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# Lymphocyte Apoptosis, Proliferation and Cytokine Synthesis Pattern in Children with *Helicobacter pylori* Infection

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## 1. Introduction

*Helicobacter pylori (H. pylori)* infection is usually acquired in early childhood. The majority of the infected children do not suffer from acute inflammatory complications and a few develop severe diseases such as peptic ulcer (Queiroz et al., 1991), mucosal atrophy, gastric carcinoma or MALT lymphoma (Guarner et al., 2003). The cellular basis for the mild gastric inflammatory changes in children with *H. pylori* infection is poorly understood. The few available studies in the *H. pylori*-infected children have revealed low expression of proinflammatory cytokines in the gastric mucosa (Bontems et al., 2003; Lopes et al., 2005), a rather low humoral systemic immune response manifested by *H. pylori*-specific IgG and IgA antibodies (Soares et al., 2005), and a high local Treg cell response (Harris et al., 2008).

In contrast to some studies on circulating T-lymphocyte distribution and activation in pediatric *H. pylori* infection (Helmin-Basa et al., 2011, Soares et al., 2005), estimation of apoptosis in different T lymphocyte subpopulations has not been evaluated separately in the peripheral blood of *H. pylori*-infected and non-infected children with gastritis. There is also the paucity of information regarding the *H. pylori*-specific peripheral cellular responses to live *H. pylori* carrying the cagA gene (*H. pylori cagA*+) which encodes an immunodominant 120-128 kD protein in the pediatric group.

As in the case of other intestinal microflora, *H. pylori* colonization of gastric mucosa results in a close and permanent bacterial contact with gut-associated lymphoid tissues (Acheson &

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Luccioli, 2004). It leads to the stimulation of specific T cell responses induced by bacterial antigens, presented to the T cells by bacteria-loaded dendritic cells. This route of immunization leads to the generation of a T cell memory pool that is able to recognize a broad spectrum of bacterial antigens and that accumulates mainly in the lamina propria of the gastric mucosa (Hatz et al., 1996). However, because at least a part of this T cell population also enters the circulation the peripheral blood mononuclear cells (PBMCs) contain "memory" T cells which can respond by proliferation and cytokine expression to numerous bacterial microflora antigens, including entire *H. pylori* cells or their various products (Jacob et al., 2001; Windle et al., 2005). Since the presence of the *cagA* gene in *H. pylori* strains has commonly been associated with strong pro-inflammatory action (Peek et al., 1995), the *H. pylori cagA*+ strains were used here as PBMC inducers in order to stimulate lymphocyte apoptosis, proliferation, and cytokine expression. To the best of our knowledge this is the first report in which *H. pylori cagA*+ strains were used as PBMC stimulators in *H. pylori*-infected and noninfected children with gastritis. The PBMC model system has been used extensively for testing the immunomodulatory properties of a broad array of both Gram-positive and Gram-negative bacteria and their products, such as lipopolysaccharide (LPS), peptidoglycans and teichoic acid (Hessle et al., 1999, 2000; Karlsson et al., 2002). Using a PBMC model system, we studied live *H. pylori cagA*+-mediated acquired and innate cellular responses in children with gastritis. Innate responses included IL-12p40, IFN-gamma, TNF-alpha, IL-6 and IL-10 secretion levels, and the acquired response was assessed by *H. pylori*-induced lymphocyte proliferation. Since immune responses are strictly associated with lymphocyte apoptosis; therefore, the spontaneous and *H. pylori*-induced lymphocyte apoptosis were also tested.

The aim of this study was to assess the distribution and apoptosis of the most prevalent lymphocytes' subpopulations in the blood, and in addition to evaluate *H. pylori*-induced lymphocyte apoptosis, proliferation, and cytokine synthesis pattern (IL-12p40, IFN-gamma, TNF-alpha, IL-6 and IL-10) in *H. pylori*-infected and noninfected children with gastritis with the goal of comparing the results with those obtained from the control group formed by dyspeptic noninfected patients without gastritis.

## 2. Materials and methods

### 2.1 Patients

The study was undertaken according to Helsinki declaration with approval from the ethics committee of Collegium Medicum Nicolaus Copernicus University in Bydgoszcz, Poland. Informed consent was obtained from all the parents of patients and patients older than 16 years of age.

A total of 136 consecutive subjects older than 8 years of age with dyspeptic symptoms and residing in the Kuivia-Pomeranin district of Poland were included in this study. Exclusion criteria included: 1) previous diagnosis of other inflammatory disease, such as celiac disease, inflammatory bowel disease or allergy; 2) gastric perforation or hemorrhage, history of surgery, bleeding disorders, or evidence of other clinical conditions or intestinal parasites.

Each subject underwent an endoscopic examination of the upper part of the gastrointestinal tract as a result of reporting permanent abdominal pain. Three antral biopsies were taken

from each patient. One biopsy specimen was subjected to a rapid urease test; the other two specimens were formalin-fixed and embedded in paraffin, sectioned and stained with hematoxylin and eosin for histological analysis, and Giemsa modified by Gray stain for *H. pylori* detection. Biopsy specimens were graded for gastritis by two independent pathologists according to the updated Sydney system. The histological variables (the presence and density of mononuclear and polymorphonuclear cells, glandular mucosa atrophy, intestinal metaplasia, and *H. pylori* colonization) were scored on a four-point scale: 0, none; 1, mild; 2, moderate; and 3, marked.

The urease test was performed in every patient. *H. pylori* infection was also excluded in every subject by performing the [$^{13}$C] urea breath test within one week of undergoing endoscopy. $^{13}$C concentration was measured with an infra-red radiation analyzer (OLYMPUS Fanci 2) with 4‰ assumed as the cutoff point.

A patient was considered *H. pylori*-infected when the [$^{13}$C] urea breath test plus either the rapid urease test or microscopic evaluation were positive for *H. pylori*. When the results of all three tests were negative a patient was considered noninfected. Fifty-nine patients fulfilled the criteria for *H. pylori* positivity, while 77 patients fulfilled the criteria for *H. pylori* negativity. None of the patients had ulcer disease or macroscopic lesions of the duodenal mucosa in endoscopic examination.

Patients were divided into three groups:

1. Control group – 15 children (7 boys and 8 girls) aged 8-15 years (median age 14 years) without gastritis on histological examination and without *H. pylori* infection;
2. Noninfected children with gastritis (*Hp*-) – 62 children (20 boys and 42 girls) aged 8-18 years (median age 14 years) with recognized gastritis but without *H. pylori* infection;
3. *H. pylori*-infected children with gastritis (*Hp*+) – 59 children (28 boys and 31 girls) aged 8-18 years (median age 14 years) with recognized gastritis and *H. pylori* infection;

### 2.2 Bacteria

*H. pylori* strain 25A *cagA*-positive (*H. pylori cagA*+) was obtained from the Department of Microbiology and Clinical Immunology, Children's Memorial Hospital, Warsaw, Poland. Bacteria were washed twice with PBS pH 7.2 and adjusted to a density of 21 x $10^8$ cells ml$^{-1}$ in PBS.

