Journal for ImmunoTherapy of Cancer

Cell surface marker-based capture of neoantigen-reactive CD8$^+$ T-cell receptors from metastatic tumor digests

Praveen D Chatani, Frank J Lowery, Neilesh B Parikh, Kyle J Hitscherich, Rami Yossef, Victoria Hill, Jared J Gartner, Biman Paria, Maria Florentin, Satyajit Ray, Alakesh Bera, Maria Parkhust, Paul Robbins, Sri Krishna, Steven A Rosenberg

To cite: Chatani PD, Lowery FJ, Parikh NB, *et al*. Cell surface marker-based capture of neoantigen-reactive CD8$^+$ T-cell receptors from metastatic tumor digests. *Journal for ImmunoTherapy of Cancer* 2023;**11**:e006264. doi:10.1136/jitc-2022-006264

► Additional supplemental material is published online only. To view, please visit the journal online (http://dx.doi.org/10.1136/jitc-2022-006264).

Accepted 23 April 2023

Surgery Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, USA

Correspondence to
Dr Sri Krishna;
sri.krishna@nih.gov

Dr Frank J Lowery;
frank.lowery@nih.gov

Dr Steven A Rosenberg;
sar@mail.nih.gov

ABSTRACT

Background Cellular immunotherapies using autologous tumor-infiltrating lymphocytes (TIL) can induce durable regression of epithelial cancers in selected patients with treatment-refractory metastatic disease. As the genetic engineering of T cells with tumor-reactive T-cell receptors (TCRs) comes to the forefront of clinical investigation, the rapid, scalable, and cost-effective detection of patient-specific neoantigen-reactive TIL remains a top priority.

Methods We analyzed the single-cell transcriptomic states of 31 neoantigen-specific T-cell clonotypes to identify cell surface dysfunction markers that best identified the metastatic transcriptional states enriched with antitumor TIL. We developed an efficient method to capture neoantigen-reactive TCRs directly from resected human tumors based on cell surface co-expression of CD39, programmed cell death protein-1, and TIGIT dysfunction markers (CD8$^+$ TIL^{TP}).

Results TIL^{TP} TCR isolation achieved a high degree of correlation with single-cell transcriptomic signatures that identify neoantigen-reactive TCRs, making it a cost-effective strategy using widely available resources. Reconstruction of additional TIL^{TP} TCRs from tumors identified known and novel antitumor TCRs, showing that at least 39.5% of TIL^{TP} TCRs are neoantigen-reactive or tumor-reactive. Despite their substantial enrichment for neoantigen-reactive TCR clonotypes, clonal dynamics of 24 unique antitumor TIL^{TP} clonotypes from four patients indicated that most in vitro expanded TIL^{TP} populations failed to demonstrate neoantigen reactivity, either by loss of neoantigen-reactive clones during TIL expansion, or through functional impairment during cognate neoantigen recognition.

Conclusions While direct usage of in vitro-expanded CD8$^+$ TIL^{TP} as a source for cellular therapy might be precluded by profound TIL dysfunction, isolating TIL^{TP} represents a streamlined effective approach to rapidly identify neoantigen-reactive TCRs to design engineered cellular immunotherapies against cancer.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Antitumor T-cell reactivity is an integral component of immunotherapy, with key focus on identification of neoantigen-reactive T-cell receptors (TCRs). Enrichment for tumor-reactive T cells has been achieved by sorting T cells co-expressing two or more markers of dysfunction, as well as gene signatures, but a more refined surface marker-based sort may enhance the ability and efficiency of enrichment of neoantigen-reactive TCRs from human tumor-infiltrating lymphocytes (TIL).

WHAT THIS STUDY ADDS

⇒ The isolation of TIL^{TP} from human tumors provides an efficient and high-fidelity method to identify neoantigen-reactive TCRs.

⇒ Despite high enrichment of neoantigen-reactive clones within TIL^{TP}, the populations themselves are terminally differentiated and dysfunctional to be used as a direct cell therapy.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ The ability to capture neoantigen-reactive TCRs by isolating TIL^{TP}, as shown in this study, represents a rapid and effective method to identify neoantigen-reactive TCRs, which have the potential to be used to genetically engineer targeted, personalized T-cell therapies against cancer.

INTRODUCTION

Immunotherapies such as checkpoint blockade and cellular therapies have expanded the landscape of treatment strategies for patients with advanced metastatic cancers.[1] Studies have shown that mutational burden and, consequently, neoantigen load presented by the tumor are associated with response to immune checkpoint blockade (ICB) and adoptive cell therapy (ACT).[2 3] Multiple lines of evidence suggest that response to immunotherapy is at least in part attributable to recognition of patient-specific neoantigens by autologous tumor-infiltrating lymphocytes (TIL).[4–9]

Cellular immunotherapies such as ACT using autologous TIL combined with systemic interleukin-2 (IL-2) are capable of inducing

durable regression of epithelial cancers in patients with chemotherapy-refractory metastatic disease,[5 10–13] a population with a response rate of less than 10% to ICB immunotherapies. Success of immunotherapy regimens, including ACT, is influenced by T cell-intrinsic factors, including cell differentiation states, self-renewing capability, and resistance to tumor microenvironment inhibition, as well as tumor-specific factors, such as mutational burden, human leukocyte antigen (HLA) expression, and tumor clonality.[3 14 15] Notwithstanding, antitumor T-cell reactivity remains a common denominator to successful immunotherapies; therefore, rapid and reliable identification of the repertoire of tumor-reactive T-cell receptor (TCR) clones remains a top priority for immune monitoring and therapeutic targeting. However, the inflammatory nature of tumor progression draws non-specific 'bystander' T cells into the tumor environment, making the isolation, analysis, and specific expansion of tumor-reactive TIL challenging.[16 17]

Analyses of intratumoral lymphocyte populations have shown that neoantigen-specific TIL are enriched in programmed cell death protein-1 (PD-1)$^+$ and CD39$^+$ expressing T cell subsets, likely secondary to oligoclonal expansion that occurs on tumor antigen recognition in vivo.[17–21] While expression of a single inhibitory molecule such as PD-1, TIM3, or LAG3 is not restricted to tumor-reactive TIL, enrichment for tumor-reactive T cells has been achieved by sorting T cells co-expressing two or more markers of dysfunction.[17 18] We and others have recently identified common gene programs expressed in CD4$^+$ and CD8$^+$ antitumor, neoantigen-reactive T cells (termed 'NeoTCR4 and NeoTCR8 gene signatures', respectively, in this study) that enabled us to prospectively identify antitumor, neoantigen-reactive TCR clonotypes with high accuracy in a multidimensional transcriptomic analysis of tumor-reactive TIL in patients with metastatic cancer.[22–27] The NeoTCR8 transcriptomic signature consists of several markers of T-cell dysfunction, including *ENTPD-1* (CD39), *PDCD1* (PD-1), and *TIGIT*.[22]

The differentiation state, proliferative potential, and effector function of tumor-reactive T cells identified in fresh tumor samples, however, appears to generally limit their ability to expand both in vitro and in vivo, which represents a challenge for the generation of T cells for adoptive immunotherapy.[28] In a recent study of the infusion products administered as ACT to patients with metastatic melanoma, we reported that antitumor TIL predominantly expressed CD39 and CD69, markers that appeared to be associated with T-cell dysfunction, and T cells that expressed these markers had poor persistence post-ACT in patients. In contrast, neoantigen-specific TIL lacking these dysfunction markers (CD39$^-$ CD69$^-$) maintained T-cell stemness, had superior proliferative and tumor regression capabilities, and persisted long-term in patients post ACT-treatment.[14] Nevertheless, optimal enrichment of the starting antitumor TIL population might streamline cell therapy efforts, potentially minimizing the outgrowth of non-reactive TCR clones during in vitro expansion, as suggested by prior studies.[28 29]

Recent studies have shown that peripheral blood lymphocytes (PBL) from patients engineered to express antitumor, neoantigen-reactive TCRs can mediate durable regressions in solid tumors.[13 30 31] In this study, we evaluated the sensitivity and specificity of detection of neoantigen-reactive CD8$^+$ T cells that could be achieved from tumor digest samples from patients with metastatic epithelial cancers by sorting T cells that co-expressed PD-1, CD39, and TIGIT, and compared this method to an effective, but resource-dependent, transcriptomic approach. Subsequently, we further tested the ability of TILs that co-expressed PD-1, CD39, and TIGIT to sustain reactivity following in vitro expansion under conditions mimicking our clinical TIL products used for ACT immunotherapy. Our findings demonstrate that TIL co-expressing PD-1, CD39, and TIGIT were highly enriched for tumor reactivity, and sorting based on surface marker co-expression correlates with single-cell transcriptomic states of antitumor TIL. However, dominant clones in this population lack the proliferative capacity to survive a traditional in vitro expansion and thus have limited potential as a cell therapy product. Thus, the sorting of TIL using PD-1, CD39, and TIGIT can potentially facilitate the rapid isolation of CD8$^+$ antitumor neoantigen-reactive TCRs for use in cancer cellular therapies.

METHODS

Tumor samples

All tumor samples and antigen presenting cells used in this study were obtained from patients enrolled on NCT00068003 and NCT00001823 National Institutes of Health (NIH) clinical protocols, both approved by the Institutional Review Board of the National Cancer Institute. Informed consent was obtained and documented in accordance with the Declaration of Helsinki. Patients were required to be 18 years of age or older, to have measurable metastatic disease, to be of good Eastern Cooperative Oncology Group (ECOG) performance status, and to be free of systemic infections. Metastatic tumor samples were obtained from patients who had been off therapy for at least 1 month at the time of metastasectomy (online supplemental table 1). Patients had undergone a wide range of prior therapies relevant to their particular tumor histology including surgery, chemotherapy, radiotherapy, or immunotherapy. Tumor samples were processed as previously described[32] using a combination of mechanical separation, enzymatic digestion, and gentleMACS dissociation technology (Miltenyi Biotec); single-cell suspensions were cryopreserved until analysis. Thawed digest samples were rested overnight in media with DNAse (Genentech), without any cytokines, prior to isolation of a lymphocyte-dense fraction over a Ficoll-Hypaque gradient (LSM; ICN Biomedicals).

