Original Article

Associations between peripheral blood lymphocyte subsets and clinical outcomes in patients with lung cancer treated with immune checkpoint inhibitor

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Background: This study aimed to estimate peripheral blood lymphocyte subsets and programmed death receptor-1 positive (PD-1+) proportions of T cells, and their impact on progression free survival (PFS) and radiological response in lung cancer.

Methods: From May 2018 to April 2020, 34patients of the Henan Tumor Hospital who were diagnosed with advanced lung cancer were recruited to this study. Peripheral blood lymphocyte subsets and PD-1+ proportions of T cells were assessed by flow cytometry before and after treatment with immune checkpoint inhibitors (ICIs). The associations among these parameters, and PFS and clinical response were estimated by survival analysis and Fishers' exact test, respectively.

Results: Several lymphocyte variables and biomarkers were found to be correlated with PFS and tumor response, as assessed using the Response Evaluation Criteria in Solid Tumors (RECIST). In all 34 lung cancer participants and a subgroup of 28 participants with non-small cell lung cancer (NSCLC), higher levels of natural killer (NK) cells and higher CD4+/CD8+ cell ratios before the ICIs treatment were associated with longer PFS. Moreover, CD4+ T cells were significantly correlated with radiological response in all 34 lung cancer participants. Of the 28 NSCLC participants, those with higher levels of CD4+ T cells, CD4+/CD8+ cell ratios, absolute numbers of NK cells, and lower levels of regulatory T cells (Tregs)before treatment had better tumor response. After 2 cycles of combined ICIs treatment, both the absolute numbers of CD4+ T cells and CD45+ lymphocytes were statistically associated with PFS after being adjusted for gender and neutrophil-lymphocyte ratio (NLR) [hazard ratio (HR) =0.23, P=0.015; HR=0.30, P=0.032, respectively]. The absolute numbers of CD45+, CD3+, and CD4+ T lymphocytes were associated with radiological response treated by ICIs (P=0.038).

Conclusions: Our results suggested that the absolute number of NK cells and CD4+/CD8+ cells ratio before treatment could predict longer PFS and better radiological response in lung cancer patients treated with ICIs combination therapy. In addition, Tregs, as well as the other parameters in lymphocyte subsets, may also predict response.

Keywords: Immune checkpoint inhibitor (ICIs); programmed death receptor-1 (PD-1); lung cancer; peripheral blood lymphocyte subsets

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Introduction

Over the past few years, immune checkpoint inhibitors (ICIs) have been considered the most significant improvement in tumor treatment. Antibodies against programmed death receptor-1 (PD-1)/PD-1 ligand 1 (PD-L1) showed promising efficiency in certain kinds of solid tumor, including non-small cell lung cancer (NSCLC) (1) and small cell lung cancer (SCLC) (2,3). It has been recommended to assess the status of PD-L1 in tumor tissue when considering treatment options for NSCLC (4). Other biomarkers such as tumor mutation burden (TMB) in tumor tissue and in circulating peripheral blood have returned conflicting results on predicting lung cancer treated by ICIs (5). There is a lack of dynamic and longitudinal methods to monitor the efficiency of ICIs, and developing such methods may become the main focus of study for ICIs.

Liquid biopsy was considered a suitable non-invasive and dynamic method to predict and evaluate the clinical outcomes of ICIs. The strategy for identifying tumor-reactive T cells in peripheral blood is to focus on the cytotoxic CD8+ T cells that express PD-1, a well-known marker for previously activated or exhausted T cells. Tumor neoantigen-specific T cell subpopulations could be isolated from the peripheral blood of tumor patients (6-8). Besides, a recent study indicated that the distribution of peripheral blood lymphocyte subsets and expression of PD-1 on T cells before treatment might predict the outcome of Nivolumab in NSCLC patients. Assessing the pre-treatment levels of exhausted T cells as well as their decrease upon treatment may also predict response and clinical outcome (9). Chemotherapy might also regulate the proportions of immune cells in peripheral blood (10). Some preclinical studies have suggested that oral anti-angiogenic agents could optimize the efficiency of ICIs (11,12). The combination of ICIs with chemotherapy or oral anti-angiogenic agents has promising potential for the clinical treatment of tumors in the clinical practice (1,13-15). The potential prediction roles of peripheral blood lymphocyte subsets and expression of PD-1 on T cells are still unknown and need to be clarified for lung cancer patients treated by ICIs in combination therapies.

To estimate the potential prediction of peripheral blood lymphocyte subsets on lung cancer patients using ICIs combinations in the real world, flow cytometer was used to detect 12 parameters before and after the ICIs treatment. We hypothesized that the proportions of peripheral blood lymphocyte subsets and PD-1 expression on T cells during treatment may help to predict the clinical outcomes in lung cancer patients. In the present study, we performed flow cytometry analysis to investigate the different lymphocyte subsets before and after ICIs treatment, including ICIs combined with anti-angiogenic agents, ICIs combined with chemotherapy, and so on. We present the following article in accordance with the MDAR checklist (available at http://dx.doi.org/10.21037/apm-21-163).

