GASTRIC TH17 RESPONSE TO HELICOBACTER PYLORI INFECTION IN CHILDREN

by

SHELTON W. WRIGHT

PHILLIP D. SMITH, COMMITTEE CHAIR
DALE J. BENOS
PAUL R. HARRIS
ROBINNA G. LORENZ
LESLEY E. SMYTHIES

A THESIS
Submitted to the graduate faculty of The University of Alabama at Birmingham, in partial fulfillment of the requirements for the degree of Master of Science

BIRMINGHAM, ALABAMA

2010
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>iv-v</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>viii</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>HELICOBACTER PYLORI GASTRITIS IN CHILDREN IS ASSOCIATED WITH A REGULATORY T CELL RESPONSE</td>
<td>3</td>
</tr>
<tr>
<td>TH17 CELL RESPONSE TO HELICOBACTER PYLORI INFECTION IS DOWN-REGULATED IN CHILDREN</td>
<td>29</td>
</tr>
<tr>
<td>CONCLUSIONS</td>
<td>61</td>
</tr>
<tr>
<td>GENERAL LIST OF REFERENCES</td>
<td>65</td>
</tr>
<tr>
<td>APPENDIX: IRB APPROVAL</td>
<td>67</td>
</tr>
</tbody>
</table>
GASTRIC TH17 RESPONSE TO HELICOBACTER PYLORI INFECTION IN CHILDREN

SHELTON W. WRIGHT

BASIC MEDICAL SCIENCES

ABSTRACT

Background & Aims: Helicobacter pylori infection in children infrequently causes gastroduodenal mucosal ulceration. Since H. pylori induces T cell (Th1/Th17)-dependent gastric inflammation in adults and T regulatory (Treg) cells suppress T cell-dependent pathology, we evaluated Treg and Th17 cell responses in H. pylori-infected children and adults. Methods: Gastric tissue from children and adults with abdominal symptoms in Santiago, Chile was evaluated prospectively for H. pylori, histopathology and Treg and Th17 activity using immunoassay and real-time polymerase chain reaction. Results: Children and adults were colonized with similar levels of the bacteria and all bacterial isolates were genotypically similar. However, the level of gastritis in the infected children was reduced compared to infected adults (P<0.05). Coincident with reduced gastric inflammation, the number of Treg cells and levels of Treg cytokines (TGF-β1 and IL-10) were markedly increased in the gastric mucosa of H. pylori-infected children compared to that of infected adults (p<0.03 and p<0.05, respectively). Also, H. pylori infection in the children was associated with markedly elevated levels of gastric TGF-β1 and IL-10 mRNA but reduced levels of Th17 mRNA. Infected children had lower levels of IL-17+, CD3+ cells in their gastric mucosa compared to infected adults (P<0.05). Importantly, gastric TGF-β1 in H. pylori-infected children localized...
predominantly to mucosal CD25$^+$ and Foxp3$^+$ cells, indicating a Treg source for the TGF-β1. **Conclusions:** Gastric pathology is reduced and local Treg cell responses are increased in *H. pylori*-infected children compared to infected adults. Furthermore, Th17 cell responses to *H. pylori* infection are reduced in children compared to adults, suggesting that gastric Treg cell responses down-regulate the inflammation and ulceration induced by *H. pylori* in children.

Keywords: *H. pylori* infection, Regulatory T-cells, T-helper 17 cells
ACKNOWLEDGEMENTS

This project would not have been possible without the support of the William J Fulbright Scholars program, the Chilean Fulbright Commission as well as the Alpha Omega Alpha Carolyn L Kuckein Student Research Fellowship. I would like to specifically thank Carolina Serrano, Andrea Villagrán, Elsa Bruce, Ingrid Mansilla and Alejandro Venegas, PhD as well as the Pontificia Universidad Católica de Chile in Santiago, Chile. At the University of Alabama at Birmingham, the assistance of Diane Bimczok, DVM, PhD, Kayci Huff, Lois Musgrove, Meg Barnum and Jamie McNaught was crucial. The opinions and efforts of my thesis committee, consisting of my mentor, Phillip D. Smith, MD, Paul R. Harris, MD, Dale J. Benos, PhD, Lesley E. Smythies, PhD and Robin G. Lorenz, MD, PhD have been extraordinary and without their guidance and support this would not have been possible. Finally, the patience and guidance of my program director, Peter R. Smith, PhD, have been unending and inspiring.
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>HELICOBACTER PYLORI GASTRITIS IN CHILDREN IS ASSOCIATED WITH A REGULATORY T CELL RESPONSE</td>
<td></td>
</tr>
<tr>
<td>1   Characteristics of the Chilean study population</td>
<td>23</td>
</tr>
<tr>
<td>2   Frequency of inflammatory changes in Chilean children and adults with and without <em>H. pylori</em> infection</td>
<td>24</td>
</tr>
<tr>
<td>TH17 CELL RESPONSE TO HELICOBACTER PYLORI INFECTION IS DOWN-REGULATED IN CHILDREN</td>
<td></td>
</tr>
<tr>
<td>1   Frequency of inflammatory changes in Chilean children and adults with and without <em>H. pylori</em> Infection</td>
<td>52</td>
</tr>
<tr>
<td>2   Frequency and genotype of <em>vacA</em> and <em>cagA</em> isolated from <em>H. pylori</em> infecting Chilean children and adults</td>
<td>52</td>
</tr>
<tr>
<td>3   IL-17 and CD3 fluorescence and overlap in the gastric mucosa</td>
<td>53</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

## HELICOBACTER PYLORI GASTRITIS IN CHILDREN IS ASSOCIATED WITH A REGULATORY T CELL RESPONSE

1. Histological features of childhood and adult *H. pylori* infection ........................................ 25

2. CD25⁺Foxp3⁺ cells in the gastric mucosa of children and adults infected with *H. pylori* .......................................................................................................................... 26

3. Regulatory cytokine protein and mRNA in the gastric mucosa of *H. pylori*-infected and uninfected children and adults ............................................................................. 27

4. Gastric Treg cells are a source of TGF-β1 ........................................................................ 28

## TH17 CELL RESPONSE TO HELICOBACTER PYLORI INFECTION IS DOWN-REGULATED IN CHILDREN

1. Histological features of childhood and adult *H. pylori* infection ........................................ 54

2. Phylogenetic structure of Chilean *H. pylori* strains .......................................................... 55

3. Effector cytokine mRNA expression in the gastric mucosa of *H. pylori*-infected and uninfected children and adults ............................................................................. 56-7

4. Adults with *H. pylori* infection express Th17 cells .......................................................... 58

5. IL-17⁺ and CD3⁺ cells in the gastric mucosa of *H. pylori*-infected and uninfected children and adults ....................................................................................... 59-60
INTRODUCTION

*Helicobacter pylori*, a flagellated, gram-negative bacteria, is unique in its ability to colonize the human stomach\(^1\). The organism has infected humans for thousands of years, evidenced by its extraction from Andean mummies\(^2,3\). Despite the potential for mutually beneficial coadaptation, modern *H. pylori* infection is associated with significant pathologic sequelae in its host. Worldwide, *H. pylori* is the most common cause of gastritis, peptic ulcer disease and gastric adenocarcinoma. These various pathologic sequelae are often attributed to a chronic inflammatory response, especially in the gastric antrum, leading to the erosion of the gastric mucosa and increasing the probability of mutation acquisition and ultimately neoplasia\(^1\). While infection rates in the United States have steadily decreased over the last century, prevalence in developing countries remains relatively high\(^4\). Likewise, in South America, gastric adenocarcinoma remains a leading cause of cancer mortality\(^5\). Infection in developing countries often occurs in children, in direct contrast to developed countries\(^6,7\). Chronic infection with *H. pylori* is directly associated with increased morbidity\(^1\). While *H. pylori* is an important cause of gastritis in children, ulceration in this population is relatively rare, including in developing countries\(^8,9\). The exact nature of the pediatric response to the organism has received little investigative attention.

The robust inflammatory response associated with *H. pylori* infection and its related pathologies is T-cell mediated, with Th1 and Th17 cells playing an important role
in activating the inflammatory response\textsuperscript{10-14}. Through several different virulence factors, such as CagA and VacA, \textit{H. pylori} simultaneously activates this pro-inflammatory response to infection while also mitigating its eradication\textsuperscript{1,15}. Interactions with the host immune system allow the organism to chronically colonize the gut mucosa as well as propagate the erosion of the gastric epithelium. The development of lymphoid follicles in response to \textit{H. pylori} infection is common and further drives the cycle of inflammation\textsuperscript{1}. Importantly, most evidence regarding the interaction between host and bacteria has focused on animal models as well as human adults. \textit{H. pylori} infection in children is rare in the United States, making the study of this interaction between pediatric host and the organism difficult\textsuperscript{16}. Nevertheless, uncovering the relationship between this young population and the organism is of crucial importance as the host response in children appears attenuated compared to that in adults. Understanding the pediatric response to \textit{H. pylori} infection may provide important clues regarding the evolution of the host/organism interaction. Lastly, eradication of the organism is difficult and may not be a feasible option to address the global burden of the disease\textsuperscript{1}. Discerning the mechanisms involved in the pathologic host response to infection can lead to the development of therapeutic targets aimed at modulating that response.
HELICOBACTER PYLORI GASTRITIS IN CHILDREN IS ASSOCIATED WITH A REGULATORY T CELL RESPONSE

by

PAUL R. HARRIS, SHELTON W. WRIGHT, CAROLINA SERRANO, FRANCISCA RIERA, IGNACIO DUARTE, JAVIERA TORRES, ALFREDO PENA, ANTONIO ROLLAN, PAOLA VIVIANI, ERNESTO GUIRALDES, JULIA M. SCHMITZ, ROBIN G. LORENZ, LEA NOVAK, LESLEY E. SMYTHIES AND PHILLIP D. SMITH


Copyright 2008 by AGA Institute

Used by permission

Format adapted for thesis
ABSTRACT

Background & Aims: Helicobacter pylori infection in children infrequently causes gastroduodenal mucosal ulceration. Since H. pylori induces T cell (Th1)-dependent gastric inflammation in adults and T regulatory (Treg) cells suppress T cell-dependent pathology, we evaluated gastric histopathology and Treg cell responses in H. pylori-infected children and adults. Methods: Gastric tissue from 36 children and 79 adults with abdominal symptoms in Santiago, Chile was evaluated prospectively for H. pylori bacteria and histopathology using the Sydney classification and Treg responses using immunoassay, immunohistochemistry and real-time PCR. Results: Eighteen (50%) of the children and 51 (65%) of the adults were infected with H. pylori. Children and adults were colonized with similar levels of H. pylori. However, the level of gastritis in the children was substantially reduced compared to that of the adults (p<0.05). Coincident with reduced gastric inflammation, the number of Treg cells and levels of Treg cytokines (TGF-β1 and IL-10) were markedly increased in the gastric mucosa of H. pylori-infected children compared to that of infected adults (p<0.03 and p<0.05, respectively). Also, H. pylori infection in the children was associated with markedly elevated levels of gastric TGF-β1 and IL-10 mRNA. Importantly, gastric TGF-β1 in H. pylori-infected children localized predominantly to mucosal CD25+ and Foxp3+ cells, indicating a Treg source for the TGF-β1. Conclusions: Gastric pathology is reduced and local Treg cell responses are increased in H. pylori-infected children compared to infected adults, suggesting that gastric Treg cell responses down-regulate the inflammation and ulceration induced by H. pylori in children.
INTRODUCTION

*Helicobacter pylori* is the most common pathogen of the gastrointestinal tract worldwide and the predominant cause of chronic gastritis, gastroduodenal ulceration and gastric malignancy\(^1\). In developing countries, up to 80% of children are infected with the bacterium\(^2\), whereas in developed countries children are infrequently infected\(^3,4\). Prevalence rates of *H. pylori* are particularly high in indigenous peoples of South America\(^5\). In Chile, the organism is acquired at an early age\(^6\), the majority of adolescents are *H. pylori*-seropositive\(^7,8\), and gastric adenocarcinoma is the leading cause of cancer-related mortality\(^9\).

