

Prevalence of Th17 and Treg cells in gastric cancer patients and its correlation with clinical parameters

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Abstract. Th17 cells and CD4⁺CD25⁺ regulatory T (Treg) cells have been reported to share reciprocal developmental pathways but exhibit opposite effects, and the balance between them controls inflammation and autoimmune diseases. However, information regarding Th17/Treg cells in cancer-bearing hosts is still limited. In the present study, we investigated the distribution of Th17 cells in relation to Treg cells in gastric cancer patients, and evaluated how the imbalance in Th17/Treg cells in gastric cancer correlates with clinical and pathological parameters. We observed that the accumulation of Th17 and Treg cells in the tumor microenvironment was gradually increased according to disease progression, leading to an imbalance in Th17/Treg cells in gastric cancer patients. TGF- β and interleukin (IL)-6 present in the gastric cancer microenvironment promoted the differentiation and expansion of Th17 cells, and increased numbers of Th17 cells promoted tumor progression through promotion of inflammation by secretion of IL-17. Treg cells promoted tumor progression by helping cancer cells escape from host immunosurveillance by secreting TGF- β , and a high level of TGF- β in the tumor microenvironment promoted differentiation and expansion of Treg cells. In conclusion, the imbalance in Th17/Treg cells was involved in the development and progression of gastric cancer. A better understanding of the nature, regulation, and function of Th17 and Treg cells in tumor immunity may aid in the development of novel and effective immunotherapy for gastric cancer.

Introduction

Gastric cancer is one of the most common malignant diseases and contributes to a significant number of cancer-related

deaths throughout the world. The precise pathogenesis of gastric cancer remains unclear, yet it has been correlated to many factors such as eating habits, environmental factors, hereditary predisposition, chronic gastritis, gastric polyps, gastric mucosa obform hyperplasy and *Helicobacter pylori* infection. Owing to the complicated pathogenesis, there are no specific strategies for dealing with gastric cancer. Although efforts have been made to introduce combination treatment strategies such as surgery combined with chemotherapy or chemoradiotherapy, the control of advanced gastric cancer remains extremely difficult. Exploring new effective ways to cure gastric cancer has become an imminent issue to be solved. It has been shown that the grade of tumor-infiltrating T lymphocytes is correlated with a favorable outcome in cancer patients. Therefore, it is important to understand the mechanisms of immunoregulation in gastric cancer, in order to develop novel treatment strategies or to improve the efficacy of standard therapy (1).

Recently, Th17 and regulatory T (Treg) cells have been defined as two distinct CD4⁺ T subsets from Th1 and Th2 cells, on the basis of their pattern of cytokine production and functions (2). Th17 cells are characterized as interleukin (IL)-17-producing CD4⁺ T cells which also produce IL-21, IL-22 and IL-26 (3,4). Retinoid orphan nuclear receptor (RORc), which encodes the human ortholog of mouse ROR γ t, is a specific transcription factor for Th17-cell lineage differentiation (5,6). TGF- β , IL-6, IL-23 and IL-1 β have been reported to collectively mediate human Th17 cell differentiation *in vitro* (7-9). Despite the important role of Th17 cells in host protection against infectious pathogens and in the pathogenesis of various inflammatory and autoimmune diseases, their prevalence and function in human cancer is still under investigation (10-12). Although several studies have shown the presence of Th17 cells in several types of human cancers, the mechanism of accumulation of Th17 cells or their generation and functional role in the tumor microenvironment remain largely unknown.

Treg cells, which are characterized by expression of Foxp3 in the nucleus, are functionally immunosuppressive subsets of T cells. They play important roles in maintaining tolerance to self components by contact-dependent suppression or by the release of anti-inflammatory cytokines, IL-10 and TGF- β . The number or functional abnormalities in Treg cells may lead

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to autoimmune disease or tumor development. Accumulating evidence suggests that increased number of Treg cells in tumor infiltrating lymphocytes (TILs) or peripheral blood mononuclear cells (PBMCs) is one of the reasons for impaired antitumor immunity in cancer-bearing hosts (13). In addition, the high number of Treg cells in several human tumors was reported to be correlated with poor prognosis and decreased survival (14,15).

Although Th17 and Treg cells play different roles in the pathogenesis of diseases, reciprocal developmental pathways for their generation have been demonstrated. Naïve T cells exposed to TGF- β upregulate Foxp3 and become induced Treg cells; however, when cultured with TGF- β and IL-6, naïve T cells generate Th17 cells with pathogenic activities (16). It has been shown that the balance between Treg and Th17 cells is a key factor that regulates helper T-cell function in autoimmune disease and graft versus host disease (GVHD). However, there is limited information regarding the balance between Treg and Th17 cells in cancer patients. To evaluate whether this balance is disrupted in gastric cancer patients, and assess the possible role of Th17/Treg in the pathogenesis and progression of gastric cancer, we investigated the distribution of Th17 cells in relation to Treg cells in gastric cancer patients, and evaluated how the imbalance in Th17/Treg cells correlates with clinical and pathological parameters.

