

# Ceramide and Toll-Like Receptor 4 Are Mobilized into Membrane Rafts in Response to *Helicobacter pylori* Infection in Gastric Epithelial Cells

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*Helicobacter pylori* infection is thought to be involved in the development of several gastric diseases. Two *H. pylori* virulence factors (vacuolating cytotoxin A and cytotoxin-associated gene A) reportedly interact with lipid rafts in gastric epithelial cells. The role of Toll-like receptor (TLR)-mediated signaling in response to *H. pylori* infection has been investigated extensively in host cells. However, the receptor molecules in lipid rafts that are involved in *H. pylori*-induced innate sensing have not been well characterized. This study investigated whether lipid rafts play a role in *H. pylori*-induced ceramide secretion and TLR4 expression and thereby contribute to inflammation in gastric epithelial cells. We observed that both TLR4 and MD-2 mRNA and protein levels were significantly higher in *H. pylori*-infected AGS cells than in mock-infected cells. Moreover, significantly more TLR4 protein was detected in detergent-resistant membranes extracted from *H. pylori*-infected AGS cells than in those extracted from mock-infected cells. However, this effect was attenuated by the treatment of cells with cholesterol-usurping agents, suggesting that *H. pylori*-induced TLR4 signaling is dependent on cholesterol-rich microdomains. Similarly, the level of cellular ceramide was elevated and ceramide was translocated into lipid rafts after *H. pylori* infection, leading to interleukin-8 (IL-8) production. Using the sphingomyelinase inhibitor imipramine, we observed that *H. pylori*-induced TLR4 expression was ceramide dependent. These results indicate the mobilization of ceramide and TLR4 into lipid rafts by *H. pylori* infection in response to inflammation in gastric epithelial cells.

*Helicobacter pylori*, a spiral Gram-negative microaerophilic bacterium, can colonize gastric epithelial cells and is thought to infect approximately half of the human population (37, 43). *H. pylori* infection is associated with several clinical pathologies, including gastritis, peptic ulcer diseases, gastric adenocarcinoma, and mucosa-associated lymphoid tissue (MALT) lymphoma (6, 44).

Persistent infection of the gastric mucosa by *H. pylori* can induce nuclear factor  $\kappa$ B (NF- $\kappa$ B) activation and proinflammatory cytokine secretion, including interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-6, IL-8, and tumor necrosis factor alpha (TNF- $\alpha$ ) secretion (8, 21). Increased IL-8 secretion is associated with inflammatory severity in patients with *H. pylori*-induced gastritis (3). Furthermore, translocation of the protein encoded by *H. pylori* cytotoxin-associated gene A (CagA) leads to activation of IL-8 transcription through the NF- $\kappa$ B signaling pathway (8), suggesting that CagA plays a crucial role in *H. pylori*-induced inflammation in gastric epithelial cells. Moreover, several *H. pylori* virulence factors, including vacuolating cytotoxin (VacA), urease, and lipopolysaccharide (LPS), contribute to pathogenesis (10, 17, 36).

Among these bacterial virulence factors, VacA was the first to be isolated from detergent-resistant membranes (DRMs), commonly used to identify lipid rafts (16, 45, 48). Lipid rafts are microdomains within membranes that contain large amounts of cholesterol, phospholipids, and glycosylphosphatidylinositol (GPI)-anchored proteins (9, 22). Notably, cholesterol-rich microdomains are generally utilized by other bacterial toxins for entry or oligomerization (1, 42).

A recent study indicated that sphingomyelin is a novel VacA receptor in lipid rafts (20). Similarly, translocation of CagA is associated with lipid rafts and has been shown to be important for the CagA-induced pathogenesis of cells (30, 40). These studies suggest that cholesterol-rich membrane microdomains provide an essential ligand for toxin binding and may efficiently enhance *H. pylori*-induced pathogenesis of host cells.

Toll-like receptors (TLRs), as pattern recognition receptors, recognize conserved microbial components (2, 24, 39). For instance, TLR2 has been found to recognize lipoproteins and peptidoglycans in bacterial cell walls (49, 54). Moreover, TLR4 recognizes LPS of Gram-negative bacteria and activates NF- $\kappa$ B and activator protein-1 (AP-1) (13, 50, 62). Several reports have suggested that TLR2 contributes to *H. pylori* LPS-induced signaling

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(33, 57, 60). Other studies support the notion that *H. pylori*-induced signal transduction is mediated through TLR4 (25, 47, 53, 61). However, previous studies have shown that immune responses to intact *H. pylori* may not involve TLR4 (5, 15). Therefore, it remains unclear which TLRs mediate signal transduction during *H. pylori* infections.

Glycosphingolipids on host cells can be utilized as receptors for *H. pylori* adhesion (26, 46). A previous study showed that ceramide, the lipid portion of glycosphingolipids, is required for recognition of glycosphingolipids by *H. pylori* (55). Ceramide, generated from sphingomyelin by sphingomyelinase, localizes abundantly in membrane rafts and is involved in the regulation of apoptosis, cellular stress responses, and cell differentiation (19). Additionally, several pathogens generate ceramide-enriched membrane platforms from small primary rafts, and these platforms can serve as entry portals or otherwise facilitate pathogen infection (18). Although a number of molecules and various membrane compartments have been shown to participate in *H. pylori*-induced inflammation, the potential roles of TLR4 or ceramide mobilization into cell rafts, which may modulate inflammatory responses in *H. pylori*-infected cells, require further investigation.

In the present study, we examined whether lipid rafts are involved in the induction of ceramide secretion and TLR4/MD-2 expression during *H. pylori* infection of gastric adenocarcinoma epithelial cells. In addition, we investigated whether disruption of cholesterol-rich microdomains influences the levels of ceramide and TLR4 in the membrane, as well as *H. pylori*-induced IL-8 production. Our results provide insight into the molecular mechanisms underlying the function of lipid rafts, which serve as a platform for the clustering of ceramide and TLR4, crucial components for IL-8 secretion during *H. pylori* infection.

## MATERIALS AND METHODS

**Reagents and antibodies.** Rabbit anti-TLR4 polyclonal antibody (H80) and anti-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-MD-2 (ab24182) was purchased from Abcam (Cambridge, MA). Mouse anti-caveolin-1 and anti-transferrin receptor (anti-TfR; anti-CD71) monoclonal antibodies were purchased from BD Pharmingen (San Jose, CA). Mouse monoclonal anti-ceramide (15B4), fluorescein isothiocyanate (FITC)-conjugated LPS, lovastatin, methyl- $\beta$ -cyclodextrin (M $\beta$ CD), nystatin, and imipramine were purchased from Sigma-Aldrich (St. Louis, MO). Alexa Fluor 647-conjugated cholera toxin subunit B (CTX-B), Alexa Fluor 488-conjugated goat anti-rabbit IgG, Alexa Fluor 568-conjugated goat anti-mouse IgM, 4',6-diamidino-2-phenylindole (DAPI), and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA). Luciferase substrate and a  $\beta$ -galactosidase expression vector were purchased from Promega (Madison, WI). An *IL-8* promoter construct (IL-8/wt construct; nucleotides -162 to +44) was a kind gift of Chih-Hsin Tang of the Department of Pharmacology, China Medical University (14).

**Cell and bacterial cultures.** Human AGS cells (ATCC CRL 1739) were cultured in F12 medium (Invitrogen). MKN45 cells were cultured in Dulbecco's minimum essential medium (Invitrogen). TSGH9201 and SC-M1 cells were cultured in RPMI 1640 medium (Invitrogen). All culture media were supplemented with 10% complement-inactivated fetal bovine serum (HyClone, Logan, UT) and maintained at 37°C. For transient transfection, AGS cells were cultured in 12-well plates and incubated with 500  $\mu$ l Opti-MEM (Invitrogen), 1  $\mu$ g reporter gene, and 1  $\mu$ l Lipofectamine 2000 (Invitrogen) for 6 h at 37°C. Transfected cells were then cultured in complete medium for 24 h before further analysis. *H. pylori* 26695 (ATCC 700392) was recovered from frozen stocks on brucella agar plates (Becton

Dickinson, Franklin Lakes, NJ) containing 10% defibrinated sheep blood. *H. pylori* was cultured as described previously (31).

