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Divergent transcriptional states and kinetics of circulating tumor-infiltrating lymphocyte repertoires with highly homologous T-cell receptor sequences in a patient during immunotherapy

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ABSTRACT

Evidence has shown that T-cell receptors (TCRs) that recognize the same epitopes may not be the exact TCR clonotypes but have slightly different TCR sequences. However, the changes in the genomic and transcriptomic signatures of these highly homologous T cells during immunotherapy remain unknown. Here, we examined the evolutionary features in circulating TCR clonotypes observed in tumors (tumor-infiltrating lymphocyte (TIL)-TCRs) by combining single-cell RNA/TCR sequencing of longitudinal blood samples and TCR sequencing of tumor tissue from a patient treated with anti-cytotoxic T-lymphocyte-associated protein 4/programmed cell death protein-1 therapy. We found frequent circulating $CD8^+$ TIL-TCRs with identical complementarity determining region 3 (CDR3)α amino acid sequences but quasi-identical CDR3β and TCR α/β (TRA/TRB) sequences. Despite their sequence similarities, these highly homologous TIL-TCRs responded differently to immunotherapy, and exhibited distinct transcriptional signatures that were uniquely distinguished by the expression of *GZMK*. Overall, the expression of *IFNG* in $CD8^+$ T-cell subsets including highly homologous TIL-TCRs increased when the patient achieved a response, but gradually decreased as the patient developed acquired resistance. Our findings provide insight into the cross-talk between T cells in the tumor microenvironment and those in the blood, and highlight that $CD8^+$ T cells with highly homologous TCR sequences might display divergent transcriptional states and kinetics in response to immunotherapy.

INTRODUCTION

The emergence of immune checkpoint inhibitors (ICIs) has revolutionized cancer therapy; however, the response to these treatments remains variable among patients. Significant efforts have been made to characterize the cellular and molecular signatures of immune cells, and single-cell sequencing technologies have provided an unparalleled opportunity to elucidate the functional heterogeneity of T cells in the tumor microenvironment and peripheral blood (PB). These studies have identified distinct T-cell subsets, exhausted phenotypes, and clonal expansions that correlate with treatment outcomes.[1–4] However, the interplay between circulating and tumor-residing T cells during immunotherapy remains elusive.

By combining bulk T-cell receptor sequencing (TCR-seq) of tumor tissue and single-cell RNA sequencing (scRNA-seq)/TCR-seq of circulating T cells from sequentially obtained blood samples from the same patient who received a long-term benefit from chemo-immunotherapy, we recently reported notable alterations in the genomic and transcriptomic immune profiles of circulating T cells containing highly abundant TCR clonotypes observed in the tumor (tumor-infiltrating lymphocyte (TIL)-TCRs).[5] This approach also revealed that clonally expanded circulating TIL-TCRs at the early stages of treatment were enriched in signatures associated with terminal effector differentiation, characterized by the high expression of *CX3CR1*, and that the increased frequency of $CX3CR1^+$ $CD8^+$ cells correlated with response to chemo-immunotherapy and better prognosis in patients with non-small cell lung cancer (NSCLC).[5] These findings underscore the potential advantage of multiomics approaches in identifying a blood-based predictor of response to immunotherapy.

Here, we employed multiomics approaches to elucidate the longitudinal transcriptomic features and kinetics of TIL-TCRs in a patient with NSCLC who showed a response followed by disease progression to anti-CTLA-4/PD-1 therapy. We found distinct $CD8^+$ TIL-TCR

clusters that showed reciprocal kinetics in response to ICI therapy. Furthermore, we identified frequent circulating TIL-TCRs that shared the same complementarity determining region 3 (CDR3)α amino acid sequence but quasi-identical CDR3β and TCRα/β nucleotide sequences (TRA/TRB). Unexpectedly, despite the similarity of sequences, these highly homologous TIL-TCRs displayed divergent transcriptomic features, uniquely characterized by *GZMK* expression.

METHODS

A full description is supplied in online supplemental methods. In brief: informed consent was obtained from the patient for the collection and storage of blood and tumor samples, and review of clinical records. Isolation and storage of peripheral blood mononuclear cells (PBMCs) and the scRNA-seq analyses were performed as previously described.[5 6] Antibodies used in this study are described in online supplemental table 1. For TCR-seq DNA from formalin-fixed, paraffin-embedded tumor biopsy samples were profiled using the ImmunoSEQ immune profiling platform (Adaptive Biotechnologies) and analyzed as previously described.[5–7] Raw and processed scRNA/TCR-seq data supporting the findings of this study were deposited in the National Center for Biotechnology Information Gene Expression Omnibus under accession number GSE266035. Statistical analysis was performed using unpaired or paired t-tests for comparisons between the two groups. All tests were two-sided and p<0.05 was considered statistically significant. Statistical analysis was performed using GraphPad Prism V.10.1.2 (GraphPad Software).

