

IL-17A-producing T cells and associated cytokines are involved in the progression of gastric cancer

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Abstract. Interleukin-17A-producing T cells (IL-17A⁺ T) (IL-17A⁺CD4⁺ Th17, IL-17A⁺CD8⁺ Tc17 and IL-17A⁺ γ δ T17 cells) and associated cytokines (IL-17A, IL-23 and IL-1 β) play crucial roles in inflammation-associated diseases, such as infection, autoimmunity and tumors. Th17 cells promote human gastric cancer (GC), although the source of intracellular IL-17A and the roles of Tc17 and γ δ T17 cells remain poorly understood. In this study, the frequencies of circulating Th17 and γ δ T17 cells in patients with GC were found to be significantly increased compared to those in healthy donors; however, Tc17 cells were decreased in these patients, and a negative relationship was found between the frequencies of Th17 and Tc17 cells. Moreover, the cytokine IL-17A was found to be produced mainly by Th17 cells in human peripheral blood. Similarly, serum cytokine levels and relative mRNA expression levels of IL-17A, IL-23 and IL-1 β were significantly increased in patients with GC, and the frequency of Th17 cells was closely associated with serum IL-17A concentrations in patients with GC. Additionally, Th17 cells and associated cytokines were present at significantly different levels during the progression and metastasis of GC, as were Tc17 and γ δ T17 cells. Taken together, these findings suggest that IL-17A⁺ T cells and associated cytokines might play crucial roles in human GC progression and metastasis and thus represent potential targets for treatment.

Introduction

Gastric cancer (GC) is one of the most common and fatal malignancies worldwide. According to Globocan 2012, GC is considered the fifth most common malignancy after lung, breast, colorectum and prostate malignancies (1). It is estimated that >70% of GC cases occur in developing countries such as China, Japan and Korea (1,2). Additionally, GC is associated with a high mortality case and is the third leading cause of cancer-related death; the fatality rate is ~75% of total GC cases worldwide, representing an important public health burden (1,3).

Chronic inflammation-related carcinomas are closely associated with chronic infections, chemical and physical factors, and autoimmune and inflammatory responses of uncertain etiology (4-8). The development, progression and metastasis of GC are closely associated with chronic inflammation associated with *Helicobacter pylori* infection, the host immune system and other agents (9-12). Inflammation was first linked to cancer by Rudolf Virchow in 1863 (4). Persistent inflammation can induce the formation of cancer cells, and inflammatory cells, cytokines and chemokines in the tumor microenvironment can contribute to cancer growth, invasion and metastasis and to the transformation to an immunosuppressive environment in malignant disease (7,8,13).

Recently, CD4⁺ T helper (Th17) cells, a new subtype of T cells and its associated inflammatory cytokines, such as interleukin-17A (IL-17A), IL-23 and IL-1 β , play critical roles in the development and metastasis of various tumors including GC, colorectal cancer (CRC), hepatocellular carcinoma (HCC) and ovarian cancer (14-19). IL-17A production has been found not only in Th17 cells but also in other immune cells, including mast cells, IL-17A-secreting γ δ T (γ δ T17) cells, IL-17A-secreting CD8⁺ T (Tc17) cells and NKT cells (20-24). These immune cells have also been suggested to predominately produce IL-17A in other tumor types or in the same tumor type in different species (25-28). Mast cells but not T cells or macrophages mainly secrete IL-17A in esophageal squamous cell carcinoma tissues and in GC tissue (21,25). Additionally, intracellular IL-17A is produced predominantly by Tc17 cells in human HCC tissue but is mainly produced by Th17 cells in the peripheral blood

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of human HCC patients (26,27). Although $\gamma\delta$ T17 cells are the main cellular source of IL-17A in human CRC, it has been suggested that IL-17A is mainly secreted by Th17 cells in murine colon cancer models (16,28). Similarly, mast cells are the main source of IL-17A in human GC tissue (21); however, the source of IL-17A-producing cells in peripheral blood from human GC patients remains poorly defined. Recent studies have shown that increased percentages of Th17 cells, Tc17 cells and their associated cytokines (such as IL-17A, IL-23, IL-6 and IL-22) promote GC development and progression (15,29,30). However, the roles of $\gamma\delta$ T17 cells have not been elucidated in human and mouse GC.

Here, we found that the frequencies of Th17 and $\gamma\delta$ T17 cells were significantly higher in peripheral blood from patients with GC than in peripheral blood from healthy donors (HDs); however, Tc17 cell numbers were lower in patients with GC. Moreover, Th17 cells were found to be the main source of intracellular IL-17A-secreting cells, and the second most important source was Tc17 cells; $\gamma\delta$ T17 cells represented a weak source of these cells in peripheral blood obtained from patients with GC and HDs. In addition, the expression levels of IL-17A and its associated cytokines (such as IL-23 and IL-1 β) in serum and the relative mRNA levels in human peripheral blood mononuclear cells (PBMCs) obtained from patients with GC were markedly higher than in those obtained from HDs, and these IL-17A-producing T cells and associated cytokines were significantly associated with partial clinical pathological characteristics of the patients with GC. Finally, we speculated that Th17 cells, Tc17 cells, $\gamma\delta$ T17 and IL-17A-associated cytokines may be involved in the progression and metastasis of GC in patients.

Materials and methods

Patients and specimens. The present study included 47 patients (32 males and 15 females) with GC who underwent surgical operations at the Second Affiliated Hospital, Soochow University from March to October, 2014. The patients did not receive anticancer treatment (such as chemotherapy and radiotherapy) before surgery. Patients with autoimmune diseases or viral infections were excluded from the study. The clinical characteristics of the studied 47 patients with GC and 35 HDs are summarized in Table I. Tumor stage was identified according to the International Union Against Cancer TNM Classification for GC. Tumor tissues were obtained from resected GCs and embedded in paraffin. Serum and peripheral blood specimens were collected from these patients before surgery and from HDs. In addition, written informed consent was obtained from all individuals in accordance with the Declaration of Helsinki (1964); the local Medical Ethics Committee of the Second Affiliated Hospital of Soochow University approved the study.

