CCL28 Is Increased in Human Helicobacter pylori-Induced Gastritis and Mediates Recruitment of Gastric Immunoglobulin A-Secreting Cells

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Helicobacter pylori infection gives rise to an active chronic gastritis and is a major risk factor for the development of duodenal ulcer disease and gastric adenocarcinoma. The infection is accompanied by a large accumulation of immunoglobulin A (IgA)-secreting cells in the gastric mucosa, and following mucosal immunization only H. pylori-infected volunteers mounted a B-cell response in the gastric mucosa. To identify the signals for recruitment of gastric IgA-secreting cells, we investigated the gastric production of CCL28 (mucosa-associated epithelial chemokine) and CCL25 (thymus-expressed chemokine) in H. pylori-infected and uninfected individuals and the potential of gastric B-cell populations to migrate toward these chemokines. Gastric tissue from H. pylori-infected individuals contained significantly more CCL28 protein and mRNA than that from uninfected individuals, while CCL25 levels remained unchanged. Chemokine-induced migration of gastric lamina propria lymphocytes isolated from patients undergoing gastric resection was then assessed using the Transwell system. IgA-secreting cells and IgA+ memory B cells from H. pylori-infected tissues migrated toward CCL28 but not CCL25, while the corresponding cells from uninfected patients did not. Furthermore, IgG-secreting cells from H. pylori-infected patients did not migrate to CCL28 but instead to CXCL12 (SDF-1α). However, chemokine receptor expression did not correlate to the migratory pattern of the different B-cell populations. These studies are the first to show increased CCL28 production during gastrointestinal infection in humans and provide an explanation for the large influx of IgA-secreting cells to the gastric mucosa in H. pylori-infected individuals.

Helicobacter pylori is a gram-negative bacterium that infects the human stomach and duodenum and gives rise to active chronic gastritis including the formation of lymphoid follicles (15, 20). The infection is widespread and is associated with the development of gastric and duodenal ulcer disease as well as gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma. The H. pylori-infected human stomach mucosa contains increased numbers of neutrophils, macrophages, dendritic cells, T cells, and B cells (5, 11, 15). In particular, H. pylori infection gives rise to a large accumulation of immunoglobulin A (IgA)-secreting cells in the gastric mucosa, many of which are specific for H. pylori virulence factors (28). In parallel, a systemic IgG and IgA response is mounted. However, despite strong immune responses, the bacteria are rarely eliminated from the stomach, and the infection is usually lifelong. As a prerequisite for the development of a vaccine against H. pylori, we have previously investigated the migration of vaccine-specific antibody-secreting cells (ASC) to the stomach mucosa following mucosal immunization with an inactivated cholera vaccine (27, 38). In these studies, vaccine-specific IgA and sometimes IgG responses could be detected only in the gastric mucosae of H. pylori-infected individuals and not in those of uninfected individuals. In contrast, both groups displayed similar and robust responses to the vaccine in the upper small intestine. It is not yet known whether the lymphoid follicles formed during H. pylori-induced gastritis can support local antigen presentation and B-cell differentiation to antibody-secreting plasma cells. We could show, however, that gastric IgA responses were not dependent on local antigen uptake and processing but were caused by increased recruitment of circulating plasma cell precursors (38).

Tissue-specific lymphocyte homing to gastrointestinal mucosal tissues is dependent on the expression of the mucosal homing receptor integrin α4β7 (8). α4β7 interacts with the mucosal addressin cellular adhesion molecule-1 (MAdCAM-1), which is expressed by endothelial cells in Peyer’s patches and the gastrointestinal mucosa (4, 7). Our previous studies have shown that B and T lymphocytes activated by antigens present on the gastric mucosa express integrin α4β7 (37). Furthermore, animal experiments demonstrate that α4β7-MAdCAM-1 interactions are necessary for vaccine-induced protection against H. pylori infection (30). However, our work also showed that MAdCAM-1 was similarly expressed on gastric endothelial cells from both H. pylori-infected and uninfected individuals (37), and thus MAdCAM-1 density could not explain the recruitment of IgA-secreting cells to H. pylori-infected stomach mucosa.

In addition to adhesion molecules, chemokines play an important role in leukocyte trafficking to different organs (9, 22).