RESULTS

Early on-treatment changes in the frequency and clonality of circulating CD8$^+$ T-cell subsets in a patient with NSCLC treated with anti-CTLA-4/PD-1 therapy

A patient in 60s with a history of unresectable stage IIIB squamous cell carcinoma of the right hilum without actionable mutations was found to have a relapse of lung cancer in the hilar, mediastinal and left supraclavicular lymph nodes 10 months after treatment with chemotherapy and radiation therapy, and underwent treatment with every 2-week nivolumab and ipilimumab. After three cycles of treatment at 6 weeks, the patient developed productive cough and dyspnea, and the CT scan showed decreased right hilar and mediastinal adenopathy, but new nodular lesions in the right lung (online supplemental figure 1). Although it was unclear whether the new lesions in the right lung were due to infection, inflammation, progression of lung cancer, or pseudoprogression, the patient was treated with a short course of antibiotics and prednisone 40 mg for 5 days for possible pneumonia or toxicity before receiving the fourth cycle of anti-CTLA-4/PD-1 therapy at 8 weeks. Subsequently, the new right lung lesions gradually disappeared, and the patient eventually achieved a partial response to ICI therapy (online supplemental figure 1).

We evaluated the frequency of circulating CD8$^+$ T-cell subsets expressing CX3CR1, Ki67, and PD-1/Ki67 (online supplemental figure 2), which were previously reported to be predictive of response in patients treated with ICI therapy.[5 6 8 9] We found that the frequency of PB CX3CR1$^+$ CD8$^+$ T cells substantially increased at 4 weeks and remained high at ≥20% increase from the baseline, except when ICI therapy was skipped and antibiotics and steroids were administered at 6 weeks (figure 1A). In contrast, the frequency of Ki-67$^+$ subsets in PB CD8$^+$ T cells and PD-1$^+$ CD8$^+$ T cells transiently increased at 4 weeks and returned to baseline at 8 weeks (figure 1B).

We have previously shown an increase in TCR clonality in peripheral CD27$^+$ CX3CR1$^+$ CD8$^+$ T cells in response to anti-CTLA-4/PD-1 antibodies in mice[6]; however, this was not confirmed in patients. To this end, we identified three subsets of PB CD8$^+$ T cells stratified by CX3CR1 and/or CD27 expression in this patient (figure 1C and online supplemental figure 3A), and isolated each subset by flow sorting (online supplemental figure 3B) for bulk TCR-seq. We found that TCR clonality in CD27$^+$ CX3CR1$^+$ CD8$^+$ T cells increased over twofold after two cycles of anti-CTLA-4/PD-1 therapy at 4 weeks (figure 1D).

Longitudinal single-cell profiling of circulating T cells identified expanding and contracting clusters during response and resistance to anti-CTLA-4/PD-1 therapy

We recently developed a multiomics platform, combining TCR-seq of tumor tissue and scRNA/TCR-seq of serial blood samples to evaluate changes in the transcriptional states of TIL-TCRs in PB.[5] We applied this approach in this patient and flow-sorted CD45$^+$CD3$^+$ live cells (online supplemental figure 4A, B) from cryopreserved PBMC samples not only at pretreatment and early on-treatment (4, 8, and 12 weeks), but also at 24 and 32 weeks when the patient had stable disease and progression, respectively. This yielded data for 14,044 high-quality cells after stringent filtering (pretreatment, 2,323 cells; 4 weeks, 1,133 cells; 8 weeks, 2,559 cells; 12 weeks, 2,422 cells; 24 weeks, 3,688 cells; and 32 weeks, 1,919 cells) (online supplemental figure 4C, D).

We first examined CD8$^+$ T-cell subsets and identified 14 distinct lymphocyte clusters (cluster (C)0–13) using unsupervised clustering analysis (figure 2A, online supplemental figure 5A,B and online supplemental data 1). Within the clusters expressing *CD8A*, we found a markedly increased frequency of C2 and C7 expressing *IFNG* at 12 weeks (figure 2B–F). However, the frequency of these clusters gradually decreased as the patient developed acquired resistance to ICI therapy at 24–32 weeks (figure 2B and F). Notably, C2 and C7 were characterized by high expression of orphan nuclear receptor *NR4A2* which is an essential transcription factor for the production of inflammatory cytokines.[10] Overall, the expression of *IFNG* in *CD8A*$^+$ T-cell subsets became higher as early as 4 weeks (p<0.00001 vs pretreatment, online supplemental

