



The Expression and Relationship of Tc17 Cells and Interleukin-27 in Local and Systemic Environment of Non-small Cell Lung Cancer

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Abstract

Objective: Tc17 cells and Interleukin-17 (IL-17) are involved in the development of lung cancer. IL-27 has been suggested as a heterodimeric cytokine for influencing T cell activities. However, their distributions, biological function as well as the relationship are still lacking. In this study, we investigated the expression of IL-27 and its association with Tc17 cells in Non-Small Cell Lung Cancer (NSCLC) patients.

Methods: Using flow cytometry, qPCR, and ELISA, the frequency of Tc17 cells and the expression of IL-27 and IL-17 from peripheral blood, tumor tissue, and peritumoral tissue of NSCLC patients and from peripheral blood of healthy controls were evaluated. The relationships among Tc17 cells, IL-27, and IL-17 were analyzed.

Results: The frequency of Tc17 cells and the expression of IL-27 were markedly decreased in peripheral blood from NSCLC patients compared with that in controls; the expression of IL-17A protein and mRNA in serum was markedly increased in NSCLC patients. In addition, the frequency of Tc17 cells was higher in peritumoral tissues than in tumor specimens and peripheral blood. Whereas the expression of IL-17 and IL-27 was higher in tumor specimens than those in peritumoral tissues and corresponding serum. Furthermore, the expression of IL-27 in tumor specimens and peripheral blood was positively correlated with Tc17 cells numbers, but not IL-17 expression.

Conclusion: Collectively, IL-27, IL-17, and Tc17 cells are involved in NSCLC immunity and elicit protumor or anti-tumor respectively.

Keywords: Interleukin-27; Interleukin-17; Tc17 cell; Inflammation; Non-small cell lung cancer

Introduction

Lung cancer is the leading cause of cancer-related mortality in the world, and effective treatments are still needed. According to the histological features, lung cancer is divided into Small Cell Lung Cancer (SCLC) and Non-Small Cell Lung Cancer (NSCLC). NSCLC accounts for approximately 80% or more of cases and its five-year survival is only 15% [1]. Tumor Microenvironments (TME), which consist of a mix of cellular and non-cellular components including many types of immune cells, tumor cells, and molecules, play a critical role in the malignant. However, the complex relationship between cells and related cytokines within the TME is still unclear. Reportedly, CD8+ T cells are important TILs with variable antitumor properties, which produce IFN- γ and express granzyme B and perforin to kill the target cells [2].

Lung cancers with high CD8+ T cell density are thought to have a better prognosis [3]. In recent years, subsets of IL-17+ CD8+ T cells (Tc17 cells) played important roles in the pathogenesis of several infections and autoimmune diseases [4,5], have been rarely studied in lung cancer. A report showed that circulating Tc17 cells were reduced in patients with lung adenocarcinoma, especially those with tumor invasion and distant metastasis [6]. An additional study indicated that IL-17 directly or indirectly promotes tumor angiogenesis and cell proliferation [7]. Despite these observations, the contribution of Tc17 cells to the immunopathology of lung cancer is unclear. After adoptive transfer of highly purified, tumor-specific, *in vitro* differentiated Tc17 cells to tumor hosts, the transferred Tc17 cells could completely convert to Tc1 cells, which could be accumulated to a greater extent and substantial pulmonary pathology or the regression of established tumors [8,9].

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These findings may be explained by the existence of the functional plasticity of Tc17 cells.

The Interleukin-27 (IL-27), a member of the IL-6/IL-12 family identified in 2002 [10], is a heterodimer composed of Epstein-Barr virus-induced gene 3 (EBI3) and L-27p28. IL-27 is mainly produced by activated antigen-presenting cells, such as Dendritic Cells (DCs), monocytes, and macrophages, and exerts proinflammatory and anti-inflammatory in several autoimmune diseases, and malignant diseases by binding to a heterodimeric receptor composed of IL-27R α (also known as WSX1 or TCCR) and the common cytokine receptor subunit gp130 [10]. Accumulating evidence has showed that IL-27 possesses potent antitumor activity against various tumor models through different mechanisms, including inhibition of tumor proliferation and angiogenesis, and regulation of antitumor immunity mediated by CD8⁺ T cells [11]. However, data concerning their biological function as well as the relationship between IL-27 and Tc17 cells are still lacking. This study aimed to investigate the expression of IL-27 and its association with Tc17 cells in NSCLC patients.

