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Short communication

A regulatory instead of an IL-17 T response predominates in *Helicobacter pylori*-associated gastritis in children

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Abstract

Th17 cells seem to have an important role in the efficacy of vaccines against *Helicobacter pylori*. Because children are a target group for human vaccination and Th17/T_{reg} cells have intrinsically linked and antagonic commitments, we compared the gastric levels of Th17- and T_{reg}-associated cytokines of children and adults. IL-6, IL-10 and TGF-β1 levels and Foxp3$^+$ cell numbers were higher, but IL-1β, IL-17A and IL-23 were lower in infected children than in infected adults. In conclusion T_{reg} instead of Th17 cell response to *H. pylori*-infection predominates in children.

Keywords: *Helicobacter pylori*; Th17; T_{reg}; Children; Adults

1. Introduction

Helicobacter pylori is a well recognized gastric pathogen that infects more than 50% of the world's population. The infection is acquired predominantly in childhood, persists throughout life and predisposes to severe diseases such as peptic ulcer or gastric carcinoma in adulthood [1]. Most infected children do not develop complication; but, the immunological events that take place in the child gastric mucosa might be decisive in the immune response and might determine the final infection's outcome in adulthood.

The exposure to bacterial antigens induces in the host the innate immune response that strongly participates in the development of the adaptive immunity by activating T lymphocytes to differentiate into T helper (Th) effector cells categorized mainly by the cytokines they produce. The Th1 cell subset protects the host against intracellular bacteria and the recently discovered proinflammatory Th17 cells are involved in the protection against extracellular bacteria [2]. The Th1 cell response to *H. pylori* infection has been largely studied [3–6]; but, although *H. pylori* is an extracellular pathogen, there are few studies evaluating the Th17 cell response to the infection [6–8]. It has also to be emphasized, that in mouse models, Th17 cells are considered to have an important role in the efficacy of vaccines [9–11], which indicates that Th17 cell subset needs to be better investigated,

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especially in children, who are the target group for vaccination. The development of Th17 cells in human beings depends on a cytokine milieu rich in IL-1β, IL-6 and TGF-β that initiates the differentiation process and IL-23 that participates in the expansion and maintenance of the Th17 cells [12,13]. Although apparently paradoxical, TGF-β participates in both Th17 and T_{reg} cell differentiation. In the former by activating the transcription factor RORγt (retinoid-related orphan receptor γ) or RORc, the human homolog of RORγt, and in the latter, by activating the Foxp3 (forkhead box 3/winged helix) transcription factor [14–16]. Because T_{reg} limits bacterium elimination and T_{reg}/Th17 cell commitments are intrinsically linked, we aimed to determine the gastric levels of the proinflammatory Th17 cell signature cytokine, IL-17A, and the cytokines associated with Th17 and T_{reg} cell differentiation in children comparing the results with those obtained in adults.

2. Materials and methods

This study was approved by the Ethics Committee of the Universidade Federal de Minas Gerais, Belo Horizonte, Brazil. Signed informed consent to participate was obtained from the adults as well as from the children (whenever possible) and their parents.

2.1. Study population

We studied prospectively 245 children, 142 *H. pylori*-negative (62 females, 9.5 ± 3.4 years, range 1–18 years) and 103 *H. pylori*-positive (43 females, 10.6 ± 3.4 years, range 2–18 years). We also included 140 adults, 40 *H. pylori*-negative (20 females, 42.2 ± 15.1 years, range 19–69 years) and 100 *H. pylori*-positive (63 females, 52.3 ± 16.2 years, range 19–87 years) who underwent endoscopy to clarify the origin of symptoms referable to the upper gastrointestinal tract. Patients with peptic ulcer, gastric cancer and other complications and those who received antimicrobial drugs, anti-cholinergic and anti-inflammatory agents or proton pump inhibitors for at least 30 days before endoscopy were not included. All patients were natives of the Minas Gerais state with the same genetic background, approximately 33% of Portuguese, 33% of Amerindian and 33% of African ancestry, homogenously present in each subject [17]. Biopsy specimens were obtained from the antral and oxyntic gastric mucosa of all patients for evaluation of the *H. pylori* status and histological parameters and from the antral mucosa for T_{reg} $Foxp3^+$ immunohistochemistry and cytokine concentration determination.

