Published in final edited form as:

Helicobacter. 2019 June ; 24(3): e12583. doi:10.1111/hel.12583.

Changes of tight junction and interleukin-8 expression using a human gastroid monolayer model of *Helicobacter pylori* infection

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Abstract

Background: Lack of a model that mirrors *Helicobacter pylori*-induced gastric mucosal inflammation has hampered investigation of early host-bacterial interactions. We used an ex vivo model of human stomach, gastric epithelial organoid monolayers (gastroid monolayers) to investigate interactions of *H. pylori* infection and the apical junctional complex and interleukin-8 (IL-8) expression.

Method: Morphology of human antral mucosal gastroid monolayers was evaluated using histology, immunohistochemical (IHC) staining, and transmission electron microscopy (TEM). Functional and gross changes in the apical junctional complexes were assessed using transepithelial electrical resistance (TEER), cytotoxicity assays, and confocal laser scanning microscopy. IL-8 expression was evaluated by real-time quantitative PCR and ELISA.

Results: When evaluated by IHC and TEM, the morphology of gastroid monolayers closely resembled in vivo human stomach. Following inoculation of *H. pylori*, TEER transiently declined

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AUTHOR CONTRIBUTIONS

KM contributed to confocal laser scanning fluorescence microscopy and transepithelial electrical resistance. TU contributed to histopathologic and immunohistochemical analysis and transmission electron microscope. ST and HU contributed to reverse transcription PCR and real-time quantitative PCR. XZ contributed to the management of gastroid. JK contributed to the management of *H. pylori*. MKE, DYG, and YY supervised and advised for this study and wrote the manuscript.

DISCLOSURES OF INTERESTS

The authors have no disclosures or other conflicts of interest to report.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

(up to 51%) in an *H pylori* density-dependent manner. TEER recovered by 48 hours post-infection and remained normal despite continued presence and replication of *H pylori*. Confocal scanning microscopy showed minimal disruption of zonula occludens-1 or E-cadherin structure. IL-8 production was unchanged by infection with either CagA-positive or CagA-negative *H pylori* and JNK and MEK inhibitors did not suppress IL-8 production, whereas p38 and IKK inhibitor significantly did.

Conclusion: Human gastroid monolayers provide a model for experimental *H pylori* infection more consistent with in vivo human infections than seen with typical gastric epithelial cell lines. This ex vivo system should lead to better understanding of *H pylori* host-pathogen interactions.

Keywords

cag pathogenicity island; *Helicobacter pylori*; interleukin-8; organoid; tight junction; virulence factor

1 | INTRODUCTION

Helicobacter pylori (*H pylori*) is a human pathogen involved in the pathogenesis of gastritis, gastroduodenal ulcers, and gastric cancer.^{1,2} The severity of inflammation and the risk of gastric cancer are modulated by bacterial virulence factors such as CagA, VacA, OipA, and DupA.³ Most experimental observations have involved nonpolarized gastric cancer cell lines or animal models and neither reliably replicates the human in vivo bacteria-host interactions. *Helicobacter pylori* infection in animal models also often fails to reflect findings in humans.⁴

Gastrointestinal organoids produced from stomach tissue called mini-stomachs were originally developed using mouse tissue and can now be produced using human gastric biopsies. Gastric organoids or gastroids were developed by Bartfeld et al⁵ and typically contain the full complement of epithelial cell types present in normal stomach. They can be cultured semi-permanently, are stable, and can be revived after freezer storage. This multicellular model has many advantages compared to cell lines developed from tumor-derived cells^{6,7} as the organoids contain polarized differentiated normal human cells without malignant alterations. However, in the 3-dimensional (3D) model, the lumen is contained within a “ball” of cells such that luminal infection requires microinjection and produces infection in a closed space. With that model, it is also possible that some of the *H pylori* inoculum can also infect from the basal side rather than interact exclusively with the apical cell side. In contrast, 2D cultures are physiologic and infection with *H pylori* is both simple and reliable. Previous studies have suggested that 2D polarized monolayers derived from human stomach are a suitable ex vivo model for *H pylori* infection.⁸

The normal stomach has a tight epithelium that maintains a high transmucosal potential difference despite the presence of *H pylori* infection (ie, as seen in patients with duodenal ulcer disease).⁹ Gastric intercellular junctions include the tight junction, adherens junction, desmosomal junction, and gap junction. The tight junction is the most apical site over the adherens junction, which together form the apical junction complex that plays important roles in the regulation of paracellular flux, cell polarity, and cell proliferation and forms a barrier to pathogens. In vitro studies using cell lines (non-organoids) have reported that *H*

pylori adheres to the cell-to-cell junctions of gastric epithelial cells and can disrupt the mucosal barrier.^{10,11} These observations are at odds with observations that *H pylori*-infected peptic ulcer patients maintain a normal gastric potential and normal intragastric permeability.⁹

The tight junction complex is composed of zonula occludens-1 (ZO-1), occludin, claudin, and junctional adhesion molecules (JAMs). Recent studies using a spheroidal mouse gastroid model reported that *H pylori* infection induced aberrant redistribution of the tight junction protein; claudin-7, from the cellular margin to focal accumulation in the cytosol.¹² Other studies using human gastric cancer cell lines have reported disruption of the adherens junction by *H pylori* via translocation of adherens junction proteins such as E-cadherin, β -catenin, and p-120.^{13–16} While studies using gastric biopsies have occasionally shown *H pylori* within gastric cells and invading the intercellular space without disruption of tight junction,¹⁷ the dynamics of these interactions are impossible to study using biopsy material, resections, or gastric lymph nodes.^{17,18} Here, we used the human ex vitro 2D antral polarized monolayer model (termed gastroid monolayers in this study) derived from spheroidal gastroid to investigate the apical junction complex during the early phase of *H pylori* infection.

The initiation of inflammation, migration, and activation of inflammatory cells into the gastric mucosa involve the expression of proinflammatory cytokines, especially interleukin-8 (IL-8) induced by gastric epithelial cells.¹⁹ The *cag* pathogenicity island (PAI) includes genes that encode a type IV secretion system (T4SS) that, after attachment to cells, injects CagA using the needle system. Recent studies have suggested that CagA is not injected apically, but rather is injected into the basal surface of cells.²⁰ Studies using a mouse gastroid monolayer model suggested that cell proliferation depended on the presence of CagA.^{12,21,22} Although previous studies have assessed the effect of *H pylori* virulence factors on induction of IL-8 using human spheroidal gastroid,⁵ none have used gastroid monolayers. Here, we compared IL-8 expression following infection with *H pylori* wild-type (*wt*), *cag* PAI-deleted mutants (*cag* PAI), or other clinical strains using gastroid monolayers as well as conventional cancer cell lines.