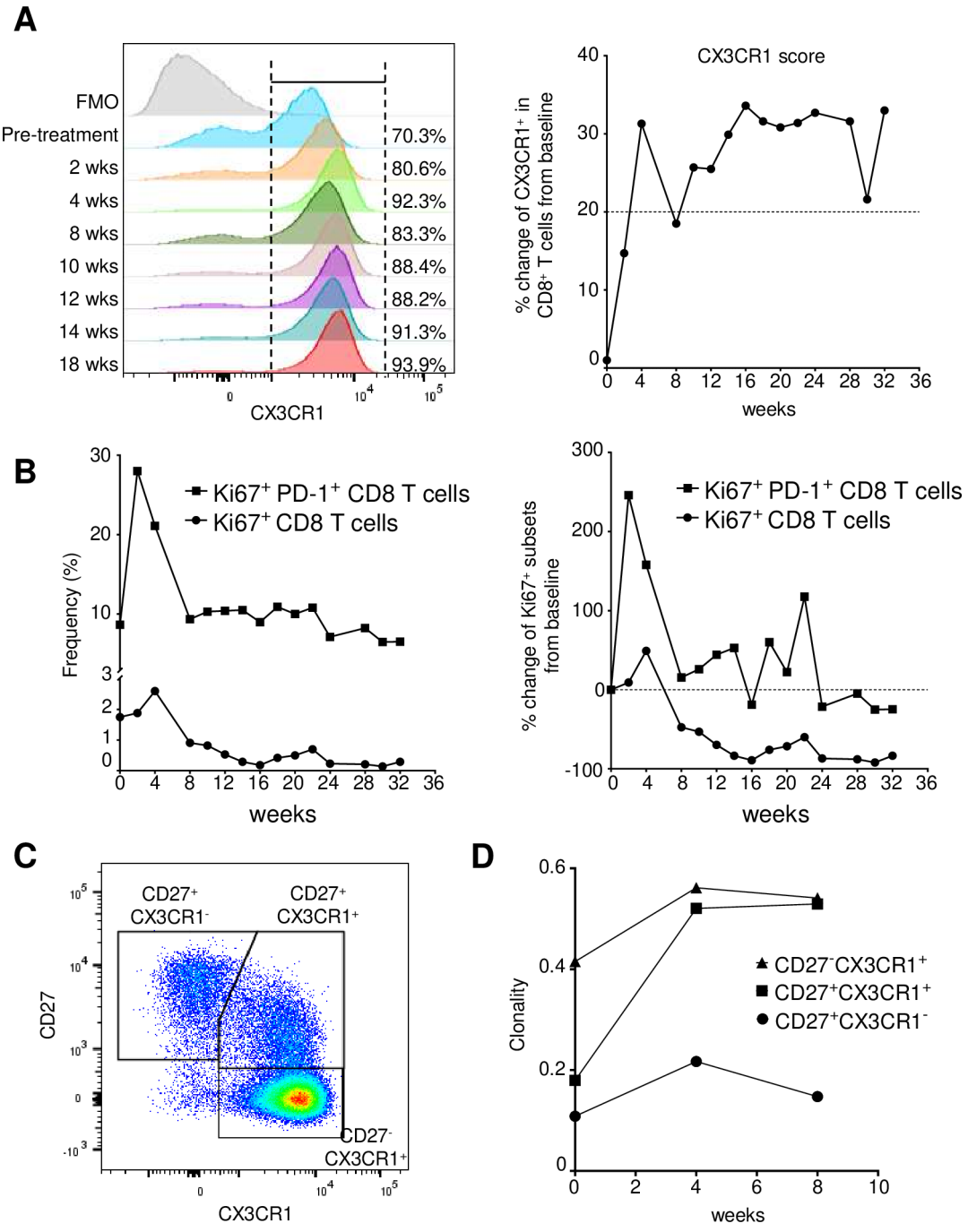

Figure 1 Early on-treatment change in the frequency and clonality of circulating CD8$^+$ T-cell subsets in a patient with non-small cell lung cancer treated with anti-CTLA-4/PD-1 therapy. (A) The expression of CX3CR1 in peripheral blood CD8$^+$ T cells (left), and % change of CX3CR1$^+$ subsets in CD8$^+$ T cells from baseline (CX3CR1 score) at different specified time points (right). (B) Frequency (left) and % change from baseline (right) of Ki67$^+$ PD-1$^+$ and Ki67$^+$ CD8$^+$ T cells at different time points. (C) Representative flow-cytometric plot of three subsets (CD27$^+$ CX3CR1$^-$, CD27$^+$ CX3CR1$^+$, and CD27$^-$ CX3CR1$^+$) of peripheral blood CD8$^+$ T cells. (D) T-cell repertoire clonality of the three subsets in (C) after anti-CTLA-4/PD-1 therapy. CTLA-4, cytotoxic T-lymphocyte associated protein 4; PD-1, programmed cell death protein-1.

Data 2A and online supplemental figure 6A), returned to the baseline after the patient was treated with antibiotics and steroids at 8 weeks (online supplemental data 2B), but increased again at 12 weeks ($p<0.00001$ vs pretreatment, online supplemental data 2C). However, *IFNG* expression decreased at 24 and 32 weeks ($p<0.02$ vs 12 weeks, online supplemental data 2D, E and online supplemental figure 6B).

