JEM

Supplemental material

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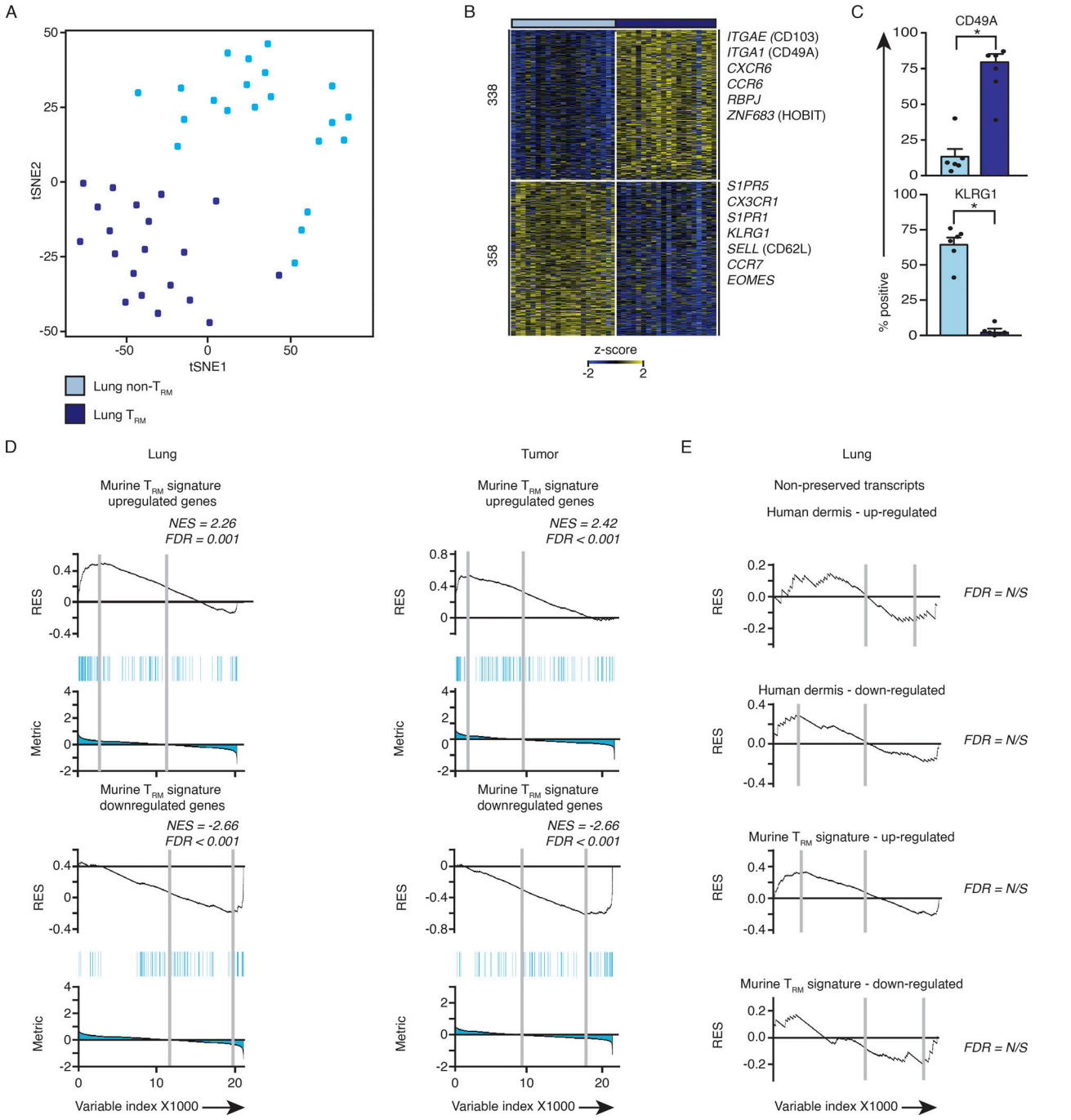

Figure S1. **Validation of T_{RM} phenotype. (A)** tSNE plot of lung T_{RM} ($CD103^+$) and non-T_{RM} ($CD103^-$) CTLs. Each symbol represents an individual patient sample (n = 21 non-T_{RM}; n = 20 T_{RM}). **(B)** RNA-seq analysis of transcripts (one per row) expressed differentially between lung T_{RM} and lung non-T_{RM} (pairwise comparison; change in expression of twofold with an adjusted P value of ≤0.05 [DESeq2 analysis; Benjamini–Hochberg test]), presented as row-wise z-scores of TPM counts. Each column represents an individual sample; key known T_{RM} or non-T_{RM} transcripts are indicated. **(C)** Flow cytometry analysis of the expression of CD49A and KLRG1 versus that of CD103 among live and singlet-gated $CD14^-CD19^-CD20^-CD45^+CD3^+CD8^+$ cells obtained from lung. Calculated as a frequency of $CD103^+$CTLs or $CD103^-$CTLs that express the indicated surface marker (*, P ≤ 0.05, n = 6; Wilcoxon rank-sum test). Bars represent the mean and t-lines