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Two subsets of stem-like CD8⁺ memory T cell progenitors with distinct fate commitments in humans

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10
11 **ABSTRACT**

12 T cell memory relies on the generation of antigen-specific progenitors with stem-like properties.
13 However, the identity of these progenitors has remained unclear, precluding a full understanding
14 of the differentiation trajectories that underpin the heterogeneity of antigen-experienced T cells.
15 We used a systematic approach guided by single-cell RNA sequencing data to map the
16 organizational structure of the human CD8⁺ memory T cell pool under physiological conditions.
17 We identified two previously unrecognized subsets of clonally, epigenetically, functionally,
18 phenotypically, and transcriptionally distinct stem-like CD8⁺ memory T cells. Progenitors
19 lacking the inhibitory receptors programmed death-1 (PD-1) and T cell immunoreceptor with Ig
20 and ITIM domains (TIGIT) were committed to a functional lineage, whereas progenitors
21 expressing PD-1 and TIGIT were committed to a dysfunctional, exhausted-like lineage.
22 Collectively, these data revealed the existence of parallel differentiation programs in the human
23 CD8⁺ memory T cell pool, with potentially broad implications for the development of
24 immunotherapies and vaccines.

1 MAIN TEXT

2 Antigen recognition by CD8⁺ naive T cells initiates a program of clonal expansion and
3 effector differentiation that leads to the clearance of infected or malignant cells and the
4 subsequent formation of heterogeneous memory populations that confer durable immunity¹.
5 These memory populations are thought to be organized in a developmental hierarchy, according
6 to which stem cell memory T (T_{SCM}) cells self-renew and generate long-lived central memory T
7 (T_{CM}) cells and short-lived effector memory T (T_{EM}) cells²⁻⁶. However, the mechanisms that
8 underlie the enhanced multipotency of T_{SCM} cells relative to T_{CM} cells have not been clearly
9 defined in molecular terms⁷.

10 Memory T cell differentiation can become corrupted under conditions of persistent
11 antigenic stimulation, as observed during chronic viral infections and progressive malignancies,
12 which promote a state of T cell exhaustion, characterized by an orderly loss of effector functions,
13 impaired proliferation, and the upregulation of inhibitory receptors⁸. This dynamic process
14 occurs over a period of weeks after the initial priming event^{9,10} and involves the genome-wide
15 accumulation of epigenetic modifications^{11,12}. Recent studies have shown that exhausted T (T_{EX})
16 cell populations are developmentally and functionally heterogeneous, incorporating stem-like
17 progenitors that express T cell factor 1 (TCF1) which give rise to highly differentiated T_{EX} cells
18 that are constitutively dysfunctional and lack TCF1¹³⁻¹⁶. Importantly, the therapeutic benefits of
19 immune checkpoint blockade in the context of chronic viral infections and various cancers are
20 thought to operate via these TCF1⁺ progenitors, which appear susceptible to interventions that
21 specifically target the inhibitory receptor programmed death-1 (PD-1)^{13,15,17-20}.

22 Current evidence therefore suggests that exhausted and functional memory T cells arise
23 from separate populations of stem-like progenitors committed to distinct fates. However, the

1 precise nature of these stem-like progenitors, which shape the adaptive immune response and
2 influence the outcome of many globally relevant diseases, has remained obscure. In this study,
3 we used a comprehensive and unbiased approach to map the origins of dysfunctional and
4 functional human CD8⁺ memory T cells. Our data identified two distinct subsets of CCR7⁺
5 progenitors in healthy individuals, distinguished by the expression of PD-1 and T cell
6 immunoreceptor with Ig and ITIM domains (TIGIT). Progenitors committed to the generation of
7 dysfunctional, exhausted-like progeny expressed both of these inhibitory receptors, whereas
8 progenitors committed to the generation of a more functional progeny lacked both of these
9 inhibitory receptors. Differential inclusion of the transcriptionally distinct PD-1⁺ TIGIT⁺ subset
10 also explained most of the differences between T_{SCM} and T_{CM} cells, providing a clearer view of
11 human CD8⁺ memory T cell differentiation.

1 RESULTS

2 **Two subsets of stem-like CD8⁺ memory T cell progenitors exist in humans.** We initially used
3 single-cell RNA sequencing (scRNA-seq; 10X Genomics Platform) to characterize the full
4 spectrum of human CD8⁺ memory T cells in peripheral blood (PB) samples obtained from
5 healthy donors (n = 4) (**Supplementary Table 1**). A total of 31,640 cells were isolated for this
6 purpose via fluorescence-activated cell sorting (FACS) based on the expression of CD95 (**Fig. 1a**
7 and **Supplementary Fig. 1a**), which identifies a vast majority of all memory T cells in humans⁴.
8 Bioinformatic analysis of gene expression mapped in two dimensions via Uniform Manifold
9 Approximation and Projection (UMAP)²¹ identified 14 distinct clusters (denoted individually as
10 C) (**Fig. 1b**). C1, C4, C5, and C7 were uniformly distant from the other cell populations and
11 expressed high levels of *KLRB1*, which encodes CD161, and *IL7R*, which encodes the
12 interleukin (IL)-7 receptor (IL-7R), also known as CD127. These clusters were therefore derived
13 from mucosal associated invariant T (MAIT) cells²². An intermediate cluster, C9, which
14 comprised less than 2% of all cells (**Fig. 1c**), overexpressed *TRDC* and *TRGC1*, which encode
15 the constant regions of the T cell receptor (TCR) δ and γ chains, respectively, suggesting the
16 expression of $\gamma\delta$ rather than $\alpha\beta$ TCRs.

17 scRNA-seq further identified seven different clusters related to conventional memory T
18 cells (**Fig. 1b**), the most abundant of which were C0, C2, C3, C6, and C10 (**Fig. 1c**). C2, C6, and
19 C10 expressed genes associated with early differentiated memory T cells, including *CCR7*,
20 *LEF1*, *SELL*, which encodes L-selectin (CD62L), and *FOXP1* (**Fig. 1b,d** and **Supplementary**
21 **Table 2**). In contrast, the highly abundant C0 overexpressed multiple effector transcripts,
22 including *GZMK* and *GZMM*, which encode serine proteases termed granzymes, *IFNG*, which
23 encodes interferon (IFN)- γ , the chemokine (C-C motif) ligands *CCL4* and *CCL5*, and genes

1 encoding human leukocyte antigen (HLA) class II molecules, consistent with the identification
2 of effector memory T (T_{EM}) cells (**Fig. 1b,d** and **Supplementary Table 2**). C3 displayed a gene
3 expression profile reminiscent of terminal effector T (T_{TE}) cells, featuring high levels of *GZMB*,
4 *GNLY*, *NKG7*, *ZEB2*, and *GZMA* (**Fig. 1b,d** and **Supplementary Table 2**). A lack of signature
5 transcripts precluded the identification of C8 based on current knowledge of the T cell
6 differentiation pathway (**Supplementary Table 2**).

7 To explore the heterogeneity of the *CCR7*⁺ memory T cell pool, we focused on C2 and C6,
8 because C10 comprised only ~1% of all sorted cells (**Fig. 1c**). We identified 160 differentially
9 expressed genes (DEGs) between C2 and C6 (**Supplementary Table 3**). C2 expressed higher
10 levels of effector molecules, including *CCL5*, *GZMK*, *GNLY*, *GZMA*, *JUN*, *GZMM*, *HOPX*,
11 *IKZF3*, *RUNX3*, and *PRF1*, which encodes perforin, whereas C6 expressed higher levels of
12 *IL6R*, *LTB*, *LEF1*, *NOSIP*, *GATA3*, and *SELL* (**Fig. 1e** and **Supplementary Table 3**). We then
13 used anchor genes selected from the most prominent DEGs to compute transcriptional modules
14 associated with memory differentiation (correlated with *CCR7* and *LEF1*), quiescence
15 (correlated with *FOXPI*), or cytotoxicity and terminal effector differentiation (correlated with
16 *GZMK/GZMB* and *ZEB2*, respectively) among the five conventional memory T cell clusters
17 (C0, C2, C3, C6, and C10). Using this approach, we found that C2 and C10 were similar,
18 exhibiting intermediate memory and effector scores, whereas C6 was skewed toward a high
19 memory score and C0 and C3 were skewed toward high effector scores (**Fig. 1f**). scRNA-seq
20 analysis therefore identified four major subsets of conventional CD8⁺ memory T cells, namely
21 early differentiated *CCR7*⁺ *GZMK*⁻ (C6) and *CCR7*⁺ *GZMK*⁺ (C2), T_{EM}-like *CCR7*⁻ *GZMK*^{hi}
22 (C0), and T_{TE}-like *GZMB*⁺ (C3).