Cell isolation and flow cytometric analysis. Human PBMCs were isolated from the fresh peripheral blood of all individuals using Ficoll-Hypaque density gradient centrifugation and transferred to sterile tubes containing RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin (Invitrogen, Carlsbad, CA, USA). The PBMCs

Table I. Clinical features of the patients with GC and healthy donors.

Features	GC patients	Healthy donors
Gender		
Male	32	21
Female	15	12
Age (years)		
Median	59	57
Range	32-76	30-75
Histologic grade		
Well/moderate	29	
Poor	18	
Tumor stage		
T1	6	
T2	24	
T3	13	
T4	4	
Lymphoid nodal status		
N0	27	
N1	13	
N2	5	
N3	2	
Distant metastasis status		
M0	44	
M1	3	
TNM stage		
I	22	
II	6	
III	13	
IV	6	

The stages of tumors were identified according to International Union Against Cancer TNM Classification for gastric cancer.

(1×10^6 /cells) were stimulated for 5 h with 50 ng/ml phorbol 12-myristate 13-acetate (PMA), 1 μ g/ml ionomycin and 500 ng/ml monensin (eBscience, San Diego, CA, USA) in 24-well plates. Subsequently, the cells were harvested and washed twice in phosphate-buffered saline (PBS). When analyzing Th17, Tc17 and $\gamma\delta$ T17 cells, the cells were stained with phycoerythrin (PE)-conjugated anti-human CD3, fluorescein isothiocyanate (FITC)-conjugated anti-human $\gamma\delta$ TCR, allophycocyanin (APC)-conjugated anti-human CD8 and Pacific Blue-conjugated anti-human CD4 antibodies at 4°C for 30 min. After surface staining, the cells were incubated with PerCP-Cy5.5-conjugated anti-human IL-17 antibody after fixation and permeabilization according to the manufacturer's protocols. Isotype-matched antibody controls were used in all procedures. The stained cells were analyzed using a BD FACSVerse flow cytometer (FCM) and FlowJo software, version 7.6.5 (TreeStar, San Carlos, CA, USA). All antibodies were obtained from Biolegend (San Diego, CA, USA).