Antibodies, cell sorting, and transcriptomic analysis

Fluorescently conjugated antibodies were purchased from BD Bioscience (CD3 (OKT3), CD4 (OKT4),

Table 1 Known-reactive TCR clones in 10× transcriptomic analysis (figure 1)

Patient	CDR3b	TCR reactivity	Cells with clonotype in UMAP
4323			
	CASSPRVPNTEAFF	HIATL1	75
	CATSGTGHSYEQYF	PPP2R1A	34
	CATSDTGRPYEQYF	PPP2R1A	31
	CATSATGRSYEQYF	PPP2R1A	25
	CASSELAGGSGANVLTF	PPP2R1A	7
	CASSLDADGRSRETQYF	HIATL1	4
	CSAATWAINQPQHF	PPP2R1A	3
	CASSLYTEKWAGYTF	PPP2R1A	1
	CASSQSDRVWNTEAFF	PPP2R1A	1
	CATSDTGLPYEQYF	PPP2R1A	1
	CATSDTGKPYEQYF	PPP2R1A	1
		Total known-reactive	**183**
4400			
	CASSELAGGNYNEQFF	PDX tumor	13
	CASSQDPGARDYGYTF	PDX tumor	13
	CASSLLATGNFNEQFF	PDX tumor	11
	CASSFPASGGAVDNEQFF	PDX tumor	7
	CAWSRVRPNEQFF	PDX tumor	6
	CASTTSPGPEYGYTF	PDX tumor	6
	CASSPGSGGTDTQYF	PDX tumor	5
	CAWSMGAGYEQYF	PDX tumor	5
	CAWSAGSSYEQYF	PDX tumor	5
		Total known-reactive	**71**
4385			
	CASSQDVTSEWVDTIYF	KRAS	2
	CASSMMNTEAFF	TMG6	1
		Total known-reactive	**3**
4317			
	CASSIWATNTGELFF	AURKAIP1	41
	CASSAGAEQYF	PIK3CA	25
	CASNSGGPTVEQYF	PIK3CA	15
	CSVVLASGDEEEQYF	AURKAIP1	15
	CSVEDGGRDTGELFF	AURKAIP1	2
	CASSPGGFEAFF	PIK3CA	1
		Total known-reactive	**99**
4382	CASSEAGASNYGYTF	SMARCA4	36
	CASSQSTGALETQYF	SMARCA4	18
	CASSEAGASQETQYF	SMARCA4	1
		Total known-reactive	**55**

PDX, patient-derived xenograft; TCR, T-cell receptor; UMAP, Uniform Manifold Approximation and Projection.

CD8 (RPA-T8), CD137 (4B4-1)) and BioLegend (PD-1 (EH12.2H7), CD39 (A1), TIGIT (A15153G), and integrin αE (Ber-ACT8, CD103)). An overview of the transcriptomic experimental pipeline is shown in figure 1A. Single-cell tumor digest samples were thawed and rested overnight in media without cytokines and, following Ficoll-Hypaque separation (LSM; ICN Biomedicals) were stained and sorted by flow cytometry with a Sony SH800S multi-application cell sorter (SONY Biotechnology). Gates for sorting were set according to isotype controls.

An example of the sorting strategy is shown in online supplemental figure 1.

For single-cell transcriptomic T-cell analyses, bulk $CD4^+$ and $CD8^+$ TIL from tumors 4317, 4323, 4382, 4385, and 4400 were sorted and single-cell gene expression libraries were sequenced on a NextSeq 550 sequencer with output processed using the Cell Ranger V.3.0 pipeline (10× Genomics, USA) and analysis completed with R package Seurat V.4 as previously described.[14 22] Briefly, Cell Ranger count and VDJ were used under default conditions to generate h5 files which were used as input to Seurat gene expression pipeline. High quality cells with at least 250 detected genes, less than 20% mitochondrial RNA content and unique molecular identifier (UMIs) greater than or equal 500 were retained for downstream analysis. Low expressed genes, with total UMI count across all cells less than 4, and TRAV, TRBV genes were removed prior to analysis. For individual patient TIL single-cell RNA (scRNA), elbowplots were used to determine optimal PC cut-off for clustering. For integration of $CD8^+$ TIL from the five patients, a combined R object encompassing all five samplers was created, Seurat SCTransform function was performed. For gene signature analyses (NeoTCR8 and other single and multiple gene combinations), single-cell gene set enrichment analysis (scGSEA) was performed as described previously,[19 22] and cells scoring within the top decile (90th percentile) were used for downstream analysis.

Single-cell sorting and single-cell RT-PCR

Single-cell sorting into 96-well plates for TCRα/β pair sequencing was performed first for $CD8^+CD39^+PD\text{-}1^+TIGIT^+$ 'triple-positive' TIL (CD8 TIL^{TP}) cells using a Sony SH800S cell sorter (SONY Biotechnology). Cells were sorted directly into a 96-well plate with reverse transcription-polymerase chain reaction (RT-PCR) buffer (CellsDirect One-Step qRT-PCR kit; Thermo Fisher) and TCR sequences from the sorted single cells were obtained by a series of two nested PCR reactions, previously described in detail.[33] Briefly, a shared initial PCR reaction was performed with the following cycling conditions: 50°C for 15 min, 95°C for 2 min, 18 cycles of 95°C for 15 s, 55°C for 30 s, 72°C for 1 min, and incubation at 4°C. The second PCR was performed separately for TCRα and TCRβ chains using dedicated nested primers for either TCRα or TCRβ targeting extended CDR3 regions of both chains (cycling conditions: 95°C for 7 min, 5 cycles of 95°C for 15 s, 65°C for 15 s, 72°C for 30 s, 5 cycles of 95°C for 15 s, 60°C for 15 s, 72°C for 30 s, 40 cycles of 95°C for 15 s, 65°C for 15 s, 72°C for 30 s, 72°C for 7 min, and final 4°C incubation). The PCR products were purified and sequenced by Sanger methods using internally nested Cα and Cβ primers.

The output of Sanger sequencing was used to reconstruct CD8 TIL^{TP} TCRα/β pairs for testing against tumor-specific neoantigens (described below), and as a surrogate for pre-REP clone-frequency within the corresponding population. Clones were selected for reconstruction if they appeared two or more times in the 96-well plate. TCR sequences that were seen once but also appeared within the NeoTCR8 cluster by transcriptomic analysis were similarly in silico reconstructed and cloned. In several instances, TCRα/β pairs were unable to be reconstructed due to failures of Sanger sequencing methods.

Rapid expansion and functional assessment of TIL populations

Following the sort for TCRα/β sequencing of the TIL^{TP} populations, $CD8^+$ TIL^{TP} and bulk $CD8^+$ TIL were sorted into separate tubes. Immediately after sorting, cells (6,000–15,000 $CD8^+$ TIL^{TP}) were plated in a G-Rex 24-well cell culture plate (Wilson Wolf, Minnesota, USA) under 'Rapid Expansion Protocol'[34] conditions: IL-2 (6000 IU/mL), anti-CD3 (OKT3, 50 ng/mL), and 1×10^7 irradiated healthy-donor PBL. Cells were cultured for 2 weeks with media changes and expansions performed based on individual population requirements. Fourteen days after REP, 2×10^4 cells from each population were co-cultured with patient-derived xenograft (PDX) tumor line and antigen-presenting cells that were either electroporated with tandem minigene (TMG) constructs or peptide-pulsed with candidate 25mer neoantigens, as previously described.[35] Briefly, up to 17 non-synonymous mutations identified by whole-exome sequencing and RNA sequencing, each flanked by 12 amino acids of the wild-type protein sequence, were genetically fused together to generate a TMG construct. Constructs were codon-optimized, synthesized, and cloned into pcDNA3.1/V5 by GenScript. Purified linear plasmid DNA was used to synthesize messenger RNA (mRNA) by IVT (mMESSAGE mMACHINE T8 Ultra kit; Life Technologies), which was then purified and adjusted to a concentration of 1 µg/µL. Crude long peptides (25mers) were synthesized and pooled for in vitro screening of T cells. For screening, in addition to those electroporated with TMG constructs, separate autologous antigen-presenting cells were peptide pulsed for 2 hours with 10 µg/mL of pooled long peptides (25mers) before co-culture. In parallel to patient-specific neoantigen and PDX tumor stimulation, post-REP $CD8^+$ TIL^{TP} populations were stimulated overnight with CD3/CD28 Dynabeads (Thermo Fisher Scientific), after-which intracellular cytokine staining was done and analysis performed with FACSymphony (BD Biosciences).

Sequencing of post-REP populations and post-REP reactive TCRs

On day 14 post-REP, in parallel with functional assessment via co-culture, 1×10^5 T cells from bulk-sorted populations ($CD8^+$ TIL^{TP} and $CD8^+$) were pelleted, snap-frozen, and sent to adaptive technologies for

genomic DNA extraction and ImmunoSEQ TCRβ survey sequencing.

Following overnight co-culture, T cells upregulating CD137 greater than two times background (as determined by co-culture with vehicle [dimethyl sulfoxide, DMSO]-treated or irrelevant-TMG-electroporated antigen-presenting cells [APCs]) underwent single-cell sorting from a duplicate co-culture into a 96-well plate using the Sony SH800S cell sorter (SONY Biotechnology); Sanger sequencing was used to determine TCRα/β pairs for reconstruction and testing.

TCR reconstruction, cloning into expression vectors, and TCR expression evaluation

The resulting PCR product of Sanger sequencing contains the full CDR3 region of matching TCRα and TCRβ genes which, when analyzed with IMGT/V-Quest tool (http://www.imgt.org/), can identify the TRAV and TRBV families with the highest likelihood to contain the segment found with our pairing methods. Full-length TRAV and TRBV regions were reconstructed using the IMGT database, and a modified murine TRAC and TRBC sequence was used for improved stability and avoidance of mismatches within the endogenous human TCR on transduction into human T cells. The full-length TCR coding sequence was aligned for expression as a single mRNA, with a 2A peptide between the TCRα and TCRβ chains, and subcloned into the pMSGV1 vector by GenScript. Only sequences with CDR3β regions beginning with cysteine and ending with phenylalanine, without intervening stop codons, as determined by IMGT/V-Quest analysis were used for TCR reconstruction.

Co-transfection of 293GP cells with pRD114 packaging plasmid and pMSGV1 expressing the TCR-of-interest resulted in the generation of retroviral supernatants, which were collected at 72 hours post-transfection. In parallel to transfection, PBLs from a healthy donor were stimulated in culture media with anti-CD3 (OKT3, 50 ng/mL) and IL-2 (300 IU/mL). Retroviral particles were then transduced into activated lymphocytes using spinoculation techniques previously described.[36 37] TCR transduction was confirmed using anti-mouse TCRβ fluorescent antibody at day 7 post-transduction, as well as at the time of reactivity testing via co-culture with patient-specific APCs and PDX tumor (14 days post-transduction).

