

# Phenotype of Circulating Tumor-Reactive T Cells Predicts Immune Checkpoint Inhibitor Response in Non-Small Cell Lung Cancer

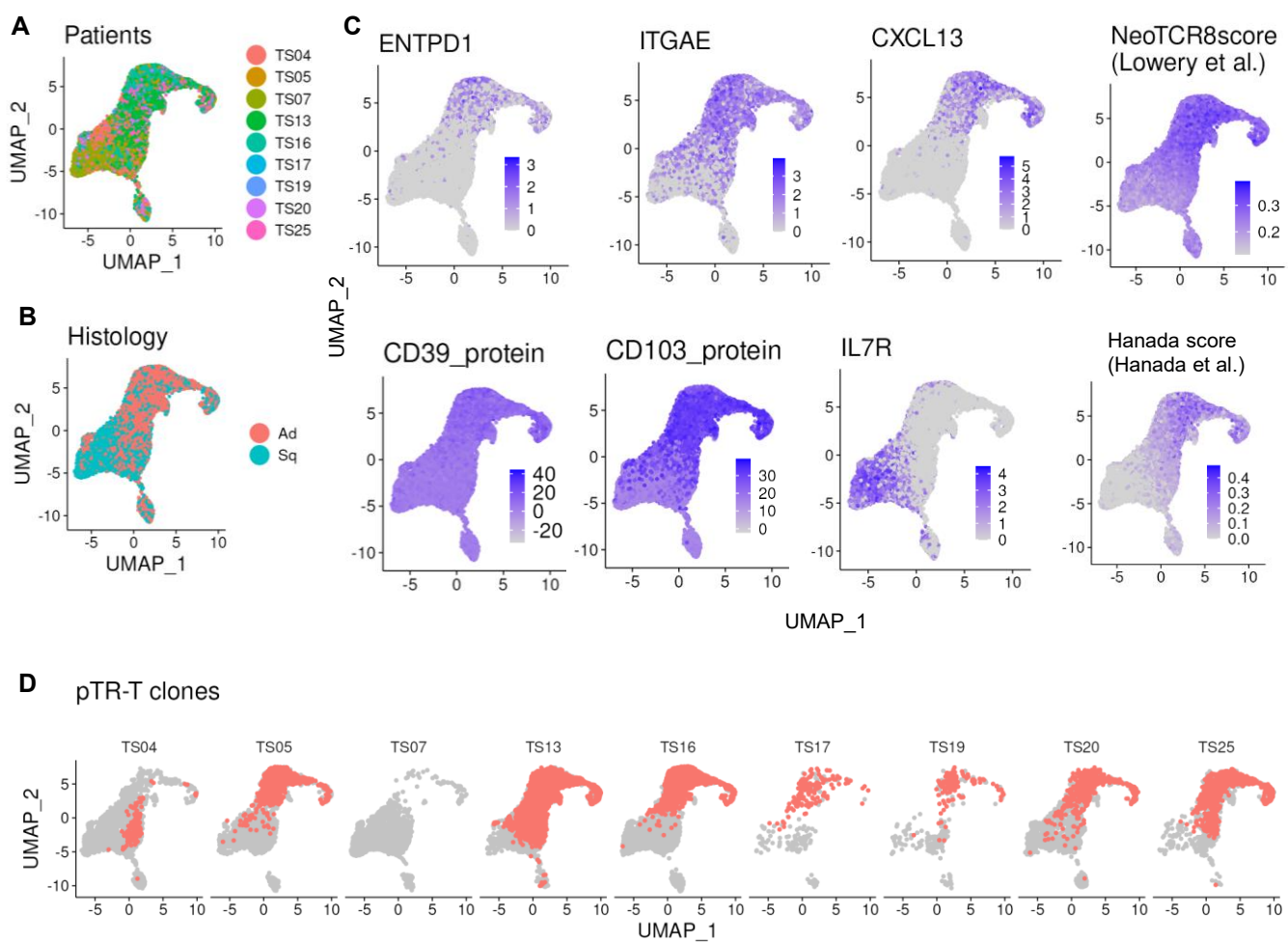
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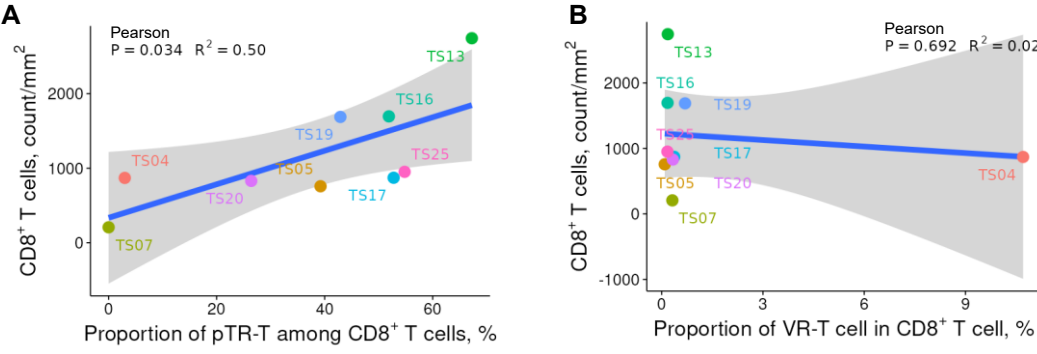
### Supplementary Figure 1. Detection of TR-Ts from high-resolution single-cell analysis

(A) UMAP plot of CD8<sup>+</sup> TILs from patients with NSCLC group by patients and (B) histological subtype (Ad: adenocarcinoma; Sq: squamous cell carcinoma).

(C) Expression of previously reported marker genes (*ENTPD1*, *CXCL13*, and *ITGAE*), marker proteins (CD39 and CD103), and gene signatures (Lowery et al.<sup>23</sup> and Hanada et al.<sup>26</sup>) predictive of tumor-reactive T cells (pTR-Ts). *ENTPD1* encodes CD39, and *ITGAE* encodes CD103.