### 2.3 Isolation of cells

At the time of endoscopy 10 ml of venous blood was obtained for immunologic testing from each patient. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by Isopaque-Ficoll (Lymphoprep, Nycomed Pharma AS, Oslo, Norway) gradient centrifugation according to the procedure laid down by the manufacturer. Isolated PBMCs were resuspended in a PBS or culture medium made up of RPMI 1640 (Gibco, Paisley, UK) supplemented with 5% heat-inactivated human ABRh+ serum and gentamicin (40 μg ml$^{-1}$).

### 2.4 Phenotyping of cell surface antigens

Freshly isolated PBMCs were stained with fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-conjugated mouse anti-human monoclonal antibodies (BD Biosceinces,

New York, USA), specific for cell-surface markers with the following combinations: CD3/CD4, CD3/CD8, CD4/CD45RA, CD4/CD45RO, CD8/CD45RA, CD8/CD45RO. Simultest LeucoGate, (CD45-FITC/CD14-PE), and isotype control (IgG1-FITC/IgG2-PE) antibodies (BD Biosceinces, San Diego, CA, USA) were included for each staining panel. Fluorescent staining was performed as described (Helmin-Basa et al., 2011). Briefly, 1 x $10^6$ PBMCs in PBS were incubated with the indicated monoclonal antibodies for 20 min at RT in the dark. After one washing in PBS cell samples were submitted to the flow cytometric method for quantification of apoptosis using 7-Amino-Actinomycin D staining.

### 2.5 7-amino-actinomycin D (7-AAD) staining

After cell surface labeling, samples (1x$10^6$ cells) were incubated for 5-10 min at RT in the dark with 5 µl (0.25 µg) 7-AAD (BD Biosciences, San Diego, CA, USA). Then the samples (20 000 events per sample) were acquired on a FACScan flow cytometry (BD Biosciences, San Diego, CA, USA) equipped with a single 488 nm Argon laser, recorded in list mode and registered on logarithmic scales. 7-AAD emission was detected in the FL-3 channel (>650 nm). Analysis was performed with a Macintosh computer with the BD CellQuest Software (BD Biosciences).

### 2.6 Annexin V and propidum iodide staining in PBMC culture

After 72 hours culture of PBMCs (3 x $10^6$ $ml^{-1}$) with *H. pylori cagA*+ (6.25 x $10^6$ bacteria $ml^{-1}$), tetanus toxoid (TT, 5 UI $ml^{-1}$, Sigma-Aldrich) or without the stimulator, the percentage of live and dead lymphocytes were evaluated by flow cytometry (FACScan, BD Biosciences), using FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, San Diego CA, USA). Briefly, 5 µl of Annexin V-FITC was added to 1 x $10^5$ cells $ml^{-1}$, incubated for 20 minutes at RT in the dark, washed, and the cells were resuspended in 190 µl of binding buffer. Then 10 µl of propidium iodide was added 5-10 min before analysis by FACScan flow cytometry. The lymphocytes were gated and the live, apoptotic and dead cells were displayed on an FL-1 versus FL-3 fluorescence dot plot. Annexin $V^-/PI^-$ cells represent live cells, the Annexin $V^+/PI^-$ cells represent early apoptotic cells, the Annexin $V^+/PI^+$ cells are late apoptotic cells, and the Annexin $V^-/PI^+$ represent dead cells.

### 2.7 Proliferation assay in PBMC culture

The assay was made according to the procedure described previously (Gackowska et al., 2006, Michalkiewicz et al., 2003). Briefly, freshly isolated PBMCs were diluted in culture medium to a final concentration of 3 x $10^6$ $ml^{-1}$ and transferred in a volume of 100 µl (3 x $10^5$) to flat-bottomed 96–well microplates (Costar, Cambridge, UK). Subsequently, 100 µl of culture medium alone (control) or containing live *H. pylori cells cagA*+ (6.25 x $10^6$ cells $ml^{-1}$) or 20 µl of Concanavalin A (Con A 12.5 µg $ml^{-1}$, Sigma-Aldrich) or tetanus toxoid (TT 5 UI $ml^{-1}$, Sigma-Aldrich) was added. The cells were cultured in triplicates for seven (stimulation with *H. pylori cagA*+ and TT) or three (stimulation with Con A) days. Lymphocyte proliferation was assessed by pulsing the cells with 1 µCi $^3H$ thymidine (Amersham, Little Chalfont, UK) for the last 16 hours of the incubation period. The cultures were then harvested onto glass filter strips using an automated multisample harvester (Skatron, Lier, Norway) and analysed for $^3H$-thymidine incorporation by liquid scintillation counting. Data

is given as the stimulation index [SI], calculated by dividing the cpm obtained after stimulation by the cpm in corresponding cultures without a stimulator.

### 2.8 Cytokine assay in PBMC culture

Cytokine concentration (IL-12p40, IFN-gamma, TNF-alpha, IL-6 and IL-10) in cell culture supernatants was determined after 72 hours of *H. pylori cagA*+ or Con A stimulation of PBMCs (3 x $10^6$ $ml^{-1}$) using ELISA (Opt-EIA system, BD Biosciences, San Diego CA, USA), according to the manufacturer's procedure. Dose-response experiments performed for each cytokine indicated that the maximal secretion was obtained with 6.25 x $10^6$ bacteria $ml^{-1}$, corresponding to a ratio of 2:1 (bacteria to PBMC) and 12.5 µg $ml^{-1}$ of Con A that also induced the optimal proliferative response. For analysis of spontaneous cytokine secretion the cells were cultured alone in complete medium for 72 hours.

### 2.9 Statistical analyses

Data was expressed as means with standard deviation [SD] or medians with 95% confidence intervals (CI). The results were compared using the paired Student's *t* test or the Mann-Whitney's *U*-test as appropriate, depending on the normality of the data distribution, with STATISTICA for Windows release 5.0 (StatSoft, Tulusa, OK, USA) For normal distribution, variables were analyzed by the Kolmogorov-Smirnov test with Lilieforse correction. Statistical significance was considered as $p < 0.05$. The gastric inflammation was correlated with the percentage of positive cells using Spearman's coefficient of rank correlation. The level of significance was set at $p < 0.05$.