Target cell recognition functional assay

Following overnight co-culture, T cells were stained with anti-CD3, anti-CD8, anti-CD4, anti-CD137, and anti-murine TCRβ fluorescent antibodies to detect transduced TCRs. Target recognition by T cells was assessed using 41BB (CD137) upregulation via FACSymphony (BD Biosciences) and interferon gamma (IFN-γ) release captured by ELISpot analysis. T cells with IFN-γ release or CD137 upregulation more than twice the background (as determined by co-culture with DMSO or irrelevant-TMG) were considered reactive. Data were analyzed with FlowJo software (Tree Star).

PDX and tumor cell line preparation

All PDX tumors were implanted on mice approved under the Institutional Review Board, NIH, animal protocol number SB 194. Fresh human tumor samples from patients were mechanically separated into fragments and implanted subcutaneously at the flank of NOD-*scid*-IL2rg^{null} (NSG, NCI Frederick) mice using a 20-gauge needle. Tumor growth was monitored weekly and PDX were harvested when their sizes were greater than 1 cm in dimension. The use of animals for this study was approved by the National Cancer Institute Animal Care and Use Committee. Freshly harvested PDX were fragmented and dissociated in a gentleMACS C-tube containing 20 mL of tissue culture media (RPMI 1640 with 1×of non-essential amino acid, sodium pyruvate, GlutaMAX, penicillin/streptomycin, gentamicin, fungizone and 2-mercaptoethanol; Thermo Fisher Scientific). The resulting cell suspension was then run through a 100 µm cell strainer and washed with culture media before being plated in tissue culture flasks. Media was changed every 3–7 days and cells were split when confluence exceeded 70%. All tumor cell lines were screened for pan-HLA class I and HLA-DR expression by flow cytometry (anti-pan HLA class I antibody W6/32 and anti-human DR antibody). Additional markers such as EpCAM and E-cadherin were also examined. Targeted neoantigens were confirmed by sequencing gene-specific PCR products from complementary DNA or genomic DNA of tumor lysate.

Statistical analysis

Wilcoxon rank-sum test was used to determine the statistical significance of TCR clonotype data comparisons. Linear regression was used to compare the efficiency of TIL^{TP} FACS-sorted populations to capture clones within NeoTCR8 TIL cluster from patient TIL scRNA. For clonal dynamics during in vitro expansion comparing TCR clonotypes from individual patient tumors, relevant populations were compared using two-tailed paired t-tests assuming unequal variance. P values of 0.05 or less were considered statistically significant. Statistical calculations were performed with Prism program V.9.0 (GraphPad Software).

RESULTS

Single-cell transcriptomic analysis of patient TIL reveals ENTPD-1 (CD39), PDCD1 (PD-1), and TIGIT co-expression on tumor-reactive T cells

We performed single-cell transcriptomic and TCR sequencing analysis of CD8$^+$ TIL from five metastatic human tumor samples with known neoantigen-reactive TCRs (NeoTCRs) (table 1, figure 1A,B). In these 5 TIL samples, 31 neoantigen-specific TIL clonotypes (table 1) were largely enriched in a single transcriptomic state (cluster 7, 38% out of all neoantigen-specific TIL) which corresponded to the NeoTCR8 transcriptomic signature (figure 1B, online supplemental figure 2). Relative to other TIL states,

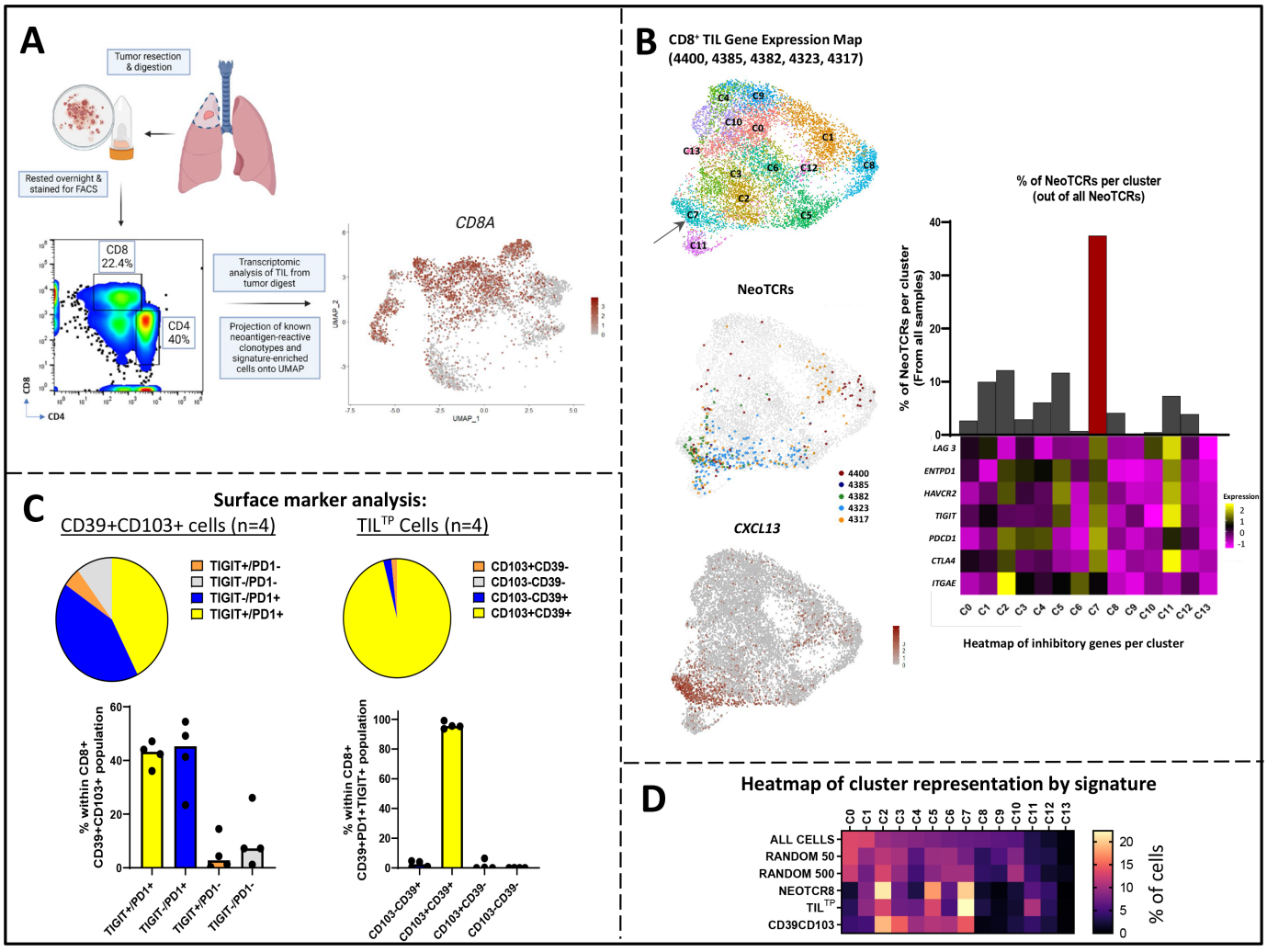


Figure 1 (A) CD8+ TIL populations were isolated from fresh tumor digest. Single-cell transcriptomic analysis was performed on the patient's TIL from tumor digest and known neoantigen-reactive clonotypes and signature-enriched cells were projected onto a Uniform Manifold Approximation and Projection (UMAP). (B) Integrated single-cell transcriptomic analysis showing UMAP-based projection of CD8+ TIL states from five patient samples (4400, 4385, 4382, 4323, 4317; left, top); cluster 7 (C7) indicated by arrow. Back-projection of 31 known neoantigen-reactive TCRs (NeoTCR) clones onto UMAP plot (left, middle). UMAP transcriptomic map of CD8+ TIL shows NeoTCR8 transcriptomic state showing co-localization of *CXCL13*-expressing cells (left, bottom). Bar graph indicating the percentage of known neoantigen-reactive TCRs (NeoTCRs) within each cluster, with the bar for NeoTCR8 state C7 denoted in red (right, top). Heatmap indicating average transcriptomic expression of inhibitory genes within each cluster (right, bottom). (C) Surface marker analysis of CD8+ CD39+CD103+ (double positive) and 'triple positive' (CD8+ PD-1+ TIGIT+ CD39+) TILTP cells of four patients (4400, 4385, 4323, 4393). Pie chart of proportion of cells expressing TIGIT and PD-1 within the CD39+CD103+ TIL subset; bar graph with the median of individual patient data (n=4) represented by black dots (left). Pie chart of proportion of cells expressing CD39 and CD103 within the TILTP subset; bar graph with the median of individual patient data represented by black dots (right). (D) Heatmap showing the relative frequency of cells within each cluster from single-cell RNA from figure 1B, that can be identified by the top decile within each signature. 'All Cells' includes all cells present in the transcriptomic UMAP. 'Random50' and 'Random500' represent signatures derived from random genes sets of 50 and 500 genes as negative control signatures, respectively. TCR, T-cell receptor; TIL, tumor-infiltrating lymphocytes.

NeoTCR8 cluster 7 displayed higher gene expression of TIL dysfunction markers *ENTPD-1* (CD39), *TIGIT*, *PDCD1* (PD-1), as well as *CXCL13*, a hallmark of dysfunctional human TIL,[22–27] with relatively lower expression of tissue-residency marker *ITGAE* (CD103) (figure 1B).

Given the previously reported importance of cell surface protein expression of CD39 and CD103 in defining neoantigen-specific, antitumor TIL,[17] we performed a cell surface protein expression analysis on CD39+CD103+ and CD39+ PD-1+ TIGIT+ TIL (TILTP) subsets (figure 1C, online supplemental figure 1). The vast majority (>95%) of TILTP co-expressed CD39 and CD103, with <2% comprizing of CD39+ CD103− and other TIL subsets (figure 1C, left). In contrast, only 43% of CD39+CD103+ TIL concomitantly co-expressed PD-1 and TIGIT, with 7.2% being negative for both markers (figure 1C, right). These results suggest that TILTP populations might represent a more terminally differentiated dysfunctional subset within the previously reported CD39+CD103+ population of antitumor TIL.[17]

To study the efficacy of using the co-expression of CD39, PD-1, and TIGIT in identifying tumor-reactive T cells compared with previously reported dysfunction markers, we evaluated the performance of gene signatures composed of individual or multiple genes in capturing known NeoTCR clonotype-positive cells as well as calling cells within the NeoTCR-enriched cluster 7 by ssGSEA (figure 1D, online supplemental figure 2). These analyses demonstrate that TIL^{TP} gene signature captured cells within the NeoTCR8 TIL state representing cluster 7 better than CD39^{+}CD103^{+} gene signature (figure 1D). Additionally, the NeoTCR8 signature performed best in identifying known NeoTCRs from the five patient TILs, with the TIL^{TP} signature performing second-best (online supplemental figure 2 right). Except for CD39, individual markers (PD-1, TIGIT, and CD103) did not perform as well in identifying known NeoTCR clonotypes or cells from cluster 7 but were improved by the inclusion of multiple markers (online supplemental figure 2, bottom). Together, our results suggest that multiple dysfunction markers expressing TIL^{TP} gene signature can identify the subset of highly dysfunctional antitumor TIL corresponding to NeoTCR8 transcriptomic state.

