Increased Populations of Regulatory T Cells in Peripheral Blood and Tumor-Infiltrating Lymphocytes in Patients with Gastric and Esophageal Cancers

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ABSTRACT

Purpose: **It is well known that tumor-infiltrating lymphocytes (TILs) and, to a lesser extent, peripheral blood lymphocytes from patients with advanced-stage cancer have a poor immune response. Regulatory T cells (T-regs), characterized by coexpression of CD4 and CD25 markers, can inhibit the immune response mediated by CD4+/CD25− and CD8+ T cells. In the present study, we evaluated the prevalence of T-regs in peripheral blood and TILs in patients with gastric and esophageal cancers.**

Experimental Design: **The population of CD4+/CD25+ cells as a percentage of total CD3+ cells was evaluated by flow cytometric analysis with triple-color staining. To assess the functional activity of CD4+/CD25+ cells, CD4+/CD25+ or CD4+/CD25− cells were purified from peripheral blood mononuclear cells with magnetic beads. The cytokine production [interleukin (IL)-10 and IFN-γ] from the CD4+/CD25+ cells in response to anti-CD3 stimulation was evaluated. Also, the antiproliferative function of CD4+/CD25+ cells was measured by evaluating the proliferative activity of CD4+/CD25− cells in response to anti-CD3 plus anti-CD28 in the presence of autologous CD4+/CD25+ cells.**

Results: **The prevalence of peripheral blood CD4+/CD25+ cells in both gastric (n = 20; 14.2 ± 4.9%) and esophageal cancer patients (n = 10; 19.8 ± 6.9%) was significantly higher than that in healthy donors (n = 16; 7.2 ± 2.1%). The population of CD4+/CD25+ cells in the TILs of gastric cancer patients with advanced disease (19.8 ± 4.5%) was significantly higher than that in TILs of patients with early-stage disease (4.8 ± 2.1%) or that in intraepithelial lymphocytes of normal gastric mucosa (4.0 ± 1.2%). As a functional consequence, CD4+/CD25+ cells did not produce IFN-γ, whereas CD4+/CD25− cells secreted IFN-γ. Moreover, CD4+/CD25+ cells produced large amounts of IL-10, whereas CD4+/CD25− cells secreted little IL-10. The proliferation of CD4+/CD25− cells was inhibited in the presence of CD4+/CD25+ cells in a dose-dependent manner, confirming that CD4+/CD25+ has an inhibitory activity corresponding to T-regs.**

Conclusions: **The populations of CD4+/CD25+ T-regs in peripheral blood and TILs in patients with gastric and esophageal cancers were significantly higher in comparison with those in healthy donors or normal mucosa.**

INTRODUCTION

It is well known that TILs[2] and, to a lesser extent, PBLs from patients with advanced-stage cancer have a poor immune response (1). This tumor-induced immunosuppression includes diminished responses to recall antigens (2), decreased proliferative T-cell responses and loss of cytokine production (3, 4), and defective signal transduction in T cells and natural killer cells (5, 6). There is also evidence for increased apoptosis among CD8+ T cells in PBLs from cancer patients and mice with experimental tumors (7–9). We reported recently that peripheral blood T cells from gastric cancer patients simultaneously exhibited an elevated caspase-3 activity, an increased degree of T-cell apoptosis, down-regulation of T cell receptor ζ molecules, and impaired cytokine production (9).

Several mechanisms may account for the T-cell dysfunction observed locally in the tumor or in the circulation of individuals with cancer. These include Fas-Fas ligand interaction leading to T-cell apoptosis, which has been shown to involve caspase-3-mediated cleavage of TCRζ molecules (10). Reactive oxygen species produced by myelomonocytic cells have recently emerged as a potentially important immunosuppressive mechanism for T cells in tumor-bearing individuals (11, 12). We showed recently that the hydrogen peroxide secreted from macrophages in tumor-draining lymph nodes in gastric cancers could induce T-cell dysfunction (13).

