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Research Article

Keywords:

Posted Date: October 8th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-11025245/v1>

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Additional Declarations: No competing interests reported.

Spatial Tumor Engagement and Functionality of Memory CD8+ T Cells Predicts Progression-Free and Overall Survival in Advanced Non-Small Cell Lung Cancer

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ABSTRACT

Introduction: Spatial organization and functionality of tumor-infiltrating lymphocytes may provide prognostic information beyond immune-cell abundance alone in advanced non-small cell lung cancer (NSCLC). We investigated whether multiplex immunophenotyping of tumor-proximal CD8+ and CD4+ immune engagement, according to naive (CD45RA+) or memory-like (CD45RO+) cell states, identify clinically relevant immune architectures in patients treated with first-line immune checkpoint inhibitor (ICI)-based therapy.

Methods: This retrospective cohort included 39 patients with stage IIIB–IV NSCLC treated between 2019 and 2022 with first-line pembrolizumab monotherapy or chemo-immunotherapy. Formalin-fixed paraffin-embedded tumor samples were analyzed by multiplex immunophenotyping (CellScape™) for CD4, CD8, CD45RO, CD45RA, granzyme B and PanCK-based tumor compartments. Patient-level immune-cell densities and nearest PanCK-distance metrics were quantified after core aggregation. A tumor engagement index (TEI) was defined as phenotype density divided by the upper-quartile nearest PanCK distance, integrating immune abundance with tumor proximity. Patients were stratified by predefined progression-free survival (PFS; 6-month cutoff) and overall survival (OS; 12-month cutoff).

Results: CD8+CD45RO+ cells showed the strongest tumor-proximal immune phenotype and the most consistent association with favorable outcome. Higher final CD8+CD45RO+ TEI was associated with prolonged PFS and OS in Kaplan–Meier analyses (median PFS, 17 vs 6 months; median OS, 48.0 vs 15.4 months). Neither CD4+, nor CD45RA+ phenotypic dominance showed significant influence on clinical outcomes. In Cox models adjusted for

sex, treatment group, metastatic burden and PD-L1 status, spatial TEI remained independently associated with both PFS (HR 0.09, 95% CI 0.02–0.44, $p=0.003$) and OS (HR 0.10, 95% CI 0.02–0.42, $p=0.002$). Elastic-net Cox analysis supported CD8+CD45RO+ TEI as the dominant stable spatial feature, with high bootstrap selection frequency for both PFS and OS.

Conclusion: A tumor-proximal CD8+CD45RO+ immune architecture identifies a favorable spatial immune phenotype far exceeding the predictive power of mere CD8+ density and PD-L1 expression in advanced NSCLC treated with first-line immunotherapy-based regimens.

INTRODUCTION

Lung cancer remains the leading cause of cancer-related mortality worldwide, with non-small cell lung cancer (NSCLC) accounting for most cases [1]. In advanced NSCLC without actionable oncogenic drivers, first-line immune checkpoint inhibitor (ICI)-based therapy has become a central treatment strategy. Pembrolizumab monotherapy provides durable benefit in PD-L1-high tumors, while pembrolizumab plus platinum-based chemotherapy improves outcomes across nonsquamous and squamous metastatic NSCLC and across PD-L1 strata [2–4]. Atezolizumab has also demonstrated first-line activity in PD-L1-selected disease [5].

Despite these advances, patient-level benefit remains heterogeneous [6]. PD-L1 immunohistochemistry is the most widely implemented tissue biomarker for ICI selection, but its predictive performance is limited by biological heterogeneity, assay-related variability, dynamic regulation and incomplete negative predictive value [6, 7]. This has sustained interest in tissue biomarkers that also capture the organization of the tumor immune microenvironment (TME), apart from tumor-intrinsic features. In advanced NSCLC, this challenge is intensified by the frequent reliance on small biopsies or cytology-derived material, where histological diagnosis, molecular profiling and PD-L1 testing compete for limited tumor tissue [8, 9]. Methods that extract multidimensional biological information from scarce formalin-fixed paraffin-embedded FFPE samples therefore have particular translational value [8, 9]. The TME reflects the combined influence of immune-cell abundance, differentiation state, spatial localization and direct relationships with tumor-cell compartments, together [10]. The concept of immune contexture has established that immune-cell type, density, functional orientation and spatial distribution can shape prognosis and therapeutic efficacy across solid tumors [11, 12]. Spatial tissue profiling extends this principle by preserving the architecture of tumor-immune interactions, enabling distinction between immune-inflamed, -excluded or -cold states that may be obscured by bulk assays or dissociative single-cell methods [13].

CD8+ T cells represent a central effector compartment in antitumor immunity and have repeatedly been associated with favorable outcome in NSCLC, although density alone does not fully capture immune competence or treatment relevance [14–16]. Within T-cell phenotyping, CD45RA and CD45RO provide a pragmatic differentiation axis, broadly separating RA-enriched naive or terminally differentiated compartments from RO-enriched antigen-experienced memory compartments [17–19]. Upon encountering their cognate antigen, naive CD45RA+ T cells require prolonged activation to initiate a primary response, whereas CD45RO+ memory cells undergo rapid clonal expansion and immediate cytokine production upon antigen re-exposure [20]. Across solid tumors, CD45RO+ tumor-infiltrating

memory T cells have been associated with improved survival, as a reliable marker of tissue-level adaptive immune competence [21, 22]. Spatial positioning may be especially important for interpreting these phenotypes in ICI-treated NSCLC. Pre-existing CD8+ T cells at the invasive tumor margin have been linked to response to PD-1 blockade, indicating that location can modify the clinical meaning of T-cell abundance [23, 24]. Multiplex immunofluorescence studies in NSCLC have further shown that simultaneous quantification of immune-cell phenotypes and their spatial relationships can reveal clinically relevant immune architectures not accessible by single-marker immunohistochemistry [25, 26].

The present study used a 22-plex CellScape™ multiplex imaging panel on FFPE tissue microarrays (TMAs) from patients with advanced NSCLC treated with first-line ICI-based therapy. The cohort included matched clinical follow-up with both progression-free survival (PFS) and overall survival (OS) endpoints, with at least 4 years of follow-up available for all patients, providing a clinically mature framework for evaluating spatial immune biomarkers beyond short-term response metrics. We focused on CD4+ and CD8+ lymphocyte phenotypes defined by CD45RO and CD45RA status, quantified their densities, tumor proximity and lineage-restricted differentiation balance, and developed a tumor engagement index integrating immune-cell abundance with distance to tumor cells. Our primary objective was to determine whether tumor-proximal memory-like CD8+ immune architecture, particularly CD8+CD45RO+ tumor engagement, identifies a clinically favorable spatial phenotype and provides prognostic information beyond immune-cell density and routine clinicopathological covariates.

MATERIALS AND METHODS

Study population

This retrospective cohort included 39 patients with inoperable stage IIIB–IV non-small cell lung cancer (NSCLC) treated with first-line standard-of-care immune checkpoint inhibitor (ICI)-based therapy between 2019 and 2022. Patients received either pembrolizumab (PEM) monotherapy or chemo-immunotherapy according to the PEM–CDDP protocol, based on routine clinical indication. Formalin-fixed paraffin-embedded (FFPE) tumor tissue and matched clinicopathological follow-up data were available for all included cases. Patients were stratified according to progression-free survival (PFS) using a predefined 6-month cutoff [27], resulting in 22 long-PFS and 17 short-PFS cases. Overall survival (OS) was evaluated using a predefined 12-month cutoff. The follow-up period for all included patients was 48 months. Clinical variables collected for cohort characterization and downstream modeling included sex, treatment regimen, metastatic burden, PD-L1 tumor proportion score (TPS), PFSI, and OS. PD-L1 TPS was classified as high ($\geq 50\%$) or low ($< 50\%$) according to routine pathological reporting. COPD status was recorded descriptively. Metastatic burden was categorized as lymph-node-only disease, single distant organ/site involvement, or multiple distant sites. All recruited patients were assessed as Eastern Cooperative Oncology Group (ECOG) 0 or 1 at diagnosis and sampling. **Table 1.** shows clinicopathological characteristics of the patient cohort.

TMA preparation

Representative areas of the formalin-fixed, paraffin-embedded (FFPE) tissue blocks were selected by a pulmonary pathologist (I.K.) based on haematoxylin and eosin (H&E) staining and relevant immunohistochemical markers (TTF-1, p40, and CK7). Tissue microarrays (TMAs) were constructed using the TMA Master instrument (3DHitech, Budapest, Hungary). Each TMA block consisted of 5 × 9 cores (2 mm diameter). In cases where sufficient tissue was available, two or three cores were sampled per case. Non-neoplastic control tissues, including liver, lymph node, tonsil, and spleen, were included as controls in each TMA block.

CellScape multiplex imaging

Multiplex immunofluorescence imaging was performed on FFPE TMA sections using the CellScape™ platform (Canopy Biosciences) according to the manufacturer's workflow for cyclic high-plex spatial protein detection [28, 29]. Briefly, tissue sections were processed for multiplex immunofluorescence staining and subjected to sequential automated cycles of antibody incubation, fluorescence image acquisition and signal removal, allowing repeated detection of protein markers on the same tissue section while preserving spatial registration. The applied 22-plex panel included DAPI, ATP1A1, Lamin B1, B2M, CD20, CD56, CD3, PD-1/CD279, PD-L1/CD274, CD4, CD45, CD19, Granzyme B, CD8, CD45RO, CD38, Ki67, CD68, CD45RA, PanCK, CD163 and FoxP3. Image acquisition was performed across TMA cores using the integrated CellScape imaging system. The resulting multiplex image sets were generated as spatially registered marker channels and used for downstream tissue segmentation, cell detection, immune-cell phenotyping and spatial analysis.

Image analysis workflow

Multiplex image analysis was performed in QuPath (v0.6.3) using a standardized TMA workflow [30]. Image files were imported as multichannel fluorescence images, and individual TMA cores were annotated and reviewed as separate analytical regions. Cores were excluded from downstream analysis when they showed major tissue loss, extensive folding, detachment, severe staining artefacts, out-of-focus acquisition, insufficient viable tissue or non-representative tissue content. All retained cores were analyzed using the same software-based workflow to maintain comparability across samples.

