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ORIGINAL ARTICLE

Analysis of peripheral blood lymphocyte phenotypes and Th1/Th2 cytokines profile in the systemic immune responses of *Helicobacter pylori* infected individuals

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ABSTRACT

***H. pylori* elicits specific humoral and cellular immune responses in the mucosal immune system. However, the type and extent of T lymphocyte response in the systemic immune system is not clear for *H. pylori* positive patients. In this study, peripheral blood T lymphocyte phenotypes and serum Th1/Th2 based cytokines of 32 *H. pylori* positive patients were analyzed and compared to those of healthy controls. While $\alpha\beta$ TCR$^+$ lymphocytes and their phenotype analysis were not significantly different to those of healthy controls, the percentage of pan $\gamma\delta$ TCR$^+$ lymphocytes was up to 2.4 times greater in the *H. pylori* positive group then in healthy controls. Furthermore, significant increases in IL-10 concentrations in serum samples of *H. pylori* patients indicated that their immune systems had switched toward a Th2 type immune response. The correlation between phenotype and type of T cell response in the peripheral blood during *H. pylori* infection is discussed.**

Key words cytokines, gamma delta T cells, *helicobacter pylori*, immunophenotyping.

H. pylori is a gram negative, microaerophilic and motile bacterium that causes persistent infection in the human gastrointestinal tract, especially the stomach, starting at an early age (1). It induces persistent infection in gastric mucosa, resulting not only in chronic gastritis which is asymptomatic in most individuals; but also in an association with pathologic development of gastric and duodenal ulcers. More seriously, it has also been associated with gastric adenocarcinoma, mucosa associated lymphoid tissue lymphoma and primary gastric non-Hodgkin's lymphoma (2, 3).

H. pylori infection induces vigorous humoral and cellular immune responses. Neutrophils, mast cells, eosinophils and B- and T- lymphocytes are all involved in that inflammatory process (4). Infection mediated chemokines and pro-inflammatory cytokines secreted by gastric epithelial cells stimulate the migration of granulocytes, monocytes and lymphocytes into the inflamed mucosa which results in more severe inflammatory pathology (5–7). In the case of the humoral immune response, infiltrating B lymphocytes and plasma cells give rise to *H. pylori* specific IgA and IgG antibodies. CD4$^+$ T cells both help B lymphocytes to produce antibody, and induce inflammatory processes in the gastric mucosa by producing high amount of IFN-γ (8, 9). It has been shown that increased numbers of activated CD8$^+$ T cells also accumulate in the gastric mucosa.

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List of Abbreviations: CD, cluster of differentiation; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; HLA-DR, human leukocyte antigen DR-1; Hp (+), *H. pylori* positive; Hp (−), *H. pylori* negative; *H. pylori Helicobacter pylori*; IFN, interferon; Ig, immunoglobulin; Il, interleukin; NK, natural killer; PC5, phycoerytrincyanin5; PE, phycoerythrin; TCR, T cell receptor; TCR$^+$, T cell receptor positive; TGF-β, transforming growth factor beta; Th, T-helper cell; TNF-α, tumor necrosis factor alpha

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The participation of mucosal $CD8^+$ T cells in the inflammatory process may lead to development of more severe disease outcomes as described above (10, 11).

Although *H. pylori* specific IgG or IgA antibodies can be detected in the peripheral blood in the early stages of infection, it is a matter of debate as to whether circulating lymphocytes are increased and activated by *H. pylori* infection. On the one hand, some researchers have reported that peripheral blood lymphocytes of infected individuals show neither proliferation nor activation signals, and that peripheral blood lymphocytes isolated from patients with duodenal ulcer and chronic antral gastritis show no significant alteration in systemic immune system in response to *H. pylori* (12, 13). On the other hand, other groups have reported that stimulation of peripheral blood lymphocytes with distinct *H. pylori* antigenic fragments induces not only proliferation but also activation of $CD4^+$ and $CD8^+$ T lymphocytes (14–16).

Another debate concerns the type of T cell response both in the gastric mucosa and the periphery. Some groups have shown that the T cell response to *H. pylori* is dominated by a Th1 type T cell response in the gastric mucosa. The consequent release of IFN-γ by Th1 cells induces expression of pro-inflammatory cytokines TNF-α, IL-12 and IL-18 by T lymphocytes and granulocytes in the gastric mucosa (17, 18). However, a regulatory T cell response which dominates secretion of IL-4, IL-10 (19) and TGF-β (20) to down-regulate the inflammatory response and reduce bacterial load has also been reported. Despite the wide range of T cell responses in gastric mucosa, their impact on the systemic immune system is not known.