2 | MATERIALS AND METHODS

2.1 | Human tissue materials for spheroidal gastroids

Human spheroidal gastroids were obtained from the Gastrointestinal Experimental Model Systems (GEMS) Core in Texas Medical Center. Spheroidal gastroids were developed from gastric tissue obtained from the antrum of adults without *H pylori* infection during routine sleeve gastrectomy at Baylor College of Medicine. Gastric gland isolation from the tissue samples was performed as previous described.^{5,23–25}

2.2 | Gastroid medium

Three different types of media were used to establish, maintain, and differentiate gastroids.^{5,23–25} Complete medium without growth factors (CMGF–) contained advanced DMEM/F-12 medium supplemented with 100 U/mL penicillin/streptomycin, 10 mmol/L HEPES

buffer, and 1% GlutaMAX (all from Thermo Fisher Scientific, Waltham, MA). High Wnt complete medium with growth factors (High Wnt CMGF+) for gastroid growth was composed of 50% CMGF+ and 50% Wnt-conditioned medium. CMGF+ contained 15% CMGF- supplemented with 50% Wnt3A-conditioned medium produced from ATCC CRL-2647 cells (ATCC, Manassas, VA), 20% R-spondin1-conditioned medium (R-spondin1-producing cells [RSPO1]; kindly provided by Calvin Kuo, Palo Alto, CA), 10% Noggin-conditioned medium, 1× B27 supplement (Thermo Fisher Scientific), 1× N2 supplement (Thermo Fisher Scientific), 50 ng/mL recombinant mouse epidermal growth factor (Thermo Fisher Scientific), 1 mmol/L N-acetylcysteine (Sigma-Aldrich, St. Louis, MO), 10 µmol/L SB202190 (Sigma-Aldrich), 10 nmol/L gastrin I (Sigma-Aldrich), 10 mmol/L nicotinamide (Sigma-Aldrich), 150 ng/mL recombinant human FGF-10 (PEPROTECH, Rocky Hill, NJ), and 500 nmol/L A-83-01 (Tocris Bioscience, Bristol, UK).

Differentiation medium (DM) for gastroids was basically the same as CMGF+ medium in 5% Noggin-conditioned medium, but did not include Wnt3A, R-spondin1-conditioned medium, nicotinamide, and SB202190.

2.3 | Human spheroidal gastroid culture, transformation from spheroid to monolayer, and differentiation

Each 24-well plate containing approximately 50–100 glands embedded in Matrigel (phenol red-free, growth factor-reduced; Corning, Corning, NY) was cultured in High Wnt media at 37°C with 5% CO₂ to develop spheroidal gastroids. We confirmed spheroidal gastroids underwent self-renewal before experiments. Spheroidal gastroids with passage number 5–20 were used for all experiments, and High Wnt CMGF+ medium was exchanged every 2 days.

Preparation of gastroid monolayers and infection of the cells with *H. pylori* were adapted from previous reports.^{24–26} The spheroidal gastroid cultures established from glands were embedded in phenol red-free, growth factor-reduced Matrigel (Corning) and were cultured in High Wnt CMGF+ media for 7 days until the cell number was sufficient for expansion for gastroid monolayers. Spheroidal gastroids were washed with 500 µL of 0.5 mmol/L EDTA in 1× PBS (without Mg and Ca; Thermo Fisher Scientific) per well. After removal of supernatants, the spheroidal gastroids were dissociated with 500 µL of 0.05% trypsin/0.5 mmol/L EDTA (Thermo Fisher Scientific) at 37°C for 4 minutes. The trypsin was inactivated by adding 1 mL CMGF-media containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific). The spheroidal gastroids were pipetted vigorously approximately 50 times using micropipette P1000 tips and passed through a wet 40-µm cell strainer (Corning) to separate single cells from cell clumps. These cells were then centrifuged at 400 *g* for 5 minutes at room temperature. Afterward, the medium was removed and the cell pellets were suspended in 100 µL of CMGF+ media containing 10 µmol/L of ROCK inhibitor Y-27632 (Sigma-Aldrich). Each 96-well and 6.5-mm transwell with 0.4 µm pore polyester membrane insert (Corning) was coated with 2.5% Matrigel diluted in ice-chilled PBS and incubated for 30 minutes at 37°C. Suspensions from spheroidal gastroids were seeded onto the Matrigel-coated 96-well plates or transwells and were cultured in CMGF+ with Y-27632 media for 2 days to be monolayers. Then, the DM was exchanged every 2 days for differentiation. The monolayers were cultured in DM until day 7 to obtain well-differentiated gastroid

monolayers. DM was changed every 2 days. Experiments were performed with transwells for Figures 1–4 and Figures S1, S2 and 96-well plates for Figures 5 and 6, respectively.

2.4 | *H pylori* culture and infection

The *H pylori* strains used included high and low virulent stains from our stocks at Baylor College of Medicine. We used strain TN2GF4 (wt: *cag* PAI-positive, *vacA*-s1m1 genotype; VacA-producing, *oipA* “on” [OipA-producing], *babA*-positive), its *cag* PAI-deleted mutant, and several clinical isolates. Clinical isolate 401C from Colombia was *cag* PAI-negative, *vacA*-s2m2 genotype (VacA non-producing), *oipA* “off” (OipA non-producing), and *babA*-negative.²⁷ We also used several clinical isolates similar to strain 401C; strains COL153 and COL443 from Colombia and strains BGD76 from People’s Republic of Bangladesh were all *cag* PAI-negative, *vacA*-s2m2, *oipA* “off,” and *babA*-negative. All strains in this study had the *htrA* gene. These strains were used from our stocks and grown on brain heart infusion broth agar (Becton Dickinson, Sparks, MD) containing 7% defibrinated horse blood for 72 hours. Before infection, bacteria were grown with brain heart infusion broth with 10% FBS under micro-aerophilic conditions at 37°C overnight with shaking. Bacteria (spiral) were washed with phosphate-buffered saline (PBS; pH 7.4) and resuspended in PBS before infection. To adjust bacterial density and check viability, OD600 and colony-forming units (CFUs) were used in all experiments. To quantify the number of cells in each well to calculate multiplicity of infection (MOI), spheroidal gastroids were dissociated into single-cell suspensions with 0.05% trypsin/0.5 mmol/L EDTA for 5 min at 37°C. The MOI was adjusted ranging from 10 to 100. All experiments were performed using well-differentiated gastroid monolayers. Gastroid monolayers were routinely maintained in DM to ensure differentiation prior to *H pylori* infection by day 12. Antibiotics were removed from the maintenance medium by washing with medium three times before infection. Infection was done with antibiotic-free medium. Inoculation was performed by placing the *H pylori* suspension into the upper side of the transwells. The infected cells were incubated at 37°C in a 5% CO₂ humidified incubator.

2.5 | Statistical analysis

All mean values and standard errors were calculated from at least triplicate samples. One-way analysis of variance followed by Student’s *t* test and Dunnett’s multiple comparisons was used as appropriate for statistical analysis using JMP 10.0 software (SAS, Cary, NC, USA). All *P* values of <0.05 were accepted as statistically significant (**P* < 0.01, ***P* < 0.05).

2.6 | Supplemental table, materials and methods

The readers are referred to Supplement table 1, the Supporting information Materials and Methods section for conventional epithelial cancer cell lines, inhibitor experiments, histopathologic and immunohistochemical analysis, transmission electron microscope (TEM), confocal laser scanning fluorescence microscopy, IL-8 protein expression by ELISA, measurement of transepithelial electrical resistance (TEER), cytotoxicity assay, and IL-8 mRNA expression by reverse transcription PCR and real-time quantitative PCR.