Materials and methods

Patients. Peripheral blood was collected from 45 patients with gastric cancer and 20 healthy volunteers. Lymph nodes and tumor tissues of the stomach were collected during surgery. None of the patients received radiotherapy, chemotherapy, or other medical interventions before the study. The present study was approved by the Ethics Committee of The Fourth Hospital of Hebei Medical University and written informed consent was obtained from all individuals.

Cell preparations. PBMCs were isolated by standard Ficoll-Hypaque density centrifugation of heparinized peripheral blood from the studied subjects.

Flow cytometric analysis of Th17. To analyze the prevalence of Th17 cells, IL-17-producing CD4⁺ lymphocytes were detected. PBMCs (2x10⁶) were incubated in the presence of 50 ng/ml PMA, 1 μ g/ml ionomycin and 10 μ g/ml brefeldin A (all from Sigma-Aldrich, St. Louis, MO, USA) for 4 h at 37°C. The cells were stained with FITC anti-human CD4 at 4°C for 30 min. After the surface staining, the cells were stained with phycoerythrin (PE) anti-human IL-17A for Th17 detection after fixation and permeabilization according to the manufacturer's directions. Isotype-matched antibodies were used as controls. All of the Abs and reagents were purchased from eBioscience (San Diego, CA, USA). The number of IL-17⁺ cells on the gating of CD4⁺ cells was evaluated, and the frequency of Th17 cells was expressed as a percentage of the total CD4⁺ cells.

Flow cytometric analysis of Treg cells. CD4⁺CD25⁺FoxP3⁺ cells as Treg cells were measured using a human regulatory T-cell staining kit (eBioscience) according to the manufacturer's protocol. Briefly, the single-cell suspension was incubated

Table I. Primers for PCR.

Gene	Sense primer	Antisense primer
RORc	5'-gcaacagcagcaacaggaa-3'	5'-ccaaggtgtaggtagggtat-3'
IL-17	5'-gccatagtgaggcaggaa-3'	5'-gtgaggtggatcggtgtag-3'
IL-23	5'-ctccctgatagccctgtgg-3'	5'-tgaagcggagaaggagacg-3'
IL-21	5'-gattagaatgcgtcaact-3'	5'-cctttcattgttctctga-3'
IL-22	5'-aagaagtgtgttcctc-3'	5'-catgtgcttagcctgttg-3'
FoxP3	5'-gctggtgctggagaaggag-3'	5'-cggatgatgccacagatgaa-3'
IL-10	5'-tgagaaccaagaccagac-3'	5'-cattcttccctgctccac-3'
TGF- β	5'-cccacaacgaaatctatgaca-3'	5'-gctgaggtatcggcaggaa-3'
IL-1 β	5'-acgaaatctccgaccacca-3'	5'-gcaggaaccagcatctt-3'
IL-6	5'-atcatcactgtcttttggag-3'	5'-ctggctgttctcactactc-3'
β -actin	5'-taagacctcaaccccag-3'	5'-gccatctattgctcgaagtc-3'

RORc, retinoid orphan nuclear receptor; IL, interleukin.

with a cocktail of anti-CD4-FITC monoclonal antibody and anti-CD25-APC monoclonal antibody for 30 min at 4°C. After fixation/permeabilization, the cells were blocked by normal rat serum and stained using anti-FoxP3-PE monoclonal antibody or PE-conjugated rat IgG2a used as the isotype control for 45 min at 4°C. The number of FoxP3⁺ cells on the gating of CD4⁺ cells was evaluated and the frequency of Th17 cells was expressed as a percentage of the total CD4⁺ cells.

Immunohistochemical staining. Paraffin-embedded samples were cut into 4- μ m sections, which were then processed for double staining immunohistochemistry. Briefly, the deparaffinized and hydrated sections were depleted of endogenous peroxidase activity by adding methanolic H₂O₂ for 20 min. After blocking the samples with normal goat serum for 45 min, each section was stained with mouse anti-human CD4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. The samples were incubated with biotinylated goat anti-mouse IgG at 37°C for 30 min, and then incubated with streptavidin-horseradish peroxidase complex for 30 min followed by incubation with 3,3'-diaminobenzidine. Subsequently, the sections were incubated with rabbit anti-human IL-17 antibody (Proteintech, Chicago, IL, USA) or rabbit anti-human Foxp3 antibody (Santa Cruz Biotechnology), respectively, followed by alkaline phosphatase anti-rabbit IgG, and then the sections were stained with alkaline phosphatase red. The sections were counterstained with hematoxylin.

