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## Down-regulated Th17 Responses Are Associated with Reduced Gastritis in *Helicobacter pylori*-infected Children

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### Abstract

*Helicobacter pylori* induces less gastric inflammation in children than adults. Here we investigated whether this reduced inflammation involves dysregulated Th17 responses. *H. pylori*-infected children and adults in Santiago, Chile had similar levels of *H. pylori* colonization, proportions of bacteria containing *cagA* and *s1/s2 vacA* markers of virulence and strain genotypes (predominantly hpEurope), but the children had significantly reduced levels of gastric inflammation and neutrophil infiltration. The reduced neutrophil accumulation in infected children was accompanied by significantly fewer gastric Th17 cells and significantly lower levels of IL-17-specific mRNA and protein compared to infected adults. The gastric mucosa of *H. pylori*-infected children also contained higher numbers of IL-10<sup>+</sup> cells and increased levels of both IL-10 and Foxp3 mRNA compared to that of infected adults. Thus, reduced gastric inflammation, including diminished neutrophil accumulation, in *H. pylori*-infected children compared with infected adults is likely due to down-regulated gastric Th17/IL-17 responses as a consequence of enhanced mucosal regulatory T cell activity in the children.

### INTRODUCTION

The inflammation associated with *H. pylori* infection is mediated by gastric mucosal T helper 1 (Th1) cells through the release of IFN- $\gamma$ , based on studies in animal models<sup>1–3</sup> and

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adult humans<sup>4-8</sup>. Study of the infection in adult subjects addresses late *H. pylori*-induced mucosal responses, whereas the study of *H. pylori* infection in children, which has received little investigative attention, offers the opportunity to investigate early mucosal responses to the bacteria. To begin to elucidate the early, possibly age-related, mucosal events in *H. pylori* infection, we<sup>8,9</sup> and others<sup>10</sup> have shown that childhood *H. pylori* infection is associated with significantly reduced levels of gastric inflammation and ulceration compared to adults. We also have shown that the gastric mucosa of *H. pylori*-infected children contains increased numbers of regulatory T (Treg) cells compared to the mucosa of infected adults<sup>8</sup>, suggesting Treg participation in the reduced Th1-mediated gastritis and ulceration in the children. In this connection, emerging evidence suggests that Treg-mediated immune regulation in humans may contribute to *H. pylori* persistence<sup>11,12</sup> and that inadequate or absent Treg responses in adult humans and mice are associated with increased mucosal inflammation during *H. pylori* infection<sup>12-15</sup>.

Recent studies also have shown that the levels of IL-17 are increased in the gastric mucosa of mice infected with *H. pylori*<sup>16-18</sup>, suggesting Th17 cells participate in *H. pylori* gastritis, at least in mice. Indeed, IL-17 is one of the earliest cytokines detected in the gastric mucosa of *H. pylori*-infected mice<sup>17</sup>. IL-17 mediates the recruitment and activation of polymorphonuclear neutrophils, a key cellular element in the inflammatory lesion associated with *H. pylori* infection<sup>19</sup>. Here we extend our study of the immunopathogenesis of *H. pylori* gastritis in children as a window into the early cellular responses to the infection by investigating gastric Th17 responses in *H. pylori*-infected children and adults living in Chile, where *H. pylori* is endemic.

## RESULTS

### Reduced levels of gastric inflammation in *H. pylori*-infected children compared with infected adults

To elucidate the immune mechanisms that initiate inflammation in early *H. pylori* infection, we first evaluated gastric tissue specimens from children and adults with or without *H. pylori* infection for inflammation. *H. pylori* infection was associated with gastric inflammation in both children and adults compared to their non-infected peers (each  $P=0.0002$ ) (Figure 1a). However, the mean level of inflammation in the infected children was significantly less compared to that of the infected adults ( $P=0.002$ ), based on histologic evaluation of coded biopsy specimens using the modified Sydney classification. The same biopsy specimens were evaluated for *H. pylori* colonization. The mean level of *H. pylori* colonization in the gastric mucosa of the children and adults was nearly identical (Figure 1b), despite the reduced level of gastritis in the children. Importantly, at each level of bacterial colonization, *H. pylori*-infected children displayed a reduced histology score compared to that of infected adults ( $P=0.018$ ) (Figure 1c), indicating that the reduced mean histology score in the children (Figure 1a) was not the consequence of a disproportionately lower amount of inflammation in the children or an excessively higher amount of inflammation in the adults for any single level of colonization. Rather, *H. pylori* induced a proportionally lower inflammatory response in children compared to adults at each level of colonization. In addition, the amount of neutrophil infiltration into the gastric mucosa, based on the neutrophil score, was reduced at each level of colonization ( $P=0.01$ ) (Figure 1d).

