

Supplementary Information for:

CX3CR1⁺ CD8⁺ Cytotoxic T Cells Drive Tumor Killing in Esophageal Squamous Cell Carcinoma During Neoadjuvant Therapy

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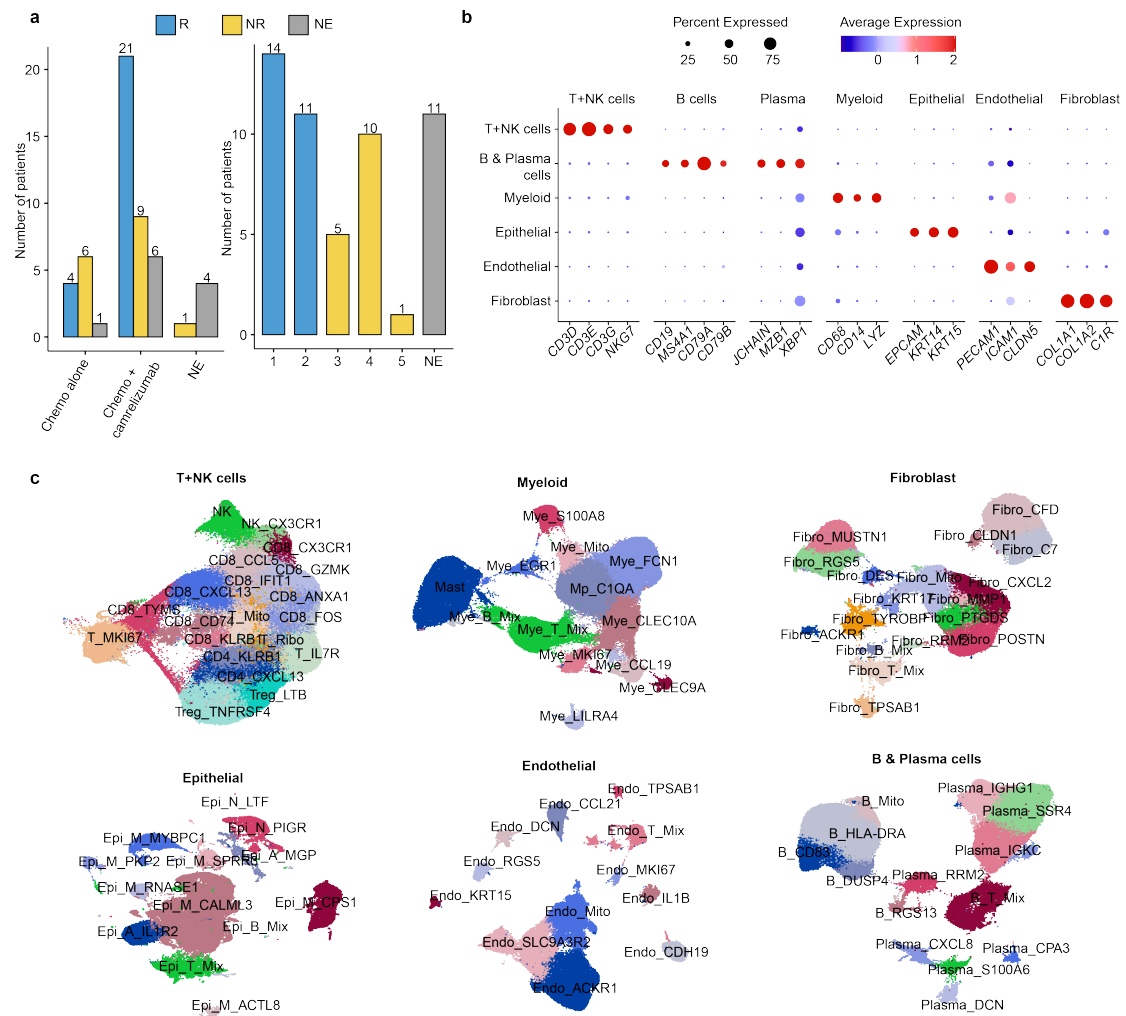
