Effects of modified FOLFOX-6 chemotherapy on cellular immune function in patients with gastric cancer

LIANG WANG[1], DONGER ZHOU[1], HAITAO REN[2] and YAN CHEN[3]

Departments of [1]Hepatobiliary-Pancreatic Surgery and [2]Burns and Wound Center, The Second Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, Zhejiang 310009; [3]Emergency Department, The Second Affiliated Hospital of Dalian Medical University, Dalian, Liaoning 116023, P.R. China

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Abstract. Tumor immunosuppression serves an important role in the occurrence and development of gastric cancer. However, the effect of chemotherapy on the immune function of patients remains unclear. The present study aimed to investigate changes in cellular immune function and regulatory T cells (T_{regs}) in patients with gastric cancer prior to and following chemotherapy. In the peripheral blood of patients with gastric cancer, the percentage of $CD4^+$ T cells was substantially decreased compared with that of healthy controls (11.39±5.91 vs. 22.34±3.37%, respectively; $P<0.05$). High frequencies of $CD8^+$ T cells and T_{regs} were also observed in the peripheral blood of patients. Although the number of T cells decreased following chemotherapy (the proportions of $CD4^+$ and $CD8^+$ cells were 8.99±7.31 and 16.00±4.51%, respectively), the ratio of $CD4^+/CD8^+$ T cells increased (0.31±0.17 vs. 0.56±0.22; $P<0.05$). Furthermore, the level of C-C motif chemokine ligand 20 (CCL20) was increased in patients prior to chemotherapy compared with healthy controls. As the sole receptor for CCL20, a high level of expression of C-C motif chemokine receptor 6 on circulating T_{regs} was also identified in the patients, which decreased following chemotherapy. These results suggest that chemotherapy may efficiently promote cellular immune function and inhibit immunosuppression in patients with gastric cancer.

Correspondence to: Dr Haitao Ren, Department of Burns and Wound Center, The Second Affiliated Hospital, School of Medicine, Zhejiang University, 88 Jiefang Street, Hangzhou, Zhejiang 310009, P.R. China
E-mail: rht@zju.edu.cn

Dr Yan Chen, Emergency Department, The Second Affiliated Hospital of Dalian Medical University, 467 Zhongshan Road, Dalian, Liaoning 116023, P.R. China
E-mail: chenymeta@163.com

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Introduction

Gastric cancer is the second leading cause of cancer-associated mortality in China (1). Due to a lack of screening protocols, the majority of patients often present with locally advanced disease or even metastasis at initial diagnosis. As surgery alone is not sufficient to guarantee curative treatment in patients with advanced disease, adjuvant therapies must be considered in order to increase the possibility of cure in surgically treated patients with a high risk of recurrence (2,3). While chemotherapy has been widely used in the clinical treatment of gastric cancer, its effects on the immune status of patients remain unknown.

Tumor immune tolerance serves an important role in contributing to the occurrence and development of gastric cancer (4). Previous studies have indicated that the outcome of an immune response towards a tumor is primarily determined by the type of immune response elicited (5,6). Regulatory T cells (T_{regs}) have been demonstrated to induce tumor immunosuppression and therefore have a prominent role in the development of cancer (7-9). Previous studies have demonstrated that the level of T_{regs}, which are usually evaluated by immunohistochemical quantification of Forkhead box protein P3 (FoxP3)-positive T cells, are associated with a poor outcome (10-13).

The present study explored the effect of folinic acid (FA)/fluorouracil (5-FU)/oxaliplatin (FOLFOX-6) chemotherapy on T_{regs} and the immune function of patients with gastric cancer prior to and following chemotherapy. It was indicated that chemotherapy may inhibit the secretion of C-C motif chemokine ligand 20 (CCL20) to prevent the migration of T_{regs}, thereby assisting in enhancing the antitumor immune response.