Materials and Methods

Study subjects

25 NSCLC patients (male 17, female 8, age range: 34 to 72 yrs) admitted to the Department of Respiratory Medicine at the Eighth People's Hospital, and 16 sex- and age-matched healthy individuals from December 2018 to March 2019 were enrolled in this study. All patients were histologically confirmed with NSCLC by two pathologists. All cases with NSCLC were staged according to the 8th edition of the Tumor, Node, and Metastasis (TNM) classification for lung cancer [12]. Histologically, 13 patients had squamous cell carcinoma and 12 patients had adenocarcinoma. There were 5 patients in stage I, 15 patients in stage II, and 5 patients in stage IIIa. The patients were excluded if they had autoimmune disease, active or chronic inflammatory conditions, or current treatment with any immunomodulatory drugs. None of the patients previously received any anticancer therapy, including radiotherapy, chemotherapy, immunosuppressive, or other medical interventions. The study protocol was approved by the Medical Ethical Committee of the Eighth People's Hospital and the First Affiliated Hospital of Guangxi Medical University (Nanning, China), and informed consent was obtained from all subjects.

Sample collection and processing

Peritumoral tissues corresponded to non-infiltrated lung situated at 10 cm from the tumor. Tumor specimens and peritumoral tissues were obtained from subjects at the time of the surgery. The tissues were divided for the detection of mRNA and protein (stored at -800°C) and for isolation of lymphocytes. Lung tissues were mechanically dissected and cell suspensions were obtained after enzymatic digestion using 100 IU/ml type I DNase (Sigma-Aldrich) and 1 mg/ml collagenase A (Roche Diagnostics) at 37°C with 5% CO₂ for 1h in serum free RPMI 1640 medium. The tumor suspension was then filtered through a wire grid, and the cells were washed 3 times with HBSS (Media- tech). Lymphocytes were purified on a Ficoll density gradient. The tissue supernatants were frozen at -80°C and preserved until further processing.

Ten milliliters of venous blood were collected from the subjects before any anticancer treatment, and serum was isolated by centrifugation for 15 min at 800× g 4°C and stored at -80°C for the

detection of cytokines. Peripheral Blood Mononuclear Cells (PBMCs) of heparinized peripheral blood from all individuals were isolated by Ficoll-Hypaque gradient centrifugation.

Flow cytometry analysis

To analyze the prevalence of Tc17 cells in the peritumoral tissues, tumoral tissues, and PB, the expression of Tc17 cells was determined using Fluorescence-Activated Cell Sorter (FACS), after surface staining or intracellular staining with anti-human-specific Abs conjugated with either fluorescein isothiocyanate. These human antibodies included PE-Cy5-conjugated anti-CD8 and PE-conjugated anti-IL-17A as well as isotype matched antibodies. All antibodies used in the flow cytometric analysis were obtained from BD Biosciences (San Diego, CA, USA) and isotype-matched antibody controls were used in all procedures. In brief, PBMCs and cells from tumor tissue or peritumoral tissue were stimulated with Phorbol Myristate Acetate (PMA, 25 ng/mL, Sigma Aldrich, USA) and ionomycin (1 g/mL, Sigma-Aldrich, USA) in the presence of GolgiStop (BD Biosciences) for 4 h. Then, the cells, fixed and permeabilized with the eBioscience fixation/permeabilization and permeabilization buffers, were stained with fluorescent antibodies against CD8 and IL-17A at room temperature according to the manufacturer's instructions. The stained cells were analyzed by flow cytometric analysis using a FACS Calibur flow cytometer (BD Biosciences) with FCS Express V4 software.

