Ceramide and TLR4 are mobilized into membrane rafts in response to *Helicobacter pylori* infection in gastric epithelial cells

Running title: Mobilization of ceramide and TLR4 to signal *H. pylori* infection

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ABSTRACT

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) are reportedly interacted with lipid rafts in gastric epithelial cells. The role of Toll-like receptors (TLRs)-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 indicated that both TLR4 and MD-2 mRNA and protein levels were significantly higher in *H. pylori* infected AGS cells relative to mock-infected cells. Moreover, significantly more TLR4 protein was detected in detergent-resistant membranes extracted from *H. pylori* infected AGS cells compared to those of 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 it 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.

Keywords: *Helicobacter pylori*, Toll-like receptor 4, ceramide, cholesterol, interleukin-8

INTRODUCTION

Helicobacter pylori, a spiral Gram-negative micro-aerophilic 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 (NF)-κB activation and proinflammatory cytokine secretion, including interleukin (IL)-1β, IL-6, IL-8, and TNF-α 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-κB 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-κB and activator protein-1 (AP)-1 (13, 50, 62). Several reports have suggested that TLR2 contributes to *H. pylori* LPS-induced signaling (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 an 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, anti-transferrin receptor (anti-TfR; anti-CD71) monoclonal antibodies were purchased from BD Pharmingen (San Jose, CA). Mouse monoclonal anti-ceramide (15B4), FITC-conjugated LPS, lovastatin,

methyl-β-cyclodextrin (MβCD), 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 β-galactosidase expression vector were purchased from Promega (Madison, WI). The *IL-8* promoter construct (IL-8/wild type; nucleotides −162 to +44) was a kind gift of Dr. 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 in 500 μl of OPTI-MEM (Invitrogen), 1 μg reporter gene, and 1 μl 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% sheep de-fibrinogen blood. *H. pylori* was cultured as described (31).

Quantitative real-time reverse transcription–PCR. Total RNA was isolated from cells using TRIzol reagent (Invitrogen), and 1 μg of total RNA was reverse transcribed into cDNA using the oligo(dT) primer. Quantitative real-time PCR using SYBR Green I Master Mix and a model 7900 Sequence Detector System was conducted according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). After pre-incubation at 50ºC for 2 min and 95ºC for 10 min, PCR was performed with 40 cycles of 95ºC for 10 s and 60ºC for 1 min. The threshold was set above the non-template control background and within the linear phase of target gene amplification in order to calculate the cycle number at which the transcript was detected (denoted as C_T). The oligonucleotide primers used corresponded to human TLR4 (forward 5'-ACAACCTCCCCTTCTCAACC-3' and reverse 5'-TGAGATGTCCAATGGGGAAG-3') and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; forward 5'-CCCCCAATGTATCCGTTGTG-3' and reverse 5'-TAGCCCAGGATGCCCTTTAGT-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% (v/v) Triton X-100 and incubated at 4°C for 30 min. Cell lysates were centrifuged at 18,000 × *g* at 4°C for 30 min to separate detergent-soluble and -resistant fractions as described (51). Each fraction was assessed by

147 western blotting.

148

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

157

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

164

165 **Reporter activity assay.** AGS cells were grown to 90% confluency in 12-well plates and
166 transfected with IL-8/wt reporter construct using Lipofectamine 2000 (Invitrogen). After 16 h, cells

were infected with *H. pylori* in the absence or presence of MβCD, lovastatin, anti-TLR4, or anti-ceramide for 6 h. To prepare total cell lysates, 100 μl of reporter lysis buffer (Promega) was added to each well, and cells were scraped from dishes. An equal volume of luciferase substrate (Promega) was 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 β-galactosidase activity generated from a co-transfected β-galactosidase expression vector (Promega).

Transfection of siRNA. TLR4 (ON-TARGET*plus* siRNA 7099) and control siRNA (sc-37007) were purchased from Thermo Fisher Scientific (Lafayette, CO) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. AGS cells were transfected with siRNAs (50 nM) using 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 of cells, AGS cells (0.2×10^6) were seeded on coverslips in six-well plates and incubated for 16 h. Cells were infected with *H. pylori* at a 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× objective (oil immersion, aperture 1.3). The quantification of fluorescence intensity was analyzed by ImageJ (7).

