

# T lymphocyte subsets and PD-1 expression on lymphocytes in peripheral blood of patients with non-small cell lung cancer

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## Abstract

The incidence rate and mortality rate of lung cancer (LC) are very high. This study aimed to analyze the T lymphocyte subsets and programmed death-1 (PD-1) expression on lymphocytes in the peripheral blood of non-small cell lung cancer (NSCLC) patients and explore whether there were changes in cellular immunity in NSCLC. Peripheral blood samples were collected from newly diagnosed NSCLC patients and healthy individuals. The T lymphocyte subsets and PD-1 expression were evaluated using flow cytometry. Single-sample gene set enrichment analysis (ssGSEA) was performed to explore the correlations of PD-1 expression with infiltration patterns for tumor-infiltrating T immune cells. By flow cytometry, two populations of lymphocytes in NSCLC patients were observed. Apart from a population of normal volume lymphocytes (Lym1), the other population had larger volume and more particles (Lym2). Compared with the healthy group, the proportion of CD4+ T cells and PD-1 expression on Lym1 was higher, and that of CD8+ T cells was lower in the NSCLC group. In the NSCLC group, the proportions of CD3+ T cells, CD8+ T cells, CD4+CD8+ T (DPT) cells, and PD-1 expression were higher on Lym2 than those on Lym1 ( $P < .05$ ). ssGSEA showed that tumor-infiltrating immune T cells were positively correlated with PD-1 expression. The PD-1 expression on lymphocytes increased in recurrent patients who treated with PD-1 inhibitor. Lym2 may be tumor-infiltrating lymphocytes (TILs) which upregulated PD-1 expression in NSCLC. PD-1 expression on lymphocytes may be used as a recurrence indicator for NSCLC patients treated with PD-1 inhibitors.

**Abbreviations:** NSCLC = non-small cell lung cancer, PD-1 = programmed death-1, PD-L1 = programmed death ligand-1, TILs = tumor-infiltrating lymphocytes.

**Keywords:** non-small cell lung cancer, programmed death 1, recurrent, T lymphocyte subsets, tumor-infiltrating immune cells

## 1. Introduction

Lung cancer (LC), one of the most malignant cancers, is the leading cause of cancer-related mortality.<sup>[1]</sup> Non-small cell lung cancer (NSCLC) is the most important pathological type, accounting for approximately 85% of LC.<sup>[2]</sup> Several therapeutic treatment modalities are available for NSCLC patients, including surgery, chemotherapy, radiotherapy, immunotherapy, and targeted therapy.<sup>[3]</sup> Although these treatments can improve patient prognosis, the 5-year survival rate for advanced-stage NSCLC remains low.<sup>[4]</sup> Programmed death-1 (PD-1, also termed CD279) is an immune checkpoint receptor expressed in activated immune cells that negatively regulate immune responses. It can promote immune tolerance by downregulating the immune system response and inhibiting T-cell inflammatory

response, promoting tumor cell immunosurveillance evasion.<sup>[5,6]</sup> The overexpression of programmed death ligand-1 (PD-L1) on tumor cells is a major immune-suppressive mechanism that acts via PD-1/PD-L1 axis.<sup>[7]</sup> PD-L1 expressed on tumor cells interacts with PD-1 expressed on activated immune cells, and enable tumor cells to escape T cell-mediated immunosurveillance.<sup>[8]</sup> Chronic stimulation leads to PD-1 overexpression on CD8+ T cells, leading to CD8+ T cell exhaustion and dysfunction.<sup>[9]</sup> PD-1 inhibitors can reactivate the immune system and enhance tumor treatment efficacy.<sup>[10]</sup> The FDA has approved some PD-1 and PD-L1 inhibitors for treating solid tumors. However, there are difficulties in their application as not all NSCLC patients are sensitive to PD-1/PD-L1 inhibitors, or patients may eventually develop drug resistance. Thus, determining the suitable population for immunotherapy and finding effective monitoring

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The study was approved by the Zhejiang Province Hospital of Traditional Chinese Medicine (2020-K-267-01). All studies were performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

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biomarkers that can effectively predict the efficacy of PD-1 inhibitors is imperative.