(D) Distribution of pTR-Ts in TILs stratified by patients

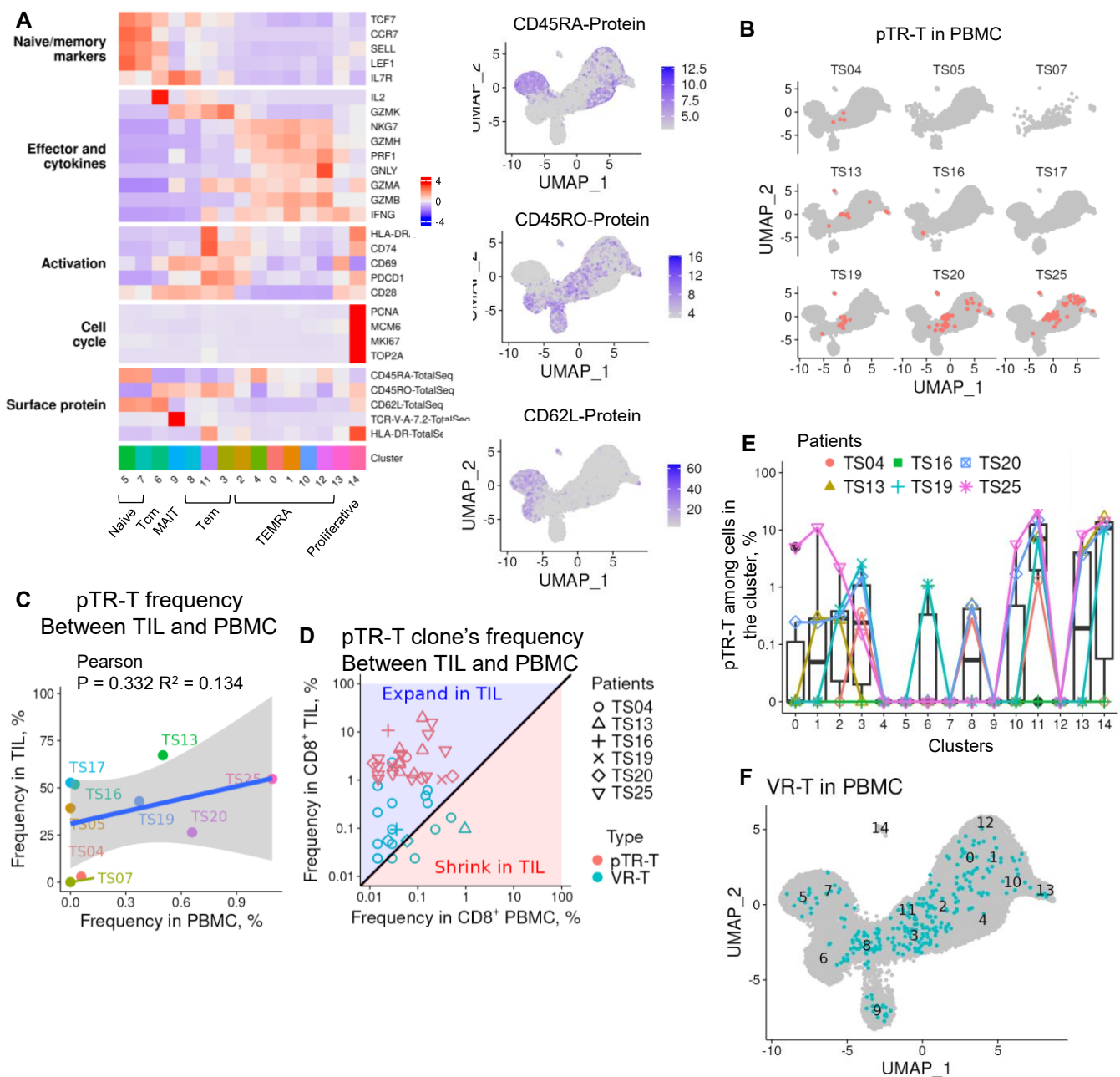
UMAP: uniform manifold approximation and projection; TILs: tumor-infiltrating lymphocyte; Sq: squamous cell carcinoma; Ad: adenocarcinoma.



**Supplementary Figure 2. Correlation between proportion of pTR-Ts and VR-T and CD8<sup>+</sup> T cell infiltration in TILs**

(A) Proportion of pTR-Ts among total CD8<sup>+</sup> TILs is positively associated with total CD8<sup>+</sup> T cell infiltration in TILs assessed by pathology specimens.

(B) Proportion of viral-reactive T cells (VR-Ts) among total CD8<sup>+</sup> TILs is not correlated with total CD8<sup>+</sup> T cell infiltration in TILs assessed by pathology specimens.



### Supplementary Figure 3. Detection of pTR-Ts in PBMCs

(A) Heatmap of cluster-defining gene and surface protein expression (left). Expression of cluster-defining proteins on UMAP (right). MAIT, mucosal-associated invariant T cell; Tcm, central memory T cell; Tem, effector memory T cell; TEMRA, terminally differentiated effector memory T cells re-expressing CD45RA

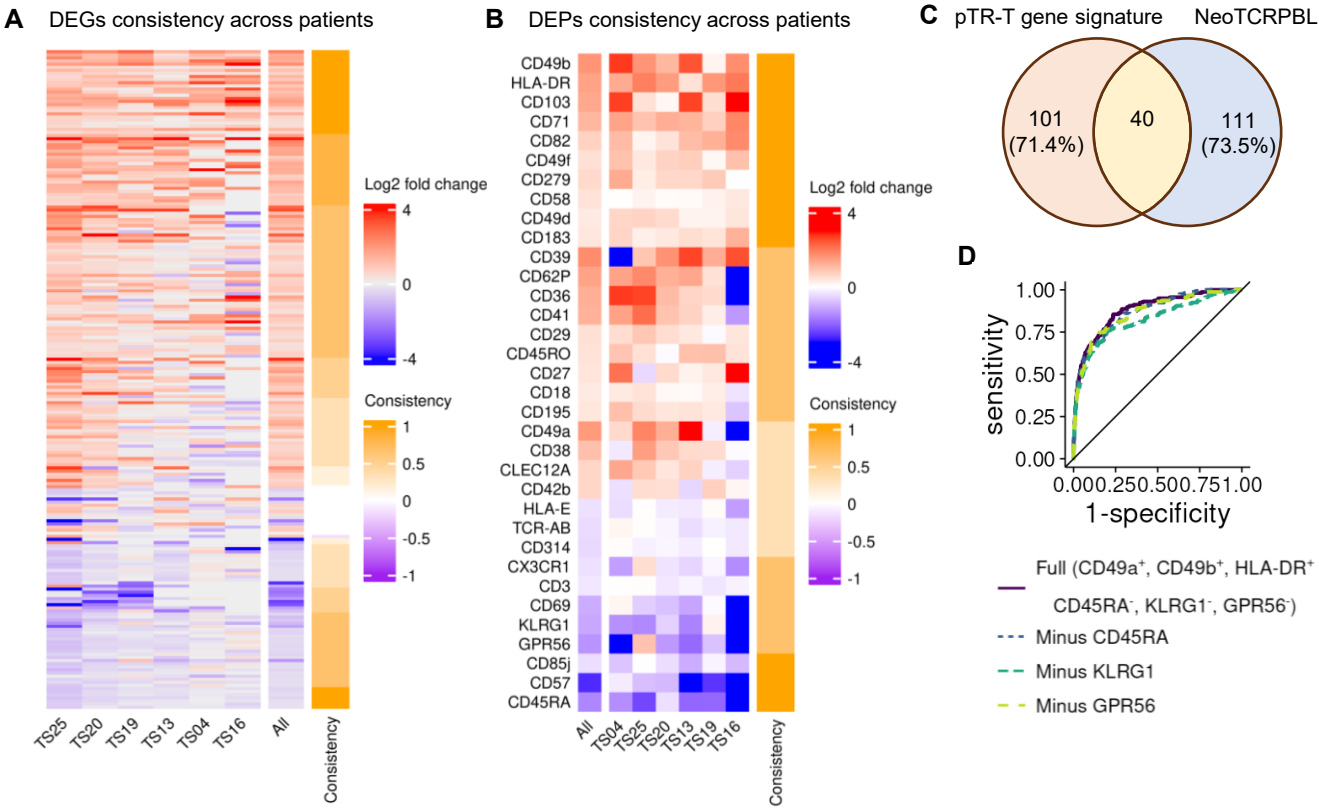
(B) Distribution of pTR-Ts in PBMCs stratified by patients

(C) Comparison of the frequency of pTR-Ts in TIL and PBMC

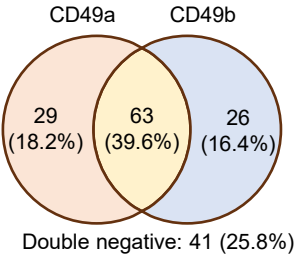
(D) pTR-T and VR-T clone frequency between PBMC and TIL. Only clones detected in both TIL and PBMC were plotted

(E) For peripheral CD8<sup>+</sup> T cell analysis, the proportions of pTR-Ts in each cluster were plotted, stratified by patient

(F) Circulating VR-Ts mapped onto the UMAP of peripheral CD8<sup>+</sup> T cells.



