Regulation of IL-18 in Helicobacter pylori Infection

Kazuyoshi Yamauchi, Il-Ju Choi, Hong Lu, Hiroaki Ogihara, David Y. Graham and Yoshio Yamaoka

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The gastric mucosal immune response is thought to be comprised predominantly of the Th1 type; however, there are limited data regarding the role of IL-18 in Helicobacter pylori-induced inflammation. We investigated IL-18 levels in gastric mucosal biopsy specimens as well as in isolated gastric epithelial cells and lamina propria mononuclear cells. We also investigated IL-18 levels in gastric epithelial cells and the monocyte cell line THP-1 cocultured with H. pylori. In both systems, IL-18 levels were markedly enhanced in H. pylori-infected epithelial cells and monocytes. IL-18 levels in H. pylori-infected gastric mucosa were well correlated with the severity of gastric inflammation, confirming that H. pylori-induced IL-18 plays an important role in gastric injury. Virulence factors of H. pylori; the cag pathogenicity island and OipA affected IL-18 induction in different manners. Up-regulation of IL-18 mRNA/protein in epithelial cells was dependent on both virulence factors. Interestingly, up-regulation of IL-18 mRNA in monocytes was independent of both factors, whereas IL-18 protein was OipA dependent – cag pathogenicity island independent, indicating that OipA regulates IL-18 induction in monocytes at the posttranscriptional level. IL-18 levels in the gastric biopsy specimens showed similar patterns to those in lamina propria mononuclear cells with respect to virulence factors, suggesting that submucosal monocytes/macrophages are the main source of IL-18 induced by H. pylori infection. H. pylori appeared to regulate the ERK/JNK→AP-1 pathway in both cell types. In addition, OipA and its related p38 pathway may be closely involved in IL-18 induction in H. pylori-infected gastric mucosa and may contribute to gastric injury. The Journal of Immunology, 2008, 180: 1207–1216.

The Helicobacter pylori infection is associated with infiltration of neutrophils, lymphocytes, monocytes, and plasma cells into the gastric mucosa. The gastric mucosal immune response is thought to consist predominantly of the Th1 type associated with a significant increase in IFN-γ secreting T cells (1–4). This notion also is supported by the fact that H. pylori-infected IFN-γ-knockout mice develop minimal pathological changes (5–7).

IL-18, previously known as an IFN-γ-inducing factor, is a potent proinflammatory cytokine in the IL-1 superfamily (8, 9) that promotes the production of IFN-γ from Th1, B, and NK cells in synergy with IL-12 (10–12). IL-18 also acts as a costimulant for Th1 cells to augment the production of IFN-γ, IL-2, and granulocyte macrophage CSF (11, 13). IL-18-deficient mice failed to develop protection after oral immunization with lyssates and cholera toxin adjuvant, indicating the importance of IL-18 in protection (14). Well-protected wild-type mice showed moderate gastric inflammation; whereas unprotected IL-18-deficient mice had less gastric inflammation. Therefore, we hypothesized that IL-18 plays an important role in the pathogenesis of H. pylori-induced gastroduodenal disease.

There are limited data regarding the role of IL-18 in H. pylori-induced inflammation in humans. Tomita et al. (15) reported that antral IL-18 mRNA levels were up-regulated in H. pylori infection, whereas mature IL-18 protein was present in mucosa of both infected and uninfected subjects as measured by immunoblot analyses. In contrast, Fera et al. reported that antral H. pylori infection was associated with IL-18 production as determined by immunohistochemistry; whereas IL-18 mRNA was expressed irrespective of H. pylori infection (16). The reason for the discrepancy is unknown; however, one reason might be due to the nonquantitative nature of analyses of mRNA and protein levels.

H. pylori strains containing an intact cag pathogenicity island (PAI)4, which encodes the type IV secretion system, and the outer inflammatory protein (OipA), which encodes the outer membrane protein, have been reported to increase the risk of more severe clinical outcomes (17, 18). Tomita et al. (15) reported that antral IL-18 mRNA levels were independent of the presence or absence of the cag PAI; however the relationship between OipA and IL-18 is not understood.

The current study was designed to test the hypothesis that IL-18 is biologically important in H. pylori-infected mucosa as determined by quantitative analyses using real-time RT-PCR and ELISA. We measured mucosal IL-18 levels in human gastric mucosa as well as in isolated gastric epithelial cells and lamina propria mononuclear cells (LPMC) in relation to H. pylori infection and its virulence factors (cag PAI and OipA), and correlated IL-18 levels with gastric mucosal inflammation. We also examined in

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1 Department of Medicine-Gastroenterology, Michael E. DeBakey Veterans Affairs Medical Center and Baylor College of Medicine, Houston, TX 77030; 2Research Institute and Hospital, National Cancer Center, Gyeonggi, Republic of Korea; and 3Department of Gastroenterology, Renji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai Institute of Digestive Disease, Shanghai, China
4 Abbreviations used in this paper: PAI, pathogenicity island; LPMC, lamina propria mononuclear cell; DTF, dithiothreitol; MNC, mononuclear cell; PMN, polymorphonuclear leukocyte; MOI, multiplicity of infection.
vitro IL-8 expression in both gastric epithelial cells and the monocytes in response to *H. pylori* infection in addition to the signaling pathways regulating *H. pylori*-infection induction of IL-18.

