T cell invigoration is associated with the clinical response to anti-PD-1 based immunotherapy in non-small cell lung cancer

Hui Wu
Lishui Central Hospital and Fifth Affiliated Hospital of Wenzhou Medical College

Gui zhen Weng
Fujian Medical University Union Hospital

Lina Sun
Lishui Central Hospital and Fifth Affiliated Hospital of Wenzhou Medical College

Zhang chi Pan
Fujian Medical University Union Hospital

Lu Zhang
Fujian Medical University Union Hospital

Qiang Chen
Fujian Medical University Union Hospital

Chun mei Shi (✉ shichunmei@tom.com)
Fujian Medical University Union Hospital

Research Article

Keywords: non-small cell lung cancer, immunotherapy, T cell invigoration, prognosis

Posted Date: June 8th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3021178/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License
Abstract

**Purpose:** Immune checkpoint inhibitors (ICIs) have been developed for clinical application and proven effective for non-small cell lung cancer (NSCLC). Blockade of the programmed cell death 1 (PD-1) protein can partially reinvigorate circulating exhausted-phenotype CD8+ T cells (Tex cells) in preclinical models, however the clinical implication in anti-PD-1 based immunotherapy in NSCLC is unknown.

**Methods:** Serum specimens were obtained before and during treatment from 145 patients with NSCLC patients who received anti-PD-1 treatment and their prognosis was followed-up. Indicators such as cell subpopulations, T cell invigoration were detected by clinical laboratory testing. Kaplan-Meier analysis was performed to draw survival curves and Cox regression analysis to confirm the independent prognostic factors of NSCLC patients.

**Results:** The expressions of Ki-67 in PD-1+/CD8+ T cells in most NSCLC patients (97 of 145 cases) increased after treatment. The responding Ki-67+/CD8+ T cell population was mainly CD45RAlo CD27hi, containing cells with high expression of CTLA-4, PD-1, and 2B4 and low expression of NKG2-D (P<0.0001). The maximum fold change of Ki-67+/PD-1+/CD8+T cells in treatment cycles and the tumor burden determined by imaging were associated with better progression-free survival (PFS) and overall survival (OS). A Ki-67 expression to tumor burden ratio greater than 0.6 at the 1st cycle of anti-PD-1 immunotherapy was associated with improvement of PFS and OS (P<0.05).

**Conclusion:** Activation of circulating Tex cells before therapy related to tumor burden may be associated with clinical efficacy of anti-PD-1 immune therapy in NSCLC.

Introduction

Immunotherapy has produced clinical reactions in patients with advanced melanoma, non-small cell lung cancer (NSCLC), and other tumor types, achieving long-term disease control and few adverse events(Chow et al. 2022). In particular, monoclonal antibodies represented by PD-1/PD-L1 inhibitors have achieved great success in the treatment of solid tumors, and the incidence of serious adverse reactions are low, making anti-PD-1 treatment one of the most promising immunotherapy options(Nielsen et al. 2022). Drugs targeting PD-1/PD-L1 can reactivate the body’s anti-tumor immunity and achieve long-term efficacy against a variety of tumors. However, it is still unclear how to predict the sensitivity of patients to treatment and improve the response rate to anti-PD-1 therapy. This therapy can have serious side effects, such as cardiac toxicity, bullous pemphigoid, thyroid dysfunction, Diarrhoea, etc. And biomarkers could help to select patients who are most likely to benefit(Ramos-Casals et al. 2020).

Tumor infiltrating T cells can be used as a positive prognostic indicator for various cancers(Paijens et al. 2021). In some cases, the expression of PD-L1 in tumors is also related to the T cell response(Freeman et al. 2000; Li et al. 2022); some studies have suggested that NSCLC patients with a high tumor mutation burden (TMB) are more likely to benefit from PD-1 monoclonal antibody therapy(Latchman et al. 2001). However, these biomarkers need to be specifically analyzed in tumor tissue samples. The acquisition of
tumor tissue samples can be complex, and the ability to predict the effect of treatment is still not ideal. Other immunotherapeutic markers, including circulating tumor DNA (ctDNA) (Abbosh et al. 2023), TMB detection on circulating tumor cell (CTC), and expression of PD-L1 (De Marchi et al. 2021), are also being actively explored. In this study, we detected and recorded serum indexes before or during immunotherapy to reflect the immune status of patients, and further explored how these immune indexes could predict the anti-PD-1 therapy immune effect of patients with non-small cell lung cancer.