2.2. H. pylori *status*

H. pylori status was evaluated by culture, preformed urease test, carbolfuchsin-stained histological section, polymerase chain reaction (PCR) for *ure*A, and ^{13}C-urea breath test as previously described [18]. Patients were considered *H. pylori*-positive when culture was positive or at least two of the other tests were positive and *H. pylori*-negative when the results of all tests were negative.

2.3. DNA extraction

Tissue and bacterial culture DNA was extracted with QIAamp® DNA mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's recommendations. The presence of the *ure*A *H. pylori* specific gene was evaluated, according to Clayton et al. [19].

2.4. Histology and evaluation of the gastric H. pylori density

Fragments from the antral and oxyntic mucosa were fixed in 10% formalin and embedded in paraffin wax, and 4-μm-thick histological sections were stained with hematoxylin and eosin and with carbolfuchsin for histological analysis and spiral bacterium evaluation, respectively. The sections were analyzed according to the revised Sydney System [20]. Mononuclear and polymorphonuclear cell infiltrations as well as intestinal metaplasia, atrophy and spiral bacterium density were graded as absent (0), mild (1), moderate (2), or marked (3).

2.5. Determination of the gastric cytokine levels

Two antral biopsy fragments were immediately placed into cryotubes, frozen in liquid nitrogen and stored at −80 °C until used. Aliquots of homogenate supernatant in 1.5 mL PBS, pH 7.4 containing 2 μg/mL aprotinin were obtained by centrifugation (10,000 g for 10 min). The total protein concentration was measured by Bradford's method. The gastric levels of the cytokines involved in the Th17 and T_{reg} cell commitment, IL-1β, IL-6, IL-10, IL-17A, IL-23, and TGF-β1 (after activation), were assayed in duplicate by ELISA (Biosource, Camarillo, CA). The cytokine mucosal levels were expressed as picogram of cytokine per milligram of protein (pg/mg protein). IL-1β and IL-6 levels were evaluated by ultra sensitive kits. The minimum detectable levels of the cytokines are 0.06 pg/mL (IL-1β), 104 fentogram per milliliter − fg/mL (IL-6), 0.2 pg/mL (IL-10), 2.0 pg/mL (IL-17A), 4.0 pg/mL (IL-23) and 15.6 pg/mL (TGF-β1). All values below the detection levels were regard as undetectable and were ascribed the value zero.

2.6. T_{reg} Foxp3+ cell number

The T_{reg}Foxp3 cell number was assessed in formalin-fixed paraffin-embedded sections of the antral mucosa of 70 (40 *H. pylori* positive) children and 50 (30 *H. pylori* positive) adults, who were randomly selected, by conventional immunohistochemistry using as primary antibody 1:35 diluted mouse anti-human Foxp3 IgG (mAbcam 22509, Abcam, Cambridge, UK) with modifications including incubation with Novocastra Post Primary Block for 30 min and with NovoLink polymer for 30 min (Novocastra Laboratories Ltd, São Paulo, Brazil).

Sections were counterstained with Meyer's hematoxycillin and then mounted. Fragments of inflamed ileum from patients with active Crohn's disease were included as positive control and slides containing tissue sections without addition of primary antibody, as negative control. The mononuclear cells with the nucleus stained in brown were considered positive for Foxp3. The number of Foxp3-positive cells (proportional to the number of all lymphocytes) was evaluated in 20 representative visual fields at a magnification of 1,000× in an Olympus CX41RF microscope.

