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Disturbed Th17/Treg Balance in Patients with Non-small Cell Lung Cancer

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Abstract—The fine balance of T help-17 (Th17)/regulatory T(Treg) cells is crucial for maintenance of immune homeostasis. However, there is little information concerning the role played in non-small cell lung cancer (NSCLC) by Th17/Treg cells. The objective of this study was to investigate the variation of Th17 and Treg cells in the peripheral blood of patients with NSCLC. Blood samples were collected from 19 patients with NSCLC and 19 healthy donors. Samples were processed to detect $CD4^+IL\text{-}17^+$ Th17 cells and $CD4^+CD25^+Foxp3^+$ Treg cells by flow cytometry, and related gene expressions were assessed by real-time quantitative polymerase chain reaction. The concentrations of interleukin (IL)-1β, IL-6, IL-10, IL-17, IL-23, and transforming growth factor-beta (TGF-β1) were also measured by enzyme-linked immunosorbent assay analysis (ELISA). The frequency of circulating Th17 cells and Treg cells was increased in samples derived from patients with NSCLC, accompanied by the upregulation of Foxp3 and RORγt. However, a negative correlation between Treg cells and Th17 cells was found in patients with NSCLC. Additionally, the Th17/Treg ratio and the related cytokines were also significantly higher in patients with NSCLC than in healthy controls. Furthermore, the frequency of Th17 cells was positively correlated with IL-1β, IL-6, and IL-23 in patients with NSCLC, and the frequency of Treg cells was positively correlated with TGF-β1 and IL-10. More importantly, the Th17/Treg ratio was positively correlated with the CEA concentrations in patients with NSCLC. Our data indicated that Th17 and Treg subset are involved in the immunopathology of NSCLC. Distinct cytokine environment might play a key role in the differentiation of the Th17 and Treg cells in NSCLC. Reconstituting an adequate balance between Th17 and Treg may be beneficial in the treatment of NSCLC.

KEY WORDS: Th17 cell; treg cell; differentiation; cytokine; imbalance; non-small cell lung cancer.

INTRODUCTION

Lung cancer is a leading cause of cancer-related mortality in the world. Non-small cell lung cancer (NSCLC) accounts for nearly 80 % of cases and with the 13 % overall 5-year survival rate [1]. The primary limitation in the effective treatment of NSCLC is an incomplete understanding of its specific cellular and molecular pathogenic elements. Accumulating evidence has shown that tumor infiltrated lymphocytes, especially CD4+ helper T cells, are present in the lungs of patients with non-small cell lung cancer [2]. Naïve CD4+ T helper precursor cells become activated and differentiate into Th1, Th2, Th17, and regulatory T cells (Tregs) after encountering a specific antigen [3, 4]. Traditionally, research in lung cancer immunity has focused almost exclusively on Th1/Th2 cell balance [5]. Recently, the identification of Th17 cells and Treg cells changes the classical Th1/Th2 paradigm of T helper

Highlights • Increased frequency of Th17 cells and Treg cells presented in peripheral blood of NSCLC patients.

• Disturbed Th17/Treg balance is involved in the immunopathology of NSCLC.

• Distinct cytokine environment might play a key role in the differentiation of the Th17 and Treg cells in NSCLC.

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cell differentiation [4, 6, 7]. Furthermore, the balance between Th17 and Treg may be important in the development/prevention of inflammatory and autoimmune disease [8].

Th17 cells are characterized by the synthesis of IL-17A, IL-17F, IL-21, IL-22, and IL-26 and express the key transcription factor: retinoic acid receptor-related orphan receptor γt (RORγt) [9–12]. With the induction of IL-1, IL-6, and IL-21, naïve CD4+ T cells can differentiate into Th17 cells [11]. Th17 cells and their hallmark cytokines are involved in both pro- and anti-tumorigenic processes [8]. While Tregs expressing the forkhead transcription factor (Foxp3) have an anti-inflammatory role and maintain tolerance to self components by releasing anti-inflammatory cytokines like IL-10 and TGF-β1 [9, 13]. Within the tumor microenvironment, Tregs may serve to suppress anticancer cell immunity [14]. Distinct from Th17 cells, Tregs are reported to be generated from naïve T cells by TGF-β [9, 13]. Notably, Tregs can promote Th17 cells development by means of TGF-β and IL-6 secretion [15]. With time, the relationship has become increasingly complex and more closely intertwined. In the present study, we were prompted to investigate whether the balance of Th17 and Treg responses as well as the related cytokine productions is dysregulated in NSCLC.

MATERIALS AND METHODS

Subjects

This study was approved by the Medical Ethical Committee of the First People's Hospital of Nanning (Nanning, China) and the Medical Ethical Committee of the Eighth People's Hospital of Nanning (Nanning, China), and informed written consent was obtained from all individuals. Peripheral blood samples were collected from 19 patients (male 13, female 6, age range 37–68 years) with newly diagnosed non-small cell lung cancer and 19 healthy volunteers (male 12, female 7, age range 41–65 years). The diagnosis of lung cancer was established by demonstration of malignant cells on biopsy specimen. Histological evaluation was based on the World Health Organization criteria. Tumor stage was evaluated according to the International Union against Cancer TNM classification system. The clinicopathological characteristics are summarized in Table 1. At the time of sample collection, none of the patients had received radiotherapy, chemotherapy, or other medical interventions.