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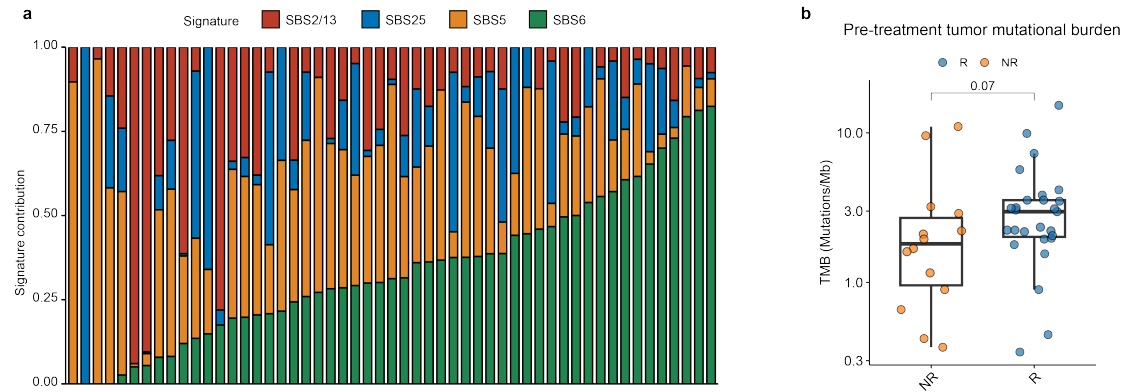
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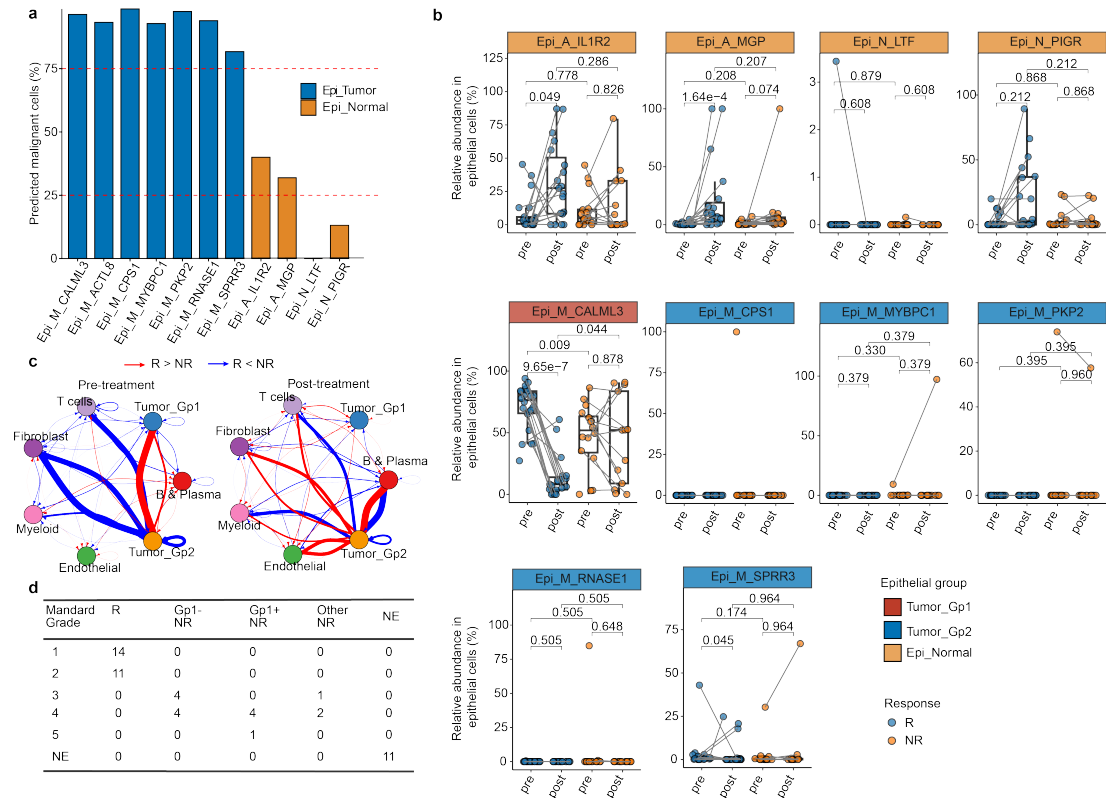
Supplementary Figures