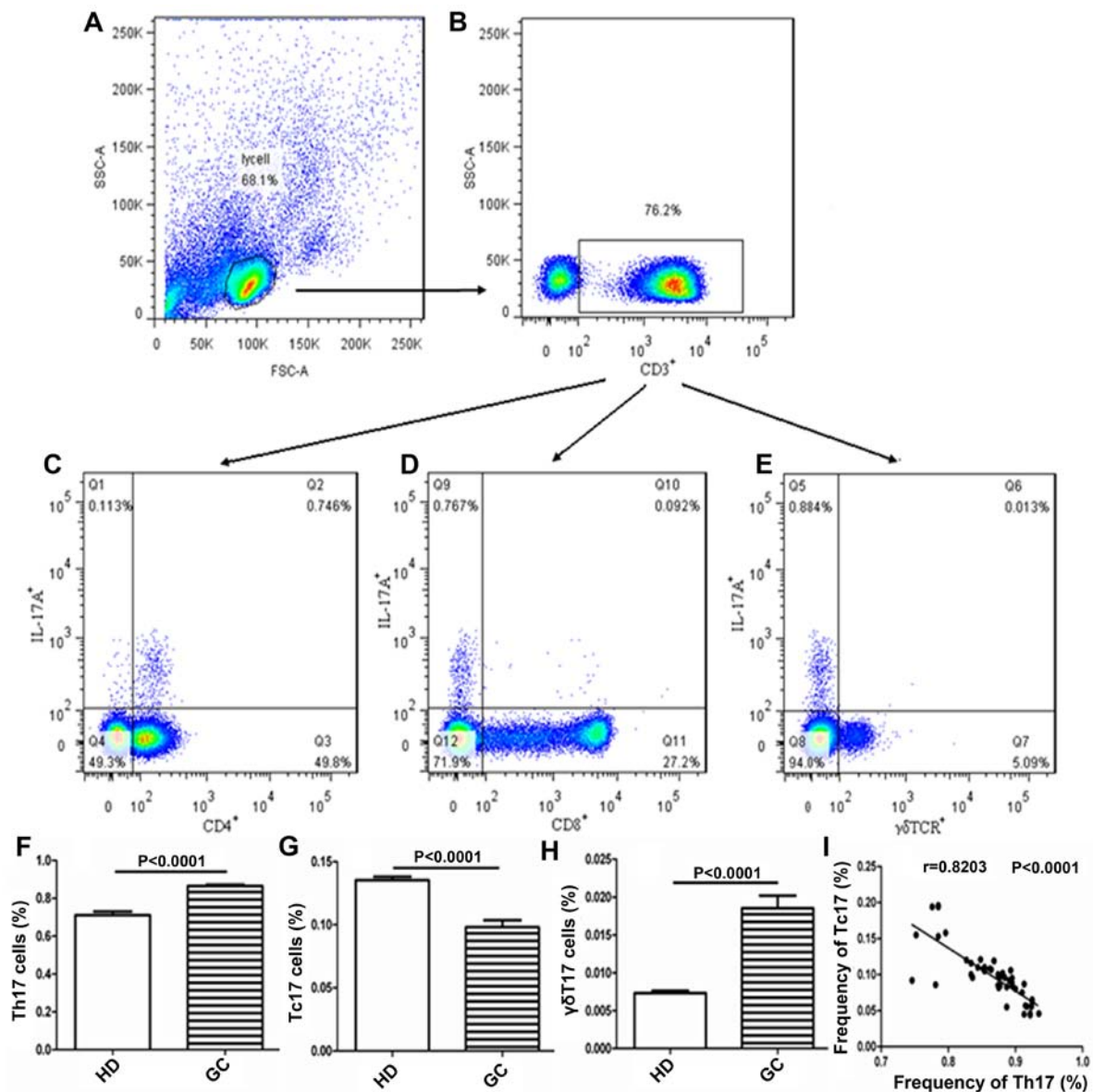


Figure 1. The frequencies of circulating Th17, Tc17 and $\gamma\delta$ T17 cells in patients with GC. Peripheral blood mononuclear cells (PBMCs) obtained from patients with GC and HDs were stained with labeled antibodies and analyzed using flow cytometry as described in Materials and methods. The cells were gated initially on living lymphocytes (A) and then on CD3⁺ T cells (B). (C) Representative plots of (C) IL-17A⁺CD4⁺ T (Th17), (D) IL-17A⁺CD8⁺ T (Tc17) and (E) IL-17A⁺ $\gamma\delta$ TCR⁺ T ($\gamma\delta$ T17) cells in total CD3⁺ T cells. (F) Th17, (G) Tc17 and (H) $\gamma\delta$ T17 cells presented as percentages of total CD3⁺ T cells from patients with GC and HDs. (I) Correlation between the frequency of Th17 cells and Tc17 cells in patients with GC. The data shown represent means \pm standard deviation. $P < 0.05$ was considered to represent statistical significance.

Analysis of serum cytokines. Serum cytokines were tested in all individuals using the Bio-Plex Pro Human Th17 Cytokine Panel kit (Bio-Rad Laboratories, Shanghai, China). The cytokines comprised IL-1 β , IL-17A and IL-23. Multiplex cytokine analysis was performed according to the manufacturer's protocols. Briefly, 50 μ l of diluted (1:1) beads that were coated with anti-human antibodies raised against the tested cytokine antigen were mixed with 150 μ l of each diluted sample (50 μ l of serum, standards or controls were diluted with 100 μ l of dilution buffer) and then incubated for 1 h at room temperature with vigorous shaking. After washing the plate three times, 25 μ l of biotinylated detection antibodies was added and incubated for 30 min. After a wash cycle, 50 μ l streptavidin (SA)-PE was added to the beads, and the mixture was incubated for 10 min. After washing the plate three times, the bead mixtures

were resuspended in 125 μ l assay buffer and measured using a Bio-Plex 200 system (Bio-Rad Laboratories, Inc., Munich, Germany). All samples were detected in triplicate. The data were analyzed using BioPlex Manager 6.1 software (Bio-Rad Laboratories, Inc.)

RNA isolation and quantitative real-time PCR (qRT-PCR). To analyze the mRNA expression of IL-1 β , IL-17A and IL-23, total RNA was extracted from human PBMCs using TRIzol (Invitrogen) after stimulating the cells for 5 h with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) and 1 μ g/ml ionomycin. cDNA was synthesized using reverse transcription reagent kits (Takara, Dalian, China) according to the manufacturer's instructions. Real-time PCR was performed in triplicate using the QuantiFastTM SYBR-Green PCR kit (Qiagen, Hilden,

Germany) and an ABI 7500 analysis system (Applied Biosystems, Foster, CA, USA). The amplification conditions were as follows: 5 min at 95°C (denaturation); then, 40 cycles of 95°C for 10 sec and 60°C for 30 sec; the fluorescence was recorded at 60°C. The primer sequences used were as follows: IL-1 β forward, 5'-CCACAGACCTTCCAGGAGA ATG-3' and IL-1 β reverse, 5'-GTGCAGTTCAGTGATCGTA CAGG-3'; IL-17A forward, 5'-CGGACTGTGATGGTCAAC CTGA-3' and IL-17A reverse, 5'-GCACTTTGCCTCCAGTCA TCACA-3'; IL-23p19 forward, 5'-GAGCCTTCTCTGCTCCCTGATA-3' and IL-23p19 reverse, 5'-GACTGAGGCTTGGAATCTG CTG-3'; β -actin forward, 5'-TGGAATCCTGTGGCATCCA TGAAAC-3' and β -actin reverse, 5'-TAAAACGCAGCTCA GTAACAGTCCG-3'. The data were analyzed using ABI 7500 software (Applied Biosystems).

Statistical analysis. One-way ANOVA analysis was performed to confirm the statistical significance of differences among the groups. Data are presented as the means \pm standard deviation (SD). Between-group comparisons were performed using Student's t-test. For non-parametric data, the Mann-Whitney U test was performed between the two studied groups. Correlations between variables were determined using Spearman's correlation coefficient. $P < 0.05$ was considered to indicate a statistically significant difference. The data were analyzed using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA).

Results

Clinical characteristics of the patients with GC. The clinical characteristics of the patients with GC are presented in Table I. Forty-seven patients, including 32 males and 15 females, were recruited in this study. The age of the patients ranged from 32 to 76 years. Twenty-nine patients presented with well/moderate histology, whereas 18 presented with severe histology. Twenty-seven patients had no lymphoid nodal metastasis, although 13 were classified as N1, 5 were classified as N2 and 2 were classified as N3. Moreover, most patients (93.6%, 44/47) had no distant metastasis, but 3 were classified as M1. Additionally, there were 63.8% (30) cases of tumor stage T1+T2 and 17 cases of stage T3+T4 according to the 7th edition of the American Joint of Committee on Cancer. Of the cases with GC, 22, 6, 13 and 6 were classified as stages I, II, III and IV, respectively according to the TNM classification for GC.