### *H. pylori* from infected children and adults display similar genetic features

To determine whether the reduced inflammation in *H. pylori*-infected children could reflect infection by less virulent bacteria, we characterized the *H. pylori* isolated from 12 of the 15 infected children and all 7 of the infected adults for the presence of *cagA* and *vacA* alleles and for phylogeographic origin. Among these isolates, the proportion of bacteria that carried

*cagA* and *vacA* (type s1/s2) markers of virulence was similar in infected children and adults ( $P=0.2$  and  $0.1$ , respectively) (Table 1). All of the strains containing s1 forms of *vacA* were classified as subtype s1b. *H. pylori* isolated from the children and adults also were subjected to nucleotide sequence analysis of the *ureI*, *muty*, *efp*, *ppa*, *atpA* and *trpC* loci and then compared to 331 reference strains to assess phylogenetic similarity. As shown in the phylogenetic tree (Figure 2), all 12 of the isolates from children and 5 of the 7 isolates from adults were of European origin<sup>20,21</sup>, suggesting that hpEurope is the predominant *H. pylori* genotype present in this region of Chile. Thus, the Chilean children and adults in our study were colonized by *H. pylori* with similar *cagA* and *vacA* profiles and predominantly of the hpEurope genotype, indicating that host, not bacterial, factors contribute to the reduced gastritis in children.

### Reduced frequency of Th17 cells and IL-17 response in the gastric mucosa of *H. pylori*-infected children compared to adults

Since *H. pylori* gastritis is mediated by Th1<sup>1-8</sup> and possibly Th17<sup>16-18, 22</sup> pathway responses, and since *H. pylori*-infected children display a reduced gastric Th1 response<sup>8</sup>, we next investigated whether infected children also have a reduced gastric Th17 response. To determine the prevalence of Th17 cells in gastric mucosa, we used immunofluorescence to analyze gastric biopsies from *H. pylori*-infected children and adults for CD3<sup>+</sup> T cells that expressed IL-17 (Figure 3a). IL-17 expression was detected predominantly in CD3<sup>+</sup> cells in the lamina propria and occasionally in CD3<sup>-</sup> cells in the epithelium and lamina propria (not shown). The proportion of CD3<sup>+</sup> T cells that expressed IL-17 in the gastric lamina propria of *H. pylori*-infected adults was significantly increased compared to that of both non-infected adults ( $P=0.008$ ) and infected children ( $P=0.024$ ) (Figure 3b). In contrast, the proportion of gastric IL-17<sup>+</sup>/CD3<sup>+</sup> T cells in infected children was not significantly increased compared that of non-infected children (Figure 3b). Thus, the number of Th17 cells in the gastric mucosa of *H. pylori*-infected children is significantly lower than that of infected adults.

Because Th17-derived IL-17 drives the recruitment and activation of neutrophils in the intestinal mucosa, we next measured the IL-17 response in gastric tissue specimens from the children and adults. *H. pylori* infection in the children did not induce increased gastric IL-17 mRNA expression, whereas infection in the adults was associated with a several-fold increase in IL-17 mRNA ( $P=0.03$ ) (Figure 4a). Consistent with these results, the level of IL-17 mRNA in the children did not correlate with the magnitude of *H. pylori* colonization ( $r=-0.09$ ) compared to the moderate positive correlation between the levels of IL-17 mRNA and colonization in the adults ( $r=+0.3$ ). Consequently, the expression of IL-17 mRNA in the mucosa of *H. pylori*-infected children was significantly lower than that of infected adults ( $P=0.008$ ) (Figure 4a). Among the subjects whose gastric tissue specimens provided sufficient total protein to analyze for IL-17 protein, gastric tissue from *H. pylori*-infected adults, but not infected children, had higher levels of IL-17 protein compared to that of uninfected subjects ( $P=0.04$ ) (Figure 4b), and, consistent with these findings, the level of IL-17 protein in the infected children was significantly reduced compared to that of infected adults ( $P=0.02$ ). The level of IFN- $\gamma$  mRNA in the gastric tissue of infected children also was significantly lower than that of similarly infected adults (data not shown;  $P=0.02$ ), corroborating our earlier finding of a reduced Th1 response in *H. pylori*-infected children<sup>8</sup>. Thus, the gastric IL-17 response, similar to the IFN- $\gamma$  response, was significantly lower in *H. pylori*-infected children compared to the response of infected adults.

### Mucosal neutrophil infiltration correlates with IL-17 mRNA in *H. pylori*-infected adults but not children

Since polymorphonuclear neutrophil accumulation is a central feature of *H. pylori* gastritis and can be induced by IL-17, we investigated the relationship between tissue IL-17

responses and neutrophil accumulation in the gastric mucosa of *H. pylori*-infected children and adults. Among the 15 children with *H. pylori* infection, only 3 of 15 (20%) had high levels of neutrophil infiltration (neutrophil score 2–3) into the gastric mucosa, whereas, among the 7 adults with infection, 4 (57%) had similar amounts of high neutrophil infiltration (Figure 5). Moreover, among the infected children with high neutrophil infiltration, the level of IL-17 mRNA was significantly reduced compared to that of infected adults with the same amount of neutrophil infiltration ( $P=0.004$ ). Thus, in the gastritis of *H. pylori*-infected children (Figure 1a), the decrease in neutrophil accumulation was associated with a significantly reduced mucosal IL-17 response.