In this study, we analyzed T lymphocyte subsets in peripheral blood of NSCLC patients and healthy subjects using flow cytometry. A small proportion of lymphocytes with larger volume and more particles were observed in the peripheral blood of NSCLC patients but rarely in healthy subjects. Subsequently, we analyzed these two lymphocyte subsets in NSCLC. The appearance of CD4+CD8+ DPT cells and increased expression of PD-1 on lymphocytes with larger volume and more particles suggested that these cells may be involved in immune regulation of NSCLC. Since there are no laboratory indicators with high diagnostic efficiency for early LC, the analysis of lymphocytes in peripheral blood may provide a new auxiliary index for the early diagnosis of NSCLC. In addition, the PD-1 expression on lymphocytes increased when relapsed. It may also provide monitoring biomarkers for NSCLC patients treated with PD-1 inhibitors.

## 2. Materials and methods

### 2.1. Patients and controls

Thirty-nine patients, who were newly diagnosed with NSCLC in the Respiratory Department of the Zhejiang Province Hospital of Traditional Chinese Medicine, Hangzhou, Zhejiang, China, were enrolled in the NSCLC group between January 2020 and December 2020 (Table 1), whereas 31 patients who came to our hospital for general physical examination were selected as the healthy control group. This study was approved by the Zhejiang Province Hospital of Traditional Chinese Medicine (2020-K-267-01), and informed consent was obtained from all participants.

The following inclusion criteria were used for enrolling study subjects: NSCLC patients diagnosed according to the National Comprehensive Cancer Network guidelines for diagnosis and treatment of primary LC (Version 3.2018), NSCLC patients who had not undergone any surgery, radiotherapy, chemotherapy, targeted therapy, or immunotherapy, and healthy controls with no obvious lung abnormalities, [low-dose spiral chest CT (LDCT) revealing no nodules, inflammation, or lung tumors]. The exclusion criteria were as follows: patients with other tumors, patients under 18 years of age, and patients with other systemic diseases, including inflammation and autoimmune diseases.

### 2.2. T lymphocyte subsets and PD-1 expression

We performed flow cytometry to detect T lymphocyte subsets and PD-1 expression in NSCLC patients and healthy

individuals. Cells with CD3+CD45+ phenotype represented the total T cells, those with CD3+CD4+ phenotype represented CD4+ T helper cells, while cells with CD3+CD8+ phenotype represented CD8+ T cytotoxic cells. The monoclonal antibodies, CD279-PE, CD45-FITC, CD4-APC, CD3-PerCP-Cy5.5, and CD8-PE-Cy7, were procured from BD Biosciences (USA). Briefly, peripheral blood samples (2 mL) were collected in ethylenediaminetetraacetic acid (EDTA) collection tubes and used for detection within 24 hours. Next, 40  $\mu$ L peripheral blood samples and 10  $\mu$ L monoclonal antibodies were mixed and incubated for 15 to 20 minute at 25°C in the dark. A buffer containing 0.8% NH<sub>4</sub>Cl and 0.1% KHCO<sub>3</sub> (pH 7.1-7.4) was used for hemolysis. The cells were washed with phosphate-buffered saline (PBS), resuspended in 200  $\mu$ L PBS, and analyzed using a flow cytometer (FACSCanto™ II, BD, USA) and MultiSET software.

CD45 was used to label leukocytes and lymphocyte populations were distinguished by flow cytometry.

The forward scattered (FSC) light represents the size of cells and the side scattered (SSC) light represents the granularity of the cells. In NSCLC, there were two distinct populations of lymphocytes, one population with the same volume and particles as normal lymphocytes was defined as Lym1, and the other population with larger volume and more particles than normal lymphocytes was defined as Lym2.