1 To confirm these findings at the protein level, we designed a high-dimensional flow
2 cytometry panel based on the cluster signature markers CCR7, LEF1, CD161, GZMB, and
3 GZMK (**Fig. 1g**). This panel was also equipped to detect memory and effector differentiation
4 markers (CD27, CD28, CD45RO, CD127, and T-bet), activation markers (CD38 and HLA-DR),
5 inhibitory receptors (PD-1 and TIGIT), and markers of tissue residency (CD69 and CD103) in
6 CD8⁺ T cells isolated from the peripheral blood and tissues (**Supplementary Table 1**). In line
7 with the scRNA-seq data, UMAP analysis revealed that CD161^{hi} MAIT cells were largely
8 distinct from other CD95⁺ memory T cells (**Fig. 1h**). Among the effector subsets, CCR7⁻
9 GZMK^{hi} cells expressed cytolytic molecules and generally lacked the memory markers LEF1,
10 CD27, CD28, and CD127, whereas GZMB⁺ cells also expressed granulysin (GNLY) and
11 relatively high levels of T-bet (**Fig. 1h**). In contrast, the CCR7⁺ GMZK⁻ subset expressed
12 relatively high levels of LEF1, CD27, CD28, and CD127 and lacked effector molecules,
13 activation markers, and inhibitory receptors, whereas the CCR7⁺ GMZK⁺ subset expressed
14 intermediate levels of LEF1 alongside PD-1 and TIGIT, which were not detected in the scRNA-
15 seq analysis, together with relatively high levels of CD27, CD28, and CD127 (**Fig. 1h**). These
16 subsets displayed variable expression of CD45RO (**Fig. 1h**). As expected, CD69⁺ CD103⁺ cells
17 were only detected in tissues (**Fig. 1h**). A survey of different tissue sites revealed that CCR7⁺
18 GZMK⁻ PD-1⁻ TIGIT⁻ and CCR7⁺ GZMK⁺ PD-1⁺ TIGIT⁺ cells were relatively abundant in PB,
19 lymph nodes (LNs), and bone marrow (BM), whereas CCR7⁻ GZMK^{hi} cells were ubiquitous,
20 and GZMB⁺ cells predominated in PB and lung tissue (**Fig. 1i**).

21 Collectively, these data identified CCR7⁺ GZMK⁻ and CCR7⁺ GMZK⁺ cells as distinct
22 entities in the early differentiated CD8⁺ memory T cell pool and further showed that these
23 subsets could be distinguished by the expression of PD-1 and TIGIT.

1
2 **Exhausted-like CD8⁺ memory T cell progenitors express GZMK, PD-1, and TIGIT.**

3 Heterogeneity in the early differentiated memory T cell pool became apparent with the
4 identification of multipotent T_{SCM} cells^{4,23}. These cells exhibit a CCR7⁺ CD45RO⁻ CD95⁺
5 phenotype, in contrast to T_{CM} cells, which exhibit a CCR7⁺ CD45RO⁺ CD95⁺ phenotype.
6 However, our scRNA-seq-guided flow cytometric analyses demonstrated that the CCR7⁺
7 GZMK⁻ PD-1⁻ TIGIT⁻ and CCR7⁺ GZMK⁺ PD-1⁺ TIGIT⁺ subsets could not be distinguished
8 via the expression of CD45RO (**Fig. 1h**). To place these findings in context, we investigated the
9 expression of PD-1 and TIGIT among classically defined T_{SCM} and T_{CM} cells. We found that 9.1
10 ± 1.3% of T_{SCM} cells and 22.1 ± 2.3% of T_{CM} cells (mean ± SEM) expressed both PD-1 and
11 TIGIT (**Fig. 2a,b**). Manual gating of the flow cytometry data confirmed that PD-1 and TIGIT
12 were preferentially expressed by CCR7⁺ GZMK⁺ cells (**Supplementary Fig. 1b**).

13 On the basis of these results, we hypothesized that differential inclusion of the
14 transcriptionally distinct CCR7⁺ GZMK⁺ PD-1⁺ TIGIT⁺ subset could explain some of the
15 previously reported differences between T_{SCM} and T_{CM} cells. To test this possibility, we analyzed
16 the transcriptomes of T_{SCM} and T_{CM} cells after depletion of the CCR7⁺ PD-1⁺ TIGIT⁺ (GZMK⁺)
17 population, hereafter termed T progenitor exhausted-like (T_{PEX}) (**Supplementary Fig. 1a** and
18 **Supplementary Fig. 2**). In line with our hypothesis, T_{SCM} and T_{CM} cells depleted of T_{PEX} cells
19 were very similar at the transcriptional level and could only be distinguished on the basis of eight
20 DEGs (adjusted *P* value < 0.01) (**Supplementary Fig. 2** and **Supplementary Table 4**). One of
21 these DEGs was *HNRNPLL*, which encodes heterogeneous nuclear ribonucleoprotein L-like, a
22 master regulator of alternative splicing responsible for the expression of CD45RO²⁴, which is
23 commonly used as a phenotypic marker to differentiate between T_{SCM} and T_{CM} cells²⁵. In

1 contrast, T_{PEX} cells were largely distinct, featuring lower expression levels of *SATB1*, which
2 encodes a negative regulator of PD-1 expression²⁶, *MYC*, *DPP4*, which encodes CD26, *IL6ST*,
3 *LEF1*, *IL6R*, and *NT5E* and higher expression levels of transcription factor (TF) genes recently
4 associated with T cell exhaustion, including *TOX*²⁷⁻³², *EOMES*³³, and *MAF*³⁴, and other genes
5 associated with effector differentiation and cytolytic activity, including *ZEB2*, *GZMK*, *GZMA*,
6 *TBX21*, *PRF1*, *IFNG*, and *NKG7* (**Fig. 2c** and **Supplementary Table 4**). As expected, *PDCDI*,
7 which encodes PD-1, and *TIGIT* were also expressed at high levels, validating the integrity of
8 cell isolation via FACS. Several other genes previously found to distinguish T_{SCM} from T_{CM} cells
9 were also identified among these DEGs⁴ (**Fig. 2c** and **Supplementary Table 4**). In line with the
10 transcriptional data, T_{PEX} cells stimulated with anti-CD3 plus CD28 and a combination of
11 effector (IL-2 plus IL-12) or homeostatic cytokines (IL-7 plus IL-15) proliferated less vigorously
12 than PD-1⁻ TIGIT⁻ T_{SCM} and T_{CM} cells under identical conditions (**Fig. 2d**). However, all three
13 subsets proliferated similarly and remained phenotypically stable in response to IL-15,
14 suggesting equivalent self-renewal capabilities (**Supplementary Fig. 3a,b**). Accordingly, T_{SCM}
15 and T_{CM} cells were better defined by the CCR7⁺ PD-1⁻ TIGIT⁻ phenotype, hereafter termed
16 stem-like T (T_{STEM}), whereas early differentiated memory cells with dysfunctional, exhausted-
17 like traits were characterized by the CCR7⁺ PD-1⁺ TIGIT⁺ phenotype (T_{PEX}). Of note, the gene
18 expression profiles of T_{STEM} and T_{PEX} cells overlapped significantly with those of C6 and C2,
19 respectively ($P < 0.01$ for each comparison using a hypergeometric test), confirming the shared
20 identity of subsets analyzed via scRNA-seq and flow cytometry (**Fig. 1e**).