3 | RESULTS

3.1 | Morphological changes of human gastroid monolayers dependent on differentiation status

Bartfeld et al⁵ previously showed that progenitor cells can be directed from the gland domain toward the pit lineage by manipulating Wnt3A concentration in the culture medium. Schlaermann et al²⁸ described that gastric spheroids constituted an undifferentiated stage and it was possible to differentiate differentiated one by withdrawing Wnt3A and RSPO1. Using their methods,^{8,28} we studied both undifferentiated (with Wnt3A and RSPO1) and differentiated (withdrawal of Wnt3A and RSPO1) gastroid monolayers.

We examined the morphology of undifferentiated and differentiated human gastroid monolayers, between passage number 5-20 in the following studies. The H&E-stained undifferentiated gastroid monolayers showed unclear cell polarity, irregularly aligned multilayer formation, and little mucus on the cells and had high nucleus/cytoplasm ratios (Figure 1A), all features of immature cells.²⁹ Differentiated gastroid monolayers were covered by rich mucus on the apical surface and formed a regular monolayer with obvious polarity (Figure 1B). In their gastroid monolayers which we analyzed over 30 samples, all sections showed the same polarity. Additionally, the nucleus morphology was small and round and the outline of the nucleus was similar to gastric mucosa in vivo (Figure 1B).

Immunohistochemical staining for HGM45 for gastric epithelial and mucin markers showed markedly higher expression in differentiated than undifferentiated gastroid monolayers (Figure 1C,D). Expression of MUC6, a pyloric gland cell marker, was absent in differentiated gastroid monolayers, but was weakly positive in undifferentiated gastroid monolayers (Figure 1E,F). HGM45 and MUC5AC are the same gastric epithelial and mucin markers, and some previous studies showed that the expression of MUC5AC and MUC6 depended on Wnt3A and RSPO1.^{5,8,28} Those studies also mention that withdrawing Wnt3A and RSPO1 changed undifferentiated gastroid monolayers (deep basal mucosa) to differentiated ones (gastric surface mucosa). Expression of MUC2, an intestinal marker, was negative in both differentiated and undifferentiated gastroid monolayers (Figure 1G,H). Alcian blue and AE1/AE3, and mucous and epithelial markers, respectively, were positive in both differentiated and undifferentiated gastroid monolayers (Figure 1I-L). TEM clearly showed that the monolayer of differentiated gastroid monolayers had cell polarity and mucus (Figure 1M) and microvilli on the apical side (Figure 1N).

3.2 | Effect of *H. pylori* infection on gastroid monolayers and apical junctional complex

We investigated the morphologic changes of differentiated gastroid monolayers infected with *H. pylori* by H&E stain and TEM. Characteristically, the mucous area in gastroid monolayers seemed to be decreased after infection (Figures 1B and 2A,B), which was consistent with mucous secretion caused by *H. pylori* infection. *Helicobacter pylori* seemed to attach to the cells mainly in areas with mucus (Figure 2C,D).

TEER of the gastroid monolayers on the transwell was measured on the day 0 (seeding day), day 2, and day 4 to confirm the differentiation status before infection (Figure 3A). The TEER at day 0, seeding day, was low ($57.8 \pm 10.7 \Omega \text{ cm}^2$) due to immature intercellular

junctions or low cell confluency. TEER reached $698.4 \pm 40 \Omega \text{ cm}^2$ on day 2 consistent with confluent cells with functional apical junctional complexes (Figure 3A, $P < 0.01$). Also, the TEER of gastroid monolayers on day 4 was $673.2 \pm 40 \Omega \text{ cm}^2$, consistent with confluent cells with robust apical junctional complexes (Figure 3A, $P < 0.01$). We chose day 4 to begin infection in our experiments.

To evaluate whether the tight junction of the apical junctional complex was disrupted in association with *H. pylori* infection, 12,30,31 gastroid monolayers were co-cultured with *H. pylori* and the TEER of the infected gastroid monolayers was measured to assess the barrier function of the tight junctions. The TEER in infected cells declined at 24 hpi, especially TEER of gastroid monolayers infected with *H. pylori* at and MOI 100, fell to $353.7 \pm 8 \Omega \text{ cm}^2$, which although still high was significantly lower than the uninfected controls ($691.9 \pm 34 \Omega \text{ cm}^2$) (Figure 3B, $P < 0.01$). However, the TEER recovered by 48 hpi and remained normal and similar to uninfected cells (Figure 3B). Additionally, the TEER of the infection group at 96 hpi was the same as uninfected gastroid monolayers, which led us to conclude that *H. pylori* infection did not cause permanent tight junction disruption.

Lactate dehydrogenase (LDH) release into the culture medium from damaged gastroid monolayers was analyzed to assess whether the transient loss of TEER was associated with a change in viability of the cells (Figure 3C). The LDH assay was carried out with the supernatants from the same transwells in which cells were subjected to the TEER measurement (Figure 3B). There was no significant difference in cell viability among all groups at 24 hpi even when the lowest TEER was present in the infected group. No difference in LDH release was observed between infection and control groups until 72 hours (Figure 3C). These results confirmed that the reduction in TEER was not associated with loss of viability of more than a small proportion of *H. pylori*-infected cells.

To evaluate changes in the epithelial barrier of *H. pylori*-infected cells, immunofluorescence staining of confluent monolayers was performed to visualize ZO-1 (tight junction protein) and E-cadherin (adherens junction protein) (Figure 4) at 96 hpi. As shown below, we also measured protein levels and found that gastroid monolayers continued to produce IL-8 until 96 hours after infection. These data confirmed that *H. pylori* remained alive on the gastroid monolayers for at least 96 hours. We also confirmed that *H. pylori* was closely localized with villin as previously described,^{32,33} which was abundant on the surface of microvilli, showing that *H. pylori* attached to the surface of gastroid monolayers (Figure 4A). In uninfected gastroid monolayers, ZO-1 and E-cadherin were observed as well-organized bands surrounding individual cells (Figure 4B,C, upper panels “Mock”). Dispersal of ZO-1 was observed in some of the infected cells detected by staining with anti-*H. pylori* antibodies; however, the other cells maintained intact ZO-1 structures (Figure 4B, lower panels “infected”). In contrast to ZO-1, E-cadherin structure was not affected by *H. pylori* infection (Figure 4C, lower panels “infected” and Figure S2). The Z-stacked images of the infected cells stained with antibodies against ZO-1 and E-cadherin showed that both ZO-1 and E-cadherin structures were intact despite *H. pylori* infection (Figure 4D,E). These experiments suggest that *H. pylori* infection resulted in at most small changes in the tight junction but not in the adherens junction of gastroid monolayers.