Table 1. Patient Characteristics

	Healthy control	NSCLC patient
Age (mean±SD years)	52.63±7.492	53.00±8.950
Gender (male/female)	12:7	13:6
Histological type		
Adenocarcinoma		6
Squamous cancer		9
Others		4
TNM stage		
I–II		14
III		5

There is no statistically significant difference for age and sex between healthy controls and NSCLC patients

Sample Collection and Processing

Thirty milliliter venous blood samples were collected and were divided into three parts. Approximately 10 ml of venous blood were removed erythrocytes with RBC lysis buffer (Sigma-Aldrich) for 10 min at room temperature, and the remaining cells were washed twice with cold PBS and centrifuged at 1200 rpm for 10 min. Fresh peripheral-blood mononuclear cells (PBMCs) were used for flow cytometric analysis within 1 h. Approximately 10 ml of venous blood were separated serum for enzyme-linked immunosorbent assay analysis (ELISA), and the rest were separated serum for real-time quantitative polymerase chain reaction (RT-qPCR).

Immunofluorescence Labeling and Flow Cytometry

The expression markers on T cells were determined by flow cytometry after surface staining or intracellular staining with anti-human-specific Abs conjugated with phycoerythrin cyanine-5.5 (PE-Cy5.5), Allophycocyanin (APC), phycoerythrin (PE), and Alexa Fluor® 488 (Alexa Fluor® 488). The human Abs included anti-human CD4, anti-human CD25, anti-human IL-17, and anti-human Foxp3 monoclonal Abs (MAbs), which were purchased from BD Biosciences or eBioscience (San Diego, CA, USA). According to the manufacturer's instructions, PBMCs were stimulated with phorbol myristate acetate (PMA, 25 ng/ml, Sigma-Aldrich, USA) and ionomycin (1 μg/ml, Sigma-Aldrich, USA) in the presence of GolgiStop (BD Biosciences) for 4 h. The cells were washed and then fixed/permeabilized in the eBioscience fixation/permeabilization and permeabilization buffers and stained with fluorescent antibodies against CD4, CD25, IL-17, and Foxp3. Appropriate isotype controls were performed for each experiment to enable correct compensation and confirm antibody specificity. Flow cytometry was performed on a BD FACS Calibur flow cytometer and analyzed by using FlowJo software (Tristar, USA).

Real-Time Quantitative PCR

For quantitative real-time polymerase chain reaction (RT-qPCR) analysis of mRNA expression of the Foxp3 and RORγt in peripheral blood, total RNA was extracted from samples with TRIzol (Invitrogen-Life Technologies) according to the manufacturer's instructions. cDNA was prepared using oligo (dT) primers (RevertAid™ First Strand cDNA Synthesis Kit, Fermenta). Quantitative RT-PCR was performed by duplicate with SYBR GreenIusing a LightCycler (iCycler IQ, BioRad, American) according to the manufacturer's instructions, and using the following primers: Foxp3: 5′-GCCAGAATGACCAGATTGTGCT T-3′ and 5′-AAGGCACTTAGGGAGTGGGAGA-3′; RORγt: 5′-GCCAGAATGACCAGATTGTGCTT-3′, and 5′-AAGGCACTTAGGGAGTGGGAGA-3′; β-actin: 5′-ACACTGTGCCCATCTACG-3′, and 5′-TGTCACGCAC GATTTCC-3′. The identity of the amplified products were examined using 12 % poly-acrylamide gel electrophoresis and melt curve analysis, and the ratios of each gene product to β-actin product were used as indices of FoxP3 and RORγt mRNA expression.

Measurement of Cytokines

The concentrations of cytokines, including IL-1β, IL-6, IL-10, IL-17, IL-23, and TGF-β1 in serum were measured by ELISA kits according to the manufacturer's protocols (all kits were purchased from R&D Systems, Minneapolis, MN, USA). All samples were assayed in duplicate.

Statistical Analysis

Quantitative data were expressed as the mean±standard deviation. Nonparametric Mann-Whitney *U* test and Spearman's correlation were used for statistical analyses. A value of $p<0.05$ was considered statistically significant. All statistical analyses were performed by using SPSS statistical software version 16 (SPSS Inc., Chicago, IL, USA).

RESULTS

Th17 Cells and Tregs in PBMCs from Patients with NSCLC

We first analyzed the frequency of $CD4^+IL\text{-}17^+$ Th17 cells and $CD4^+CD25^+Foxp3^+$ Treg cells in PBMCs from patients with NSCLC by using flow cytometry. The quantitative results are shown in Fig. 1. The percentage of Th17 cells was significantly increased in patients with NSCLC compared with healthy controls (3.73±0.72 *versus* 0.71±0.21 %; $p<0.001$) (Fig. 1a). Similar to Th17 cells, the percentage of Tregs was also significantly increased in NSCLC patients compared with healthy controls (11.13± 1.62 *versus* 6.92±1.44 %; $p<0.001$) (Fig. 1b).

RORγt was described as an important transcription factor for the development of Th17, while Foxp3 was described as the most specific transcription factor in Tregs. Since increased the percentage of Th17 and Treg cells in the peripheral blood of patients with NSCLC, we thus investigated the mRNA expression of RORγt and Foxp3 by RT-qPCR. As expected, we found much higher levels of RORγt and Foxp3 mRNA in patients with NSCLC compared to healthy donors (3.95±0.54 *versus* 2.40±0.18; 1.89±0.13 *versus* 0.63 ±0.10, respectively, both $p<0.001$) (Fig. 2a, b). These results indicate that Th17 cells and Treg cells may play a central role in the immunopathology of NSCLC.