**Quantitative real-time reverse transcription-PCR.** Total RNA was isolated from cells by use of TRIzol reagent (Invitrogen), and 1  $\mu$ g of total RNA was reverse transcribed into cDNA by use of an oligo(dT) primer. Quantitative real-time PCR using SYBR green I master mix and a model 7900 sequence detection system was conducted according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). After preincubation at 50°C for 2 min and 95°C for 10 min, PCR was performed with 40 cycles of 95°C for 10 s and 60°C for 1 min. The threshold was set above the nontemplate control background and within the linear phase of target gene amplification in order to calculate the cycle number at which the transcript was detected (the threshold cycle [ $C_T$ ]). The oligonucleotide primers used corresponded to human TLR4 (forward, 5'-ACAACC TCCCCTTCTCAACC-3'; and reverse, 5'-TGAGATGTCCAATGGGA AG-3') and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward, 5'-CCCCCAATGTATCCGTTGTG-3'; and reverse, 5'-TAGC CCAGGATGCCCTTTAGT-3'). All oligonucleotide primers were synthesized by Invitrogen.

**Isolation and analysis of proteins in detergent-resistant fractions.** To isolate detergent-soluble and -resistant fractions, AGS cells were lysed with ice-cold buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA) containing 1% (vol/vol) Triton X-100 and incubated at 4°C for 30 min. Cell lysates were centrifuged at 18,000  $\times$  g at 4°C for 30 min to separate detergent-soluble and -resistant fractions as described previously (51). Each fraction was assessed by Western blotting.

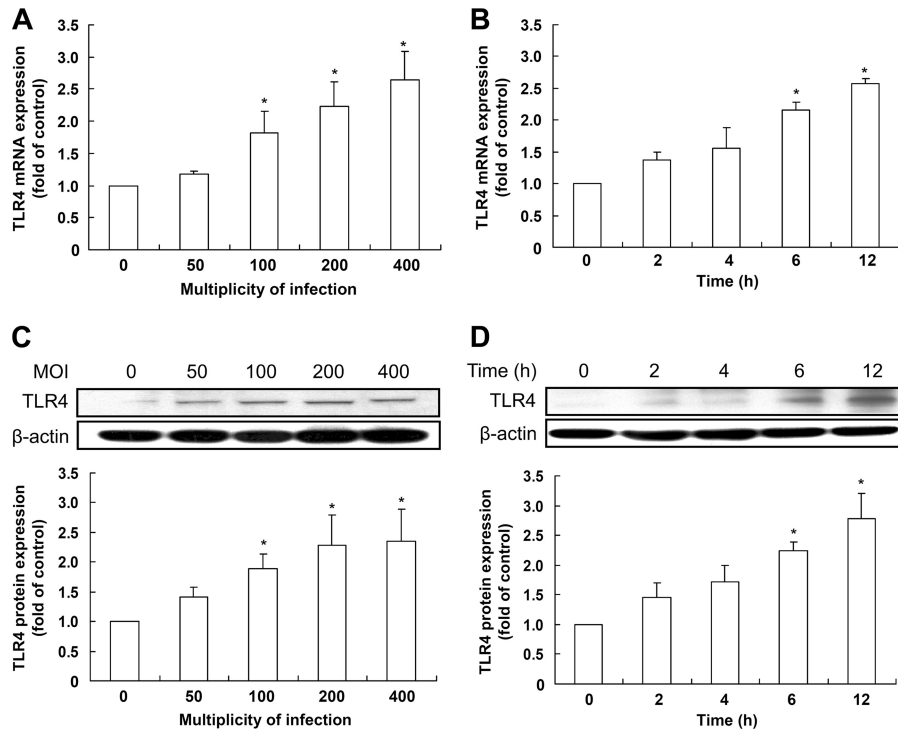
**Western blot analysis.** *H. pylori*-infected cells were washed three times with phosphate-buffered saline (PBS) and then boiled in SDS-PAGE sample buffer for 10 min. The samples were then resolved by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). The membranes were incubated with rabbit anti-TLR4 or mouse anti-caveolin-1 or anti-TfR (anti-CD71) at room temperature for 1 h. The blots were washed and then incubated with horseradish peroxidase-conjugated secondary antibody (Millipore). The proteins of interest were detected using ECL Western blotting detection reagents (GE Healthcare, Piscataway, NJ) and visualized using X-ray film (Kodak, Rochester, NY).

**Flow cytometry analysis.** Cells infected at various multiplicities of infection (MOIs) with *H. pylori* at different times points were harvested and fixed with ice-cold 70% ethanol for 1 h. Cells were then stained with 200 ng/ml FITC-conjugated LPS (B4), anti-ceramide, and an isotype control. The stained cells were subjected to flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) and were analyzed using Cell Quest software (Becton Dickinson). All samples were examined in triplicate for at least three independent experiments.

**Reporter activity assay.** AGS cells were grown to 90% confluence in 12-well plates and transfected with the *IL-8*/wt reporter construct by use of Lipofectamine 2000 (Invitrogen). After 16 h, cells were infected with *H. pylori* in the absence or presence of M $\beta$ CD, lovastatin, anti-TLR4, or anti-ceramide for 6 h. To prepare total cell lysates, 100  $\mu$ l of reporter lysis buffer (Promega) was added to each well, and cells were scraped from dishes. Equal volumes of luciferase substrate (Promega) were added to all samples, and luminescence was measured using a microplate luminometer (Biotek, Winooski, VT). Luciferase activity was normalized to transfection efficiency, which was determined by the  $\beta$ -galactosidase activity generated from a cotransfected  $\beta$ -galactosidase expression vector (Promega).

**Transfection of siRNA.** TLR4 (On-Targetplus siRNA 7099) and control (sc-37007) small interfering RNAs (siRNAs) were purchased from Thermo Fisher Scientific (Lafayette, CO) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. AGS cells were transfected with siRNAs (50 nM) by use of Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

**Immunofluorescence labeling and confocal microscopic analysis.** To visualize the localization of *H. pylori*, TLR4, and ceramide in lipid rafts



**FIG 1** *H. pylori* induces TLR4 expression in gastric epithelial cells. AGS cells were infected with *H. pylori* for 6 h at different MOIs (A and C) or at an MOI of 100 for the indicated times (B and D). Total RNA and cell lysates were prepared to measure TLR4 mRNA and protein expression levels by quantitative real-time PCR (A and B) and Western blot analysis (C and D), respectively. (A and B) TLR4 mRNA expression was measured by quantitative real-time PCR, and GAPDH was used as an internal control. (C and D) Protein expression levels were quantified by densitometric analysis and normalized to the  $\beta$ -actin level. The data are presented as means  $\pm$  standard deviations for 3 independent experiments. Statistical significance was evaluated using Student's *t* test (\*,  $P < 0.05$ ).

of cells, AGS cells ( $0.2 \times 10^6$ ) were seeded on coverslips in six-well plates and incubated for 16 h. Cells were infected with *H. pylori* at an MOI of 100 for 6 h and then washed three times with PBS and fixed with 3.7% paraformaldehyde for 1 h. The cells were then permeabilized with 0.1% Triton X-100 for 30 min and stained with Alexa Fluor 647-conjugated CTX-B. To label TLR4 and ceramide, samples were incubated for 30 min with rabbit polyclonal anti-TLR4 and mouse monoclonal anti-ceramide followed by Alexa Fluor 488-conjugated goat anti-rabbit IgG and Alexa Fluor 568-conjugated goat anti-mouse IgM, respectively. The stained cells were then analyzed under a confocal laser scanning microscope (LSM 510; Carl Zeiss, Göttingen, Germany) with a 100 $\times$  objective (oil immersion; numerical aperture, 1.3). The quantification of fluorescence intensity was performed by ImageJ (7).

