

Expression of CD25^{high} Regulatory T Cells and PD-1 in Gastric Infiltrating CD4⁺ T Lymphocytes in Patients with *Helicobacter pylori* Infection[∇]

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We observed by flow cytometry that the frequency of both gastric infiltrating Tregs and PD-1-positive CD4 T cells is correlated with the density of *Helicobacter pylori*, suggesting that cellular immunity against this pathogen is inhibited.

Helicobacter pylori, a common human pathogen, infects about 50% of the world's population (9, 10). Many studies have confirmed that the pathogenesis of *H. pylori* infection is probably due largely to the host immune system, particularly the cellular immune response. However, the mechanism by which *H. pylori* negatively regulates the immune response against the bacteria is not completely understood.

In recent years, regulatory T cells (Tregs) and related negative regulatory molecules have been a focus of research on the immune mechanisms of diseases. Evidence indicates that removal or reduction of Tregs can enhance immune responses against infectious microbes (1, 2, 5, 6, 8, 11–13, 16). Expression of programmed cell death 1 (PD-1, also called CD279), a negative regulatory molecule in the CD28/B7 family, is induced on CD4⁺ T cells, CD8⁺ T cells, natural killer T cells, B cells, and activated monocytes during the course of many diseases. B and T lymphocyte attenuator (BTLA), a newly discovered member of the CD28 family of costimulatory molecules, may have inhibitory function similar to that of PD-1.

The PD-1 signaling pathway is involved in chronic bacterial infection and may be a potential therapeutic target. However, the role of BTLA in *H. pylori* infection has not been investigated. In this study, we analyzed the frequency of gastric infiltrating CD4⁺ CD25^{high} Tregs in patients with *H. pylori* infection, as well as the expression of PD-1 and BTLA on gastric infiltrating CD4⁺ T cells, and their relationships with *H. pylori*-related clinical markers.

Biopsy specimens of the gastric antrum were obtained from patients undergoing gastric endoscopy for dyspepsia at China

Medical University Hospital. There were 38 *H. pylori*-infected subjects enrolled in this study. Peripheral blood samples from the same patients served as the control. The presence of *H. pylori* infection was determined by histopathologic examination (including Giemsa staining) and/or by a positive result for a rapid urease test performed on at least one additional biopsy sample. The density of *H. pylori* was analyzed as suggested by the updated Sydney system (4): 0, absent; 1, mild; 2, moderate; and 3, marked. The degree of inflammation present in the histological specimens was also classified according to the updated Sydney system. Biopsy specimens of the gastric antrum were collected into sterile collection medium (calcium- and magnesium-free Hanks' balanced salt solution [HBSS] with 5% fetal calf serum and penicillin plus streptomycin). The collected tissues were immediately placed in ice-cold RPMI 1640 complete medium (Gibco BRL, Gaithersburg, MD) which contained 10% fetal calf serum supplemented with penicillin (50 IU/ml), streptomycin (50 µg/ml), L-glutamine (2 mM), and sodium pyruvate (1 mM). Gastric infiltrating lymphocytes were isolated using a modified protocol according to a reported technique (15).

Flow cytometry analysis was performed using a technique described previously (14). The CD4⁺ T cells were gated from the forward scatter (FSC) low versus CD4⁺ T cells, which correspond to the lymphocyte population according to our previous data (15). All data were analyzed using SPSS version 13.0 for Windows (SPSS Inc., Chicago, IL). Mann-Whitney U tests were used for comparison between groups. Pearson correlation tests were performed for correlation analysis. All tests were two tailed, and *P* values of <0.05 were considered significant.

Initially, we determined the percentage of CD25⁺ T cells in the total CD4⁺ T-cell population in each of the 38 patients with *H. pylori* infection (Fig. 1A). The CD4⁺ CD25^{high} population represented 3.37 to 12.78% of CD4⁺ T cells, and the CD4⁺ CD25⁺ T cell population represented 12.00 to 31.02%