3.3 | Interleukin-8 expression from gastroid monolayers infected with *H pylori*

Interleukin-8 is a chemoattractant for neutrophils and T lymphocytes thought to be in part responsible for the histologic inflammation seen in vivo *H pylori* infection.^{3,34} *Helicobacter pylori* stimulated *IL-8* mRNA expression in a time-dependent manner with maximal levels at 3 hpi in 96-well plates (Figure 5A). Additionally, IL-8 protein levels in both infection groups (MOI 10 and 100) were significantly higher than uninfected controls in each time course in transwells (Figure 5B). Infected gastroid monolayers continued to release IL-8 constantly from 0 to 96 hpi in an MOI-dependent manner (Figure 5B). Thus, human gastroid monolayers were able to sustain chemokine release which is similar to what occurs in chronic *H pylori* infections.

CagA-containing *H pylori* are associated with an increase in the inflammatory response as mentioned above. In agreement with previous studies using gastric cell lines, IL-8 protein levels in infected groups were significantly higher than in uninfected controls and that in TN2GF4 *wt* were also significantly higher than that in TN2GF4 *cag* PAI when using AGS or MKN28 cells (Figure 5C). Next, we compared *IL-8* mRNA expression induced by gastroid monolayers with *H pylori* strains TN2GF4 *wt* and TN2GF4 *cag* PAI to examine the response to the presence of the virulence factor. The *IL-8* mRNA level in TN2GF4 *cag* PAI infection showed no significant change compared to that with TN2GF4 *wt* at 3 hpi (Figure 5D). IL-8 protein levels in infected gastroids were significantly higher than in uninfected controls; however, they were similar to IL-8 levels from gastroid monolayers co-cultured with strains TN2GF4 *wt* and *cag* PAI using transwells (Figure 5E). We interpret this to mean that the mechanisms of IL-8 production in gastroid monolayers differ from that present in gastric cancer cell lines.

Furthermore, we investigated a role of virulence factors for IL-8 production using several clinical isolates with lower virulence (*cag* PAI-negative, *vacA*-s2m2, *oipA* “off,” and *babA*-negative). Surprisingly, although these strains do not induce IL-8 from MKN28 cells, they stimulated large amounts of IL-8 from gastroid monolayers (Figure 6A). These data suggest that the *cag* PAI/CagA, VacA, OipA, and BabA may not be directly involved in IL-8 production from gastroid monolayers.

To investigate the differences in IL-8 expression between gastroid monolayers and gastric cancer cell lines, we evaluated the effects of inhibitors of different signal transduction pathways for IL-8 production. We used each inhibitor (SB203580: p38 inhibitor, SC-514: IKK inhibitor, SP600125: JNK inhibitor, and U-0126: MEK inhibitor, each 10 μ mol/L) with gastroid monolayers infected with strain TN2GF4 *wt*. As previous studies had shown, all inhibitors influenced IL-8 production from gastric cancer cell lines (Figure 6B). However, JNK and MEK inhibitors had no effect on IL-8 production from gastroid monolayers and p38 and IKK inhibitors significantly reduced IL-8 production (Figure 6C). We obtained the same results using inhibitors at higher concentration (50 μ mol/L, data not shown). Overall, we concluded that IL-8 signaling pathways differ between gastroid monolayers and gastric cancer cell lines.

4 | DISCUSSION

Using a robust ex vivo model of human *H. pylori* infection, we showed that the results of *H. pylori* infection of human gastroids differed from those obtained with gastric epithelial cancer cell lines. For example, *H. pylori*-infected epithelial cells of gastroid monolayers maintained their normal apical junctional complex morphology and TEER. In addition, IL-8 expression from gastroid monolayers was independent of the presence or absence of what are considered traditionally as *H. pylori* virulence factors.

Apical junctional complex disruption is histological feature of gastric cancer, and prior vitro studies have suggested this might also be associated with *H. pylori* infection,^{10,12,30} especially, in studies using non-human non-gastric cells. For example, in the canine kidney cell line MDCK,³⁵ the presence of CagA has been reported to be associated with disruption of cell adhesion, cell polarity, and inhibition of migration related to dislocation of ZO-1. In that model, PAR1/MARK kinase targets as CagA-induced tight junction disruption, loss of epithelial cell polarity, and reduced TEER with the MDCK monolayer. In addition, possibly because CagA can physically interact with E-cadherin, E-cadherin was reported to translocate from the membrane to the cytoplasm of epithelial cells.^{22,36} However, these results were not confirmed in another study which showed no change of ZO-1 protein expression level irrespective of *H. pylori* infection or the presence of CagA.³⁷ Previous TEER studies with *H. pylori* infection of gastric cancer epithelial cell lines, N87 and MKN28, showed that while wild-type *H. pylori* infection decreased epithelial barrier function (ie, TEER), TEER was also reduced by *cagA*, *cagE*, *vacA*, and *urease B* knockout strains (ie, the TEER changes were independent of what are considered the main virulence factors).^{30,31} For example, *H. pylori* strain 26695 *wt*, and the *vacA* and the *cagA* mutant strains induced a significant TEER decreased at both 24 hours (691-776 Ω cm²) and 48 hours (436-519 Ω cm²) compared with uninfected monolayers (1110 and 11 096 Ω cm²).³¹ In Figure 3B, we show that the TEER rapidly recovered even with TN2GF4 wild-type infection consistent with the notion that the apical junctional complex of gastroid monolayers is robust and confirming that the TEER of gastroid monolayers was not altered by these virulence factors. The TEER range differs depending on the cell type; the maximum TEER reported has been 470 Ω cm² on mucosoid⁸ vs, 80-1400 Ω cm² on conventional cell lines.^{30,31} Studies^{30,31} with conventional cell lines showed that TEER approximately dropped 30%-50% permanently compared with base levels during 8-48 hours co-culture with *H. pylori*. However, conventional cell lines have been examined for only as long as 48 hours. The present study used polarized normal human monolayers and found that although ZO-1 changed during *H. pylori* infection, ZO-1 on the tight junction remained intact, or rapidly recovered, as assessed by changes in TEER which is consistent with in vivo studies of TEER (Figures 3B,D and 4B). The adherens junction also remained unaffected despite the presence of CagA in the intercellular space (Figure 4C,E and Figure S2 and Figure 3B). Although prior studies lasted up to 48 hours,^{10,14,16,30,35,37-39} our studies confirmed that the apical junctional complex remained relatively preserved and, as noted, the TEER recovered after temporally dropping which is in agreement with vivo studies.⁴⁰ These results suggest the gastroid monolayer model responds similarly to normal gastric epithelial cells in vivo.

The epithelial mucosa is programmed to rapidly heal and reseal the barrier including the tight junctions.⁴¹ Our results are similar to that reported in normal stomach after the introduction of a gastrotoxin such as aspirin which is associated with a rapid but reversible fall in TEER and rapid healing of the mucosal damage.⁴² Repeated insults also result in adaptation such that the gastric mucosa becomes resistant to further insults. Examination of gastric mucosal tissues obtained from *H pylori*-infected patients also showed that almost all tight junctions remain intact, even in the presence of *H pylori* infection.¹⁷ In the *H pylori*-infected stomach, there is considerable trafficking of polymorphonuclear (PMN) cells through the tight junctions which provides a potential site for *H pylori* to enter the intercellular space.⁴³ Recent in vitro studies reported that *H pylori* released the serine protease HtrA, which opens cell-to-cell junctions through cleaving epithelial junctional proteins including occludin, claudin-8, and E-cadherin and allows *H pylori* to reach the basolateral membranes where it can inject CagA in polarized conventional cell monolayers.⁴⁴ On the other hand, in vivo study of gastric biopsy specimens during chronic *H pylori* infection has shown no adverse effects on E-cadherin and β -catenin.⁴⁵ E-cadherin in our model was unchanged after infection consistent with finding using the vivo model.⁴⁵ Overall, the data support the notion that apical junctional complex in human gastroid monolayers is robust, and while it may temporarily open, the injury is reversible rather than leading to a permanent disruption.