**E** CD49a/b expression in pTR-T



**Supplementary Figure 4. Establishment of the pTR-T gene signature and TRTPBL signature**

(A) Differentially expressed genes between pTR-Ts and other CD8+ T cells across patients. “All” represents results aggregated from all patient samples, which correspond to the cell-level analysis shown in Figure 4D. Consistency was evaluated based on the direction of log<sub>2</sub> fold change, where a value of 1 indicates that the gene was regulated in the same direction (either up or down) across all patients (see Methods).

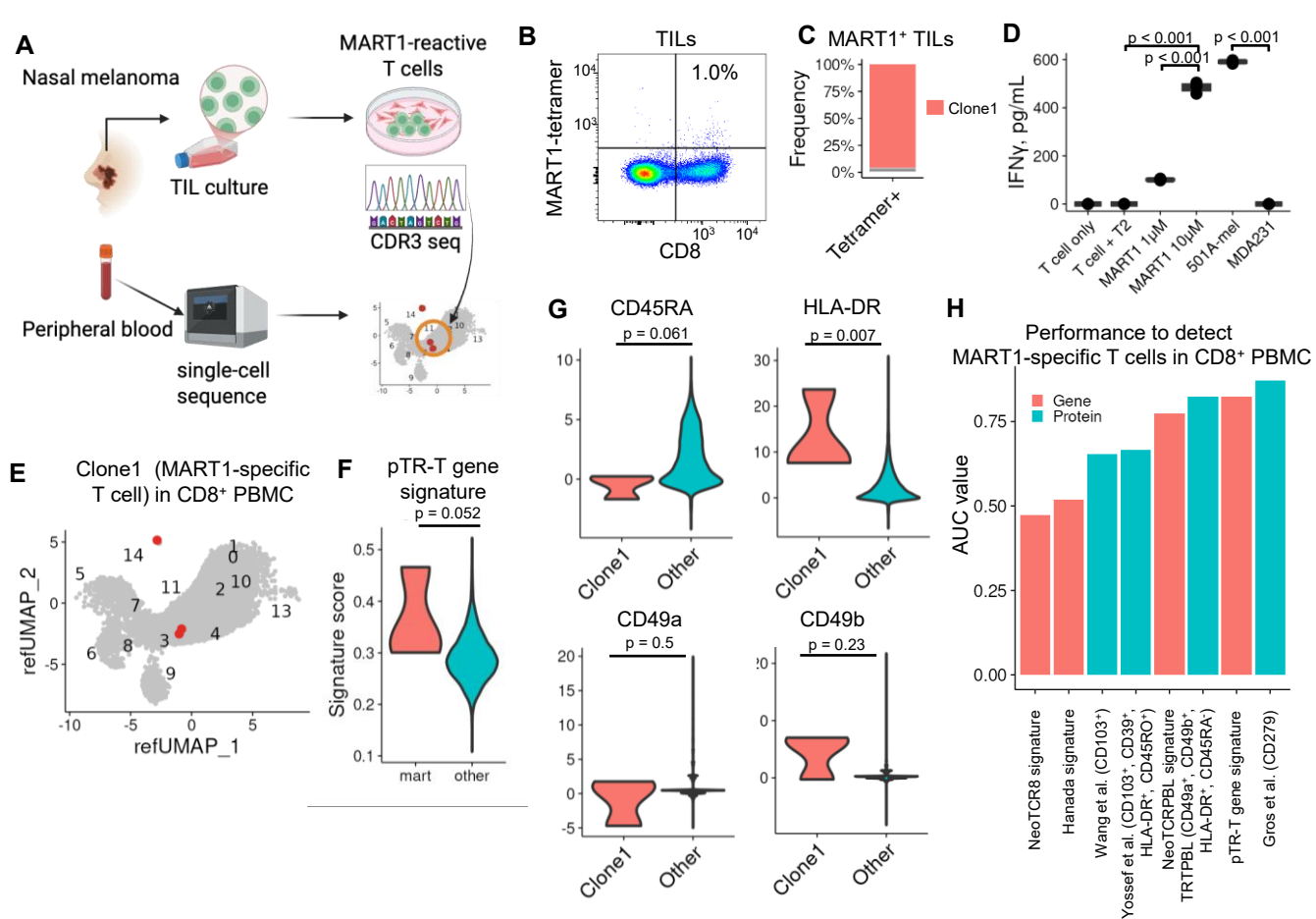
(B) Differentially expressed proteins between pTR-Ts and other CD8+ T cells across patients. “All” represents results aggregated from all patient samples, which correspond to the cell-level analysis shown in Figure 4E. Consistency was calculated using the same method described in Supplementary Figure 4A.

(C) Shared and distinct genes between the pTR-T gene signature and the NeoTCRPBL signature<sup>16</sup>.

(D) ROC analysis for detecting circulating pTR-Ts based on protein marker combinations. The full marker set including CD49a, CD49b, HLA-DR, CD45RA, GPR56 and KLRG1 is shown as a red line. Combinations excluding CD45RA, KLRG1, and GPR56 are shown as green, blue, and purple dashed lines, respectively.

(E) Expression of CD49a and CD49b in pTR-Ts (159 cells)

DEG: differentially expressed gene; DEP: differentially expressed protein; ROC: receiver operating characteristic



## Supplementary Figure 5. Validation of the TRTPBL marker in a melanoma patient

(A) TILs from a melanoma patient were cultured with the MART1 peptide to establish a MART1-specific clone line. In parallel, CD8<sup>+</sup> TILs and PBMCs from the same patient were subjected to single-cell sequencing. Created in BioRender. Ito, K. (2025) <https://BioRender.com/jssrzfr>

(B) Frequency of MART1 tetramer-positive cells in the fresh tumor digest

(C) Single-cell TCR sequencing of MART1 tetramer-positive TILs reveals single clone (Clone1) accounted for more than 95% of the population

