

1 Acute Myeloid Leukemia Skews Therapeutic WT1-specific CD8 TCR-T Cells 2 Towards an NK-like Phenotype that Compromises Function and Persistence

3 *Francesco Mazziotta*^{1,2,3}, *Lauren E. Martin*¹, *Daniel N. Eagan*^{2,4}, *Merav Bar*^{2,4,5}, *Sinéad Kinsella*¹, *Kelly G. Paulson*^{2,4}, *Valentin*
4 *Voillet*^{6,7}, *Miranda C. Lahman*¹, *Daniel Hunter*¹, *Thomas M. Schmitt*^{1,2}, *Natalie Duerkopp*^{1,2}, *Cecilia Yeung*², *Tzu-Hao Tang*¹,
5 *Raphael Gottardo*^{8,9,10,11}, *Yuta Asano*¹, *Elise C. Wilcox*¹, *Bo Lee*¹, *Tianzi Zhang*¹, *Paolo Lopodote*¹², *Livius Penter*^{13,14,15}, *Catherine*
6 *J Wu*¹³, *Filippo Milano*^{2,3}, *Philip D. Greenberg*^{1,2,16*}, *Aude G. Chapuis*^{1,2,3,4*}

7 *Contributed equally

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9 1 Program in Immunology, Fred Hutchinson Cancer Center, Seattle, WA, USA.
10 2 Translational Sciences and Therapeutics Division, Fred Hutchinson Cancer Center, Seattle, WA, USA.
11 3 Immunotherapy Integrated Research Center, Fred Hutch Cancer Center, Seattle, WA, USA.
12 4 Division of Medical Oncology, University of Washington, Seattle, WA, USA.
13 5 Bristol Myers Squibb.
14 6 Vaccine and Infectious Disease Division, Fred Hutchinson Cancer Center, Seattle, WA, USA.
15 7 Cape Town HVTN Immunology Laboratory, Hutchinson Centre Research Institute of South Africa, Cape Town, South Africa.
16 8 Biomedical Data Science Center, Lausanne University Hospital.
17 9 University of Lausanne, Lausanne, Switzerland.
18 10 Agora Translational Research Center, Lausanne, Switzerland
19 11 Swiss Institute of Bioinformatics, Lausanne, Switzerland
20 12 Department of Medicine, St. Elizabeth's Medical Center, Boston University, Boston, MA, USA.
21 13 Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA, USA.
22 14 Department of Hematology, Oncology, and Tumorimmunology, Campus Virchow Klinikum, Charité - Universitätsmedizin Berlin, Corporate
23 Member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Berlin, Germany
24 15 Berlin Institute of Health at Charité-Universitätsmedizin Berlin, BIH Biomedical Innovation Academy, BIH Charité Digital Clinician Scientist
25 Program, Berlin, Germany
26 16 Departments of Immunology and Medicine, University of Washington, Seattle, WA, USA.

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29
30
31 **Corresponding Author:** Aude G. Chapuis, MD
32 Division of Hematology and Oncology, University of Washington
33 Fred Hutchinson Cancer Center
34 Phone: 206-667-4369; E-mail: achapuis@fredhutch.org

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55 **Data sharing**
56 For original data, please contact fmazziot@fredhutch.org and achapuis@fredhutch.org. Raw data will be deposited and made publicly available as
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59

60 **Abstract**

61 Acute myeloid leukemia (AML) that is relapsed and/or refractory post-allogeneic hematopoietic cell
62 transplantation (HCT) is usually fatal. In a prior study, we demonstrated that AML relapse in high-risk
63 patients was prevented by post-HCT immunotherapy with Epstein-Barr virus (EBV)-specific donor CD8⁺
64 T cells engineered to express a high-affinity Wilms Tumor Antigen 1 (WT1)-specific T-cell receptor (T_{TCR-}
65 c₄). However, in the present study, infusion of EBV- or Cytomegalovirus (CMV)-specific T_{TCR-C4} did not
66 clearly improve outcomes in fifteen patients with active disease post-HCT. TCR_{C4}-transduced EBV-specific
67 T cells persisted longer post-transfer than CMV-specific T cells. Persisting T_{TCR-C4} skewed towards
68 dysfunctional natural killer-like terminal differentiation, distinct from the dominant exhaustion programs
69 reported for T-cell therapies targeting solid tumors. In one patient with active AML post-HCT, a sustained
70 T_{TCR-C4} effector-memory profile correlated with long-term T_{TCR-C4} persistence and disease control. These
71 findings reveal complex mechanisms underlying AML-induced T-cell dysfunction, informing future
72 therapeutic strategies for addressing post-HCT relapse.

73 Introduction

74 Relapsed/refractory acute myeloid leukemia (AML) after allogeneic hematopoietic cell
75 transplantation (HCT) poses a major therapeutic challenge,^{1,2} with 2-year overall survival (OS) rate below
76 20% and only 4% if relapse occurs within six months post-HCT.¹⁻³ Salvage therapies, including intensive
77 chemotherapy, donor lymphocyte infusions (DLI), and second HCTs have shown limited efficacy,
78 underscoring the urgent need for novel therapies.

79 The Wilms' Tumor 1 (WT1) protein is an attractive AML immunotherapy target,⁴ as WT1
80 overexpression promotes proliferation and oncogenicity.⁵⁻⁷ In a phase I/II trial, targeting WT1 in the post-
81 HCT setting, we genetically modified matched donor CD8⁺ T cells to express a high-affinity WT1-specific
82 T cell receptor (TCR_{C4}) specific for the HLA-A*0201-restricted WT1₁₂₆₋₁₃₄ epitope. To minimize the
83 potential for graft-versus-host disease (GVHD) mediated by infused donor cells,⁸ Epstein-Barr virus
84 (EBV)-specific or cytomegalovirus (CMV)-specific (in EBV-negative donors) CD8⁺ T cells were
85 transduced (T_{TCR-C4}). Prophylactic infusion of EBV-specific T_{TCR-C4} in patients without detectable disease,
86 but at high relapse risk post-transplant (Arm 1), yielded 100% relapse-free survival in 12 patients at a
87 median follow-up of 44 months, and 54% in concurrent controls.⁹ However, in 15 patients with prior
88 evidence of disease post-HCT discussed here, infused EBV-specific or CMV-specific T_{TCR-C4} did not yield
89 a superior overall survival compared to historical controls.^{1,2,10,11}

90 The persistence of functional antigen-specific T cells is required for sustained immunotherapy
91 efficacy.¹²⁻¹⁴ In solid tumors and lymphomas, reduced persistence generally correlates with T-cell
92 exhaustion,^{12,15,16} characterized by markers like PD-1, CTLA-4, Tim3, LAG-3, BTLA and/or TIGIT.¹⁷
93 Targeting these immune-inhibitory receptors with checkpoint-blocking antibodies mitigates exhaustion.^{18,19}
94 In AML, recent studies have questioned the presence of T-cell exhaustion,²⁰ proposing alternative
95 mechanisms of immune dysfunction.^{21,22} Natural killer-like (NKL) markers expressed on CD8⁺ T cells have
96 correlated with adverse outcomes, suggesting that NKL skewing may contribute to T-cell dysfunction.²²⁻²⁴
97 However, whether AML directly induces terminally differentiated, dysfunctional antigen-specific T cells
98 remains unclear.²⁵

99 Understanding the mechanisms of dysfunction that are operative in AML is critical for designing
100 strategies to overcome the current limitations observed in T-cell therapies. Adoptive transfer of T_{TCR-C4} in
101 refractory or relapsed AML patients has provided a unique opportunity to track AML-specific T cells,
102 elucidate AML-induced T cell states, identify the mechanisms responsible for T-cell dysfunction, and
103 inform the design of effective anti-AML therapies.

104 Results

105 **T_{TCR-C4} is safe, well tolerated, and produces comparable outcomes to conventional treatments for** 106 **high-risk relapsed/refractory AML patients post-HCT.**

107 From December 2012 through March 2020, 15 HLA-A2-expressing patients with relapsed and/or
108 refractory AML post-HCT were enrolled on trial NCT01640301 (**Table 1, Extended data Fig. 1**). The
109 median age at diagnosis was 40. Pre-HCT, 27% of patients had secondary or treatment-related AML, at
110 diagnosis 47% were adverse risk, and 53% favorable/intermediate risk per European LeukemiaNet (ELN)
111 stratification (**Supplementary Table 1**).²⁶ Post-HCT, four patients had measurable residual disease (MRD)
112 by ~28 days (refractory), two relapsed by 3 months, and nine relapsed after day 100. The median time to
113 relapse was 496 days post-HCT. Thirteen patients received salvage therapy before T_{TCR-C4}, including six
114 who underwent a second HCT. Within a median of two weeks before infusion, two patients had overt
115 disease, five were MRD-positive and the rest had no evaluable disease (NED) (**Extended data Fig. 2**). Ten
116 patients received EBV-specific T_{TCR-C4} and five received CMV-specific T cells (**Table 1**). Patients received
117 one to four infusions depending on their place in the T_{TCR-C4} dose escalation (**Extended data Fig. 3**).

118 As in Arm 1,⁹ T_{TCR-C4} infusions were safe and well tolerated (**Supplementary Table 2**). The
119 incidence of acute and chronic GVHD (aGVHD, cGVHD) was lower compared to DLIs or second HCTs
120 as salvage strategies,^{11,27} with only one patient (7%) developing grade 3 aGVHD and three (20%)
121 developing cGVHD post-T_{TCR-C4} infusion. Post-infusion cGVHD included one grade 3, one grade 2 and
122 one grade 1 cGVHD. However, the onset of cGVHD occurred when T_{TCR-C4} constituted <1% of the
123 peripheral blood (PB) CD8⁺ T cells, making the infusion of T_{TCR-C4} with an endogenous virus-specific TCR
124 the unlikely cause of cGVHD.

125 Among the patients enrolled (**Table 1**), four showed no overt relapse and/or MRD following T_{TCR-}
126 _{C4} infusion(s) (**Extended data Fig. 4**) supporting T_{TCR-C4}'s potential biologic activity. However, when all
127 15 patients were analyzed together, the median OS was 242 days, the 2-year OS was 33%, and the 3-year
128 OS was 20% (**Extended data Fig. 5**), indicating that these individual outcomes did not translate into a
129 significant survival advantage over historical salvage treatments.^{1,2,10,11} For example, DLI post-HCT relapse
130 showed a 2-year OS of 21% (±3%) from relapse and 56% (±10%) from DLI in cases of remission or with
131 a favorable karyotype, dropping to 15% (±3%) in aplasia or active disease.²⁷

133 **Virus-specific substrate T cells and AML presence at the time of infusion are major determinants of** 134 **T_{TCR-C4} persistence**

135 In our cohort, baseline PB WT1-specific tetramer⁺ T cells were low (**Fig. 1A** – red arrows, day 0)
136 indicating limited endogenous WT1-specific T cells. By day 28 post-infusion, four patients showed T_{TCR-}
137 _{C4} levels exceeding 3% (persistence threshold) of the CD8⁺ T cells, with three maintaining these levels

138 beyond day 100. Higher frequencies correlated with increased absolute T_{TCR-C4} counts (**Extended data Fig.**
139 **6A**). Patients with T_{TCR-C4} frequencies below 3% were eligible for additional infusions (two infusions in
140 6/15 patients, >2 infusions in 4/15 patients) (**Table 1**). However, more than two infusions did not
141 significantly increase persistence (**Fig. 1B**).

142 We next explored the contribution of substrate cell virus-specificity to post-infusion persistence.
143 Within 28 days, EBV-specific T_{TCR-C4} were more abundant than CMV-specific T_{TCR-C4} , which remained
144 below the persistence threshold (**Fig. 1C**). These differences endured when EBV-specific T_{TCR-C4} recipients
145 from Arm 1 were included (**Extended data Fig. 6B**).⁹ As central-memory $CD8^+$ T cells (Tcm) have shown
146 improved persistence compared to more differentiated phenotypes post-transfer,²⁸ we sought to explore
147 whether differences in endogenous virus-specific cells could recapitulate these findings. We analyzed the
148 phenotypes of EBV- and CMV-specific T cells using mass cytometry data²⁹ from 143 healthy individuals
149 and cancer patients. Multidimensional scaling (MDS) showed segregation of CMV- and EBV-specific
150 $CD8^+$ T cells along the first dimension (MDS dim.1) suggesting distinct T-cell differentiation states
151 between these groups (**Extended data Fig. 7A**). Next, Flow Self-Organizing Maps (FlowSOM)³⁰
152 metaclustering identified four $CD8^+$ T-cell subsets: naïve ($CD95^+, CD45RA^+, CCR7^+, CD27^+, CD28^+$),
153 effector memory (Tem) ($CD95^+, CD45RA^-, CD45RO^+, CD27^+, CD28^+, CD127^+$), Tcm ($CD95^+, CD45RA^-$
154 , $CD45RO^+, CCR7^+, CD27^+, CD28^+, CD127^+$), $CD45RA^+$ effector memory (Temra) ($CD95^+, CD45RA^+$,
155 $CD45RO^-, CD57^+, KLRG1^+$) (**Extended data Fig. 7B**).^{31,32} Differential abundance analysis revealed a
156 significant (false discovery rate/FDR < 0.05) increase of Temra cells in CMV-specific T cells and of naïve,
157 Tcm and Tem in EBV-specific T cells (**Fig. 1D**). These results suggest that the Temra state of CMV-specific
158 substrate cells compromises post-infusion persistence, whereas EBV-specific cells, derived from less
159 differentiated precursors, facilitate persistence.

160 Persistence varied among EBV-specific T_{TCR-C4} recipients, (**Fig. 1C, Extended data Fig. 6A**), with
161 only three of ten (4, 8 and 27) exhibiting long-term persistence (>100 days post-infusion) (**Fig. 1A,**
162 **Extended data Fig. 8A**). We reasoned that persistence was influenced by AML high-risk factors, including
163 post-transplant remission duration and disease burden at the time of T_{TCR-C4} infusion.³ Patient 1 was
164 excluded from this analysis due to unavailable WT1-expression data. Six of the remaining patients did not
165 show long-term persistence: four (5,7,14, and 19) had detectable disease (MRD or overt) within two weeks
166 of T_{TCR-C4} infusion, and two (23, 26) who were disease-free at infusion, had relapsed within 3 months post-
167 HCT, suggesting difficult-to-control disease. Statistical testing revealed a trend (Fisher's exact test, $p =$
168 0.08) linking reduced persistence with “aggressive” disease (defined as having detectable disease pre-
169 infusion or early relapse post-HCT). Patient 8, initially categorized as “aggressive” because MRD-positive
170 before the first infusion, became MRD-negative through salvage therapy before the second infusion,
171 achieving the longest observed T_{TCR-C4} persistence. Reclassifying this patient as “non-aggressive”, renders

172 the association between disease aggressiveness and persistence statistically significant ($p < 0.05$). These
173 findings suggest that aggressive disease post-HCT, independently of virus-specificity, was associated with
174 reduced T_{TCR-C4} persistence *in vivo*.

175

176 **Long-term persistent T_{TCR-C4} acquire NKL/terminal differentiation markers associated with** 177 **progressive loss of function *in vivo***

178 To investigate the fate of T_{TCR-C4} post-transfer in patients with persistent T_{TCR-C4} , and compare these
179 with endogenous (TCR_{C4}) T cells, we designed a 24-color spectral flow-cytometry panel (**Supplementary**
180 **Table 3**). PB was analyzed at ~ 1 (T1), ~ 7 (T2), 28 (T3) days, and ~ 4 months (T4) post-transfer
181 (**Supplementary Table 4**). FlowSOM clustering revealed five $CD8^+$ T-cell states categorized into three
182 higher-level clusters (**Fig. 2A, top dendrogram**): naïve-/cm-like cells (**Fig. 2A, dark green**) ($CD45RA^+$,
183 $CCR7^+$, $CD27^+$, $CD28^+$); Tem subgroup, including endogenous $CD8^+$ Tem ($CD28^+$, $CD27^+$, $Ki67^+$, $CD38^+$,
184 $TIGIT^+$, $PD1^+$, $Tbet^+$) (**Fig. 2A, violet**), and phenotypically similar T_{TCR-C4} _Tem (tetramer⁺) (**Fig. 2A, blue**);
185 Temra subgroup of endogenous $CD8^+$ T cells ($CD45RA^+$, $CD57^+$, $KLRG1^+$, $GZMB^+$) (**Fig. 2A, light green**)
186 and closely clustered T_{TCR-C4} _Temra (**Fig. 2A, red**). Two-dimensional Uniform Manifold Approximation
187 and Projection (UMAP) revealed a progressive increase in T_{TCR-C4} _Temra and decline in T_{TCR-C4} _Tem from
188 T1 to T4 (**Fig. 2B**). T_{TCR-C4} _Temra expressed minimal Ki67 compared to T_{TCR-C4} _Tem (**Fig. 2A**), enabling
189 manual gating of these two subsets (**Fig. 2C**). Over time, T_{TCR-C4} _Temra significantly increased, while T_{TCR-}
190 $c4$ _Tem decreased ($p < 0.05$) (**Fig. 2D**), suggesting differentiation from proliferative T_{TCR-C4} _Tem to non-
191 proliferative T_{TCR-C4} _Temra. T_{TCR-C4} _Temra predominantly expressed cytotoxic/KLR markers ($KLRG1$,
192 $CD57$, $GNLY$), previously linked to T-cell dysfunction in AML,²¹⁻²³ rather than classical T-cell exhaustion
193 markers ($Tim3$, $PD1$, and $TIGIT$) (**Fig. 2E**).^{17,33} Stratification by timepoint revealed progressive increase
194 of the cytotoxic subset versus the exhausted subset (**Fig. 2F**). This phenotypic shift coincided with a
195 detectable but slow decline in T_{TCR-C4} $CD8^+$ T cells producing $IFN\gamma$ and $TNF\alpha$ (**Fig. 2G, Extended data**
196 **Fig. 8B**). Thus, long-term persisting EBV-specific T_{TCR-C4} skew towards a distinct NKL phenotype linked
197 to functional decline.

198

199 **T_{TCR-C4} largely represent an intermediate state between effector memory and NK-like/terminally** 200 **differentiated cells.**

201 To explore the relationship between $CD8^+$ endogenous T-cell and T_{TCR-C4} states, we performed
202 single-cell RNA sequencing (scRNAseq) on available PB and bone marrow (BM) samples from patients
203 with T_{TCR-C4} exceeding 3% of $CD8^+$ T cells at least until day 28 post-infusion. This analysis included two
204 prophylactic⁹ and five treatment-arm patients (**Supplementary Table 5**).

205 Unsupervised clustering of PB CD8⁺ T cells (n = 24,472) identified 13 clusters, with the TCR_{C4}
206 transgene expressed across several clusters, but primarily in cluster 2 (**Fig. 3A**). Using the marker-based
207 purification algorithm scGate,³⁴ we identified TCR_{C4}⁺ cells (**Fig. 3B**) labeled as T_{TCR-C4}, while endogenous
208 (TCR_{C4}⁻) T cells included clusters 5 and 6, labeled as naïve-/cm-like, expressing *CCR7*, *SELL*, *TCF7*, *LEF1*,
209 *IL7R*; clusters 2, 4 and 11, labeled as Tem, expressing genes associated with activation (*CD69*, *TIGIT*,
210 *GZMK*); cluster 12, labeled as interferon signaling genes (ISG), expressing *ISG15*, *ISG20*, *IRF7*, *IFI6*;
211 clusters 9 and 10, labeled as T memory/proliferative (Tmem/prolif), expressing proliferation (*MKI67*,
212 *MCM5*, *MCM7*) and memory (*CD27*) genes; clusters 0,1,3,7, and 8, labeled as NKL/Temra, expressing
213 genes associated with cytotoxicity/NKL (*KLRF1*, *KIR3DL1*, *NKG7*, *FCGR3A*, *GZMB*, *KLRD1*, *PRF1*)
214 (**Fig. 3A-C, Extended data Fig. 9A-C, Supplementary Table 6**).