Quantitative real-time RT-PCR. Tumor samples were snap-frozen in liquid nitrogen. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Complementary DNA was synthesized from total RNA (0.5 μ g) using the ReverAid™ First Strand cDNA Synthesis kit (Takara Biotechnology, Dalian, China). Subsequently, quantitative real-time PCR was performed in an ABI PRISM 7500 Cycloer (Applied Biosystems, Warrington, UK) using the SYBR Premix Ex Taq™ II kit (Takara Biotechnology) for 40 cycles (95°C for 5 sec, 60°C for 31 sec) after an initial 30-sec incubation at 95°C. The expression levels of RORc, IL-17, IL-23, IL-21, IL-22, TGF- β , IL-1 β , IL-6, Foxp3 and IL-10 were determined

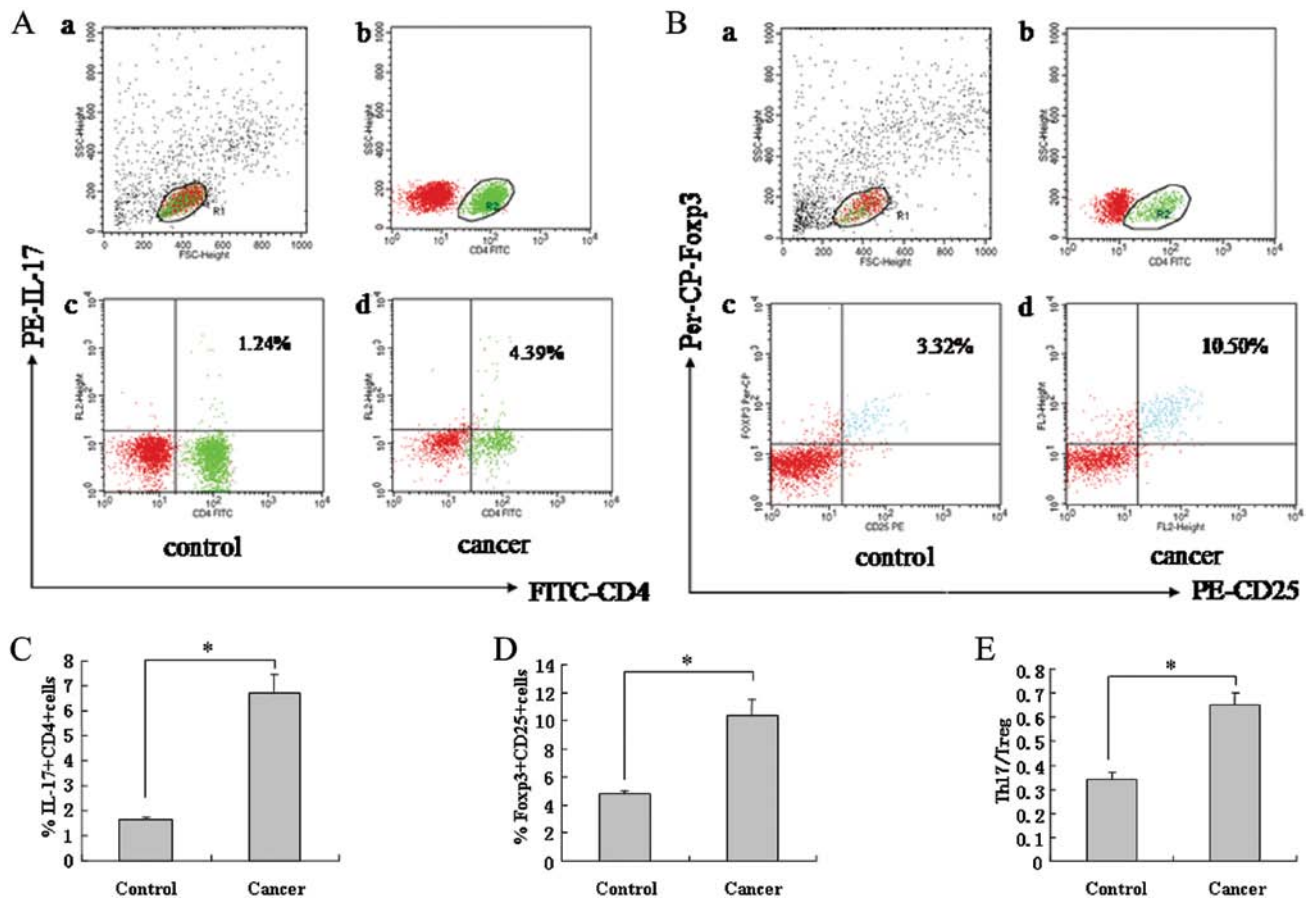


Figure 1. Prevalence of Th17 and regulatory T (Treg) cells in gastric cancer patients. For Th17 cell analysis, peripheral blood mononuclear cells (PBMCs) from all subjects were stimulated with PMA, ionomycin, and brefeldin A for 4 h, and then stained with labeled antibodies. As for Treg cells, PBMCs from all subjects were stained with labeled antibodies. (A) The frequency of Th17 cells in controls and gastric cancer patients. (a) Lymphocytes were gated by flow cytometry. (b) CD4⁺ T subsets were gated by flow cytometry. (c and d) The proportion of Th17 cells to CD4⁺ subsets from representative control and gastric cancer patient. The percentage of positive cells is shown in each panel. (B) The frequency of Treg cells in controls and gastric cancer patients. (a) Lymphocytes were gated by flow cytometry. (b) CD4⁺ T subsets were gated by flow cytometry. (c and d) The proportion of Foxp3 and CD25 double staining cells to CD4⁺ subsets from representative control and gastric cancer patient. The percentage of positive cells is shown in each panel. (C and D) Percentage of Th17 and Treg cells in gastric cancer patients. Significantly increased percentage of Th17 and Treg cells gated on CD4⁺ subjects, was found in gastric cancer patients when compared to the controls ($P < 0.05$). (E) The proportion of Th17/Treg in gastric cancer patients. Obviously increased proportion of Th17/Treg was found in gastric cancer patients when compared to the controls ($P < 0.05$).

from the cycle threshold values normalized to β -actin using the relative standard curve method. Primers used in the study are shown in Table I.

Statistical analysis. Data analysis was performed using SPSS 13.0. Values are expressed as means \pm SD. The Student's t-test and ANOVA analysis were used to compare the significance of differences between experimental groups. Correlations were evaluated using linear correlation analysis. The adjusted partial regression coefficient was determined using multiple linear regression. P-values < 0.05 were considered statistically significant.

Results

