Original Article
Exploring the evolution of T cell function and diversity across different stages of non-small cell lung cancer

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Abstract: The immune system plays a key role in detecting and fighting cancerous tumors. T cells are a crucial component in both natural and therapeutic cancer immunoediting responses, but it is unclear if they are the primary agents of these processes. In this study, patients with lung lesions detected by CT scan were selected, and their peripheral blood samples were analyzed for T cell population and serum cytokines/chemokines. T cell subtypes (CD3, CD4, CD8, CD27, CD28, CD45, CD45RA, CD57, CCR7, and PD1) and serum cytokines/chemokines (IL-2, IL-6, IL-10, IFN-γ, TGF-β, TNFα, CXCL1, CXCL9, and CXCL12) were measured by flow cytometry and analysis before surgical resection or other cancer treatments. The frequency of T cell subpopulations in patients with lung cancer (n = 111) corresponded to those seen in patients with T cell exhaustion. As lung cancer progressed, the proportion of effector memory T cells decreased, while the proportion of naive T cells, PD-1, CD57+, CD28+CD27+, CD45RA+, and CD3+CD4+CCR7 increased. Circulating CD8+PD1+ T cells were positively correlated with intra-tumoral PD-L1 expression. Concurrently, serum levels of IL-2, TGF-β, and CXCL9 decreased, while IL-6, IL-10, IFN-γ, and CXCL12 increased during the progression of lung cancer. In conclusion, T cell dysfunction is associated with cancer progression, particularly in advanced-stage lung cancer, and cancer immunoediting will provide early-stage cancer detection and further therapeutic strategies.

Keywords: T cell immunity, T cell exhaustion, lung cancer, immunosurveillance, immunoediting

Introduction

The immune system plays a crucial role in the surveillance of tumors. It was first proposed in the early 1900s that the immune system may prevent the formation of tumors [1]. Later, it was discovered that the immune system recognizes newly forming tumors through the expression of tumor-specific neo-antigens, which aid in their elimination [2]. The immune system's reaction to these neo-antigens is referred to as the immunological reaction against cancer cells, which led to the development of the theory of immune surveillance [3-7]. The discovery of the role of interferon-γ (IFN-γ) in promoting the rejection of transplanted tumor cells further highlighted the importance of the immune system in cancer prevention [8]. Studies have shown that mice lacking T cells, B cells, and natural killer T (NKT) cells are more susceptible to carcinogen-induced and spontaneous primary tumor formation [9, 10], supporting the vital role of the immune system in cancer prevention.

The process of cancer immunoediting is complex and can result in three potential outcomes: tumor elimination, equilibrium with the immune

system, and escape from immune control [11]. Tumor cells that are in equilibrium may no longer be recognized by the adaptive immune system, become resistant to immune effector mechanisms, or create an immunosuppressive environment within the tumor microenvironment. These tumor cells may then enter an escape phase, where their growth is no longer blocked by the immune system, leading to the development of cancer [12].

T cell exhaustion is a specific state of T cell dysfunction found in patients with chronic infection or cancer, characterized by decreased cytokine expression and limited ability to function. Recent studies have shown that T cell exhaustion and dysfunction in the tumor microenvironment is a defining feature of cancer [13]. Dysfunctional CD8+ T cells in cancer are identified by increased expression of inhibitory receptors, such as programmed cell death 1 (PD-1) and reduced production of effector cytokines, such as interleukin-2 (IL-2), IFN-γ and tumor necrosis factor-alpha (TNF-α) [14]. In addition to regulating tumor immunity, CXC chemokine families also play a role in important aspects of tumor biology such as cell growth, angiogenesis and metastasis [15].

The cancer immunoediting hypothesis was initially formulated through studies of tumor development in mice, but there is now evidence that it also occurs in humans and can influence the progression of cancer in patients. T cells play a crucial role in both natural and therapeutic cancer immunoediting responses. However, the role of T cell immunity and dysfunction in different stages of lung cancer has not been clarifed. In order to understand which immune effector processes drive cancer progression, this study aimed to identify the T cell response and expression of important cytokines/chemokines at different stages of lung cancer to demonstrate cancer immunoediting by immune effector processes.

Material and methods

Study population and patient assessment

Lung lesions were identified in patients who underwent chest computed tomography (CT) scans as part of their lung cancer screening or diagnosis. A definitive diagnosis was made based on pathology reports of biopsy or surgical resection samples. NSCLC was confirmed through histological or pathological examination. Lung cancer stage was determined according to the TNM Classification of Malignant Tumours, 8th edition [16]. Early-stage lung cancer was defined as carcinoma in situ (Tis), minimally invasive adenocarcinoma (T1mi), stage I and stage II, while advanced stage lung cancer included stage III and stage IV.

The inclusion criteria for this study were patients diagnosed with NSCLC, aged 20 years or older, with an Eastern Cooperative Oncology Group (ECOG) performance status of less than 2, and adequate hematological and biochemical parameters. The exclusion criteria were patients without pathology results, previous malignancies, non-NSCLC, prior anticancer treatment, concurrent autoimmune disease, and active infectious disorders including bacterial, fungal or viral infections. Demographic and medical information was collected and analyzed, including age, gender, smoking status, clinical diagnosis, histology type, and lung cancer stage.

The study was conducted at National Taiwan University Hospital Yunlin Branch between 2017 and 2019 and was approved by the Institutional Review Board of National Taiwan University Hospital (201611009RINB). All methods were carried out in accordance with relevant guidelines and regulations.

Intra-tumoral programmed death ligand 1 (PD-L1) expression

Immunohistochemistry (IHC) staining was used to evaluate PD-L1 expression. The assay used was 22C3 (pharmDx Assay; Agilent/Dako) and the testing platform was the Autostainer Link 48 system. Detection was performed using the EnVision FLEX visualization system. Specimens were considered adequate if they had at least 100 viable tumor cells. Results were reported as a percentage of tumor proportion score.

Flow cytometry for detection of peripheral T cell differentiating

Peripheral blood samples (2 ml) were collected prior to surgical resection or anticancer treatment. Samples were collected in sterile heparinized vials and were analyzed immediately. Aliquots of heparinized whole blood (200 μl) were incubated with antibodies for 15 minutes at room temperature. DuraClone IM panels

(Beckman Coulter) were used to identify cell subpopulations in human whole blood samples. The IM T cell subsets Tube is a 10-color, 10-monoclonal antibody reagent that includes CD45RA, CCR7, CD28, PD1, CD27, CD4, CD8, CD3, CD57, and CD45 to identify common extracellular markers of different cell subsets present in whole blood specimens.

200 µl of peripheral blood was added to a DuraClone IM T Cell Subset dry reagent tube (Beckman Coulter). The cells were mixed for 8 seconds, incubated for 15 minutes at room temperature (RT) in the dark, and red blood cells were lysed with 2 mL of VersaLyse Lysing solution plus 50 µl of IOTest 3 Fixative Solution (Beckman Coulter). After 20 minutes of incubation at RT, the suspension was centrifuged at 200×g for 5 minutes, the supernatant was discarded, and the pellet was resuspended in 3 mL of 1× PBS. Following an additional centrifugation step, the cell pellet was resuspended in 500 µl of 1× PBS for subsequent analysis on a 13 color/3 laser CytoFLEX flow cytometry system (Beckman Coulter).

CD45 was used as a gating marker along with the side scatter properties of the cells to identify lymphocytes. CD3, CD4, and CD8 were included to identify all T cells and distinguish between T helper and cytotoxic T cells, respectively. Co-expression of CD45RA and CCR7 identifies naïve T cells, while the lack of expression of both CD45RA and CCR7 defines effector memory T cells (TEM). Central memory T cells (TCM) are characterized by CCR7 expression and the absence of the CD45RA isoform. Terminally differentiated effector T cells have a CD45RA+ CCR7- phenotype [17].