CD39, PD-1, and TIGIT co-expression enriches for NeoTCR8 clonotypes in CD8^{+} TIL

Given that the neoantigen-specific TIL scRNA clusters from the five patient TILs demonstrated enrichment of PD-1, TIGIT, and CD39, we characterized the TIL^{TP} clonotypic repertoire by developing a flow cytometry-based gating strategy for single-cell sorting (figure 2, online supplemental figure 1). TIL^{TP} phenotypic analysis was performed on tumor digest samples derived from nine metastatic epithelial cancers. There was wide variability in the cell surface protein expression of CD39, PD-1, and TIGIT between samples (figure 3A). While we observed high levels of PD-1 expression across all patients, except for 4420, TIL^{TP} populations were limited by either the CD39 or TIGIT expression. The median percentage of TIL^{TP} T cells across all nine patients was 14.3% (range, 1.7–29.7%), with median percentages of PD-1, CD39, and TIGIT expression of 61.7% (range, 17.8–78.7%), 25.3% (range, 6.02–53.9%), and 26.8% (range, 10.6–62.0%), respectively.

We studied five tumors in further detail, focusing on clones isolated from TIL^{TP} populations. A total of 120 unique CDR3β sequences were captured by single-cell sorting of CD8^{+} TIL^{TP} T cells, with a median of 37.3% unique TCRβ clones (range, 25.7–90.1%) sequenced from each tumor suggesting oligoclonal expansion (figure 3B). To investigate if clones enriched by TIL^{TP} FACS-sorting represent T-cell clonotypes prevalent within the transcriptomic cluster best enriched by the NeoTCR8 signature signature,[22] we analyzed the relative frequency of clonotypes captured by both the TIL^{TP} plate sort as well as the *CXCL13* expressing NeoTCR8 cluster C7 of the transcriptomic analysis (figure 3C, figure 1B, online supplemental figure 3). We observed a strong correlation between the two methods of enrichment (figure 3C, R^2=0.42, slope=0.60, p<0.0001), suggesting that dominant TCR clonotypes enriched by TIL^{TP} FACS-sorting largely represent T-cell clones found within the dysfunctional NeoTCR8 transcriptomic state.[22]

High-frequency CD8^{+} TIL^{TP} TCR clonotypes display tumor and neoantigen reactivity

To evaluate whether sorted CD8^{+} TIL^{TP} populations are enriched for antitumor neoantigen-specific clonotypes from patient tumors, we analyzed both known NeoTCR clonotypes and prospectively reconstructed other TIL^{TP} clonotypes by single-cell PCR/TCR reconstruction[19] and retrovirally transduced them into healthy donor PBL for testing against tumor-specific candidate neoantigens and PDX when available (figure 3D). Of the 120 unique CDR3β sequences detected across TIL^{TP} cells from 5 tumors, 39 TCR clones (33.3%) were either already known or met prospective selection criteria for reconstruction based on frequency by plate sorting and presence of a matched TCRα chain. Following reconstruction and testing of previously unknown TCRs, of the 120 unique CDR3β sequences, 19 (48.7% of tested clones, 15.8% of total clones) TIL^{TP} TCR clones displayed neoantigen (n=14, 35.9% of tested clones, 11.7% of total clones) or only autologous tumor reactivity (n=5, 12.8% of tested clones, 4.2% of total clones).

Figure 3D summarizes the reactivity of top CD8^{+} TIL^{TP} clones in four of five patients where TCR clonotypes were either previously known or reconstructed for neoantigen and PDX tumor testing. In TIL^{TP} sorted cells from tumor 4323, 7 of the top 10 CD8^{+} TIL^{TP} TCR clones, totaling approximately 82.9% of the pre-REP population, were known neoantigen-reactive clones. Of the reactive TCR clones, two targeted mutant HIATL1^{G380V} and five TCRs targeted mutant PPP2R1A^{L487S}.

In tumor 4382, two of the top eight CD8^{+} TIL^{TP} TCR clones recognized a patient-specific neoantigen (SMARCA4^{R973W}), comprizing approximately 20.9% of the sorted population. However, multiple TCRs were unable to be reconstructed for testing due to failures of Sanger sequencing methods (denoted as 'Not made' in figure 3D). In tumor 4400, 5 of the top 10 CD8^{+} TIL^{TP} TCR clones were previously shown to be reactive to the autologous PDX tumor (but not against screened candidate neoantigens), comprizing approximately 13.7% of sorted CD8^{+} TIL^{TP} cells (figure 3D). The exact antigenic target of these TCRs has not been determined and all TCR-transduced T cells were non-reactive against patient tumor-specific or PDX-specific mutations in peptide pools and TMGs, suggesting that TIL^{TP} clonotypes also encompass orphan antitumor T-cell receptors as previously reported.[22 38] In tumor 4317, 5 of the top 11 CD8^{+} TIL^{TP} TCR clones, representing approximately 27.5% of sorted CD8^{+} TIL^{TP} cells, were neoantigen-reactive. Of these TCRs, two

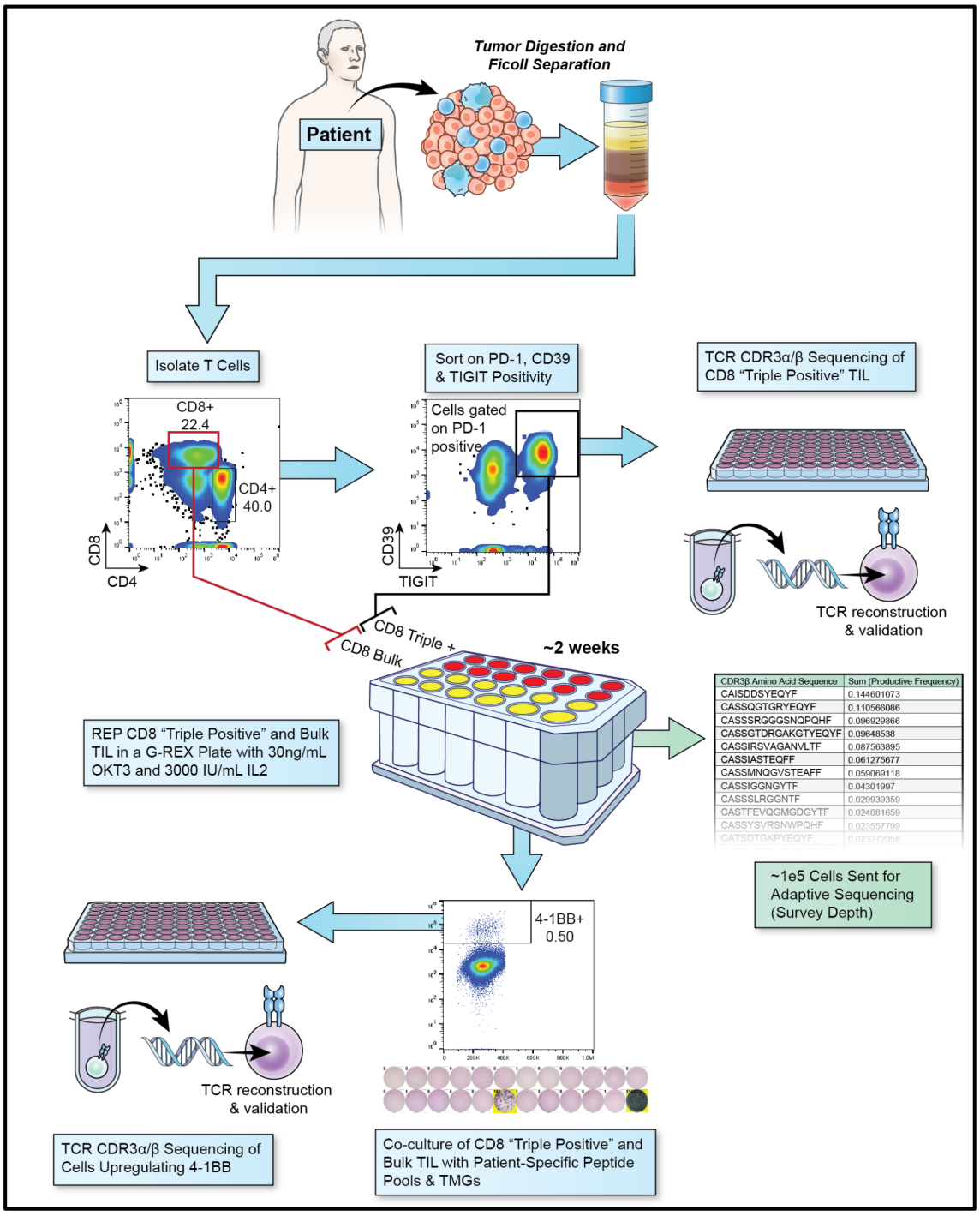


Figure 2 Overview of experimental pipeline for isolating, sequencing, expanding, and testing TIL^{TP} cells from single-cell suspensions of epithelial tumors. Single-cell PCR plates of pre-REP TIL^{TP} populations were performed to capture candidate neoantigen-reactive clones for reconstruction and testing. Survey sequencing was performed on post-REP TIL^{TP} to analyze clonal dynamics of TIL^{TP} clones before and after in vitro REP expansion. In vitro expanded post-REP TIL^{TP} populations were analyzed for the presence of known neoantigen-reactive clones and to identify new neoantigen-specific TCRs. PD-1, programmed cell death protein-1; REP, rapid expansion protocol; TCR, T-cell receptor; TIL, tumor-infiltrating lymphocytes.

were reactive against mutant PIK3CA^{P449T} and three against mutant AURKAIP1^{R164H}. Aside from TCR #9 (vs PIK3CA, figure 3D) all TCRs in this patient were previously undetected through traditional screening methods of in vitro-expanded TIL fragments. In tumor 4385, of the top nine dominant CD8^{+} TIL^{TP} clonotypes, eight TCRs were reconstructed and all failed to react against patient-specific candidate neoantigens and PDX tumor (online supplemental figure 4). Of note, this patient's TIL^{TP} population was remarkably oligoclonal, with the top three clones comprizing approximately 59.2% of all cells and demonstrating corresponding enrichment within the NeoTCR8 cluster by scRNA analysis.