### Increased levels of IL-10 and Foxp3 mRNA in the gastric mucosa of *H. pylori*-infected children compared to infected adults

To elucidate the mechanism by which the Th17 response might be down-regulated in children infected with *H. pylori*, we analyzed the same gastric tissue specimens for evidence of Treg cell responses. As shown in Figure 6a, gastric *H. pylori* infection in children was associated with a significant increase in the mucosal level of mRNA for the down-regulatory cytokine IL-10 compared to that of non-infected children ( $P=0.03$ ). In contrast, *H. pylori* infection in adults was not associated with increased levels of IL-10 mRNA. Importantly, the gastric mucosa of *H. pylori*-infected children contained significantly higher levels of IL-10 mRNA compared to that of infected adults ( $P=0.03$ ). Consistent with the increased gastric IL-10 response in *H. pylori*-infected children, IL-10<sup>+</sup> cells were identified throughout the lamina propria, albeit at low frequency (Figure 6b). IL-10<sup>+</sup> cells also were identified in the deep regions of the gastric glands, but more frequently than in the lamina propria, of infected children (Figure 6b). The cells that expressed IL-10 in the lamina propria were exclusively CD3<sup>+</sup> T cells and not HLA-DR<sup>+</sup> cells, whereas the IL-10-expressing cells in the glands were either CD3<sup>+</sup> T cells or cytokeratin<sup>+</sup> epithelial cells (Figure 6b).

The gastric mucosa of *H. pylori*-infected children also had higher levels of mRNA for the Treg transcription factor Foxp3 than that of both non-infected children and infected adults (Figure 6c), although the increase did not reach statistical significance. In addition, the Foxp3 and IL-17 mRNA levels in infected children exhibited a negative correlation ( $r=-0.08$ ) compared to the positive, albeit not significant, correlation in adults ( $r=+0.25$ ). Together, the increased IL-10 response and prominent Foxp3 response are consistent with an enhanced Treg response in the gastric mucosa of *H. pylori*-infected children.

## DISCUSSION

To elucidate the mucosal regulation of *H. pylori* infection in children, which provides a window into the early host response to the bacteria, we characterized the bacteria, associated cellular infiltrate and mucosal cytokine response to the infection in children (and adults) residing in Santiago, Chile. Using pediatric and adult cohorts completely different from our previous study<sup>8</sup>, we first confirmed that *H. pylori*-infected children have reduced gastric inflammation compared to infected adults, despite similar mean levels of *H. pylori* colonization. Importantly, inflammation in the children was reduced at each level of bacterial colonization compared to that of adults, suggesting an overall down-regulation of the immune-mediated response to *H. pylori* in children. In addition, sequence analysis showed that the bacteria isolated from infected children and adults had similar *cagA* and *vacA* (s1b and s2) gene profiles and, in the first genotype analysis of *H. pylori* in Chile, that the bacteria were exclusively of European phylogeographic origin. Although genetic changes due to homologous sequence recombination commonly occur in *H. pylori* over time<sup>23</sup>, our gene expression studies indicate that differences in common virulence factors and bacterial strains are not the cause of the reduced inflammation in *H. pylori*-infected children compared to infected adults residing in this highly endemic region of the world.

The presence of only hpEurope strains in our subjects is consistent with the European ancestry in approximately two-thirds of the Chilean population, especially that of the cities<sup>24</sup>. Amerindians or persons of Amerindian admixture constitute most of the remaining third and persons of African descent less than 0.1% of the population<sup>25</sup>. In other regions of South America, persons of African ethnicity represent a substantial proportion of the population. In Colombia, for example, persons with at least some African ancestry represent 21% of the population and are concentrated along the Caribbean and Pacific coasts<sup>26</sup>. In the Pacific coast region, up to two-thirds of the inhabitants were recently shown to be colonized by hpAfrica1 strains<sup>27</sup>, likely introduced there in the 19<sup>th</sup> century through the importation of slaves infected with African strains<sup>20, 21</sup>. Importantly, strains of African origin dominated in the coastal residents and were less strongly associated with gastric cancer than European strains in the mountain residents<sup>27</sup>. In contrast, the high prevalence of only hpEurope strains in the Chilean children reported here assures prolonged exposure to hpEurope strains as the children develop into infected adults, which together with the high frequency of cancer-associated *cagA*<sup>+</sup> and *vacA-s1*<sup>+</sup> genotypes<sup>28, 29</sup>, likely contributes to the high rate of gastric cancer in the Chilean population.