Methods

Study population and assessments of clinical outcomes

This retrospective study included patients with advanced NSCLC who were receiving PD-1 inhibitors in the Medical Oncology and Immunotherapy Departments of the Henan Tumor Hospital from May 2018 to April 2020. The main eligibility criteria for treatment with PD-1 inhibitors (including pembrolizumab, nivolumab, sintilimab, camrelizumab, tislelizumab, and toripalimab) included histological diagnosis of advanced lung cancer (stage IV NSCLC and extensive stage SCLC), Eastern Cooperative Oncology Group Performance Status (ECOG-PS) ≤2. The major exclusion criteria were the need for systemic corticosteroid treatment at a dose >10 mg/day of prednisone (or equivalent), and the uncontrolled brain metastases. Eligible patients could have received PD-1 inhibitor alone, chemotherapy combined with PD-1 inhibitor, or oral anti-angiogenesis drugs (including anlotinib and apatinib). The neutrophil-lymphocyte ratio (NLR) was defined as the neutrophil count divided by the lymphocyte count, and was included on account of previous support for its prognostic effect (16). After exclusion of patients with incomplete follow-up information, a total of 34 participants remained for the PFS analysis. This retrospective study was approved by the institutional review board of Henan Tumor Hospital and performed in compliance with Helsinki Declaration (as revised in 2013). Before the initiation of any study-related procedures, written informed consent was given by each participant.

Response assessment

The participants underwent a therapeutic effect assessment every 2 cycles of PD-1 inhibitor. Radiological response assessment was performed using the Response Evaluation Criteria in Solid Tumors (RECIST) v. 1.1; moreover, because RECIST may underestimate the evaluation of therapeutic effect of ICIs, an extra response assessment was performed using the immune-related response criteria

(irRC). The best overall response (BOR) during the whole treatment with ICIs was recorded for both RECIST and irRC according to the following groups: complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD). For purposes of our analyses, participants were then allocated to: (I) responder group, which included all patients with CR or PR or (II) no responder group, which included all patients with SD or PD. The PFS was calculated from the date that treatment began to the first instance of tumor progression according to RECIST (RECIST-PFS) or irRC (irRC-PFS), date at last clinical examination, date of death of any cause before tumor progression, or the last contact date.

Blood collection and flow cytometry

Peripheral blood cells (2mL) were collected from participants who had provided written informed consent before their first treatment with a PD-1 inhibitor. A total of 3 panels were designed for flow cytometry. The CD3-4-8 panel was used to detect the ratio of CD3, CD4, and CD8 in CD45+ lymphocytes and the expression of PD-1 on these cells. In the NK+B+ Trucount panel, BD Trucount tubes (Becton, Dickinson and Co., San Jose, CA, USA) were used to detect the proportion and absolute counts of NK and B cells in peripheral blood lymphocytes; meanwhile, the absolute counts of CD45+ lymphocytes and CD3+ T cells were determined in this panel. Finally, regulatory T cells (Tregs) in CD4+ T cells was detected in the Tregs panel with the phenotype of CD25+ and CD127. According to the absolute count of CD3+T cells in the NK+B+ Trucount panel and the proportion of CD4+and CD8+T cells in CD3+T cells in the CD3-4-8 panel, we calculated the absolute number of CD4+ and CD8+ T cells in peripheral blood. The representative gating strategy and the expression of PD-1 on CD45+ T cells, CD3+ T cells, CD8+ T cells, CD4+ T cells from one representative patient were shown in *Figure 1*. The detailed operational procedures and related materials are provided in the Supplementary documents.

Statistical analysis

Descriptive statistics were used to summarize the investigatory findings of the 12 variables tested from peripheral blood lymphocytes and participant characteristics. The median values were set as the cutoff values for the higher and lower groups. The median survival time was calculated using the Kaplan-Meier method, and the survival curves were compared using log-rank test. Multivariate Cox proportional hazards regression models were conducted to test the effect of each variable [in terms of hazards ratios (HRs) and their corresponding 95% confidence intervals (CIs)] on PFS with or without adjustment for selected factors by using the univariate Cox proportional hazards regression models (for PFS, 2 variables were adjusted: gender and NLR). All tests were 2-sided, and a P value <0.05 was considered statistically significant. All data were analyzed with R version 4.0.3 software (https://www.r-project.org/), if not mentioned elsewhere. The survival curve and box plot were performed by Prism GraphPad Software 8.0(San Diego, CA, USA).

Results

Participant characteristics and clinical outcomes

A total of 34 patients were enrolled in this study. The baseline clinical and histology characteristics are listed in *Table 1*. Epidermal growth factor receptor EGFR gene mutations were detected by next-generation sequencing (NGS) in 11 participants and only 2 participants were EGFR gene sensitive mutation positive. Both EGFR gene mutation participants received ICIs after the EGFR-tyrosine kinase inhibitor (TKI) treatment and did not have T790M mutation. The protein expressions of PD-L1 were tested by immunohistochemistry in 14 participants, of which 9 were positive. The tumor response rate was 33.3% (3 PR) , the median PFS was 17.8 months in the 9 positive patients; and 20% (1 PR) and 12.3 months in the 5 negative patients. These results were not significant statistically for the small sample. In this study, 10 patients had immune related adverse events, including immune related hypothyroidism (n=6), immune related livery injury (n=1), immune related thrombocytopenia (n=1), immune related hyperthyroidism (n=1), immune related skin rash (n=1).