2.7. Statistical analysis

Data were analyzed with SPSS (SPSS Inc., Chicago, IL) statistical software package version 17.0. In addition to the visual examination of the histograms and box plots, the Kolmogorov–Smirnov goodness-of-fit was used to assess the normality of the data. When significant departures from normality were detected the data were log transformed and became normally distributed. The two-tailed Student's t test was used to compare the sub groups of patients. The scores of mononuclear and polymorphonuclear cells and of *H. pylori* density were compared by the two-tailed Mann Whitney U test. Correlations were evaluated by the Pearson's correlation test (continuous data) or Spearman's correlation test (scores). The adults and children were compared in regard to the presence of gastric atrophy and intestinal metaplasia by the two-tailed χ^2 or Fisher test. The level of significance was set at $p < 0.05$.

3. Results

3.1. Population

No difference in the sex frequency was observed between infected and uninfected children ($p = 0.65$) and adults ($p = 0.28$); but, the mean age was higher in *H. pylori*-positive children ($p = 0.002$) and adults ($p = 0.005$) than in the negative groups.

The score of the antral and corpus mononuclear and polymorphonuclear cells was higher in *H. pylori*-positive than in *H. pylori*-negative children and adults. The degree of mononuclear and polymorphonuclear cells in the antrum and in the corpus was significantly higher in infected-adults than in infected children (Table 1). Also, atrophy ($n = 6$, $p = 0.01$) and intestinal metaplasia ($n = 5$, $p = 0.03$) were observed only in the corpus gastric mucosa of adults.

The density of *H. pylori* in the antrum did not differ ($p = 0.98$) between infected children (median: 2.0, range: 0–3) and adults (median: 2.0, range: 1–3).

3.2. Cytokine levels in the gastric mucosa of children and adults

3.2.1. H. pylori-*negative group*

In the *H. pylori*-negative group, IL-1β, IL-6, IL-10, IL-17A, IL-23 and TGF-β1 were naturally expressed in the gastric mucosa of 44.4%, 3.5%, 52.8%, 86.6%, 12.7% and 59.2% of the children and in the gastric mucosa of 97.5%, 82.5%, 42.5%, 100%, 22.5% and 100% of the adults, respectively. When children and adults were compared, the mean gastric levels (pg/mg of protein) of IL-1β (19.2 ± 56.9 vs. 227.5 ± 112.4, $p < 0.001$), IL-6 (2.8 ± 15.3 vs. 21.0 ± 14.5, $p < 0.001$), IL-17A (152.8 ± 98.2 vs. 192.9 ± 124.5, $p = 0.03$), IL-23 (26.2 ± 73.6 vs. 34.7 ± 67.0, $p = 0.05$) and TGF-β1 (1101.7 ± 1194.3 vs. 3742.1 ± 1601.3, $p < 0.001$) were significantly lower, but IL-10 levels (34.5 ± 43.9 vs. 18.0 ± 24.3, $p < 0.001$) were higher in the children than in the adults.

Table 1
Histological comparison of the gastric mucosa of *Helicobacter pylori*-positive children ($n = 103$) and adults ($n = 100$).

Inflamation	Absent	Mild	Moderate	Marked	p
	n (%)	n (%)	n (%)	n (%)	
Antrum MN cells					
Children	5 (4.9)	30 (29.1)	63 (61.1)	5 (4.9)	<0.001
Adults	1 (1.0)	16 (16.0)	67 (67.0)	16 (16.0)	
Antrum PMN cells					
Children	14 (13.6)	53 (51.5)	34 (33.0)	2 (1.9)	=0.01
Adults	5 (5.0)	53 (53.0)	36 (33.0)	6 (6.0)	
Corpus MN cells					
Children	5 (4.8)	72 (69.9)	22 (21.4)	4 (3.9)	<0.001
Adults	3 (3.0)	47 (47.0)	41 (41.0)	9 (9.0)	
Corpus PMN cells					
Children	28 (27.2)	62 (60.2)	9 (8.7)	4 (3.9)	<0.001
Adults	18 (18.0)	57 (57.0)	19 (19.0)	6 (6.0)	

n, number; MN, mononuclear; PMN, polymorphonuclear.