Supplementary Fig. 1. Cohort annotation and single-cell annotation of major cell types and subclusters. **a** Distribution of treatment assignment, pathological response status, and Mandard tumor regression grades in the scRNA-seq cohort. NE indicates cases not evaluable for pathological response. **b** Dot plot of marker gene expression by cell type. Dot size indicates the proportion of expressing cells, and color intensity indicates mean expression. **c** UMAP visualization of transcriptionally distinct subclusters identified within each major cell compartment.

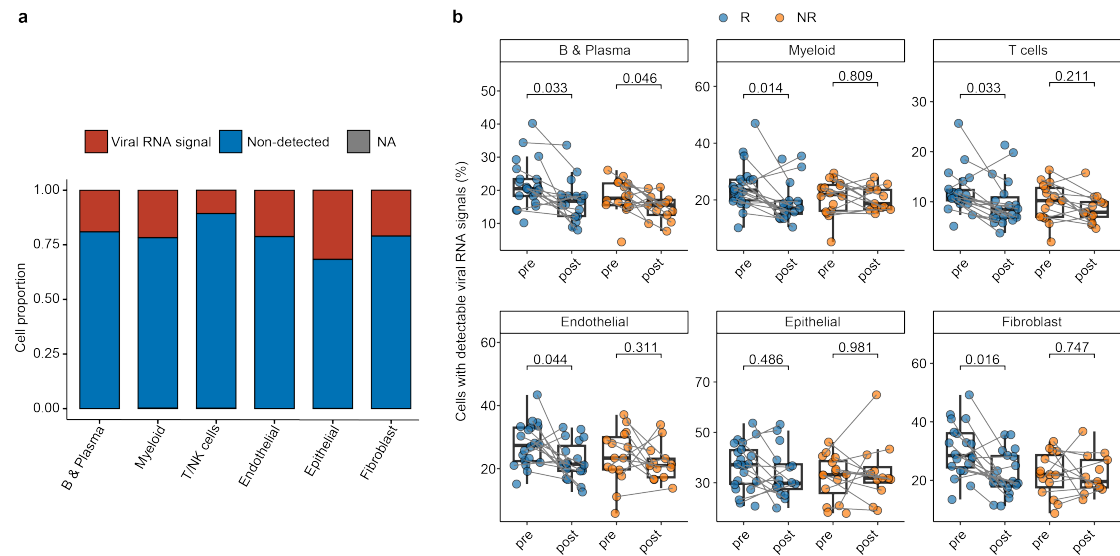


Supplementary Fig. 2. Mutational signatures and tumor mutational burden in ESCC WES samples. **a** Relative contribution of COSMIC v3 mutational signatures in WES tumor samples. Each bar represents one tumor sample, and samples are ordered by increasing SBS6 contribution. **b** Pre-treatment tumor mutational burden in responders (R; $n = 27$) and non-responders (NR; $n = 14$) with available WES data and evaluable pathological response. Each dot represents one patient sample. Box plots show the median, interquartile range and whiskers extending to $1.5 \times$ the interquartile range. The P value was calculated using a two-sided unpaired Wilcoxon rank-sum test.

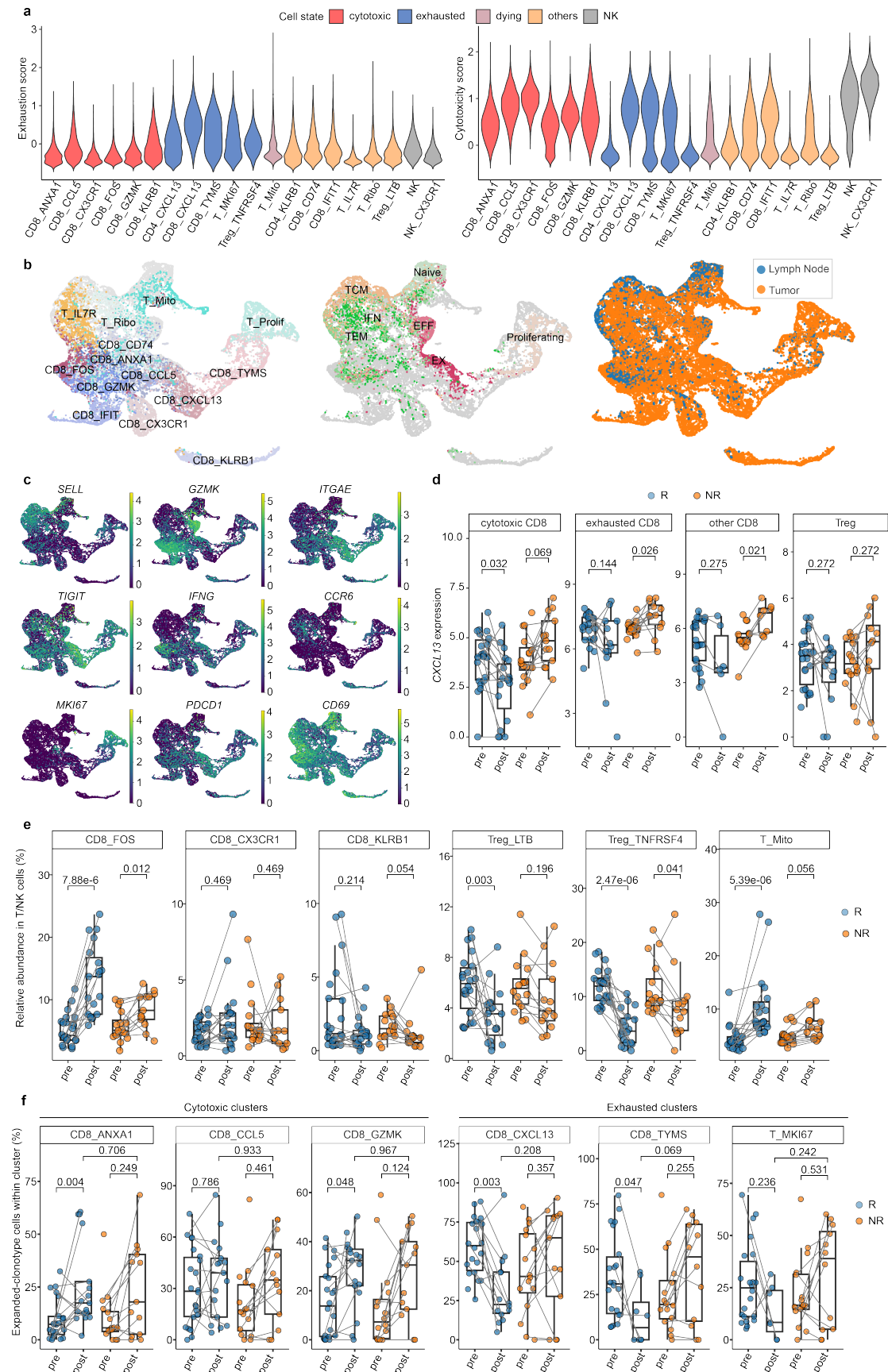


Supplementary Fig. 3. Epithelial subcluster annotation and treatment-associated changes.

a Malignance ratio of epithelial subclusters, defined as the proportion of abnormal cells (inferCNV) in each subcluster. **b** Relative abundance of normal (low/medium malignancy ratios) and tumor cells (high malignancy ratio) among epithelial cells before and after treatment in responders (R; pre, $n = 22$; post, $n = 19$) and non-responders (NR; pre, $n = 16$; post, $n = 13$). **c** Differential cell-cell interaction networks before and after treatment. Red and blue edges indicate stronger interactions in responders and non-responders, respectively; edge width reflects the magnitude of the difference. **d** Contingency table showing the distribution of Mandard tumor regression grades across response and epithelial-group categories. Gp1+NR and Gp1-NR indicate non-responders with or without post-treatment Tumor_Gp1 enrichment, respectively. NE indicates cases not evaluable for pathological response. Each dot represents one patient sample; box plots show the median, interquartile range and whiskers extending to $1.5 \times$ the interquartile range; P values were calculated by two-sided unpaired Wilcoxon rank-sum tests (**b**).



