Clin. Lab. 2022;68:XXX-XXX

ORIGINAL ARTICLE

Role of Peripheral Blood T Helper 17 Cells in Non-Small Cell Lung Cancer of Different Clinical Stages, Pathological Types and Differentiation Degrees

Kai Fu, Xuefeng Wang, Jingbo Li, Ju Wang

Department of Thoracic Surgery, The First Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang Province, China

SUMMARY

Background: **We aimed to explore the roles of T helper 17 (Th17) cells and cytokines interleukin 17 (IL-17), IL-6, IL-4, transforming growth factor β (TGF-β) and γ-interferon (IFN-γ) in the peripheral blood of patients with non-small cell lung cancer (NSCLC) of different clinical stages, pathological types, and differentiation degrees.**
Methods: **A total of 120 NSCLC patients admitted and 80 healthy people receiving physical examinations from June 2019 to October 2021 were enrolled as the case group (TNM stage: I, II, III and IV; pathological type: adenocarcinoma and squamous cell carcinoma; differentiation degree: low and moderate-high) and the control group, respectively. The serum levels of IL-17, IL-6, IL-4, TGF-β and IFN-γ were measured using an enzyme-linked immunosorbent assay. Th17 cells were counted by flow cytometry.**
Results: **The case group had higher levels of Th17 cells, IL-17, IL-6, IL-4, and TGF-β and lower IFN-γ level in the peripheral blood than those of the control group ($p < 0.05$). NSCLC patients with different TNM stages had significantly different levels of Th17 cell, IL-17, IL-6, TGF-β, and IFN-γ ($p < 0.05$). The expression levels of Th17 and IL-6 in patients with lowly differentiated NSCLC were lower than those of patients with moderately and highly differentiated NSCLC, while the IFN-γ expression level followed an opposite trend ($p < 0.05$). The count of Th17 cells in the peripheral blood of NSCLC patients had significantly positive correlations with IL-17, IL-6, IL-4, and TGF-β levels, but negative correlation with IFN-γ level ($p < 0.05$).**
Conclusions: **The count of Th17 cells increases significantly in NSCLC patients and has correlations with TNM stage and differentiation degree.**
(Clin. Lab. 2022;68:xx-xx. DOI: 10.7754/Clin.Lab.2022.220116)

Correspondence:
Xuefeng Wang
Department of Thoracic Surgery
The First Affiliated Hospital of
Harbin Medical University
Harbin 150001, Heilongjiang Province
China
Email: wangxffahhmu@ecu-edu.cn

Manuscript accepted April 17, 2022

KEYWORDS

non-small cell lung cancer, T helper 17 cell, cytokine

INTRODUCTION

As a malignancy severely endangering human health and life in the world today, lung cancer has a significantly increasing incidence rate in many countries. In China, the incidence rate of lung cancer ranks first among malignant tumors in many cities, and the estimated deaths from lung cancer will be up to 900,000 people every year [1]. There are no specific symptoms and signs in patients with early lung cancer, and most patients have had metastases at diagnosis. According to a recent report, the 1-year survival rate of advanced

lung cancer is only about 40%, with a median survival time of 15.47 months for males and 9.25 months for females [2]. T helper 17 (Th17) cells, a novel kind of helper lymphocytes, are classified into Th1 and Th2 cells. Mature Th1 cells mostly excrete interleukin 2 (IL-2), γ-interferon (IFN-γ), tumor necrosis factor β (TNF-β), and TNF-α and mediate cellular immunity. Th2 cells mainly secrete IL-4, IL-5, IL-6, and IL-10 and manage fluid immunity [3,4]. Th17 cells are able to specifically secrete IL-17 and have close associations with (TGF-β) and IL-6 [5]. Besides, they mediate the inflammatory response of the body and participate in the development and progression of autoimmune diseases, tumors, and so on, and determine the outcome and prognosis of diseases [6,7]. In addition, they are closely correlated with the development and progression of tumors [8,9]. In this study, investigation was conducted on the expression level and significance of Th17 cells in the peripheral blood and cytokines IL-17, IL-6, IL-4, TGF-β, and IFN-γ of patients with non-small cell lung cancer (NSCLC) of different clinical stages, pathological types, and differentiation degrees, providing guidance for prevention, diagnosis, and treatment.