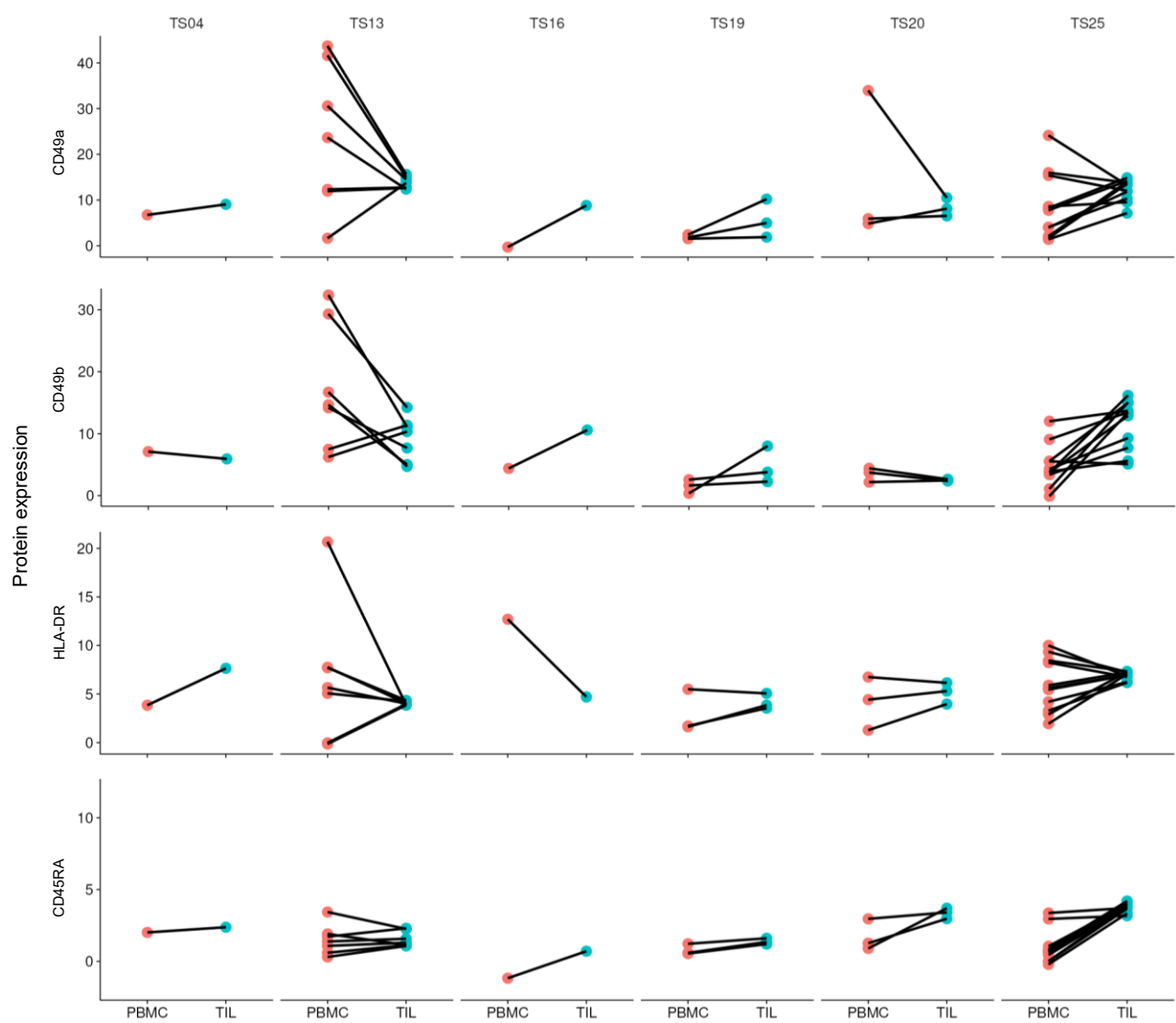
(D) MART1 specific IFN- $\gamma$  production from Clone1. Clone 1 was cocultured for 24 hours with T2 cells pulsed with HLA-A\*0201-restricted MART1 peptide (MART1 1  $\mu$ M and 10  $\mu$ M) or a MART-1 expressing melanoma cell line (501Amel). IFN- $\gamma$  levels in the supernatants were measured by ELISA. Negative controls included Clone 1 alone (T cell only), Clone 1 with unpulsed T2 cells (T cell + T2) and Clone 1 with a MART1 negative cell line (MDA231). (technical replicates,  $n = 3$ , two-sided unpaired  $t$ -test)

(E) Clone1 was projected onto in UMAP of CD8<sup>+</sup> PBMC. Clone1 was located within the cluster 3 and 14.

(F) pTR-T signature scores (Figure 2D and G) of Clone 1 and other CD8<sup>+</sup> T cells in PBMCs (two-sided unpaired Wilcoxon rank-sum test)

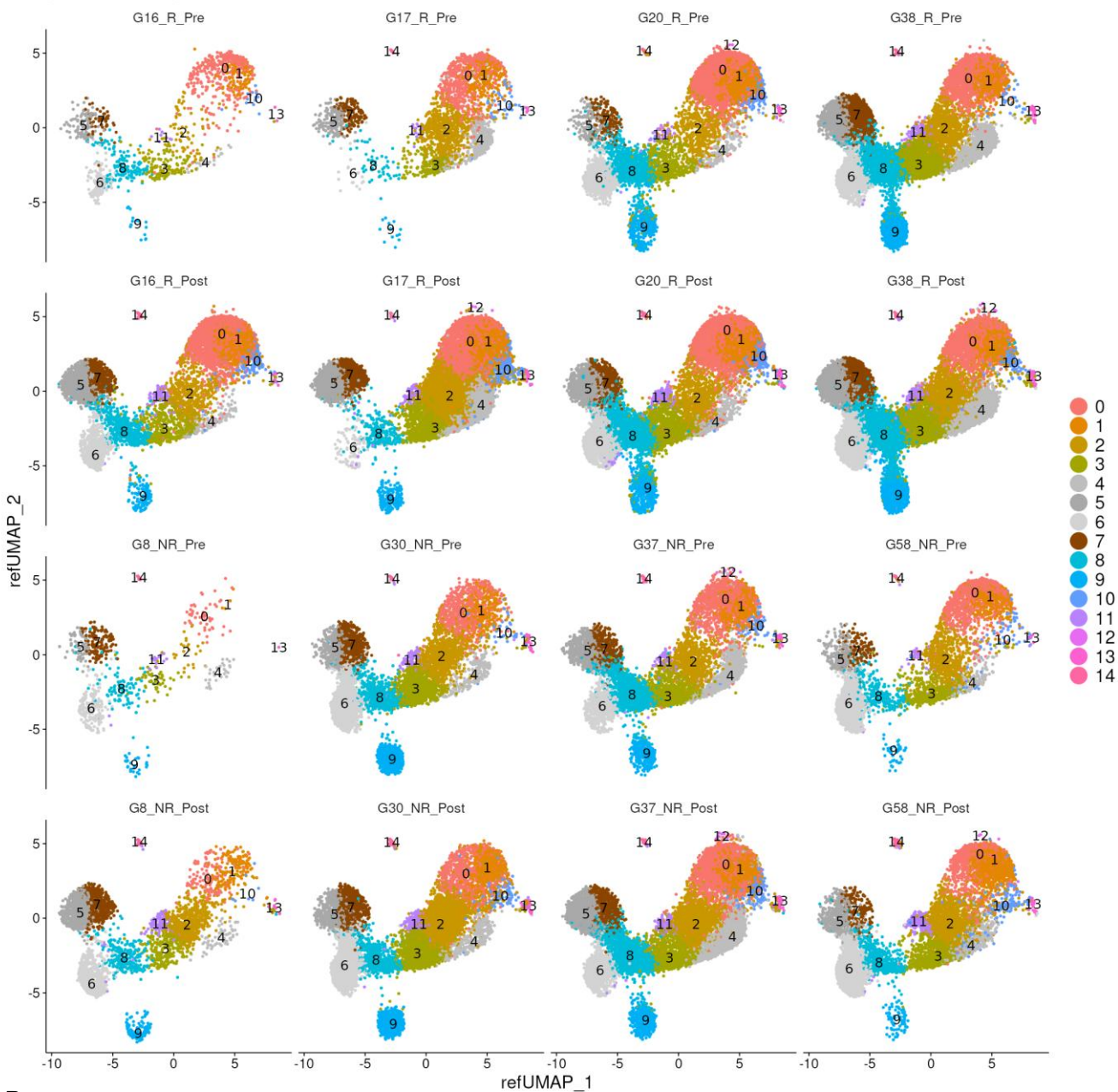
(G) Expression of CD45RA, HLA-DR, CD49a, and CD49b on Clone1 and other CD8<sup>+</sup> T cells in PBMC (two-sided unpaired Wilcoxon rank-sum test)