IL-17 is the signature cytokine produced by Th17 cells and has a mediator role in the inflammation associated with certain autoimmune diseases and host defense against bacterial and fungal pathogens, particularly at mucosal surfaces<sup>30-32</sup>. Therefore, we characterized the mucosal Th17/IL-17 response in children and adults with *H. pylori*-associated gastritis. Whereas no differences in the frequency of Th17 cells were detected in the gastric mucosa of children and adults without *H. pylori* infection, children infected with the bacteria had significantly fewer gastric Th17 cells and significantly lower levels of gastric IL-17-specific mRNA and protein compared to that of similarly infected adults, indicating a potent reduction in the mucosal Th17 response in the children. The gastric mucosa of the infected children also contained less IFN- $\gamma$  mRNA, corroborating our earlier finding of a reduced Th1 response in *H. pylori*-infected children<sup>8</sup>. These findings indicate that adult *H. pylori* gastritis is the consequence of both Th17 and Th1 immune-mediated inflammatory pathways and that both pathways are down-regulated in the gastric mucosa of infected children.

Within the gastric inflammatory lesion of *H. pylori*-infected children, neutrophil infiltration was significantly lower and was associated with a reduced IL-17 response compared to that of infected adults. Thus, IL-17-producing cells in *H. pylori*-infected gastric mucosa likely contribute to local neutrophil accumulation, but this contribution is significantly less in children. *H. pylori* infection in the children also was accompanied by higher mucosal levels of mRNA for the transcription factor Foxp3 and the down-regulatory cytokine IL-10, consistent with an enhanced Treg response that may down-regulate the Th17-mediated gastric inflammation in the children. The increased IL-10 response is consistent with the finding of higher numbers of IL-10<sup>+</sup> cells among CD3<sup>+</sup> T cells in the lamina propria and epithelium of *H. pylori*-infected children. In addition to the presence of IL-10<sup>+</sup>CD3<sup>+</sup> T cells in the epithelium, we also detected IL-10<sup>+</sup>cytokeratin<sup>+</sup> cells in the epithelium, consistent with an epithelial source of IL-10 that has been reported by others<sup>33</sup>. In mice, the reciprocal polarization between Treg and Th17 cells is associated with inflammation of the central nervous system<sup>34, 35</sup> and colon<sup>36</sup> and commensal microbiota in the small intestine<sup>37</sup>. In the mouse stomach, Kao et al<sup>18</sup> and Zhang et al<sup>38</sup> showed that *H. pylori*-induced dendritic cells (DCs) skew the Th17/Treg balance toward a Treg-biased response that suppresses Th17 immunity through a CagA- and VacA-independent, TGF- $\beta$ - and IL-10-dependent mechanism. In support of these findings, we recently showed that *H. pylori* was capable of stimulating human gastric DCs to produce IL-10<sup>39, 40</sup>, potentially supplementing Treg suppression of inflammation in the gastric mucosa. Although we have shown that macrophages in normal human intestinal mucosa are incapable of TGF- $\beta$  and IL-10



production<sup>41, 42</sup>, the ability of human gastric macrophages to produce down-regulatory cytokines is not known.

Our finding that *H. pylori*-infected children have down-regulated Th17 responses in the gastric mucosa has important implications for the development of an effective *H. pylori* vaccine. First, in animal model studies, IL-17 has been implicated as a key mediator of vaccine-induced reduction in *H. pylori* infection<sup>43–45</sup> through the stimulation of local chemokines, including MIP-2, KC and LIX, which recruit neutrophils to the gastric mucosa<sup>43</sup>. Second, since children will be an especially important target population for *H. pylori*-specific vaccination in resource-poor nations, reduction in the gastric IL-17 response in children infected with *H. pylori* is a potentially confounding issue that will need to be addressed in the development of such a vaccine. Thus, the Th17/IL-17 results presented here implicate discordant regulation of the gastric mucosal response of children and adults to *H. pylori*. Further elucidation of the immune-mediated mechanism(s) of *H. pylori*-induced mucosal inflammation during early host development will provide important information for the design of more effective intervention strategies against this challenging pathogen, especially for children in countries with high rates of *H. pylori* infection and gastric cancer.

## METHODS

### Patients

Consecutive subjects with abdominal symptoms residing in Santiago, Chile, including 30 children less than 13 years of age and 16 adults greater than 18 years of age, were evaluated in this IRB-approved study. The mean age of the children was 10.4  $\pm$  2.9 years old and the adults 48  $\pm$  13.1 years old. Female gender dominated in both the children (62%) and the adults (69%). All subjects were of European ethnicity, and the mean socioeconomic levels based on the Department of Economy criteria were similar for the groups. Acceptance into the study was based on the presence of symptoms of recurrent abdominal pain, burning abdominal discomfort, hematemesis or chronic vomiting. Exclusion criteria included (a) use of antibiotics, antacid, H<sub>2</sub>-blocker, proton-pump inhibitor, bismuth compound, non-steroidal anti-inflammatory drug or immunosuppressive agent during the two weeks prior to endoscopy; (b) presence of an autoimmune disease; and (c) stool exam positive for ova or parasites.

### Assessment of *H. pylori* infection

Subjects provided six antral biopsies by esophagogastroduodenoscopy for the following studies: (1) Rapid urease test (Rapid Urea Test; Bios Chile, Santiago, Chile) to detect *H. pylori*; (2) Histologic analysis and microscopic detection of *H. pylori*; (3) Genotype analysis; (4) Immunofluorescence studies; (5) Cytokine protein determination; and (6) Analysis of cytokine gene expression. A study subject was judged colonized with *H. pylori* if either the rapid urease test or the microscopic evaluation was positive for the bacteria.

