Increased Prevalence of Regulatory T-Cells in the Peripheral Blood of Patients with Gastrointestinal Cancer

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Abstract. *Background: Although recent studies have shown that FoxP3 represent the most specific Treg marker only a few studies have reported on the presence of FoxP3$^+$Treg in peripheral blood. Patients and Methods: Peripheral blood mononuclear cells (PBMC) were harvested from 37 healthy volunteers and 94 patients with gastrointestinal cancer. The prevalence of Treg co-expressing CD4$^+$FoxP3$^+$ was analyzed using flow cytometry. Results: The prevalence of Treg in the peripheral blood of gastrointestinal cancer patients was significantly higher than that in healthy volunteers (p=0.012). In early stage I cancer, Treg levels tended to be higher than those in healthy volunteers (p=0.069); these levels were significantly reduced after tumor resection (p=0.0027). Conclusion: The prevalence of Treg was increased in patients with gastrointestinal cancer, even in the early stages of the disease. Since Treg levels decreased after curative resection, it is possible that tumor cells may have induced and expanded the Treg pool.*

In the last 10 years, many reports have demonstrated the beneficial role of regulatory T-cells (Treg) in preventing pathological immune responses in autoimmune diseases, transplantation, graft-versus-host diseases and allergies (1-3). On the other hand, increased levels of Treg in cancer patients prevent an effector cell response. In cancer patients, increases in CD4$^+$CD25$^+$ Treg are observed in the peripheral blood and in the population of tumor-infiltrating lymphocytes (4,5). In murine tumor models, depletion of CD4$^+$CD25$^+$ Treg before tumor inoculation promotes tumor rejection and inhibition of tumor growth (6,7). These results suggest that CD4$^+$CD25$^+$ Treg may be induced in cancer-bearing hosts and that this may suppress antitumor immune responses.

Although the immunoregulatory activity of CD4$^+$CD25$^+$ Treg has been well established, knowledge about the generation and localization of the suppressive activity is limited. Because CD25 is an activation marker of T lymphocytes, it is difficult to discriminate Treg from activated T lymphocytes. Recent studies have shown that the forkhead/winged helix transcription factor, FoxP3, is specifically expressed on Treg and regulates their development and function. FoxP3 is not simply a marker of activation since CD4$^+$CD25$^-$ T-cells do not express FoxP3 after activation (8-10). This suggests that FoxP3 can identify Treg not only in steady-state conditions but also in tumor-bearing hosts and in inflammatory and allergic diseases. As such, it is thought that FoxP3 represents the most specific Treg marker.

There have been few studies on FoxP3$^+$ Treg in the peripheral blood in malignant states. In this study, we evaluated the prevalence of CD4$^+$FoxP3$^+$ Treg in the peripheral blood of patients with gastrointestinal cancer is evaluated.

Patients and Methods

Patient recruitment. A total of 94 patients with gastrointestinal cancer (esophageal, n=19; gastric, n=37; cholangio-pancreatic, n=11; and colorectal, n=27) treated at Yamaguchi University Hospital from October 1, 2005 to May 9, 2008 were enrolled in this study. Patient characteristics are described in Table I. Patients who had elevated inflammatory parameters, as denoted by a white blood cell count of more than 10×10^9/L or a CRP of more than 5 mg/dL, viral infection or autoimmune diseases were excluded from the study. None of the patients received radiotherapy, chemotherapy or pharmacological intervention before the study. Staging was performed according to the TNM classification for gastrointestinal cancer (11). In order to clarify the prevalence of CD4+FoxP3+ Treg in the peripheral blood in the presence or absence of a tumor, preoperative and postoperative (as defined by more than 2 months after the operation) levels were compared in patients with stage I disease.

As controls, 37 healthy volunteers were also enrolled. The average age of individuals in the control group was 63±2 (mean±SE) years old, while the age of those with gastrointestinal malignancy was 65±1 years old (esophageal, 65±3; gastric, 66±2;

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Table I. *Patient characteristics*.