T-regs, characterized by coexpression of CD4 and CD25 markers, are thought to be a functionally unique population of T cells and function to maintain immune homeostasis (14, 15). Of note, T-regs can inhibit the immune response mediated by CD4+/CD25− and CD8+ T cells because it was reported that

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[2] The abbreviations used are: TIL, tumor-infiltrating lymphocyte; T-reg, regulatory T cell; PBMC, peripheral blood mononuclear cell; IL, interleukin; IEL, intraepithelial lymphocyte; PBL, peripheral blood lymphocyte; mAb, monoclonal antibody; TNM, tumor-node-metastasis.

Table 1 Patients characteristics

	Gastric cancer ($n = 20$)	Esophageal cancer ($n = 10$)	Healthy individuals ($n = 16$)
Gender (male:female)	14:6	9:1	12:4
Age (yrs)	68 ± 11	70 ± 9	62 ± 18
TNM stage[a]			
I	11	1	
II	2	1	
III	4	6	
IV	3	2	

[a] Stage according to the TNM classification for gastric cancer (UICC).

T-regs play an important role in preventing allograft rejection, graft-*versus*-host disease, and autoimmune disease (16, 17). In addition, patients and experimental models with cancer showed that T-regs down-regulated the activity of effector function against tumors, resulting in T-cell dysfunction in cancer-bearing hosts (18, 19). These observations led us to the hypothesis that tumor-bearing hosts with advanced cancers have an increased population of T-regs, which might inhibit the tumor-specific T-cell response. In fact, an increased population of T-regs was reported in patients with ovarian cancer (20), lung cancer (21), and breast cancer (22). However, there are no previous reports describing T-regs in gastric and esophageal cancers.

In the present study, we evaluated the prevalence of T-regs in peripheral blood and TILs in patients with gastric and esophageal cancers and, furthermore, performed functional analysis to confirm their suppressive function.

MATERIALS AND METHODS

Patients. Peripheral blood was collected from 20 patients with gastric cancer, 10 patients with esophageal cancer, and 16 healthy donors. None of the patients received surgery, radiotherapy, chemotherapy, or other medical interventions during this study. Characteristics of the study subjects are summarized in Table 1. This study was approved by the ethical committee of University of Yamanashi, and written informed consent was obtained from all individuals.

Cell Preparation. PBMCs were isolated with a Ficoll (Amersham, Uppsala, Sweden) density gradient.

For separation of CD4+/CD25+ or CD4+/CD25− cells, PBMCs were further separated with a Macs CD4 Multisort Kit and CD25 Microbeads (Miltenyi Biotec) using magnetic separation columns according to the manufacturer's guidelines. The enriched cells were >93% CD4+/CD25+ or CD4+/CD25− cells as determined by flow cytometry.

For isolation of TILs and IELs, tumor specimens and normal gastric mucosa in the same patients were collected at surgery and minced into 1-mm pieces, followed by enzyme digestion with 1 mg/ml collagenase (Sigma-Aldrich, St. Louis, MO), 2.5 units/ml hyaluronidase (Sigma-Aldrich), and 0.1 mg/ml DNase (Sigma-Aldrich) for 2 h.

Flow Cytometric Analysis. PBMCs, TILs, or IELs were stained for cell surface molecules to determine their immunophenotype with anti-CD25-FITC, anti-CD152-PE, anti-CD45RO-PE, anti-CD4-PerCP, and anti-CD3-APC antibodies (DAKO, Glostrup, Denmark). Triple- or four-color flow cytometry was performed using FACSCalibur (Becton Dickinson, San Jose, CA). Cells were analyzed using CellQuest software.

Cytokine Production Assay. Purified CD4+/CD25+ or CD4+/CD25− cells (1×10^5 cells) were placed on anti-CD3 mAb (10 ng/ml; DAKO)-coated 96-well flat-bottomed plates (Becton Dickinson, Franklin Lakes, NJ) and cultured in 200 μl of AIM-V medium (Life Technologies, Inc.) at 37°C for 24 h. The culture supernatants were then harvested and tested for cytokine production using Quantikine human IFN-γ or IL-10 ELISA kit (R&D Systems, Minneapolis, MN) according to the protocols provided by the manufacturer.