The pathogenesis of *H. pylori* disease is initiated by the interaction between bacterial virulence factors and host mucosal cells, leading to the local production of pro-inflammatory mediators and the inflammatory cascade. Gastric inflammation is present in all colonized persons, but some investigators\(^10-12\) have reported that *H. pylori*-associated histopathology in children is variably reduced compared to that of adults. In Peru, for example, both the prevalence and severity of *H. pylori*-associated chronic gastritis is significantly less in children than in adults\(^13\).

In adult humans and animal models, *H. pylori* drives a local Th1 response that promotes gastric mucosal inflammation\(^14-20\). In contrast, the pathogenesis of the inflammatory response to *H. pylori* in children has received little investigative attention. Here we investigated the relationship between T regulatory (Treg) cells, which down-modulate T cell proliferation and function\(^21-24\), and the apparent reduced inflammatory response to *H. pylori* in children. We studied this relationship in *H. pylori*-infected subjects residing in Santiago, Chile, where *H. pylori* infection is endemic in children.
MATERIALS AND METHODS

Patients. One hundred and fifteen consecutive subjects with abdominal symptoms residing in Santiago, Chile, including 36 children ≤12 years of age and 79 adolescent and adult subjects >12 years of age, were enrolled in this institutional review board-approved study. Since only three subjects were adolescents, hereafter the group >12 years of age is referred to as adults. Criteria for patient inclusion were symptoms suggestive of peptic disease, including recurrent abdominal pain, burning abdominal discomfort, hematemesis or chronic vomiting. Exclusion criteria included a history of antibiotic, antacid, H₂ blocker, proton-pump inhibitor, bismuth compound or non-steroid anti-inflammatory drug usage during the previous four weeks. Each subject or the subject’s parents provided the clinical history. If the subject fulfilled the inclusion criteria, they were invited to participate in the study. Patients’ socioeconomic status was classified according to a locally validated version of the Graffar score. Written informed consent was obtained from either the patient or the patients’ parents or guardian.

Assessment of *H. pylori* infection. Each subject underwent serum collection and standard esophagogastroduodenoscopy. Serum samples were analyzed for antibodies to whole *H. pylori* and CagA by ELISA with OD cut-off values for the serological assays set at 1.0 for IgG and IgA anti-*H. pylori* antibodies and 0.35 for IgG anti-CagA antibodies, as previously reported. The CagA analysis was performed to determine whether histology or Treg findings related specifically to the presence of CagA. Based on our previous observation that *H. pylori*-associated gastritis of the corpus was present only when antral gastritis was present, we obtained six biopsy specimens from the
gastric antrum. One specimen was subjected to rapid urease test (Rapid Urea Test, Bios Chile, Santiago, Chile) for the detection of \textit{H. pylori}; a second specimen was formalin-fixed, paraffin-embedded, sectioned and treated with hematoxylin and eosin (H&E) for histological analysis, Warthin-Starry silver stain for \textit{H. pylori} detection, and antibodies to CD25, Foxp3 and TGF-\(\beta\)1 for immunohistochemical analysis; a third specimen was used for real-time PCR analysis; a fourth specimen was snap frozen for immunofluorescence; and two additional specimens were prepared together for cytokine protein determinations. A subject was considered colonized by \textit{H. pylori} when the serologic test plus either the rapid urease test or microscopic evaluation were positive for \textit{H. pylori}.

**Evaluation of gastric \textit{H. pylori} colonization and histopathology.** Serial sections of H&E-stained gastric tissue were evaluated independently by two pathologists who were blinded to the results of other assays. To quantify the levels of \textit{H. pylori} colonization and gastric histopathology, coded tissue sections from each biopsy specimen (five random fields in each of three tissue sections per biopsy) were graded according to the Sydney classification\textsuperscript{27} for \textit{H. pylori} colonization and the presence of polymorphonuclear and mononuclear cell infiltrations, number of lymphoid follicles, mucosal atrophy and intestinal metaplasia. A colonization score was calculated on the basis of \textit{H. pylori} density (0 absent, 1 mild, 2 moderate, 3 intense)\textsuperscript{27} and a histology score was calculated by adding the mean individual scores for each parameter to quantify the level of gastritis. In addition, we determined the frequency of each parameter and endoscopically identified ulceration in the children and adults. Intraobserver variation between pathologists was negligible, as previously established by kappa statistical analysis\textsuperscript{28}. 
Identification and enumeration of gastric Treg cells. Frozen sections (5 μm) were fixed by irradiation in 0.5% paraformaldehyde/5% Kryofix in 0.1 M phosphate buffer, pH 7.4 (60 sec at 650 W), rinsed in PBS (15 min), incubated with 3% hydrogen peroxide (45 min) to eliminate endogenous peroxidase activity, blocked with casein protein (1 hr, CSA System; DAKO) and incubated sequentially with mouse anti-human Foxp3 (0.08 mg/ml, 15 hr at 4°C; Abcam Inc, DAKOCytomation CSA System with tyramide), FITC-streptavidin (DAKO), mouse anti-human CD25-PE (1:50, 30 min at RT; BD Biosciences Pharmingen, San Diego, CA) and counterstained with DAPI (Calbiochem, San Diego, CA) for 5 min at 25°C.

Formalin-fixed serial sections (5 μm) were deparaffinized, rehydrated and heated in 10 mM sodium citrate buffer, pH 6.0 (5 min, 95°C) and cooled for 20 min. Slides were transferred to water (5 min), blocked as above, and incubated with rabbit anti-human TGF-β1 (0.05 mg/ml, Santa Cruz Biotechnology, Santa Cruz, CA) or goat anti-human Foxp3 (0.01 mg/ml, Abcam Inc, Cambridge, MA) for 15 hr at 4°C. Sections then were washed in PBS, incubated with HRP-conjugated donkey anti-rabbit IgG or donkey anti-goat IgG (1:500, 30 min at 25°C; both from Santa Cruz Biotechnology), followed by diaminobenzidine- or fuscein red-substrate/chromagen solution (DAKO) and counterstained with hematoxylin. For control tissue, biopsies of inflamed ileum from patients with active Crohn’s disease were prepared in the same manner; antibody controls were included with each staining experiment.

To quantify the number of Treg cells, gastric biopsies from nine *H. pylori*-infected children and nine *H. pylori*-infected adults were examined for Foxp3+ cells. The mean number of Foxp3+ cells/1,000 mononuclear leukocytes (MNL)/biopsy was
determined by enumerating the number of Foxp3-stained cells per grid, the number of mononuclear cells per grid and the number of grids per biopsy.

**Cytokine protein determination.** Two antral biopsies were used to measure tissue cytokine levels. The biopsies were homogenized using a tissue homogenizer (Biospec, Bartlesville, OK) in 750 µl PBS. After centrifugation (12,000g, 5 min at 4°C), the supernatants were collected and frozen (-70°C) until assayed. The levels of regulatory cytokines IL-4, IL-13, IL-10 and TGF-β1 were measured by ELISA (R&D Systems, Minneapolis, MN). For the measurement of TGF-β1, supernatant was first acid-activated according to the manufacturer’s recommendation. Protein levels were measured using the bicinchoninic acid method (Pierce, Rockford, IL), and total protein concentrations were expressed as mg/ml. The final cytokine concentrations in the biopsied tissue were expressed as pg/mg of protein.

**Real-time PCR analysis for cytokine gene expression.** Total RNA was isolated using the Qiagen RNeasy (Qiagen, Valencia, CA) Minikit and the RNA reverse transcribed into first-strand cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Pensberg, Germany). Real-time reverse transcription PCR (RT-PCR) was performed with Applied Biosystems Assays-On-Demand primer/probe sets and TaqMan Universal PCR Mix (PE Applied Biosystems; Foster City, CA) using a Stratagene MX3000P real-time PCR machine. The following primer/probe sets were used: 18S housekeeping gene (Hs99999901_s1; Genebank ID# X03205.1); TGF-β1 gene (Hs99999918_m1; Genebank ID# X02812.1); IL-10 gene (Hs00174086_m1; Genebank ID# M57627.1); and Foxp3 gene (Hs00203958_m1; Genebank ID# AF277993.1). The level of gene expression was calculated using the “delta-delta Ct” relative quantitation
method as detailed by Applied Biosystems instructions. Briefly, the relative expression (ΔCt) of each target gene was determined by comparing its crossing threshold (Ct) with the Ct for 18S as the reference (housekeeping) gene. The difference in the mean ΔCt of each group and its control group (ΔΔCt) was used to determine the fold change (2 ΔΔCt) in mRNA expression\textsuperscript{30,31}.

**Statistical analysis.** Comparisons between groups were performed using Student’s t test and ANOVA with Tukey’s pairwise comparisons for continuous data. Categorical data was analyzed using a chi-square test and Fisher’s exact test. Statistical significance was defined as a p value less than 0.05.

**RESULTS**

**Patients.** The 115 study subjects, including 36 children and 79 adults, displayed similar gender distributions, socioeconomic status and indications for endoscopy (Table 1). Eighteen children (50\%) and 51 adults (65\%) were infected with *H. pylori*, based on the presence of circulating anti-*H. pylori* antibodies and *H. pylori* in the gastric mucosa. Among the *H. pylori*-infected children, 12 (66.7\%) were CagA\textsuperscript{+}, whereas among the *H. pylori*-infected adults, 33 (65.0\%) were CagA\textsuperscript{+}. CagA titers for the two groups were similar (0.434 and 0.570, p=0.114). In the children, the presence of CagA correlated with polymorphonuclear cell infiltrate (p=0.0006) and mononuclear cell infiltrate (p=0.0001) and, in the adults, with polymorphonuclear cell infiltrate (p=0.02) and mononuclear cell infiltrate (p=0.004). CagA antibody levels were significantly higher in patients with active or treated peptic ulcer disease compared to those with chronic gastritis only (0.932 vs. 0.521; p<0.001). The presence of CagA did not correlate with increased cytokine levels.
Marked reduction in gastritis in *H. pylori*-infected children compared to infected adults. The antral mucosa of *H. pylori*-infected children typically showed minimal polymorphonuclear and mononuclear cell infiltration, no lymphoid follicles and intact epithelium (Fig. 1A) compared to that of infected adults, whose mucosa usually showed dense polymorphonuclear and mononuclear cell (including plasma cell) infiltration, lymphoid follicles and reactive epithelial changes (Fig. 1B). To quantify these findings, we next evaluated coded gastric tissue sections from the *H. pylori*-infected and uninfected subjects for the levels of both *H. pylori* and inflammation. Based on visual assessment of bacteria, the level of *H. pylori* colonization in the gastric tissue specimens from the infected children was similar to that of infected adults (Fig. 1C). Importantly, the level of inflammation in the same gastric specimens, reflected in the histology scores based on the Sydney classification, was greater for the *H. pylori*-infected children and adults compared to that of uninfected children and adults (both p<0.05) (Fig. 1D). However, the mean histology score for the *H. pylori*-infected children was significantly less than that of the infected adults (2.1 ± 0.4 vs. 2.9 ± 0.6, p<0.05) (Fig. 1D), indicating a lower level of gastritis in *H. pylori*-infected children compared to infected adults.