3.2.2. H. pylori-*positive group*

All cytokines, but IL-23 (detected in 86.4% of children and 82.0% of adults), were detected in the gastric mucosa of all *H. pylori*-positive children and adults.

The gastric levels of IL-6 ($p < 0.001$), IL-10 ($p < 0.001$) and TGF-β1 ($p = 0.04$) were significantly higher in the gastric mucosa of children than in adults. Otherwise, IL-1β ($p < 0.001$), IL-17A ($p < 0.001$) and IL-23 ($p = 0.001$) gastric levels were significantly higher in the adults than in the children (Fig. 1).

3.3. Comparison of the gastric cytokine levels between H. pylori-*positive and -negative patients*

The gastric concentrations of all cytokines were significantly higher in infected than in non-infected children ($p < 0.001$ for all) (Fig. 2A) and adults ($p = 0.004$ for IL-1β and $p < 0.001$ for the other cytokines) (Fig. 2B).

An 11.5-, 307.1-, 20.6-, 3.2-, 15.8- and 7.1-fold increased gastric levels of IL-1β, IL-6, IL-10, IL-17A, IL-23 and TGF-β1, respectively, was observed in infected children when compared with non-infected ones.

The concentration of IL-1β, IL-6, IL-10, IL-17A, IL-23 and TGF-β1 were 1.3-, 18.6-, 6.3-, 4.2-, 17.5- and 1.8-fold

