ORIGINAL RESEARCH

T Cell Invigoration is Associated with the Clinical Response to Anti-PD-1-Based Immunotherapy in Non-Small Cell Lung Cancer

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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 prognoses were followed-up. Indicators such as cell subpopulations, T cell invigoration were detected by clinical laboratory testing. Survival curves were estimated by the Kaplan-Meier method, Cox regression analysis was used to identify factors associated with prognoses 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 may be associated with survival. Patients with higher Ki-67 expression on PD-1+CD8+ T-cells (pretreatment) had statistically significant increased progression-free survival (PFS). 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 overall survival ($P < 0.05$).

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

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

Introduction

Lung cancer is a global health problem, among which NSCLC accounts for 80%–85%.[1] With increasing awareness of immune escape mechanisms, immunotherapy has become an effective treatment method for NSCLC patients. Immunotherapy has produced clinical reactions in patients with NSCLC, advanced melanoma, and other tumor types, achieving long-term disease control and few adverse events.[2] Specifically, monoclonal antibodies, such as PD-1/PD-L1 inhibitors, have exhibited remarkable success in treating solid tumors, with a low incidence of severe adverse reactions, thereby establishing anti-PD-1 treatment as a highly promising immunotherapy option.[3] 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. Therefore, predictive biomarkers that identify which patients derive benefit and toxicity from anti-PD-1 therapy are needed.[4]

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The presence of tumor infiltrating T cells can be used as a positive prognostic indicator for various cancers.[5] In some cases, the expression of PD-L1 in tumors is also related to the T cell response;[6,7] 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.[8] 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),[9] TMB detection on circulating tumor cell (CTC), and expression of PD-L1 on CTC,[10] are also being actively explored. Though such markers appear to be helpful in predicting response in specific tumor types, none of them have been evaluated prospectively as a pan-tumor biomarker. Patients whose mismatch repair capacity was deficiency or with microsatellite instability (MSI), however, may not respond to immunotherapy with anti-PD-1 monoclonal antibodies.[11] This study aimed to assess the immune status of patients undergoing immunotherapy by detecting and recording serum indexes, and subsequently investigate the potential of these immune indexes to predict the immune response to anti-PD-1 therapy in patients with NSCLC.

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 was 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 monthly. 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 an intravenous dose 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 obtained informed consent using a Cancer Institute Institutional Review Board approved protocol. Patients receiving immunotherapy before enrollment,or patients with autoimmune diseases, unclear pathological diagnosis, active brain metastasis,no measurable target lesions were excluded. All the patients provided written informed consent before enrollment. 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:2023KY096). We confirm that our study complies with the Declaration of Helsinki.

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.[12]

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). 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.

Table 1 Relationship Between T-Cell Invigoration to Tumour Burden Ratio and Clinicopathological Features in 145 Cases of NSCLC

| Clinicopatholo-gical features	N	T-Cell Invigoration to Tumour Burden Ratio		χ^2	P
		Low	High		
Gender				0.444	0.505
Male	103	24	79		
Female	42	12	30		
Age (years)				0.604	0.437
≧60	41	12	29		
<60	104	24	80		
Smoking history				2.228	0.135
Never	65	20	45		
Ever	80	16	64		
Histologic subtype				8.391	0.078
ADC	75	15	60		
SqCC	34	13	21		
ASC	12	1	11		
PC	4	0	4		
NSCLC PD	20	7	13		
Metastasis				3.574	0.059
Yes	84	16	68		
No	61	20	41		
ECOG				12.126	<0.001*
>1	88	13	75		
≤1	57	23	34		
Response to PD-1 blockade				9.666	0.002*
Clinical benefit	115	22	93		
Non-responder	30	14	16		

Note: *Significance with P<0.05.
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.