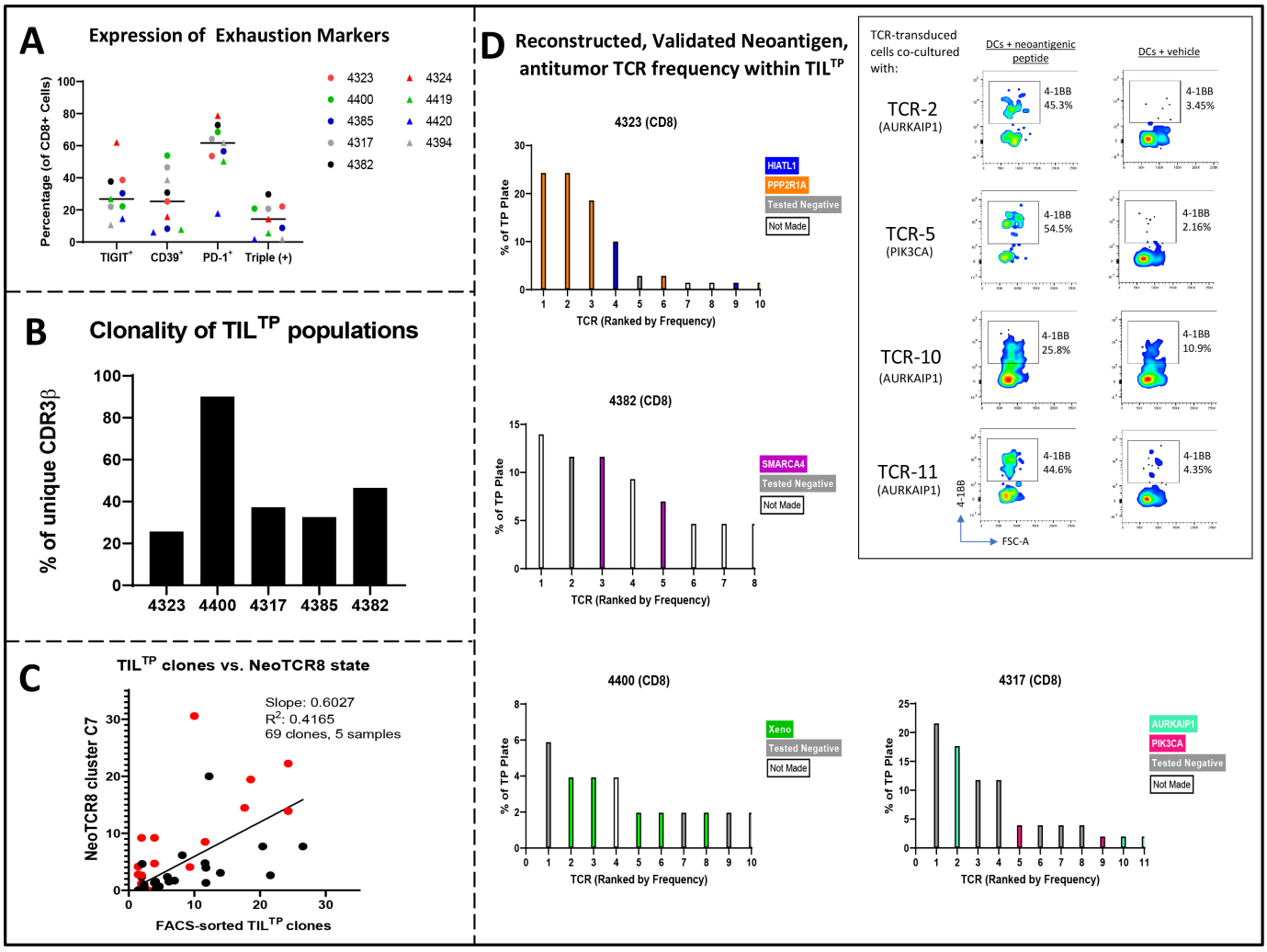
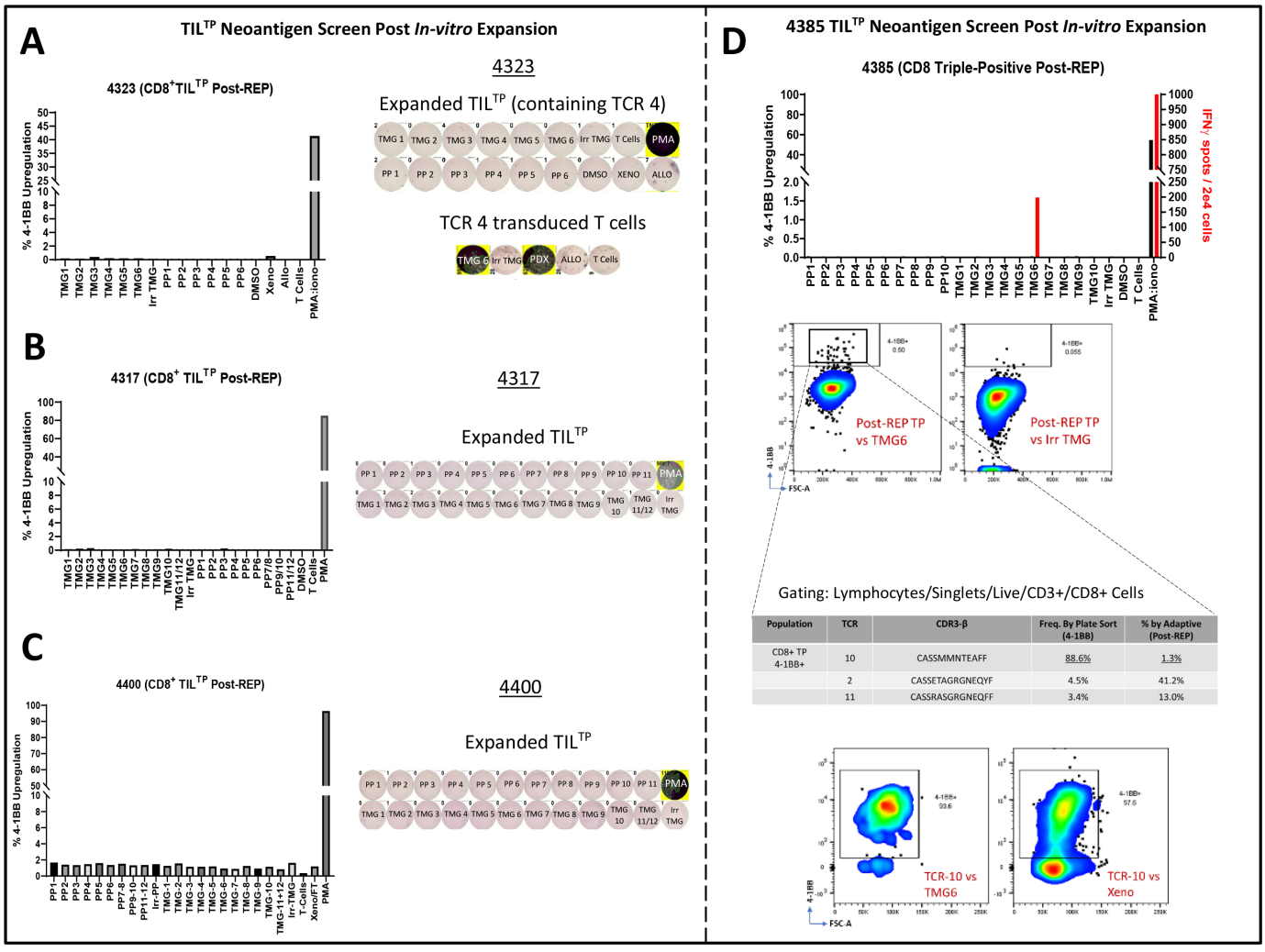


Figure 3 (A) TIGIT, CD39, and PD-1 expression by flow cytometry across nine T-cell samples isolated from tumor digest demonstrates variability in the surface expression of dysfunction markers between patients. Median PD-1 % across the samples is higher than any other single marker (61.7%). (B) Clonality of CD8+ TILTP populations in each patient. Y-axis reflects the percentage of unique CDR3β clonotypes with respect to the number of legible TCRs sequenced. (C) Correlation of 69 clones from five patient TIL samples within the NeoTCR8 cluster by single-cell sequencing compared with their frequencies within the TILTP compartment (R^2=0.4165).[22] (D) TILTP sequenced from each patient's tumor digest, ranked by frequency based on percentage of 96-well plate occupied. Reconstructed and experimentally vetted neoantigen-reactive and tumor-reactive TCR clonotypes are indicated. For patient 4317, FACS plots of activated TCR-transduced T cells (4-1BB+cells of CD8+ mTCR+ cells) co-culture results for TCR-2, TCR-5, TCR-10, and TCR-11 are shown with corresponded listed antigens (AURKAIP and PIK3CA), alongside pertinent negative controls. Unevaluated clones were unable to be reconstructed due to technical difficulties in Sanger sequencing. PD-1, programmed cell death protein-1; TCR, T-cell receptor; TIL, tumor-infiltrating lymphocytes.

In vitro-expanded CD8+ TILTP demonstrate limited functional tumor and neoantigen reactivity despite presence of known reactive clones

Identifying autologous T-cell populations from patient tumors that can be directly enriched and expanded for antitumor activity remains an important goal for T cell-based clinical cancer immunotherapies.[4] Given the substantial enrichment of antitumor neoantigen-reactive T-cell clonotypes from patient tumors within the TILTP subset, we evaluated the ability of TILTP to expand and retain tumor antigen-specific functions in vitro, as this could potentially serve as an effective cell therapy treatment product.

