

Single-Cell Profiling of CD8⁺ T Cells in Acute Myeloid Leukemia Reveals a Continuous Spectrum of Differentiation and Clonal Hyperexpansion

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ABSTRACT

Comprehensive investigation of CD8⁺ T cells in acute myeloid leukemia (AML) is essential for developing immunotherapeutic strategies beyond immune checkpoint blockade. Herein, we performed single-cell RNA profiling of CD8⁺ T cells from 3 healthy bone marrow donors and 23 newly diagnosed (NewlyDx) and 8 relapsed/refractory (RelRef) patients with AML. Cells coexpressing canonical exhaustion markers formed a cluster constituting <1% of all CD8⁺ T cells. We identified two effector CD8⁺ T-cell subsets characterized by distinct cytokine and metabolic profiles that were differentially enriched in NewlyDx and RelRef patients. We refined a 25-gene CD8-derived signature correlating with therapy resistance, including genes associated with activation, chemoresistance, and terminal differentiation. Pseudotemporal trajectory analysis supported enrichment of a terminally differentiated state in CD8⁺ T cells with high CD8-derived signature expression at relapse or

refractory disease. Higher expression of the 25-gene CD8 AML signature correlated with poorer outcomes in previously untreated patients with AML, suggesting that the *bona fide* state of CD8⁺ T cells and their degree of differentiation are clinically relevant. Immune clonotype tracking revealed more phenotypic transitions in CD8 clonotypes in NewlyDx than in RelRef patients. Furthermore, CD8⁺ T cells from RelRef patients had a higher degree of clonal hyperexpansion associated with terminal differentiation and higher CD8-derived signature expression. Clonotype-derived antigen prediction revealed that most previously unreported clonotypes were patient-specific, suggesting significant heterogeneity in AML immunogenicity. Thus, immunologic reconstitution in AML is likely to be most successful at earlier disease stages when CD8⁺ T cells are less differentiated and have greater capacity for clonotype transitions.

Introduction

Acute myeloid leukemia (AML) is a clonal, immature myeloid cell disorder (1). Up to 40% of newly diagnosed (NewlyDx) AML cases are refractory to first-line therapy, and up to 50% of patients who experience remission have relapses within 1 year (2). Relapsed/refractory (RelRef) patients have dismal outcomes following therapy failure, with median survival under 6 months (3). Thus, new therapeutic approaches are needed to improve outcomes and maintain durable remissions.

Immunotherapy via immune checkpoint blockade has resulted in long-lasting responses for several solid tumors, but has only produced modest clinical improvements in AML (4–7). The curative potential of allogeneic stem cell transplantation mediated via the antileukemic activity of donor T cells demonstrates that T cells can be weaponized to eradicate leukemia (8). Furthermore, donor lymphocyte infusions can augment T-cell activity against leukemia and maintain complete remissions (8). Therefore, despite the immunosuppressive milieu, AML cells are susceptible to T cell-mediated killing, but the conditions augmenting T-cell function are poorly understood.

CD8⁺ T cells, the main mediators of the patient response to checkpoint blockade, are essential for effective antitumor immunity, and their composition and number impact therapeutic responses (9–11). Single-cell RNA sequencing (scRNA-seq) has provided unique insights into CD8⁺ T-cell composition and cellular states, entwined with cancer survival (11). Furthermore, coupling scRNA-seq with single-cell T-cell receptor sequencing (scTCR-seq) analysis reveals underlying complexities characterizing CD8⁺ T-cell transitions across different phenotypes (11). Extensive studies of solid tumors identifies CD8⁺ T-cell dysfunctional states, largely characterized by upregulation of immune-inhibitory receptors such as PD-1 and CTLA-4 (10). Some of these properties are shared across tumors, but tumor-specific paths to dysfunction exist (12). Thus, thorough the evaluation of CD8⁺ T-cell states in AML patients' bone marrow, where CD8⁺ T cells and leukemic cells coexist, is needed. A recent study demonstrates that CD8⁺ T cells may enter a senescent-like state, exhibiting impaired functionality in AML (13). Other researchers demonstrate that the number of CD8⁺ T cells coexpressing multiple

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checkpoint genes, which is usually associated with classic exhaustion, is low (14).

For immune-based therapies for AML to be effective, a deeper understanding of CD8⁺ T cells is critical. Therefore, we performed paired scRNA-seq and scTCR-seq of CD8⁺ T cells in healthy adult bone marrow donors and adult NewlyDx and RelRef patients with AML to identify cell states associated with therapy resistance, delineate CD8 AML signatures, and characterize the clonal architecture of CD8⁺ T-cell repertoires.

Materials and Methods

Human participants and sample collection

A total of 31 adult patients (≥18 years old) with AML and 3 healthy adult donors seen at The University of Texas MD Anderson Cancer Center were included in this study. Baseline data from 8 RelRef patients, which qualified for a previously reported clinical trial was used (ClinicalTrials.gov identifier: NCT02397720; ref. 15). The 23 NewlyDx patients were selected on the basis of sample availability. Written informed consent was obtained from all participants, and the study was conducted in accordance with the Declaration of Helsinki and had MD Anderson Institutional Review Board approval. Bone marrow biopsy samples were collected prior to initiation of treatment. Samples were freshly frozen in freezing medium containing 20% fetal calf serum (FCS; catalog no. F0900-050; GenDEPOT) and 10% dimethyl sulfoxide (DMSO, catalog no. D8418; Sigma-Aldrich) in DMEM (catalog no. 11965; Gibco) and then stored in liquid nitrogen until thawing and library preparation.

Sample preparation

Frozen bone marrow mononuclear cells stored in liquid nitrogen were retrieved immediately before library preparation. To maximize cellular viability recovery, samples were processed in batches using a thawing protocol. Briefly, cells were gently thawed in a water bath at 37°C for 5 minutes until partially thawed and immediately placed on ice. Next, cells were gently transferred to 10-mL room temperature media [10 mL of RPMI1640 (catalog no. R8758; Sigma) supplemented with 20% FCS] and centrifuged (453 × g for 5 min). After centrifugation, supernatants were disposed, and the cell pellets were carefully resuspended in 10-mL thawing media [RPMI1640 supplemented with 20% FCS containing 500 µL of heparin (stock 2 mg/mL, catalog no. 9041-08-1; Sigma-Aldrich), 20 µL of DNase (stock 1 µg/mL, catalog no. 89835; Thermo Fisher Scientific), and 200 µL of MgSO₄ (stock 200 mmol/L; Sigma-Aldrich)] and incubated at 37°C for 15 minutes to digest the DNA released from dead cells. Following incubation, cells were centrifuged and gently washed twice in 2 mL of 0.04% BSA (catalog no. A7906; Sigma) in PBS (catalog no. 21-040-CV; Corning). Finally, to remove cell clumps, cells were filtered with a 35-µm mesh strainer directly into BD Falcon 5-mL round-bottom tubes with cell strainer caps (catalog no. 352235; Corning). After transferring cells, 10 µL of cell suspension was stained with 10 µL of 0.4% trypan blue, counted, and assessed for viability using a standard hemocytometer and light microscopy. To adjust the cell density, cells were centrifuged and resuspended in the desired amount of PBS, as specified below.

scRNA-seq and paired T-cell receptor sequencing

scRNA-seq libraries were generated using a Chromium Controller and Chromium Single Cell 5' (v5.1) reagent kits (catalog no. 10000204; 100263 10x Genomics). All reagents and primers necessary for library preparation were provided in the kits. In brief, cells were concentrated to 1,000 cells per µL and loaded into each channel to generate single-

cell gel Bead-in-Emulsions, resulting in mRNA barcoding of an expected 5,000 single cells for each sample. After reverse transcription, gel Bead-in-Emulsions were broken, and single-strand cDNA was cleaned up with DynaBeads. cDNA was amplified, and barcoded cDNA was fragmented, A-tailed, ligated with adaptors, and amplified via index PCR. cDNA and constructed libraries were assessed using High-Sensitivity D5000 DNA ScreenTape analysis (catalog no. 5067-5592; Agilent Technologies) and a Qubit dsDNA HS Assay Kit (catalog no. Q32851; Thermo Fisher Scientific). Sequencing was conducted using an Illumina NovaSeq sequencer with 2 × 100 bp paired reads to reach a depth of at least 50,000 read pairs per cell. A Chromium Single Cell V(D)J Enrichment Kit (catalog no. 100252; 10x Genomics) was used to enrich the immune repertoire TCR transcripts. Single-cell V(D)J-enriched libraries were sequenced using an Illumina HiSeq 4000 system at 2 × 150 bp with a minimum depth of 5,000 read pairs per cell.

scRNA-seq analysis

Raw scRNA-seq data were demultiplexed, aligned to the human transcriptome reference GRCh38, and gene read count matrices using the Cell Ranger single cell software suite V3.0 (10x Genomics). Matrices were imported into R using Seurat V4.3, with genes detected in fewer than three cells and cells with fewer than 250 genes or 500 features filtered out and excluded from subsequent analysis. Cells with >10% mitochondrial-derived transcripts were also filtered. Potential T-cell clusters were identified using expression of *CD3D*, *CD3E*, and *CD3G*, and CD8⁺ T cells were separated via expression of *CD8A* and *CD8B*. Possible doublets were removed using the DoubletFinder R package, by identifying clusters with aberrantly high gene counts, and by identifying T-cell clusters that coexpressed *CD4* and *CD8*.

The filtered read matrix was log-normalized using the Seurat function “NormalizeData,” followed by identification of highly variable features. To avoid biasness of TCR gene expression, TCR genes were not included in the highly variable genes. An elbow plot was generated to identify the number of significant principal components to include for analysis ($n = 50$), and different resolution parameters were applied for unsupervised clustering. Batch effects were corrected using the Harmony R package prior to clustering analysis. Further dimensional reduction was performed using Uniform Manifold Approximation and Projection (UMAP). All software packages used for analysis are listed in Supplementary Table S1, and all scRNA-seq patient cohorts are listed in Supplementary Table S2 (12, 16, 17).

Determination of major cell types and cell states

Differentially expressed genes for each cell cluster were identified using the FindAllMarkers function in the Seurat R package (Supplementary Table S4). The 50 most significant genes for each cluster by adjusted *P* value and a curated set of canonical T-cell markers were used to determine the CD8 T-cell type (18). For exhaustion analysis, the markers chosen were from a previous pan-cancer T-cell analysis: *PDCD1*, *TOX*, *CXCL13*, *TIGIT*, *CTLA4*, *TNFRSF9*, *HAVCR2*, and *LAG3* (12).

Differential abundance testing

Differential abundance testing was performed using the R package miloR (version 1.4). The harmonized dimensional reduction was used to construct a k-nearest neighbor graph of partially overlapping neighborhoods. Differential abundance was tested across all neighborhoods using the patient group as testing variable (healthy donor, NewlyDx, and RelRef). A FDR procedure was used to account for multiple hypothesis testing.

Trajectory analysis

The monocle 3 R package was used as an independent unsupervised clustering tool for trajectory analysis. The Seurat object was converted into a cell dataset object for use in the monocle3 pipeline. Batch effects were corrected using the `align_cds` function. The clustering determined using Monocle 3 was projected using UMAP via the `cluster_cells` and `reduce_dimensions` functions. Pseudotime was determined using the `learn_graph` and `order_cells` functions. The UMAP coordinates and pseudotime information were imported into the CD8 Seurat object for further analysis.