Correlation Analysis of Th17 and Treg Cells in Peripheral Blood of Patients with NSCLC

We determined the relationships of Th17 and Treg cell-associated factors in peripheral blood of patients with NSCLC. The expression levels of RORγt mRNA were significantly and positively correlated with Th17 cells numbers ($r=1.000$, $p<0.001$) (Fig. 3a). Similarly, the expression levels of Foxp3 mRNA were also significantly and positively correlated with Tregs numbers ($r=0.756$, $p<0.001$) (Fig. 3b). Of note, the frequency of Th17 cells in peripheral blood of NSCLC patients was significantly and negatively correlated with the frequency of Tregs ($r=-0.841$, $p<0.001$) (Fig. 3c). A negative correlation was also found between RORγt mRNA and Foxp3 mRNA ($r=-0.785$, $p<0.001$) (Fig. 3d). Thus, these results indicate that the balance of Th17/Treg might be broken in patients with NSCLC.

Serum IL-1β, IL-6, IL-10, IL-17, IL-23, and TGF-β1 Levels in Patients with NSCLC

Th17 cells are reported to be generated from naïve T cells by IL-1β, IL-6, and TGF-β1, and are expanded and stabilized further by IL-23 [11]. TGF-β1 is also the crucial cytokine involved in the differentiation of naïve T cells into Tregs and is needed for Tregs expansion [9, 13]. Thus, we analyzed the levels of cytokines involved in the differentiation of Th17 cells and Treg cells, and we found that patients with NSCLC had an increased serum concentrations of IL-1β, IL-6, IL-23, and TGF-β1 compared with healthy subjects (IL-1β, 221.78±40.13 *versus* 84.94±25.12 pg/ml; IL-6, 123.20± 17.80 *versus* 42.61±6.28 pg/ml; IL-23, 231.68±31.61 *versus* 80.25±19.12 pg/ml; TGF-β1. 276.77±51.86 *versus* 125.85± 24.02 pg/ml, respectively, $p<0.05$) (Fig. 4a–d). In addition,

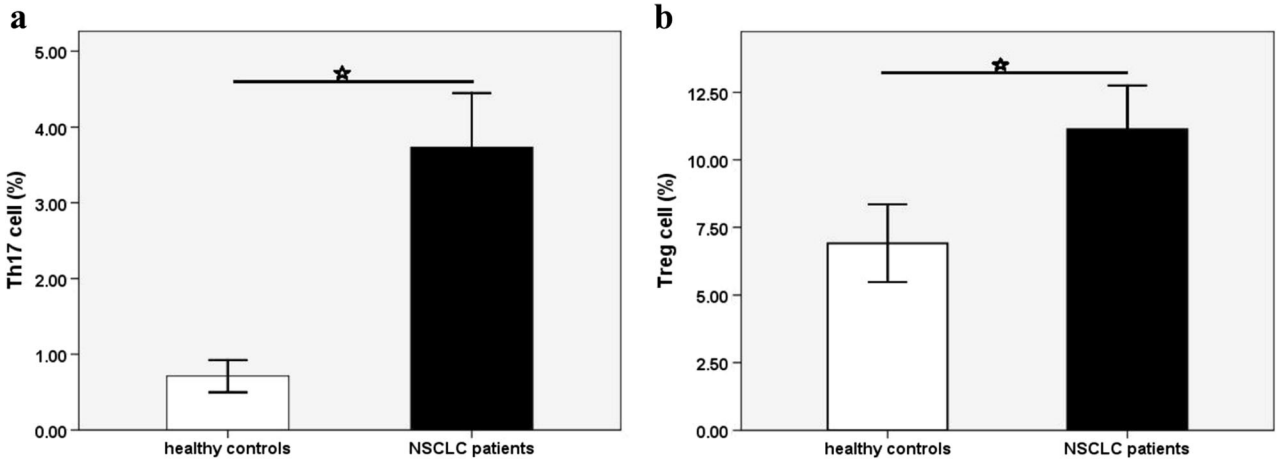

Fig. 1. The percentage of $CD4^+IL\text{-}17^+$ Th17 and $CD4^+CD25^+Foxp3^+$ Treg cells was measured by flow cytometry, The proportion of Th17 cells (**a**) was significantly increased in NSCLC patients compared with healthy controls. The proportion of Treg cells (**b**) was significantly increased in NSCLC patients compared with healthy controls. Results are expressed as % (mean±SD). $n=19$; $^*p<0.001$.

serum levels of IL-10 and IL-17 were also significantly augmented in patients with NSCLC compared with healthy controls (77.82±12.46 *versus* 38.74±14.09 pg/ml) (394.17±38.28 *versus* 146.07±18.85 pg/ml, respectively, $p<0.01$) (Fig. 4e, f).