### 2.3. Correlation analysis of immune infiltration

The LUAD-TCGA database (July 8, 2022) including clinical information and processed RNA-sequencing expression (level 3) data were downloaded from The Cancer Genome Atlas (TCGA) (<https://portal.gdc.cancer.gov/>). A total of 535 tumor samples were collected for analyze immune infiltration. GSEA package was used to assess the correlations of PD-1 expression with infiltration patterns for tumor-infiltrating T immune cells using ssGSEA algorithms.

### 2.4. Statistical analysis

Statistical analysis was performed using SPSS software version 23.0 (IBM Corp., Armonk, NY). The variables are presented as median (interquartile range [IQR]) if they were not normally distributed. The two groups were compared using Mann – Whitney *U*-tests. The R package ggplot2 (R software, version 3.6.3) was used for data visualization. Results with *P*-value < .05 were considered statistically significant.

## 3. Results

### 3.1. T lymphocyte subsets and PD-1 expression in NSCLC and healthy groups

T lymphocyte subsets and PD-1 expression in peripheral blood of the NSCLC patients and healthy individuals were analyzed using flow cytometry. We found a subgroup of lymphocytes with larger volumes and more particles in the NSCLC group that was rarely observed in the healthy group; these were defined as “Lym2.” Lym2 were a group of lymphocytes with large aniline granules in the cytoplasm. These Lym2 included lymphocytes with CD3+CD4+, CD3+CD8+, and CD3+CD4+CD8+ phenotype and had enhanced expression of PD-1 (CD279) (Fig. 1).

### 3.2. Proportions of peripheral blood lymphocytes in leukocytes

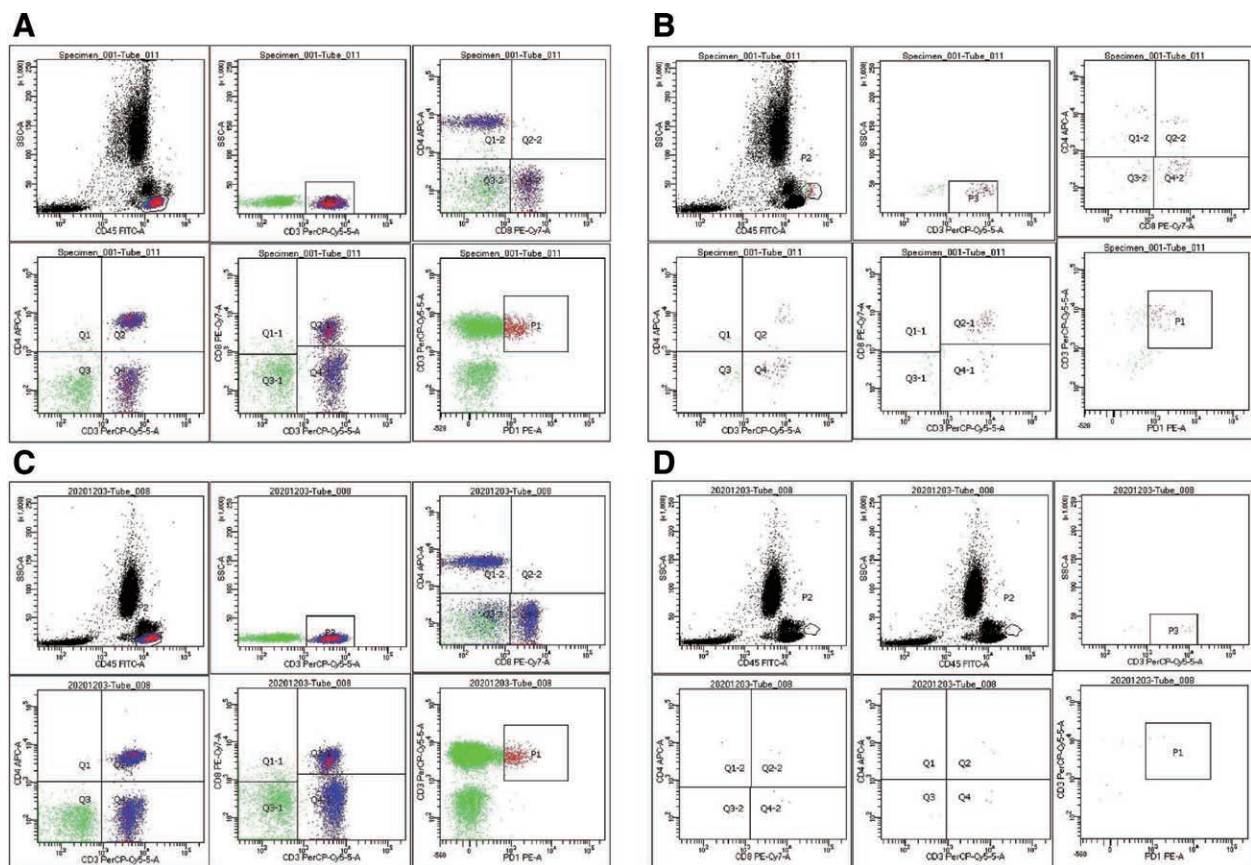
The proportions of Lym1 and Lym2 in leukocytes were compared between the NSCLC and healthy groups. The median