Materials and methods

Patients and specimens. The clinical records of 38 patients with gastric cancer who underwent curative surgical intervention of primary tumors between June 2011 and September 2014 at the Second Affiliated Hospital, Zhejiang University School of Medicine (Hangzhou, China) were collected. Written informed consent was obtained from the patients and the study was approved by the Ethics Committee of the Second Affiliated Hospital. Tumor stages were classified according to the 7th edition of the American Joint Committee on Cancer Tumor Node Metastasis Classification (14). Patients treated

with neoadjuvant chemotherapy were excluded. Blood was obtained from patients before and after chemotherapy and healthy control.

Chemotherapy. Venous blood (3 ml) was obtained from patients with gastric cancer (n=38) and from healthy individuals (n=31), using an EDTA K2 anticoagulant mining vessel (BD Biosciences, Franklin Lakes, NJ, USA). Blood was obtained from the gastric cancer patients 1 day prior to chemotherapy and 1 month following chemotherapy. Chemotherapy consisted of 65 mg/m^2 intravenously (IV) oxaliplatin on day 1, 200 mg/m^2 FA IV as a 2 h infusion, followed by 400 mg/m^2 bolus 5-FU IV and a 22 h infusion of 5,600 mg/m^2 5-FU IV on days 1 and 2, every 2 weeks (15). All patients who were included in the present study completed at least three cycles of chemotherapy.

Flow cytometry. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density-gradient (GE Healthcare, cat. no. 17-1440-03) centrifugation (400 x g for 30 min at 18˚C). Antibodies specific for mouse FITC-conjugated anti-cluster of differentiation (CD)4 (clone RM4-5, cat. no. 317407), mouse PE-conjugated anti-CD25 (clone BC96, cat. no. 302605), mouse APC-conjugated anti-interleukin receptor 7 (CD127; clone A019D5, cat. no. 351315), mouse APC/CY7-conjugated anti-CC-motif chemokine receptor 6 (CCR6; clone G034E3, cat. no. 353431) and mouse PE/CY7-conjugated anti-IFN-γ (clone B27, cat. no. 506517) were purchased from BioLegend, Inc., (San Diego, CA, USA). A total of 5 μl antibody was added per million cells in 100 μl staining Buffer (BioLegend, Inc.; cat. no. 420201). IFN-γ was detected by the Intracellular Cytokine Staining™ kit from BD Pharmingen (BD Biosciences), according to the manufacturer's protocol, and was quantified by flow cytometry.

PBMCs were blocked with Fc Receptor Blocking Solution at room temperature for 5 min (BioLegend, Inc.; cat. no. 422301). After 10 min, red cells were removed by lysis buffer (BioLegend, Inc.; cat. no. 420301). Then, cells were incubated with antibody at 4˚C for 30 min, washed once with PBS. The cells were resuspended in 0.5 ml PBS. Flow cytometric analysis was performed on a BD FACSCanto-II instrument (BD Biosciences). The data was analyzed by FlowJo V10 (FlowJo LLC, Ashland, OR, USA).

ELISA. CCL20 was detected by a CCL20 ELISA kit (cat. no. DM3A00; R&D Systems, Inc., Minneapolis, MN, USA), following the manufacturer's protocol.

Statistical analysis. The data were analyzed using the Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA). Continuous variables are presented as the mean ± standard deviation, and compared using the unpaired Student's t-test between two groups or one-way analysis of variance between multiple groups followed by Tukey's post-hoc test. Dichotomous variables were compared using a χ^2 test or Fisher's exact test. P<0.05 was considered to indicate a statistically significant difference.