215 To assess transcriptional similarities among cell states, we performed principal component
216 analysis, positioning naïve-/cm-like and NKL/Temra as differentiation spectrum extremes, with T_{TCR-C4} as
217 the intermediate state between these two extremes (**Fig. 3C**).

218 To ensure unbiased labeling of CD8⁺ T cells, we constructed a scRNAseq reference atlas
219 comprising 109,051 CD8⁺ T cells, using a published scRNAseq dataset of tumor-infiltrating lymphocytes.³⁵
220 We aligned and projected our scRNAseq dataset onto the reference atlas (**Extended data Fig. 9B**),
221 confirming the correspondence between the transcriptional states in our dataset and the reference.
222 Differential gene expression (DGE) analysis using manually curated markers further supported this
223 annotation (**Supplementary Table 7, Extended data Fig. 9C**).^{31,32,36,37}

224 Consistent with previous work,³² we observed a progressive reduction of stem-like markers (*IL7R*,
225 *TCF7*) from naïve/cm-like cells through Tem and NKL/Temra cells, while activation markers (*CD69*,
226 *GZMK*) peaked in Tem. NKL markers, including KLR-exhaustion markers (*SIPR5*, *ZEB2*),^{38,39} were
227 highest in NKL/Temra. T_{TCR-C4} expressed activation and NKL markers, reflecting an intermediate state
228 between Tem and NKL/Temra (**Extended data Fig. 9D**).

229 Monocle⁴⁰ trajectory inference confirmed T_{TCR-C4} as an intermediate state between Tem and the
230 terminal NKL/Temra state (**Fig. 3E-F**), and removing the TCR_{C4} transgene did not alter this differentiation
231 trajectory (**Extended data Fig. 10A-B**). Single-gene expression over pseudotime revealed early peaks of
232 naïve/stem-like markers (*CCR7*, *IL7R*, *TCF7*, *SELL*, *LEF1*) (**Fig. 3G, first row**), naïve/memory (*CD27*,
233 *CD28*) and activation markers (*GZMK*, *CD69*), with the latter remaining high throughout mid-pseudotime
234 before declining. (**Fig. 3G, second row**). TCR_{C4} expression peaked at mid-pseudotime, but remained
235 expressed until the end of pseudotime. NKL/Temra-associated genes (*PRF1*, *KLRG1*, *NKG7*, *ZEB2*,
236 *SIPR5*) peaked at the end of pseudotime (**Fig. 3G, third row**). RNA velocity inference⁴¹ supported the
237 spectrum of differentiation from naïve-like to Tem and T_{TCR-C4}, culminating in NKL/Temra cells (**Fig. 3H**).

238 Overall, our findings suggest that T_{TCR-C4} largely existed in an intermediate state between the Tem
239 phenotype and the NKL/Temra stage.

240

241 **AML drives T_{TCR-C4} towards NKL/terminal differentiation instead of the dominant exhaustion**
242 **pattern associated with T cells in solid tumors.**

243 To investigate AML's impact on the differentiation of endogenous $CD8^+$ T cells and T_{TCR-C4} ,
244 patients samples were grouped as AML- (no AML detected, including two prophylactic arm cases),⁹ or
245 AML+ (blasts evident in BM and/or PB) (**Supplementary Table 8**). The increase of ISG and NKL/Temra
246 in AML+ and T_{TCR-C4} in AML- (**Fig. 4A**) was significant (**Fig. 4B**), with a wide confidence interval for ISG,
247 making the magnitude of this difference unclear. T_{TCR-C4} overexpressed NKL/Temra genes in AML+
248 compared to AML- (**Fig. 4C**), suggesting that AML blasts may induce a transcriptional shift towards
249 NKL/Temra in T_{TCR-C4} and endogenous T cells.

250 Following previously described methodology,⁴² we exposed *in vitro* $CD8^+$ transgenic WT1-specific
251 T cells targeting the HLA A*0201-restricted WT1₃₇₋₄₅ epitope⁴³ ($T_{TCR37-45}$) to high-WT1-expressing HLA-
252 A*0201-transduced K562 acutely transformed chronic myelogenous leukemia (**Extended data Fig. 11A**).
253 As K562 primarily express standard proteasomes and are not readily lysed by T_{TCR-C4} targeting the
254 immunoproteasome-specific WT1₁₂₆₋₁₃₄ peptide, we used $T_{TCR37-45}$ due to its proteasome-agnostic WT1-
255 derived peptide recognition.⁴³ Exposing $T_{TCR37-45}$ to fresh K562 every 3-4 days at stable effector-to-target
256 (E:T) ratios (1:1, 1:4) caused initial tumor lysis (**Extended data Fig. 11B**), but control was lost by day 13,
257 marked by a declining $T_{TCR37-45}$ -to-tumor ratio (**Extended data Fig. 11C**). Irrelevant TCR-expressing T
258 cells exerted no control (**Extended data Fig. 11D**). Bulk-RNA sequencing on sorted T cells found increased
259 (FDR <0.05) naïve/stem-like genes (*IL7R*, *TCF7*, *LTB*, *LEF1*, *SELL*) at day 0, followed at day 13 by
260 increased effector (*CCL5*, *GZMK*), activation/exhaustion (*CD69*, *LAG3*, *CTLA4*, *PDCDI*), and
261 NKL/Temra markers (*KLRB1*, *ENTPDI*, *KLRC1*, *KLRC2*, *NCAMI*) (**Fig. 4D-E**). By day 23, the
262 activation/exhaustion markers decreased, while NKL/Temra persisted or increased (**Fig. 4D-E**). To
263 integrate these findings with the scRNAseq dataset, we performed a gene-set enrichment analysis with the
264 top 50 differentially expressed genes from each $CD8^+$ subset identified in scRNAseq, including naïve-/cm-
265 like, Tem, NKL/Temra, T_{TCR-C4} , Tmem/prolif and ISG from the scRNAseq dataset. We also included a
266 manually curated exhaustion signature (**Supplementary Table 9**). Exhaustion and Tem signatures
267 exhibited enrichment in later timepoints, yet there was no further increase at day 23 versus day 13 (**Fig.**
268 **4F**). In contrast, the NKL/Temra increased between days 13 and 23 corresponding to $T_{TCR37-45}$ loss of tumor
269 control (**Extended data Fig. 11C**).

270 We next projected scores from published NKL dysfunction signatures^{22,38,39} onto our UMAP,
271 revealing overlaps that link the NKL/Temra $CD8^+$ T cells we identified with an end-term dysfunctional

272 differentiation subset (**Extended data Fig. 12**). To confirm that AML-exposed T cells skewed towards a
273 NKL/Temra rather than exhausted (Tex) phenotype, we projected onto a scRNAseq reference atlas a BM
274 AML CD8⁺ T-cell dataset, compiled from published sources,^{20,22,23,44,45} alongside datasets representing
275 pancreatic,⁴⁶ melanoma,⁴⁷ and lung⁴⁸ solid tumors (**Fig. 4G-H**).³⁵ Unlike solid tumors, which included a
276 Tex cluster, AML datasets aligned with our spectral flow-cytometry and scRNAseq findings confirming the
277 absence of Tex and the presence of NKL/Temra cells (**Fig. 4G, Extended data Fig. 13**). Although these
278 studies did not specifically analyze tumor-antigen reactive T cells, the findings collectively suggest that the
279 NKL (versus exhaustion) signature is intricately associated with AML-induced T-cell dysfunction.

280

281 **Prolonged azacitidine exposure enhances self-renewal and Tcm features of T_{TCR-C4}, supporting T_{TCR-}** 282 **C₄-mediated AML control**

283 Given the link between NKL differentiation and AML-induced T-cell dysfunction, we explored the
284 state of T_{TCR-C4} in patient 8, the only patient of 4 (**Extended data Fig. 4**) who exhibited prolonged T_{TCR-C4}
285 and MRD persistence indicative of disease control despite incomplete leukemia clearance, enabling T_{TCR-}
286 C₄/AML interplay analysis. This female patient underwent a non-myelo-ablative (Flu/3Gy-TBI) HCT from
287 a male donor, with MRD (0.26% blasts by flow cytometry) detected 28 days post-HCT (**Fig. 5A**) and
288 received a T_{TCR-C4} (10¹⁰ cells/m²) 77 days post-HCT. AML blasts expressed detectable but limited WT1
289 protein pre-HCT (**Extended data Fig. 14**). scRNAseq at day 49 post-infusion revealed circulating CD34⁺
290 female-origin blasts (XIST⁺) expressing WT1 (**Fig. 5E-F**). Persistent MRD and limited T-cell persistence
291 at day 63 post-infusion (T cells declined to only 0.1 multimer⁺ CD8⁺ T cells/μl on day 60), (**Fig. 5B, 5C-**
292 **red arrow, 5D**) prompted azacitidine treatment (five days every 28 days). Post-azacitidine, T_{TCR-C4} counts
293 increased (~50 multimer⁺ CD8⁺ T cells/ul; >3% CD8 multimer⁺ T cells) without additional T_{TCR-C4} (**Fig.**
294 **5D**), leading to azacitidine discontinuation after seven cycles when no AML was detected. However, AML
295 recurrence at day 414 post-infusion triggered chemotherapy (mitoxantrone, etoposide and cytarabine),
296 additional azacitidine cycles followed by a second T_{TCR-C4} (10¹⁰ cells/m²) infusion (**Fig. 5A**). Azacitidine
297 was held post-infusion but restarted upon MRD detection 162 days post-second T_{TCR-C4} infusion, continuing
298 for over 20 monthly cycles, maintaining marrow MRD below 1% for 21 months (641 days, from day 868
299 to day 1509) before progression was detected on day 1546 (**Fig. 5A-B**). During this 21-month period, blasts
300 were detectable via scRNAseq in BM but not in PB (**Fig. 5F, Extended data Fig. 15A-B**). Throughout the
301 azacitidine cycles, platelet counts decreased (~100 x 10³ platelets/μl) mid-cycle before rising (~300 x 10³
302 platelets/μl) immediately before the next cycle, consistent with transient azacitidine-mediated
303 myelosuppression. Neutrophil counts, however, paradoxically increased mid-cycle (~4.5 x 10³
304 neutrophils/μl) and decreased (~1.6 x 10³ neutrophils/μl) just before the next cycle, suggesting that
305 treatment-related AML contraction facilitated enhanced mid-cycle neutrophil production despite

306 azacitidine's myelosuppressive effect (**Extended data Fig. 15C**). This 21-month response exceeds the
307 typical 4-month median for post-HCT azacitidine treatment,⁴⁹ suggesting that persisting T_{TCR-C4} plus
308 azacitidine contributed to long-term disease control.

309 T_{TCR-C4} transcriptomics were analyzed to identify associations with disease control. We scored
310 genes associated with self-renewal and NK-like profiles and found that at earlier timepoints (d49, d256,
311 and d405 post-first infusion), T_{TCR-C4} were predominantly skewed towards an NKL/Temra transcriptional
312 state. However, at later timepoints (d1322, d1343) although T_{TCR-C4} remained along the same differentiation
313 trajectory (from less differentiated states to NKL/Temra T_{TCR-C4}), they exhibited fewer NKL features, and
314 showed an enrichment of self-renewal markers (**Fig. 5G-I**). scTCRseq analysis of PB at days 265, 405,
315 1322, 1343 post-first T_{TCR-C4} infusion revealed progressive clonal expansion of T_{TCR-C4} (**Fig. 5K**). T_{TCR-C4}
316 clonal expansion peaked during the 21 months of continued azacitidine treatment, suggesting that during
317 this phase, T_{TCR-C4} were capable of both self-renewal, which in turn facilitated their long-term persistence,
318 and antigen recognition, which triggered further expansion and disease control. Similarly, BM scRNAseq
319 revealed clonal expansion of T_{TCR-C4} between d1322 and d1344 post-first infusion (**Fig. 5L**) despite no
320 additional T_{TCR-C4}. These findings indicate that azacitidine-exposed T_{TCR-C4}, retained a self-renewing (versus
321 NKL) features, which enabled their long-term persistence and enhanced AML-targeting efficacy that may
322 have contributed to long-term disease control (**Fig. 5M**).

323

324 Discussion

325 We previously demonstrated that EBV-specific T_{TCR-C4} infusion prevented AML recurrence in
326 patients at high risk of post-HCT relapse.⁹ In 15 AML patients who received T_{TCR-C4} post-HCT relapse, we
327 observed indirect anti-leukemic activity, but no survival advantage in those receiving EBV- or CMV-
328 specific T_{TCR-C4} . We analyzed the AML/ T_{TCR-C4} interplay to investigate how AML affects antigen-specific T
329 cells, and identified T-cell characteristics associated with AML control in one case.

330 The characteristics of substrate cells from which T-cell products are derived can impact post-
331 transfer persistence. In murine and macaque models, antigen-specific Tcm-like $CD8^+$ T cells persist long-
332 term post-adoptive transfer, unlike Temra-like cells.^{9,28} In our study, T_{TCR-C4} from EBV-specific persisted
333 longer compared to CMV-specific substrate cells which may reflect the distinct biology of EBV and
334 CMV.^{50,51} CMV reactivates periodically, requiring rapidly activated responses that promote Temra
335 differentiation,^{52,53} while EBV tends to remain quiescent, preserving Tcm phenotypes.⁵⁴⁻⁵⁶ While
336 reactivation frequency plays a role, the reactivation microenvironment is also critical. CMV reactivates in
337 non-professional antigen-presenting cells, including fibroblasts, which lack co-stimulatory signals and
338 favor Temra differentiation; EBV reactivates in lymphoid tissues B cells, providing co-stimulation that may
339 preserve the Tcm phenotype.^{57,58} Our analysis of an independent mass-cytometry dataset²⁹ confirmed that
340 EBV-specific cells better maintain self-renewing features than CMV-specific cells. Taken together these
341 findings establish that self-renewal may predict prolonged T-cell persistence.^{9,28} However, EBV-specific
342 T_{TCR-C4} were unable to sustain long-term responses in all patients, suggesting influence by non-intrinsic
343 AML-related factors.

344 Despite efforts to investigate T cell dysfunction in AML,^{20,21,23} several critical questions have
345 remained unanswered,²⁵ mainly due to the challenge of identifying AML-specific T cells. In this study,
346 infused T_{TCR-C4} offered the possibility of examining AML-specific T cell and AML biology. We found that
347 T-cell exhaustion is not the primary cause of AML-specific T-cell dysfunction.^{20,21,23} Instead, T_{TCR-C4} with
348 exhaustion markers (PD1, TIGIT, Tim3) also expressed activation markers (CD38, CD69) and proliferation
349 (Ki67), indicating an effector-like T-cell state. Overall, this suggests that AML-exposed T cells follow a
350 differentiation trajectory from effector-memory to NKL cells, compromising T_{TCR-C4} cell persistence and
351 function.

352 AML's distinct properties, including myeloid cells immunosuppressive effects⁵⁹ via production of
353 reactive oxygen species and other soluble factors, may distinctly redirect T cells toward functional
354 impairment that may preclude transitioning to a classical exhausted phenotype and explain the observed
355 NKL skewing in T_{TCR-C4} and endogenous $CD8^+$ T cells. The absence of classical exhausted cells in AML
356 has several implications. In solid tumors, chronic antigen stimulation induces a multi-step epigenetic shift
357 of $CD8^+$ T cells into exhaustion,⁶⁰ starting from $PD1^+TCF1^+$ precursor/progenitor exhausted cells,^{23,24}

358 whose frequency correlates with response to checkpoint inhibitors.^{9,28} In contrast, our AML findings suggest
359 that exhaustion markers reflect recent T cell activation rather than true exhaustion, which may also explain
360 the limited efficacy of checkpoint inhibitors in AML.^{20,23,24,61} The presence of a distinct dysfunctional
361 program supports new immunotherapy strategies to enhance the efficacy of anti-AML adoptive T-cell
362 therapy, preventing T-cell dysfunction. For example, knocking out ID3 and SOX4 transcription factors in
363 chimeric antigen receptor-engineered (CAR)-T cells *in vitro* reduces NKL skewing and enhances effector
364 functions.⁴² Whether these strategies can improve cell therapy in AML *in vivo* remains to be determined.

365 Another strategy to reduce NKL skewing of T_{TCR-C4}, as suggested by the outcome in one of our
366 treated patients, could involve the hypomethylating agent azacitidine. While azacitidine has direct anti-
367 leukemia effects,⁶² the prolonged disease control observed in our patient was unusual prompting further
368 investigation into the mechanisms at play. Azacitidine administration correlated with clonal expansion and
369 persistence of central memory, as opposed to NKL differentiated T_{TCR-C4}, leading to a prolonged equilibrium
370 between anti-leukemic T_{TCR-C4} and AML MRD. Before the first T cell infusion in this patient, low AML
371 WT1 expression likely did not activate T_{TCR-C4}, causing their near disappearance. However, after azacitidine
372 introduction, T_{TCR-C4} frequencies increased, likely driven by azacitidine-induced WT1 expression.⁶³
373 Intermittent T_{TCR-C4} activation by WT1 presentation on AML cells transiently induced by cycles of
374 azacitidine with subsequent temporary clearance of AML may have helped sustain T_{TCR-C4} persistence and
375 response to stimulation. Other studies have shown that azacitidine can have negative effect on T regulatory
376 cells⁶⁴ and boosts CAR T cell toxicity towards AML.⁶⁵ Further investigation is needed to determine if
377 azacitidine acts preferentially on leukemia cells by increasing WT1 expression thereby promoting antigen-
378 recognition and/or T_{TCR-C4} effector functions. However, taken together, these findings support azacitidine as
379 a favorable adjunct to T cell immunotherapy.

380 Paradoxically, our ability to track dysfunction was limited to patients with persisting T_{TCR-C4}, where
381 T cells were relatively more functional. We hypothesize that patients with more aggressive disease
382 experience accelerated T-cell dysfunction and death rather than establishment of progenitor cells capable
383 of self-renewal; however we were unable to formally test this in our study. Despite a limited sample size,
384 our findings on the nature of T-cell dysfunction in AML were confirmed in larger cohorts of endogenous
385 CD8⁺ T cells, though further validation in larger trials could provide more detail. Additionally, our
386 interpretation of the effect of azacitidine on T_{TCR-C4} is based on a single patient. To address this limitation,
387 we are initiating a clinical trial of co-administration of azacitidine and TCR-T cells.

388 Our study illuminates some of the complex mechanisms underlying AML-induced T cell
389 dysfunction and strengthens support for a distinct pathway outside the traditional paradigm of T cell
390 exhaustion, emphasizing the need to address this unique dysfunction in future immunotherapy strategies.

391

392 **Methods**

393

394 **Clinical protocol**

395 The trial was approved by the Fred Hutchinson Cancer Center (FHCC) Institutional Review Board, the US
396 Food and Drug Administration and the National Institutes of Health Recombinant DNA Advisory
397 Committee. It was registered at ClinicalTrials.org under the identifier NCT01640301. Eligible participants
398 included ‘high-risk’ AML patients with relapsed or refractory disease (overt or MRD) post-HCT along with
399 their fully HLA-matched (10 of 10) related or unrelated donors expressing HLA A*0201 (HLA-A2).