To further confirm these cytokines correlated with the two T cell subsets, we did the correlation analysis in peripheral blood of NSCLC group. As shown in Fig. 5, there was a significantly positive correlation between the percentage of Th17 cells and the levels of IL-1β, L-6, and IL-23 ($r=0.659$, $p<0.01$, Fig. 5a; $r=0.912$, $p<0.001$, Fig. 5b; $r=0.615$, $p<0.01$, Fig. 5c). Similarly, as shown in Fig. 5d, there was a positive correlation between percentages of Tregs and TGF-β1 levels ($r=0.941$, $p<0.001$). By contrast, no clear correlation was found between the percentage of Treg cells and the levels of IL-1β, L-6, and IL-23 (data not shown). In addition, although a trend was noted toward the positive correlation between the percentage of Th17 cells and the levels of TGF, it was not statistically significant (data not shown).

Circulating CEA and Th17/Treg in Patients with NSCLC

The levels of CEA were markedly increased in NSCLC group (11.44±7.30) compared with those in

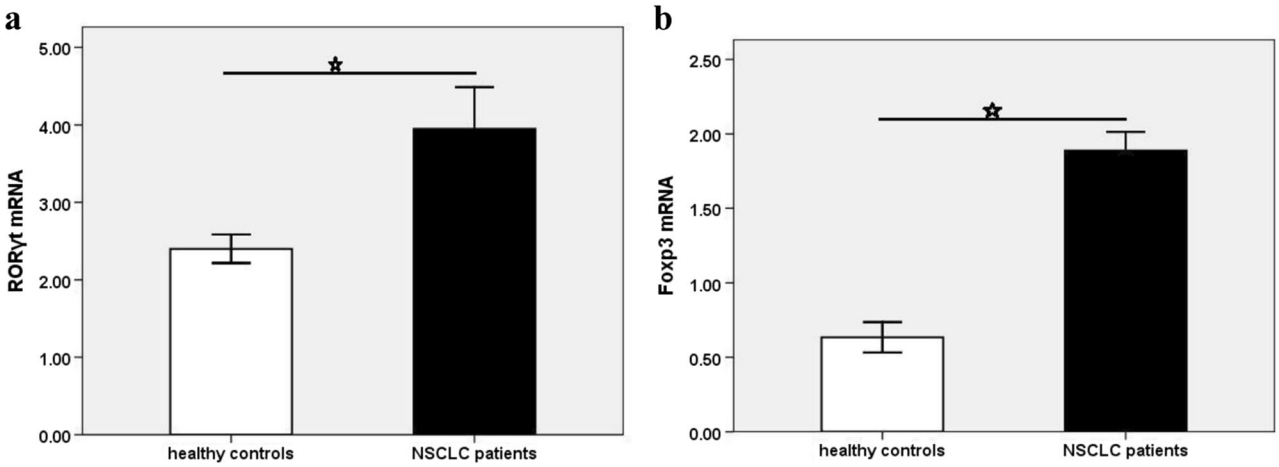

Fig. 2. The mRNA expression of ROR γt and Foxp3 in peripheral blood was measured by using RT-QPCR. The mRNA expression of ROR γt (**a**) was significantly higher in NSCLC patients than healthy controls. The mRNA expression of Foxp3 (**b**) was significantly higher in NSCLC patients than healthy controls. Results are expressed as (mean±SD). $n=19$; $^*p<0.001$.