Gene regulatory network analysis

For each of the six clusters in Group 1 and Group 2 [EM1, EM2, CTL1, CTL2, natural killer (NK)-like1, and NK-like2], 35% of cells were subsampled. The resulting output was used in the SCENIC gene regulatory network (regulon) algorithm in R. Coexpression modules for transcription factors were identified following the standard SCENIC pipeline, which was followed by regulon selection based on analysis of DNA motifs. Regulons were scored using the AUCell R package to quantify the network activity in each cell. The regulon scores were projected onto *t*-distributed stochastic neighbor embedding (tSNE) plots for visualization. The regulon specificity scores were calculated for each cluster to identify cluster-specific regulators.

Pathway enrichment analysis

Hallmark and Gene Ontology Biological Process gene sets were downloaded from the Molecular Signature Database using the `enrichR` R package. Next, single-sample gene set variation analysis of the scRNA-seq data was performed. The `gsva` function in the GSVA R package was used to calculate pathway scores.

Cytokine signaling analysis

Mean log-transformed expression data for each cluster were calculated for all genes and uploaded to the Cytokine Signaling (CytoSig) database for processing (<https://cytosig.ccr.cancer.gov/>). Results were downloaded and imported into R for heat map generation and statistical analysis.

Gene signature scoring

To score the pathway signatures and CD8-derived score, the AUCell R package was applied to each cell for the gene set signature in question. For scoring of ranked differential gene expression, the `fgsea` R package was used to perform single-sample gene set analysis.

Survival analysis

LogTPM-normalized RNA-seq data from patients with AML from The Cancer Genome Atlas (TCGA) LAML downloaded from the NIH genomic data commons (GDC) and a newly diagnosed MD Anderson cohort (GSE165656) were used to calculate survival statistics for adult patients with AML (19, 20). Patients with acute promyelocytic leukemia were excluded, leaving 158 TCGA LAML patients and 89 MD Anderson patients. Normalization for CD8 cells was done by adding CD8A to the CD8-derived signature.

To calculate survival information for these independent bulk AML RNA-seq cohorts, the `survminer` R package was used. Overall survival duration was defined as the time from diagnosis to death or most recent follow-up visit. Kaplan–Meier curves were generated using the `survival` package, with data split by the median CD8-derived score. Univariate and multivariate HRs were calculated using a Cox proportional hazards model with the `survivalAnalysis` R package. The cov-

ariates in the multivariate analysis were blast percentage, patient age, European LeukemiaNet (ELN) 2017 cytogenetic classification, and CD8-derived score.

scRNA-seq analysis for additional datasets

Data from a pan-cancer T-cell study assessing exhaustion were obtained from Zenodo (Record 5461803; ref. 12). Cancer types included for comparison were basal cell carcinoma (BCC, 9 samples), B-cell lymphoma (BCL, 2 sample), breast cancer (BRCA, 14 samples), cholangiocarcinoma (CHOL, 4 samples), colorectal cancer (CRC, 16 samples), esophageal cancer (ESCA, 6 samples), fallopian tube carcinoma (FTC, 1 sample), hepatocellular carcinoma (HCC, 14 samples), head and neck squamous cell carcinoma (HNSCC, 5 samples), lung cancer (LC, 28 samples), melanoma (MELA, 36 samples), multiple myeloma (MM, 3 samples), nasopharyngeal carcinoma (NPC, 10 samples), ovarian cancer (OV, 1 sample), pancreatic cancer (PACA, 15 samples), renal carcinoma (RC, 11 samples), squamous cell carcinoma (SCC, 4 samples), stomach adenocarcinoma (STAD, 5 samples), thyroid carcinoma (THCA, 10 samples), and uterine corpus endometrial cancer (UCEC, 9 samples). The exhaustion frequency information provided by the authors was used for this comparison. Data from 35 samples from 16 AML patients and 4 healthy donors reported in Van Galen and colleagues (GSE116256) and 21 samples from 6 patients with AML reported in Petti and colleagues (Zenodo Record 4905250) were downloaded and processed using the parameters reported in the original studies with Seurat V4.3 (16, 17).

TCR repertoire analysis

TCR V(D)J sequence assembly and paired clonotype determination were performed using Cell Ranger. Diversity analysis was performed using the `alphaDiversity` (Hill Diversity) and `rankAbundance` functions in the `alakazam` R package. The `scRepertoire` R package was used to generate figures showing basic T-cell repertoire information and to integrate scTCR and scRNA data into a single Seurat object. The `getCirclize` function from `scRepertoire` was used to extract unique and shared clonotype information from the Seurat object. STARTRAC analysis of state transition was done using the `scRepertoire` package. Additional figures and analysis were generated using in-house R scripts. For evaluation of transitions between cell clusters, the Morisita Index of Dispersion was used. This index measures the similarity of a dataset, with values ranging from 0 (no similarity) to 1 (full similarity).

Annotating TCR specificity groups

To characterize the TCR repertoire specificity, data from two gold-standard databases with known antigen-specificity data—VDJdb and McPAS-TCR—were leveraged (21, 22). Database entries were selected considering CDR3 β clonotypes associated with HLA class I antigens, including data derived from infections and cancer-associated samples. VDJdb and McPAS-TCR data were clustered together with AML-related repertoires. Clustering was carried out with the GLIPH2 algorithm using default parameters (23). The significant clusters were filtered out based on `final_score` of up to 0.05 and the presence of at least one AML-related CDR3 β clonotype.

Next, an in-house R script was developed to annotate AML-related CDR3 β clonotypes regarding four specificity categories: pathogen-related clonotypes, cancer-related clonotypes, and clonotypes with ambiguous and unknown specificity. The specificity categories were associated with known antigen-specificity abundance. For instance, clusters highly populated with pathogen-specificity clonotypes were

labeled as pathogen-related. In contrast, clusters predominantly composed of known cancer-associated clonotypes were labeled cancer-related. The ambiguous specificity category was related to mixed clusters (i.e., equally populated by cancer- and pathogen-related clonotypes). Finally, the unknown specificity category represents AML-related clonotypes not clustered with VDJdb and McPAS-TCR clonotypes and were exclusive in our cohort. Furthermore, the specificity annotation was compared in healthy donors and NewlyDx and RelRef patients. The CDR3 β clonotype shareability was analyzed using the UpSetR R package (version 1.4.0).

Data visualization

Heat maps were constructed using the ComplexHeatmap and pheatmap R packages. Plots were constructed using the ggplot2 R package.

Statistical analysis

Nonparametric statistical tests were used for statistical testing. Statistical differences were calculated using the paired Wilcoxon rank sum test and Kruskal–Wallis test. *P* values less than 0.05 were considered significant. For multiple hypothesis testing, a FDR of $q < 0.05$ was used. Pearson correlation via the corplot R package was used for gene expression correlation analysis. Statistical analysis was performed using base R packages and the rstatix R package.

Data availability statement

All newly generated sequencing data will be released for public access via Zenodo (Record 7883681; <https://zenodo.org/record/7883681#.ZFqHXOzMJzw>). Data on RelRef patients are available at the Gene Expression Omnibus using the accession number

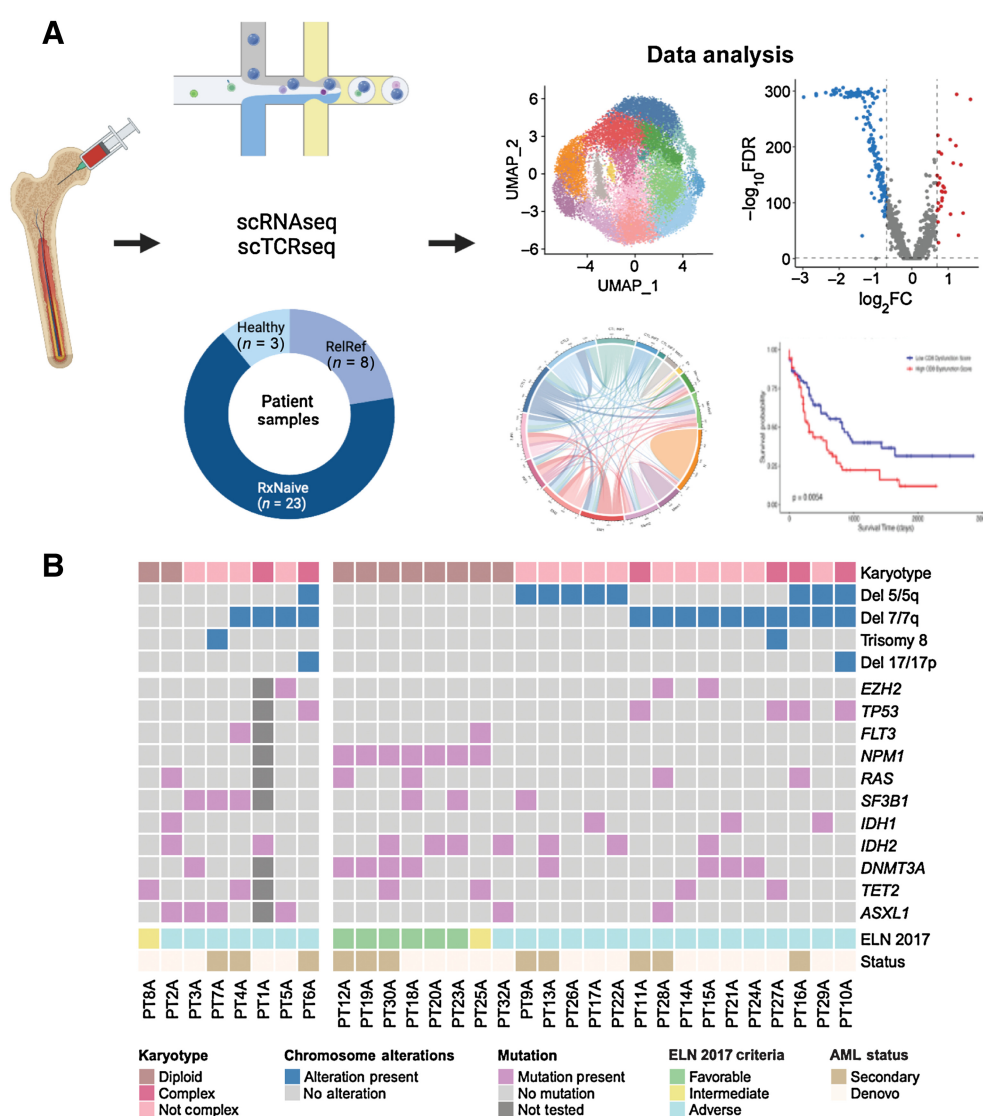
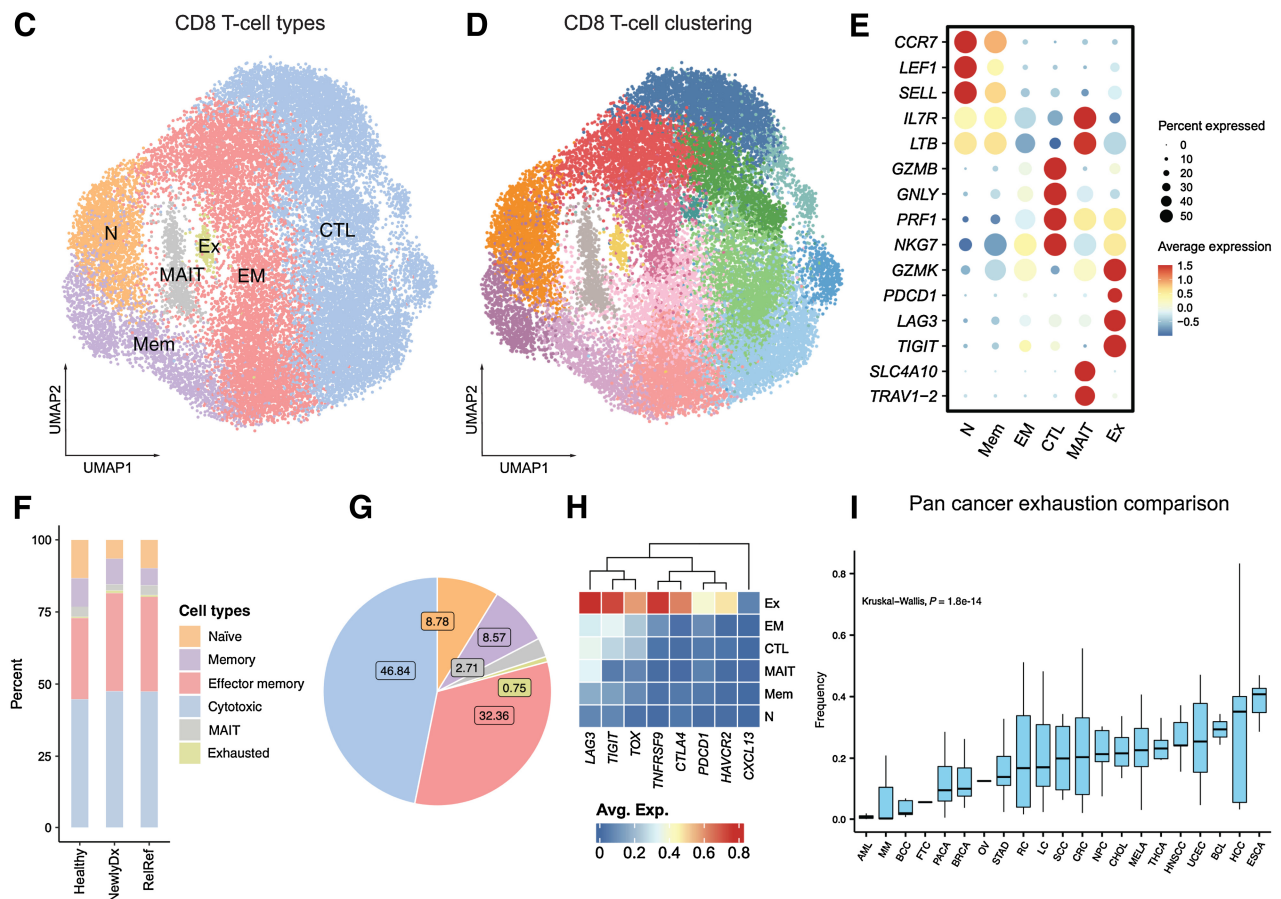