**Table 1**

**The clinical feature of the NSCLC group.**

	Characteristic	NSCLC, %(n)
Pathological classification	Adenocarcinoma	89.74% (35)
	Squamous carcinoma	10.26% (4)
T stage, %(n)	Tis	15.38% (6)
	T1	53.85% (21)
	T2	15.38% (6)
	T3	2.56% (1)
	T4	12.82% (5)
N stage, %(n)	N0	74.36% (29)
	N1	2.56% (1)
	N2	7.69% (3)
	N3	15.38% (6)
M stage, %(n)	M0	89.74% (35)
	M1	10.26% (4)
Typical respiratory symptoms, %(n)	Cough	23.08% (9)
	Expectoration	10.26% (4)
	Chest tightness	5.13% (2)

NSCLC = non-small cell lung cancer.



**Figure 1.** Flow cytometric evaluation of T lymphocyte subsets and PD-1 expression on lymphocytes present in peripheral blood. (A) T lymphocyte subsets and PD-1 expression on Lym1 in NSCLC group. (B) T lymphocyte subsets and PD-1 expression on Lym2 in NSCLC group. (C) T lymphocyte subsets and PD-1 expression on Lym1 in healthy group. (D) T lymphocyte subsets and PD-1 expression on Lym2 in healthy group. NSCLC = non-small cell lung cancer, PD-1 = programmed death 1.

Lym1 percentage was 16.00 (12.15, 20.25) in the NSCLC group and 26.20 (23.10, 32.45) in the healthy group. There was a significant difference in the Lym1 percentage between the two groups ( $P < .05$ ). The median Lym2 percentage in the NSCLC group was 0.30 (0.20, 0.65) and 0.00 (0.00, 0.00) in the healthy group, and a significant difference was observed in Lym2 percentage between the two groups ( $P < .05$ ) (Fig. 2).

### 3.3. T lymphocyte subsets and PD-1 expression on Lym1 in NSCLC and healthy groups

T lymphocyte subsets and PD-1 expression were compared between NSCLC and healthy groups by gating Lym1. Significant differences were noted in percentages of Lym1 cells with CD3+CD4+ and CD3+CD8+ phenotypes, and PD-1 expression between the two groups ( $P < .05$ ), but not between the percentages of Lym1 cells with CD3+CD45+ and CD3+CD4+CD8+ phenotype ( $P > .05$ ) (Fig. 3).

