



**HAL**  
open science

## **Chemokines and Antimicrobial Peptides Have a *cag*-Dependent Early Response to *Helicobacter pylori* Infection in Primary Human Gastric Epithelial Cells**

Pascale Mustapha, Isabelle Petit-Paris, Magali Garcia, Cong Tri Tran, Julie Cremniter, Martine Garnier, Jean-Pierre Faure, Thierry Barthes, Ivo G. Boneca, Franck Morel, et al.

### ► To cite this version:

Pascale Mustapha, Isabelle Petit-Paris, Magali Garcia, Cong Tri Tran, Julie Cremniter, et al.. Chemokines and Antimicrobial Peptides Have a *cag*-Dependent Early Response to *Helicobacter pylori* Infection in Primary Human Gastric Epithelial Cells. *Infection and Immunity*, 2014, 82 (7), pp.2881 - 2889. 10.1128/IAI.01517-13 . hal-01699819

**HAL Id: hal-01699819**

**<https://hal.science/hal-01699819v1>**

Submitted on 5 Feb 2018

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

## Chemokines and Antimicrobial Peptides Have a *cag*-Dependent Early Response to *Helicobacter pylori* Infection in Primary Human Gastric Epithelial Cells

Pascale Mustapha, Isabelle Paris, Magali Garcia, Cong Tri Tran, Julie Cremniter, Martine Garnier, Jean-Pierre Faure, Thierry Barthes, Ivo G. Boneca, Franck Morel, Jean-Claude Lecron, Christophe Burucoa and Charles Bodet  
*Infect. Immun.* 2014, 82(7):2881. DOI: 10.1128/IAI.01517-13.  
Published Ahead of Print 28 April 2014.

---

Updated information and services can be found at:  
<http://iai.asm.org/content/82/7/2881>

---

### SUPPLEMENTAL MATERIAL

*These include:*

[Supplemental material](#)

### REFERENCES

This article cites 39 articles, 13 of which can be accessed free at: <http://iai.asm.org/content/82/7/2881#ref-list-1>

### CONTENT ALERTS

Receive: RSS Feeds, eTOCs, free email alerts (when new articles cite this article), [more»](#)

---

---

Information about commercial reprint orders: <http://journals.asm.org/site/misc/reprints.xhtml>  
To subscribe to to another ASM Journal go to: <http://journals.asm.org/site/subscriptions/>

---

# Chemokines and Antimicrobial Peptides Have a *cag*-Dependent Early Response to *Helicobacter pylori* Infection in Primary Human Gastric Epithelial Cells

Pascale Mustapha,<sup>a</sup> Isabelle Paris,<sup>a,b</sup> Magali Garcia,<sup>a,b</sup> Cong Tri Tran,<sup>a,b</sup> Julie Cremniter,<sup>a,b</sup> Martine Garnier,<sup>a,b</sup> Jean-Pierre Faure,<sup>b</sup> Thierry Barthes,<sup>c</sup> Ivo G. Boneca,<sup>d,e</sup> Franck Morel,<sup>a</sup> Jean-Claude Lecron,<sup>a,b</sup> Christophe Burucoa,<sup>a,b</sup> Charles Bodet<sup>a</sup>

Laboratoire Inflammation, Tissus Épithéliaux et Cytokines (LITEC—EA 4331), Université de Poitiers, Poitiers, France<sup>a</sup>; Centre Hospitalier Universitaire de Poitiers, Poitiers, France<sup>b</sup>; Polyclinique de Poitiers, Poitiers, France<sup>c</sup>; Institut Pasteur, Unité de Biologie et Génétique de la Paroi Bactérienne, Paris, France<sup>d</sup>; INSERM, Equipe Avenir, Paris, France<sup>e</sup>

*Helicobacter pylori* infection systematically causes chronic gastric inflammation that can persist asymptotically or evolve toward more severe gastroduodenal pathologies, such as ulcer, mucosa-associated lymphoid tissue (MALT) lymphoma, and gastric cancer. The *cag* pathogenicity island (*cag* PAI) of *H. pylori* allows translocation of the virulence protein CagA and fragments of peptidoglycan into host cells, thereby inducing production of chemokines, cytokines, and antimicrobial peptides. In order to characterize the inflammatory response to *H. pylori*, a new experimental protocol for isolating and culturing primary human gastric epithelial cells was established using pieces of stomach from patients who had undergone sleeve gastrectomy. Isolated cells expressed markers indicating that they were mucin-secreting epithelial cells. Challenge of primary epithelial cells with *H. pylori* B128 underscored early dose-dependent induction of expression of mRNAs of the inflammatory mediators CXCL1 to -3, CXCL5, CXCL8, CCL20, BD2, and tumor necrosis factor alpha (TNF- $\alpha$ ). In AGS cells, significant expression of only CXCL5 and CXCL8 was observed following infection, suggesting that these cells were less reactive than primary epithelial cells. Infection of both cellular models with *H. pylori* B128 $\Delta$ cagM, a *cag* PAI mutant, resulted in weak inflammatory-mediator mRNA induction. At 24 h after infection of primary epithelial cells with *H. pylori*, inflammatory-mediator production was largely due to *cag* PAI substrate-independent virulence factors. Thus, *H. pylori* *cag* PAI substrate appears to be involved in eliciting an epithelial response during the early phases of infection. Afterwards, other virulence factors of the bacterium take over in development of the inflammatory response. Using a relevant cellular model, this study provides new information on the modulation of inflammation during *H. pylori* infection.

It is now widely established that infection with *Helicobacter pylori* is the leading cause of chronic gastritis, duodenal ulcers, and gastric cancer. This Gram-negative bacterial pathogen selectively colonizes the human gastric epithelium, thereby inducing an inflammatory response characterized by massive neutrophil infiltration and large-scale production of cytokines and chemokines, mainly interleukin 8 (IL-8) (CXCL8).

The *cag* pathogenicity island (*cag* PAI) is one of the most widely studied virulence factors of *H. pylori*. This 37-kb DNA segment encodes a type IV secretion system (TIVSS) that delivers virulence factors such as the CagA protein as well as fragments of peptidoglycan (PG) into host cells (1–3). CagM is a 43.7-kDa protein which is also encoded by the *cag* PAI. Along with CagX and CagT, CagM forms an outer membrane-associated TIVSS subcomplex (4). Systematic mutagenesis of *cag* PAI genes showed that *H. pylori*  $\Delta$ cagM strains cannot perform efficient translocation of CagA or PG into epithelial cells (3). CagA and CagM are consequently important for assessing the virulence of *H. pylori* strains.

In order to study the interactions between the *cag* PAI and epithelial cells, several cell line models derived from gastric adenocarcinoma, such as the AGS and KATO-3 cell lines, have been used. Even though these models have clarified many aspects of the changes triggered by *H. pylori* at the cellular level, such as establishment of the inflammatory response, at a later time these cell lines were found to have lost some of the key elements in signaling pathways involved in cellular responses to *H. pylori* infection. For example, the AGS cell line lacks Toll-like receptor 2 (TLR2) and

the MD2 cofactor, which is essential for lipopolysaccharide (LPS) recognition by TLR4 (5).

As a result, in order to obtain a cellular model more relevant for defining and characterizing the pathophysiological processes associated with *H. pylori* infection, development of primary human epithelial cell models was considered necessary. Even though primary culture of human gastric epithelial cells has been limited, mainly on account of the unavailability of sufficient quantities of human gastric tissue and the difficulty of isolating and expanding these cells, a few models have been developed using organ donors (6), fetal tissue (7), and gastric biopsy specimens (8). Isolation of gastric epithelial cells from organ donors was used to study the characteristics of attachment of *H. pylori* to cultured cells (9) and its effects on gastrin secretion (10). In another study, Basque et al. developed a primary epithelial cell model from legally aborted

Received 26 November 2013 Returned for modification 20 December 2013

Accepted 10 April 2014

Published ahead of print 28 April 2014

Editor: B. A. McCormick

Address correspondence to Charles Bodet, charles.bodet@univ-poitiers.fr.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/IAI.01517-13>.