Consistent with the higher frequency of PB CX3CR1$^+$ CD8$^+$ T cells in this patient (figure 1A), *CX3CR1* was expressed by the majority of *CD8A*$^+$ T-cell clusters, except for C6 and C9 (figure 2E). These two clusters uniquely

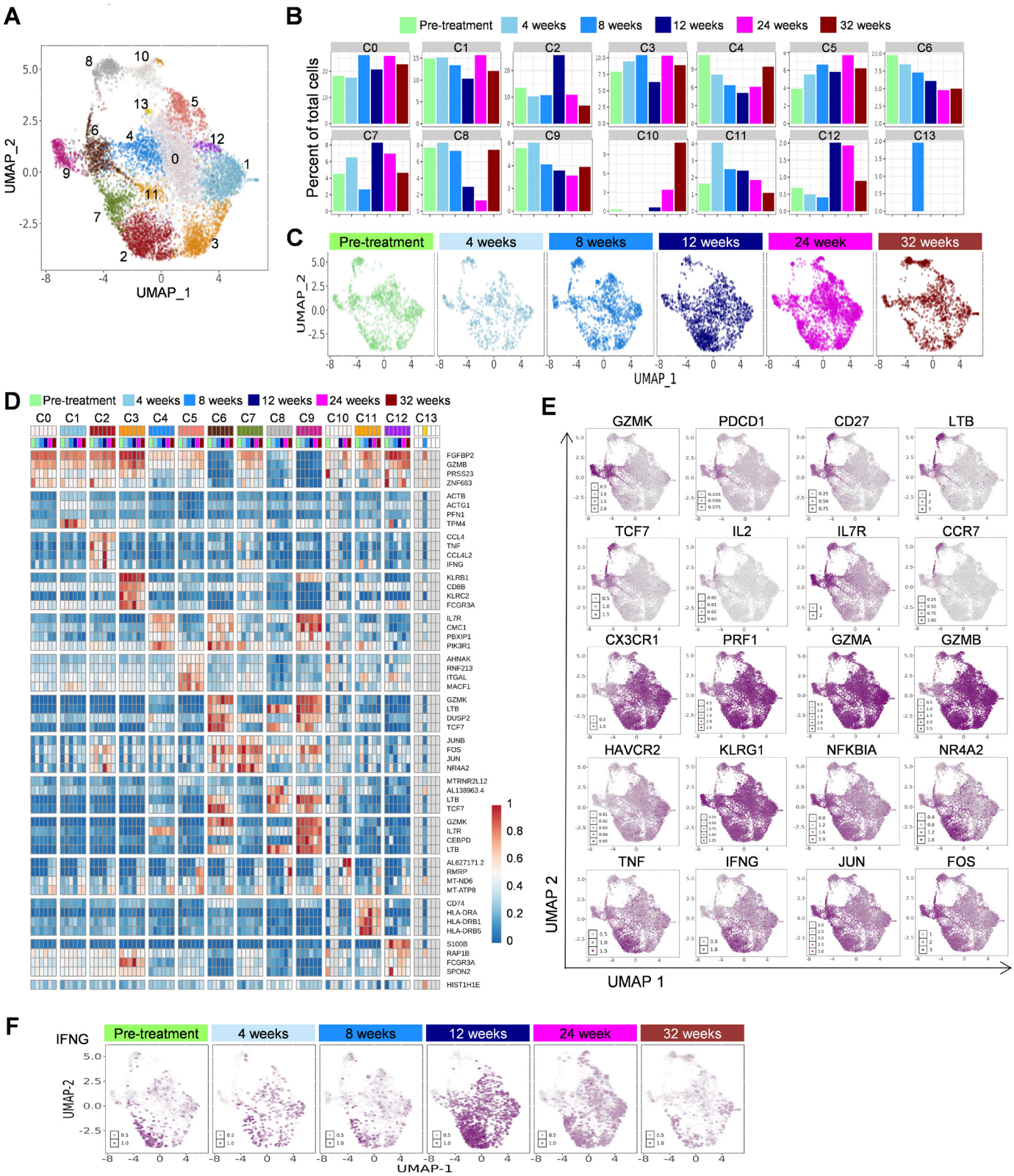

Figure 2 Longitudinal single-cell profiling of circulating T cells identified expanding and contracting clusters during response and resistance to anti-cytotoxic T-lymphocyte associated protein 4/programmed cell death protein-1 therapy. (A) UMAP plot of *CD8A*$^+$ T-cell subsets determined from single-cell RNA sequencing of total T cells in peripheral blood, cells colored by unsupervised cluster classification. (B) Frequency of each CD8$^+$ T-cell cluster in peripheral blood over time as indicated. (C) UMAP plots of *CD8A*$^+$ T cells over time. (D) Heatmap of top cluster-specific genes identified in peripheral blood *CD8A*$^+$ T-cell clusters. (E) Expression profiles of select genes across cells over time. (F) Expression profile of *IFNG* across cells over time. Expression levels are color-coded: gray, not expressed; purple, expressed. UMAP, Uniform Manifold Approximation and Projection.