Supplementary Fig. 4. Distribution of cells with detectable viral RNA signals across major cell types. **a** Proportion of cells with detectable viral RNA signals, non-detected cells, and cells with unavailable detection status (NA) across major cell types. **b** Percentage of cells with detectable viral RNA signals in major cell compartments before and after treatment in responders (R) and non-responders (NR). Only samples with at least 50 cells in the corresponding compartment were included; exact sample sizes are provided in the Source Data. Each dot represents one patient sample. Box plots show the median, interquartile range and whiskers extending to $1.5 \times$ the interquartile range. *P* values were calculated using two-sided unpaired Wilcoxon rank-sum tests with Benjamini–Hochberg correction.

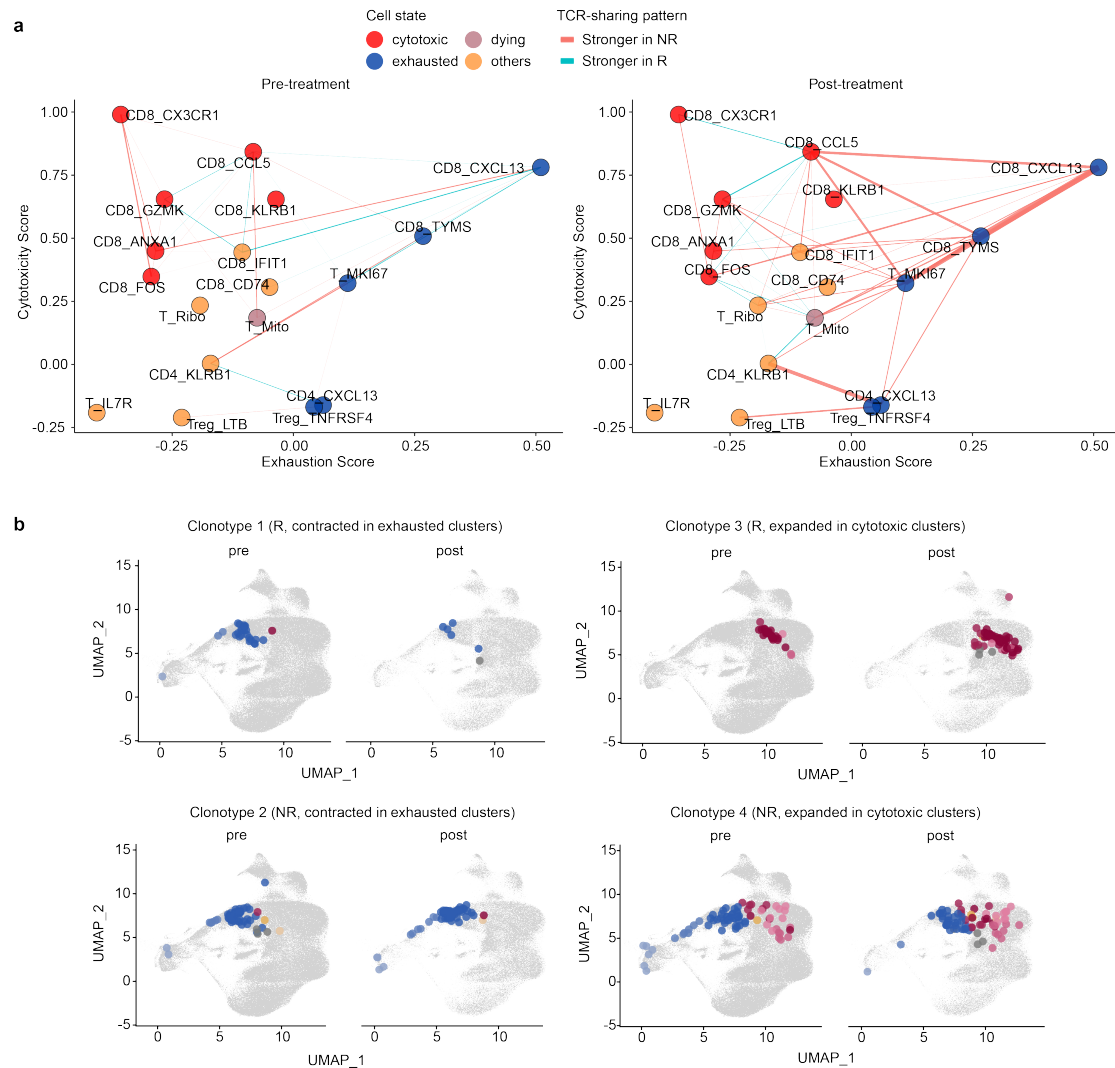


Supplementary Fig. 5. T/NK-cell state annotation, relative abundance, and cluster-level TCR clonal enrichment. **a** Violin plot for exhaustion and cytotoxicity scores across T/NK-cell