21 Flow cytometric analyses demonstrated that T_{STEM} cells expressed CD26 and LEF1 more
22 commonly and CCR5 and Eomes less commonly than T_{PEX} cells (**Fig. 2e**). Gene set enrichment
23 analysis (GSEA) further revealed that T_{STEM} cells were characterized by transcripts associated

1 with the naive state, quiescence, oxidative phosphorylation, the Wnt³⁵ and Notch signaling
2 pathways³⁶, and proteasome activity³⁷, whereas T_{PEX} cells were characterized by transcripts
3 associated with the TGF- β signaling pathway³⁸, potassium regulation³⁹, and other mechanistic
4 correlates of exhaustion, including the PD-1^{hi} state (**Fig. 2f**). Transcripts associated with the cell
5 cycle and the TCR and mTOR signaling pathways, collectively suggesting a predisposition to
6 antigen-driven proliferation and effector differentiation, were also upregulated in T_{STEM} versus
7 T_{PEX} cells (**Fig. 2f**). Previous analyses have shown that progenitor exhausted CD8⁺ T cells from
8 tumors express stem-like genes along with *PDCDI*, *TIGIT* and *GZMK*¹⁶, thereby suggesting a
9 shared identity with T_{PEX} cells. Indeed, the transcriptional features of T_{PEX} cells aligned closely
10 with those reported previously for progenitor exhausted-like (*CCR7*^{hi} *GZMK*^{hi}) but not memory-
11 like CD8⁺ T cells (*CCR7*^{hi} *GZMK*^{lo}) isolated from melanomas¹⁸, whereas the opposite result was
12 obtained in a parallel analysis of T_{STEM} cells ($P < 0.05$ for each comparison using a
13 hypergeometric test) (**Supplementary Table 4**).

14 Collectively, these data revealed that T_{SCM} and T_{CM} cells were largely homogenous after
15 depletion of the T_{PEX} subset, indicating a need to refine current models of CD8⁺ memory T cell
16 differentiation.

17
18 **T_{STEM} cells are functionally superior to T_{PEX} cells.** To validate our transcriptional and
19 phenotypic data, we compared the functional properties of FACS-purified T_{STEM} and T_{PEX} cells.
20 In response to TCR-dependent stimulation with Staphylococcal enterotoxin B (SEB), T_{STEM} cells
21 upregulated CD25 and CD69 to a greater extent than T_{PEX} cells (**Fig. 3a**), and activated CD25⁺
22 CD69⁺ T_{STEM} cells expressed higher levels of T-bet than activated CD25⁺ CD69⁺ T_{PEX} cells (**Fig.**
23 **3b**). Likewise, T_{STEM} cells produced cytokines at higher frequencies (IL-2 and TNF) and at

1 higher levels on a per cell basis (IFN- γ , IL-2, and TNF) than T_{PEX} cells in response to stimulation
2 with anti-CD3 plus CD28 (**Fig. 3c–e**). No clear differences were observed between T_{STEM} and
3 T_{PEX} cells with respect to degranulation, measured via the surface mobilization of CD107a (**Fig.**
4 **3c–e**). In response to TCR-independent stimulation with phorbol myristate acetate (PMA) and
5 ionomycin, however, T_{PEX} cells produced IFN- γ and TNF and mobilized CD107a at much higher
6 frequencies than T_{STEM} cells, the functional superiority of which was therefore limited to
7 conditions that mimicked antigen recognition events (**Fig. 3f,g**).

8 To determine the in vivo relevance of these observations, we performed serial adoptive
9 cell transfers (ACTs) in NOD.Cg-*Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ* (NSG) humanized mice (**Fig. 3h**). T_{EM}
10 cells failed to repopulate these mice efficiently after the first ACT (not shown), as reported
11 previously⁴. Although the early memory subsets both expanded to similar numbers in primary
12 hosts (not shown), T_{STEM} cells proliferated more rapidly in PB (**Fig. 3i**) and repopulated the
13 spleen more efficiently in secondary hosts compared with T_{PEX} cells (**Fig. 3j**). Of note, the
14 suboptimal proliferative capabilities of T_{PEX} cells observed in vitro and in vivo were not
15 associated with differences in telomere length relative to T_{STEM} cells, whereas T_{EM} cells harbored
16 shorter telomeres ex vivo compared with either T_{STEM} or T_{PEX} cells (**Supplementary Fig. 3c**).

17 Collectively, these data showed that T_{STEM} cells were functionally superior to T_{PEX} cells,
18 both under homeostatic conditions and in response to stimulation via the TCR.

19
20 **T_{PEX} cells are committed to a terminally dysfunctional state.** Epigenetic regulation plays a
21 key role in T cell fate decisions⁴⁰. We therefore employed the Assay for Transposase-Accessible
22 Chromatin using sequencing (ATAC-seq) to compare the open chromatin landscapes of T_{STEM}
23 and T_{PEX} cells in terms of differentially accessible regions (DARs). Naive and T_{EM} cells were

1 analyzed in parallel as lineage controls. Principal component analysis (PCA) revealed that T_{STEM}
2 and T_{PEX} cells were globally similar, although T_{STEM} cells mapped toward the naive subset,
3 whereas T_{PEX} cells mapped toward the T_{EM} subset (**Fig. 4a**). However, we also identified a total
4 of 13,414 DARs between T_{STEM} and T_{PEX} cells (**Fig. 4b**). Genes associated with T cell
5 dysfunction (*e.g.*, *TOX*, *TOX2*, *TIGIT*, *PDCD1*, *NFATC2*, and *MAF*), terminal differentiation
6 (*e.g.*, *ZEB2* and *BATF*), and other immune-related processes previously identified at the mRNA
7 level (*e.g.*, *EOMES* and *GZMA*) were more accessible in T_{PEX} versus T_{STEM} cells (**Fig. 4b,c**). In
8 contrast, genes associated with T cell memory (*e.g.*, *LEF1*, *SELL*, *CCR7*, *BACH2*, and *SATB1*)
9 and effector functions (*e.g.*, *GZMB* and *RORA*⁴¹) were more accessible in T_{STEM} versus T_{PEX} cells
10 (**Fig. 4b,c**). Computational analysis of these DARs further identified differentially accessible TF
11 binding motifs (TFBMs). Motifs linked to TFs associated with thymocytes and naive and early
12 memory cells (*RUNX2*, *RUNX1*, *LEF1*, and *FOXP1*), effector differentiation (*RORA*)⁴¹, and
13 cytokine signaling (*STAT5*, *STAT4*, and *STAT1*) were enriched in T_{STEM} versus T_{PEX} cells,
14 whereas the *TBX21* (T-bet), *EOMES*, and combined *TBOX:SMAD* motifs were enriched in
15 T_{PEX} versus T_{STEM} cells (**Fig. 4d**).

16 The chromatin accessibility data suggested that T_{PEX} cells were predisposed to the
17 generation of dysfunctional progeny and susceptible to the inhibitory effects of TGF- β signaling
18 via SMADs. Accordingly, T_{PEX} cells proliferated to a lesser extent and produced less *GZMB*
19 than T_{STEM} cells in response to stimulation with anti-CD3 plus CD28 and IL-15 (**Fig. 4e**). The
20 addition of TGF- β further inhibited these responses, especially the production of *GZMB*, in
21 parallel cultures of T_{PEX} cells, whereas minimal effects were observed in parallel cultures of
22 T_{STEM} cells (**Fig. 4e**). Importantly, most T_{PEX} cells retained a PD-1⁺ TIGIT⁺ phenotype after
23 stimulation with anti-CD3 plus CD28 in the presence of the effector cytokines IL-2 and IL-12

1 (Fig. 4f,g) or the homeostatic cytokines IL-7 and IL-15 (Supplementary Fig. 3d). In contrast,
2 T_{STEM} cells generated all possible combinations of phenotypes defined by PD-1 and TIGIT (Fig.
3 4f,g). We then used RNA-seq to profile the transcriptomes of T_{STEM} and T_{PEX} cells after
4 stimulation with anti-CD3 plus CD28 in the presence of IL-2 and IL-12 (Fig. 4h). Activated
5 T_{STEM} cells overexpressed the memory-related genes *BACH2*, *ID3*, *IL2*, and *SATB1* alongside the
6 effector-related genes *IRF8*, *RORC*, *GPLY*, *XBPI*, *IL26*, and *IL23R*, whereas activated T_{PEX} cells
7 overexpressed the dysfunction/exhaustion-related molecules *TOX*, *PDCD1*, *TIGIT*, *MAF*, and
8 *CXCL13*⁴², together with various chemokine genes, *IKZF3*, which encodes an inhibitor of IL-2
9 production, *SMAD3*, and genes associated with cytolytic activity, including *GZMK*, *GZMH*, and
10 *GZMA* (Fig. 4h and Supplementary Table 5). Some of these genes were also differentially
11 expressed between the corresponding subsets in ex vivo analyses (Fig. 2c). GSEA further
12 demonstrated that activated T_{STEM} cells were preferentially enriched for gene sets associated with
13 early differentiation and proliferation, whereas activated T_{PEX} cells were preferentially enriched
14 for gene sets associated with the TGF- β and PD-1 signaling pathways and exhaustion in the
15 tumor microenvironment¹⁸ (Supplementary Fig. 4a). PCA of ATAC-seq data from paired ex
16 vivo and activated samples revealed that stimulation profoundly altered the chromatin
17 accessibility landscape in T_{STEM} and T_{PEX} cells (Fig. 4i). However, the major epigenetic
18 differences between these subsets in the ex vivo state were maintained after stimulation (Fig. 4i),
19 both at the level of specific genes (Supplementary Fig. 4b) and in terms of enrichment for
20 particular TFBMs (Supplementary Fig. 4c).