MATERIALS AND METHODS

This study was approved by the ethics committee of the hospital and conducted after obtaining the signed informed consent from enrolled subjects. A total of 120 blood samples from NSCLC patients admitted from June 2019 to October 2021 (case group) were retrospectively analyzed. Inclusion criteria: patients newly diagnosed as primary NSCLC by cytology or pathology, and not undergoing surgery, radiotherapy, chemotherapy, molecular targeting, and immunobiological treatment before blood drawing were included as the case group (n = 120). The people with good health and no relationships with all subjects were included as the control group (n = 80). Exclusion criteria: patients with chronic obstructive pulmonary disease, bronchial asthma, various acute and chronic infections, and autoimmune diseases were excluded.

The case group included 71 males and 49 females aged 34 - 78 (56.89 ± 6.54) years old. As for tumor-node-metastasis (TNM) stage, there were 30 cases of stage I, 34 cases of stage II, 44 cases of stage III, and 12 cases of stage IV. In terms of pathological type, 69 cases were adenocarcinoma and 51 cases were squamous cell carcinoma. For the differentiation degree of cancer tissues, 54 cases were lowly differentiated and 66 cases were moderately and highly differentiated. Among 80 people receiving physical examinations in the same period of the control group, 48 were males and 32 were females, who were aged 35 - 67 (56.84 ± 6.48) years old. The gender ratio and age showed no significant difference between the two groups ($p > 0.05$).

FACSCalibur flow cytometer (BD, USA) was employed for flow cytometry. Phycoerhthrin-Cy5 conjugate (PE-Cy5)-labeled anti-human cluster of differentiation 3 (CD3) antibody, orange fluorescein isothiocyanate (FITC)-labeled anti-human CD8 antibody, and PE-labeled anti-human IL-17A antibody were all bought from eBioscience (USA). Phorbol-12-myristate-13-acetate (PMA) and ionomycin were acquired from Sigma (USA). Enzyme-linked immunosorbent assay (ELISA) kits for human IL-17, IL-6, IL-4, TGF-β, and IFN-γ were provided by R&D (USA).

Peripheral venous blood (10 mL) was collected with a heparin anticoagulation tube, followed by separation of mononuclear cells. Next, the cells were stimulated and cultured with PMA and ionomycin, and 2 mL of hemolysin was added to lyse red blood cells. Ten minutes later, the cells were centrifuged, and the supernatant was discarded, followed by addition of 100 μL of fixative solution and fixation at room temperature for 10 minutes. Afterwards, 2 mL of phosphate buffered saline (PBS) was added to the resulting solution, mixed and centrifuged, followed by the discarding of the supernatant. Then, 100 μL of rupture fluid, PE-Cy5 labeled anti-human CD3 antibody, FITC labeled anti-human CD8 antibody, and PE-labeled anti-human IL-17A antibody were added successively, shaken, mixed well, and placed in a dark place at room temperature for 30 minutes. Then, 2 mL of PBS was added, followed by centrifugation and removal of the supernatant. Afterwards, 0.5 mL of 1% paraformaldehyde was added to each tube for fixation, and the resulting product was loaded on FACSCalibur flow cytometer for measurement.

The serum levels of IL-17, IL-6, IL-4, TGF-β, and IFN-γ were measured using ELISA in accordance with the kits' instructions. Briefly, specimens and standards of different concentrations were added to corresponding wells and then cultured. Next, the plate was washed, and specific enzyme solution was added, followed by plate washing. After color developing solution was added, the reaction was terminated, followed by testing.