In chronic infection, *H pylori* are commonly seen adhering to the apical junctional complex of gastric epithelial cells.¹⁰ It has been suggested that this site is preferred as it is likely the pathway for nutrients to pass through the paracellular pathway along with PMNs.⁴³ In human in vivo studies, *H pylori* has been reported to enter in the gastric epithelium or deep stroma through the tight junction¹⁷ or intercellular space as visualized by TEM.⁴⁶ While we found CagA protein in the intercellular space, we were unable to find *H pylori* themselves “under” the tight junction using confocal laser fluorescence microscopy or TEM (Figures 2A–D and 4D,E). Our model differs from the normal stomach in which the lumen is often highly acidic causing the bacteria to seek regions where the pH is highest. Also in our study, *H pylori* specifically attached to areas of mucous area as seen by TEM (Figure 2C,D). A study using gastric mucosa which is an ex vivo model like our gastroid monolayers, Boccellato et al⁸ reported the surface of the mucus was a barrier against infection. Mucous production is another feature of this model that is similar to in vivo conditions; conversely, cancer cell lines used for *H pylori* experiments, such as AGS cells, lack mucous expression.⁴⁷

IL-8, a potent neutrophil chemotactic and activating peptide, is thought to be indispensable for the migration and activation of inflammatory cells into the gastric mucosa. Most prior in vitro studies have shown that the level of IL-8 expression depends on CagA and/or *cag* PAI,^{48,49} and this has been confirmed in studies with AGS and MKN28 cells (Figure 5C). IL-8 expression in gastroid monolayers with *H pylori* infection differed from IL-8 expression in cell lines as it was not influenced by CagA and/or *cag* PAI (Figure 5D,E) suggesting the presence of other signaling pathways. This finding was also suggested in a previous human spheroidal gastroids study.⁵ Previous studies reported that CagA was efficiently phosphorylated in host integrin-containing basolateral sites and integrin acts as a receptor with an integrin-specific bacterial adhesin, CagL protein. Unlike many established

unpolarized cell lines, gastroid monolayers contain integrin at the basolateral surface similar to normal gastric epithelium in vivo. As gastroid monolayers maintained polarity and robustness of tight junction or adherens junction even after *H pylori* infection, it was difficult for the bacteria to approach to the integrin-containing basolateral surface and the attachment was largely restricted to the apical surface and appeared *cag* PAI independent. These data suggest that the interaction of CagA with normal polarized cells and unpolarized cells differs. However, another study showed that CagA could induce morphological change, such as the “hummingbird” phenotype, in human gastroid monolayers.²⁸ And that proliferation of spheroidal gastroids with *H pylori* wt was significantly greater than that with *cagA*.^{21,22} The IL-8 signaling pathway is involved in at least 20 signaling cascades.⁴⁹ The central downstream targets are NF- κ B and AP-1. In our study, signaling pathways through only IKK (NF- κ B) activation and p38 in AP-1 activation were related to IL-8 expression from gastroid monolayers, whereas cancer cell lines also involved JNK and MEK. Another study showed that heat-killed *H pylori* was able to induce IL-8 from the spheroidal gastroids.⁵ The factor(s) other than the well-known virulence factors such as CagA which are involved in IL-8 induction in gastroid monolayers remain to be determined.

Our study has several limitations. First, gastroids are composed of highly polarized cells which differentiate into the cell lineages depending on the tissue of origin. Two key factors are Wnt signal strength and origin of the samples. The cell composition of gastroids will also differ depending on the anatomical region of stomach from which the tissue is obtained.^{29,50} Gastric units in antral epithelium are simpler than that in the corpus and contain only two types of mucous cells, the mucus-secreting-pit cells and intermediate cells between chief cells and mucous neck cells; both of these cells are mucous cells.⁵⁰ Endocrine cells are present but very rare. In this study, the gastroids were established from biopsy specimens from the antrum based on the fact that in vivo *H pylori* primarily and preferentially colonize antral mucosa.⁵¹ It is unknown whether antral organoids from another individual might have produced different results. Second, gastroid monolayers are gastric epithelial cell model that lacks innervation, blood vessels, immune cells, or stroma cells which may be critical for inflammatory signals and paracrine signaling. Third, in the mouse model, it has been shown that *H pylori* can infect both differentiated epithelial pit cells and short-lived in superficial cells as well as progenitor or long-lived stem cells which live in deep gastric gland.⁵² The gastroid model used cells with a uniform degree of well-differentiated epithelial cells which differs from the in vivo model where there are various degrees of cell differentiation in the epithelial and deep sites. Fourth, we did not quantify the number of the *H pylori* 96 hours post-infection in our study. *Helicobacter pylori* generally cannot survive in FBS-free/Brucella broth-free medium, but have been reported to survive in co-culture with conventional host cells and medium as they are able to require the appropriate nutrition from the apical surface of epithelium.⁵³ It has also been noted that in the mucosoid system, *H pylori* can survive for several weeks.⁸ These data are consistent with our hypothesis that the bacteria in our co-culture system survive and are not killed by either the gastroid monolayer or medium during at least the 96-hours post-infection period. The ability of *H pylori* to survive for longer periods should be useful to clarify the mechanism of persistent infection of *H pylori*.

In conclusion, we used human gastroid monolayers as experimental *H. pylori* infection model and examined the effects of the infection on apical junctional complex and IL-8 expression. Our results tended to mirror in vivo finding in infected patients. The ex vivo gastroid monolayer model provides a new method to investigate *H. pylori* infection using healthy, untransformed human cells infected in 2D model system that allows one to examine the interplay between the host and the pathogen. The model should facilitate further research in both the early and chronic stages of *H. pylori*. This model has a great potential promise for future research as a standard to use not only host-microbe infection, but also personalized therapeutics, regenerative medicine, disease modeling such as hereditary disease or cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENT

We would like to thank G. R. van den Brink, Amsterdam, The Netherlands, for providing Noggin-producing cells and Hidetoshi Okabe, Misumi Kasagi, and Saya Tomonaga, Oita, Japan, for helping in the histopathologic and immunohistochemical analysis.

Funding information

This study was funded by grants from the National Institutes of Health (DK62813; YY, P30 DK56338 and U19 AI116497; MKE) and the Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan (221S0002, 16H06279, 16H05191, and 18KK0266) (YY). It was also supported by the Japan Society for the Promotion of Science (JSPS) Institutional Program for Core-to-Core Program; B. Africa-Asia Science Platform (YY).