21 To assess the in vivo relevance of these findings, we employed a stringent ACT protocol
22 in which T_{STEM} and T_{PEX} cells were redirected using a chimeric antigen receptor (CAR) targeting
23 CD19 and transferred in the absence of autologous CD4⁺ T cells or exogenous cytokines into

1 NSG mice previously injected with the acute lymphoblastic leukemia cell line NALM6. In line
2 with the in vitro data, T_{STEM} cells displayed enhanced control of leukemic burden compared with
3 T_{PEX} cells at multiple time points after ACT (**Fig. 4j**).

4 Collectively, these data indicated that T_{STEM} cells were relatively resistant to exhaustion,
5 facilitating more efficient control of tumor growth in vivo compared with T_{PEX} cells, which were
6 hardwired to a dysfunctional signature.

7
8 **T_{PEX} cells are abundant in persistent infections and clonally distinct from T_{STEM} cells.** CD8⁺
9 T cell dysfunction and exhaustion develop in response to persistent antigenic stimulation via
10 cognate TCRs⁴³. We therefore reasoned that acute viral infections would preferentially generate
11 antigen-specific T_{STEM} cells, whereas chronic viral infections would preferentially generate
12 antigen-specific T_{PEX} cells. A single round of yellow fever virus (YFV) vaccination is known to
13 induce long-lived memory cells with an early differentiated T_{SCM}-like CCR7⁺ CD45RA⁺ (or
14 CD45RO⁻) CD95⁺ phenotype^{44,45}. To determine the composition of these T_{SCM}-like populations
15 in terms of CCR7⁺ PD-1⁻ TIGIT⁻ T_{STEM} and CCR7⁺ PD-1⁺ TIGIT⁺ T_{PEX} cells, we compared
16 publicly available gene expression data from vaccinated individuals⁴⁵ with our RNA-seq data
17 (**Fig. 2c**). In line with our hypothesis, we found that YFV-specific CCR7⁺ CD45RA⁺ CD95⁺
18 cells analyzed years after vaccination were transcriptionally related to T_{STEM} but not T_{PEX} cells
19 (**Fig. 5a**).

20 To extend these findings, we used peptide-HLA class I tetramers in conjunction with
21 mass cytometry (CyTOF) to investigate the phenotypic characteristics of CCR7⁺ CD8⁺ memory
22 T cells specific for acute (influenza virus or rotavirus) or chronic viruses (cytomegalovirus
23 [CMV] or Epstein-Barr virus [EBV]) in healthy donors (n = 3) and HIV⁺ patients (n = 2).

1 Signature markers of T_{PEX} cells, namely PD-1, TIGIT, GZMK, GZMA, and CCR5, were
2 expressed at higher levels among EBV-specific and, to a lesser extent, CMV-specific versus
3 influenza virus-specific and rotavirus-specific CD8⁺ T cells (**Fig. 5b,c** and **Supplementary Fig.**
4 **5a,b**). Chronic virus-specific CD8⁺ T cells also overexpressed 2B4. Moreover, high-avidity
5 CMV-specific CD8⁺ T cell populations, selectively identified using a double point-mutated
6 peptide-HLA class I tetramer⁴⁶, incorporated T_{PEX} cells at frequencies equivalent to those
7 detected among the corresponding total CMV-specific CD8⁺ T cell populations, suggesting that
8 persistent antigenic drive rather than signal strength determined the acquisition and maintenance
9 of dysfunctional, exhausted-like traits⁴³ (**Supplementary Fig. 5c**).

10 In further experiments, we used a high-throughput approach (TCR-seq) to profile the
11 clonotypic repertoires of T_{STEM} and T_{PEX} cells. As expected, these early differentiated subsets
12 exhibited similarly diverse repertoires, measured via the normalized Shannon-Weiner index,
13 whereas the corresponding T_{EM} subsets exhibited comparatively less diverse repertoires (**Fig.**
14 **5d**). An additional estimator based on abundance, the Chao1 index, which accounts for the
15 distribution of infrequent clonotypes, revealed similar trends and further identified greater levels
16 of diversity among the T_{STEM} subsets compared with the T_{PEX} subsets, potentially reflecting
17 distinct broadness of specificities (**Supplementary Fig. 5d**). Although the stem-like subsets both
18 shared clonotypes with the corresponding T_{EM} subsets, minimal repertoire overlap was detected
19 between T_{STEM} and T_{PEX} cells, quantified in terms of the absolute numbers (**Fig. 5e**) or
20 normalized counts of shared clonotypes (**Fig. 5f**), suggesting distinct spectra of recognized
21 antigens. Comparable data were obtained using two additional metrics, F2, which accounts for
22 the size of each clonotype (**Supplementary Fig. 5e**), and R, which estimates correlations of
23 clonotype frequencies (**Supplementary Fig. 5f**).

1 Collectively, these data revealed that T_{STEM} and T_{PEX} cells were clonally distinct and
2 committed to parallel differentiation programs, the relative prevalence of which was determined
3 by the dynamics of antigen exposure within any given specificity (**Fig. 5g**).

1 DISCUSSION

2 In this study, we used an unbiased approach guided by scRNA-seq to capture the
3 extensive heterogeneity that exists in the human CD8⁺ memory T cell pool under physiological
4 conditions. We identified two previously unrecognized subsets of stem-like CD8⁺ memory T
5 cells, neither of which corresponded with previous descriptions of early differentiated
6 progenitors based on the expression of CCR7, CD45RA/RO, and CD95. These subsets were
7 defined by core transcriptional signatures that could be distilled phenotypically into simple
8 profiles, namely CCR7⁺ PD-1⁻ TIGIT⁻ (T_{STEM} cells) and CCR7⁺ PD-1⁺ TIGIT⁺ (T_{PEX} cells).
9 Moreover, the distinct gene expression profiles of T_{SCM} and T_{CM} cells were mostly attributable to
10 the differential inclusion of T_{PEX} cells, indicating a need for refined models to understand the
11 process of human CD8⁺ memory T cell differentiation.

12 T_{STEM} cells proliferated vigorously in response to activation and generated a diverse array
13 of memory and effector progeny, collectively enabling functionally superior immunity in vivo.
14 Of note, the ex vivo frequencies of T_{STEM} cells were sufficient to overcome current limitations
15 associated with the relative paucity of T_{SCM} cells, potentially facilitating immunotherapies that
16 rely on specificity redirection by providing an alternative source of progenitors with self-renewal
17 capabilities and a propensity for effector differentiation. In contrast, T_{PEX} cells were committed
18 to the generation of progeny with reduced functionality and proliferated weakly in response to
19 activation, at least via the TCR. Importantly, T_{STEM} and T_{PEX} cells were also clonally,
20 epigenetically, and transcriptionally distinct, suggesting a branching point in the early memory
21 compartment associated with the initial antigen recognition event(s). This interpretation was
22 supported by the observation that persistent antigenic stimulation was preferentially associated
23 with the development of T_{PEX} cells. On the basis of these findings, we propose a revised model

1 of T cell differentiation, according to which T_{PEX} cells become hardwired to a dysfunctional-like
2 signature after immune activation and effector differentiation, compatible with the generation of
3 a parallel lineage⁴⁷ defined by genome-wide epigenetic modifications^{48,49}, whereas T_{STEM} cells
4 remain multipotent and relatively resistant to exhaustion, resulting in enhanced functionality and
5 protective immunity in vivo. This model will likely need further refinement to accommodate a
6 degree of plasticity within the T_{STEM} subset, given that initially functional CD8⁺ memory T cells
7 can become exhausted as a result of continuous exposure to high-dose antigen in mice
8 chronically infected with LCMV⁵⁰.