## 3. Results

### 3.1 Gastric mucosa histology

The intensity and activity of antral gastritis were greater in the *H. pylori*-infected children (*Hp*+) as compared to children with gastritis where *H. pylori* infection was excluded (*Hp*-) ($p < 0.05$), (Table 1). Neither atrophy nor intestinal metaplasia were seen in children's gastric mucosa.

| Children | Antral mucosa | | | | |
|---|---|---|---|---|---|
| | MN cells (intensity) | PMN cells (activity) | Atrophy | Intestinal metaplasia | *H. pylori* |
| *Hp*+ | 2 (1-3) | 0 (0-2) | 0 | 0 | 1 (0-3) |
| *Hp*- | 2 (0-3) | 0 (0-1) | 0 | 0 | 0 |
| Control group | 0 | 0 | 0 | 0 | 0 |

*Hp*+, *H. pylori*-infected children; *Hp*-, noninfected children; MN: mononuclear cells; PMN: polymorphonuclear cells.

Table 1. Gastric mucosa histology of *Hp*+ and *Hp*- children, and control group (median score according to the update Sydney system: 0, none; 1, mild; 2, moderate; 3, marked)

### 3.2 The distribution of T-cell subsets in peripheral blood

There was an equal percentage of $CD4^+$ and $CD8^+$ T cell subsets in peripheral blood in the *Hp*+ and *Hp*- children and the control group (Table 2). However, the naive $CD8^+$ T-cell subset ($CD45RA^+$) was higher in the *Hp*+ children when compared to the control group ($p < 0.01$). In *Hp*- children, the proportion of memory $CD8^+$ T-cell subset ($CD45RO^+$) was increased ($p = 0.01$, *versus* control group). However, there was no correlation between the percentage of naive and memory T-cell subsets and the gastric inflammation scores in *Hp*+ and *Hp*- children.

| T lymphocyte subsets | | Means ± SD | | |
|---|---|---|---|---|
| | | *Hp*+ children (n = 13) | *Hp*- children (n = 15) | Control group (n = 12) |
| CD3+CD4+ | % | 39.95±4.78 | 36.96±6.76 | 38.98±5.92 |
| CD3+CD8+ | % | 28.89±5.72 | 27.57±6.61 | 27.04±3.91 |
| CD4+CD45RA+ | % | 29.51±8.62 | 26.31±6.15 | 22.95±0.65 |
| CD4+CD45RO+ | % | 16.74±6.50 | 16.37±0.58 | 15.21±4.10 |
| CD8+CD45RA+ | % | 34.95±1.72[a] | 29.72±7.17 | 25.24±1.34 |
| CD8+CD45RO+ | % | 10.44±2.78 | 9.91±0.52[a] | 6.76±1.79 |

*Hp*+, *H. pylori*-infected children; *Hp*-, noninfected children; SD, standard deviation. Statistically significant differences (Student's *t* test): *versus* controls ([a]$p < 0.05$).

Table 2. The distribution of T-cell subsets in peripheral blood of *Hp*+ and *Hp*- children and the control group

### 3.3 Apoptosis of T-cell subsets in peripheral blood

Flow cytometric quantification of apoptotic T cell subsets in freshly isolated PBMCs showed significantly more apoptotic $CD4^+$ and $CD8^+$ T cells in both *Hp*+ ($p < 0.01$ and $p < 0.01$) and

| T lymphocyte subsets | | Means ± SD | | |
|---|---|---|---|---|
| | | *Hp*+ children (n = 13) | *Hp*- children (n = 15) | Control group (n = 12) |
| $CD4^+$ | % | 10.48 ± 5.80[a,b] | 4.50 ± 2.12[a] | 2.13 ± 2.00 |
| $CD8^+$ | % | 8.46 ± 4.34[a,b] | 5.01 ± 1.92[a] | 1.48 ± 1.27 |
| CD4+CD45RA+ | % | 5.24 ± 2.16[a] | 4.62 ± 1.58[a] | 0.36 ± 0.07 |
| CD4+CD45RO+ | % | 5.11 ± 1.94[a] | 7.37 ± 1.42[a] | 0.55 ± 0.34 |
| CD8+CD45RA+ | % | 4.69 ± 0.79[a] | 5.45 ± 1.85[a] | 0.68 ± 0.21 |
| CD8+CD45RO+ | % | 6.12 ± 2.44 | 5.76 ± 1.51 | 4.17 ± 1.55 |

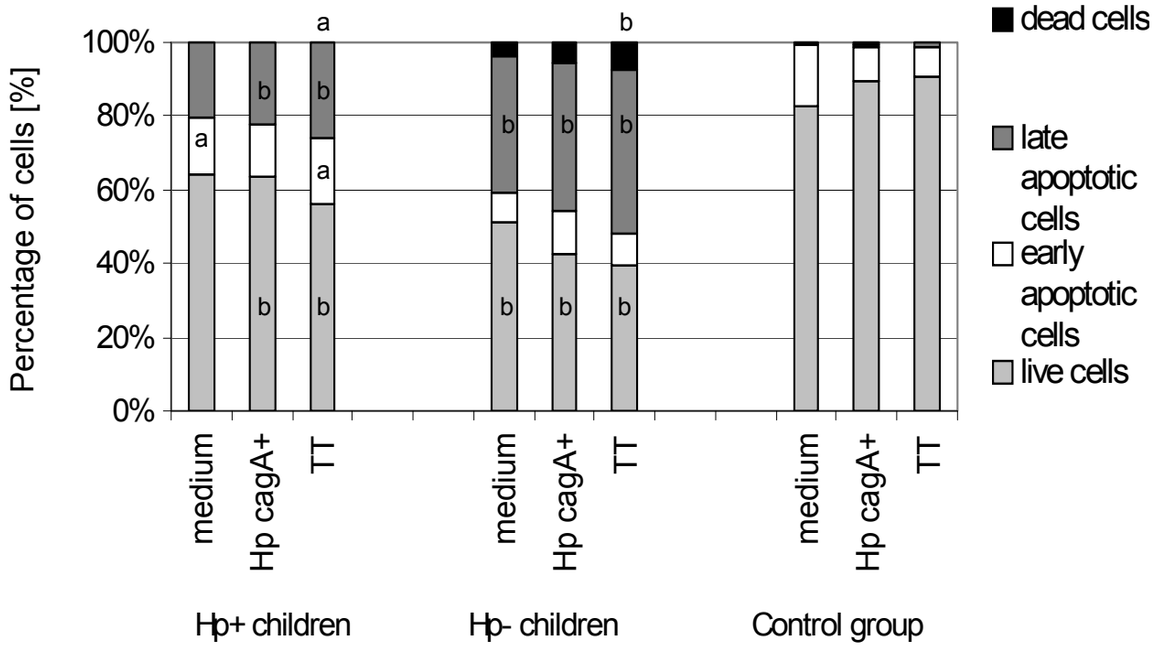
*Hp*+, *H. pylori*-infected children; *Hp*-, noninfected children; SD, standard deviation. Statistically significant differences (Student's *t* test): *versus* controls ([a]$p < 0.05$), *versus Hp*- children ([b]$p < 0.05$).