Table 1 CDR3α/β amino acid and TCRα/β nucleotide sequence (TRA/TRB) of frequent circulating tumor-infiltrating lymphocyte T-cell repertoires (TIL-TCRs)

Clone	CDR3α	TRA
#1	CAVSDRSGGGADGLTF	TGTGCTGTGAGTGATCGTTCAGGAGGAGGTGCTGACGGACTCACCTTT
#2	CAVSDRSGGGADGLTF	TGTGCTGTGAGTGATCGATCAGGAGGAGGTGCTGACGGACTCACCTTT
#3	CAVSDRSGGGADGLTF	TGTGCTGTGAGTGATCGGTCAGGAGGAGGTGCTGACGGACTCACCTTT
#4	CAFMGYNNNDMRF	TGTGCCTTTATGGGGTACAATAACAATGACATGCGCTTT
#5	CAFMGYNNNDMRF	TGTGCCTTTATGGGCTACAATAACAATGACATGCGCTTT
#6	CAFMGYNNNDMRF	TGTGCTTTTATGGGGTACAATAACAATGACATGCGCTTT
Clone	**CDR3β**	**TRB**
#1	CASSLGLNYEQYF	TGTGCCAGCAGTTTAGGGCTGAACTACGAGCAGTACTTC
#2	CASSLGVHYEQYF	TGTGCCAGCAGTTTAGGGGTCCATTACGAGCAGTACTTC
#3	CASSLGLHYEQYF	TGTGCCAGCAGTTTGGGACTCCACTACGAGCAGTACTTC
#4	CASSSRGTGELFF	TGTGCCAGCAGTTCCAGGGGGACCGGGGAGCTGTTTTTT
#5	CASSSRGTGELFF	TGTGCCAGCAGTTCCCGTGGGACCGGGGAGCTGTTTTTT
#6	CASSYRGTGELFF	TGTGCCAGCAGTTACAGGGGTACCGGGGAGCTGTTTTTT

CDR3, complementarity determining region 3.

expressed high levels of *GZMK*, and gradually decreased on initiation of ICI therapy (figure 2B). Both C6 and C9 cells expressed *PDCD1* and genes associated with memory T-cell differentiation, *LTB*, *CD27*, *IL7R*, and *TCF7* (encoding TCF1). Furthermore, similar to C2 and C7, C6 and C9 expressed NF-κB target gene, *NFKBIA* (encoding the repressor of NF-κB, IκBα), and activating protein-1 family transcription factors, *FOS* and *JUN*, indicating recently activated, less-differentiated effector T cells.[11 12]

Divergent gene expression and kinetics of circulating TIL repertoires with highly homologous TCR sequences during anti-CTLA-4/PD-1 therapy

Next, we performed bulk TCR-β-seq of tumor tissue and identified the frequent CDR3β sequences, CASSLGLNYEQYF and CASSSRGTGELFF (online supplemental data 3), which were the top two most frequent circulating T cells in scRNA/TCR-seq data before treatment (clone #1 and #4 in table 1 and online supplemental data 4, 5). We then evaluated changes in the frequency of these TIL-TCRs in PB during immunotherapy. Both clonotypes were detected at a high frequency in PB throughout the treatment; however, their kinetics were different (figure 3A). The CASSLGLNYEQYF (#1 in table 1) substantially expanded at 4 weeks ($p<0.0002$ vs pretreatment, online supplemental data 6A and online supplemental figure 7), and remained high throughout the treatment (figure 3A). In contrast, CASSSRGTGELFF (#4 in table 1) markedly decreased at 4 weeks ($p=0.0273$ vs pretreatment, online supplemental data 6A and online supplemental figure 7), and remained low until 12 weeks (figure 3A). Overall, TCR overlap score of all circulating T cells between samples was low between pretreatment and 4 weeks, but remained stable after 4 weeks (figure 3B), suggesting that reshaping of $CD8^+$ T-cell repertoires occurred early on-treatment.

When we evaluated CDR3α amino acid sequences, we found that CASSLGLNYEQYF (#1 in table 1) shared the same CDR3α sequence with the highly homologous CDR3β clonotypes, CASSLGVHYEQYF and CASSLGLHYEQYF (#2 and #3 in table 1). Despite the similarity of their CDR3β sequences, these TCR clonotypes responded differently to ICI therapy (figure 3A). Particularly, CASSLGLNYEQYF and CASSLGVHYEQYF (#1 and #2 in table 1) clonally expanded and contracted over time, respectively (figure 3A, online supplemental figure 7 and online supplemental data 6A-E). In addition, their transcriptomic signature was also different, where CASSLGVHYEQYF (#2 in table 1) had a higher frequency of *GZMK*-expressing clusters, C6 and C9, than CASSLGLNYEQYF (#1 in table 1) (figure 3C–E, online supplemental figure 8 and online supplemental data 7). The relative frequency of $IFNG^+$ C2 and/or C7 in CASSLGLNYEQYF (#1 in table 1) increased at 12 weeks, but gradually decreased at 24–32 weeks (figure 3C and online supplemental figure 8).