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 value 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 χ^2 log-rank value of 8.83 (P < 0.05)[9] (Figure 1a–c). 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 of serum indicators and clinicopathologic factors between groups of patients. Correlation analyses were done with Pearson or Spearman tests, as indicated. 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.
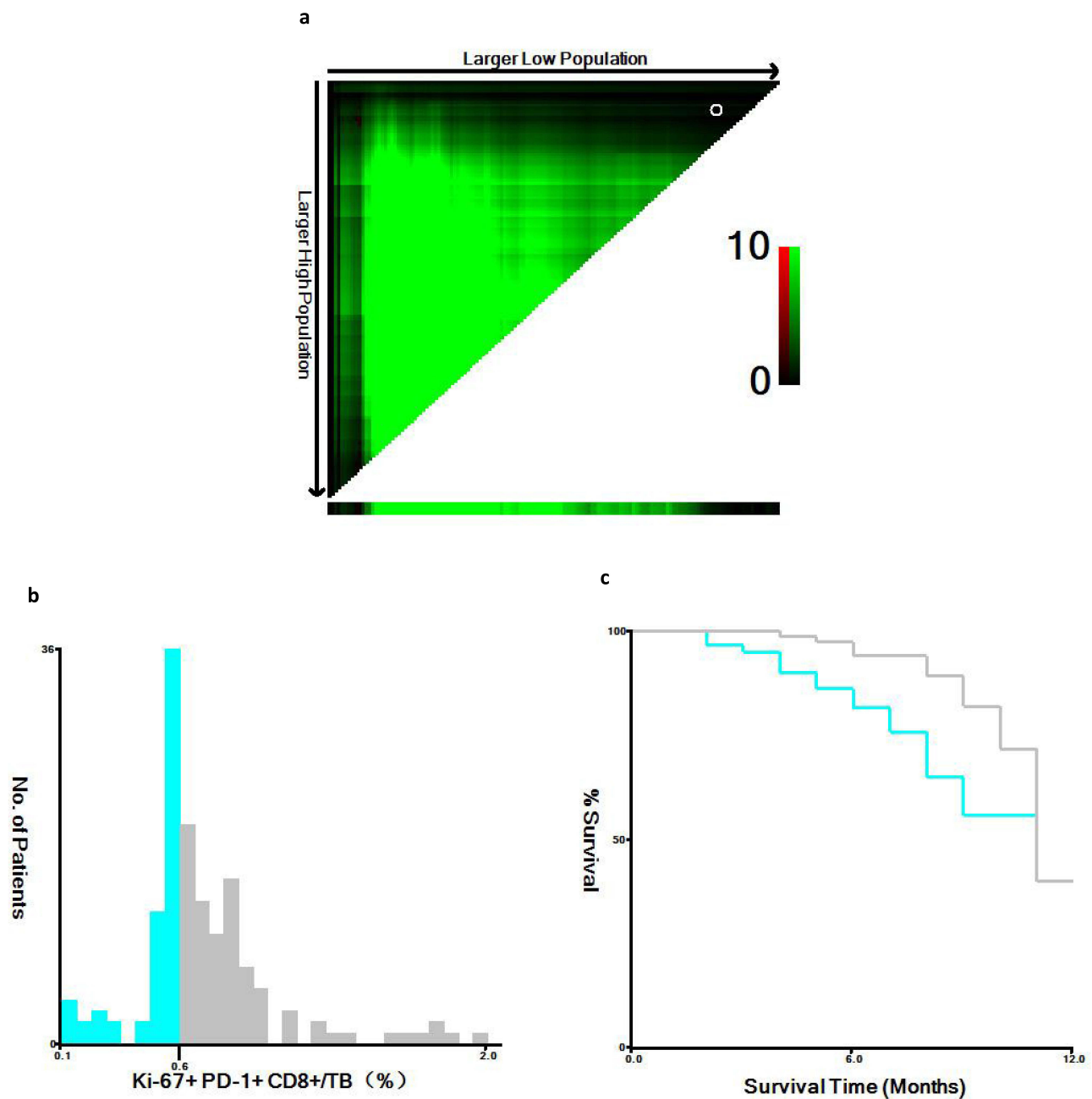
Figure 1 X-tile analysis of PFS performed using patient data to determine the optimal cut-off value for ratio of Ki-67+PD-1+CD8+T-cells to tumor burden.
Notes: (**a**) The X-axis represents all potential cut-off values from low to high (left to right) that define a low subset, whereas the Y-axis represents 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 tumor progression, and green coloration represents direct associations. The optimal cut-off values highlighted by the black circles (**a**) are shown in the histograms of the entire cohort (**b**). Kaplan–Meier plot is displayed (**c**), 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.