9 The acquisition of dysfunctional traits associated with exhaustion was not accompanied
10 by a substantial loss of memory-like features in the T_{PEX} subset. Instead, these characteristics
11 were found to coexist in individual cells, potentially indicating functional adaptation to persistent
12 antigenic stimulation^{3,16}. Such adaptations may be necessary in this context to maintain a diverse
13 repertoire and simultaneously minimize the risk of immunopathology^{28,30,47}. Further studies will
14 nonetheless be required to address these issues in patients with progressive malignancies or
15 uncontrolled viral infections. In contrast, activated T_{STEM} cells coexpressed stem-like and
16 effector genes, consistent with the notion of a functionally sustainable hybrid state³.
17 Accordingly, we propose that T_{STEM} cells represent a naturally occurring lineage with optimal
18 features for the induction of potent long-term immunity.

19 In summary, we have identified two subsets of human stem-like CD8⁺ memory T cell
20 progenitors with distinct fate commitments and lineage relationships. Although further work is
21 required to characterize the molecular mechanisms that underlie the early dichotomy between
22 T_{STEM} and T_{PEX} cells, we anticipate that such efforts will reveal novel targets for therapeutic

1 interventions designed to inhibit or reverse the process of exhaustion, with obvious implications
2 for the treatment of persistent infections and various cancers.

3

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16 **AUTHOR CONTRIBUTIONS**

17 G.G. and E.L. conceived the study; G.G., G.D.S., E.M.C.M., S.P., C.M., T.M.B., A.N.D., M.M., E.S., G.A., F.D.P.,
18 V.Z., A.S., B.C., F.S.C., A.A., C.P., S.P., L.G., R.E.J., D.M.B., E.G., S.L.L., and K.L. performed experiments; G.G.,
19 G.D.S., E.M.C.M., S.P., T.M.B., A.N.D., D.M.B., D.M.C., E.W.N., M.C., and E.L. analyzed data; D.M., S.K.B.,
20 B.A.Y., and D.A.P. provided critical expertise and resources; E.L. supervised the study; G.G., D.A.P., and E.L.
21 wrote the manuscript. All authors contributed intellectually and approved the manuscript.

22 **COMPETING INTERESTS**

23 The Laboratory of Translational Immunology receives reagents in kind as part of a collaborative research agreement
24 with BD Biosciences (Italy). L.G. and E.L. are inventors on a patent describing methods for the generation and
25 isolation of T_{SCM} cells. L.G. has consulting agreements with Lyell Immunopharma and Advaxis Immunotherapies.
26 The other authors have no conflicts of interest to disclose.

1

2 **ADDITIONAL INFORMATION**

3 Supplementary Figs. 1 to 5

4 Supplementary Tables 1 to 7

5

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FIGURE LEGENDS

Fig. 1. Heterogeneity of the human CD8⁺ memory T cell pool. **a**, Strategy for the isolation of CD8⁺ memory T cells from PB via FACS. **b**, UMAP plot showing the distribution of 31,640 cells (n = 4 donors). Cluster labels indicate selected DEGs. **c**, Histogram plot showing the median frequency of each cluster obtained in **b**. The dashed line is set at 1%. **d**, Balloon plot showing the average expression levels and expression frequencies of selected genes in each cluster obtained in **b**. **e**, Balloon plot showing the average expression levels and expression frequencies of selected genes in C2 versus C6. **f**, Bivariate plots depicting transcriptional module scores correlated with specific genes for selected clusters obtained in **b**. **g**, Flow cytometric gating strategy for the identification of CD8⁺ memory T cell subsets. Representative data are shown from PB. **h**, UMAP plot showing the expression of selected markers among CD8⁺ memory T cells isolated from different tissues (n = 6 donors per tissue with matched peripheral blood samples). Top left: overlays of the cell populations identified in **g**. **i**, Dot plot showing the tissue-specific frequencies of each subset identified in **g**. PB, peripheral blood; LN, lymph node; BM, bone marrow. Each dot represents one donor (n = 6 per tissue with matched peripheral blood samples). Bars indicate mean ± SEM. Statistics were calculated only for the GZMK⁻ and GZMK⁺ populations. **P* < 0.05, ***P* < 0.01 (two-tailed unpaired t-test for GZMK⁺ in PB versus LN and lung versus LN, two-tailed Mann-Whitney U test for all other comparisons).

Fig. 2. Identification of stem-like CD8⁺ memory T cell progenitors with differential expression of GZMK, PD-1, and TIGIT. **a**, Flow cytometric gating strategy for the identification of PD-1⁺ TIGIT⁺ cells in the CD8⁺ naive (CCR7⁺ CD45RO⁻ CD95⁻), T_{SCM} (CCR7⁺ CD45RO⁻ CD95⁺), and T_{CM} compartments (CCR7⁺ CD45RO⁺ CD95⁺). Numbers indicate percentages in the drawn gates. **b**, Dot plot summarizing the data obtained as in **a**. Each

1 dot represents one donor (n = 20 from two independent experiments). Bars indicate mean ±
2 SEM. **** $P < 0.0001$ (one-way repeated measures ANOVA). **c**, Heatmap showing DEGs
3 (adjusted P value < 0.01) for the indicated CD8⁺ memory T cell subsets (n = 5 donors). Labels
4 highlight genes associated with memory or effector differentiation or exhaustion. Significance
5 was evaluated using edgeR analysis with glmQLFTest and Benjamini-Hochberg correction. **d**,
6 Proliferation of the indicated CD8⁺ memory T cell subsets in response to stimulation with anti-
7 CD3 plus CD28 for 4 d in the presence of IL-2 and IL-12 (n = 11 donors from six independent
8 experiments) or IL-7 and IL-15 (n = 5 donors from three independent experiments). Index
9 calculations were based on the dilution of carboxyfluorescein succinimidyl ester (CFSE). Each
10 dot represents one donor. Bars indicate mean ± SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (two-
11 tailed Wilcoxon signed rank test for each population versus T_{EM} in the presence of IL-2 and IL-
12 12, two-tailed paired t-test for all other comparisons). **e**, Representative flow cytometric analysis
13 of T_{STEM} (CCR7⁺ PD-1⁻ TIGIT⁻) and T_{PEX} cells (CCR7⁺ PD-1⁺ TIGIT⁺) showing the expression
14 of markers selected from the DEGs identified in **c**. Numbers indicate percentages in the drawn
15 gates. Similar data were obtained from other donors (n = 5 for LEF1 and CCR5, n = 4 for CD26
16 and Eomes). **f**, GSEA based on 1,000 permutations showing manually curated signatures that
17 differed significantly (adjusted P value < 0.05) between T_{STEM} and T_{PEX} cells. NES, normalized
18 enrichment score.