(H) Performance comparison of individual markers for detecting MART1-specific T cells within total CD8<sup>+</sup> T cells in PBMCs. AUC values are shown.

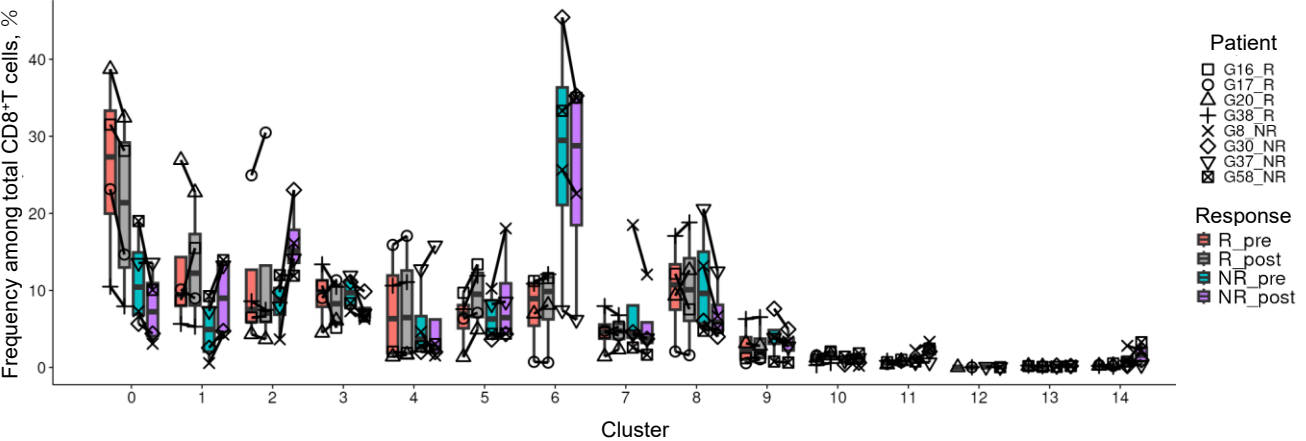


**Supplementary Figure 6. TRTPBL markers between pTR-Ts in PBMCs and TILs**  
Averaged clone-level protein expression of TRTPBL markers (CD49a, CD49b, HLA-DR, and CD45RA) between pTR-Ts in PBMCs and those in TILs stratified by patient. Each dot and line represents a clonotype.