Determination of IL-8 secretion. The concentration of IL-8 was determined by enzyme-linked immunosorbent assay (ELISA). AGS cells were pre-treated with MβCD, lovastatin, anti-TLR4, or anti-ceramide and then infected with *H. pylori* at a 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. The Student's *t*-test was used to calculate the statistical significance of experimental differences between two groups. Multiple testing was corrected using the Bonferroni correction. The co-localization of TLR4 and GM1 was analyzed by ImageJ (7) and quantified using the Pearson's correlation coefficient. The difference was considered significant when $P < 0.05$. Statistical analyses were carried out using 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 co-cultured 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 in an MOI- and time-dependent manner following infection (Fig. 1C and D). After adjusting the results using the Bonferroni correction, no significant differences in TLR4 expression were observed at low MOI (0–50) or short duration (0–4 h) of *H. pylori* infection. These results suggest that *H. pylori* infection of gastric epithelial cells induced TLR4 expression and that this induction required sufficient bacterial load and duration of infection.

As 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 (P value must be less than 0.0125) were observed in cells infected with *H. pylori* at low MOI (0–50) or short duration (0–4 h), similar to the trend observed in Fig. 1.

Cells were then transfected with TLR4 or control siRNA for 24 h. As indicated in figure 3A, the transfection of cells with TLR4 siRNA significantly reduced the level of TLR4 mRNA.

Quantitative real-time PCR and western blot analysis showed that *H. pylori*-induced TLR4 RNA and protein expression was suppressed by transfection with TLR4 siRNA (Fig. 3). Our data therefore confirm 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 MβCD followed by infection with *H. pylori* for 6 h. As shown in Fig. 4A, pretreatment of cells with MβCD 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, transferrin receptor (TfR), was enriched in the soluble fraction (S) of the membrane, whereas a raft-associated protein, cholera toxin subunit B (CTX-B) that binds to the ganglioside GM1, was enriched in the detergent-resistant membrane (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 MβCD (Fig. 4B).

We then analyzed whether *H. pylori*-induced TLR4 expression required cholesterol-rich membrane microdomains. As shown in Fig. 4C and 4D, *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 MβCD followed by *H. pylori* infection, the expression of TLR4 was significantly reduced to baseline levels (MCF = 14.8). These data show that disrupting lipid rafts by

pretreating AGS cells with MβCD led to a reduction in *H. pylori*-induced TLR4 expression in the DRM fraction, suggesting that lipid rafts play an important role in *H. pylori*-triggered TLR4 expression.

We then directly analyzed whether TLR4 co-localized 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 co-localized with the raft marker GM1 in the absence of *H. pylori* infection (Fig. 5, upper panel). However, remarkably greater mobilization of TLR4 and GM1 was observed at sites of *H. pylori* infection (Fig. 5, lower panel). The co-localization of TLR4 and GM1-raft signals showed significant correlation in *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 co-localized with the raft marker GM-1, 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 C2-ceramide or *H. pylori*. C2-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 expressions 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, B). In parallel, *H. pylori* significantly elevated ceramide expression in these cells, as was determined by flow cytometric analysis (Fig. 7C). These results suggest that *H. pylori* infection induces TLR4/MD-2 and ceramide expressions 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 the 2 raft-disruption agents, MβCD 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 stimulated 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 co-localization of ceramide and TLR4 around the cell surface was evident, but decreased in non-infected AGS cells compared to infected cells (Fig. 9A–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–H, arrows). Adhered *H. pylori* (stained with DAPI)

284 co-localized with ceramide and TLR4 as shown in merged DAPI/TLR4/ceramide images obtained
285 by confocal microscopy *z*-section analysis (Fig. 9I–L). These results indicate that both ceramide
286 and TLR4 are mobilized into lipid rafts upon *H. pylori* infection.

287 **Mobilization of ceramide and TLR4 into lipid rafts by *H. pylori* induces IL-8 activation.**
288 IL-8 secretion was significantly increased on exposure of AGS cells to C2-ceramide at 2.5–5 μM
289 (Fig. 10A). This finding remained significant after adjustment for Bonferroni correction for
290 multiple testing ($P < 0.0125$). This is consistent with a previous report by Masamune *et al*. that
291 ceramide plays a crucial role in *H. pylori*-induced IL-8 expression (38). We then tested whether the
292 3 components–TLR4, ceramide, and lipid raft integrity–were essential for IL-8 secretion in *H.*
293 *pylori*-infected cells. The human *IL-8* promoter-Luc construct, IL-8/wt, containing both AP-1 and
294 NF-κB sites, was transfected into AGS cells. Following transfection, the cells were infected with *H.*
295 *pylori* and then subjected to luciferase activity assays. Culture supernatants of *H. pylori*-infected
296 cells were harvested for IL-8 secretion analysis. As shown in Fig. 10B and C, *IL-8* promoter activity
297 and IL-8 secretion were strongly induced in *H. pylori*-infected AGS cells.