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The mucosa-associated epithelial chemokine CCL28 (mucosa-associated epithelial chemokine) is a common mucosal chemokine which is constitutively expressed by epithelial cells in most mucosal sites (34, 40). The second mucosal chemokine, CCL25 (thymus-expressed chemokine), on the other hand, is mainly expressed by epithelial cells in the small intestine (23, 35, 41). Although produced by epithelial cells, these chemokines are enriched by endothelial cells and presented to migrating lymphocytes on the apical side (18, 22). Both CCL28 and CCL25 have recently been shown to be essential for lymphocyte migration to gastrointestinal tissues. CCL28 attracts IgA ASC, but not IgG or IgM ASC, from both intestinal and extraintestinal mucosal tissue, while CCL25 preferentially attracts IgA ASC from the small intestine and its draining lymphoid tissues, as well as α4β7+ T cells (6, 18, 25, 35, 41). Since α4β7+/MAdCAM-1 interactions did not seem to explain the increased B-cell migration to the H. pylori-infected gastric mucosa, we hypothesized that it might instead be mediated by altered chemokine production. These considerations prompted us to investigate the gastric production of CCL25 and CCL28 in H. pylori-infected and uninfected individuals, as well as the potential of gastric ASC and memory B cells to migrate toward these chemokines.

MATERIALS AND METHODS

Volunteers, patients, and specimen collection. This study was performed following approval from the human research ethics committee of the Medical Faculty, Göteborg University, and all participants gave informed consent to participate. Eight H. pylori-infected volunteers (two females and six males, aged 26 to 60 years) and eight uninfected volunteers (three females and five males, aged 24 to 34 years) were recruited from blood donors at Sahlgrenska University Hospital by serological screening. H. pylori infection was subsequently confirmed or excluded by culture on Scirrow plates and serology (16). Ten antrum biopsy samples were collected from each volunteer by endoscopy.

Three biopsy samples were immediately embedded in OCT compound (Tissue-Tek; Sakura Finetek, Zoeterwoude, The Netherlands) and frozen in liquid nitrogen for immunofluorescence analysis, two biopsy samples were immediately frozen in liquid nitrogen for RNA purification, and four biopsy samples were collected on ice for protein extraction. The last biopsy sample was fixed in formalin, and gastritis and the presence of Helicobacter-like organisms (HLO) were graded by an experienced histopathologist using the updated Sydney system (12).

Gastric tissues from 14 individuals (7 females and 7 males, aged 30 to 80 years) undergoing gastric resection due to gastric adenocarcinoma (n = 7), severe gastric dysplasia (n = 1), duodenal adenocarcinoma (n = 1), bile duct carcinoma (n = 1), endocrine gastric stromal tumor (GIST; n = 1), pancreatic carcinoma (n = 2), or chronic pancreatitis (n = 1) were used to isolate gastric lymphocytes for migration experiments. Directly after gastrectomy, a strip of gastric tissue encompassing antrum and corpus mucosa was collected. In patients undergoing gastrectomy as previously described (26). Briefly, the epithelium was removed by incubation in Hanks’ balanced salt solution containing EDTA and dithiothreitol. Thereafter, the remaining tissue was incubated for 2 h at 37°C in collagenase and DNase to release LPMNC. The concentration and integrity of the RNA were measured by use of NanoDrop and by gel electrophoresis. cDNA was synthesized using 600 ng total RNA and oligo(dT) primers with the Omniscript RT-PCR kit (Qiagen, Hilden, Germany) in a total volume of 20 μl, as described by the manufacturer. The cDNA was stored at −20°C. CCL25 primers were designed by Primer3 software (forward primer, CCATCGTGGCCTTGGCTGTCTGTG; reverse primer, GCCGTATGTTCCGCTTGCCTG). CCL25 and hypoxanthine phosphoribosyltransferase (HPRT) primers were used as previously described (34, 36). All primers were ordered from MGW-biotech (Ebersberg, Germany). RT-PCR was performed by multiplex PCR using either the CCL25 or CCL28 primers in combination with the HPRT primers according to standard procedures for 35 cycles. The expression of CCL25 and CCL28 was expressed as the ratio of the optical density of gastric ASC and memory B cells to migrate toward these chemokines.