Frequency of circulating Th17, Tc17 and $\gamma\delta$ T17 cells in peripheral blood from patients with GC. The frequencies of circulating Th17, Tc17 and $\gamma\delta$ T17 cells in gated CD3⁺ T cells in the PBMCs obtained from patients with GC were detected to explore their roles in the patients with GC (Fig. 1A-I). The frequencies of circulating Th17 and $\gamma\delta$ T17 cells were significantly higher in patients with GC than in the HDs (Fig. 1F and H), although the frequency of circulating Tc17 cells was notably lower in patients with GC than in the HDs (Fig. 1G). Additionally, the frequency of Th17 cells was negatively associated with that of Tc17 cells in the patients with GC (Fig. 1I). Further analysis indicated that there was no significant difference in regards to the frequency of Th17 cells

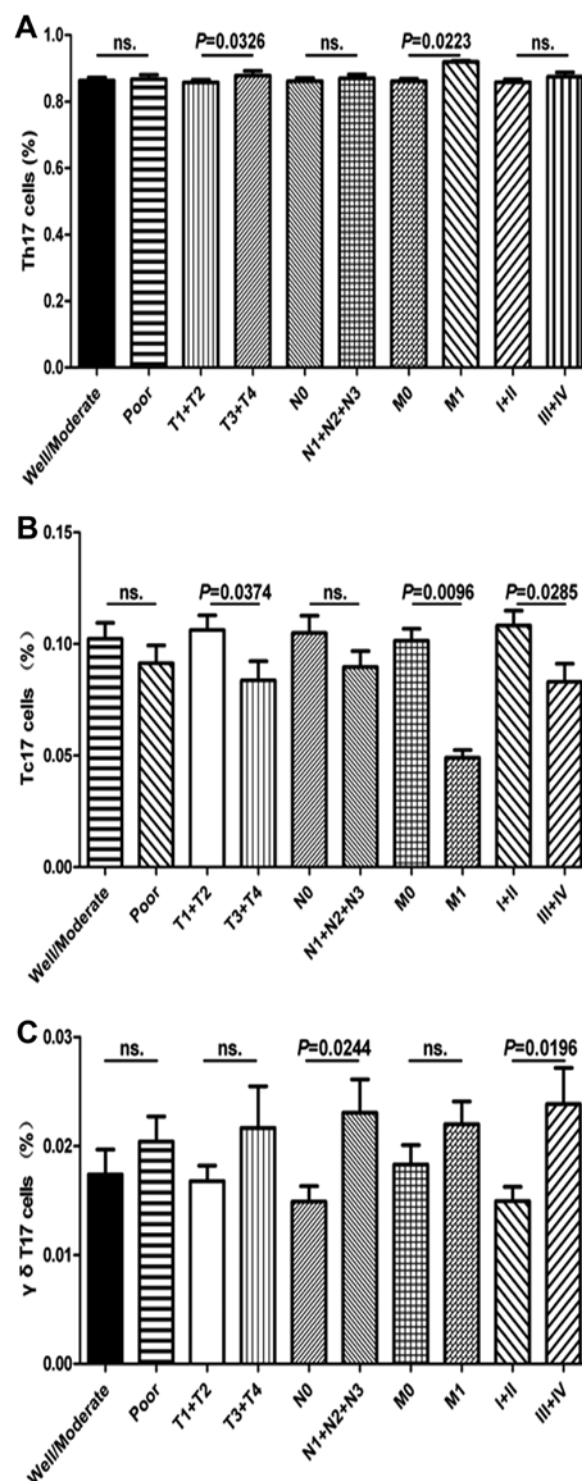


Figure 2. Analysis of the correlation of circulating cells in patients with GC with different tumor characteristics. (A) The percentage of Th17 cells was compared in patients according to tumor differentiation (good/moderate vs. poor), depth of tumor invasion (T1+T2 vs. T3+T4), lymphoid nodal metastasis (N0 vs. N1+N2+N3), distant metastasis (M0 vs. M1) and TNM stage (I+II and III+IV). (B) The percentage of Tc17 cells was compared to these same parameters. (C) The percentage of $\gamma\delta$ T17 cells was compared to these same parameters. The data shown represent means \pm standard deviation. $P < 0.05$ was considered to represent statistical significance; ns., no significant difference.

in regards to clinicopathologic features such as tumor differentiation (well/moderate vs. poor), TNM stage (I+II and III+IV) and lymphoid nodal metastasis (N0 vs. N1+N2+N3); however,