Materials and Methods

Patients

The study ran from November 2019 to October 2021 at the Department of Oncology, Fujian Medical University Union Hospital and included 145 patients with unresectable or metastatic non-small cell lung cancer who were treated with anti-PD-1 therapy. The deadline for follow-up is April 2022. Patients were stratified as responders (n=56) or non-responders (n=89) to anti-PD-1 therapy with Pembrolizumab according to their subsequent clinical response based on tumor burden assessed by imaging at four-week intervals. We assessed immune cell populations by flow cytometry to identify specific immune cell subsets. The Ki-67 expression was used as an indicator of T cell activation and proliferation. Principal component analysis (PCA) was carried out to select biomarkers to determine which cell populations could best describe the difference in cell frequency between responders and non-responders. Patients received treatment with up to three intravenous doses every third week. A total number of 145 patients from whom serum samples were obtained at baseline (before the start of treatment), at week three (before the second treatment), and at week six (before the third treatment) were included in the study.

Patients started therapy on week one and were followed-up every third week during treatment. The clinical data of the patients is shown in Table 1. Peripheral blood mononuclear cells (PBMCs) were obtained from tumor patients and from twenty healthy volunteers after they gave informed consent using a Cancer Institute Institutional Review Board approved protocol. Patients with recent immunotherapy, autoimmune diseases, unclear pathological diagnosis, active brain metastasis, or no measurable target lesions were excluded. All the patients provided written informed consent. The experiments using human tissue samples were approved by the Clinical Research Ethics Committee of Fujian Medical University Union Hospital (Medical Ethics Committee approval number: 2019KY096).

Assessment of tumor burden and response

Total measurable tumor burden was defined as the sum of the long axis of all measurable lesions (no more than five lesions and no more than two lesions per organ) reported on the pre-therapy imaging reports. Assessment of the clinical response and tumor burden was performed independently in a blinded fashion. The clinical response to anti-PD-1 therapy was determined as the best response based on immune related RECIST (irRECIST) using unidimensional measurements (Nishino et al. 2013).

Flow cytometry for lymphocyte subset analyses
Cryopreserved PBMC samples from pretreatment and cycles 1-3 (weeks 3-9) were thawed and labeled with a master mix of antibodies for surface antigens including CD3, CD4, CD8, CD56, NKG2D, CD25, CD127, HLA-DR, CD14, CD16, CD45RO, CD62L, CTLA-4, PD-1, CD45RA, and CD27 (Biolegend) and intracellular expression of Ki-67 (Biolegend). Permeabilization was performed using the FIX & PERM™ Cell Permeabilization Kit (Invitrogen). The PD-1 on post Pembro treatment specimens was detected using anti-human IgG4 PE (Biolegend). Pretreatment samples were pretreated with 25µg/ml Pembro in vitro for 30 min at 37.0°C, washed twice and stained with a standard antibody mix. Cells were resuspended in 1% paraformaldehyde until analysis on a BD FACSCanto II cytometer and analyzed using FlowJo.

Definition of the cut-off values

The X-tile program (http://medicine.yale.edu/lab/rimm/research/software.aspx) was used to determine the optimal cut-off values of Ki-67+PD-1+CD8+ T cells/TB ratio for Progression-Free-Survival (PFS). The Ki-67+PD-1+CD8+ T cells/TB ratio cut-off value for PFS was 0.6 with maximum χ² log-rank values of 8.83 (P<0.05) [9] (Figure 1). Therefore, patients were categorized into two groups: 61 patients with low value (≤0.6) and 84 patients with high value (>0.6).

Statistical analysis

Chi-square, Fisher exact, or unpaired Student’s t-tests were used to compare the differences between serum indicators and clinicopathologic factors of groups of patients. Correlation analyses were done with Pearson or Spearman tests, as indicated. Cohen's kappa coefficient was employed to analyze the consistency of the test results. Survival curves were estimated using the Kaplan-Meier method and were compared using a log-rank test. A multivariate Cox regression analysis was carried out to determine whether the different variables were associated with PFS and OS. Multivariate analysis was performed using the variables that showed significant univariate relationships with PFS and OS. All data analyses were performed using R software (version 3.6.1), SPSS version 24.0 (SPSS, Chicago, IL, USA), GraphPad Prism version 8.0 (GraphPad Software, La Jolla, CA, USA), and X-tile version 3.6.1 (Yale University, New Haven, CT, USA). Statistical significance was defined as P<0.05.

