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Original Article

Th17/Treg cell imbalance in patients with early esophageal squamous cell carcinoma

Da-Yu Kuai[1*], Juan Cheng[2*], Rui-Chuang Yang[2], Tong Liu[3], Hui Liu[3], Feng-Ge Liu[3], Chun-Xia Gao[1], Shen Xin[1], Jing Li[1], Sheng-Tao Zhu[4], Shu-Tian Zhang[4], Bao-Hong Xu[1]

[1]Department of Gastroenterology, Beijing Luhe Hospital, Capital Medical University, Beijing, China; [2]Beijing 302 Hospital, Beijing, China; [3]Department of Pathology, Beijing Luhe Hospital, Capital Medical University, Beijing, China; [4]Department of Gastroenterology, Beijing Friendship Hospital, Capital Medical University, Beijing Key Laboratory of Digestive Diseases and Precancerous Lesions, Beijing, China. [*]Equal contributors.

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Abstract: The role of Th17 and Treg cells in esophageal squamous cell carcinoma (ESCC) remains unknown. This study aims to investigate the imbalance between Th17 and Treg cells in patients with early ESCC and its significance. Flow cytometry was performed to determine the percentage of Th17 and Treg cells in the peripheral blood of ESCC patients, and immunohistochemistry was utilized to detect the expression of forkhead box protein 3 ($FOXP3^+$) and interleukin (IL)-17 in the tissue specimens from 30 patients with early ESCC and 30 normal human controls. The proportion of Th17 and Treg cells in peripheral blood of the ESCC patients was 1.34±0.30% and 3.03±1.13%, respectively, and in the normal controls the values were 0.52±0.18% and 1.97±0.92%, respectively. These differences were statistically significant ($P<0.05$). The Th17/Treg ratio in the ESCC patients was higher than in the normal control group (0.50±0.20 and 0.31±0.13, respectively, $P<0.05$). Compared with the peri-tumoral tissues and the tumor-free esophageal tissues, the fresh tumor tissues showed significantly higher expression of IL-17 (163.64±53.9, 98.19±25.27 and 44.10±17.86, respectively, $P<0.01$), and $FoxP3^+$ cells (115.45±31.74, 92.73±16.19 and 69.09±26.63, respectively, $P<0.05$). In addition, the ratio of the Th17/Treg cells in the fresh tumor tissues was higher than in the peri-tumoral tissues and the tumor-free esophageal tissues (1.45±0.45, 1.08±0.30 and 0.70±0.30, respectively, $P<0.05$). In conclusion, the imbalance between the Th17 and Treg cells demonstrated here might play an important role in the pathogenesis of ESCC.

Keywords: Esophageal squamous cell carcinoma, interleukin-17, forkhead box protein 3, flow cytometry, immunohistochemistry

Introduction

Esophageal cancer is the sixth most common cause of cancer-related death and the eighth most commonly diagnosed cancer worldwide with a 5-year survival rate of 10% to 15% [1-3]. The predominant histological types of esophageal cancer are squamous cell carcinoma (SCC) and esophageal adenocarcinoma (EAC), which account for more than 95% of all cases of esophageal carcinoma. ESCC is the predominant histological type of esophageal cancer in China [4]. The etiology of ESCC is multifactorial, but alcohol consumption and cigarette smoking are the leading risk factors [5, 6]. Some studies show that in the esophageal cancer high-risk population, the number of immune inflammatory cells infiltrating in the mucous membrane is correlated with the stage of epithelial proliferation, and local inflammation might contribute to the generation and development of esophageal cancer [7]. Therefore, it is important to understand the precise mechanism by which the immune system is modulated in ESCC patients.

There is growing evidence that $CD4^+$ T-helper (Th) cells play an important role in maintaining the immune responses against cancer [8, 9], and it is unclear to what extent these cells are involved in the pathogenesis of ESCC. Th17 cells are a novel subset of interleukin IL-17-producing $CD4^+$ T cells [10] that play a crucial role in inflammation and autoimmune disease

[11-13]. Th17 cells also accumulate in tumors, such as hepatocellular carcinoma (HCC) [14], gastric cancer [15], melanoma, breast and colon cancers [16]. These findings suggest that Th17 cells may contribute to the immunopathogenesis of many types of cancers.