Cell segmentation

Cell segmentation was performed using the nuclear counterstain channel as the primary segmentation reference. Detection parameters were optimized to capture lymphocyte-sized and tumor-cell-sized nuclei while minimizing over-segmentation of clustered nuclei and exclusion of small immune cells. Segmented nuclei were expanded to approximate whole-cell boundaries, thereby allowing marker intensity extraction from cell-associated regions in addition to nuclear measurements. For each detected cell, QuPath-derived intensity features were extracted from the relevant fluorescence channels, including PanCK for epithelial tumor-cell identification and CD4, CD8, CD45RO, CD45RA and Granzyme B for lymphocyte subset classification. Measurements were retained at the single-cell level and linked to the corresponding core annotation for downstream phenotype-level aggregation.

Phenotypization

Marker positivity was assigned using predefined intensity thresholds established by visual inspection of representative positive and negative staining patterns across the TMA. Thresholds were selected separately for each marker channel, taking into account marker-specific subcellular localization, staining distribution, background signal and expected biological expression patterns. CD4, CD8, CD45RO, CD45RA and Granzyme B thresholds were applied to segmented cell measurements to identify lymphocyte phenotypes, while PanCK positivity was used to define epithelial tumor cells and to distinguish tumor from non-tumor cellular compartments. The same thresholding rules were applied uniformly to all evaluable cores after parameter optimization. Cellular phenotyping was performed using custom Groovy scripts in QuPath (Supplementary material). Single-cell marker measurements were accessed programmatically, and logical marker combinations were applied to assign each detected cell to a defined phenotypic class. Single-positive lymphocyte classes included CD4+, CD8+, CD45RO+ and CD45RA+ cells. Combined lymphocyte phenotypes included CD8+CD45RO+, CD8+CD45RA+, CD4+CD45RO+, CD4+CD45RA+ and CD8+CD45RO+Granzyme B+ cells. Cells fulfilling criteria for higher-order combined phenotypes were assigned according to a predefined priority structure, with biologically more specific multi-marker phenotypes prioritized over broader single-marker categories. For each TMA core, phenotype-specific cell counts were calculated from the final classified detection set. Cell densities were derived by normalizing counts to the analyzed tissue area of the corresponding core annotation. Core-level outputs included total cell number, marker-positive cell counts, combined phenotype counts and phenotype densities. These measurements were exported from QuPath for statistical analysis at core and patient level. All phenotypization codes are available in the Supplementary materials.

Proximity measurements

Proximity measurements were performed after rule-based cellular phenotyping using centroid-based nearest-neighbor distances between immune phenotypes and PanCK-positive tumor cells within each TMA core annotation. For each cell object, marker positivity was assigned from the same shared all-cell detection table using predefined intensity thresholds. PanCK-positive cells were used as the tumor-cell reference population. For each phenotype-positive immune population P, including CD8+ and CD8+CD45RO+ cells, the Euclidean centroid-to-centroid distance from each immune cell i in P to the nearest PanCK-positive tumor cell was calculated, yielding the immune-to-tumor proximity distribution d(i, PanCK). Distances were converted to micrometers using image pixel calibration. For each phenotype P, the 75th percentile of the immune-to-PanCK nearest-neighbor distance distribution was used as the core proximity metric. This percentile-based summary was prioritized over a fixed 50 μ m proximity threshold because the latter showed limited dynamic range in the preliminary subset. The abundance-adjusted tumor-engagement index (TEI) was defined as:

$$TEI_P = \frac{\text{density}_P}{Q_{75}[d(i, \text{PanCK})]}$$

where density_P is the phenotype-positive cell density per mm² and Q₇₅[d(i, PanCK)] is the 75th percentile immune-to-PanCK nearest-neighbor distance for phenotype P. Thus, higher TEI values indicate an immune phenotype that is both more abundant and more tumor-

proximal. Core-level results were aggregated at patient level by summing phenotype counts and analyzed tissue areas to derive patient-level density, while distance-based metrics were summarized across the measured cores for each patient. For graphical spatial-state mapping, phenotype density was plotted against Q75 distance, and cases were categorized by cohort-level median splits into engaged, immune-rich but displaced, sparse but proximal and cold/distal states. Phenotype-specific proximity metrics were calculated only for patient-level observations with at least 30 phenotype-positive cells after core aggregation, to avoid unstable nearest-neighbor estimates driven by sparse cell counts.

Statistical analyses

Data aggregation and clinical endpoints

All image-derived measurements were first generated at TMA core level and then aggregated at patient level before clinical association analyses. Phenotype counts were summed across evaluable cores from the same patient, and phenotype densities were calculated by dividing summed cell counts by the summed analyzed tissue area. PFS was defined from treatment initiation to radiological or clinical progression or death, and overall survival was defined from treatment initiation to death from any cause. Patients without an event were censored at last follow-up. For categorical outcome analyses, patients were stratified using predefined clinically relevant cutoffs into long versus short PFS at 6 months [27, 31] and long versus short overall survival at 12 months [32, 33].

Clinicopathological and immune phenotype comparisons

Clinicopathological variables were summarized using descriptive statistics and compared between outcome groups. Continuous variables were reported as median with interquartile range or mean with standard deviation, as appropriate, and categorical variables were reported as counts and percentages. Between-group comparisons were performed using Mann–Whitney U tests for continuous variables and Fisher's exact or χ^2 tests for categorical variables, depending on expected cell counts. Immune phenotype analyses included CD4+, CD8+, CD45RO+, CD45RA+, CD8+CD45RO+, CD8+CD45RA+, CD4+CD45RO+, CD4+CD45RA+ and CD8+CD45RO+Granzyme B+ cell densities. Density-based immune features and derived spatial metrics were compared between PFS6 and OS12 outcome strata using two-sided non-parametric tests.

Survival modeling, feature selection and reporting

Associations with survival were evaluated using Kaplan–Meier curves, log-rank tests and Cox proportional hazards regression. Univariable Cox models were used to screen image-derived features, followed by multivariable Cox models adjusted for clinically relevant covariates, including sex, treatment modality, metastatic burden and PD-L1 TPS. COPD was reported descriptively but excluded from primary multivariable modeling because of missingness. Penalized Cox regression with elastic-net regularization was used for multivariable spatial feature selection and internal robustness assessment. Model performance was evaluated using concordance indices, leave-one-out cross-validation (LOOCV), feature-selection stability and permutation testing. Elastic-net models used $\alpha=0.5$ and L1 ratio=0.7. Model performance was summarized using Harrell's C-index and compared with 500 outcome-permuted null models. Feature stability was estimated using

500 bootstrap resamples, with features considered selected when the absolute standardized coefficient exceeded 0.001. All statistical tests were two-sided, and p values below 0.05 were considered statistically significant unless otherwise specified. Analyses and visualizations were performed using Python.

RESULTS

This study included 39 patients with advanced-stage NSCLC treated with first-line ICI-based therapy and available FFPE TMA material for multiplex spatial profiling. Baseline clinicopathological characteristics according to the predefined PFS groups are summarized in **Table 1**. **Figure 1A** outlines the study workflow, from cohort selection and CellScape 22-plex imaging to QuPath-based single-cell phenotyping and patient-level spatial modeling. Representative multiplex images and phenotype maps illustrate whole TMA core morphology (bronchoscopic and core biopsies, **Fig. 1B and C**), PanCK-defined tumor compartments and spatially resolved CD45RO- and CD45RA-related lymphocyte phenotypes used for downstream quantitative analyses (**Fig. 1D and E**). **Figure 1F** shows color code for included cellular phenotypes in phenotype maps.

Table 1. Baseline clinical covariates according to PFS group

Covariate	Long PFS patients (n=22)	Short PFS patients (n=17)	p-value
Sex, n (%)			0.314
Male	16 (72.7%)	9 (52.9%)	
Female	6 (27.3%)	8 (47.1%)	
Therapy, n (%)			0.106
IO-mono	13 (59.1%)	5 (29.4%)	
CHT-IO	9 (40.9%)	12 (70.6%)	
PD-L1 TPS, n (%)			1.000
High	10 (45.5%)	9 (52.9%)	
Low	7 (31.8%)	8 (47.1%)	
Unknown	5 (22.7%)	0 (0.0%)	
COPD, n (%)			0.441
Yes	13 (59.1%)	8 (47.1%)	
No	4 (18.2%)	6 (35.3%)	
Unknown	5 (22.7%)	3 (17.6%)	
Metastatic burden, n (%)			0.048
LN-only	5 (22.7%)	0 (0.0%)	
Single distant organ/site	15 (68.2%)	11 (64.7%)	
Multiple distant sites	2 (9.1%)	5 (29.4%)	

Covariate	Long PFS patients (n=22)	Short PFS patients (n=17)	p-value
Unknown	0 (0.0%)	1 (5.9%)	

Values are shown as n (%). P-values were calculated using Fisher's exact test for binary covariates and a permutation chi-square test for metastatic-burden categories. Unknown PD-L1, COPD and metastatic-burden values are displayed but excluded from the corresponding p-value calculation.

Spatial imaging reveals CD45RO- and CD45RA-related T-cell phenotypes

Across the cohort, the quantified immune landscape showed broad detectability and substantial inter-patient heterogeneity across the studied phenotypes. Total CD45RO⁺ cells represented the most abundant measured immune phenotype, with a median density of 439 cells/mm², followed by CD4⁺ cells at 360 cells/mm² and CD8⁺ cells at 157 cells/mm². CD45RA⁺ cells were also consistently detected, but at a lower median density of 147 cells/mm² (**Fig. 1G**). Within lineage-restricted subsets, CD4⁺CD45RO⁺ cells were more abundant than CD8⁺CD45RO⁺ cells, with median densities of 113 and 29 cells/mm², respectively, while RA-positive double-positive subsets were less frequent, particularly CD8⁺CD45RA⁺ cells, with a median density of 11 cells/mm² (**Fig. 1G**). All major phenotypes were detectable in all patients except CD8⁺CD45RO⁺-related measures, which were detectable in 97.4% of cases. Correlation analysis showed that CD45RO density correlated with both CD4⁺CD45RO⁺ and CD8⁺CD45RO⁺ densities, while CD45RA density correlated strongly with CD4⁺CD45RA⁺ and CD8⁺CD45RA⁺ subsets. The RO ratios within CD4⁺ and CD8⁺ lineages were positively coupled, but showed inverse associations with RA-positive compartments, consistent with a shared differentiation balance across T-cell lineages (**Fig. 1H**).

