Supplementary Materials for

Clonotype-resolved T-cell response monitoring via repertoire-wide multidimensional TCR decoding

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Materials and Methods:

Cell lines

Cell lines, unless specified otherwise, were acquired from ATCC. K562, MC38, and Jurkat-related cell lines are cultured in RPMI medium (Gibco), supplemented with 10% FBS (Gibco), 100 IU/mL penicillin (Gibco), and 100 μg/mL streptomycin (Gibco). The HEK293T cell line is maintained in DMEM (Gibco), supplemented with 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin. 1G4 Jurkat cell lines were generated via lentiviral transduction based on ΔTCR Jurkat (ATCC, J.RT3-T3.5), and the K562-A*02:01 cell line was similarly developed using lentiviral transduction based on the K562 cell line. GP100 Jurkat cell lines and T2-BFP were provided by Beijing CorreGene Biotechnology (Beijing, China). All cell cultures were kept at 37°C in a 5% CO_2 atmosphere.

Mice

C57BL/6J mice were sourced from GemPharmatech (Nanjing, China), and OT-1 mice were obtained from Charles River. Experiments employed both male and female mice, aged between 6 and 12 weeks, across all experimental conditions. All purchased mice were specific pathogen-free (SPF) animals and were bred or housed under a clean environment. Ethical approval for all animal experiments was granted by the Animal Ethics and Welfare Committee (AEWC, Approval No. IACUC-2012003).

Human sample acquisition

Human lung cancer-related tissues and PBMCs were obtained from the biobank of Jiangsu Cancer Hospital (Jiangsu Institute of Cancer Research & The Affiliated Cancer Hospital of Nanjing Medical University) with appropriate written consent and approval from the Ethics Committee of Jiangsu Cancer Hospital (protocol 2022K-K006). Human bladder cancer-related tissues and PBMCs were obtained from Nanjing Drum Tower Hospital, the Affiliated Hospital of Nanjing University Medical School. The bladder cancer-associated part in the present study was approved by the Ethics Committee of Nanjing Drum Tower Hospital in accordance with the Declaration of Helsinki (protocol 2021-394-01). Written informed consent was obtained from all participants prior to sample collection.

Human tumor-related tissues were obtained through surgical resection from treatment-naïve patients who had not received prior chemotherapy or immunotherapy to minimize inter-donor variability. In this study, lung cancer samples consisted of lung adenocarcinoma, and bladder cancer samples comprised urothelial carcinoma. For each patient, tumor tissue, tumor-draining lymph nodes (dLNs), and peripheral blood samples were collected during surgery. For lung cancer patients, tumor-adjacent normal lung regions located more than 5 cm from the tumor site were also collected. Following resection, all tissue specimens were preserved in T-cell medium at 4°C and transported to the laboratory within 3 hours.

For the lung cancer, tumor-infiltrating lymphocyte (TIL) samples included a total of 7 donors for both $CD4^+$ and $CD8^+$ T-cell analyses, with one donor each missing either the NT or Un group; for dLN samples, a total of 5 donors were analyzed for both $CD4^+$ and $CD8^+$ T cells. For the bladder cancer, the $CD8^+$ TIL samples involved a total of 3 donors, while the remaining experimental groups comprised 2 donors.

Plasmid cloning and construction

In the study, the TCR constructs, comprising α and β chains linked via a P2A peptide, and the HLA-A*02:01 construct, where β2-microglobulin is connected to HLA-A*02:01 through GS linkers, were synthesized by GenScript and subsequently cloned into the lentiviral vector pLV (Addgene no. 36084) for subsequent lentivirus packaging. For the construction of the retroviral vectors encoding the TCR, the synthesized TCR sequences (α and β chains linked via a T2A peptide) were directly cloned into the retroviral vector MIGR1 which carries a gene encoding green fluorescent protein (GFP) (Addgene no. 75021).

Primary T cells and immature bone marrow dendritic cells (iDCs) preparation

Naïve $CD8^+$ T cells were isolated from the spleens of OT-1 mice or C57BL/6J mice using a $CD8^+$ T cell isolation kit (STEMCELL Technologies). Subsequent to isolation, T cells were maintained in T cell medium, comprising RPMI 1640 supplemented with 10% FBS, 1 mM sodium pyruvate (Gibco), 50 μM β-ME (Gibco), 10 mM HEPES (Gibco), 1x MEM NEAA (Gibco), 100 IU/mL penicillin, and 100 μg/mL streptomycin. All cell cultures were kept at 37°C in a 5% CO_2 atmosphere. Naïve $CD8^+$ OT-1 T cells were first activated for 48 h (day 0–2) on plates coated with anti–CD3 (2 μg/mL, BioXCell) and in the presence of anti–CD28 (0.5 μg/mL, BioXCell) and IL-2 (100 IU/mL, T&L Biological Technology, Beijing, China). For (central) memory-like T cell condition, cells were cultured in IL-15 (10 ng/mL, PeproTech) and IL-7 (10 ng/mL, PeproTech) until day 8. Memory-like cells were identified as $CD44^+$ $CD62L^+$. For the exhausted T cell condition, cells were maintained with continued plate-coated anti–CD3 (2 μg/mL) and IL-2 (100 IU/mL) until day 8. Exhausted cells were defined by expression of $TIM\text{-}3^+$ $PD\text{-}1^+$ $LAG\text{-}3^+$.

Bone marrow was harvested from the femurs and tibias of C57BL/6J mice, followed by erythrocyte lysis. Subsequently, the purified bone marrow cells were cultured in T cell medium, supplemented with mGM-CSF (20 ng/mL, T&L Biological Technology, Beijing, China), for 8 to 13 days, refreshing the medium every 2–3 days. After 8 days, nonadherent and loosely adherent cells, identified as iDCs, were harvested and gently washed with DPBS for further experiments.

Lentiviral production and transduction

To produce lentiviruses for the transduction of Jurkat and K562 cell lines, HEK293T cells were cultured in 10 cm dishes and transfected per dish with a viral expression vector (5.625 μg), pMD2.G (1.875 μg, Addgene no. 12259), pRSV-Rev (4.5 μg, Addgene no. 12253), pMDLg/pRRE (4.5 μg, Addgene no. 12251) and PEI (50 μg, Beyotime Biotechnology, Shanghai, China) in 1 mL Opti-MEM (Gibco). The medium was replaced with fresh medium following an 8 h incubation. After 48 h, the supernatant was collected, filtered through a 0.45 μm filter, aliquoted, and stored at −80°C until use.

For the establishment of cell lines overexpressing relevant genes, 150 μL of lentivirus, 3×10^5 target cells, and polybrene (at a final concentration of 8 μg/mL, Beyotime Biotechnology) were added to each well of a 24-well plate and the volume was brought up to 600 μL with culture medium. This was followed by centrifugation (32°C, 1,000 × g, 90 min) and an 18 h incubation. Subsequent to this, the viral medium was removed, cells were cultured in fresh medium, and analyzed via flow cytometry after 2 days, using anti–mouse TCR β chain-PE (anti–mTRBC-PE, clone H57-597) and anti–HLA-A2-APC (clone BB7.2) for detecting TCR expression in TCR Jurkat and HLA-A*02:01 expression in K562-A*02:01 cells, respectively.

Retroviral production and mouse primary T cell transduction

To generate retroviruses for the infection of mouse primary cells, HEK293T cells were cultured in 10 cm dishes and transfected per dish with a retroviral expression vector (25 μg), pCL-Eco (5 μg, Addgene no. 12371), and PEI (50 μg) in 1 mL Opti-MEM. After an 8 h incubation, the medium was replaced with fresh medium. After 48 h, the supernatant was harvested, filtered through a 0.45 μm filter, and treated with Retro-X™ Concentrator (Takara) for overnight concentration. This was then followed by centrifugation at 3,000 × g and 4℃ for 50 min to achieve a 20× concentration, which was then stored at -80℃ for future use. 100 μL concentrated retrovirus was added to 1×10^6 activated T cells in a 24-well plate, maintaining a final polybrene concentration of 8 μg/mL. Following centrifugation at 1,000 × g and 33℃ for 90 min, the supernatant was removed and cells were resuspended in T cell medium containing 100 IU/mL IL-2 (T&L Biological Technology). After 48 h, cells were harvested and analyzed via flow cytometry, with GFP expression utilized to confirm the efficacy of transduction.

General protocol for flow cytometry and cell sorting

Following specific experimental condition treatments, cells were initially incubated with Fc blocking reagent (BioLegend) and Zombie-Aqua™ (BioLegend) at room temperature for 10 min. Subsequently, cells were stained on ice for 30 min with the corresponding flow cytometry antibody panel. Post-staining, cells underwent two washes with FACS buffer consisting of DPBS supplemented with 2% FBS and 1 mM EDTA (Gibco). Cells were acquired on an Agilent NovoCyte Quantum flow cytometer and subsequently analyzed. All the antibodies for flow cytometric analysis and DNA-barcoded antibodies in this paper were purchased from BioLegend if not otherwise specified.

For cell sorting experiments, following the Fc blocking and Zombie-Aqua™ treatment, and staining with the relevant flow cytometry antibody panel on ice, cells were washed twice with FACS buffer and then subjected to sorting for specific marker expression using BD FACSAria™ III Sorter.

PBMCs and monocyte-derived DCs (MoDCs) preparation

Peripheral blood mononuclear cells (PBMCs) from patients were isolated using SepMate™ (STEMCELL Technologies) density-gradient centrifugation and collected PBMCs were used directly or cryopreserved in FBS with 10% DMSO at −80℃. For monocyte isolation, $CD14^+$ monocytes were collected from PBMCs by the EasySep™ Human CD14 Positive Selection Kit II (STEMCELL Technologies) according to manufacturer's protocol and used for the differentiation of MoDCs directly.

For MoDCs differentiation, collected $CD14^+$ monocytes were cultured in T cell medium with hGM-CSF (100 ng/mL, PeproTech) and hIL-4 (125 ng/mL, PeproTech) at 1×10^6 cells/mL, fresh medium and cytokines were added every two days. On day 5, cells were harvested and checked for the expression of CD14, CD209, CD11c and CD83, immature MoDCs were identified as $CD14^-$, $CD83^-$, $CD209^+$, and $CD11c^+$.

Human tissue dissociation for single cell suspension

To prepare single-cell suspension, tumor tissues (lung cancer and bladder cancer), normal lung tissues and dLNs (lung cancer and bladder cancer) were washed twice with FACS buffer and cut into 1–2 mm fragments in a 6 cm dish. The tissues were then resuspended in digesting buffer, which

contained RPMI 1640 supplemented with collagenase I/II/IV (1 mg/mL, BioFroxx) and DNase (50 IU/mL, Sigma-Aldrich) but excluding FBS. Tumor tissues and dLNs were digested for 30 min at 37℃, tumor-adjacent normal lung tissues were digested for at least 1 h. After enzymatic digestion, digesting buffer was removed by centrifugation, and the remaining tissues were mechanically dissociated, and all cell suspension was filtered through a 40 μm cell strainer. The cell suspension was then washed twice with cold T cell medium followed by erythrocyte lysis if necessary and further washed twice to get final single cell suspension.

General protocol for T-SCOPE-based antigen-responsive T cell recording

In this part, general protocol for T-SCOPE was modified by previously published methods PhoXCELL[1].

mgSrtA-mediated ligation

Expression and purification of mgSrtA, as well as the synthesis of DBF-LPETGG, have been described previously[1,2]. Bait cells were resuspended to a density of 1×10^6 cells/mL in FACS buffer and incubated with 100 μM DBF-LPETGG and 10 μM mgSrtA at 37℃ for 30 min. Following the reaction, cells were washed three times with FACS buffer, and catalyst loading was assessed using flow cytometry. Cells were reserved for subsequent experiments.

T-SCOPE capture

DBF-functionalized bait cells (1×10^5) were resuspended in 100 μL of T cell medium and primed with specific antigen at 37℃ or unprimed. After a 1 h incubation, cells were washed with FACS buffer to remove excess peptides and then resuspended in T cell medium. Next, T cells and DBF-bait cells were quantified via flow cytometry, mixed and co-incubated in T cell medium at 37℃ for 2 h unless otherwise mentioned. Subsequently, cells were treated with 100 μM NH_2-Biotin (CAS: 138529-46-1, 9dingchem, Shanghai, China) or BHy (Biotin-PEG_3-hydrazide, CAS: 1381861-94-4, Confluore, Xi'an, China) and exposed to 520 nm laser light for 10 min on ice. Following three washes with FACS buffer, cells were harvested and subjected to flow cytometric analysis.

In this paper, three bait-prey cell pairs were used for T-SCOPE labeling: HLA-A*02:01 expressed K562 (K562-A*02:01) or T2-BFP and 1G4-TCR expressed Jurkat cells; T2-BFP and GP100-TCR expressed Jurkat cells; iDCs and OT-1 T cells. 100 nM NY-ESO-$1_{157\text{-}165}$ (SLLMWITQC, SLL-1G4) was used as the specific antigen for 1G4 Jurkat cells labeled by K562-A*02:01 or T2-BFP; 10 nM $gp100_{280\text{-}288}$ 288V (YLEPGPVTV, YLE-GP100) was used as the specific antigen for GP100 Jurkat cells labeled by T2-BFP. In these two systems, bait-prey cells were mixed as 2:1 (bait cells: prey cells) and co-cultured for 2 h and NH_2-Biotin was used as probe. For labeling of OT-1 T cells, 100 nM ovalbumin $(OVA)_{257\text{-}264}$ (SIINFEKL, N4) or variant peptides (SIITFEKL, T4; SIIVFEKL, V4) were used as specific antigens to be pulsed on DBF-functionalized iDCs, and antigen-primed DBF-iDCs were mixed with OT-1 T cells at a ratio of 2:1 for 2 h co-culturing. BHy here was used as the probe, which has been proved a higher labeling efficiency in singlet oxygen-mediated cell surface proximity labeling reaction[3]. The staining panel was as follows: for 1G4 Jurkat-K562-A*02:01, Zombie-Aqua™, anti–mTRBC-PE, streptavidin-PE/Cy7, and anti–hCD69-APC (clone FN50); For GP100 Jurkat-T2-BFP, Zombie-Aqua™, streptavidin-APC (SA-APC), and anti–hCD69-PE (clone FN50); for DC-OT-1, Zombie-Aqua, anti–mCD8-PB (clone 53-6.7), SA-APC, anti–mCD69-PE (clone H1.2F3) or anti–Nur77-PE (clone 12.14) for exhausted T cells. All the peptides using in this paper were synthesized from GenScript.

pMHC-pentamer staining of T cells

Following resuspension in FACS buffer, OT-1 T cells and 1G4 Jurkat cells were stained with PE-conjugated H-2K^b/OVA$_{257-264}$ MHC pentamer (N4-pentamer, ProImmune) or A*02:01/NY-ESO-1$_{157-165}$ MHC pentamer (SLL-pentamer, ProImmune) at room temperature for 20 min respectively, following the manufacturer's protocol. After the co-incubation, Zombie-Aqua^{TM} and cell surface staining antibodies were added, and the cells were stained on ice for 30 min. For 1G4 Jurkat cells, cells were stained with anti–hCD69-APC. For OT-1 T cells, cells were stained with anti–mCD8-AF700 (clone 53-6.7), anti–mCD19-FITC (clone 1D3/CD19) and anti–mCD69-PerCP/Cy5.5 (clone H1.2F3). Pentamer+ OT-1 T cells were gated by mCD8$^+$, mCD19$^-$ and pentamer+. The stained cells were then analyzed using flow cytometry or cell sorting.

