermore, TLR2-diminished cells also exhibited a low response to *H. pylori*-LPS stimulation, although TLR4 expression was unchanged in these cells. The observations suggest that induced TLR2 cooperates with TLR4 to amplify the innate immune response to *H. pylori*-LPS in GSM06 cells.

Such TLR2 responsiveness to *H. pylori*-LPS was particular to this pathogen, as demonstrated by the NO production assay: 1) TLR4-diminished cells lost the responsiveness to *E. coli*-LPS, but not to PAM3CSK4, and 2) TLR2-diminished cells conversely responded to *E. coli*-LPS, but not to PAM3CSK4. Thus declines in TLR2 or TLR4 expressions, respectively, deteriorated specific responses to corresponding ligands PAM3CSK4 or *E. coli*-LPS; therefore, the reduced responsiveness to *H. pylori*-LPS in TLR2-diminished cells may indicate a possible interaction between TLR2 and *H. pylori*-LPS. So far, no proof has been provided that TLR2 specifically binds to *H. pylori*-LPS; nevertheless, the possibility remains controversial, i.e., Smith et al. (28) reported with in vitro transfectants of gastric epithelial cell lines that TLR2 and TLR5, but not TLR4, are required for *H. pylori*-LPS-induced NF-κB activation. Differently, since repurification of LPS may eliminate signaling through both human and murine TLR2 (10), it seems possible that *H. pylori*-LPS employed in the present study may still retain other *H. pylori* components that react with TLR2 during the extraction process. Regardless of what pathogen contained in the *H. pylori* organism is able to interact with TLR2, this receptor is considered to play important roles in innate immune responses to *H. pylori* infection in gastric mucosa (17, 18, 28).

*H. pylori*-LPS itself is weak in pathogenic activity, with 1,000- to 10,000-fold less potency than other enterobacterial LPS (20–22); nevertheless, the interaction between *H. pylori*-LPS and TLR4 plays a significant role in the initiation and development of various pathological statuses in *H. pylori*-infected gastric mucosa, i.e., mitogen oxidase 1, which is known to play crucial roles in innate immune responses of gastric epithelium, was upregulated in guinea pig gastric pit cells by *H. pylori*-LPS-initiated TLR4 signaling (13). Furthermore, a previous in vivo study demonstrated that TLR4-intact C3H/He mice showed severe atrophic gastritis in response to *H. felis* infection, but that TLR4-deficient C3H/HeJ mice...
showed minimal atrophic gastritis with much reduced macrophage infiltration in the gastric mucosa, despite a heavy colonization of the bacteria (26). In contrast, although *H. pylori*-LPS can activate macrophages in a TLR4-dependent manner, Mandell et al. (17) pointed out the possibility that TLR2 might act as a dominant innate immune receptor for recognition of whole *H. pylori* organisms, because *H. pylori* may contain abundant components with a high affinity for TLR2, of which expression levels are regulated by *cag* pathogenicity island-encoded genes. Considering all of these observations together, the induction of TLR2 by TLR4 signaling initiated by *H. pylori*-LPS possibly indicates a positive correlation between TLR2 and TLR4 to intensify the innate immune responses of gastric epithelial cells against *H. pylori* infection.

Finally, our immunohistochemical data demonstrate that the expression of TLR2 was very weak in noninflected gastric mucosa, but clearly increased on the apical surfaces of gastric mucosal epithelial cells in *H. pylori*-infected patients. Concerning TLR4 expression in human gastric mucosa, Schmauss et al. (27) reported that TLR4 expression was identified at the apical and basolateral pole of human gastric epithelium and that its intensity tended to be strong in *H. pylori*-infected gastric mucosa. Controversially, a previous study with a cDNA microarray system reported that only TLR2 was not upregulated in *H. pylori*-infected gastric mucosa, suggesting a selective expression pattern of TLRs against this gram-negative bacterium (33). The difference from our data may be derived from the fact that increased TLR2 expression is limited to the apical surface of *H. pylori*-infected gastric mucosa or the fact that there are controversial results of TLR2 regulation in *H. pylori*-infected gastric mucosa. Contrary to this, some adapter proteins such as TLR4, adaptor Spins, and Grant 15087212 from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

**REFERENCES**