400 **Patient selection**

401 HLA-A2 genotype was confirmed by high-resolution typing before enrollment. Exclusion criteria included:
402 refractory central nervous system disease, HIV seropositivity, grade ≥ 3 GVHD and no available
403 CMV/EBV-seropositive matched donor. The sample size for this study was not based on formal power
404 calculations, but on feasibility, the potential to provide descriptive information, determine whether further
405 study was warranted and evaluate toxicity.

406 **Treatment plan**

407 Isolation of TCR_{C4}, lentiviral vector construction, and T_{TCR-C4} generation were performed as previously
408 described.⁹ Patients were eligible to receive a first infusion of T_{TCR-C4} after demonstrating relapse (overt or
409 MRD) at any time post allogeneic HCT. Patients 26,27,28 received lymphodepleting treatment before the
410 first T_{TCR-C4} infusion with cyclophosphamide (300 mg/m² IV) and fludarabine phosphate (30 mg/m² IV)
411 daily on days -4 to -2. A second T_{TCR-C4} infusion was administered only if the frequency of T_{TCR-C4} was <3%
412 of total peripheral CD8⁺ T cells. Patients 5,7,19,23,27 received 1 infusion, patients 4,8,15,26,28 received 2
413 infusions, patient 1 received 1 infusion, the remainder received 4 infusions (**Table 1**). Patients were
414 monitored for toxicities, based on Common Toxicity Criteria v.4.0.

415 **Assessment of disease status**

416 Morphology, multiparameter flow cytometry, standard cytogenetics or genomic technologies were routinely
417 performed on bone marrow aspirates and peripheral blood samples that were obtained from all patients.
418 Any level of residual disease was considered to indicate positivity for MRD.

419 **T cell tracking by WT1 peptide/HLA (pHLA) tetramers**

420 WT1 pHLA-specific tetramers (produced by the FHCC Immune Monitoring Core Facility) were used to
421 detect T_{TCR-C4} in PBMCs collected after infusions, with a staining sensitivity of 0.01% of total CD8⁺ T cells,
422 as previously described.⁹ T_{TCR-C4} percentages were calculated using FlowJo v.10 (Treestar).

423 **Patient outcomes and survival analysis**

424 Kaplan-Meier OS curves were estimated using the *survminer* (v0.4.9) and *survival* (v3.3-1) packages in R.
425 OS was calculated from the date of first T_{TCR-C4} infusion to the date of death or censoring. Outcomes of
426 responding patients were represented using a swimmerplot. Day 0 was defined as the post-HCT relapse

427 date or, for HCT-refractory patients, as day 28 post-HCT, at which timepoint persistent disease was
428 observed. R package *swimplot* (v1.2.0) was used for visualization.

429 **Analysis of T_{TCR-C4} persistence**

430 Persistence of TCR-T cells (%T_{TCR-C4}) over time was visualized using *ggplot2* (v3.4.4) R package.
431 Wilcoxon rank-sum tests were used for pairwise comparisons between T_{TCR-C4} with different virus-
432 specificity (EBV vs. CMV) over time. Kruskal-Wallis tests were applied for multi-group comparisons.
433 Patients were categorized based on disease status prior to infusion: those with detectable MRD or overt
434 disease before T_{TCR-C4} infusion, or those who relapsed within 3 months post-HCT, were classified as having
435 "aggressive" disease. Patients without these risk factors were considered "non-aggressive." One patient was
436 excluded from the analysis due to the unavailability of WT1-expression data, which could confound
437 interpretation of the persistence results. Fisher's exact test was used to evaluate the association between
438 disease aggressiveness and T_{TCR-C4} persistence. The test was chosen due to the small sample size and binary
439 classification of persistence (long-term vs. short-term) and disease aggressiveness (aggressive vs. non-
440 aggressive). Statistical significance was assessed with a p-value threshold of 0.05.

441 **Flow-cytometry**

442 Cryopreserved PBMCs were thawed and allowed to rest overnight in RPMI medium supplemented with
443 10% fetal bovine serum (R10). The cells underwent stimulation with a cocktail containing the WT1₁₂₆₋₁₃₄
444 peptide at a final concentration of 1 µg/ml in R10 and intracellular cytokine staining using a 15-color
445 staining panel, as previously described.⁹ Flow cytometry was conducted on an LSRII instrument (Becton
446 Dickinson) with data acquisition using FACS-Diva software v.8.0.1. Flow cytometry data were
447 subsequently analyzed using FlowJo v.10 (Treestar). Finally, the percentage of Tetramer⁺cytokine⁺ cells
448 gated on the CD8⁺ T cell population was determined.

449 **Spectral flow cytometry**

450 Post-infusion PB samples were analyzed using a 5-laser Cytex Aurora. Antibodies used are listed in
451 **Supplementary Table 1**. T_{TCR-C4} were identified by binding to the APC dye-labeled HLA-A2:WT1126-
452 134 tetramer. Spectral flow-cytometry data were biexponentially transformed, compensated and
453 preprocessed (aggregates and dead cell removal) in FlowJo V10 (TreeStar). Pregated CD8⁺ T cells were
454 exported from FlowJo and loaded in R (v4.3.2). First we created a flowSet using *flowCore* (v2.12.2)⁶⁶ and
455 subsequently analyzed the data using *CATALYST* (v1.24.0).⁶⁷

456 Ki67 was then used to manually gate subsets of interest using the FlowJo software. Statistical analysis
457 included Kruskal-Wallis to compare T_{TCR-C4}_Temra and T_{TCR-C4}_Tem percentages over time, and
458 Wilcoxon test for comparing KLRG1⁺CD57⁺GNLY⁺ and Tim3⁺PD1⁺TIGIT⁺ T_{TCR-C4}_Temra.

459 **In-silico mass cytometry validation**

460 Mass cytometry files used to generate an atlas of virus-specific CD8⁺ T cells²⁹ were downloaded from
461 <https://zenodo.org/records/8330231>. Data were analyzed using *flowCore* and *CATALYST* R packages as
462 specified above.

463 **Single Cell RNA Sequencing**

464 Patients with persisting PB T_{TCR-C4} (> 3% of the total CD8⁺ T cells) at least until day 28 after infusion were
465 selected for scRNAseq analysis. Available PBMCs or BMMCs were thawed, washed and loaded on a 10x
466 Chromium Controller based on the 3' Chromium or 5' Chromium Single Cell V(D)J Reagent Kit manual
467 (10x Genomics). Library preparation was performed as per manufacturer's protocol with no modifications.
468 Library quality was confirmed by TapeStation High Sensitivity (Agilent, evaluates library size), Qubit
469 (Thermo Fisher, evaluates dsDNA quantity), and KAPA qPCR analysis (KAPA Biosystems, evaluates
470 quantity of amplifiable transcript). Samples were mixed in equimolar fashion and sequenced on an Illumina
471 HiSeq 2500 rapid run mode according to the standard 10X Genomics protocol. TCR target enrichment, 5'
472 gene expression library, and TCR library were carried out to the 5' Chromium Single Cell V(D)J Reagent
473 Kit manual (10x Genomics). The 10X Genomics software Cell Ranger (v2.0.0) was used to process the raw
474 data FASTAQ files with default parameters. The EmptyDrops method⁶⁸ was used to identify cells with low
475 RNA contents. The "count" function was used to perform alignment, filtering, barcode counting and UMI
476 counting. Reads were aligned to the hg38 human reference genome (Ensembl) and the known transgene
477 codon-optimized sequence using Spliced Transcripts Alignment to a Reference (STAR).⁶⁹
478 For V(D)J sequencing assembly and paired clonotype calling, we used CellRanger "vdj" function. This
479 function leverages Chromium cellular barcodes and UMIs to assemble V(D)J transcripts for each cell.
480 CellRanger V(D)J calling produces an output named "filtered_contig_annotations.csv" for each sample,
481 which lists CDR3 amino acid and nucleotide sequences for single cells identified by their barcodes.

482 **scRNAseq quality control and subsetting**

483 The filtered feature matrices generated by the CellRanger pipeline were used for downstream quality
484 control (QC) and analyses. We used the function read10xCounts from the R package *DropletUtils* (version
485 1.14.2) to load the CellRanger output in R as a SingleCellExperiment⁷⁰ object. Doublet cells filtering was
486 performed on each sample using the *scds* package (v1.10.0).⁷¹ QC and filtering were conducted using the
487 *scater* R package (v1.22.0)⁷².⁷¹ Genes not detected across all the cells were removed and cells were filtered
488 based on feature counts, the percentage of mitochondrial and ribosomal genes, and the number of expressed
489 features. Cells with values beyond a specific threshold, between 1 and 3.5 median absolute deviations
490 (MAD) from the median, were excluded. These MAD thresholds were established according to the quality
491 of each sample. Features with a count greater than 1 in at least 3 cells were retained for downstream analysis.
492 We then split cells by sample in 15 datasets, normalized, found the 2000 most variable genes and scaled for
493 each dataset using *Seurat* (v4.3.0.9001) SplitObject, NormalizeData, FindVariableFeatures and ScaleData

494 respectively.^{73,74} For batch correction, we used the FindIntegrationAnchors and IntegrateData functions
495 from *Seurat*. The integrated dataset was then used for scaling (ScaleData) and dimensionality reduction
496 (PCA and UMAP) using RunPCA and RunUMAP, respectively. UMAP dimension reduction and clustering
497 were computed using the first 20 principal components (PCs). The number of PCs capturing most of the
498 variation in our data was selected using *Seurat* function ElbowPlot which visualizes the standard deviation
499 of each PC. Clusters were identified via shared-nearest-neighbor-based (SNN) clustering and further
500 analyzed at a resolution of 0.6. CD8⁺ cell subset was identified using *scGate* (v1.0.1)³⁴ and subsequently
501 extracted with Seurat subset function.

502 **scRNAseq CD8⁺ endogenous and T_{TCR-C4} analysis**

503 The CD8⁺ subset was used to find the 2000 most variable features (FindVariableFeatures), scale the data
504 (ScaleData), and run dimensionality reduction (runPCA and runUMAP). SNN was used for clustering with
505 a resolution of 0.5 for downstream analysis. Differential gene expression across clusters or condition (e.g.
506 AML(+) vs AML(-)) was computed using *Seurat* FindAllMarkers with default parameters. TCR_{C4}-
507 transgene⁺ cells were identified using *scGate* (v1.0.1). For visualization purposes, we used Seurat built-in
508 functions alongside *ComplexHeatmap* (v2.15.1)⁷⁵, *scCustomize* (v1.1.0.9001) ([https://github.com/samuel-](https://github.com/samuel-marsh/scCustomize)
509 [marsh/scCustomize](https://github.com/samuel-marsh/scCustomize)) and *SCP* (<https://github.com/zhanghao-njmu/SCP>). The pan-cancer CD8⁺ single-cell
510 reference atlas was built using the CD8.thisStudy_10X.seu.rds file³⁵ downloaded from Zenodo and
511 processed using the make.reference function from the *ProjectTILs* R package (v3.2.0).⁷⁶ We then used
512 Run.Projectils function, from the same package, to project the cell states from the query dataset onto the
513 reference.

514 Cell trajectory inference was computed using the *Monocle* R package (v1.3.4), with UMAP used as
515 dimensionality reduction. Single-gene expression patterns along the pseudotime were visualized using the
516 plot_genes_in_pseudotime function in *Monocle*. RNA velocity was conducted by exporting the CD8⁺ T-
517 cell Seurat object as an h5ad file using the R package *seurat-disk* (v0.0.0.9020)
518 (<https://github.com/mojaveazure/seurat-disk>), loading this file in Python (v3.8.14) as AnnData object,⁷⁷ and
519 estimating velocities with the *scvelo* Python package (v0.2.4)⁴¹ using the deterministic model.⁷⁷

520 We classified patients as AML(+) or AML(-) based on the presence of detectable blasts in BM or PB. Three
521 patients lacked BM or PB evaluations at one timepoint each. For two of these patients, we used the donor-
522 recipient (male-female) sex mismatch. Patient 8 at day 49 exhibited cells expressing the female-specific
523 gene *XIST* alongside the AML-associated genes *CD34* and *WT1* (**Extended data Fig. 15A**); similarly,
524 patient 25 expressed *XIST* in a *RPS4Y1* (male-specific gene) negative region at days 7 and 28, thus classified
525 as AML(+) at these timepoints (**Extended data Fig. 15B**). Patient 4 was categorized as AML(-) at day 100
526 and AML(+) at day 581, as previously reported.⁴³

527 To assess the significance of differences in cell proportions per CD8⁺ cell state between groups (AML(+) vs AML(-)), a permutation test was applied. Specifically, the `permutation_test` and `permutation_plot` functions from the R package *scProportionTest* (version 0.0.0.9000) (<https://github.com/rpolICASTRO/scProportionTest>) were used with default parameters.

531 For scRNAseq *in-silico* validation, we compiled scRNAseq datasets from independent studies on AML^{20,22,23,44,45}, lung cancer⁴⁸, pancreatic cancer⁴⁶ and melanoma.⁴⁷ CD8⁺ T cells were identified and extracted using the *scGate*³⁴ R package. These cells were then projected onto the pan-cancer CD8⁺ T cell reference atlas³⁵ using *ProjecTILs* R package.

535 The TCR repertoire was analyzed using the R package *scRepertoire* (v1.10.1).⁷⁸ Initially the degree of single cells clonal expansion was defined based on the number of cells sharing the same clonotype: Hyperexpanded ($100 < X \leq 500$), Large ($20 < X \leq 100$), Medium ($5 < X \leq 20$), Small ($1 < X \leq 5$), Single ($0 < X \leq 1$) The function `occupiedscRepertoire` was used to visualize the degree of clonal expansion by cell-state over time.

540 **In-vitro chronic antigen stimulation model**

541 The in-vitro dysfunction model was established as previously described.⁴² The WT1⁺ K562 tumor cell line was transduced with lentiviral constructs to express HLA-A*02:01 and GFP, then sorted for purity using the Sony MA900 cell sorter. Cells were then cultured in media consisting of IMDM with GlutaMAX (Gibco, Life Technologies, #31980030) with 10% FBS, and 1% of penicillin/streptomycin.

545 T_{TCR37-45} cells were obtained following previously published protocols.⁴³ We selected TCR-T cells targeting WT1₃₇₋₄₅ as K562 cell line primarily express the standard proteasome and is not lysed by T_{TCR-C4} targeting WT1₁₂₆₋₁₃₄.⁴³ T cells expressing an irrelevant virus-specific TCR were used as negative control. Co-cultures of T cells with K562 cells were established at a 1:1 and 1:4 E:T ratio (2.5×10^5 T cells, 2.5×10^5 or 1×10^6 tumor cells, respectively). After 3-4 days of coculture, 250 μ l of the cell suspension was used for T cell counting and flow-cytometry staining. The remaining cell suspension was spun down, and cells were resuspended in fresh media. A Novocyte 3 lasers flow cytometer was used to quantify GFP⁺ tumor cells and T cells and to maintain constant E:T ratios by reseeding K562 cells. Notably, during the peak of T-cell expansion (day 9-13), the volumes of the cocultures were reduced to ensure the reseeding of an adequate number of tumor cells, thereby maintaining constant E:T ratios. This protocol was followed for 23 days.

555 **Bulk RNA sequencing**

556 Bulk RNA sequencing was performed using BGISEQ-500 platform at BGI Genomics. Briefly, total RNA was extracted using the Qiagen RNeasy Micro Kit according to the manufacturer's instructions. For the construction of low input polyA mRNA-seq libraries, the *SMARTseq* (v4) Package was used. Sequencing was performed on a DNBseq T7 machine (MGI) with paired-end 150 bp reads, generating 30M raw reads per sample. Raw sequencing data were filtered and trimmed using the software Soapnuke developed by

561 BGI Genomics. The filtered reads were then aligned to the reference transcriptome using *Bowtie2* (v2.2.5).
562 Gene read counts were subsequently generated from the alignment results using *RSEM* (v1.2.8).
563 To assess differences over time across conditions (T_{TCR37-45_D0}, K562_D14, and K562_D23), we used the
564 likelihood ratio test (LRT) with a full model of ~Condition and a reduced model including only the intercept
565 (reduced = ~ 1). Normalized counts were obtained using the rlog transformation, and differential expression
566 analysis was performed using the R package *DESeq2* (v1.42.0). We manually curated a list of genes of
567 interest (**Supplementary Table 10**) and filtered the results to retain only those genes that were significant
568 ($p_{adj} < 0.05$) with an absolute log₂ fold-change > 1). For visualization purposes, average normalized counts
569 of biological replicates were calculated using the *avereps* function from the R package *limma* (v3.56.2) and
570 visualized using the R package *heatmap* (v1.0.12). To visualize the expression patterns of manually
571 curated gene signatures, we calculated z-scores for the averaged normalized counts across conditions
572 (T_{TCR37-45_D0}, K562_D14, and K562_D23). Finally, to compare the enrichment of scRNAseq-derived gene
573 signatures (top 50 differentially expressed genes) and of a manually curated exhaustion signature across
574 conditions, we used the *hciR* (v1.7) function *fgsea_all* with default parameters and *plot_fgsea* for plotting.
575 .

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- 759

760 **Acknowledgements**

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770

771 **Contributions**

772 Conception and design were performed by P.D.G., A.G.C., F.M., M.B., and T.M.S. Collection and assembly
773 of data were carried out by F.M., L.M., A.G.C., D.N.E., M.B., V.V., D.H. Data analysis and interpretation
774 were performed by F.M., L.M., T.T., V.V., R.G., F.M., A.G.C., and P.D.G. All of the authors contributed to
775 the writing of the manuscript. Final approval of the manuscript was given by all of the authors.