**Materials and Methods**

**Patients and samples**

Antral gastric mucosal samples were collected from *H. pylori*-infected Colombian patients with nonulcer dyspepsia. The samples included patients infected with *cag* PAI-positive/OipA-positive *H. pylori* isolates, *cag* PAI-negative/OipA-negative isolates, *cag* PAI-negative/OipA-positive isolates, and *cag* PAI-positive/OipA-negative isolates. *H. pylori*-positive isolates were evaluated by culture using standard methods as previously described (19). Multiple colonies on the plates were harvested en masse and three to six single colonies also were harvested. Genomic DNA and total proteins were extracted using a commercially available DNA extraction kit (Qiagen) and bacterial protein extraction kit (Pierce), respectively. The *cag* PAI status was determined by PCR as previously described (17). OipA status was determined by immunoblot as previously described (18). Controls consisted of gastric mucosal biopsies without *H. pylori* infection. The patients were proven to be *H. pylori*-negative by a combination of serology, histology, rapid urease test, and culture. The biopsy specimens were obtained under protocols approved by the ethics committees in Universidad Nacional de Colombia (Bogota, Colombia) and informed consent was obtained from all patients.

**Isolation of gastric epithelial cells and lamina PIMC**

In addition to the whole antral biopsy specimens, isolated gastric epithelial cells and gastric PIMC from some Colombian patients were used for examining the IL-18 mRNA levels. Gastric epithelial cells were isolated from freshly obtained antral biopsy specimens from patients with and without *H. pylori* infection as previously described (20). In brief, biopsy specimens were washed in HBSS at 4°C, cut into small pieces (0.4 mm) and washed in 10 ml of HBSS with 0.5 mM DTT for 5 min at 4°C under constant stirring. Tissue was transferred and incubated with chelating buffer (27 mM trisodium citrate, 5 mM Na2HPO4, 96 mM NaCl, 8 mM KH2PO4, 1.5 mM KCl, 55 mM s-tosibitol, and 44 mM sucrose) for 7 min at 4°C. The supernatant was collected and centrifuged at 1,000 *g* for 10 min. After washing in calcium- and magnesium-free HBSS (Invitrogen), the pellet was resuspended in a 40% BCA protein assay reagent (Pierce) and IL-18 protein levels in the supernatant were determined by ELISA detects mature IL-18; pro-IL-18 showed 1% cross-reactivity.

**Quantitative analyses for IL-18 mRNA in the gastric mucosa**

Total RNA was isolated from whole gastric biopsy specimens using RNeasy Mini kits (Qiagen). An aliquot containing 0.2 µg of total RNA was used for the reverse transcription reaction, which was conducted using the Quotisy PCR (50 µl) were prepared by mixing 5 µl of synthesized cDNA solution, 2× TaqMan Universal Master mix (Applied Biosystems), 500 nM of each primer, and 25 µl of the TaqMan probe. The samples were placed in the analyzer and PCR was conducted according to the manufacturer’s instructions. To normalize the IL-18 mRNA expression level, GAPDH mRNA also was quantified in the same reactions using TaqMan GAPDH controls (Applied Biosystems). The expression levels of IL-18 mRNA were expressed as the ratio of IL-18 mRNA to GAPDH mRNA.

**Cell culture**

The human gastric epithelial cancer cell line AGS and human monocyte cell line THP-1 were obtained from American Type Culture Collection. Human gastric epithelial cancer cell lines MKN1, MKN7, MKN28, MKN45, and KATOIII were obtained from Riken Cell Bank (Tsukuba, Japan). Cell lines, except for KATOIII, were routinely maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated FBS (Invitrogen), penicillin (100 U/ml), and streptomycin (100 µg/ml) in 5% CO2-air at 37°C to 80% confluence; 20% heat-inactivated FBS was used for KATO III cells.

We also used primary gastric epithelial cells that were isolated enzymatically from uninfected adult human stomachs (noncancer containing normal appearing mucosa) as previously described (23).

**Histology**

The surface mucosal layer was removed with a razor blade, immediately minced and then incubated in Ham’s F-12 culture medium containing collagenase (0.2 mg/ml) (Invitrogen) for 10 min. Cells from the final incubation were washed and cultured in Ham’s F-12 medium supplemented with 10% FBS and streptomycin (300 µg/ml) in 5% CO2-air at 37°C. Epithelial cells were cultured in a 24-well collagen-coated dish at a final concentration of 106 cells/ml for 24 h before use. Cultured cells formed subconfluent monolayers within 24 h of the inoculation. Approximately 99% of cultured cells in the monolayers had periodic acid-Schiff-positive material in the cytoplasm confirming that the population consisted of mucus producing epithelial cells with minimal contamination by other cells.

**Quantitative analyses for IL-18 protein in the gastric mucosa using ELISA and immunoblot**

IL-18 protein levels were determined in antral mucosal biopsy specimens. Gastric antral mucosal biopsy specimens were placed immediately in 200 µl PBS (pH 7.4), frozen on dry ice and stored at −80°C until use. Samples were homogenized using a tissue homogenizer (Kontes), and aliquots of supernatants from the homogenized tissues were obtained by centrifugation (10,000 × g for 10 min); total protein in the supernatants was measured by BCA protein assay reagent (Pierce) and IL-18 protein levels in the supernatants were determined by ELISA (Medical & Biological Laboratories). In our laboratory, the ELISA sensitivity for IL-18 was ~10 pg/ml. The ELISA detects mature IL-18; pro-IL-18 showed <1% cross-reactivity. The mucosal IL-18 levels were expressed as pg/mg protein.