Real-time quantitative PCR

Total RNA was extracted from samples using the TRIzol (Invitrogen)/chloroform method according to the manufacturer's instructions. The first-strand cDNA was synthesized using oligo (dT) primers (RevertAid™ First Strand cDNA Synthesis Kit, Fermenta). Each primer was entered into a NCBI BLAST search to ensure specificity for target mRNA transcription. Real-time quantitative PCR was performed in triplicate on the Light Cycler (Bio-Rad, American) using SYBR Green PCR kit using an Applied Biosystems 7900 instrument. The following primer pairs were used: IL-27: 5' - T G C C A G G A G T G A A C C T G T A C C - 3', and 5' - C G T G G T G G A G A T G A A G C A G A - 3'; IL-17: 5' - G G A A T C T C C A C C G C A A T G A G - 3', and 5' - A C A C C A G T A T C T T C T C C A G G C - 3'; β -actin: 5' - A C A C T G T G C C C A T C T A C G - 3', and 5' - T G T C A C G C A C G A T T T C C - 3'. The identities of the amplified products were examined using 12% polyacrylamide gel electrophoresis and melt curve analysis, and the ratios of each gene product to β -actin product were used as indices of IL-27 and IL-17 mRNA expression.

Cytokines measurement

IL-17 and IL-27 levels in the cell-free supernatants of tumor specimens and peritumoral tissues, as well as corresponding serum were tested using ELISA kits according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). All samples were tested in triplicate.

Statistical analysis

The data were presented as the mean \pm standard deviation. Differences among groups were compared by unpaired Student's t-test or one-way ANOVA. For nonparametric data, the Mann Whitney U test was performed between groups. Correlation analysis was performed using Spearman's rank correlation coefficient. Analysis was performed by using a SPSS statistical software (version 22.0, SPSS Inc., Chicago, IL, USA), and $p < 0.05$ was considered significant.

Results

Percentages of Tc17 cells in lung tissues and peripheral blood from NSCLC patients

To investigate the role of Tc17 cells in the development of NSCLC, we first determined the percentages of circulating Tc17 cells in the peripheral blood from all individuals (Figures 1A-1D). In healthy controls, the mean percentages of Tc17 cells in the peripheral blood were $5.03 \pm 0.37\%$ of all CD8+ T cells. In contrast, peripheral blood of NSCLC patients exhibited a 1.46 fold decrease in IL-17+ CD8+ T cells, which is highly significant ($p < 0.001$) as compared with healthy controls (Figure 2A). We further determined the percentages of Tc17 cells in tumor specimens and peritumoral tissues, as well as corresponding peripheral blood. The percentages of Tc17 cells were significantly higher in peritumoral tissues, compared to that in peripheral blood and in tumor tissues from NSCLC patients (all $p < 0.001$) (Figure 2B). No significant difference was found between the percentages of Tc17 cells in peripheral blood and that in tumor tissues ($p > 0.05$) (Figure 2B).

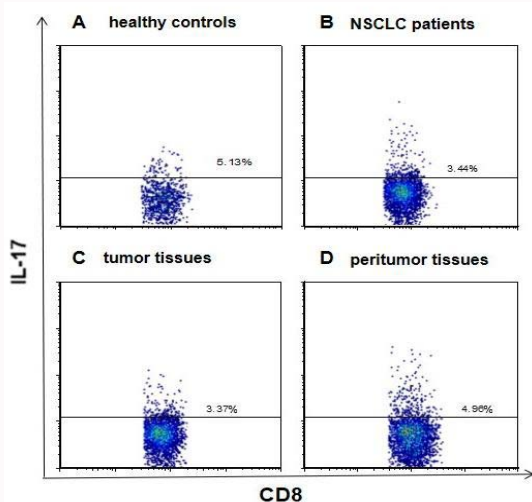


Figure 1: The frequency of CD8+ IL-17+ (Tc17) cells in tumor specimens and peritumoral tissues, as well as corresponding peripheral blood was measured by flow cytometry. (a) Representative FACS staining for IL-17A in gated CD8+ T cells in PBMCs from healthy controls; (b) Representative FACS staining for IL-17A in gated CD8+ T cells in PBMCs from patients with NSCLC; (c) Representative FACS staining for IL-17A in gated CD8+ T cells in tumor specimens from patients with NSCLC; (d) Representative FACS staining for IL-17A in gated CD8+ T cells in peritumoral tissues from patients with NSCLC.