REFERENCES

1. Graham DY. *Helicobacter pylori* infection in the pathogenesis of duodenal ulcer and gastric cancer: a model. *Gastroenterology*. 1997;113:1983–1991. [PubMed: 9394739]
2. Peek RM Jr, Blaser MJ. *Helicobacter pylori* and gastrointestinal tract adenocarcinomas. *Nat Rev Cancer*. 2002;2:28–37. [PubMed: 11902583]
3. Yamaoka Y. Mechanisms of disease: *Helicobacter pylori* virulence factors. *Nat Rev Gastroenterol Hepatol*. 2010;7:629–641. [PubMed: 20938460]
4. Burkitt MD, Duckworth CA, Williams JM, et al. *Helicobacter pylori*-induced gastric pathology: insights from in vivo and ex vivo models. *Dis Model Mech*. 2017;10:89–104. [PubMed: 28151409]
5. Bartfeld S, Bayram T, van de Wetering M, et al. In vitro expansion of human gastric epithelial stem cells and their responses to bacterial infection. *Gastroenterology*. 2015;148:126–136 e6. [PubMed: 25307862]
6. Dedhia PH, Bertaux-Skeirik N, Zavros Y, et al. Organoid models of human gastrointestinal development and disease. *Gastroenterology*. 2016;150:1098–1112. [PubMed: 26774180]
7. Gillet JP, Varma S, Gottesman MM. The clinical relevance of cancer cell lines. *J Natl Cancer Inst*. 2013;105:452–458. [PubMed: 23434901]
8. Boccellato F, Woelffling S, Imai-Matsushima A, et al. Polarised epithelial monolayers of the gastric mucosa reveal insights into mucosal homeostasis and defence against infection. *Gut*. 2018;68:400–413.
9. Rabassa AA, Goodgame R, Sutton FM, Ou CN, Rognerud C, Graham DY. Effects of aspirin and *Helicobacter pylori* on the gastroduodenal mucosal permeability to sucrose. *Gut*. 1996;39:159–163. [PubMed: 8977334]
10. Amieva MR, Vogelmann R, Covacci A, et al. Disruption of the epithelial apical-junctional complex by *Helicobacter pylori* CagA. *Science*. 2003;300:1430–1434. [PubMed: 12775840]

11. Wroblewski LE, Peek RM Jr. Targeted disruption of the epithelial-barrier by *Helicobacter pylori*. *Cell Commun Signal*. 2011;9:29. [PubMed: 22044698]
12. Wroblewski LE, Piazzuelo MB, Chaturvedi R, et al. *Helicobacter pylori* targets cancer-associated apical-junctional constituents in gastroids and gastric epithelial cells. *Gut*. 2015;64:720–730. [PubMed: 25123931]
13. Suzuki M, Mimuro H, Suzuki T, et al. Interaction of CagA with Crk plays an important role in *Helicobacter pylori*-induced loss of gastric epithelial cell adhesion. *J Exp Med*. 2005;202:1235–1247. [PubMed: 16275761]
14. Murata-Kamiya N, Kurashima Y, Teishikata Y, et al. *Helicobacter pylori* CagA interacts with E-cadherin and deregulates the beta-catenin signal that promotes intestinal transdifferentiation in gastric epithelial cells. *Oncogene*. 2007;26:4617–4626. [PubMed: 17237808]
15. Ogden SR, Wroblewski LE, Weydig C, et al. p120 and Kaiso regulate *Helicobacter pylori*-induced expression of matrix metalloproteinase-7. *Mol Biol Cell*. 2008;19:4110–4121. [PubMed: 18653469]
16. Oliveira MJ, Costa AM, Costa AC, et al. CagA associates with c-Met, E-cadherin, and p120-catenin in a multiproteic complex that suppresses *Helicobacter pylori*-induced cell-invasive phenotype. *J Infect Dis*. 2009;200:745–755. [PubMed: 19604117]
17. Necchi V, Candusso ME, Tava F, et al. Intracellular, intercellular, and stromal invasion of gastric mucosa, preneoplastic lesions, and cancer by *Helicobacter pylori*. *Gastroenterology*. 2007;132:1009–1023. [PubMed: 17383424]
18. Ito T, Kobayashi D, Uchida K, et al. *Helicobacter pylori* invades the gastric mucosa and translocates to the gastric lymph nodes. *Lab Invest*. 2008;88:664–681. [PubMed: 18475258]
19. Yamaoka Y, Kodama T, Kita M, Imanishi J, Kashima K, Graham DY. Relation between clinical presentation, *Helicobacter pylori* density, interleukin 1beta and 8 production, and cagA status. *Gut*. 1999;45:804–811. [PubMed: 10562576]
20. Mimuro H, Berg DE, Sasakawa C. Control of epithelial cell structure and developmental fate: lessons from *Helicobacter pylori*. *Bioessays*. 2008;30:515–520. [PubMed: 18478526]
21. McCracken KW, Catá EM, Crawford CM, et al. Modelling human development and disease in pluripotent stem-cell-derived gastric organoids. *Nature*. 2014;516:400–404. [PubMed: 25363776]
22. Bertaux-Skeirik N, Feng R, Schumacher MA, et al. CD44 plays a functional role in *Helicobacter pylori*-induced epithelial cell proliferation. *PLoS Pathog*. 2015;11:e1004663.
23. Sato T, Stange DE, Ferrante M, et al. Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. *Gastroenterology*. 2011;141:1762–1772. [PubMed: 21889923]
24. Saxena K, Blutt SE, Ettayebi K, et al. Human intestinal enteroids: a new model to study human rotavirus infection, host restriction, and pathophysiology. *J Virol*. 2016;90:43–56. [PubMed: 26446608]
25. Ettayebi K, Crawford SE, Murakami K, et al. Replication of human noroviruses in stem cell-derived human enteroids. *Science*. 2016;353:1387–1393. [PubMed: 27562956]
26. VanDussen KL, Marinshaw JM, Shaikh N, et al. Development of an enhanced human gastrointestinal epithelial culture system to facilitate patient-based assays. *Gut*. 2015;64:911–920. [PubMed: 25007816]
27. Ito K, Yamaoka Y, Ota H, El-Zimaity H, Graham DY. Adherence, internalization, and persistence of *Helicobacter pylori* in hepatocytes. *Dig Dis Sci*. 2008;53:2541–2549. [PubMed: 18320323]
28. Schlaermann P, Toelle B, Berger H, et al. A novel human gastric primary cell culture system for modelling *Helicobacter pylori* infection in vitro. *Gut*. 2016;65:202–213. [PubMed: 25539675]
29. Mills JC, Shivdasani RA. Gastric epithelial stem cells. *Gastroenterology*. 2011;140:412–424. [PubMed: 21144849]
30. Wroblewski LE, Shen LE, Ogden S, et al. *Helicobacter pylori* dysregulation of gastric epithelial tight junctions by urease-mediated myosin II activation. *Gastroenterology*. 2009;136:236–246. [PubMed: 18996125]
31. Fiorentino M, Ding H, Blanchard TG, Czinn SJ, Sztein MB, Fasano *Helicobacter pylori*-induced disruption of monolayer permeability and proinflammatory cytokine secretion in polarized human gastric epithelial cells. *Infect Immun*. 2013;81:876–883. [PubMed: 23297384]