### 3.4. T lymphocyte subsets and PD-1 expression on Lym1 and Lym2 cells in NSCLC group

T lymphocyte subsets and PD-1 expression were compared between Lym1 and Lym2 in NSCLC group. We found that the percentages of cells with CD3+CD45+, CD3+CD8+, and CD3+CD4+CD8+ phenotype as well as PD-1 expression were significantly higher on Lym2 than on Lym1 ( $P < .05$ ) (Fig. 4).

### 3.5. Correlation analysis of immune infiltration

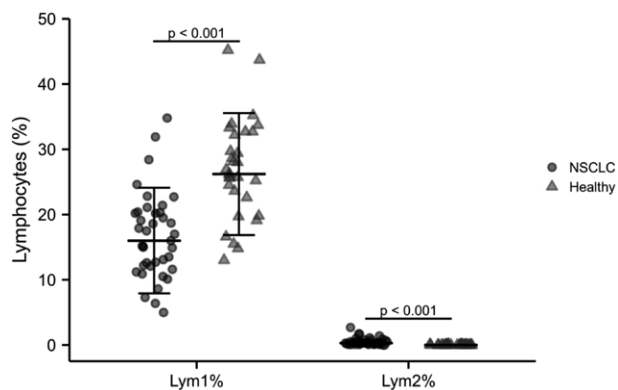
The correlation of PD-1 and T cells was analyzed in tumor tissues. PD-1 and total T cells: Spearman correlation coefficient  $R = 0.735$  ( $P < .05$ ), PD-1 and CD4+ T cells: Spearman correlation coefficient  $R = 0.363$  ( $P < .05$ ), PD-1 and CD8+ T cells: Spearman correlation coefficient  $R = 0.484$  ( $P < .05$ ) (Fig. 5).

### 3.6. PD-1 expression on lymphocytes before and after relapse using PD-1 inhibitor

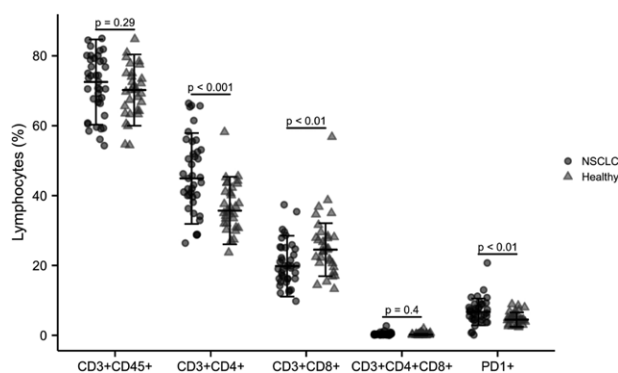
PD-1 expression in a NSCLC patient who treated with PD-1 inhibitor was detected. When using PD-1 inhibitor, there was no PD-1 expression on peripheral blood lymphocytes, including Lym1 and Lym2. However, four months later, the patient relapsed. The PD-1 expression on lymphocytes increased, and reached 6.10% on Lym1 and 29.00% on Lym2, respectively.

## 4. Discussion

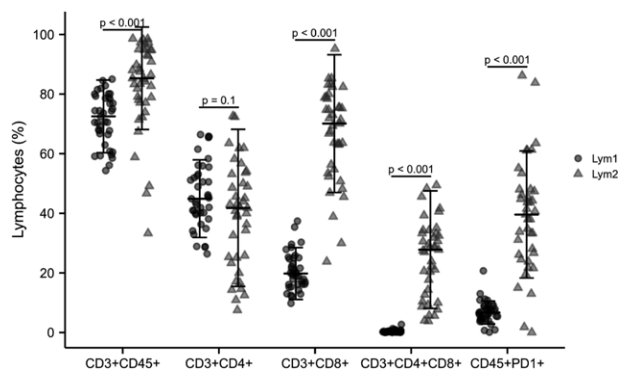
NSCLC is the primary pathological type of LC, with higher incidence and poor prognosis<sup>[11]</sup> PD-1 expressed on tumor cells interacts with PD-L1 expressed on immune cells as an immune checkpoint molecule, resulting in immune cell deactivation and tumor cells evading immune surveillance.<sup>[10]</sup> In the tumor microenvironment (TME), the upregulation of T cell checkpoint molecules leads to the failure of T cells to proliferate, under activation, and secrete cytokines to destroy tumor cells.<sup>[12]</sup>