298 Our results indicated that MβCD stimulated NF-κB reporter activity as well as IL-8 production.
299 Therefore, previous studies have used lovastatin, an inhibitor of HMG-CoA reductase, to reduce the
300 concentration of endogenous cholesterol and thereby disrupt the rafts, which has no effect on
301 NF-κB or IL-8 activation (30, 40). Hence, lovastatin was employed in the following experiments.
302 To examine whether lipid raft integrity was crucial for *H. pylori*-induced IL-8 production, AGS
303 cells were pretreated with lovastatin prior to infection with *H. pylori*. Lovastatin treatment

304 significantly attenuated *IL-8* promoter activity and IL-8 secretion in *H. pylori*-infected cells (Fig.
305 10B and C). Similarly, the attenuation occurred when cells were pretreated with either
306 anti-ceramide or anti-TLR4 followed by *H. pylori* infection. Moreover, both *IL-8* promoter activity
307 (Fig. 10D) and IL-8 secretion (Fig. 10E) were significantly reduced by transfection of cells with
308 TLR4 siRNA prior to infection with *H. pylori*. Thus, these results suggest that modulation of
309 cellular cholesterol levels reduces the mobilization of ceramide and TLR4 into cholesterol-rich
310 microdomains, thereby attenuating *H. pylori*-induced IL-8 activation.
311

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 up-regulate 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 show that TLR4 and MD-2 expression in gastric epithelial cells were 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 signaling rather than TLR4 (52, 60). The reasons for these 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 infection, the cell lines assessed, and the infection times utilized vary 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). Further, we employed 3 additional gastric-epithelium-derived cell lines (MKN45, TSGH9201, and SC-M1) to validate that *H. pylori* can up-regulate TLR4 and MD-2 expression (Fig. 7), and obtained data in accordance with findings previously reported by Ishihara *et al.* (25).

In this study, we demonstrated that up-regulation of TLR4 by *H. pylori* was associated with increased secretion of ceramide (Fig. 6), which has been shown to activate NF-κB and IL-8 production 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). Increased ceramide levels lead to the formation of ceramide-enriched membrane rafts and activation of specific signaling pathways including proinflammatory cytokine release (18). These studies support our finding that *H. pylori* infection induced mobilization of ceramide into lipid rafts, whereby it may activate TLR4 signaling and contribute to induction of IL-8 secretion.

The DRM fractionation data in our current study provides 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,

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

358 Our results indicated that *H. pylori* infection of AGS cells induced not only TLR4 mRNA and
359 protein expression but also secretion of the proinflammatory cytokine IL-8. We found that treatment
360 of AGS cells with anti-TLR4 or TLR4 siRNA inhibited *H. pylori*-induced *IL-8* promoter activity
361 and IL-8 production (Fig. 10D-E). The *IL-8* promoter contains NF-κB and AP-1 binding sites (14),
362 and thus our results implicate a possible connection between *H. pylori* infection and
363 NF-κB-mediated activation of *IL-8*. However, IL-8 secretion was not completely impaired by the
364 pretreatment of cells with anti-TLR4 or TLR4 siRNA. This may be explained by another pathway
365 which involved in *H. pylori* CagA-induced IL-8 production (8). Although this is not direct evidence
366 for TLR4 as the major receptor for signaling pathways induced by *H. pylori*, close associations
367 between *H. pylori*, TLR4, and IL-8 secretion suggest the possible involvement of TLR4 in *H.*
368 *pylori*-induced responses. Constitutive expression of TLR4 contributed by serving as a receptor for
369 *H. pylori* binding (53) and also by acting as a potent receptor involved in the response to *H. pylori*
370 LPS-induced activation of cytoplasmic NF-κB and the subsequent activation of the *IL-8* promoter
371 (25). Together these data support our hypothesis that TLR4 is crucial for inducing IL-8 activation in

372 response to *H. pylori* infection.