Reduced frequency of gastric inflammatory changes in children infected with *H. pylori* compared to infected adults. Having detected a reduced intensity of inflammation in the gastric mucosa of *H. pylori*-infected children compared to that of infected adults, we next determined the frequency of gastric inflammatory changes and ulceration in the *H. pylori*-infected children compared to infected adults (Table 2). Among the 18 *H. pylori*-infected children, 9 (50%) displayed gastric polymorphonuclear
cell infiltration, 14 (78%) mononuclear cell accumulation and 6 (33%) lymphoid follicles, in contrast to the 51 infected adults, among whom 37 (73%) showed polymorphonuclear cell infiltration, 45 (88%) mononuclear cell accumulation and 21 (41%) lymphoid follicles (p<0.05 for each). Atrophy of the gastric mucosa was present in only six patients, one child and five adults. Intestinal metaplasia in the gastric antrum was present in none of the infected children and one infected adult. Importantly, among the infected children, the proportion with gastric ulceration (4/18) was substantially less than the proportion of infected adults with ulceration (24/51, p<0.05). Thus, gastric inflammatory changes and ulceration were significantly less frequent in *H. pylori*-infected children than in infected adults colonized with similar levels of the bacteria.

**Increased prevalence of CD25⁺Foxp3⁺ cells in the gastric mucosa of *H. pylori*-infected children compared to infected adults.** We next sought to determine a mechanism for the reduced gastritis in the children infected with *H. pylori*. Since *H. pylori* inflammation is T cell-dependent¹⁴⁻²⁰ and Treg cells down-modulate T cell-mediated inflammation²¹⁻²⁴, we first determined whether the gastric mucosa of *H. pylori*-infected children contains increased numbers of Treg cells. A representative gastric tissue section from an *H. pylori*-infected adult and an infected child show increased numbers of CD25⁺Foxp3⁺ in the tissue from the child compared to that of the adult (**Fig. 2A**). Among nine children and nine adults with similar levels of *H. pylori* colonization, cells with nuclear Foxp3⁺ staining (**Fig. 2B inset**) were four-fold more prevalent in the gastric mucosa of the children than the adults (0.87 Foxp3⁺ cells/1,000 MNLs/biopsy vs 0.21 Foxp3⁺ cells/1,000 MNLs/biopsy, p<0.03) (**Fig. 2B**). These findings indicate that
the reduced inflammation in *H. pylori*-infected children is associated with an increased prevalence of gastric Treg cells.

**Increased levels of gastric TGF-β1 and IL-10 in *H. pylori*-infected children compared to infected adults.** To determine whether the increased number of Tregs in the gastric mucosa of *H. pylori*-infected children was associated with increased levels of Treg cytokines (TGF-β1 and IL-10), we next quantified T cell-derived cytokines in the antral mucosa of the infected and uninfected children and adults. The level of TGF-β1 in the gastric mucosa of *H. pylori*-infected children was more than two-fold higher than the level in the gastric mucosa of infected (and uninfected) adults (p<0.01) and significantly higher than the level in the mucosa of uninfected children (p<0.05) (Fig. 3A). Interestingly, the level of TGF-β1 in the gastric mucosa of uninfected children was nearly twice as high as that of uninfected adults (p<0.05), suggesting increased endogenous Treg cell activity in the gastric mucosa of children. Because Treg cells also produce the immunoregulatory cytokine IL-10, we also determined the level of IL-10 in gastric tissue from *H. pylori*-infected and uninfected children and adults. The level of IL-10 in the antral mucosa of *H. pylori*-infected children was two-fold more than the level in infected (and uninfected) adults (p<0.05) and nearly three-fold more than that of uninfected children (p<0.01) (Fig. 3A). In contrast, the levels of gastric IL-4 and IL-13, important Th2 cytokines, were equivalent among the groups of subjects, suggesting that the IL-10 cytokine response was not the consequence of an enhanced Th2 response. Consistent with these findings, gastric TGF-β1- and IL-10-specific mRNA increased 6-fold and 14-fold, respectively, in the *H. pylori*-infected children compared to the uninfected children (Fig. 3B). In sharp contrast, gastric TGF-β1- and IL-10-specific mRNA increased only
3-fold and 2-fold, respectively, in the infected adults compared to uninfected adults. However, the level of IFN-\(\gamma\) mRNA, which was not increased in the infected children, was increased 5-fold in the infected adults. These findings indicate an increased Treg and reduced Th1 response in the gastric mucosa of infected children and the reverse in the gastric mucosa of infected adults.

**CD25\(^+\) and Foxp3\(^+\) cells are the predominant source of TGF-\(\beta1\) in the gastric tissue of \(H.\) pylori-infected children.** We previously showed that epithelial cells and lamina propria mast cells are the major source of TGF-\(\beta1\) in the intestinal mucosa of healthy adults\(^1\). To determine the source of the TGF-\(\beta1\) in the gastric mucosa during \(H.\) pylori infection, we used immunofluorescence and immunohistochemistry to identify TGF-\(\beta1\)-producing cells and Foxp3\(^+\) cells in the gastric mucosa. In the gastric tissue of \(H.\) pylori-infected children, TGF-\(\beta1\) in the lamina propria localized to CD25\(^+\) and Foxp3\(^+\) cells (Fig. 4). Low levels of TGF-\(\beta1\) also were detected in the epithelial cells of \(H.\) pylori-infected (Fig. 4) and uninfected subjects, consistent with our earlier studies\(^32\). Whereas gastric TGF-\(\beta1\) in uninfected subjects was produced mainly by epithelial cells, the TGF-\(\beta1\) in the gastric mucosa of \(H.\) pylori-infected subjects was detected predominantly in Treg cells.
DISCUSSION

*H. pylori* is acquired predominantly in early childhood in inhabitants of developing countries, such as those of South America, where the seroprevalence of *H. pylori* in asymptomatic children ranges between 25%-78%\(^2\). In Chile, the prevalence of *H. pylori* in asymptomatic children, is generally lower than in other South American countries\(^7\), although among Chilean children with abdominal symptoms up to 86% are infected with the bacteria\(^26\). In sharp contrast to children in South America, asymptomatic young children in developed countries are infrequently infected with *H. pylori*\(^4\) and only 11% of symptomatic children are infected\(^33\). Thus, children residing in developing countries such as Chile offer a unique opportunity to investigate the host response to *H. pylori* in the early stage of infection.

In our study of *H. pylori*-infected subjects in Chile, the intensity of the gastritis in infected children was substantially less than that of infected adults, despite equivalent levels of *H. pylori* colonization. In addition, fewer infected children displayed gastric histopathology than infected adults. Importantly, *H. pylori*-infected children had significantly less ulceration than infected adults, consistent with the findings of others\(^10,34,35\). Differences in gastric histopathology between *H. pylori*-infected children and infected adults could not be attributed to differences in socioeconomic status or the distribution of CagA\(^+\) bacteria, since the children and adults had similar Graffar scores (a measure of socioeconomic status) and high levels of CagA seropositivity. Although the children may have had their infection for a shorter period of time than the adults, shorter duration of infection would not account for the reduced gastritis in the children, since the onset of *H. pylori*-induced histopathology is relatively rapid\(^36\). Therefore, we
investigated whether the reduced inflammation in *H. pylori*-infected children could be the consequence of an enhanced Treg cell response capable of down-modulating *H. pylori*-induced inflammation, which is T-cell driven\(^ {37} \). We report here that the antral mucosa of *H. pylori*-infected children contained significantly more Treg cells and higher levels of protein and mRNA for the Treg cell cytokines TGF-β1 and IL-10 compared to that of infected adults (and uninfected children). The TGF-β1 localized predominantly to CD25\(^ + \) and Foxp3\(^ + \) cells in the lamina propria in infected children, implicating *H. pylori*-induced Treg cells as a major source of the TGF-β1. Conversely, the gastric mucosa of infected children displayed no increase in IFN-γ mRNA compared to infected adults. Interestingly, the childhood acquisition of *H. pylori* is associated with reduced risks for asthma and allergy\(^ {38} \), possibly due to allergen induction of systemic Treg cells that localize to the mucosae, as reported in a mouse model of dust mite-induced asthma\(^ {39} \).

The findings presented here suggest that local Treg cell activity in children down-regulates the Th1-mediated inflammation typical of *H. pylori*-associated gastritis in adult hosts\(^ {14-20} \). This down-regulation is consistent with the 10-fold lower level of IFN-γ, a key Th1 cytokine, in the gastric mucosa of children compared to that of adults reported by Bontems et al.\(^ {36} \). In this connection, Th1 cells are reportedly more susceptible than Th2 cells to Treg suppression\(^ {40} \). In support of these concepts, Raghavan et al.\(^ {41} \) showed in a mouse model of *H. pylori* infection that the absence of Treg cells is associated with increased gastric pathology, and Lundgren et al.\(^ {42} \) showed that circulating *H. pylori*-specific Treg cells suppress memory T-cell responses to *H. pylori* in infected persons. In addition, Kullberg et al.\(^ {43} \) and Maloy et al.\(^ {44} \) have shown that Treg cells down-modulate *H. hepaticus*-induced colonic inflammation in a mouse model of inflammatory bowel
disease through the release of TGF-β1 and IL-10. Our findings indicate that a Treg-mediated response is also active in the gastric mucosa of children during *H. pylori* infection. Whether this Treg response is associated with the young age of the host(s) or early *H. pylori* infection warrants further investigation.

*H. pylori* has long been appreciated as a major cause of antral gastritis in children\(^4\). Our results raise the possibility that early events in *H. pylori*-infected gastric mucosa play an important role in the progression of *H. pylori* inflammatory disease in young hosts, limiting more severe clinical outcomes such as ulceration. Understanding how *H. pylori* induces Treg cells in early infection and how Th1 cells appear to eventually override the Treg cell influence on *H. pylori*-induced inflammation in adults warrant critical investigation. Promoting or prolonging Treg cell responses could serve to blunt the inflammatory response associated with *H. pylori* colonization and possibly the sequelae of long-term infection such as gastric cancer. This is particularly relevant to developing countries such as Chile, where gastric cancer is the leading cause of cancer-related mortality\(^9\) and where early *H. pylori* infection has been related to the development of gastric adenocarcinoma\(^4\).