32. Rieder G, Tessier AJ, Qiao XT, Madison B, Gumucio DL, Merchant JL. *Helicobacter*-induced intestinal metaplasia in the stomach correlates with Elk-1 and serum response factor induction of villin. *J Biol Chem*. 2005;280:4906–4912. [PubMed: 15576363]
33. Li Q, Jia Z, Wang L, et al. Disruption of Klf4 in villin-positive gastric progenitor cells promotes formation and progression of tumors of the antrum in mice. *Gastroenterology*. 2012;142:531–542. [PubMed: 22155367]
34. Yamaoka Y, Kudo T, Lu H, Casola A, Brasier AR, Graham DY. Role of interferon-stimulated responsive element-like element in interleukin-8 promoter in *Helicobacter pylori* infection. *Gastroenterology*. 2004;126:1030–1043. [PubMed: 15057743]
35. Bagnoli F, Buti L, Tompkins L, Covacci A, Amieva MR. *Helicobacter pylori* CagA induces a transition from polarized to invasive phenotypes in MDCK cells. *Proc Natl Acad Sci U S A*. 2005;102:16339–16344. [PubMed: 16258069]
36. Wroblewski LE, Peek RM Jr, Wilson KT. *Helicobacter pylori* and gastric cancer: factors that modulate disease risk. *Clin Microbiol Rev*. 2010;23:713–739. [PubMed: 20930071]
37. Krueger S, Hundertmark T, Kuester D, Kalinski T, Peitz U, Roessner A. *Helicobacter pylori* alters the distribution of ZO-1 and p120ctn in primary human gastric epithelial cells. *Pathol Res Pract*. 2007;203:433–444. [PubMed: 17509776]
38. Saadat I, Higashi H, Obuse C, et al. *Helicobacter pylori* CagA targets PAR1/MARK kinase to disrupt epithelial cell polarity. *Nature*. 2007;447:330–333. [PubMed: 17507984]
39. Weydig C, Starzinski-Powitz A, Carra G, Löwer J, Wessler S. CagA-independent disruption of adherence junction complexes involves E-cadherin shedding and implies multiple steps in *Helicobacter pylori* pathogenicity. *Exp Cell Res*. 2007;313:3459–3471. [PubMed: 17692843]
40. Terres AM, Pajares JM, Hopkins AM, et al. *Helicobacter pylori* disrupts epithelial barrier function in a process inhibited by protein kinase C activators. *Infect Immun*. 1998;66:2943–2950. [PubMed: 9596771]
41. Russo JM, Florian P, Shen L, et al. Distinct temporal-spatial roles for rho kinase and myosin light chain kinase in epithelial purse-string wound closure. *Gastroenterology*. 2005;128:987–1001. [PubMed: 15825080]
42. Baskin WN, Ivey KJ, Krause WJ, et al. Aspirin-induced ultrastructural changes in human gastric mucosa: correlation with potential difference. *Ann Intern Med*. 1976;85:299–303. [PubMed: 962220]
43. Graham DY. Polymorphonuclear leukocytes traffic into the gastric mucosa and through the gastric mucosal barrier in *H. pylori* infection: is that bad? In: Hunt RH, Tytgat G, eds. *Helicobacter pylori*. Dordrecht, the Netherlands: Springer; 1994:214–221.
44. Tegtmeyer N, Wessler S, Necchi V, et al. *Helicobacter pylori* employs a unique basolateral type IV secretion mechanism for CagA delivery. *Cell Host Microbe*. 2017;22:552–560 e5. [PubMed: 29024645]
45. Bebb Jr, Leach L, Zaitoun A, et al. Effects of *Helicobacter pylori* on the cadherin-catenin complex. *J Clin Pathol*. 2006;59:1261–1266. [PubMed: 16679349]
46. Petersen AM, Krogfelt KA. *Helicobacter pylori*: an invading microorganism? A review. *FEMS Immunol Med Microbiol*. 2003;36:117–126. [PubMed: 12738380]
47. Pompaiah M, Bartfeld S. Gastric organoids: an emerging model system to study *Helicobacter pylori* pathogenesis. *Curr Top Microbiol Immunol*. 2017;400:149–168. [PubMed: 28124153]
48. Brandt S, Kwok T, Hartig R, et al. NF-kappaB activation and potentiation of proinflammatory responses by the *Helicobacter pylori* CagA protein. *Proc Natl Acad Sci U S A*. 2005;102:9300–9305. [PubMed: 15972330]
49. Backert S, Naumann M. What a disorder: proinflammatory signaling pathways induced by *Helicobacter pylori*. *Trends Microbiol*. 2010;18:479–486. [PubMed: 20863705]
50. Hoffmann W. Regeneration of the gastric mucosa and its glands from stem cells. *Curr Med Chem*. 2008;15:3133–3144. [PubMed: 19075658]
51. Alzahrani S, Lina TT, Gonzalez J, et al. Effect of *Helicobacter pylori* on gastric epithelial cells. *World J Gastroenterol*. 2014;20:12767–12780. [PubMed: 25278677]

52. Sigal M, Rothenberg ME, Logan CY, et al. *Helicobacter pylori* activates and expands Lgr5(+) stem cells through direct colonization of the gastric glands. *Gastroenterology*. 2015;148:1392–1404 e21. [PubMed: 25725293]
53. Tan S, Tompkins LS, Amieva MR. *Helicobacter pylori* usurps cell polarity to turn the cell surface into a replicative niche. *PLoS Pathog*. 2009;5:e1000407.

FIGURE 1.

Morphological changes of human gastroid monolayers dependent on differentiation status. Gastroid monolayers cultured in CMGF+ media (undifferentiated) and that in DM (differentiated) for 12 d each were processed as described in the Material and Methods. (A and B), H&E staining; (C and D), HGM45 (gastric crypt epithelial marker); (E and F), MUC6 (pyloric gland cell marker); (G and H), MUC2 (intestinal mucin marker); (I and J), Alcian blue staining; (K and L), AE1/AE3 staining (cytokeratin). Gastroid monolayers cultured in DM (differentiated) were processed at 80-nm-thick samples with negative-

stained sections and observed by TEM at $\times 3000$ (M) and $\times 10\,000$ (N) magnifications. (A) The polarity is unclear with multilayer and few mucus in undifferentiated gastroid monolayers. (B) Each gastroid monolayer had mucus in top and monolayer lined regularly with clear nuclei in differentiated gastroid monolayers. (C, E, G, I, and K): undifferentiated gastroid monolayers and (D, F, H, J, and L): differentiated gastroid monolayers. (C-D) HGM45, which is gastric crypt epithelial markers, showed strongly positive staining in differentiated gastroid monolayers compared to undifferentiated gastroid monolayers. (E, F) MUC6, pyloric gland cell marker, is negative in differentiated gastroid monolayers but weak positive in undifferentiated gastroid monolayers. (G, H) MUC2, which is intestinal marker, is negative in both gastroid monolayers. (I-L) Alcian blue and AE1/AE3, which is epithelial marker and mucous marker, are positive in both gastroid monolayers. (M) Gastroid monolayers had polarity and mucus, lined regularly as monolayer (TEM original magnification $3000\times$). (N) There is microvillus or apical junctional complex on the top of each gastroid monolayer (TEM original magnification $10\,000\times$). Scale bars in (M) and (N) are 10 and 2 μm , respectively. Above all, differentiated gastroid monolayers had the feature as gastric mucosa in vivo

FIGURE 2.