373 Several studies have reported that bacteria utilize lipid rafts or raft-associated molecules for
374 adhesion to or invasion of host cells (11, 29, 34, 59). With *H. pylori*, previous studies have shown
375 that cholesterol-rich microdomains are required for VacA binding, delivery, and intoxication of
376 cells (20, 28, 41, 48). Our previous studies demonstrated that translocation of CagA and the
377 internalization of *H. pylori* in gastric epithelial cells is significantly reduced upon treatment of cells
378 with MβCD, thereby decreasing the pathogenesis induced by the bacteria (30, 32). Moreover, we
379 recently reported that the glucosylation of cholesterol in *H. pylori* is crucial for efficient *cag* type
380 four secretion system (TFSS)-associated activity, which may due to the reorganization of
381 membrane architecture (58). Here we showed that *H. pylori*-induced release of ceramide, and
382 expression of TLR4, as well as secretion of IL-8, was attenuated upon depletion of cholesterol or by
383 inhibition of cholesterol synthesis. These data support the notion that the integrity of lipid rafts is
384 essential for *H. pylori*-induced pathogenesis of host cells, as suggested by previous studies (23, 30,
385 40).

386 In conclusion, we have demonstrated that infection of gastric epithelial cells with *H. pylori*
387 induced TLR4/MD-2 mRNA and protein expression. Furthermore, *H. pylori* facilitated the
388 mobilization of ceramide and TLR4 into lipid rafts, where both of which contribute to NF-κB
389 activation and IL-8 production. Together these results shed light on the molecular mechanisms of *H.*
390 *pylori*-induced pathogenesis of host cells.

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552 ## FIGURE LEGENDS

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

562

563 **FIG. 2. Involvement of MD-2 expression in response to *H. pylori* infection in AGS cells.** AGS
564 cells were infected with *H. pylori* at various MOI from 0 to 400 for 6 h (A) or at an MOI of 100 for
565 the indicated times (B). Expression levels of MD-2 were determined by western blot analysis.
566 Protein expression levels were quantified using densitometric analysis, normalized to β-actin, and
567 presented as the mean ± standard deviation derived from 3 independent experiments. Statistical
568 significance was determined using the Student's *t*-test (*$P < 0.05$).

569

570 **FIG. 3. Infection of *H. pylori* induces TLR4 and MD-2 expression.** AGS cells were transfected
571 with TLR4 or control siRNA for 24 h followed by infected with or without *H. pylori* for 6 h. TLR4

572 mRNA levels were assessed using semi-quantitative reverse-transcriptase PCR (A) and quantitative
573 real-time PCR (B). The protein levels of TLR4 and MD-2 were examined using western blot
574 analysis (C). Protein expression levels of TLR4 were quantified with densitometric analysis and
575 normalized to β-actin (D). Results are expressed as the mean ± standard deviation. *$P < 0.05$
576 compared to control siRNA with mock infection group; $^{\#}P < 0.05$ compared to control siRNA with
577 *H. pylori* infected group.
578
579 **FIG. 4. Involvement of lipid rafts in *H. pylori*-induced TLR4 expression in gastric epithelial**
580 **cells.** AGS cells were pretreated with medium alone or 5 mM methyl-β-cyclodextrin (MβCD) at
581 37°C for 30 min. Cells were then washed and incubated with *H. pylori* at an MOI of 100 for 6 h.
582 (A) TLR4 mRNA level was evaluated by quantitative real-time PCR, and GAPDH was used as
583 an internal control. (B) Detergent-resistant membrane (DRM) and -soluble (S) fractions were
584 prepared from cells that were subjected to cold-detergent extraction using 1% Triton X-100
585 followed by centrifugation. Each fraction was subjected to western blot or dot blot analysis using
586 antibodies against transferrin receptor (TfR) or cholera toxin subunit B (CTX-B)-conjugated to
587 horseradish peroxidase, or an antibody specific to TLR4. (C) After infection with *H. pylori* for 6
588 h, cells were probed with control FITC-conjugated anti-mouse IgG (gray histograms) or
589 FITC-conjugated LPS (white histograms). The FITC fluorescence level was analyzed by flow
590 cytometry. (D) The numbers represent the mean channel fluorescence (MCF) and the quantitative
591 results represent the mean and standard deviation of 3 independent experiments. An asterisk

indicates $P < 0.05$ compared to untreated MβCD control group, as determined by Student's *t*-test.