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 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 2a and 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 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 3a–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 tumor patients with higher Ki-67 expression on PD-1+CD8+ T-cells (pretreatment) showed a statistically significant improvement in progression-free survival (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
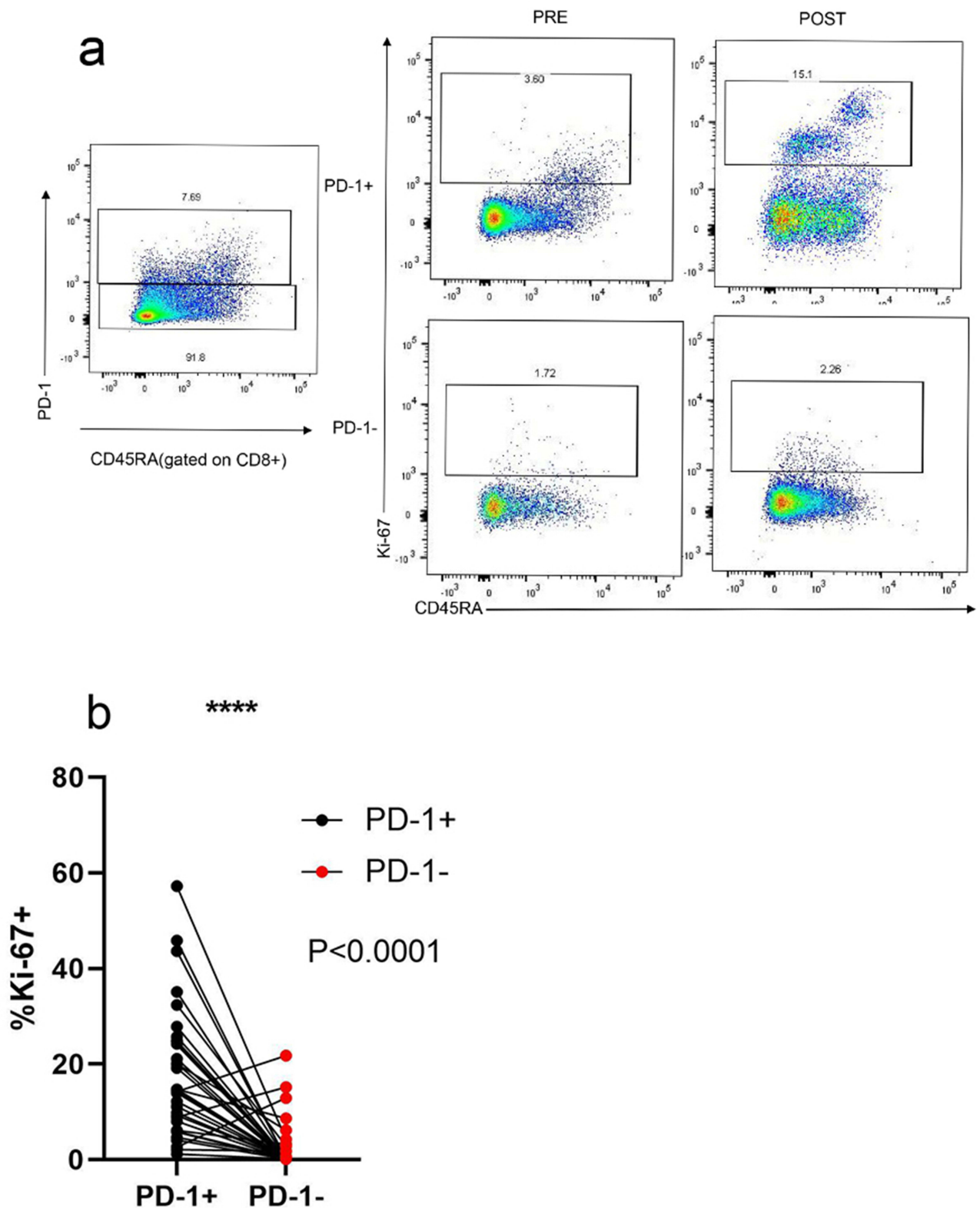
Figure 2 Expression of Ki-67 of human PD-1+/CD8+ T-cells and PD-1-/CD8+ T-cells was detected by flow cytometry.
Notes: PBMCs were probed for surface expression of human CD8, CD45RA, and PD-1. Cells were also probed to measure intracellular expression of Ki-67. Gating strategy (**a**) and frequencies of T-cell phenotypes (PD-1+/Ki-67+ and PD-1-/Ki-67+) (**b**) are described. (****P < 0.0001).

Table 2 Cox Regression Analysis of Univariate Analysis and Multivariate Analysis for Overall Survival in NSCLC Patients

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

Note: *Significance with P<0.05.