Immunofluorescence detection of CCL28. The expression of CCL28 was detected using immunofluorescent staining. Cryosect tissue sections (8 μm) from three biopsy samples from every volunteer were fixed in ice-cold acetone. Endogenous peroxidase was blocked with glucose oxidase (Sigma-Aldrich) followed by blocking of biotin in the tissue ( Molecular Probes, Invitrogen, Carlsbad, CA). Thereafter, the slides were incubated with mouse IgG anti-CCL28 (R&D Systems) or with mouse IgG1 as the negative control. Primary antibodies were used at optimal dilutions in phosphate-buffered saline with 0.05% Tween at room temperature for 1 h. The samples were then incubated with goat anti-mouse IgG1 conjugated to AlexaFluor 594 (Molecular Probes) followed by detection using tyramide amplification. Finally, slides were mounted using a DAPI (4′,6′-diamidino-2-phenylindole)-containing mounting medium.

Detection of CCL25 and CCL28 mRNA in gastric tissue samples. The expression of CCL25 and CCL28 mRNA in gastric tissues was assessed by reverse transcriptase PCR (RT-PCR). Total RNA was purified by use of a total RNA extraction kit for mammalian RNA (Sigma Aldrich) and DNase treated by use of a DNase 1 kit and DNase I digestion. RNA was used to remove residual genomic DNA. The concentration and integrity of the RNA were measured by use of NanoDrop and by gel electrophoresis. cDNA was synthesized using 600 ng total RNA and oligo(dT) primers with the Omniscript RT-PCR kit (Qiagen, Hilden, Germany) in a total volume of 20 μl, as described by the manufacturer. The cDNA was stored at −20°C. CCL25 primers were designed by Primer3 software (forward primer, CCATCGTGGCCTTGGCTGTCTGTG; reverse primer, GCCGTATGTTCCGCTTGCCTG). CCL25 and hypoxanthine phosphoribosyltransferase (HPRT) primers were used as previously described (34, 36). All primers were ordered from MGW-biotech (Ebersberg, Germany). RT-PCR was performed by multiplex PCR using either the CCL25 or CCL28 primers in combination with the HPRT primers according to standard procedures for 35 cycles. The expression of CCL25 and CCL28 was expressed as the ratio of the optical density of gastric ASC and memory B cells to migrate toward these chemokines.

Isolation of gastric LPMNC. Lamina propria mononuclear cells (LPMNC) were isolated from gastric tissue from H. pylori-infected and uninfected patients undergoing gastrectomy as previously described (26). Briefly, the epithelium was removed by incubation in Hanks’ balanced salt solution containing EDTA and dithiothreitol. Thereafter, the remaining tissue was incubated for 2 h at 37°C in collagenase and DNase to release LPMNC, which were then run through a nylon mesh to remove remaining tissue fragments. Isolated LPMNC were resuspended in Iscove’s medium containing 5% fetal calf serum, 50 μg/ml of gentamicin, and 3 μg/ml of 1-glutamine at 105 cells/ml and were stained for flow cytometry analysis (see below) or kept overnight at 37°C in a humidified atmosphere containing 5% CO2 before migration assays.

Chemotaxis assay. B-cell migration toward CCL25, CCL28, and the positive chemotaxis agent CCL19 were measured using the transwell chemotaxis system (CCL25: 5 μg/ml, CCL28: 3 μg/ml, CCL19: 10 μg/ml). Cells were plated at a density of 1×10^5 cells/ml in RPMI-1640 medium with 0.5% fetal calf serum and 50 μg/ml of gentamicin. The upper chamber was loaded with 100 μl of cell suspension and the lower compartment contained 600 μl of medium, with or without CCL25 (5, 2.5, or 1.2 μg/ml), CCL28 (5, 2.5, or 1.2 μg/ml), or CCL19 (1.8 μg/ml). The experiments were performed in duplicate, and chemokine concentrations were determined by a protein assay kit (Bio-Rad, Hercules, CA). Total IgA concentrations in the tissue extracts were determined in ELISA as previously described (3). Purified human IgA was used to construct a standard curve, and the tissue extracts were diluted 100-fold before analysis.