Increased frequency of circulating Th17 in gastric cancer patients. PBMCs in patients with gastric cancer and in healthy donors were examined for the frequency of Th17 cells. The population of Th17 cells as a percentage of total CD4⁺ cells was evaluated by flow cytometric analysis. Representative dotplots are shown in Fig. 1A. Summarized data from all individuals

indicated that the percentage of Th17 cells was significantly increased in patients with gastric cancer when compared to healthy donors (Fig. 1C).

We next sought to determine whether this Th17 skewing was associated with clinicopathologic status including gender, age, TNM stage, differentiation, metastatic lymph nodes, tumor factors, invasive depth, blood vessel invasion, intravascular cancer embolus and gastric cancer-related tumor antigens in gastric cancer patients. As shown in Table II, specifically, the frequency of Th17 cells was positively correlated with TNM stage and lymph node metastases. There was no association between Th17 frequency and other studied factors.

Increased frequency of circulating Treg cells in gastric cancer patients. To analyze the prevalence of Treg, CD4⁺CD25⁺FoxP3⁺ cells were examined by flow cytometry and expressed as a percentage of the total CD4⁺ cells. Representative dotplots are shown in Fig. 1B. Summarized data from all individuals showed that the frequency of Treg cells in PBMCs of gastric cancer patients was significantly higher than that in healthy donors (Fig. 1D). The association

Table II. Association between Th17, Treg cells or Th17/Treg in peripheral blood and clinicopathologic factors of gastric cancer patients.

Clinical characteristic	Evaluation	N=45	P-value		
			Th17	Treg	Th17/Treg
Gender			0.754	0.674	0.917
Male	0	36			
Female	1	9			
Age, years			0.963	0.884	0.890
<60	0	24			
≥60	1	21			
TNM stage			0.002 ^a	0.034 ^a	0.110
I	1	15			
II	2	9			
III	3	9			
IV	4	12			
Differentiation			0.176	0.015 ^a	0.165
Well	0	14			
Poor	1	31			
Metastatic lymph node			0.017 ^a	0.042	0.036 ^a
Yes	1	18			
No	0	27			
Size of tumor (cm)			0.262	0.279	0.476
<4	0	19			
≥4	1	26			
Invasive depth (mm)			0.507	0.572	0.291
<15	0	17			
≥15	1	28			
Blood vessel invasion			0.077	0.134	0.237
Yes	1	11			
No	0	34			
Intravascular cancer embolus			0.508	0.519	0.623
Yes	1	9			
No	0	36			
Gastric cancer-related tumor antigens					
CA50 (0.21-10 IU/ml)			0.903	0.972	0.431
<10	0	37			
≥10	1	8			
CEA (0-5 ng/ml)			0.321	0.465	0.936
<5	0	40			
≥5	1	5			
CA199 (0-27 U/ml)			0.570	0.917	0.274
<27	0	29			
≥27	1	6			
CA724 (0-6.9 U/ml)			0.309	0.274	0.268
<6.9	0	31			
≥6.9	1	15			

Multiple regression analysis, ^aP<0.05 compared with the control group. Treg, regulatory T cells.

between expression of Treg and clinical/pathologic parameters showed that the frequency of Treg cells was positively correlated with TNM stage and tumor differentiation, and there was no association between the frequency of Treg and other studied factors (Table II).