Table 3. Apoptosis in T-cell subsets in peripheral blood of *Hp*+ and *Hp*- children and the control group

*Hp*- ($p = 0.01$ and $p < 0.01$) children as compared with controls, but the percentages of apoptotic $CD4^+$ and $CD8^+$ T cells were higher in the former group ($p < 0.01$ and $p = 0.02$) (Table 3). Moreover, we found that the naive $CD4^+$ and $CD8^+$ T-cell subsets ($CD45RA^+$) and memory $CD4^+$ T-cell subset ($CD45RO^+$) showed an increased apoptosis in both *Hp*+ ($p = 0.02$, $p < 0.01$ and $p = 0.01$) and *Hp*- ($p < 0.01$, $p = 0.02$ and $p < 0.01$) children, but with no statistically significant differences between the groups.

### 3.4 The apoptosis of lymphocytes in *H. pylori*-induced PBMCs

The level of lymphocyte early apoptosis was unchanged in *H. pylori*- and TT-stimulated culture of PBMCs as compared with medium in both the *Hp*+ and *Hp*- children. However, *Hp*+ children had a higher level of spontaneous and TT-induced early apoptosis of lymphocytes than the *Hp*- ones ($p = 0.02$ and $p < 0.01$) (Fig. 1). Lymphocyte late apoptosis in *H. pylori*- and TT-induced PBMCs was equally high in both *Hp*+ and *Hp*- children (*Hp*+: $p = 0.01$ and $p = 0.09$; *Hp*-: $p = 0.01$ and $p = 0.01$) when compared to the controls. Moreover, both *Hp*+ and *Hp*- children had a low proportion of live lymphocytes in PBMC culture with *H. pylori* and TT (*Hp*+: $p = 0.05$ and $p < 0.01$; *Hp*-: $p = 0.01$ and $p = 0.02$), with no significant differences between the groups. However, only *Hp*- children have high percentage of dead (necrotic) cells in culture PBMC especially after TT stimulation ($p < 0.01$ *versus* *Hp*+ children and controls).




*Hp*+, *H. pylori*-infected children; *Hp*-, noninfected children; *Hp*, *H. pylori*; TT, tetanus toxoid. Results represent median values obtain from n independent experiments: *Hp*+ children and controls: n = 6; *Hp*- children: n = 5. Statistically significant differences (Mann-Whitney's *U*-test): *versus* *Hp*- children ($^a p < 0.05$), *versus* controls ($^b p < 0.05$).

Fig. 1. The apoptosis of lymphocytes in PBMC culture in *Hp*+ and *Hp*- children and the control group

### 3.5 The proliferative response in *H. pylori*-induced PBMCs

The changes in the mode of lymphocyte proliferation involved: a) lower lymphocyte response to *H. pylori* in the *Hp*+ children than in the *Hp*- ones ($p = 0.03$) and control group ($p < 0.01$); b) increased response to Con A in the *Hp*+ as compared to the *Hp*- children ($p = 0.02$) and control group ($p = 0.04$). However, lymphocytes of *Hp*+ and *Hp*- children and control group did not differ in their response to TT (Table 4).

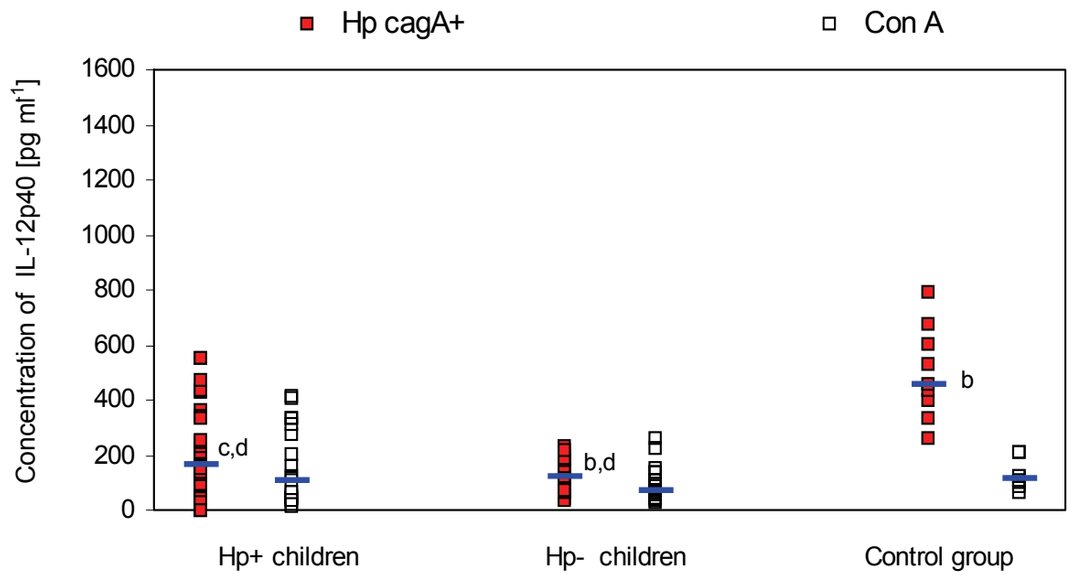
| Type of stimulator | Medians [CI] | | |
|---|---|---|---|
| | *Hp*+ children (n = 46, 14 or 8) | *Hp*- children (n = 59, 21 or 13) | Control group (n = 13) |
| *H. pylori cagA*+ | 3.28[a,b] [1.55/26.09] | 6.29 [3.50/45.68] | 10.48 [8.26/24.16] |
| Concanavalin A | 312.12[a,b] [190.63/513.91] | 169.12 [111.44/199.25] | 121.52 [90.60/181.82] |
| Tetanus toxoid | 7.77 [0.45/18.02] | 4.27 [3.06/25.68] | 2.44 [0.71/5.01] |

*Hp*+, *H. pylori*-infected children; *Hp*-, noninfected children; CI, confidence intervals. Statistically significant differences (Mann-Whitney's *U*-test): *versus* controls ([a]$p < 0.05$), *versus Hp*- children ([b]$p < 0.05$).