SPSS 26.0 software was utilized for statistical analysis. The measurement data were expressed as mean ± standard deviation ($\bar{x} \pm s$) and compared between two groups using a *t*-test and among groups by analysis of variance. For the data with significance in analysis of variance, the LSD or Dunnett's T3 test was conducted for further pairwise comparison. The numerical data were expressed as ratio and subjected to the χ^2 test. Spearman's analysis was employed to study correlations. $p < 0.05$ suggested a statistically significant difference.

RESULTS

The case group exhibited higher levels of Th17 cells, IL-17, IL-6, IL-4, and TGF-β and a lower IFN-γ level in the peripheral blood in contrast with the control group ($p < 0.05$) (Table 1).

The level of Th17 cells was significantly different among NSCLC patients with different TNM stages ($p <$

Table 1. Levels of Th17 cells and related cytokines.

Indicator	Case group (n = 120)	Control group (n = 80)	p
Th17 (%)	3.81 ± 0.29	1.84 ± 0.19	< 0.001
IL-17 (pg/mL)	8.76 ± 0.54	4.31 ± 0.51	< 0.001
IL-6 (pg/mL)	13.27 ± 1.12	5.24 ± 0.56	< 0.001
IL-4 (pg/mL)	10.61 ± 1.02	5.62 ± 0.46	< 0.001
TGF-β (ng/mL)	26.23 ± 1.76	20.03 ± 1.01	< 0.001
IFN-γ (ng/mL)	21.73 ± 1.45	27.84 ± 2.04	< 0.001

Table 2. Correlations of levels of Th17 cells and related cytokines with clinicopathologic characteristics.

	Stage I (n = 50)	Stage II (n = 34)	Stage III (n = 44)	Stage IV (n = 12)	F	p
Th17 (%)	3.21 ± 0.21	3.86 ± 0.11 *	3.94 ± 0.11 **, #	3.96 ± 0.11 **, #	55.654	< 0.001
IL-17 (pg/mL)	8.34 ± 0.51	8.70 ± 0.34 *	8.94 ± 0.32 **	8.83 ± 0.34 **	11.231	< 0.001
IL-6 (pg/mL)	11.08 ± 1.31	13.89 ± 0.51 **	13.76 ± 0.59 **	13.46 ± 0.76 **	31.221	< 0.001
IL-4 (pg/mL)	11.03 ± 1.08	10.66 ± 0.78	10.35 ± 1.02	10.31 ± 0.38	0.982	> 0.05
TGF-β (ng/mL)	23.89 ± 1.08	25.92 ± 1.23 **	26.76 ± 1.57 **	27.12 ± 1.38 **	9.297	< 0.001
IFN-γ (ng/mL)	23.76 ± 1.32	22.11 ± 1.13 **	21.19 ± 1.34 **, #	20.16 ± 0.41 **, ##	11.432	< 0.001

*- p < 0.05 vs. stage I, ** - p < 0.001 vs. stage I, #- p < 0.05 vs. stage II, ## - p < 0.05 vs. stage II.

Table 3. Levels of Th17 cells and related cytokines in NSCLC patients with different pathological types.

	Adenocarcinoma (n = 69)	Squamous carcinoma (n = 51)	p
Th17 (%)	3.84 ± 0.28	3.75 ± 0.31	> 0.05
IL-17 (pg/mL)	8.82 ± 0.46	8.69 ± 0.45	> 0.05
IL-6 (pg/mL)	13.41 ± 1.18	13.15 ± 1.45	> 0.05
IL-4 (pg/mL)	10.65 ± 1.02	10.54 ± 1.09	> 0.05
TGF-β (ng/mL)	26.13 ± 1.78	25.87 ± 1.84	> 0.05
IFN-γ (ng/mL)	21.46 ± 1.45	22.29 ± 1.79	> 0.05

Table 4. Levels of Th17 cells and related cytokines in NSCLC patients with different differentiation degrees.