**Determination of IL-8 secretion.** The concentration of IL-8 was determined by enzyme-linked immunosorbent assay (ELISA). AGS cells were pretreated with M $\beta$ CD, lovastatin, anti-TLR4, or anti-ceramide and then infected with *H. pylori* at an MOI of 100 for 24 h. The IL-8 concentration in AGS cell culture supernatants was determined using a sandwich ELISA kit (R&D Systems) according to the manufacturer's instructions (30).

**Statistical analysis.** Student's *t* test was used to calculate the statistical significance of experimental differences between two groups. Multiple testing results were corrected using the Bonferroni correction. The colocalization of TLR4 and GM1 was analyzed by ImageJ (7) and quantified using Pearson's correlation coefficient. The difference was considered significant when the *P* value was  $< 0.05$ . Statistical analyses were carried out using the SPSS program (version 11.0; SPSS Inc., Chicago, IL).

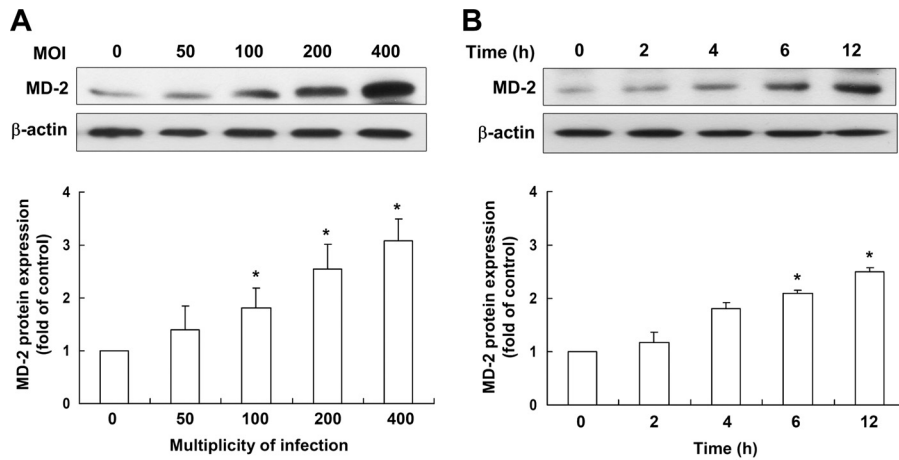
## RESULTS

***H. pylori* infection induces TLR4 expression in AGS cells.** To examine whether *H. pylori* infection induced TLR4 expression in our experimental system, human AGS cells were cocultured with

*H. pylori*. Induction of TLR4 mRNA expression was dependent on the MOI and duration of *H. pylori* infection, as determined by quantitative real-time reverse transcription-PCR (Fig. 1A and B). Furthermore, as expected, *H. pylori*-induced TLR4 protein expression increased following infection, in an MOI- and time-dependent manner (Fig. 1C and D). After adjusting the results using the Bonferroni correction, no significant differences in TLR4 expression were observed at low MOIs (0 to 50) or for short durations (0 to 4 h) of *H. pylori* infection. These results suggest that *H. pylori* infection of gastric epithelial cells induces TLR4 expression and that this induction requires a sufficient bacterial load and duration of infection.

Since TLR4-mediated signaling is reportedly coupled to MD-2 expression (50), we performed Western blot analysis to determine the protein expression of MD-2 during infection. As shown in Fig. 2, infection of cells with *H. pylori* increased the expression of MD-2 in AGS cells, in an MOI- and time-dependent manner. After Bonferroni correction was applied to these values to determine significance, no significant changes (the *P* value must be less than 0.0125) were observed in cells infected with *H. pylori* at low MOIs (0 to 50) or for short durations (0 to 4 h), similar to the trend observed in Fig. 1.

Cells were then transfected with TLR4 or control siRNA for 24 h. As indicated in Fig. 3A, the transfection of cells with TLR4 siRNA significantly reduced the level of TLR4 mRNA. Quantitative real-time PCR and Western blot analyses showed that *H. pylori*-induced TLR4 RNA and protein expression was suppressed by transfection with TLR4 siRNA (Fig. 3). Our data therefore con-



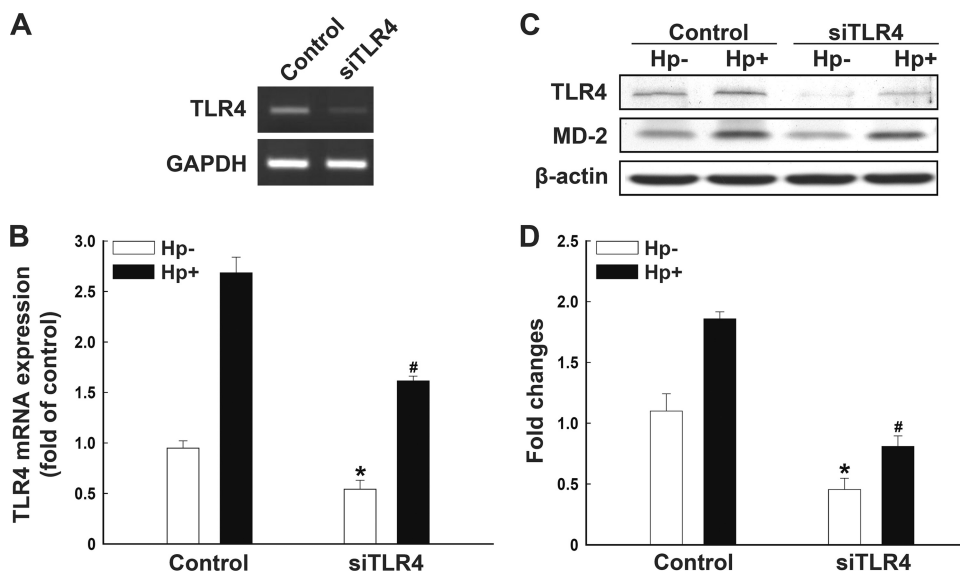
**FIG 2** Involvement of MD-2 expression in response to *H. pylori* infection in AGS cells. AGS cells were infected with *H. pylori* at various MOIs from 0 to 400 for 6 h (A) or at an MOI of 100 for the indicated times (B). Expression levels of MD-2 were determined by Western blot analysis. Protein expression levels were quantified using densitometric analysis, normalized to the  $\beta$ -actin level, and presented as means  $\pm$  standard deviations for 3 independent experiments. Statistical significance was determined using Student's *t* test (\*,  $P < 0.05$ ).

firm the involvement of TLR4/MD-2 expression in the signaling cascade induced by *H. pylori* infection.