Expression of IL-27 and IL-17 levels in lung tissues and peripheral blood from patients with NSCLC

As shown in Figure 3A, serum IL-27 levels were significantly decreased in patients with NSCLC as compared to normal controls ($p < 0.001$). The protein levels of IL-27 in tumor specimens were further lower than in peritumoral tissues and peripheral blood (all $p < 0.001$) (Figure 3B). No significant difference was found between the protein levels of IL-27 in peripheral blood and that in peritumoral tissues ($p > 0.05$) (Figure 3B). In contrast, the levels of IL-17A in the serum of patients with NSCLC were much higher than those in the healthy controls ($p < 0.001$) (Figure 3C). IL-17A concentration in tumor tissue was further higher as compared to serum levels and peritumoral tissue levels (all $p < 0.001$) (Figure 3D). No significant difference was found between the IL-17 levels in peripheral blood and that in peritumoral tissues ($p > 0.05$) (Figure 3D).

The mRNA expression of IL-27 and IL-17 in lung tissues and peripheral blood from patients with NSCLC

As expected, Figure 4A indicated that the mRNA levels of IL-27 in the serum of patients with NSCLC were markedly lower than those in the healthy subjects ($p < 0.001$). The mRNA levels of IL-27 were further lower in tumor specimens than in peritumoral tissues and peripheral blood (all $p < 0.001$) (Figure 4B). No significant difference was found between the mRNA levels of IL-27 in peritumoral tissues and that in peripheral blood ($p > 0.05$) (Figure 4B). In contrast, the mRNA levels of IL-17A in the serum of patients with NSCLC were markedly higher than those in the healthy subjects ($p < 0.001$) (Figure 4C). The mRNA levels of IL-17A were also further higher in tumor specimens than in peritumoral tissues and peripheral blood ($p < 0.001$) (Figure 4D). No significant difference was found between the IL-17 mRNA in peritumoral tissues and that in peripheral blood ($p > 0.05$) (Figure 4D).

Correlations between the levels of Tc17 cells and IL-27 as well as IL-17 in NSCLC patients

Since IL-27 heterodimer influences CD8+ T cell activity, it is reasonable to investigate the association between IL-27 and Tc17 cells. We observed a significant positive correlation between the proportion of Tc17 cells and IL-27 concentration in the tumor specimens and peripheral blood (all $p < 0.001$) (Figure 5A and 5B). We did not observe this correlation in the peritumoral tissues ($p > 0.05$). Similarly, there was also a positive significant correlation between the proportion of Tc17 cells and IL-27 mRNA levels in the tumor specimens and peripheral blood (all $p < 0.001$) (Figure 5C and 5D). However, this association did not occur in peritumoral tissues

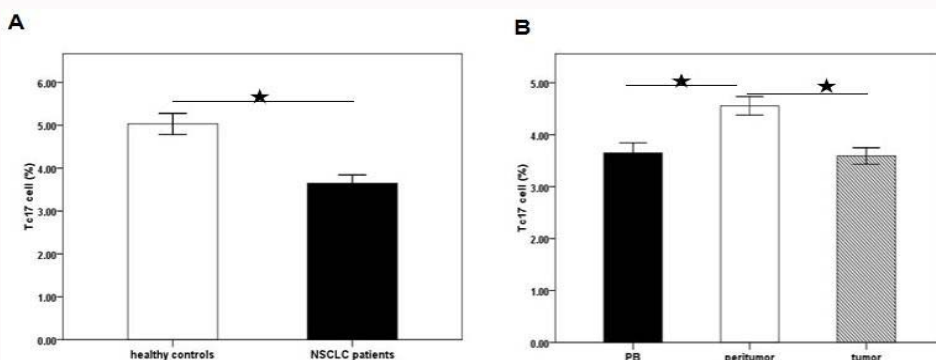


Figure 2: The frequency of CD8+ IL-17+ Tc17 cells was measured by using flow cytometry. The proportion of Tc17 cells (a) was significantly decreased in the peripheral blood from NSCLC patients compared with healthy controls. The proportion of Tc17 cells (b) was significantly higher in peritumoral tissues than in tumor specimens and corresponding peripheral blood samples NSCLC patients. * $p < 0.001$ was considered significant.