Primary	TNM Stage*				
	I	II	III	IV	Total number
Esophageal cancer	2	11	1	5	19
Gastric cancer	23	3	5	6	37
Colorectal cancer	9	6	8	4	27
Cholangio-pancreatic cancer	4	2	1	4	11
Total number	38	22	15	19	94

*Stage according to the TNM classification for various gastrointestinal cancers (UICC).

cholangio-pancreatic, 65±3; and colorectal, 65±2). There was no statistical difference in the mean age between the two groups.

Written informed consent was obtained from all patients and the study protocol was approved by the Institutional Review Board for Human Use of Yamaguchi University School of Medicine.

Isolation of PBMC and immunofluorescence labeling. Blood samples (10 mL) were collected from patients and healthy donors in sterile heparinized containers. Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation on Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden). PBMC were then harvested, washed with Dulbecco's phosphate buffered saline (D-PBS) and incubated with Energy Coupled Dye Phycoerythrin-Texas Red (ECD)-labeled anti-human CD4, fluorescein isothiocyanate (FITC)-labeled anti-human CD25 antibodies, as well as the corresponding isotype control antibodies (all from Beckman Coulter, Miami, FL, USA) by incubating them for 30 minutes at room temperature. Cells were then incubated for 15 minutes at 4˚C with normal rat serum and permeabilization buffer to prevent nonspecific binding to Fc receptors before incubation with rat phycoerythrin (PE)-labeled anti-human FoxP3 antibody (eBioscience, San Diego, CA) and the appropriate isotype controls (Beckman Coulter) for 30 minutes at 4˚C. Cells were then washed and resuspended in 1% paraformaldeyde (PFA) in D-PBS and stored at 4˚C in the dark until flow cytometric analysis.

Flow cytometry. Triple color flow cytometry was performed using an Epics-XL flow cytometer (Beckman Coulter). In order to identify Treg, lymphocytes were gated on the basis of forward *vs*. side scatter profile followed by gating of $CD4^+$ T-cells. $CD4^+$ cells were then analyzed for CD25 and FoxP3 expression. A stringent gating criteria was used, setting gates at the 0.5% level of the respective isotype control to identify cells positive for Treg cell markers. On reanalysis, the forward and side scatter properties of $CD4^+CD25^{high}$ cells were not appreciably different from those of the $CD4^+CD25^-$ population, indicating that these cell populations were similar in size.

Statistical analysis. All data are shown as mean±standard error (SE). Comparisons between the control group and cancer-bearing group were performed using Student's *t*-test. A *p*-value of less than 0.05 was defined as statistically significant. Analysis was performed using Windows XP and Stat View 5.0 software (SAS Institute, Berkley, CA).

Results

The prevalence of $CD4^+FoxP3^+$ Treg in cancer patients was significantly higher than in healthy volunteers. There was no significant difference in the prevalence of $CD4^+CD25^+$ cells (healthy group *vs*. cancer group: 6.76±0.57% *vs*. 7.53±0.39%, p=0.27) and $CD4^+CD25^{high}$ cells (healthy group *vs*. cancer group: 0.63±0.05% *vs*. 0.46±0.04%, p=0.68) between healthy donors and cancer patients (Figure 1a, b). However, the percentage of $CD4^+FoxP3^+$ Treg in cancer patients was significantly higher than in healthy volunteers (healthy group *vs*. cancer group: 0.63±0.05% *vs*. 0.88±0.06%, p=0.012) (Figure 1c).

The prevalence of $CD4^+FoxP3^+$ Treg in patients with different gastrointestinal cancers is shown in Figure 2. Compared with healthy volunteers, the population of $CD4^+FoxP3^+$ Treg was significantly increased in patients with esophageal (0.91±0.14%, p=0.028), cholangio-pancreatic (1.03±0.20%, p=0.0056) and colorectal cancer (0.88±0.11%, p=0.029). In gastric cancer patients, there was a trend toward higher percentages of $CD4^+FoxP3^+$ Treg (0.82±0.09%, p=0.057).