*H. pylori* has infected humans for millennia, having been identified in 3,000 year old South American mummies\(^4\). In native South American peoples, the seroprevalence for *H. pylori* does not correlate with the length of contact with the outside world\(^5\), suggesting that *H. pylori* is indigenous to South American Indians and was not introduced by contact with outsiders. Thus, the gastric Treg activity in *H. pylori*-infected children may reflect a homeostatic mechanism by gut-associated lymphoid tissue (GALT) that evolved in response to early colonization of the human species. The loss of Treg
responses in the gastric mucosa of adults, however, is currently unexplained and different from that observed in chronically infected mice\textsuperscript{48}. Nevertheless, the increased level of TGF-\(\beta\)1 in the gastric mucosa of uninfected children compared to uninfected adults shown here is consistent with a pre-existing or higher “set-point” for Treg responses in children. Elucidating the role of Treg activity in \textit{H. pylori}-infected children will provide new insights into \textit{H. pylori} pathogenesis in early infection.
REFERENCES


Table 1. Characteristics of the Chilean study population

<table>
<thead>
<tr>
<th></th>
<th>≤ 12 yr (n = 36)</th>
<th>&gt; 12 yr (n = 79)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (years± SD)</td>
<td>10.7±1.9</td>
<td>26.2±15.9*</td>
</tr>
<tr>
<td>Gender (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>22 (61)</td>
<td>49 (57)</td>
</tr>
<tr>
<td>Male</td>
<td>14 (39)</td>
<td>36 (43)</td>
</tr>
<tr>
<td>Socioeconomic status (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low (Graffar 1, 2)</td>
<td>20 (56)</td>
<td>27 (34)</td>
</tr>
<tr>
<td>Medium (Graffar 3)</td>
<td>12 (33)</td>
<td>34 (43)</td>
</tr>
<tr>
<td>High (Graffar 4, 5)</td>
<td>4 (11)</td>
<td>18 (23)</td>
</tr>
<tr>
<td>Indication for endoscopy (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recurrent abdominal pain</td>
<td>21 (58)</td>
<td>43 (54)</td>
</tr>
<tr>
<td>Burning abdominal discomfort</td>
<td>6 (17)</td>
<td>9 (11)</td>
</tr>
<tr>
<td>Hematemesis</td>
<td>3 (8)</td>
<td>11 (14)</td>
</tr>
<tr>
<td>Chronic vomiting</td>
<td>2 (6)</td>
<td>2 (3)</td>
</tr>
<tr>
<td>Other</td>
<td>2 (6)</td>
<td>8 (10)</td>
</tr>
<tr>
<td><em>H. pylori</em> infection (%)</td>
<td>18 (50)</td>
<td>51 (65)</td>
</tr>
</tbody>
</table>

*p < 0.05
Table 2. Frequency of inflammatory changes in Chilean children and adults with and without *H. pylori* infection

<table>
<thead>
<tr>
<th></th>
<th>Children (≤ 12 yr)</th>
<th>Adults (&gt; 12 yr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H. pylori (-) n=18</td>
<td>H. pylori (+) n=18</td>
</tr>
<tr>
<td>Polymorphonuclear cell infiltration</td>
<td>0</td>
<td>9 (50%)&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mononuclear cell infiltration</td>
<td>1 (6)</td>
<td>14 (78)&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lymphoid follicles</td>
<td>2 (11)</td>
<td>6 (33)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Atrophy</td>
<td>0</td>
<td>1 (6)</td>
</tr>
<tr>
<td>Intestinal metaplasia</td>
<td>0</td>
<td>(0)</td>
</tr>
<tr>
<td>Ulceration</td>
<td>0</td>
<td>4 (22)&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> p < 0.05 for comparison between infected children and infected adults
<sup>b</sup> p < 0.001 for comparison between infected children and non-infected children
<sup>c</sup> p < 0.05 for comparison between infected and non-infected adults
Figure 1. Histological features of childhood and adult *H. pylori* infection. (A) A representative gastric tissue section from an *H. pylori*-infected child shows minimal polymorphonuclear and mononuclear cell infiltration in contrast to (B) a representative gastric tissue section from an infected adult, which shows dense polymorphonuclear and mononuclear cell infiltration, a lymphoid follicle and epithelial regeneration (mag 200x). (C) Levels of *H. pylori* colonization in coded gastric tissue specimens from *H. pylori*-infected children (n=18) and adults (n=51) were similar, based on colonization scores. (D) Analysis of the same gastric tissue specimens in C showed the *H. pylori*-infected children had less severe gastric inflammation than the infected adults, reflected in a lower histology score.
Figure 2. CD25^+Foxp3^+ cells in the gastric mucosa of children and adults infected with *H. pylori*. (A, left panels) For control staining, serial sections of ileal mucosa from a patient with Crohn’s disease were stained with mouse anti-CD25 antibodies followed by green fluorescence-labeled goat anti-mouse IgG antibodies, or mouse anti-Foxp3 antibodies followed by red fluorescence-labeled goat anti-mouse IgG antibodies, and examined by confocal microscopy. Merge of the upper and middle panels shows lamina propria CD25^+ cells were also Foxp3^+ (*A, right panels*) Gastric mucosa from an *H. pylori*-infected adult and child stained similarly with anti-CD25 and then anti-Foxp3 antibodies as above and examined by confocal microscopy shows a CD25^+Foxp3^+ cell in the adult gastric mucosa and multiple CD25^+Foxp3^+ cells in the gastric mucosa of the child. (B) Foxp3^+ cells coded in gastric biopsies from nine adults and nine children with *H. pylori* gastritis were enumerated as described in the Materials and Methods. The number of Foxp3^+ cells in the gastric mucosa of infected children was 4-fold more prevalent than in the gastric mucosa of the adults (p<0.03). Inset shows typical Foxp3^+ cells with nuclear location of the transcription factor.
Figure 3. Regulatory cytokine protein and mRNA in the gastric mucosa of *H. pylori*-infected and uninfected children and adults. (A) Gastric biopsies from the subjects represented in Fig. 1 were homogenized, centrifuged and the supernatants analyzed by immunosorbent and bicinchoninic acid assays for cytokine and protein levels, respectively. Gastric levels of TGF-β1 and IL-10 were significantly higher in the infected children compared to the adults (a,e = p<0.01; b,c,d = p<0.05). (B) Cytokine mRNA expression in the gastric mucosa of *H. pylori*-infected children compared to uninfected children and in the gastric mucosa of infected adults compared to uninfected adults was analyzed by real-time PCR and expressed as fold-differences. TGF-β1 and IL-10 in mRNA levels were substantially higher in the infected versus uninfected children compared to the adults; conversely, IFN-γ mRNA was unchanged in the infected versus uninfected children and sharply elevated in the infected adults.
Figure 4. Gastric Treg cells are a source of TGF-β1. Gastric tissue sections from a child infected with *H. pylori* were analyzed by immunofluorescence and immunohistochemistry for CD25, TGF-β1 and Foxp3 as described in the Materials and Methods. Gastric mucosa shows colocalization of CD25 and TGF-β1 (Left panels) and colocalization of Foxp3 and TGF-β1 in a lamina propria mononuclear cell (Right upper panel) but TGF-β1 only in crypt epithelial cells (Right lower panel).
TH17 CELL RESPONSE TO HELICOBACTER PYLORI INFECTION IS DOWN-REGULATED IN CHILDREN

by

SHELTON W WRIGHT, CAROLINA SERRANO, DIANE BIMCZOK, ANDREA VILLAGRAN, INGRID MANSILLA, ELSA BRUCE, MANUEL ALVAREZ, ALEJANDRO VENEGAS, LESLEY E. SMYTHIES, PAUL R. HARRIS AND PHILLIP D. SMITH

In preparation for *Gastroenterology*

Format adapted for thesis
ABSTRACT

**Background & Aims:** *Helicobacter pylori* infection in children infrequently causes gastroduodenal ulceration and is associated with increased local regulatory T-cell (Treg) activity. Because of the reciprocal relationship of Treg and IL-17-producing T-helper cells (Th17), we evaluated the mucosal Th17 response in *H. pylori*-infected children and adults. **Methods:** Gastric tissue from 36 children and 25 adults with abdominal symptoms in Santiago, Chile was evaluated prospectively for *H. pylori*, histopathology and Th17 activity using immunoassay and real-time polymerase chain reaction. *H. pylori* isolates were genotypically analyzed. **Results:** Nine (24%) of the children and 8 (32%) of the adults were infected with *H. pylori*. Children and adults were colonized with similar levels of the bacteria and all bacterial isolates were genotypically similar. However, the level of gastritis in the infected children was reduced compared to infected adults (*P*<0.05). Associated with the reduced gastritis, infected children had reduced mRNA expression of Th17 cytokine (IL-17) as well as IFN-γ (*P*<0.05). Furthermore, infected children had lower levels of IL-17⁺, CD3⁺ cells in their gastric mucosa compared to infected adults (*P*<0.05). **Conclusions:** Th17 cell responses to *H. pylori* infection are reduced in children compared to adults, suggesting that Treg cells may down-modulate Th17 activity in children, decreasing inflammation induced by *H. pylori* in adults.
INTRODUCTION

*Helicobacter pylori* is the most common gastrointestinal pathogen worldwide and is the primary cause of gastritis, peptic ulcer disease and gastric adenocarcinoma\(^1\). *H. pylori* infection is relatively common among adults in the United States\(^2\), though infection in children is relatively rare\(^3,4\). In developing countries, *H. pylori* infection is common in both children and adults, with approximately 80% of children under the age of 10 infected in some South American countries\(^5,6\). Gastric ulceration during pediatric *H. pylori* infection is rare with children experiencing reduced symptoms and decreased gastric histopathology compared to adults\(^7,8,9,10\). In Chile, the bacterium is acquired during childhood and gastric adenocarcinoma is a leading cause of cancer-related mortality\(^11,12\).

In adults, *H. pylori* infection promotes a mucosal Th1 response, driving local inflammation by interactions between the host’s mucosal immune response and the bacteria’s multiple virulent products\(^13-15\). A prolonged inflammatory response is implicated in the *H. pylori*-associated sequelae of gastritis, gastroduodenal ulceration and, ultimately, neoplasia\(^1\). Conversely, the pathogenesis of *H. pylori* disease in children has received little investigative attention. Previously, we reported a heightened regulatory T-cell (Treg) response to *H. pylori* infection in children\(^9\). The up-regulation of Tregs in infected children is associated with reduced histologic and macroscopic gastric inflammation. This finding indicates a possible down-modulation of the inflammatory response associated with *H. pylori* infection in children.