Inhibitor perturbation

To investigate the potential impact of inhibitors on pentamer binding, T cells were co-incubated with inhibitory molecules, maintaining final concentrations of inhibitors, respectively. T cell density was kept within 0.8-1.0 × 10^6 cells/mL. Following incubation at 37℃ for 1 h in T cell medium (BAPTA-AM treated in FACS buffer), pentamer staining was performed. Final concentration of inhibitors: Dasatinib (100 nM, CAS: 302962-49-8, Selleck), Cytochalasin D (10 μM, CAS: 22144-77-0, MCE), RK24466 (10 μM, CAS: 213743-31-8, MCE), PP2 (10 μM, CAS: 172889-27-9, Selleck), Ruxolitinib (1 μM, CAS: 941678-49-5, Selleck), AZD1480 (1 μM, CAS: 935666-88-9, Selleck), BAPTA-AM (10 μM, CAS: 126150-97-8, Selleck).

For examining the impact of these inhibitors on T cell response and T-SCOPE labeling efficiency, the T cells were co-incubated with the inhibitors in T cell medium, maintaining the specified concentrations for 1 h. This was followed by a co-incubation with DBF-functionalized bait cells, under the same inhibitor concentration conditions, to perform T-SCOPE labeling as previously described. For BAPTA-AM, T cells were treated and labeled in FACS buffer.

T-SCOPE workflow in mixed TCR-Jurkat pool

1G4 Jurkat mixed pool

To construct a 1G4 Jurkat mixed pool, 1G4-3 Jurkat cells were stained at room temperature for 10 min with 1 μM CFSE (4A Biotech, Beijing, China) in FACS buffer, while 1G4-2 Jurkat cells were stained with 1 μM cell proliferation dye eFluor^{TM} 450 (Invitrogen) for the same duration. After washing three times with FACS buffer, we mixed 1G4-2, 1G4-3, and untreated ΔTCR Jurkat cells at specific ratios to create the 1G4 Jurkat mixed pool. Next, we co-incubated the mixed pool with DBF-T2 cells at a ratio of 1:1, either peptide-primed with 100 nM SLL-1G4 or left unprimed. Subsequent to co-incubation, the Jurkat mixed pool was labeled according to the standard T-SCOPE labeling protocol. The cells were ultimately harvested and analyzed via flow cytometry, employing a staining panel including Zombie-Aqua^{TM}, anti–mTRBC-PE, SA-APC, and anti–hCD69-PE/Cy7.

GP100 Jurkat mixed pool

To construct a GP100 Jurkat mixed pool, gp-2 Jurkat cells were stained with 1 μM eFluor^{TM} 450 in FACS buffer at room temperature for 10 min, while gp-3 Jurkat cells were similarly stained with 1 μM CFSE. Following staining, cells were washed three times with FACS buffer. Subsequently, gp-2, gp-3, untreated gp-1, and ΔTCR Jurkat cells were mixed in specific ratios to achieve the final GP100 Jurkat mixed pool. T-SCOPE for the GP100 Jurkat mixed pool with DBF-T2-BFP followed the general protocol described above. The flow cytometry staining panel consisted of Zombie-

Aqua™, anti–hCD3-PE, and SA-APC. Gating strategies for different analytical logic are shown in Supplementary Fig. S6.

TCR functional avidity validation

To validate the functionality of the TCRs selected from the SIINFEKL-specific T cell pool using T-SCOPE, various TCR-Jurkat cells were constructed according to previously established protocols. TCR-Jurkat cells (1×10^4) were co-cultured with MC38 cells (2×10^4) in 100 μL of T cell medium, along with SIINFEKL peptide at various final concentrations (10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11}, 10^{-12} mol/L). After an 18 h co-incubation, the cells were washed twice with FACS buffer and subsequently analyzed via flow cytometry. The staining panel consisted of Zombie-Aqua™, anti–mTRBC-PE, and anti–hCD69-APC.

To evaluate the tumor cell recognition and cytotoxicity of the screened TCR-1, we packaged corresponding retrovirus for infecting primary murine $CD8^+$ T cells, resulting in the generation of TCR-T cells expressing either OT-1 TCR or TCR-1. Stable luciferase-expressing MC38-luciferase cells were seeded in a 96-well plate at 10,000 cells per well. After 24 h, TCR-T cells were added at specified effector-to-target ratios of 0.5:1, 1:1, 2:1, 4:1, and 8:1, along with the SIINFEKL antigen peptide at a final concentration of 100 nM. After a 24 h co-incubation, luciferase activity was quantified using the Bio-lite assay kit (Vazyme Biotech, Nanjing, China), in accordance with the manufacturer's instructions.

Bulk RNA-seq and analysis

Fluorescence-activated cell sorting was employed to sort SLL-1G4-primed DBF-K562-A*02:01-labeled 1G4 Jurkat Bio+ and Pentamer-labeled 1G4 Jurkat pentamer+ subgroups. Labeling protocol followed the general protocol as described above. Post-sorting, cells were washed twice with DPBS, followed by cell lysis and mRNA capture using the AccuraCode® HTP OneStep RNAseq Kit (Singleron Biotechnologies, Nanjing, China) according to the manufacturer's protocol. RNA-seq libraries were constructed via reverse transcription (RT) and PCR amplification. High-throughput sequencing was carried out on the NovaSeq6000 platform, with the initial data processed using AccuraCode (v 1.0.0) to derive detailed gene expression matrices.

mRNA analyses were conducted in R. Principal Component Analysis (PCA) was performed using the gmodels package (v 2.18.1.1). Differential gene expression between groups was assessed using DESeq2[4] with significance thresholds set at p value < 0.05 and $|\log_2(\text{Fold Change})| > 1$. Heatmaps were generated using pheatmap (v 1.0.12). Volcano plots and cnetplot visualizations were generated using ggplot2 (v 3.4.1) and clusterProfiler, respectively. GSVA-based gene-set scores were calculated using the GSVA package (v 1.46.0) with TCR signaling transcription factor-related gene sets (Supplementary Table S1).

SIINFEKL-TCR pool preparation

SIINFEKL-specific TCR pool was obtained from mice immunized with OVA protein. C57BL/6 mice were immunized by a prime-boost strategy. For the initial OVA protein immunization, 12 mice were immunized intraperitoneally with OVA protein (50 μg, Sangon Biotech, Shanghai, China) and MnJ adjuvant (50 μg, MnStarter Biotechnology, Jiangsu, China), which were suspended together in DPBS with a final volume of 100 μL. Next, mice were boosted on day 7 and day 14 following the same protocol. On day 21, the mice were sacrificed, and splenocytes were isolated from the spleens

followed by erythrocyte lysis. Approximately 2×10^7 splenocytes were collected from each mouse and stained with N4-pentamer to primarily confirm the proportion of SIINFEKL-responsive T cells. For each mouse, approximately 3% of $CD8^+$ T cells were detected as pentamer+ SIINFEKL-specific T cells.

To obtain SIINFEKL-specific T cell pool, splenocytes from 12 mice were mixed, and $CD8^+$ T cells were isolated using EasySep™ Mouse $CD8^+$ T Cell Isolation Kit (STEMCELL Technologies), following the manufacturer's protocol. Collected $CD8^+$ T cells were stained with N4-pentamer, followed by anti–mCD19-FITC and anti–mCD8-AF700 staining after Fc blocking and Zombie Aqua™ staining, then pentamer+ and pentamer− $CD8^+$ T cells were sorted separately by FACS. 1×10^6 $CD8^+$ T cells were kept as the control group without pentamer sorting. Freshly isolated or unsorted $CD8^+$ T cells were resuspended in T cell medium with IL-2 (200 IU/mL) and soluble anti–mCD28 (0.5 μg/mL), then seeded in the anti–mCD3 pre-treated plates separately at $0.5\text{-}1 \times 10^6$ cells/mL for activation. After 72 h of culturing, cell medium was removed followed by washing twice with cold FACS buffer. For T cell resting, activated T cells were resuspended in T cell medium with IL-2 (20 IU/mL), IL-7 (10 ng/mL), and IL-15 (10 ng/mL) at 1×10^6 cells/mL and cultured for 8-9 days, fresh medium and cytokines were added every two days. Before further experiments, the rested T cells were counted, and the $CD8^+$ proportion, cell viability, pentamer+ proportion were checked by flow cytometric analysis. The prepared SIINFEKL-TCR pool was mixed thoroughly and divided into four groups, three of which were used individually for T-SCOPE-seq, Re-stimu-seq, and pentamer-seq.

T-SCOPE-seq workflow within SIINFEKL-TCR pool

Before performing T-SCOPE-seq, we first tested the T-SCOPE labeling of the SIINFEKL-TCR pool. For the test, T cells from pentamer+, pentamer− and unsorted group were counted, and 20,000 living cells were taken for intercellular labeling test. DBF-iDCs as bait cells were primed with SIINFEKL peptide (0.1 μM or 1 μM for testing, and 1 μM for T-SCOPE-seq) or left unprimed in T cell medium at 37°C for 1 h. Antigen-primed DBF-iDCs were mixed with prepared $CD8^+$ T cells from each group at a ratio of 3:1(iDCs: $CD8^+$ T cells) and T cells were labeled with BHy probe (100 μM) on ice after 2 h incubation at 37°C. After three washes, cells were collected for flow cytometric analysis. If SIINFEKL-responsive T cells were successfully detected in the T cell pool, it was ready for T-SCOPE-seq.

For T-SCOPE-seq, T cells from SIINFEKL-TCR pool were first T-SCOPE labeled as described above, and samples of N4 group and unprimed group were labeled by CLindex™ Sample Multiplexing Kit[5] (SMK, Singleron Biotechnologies, Nanjing, China) according to manufacturer's protocol. Then, cells were washed twice and counted. Cells from N4 group and unprimed group were mixed as a ratio of 5:1. Next, mixed cells were incubated with mouse Fc-blocker and Zombie Aqua™ for 10 min, stained with TotalSeq™-A0952 PE Streptavidin (v/v = 50:1) and anti–mCD8-AF700 for 30 min on ice followed by two washes. $CD8^+$ T cells were sorted from the mixture for single-cell sequencing analysis.

For library preparation, $CD8^+$ T cells were counted and performed on Singleron Matrix® using sCircle™ Single Cell Full Length Immunoreceptor Library Kit (Singleron Biotechnologies, Nanjing, China). Briefly, single-cell isolation, lysis and reverse transcription were performed according to the manufacturer's protocol. To apply 10x role streptavidin barcode in Singleron system, 10x primer-A (TGGAGTTCAGACGTGTGCTCTTCCGATCTCCTTGGCACCCGAGAATTCCA

) was additionally added during cDNA amplification. After cDNA amplification, feature barcoded library for Totalseq™-A0952 PE Streptavidin and SMK tag cDNAs were then separated from transcriptomes by size selection. Each sample yielded an RNA expression library, a V(D)J library, and two feature-barcoded biotin and SMK tag libraries. The RNA expression library and VDJ library construction followed the protocol of sCircle™, while feature barcoded library construction followed the protocol of CLindex™, respectively. Further data were analyzed using the Celescope platform and R.

Workflow of Re-stimu-seq and pentamer-seq

For Re-stimu-seq, T cells from SIINFEKL-TCR pool were mixed with DCs at a ratio of DC:T cells=3:1. 1 μM SIINFEKL peptide was added in culture medium of N4 group and an equal volume of DMSO was added in unprimed group. After 18 h co-culturing, cells were harvested, washed twice and labeled with SMK tag following the protocol of CLindex™ kit. Then, cells were washed twice and counted. Cells from N4 group and unprimed group were mixed at a ratio of 5:1. Next, $CD8^+$ T cells were sorted from the mixture for single-cell sequencing analysis.

For pentamer-seq, T cells from the SIINFEKL-specific TCR pool were first incubated with mouse Fc-blocker and Zombie Aqua™ viability dye. Cells were then stained with PE-conjugated N4-pentamer, followed by incubation with anti–mCD19-FITC, anti–mouse CD8-AF700 and TotalSeq™-A0911 anti-PE antibody (pentamertag, BioLegend; v/v = 50:1 for staining) for 30 minutes on ice. After two washes, $CD8^+$ T cells were sorted for subsequent single-cell sequencing analysis.

Single-cell-based sequencing for Re-stimu-seq and pentamer-seq was adapted from T-SCOPE-seq. Sorted $CD8^+$ T cells were processed on the Singleron Matrix® using the sCircle™ kit, and after cDNA amplification, feature barcoded libraries for SMK tags (Re-stimu-seq) and DNA-barcoded anti-PE (pentamer-seq) were separated from transcriptomes. RNA expression and TCR VDJ libraries followed the sCircle™ protocol, while feature barcoded libraries were prepared with the CLindex™ protocol.

General single-cell sequencing analysis workflow

Single-cell sequencing data processing

Data obtained from the Singleron matrix platform were initially processed using the Celescope pipeline. Single-cell RNA-seq reads were aligned to the mouse reference genome (mm10), while TCR-seq reads were mapped to a VDJ-compatible reference (refdata-cellranger-vdj-GRCm38-alts-ensembl-5.0.0), generating a count matrix for downstream analyses. SMK tag reads were processed within Celescope for UMI counting, with unprimed, N4, multiplet, and undetermined cells defined directly based on the sample barcode and SMK tags.

For quality control and filtering, we calculated the percentage of mitochondrial transcripts for each cell (percent.mt) and excluded cells with fewer than 200 detected genes or greater than 10% mitochondrial RNA reads. The remaining expression values were normalized to 10,000 transcripts per cell and log-transformed using the *NormalizeData* function. Effects of mitochondrial content were regressed out using the *ScaleData* function (vars.to.regress = 'percent.mt'), and the scaled residuals were used for subsequent dimensionality reduction and clustering.

For dimensionality reduction, we selected the top 2,000 variable genes based on cell-to-cell variation using the *FindVariableFeatures* function (selection.method = 'vst'). PCA was performed

using the *RunPCA* function on the scaled data, and the appropriate number of principal components was determined via the *ElbowPlot* function. Non-linear dimensionality reduction was performed using t-SNE (*RunTSNE* function, dims = 1:10). Clustering was conducted using the *FindNeighbors* (dims = 1:10) and *FindClusters* (resolution = 0.5) function.