Cell Proliferation Assay. Purified CD4+/CD25− cells (1.25×10^4 cells) were incubated with the indicated ratio of autologous CD4+/CD25+ cells on anti-CD3 mAb (10 ng/ml; DAKO)-coated 96-well round-bottomed plates (Becton Dickinson) in the presence of an anti-CD28 mAb (10 μg/ml; PharMingen, San Diego, CA). Cell proliferation was measured by incorporation of [^{3}H]thymidine (1 μCi/well; Moravek Biochemicals, Inc.). The cells were harvested after 16 h, and thymidine incorporation was expressed as cpm.

Statistical Analysis. Differences between the values were determined using Student's *t* test. Significance was determined as $P < 0.05$.

RESULTS

Increased Populations of CD4+/CD25+ T Cells in Peripheral Blood of Patients with Gastric and Esophageal Cancers. PBLs in patients with gastric ($n = 20$) and esophageal cancers ($n = 10$) and in healthy donors ($n = 16$) were examined for the prevalence of CD4+/CD25+ T cells. The population of CD4+/CD25+ cells as a percentage of total CD3+ cells or total CD4+ cells was evaluated by flow cytometric analysis with triple-color staining. Representative flow cytometric data showed that the populations of CD4+/CD25+ T cells after gating for CD3+ cells were increased in the patients with gastric and esophageal cancers in comparison with those in healthy donors (Fig. 1*A*). Summarized data from all individuals indicated that the prevalence of peripheral blood CD4+/CD25+ in total CD3+ cells in both gastric (14.2 ± 4.9%) and esophageal (19.8 ± 6.9%) cancer patients was significantly higher than that in healthy donors (7.2 ± 2.1%), as shown in Fig. 1*B*. Furthermore, the population of CD4+/CD25+ T cells in esophageal cancer patients was significantly increased compared with that in gastric cancer patients (Fig. 1*B*). Similarly, the prevalence of peripheral blood CD4+/CD25+ in total CD4+ cells in both gastric (18.8 ± 4.1%) and esophageal (26.5 ± 4.9%) cancer patients was significantly higher than that in healthy donors (8.9 ± 3.5%; $P < 0.05$).

Furthermore, we analyzed cell surface markers such as CTLA-4 (CD152) and CD45RO on CD4+/CD25+ T cells in cancer patients. The expression of CTLA-4 and CD45RO was analyzed in the gated population of CD4+/CD25+ T cells. Representative flow cytometric data from a gastric cancer patient showed that most of CD4+/CD25+ T cells coexpressed CTLA-4 and CD45RO molecules (Fig. 2).