Treg cells, which are characterized by their constitutive expression of CD25 and FoxP3$^+$ and by their function in immunological suppression [17-20], are considered a T cell subset that are relevant to immune-mediated diseases in humans. Tregs are characterized by their role in immune suppression and are essential for the maintenance of peripheral tolerance and to control the immune response by direct contact inhibition and the secretion of inflammatory cytokines, such as IL-10 and transforming growth factor-beta. Foxp3$^+$, the most specific marker of Tregs, controls the development of the Tregs.

Immunopathogenesis in tumors is complex. Recently, we found that Th17 cells play an important role in the process that causes damage during tumor disease. On the contrary, Tregs play a role in immunosuppression. Further study demonstrated that the balance of the Th17/Treg ratio is involved in the occurrence of tumors. However, little is known about the dynamic variations of both the Th17 and the Treg cells in ESCC. In addition, whether the circulating Treg population is decreased or increased in esophageal carcinoma remains controversial based on published results. To address these issues, Th17 and FoxP3$^+$ Treg cells, both in the peripheral circulation and in esophageal carcinoma tissue lesions, were evaluated during the early stage of ESCC. The ratios and functional influence between these cells were also analyzed.

Materials and methods

Study subjects

The study protocol was approved by the Ethics Committee of Beijing Luhe Hospital, the Affiliated Hospital of Capital Medical University, and all of the subjects signed an agreement to participate in the study. Peripheral blood samples were obtained from 30 patients with early ESCC and 30 age- and gender-matched healthy donors from June 2011 to June 2015. Fresh tumor tissues, peri-tumoral tissues and tumor-free esophageal tissues (at least a 2-cm distance from the tumor site) were isolated from the resected tumor specimens of the 30 patients with esophageal cancer. All of the patients' pathology was confirmed at the Department of Pathology, Beijing Luhe Hospital, the Affiliated Hospital of Capital Medical University. None of the patients received anticancer therapy before sampling. None of the patients had a significant infection, immune suppression, renal, hepatic or other medical diseases.

Flow cytometric analysis

The peripheral blood samples were collected in sodium-heparin vacutainer tubes, and the peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient centrifugation. FITC mouse anti-human CD3, PerCP CY5.5 mouse anti-human CD8, FITC mouse anti-human CD4, APC mouse anti-human CD25, APC mouse anti-human IFN-γ, PE mouse anti-human FoxP3 and PE mouse anti-human IL-17 antibodies were purchased from BD PharMingen (San Diego, CA, USA).

For Th17 detection, the cells were activated with 100 μg/ml phorbol 12-myristate 13-acetate (PMA, SIGMA, USA), 1 μg/ml ionomycin (SIGMA) and 0.1 mg/ml Protein Transport Inhibitor (Containing Monensin) (BD GolgiStopTM, USA) for 6 h at 37°C and at 5% CO_2. The cells were then washed in PBS and surface-labeled with FITC mouse anti-human CD3 and PerCP CY5.5 mouse anti-human CD8. Following the surface staining, the cells were fixed and permeabilized using the IntraPrep Permeabilization Reagent (Beckman Coulter Inc., Fullerton, CA, USA) and were then stained with APC mouse anti-human IFN-γ and PE mouse anti-human IL-17. The labeled cells were washed and analyzed with a BD FACSCanto™ II flow cytometer (Becton-Dickinson, San Diego, CA, USA) using the FACSDiva™ software (Becton-Dickinson San Jose, CA, USA).