The co-stimulatory molecules CD27 and CD28 were used to further define T cell populations. Naive T cells and TCM express both CD27 and CD28. Effector memory T cells were further divided into early (CD27+CD28+), intermediate (CD27+CD28-), and late (CD27-CD28-) TEM. CD57 expression on T cells has been shown to correlate with a state of dysfunction and decreased proliferative potential. PD-1 is a mediator of inhibitory signals in T cells that promote immunologic tolerance. The combination of CD57 and PD-1 allows for the distinction between exhausted T cells (PD-1+CD57+/-) and terminal effector CD8+ T cells (PD-1-CD57+) [17].

Multiplex ELISA analysis of cytokines and chemokines

Levels of human serum cytokines/chemokines were analyzed using the LEGENDplex Custom 9 plex (IL-2, IL-6, IL-10, IFN-γ, TGF-β1, TNFα, CXCL1 (GROα), CXCL9 (MIG), CXCL12 (SDF-1)) cytokine panel kit (BioLegend, San Diego, CA). The serum samples were diluted in assay buffer and cytokine levels were measured according to the manufacturer's protocol (BD FACS Canto II; BD Biosciences, San Jose, CA). The data was quantified using LEGENDplex analysis software. Supplementary Table 1 provided a detailed listing of the relationship between T cell subtypes and immune molecules.

Statistical analyses

Continuous variables were reported as either mean with standard deviation (SD) or median with range, while categorical variables were expressed as percentages of the respective groups. Differences among healthy normal, benign lesion, early and advanced-stage NSCLC in T cell subsets were assessed through independent T-tests and one-way analysis of variance (ANOVA), followed by LSD for multiple mean comparisons. Linear regression, along with a 95% confidence interval (CI), was employed to determine the correlation of T cell subsets and to estimate the association between circulating T cell PD1 subset and intratumoral PD-L1 expression. Statistical analyses were conducted using SPSS version 22.0 (SPSS Inc., IBM, USA), with *P*-values < 0.05 considered statistically significant.

Results

Clinical characteristics

A total of 182 patients were included in the study. Patients had a median of age 58 years (range: 24-89), 51.1% (n = 93) were male, and 25.8% (n = 47) had a history of ever or current cigarette smoking (clinical characteristics are demonstrated in **Table 1**). A total of 46 (25.3%) patients were considered to be healthy (normal), 25 (13.7%) had benign lesions, including inflammation (n = 12), infectious disease (n = 8), lymph node (n = 3), cyst (n = 1), and hamartoma (n = 1). Fifty-two patients (28.6%) had early-stage lung cancer, including Tis (n = 8), stage Ia (n = 37) (T1mi, n = 7; T1a, n = 19; T1b,

Table 1. Basic characteristics of the study population

	Group			
	Healthy normal (n = 46)	Benign lesion (n = 25)	Early NSCLC (n = 52)	Advanced NSCLC (n = 59)
Age, yeas, median (range)	47.5 (38-72)	54 (24-76)	56 (34-84)	70 (44-89)
Gender				
Male (%)	23 (50)	16 (64.0)	20 (38.5)	34 (57.6)
Female (%)	23 (50)	9 (36.0)	32 (61.5)	25 (42.4)
Smoking status				
Never (%)	42 (91.3)	18 (72.0)	41 (78.8)	34 (57.6)
Current or ever-smoker (%)	4 (8.7)	7 (28.0)	11 (21.2)	25 (42.4)

NSCLC: non-small cell lung cancer.

Table 2. The difference of histology type, stage, driver mutation and PD-L1 expression in non-small cell lung cancer (NSCLC) patients

	Early NSCLC (n = 52)	Advanced NSCLC (n = 59)
Histology type		
Adenocarcinoma	51 (98.1)	46 (78.0)
Squamous cell carcinoma	1 (1.9)	10 (17.0)
Adenosquamous carcinoma	0	2 (3.4)
Lymphoepithelioma-like carcinoma	0	1 (1.6)
Stage		
0	8 (15.4)	
IA	37 (71.2)	
IB	5 (9.6)	
IIA	0	
IIB	2 (3.8)	
IIIA		8 (13.5)
IIIB		9 (15.3)
IIIC		3 (5.1)
IV		39 (66.1)
M1a		14
M1b		15
M1c		10
Driver mutation		
EGFR		24 (40.7)
ALK		2 (3.4)
Wild type		27 (45.7)
Not detection		6 (10.2)
PD-L1 expression		
≥ 50%		14 (23.7)
< 50%		36 (61.0)
Not detection		9 (15.3)

NSCLC: non-small cell lung cancer; EGFR: epidermal growth factor receptor; ALK: anaplastic lymphoma kinase; PD-L1: programmed death-ligand 1.

n = 6; T1c, n = 5), stage Ib (n = 5) (T2a), and stage IIb (n = 2) (T2bN1, n = 1; T3N0, n = 1). The remaining 59 patients (32.4%) had advanced stage lung cancer (stage IIIA, n = 8; stage IIIB, n = 9; stage IIIC, n = 3; stage IV, n = 39) (**Table 2**).

Altered proportion of T cells with immunosurveillance in lung cancer

Multi-color flow cytometry was utilized to analyze the highly heterogeneous human T cell compartment among study participations (**Figure 1**). As demonstrated with flow cytometry, patients with lung cancer had a significantly higher proportion of the following markers compared with healthy normal/benign patients (healthy normal/benign vs. lung cancer; **Figure 2**): CD3+CD4 central memory T cells (39.2 ± 16.2 vs. 43.8 ± 14.5, P = 0.048), CD3+CD4+ naïve T cell (10.1 ± 15.4 vs. 22.0 ± 17.3, P < 0.001), CD3+CD8+ effector T cell (17.9 ± 15.2 vs. 30.7 ± 17.3, P < 0.001), CD4+CD28+CD27+ T cell (39.6 ± 29.1 vs. 70.0 ± 27.3, P < 0.001), CD8+CD28+CD27+ T cell (19.3 ± 23.8 vs. 41.2 ± 24.3, P < 0.001), CD4+PD1+ T cell (13.5 ± 7.2 vs. 18.7 ± 9.2, P < 0.001), CD8+PD1+ T cell (18.1 ± 13.5 vs. 25.2 ± 16.1, *P* = 0.002), CD4+CD57+ T cell (11.8 ± 19.5 vs. 21.0 ± 19.9, P = 0.002), CD8+CD57+ T cell (32.3 ± 19.7 vs. 45.2 ± 21.3, P < 0.001), CD3+CD4+CD45RA+ T cell (14.1 ± 21.6 vs. 23.3 ± 17.9, P = 0.002), and CD3+CD4+CCR7+ T cell (50.6 ± 22.7 vs. 69.8 ± 24.6, P < 0.001).

Patients with lung cancer had a significantly lower proportion of the following markers compared with healthy normal/benign patients (healthy normal/benign vs. lung cancer; **Figure 2**): CD3+CD8+ (25.4 ± 9.8 vs. 22.1 ± 9.9, P = 0.028), CD3+CD4+ effector memory T cell (47.2 ± 21.3 vs. 32.9 ± 23.1, P < 0.001), CD3+CD8+ effector memory T cell (56.2 ± 21.7 vs. 40.4 ± 22.7, P < 0.001). Moreover, the proportions of CD3+CD4+ T cell, CD3+CD4+ effector T cell, CD3+CD8+ central memory T cell, CD3+CD8+ Naïve T cell, CD4+CD27+ T cell, CD4+CD28+ T cell, CD8+CD27+ T cell, and CD8+CD28+ T cell had no significant difference between healthy normal/benign lesions and lung cancer patients (Supplementary Figure 1).