For decoding of the biotin signal, reads of DNA-barcoded Streptavidin (biotintag) were also primarily analyzed by the Celescope platform for UMI counting following the designed rule. Next, detailed analysis of the Celescope-generated matrix was performed using R and Seurat v4 package[6]. Firstly, the transcriptome and biotintag were integrated into a single Seurat object. Then, the transcriptomes were independently normalized and scaled, while the biotintags were subjected to logarithmic normalization using the following formula: $x_i = \log_{10}(c_i + 1)$, where x represents the normalized data for the biotintag, and c denotes the original count for the biotintag. The pentamertags in pentamer-seq were processed and analyzed using the same computational approach as described for the biotintag.

For scTCR-seq data processing, the filtered contig annotation matrices obtained from the Celescope output were imported into R. The scRepertoire package (v 1.3.5) was employed for the annotation and quantification of TCR clonotypes. TCR clones were defined as the amino acid sequence of their unique complementarity-determining region 3 (CDR3) sequences of both α and β chains. A TCR clonotype that matched only with the CDR3 α or β chain was defined as a new clonotype. Specifically, if a TCR clonotype (A) was matched to only a single CDR3 chain (α or β), but the matched CDR3 sequence was identical to that of a double-chain–matched clonotype (B), clonotype (A) was considered a distinct clonotype rather than being assigned to clonotype (B). For most analysis, cells whose TCR clone size was less than 3 were excluded.

TScore definition

To establish a generalized and transferable strategy for monitoring clonotype-resolved T-cell potency while eliminating batch effects, TScore was defined and calculated via the following three standardized steps: First, to consistently define biotin-positive (Bio+) T cells across independent experiments, the threshold is determined by mapping the gating ratio from wet-lab flow cytometry directly onto the dry-lab single-cell sequencing distribution. Specifically, the exact percentage of positive cells identified via flow cytometry is utilized to calculate the corresponding percentile cutoff for the single-cell sequencing biotin tag values. Cells exceeding this mathematically mapped cutoff are then categorized as Bio+ T cells. Second, T cells were re-grouped into distinct clonotypes using their unique TCR sequences as barcodes. For each individual TCR clonotype, both the percentage of Bio+ cells (Bio+%) and the average normalized intensity (MeanBiotintag) were calculated. To ensure statistical robustness, cells without matched TCR sequences were excluded, and clonotypes with a clone size of less than 3 were filtered out. Third, the TScore for each qualified TCR clonotype was mathematically calculated using the following formula: TScore = MeanBiotintag * (1 + Bio+%). Related information of labeled SIINFEKL-TCR pool was summarized in Supplementary Table S2.

For T-SCOPE-seq in SIINFEKL-TCR pool, based on the gating ratios obtained from flow cytometry (unprimed: 5.58%; N4-primed: 23.8% and 23.1%), a normalized biotin tag cutoff of 1.623 was selected to capture a matching 23.5% Bio+ population within the N4 group in the single-cell sequencing dataset. Individual T cells with values above this cutoff are definitively identified as the Bio+ population. To illustrate the calculation of the TScore, we present a specific TCR clone (CATEAGYQNFYF_CASSLGGSYEQYF) as a representative example. This individual TCR clone

comprises a total of 10 T cells. Among them, 2 T cells exhibit a normalized biotintag value greater than the 1.623 threshold, yielding a Bio+ ratio of 0.2 (20%). The average normalized biotintag value across all 10 T cells within this clone is 1.334791. Consequently, the TScore for this specific TCR clone is calculated using our formula as follows: TScore=1.334791 * (1 + 0.2) = 1.601749.

Pentamer+ TCR definition in pentamer-seq

To define Pentamer+ TCRs, cells were first re-grouped by TCR barcodes, and the mean pentamertag level was calculated for each group. Based on the ranked mean pentamertag levels, TCRs with values greater than 2.0 were defined as Penta+ TCRs, while those with values below 2.0 were defined as Penta− TCRs. For the integrative analysis of pentamer-seq and T-SCOPE-seq, TCR sequence information together with clone size, pentamertag level, and TScore from both datasets were combined. TCRs with clone sizes greater than 2 in both datasets were selected for subsequent detailed analyses (Supplementary Fig. S8e and Table S3).

Combined analysis of T-SCOPE-seq and Re-stimu-seq

Gene expression matrices were generated using the methods described in *General single-cell sequencing analysis workflow*. To integrate cells from T-SCOPE-seq and Re-stimu-seq into a common space, batch effects were corrected using the *Harmony algorithm* (group.by.vars = 'Sample', dims.use = 1:15). The resulting batch-corrected matrix underwent non-linear dimensionality reduction via t-SNE, followed by clustering, marker gene identification, and scTCR-seq data processing, as previously described.

To define TScore in the combined space, TScore for each TCR was first calculated in T-SCOPE-seq as outlined earlier. In Re-stimu-seq, TCRs shared with T-SCOPE-seq were assigned the same TScore, while those not shared were excluded. TCRs were ranked based on their TScore values and divided into four rank groups: TCRs-I (TScore > 2.5), TCRs-II (2.5 ≥ TScore > 2.0), TCRs-III (2.0 ≥ TScore > 1.5), and TCRs-IV (TScore < 1.5). To assess correlations between TScore and gene expression, Spearman's rank correlation (ρ) was performed using the *correlatePairs* function from the Scran package (v1.22.1).

Differentially expressed genes (DEGs) between TCRs-I in T-SCOPE-seq and Re-stimu-seq were identified using the *FindMarkers* function in Seurat. Upregulated genes were selected for Gene Ontology (GO) enrichment analysis using the clusterProfiler (v4.18.4, R package) with the enriched functional genes summarized into gene sets (Supplementary Table S4). For feature score calculation, TCRs detected in both T-SCOPE-seq and Re-stimu-seq (with a clone size > 2 in both assays) were selected. Gene set activity scores were then computed using AUCell (v1.13.1, R package) for each gene set.

T-SCOPE workflow in human samples

T cell preparation

To enrich TILs, tumor single cell suspension was subjected to a discontinuous Percoll gradient (GE Healthcare) centrifugation, where TILs and tumor cells were separated from different layers and collected individually. For T cell purification from dLNs, dLNs single cell suspension was also subjected to Percoll gradient centrifugation and T cells from lymphocyte layer were collected.

The collected T cells were washed twice with cold FACS buffer and cultured in complete T cell medium containing IL-2 (2,000 IU/mL for TILs and 100 IU/mL for T cells from dLNs), 10 ng/mL

IL-7 and 10 ng/mL IL-15 at 1-2 × 10^6 cells/mL.

Lysates preparation

Tumor cells isolated by Percoll gradients and cells from tumor-adjacent normal lung tissues were collected after centrifugation, washed twice with FACS buffer, and counted, followed by resuspending in complete T cell medium at 5-10 × 10^6 cells/mL. To lyse cells, cell suspension was subjected to five rapid freeze-thaw cycles in liquid nitrogen and 37℃ water bath, followed by centrifugation at 5,000 × g for 5 min. The supernatant was collected as cell lysates of tumor and normal lung tissue and stored at −80℃ for antigen priming.

T-SCOPE protocol for tumor-responsive T cells monitoring in human samples

For antigen priming, immature MoDCs were primed with tumor lysates, normal tissue lysates as described above or unprimed overnight, followed by washing with cold FACS buffer and counting. Next, antigen-primed MoDCs were conjugated with DBF on cell surface to generate DBF-DCs by mgSrtA-mediated ligation following the general protocol described above.

After antigen priming and T cells preparation, DBF-DCs and T cells were resuspended in complete T cell medium followed by cell counting. Then, tumor lysate-primed (TL), normal tissue lysate-primed (NT) and unprimed (Un) DBF-DCs were mixed with T cells individually at the ratio of DBF-DCs : total T cells = 2:1, and cultured at 37℃. After 2 h co-culturing, 100 μM NH_2-Biotin probe was carefully added to the culture system, and cells were subjected to 520 nm light irradiation for 10 min on ice. For lung cancer sample for T-SCOPE-seq, Con. group was also added NH_2-Biotin probe and subjected to light irradiation together with other groups, where only TILs were added to the culture system without DBF-DCs mixed. After centrifugation, cell medium was removed, and cells were washed three times at least by cold FACS buffer for further flow cytometric analysis or cell sorting.

For flow cytometric analysis, the labeled cell mixtures were stained with anti–hCD3-FITC, anti–hCD4-PB, anti–hCD8a-PE and SA-APC after Fc blocking and Zombie Aqua™ staining.

T-SCOPE-seq workflow and analysis for human lung cancer TILs

For T-SCOPE-seq workflow with human sample, one lung cancer TIL sample was first labeled, steps of T-SCOPE labeling, antibody staining and results testing were the same as those described above, for which about 1/20 labeled cells were removed after washing for antibody staining and flow cytometric analysis.

Cell Sorting and library preparation

In human T-SCOPE-seq workflow, we expanded our method to droplet-based single-cell RNA-seq and biotintag and SMK tags were designed as the capturing role for commercially available 5′ gel bead-in-emulsions (GEM) beads. After labeling result testing, sample of Con. group was labeled with 5′ SMK tag following the manufacturer's protocol of CLindex™. ssDNA sequence of 5′ SMK tag was designed as the TotalseqC (BioLegend) rules and compatible with 5′ 10x genomics platform, and synthesis of 5′ SMK tag was followed as our previously published protocol[5]. Then, Con. group cells were washed twice and both two group cells were counted. Cells from TL group and Con. group were mixed at a ratio of 2:1. Next, mixed cells were incubated with human Fc-blocker and Zombie Aqua™, stained with TotalSeq™-C0951 PE Streptavidin (v/v = 50:1) and anti–hCD3-FITC for 30 min on ice followed by two washes. $CD3^+$ T cells were sorted from the mixture for single-cell sequencing analysis.

For T-SCOPE-seq in human samples, we generated single-cell-based sequencing with 10x genomics

5′ single-cell RNA-seq strategy. Counted $CD3^+$ T cells were processed on 10x Genomics Next GEM platform, followed by mRNA capturing, reverse transcription, cDNA amplification and feature barcoded cDNA separation. For each sample, RNA expression library, VDJ library and feature barcoded library were all constructed following the protocol of 10x Chromium Single Cell V(D)J Enrichment Kit, in which feature barcoded library of TotalSeq™-C0951 PE Streptavidin and 5′ SMK tag was performed as part of the Cell Surface Protein Library Construction steps. Further data were analyzed using the Cellranger platform and R.

Single-cell sequencing data processing in human samples

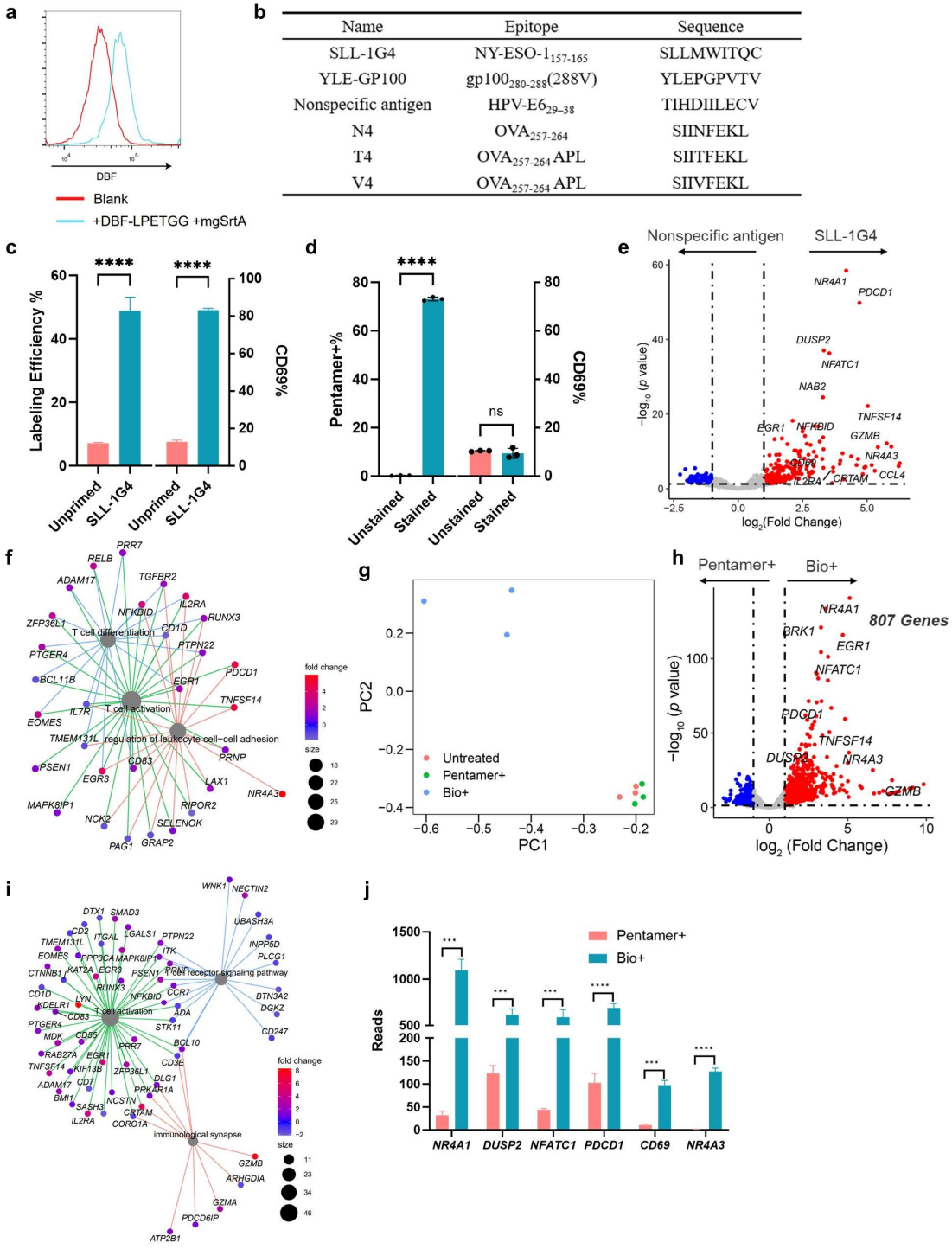
Data processing followed previously described methods in *General single-cell sequencing analysis workflow* unless otherwise instructions. For 10x Genomics platform, data obtained underwent initial processing using Cellranger, and human single-cell TCR-seq reads were mapped to the VDJ compatible reference (refdata-cellranger-vdj-GRCh38-alts-ensembl-5.0.0). The transcriptome, SMK tag and biotintag were integrated into a single Seurat object, and the transcriptomes were independently normalized and scaled. For SMK tag analysis, reads of SMK tag were primarily analyzed by Cellranger platform for UMI counting following the designed rule, and subjected to logarithmic normalization according to the method normalized for biotintag. After comparing SMK tag level in each cell, $\log_{10}$(SMKtag) level of 5.20 was chosen to gate control group cells (Con., over 5.20) from tumor lysate-primed group cells (TL, less than 5.20). To define Bio+ T cells, biotintag level 1.82 was chosen to gate Bio+ T cells from Bio− T cells (Bio+% = 27.5% in $CD8^+$ T cells). TScore definition of $CD8^+$ T cell TCRs was followed the protocol previously described and summarized in Supplementary Table S5. Gene expression matrix analysis was similar to that was previously described. For special annotation in human sample process, cells were not filtered before dimensionality reduction. After reducing dimensionality, matrix was used to non-linear dimensional reduction by *RunTSNE* (dims = 1:15) function, and cells were clustered using the *FindNeighbors* (dims = 1:15) and *FindClusters* (resolution = 0.9) functions. *FindAllMarkers* function in Seurat v4 package was used to find out highly expressed genes in each cluster. Cell types were annotated according to the expression of canonical markers in each cluster. After cell type annotation, clusters of low-quality cells, myeloid cells and γδ T cells were filtered.