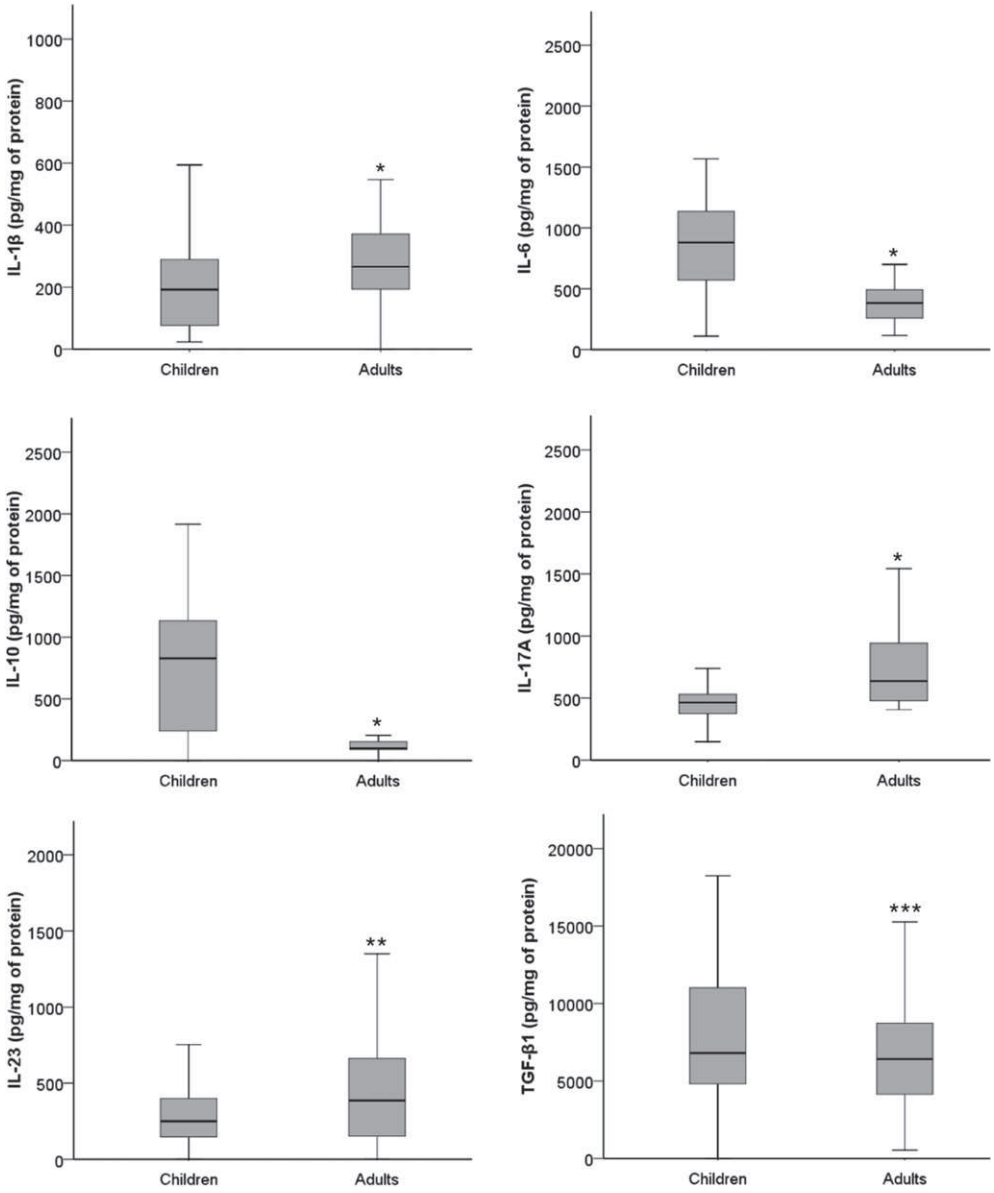

Fig. 1. Box plots representing the comparison of the antral gastric cytokine concentrations (pg/mg of protein) between *H. pylori*-positive children ($n = 103$) and adults ($n = 100$). The upper and lower limits of the boxes represent the 75th and 25th percentiles, respectively. The horizontal bar across the box indicates the median and the capped bars indicate the minimum and maximum data values. The data were analyzed by the two-tailed Student's *t*. $^{*}P < 0.001$, $^{**}P = 0.001$ and $^{***}P = 0.04$.

increased, respectively, in the gastric mucosa of *H. pylori*-positive adults when compared with the bacterium-negative ones.

3.4. Foxp3 cells

The number of Foxp3^{+} cells was significantly higher in the antral mucosa of *H. pylori*-positive than in -negative children and adults ($p < 0.001$ for both) (Fig. 3A and B).

When *H. pylori*-positive children and adults were compared, the number of Foxp3^{+} cells was significantly higher ($p < 0.001$) in the antral mucosa of children (Fig. 3A and B). Otherwise, no difference ($p > 0.64$) was observed between *H. pylori*-negative adults and children (Fig. 3B).

3.5. Correlation between Foxp3, histology and cytokine concentration

In the infected patients, the number of Foxp3^{+} cells in the antral mucosa was negatively correlated with the antrum mononuclear cell infiltration in children ($r = -0.40$, $p = 0.03$) and adults ($r = -0.34$, $p = 0.05$). A negative correlation between the number of Foxp3 cells and the score of polymorphonuclear cells in the antrum of children was also observed ($r = -0.32$, $p = 0.05$).