Increased proportion of Th17/Treg cells in gastric cancer patients. We also observed the proportion of Th17/Treg in

gastric cancer patients. As shown in Fig. 1E, the proportion of Th17/Treg cells was significantly increased in gastric cancer patients when compared to healthy donors. In addition, compared with the gastric cancer patients without lymph node metastasis, a significantly increased ratio of Th17/Treg was found in patients with lymph node metastasis. There was no statistical correlation between the Th17/Treg ratio and other clinical/pathologic parameters (Table II).

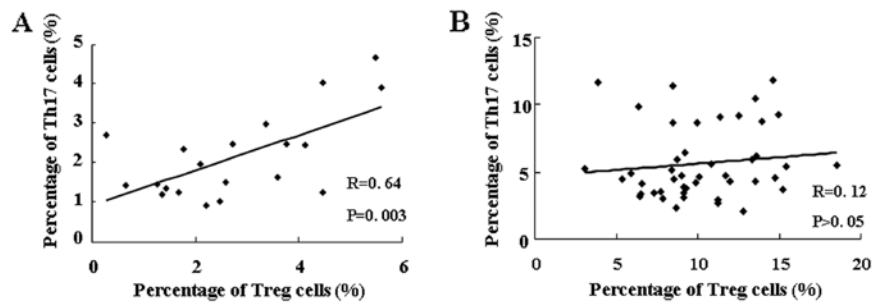


Figure 2. Correlation between Th17 and regulatory T (Treg) cells in gastric cancer patients. (A) A positive correlation between the frequency of Th17 and Treg cells was found in the healthy controls ($R=0.64$, $P=0.003$). (B) No statistically significant correlation between Th17 and Treg cells in gastric cancer patients was found.

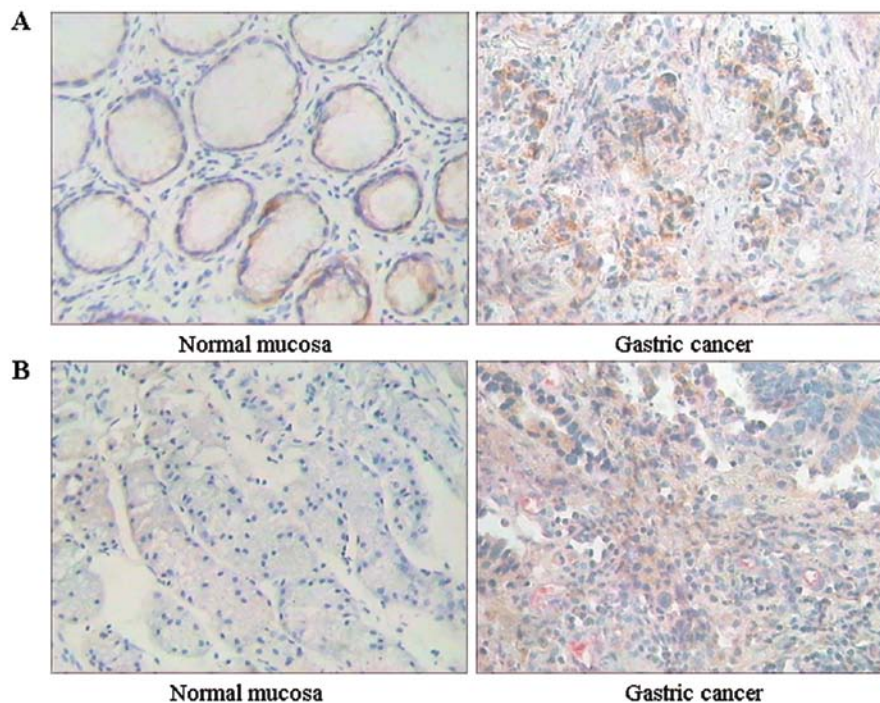


Figure 3. Immunohistochemical staining for CD4/interleukin (IL)-17 or CD4/Foxp3 in gastric cancer. Magnification, x400. (A) Representative immunostaining for CD4/IL-17 is shown for normal gastric mucosa and gastric cancer. (B) Representative immunostaining for CD4/Foxp3 is shown for normal gastric mucosa and gastric cancer. Summarized data indicated that the frequency of CD4⁺IL-17⁺ or CD4⁺FoxP3⁺ cells was frequently observed in gastric cancer.