the SEM, and symbols represent data from individual samples. **(D)** GSEA of the murine composite T_{RM} signature in the transcriptome of T_{RM} versus non-T_{RM}. Top: RES for the gene set, from most enriched genes at left to most underrepresented at right. Middle: Positions of gene set members (blue vertical lines) in the ranked list of genes. Bottom: Value of the ranking metric. Values above the plot represent the NES and FDR-corrected significance value in CTLs isolated from lung and tumor samples. **(E)** GSEA of the lung T_{RM} versus non-T_{RM} cells for nonpreserved transcripts (in Fig. 1, B and C; as per D; N/S, not significant).

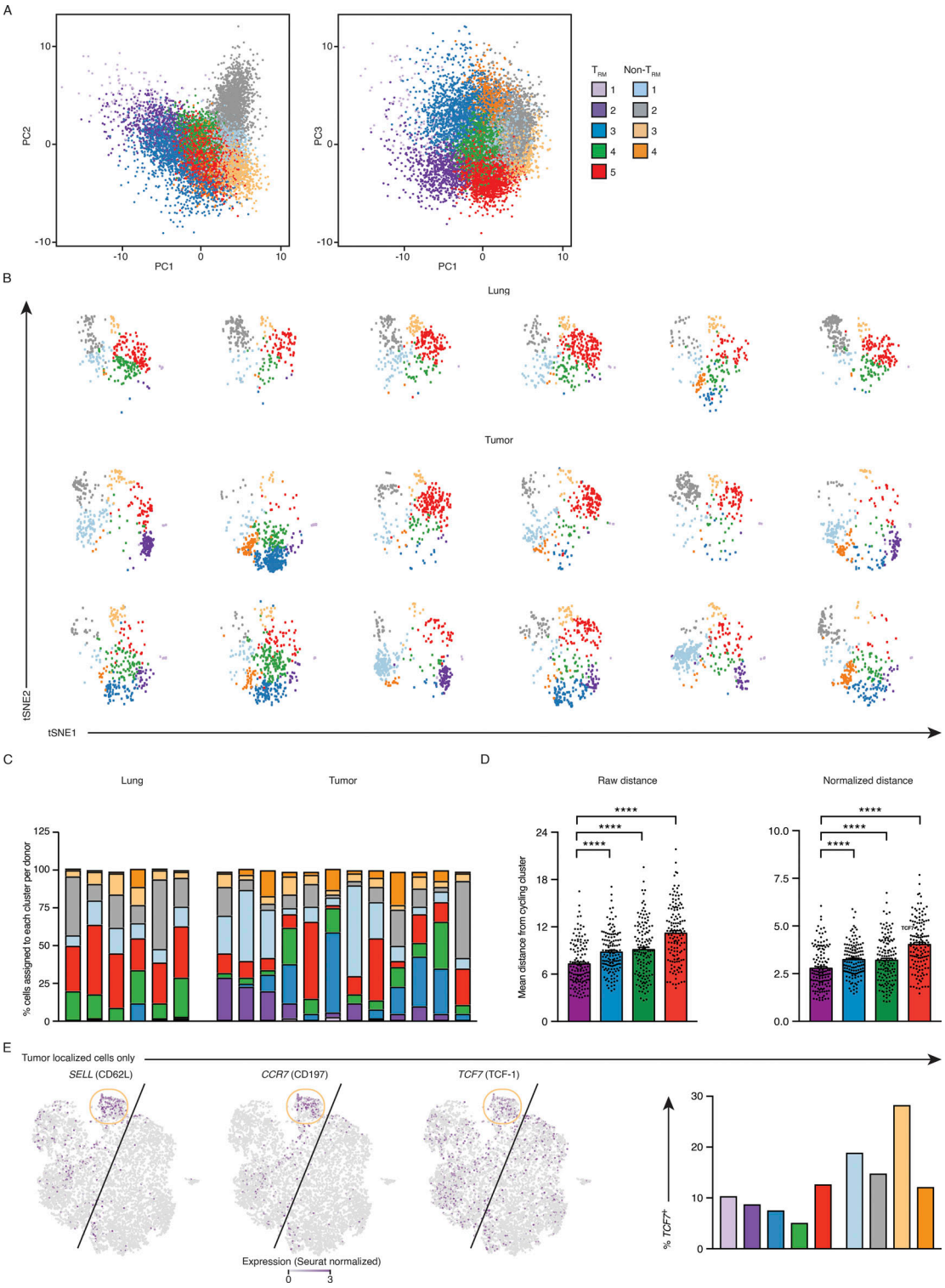

Figure S2. **T_{RM} cells cluster into four major subtypes. (A)** PCA of the single-cell transcriptomes, where each point represents a cell that is colored as per the cluster assignment in Fig. 3; numbers along perimeter indicate PCs (PC1–PC3). **(B)** tSNE visualization of single-cell transcriptomes, shown per donor, obtained from 12 tumors and 6 matched normal lung samples. Each symbol represents a cell; color indicates Seurat clustering of cells, as per Fig. 3 B**,** identifying nine clusters. **(C)** Breakdown of cells assigned to each cluster in each donor, separated by tissue type of origin (colored as per Fig. 3 B). **(D)** The distance in PC space between a cell assigned to cluster 1 compared with the mean of cells assigned into the other clusters (colored as per Fig. 3 B). The difference was calculated with the raw (left) and z-score–normalized (right) distances; bars represent the mean distance to each of the other clusters, t-lines represent SEM, and symbols represent individual cells in cluster 1 (****, P ≤ 0.0001; *n* = 135 cells; Wilcoxon rank-sum test). **(E)** Left: Seurat-normalized expression of indicated transcripts identified as differentially enriched in the non-T_{RM} cluster 3 (colored as per Figs. 3 B and S3 A), overlaid across the tSNE plot, with expression levels represented by the color scale. Right: Percentage of cells expressing *TCF7* transcripts in each T_{RM} cluster (as per Fig. 3 B), where positive expression was defined as >1 Seurat-normalized count.