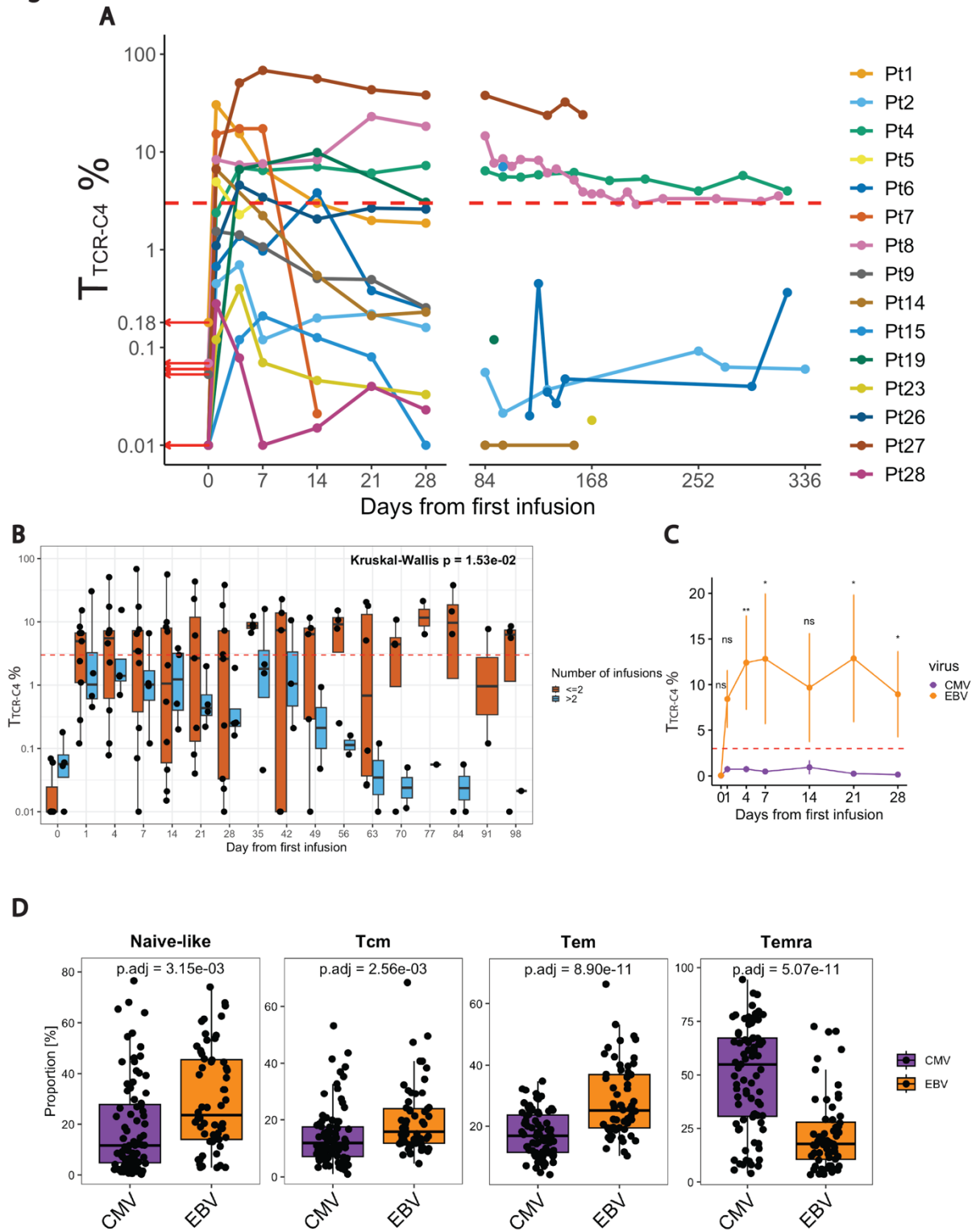
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777 **Table 1**

Pt id	M/F	AML WT1 expression	Disease status 28 days post-HCT	Number of HCT before T _{TCR-C4} infusion	Salvage therapy before T _{TCR-C4} infusion	Lymphodepletion	Disease status at 1st T _{TCR-C4} infusion	Days between salvage and T _{TCR-C4} infusion	Virus specificity	T _{TCR-C4} infusions received
1	M	NA	NED	1	Yes	No	NED	87	EBV	3
2	F	Yes	NED	2	Yes	No	MRD	60	CMV	4
4	M	Yes	NED	2	Yes	No	NED	89	EBV	2
5	M	Yes	MRD	1	No	No	Overt		EBV	1
6	F	NA	NED	2	Yes	No	NED	59	CMV	4
7	F	No	MRD	1	Yes	No	MRD	33	EBV	1
8	F	Yes	MRD	1	No	No	MRD		EBV	2
9	F	Yes	MRD	1	Yes	No	MRD	52	CMV	4
14	M	Yes	NED	2	Yes	No	MRD	61	EBV	2
15	F	No	NED	2	Yes	No	NED	110	CMV	2
19	F	Yes	NED	1	Yes	No	Overt	243	EBV	1
23	M	Yes	NED	1	Yes	No	NED	246	EBV	1
26	F	Yes	NED	1	Yes	Yes	NED	53	EBV	2
27	F	Yes	NED	1	Yes	Yes	NED	178	EBV	1
28	M	Yes	NED	2	Yes	Yes	NED	31	CMV	2

778 **Table 1 Clinical characteristics of AML patients receiving T_{TCR-C4} infusions.** This table summarizes the clinical data of
 779 patients with AML relapsed/refractory after HCT, who received T_{TCR-C4} infusions.
 780 *AML: Acute Myeloid Leukemia, HCT: Hematopoietic Cell Transplantation, NED: No Evidence of Disease, MRD: Measurable*
 781 *Residual Disease, EBV: Epstein-Barr Virus, CMV: Cytomegalovirus.*
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Figure 1

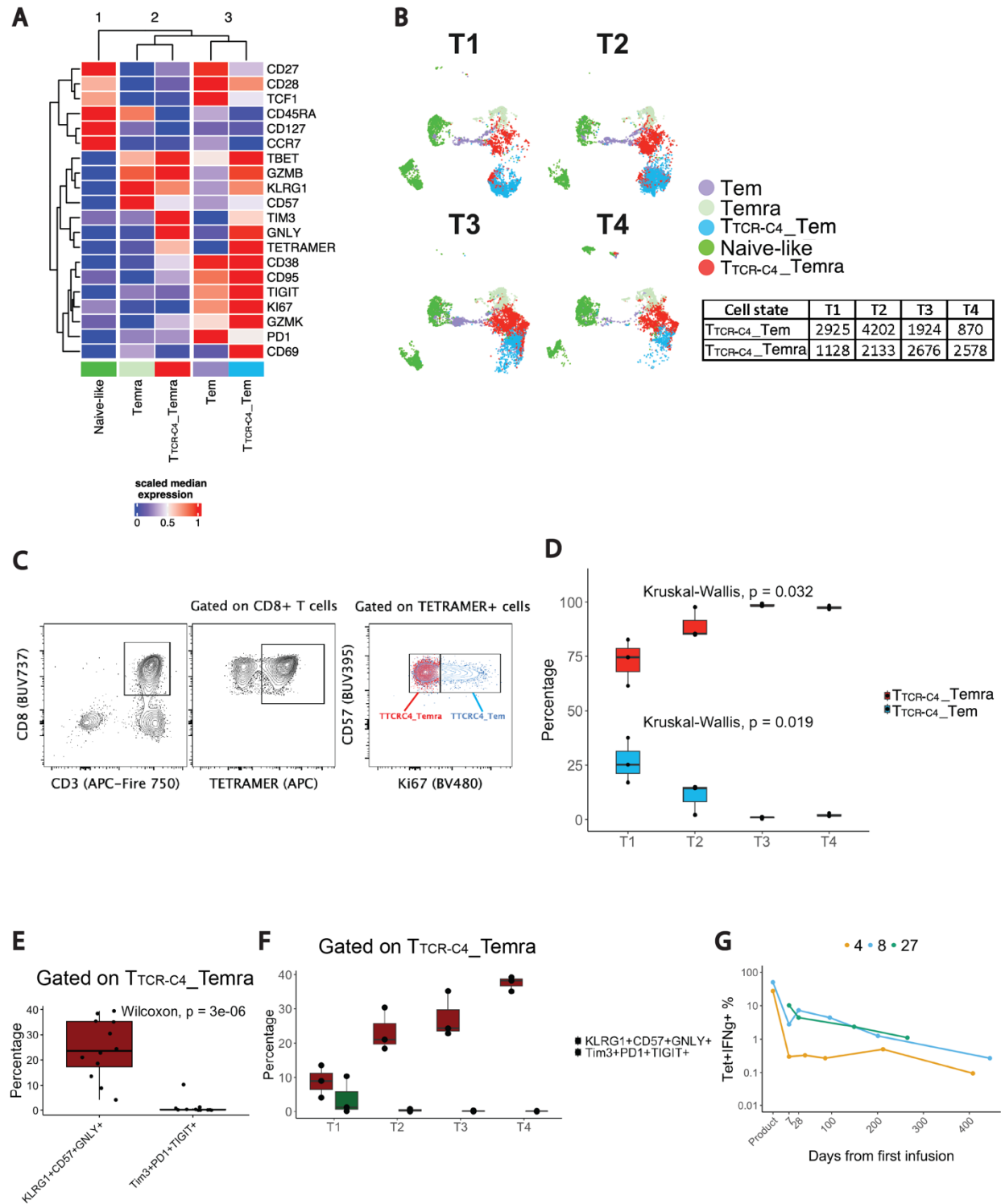


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Fig. 1 Virus and time-dependent terminal differentiation skewing of T_{TCRC4} . (A) Line plot showing the percentage (log scale) of T_{TCRC4} in PBMCs collected after the first T_{TCRC4} infusion for all patients ($n=15$), with data points representing individual samples at different timepoints post-infusion. Each patient is represented by a distinct color, and red arrows indicate the percentage of WT1-specific $CD8^+$ T cells already present at day 0. (B) Boxplots comparing the percentage of T_{TCRC4} (log scale, y-axis) after the first infusion (x-axis), colored by the number of infusions (light blue for ≤ 2 infusions; dark orange > 2 infusions). The

790 horizontal dashed red line indicates the 3% threshold used to define persisting T_{TCRC4} cells. Statistical significance was determined
791 using Kruskal-Wallis test, with $p < 0.05$ considered significant. **(C)** Line plot illustrating the percentage of T_{TCRC4} (y-axis) cells
792 derived from EBV-specific substrate cells (dark orange) or CMV-specific substrate cells (dark violet) over a 28-day period (x-axis)
793 after the first infusion. Statistical significance was determined using Wilcoxon Rank Sum test. (* $p < 0.05$, ** $p < 0.01$) **(D)** Boxplots
794 showing differential abundance analysis of CMV-specific (dark violet) and EBV-specific (dark orange) CD8⁺ T-cell subsets derived
795 from the analysis of mass cytometry data.²⁹ CD8⁺ T-cell subsets are defined based on marker co-expression showed in Extended
796 data Fig. 7B. A generalized linear model was used to compute differential cluster abundance between the two conditions (CMV
797 vs. EBV), with adjusted p-values (threshold for significance of $p < 0.05$) displayed above each comparison.
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Figure 2

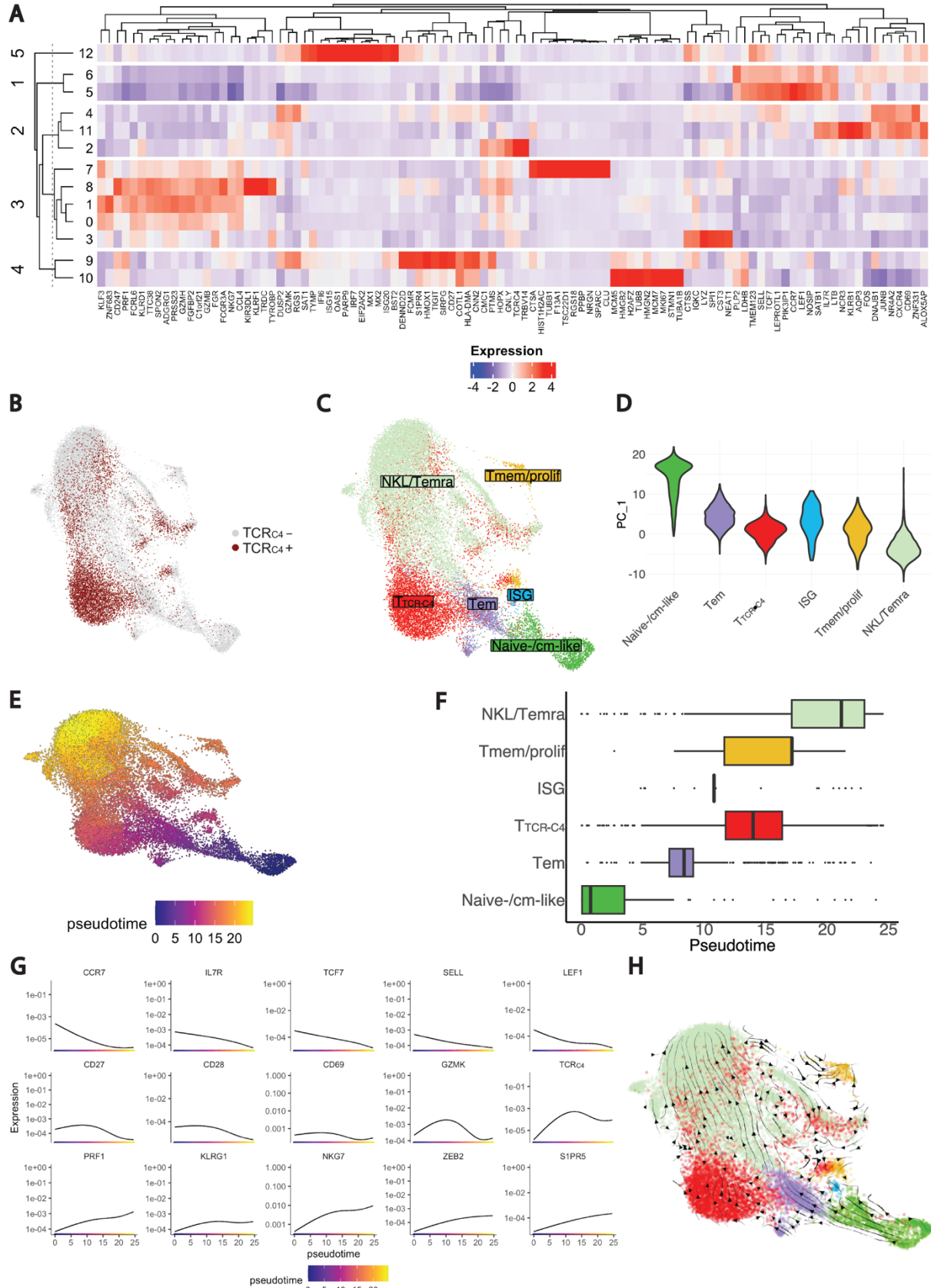


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Fig. 2 CD8⁺ T cell subset phenotypes and functional states over time (A) Heatmap showing fluorescence intensity values of 20 markers across five CD8⁺ PB subsets annotated as Naïve-like (CD45RA, CCR7, CD27, CD28), Tem (CD28, CD27, Ki67, CD38, TIGIT, PD1, Tbet), T_{TTCRC4}_Tem (TETRAMER, CD28, Ki67, CD38, TIGIT, GZMB, Tbet, KLRG1, GNLY), Temra (CD45RA, CD57, KLRG1, GZMB, Tbet), T_{TTCRC4}_Temra (TETRAMER, GNLY, KLRG1, TIM3, GZMB, Tbet). The median marker expression identifies the markers that characterize each subset with red indicating high expression and blue low. The data were scaled after

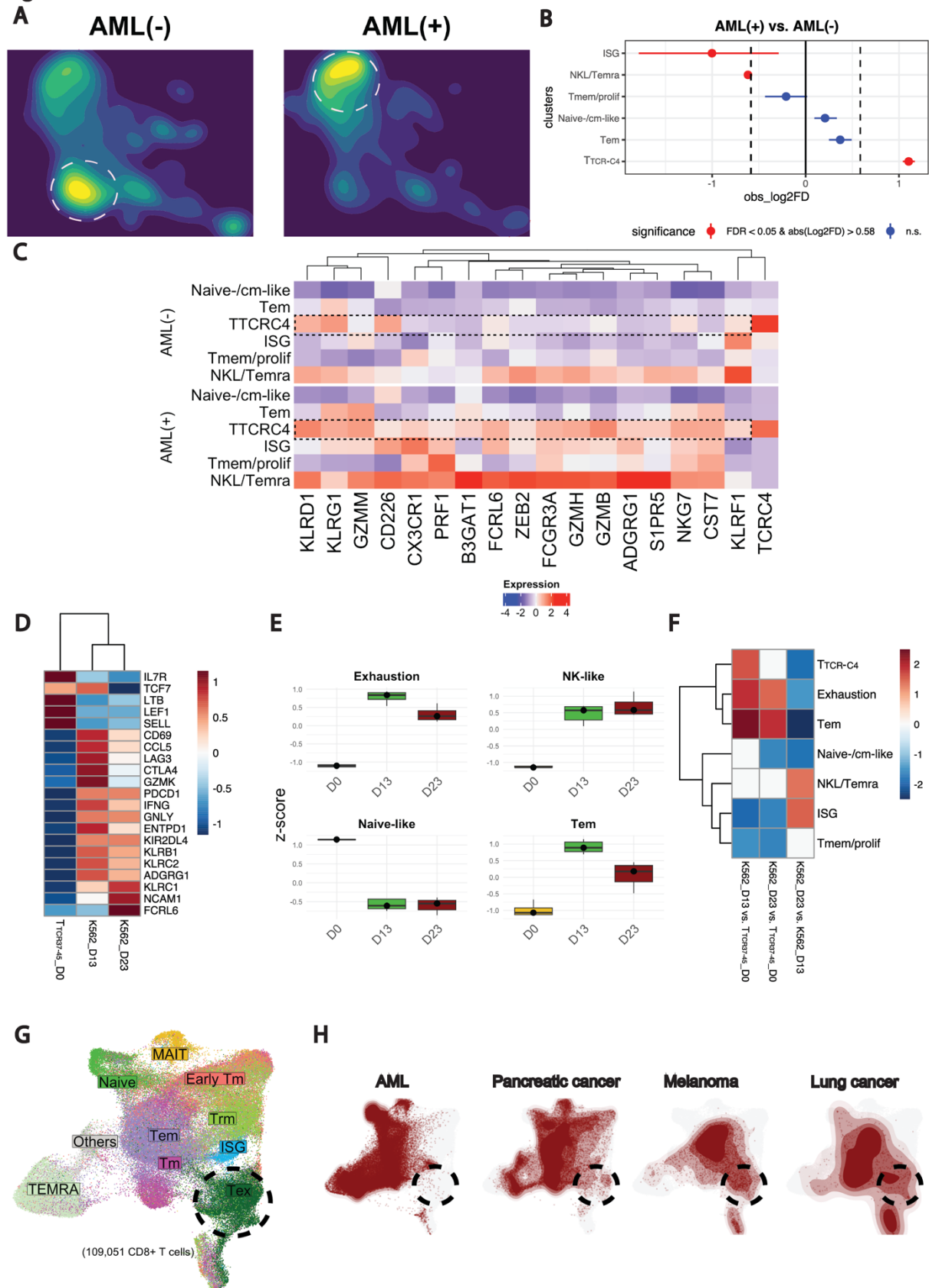
805 aggregation to highlight population-level differences between tetramer⁺ and tetramer⁻ samples, ensuring a clear visualization of
806 marker expression patterns across subsets. Hierarchical clustering on the left dendrogram groups markers based on their expression
807 patterns, revealing co-occurring markers across subsets, while the top dendrogram shows relationships between subsets based on
808 their marker profiles, revealing similarities and differences. The K-means algorithm was applied to further group the clusters by
809 similarity into three groups marked by the numbers (1,2,3) at the top of the heatmap. Data were derived from spectral flow-
810 cytometry analysis. **(B)** UMAP plots showing the two-dimensional distribution of the annotated CD8⁺ T-cell subsets, with the plots
811 colored according to the subset annotation and split by timepoint. The table shows the absolute numbers of T_{TCR-C4_Tem} and T<sub>TCR-
812 C4_Temra</sub> cells at each timepoint (T1, T2, T3, T4). **(C)** Contour plots illustrating the gating strategy for T_{TCR-C4_Tem} (blue) and
813 T_{TCR-C4_Temra} (red). This strategy starts by identifying the CD3⁺CD8⁺ cell population, followed by selecting tetramer⁺ CD8⁺ cells.
814 Cells are then gated based on Ki67 expression, with Ki67⁻ cells (red) classified as T_{TCR-C4_Temra} and Ki67⁺ cells (blue) as T<sub>TCR-
815 C4_Tem</sub>. This gating strategy is based on the marker expression shown in the heatmap in Fig. 2A. **(D)** Boxplots depicting the
816 percentage (y-axis) of T_{TCRC4_Tem} and T_{TCRC4_Temra} populations identified in Fig. 2C over time (x-axis, from T1 to T4). Statistical
817 significance was assessed using Kruskal-Wallis test (threshold for significance: p < 0.05). **(E)** Boxplot showing the percentage (y-
818 axis) of cytotoxic KLRG1⁺, CD57⁺, GNLY⁺ (dark red) compared to exhausted TIM3⁺, PD1⁺, TIGIT⁺ (dark green) T cells among
819 T_{TCRC4_Temra}. Statistical significance was assessed using the Wilcoxon rank-sum test, with a significance threshold of p < 0.05.
820 **(F)** Boxplots displaying the percentage (y-axis) of cytotoxic (dark red) compared to exhausted (dark green) T cells among
821 T_{TCRC4_Temra} over time (x-axis, T1 to T4). **(G)** Line plot showing the T_{TCRC4}⁺IFNγ⁺ cells following WT1-peptide stimulation. The
822 x axis represents the days from the first infusion, while the y axis indicates the percentage of tetramer⁺IFNγ⁺ cells (log10 scale)
823 gated on CD8⁺ T cells.
824