Copyright © 2014, American Society for Microbiology. All Rights Reserved.

doi:10.1128/IAI.01517-13

fetuses (7), and it was used to study the secretion of pepsinogen and gastric lipase (11) and to examine the effects of growth factors on cell proliferation and regeneration (12). In a third approach, Smoot et al. developed a protocol for primary culture of epithelial cells using gastric biopsy specimens (8). Using the same model, Bäckhed et al. reported that *H. pylori* infection of primary cells induced a regulated production of IL-6, IL-8, and tumor necrosis factor alpha, whereas infection of cell lines resulted in only IL-8 production (13). A CagA-dependent induction of cytoskeletal rearrangements and alterations in cell junctions was likewise reported with regard to this model (14). However, given the low yield in primary cells, these partial results needed to be complemented by parallel studies on cell lines. And even though the aforementioned models have clearly provided valuable insights into the functioning of normal gastric epithelial cells, until now the inflammatory response associated with *H. pylori* has remained poorly investigated in primary cells. The aim of this study was to characterize *H. pylori*-associated inflammatory reactions by applying a new protocol allowing the isolation and culture of human primary gastric epithelial cells (PGEC) using normal gastric tissue obtained from obese subjects who underwent gastric sleeve surgery. Isolation of PGEC from large pieces of human stomach is an original approach that has contributed to the development of a pertinent *in vitro* cell model facilitating study of the expression of the antimicrobial peptides, chemokines, and cytokines induced by *H. pylori*.

## MATERIALS AND METHODS

**Human stomach samples.** The use of stomach samples for this study was approved by the ethics committee of Poitiers Hospital. After the provision of fully informed consent, normal human antra were obtained from *H. pylori*-negative subjects who had undergone gastric sleeve surgery.

**Isolation and culture of PGEC from human stomach.** Pieces of antrum were thoroughly washed with phosphate-buffered saline solution (PBS) (Gibco) to remove blood and mucus and then cut along the surgical crease. Fat and muscle layers were discarded. The mucosa was carefully dissected from underlying submucosa and minced into fragments of about 2 mm<sup>3</sup> using scalpel blades. Tissues were digested in 0.5 mg/ml of collagenase B (Roche), 2.5 mg/ml of pronase (Roche), and 3 U/ml of dispase (Sigma-Aldrich) in Ham's F12/DMEM (Gibco) for 40 min at 37°C and then recovered using a 500- $\mu$ m Nitex mesh. The tissue was further digested by two sequential digestions in trypsin–0.05% EDTA (Gibco) for 15 min each at 37°C. The cell suspension collected was then filtered through a 250- $\mu$ m Nitex mesh. Cells were washed twice, centrifuged at 400  $\times$  g for 5 min, and filtered again through a 100- $\mu$ m Nitex mesh. Primary human gastric cells were seeded at a density of 4  $\times$  10<sup>5</sup> cells/well in 24-well collagen I-coated culture plates (Becton, Dickinson). The culture medium consisted of Ham's F-12–Dulbecco's modified essential medium (DMEM) (vol/vol) (Gibco) supplemented with 10% fetal calf serum (FCS; Sigma-Aldrich), 0.5 ng/ml of epidermal growth factor (EGF; Gibco), 15 ng/ml of hepatocyte growth factor (HGF; Miltenyi Biotec), 50 U/ml of penicillin, and 50  $\mu$ g/ml of streptomycin (Gibco). The cultures were incubated at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> before infection assays with *H. pylori*.

**Human gastric cell line culture.** AGS cells (ATCC CRL 1739) were cultured in 75-cm<sup>2</sup> flasks in DMEM supplemented with 10% (vol/vol) FCS, 50 U/ml penicillin, and 50  $\mu$ g/ml streptomycin. AGS cells were seeded at a density of 4  $\times$  10<sup>5</sup> cells/well in 24-well culture plates. The cultures were maintained at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>.

**Bacterial culture.** The *H. pylori* strains used throughout the study were B128 and B128 $\Delta$ cagM. *H. pylori* B128 (*cagA vacA s1/m2*) was isolated from a gastritis patient (15). This strain contains an entirely functional *cag*

PAI. The isogenic mutant B128 $\Delta$ cagM was obtained by natural transformation, allelic exchanges, and insertion of a chloramphenicol resistance cassette (16). PCR of the *cagM* gene empty site was performed to confirm the mutagenesis. Deletion of the *cagM* gene renders *H. pylori* TIVSS dysfunctional in such a way that B128 $\Delta$ cagM strains become unable to perform efficient translocation of CagA or peptidoglycan into epithelial cells (16). *H. pylori* strains were routinely cultured on Skirrow's medium (Oxoid) and incubated for 48 h at 37°C under microaerobic conditions using CampyGen bags (Oxoid). For cell infection assays, suspensions of *H. pylori* B128 and B128 $\Delta$ cagM were prepared in the cell culture medium using 24-h bacterial cultures. Bacteria were added to cells at a multiplicity of infection (MOI) equal to 10 or 100 bacteria per cell. Bacterial concentrations were determined by measuring the optical density of the culture at 600 nm. In addition, CFU counts of *H. pylori* were performed.

**Cell infection assays with *H. pylori*.** Primary human gastric epithelial cells and AGS cells were incubated in Ham's F-12–DMEM culture medium without growth factors, FCS, or antibiotics for 12 h prior to infection. Cell monolayers were then infected with *H. pylori* B128 or B128 $\Delta$ cagM for 3 h or 24 h at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. Culture supernatants were collected, centrifuged (400  $\times$  g, 5 min, 20°C) and stored at –80°C until used. Cultures without bacteria were used as controls. To assess the involvement of epidermal growth factor receptor (EGFR) signaling in inflammatory reactions induced by *H. pylori*, PGEC cells were pretreated for 2 h with tyrphostin AG1478 (2  $\mu$ mol/liter; Sigma-Aldrich), a specific inhibitor of EGFR tyrosine kinase, before they were infected by *H. pylori*.

**RNA extraction.** Total RNA was extracted from frozen gastric biopsy specimens using a MagNA Pure compact system and an RNA isolation kit (Roche) according to the manufacturer's instructions. Total RNA extraction from primary epithelial cells and AGS cells was performed using the NucleoSpin XS RNA extraction kit according to the manufacturer's instructions (Macherey-Nagel). RNA was eluted in 10  $\mu$ l of RNase-free H<sub>2</sub>O supplemented with 40 units of RNaseout (Invitrogen). RNA concentrations and purity were determined using the Nanodrop 2000 spectrophotometer (Thermo Scientific) and visualized using 0.8% (wt/vol) agarose gel electrophoresis containing 0.5  $\mu$ g/ml of GelRed (Fluoroprobes).

**Reverse transcription and real-time PCR analysis.** Total RNA (2  $\mu$ g) was reverse transcribed using a SuperScript II kit (Invitrogen) according to the manufacturer's instructions. Quantitative RT-PCR was performed in 96-well plates using a LightCycler-FastStart DNA MasterPlus SYBR green I kit (Roche) on LightCycler 480 (Roche). Reaction mixtures consisted of 1 $\times$  DNA master mix (Applied Biosystems), 1  $\mu$ M concentrations of forward and reverse primers designed using Primer 3 software, and 12.5 ng of cDNA template in a total volume of 10  $\mu$ l. PCR conditions were as follows: 5 min at 95°C and 40 amplification cycles comprising 20 s at 95°C, 15 s at 64°C, and 20 s at 72°C. Samples were normalized with regard to two independent control housekeeping genes (encoding glyceraldehyde phosphodehydrogenase and  $\beta$ 2-microglobulin) and reported according to the  $\Delta\Delta C_T$  method as RNA fold increase:  $2^{-\Delta\Delta C_T} = 2^{-(\Delta C_T \text{ stimulated} - \Delta C_T \text{ unstimulated})}$ .

**Gastric biopsy samples.** Patients with dyspeptic symptoms scheduled for upper gastrointestinal endoscopy were prospectively enrolled in the study. The use of gastric tissue samples was approved by the local ethics committee (CPP, CHU de Poitiers, protocol 09.10.23). Under fully informed consent, 15 *cagA*-positive gastric biopsy specimens, 15 *cagA*-negative gastric biopsy specimens, and 15 normal mucosa biopsy specimens were collected.

***H. pylori* detection and determination of *cag* PAI status.** *H. pylori* detection in human gastric biopsy specimens was performed by bacterial culture and Scorpion PCR as previously described (17). *H. pylori* strains from human biopsy specimens were cultured, and 24-h cultures were used for DNA extraction using a MagNA Pure compact system and DNA isolation kit (Roche) according to the manufacturer's instructions. A PCR was then performed to determine *cagA* and *cagM* status as previously described (18, 19). In clinical isolates, the presence of *cagA* and *cagM* was used as a marker of the presence of the *cag* PAI.



**Enzyme-linked immunosorbent assay (ELISA).** Levels of CXCL1, CXCL5, CXCL8, BD2, and BD3 in cell culture supernatants were determined in duplicates using human ELISA development kits (R&D Systems for CXCL1 and BD3 and Peprotech for CXCL8, CXCL5, and BD2) in accordance with the manufacturers' specifications.