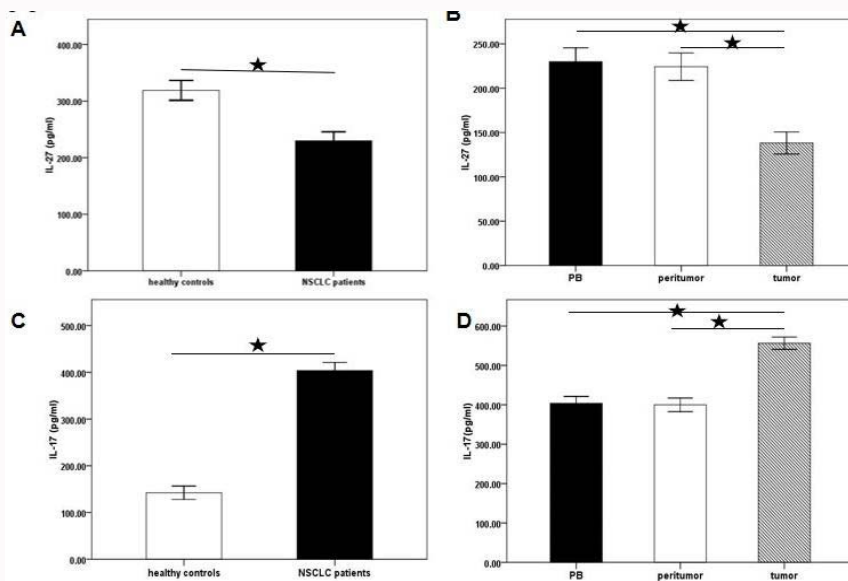


Figure 3: The expressions of IL-27 and IL-17 protein in peripheral blood from healthy controls and in the tumor specimens, peritumoral tissues and corresponding peripheral blood from NSCLC patients were measured by using ELISA. (a) The IL-27 protein levels in serum were significantly decreased in patients with NSCLC as compared to healthy controls. (b) The protein levels of IL-27 were lower in tumor specimens than in peritumoral tissues and peripheral blood. (c) The IL-17A protein levels in serum were significantly higher in patients with NSCLC as compared to healthy controls. (d) The protein levels of IL-17A were higher in tumor specimens than in peritumoral tissues and peripheral blood. * p<0.001 was considered significant.

