

Regulatory T cells percentage in peripheral blood before and after eradication of *Helicobacter pylori*

Yuka Satoh¹, Hatsue Ogawara^{1*}, Osamu Kawamura², Yasuyuki Shimoyama³,
Motoyasu Kusano³, Akihiko Yokohama⁴, Takayuki Saitoh⁵, Hiroshi Handa⁶,
Norifumi Tsukamoto⁷, Hirokazu Murakami⁵

¹Faculty of Health Sciences, Gunma Paz College, Takasaki, Japan; *Corresponding Author: hmura@gunma-u.ac.jp

²Department of Medicine and Molecular Science, Gunma University Graduate School of Medicine, Maebashi, Japan

³Department of Endoscopy and Endoscopic Surgery, Gunma University Hospital, Maebashi, Japan

⁴Department of Blood Transfusion Service, Gunma University Hospital, Maebashi, Japan

⁵Department of Laboratory Sciences, Course of Health Sciences, Gunma Graduate School of Health Sciences, Maebashi, Japan

⁶Department of Medicine and Clinical Science, Gunma Graduate School of Medicine, Maebashi, Japan

⁷Oncology Center, Gunma University Hospital, Maebashi, Japan

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ABSTRACT

Helicobacter pylori (*H. pylori*) induces gastro-duodenal diseases and vigorous humoral and cellular immune abnormalities. In order to clarify the immunological changes before and after eradication of *H. pylori*, the percentages and ratios of the following cells in the peripheral blood of 32 *H. pylori*-infected patients and 25 control subjects were analyzed: CD4+ T cells, CD8+ T cells, T helper 1 cells (Th1), T helper 2 cells (Th2), CD4+CD25+ T cells, Foxp3+ regulatory T cells (Treg), CD4/CD8 ratio, and Th1/Th2 ratio. CD4/CD8 ratio was significantly higher in *H. pylori*-infected patients before (mean ± SD, 2.9 ± 1.9) and after (mean ± SD, 2.8 ± 1.6) eradication of *H. pylori* than in control subjects (mean ± SD, 2.1 ± 0.9). The percentage of Th2 cells was significantly higher in *H. pylori*-infected patients (mean ± SD, 2.6 ± 1.1) than in control subjects (mean ± SD, 1.9 ± 1.1; $p < 0.02$). The percentage of Th2 cells after eradication of *H. pylori* (mean ± SD, 2.3 ± 1.4) was lower than that before eradication. There was no significant difference between control subjects (mean ± SD, 4.1% ± 1.5%) and patients before *H. pylori* eradication (mean ± SD, 4.5% ± 2.4%) in the percentage of Tregs, but the percentage was significantly higher in patients after *H. pylori* eradication (mean ± SD, 5.2% ± 2.6%) than in control subjects. The function of peripheral induced Tregs was reported to sup-

press the excessive immune reaction in chronic inflammation. These data suggest that Tregs may proliferate and be activated to suppress the activation of humoral immunity in *H. pylori*-infected patients, and these changes continue after 3 months or later of successful eradication of *H. pylori*.

KEYWORDS

Helicobacter pylori; Regulatory T Cell; Th1 Cell; Th2 Cell

1. INTRODUCTION

Helicobacter pylori (*H. pylori*) is the most common pathogen of the gastrointestinal tract worldwide and the predominant cause of chronic gastritis, gastroduodenal ulcer, gastric lymphoma, and gastric cancer [1-3]. *H. pylori* is known to induce vigorous humoral and cellular immune responses. In gastric mucosa, *H. pylori* induces recruitment of CD4+ and CD8+ T cells and is suspected to be related to the pathogenesis of gastric inflammation [4]. In addition, *H. pylori*-induced pathology largely depends on Th1 cell-mediated responses and Th1 cytokines like IFN-gamma [5]. In the peripheral blood of *H. pylori*-infected patients, conflicting data have been reported on the B and T cell subsets. Some investigators reported that there were no significant changes in the percentages of B, CD4+ T, CD8+ T, or NK cells [6,7]. On the other hand, Soares *et al* reported that CD4+ T cells increased in *H.*

pylori-infected patients [8]. We have reported that an increased CD4/CD8 ratio and a decreased Th1/Th2 ratio were observed in *H. pylori*-infected patients. Based on these data, we concluded that the activation of humoral immunity and Th2 polarization in peripheral blood were characteristic features of *H. pylori*-infected patients [9]. In this study, the peripheral blood T cell subsets before and after eradication of *H. pylori* were analyzed to determine the influence of *H. pylori* infection on the systemic immune response.