The cohort showed a consistent RO-skewed differentiation pattern within both T-cell lineages. The median CD45RO:CD45RA ratio was 3.07 within CD8⁺ cells and 2.56 within CD4⁺ cells (**Fig. 1I**). Parent-normalized analyses confirmed that RO-positive fractions exceeded RA-positive fractions in both compartments: CD8 RO fraction 0.235 versus CD8 RA fraction 0.094, and CD4 RO fraction 0.281 versus CD4 RA fraction 0.093. These differences were significant in paired analyses, particularly for the CD4 lineage (**Fig. 1J**). Lineage-coupling analysis showed that lineage-restricted CD45RO-positive subsets scaled positively with their parent compartments. CD4 density correlated strongly with CD4⁺CD45RO⁺ density (Spearman $\rho=0.78$, $p<0.001$), while CD8 density also correlated with CD8⁺CD45RO⁺ density ($\rho=0.59$, $p<0.001$) (**Fig. 1K and L**). Overall, these data indicate that the cohort is characterized by measurable, heterogeneous, and predominantly RO-skewed adaptive immune states, supporting subsequent outcome-focused analyses of memory-associated T-cell phenotypes. Density distributions of all measured cellular phenotypes are shown on histograms in **Supplemental Figures 1 and 2**.

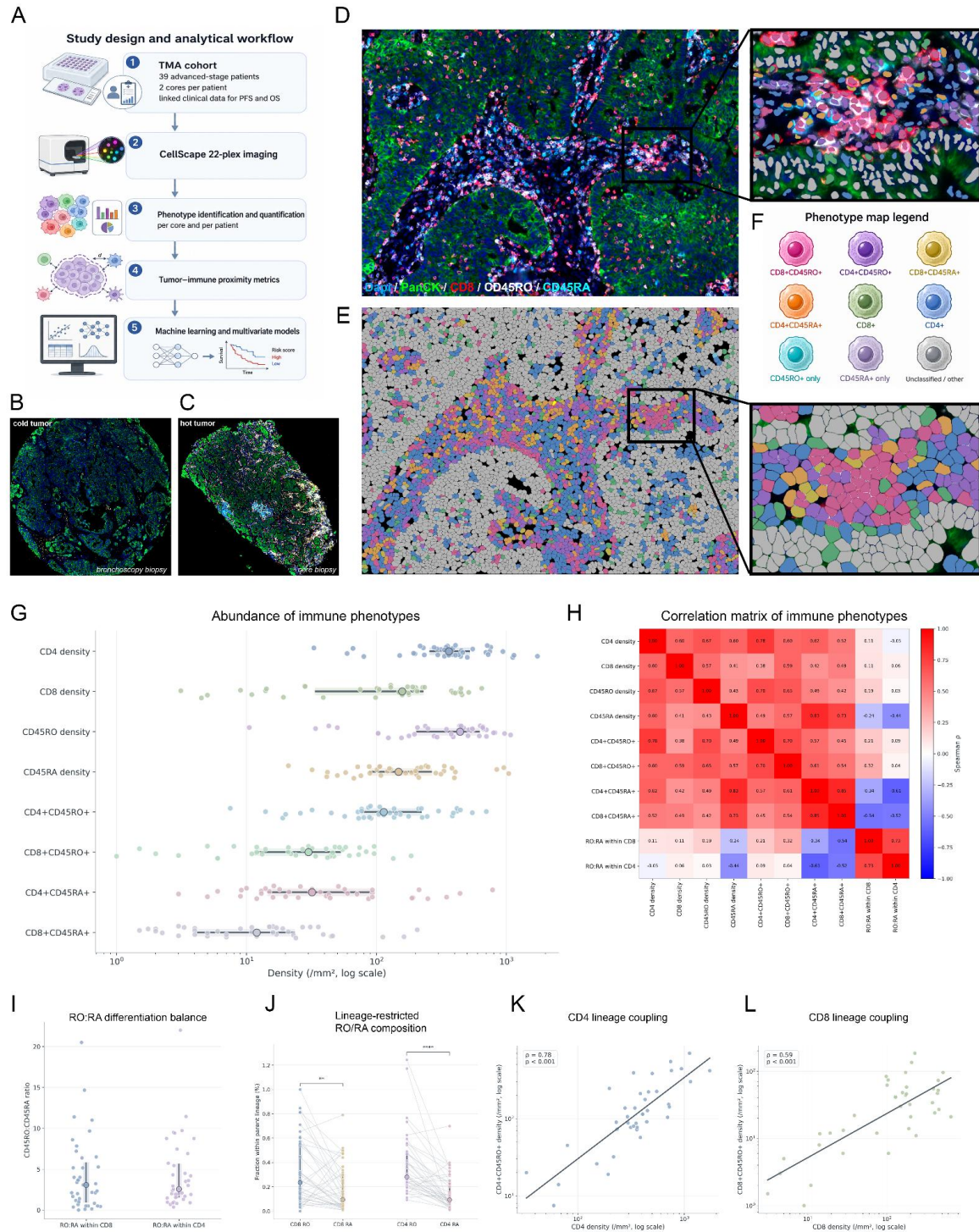


Figure 1. Study design and whole-cohort T-cellular phenotype landscape according to CD45RO/RA. (A) shows study design and analysis workflow. (B) and (C) show representative images of whole cores (cold tumor, bronchoscopy biopsy (B) and hot tumor, core biopsy (C) with all used biomarker channels included. (D) shows representative area from hot tumor at stroma-tumor engagement site, inset with overlaid cellular segmentation and phenotypes color coded. (E) shows the same area with cellular phenotype map with legend in (F). Patient-level aggregated immune metrics were visualized across the full cohort, where (G) shows density distributions of total CD4⁺, CD8⁺, CD45RO⁺, CD45RA⁺ and lineage-restricted double-positive phenotypes on a log-scaled cells/mm² axis; dots represent individual patients, with median and interquartile range overlaid. (H) shows a Spearman correlation matrix for basic density variables and RO:RA ratios, excluding fraction variables. Correlation coefficients are displayed within cells; color encodes Spearman ρ from negative to positive

correlation. (I) shows CD45RO:CD45RA ratio distributions within CD8⁺ and CD4⁺ lineages. (J) compares parent-normalized CD45RO⁺ and CD45RA⁺ fractions within CD8⁺ and CD4⁺ cells using paired patient-level values; paired Wilcoxon signed-rank tests were applied. (K) and (L) show outcome-independent lineage-coupling scatterplots relating total CD4⁺ density to CD4⁺CD45RO⁺ density and total CD8⁺ density to CD8⁺CD45RO⁺ density, respectively. Each dot represents one patient; both axes are shown on a log scale. Association strength was assessed by Spearman correlation.

CD45RO- and CD45RA-related T-cell phenotypes and clinical outcomes

Representative CellScape multi-channel images illustrate spatially resolved CD8⁺, CD4⁺, CD45RO⁺ and CD45RA⁺ immune-cell patterns in relation to PanCK-defined tumor compartments, together with lineage-restricted CD8/CD45RO, CD8/CD45RA, CD4/CD45RO and CD4/CD45RA phenotypes (**Fig. 2A**). Patients with long PFS showed higher CD8⁺ T-cell density than patients with short PFS (median 176.5 vs 38.0 cells/mm²; $p=0.0154$), whereas CD4⁺ density (372.0 vs 360.0 cells/mm²; $p=0.8428$), total CD45RO⁺ density (456.9 vs 195.5 cells/mm²; $p=0.0767$) and total CD45RA⁺ density (174.5 vs 125.0 cells/mm²; $p=0.4702$) did not differ significantly (**Fig. 2B**).