Recent studies implicate Th17 cells in the inflammatory response of adults and animal models to *H. pylori* infection\(^16-18\). Under physiologic conditions, Th17 cells can help support the epithelium against pathogens. However, excess Th17 activity can lead
to chronic inflammation and has been implicated in several gastro-intestinal autoimmune diseases, including Crohn’s disease\textsuperscript{19,20}. Naïve T-helper cell exposure to specific cytokines (IL-6 and TGF-β) and the activation of the transcription factor retinoic acid receptor-related orphan receptor α (RORα) are critical in steering the differentiation pathway towards either Treg or Th17 development\textsuperscript{21,22}. The direct regulatory interaction between these two T cell populations, as well as their divergent lineage, indicate that the Treg/Th17 balance may play a critical role in the development of a pro-inflammatory response to infection. Children, with a robust regulatory T cell response, may down-modulate a Th17 response to infection. Here we extend our previous findings regarding increased Treg activity in \textit{H. pylori}-infected children by investigating the relationship between Th17 cells and the reduced inflammatory response to \textit{H. pylori} infection in children.
MATERIALS AND METHODS

Patients. Sixty-one consecutive subjects with abdominal symptoms residing in Santiago, Chile and the surrounding area, including 36 children less than 12 years of age and 25 adults greater than 12 years of age who present for esophagogastroduodenoscopy were enrolled in this institutional review board-approved study as previously described. Criteria for patient inclusion were symptoms of peptic disease, including recurrent abdominal pain, burning abdominal discomfort, hematemesis or chronic vomiting. Exclusion criteria included a history of antibiotic, antacid, H2-blocker or proton-pump inhibitor, bismuth compound or non-steroidal anti-inflammatory drug usage in the previous 2 weeks as well as history of any type of autoimmune disease. Patients’ clinical history was provided by the patient or parent/guardian. Patients’ socioeconomic status was classified according to a locally validated version of the Graffar score. Written informed consent was obtained from either the patient or the patient’s parent/guardian.

Assessment of H. pylori infection. Each patient underwent serum collection and esophagogastroduodenoscopy. Eleven antral biopsies were obtained as previously described. One specimen was subjected to a rapid urease test (Rapid Urea Test; Bios Chile, Santiago, Chile) to detect the presence of H. pylori. Two specimens were formalin-fixed, paraffin-embedded, sectioned and treated with hematoxylin and eosin (H&E) for histologic analysis and Warthin-Starry silver stain for H. pylori detection. A subject was considered to be colonized with H. pylori if either the rapid urease test or the microscopic evaluation were positive for H. pylori.
Evaluation of gastric *H. pylori* colonization and histopathology. Serial sections of the H&E treated gastric specimens were evaluated by a pathologist blinded to the results of other assays. Tissue sections were graded according to the updated Sydney classification for the level of inflammation\(^\text{24}\). Briefly, 5 random fields in each of the 3 tissue sections per biopsy were graded for the presence and level of polymorphonuclear and mononuclear cell infiltrations, number of lymphoid follicles, mucosal atrophy and intestinal metaplasia. A mean histology score was calculated by adding the mean individual scores for each parameter to quantify the level of gastritis. Tissue sections stained with Warthin-Starry silver stain were evaluated for *H. pylori* colonization. A colonization score was calculated based on *H. pylori* density (0=absent, 1=mild, 2=moderate, 3=intense). In addition, the frequency of each inflammatory parameter as well as endoscopically identifiable ulceration was recorded.

Genotypic identification of *H. pylori* isolates. One gastric biopsy was placed in brucella broth immediately frozen in liquid nitrogen. Specimens were subsequently cultured on plates with brucella agar and 5% horse blood with an *H. pylori*-selective supplement (Dent), containing vancomycin (10 mg/liter), trimethroprim lactate (5 mg/liter), cefsulodin (5 mg/liter), and amphotericin B (5 mg/liter). The plates were cultured in a carbon-dioxide infused incubator for up to 7 days with subsequent single colony transference to a new plate and incubation repeated twice more. A single colony on the third plate in the transfer series was identified as *H. pylori* using a rapid urease test (Rapid Urea Test, Bios Chile, Santiago, Chile). DNA from the remaining colonies was extracted using the Qiagen DNeasy (Qiagen, Valencia, Ca) Minikit. DNA levels were quantified by spectrophotometry. Using primer sequences for 6 *H. pylori* housekeeping
genes (*ureI*, *mutY*, *efp*, *ppa*, *atpA* and *trpC*) as well as two pathogenic genes (*cagA* and *vacA*), bacterial gene sequences were amplified by PCR, as previously described\textsuperscript{25-27}. Strains without *cagA* amplification by PCR were confirmed as *cagA* negative by amplifying the *cag* pathogenicity island empty site as previously described\textsuperscript{28}. PCR products were subsequently purified using the Wizard SV gel and PCR Clean-up kit (Promega, Fitchburg, WI) and stored at -20\(^0\)C. PCR products for all 8 gene sequences were sequenced by the University of Alabama at Birmingham DNA Sequencing and Analysis Core.

To analyze the relationship between strains of *H. pylori* infecting patients in this study and strains from international *H. pylori* isolates, we searched a database for multilocus sequence typing (MLST) \url{http://pubmlst.org/helicobacter} for 31 isolates with appropriate geographic and species-specific distribution. Concatenated nucleotide sequences for 6 *H. pylori* MLST loci (*ureI*, *mutY*, *efp*, *ppa*, *atpA* and *trpC*) from 8 isolates infecting children and 3 isolates infecting adults as well as the 31 isolates retrieved from the MLST database were analyzed using Mega4 software\textsuperscript{29}. Sequences were aligned using the ClustalW algorithm and phylogenetic relationships were produced using the Kimura 2-parameter model for nucleotide substitution and neighbor-joining clustering. A phylogenetic structure was constructed as a bootstrap consensus tree inferred from 1000 replicates.

To analyze the s-region of *vacA*, sequence data from 8 infected children and 4 infected children was compared to variants of four known *vacA* s-region subtypes (s1a, s1b, s1c and s2). After comparative analysis, each sequence was classified accordingly\textsuperscript{30}.  

35
Consensus sequences of cagA were translated into amino acid sequences using Mega4. As previously described, sequences were categorized based on the C or D repeat sequences present in the EPIYA motif\textsuperscript{31,32}.

**Identification and enumeration of gastric Th17 cells.** One gastric specimen was snap frozen in Tissue Freezing Media (TBS, Durham, NC) for immunofluorescence. Frozen sections of 5 \(\mu\)m were fixed by acetone (10 min), rinsed in PBS with .005\%Tween, blocked in casein protein (30 min at room temperature, CSA system; DAKO), blocked and incubated sequentially with goat anti-human IL-17 (10 \(\mu\)g/ml; R&D Systems, Minneapolis, MN) and rabbit anti-human CD3 (1:300, Sigma-Aldrich, St. Louis, MO) for 4 h at room temperature, Cy3 donkey anti-goat IgG (1:200, Jackson Immunity, West Grove, PA) and fluorescein isothiocyanate-streptavidin donkey anti-rabbit IgG (1:50, Jackson Immunity, West Grove, PA) for 30 min at room temperature, and counterstained with DAPI (Calbiochem, San Diego, CA) for 5 min at 25\(^\circ\)C. Antibody controls were included with each staining experiment.

To quantify the number of Th17 cells, gastric biopsies from 8 \(H.\ pylori\)-infected children, 8 \(H.\ pylori\)-infected adults and 5 uninfected children and 5 uninfected adults were examined for IL-17\(^+\), CD3\(^+\) cells. Digital image analysis was performed using ImageJ software (version 1.43, National Institute of Health, http://rsbweb.nih.gov/ij/). Density of red (IL-17) and green (CD3) cells in the lamina propria was determined as percent positive pixels per area using the “analyze particles” tool. Colocalization of red and green pixels was determined using the JaCOP plugin (Mander’s colocalization coefficients)\textsuperscript{33}. Images were thresholded to exclude background staining and regions of
interest were set to exclude surface and glandular epithelial cells. Three or more independent areas from 2 slides per specimen were analyzed.

**Real-time PCR analysis for cytokine gene expression.** One gastric specimen was immediately frozen in liquid nitrogen and kept at -80\(^\circ\)C. Samples were then submerged in a liquid nitrogen-filled RNase-free plastic mortar and homogenized with an RNase-free plastic pestle. Total RNA was extracted using the Qiagen RNeasy (Qiagen, Valencia, CA) Minikit, and RNA concentration and purity were determined by spectrophotometry. RNA was reverse transcribed into first-strand cDNA using the Superscript II cDNA synthesis kit (Invitrogen, Carlsbad, CA). Real-time reverse transcription PCR (RT-PCR) of cDNA was performed with custom-made primer sets and SYBR green PCR mix using either an Applied Biosystems 7500 Real-time PCR system or a Stratagene MX3000P real-time PCR machine. The following forward and reverse primer pair for IL-17 (F: ACCAATCCCCAAAAGGTCTTC, R: GGGGACAGAGTTTCATGTGGT) and IFN-\(\gamma\) (F: GAATTGGAAAGAGGAGTGAC, R: TGTATGGCTTTGCGTTGGAC) were used for amplification. For a reference gene, GAPDH forward and reverse primers were used (F: AACCTGCCAAATATGATGAC, R: GTTGTCATACCAGGAAATGAG). Standard curves were developed by inserting vector plasmids of PCR products into *E. coli* using the pGEM-T Easy Vector System (Promega, Litchburg, WI), extracting total DNA using the Qiagen DNeasy (Qiagen, Valencia, CA) minikit, amplifying the DNA using PCR and then purifying the resulting PCR products using the Wizard SV gel and PCR Clean-up kit (Promega, Fitchburg, WI). mRNA levels were analyzed by comparing fold change differences of the level of
detection by the real-time PCR machine as well as by using the developed standard curve to quantify cytokine levels in pg/µl, with GAPDH as a reference gene in both cases.

Statistical analysis. Comparisons between groups were performed using the Student t test of variance with the Welch correction and Mann Whitney U test. Statistical significance was defined as a P value of less than .05.

RESULTS

Reduction frequency of gastric mucosal atrophy in H. pylori-infected children compared with infected adults. To determine the level of inflammation in the antral mucosa associated with H. pylori disease, we evaluated gastric tissue sections from H. pylori-infected and uninfected children and adults for the frequency of inflammatory changes and ulceration (Table 1). Among 9 H. pylori-infected children, 8 (89%) showed gastric polymorphonuclear cell infiltration, 8 (89%) showed mononuclear cell infiltration, and 7 (88%) showed lymphoid follicles. Among 8 H. pylori-infected adults, 8 (100%) showed gastric polymorphonuclear cell infiltration, 8 (89%) showed mononuclear cell infiltration, and 7 (88%) showed lymphoid follicles. Atrophy of the gastric mucosa was not present in any children but was found in 5 (63%) of the H. pylori-infected adults (P<0.05). Intestinal metaplasia was found in none of the H. pylori-infected children but was present in 3 (38%) of the H. pylori-infected adults(P<0.05). Finally no ulceration was observed endoscopically in any of the children but was seen in 3 (18%) of the uninfected adults and 1 (13%) of the infected adults. Overall these findings indicate that mucosal atrophy, an important feature of gastritis associated with gastric neoplasia, as well as intestinal metaplasia are observed significantly less frequently in H. pylori-infected children compared to infected adults.
Reduced gastritis in *H. pylori*-infected children compared with infected adults. Having detected similar rates of several inflammatory markers, in the presence of decreased atrophy, in the gastric mucosa of *H. pylori*-infected children compared to infected adults, we next quantified the level of inflammation in these same gastric samples. The level of gastritis is reflected in the histology scores based on the updated Sydney classification. The histology scores of both *H. pylori*-infected children and adults were greater than the scores of their uninfected counterparts (P<0.05) (Fig. 1). Importantly, the mean histology score of *H. pylori*-infected adults was significantly higher than that of infected children (P<0.05). Furthermore, uninfected adults had similar scores to uninfected children, indicating that while *H. pylori*-infected children have lower levels of gastritis compared to infected adults, uninfected adults do not have elevated baseline gastritis.