We also performed immunoblot for IL-18 protein in the purified gastric epithelial cells and PIMC using anti-rabbit IL-18 polyconal Ab (Santa Cruz Biotechnology), which detects both the precursor 24 K pro-IL-18 and 18 K mature IL-18. Protein extraction and immunoblotting were performed using standard techniques. For semiquantitative analysis, x-ray films were scanned and quantified using Image J 1.36 software (http://rsbweb.nih.gov/ij/) from the National Institutes of Health. The density of mature IL-18 was normalized to β-actin for quantitative analyses.
In some experiments, heat-killed *H. pylori* were used at the same MOI. *H. pylori* were heat-killed by boiling for 15 min in saline, followed by centrifugation; the supernatant or pellet of killed *H. pylori* was used. Killing was confirmed by lack of growth on blood agar plates. In some experiments, the same concentration of live bacteria was added to the upper well of a transwell plate (Falcon); the lower well contained gastric epithelial cells or THP-1 cells. In some experiments, human cells were pretreated with U0126 (a specific inhibitor of MAPK/ERK1/2), SB203580 (a specific inhibitor of p38), or the proteasome inhibitor MG-132, which inhibits NF-κB activation. We used 2 μM of each inhibitor according to our previous determination of optimal concentrations (23) as well as from our preliminary experiments (data not shown). All inhibitors were purchased from Calbiochem.

**Analyses for IL-18 protein levels from human cells cocultured with *H. pylori* using ELISA and immunoblot**

Exponentially growing *H. pylori* were added to the human cells in 24-well plates for 0 to 36 h and IL-18 protein levels released from human cells were assessed in duplicate using an ELISA and immunoblot, as described above.

**Quantitative analyses for IL-18 mRNA levels from human cells cocultured with *H. pylori* using real-time RT-PCR**

Exponentially growing *H. pylori* were added to human cells in 6-well plates for 0 to 24 h and total RNA was isolated from cocultured cells using RNeasy Mini kits (Qiagen). An aliquot containing 1 μg of total RNA was used for the reverse transcription reaction and IL-18 mRNA levels was measured by real-time RT-PCR as above. The expression levels of IL-18 mRNA were expressed as the ratio of IL-18 mRNA to GAPDH mRNA.

**Luciferase plasmid and luciferase reporter gene assays**

The pGL3–1375, 1375 bp 5′ flanking region of the human IL-18 promoter, and its deletion constructs, linked to the luciferase reporter gene of the pGL3 basic plasmid (Promega) were gifts from Dr. Koyama (University Hospital Frankfurt, Frankfurt, Germany) (27–29). AGS cells (plated into 24-well dishes at 10^6/well) were transiently transfected with 500 ng of total RNA using poly (dI-dC), and 40,000 cpm of 32P-labeled double-stranded oligonucleotide in a total volume of 20 μl. The nuclear proteins were incubated with the probe for 30 min at room temperature and then electrophoresed on 6% non-denaturing polyacrylamide gels in Tris-borate-EDTA buffer (22 mM Tris-HCl, 22 mM boric acid, 0.25 mM EDTA, pH 8). After electrophoresis, gels were dried and exposed for autoradiography using Kodak XAR film at −80°C and intensifying screens.

The competition assays were performed by the addition of 5.0 pmol/L unlabeled competitor into the reaction mixture at the time of probe addition. In the gel shift assays, commercial Abs (anti-p50, anti-p60, and anti-c-Jun) (Santa Cruz Biotechnology) were added to the reaction mixtures, followed by incubation on ice for 1 h.

**Table I. Probes used in electrophoretic mobility shift assays**

<table>
<thead>
<tr>
<th>Transcriptional Regulatory Site of Human IL-18 Promoter</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>AP-1</td>
<td>sense, 5′-CTATTTCACCTTGGACTCATATGATTTC-′3′</td>
</tr>
<tr>
<td>AP-1</td>
<td>anti-sense, 5′-GAATAACATAGGACTCCAAATGGTAAG-′3′</td>
</tr>
<tr>
<td>NF-κB</td>
<td>sense, 5′-TTGGAGAAGACGGAAAAATCGCTGAAAG-′3′</td>
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<tr>
<td>NF-κB</td>
<td>anti-sense, 5′-CTCCACCTCCCTCTCAGCACTCTC-′3′</td>
</tr>
<tr>
<td>PU.1</td>
<td>sense, 5′-CTCCACCTCCCTCTCAGCACTCTC-′3′</td>
</tr>
<tr>
<td>PU.1</td>
<td>anti-sense, 5′-GGAGAAGAAATGGAGAAGGTGGAG-′3′</td>
</tr>
</tbody>
</table>

**EMSA analysis**

Nuclear extracts of infected and uninfected human cells were prepared using hypotonic/nonionic detergent lysis as previously described (30). The concentration of extracted nuclear proteins was determined using the BCA protein assay reagent (Pierce) and equal amounts were used to bind to duplex oligonucleotides corresponding to the IL-18 specific PU.1, NF-κB, and AP-1 binding sites. The oligonucleotides used in this study are summarized in Table I. Binding reactions contained 10–15 μg total protein, 5% glycerol, 12 mM HEPES, 80 mM NaCl, 1 mM DTT, 5 mM MgCl₂, 0.5 mM EDTA, 1 μg of poly (dI-dC), and 40,000 cpm of 32P-labeled double-stranded oligonucleotide in a total volume of 20 μl. The nuclear proteins were incubated with the probe for 30 min at room temperature and then electrophoresed on 6% nondenaturing polyacrylamide gels in Tris-borate-EDTA buffer (22 mM Tris-HCl, 22 mM boric acid, 0.25 mM EDTA, pH 8). After electrophoresis, gels were dried and exposed for autoradiography using Kodak XAR film at −80°C and intensifying screens.