For the intracellular staining with FoxP3$^+$, the cells were first stained with FITC mouse anti-human CD4 and APC mouse anti-human CD25 and were then permeabilized and fixed using Becton-Dickinson fix/perm (Becton-Dickinson, San Diego, CA, USA) according to the manufacturer's instructions. PE mouse anti-human FoxP3 was added after the permeabilization for 40 min. The cells were fixed in paraformaldehyde, and the flow cytometric analyses were

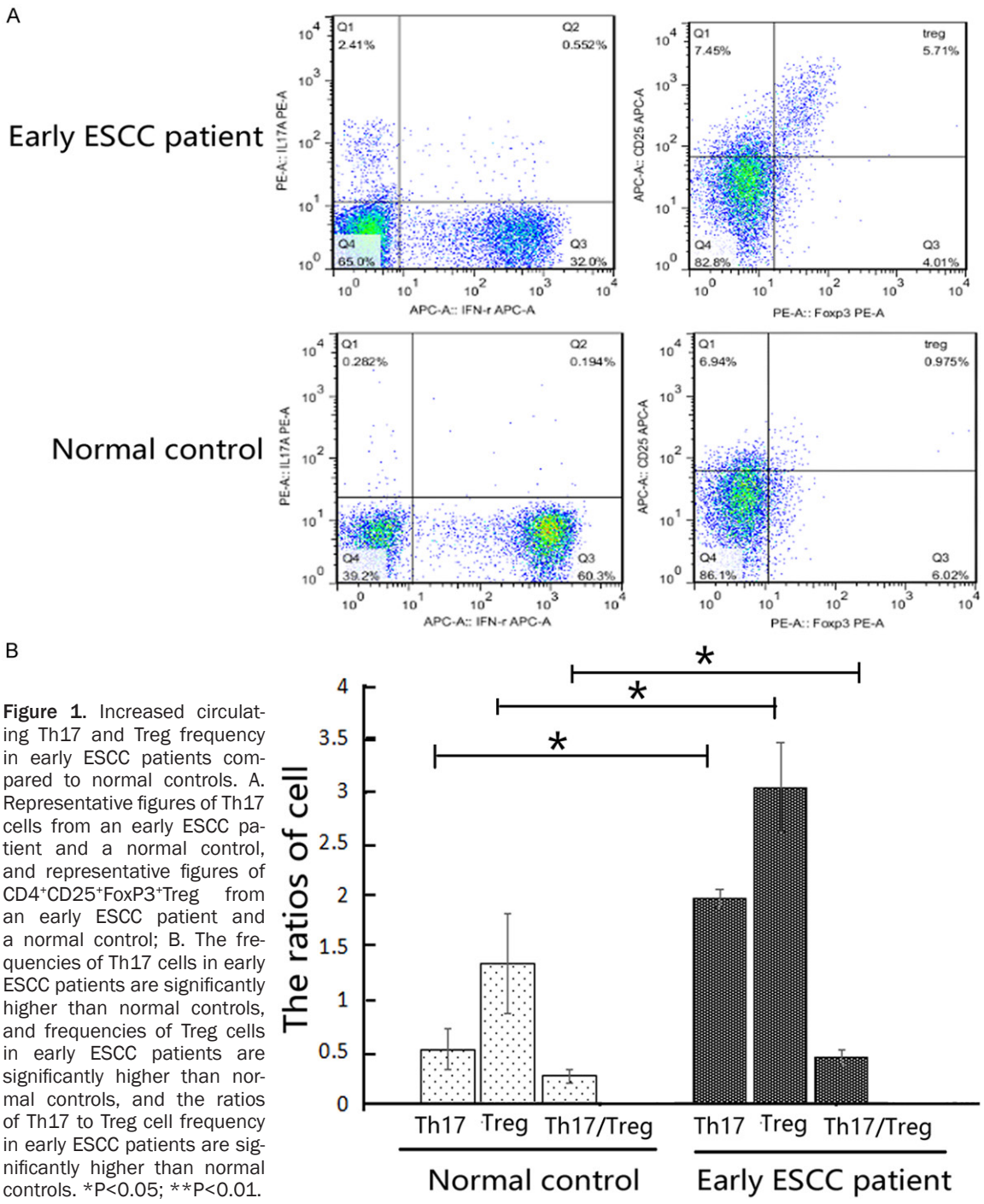

Figure 1. Increased circulating Th17 and Treg frequency in early ESCC patients compared to normal controls. A. Representative figures of Th17 cells from an early ESCC patient and a normal control, and representative figures of $CD4^+CD25^+FoxP3^+$Treg from an early ESCC patient and a normal control; B. The frequencies of Th17 cells in early ESCC patients are significantly higher than normal controls, and frequencies of Treg cells in early ESCC patients are significantly higher than normal controls, and the ratios of Th17 to Treg cell frequency in early ESCC patients are significantly higher than normal controls. *P<0.05; **P<0.01.

performed using a BD FACSCanto™ II and the FACSDiva™ software (Becton Dickinson, San Jose, CA, USA).