The prevalence of $CD4^+FoxP3^+$ Treg in cancer patients with different disease severity as determined by TNM staging classification is shown in Figure 3. Compared with healthy volunteers, the percentage of $CD4^+FoxP3^+$ Treg in patients with stage IV cancer (1.20±0.17%, p=0.0001) was significantly increased. The percentage of $CD4^+FoxP3^+$ Treg was higher in patients with stage III disease (0.73±0.12%) than in healthy volunteers, but this was not significant (p=0.35). There was a tendency towards higher levels of $CD4^+FoxP3^+$ Treg in patients with stage I (0.80±0.08%, p=0.069) and stage II cancer (0.84±0.11%, p=0.058).

After curative resection for stage I disease, $CD4^+FoxP3^+$ Treg levels were significantly reduced (preoperative *vs*. postoperative: 0.80±0.08% *vs*. 0.58±0.04%, p=0.0027); these levels were similar to those in healthy volunteers (0.63±0.05%, p=0.45) (Figure 4).

Discussion

It has been demonstrated that the prevalence of $CD4^+FoxP3^+$ Treg in gastrointestinal cancer patients was significantly higher than healthy volunteers, but this was not observed with the population of $CD4^+CD25^+$ and $CD4^+CD25^{high}$ T lymphocytes. Specifically, the population of $CD4^+FoxP3^+$ Treg was significantly increased in stage IV disease. In stage I cancer, Treg levels tended to be higher than in healthy controls but their levels were significantly reduced after curative tumor resection.

In 1995, Sakaguchi *et al*. described and characterized a population of T lymphocytes that co-express CD4 and CD25 (12). Since then, numerous studies have documented an