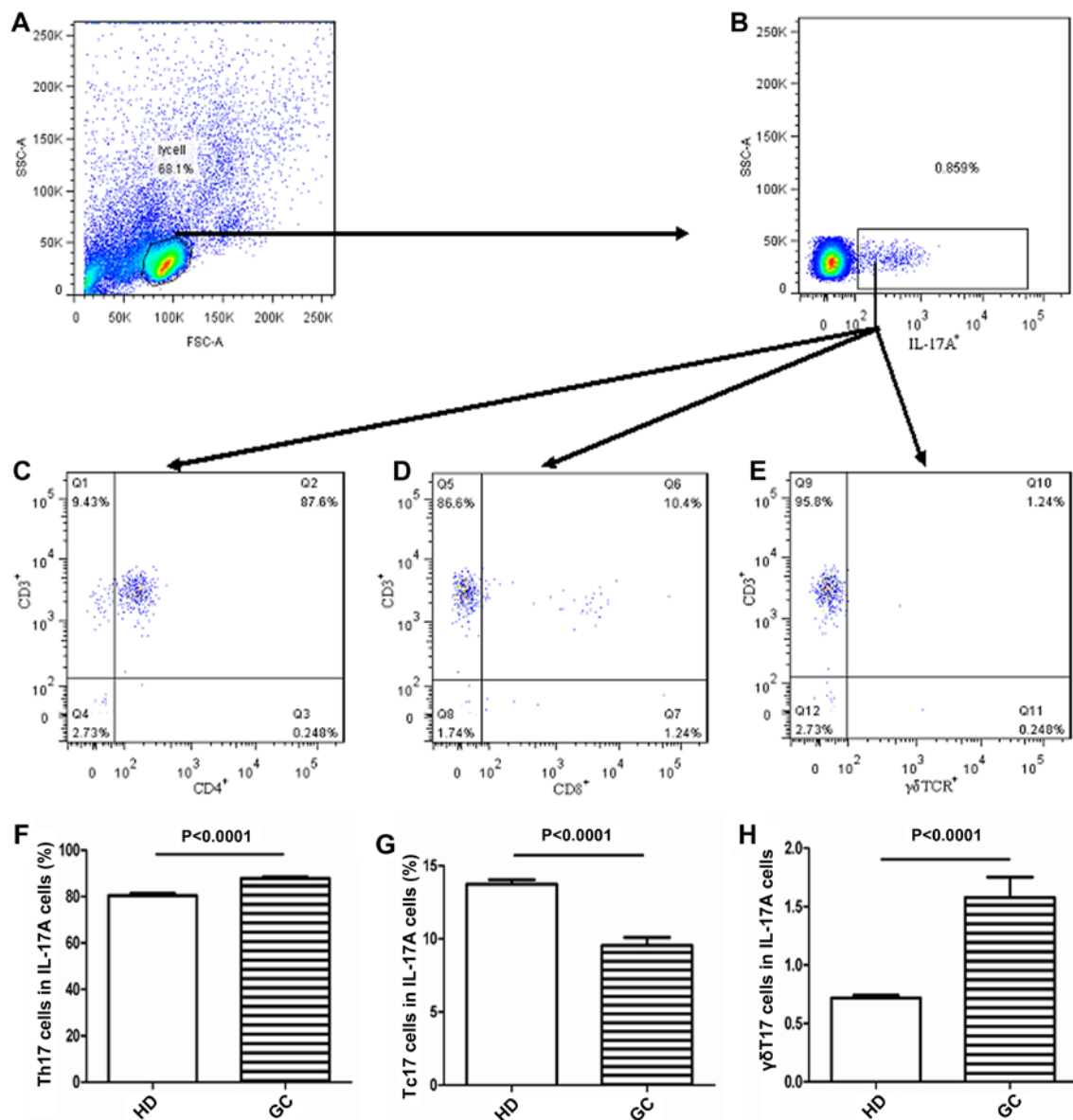


Figure 3. Percentages of Th17, Tc17 and $\gamma\delta$ T17 cells in IL-17A cells. Peripheral blood mononuclear cells (PBMCs) obtained from patients with GC and from HDs were stained with labeled antibodies and analyzed using flow cytometry as described in Materials and methods. The cells were gated initially on living lymphocytes (A) and then on intracellular IL-17A⁺ cells (B). Representative plots of (C) IL-17A⁺CD4⁺ T (Th17), (D) IL-17A⁺CD8⁺ T (Tc17), (E) IL-17A⁺ $\gamma\delta$ TCR⁺ T ($\gamma\delta$ T17) cells in the intracellular IL-17A⁺ cells. The percentages of (F) Th17, (G) Tc17 and (H) $\gamma\delta$ T17 cells in total intracellular IL-17A⁺ cells obtained from patients with GC and from HDs. The data shown represent means \pm standard deviation. P<0.05 was considered to represent statistical significance.

a significant association with tumor invasion (T1+T2 vs. T3+T4) and distant metastasis (M0 vs. M1) was observed in patients with GC (Fig. 2A). The percentage of $\gamma\delta$ T17 cells in patients with GC with lymphoid nodal metastasis (N1+N2+N3) was significantly higher than that in patients with GC with no lymphoid nodal metastasis (N0), similar to TNM stage (I+II and III+IV); however, no significant difference was found between other clinical characteristics and the percentage of $\gamma\delta$ T17 cells, respectively (Fig. 2C). The percentage of Tc17 cells in patients with III+IV stage GC was significantly lower than that in patients with I+II stage GC; similar findings were observed for some clinical features, such as distant metastasis (M0 vs. M1) and depth of tumor invasion (T1+T2 vs. T3+T4), but not for lymphoid nodal metastasis (N1+N2+N3) or tumor differentiation (good/moderate vs. poor) (Fig. 2B), respectively. These

findings indicate that the patients with GC generally had higher populations of circulating Th17 and $\gamma\delta$ T17 cells and a decreased percentage of circulating Tc17 cells in peripheral blood, which were involved in the progression of GC in the patients.

The principal source of intracellular IL-17A cells in peripheral blood. Since circulating Th17, Tc17 and $\gamma\delta$ T17 cells play important roles in patients with GC, we determined the principal source of intracellular IL-17A cytokines in peripheral blood obtained from the patients with GC by measuring the frequencies of circulating Th17, Tc17 and $\gamma\delta$ T17 cells in gated intracellular IL-17A cells obtained from patients with GC using FCM (Fig. 3A-E). The proportion of circulating Th17 cells was highest in intracellular IL-17A cells