To this end, from the four patients' tumors, we assessed functional antigenic recognition and clonotype frequencies of TILTP -enriched TCR clonotypes prior to and post in vitro REP (post-REP) to study the clonal dynamics of TILTP (figures 4,5). TILTP populations were subject to in vitro expansion similar to our clinical protocols, as detailed above (Materials and Methods). At 14 days post-REP, in 3/4 patients analyzed, CD8+ TILTP did not demonstrate reactivity against patient-specific candidate neoantigens by IFN-γ secretion or 4-1BB upregulation despite demonstrating functionality at the bulk population level in response to phorbol myristate acetate/ionomycin stimulation (figure 4A–C). The lack of



Population	TCR	CDR3-β	Freq. By Plate Sort (4-1BB)	% by Adaptive (Post-REP)
CD8+ TP 4-1BB+	10	CASSMMNTEAFF	88.6%	1.3%
	2	CASSETAGRGNEQYF	4.5%	41.2%
	11	CASSRASGRGNEQFF	3.4%	13.0%

Figure 4 Co-culture of TCRs reconstructed from CD8+ TIL^{TP} with tumor-specific TMGs and peptide pools failed to demonstrate reactivity via upregulation of 4-1BB and IFN-γ secretion detected by ELISpot assays in samples (A) 4323, (B) 4317, and (C) 4400. For 4323 TIL, inset also shows TMG-recognition by TCR4-transduced PBL in the same experiment (present within pre-REP TIL^{TP}) indicating poor functional response of the same clonotype within TIL^{TP} (D) Co-culture of in vitro-expanded post-REP CD8+ TIL^{TP} with tumor-specific TMGs and peptide pools demonstrated reactivity to TMG6 via IFN-γ release and low-level 4-1BB upregulation. CDR3β sequencing of 4-1BB-upregulating clonotypes resulted in the reconstruction and identification of the reactive TCR, which also recognized the patient-specific PDX tumor. Allo, Allogeneic control PDX; DMSO, control for peptide; IFN, interferon; Irr TMG, irrelevant control TMG; PMA, phorbol myristate acetate; PP, peptide pools; REP, rapid expansion protocol; TCR, T-cell receptor; TIL, tumor-infiltrating lymphocytes; TMG, tandem minigene.

recognition was not due to lack of TCR specificity since neoantigen-reactive TCR-transduced T cells (eg, HIATL1 neoantigen-specific TCR four from patient 4323) in the same experiment demonstrated neoantigen and tumor recognition (figure 4A). In the one patient where neoantigen reactivity was still detected in the in vitro-expanded TIL^{TP} (4385, figure 4D), ~0.5% of the post-REP CD8+ TIL^{TP} upregulated CD137 in response to TMG6. Single-cell sorting and Sanger sequencing led to the identification of a single dominant, previously unknown TCR clonotype (CASSMMNTEAFF) that, on reconstruction and testing, demonstrated neoantigen reactivity against both TMG6 and PDX tumor line (figure 4D). TCRβ survey sequencing of the post-REP TIL^{TP} population showed expansion of this reactive clone to 1.3% of the total T cells, despite being below the limit of detection in the pre-REP sort in that it was not observed within the plate-sorted clonotypes (table 2).

To understand the lack of functional neoantigen reactivity in TIL^{TP} populations which initially contained and enriched for neoantigen-reactive TCR clonotypes (figure 4), we analyzed the TCR repertoire of TIL^{TP} relative to bulk CD8+ cells, as well as before and after in vitro REP expansion (figure 5A). Evaluation of the frequency of 19 neoantigen/tumor-reactive TIL^{TP} clones from the four patients prior to and following TIL^{TP} sorting revealed that antitumor TCR clones were highly enriched in the pre-expansion CD8+ TIL^{TP} populations relative to the bulk CD8+ TIL population (average fold enrichment from four patients=4.5, p=0.0018, paired t-test, figure 5B, table 1). However, following in vitro expansion, in three out of the four patient TIL^{TP} populations, antitumor

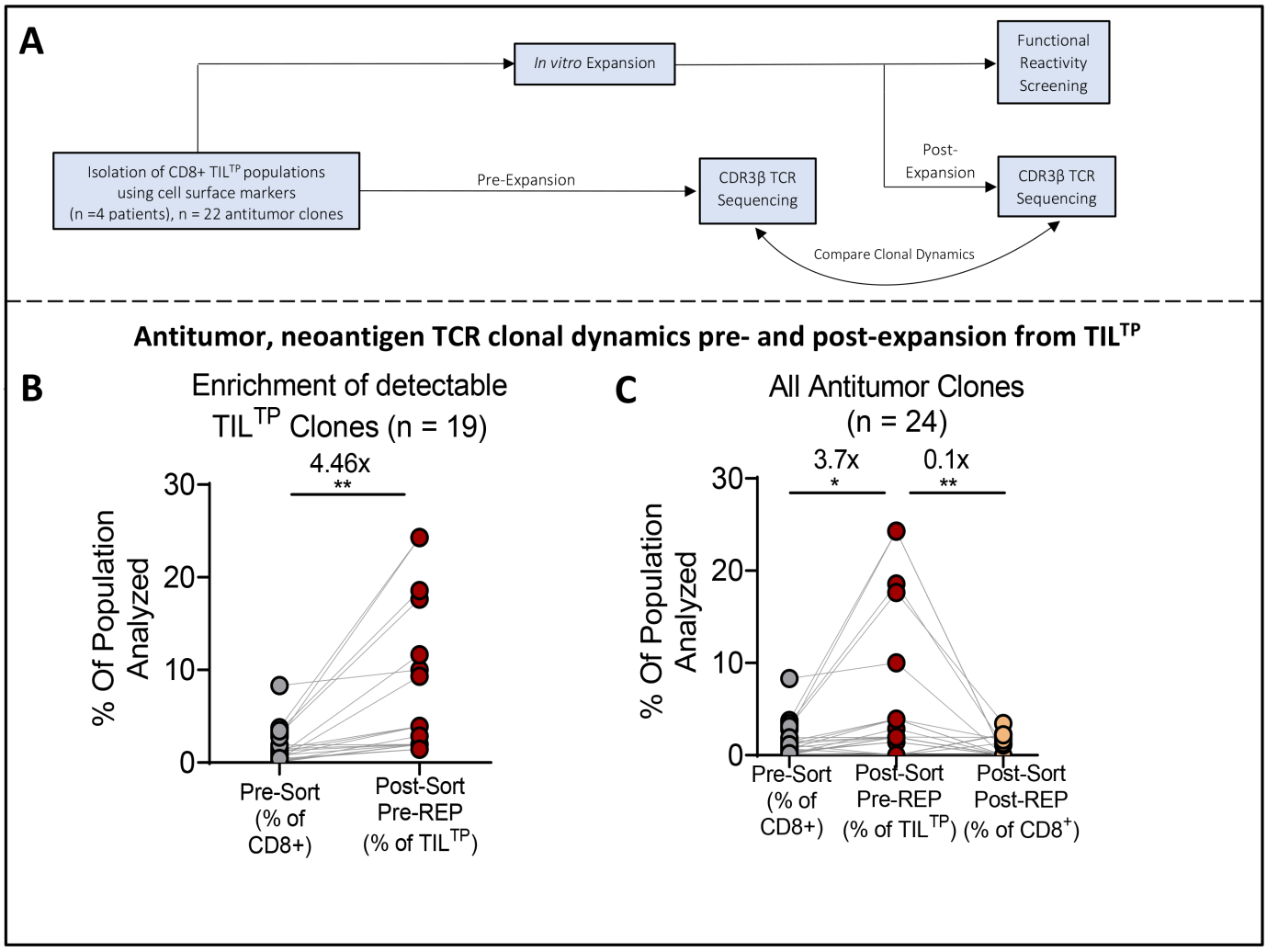


Figure 5 (A) Workflow for assessing clonal dynamics of antitumor or neoantigen-specific TCR clonotypes before and after in vitro expansion of CD8+ TILTP populations. (B) In vitro tracking of the percentage of neoantigen-reactive and antitumor clonotypes (n=19 clones, samples 4323, 4317, 4400, 4385, and 4382) among CD8+ TILTP T cells relative to bulk CD8+ TIL in tumor digest. Only clones that were detectable in a TILTP FACS-based plate sort (96 wells) are included in this analysis. (C) In vitro clonal tracking of all antitumor, neoantigen-reactive clones (n=24) among bulk CD8+ TIL (left), CD8+ TILTP (middle), and post-REP CD8+ TILTP (right) for samples 4323, 4317, 4400, and 4385. The majority of known neoantigen-reactive clonotypes are enriched by triple-positive sorting but are unable to expand in the REP. P values indicate paired t-test between bulk versus pre-expansion (pre-REP) populations, and pre-REP versus post-expansion (post-REP) TILTP populations. Numbers above indicate fold-expansion between the populations analyzed. * = p<0.05 ** = p<0.01. REP, rapid expansion protocol; TCR, T-cell receptor; TIL, tumor-infiltrating lymphocytes.

TCR clonotypes were not maintained by the end of an REP, as the average 3.7-fold enrichment of all known 24 antitumor TCR clones in four patients (p=0.0106, paired t-test) fell to 0.1 post-REP (p=0.0074, paired t-test, figure 5C, table 1). Despite a relative lack of clonal maintenance in the three patient TILTP populations (4323, 4400, and 4317) with no demonstrable neoantigen reactivity in their in vitro-expanded TILTP, known reactive TCRs still comprised 3.45%, 0.013%, and 9.27% of post-REP CD8+ TILTP, respectively, suggesting that lack of functional neoantigen recognition was not solely due to complete loss of neoantigen-specific T-cell clones during in vitro expansion in at least two patient TILTP (figure 5C). To understand if in vitro expanded post-REP TILTP subsets had broad functional defects in responding to TCR stimulation, we compared the intracellular cytokine levels of IFN-γ, tumor necrosis factor-α, and IL-2 between bulk CD8+ TIL and CD8+ TILTP after in vitro expansion. Intracellular cytokine staining detected no significant differences in cytokine expression between bulk and TILTP post-REP CD8+ TIL following overnight stimulation with CD3/CD28 Dynabeads (Thermo Fisher Scientific, online supplemental figure 5). This data in conjunction with specific neoantigen functional testing from the four patient TILTP populations (figure 4A–C) suggests that while some cells within the bulk TILTP expanded populations are capable of cytokine secretion, lack of IFN-γ secretion in our neoantigen screening assays is due to the functional impairment of antitumor neoantigen-specific clones on their cognate neoantigenic stimulation.