19
20 **Fig. 3. Functional properties of T_{STEM} and T_{PEX} cells.** **a**, Representative flow cytometric
21 analysis of FACS-purified T_{STEM}, T_{PEX}, and T_{EM} cells showing the expression of CD25 and
22 CD69 before (us, unstimulated) and after stimulation with SEB for 24 h. Similar data were
23 obtained from other donors (n = 4). Numbers colored to match each subset indicate percent

1 marker⁺ cells with the corresponding median fluorescence intensity (MFI) in brackets. **b**, Dot
2 plot showing the expression of T-bet among CD25⁺ CD69⁺ T_{STEM}, T_{PEX}, and T_{EM} cells before
3 (us) and after stimulation as in **a**. Data are shown in terms of MFI. Each dot represents one donor
4 (n = 5 from two independent experiments). Bars indicate mean ± SEM. **P* < 0.05 (two-tailed
5 paired t-test). **c**, Representative flow cytometric analysis showing the expression of CD107a,
6 IFN-γ, IL-2, and TNF among T_{STEM}, T_{PEX}, and T_{EM} cells stimulated with anti-CD3 plus CD28
7 for 12 h. Numbers indicate percentages in the drawn gates. **d**, Dot plot summarizing the data
8 obtained as in **c**. Each dot represents one donor (n = 7 from four independent experiments). Bars
9 indicate mean ± SEM. ***P* < 0.01 (two-tailed paired t-test). **e**, Dot plot showing the function⁺
10 populations identified in **d** in terms of MFI. Each dot represents one donor (n = 3 from four
11 independent experiments for CD107a, n = 7 from four independent experiments for IFN-γ, IL-2,
12 and TNF). Bars indicate mean ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (two-tailed paired t-
13 test). **f**, Representative flow cytometric analysis showing the expression of CD107a, IFN-γ, IL-2,
14 and TNF among T_{STEM}, T_{PEX}, and T_{EM} cells after stimulation of magnetically-enriched CD8⁺ T
15 cells with PMA and ionomycin for 3 h. Numbers indicate percentages in the drawn gates.
16 Subsets were gated as CCR7⁺ GZMK⁻ (T_{STEM}), CCR7⁺ GZMK⁺ (T_{PEX}), or CCR7⁻ CD45RO⁺
17 CD95⁺ (T_{EM}). **g**, Dot plot summarizing the data obtained as in **f**. Each dot represents one donor
18 (n = 6 from three independent experiments). Bars indicate mean ± SEM. **P* < 0.05; ***P* < 0.01;
19 ****P* < 0.001 (two-tailed paired t-test). **h**, Schematic layout of the serial transfer experiments. **i**,
20 Line chart showing the absolute numbers of CD8⁺ T cells in PB on days 11, 18, and 28 after
21 transfer of T_{STEM} or T_{PEX} cells into secondary NSG recipients. Data were pooled from two
22 independent experiments (total n = 8 mice). Bars indicate mean ± SEM. **P* = 0.0117 (two-way
23 ANOVA). **j**, Dot plot showing the absolute numbers of CD8⁺ T cells in spleen on day 28 after

1 transfer of T_{STEM} or T_{PEX} cells into secondary NSG recipients. Each dot represents one mouse (n
2 = 8 from two independent experiments). Bars indicate mean ± SEM. **P* = 0.0148 (two-tailed
3 Mann-Whitney U test).

4
5 **Fig. 4. Fate commitments of T_{STEM} and T_{PEX} cells.** **a**, PCA plot showing the top 1,000
6 hypervariable peaks obtained from *ex vivo* ATAC-seq analysis (adjusted *P* value < 0.01) of
7 T_{STEM}, T_{PEX}, and T_{EM} cells. Each dot represents one donor (n = 3). **b**, Heatmap showing DARs.
8 Labels highlight accessible genes associated with memory or effector differentiation or
9 exhaustion. **c**, Representative genomic regions showing the ATAC-seq profiles of *TOX*, *TIGIT*,
10 and *SATB1* in T_{STEM} and T_{PEX} cells. DARs are highlighted in purple. **d**, TFBMs enriched among
11 the DARs shown in **b**. Enrichment was assessed using a one-sided hypergeometric test in
12 HOMER with correction for FDR. **e**, Representative overlay histograms showing CFSE dilution
13 (left) and GZMB expression profiles (right) for T_{STEM} and T_{PEX} cells stimulated with anti-CD3
14 plus CD28 and IL-15 for 3 d in the absence or presence of TGF-β. Unstimulated controls are
15 shown for comparison. Similar data were obtained from other donors in the absence (n = 8 from
16 four independent experiments) or presence of TGF-β (n = 6 from four independent experiments).
17 PI, proliferation index. **f**, Representative flow cytometric analysis of T_{STEM} and T_{PEX} cells
18 showing the expression of PD-1 and TIGIT after stimulation with anti-CD3 plus CD28 for 4 d in
19 the presence of IL-2 and IL-12. Numbers indicate percentages in the drawn gates. **g**, Bar graph
20 summarizing the data obtained as in **f** (n = 5 donors from three independent experiments). Bars
21 indicate mean ± SEM. ***P* < 0.01, ****P* < 0.001 (two-tailed Mann-Whitney U test). **h**, Heatmap
22 showing selected DEGs (adjusted *P* value < 0.05) for T_{STEM} and T_{PEX} cells stimulated as in **f** (n =
23 4 donors). Significance was evaluated using edgeR analysis with glmQLFTest and Benjamini-

1 Hochberg correction. **i**, PCA plot as in **a** comparing T_{STEM} and T_{PEX} cells before and after
2 stimulation as in **f**. **j**, Top: schematic layout of the adoptive transfer experiment. Bottom: time
3 series plot showing the growth of NALM6 cells in NSG mice (n = 5/group) adoptively
4 transferred with T_{STEM} or T_{PEX} cells expressing a CAR specific for CD19 (CAR19). RLU,
5 relative light unit; UT, untransduced CD3⁺ cells. Follow-up was stopped when RLU values $\geq 10^6$
6 were observed in more than 75% of mice in one of the treated groups. Bars indicate mean \pm
7 SEM. **P* < 0.05 (two-tailed unpaired t-test for CAR19 T_{STEM} versus CAR19 T_{PEX} on day 17,
8 two-tailed Mann-Whitney U test for all other comparisons).

9
10 **Fig. 5. Antigen specificity and clonal identity of T_{STEM} and T_{PEX} cells.** **a**, GSEA of the YFV-
11 specific CD8⁺ memory T cell signature⁴⁵ in T_{STEM} versus T_{PEX} cells. **b**, Representative CyTOF
12 analysis showing the expression of GZMK, GZMA, PD-1, TIGIT, CCR5, and 2B4 among
13 CCR7⁺ virus-specific CD8⁺ T cell populations from healthy (n = 3) and HIV⁺ donors (n = 2). **c**,
14 Dot plots summarizing the data obtained as in **b**. Epitopes derived from influenza virus (n = 6)
15 and rotavirus (n = 1) were pooled for simplicity. Comparative data are shown for the
16 corresponding total CD8⁺ T cell populations. Each dot represents one specificity in one donor (n
17 = 3 healthy donors, n = 2 HIV⁺ donors). Bars indicate mean \pm SEM. **P* < 0.05, ***P* < 0.01, ****P*
18 < 0.001 (two-tailed Mann-Whitney U test for CCR5 comparisons, two-tailed unpaired t-test for
19 all other marker comparisons). HD, healthy donor. **d**, Dot plot showing the normalized Shannon-
20 Wiener index for TCR β repertoires obtained from the T_{STEM}, T_{PEX}, and T_{EM} subsets. Each dot
21 represents one donor (n = 6). Bars indicate median values. ***P* < 0.01 (two-tailed paired t-test
22 with Bonferroni correction). **e**, Venn diagram showing the numbers of shared and unique
23 clonotypes among T_{STEM}, T_{PEX}, and T_{EM} cells from a representative donor. Similar data were

1 obtained from other donors ($n = 5$). Analysis was restricted to the top 3,000 clonotypes. **f**, Dot
2 plot summarizing the pairwise comparisons among subsets illustrated in **e**. D metric in VDJtools.
3 Each dot represents one donor ($n = 6$). Bars indicate median values. $*P < 0.05$, $**P < 0.01$ (two-
4 tailed paired t-test with Bonferroni correction). **g**, Proposed model showing the origins and
5 differentiation trajectories of T_{STEM} and T_{PEX} cells.

6

1 **METHODS**

2 **Study approval.** The use of human samples was approved by the Humanitas Clinical and Research Center
3 Institutional Review Board under the following protocols: buffy coats from healthy donors (28/01/2016), LNs and
4 PB from patients with head and neck cancer (700/2010), adjacent cancer-free lung tissue and PB from patients with
5 non-small cell lung cancer (1501), and BM and PB from healthy donors (1397). Healthy and HIV⁺ donors from the
6 Fred Hutchinson Cancer Research Center were obtained via the HIV Vaccine Trials Network (HVTN). All donors
7 provided written informed consent in accordance with the Declaration of Helsinki. Mice were housed and bred in a
8 specific pathogen-free animal facility and treated in accordance with the European Union Guideline on Animal
9 Experiments. Mouse protocols were approved by the Italian Ministry of Health, the Humanitas Institutional Animal
10 Care and Use Committee (256/2015-PR), and the San Raffaele Institutional Animal Care and Use Committee (646).