**A** predicted.soup\_snn\_res1



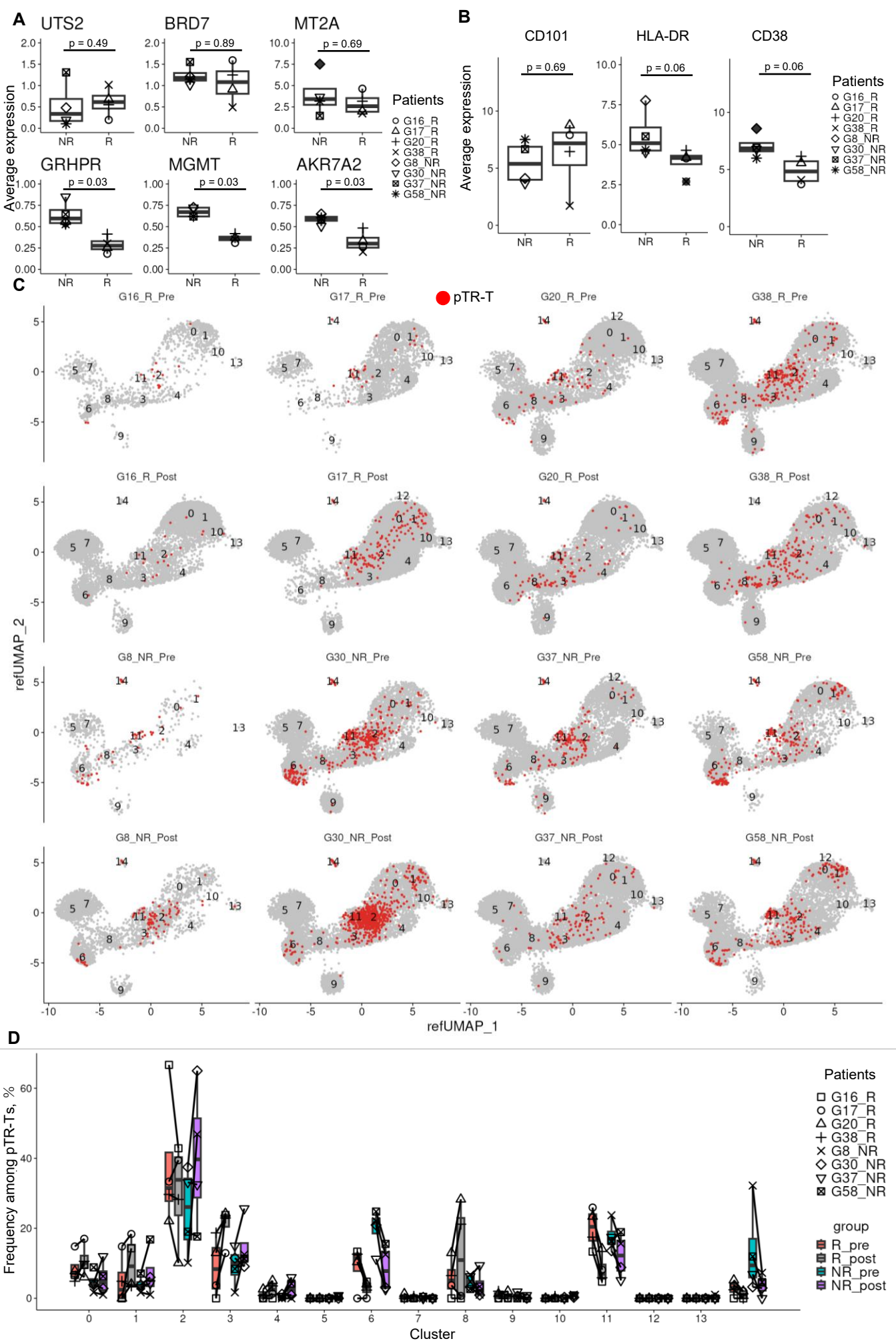
**B**



**Supplementary Figure 7. Single cell analysis from 8 patients who received ICIs**

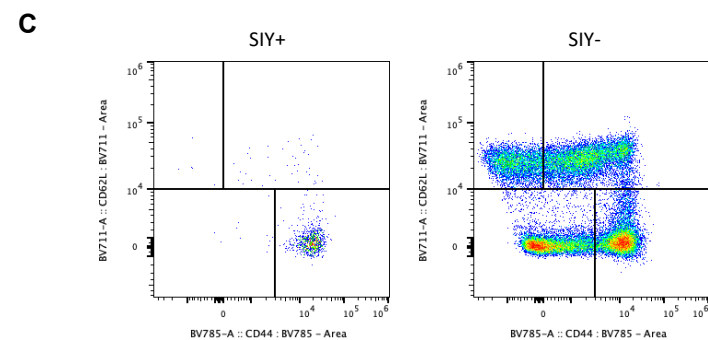
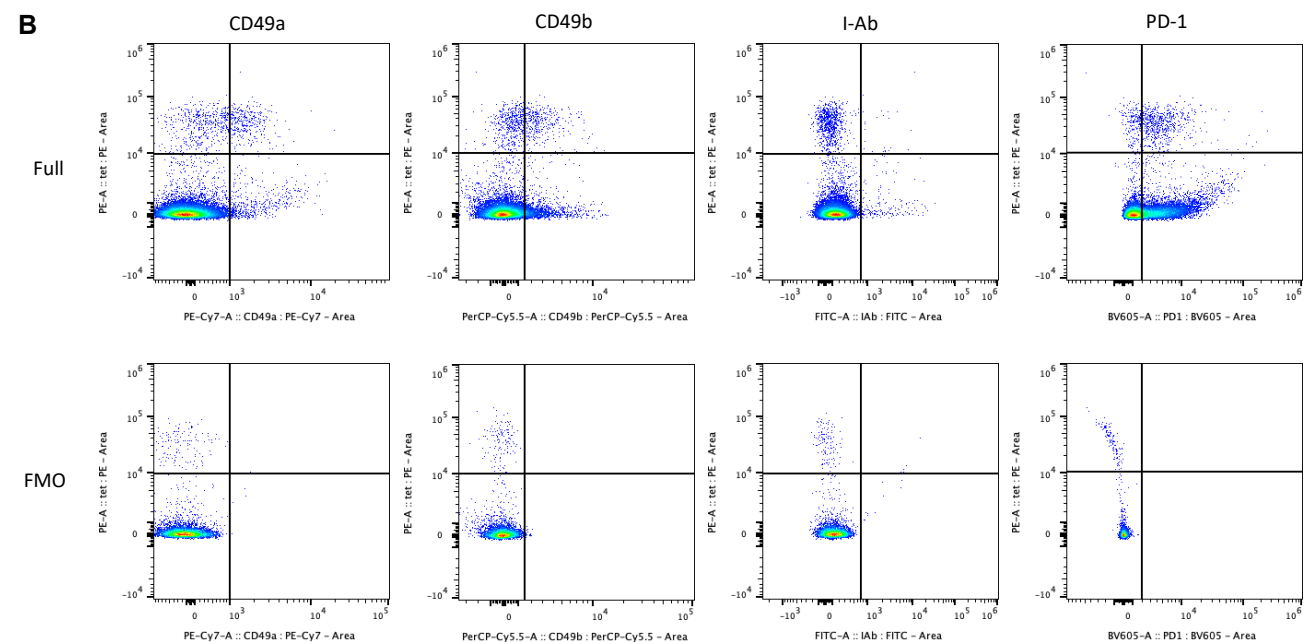
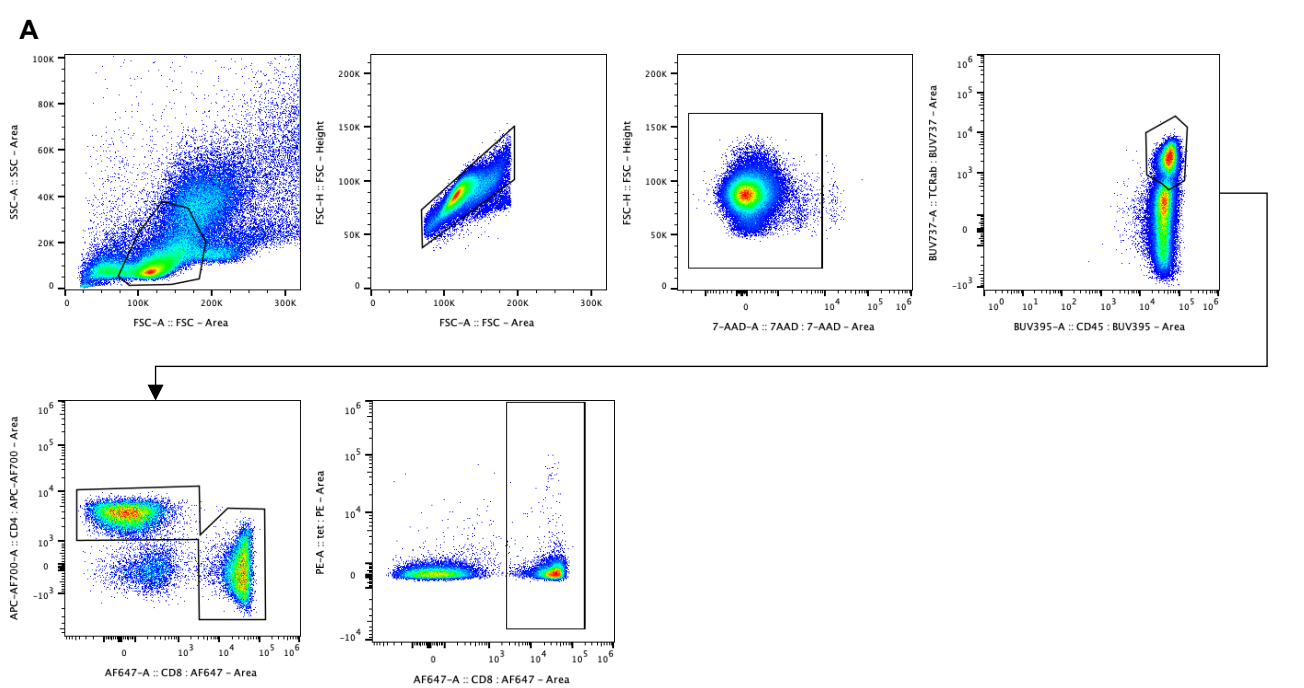
(A) Pre and post treatment single-cell CD8<sup>+</sup> PBMCs from 8 patients who received immune-checkpoint therapy were projected onto the UMAP image (Figure 2A)

(B) Distribution of total CD8<sup>+</sup> T cells across clusters in each patient, shown separately by treatment phase (pre- and post-treatment) and response status. In the box plots, the center line indicates the median; the box represents the interquartile range (25th–75th percentiles); whiskers extend to the most extreme values within  $1.5 \times$  the interquartile range.