Figure 3



826 **Fig. 3 Single cell transcriptomic analysis of endogenous, T_{TCRC4} CD8⁺ T-cell states and their differentiation dynamics. (A)**
827 Heatmap showing the top 10 differentially expressed genes identified within each cluster. The “top 10” refers to the 10 genes with
828 the most significant differential expression across the identified clusters. The dendrogram on the left displays the similarity between
829 the 13 clusters, which were determined through unsupervised clustering based on gene co-expression patterns. The top dendrogram
830 shows the relationships between the genes based on their expression patterns. To further refine this clusters, a K-means algorithm
831 was applied to group the 13 clusters into 5 main categories, labeled 1 to 5 on the left side of the heatmap. Blue represents low gene
832 expression, while red indicates high gene expression. **(B)** UMAP plot illustrating the distribution of CD8⁺ T cells (endogenous and
833 T_{TCRC4}⁺) on a two-dimensional space. T_{TCRC4}⁺ cells were identified using scGate,³⁴ an R package which scores cells based on
834 TCR_{C4} expression and defines thresholds to classify cells as positive or negative for the population of interest. TCR_{C4}⁺ cells are
835 colored in dark red, while TCR_{C4}⁻ cells (endogenous) are represented in light grey. **(C)** UMAP plot displaying the two-dimensional
836 distribution of annotated CD8⁺ T-cell transcriptional states, colored by subset. **(D)** Violin plot illustrating the distribution of CD8⁺
837 T-cell subsets identified through gene expression (Fig. 3A) and TCR_{C4} score (Fig. 3B) along the principal component 1 (PC_1)
838 axis. Each violin represents a different CD8⁺ T-cell state. The proximity of each subset along PC_1 (y-axis) indicates transcriptional
839 similarity, with subsets closer together showing more similar gene expression profiles **(E)** UMAP plot illustrating the inferred
840 developmental trajectory of CD8⁺ T-cell transcriptional states, as predicted by Monocle.⁷⁹ The color scale, ranging from blu to
841 orange/yellow, represents the pseudotime, where blue indicates earlier developmental stages and orange/yellow indicates later
842 stages of the trajectory. **(F)** Boxplot showing the distribution of the 5 CD8⁺ T-cell subsets (y-axis) along the pseudotime (x-axis).
843 The position of the boxes along the pseudotime axis provides context for how the subsets are positioned within the developmental
844 trajectory, with subsets at lower pseudotime values representing earlier stages of differentiation, while those at higher pseudotime
845 values correspond to later stages. **(G)** Line plots displaying the smoothed gene expression of selected genes characterizing the 5
846 CD8⁺ T-cell subsets along the pseudotime. These plots illustrate the dynamic changes in gene expression (y-axis) as cells progress
847 along the inferred trajectory (x-axis). The smoothed curves show how the expression of each gene varies at different pseudotime
848 points. **(H)** UMAP plot colored by the annotated CD8⁺ subsets overlaid with the predicted velocity stream computed through
849 scVelo.⁴¹ The velocity streams represent the predicted direction and magnitude of gene expression changes for each individual cell,
850 providing insights into the dynamic transitions between cellular states. These streams highlight the likely trajectories cells follow
851 as they evolve over time, offering a predictive view of future cellular states based on current transcriptional dynamics.
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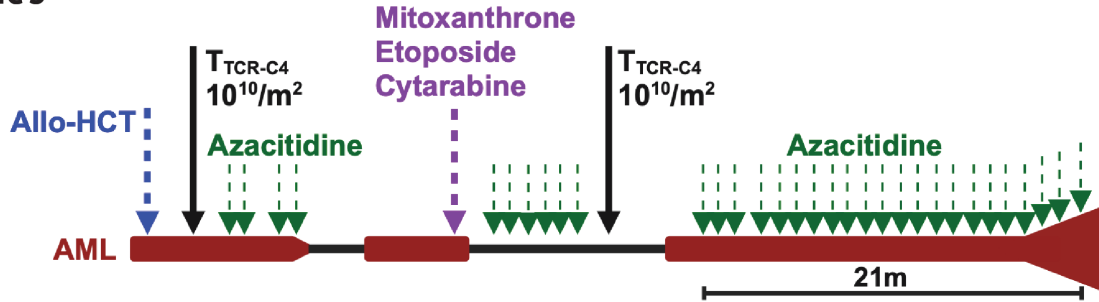
Figure 4



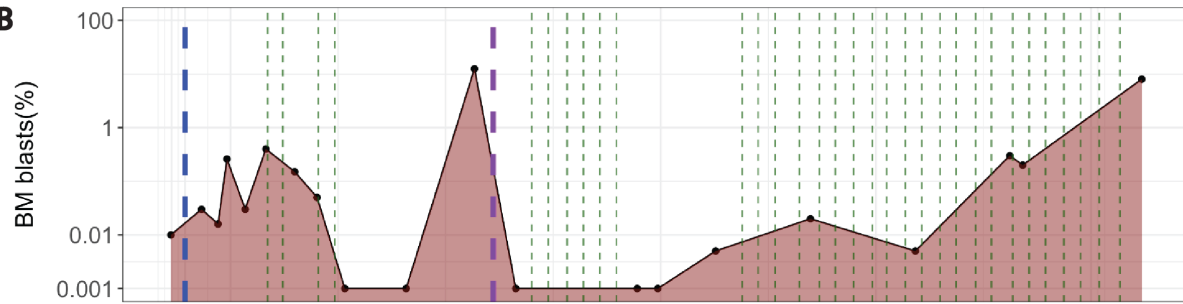
854 **Fig. 4 AML induces T_{TCRC4} NKL/Temra differentiation skewing.** (A) UMAP plot of CD8⁺ T cells colored by density and split
855 by group (AML(-) and AML(+)). AML(+) samples are those in which leukemic cells were detected in BM or PB, while AML(-)
856 samples lack detectable leukemic cells. The plot shows the two-dimensional distribution of CD8⁺ T cells, with color intensity
857 representing cell density, where blue indicates lower density and yellow indicates higher density. Dashed lines highlight areas of
858 highest cell density in both AML(+) and AML(-) groups. (B) Point-range plot showing the pairwise (AML(+) vs. AML(-))
859 proportional difference for each CD8⁺ T-cell subset. Colors indicate the statistical significance (red: FDR < 0.05, blue: FDR ≥
860 0.05); vertical dashed lines mark the absolute value of log2FD cutoff for significance. (C) Heatmap displaying DGE of NKL genes
861 across the CD8⁺ T-cell subsets split horizontally by AML(-) vs. AML(+), with each condition showing the gene expression patterns
862 for CD8⁺ T-cell subsets. Blue indicates low expression, while red indicates high expression. Dashed boxes highlight NKL/Temra
863 genes, emphasizing their relative expression across the two conditions. (D) Heatmap depicting significant DEGs across three
864 conditions: T_{TCR37-45_D0} (T_{TCR37-45}-only), K562_D13 (T_{TCR37-45} T cells after 13 days of coculture with K562 AML cell line),
865 K562_D23 (T_{TCR37-45} T cells after 23 days of coculture with K562 AML cell line). Significance was determined using the Likelihood
866 Ratio Test in DESeq2,⁸⁰ with genes filtered for adjusted p-value (FDR < 0.05). Color intensity represents relative gene expression,
867 with blue indicating low expression and red indicating high expression. (E) Boxplots illustrating the z-score of Exhaustion, NK-
868 like, Naïve-like and Tem signatures across the three conditions (co-culture timepoints). (F) Heatmap illustrating the enrichment of
869 scRNAseq-derived (TTCRC4, Tem, Naïve.like, NKL.Temra, ISG, Tmem.prolif) and manually curated exhaustion markers across
870 three conditions: K562_D13 vs. T_{TCR37-45_D0}, K562_D23 vs. T_{TCR37-45_D0}, K562_D23 vs. K562_D13. The signatures shown are
871 derived from the top 50 DEGs for each subset from scRNAseq dataset. Additionally, a manually curated exhaustion signature is
872 included. Each row represents the relative enrichment of these gene signatures in the comparison of interest, while the color
873 intensity indicates the degree of enrichment: red indicates high enrichment, while blue indicates low enrichment. The comparison
874 group correspond to timepoint during coculture with the K562 cell line. T_{TCR37-45} represents the baseline (T_{TCR37-45}-only condition)
875 (G) UMAP plot showing the distribution of CD8⁺ T-cell states from a published independent dataset used as reference atlas.³⁵
876 Dashed line indicates the UMAP coordinates of Tex CD8⁺ T-cell subset. (H) UMAP plot of CD8⁺ T-cell reference atlas (light grey)
877 overlaid with projections (dark red) of CD8⁺ T cells collected from various independent published scRNAseq datasets (queries) of
878 AML^{20,22,23,44,45}, pancreatic cancer,⁴⁶, melanoma,⁴⁷ and lung cancer.⁴⁸ Dashed line indicates the area corresponding to Tex.
879

Figure 5

A



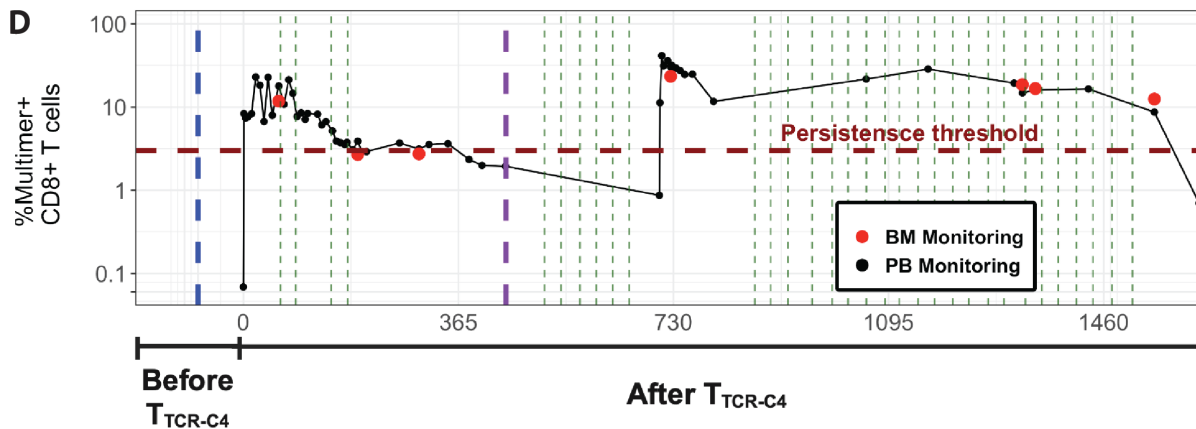
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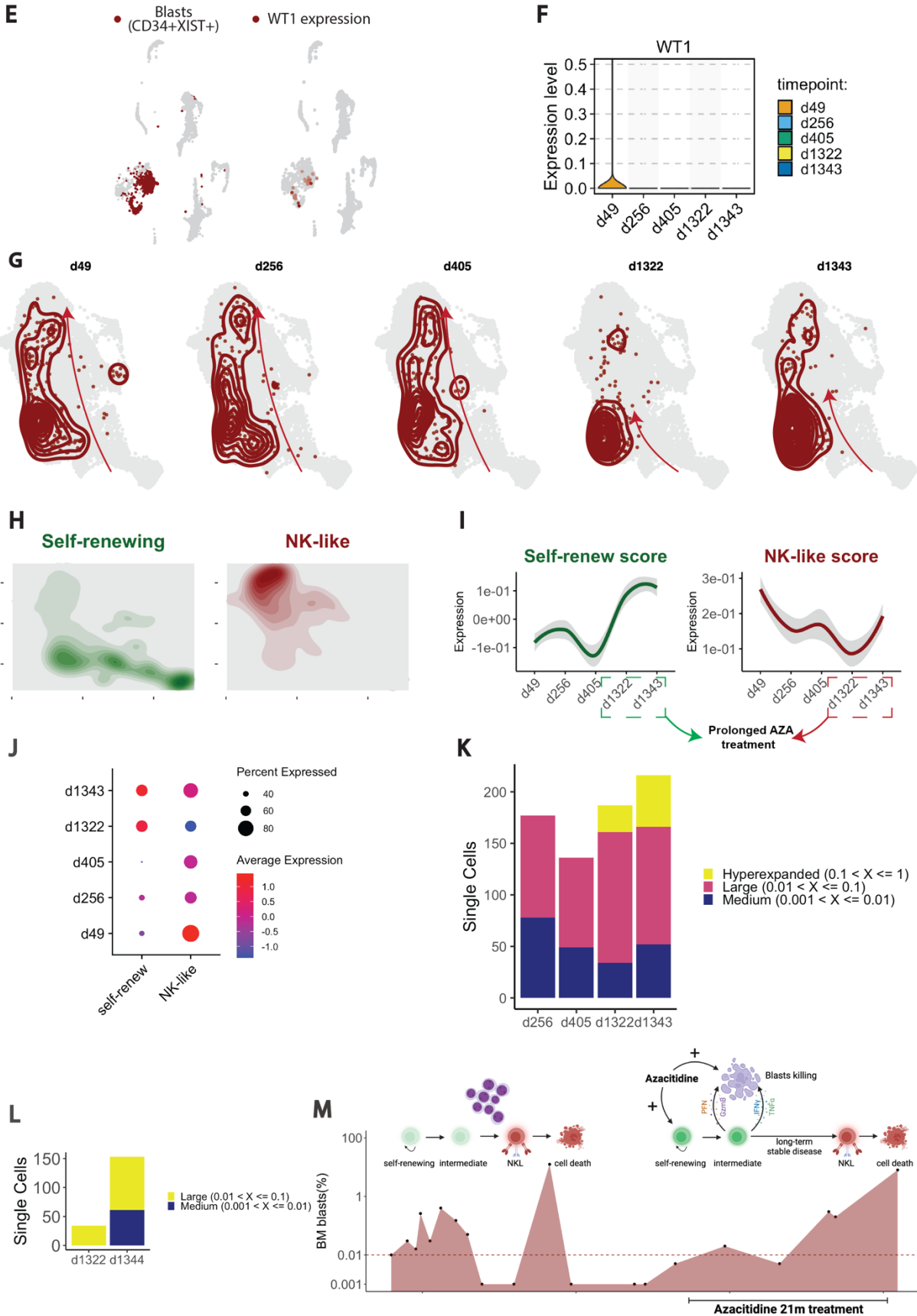
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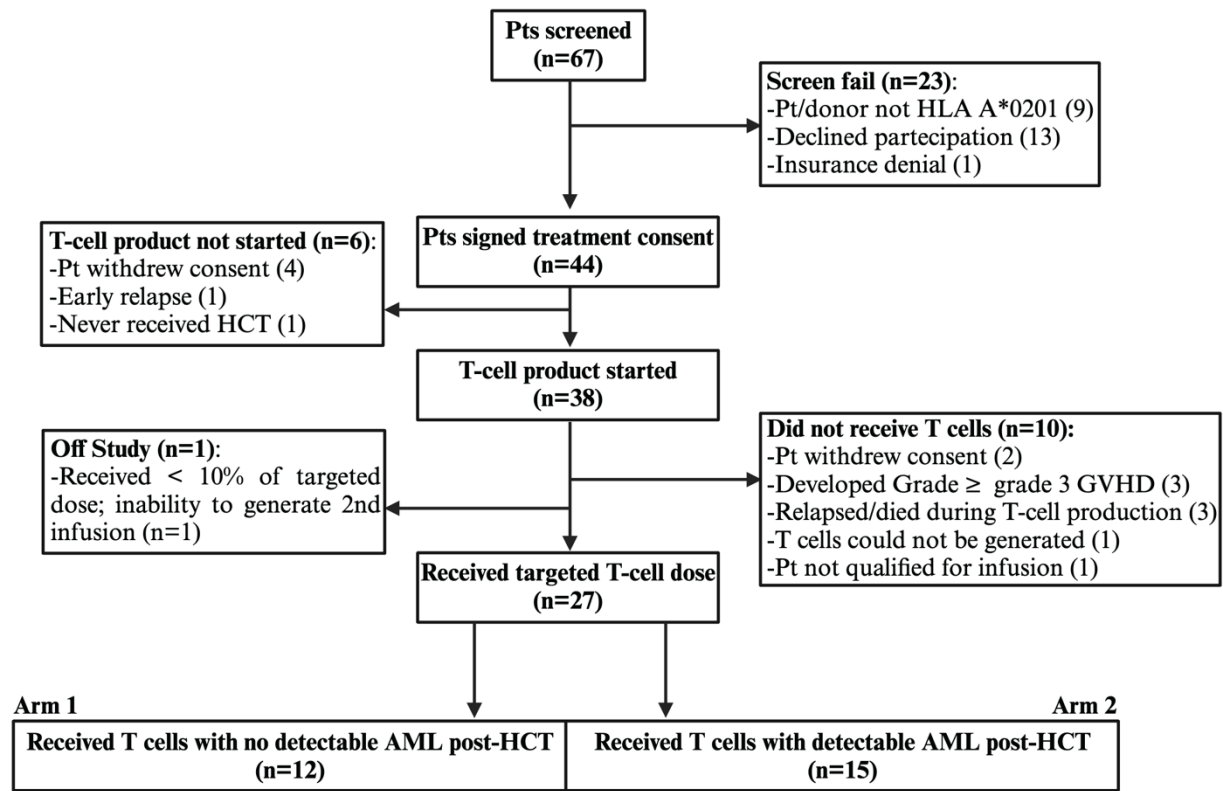