**Immunocytofluorescence.** Primary gastric epithelial cells grown in 24-well plates for 4 days were collected using trypsin–0.05% EDTA and deposited by cytocentrifugation ( $250 \times g$ , 6 min) on slides at a density of  $5 \times 10^4$  cells/slide and then stored at  $-20^\circ\text{C}$  before completion of protein expression studies by immunofluorescence. After thawing, cells were fixed in a 4% paraformaldehyde–PBS bath, pH 7.4, for 20 min at room temperature. Saturation of nonspecific sites was performed for 30 min at room temperature using 5% donkey serum–PBS for cytokeratin 18 (CK18) labeling and 5% goat serum–PBS for mucin 1 (MUC1), MUC5AC, MUC6, and trefoil factor 1 (TFF-1) labeling. Incubation with specific primary antibodies (Ab) for each antigen was then performed in a humid chamber for 2 h at room temperature. The primary mouse monoclonal Ab anti-human CK18 and MUC6 (sc-58727 and sc-33668, respectively; Santa Cruz Biotechnology) were used at 2  $\mu\text{g}/\text{ml}$  in 1% donkey serum–PBS, and the primary rabbit polyclonal Ab anti-human MUC1, MUC5AC, and TFF-1 (sc-15333, sc-20118, and sc-28925, respectively; Santa Cruz Biotechnology) were used at 4  $\mu\text{g}/\text{ml}$  in 1% goat serum PBS. Incubation with donkey anti-mouse IgG secondary Ab coupled to Alexa-Fluor 488 (Invitrogen) or goat anti-rabbit IgG secondary Ab coupled with rhodamine-RedX (Jackson ImmunoResearch) was carried out for 45 min in a dark humid chamber at room temperature. In addition, omission of the first antibody was used as a negative control. After successive washes, slides were mounted with coverslips using a 4',6-diamidino-2-phenylindole (DAPI) mounting medium (Santa Cruz Biotechnology). The slides were then observed by confocal microscopy using an Olympus Fluoview FV1000 microscope (Olympus).

**Statistical analysis.** The data presented constitute the averages obtained from independently performed experiments with a standard error of the mean (SEM). Statistical analysis of significance was carried out using Kruskal-Wallis one-way analysis of variance (ANOVA) followed by Dunn's comparison between groups. *P* values of  $\leq 0.05$  were considered significant.

## RESULTS

**Expression of CK18, MUC1, MUC5AC, MUC6, and TFF1 in cultivated PGEC.** Preliminary evaluation of enzymatic digestion duration, adhesion, and culture medium conditions was needed in order to provide an environment suitable for growing PGEC *in vitro*. Several filtration and washing steps performed between and after digestion were needed in order to remove dissolved collagen, scraps, and highly viscous mucus that prevented cell adhesion. Proliferation rate was highly dependent on the initial cellular density, which was set to  $4 \times 10^5$  cells at seeding, and rat tail collagen I coating was required for cell adhesion. A mix of Ham's F-12–DMEM (vol/vol) containing 10% heat-inactivated FCS ensured cell expansion; supplementation with EGF and HGF significantly increased the proliferation rate. Within the initial 24 h of culture, multiple clusters of small flat polygonal cells could be observed. The colonies spread out and formed a subconfluent layer 3 days later and could be maintained in culture for up to 8 days. To confirm the nature of the cultured primary gastric cells, an immunofluorescence study of the CK18 epithelial marker, TFF1, and gastric mucins (MUC1, MUC5AC, and MUC6) was performed. PGEC and AGS cells stained positive for CK18, MUC1, MUC5AC, and TFF1 (Fig. 1). PGEC cells stained negative for MUC6 (data not shown). Positive staining for CK18, MUC1, MUC5AC, and TFF1 in isolated primary cells indicated that they are gastric surface mucous epithelial cells. In addition,

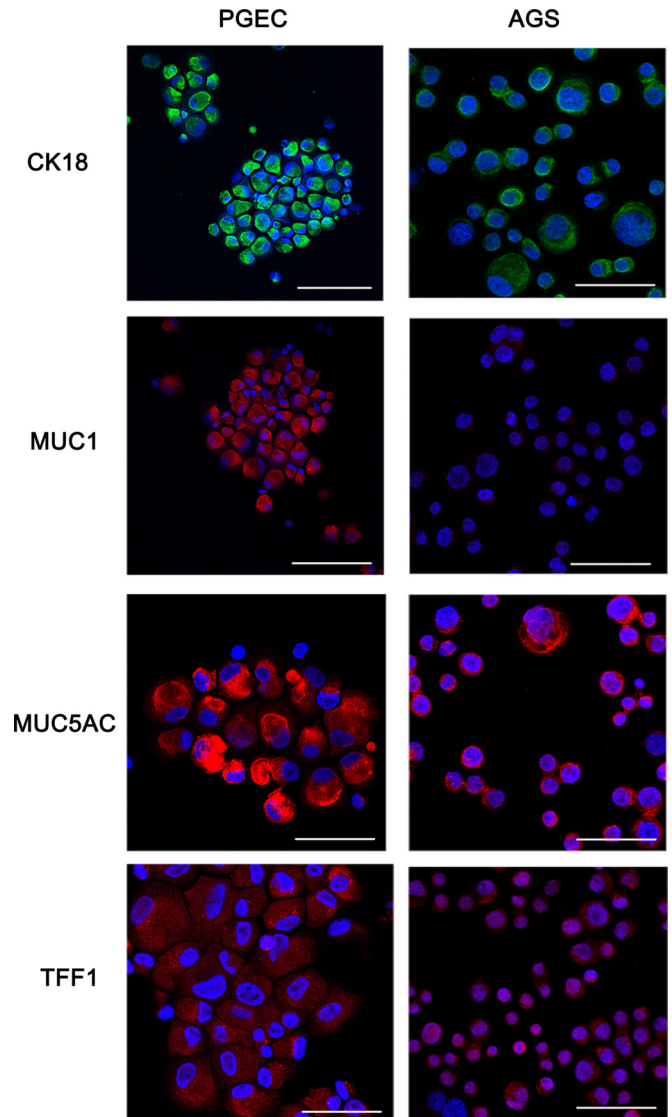
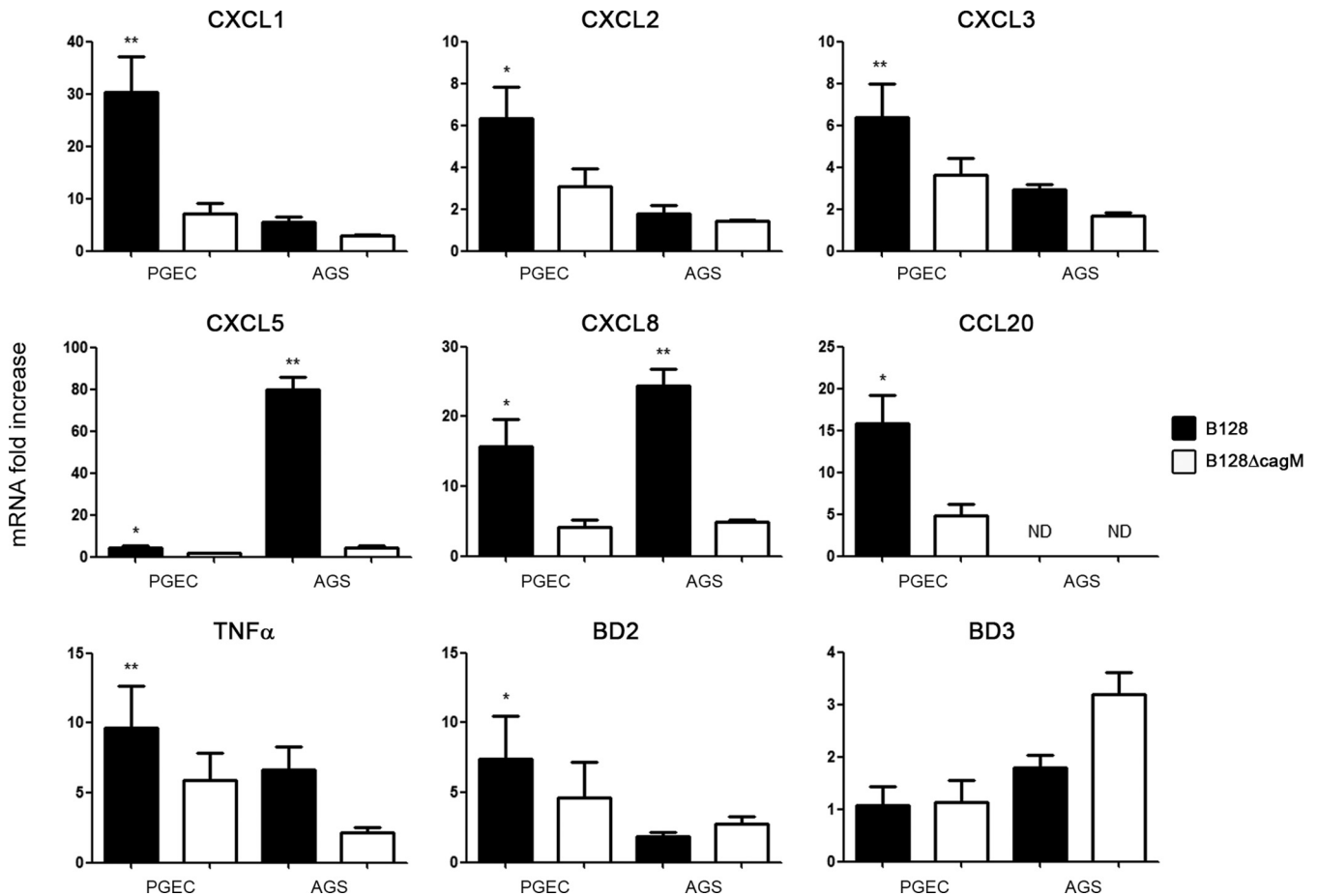