Table 4. PBMC proliferative response in *Hp*+ and *Hp*- children and the control group

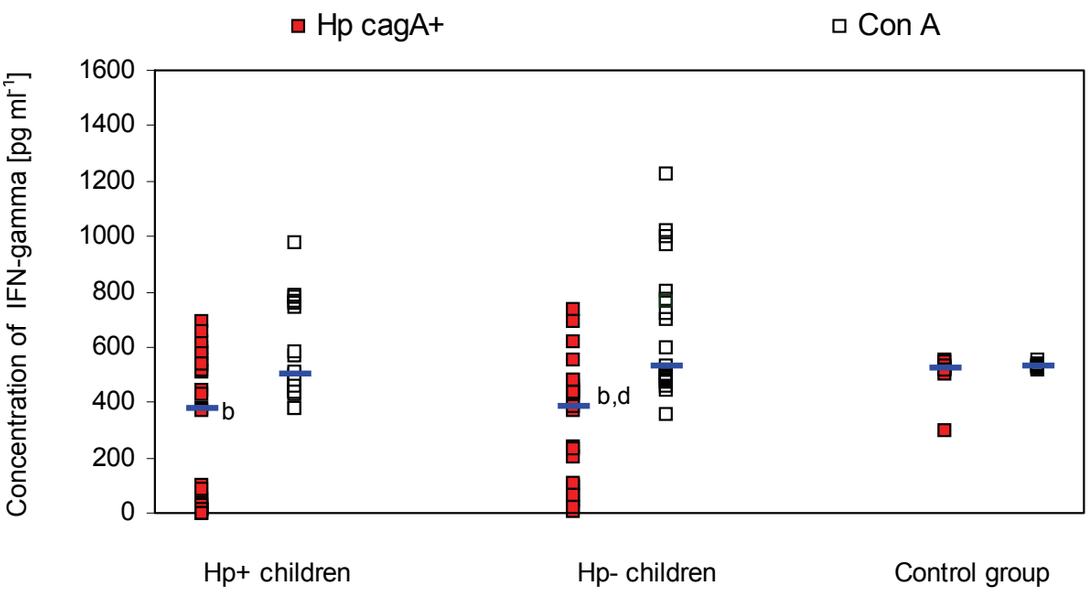
### 3.6 The pattern of cytokine expression in *H. pylori*-induced PBMCs

The levels of *H. pylori*- or Con A-induced cytokines did not exceed the control values in both *Hp*+ and *Hp*- children. However, the profile of cytokine synthesis showed a different pattern: 1) IL-12p40 synthesis (Fig. 2): *H. pylori* was a poor IL-12p40 inducer both in the *Hp*+ and *Hp*- children ($p < 0.01$). However, in the *Hp*+ children production of IL-12p40 was higher than in the *Hp*- ones ($p = 0.02$). In contrast, Con A stimulation gave rise to a similar IL-12p40 level in both groups of children. Finally, the IL-12p40 synthesis profile in the *Hp*- children was similar to that of the control group with a better response to *H. pylori* than to Con A ($p = 0.04$ and $p < 0.01$). 2) IFN-gamma synthesis (Fig. 3): *H. pylori*-induced IFN-gamma synthesis remained unchanged in the *Hp*+ children, but it was slightly lower in the *Hp*- ones ($p = 0.01$) as compared with the control group. Con A was a significantly better IFN-gamma inducer than *H. pylori* in both *Hp*+ and *Hp*- children with no significant differences between the two groups. 3) TNF-alpha synthesis (Fig. 4): In the *Hp*- children, *H. pylori* and Con A-induced TNF-alpha levels were lower than in the control group ($p < 0.01$ and $p = 0.01$). However, Con A was a stronger inducer of TNF-alpha in the *Hp*+ children than in the *Hp*- ones ($p = 0.02$). 4) IL-6 synthesis (Fig. 5): *H. pylori* was a poorer IL-6 inducer in the *Hp*+ children than in the *Hp*- ones ($p = 0.03$) and control group ($p = 0.03$); this tendency was maintained with Con A being a worse stimulator of IL-6 in the *Hp*+ than in the *Hp*- children ($p < 0.01$). 5) IL-10 synthesis (Fig. 6): *H. pylori* was a better IL-10 inducer in the *Hp*+ than in *Hp*- children ($p = 0.03$). However, in both the *Hp*- children and control group Con A was a better IL-10 inducer than *H. pylori* ($p = 0.05$).



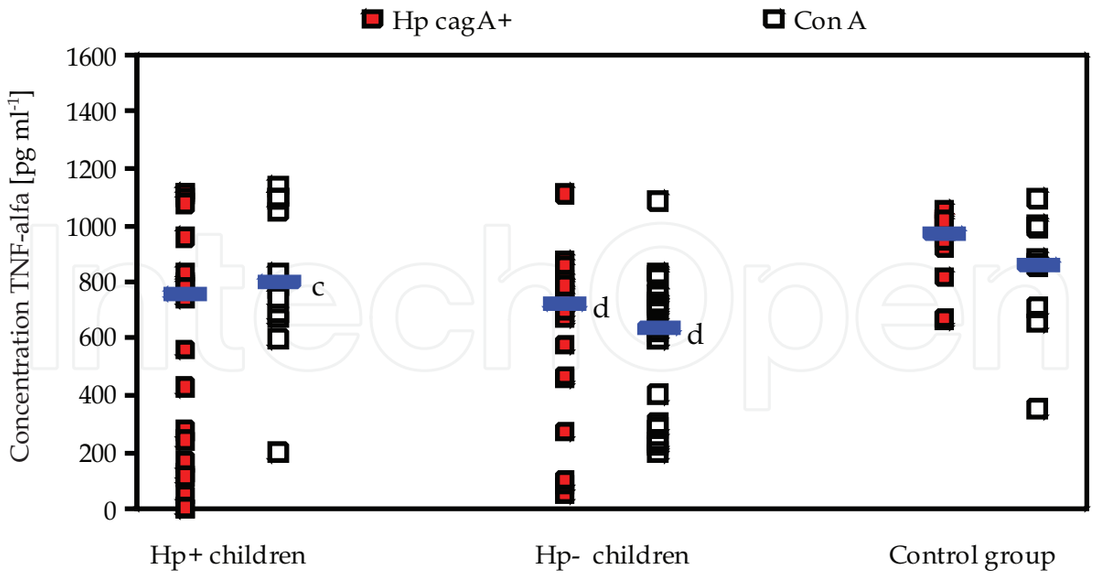

*Hp*, *H. pylori*; *Hp*+, Con A, Concanavalin A, *Hp*+, *H. pylori*-infected children; *Hp*-, noninfected children. Blue horizontal bars represent medians obtained from n independent experiments: *Hp*+ children: n = 29; *Hp*- children: n = 23; controls: n = 9. Statistically significant differences (Mann-Whitney's *U*-test): *versus* Con A stimulation. ($^{b}p < 0.05$), *versus Hp*- children ($^{c}p < 0.05$), *versus* controls ($^{d}p < 0.05$).