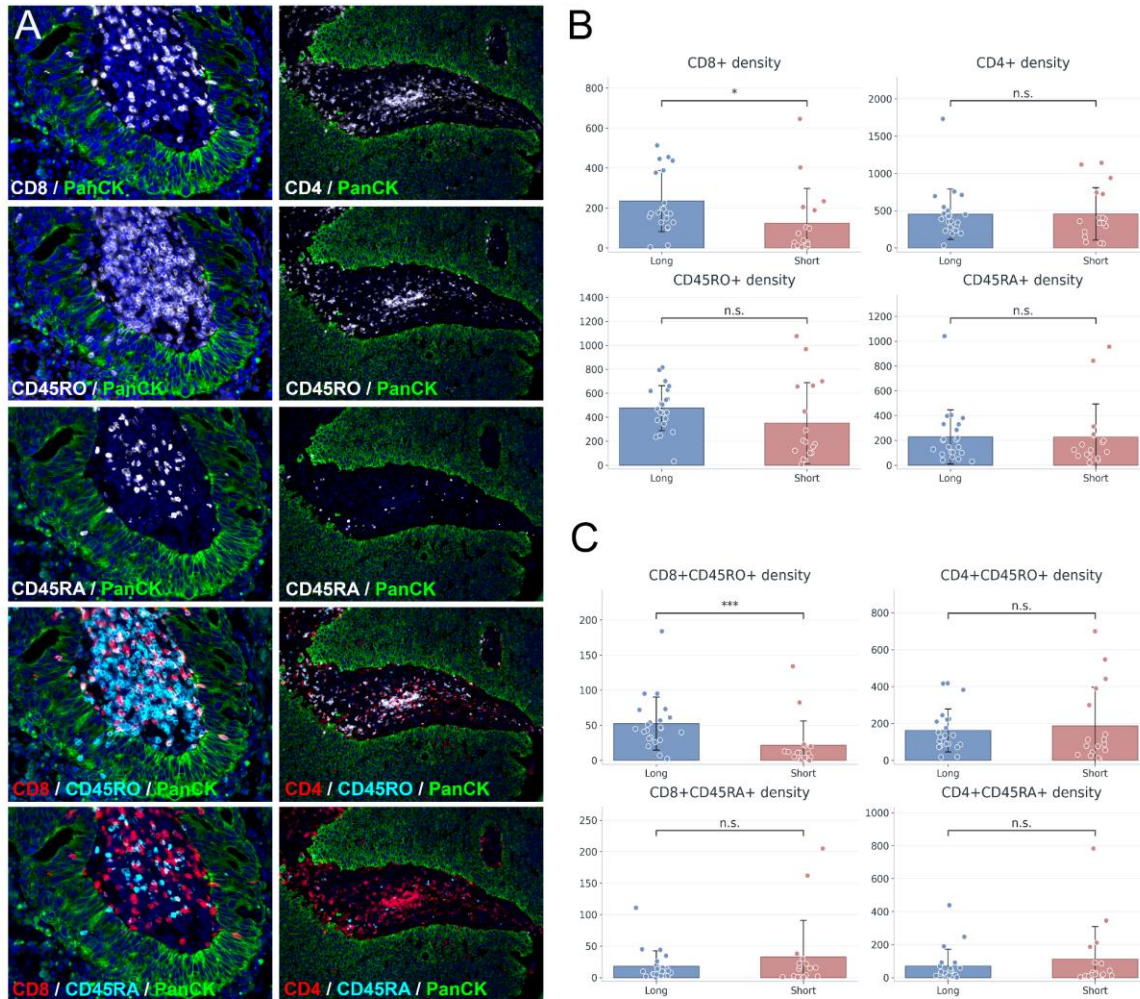


Figure 2. CD45RO- and CD45RA-related T-cell phenotypes according to PFS group. (A) Representative CellScape multiplex images illustrate PanCK-defined tumor compartments together with CD8, CD4, CD45RO and CD45RA signal patterns, including lineage-restricted CD8/CD45RO, CD8/CD45RA, CD4/CD45RO and CD4/CD45RA overlays. (B-C) Patient-level phenotype densities were compared between long-PFS and short-

PFS groups using boxplots with individual patient values overlaid. (B) Among basic immune densities, CD8+ cell density was significantly higher in long-PFS patients, whereas CD4+, total CD45RO+ and total CD45RA+ densities did not differ significantly between groups. (C) Among lineage-restricted double-positive phenotypes, CD8+CD45RO+ density showed the strongest group difference and was enriched in long-PFS patients, while CD4+CD45RO+, CD8+CD45RA+ and CD4+CD45RA+ densities were not significantly different. Y-axis shows patient-level phenotype density in cells/mm², calculated by dividing the summed phenotype-positive cell count across evaluable TMA cores by the corresponding summed analyzed tissue area. Statistical significance is indicated above each comparison; n.s., not significant; *p<0.05; ***p<0.001.

Among lineage-restricted phenotypes, CD8+CD45RO+ density showed the strongest PFS-associated difference and was higher in the long-PFS group (median 45.5 vs 11.0 cells/mm²; p=0.00049; FDR=0.0069), while CD4+CD45RO+ (131.0 vs 87.3 cells/mm²; p=0.4789), CD8+CD45RA+ (10.0 vs 15.0 cells/mm²; p=1.0000) and CD4+CD45RA+ densities (38.0 vs 30.0 cells/mm²; p=0.9323) were not significantly different (**Fig. 2C**). Ratio and fraction analyses further showed higher CD45RO ratios in long-PFS patients within both CD8+ cells (median 3.65 vs 0.67; p=0.00222; FDR=0.0103) and CD4+ cells (median 3.55 vs 1.73; p=0.0219), whereas the CD8+CD45RA+ fraction within CD8+ cells was higher in short-PFS patients (median 0.046 vs 0.250; p=0.00144; FDR=0.0101). CD8+CD45RO+/CD8+ (0.252 vs 0.185; p=0.5615), CD4+CD45RO+/CD4+ (0.374 vs 0.262; p=0.2755) and CD4+CD45RA+/CD4+ fractions (0.087 vs 0.127; p=0.3723) were not significantly different (**SFig. 3**). Together, these analyses identified CD8+CD45RO+ abundance and a higher CD45RO balance, particularly within the CD8+ lineage, as the main T-cell features associated with longer PFS.

In OS12 group comparisons, long-OS patients showed higher total CD45RO+ density than short-OS patients (median 456.94 vs 206.00 cells/mm²; p=0.03485), while CD8+ density showed a non-significant trend in the same direction (174.5 vs 97.5 cells/mm²; p=0.07208). CD4+ density (395.75 vs 352.00 cells/mm²; p=0.42770) and CD45RA+ density (174.5 vs 121.0 cells/mm²; p=0.56151) were not significantly different (**Fig. 3A**). Among lineage-restricted double-positive phenotypes, CD8+CD45RO+ density was higher in long-OS patients (44.0 vs 12.19 cells/mm²; p=0.01543), whereas CD4+CD45RO+ (136.0 vs 86.0 cells/mm²; p=0.17396), CD8+CD45RA+ (9.0 vs 15.0 cells/mm²; p=0.59027) and CD4+CD45RA+ densities (38.0 vs 30.0 cells/mm²; p=0.96611) were not significantly associated with OS group (**Fig. 3B**). The CD45RO ratio within CD8+ cells was also higher in long-OS patients (3.83 vs 0.83; p=0.00192), while the CD8+CD45RA+ fraction within CD8+ cells was higher in short-OS patients (0.046 vs 0.200; p=0.00956) (**SFig. 4**). Overall, OS comparisons reproduced the main PFS pattern by consistently implicating CD8+CD45RO+ enrichment and a higher CD8-lineage CD45RO balance as favorable features, while differing mainly in that total CD45RO+ density reached significance for OS, whereas total CD8+ density was more clearly separated in the PFS comparison.

Median-cutoff survival analyses supported CD8+CD45RO+ density as the most consistent immune feature across endpoints. High CD8+CD45RO+ density was associated with improved PFS (HR=0.21, 95% CI 0.08–0.54; log-rank p=0.000322) and OS (HR=0.21, 95% CI 0.08–0.53; log-rank p=0.000359). High CD8+ density was significantly associated with OS but not PFS (OS HR=0.38, 95% CI 0.16–0.90; log-rank p=0.02286; PFS HR=0.45, 95% CI 0.19–1.08; log-rank p=0.06135). A high CD45RO ratio within CD8+ cells was also associated with improved OS (HR=0.31, 95% CI 0.13–0.77; log-rank p=0.00803), whereas a

high CD8+CD45RA+ fraction within CD8+ cells was associated with shorter OS (HR=3.41, 95% CI 1.35–8.62; log-rank p=0.005996) (**Fig. 3C and D**).

Granzyme B-associated memory-like CD8+ phenotyping (**Fig. 3E**) further supported the outcome association of cytotoxic CD8+CD45RO+ cells. CD8+CD45RO+Granzyme B+ density was higher in long-PFS patients than in short-PFS patients (median 21.5 vs 6.0 cells/mm²; p=0.00262) and also higher in long-OS patients than in short-OS patients (23.5 vs 8.0 cells/mm²; p=0.00474) (**Fig. 3F**). In median-cutoff Kaplan–Meier analyses, high CD8+CD45RO+Granzyme B+ density was associated with improved PFS (log-rank p=0.03463) and OS (log-rank p=0.00391) (**Fig. 3G**). The directional heatmap summarized these patterns across binary comparisons and survival analyses, identifying CD8+CD45RO+ density as the strongest and most consistent favorable feature, while CD8+CD45RA+ fraction showed the most consistent adverse direction (**Fig. 3H**). **Supplementary Figure 5** shows additional KM curves for all remaining cellular phenotypes, fractions and ratios.