	Lowly differentiated (n = 54)	Moderately and highly differentiated (n = 66)	p
Th17 (%)	3.65 ± 0.34	3.91 ± 0.18	< 0.05
IL-17 (pg/mL)	8.65 ± 0.49	8.82 ± 0.34	> 0.05
IL-6 (pg/mL)	12.78 ± 1.67	13.72 ± 0.81	< 0.05
IL-4 (pg/mL)	10.48 ± 1.12	10.64 ± 0.89	> 0.05
TGF-β (ng/mL)	25.45 ± 2.09	26.43 ± 1.65	> 0.05
IFN-γ (ng/mL)	22.43 ± 1.56	21.34 ± 1.34	< 0.05

0.05). Such a level was significantly higher in stage II, III, and IV patients than that in stage I patients ($p < 0.05$). It was also significantly higher in stage III and IV patients than that in stage II patients ($p < 0.05$). Besides, a significant difference was found in IL-17 level among NSCLC patients with different TNM stages ($p < 0.05$). The IL-17 level significantly rose in stage II, III, and IV patients compared with that in stage I patients ($p < 0.05$). In addition, the IL-6 level exhibited significant differences among NSCLC patients with different TNM stages ($p < 0.05$). In contrast with stage I patients, stage II, III, and IV patients had a significantly elevated IL-6 level ($p < 0.05$). However, no significant difference was found in IL-4 level among NSCLC patients with different TNM stages ($p > 0.05$). The level of TGF-β was significantly different among NSCLC patients with different TNM stages ($p < 0.05$). The level of TGF-β was significantly higher in stage II, III and IV patients than that in stage I patients ($p < 0.05$). The level of IFN-γ displayed significant differences among NSCLC patients with different TNM stages ($p < 0.05$). In comparison with stage I patients, stage II, III and IV patients had a significantly declined IFN-γ level, and the difference was of statistical significance ($p < 0.05$). The level of IFN-γ was significantly lower in stage III and IV patients than that in stage II patients ($p < 0.05$) (Table 2). The levels of Th17 cells, IL-17, IL-6, IL-4, TGF-β, and IFN-γ had no significant differences among NSCLC patients with different pathological types ($p > 0.05$) (Table 3).

Patients with moderately and highly differentiated NSCLC exhibited a significantly higher level of Th17 cells and a significantly lower level of IL-6 than patients with lowly differentiated NSCLC ($p < 0.05$). The level of IFN-γ was also significantly different among patients with different differentiation degrees ($p < 0.05$), which significantly declined in patients with moderately and highly differentiated NSCLC in contrast with patients with lowly differentiated NSCLC. However, no significant differences were detected in the levels of IL-17, IL-4, and TGF-β among patients with different differentiation degrees ($p > 0.05$) (Table 4).

The level of Th17 cells was significantly positively related to the levels of IL-17 ($r = 0.713$, $p < 0.05$), IL-6 ($r = 0.802$, $p < 0.05$), TGF-β ($r = 0.756$, $p < 0.05$), and IL-4 ($r = 0.467$, $p < 0.05$), but it was significantly negatively associated with IFN-γ level ($r = -0.689$, $p < 0.05$) in the peripheral blood of NSCLC patients.