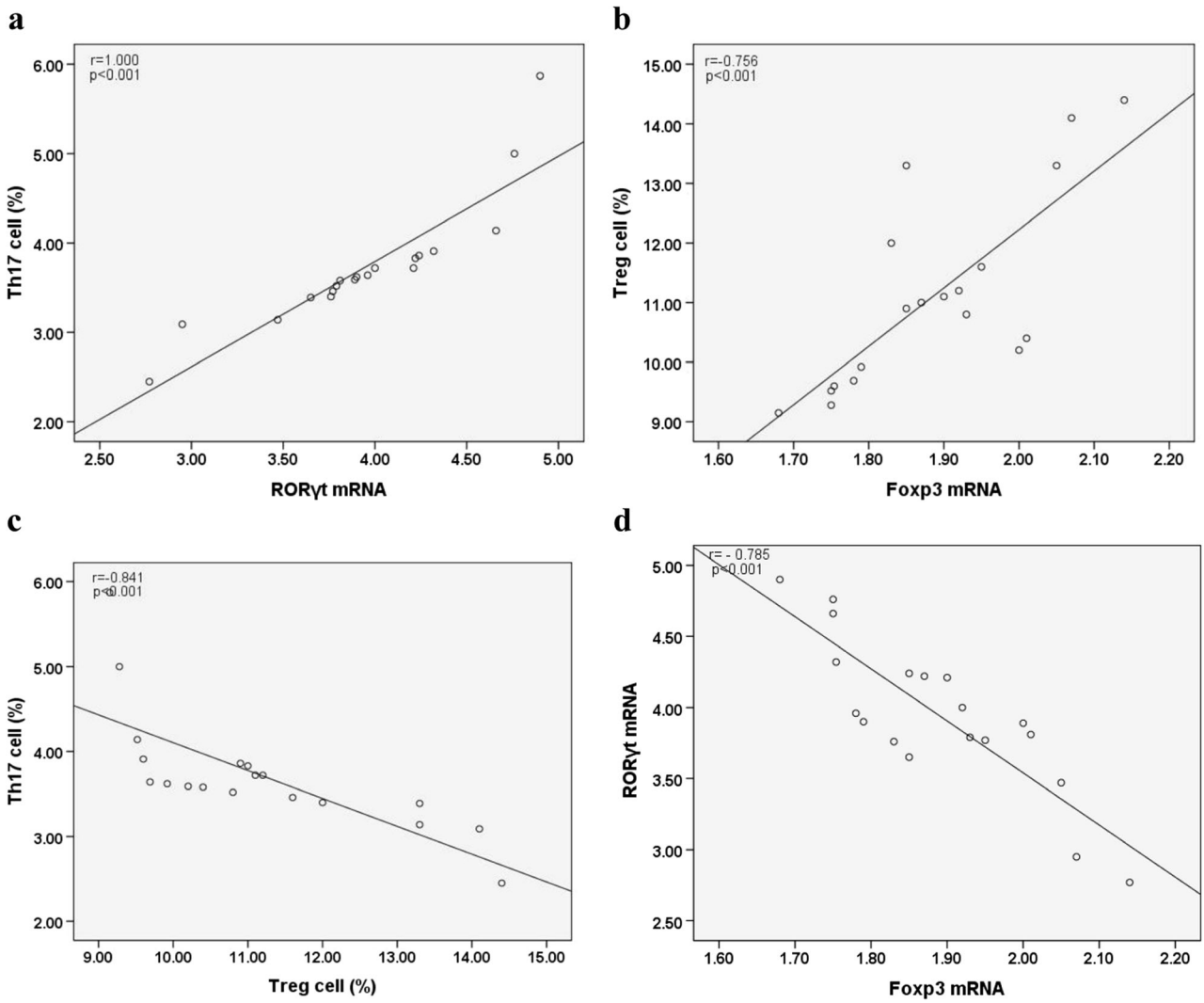

Fig. 3. Correlations between **a** the frequency of Th17 cells and ROR γt mRNA, **b** the frequency of Treg cells and Foxp3 mRNA, **c** the frequency of Th17 cells and the frequency of Treg cells, and **d** ROR γt mRNA and Foxp3 mRNA in NSCLC patients. Data were determined by Spearman's rank correlation coefficients. *$p<0.001$.

healthy group (1.24±1.05). To further explore the significance of circulating Th17 and Treg cells in NSCLC, we next calculated the Th17/Treg ratio of patients with NSCLC and healthy controls. As shown in Fig. 6a, the ratio of Th17 to Treg was significantly higher in NSCLC patients (0.34±0.06) than in controls (0.08±0.03) ($p<0.001$). Of interest, compared to control subjects, the percentage of Tregs in peripheral blood was generally higher than those of Th17 cells. Moreover, the Th17/Treg ratio was positively correlated with the CEA concentrations in patients with NSCLC ($r=0.551$, $p<0.05$) (Fig. 6b).

DISCUSSION

We present data demonstrating both enhanced Th17 and Treg response in peripheral blood of patients with NSCLC compared with those in healthy controls. More important, the increased frequency of Th17 cells was positively correlated with the levels of IL-1β, IL-6, and IL-23, whereas the increased frequency of Tregs was positively correlated with the levels of TGF-β1. Interestingly, the Th17/Treg ratio was much higher in patients with NSCLC than in healthy controls, with a negative correlation between Tregs and Th17 cells. Although the mechanism(s)