In view of the role of *H. pylori* in the pathogenesis of persistent bacterial infection, it is of interest to study the effect of *H. pylori* infection on the phenotypic distribution of lymphocytes, as well as on the pattern of cytokines involved both in inflammatory and anti-inflammatory T cell responses. Hence, it is reported here that *H. pylori* infection leads to expansion of pan gamma delta TCR positive T cells groups in the peripheral blood circulation, and that cytokine concentrations display a shift toward a Th2 type immune response as evidenced by high concentrations of IL-10 in the peripheral blood.

MATERIALS AND METHODS

Patients and collection of samples

The study was approved by the Ethical Committee for Human Research, Faculty of Medicine, Zonguldak Karaelmas University, Zonguldak, Turkey.

We studied 32 Hp (+) patients (10 men and 22 women; mean age 37 years; range 19–55) who underwent investigation of gastric complaints and 15 Hp (−) uninfected volunteers (6 men and 9 women; mean age 40 years, range 22–56).

The Hp (+) patients had not received antimicrobial, anticholinergic or anti-inflammatory agents, proton pump inhibitors or H2 receptor antagonists before endoscopy. At the time of endoscopy venous blood samples were obtained from each patient to evaluate *H. pylori* specific IgA and IgG antibodies, phenotypes and cytokine concentrations. Serum samples were assayed for *H. pylori* specific IgA and IgG antibodies by using a commercial ELISA test according to the manufacturer's instructions. (DIA.PRO Diagnostic Bioprobes, Milano Italy). The *H. pylori* status of patients was also assessed by the 14C-urea breath test within a week after endoscopy. Members of the Hp (+) group were identified on the basis of the 14C-urea breath test, positive *H. pylori* specific IgA and IgG and their endoscopy reports. According to endoscopy reports all Hp (+) patients had chronic antral gastritis. Since endoscopy was not considered to be an ethical procedure for asymptomatic subjects, control Hp (−) healthy controls were identified on the basis of being without symptoms of gastrointestinal system and were confirmed to be negative for Hp specific IgA, IgG and 14C urea breath tests.

Flow cytometry analysis

Mouse anti-human monoclonal antibodies specific for $IgG_{1,2a,}$ CD14, CD45, CD45RO, CD45RA CD3, CD19, CD4, CD8, HLA-DR, CD27, $CD16^+56^+$, pan TCR $\gamma\delta$ were purchased from either BD Bioscience (San Jose, CA, USA) or Beckman Coulter (Immunotech, Marseille, France). Peripheral blood lymphocyte phenotypes were analyzed following the immunofluorescence procedure recommended by Becton Dickinson. Briefly, peripheral blood samples were incubated with 10 μl FITC, PE or phycoerytrincyanin5 labeled mouse anti-human monoclonal antibodies in the dark for 15 min at room temperature. Erythrocytes were lysed in ImmunoPrep solutions maintained for Coulter TQ Prep (Beckman Coulter, Miami, CA, USA) according to the manufacturer's instructions. A Beckman Coulter Epics XL.MCL, four-color, equipped with computer software Expo 32 ADC (Beckman Coulter) was used to assess the phenotype analysis of lymphocytes. For each analysis, 50 000 cells were collected.

Cytokine measurement

Cytokine concentrations in the serum samples of Hp (+) patients and Hp (−) healthy controls were determined by using the commercially available fluorescent bead immunoassay; FlowCytomix human Th1/Th2 11 plex kit (BenderMed Systems, GmbH, Vienna, Austria) according to the manufacturer's instructions. Briefly; serum samples or serial diluted standards were suspended with

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fluorescent beads and coated with monoclonal antibodies specific to distinct cytokines, together with cytokine specific biotin conjugated monoclonal antibodies. Following two hr incubation, beads were washed twice and incubated with PE labeled Streptavidin. Forward and Side scatter voltages were adjusted with specific set up beads, a 675 nm (fluorescent 4) detector was used for bead differentiation and a 575 nm (fluorescent 2) detector for bead quantification. Standard curves were determined for each cytokine from a range of 27.44–20 000 pg/mL. The lower limits of detection for all cytokines according to the manufacturer are between 0.8–20 pg/mL. Samples were analyzed at EPICS-XL MCL flow cytometry (Beckman Coulter). The concentrations were assessed by using FlowCytomix Pro 2.1 software (Bender MedSystems). For each analysis 3000 beads were collected.