As previously described, TScore was defined for $CD8^+$ TIL TCRs (clone size > 2), and the corresponding TScore values for these T cells were visualized on t-SNE plots. To assess gene expression changes before and after T-SCOPE labeling, TCRs with a clone size greater than 20 were selected. Differential expression analysis was performed to compare the same TCRs between the Con. and TL group (Supplementary Fig. S13d). Functional categories from GO enrichment analysis of upregulated genes were summarized into gene sets (Supplementary Table S6). Gene set activity scores and tumor-specific gene signature scores were calculated separately using AUCell.

Statistical analysis

Statistical analyses were performed using R and GraphPad Prism. Data are presented as mean ± SEM unless otherwise indicated, with n = 3 biological replicates per group. For **Fig. 1**, two-tailed unpaired *t*-tests were used in panels **c**, **d**, and **j**, and statistical correlation analysis in panel **o** was assessed by linear regression. The strength of the association was quantified by the Pearson correlation coefficient (*r*), and the statistical significance of the regression model was determined using an ANOVA *F*-test via analysis of variance. Information on other specific statistical tests for each analysis is provided in the corresponding figure legends and/or method descriptions. Statistical

significance was defined as ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.



Supplementary Fig. S1 T-SCOPE labeling induces activation-associated transcriptional programs.

a Flow cytometric plot showing the mgSrtA-mediated ligation of DBF-LPETGG on K562-A*02:01. Fluorescent signal of DBF was detected by the FITC channel of flow cytometry.

b Summary of epitope sequences used in this study.

c Bar plot of the T-SCOPE-yielded labeling efficiency (left) and expression level of CD69 (right) on 1G4-3 Jurkat cells when labeled by DBF-K562-A*02:01 primed SLL-1G4 or unprimed. For T-SCOPE, DBF-K562-A*02:01 were peptide-pulsed, co-cultured with 1G4-3 Jurkat at 2:1 (bait: prey) for

2 h at 37°C, then irradiated at 520 nm for 10 min labeling on ice before acquisition. Two-tailed unpaired *t*-test.

d Bar plot of the staining of SLL-pentamer (left) and expression level of CD69 (right) on 1G4-3 Jurkat cells. Two-tailed unpaired *t*-test.

e Volcano plot of differentially expressed genes of 1G4-3 Jurkat cells when stimulated by DBF-K562-A*02:01 primed SLL-1G4 (right, red) and nonspecific antigen (left, blue). Significance was determined as p value < 0.05 and $|\log_2(\text{Fold Change})| > 1$.

f Gene ontology (GO) enrichment analysis of upregulated genes in antigen-responsive labeled 1G4-3 Jurkat cells (in **e**). Three representative enriched functions are shown here, including T cell activation, T cell differentiation, and regulation of leukocyte cell-cell interaction.

g PCA (Principal Component Analysis) of 1G4-3 Jurkat cells transcriptomes post T-SCOPE labeling (Bio+, green), SLL-pentamer staining (pentamer+, blue), or untreated (pink). Dots represent samples of the three different populations, each analyzed across three biological replicates. Axes PC1 and PC2 denote the principal sources of variation.

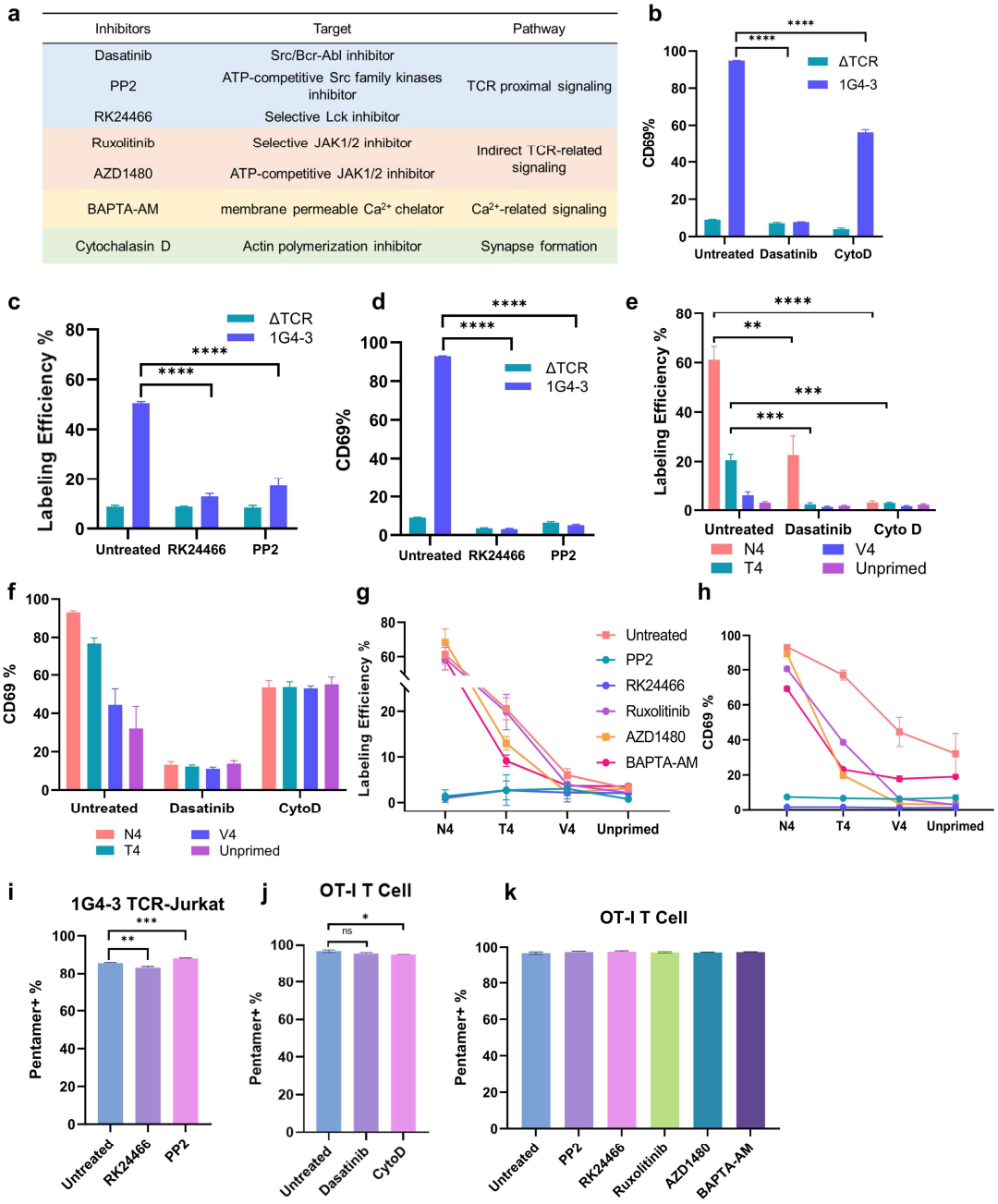
h Volcano plot illustrating differentially expressed genes (DEGs) between Bio+ (red) and SLL-pentamer+ (blue) 1G4-3 Jurkat cells, based on bulk RNA-seq of FACS-sorted Bio+ and pentamer+ populations. Significance was determined as p value < 0.05 and $|\log_2(\text{Fold Change})| > 1$ was considered significant.

i GO enrichment analysis of upregulated genes in Bio+ 1G4-3 Jurkat cells compared with pentamer+ 1G4-3 Jurkat cells (in **h**).

j Expression levels of representative genes related to TCR signaling induced by T-SCOPE labeling and SLL-pentamer staining in 1G4-3 Jurkat cells, quantified from bulk RNA-seq. Two-tailed unpaired *t*-tests.

The data are presented as the mean ± standard error of the mean across three biological replicates per group in parts **c**, **d**, **j**.

The sequencing data presented in parts **e** and **h** represented three biological replicates, with the mean expression level calculated for each group.



Supplementary Fig. S2 Perturbation of TCR signaling impairs T-SCOPE labeling.

a Summary table showing the information of inhibitors used for T cell signaling perturbation.

b Bar plot showing CD69 expression levels on 1G4-3 Jurkat cells after inhibitor treatment (dasatinib and CytoD), following co-incubation with SLL-1G4-primed DBF-K562-A*02:01. Two-tailed unpaired *t*-tests.

c Bar plot showing the antigen-responsive capturing of 1G4-3 Jurkat cells after TCR proximal signaling perturbation (RK24466 and PP2). Two-tailed unpaired *t*-tests.

d Bar plot showing CD69 expression levels on 1G4-3 Jurkat cells after inhibitor treatment (RK24466 and PP2), following co-incubation with SLL-1G4-primed DBF-K562-A*02:01. Two-tailed unpaired *t*-tests.

e Bar plots showing the T-SCOPE-labeled Bio+ proportion of mouse OT-1 T cells after a 1 h pre-incubation at 37°C with dasatinib and Cyto D. OT-1 T cells were labeled by mouse immature DCs

(iDCs) primed with 100 nM SIINFEKL peptide variants (N4: SIINFEKL, T4: SIITFEKL, V4: SIIVFEKL). Two-tailed unpaired *t*-tests.

f Summary of flow cytometric analysis of T cell activation (expression of CD69) when OT-1 T cells were disrupted by dasatinib and CytoD.

g, h Summary of antigen-responsive capturing (**g**) and T cell response (expression of CD69, (**h**)) when OT-1 T cells perturbed with TCR proximal signaling inhibitors (PP2 and RK24466) and indirect TCR signaling inhibitors (Ruxolitinib, AZD1480 and BAPTA-AM).

i Bar plot showing the SLL-pentamer+ proportion of 1G4-3 Jurkat cells after TCR proximal signaling perturbation (RK24466 and PP2). Two-tailed unpaired *t*-tests.

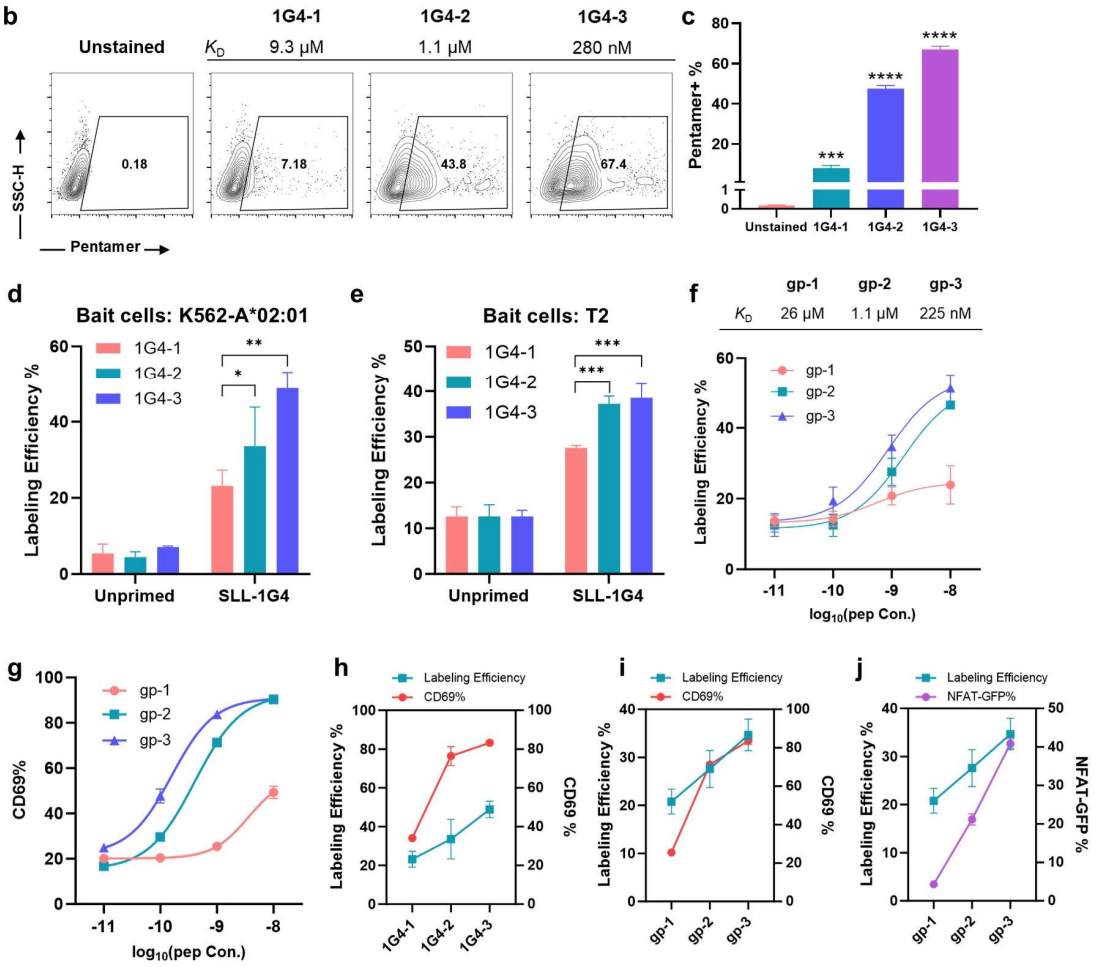
j Bar plot showing the N4-pentamer+ proportion of OT-1 T cells after the same inhibitor treatment in **e**. Two-tailed unpaired *t*-tests.

k Bar plot showing the N4-pentamer+ proportion of OT-1 T cells after inhibitors perturbation, as used in **g** and **h**.

The data are presented as the mean ± standard error of the mean across three biological replicates per group in parts **b-k**.

a

Name	CDR3α	CDR2β	CDR3β
1G4-1	CAVRPTSGGSYIPT	GAGI	CASSYVGNTGELFF
1G4-2	CAVRPTSGGSYIPT	GIGI	CASSYVGNTGELFF
1G4-3	CAVRPTSGGSYIPT	AIGI	CASSYVGNTGELFF
gp-1	CATDGDTPLVFGKG	SQIVND	CASSIGGPYEQYF
gp-2	CATDGDTPLVFGKG	SQIVND	CASSWGAPYEQYF
gp-3	CATDGDTPLVFGKG	SWITGD	CASSIGGPYEQYF



Supplementary Fig. S3 T-SCOPE labeling correlates with TCR avidity across multiple systems.

a Summary table of relevant TCR sequences.