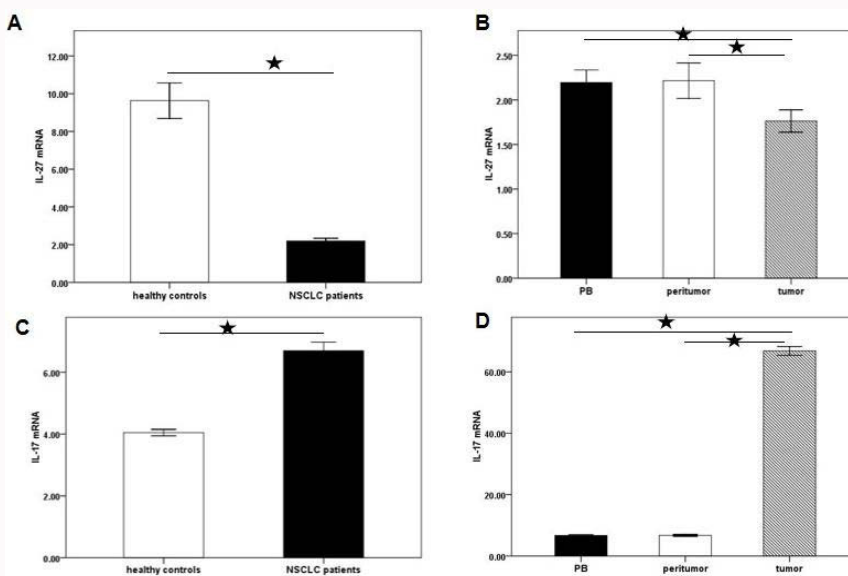


Figure 4: The expressions of IL-27 and IL-17 mRNA in peripheral blood from healthy controls and in the tumor specimens, peritumoral tissues and corresponding peripheral blood from NSCLC patients were measured by qPCR. (a) The mRNA levels of IL-27 in serum were significantly lower in patients with NSCLC than those in the healthy controls. (b) The mRNA levels of IL-27 were lower in tumor specimens than in peritumoral tissues and peripheral blood. (c) The mRNA levels of IL-17 in serum were significantly higher in patients with NSCLC than those in the healthy controls. (d) The mRNA levels of IL-17 were higher in tumor specimens than in peritumoral tissues and peripheral blood. * p<0.001 was considered significant.

(p>0.05). Besides, since IL-17A produced by several other immune cells, including Th17 cells, Tc17 cells, natural killer T cells, and mast cells, have been shown to be involved in both inflammation and immune responses in lung cancer, the relationship between the levels of IL-17A and IL-27 in the NSCLC patients were also analyzed. However, no correlation between IL-17A and IL-27 expression was found in the tumor specimens, peritumoral tissues, and peripheral blood (all p>0.05).

Discussion

Evidence has shown the dual roles of T cells and associated

cytokines in the development of lung cancer [13,14]. Although IL-27 could limit many facets of T-cell-mediated pathology by promoting proliferation and cytotoxic functions of CD8 T cells [15], the knowledge of the relationship between levels of IL-27 and Tc17 cells in the lung cancer environment is limited. It is also known that changes in the lung cancer environment are different than those observed in the systemic environment. To the best of our knowledge, it is the first study that identified IL-27 and Tc17 cells in lung cancerous, peritumoral tissues, and corresponding peripheral blood and evaluated the relationship between IL-27 and Tc17 cells in lung cancer patients. Here, we found that the frequency of Tc17 cells