Fig. 2. Concentration of IL-12p40 in culture supernatants of PBMC in *Hp*+ and *Hp*- children and the control group



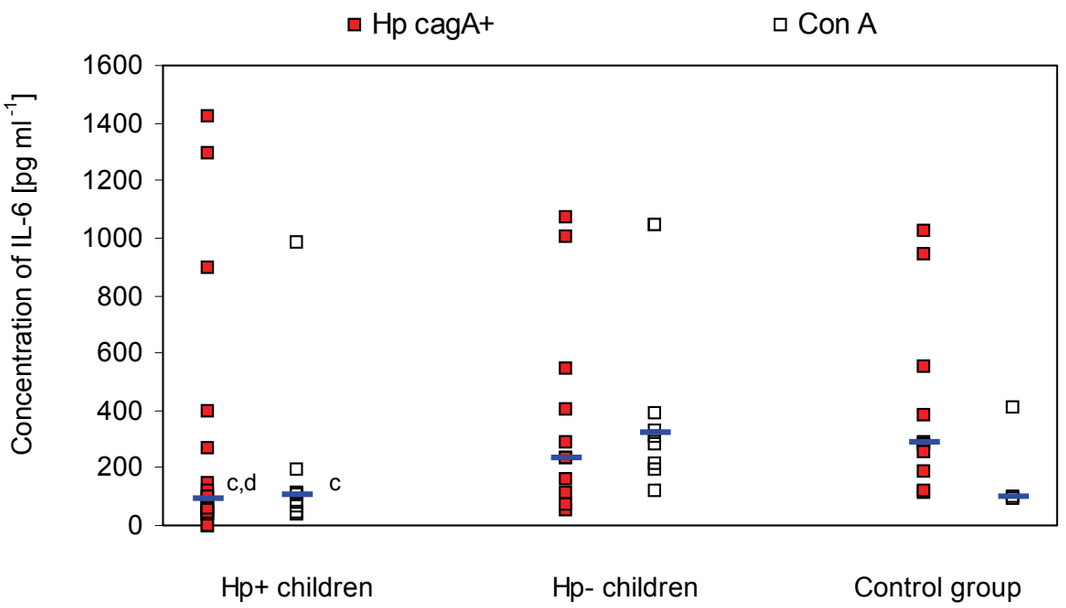

*Hp*, *H. pylori*; *Hp*+, Con A, Concanavalin A, *Hp*+, *H. pylori*-infected children; *Hp*-, noninfected children. Blue horizontal bars represent median values obtained from n independent experiments: *Hp*+ children: n = 27; *Hp*- children: n = 27; controls: n = 9. Statistically significant differences (Mann-Whitney's *U*-test): *versus* Con A stimulation. ($^{b}p < 0.05$), *versus Hp*- children ($^{c}p < 0.05$), *versus* controls ($^{d}p < 0.05$).

Fig. 3. Concentration of IFN-gamma in culture supernatants of PBMC in *Hp*+ and *Hp*- children and the control group



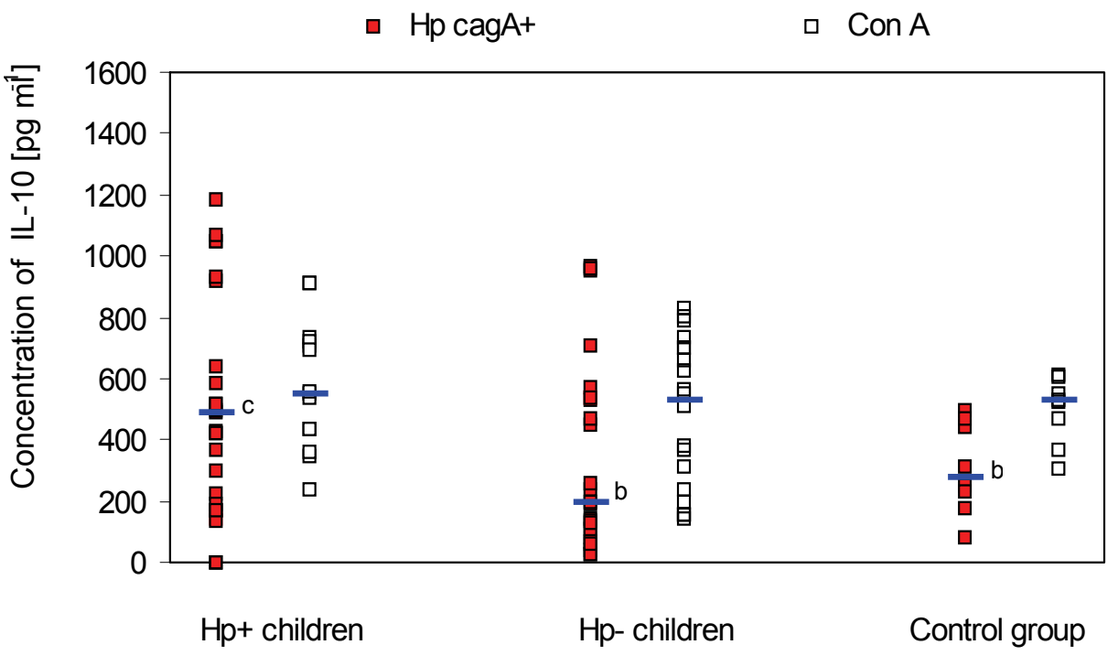

*Hp*, *H. pylori*; *Hp*+, Con A, Concanavalin A, *Hp*+, *H. pylori*-infected children; *Hp*-, noninfected children. Blue horizontal bars represent median values obtained from n independent experiments: *Hp*+ children: n = 19; *Hp*-children: n = 22; controls: n = 10. Statistically significant differences (Mann-Whitney's *U*-test): *versus Hp*- children ($^{c}p < 0.05$), *versus* controls ($^{d}p < 0.05$).

Fig. 4. Concentration of TNF-alfa in culture supernatants of PBMC in *Hp*+ and *Hp*- children and the control group




*Hp*, *H. pylori*; *Hp*+, Con A, Concanavalin A, *Hp*+, *H. pylori*-infected children; *Hp*-, noninfected children. Blue horizontal bars represent median values obtained from n independent experiments: *Hp*+ children: n = 21; *Hp* children: n = 12; controls: n = 11. Statistically significant differences (Mann-Whitney's *U*-test): *versus Hp*- children ($^{c}p < 0.05$), *versus* controls ($^{d}p < 0.05$).