FIG 1 Immunodetection of CK18, MUC1, MUC5AC, and TFF1 in PGEC cells and in AGS cells. Scale bar, 50  $\mu\text{m}$ .

tion, levels of MUC1 and TFF1 proteins appeared to be higher in PGEC than in AGS cells. This finding was in agreement with MUC1 and TFF1 mRNA expression levels detected in both cellular models (data not shown).

**Dose-dependent induction of chemokines, cytokines, and antimicrobial peptides in PGEC infected with *H. pylori*.** PGEC were infected with *H. pylori* B128 at MOIs of both 10 and 100 for 3 h and the expression of a panel of cytokines (TNF- $\alpha$  and IL-1- $\beta$ ), chemokines (CXCL1, CXCL2, CXCL3, CXCL5, CXCL8, and CCL20), and antimicrobial peptides (S100A7, S100A8, S100A9, BD1, BD2, and BD3) was then analyzed by reverse transcription-quantitative PCR (RT-qPCR) analysis (see Fig. S1 in the supplemental material). Significant enhanced mRNA expression by PGEC of CXCL1 to -3, CXCL5, CXCL8, CCL20, TNF- $\alpha$ , and BD2 was observed following *H. pylori* infection at an MOI of 100 compared to uninfected controls (see Fig. S1). Under these conditions, IL-1- $\beta$ , S100A7, S100A8, S100A9, and BD1 expression was not



**FIG 2** Gene expression of PEGC and AGS cells stimulated by *H. pylori*. Cells were infected by *H. pylori* B128 or B128ΔcagM at an MOI of 100 for 3 h. RT-qPCR analysis was carried out on total RNA of independent cultures from 5 different patients. mRNA expression levels are expressed as the fold increase above unstimulated cultures. Data are means and SEM. Statistically significant differences in inflammatory mediator production between infected cells and uninfected cells as well as between cells stimulated with *H. pylori* B128 and those stimulated with B128ΔcagM were analyzed. \*,  $P < 0.05$ , and \*\*,  $P < 0.01$ , compared to the uninfected control. ND, not detected.

detected, whereas BD3 expression was unchanged (data not shown). Expression of CXCL1 to -3, CXCL5, CXCL8, CCL20, TNF- $\alpha$ , and BD2 mRNAs after coculture of PEGC with *H. pylori* at an MOI of 10 was slightly but not significantly upregulated (see Fig. S1).

**An early cag PAI-dependent induction of inflammatory mediators in PEGC.** In order to establish a transcriptional inflammatory profile comparing primary epithelial cells and AGS cells, and also to investigate the role of *H. pylori*'s functional TIVSS in eliciting the inflammatory response, the expression of inflammatory mediators induced by *H. pylori* B128 and B128ΔcagM at an MOI of 100 was further compared in PEGC and in AGS cells after 3 h of infection (Fig. 2). Whereas stimulation of primary epithelial cells with *H. pylori* B128 induced significant expression of CXCL1 to -3, CXCL5, CXCL8, CCL20, TNF- $\alpha$ , and BD2 mRNAs compared to unstimulated controls, no significant induction of any of these mediators was observed after stimulation with *H. pylori* B128ΔcagM (Fig. 2). However, statistically significant differences in inflammatory mediator expression were not observed between infections with the two bacterial strains. In AGS cells, the *H. pylori* response profile is pronouncedly different. Among the inflammatory mediators, only CXCL5 and CXCL8 mRNA showed signifi-

cantly higher expression compared to uninfected controls following *H. pylori* B128 infection, while no significant induction of any of these mediators was observed after coculture with *H. pylori* B128ΔcagM (Fig. 2). CCL20 mRNA expression was not detected in AGS cells upon infection. On the other hand, *H. pylori*-induced expression of CXCL1 to -3, TNF- $\alpha$ , and BD2 mRNAs was higher in PEGC than in AGS cells. At 24 h postinfection, the two cellular models expressed similar profiles of inflammatory mediators compared to an earlier time of infection (Fig. 3), but cag PAI-dependent induction of inflammatory mediators was not observed. In addition, chemokine expression levels induced by *H. pylori* were lower than those observed after 3 h of infection. In contrast, BD2 and BD3 mRNA expression induced by *H. pylori* was higher at 24 h postinfection. Significantly enhanced expression of BD2 mRNA was observed in PEGC following *H. pylori* B128 and B128ΔcagM infection (Fig. 3). In addition, the role of the EGFR pathway in chemokine expression of PEGC induced by *H. pylori* was evaluated using AG1478, a specific inhibitor of EGFR tyrosine kinase (see Fig. S2 in the supplemental material). This inhibitor reduced CXCL1, -5, and -8 mRNA expression induced by both bacterial strains at 3 h postinfection. AG1478 does not affect bacterial viability in the absence of host cells (data not