Results

Distribution of T cell subsets in the peripheral blood of patients with gastric cancer. A total of 38 patients were enrolled in the present study, including 23 males and 16 females. The age of the patients ranged from 43 to 78 years, with a median age of 64 years. A total of 31 patients had grade 0-1 ECOG performance status; the other 7 patients had grade 2 ECOG performance status (16) (Table I). The proportions of CD4$^+$ and CD8$^+$ T cells in the peripheral blood of patients with gastric cancer and normal control individuals were compared. The percentage of CD4$^+$ T cells was significantly decreased compared with that of the normal controls (22.34±3.37 vs. 11.39±5.91%; P=0.03). However, the percentage of CD8$^+$ T cells was increased in the patients with gastric cancer compared with the controls (36.81±5.33 vs. 29.84±2.01%, respectively; P=0.01; Fig. 1; Table II). In addition, the ratio of CD4$^+$ T cells to CD8$^+$ T cells was significantly decreased in the patient group compared with the healthy control group (P=0.005; Table II). These results indicated that the patients with gastric cancer exhibited impaired cellular immune function compared with the

Table I. Clinical data of the 38 patients with gastric cancer.

Characteristics	Value
Age, years; median (range)	64 (43-78)
Sex, n	
Male	23
Female	16
AJCC stage, n	
0	1
I	7
II	11
III	19
IV	0
Performance status, n	
ECOG 0-1	31
ECOG-2	7
Histopathological type, n	
Intestinal	24
Diffuse	12
Unknown	2

UICC, Union for International Cancer Control; ECOG, Eastern Cooperative Oncology Group.

Table II. T cell subsets in PBMCs from patients and controls.

Group	Patients (n=38)	Healthy controls (n=31)	P-value
CD4$^+$/PBMC (%)	11.39±5.91	22.34±3.37	0.032[a]
CD8$^+$/PBMC (%)	36.81±5.33	29.84±2.01	0.010[a]
CD4$^+$/CD8$^+$	0.31±0.17	0.74±0.13	0.005[a]

[a]Statistically significant (P<0.05). PBMCs, peripheral blood mononuclear cells; CD, cluster of differentiation.

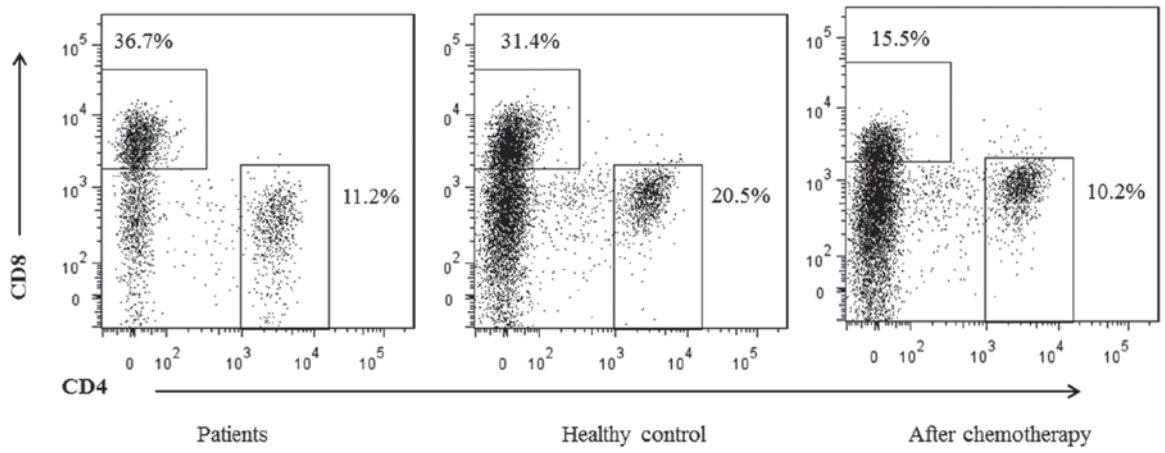

Figure 1. Proportion of $CD4^+$ and $CD8^+$ T cells in the peripheral blood from patients with gastric cancer (prior to and following chemotherapy), and from the control group. CD, cluster of differentiation.

Table III. T cell subsets in PBMCs between patients prior to and following chemotherapy.