DISCUSSION

Th17 cells are vital players in the development and progression of various diseases, as well as the maintenance of immune homeostasis, whose discovery covers the shortages of Th1/Th2 mediated effect mechanisms. Deeply researching their differentiation, physiological and pathological functions and regulatory mechanisms is of theoretical and practical significance for vaccine design and studies on infectious diseases, autoimmune diseases, tumors, and transplant rejection. Besides, Th17 cells have independent differentiation and developmental regulation mechanisms and specifically secrete IL-17 effector factors [10,11]. IL-17, a crucial player in the development and progression of tumors, facilitates tumorigenesis. IL-17 significantly increases the number and density of microvessels in tumors mainly through its chemotactic effect on vascular endothelial cells, its up-regulation of the expression of pro-angiogenic factors, and its promotion of angiogenesis by cooperating with other angiogenic factors and, thereby, indirectly expediting the growth, metastasis, and infiltration of tumors [12]. It was found in this study that the level of IL-17 was significantly higher in the peripheral blood of NSCLC patients than that in the healthy control group. According to analysis on the difference of IL-17 level among patients with different TNM stages of NSCLC, in contrast with that in stage I patients, the IL-17 level rose significantly in stage II, III, and IV patients. However, such a level showed no significant difference among patients with different types and differentiation degrees of NSCLC, which is in line with the findings of a study conducted by Kirshberg et al. [10]. When the immune function declines, the probability of tumor occurrence increases. Meanwhile, when tumor progresses, the immune function is inhibited. As a result, the onset and progression of tumors are promoted, and proinflammatory cytokine IL-17 recruits granulocytes, thereby facilitating the release of inflammatory factors from various cells. It also participates in the occurrence of inflammatory diseases, autoimmune diseases, and tumors [13]. Possibly, the level of IL-17 increases with the progression of disease.

The differentiation of Th17 cells is regulated by factors such as TGF-β and IL-6, and the combined action of TGF-β and IL-6 in the tumor microenvironment can induce such differentiation processes. TGF-β and IL-6 are factors triggering the differentiation of Th17 cells. If TGF-β and IL-6 coexist, activated $CD4^+$ T cells will activate the specific transcription factor retinoic acid-related orphan receptor γt (ROR-γt) through signal transduction and conduction by the activator of transcription 3 (STAT3) signal pathway. ROR-γt enables activated $CD4^+$ T cells to differentiate into Th17 cells [14,15]. An *in vitro* culture study denoted that the absence of TGF-β or addition of IL-6 receptor antibody is capable of blocking the induction of Th17, resulting eventually in a reduced proportion of Th17 [16]. In this study, the levels of TGF-β and IL-6 were significantly higher in the peripheral blood of NSCLC patients than those in the healthy control group, and significantly positively related to the level of Th17 cells, consistent with the research findings of Veldhoen et al. [14].

IFN-γ and IL-4, major effector cytokines, are produced by Th1 and Th2 cells, respectively. The presence of IFN-γ or IL-4 represses the differentiation of Th17 cells. If IFN-γ exists, the STAT-1 signaling pathway will be activated, and T-bet, a specific transcription fac-

tor of Th1 cells, will be induced to differentiate into Th1. If IL-4 exists, STAT-6 will be activated by activated $CD4^+$ T cells, and GATA-3, a specific transcription factor of Th2 cells, will be induced to differentiate into Th2 [17-19].

CONCLUSION

In this study, the level of IL-4 was significantly elevated in the peripheral blood of NSCLC patients compared with that in the healthy control group, and it had a significantly positive correlation with the level of Th17 cells. However, the level of IFN-γ significantly declined in the peripheral blood of NSCLC patients in comparison with that in the healthy control group, and was significantly negatively correlated with the level of Th17 cells. The results of this study are in accord with results of a previous study [20] that the immune cells in the peripheral blood of lung cancer patients have the advantage of Th2-type immune response, with Th1/Th2 drifting. We postulate that the progression of lung cancer can be inhibited by down-regulating Th17 and regulating the expression of related cytokines, providing novel insights into the immunotherapy for lung cancer.

Source of Funds:
This study was financially supported by Young Reserve Talent Project of Harbin Science and Technology Bureau (No. 2016RAQXJ170).

Declaration of Interest:
The authors declare no conflict of interest.