Results

T-cell invigoration to tumour burden ratio and Clinicopathological Features

The relationship between T-cell invigoration to tumor burden ratio and clinicopathological characteristics of the 145 NSCLC patients is summarized in Table 1. The high level of T-cell invigoration to tumour burden ratio was observed in about 75.2% (109/145) of the NSCLC patients. Statistical analysis revealed that T-cell invigoration to tumour burden ratio was associated with ECOG (P<0.001) and response to PD-1 blockade (P=0.002). In contrast, there were no significant differences in terms of age, gender, Smoking history, Histologic subtype, metastasis.
Table 1. Relationship between T-cell invigoration to tumour burden ratio and clinicopathological features in 145 cases of NSCLC.

<table>
<thead>
<tr>
<th>Clinicopathological features</th>
<th>N</th>
<th>T-cell invigoration to tumour burden ratio</th>
<th>$\chi^2$</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Low</td>
<td>High</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>male</td>
<td>103</td>
<td>24</td>
<td>79</td>
<td>0.444</td>
</tr>
<tr>
<td>female</td>
<td>42</td>
<td>12</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Age [years]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\geq 60$</td>
<td>41</td>
<td>12</td>
<td>29</td>
<td>0.604</td>
</tr>
<tr>
<td>&lt;60</td>
<td>104</td>
<td>24</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>Smoking history</td>
<td></td>
<td></td>
<td></td>
<td>2.228</td>
</tr>
<tr>
<td>Never</td>
<td>65</td>
<td>20</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>Ever</td>
<td>80</td>
<td>16</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>Histologic subtype</td>
<td></td>
<td></td>
<td></td>
<td>8.391</td>
</tr>
<tr>
<td>ADC</td>
<td>75</td>
<td>15</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>SqCC</td>
<td>34</td>
<td>13</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>ASC</td>
<td>12</td>
<td>1</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td>4</td>
<td>0</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>NSCLC PD</td>
<td>20</td>
<td>7</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>metastasis</td>
<td></td>
<td></td>
<td></td>
<td>3.574</td>
</tr>
<tr>
<td>Yes</td>
<td>84</td>
<td>16</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>61</td>
<td>20</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>ECOG</td>
<td></td>
<td></td>
<td></td>
<td>12.126</td>
</tr>
<tr>
<td>1</td>
<td>88</td>
<td>13</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>$\leq 1$</td>
<td>57</td>
<td>23</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>Response to PD-1 blockade</td>
<td></td>
<td></td>
<td></td>
<td>9.666</td>
</tr>
<tr>
<td>Clinical benefit</td>
<td>115</td>
<td>22</td>
<td>93</td>
<td></td>
</tr>
<tr>
<td>Non-responder</td>
<td>30</td>
<td>14</td>
<td>16</td>
<td></td>
</tr>
</tbody>
</table>
Abbreviations: ECOG, Eastern Cooperative Oncology Group. ADC adenocarcinoma, SqCC squamous cell carcinoma, ASC adenos-quamous carcinoma, PC pleomorphic carcinoma, NSCLC PD non-small cell lung cancer, poorly differentiated, PD-1 programmed-cell death-1, PD-L1 programmed-cell death ligand-1.*Significance with P<0.05.

Table 2  Cox Regression Analysis of Univariate Analysis and Multivariate Analysis for overall survival in NSCLC Patients

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Univariate Analysis</th>
<th></th>
<th>Multivariate Analysis</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>P-value</td>
<td>HR (95% CI)</td>
<td>P-value</td>
</tr>
<tr>
<td>Gender</td>
<td>0.705 (0.337-1.479)</td>
<td>0.356</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age ≥60 yr vs. &lt; 60 yr</td>
<td>1.006 (0.980-1.033)</td>
<td>0.653</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking history</td>
<td>0.770 (0.413-1.436)</td>
<td>0.411</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ECOG</td>
<td>0.333 (0.181-0.613)</td>
<td>&lt;0.001*</td>
<td>0.467 (0.238-0.916)</td>
<td>0.027*</td>
</tr>
<tr>
<td>Metastasis</td>
<td>1.650 (0.843-3.231)</td>
<td>0.144</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ki-67+PD-1+CD8+ T cells</td>
<td>0.877(0.772-0.996)</td>
<td>0.043*</td>
<td>0.958 (0.839-1.092)</td>
<td>0.519</td>
</tr>
<tr>
<td>Ki-67+PD-1+CD8+ T cells/TB ratio</td>
<td>0.086(0.020-0.367)</td>
<td>0.001*</td>
<td>0.171 (0.040-0.733)</td>
<td>0.017*</td>
</tr>
</tbody>
</table>

Note: *Significance with P<0.05.