Similar bacterial colonization of *H. pylori*-infected children compared to infected adults. In order to determine whether the experienced reduction in gastric inflammation is due to differences in the infecting bacteria and not the host response, we evaluated coded gastric tissue sections from the *H. pylori*-infected and uninfected subjects for levels of *H. pylori*. Based on visual assessment of the bacteria, the level of *H. pylori* colonization in the gastric specimens from the infected children was similar to that of infected adults (2.2±0.28 vs. 1.5±0.27). Because of the diverse heterogeneity of *H. pylori*, especially in South America, and the variability of strain pathogenicity, as well as a lack of genotypic data regarding Chilean strains of *H. pylori*, we next analyzed the genotypic variability of the *H. pylori* isolates infected study subjects. Using MLST, isolates of *H. pylori* infecting study subjects were genotypically compared to globally
representative strains of *H. pylori* (Fig. 2)\(^{25}\). All study strains were classified in the European population (hp-Europe) of *H. pylori* with no strains categorized into either the hp-Africa1 or the more pathogenic hp-EastAsia population. Because of the high degree of strain variability in some South American countries\(^{34,35}\), we next analyzed the genotypes of *vacA* and *cagA* to ensure that a European strain of *H. pylori* in Chile had not acquired a more pathogenic form of one of these genes. Specific tyrosine residues within repeating Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs on *cagA* were analyzed and classified according to the presence of Western type (ABC, ABCC) regions or Eastern-type (ABD) regions\(^{31,32}\). In a limited sample, 3 *cagA* genes were analyzed (2 in children, 1 in an adult) and all were classified as Western forms of *cagA* (either ABCC or ABC) (Table 2). Importantly, based on PCR detection of the *cagA* gene, 3 (37%) of strains infecting children were *cagA* positive which was similar to that of infected adults, 2 (50%). All strains that did not amplify a *cagA* PCR product were determined to not have the *cagA* gene by amplification of the *cagA* empty site. Finally, the s-region of the *vacA* gene was analyzed, compared to known variable types and categorized as s2, s1b, s1a, or s1c (listed in increasing order of presumed pathogenic potential)\(^{30,36,37}\). In infected children, 3 (37%) of the *vacA* genes contained s1b regions compared to 2 (50%) of infected adults. Five (63%) of infected children had strains with an s2 region compared to 2 (50%) of infected adults. Taken together, these findings indicate that *H. pylori*-infected children and adults were colonized with similar levels of *H. pylori* and were infected with bacteria sharing similar genetic variability as well as similar pathogenic potential.
**Decreased levels of gastric IL-17 and IFN-γ in *H. pylori*-infected children compared with infected adults.** Having determined that the *H. pylori*-infected children have reduced gastritis with similar bacterial colonization, we next sought to determine the role of various local inflammatory responses to infection. As we have previously shown that *H. pylori*-infected children have increased Treg activity, and because Treg’s have a reciprocal relationship with Th17 cell differentiation—an important mediator of inflammation in *H. pylori*-infected adults—we next sought to determine whether the gastric mucosa of *H. pylori*-infected children expresses less Th17- and Th1-related cytokines compared to infected adults. To determine levels of cytokine expression, we quantified mRNA expression of IL-17 (Th17) and IFN-γ (Th1) in the antral mucosa of infected and uninfected children and adults. When normalized to the reference gene GAPDH, levels of IL-17 were significantly higher in *H. pylori*-infected adults compared to infected children (*P*<0.05) (**Fig. 3A**). Interestingly, while *H. pylori*-infected adults had significantly higher levels of IL-17 mRNA expression compared uninfected adults (*P*<0.05), infected children and uninfected children and adults all had similar levels of IL-17. Levels of IFN-γ mRNA, a Th1 effector cytokine, were also significantly greater in *H. pylori*-infected adults compared to infected children (*P*<0.05) (**Fig. 3B**). Furthermore, no differences were observed in mRNA levels of IFN-γ between infected children and uninfected children and adults. This same data can be represented as fold differences of infected children and adults compared to their uninfected counterparts (**Fig. 3C**). *H. pylori*-infected adults expressed almost 3-fold more IL-17 mRNA compared to uninfected adults, compared to only the 1-fold increase in IL-17 mRNA expression seen in infected children compared to uninfected children. Furthermore, supporting our
previous findings, infected adults expressed nearly 30-fold more IFN-γ mRNA over uninfected adults, compared to only a 2-fold increase in infected children over uninfected children. Taken together, these findings indicate that *H. pylori*-infected children express less Th17-related cytokine in their gastric mucosa compared to infected adults.

**Decreased prevalence of IL-17\(^+\),CD3\(^+\) Cells in the gastric mucosa of *H. pylori*-infected children compared to infected adults.** To determine whether the low levels of IL-17 in the gastric mucosa in response to *H. pylori*-infection children is associated with reduced number of Th17 cells, we next sought to quantify the prevalence of IL-17\(^+\),CD3\(^+\) cells in the gastric mucosa of *H. pylori*-infected and uninfected children and adults. In the gastric mucosa of *H. pylori*-infected adults, IL-17 colocalized to CD3\(^+\) cells, as seen in a representative section (Fig. 4). In order to quantify this observed colocalization in study subjects, we analyzed specimen images using ImageJ software. In *H. pylori*-infected adults, significantly higher area fractions of gastric lamina propria (with epithelial cells excluded) were positive for IL-17 (Fig. 5A)\( (P=.003)\) as well as CD3 (Fig. 5B) \( (P<0.05)\) compared to infected children (Table 3). IL-17 overlap with CD3, as determined by Manders’ colocalization coefficients, was significantly increased in infected adults compared to infected children. Interestingly, infected children had significantly higher area fractions for IL-17 \( (P<0.05)\) but not CD3 compared to uninfected children. Furthermore, cells positive for IL-17 in infected children did not colocalize to those positive for CD3. Take together, these findings indicate a lower number of IL-17\(^+\),CD3\(^+\) cells are present in the lamina propria of *H. pylori*-infected children compared to infected adults.
DISCUSSION

*H. pylori* infection serves as a major cause of global morbidity and mortality, especially in developing countries\textsuperscript{11,12}. In South America, infection occurs at a relatively young age, with broad ranges of seroprevalence between 30-80%\textsuperscript{10}. In Chile, up to 86% of children with abdominal symptoms consistent with gastritis are infected with the bacteria\textsuperscript{38}. *H. pylori* infection is rare in children in developed countries\textsuperscript{4} and only 11% of those with abdominal symptoms are infected with *H. pylori*\textsuperscript{39}. Previously, we identified an association between a gastric Treg response to *H. pylori* infection in Chilean children and low levels of gastric histopathology\textsuperscript{9}. Therefore, Chilean children serve as a unique and ideal population to study the gastric mucosal immune response to *H. pylori* infection.

In our study, using a nearly identical recruitment strategy to our previous report, we extend our findings that the intensity of the gastritis in *H. pylori*-infected children is significantly reduced compared to infected adults, even though both groups had similar levels of colonization. Furthermore, gastric mucosal atrophy and intestinal metaplasia were significantly more frequent in *H. pylori*-infected adults. In order to determine whether the reduced gastric histopathology in *H. pylori*-infected children is due to the strain of bacteria and not the host immune response, we genotypically identified the strains of *H. pylori* infecting both adults and children in this study. By using three different methodologies for genotyping the bacterium, we show that the strains of *H. pylori* infecting the children and adults are similar in respect to highly conserved gene variability as well as in terms of two pathogenic genes, *cagA* and *vacA*. Furthermore, rates of *cagA* positivity, regardless of genotype, were similar between the two groups. Because our prior study showed that the reduced gastric histopathology in children is
associated with an increased mucosal Treg response, we investigated whether a Th17 response, which can be directly affected by Treg differentiation, is reduced in *H. pylori*-infected children compared to infected adults. Here, we report that the antral mucosa of *H. pylori*-infected children contains significantly lower levels of mRNA for the Th17 cytokine IL-17. By immunofluorescence, the gastric mucosal lamina propria of *H. pylori* infected children had significantly fewer IL-17^{+} and CD3^{+} cells compared to infected adults. Furthermore, colocalization of the IL-17 with CD3 occurred significantly less in *H. pylori*-infected children compared to infected adults, indicating the presence of fewer IL-17^{+}, CD3^{+} cells.

The findings here suggest that the robust Th17 response associated with *H. pylori* disease in adults is relatively down-regulated in infected children. Evidence of Th17 down-regulation supports the role of Treg cell activity in modulating the inflammatory response to *H. pylori* infection. Furthermore, this is the first study to show an inverse relationship between gastric histopathology and a Th17 response in *H. pylori*-infected children. This mediation of a Th17 response is supported by evidence regarding the interaction between Th17 and Treg cell differentiation. Th17 differentiation is thought to be initiated by IL-6 and IL-21-mediated signal transducer and activator of transcription 3 (STAT3)^{21}. STAT3 subsequently induces expression of the transcription factor retinoic-acid-receptor-related orphan receptor-α and -γ (RORα, RORγt). These transcription factors activate the Th17-cell-associated gene-expression program with subsequent effector cytokine development including IL-17, IL-21 and IL-22^{21}. Recent studies indicate that synergistic activation of IL-6 and TGF-β, a Treg-associated cytokine, are required for the differentiation of a Th17 cell from an activated T cell^{40-42}. Tregs have
also been shown to facilitate Th17 differentiation from naïve T cells and the addition of exogenous TGF-β can replace the Treg role in differentiation\textsuperscript{40}. Furthermore, while TGF-β is a Treg-associated cytokine, TNF and IL-1 both increase Th17 differentiation in the presence of both TGF-β and IL-6\textsuperscript{40}. While TGF-β is necessary for Th17 differentiation, numerous Th17-related cytokines have directly antagonistic effects on Treg differentiation. IL-6 as well as IL-21, a Th17 effector cytokine involved in Th17 differentiation and induced by IL-6\textsuperscript{22}, inhibit the expression TGF-β-induced factor forkhead box P3 (Foxp3), preventing Treg differentiation\textsuperscript{43}. Conversely, Treg-associated factors can directly inhibit Th17 activity as well\textsuperscript{22}. IL-2, important in the development and peripheral expansion of Tregs, induces STAT5 expression which antagonizes STAT3 and promotes Treg generation. TGF-β-induced Foxp3 inhibits Th17 differentiation by antagonizing ROR\textgamma\textsuperscript{22}. Finally, retinoic acid appears to play a role in inducing Foxp3 and inhibiting Th17 cell differentiation\textsuperscript{44}. Thus, a reciprocal relationship between Th17 cell and Treg cell activity appears important in their respective developments. Our findings support this conclusion by showing a reduced Th17 response in a pediatric population previously shown to have a robust Treg response.