Figure 1.

Study design and single-cell characterization of CD8⁺ T cells in AML. **A**, Schematics of the experimental study design. Bone marrow aspirates were taken from 3 healthy donors and 23 NewlyDx and 8 RelRef patients. They underwent paired single-cell 5' RNA and TCR sequencing followed by computational analysis. **B**, Oncoprint summarizing the cytogenetics of the 8 RelRef (left) and 23 NewlyDx (right) patients. Secondary AML refers to transformed myelodysplastic syndrome AML and therapy-related AML. (Continued on the following page.)

**Figure 1.**

(Continued.) **C** and **D**, UMAP visualization of CD8⁺ T cells by **C**, CD8 cell type and **D**, Seurat-determined clusters. **E**, Dot plot of the canonical markers used to define CD8⁺ T-cell types. **F** and **G**, Frequency of CD8 cell types by **F**, participant and **G**, overall. **H**, Heat map of exhaustion marker expression across CD8⁺ T-cell types. **I**, Box and whisker plot depicting relative abundance of exhausted cells in AML (this study) and 20 other tumor types (15). Boxes identify quartiles, horizontal lines represent median, and whiskers identify minimum and maximum values. Data analyzed using the Kruskal-Wallis test.

GSE198052. All codes are available at https://github.com/abbaslab/2023_Single-Cell-CD8-Profiling-in-AML.

Results

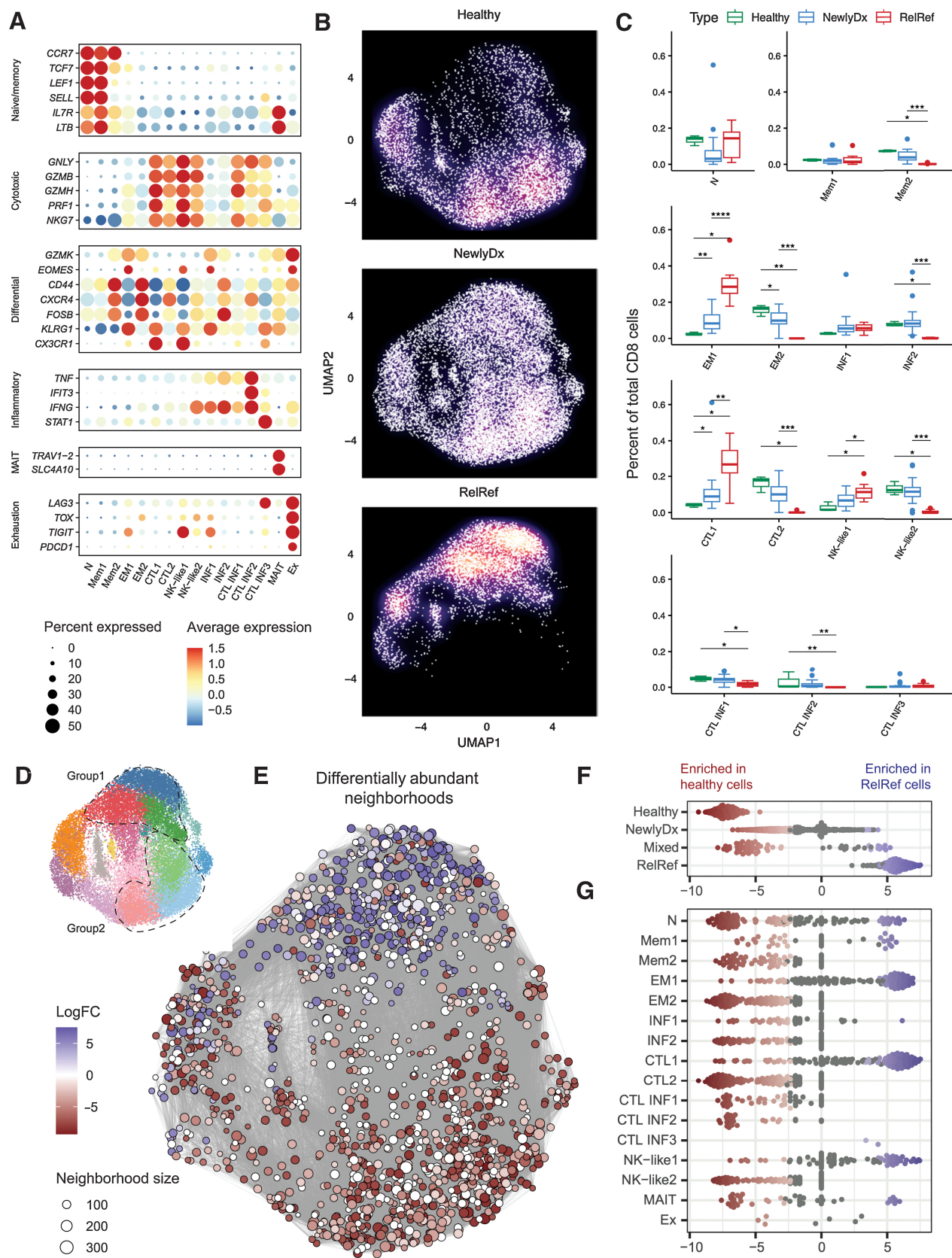
Paucity of CD8⁺ T cells expressing immune-inhibitory receptors in patients with AML

We performed paired scRNA-seq and scTCR-seq of bone marrow mononuclear cells from 3 healthy donors and 23 NewlyDx patients with AML. We also compiled previously reported paired scRNA-seq and scTCR-seq data for eight RelRef patients with AML Aat relapse or refractory disease (Fig. 1A; ref. 15). The participants' clinical and demographic characteristics are summarized in Fig. 1B and Supplementary Table S3. In brief, median age at AML diagnosis was 72 (range, 52–88 years), and 77% were male. Eight NewlyDx (35%) and three RelRef (38%) patients had secondary AML. Of these, 2 and 3 patients, respectively, received myeloid-directed therapy prior to AML diagnosis. The most common mutations in all 31 participants were *IDH2* and *DNMT3A* mutations (9 patients each). Eleven (35%) and 20 (65%) patients had diploid and nondiploid cytogenetics, respectively. Of those with nondiploid cytogenetics, 10 had a deletion in chromosome

7/7q (7 NewlyDx, 3 RelRef patients), and 4 had double deletions in 5/5q and 7/7q (3 NewlyDx, 1 RelRef patient). In addition, 22 patients had an ELN 2017 risk classification of adverse. Collectively, our cohort spans different clinical, molecular, and cytogenetic subgroups representative of the real-world diversity among patients with AML.

After quality control, doublet removal, and batch correction (Supplementary Figs. S1 and S2), we filtered 192,667 cells, 51,219 of which were T cells (Supplementary Fig. S3). We refined 23,320 CD8⁺ T cells by identifying clusters expressing *CD3* and *CD8A* (Supplementary Fig. S3), 81% of which were newly sequenced in this study. Using canonical gene expression patterns (15, 24–30), we identified 16 clusters from six major CD8⁺ T-cell types: naïve (N; *n* = 1), memory (Mem; *n* = 2), effector memory (EM; *n* = 4), cytotoxic (CTL; *n* = 7), mucosal-associated invariant T cell (MAIT; *n* = 1), and exhausted (Ex; *n* = 1; Fig. 1C–E; Supplementary Fig. S4A). The most abundant cell type was CTL in healthy donor (44.8%) and NewlyDx (47.5%) and RelRef (47.4%) patient bone marrow (Fig. 1F; Supplementary Fig. S4B).

Of note, the Ex cluster, as defined by the high coexpression of immune-inhibitory receptors, constituted 0.75% of all CD8⁺ T cells. The relative abundance, scaled for number of cells per patient



group, did not differ among healthy donors and NewlyDx and RelRef patients ($P = 0.74$), suggesting that cluster distribution was independent of patient cell number (Fig. 1G; Supplementary Fig. S5A). We also observed higher correlation of exhaustion marker expression in the Ex cluster than in the other cell types (Supplementary Fig. S5B). In addition, immune-inhibitory receptor profiling demonstrated that the Ex cluster had the highest relative expression of these genes (Supplementary Fig. S6). To further investigate the paucity of exhausted cells, we analyzed CD8⁺ T cells from previously published AML scRNA-seq datasets (16, 17). Similarly, those datasets had a small cluster marked by high coexpression of immune-inhibitory genes (Supplementary Figs. S7, S8A, and S8B). We next compared the relative abundance of exhausted CD8⁺ T cells from the present study with that of 20 other tumors using the same exhaustion definition (12): coexpression of *PDCD1*, *TOX*, *CXCL13*, *TIGIT*, *CTLA4*, *TNFRSF9*, *HAVCR2*, and *LAG3* (Fig. 1H). Patients with AML had significantly lower relative abundance of CD8⁺ Ex cells [$P = 1.8 \times 10^{-14}$ (Kruskal–Wallis test; Fig. 1I; Supplementary Fig. S8C)]. These data support a lower frequency of CD8⁺ T cells coexpressing canonical immune inhibitory-related markers in patients with AML.