### Evaluation of gastric *H. pylori* histopathology and colonization

Serial sections of formalin-fixed, paraffin-embedded, H&E-stained gastric tissue specimens were evaluated for inflammation and bacterial colonization by two pathologists without knowledge of other results. Briefly, 5 random fields in 3 tissue sections per biopsy were scored (0–3) according to the modified Sydney classification system<sup>46</sup> for the level of neutrophils and mononuclear cells, number of lymphoid follicles, mucosal atrophy and intestinal metaplasia. Thereafter, a histology score reflecting the level of inflammation<sup>8</sup> was determined by adding the score of each parameter. Similarly, a neutrophil score reflected the level of neutrophils in 5 random fields in 3 sections per coded biopsy with 0=absent,

1=mild, 2=moderate and 3=high. A colonization score was calculated based on *H. pylori* density (0=absent, 1=mild, 2=moderate, 3=intense).

### ***H. pylori* virulence factor and genotype analysis**

Gastric biopsies were placed in Brucella broth, frozen immediately in liquid nitrogen and subsequently cultured on plates with Brucella agar, 5% horse blood and *H. pylori*-selective supplement (Dent; Oxoid, Basingstoke, UK). The plates were cultured for 7 days in 10% CO<sub>2</sub> and then passaged three times. A single colony was identified as *H. pylori* using colony morphology and the rapid urease test (Rapid Urea Test, Bios Chile, Santiago, Chile). DNA from the remaining colonies was extracted using the Qiagen DNeasy Minikit (Qiagen, Valencia, CA) and quantified by spectrophotometry. Virulence factor *cagA* was amplified by PCR, as previously described<sup>47</sup>. Strains without *cagA* amplification by PCR were confirmed as *cagA* negative by amplifying the *cag* pathogenicity island empty site as previously described<sup>48</sup>. To analyze the s-region of *vacA*, the nucleotide sequences of PCR products were compared to variants of four known *vacA* s-region subtypes (s1a, s1b, s1c and s2)<sup>49</sup>.

Nucleotide sequences for *H. pylori* housekeeping genes (*ureI*, *mutY*, *efp*, *ppa*, *atpA* and *tpcC*) also were amplified by PCR<sup>20, 50</sup>. PCR products were subsequently purified using the Wizard SV gel and PCR Clean-up kit (Promega, Fitchburg, WI) and stored at -20°C. PCR products were sequenced by the University of Alabama at Birmingham DNA Sequencing and Analysis Core. To determine the relationship between strains of *H. pylori* infecting patients in this study and globally distributed *H. pylori* isolates, we retrieved data from a multi-locus sequence typing (MLST) database (<http://pubmlst.org/helicobacter>) for 331 reference isolates with appropriate geographic distribution. Concatenated nucleotide sequences for *H. pylori* MLST loci (*ureI*, *mutY*, *efp*, *ppa*, *atpA* and *tpcC*) from seven isolates obtained from children and three isolates obtained from adults as well as the 331 isolates retrieved from the MLST database were analyzed using Mega4 software<sup>51</sup>. Sequences were aligned using the ClustalW algorithm and phylogenetic relationships were produced using the Kimura two-parameter model for nucleotide substitution and neighbor-joining clustering. A phylogenetic structure was constructed as a bootstrap consensus tree inferred from 1000 replicates.

### **Identification and enumeration of gastric Th17 cells**

Snap frozen biopsies were cut into 5 µm sections, fixed in acetone (10 min) and rinsed in PBS with 0.05% Tween. After blocking in Dako serum-free protein block for 30 min at room temperature, the sections were incubated with goat anti-human IL-17 (10 µg/mL; R&D Systems, Minneapolis, MN) and rabbit anti-human CD3 (1:300; Sigma-Aldrich, St. Louis, MO) for 4 h at room temperature, followed by incubation with Cy3 donkey anti-goat IgG (1:200; Jackson Immunity, West Grove, PA) and fluorescein isothiocyanate (FITC) donkey anti-rabbit IgG (1:50; Jackson Immunity, West Grove, PA) for 30 min at room temperature. Isotype-matched, irrelevant antibodies were included as controls with each staining experiment. Cell nuclei were labeled with DAPI. Sections were mounted, sealed and stored at 4°C until analysis by fluorescence microscopy (Nikon Eclipse T2000-U, equipped with a CoolSnap ES digital camera and NIS Elements BR2.30 software). The proportion of IL-17<sup>+</sup> cells in the CD3<sup>+</sup> T cell population was determined on digital images by counting CD3<sup>+</sup> cells with and without co-expression of IL-17 using ImageJ1.46R software (Wayne Rasband, National Institutes of Health, USA, <http://imagej.nih.gov/ij>). Regions of interest were set to exclude surface and glandular epithelial cells. Three or more randomly selected areas from two slides per specimen were analyzed by two independent investigators.