## **Supplementary Figure 8. Expression and differentiation of pTR-Ts between responder and non-responder**

- (A) Patient-level expression of differentially expressed genes (Figure 4C) between responders' and non-responders' pTR-Ts in pre-treatment samples (unpaired two-sided Wilcoxon rank-sum test).
- (B) Patient-level expression of differentially expressed proteins (Figure 4D) between responders' and non-responders' pTR-Ts in pre-treatment samples (unpaired two-sided Wilcoxon rank-sum test).
- (C) Patient-level pTR-T distribution shown separately by treatment phase (pre- and post-treatment)
- (D) Distribution of pTR-T cells across clusters in each patient, shown separately by treatment phase and response status. The proportion of pTR-T cells within each cluster is displayed for individual patients. In the box plots, the center line indicates the median; the box represents the interquartile range (25th–75th percentiles); whiskers extend to the most extreme values within  $1.5 \times$  the interquartile range.

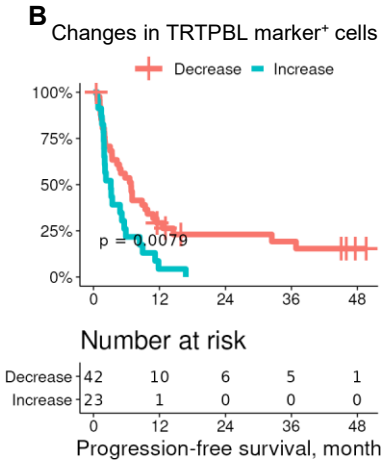
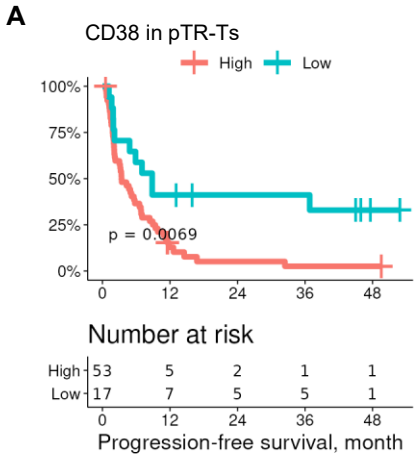


**Supplementary Figure 9. Gating strategies used for mouse PBMCs.**

(A) Gating strategy to analyze CD8<sup>+</sup> T cells in PBMCs from C57BL/6N mice inoculated with a murine melanoma cell line expressing the artificial antigen SIY (Bpmel-1-SIY), treated with anti-PD-L1 antibody (80 µg/mouse) on day 7, and sacrificed on day 11

(B) Gating strategy to detect marker-expressed cells in SIY tetramer-positive cells (top). Fluorescent minus one samples (bottom) were used to determine cut-off values, related to Figure 5B

(C) Gating strategy to determine naïve (CD62L<sup>+</sup>, CD44<sup>-</sup>), central memory (CD62L<sup>+</sup>, CD44<sup>+</sup>), and effector memory (CD62L<sup>-</sup>, CD44<sup>+</sup>) cells in SIY tetramer-positive cells (left) and negative cells (right)



**Supplementary Figure 10**

(A) Progression-free survival stratified by the proportion of CD38<sup>+</sup> cells in TRTPBL marker<sup>+</sup> CD8<sup>+</sup> T cells (log-rank test)

(B) Progression-free survival stratified by changes in TRTPBL marker<sup>+</sup> CD8<sup>+</sup> T cells (log-rank test)

**Supplementary Table 1. Patient characteristics of paired PBMC and TILs cohort, related to Figure 1**

| Patient ID | Sex | Age | Histology      | Smoking status   | Driver mutation    | PDL1 TPS | Clinical stage        | Pathological stage | Number of cells (TIL) | Number of cells (PBMC) |
|------------|-----|-----|----------------|------------------|--------------------|----------|-----------------------|--------------------|-----------------------|------------------------|
| TS4        | M   | 71  | Squamous       | Smoker/Ex-smoker | No                 | NA       | cT2aN0M0<br>StageIB   | pT2aN0             | 4,212                 | 6,895                  |
| TS5        | M   | 71  | Squamous       | Smoker/Ex-smoker | No                 | 1-49%    | cT1cN0M0<br>StageIA3  | pT1cN1             | 2,243                 | 3,368                  |
| TS7        | M   | 75  | Squamous       | Smoker/Ex-smoker | No                 | 1-49%    | cT2aN0M0<br>StageIB   | pT2aN1             | 5,390                 | 200                    |
| TS13       | M   | 69  | Adenocarcinoma | Smoker/Ex-smoker | No<br>KRAS         | ≥50%     | cT2bN0M0<br>stage IIA | pT3N0              | 7,181                 | 2,396                  |
| TS16       | M   | 79  | Adenocarcinoma | Non-smoker       | p.Gly12Ala         | <1%      | cT2aN0M0<br>StageIB   | pT1cN0             | 6,335                 | 8,323                  |
| TS17       | F   | 76  | Squamous       | Smoker/Ex-smoker | No                 | ≥50%     | cT1bN0M0<br>StageIA2  | pT1bN0             | 271                   | 6,890                  |
| TS19       | M   | 54  | Adenocarcinoma | Non-smoker       | p.Gly12Cys<br>KRAS | ≥50%     | cT1cN0M0<br>StageIA3  | pT1cN0             | 289                   | 3,483                  |
| TS20       | M   | 64  | Adenocarcinoma | Smoker/Ex-smoker | KRAS<br>p.Gln61His | 1-49%    | cT1bN0M0<br>StageIA2  | pT1aN0             | 1,818                 | 8,342                  |
| TS25       | M   | 53  | Squamous       | Smoker/Ex-smoker | No                 | 1-49%    | cT3N1M0<br>StageIIIA  | pT3N0              | 1,770                 | 6,675                  |