Disparate clustering in effector CD8⁺ T cells in NewlyDx and RelRef patients

To examine CD8 cellular states, we analyzed gene expression in the N, Mem, Ex, MAIT, EM (*GZMK*⁺, low cytotoxic genes), and CTL (*GZMK*⁺, high cytotoxic genes *PRF1*, *GNLY*, and *GZMB*) clusters (Fig. 2A; Supplementary Table S4). Two EM clusters demonstrated differential expression of the EM markers *EOMES* (EM1) and *CD44* (EM2; ref. 31). The other two EM clusters had higher expression of inflammatory markers, including *IFNG* and *TNF* (INF1 and INF2). Similarly, two CTL clusters were characterized by expression of *CX3CR1* (CTL1) or *CD44* (CTL2). Also, two CTL clusters had low killer-cell Ig-like receptor expression (KIR; Fig. 2A; Supplementary Fig. S9A), indicating an NK-like regulatory phenotype (32), and *CX3CR1/TIGIT* (NK-like1) or *IFNG* (NK-like2) expression (Supplementary Fig. S9B). Three CTL clusters (6.6% of all cells) expressed *IFNG*, *TNF*, or IFN-stimulated genes (CTL INF1, INF2, and INF3), suggesting an inflammatory phenotype (Fig. 2A). These results demonstrated a heterogeneous CD8⁺ effector population in AML. Immune-inhibitory and exhaustion marker profiling revealed varied expression across cell types that cannot be resolved at a group level (Supplementary Fig. S9C). Thus, we observed that whereas immune checkpoint genes were expressed across different cell types, only a single cluster (Ex) had high coexpression of these markers (Supplementary Figs. S6 and S9D).

UMAP-based density plots revealed coclustering of N and Mem cells from healthy donors and NewlyDx and RelRef patients (Fig. 2B), with no significant differences in the relative abundance of these populations ($P = 0.15$ and 0.19 , respectively; Fig. 2C).

To account for differences in cell number between NewlyDx and RelRef patients (13,398 cells vs. 4,397 cells), we randomly subsampled 4,397 cells from NewlyDx patients, revealing clustering and distribution similar to that shown in Fig. 1D (Supplementary Fig. S10A and S10B), suggesting that the observed findings were biologically related.

CD8⁺ effector cells (EM and CTL) from NewlyDx patients were distributed across effector clusters (Fig. 2B), whereas those from RelRef patients were concentrated in three clusters (EM1, CTL1, and NK-like1), collectively known as Group 1 (Fig. 2D). These clusters exhibited high expression of the inhibitory receptor *KLRG1*, which is usually expressed in antigen-experienced and senescent cells (33), compared with the Group 2 (EM2, CTL2, and NK-like2) clusters (Fig. 2A and D). Conversely, Group 2 clusters were enriched in the cell-migratory receptor *CXCR4* and AP-1 transcription factor *FOSB*. The relative abundance of NewlyDx and RelRef cells in these clusters differed markedly. Only 1.8% of RelRef CD8⁺ T cells contributed to the Group 2 clusters, whereas 23.7% of NewlyDx cells contributed to the Group 1 clusters [$P = 2.2 \times 10^{-16}$ (Kruskal–Wallis test); Fig. 2C; Supplementary Fig. S9C]. Group 1 clusters had a higher relative abundance of RelRef than NewlyDx cells (CTL1, $P = 0.009$; NK-like1, $P = 0.04$; EM1, $P = 0.000002$). Group 2 clusters were significantly more abundant in NewlyDx than in RelRef patients (CD8 EM2, $P = 0.0007$; CD8 CTL2, $P = 0.0008$; CD8 NK-like2, $P = 0.001$; Fig. 2C). Therefore, whereas Groups 1 and 2 clusters had similar phenotypic markers, distinct clusters emerged, suggesting heterogeneous functional states.

Although cluster-based analysis can identify cells with related expression, it may obscure biologically relevant perturbations and variations within cell types. To investigate these perturbations, we used miloR (34), a generalized linear model that assigns cells to localized, partially overlapping neighborhoods without clustering them, to identify neighborhoods based on patient group (Fig. 2E; Supplementary Fig. S11). MiloR identified 1,549 total neighborhoods, 890 (57.5%) of which were differentially abundant in healthy donors and NewlyDx and RelRef patients (FDR, <10%; Fig. 2F). NewlyDx cells made up most nondifferential neighborhoods and were heterogeneously distributed between healthy donors and RelRef patients, suggesting a diverse spectrum of phenotypes in this group. In addition, we labeled neighborhoods by cluster, revealing that N and Mem1 clusters were more evenly enriched for neighborhoods from the entire cohort (Fig. 2G). However, effector clusters had a gradient of neighborhood distributions. Specifically, 54.3% of healthy donor neighborhoods and 41.9% of NewlyDx neighborhoods were enriched in Group 2 clusters, whereas 78.1% of RelRef neighborhoods were enriched in Group 1 clusters (Fig. 2G), consistent with results of our relative abundance analysis (Fig. 2B and C). Thus, we discovered a distinct differential abundance of healthy donor and patient cells within clusters, pointing to distinct transcriptional and functional states separating Groups 1 and 2 cells.

Figure 2.

Characterization of CD8⁺ T-cell subsets among 3 healthy donors and 23 NewlyDx and 8 RelRef patients. **A**, Dot plot of the expression of signature genes in the 16 cell clusters. Gene category is listed on the y-axis. "Differential" genes are those which can be used to separate clusters of the same group. **B**, UMAP density plot depicting cellular concentration (represented by color intensity) across participants. **C**, Box plots of relative abundance by cluster identifying significant differences in cell distribution by patient group [* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ (Wilcoxon rank sum test with Benjamini–Hochberg correction)]. Boxes identify quartiles, horizontal lines represent median, and whiskers identify minimum and maximum values. **D**, UMAP visualization of Group 1 (top: EM1, CTL1, and NK-like1) and Group 2 (bottom: EM2, CTL2, and NK-like2) clusters. **E**, Neighborhood graph of differential abundance testing results using miloR. Neighborhood colors represent the log fold-change between healthy donor (red) and RelRef patient (blue) cells. White neighborhoods are nondifferential (FDR, 10%). Edges link cells shared by neighborhoods. **F** and **G**, Distribution of the neighborhoods shown in **E** across **F**, participant groups and **G**, clusters.

Pseudotemporal analyses reveals a continuous spectrum of differentiation in CD8⁺ T cells

We evaluated CD8 cellular states across time using Monocle 3-based pseudotemporal trajectory analysis (Fig. 3A) and Slingshot, a tree-fitting model with pseudotime estimation, to model lineage transitions and CD8⁺ T-cell differentiation. Both methods had significantly high concordance ($r = 0.81$, $P = 2.2 \times 10^{-16}$) for pseudotime (Supplementary Fig. S12A), allowing for further downstream analysis.

Effector cells (EM and CTL) demonstrated a continuum across pseudotime (Fig. 3B and C; Supplementary Fig. S12B), with high coexpression of *CXCR4* and *FOSB* in earlier pseudotime compared with higher expression of *KLRG1* and the terminal differentiation marker *CX3CR1* (35) in later pseudotime (Fig. 3D; Supplementary Fig. S12C). We also observed a significant negative correlation between *CXCR4* and *CX3CR1* expression ($r = -0.68$, $P = 2.2 \times 10^{-7}$; Fig. 3E). These findings suggested distinct effector states with dynamic changes

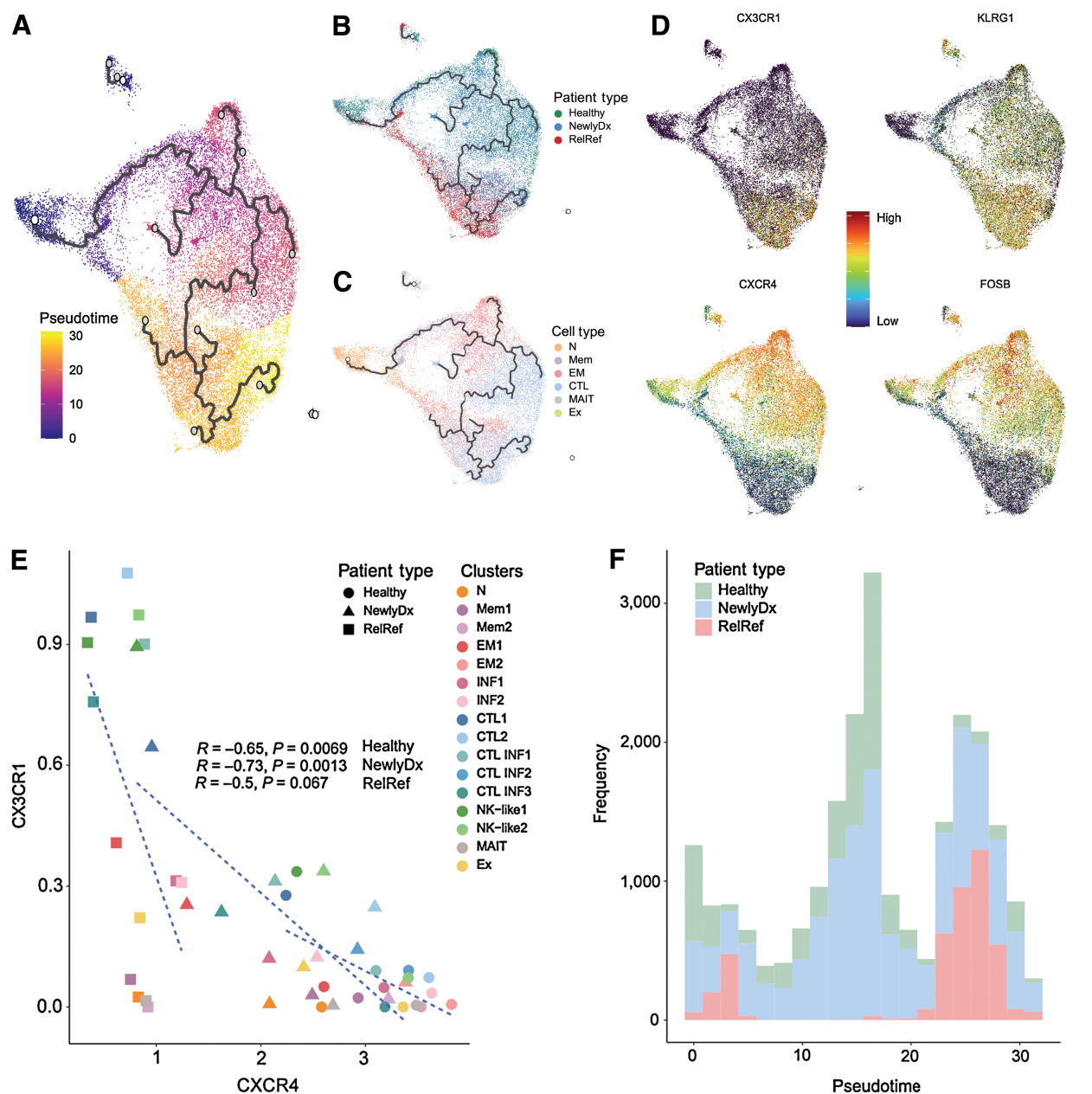


Figure 3.