Protein extraction from gastric tissue specimens. Four antral biopsy samples from each subject were incubated in 600 μl phosphate-buffered saline containing 2% saponin, 100 mg/ml soybean trypsin inhibitor, 350 mg/ml phenylmethylsulfonyl fluoride, and 0.1% bovine serum albumin (all from Sigma Aldrich, St Louis, MO) overnight at 4°C. Each suspension was centrifuged at 13,000 x g for 5 min, and the supernatants were collected and frozen at −70°C until used for chemokine analyses.

Detection of chemokines and antibodies in tissue extracts. The concentrations of CCL25 and CCL28 were determined by ELISA. CCL28 was determined using the Quantikine ELISA kit and thymus-expressed chemokine was determined using Duoset ELISA (both from R&D Systems, Abingdon, United Kingdom) according to the manufacturer’s instruction. The detection limit for CCL25 was 30 pg/ml and for CCL28 was 10 pg/ml. Chemokine concentrations were related to the total protein concentration in the respective samples, which were determined by a protein assay kit (Bio-Rad, Hercules, CA).
the IgA- and IgG-secreting cells by enzyme-linked immunospot assay (ELISPOT assay), and the rest was used for flow cytometry analysis.

Analysis of IgA and IgG ASC migration by ELISPOT assay. IgA- and IgG-secreting cells that migrated toward CCL28, CCL25, or CXCL12 were detected in two-color ELISPOT assays as previously described (10, 28). Wells were coated with goat antibodies to the F(ab)_2 fragment of human IgG (Jackson Immunoresearch Laboratories, Inc., West Grove, PA). Fifty microliters of cell suspension was added to each well and the experiments were performed in duplicate. Cells were incubated overnight at 37°C in a humidified atmosphere containing 5% CO_2. The assay was developed by the addition of horseradish peroxidase-conjugated goat antibodies to human IgA and alkaline phosphatase-conjugated goat antibodies to human IgG (Southern Biotech, Birmingham, AL) for 4 h followed by chromagen substrates. Frequencies of IgA and IgG ASC, represented by spots, were determined under low magnification with a stereomicroscope. Untreated LPMNC were assayed in parallel as a positive control, and incubation of these cells for 2.5 h with the respective chemokines did not influence ASC frequencies or the amounts of IgA secreted into the medium (data not shown).

Flow cytometry analyses. Half of the migrating cells collected from Transwell experiments were used to enumerate memory B cells by flow cytometry using true-count beads as previously described (21). Memory B cells were defined as small resting lymphocytes that had undergone isotype switching to IgA or IgG. In addition, most IgA^+ and IgG^+ cells coexpress CD27 (21), confirming their memory cell status. The expression of IgA and IgG on the cell surface was determined by fluorescein isothiocyanate-labeled rabbit anti-IgA and -IgG antibodies (Dako Cytomation, Solna, Sweden) and was combined with phycoerythrin-labeled anti-CD3, peridinin chlorophyll protein-labeled anti-CD19, and allophycocyanin (APC)-labeled anti-CD69 antibodies. Appropriate isotype control antibodies were used to determine unspecific staining. Cells were analyzed on a FACSCalibur using CellQuest and FlowJo software (Becton Dickinson, San Jose, CA).

In addition, chemokine receptor expression by IgA^+ and IgG^+ memory B cells and plasmablasts (identified as large CD3^−/H11006 CD38^hi^/H11006 cells located within mucosal lymphoid tissues) was examined. Chemokine receptor expression was visualized using a biotinylated mouse monoclonal antibody to CCR9 (clone 3D3, kindly provided by D. Picarella, Millennium Inc., Cambridge, MA) followed by APC-conjugated streptavidin, APC-labeled anti-CCR10, and anti-CXCR4 antibodies (R&D Systems).

Statistical analyses. Comparisons between H. pylori-infected and uninfected subjects were performed using the two-tailed Mann-Whitney U test. P values of <0.05 were considered statistically significant. Correlation was evaluated using the two-tailed Pearson test.

RESULTS

Inflammation and bacterial load. To investigate the expression of CCL25 and CCL28 in gastric tissues, biopsy samples were collected from both asymptomatic H. pylori-infected and uninfected individuals. Biopsy samples from uninfected subjects were histologically normal without inflammation or HLO. In contrast, active chronic inflammation and HLO were observed for biopsy samples from the antrum of all H. pylori-infected subjects. The mean chronic inflammation score was 1.9 ± 0.4 (mean ± standard deviation) and the mean active inflammation score was 1.1 ± 0.8. The mean HLO score was 1.9 ± 0.4. No atrophy or intestinal metaplasia was seen for any of the subjects.