The median number of ICI administrations was 6 cycles, with a range of 2–17 cycles. Overall, 6 participants had died at the last contact. The median PFS and overall survival (OS) were 9.95 and 24.07 months, respectively. The best of response in RECIST was reported as follows: PR =19, SD =10, PD =5; no complete responses were observed. The associations between the 34 participant characteristics and PFS were tested by univariate and multivariate analysis. However, we did not find statistical significance in the univariate analysis (data not shown). In the multivariate analysis, gender and NLR were significantly associated with

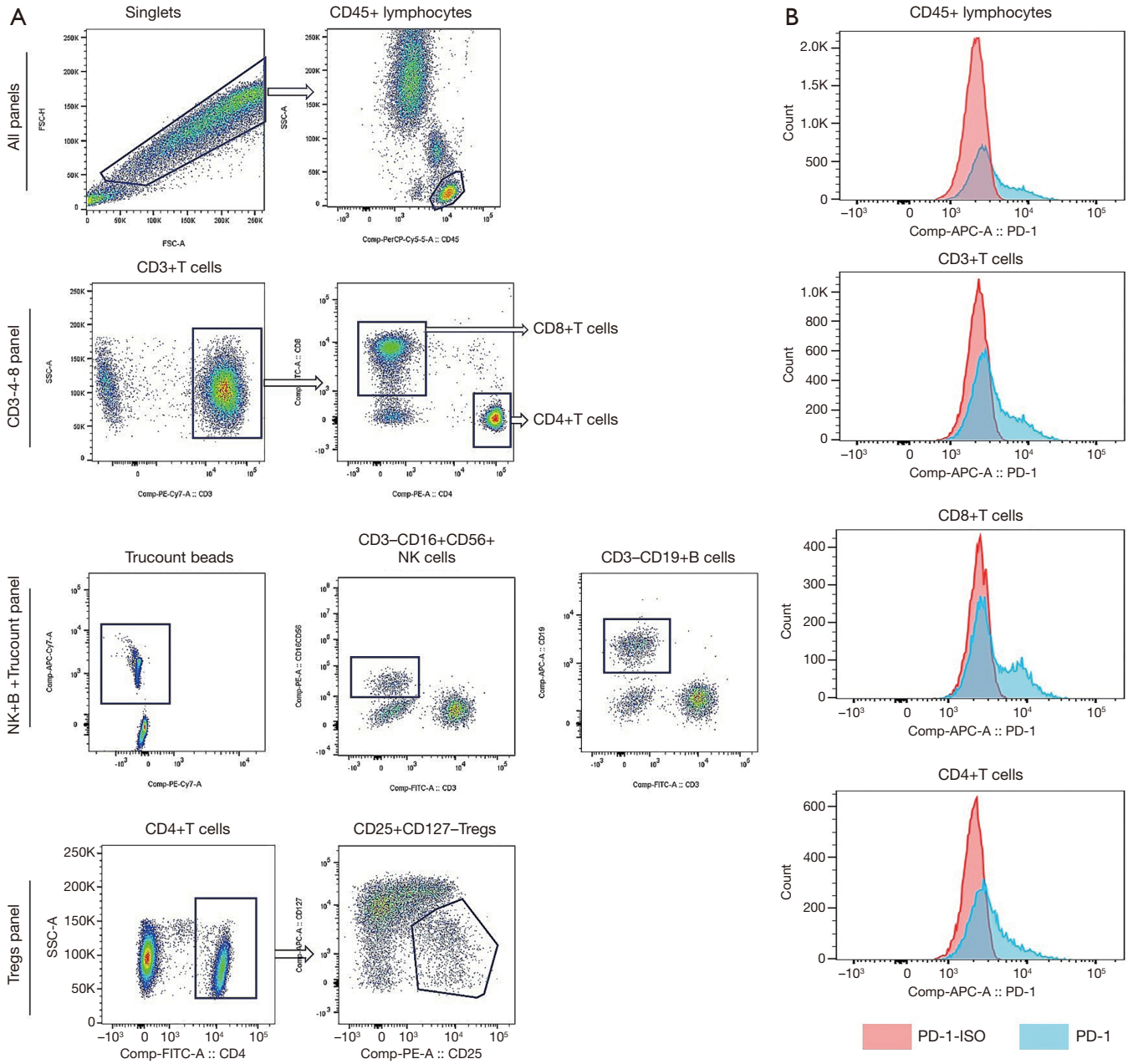

Figure 1 Identification the subgroups and PD-1 expression of lymphocytes in blood of lung cancer participants by flow cytometry. Flow cytometry of lymphocytes in blood of lung cancer participants (A) Representative gating strategy: As shown in the top row, all data files except the bead events gated for absolute counts of lymphocytes were first preprocessed to remove adhesion cells by FSC-A and FSC-H and then circle the CD45+ lymphocytes. CD3-4-8 panel was used to identify CD3+, CD4+ and CD8+ T cells. NK+B+ Trucount panel was used for identifying NK and B cells and determining absolute counts of lymphocytes in bloods. Tregs panel was used to characterize Tregs in CD4+T cells (CD4+CD25+CD127–). (B) The expression of PD-1 on CD45+ T cells, CD3+ T cells, CD8+ T cells, CD4+ T cells from one representative patient.

Table 1 Summary of clinical and pathological participant characteristics

Participant characteristic	N	%
Gender		
Male	28	82.4
Female	6	17.6
Age		
<65	18	52.9
≥65	16	47.1
Smoker		
Yes	23	67.6
No	11	32.4
Histology		
Adenocarcinoma	16	47.1
Squamous cell lung cancer	12	35.3
Small cell lung cancer	6	17.6
Treatment line		
First and second line	24	70.6
Third line and beyond	10	29.4
Brain metastasis		
No	24	70.6
Yes	10	29.4
Live metastasis		
No	28	82.4
Yes	6	17.6
Bone metastasis		
No	28	82.4
Yes	6	17.6
Treatment model		
ICIs alone	14	41.2
ICIs plus	20	58.8
Best treatment response		
Response	18	52.9
No response	16	47.1
NLR		
Low	17	50.0
High	17	50.0

ICIs, immune checkpoint inhibitors; NLR, neutrophil-lymphocyte ratio.