**Results**

Mucosal IL-18 protein levels in gastric mucosa

We initially examined 124 *H. pylori*-infected Colombian patients with nonulcer dyspepsia including 111 patients whose cag PAI and OipA status as determined using multiple colonies was identical with the status determined using single colonies from the same patient. Among these patients, 82 were infected with *cag* PAI-positive/OipA-positive isolates, 6 with *cag* PAI-negative/OipA-positive isolates, 5 with *cag* PAI-positive/OipA-negative isolates, and 18 with *cag* PAI-negative/OipA-negative isolates. Of these, we arbitrarily selected 20 patients infected with *cag* PAI-positive/OipA-positive isolates and 10 with *cag* PAI-negative/OipA-negative isolates, as well as all 11 patients with *cag* PAI-negative/OipA-positive or *cag* PAI-positive/OipA-negative isolates.

Gastric mucosal IL-18 protein levels were significantly higher in *H. pylori*-positive specimens (243 (131–370) pg/mg protein) than in *H. pylori*-negative specimens (21.2 (6.0–24.0) pg/mg protein) (*p < 0.001*). Importantly, among *H. pylori* infected patients, mucosal IL-18 protein levels were similar between *cag* PAI-positive/OipA-positive isolates (344 (173–452)) and *cag* PAI-negative/OipA-negative isolates (278 (232–765)) (Fig. 1A). In contrast, *cag* PAI-positive/OipA-negative isolates and *cag* PAI-negative/OipA-negative isolates induced less IL-18 (198 (45.1–225) and 232 (104–326)) compared with *cag* PAI-positive/OipA-positive isolates (*p = 0.09* for both). However, IL-18 levels were still significantly higher in *cag* PAI-negative/OipA-negative specimens than in *H. pylori*-negative isolates (*p < 0.001*), indicating that *H. pylori* factors other than OipA and *cag* PAI likely also are involved in IL-18 induction.

We next examined IL-18 protein levels in isolated gastric epithelial cells and LPMC. For this purpose, we used five patients infected with *cag* PAI-positive/OipA-positive isolates, two with *cag* PAI-negative/OipA-positive isolates, two with *cag* PAI-positive/OipA-negative isolates, five with *cag* PAI-negative/OipA-negative isolates as well as five without *H. pylori* infection. Our attempts to isolate gastric epithelial cells and LPMC from additional samples with *cag* PAI-negative/OipA-positive isolates
without H. pylori with the levels of samples infected with t due to low cell viability. Due to the small number of cells, it was difficult to measure secreted IL-18 levels by ELISA; therefore, Immunoblot analyses showed that all the gastric epithelial cells and LPMC were able to produce pro-IL-18 but were not able to process the precursor to the bioactive peptide, except for KATOIII cells (data not shown). Therefore, we examined the effect of IL-18 induction from cells cocultured with H. pylori on gastric epithelial cells.

Induction of IL-18 from cells cocultured with H. pylori

The ability of six gastric epithelial cancer cell lines to produce IL-18 was examined following coculture with H. pylori. Surprisingly, IL-18 protein levels were below the level of detection with all six gastric epithelial cancer cells irrespective of the presence or absence of H. pylori infection (MOI of 0–500) during observation periods of up to 36 h (data not shown). IL-18 is synthesized as a precursor 24 K pro-IL-18 and must be cleaved by caspase-1 to produce the mature bioactive peptide (31, 32). Caspase-1 adaptors ASC (apoptosis-associated speck-like protein containing a caspase activation and recruitment domain) also are essential for maturation of IL-18 (33). For example, AGS cells lack ASC and they cannot produce the mature IL-18 protein (our unpublished data). Immunoblot analyses showed that all the gastric cell lines tested were able to produce pro-IL-18 but were not able to process the precursor to the bioactive peptide, except for KATOIII cells (data not shown). Therefore, we examined the effect of H. pylori on IL-18 production from primary (noncancer) gastric epithelial cells.

Mucosal IL-18 mRNA levels in gastric mucosa

Antral mucosal IL-18 mRNA levels from the whole biopsy specimens correlated well with protein levels irrespective of H. pylori status (R = 0.84; p < 0.001 for H. pylori-positive cases and R = 0.61; p = 0.005 for H. pylori-negative cases). Accordingly, mucosal IL-18 mRNA levels were significantly higher in H. pylori-positive specimens (102 (82.2–114)) than in H. pylori-negative specimens (23.2 (12.4–39.8)) (p < 0.001) (Fig. 1C). However, among H. pylori infected patients, mucosal IL-18 mRNA levels were independent of the cag PAI and OipA status. Overall, IL-18 protein levels in the whole biopsy specimens were dependent of OipA status; whereas IL-18 mRNA levels were not.