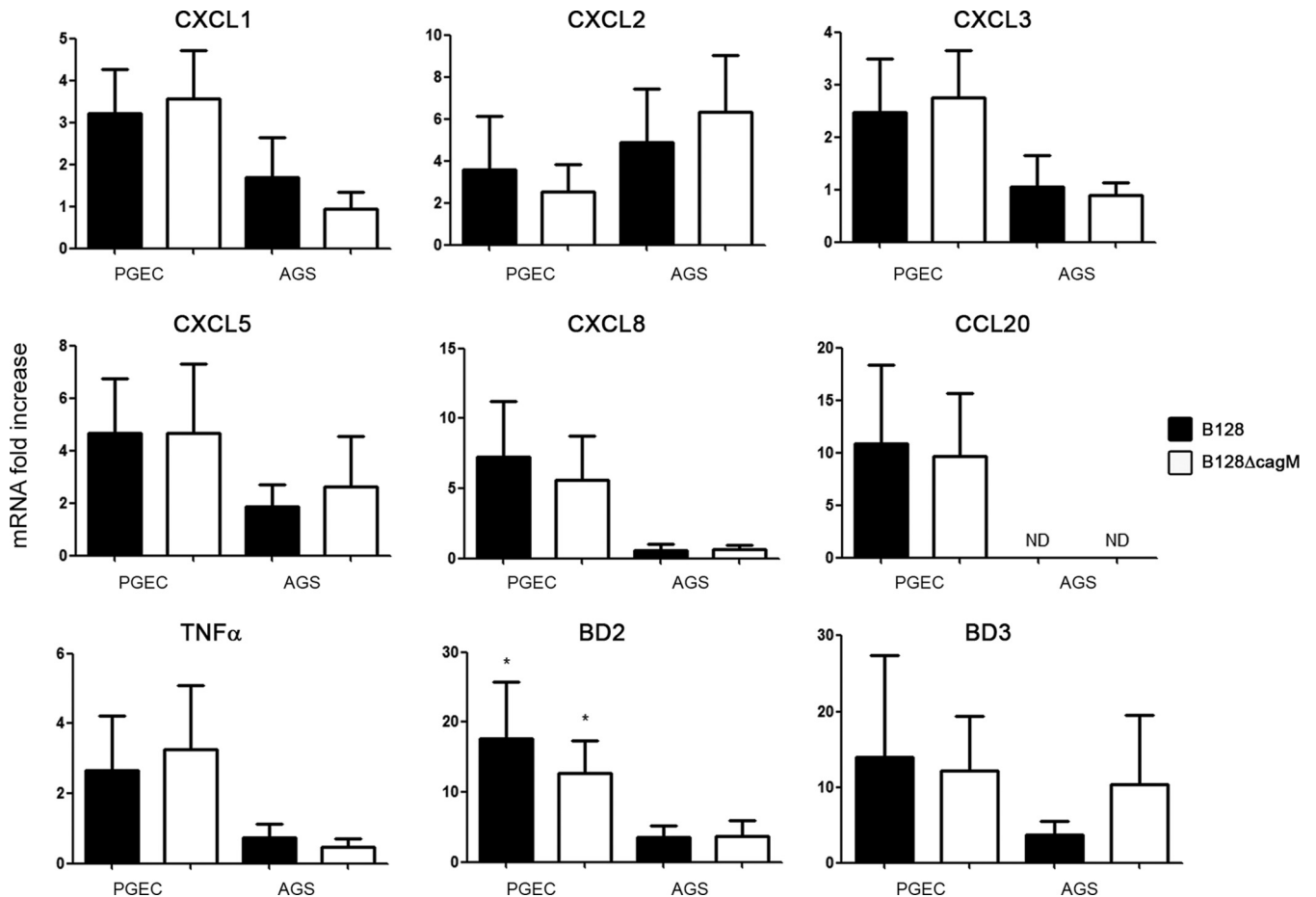


FIG 3 Gene expression of PGEC and AGS cells stimulated by *H. pylori*. Cells were infected by *H. pylori* B128 or B128ΔcagM at an MOI of 100 for 24 h. RT-qPCR analysis was carried out on total RNA of 5 independent cultures from 5 different patients. mRNA expression levels are expressed as the fold increase above unstimulated cultures. Data are means and SEM. \*,  $P < 0.05$  compared to uninfected control. ND, not detected.

shown). After 24 h of infection, inhibition of the EGFR had no further impact on chemokine expression (data not shown).

**A *cag* PAI-independent production of CXCL1, CXCL5, and CXCL8.** CXCL1, CXCL5, CXCL8, BD2, and BD3 production was quantified by ELISA in PGEC and AGS supernatants after 24 h of infection with *H. pylori* B128 or B128ΔcagM at an MOI of 100 (Fig. 4). Production of CXCL1, CXCL5, and CXCL8 was significantly higher in *H. pylori* B128 and B128ΔcagM-infected PGEC than in uninfected cells. The amounts of chemokine produced were similar when cells were stimulated with either the wild-type strain or the TIVSS-deficient strain at 24 h postinfection. In contrast, significant production of CXCL8 alone was observed in AGS cells infected with *H. pylori* B128 and the protein levels of these three chemokines were lower than those of primary cells. BD2 and BD3 protein levels could not be detected (below 8 and 64 pg/ml, respectively) either in PGEC or in AGS culture supernatants.

**Chemokine and BD2 mRNA expression during *H. pylori* gastritis.** CXCL1 to -3, CXCL5, CXCL8, and BD2 mRNA expression in the gastric mucosa of 30 patients infected with either *H. pylori* *cagM*-positive or *cagM*-negative strains was examined in order to analyze the induction of these mediators according to *cag* PAI status compared to normal gastric mucosa. All the *cagM*-positive strains were positive for *cagA*, and all the *cagM*-negative strains

were negative for *cagA*. CXCL2, CXCL3, CXCL5, CXCL8, and BD2 mRNA expression was significantly higher in *H. pylori* *cagM*-positive mucosa than in normal mucosa, but CXCL1 mRNA levels in noninfected and infected patients were similar (Fig. 5). In patients infected with *cagM*-negative strains, only CXCL2, CXCL5, and CXCL8 mRNA expression significantly increased compared to that in normal gastric mucosa.

## DISCUSSION

In this study, a novel and reproducible experimental protocol based on sequential enzymatic digestions was developed to isolate gastric epithelial cells from human stomach samples generated after sleeve gastrectomy. Isolation and expansion of PGEC has always been difficult, partially due to the fragility of these cells and to the lack of sufficient quantities of gastric material. Gastric sleeve operations allow the recovery of the large portions of normal gastric tissue needed to obtain a high cell yield. However, several attempts and optimization steps were needed before it was possible to define and set up culture conditions conducive to growth of the PGEC. Other cellular models, most of them derived from gastric adenocarcinoma, such as the AGS, KATO-3, and MKN-45 cell lines, had been employed to study the cellular changes triggered by *H. pylori*. However, because of their tumoral origin, they may not

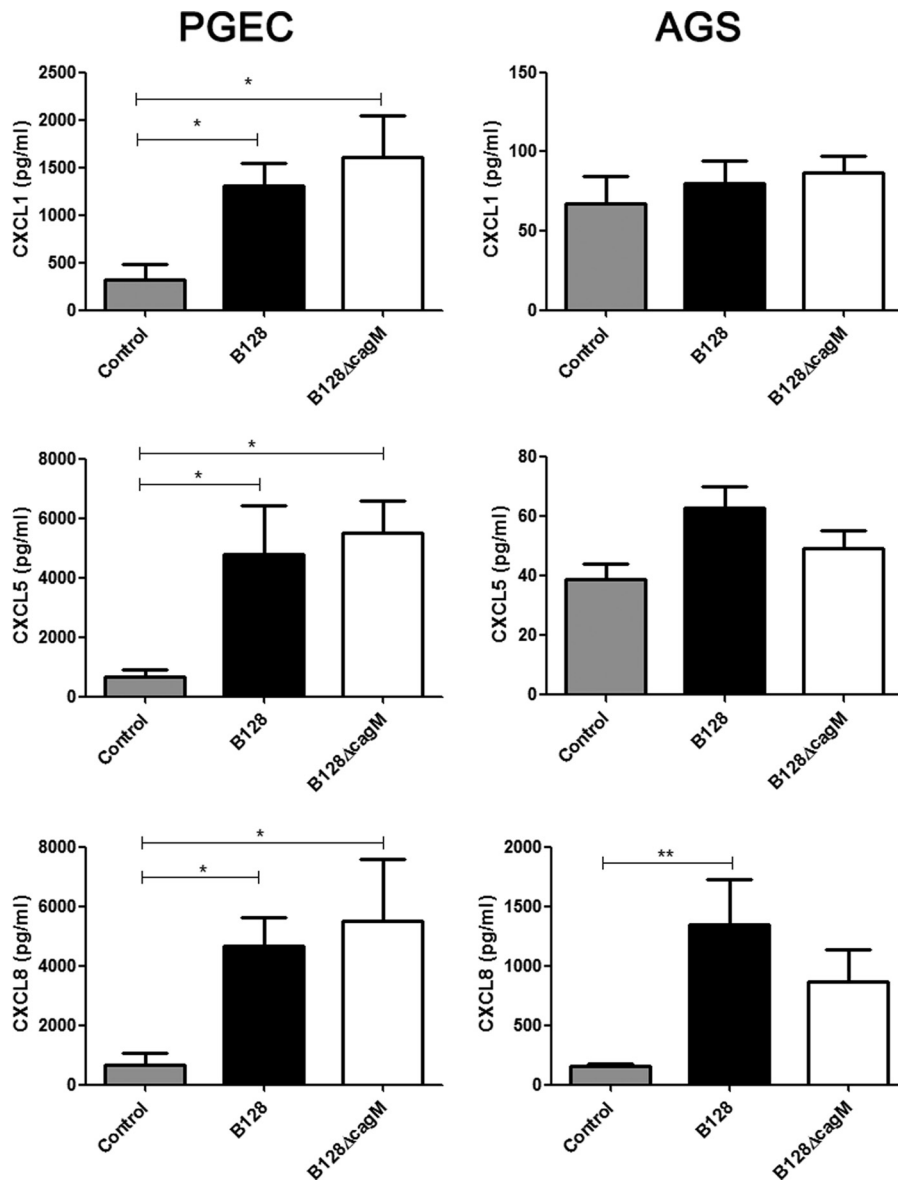


FIG 4 CXCL1, CXCL5, and CXCL8 production by PGEC and AGS cells after 24 h infection with *H. pylori* B128 and B128ΔcagM at an MOI of 100. Protein concentrations were measured in culture supernatants of independent cell cultures from 7 different patients by ELISAs. Data are means and SEM. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

be considered bona fide counterparts of the normal gastric epithelial cells required in study of *H. pylori* response. The PGEC obtained with our protocol were mucus-secreting cells as shown by immunodetection of MUC1, MUC5AC, and TFF-1, highly glycosylated proteins specifically present in mucous cells of the antrum and the fundus (20, 21). PGEC stained negative for MUC6, whose expression is known to be localized in the lower part of the gastric pits (22). It was shown that gastric epithelial cells specifically express only one of the MUC5AC and MUC6 mucins and that *H. pylori* is very closely associated with epithelial cells that produce MUC5AC (22). These findings suggest that PGEC are relevant for study of *H. pylori* infection. Compared to AGS cells, PGEC expressed higher levels of the proteins MUC1 and TFF1, both of which play an important role in regulation of inflammatory signaling (23, 24). Most notably, it was shown that MUC1 regulates

CXCL8 production by gastric epithelial cells in response to *H. pylori* (23). The aim of this work was to provide a comparative inflammatory profile study involving PGEC and AGS cells after *H. pylori* infection.