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 6a) indicated that the ratio of Tex cell (PD-1+/CD8+ T-cells) invigoration to tumor burden might be related to the clinical results. Before or during the 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 6b). After the 1st treatment course, the ratio of Tex cell invigoration to tumor burden greater than 0.6 was associated with the ORR and the improvement of PFS, as shown in Figure 6a and 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 ratio 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 6c (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. Despite the broad approavals in both solid and liquid tumors in neoadjuvant therapy, adjuvant therapy, or metastatic tumor therapy, indication Checkpoint Inhibitor (CPI), akin to cytotoxic and targeted therapies often benefit less than half of patients exposed. Biomarkers including the anatomic location of metastases, PD-L1 expression, TMB, circulating tumor DNA (ctDNA),[13] and PD-L1 expression on circulating tumor cells (CTC)[14] 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.[15] Ki-67 has been used as a marker of cell proliferation and T cell regeneration in mouse models with immune checkpoint blockade[16] and in patients receiving anti-CTLA-4 therapy combined with radiotherapy.[17] 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,[16,17] and produce a positive clinical response in human cancers.[18] 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 blood can predict the response of solid tumor to anti-PD-1 therapy.[19] Early clinical intervention, such as immunotherapy or targeted therapy, can release T-cells to target tumor cells and limit tumor growth. Different from other cancer therapies, these T-cells present more favorable characteristics, including specificity, adaptability, and memory.[20,21]
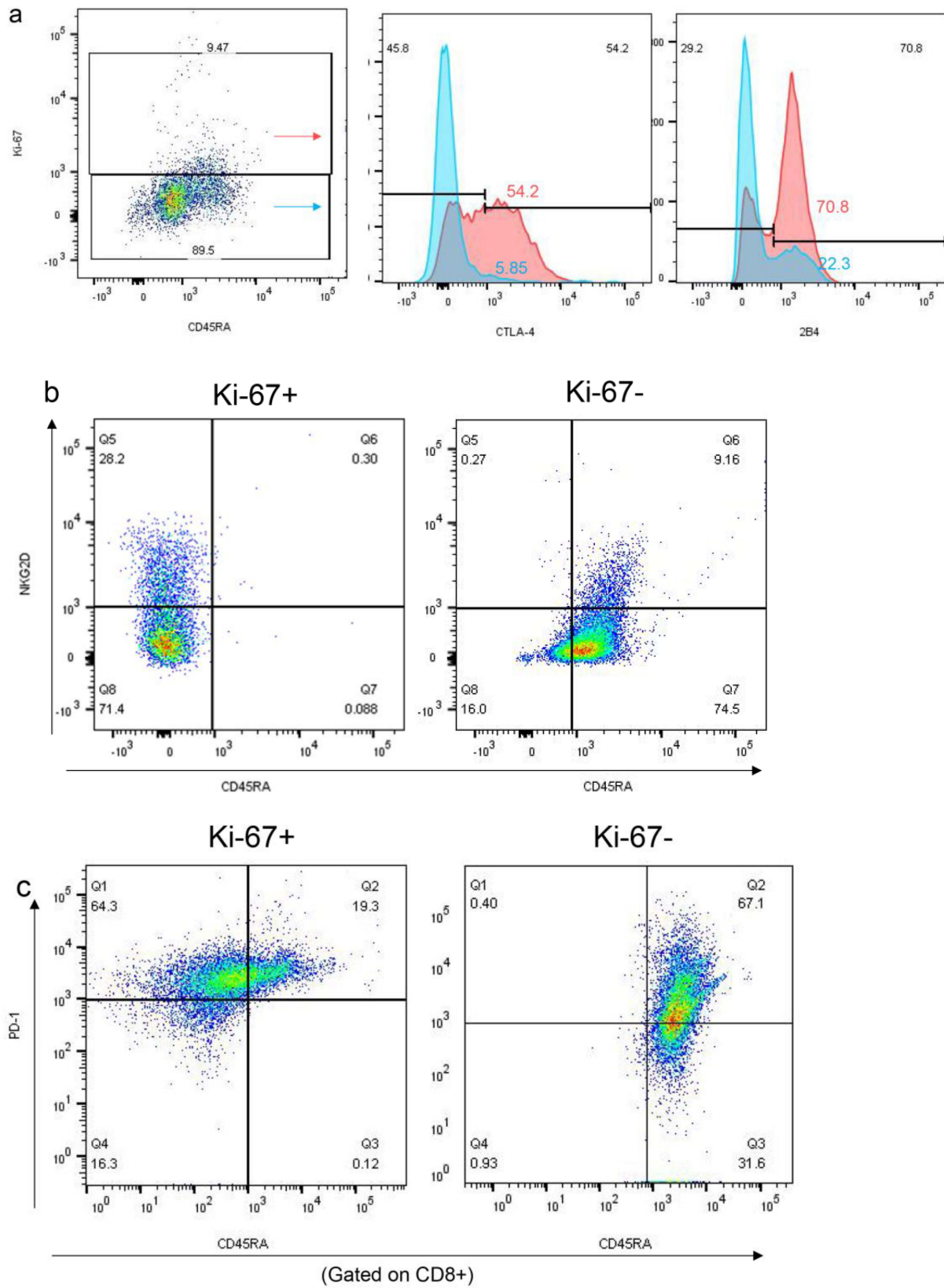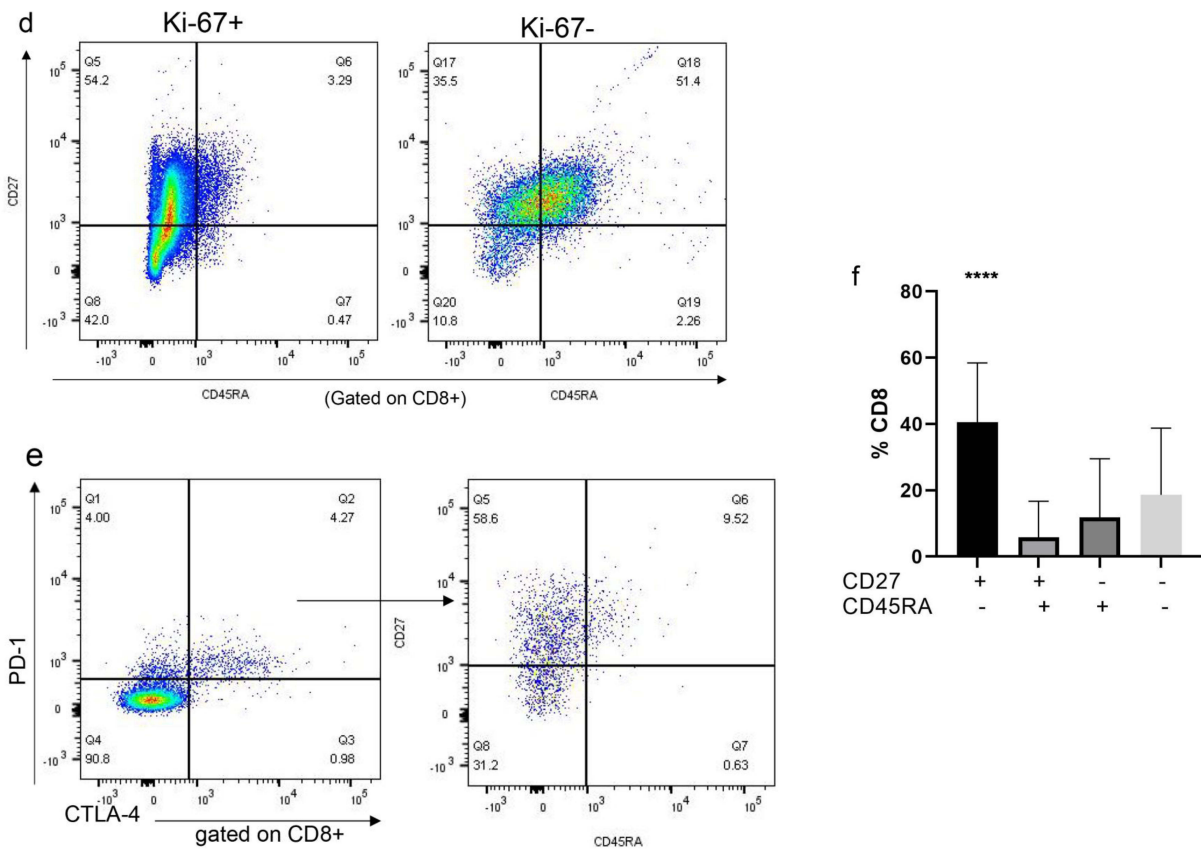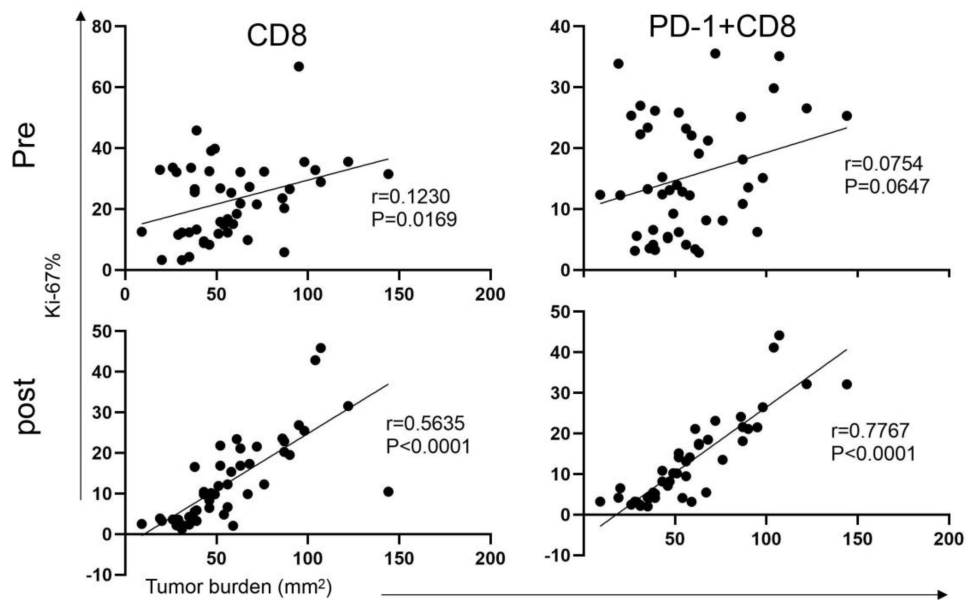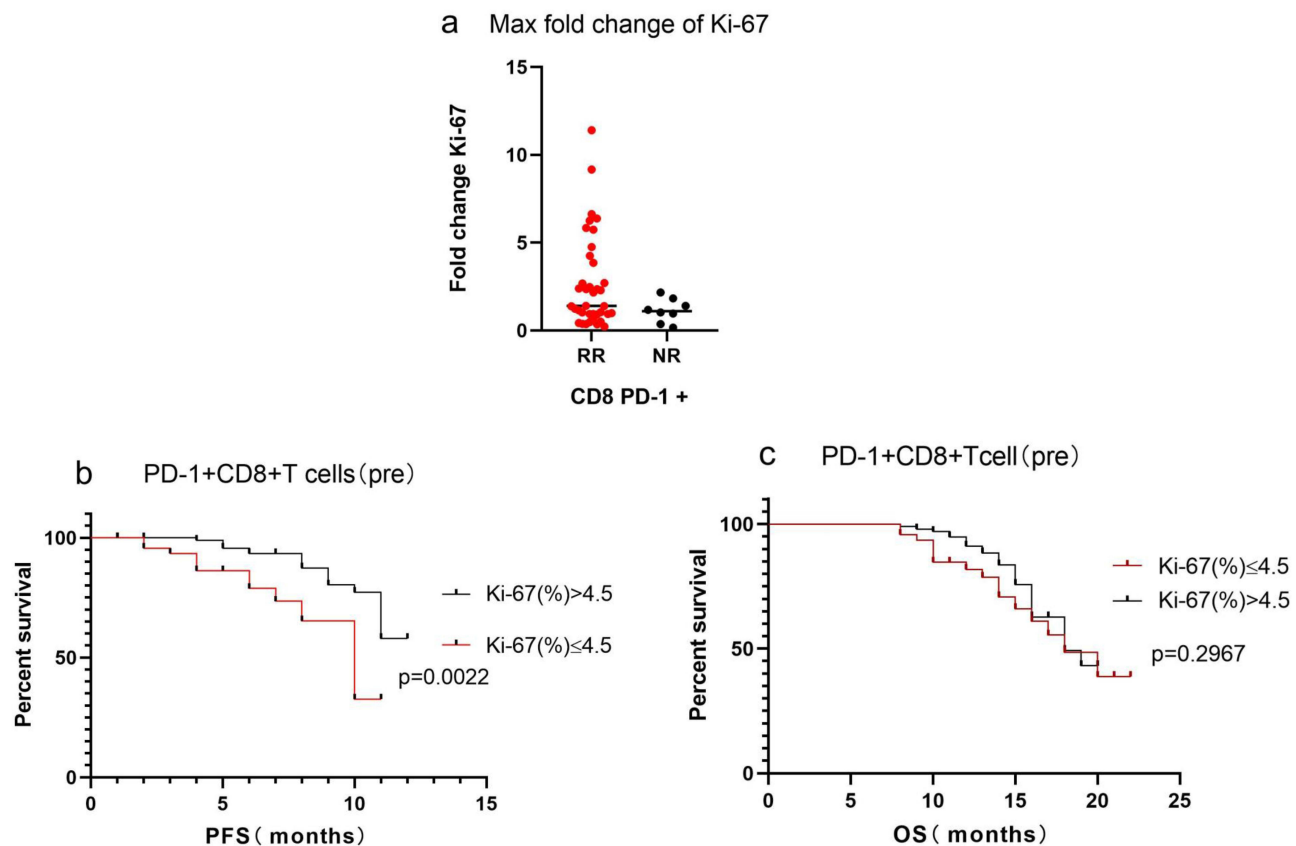
Figure 3 Continued.