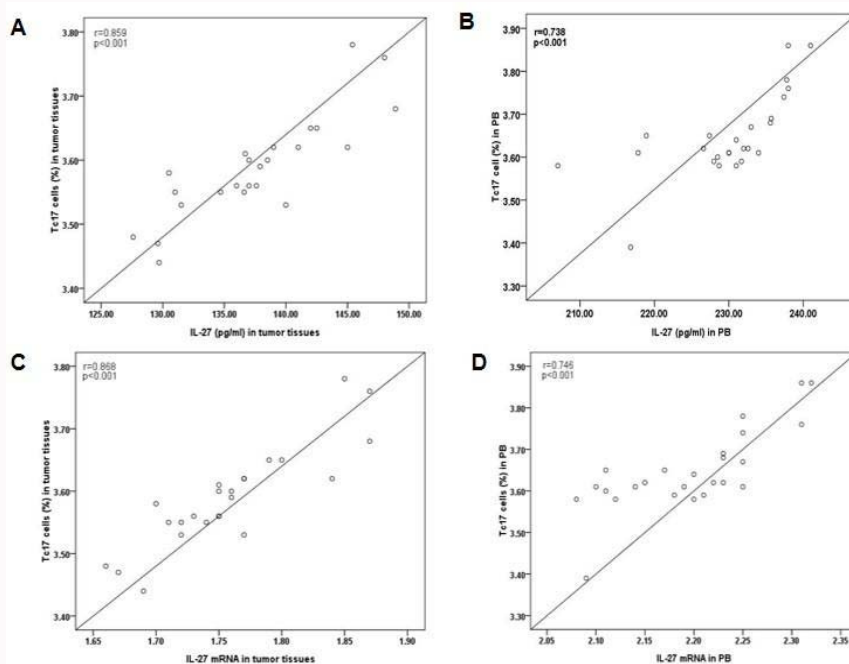


Figure 5: Correlations between (a) the frequency of Tc17 cells and IL-27 protein levels in the tumor specimens, (b) the frequency of Tc17 cells and IL-27 protein levels in the peripheral blood from NSCLC patients, (c) the frequency of Tc17 cells and IL-27 mRNA in the tumor specimens, (d) the frequency of Tc17 cells and IL-27 mRNA in the peripheral blood from NSCLC patients. Data were determined by Spearman's rank correlation coefficients.

and the expression of IL-27 were markedly decreased in peripheral blood from NSCLC patients compared with that in controls. Whereas the serum expression of IL-17A protein and mRNA was markedly increased in patients with NSCLC. In addition, the frequency of Tc17 cells was higher in peritumoral tissues than in local lung cancer environment and systemic response. Whereas the expression of IL-17A and IL-27 was higher in tumor specimens. Moreover, the levels of IL-27 in tumor specimens and peripheral blood from patients were positively correlated with Tc17 cells, but not IL-17A. These findings suggest that Tc17 cells, IL-17A, and IL-27 play important roles in protumor or antitumor immune responses in NSCLC.

Tc17 cells and IL-17A play various roles in inflammation, cancer, and autoimmune diseases [6,16,17]. More recent studies further indicate that Tc17 cells and IL-17A are involved in the initiation, progression, and metastasis of human lung cancer and animal models [7,17,18]. However, their roles in the development of lung cancer are conflicting. Bao et al. [6] reported that the frequency of Tc17 cells in peripheral blood of patients with lung adenocarcinoma was lower than that in healthy controls. After tumor resection, the frequency of Tc17 cells was inversely increased. Song L et al. also reported that reduced peripheral Tc17 cells in NSCLC patients were significantly associated with unfavorable 5-year OS [19]. Using a murine tumor model, the Yu Y group showed that adoptive transfer of tumor-specific Tc17 cells reduced large established tumors [9]. On the contrary, Chen et al. [20] reported that the increased number of Tc17 cells in patients with NSCLC was correlated with poor prognosis. It implicated that Tc17 cells elicit protumor effects. As in the controversial role of Tc17 cells in the lung cancer immunity, IL-17A also showed a paradoxical role. Previous studies have reported that IL-17A production was significantly elevated in peripheral blood of NSCLC, and IL-17A was associated with poor prognosis [20,21]. Notably, transfection of IL-17 into tumor cells augmented the progression of the disease in nude mice. IL-17 promotes NSCLC angiogenesis via the STAT3/GIV

signaling pathway [22]. Contrary to these results, Zhao et al. [23] have shown that there is a significant decrease in serum IL-17 expression in NSCLC patients while compared to that in healthy patients, IL-17A deficiency reduces lung tumor latency and promotes metastasis in Pts4d/d mice [24]. Therefore, Tc17 cells and IL-17A-related cytokine may function as a double-edged sword in lung cancer. In our study, we found decreased percentage of Tc17 cells in peripheral blood of NSCLC patients as compared with healthy controls, while significant higher serum levels of IL-17 in NSCLC patients, suggesting that IL-17A dominantly act to induce protumor immunity, whereas Tc17 cells elicit anti-tumor immunity effects. Combined with our previous study [21], data showed that the expression of IL-17A was significantly positively associated with the frequency of Th17 cells, but was not related to the frequency of Tc17 cells, suggesting that Tc17 cells were not the main source of intracellular IL-17A in NSCLC patients. Notably, these investigations only focus on the systemic immune response. In the present study, we further studied the distribution and regulation of Tc17 cells and IL-17A in tumor tissues and adjacent normal lung tissues. These results showed that the percentages of Tc17 cells were higher in peritumor lung tissues than in tumor tissues and peripheral blood, and the IL-17A expression was even higher in tumor specimens than in peritumoral tissues and peripheral blood, demonstrating the protumor ability of IL-17A further increased and the anti-tumor ability of Tc17 cells further decreased at the local tumor site.