Induction by *H. pylori* of PGEC expression of CXCL1 to -3, CXCL5, CXCL8, CCL20, TNF- $\alpha$ , and BD2 was more pronounced with the wild-type strain than with the CagM mutant at 3 h postinfection, whereas this effect was not observed after 24 h of infection. This finding suggests that the *H. pylori* TIVSS plays a major role in the early induction of these inflammatory mediators during infection. In AGS cells, significant induction of CXCL5 and CXCL8 mRNA expression was the only induction observed following 3 h of stimulation with *H. pylori* B128. These results suggest that PGEC were more reactive to *H. pylori* cag PAI than AGS cells. However, production by PGEC of similar levels of CXCL1,



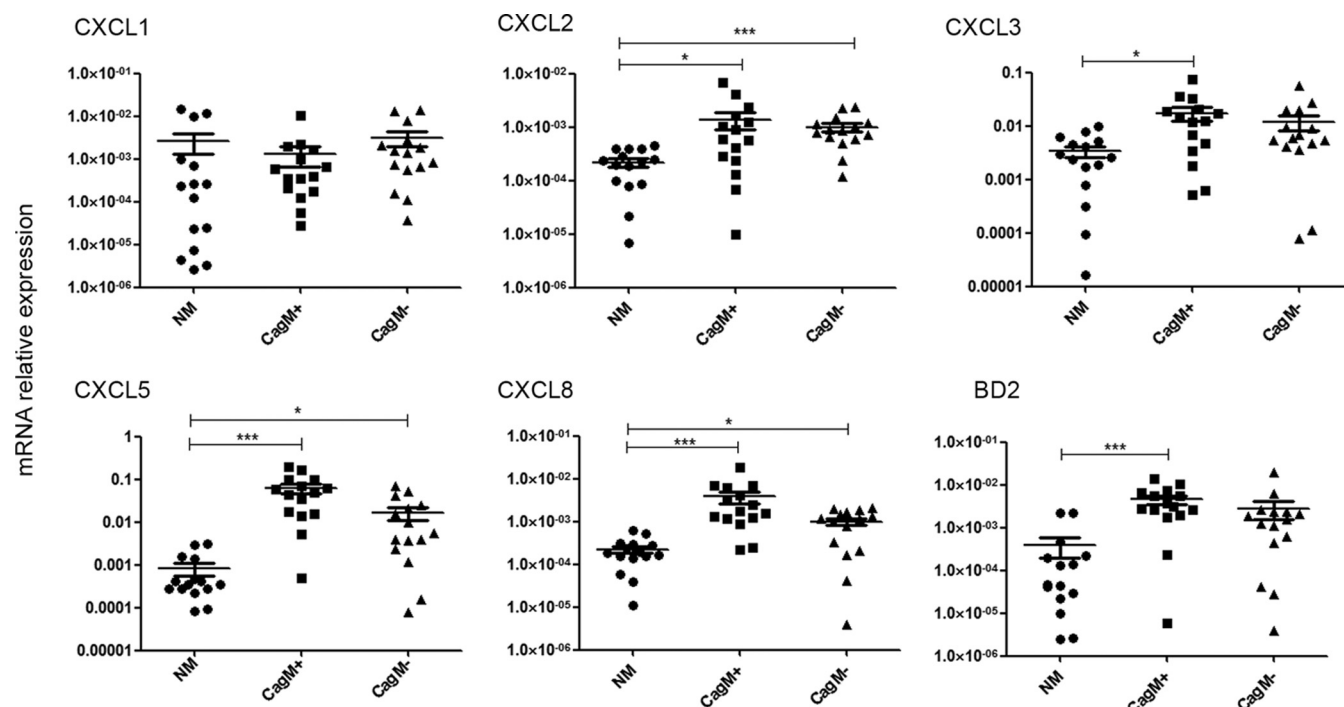


FIG 5 CXCL1 to 3, CXCL5, CXCL8 and BD2 mRNA expression in 15 normal mucosa (NM) and in 15 CagM-positive and 15 CagM-negative gastric mucosa from patients with *H. pylori* gastritis. mRNA expression levels were normalized using housekeeping genes and expressed as relative expression. Error bars indicate the SEM. \*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$ .

CXCL5, and CXCL8 following stimulation with *H. pylori* B128 and B128 $\Delta$ cagM at 24 h postinfection suggest that other virulence factors are involved in chemokines production, and that they compensate for the early mRNA induction specifically associated with functional *cag* PAI. The similar levels of chemokines mRNA induced by both *H. pylori* strains after 24 h of stimulation support this hypothesis. In addition, results of EGFR inhibition in PGEC show that the EGFR pathway is involved in chemokine induction by both *H. pylori* strains, suggesting a *cag* PAI-independent mechanism. In agreement, we showed that CXCL5 and CXCL8 mRNA expression was significantly higher in the gastric mucosa of patients infected with *H. pylori* compared to normal mucosa, but no significant difference was observed between patients infected with *cagA*-positive strains and those infected with *cagA*-negative strains. CXCL1, -2, -3, -5, and -8 proteins are neutrophil-activating chemokines contributing to establishment of a chemotactic gradient during inflammation, and CCL20 plays an important role in the homing of lymphocytes and dendritic cells to sites of inflammation. Infection with *H. pylori* induces production of a large panel of chemokines by PGEC that leads to infiltration toward the gastric mucosa of inflammatory cells involved in the pathophysiological process of the infection. Thus, these cells can allow the development of relevant *ex vivo* coculture models, for example with dendritic cells or lymphocytes, as they express CCL20 in response to *H. pylori* infection.

CXCL8 expression during *H. pylori* infection has long been described as being directly linked to CagA translocation into epithelial cells (25, 26). Actually, CagA is not essential for induction of this chemokine since CagA-deficient strains of *H. pylori* can likewise induce CXCL8 production by several cell lines at levels similar to those of wild-type strains (1, 27). In fact, it is the pres-

ence of a functional TIVSS that plays a major role in the induction of CXCL8 and other proinflammatory cytokines during *H. pylori* infection through NF- $\kappa$ B or AP-1 activation (28). Several *cag* PAI-dependent but *cagA*-independent NF- $\kappa$ B activation mechanisms have recently been suggested, involving at least six different signaling pathways (28). One of them is the internal recognition of translocated PG through TIVSS by the Nod1 protein, which activates NF- $\kappa$ B signaling and thereby induces CXCL8 production (16). An alternative explanation for chemokine production by gastric epithelial cells stimulated by B128 $\Delta$ cagM strain is that since TIVSS promotes intimate interactions between the bacteria and epithelial cells, endocytosis of some *H. pylori* products, such as PG, is facilitated. This hypothesis was established by Viala et al. to explain detection of radioactively labeled PG inside AGS cells exposed to  $\Delta$ cagM *H. pylori* mutants (16). Furthermore, it seems that *H. pylori* can ensure PG translocation inside host cells through the outer membrane vesicles that are constantly released by the bacterium (29). Recently, it was suggested that CagL, via interactions with host integrins, can trigger proinflammatory responses independently of CagA translocation or NOD1 signaling (30). As a result, the TIVSS apparatus *per se* could elicit host proinflammatory responses independently of its substrates (30). Finally, chemokine production during *H. pylori* infection could also be induced by other bacterial virulence factors. For example, Beswick et al. showed that the interaction of urease with the surface receptor CD74 can activate NF- $\kappa$ B, which induces CXCL8 production (31). Smith et al. reported that TLR2 and TLR5 are required for *H. pylori*-induced NF- $\kappa$ B activation and chemokine expression by epithelial cells (5). It has also been suggested that *H. pylori*-induced expression of TLR2 and TLR5 can qualitatively shift *cag* PAI-dependent to *cag* PAI-independent proinflamma-

tory signaling pathways, leading to CXCL8 production in HEK293 cells transfected with TLR2 and TLR5 (32). The TLR2 ligand of *H. pylori* is controversial. Indeed, it has also been suggested that lipopolysaccharide functions as a TLR2 ligand and induces CXCL1 to -3 and CCL20 expression in MKN45 cells (33). Yet another study suggested that *H. pylori* LPS does not activate TLR2 even at high concentrations of LPS (34). On the other hand, *H. pylori* heat shock protein 60 has been reported to induce CXCL8 via a TLR2 pathway in monocytes (35). In addition, EGFR seems to play an important role in the inflammatory reaction of PGEC; this pathway can be activated by *H. pylori* independently of the presence of a functional *cag* PAI, as previously reported (36). Our data suggest that gastric epithelial cells can secrete chemokines via *cag* PAI-independent pathways, which have been shown to play a major role in *H. pylori*-induced delayed inflammatory response.