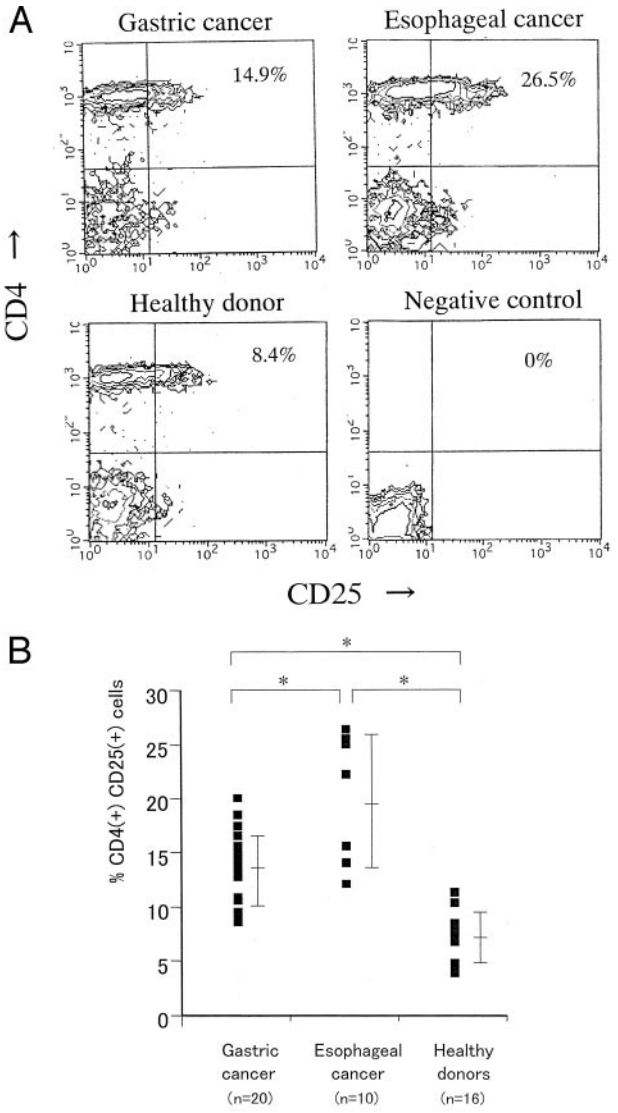

Fig. 1 Increased populations of CD4+/CD25+ T cells in peripheral blood of patients with gastric and esophageal cancers. The population of CD4+/CD25+ cells as a percentage of total CD3+ cells in the PBMCs was evaluated by flow cytometric analysis with triple-color staining. Representative flow cytometric data (*A*) and summarized data from all individuals (*B*) showed that the population of CD4+/CD25+ T cells was increased in patients with gastric and esophageal cancers in comparison with that in healthy donors. A negative control for staining is included in *A*. ∗, $P < 0.05$.

The CD4+/CD25+ T Cells Separated from Patient PBLs Corresponded Functionally to T-regs. To perform functional analysis of CD4+/CD25+ T cells, CD4+/CD25+ and CD4+/CD25− cells were purified from the PBLs in gastric cancer patients ($n = 15$) and healthy donors ($n = 9$) with magnetic beads. The purity of CD4+/CD25+ was always >93%. Both CD4+/CD25+ and CD4+/CD25− cells were stimulated with immobilized anti-CD3 mAbs, and their supernatants were examined for IFN-γ or IL-10 content. As shown in Fig. 3*A*, CD4+/CD25+ cells derived from gastric cancer patients did not produce IFN-γ, whereas CD4+/CD25− cells secreted IFN-γ. Moreover, CD4+/CD25+ cells did produce large amounts of IL-10, but CD4+/CD25− cells secreted a little IL-10. Similarly, CD4+/CD25+ cells derived from healthy donors did produce large amounts of IL-10 but did not produce IFN-γ (Fig. 3*B*).

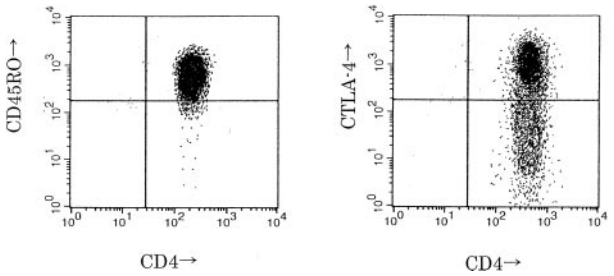

Fig. 2 The expression of CTLA-4 (CD152) or CD45RO on CD4+/CD25+ T-regs. Representative data from PBLs in a gastric cancer patient showed the expression of CTLA-4 (CD152) or CD45RO after gating of CD4+/CD25+ T cells.

Next, we assessed the antiproliferative function of CD4+/CD25+ by evaluating the proliferative activity of CD4+/CD25− cells in response to anti-CD3 plus anti-CD28 in the presence of autologous CD4+/CD25+ cells ($n = 6$). The proliferation of CD4+/CD25− cells was inhibited in the presence of CD4+/CD25+ cells in a dose-dependent manner (Fig. 3*C*). Thus, CD4+/CD25+ T cells separated from patient PBLs showed a suppressor function and corresponded functionally to T-regs.