CCL25 and CCL28 content in H. pylori-infected and uninfected gastric tissue. Total proteins were extracted from gastric biopsy samples collected from H. pylori-infected and uninfected individuals, and CCL25 and CCL28 levels were determined by ELISA. These analyses showed that gastric tissue from H. pylori-infected individuals contained significantly more (P < 0.001) CCL28 than did that from individuals not infected with H. pylori (Fig. 1A). On average, H. pylori-infected tissues contained 2.8 times more CCL28 than did uninfected tissues (33 ± 21 pg/mg protein compared to 12 ± 3 pg/mg). Since the H. pylori-infected group contained some individuals that were older than the uninfected volunteers, we also investigated if there was a correlation between CCL28 concentration and age in the H. pylori-infected group. This was, however, not the case (r = 0.457, P > 0.05). The CCL25 concentrations were more variable than CCL28, but there was no difference between the groups as a whole (Fig. 1B).

In order to localize CCL28 protein within the gastric mucosa, immunofluorescence staining of CCL28 was performed on frozen tissues from the same individuals. These analyses revealed CCL28 reactivity exclusively in the epithelia in all individuals except two uninfected volunteers for whom no CCL28 staining could be detected. The staining was, however, always more intense in the H. pylori-infected individuals (Fig. 2). The CCL28 staining was cytoplasmatic and found mainly in the deep zone of the antral glands in the uninfected volunteers. In the H. pylori-infected individuals, on the other hand, CCL28 was also detected in the epithelium of the neck region and the surface epithelium.

CCL25 and CCL28 mRNA in H. pylori-infected and uninfected gastric tissue. To validate the results for CCL25 and CCL28 protein expression, we used RT-PCR to examine CCL25 and CCL28 mRNA in gastric tissues from the same subjects and compared the results to those for the housekeeping gene HPRT. These assays showed a significantly high CCL28 expression (P < 0.001) in H. pylori-infected gastric tissue compared to uninfected tissue (Fig. 3A and B). In contrast, there was no difference between infected and uninfected tissue with regard to CCL25 expression (Fig. 3C).

FIG. 1. CCL28 and CCL25 concentrations in tissue extracts. Total proteins were extracted from gastric biopsy samples collected from H. pylori-infected (Hp+) and uninfected (Hp−) subjects. Biopsy samples were collected from infected and uninfected subjects. The concentration of CCL28 (A) and CCL25 (B) in the tissue extracts were determined by ELISA and related to the total protein concentration in the respective samples. Circles represent individual values and horizontal bars the median of each group. ***, P < 0.001.
IgA content in *H. pylori*-infected and uninfected gastric tissue. Total IgA concentrations were then determined by ELISA in the gastric tissue extracts previously used for chemokine detection. As previously reported (3), *H. pylori*-infected subjects had higher levels of gastric IgA than did the uninfected. On average, tissue extracts from *H. pylori*-infected subjects contained 19.3 ± 13.0 μg IgA per mg protein (mean ± standard deviation), and extracts from uninfected subjects contained 2.8 ± 0.7 μg/mg. Furthermore, when the IgA concentrations in the gastric extracts from infected individuals were plotted against CCL28 concentrations, there was a significant positive correlation ($r = 0.897$, $P < 0.01$) (Fig. 4).

Chemokine receptor expression on gastric B-cell subsets. The expression of CCR9, CCR10, and CXCR4, the receptor for CXCL12, on gastric B-cell subsets was determined using flow cytometry. Within the population of small, resting naïve, and memory gastric lymphocytes, 46% ± 10% were IgA$^+$ and 6% ± 4% IgG$^+$ in the infected individuals and 43% ± 28% IgA$^+$ and 12% ± 10% IgG$^+$ in uninfected subjects. More than half of the IgA$^+$ and IgG$^+$ memory B cells expressed CCR9, regardless of whether they were isolated from *H. pylori*-infected or uninfected tissue (Table 1). Furthermore, the majority of memory cells expressed CCR10, and there were no large differences between IgA$^+$ and IgG$^+$ cells or between infected and uninfected individuals (Table 1). The expression of CXCR4 was generally high for the memory B cells, with a somewhat higher expression for cells from *H. pylori*-infected patients.