Stratification of therapy response

We found that CD4+ T cells, CD8+ T cells, NK cells, and myeloid cells may reflect the differences between the response and non-response groups of patients; the Kaiser-Meyer-Olkin Measure of Sampling Adequacy (KMO) was 0.854 and the P-value of Bartlett's Test of Sphericity was <0.01. The analysis of CD4+ T cell markers in responders showed that CTLA-4, HLA-DR, NKG2D, and Ki-67 were up-regulated after treatment. Similarly, the CD8+ T cells in the responders showed high expression of CD45RO, CD45RA, CTLA-4, CD62L, NKG2D, and Ki-67.

Selection of serum markers in the early stage of immunotherapy

The immune parameters related to PFS were analyzed by Cox regression analysis after one cycle of treatment to evaluate the short-term efficacy in the response group and non-response group. Univariate
analysis suggested that the following phenotypes might be related to PFS (P<0.05): ratio of Ki-67+ to CD8+ T-cells %, ratio of Ki-67+ to PD-1+/CD8+ T-cells %, ratio of Ki-67+ to PD-1+/CTLA-4+/CD4+ T-cells %, ratio of Ki-67+ to PD-1+CTLA-4+CD8+ T-cells %, ratio of Ki-67+ to PD-1+CD127+CD4+T-cells %, CD4+CD25+CD127low T-cells %, CD3+CD4+CD45RO+ T-cells %, CD3+CD8+CD45RO+ T-cells %, CD14+CD16-HLA-DRhi T-cells %, CD8+CD45RO+CD62L+ T-cells %. Multivariate analysis of these factors (significant in univariate analysis) showed that ratio of Ki-67+ to CD8+ T-cells %, ratio of Ki-67+ to PD-1+/CD8+ T-cells %, ratio of Ki-67+ to PD-1+CTLA-4+CD4+T-cells %, ratio of Ki-67+ to PD-1+CTLA-4+CD8+ T-cells %, CD3+CD8+CD45RO+ T-cells % might be independently associated with PFS (P<0.05).

**Predictive analysis of T cell biomarkers on anti-PD-1 immune response**

The expression of Ki-67 in PD-1+/CD8+ T-cells in healthy volunteers changed only 1.2-folds ± 0.15 (P>0.05). Compared with healthy donors, Ki-67 expression was higher in CD8+ T-cells in tumor patients (P<0.0001), mainly in the PD-1+/CD8+ T-cell subset (P<0.0001), indicating a pre-existing immune response.

Peripheral blood collected before the anti-PD-1 immunotherapy was analyzed for CD8+ T cell numbers and phenotype. The increase of Ki-67 expression was most significant in PD-1+/CD8+ T-cells versus PD-1-/CD8+ T-cells (P<0.0001, Figure 2 a-b). Compared with the PD-1 negative subgroup, the Ki-67 expression of the PD-1+ subgroup reached its peak after one course of treatment.

On univariate analyses, gender, age ≥ 60 yr, smoking history, metastasis, ECOG, Ki-67+PD-1+CD8+ T cells, Ki-67+PD-1+CD8+ T cells/TB ratio were detected as potential poor prognostic factors for OS. Among them, ECOG (hazard ratio [HR], 0.333; 95% CI, 0.181 to 0.613; p<0.001), Ki-67+PD-1+CD8+ T cells (HR, 0.877; 95% CI, 0.772 to 0.996; p=0.043) and Ki-67+PD-1+CD8+ T cells/TB ratio (HR, 0.086; 95% CI, 0.020 to 0.367; p=0.001) were independent prognostic factors for OS (Table 2). Multivariate regression analysis revealed that low ECOG score and low Ki-67+PD-1+CD8+ T cells/TB ratio were independent poor prognosis factors for NSCLC patients.

To accurately track the biological indicators for predicting the efficacy of anti-PD-1 immunotherapy we continued to evaluate the cell subsets co-expressing PD-1 and other inhibitory receptors. Combined with the screening of immune parameters we found that compared to the Ki-67-/CD8+ T-cells, the Ki-67+/CD8+/T-cell population was mainly CD45RAlo and CD27hi, with high expression of CTLA-4, PD-1, and 2B4 and low expression of NKG2-D (P<0.0001, Figure 3 a-f).