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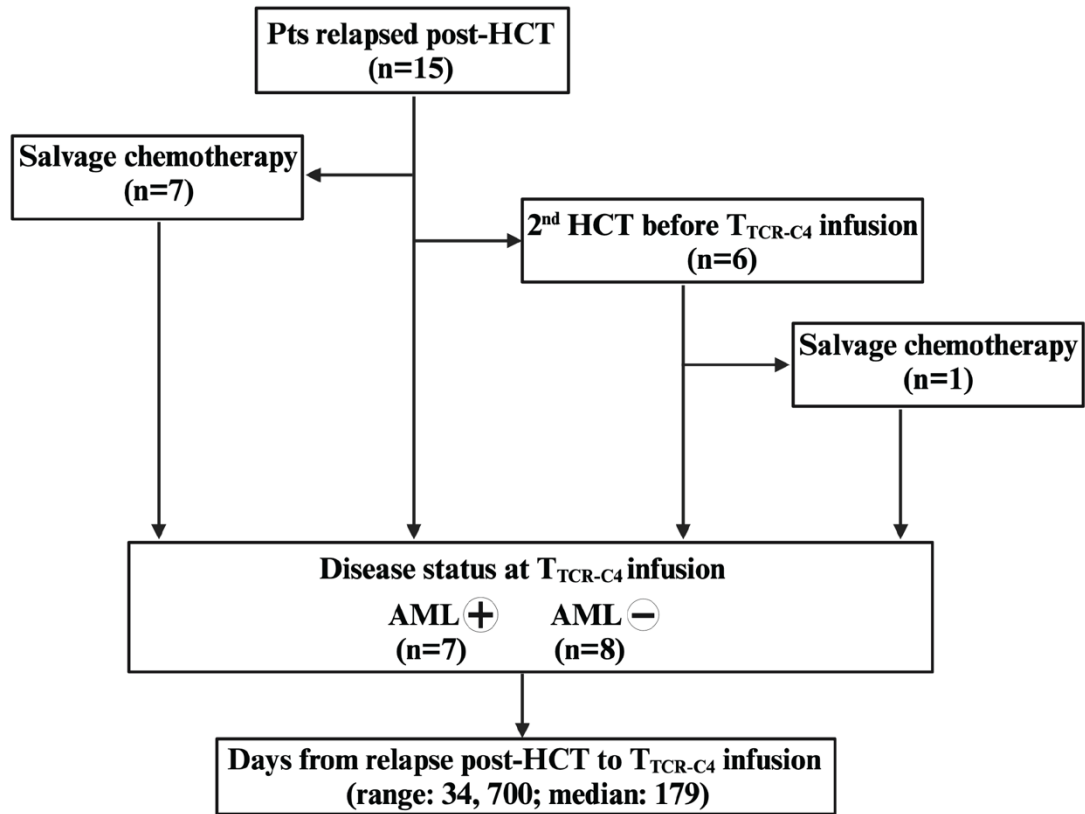
882 **Fig. 5 Azacitidine as a potential boost for T_{TCR-C4} *in vivo* function and persistence (A)** Timeline of patient's treatment regimen.
883 Dark red highlights timeframes with detectable BM AML (B) Percent of BM AML blasts (y-axis, log scale) by multiparametric
884 flow cytometry (MFC) at specific timepoints (black dots, dark red shaded area). The horizontal dashed line represents the sensitivity
885 limit of MFC. (C) Multimer⁺ cells/ μ l and (D) Percent multimer⁺ of CD8⁺ T cells in PB (black dots) and BM (red dots) collected
886 before and after T_{TCR-C4} infusions. The red arrow indicates the lack of T_{TCR-C4} persistence before the start of Azacitidine (E) UMAP
887 plots showing a blast score (dark red) calculated based on the co-expression of *CD34* and *XIST* (a female-specific gene) in a patient
888 previously transplanted with a sex-mismatched donor (female patient, male donor). The score was used to identify cells expressing
889 a high blast score. Additionally, *WT1* expression across all cell populations is shown. Cells with high expression of each marker
890 are highlighted in red, while other cells appear in grey. (F) Violin plot of the *WT1* expression (y-axis) across the timepoints analyzed
891 after first infusion of T_{TCR-C4} (x-axis, d265, d405, d1322, d1343). Violin plots are colored by timepoint. (G) UMAP plots display
892 the distribution of total PB CD8⁺ T cells from the scRNAseq dataset, with the T_{TCR-C4} subset from patient 8 highlighted in dark red.
893 Single-cell points represent individual cells, while kernel density contours (also in dark red) depict the density of T_{TCR-C4} cells
894 within the CD8⁺ T cell landscape. The plots are stratified by timepoints post-infusion: (day 49, day 256, day 405, day 1322, and
895 day 1343). This series illustrates the spatiotemporal dynamics of the T_{TCR-C4} subset, showing its distribution in relation to the
896 broader PB CD8⁺ T cell population and highlighting potential transcriptional changes over time. Red arrows were added manually
897 to indicate the different skewing of T_{TCR-C4} across the timepoints examined. (H) UMAP plots showing the distribution of PB CD8⁺
898 T cells, with the density contours highlighting cells with high self-renewing (dark green) and NK-like (dark red) scores. The self-
899 renewing score was calculated based on a set of stem-like/survival genes (*TCF7*, *LEF1*, *SELL*, *CCR7*, *BCL2*, *IL7R*, *CD27*, *CD28*)
900 and the NK-like score was based on NK-associated genes (*ZEB2*, *SIPR5*, *CX3CR1*, *KLRG1*, *NKG7*, *FCRL6*, *KLRD1*, *ADGRG1*).
901 The plots are color-coded by score (light grey for the background, darker shades indicating higher scores). Cells with scores above
902 the 75th percentile for each gene set are highlighted with density in dark green for high self-renewing score cells and dark red for
903 high NK-like score cells. (I) The plot shows the temporal changes in the self-renew (left) and NK-like (right) scores of T_{TCR-C4}
904 from patient 8. The smoothed trends of the scores were calculated using LOESS regression at multiple timepoints. The dark green
905 (self-renew) and dark red (NK-like) lines represent the smoothed score trends, with shaded areas indicating the confidence interval.
906 The y-axis reflects the score expression values. (J) Dot plot displaying the expression of the self-renew and NK-like scores across
907 timepoints post-infusion. The size of each dot corresponds to the expression level of the respective score and the color indicates
908 the relative intensity of the expression. (K) Stacked plot showing cell count of T_{TCR-C4} in specific clonal frequency ranges over time
909 (d256, d405, d1322, d1343). Colors indicate the clonal frequencies. (L) Stacked plot showing cell count for BM T_{TCR-C4} within
910 specific clonal frequency ranges at d1322 and d1343. Colors indicate the clonal frequencies. (M) Cartoon (created with Biorender)
911 showing the influence of blasts and azacitidine on T_{TCR-C4} . The red area under the curve represents the blasts percentage (y-axis)
912 over time (x-axis). Blasts induce T_{TCR-C4} skewing towards NKL and cell death. However, prolonged exposure to azacitidine allows
913 T_{TCR-C4} to maintain self-renewal, facilitating persistence and long-term disease control.

Extended data Fig. 1



Extended data Fig. 1: Flow diagram of patients enrolled on the clinical study. Follow-up of the 67 patients screened for participation in the study and the 44 patients who signed the informed consent. Patients with no detectable disease post-HCT were assigned to the Prophylactic Arm¹, while those with evidence of disease post-HCT were treated in the Treatment Arm described here. A total of 44 pt/donor pairs were enrolled pre-HCT, TCR_{C4} transduced cells were initiated for 38 patients, among whom 12 disease-free (Arm 1) and 15 relapsed/refractory (Arm 2) patients received TCR_{C4} transduced cells. An additional patient was considered not evaluable for response outcomes as this patient received <10 % of the targeted dose and was unable to receive further infusions due to inability to generate additional T cells from the donor.

Extended data Fig. 2

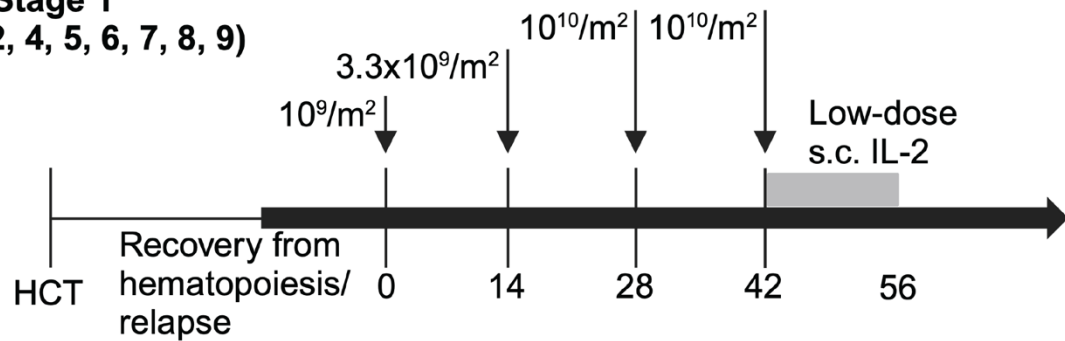


Extended data Fig. 2: Flow diagram of patients' treatment history. The cohort included 15 patients who relapsed after HCT. 7 patients received salvage chemotherapy before the infusion of T_{TCR-C4}, while 6 patients had two HCTs before the infusion, and 1 patient among them also received salvage chemotherapy. At the time of TCR-C4 infusion, 7 patients had detectable AML, while 8 patients were in remission (AML-negative). The time from relapse post-HCT to TCR-C4 infusion ranged from 34 to 700 days, with a median of 179 days.

Extended data Fig. 3

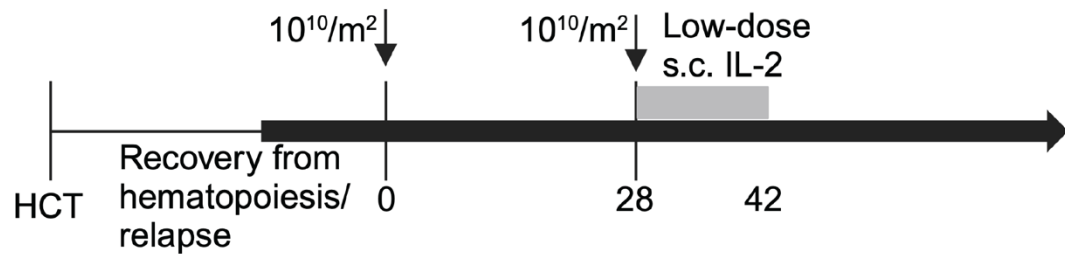
Arm 2, Stage 1

(Pts 1, 2, 4, 5, 6, 7, 8, 9)



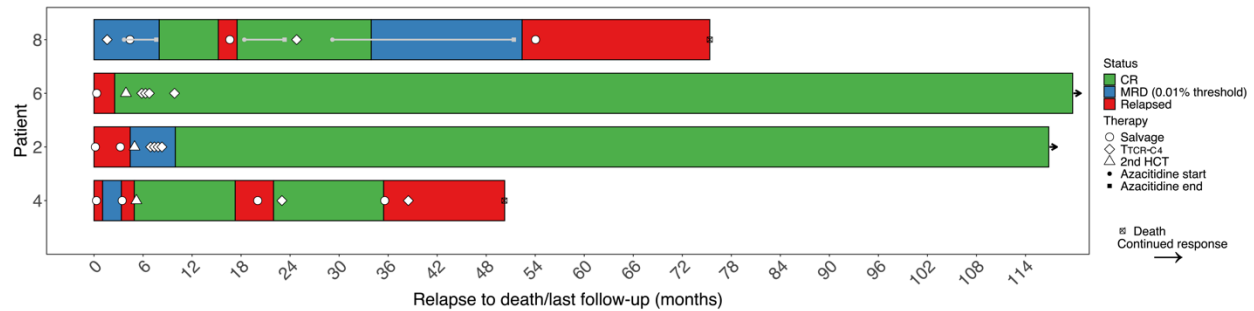
Arm2, Stage 2

(Pts 14, 15, 19, 23, 26, 27, 28)



Extended data Fig. 3. Study schema. The first 8 patients who received T_{TCR-C4} after post-HCT relapse (Treatment Arm, Stage1) received 4 escalating infusions of 10^9 - $10^{10}/m^2$ 14 days apart, the last infusion followed by s.c. low-dose IL2 for 14 days. After safety of the highest dose was established, all subsequent patients received 2 infusions of $10^{10}/m^2$ T_{TCR-C4} 28 days apart, the second infusion followed by s.c. low-dose IL2 for 14 days.

Extended data Fig. 4



Extended data Fig. 4. Swimmer Plot of Patient Responses.

The swimmer plot illustrates the response duration for patients enrolled in the trial. Time 0 represents the relapse or persistent disease after the first HCT that qualified patients for trial inclusion. The end of each bar indicates the date of death or last follow-up, with arrows marking ongoing responses. "Salvage" denotes any salvage strategy, including chemotherapy, radiotherapy, or intrathecal treatment. Salvage with a second HCT is marked by a triangle.

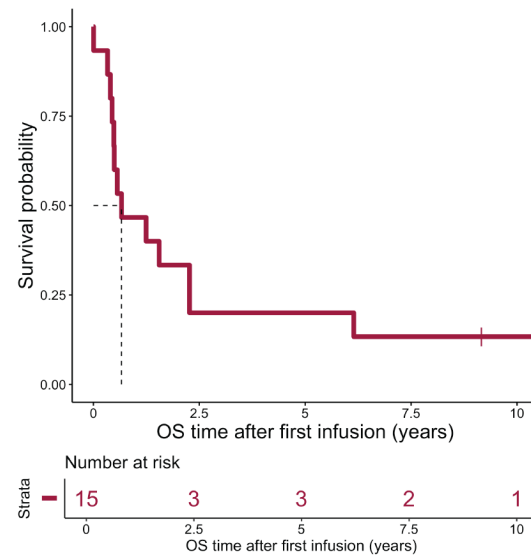
Patient 8: A patient who had MRD post HCT and received T_{TCR-C4} with MRD. She later underwent azacitidine salvage treatment for persistent MRD (duration shown as a gray line). The patient then relapsed, underwent additional salvage chemotherapy, azacitidine, and received a second T_{TCR-C4} infusion. Following this, she experienced prolonged MRD+ stable disease with detectable long-term persisting PB T_{TCR-C4}. Eventually, the disease progressed, and she relapsed and died (see **Results** for more details).

Patient 6: A patient who received salvage chemotherapy followed by a second HCT which resulted in no evaluable disease. Subsequently, she received four T_{TCR-C4} infusions. The patient was alive at the last follow-up.

Patient 2: A patient who, after salvage treatment, underwent a second HCT while in overt disease. She remained MRD-positive 28 days post-second-HCT. She received four T_{TCR-C4} infusions. Eventually, she achieved a MRD negative status. The patient was alive at the last follow-up.

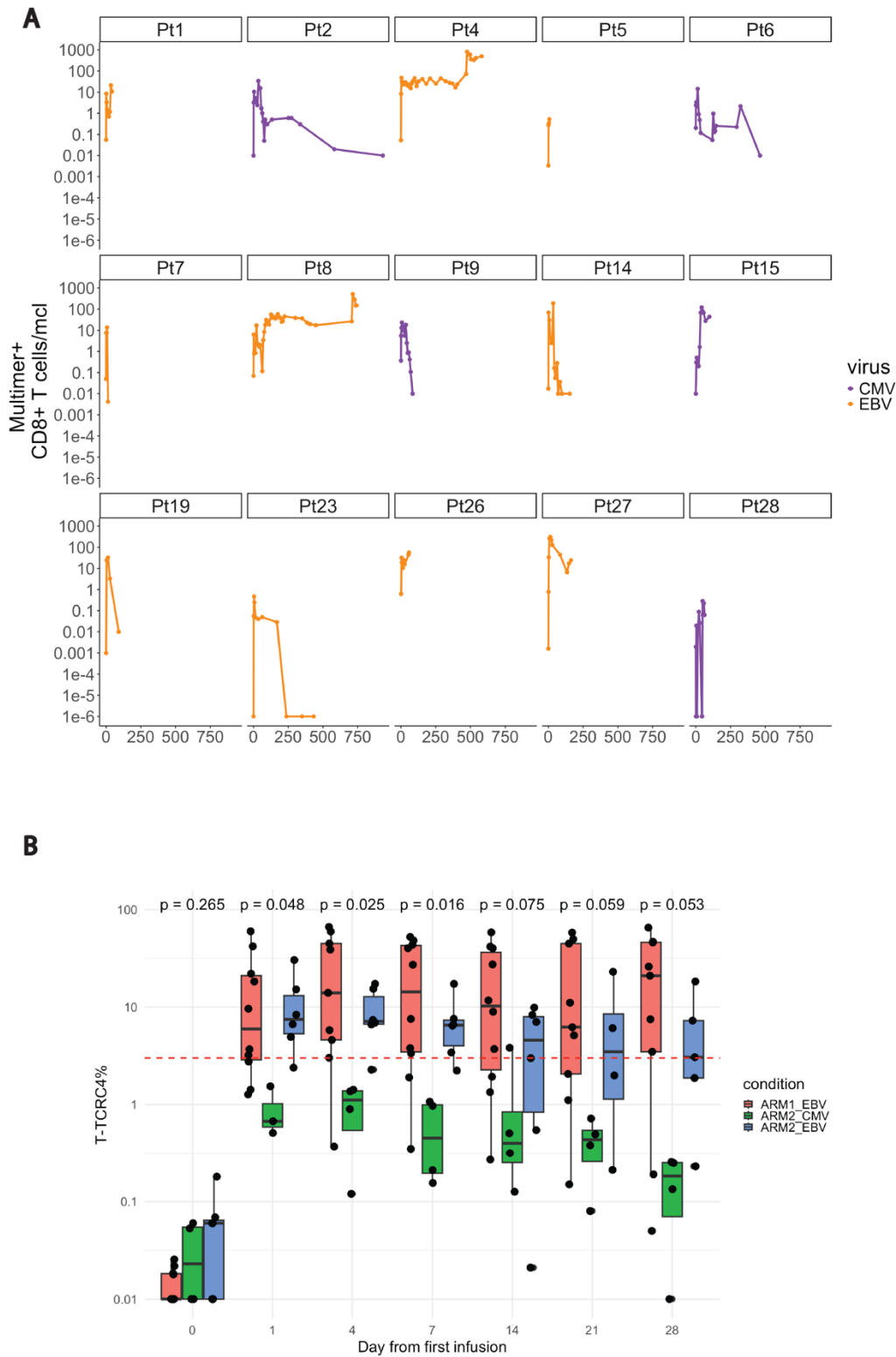
Patient 4: A patient who achieved a complete remission after a second HCT. However, after ~15 months, he experienced extramedullary relapse, treated with azacitidine and radiotherapy which achieved no evaluable disease. He maintained remission for ~1 year after the first T_{TCR-C4} infusion. Subsequently, he relapsed extramedullary and was refractory to an additional T_{TCR-C4} infusion. Further details on this case and mechanisms of AML escape have been described previously.²

Extended data Fig. 5



Extended data Fig. 5. Overall survival of AML patients treated with T_{TCRC4} T cells. Kaplan Meier estimate of overall survival (OS) of 15 AML patients with evidence of disease post-HCT, who were subsequently treated with T_{TCRC4} T cells

Extended data Fig. 6

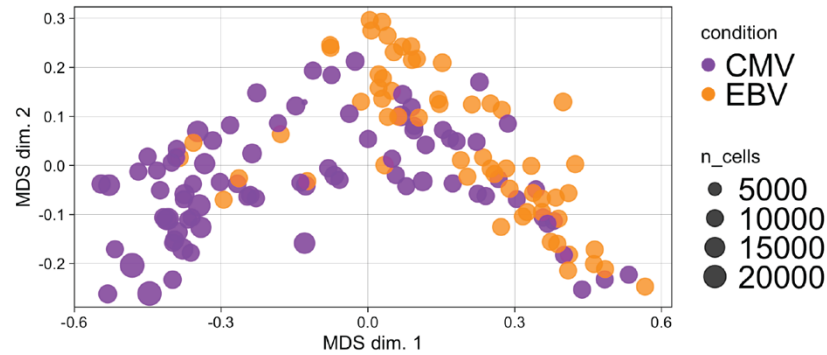


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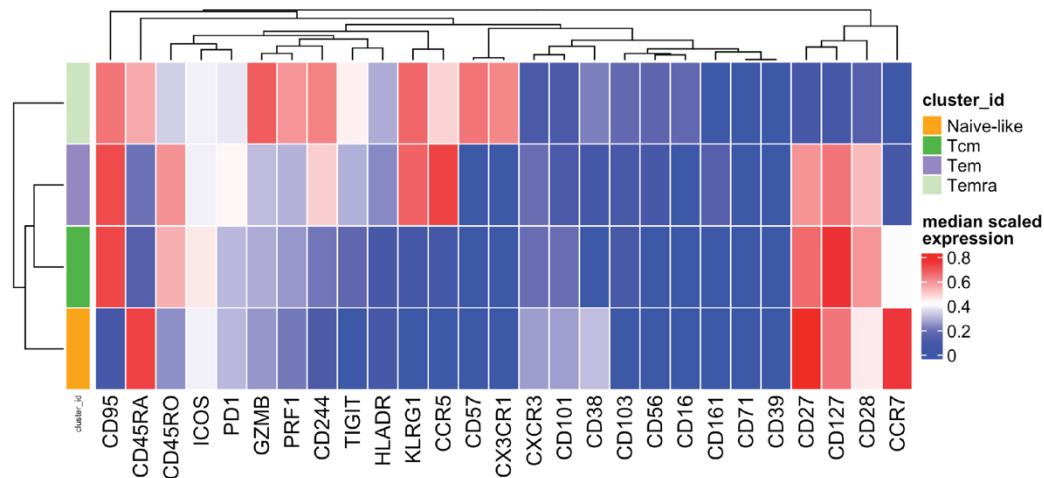
Boxplots comparing the percentage of EBV-specific T_{TCR-C4} cells in Arm 1 (red) and Arm 2 (blue) with the percentage of CMV-specific T_{TCR-C4} cells in Arm 2 (green) across post-infusion timepoints. The y-axis indicates the percentage (log scale) of T_{TCR-C4} cells, while the x-axis represents the time post-infusion. Data were derived from flow-cytometry analysis. The horizontal dashed red line indicates the 3% threshold used to define persisting T_{TCR-C4} cells. The y-axis is displayed on a log₁₀ scale. Statistical comparison between all three groups at each time point was performed using a Kruskal-Wallis test, with p-values displayed above the corresponding boxplots. Statistical significance was defined as $p < 0.05$.