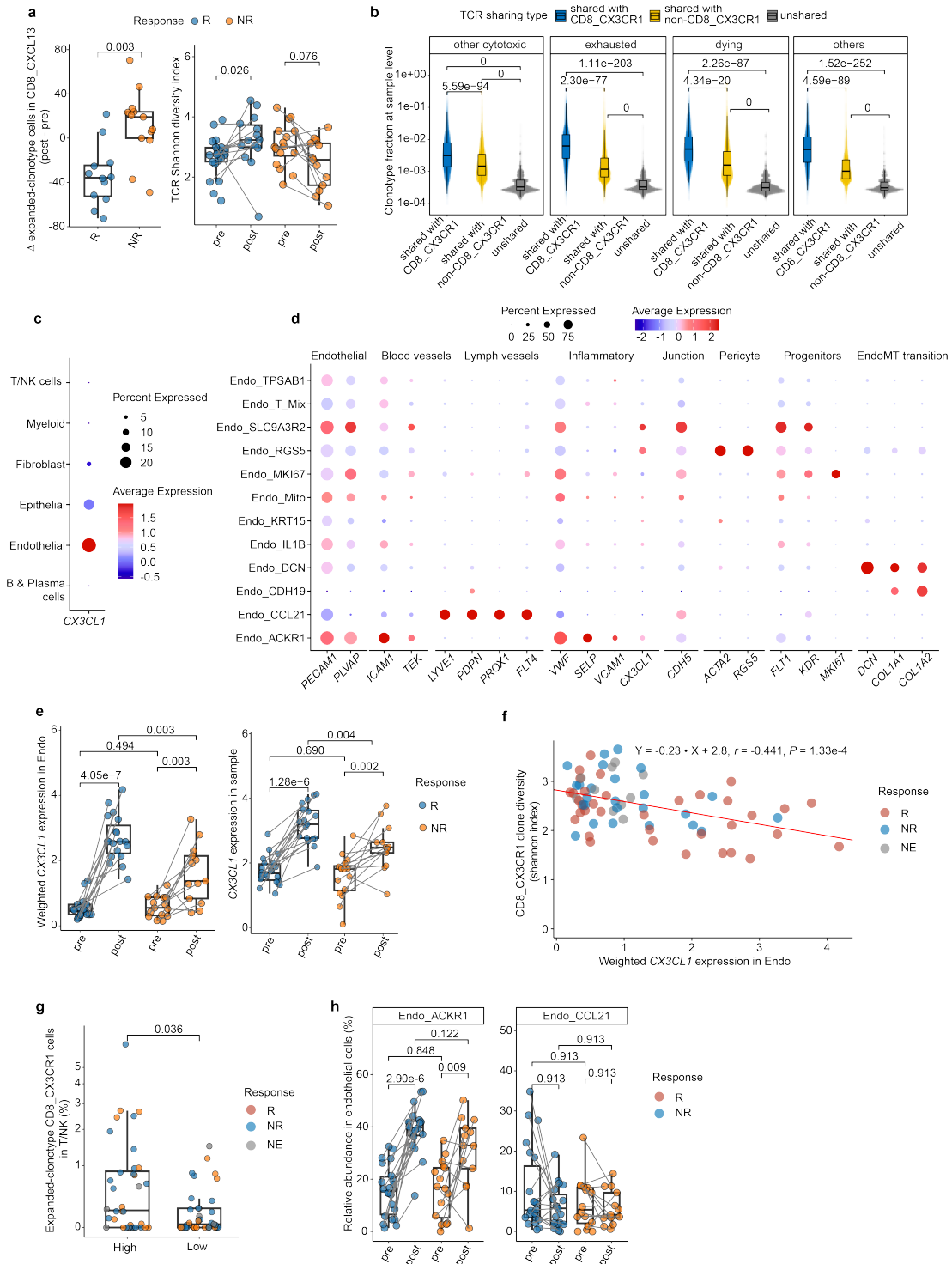
clusters. **b** UMAP illustration of T/NK-cell states from ESCC tumor and lymph node dataset. Colored by cluster or origin. **c** UMAP feature plots showing expression of representative T-cell state marker genes. **d** Pseudobulk *CXCL13* expression in cytotoxic/exhausted CD8⁺ T cells, NK cells, other CD8⁺ T cells, and Tregs. Only sample–cell-group combinations containing at least 50 cells were included. **e** Relative abundance of six T-cell clusters within the T/NK-cell compartment before and after treatment in responders (R: pre, $n = 22$; post, $n = 19$) and non-responders (NR: pre, $n = 16$; post, $n = 13$). **f** Cluster-level TCR clonal enrichment in major cytotoxic and exhausted CD8⁺ T-cell states. Only sample–cluster combinations containing at least 10 cells were included. Exact sample sizes for **d** and **f** are provided in the Source Data. In **d–f**, each dot represents one patient sample. Box plots show medians, interquartile ranges and whiskers extending to 1.5× the interquartile range. P values were calculated using two-sided Wilcoxon rank-sum tests with Benjamini–Hochberg correction.