Relationship between mucosal IL-18 levels and gastric injury

The above data clearly showed that H. pylori induced IL-18 in the gastric mucosa. We next examined whether the IL-18 induced by H. pylori infection was related to gastric injury. Because tissue samples from all uninfected patients studied had normal histology (i.e., no PMN infiltration, no presence of atrophy and no to very mild MNC infiltration), we only compared samples obtained from H. pylori infected patients. IL-18 protein levels strongly correlated with MNC and PMN infiltration (p < 0.001 and in trend) (Table II). Cellular infiltration was closely related to OipA and cag PAI status (data not shown); accordingly, IL-18 mRNA levels only weakly correlated with MNC and PMN infiltration (p = 0.004 and p = 0.028, respectively) (Table II). IL-18 levels did not correlate with gastric atrophy (data not shown).

Table II. IL-18 and cellular infiltration in H. pylori-infected tissue

<table>
<thead>
<tr>
<th>Histology Score (no.)</th>
<th>IL-18 Protein (pg/mg protein)</th>
<th>IL-18 mRNA (IL-18/GAPDH × 10⁴)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MNC infiltration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (14)</td>
<td>80 (23–232)</td>
<td>84 (65–105)</td>
</tr>
<tr>
<td>2 (16)</td>
<td>233 (213–336)</td>
<td>105 (88–116)</td>
</tr>
<tr>
<td>3 (11)</td>
<td>468 (380–804)</td>
<td>120 (102–134)</td>
</tr>
<tr>
<td>p value</td>
<td>&lt;0.001</td>
<td>0.004</td>
</tr>
<tr>
<td>PMN infiltration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 (15)</td>
<td>104 (24–232)</td>
<td>90 (65–109)</td>
</tr>
<tr>
<td>1 (10)</td>
<td>222 (198–326)</td>
<td>108 (88–118)</td>
</tr>
<tr>
<td>2 (7)</td>
<td>357 (314–683)</td>
<td>115 (93–119)</td>
</tr>
<tr>
<td>3 (9)</td>
<td>468 (370–661)</td>
<td>117 (98–143)</td>
</tr>
<tr>
<td>p value</td>
<td>&lt;0.001</td>
<td>0.028</td>
</tr>
</tbody>
</table>

* The p value was calculated by Kruskal-Wallis test.

Acknowledgments

This work was supported in part by Grants-in-Aid from the Ministry of Health, Labor, and Welfare of Japan (09.2752) and the Ministry of Education, Science, Sports, and Culture (14570230) and the Japan Society for the Promotion of Science (13000205) (K.H.). We also thank Dr. C. Miller (Columbia University) for valuable comments on this manuscript.
primary gastric epithelial cells (Fig. 2A). The increase in IL-18 production was directly proportional to the MOI peaking at an MOI of 100 and then decreasing at MOIs of 200 or greater, probably due to toxicity of *H. pylori* at high density. The induction levels were reproducible at an MOI of 100 and subsequent experiments used that MOI. IL-18 protein reached maximal levels at ~18 h post inoculation and then plateaued during an observation period of 36 h in THP-1 cells (Fig. 2B).

To investigate the effects of virulence factors of *H. pylori* on the IL-18 production, we cocultured cells with oipA mutants or with cag PAI mutants (Fig. 3). IL-18 was not induced from primary gastric epithelial cells cocultured with either the oipA mutants or the cag PAI mutants whereas IL-18 induction was similar to wild type in THP-1 cells using the cag PAI mutants (121.5 ± 12 pg/ml) and was slightly, but significantly decreased with the oipA mutants (94.3 ± 8 pg/ml) (*p* < 0.01 vs live wild-type strains). The data from in vitro coculturing experiments using gastric epithelial cells and THP-1 cells were identical with the data from in vivo measurement of IL-18 protein using isolated gastric epithelial cells and LPMC, respectively.

To examine whether IL-18 induction required live *H. pylori*, we cocultured cells with heat-killed *H. pylori* for 18 h (Fig. 3). No IL-18 protein was produced from primary gastric epithelial cells but IL-18 protein was induced from THP-1 cells by heat-killed *H. pylori* (112.3 ± 6 pg/ml). The levels were slightly lower than those achieved with live wild-type *H. pylori* (135.4 ± 6 µg/ml) (Fig. 3), clearly showing that unknown heat-resistant factors in *H. pylori* unrelated to cag PAI and OipA also are involved in IL-18 induction by THP-1 cells.

We used a polyester membrane to separate the bacteria from the cells thus preventing direct *H. pylori*-cell interaction. Prevention of bacterial-cell attachment resulted in the prevention of IL-18 induction from both primary gastric epithelial cells and THP-1 cells (Fig. 3) suggesting that a direct interaction was necessary for IL-18 production irrespective of the cell type.

### IL-18 mRNA expression from cells cocultured with *H. pylori*

To test whether IL-18 mRNA expression paralleled changes in IL-18 protein levels, we used quantitative RT-PCR on the six gastric epithelial cell lines and the primary gastric epithelial cells cocultured with *H. pylori* (MOI of 100) were performed 3 h post inoculation. IL-18 mRNA was significantly up-regulated by *H. pylori* infection in four of the six gastric cell lines and in the primary gastric epithelial cells (Fig. 4A). Cell viability of primary gastric epithelial cells was ~96% during the coculturing experiments (data not shown). Due to the limitations associated with primary gastric epithelial cells (e.g., difficulty obtaining sufficient cell numbers), we used AGS cells in subsequent experiments based on the fact that the mRNA in AGS cells was similar to those obtained from primary cells. An additional advantage to the use of AGS cells is that they are easily transfected with plasmids whereas this proved difficult with primary cells, MKN1, and MKN7 cells (data not shown). Transfection also was not successful in THP-1 cells (data not shown).