### Identification of gastric IL-10 cells

Biopsies fixed in 4% formalin, embedded in paraffin and cut in 7µm sections were rehydrated and rinsed in water. Sections were subjected to antigen retrieval (citrate buffer, DAKO Target Retrieval Solution; 20 min >92°C by microwave), endogenous peroxidase inhibition and casein protein blocking (each 20 min, room temperature, CSA System DAKO), and incubated overnight at 4°C with primary antibody (DAKO Antibody Diluent with Background Reducing Components). Primary antibodies included a) mouse anti-human IL-10 IgG2b (1:200, Santa Cruz); b) rabbit anti-human CD3 IgG (1:400, Sigma-Aldrich); c) mouse anti-human HLA-DR IgG1 (1:20, BD Biosciences); and d) mouse anti-human cytokeratin IgG1 (1:50, Cell Signaling). IL-10 positive cells were identified by tyramide signal amplification (1:100, goat anti-mouse IgG2b-HRP; 1:50, biotinylated tyramide; 1:1000, streptavidin-HRP, PerkinElmer) followed by diaminobenzidine staining and counterstained with hematoxylin, or by tyramide signal amplification as above using tyramide-FITC (1:50, Molecular Probes 488). Nuclei were counterstained with DAPI (1:500). CD3<sup>+</sup> cells were detected with donkey anti-rabbit IgG (1:400, Jackson Immunity); HLA-DR<sup>+</sup> and cytokeratin<sup>+</sup> cells were detected with goat anti-mouse IgG1 (1:100, Molecular Probes 594) and nuclei were counterstained with DAPI. Control slides were included with every experiment (n=12). Sections were stored and analyzed as above.

### Cytokine protein determination

Biopsy specimens were homogenized in 750 µL PBS, centrifuged (12,000g, 5 min, 4°C) and the supernatant collected and frozen (-70°C) until assayed. The concentration of IL-17 was measured by ELISA (R&D Systems, Minneapolis, MN) and expressed as pg/mg protein. Total protein was measured using the bicinchoninic acid method (Pierce, Rockford, IL) and expressed as µg/mL.

### Real-Time RT-PCR analysis for cytokine gene expression

Gastric specimens were snap frozen in liquid nitrogen and kept at -80°C until processed. Total RNA was extracted using the Qiagen RNeasy Minikit (Qiagen, Valencia, CA). RNA concentration and purity were determined by spectrophotometry, and RNA integrity was assessed by agarose gel electrophoresis. RNA then was treated with DNase I (Invitrogen, Carlsbad, CA) for 15 min, heat-inactivated at 65°C to avoid genomic DNA amplification during real-time PCR and reverse transcribed into first-strand cDNA using the affinityScript qPCR cDNA synthesis kit (Agilent Technologies Inc, Santa Clara, CA) with oligo dT as primers. Real-time PCR was performed on duplicate cDNA samples with custom-made primer sets using Brilliant II SYBR green QPCR master mix in an Mx3000p Real-Time PCR System machine (both from Agilent Technologies). The following primer pairs for IL-17 (FW: 5'ACCAATCCCAAAGGTCCTC3'; RV: 5'GGGGACAGAGTTCATGTGGT3'); IL-10 (FW: 5'GTGATGCCCAAGCTGAGA3'; RV: 5'CACGGCCTTGCTCTTGT3'); Foxp3 (FW: 5'AGAAGCAGCGGACACTCAAT3'; RV: 5'GAAAGGAGGATGGACGAACA3'); IFN-γ (FW: 5'GAATTGGAAAGAGGAGAGTGAC3'; RV: 5'TGTATTGCTTTGCGTTGGAC3') and GAPDH (reference gene) (FW: 5'AACCTGCCAAATATGATGAC3'; RV: 5'GTTGTCATACCAGGAAATGAG3') were used for amplification.

PCR fragments for each cytokine and the reference gene obtained by RT-PCR were cloned into the pGEM-T Easy Vector System (Promega, Litchburg, WI). Plasmids extracted from selected clones using the Wizard SV miniprep DNA purification systems (Promega, Fitchburg, WI) containing expected inserts were quantified by spectrophotometry. Serial dilutions of the vector-insert were used as standard curves to quantify the levels of gene expression. mRNA levels were analyzed by comparing the differences in fold change in cytokine mRNA normalized to GAPDH mRNA.



## Statistical analysis

Comparisons between groups were performed using Student's *t*-test or the Mann-Whitney *U* test when appropriate for continuous data. Categorical data were analyzed using the Chi-square test and the Fisher exact test. Spearman's rank correlation and the Goodness-of-Fit test were used to analyze relationships, although only the Spearman correlation coefficient is presented as analyses were uniformly consistent with each other. Comparison of curve fit values was performed using an F-test. Statistical significance was defined as a *P* value of less than .05.