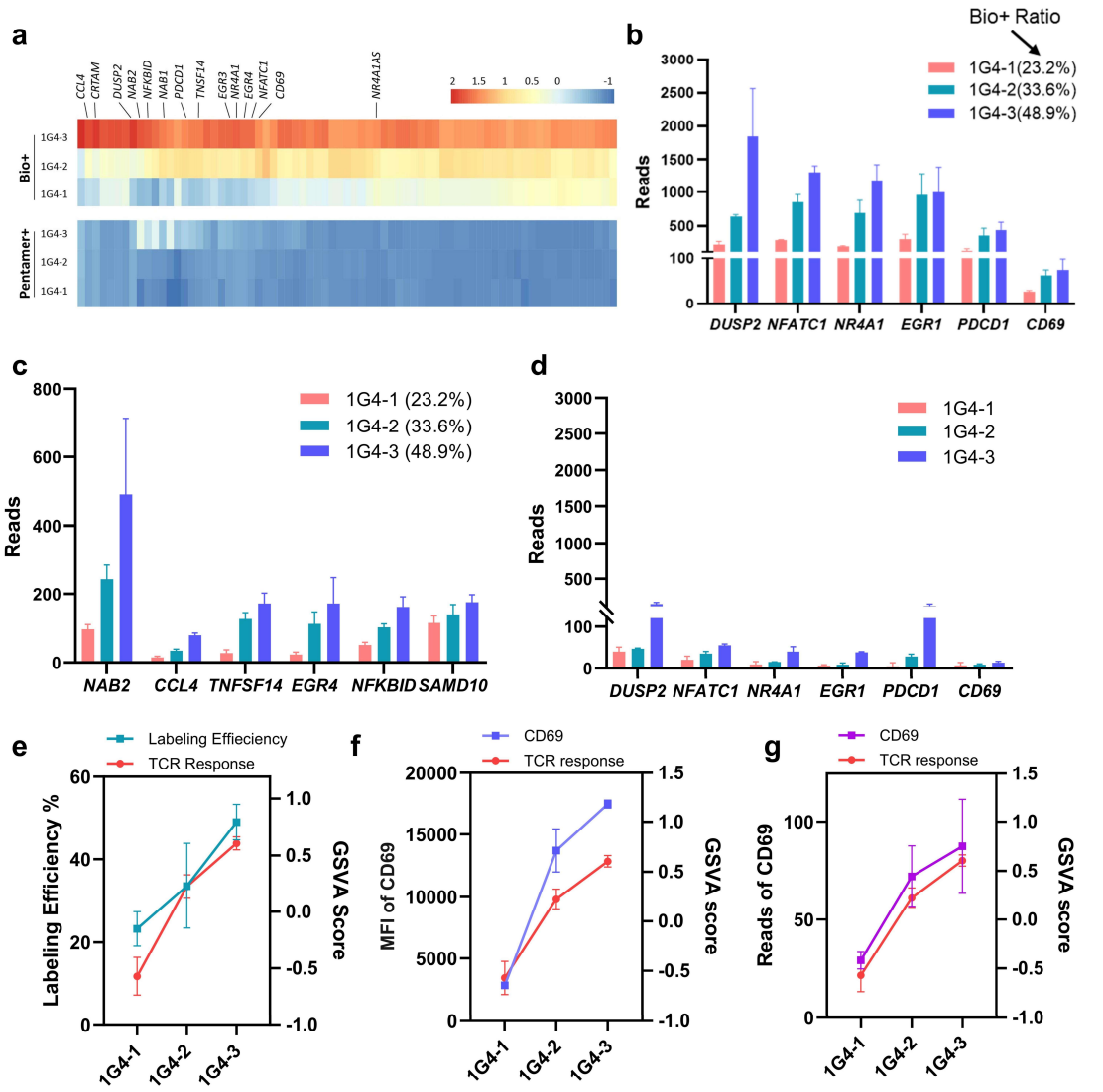
b Flow cytometry analysis of the proportion of SLL-pentamer+ cells in variant 1G4 Jurkat populations.

c Bar plot showing the result of SLL-pentamer+ proportions in variant 1G4 Jurkat cells. Statistical significance was determined relative to Unstained within each condition. Two-tailed unpaired *t*-tests.

d, e Bar plots showing the T-SCOPE-induced biotinylation ratio of 1G4 Jurkat cells by DBF-K562-A*02:01 (**d**) or T2 (**e**) primed with 100 nM SLL-1G4 epitope or unprimed, using 1G4-1, 1G4-2 and 1G4-3 separately under identical labeling conditions. Two-tailed unpaired *t*-tests.

f Dose-response curves showing the T-SCOPE-labeled Bio+ ratio on Jurkat cells with GP100-specific TCRs (GP100 Jurkat cells henceforth). YLE-GP100-primed DBF-T2 cells were co-cultured with GP100 Jurkat cells at a 2:1 (bait: prey) ratio for 2 h at 37°C, and then labeled for 10 min on ice under 520 nm irradiation. YLE-GP100 was titrated at 10 nM, 1 nM, 100 pM, and 10 pM.

g Curve graph showing the expression of CD69 on GP100 Jurkat cells induced by YLE-GP100-primed DBF-T2. YLE-GP100 (10 nM, 1 nM, 100 pM, 10 pM) were presented on T2 followed by co-culture with GP100 Jurkat cells.
h, i Line graphs showing that T-SCOPE labeling efficiency correlates with T cell activation, as measured by CD69 expression, in 1G4 Jurkat cells labeled by SLL-1G4–primed (10 nM) K562 cells (**h**) and GP100 Jurkat cells labeled by YLE-GP100–primed (1 nM) T2 cells (**i**).
j Line graph showing that T-SCOPE labeling efficiency was correlated with NFAT–GFP reporter expression when GP100 Jurkat cells stably expressing an NFAT–GFP reporter were stimulated by YLE-GP100–primed (1 nM) T2 cells. The GFP signal (mean fluorescence intensity [MFI]) was quantified by flow cytometry under the same co-culture and labeling conditions as in **i**.
The data were presented as the mean ± standard error of the mean across three biological replicates per group in parts **c-j**.



Supplementary Fig. S4 T-SCOPE labeling associates with graded transcriptional activation programs.

a Gene expression profiles highlight key genes associated with TCR activation in Jurkat cells expressing 1G4 TCRs of various avidities. For comparative analysis, 1G4 Jurkat cells were subjected either to T-SCOPE labeling or to SLL-pentamer staining, and the corresponding Bio+ and SLL-pentamer+ populations were FACS sorted for bulk RNA-seq. Representative DEGs are shown in the heatmap.

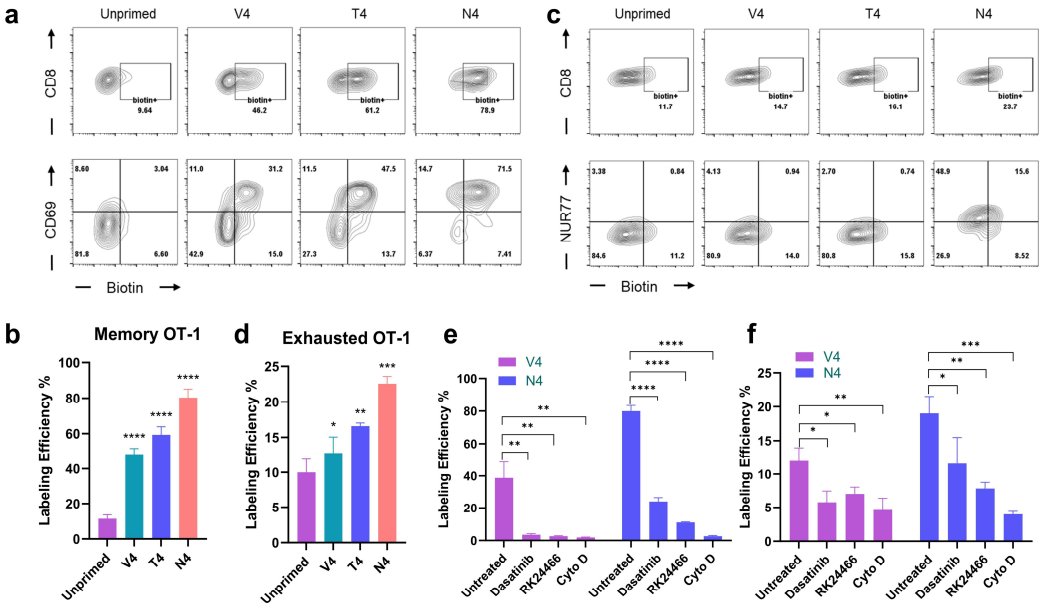
b Summary of the expression of representative genes related to TCR activation induced by T-SCOPE labeling, which was correlated with the avidity of each 1G4 TCR.

c Bar plot showing the expression levels of selected genes induced by T-SCOPE, correlated with the avidity of each 1G4-TCR. Significant differences ($p < 0.05$) relative to 1G4-1 TCR were observed in *NAB2*, *CCL4*, *TNFSF14*, *EGR4*, and *NFKBID*. Two-tailed unpaired *t*-tests.

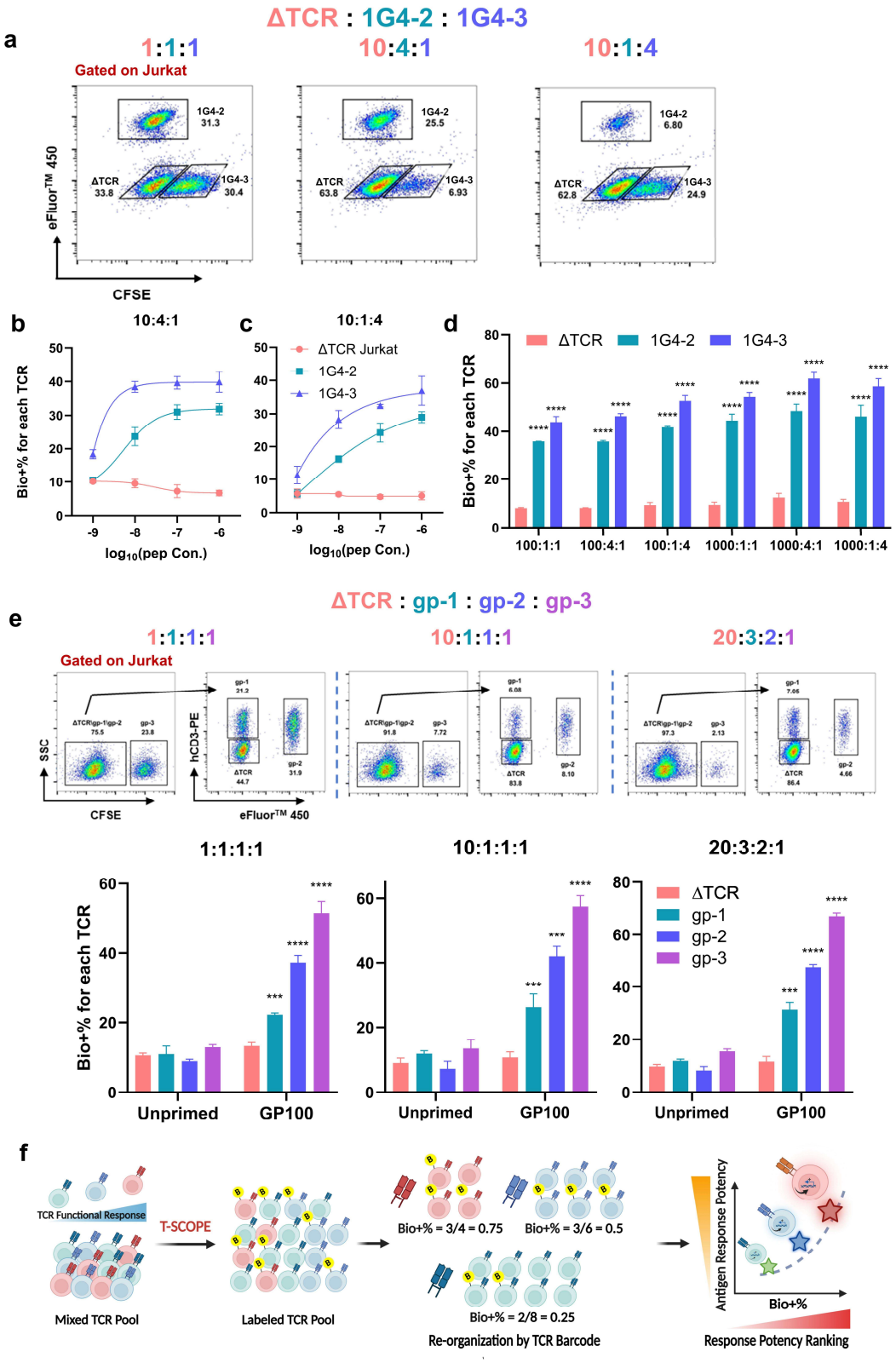
d Bar plot showing the expression levels of selected genes induced by SLL-pentamer staining. Significant differences ($p < 0.05$) relative to 1G4-1 TCR were observed in *PDCD1*. Two-tailed unpaired *t*-tests.

e Line graph showing the percentage of Bio+ 1G4 Jurkat cells of various avidities after T-SCOPE labeling and TCR response scoring, using a manually curated TCR-response gene set for scoring. The gene set is summarized in Supplementary Table S1.

f Line plot showing the TCR response score and Bio+% of 1G4 Jurkat cells with various avidities after T-SCOPE labeling, analyzed based on GSVA.
g Line plot showing the TCR response score and surface protein expression of CD69 on 1G4 Jurkat cells with various avidities after T-SCOPE labeling, analyzed based on GSVA.
The data are presented as the mean ± standard error of the mean across three biological replicates per group in parts **b-g**.
The sequencing data presented in part **a** represented three biological replicates, with the mean expression level calculated for each group.



Supplementary Fig. S5 T-SCOPE labeling of memory-like and exhausted OT-1 T cells.
a-d Representative flow cytometric plots (**a**, **c**) and summarized bar plots (**b**, **d**) showing T-SCOPE labeling of (central) memory OT-1 (**a**, **b**) and exhausted OT-1 (**c**, **d**) T cells. DBF-iDCs were pulsed with 100 nM N4, T4, V4 peptides or not (unprimed), followed by co-incubation with OT-1 T cells. Statistical significance was determined relative to Unprimed within each condition in (**b**, **d**). Two-tailed unpaired *t*-tests.
e, f Bar plots showing that T-SCOPE capturing of memory (**e**) and exhausted (**f**) OT-1 T cells by N4-primed DBF-iDCs can also be decreased by TCR proximal signaling and synapse formation perturbation. Two-tailed unpaired *t*-tests.
The data are presented as the mean ± standard error of the mean across three biological replicates per group in parts **b, d, e, f**.