Similar to the protein levels, IL-18 mRNA levels in both AGS and THP-1 cells increased in proportion to the MOI and peaked at an MOI of 100, then decreased at MOIs of 200 or greater (data not shown). Although AGS cells spontaneously expressed IL-18 mRNA, IL-18 mRNA levels in both cell types significantly increased following *H. pylori* infection (MOI of 100) reaching maximal levels within 3–6 h and then gradually decreasing to a baseline (Fig. 4B). Subsequent experiments examining IL-18 mRNA levels used an MOI of 100 and coculturing for 3 h.

As with IL-18 protein levels, IL-18 mRNA levels in AGS cells did not increase following infection with the oipA mutants (1.0 ± 0.2-fold induction of uninfected control) or the cag PAI mutants (1.0 ± 0.2-fold induction of uninfected control) (Fig. 5). IL-18
in AGS cells were significantly reduced when using killed H. pylori infected control; (0.9-fold induction relative to uninfected control in AGS cells (3.9-fold induction relative to uninfected control in THP-1 cells). To investigate the signal transduction pathways involved in H. pylori-associated IL-18 induction, we preincubated cells with chemical inhibitors or DMSO (for the infected control) at 37°C for 1 h. We inhibited the interaction of wild-type H. pylori strains (MOI of 100) with cells using a polyester membrane to inhibit any direct bacteria-cell interaction. After 3-h coculture, IL-18 protein levels released from human cells were determined by absolute quantitative real-time RT-PCR. The expression levels of IL-18 mRNA were expressed as the ratio of IL-18 mRNA to GAPDH mRNA. At least three independent cocultures were performed and each was measured in triplicate. Data are expressed as mean ± SE and p value was calculated by nonpaired t test. mRNA levels in THP-1 cells were similar with live H. pylori irrespective the presence or absence of functional OipA or the cag PAI. Overall, IL-18 protein levels, but not IL-18 mRNA levels, were lower using the oipA mutants in THP-1 cells, suggesting that OipA may play a role in regulating the posttranscriptional levels in THP-1 cells.

Similar to the results with IL-18 protein production from primary gastric epithelial cells, H. pylori-induced IL-18 mRNA levels in AGS cells were significantly reduced when using killed H. pylori (0.9 ± 0.1-fold induction of uninfected control). In contrast, IL-18 mRNA was induced in THP-1 cells (3.9 ± 0.1-fold induction of uninfected control) at only slightly lower levels than those associated with live wild-type H. pylori (5.0 ± 0.2-fold induction of uninfected control; p = 0.2) (Fig. 5). Inhibition of direct H. pylori-cell interactions completely prevented induction of IL-18 mRNA synthesis in AGS cells and THP-1 cells (Fig. 5).

**Effect of inhibition of MAPK and NF-κB pathways on IL-18 induction**

To investigate the signal transduction pathways involved in H. pylori-associated IL-18 induction, we preincubated cells with chemical inhibitors or DMSO (for the infected control) at 37°C for 1 h followed by infection with wild-type H. pylori. U0126, an inhibitor of MEK1/2 and MG-132, an inhibitor of NF-κB significantly inhibited the induction of IL-18 protein and IL-18 mRNA in both gastric epithelial cells (primary cells for protein and AGS cells for mRNA) and THP-1 cells (Fig. 6). SB203580, an inhibitor of p38, also significantly inhibited the production of IL-18 protein both in primary gastric epithelial cells and THP-1 cells; whereas SB203580 had no effect on IL-18 mRNA levels in either AGS cells or THP-1 cells. These data suggest that p38 plays a role at the posttranscriptional level.

The fact that IL-18 protein levels, but not IL-18 mRNA levels in THP-1 cells were reduced following infection with the oipA mutants or the p38 inhibitor suggests a possible relationship between OipA and the p38 pathway. This result also is consistent with prior studies showing that in gastric epithelial cells, OipA, but not the cag PAI induced the p38 pathway (23, 26). To test the hypothesis that OipA plays a role in p38 induction in THP-1 cells, we preincubated cells with chemical inhibitors or DMSO at 37°C for 1 h followed by infection with the oipA mutants. IL-18 protein levels induced by the oipA mutants were unchanged by adding the SB203580 (102.6 ± 7 pg/ml for oipA mutants with DMSO and 96.0 ± 7 pg/ml for oipA mutants without DMSO) consistent with the proposed relationship between OipA and p38 pathway in THP-1 cells. We also used immunoblot analyses with phospho-p38 Ab and total p38 Ab (both from Cell Signaling Technology) to confirm that wild-type H. pylori but not the oipA mutants induced phosphorylation of p38 in THP-1 cells (our unpublished data).