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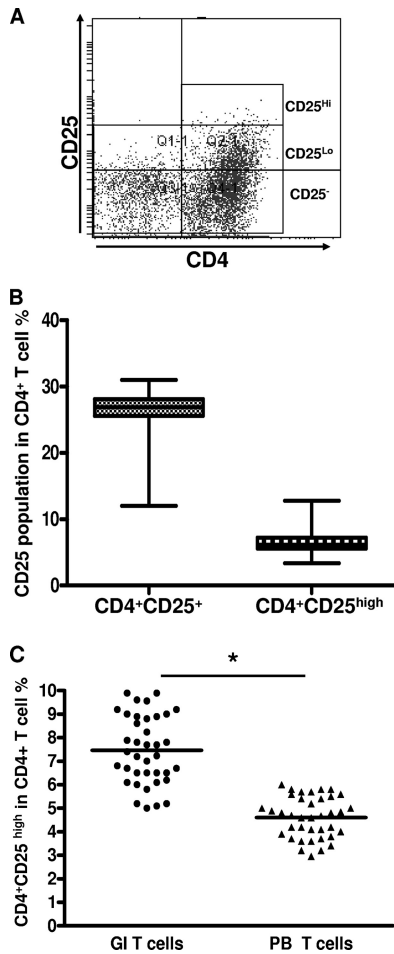


FIG. 1. Frequency of gastric infiltrating/peripheral blood CD4⁺ CD25⁺ regulatory T cells (Tregs) in patients with *H. pylori* infection. (A) Gastric infiltrating CD4⁺ T cells were separated into CD25^{high}, CD25^{low}, and CD25⁻ T-cell subsets, as defined by the fluorescence intensity of CD25 obtained using isotypic control antibody. (B) Frequency of gastric infiltrating CD4⁺ CD25^{high} and total gastric infiltrating CD4⁺ CD25⁺ T cells in patients with *H. pylori* infection. Data are expressed as box plots in which the horizontal lines represent the 25th, 50th, and 75th percentiles of the measured frequencies of Tregs. (C) Percentages of gastric infiltrating (GI)/peripheral blood (PB) CD4⁺ CD25^{high} Tregs in patients with *H. pylori* infection. The horizontal bars indicate the median percentages of Tregs. The individual frequency for each subject included in the analysis is shown. Significant differences were calculated using the Mann-Whitney U test. *, *P* < 0.05.

of CD4⁺ T cells in gastric infiltrating lymphocytes (Fig. 1B). These data indicate that the frequency of CD4⁺ CD25^{high} Tregs in gastric infiltrating lymphocytes (mean ± standard deviation [SD], 7.38% ± 2.02%) was significantly higher than that in peripheral blood lymphocytes (mean ± SD, 4.58% ± 1.04%) (*P* = 0.008) (Fig. 1C).

PD-1 and BTLA are important negative-immune-regulatory molecules. The percentage of CD4⁺ PD-1⁺ T cells in gastric infiltrating lymphocytes (Fig. 2A) (mean ± SD, 39.62% ± 10.09%) was significantly higher than that in peripheral blood lymphocyte controls (mean ± SD, 26.64% ± 10.47%) (*P* = 0.0012) (Fig. 2B). However, the percentage of CD4⁺ BTLA⁺ T cells in gastric infiltrating lymphocytes (Fig. 2A) (mean ±

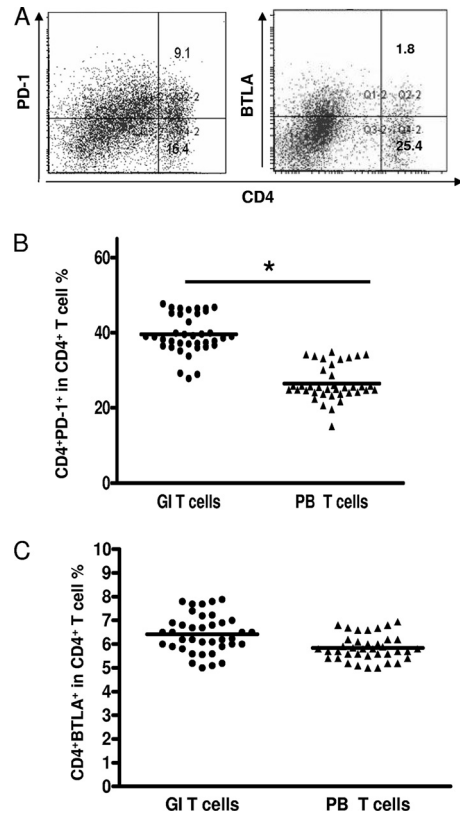


FIG. 2. PD-1 and BTLA expression in gastric infiltrating/peripheral blood CD4⁺ T cells in patients with *H. pylori* infection. (A) Surface expression of PD-1 and BTLA on gastric infiltrating CD4⁺ T cells in one patient with *H. pylori* infection. (B and C) Percentages of CD4⁺ PD-1⁺ and CD4⁺ BTLA⁺ gastric infiltrating (GI)/peripheral blood (PB) T cells in patients with *H. pylori* infection. Horizontal bars indicate the median percentages of CD4⁺ PD-1⁺ T cells and CD4⁺ BTLA⁺ T cells. The individual frequency for each subject included in the analysis is shown. Significance was calculated using the Mann-Whitney U test. *, *P* < 0.05.