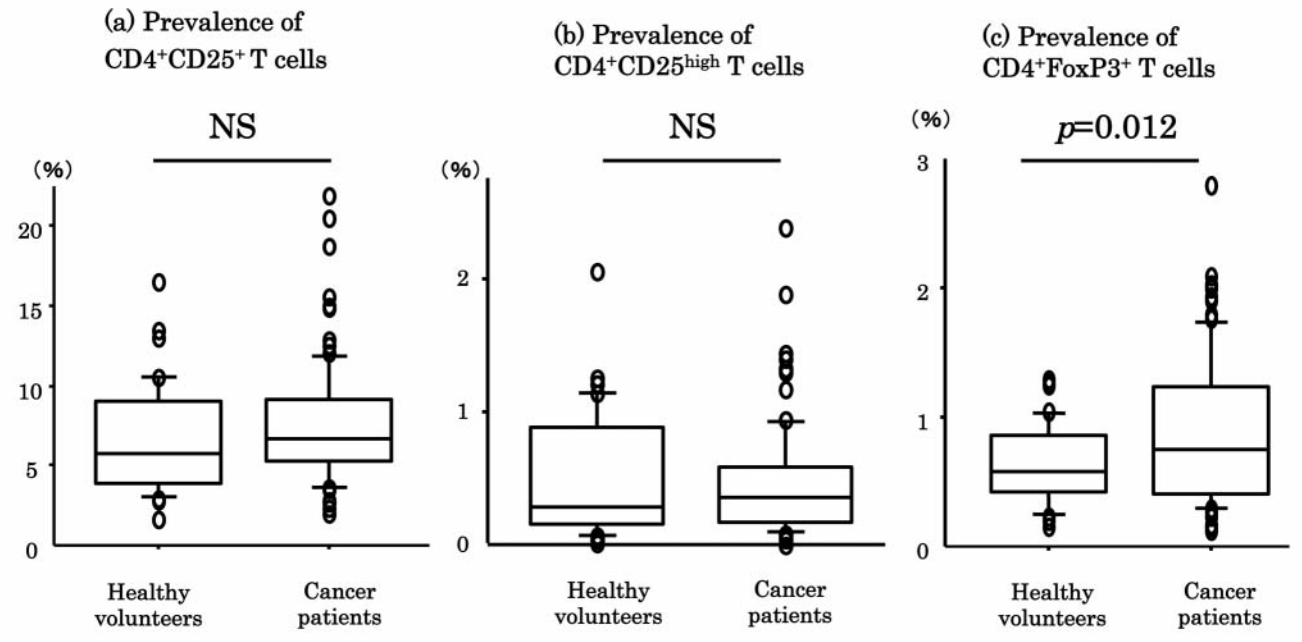

Figure 1. *Comparison of the prevalence of CD4+CD25+ (a), CD4+CD25high (b) and CD4+FoxP3+ Treg (c) in the peripheral blood of cancer patients and healthy volunteers. There was no significant difference in the levels of CD4+CD25+ (a) and CD4+CD25high T lymphocytes (b) in cancer patients compared with healthy volunteers. The population of CD4+FoxP3+ Treg in cancer patients was significantly higher than in healthy volunteers (p=0.012) (c).*

increase of $CD4^+CD25^+$ T-cells in the peripheral blood of patients with malignancy, including head and neck (13), esophageal (7, 14), gastric (6, 7, 14, 15), colorectal (13, 15), gall bladder (13, 15), pancreatic (13, 15, 16), ovarian (17), lung (18, 19), breast (16), skin (10), Hodgkin lymphoma (18, 20) and B-cell chronic lymphocytic leukemia (21). However, CD25 is not an optimal marker to define Treg since activated T-cells also express CD25. In this study, there was no significant difference in the prevalence of $CD4^+CD25^+$ and $CD4^+CD25^{high}$ T lymphocytes between healthy individuals and cancer patients. Since 2004, FoxP3 has been reported to be the most specific Treg cell marker (8, 9, 22, 23). Experiments with FoxP3-overexpressing transgenic or FoxP3 gene-deleted mice have shown that FoxP3 is a master control gene for the development and function of natural $CD4^+CD25^+$ Treg (24-27). Thus, FoxP3 is thought to be a suitable single marker for detecting Treg. In contrast to the murine system, defining human Treg has been more difficult, and assessment of the specificity of FoxP3 has not been performed in many of the early studies (28).

In this study, the prevalence of $CD4^+FoxP3^+$ Treg in esophageal, cholangio-pancreatic and colorectal cancer was significantly increased compared with healthy individuals. Curiel *et al*. demonstrated that $CD4^+CD25^+FoxP3^+$ Treg suppressed tumor-specific T-cell immunity in ovarian cancer, contributed to tumor growth and accumulated during disease progression (29). Furthermore, increased levels of Treg were associated with a high death hazard ratio and reduced survival. For patients with squamous cell carcinoma of the head and neck, a significantly elevated level of $FoxP3^+GITR^+$ Treg was shown (30). These Treg were significantly more sensitive to apoptosis than non-Treg, which might hint at a rapid turnover in the peripheral circulation. Increased numbers of Treg have also been reported in the peripheral blood and in the population of tumor infiltrating lymphocytes in patients with hepatocellular carcinoma (31).

There are suggestions that the extent of Treg increase in malignancy is influenced by disease severity. Increased Treg in the peripheral blood has been reported in advanced gastrointestinal tumors (14). In this study, $CD4^+FoxP3^+$ Treg were significantly increased in patients with stage IV cancer. In contrast to the numerous reports on increased Treg in advanced cancers, there have been few reports on Treg levels in early cancers. Aschley *et al*. demonstrated that Treg were increased in patients with early stage prostate cancer (32). Here, a trend toward higher levels of Treg were observed in patients with stage I and stage II cancer compared with healthy individuals. This suggests that levels of Treg increase in the peripheral blood from an early stage in gastrointestinal malignancies. In addition, it was observed that elevated levels of Treg in the peripheral blood of early stage cancer patients decreased significantly after tumor resection. Kono *et al*. reported that elevated numbers of $CD4^+CD25^+$ Treg