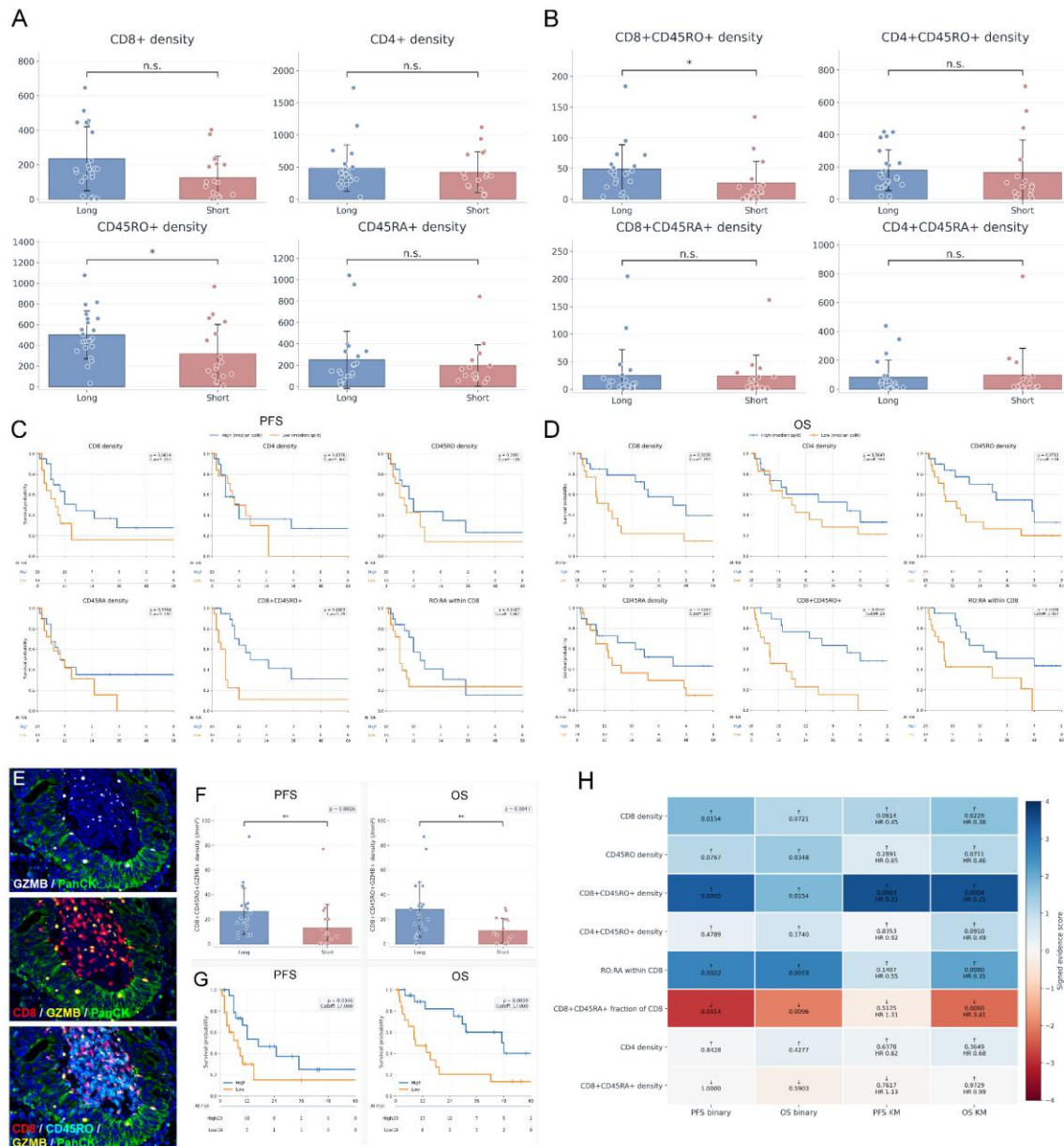


Figure 3. Outcome-associated CD45RO- and CD45RA-related T-cell phenotypes and cytotoxic memory-like CD8+ subsets. (A) Patient-level OS12 group comparisons of basic immune-cell densities, including CD8+, CD4+, total CD45RO+ and total CD45RA+ cells. (B) OS12 group comparisons of lineage-restricted double-positive phenotypes, including CD8+CD45RO+, CD4+CD45RO+, CD8+CD45RA+ and CD4+CD45RA+ cells. Boxplots show median and interquartile range with individual patient values overlaid; long-OS and short-OS groups were compared using two-sided Mann–Whitney U tests. Y-axis shows patient-level phenotype density in cells/mm², calculated by dividing the summed phenotype-positive cell count across evaluable TMA cores by the corresponding summed analyzed tissue area. (C–D) Kaplan–Meier analyses for selected immune biomarkers dichotomized at the cohort median, shown separately for PFS and OS; p values were calculated using log-rank tests and hazard ratios were estimated by Cox proportional hazards regression. (E) Representative CellScape multiplex images showing Granzyme B, CD8 and CD45RO signal distribution in relation to PanCK-defined tumor compartments. (F) Binary PFS and OS group comparisons of CD8+CD45RO+Granzyme B+ cell density. (G) Kaplan–Meier analyses of CD8+CD45RO+Granzyme B+ density dichotomized at the cohort median. (H) Directional comparison heatmap summarizing binary outcome associations and median-cutoff survival analyses across selected immune biomarkers. Color encodes signed evidence strength according to direction $\times -\log_{10}(p)$, capped at 4; arrows indicate the direction associated with longer survival. n.s., not significant; *p<0.05; **p<0.01.

Spatial tumor engagement of CD8+CD45RO+ cells is associated with improved PFS and OS

We next quantified the spatial relationship between CellScape-derived immune phenotypes and PanCK-defined tumor compartments, summarizing single-cell proximity measurements at patient level and integrating immune-cell abundance with tumor proximity through the tumor-engagement index (TEI, **Fig. 4A**). Spatial proximity analysis showed a non-random hierarchy of immune-cell positioning relative to PanCK+ tumor cells (Friedman $\chi^2=36.38$, $p=7.96 \times 10^{-7}$). The most tumor-proximal phenotypes were CD8+CD45RO+ and total CD8+ cells, whereas CD8+CD45RA+ and CD4+CD45RA+ cells were the most distal compartments (median nearest PanCK+ distance: CD8+CD45RO+, 16.66 μm ; CD8+, 16.84 μm ; CD4+, 20.61 μm ; CD4+CD45RO+, 23.36 μm ; CD8+CD45RA+, 27.11 μm ; CD4+CD45RA+, 30.34 μm , **Fig. 4B**). This lineage-restricted RO-versus-RA contrast was retained at the Q75 distance level, with CD8+CD45RO+ cells closer to tumor than CD8+CD45RA+ cells (24.46 vs 36.17 μm ; paired Wilcoxon $p=0.00011$) and CD4+CD45RO+ cells closer than CD4+CD45RA+ cells (30.17 vs 41.30 μm ; $p=0.00664$) (**Fig 4C and D**).

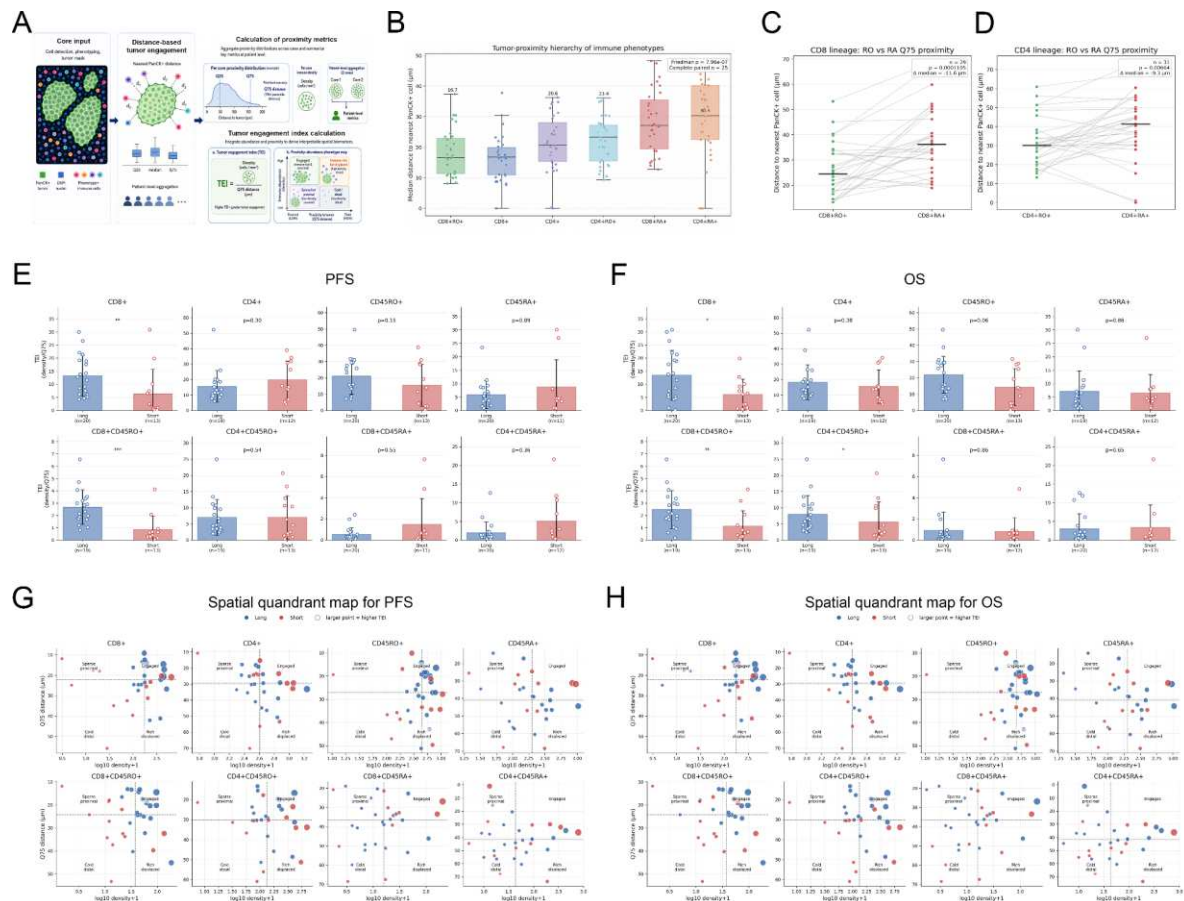


Figure 4. Spatial tumor proximity and tumor-engagement index analyses of CD45RO- and CD45RA-related immune phenotypes. (A) Schematic overview of the spatial proximity analysis workflow, including whole-core CellScape/QuPath-derived cellular input, centroid-based nearest-neighbor distance calculation to PanCK+ tumor cells, core- and patient-level aggregation, and derivation of tumor-engagement outputs. (B) Patient-level median nearest PanCK+ distances were summarized across the six principal immune phenotypes included in the descriptive spatial analysis: CD8+, CD4+, CD8+CD45RO+, CD4+CD45RO+, CD8+CD45RA+ and CD4+CD45RA+. Boxes indicate interquartile range, center lines the median and overlaid points of individual patients; phenotype ordering was assessed using a Friedman test across complete paired cases. Lower values indicate closer tumor proximity. (C,D) Paired lineage-restricted CD45RO-versus-CD45RA comparisons were performed using Q75 nearest PanCK+ distance within CD8+ and CD4+ cells, respectively; paired Wilcoxon signed-rank tests were used. (E,F) Tumor-engagement index (TEI) values were compared between long and short PFS groups and between long and short OS groups for each phenotype. TEI integrates phenotype density and Q75 tumor proximity, with higher values indicating a denser and more tumor-proximal immune compartment. Boxplots show group distributions with individual patients overlaid; Mann-Whitney U tests were applied. The y-axis shows patient-level phenotype density in cells/mm², calculated by dividing the summed phenotype-positive cell count across evaluable TMA cores by the corresponding summed analyzed tissue area. (G,H) Spatial quadrant maps show phenotype density on the x-axis and Q75 nearest PanCK+ distance on the y-axis for PFS and OS groups, respectively. Point size is proportional to TEI. Dashed lines indicate cohort medians and define four spatial states: engaged, sparse proximal, rich displaced and cold distal.

TEI analyses showed that outcome-associated spatial signals were concentrated in CD8-related compartments. In the PFS comparison, long-PFS patients had higher CD8+ TEI than short-PFS patients (median 12.20 vs 2.28; $p=0.00756$), with the strongest separation observed for CD8+CD45RO+ TEI (2.33 vs 0.58; $p=0.000198$). CD4+, CD45RO+, CD45RA+, CD4+CD45RO+, CD8+CD45RA+ and CD4+CD45RA+ TEI values were not significantly different by PFS group (**Fig. 4E**). In the OS comparison, long-OS patients also showed higher CD8+ TEI (12.70 vs 6.20; $p=0.03737$) and CD8+CD45RO+ TEI (2.16 vs 0.65;

p=0.00908), with an additional weaker association for CD4+CD45RO+ TEI (6.39 vs 2.49; p=0.04602). Total CD45RO+ TEI showed a borderline OS-associated trend (19.58 vs 13.42; p=0.06280) (**Fig. 4F**). Spatial quadrant maps were consistent with the TEI comparisons. For CD8+ and particularly CD8+CD45RO+ cells, long-PFS and long-OS cases were more frequently positioned in the engaged quadrant, reflecting the combined presence of higher phenotype density and shorter Q75 tumor distance). Short-outcome cases were more often distributed toward cold-distal or rich-displaced regions. RA-positive compartments showed greater overlap between outcome groups, indicating that the most reproducible spatial outcome signal was the tumor-engaged CD8+CD45RO+ phenotype (**Fig 4G and H**). Compared with PFS, OS quadrant maps showed a largely concordant but slightly broader spatial pattern: CD8+ and CD8+CD45RO+ engagement remained the dominant favorable configuration, while CD4+CD45RO+ showed a weaker additional OS-associated shift, whereas RA-positive compartments remained poorly separated across both endpoints.