Trajectory and gene regulatory network analysis of CD8⁺ T cells. **A–D**, Monocle 3-based UMAP visualization of pseudotime across CD8 cells (**A**), participant groups (3 healthy donors, 23 NewlyDx, 8 RelRef; **B**), and CD8⁺ T-cell types (**C**). **D**, Expression of the *CX3CR1*, *KLRG1*, *FOSB*, and *CXCR4* genes in Monocle 3-based UMAP coordinates. **E**, Pearson correlation between *CXCR4* and *CX3CR1* expression across clusters for each participant group. The *P* values for NewlyDx ($P = 0.0013$) and Healthy ($P = 0.0069$) patients are significant ($P < 0.05$). **F**, Histogram of the frequency of cells from the 3 participant groups across pseudotime. (Continued on the following page.)

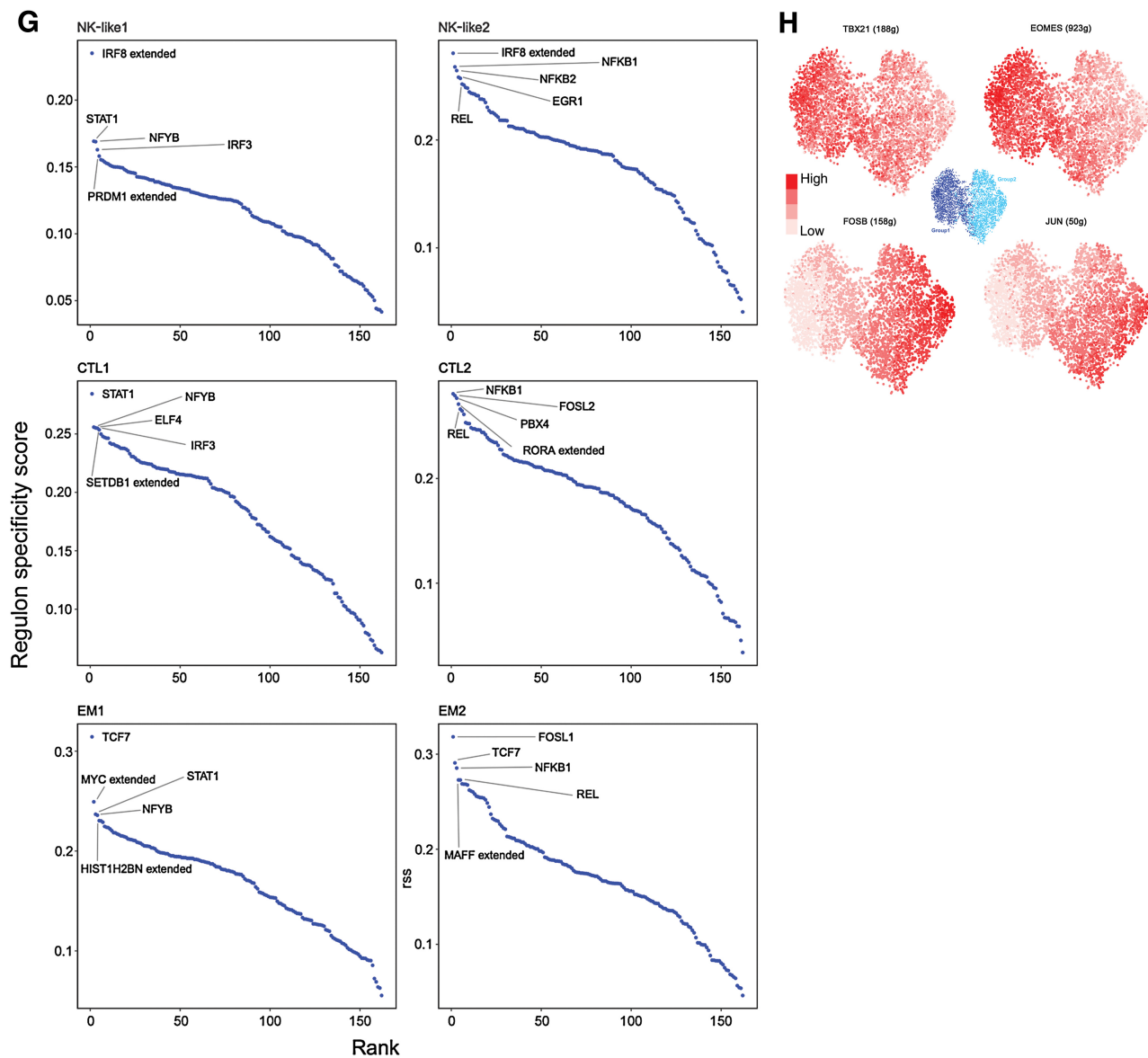


Figure 3.

(Continued.) **G**, Scatter plot of regulon specificity scores from SCENIC-based transcription network analyses for the CTL1, CTL2, EM1, EM2, NK-like1, and NK-like2 effector clusters. The top five regulons for each cluster are labeled. **H**, Transcription factor activity in six effector clusters as determined using SCENIC. The central UMAP shows the localization of cells from Group 1 and Group 2 clusters.

in transcriptional profiles associated with homeostasis, effector function, and terminal differentiation. We next evaluated CD8⁺ T-cell abundance from different patient groups across pseudotime. Cells from NewlyDx patients were more evenly distributed across pseudotime, indicating a continuous, dynamic state of differentiation. However, the majority (91.3%) of RelRef CD8⁺ effector cells grouped in the last third of pseudotime, suggesting a distinct state of terminal differentiation (Fig. 3F; Supplementary Fig. S12D).

Because CD8 cellular states are primarily modulated via transcription factors (36), we applied SCENIC (37) to determine the gene regulatory networks associated with Group 1 and Group 2 clusters. Group 1 clusters had the highest activity of *NFYB* and *STAT1* regulons,

which relate to cell longevity and IFN α production (Fig. 3G; refs. 38, 39). In addition, the *TBX21* and *EOMES* regulons were enriched in Group 1 clusters, consistent with their role in terminal differentiation (Fig. 3H; Supplementary Fig. S13A; ref. 40). TNF α signaling-related *NFKB1* and *REL* were among the top regulons in Group 2 clusters (Fig. 3G). Furthermore, Group 2 demonstrated higher activity of *JUN* and *FOS* regulons (AP-1 family; Fig. 3H; Supplementary Fig. S13B), which are downstream from TCR activation and associated with immune checkpoint regulation (41). These results suggest that chronic IFN α stimulation, concurrent with down-regulation of TNF α and AP-1 signaling, associates with the persistence of terminally differentiated cells at therapy resistance.

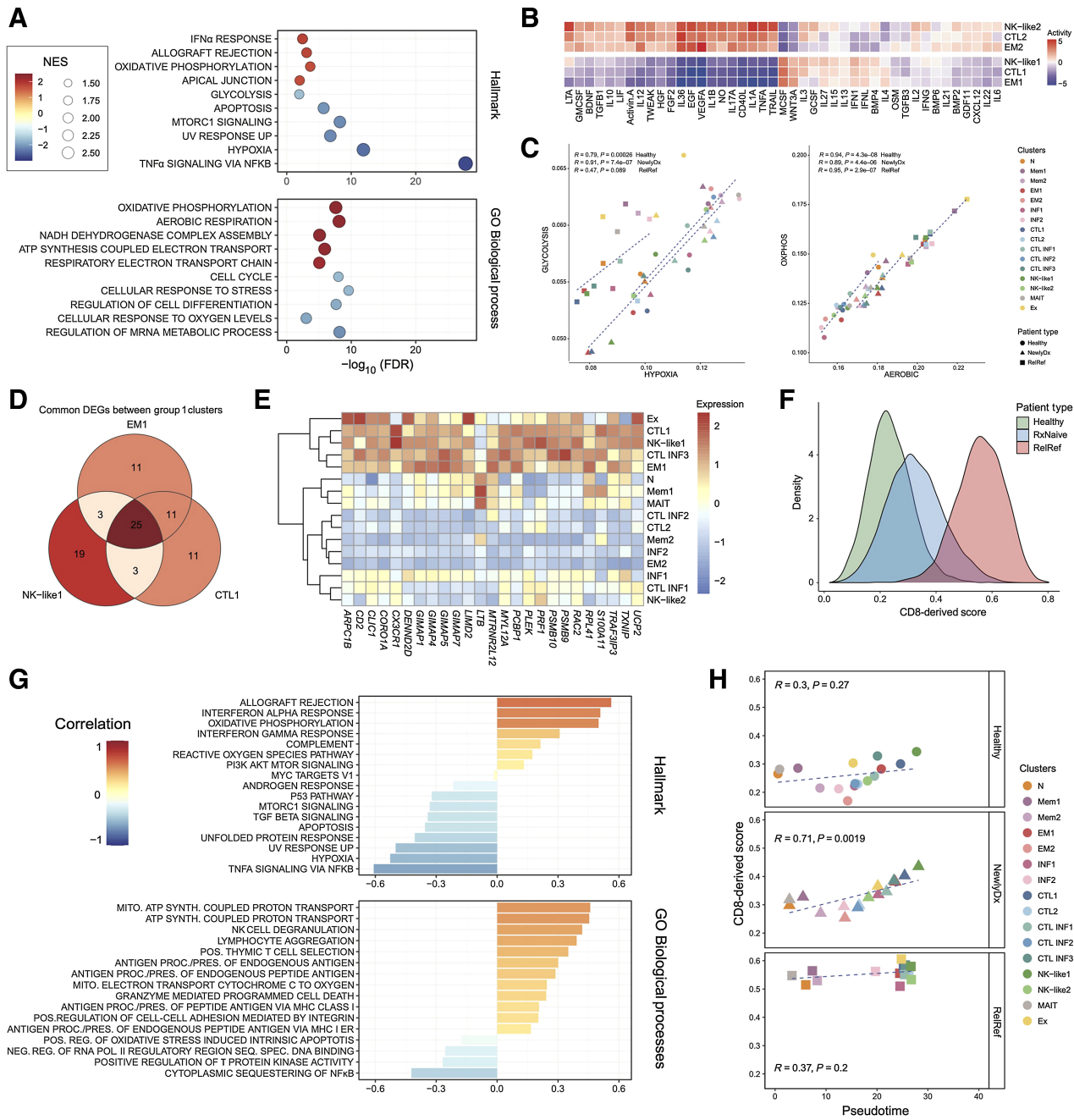


Figure 4. Differential gene expression analysis identifies metabolic changes and a CD8-derived signature. **A**, Hallmark and GOBP pathway enrichment based on differential expression in Group 1 and Group 2 clusters. Positive normalized enrichment scores associated with Group 1 clusters. **B**, Cytokine signaling activity analysis of Group 1 (bottom) and Group 2 (top) clusters using CytoSig. Signaling pathways names are on the x-axis. **C**, Left: Pearson correlation of aerobic respiration with oxidative phosphorylation; Right: Pearson correlation of hypoxia with glycolysis by cluster and patient type. **D**, Venn diagram of the overlap of the top 50 genes in Group 1 clusters. **E**, Heat map of the average expression of the 25 genes in the CD8-derived signature by cluster. **F**, Density plot of the distribution of cells from each participant group across an increasing CD8-derived score, as calculated by AUCell. **G**, Bar plot of the most positive (red) and negative (blue) significant correlations between CD8-derived score and Hallmark and GOBP pathway score as calculated using AUCell. **H**, CD8-derived score across pseudotime averaged by cluster for 3 healthy donors, 23 NewlyDx patients, and 8 RelRef patients, with Pearson correlation. The threshold for significance used was $P < 0.05$.