Within the large-lymphocyte fraction, chemokine receptor expression was analyzed for plasmablasts, defined as large CD3$^-$ CD38$^+$ cells expressing IgA or IgG on the surface. A majority of cells expressed IgA in both *H. pylori*-infected and uninfected individuals (68% ± 10% in infected and 62% ± 9% in uninfected), whereas 7% ± 7% expressed IgG in the infected individuals and 6% ± 6% of cells did so in the uninfected. A majority of IgA$^+$ plasmablasts expressed CCR9, and there was also a very high expression of CCR10 on IgA$^+$ plasmablasts, with no difference between cells from infected and uninfected individuals (Table 1). CXCR4 expression was lower and more variable on plasmablasts than on memory cells, but no differences were seen between *H. pylori*-infected and uninfected volunteers. The expression of chemokine receptors on IgG$^+$ plasmablasts was hard to evaluate, since there were often frequencies of cells too low to allow proper analysis.

Chemokine-induced migration of gastric ASC. Previous studies have shown that *H. pylori* infection results in a large accumulation of IgA-secreting cells in the human gastric mucosa both during steady state and following mucosal immunizations (27, 38). We therefore examined if gastric cells from *H. pylori*-infected and uninfected subjects would respond to

FIG. 2. Immunofluorescent staining of CCL28 in gastric tissues. Gastric biopsy samples collected from *H. pylori*-infected and uninfected subjects were cryopreserved, stained with antibodies to CCL28, and mounted in a DAPI-containing mounting medium. Representative staining from one *H. pylori*-negative individual (A) and one *H. pylori*-infected individual (B and C) is shown. (D) Isotype control for the infected individual. CCL28 is shown in red, cell nuclei are in blue, and the scale bar indicates 50 μm. Arrows in panel A indicate CCL28 staining.
CCL25 or CCL28. LPMNC isolated from tissue collected at gastrectomy surgery were allowed to migrate in the Transwell system toward CCL25, CCL28, or the positive control, CXCL12 (SDF-1c), known to recruit IgG ASC (32), and ASC frequencies among the migrating cells were analyzed by ELISPOT assay.

Gastric IgA-secreting cells from all *H. pylori*-infected subjects migrated toward CCL28, while no such responses were seen among cells from uninfected individuals (*P* < 0.01 comparing infected and uninfected individuals) (Fig. 5A). In contrast, there was no IgG ASC response in any of the patient groups to CCL25 (Fig. 5B). Likewise, there was no response to CCL25 on any of the patient groups. Both IgA- and IgG-secreting gastric cells from *H. pylori*-infected, but not uninfected, individuals responded to the positive control, CXCL12, but only the IgG ASC response was significantly different (*P* < 0.05) between infected and uninfected patients (Fig. 5A and B).

**Chemokine-induced migration of gastric IgA+ and IgG+ memory B cells.** In parallel to ASC detection, we also analyzed the migration of IgA+ and IgG+ memory B cells from the same individuals by flow cytometry. IgA+ memory B cells from *H. pylori*-infected individuals responded significantly (*P* < 0.05) to CCL28 compared to uninfected subjects but not to the same extent as ASC (Fig. 6A). While the median response of IgA-secreting cell migration to CCL28 was more than 10-fold in relation to spontaneous migration, the IgA+ memory cells had only a 2-fold-increased response against CCL28.

In contrast, IgG+ memory B cells did not respond to CCL28 (Fig. 6B). Similar to what was seen for the ASC response, CCL25 did not induce any migration of memory B cells, except in two individuals. The positive control, CXCL12, induced a robust migration of both IgA+ and IgG+ memory B cells isolated from infected individuals. Gastric memory B cells from uninfected subjects, on the other hand, did not respond to CXCL12 (*P* < 0.05 and *P* < 0.01 compared to infected individuals) (Fig. 6A and B).