Statistical analysis

Statistical analysis was performed with two-tailed Mann Whitney U and Pearson Correlation tests with $p < 0.05$ considered as statistically significant according to the SPSS 10.0 software program.

RESULTS

Prior to phenotypic analysis of peripheral blood samples, lymphocyte, monocyte and granulocyte distributions were assessed by forward and side scatter analysis. The granulocyte frequency was significantly higher in Hp (+) as compared to Hp (−) subjects ($P < 0.05$; Fig. 1). While the Hp (+) group showed 32.63% lymphocytes, 6.03% monocytes and 57.13% granulocytes, the Hp (−) group showed 39.8% lymphocytes, 6.9% monocytes and 49.0% granulocytes (Fig. 1).

Phenotypic distribution of lymphocytes

Phenotypic distribution of B and T lymphocytes among lymphocyte gates are demonstrated in Figure 2. Individuals infected with *H. pylori* did not show a higher percentage of $CD19^+/CD3^-$ B lymphocytes and $CD3^+/CD19^-$ T lymphocytes in comparison to non-infected subjects (Fig. 2a). Moreover, analysis of percentages of T lymphocyte sub-groups showed that the frequencies of $CD4^+$ and $CD8^+$ lymphocytes are comparable in Hp (+) and Hp (−) groups (Fig. 2b). A similar phenomenon was also observed for the CD4/CD8 ratio (Table 1). According to analysis of the activation marker HLA-DR^+, there was no significant increase in the frequency of activated T lymphocytes over $CD3^+$ T cells (Table 1).

Since peripheral blood is known to contain both naïve, memory and effector $CD4^+$ and $CD8^+$ T cells (21, 22), the frequencies of T lymphocyte subpopulations were investigated in both Hp (+) and Hp (−) groups. In the case of $CD4^+$ T cells, naïve and memory $CD4^+$ cells were investigated according to the expression levels of CD45RA (naïve) and CD45RO (activated/memory), since the structure of CD45RA switches to CD45RO according to the activation status of T cells (21). As shown in Figure 3a, there were no significant differences between infected and non-infected healthy controls on naïve and memory CD4 T lymphocyte frequencies. The frequencies of $CD8^+$ T lymphocyte subgroups were evaluated as naïve ($CD45RA^+CD27^+$), effector ($CD45RA^+CD27^-$) and memory ($CD45RA^-$ $CD27^{+/-}$) cells. The results show that frequencies of the naïve, memory and effector $CD8^+$ T cell subpopulations were comparable in peripheral blood of Hp (+) and Hp (−) individuals (Fig. 3b, c).

Other lymphocyte groups; NK and NK-T cells; which are phenotypically and functionally distinct from B and T

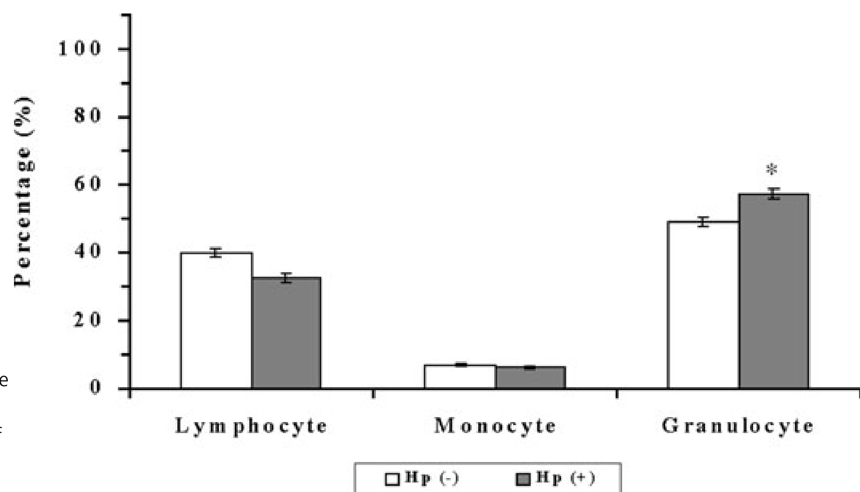

Fig. 1. Distribution of mononuclear cells in peripheral blood samples of Hp (−) (white bar) and Hp (+) (grey bar) subjects. According to forward and side scatter analysis in flow cytometry, the mean percentages of lymphocytes, monocytes and granulocytes were calculated. Data are presented as the mean ± S.E. *indicates statistical significance of granulocyte percentage in Hp (+) group. The actual p value was $p = 0.01$.