Furthermore, expression of antimicrobial peptides in response to *H. pylori* has been investigated as part of the innate response and defense upon infection. Early *cag* PAI-dependent BD2 mRNA expression was observed in PGEC infected with *H. pylori*, whereas no significant induction of this peptide was observed in AGS cells. In agreement with this result, BD2 mRNA expression was higher in the gastric mucosa of patients infected with *cagA*-positive strains than in that of patients infected with *cagA*-negative strains, a finding suggesting *cag* PAI-dependent induction of this peptide. BD2 was shown to be induced in the gastric epithelial tissues during *H. pylori* infection (37). Furthermore, *cag* PAI-dependent induction of BD2 was also observed in MKN45 cells infected with *H. pylori* (38). The binding of BD2 on the surface of *H. pylori* cells was recently shown (39), and it is a phenomenon that may explain the absence of detection of BD2 proteins in the cell culture supernatants. Our results support the view that BD2 is expressed in response to early and chronic *H. pylori* infection and that the *cag* PAI is involved in this antimicrobial peptide mRNA induction.

This new culture model of primary human gastric epithelial cells using pieces of stomach enabled the development of an *H. pylori* infection model and characterization of the inflammatory profile generated upon infection. The cellular response of PGEC was reproducible despite the different genetic backgrounds of the patients included in the study. Furthermore, the inflammatory response of PGEC upon infection with *H. pylori* is closer to the *in vivo* response characteristic of infected gastric mucosa than was that of previously described cell lines, whose response could be altered due to their tumoral physiology. The model also supported the hypothesis of involvement of the *H. pylori* TIVSS in eliciting an inflammatory response at early phases of infection. At a later phase, our data supported the view that inflammatory mediator expression and production are largely due to the *cag* PAI substrate independent virulence factors. This largely novel human gastric epithelial cell model could be used to investigate the interactions of stomach epithelium with inflammatory cells and to study other functional aspects of infection with *H. pylori* in order to better characterize the pathological processes associated with gastric infections.

#### ACKNOWLEDGMENTS

This study was supported in part by a grant from the French Ministry of Health (PHRC Pylorikine). Work in the Ivo G. Boneca laboratory was supported by an ERC Starting Grant (PGNfromSHAPetoVIR no. 202283).

We thank Emilie Peyrot and Damien Chassaing for technical assistance and Jeffrey Arsham for English revision of the paper.

There are no conflicts of interest to disclose.

#### REFERENCES

- Censini S, Lange C, Xiang Z, Crabtree JE, Ghiara P, Borodovsky M, Rappuoli R, Covacci A. 1996. *cag*, a pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated virulence factors. Proc. Natl. Acad. Sci. U. S. A. 93:14648–14653. <http://dx.doi.org/10.1073/pnas.93.25.14648>.
- Covacci A, Censini S, Bugnoli M, Petracca R, Burroni D, Macchia G, Massone A, Papini E, Xiang Z, Figura N, Rappuoli R. 1993. Molecular characterization of the 128-kDa immunodominant antigen of *Helicobacter pylori* associated with cytotoxicity and duodenal ulcer. Proc. Natl. Acad. Sci. U. S. A. 90:5791–5795. <http://dx.doi.org/10.1073/pnas.90.12.5791>.
- Fischer W, Puls J, Buhrdorf R, Gebert B, Odenbreit S, Haas R. 2001. Systematic mutagenesis of the *Helicobacter pylori* *cag* pathogenicity island: essential genes for CagA translocation in host cells and induction of interleukin-8. Mol. Microbiol. 42:1337–1348. <http://dx.doi.org/10.1046/j.1365-2958.2001.02714.x>.
- Fischer W. 2011. Assembly and molecular mode of action of the *Helicobacter pylori* Cag type IV secretion apparatus. FEBS J. 278:1203–1212. <http://dx.doi.org/10.1111/j.1742-4658.2011.08036.x>.
- Smith MF, Jr, Mitchell A, Li G, Ding S, Fitzmaurice AM, Ryan K, Crowe S, Goldberg JB. 2003. Toll-like receptor (TLR) 2 and TLR5, but not TLR4, are required for *Helicobacter pylori*-induced NF-kappa B activation and chemokine expression by epithelial cells. J. Biol. Chem. 278:32552–32560. <http://dx.doi.org/10.1074/jbc.M305536200>.
- Campos RV, Buchan AM, Meloche RM, Pederson RA, Kwok YN, Coy DH. 1990. Gastrin secretion from human antral G cells in culture. Gastroenterology 99:36–44.
- Basque JR, Chailier P, Perreault N, Beaulieu JF, Menard D. 1999. A new primary culture system representative of the human gastric epithelium. Exp. Cell Res. 253:493–502. <http://dx.doi.org/10.1006/excr.1999.4711>.
- Smoot DT, Sewchand J, Young K, Desbordes BC, Allen CR, Naab T. 2000. A method for establishing primary cultures of human gastric epithelial cells. Methods Cell Sci. 22:133–136. <http://dx.doi.org/10.1023/A:1009846624044>.
- Heczko U, Smith VC, Mark Meloche R, Buchan AM, Finlay BB. 2000. Characteristics of *Helicobacter pylori* attachment to human primary antral epithelial cells. Microbes Infect. 2:1669–1676. [http://dx.doi.org/10.1016/S1286-4579\(00\)01322-8](http://dx.doi.org/10.1016/S1286-4579(00)01322-8).
- Richter-Dahlfors A, Heczko U, Meloche RM, Finlay BB, Buchan AM. 1998. *Helicobacter pylori*-infected human antral primary cell cultures: effect on gastrin cell function. Am. J. Physiol. 275:393–401.
- Basque JR, Menard D. 2000. Establishment of culture systems of human gastric epithelium for the study of pepsinogen and gastric lipase synthesis and secretion. Microsc. Res. Tech. 48:293–302. [http://dx.doi.org/10.1002/\(SICI\)1097-0029\(20000301\)48:5<293::AID-JEMT6>3.0.CO;2-A](http://dx.doi.org/10.1002/(SICI)1097-0029(20000301)48:5<293::AID-JEMT6>3.0.CO;2-A).
- Tetreault MP, Chailier P, Rivard N, Menard D. 2005. Differential growth factor induction and modulation of human gastric epithelial regeneration. Exp. Cell Res. 306:285–297. <http://dx.doi.org/10.1016/j.yexcr.2005.02.019>.
- Bäckhed F, Rokbi B, Torstensson E, Zhao Y, Nilsson C, Seguin D, Normark S, Buchan AM, Richter-Dahlfors A. 2003. Gastric mucosal recognition of *Helicobacter pylori* is independent of Toll-like receptor 4. J. Infect. Dis. 187:829–836. <http://dx.doi.org/10.1086/367896>.
- Lai YP, Yang JC, Lin TZ, Lin JT, Wang JT. 2006. *Helicobacter pylori* infection and CagA protein translocation in human primary gastric epithelial cell culture. Helicobacter 11:451–459. <http://dx.doi.org/10.1111/j.1523-5378.2006.00438.x>.
- Peek RM, Jr, Thompson SA, Donahue JP, Tham KT, Atherton JC, Blaser MJ, Miller GG. 1998. Adherence to gastric epithelial cells induces expression of a *Helicobacter pylori* gene, *iceA*, that is associated with clinical outcome. Proc. Assoc. Am. Physicians 110:531–544.
- Viala J, Chaput C, Boneca IG, Cardona A, Girardin SE, Moran AP, Athman R, Memet S, Huerre MR, Coyle AJ, DiStefano PS, Sansonetti PJ, Labigne A, Bertin J, Philpott DJ, Ferrero RL. 2004. Nod1 responds to peptidoglycan delivered by the *Helicobacter pylori* *cag* pathogenicity island. Nat. Immunol. 5:1166–1174. <http://dx.doi.org/10.1038/nri1131>.
- Buruco C, Garnier M, Silvain C, Fauchere JL. 2008. Quadruplex