**DISCUSSION**

*Helicobacter pylori* infection results in a large accumulation of IgA-secreting cells in the human gastric mucosa both during steady state and following mucosal immunizations. The chemokines CCL28 and CCL25 have recently been shown to be essential for lymphocyte migration to gastrointestinal tissues (6, 18, 25, 35, 41), and we therefore evaluated their contribution to B-cell migration into *H. pylori*-infected gastric tissues.

Our results strongly suggest that CCL28 contributes to effector B-cell recruitment to the gastric mucosa in *H. pylori*-associated gastritis. We could show increased concentrations of CCL28 protein in gastric tissues from *H. pylori*-infected individuals, as well as increased CCL28 mRNA expression. However, with this limited material we cannot completely rule out the possibility that the age of the volunteers also contributed to the higher CCL28 production seen for *H. pylori*-infected individuals. It is worth noting that there is a baseline production of CCL28 in the stomach of *H. pylori*-negative individuals, confirming previous studies of CCL28 mRNA expression in human tissues (34, 40). Furthermore, immunofluorescence analyses showed increased epithelial expression of CCL28 in *H. pylori*-infected individuals. Total proteins were extracted from gastric biopsy samples collected from *H. pylori*-infected subjects. The concentrations of CCL28 and IgA in the tissue extracts were determined by ELISA and related to the total protein concentrations in the respective samples. Symbols represent individual values, and the line represents the best linear approximation of the relationship between the IgA and CCL28 concentrations. Pearson’s coefficient of correlation, *r* = 0.897.
pylori-infected subjects, but again a low baseline CCL28 expression was seen for most of the H. pylori-negative subjects. Therefore, these studies bring forward the concept that in parallel to being constitutively expressed at most mucosal surfaces, CCL28 production can also be induced by mucosal infections, thereby probably enabling the recruitment of additional IgA-secreting cells from the circulation. Indeed, even in this limited material, there was a correlation between CCL28 and IgA concentrations in the gastric tissue of H. pylori-infected subjects. The hypothesis of inducible CCL28 expression is further supported by a recent study by Ogawa et al. (33) showing increased CCL28 levels in inflamed colonic tissue from patients suffering from ulcerative colitis. In the case of H. pylori infection, it is not possible at this stage to determine if the increased CCL28 expression is a direct effect of H. pylori bacteria on epithelial cells or if proinflammatory mediators in the tissue influence CCL28 expression. Since the study by Ogawa et al. (33) demonstrated that both proinflammatory cytokines and bacteria can induce CCL28 in colonic cell lines, a combination of the two seems most likely.

### TABLE 1. CCR9, CCR10, and CXCR4 expression for gastric B-cell subsets isolated from gastric mucosae of H. pylori-infected and uninfected individuals

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<tr>
<th>Mucosa</th>
<th>% of indicated cell type (mean ± SD) expressing indicated chemokine receptora</th>
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<tr>
<td></td>
<td>CCR9⁺</td>
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<tr>
<td>H. pylori⁺</td>
<td>64 ± 15</td>
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* n = 3 to 7 in the infected group; n = 3 to 5 in the uninfected group.

**FIG. 5.** Chemokine-induced migration of gastric ASC. Lamina propria lymphocytes isolated from gastric tissue collected from H. pylori-negative (open symbols) and -infected (black symbols) patients undergoing gastrectomy were allowed to migrate toward optimal concentrations of CCL25 (7.2 μg/ml), CCL28 (5 μg/ml), and CXCL12 (0.3 μg/ml). The frequencies of IgA-secreting (A) and IgG-secreting (B) cells among the migrating cells were determined by ELISPOT assay and related to the spontaneous migration of the same subset without any chemokines. Symbols represent individual values and horizontal bars the median of each group. *, P < 0.05; **, P < 0.01.

**FIG. 6.** Chemokine-induced migration of gastric memory B cells. Lamina propria lymphocytes isolated from gastric tissue collected from H. pylori-negative (open symbols) and -infected (black symbols) patients undergoing gastrectomy were allowed to migrate toward optimal concentrations of CCL25 (7.2 μg/ml), CCL28 (5 μg/ml), and CXCL12 (0.3 μg/ml). The frequencies of IgA⁺ (A) and IgG⁺ (B) B cells among the migrating cells were determined by flow cytometry and related to the spontaneous migration of the same subset without any chemokines. Symbols represent individual values and horizontal bars the median of each group. *, P < 0.05; **, P < 0.01.
In contrast to what was seen for CCL28, CCL25 expression levels were similar in subjects with and without *H. pylori* infection. Almost all subjects had detectable levels of CCL25 in the gastric mucosa, although these levels were generally much lower than what we have previously detected in small intestinal tissue extracts (C. Lindholm and M. Quinding-Järbrink, unpublished observations). Therefore, changes in CCL25 expression do not seem to mediate lymphocyte influx during *H. pylori*-induced gastritis.