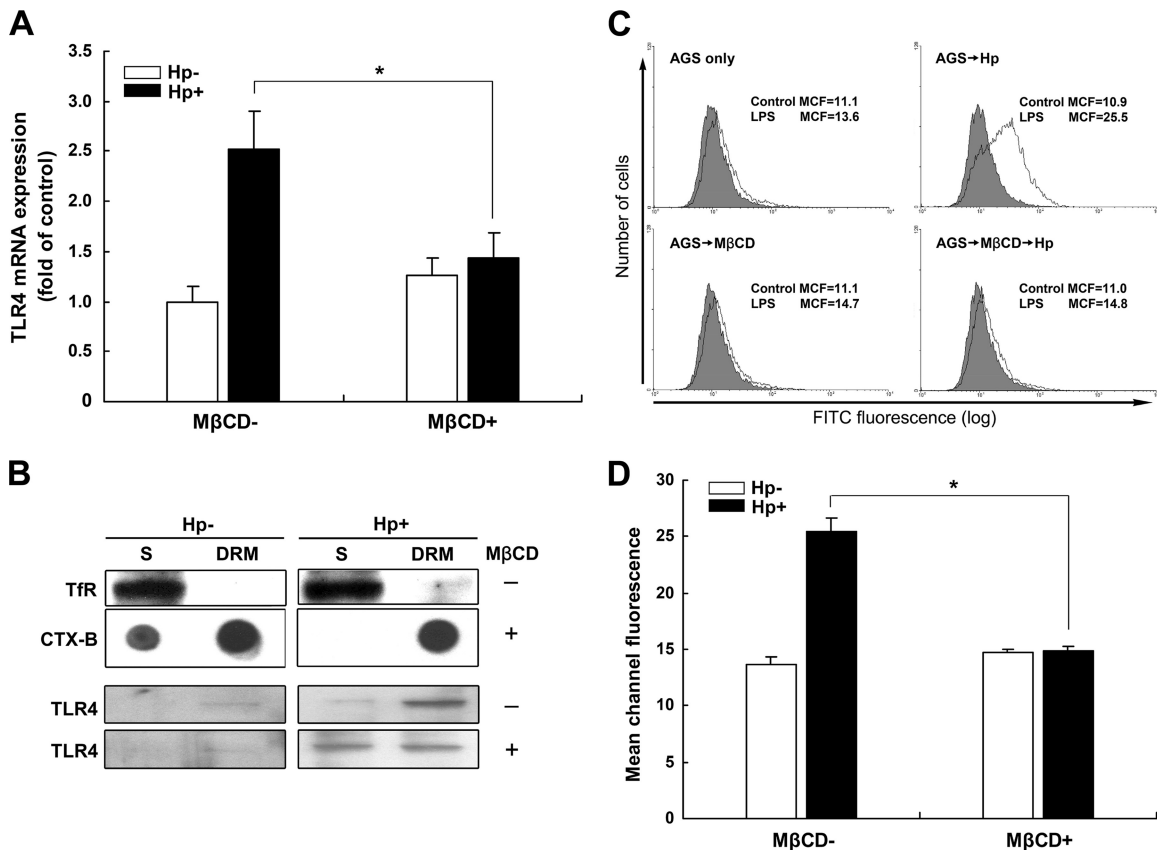
**Involvement of lipid rafts in *H. pylori*-induced TLR4 expression.** We then explored whether lipid rafts play a crucial role in *H. pylori*-induced TLR4 expression. AGS cells were pretreated with or without M $\beta$ CD, followed by infection with *H. pylori* for 6 h. As shown in Fig. 4A, pretreatment of cells with M $\beta$ CD inhibited *H. pylori*-induced TLR4 mRNA expression. We then examined whether *H. pylori*-enhanced TLR4 expression occurs in cholesterol-rich membrane microdomains, also called lipid rafts. Immunoblotting showed that a non-raft-associated protein, TfR, was enriched in the soluble fraction (S) of the membrane, whereas a raft-associated protein, CTX-B, which binds to the ganglioside GM1, was enriched in the

DRM fraction (Fig. 4B). Upon infection with *H. pylori*, TLR4 was abundantly localized in the DRM fraction. However, a portion of TLR4 translocated from the DRM fraction to the detergent-soluble fraction after pretreatment of cells with M $\beta$ CD (Fig. 4B).

We then analyzed whether *H. pylori*-induced TLR4 expression required cholesterol-rich membrane microdomains. As shown in Fig. 4C and D, *H. pylori* infection elicited an increase in TLR4 expression; the mean channel fluorescence (MCF) for FITC-conjugated LPS was 25.5. After treatment of cells with M $\beta$ CD followed by *H. pylori* infection, the expression of TLR4 was significantly reduced, to the baseline level (MCF = 14.8). These data show that disrupting lipid rafts by pretreating AGS cells with M $\beta$ CD led to a reduction in *H. pylori*-induced TLR4 expression in



**FIG 3** Infection of *H. pylori* induces TLR4 and MD-2 expression. AGS cells were transfected with TLR4 or control siRNA for 24 h, followed by infection (Hp+) or no infection (Hp-) with *H. pylori* for 6 h. TLR4 mRNA levels were assessed using semiquantitative reverse transcriptase PCR (A) and quantitative real-time PCR (B). (C) The protein levels of TLR4 and MD-2 were examined using Western blot analysis. (D) Protein expression levels of TLR4 were quantified by densitometric analysis and normalized to the  $\beta$ -actin level. Results are expressed as means  $\pm$  standard deviations. \*,  $P < 0.05$  compared to control siRNA-treated, mock-infected group; #,  $P < 0.05$  compared to control siRNA-treated, *H. pylori*-infected group.



**FIG 4** Involvement of lipid rafts in *H. pylori*-induced TLR4 expression in gastric epithelial cells. AGS cells were pretreated with medium alone or 5 mM MβCD at 37°C for 30 min. Cells were then washed and incubated with *H. pylori* at an MOI of 100 for 6 h. (A) The TLR4 mRNA level was evaluated by quantitative real-time PCR, and GAPDH was used as an internal control. (B) Detergent-resistant (DRM) and detergent-soluble (S) membrane fractions were prepared from cells that were subjected to cold detergent extraction using 1% Triton X-100 followed by centrifugation. Each fraction was subjected to Western blot or dot blot analysis using antibodies against transferrin receptor (TfR) or cholera toxin subunit B (CTX-B), conjugated to horseradish peroxidase, or an antibody specific to TLR4. (C) After infection with *H. pylori* for 6 h, cells were probed with control FITC-conjugated anti-mouse IgG (gray histograms) or FITC-conjugated LPS (white histograms). The FITC fluorescence level was analyzed by flow cytometry. (D) The numbers represent the mean channel fluorescence (MCF), and the quantitative results represent the means and standard deviations for 3 independent experiments. \*,  $P < 0.05$  compared to untreated MβCD control group, as determined by Student's *t* test.

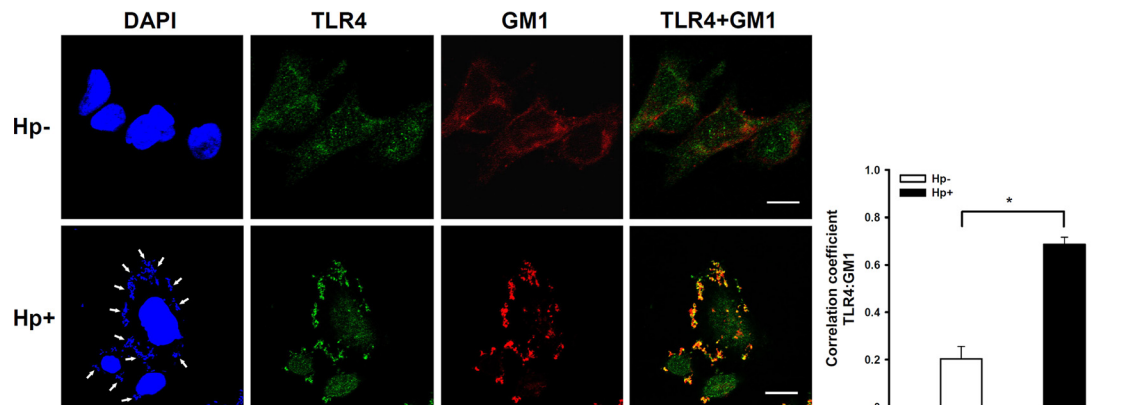
the DRM fraction, suggesting that lipid rafts play an important role in *H. pylori*-triggered TLR4 expression.