Group	Prior to chemotherapy	Following chemotherapy	P-value
$CD4^+$/PBMC, %	11.39±5.91	8.99±7.31	0.021[a]
$CD8^+$/PBMC, %	36.81±5.33	16.00±4.51	0.001[a]
$CD4^+/CD8^+$ ratio	0.31±0.17	0.56±0.22	0.001[a]

[a]Statistically significant (P<0.05). PBMCs, peripheral blood mononuclear cells; CD, cluster of differentiation.

Table IV. Distribution of $CD4^+$ T cell subsets in peripheral blood mononuclear cells between patients and controls.

Group	Patients (n=38)	Healthy controls (n=31)	P-value
IFN-$\gamma^+/CD4^+$ (%)	13.15±3.99	37.7±4.41	0.033[a]
$T_{reg}/CD4^+$ (%)	18.33±2.51	1.5±0.31	0.002[a]

[a]Statistically significant (P<0.05). IFN-γ, interferon-γ; CD, cluster of differentiation; T_{reg}, T regulatory cells.

healthy controls. The proportions of $CD4^+$ T cells and $CD8^+$ T cells in the PBMCs of patients with gastric cancer prior to and following chemotherapy were additionally assessed by flow cytometric analysis (Fig. 1). Prior to chemotherapy, the $CD4^+$ and $CD8^+$ T cells accounted for 11.39±5.91% and 36.81±5.33%, respectively, in the patient group. Although the number of T cells decreased following chemotherapy (the proportions of $CD4^+$ and $CD8^+$ cells were 8.99±7.31% and 16.00±4.51%, respectively), the ratio of $CD4^+/CD8^+$ T cells was increased compared with the ratio prior to chemotherapy [0.56±0.22 vs. 0.31±0.17, respectively (P<0.05); Table III].

Table V. Distribution of $CD4^+$ T cell subsets in peripheral blood mononuclear cells between patients prior to and following chemotherapy.

Group	Prior to chemotherapy	Following chemotherapy	P-value
IFN-$\gamma^+/CD4^+$ (%)	13.15±3.99	20.1±5.79	0.001[a]
$T_{reg}/CD4^+$ (%)	18.33±2.51	5.5±4.11	0.001[a]

[a]Statistically significant (P<0.05). IFN-γ, interferon-γ; CD, cluster of differentiation; T_{reg}, T regulatory cells.

Effect of chemotherapy on T cell subsets. To evaluate the effect of chemotherapy on the function of the T cells, the production of interferon (IFN)-γ by $CD4^+$ T cells in patients was investigated. It was identified that the percentage of IFN-γ^+ T cells from the $CD4^+$ T cells was decreased in the patient cohort compared with healthy controls (Table IV), but was increased following chemotherapy compared with prior to chemotherapy (Fig. 2; Table V).

Furthermore, whether the chemotherapy affected immunosuppressive T_{regs} cells was examined. Liu *et al* (17) suggested that CD127 expression is inversely correlated with FoxP3 in T_{regs} cells; thus, CD127 was examined instead of FoxP3 in the present study (Fig. 3). It was identified that the proportion of T_{regs} was significantly increased in the patients vs. healthy controls (Table IV), but decreased following chemotherapy compared with prior to chemotherapy (Table V). These results suggest that chemotherapy may promote antitumor immunity and partially restore cellular immune function in patients with gastric cancer.

CCL20 is required for the recruitment of CCR6$^+$ T_{regs} in patients with gastric cancer. It has been established that the CCL20/CCR6 signal mediates the migration of T_{regs} to the tumor microenvironment in human liver cancer (11,18). To identify whether this occurred in gastric cancer, the concentration of CCL20 in 30 patients and 30 healthy controls from the original cohort was detected. The results indicated that the level of CCL20 was significantly increased prior to