The association of circulating T cell PD1 expression and intra-tumoral PD-L1 expression

In patients with advanced lung cancer (n = 50), intra-tumoral PD-L1 expression was evaluated. Circulating CD4+PD1+ T cells were positively correlated with intra-tumoral PD-L1 expression (R: 0.11, B = 0.47, *P* = 0.442). CD8+PD1+ was also positively correlated with intra-tumoral PD-L1 expression (R: 0.02, B = 0.02, *P* = 0.874).

These observations were not significant statistically different (Supplementary Figure 2).

Expression of cytokines and chemokines recognized as T cell dysfunction in lung cancer

Cytokine and chemokine expression was evaluated in 40 healthy normal patients, 8 with benign lesions, 35 early-stage lung cancer patients, and 39 advanced stage lung cancer patients assessed using Multiplex ELISA (pg/ml).

Patients with lung cancer had a significantly higher expression of the following markers compared with healthy normal/benign patients (healthy normal/benign vs. lung cancer; **Figure 3**): IL-6 (18.3 ± 16.0 vs. 24.2 ± 19.7, *P* = 0.016), IL-10 (3.3 ± 5.6 vs. 14.1 ± 13.0, *P* < 0.001), and IFN-γ (41.3 ± 55.0 vs. 129.5 ± 102.7, *P* < 0.001).

In contrast, patients with lung cancer had a significantly lower expression of the following markers compared with healthy normal/benign patients (healthy normal/benign vs. lung cancer; **Figure 3**): IL-2 (27.1 ± 11.6 vs. 8.9 ± 9.5, *P* < 0.001), TGF-β (40.1 ± 29.5 vs. 14.5 ± 26.0, *P* < 0.001), CXCL9 (130.5 ± 153.3 vs. 42.9 ± 49.8, *P* < 0.001), and CXCL12 (973.3 ± 671.9 vs. 1215.1 ± 580.6, *P* = 0.003). Otherwise, expression of TNFα and CXCL1 had no significant difference between healthy normal/benign lesions and lung cancer patients (Supplementary Figure 3).