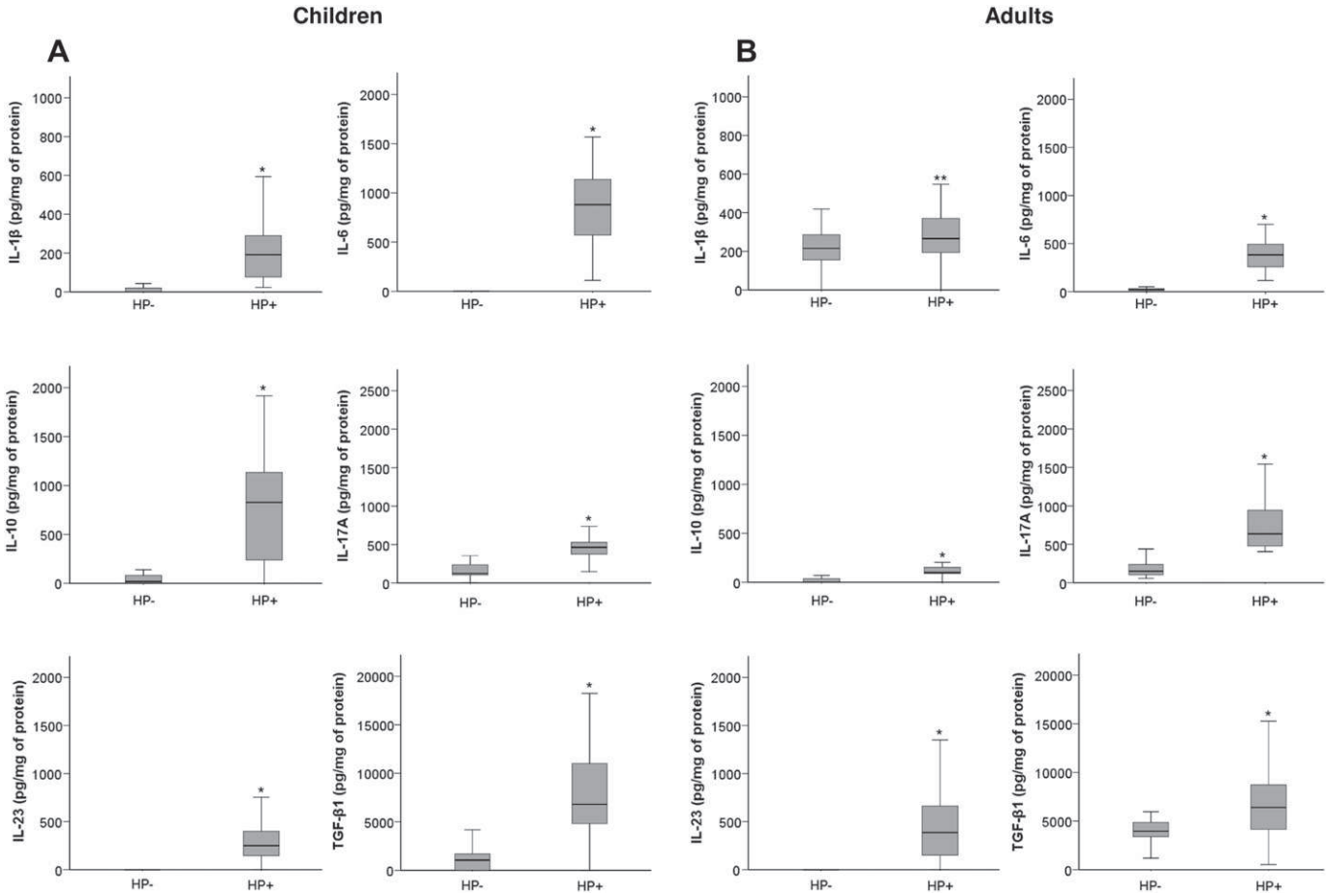

Fig. 2. A-Box plots representing the comparison of the antral gastric cytokine concentrations (pg/mg of protein) between *H. pylori*-positive children ($n = 103$) with children without *H. pylori* infection ($n = 142$). The upper and lower limits of the boxes represent the 75th and 25th percentiles, respectively. The horizontal bar across the box indicates the median and the capped bars indicate the minimum and maximum data values. The data were analyzed by the two-tailed Student's *t* test. $*P < 0.001$. 2B-Box plots representing the comparison of the antral gastric cytokine concentrations (pg/mg of protein) between *H. pylori*-positive ($n = 100$) and -negative ($n = 40$) adults. The upper and lower limits of the boxes represent the 75th and 25th percentiles, respectively. The horizontal bar across the box indicates the median and the capped bars indicate the minimum and maximum data values. The data were analyzed by the two-tailed Student's *t*. $*P < 0.001$, $**P = 0.004$.