Another highly frequent TIL-TCRs, CASSSRGTGELFF (#4 and #5 in table 1), shared the same CDR3α sequence (CAFMGYNNNDMRF) as CASSYRGTGELFF (#6 in table 1). Of these, clones #4 and #5 had identical CDR3α/β amino acid sequences and similar TRA and TRB nucleotide sequences, but they exhibited distinct levels of *GZMK* expression and reciprocal responses to ICI therapy at 4 weeks (figure 3A and C–E and online supplemental data 8). Similar to clone #2, clone #4 which contains *GZMK*-expressing C6, decreased on the initiation of immunotherapy. Of note, despite heterogeneous transcriptional states in each TIL-TCR clone (figure 3C), the majority of these $CD8^+$ T cells expressed *CX3CR1* (figure 3F). Interestingly, regardless of their baseline transcriptional states, C2 and/or C7 expressing *IFNG* decreased between 12 and 32 weeks in all six highly

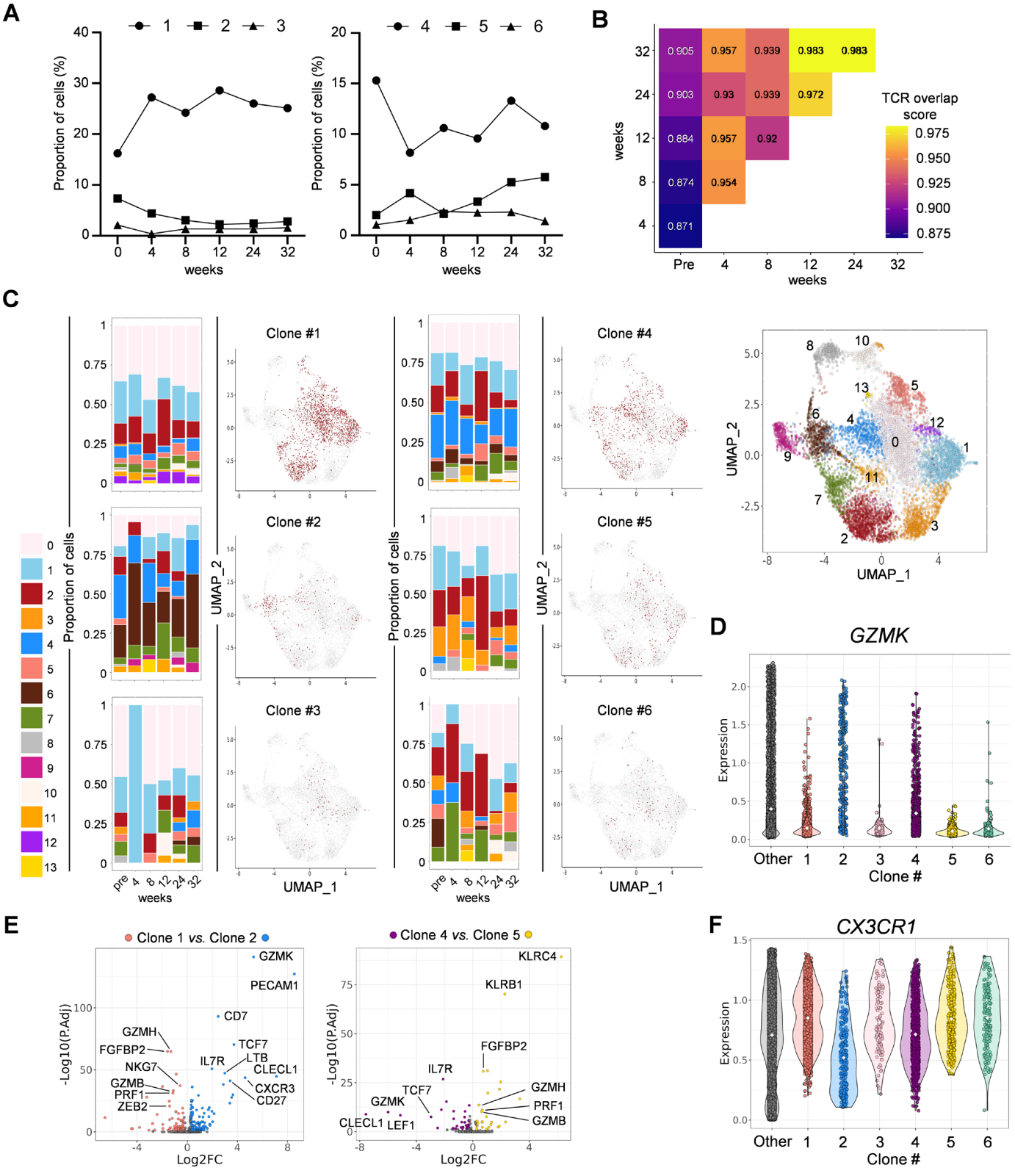

Figure 3 Divergent gene expression and kinetics of circulating TIL repertoires with highly homologous TCR sequences during anti-cytotoxic T-lymphocyte associated protein 4/programmed cell death protein-1 therapy. (A) Relative frequency of circulating tumor-infiltrating lymphocyte T-cell repertoires (TIL-TCRs) #1–6 described in table 1. (B) TCR complementarity determining region 3β repertoire similarity determined by Morisita's Index between blood samples. (C) UMAP plots (right) and cluster distribution of TIL-TCRs over time (left) in peripheral blood. (D) *GZMK* expression of cells over time within each selected clonotype. (E) Volcano plots showing enrichment of differentially expressed genes between TIL-TCR clones #1 and #2 (left), and #4 and #5 (right) over time. (F) *CX3CR1* expression of cells over time within each selected clonotype. UMAP, Uniform Manifold Approximation and Projection