We then directly analyzed whether TLR4 colocalized in lipid rafts at sites of *H. pylori* infection by confocal microscopy. The analysis of the distribution of fluorescence intensity signals showed that faint TLR4 staining colocalized with the raft marker GM1 in the absence of *H. pylori* infection (Fig. 5, upper panels). However, remarkably greater mobilization of TLR4 and GM1 was observed at sites of *H. pylori* infection (Fig. 5, lower panels). The colocalization of TLR4 and GM1-raft signals showed significant correlation for *H. pylori*-infected cells (Pearson coefficient of 0.71;  $P < 0.01$ ) (Fig. 5, right panel). Our data clearly indicate that *H. pylori* infection and TLR4 expression colocalized with the raft marker GM1, which stained the cell membrane. These results further support the hypothesis that reciprocal elicitation and mobilization of TLR4 into membrane rafts occur in response to *H. pylori* infection.

***H. pylori*-induced TLR4 expression is dependent on ceramide release.** To examine whether *H. pylori*-elicited TLR4 signaling depends on ceramide secretion, AGS cells were treated with exogenous  $C_2$ -ceramide or *H. pylori*.  $C_2$ -ceramide increased both

TLR4 mRNA and protein expression, in a dose-dependent manner (Fig. 6A and B). Furthermore, flow cytometry showed that infection of AGS cells with *H. pylori* increased ceramide release (Fig. 6C). We further analyzed the expression of ceramide and TLR4 following *H. pylori* infection in AGS and 3 other gastric epithelial cell lines (MKN45, TSGH9201, and SC-M1). The effects of *H. pylori* on TLR4 and MD2 protein expression were evaluated by Western blot analysis. Infection with *H. pylori* induced different levels of TLR4 and MD-2 expression in different gastric epithelial cells (Fig. 7A and B). In parallel, *H. pylori* significantly elevated ceramide expression in these cells, as determined by flow cytometric analysis (Fig. 7C). These results suggest that *H. pylori* infection induces TLR4/MD-2 and ceramide expression not only in AGS cells but also in other gastric epithelium-derived cell lines.

The involvement of lipid rafts in the induction of ceramide and TLR4 by *H. pylori* infection was further investigated. Pretreatment of cells with imipramine, an acid sphingomyelinase inhibitor, resulted in significantly attenuated *H. pylori*-induced ceramide release ( $P < 0.05$ ) (Fig. 8A) and reduced TLR4 expression (Fig. 8B). These phenomena were evidently dependent on lipid raft integrity, as disruption of lipid rafts with either of 2 raft disruption



**FIG 5** Mobilization of TLR4 into cholesterol-rich microdomains at sites of *H. pylori* infection. AGS cells were infected with *H. pylori* (or not infected) at 37°C for 6 h. Cells were fixed and stained with anti-TLR4 (green), with Alexa Fluor 647-conjugated CTX-B to visualize GM1 (red), or with DAPI (blue) to visualize bacteria (arrows) and the cell nucleus. Samples were analyzed by confocal microscopy. The colocalization of TLR4 with GM1 appears yellow in the overlay. The colocalization of fluorescence for TLR4 and GM1 signals was quantified using the Pearson correlation coefficient, and the data are presented in the right panel. Statistical significance was determined for 3 independent experiments. \*,  $P < 0.01$ . Bars, 10  $\mu\text{m}$ . Hp<sup>-</sup>, not treated with *H. pylori*; Hp<sup>+</sup>, treated with *H. pylori*.

agents, M $\beta$ CD and nystatin, significantly attenuated *H. pylori*-stimulated ceramide release and reduced TLR4 expression (Fig. 8). These results suggest that *H. pylori* infection of AGS cells stimulates TLR4 expression via ceramide release, which is dependent on lipid rafts.

**Ceramide and TLR4 are mobilized into lipid rafts upon *H. pylori* infection.** The localization of ceramide and TLR4 in *H. pylori*-infected AGS cells was then visualized by confocal microscopy. The colocalization of ceramide and TLR4 around the cell surface was evident but decreased in noninfected AGS cells compared to infected cells (Fig. 9A to D). In *H. pylori*-infected AGS cells, more apparent punctate aggregates of ceramide and TLR4 were noted at sites where *H. pylori* adhered to the cell surface (Fig. 9E to H, arrows). Adhered *H. pylori* cells (stained with DAPI) colocalized with ceramide and TLR4, as shown in merged DAPI-TLR4-ceramide images obtained by confocal microscopy z-section analysis (Fig. 9I to L). These results indicate that both ceramide and TLR4 are mobilized into lipid rafts upon *H. pylori* infection.

**Mobilization of ceramide and TLR4 into lipid rafts by *H. pylori* induces IL-8 activation.** IL-8 secretion was significantly increased on exposure of AGS cells to C<sub>2</sub>-ceramide at 2.5 to 5  $\mu\text{M}$  (Fig. 10A). This finding remained significant after adjustment by the Bonferroni correction for multiple testing ( $P < 0.0125$ ). This is consistent with a previous report by Masamune et al. that ceramide plays a crucial role in *H. pylori*-induced IL-8 expression (38). We then tested whether the 3 components—TLR4, ceramide, and lipid raft integrity—were essential for IL-8 secretion in *H. pylori*-infected cells. A human IL-8 promoter-Luc reporter construct (IL-8/wt construct) containing both AP-1 and NF- $\kappa$ B sites was transfected into AGS cells. Following transfection, the cells were infected with *H. pylori* and then subjected to luciferase activity assays. Culture supernatants of *H. pylori*-infected cells were harvested for IL-8 secretion analysis. As shown in Fig. 10B and C, IL-8 promoter activity and IL-8 secretion were strongly induced in *H. pylori*-infected AGS cells.

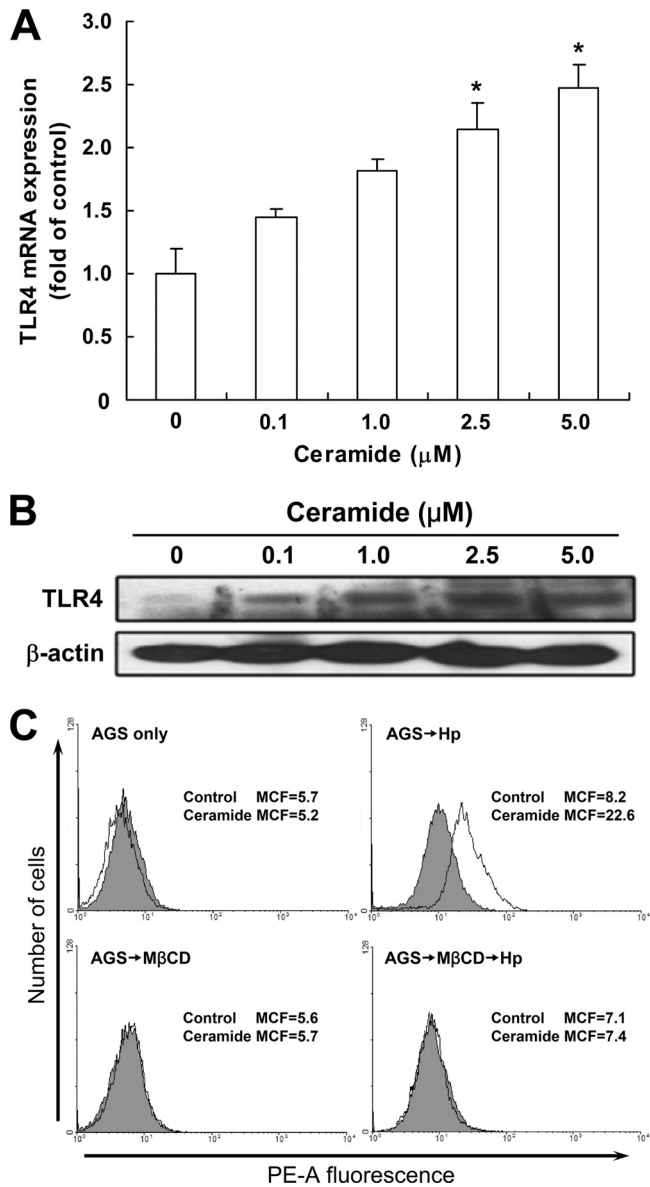
Our results indicated that M $\beta$ CD stimulated NF- $\kappa$ B reporter activity as well as IL-8 production. Previous studies have used lovastatin, an inhibitor of 3-hydroxy-3-methyl-glutaryl-coen-