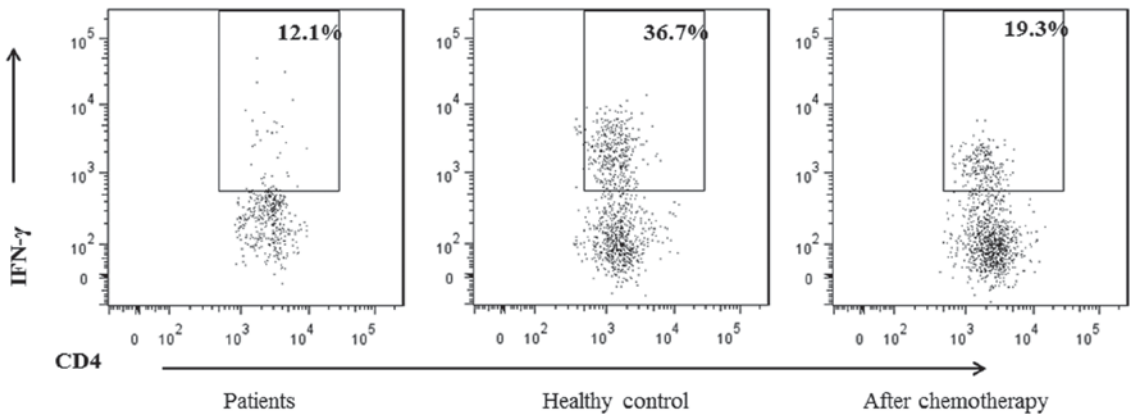

Figure 2. IFN-γ production in $CD4^+$ T cells in the peripheral blood of patients with gastric cancer (prior to and following chemotherapy) and the control group. CD, cluster of differentiation; IFN-γ, interferon-γ.

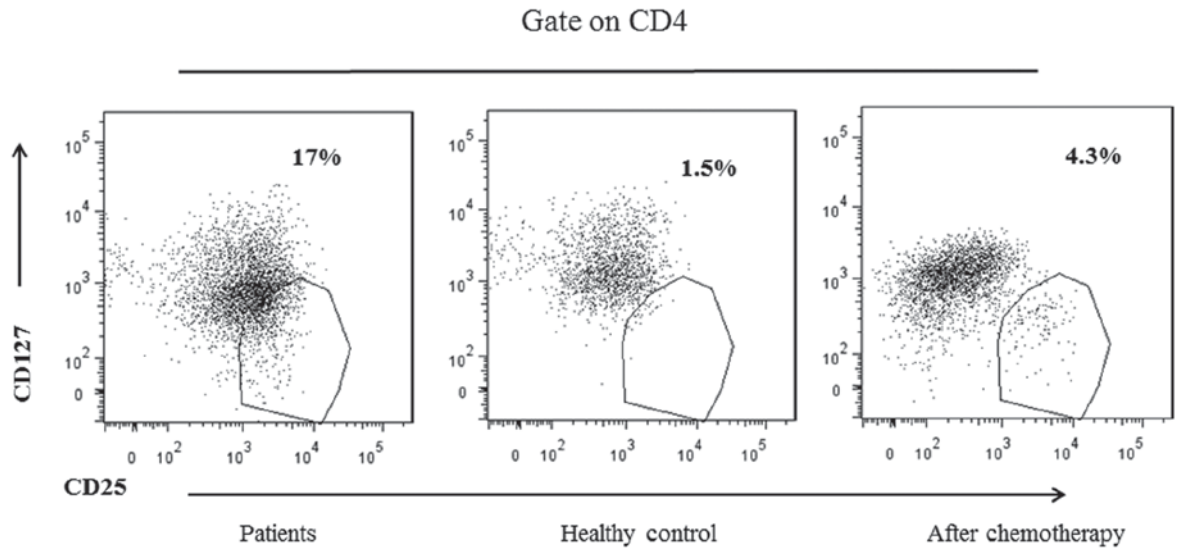

Figure 3. Percentage of $CD25^+/CD127^-/CD4^+$ T cells in the peripheral blood of patients with gastric cancer (prior to and following chemotherapy) and the control group. CD, cluster of differentiation.