Correlation between Th17 and Treg cells. Next, we evaluated the relationship between Th17 and Treg cells in gastric cancer patients and in healthy donors. As shown in Fig. 2, the frequencies of Th17 and Treg cells were positively correlated in the healthy donors, but not correlated in the gastric cancer patients.

Distribution of Th17 and Treg cells in gastric cancer tissue. To further evaluate the frequency of Th17 and Treg cells in gastric cancer tissue, double staining immunohistochemistry (CD4 and IL-17 for Th17, or CD4 and Foxp3 for Treg) was performed. Notably, representative data revealed that CD4⁺IL-17⁺ or CD4⁺FoxP3⁺ cells were frequently observed in gastric cancer in comparison to the controls (Fig. 3).

mRNA expression of RORc, IL-17, IL-23, IL-21, IL-22, TGF- β , IL-1 β , IL-6, Foxp3 and IL-10 in gastric cancer tissue. To

further analyze the mechanism of Th17 and Treg accumulation in gastric cancer tissue or the relationship between the immune-imbalance of Th17/Treg and their differentiation or function in gastric cancer tissues, we also examined the mRNA expression levels of RORc, IL-17, IL-23, IL-21, IL-22, TGF- β , IL-1 β , IL-6, Foxp3 and IL-10 in gastric cancer tissue by real-time RT-PCR. As shown in Fig. 4, mRNA expression levels of RORc, IL-17, IL-21, TGF- β , IL-6, Foxp3 and IL-10 in gastric cancer were significantly increased in comparison to the controls. The correlation between RORc and cytokines showed that mRNA expression of RORc did correlate with mRNA expression of IL-17, IL-6, TGF- β and IL-23, but did not correlate with mRNA expression of IL-21, IL-22 and IL-1 β (Fig. 5). The correlation between Foxp3 and TGF- β or IL-10 showed that mRNA expression of Foxp3 did correlate with mRNA expression of TGF- β , but did not correlate with mRNA expression of IL-10 (Fig. 6).

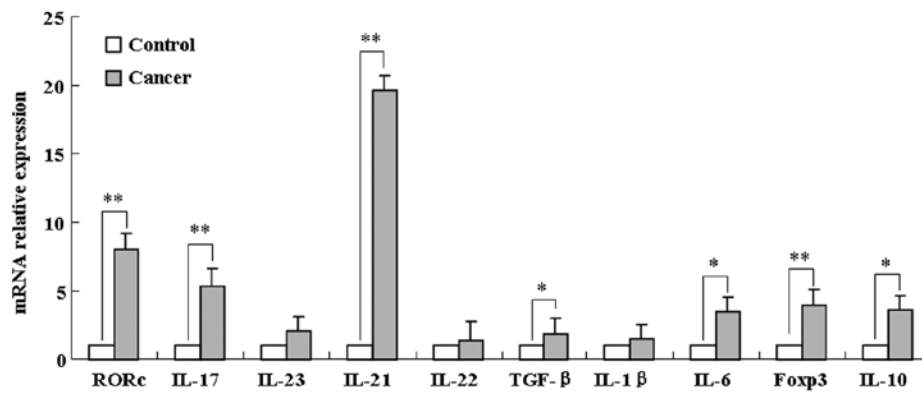


Figure 4. mRNA expression of cytokines and transcription factors in gastric cancer. Total RNA was extracted from gastric cancer and control tissues. The mRNA relative expression of indicated cytokines and specific transcription factors was analyzed by quantitative RT-PCR using the $\Delta\Delta C_t$ method with β -actin mRNA as an internal control. Data are expressed as mean \pm SD. * $P < 0.05$, ** $P < 0.01$ vs. control.