Table 2 Frequency of known-reactive TCR clones in pre-REP and post-REP CD8+TIL^{TP} populations. Pre-REP frequencies taken from transcriptomic analysis of bulk TIL (n.d., not detected, <0.001%). Post-REP TIL n/a for sample 4382

Patient	TCR	CDR3b	TCR reactivity	Pre-REP frequency (% of CD3+)	Pre-REP enrichment (% of TP)	Post-REP frequency (% by CDR3b seq)
4323						
	1	CATSGTGHSYEQYF	PPP2R1A	3.77	24.29	0.002
	2	CATSATGRSYEQYF	PPP2R1A	2.77	24.29	0.05
	3	CATSDTGRPYEQYF	PPP2R1A	3.43	18.57	1.1
	4	CASSPRVPNTEAFF	HIATL1	8.31	10.00	n.d.
	6	CASSQLTGAETQYF	PPP2R1A	0.11	2.86	n.d.
	9	CASSLDADGRSRETQYF	HIATL1	0.44	1.43	n.d.
	10	CSAATWAINQPQHF	PPP2R1A	0.33	1.43	n.d.
	(11)	CATSDTGKPYEQYF	PPP2R1A	0.11	–	2.3
	(12)	CATSDTGLPYEQYF	PPP2R1A	0.11	–	0.002
			Total known-reactive	**19.38**	**82.86**	**3.45**
4400						
	2	CASSQDPGARDYGYTF	PDX tumor	1.48	3.39	n.d.
	3	CAWSRVRPNEQFF	PDX tumor	0.68	3.39	n.d.
	5	CASSELAGGNYNEQFF	PDX tumor	1.48	1.69	n.d.
	6	CASSLLATGNFNEQFF	PDX tumor	1.26	1.69	n.d.
	8	CASSFPASGGAVDNEQFF	PDX tumor	0.80	1.69	0.003
	(15)	CASTTSPGPEYGYTF	PDX tumor	0.68	–	0.01
			Total known-reactive	**6.39**	**13.73**	**0.01**
4385						
	10	CASSMMNTEAFF	TMG6	0.08	–	1.30
			Total known-reactive	**0.08**	–	**1.30**
4317						
	2	CASSIWATNTGELFF	AURKAIP1	3.11	17.65	3.45
	5	CASNSGGPTVEQYF	PIK3CA	1.14	3.92	1.67
	9	CASSAGAEQYF	PIK3CA	1.90	1.96	1.94
	10	CSVVLASGDEEEQYF	AURKAIP1	0.15	1.96	n.d.
	11	CSVEDGGRDTGELFF	AURKAIP1	0.08	1.96	n.d.
	(12)	CASSPGGFEAFF	PIK3CA	1.14	–	2.22
			Total known-reactive	**7.51**	**27.45**	**9.27**
4382						
	2	CASSEAGASNYGYTF	SMARCA4	0.82	11.63	n/a
	4	CASSQSTGALETQYF	SMARCA4	0.41	9.30	n/a
			Total known-reactive	**1.23**	**20.93**	**n/a**

FACS, fluorescence-associated cell sorting; PDX, patient-derived xenograft; REP, rapid expansion protocol; TIL, tumor-infiltrating lymphocytes; TMG, tandem minigene .

DISCUSSION

ACT with TIL and their TCRs recognizing patient-specific neoantigens can mediate durable regression of metastatic epithelial cancers.[5 10–13 15 31] Tumor-reactive TCRs expressed by TIL can be isolated, retrovirally transduced into autologous PBL, and administered as ACT,

an approach currently under clinical investigation by the NCI Surgery Branch in clinical trial NCT03412877. This emphasizes the value of swift, and early, TCR identification for the treatment of patients with metastatic cancers often characterized by rapidly progressive visceral disease. In this study, we demonstrate quick and reliable identification of tumor-reactive CD8$^+$ TIL based on CD39, PD-1, and TIGIT co-expression using widely available methodology.

Although numerous combinations of cell surface markers have demonstrated the ability to enrich for tumor-reactive T cells,[17 18] identifying optimal marker combinations for the selection of tumor-reactive T cells and their receptors remains an open question. We leveraged a previously described high-dimensional transcriptomic analysis of TIL from patients with metastatic epithelial cancers to uncover CD39, PD-1, and TIGIT protein co-expression as common to antitumor T-cell clones.[22] While single-cell transcriptomic analysis offers substantially more granularity when examining T-cell populations, it is computationally intensive and precludes subsequent use of the cells of interest for experimental studies. TIL^{TP} sorting dispenses with transcriptomic analysis and is therefore amenable for wider usage including experimental manipulation. Additionally, in samples with very low frequency of antitumor TIL, or when plate-based TCR sequencing methods are not available, FACS enrichment of TIL^{TP} followed by single-cell transcriptomic sequencing might improve the identification of additional clonotypes.

Enrichment of T-cell clones by TIL^{TP} sorting demonstrated a strong correlation with that of its parent NeoTCR8 gene-signature and, on validation by TCR reconstruction, demonstrated specificity of at least 39.5% for neoantigen-reactive TCR identification. This is likely a conservative estimate, since we did not reconstruct and screen every TCR clonotype present in the TIL^{TP} subset, nor did we screen for every possible tumor antigen represented in the tumor. Additionally, given the presence of autologous tumor-reactive but neoantigen non-reactive TCR clonotypes within TIL^{TP} (five PDX-reactive TCRs from 4400 TIL, figure 3D), our calculation of antitumor TCRs is also impacted by the broader landscape of tumor antigens, beyond the neoantigens that we screened in this study.[22 24 25] Despite these limitations, sorting TIL^{TP} achieved as much as 82.9% enrichment for neoantigen reactivity in a single patient (4323) and a median enrichment of 20.9% across all patients (figure 3D). We demonstrate that neoantigen-reactive CD8$^+$ TCR clonotypes can be defined by the TIL^{TP} subset in every patient we analyzed, including instances of non-clonally expanded singleton clones captured on the TIL^{TP} plate being neoantigen-reactive (TCRs 9 and 10 from 4323, TCRs 5, 6, and 8 from 4400, and TCRs 9, 10, and 11 from 4317, respectively (table 2), suggesting potential advantage of direct sorting and identification of antitumor TCRs for use in cell therapies. Given that CD39 and TIGIT expression alternated as the limiting factor of patients' TIL^{TP} populations (figure 3A), and PD-1$^+$CD39$^+$ populations were more enriched for neoantigen reactivity than their single-positive counterparts (figure 1D), our findings are congruent with prior studies demonstrating superior enrichment of tumor reactivity with the addition of markers beyond PD-1 and validated our use of three markers to achieve optimal enrichment.[18] Additionally, because we relied on TCR reconstruction and engineering into healthy donor PBL rather than selection, growth, and expansion of this subset, this methodology overcomes limitations of functional experimental screens done on in vitro-expanded TIL material obtained from patients.

Prior studies have demonstrated the enrichment of tumor reactivity among oligoclonally expanded T cells expressing cell surface markers of dysfunction (eg, PD-1, or CD39$^+$CD103$^+$), though the ability of these cells to undergo expansion into a viable clinical TIL treatment product mediating durable tumor regressions remains to be evaluated.[17–19 21 39] Our phenotypic analyses by cell surface profiling, as well as mapping of known neoantigen-reactive TCR clones to the scRNA states reveal that TIL^{TP} likely represents a more tightly defined subset within the CD39$^+$CD103$^+$ TIL compartment, highly enriched for tumor reactive NeoTCRs, but more terminally differentiated, dysfunctional than the CD39$^+$CD103$^+$population as a whole (figure 1B–D).[17] This might in part explain the impaired proliferative potential and lack of functional neoantigen recognition in in vitro-expanded cultures (figure 4).

While four of five CD8$^+$ TIL^{TP} populations were demonstrably enriched for tumor reactivity, expanded TIL from three of four samples failed to maintain reactivity through a rapid expansion. CDR3β sequencing of in vitro-expanded post-REP populations confirmed a significant loss, but not absence of, reactive TCRs; known reactive clones comprised up to 9.27% of CD8$^+$ TIL^{TP} cells after in vitro REP (figure 5C, table 2). The robust post-stimulation cytokine response exhibited at the bulk level by post-REP CD8$^+$ TIL^{TP} populations, comparable to that of paired bulk TIL controls (online supplemental figure 5), is very likely driven by the bystander T cells that grow out in the REP since antigen-specific stimulation of the same populations demonstrated no IFN-γ secretion in our screens.

Taken together, these results suggest that in epithelial tumors, while TIL^{TP} can consistently and selectively enrich tumor reactivity, they might lack the proliferative potential to serve as an enriched cell therapy product. These findings are consistent with our prior studies in melanoma, in which both responders and non-responders to TIL therapy had no differences in the terminally differentiated dysfunctional neoantigen-specific CD39$^+$ TIL subset.[14] Rather, TIL-ACT responses were associated with a smaller pool of less differentiated CD39-negative stem-like TILs that were not enriched for neoantigen reactivity.[14]

While these findings strengthen prior claims of impaired effector function in post-expansion PD-1$^+$ and

CD39$^+$ TIL, the selective expansion of non-dominant tumor-reactive clones in all four post-REP CD8$^+$ TIL^{TP} populations, and demonstration of post-REP reactivity in one sample, alludes to a spectrum of dysfunction within endogenous TIL^{TP}.[14] In the sample with post-REP TIL^{TP} reactivity (4385), tumor recognition was driven by a non-dominant clone that was below the level of detection in the initial sort from tumor digest. Transcriptomic analysis of such unique TIL^{TP} prior to the REP may reveal key phenotypic differences that enable them to expand and react, although capturing them in sufficient quantities represents a significant challenge.

In this study, we evaluated cell surface markers enriching neoantigen reactivity within CD8$^+$ T cells from metastatic epithelial tumor deposits. Our screening methods did not include the examination of cancer germline antigens and other potential tumor-associated antigens when assaying the reactivity of TIL^{TP} populations, which may have provided a better understanding of the intratumoral TCR repertoire. In our study, we made no attempts at expanding TIL^{TP} by modifications to the standard clinical in vitro REP expansion, or prolonged rest as suggested by some chimeric antigen receptor expansion studies,[40] or via pharmaceutical intervention targeting epigenetic[41] or genetic[42] engineering of antitumor neoantigen-reactive T cells during their in vitro expansion for cell therapy. The dysfunctional state of TIL^{TP} may also be amenable to epigenetic reprogramming, as suggested by some murine models[43 44]. These remain important avenues to pursue in future studies to evaluate the potential direct use of TIL^{TP} based cell therapies.

These data demonstrate that while CD8$^+$PD-1$^+$CD39$^+$TIGIT$^+$ TIL serve as a highly efficient and reliable source of tumor-reactive TCRs, the cells themselves are phenotypically exhausted and offer limited utility as a treatment product. In an era of ACT where genetically engineered PBL can mediate regression of metastatic cancers[13 30] TIL^{TP} sorting enables rapid identification of TCR sequences from tumor-reactive TIL using readily available and affordable technology, saving patients precious time in pursuit of treatment.

Twitter Rami Yossef @YosephRami and Sri Krishna @tellkrish

Acknowledgements We thank the Surgery Branch TIL Laboratory and clinical team for generating TIL, and patients enrolled in our clinical protocols. We acknowledge Sivasish Sindiri for advice with computational analysis and Dr Zhiya Yu for her development of the patient-derived xenograft tumor models described herein. This work used the computational resources of the NIH HPC Biowulf cluster (http://hpc.nih.gov). We also thank NIDAP for providing additional computational support and the CCR Genomics Core for next-generation sequencing support.