Discussion

Our study aimed to investigate the role of T cell dysfunction in cancer immunosurveillance, specifically in NSCLC patients. Using flow cytometry, we analyzed the peripheral blood of patients with lung cancer and benign lesions, and found that as the cancer progressed, the proportion of effector memory T cells decreased while the proportion of naive T cells, PD-1 positive cells, CD57+ cells, CD28+CD27+ cells, CD45RA+ cells, and CCR7+ cells increased. Concurrently, we observed changes in the levels of various cytokines in the patients' blood, with decreased levels of IL-2, TGF-β, and CXCL9 and increased levels of IL-6, IL-10, IFN-γ, and CXCL12. Our findings suggest that T cell exhaustion is associated with cancer progression, particularly in advanced stage NSCLC, providing
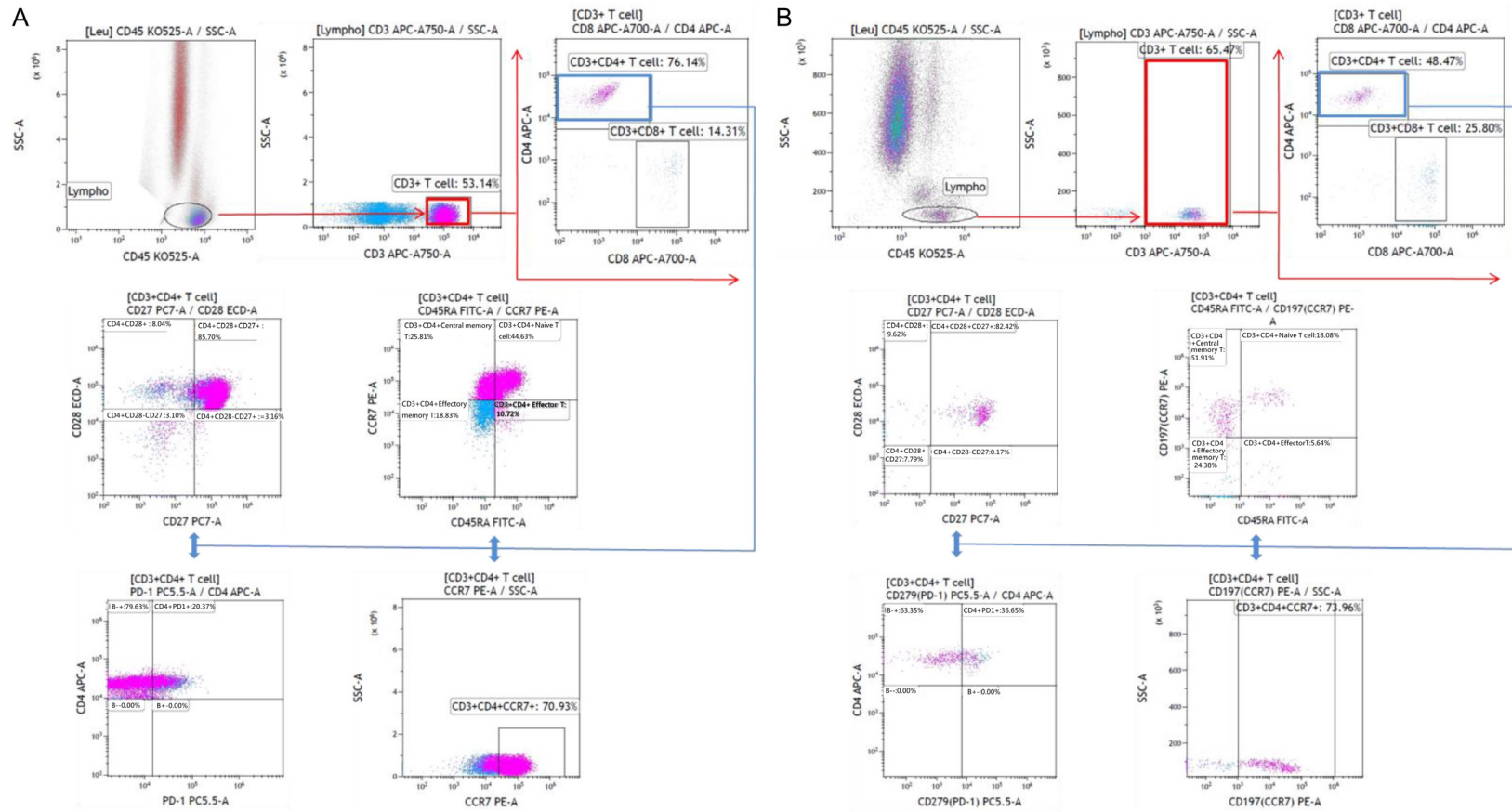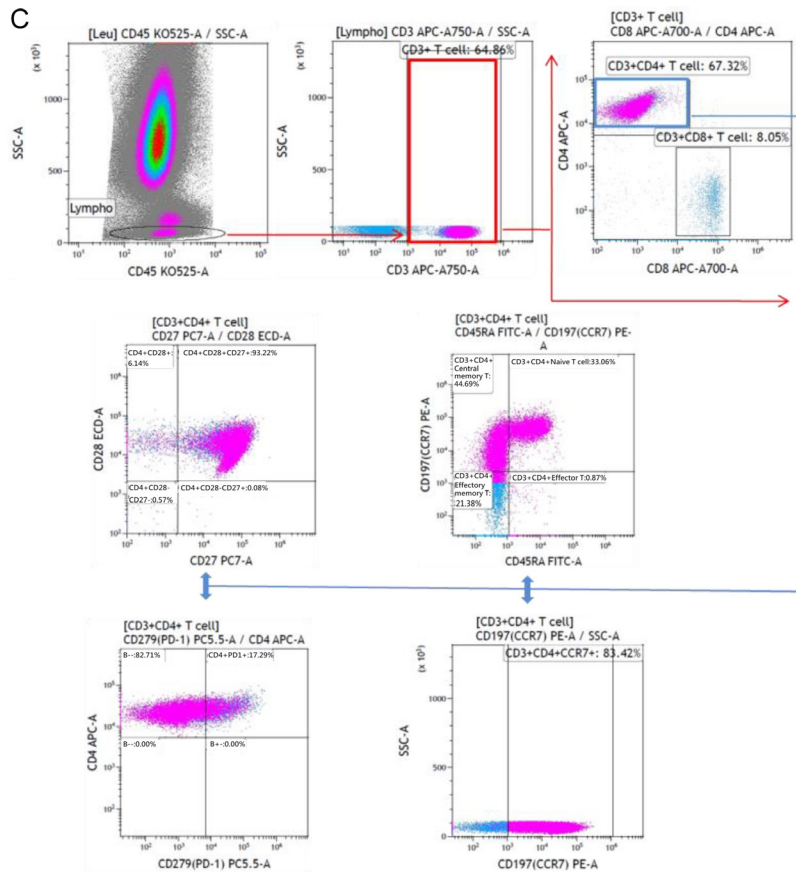
Figure 1. The DuraClone IM T Cell Subset dry reagent kit (CD45RA-FITC, CD197-PE, CD28-ECD, CD279-PC5.5, CD27-PC7, CD4-APC, CD8-Alexa-Fluor 700, CD3-APC-Alexa-Fluor 750, CD57-Pacific Blue, CD45-Krome Orange) were used to identify cell compartments in human whole blood samples using a 13 color/3 laser CytoFLEX Flow Cytometer with 488 nm/638 nm/405 nm laser configuration (Beckman Coulter). Patients were: (A) Benign lesion (patient No. 44); (B) Early stage non-small cell lung cancer (NSCLC) (patient No. 142); (C) Advanced stage NSCLC (patient No. 118).
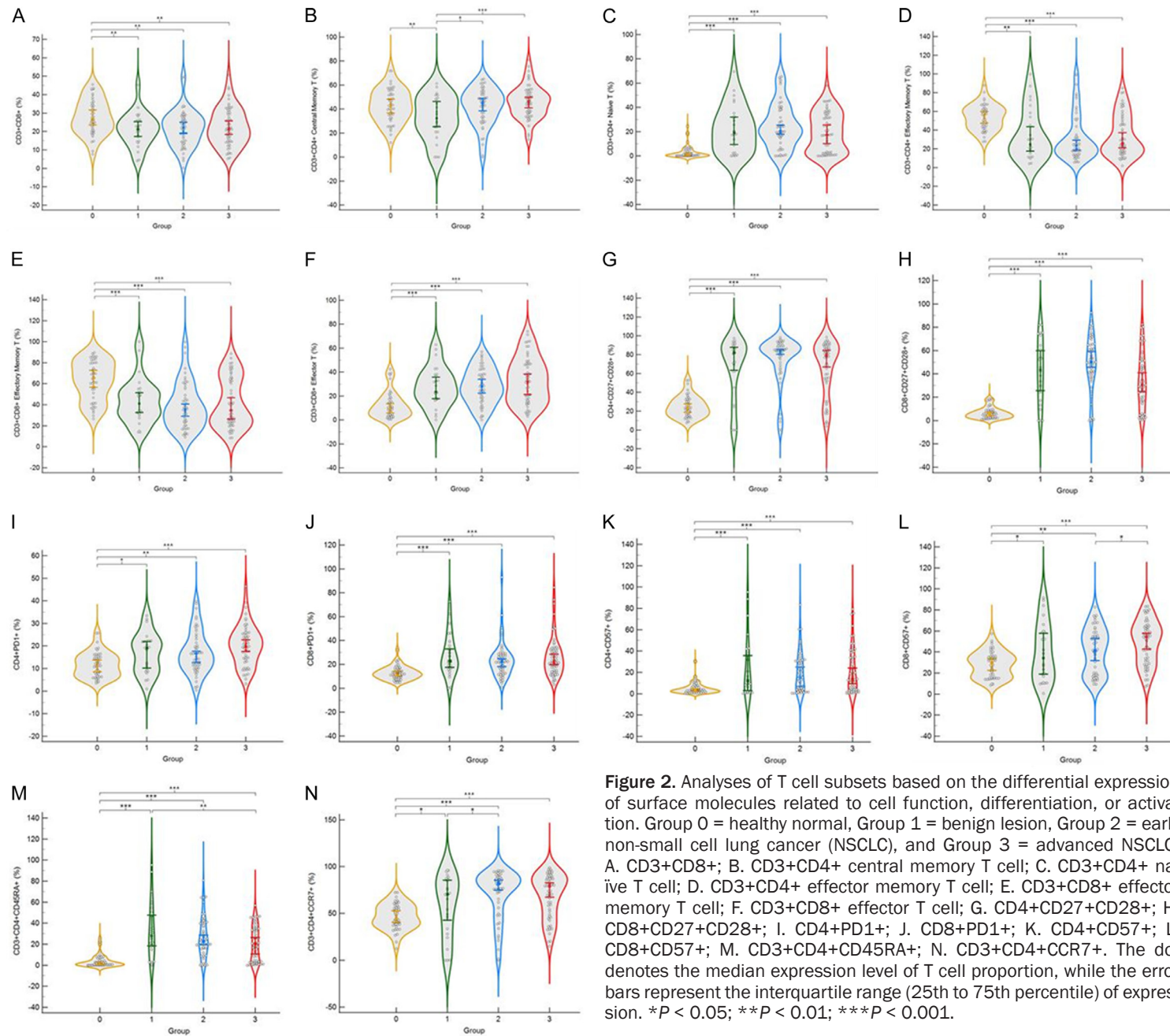
Figure 2. Analyses of T cell subsets based on the differential expression of surface molecules related to cell function, differentiation, or activation. Group 0 = healthy normal, Group 1 = benign lesion, Group 2 = early non-small cell lung cancer (NSCLC), and Group 3 = advanced NSCLC. A. CD3+CD8+; B. CD3+CD4+ central memory T cell; C. CD3+CD4+ naïve T cell; D. CD3+CD4+ effector memory T cell; E. CD3+CD8+ effector memory T cell; F. CD3+CD8+ effector T cell; G. CD4+CD27+CD28+; H. CD8+CD27+CD28+; I. CD4+PD1+; J. CD8+PD1+; K. CD4+CD57+; L. CD8+CD57+; M. CD3+CD4+CD45RA+; N. CD3+CD4+CCR7+. The dot denotes the median expression level of T cell proportion, while the error bars represent the interquartile range (25th to 75th percentile) of expression. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.
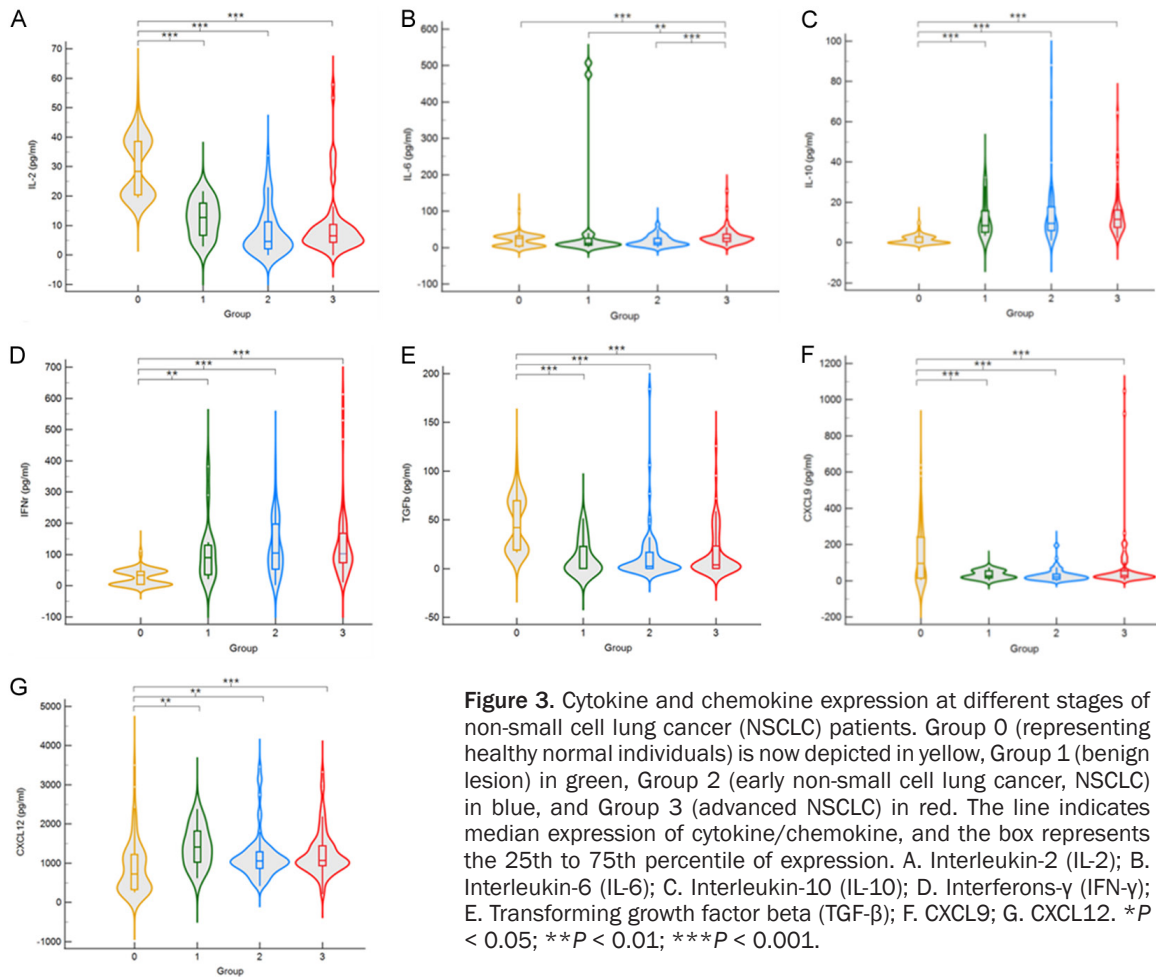
Figure 3. Cytokine and chemokine expression at different stages of non-small cell lung cancer (NSCLC) patients. Group 0 (representing healthy normal individuals) is now depicted in yellow, Group 1 (benign lesion) in green, Group 2 (early non-small cell lung cancer, NSCLC) in blue, and Group 3 (advanced NSCLC) in red. The line indicates median expression of cytokine/chemokine, and the box represents the 25th to 75th percentile of expression. A. Interleukin-2 (IL-2); B. Interleukin-6 (IL-6); C. Interleukin-10 (IL-10); D. Interferons-γ (IFN-γ); E. Transforming growth factor beta (TGF-β); F. CXCL9; G. CXCL12. *P < 0.05; **P < 0.01; ***P < 0.001.