**Figure 2.** Comparative analysis of lymphocyte proportion in leukocytes between NSCLC and healthy groups. NSCLC = non-small cell lung cancer.



**Figure 3.** Comparative analysis of T lymphocyte subsets and PD-1 expression on Lym1 between NSCLC and healthy groups. NSCLC = non-small cell lung cancer, PD-1 = programmed death 1.

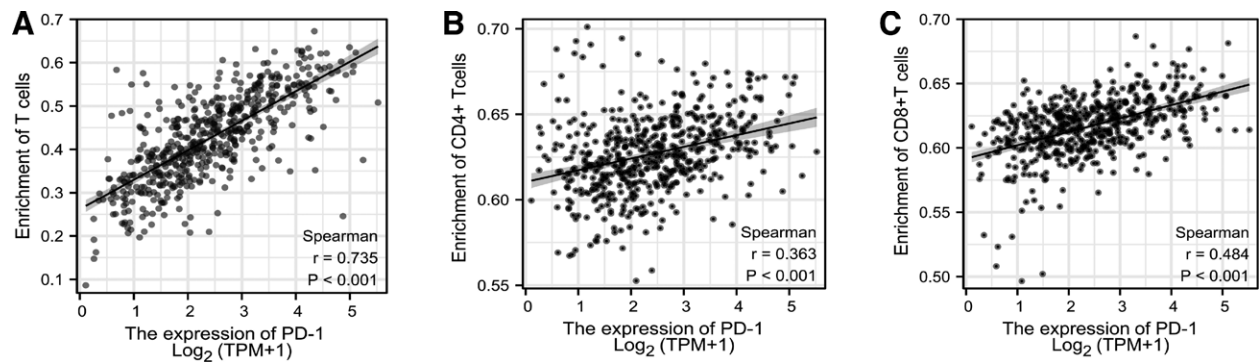


**Figure 4.** Comparative analysis of T lymphocyte subsets and PD-1 expression between Lym1 and Lym2 in NSCLC group. NSCLC = non-small cell lung cancer, PD-1 = programmed death 1.

Some PD-1/PD-L1 inhibitors have been approved by the FDA to treat solid tumors, such as melanoma and NSCLC, as they significantly improve patient survival. However, PD-1 inhibitors are not effective for all NSCLC patients, as the patients may be either be insensitive to these inhibitors or develop resistance during immunotherapy. Factors affecting the efficacy of PD-1/PD-L1 inhibitors include accumulation of CD4+foxp3-PD-1<sup>hi</sup> tumor cells that inhibit T cell function.<sup>[13]</sup> Additionally, myeloid-derived immune cells, such as monocytes and DC cells, can be used as biomarkers to monitor the efficacy of PD-1 inhibitor.<sup>[14,15]</sup> In this study, we found a population of lymphocytes

with larger volumes and more particles in NSCLC patients. We explore whether the phenotype and PD-1 expression of these lymphocytes were consistent with those of the normal volume lymphocytes.