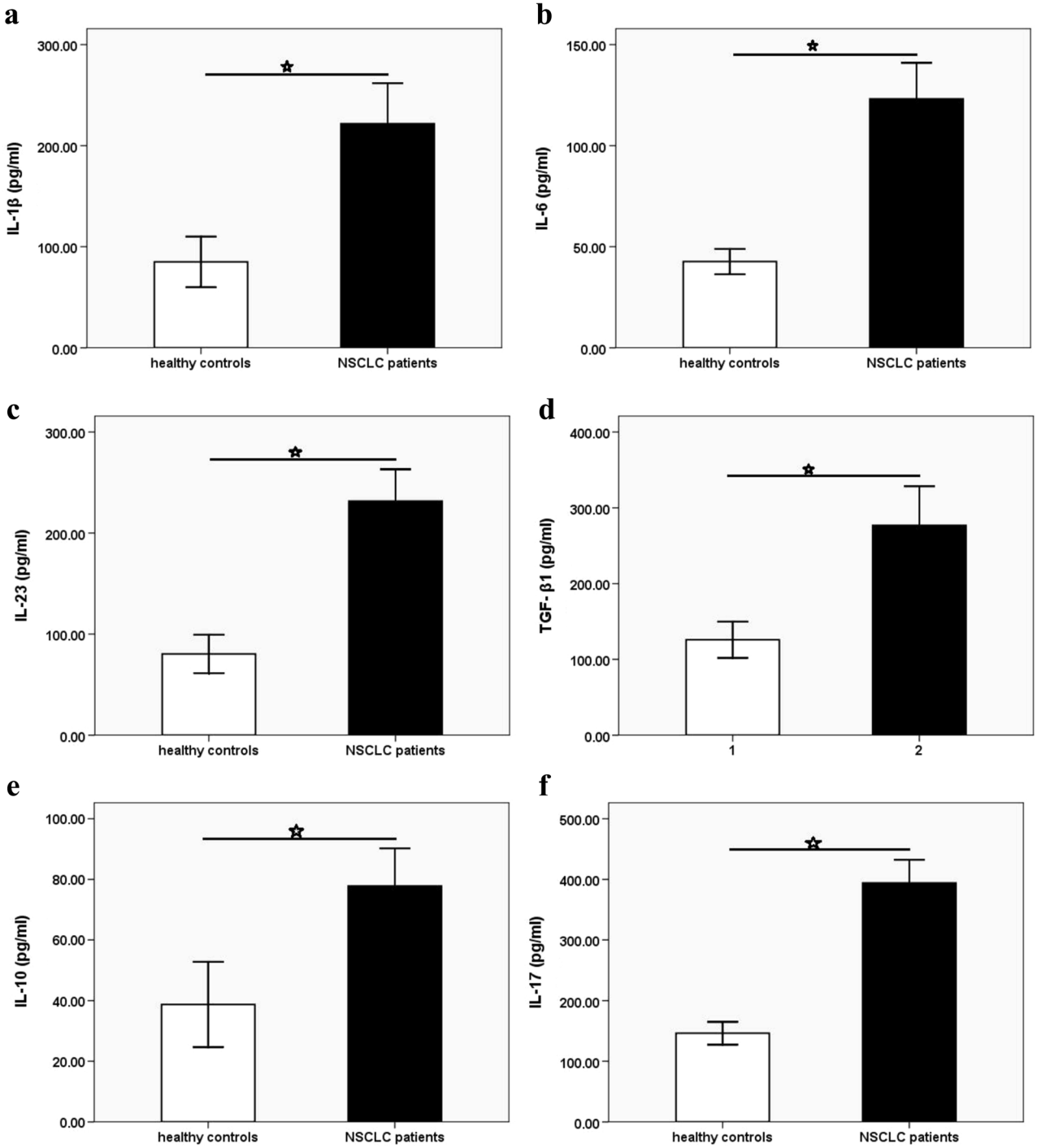

Fig. 4. The levels of IL-1β, IL-6, IL-23, TGF- β1, IL-10, and IL-17 were measured by using ELISA. Serum IL-1β levels (**a**) were significantly increased in NSCLC patients as compared to healthy controls, IL-6 levels (**b**) in serum were significantly higher in NSCLC patients than healthy controls, IL-23 levels (**c**) in serum were significantly higher in NSCLC patients than healthy controls, TGF- β1 levels (**d**) in serum were significantly higher in NSCLC patients than healthy controls, IL-10 levels (**e**) in serum were significantly higher in NSCLC patients than healthy controls, and IL-17 levels (**f**) in serum were significantly higher in NSCLC patients than healthy controls. Results are expressed as pg/ml (mean±SD). $n=19$; *$p<0.001$.