\textit{H. pylori} is well-characterized as a member of the stomach flora for a large percentage of the world’s children\textsuperscript{12,45}. Furthermore, the bacterium is known to produce antral gastritis in children\textsuperscript{7}. Our study provides further evidence that the milieu of the pediatric gastric mucosal immune system is unique and distinctly different from adults in its response to infection. Understanding how this response changes over time, in terms of years of infection as well as the developmental age of the host, can provide new insights into \textit{H. pylori} pathogenesis. Prior studies indicate that \textit{H. pylori}-infected children not
only have increased Treg activity compared to adults but that the pediatric Treg response is also more effective in reducing the Th1 response\textsuperscript{9}. Interestingly, patients with gastric adenocarcinoma have been shown to have increased levels of peripheral blood Treg’s, and increased Tregs are associated with poorer prognosis\textsuperscript{46}, indicating that an ineffective Treg response may reduce an inflammatory response enough to prevent eradication but not enough to prevent neoplastic development. \textit{H. pylori} has infected humans for thousands of years, with polymorphisms in the \textit{H. pylori} genome being used to track human migration\textsuperscript{33}. Therefore, a Treg response to \textit{H. pylori} may be an evolutionary byproduct of coadaptation between the bacterium and its host. Furthermore, \textit{H. pylori} infection in mice can induce a systemic Treg response which may account for the increases in asthma and atopy after \textit{H. pylori} eradication\textsuperscript{47}. While a systemic Treg response may prolong \textit{H. pylori} infection and increase relative risk for adenocarcinoma development, this modulation of the immune system may still provide the host with a relative survival benefit.

The interaction between Treg and Th17 cell lines, above the global down-modulating effects of Treg activity, may play a crucial role in the progression from the low levels of inflammation observed in the pediatric population to the profound pro-inflammatory response observed in \textit{H. pylori}-infected adults. The elevated levels of TGF-\textbeta in \textit{H. pylori}-infected adults may drive the production of Th17 cells in the absence of any negative regulators. Therefore, understanding which of the many mechanisms involved in the reciprocal relationship between Th17 and Treg cells acts as the “switch” from a Treg-driven response in children to a Th1/Th17 mediated response in adults is crucial in order to determine appropriate therapeutic targets. Elucidating the interaction
between Treg and Th17 activity in the natural history of *H. pylori* infection will provide important new insights for understanding the sequelae of *H. pylori* pathogenesis.
REFERENCES


Table 1. Frequency of inflammatory changes in Chilean children and adults with and without *H. pylori* infection

<table>
<thead>
<tr>
<th></th>
<th>Children</th>
<th>Adults</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>H. pylori (-)</em></td>
<td><em>H. pylori (+)</em></td>
</tr>
<tr>
<td>Polymorphonuclear cell infiltration</td>
<td>n=28</td>
<td>n=9</td>
</tr>
<tr>
<td></td>
<td>2 (7%)</td>
<td>8 (89%)*</td>
</tr>
<tr>
<td>Mononuclear cell infiltration</td>
<td>9 (32)</td>
<td>8 (89%)*</td>
</tr>
<tr>
<td>Lymphoid follicles</td>
<td>9 (32)</td>
<td>7 (88)</td>
</tr>
<tr>
<td>Atrophy</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Intestinal metaplasia</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ulceration</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*a* *p*<0.05 for comparison between infected and uninfected children

*b* *p*<0.05 for comparison between adults and uninfected children

Table 2. Frequency and genotype of *vacA* and *cagA* isolated from *H. pylori* infecting Chilean children and adults

<table>
<thead>
<tr>
<th></th>
<th>Children</th>
<th>Adults</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>H. pylori (+)</em></td>
<td><em>H. pylori (+)</em></td>
</tr>
<tr>
<td></td>
<td>n=8</td>
<td>n=4*</td>
</tr>
<tr>
<td><em>cagA</em> positive</td>
<td>3 (37%)</td>
<td>2 (50%)</td>
</tr>
<tr>
<td>ABC</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>ABCC</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><em>vacA</em> positive</td>
<td>8 (100)</td>
<td>4 (100)</td>
</tr>
<tr>
<td>s1b</td>
<td>3 (37)</td>
<td>2 (50)</td>
</tr>
<tr>
<td>s2</td>
<td>5 (63)</td>
<td>2 (50)</td>
</tr>
</tbody>
</table>

*Two *cagA* PCR products were isolated from adult specimens. However, only 1 adult *cagA* PCR product was subsequently sequenced.*
Table 3. IL-17 and CD3 Fluorescence and Overlap in the Gastric Mucosa

Fluorescence is expressed in terms of mean % area as seen graphically in Figure 5A,B with standard error of the mean. Mean Mander’s coefficient expressing colocalization is listed as overlap coefficient where IL-17 colocalized to CD3 or CD3 colocalized to IL-17 with standard error of the mean.

<table>
<thead>
<tr>
<th></th>
<th>Fluorescence</th>
<th></th>
<th>Overlap Coefficient</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% IL-17 Area</td>
<td>SEM</td>
<td>% CD3 Area</td>
<td>SEM</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Children</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninfected</td>
<td>0.15</td>
<td>0.06</td>
<td>3.22</td>
<td>1.13</td>
</tr>
<tr>
<td><em>H. pylori-</em></td>
<td>0.49&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.11</td>
<td>4.32</td>
<td>0.87</td>
</tr>
<tr>
<td>infected</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adults</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninfected</td>
<td>1.02</td>
<td>0.24</td>
<td>4.56</td>
<td>0.96</td>
</tr>
<tr>
<td><em>H. pylori-</em></td>
<td>1.34&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.19</td>
<td>8.30</td>
<td>1.63</td>
</tr>
<tr>
<td>infected</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>IL-17 on CD3</th>
<th>Mean</th>
<th>SEM</th>
<th>CD3 on IL-17</th>
<th>Mean</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.271</td>
<td>0.109</td>
<td>0.068</td>
<td>0.351</td>
<td>0.056</td>
<td>0.147</td>
</tr>
<tr>
<td>0.068</td>
<td>0.036</td>
<td></td>
<td>0.147</td>
<td>0.058</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>P<0.003 for comparison between infected adults and uninfected children

<sup>b</sup>P<0.05 for comparison between infected children and non-infected children

<sup>*</sup>P<0.05 for comparison between infected adults and non-infected children
Figure 1. Histological features of childhood and adult *H. pylori* infection. Analysis of gastric specimens showed that *H. pylori*-infected children had less severe gastritis compared to infected adults, as demonstrated by the histology score, a combination of five inflammatory features.
Figure 2. Phylogenetic structure of Chilean *H. pylori* strains. Chilean strains isolated from study subjects (denoted by black diamonds; ChP=child host, ChA=adult host) were compared to representative strains isolated from patients in diverse geographic locations. Strain designations and the countries where strains were isolated are listed. The nucleotide sequences of the concatenated MLST loci (*ureI, mutY, efp, ppa, atpA* and *trpC*) were aligned and compared. Neighbor-joining trees were constructed based on distances estimated by the Kimura 2-parameter model of nucleotide substitution. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the strains analyzed. The tree is drawn to scale, with the branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Major strain populations are noted as *hp-Europe*, *hp-Africa1* and *hpEastAsia* with subgroups *hspEAsia* and *hspAmerind*. All 10 *H. pylori* strains were classified in *hp-Europe*. 
Figure 3. Effector cytokine mRNA in the gastric mucosa of *H. pylori* infected and uninfected children and adults. (A, B) Cytokine mRNA expression in the gastric mucosa normalized to expression of GAPDH, a housekeeping gene. (A) IL-17 cytokine expression is *H. pylori*-infected was significantly higher compared to infected children and uninfected adults. IL-17 mRNA expression in infected and uninfected children was similar. (B) IFN-γ cytokine expression in *H. pylori* infected adults was significantly higher compared to infected children. *H. pylori*-infected children expressed similar levels of mRNA compared to uninfected children. (C) The same data set as seen in (A, B) normalized to uninfected subjects and represented as fold differences of cytokine mRNA expression in infected children and infected adults compared to their uninfected counterparts. *H. pylori*-infected adults expressed substantially higher levels of IL-17 and IFN-γ over uninfected adults compared to infected children relative to uninfected children.
Figure 4. Adults with *H. pylori* infection express Th17 cells. Gastric tissue sections from *H. pylori* infected and uninfected children and adults were analyzed by immunofluorescence for IL-17 and CD3, as described in the Methods section. A gastric mucosal section of an *H. pylori*-infected adult shows colocalization of cytoplasmic IL-17 with membranous CD3. In the merge panel, an IL-17CD3+ cell is also identified.
Figure 5. IL-17+ and CD3+ cells in the gastric mucosa of *H. pylori* infected and uninfected children and adults. Gastric biopsy sections from 8 infected children and adults and 5 uninfected children and adults were stained with goat anti-IL-17 antibodies or goat anti-CD3 antibodies as described in the Methods sections. Images were analyzed using ImageJ software, thresholded to reduce background interference and area fractions calculated as % pixels of antibody per total pixel area were recorded. (A) *H. pylori*-infected adults had significantly more IL-17+ cells in the gastric mucosa lamina propria compared to infected children. *H. pylori*-infected children had significantly more IL-17+ cells in the lamina propria compared to uninfected children. (B) *H. pylori*-infected adults had significantly more CD3+ cells in the gastric mucosa compared to infected children.
CONCLUSIONS

In South America, *H. pylori* infection occurs at a relatively young age\(^{17,18}\). Globally, *H. pylori* infection serves as a major cause of global morbidity and mortality, especially in developing countries\(^1\). In Chile, up to 86% of children with abdominal symptoms consistent with gastritis are infected with the bacteria\(^{19}\). Importantly, ulceration in children is rare\(^8\). Furthermore, while considerable evidence exists concerning the mucosal immune response of adults and animal models to *H. pylori* infection, little investigative attention has explored the basis for both the decrease in symptoms as well as the reduced pathology observed in infected children.

We explored the pediatric response to *H. pylori* infection by utilizing a population of infected children and adults in Santiago, Chile. Through this population, we identified an association between a gastric Treg response to *H. pylori* infection in Chilean children and low levels of gastric histopathology. *H. pylori* infection in children was not associated with the pro-inflammatory Th1 response characterized in adults. Rather, children responded to infection with a local increase in gastric Treg’s as well as Treg-related cytokines, TGF-\(\beta\) and IL-10. Furthermore, the TFG-\(\beta\) localized to CD25\(^+\), Foxp3\(^+\) cells in the gastric mucosa, indicating that Treg’s are responsible for this local increase in TGF-\(\beta\). Importantly, levels of IFN-\(\gamma\), a Th1-related cytokine were relatively decreased in infected children compared to infected adults.