Figure 3 CD8 T-cells responding to anti-PD-1 therapy display an exhausted phenotype.
Notes: (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 < 0.0001).


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. (**b** and **c**) Kaplan-Meier estimates of progression-free survival and overall survival analysis according to Ki-67 expression in PD-1+/CD8+ T-cells.

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.[20] These activated CD8+ T-cells, which become dysfunctional or exhausted, often fail to eradicate tumors. Tex cells exhibit weaker effector function and altered differentiation patterns, as they are actively suppressed by inhibitory receptors such as PD-1, in contrast to effector T-cells and memory CD8+ T-cells.[22] The circulating Tex cells express multiple inhibitory receptors, including PD-1, and blocking PD-1 in vivo can improve the activity of these cells.[16]

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.[23] A higher tumor burden was associated with more Ki-67+/CD8+T cells after treatment, and this correlation ran through the treatment process. This indicated that the pre existing CD8+T-cell response increased through anti PD-1 therapy. In addition, the maximum fold change of Ki-67 expression on PD-1+/CD8+T cells may be related to recent efficacy. 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' proliferation, which can be detected in peripheral blood circulation within a few weeks after treatment, was initiated by these inhibitory signals blocking the T-cells. T-cells would then migrate to the tumor or inflammatory sites and mediate the level of cytokines participating in immune regulation.
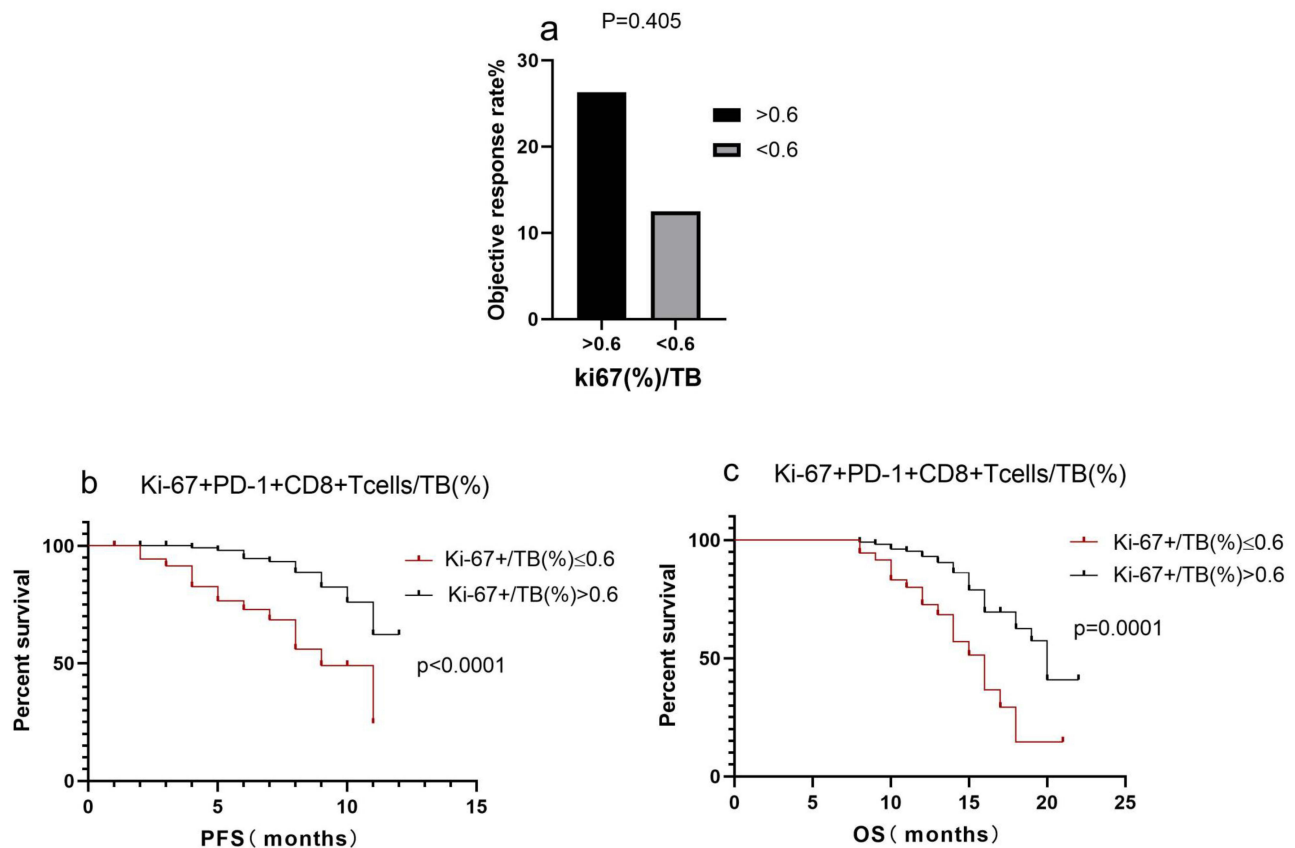
Figure 6 (**a**) Objective response rate for high and low Ki-67 to tumor burden ratio. (**b** and **c**) Kaplan-Meier progress free survival and overall survival analysis for high versus low post-treatment Ki-67 expression to tumor burden ratio.