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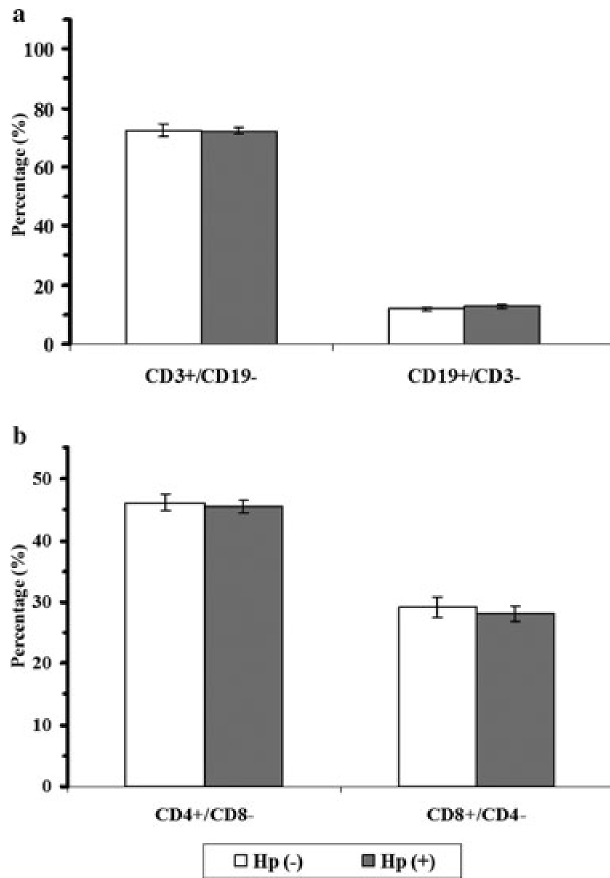

Fig. 2. Immunophenotyping profile of peripheral blood lymphocytes in Hp (−) (white bar) and Hp (+) (grey bar) subjects. (a) T- ($CD3^+/CD19^-$) and B- ($CD19^+/CD3^-$) lymphocytes percentages; (b) $CD4^+$ and $CD8^+$ T cell percentages are presented as the mean ± S.E.

Table 1. Comparison of CD4/CD8 ratio, activated T cells percentages and ratio of activated and naïve CD4 T cell population in peripheral blood samples of Hp(−) and Hp(+) subjects. Activated T cells were identified as $CD3^+HLA\text{-}DR^+$ population

Population	Hp (−)	Hp (+)
CD4/CD8 Ratio	1.64 ± 0.11	1.71 ± 0.087
Percentages of $CD3^+HLA\text{-}DR^+$	4.46 ± 0.52%	5.03 ± 0.46%
$CD4^+CD45RO^+/CD4^+CD45RA^+$ Ratio	2.001 ± 0.47	1.84 ± 0.25

lymphocytes, were investigated. NK cells were identified as $CD3^-CD16^+CD56^+$ and NK-T cells were identified as $CD3^+CD16^+CD56^+$. While the frequency of NK cells in the Hp (+) group was lower than in the Hp (−) group, the frequency of NK-T cells was higher in the Hp (+) group (Fig. 4). However; these differences were not statistically significant ($P > 0.05$).

Interestingly, when the distribution of another T lymphocyte group, that carrying the gamma delta T cell receptor, was investigated the frequencies in Hp (+) subjects were 2.35 times greater in comparison to Hp (−) individuals ($P < 0.05$). (Fig. 4.)