**Effect of H. pylori on the IL-18 promoter activity**

To confirm our hypothesis that the induction in IL-18 mRNA associated with H. pylori infection was due to transcriptional regulation at the promoter level, we transfected the pGL3–1375 plasmid into AGS cells followed by the infection of wild-type H. pylori, oipA mutants or cag PAI mutants (Fig. 7A). Wild-type H. pylori significantly induced luciferase activity of the IL-18 promoter (1.6 ± 0.1-fold induction of uninfected control; p < 0.001). Similar to the results for IL-18 mRNA expression, the IL-18 promoter activity in AGS cells was not increased with heat-killed...
IL-18 promoter involved in the induction of IL-18 by H. pylori infection. Human cells were incubated with SB203580, U0126, or MG-132 for 1 h and subsequently cocultured with wild-type H. pylori strains at an MOI of 100 for 20 h (A) or 3 h (B). IL-18 protein levels released from human cells were determined by ELISA (A) and IL-18 mRNA expression levels by absolute quantitative real-time RT-PCR normalized to GAPDH mRNA. At least three independent cocultures were performed and each was measured in triplicate. Data are expressed as mean ± SE. *p < 0.01 and **p < 0.001 compared with wild-type H. pylori infected controls without inhibitors by nonpaired t test.

H. pylori or by the prevention of an H. pylori-AGS cells interaction. The promoter activity was significantly decreased using the cag PAI mutants and the oipA mutants (1.2 ± 0.3-fold and 1.3 ± 0.1-fold induction of uninfected control, respectively) compared with wild-type H. pylori. However, both mutants significantly induced luciferase activity compared with the uninfected control (p < 0.001 for oipA mutants and p < 0.05 for cag PAI mutants). This result differed from results of IL-18 mRNA expression suggesting that OipA and the cag PAI partially affect the expression of IL-18 mRNA after promoter activation. The basal promoter activity was relatively low. However, there were low interexperimental variations of IL-18 promoter activity and the addition of 1 mM sodium butyrate (positive control) resulted in a 2.1-fold induction of IL-18 promoter activity compared with uninfected AGS cells (data not shown).

To confirm the signaling transduction pathways involved in H. pylori-induced IL-18 promoter activation, we preincubated the pGL3–1375-transfected AGS cells with chemical inhibitors or DMSO (for the infected control) at 37°C for 1 h followed by the wild-type H. pylori infection. Consistent with the results of real-time RT-PCR for IL-18 mRNA, H. pylori-inducible luciferase activity was not increased by preincubation of U0126 or MG-132, but was increased by that of SB203580 (data not shown).

Effect of the IL-18 promoter deletions on H. pylori-inducible luciferase activity

To define the necessary transcriptional regulatory region of the IL-18 promoter involved in the induction of IL-18 by H. pylori infection, a series of deletion constructs derived from pGL3–1375 were transfected into AGS cells followed by the infection of wild-type H. pylori, pGL3–972 (−972 to −1) contained binding sites for STAT, NF-κB, OCT-1, SP2, and PU.1 with deletion of the AP-1 binding site. pGL3–515 (−515 to −1) has a further deleted STAT binding site, pGL3–315 (−315 to −1) had a further deleted NF-κB binding site, pGL3–108 (−108 to −1) had a further deleted OCT-1 binding site, pGL3–67 (−67 to −1) had a further deleted SP2 binding site, and pGL3–25 (−25 to −1) had an additionally deleted PU.1 binding site. H. pylori inducible luciferase activity was reduced in all transfectants (Fig. 7B). Luciferase activity for pGL3–972 (−972 to −1)
We showed for the first time that IL-18 levels were up-regulated both in the gastric epithelial cells and LPMC isolated from *H. pylori*-infected gastric mucosa. Importantly, the behavior of gastric epithelial cells and monocytes differed markedly in response to the virulent factors of *H. pylori*; *cag* PAI and OipA. Previous studies (23, 25, 26) have suggested that both *cag* PAI and OipA were involved in the induction of various cytokines and chemokines in the gastric mucosa, such as IL-6, IL-8, and RANTES cells. Although the relationship between OipA and IL-8 remains unclear in epithelial cells (36), there is agreement that the *cag* PAI does not play a role in the induction of these cytokines in THP-1 cells (37). As shown here, both *cag* PAI and OipA were involved in IL-18 induction from gastric epithelial cells; whereas the *cag* PAI was not involved in IL-18 induction in monocytes, which was confirmed both by direct measurement of IL-18 in the isolated LPMC and measurement of IL-18 induced by coculturing of *H. pylori* with THP-1 cells. Of interest, OipA was involved in induction of IL-18 protein, but not in IL-18 mRNA in monocytes. In addition, we showed that OipA was involved in activation of the p38 pathway and that the p38 pathway also regulated IL-18 induction at a posttranscriptional level in both gastric epithelial cells and in monocytes. This effect possibly is related to p38 functioning as an mRNA stabilizer (38, 39). Further studies will be necessary to examine the details of the OipA→p38 pathways in mRNA stabilization as well as other posttranscriptional regulation events.