CD8+CD45RO+ tumor engagement provides independent prognostic value

To evaluate whether the spatially derived TEI provided additional discriminatory information beyond immune-cell abundance alone, we performed an incremental-value analysis for the two biologically prioritized phenotypes, CD8+ and CD8+CD45RO+ cells. The analysis showed that TEI consistently improved discrimination compared with density alone and was the best-performing configuration across all tested phenotype–endpoint combinations (**Fig. 5A and B**). For PFS, TEI increased LOOCV AUC from 0.68 to 0.75 for CD8+ cells and from 0.81 to 0.87 for CD8+CD45RO+ cells. For OS, TEI increased AUC from 0.58 to 0.67 for CD8+ cells and from 0.62 to 0.72 for CD8+CD45RO+ cells (**Fig. 5A and B**). In each case, TEI outperformed both Q75 alone and the additive density-plus-Q75 model, supporting the use of a compact abundance–proximity composite rather than either component alone.

Kaplan–Meier analyses using the final CD8+CD45RO+ TEI confirmed its association with clinical outcome. Patients with high TEI had longer PFS than patients with low TEI (log-rank p=0.0009, **Fig. 5C**), and the same dichotomization was also associated with longer OS (log-rank p=0.0404, **Fig. 5D**). In multivariable Cox models adjusted for sex, treatment modality, metastatic burden and PD-L1 TPS, final spatial TEI remained independently associated with both PFS and OS. Higher TEI was associated with markedly lower progression risk (HR 0.09, 95% CI 0.02–0.44; p=0.003) and lower mortality risk (HR 0.10, 95% CI 0.02–0.42; p=0.002), whereas the included clinical covariates did not reach statistical significance (**Fig. 5E–F**).

Elastic-net Cox analysis further supported CD8+CD45RO+ TEI as the dominant stable spatial feature. The observed model C-index was 0.80 for PFS, exceeding the outcome-permuted null distribution (permutation p=0.030), and 0.72 for OS, with a non-significant permutation result (p=0.273) (**Fig. 5G**). In bootstrap stability analysis, CD8+CD45RO+ TEI showed the highest selection frequency for both endpoints (PFS, 97%; OS, 100%) and was consistently selected with a negative coefficient, indicating association with lower hazard. Other TEI metrics and clinical covariates showed lower and less consistent selection frequencies (**Fig. 5H**). Waterfall diagram shows TEI-related relative risk and binary clinical variables for all patients in **Supplementary Figure 6**.

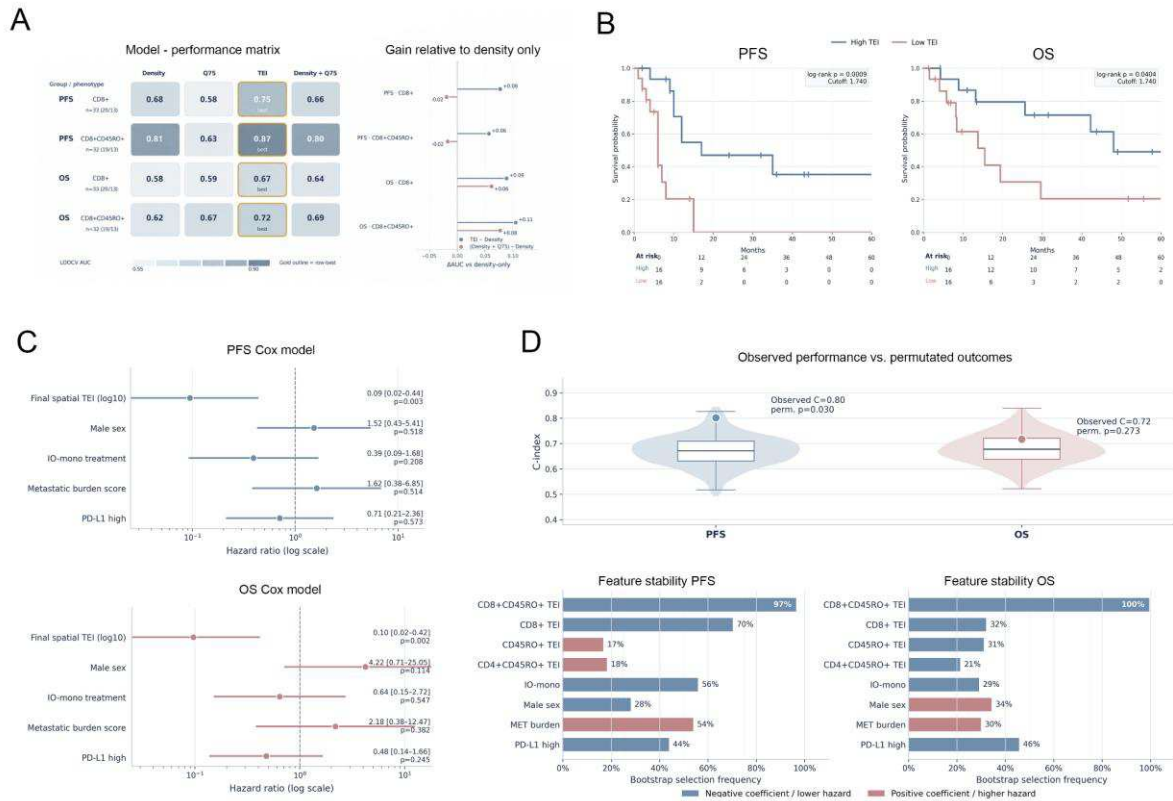


Figure 5. Spatial tumor-engagement index (TEI) model and clinical validation analyses. (A) Incremental-value analysis of the TEI compared with density-only, Q75-only and density-plus-Q75 models for CD8+ and CD8+CD45RO+ phenotypes. Model performance was estimated using leave-one-out cross-validated AUC within the same TEI-eligible patient subsets for each phenotype and endpoint; the best-performing configuration within each row is highlighted. The right panel shows the AUC gain of TEI and density-plus-Q75 models relative to the density-only baseline. (B) Kaplan–Meier analyses of the final CD8+CD45RO+ TEI, dichotomized into high and low groups using the predefined median cutoff. PFS and OS were compared using log-rank tests, with risk tables shown below each curve. (C) Multivariable Cox proportional hazards models for PFS and OS, including final spatial TEI, sex, treatment modality, metastatic burden and PD-L1 TPS high/low status; COPD was not included because of missingness. Hazard ratios are shown on a log scale with 95% confidence intervals. (D) Elastic-net Cox stability and permutation analysis including selected TEI metrics and clinical covariates. Observed C-indices were compared with outcome-permuted null distributions, and bootstrap selection frequencies summarize feature stability; negative coefficients indicate association with lower hazard.

DISCUSSION

The clinical value of tissue immune biomarkers in advanced NSCLC increasingly depends on whether they can describe therapeutically relevant tumor-immune organization rather than immune-cell abundance alone. ICI efficacy is shaped by the local balance between tumor antigenicity, effector T-cell recruitment, suppressive cellular programs and physical access to malignant cells; therefore, spatially preserved tissue analysis can add biological information that is lost when immune phenotypes are measured only as bulk density or non-localized expression signals [10, 34]. This is particularly relevant in advanced NSCLC, where treatment decisions still rely heavily on PD-L1 expression despite heterogeneous clinical benefit, and where small FFPE samples often represent the only available tumor material for biomarker development [6, 9]. In this context, high-plex spatial biology provides a practical

translational opportunity: it can define multiple immune-cell phenotypes, differentiation state and tumor proximity within the same tissue section, thereby converting limited diagnostic material into a multidimensional description of tumor-immune engagement.

In the present study, we identified a consistent favorable signal centered on CD8+CD45RO+ memory T-cells. Patients with Long PFS showed higher CD8+ density, but the most reproducible difference was observed for CD8+CD45RO+ density, while CD4-related and CD45RA-related double-positive phenotypes were not significantly associated with PFS. OS analyses reproduced the same main pattern, with higher total CD45RO+ and CD8+CD45RO+ densities in long-OS patients and a concordant favorable direction for CD8+ cells. Ratio and fraction analyses supported this by showing a higher CD45RO balance within the CD8 lineage in favorable outcome groups and a higher CD8+CD45RA+ fraction among patients with shorter survival. Granzyme B-associated analyses further indicated that the favorable CD8+CD45RO+ phenotype was enriched for a cytotoxic component, linking the memory-like phenotype to a plausible effector function.

These findings are broadly consistent with the established prognostic role of T-cell infiltration in NSCLC. Earlier tissue-based studies and meta-analyses reported favorable associations for CD8+ TIL density, particularly in stromal or tumor-nest compartments, although the magnitude of effect varies across cohorts, histological subtypes, scoring systems and treatment contexts [35–39]. Kaira et al. showed that, in advanced NSCLC treated with first-line pembrolizumab, stromal CD4+ TILs were associated with PFS, while intratumoral CD8+ TILs independently predicted OS [40]. Our data are aligned with these observations, but also refine them by indicating that total CD8 density is less informative than the phenotypic identity of the CD8 compartment, particularly the enrichment of CD45RO-positive memory-like CD8+ cells. This is in line with NSCLC studies showing that clinically relevant CD8+ TIL biology depends on functional and differentiation state, including PD-1-low cytotoxic TILs, tissue-resident memory-like CD8+ populations and progenitor-like exhausted T-cell states, rather than total CD8 abundance alone [41–45]. The CD45RO signal is additionally supported by solid-tumor and lung adenocarcinoma data linking CD45RO+ memory TILs to favorable outcome [22, 46]. The main contrast with some previous reports is that CD4+ density and CD4+CD45RO+ density were not dominant favorable density biomarkers in our cohort. This may reflect endpoint differences, treatment setting, sample size, or the fact that CD4 compartments are biologically heterogeneous and include helper, regulatory and exhausted-like states that cannot be resolved by CD4/CD45RO/CD45RA markers alone.