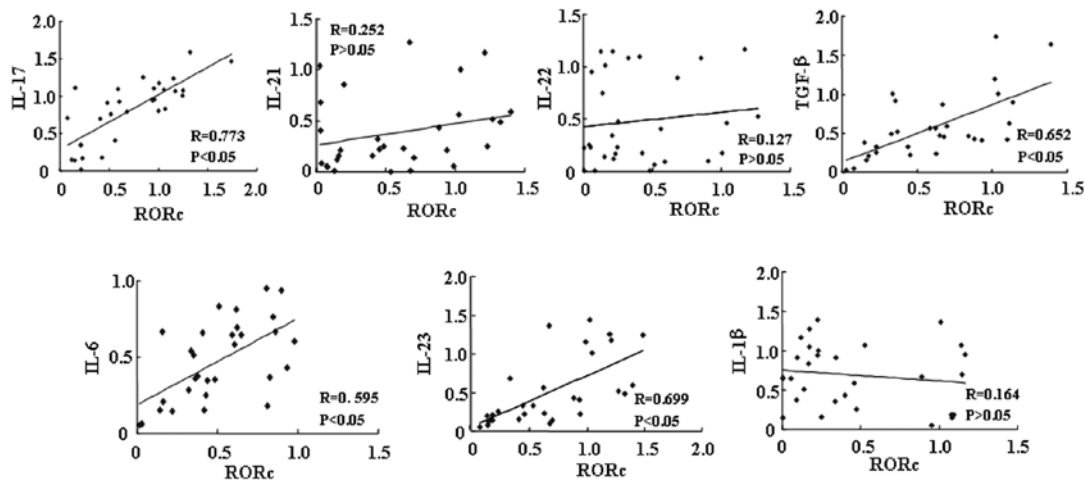


Figure 5. A linear correlation dependability analysis of retinoid orphan nuclear receptor (RORc) mRNA expression and interleukin (IL)-17, IL-21, IL-22, IL-6, IL-23 and IL-1 β mRNA expression in gastric cancer. The mRNA expression of RORc was positively correlated with the mRNA expression of IL-17, IL-6, TGF- β and IL-23, but did not correlate with mRNA expression of IL-21, IL-22 and IL-1 β .

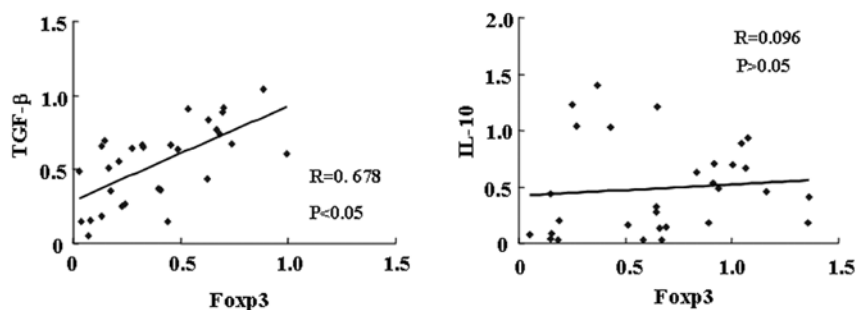


Figure 6. A linear correlation dependability analysis of Foxp3 mRNA expression and TGF- β and interleukin (IL)-10 mRNA expression in gastric cancer. The mRNA expression of Foxp3 was positively correlated with mRNA expression of TGF- β , but did not correlate with mRNA expression of IL-10.

Discussion

Th17 and Treg cells are two newly identified important subsets of T helper cells. The Th17/Treg balance controls autoimmunity and inflammation and has been found to play an important role in the pathogenesis of tumor disease. To assess whether this balance is disturbed in gastric cancer patients, we determined the distribution of Th17 and Treg cells in gastric cancer

patients, and evaluated how the imbalance of Th17/Treg cells correlates with clinical and pathological parameters.

There have been several reports describing Th17 cells in both murine and human tumors, but the nature and role of Th17 cells in cancer immunity remains elusive. Previous investigations demonstrated that the levels of Th17 cells were significantly increased in peripheral blood, malignant ascites fluid and tumor tissues in human ovarian, renal and

pancreatic malignancies (10,11,17,18). In the present study, we reconfirmed an increased number of Th17 cells in the PBMCs of patients with gastric cancer. Moreover, an increased population of Th17 cells was associated with TNM stage and lymph node metastases. Compared with patients with early stage disease, patients with advanced disease showed a significantly higher percentage of Th17 cells. Compared with patients without lymph node metastases, patients with lymph node metastases exhibited a significantly higher percentage of Th17 cells. These results are consistent with the findings of Zhang *et al* (9), which indicated that Th17 cells may contribute to the pathogenesis of gastric cancer.

Zhang *et al* also demonstrated that the levels of Th17 and Treg cells were higher in hepatocellular carcinoma than in the corresponding non-tumor tissues. Our immunohistochemical staining results showed that CD4/IL-17 double stained lymphocytes were more frequently observed in gastric cancer tissues than in their corresponding non-tumor tissues, which was consistent with our flow cytometric results that the frequency of Th17 cells was increased in PBMCs.

Since their discovery, Treg cells have gained much attention in tumor immunity, due to their strong immune suppressive activity on T cell responses. Accumulating evidence demonstrates that the levels of Foxp3⁺ Treg cells are increased in cancer patients, and the high number of Treg cells is associated with poor survival (14,15,19-22). In addition, research regarding Treg cells in ovarian and prostate cancer has demonstrated that Treg cells exist in a markedly higher proportion in PBMCs, and that a significant difference in the prevalence of Treg cells between the early and advanced disease stages was found (11,23). Consistently, our data showed that the frequencies of Treg cells in PBMCs were prominently increased in patients with gastric cancer. Moreover, the increased number of Treg cells was associated with TNM stage and the degree of tumor differentiation. Lymphocyte migration is modulated by chemokine receptor interaction. Previous studies have reported that the majority of tumor-associated Treg cells express lymphoid homing receptor CD62L, CCR4 and CCR6, which induce a gradual increase in Treg cells in tumors during disease progression (23,24). This notion was supported by our study that the percentage of Treg cells was obviously increased in gastric cancer tissue. In addition, we also found that the frequency of Treg cells was positively correlated with the degree of tumor differentiation. These results suggest that suppression of immunity by Treg cells may be an impediment to gastric cancer therapy.