In order to identify biological indicators that predict the efficacy of anti PD-1 immunotherapy, we investigated cell subpopulations co-expressing PD-1 and other inhibitory receptors. Through screening of immune parameters, we found that the main responsive Ki-67+/CD8+T cell population. 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.[24,25]

It is known that PD-1 is expressed by Tex cells, effector cells, effector memory CD8+ T-cells and central memory CD8+ T-cells.[26,27] In this study, compared with the above T cell subsets, the expression of Ki-67 and PD-1+ 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.[28] 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.[29] 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.[28] Memory T cells have a CD45RO+ phenotype,[30] and secrete IFN-γ, CCL4, XCL1, and other cytokines to kill tumor cells directly or indirectly.[31] In this study, we also found similar correlations that require further confirmation. We tested some cytokines in the patients' peripheral blood and found that patients receiving anti PD-1 treatment had systemic inflammation before treatment. After one cycle of anti PD-1 treatment, the cytokine IFNγ in the plasma raised and there was a certain consistency with the increase of PD-1+CTLA-4+CD8+T cells and the Invigoration of Tex cell.

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. We controlled for this bias by extending the observation time and follow-up frequency, and if necessary, comparing the survival distribution between the exposed and non-exposed groups, using the AFT model.[32] Second, a limitation of this study is that detailed subgroup analyses of PFS and OS were limited by sample size.

To summarize, our study identified the peripheral blood biomarkers of NSCLC patients related to the immune efficacy of anti-PD-1 therapy. 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.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no competing interests.

References

1. Siegel RL, Miller KD, Fuchs HE, Jemal A. Cancer statistics, 2022. *CA Cancer J Clin*. 2022;72(1):7–33. doi:10.3322/caac.21708
2. Chow A, Perica K, Klebanoff CA, Wolchok JD. Clinical implications of T cell exhaustion for cancer immunotherapy. *Nat Rev Clin Oncol*. 2022;19 (12):775–790. doi:10.1038/s41571-022-00689-z
3. Nielsen DL, Juhl CB, Chen IM, Kellermann L, Nielsen OH. Immune checkpoint inhibitor-induced diarrhea and colitis: incidence and management. A systematic review and meta-analysis. *Cancer Treat Rev*. 2022;109:102440. doi:10.1016/j.ctrv.2022.102440
4. Ramos-Casals M, Brahmer JR, Callahan MK, et al. Immune-related adverse events of checkpoint inhibitors. *Nat Rev Dis Primers*. 2020;6(1):38. doi:10.1038/s41572-020-0160-6
5. Paijens ST, Vledder A, de Bruyn M, Nijman HW. Tumor-infiltrating lymphocytes in the immunotherapy era. *Cell Mol Immunol*. 2021;18(4):842–859. doi:10.1038/s41423-020-00565-9
6. Freeman GJ, Long AJ, Iwai Y, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med*. 2000;192(7):1027–1034. doi:10.1084/jem.192.7.1027
7. Li W, Wu F, Zhao S, Shi P, Wang S, Cui D. Correlation between PD-1/PD-L1 expression and polarization in tumor-associated macrophages: a key player in tumor immunotherapy. *Cytokine Growth Factor Rev*. 2022;67:49–57. doi:10.1016/j.cytogfr.2022.07.004
8. Latchman Y, Wood CR, Chernova T, et al. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat Immunol*. 2001;2(3):261–268. doi:10.1038/85330
9. Abbosh C, Frankell AM, Harrison T, et al. Tracking early lung cancer metastatic dissemination in TRACERx using ctDNA. *Nature*. 2023;616 (7957):553–562. doi:10.1038/s41586-023-05776-4
10. De Marchi P, Leal LF, Duval da Silva V, da Silva E, Cordeiro de lima VC, Reis RM. PD-L1 expression by Tumor Proportion Score (TPS) and Combined Positive Score (CPS) are similar in non-small cell lung cancer (NSCLC). *J Clin Pathol*. 2021;74(11):735–740. doi:10.1136/jclinpath-2020-206832
11. Le DT, Durham JN, Smith KN, et al. Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade. *Science*. 2017;357 (6349):409–413. doi:10.1126/science.aan6733
12. Nishino M, Giobbie-Hurder A, Gargano M, Suda M, Ramaiya NH, Hodi FS. Developing a common language for tumor response to immunotherapy: immune-related response criteria using unidimensional measurements. *Clin Cancer Res*. 2013;19(14):3936–3943. doi:10.1158/1078-0432. CCR-13-0895
13. Khagi Y, Goodman AM, Daniels GA, et al. Hypermutated circulating tumor DNA: correlation with response to checkpoint inhibitor-based immunotherapy. *Clin Cancer Res*. 2017;23(19):5729–5736. doi:10.1158/1078-0432.CCR-17-1439