**The Prognostic Value of the activation of CD8+ T cells and tumor burden in NSCLC patients receiving immunotherapy**

This study developed a practical approach to estimate tumor burden using all measurable tumor lesions on a pretreatment imaging scan. Higher tumor burden was associated with more Ki-67+/CD8+T-cells before treatment. This correlation could also be detected after treatment and became stronger, as shown
in Figure 4, indicating that the pre-existing CD8+ T-cell response related to tumor burden was enhanced by anti-PD-1 treatment. The maximum fold change of the Ki-67 expression rate of PD-1+/CD8+ T-cells in the response group was higher than that in the non-response group (P<0.0001) (Figure 5a). The progression-free survival of patients with higher Ki-67 expression on PD-1+CD8+ T-cells was statistically significant (mPFS 8.6 vs. 10.8 months; log-rank test P-value=0.001). The cut-off value of the Ki-67 expression rate obtained by X-tile software was 4.5, as shown in Figure 5b. The overall survival of patients with higher Ki-67 expression on PD-1+CD8+ T-cells was not significantly longer (mOS 17.4 vs. 17.5 months; log-rank test P-value=0.297) with the same cut-off value of Ki-67, as shown in Figure 5c.

Patients with a longer PFS usually had a lower tumor burden. The ORR comparison of Ki-67 to tumor burden ratio (Figure 6 a) indicated that the ratio of Tex cell (PD-1+/CD8+ T-cells) increase to tumor burden might be related to the clinical results. Even after treatment, the number of Ki-67+/PD-1+/CD8+ T-cells may be related to the clinical outcome. The higher the ratio of Ki-67+PD-1+CD8+T-cells to tumor burden, the better the clinical outcome (Figure 6 b). After the 1st treatment course, Ki-67 and a tumor burden ratio greater than 0.6 was associated with the ORR and the improvement of PFS, as shown in Figure 6a-b (mPFS 8.4 vs. 10.9 months; log rank test P-value<0.0001, the cut-off value of Ki-67 to tumor burden was 0.6 by X-tile analysis). And a ratio greater than 0.6 was associated with the improvement of OS, as shown in Figure 6 c (mOS 15.3 vs. 18.8 months; log rank test P-value<0.0001).

Discussion

Anti-PD-1 therapy has improved survival in subgroups of patients with NSCLC, but no biomarker is currently available for predicting treatment benefit. Biomarkers including the anatomic location of metastases, PD-L1 expression, TMB, circulating tumor DNA (ctDNA) (Khagi et al. 2017), and PD-L1 expression on circulating tumor cells (CTC) (Yue et al. 2018) may have a role to play in predicting the efficacy of immunotherapy. In the present study we investigated the predictive value of peripheral blood T cell invigoration in NSCLC patients receiving anti-PD-1 treatment. The Ki-67 expression to tumor burden ratio greater than 0.6 at the 1st course of anti-PD-1 immunotherapy was associated with an improved survival.