Immunohistochemical staining

The paraffin-embedded, formalin-fixed esophageal mucosa tissues were cut into 4-μm sections and were placed on polylysine-coated slides. Ag retrieval was achieved by pressure cooking for 10 min in EDTA (pH 8.0). Antibodies for anti-human FoxP3 (eBiosciences, San Diego, CA, USA) or goat anti-human IL-17 (R&D Systems, Minneapolis, MN, USA) and biotinylated donkey anti-mouse Ig or biotinylated rabbit anti-goat Ig (Zhongshan Goldenbridge Biotech, Beijing, China) were used for the FoxP3 and IL-17 staining, respectively. The substrate was 3-amino-9-ethyl-carbazole (AEC) (red color) followed by counterstaining with hema-

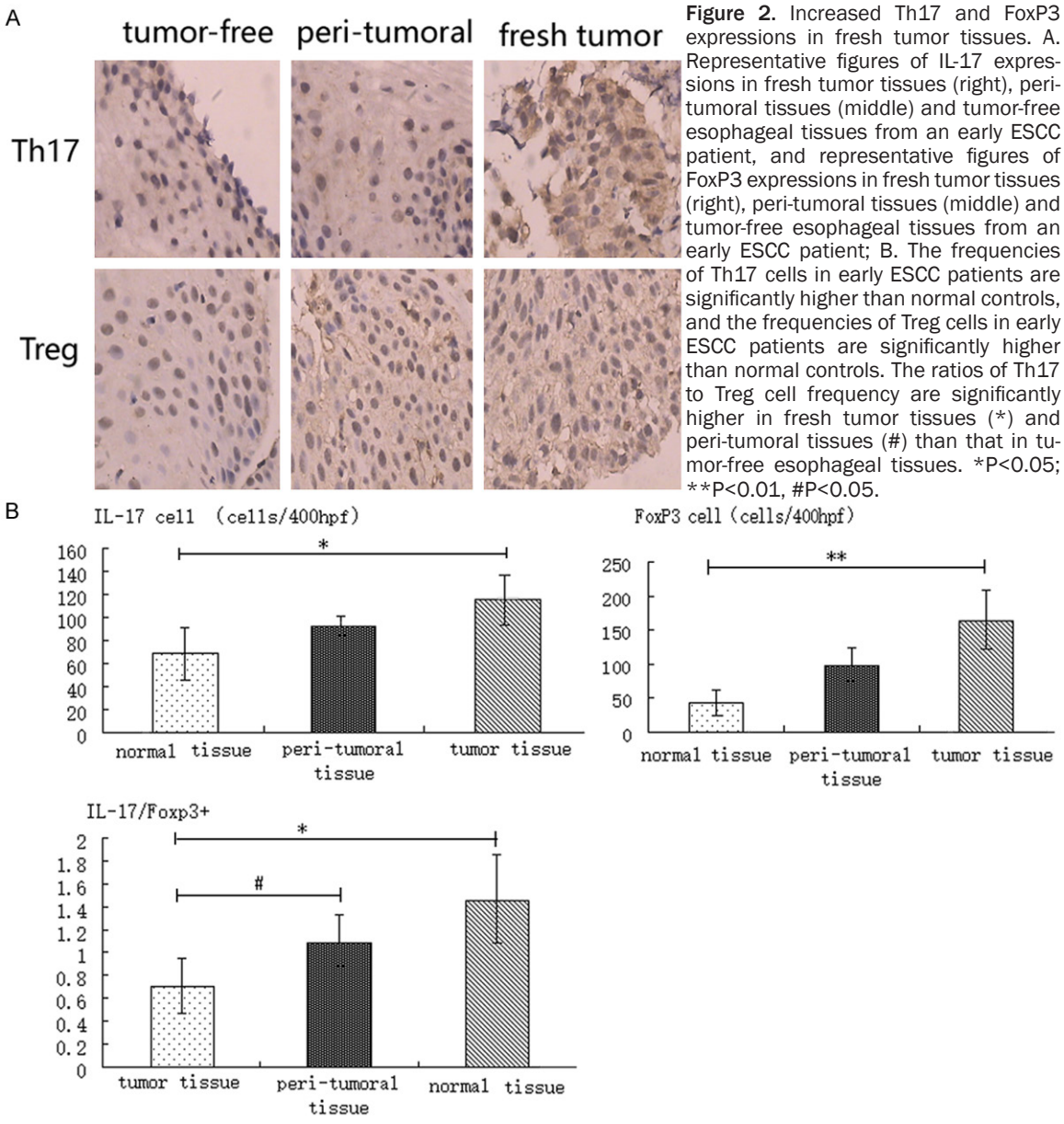

Figure 2. Increased Th17 and FoxP3 expressions in fresh tumor tissues. A. Representative figures of IL-17 expressions in fresh tumor tissues (right), peri-tumoral tissues (middle) and tumor-free esophageal tissues from an early ESCC patient, and representative figures of FoxP3 expressions in fresh tumor tissues (right), peri-tumoral tissues (middle) and tumor-free esophageal tissues from an early ESCC patient; B. The frequencies of Th17 cells in early ESCC patients are significantly higher than normal controls, and the frequencies of Treg cells in early ESCC patients are significantly higher than normal controls. The ratios of Th17 to Treg cell frequency are significantly higher in fresh tumor tissues (*) and peri-tumoral tissues (#) than that in tumor-free esophageal tissues. *P<0.05; **P<0.01, #P<0.05.

toxylin for single staining. Two independent observers who were blinded to the patients quantified the lymphocytes by analyzing 10 different high-powered fields (HPF × 400).

Statistical analysis