Contributors FJL, SAR, and SK conceived the study and designed experiments. JJG and PR performed bioinformatic analysis of generating patient exome sequencing data and designed the tandem mini-gene constructs for neoantigen screening. PDC, FJL, SK, RY, NBP, KJH, VH, BP, MF, SR, AB, and MP performed experiments. PDC, FJL, SK, RY, and NBP performed data analysis. PDC, FJL, SK, NBP, and SAR wrote the manuscript with input from all authors. SAR is the guarantor for this manuscript.

Funding This research was supported by the Center for Cancer Research intramural research program of the National Cancer Institute, National Institutes of Health. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the US Government.

Competing interests FJL, SK, PR, and SAR are listed on an international patent application filed based on the NeoTCR8 signature described in this study. All other authors declare no competing interests.

Patient consent for publication Consent obtained directly from patient(s).

Ethics approval Not applicable.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data may be obtained from a third party and are not publicly available. TIL scRNA from select patients can be accessed via the NCBI database of Genotypes and Phenotypes (dbGaP) Study Accession: phs002748.v1.p1.

ORCID iDs
Frank J Lowery http://orcid.org/0000-0002-4620-5293
Paul Robbins http://orcid.org/0000-0002-1260-8123
Sri Krishna http://orcid.org/0000-0003-4994-3758

REFERENCES

1 Tran E, Robbins PF, Rosenberg SA. "final common pathway'' of human cancer immunotherapy: targeting random somatic mutations. *Nat Immunol* 2017;18:255–62.
2 Colli LM, Machiela MJ, Myers TA, *et al*. Burden of nonsynonymous mutations among TCGA cancers and candidate immune checkpoint inhibitor responses. *Cancer Res* 2016;76:3767–72.
3 Yarchoan M, Hopkins A, Jaffee EM. Tumor mutational burden and response rate to PD-1 inhibition. *N Engl J Med* 2017;377:2500–1.
4 Parkhurst MR, Robbins PF, Tran E, *et al*. Unique neoantigens arise from somatic mutations in patients with gastrointestinal cancers. *Cancer Discov* 2019;9:1022–35.
5 Zacharakis N, Chinnasamy H, Black M, *et al*. Immune recognition of somatic mutations leading to complete durable regression in metastatic breast cancer. *Nat Med* 2018;24:724–30.
6 Prickett TD, Crystal JS, Cohen CJ, *et al*. Durable complete response from metastatic melanoma after transfer of autologous T cells recognizing 10 mutated tumor antigens. *Cancer Immunol Res* 2016;4:669–78.
7 Zhou J, Dudley ME, Rosenberg SA, *et al*. Persistence of multiple tumor-specific T-cell clones is associated with complete tumor regression in a melanoma patient receiving adoptive cell transfer therapy. *J Immunother* 2005;28:53–62.
8 Andersen R, Donia M, Ellebaek E, *et al*. Long-Lasting complete responses in patients with metastatic melanoma after adoptive cell therapy with tumor-infiltrating lymphocytes and an attenuated IL2 regimen. *Clin Cancer Res* 2016;22:3734–45.
9 Levi ST, Copeland AR, Nah S, *et al*. Neoantigen identification and response to adoptive cell transfer in anti-PD-1 naïve and experienced patients with metastatic melanoma. *Clin Cancer Res* 2022;28:3042–52.
10 Tran E, Robbins PF, Lu Y-C, *et al*. T-Cell transfer therapy targeting mutant KRAS in cancer. *N Engl J Med* 2016;375:2255–62.
11 Tran E, Turcotte S, Gros A, *et al*. Cancer immunotherapy based on mutation-specific CD4+ T cells in a patient with epithelial cancer. *Science* 2014;344:641–5.

12 Zacharakis N, Huq LM, Seitter SJ, *et al*. Breast cancers are immunogenic: immunologic analyses and a phase II pilot clinical trial using mutation-reactive autologous lymphocytes. *J Clin Oncol* 2022;40:1741–54.
13 Kim SP, Vale NR, Zacharakis N, *et al*. Adoptive cellular therapy with autologous tumor-infiltrating lymphocytes and T-cell receptor-engineered T cells targeting common p53 neoantigens in human solid tumors. *Cancer Immunol Res* 2022;10:932–46.
14 Krishna S, Lowery FJ, Copeland AR, *et al*. Stem-Like CD8 T cells mediate response of adoptive cell immunotherapy against human cancer. *Science* 2020;370:1328–34.
15 Axelrod ML, Cook RS, Johnson DB, *et al*. Biological consequences of MHC-II expression by tumor cells in cancer. *Clin Cancer Res* 2019;25:2392–402.
16 Simoni Y, Becht E, Fehlings M, *et al*. Bystander CD8+ T cells are abundant and phenotypically distinct in human tumour infiltrates. *Nature* 2018;557:575–9.
17 Duhen T, Duhen R, Montler R, *et al*. Co-Expression of CD39 and CD103 identifies tumor-reactive CD8 T cells in human solid tumors. *Nat Commun* 2018;9:2724.
18 Gros A, Robbins PF, Yao X. Pd-1 identifies the patient-specific CD8PD-1 identifies the patient-specific CD8. *J Clin Invest* 2014;124:2246–59.
19 Pasetto A, Gros A, Robbins PF, *et al*. Tumor- and neoantigen-reactive T-cell receptors can be identified based on their frequency in fresh tumor. *Cancer Immunol Res* 2016;4:734–43.
20 Yossef R, Tran E, Deniger DC, *et al*. Enhanced detection of neoantigen-reactive T cells targeting unique and shared oncogenes for personalized cancer immunotherapy. *JCI Insight* 2018;3:e122467.
21 Kortekaas KE, Santegoets SJ, Sturm G, *et al*. Cd39 identifies the CD4+ tumor-specific T-cell population in human cancer. *Cancer Immunol Res* 2020;8:1311–21.
22 Lowery FJ, Krishna S, Yossef R, *et al*. Molecular signatures of antitumor neoantigen-reactive T cells from metastatic human cancers. *Science* 2022;375:877–84.
23 Caushi JX, Zhang J, Ji Z, *et al*. Author correction: transcriptional programs of neoantigen-specific TIL in anti-pd-1-treated lung cancers. *Nature* 2021;598:E1.
24 Oliveira G, Stromhaug K, Klaeger S, *et al*. Phenotype, specificity and avidity of antitumour CD8+ T cells in melanoma. *Nature* 2021;596:119–25.
25 Oliveira G, Stromhaug K, Cieri N, *et al*. Landscape of helper and regulatory antitumour CD4+ T cells in melanoma. *Nature* 2022;605:532–8.
26 Hanada K-I, Zhao C, Gil-Hoyos R, *et al*. A phenotypic signature that identifies neoantigen-reactive T cells in fresh human lung cancers. *Cancer Cell* 2022;40:479–93.
27 Zheng C, Fass JN, Shih Y-P, *et al*. Transcriptomic profiles of neoantigen-reactive T cells in human gastrointestinal cancers. *Cancer Cell* 2022;40:410–423.
28 Poschke IC, Hassel JC, Rodriguez-Ehrenfried A, *et al*. The outcome of ex vivo TIL expansion is highly influenced by spatial heterogeneity of the tumor T-cell repertoire and differences in intrinsic in vitro growth capacity between T-cell clones. *Clin Cancer Res* 2020;26:4289–301.
29 Fernandez-Poma SM, Salas-Benito D, Lozano T, *et al*. Expansion of tumor-infiltrating CD8+ T cells expressing PD-1 improves the efficacy of adoptive T-cell therapy. *Cancer Res* 2017;77:3672–84.
30 Leidner R, Sanjuan Silva N, Huang H, *et al*. Neoantigen T-cell receptor gene therapy in pancreatic cancer. *N Engl J Med* 2022;386:2112–9.
31 Robbins PF, Morgan RA, Feldman SA, *et al*. Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. *J Clin Oncol* 2011;29:917–24.
32 Gros A, Turcotte S, Wunderlich JR, *et al*. Myeloid cells obtained from the blood but not from the tumor can suppress T-cell proliferation in patients with melanoma. *Clin Cancer Res* 2012;18:5212–23.
33 Paria BC, Levin N, Lowery FJ, *et al*. Rapid identification and evaluation of neoantigen-reactive T-cell receptors from single cells. *J Immunother* 2021;44:1–8.
34 Jin J, Sabatino M, Somerville R, *et al*. Simplified method of the growth of human tumor infiltrating lymphocytes in gas-permeable flasks to numbers needed for patient treatment. *J Immunother* 2012;35:283–92.
35 Gros A, Tran E, Parkhurst MR, *et al*. Recognition of human gastrointestinal cancer neoantigens by circulating PD-1+ lymphocytes. *J Clin Invest* 2019;129:4992–5004.
36 Yang S, Rosenberg SA, Morgan RA. Clinical-scale lentiviral vector transduction of PBL for TCR gene therapy and potential for expression in less-differentiated cells. *J Immunother* 2008;31:830–9.
37 Morgan RA, Dudley ME, Wunderlich JR, *et al*. Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science* 2006;314:126–9.
38 Gee MH, Han A, Lofgren SM, *et al*. Antigen identification for orphan T cell receptors expressed on tumor-infiltrating lymphocytes. *Cell* 2018;172:549–563.
39 Inozume T, Hanada K-I, Wang QJ, *et al*. Selection of CD8+PD-1+ lymphocytes in fresh human melanomas enriches for tumor-reactive T cells. *J Immunother* 2010;33:956–64.
40 Weber EW, Parker KR, Sotillo E, *et al*. Transient rest restores functionality in exhausted CAR-T cells through epigenetic remodeling. *Science* 2021;372:eaba1786.
41 Henning AN, Roychoudhuri R, Restifo NP. Epigenetic control of CD8+ T cell differentiation. *Nat Rev Immunol* 2018;18:340–56.
42 Gautam S, Fioravanti J, Zhu W, *et al*. The transcription factor c-Myb regulates CD8+ T cell stemness and antitumor immunity. *Nat Immunol* 2019;20:337–49.
43 Yates KB, Tonnerre P, Martin GE, *et al*. Epigenetic scars of CD8+ T cell exhaustion persist after cure of chronic infection in humans. *Nat Immunol* 2021;22:1020–9.
44 Abdel-Hakeem MS, Manne S, Beltra J-C, *et al*. Author correction: epigenetic scarring of exhausted T cells hinders memory differentiation upon eliminating chronic antigenic stimulation. *Nat Immunol* 2021;22:1465.