Compared with the healthy individuals, the percentage of Lym1 decreased while the percentage of Lym2 increased in the NSCLC group. Since Lym2 was almost absent in healthy individuals, we compared the T lymphocyte subsets and PD-1 expression on Lym1 between NSCLC and healthy groups. The proportion of CD4+ T helper cells and PD-1 expression was higher and the proportion of CD8+ T cytotoxic cells was lower in NSCLC group than in healthy group ( $P < .05$ ). T helper cells and T cytotoxic cells prevent tumor progression by targeting tumor-associated antigens.<sup>[16]</sup> CD4+ T helper cells assist CD8+ cytotoxic T cell-mediated tumor killing, and a high proportion of activated CD8+ cytotoxic T cells predicts good prognosis.<sup>[16,17]</sup> There was evidence that the percentages of CD4+ T cells and natural killer T (NKT) cells increased within one week after tumor inoculation.<sup>[18]</sup> In our study, Lym1 accounted for the majority proportion of lymphocytes in NSCLC. The NSCLC group had increased percentage of CD4+ T helper cells in Lym1, possibly due to tumor antigen stimulation, which makes naive CD4+ T cells get activated, proliferate, and differentiate into cells with different effector phenotypes. Moreover, decreased proportion of CD8+ cytotoxic T cells, while increased PD-1 expression in the NSCLC group, suggested T cell depletion in TME. In the NSCLC group, the proportions of CD8+ T cytotoxic cells, CD4+CD8+ DPT cells, and PD-1 expression on Lym2 were higher than those on Lym1 ( $P < .05$ ). Different distribution frequencies were observed for Lym1 and Lym2 subsets in NSCLC. In Sanmamed study, they identified a burned-out CD8+ tumor-infiltrating lymphocytes (TILs) in TME in NSCLC, which highly expressed PD-1.<sup>[19]</sup> The phenotypes of Lym2 in our study were similar to these burned-out TILs, including high proportion of CD8+ and PD-1 expression. In addition, we analyzed the correlation between tumor infiltrating T lymphocytes and PD-1 in tumor tissues, and the results showed that there was a linear positive correlation between tumor infiltrating T lymphocytes and PD-1 expression. In Akiko Arakawa et al study, they identified TCR rearrangements of circulating T cells and TILs by sequencing and found that there were some common TCR rearrangements between peripheral blood T lymphocytes and TILs.<sup>[20]</sup> Considering that our Lym2 highly expressed PD-1, we speculated that Lym2 may contain a subgroup of burned-out TILs in TME. The PD-1 overexpression on lymphocytes in the NSCLC group, especially on Lym2 subset, indicated that Lym2 subset may participate in tumor immune escape via upregulating the expression of immune checkpoint. Interestingly, CD4+CD8+ DPT cells were observed in Lym2 subset. DPT cells were previously considered precursors of mature T cells.<sup>[21]</sup> Recently, it has been proposed that CD4+CD8+ DPT cells are involved in immune response to chronic viral infections, autoimmune diseases, and tumors.<sup>[22]</sup> Caraballo et al reported increased proportion of CD4+CD8+ DPT cells, and overexpression of PD-1 and T cell immunoglobulin and mucin-domain-containing molecule-3 (Tim-3) in chronic viral infections.<sup>[23]</sup> Additionally, increased number of circulating CD4+CD8+ DPT cells was observed in urinary tumors and Hodgkin's lymphoma.<sup>[24]</sup> Bo Zheng et al analyzed the trajectory and function of PD-1<sup>high</sup>CD4+CD8+ DPT cells in hepatocellular carcinoma by single cell flow cytometry and transcriptome sequencing, and found that these DPT cells are closely related to tumor, share ancestors with depleted CD8+ T cells, and have higher expression similarity and closer pathological localization with PD-1+CD8+ T cells.<sup>[25]</sup> To sum up, we found that the proportion of CD8+ T in the circulation of NSCLC patients decreased, which was likely due to the depletion of CD8+ T cells caused by tumors. At the same time, a subset of large granular and bulky T lymphocytes appeared in



**Figure 5.** The correlation analysis result of PD-1 and T cell in tumor tissues. (A) Correlation between PD-1 and total T cells. (B) Correlation between PD-1 and CD4 + T cells. (C) Correlation between PD-1 and CD8 + T cells. NSCLC = non-small cell lung cancer, PD-1 = programmed death 1.