Supplementary Fig. S6 T-SCOPE resolves TCR avidity in a mixed TCR pool.

a Representative flow cytometric plots showing the detailed gating strategy for each subpopulation and validating the indicated mixing proportions of different 1G4 Jurkat cells.

b, c Curve graphs showing the T-SCOPE-defined Bio+% for 1G4-2, 1G4-3, and ΔTCR Jurkat cells within the model TCR pool. T2 cells were used as APCs pulsed with different antigen concentrations, and Jurkat cells expressing 1G4-2, 1G4-3, or ΔTCR were mixed at varying ratios to generate

different T-cell pools. ΔTCR Jurkat cells with the TCR β chain knocked out served as a negative control.

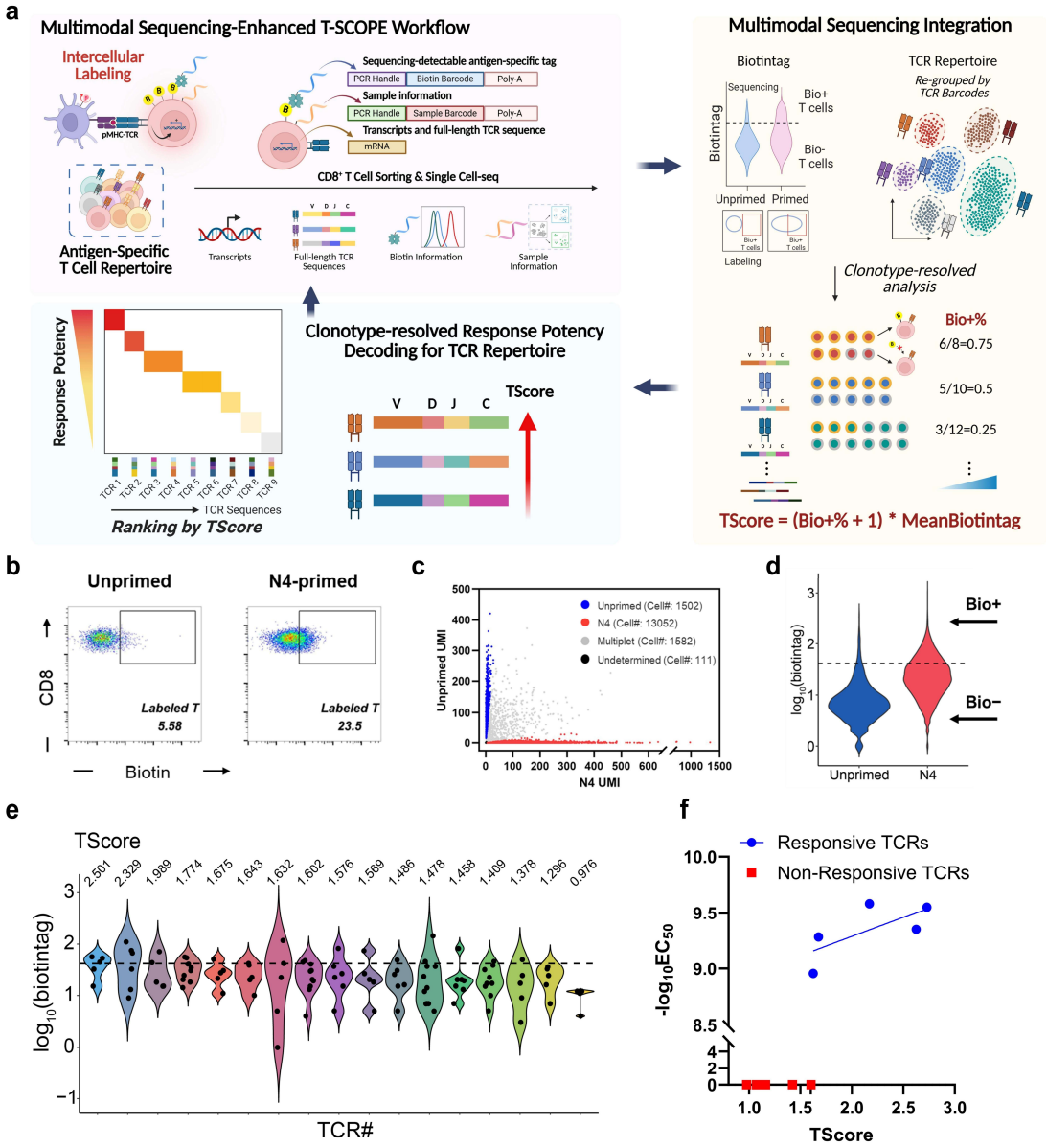
d Bar plot showing the T-SCOPE-defined Bio+% for 1G4-2, 1G4-3, and ΔTCR Jurkat cells within the model TCR pool under different TCR mixing ratios (ΔTCR:1G4-2:1G4-3), as indicated on the x-axis. Statistical significance was determined relative to ΔTCR under each condition. Two-tailed unpaired *t*-tests.

e Representative flow cytometric plots showing the detailed gating strategy for each subpopulation and validating the indicated mixing proportions of different GP100 Jurkat cells (top).Bar plots displaying the T-SCOPE-labeled Bio+% for GP100 Jurkat cells within GP100 TCR pool, comprising varying proportions of gp-1, gp-2, and gp-3 TCR Jurkat, and ΔTCR Jurkat, which marked by different colors (bottom). Statistical significance was determined relative to ΔTCR within each condition. Two-tailed unpaired *t*-tests.

f Schematic illustration of T-SCOPE ranking TCR functional responses within a T-cell pool based on the Bio+ ratio.

The data were presented as the mean ± standard error of the mean across three biological replicates per group in parts **b-e**.

Part **f** was created by BioRender.



Supplementary Fig. S7 T-SCOPE-seq defines clonotype-level TScore within SIINFEKL-specific TCR repertoire.

a Schematic illustration of the T-SCOPE-seq workflow and multimodal signal integration for defining the TScore within a TCR pool. In this workflow, the T-SCOPE-induced Bio tag is converted into a sequencing-readable DNA barcode using DNA-barcoded streptavidin. Distinct DNA sample barcodes are conjugated to the cell surface to distinguish the unprimed and N4-primed (labeled) groups. For signal translation into a bead-based single-cell capture method, the Bio and sample barcodes are inserted separately between the PCR handle and poly-A. After sequencing, multimodal information for the transcript, TCR, Bio, and sample are integrated, with cells in the N4 group separated from those in the unprimed group using the SMK tag, and Bio+ cells are defined according to the detected biotintag level. Then, cells in the N4 group are regrouped by TCR sequence, and the TScore for each TCR is computed as follows: TScore = (Bio+% + 1) × MeanBiotintag.

b Representative plots showing the T-SCOPE labeling results of the SIINFEKL-TCR pool derived from OVA-immunized C57BL/6J mice. DBF-labeled iDCs, either pulsed with SIINFEKL peptide

(1 μM) or unprimed, were used to capture the SIINFEKL-TCR pool. The iDCs and $CD8^+$ T cells were co-cultured at 3:1 (iDCs: T cells) for 2 h at 37°C, after which they were irradiated at 520 nm for 10 min on ice.

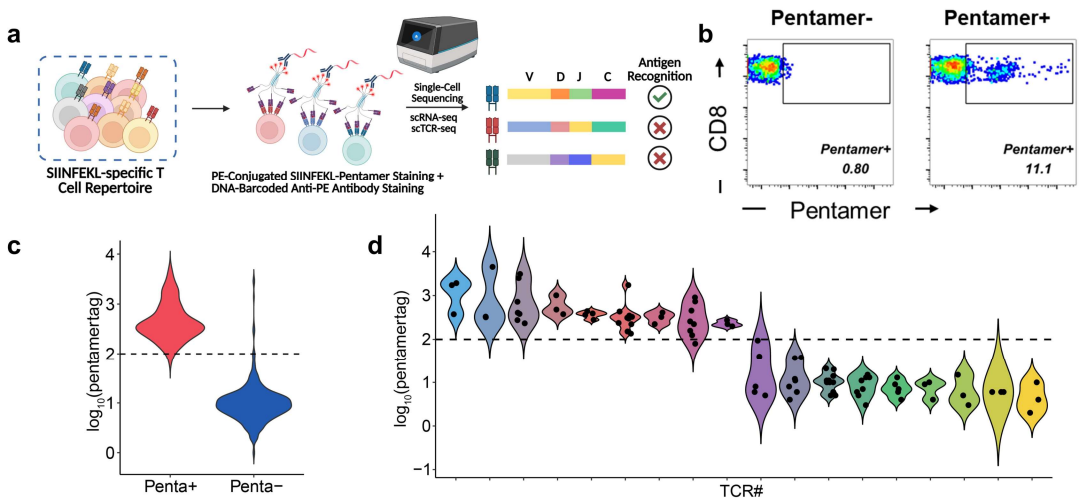
c The unique molecular identifier (UMI) counts of N4-primed (*x*-axis) and unprimed (*y*-axis) cells associated with each SMK barcode of a given cell (dot).

d Violin plot showing the detection level of Bio in unprimed and N4-labeled T cells. Cells in the N4 group with Bio levels above 1.623 were classified as Bio+, while those below this threshold were defined as Bio−.

e Violin plot showing the $\log_{10}$(biotintag) distribution of representative TCRs, based on biotin detection levels in cells expressing the same TCR. The proportion of Bio+ cells was used to calculate Bio+%, and the mean value of $\log_{10}$(biotintag) was defined as MeanBiotintag for each TCR.

f Scatterplot showing the correlation between the TScore and functional avidity (EC_{50}) for representative SIINFEKL-TCRs. TCRs were engineered on ΔTCR Jurkat cells and co-incubated with H-$2K^b$–expressing mouse MC38 cells at a 1:1 ratio for 18 h under a peptide titration of 10^{-5}–10^{-12} M SIINFEKL peptide. CD69 expression was quantified as the MFI, and EC_{50} values were calculated from CD69 dose-dependent response curves. Non-responsive TCRs without a calculable EC_{50} were plotted at $-\log_{10}(EC_{50}) = 0$ (red). Ten TCRs were tested, showing a positive trend between the TScore and functional avidity among the five responsive TCRs (TScore > 1.62, $p = 0.1933$).

Part **a** was created by BioRender.



e

No.	CDR3 α_β	log_{10}(pentamertag)	Clone Size in pentamer-seq	Penta_Category	Clone Size in T-SCOPE-seq	TScore
1	NA_CASSRTGKVFF	2.821450771	7	Penta+	4	2.704637
2	CALGDYNYAQGLTF_CASSDRHNYAEQFF	2.4927876	3	Penta+	3	2.170597
3	CAAGAATGYQNFYF_CASSFPDWGGPGQNTLYF	2.497997282	11	Penta+	5	1.67512
4	NA_CASSFPDWGGPGQNTLYF	2.430289763	8	Penta+	3	1.326637
5	CAMREGDNYNVLYF_CASSLRGNYAEQFF	0.8177584	6	Penta-	3	2.854637
6	CALTGNTGKLIF_CASSPDRANTGQLYF	1.002568545	11	Penta-	4	2.406734
7	NA_CASSPGGADTGQLYF	0.7999388	4	Penta-	4	2.346343
8	CAVSAAGNTGKLIF_CASIQGGALYF	0.933113533	3	Penta-	4	1.68399
9	CAVRNNNNAPRF_CASSLVGDSDYTF	0.92165905	4	Penta-	4	1.614454
10	CAVEATGGNNKLTF_CASGDNAEQFF	0.9854772	4	Penta-	4	1.613674
11	CAASNYAQGLTF_CASSRDLGGGNTEVFF	1.188396683	6	Penta-	4	1.606212
12	NA_CASSDLLGHQNTLYF	0.802746667	3	Penta-	7	1.45792
13	CALSDRFVGDNSKLIW_CAWSPGRGDTGQLYF	0.984128425	4	Penta-	4	1.438556
14	NA_CASSRQGEYAEQFF	0.7611004	3	Penta-	3	1.377931
15	CVLGDTNAYKVIF_CASSFPNSGNTLYF	0.962315	5	Penta-	4	1.270373
16	CAMRTNYGNEKITF_CASSLRANTEVFF	1.007476167	3	Penta-	3	1.228998
17	NA_CASGEDWGSQNTLYF	0.80103	4	Penta-	3	1.188301
18	CAASSSAGNKLTF_CASSPEQSAETLYF	1.125797825	4	Penta-	4	1.160673
19	CAMREGEKGGRALIF_CASGHWGGGAETLYF	0.8450528	4	Penta-	4	1.072174
20	CAMREVDSNYQLIW_CASSLDYEQYF	1.026393767	3	Penta-	3	0.971095

Supplementary Fig. S8 Pentamer-seq comparison with T-SCOPE-seq in the SIINFEKL-specific TCR pool.

a Schematic illustration of pentamer-seq.