zyme A (HMG-CoA) reductase, to reduce the concentration of endogenous cholesterol and thereby to disrupt rafts, which has no effect on NF- $\kappa$ B or IL-8 activation (30, 40). Hence, lovastatin was employed in the following experiments. To examine whether lipid raft integrity was crucial for *H. pylori*-induced IL-8 production, AGS cells were pretreated with lovastatin prior to infection with *H. pylori*. Lovastatin treatment significantly attenuated IL-8 promoter activity and IL-8 secretion in *H. pylori*-infected cells (Fig. 10B and C). Similarly, the attenuation occurred when cells were pretreated with either anti-ceramide or anti-TLR4 followed by *H. pylori* infection. Moreover, both IL-8 promoter activity (Fig. 10D) and IL-8 secretion (Fig. 10E) were significantly reduced by transfection of cells with TLR4 siRNA prior to infection with *H. pylori*. Thus, these results suggest that modulation of cellular cholesterol levels reduces the mobilization of ceramide and TLR4 into cholesterol-rich microdomains, thereby attenuating *H. pylori*-induced IL-8 activation.

## DISCUSSION

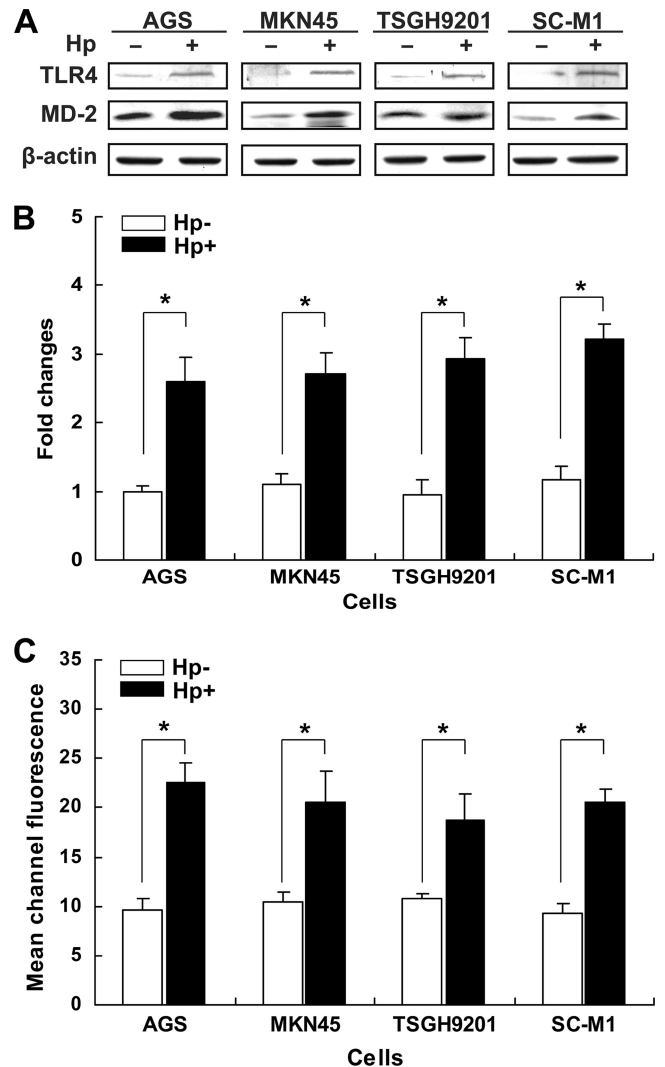
TLRs are transmembrane proteins that have been found to play a critical role in the recognition of microbial components (2, 24, 39). LPS derived from Gram-negative bacteria can be recognized by TLR4 and activated MD-2 to signal cell responses (50). Several studies have shown that *H. pylori* and LPS alone have the capacity to upregulate TLR4 expression in gastric epithelial cells (27, 53). In addition, patients with *H. pylori* infection reportedly exhibit definite TLR4 expression in the gastric mucosa (4, 47). Together, these reports suggest that TLR4 may contribute to inflammatory responses induced by *H. pylori*. Consistent with these findings, our results showed that TLR4 and MD-2 expression in gastric epithelial cells was upregulated upon *H. pylori* infection (Fig. 1 and 2). Transfection of cells with TLR4 siRNA followed by infection with *H. pylori* reduced TLR4 expression (Fig. 3) and attenuated IL-8 activation (Fig. 10).

Controversy remains, however, over which TLRs are involved in *H. pylori*-induced pathogenesis. Several studies have reported that immune responses to intact *H. pylori* may not involve TLR4 (5, 15). In addition, LPS derived from *H. pylori* reportedly induces TLR2 rather than TLR4 signaling (52, 60). The reasons for these



**FIG 6** *H. pylori* activates ceramide release in lipid rafts. AGS cells were treated with various concentrations of ceramide for 24 h. Total RNA and cell lysates were prepared to measure TLR4 mRNA and protein expression levels by quantitative real-time PCR (A) and Western blot analysis (B), respectively. The mRNA and protein expression levels were normalized to GAPDH and  $\beta$ -actin, respectively. (C) AGS cells were pretreated with medium alone or 5 mM M $\beta$ CD for 30 min and then incubated with *H. pylori* at an MOI of 100 for 6 h. Cells were then stained with anti-ceramide (white histograms) or isotype control IgM (gray histograms), followed by Alexa Fluor 568-conjugated goat anti-mouse IgM, and the fluorescence intensity was assessed by flow cytometry. Results are expressed as means  $\pm$  standard deviations. \*,  $P < 0.05$  compared with untreated control. MCF, mean channel fluorescence; M $\beta$ CD, methyl- $\beta$ -cyclodextrin.

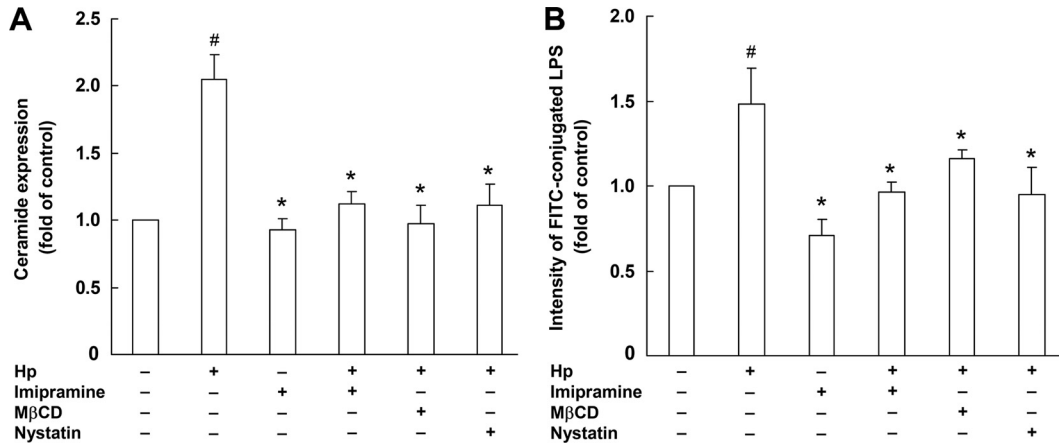
discrepancies may potentially be explained by variations in the bacterial strains or experimental systems employed in the studies. We used the laboratory strain *H. pylori* 26695 (56), unlike various other studies that have used clinical isolates (5, 15, 52, 60), as substantial heterogeneity exists across clinical isolates. In addition, experimental parameters such as the multiplicity of infec-