Our proximity analyses extended the density findings by showing that immune-cell localization relative to PanCK+ tumor cells was non-random and outcome-relevant. CD8+CD45RO+ cells and total CD8+ cells were the most tumor-proximal phenotypes, whereas CD8+CD45RA+ and CD4+CD45RA+ cells were more distal. Importantly, the RO-versus-RA proximity difference was also evident when using the upper-quartile (Q75) nearest-tumor distance, indicating that CD45RO-positive subsets were more broadly shifted toward the tumor compartment rather than being defined only by a small number of highly tumor-proximal cells. To capture tumor engagement as a single interpretable biomarker, we established the tumor engagement index (TEI) metric to combine immune-cell abundance with the spatial accessibility of the corresponding phenotype to PanCK+ tumor cells. TEI integrated density and Q75 proximity and consistently improved discrimination beyond density alone for CD8+ and CD8+CD45RO+ phenotypes across PFS and OS. The final

CD8+CD45RO+ TEI separated survival curves, remained independently associated with PFS and OS after adjustment for sex, treatment modality, metastatic burden and PD-L1 status, and showed the highest stability in elastic-net Cox models.

Pre-existing CD8+ cells at the tumor margin have been linked to response to PD-1 blockade, and spatial heterogeneity of TIL subsets has been associated with PD-1-axis sensitivity in NSCLC [23, 24]. Similarly, quantitative spatial studies have shown that TIL architecture, tumor-immune cell proximity and T-cell subset interactions are associated with recurrence or survival in NSCLC [47–49]. Recent work on CD103+CD8+ tissue-resident memory cells further supports the concept that functional state and spatial distribution jointly shape prognostic information [50]. The novelty of our study lies in extending memory T-cell biology in NSCLC beyond abundance-based assessment toward a spatially resolved and clinically anchored framework of tumor immune engagement. The availability of OS data after several years of follow-up strengthens the clinical relevance of this cohort, as mature survival endpoints remain relatively uncommon in spatial biomarker studies of advanced NSCLC treated with first-line ICI-based therapy. This allowed us to test whether the CD8+CD45RO+ tumor-engagement signal extended beyond early disease-control metrics and retained prognostic relevance for long-term survival. Furthermore, a 6-month PFS cutoff was selected because prior NSCLC PD-1 biomarker studies have used durable clinical benefit, defined by objective response or stable disease lasting beyond 6 months, to capture clinically meaningful disease control that may be missed by RECIST response alone; this is also consistent with the recognized limitations of conventional RECIST assessment under immunotherapy [51, 52].

Several limitations should be considered. This was a retrospective, single-cohort study with a limited sample size, and the number of TEI-eligible patients was further constrained by phenotype-specific abundance and distance requirements. Although PFS and OS endpoints were available and clinically relevant, the analyses remain exploratory and require external validation in an independent ICI-treated advanced NSCLC cohort. Tissue microarrays enabled standardized high-plex profiling but cannot fully represent whole-tumor spatial heterogeneity, invasive-margin architecture or regional immune exclusion across the entire lesion. The phenotyping strategy intentionally prioritized reproducible rule-based CD4/CD8/CD45RO/CD45RA/Granzyme B states, but it did not fully resolve exhaustion, residency, clonality, antigen specificity, macrophage-mediated exclusion, stromal barriers or tertiary lymphoid structure context. Finally, TEI is a deliberately simple composite; its cutoffs, mathematical form and robustness across imaging platforms, segmentation pipelines and tissue sampling schemes should be tested prospectively.

CONCLUSION

In conclusion, this study identifies tumor-proximal CD8+CD45RO+ immune architecture as the dominant favorable spatial phenotype in advanced NSCLC treated with first-line ICI-based therapy. CD8+CD45RO+ density was the most consistent non-spatial immune feature, but integrating abundance with tumor proximity through TEI provided additional prognostic information and remained associated with both PFS and OS independently of routine clinical covariates and PD-L1 status. These findings support the concept that clinically informative tissue biomarkers in ICI-treated NSCLC should capture not only which immune cells are present, but whether favorable effector-memory-like T-cell compartments are positioned close enough to tumor cells to represent true tumor engagement.

DATA AVAILABILITY

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki and was approved by the competent institutional/regional ethics committee (approval number: ETT TUKÉB BM/30301-1/2023). The use of archived tumor material and retrospective clinical data was performed according to institutional and national regulations for retrospective tissue-based research. The requirement for informed consent was handled in accordance with the applicable ethical approval and local regulatory framework.

Contributions:

David Dora: Conceptualization, Investigation, Methodology, Software, Data curation, Formal analysis, Visualization, Resources, Writing – original draft. Writing – review & editing. **Ildikó Krencz:** Methodology, Software, Validation, Supervision, Writing – review & editing. **Anna Sebestyén:** Resources, Project administration, Supervision, Writing – review & editing. **Fatime Szalai:** Methodology, Data curation, Writing – review & editing. **Brigitta Roskó:** Methodology, Data curation, Visualization, Validation, Writing – review & editing. **Yahya Kamel:** Data curation, Validation, Writing – review & editing. **Réka Bujdosó:** Data curation, Validation, Writing – review & editing. **Péter Király:** Data curation, Validation, Supervision, Writing – review & editing. **Zoltan Lohinai:** Conceptualization, Resources, Supervision, Project administration, Writing – original draft. Writing – review & editing. **Gabriella Galfy:** Resources, Supervision, Project administration, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. All authors agree to submit the article for publication.

FUNDING

ZL and DD acknowledge funding from the Hungarian National Research, Development and Innovation Office (OTKA-FK, #146775 and OTKA-PD, #142287).

REFERENCES

1. Bray F, Laversanne M, Sung H, et al. Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin.* 2024;74:229–263. doi:10.3322/caac.21834.
2. Reck M, Rodríguez-Abreu D, Robinson AG, Hui R, Csőszi T, Fülöp A, et al. Five-year outcomes with pembrolizumab versus chemotherapy for metastatic non-small-cell lung

- cancer with PD-L1 tumor proportion score $\geq 50\%$. *J Clin Oncol*. 2021;39(21):2339–2349. doi:10.1200/JCO.21.00174.
3. Garassino MC, Gadgeel S, Speranza G, et al. Pembrolizumab plus pemetrexed and platinum in nonsquamous non-small-cell lung cancer: 5-year outcomes from the phase 3 KEYNOTE-189 study. *J Clin Oncol*. 2023;41:1992–1998. doi:10.1200/JCO.22.01989.
 4. Novello S, Kowalski DM, Luft A, et al. Pembrolizumab plus chemotherapy in squamous non-small-cell lung cancer: 5-year update of the phase 3 KEYNOTE-407 study. *J Clin Oncol*. 2023;41:1999–2006. doi:10.1200/JCO.22.01990.
 5. Herbst RS, Giaccone G, de Marinis F, et al. Atezolizumab for first-line treatment of PD-L1-selected patients with NSCLC. *N Engl J Med*. 2020;383:1328–1339. doi:10.1056/NEJMoa1917346.
 6. Doroshov DB, Bhalla S, Beasley MB, et al. PD-L1 as a biomarker of response to immune-checkpoint inhibitors. *Nat Rev Clin Oncol*. 2021;18:345–362. doi:10.1038/s41571-021-00473-5.
 7. Sholl LM, Hirsch FR, Hwang D, et al. Biomarkers of response to checkpoint inhibitors beyond PD-L1 in lung cancer. *Mod Pathol*. 2022;35(Suppl 1):66–74.
 8. Penault-Llorca F, Kerr KM, Garrido P, et al. Expert opinion on NSCLC small specimen biomarker testing. *Virchows Arch*. 2022;481:335–350.
 9. Kerr KM, Bibeau F, Thunnissen E, et al. Optimizing tissue stewardship in non-small cell lung cancer. *Histopathology*. 2024. doi:10.1111/his.15078.
 10. Binnewies M, Roberts EW, Kersten K, Chan V, Fearon DF, Merad M, et al. Understanding the tumor immune microenvironment (TIME) for effective therapy. *Nat Med*. 2018;24(5):541–550. doi:10.1038/s41591-018-0014-x.
 11. Fridman WH, Pagès F, Sautès-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer*. 2012;12:298–306. doi:10.1038/nrc3245.
 12. Galon J, Bruni D. Approaches to treat immune hot, altered and cold tumours with combination immunotherapies. *Nat Rev Drug Discov*. 2019;18:197–218. doi:10.1038/s41573-018-0007-y.
 13. Fu T, Dai LJ, Wu SY, et al. Spatial architecture of the immune microenvironment orchestrates tumor immunity and therapeutic response. *J Hematol Oncol*. 2021;14:98. doi:10.1186/s13045-021-01103-4.
 14. Bremnes RM, Busund LT, Kilvær TL, et al. The role of tumor-infiltrating lymphocytes in development, progression, and prognosis of non-small cell lung cancer. *J Thorac Oncol*. 2016;11:789–800. doi:10.1016/j.jtho.2016.01.015.
 15. Schalper KA, Brown J, Carvajal-Hausdorf D, McLaughlin J, Velcheti V, Syrigos KN, et al. Objective measurement and clinical significance of TILs in non-small cell lung cancer. *J Natl Cancer Inst*. 2015;107(3):dju435. doi:10.1093/jnci/dju435.
 16. Thommen DS, Koelzer VH, Herzig P, Roller A, Trefny M, Dimeloe S, et al. A transcriptionally and functionally distinct PD-1+ CD8+ T cell pool with predictive potential in non-small-cell lung cancer treated with PD-1 blockade. *Nat Med*. 2018;24(7):994–1004. doi:10.1038/s41591-018-0057-z.
 17. Sallusto F, Lenig D, Förster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature*. 1999;401:708–712. doi:10.1038/44385.
 18. Farber DL, Yudanin NA, Restifo NP. Human memory T cells: generation, compartmentalization and homeostasis. *Nat Rev Immunol*. 2014;14:24–35. doi:10.1038/nri3567.