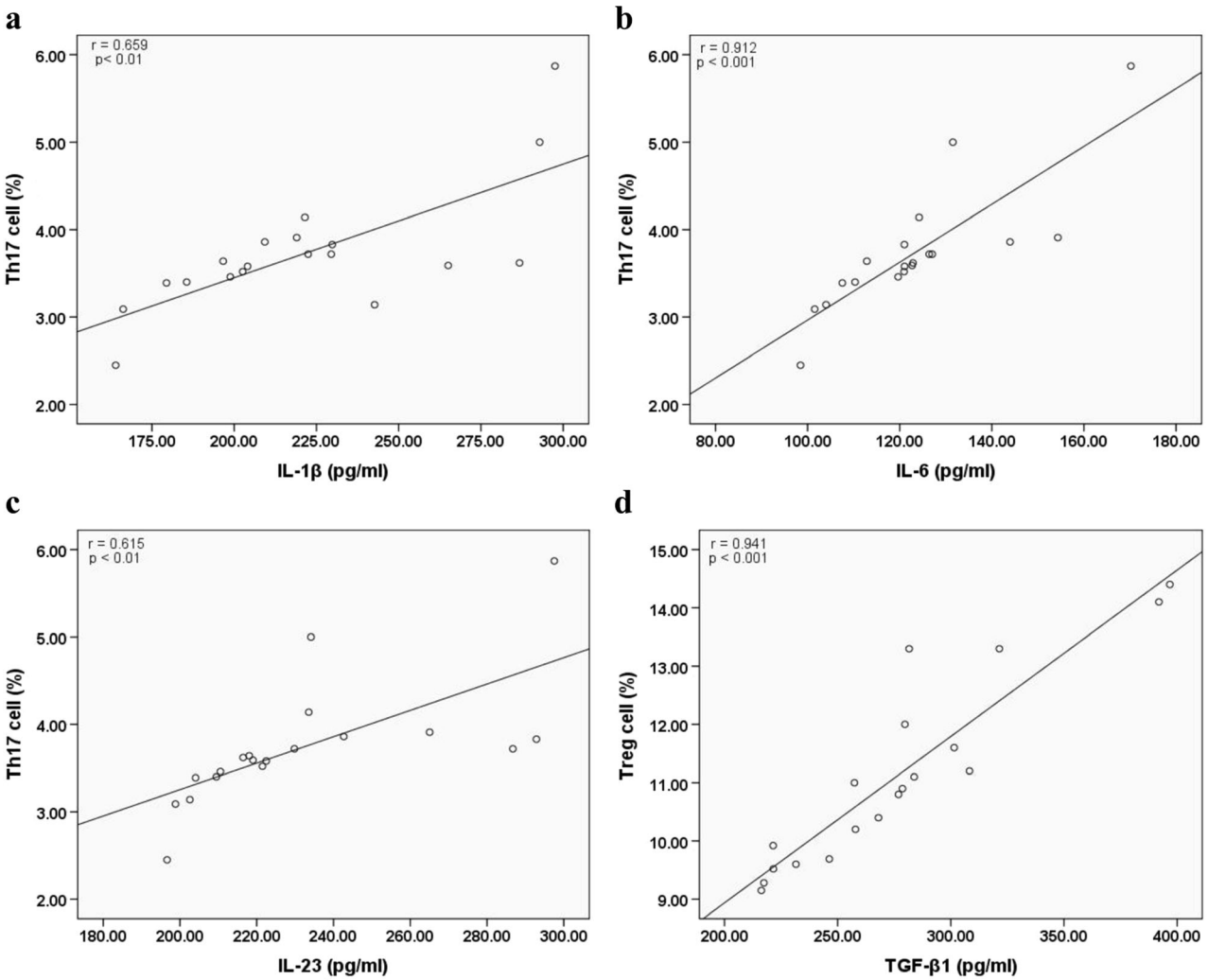

Fig. 5. Correlations between (**a**) the frequencies of Th17 cells and levels of IL-1β, (**b**) the frequencies of Th17 cells and levels of IL-6, (**c**) the frequencies of Th17 cells and levels of IL-23, and (**d**) the frequencies of Treg cells and levels of TGF-β1 in NSCLC patients. Data were determined by Spearman's rank correlation coefficients.

for these findings remains to be determined, our results indicate that the Th17 and Treg cell balance are dysregulated in NSCLC, and there could be a dynamic interaction between Th17 and Treg cells in the NSCLC microenvironment.

Th17 cell is now defined as a separate CD4+ T cell subset distinct from Th1 and Th2 cells, which is involved in the pathogenesis of different inflammatory and autoimmune diseases [8]. More recently, several studies have shown that Th17 cell is involved in the pathogenesis of lung cancer [16, 17]. However, the role of the subset in lung cancer is somewhat controversial. Previous studies showed that a higher level of IL-17A mRNA and protein expression was noted in lung CD4+ T cells from NSCLC patients as compared to healthy controls [18, 19]. Overexpression of IL-17 in tumor cell lines promotes angiogenesis and tumor growth when the tumors are implanted in immune-compromised mice [20]. These reports revealed that IL-17-mediated responses promote tumor development, whereas another recent report indicated that tumor growth in subcutaneous tissue and lung tumor metastasis are enhanced in IL-17−/− mice [21, 22]. It implicates that IL-17-mediated responses are protective against tumor development. In this study, we found that the Th17 cells were increased in peripheral blood of patients with NSCLC, accompanied by the upregulation of IL-17 and RORγt. Additionally, Th17 cell percentage and RORγt mRNA were positively correlated in patients with NSCLC. Thus,

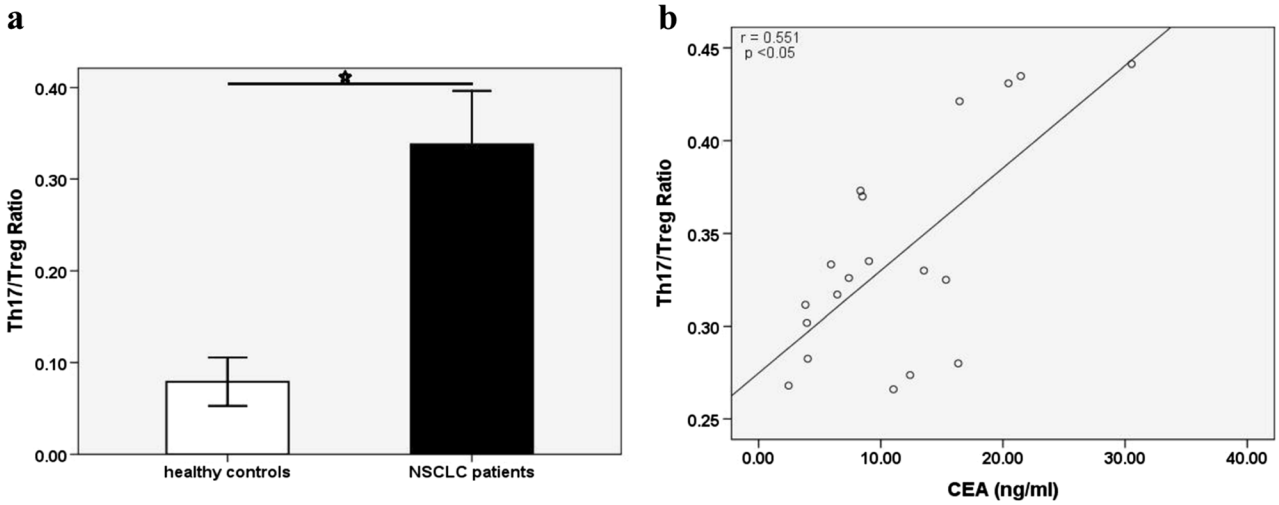

Fig. 6. The Th17/Treg ratio correlates with CEA concentrations (**a**) the Th17/Treg ratio was significantly higher in NSCLC patients than in controls, (**b**) correlation between the Th17/Treg ratio and CEA concentrations in patients with NSCLC.

these results indicate that the inflammatory function of Th17 might play important roles in the pathogenesis of lung cancer.