insight into potential early-stage cancer detection and therapeutic strategies.

T Cells, specifically T cells, play a crucial role in fighting cancer. However, in patients with malignancies, T cells become less effective due to a state of dysfunction or exhaustion. This is characterized by the prolonged expression of inhibitory receptors and a distinct transcriptional state from functional effector or memory T cells. Memory T cells have been found to be crucial in maintaining T cell persistence and the effectiveness of tumor immunotherapy. They differentiate into effector and central memory T cell subsets after antigenic stimulation [18, 19]. Tumor-specific central memory T cells have been found to selectively target and attack tumors by developing homing capabilities to the tumor site and cytotoxic abilities that lead to tumor regression in xenotransplant mouse models [20]. Studies have also demonstrated that central memory T cells have a greater

capacity for proliferation, reinforcing their potential effectiveness in targeting and eliminating tumor cells [21-23]. However, it has also been proposed that prolonged exposure to antigens may result in corrupt or altered CD8+ T memory cells, skewing the antitumor response towards non-responsiveness [21, 23]. In our study, we found that the proportion of central memory T cells and effector T cells were higher in patients with non-small cell lung cancer (NSCLC) compared to healthy controls and those with benign lesions. However, a lower proportion of effector memory T cells were present in NSCLC patients. These results suggest that tumor neo-antigens may stimulate T cell proliferation, but cancer also leads to T cell dysfunction at different stages.

In cancerous environments, T cells can become dysfunctional as a result of persistent antigen exposure. These dysfunctional T cells are characterized by a reduced ability to proliferate,

decreased effectiveness in performing their functions, and an overexpression of multiple inhibitory receptors [24-26]. Recent studies have shown that immune checkpoints or co-inhibitory receptors, such as CTLA-4 and PD-1, play a significant role in regulating T cell responses. These receptors, when overexpressed on effector T cells, have been proven to be effective targets for cancer treatment [27, 28]. Our analysis revealed that elevated expression of PD-1 on the surface of T cells in patients with advanced stage non-small cell lung cancer (NSCLC) is an indicator of T cell exhaustion and dysfunction in the progression of lung cancer.

It has been shown that CD28 and CD27 co-stimulation is necessary during primary T cell immune responses and supports T cell proliferation and survival. The downregulation of CD28, a costimulatory molecule, is a characteristic of senescent T cells found in various solid tumors [29]. A recent study has shown that PD-1 primarily suppresses T cell function by deactivating CD28 signaling, indicating that costimulatory pathways may play a critical role in regulating the function and response of effector T cells to anti-PD-L1/PD-1 therapy [30]. CD27 is a member of the tumor necrosis factor (TNF) receptor superfamily and its ligand CD70 is expressed on antigen-presenting cells. When CD27 interacts with CD70 through T-cell receptor (TCR) stimulation, it leads to T-cell activation, proliferation, survival, and maturation of effector capacity and T-cell memory [31]. Administering anti-CD27 antibody in patients with advanced solid tumors has been observed to result in biologic activity consistent with CD27 stimulation, including chemokine induction, T-cell stimulation, and depletion of regulatory T cells [32]. Our results showed that CD27+CD28+ T cells are elevated in patients with non-small cell lung cancer (NSCLC), however, the downregulation of costimulatory molecule-associated signaling pathways need further investigation.

C-C chemokine receptor 7 (CCR7) is a crucial chemokine receptor involved in T cell and dendritic cell migration towards cells producing CCL19 and CCL21, and therefore, it regulates the homeostasis of effector and memory T cells [33]. Activation of the CCR7 receptor by its ligands in cancer cells initiates various signaling pathways linked to cell migration and metastasis in lung cancer [34, 35]. Targeting

specific chemokine receptors has shown considerable potential in the diagnosis and treatment of certain cancers [36].

PD-1 is a crucial immune checkpoint molecule expressed on both activated and exhausted T-cells [37]. Its ligand PD-L1 is expressed on tumor cells, and the interaction between PD-1 and PD-L1 provides a negative signal for antigen-induced T-cell activation [38]. This T-cell inactivation caused by PD-1/PD-L1 is thought to contribute to immunosuppression at the tumor site [36]. Studies have examined PD-1 expression on tumor-infiltrating T-cells, and its correlation with prognosis and response to anti-PD-1 therapy has been investigated in lung cancer [39-42]. Currently, the direct evaluation of PD-L1 expression is considered the best available biomarker for predicting the tumor response and survival [43, 44]. A recent study reported that expression of PD-1/PD-L1 and PD-L2 in peripheral T-cells were increased in patients with advanced non-small cell lung cancer (NSCLC) [45], and expression of PD-1 on CD4+ T cells in peripheral blood was associated with poor clinical outcome in the same patient population [46]. However, no correlation was found between PD-L1 expression in lung cancer tissues and PD-1 expression in CD4+ or CD8+ T cells from patients with advanced NSCLC [46]. Our study showed that circulating T cell PD-1 expression was associated with lung cancer progression. Therefore, the association between peripheral T cell PD-1 and intra-tumoral PD-L1 expression needs to be further explored before it can be used as a clinical biomarker for anti-cancer immunotherapy.