Supplementary Fig. 6. Chemokine and chemokine-receptor expression patterns associated with T-cell states. **a** Chemokine receptor expression in pseudo-bulk cytotoxic CD8⁺ T-cell clusters (R: pre, $n = 22$; post, $n = 19$; NR: pre, $n = 16$; post, $n = 13$) and exhausted CD8⁺ T-cell clusters (R: pre, $n = 22$; post, $n = 12$; NR: pre, $n = 16$; post, $n = 12$). **b** Pseudobulk chemokine expression in whole samples before and after treatment (R: pre, $n = 22$; post, $n = 19$; NR: pre, $n = 16$; post, $n = 13$). **c** Expression of *CXCL12* in pseudo-bulk of major cell types. Exact sample sizes are provided in the Source Data. Each dot represents one patient sample. Box plots show medians, interquartile ranges and whiskers extending to 1.5 $\times$ the interquartile range. P values were calculated using two-sided Wilcoxon rank-sum tests with Benjamini–Hochberg correction (**a–c**).

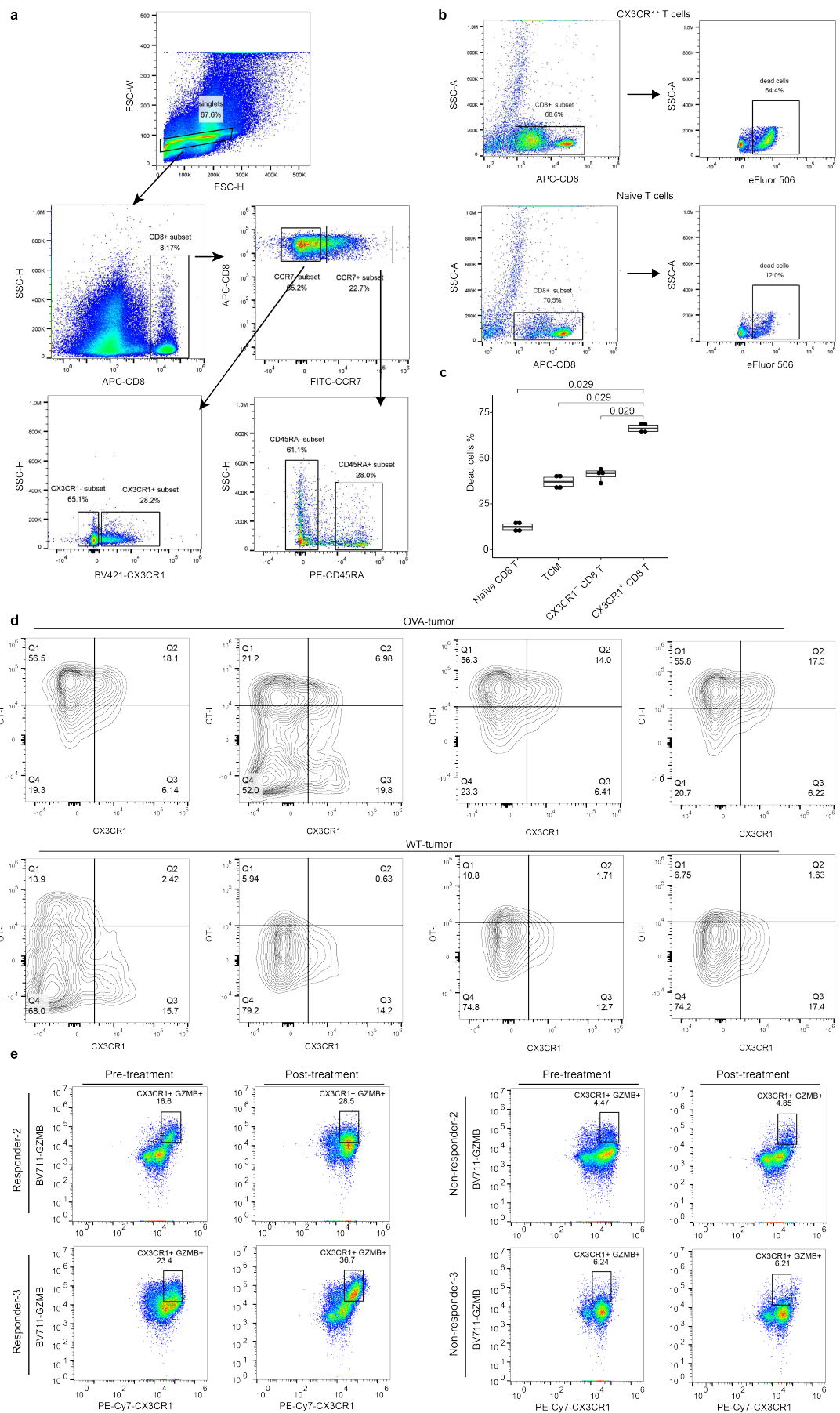


Supplementary Fig. 7. Paired pre- and post-treatment TCR clonal replacement dynamics.
a T-cell state maps before and after treatment. Lines denote TCR sharing score differences between R and NR patients at baseline (left) and post-treatment (right). Red and blue indicate stronger sharing in NR and R, respectively. Line width indicates difference magnitude. **b** UMAP visualization of key contracted/expanded clonotypes by response status.