11 **Cells.** Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats via density gradient
12 separation and either used fresh or cryopreserved in fetal bovine serum (FBS) supplemented with 10% dimethyl
13 sulfoxide (DMSO). Tissue samples were processed as described previously^{16,51,52}. Total CD8⁺ T cells were enriched
14 via negative magnetic separation using an EasySep Human CD8⁺ T Cell Isolation Kit (Stem Cell Technologies) or a
15 MojoSort Human CD8⁺ T Cell Isolation Kit (BioLegend). Total CD3⁺ T cells were enriched via negative magnetic
16 separation using a MojoSort Human CD3⁺ T Cell Isolation Kit (BioLegend). CD8-depleted PBMCs were obtained
17 via negative magnetic separation using CD8 MicroBeads (Miltenyi Biotec). The human NALM-6 cell line (DSMZ)
18 was tested for *Mycoplasma* (Eurofins Genomics) and transduced with a lentiviral vector encoding secreted luciferase
19 (Lucia⁺/NGFR⁺/NALM-6)⁵³.

20 **Flow cytometry and cell sorting.** High-dimensional flow cytometry was performed as described
21 previously⁵⁴. Dead cells were excluded from all analyses using Zombie Aqua (BioLegend). Fluorochrome-
22 conjugated monoclonal antibodies were purchased from commercial vendors (**Supplementary Table 6**). All
23 reagents were titrated prior to use to determine optimal concentrations. Cells were fixed/permeabilized for
24 intracellular analyses using a Cytotfix/Cytoperm Kit (BD Biosciences). Transcription factors and intranuclear
25 molecules were measured in conjunction with a FoxP3 Transcription Factor Staining Buffer Set (eBioscience) or a
26 Transcription Factor Buffer Set (BD Biosciences). Cell proliferation was determined by measuring the progressive
27 dilution of CFSE (Thermo Fisher Scientific). The proliferation index (PI) was calculated as follows: (MFI of the
28 non-proliferating fraction / MFI of the proliferating fraction) × (% cells with diluted CFSE)¹⁶. Samples were

1 acquired using a FACSymphony A5 or an LSR Fortessa equipped with FACSDiva software version 8.0.1 (all from
2 BD Biosciences). Electronic compensation was performed using single-stained controls prepared with antibody-
3 capture beads (BD Biosciences)⁵⁴. T cell subsets were sorted to purity using a FACSAria III (BD Biosciences) as
4 shown in **Supplementary Fig. 1a**.

5 Flow cytometry standard (FCS) 3.0 files were imported into FlowJo software version 9 (FlowJo LLC). A
6 conventional gating strategy was used to remove aggregates and dead cells, and 5,000 CD95⁺ bulk CD8⁺ memory T
7 cells per sample (**Supplementary Table 1**) were exported into FlowJo software version 10 (FlowJo LLC). Data
8 were then biexponentially transformed and exported for further analysis in Python version 3.7.3 using a custom-
9 written script incorporating PhenoGraph retrieved from the scikit-learn package
10 (<https://github.com/luglilab/Cytophenograph>). Tissue samples were labeled with a unique computational barcode for
11 further identification, converted into comma separated (CSV) files, and concatenated in a single matrix using the
12 merge function in pandas (<https://pandas.pydata.org/>). The K value, indicating the number of nearest neighbors
13 identified in the first iteration of the algorithm, was set to 1,000. UMAP was retrieved from Python. Data were
14 visualized using FlowJo version 10 (FlowJo LLC).

15 **Peptide-HLA class I tetramers.** Biotinylated wildtype and D227K/T228A (KA) HLA-A*0201 complexes
16 refolded with CMV pp65₄₉₅₋₅₀₃ NLVPMVATV (NV9) were multimerized with streptavidin-PE (Sigma-Aldrich) as
17 described previously⁵². Cells were stained with each tetramer at a concentration of 5 µg/ml for 15 min at 37°C.

18 **Mass cytometry by time of flight (CyTOF).** Cryopreserved PBMCs from three healthy donors and two
19 HIV⁺ donors were obtained from the HVTN. Purified monoclonal antibodies were purchased from commercial
20 vendors (**Supplementary Table 7**) and labeled according to the Maxpar Antibody Labeling Kit Protocol (Fluidigm).
21 Streptavidin was produced and labeled as described previously⁵⁵. Myc-tagged peptide-HLA class I monomers were
22 synthesized and biotinylated as described previously (**Supplementary Table 7**)⁵⁶. Peptide-HLA class I tetramers
23 were generated via the addition of heavy metal-labeled streptavidins in a triple coding scheme and used to stain cells
24 in a cocktail format as described previously⁵⁵. All reagents were titrated prior to use to determine optimal
25 concentrations. Antibody staining, live/dead discrimination, and DNA staining were performed as described
26 previously⁵⁷.

27 **Cell culture and stimulation conditions.** Cells were cultured in RPMI 1640 medium supplemented with
28 10% FBS, 1% penicillin/streptomycin, and 2 mM L-glutamine. To induce cytokine production, magnetically-

1 enriched CD8⁺ T cells were stimulated for 3 h with phorbol 12-myristate 13-acetate (PMA, 10 ng/ml) and
2 ionomycin (500 ng/ml) (both from Sigma-Aldrich) in the presence of anti-CD107a (clone H4A3, BD Biosciences)
3 and the protein transport inhibitors GolgiPlug (brefeldin A, 1 µl/ml, BD Biosciences) and GolgiStop (monensin,
4 0.67 µl/ml, BD Biosciences). Subsets were identified among bulk CD8⁺ memory T cells by gating as specified in the
5 legend to **Fig. 3f**. Alternatively, FACS-purified CD8⁺ T cell subsets were stimulated for 12 h with anti-CD3 plus
6 CD28 DynaBeads (bead-to-cell ratio 1:2, Thermo Fisher Scientific). To evaluate the expression of activation
7 markers and T-bet, FACS-purified CD8⁺ T cell subsets were mixed with CD8-depleted autologous PBMCs (cell-to-
8 cell ratio 1:4) and stimulated for 24 h with SEB (1 µg/ml, Sigma-Aldrich). To evaluate differentiation and
9 proliferation, FACS-purified CD8⁺ T cell subsets were stimulated for 3 or 4 d with anti-CD3 plus CD28 DynaBeads
10 (bead-to-cell ratio 1:2, Thermo Fisher Scientific) in combination with various cocktails of human cytokines,
11 including TGF-β, IL-2, IL-7, IL-12, and IL-15 (each at 10 ng/ml, Peprotech). To evaluate self-renewal capacity,
12 FACS-purified CD8⁺ T cell subsets were stimulated for 10 d with IL-15 (25 ng/ml, Peprotech). Unstimulated
13 samples were used as controls in all assays.

14 **scRNA-seq.** FACS-purified CD95⁺ CD8⁺ T cells were resuspended in 1 ml of PBS^{-/-} containing 0.04%
15 bovine serum albumin, washed twice by centrifugation at 450 rcf for 7 min, resuspended in 100 µl of the same
16 medium, and counted using a Countess II Automatic Cell Counter (Thermo Fisher Scientific). Approximately
17 20,000 cells from each sample were then loaded into one channel of Single Cell Chip A using a Chromium Single
18 Cell 3' v2 Reagent Kit (10X Genomics). After capture and lysis, cDNA was synthesized and amplified over 14
19 cycles according to the manufacturer's protocol (10X Genomics). Libraries were prepared from 50 ng of amplified
20 cDNA. Sequencing was performed using a NovaSeq 6000 System (Illumina). An average sequencing depth of at
21 least 50,000 reads/cell was obtained for each sample.

22 **scRNA-seq data analysis.** Sample demultiplexing, barcode processing, and unique molecular identifier
23 (UMI) counting were performed using Cell Ranger version 2.1.1 (10X Genomics). Briefly, raw base call files were
24 demultiplexed in FASTQ format using the "cellranger mkfastq" pipeline, and the "cellranger count" pipeline was
25 run with "--transcriptome=refdata-cellranger-GRCh38-1.2.0" for each sample. Outputs from "cellranger count" were
26 concatenated in a single matrix. Libraries were then normalized to an identical sequencing depth using the
27 "cellranger aggr" pipeline. Pooled data were imported into R version 3.5.1 using Seurat version 3.0.1 (ref. 58).
28 Genes detected in less than three cells or cells containing less than 200 features were excluded from the analysis.