2. PATIENTS AND METHODS

2.1. Patients

This study was performed with approval from the Human Research Ethical Committee of Gunma University Hospital, and all patients and volunteers gave their informed consent. Thirty-two *H. pylori*-infected patients diagnosed at Gunma University Hospital between 2007 and 2012 were included in this study. The median age of the patients was 58.8 years (range, 22 - 81 years), and the male/female ratio was 26/6. *H. pylori* infection was established by the following criteria: positive tests for urease breath test and/or biopsy sample of the stomach. All patients were treated with lansoprazole 30 mg bid, amoxicillin hydrate 750 mg bid, and clarithromycin 200 mg bid for 7 days, and 29 patients achieved successful eradication. The other 3 patients failed to achieve *H. pylori* eradication and were consequently treated with rabeprazole 10 mg bid, amoxicillin hydrate 750 mg bid, and metronidazole 250 mg bid for 7 days; complete eradication of *H. pylori* was achieved after the second eradication therapy. The completion of eradication was confirmed by the urease breath test and/or serum anti-*H. pylori* IgG antibody (Eiken Chemical Co., Ltd, Tokyo, Japan).

Controls were obtained from 25 *H. pylori*-uninfected donors who were negative for serum anti-*H. pylori* IgG antibody. The male/female ratio of controls was 1.1 (13/12), and their median age was 43.4 years (range, 22 - 65 years). The clinical data for study subjects are shown in **Table 1**. The analyses of peripheral blood T lymphocyte subsets were performed before and at least 3 months after eradication of *H. pylori*.

2.2. Measurement of the CD4/CD8 Ratio

Peripheral blood cells were collected using ethylenediaminetetraacetic acid-3K. Lymphocytes were labeled using the following monoclonal antibodies: CD45-FITC/CD4-RD1/CD8-ECD/CD3-PC5 (Cyto-Stat tetraCHROME monoclonal antibodies, BD Pharmingen, San Jose, CA), and subsets were analyzed by 3-color flow cytometer using an EPICS XL System II (Beckman Coulter, Fullerton, CA).

Table 1. Characteristics of participants in this study.

	Control	Before eradication	After eradication
Cases (n)	25	32	
Sex (male:female)	(13:12)	(26:6)	
Age median: range (year)	43.4:22 - 65	58.8:22 - 81	
Hemoglobin (g/dl)	13.7 ± 1.2	14.0 ± 1.2	14.1 ± 1.2
Hematocrit (%)	41.2 ± 3.9	41.4 ± 3.5	41.8 ± 3.6
WBC (×10 ⁹ /l)	5.8 ± 1.6	6.5 ± 2.2	6.2 ± 2.3
Neutrophils (×10 ⁹ /l)	3.4 ± 1.3	4.0 ± 1.7	3.6 ± 1.9*
Monocytes (×10 ⁹ /l)	0.33 ± 0.12	0.37 ± 0.14	0.36 ± 0.14
Lymphocytes (×10 ⁹ /l)	1.8 ± 0.5	1.8 ± 0.5	1.9 ± 0.6
Platelets (×10 ⁹ /l)	231 ± 51	235 ± 56	222 ± 56*

Mean ± SD, *p values < 0.05 as compared with before eradication.

2.3. Measurement of the Th1/Th2 Ratio

Whole heparinized blood was mixed with an equal volume of RPMI1640 medium and incubated at 37°C with 7% CO₂ for 4 hours with 25 ng/mL phorbol 12-myristate 13-acetate (Sigma-Aldrich, St. Louis, MO), 1 µg/mL ionomycin, and 10 µg/mL Brefeldin A (Sigma-Aldrich). After treatment with FACS lysing solution and FACS permeabilizing solution (BD Biosciences, San Jose, CA), cells were stained at 4°C for 30 min with antihuman CD4-PE-Cy5 (BD Pharmingen), FastImmune fluorescein isothiocyanate (FITC)-labeled antihuman IFN-γ, and phycoerythrin-(PE-) labeled antihuman IL-4 (BD Biosciences). FastImmune IgG2a FITC/IgG1 PE isotype controls (BD Biosciences) were used as negative controls for human IFN-γ and IL-4. Three-color flow cytometric analysis was performed on FACS Calibur flow cytometer using Cell Quest software (BD Biosciences).