Extended data Fig. 7

A

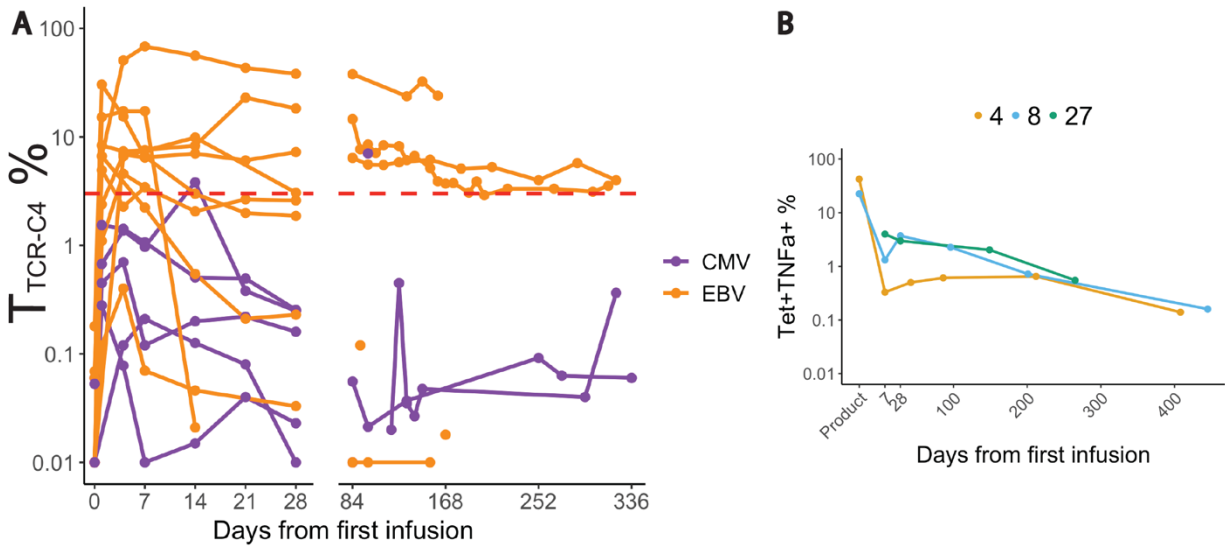


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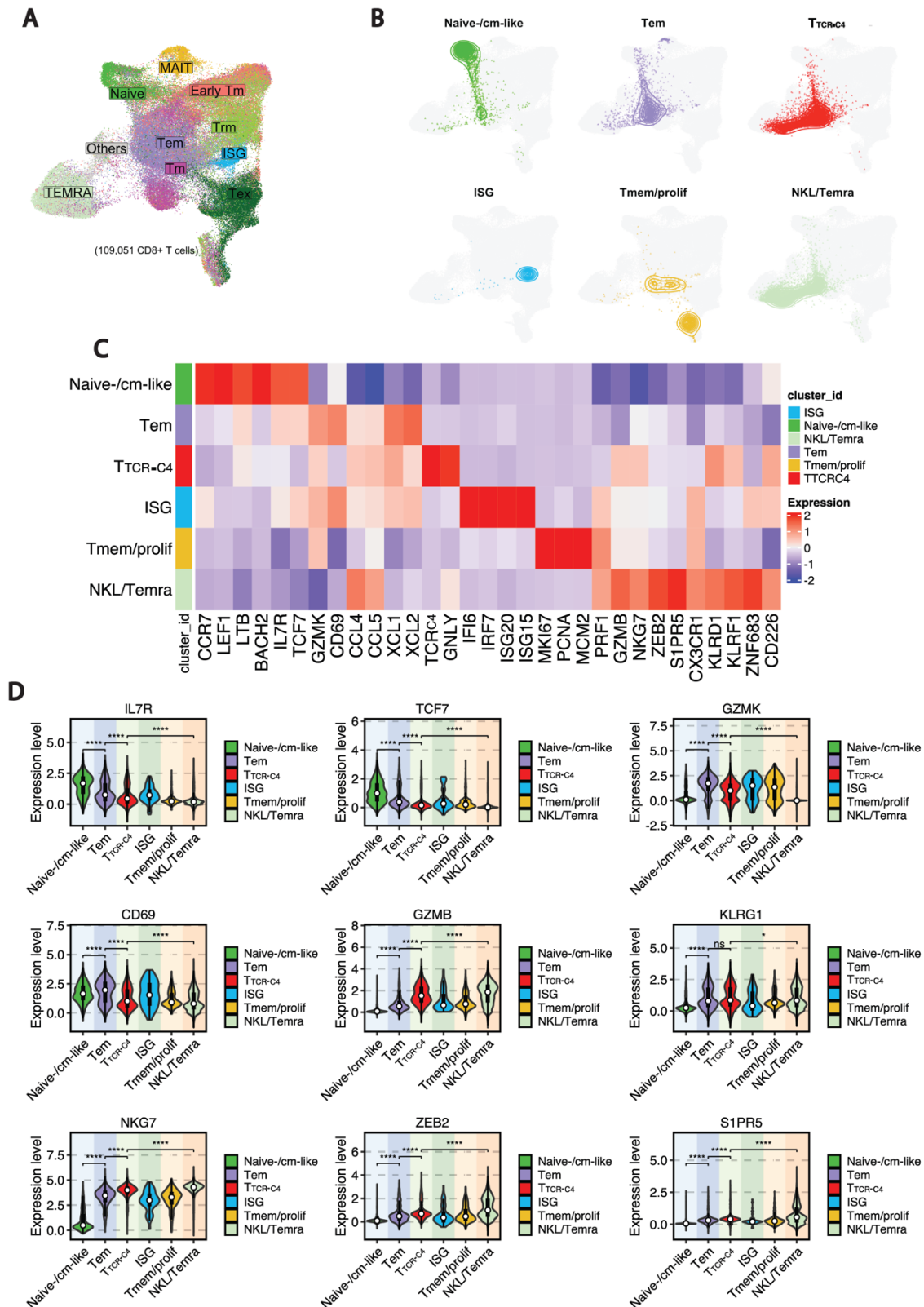
Extended data Fig. 7. EBV and CMV specific CD8⁺ T-cell states. (A) Multidimensional (MDS) scaling plot illustrates the distribution of samples based on the expression of top varying markers, which are those markers showing the most variation in expression levels across the dataset. These markers are selected for their ability to highlight meaningful differences between samples or conditions. In this context, samples associated with CMV (violet dots) and EBV (orange dots) are plotted along the first two MDS dimensions (MDS dim. 1, MDS dim. 2). This unsupervised method visualizes sample clustering based on marker expression, with dissimilarities calculated using the median expression of markers across all cells in each sample. The plot provides an overview of variance and potential clustering patterns among the samples. (B) Heatmap illustrating the median expression levels of 27 markers across four PB CD8⁺ T cell subsets: Naïve-like (CD95⁻, CD45RA⁺, CCR7⁺, CD27⁺, CD28⁺), Effector Memory (Tem) (CD95⁺, CD45RA⁻, CD45RO⁺, CD27⁺, CD28⁺, CD127⁺), Central Memory (Tcm) (CD95⁺, CD45RA⁻, CD45RO⁺, CCR7⁺, CD27⁺, CD28⁺, CD127⁺), and Effector Memory RA⁺ (Temra) (CD95⁺, CD45RA⁺, CD45RO⁻, CD57⁺, KLRG1⁺). The data are scaled to normalize marker expression across the dataset before clustering, ensuring each marker contributes proportionally to cluster definitions while minimizing the impact of extreme values or outliers. Blue represents lower expression, and red indicates higher expression. Hierarchical clustering on the top dendrogram groups markers based on their expression patterns, revealing co-occurring markers across subsets, while the clustering on the left shows relationships between subsets based on their marker profiles, highlighting their similarities.

Extended data Fig. 8



Extended data Fig. 8 Persistence and functionality of T_{TCR-C4} post-first infusion (A) Line plot showing the percentage (log scale) of T_{TCR-C4} in PBMCs collected after the first infusion across all patients ($n=15$), colored by virus-specificity (violet = CMV; orange = EBV), derived from flow-cytometry data **(B)** Line plot showing the $T_{TCR-C4}^{+}TNF\alpha^{+}$ cells following WT1-peptide stimulation. The x axis represents the days from the first infusion, while the y axis indicates the percentage (log10 scale) of Tetramer⁺ TNF α ⁺ gated on CD8⁺ T cells, based on flow cytometry data

Extended data Fig. 9

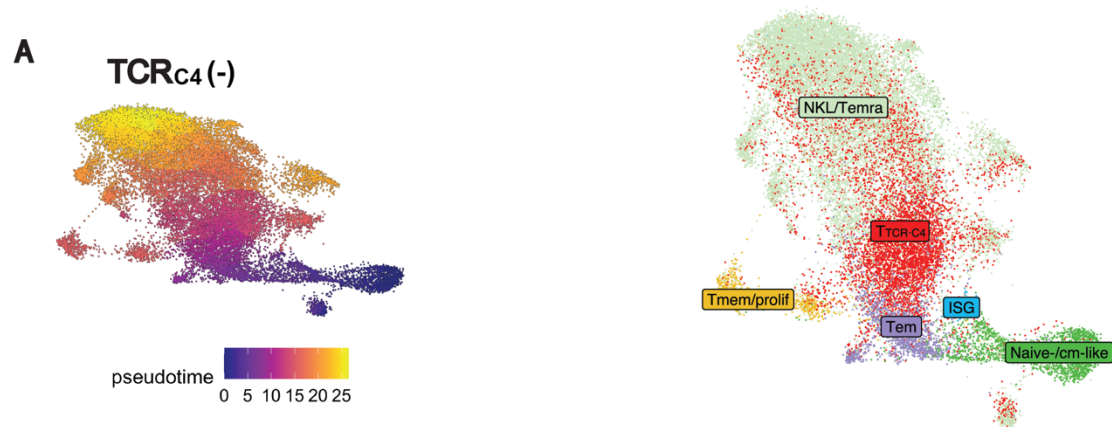


Extended data Fig. 9. Identification of transcriptional endogenous and T_{CR-C4} $CD8^+$ T-cell states. (A) UMAP plot showing the distribution of

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CD8⁺ T-cell states from a published independent dataset³ used as a reference atlas. **(B)** The UMAP projections of each T-cell state defined in Fig. 3C are overlaid onto the reference atlas as contour plots. Contours corresponding to each subset from the query are color-coded as in Fig. 3C, allowing for easy visualization of the distribution of each subset within the reference atlas. **(C)** Heatmap showing the differential expression of manually curated genes (Supplemental Table 7) across the five annotated CD8⁺ T-cell subsets. Blue and red indicate the relative expression levels of each marker within each subset, with blue representing lower expression and red indicating higher expression. **(D)** Violin plots displaying the expression levels (y-axis) of stem-like (*IL7R*, *TCF7*), activation (*GZMK*, *CD69*), and cytotoxicity/NK-like (*GZMB*, *KLRG1*, *NKG7*, *ZEB2*, *SIPR5*) markers across CD8⁺ T-cell transcriptional states (x-axis). Statistical significance was assessed using the Wilcoxon rank sum test. Asterisks indicate the following thresholds of significance: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

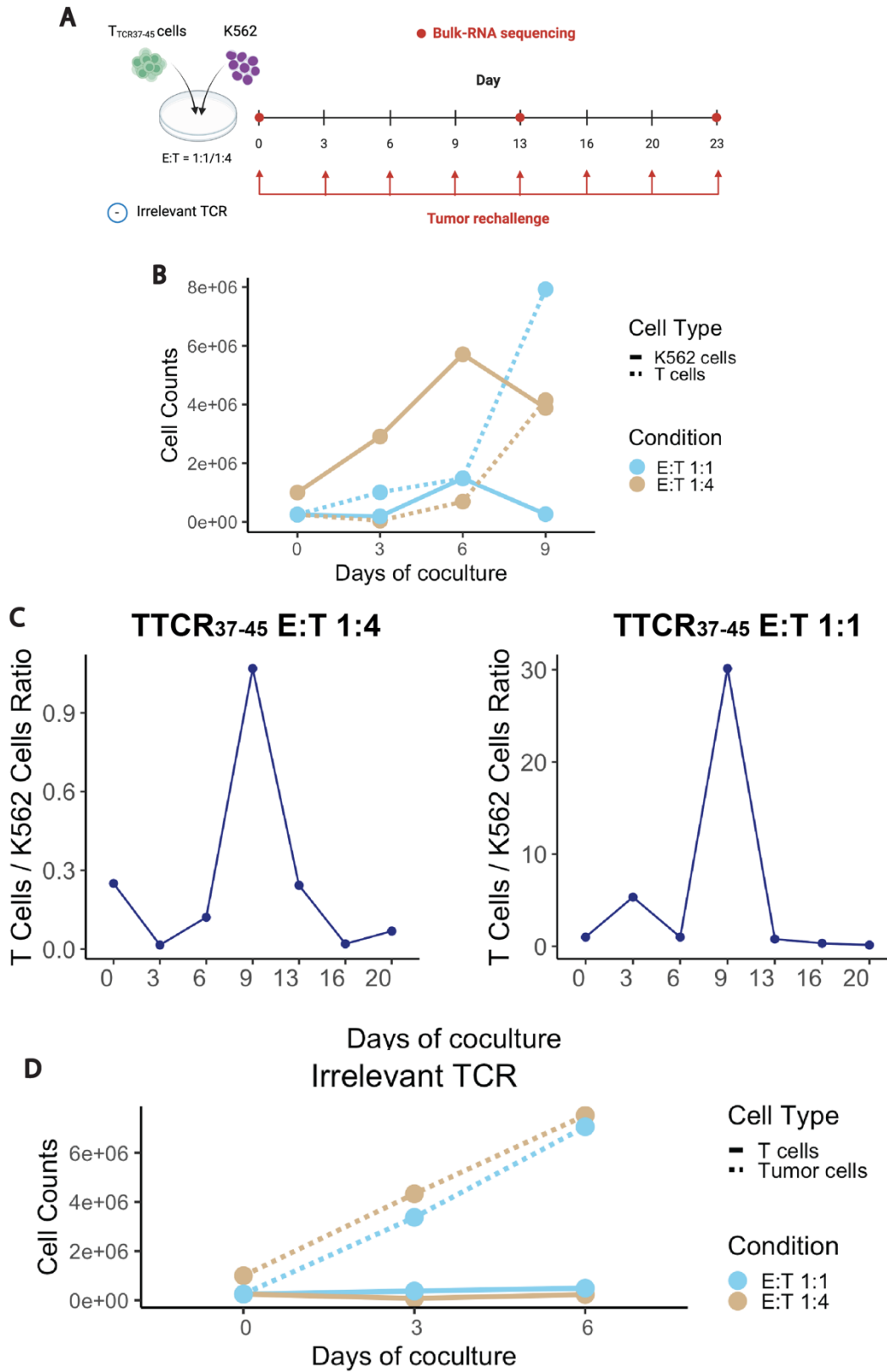
Extended data Fig. 10



Extended data Fig. 10. CD8⁺ transcriptional states after TCR_{C4} removal. (A) UMAP plot depicting the inferred developmental trajectory of CD8⁺ T-cell transcriptional states, as predicted by Monocle, following the removal of the TCR_{C4} transcript. The color scale, ranging from blue to orange/yellow, represents the pseudotime, where blue indicates earlier developmental stages and orange/yellow indicates later stages of the trajectory.

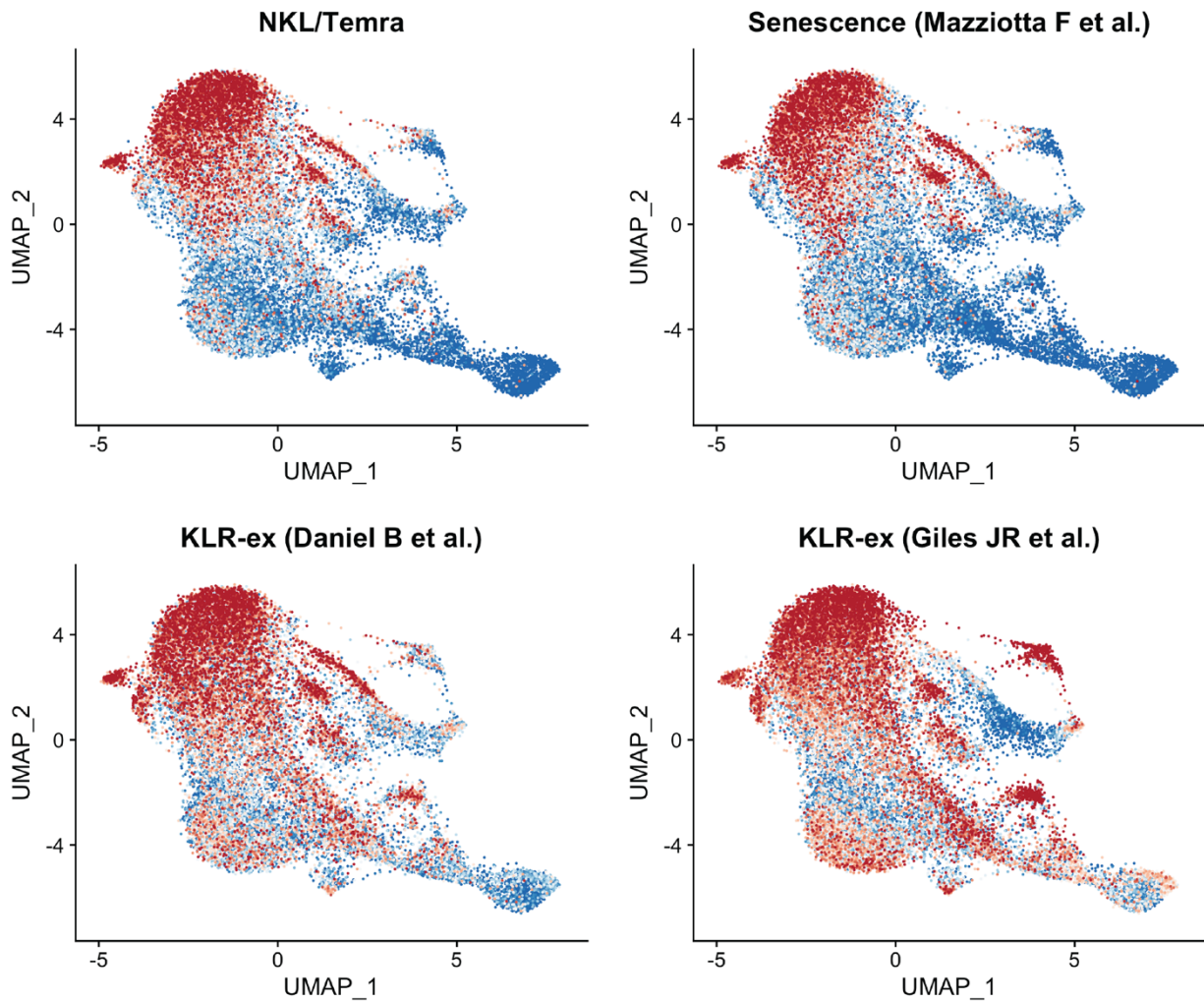
(B) UMAP plot showing the distribution of CD8⁺ T-cell transcriptional states after TCR_{C4} removal, with the same coordinates as panel A. The T-cell subsets are labeled according to the clustering defined in Fig. 3D, providing context for how the subsets are positioned within the developmental trajectory in A.