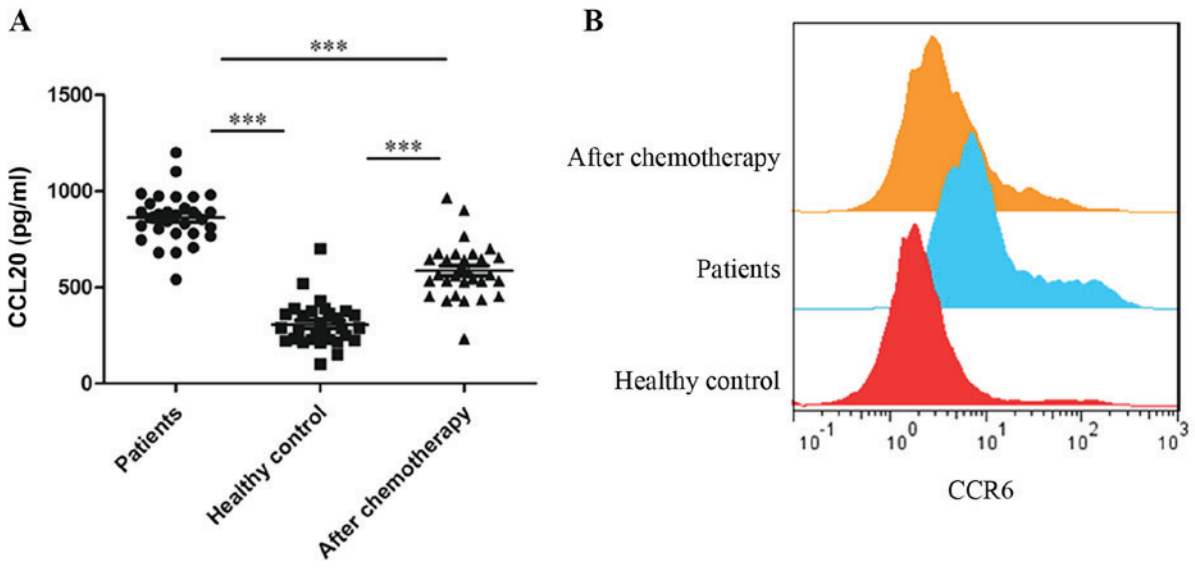

Figure 4. Expression levels of (A) CCL20 and (B) CCR6 in the peripheral blood of patients with gastric cancer (prior to and following chemotherapy) and the control group. CCL20, C-C motif chemokine ligand 20; CCR6, C-C motif chemokine receptor 6. Data are compared using the unpaired t-test ($^{***}P<0.001$).

chemotherapy in patients compared with the healthy controls (Fig. 4A). After chemotherapy, the expression of CCL20 significantly decreased. CCR6 has been established as the sole receptor for the chemokine CCL20 (11). As expected, it was identified that the expression of CCR6 on T_{regs} was significantly decreased following chemotherapy ($P<0.01$; Fig. 4B). These data suggested that CCL20 was required for the recruitment of $CCR6^+$ T_{regs} in patients with gastric cancer.

Discussion

Due to the low chemotherapeutic sensitivity of gastric cancer, surgery and chemotherapy is the primary course of treatment, and gastric cancer has a 5-year overall survival rate of 30-60% in surgically curable cases (19,20). The survival benefits of chemotherapy are well-recognized, but the effect of chemotherapy on immune cells remains unknown. Tumors are believed to be controlled by the immune system through a process termed immunosurveillance, which includes an equilibrium phase and eventual immune escape. The results from a previous study using a mouse model indicated that chemotherapy may selectively inhibit T_{regs}, while sparing effector T cells (21). Of the compounds included in the chemotherapy regimens of the present study, oxaliplatin and 5-FU are hypothesized to induce immunogenic cell death and partially deplete or transiently inactivate inhibitory immune cells (22). Therefore, we hypothesized that chemotherapy may result in immunomodulation in patients, suppressing the inhibitory immune cell function.