Th1/Th2 based cytokine analysis during *H. pylori* infection

In order to investigate whether *H. pylori* infection has an effect on cytokines that are able to route the T cell response toward an inflammatory Th1 type or anti-inflammatory Th2 type response, the concentrations of nine different cytokines in serum samples of infected and non-infected individuals were analyzed. Those nine cytokines consisted of pro-inflammatory (IL-8, IL-1β, TNF-α), Th1 type inflammatory (IL-2, IFN-γ, IL-12p70) and Th2 anti-inflammatory (IL-10, IL-4, IL-6) type cytokines group. The results are presented in Figure 5. As shown, *H. pylori* infection did not lead to secretion of pro-inflammatory cytokines IL-8, IL-1β and TNF-α into the systemic circulation in any significant way (Fig. 5a). A similar phenomenon was also observed when Th1 type cytokines were analyzed. The concentrations of the inflammatory response type cytokines IL-2, IFN-γ and IL-12p70 were similar for both groups (Fig. 5a). In the case of Th2 cytokines, while IL-6 and IL-4 were at similar concentrations, interestingly IL-10 was significantly higher (8.8 times) in the Hp (+) group in comparison to non infected subjects Fig. 5b) ($P < 0.05$).

DISCUSSION

In the present study the phenotypic distribution of peripheral blood lymphocytes, and the balance between Th1-Th2 T cell responses in the systemic immune system of *H. pylori* infected individuals, was investigated. Our data demonstrates a significant expansion of granulocytes, gamma delta TCR^+ T lymphocytes and high concentrations of IL-10 in the peripheral blood of Hp (+) subjects in comparison to healthy controls. No differences were detected between Hp (+) and Hp (−) subjects in regard to the frequencies of $CD3^+$, $CD19^+$, $CD4^+$, $CD8^+$ T cells and their subsets. Neither pro-inflammatory nor inflammatory cytokines were increased in Hp (+) patients. Accordingly, our data address three main points about the systemic immune response during *H. pylori* infection.

Firstly, many studies have reported that *H. pylori* infection results in massive infiltration of mononuclear cells into the gastric mucosa. However, the pathogen is rarely eliminated and, in the absence of treatment, infection persists for life. That is because, during persistent infection, the pathogen may induce either inflammatory or regulatory cellular immune responses in the gastric mucosa (2, 3). In order to determine the impact of that local immune

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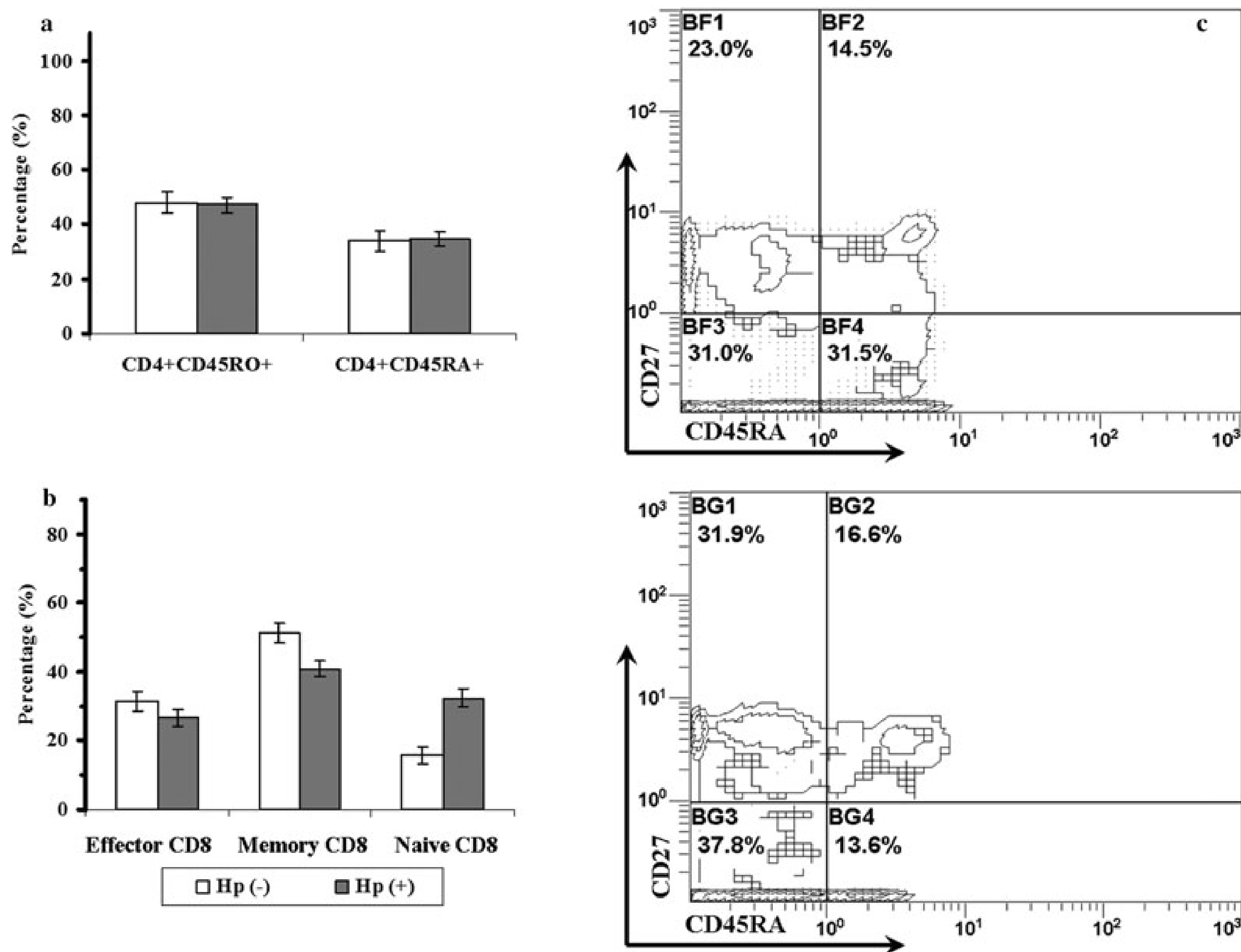