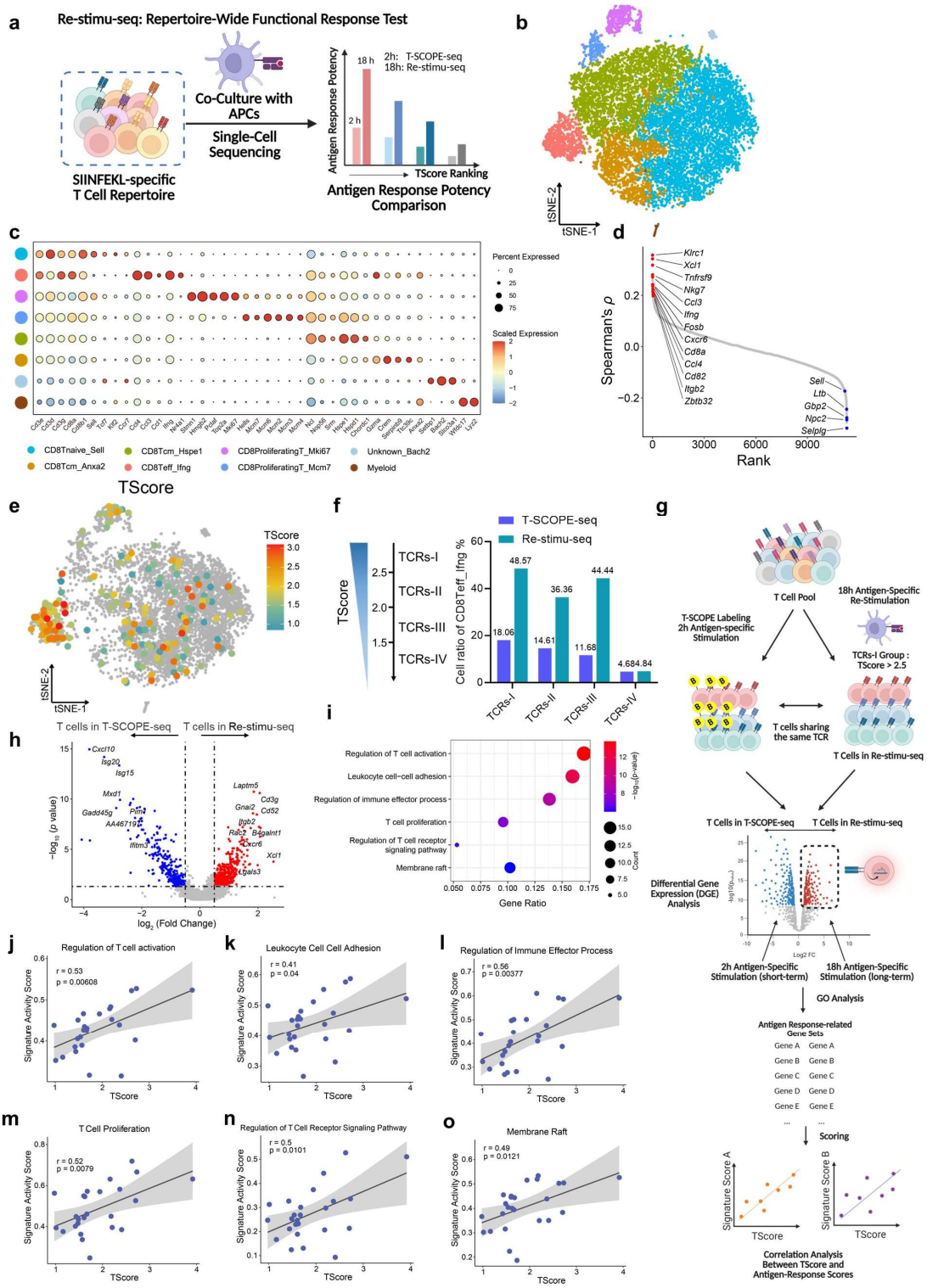
b Flow cytometric plots showing the N4-pentamer staining results of SIINFEKL-TCR pool.

c Violin plot showing the detection levels of pentamertag in pentamer-seq. TCRs with a mean log_{10}(pentamertag) value ≥ 2.0 were defined as Penta+, while those with a mean value < 2.0 were defined as Penta−.

d Violin plot showing the log_{10}(pentamertag) distribution of representative TCRs, based on pentamertag detection levels in cells expressing the same TCR.

e Table summarizing the combined TCR analysis of pentamer-seq and T-SCOPE-seq, presenting clone size information and corresponding pentamertag intensity and TScore for overlapping TCR clonotypes identified by both assays.

Part **a** was created by BioRender.



Supplementary Fig. S9 Re-stimu-seq validates TScore-associated antigen response programs.

a Schematic illustration of combined analysis of T-SCOPE-seq with Re-stimu-seq for the same SIINFEKL-TCR pool, for which the TScore of TCRs was defined in the T-SCOPE assay and compared to the T cell response analysis conducted for the same TCR rank group across two assays. Re-stimu-seq was performed by co-culturing the same SIINFEKL-specific T-cell pool with iDCs primed with 1 μM SIINFEKL for 18 h, followed by sorting $CD8^+$ T cells for single-cell sequencing.

b t-SNE plot showing combined expression profiles of T cells in T-SCOPE-seq and Re-stimu-seq. Immune cell subsets, defined by 8 unique clusters, were annotated and marked by color code and shown in **c**.

c Bubble plot showing normalized expression of selected marker genes for the main subtypes defined in **b**. Bubble color represents mean expression level, and bubble size indicates the proportions of cells expressing the genes.

d Correlation (Spearman's ρ) between TScore and normalized gene expression, calculated for each gene over T cells defined by TScore in the Re-stimu-seq data. Selected up-correlated and down-correlated genes were highlighted separately.

e t-SNE plot showing the TScore distribution of T cells in the Re-stimu-seq data. The TScore was defined using T-SCOPE-seq data and mapped onto Re-stimu-seq data using identical TCR sequences. Cells are colored by their TScores.

f Bar plot showing the proportion of cluster CD8Teff_Ifng among the T cells in T-SCOPE-seq and Re-stimu-seq datasets grouped by TScore.

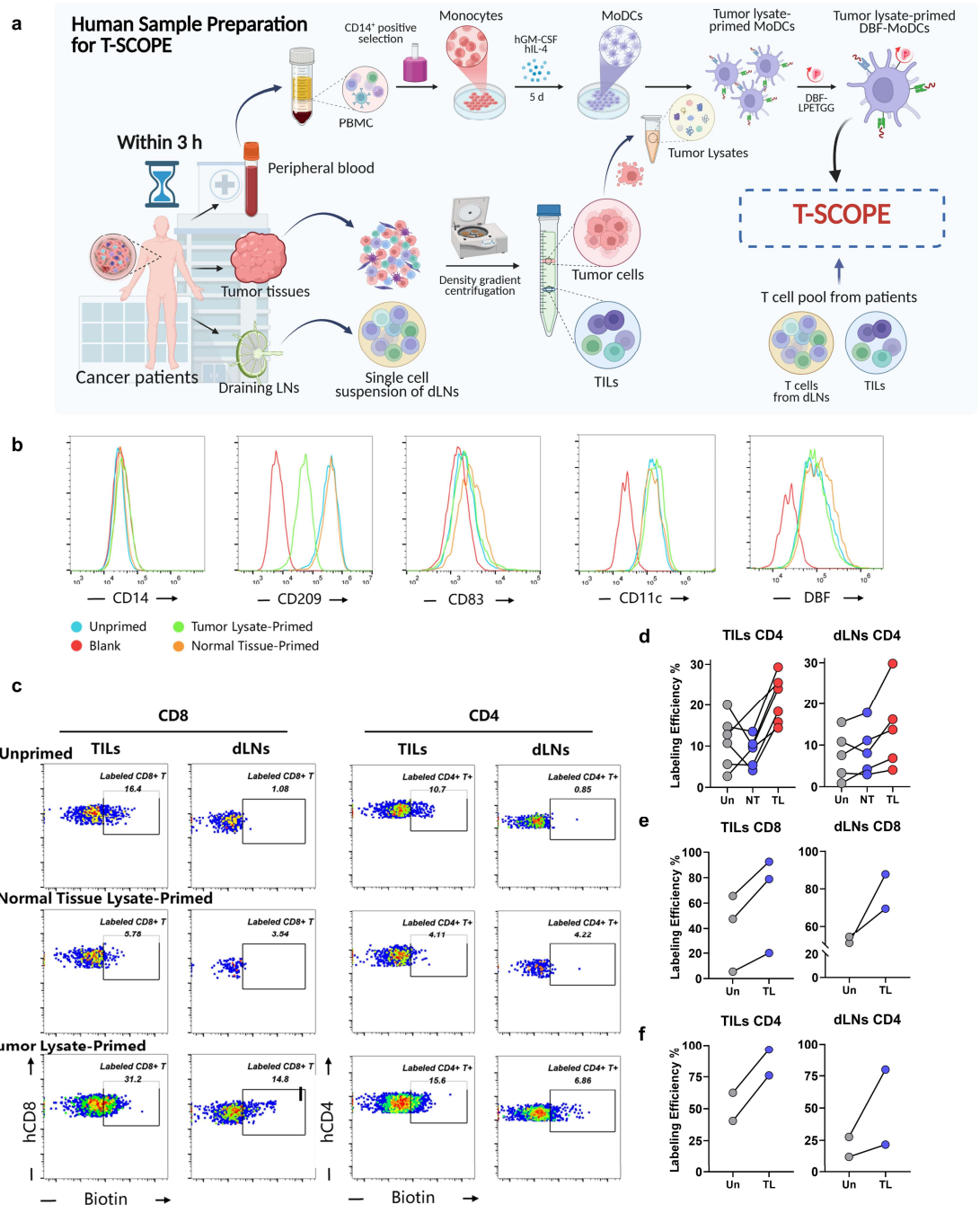
g Schematic overview of the analytical workflow used to establish the correlation between TScore and antigen-induced activation potency from single-cell sequencing data. Differential gene expression analysis was performed on high-TScore TCRs between T cells from T-SCOPE-seq and Re-stimu-seq. Genes upregulated in Re-stimu-seq were subjected to subsequent GO enrichment analysis. The genes associated with the enriched GO terms were grouped into corresponding functional gene sets, which were then used to calculate a functional score for each TCR. Finally, the relationship between the functional score and the TScore was analyzed.

h Volcano plot showing differentially expressed genes of TCRs-I T cells (TScore > 2.5) between T-SCOPE-seq and Re-stimu-seq. Significance was determined as p value < 0.05 and $|\log_2(\text{Fold Change})| > 0.5$.

i GO enrichment analysis of the top 100 upregulated DEGs between the Re-stimu-seq and T-SCOPE-seq datasets in TCRs-I T cells (TScore > 2.5). Related to **h**.

j-o Scatterplots showing the correlation between TScore and activity scores for altered pathways of selected TCRs in Re-stimu-seq. Gene sets of related functional pathways were derived from GO analysis in **i** and summarized in Supplementary Table S4. Each dot represents a selected TCR found in both T-SCOPE-seq and Re-stimu-seq, with a clone size > 2 in both datasets. (**j**) T Cell Activation, (**k**) Leukocyte Cell-Cell Interaction; (**l**) Regulation of Immune Effector Process; (**m**) T Cell Proliferation; (**n**) Regulation of T Cell Receptor Signaling Pathway; (**o**) Membrane Raft. ANOVA F-tests.

Part **a** and **g** were created by BioRender.



Supplementary Fig. S10 T-SCOPE captures tumor-responsive T cells in human cancer samples.

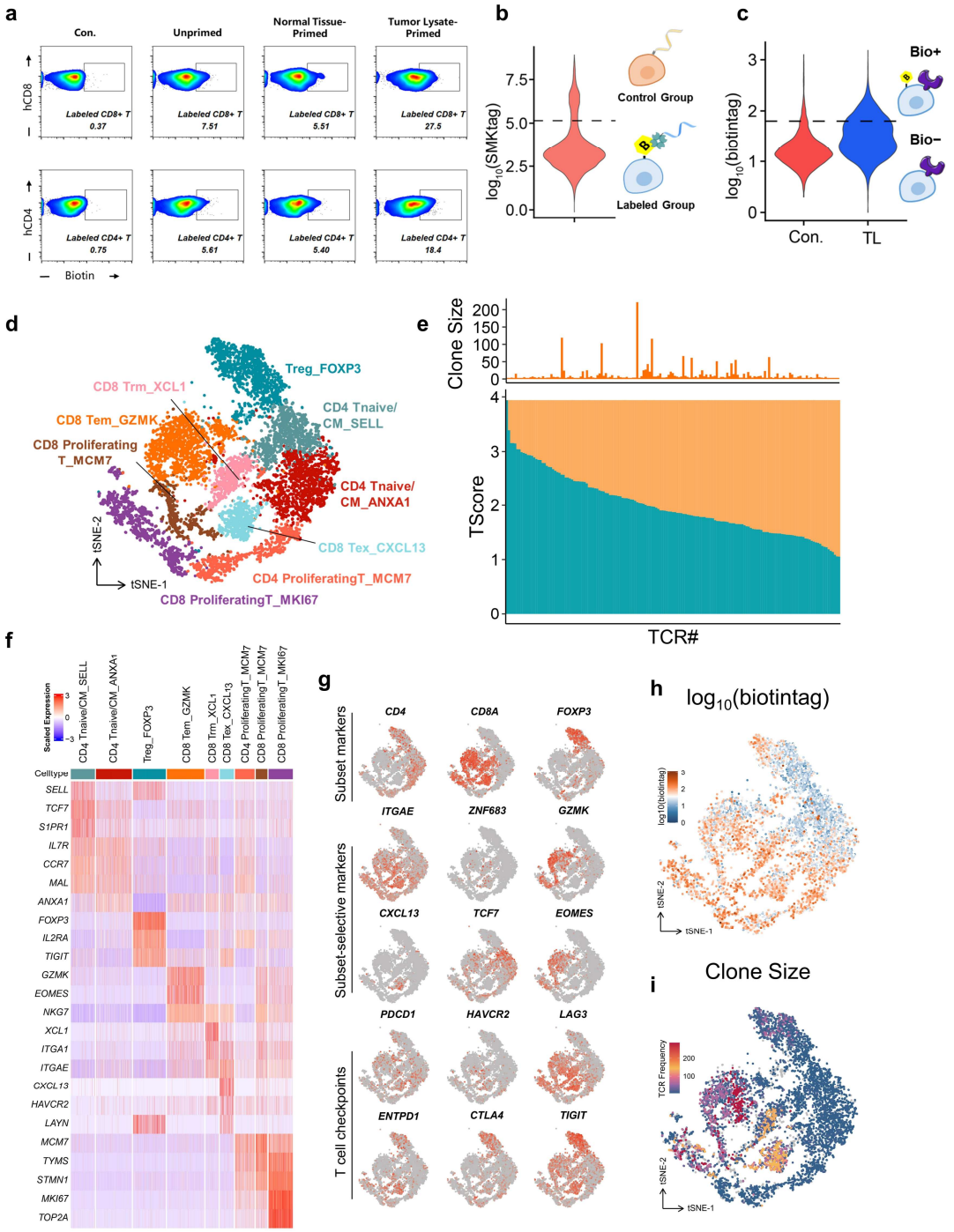
a Overview workflow of human sample preparation for T-SCOPE-mediated tumor-responsive T cell capturing in human cancer samples.

b Flow cytometric plots showing the characterization of monocyte-derived DCs used for T-SCOPE labeling in human samples.

c Representative flow cytometric plots showing tumor-responsive T cells detected by T-SCOPE in TILs and dLNs of human lung cancer.

d Summary of $CD4^+$ tumor-responsive T cells captured from TILs and dLNs in lung cancer samples via T-SCOPE. Un: unprimed group, NT: normal tissue lysate–primed group, TL: tumor lysate–primed group.

e, f Summary of simultaneous $CD8^+$(**e**) and $CD4^+$(**f**) tumor-responsive T cell capturing in TILs and dLNs of bladder cancer samples.
Part **a** was created by BioRender.