homologous TIL-TCRs (figure 3C and online supplemental figure 8).

We also evaluated the changes in the transcriptomic signature of *CD4*-expressing clusters (online supplemental figure 9A, B). Clusters expressing *FOXP3* (C5) and *NR4A2, FOS, FOSB, JUN*, and *JUNB* (C3) peaked at 12 weeks but decreased in patients with stable disease/progression (online supplemental figure 10A-E and online supplemental data 9). However, we did not observe significant changes in the diversity and frequency of TCR clonotypes in circulating *CD4*$^+$ T cells. Baseline TCR diversity was high and remained unchanged during immunotherapy (online supplemental figure 11A). In line with this, we did not identify expanded or contracted *CD4*$^+$ T-cell clones (online supplemental figure 11C).

DISCUSSION

ICI therapy can elicit dynamic changes in the tumor microenvironment and systemic immunity; however, the cross-talk between immune cells in tumors and those in the blood remains elusive. Here, we profiled the evolutionary landscape of circulating TIL-TCRs by combining scRNA/TCR-seq of prospectively collected longitudinal blood samples and TCR-seq of tumor tissue. Our findings are as follows: (1) circulating highly homologous TIL-TCRs sharing the same CDR3α sequence exhibited distinct transcriptomic signatures, uniquely distinguished by the expression of *GZMK*, and responded differently to ICI therapy; (2) CD8$^+$ clusters containing TIL-TCRs that expressed *GZMK* gradually decreased on initiation of ICI therapy; (3) reshaping of CD8$^+$ T-cell repertoires occurred early on-treatment; and (4) expression of *IFNG*$^+$ CD8$^+$ clusters including TIL-TCRs increased in response to anti-CTLA-4/PD-1 therapy but decreased over time as the patient developed acquired resistance.

Evidence has shown that TCRs recognizing the same viral antigen or neoantigen may not be the exact TCR clonotypes, but have highly homologous CDR3 amino acid sequences.[13–15] In preclinical models of ICI therapy, our study and others have demonstrated that T cells sharing similar amino acid sequence features frequently exist in PB and the tumor microenvironment.[6 16 17] However, the phenotypic and transcriptomic signatures of highly homologous T cells remain incompletely understood. In the present study, we found that circulating TIL-TCRs sharing identical CDR3α but quasi-identical CDR3β sequences display divergent transcriptional states and kinetics in response to ICI therapy. Furthermore, this was also observed in TIL-TCRs that shared identical CDR3α/β sequences, but had distinct DNA sequences (clones #4 and #5 in table 1 and figure 3). Previous studies have reported that the presence of this phenomenon, known as TCR convergence, was associated with antigen-specific T-cell response to ICI therapy[18] and that convergent TCRs are likely to be antigen-specific.[19] These findings are in line with our results demonstrating the presence of convergent circulating TIL-TCRs in a patient who responded to anti-CTLA-4/PD-1 therapy. Our study further demonstrated that these convergent CD8$^+$ TIL-TCR clones may show a reciprocal response to ICI therapy.

Our results also revealed that these convergent CD8$^+$ T-cell clones displayed different transcriptional states distinguished by the level of *GZMK* expression (figure 3D). Notably, on the initiation of ICI therapy, there was a gradual decline in *CD8A*$^+$ clusters containing TIL-TCRs that expressed *GZMK* (C6 and C9 in figure 2). This finding is consistent with our recent study, which used the same multiomics approach in patients with NSCLC treated with chemo-immunotherapy.[5] However, the cause of the decreased frequency of this subset remains unclear. In this regard, *GZMK*$^+$ *CD8A*$^+$ clusters C6 and C9 expressed *PDCD1*, *TCF7* and *IL7R* (figure 2D and E), resembling intratumoral pre-dysfunctional cells that were found to express high levels of *GZMK* and intermediate expression of inhibitory receptors and/or *TCF7* and *IL7R*.[1–4] We also reported that newly introduced CD8$^+$ T cells after effective multimodal intralesional therapy were characterized by the expression of *Gzmk* and *Tcf7* and intermediate expression of *Pdcd1* in preclinical models.[7] Therefore, it may be speculated that circulating *GZMK*$^+$ T-cell clones may be recruited to the tumor microenvironment. Future studies with TCR-seq of pretreatment and on-treatment tumor tissues and scRNA/TCR-seq analysis of PBMCs are warranted to determine the fate of circulating TIL-TCRs expressing *GZMK*.