Distinct metabolic and cytokine profiles of Group 1 and Group 2 CD8⁺ effector cells

Collectively, the differential relative abundance of Group 1 and Group 2 cells in disease states despite their similar phenotypes, coupled with distinct transcription factor profiles, merited further examination. Gene set enrichment analysis revealed significant positive enrichment in TNF α signaling via NF- κ B (Hallmark) in Group 2, whereas Group 1 had positive enrichment in IFN α signaling (Hallmark; **Fig. 4A**; Supplementary Fig. S14A; Supplementary Table S5), which aligned with transcription factor enrichment findings (**Fig. 3G and H**). To further explore the differential cytokine profiles in the two groups, we applied CytoSig, a tool that leverages public datasets to predict cytokine signaling activity using scRNA-seq data (42). Group 1 clusters had downregulation of effector cytokine pathways, such as TNF α , but greater enrichment in type 1 IFN (**Fig. 4B**). Notably, IFN α induces *EOMES* expression (40), which subsequently regulates effector memory and late differentiation states in CD8⁺ T cells (31).

Consistent with the role of metabolic rewiring in modulating CD8⁺ effector functions (10), Group 1 and Group 2 clusters exhibited differential enrichment in oxidative and metabolic profiles. Specifically, Group 1 clusters had enrichment in oxidative phosphorylation [Hallmark and Gene Ontology Biological Process (GOBP)] and aerobic respiration (Gene Ontology Biological Process; **Fig. 4A**; Supplementary Fig. S14A and S14B). These results were corroborated by CytoSig analysis, which identified enrichment in the oxidative phosphorylation-related pathways WNT3A and IL27 (43, 44) in Group 1 (**Fig. 4B**). Group 2 clusters were enriched in hypoxia (Hallmark) and glycolysis (Hallmark; **Fig. 4A**) and related pathways, such as TGF β 1 and VEGFA (45, 46). These pathways were positively correlated, with all but hypoxia-glycolysis for RelRef cells having significant *P* values (**Fig. 4C**). Hence, whereas Group 1 and Group 2 cells are phenotypically similar, they were characterized by distinct cytokine and metabolic transcriptional profiles that likely contributed to their distinct functional states.

CD8-derived 25-gene signature correlates with state of terminal differentiation

To further delineate a shared biologic program in Group 1 clusters, we conducted pairwise differential gene expression analysis of phenotypically similar clusters: EM 1/2, CTL 1/2, and NK-like 1/2 (Supplementary Fig. S14C–S14E). Gene overlap analysis of the top 50 upregulated genes in Group 1 clusters revealed 25 shared genes (**Fig. 4D**), hereafter referred to as the CD8-derived signature in AML. Immune activation genes, including *CD2* and *PRF1* (47); transcription repressor *TXNIP*, which modulates *IFNG* transcription (48); exhaustion marker *S100A11* (49); terminal differentiation marker *CX3CR1* (50); and immunoproteasome members *PSMB9* and *PSMB10* were among the upregulated genes (**Fig. 4E**). Furthermore, several genes in this signature—*CX3CR1*, *PSMB9*, *TRAF3IP3*, *TXNIP*, and the GIMAP proteins—are associated with chemotherapy resistance (51–54). Collectively, these genes suggest a CD8 state characterized by activation and terminal differentiation.

Because senescent CD8⁺ T cells are prevalent in patients with AML (13, 55), we compared the CD8-derived signature with the immune effector dysfunction (IED) and prognostic index (PI) signatures associated with senescence and CD8 dysfunction (13). We found overlap of four (*LTB*, *CD2*, *PRF1*, and *GIMAP4*), three (*LTB*, *CD2*, and *PRF1*), one (*LTB*), and no genes when compared with the IED172, IED68, PI20, and PI24, respectively. These suggest independent programs characterizing these signatures and our CD8-derived signature. We also noted higher expression of IED172, IED68, and

PI20 in Group 1 clusters (Supplementary Fig. S15A) and significant correlations of the CD8-derived signature with IED172 ($r = 0.72$; $P < 2.2 \times 10^{-16}$) and IED68 ($r = 0.7$; $P < 2.2 \times 10^{-16}$; Supplementary Fig. S15B and S15C). Thus, cells expressing the IED172 and IED68 senescence and dysfunction signatures may also express the 25-gene CD8-derived signature, suggesting dysfunction mediated via distinct transcriptional programs.

To evaluate the activity of the CD8-derived signature, we applied AUCell to identify gene expression enrichment within each cell (37). The CD8-derived signature score was highest for cells from RelRef patients, followed by NewlyDx patients ($P = 3.96 \times 10^{-8}$ [Kruskal–Wallis test]), demonstrating increased representation of this signature with disease progression (**Fig. 4F**). In a paired analysis of 5 patients with AML with over 20 evaluable CD8⁺ T cells before and after relapse (17), 2 patients had significant increases in CD8-derived score at relapse, whereas 3 had similar scores at diagnosis and relapse (Supplementary Fig. S16). Of note, all cases relapsed, suggesting that the baseline state likely contributes to relapse.

We next examined pathway correlation with the CD8-derived signature. Lower signature scores correlated with hypoxia, TNF α signaling, and apoptosis, whereas higher scores correlated with oxidative phosphorylation, IFN response, MHC class I antigen presentation, high energy usage, and cytolytic activity (**Fig. 4G**; Supplementary Fig. S17). Collectively, these data indicate that the 25-gene signature represents the cytotoxic, terminally differentiated state enriched in RelRef patients.

Assessment of the CD8-derived signature across pseudotime revealed an increasing score in the cells of NewlyDx patients ($r = 0.71$; $P = 0.0019$; **Fig. 4H**). However, cells from RelRef patients had higher scores ($P = 4.4 \times 10^{-7}$), were nonsignificantly positively correlated with pseudotime ($r = 0.37$; $P = 0.2$), and congregated in early and late pseudotime, suggesting a gradient of differentiation at therapy resistance or relapse. These findings suggest a more heterogeneous and dynamic cellular state in NewlyDx patients but a higher, static differentiation status in RelRef patients.

The CD8-derived signature score is predictive of outcomes in untreated patients with AML

Our results demonstrated a gradient of CD8⁺ effector cell differentiation in NewlyDx patients. Specifically, 39% of their effector cells clustered in the terminally differentiated Group 1 clusters, indicating existence of a *bona fide* differentiation gradient in these patients. Thus, we leveraged the *de novo* LAML TCGA cohort (19) and evaluated the expression profile of the 24 genes in the CD8-derived signature with expression data (**Fig. 5A and B**). Twenty-three had positive correlation of coexpression, suggesting similar regulatory programs (**Fig. 5A**). To determine whether the CD8-derived score was a clinically relevant biomarker, we performed single-sample gene set enrichment analysis to generate a CD8-derived score for each patient (**Fig. 5B**). Patients with higher CD8-derived scores had significantly worse outcomes ($P = 0.0022$; **Fig. 5C**). This was corroborated in a multivariate Cox proportional hazards model that included age, ELN 2017 cytogenetic risk classification, and blast count (HR, 3.40; 95% CI, 1.27–9.05; $P = 0.015$; Supplementary Table S6). In addition, we validated our findings in an independent cohort of untreated patients with AML (19), finding similar results of higher CD8-derived scores associated with worse outcomes (Supplementary Fig. S18). Furthermore, the HR positively correlated with the CD8-derived signature for each incremental score unit ($r = 0.95$; $P = 2.2 \times 10^{-16}$), consistent with a progressive state impacting outcomes (**Fig. 5D**).

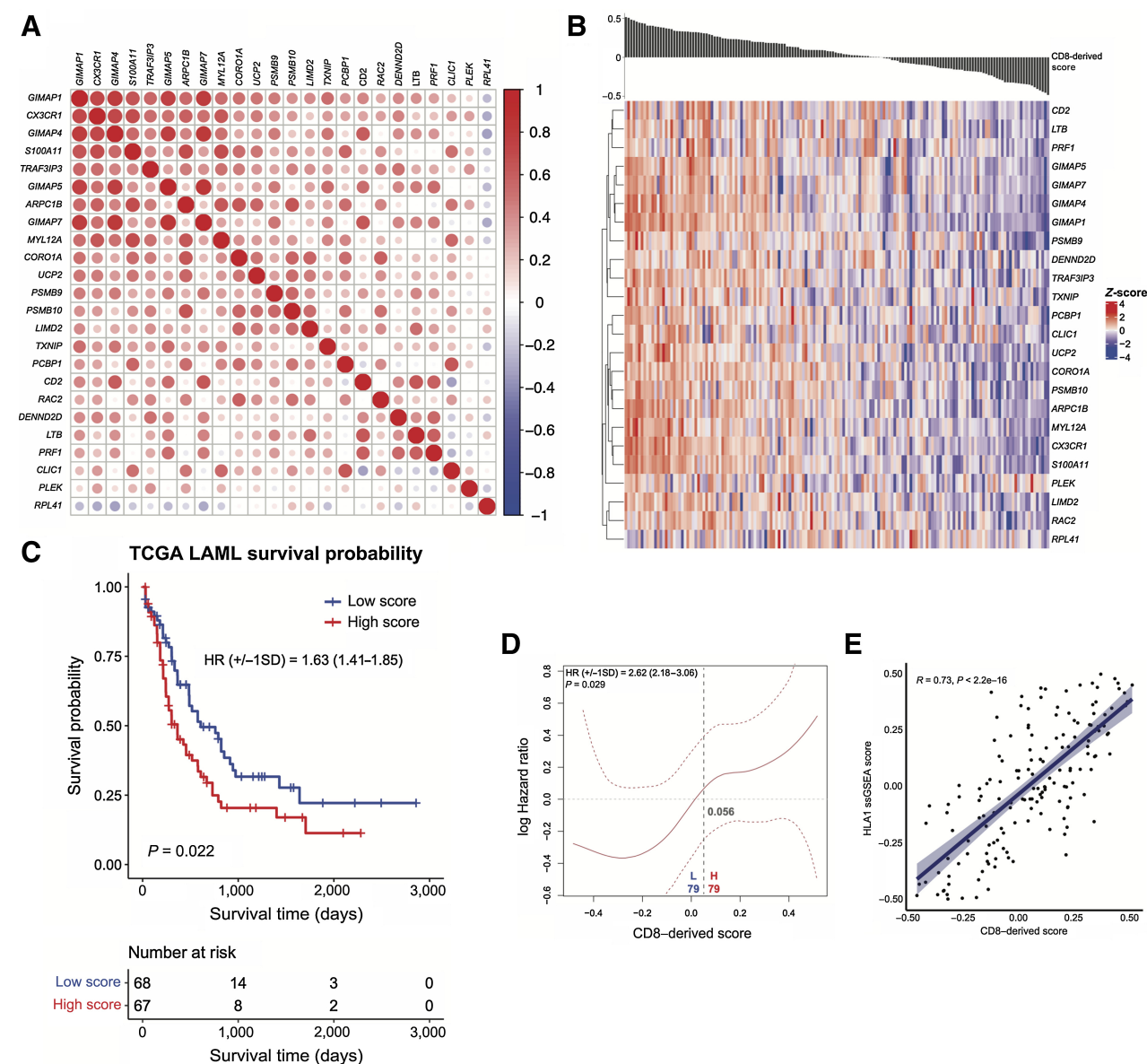


Figure 5.