T cells play a vital role in the immune system's response to tumors. When they come in contact with tumor-associated antigens, T cell receptors activate a process that directs T cell differentiation. However, when T cells are repeatedly exposed to the same antigen, significant changes in T cell activation and differentiation can lead to T cell dysfunction or exhaustion [47, 48]. Previous studies have suggested that the state of dysfunction and exhaustion in tumor-specific T cells occurs early in the course of tumor development [49]. As exhausted T cells develop, a sequence of phenotypic and functional changes occur. The first effector activity to be lost is the production of IL-2, followed by TNF-α. The ability to produce

IFN-γ, however, is more resistant to deactivation [48, 50, 51]. These changes also affect cell metabolism and lead to distinct transcriptional profiles, including the proinflammatory IL-6 and suppressive IL-10 and TGF cytokines [52]. IL-2 is a crucial cytokine in regulating the survival, proliferation, and differentiation of activated T cells and natural killer cells [53]. IL-6 is a prevalent inflammatory cytokine in the tumor microenvironment. Its excessive expression has been reported in many types of tumors, and it plays a role in promoting tumor growth by regulating multiple signaling pathways, including apoptosis, survival, proliferation, angiogenesis, invasiveness, and metastasis [54]. IL-10 is necessary for T helper cell functions, T cell immune surveillance, and the suppression of cancer-associated inflammation. By promoting tumor-specific immune surveillance and inhibiting pathological inflammation, IL-10 is becoming a key cytokine in the host's fight against cancer [55]. Our study showed that the levels of IL-6 and IL-10 increased and IL-2 decreased in lung cancer patients, indicating that the expression pattern of inflammatory cytokines in the peripheral blood of cancer patients could be a valuable biomarker for immunosurveillance.

Cytokines and chemokines play a crucial role in cancer-related inflammation and have direct and indirect effects on the growth and spread of tumor cells [56]. Tumor cells, stromal cells, and the leukocytes associated with the tumor, all contribute to the local production of chemokines within the tumor and affect systemic circulating chemokine levels. Chemokines are primarily known for inducing directed cell migration, particularly of leukocytes during inflammation. Prolonged inflammation is believed to aid in carcinogenesis by creating an ideal microenvironment for tumor cell development and growth [15, 57]. Our study found that the levels of CXCL12 increased and CXCL9 decreased in association with lung cancer progression. A CXCL1 paracrine network has been found to be related to cancer chemoresistance and metastasis. CXCL1 promotes angiogenesis and its elevated expression in non-small cell lung cancer (NSCLC) tumor tissue has been linked to cancer progression [58-60]. On the other hand, CXCL9 has been shown to regulate immune cell migration, differentiation, and activation, leading to tumor suppression. Cancer cells deficient in CXCL9 have been found to be more tumori-

genic than cancer cells expressing CXCL9 [61, 62]. CXCL12 is known to be one of the most powerful angiogenic chemokines. Studies in a mouse model have shown that target organs that are the preferred destination of human non-small cell lung cancer (NSCLC) metastases have higher levels of CXCL12 than primary tumors, indicating the presence of chemotactic gradients. These findings suggest that the CXCL12-CXCR4 biological axis plays a role in regulating the metastasis of NSCLC [63, 64].

There are several important limitations that necessitate consideration in our study. We primarily utilized flow cytometry combined with cytokine and chemokine analysis to characterize the immune landscape in non-small cell lung cancer (NSCLC) patients compared to healthy controls. While flow cytometry provides valuable information on cell surface markers and allows for the identification of distinct T-cell subsets, such as CD8+ cytotoxic T cells and regulatory T cells (Tregs), we recognize its limitations in capturing the full spectrum of functional heterogeneity within these populations. In future investigations, we will certainly consider the incorporation of single-cell RNA sequencing (scRNA-seq) or similar approaches to further dissect the functional roles of specific T-cell clusters in NSCLC. By analyzing gene expression profiles at the single-cell level, scRNA-seq offers the opportunity to identify novel T-cell subsets and delineate their unique functional properties within the tumor microenvironment. Notably, our study exhibits significant bias in its patient population concerning age, gender, smoking status, and historical background across most groups. These factors hold substantial potential to impact immunomonitoring outcomes. Additionally, a significant limitation lies in the absence of longitudinal follow-up and outcome data. The inclusion of such data would be indispensable for effectively addressing the questions that have been raised.

In conclusion, peripheral blood T cells are a diverse mixture of different functional characteristics and stages of differentiation. There is limited knowledge about the role of the immune system during the early stages of cancer and its progression to metastasis. In this study, we show that monitoring changes in peripheral effector memory T cells and their co-expression
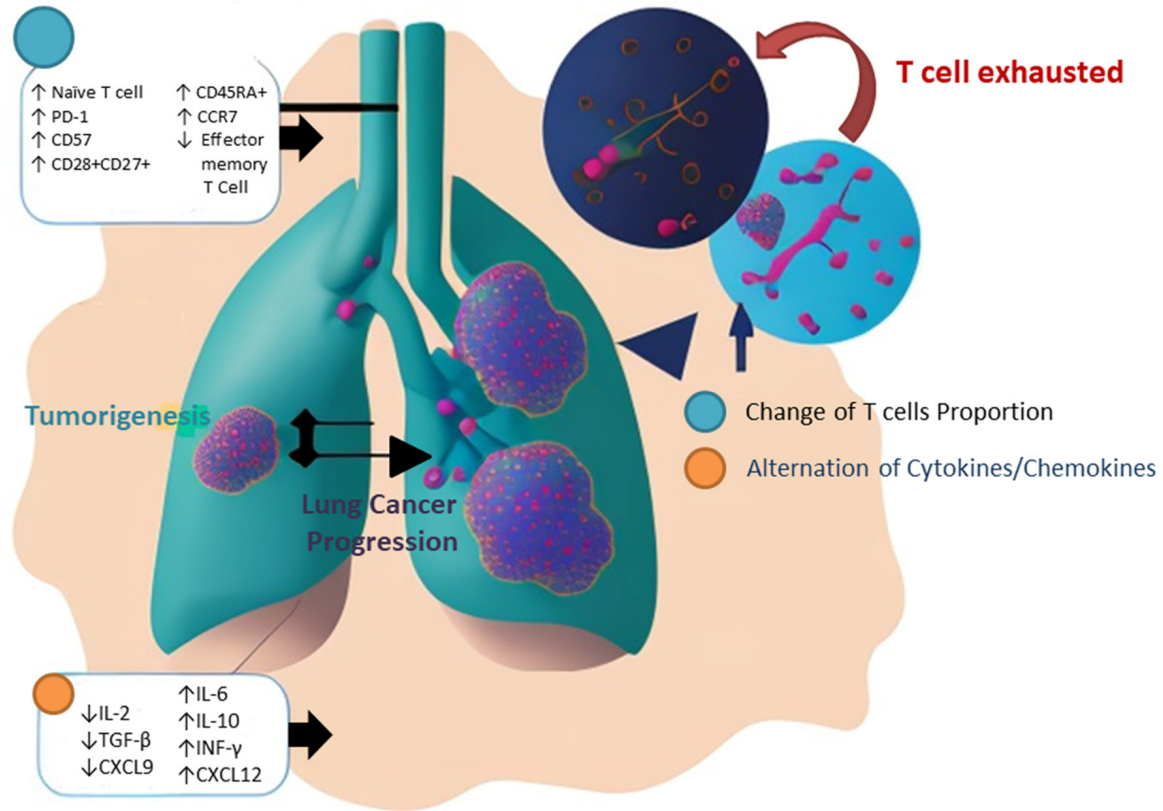
Figure 4. The immune system plays a crucial role in the fate of a tumor, from initial detection through immunosurveillance to shaping the tumor through immunoediting. As cancer develops, specific T cell populations, such as naive T cells, PD-1, CD57, CD28+CD27+, CD45RA+, and CCR7+ T cells, are eliminated. In contrast, the number of effector memory T cells decline with cancer progression. Additionally, there are changes in the levels of certain cytokines and chemokines in the blood, such as a decrease in IL-2, TGF-β, and CXCL9 and an increase in IL-6, IL-10, INF-γ, and CXCL12. These changes in T cell subpopulations and the expression of cytokines and chemokines may indicate a state of immunosenescence.