PFS (Figure S1). Hence, in the subsequent analysis, gender and NLR were selected as adjusted factors in multivariable analysis.

Peripheral blood lymphocytes subsets and PFS in 34 lung cancer patients and 28 NSCLC patients

We examined 12variables in the peripheral blood lymphocytes tests of 34 lung cancer participants, including Tregs, B cells, NK cells, CD45+ T cells, CD8+ T cells, CD4+ T cells, CD3+ T cells, CD4+/CD8+ ratio, PD-1+/CD45+ T cells, PD-1+/CD8+ T cells, PD-1+/CD8+ T cells, PD-1+/CD4+ T cells, and PD-1+/CD3+ T cells. In the survival analysis, when HR<1, the variable was a protective factor, which meant that groups with higher numbers had longer survival outcomes. The associations between the 12 variables in peripheral blood lymphocyte tests and PFS were not significant in the univariable analysis (data no shown). However, the absolute count of NK cells and CD4+/CD8+ ratio were significantly associated with PFS by multivariable analysis (forest plot shown in *Figure 2A*). The analysis of association between NK cell absolute counts and PFS by Kaplan-Meier survival curve is shown in *Figure 2B*. In the log-rank test, the median PFS in groups with lower NK cells was 8.63 months, and the higher group was 17.83 months (P=0.008). The Kaplan-Meier survival curve analysis of the association between CD4+/CD8+ cells ratio and PFS is shown in *Figure 2C*. In the log-rank test, the median PFS in the lower grouping of CD4+/CD8+ T cells ratio was 8.73 months, and the higher grouping was 11.53 months (P=0.017).

Among the 28 NSCLC participants, the association between the 12 variables in the peripheral blood lymphocytes test and PFS were not significant in the univariable analysis (data not shown). However, the absolute counts of NK cells and CD4+/CD8+ ratio were significantly associated with PFS by multivariable analysis (forest plot shown in *Figure 3A*). The Kaplan-Meier survival curve analysis of the association between the absolute count of NK cells and PFS is shown in *Figure 3B*. In the log-rank test, the median PFS in the contingent with lower counts of NK cells was 8.68 months, and the higher group was 18.37 months (P=0.013). Kaplan-Meier survival curve analysis of the association between CD4+/CD8+ cells ratio and PFS is shown in *Figure 3C*. In the log-rank test, the median PFS in the lower group of CD4+/CD8+ T cells ratio was 8.73 months, and the higher group was 18.37 months (P=0.016).

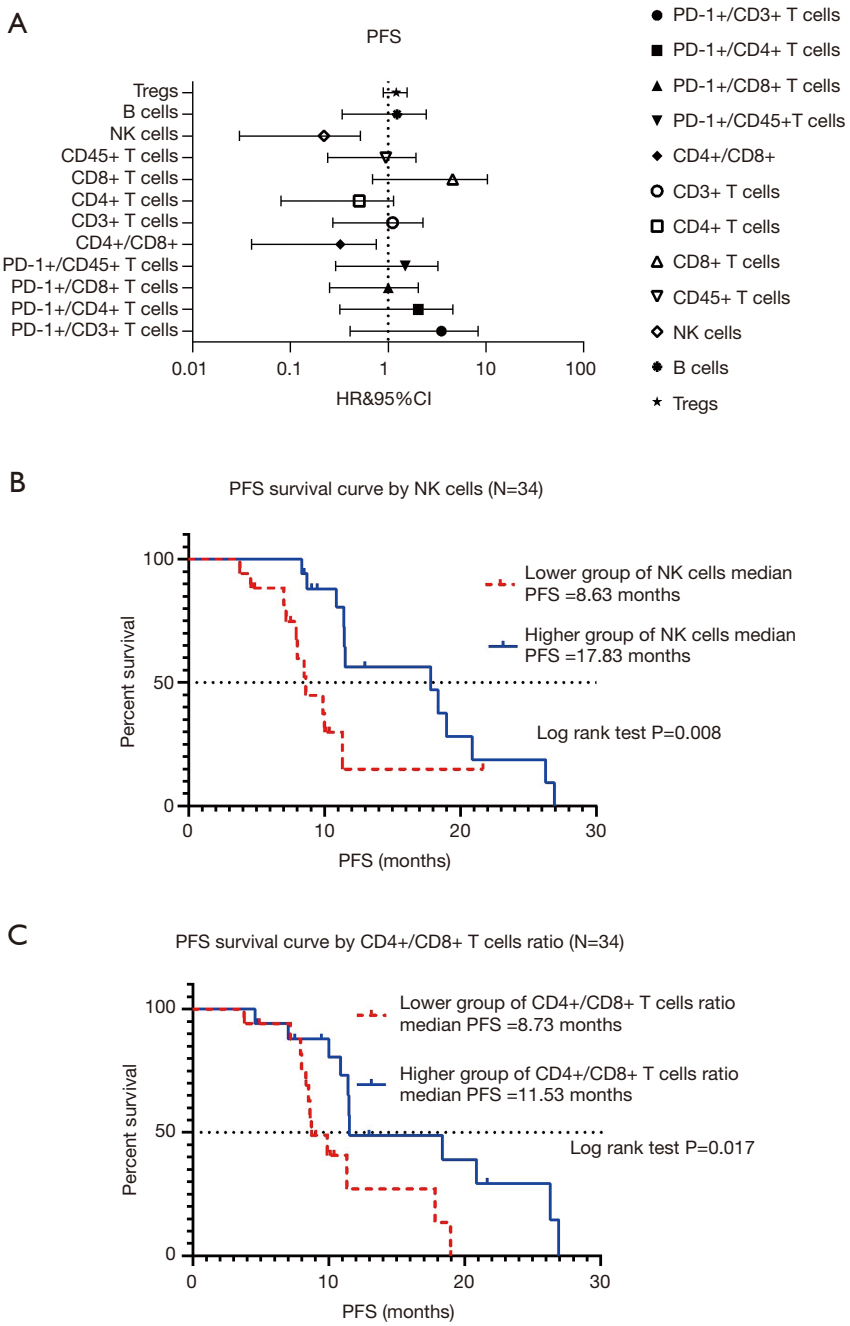

Figure 2 The associations between peripheral blood lymphocyte subsets and PFS in 34 lung cancer participants. PFS, progression free survival; NK, natural killer.