Fig. 5. Concentration of IL-6 in culture supernatants of PBMC in *Hp*+ and *Hp*- children and the control group




*Hp*, *H. pylori*; *Hp*+, Con A, Concanavalin A, *Hp*+, *H. pylori*-infected children; *Hp*-, noninfected children. Blue horizontal bars represent median values obtained from n independent experiments: *Hp*+ and *Hp*- children: n = 23; controls: n = 8. Statistically significant differences (Mann-Whitney's *U*-test): *versus* Con A stimulation. ($^{b}p < 0.05$), *versus Hp*- children ($^{c}p < 0.05$).

Fig. 6. Concentration of IL-10 in culture supernatants of PBMC in *Hp*+ and *Hp*- children and the control group

## 4. Discussion

In the present study *H. pylori*-infected and noninfected children with gastritis and the control group were compared with respect to 1) the distribution and apoptosis of T lymphocyte subsets in the blood, and 2) lymphocyte apoptosis, proliferation and cytokine synthesis pattern in the culture PBMCs. Additionally, the lymphocyte phenotypes of children with gastritis were correlated with gastric inflammation scores.

We report that the group of *H. pylori*-infected children have: 1) an elevated proportion of naive $CD8^+$ ($CD45RA^+$) T-cell population along with an increase in the percentage of apoptotic $CD4^+$ and $CD8^+$ T cells in the blood, 2) high spontaneous early lymphocyte apoptosis, low lymphocyte proliferation to *H. pylori* but a high response to Con A, and 3) slightly increased IL-10 expression along with decreased IL-6 expression in *H. pylori*-induced PBMC culture. Children with gastritis but without infection had a high percentage of memory $CD8^+$ T-cell subset ($CD45RO^+$) in the blood and a low INF-gamma and TNF-alpha in *H. pylori*-induced PBMC culture. Both *H. pylori*-infected and noninfected children with gastritis were characterized by 1) an increased percentage of apoptotic naive and memory $CD4^+$ and $CD8^+$ T-cell subsets in blood, 2) a high percentage of late apoptotic lymphocytes, along with a low proportion of live lymphocytes, and a low IL-12p40 expression in culture PBMCs.

An increase in the percentage of CD45RA-bearing $CD8^+$ T-cell subsets in the *H. pylori*-infected children may indicate that the infection with *H. pylori* induces the thymic T-cell renewal leading to the generation of T cells with high expression of CD45RA isoform. These results differ from those obtained in another study, also examining blood, which showed an unchanged proportion of circulating naive T cell subset ($CD45RA^+$) in a group of *H. pylori*-infected children [Helmin-Basa et al., 2011]. This discrepancy might result from a different number of examined children and the age of the children evaluated in previous study. However, similar changes have been observed in the gastric antral mucosa using quantitative immunohistochemistry [Maciorkowska et al., 2004].

Other differences that we have observed in *H. pylori*-infected children were the increase in the percentage of apoptotic $CD4^+$ and $CD8^+$ T lymphocytes in the blood and spontaneous early apoptosis of lymphocyte in PBMC culture. Similar changes have been observed in previous studies that evaluated the apoptosis of gastric mucosa inflammatory cells showing an increased apoptosis of $CD4^+$ T-cell subset using immunohistochemistry methods [Guarner et al., 2003, Kotłowska-Kmieć et al., 2009].

The noninfected children with gastritis had an elevated proportion of CD45RO-positive $CD8^+$ T-cell subset in the blood. These results are analogous to our previous study that also showed an increase in the percentage of circulating memory T cells with very high expression of CD45RO isoform in a group of noninfected children with gastritis [Helmin-Basa et al., 2011]. This suggests the activation of thymic–generated $CD45RA^+$ cells leading to the transition of the CD45RA isoform into CD45RO.

Additionally, our results have shown that circulating naive ($CD45RA^+$) and memory ($CD45RO^+$) T-cell subsets display high apoptosis in the *H. pylori*-infected and noninfected children with gastritis. $CD45RO^+$ cells may provide pro-inflammatory signals that contribute to the gastric inflammation. Hence, high apoptosis, especially of the memory T cells, can make the gastritis self-limited regardless of *H. pylori* infection.

We also found that the lymphocytes of the *H. pylori*-infected children had reduced proliferation in *H. pylori cagA*+-induced PBMC culture. These results essentially confirm some adult reports indicating that *H. pylori* infection is associated with a decreased peripheral lymphocyte response to live or killed *H. pylori* bacteria or their products (Fan et al., 1994; Malfitano et al., 2006; Lungren et al., 2003). Several mechanisms have been proposed as an explanation for this phenomenon, for example the active suppression of T and B lymphocyte proliferation potential, suppression of monocytes activation, inhibitory action of several virulent *H. pylori* products including CagA protein and VacA cytotoxin, and others (Boncristino et al., 2003; Wang et al., 2001). Profound low lymphocyte response to *H. pylori cagA*+ in infected children with high proliferation to antigen specific inducer (tetanus toxoid) and T -cell mitogen (Con A) in both the *H. pylori*-infected and noninfected children with gastritis, as well as in the controls leads to a number of conclusions. First-of-all, a low lymphocyte proliferation in response to *H. pylori* induction selectively found in the *H. pylori*-infected children is highly specific only for *H. pylori*. Secondly, the ongoing *H. pylori* infection creates conditions for *H. pylori* specific low T lymphocyte proliferation. This profile of response is also typical of gastric mucosa T cells in *H. pylori*-infected adults showing unresponsiveness to *H. pylori* antigens (Lungren et al., 2005; Ren et al., 2000) unless costimulatory cytokines are present (Ren et al., 2000).

We report here that lymphocytes of both *H. pylori*-infected and noninfected children with gastritis proved to be much more sensitive to *H. pylori* bacteria and tetanus toxoid-induced apoptosis than lymphocytes of the controls. These observations indicate that gastritis itself (independent of ongoing *H. pylori*-infection) makes peripheral lymphocytes sensitive to apoptosis. However, the higher early spontaneous lymphocyte apoptosis in *H. pylori*-infected children may suggest that ongoing *H. pylori* infection can make the lymphocyte apoptosis rate faster, possibly due to their prolonged contact with *H. pylori in vivo*. Our study of apoptotic lymphocytes in blood confirm that *H. pylori* infection increases apoptosis of circulating $CD4^+$ and $CD8^+$ T-cell subsets.