2.4. Regulatory T cell (Treg) Measurements

PE-Cy5 antihuman CD4 monoclonal antibody (BD Pharmingen) and FITC antihuman CD25 (BD Biosciences) were used for surface antigen staining. Mouse IgG1, κ-FITC (BD Pharmingen) was used as an isotype control. PE-conjugated antihuman Foxp3 (PCH101) and PE-conjugated rat IgG2a isotype control from eBiosciences (San Diego, CA) were used for intracellular Foxp3 staining according to the manufacturer's instructions. Tregs were defined as CD4+CD25+Foxp3+ cells. Measurement of Tregs was performed by 3-color flow cytometry as described above for Th1/Th2 ratio.

2.5. Complete Blood Cell Count

Complete blood cell counts were performed using a

total blood analyzer (ADVIA 120, Siemens, Tarrytown, NY).

2.6. Statistical Analysis

Data analysis was performed using SPSS statistics 17.0 (IBM, Armonk, NY). Comparisons of control and patients before eradication were performed using a non-parametric test (Mann-Whitney U test). Comparisons of patients before and after eradication were performed using a non-parametric test (Wilcoxon test). P values <0.05 were considered significant.

3. RESULTS

3.1. Clinical Data of Control Subjects and Patients before and after *H. pylori* Eradication (Table 1)

White blood cell, neutrophil, monocyte, lymphocyte, and platelet counts were statistically identical in *H. pylori*-infected patients and control subjects. The white blood cell count tended to decrease after eradication of *H. pylori* (mean \pm SD, $6.2 \pm 2.4 \times 10^9/L$) compared to that before eradication (mean \pm SD, $6.5 \pm 2.2 \times 10^9/L$; $p = 0.06$). The neutrophil count was significantly lower after eradication of *H. pylori* (mean \pm SD, $3.6 \pm 1.9 \times 10^9/L$) compared to that before eradication (mean \pm SD, $4.0 \pm 1.7 \times 10^9/L$; $p < 0.02$). The platelet count was significantly lower after eradication of *H. pylori* (mean \pm SD, $222 \pm 56 \times 10^9/L$) than before eradication (mean \pm SD, $235 \pm 56 \times 10^9/L$; $p = 0.04$). The hemoglobin level and monocytes and lymphocyte counts showed no significant differences between before and after *H. pylori* eradication.

3.2. CD4/CD8 Ratio before and after Eradication of *H. pylori*

The CD4/CD8 ratio was significantly higher in *H. pylori*-infected patients (mean \pm SD, 2.9 ± 1.9) than in control subjects (mean \pm SD, 2.1 ± 0.9 ; $p < 0.05$). The ratio after eradication of *H. pylori* (mean \pm SD, 2.8 ± 1.6) was also significantly higher than in control subjects ($p < 0.02$) (Figure 1).

3.3. The Percentage of Th1 Cells and Th2 Cells, and the Th1/Th2 Ratio

There was no significant difference in the percentage of Th1 cells between before and after eradication of *H. pylori* (mean \pm SD, 21.4 ± 7.5 vs. 20.4 ± 9.1) (Figure 2(a)). The percentage of Th2 cells was significantly higher in *H. pylori*-infected patients (mean \pm SD, 2.6 ± 1.1) than in control subjects (mean \pm SD, 1.9 ± 1.1 ; $p < 0.02$). The percentage of Th2 cells after eradication of *H. pylori* (mean \pm SD, 2.3 ± 1.4) was lower than before era-