In this study, the expression of *IFNG* in *CD8A*$^+$ T-cell subsets including highly homologous TIL-TCRs increased when the patient achieved a response, but gradually decreased as the patient developed acquired resistance. Interferon (IFN)-γ enhances immune function by inducing the expression of surface major histocompatibility complex class I and facilitates the presentation of tumor antigens.[20] Therefore, the generation of IFN-γ-producing T cells may have been associated with the clinical response to ICI therapy in this patient. Our study further demonstrated that the relative frequency of *IFNG*$^+$ CD8$^+$ T cells decreased as the patient developed resistance to ICI therapy. Because *IFNG*$^+$ CD8$^+$ T cells are endowed with superior effector function compared with *IFNG*$^-$ CD8$^+$ T cells,[21] our results merely indicate that the frequency of this subset correlates with resistance as well as response to ICI therapy. However, opposing functions of IFN-γ signaling have been described during ICI therapy.[22] Therefore, it remains unclear whether *IFNG*$^+$ CD8$^+$ T cells cause the acquired resistance. Additional studies are needed to decipher the role of *IFNG*$^+$ CD8$^+$ T cells in circulation and tumors.

Single-cell profiling of PB CD8$^+$ T cells and their TCR sequences identified remarkable heterogeneous transcriptional states in each circulating TIL-TCR at pretreatment as well as on-treatment. Although some circulating TIL-TCR clones such as clones #2 and #4 contained unique clusters expressing *GZMK*, the majority of frequent circulating TIL-TCRs expressed *CX3CR1* (figure 3F). This finding is in line with our previous preclinical study

demonstrating the high degree of TCR sequence similarity between CD8+ TILs and peripheral CX3CR1+ CD8+ T cells.[6] Furthermore, our patient in this study had an early on-treatment increase of circulating CX3CR1+ CD8+ T cells (figure 1A) expressing genes associated with terminally differentiated T cells (figure 2E) in agreement with previous studies.[5 6 23 24]

Our study has several limitations. First, due to the limited availability of tumor and sequential blood samples from patients who had an initial response and developed acquired resistance, we had only one patient in the current study. The intratumor heterogeneity of TIL-TCRs may not have been comprehensively captured as DNA was not extracted from the entire tumor for TCR-seq. It is unknown whether clonally expanded TIL-TCRs in PB also expanded within the tumor during immunotherapy due to the lack of on-treatment tumor tissue from this patient. Additionally, as the patient received combined anti-CTLA-4/PD-1 checkpoint therapy, it is uncertain whether the results in the study were attributable to anti-CTLA-4 antibody, anti-PD-1 antibody, or both. Moreover, it remains unclear why our study did not detect expanded or contracted CD4+ T cells, despite evidence that anti-CTLA-4 therapy can target CD4+ T cells.[25] Additional studies are necessary to elucidate the plasticity of circulating CD4+ T cells including TIL-TCRs, in response to immunotherapy.

Finally, the identification of T cells sharing identical CDR3α amino acid sequences but quasi-identical CDR3β and TCRα/β sequences in our study was facilitated by the advances in scRNA/TCR-seq technology, which allowed us to sequence both TCRα and TCRβ rearrangements simultaneously. The incidence of this phenomenon remains unclear because these findings could have been overlooked if TCR-seq had been performed for either TCRα or TCRβ chain alone. Moreover, integrating TCR-seq of tumor tissue allowed us to identify tumor-residing T-cell clones within the broad and diverse T-cell repertoires in PB, and reveal the potential antigen specificity of convergent T cells.[19] Future studies employing multiomics approaches on samples from additional patients undergoing immunotherapy are expected to yield potential predictive insights into circulating highly homologous TIL-TCRs.

In summary, our work has revealed that frequently circulating TIL-TCRs with highly homologous TCR nucleotide sequences displayed divergent transcriptional states and response kinetics in a patient undergoing anti-CTLA-4/PD-1 therapy. These results provide insight into the development of TCR sequence-based predictive markers for response to immunotherapy.

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Contributors RK performed experiments, analyzed data and wrote the manuscript. ML performed bioinformatics analyses and revised the manuscript. TH, TY, and HK performed experiments, analyzed data, and revised the article. HC and GKD recruited patients, assisted in patient outcome analysis, and reviewed the manuscript. BHS assisted in the acquisition of patient samples and revised the manuscript. FI developed the concept, managed the project, analyzed data, revised the manuscript coordinated author activities, provided final approval of the version to be submitted, and is the guarantor of the study.

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Competing interests No, there are no competing interests.

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

Ethics approval This study was reviewed and approved by the Institutional Review Board of the Roswell Park Comprehensive Cancer Center (approval number: I 188310) in accordance with the Declaration of Helsinki. Participants gave informed consent to participate in the study before taking part.

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