Fig. 3. Distribution of $CD4^+$ and $CD8^+$ sub-groups in peripheral blood. $CD4^+$ T cell sub populations (a) were identified in the gated $CD4^+$ T cell population by using anti- CD45RO (activated/memory cells) and anti-CD45RA (naïve cells). $CD8^+$ T cell sub populations (b) were identified in the pre-gated $CD8^+$ T cell population by using anti-CD45RA and anti-CD27: $CD45RA^+CD27^-$ (effector cells), $CD45RA^+CD27^+$ (naïve cells), $CD45RA^-$ $CD27^{+/-}$ (memory cells). (c) Representative flow cytometry figures of $CD8^+$ sub-populations in Hp (−) and Hp (+) subjects are shown.

response on mononuclear cells in the systemic circulation, increase in peripheral blood mononuclear cells, T-cells and their sub-groups and the expression status of activation markers have been investigated by several groups. Kondo *et al.* have analyzed peripheral blood mononuclear cell counts; specifically neutrophil and monocyte counts, in the peripheral blood of Hp (+) patients. They observed an increase in neutrophil and monocyte counts and therefore concluded that *H. pylori* infection plays a role in systemic disorders (23). Besides that, Karttunen *et al.* observed a significant increase in peripheral blood basophiles in *H. pylori* patients (24). According to our data *H. pylori* infection induces a significant expansion in the granulocyte population, however we do not know which type of granulocytes are involved nor at which phase of the disease their proportion increases.

With respect to the analysis of peripheral blood T cells; Yuceyar *et al* have investigated the expansion of peripheral blood lymphocytes and the expression of activation markers in *H. pylori* associated duodenal ulcer and chronic antral gastritis. They observed neither expansion nor activation of T- and B- lymphocytes and NK cells in peripheral blood samples from Hp(+) subjects (12). Conversely, Soares *et al.* have investigated co-stimulatory marker CD28 expression on $CD4^+$ and $CD8^+$ T cells together with activation marker HLA-DR. They observed an increase in the number of $CD3^+$ and $CD4^+$ cells, and significant expression of co-stimulatory marker CD28 over $CD4^+$ and $CD8^+$ T cells in the Hp (+) group. Moreover, they did not detect an increase in the expression of HLA-DR on T cells, in agreement with other studies and the present data (25). With respect to the effector function