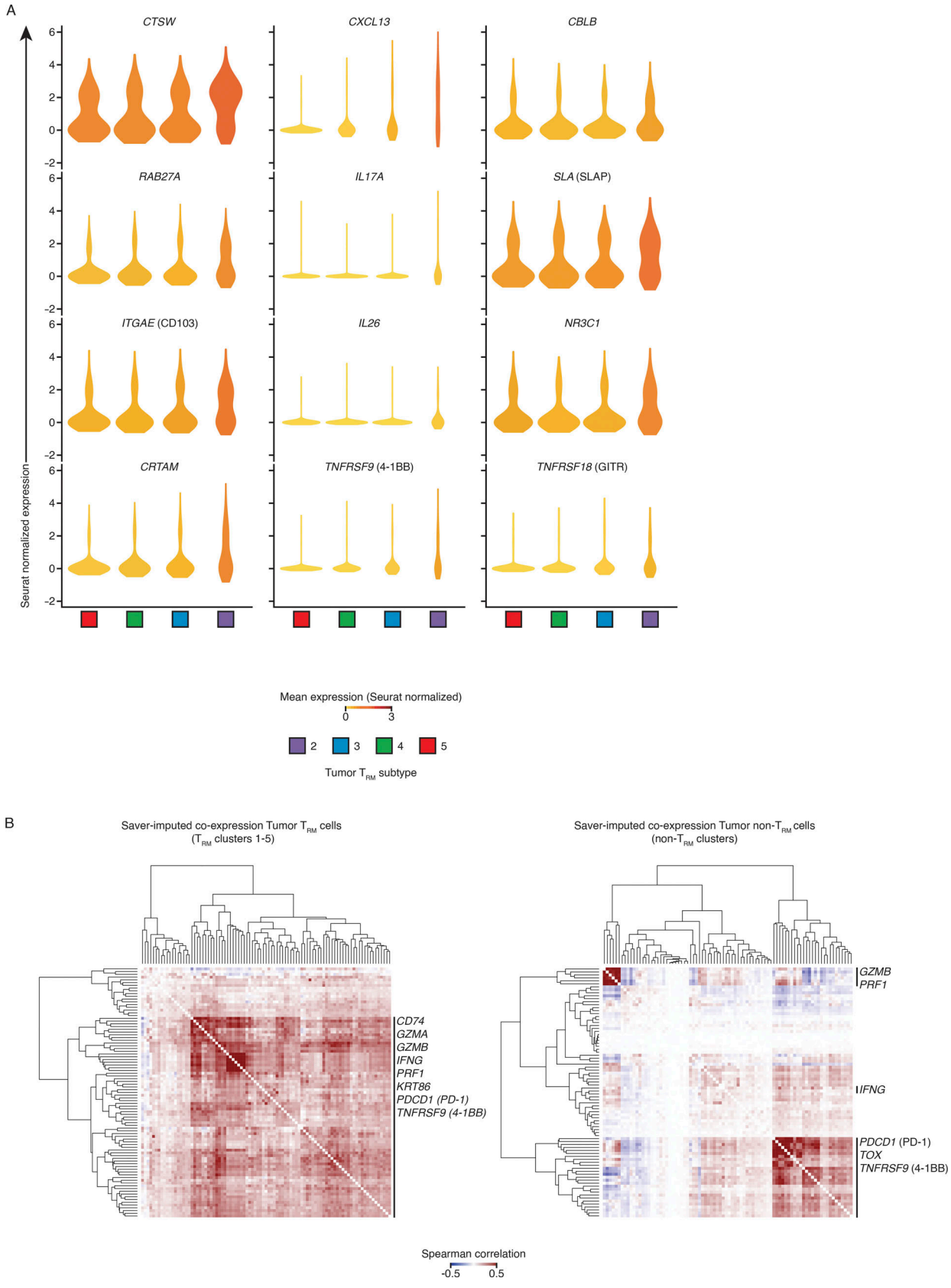

Figure S3. **Tumor T_{RM} cells are enriched for transcripts associated with enhanced antitumor features. (A)** Violin plot of expression of indicated transcripts; shape represents the distribution of expression among cells, and color represents average expression, calculated from the Seurat-normalized counts. **(B)** SAVER-imputed spearman coexpression analysis of genes whose expression is enriched in the *TIM-3*$^{+}$*IL7R*$^{-}$ T_{RM} cluster (Fig. 4 A) in tumor T_{RM} and non-T_{RM} clusters, respectively; the matrix is clustered according to complete linkage.