Peripheral blood lymphocyte subsets and radiological response in all lung cancer participants and NSCLC participants

Next, we investigated the correlation between baseline peripheral blood lymphocyte subsets and radiological response. Notably, analyses using the irRC criteria did not show any differences from those using RECIST 1.1. Among the 34 lung cancer participants, only the absolute value of CD4+ T cells at baseline was profoundly associated with radiological response by unpaired t-test (P=0.018), and is shown in *Figure 4*.

Among the 28 NSCLC participants, in agreement with

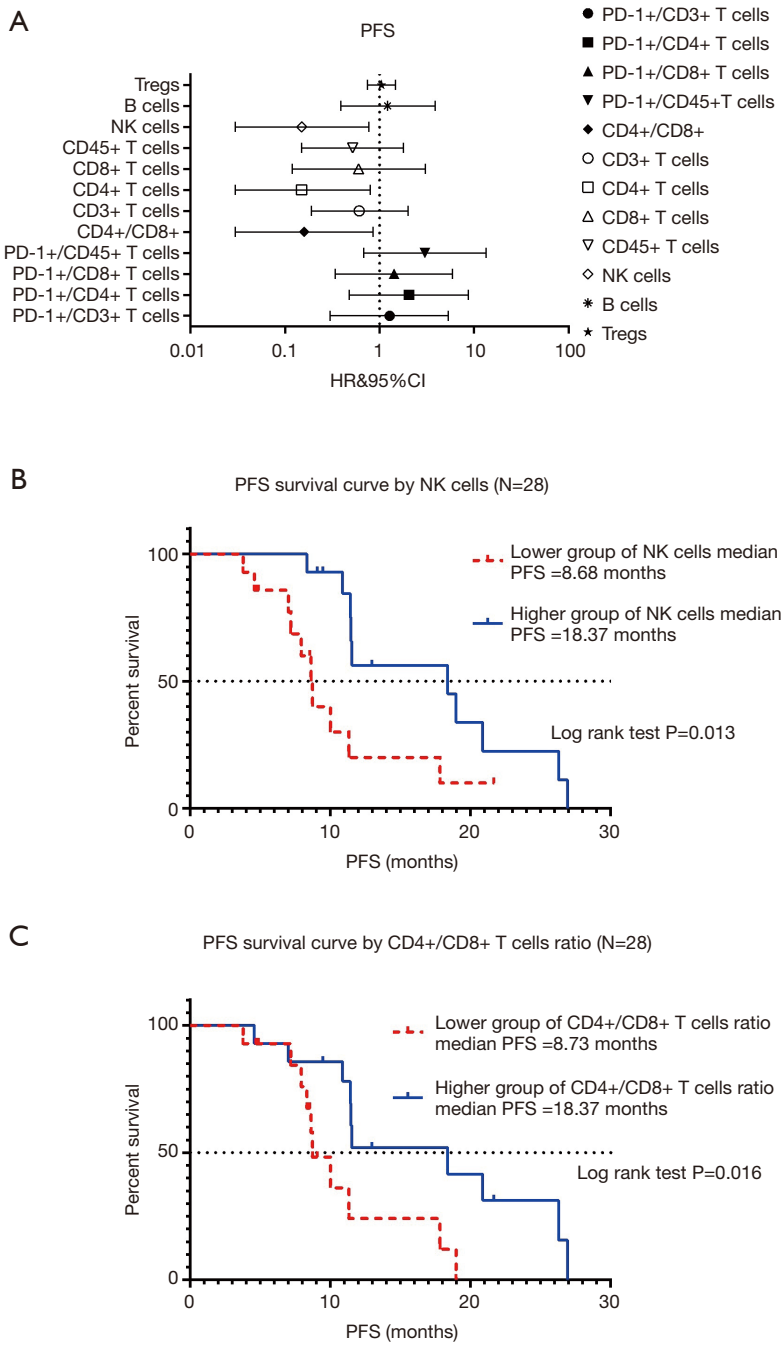

Figure 3 The associations between peripheral blood lymphocytes subsets and PFS in 28 NSCLC patients. PFS, progression free survival; NSCLC, non-small cell lung cancer.

our data correlating peripheral blood lymphocyte subsets and PFS, higher numbers of NK cells and CD4+/CD8+ ratios were statistically associated with radiological response by unpaired t-test (P=0.006 and P=0.030, respectively). In addition, higher grouping of CD4+ T cells and lower grouping of Tregs were significantly associated with radiological response in the unpaired t-test (P=0.008 and P=0.019), respectively (*Figure 5*).