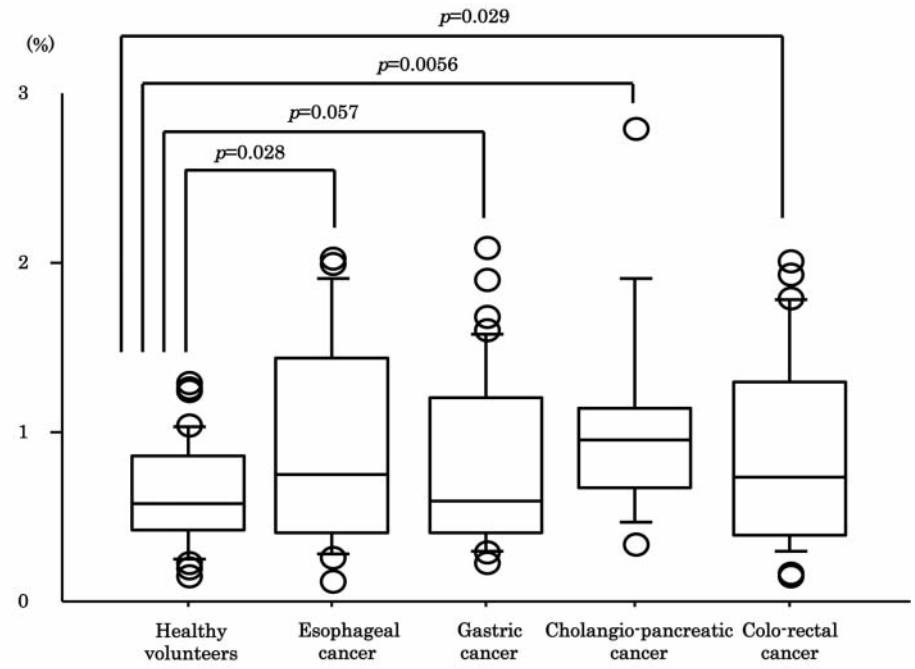

Figure 2. *The prevalence of CD4+FoxP3+ Treg in different gastrointestinal cancers. CD4+FoxP3+ Treg levels were significantly increased in the peripheral blood of patients with esophageal, cholangio-pancreatic and colorectal cancer compared with healthy individuals. In gastric cancer patients, there was a trend toward higher numbers of CD4+FoxP3+ Treg.*