In general, our results concerning lymphocyte apoptosis are partly in agreement with other reports suggesting that *H. pylori*-induced apoptosis could be the reason for modest *H. pylori*-induced proliferation of T cells in *H. pylori*-infected subjects (Schmees et al., 2007). Some authors suggest that the apoptotic mechanism possibly involves Fas-bearing T cells through induction of FasL expression (Wang et al., 2000, 2001). Also, gastric T cells have been reported to express FasL and undergo apoptosis *in situ* following *H. pylori* infection as has also been shown previously (Galgani et al., 2004). The role of *cag* PAI in induction of T cell death through Fas/FasL interaction has also been strongly suggested on the basis that *cag* PAI-deficient strains of *H. pylori* were not able to induce apoptosis in T cells (Wang et al., 2001). In line with these results, Galgani e*t al*. (Galgani et al., 2007) have shown that *cagA*+ strains of *H. pylori* were highly effective inducers of apoptosis in human monocytes, but not in monocyte-derived dendritic cells. Our results also suggest that at least in the model of *H. pylori*-induced PBMCs *cagA* expression in the stimulating bacteria affected lymphocyte apoptosis levels.

Low *H. pylori*-induced IL-12p40 production found in the PBMCs of *H. pylori*-infected children may result either from limited number of IL-12 producing cells or from the defects in IL-12 regulation. Gastritis itself also limits the PBMC abilities in terms of IL-12p40 synthesis since *H. pylori*-activated PBMCs of noninfected children with gastritis also showed lower IL-12p40 production than controls; however, the values were higher than those of infected subjects. This observation may indicate that children gastritis itself, regardless of *H. pylori* infection, down-regulates IL-12p40 expression in *H. pylori*-induced PBMCs, and that *H. pylori*-infection makes this process more profound. Gram-negative bacteria have been found to induce rather low IL-12 synthesis in PBMCs but high IL-10 levels (Hessle et al., 1999, 2000). In this model system IL-12 is produced mainly by monocytes and is up-regulated by T cells, since their removal from PBMCs decreases the bacteria-induced IL-12 production (Hessle et al., 1999). Low IL-12p40 levels found in the *H. pylori cagA*+-induced PBMCs of the *H. pylori*-infected children and in a lesser degree also in the noninfected children with gastritis may result from two processes. Firstly, a high apoptosis of $CD4^+$ T cells participating in the up-regulation of IL-12 production by monocytes, and secondly, hypo-reactivity and/or apoptosis of monocytes directly induced either by *H. pylori* action (Galgani et al. 2004), or indirectly mediated by a relative excess of IL-10 (D'Andrea et al., 1993). Low IL-12 production may play a role in a complex and still poorly understood mechanism of rather limited *H. pylori*-mediated pro-inflammatory responses usually observed in children (Bontems at al. 2003, Lopes et al. 2005).

We have found that the production of INF-gamma decreased in noninfected children with gastritis but not in *H. pylori*-infected ones. This is in contrast with the IL-12p40 findings, and

indirectly suggests that in the PBMC model system, *H. pylori cagA*+-induced IFN-gamma production essentially does not depend on IL-12p40. This observation confirms the results of other researchers showing that PBMC stimulation with Gram-negative bacteria results in a rather low and separately-independent IFN-gamma and IL-12 expression (Hessle et al., 2000).

In the *H. pylori*-infected children, but not in the noninfected ones, a relatively high *H. pylori cagA*+-mediated IFN-gamma synthesis was connected with relatively elevated IL-10 production (both responses were on the level of the controls). This observation may indicate a possible role of IL-10 in the down-regulation of IFN-gamma in the course of *H. pylori*-infection in children. This issue has been studied extensively by others (Holck et al., 2003). *H. pylori* bacteria (live or killed) and their antigens have been found to induce a higher IL-10 production in PBMCs of *H. pylori*-infected than noninfected adults (Haeberle et al., 1997; Jakob et al., 2001; Windle et al., 2005).

To the best of our knowledge, our report is the first showing a relative increase in IL-10 production in culture PBMCs of *H. pylori*-infected children. These results are consistent with previous findings, indicating that *H. pylori* infection is related to a high expression of IL-10 in the gastric mucosa both on mRNA and protein level (Bodger et al., 2001; Hida et al., 1999). IL-10 has long been considered a potent anti-inflammatory cytokine strongly implicated in *H. pylori* infection (Bodger et al., 2001). On the one hand it may protect from the harmful effects of potentially pro-inflammatory responses induced by *H. pylori,* but on the other, it may inhibit the protective mechanisms of immune responses directed against *H. pylori* antigens.

We also found that *H. pylori cagA*+ was a much lower IL-6 inducer in the culture PBMCs of *H. pylori*-infected than noninfected children with gastritis. Diminished IL-6 induction may play a role in the hypo-responsiveness of PBMCs to *H. pylori* stimulation in infected children. In our model system IL-6 is produced both by T cells and monocytes. IL-6 is a known strong co-stimulatory cytokine that plays a significant role in providing second signal to antigen-induced T cells (Shi et al., 1989).

In this study, *H. pylori* evoked a similar activity in stimulating TNF secretion in PBMCs of both *H. pylori*-infected and noninfected children. However, in the former group there was a tendency to a slightly lower TNF-alpha level. This partially confirms the findings of earlier studies in the stomach in which *H. pylori*-infected children and adults showed a similar concentration of this cytokine (Bontems et al., 2003). Unchanged or slightly decreased production of this cytokine in *H. pylori cagA*+-mediated PBMCs may also be contributing to the limited pro-inflammatory responses in children with gastritis.

## 5. Conclusion

In conclusion, *H. pylori* infection in children results in: a) increased percentage of peripheral blood memory $CD8^+$ T cells, b) high apoptosis of circulating $CD4^+$ and $CD8^+$ T-cell subsets, c) *H. pylori*-specific-peripheral hypo-responsiveness (low lymphocyte proliferation and IL-12p40 expression), and d) unchanged *H. pylori* dependent pro- inflammatory responses (IFN-gamma, TNF-alfa, respectively), associated with a high expression of IL-10 but low expression of IL-6. This response pattern, together with a high T-cell subsets apoptosis, may

protect from more aggressive forms of *H. pylori*-induced inflammation, but on the other hand may also participate in the failure of eliminating the infection.

## 6. Acknowledgment

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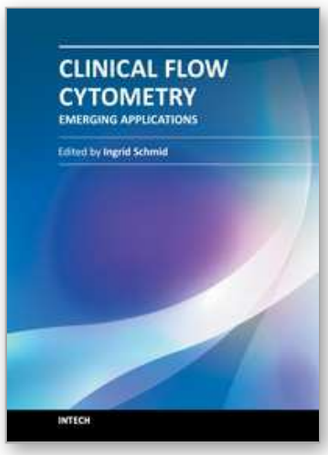


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