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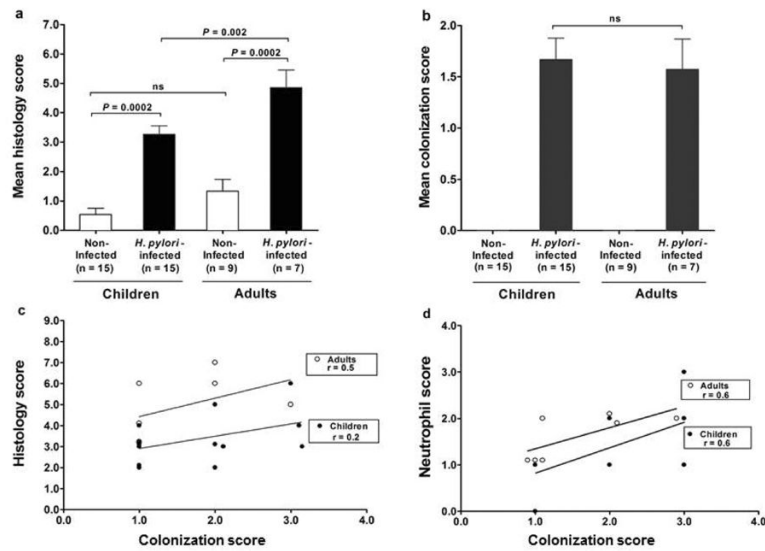
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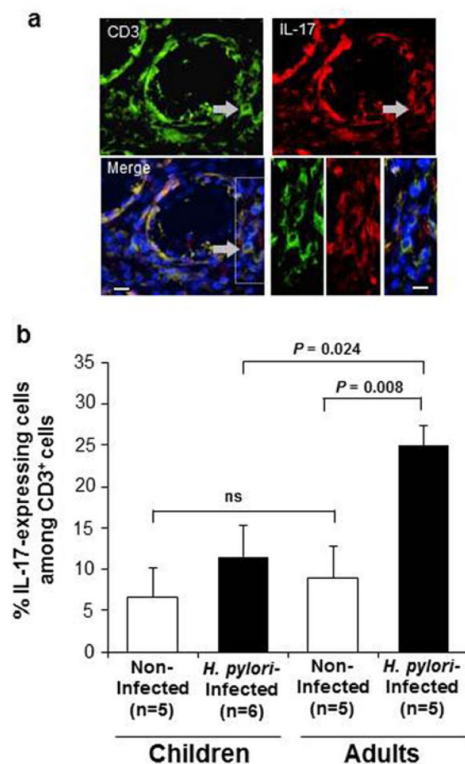


**Figure 1.**

Levels of gastric inflammation and *H. pylori* colonization in children and adults. (a) *H. pylori* induced inflammation in the gastric mucosa of both children and adults, but inflammation was significantly less severe in the infected children. (b) Levels of *H. pylori* colonization in gastric mucosa were similar in infected children and adults. Levels of gastric inflammation and colonization are presented as the histology score and colonization score (mean  $\pm$  SEM), respectively (see Materials and Methods). *H. pylori*-infected children had reduced levels of (c) gastric inflammation based on the histology score and (d) neutrophil infiltration based on the neutrophil score compared to infected adults. (For comparison of lines in c  $P=0.018$  and d  $P=0.01$ ).