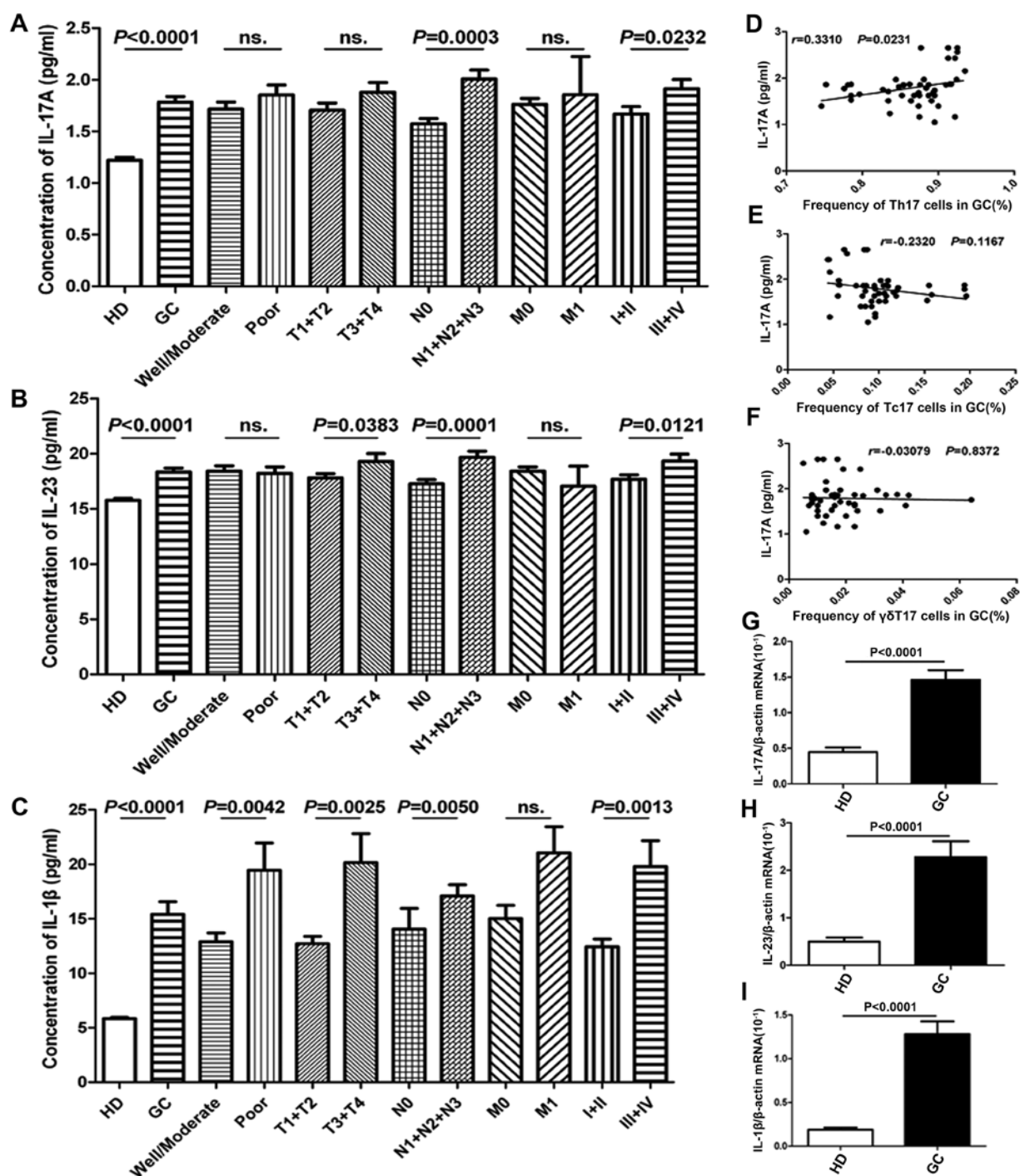


Figure 4. Expression levels of the cytokines IL-17A, IL-23 and IL-1β in serum and PBMCs. Serum (A) IL-17A (B) IL-23 and (C) IL-1β concentrations in HDs and in patients with GC according to different tumor characteristics. Relationships between IL-17A levels and the frequency of circulating (D) Th17, (E) Tc17 and (F) γδT17 cells. Relative (G) IL-17A, (H) IL-23 and (I) IL-1β mRNA expression levels in PBMCs. $P < 0.05$ was considered to represent statistical significance; ns., no significant difference.

of peripheral blood cells from patients with GC or the HDs (Fig. 3C). The second-largest population of intracellular IL-17A cells were circulating Tc17 cells (Fig. 3D) and the lowest population of circulating Tc17 cells was γδT17 cells (Fig. 3E). Moreover, the percentages of intracellular IL-17A cells represented by circulating Th17 and γδT17 cells in peripheral blood were significantly higher in patients with GC than in HDs (Fig. 3F and H). In contrast, the percentage of intracellular IL-17A cells represented by circulating Tc17

obtained from patients with GC was obviously lower than that in the HDs (Fig. 3G). These data imply that circulating Th17 cells represent the main source of intracellular IL-17A cytokine in peripheral blood and that the percentages of circulating Th17 and γδT17 cells were notably increased in patients with GC than in the HDs.

Expression of IL-17A and associated cytokines in patients with GC. Since circulating Th17, Tc17 and γδT17 cells were

found to be an important source of intracellular IL-17A cytokines in peripheral blood, we further detected the expression levels of IL-17A and its associated cytokines (IL-23 and IL-1 β) in serum and PBMCs obtained from patients with GC and from HDs (Fig. 4A-G). The concentrations of the cytokines IL-17A, IL-23 and IL-1 β were significantly higher in sera obtained from patients with GC than in sera obtained from the HDs (Fig. 4A-C). Moreover, significant associations were found between IL-17A levels and various clinical pathological characteristics including lymphoid nodal metastasis (N0 vs. N1+N2+N3) and TNM stage (I+II and III+IV), respectively. However, no significant association between IL-17A concentration and tumor histopathology (well/moderate vs. poor), depth of tumor invasion (T1+T2 vs. T3+T4) or distant metastasis (M0 vs. M1) was observed in the patients with GC (Fig. 4A). Similar findings regarding IL-23 and IL-1 β concentrations were observed in patients with GC (Fig. 4B and C). Additionally, serum IL-23 and IL-1 β levels were markedly different between patients with GC at stages T1+T2 and T3+T4 and IL-1 β levels were also significantly different between good/moderate and poor tumor histopathology (Fig. 4B and C). Moreover, serum IL-17A concentrations were markedly associated with Th17 cell frequency in patients with GC but not with Tc17 and $\gamma\delta$ T17 cell frequencies (Fig. 4D-F). In addition, the mRNA expression levels of IL-1 β , IL-17A and IL-23 were notably higher in PBMCs in patients with GC than in those of the HDs (Fig. 4G-I).