References:

1. Mao Y, Yang D, He J, Krasna MJ. Epidemiology of Lung Cancer. Surg Oncol Clin N Am 2016;25:439-45. (PMID: 27261907)

2. Zhou M, Wang H, Zeng X, et al. Mortality, morbidity, and risk factors in China and its provinces, 1990 - 2017: a systematic analysis for the Global Burden of Disease Study 2017. Lancet 2019; 394:1145-58. (PMID: 31248666)

3. Harrington LE, Hatton RD, Mangan PR, et al. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat Immunol 2005;6:1123-32. (PMID: 16200070)

4. Wu HJ, Ivanov II, Darce J, et al. Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. Immunity 2010;32:815-27. (PMID: 20620945)

5. Michalak-Stoma A, Bartosinska J, Kowal M, Juszkiewicz-Borowiec M, Gerkowicz A, Chodorowska G. Serum levels of selected Th17 and Th22 cytokines in psoriatic patients. Dis Markers 2013; 35:625-31. (PMID: 24288431)

6. Martin-Orozco N, Muranski P, Chung Y, et al. T helper 17 cells promote cytotoxic T cell activation in tumor immunity. Immunity 2009;31:787-98. (PMID: 19879162)

7. Crome SQ, Wang AY, Levings MK. Translational mini-review series on Th17 cells: function and regulation of human T helper 17 cells in health and disease. Clin Exp Immunol 2010;159:109-19. (PMID: 19912252)

8. Punt S, Langenhoff JM, Putter H, Fleuren GJ, Gorter A, Jordanova ES. The correlations between IL-17 vs. Th17 cells and cancer patient survival: a systematic review. Oncoimmunology 2015; 4:e984547. (PMID: 25949881)

9. Liu Y, Guo QF, Chen JL, et al. Correlations between alterations of T-helper 17 cells and treatment efficacy after concurrent radiochemotherapy in locally advanced cervical cancer (stage IIB-IIIB): a 3-year prospective study. Chin Med J (Engl) 2021;134: 954-62. (PMID: 33840740)

10. Kirshberg S, Izhar U, Amir G, et al. Involvement of CCR6/CCL20/IL-17 axis in NSCLC disease progression. PLoS One 2011;6:e24856. (PMID: 21949768)

11. Chen X, Wan J, Liu J, et al. Increased IL-17-producing cells correlate with poor survival and lymphangiogenesis in NSCLC patients. Lung Cancer 2010;69:348-54. (PMID: 20022135)

12. Lu Y, Lin H, Zhai K, Wang X, Zhou Q, Shi H. Interleukin-17 inhibits development of malignant pleural effusion via interleukin-9-dependent mechanism. Sci China Life Sci 2016;59:1297-304. (PMID: 27535421)

13. Onishi RM, Gaffen SL. Interleukin-17 and its target genes: mechanisms of interleukin-17 function in disease. Immunology 2010; 129:311-21. (PMID: 20409152)

14. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. Immunity 2006;24:179-89. (PMID: 16473830)

15. Lin ZW, Wu LX, Xie Y, et al. The expression levels of transcription factors T-bet, GATA-3, RORγt and FOXP3 in peripheral blood lymphocyte (PBL) of patients with liver cancer and their significance. Int J Med Sci 2015;12:7-16. (PMID: 25552913)

16. Wang X, Wang L, Mo Q, Dong Y, Wang G, Ji A. Changes of Th17/Treg cell and related cytokines in pancreatic cancer patients. Int J Clin Exp Pathol 2015;8:5702-8. (PMID: 26191284)

17. Shahid A, Bharadwaj M. The connection between the Th17 cell related cytokines and cancer stem cells in cancer: Novel therapeutic targets. Immunol Lett 2019;213:9-20. (PMID: 31278971)

18. Stockinger B, Omenetti S. The dichotomous nature of T helper 17 cells. Nat Rev Immunol 2017;17:535-44. (PMID: 28555673)

19. Dong C. TH17 cells in development: an updated view of their molecular identity and genetic programming. Nat Rev Immunol 2008;8:337-48. (PMID: 18408735)

20. Li J, Wang Z, Mao K, Guo X. Clinical significance of serum T helper 1/T helper 2 cytokine shift in patients with non-small cell lung cancer. Oncol Lett 2014;8:1682-6. (PMID: 25202391)