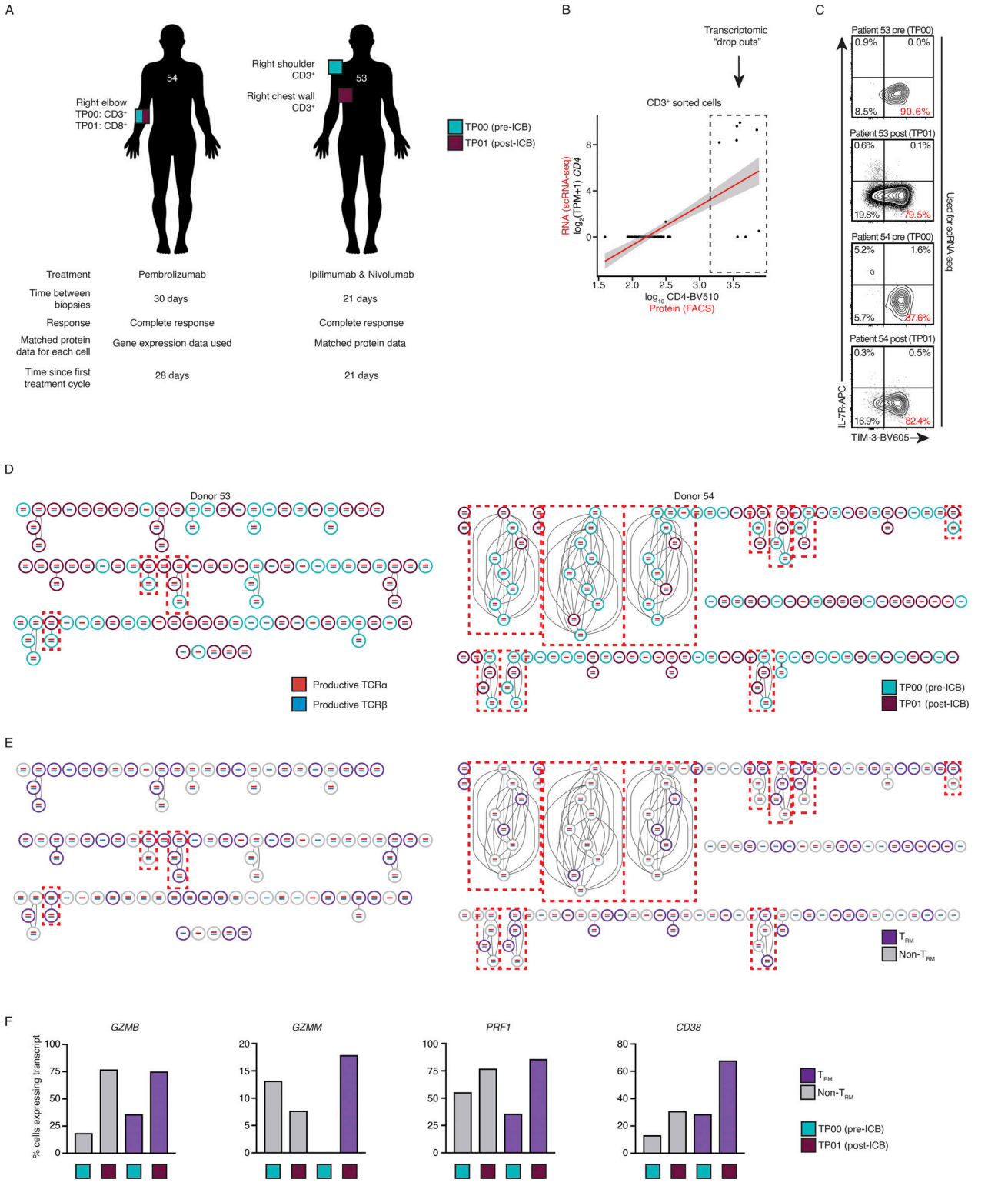

Figure S4. **Single-cell transcriptome analysis of CTLs from anti-PD-1 responders. (A)** Schematic representation of clinical details and cells sorted for the patients selected for study. TP, time point; ICB, immune-checkpoint blockade. **(B)** Example of in silico removal of CD4$^+$ cells, highlighting the transcriptomic dropouts (single-cell RNA-seq [scRNA-seq]). The dashed line corresponds to the CD4$^+$ cells removed. **(C)** Flow cytometry analysis of the expression of TIM-3 versus that of IL-7R in live, singlet CD14$^-$CD19$^-$CD20$^-$CD4$^-$CD45$^+$CD3$^+$CD8$^+$CD103$^+$ cells obtained from patients responding to anti-PD-1 therapy both before and after therapy (n = 2 donors at two time points, as per A). **(D)** A clonotype network graph of cells from patient 53 and 54 (A), highlighting the time point from which the cells were isolated. Cells highlighted through a dashed line correspond to shared clonotypes across time points. **(E)** A clonotype network graph (as per D), highlighting the T_{RM} cells and non-T_{RM} cells, marked in purple and black, respectively. Cells were assigned based on protein expression of CD103; alternatively, if cell-specific protein expression was not available, cells with >10 TPM counts expression of *ITGAE* (*CD103*), *RBPJ*, or *ZNF683* (*HOBIT*) were considered T_{RM} cells. **(F)** Percentage of cells expressing the indicated transcripts in each population, where T_{RM} cells were identified as per D and E.

Tables S1–S12 are provided online in a .zip file containing separate Excel files. Table S1 lists clinical and histopathological characteristics of patients used in this study. Table S2 contains a list of differentially expressed genes in lung T_{RM} versus non-T_{RM} cells. Table S3 contains gene lists utilized for GSEA and preservation analysis of T_{RM} signatures from published datasets. Table S4 contains