14. Yue C, Jiang Y, Li P, et al. Dynamic change of PD-L1 expression on circulating tumor cells in advanced solid tumor patients undergoing PD-1 blockade therapy. *Oncoimmunology.* 2018;7(7):e1438111. doi:10.1080/2162402X.2018.1438111

15. Tarighati E, Keivan H, Mahani H. A review of prognostic and predictive biomarkers in breast cancer. *Clin Exp Med.* 2023;23(1):1–16. doi:10.1007/s10238-021-00781-1

16. Blackburn SD, Shin H, Haining WN, et al. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nat Immunol.* 2009;10(1):29–37. doi:10.1038/ni.1679

17. Twyman-Saint Victor C, Rech AJ, Maity A, et al. Radiation and dual checkpoint blockade activate non-redundant immune mechanisms in cancer. *Nature.* 2015;520(7547):373–377. doi:10.1038/nature14292

18. Topalian SL, Drake CG, Pardoll DM. Immune checkpoint blockade: a common denominator approach to cancer therapy. *Cancer Cell.* 2015;27 (4):450–461. doi:10.1016/j.ccell.2015.03.001

19. Kim KH, Cho J, Ku BM, et al. The first-week proliferative response of peripheral blood PD-1+CD8+ T cells predicts the response to anti-PD-1 therapy in solid tumors. *Clin Cancer Res.* 2019;25(7):2144–2154. doi:10.1158/1078-0432.CCR-18-1449

20. Rizvi NA, Hellmann MD, Snyder A, et al. Cancer immunology. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. *Science.* 2015;348(6230):124–128. doi:10.1126/science.aaa1348

21. Sharma P, Allison JP. Immune checkpoint targeting in cancer therapy: toward combination strategies with curative potential. *Cell.* 2015;161 (2):205–214. doi:10.1016/j.cell.2015.03.030

22. Pauken KE, Wherry EJ. Overcoming T cell exhaustion in infection and cancer. *Trends Immunol.* 2015;36(4):265–276. doi:10.1016/j.it.2015.02.008

23. Barber DL, Wherry EJ, Masopust D, et al. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature.* 2006;439 (7077):682–687. doi:10.1038/nature04444

24. Paley MA, Kroy DC, Odorizzi PM, et al. Progenitor and terminal subsets of CD8+ T cells cooperate to contain chronic viral infection. *Science.* 2012;338(6111):1220–1225. doi:10.1126/science.1229620

25. Wherry EJ, Blattman JN, Murali-Krishna K, van der Most R, Ahmed R. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *J Virol.* 2003;77(8):4911–4927. doi:10.1128/JVI.77.8.4911-4927.2003

26. Bengsch B, Seigel B, Ruhl M, et al. Coexpression of PD-1, 2B4, CD160 and KLRG1 on exhausted HCV-specific CD8+ T cells is linked to antigen recognition and T cell differentiation. *PLoS Pathog.* 2010;6(6):e1000947. doi:10.1371/journal.ppat.1000947

27. Khan O, Giles JR, McDonald S, et al. TOX transcriptionally and epigenetically programs CD8(+) T cell exhaustion. *Nature.* 2019;571(7764):211–218. doi:10.1038/s41586-019-1325-x

28. Crespo J, Wu K, Li W, et al. Human naive T cells express functional CXCL8 and promote tumorigenesis. *J Immunol.* 2018;201(2):814–820. doi:10.4049/jimmunol.1700755

29. Hang J, Huang J, Zhou S, et al. The clinical implication of CD45RA+ naïve T cells and CD45RO+ memory T cells in advanced pancreatic cancer: a proxy for tumor biology and outcome prediction. *Cancer Med.* 2019;8(3):1326–1335. doi:10.1002/cam4.1988

30. Farber DL, Yudanin NA, Restifo NP. Human memory T cells: generation, compartmentalization and homeostasis. *Nat Rev Immunol.* 2014;14 (1):24–35. doi:10.1038/nri3567

31. Brewitz A, Eickhoff S, Dähling S, et al. CD8(+) T cells orchestrate pDC-XCR1(+) dendritic cell spatial and functional cooperativity to optimize priming. *Immunity.* 2017;46(2):205–219. doi:10.1016/j.immuni.2017.01.003

32. Hernán MA, Cole SR, Margolick J, Cohen M, Robins JM. Structural accelerated failure time models for survival analysis in studies with time-varying treatments. *Pharmacoepidemiol Drug Saf.* 2005;14(7):477–491. doi:10.1002/pds.1064