The CD8-derived score is predictive of patient outcomes. **A**, Pearson correlation of genes composing the CD8-derived signature in TCGA LAML data. Color saturation indicates correlation strength, whereas the color reflects the correlation direction. **B**, Heat map of CD8-derived signature gene expression in the TCGA LAML cohort ($n = 158$). Patients were organized by progressive CD8-derived score (top). **C**, Kaplan-Meier survival curve for the TCGA LAML cohort ($n = 135$ patients with survival information). Patients were put in two equal groups using the median CD8-derived score. **D**, Changes in HR as the CD8-derived score increased. The solid line represents the HR at different CD8-derived scores, and the dotted red lines represent the 95% confidence interval (CI). The vertical line represents the median HR separating the patient groups (L: low score, $n = 79$; H: high score, $n = 79$). **E**, Scatter plot of the correlation between the HLA1 gene signature and CD8-derived score. Shaded area represents 95% CI. The threshold for significance used was $P < 0.05$.

Because the CD8-derived signature correlated with MHC class I antigen-presenting signatures (Fig. 4G), we assessed the correlation between the CD8-derived signature and HLA class I gene expression in the TCGA LAML cohort (Fig. 5E). We saw a significant positive correlation ($r = 0.73$; $P = 2.2 \times 10^{-16}$) between the signature and HLA gene expression. These suggests that in patients with AML, an advanced differentiation state associates with increased potential for antigen presentation via HLA class I and chronic stimulation.

CD8⁺ T cells from RelRef patients are characterized by hyperexpansion and reduced plasticity

A total of 17,701 CD8⁺ T cells from 6,853 clonotypes had paired TCR-sequencing data, of which 76% harbored at least one pair of productive TCR α and β chains, 68% of which were clonal (defined as TCR pairs found in at least two cells; Supplementary Fig. S19A and S19B). Clonal diversity was highest in healthy donors followed by NewlyDx and RelRef patients (Fig. 6A; Supplementary Fig. S19C). Furthermore, N cells had the highest percentage of unique clonotypes,

whereas CTL cells had the lowest (Supplementary Fig. S19D). Conversely, the highest expansion indices of clonal rank abundance were in RelRef patients, followed by NewlyDx patients and healthy donors (Fig. 6B).

We next evaluated the frequency of clonotypes, from those expressed in a single cell (rare) to those expressed in over 100 cells (hyperexpanded). Thirty-four percent of clonotypes in RelRef patients were hyperexpanded, compared with 8% and 3% in NewlyDx patients and healthy donors, respectively [$P = 2.2 \times 10^{-16}$ (Kruskal–Wallis test); Fig. 6C]. Projection of clonotype expansion onto the CD8 UMAP revealed congregation of hyperexpanded clonotypes in Group 1 clusters (Fig. 6D). Furthermore, hyperexpanded cells exhibited a significantly higher CD8-derived signature score [$P = 0.008$ (Kruskal–Wallis test); Fig. 6E]. These data supported oligoclonal expansion at therapy resistance, likely owing to chronic antigen exposure.

We next sought to identify clonotypes within patient groups that recognized the same antigen. Clonotype sharing demonstrated significant differences among all three participant groups (healthy–NewlyDx, $P = 6.46 \times 10^{-7}$; healthy–RelRef, $P = 2.98 \times 10^{-4}$; NewlyDx–RelRef, $P = 1.48 \times 10^{-15}$; Fig. 6F). Analysis of antigen sharing and clonal overlap using the Morisita index identified increased clonotype sharing among effector clusters, which was markedly lower for N, Mem, and MAIT (Fig. 6F and G). Naïve cells did not overlap with any other cluster in healthy donor cells, which was not the case for NewlyDx or RelRef patient cells. Furthermore, RelRef cells had the most overlap among clusters, particularly CTL clusters, whereas NewlyDx repertoires had greater overlap in the EM compartment. In healthy donors, overlap was mostly confined to CTL clusters (Fig. 6G), likely suggesting expansion of activated CTL cells.

To determine whether shared antigens are related to cellular transitions, we performed STARTRAC transition analysis (56), which revealed that RelRef patient cells had the lowest transition probabilities (transition indices: 0.661, 0.982, and 0.906 in RelRef and NewlyDx patients and healthy donors, respectively), likely because of higher percentages of terminally differentiated and hyperexpanded cells [$P = 0.001$ (Kruskal–Wallis test)]. NewlyDx patients exhibited the most variability in score, which may relate to the heterogeneity of AML, resulting in diverse CD8⁺ T-cell responses (Fig. 6H). Overall, clusters occurring earlier in differentiation and pseudotime had less transitional potential than effector clusters. Therefore, CD8⁺ T cells from RelRef patients had the most shared antigens but the fewest transitions, suggesting chronic antigen stimulation but reduced plasticity when transitioning between states.

Specificity analysis of CD8⁺ clonotypes

We next leveraged immune repertoires to evaluate the predicted shared antigenicity among tumor-infiltrating CD8⁺ T cells in AML. A total of 13,301 cells were analyzable following quality assessment using GLIPH2 for comparison with known clonotypes in the VDJdb (22) and McPAS-TCR databases (21). We classified clonotypes into four groups based on the predicted specificity: pathogen-specific, cancer-specific, ambiguous (both cancer- and pathogen-related), and unknown. Notably, RelRef clonotypes were more abundant in the unknown specificity group, suggesting prevalence of patient-specific clonotypes at later disease stages (Fig. 7A). When evaluating clonotype specificity by CD8⁺ T-cell type, clonotypes of unknown specificity were more prevalent in EM and CTL cells [$P = 0.0005$ (Fisher exact test); Fig. 7B]. In addition, interrogating clonotypes in the context of clonal expansion demonstrated that the majority of unknown clonotypes were in clonal cells [$P = 0.0005$ (Fisher exact test); Fig. 7C; Supplementary Fig. S20].

We next evaluated whether patients with AML shared clonotypes. We found 2,645 unique clonotypes, 12 of which were shared by NewlyDx and RelRef patients (Fig. 7D, inset). The clonotype specificity distribution differed significantly among the groups ($P = 4.5 \times 10^{-15}$). All shared clonotypes were pathogen-related except for one unknown clonotype shared by a NewlyDx and RelRef patient (Fig. 7D). Although these patients did not share known mutations or cytogenetic abnormalities, this unknown clonotype may represent activity against a cancer-specific antigen.

We then sought to identify any predicted antigen sharing within the patient groups. Of the 62% of RelRef cells with associated clonotype specificity data, 65% were clonotypes of unknown specificity. Two RelRef patients shared one pathogen-specific clonotype, and two others shared one unknown clonotype (Fig. 7E). Notably, the latter two patients had an *IDH2* mutation. Also, of the 55% of NewlyDx cells with specificity data, 63% were clonotypes of unknown specificity, none of which were shared among patients. Seven clonotypes were shared by 2 patients (six pathogen-related and one ambiguous), and one pathogen-related clonotype was shared by three patients (Fig. 7F).

Discussion

The composition of CD8⁺ T cells, particularly effector cells that mediate cancer cell eradication, impacts therapeutic responses and patient outcomes (9, 10, 13). In solid tumors, CD8⁺ T-cell dysfunction is mediated via an exhausted phenotype and upregulation of immune-inhibitory receptors (12). However, the dynamics of CD8⁺ T-cell dysfunction vary across cancers (12, 15). Thus, extrapolating CD8 biologic characteristics from solid tumors neglects AML-specific features. Indeed, checkpoint blockade therapy was not as successful in AML as in solid tumors (5–7, 57), suggesting mechanisms of exhaustion and dysfunction other than canonical immune-inhibitory receptors.

Several lines of evidence corroborate a *bona fide* dysfunctional state of CD8⁺ T cells in patients with AML. Host immune cells fail to prevent AML emergence despite their coexistence in the bone marrow. Absolute percentages of T cells in healthy donors and patients with AML are not significantly different (58), suggesting a qualitative disorder. In addition, functional studies of CD8⁺ T cells from patients with AML reveals a blunted immune response to stimulatory signals and inability to form immunologic synapses with antigen-presenting cells (55, 58). Our comparison of the frequency of exhausted cells in AML cases with those in 20 other tumors revealed a dearth of *in silico*-predicted exhausted-state CD8⁺ T cells with AML (12). Because responses to immune checkpoint blockade targeting CD8⁺ T cells correlate with immune-inhibitory target expression (10), our findings may clarify the limited efficacy of checkpoint blockade therapy for myeloid malignancies in recent clinical trials (4, 57, 59).

The distinctive clustering of phenotypically similar cells across disease states suggests unique transcriptional programs in effector populations of RelRef CD8⁺ T cells. This led to identification of a CD8-derived signature comprising 25 genes involved in activation, oxidative phosphorylation, TCR signaling, terminal differentiation, and exhaustion, and enriched in terminally differentiated cells. Some evidence suggests that the CD8-derived signature is related to dysfunction. The CD8-derived signature positively correlated with senescence and dysfunction signatures in AML, suggesting that dysfunctional cells coexpress the 25-gene CD8-module. Higher expression of the CD8-derived signature correlated with worse clinical outcomes, supporting a *bona fide* dysfunctional state in CD8⁺ T cells of patients with AML informing clinical outcomes. Cells with higher CD8-derived signature score were also enriched in oxidative phosphorylation and

IFN signaling, which are associated with immunotherapy resistance (60, 61). The CD8-derived signature also correlated with markers of terminal differentiation. Several studies support that terminally differentiated cells are also dysfunctional (35, 62, 63). However, functional studies are needed to confirm the significance of cells enriched for this signature.