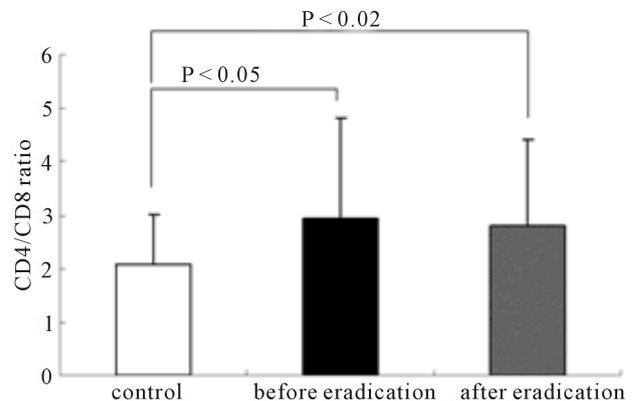


Figure 1. Comparison of CD4/CD8 ratios. White, black, and oblique line columns represent the CD4/CD8 ratios of control subjects, *H. pylori*-infected patients before eradication, and *H. pylori*-infected patients after eradication, respectively. The ratio is significantly higher in *H. pylori*-infected patients before and after eradication (mean \pm SD, 2.9 ± 1.9 and 2.8 ± 1.6) than in control subjects (mean \pm SD, 2.1 ± 0.9 ; $p < 0.05$ and $p < 0.02$, respectively). There is no significant difference between before and after *H. pylori* eradication. Data are given as means \pm SD.

dication (mean \pm SD, 2.6 ± 1.1) (Figure 2(b)). The Th1/Th2 ratio was lower in *H. pylori*-infected patients than in control subjects (mean \pm SD, 11.9 ± 12.5 vs. 13.3 ± 8.8); however there was no significant difference. The ratio after eradication of *H. pylori* (mean \pm SD, 14.7 ± 13.4) was higher than before eradication of *H. pylori* (mean \pm SD, 13.3 ± 8.8) (Figure 3); however there was no significant difference.

3.4. Percentage CD4+CD25+ and CD4+CD25+ Foxp3+ T Cells

The percentage of CD4+CD25+ T cells was significantly higher in patients before eradication of *H. pylori* (mean \pm SD, $14.8\% \pm 6.0\%$) than in control subjects (mean \pm SD, $12.2\% \pm 6.6\%$; $p < 0.05$). The percentage was also significantly higher after eradication of *H. pylori* (mean \pm SD, $17.4\% \pm 6.6\%$) than in control subjects ($p < 0.01$) (Figure 4(a)). This population includes both activated CD4+ T cells and Tregs and, thus, changes in Tregs could be masked in this impure population. To measure the percentages of Tregs, the frequency of CD4+CD25+ Foxp3+ T cells was monitored. There was no significant difference in the percentage of Tregs between control subjects (mean \pm SD, $4.1\% \pm 1.5\%$) and patients before eradication of *H. pylori* (mean \pm SD, $4.5\% \pm 2.4\%$). The percentage was significantly higher in patients after eradication of *H. pylori* (mean \pm SD, $5.2\% \pm 2.6\%$) than in control subjects (Figure 4(b)).

4. DISCUSSION

In addition to gastrointestinal disease, *H. pylori* is associated with several systemic autoimmune diseases

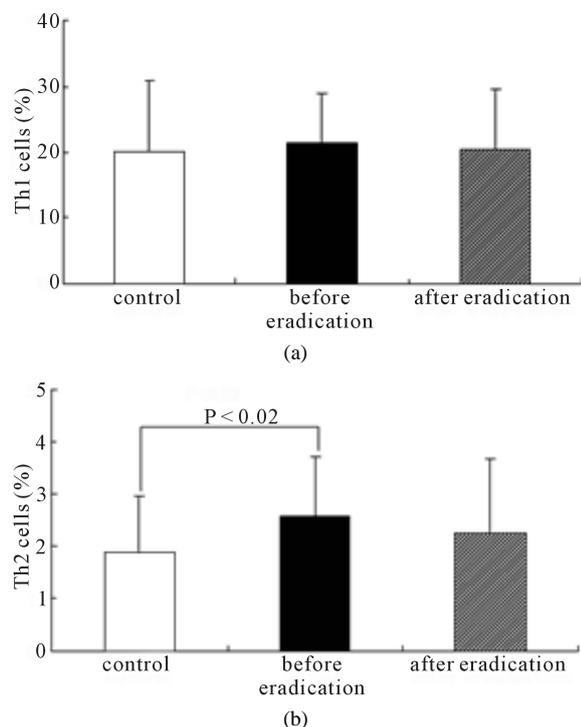


Figure 2. White, black, and oblique line columns represent the percentages of Th1 cells and Th2 cells of control subjects, *H. pylori*-infected patients before eradication, and *H. pylori*-infected patients after eradication, respectively. Data are given as means \pm SD. (a) Comparison of the percentage of Th1 cells. There are no significant differences among control subjects, *H. pylori*-infected patients before eradication, and *H. pylori*-infected patients after eradication. (b) Comparison of the percentage of Th2 cells. The percentage is significantly higher in *H. pylori*-infected patients (mean \pm SD, 2.6% \pm 1.1%) than in control subjects (mean \pm SD, 1.9% \pm 1.1%; $p < 0.02$). There is no significant difference in the percentage of Th2 cells before and after eradication of *H. pylori*.