the peripheral blood. Different from the phenotype of normal T lymphocytes, this subset of T lymphocytes not only highly expressed PD-1, but also had CD4+CD8+ DPT cells. We speculated that this subgroup of PD1+CD4+CD8+DPT cells might be TILs, which originated from the depleted CD8+ T cells caused by tumors. Circulating CD4+CD8+ DPT cells can trigger Th2 polarization of naive CD4+ T cells and inhibit Th1 induction, indicating that CD4+CD8+ DPT cells exert immunomodulatory effects; however, the regulatory mechanism underlying this effect has not been studied.<sup>[20]</sup> Furthermore, count of CD4+CD8+ DPT cells expressing exhaustion markers is increased significantly in patients with renal cell carcinoma.<sup>[26]</sup> Taken together, Lym2 was present in NSCLC but rare in healthy individuals. Its high expression of PD-1 and the presence of DPT cells further proved that Lym2 was closely related to NSCLC. Further studies evaluating immune functions of CD4+CD8+ DPT cells could further help understanding this new tumor immune regulation mechanism. In addition, in relapsed patients treated with PD-1 inhibitor, we found that the PD-1 expression on peripheral blood lymphocytes increased significantly. Detecting the PD-1 expression on lymphocytes may help us to monitor the recurrence.

In conclusion, we analyzed T lymphocyte subsets and PD-1 expression in peripheral blood of NSCLC patients and healthy controls. Two populations of lymphocytes were determined in NSCLC, and the phenotypes of these two populations were inconsistent. Apart from a population of normal volume lymphocytes, lymphocytes with larger volume and more particles (Lym2) were observed. Lym2 overexpressed PD-1 and included a subset of CD4+CD8+ DPT cells that are reportedly involved in the tumor immunomodulatory mechanism; moreover, PD-1 overexpression helps tumor cells evade immune surveillance. Based on our collective findings, we inferred that the Lym2 in NSCLC, which may contain TILs in TME, may be closely related to immune regulation of tumors. Additionally, when the NSCLC patient treated with PD-1 inhibitor relapsed, the PD-1 expression on lymphocytes increased. Perhaps PD-1 expression on lymphocytes can be used as a recurrence monitoring index. However, our study has some deficiencies. In view of the high price of monoclonal antibodies, the cases we enrolled were not very large. In addition, we did not compare and analyze whether there were differences among the patients with different pathological stages, and the T lymphocyte subsets and PD-1 expression on Lym2 in peripheral blood of NSCLC patients may change with the disease progression. These results should be validated in future studies based on larger numbers of samples and NSCLC patients at different stages to confirm their clinical implications. If possible, these Lym2 need to be labeled and sorted out to further explore their function.

## 5. Conclusions

A new population of lymphocytes with larger volume and more particle content were observed in NSCLC patients. These lymphocytes overexpressed PD-1 and included CD4+CD8+ DPT cells. Both PD-1 and DPT cells are reportedly related to tumor immunomodulatory mechanisms. Therefore, these lymphocytes with larger volume and more particles may be the key players involved in tumor immune regulation. Combined with the understanding of TILs, we inferred that Lym2 may contain TILs in TME. In future research, we can sort these cells and analyze whether they contain other types of cells except TILs by single cell sequencing. In addition, with the recurrence in those NSCLC patients treated with PD-1 inhibitor, the PD-1 expression on lymphocytes increased. PD-1 expression on lymphocytes can be used as a recurrence monitoring index.

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## Author contributions

CHX and LW were responsible for patient enrollment and sample collection according to the inclusion criteria. YYM carries out sample detection and data analysis. CTT was a major contributor in writing the manuscript.

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**Formal analysis:** Haixin Chen.

**Methodology:** Wei Lu.

**Software:** Wei Lu.

**Project administration:** Yimin Yao.

**Visualization:** Tingting Chen.

**Writing – review & editing:** Tingting Chen.

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