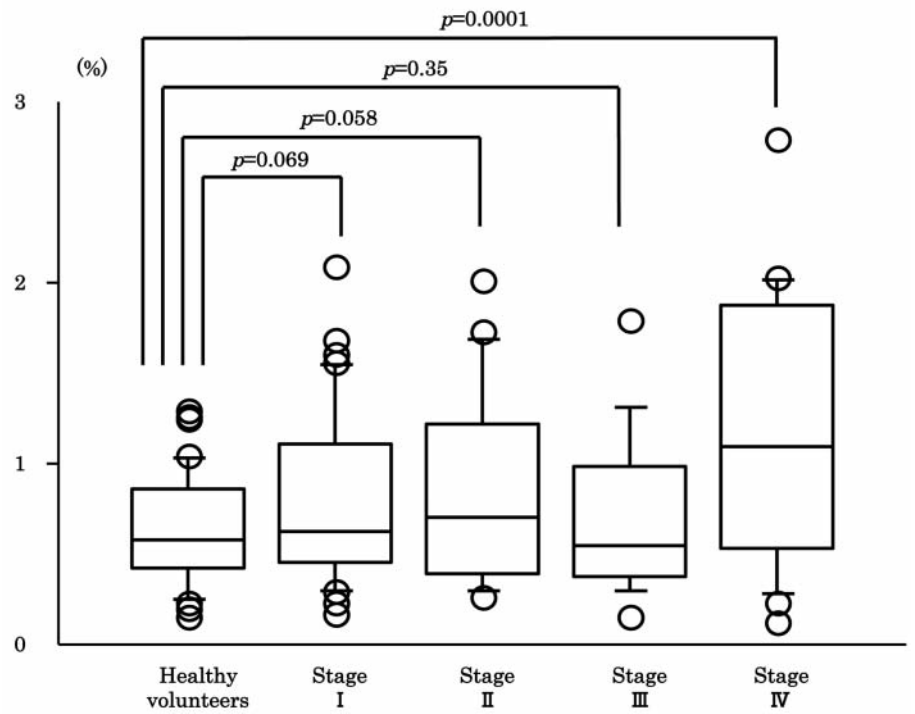

Figure 3. *The prevalence of CD4+FoxP3+ Treg in the peripheral blood of gastrointestinal cancer patients with different disease severity. CD4+FoxP3+ Treg levels in patients with stage IV cancer were significantly increased. Treg levels in patients with stage III cancer were high, but were not statistically significant. There was a trend towards increased percentage of CD4+FoxP3+ Treg in patients with stage I and stage II disease compared with healthy volunteers.*

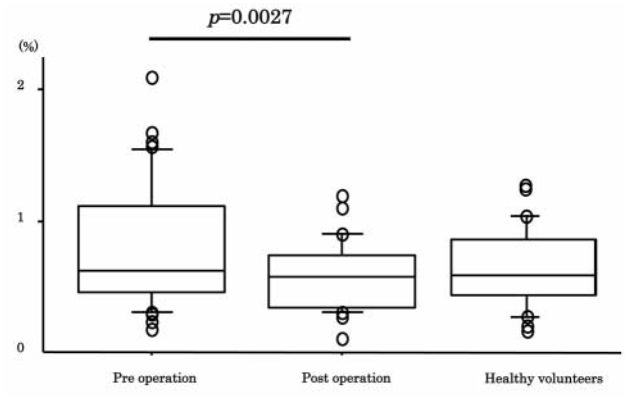

Figure 4. *Change in CD4$^+$Foxp3$^+$ Treg levels in the peripheral blood of patients with stage I disease after tumor resection. After curative resection of stage I cancers, CD4$^+$FoxP3$^+$ Treg levels were significantly reduced and were similar to those in healthy volunteers.*

were significantly reduced after curative resection but were increased again in cases of disease relapse (14). These findings suggest a close relationship between tumor growth and Treg levels.

Possible mechanisms for the induction of Treg in cancer include the specific expansion of Treg by cancer-derived factors or as a physiological defense phenomenon against inflammation induced by cancer. It has recently been shown that tumor cells and microenvironmental macrophages produce the chemokine CCL22, which mediated the trafficking of Treg to the tumor (29). In addition, other soluble factors have been identified in a variety of malignancies that induce Treg, including TGF-β, IL-10 and H-ferritin (33-37). Wolf *et al*. showed that the increased population of Treg in the peripheral blood of cancer patients was due to active proliferation rather than redistribution from secondary lymphoid organs or the bone marrow (38). Furthermore, Hiraoka *et al*. (39) reported that the prevalence of Treg was significantly increased in the stroma of pancreatic invasive ductal carcinomas compared to the stroma of non-neoplastic inflammation of the pancreas. Thus, apart from the peripheral blood, Treg may be induced at the actual tumor site. Further studies are warranted to elucidate the details regarding the induction and activity of Treg in cancer patients.

In conclusion, this study demonstrated an increase in CD4$^+$FoxP3$^+$ Treg in the peripheral blood in gastrointestinal cancers, even in early stages of the disease. Since CD4$^+$FoxP3$^+$ Treg levels decreased after curative resection, tumor cells may have induced and expanded Treg pools.

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