**Supplementary Table 2. single cell TCR sequence of MART1 tetramer-positive CD8+ TILs, related to supplementary Figure 4C**

| Clone   | CDR3 sequence                                                                                                                                | Cell count |
|---------|----------------------------------------------------------------------------------------------------------------------------------------------|------------|
| Clone1  | TRB:TGTGCCAGCAGTTTAGCCGGGACTTTAGACTACGAGCAGTACTTC;TRA:TGTGCAGAGGGGGCAGGGCGGTACCTCAGGAACCTACAAATACATCTTT;TRA:TGTGCGTGAACGATGCAGGCAAATCAACCTTT | 402        |
| Clone2  | TRB:TGTGCCAGCAGCCAAAGCTTCAGGGGAGGGCTACAATGAGCAGTTCTTC;TRA:TGTGCAATGAGAGACTTAAATGCTGGTGGTACTAGCTATGGAAAGCTGACATTT                             | 4          |
| Clone3  | TRB:TGTGCCCCACCGGGTCTAGCGGAACTTACGAGCAGTACTTC;TRA:TGTGCAGCAGTCCATACGGGGGACAGGGAACCGAGCAGTACTTC;TRA:TGTGCATCGGGGTAGGCTTTGGGAATGTGCTGCATTGC    | 2          |
| Clone4  | TRB:TGCAGTGCAACAACCGGGAACTATGGCTACACCTTC;TRA:TGTGCTACCTATGGGGTATCTAACTTTGGAAATGAGAAATTAACCTTT                                                | 1          |
| Clone5  | TRA:TGTGCCGTGAACATAAGGATGGACATGCGCTTT                                                                                                        | 1          |
| Clone6  | TRB:TGTGCCACCGGGACAGGGGGTGGCTACACCTTC;TRA:TGTGCAGCAAAAGGAACCGGCACTGCCAGTAACTCACCTTT                                                          | 1          |
| Clone8  | TRB:TGTGCCAGCAGCCTCGGGCCCCGGTGTGTAAGCTTTCTTT;TRA:TGTGCTGCTGTTCTAGAAACCACTGGCTCTAGGTTGACCTTT                                                  | 1          |
| Clone9  | TRB:TGCGCCAGCAGCCAAATCGGACTAGCGGACAATGAGCAGTTCTTC                                                                                            | 1          |
| Clone10 | TRB:TGTGCCAGCAGCCGGATGGGCGGGGTGGCCGGGGAGCTGTTTTTT                                                                                            | 1          |
| Clone11 | TRB:TGTGCCATCAGTGAGTCGAGGTCTTCGGCCGGCGAGCAGTACTTC;TRA:TGTGGTGAACATGAGGTTGGAGAGCAGTGCTTCCAAGATAATCTTT                                         | 1          |
| Clone12 | TRB:TGCGCCAGCAGCTTGGCCCAGGGGACAGGCCACTACGAGCAGTACTTC;TRA:TGTGCTGTGAGTGGGTTTGGAAATGAGAAATTAACCTTT                                             | 1          |
| Clone13 | TRB:TGTGCCAGCAGATTACAGGGGGTGGCAACTAATGAAAACTGTTTTTT;TRA:GTGTGGTGAACCTACCTATATACAACTTCAACAAATTTTACTTT                                         | 1          |
| Clone14 | TRB:TGTGCCAGCAAAACCTTGGGGGCCTTGACACCGGGGAGCTGTTTTTT;TRA:TGTGCCGTGAAGGGGGGCCAGAAGCTGCTCTTT                                                    | 1          |
| Clone15 | TRB:TGTGCCAGCAGTTCCCCCGTACTAGCGGGATATCCTACGAGCAGTACTTC;TRA:TGTGCCGTGAATGCGAGCCAGGCAGGAAGTCTCTGATCTTT                                         | 1          |

**Supplementary Table 3. Patient characteristics of paired PBMC and TILs cohort, related to Figure 4.**

| ID  | Sex | Age at sample collection | Histology      | Smoking status   | Driver mutation | PDL1 TPS | Clinical stage     | Treatment                                 | Best objective response | Response      | Progression-free survival, days | Cell number               |
|-----|-----|--------------------------|----------------|------------------|-----------------|----------|--------------------|-------------------------------------------|-------------------------|---------------|---------------------------------|---------------------------|
| G16 | M   | 73                       | Adenocarcinoma | Smoker/Ex-smoker | No              | ≥50%     | cT3N0M1c StageIVB  | CBDCA+Pemetrexed+Pembrolizumab            | Partial response        | Responder     | 714                             | Pre: 1177<br>Post: 8651   |
| G17 | M   | 66                       | Adenocarcinoma | Smoker/Ex-smoker | No              | <1%      | cT1aN2M0 StageIIIA | CBDCA+Paclitaxel+Bevacizumab+Atezolizumab | Partial response        | Responder     | 742                             | Pre: 2767<br>Post: 17050  |
| G20 | M   | 72                       | Adenocarcinoma | Smoker/Ex-smoker | No              | 1-49%    | cT4N0M0 StageIIIB  | CBDCA+Pemetrexed+Atezolizumab             | Partial response        | Responder     | 350                             | Pre: 12381<br>Post: 24389 |
| G38 | M   | 52                       | Adenocarcinoma | Smoker/Ex-smoker | No              | 1-49%    | cT2bN1M1a StageIVA | CBDCA+Pemetrexed+Atezolizumab             | Partial response        | Responder     | 299                             | Pre: 10583<br>Post: 15621 |
| G8  | M   | 73                       | Adenocarcinoma | Smoker/Ex-smoker | No              | 1-49%    | cT1cN0M1c StageIVB | CBDCA+Pemetrexed+Atezolizumab             | Stable disease          | Non-responder | 147                             | Pre: 714<br>Post: 3843    |
| G30 | M   | 76                       | Adenocarcinoma | Smoker/Ex-smoker | No              | <1%      | cT2aN3M1a StageIVA | CBDCA+Pemetrexed+Atezolizumab             | Stable disease          | Non-responder | 130                             | Pre: 9266<br>Post: 16358  |
| G37 | M   | 50                       | Adenocarcinoma | Smoker/Ex-smoker | No              | 1-49%    | cT3N2M1c StageIVB  | CBDCA+Paclitaxel+Bevacizumab+Atezolizumab | Progressive disease     | Non-responder | 42                              | Pre: 11779<br>Post: 19492 |
| G58 | M   | 71                       | Adenocarcinoma | Smoker/Ex-smoker | No              | 1-49%    | cT1bN0M1a StageIVA | CBDCA+Abraxane+Atezolizumab               | Stable disease          | Non-responder | 170                             | Pre: 5827<br>Post: 11475  |

**Supplementary Table 4. Patient characteristics of validation cohort, related to Figure 6.**

| Variables       |                                             |             |
|-----------------|---------------------------------------------|-------------|
| Age             |                                             | 66.9 ± 11.4 |
| Sex             | Male                                        | 49 (70.0)   |
|                 | Female                                      | 21 (30.0)   |
| Smoking history | Current/past                                | 54 (77.1)   |
|                 | Non-smoker                                  | 16 (22.9)   |
| Histology       | Adenocarcinoma                              | 54 (77.1)   |
|                 | Squamous cell carcinoma                     | 16 (22.9)   |
| PDL1 TPS        | ≥50%                                        | 21 (30.0)   |
|                 | 1-49%                                       | 19 (27.1)   |
|                 | <1%                                         | 20 (28.6)   |
|                 | Unknown                                     | 10 (14.3)   |
| Treatment       | Chemotherapy + anti-PD1/PDL1 antibody       | 39 (55.7)   |
|                 | Anti-PD1/PDL1 antibody (monotherapy)        | 21 (30.0)   |
|                 | Anti-PD1/PDL1 antibody +anti-CTLA4 antibody |             |
|                 | ±chemotherapy                               | 10 (14.3)   |

Data are presented as mean ± SD or number (%).