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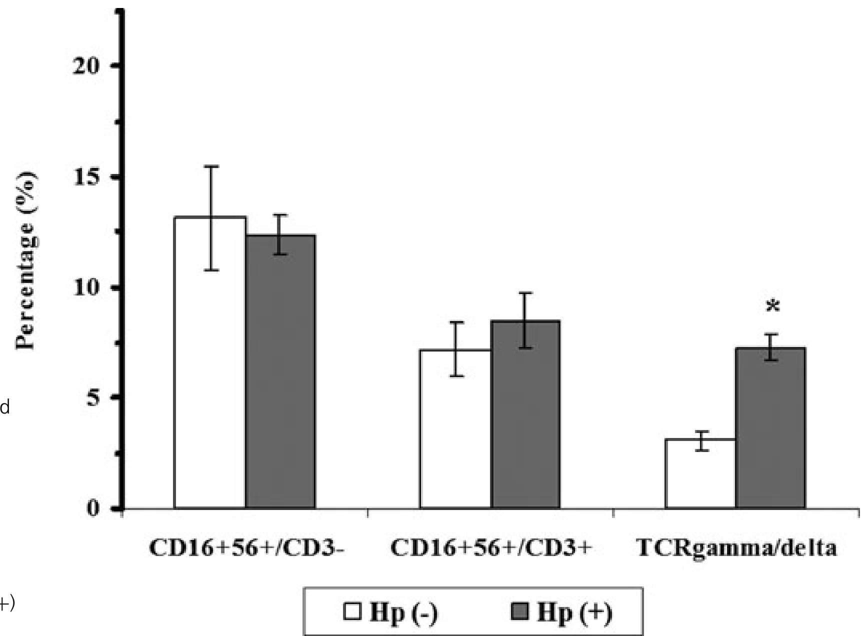
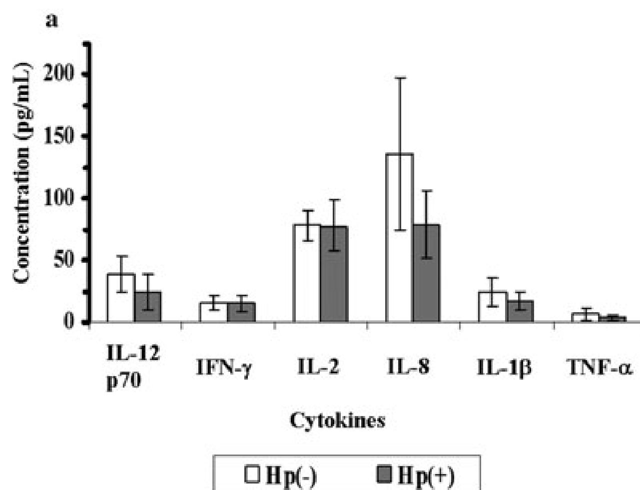


Fig. 4. Distribution of NK, NK-T cells and $\gamma\delta$ TCR$^+$ T cells in peripheral blood samples of Hp (−) and Hp (+) subjects. NK cells were identified as CD16$^+$CD56$^+$/CD3$^-$, NK-T cells were identified as CD16$^+$CD56$^+$/CD3$^+$ according to flow cytometry analysis. $\gamma\delta$ TCR$^+$ cells were identified by using pan-anti-$\gamma\delta$TCR antibody. Data are presented as the mean value of percentages ± S.E. *indicates statistical significance of $\gamma\delta$ TCR$^+$ T cell expansion in HP(+) group. The actual p value was $p = 0.001$.



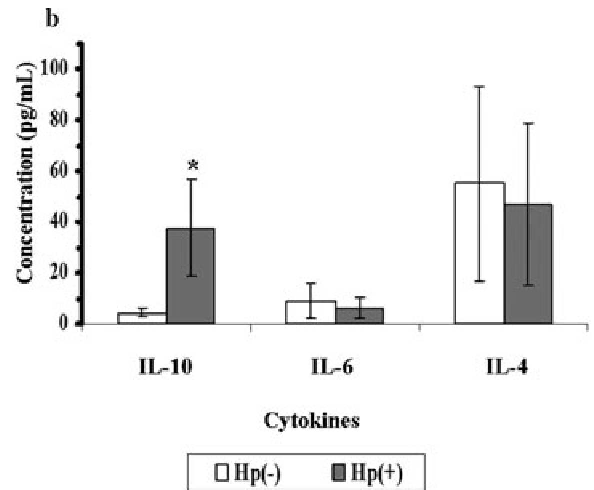


Fig. 5. Concentrations of pro-inflammatory (IL-1β, IL-8, TNF-α), inflammatory (IFN-γ, IL-12p70, IL-2) and anti-inflammatory (IL-10, IL-6, IL-4) cytokines in peripheral blood samples of Hp (−) (white bar) and Hp (+) (grey bar) subjects. The concentration of cytokine in serum samples was measured by using fluorescent bead assay. Data are the mean values of 32 Hp (+) and 15 Hp (−) ± S.E. *indicates the statistical significance of IL-10 concentrations between Hp (−) and Hp (+) groups. The actual p value was $p = 0.015$.