Increased Populations of CD4+/CD25+ T Cells in TILs with Gastric Cancers. TILs from gastric cancers and IELs from normal gastric mucosa were isolated from resected gastric cancer specimens ($n = 15$). The population of CD4+/CD25+ cells as a percentage of total CD3+ cells was evaluated by flow cytometric analysis with triple-color staining. The patients were divided into two groups: (*a*) those with early disease ($n = 7$) corresponding to stage I according to the TNM classification for gastric cancer (Union Internationale Contre le Cancer); and (*b*) those with advanced disease ($n = 8$) corresponding to stages II, III, and IV. The population of CD4+/CD25+ T cells in TILs of patients with advanced disease was significantly higher than that in TILs of patients with early disease or that in IELs of normal mucosa (Fig. 4). These results indicate that how T-regs infiltrate the tumor microenvironment depends on disease progression.

DISCUSSION

The present study contains the first evidence related to the prevalence of T-regs in gastric and esophageal cancer. We showed increased populations of CD4+/CD25+ cells in peripheral blood T cells in patients with gastric and esophageal cancers in comparison with healthy donors. Moreover, the population of CD4+/CD25+ cells in the TILs of gastric cancer was higher than that in the IELs of normal gastric mucosa. Furthermore, we confirmed that CD4+/CD25+ isolated from patient PBLs had a regulatory function by evaluating cytokine production and suppressive capacity against CD4+/CD25− cells.

There is increasing evidence that CD4+/CD25+ T-regs

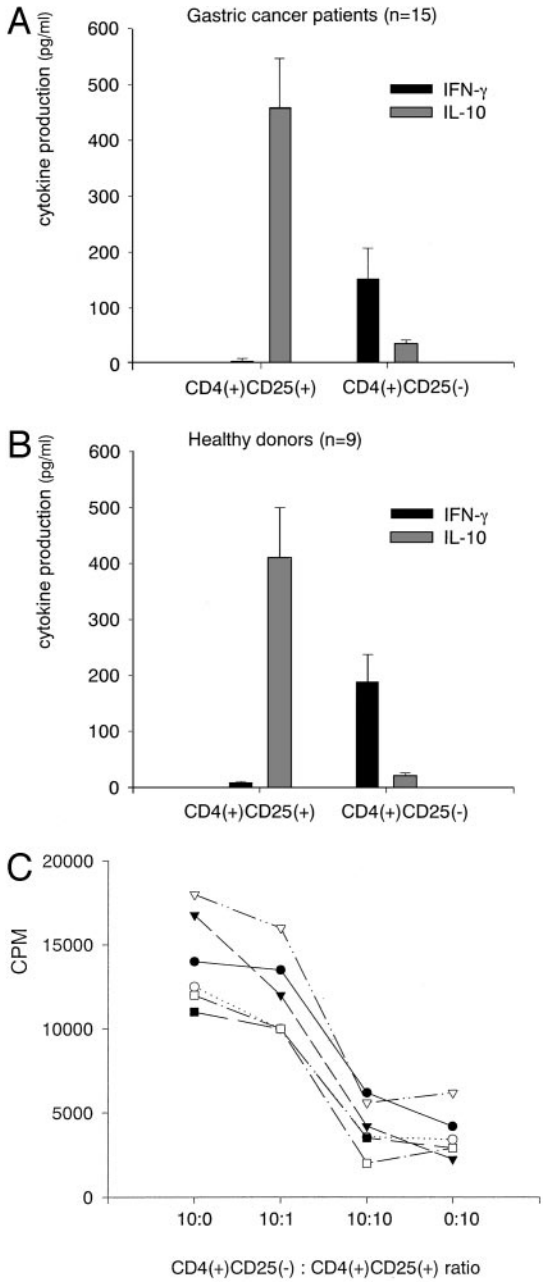

Fig. 3 Functional analysis of CD4+/CD25+ T cells. CD4+/CD25+ and CD4+/CD25− cells were purified from the PBLs in gastric cancer patients and healthy individuals with magnetic beads. Both CD4+/CD25+ and CD4+/CD25− cells were stimulated with immobilized anti-CD3 mAbs, and their supernatants were examined for IFN-γ or IL-10 content (*A* and *B*). The proliferative activity of CD4+/CD25− cells in response to anti-CD3 plus anti-CD28 in the presence of autologous CD4+/CD25+ cells is indicated as a ratio ($n = 6$; *C*).