Ki-67 is an RNA transcription factor and can be detected at all stages of the cell cycle. It is strongly expressed in proliferative cancer cells, and a positive expression in tumor cells indicates a poor prognosis(Tarighati et al. 2023). Ki-67 has been used as a marker of cell proliferation and T cell regeneration in mouse models with immune checkpoint blockade(Blackburn et al. 2009) and in patients receiving anti-CTLA-4 therapy combined with radiotherapy(Twyman-Saint Victor et al. 2015). In preclinical models the tumor burden is a key element of T-cell failure and regeneration after anti-PD-1 immunotherapy. In these models blocking the PD-1 pathway can partially activate Tex cells(Blackburn et al. 2009; Twyman-Saint Victor et al. 2015), and produce a positive clinical response in human cancers(Topalian et al. 2015). Patients with a high tumor burden have a relatively poor response to anti-PD-1 therapy, which may be caused by the lack of activated CD8+ T-cells induced by PD-1 inhibitors. Studies have shown that the first-week proliferation of PD-1+/CD8+ T-cells (Ki-67 D7/D0) in peripheral
CD8+ T-cells responding to anti-PD-1 therapy share some common characteristics with effector cells induced by a live attenuated virus, such as low expression of Bcl-2, CCR7, and CD45RA. Like the infiltrating CD8+ T-cells in tumors, the proliferating CD8+ T-cells in peripheral blood after anti-PD-1 treatment had higher co-expression of PD-1 and CTLA-4, which indicated that the regenerated CD8+ T-cells had reduced activation by co-expression of inhibitory receptors(Rizvi et al. 2015). These activated CD8+ T-cells, which become dysfunctional or exhausted, often fail to eradicate tumors. Compared with effector T-cells and memory CD8+ T-cells, the Tex cells are also actively inhibited by inhibitory receptors, including PD-1, their effector function is weaker, and their differentiation pattern has changed(Pauken and Wherry 2015). The circulating Tex cells express multiple inhibitory receptors, including PD-1, and blocking PD-1 in vivo can improve the activity of these cells(Blackburn et al. 2009). CD8+ T-cells can respond to various human cancers, especially those with a high mutation burden(Topalian et al. 2016). Various studies have suggested that PD-L1 expression on tumor cells or tumor infiltrating hematopoietic cells is related to the clinical response to PD-1 immunotherapy. Pre-existing tumor infiltrating T-cells may be a positive prognostic indicator for various anti-tumor therapies. In addition, the expression of PD-L1 in tumors has been related to the T cell response in some cases(Herbst et al. 2014; Tumeh et al. 2014).

Compared with healthy individuals, Ki-67 expression was higher in the CD8+ T-cells of tumor patients before treatment, mainly in PD-1+/CD8+ T-cell subsets, suggesting a pre-existing immune response. Clinical failure in many patients is not solely due to an inability to induce an immune response, but rather results from an imbalance between T-cell activation and tumor burden. The extent of the activation of circulating Tex cells and the pretreatment tumor burden correlate with the clinical response(Barber et al. 2006). A higher tumor burden was associated with more Ki-67+/CD8+ T-cells after treatment. This correlation can also be detected before treatment but became stronger after treatment. This suggests that the pre-existing CD8+ T-cell response is increased by anti-PD-1 treatment. Moreover, the maximum fold change of Ki-67 expression of PD-1+/CD8+ T-cells in the response group was higher than in the control group. Whether there is only one single peak of immune recovery caused by PD-1 blockade in most patients may need further follow-up observation. We found that the reaction of CD8+ T-cells in peripheral blood was not persistent and was only detected at one or two time points. These findings may indicate that PD-1+/CD8+ T-cells activated by the inhibition signal block would proliferate, which could be detected in the peripheral blood circulation within a few weeks after treatment. T-cells would then migrate to the tumor or inflammatory sites and mediate the level of cytokines participating in immune regulation.

To accurately track biological indicators for predicting the efficacy of anti-PD-1 immunotherapy, we evaluated the cell subsets co-expressing PD-1 and other inhibitory receptors. Combined with the
screening of immune parameters, we found that the main responsive Ki-67+/CD8+ T-cell population was CD45RAlo/CD27hi, with high expression of CTLA-4, PD-1, and 2B4 and low expression of NKG2-D. This constitutes part of the phenotype of exhausted T cells in the mouse model that was previously reported, and may indicate that anti-PD-1 immunotherapy can affect the regeneration of these cells (Paley et al. 2012; Wherry et al. 2003).

It is known that PD-1 is expressed by Tex cells, effector cells, effector memory, and central memory CD8+ T-cells (Bengsch et al. 2010; Khan et al. 2019). In this study, compared with the above T cell subsets, the expression of Ki-67 and PD-1+/CD8+ T-cells was higher in Tex cells. Patients with a longer PFS or OS usually have a lower tumor burden. Hierarchical cluster analysis of relevant circulating T-cell subpopulations calibrated to the pretreatment disease burden revealed that the ratio of Tex cell regeneration to tumor burden might be related to the clinical outcome.

Previous studies have shown that immature T cells and memory T cells play a key role in tumour pathogenesis and have an impact on prognosis (Crespo et al. 2018). Similar results were found in NSCLCs, a higher CD4+ naive/memory T-cell ratio associated with longer progression-free survival. The CD45RA+ immature T cells and CD45RO+ memory T cells can also be used to predict PFS and OS in advanced pancreatic cancer patients undergoing chemotherapy (Hang et al. 2019). Immature T cells expressing CD45RA are usually functionally inactive. In response to stimulation, these cells may produce high levels of chemokines, such as CXCL8, which mediates the migration of neutrophils to the tumor and promotes tumor growth (Crespo et al. 2018). Memory T cells have a CD45RO+ phenotype (Farber et al. 2014), and secrete IFN-γ, CCL4, XCL1, and other cytokines to kill tumor cells directly or indirectly (Brewitz et al. 2017).