19. Jameson SC, Masopust D. Understanding subset diversity in T cell memory. *Immunity*. 2018;48:214–226. doi:10.1016/j.immuni.2018.02.010.
20. Sallusto F, Lanzavecchia A, Araki K, Ahmed R. From vaccines to memory and back. *Immunity*. 2010;33(4):451–463. doi:10.1016/j.immuni.2010.10.008.
21. Pagès F, Berger A, Camus M, Sanchez-Cabo F, Costes A, Molitor R, et al. Effector memory T cells, early metastasis, and survival in colorectal cancer. *N Engl J Med*. 2005;353(25):2654–2666. doi:10.1056/NEJMoa051424.
22. Hu G, Wang S. Tumor-infiltrating CD45RO+ memory T lymphocytes predict favorable clinical outcome in solid tumors. *Sci Rep*. 2017;7:10376. doi:10.1038/s41598-017-11122-2.
23. Tumeh PC, Harview CL, Yearley JH, et al. PD-1 blockade induces responses by inhibiting adaptive immune resistance. *Nature*. 2014;515:568–571. doi:10.1038/nature13954.
24. Lopez de Rodas M, Nagineni V, Ravi A, Datar IJ, Mino-Kenudson M, Corredor G, et al. Role of tumor infiltrating lymphocytes and spatial immune heterogeneity in sensitivity to PD-1 axis blockers in non-small cell lung cancer. *J Immunother Cancer*. 2022;10(6):e004440. doi:10.1136/jitc-2021-004440.
25. Peng H, Wang J, Li J, et al. Profiling tumor immune microenvironment of non-small cell lung cancer using multiplex immunofluorescence. *Front Immunol*. 2021;12:750046. doi:10.3389/fimmu.2021.750046.
26. Yang G, Cai S, Hu M, et al. Functional status and spatial architecture of tumor-infiltrating CD8+ T cells are associated with lymph node metastases in non-small cell lung cancer. *J Transl Med*. 2023;21:320. doi:10.1186/s12967-023-04154-y.
27. Dora D, Ligeti B, Kovacs T, Revisnyei P, Galffy G, Dulka E, et al. Non-small cell lung cancer patients treated with anti-PD1 immunotherapy show distinct microbial signatures and metabolic pathways according to progression-free survival and PD-L1 status. *Oncoimmunology*. 2023;12(1):2204746. doi:10.1080/2162402X.2023.2204746.
28. Lin JR, Izar B, Wang S, Yapp C, Mei S, Shah PM, et al. Highly multiplexed immunofluorescence imaging of human tissues and tumors using t-CyCIF and conventional optical microscopes. *eLife*. 2018;7:e31657. doi:10.7554/eLife.31657.
29. Bruker Spatial Biology. Introducing enhanced photobleaching in cyclic immunofluorescence for spatial biology. Published October 28, 2024. <https://brukerspatialbiology.com/blog/introducing-epicif/>. Accessed 23 July 2026.
30. Bankhead P, Loughrey MB, Fernández JA, Dombrowski Y, McArt DG, Dunne PD, et al. QuPath: open source software for digital pathology image analysis. *Sci Rep*. 2017;7(1):16878. doi:10.1038/s41598-017-17204-5.
31. Dora D, Revisnyei P, Pasic A, Galffy G, Dulka E, Mihucz A, et al. Host and bacterial urine proteomics might predict treatment outcomes for immunotherapy in advanced non-small cell lung cancer patients. *Front Immunol*. 2025;16:1543817. doi:10.3389/fimmu.2025.1543817.
32. Gandhi L, Rodríguez-Abreu D, Gadgeel S, Esteban E, Felip E, De Angelis F, et al. Pembrolizumab plus chemotherapy in metastatic non-small-cell lung cancer. *N Engl J Med*. 2018;378(22):2078–2092. doi:10.1056/NEJMoa1801005.
33. Paz-Ares L, Luft A, Vicente D, Tafreshi A, Gümüş M, Mazières J, et al. Pembrolizumab plus chemotherapy for squamous non-small-cell lung cancer. *N Engl J Med*. 2018;379(21):2040–2051. doi:10.1056/NEJMoa1810865.
34. Hegde PS, Chen DS. Top 10 challenges in cancer immunotherapy. *Immunity*. 2020;52(1):17–35. doi:10.1016/j.immuni.2019.12.011.

35. Al-Shibli KI, Donnem T, Al-Saad S, Persson M, Bremnes RM, Busund LT. Prognostic effect of epithelial and stromal lymphocyte infiltration in non-small cell lung cancer. *Clin Cancer Res.* 2008;14(16):5220–5227. doi:10.1158/1078-0432.CCR-08-0133.
36. Donnem T, Hald SM, Paulsen EE, Richardsen E, Al-Saad S, Kilvaer TK, et al. Stromal CD8+ T-cell density—a promising supplement to TNM staging in non-small cell lung cancer. *Clin Cancer Res.* 2015;21(11):2635–2643. doi:10.1158/1078-0432.CCR-14-1905.
37. Geng Y, Shao Y, He W, Hu W, Xu Y, Chen J, et al. Prognostic role of tumor-infiltrating lymphocytes in lung cancer: a meta-analysis. *Cell Physiol Biochem.* 2015;37(4):1560–1571. doi:10.1159/000438523.
38. Zeng DQ, Yu YF, Ou QY, Li XY, Zhong RZ, Xie CM, et al. Prognostic and predictive value of tumor-infiltrating lymphocytes for clinical therapeutic research in patients with non-small cell lung cancer. *Oncotarget.* 2016;7(12):13765–13781. doi:10.18632/oncotarget.7282.
39. Chen B, Li H, Liu C, Xiang X, Wang S, Wu A, et al. Prognostic value of the common tumour-infiltrating lymphocyte subtypes for patients with non-small cell lung cancer: a meta-analysis. *PLoS One.* 2020;15(11):e0242173. doi:10.1371/journal.pone.0242173.
40. Kaira K, Yamaguchi O, Kawasaki T, Hashimoto K, Miura Y, Shiono A, et al. Prognostic significance of tumor infiltrating lymphocytes on first-line pembrolizumab efficacy in advanced non-small cell lung cancer. *Discov Oncol.* 2023;14:6. doi:10.1007/s12672-023-00615-4.
41. Mazzaschi G, Madeddu D, Falco A, Bocchialini G, Goldoni M, Sogni F, et al. Low PD-1 expression in cytotoxic CD8+ tumor-infiltrating lymphocytes confers an immune-privileged tissue microenvironment in NSCLC with a prognostic and predictive value. *Clin Cancer Res.* 2018;24(2):407–419. doi:10.1158/1078-0432.CCR-17-2156.
42. Djenidi F, Adam J, Goubar A, Durgeau A, Meurice G, de Montpreville V, et al. CD8+CD103+ tumor-infiltrating lymphocytes are tumor-specific tissue-resident memory T cells and a prognostic factor for survival in lung cancer patients. *J Immunol.* 2015;194(7):3475–3486. doi:10.4049/jimmunol.1402711.
43. Corgnac S, Malenica I, Mezquita L, Auclin E, Voilin E, Kacher J, et al. CD103+CD8+ TRM cells accumulate in tumors of anti-PD-1-responder lung cancer patients and are tumor-reactive lymphocytes enriched with Tc17. *Cell Rep Med.* 2020;1(7):100127. doi:10.1016/j.xcrm.2020.100127.
44. Gueguen P, Metoikidou C, Dupic T, Lawand M, Goudot C, Baulande S, et al. Contribution of resident and circulating precursors to tumor-infiltrating CD8+ T cell populations in lung cancer. *Sci Immunol.* 2021;6(55):eabd5778. doi:10.1126/sciimmunol.abd5778.
45. Koh J, Kim S, Woo YD, Song S, Yim J, Han B, et al. TCF1+PD-1+ tumour-infiltrating lymphocytes predict a favorable response and prolonged survival after immune checkpoint inhibitor therapy for non-small-cell lung cancer. *Eur J Cancer.* 2022;174:10–20. doi:10.1016/j.ejca.2022.07.004.
46. Hu Z, Gu X, Zhong R, Zhong H. Tumor-infiltrating CD45RO+ memory cells correlate with favorable prognosis in patients with lung adenocarcinoma. *J Thorac Dis.* 2018;10(4):2089–2099. doi:10.21037/jtd.2018.03.148.
47. Barua S, Fang P, Sharma A, Fujimoto J, Wistuba I, Rao AUK, et al. Spatial interaction of tumor cells and regulatory T cells correlates with survival in non-small cell lung cancer. *Lung Cancer.* 2018;117:73–79. doi:10.1016/j.lungcan.2018.01.022.

48. Corredor G, Wang X, Zhou Y, Lu C, Fu P, Syrigos K, et al. Spatial architecture and arrangement of tumor-infiltrating lymphocytes for predicting likelihood of recurrence in early-stage non-small cell lung cancer. *Clin Cancer Res.* 2019;25(5):1526–1534. doi:10.1158/1078-0432.CCR-18-2013.
49. Yang L, Cai S, Hu M, Li C, Yang G, Zhang W, et al. Functional status and spatial interaction of T cell subsets driven by specific tumor microenvironment correlate with recurrence of non-small cell lung cancer. *Front Immunol.* 2023;13:1022638. doi:10.3389/fimmu.2022.1022638.
50. Yang G, Cai S, Hu M, Li C, Yang L, Zhang W, et al. Spatial features of specific CD103+CD8+ tissue-resident memory T cell subsets define the prognosis in patients with non-small cell lung cancer. *J Transl Med.* 2024;22:27. doi:10.1186/s12967-023-04839-4.
51. Rizvi NA, Hellmann MD, Snyder A, Kvistborg P, Makarov V, Havel JJ, et al. 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.
52. Seymour L, Bogaerts J, Perrone A, Ford R, Schwartz LH, Mandrekar S, et al. iRECIST: guidelines for response criteria for use in trials testing immunotherapeutics. *Lancet Oncol.* 2017;18(3):e143–e152. doi:10.1016/S1470-2045(17)30074-8.

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