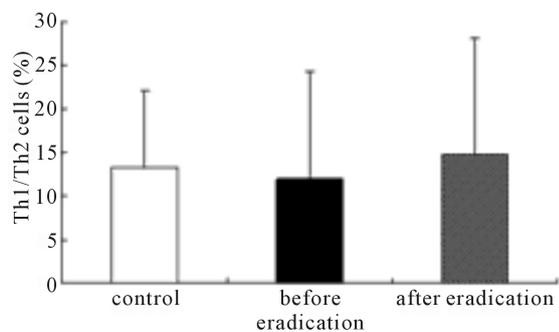


Figure 3. Comparison of the Th1/Th2 ratio. White, black, and oblique line columns represent the Th1/Th2 ratios of control subjects, *H. pylori*-infected patients before eradication, and *H. pylori*-infected patients after eradication, respectively. Data are given as means \pm SD. The Th1/Th2 ratio is lower in *H. pylori*-infected patients than in control subjects, but there is no significant difference in the Th1/Th2 ratio between before and after eradication of *H. pylori*.

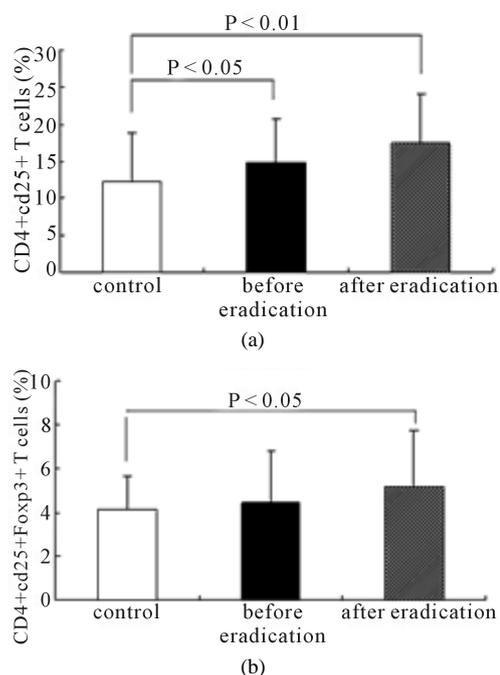


Figure 4. White, black, and oblique line columns represent the percentages of CD4+CD25+ T cells and CD4+CD25+Foxp3+ T cells of control subjects, *H. pylori*-infected patients before eradication, and *H. pylori*-infected patients after eradication, respectively. Data are given as means \pm SD. (a) Comparison of the percentage of CD4+CD25+ T cells. The percentage of CD4+CD25+ T cells is significantly higher in patients before eradication of *H. pylori* (mean \pm SD, 14.8% \pm 6.0%) than in control subjects (mean \pm SD, 12.2% \pm 6.6%; $p < 0.05$). The percentage is also significantly higher after eradication of *H. pylori* (mean \pm SD, 17.4% \pm 6.6%) than in control subjects ($p < 0.01$). Comparison of the percentage of CD4+CD25+Foxp3+ T cells. There is no significant difference between control subjects (mean \pm SD, 4.1% \pm 1.5%) and patients before *H. pylori* eradication (mean \pm SD, 4.5% \pm 2.4%). The percentage is significantly higher in patients after *H. pylori* eradication (mean \pm SD, 5.2% \pm 2.6%) than in control subjects ($p < 0.05$).

(Sjögren's syndrome [10], and systemic sclerosis [11], and organ-specific autoimmune diseases (idiopathic thrombocytopenic purpura [12], Graves' disease [13], and autoimmune pancreatitis [14]). Given this association with autoimmune diseases, we hypothesized that *H. pylori* might induce systemic immunological changes. Therefore, we compared the immune characteristics of peripheral blood of *H. pylori*-infected patients and control subjects, and we reported activation of humoral immunity and Th2 polarization in *H. pylori*-infected patients [9]. Based on these data, we suspected that these abnormalities may induce systemic autoimmune diseases.