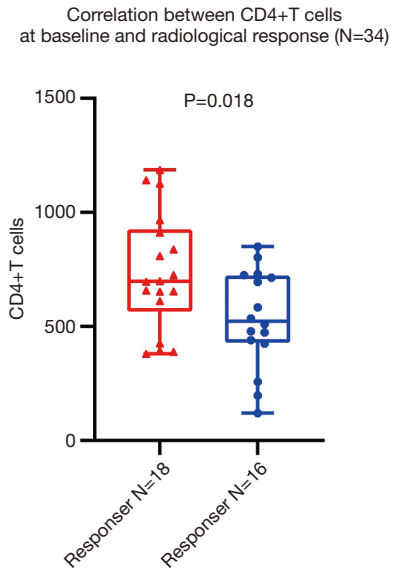

Figure 4 Correlation between CD4+ T cells at baseline and radiological response in 34 lung cancer participants.

Peripheral blood lymphocyte subsets and clinical outcomes (PFS and radiological response) in all lung cancer and NSCLC participants after 2 cycles of ICI treatments

The peripheral blood lymphocyte subsets of 33 participants were assessed after 2 cycles of ICIs treatment. In the univariate analysis, no variables were associated with PFS. In the multivariate analysis, the absolute number of CD4+ T cells and CD45+ lymphocytes were associated with PFS statistically adjusted by gender and NLR (HR=0.23, P=0.015; HR=0.30, P=0.032, respectively).

Due to the small sample size, Fisher's exact test was used to analyze the associations between radiological response and lymphocyte subsets after treatment. The absolute numbers of CD45+, CD3+, and CD4+ T lymphocytes were associated with response to treatment with ICIs (P=0.038).

Discussion

In this real-world study, we found that several variables in peripheral blood lymphocyte subsets tested prior to treatment were associated with clinical outcomes in lung cancer participants treated with ICIs. A total of 34 lung cancer patients were enrolled including 28 NSCLC patients, and they were treated with ICIs alone or ICIs combined therapy. Among the entire participant cohort, compared to those with lower levels of NK cells, participants with higher levels of NK cells had longer PFS. We also found that the CD4+/CD8+ cells ratio was associated with PFS in the entire participant group. Participants with higher CD4+/CD8+ cell ratios were found to have longer PFS. Furthermore, CD4+ T cells at baseline were significantly correlated with the radiological response in the 34 lung cancer participants. The participants with higher levels of CD4+ T cells, CD4+/CD8+ ratio, absolute numbers of NK cells, and lower levels of Tregs had better tumor responses.

The CD4+/CD8+ ratio in peripheral blood lymphocytes is an old marker in many diseases (17), and is also associated with lung cancer risk (18). Low CD4+/CD8+ ratio has been correlated with high-risk types of lung cancer (19). In a small retrospective study (20), an increasing lymphocyte ratio was associated with disease control in NSCLC patients treated with an ICIs alone. In present study, the median CD4+/CD8+ ratio of the 34 lung cancer participants was 1.44 (0.3–3.6). A higher CD4+/CD8+ ratio was associated with better PFS. In the 28 NSCLC participants, tumor response was also correlated with a higher CD4+/CD8+ ratio.

A similar study reported on 74 participants in an advanced NSCLC cohort who were treated with nivolumab and had their immune parameters assessed from peripheral blood lymphocyte subsets during treatment. They found that higher levels of CD3+, CD4+, and CD8+ T cells but lower levels of NK cells at baseline was associated with longer OS (9). In the present study, participants with higher levels of CD4+ had a trend of longer PFS but this trend was not statistically significant. Conversely, the baseline absolute numbers of NK in peripheral blood were suggested to be positively associated with longer PFS in our investigation. This data is similar to a previous report by Mazzaschi *et al.* (21). The NK cells are innate cytotoxic lymphocytes that actively prevent tumor development, growth, and metastatic dissemination in a process called cancer immunosurveillance; some chemotherapies have been created to regulate the functions of NK cells for anti-tumor immunity (22). Yang *et al.* reported that anlotinib (11), an oral multi-targeted anti-angiogenic agent, could reprogram the tumor immune microenvironment in a manner that augments anti-cancer innate immune cells, including NK cells, dendritic cells (DC), and so on. Moreover, this kind of combination might have potential additive or synergistic anti-tumor efficacy.

In the NSCLC subgroup analysis, higher levels of CD4+/CD8+ ratio, CD4+, and NK cells at baseline were associated with higher PFS, and parallel evaluation of the 3 variables was correlated with higher response. However, a