The results are expressed as the means ± SD in percentages. A Mann-Whitney test was performed to determine the statistical comparisons between the two groups. A Bonferroni test was used for multiple comparisons. The data analysis was performed on SPSS version 15.0 for Windows (SPSS Inc., Chicago, IL). P<0.05 was considered statistically significant.

Results

Proportion of Th17 cells and Treg cells in patients with early esophageal cancer in the PBMCs

To determine the distribution of the Th17 cells in the PBMCs of the patients with early ESCC, the proportion of Th17 cells, as a percentage of the total $CD4^+$ T cells, was evaluated by a flow cytometric analysis. The representative flow cytometric data demonstrating the proportion of Th17 cells in the gated $CD4^+$ cells from the PBMCs of patients with early ESCC and the normal controls is shown in **Figure 1A**. There was a

significantly higher frequency of circulating Th17 cells in the early ESCC patients compared to the normal controls (1.34±0.30% vs. 0.52±0.18%, P<0.01) (**Figure 1B**). These observations indicate that patients with ESCC have a predominantly elevated proportion of Th17 cells in their PBMCs.

The Treg cells were defined as a population of $CD4^+CD25^+FoxP3^+$ T cells and were expressed as a percentage of the total $CD4^+$ T cells. The percentage of Treg cells in the patients with early ESCC and in normal controls is shown in **Figure 1B**. There was a significant difference in the Treg cell percentage in the early ESCC patients compared to the normal controls (3.03±1.13% vs. 1.97±0.92%, P<0.05) (**Figure 1B**).

To further explore whether the Th17 cells or the Treg cells were preferentially increased in the early ESCC patients, we analyzed the ratio of the Th17 to Treg cell population found in the circulation. The ratio in the circulation was significantly increased in the early ESCC patients compared to the normal controls (0.50±0.20 vs. 0.31±0.13, P<0.05).