Morphology of differentiated gastroid monolayers changes with *Helicobacter pylori* infection. 80-nm-thick sections of differentiated gastroid monolayers infected with *H. pylori* at 6 hpi (A) and 24 hpi (B) were analyzed by H&E staining and TEM (C and D). Negative-stained sections were observed by TEM at $\times 7000$ (C) and $\times 20\,000$ (D) magnifications. (A and B) From 6 to 24 h, some mucous area had smaller on the top of gastroid monolayers compared with gastroid monolayers without infection (Figure 1B). (C) Original magnification $7000\times$ and (D) original magnification $20\,000\times$ 24 h after *H. pylori* infection.

Helicobacter pylori tried to attach and enter on mainly mucous areas on the top of gastroid monolayer mucosa

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FIGURE 3.

Drop in TEER of *Helicobacter pylori*-infected cells is independent on cell viability. (A) Gastroid monolayers in day 2 and day 4 were significantly greater than day 0 (seeding) and reached confluency. The error bars indicate the means $\pm$ SD of three independent experiments; * $P < 0.01$ day 0 vs day 2 and day 0 vs day 4. (B) Kinetics of TEER of infected cell at the different points. Differentiated gastroid monolayers cultured in transwells were infected with *H. pylori* TN2GF4 *wt* at MOI 10 (long dotted line) and MOI 100 (short dotted line). Incubation in the absence of *H. pylori* was used as control (solid line). TEER of

infected and uninfected cells was measured at indicated points. $*P < 0.01$ comparing TEER to control at 24 hpi. (C) Cell viability of infected cells. Cultured medium of differentiated gastroid monolayers in transwell at indicated points was collected before TEER measurement described above and subjected to LDH assay to assess cell viability. The cell viabilities of control, infected cells at TN2GF4 *wt* MOI 10 and MOI 100 were depicted as light gray, medium gray, and dark gray bars, respectively. There was no significant change in gastroid monolayer viability among all groups until 72 h; $*P < 0.01$ comparing cell viability to control at 96 hpi. In panels (B) and (C), data represent the mean of three wells for each point, and each error bar denotes SD

FIGURE 4.

Immunofluorescence microscopy of human gastroid monolayers infected with *Helicobacter pylori*. (A) Detection of *H. pylori* bound to the apical side of the gastroid monolayers. Differentiated gastroid monolayers cultured in transwells were infected with *H. pylori* at MOI 100 and cultured for 96 h. Cells were washed with PBS, fixed with 4% paraformaldehyde, and permeabilized with 0.5% NP-40 followed by blocking with 0.5% BSA. *H. pylori* bound to the cell surface was visualized by confocal scanning microscopy using antibodies against *H. pylori* (red) and villin (green). DAPI detects nuclei (blue). The X-

Zorthogonal image sectioned at the dotted line in the merge image is depicted as the right panel. The dotted line shown in the right panel corresponds to the height where left images were obtained. Scale bar, 10 μ m. (B and C) Detection of tight and adherens junctions in uninfected and infected cells. The differentiated gastroid monolayers cultured for 4 d in the absence or presence of *H pylori* were fixed and subjected to the microscopy as described above. Tight and adherens junctions were visualized by using antibodies against ZO-1 and E-cadherin, respectively (green, each). Infected cells were detected by using antibodies against *H pylori* and CagA. Scale bars, 20 μ m. (D and E) Detection of tight and adherens junctions in infected cells. Samples of infected cells for IF were prepared as described in (A). The tight and adherens junctions were visualized by using antibodies against ZO-1 and E-cadherin (green). *H Pylori* and CagA in infected cells were represented by red signals. The X-Z orthogonal images sectioned at the dotted lines in the merge images are depicted as the below. Scale bars, 10 μ m

FIGURE 5.

IL-8 expression of gastroid monolayers infected with *Helicobacter pylori* wt and *cag* PAI.

(A) IL-8 mRNA expression among control and infection groups in gastroid monolayers.

Helicobacter pylori infection increased gastroid monolayers IL-8 mRNA in a time-dependent manner with maximal level at 3 hpi; * $P < 0.01$ control vs TN2GF4 wt on each time. (B) IL-8 protein expression among control and infection groups in gastroid monolayer. IL-8 protein expressions in infection groups were significantly greater than control. Infection groups continued to release IL-8 protein constantly from 0 to 96 h on MOI-dependent

manner; $*P < 0.01$; $**P < 0.05$. (C) IL-8 proteins in AGS and MKN28 with TN2GF4 *wt* were significantly greater than *cag* PAI and control at 24 hpi; $*P < 0.01$. (D) Gastric monolayers, *IL-8* mRNA between TN2GF4 *wt* and *cag* PAI at 3 h post-infection. The *IL-8* mRNA was almost the same level in both strains; $*P < 0.01$. (E) Gastric monolayers, IL-8 protein between control (short dotted line), TN2GF4 *wt* (solid line), and *cag* PAI (long dotted line) until 96 h. Gastric monolayers, IL-8 protein in infected groups were significantly greater than control; however, these proteins were almost the same level in TN2GF4 *wt* and *cag* PAI; 0-24 h $**P < 0.05$ control vs TN2GF4 *wt*; 24-48 h and 48-72 h $*P < 0.01$ control vs TN2GF4 *wt* and *cag* PAI; 72-96 h $*P < 0.01$ control vs *cag* PAI, $**P < 0.05$ control vs TN2GF4 *wt*.

FIGURE 6.

IL-8 expression of gastroid monolayers infected with *Helicobacter pylori* clinical isolates or wt with signaling pathway inhibitors. (A) Clinical isolates with less virulence factors such as strains COL153, COL443, BGD76, and 401C with negative for *cag* PAI and non-producing of VacA, OipA, and BabA. Although IL-8 protein in MKN28 was the same between uninfected control and infected one, the proteins in infected gastroid monolayers were greater than that in control. (B) and (C), The effects of inhibitors in different signal transduction pathways for IL-8 production in (B) AGS, MKN28, and (C) gastroid

monolayer. Each cell was infected with TN2GF4 *wt* with inhibitors as below: SB203580 as p38 inhibitor, SC-514 as IKK inhibitor, SP600125 as JNK inhibitor, and U-0126 as MEK inhibitor by each concentration of 10 μ mol/L. (B), IL-8 production in each cell line infected with TN2GF4 *wt* was influenced by all inhibitors. (C), JNK and MEK inhibitor did not induce any effect on IL-8 production in gastroid monolayers, it indicated that p38 and IKK inhibitors reduced IL-8 protein, and it seemed that they were important key factor in signaling pathway for IL-8 production. * $P < 0.01$ TN2GF4 *wt* vs each arm. These error bars indicate the means $\pm$ SD of three independent experiments