lists of differentially expressed genes in tumor T_{RM} versus tumor non-T_{RM} cells. Table S5 lists differentially expressed genes in stimulated versus unstimulated T_{RM} and non-T_{RM} cells from both lung and tumor from cells isolated from immunotherapy treatment–naive patients. Table S6 provides TCR-seq library and clonality information from cells isolated from immunotherapy treatment–naive patients. Table S7 provides a list of genes uniquely expressed in tumor T_{RM} subsets from cells isolated from immunotherapy treatment–naive patients, a list of genes uniquely expressed in tumor CTL subsets from cells isolated from immunotherapy treatment–naive patients, and a list of differentially expressed genes in *PDCD1*$^+$ T_{RM} (clusters 2–5) versus *PDCD1*$^+$ non-T_{RM} cells (non-T_{RM} clusters 1–4) from cells isolated from immunotherapy treatment–naive patients. Table S8 lists TCR chain sequences from single-cell RNA-seq assays from cells isolated from immunotherapy treatment–naive patients. Table S9 lists differentially expressed genes in *TIM-3*$^+$ T_{RM} cells versus other T_{RM} cells from cells isolated from immunotherapy treatment–naive patients. Table S10 lists single-cell coexpression and correlation analysis of genes enriched in "cluster 2" TRM subset, and correlation analysis of protein expression levels from flow cytometry data from cells isolated from immunotherapy treatment–naive patients. Table S11 lists quantification of CD8, CD103, and TIM-3 multiplexed immunohistochemistry counts from tumor samples of lung cancer patients with $TIL^{high}T_{RM}{}^{high}$ and $TIL^{low}T_{RM}{}^{low}$ tumor status. Table S12 describes assignment of single-cell libraries into T_{RM} and non-T_{RM} cells, TCR chain sequences from single-cell RNA-seq assays, list of differentially expressed genes from cells before and after anti-PD-1, and single-cell correlation analysis after anti-PD-1 in CTLs.