with markers such as CD57+, CD28+CD27+, CD45RA, and CCR7 may aid in the detection of cancer through immunosurveillance. Specifically, the presence of PD-1 may be a strong predictive marker. Simultaneously, there was a decrease in serum levels of IL-2, TGF-β, and CXCL9, while an increase in serum levels of IL-6, IL-10, IFN-γ, and CXCL12 was observed (**Figure 4**). Further research, in the form of a longitudinal cohort study, is needed to fully understand the relationship between T cell function, cancer progression, and immunosenescence, as T cell dysfunction is thought to be a continuous process and the decline of the immune system may be influenced by specific cytokines/chemokines that also play a role in cancer progression. The identification of T cell exhaustion as part of lung cancer screening and early detection in high-risk populations may be a crucial aspect of clinical practice.

Disclosure of conflict of interest

None.

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Supplementary Table 1. Cytokine and chemokine signals of T cells in tumors

| Signal	Producer/Target	Mechanisms
IL-2	DC, activated T cells/effector T cell, Treg	CD8+ T cells depend on IL2 for sustained expansion.
IL-6	T cell, macrophage, endothelial cell, epithelial cell/ endothelial cell	IL6 trans-signaling enhances both E- and P-selectin interactions and ICAM1 dependent T-cell transmigration on tumor vessels.
IL-10	Treg, Th1, DC, macrophage, epithelial cell/Tumor-resident T-cell	IL10 directly activates and expands tumor-resident T cells without de novo infiltration from secondary lymphoid organs.
IFN-γ	Cytotoxic T cells/endothelial cell, fibroblasts, tumor cell, monocyte	Interferon-γ induces CXCR3 ligands (CXCL9, 10, 11), thus enhancing the CXCR3-mediated T-cell recruitment.
TGF-β1	Fibroblasts/Tumor epithelial cell	TGF-β is a well-known regulator of EMT. Fibroblast induced TGFβ may repro-gram peritumoral stromal fibroblasts and exhibit a fibroblast- and collagen-rich tumor, which will decrease the CD8+ T effector cell penetration in the tumor.
TNF-α	Lymphoid, mast, endothelial, fibroblast, tumor cell/ endothelial cell	TNF stimulation induces ICAM1 and VCAM1 expression on endothelial cells for T-cell extravasation.
CXCL1	Tumor cell, macrophage, neutrophil/CXCR2+ MDSC	CXCL1, 2, 5-CXCR2 signal promotes the recruitment of MDSC to tumors.
CXCL9	Endothelial, fibroblast, tumor cell, monocyte/CXCR3+ T cell	CXCL9 is induced by interferon-γ and share a receptor CXCR3, directing the migration of activated T and NK cells.
CXCL12	FAP+ CAF/CXCR4+ MDSC, Endothelial cell, T cell	CXCL12 has chemo-repulsive effects on T cells. CXCL12 promotes angiogen-esis by recruiting endothelial precursor cells. CXCL12 also recruits MDSCs to tumors.

EMT: Epithelial-mesenchymal transition; MDSCs: Myeloid-derived suppressor cells.

# Diversity T cell function in different stage lung cancer
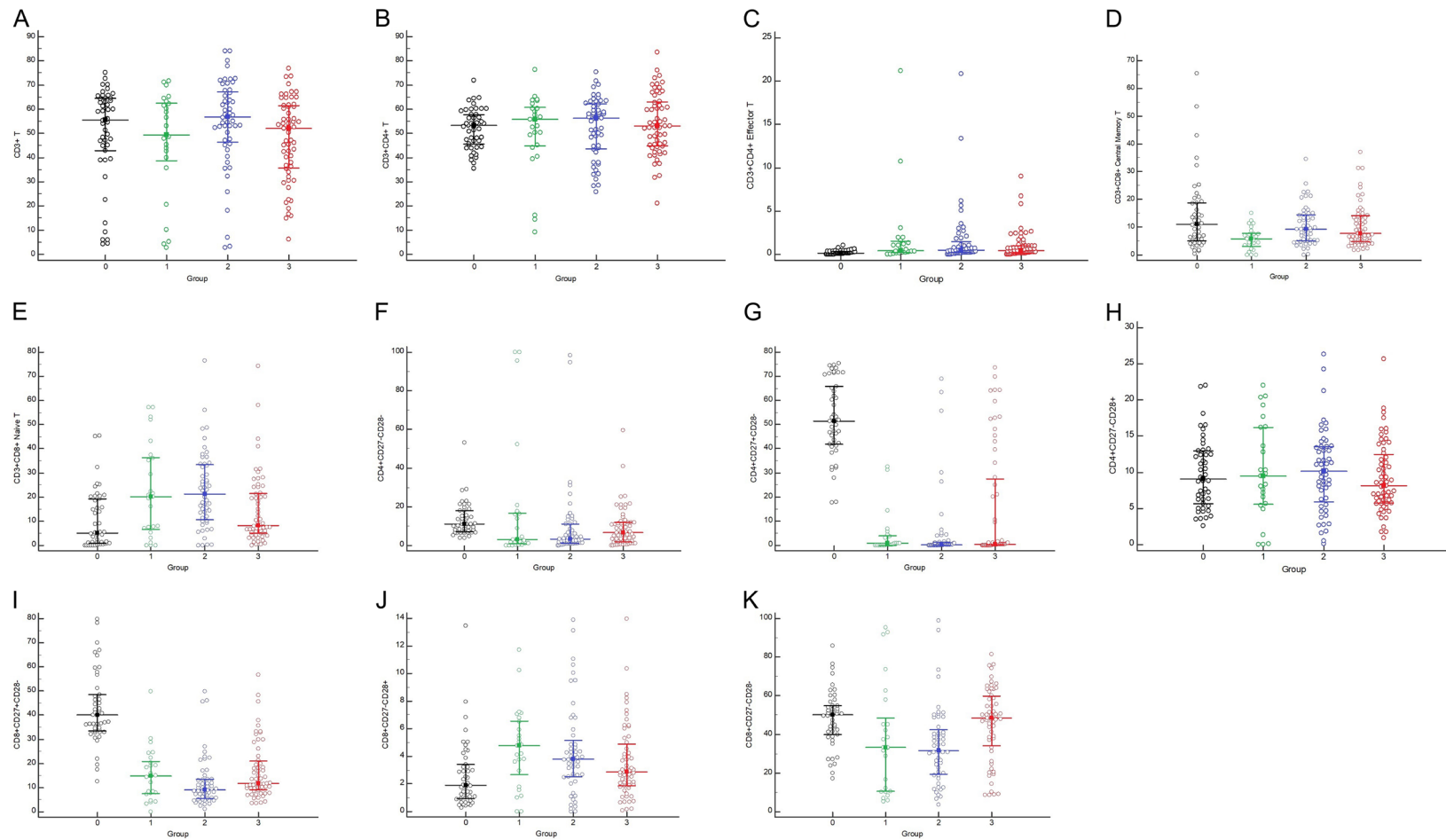
Supplementary Figure 1. Analyses of T cell subsets based on the differential expression of surface molecules related to cell function, differentiation, or activation. Group 0 = healthy normal, Group 1 = benign lesion, Group 2 = early non-small cell lung cancer (NSCLC), and Group 3 = advanced NSCLC. A. CD3+; B. CD3+CD4+; C. CD3+CD4+ effector T cell; D. CD3+CD8+ central memory T cell; E. CD3+CD8+ naïve T cell; F. CD4+CD28-CD27-; G. CD4+CD27+CD28-; H. CD4+CD27-CD28+; I. CD8+CD28-CD27-; J. CD8+CD27+CD28-; K. CD8+CD27-CD28+. The dot denotes the median expression level of T cell proportion, while the error bars represent the interquartile range (25th to 75th percentile) of expression.