The degree of terminal differentiation in NewlyDx CD8⁺ T cells linearly correlated with pseudotime, suggesting a continuous spectrum. However, CD8⁺ T cells of RelRef patients had a high baseline

differentiation status and mostly congregated at the borders of pseudotime. This is supported by immune repertoire tracking, which revealed more phenotypic transitions in CD8⁺ T cells of NewlyDx patients than in terminally committed cells of RelRef patients. Determining whether CD8 states are directly modulated by AML cells versus therapy can have therapeutic implications. High expression of *CX3CR1*, a chemokine with increased expression in chemoresistant CD8⁺ T cells in melanoma (54), in terminally differentiated cells at therapy resistance suggests that prior therapies

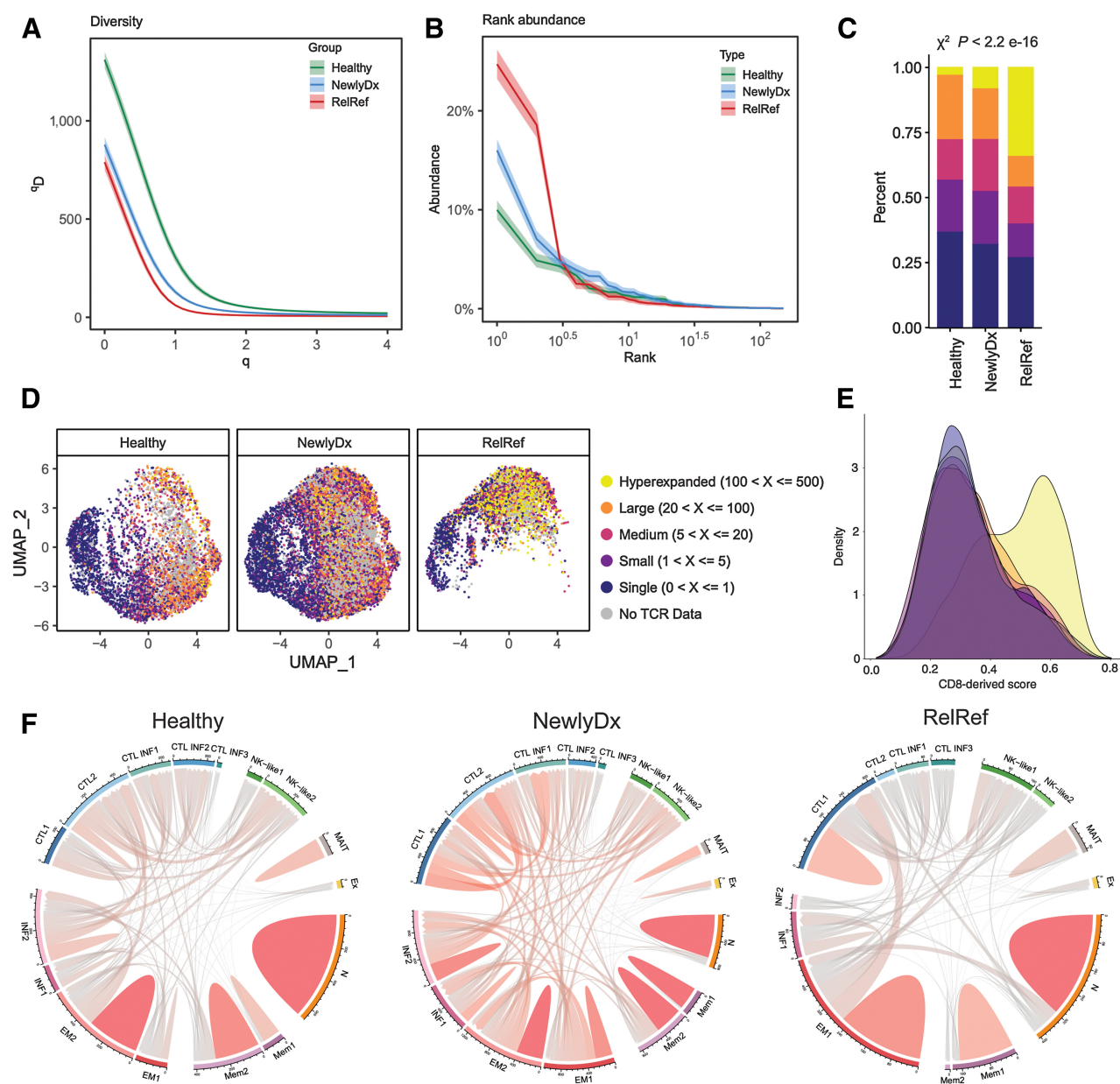
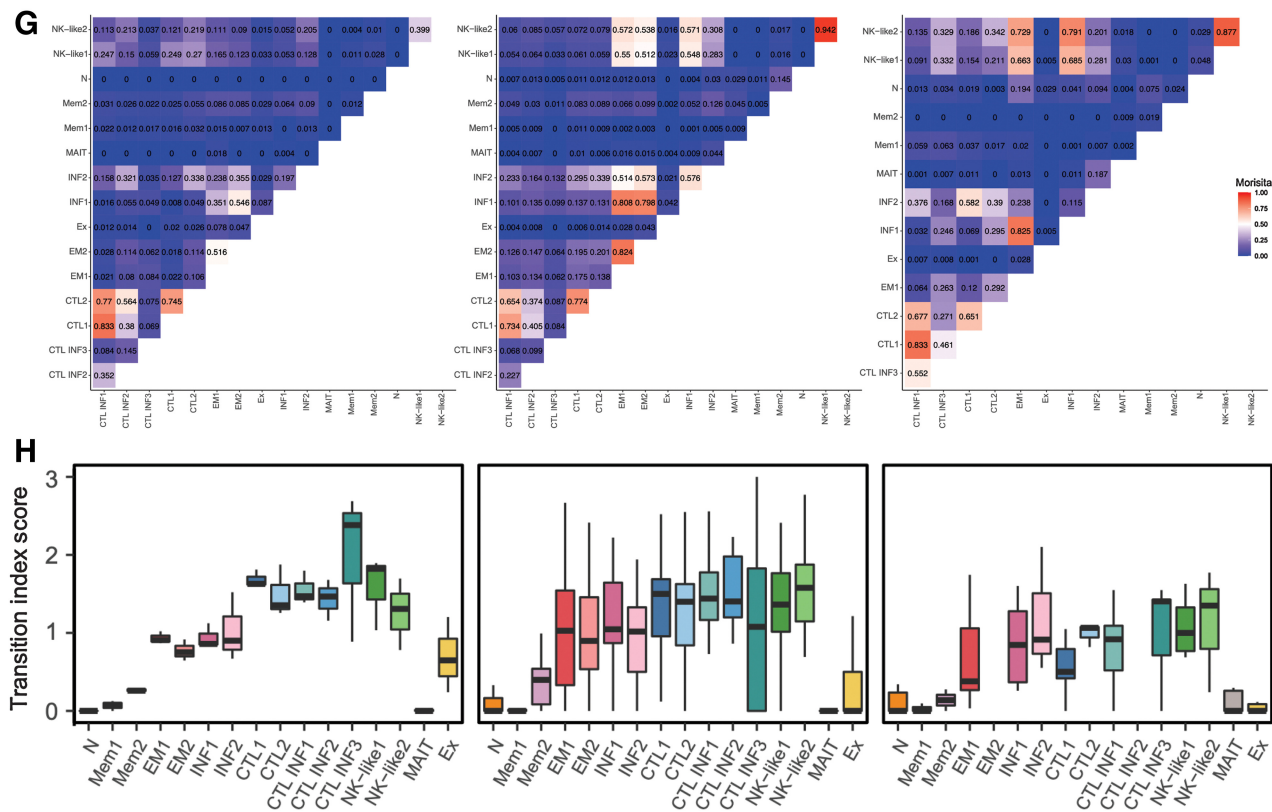


Figure 6.

CD8 repertoire analysis identifying differential clonal states. **A**, Hill diversity analysis according to participant type. **B**, Rank abundance of the top clones for each participant group, identifying how much of the repertoire top clones occupy. Shaded area represents the 95% CI. **C** and **D**, Clonal expansion dynamics across participants in a bar plot (**C**) and projected onto an scRNA-seq-based UMAP (**D**). **E**, Density plot showing the CD8-derived score across clone types. **F**, Circos plot depicting relative clonal sharing clusters according to participant group. Link color represents the relationship strength, and arrowheads show the direction of relation. Line thickness indicates the percentage of shared clones. (Continued on the following page.)

**Figure 6.**

(Continued.) **G**, Clonal overlap of clusters for each participant group using the Morisita index. Red indicates higher levels of clonal sharing between clusters, while blue indicates lower levels. The index value is displayed inside each heat map cell. **H**, STARTRAC-based transition index score for clusters across participants, quantifying clonotype state transitions for each cluster. Index is calculated on the basis of the weighted average of the observed frequency of cells across clusters based on clonotype. Boxes identify quartiles, horizontal lines represent median, and whiskers identify minimum and maximum values.

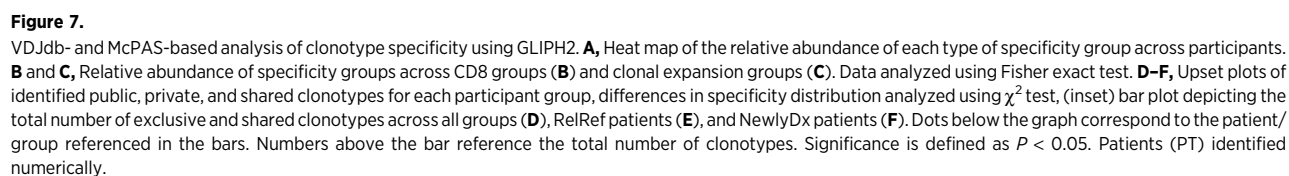
can impact CD8 cellular states. However, a direct AML-modulatory effect on CD8⁺ T cells cannot be ruled out, as increased CD8-derived signature scores substantially associated with worse outcomes in previously untreated patients with *de novo* AML in a multivariate model.

The nature and duration of TCR engagement can modulate CD8 functional states and impact their differentiation trajectory (10). A functional effector T-cell emerges in response to an acute antigen encounter. With persistent stimulation or TCR engagement in the absence of a costimulatory signal, a hyporesponsive state develops (10). Our data suggest a model whereby CD8⁺ T cells likely engage with antigens on AML cells. Supporting this, we found an enriched AP-1 transcriptional signature in effector CD8⁺ T cells in Group 2 clusters, and TCR engagement in CD8⁺ T cells activates the AP-1 complex (41). However, by virtue of *in situ* leukemogenesis, CD8⁺ T cells likely lacked a costimulatory signal for eliminating AML cells in lieu of upregulating checkpoint inhibitory receptors. High correlation of the CD8-derived score with MHC class I genes and antigen-processing pathways, *CX3CR1* expression, and clonal hyperexpansion supports a continuum with chronicity of antigen exposure that leads to terminal differentiation. Therefore, therapeutic strategies to reinvigorate CD8⁺ T cells should address reversing dysfunction beyond canonical immune checkpoint inhibition.

The paired scRNA-seq and TCR analysis provided further insight into the correlation between CD8 differentiation and clonal

expansion. Immune repertoires were largely patient-specific. Although this may be confounded by low depth of TCR-repertoire sequencing, patients with AML may not have a common neoantigen, which is supported by the low mutation burden with AML (64). Deeper sequencing of TCR repertoires may better delineate shared repertoires among patients with AML. Although our analysis highlights the differences between patient with AML groups and defines a CD8-derived signature *in silico*, experimental validation is still needed to better delineate the functional state of CD8⁺ T cells. In addition, whereas trajectory analysis can reveal differentiation states and lineage relationships, it cannot replace experimental validation (65).

Our results demonstrated some shared characteristics between of CD8⁺ T cells in AML and solid cancers. For instance, upon cancer progression, CD8 dysfunction increases while reinvigoration capacity decreases (10). However, the mechanism of dysfunction is independent of classic inhibitory receptors. Rather, we identified a 25-gene signature associated with poor outcomes and clonal hyperexpansion. Importantly, our data support that CD8⁺ T cells have a continuous dysfunctional state and is *fait accompli*: when treatment is initiated, CD8⁺ T cells are already on a spectrum of dysfunction that predicts outcomes. At therapy resistance, CD8 dysfunction progresses, clonal hyperexpansion ensues, and CD8⁺ T cells become less dynamic. Our findings suggest that immune-based



therapy for AML is most successful at early stages when CD8⁺ T cells can afford plasticity.

Authors' Disclosures

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Authors' Contributions

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