**FIG 7** Infection of *H. pylori* enhances ceramide and TLR4 expression. Cells from the indicated lines were infected (or not infected) with *H. pylori* for 6 h at 37°C. (A) Cell lysates were prepared to analyze TLR4 and MD-2 protein expression levels by Western blotting. (B) Protein expression levels of TLR4 were quantified by densitometric analysis and normalized to the  $\beta$ -actin level. (C) The cells were fixed and stained with anti-ceramide and assessed by flow cytometry to determine fluorescence intensities. \*,  $P < 0.05$  compared to each uninfected *H. pylori* group, as determined by Student's *t* test. Hp-, not treated with *H. pylori*; Hp+, treated with *H. pylori*.

tion, the cell lines assessed, and the infection times utilized varied among reports. In this study, we used *H. pylori* at an MOI of 100 and infected AGS cells for 6 h. Notably, our experimental approach was very similar to that reported by Su et al., who employed AGS cells as an assay platform and suggested that *H. pylori* contributes to activation of TLR4 expression (53). Furthermore, we employed 3 additional gastric epithelium-derived cell lines (MKN45, TSGH9201, and SC-M1) to validate that *H. pylori* can upregulate TLR4 and MD-2 expression (Fig. 7), and we obtained data in accordance with findings previously reported by Ishihara et al. (25).

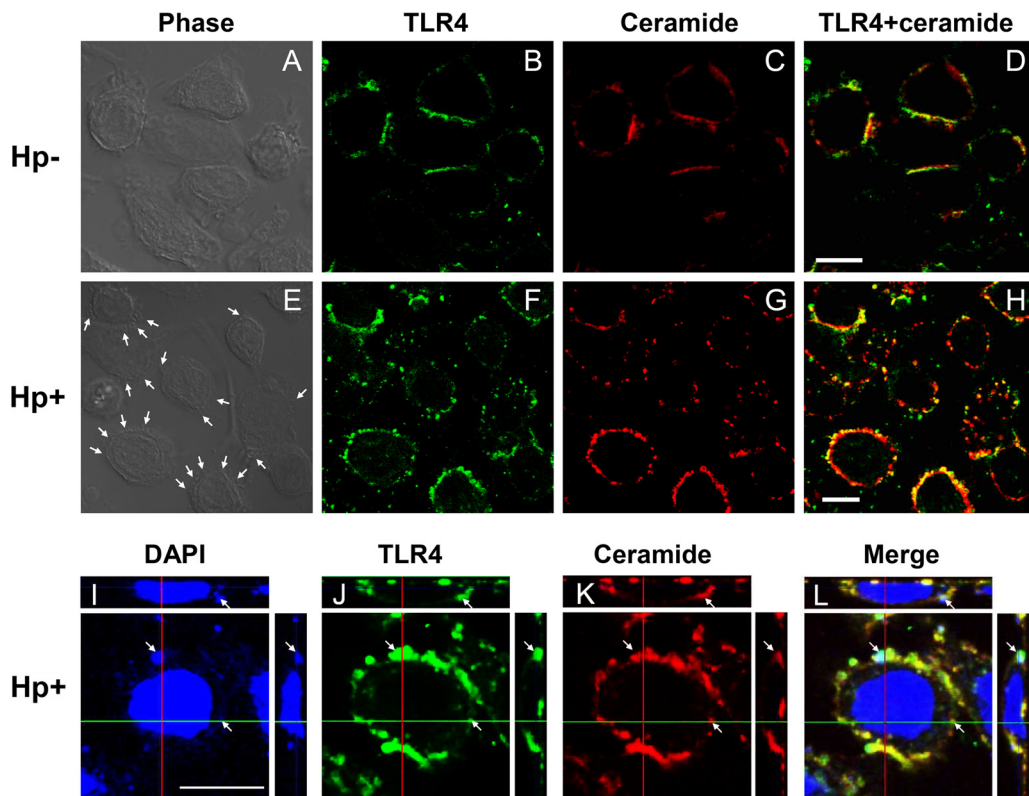
In this study, we demonstrated that upregulation of TLR4 by *H. pylori* was associated with increased secretion of ceramide (Fig. 6), which has been shown to activate NF- $\kappa$ B and IL-8 production



**FIG 8** Disruption of lipid rafts reduces *H. pylori*-induced ceramide and TLR4 expression. AGS cells were pretreated with imipramine (10 μM), MβCD (2.5 μM), or nystatin (10 μg/ml) for 1 h, followed by infection with *H. pylori* at an MOI of 100 for 6 h. The cells were fixed and stained with anti-ceramide (A) or anti-TLR4 (B), and the fluorescence intensities were then determined by flow cytometry. Results are expressed as means ± standard deviations. #, *P* < 0.05 compared with untreated control group; \*, *P* < 0.05 compared with *H. pylori*-infected group.

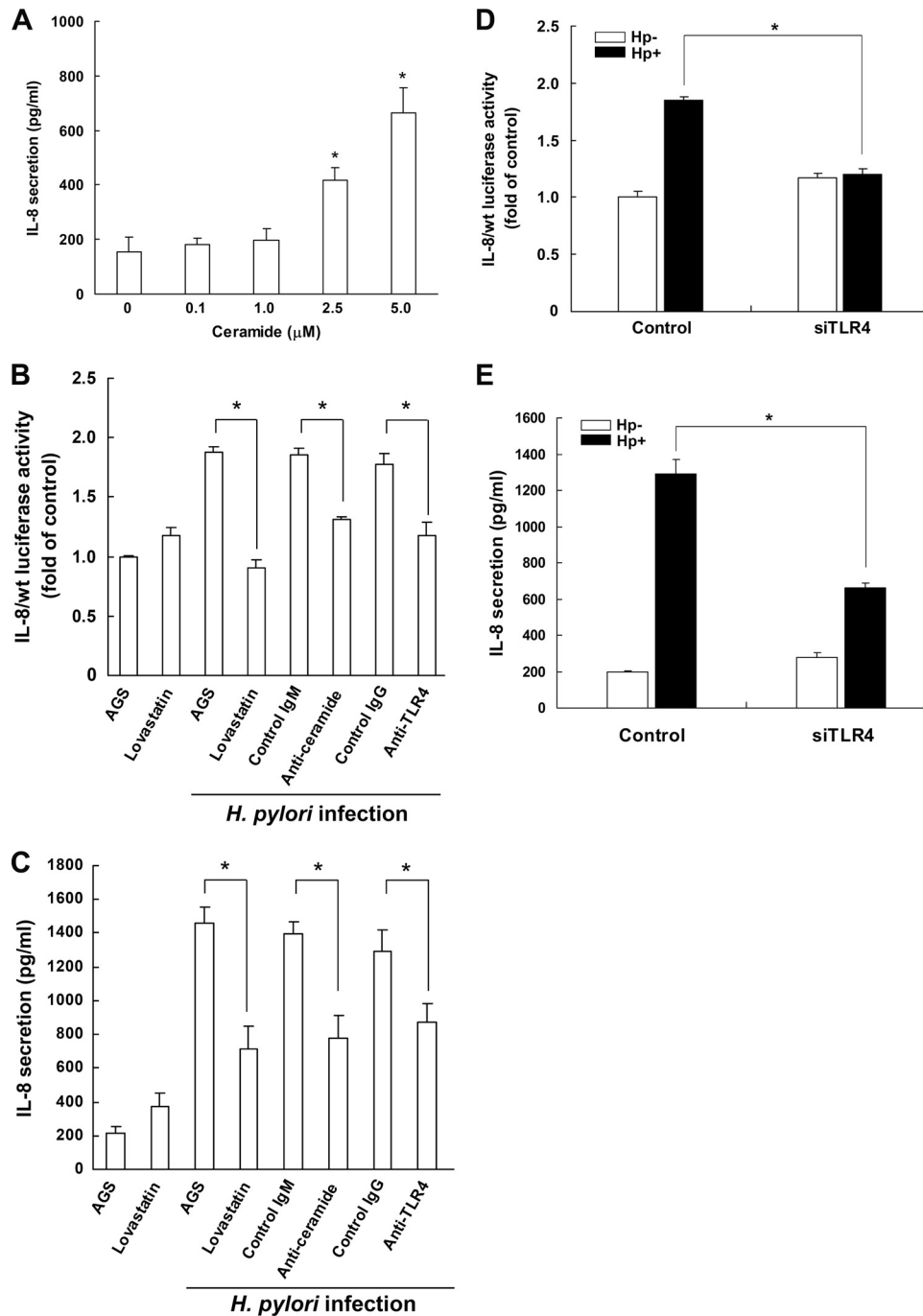
upon *H. pylori* infection (38). Ceramide plays an important role in several signaling pathways by triggering a coalescence of receptor molecules in membrane rafts (19). Strikingly, it was found that *H. pylori* harbors sphingomyelinase activity, which can hydrolyze sphingomyelin and elevate cellular ceramide levels (35). In-