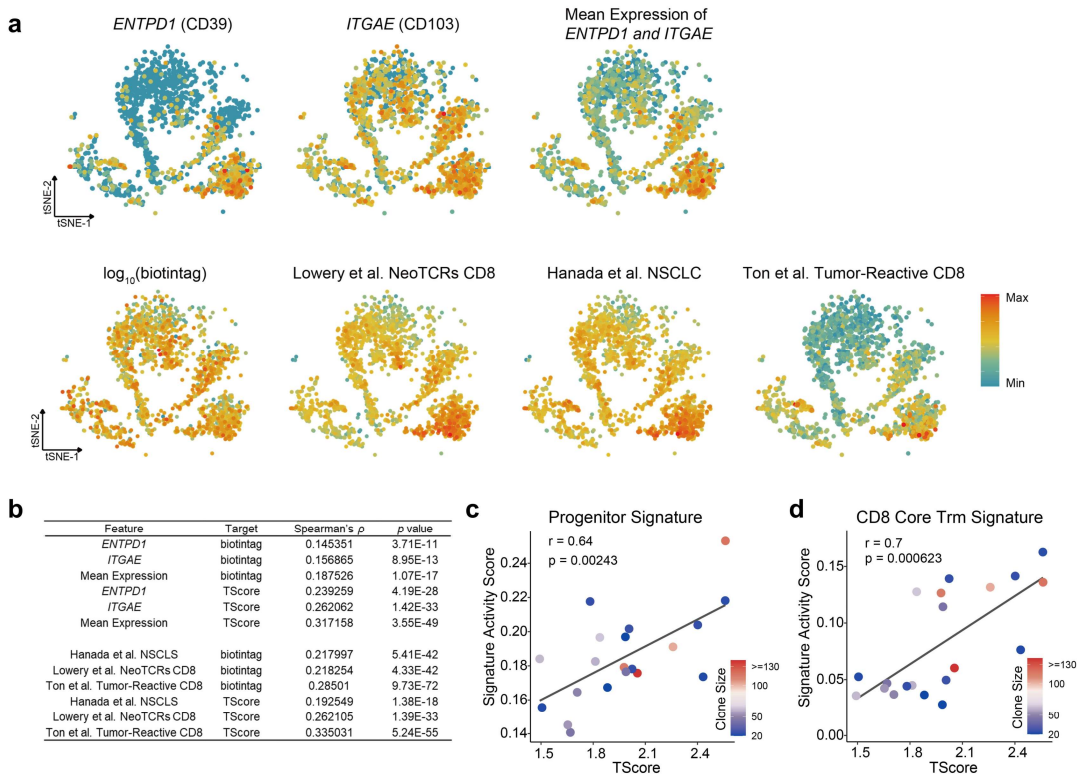


Supplementary Fig. S11 T-SCOPE-seq decodes clonotype-resolved tumor responsiveness in human TILs.

a Representative flow cytometric plots showing T-SCOPE labeling results of lung cancer samples that were further used for T-SCOPE-seq. Con. group: TILs treated under the same conditions of other T-SCOPE-labeled groups but without DBF-DCs co-culturing.

b Detection level of SMK sample barcode and labeled group separation from control group in sequencing data. Cells whose $\log_{10}$(SMKtag) over 5.2 were defined as control group cells (Con.) and those lower than 5.2 were defined as tumor lysate-primed group cells (TL).

c Detection level of biotin labeled on Con. T cells and TL T cells. Comparing biotin level in Con. group, cells whose $\log_{10}$(biotintag) were over 1.820 in TL group were defined as Bio+ T cells and those lower than 1.820 were defined as Bio− T cells.
d t-SNE plots showing the expression profiles of αβ T cells in the TL group from lung cancer patient TILs. Clusters are denoted by colors and labeled with inferred cell states. Related to **f**.
e TScore ranking of $CD8^+$ TIL TCRs (clone size > 2). Each bar represents one TCR, ordered by TScore (blue), with the corresponding clone size shown in the top panel.
f Heatmap showing DEGs found in each T cell cluster. Related to **d**.
g t-SNE plots showing expression of subset marker genes, subset-selective genes and T cell checkpoint genes.
h t-SNE plot showing biotintag level of lung cancer TILs integrated sequencing results labeled by T-SCOPE. Cells were colored by the value of $\log_{10}$(biotintag).
i t-SNE plot showing the clonal expansion size of lung cancer patient TILs colored by the clone size of their corresponding TCR clonotype.



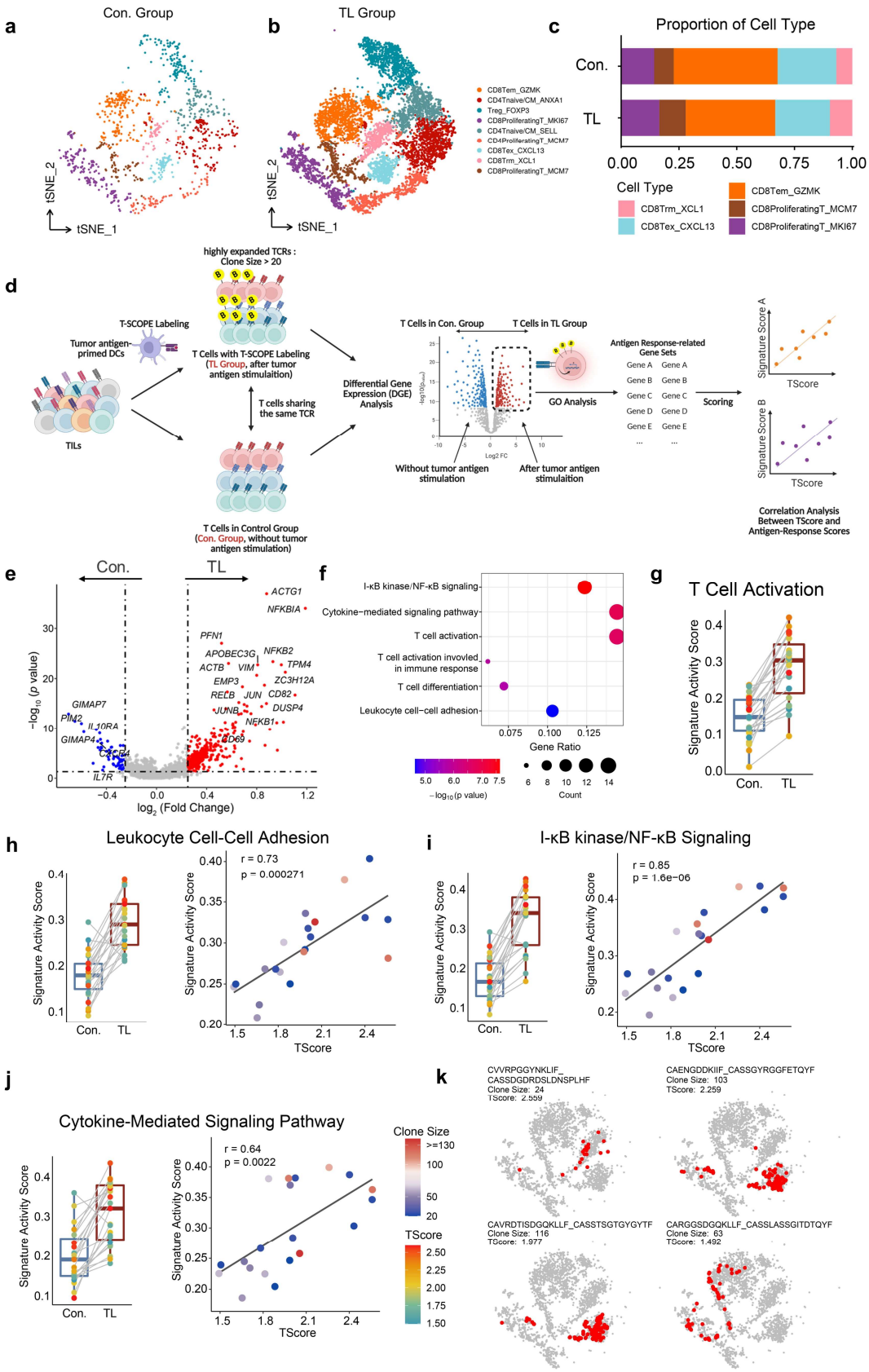
Feature	Target	Spearman's ρ	p value
ENTPD1	biotintag	0.145351	3.71E-11
ITGAE	biotintag	0.156865	8.95E-13
Mean Expression	biotintag	0.187526	1.07E-17
ENTPD1	TScore	0.239259	4.19E-28
ITGAE	TScore	0.262062	1.42E-33
Mean Expression	TScore	0.317158	3.55E-49
Hanada et al. NSCLS	biotintag	0.217997	5.41E-42
Lowery et al. NeoTCRs CD8	biotintag	0.218254	4.33E-42
Ton et al. Tumor-Reactive CD8	biotintag	0.28501	9.73E-72
Hanada et al. NSCLS	TScore	0.192549	1.38E-18
Lowery et al. NeoTCRs CD8	TScore	0.262105	1.39E-33
Ton et al. Tumor-Reactive CD8	TScore	0.335031	5.24E-55

Supplementary Fig. S12 TScore aligns with tumor-reactive markers and signature programs in CD8⁺ TILs.

a t-SNE plots showing the distribution of *ENTPD1* (CD39), *ITGAE* (CD103), mean expression, along with $\log_{10}$(biotintag) levels and tumor-specific gene signature scores in CD8⁺ TILs (clone size > 2). Tumor-specific signatures were derived from gene sets reported by Lowery et al.[7], Hanada et al.[8], and Ton et al.[9], and applied to score CD8⁺ TILs from patients with lung cancer in this study. The gene sets are summarized in Supplementary Table S7.

b Summary table showing the correlation analysis of gene expression and tumor-specific gene signature scores with biotintag intensity and TScore. Statistical correlation and the corresponding *p* value were evaluated using Spearman rank correlation analysis.

c, d Scatterplots showing the correlation between TScore and progenitor signature[10,11] (**c**) or CD8 Core Trm signature[12] (**d**) scores in our lung cancer CD8⁺ TIL sample. Each dot represented an expanded CD8⁺ TCR with a clone size > 20 in the TL group. Gene sets were summarized in Supplementary Table S7. ANOVA *F*-tests.



Supplementary Fig. S13 TScore links tumor antigen responsiveness to activation programs in human CD8⁺ TILs.

a, b t-SNE plots showing the expression profiles of Con. group (**a**) and TL group (**b**) αβ T cells in

lung cancer patient TILs. Clusters are denoted by colors and labeled with inferred cell states.
c Bar plot of the proportion of cells in the indicated phenotype clusters among the $CD8^+$ T cells (clone size > 20) in the Con. and TL groups.
d Schematic overview of the analytical workflow used to establish the correlation between TScore and antigen-induced activation potency in human lung cancer tumors. Differential gene expression analysis was performed on high-frequency TCRs (clone size > 20) between T cells before and after T-SCOPE labeling. Genes upregulated after T-SCOPE labeling were subjected to GO enrichment analysis, and genes associated with enriched GO terms were grouped into corresponding functional gene sets. These gene sets were then used to calculate a functional score for each TCR, and the correlation between the functional score and TScore was subsequently analyzed.
e Volcano plot showing the DEGs in $CD8^+$ T cells (TCR clone size >20) between the Con. and TL groups. A $p < 0.05$ and $|\log_2(\text{Fold Change})| > 0.25$ was considered significant.
f GO enrichment analysis of the upregulated genes in **e**.
g Paired box plot showing the correlation between TScores and activity scores for T cell activation pathways. Each dot represents a TCR. The box plot shows changes in activity scores between the Con. and TL groups for TCRs with a clone size greater than two in both groups. Color scheme for TScore is shown in panel **j**. Related to **Fig. 1o**.
h-j Paired box plots (left) and scatterplots (right) showing the correlation between TScore and activity scores for leukocyte cell-cell adhesion (**h**), I-κB kinase/NF-κB signaling (**i**) and cytokine-mediated signaling pathway (**j**). Each dot represented a TCR. Box plots showed activity score changes between Con. and TL groups for TCRs with a clone size > 2 in both groups. Scatterplots showed the positive correlation between TScore and activity scores for expanded $CD8^+$ TIL TCRs with a clone size > 20 in the TL group. Gene sets were summarized in Supplementary Table S6. ANOVA *F*-tests in scatterplots.
k t-SNE plots highlighting the phenotype distribution of representative TCRs with different TScores. Part **d** was created by BioRender.

	T-SCOPE	FucoID	Trogocytosis-based	pMHC-multimer	TCRAFT/RAPTR	PRECISE-seq
References	This paper	Liu, Z. et al. *Cell* 183, 1117–1133 (2020).	Li, G. et al. *Nature Methods* 16, 183–190 (2019).	Ma, K. Y. et al. *Nature Immunology* 22, 1590–1598 (2021).	Gaglione, S. A. et al. *Immunity* 59, 477-493. (2026).	Liu, S. et al. *JEM* 223, e20251779 (2026).
Main purposes	Clonotype-resolved T cell response monitoring	Tumor-reactive T cell enrichment	epitope identification of specific TCR	Antigen-specific T cell detection	TCR-antigen screening	Tumor-reactive T cell and TCR enrichment
Labeling or detection principle	DBF-mediated proximity biotinylation at cell–cell contacts	FT-mediated fucosyl-biotinylation at cell–cell contacts	Membrane-protein transfer to target cells	Direct TCR binding by fluorescent pMHC-multimers	Activation-based screening of reconstructed TCR libraries	SrtA/AP-tag-mediated proximity biotinylation at cell–cell contacts
Prior antigen knowledge	Not required	Not required	Required	Required	Required	Not required
Genetic engineering	Not required	Not required	Required for TCR/SCT-library cells	Not required	Required for TCR/reporter libraries	Required for SrtA-expressing bait cells
Unknown tumor antigen analysis	Both known and unknown antigens	Both known and unknown antigens	Antigen discovery within predefined libraries	Known antigens	Known antigens	Both known and unknown antigens
Main output	Repertoire-wide and clonotype-resolved antigen response potency	Tumor reactive T cells enrichment	epitope information of specific TCR	Antigen(s) specific T cells enrichment	TCR-antigen pairing	Tumor (antigen) response potency
Primary/clinical sample compatibility	Compatible with primary cells and patient-derived samples	Compatible with primary cells; Not used in patient-derived samples yet	Indirect; Using patient-derived TCRs after TCR reconstruction	Compatible with primary cells and patient-derived samples	Indirect; Using patient-derived TCRs after TCR reconstruction	Compatible with primary T cells for known antigens; Not directly compatible with human patient-derived samples for unknown antigens;
Single-cell RNA/TCR integration	Directly integrates biotin tag, transcriptome and paired TCR of single cell in one workflow	Downstream RNA/TCR profiling after Biotin+ sorting; Not directly coupled to scRNA/scTCR;	Not directly coupled to scRNA/scTCR	Compatible after sorting or DNA barcoded-multimer staining	Indirect; Decoupled functional validation with scRNA/scTCR integration	Indirect; Separated scRNA/scTCR profiling for Biotin+ and baseline cells
Quantitative potency readout	Clone-level TScore for functional response potency profiling	No dedicated potency score	No dedicated potency score	No functional response readout	Clone-level functional response validation	Enrichment-based potency score
Main limitations	Does not resolve cognate antigen identity	Not designed for clone-level potency ranking; Does not resolve cognate antigen identity;	Requires engineered antigen libraries and TCR reconstruction; Not applicable in tumor reactive TCR discovery and ranking;	Requires known antigens; May identify TCRs only recognize anitgens but not response	Requires TCR synthesis, TCR reconstruction and predefined antigen/HLA libraries	Requires genetically engineered bait cells; Decoupled labeling biotin information from transcriptome/TCR profiling; Does not resolve cognate antigen identity;

Supplementary Table S8 Comparison of T-SCOPE with existing tools.

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