Cellular immunity serves a key role in the antitumor immune response (23). T cells are critical in the immune regulation and surveillance involved in cellular immunity, and are divided into two major subsets: $CD4^+$ and $CD8^+$. The ratio of $CD4^+/CD8^+$ cells significantly affects the host immune function; a decreased $CD4^+/CD8^+$ ratio impairs the immune systems of patients and promotes the development of tumors (24). A previous study indicated that chemotherapy may selectively decrease naïve $CD4^+$ T cells, but preserved the activated $CD4^+$ or $CD8^+$ and memory T cells (25). However, this particular study compared the fraction of $CD4^+$ cells among the total mononuclear cells in the bone marrow of patients with that of healthy controls (13.7±5.0 vs. 22.9±6.6, respectively), which may not take into account the complex cell composition of the bone marrow. Furthermore, from the same data set, an increase of the ratio of $CD4^+/CD8^+$ (1.63 vs. 1.55) was identified in the patients received chemotherapy, compared with the healthy control (25). We hypothesize that the percentage of CD4 in peripheral blood may better reflect the immune status of patients, as there are numerous cell types existing in BM, including progenitors and naïve T cells. Consistent with this previous study, to the best of our knowledge, the present study demonstrated for the first time the effects of systemic treatment on the peripheral immune cell populations of patients with gastric cancer. Although total counts were reduced following chemotherapy, the proportion of $CD4^+/CD8^+$ cells increased compared with before chemotherapy, suggesting that chemotherapy may enhance the cellular immune function in patients with gastric cancer.

T_{regs} serve a critical role in the maintenance of self-tolerance and suppression of autoimmune disease (26,27). T_{regs} are also essential for the immunopathogenesis of cancer, in which they may prevent the anti-tumor immune response in a non-antigen-specific manner (28). There is emerging evidence suggesting that higher frequencies of T_{regs} in tumors microenvironment are associated with a poor outcome (29,30). However, the role of T_{regs} remains controversial in gastric cancer, particularly in the peripheral blood: Previous studies have demonstrated that $CD4^+CD25^+$ (T_{regs}) cells comprise 5-10% of peripheral $CD4^+$ T cells in healthy controls (11); however, this is inaccurate for the T_{regs} definition. The most specific cell marker of T_{regs} identified is the nuclear transcription factor FoxP3 (31). As Liu *et al* (17) demonstrated, CD127 expression was inversely correlated with FoxP3 expression. In the present study, the combined expression of CD4, CD25 and CD127 was used to calculate the population of T_{reg} cells out of the total $CD4^+$ population; this proportion was 1.5±0.31% in healthy controls, but was significantly increased in patients with gastric cancer (18.33±2.51%). Furthermore, the function of $CD4^+$ T cells was also investigated in patients with gastric cancer. Increased IFN-γ production following chemotherapy was identified, which is predominantly mediated by $CD4^+$ T cells subsequent to the development of antigen-specific immunity. Taken together, the data from the present study indicated that chemotherapy may effectively suppress the quantity and function of T_{regs}.

The high fraction of T_{regs} in the peripheral blood raises the question how these cells migrate to the tumor. It has been revealed that CCL20 is important in recruiting circulating T_{regs} into tumor tissue in the liver cancer (11,18). In the present study, an increase in the level of CCL20 was also observed in the patient group compared with healthy controls. Furthermore, as the only known receptor for CCL20 (32), the level of CCR6 was significantly higher in the T_{regs} of patients. Therefore, the elevation of T_{regs} is due to, at least in part, their selective migration in response to CCL20.

In conclusion, the present study suggested that chemotherapy may promote cellular immune function and inhibit immunosuppression in patients with gastric cancer. These results provide additional understanding of the mechanism of FOLFOX-6 chemotherapy on gastric cancer, and provide important insights into the immune status of patients with gastric cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HR and LW conceived the idea. LW and DZ performed the experiments. HR analyzed the data. HR, LW and DZ wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Informed consent was obtained from the patients and the study was approved by the Ethics Committee of the Second Affiliated Hospital.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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