Discussion

In the present study, we found that the frequencies of circulating Th17 and $\gamma\delta$ T17 cells were significantly higher in peripheral blood obtained from the patients with GC than in the HDs; however, the percentage of Tc17 cells was lower. Moreover, the expression levels of the associated cytokines, such as IL-17A, IL-23 and IL-1 β in serum and PBMCs obtained from patients with GC were also significantly higher than those of the HDs, and Th17, Tc17 and $\gamma\delta$ T17 cells and cytokines were partly associated with clinicopathologic characteristics such as cancer invasion, metastasis and TNM stage. Furthermore, the main source of intracellular IL-17A cytokine was Th17 cells, the second-most important source was Tc17 cells and $\gamma\delta$ T17 cells also acted as a source of intracellular IL-17A cytokine in peripheral blood obtained from patients with GC. Taken together, these findings suggest that Th17, Tc17 and $\gamma\delta$ T17 cells might play crucial roles in human GC development and metastasis.

Th17 cells play pivotal roles in inflammation and in autoimmune diseases of humans and mice, thus exacerbating the severity of diseases; they also act in host defense against pathogens (31-33). Recently, accumulating evidence has suggested that Th17 cells exhibit complex and controversial roles in the development, progression and metastasis of tumors (30,34,35). An increased prevalence of Th17 cells in peripheral blood, tumor tissue or tumor-draining lymph nodes contributes to the development, progression and/or metastasis of human cancers, such as gastric carcinoma, CRC, HCC and pancreatic cancer (16,26,30). In contrast, a high frequency of tumor-infiltrating Th17 cells is associated with improved survival in ovarian and lung carcinoma patients and with

slower progression in patients with prostate cancer (34-36). Similarly, increased numbers of Th17 cells in the tumor environment improve survival in mouse models of pancreatic and melanoma cancer (37,38). Although the frequency of Th17 cells was obviously higher in peripheral blood obtained from patients with GC, it was not directly confirmed that these cells were the main source of IL-17 in human peripheral blood.

Our results demonstrated that the frequency of circulating Th17 cells was significantly higher in PBMCs obtained from patients with GC than in that obtained from HDs, consistent with previous studies (15,21,30). Moreover, it was clear that intracellular IL-17A cytokines were mainly produced by Th17 cells obtained from the PBMCs of patients with GC and HDs. Additionally, the high percentage of circulating Th17 cells was individually associated with depth of tumor invasion (T1+T2 vs. T3+T4) and distant metastasis (M0 vs. M1) but not with other factors such as TNM stage (I+II and III+IV) and lymphoid nodal metastasis (N0 vs. N1+N2+N3). These findings suggest that Th17 cells partly participate in the progression and metastasis of human GC, a finding that is partially consistent with previous reports (15,30). The discrepancies found might be due to the numbers of recruited patients, age, lifestyle and other causative agents, such as infections, chemical and physical factors and other factors of uncertain etiology.

Similar to Th17 cells, Tc17 cells that are defined by a signature of IL-17A-secreting CD8⁺ T cells play a critical role in various diseases, such as those related to infection and autoimmunity (39-42). For example, in rhesus macaques with pathogenic simian immunodeficiency virus (SIV) infection, Tc17 cells regulate the disease progression and contribute to the disease outcome (39). Additionally, Tc17 cells can protect mice against lethal influenza infection, which is accompanied by an early enhanced influx of neutrophils into the lung (40). Conversely, Tc17 cells, but not Th17 cells, notably caused severe autoimmune colitis in a mouse model (41). In an animal model of multiple sclerosis (MS), the numbers of IL-17⁺CD8⁺ T (Tc17) cells were found to be significantly higher in active regions of MS lesions than in inactive areas and controls, suggesting that Tc17 cells are associated with active disease in MS (42). However, the clinical prevalence and role of Tc17 cells remain elusive in human and mouse tumors (43).

Recent studies indicate that Tc17 cells have important roles in the development and metastasis of tumors (44-48). Elevated proportions of Tc17 cells in patients with cervical cancers are associated with cancer progression that is accompanied by increased infiltrations of Th17 cells as well as enhanced tumor vasculogenesis and metastasis (44). Similar results were also observed in patients with endometrial carcinoma (45). In contrast, adoptive transfer of Tc17 cells was found to mediate effective antitumor immunity against an established murine melanoma model, and this depended on the effects of IFN- γ in the tumor environment (46,47).

A high proportion of Th17 and a low proportion of Tc17 cells are observed in peripheral blood obtained from patients with thyroid tumors, and these proportions are negatively correlated with tumor size, suggesting that Tc17 cells might have an antitumor role in thyroid tumors and that its effective function may not be involved with IL-17

production (48). Recently, Tc17 cell numbers were found to be elevated in peripheral CD8⁺ T cells and in tumor tissues obtained from patients with GC (29). However, increased Tc17 cell frequency was not directly shown in PBMCs obtained from patients with GC, and it was unclear whether Tc17 cells were the main source of intracellular IL-17A in patients with GC and in HDs.