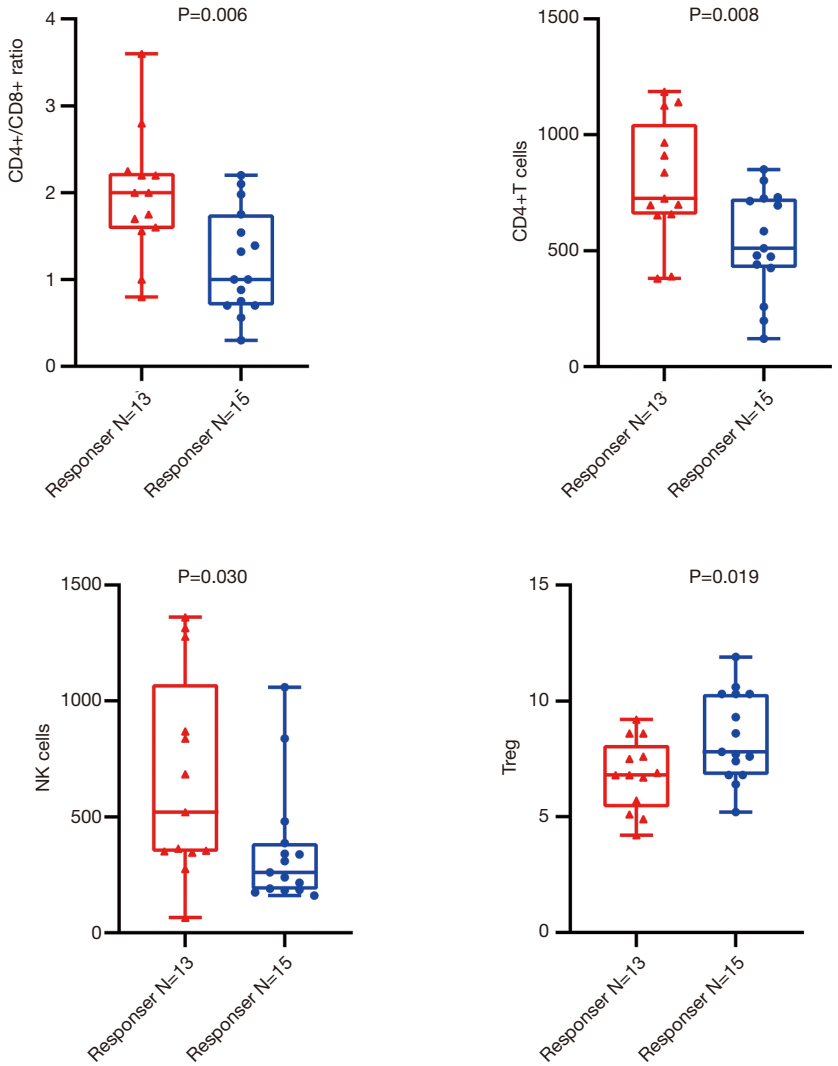

Figure 5 Correlation between CD4+/CD8+ ratio, CD4+ T cells, NK cells and Treg cells at baseline and radiological response in 28 lung cancer participants. NK, natural killer; Treg, T regulatory.

higher level of Treg was correlated with worse radiological response, which was not associated with PFS. These findings could inspire further investigation at the tumor site as opposed to the peripheral blood lymphocyte subsets.

Remarkably, our results were derived from real word clinical practice, the participants were treated with different combinations with ICIs including single ICIs (n=7), double ICIs (n=1), chemotherapy plus ICIs (n=6), and small molecular antiangiogenic drug plus ICIs (n=20). Among the 34 participants, only 14 were evaluated for the expression of PD-L1 protein in tumor tissues, of which 9 were positive and 5were negative. On account of our small sample size, we did not perform sub-group analyses based on histology and smoking status. We proceeded with multivariable analysis in Cox regression models for PFS. The NLR and gender were the major factors of adjustment. In addition, the PD-1 positive T cells at baseline were not associated with the PFS and response in 34 lung cancer participants based on peripheral blood lymphocyte subsets analysis, which was contrary to the findings of Ottonello *et al.* (9). Results from metastatic melanoma samples indicate that a higher proportion of PD-1+/CD8+ among tumor infiltrating lymphocytes (TILs) predicts higher response and longer PFS (23).

Nevertheless, the absolute numbers of circulating NK cells and the CD4+/CD8+ ratio were the main factors

affecting the PFS in cases of lung cancer receiving different ICIs combinations.

Our study had some limitations. Firstly, the participants had different kinds of treatment combinations and the sample size was small. Studies involving larger sample sizes are required to validate our results. Secondly, our present data were based on the peripheral blood lymphocyte subsets analysis at the baseline, so it might not have reflected the actual situations at the primary tumor site. And lack of further longitudinal peripheral blood samples collecting. Thirdly, the benefit to OS was the primary goal of immunotherapy for the lung cancer, and ongoing subsequent follow-up is required. Fourthly, the ICIs and treatment combinations were from different pharmaceutical corporations may be an important confounder in the analysis.

Currently, the expression levels of PD-L1protein in lung cancer tissue are defined as the main positive biomarker in clinical practice (24,25). Besides, using tumor mutation burden (TMB) in tumor tissue or peripheral blood has yielded mixed results in lung cancer, and still needs further assessment before applying it to clinical practice (26,27). Recently, STK11/LKB1 was considered a negative biomarker in ICIs treatments, regardless of KRAS status (28,29). Further investigations might focus on the tissue-based biomarkers and circulating blood, including the peripheral blood lymphocyte subsets analysis as in our study.

In spite of these limitations, our small sample size lung cancer study, mainly investigating treatment with ICIs plus small molecular anti-angiogenic drugs, provided novel immune parameters to potentially predict clinical outcomes based on the analysis of peripheral blood lymphocyte subsets.

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Footnote

Reporting Checklist: The authors have completed the MDAR checklist. Available at http://dx.doi.org/10.21037/apm-21-163

Data Sharing Statement: Available at http://dx.doi.org/10.21037/apm-21-163

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/apm-21-163). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This retrospective study was approved by the institutional review board of Henan Tumor Hospital and performed in compliance with Helsinki Declaration (as revised in 2013). Before the initiation of any study-related procedures, written informed consent was given by each participant.

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Flow cytometry staining and analysis

(I) Marked flow pipe, 100 µL blood samples (50 µL in absolute count tube) were added into each tube, and then fluorescein labeled cell membrane molecular antibodies were added according to the antibody instructions (*Table S1*), and incubated at 4 ℃ in dark for 20–30 minutes.

(II) After the staining, 1mL of red blood cell lysate was added into each tube (450ul in the absolute counter tube). After the oscillation, it was left for 10 minutes at room temperature and in dark, and then centrifuged at 300g for 5 minutes (the absolute counter tube was directly put on the machine without centrifugation).