Supplementary Fig. 8. CXCL13⁺ and CX3CR1⁺ CD8⁺ T-cell-associated clonotype abundance, TCR diversity, and CX3CL1 expression. **a** CD8_CXCL13-associated clonotype abundance in responders (R; $n = 12$) and non-responders (NR; $n = 13$; left), and TCR Shannon diversity analysis before and after treatment (R: pre, $n = 22$; post, $n = 14$; NR: pre, $n = 16$; post, $n = 13$; right). **b** Clonotype fractions of T cells stratified by TCR-sharing category with CD8_CX3CR1 cells. **c** CX3CL1 expression across major cell compartments. Dot size indicates the proportion of cells expressing CX3CL1; color intensity reflects mean expression level. **d** Dot plots showing marker-gene expression used to annotate endothelial subclusters. **e** Weighted

endothelial CX3CL1 expression (R: pre, $n = 20$; post, $n = 19$; NR: pre, $n = 15$; post, $n = 13$; left) and whole-sample CX3CL1 expression (R: pre, $n = 22$; post, $n = 19$; NR: pre, $n = 16$; post, $n = 13$; right). **f** Correlation between CD8_CX3CR1 TCR Shannon diversity and endothelial *CX3CL1* expression. **g** Frequency of expanded-clonotype CD8_CX3CR1 cells in CX3CL1-high and CX3CL1-low samples. **h** Relative abundance of Endo_ACKR1 blood vascular endothelial cells and Endo_CCL21 lymphatic endothelial cells before and after treatment in responders (pre, $n = 22$; post, $n = 19$) and non-responders (pre, $n = 16$; post, $n = 13$). Each dot in **a**, **e**, **g** and **h** represents one sample. Box plots show medians, interquartile ranges and whiskers extending to $1.5\times$ the interquartile range. P values were calculated using two-sided Wilcoxon rank-sum tests with Benjamini–Hochberg correction (**a**, **b**, **e**, **h**), a two-sided Wilcoxon rank-sum test (**g**) or two-sided Pearson correlation (**f**).



Supplementary Fig. 9. Flow-cytometry validation and functional characterization of CX3CR1⁺ CD8⁺ T cells. **a** Gating strategy for sorting CD8⁺ T-cell subsets from PBMCs for the in vitro co-culture assay. **b, c** Representative flow-cytometry plots (**b**) and quantification (**c**) of dead cells among sorted CD8⁺ T-cell subsets after in vitro activation ($n = 4$ biologically independent replicates per subset). Each dot represents one replicate. Box plots show medians, interquartile ranges and whiskers extending to 1.5× the interquartile range. P values were calculated using two-sided Wilcoxon rank-sum tests with Benjamini–Hochberg correction. **d** Representative flow-cytometry plots of CX3CR1 and OT-I expression in tumor-infiltrating CD8⁺ T cells from AKR-OVA and WT tumor-bearing mice ($n = 4$ mice per group). **e** Representative flow-cytometry plots of CX3CR1⁺GZMB⁺ CD8⁺ T-cell frequencies from the additional responders and non-responders ($n = 2$ patients per group).

Supplementary Tables

Patient ID	Sex	Age	Stage	PD-L1 CPS	Response	Mandard grade	Treatment	scRNA-seq Pre	scRNA-seq Post	scTCR-seq Pre	scTCR-seq Post	WES Pre	WES Post
P01	Female	70	ypTisN0	NA	R	1	Cam+Chemo	1	1	1	1	0	0
P02	NA	NA	NA	NA	NE	NE	NA	1	0	1	0	0	0
P03	Male	62	ypT3N1	≥ 10	NR	3	Chemo	1	1	1	1	1	0
P04	Male	67	ypT3N1	≥ 10	NR	5	Cam+Chemo	1	1	1	1	0	0
P05	Male	64	ypT2N0	$1 \leq < 10$	R	2	Cam+Chemo	1	1	1	1	1	0
P06	NA	NA	NA	NA	NE	NE	Chemo	1	0	1	0	0	0
P07	Male	52	ypT3N0	$1 \leq < 10$	NR	4	Chemo	1	1	1	1	1	0
P08	Male	69	ypT2N2	$1 \leq < 10$	R	2	Cam+Chemo	1	1	1	1	0	0
P09	Male	53	ypT3N3	≥ 10	NR	4	Cam+Chemo	1	1	1	1	1	0
P10	NA	NA	NA	NA	NE	NE	Cam+Chemo	1	0	1	0	1	0
P11	NA	NA	NA	NA	NE	NE	Cam+Chemo	1	0	1	0	1	0
P12	Male	48	ypT3N3	≥ 10	NR	4	Chemo	1	1	1	1	1	0
P13	Male	52	ypT3N0	≥ 10	NR	4	Cam+Chemo	1	1	1	1	1	1
P14	Male	67	ypT0N0	NA	R	1	Cam+Chemo	1	0	1	0	1	0
P15	Male	55	ypT2N0	$1 \leq < 10$	R	2	Cam+Chemo	1	1	1	1	0	0
P16	NA	68	ypT0N0	NA	R	1	Cam+Chemo	1	0	1	0	1	0
P17	Male	63	ypT3N3	≥ 10	NR	4	Cam+Chemo	1	1	1	1	0	0
P18	NA	NA	NA	NA	NE	NE	Cam+Chemo	1	0	1	0	0	0
P19	Male	72	ypT1aN0	NA	R	2	Cam+Chemo	1	0	1	0	1	0
P20	Male	58	ypT2N2	≥ 10	NR	4	Cam+Chemo	1	1	1	1	0	0
P21	Male	59	ypT0N0	NA	R	1	Cam+Chemo	1	1	1	1	0	0
P22	NA	NA	NA	NA	NE	NE	Cam+Chemo	1	0	1	0	1	0