Tumor progression has been considered to be the result of an interaction between different cell types. According to the reciprocal differentiation pathways of Th17 and Treg cells and the regulatory action, Th17/Treg subsets may be involved in immunomodulation, similar to the paradigm of Th1/Th2 subsets, and the imbalance in Th17/Treg cells may lead to tumor progression. This notion was supported by Zhang's *et al* results (9) and our finding that the proportion of Th17/Treg was significantly increased in gastric cancer patients when compared to healthy donors. In addition, the frequencies of Th17 and Treg cells were well correlated in the healthy donors, but not in the gastric cancer patients, which may reflect that an immune balance exists in healthy individuals but has been disrupted in gastric cancer patients. Moreover, compared

with patients without lymph node metastases, an obviously increased ratio of Th17/Treg cells was found in gastric cancer patients with lymph node metastases. These data suggest that the immune imbalance between Th17 and Treg cells may potentially play an important role in the development and progression of gastric cancer.

To further analyze the mechanism of Th17 and Treg accumulation in gastric cancer tissue or the relationship between the immune-imbalance of Th17/Treg and their differentiation or function in gastric cancer tissues, we examined the mRNA expression levels of Th17- and Treg-related cytokines. Our results demonstrated that the mRNA expression level of Th17-specific transcription factor RORc was significantly elevated in gastric cancer tissues, which was consistent with the elevation of Th17 in peripheral blood. Moreover, the mRNA expression level of RORc was positively correlated with that of IL-17, further elevated prevalence of Th17 cells was associated with higher TNM stages. We, therefore, conclude that increased expression of IL-17 in gastric cancer tissue, most likely, originates from Th17 cells, and Th17 cells may promote tumor progression by promoting inflammation through secretion of IL-17.

Recently, TGF- β , IL-1 β and IL-6 have been recognized as the most important cytokines for the initiation of Th17 cell differentiation, whereas IL-23, originally thought to be the master regulator, appears to be important for expansion and/or maintenance of the Th17 response (25-30). In the present study, we found that mRNA expression of both TGF- β and IL-6 was much higher in gastric cancer tissues than in non-cancerous adjacent tissues. Importantly, in tumor tissues, both TGF- β and IL-6 mRNA expression levels showed a strong correlation with the RORc mRNA expression level. We also examined IL-1 β and IL-23, but we did not find a correlation between them and RORc. These results suggest that TGF- β and IL-6 present in the gastric cancer microenvironment may promote differentiation and expansion of Th17 cells. Our results are consistent with data from Manel *et al* (7) and Yang *et al* (31).

Foxp3 is the characteristic transcription factor for Treg cells. At the mRNA and protein levels, CD4⁺ T cells expressing Foxp3 in the periphery are mainly CD4⁺CD25⁺ Treg cells. Treg cells suppress immune responses by a contact-dependent manner or modulate the immune balance through the release of cytokines IL-10 and TGF- β . The present study showed that mRNA expression of Foxp3, IL-10 and TGF- β was much higher in gastric cancer tissues than in non-cancerous adjacent tissues and that the high mRNA expression levels of Foxp3 and TGF- β were associated with higher TNM stage. Our data were consistent with the results from several studies demonstrating that Treg cells are increased in blood, TDLN and cancer specimens from patients with gastric cancer (32,33). Previous studies have shown that Th17 and Treg cells are synchronistically increased following tumor development, and both populations reached maximal levels in advanced tumor (2,9,11,34,35). Our data further reconfirmed this finding. Therefore, the dynamic interaction between Th17 and Treg cells may be important in the development of gastric cancer.

In conclusion, the accumulation of Th17 and Treg cells in the gastric cancer tumor microenvironment was gradually increased according to disease progression, leading to an imbalance in Th17/Treg cells in gastric cancer patients.

TGF- β and IL-6 present in the gastric cancer microenvironment promoted the differentiation and expansion of Th17 cells, and increased Th17 cells promoted tumor progression through promotion of inflammation by secretion of IL-17. Treg cells promoted tumor progression by helping cancer cells escape from host immunosurveillance by secreting TGF- β , and a high level of TGF- β in the tumor microenvironment promoted differentiation and expansion of Treg cells. A better understanding of the nature, regulation, and function of Th17 and Treg cells in tumor immunity may aid in the development of novel and effective immunotherapy for gastric cancer.

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