SD, 6.68% ± 0.18%) did not differ significantly from that in peripheral blood lymphocytes (mean ± SD, 6.2750% ± 0.3709%) (*P* = 0.085) (Fig. 2C).

We divided the *H. pylori*-positive gastric biopsy samples into three groups based on their histology scores for chronic inflammation (marked, moderate, and mild). Patients with marked inflammation had a higher mean percentage of gastric infiltrating CD4⁺ CD25^{high} Tregs than patients with moderate degree of inflammation (7.84% ± 1.07% versus 5.76% ± 1.23%, respectively; *P* = 0.0043). There were also significant differences in CD4⁺ CD25^{high} Treg populations between the marked or moderate group and the mild group (Fig. 3A). Although the level of PD-1 expression on gastric infiltrating CD4⁺ T cells in patients with marked inflammation was higher than that in patients with moderate inflammation, there was no significant difference in the number of gastric infiltrating CD4⁺ T cells expressing PD-1 between patients with marked inflammation and those with moderate inflammation (52.07% ± 11.01% versus 48.25% ± 6.77%, respectively; *P* = 0.0552). There were significant differences in percentage of gastric infiltrating CD4⁺ PD-1⁺ T cells between patients with marked

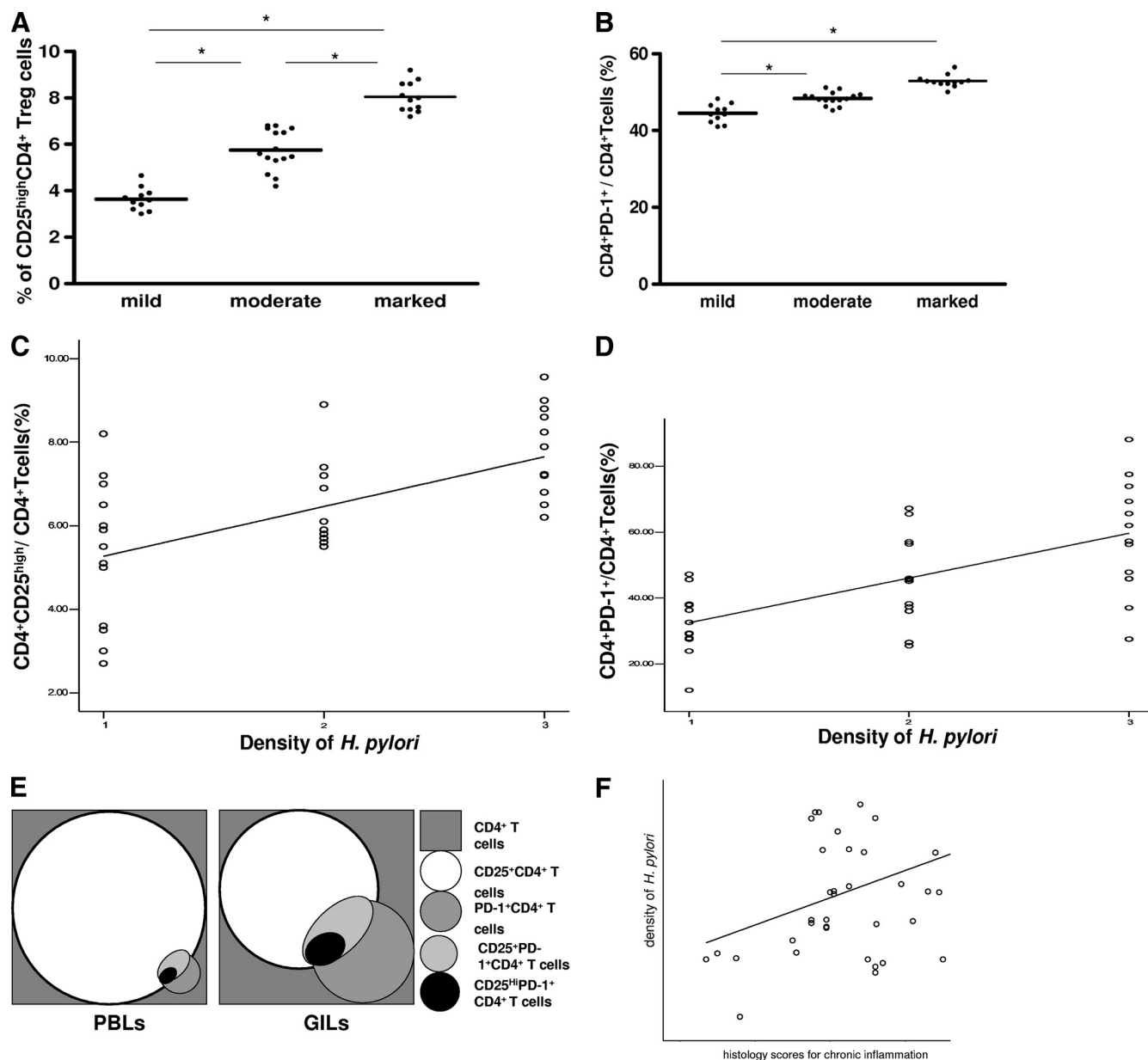


FIG. 3. Association between inflammation degree or density of *H. pylori* and gastric infiltrating CD4⁺ CD25^{high} Treg frequency or gastric infiltrating CD4⁺ PD-1⁺ T cells in patients with *H. pylori* infection. Gastric infiltrating lymphocytes were divided into three groups based on inflammation degree (A and B). Significance of differences between inflammation degree and gastric infiltrating CD4⁺ CD25^{high} Treg frequency or gastric infiltrating CD4⁺ PD-1⁺ T cells was analyzed using nonparametric Mann-Whitney U tests. The correlation between density of *H. pylori* and gastric infiltrating CD4⁺ CD25^{high} Treg frequency (C) or gastric infiltrating CD4⁺ PD-1⁺ T-cell populations (D) was analyzed using Spearman correlation analysis. (E) Correlation of gastric infiltrating (GI)/peripheral blood (PB) T cells in patients with *H. pylori* infection. The correlation between density of *H. pylori* and histology scores for chronic inflammation (F) was also analyzed using Spearman correlation analysis. *, $P < 0.05$.