- real-time PCR assay using allele-specific scorpion primers for detection of mutations conferring clarithromycin resistance to *Helicobacter pylori*. *J. Clin. Microbiol.* 46:2320–2326. <http://dx.doi.org/10.1128/JCM.02352-07>.
18. Ben Mansour K, Fendri C, Zribi M, Masmoudi A, Labbene M, Fillali A, Ben Mami N, Najjar T, Meherzi A, Sfar T, Burucoa C. 2010. Prevalence of *Helicobacter pylori* vacA, cagA, iceA and oipA genotypes in Tunisian patients. *Ann. Clin. Microbiol. Antimicrob.* 9:10. <http://dx.doi.org/10.1186/1476-0711-9-10>.
  19. Jenks PJ, Megraud F, Labigne A. 1998. Clinical outcome after infection with *Helicobacter pylori* does not appear to be reliably predicted by the presence of any of the genes of the cag pathogenicity island. *Gut* 43:752–758. <http://dx.doi.org/10.1136/gut.43.6.752>.
  20. Gott P, Beck S, Machado JC, Carneiro F, Schmitt H, Blin N. 1996. Human trefoil peptides: genomic structure in 21q22.3 and coordinated expression. *Eur. J. Hum. Genet.* 4:308–315.
  21. Guyonnet Duperat V, Audie JP, Debailleul V, Laine A, Buisine MP, Galiegue-Zouitina S, Pigny P, Degand P, Aubert JP, Porchet N. 1995. Characterization of the human mucin gene MUC5AC: a consensus cysteine-rich domain for 11p15 mucin genes? *Biochem. J.* 305:211–219.
  22. Van den Brink GR, Tytgat KM, Van der Hulst RW, Van der Loos CM, Einerhand AW, Buller HA, Dekker J. 2000. *H. pylori* colocalises with MUC5AC in the human stomach. *Gut* 46:601–607. <http://dx.doi.org/10.1136/gut.46.5.601>.
  23. Sheng YH, Triyana S, Wang R, Das I, Gerloff K, Florin TH, Sutton P, McGuckin MA. 2013. MUC1 and MUC13 differentially regulate epithelial inflammation in response to inflammatory and infectious stimuli. *Mucosal Immunol.* 6:557–568. <http://dx.doi.org/10.1038/mi.2012.98>.
  24. Soutto M, Belkhir A, Piazuolo MB, Schneider BG, Peng D, Jiang A, Washington MK, Kokoye Y, Crowe SE, Zaika A, Correa P, Peek RM, Jr, El-Rifai W. 2011. Loss of TFF1 is associated with activation of NF-kappaB-mediated inflammation and gastric neoplasia in mice and humans. *J. Clin. Invest.* 121:1753–1767. <http://dx.doi.org/10.1172/JCI43922>.
  25. Crabtree JE, Covacci A, Farmery SM, Xiang Z, Tompkins DS, Perry S, Lindley IJ, Rappuoli R. 1995. *Helicobacter pylori* induced interleukin-8 expression in gastric epithelial cells is associated with CagA positive phenotype. *J. Clin. Pathol.* 48:41–45. <http://dx.doi.org/10.1136/jcp.48.1.41>.
  26. Kim SY, Lee YC, Kim HK, Blaser MJ. 2006. *Helicobacter pylori* CagA transfection of gastric epithelial cells induces interleukin-8. *Cell. Microbiol.* 8:97–106. <http://dx.doi.org/10.1111/j.1462-5822.2005.00603.x>.
  27. Sharma SA, Tummuru MK, Miller GG, Blaser MJ. 1995. Interleukin-8 response of gastric epithelial cell lines to *Helicobacter pylori* stimulation *in vitro*. *Infect. Immun.* 63:1681–1687.
  28. Backert S, Naumann M. 2010. What a disorder: proinflammatory signaling pathways induced by *Helicobacter pylori*. *Trends Microbiol.* 18:479–486. <http://dx.doi.org/10.1016/j.tim.2010.08.003>.
  29. Kaparakis M, Turnbull L, Carneiro L, Firth S, Coleman HA, Parkinson HC, Le Bourhis L, Karrar A, Viala J, Mak J, Hutton ML, Davies JK, Crack PJ, Hertzog PJ, Philpott DJ, Girardin SE, Whitchurch CB, Ferrero RL. 2010. Bacterial membrane vesicles deliver peptidoglycan to NOD1 in epithelial cells. *Cell. Microbiol.* 12:372–385. <http://dx.doi.org/10.1111/j.1462-5822.2009.01404.x>.
  30. Gorrell RJ, Guan J, Xin Y, Tafreshi MA, Hutton ML, McGuckin MA, Ferrero RL, Kwok T. 2012. A novel NOD1- and CagA-independent pathway of interleukin-8 induction mediated by the *Helicobacter pylori* type IV secretion system. *Cell. Microbiol.* 15:554–570. <http://dx.doi.org/10.1111/cmi.12055>.
  31. Beswick EJ, Pinchuk IV, Minch K, Suarez G, Sierra JC, Yamaoka Y, Reyes VE. 2006. The *Helicobacter pylori* urease B subunit binds to CD74 on gastric epithelial cells and induces NF-kappaB activation and interleukin-8 production. *Infect. Immun.* 74:1148–1155. <http://dx.doi.org/10.1128/IAI.74.2.1148-1155.2006>.
  32. Kumar Pachathundikandi S, Brandt S, Madassery J, Backert S. 2011. Induction of TLR-2 and TLR-5 expression by *Helicobacter pylori* switches cagPAI-dependent signalling leading to the secretion of IL-8 and TNF-alpha. *PLoS One* 6:e19614. <http://dx.doi.org/10.1371/journal.pone.0019614>.
  33. Smith SM, Moran AP, Duggan SP, Ahmed SE, Mohamed AS, Windle HJ, O'Neill LA, Kelleher DP. 2011. Tribbles 3: a novel regulator of TLR2-mediated signaling in response to *Helicobacter pylori* lipopolysaccharide. *J. Immunol.* 186:2462–2471. <http://dx.doi.org/10.4049/jimmunol.1000864>.
  34. Cullen TW, Giles DK, Wolf LN, Ecobichon C, Boneca IG, Trent MS. 2011. *Helicobacter pylori* versus the host: remodeling of the bacterial outer membrane is required for survival in the gastric mucosa. *PLoS Pathog.* 7:e1002454. <http://dx.doi.org/10.1371/journal.ppat.1002454>.
  35. Zhao Y, Yokota K, Ayada K, Yamamoto Y, Okada T, Shen L, Oguma K. 2007. *Helicobacter pylori* heat-shock protein 60 induces interleukin-8 via a Toll-like receptor (TLR)2 and mitogen-activated protein (MAP) kinase pathway in human monocytes. *J. Med. Microbiol.* 56:154–164. <http://dx.doi.org/10.1099/jmm.0.46882-0>.
  36. Wallasch C, Crabtree JE, Bevec D, Robinson PA, Wagner H, Ullrich A. 2002. *Helicobacter pylori*-stimulated EGF receptor transactivation requires metalloprotease cleavage of HB-EGF. *Biochem. Biophys. Res. Commun.* 295:695–701. [http://dx.doi.org/10.1016/S0006-291X\(02\)00740-4](http://dx.doi.org/10.1016/S0006-291X(02)00740-4).
  37. Bajaj-Elliott M, Fedeli P, Smith GV, Domizio P, Maher L, Ali RS, Quinn AG, Farthing MJ. 2002. Modulation of host antimicrobial peptide (beta-defensins 1 and 2) expression during gastritis. *Gut* 51:356–361. <http://dx.doi.org/10.1136/gut.51.3.356>.
  38. Wada A, Mori N, Oishi K, Hojo H, Nakahara Y, Hamanaka Y, Nagashima M, Sekine I, Ogushi K, Niidome T, Nagatake T, Moss J, Hirayama T. 1999. Induction of human beta-defensin-2 mRNA expression by *Helicobacter pylori* in human gastric cell line MKN45 cells on cag pathogenicity island. *Biochem. Biophys. Res. Commun.* 263:770–774. <http://dx.doi.org/10.1006/bbrc.1999.1452>.
  39. Nuding S, Gersemann M, Hosaka Y, Konietzny S, Schaefer C, Beisner J, Schroeder BO, Ostaff MJ, Saigenji K, Ott G, Schaller M, Stange EF, Wehkamp J. 2013. Gastric antimicrobial peptides fail to eradicate *Helicobacter pylori* infection due to selective induction and resistance. *PLoS One* 8:e73867. <http://dx.doi.org/10.1371/journal.pone.0073867>.