Diversity T cell function in different stage lung cancer
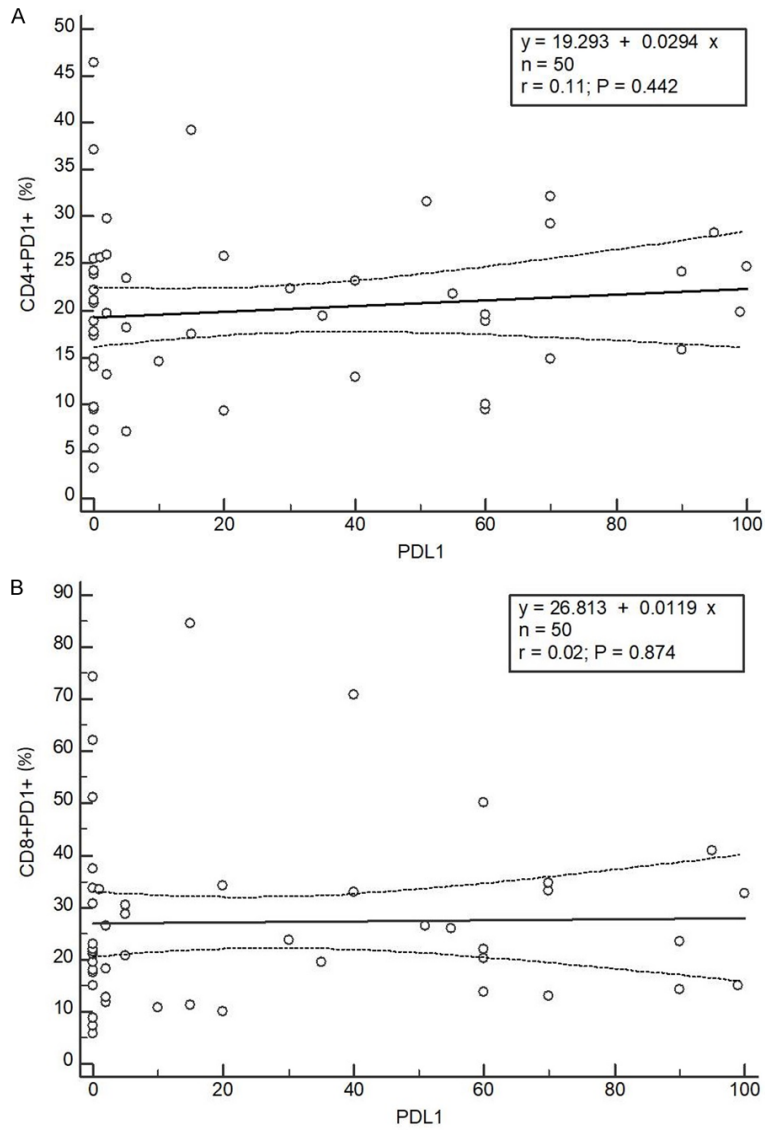
Supplementary Figure 2. Linear regression model with 95% confidence interval illustrated the relationship of peripheral T cell PD-1 expression. A. CD4+PD-1+; B. CD8+PD-1+ and intra-tumoral PD-L1 expression.

Diversity T cell function in different stage lung cancer
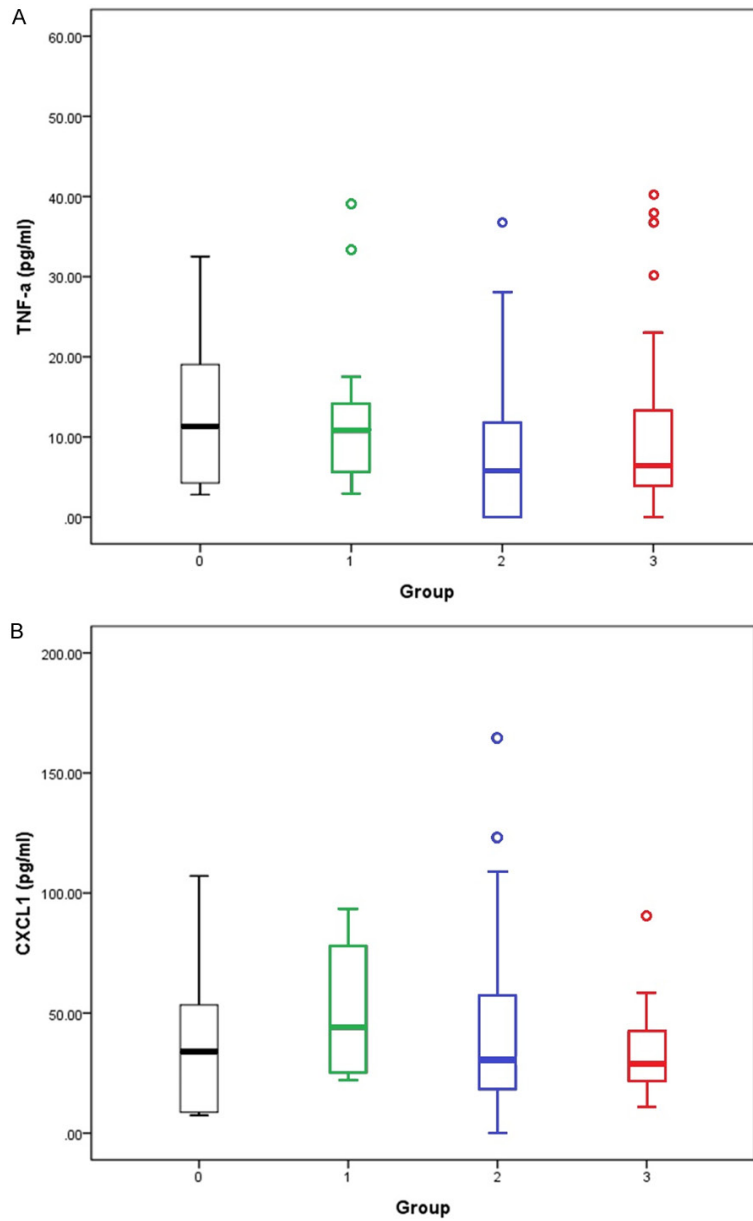
Supplementary Figure 3. Cytokine and chemokine expression at different stages of non-small cell lung cancer (NSCLC) patients. Group 0 = healthy normal, Group 1 = benign lesion, Group 2 = early NSCLC, and Group 3 = advanced NSCLC. The line indicates median expression of cytokine/chemokine, and the box represents the 25th to 75th percentile of expression. (A) Tumor necrosis factor-α (TNF-α), and (B) CXCL1.