In the *H. pylori*-positive children, the antral Foxp3$^+$ cells number positively correlated with the IL-10 antral levels ($r = 0.26$, $p = 0.05$). No other correlation between Foxp3 cells and the cytokine gastric concentrations was observed in the *H. pylori*-positive groups ($p > 0.2$ for all).

4. Discussion

Because *H. pylori* infection is usually acquired in early childhood, events that take place at this age might influence or even determine susceptibility to the infection and might also contribute to the clinical outcomes in adulthood. Therefore, a better understanding of the child's immune response to *H. pylori* infection is the first step in the development of an effective vaccine to target children. It has been recently demonstrated that the efficacy of a vaccine against *H. pylori* in mouse models relies more on Th17 than on Th1 cell response [9–11]. However, to date, we are aware of only one study evaluating IL-17 cell response to *H. pylori* in children [6]. Luzza et al. demonstrated an increased IL-17A mRNA gastric expression in *H. pylori*-positive children; but, other cytokines linked to the Th17 cell commitment were not investigated by the authors. In adults, higher levels of IL-17A and expression of IL-17A mRNA were observed in the gastric mucosa of *H. pylori*-positive than in that of *H. pylori*-negative Japanese [7] and Italian [8] patients. In the latter, increased levels of IL-23 was also observed in the gastric mucosa of *H. pylori*-positive adults and the authors also demonstrated the role of IL-23 in increasing the production of IL-17 by gastric mononuclear cells "*in vitro*".

In agreement with the above cited studies, higher IL-17A gastric levels were observed in infected than in uninfected adults and children, but significantly higher in the former. Conversely, the gastric number of T_{reg} Foxp3$^+$ cells and the IL-10 and TGF-β gastric levels in *H. pylori*-positive groups were higher in children than in adults as also observed by Harris et al. in Chile [21].

The current consensus is that the Th17 and T_{reg} cell commitments are mutually controlled. TGF-β is required for the differentiation of both Th17 and T_{reg} cells by inducing