Here, a significantly decreased frequency of Tc17 cells was observed in patients with GC and was notably associated with depth of tumor invasion and TNM stage but not with other factors, such as lymphoid nodal metastasis and tumor differentiation. Moreover, Tc17 cells were the second most important source of intracellular IL-17A cytokine in the patients with GC and in HDs. Interestingly, a negative association was found between the frequency of Th17 and Tc17 cells in patients with GC, indicating that increased percentage of Th17 cells might negatively regulate Tc17 cells in patients with GC and that Tc17 cells might exert antitumor activity in patients with GC; however, this notion requires further investigation by studying the role of Tc17 cells in human GC. Taken together, these findings indicate that a low percentage of Tc17 cells might affect the development and progression of GC. The different age, population and causative factors in our patients with GC, together with differences in the study aim, might account for the discrepancies between our results and those described in a previous study (29).

$\gamma\delta$ T17 cells, as innate cells that secrete the IL-17 cytokine, are involved in the pathogenesis of various inflammation-associated diseases in humans and mice (49,50). $\gamma\delta$ T17 cells play an effective and pivotal role in psoriasisiform plaque formation in mice and are the primary source of IL-17A cytokine but not Th17 cells (51). Additionally, a high frequency of $\gamma\delta$ T17 cells is found in the brains of mice with experimental autoimmune encephalomyelitis (EAE), and the activation of $\gamma\delta$ T17 cells can promote the expansion of Th17 cells and increase susceptibility to EAE (50). Elevated $\gamma\delta$ T17 cells exacerbate arthritis and are the main source of IL-17 cytokine in mice with collagen-induced arthritis (CIA) (52). However, the role of $\gamma\delta$ T17 cells is poorly understood in tumors.

Tumor-infiltrating IL-17 promotes the progression of skin carcinoma in mice by inducing angiogenesis and is mainly produced by $\gamma\delta$ T17 cells in T cell tumor-infiltrating lymphocytes (TILs) but not in Th17 or Tc17 cells (53). Additionally, the frequencies of Th17, Tc17 and $\gamma\delta$ T17 cells are all clearly elevated in human PBMCs and in the tumor tissues of patients with CRC (16). Moreover, $\gamma\delta$ T17 cells are the main producers of IL-17A and promote the progression of human CRC (16). Dissimilarly, Th17 cells, not $\gamma\delta$ T17 cells, are considered the main source of IL-17 and were found to improve the progression of a murine colon cancer model (28). Although the frequency of Th17 and/or Tc17 cells was obviously elevated in patients with GC (15,16,30), it has not been clearly proven that $\gamma\delta$ T17 cells are the main source of IL-17, and the role of $\gamma\delta$ T17 cells has not yet been elucidated in human GC.

Our results demonstrated that a significantly increased frequency of $\gamma\delta$ T17 cells was observed in patients with GC, and this increase was notably associated with lymphoid nodal metastasis and TNM stage but not with other factors, such as depth of tumor invasion, distant metastasis and tumor differentiation. In addition, $\gamma\delta$ T17 cells were not an abundant source

of intracellular IL-17A cytokine; rather, they were a poorer source than Th17 and Tc17 cells in patients with GC and in HDs. Taken together, these findings indicate that an increased percentage of $\gamma\delta$ T17 cells might contribute to the development and progression of GC. Moreover, human GC represents a possible cause for the low production of intracellular IL-17A from $\gamma\delta$ T17 cells, implying that various tumors might affect the phenotype and distribution of IL-17A-producing T cells differently and act as the main source of intracellular IL-17A cytokine. We will explore the role of $\gamma\delta$ T17 cells in human GC in future studies.

IL-23 and/or IL-1 β cytokines, which are secreted via innate immune cells, such as dendritic cells and macrophages, can contribute to the proliferation and differentiation of Th17, Tc17 or $\gamma\delta$ T17 cells, which promote the production of the IL-17 cytokine (54-56). Increased IL-17, IL-23 and/or IL-1 β cytokines, which are accompanied by elevated percentages of Th17, Tc17 or $\gamma\delta$ T17 cells, play important roles in inflammation-associated diseases, including those related to infection, autoimmunity and tumors (14,49,50,54). Recently, serum concentrations and mRNA expression levels of IL-17A, IL-23 and/or IL-1 β , which are associated with Th17 and Tc17 cells, have been found to be significantly increased in patients with GC compared to those in healthy individuals and are closely associated with the development of gastric carcinoma (15,21,29,30). IL-23 and IL-1 β cytokines, which are produced by tumor-activated monocytes, promote the differentiation of Tc17 cells that contribute to cancer progression in patients with GC (29).

Our results showed that serum IL-17, IL-23 and IL-1 β levels were obviously increased in patients with GC, and this increase was associated with stages of GC development, such as lymphoid nodal metastasis and the TNM stage and was partly associated with depth of tumor invasion and distant metastasis; in addition, Th17 cell percentage was positively associated with serum IL-17A concentrations in patients with GC. Additionally, the relative mRNA expression of these cytokines was significantly higher in patients with GC than in HDs, consistent with previous studies (15,21,30). These findings indicate that these increases in the inflammatory cytokines IL-17, IL-23 and IL-1 β contribute to the progression and metastasis of human GC and to the proliferation of inflammatory cells, such as Th17 cells. These inflammatory cytokines and associated inflammation-related cells play important roles in the development, progression and metastasis of human GC.

In summary, our results revealed that the frequencies of circulating Th17 and $\gamma\delta$ T17 cells were significantly higher in patients with GC than in HDs; however, the frequency of Tc17 cells, which were involved in the progression and metastasis of patients with GC, was decreased. Moreover, the intracellular IL-17A cytokine was predominantly produced by Th17 cells in human peripheral blood. Additionally, the expression levels of Th17 cell-associated cytokines, such as IL-17A, IL-23 and IL-1 β , were significantly elevated in the serum and PBMCs of patients with GC and played important roles in the progression and metastasis in the patients with GC. In future studies, a large number of patients with GC will be studied to determine the roles of Th17, Tc17 and $\gamma\delta$ T17 cells in the pathogenesis of GC.

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