We have already reported that the frequency of Th1, Th2, and Treg cells was not associated with aging [15, 16], so that we compared the frequency of these cells between *H. pylori*-infected patients and control subjects regardless of the difference of age distribution.

Kodama *et al.* reported that cultures and histological examinations of stomach were performed 6 months after eradication in *H. pylori*-infected patients and revealed a significant decrease of inflammatory activity in the stomach [17]. Watanabe *et al.* reported that the gastric atrophy due to *H. pylori* improved gradually, but moderate or marked gastric inflammation improved 6 weeks after eradication of *H. pylori* [18]. Fichman and Niv also reported rapid improvement of acute and chronic inflammation of the stomach 3 months after the eradication of *H. pylori* [19]. Based on these reports, we analyzed the immunological abnormalities before and 3 months or later after the eradication of *H. pylori* in order to determine whether the immunological abnormalities recover after successful eradication.

White blood cell, neutrophil, and lymphocyte counts have been reported to increase in *H. pylori*-infected patients [20,21]. In the present study, these counts were slightly higher in *H. pylori*-infected patients than in control subjects, and they decreased after eradication of *H. pylori*. It is suspected that this decrease was induced by the improvement of inflammation due to *H. pylori*.

Yuceyar *et al.* reported that there was no significant difference in peripheral blood lymphocyte subsets between *H. pylori*-infected patients and control subjects [7], but we have reported a decrease in the percentage of CD8+ T cells and an increase in the CD4/CD8 ratio in peripheral blood lymphocytes of *H. pylori*-infected patients [9]. We suspected that the distribution of CD8+ T cells changes in *H. pylori*-infected patients, with migration of peripheral CD8+ T cells into the gastric mucosa, based on the reports that showed CD8+ T cell accumulation in the gastric mucosa, which increases the severity of gastric inflammation. However, the increase of CD4/8 ratio sustained after successful eradication of *H. pylori*.

We also reported Th2 polarization and suspected that peripheral blood Th1 cells migrate to the gastric mucosa in *H. pylori*-infected patients, because D'Elios showed that *H. pylori*-specific Th1 cells could be cloned from *H. pylori*-infected gastric mucosa and that these cells were cytotoxic to gastric epithelial cells because they produced IFN-gamma [5].

Tregs have a crucial role in suppressing immune responses to self-antigens, preventing autoimmune diseases, and in controlling immune responses to pathogens [22]. Their local infiltration into the gastric mucosa of *H. pylori*-infected patients suggested their involvement in suppressing mucosal immune responses, contributing to the persistence of infection [23-26]. Lundgren *et al.* re-

ported that *H. pylori*-infected patients have an impaired memory CD4+ T cell response to *H. pylori* that is linked to the presence of *H. pylori*-specific CD4+CD25+ T cells in peripheral blood, which actively suppress the response [27]. In the present study, the percentage of CD4+CD25+ T cells was significantly higher in *H. pylori*-infected patients and successfully eradicated patients than in control subjects. In addition, the percentage of Treg (CD4+CD25+Foxp3+ T cells) was higher in *H. pylori*-infected patients than in control subjects, and it was significantly higher in successfully eradicated patients than in control subjects. The function of peripheral induced Tregs was reported to suppress the excessive immune reaction in chronic inflammation. These data suggest that Tregs may proliferate and be activated to suppress the activation of humoral immunity in *H. pylori*-infected patients, and these changes continue after 3 months or later of successful eradication of *H. pylori*.

We have expected the decrease of Tregs in *H. pylori*-infected patients, because *H. pylori* is associated with several systemic autoimmune diseases. Tregs were reported to be composed of natural Tregs and induced Tregs. Natural Tregs are produced by the thymus as an antigen-primed and functionally mature T cell subpopulation specialized for self-tolerance. Induced Tregs are produced by the peripheral lymph-nodes in response to the external antigens in chronic infection, and are composed of some subsets including T helper 3 (Th3) and type 1 regulatory T (Tr1) cells. The lasting increase of Tregs might be due to the difference of the subtype of Tregs, and depend on the increase of Th3 which is responsible for mucosal immunity in *H. pylori*-infected patients.

5. CONCLUSION

These data suggest that Tregs may proliferate and be activated to suppress the activation of humoral immunity in *H. pylori*-infected patients, and these changes continue after 3 months or later of successful eradication of *H. pylori*.

CONFLICT OF INTERESTS

The authors have no conflict of interest to declare.

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