Extended data Fig. 11



Extended data Fig. 11. In-vitro dysfunction coculture system. (A) Experimental design of the in-vitro model used to study chronic tumor exposure of WT1-specific T cells ($T_{TCR37-45}$ cells). The diagram shows two conditions with Effector ($T_{TCR37-45}$) to Target (K562) (E:T) ratios of 1:1 and 1:4. Red arrows indicate tumor re-challenge every 3-4 days, while red circles on the timeline mark the time points when RNA extraction for bulk-RNA sequencing was performed. An irrelevant TCR-expressing T cell line was used as a negative control. The experiment involved exposing transgenic $CD8^+$ WT1-specific T cells to HLA-A*0201-transduced K562 cells expressing high levels of WT1. This figure was realized using BioRender. (B) Line plots showing the changes in absolute cell counts (y-axis) over time (x-axis, days of coculture) for $T_{TCR37-45}$ (solid lines) and K562 tumor cells (dashed lines) in coculture at E:T ratios of 1:1 and 1:4. The lines for each condition are colored sky blue (1:4 ratio) and tan (1:1 ratio). Cell counting was performed by flow cytometry. (C) Line plots showing the T cell to tumor cell ratio (y-axis) changes at the timepoints of tumor re-challenge (x-axis). Each plot represents one condition (left, E:T 1:4; right, E:T 1:1). Cell ratios were calculated based on flow cytometry data. (D) Line plots showing the changes in absolute cell counts (y-axis) over time (x-axis, days of coculture) for T cells with irrelevant TCR (solid lines) and K562 tumor cells (dashed lines) in coculture at E:T ratios of 1:1 and 1:4. The lines for each condition are colored sky blue (1:4 ratio) and tan (1:1 ratio). Cell counting was performed by flow cytometry.

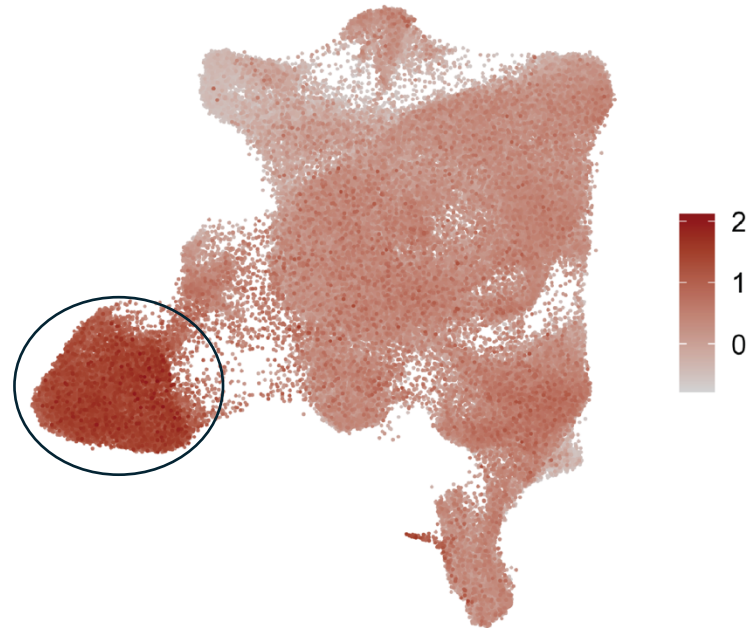
Extended data Fig. 12



Extended data Fig. 12 UMAP projection of KLR dysfunction signatures in CD8⁺ T cells. UMAP plots illustrate the spatial distribution of CD8⁺ T cells based on their expression of published KLR dysfunction gene signatures.⁴⁻⁶ These signatures, derived from prior studies, were applied to our scRNAseq dataset to calculate a score for each cell, reflecting the degree of expression of the KLR dysfunction features. The scores are visualized using a gradient, where blue indicates low expression and red indicates high expression. Regions with high expression (red) align with the subset we identified as Temra NK-like cells, suggesting a functional and phenotypic overlap within this subset.

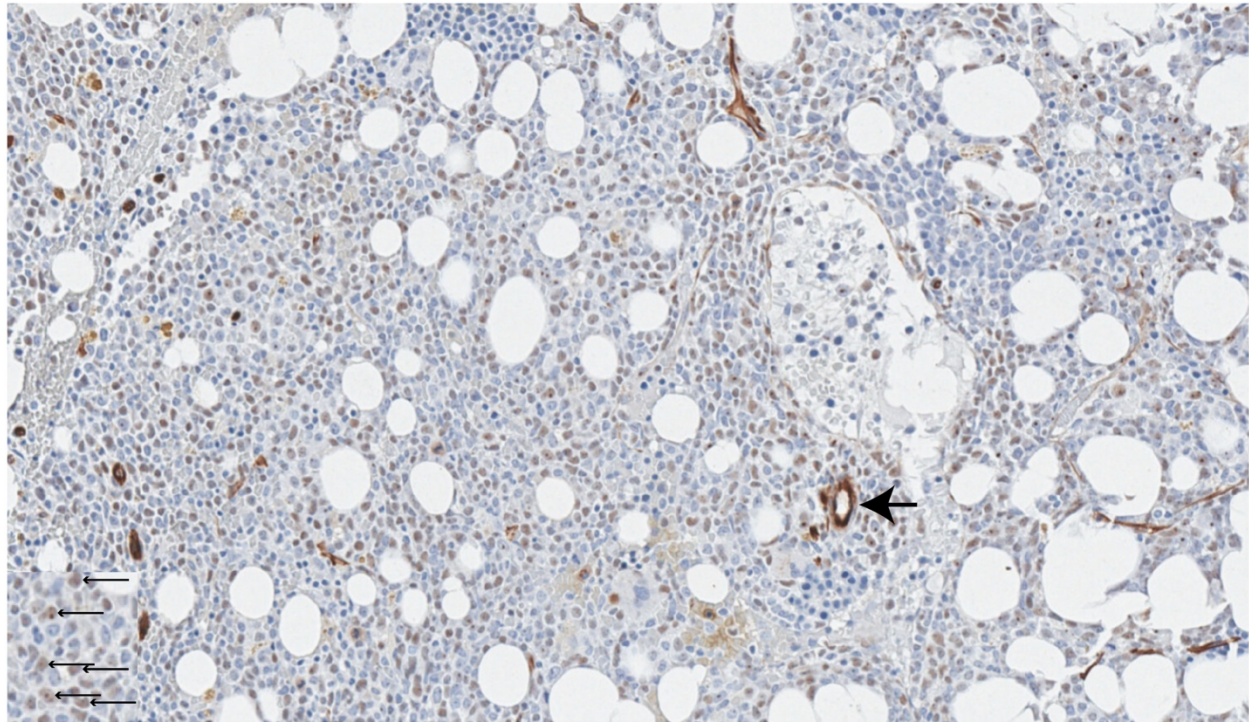
Extended data Fig. 13

NK-like score



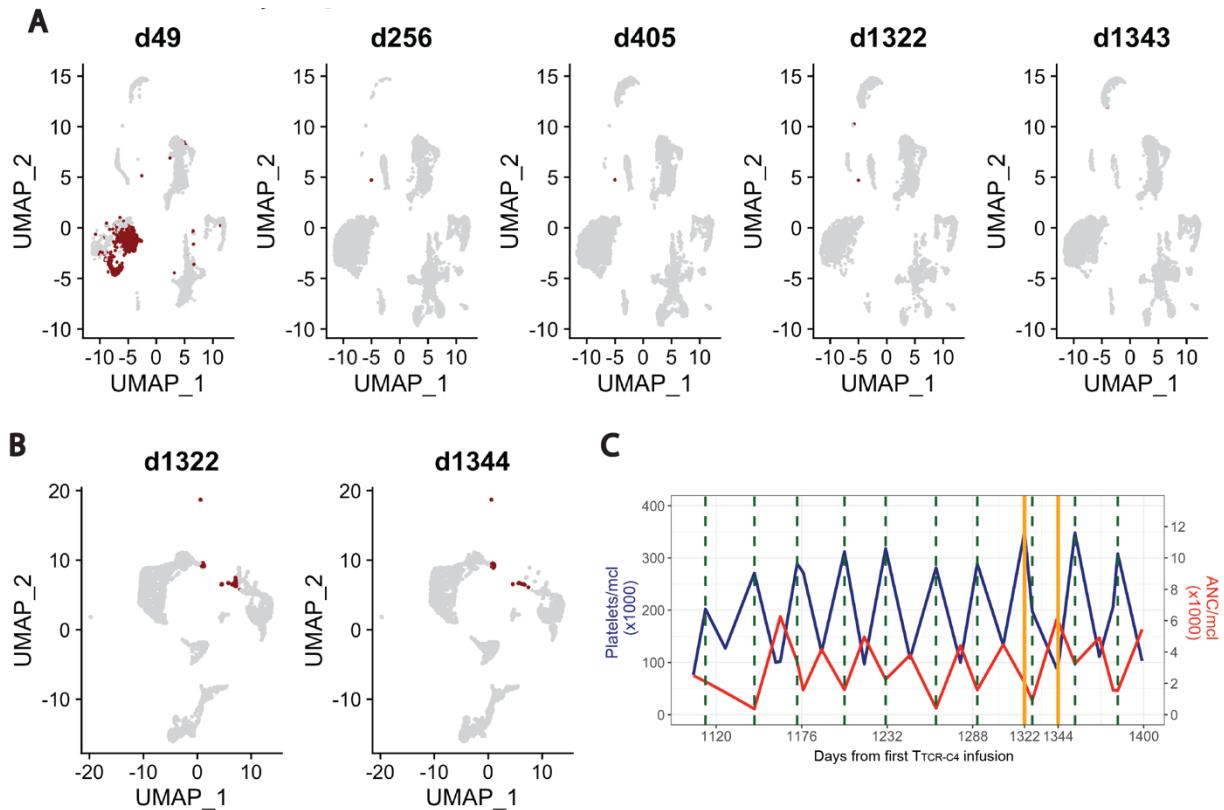
Extended data Fig. 13 UMAP projection of NK-like module score based on gene expression in a tumor-infiltrating lymphocytes reference atlas.³ UMAP plot projection showing the module score based on the expression of the following genes: *KLRG1*, *KLRD1*, *ZEB2*, *FCRL6*, *ADGRG1*, *SIPR5*, *FCGR3A*, *GZMB*, and *NKG7*. Each point represents an individual cell, and the color scale ranges from light grey (low module score) to dark red (high module score), reflecting the intensity of the gene set expression across cells. The black circle marks the area with the highest NK-like score, indicating the regions where the combined expression of NK-associated genes is most prominent.

Extended data Fig. 14



Extended data Fig. 14. Immunohistochemistry of bone marrow biopsy stained for WT1. This immunohistochemistry image shows a bone marrow biopsy stained for WT1. Black arrows indicate WT1-positive nuclei, visible as distinct dark-stained areas within the cells, which signify expression of the WT1 protein.

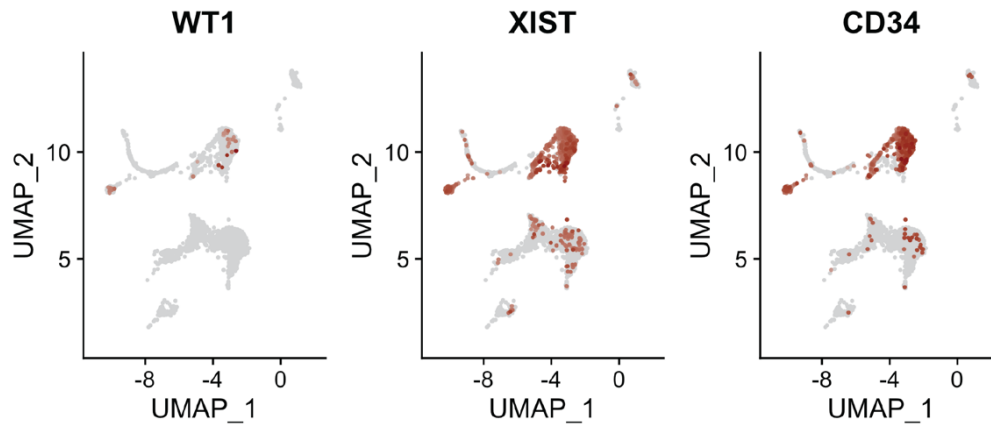
Extended data Fig. 15



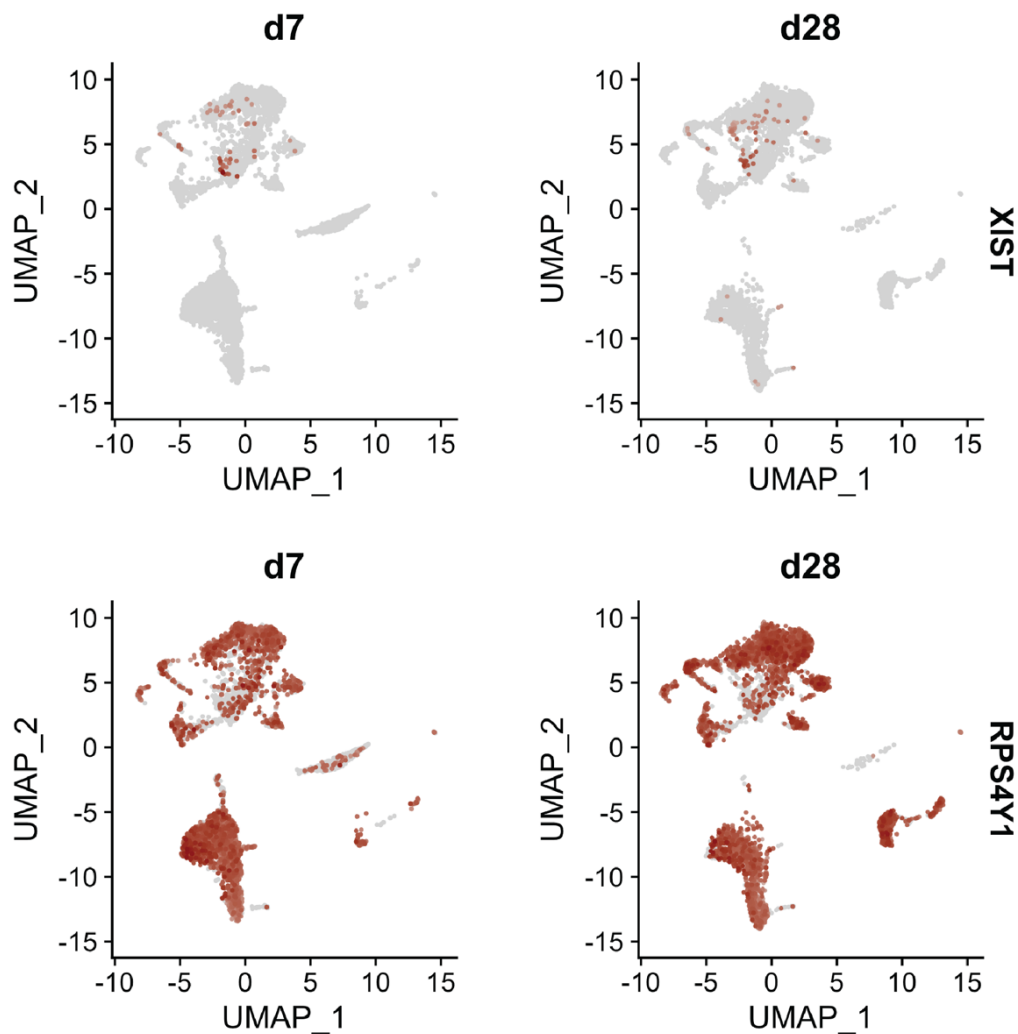
Extended data Fig. 15 Patient 8 blasts and T cells characterization (A) UMAP plot showing a blast score (dark red) calculated based on the co-expression of *CD34* and *XIST* (a female-specific gene) in a patient previously transplanted with a sex-mismatched donor (female patient, male donor). The score was used to identify cells expressing a high blast score. Each UMAP plot represents a different timepoint (day 49, 256, 405, 1322, 1343) after the first infusion, with lightgrey indicating low expression and dark red indicating high expression of the blast score. **(B)** UMAP plot showing a blast score calculated based on the co-expression of *XIST*, *CD34*, *CD33*, and *KIT*. This score was used to visualize cells with high blast scores in bone marrow samples. Each UMAP plot represents a specific timepoint (day 1322 and 1344) post-first T_{TCR-C4} infusion, **(C)** Line plot showing platelets and neutrophil absolute counts over time after the first infusion. Platelets (left y-axis, dark blue line plot, measured as platelets/ μ L \times 1000) and neutrophils (right y-axis, red line plot, measured as ANC/ μ L \times 1000) are shown in relation to the days after the first infusion (x-axis). Vertical dark green dashed lines indicate the timing of azacytidine cycles, while solid orange lines represent the timepoints when BM scRNA-seq was performed.

Extended data Fig. 16

A



B



Extended data Fig. 16 UMAP visualization of gene expression in AML patient samples. (A) UMAP plots at day 49 post-first T_{TCR- α} infusion depict the expression patterns of leukemia-associated markers *WT1* and *XIST* (a female-specific gene) in patient 8, previously transplanted with a sex-mismatched donor (female patient, male donor). Additionally, *CD34* expression across all cell populations is shown. Cells with high expression of each marker are highlighted in red, while other cells appear in grey. **(B)** UMAP plots illustrate a longitudinal analysis of leukemia-associated gene expression in patient 26, who was transplanted with a sex-mismatched donor (female patient, male donor), at days 7 (d7) and 28 (d28) post-first T_{TCR- α} infusion. *XIST* indicates leukemic cells, while *RPS4Y1* (a male-specific gene) marks non-leukemic cells. Cells with high gene expression are represented in dark red, and those with lower expression appear in light grey.

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