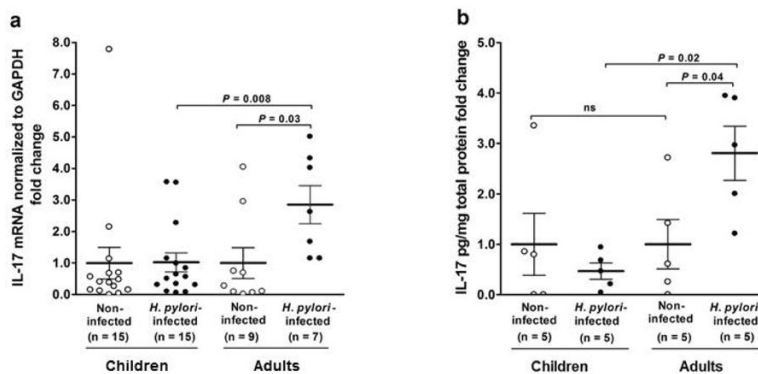






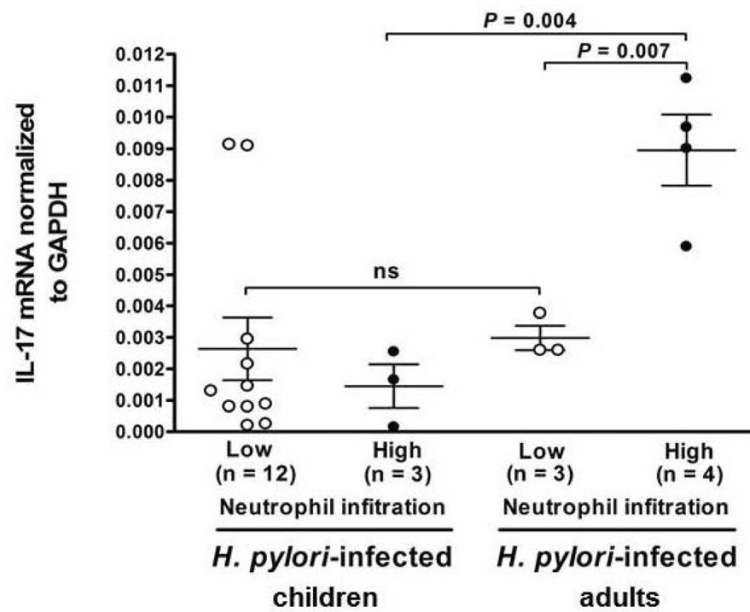
**Figure 3.**

IL-17-expressing CD3<sup>+</sup> T cells in the gastric mucosa of *H. pylori*-infected and uninfected children and adults. (a) Co-localization of CD3<sup>+</sup> and IL-17<sup>+</sup> mucosal cells in a gastric tissue section from a representative *H. pylori*-infected adult stained with goat anti-CD3 (Cy3) and goat anti-IL-17 (FITC) antibodies. Bar = 20  $\mu$ m. (b) Percentage (mean  $\pm$  SEM) of CD3<sup>+</sup> T cells that co-expressed IL-17 was significantly lower in *H. pylori*-infected children (n=6) compared to that of infected adults (n=5).



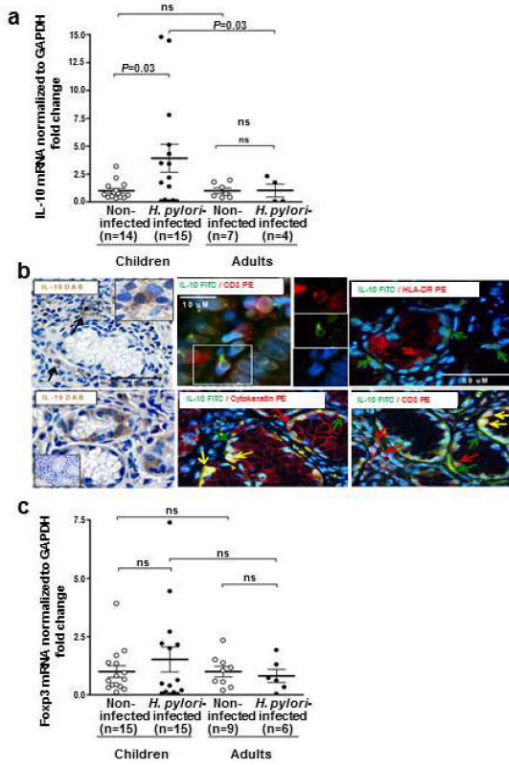
**Figure 4.**

IL-17-specific mRNA and protein in gastric mucosa of non-infected and *H. pylori*-infected children and adults. IL-17 (a) mRNA and (b) protein in *H. pylori*-infected children were significantly lower compared to that of infected adults. mRNA expression was determined by real-time PCR and normalized to the housekeeping gene GAPDH. IL-17 pg protein was determined by ELISA and normalized to total mg protein. Data are presented as fold change ( $\pm$ SEM) in *H. pylori*-infected children and infected adults compared to non-infected cohort subjects.



**Figure 5.**

Levels of gastric IL17 mRNA and polymorphonuclear neutrophil infiltration in *H. pylori*-infected children and adults. Gastric mucosa from *H. pylori*-infected children (n=15) and adults (n=7) were analyzed for neutrophil infiltration and IL-17 mRNA expression as described in the Materials and Methods. Low neutrophil infiltration = neutrophil score 0–1 and high neutrophil infiltration = neutrophil score 2–3. Gastric IL-17 mRNA responses were lower in *H. pylori*-infected children than in infected adults. Values are presented as mean IL-17 mRNA normalized to GAPDH mRNA  $\pm$  SEM.



**Figure 6.** IL-10 and Foxp3 responses in the gastric mucosa of non-infected and *H. pylori*-infected children and adults. Gastric tissue specimens were analyzed for (a) IL-10 mRNA, (b) identity of the IL-10-expressing cells and (c) Foxp3 mRNA. Levels of IL-10 and Foxp3 mRNA, determined by real-time PCR and normalized to GAPDH mRNA, were higher in *H. pylori*-infected children than infected adults. Gastric IL-10 protein, detected by immunohistochemistry (left upper and lower panels) and immunofluorescence (right panels) in an *H. pylori*-infected child, co-localized in the lamina propria in CD3<sup>+</sup> T cells and in the epithelium in cytokeratin<sup>+</sup> epithelial cells and CD3<sup>+</sup> T cells. Inset in the left lower panel shows control tissue without DAB staining. In the immunofluorescence panels, red and green arrows point to cells that stain with the indicated antibody of the same color; yellow arrows point to the merge, indicating co-localization.



**Table 1***H. pylori* virulence factors

	<u>Source of <i>H. pylori</i></u>		<i>P</i> value
	Children n=12	Adults n=7	
CagA, n (%)	11 (92)	5 (71)	0.2
VacA S alleles			0.1
s1 allele, n (%)	4 (33)	5 (71)	
s2 allele, n (%)	8 (66)	2 (29)	