Once we had established that CCL28 production was increased in *H. pylori*-associated gastritis, we asked whether gastric B cells could respond to CCL28. Indeed, gastric IgA-secreting cells displayed a robust migration toward CCL28, and this is in fact the first demonstration that human mucosal IgA-secreting cells chemotax to CCL28, a feature that was previously shown only for murine cells. However, only IgA ASC from *H. pylori*-infected patients responded to CCL28, suggesting that the few gastric IgA ASC present in uninfected individuals might have been attracted by other signals or might represent a different, nonmigrating B-cell subset. Gastric IgA-secreting cells did not migrate to CCL28, consistent with our earlier observation that the frequencies of gastric IgG-secreting cells remain unchanged during *H. pylori* infection (28). Cross-reacting antibodies, especially IgG, binding to epithelial cells have been suggested to contribute to autoimmune pathology during *H. pylori* infection (31), but based on our results these antibodies are contributed by serum rather than local IgG-secreting plasma cells.

A recent study by Kunkel et al. (24) has shown that the vast majority of gastric plasma cells express the CCL28 receptor CCR10. However, the *H. pylori* status of patients in that study was not determined. In this study, we compared chemokine receptor expressions on B cells isolated from *H. pylori*-infected and uninfected individuals, and we could show a large and uniform expression of CCR10 on both IgA^+^ and IgG^+^ gastric plasmablasts, regardless of *H. pylori* status. This finding is apparently contradictory to the distinct differences in migration between IgA ASC from infected and uninfected individuals and between IgA and IgG ASC. Clearly, additional factors that are not yet identified contribute critically to CCL28-induced migration of the gastric IgA-secreting cells. A similar phenomenon has previously been described for CXCR4-expressing plasma cells in the bone marrow, hematopoietic stem cells, and B cells (17, 19, 39).

In relation to CCL28-induced migration of gastric ASC, it is interesting that circulating IgA ASC induced by intestinal immunization respond to CCL28 in chemotaxis assays (P. Sundström and M. Quinding-Järbrink, unpublished data). This is not the case for the general pool of circulating IgA ASC (21), suggesting that most cells recruited to the gastric mucosa may be recently activated B cells originating from intestinal inducive sites. This would explain the accumulation of vaccine-specific ASC in the gastric mucosa following oral immunization and would also suggest that the use of CCL28-inducing adjuvant formulations might promote mucosal antibody responses to vaccination.

Gastric memory B cells did not respond to any larger extent to CCL25 or CCL28, as we have previously reported for circulating memory B cells (21). Instead, gastric IgA^+^ and IgG^+^ memory B cells displayed robust migration to CXCL12 in *H. pylori* infection. CXCL12 has previously been shown to be similarly expressed during *H. pylori*-negative and *H. pylori*-associated gastritis (1, 2). In another study, Mazzucchelli et al. (29) showed a large production of CXCL13 (BCA-1) in *H. pylori*-induced gastritis that was localized mainly to the lymphoid follicles. CXCL13 is expressed in most secondary lymphoid tissues, where it contributes to the recruitment of naïve B cells (9). Therefore, the combined actions of CCL28, CXCL12, and CXCL13 would enable recruitment of naïve and memory as well as effector B cells to the human *H. pylori*-infected gastric mucosa.

In conclusion, we have shown that CCL28 expression is increased in human *H. pylori*-induced gastritis and that CCL28, but not CCL25, efficiently recruits gastric IgA ASC from *H. pylori*-infected individuals. These studies are the first to show increased CCL28 production during mucosal infection in humans and provide an explanation for the large influx of IgA-secreting cells into the gastric mucosa in *H. pylori*-infected individuals, both during steady state and following active mucosal immunization.

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