(III) The cells were resuspended with PBS and centrifuged at 300g for 5 min. The cells were washed twice and added with 200 L PBS and prepared for loading.

i. Add an appropriate Staining Buffer. Adjust cell concentration to 106/100 L/Test;

ii. Labeled flow tubes were 1, 2, 3, 4, and 5 (*Table S2*). Each tube was labeled as shown in the table below. Fluorescein labeled cell membrane molecular antibodies were added according to the antibody instructions, and incubated at 4 ℃ in dark for 20–30 minutes.

iii. After the staining, the cells were resuspended with PBS (300 percp-CY5.5) for 5 minutes, and then washed twice. Cells were resuspended with 200 L PBS (10 L 7AAD (percP-CY5.5 channel detection) active dye was added into each tube.

Table S1 Flow cytometry antibody labeling details

Anti-bodies	Companies	Product No.
Tregs		
CD4-PE	Biolegend	317410
CD25-PE	Biolegend	302606
CD127-APC	Biolegend	351316
CD3-4-8, PD-1		
CD3-PE-cy7	Biolegend	344816
CD4-PE	Biolegend	317410
CD8-FITC	Biolegend	301050
PD-1-APC	Biolegend	329908
CD45-PERCP-CY5.5	BD	340953
NK,B absolute count		
CD3-FITC	Biolegend	300306
CD56-PE	Biolegend	362508
CD16-PE	Biolegend	360704
CD19-APC	Biolegend	302212
CD45-PERCP-CY5.5	BD	340953
Absolute counter tube	BD	340334

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Table S2 Flow cytometry antibody labeling details

	FITC	PE	APC	PE-cy7	APC-cy7
1*	CD4		IgG1-APC	CD3	CD8
2	CD4		PD-L1	CD3	CD8
3*	CD103	IgG1	PD-1	CD3	CD8
4*	CD103	TIM-3	IgG1	CD3	CD8
5	CD103	TIM-3	PD-1	CD3	CD8

1*, PD-L1 for continuous expression, the tube was set to a fluorescence minus one control (FMO) tube with no clustering; 3*, 4*, Tim-3 and PD-1, the cluster was not obvious in individual tumor tissues, and the two tubes were set as FMO tubes with no. 5 tubes.

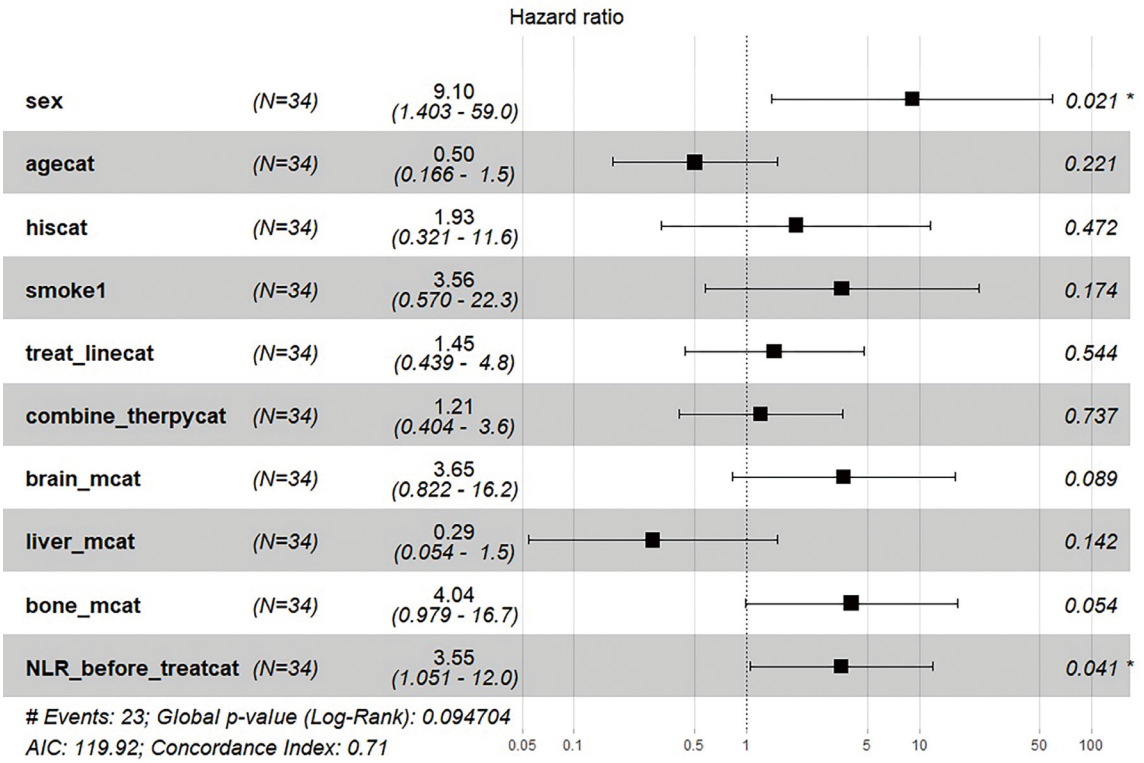

Figure S1 Forest plot for the associations between the 34 participant characteristics and PFS by Cox proportional hazards regression models. sex = gender; agecat = age category; smoke1 = smoker; treat_linecat = treat line; combine_therapycat = treatment model; brain_mcat = brain metastasis; liver_mcat = liver metastasis; bone_mcat = bone metastasis; NLR_before_treatcat = NLR.

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