inflammation or moderate inflammation and patients with mild inflammation (Fig. 3B).

We measured the density of *H. pylori* in each patient in order to investigate whether the increase in gastric infiltrating CD4⁺ CD25^{high} Tregs and gastric infiltrating CD4⁺ PD-1⁺ T cells correlated with density of *H. pylori*. Spearman analysis showed that the frequencies of gastric infiltrating CD4⁺ CD25^{high} Tregs ($r = 0.524$; $P = 0.0282$) and gastric infiltrating CD4⁺ PD-1⁺ T cells ($r = 0.431$; $P = 0.016$) were positively correlated with density of *H. pylori* (Fig. 3C and D). These results suggest

that an increased frequency of gastric infiltrating CD4⁺ CD25^{high} Tregs and gastric infiltrating CD4⁺ PD-1⁺ T cells may be associated with a weaker immune response, leading to poor bacterial clearance in patients with *H. pylori* infection. We show PD1/CD25 costaining and proportions of CD25^{high}/PD-1 double and single positive T cells to clarify the relationship in Fig. 3E. In addition, there was no correlation between histology scores for chronic inflammation and density of *H. pylori* in patients ($P = 0.096$) (Fig. 3F).

A recent study found that expression of the PD-L1 on gastric

epithelial cells after *H. pylori* exposure promotes development of CD4⁺ CD25⁺ FoxP3⁺ regulatory T cells (3). In addition, *H. pylori*-specific CD4⁺ T cells were shown to accumulate in the human *H. pylori*-infected gastric mucosa (7). Further studies are needed to clarify the relationship between PD-1 expression and high-level expression of CD25 on gastric infiltrating CD4⁺ T cells. Our findings demonstrate that the frequency of gastric infiltrating CD4⁺ CD25^{high} Tregs and PD-1 expression levels on CD4⁺ T cells were significantly increased in patients with *H. pylori* infection and that these variables were positively correlated with the degree of inflammation and the density of *H. pylori*. Tregs and PD-1, as negative regulators of immunity, may not only contribute to the persistence of chronic *H. pylori* infection but also be potential therapeutic targets for the treatment of chronic *H. pylori* gastritis patients.

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We declare no conflicts.

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