We next investigated the mechanism by which the host response to infection changes over the lifespan of the host. Th17 cells, which recent evidence indicates play an important role in the inflammatory response to adult *H. pylori*-infection\(^{13,14}\), have a distinct reciprocal relationship with Treg’s\(^{20}\). Differentiation of both cell lines requires
TGF-β, a Treg-related cytokine. However, in the presence of IL-6, differentiation is driven towards Th17 generation and Treg development is inhibited through several mechanisms\textsuperscript{20}. In order to explore the role of Th17 cells in \textit{H. pylori}-infected children, we studied a similar group of infected Chilean children and adults. By controlling for level of colonization as well as the infecting strain of \textit{H. pylori}, we showed that \textit{H. pylori}-infected children response to infection with considerably attenuated Th17 activity. The gastric mucosa of infected children contained not only significantly fewer IL-17\textsuperscript{+}, CD3\textsuperscript{+} cells, but also expressed significantly less IL-17. Furthermore, levels of IFN-γ expression in both children and adults were relatively similar in the two studies.

Since children do not respond to \textit{H. pylori}-infection with a robust Th17 response, the development of this response may be critical in the natural history of the infection. Once T-cell differentiation switches from Treg development to Th17 development, not only would the inherently pro-inflammatory effects of Th17 effector cytokines drive the immune response but, perhaps more importantly, removal of Treg down-modulation could allow for the propagation of a pro-inflammatory cascade. Furthermore, while Treg’s are known to have some activity in \textit{H. pylori}-infected adults\textsuperscript{21}, low levels of TGF-β may paradoxically drive Th17 differentiation thus subsequently inhibiting Treg differentiation. Understanding the driving forces involved in this Treg/Th17 relationship is crucial in subsequent investigations. Determining what drives the differentiation of T cells into Th17 cells in response to infection is perhaps the most important of these steps. Dendritic cells (DC) were recently implicated in promoting a Th1 response to \textit{H. pylori} in the gastric mucosa\textsuperscript{22}. Elucidating the role of DC’s in the Th17 response in adults and children, and the role of IL-6 in the Treg/Th17 balance in children, can add considerable
insight regarding the involved mechanisms. Furthermore, the roles of the Th17-related cytokines IL-21 and IL-22, transcription factors RORα and RORyt as well as the cytokines IL-1 and IL-2 may help uncover the underlying mechanisms involved in this relationship.

While understanding the manner by which the mucosal immune response to *H. pylori* infection changes with age is of considerable importance, the reasons for a differing response between children and adults can also yield important answers. As indicated previously, *H. pylori* has colonized the human stomach for thousands of years\(^2\). While the organism clearly can incite substantial pathology in its host, the possibility for a survival advantage incurred by the bacteria is intriguing\(^23\). Indeed, after eradication, rates of allergy, asthma and atopic disease increase, as well as the incidence of gastro-esophageal reflux disease and esophageal adenocarcinoma. In mice, *H. pylori* infection induces a systemic increase in Treg activity\(^23\), possibly accounting for the decrease in atopic disease through suppression of the Th2 immune response to allergens. Furthermore, the induction of a Treg response by organisms lies at the heart of the hygiene hypothesis\(^24\). Perhaps a survival benefit of *H. pylori* infection is a robust Treg response that, either with age or over time, dissipates or is inhibited. Exploring this philosophical arena is possible through animal models, comparing length of infection with host age. Another intriguing avenue includes investigating the response of populations, such as certain indigenous peoples, who may be relatively unexposed to modern hygienic techniques, which may play a role in suppressing the potentially beneficial attributes of *H. pylori* infection. Further exploration regarding the mechanism
of early *H. pylori* infection can lead to important new insights and developments regarding the pathogenicity of the organism.


APPENDIX

IRB APPROVAL FORM

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Institutional Review Board for Human Use

MEMORANDUM

TO:  Lesley E. Smyth, PhD
      Principal Investigator

FROM:  Sheila Moore, CR
      On behalf of IRB 01

DATE:  May 26, 2009

RE:  E9942708
      Mucosal TGF-β/IL-6 Axis in the Regulation of T Cell Function: Isolation of Cells from Blood and Gastrintestinal Tract of Morbidly Obese People (Mucosal TGF-
           β/IL-6 Axis in the Regulation of T Cell Function)

The IRB 01 met on May 13, 2009 and approved with limited modifications the protocol referenced above. The modifications requested are listed by number below. Please respond by memorandum to the Board, address each item by number.

This approval is contingent upon your response (a) being received in the Office of the IRB within 30 days of the date of this letter and (b) being reviewed and approved by the IRB or designated reviewer. No activity related to this protocol may occur until the IRB has issued to you a signed IRB approved form; when issued, this approval will expire and no longer be valid as May 13, 2010.

1) The IRB noted the submitted grant application to the National Institutes of Health (NIH) dated October 10, 2008, contains references to research involving other procedures, such as involving lymph nodes, which are not addressed in the purpose and methodology of this protocol. Please clarify if this grant is an "umbrella" grant and whether the research proposed in this specific protocol, Mucosal TGF-β/IL-6 Axis in the Regulation of T Cell Function: Isolation of Cells from Blood and Gastrintestinal Tract of Morbidly Obese People, represents only a portion of the inclusive grant.

2) In addition, the grant application refers to research done on individuals under the age of 18. The IRB approved this study for participants age 15 years and older.

3) Clarify the involvement of Dr. Phillip D. Smith in this study. The IRB noted Dr. Smith is listed as a key individual on the grant application. If Dr. Smith will be involved in this study, you will need to add him to items 2.e and 4 of the Human Subjects Protocol (HSP).

4) Clarify if the specimens that will be supplied by Drs. Declahan, Civer, and Eckoff can be limited to specific individuals either directly or indirectly. Please note the Office for Human Research Protections (OHRP) considers private information or specimens not to be individually identifiable when they cannot be linked to specific individuals by the investigator either directly or indirectly through coding systems.

   a. Please clarify the agreement made between you and the above-reference physicians to protect the identity of any individuals whose tissue or blood samples may be used in this research, if applicable. The information about these specimens was not included in the HSP.

   b. If specimens will be received from these individuals, the process / information must be added to the HSP.
c. This information may need to be included in Item 9 of the HSP if it is remnant biopsy or surgical material. You may also need to revise your response to Item 10, sections a. through h., of the HSP to describe how participant specimens will be obtained, labeled, and stored and how the confidentiality of these specimens will be protected.

d. If specimens will be obtained from Dr. Cover at Vanderbilt University for use in this study, you will need to provide IRB approval sign-off from Vanderbilt.

5) Clarify the involvement of Dr. Olaf Kutsche at the UAB Center for AIDS Research in this study. The CHRP considers "the study, interpretation, or analysis of the data resulting from the coded information or specimen" to constitute involvement in the conduct of the research. If Dr. Kutsche will be involved in this study you will need to add him to Items 2.e and 4 of the HSP.

6) Please clarify whether Kayci R. Huff and Diane Bimonte, listed on the UAB as UAB students or fellows who intend to use this research for a thesis, dissertation, or other publication and if they have begun any human subjects research (as defined in the UAB Policy on the Protection of Human Subjects in Research). UAB requires that the applicable approvals be in place for all projects. If these individuals will be using data or specimens from this research, you will need to clarify their research methodology so the IRB can determine if these individuals need separate IRB approval(s).

7) Item 2.e of the HSP indicates no medical supervision will be required for this research. Please clarify who will perform the tissue removal and add this individual to this item.

8) The IRB noted that you had indicated in Item 16 of the HSP that participants would receive a blood draw; yet, in the Risk and Discomfort section of the consent form you state that removal of blood will occur during surgery through the intravenous line already in place. Please clarify how blood samples will be obtained from participants and revise the appropriate documents.

9) Revise your response to Item 19 of the HSP to describe your plan for monitoring study data. Describe what data will be monitored, how often it will be monitored, who will monitor it, what data will be evaluated, and the decisions that will be made in response to the evaluation.

10) When the modifications described above are made to the HSP, you will need to submit a revised HSP—with all changes highlighted—for IRB review. This revised HSP should include a revision date on the bottom of each page and should be used when submitting materials for continuing IRB review.

11) Please clarify if laptops or other PDA will be utilized in this study and whether these devices have been encrypted as per UAB computer security policies.

The following refer to the informed consent document. The UAB IRB sample consent form is online at www.uab.edu/irb/forms/sample-consent-form.doc.

12) Add the UAB IRB protocol number (H30427008) where indicated on
   a. Page 1 and
   b. The HIPAA authorization.
13) Clarify whether tissue removal will increase the time of participant surgery and add this to the Risks and Discomforts section of the consent form, if applicable.

14) Revise the Risks and Discomforts section of the consent form to address the amount of blood taken for this study.

15) The UAB IRB has updated the text in the sample consent form. Please revise your consent form to include the updated information for the Questions section.

Please note that this protocol was approved with limited modifications and the IRB has requested additional information and changes to the informed consent form. The IRB Chair or designee will review this information and changes before formal approval of the protocol is issued. Please note that based on the information contained in your response, review by the convened IRB may be required. Enrollment may not begin until formal approval of the protocol and an IRB-stamped consent form have been issued by the IRB Office.

Upon receipt of the following, the IRB Office will issue formal approval of this protocol. Please note that you will need to provide:

1) One copy of a memorandum addressing the numbered items above,
2) One copy of the revised Human Subjects Protocol,
3) One copy of IRB approval from Vanderbilt, if applicable,
4) One copy of the revised consent form with the revisions highlighted, and
5) One copy of the revised consent form for the IRB approval stamp.
1. Contact Information
Principal Investigator's Name: Lesley Smith/les
Contact Person's Name: Mav Mosteller-Barnum
Telephone: 4-2729
Fax: 6-9113
Campus Address: Shelby Building Room 670

2. Protocol Identification
Protocol Title: Mucosal TGF-beta/IL-6 Axis in the Regulation of T Cell Function: Isolation of Cells from Blood and Gastrointestinal Tract of Morbidly Obese People
IRB Protocol Number: F009427008

Current Status of Project (check only one):
☒ Currently In Progress (Number of participants entered: 0)
☐ Study has not yet begun (No participants entered)
☐ Closed to participant enrollment (remains active)
☐ Number of participants on therapy/intervention:
☐ Number of participants in long-term follow-up only:
☐ Closed to participant enrollment (data analysis only)
☐ Total number of participants enrolled:

This submission changes the status of this study in the following manner (check all that apply):
☐ Protocol Revision
☒ Protocol Amendment
☐ Study Closed to participant entry
☐ Study Closure
☐ Other, (specify) __________

3. Reason for change
Briefly describe, and explain the reason for, the change. If normal, healthy controls are included, describe in detail how this change will affect those participants.
Include a copy of the protocol and any other documents affected by this change (e.g., consent form, questionnaire) with all the changes highlighted.
If we would like to add Shlomo Wright, a UAB medical student, to the personnel on this IRB, He may be doing experiments with some of the cells isolated from the blood and tissue obtained for this protocol.

4. Does this change revise or add a genetic or storage of samples component?
☐ Yes ☒ No

5. Does the change affect subject participation (e.g., procedures, risks, costs, location of services, etc.)?
☐ Yes ☒ No

6. Does the change affect the consent document(s)?
☐ Yes ☒ No

Include the revised consent document with the changes highlighted.
Will any participants need to be reconsented as a result of the changes?
If yes, when will participants be reconsented? __________