P23	Male	58	ypT0N0	NA	R	1	Cam+Chemo	1	0	1	0	1	0
P24	Male	NA	ypT3N0	NA	NR	4	Chemo	1	1	1	1	0	0
P25	Male	50	ypTisN0	NA	R	1	Cam+Chemo	1	1	1	1	1	1
P26	Male	62	ypT0N0	NA	R	1	Cam+Chemo	1	1	1	1	1	0
P27	Male	58	ypT1bN1	$1 \leq & < 10$	R	2	Chemo	1	1	1	1	1	0
P28	Male	58	ypT3N0	NA	R	2	Chemo	1	1	1	1	1	0
P29	NA	NA	NA	NA	NE	NE	Cam+Chemo	1	0	1	0	0	0
P30	NA	NA	NA	< 1	NE	NE	Cam+Chemo	1	0	1	0	0	0
P31	Male	69	ypT3N1	≥ 10	NR	4	NA	1	0	1	0	0	0
P32	Male	64	ypTisN0	NA	R	1	Cam+Chemo	1	0	1	0	0	0
P33	Male	51	ypT3N2	≥ 10	NR	3	Cam+Chemo	1	1	1	1	1	1
P34	Male	62	ypT1aN0	NA	R	2	Cam+Chemo	1	1	1	1	1	1
P35	NA	NA	NA	$1 \leq & < 10$	NE	NE	NA	1	0	1	0	0	0
P36	Male	62	ypT0N0	NA	R	1	Cam+Chemo	1	0	1	0	1	0
P37	NA	NA	NA	NA	NE	NE	NA	1	0	1	0	0	0
P38	Male	57	ypT1aN0	< 1	R	2	Cam+Chemo	0	1	0	1	1	0
P39	Male	52	ypT0N0	NA	R	1	Cam+Chemo	0	1	0	1	1	0
P40	Male	65	ypT2N0	≥ 10	NR	3	Chemo	1	1	1	1	1	0
P41	Male	52	ypTisN0	NA	R	1	Cam+Chemo	1	1	1	1	1	0
P42	Male	62	ypT1bN1	≥ 10	R	2	Chemo	0	1	0	1	1	0
P43	Female	68	ypTisN0	NA	R	1	Chemo	1	1	1	1	1	0
P44	Male	55	ypT1aN0	≥ 10	R	2	Cam+Chemo	1	1	1	1	1	0
P45	Male	57	ypT2N0	≥ 10	NR	3	Cam+Chemo	1	1	1	1	1	0
P46	Male	64	ypT0N0	NA	R	1	Cam+Chemo	1	1	1	1	1	0
P47	Female	63	ypT3N0	≥ 10	R	2	Cam+Chemo	1	1	1	1	1	0

P48	Female	69	ypT4aN0	≥ 10	NR	4	Cam+Chemo	1	1	1	1	1	0
P49	Male	NA	ypT3N0	NA	NR	3	Chemo	1	0	1	0	1	1
P50	Male	49	ypT3N1	≥ 10	NR	4	Cam+Chemo	1	0	1	0	1	0
P51	Male	47	ypT0N0	NA	R	1	Cam+Chemo	1	1	1	1	1	0
P52	NA	NA	NA	NA	NE	NE	NA	1	0	1	0	0	0
P53	NA	NA	NA	NA	R	2	Cam+Chemo	0	0	0	0	1	0
P54	NA	NA	NA	NA	NR	3	Cam+Chemo	0	0	0	0	1	0
P55	NA	NA	NA	NA	R	2	Cam+Chemo	0	0	0	0	1	0
P56	NA	NA	NA	NA	R	1	Chemo	0	0	0	0	1	0
P57	NA	NA	NA	NA	R	1	Cam+Chemo	0	0	0	0	1	1
P58	NA	NA	NA	NA	R	2	Cam+Chemo	0	0	0	0	1	0
P59	NA	NA	NA	NA	NR	4	Chemo	0	0	0	0	1	0
P60	NA	NA	NA	NA	NE	NE	Cam+Chemo	0	0	0	0	1	1
P61	NA	NA	NA	NA	R	1	Cam+Chemo	0	0	0	0	1	1
P62	NA	NA	NA	NA	NR	3	Chemo	0	0	0	0	1	0
P63	NA	NA	NA	NA	R	1	Cam+Chemo	0	0	0	0	1	0

Supplementary Table 1. Clinical characteristics and sequencing data availability of ESCC patients included in this study. Response was assessed using the Mandard tumor regression grading system in patients with available post-treatment surgical specimens. NA, not available; NE, not evaluable because pathological response could not be assessed; Cam+Chemo, camrelizumab plus chemotherapy; Chemo, chemotherapy alone. For scRNA-seq Pre/Post, scTCR-seq Pre/Post and WES Pre/Post, 1 indicates available data and 0 indicates unavailable data.