IL-27, mainly produced by activated antigen-presenting cells, has been involved in various cancers through its dual protumor and anti-tumor effects on tumor immunology [25]. Decreased concentration of IL-27 has been reported in lung cancer, and in many cases, decreased IL-27 level was associated with a poor cancer progression. For example, our previously published study reported that serum IL-27 levels were decreased in NSCLC patients as compared with normal controls [21]. Wang et al. [26] also reported that the mRNA and

protein levels of IL-27 were lower in peripheral blood from NSCLC patients as compared with healthy controls, and the levels of IL-27 were lower in the tumor tissues than in adjacent normal tissues. The decreased IL-27 levels in NSCLC blood samples and tumor tissues indicate that IL-27 may function as a tumor suppressor in NSCLC progression. IL-27 can directly suppress cancer cells proliferation, migration, and invasion, and enhance cancer cells apoptosis, or indirectly by stimulating different subsets of immune cells, especially cytotoxic CD8 T cells, and in turn, a more robust antitumor immune response [25]. However, a few studies suggest that IL-27 may have potential tumor-promoting effects in lung cancer. For example, IL-27 levels were elevated in bronchoalveolar lavage supernatants from NSCLC, in a relationship with Early NSCLC clinical stages [27]. IL-27 can directly enhance Treg activity by inducing CD39 expression, or indirectly increase expression of PD-1, CTLA4, LAG-3, and TIM-3 on T cells, thereby promoting tumor growth [25,28]. Our results revealed a significantly decreased expression of IL-27 protein and mRNA in peripheral blood of NSCLC patients. In addition, the expression of IL-27 protein and mRNA was even lower in tumor specimens than in adjacent normal lung tissues and peripheral blood. Therefore, IL-27 may have a significant antitumor role in NSCLC, and the antitumor ability of IL-27 is further decreased at the local tumor site.

Although IL-27, IL-17, and Tc17 cells are present in the tumor microenvironment of NSCLC, little is known about the relationships among IL-27, IL-17, and Tc17 cells. It was reported that Tc17 cells may display suppressed cytotoxic function similarly to the function of Th17 cells in antitumor immunity [29]. In addition, in our previous study, decreased levels of IL-27 negatively correlated with the numbers of Th17 cells and ROR γ mRNA [25]. Furthermore, IL-27 can inhibit the expression of Th17-specific transcription factor ROR γ [30] and IL-17 secretion from T cells [31]. Thus, we speculate that IL-27 may antagonize the function of Tc17 cells in lung cancer progression. In our present study, However, decreased IL-27 levels were positively correlated with Tc17 cells proportions in tumor mass and peripheral blood, and no association of IL-27 with IL-17 was found in NSCLC patients, suggesting that the differentiation activation of Tc17 cells may decline due to the decrease of IL-27 level in NSCLC patients, and that the higher IL-17 levels may originate from any other IL-17 producing T cells. Additionally, the differentiation of Tc17 cells cannot be directly regulated by IL-27 expression. Therefore, it will be intriguing in future studies to elucidate the underlying mechanisms of IL-27 regulating Tc17 cells responses in lung cancer.

Conclusion

In conclusion, Tc17 cells, IL-17A, and IL-27 play important roles in protumor or antitumor immune responses in NSCLC. However, there are some limitations in our study. Firstly, we did not investigate the relationship between IL-27 and the metastasis and staging of patients with NSCLC. Secondly, we did not explore the exact function of IL-27 and Tc17 cells in the development of lung cancer *in vivo* and *in vitro* experiments. Thirdly, we did not know whether the decreased Tc17 cells are required for mediating the inflammatory response in patients with NSCLC. For the last, we did not directly investigate the role of IL-27 for Tc17 induction. Answering these questions would be helpful to better understand the mechanisms involved in lung cancer progression and may lead to the development of a novel therapeutic intervention against lung cancer.

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