Tregs, which exert anti-inflammatory functions and control the local immune response, are associated with advanced tumor growth and poor prognosis in several types of malignant tumor [23–25]. Many studies have shown that tumor infiltrating Tregs were elevated in NSCLC patients as compared to healthy controls [26, 27]. These Tregs were found to secrete immunosuppressive cytokine TGF-β, which may play a role in cancer progression [28]. In addition, these Tregs have been shown to express cytotoxic lymphocyte-associated antigen-4 CTLA-4 (CD152) in mice [29], and triggering of CTLA-4 has been shown to induce TGF-β secretion [30]. In this study, we found that the Tregs were increased in peripheral blood of NSCLC patients and were accompanied by the upregulation of IL-10, TGF-β1, and Foxp3. Additionally, Tregs percentage and Foxp3 mRNA were positively correlated in patients with NSCLC. Thus, these results indicate that Tregs in NSCLC could suppress host immune responses through the release of IL-10 and TGF-β1, and therefore might contribute to NSCLC progression.

Several lines of evidence have demonstrated that the local cytokine environment is a determining factor in the differentiation of T cell subsets. The combination of IL-1β and IL-6 is a proinflammatory cytokine milieu for human Th17 generation while IL-23 is critical in maintaining and expanding Th17 [8, 11]. In this context, TGF-β1 is the inhibitor of human Th17 differentiation. However, when IL-6 is absent, TGF-β1 is capable of inducing Tregs, while IL-2 is required for the expansion and maintenance of Tregs [8, 9]. On the basis of our observation that the increased Th17 and Treg cells could be seen in peripheral blood of patients with NSCLC, we reasoned that a cytokine milieu that facilitates the differentiation of the two subsets should also be present in NSCLC microenvironments. Indeed, our data showed that the serum concentrations of IL-1β, IL-6, IL-23, and TGF-β1 were significantly higher in patients with NSCLC than those in normal control subjects. Furthermore, our data showed that the serum levels of IL-1β, IL-6, and IL-23 were positively correlated with Th17 cells, and the serum levels of TGF-β1 were positively correlated with Tregs. Our data also showed that no correlation of Treg cells with the levels of IL-1β, L-6, and IL-23 were detected. Although a trend was noted toward the positive correlation between the percentage of Th17 cells and the levels of TGF, it was not statistically significant. Taken together, in the NSCLC microenvironment, IL-1β, IL-6, or IL-23, but not TGF-β1, could promote the differentiation of Th17 cells. In contrast, TGF-β1 could promote the differentiation of Tregs. We believe that further studies are warranted to determine the mechanism underlying the generation and regulation of Th17 cells and Treg cells in NSCLC.

The balance between Treg and Th17 cells is a key factor that regulates T helper cell function in variety inflammatory autoimmune diseases, including rheumatoid arthritis, systemic lupus erythematosus, and chronic hepatitis C [31–33]. Several studies have shown that Th17 cells and Treg cells were closely related with lung cancer [25, 27, 34]. Currently, very little is known about the Th17 and Treg cell function and balance in patients with NSCLC. In

this study, we demonstrated that patients with NSCLC had a higher Th17/Treg ratio, with negative correlation between Treg cells and Th17 cells. Furthermore, the increased Th17/Treg ratio was positively correlated with the increased CEA concentrations in NSCLC patients. As mentioned above, Tregs and Th17 cells arise in a mutually exclusive manner, depending on whether they are activated in the presence of TGF-β1 or TGF-β1 plus IL-6. In addition, in the presence of strong TCR signaling and APC, Tregs could secrete IL-17 [35]. Furthermore, in the tumor microenvironment producing IL-17, Tregs could be transformed into Th17 cells, which further amplified the inflammatory response [35]. Taken together, these results suggest that lung cancer may occur as a consequence of cytokine imbalance and eventually of the Th17/Treg ratio. However, the precise mechanisms for the dynamic interaction between Th17 and Treg cells in NSCLC should be clarified in future studies.

Some limitations of this study need to be acknowledged. Firstly, the sample size of this study was too small to obtain a definitive conclusion, so we need to prove our conclusion in a larger scale of the population. Secondly, although both Th17 and Treg cells have been found to be higher in patients with NSCLC; however, their direct role in contribution to pathogenesis of NSCLC remains to be elucidated. Thirdly, although the related cytokines were higher in patients with NSCLC, the mechanisms of differentiation of Th17 cells and Treg cells in the tumor microenvironment remain largely unknown. Finally, it is also uncertain as to whether Tregs are capable of suppressing generation and differentiation of Th17 cells in lung cancer; Additional *in vitro* experiments would be required to demonstrate the regulation and mechanism of Tregs on generation and differentiation of Th17 cells in the pathogenesis of lung cancer.

In conclusion, our data showed that Th17 cells and Treg cells are involved in the perpetuation of the inflammatory immune response in NSCLC, with negative correlation between the two cell subsets, and that the factor(s) involved in balancing Th17 and Treg cell responses are dysregulated in NSCLC, suggesting a potential role for Th17/Treg imbalance in the pathogenesis of lung cancer. Restoring an adequate cytokine network and Th17/Treg balance may represent a useful tool for lung cancer treatment in the next future.

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Competing Interests. The authors declare that they have no competing interests.

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