Of interest, the behavior of gastric epithelial cells and monocytes also differed markedly in response to heat-killed *H. pylori*. Haebeler et al. (40) previously reported both live and killed *H. pylori* were able to induce IL-12 and IL-10 from peripheral blood leukocytes whereas with *H. pylori* inducing significantly more IL-10 than with live organisms. We also previously reported the release of IL-6 not IL-8 following treatment of gastric cancer cell lines with heat-killed *H. pylori* (23). It remains unclear whether the different responses of epithelial and inflammatory cells to heat-killed *H. pylori* is restricted to IL-18 or whether the phenomenon is shared by other cytokines. One difference between gastric epithelial cells and THP-1 cells relates to the fact that THP-1 cells are professional phagocytes whereas gastric epithelial cells are not (41). Although a role for some soluble *H. pylori* proteins (e.g., urease) has been suggested in relation to IL-10 induction by killed *H. pylori* (40), separation of THP-1 cells and the bacteria using polyester membranes abrogated the response, providing compelling evidence that cell-bacteria interaction rather than soluble proteins mediate the response. Overall, our data suggest that direct attachment of *H. pylori* and cells along with the participation of the *cag* PAI and OipA are required to trigger *H. pylori*-associated IL-18 induction from gastric epithelial cells. In contrast, *H. pylori* appear to interact with monocytes differently although even both live *H. pylori* and OipA were required for maximal induction of IL-18 production.

We showed that IL-18 was produced mainly from LPMC in the gastric mucosa. Although *H. pylori* generally are attached to the gastric epithelial cells, but not to infiltrating cells, it is known that *H. pylori* and/or *H. pylori* products likely have many opportunities to come into direct contact with intraepithelial monocytes in the inflamed mucosa. For example, tight junctions are opened by the movement of polymorphonuclear cells, which is thought to result in increased permeability (42). *H. pylori* also are known to invade the gastric mucosa making whole cell Ags available to phagocytic cells (43). Finally, it has been postulated from in vitro studies that *H. pylori* itself may affect tight junction activity (44). The induction patterns of IL-18 in LPMC in *H. pylori*-positive patients were similar to those in THP-1 cells directly cocultured with *H. pylori*, especially in relation to the virulence factors, suggesting that

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**Discussion**

IL-18 normally is produced from both macrophage-like cells (8, 9, 34) and from some epithelial cells, such as intestinal epithelial cells (35). This study was designed to test the hypothesis that IL-18 expression as measured in *H. pylori* infected gastric mucosa reflected expression from both epithelial cells and from *H. pylori*-associated inflammatory infiltrates. We clearly confirmed that *H. pylori* induced both IL-18 mRNA and protein in the gastric mucosa using quantitative analyses. In addition, strong correlation between mucosal IL-18 levels and gastric inflammation suggest that *H. pylori*-induced IL-18 plays an important role in gastric inflammation via direct effects of elevated IL-18 or via indirect effects of IL-18 such that effects of IFN-γ, which is a well-known proinflammatory cytokine induced by IL-18.
H. pylori-associated IL-18 induction in the gastric mucosa in vivo occurs by direct contact with intraepithelial monocytes. We showed that H. pylori up-regulated expression of IL-18 mRNA and the mature IL-18 protein in primary gastric epithelial cells whereas gastric epithelial cancer cell lines, with the exception of KATOIII cells, did not produce the mature IL-18 protein. However, we were unable to confirm recent studies suggesting that H. pylori could induce IL-18 from AGS cells (45). In fact, we were unable to detect the mature IL-18 protein from AGS cells either by ELISA or by immunoblot. Our preliminary studies showed that the AGS cells we used did not produce ASC (our unpublished data), which is essential for IL-18 maturation. IL-18 plays a critical role in the host defensive immune response against tumors (10, 46) and we speculate that mutations such as those blocking ASC function occur during carcinogenesis. Further studies will be necessary to determine whether the loss of ability to produce mature IL-18 in gastric cancer cells is biologically important.

One limitation of our study includes the use of primary gastric epithelial cells, which are unlikely to be perfectly pure cultures.Importantly, only ~1.5% of cultured cells in the monolayers did not express periodic acid-Schiff-positive material in their cytoplasm. It remains possible that the low levels of mature IL-18 produced from the primary gastric cell cultures originated from contaminating nonepithelial cells. Subsequent studies will be required that immunolocalize IL-18 within the gastric mucosa to define the relative contributions of the epithelial and inflammatory cells in terms of IL-18 expression.

We examined the transcriptional regulation of IL-18 promoter activity by luciferase reporter gene assay and EMISA. Luciferase reporter gene assay using serial deleted plasmids and EMISA showed that AP-1 played a major role in IL-18 transcription in AGS cells. It is well known that MAPK pathways, especially the ERK and JNK pathways, are involved in AP-1 activation. The fact that inhibition of the ERK pathway diminished H. pylori-related IL-18 induction, and that supershift analysis showed that c-Jun, which is downstream of JNK, bound to the AP-1 site of the IL-18 promoter, support the hypothesis that H. pylori activated ERK/JNK→AP-1 pathways is followed by IL-18 induction in AGS cells. Interestingly, the same pathways also play important roles in THP-1 cells. EMISA and chemical inhibitor experiments showed that the NF-κB pathway also is involved in IL-18 induction in both AGS cells and THP-1 cells. Overall, although actual components of H. pylori (cag PAI, OipA, or unknown heat-resistant factors) may differ between gastric epithelial cells and THP-1 cells, the major signaling pathways appear to be similar.

Finally, we also examined the binding of the transcription factor PU.1 to the IL-18 promoter. Kalina et al. (28) previously reported that the regulatory region-related to the sodium butyrate-induced expression of IL-18 in THP-1 cells was the PU.1 consensus binding site. However, we failed to detect a relationship between PU.1 binding and H. pylori-induced IL-18 induction, suggesting that the signaling pathways of H. pylori-induced IL-18 differ from that of sodium butyrate induced IL-18 in THP-1 cells.

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The authors have no financial conflict of interest.

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