Proportion of Th17 cells and Treg cells in the patients with early ESCC in the esophageal mucosa tissue

To observe the Th17 and Treg cells in the esophageal mucosa tissue, we used immunohistochemical staining to assess the expression of IL-17 and FoxP3 in fresh tumor tissues, peri-tumoral tissues and tumor-free esophageal tissues from 30 early ESCC patients (**Figure 2**). Representative immunohistochemical staining data of the Th17 cells in the carcinoma tissues, the peri-tumoral tissues and the tumor-free esophageal tissues are shown in **Figure 2A**. As shown in **Figure 2B**, the expression of IL-17 was significantly higher in the fresh tumor tissue than in the peri-tumoral tissue and the tumor-free esophageal tissue (163.64±53.9 vs. 98.19±25.27 and 163.64±53.9 vs. 44.10±17.86, respectively, P<0.01). Significant increases were also observed in the peri-tumoral tissue compared with tumor-free esophageal tissue (98.19±25.27 vs. 44.10±17.86, P<0.01). These observations indicate that patients with early ESCC have a predominantly elevated proportion of Th17 cells in their esophageal mucosa tissues.

As shown in **Figure 2A**, there was a significant difference in the expression of Treg cells in the fresh tumor tissues compared with the peri-tumoral tissues and the tumor-free esophageal tissues (115.45±31.74 vs. 92.73±16.19 and 92.73±16.19 vs. 69.09±26.63, respectively, P<0.05) (**Figure 2B**). Significant increases in the Treg cells were also observed in the peri-tumoral tissues compared with tumor-free esophageal tissues (92.73±16.19 vs. 69.09±26.63, P<0.05).

We also analyzed the ratio of the Th17 to Treg cell population in the tissue and found that the ratio was significantly increased in the early ESCC patients compared to the normal controls (1.45±0.45, 1.08±0.30 and 0.70±0.30, respectively, P<0.05) (**Figure 2B**).

Discussion

Th17 cells were recently identified as a new type of $CD4^+$ cell that are distinct from Th1 and Th2 cells by their preferential production of interleukins (IL)-17A and F and their requirement of RORγt as a key transcription factor for their differentiation [21-23]. Although some reports demonstrate a role for Th17 cells in host defense against microbes and fungus [24-26], more and more evidence, accumulating from human and mouse studies, suggests that Th17 cells are the principal mediators during the pathogenesis of autoimmune and inflammatory disorders [27, 28]. IL-17 expression is detected in biopsies from esophageal carcinoma lesions, but is not significantly found in normal tissues, suggesting that Th17 cells are involved with the pathogenesis of ESCC.

In this study, the expression of Th17 in fresh tumor tissues and in the peripheral blood from patients with ESCC was significantly higher than the control group, and both were positively correlated. The increased expression of Th17 cells in the occurrence of early ESCC might play a proinflammatory role. In the development of esophageal cancer, Th17 cells promote tumor growth, and the specific mechanism might be that the Th17 cells secret IL-17, which promotes tumor growth by promoting the formation of new blood vessels. The number of Th17 cells within the tumor and the microvascular density is positively correlated, and with the increased microvascular density the prognosis is worse. Thus, the assumption is that esophageal can-

cer uses an inflammatory reaction to escape antitumor immunity, prompting new blood vessel formation and tissue remodeling, which protects the cancer tissue [29].

According to our results, the Treg cells in the peripheral blood and the fresh tumor tissues were significantly higher than in the normal control group, which illustrates that tumor cells might recruit and induce Treg cells at the same time to adjust and supply the tumor microenvironment with factors that play a role in the resistance of anti-tumor immunity [30].

The proportion of Th17 and Treg cells in normal controls is balanced, and this balance is broken in patients with early ESCC. The percentage of Th17 and Treg cells in the peripheral blood and their tissue expression were significantly higher in patients with early ESCC. The process of developing ESCC plays an important role in promoting the imbalance between the Th17 and Treg cells, which has recently been described by several groups and suggests that either the number or the functional imbalance in the blood and tissues may be a reason for the reduced regulatory restraints and the consequent hyper proliferation of esophageal carcinoma. Although the specific mechanism is still not very clear, the current data open a new window about the immune mechanism of ESCC and also provide new ideas for the prevention and treatment of ESCC.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Bao-Hong Xu, Department of Gastroenterology, Beijing Luhe Hospital of Capital Medical University No.82 Xinhua South Street, Tongzhou District, Beijing 101149, China. Tel: +86-10-69543901; Fax: +86-10-69531069; E-mail: bhxu22@126.com

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