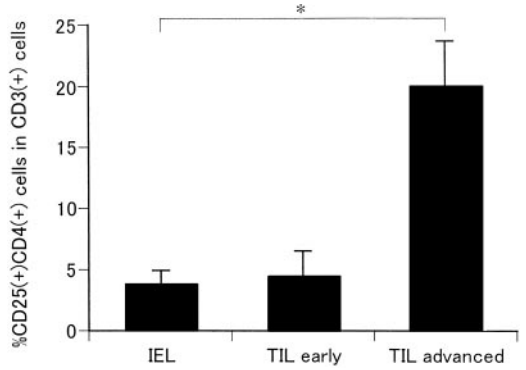

Fig. 4 Increased populations of CD4+/CD25+ T cells in TILs with gastric cancers. TILs from gastric cancers and IELs from normal gastric mucosa were isolated from resected gastric cancer specimens ($n = 15$). The population of CD4+/CD25+ cells as a percentage of total CD3+ cells was evaluated by flow cytometric analysis with triple-color staining. The patients were divided into two groups: those with early disease (*TIL early*) corresponding to stage I according to the TNM classification for gastric cancer (UICC); and those with advanced disease (*TIL advanced*) corresponding to stages II, III, and IV.

play a key role in suppressing T-cell-mediated immunity in cancer-bearing hosts, as indicated in several animal models in which the efficacy of therapeutic cancer vaccination can be enhanced by depleting T-regs (23), and adoptive transfer of T-regs impaired tumor-specific immunity, resulting in tumor progression (24). In human cancer, an increased population of T-regs has been reported in patients with lung (21), breast (22), and ovarian cancer (20). Considering the present study and previous reports, the fact that an increased population of CD4+/CD25+ T-regs is observed in peripheral blood and tumor microenvironments in patients with cancer is established.

It is well known that cell-mediated immunity in cancer-bearing hosts is suppressed by many factors (5). Many studies have reported (*a*) deficient antigen presentation by down-regulation of MHC class I expression on tumor cells, (*b*) decreased or lost expression of T-cell epitopes on tumor cells, (*c*) immunosuppressive factors derived from tumor cells, or (*d*) T-cell dysfunction in cancer-bearing hosts, including down-regulation of T-cell signaling molecules or increased induction of T-cell apoptosis (5). As an additional explanation for impaired cell-mediated immunity in cancer-bearing hosts, the increased prevalence of T-regs could be included.

There is no clear evidence for the mechanisms of induction of T-regs in cancer-bearing hosts. There are several possibilities, including specific expansion of T-regs induced by cancer-derived factors, or physiological defense phenomena against the continuous inflammation induced by cancer.

Current attempts at immunotherapy for cancer, including cancer vaccination or adoptive transfer of T cells, remain limited in their effect on the regression of established tumors (25, 26). Even if the effective CTLs are transferred adoptively to the patients or tumor-specific CTLs are generated by tumor vaccination, there are several mechanisms by which tumor cells can escape from tumor-specific T-cell surveillance in the tumor microenvironment, as described above. For example, the presence of factors such as Fas-Fas ligand interaction, oxidative metabolites, or immunosuppressive cytokines can be predicted to rapidly shut off the effector functions of CTLs (10–12). Here, the increased population of T-regs could be an additional problem to be resolved in immunotherapy for cancer. A better understanding of the underlying mechanism of T-reg regulation

or of the strategy for controlling T-regs may lead to more effective immunotherapy for cancer.

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