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), Alexa 647–conjugated CTX-B to visualize GM1 (red), or DAPI (blue) to visualize bacteria (arrows) and the cell nucleus. Samples were analyzed by confocal microscopy. The co-localization of TLR4 with GM1 appears as yellow in the overlay. The co-localization of fluorescence for TLR4 and GM1 signals was quantified using the Pearson correlation coefficient and presented in the right panels. Statistical significance was determined from 3 independent experiments, $*P < 0.01$. Scale bars, 10 μm. Hp–, not treated with *H. pylori*; Hp+, treated with *H. pylori*.

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 using quantitative real-time PCR (A) and western blot analysis (B), respectively. The mRNA and protein expression levels were normalized to GAPDH and β-actin, respectively. (C) AGS cells were pretreated with medium alone or 5 mM MβCD 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 probed with Alexa Fluor 568–conjugated goat anti-mouse IgM, and the fluorescence intensity was

612 assessed by flow cytometry. Results are expressed as the mean ± standard deviation, *$P < 0.05$
613 compared with un-treated control. MCF: mean channel fluorescence, MβCD:
614 methyl-β-cyclodextrin.
615
616 **FIG. 7. Infection of *H. pylori* enhances ceramide and TLR4 expression.** Cells from the indicated
617 lines were infected (or not infected) with *H. pylori* for 6 h at 37°C. (A) Cell lysates were prepared
618 to analyze TLR4 and MD-2 protein expression levels by western blot. (B) Protein expression levels
619 of TLR4 were quantified with densitometric analysis and normalized to β-actin. (C) The cells were
620 fixed and stained with anti-ceramide and assessed by flow cytometry for the fluorescence intensities.
621 An asterisk indicates $P < 0.05$ compared to each un-infected *H. pylori* group, as determined by
622 Student's *t*-test. Hp–, not treated with *H. pylori*; Hp+, treated with *H. pylori*.
623
624 **FIG. 8. Disruption of lipid rafts reduces *H. pylori*-induced ceramide and TLR4 expression.**
625 AGS cells were pretreated with imipramine (10 μM), MβCD (2.5 μM), or nystatin (10 μg/ml) for 1
626 h, followed by infection with *H. pylori* at an MOI of 100 for 6 h. The cells were fixed and stained
627 with anti-ceramide (A) or anti-TLR4 (B), the fluorescence intensities were then determined by flow
628 cytometry. Results are expressed as the mean ± standard deviation. $^{\#}P < 0.05$ compared with
629 untreated control; *$P < 0.05$ compared with *H. pylori*-infected group.
630
631 **FIG. 9. *H. pylori*-stimulated ceramide and TLR4 cluster in lipid rafts.** AGS cells were infected

632 with *H. pylori* (or not infected) at 37°C for 6 h. Cells were fixed and stained with (B, F, J)
633 anti-TLR4 (green), (C, G, K) anti-ceramide (red), (A, E) phase to visualize bacteria, or (I) DAPI
634 (blue) to visualize bacteria and the cell nucleus, and then analyzed by confocal microscopy. Arrows
635 indicate the co-localization of TLR4, ceramide, and *H. pylori* on infected cells. Confocal *z*-section
636 images are shown that bacteria co-localized with TLR4 and ceramide (I–L). Scale bars, 10 μm. Hp–,
637 not treated with *H. pylori*; Hp+, treated with *H. pylori*.
638
639 **FIG. 10. Activation of IL-8 by *H. pylori* is attenuated by inhibition of ceramide and TLR4 and**
640 **disruption of rafts.** (A) Ceramide stimulates IL-8 secretion in AGS cells. Cells were treated with
641 ceramide in the indicated concentrations for 24 h. The level of IL-8 in the culture supernatant was
642 determined by a standard ELISA method. (B–E) *H. pylori*-induced *IL-8* activity and IL-8 secretion
643 were influenced by pretreatment with lovastatin, anti-ceramide, anti-TLR4 and TLR4 siRNA. AGS
644 cells were transfected with IL-8/wt Luc reporter plasmid in the presence of lovastatin (50 μM),
645 anti-ceramide, or anti-TLR4 prior infection of cells with *H. pylori* at MOI 100. Cell lysates were
646 subjected to luciferase activity assays for *IL-8* activity (B), and IL-8 levels in the cell culture
647 supernatants were determined by ELISA (C). Cells were transfected with TLR4 or control siRNA
648 for 24 h followed by infection of *H. pylori*, the *IL-8* activity (D) and IL-8 production (E) were
649 analyzed using luciferase activity assay and ELISA, respectively. Data represent the mean ±
650 standard deviation of 3 independent experiments. Statistical significance was evaluated using
651 Student's *t*-test (*$P < 0.05$).