of T cells in the systemic circulation, while some studies have reported a proliferative and effective T cell response after *in vitro* stimulation with or without *H. pylori* antigen/s; some have observed no significant activation or co-stimulation response of peripheral blood lymphocyte subgroups (26, 27). Karttunen *et al.* detected a proliferative response of mononuclear cells after stimulation with intact *H. pylori*. However those responses do not correlate with infection status, since in both antibody positive (*H. pylori* infected) and antibody negative (non-infected subjects) samples showed a similar degree of T cell proliferative response. In another study Chmiela *et al.* also demonstrated that T lymphocytes from healthy volunteers show a significant proliferative response after stimulation with *H. pylori* and distinct antigenic fragments. According to our data, *H. pylori* does not induce a significant expansion of B-, T- lymphocytes (carrying $\alpha\beta$ TCR) and their subgroups.

Another conflict concerns the expansion status of $\gamma\delta$ TCR$^+$ T cell populations in the gastric mucosa during *H. pylori* infection. Trejdosiewicz *et al.* observed no significant change from normal in percentages of $\gamma\delta$ TCR$^+$

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in *H. pylori* associated gastritis (28). On the other hand, Futagami *et al.* detected accumulation of $\gamma\delta$ TCR$^+$ cells in gastric mucosa and that accumulation corresponded to an increase in IL-7 and IL-1beta in gastric mucosa (29). The present study is the first report of expansion of $\gamma\delta$ TCR$^+$ T-cells in the peripheral blood of Hp (+) patients. We think that expansion of $\gamma\delta$ T cells may be sourced by extraordinary expansion in the gastric mucosa. Nevertheless, we do not know if our expanded clone originates from gastric mucosa or peripheral blood, since our $\gamma\delta$ TCR$^+$ specific antibody's specificity covers pan-$\gamma\delta$ TCR$^+$ T cells clones.

It has been suggested that $\gamma\delta$ TCR$^+$ cells perform an autologous surveillance role, removing damaged cells by recognition of stress or heat shock proteins. Thus it is predictable that there should be an increase in $\gamma\delta$ TCR$^+$ cells as part of an inflammatory response. Additionally, both mucosal and peripheral blood $\gamma\delta$ TCR$^+$ T cells differentiate into either Th1 or Th2 cells depending on the microenvironment (30, 31), which means $\gamma\delta$ T cells can function as inflammatory or anti-inflammatory T cells. Given that *H. pylori* can induce either inflammatory or regulatory T cell responses, the expansion of that T cell population appears to be meaningful since an increased $\gamma\delta$ T cell frequency could explain the significant elevation of IL-10 in our study. On the other hand, no significant correlation between IL-10 levels and $\gamma\delta$ TCR$^+$ T cell frequencies ($r = 0.027$, $p = 0.888$) was found in the present study.

Thirdly, during *H. pylori* infection an anti-inflammatory T cell response in the systemic circulation was observed by us. That kind of T cell response could be initiated by one of the regulatory phases of persistent infection. Lundin *et al.* have demonstrated that high production of the suppressive cytokine IL-10 in *H. pylori* infected gastric adenocarcinoma patients leads to a diminished cytotoxic anti-tumor T cell response in the stomach (32). That phenomenon may contribute to tumor progression in gastric adenocarcinoma patients. The role of peripheral blood originated $\gamma\delta$ T cells on that progression should be investigated in future studies. Another view is that an anti-inflammatory T cell response can be associated with a regulatory function of T-lymphocytes during persistent infection (33). *H. pylori* makes use of some features of the host immune response to its benefit. However, Lundgren *et al.* have shown that it also induces expansion of CD4+ CD25highFoxp3+ regulatory T cells in *H. pylori* infected gastric mucosa, which weakens host lymphocyte proliferation and anti-inflammatory responses (34). Concerning that, a significant increase in IL-10 levels of Hp (+) patients may be the result of regulatory T-lymphocytes.

In conclusion, it has been shown that a regulatory T cell response is dominant in the systemic immune system during *H. pylori* infection, and that response is linked to the existence of $\gamma\delta$ TCR$^+$ T cells and high level of IL-10 in Hp (+) patients. These results could have important consequences both for understanding the role of systemic immune response during *H. pylori* infection and for protection against infection.

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