The present study has multiple limitations. First, the absence of a longer duration of follow-up to assess ongoing clinical responses remains the major limitation. This may have resulted in bias, but this is due to the high mortality from advanced cancers. Second, a limitation of this study is that detailed subgroup analyses of PFS and OS were limited by sample size. Third, cancer types and small sample studies may affect the results.

To summarize, our study identified the peripheral blood biomarkers of NSCLC patients related to the immune efficacy of anti-PD-1. Moreover, we identified that two immune related indicators were consistent with the predictive efficacy of blood-based tumor mutational burden (bTMB) in an anti-PD-1 immune response. This research adds to the understanding of the effect of immunotherapy on peripheral blood immune status of tumor patients. We can further analyze the effect of activation related genes on T cell activation. Thus, our research is of prognostic and therapeutic significance to guide immunotherapy for treating cancer.

**Abbreviations**

**NSCLC** non-small cell lung cancer  
**ICIs** Immune checkpoint inhibitors
PFS  progression-free survival  PD-1  programmed cell death 1
OS  overall survival

Declarations

Acknowledgements
Not applicable.

Authors’ contributions
LS, HW and GZW contributed to study conceptualization, data analysis, interpretation, manuscript preparation; QC and CMS contributed to designing the manuscript; HW, ZCP contributed to drafting the manuscript experimental conduct, methods, initial data analysis; HW, ZCP and LZ contributed to manuscript writing, reviewing and editing. All authors critically reviewed the manuscript. All authors read and approved the final manuscript.

Funding
This work was supported by Fujian provincial health technology project (Grant No. 2019-1-28) and Bethune. Cancer Clinical Research Project (Grant No. BCF-XD-ZL-20220118-035).

Availability of data and materials
Not applicable.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

References


37. https://doi.org/10.1038/ni.1679


**Figures**

**Figure 1**

X-tile analyses of PFS performed using patient data to determine the optimal cut-off value for ratio of Ki-67+PD-1+CD8+T-cells to tumor burden. In the left panels, the X-axis represents all potential cut-off values from low to high (left to right) that define a low subset, whereas the Y-axis represents the cut-off values from high to low (top to bottom) that define a high subset. Red coloration of a cut-off value indicates an inverse correlation with time to recurrence, and green coloration represents direct associations. The optimal cut-off values highlighted by the black circles in the left panels are shown in the histograms of the entire cohort (middle panels). Kaplan-Meier plots are displayed in the right panels, where blue represents the low subgroup and gray represents the high subgroup. The optimal cut-off value for ratio of Ki-67+PD-1+CD8+T-cells to tumor burden is 0.6 for PFS.
Expression of Ki-67 of human PD-1+/CD8+ T-cells and PD-1-/CD8+ T-cells was detected by flow cytometry. PBMCs were probed for surface expression of human CD8, CD45RA, and PD-1. Cells were also probed to measure intracellular expression of Ki-67. Gating strategy (a) and frequencies of T-cell phenotypes (PD-1+/Ki-67+ and PD-1-/Ki-67+) (b) are described. Data are presented as mean±standard error of the mean (SEM). (****P<0.0001).
Figure 3

CD8 T-cells responding to anti-PD-1 therapy display an exhausted phenotype. (a-f) Expression of the indicated markers (CTLA-4, 2B4, PD-1, CD45RA, CD27) in Ki-67+/CD8+ T-cells and Ki-67-/CD8+ T-cells at the 1st course of treatment. (**P<0.01, ****P<0.0001).
Figure 4

Pearson correlation analysis of tumor burden to Ki-67 expression in indicated cells during treatment.
Figure 5

(a) Maximum fold change in Ki-67+/PD-1+/CD8+ T-cell during the treatment cycle in responding and non-responding patients (****P<0.0001). (b-c) Kaplan-Meier estimates of progression-free survival and overall survival analysis according to Ki-67 expression in PD-1+/CD8+ T-cells.
Figure 6

(a) Objective response rate for high and low Ki-67 to tumor burden ratio. (b-c) Kaplan-Meier progress free survival and overall survival analysis for high versus low post-treatment Ki-67 expression to tumor burden ratio.