creased ceramide levels lead to the formation of ceramide-enriched membrane rafts and the activation of specific signaling pathways, including proinflammatory cytokine release (18). These studies support our finding that *H. pylori* infection induces mobilization of ceramide into lipid rafts, whereby it may



**FIG 9** *H. pylori*-stimulated ceramide and TLR4 cluster in lipid rafts. AGS cells were infected with *H. pylori* (or not infected) at 37°C for 6 h. Cells were fixed and stained with anti-TLR4 (green) (B, F, and J), anti-ceramide (red) (C, G, and K), or DAPI (blue) to visualize bacteria and the cell nucleus (I) and then analyzed by confocal microscopy or were analyzed by phase-contrast microscopy to visualize bacteria (A and E). Arrows indicate the colocalization of TLR4, ceramide, and *H. pylori* on infected cells. Confocal z-section images are shown for bacteria colocalized with TLR4 and ceramide (I to L). Bars, 10 μm. Hp-, not treated with *H. pylori*; Hp+, treated with *H. pylori*.





**FIG 10** Activation of IL-8 by *H. pylori* is attenuated by inhibition of ceramide and TLR4 and disruption of rafts. (A) Ceramide stimulates IL-8 secretion in AGS cells. Cells were treated with ceramide at the indicated concentrations for 24 h. The level of IL-8 in the culture supernatant was determined by a standard ELISA method. (B to E) *H. pylori*-induced IL-8 activity and IL-8 secretion were influenced by pretreatment with lovastatin, anti-ceramide, anti-TLR4, and TLR4 siRNA. AGS cells were transfected with IL-8/wt Luc reporter plasmid in the presence of lovastatin (50  $\mu\text{M}$ ), anti-ceramide, or anti-TLR4 prior to infection of cells with *H. pylori* at an MOI of 100. Cell lysates were subjected to luciferase activity assays to determine IL-8 activity (B), and IL-8 levels in the cell culture supernatants were determined by ELISA (C). Cells were transfected with TLR4 or control siRNA for 24 h, followed by infection with *H. pylori*, and the IL-8 activity (D) and IL-8 production (E) were analyzed using a luciferase activity assay and ELISA, respectively. Data represent the means  $\pm$  standard deviations for 3 independent experiments. Statistical significance was evaluated using Student's *t* test (\*,  $P < 0.05$ ).

activate TLR4 signaling and contribute to induction of IL-8 secretion.

The DRM fractionation data in our current study provide evidence for the localization of TLR4 with the raft marker CTX-B,

suggesting that infection by *H. pylori* triggered the coalescence of TLR4 into lipid rafts. We further observed that TLR4 expression was diminished after pretreatment of cells with imipramine (Fig. 8), which acts as an acid sphingomyelinase inhibitor, indicating

that ceramide stimulated TLR4 expression in gastric epithelial cells upon *H. pylori* infection. Our finding was consistent with previous reports that ceramide acts as a signaling intermediate between TLR4 and sphingolipid receptors, which localize abundantly in lipid rafts (12). It remains unclear, however, whether ceramide acts as a TLR4 signaling agonist upon *H. pylori* infection. Details of the connection between *H. pylori*-induced ceramide/TLR4 and lipid rafts require further research.

Our results indicate that *H. pylori* infection of AGS cells induces not only TLR4 mRNA and protein expression but also secretion of the proinflammatory cytokine IL-8. We found that treatment of AGS cells with anti-TLR4 or TLR4 siRNA inhibited *H. pylori*-induced IL-8 promoter activity and IL-8 production (Fig. 10D and E). The IL-8 promoter contains NF- $\kappa$ B and AP-1 binding sites (14), and thus our results implicate a possible connection between *H. pylori* infection and NF- $\kappa$ B-mediated activation of IL-8. However, IL-8 secretion was not completely impaired by the pretreatment of cells with anti-TLR4 or TLR4 siRNA. This may be explained by another pathway involved in *H. pylori* CagA-induced IL-8 production (8). Although this is not direct evidence for TLR4 as the major receptor for signaling pathways induced by *H. pylori*, close associations between *H. pylori*, TLR4, and IL-8 secretion suggest the possible involvement of TLR4 in *H. pylori*-induced responses. Constitutive expression of TLR4 contributes by allowing TLR4 to serve as a receptor for *H. pylori* binding (53) and also to act as a potent receptor involved in the response to *H. pylori* LPS-induced activation of cytoplasmic NF- $\kappa$ B and the subsequent activation of the IL-8 promoter (25). Together, these data support our hypothesis that TLR4 is crucial for inducing IL-8 activation in response to *H. pylori* infection.

Several studies have reported that bacteria utilize lipid rafts or raft-associated molecules for adhesion to or invasion of host cells (11, 29, 34, 59). With *H. pylori*, previous studies have shown that cholesterol-rich microdomains are required for VacA binding, delivery, and intoxication of cells (20, 28, 41, 48). Our previous studies demonstrated that translocation of CagA and the internalization of *H. pylori* in gastric epithelial cells are reduced significantly upon treatment of cells with M $\beta$ CD, thereby decreasing the pathogenesis induced by this bacterium (30, 32). Moreover, we recently reported that the glucosylation of cholesterol in *H. pylori* is crucial for efficient *cag* type 4 secretion system (TFSS)-associated activity, which may due to the reorganization of membrane architecture (58). Here we showed that *H. pylori*-induced release of ceramide and expression of TLR4, as well as secretion of IL-8, were attenuated upon depletion of cholesterol or by inhibition of cholesterol synthesis. These data support the notion that the integrity of lipid rafts is essential for *H. pylori*-induced pathogenesis of host cells, as suggested by previous studies (23, 30, 40).

In conclusion, we have demonstrated that infection of gastric epithelial cells with *H. pylori* induces TLR4/MD-2 mRNA and protein expression. Furthermore, *H. pylori* facilitates the mobilization of ceramide and TLR4 into lipid rafts, where both contribute to NF- $\kappa$ B activation and IL-8 production. Together, these results shed light on the molecular mechanisms of *H. pylori*-induced pathogenesis of host cells.

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