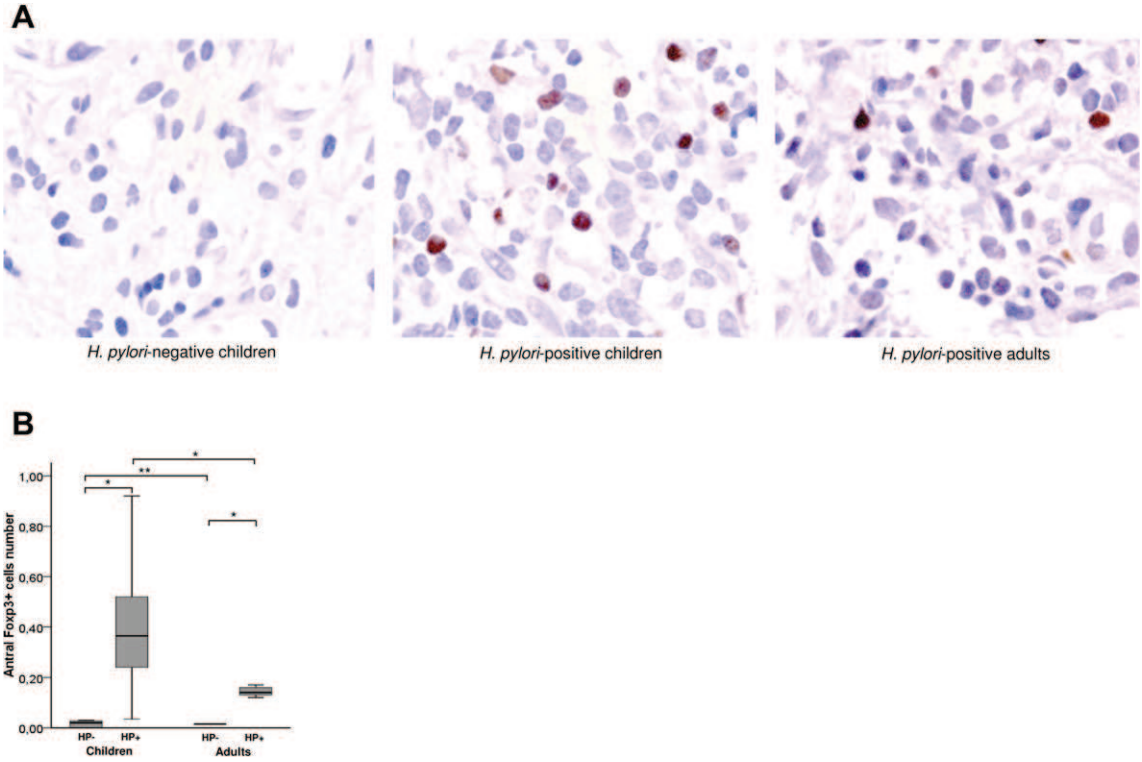

Fig. 3. A-Immunohistochemistry for T_{reg} Foxp3+ cell in the gastric mucosa of a *H. Pylori*-negative child, *H. pylori*-positive child and *H. pylori*-positive adult; brown staining indicates positive cells whereas negative cells are counterstained with hematoxylin. Magnification: 1,000×. 3B-box plots representing the comparison of the antral Foxp3 cells number between infected and non-infected children and adults. The upper and lower limits of the boxes represent the 75th and 25th percentiles, respectively. The horizontal bar across the box indicates the median and the capped bars indicate the minimum and maximum data values. The data were analyzed by the two-tailed Student's *t*. $*P < 0.001$, $**P = 0.64$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

their key transcription factors, RORγt/RORc and Foxp3, respectively [14–16]. However, in the absence of IL-6, an exclusive T_{reg} differentiation occurs as Foxp3 is able to associate with and to inhibit RORγt. Otherwise, in the presence of IL-6, this inhibition is abrogated allowing Th17 differentiation [22]. Paradoxically, the gastric concentration of IL-6 was also higher in infected children than in infected adults. One might hypothesize that these results are due to the lower gastric levels of IL-23 in children when compared with adults, which prevents the amplification/stabilization of the shifted Th17 cells. Another possibility is the higher concentration of TGF-β in the gastric milieu of infected children. At low concentrations, TGF-β synergizes with IL-6 to promote IL-23 receptor (*IL-23r*) expression favoring Th17 cell commitment. High concentrations of TGF-β; however, repress *IL-23r* expression and favor $Foxp3^+$ T_{reg} cell differentiation [23]. Alternatively, a recent study has demonstrated that IL-6 overproduction *in vivo* by an IL-6 transgenic mouse does not affect the development and function of natural T_{reg} [24].

The predominant T_{reg} instead of Th17 cell differentiation in *H. pylori*-infected children might account to the susceptibility of children to the infection as well as to the bacterium persistence. It may also explain the lower degree of mononuclear and polymorphonuclear cell gastric infiltration observed in infected children than in infected adults that seems not to be due to differences in the gastric bacterium density. It has to be highlighted that the gastric number of $Foxp3^+$ cells negatively correlated with the gastric polymorphonuclear cell infiltration in children. Of note, IL-17A participates in the recruitment and activation of polymorphonuclear cells that are considered relevant to the clearance of the *H. pylori*. In mice, the IL-17 production and the associated neutrophil infiltration seem to be essential in the bacterium clearance induced by the vaccine [10,11].

Finally, all the results we observed could not be attributed to the infection by a more virulent *H. pylori* strain because adults and children did not differ in respect to the prevalence of infection by *cag*A-positive strain (data not shown). Furthermore, no association was observed between the CagA status and the gastric cytokine concentrations (data not shown).

In conclusion T_{reg} instead of Th17 cell response to *H. pylori*-infection predominates in children.

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