1 Cells with unique feature counts less than 200 or greater than 3,500 were also filtered out, along with cells
2 containing mitochondrial counts above 10%. The resulting dataset was normalized using a global scaling method
3 converted by a scale factor (10,000) and log-transformed using the “ScaleData” function in Seurat version 3.0.1.
4 Data were then subjected to cluster analysis using standard package procedures and the “FindClusters” function in
5 Seurat version 3.0.1. Parameters were set to the first 20 principal components and a resolution of 0.6. DEGs for each
6 cluster were identified using the Wilcoxon rank sum test with default correction for multiple comparisons in Seurat
7 version 3.0.1.

8 **Anchor gene analysis of memory cell clusters from scRNA-seq data.** Six different gene modules were
9 computed from the scRNA-seq dataset using selected genes as anchors. Transcriptional scores were built by
10 calculating the mean expression profiles of the top 100 genes most correlated with the anchors (Pearson’s
11 correlation).

12 **Bulk RNA-seq.** RNA was extracted from 50,000 FACS-purified CD8⁺ T cells per subset using a Direct-Zol
13 RNA Microprep Kit (Zymo Research) and stored at -80°C. Quality control was performed using a High Sensitivity
14 RNA ScreenTape Assay with a 4200 TapeStation System (Agilent). Libraries for mRNA sequencing were prepared
15 from 5 ng of total RNA using the SMART-Seq v4 Ultra Low Input RNA Kit (Clontech-Takara). Full-length cDNAs
16 were processed using a Nextera XT DNA Library Preparation Kit (Illumina). Quality control was performed using a
17 High Sensitivity DNA ScreenTape Assay with a 4200 TapeStation System (Agilent). Libraries were then
18 multiplexed in an equimolar pool and sequenced using a NextSeq 500/550 Platform (Illumina). An average of 11
19 million single-end 75 base pair (bp) reads were generated per sample. Libraries for total RNA sequencing were
20 prepared from 1 ng of total RNA using a SMART-Seq Stranded Kit (Clontech-Takara). Quality control and
21 sequencing were performed as described for the mRNA libraries, generating an average of 103 million paired-end
22 75 bp reads per sample.

23 **Bulk RNA-seq data analysis.** Raw sequence data were quality-controlled using FastQC version 0.11.8
24 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc>). Single-end reads were aligned to the human genome
25 (GENCODE Human Release 29, reference genome sequence GRCh38/hg38) using STAR version 2.5.1b (ref. 59).
26 Alignments were performed using default parameters. Reads associated with annotated genes were counted using
27 the STAR aligner option “-quantMode geneCounts”. Differential gene expression was assessed using the edgeR
28 package version 3.20.9. Benjamini-Hochberg correction was applied to estimate the false discovery rate (FDR).

1 Paired-end reads were processed similarly after removing adapter sequences and poor-quality bases with
2 Trimmomatic version 0.36.

3 **Overrepresentation analysis.** GSEA was applied to the entire list of genes in the RNA-seq expression
4 matrix. Genes were ranked based on log₂ fold changes calculated using the edgeR package version 3.20.9. GSEA
5 was performed in preranked mode using a “classic” enrichment statistic. Gene sets of interest were retrieved from
6 collections C2 and C7 in the Molecular Signatures Database version 6.2 and integrated with those corresponding to
7 exhausted T cell clusters G6 and G9 in Sade-Feldman *et al.*¹⁸ or with those obtained via a reanalysis of the dataset in
8 Akondy *et al.*⁴⁵.

9 **Enrichment analysis.** Normalized scRNA-seq counts were downloaded from the Gene Expression Omnibus
10 (GSE120575). Analysis was restricted to cells belonging to clusters G5 or G10 as defined in Sade-Feldman *et al.*¹⁸.
11 DEGs with an adjusted *P* value < 0.01 in the pairwise comparisons of G5 versus G10 were identified using the
12 “FindAllMarkers” function in Seurat version 3.0.1. Hypergeometric tests were used to compare the G5 or G10
13 signatures with the combined T_{SCM}/T_{CM} or T_{PEX} signatures in “phyper” R.

14 **Microarray data analysis.** Normalized data matrices from Akondy *et al.*⁴⁵ were downloaded from GEO:
15 GSE26347. To identify the signatures “YF_naive vs. effector UP” and “YF_effector vs. naive UP”, the expression
16 profiles of effector CD8⁺ T cells isolated 14 d after vaccination with YF-17D (GSM837587, GSM837588,
17 GSM837589, and GSM837590) were compared with those of naive CD8⁺ T cells (GSM837584, GSM837585, and
18 GSM837586) using the limma algorithm in R version 3.34.9 (ref. 60). The gene set of interest arbitrarily included
19 the top 200 genes with the highest log fold change among DEGs with an adjusted *P* value < 0.05. All samples
20 hybridized on the Human Genome U133 Plus 2.0 Array (the second set of samples from the GSE26347 dataset)
21 were concatenated with those hybridized on the HT Human Genome U133A Array (the third set of samples from the
22 GSE26347 dataset). Probe sets were matched on both chips. Batch effects were eliminated by adjusting gene
23 expression values in the combined data matrix with the empirical Bayes method ComBat in SVA version 3.26.0 (ref.
24 61). ComBat was applied with default parameters, except for the adjustment variables, which were imputed as a
25 vector of platform type labels. To identify genes comprising the “YFV-specific memory cell signature” shown in
26 **Fig. 5a**, the expression profiles of YF-17D virus NS4B-214 epitope-specific human CD8⁺ memory T cells
27 (GSM837594, GSM837595, GSM837596, GSM837597, GSM837598, and GSM837599) were compared with those
28 of YFV-tetramer⁺ effector CD8⁺ T cells (GSM837587, GSM837588, GSM837589, and GSM837590) using the

1 limma algorithm, arbitrarily selecting the top 200 genes with the highest log fold change among DEGs with an
2 adjusted P value < 0.05 .

3 **ATAC-seq.** Libraries were prepared using a protocol adapted from Buenrostro *et al.*⁶². Briefly, 50,000
4 FACS-purified CD8⁺ T cells per subset were washed in PBS^{-/-} and resuspended in 50 μ l of lysis buffer (10 mM
5 Tris-HCl pH 7.4, 10 mM MgCl₂, 0.1% IPEGAL CA-630). Nuclei were pelleted by centrifugation for 10 min at 500
6 g and resuspended in a final reaction volume of 50 μ l comprising 1 μ l of Tn5 transposase (made in-house), 10 μ l of
7 5x transposase buffer (50 mM Tris-HCl pH 8.4, 25 mM MgCl₂), and 39 μ l of ultrapure water (Milli-Q). The reaction
8 was incubated with mixing at 300 rpm for 30 min at 37°C, supplemented with 10 μ l of clean-up buffer (900 mM
9 NaCl, 30 mM EDTA), 5 μ l of 20% SDS, 0.7 μ l of ultrapure water (Milli-Q), and 4.3 μ l of proteinase K (18.6 μ g/ μ l,
10 Thermo Fisher Scientific), and incubated for a further 30 min at 40°C. Tagmented DNA was isolated using 2x SPRI
11 Beads (Beckman Coulter) and amplified via PCR. Fragments smaller than 600 bp were isolated via negative size
12 selection using 0.65x SPRI Beads (Beckman Coulter) and purified using 1.8x SPRI Beads (Beckman Coulter).
13 Quality control was performed using a 4200 TapeStation System (Agilent) in conjunction with a Qubit 2.0
14 Fluorometer (Thermo Fisher Scientific). Libraries were then multiplexed in an equimolar pool and sequenced using
15 a NextSeq 500/550 Platform (Illumina). At least 20 million single-end 75 bp reads were generated per sample.

16 **ATAC-seq data analysis.** Read quality was assessed using FastQC version 0.11.8
17 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc>). Adaptors and poor-quality bases were trimmed using
18 Cutadapt version 1.16 (ref. 63). Samples were aligned to the human reference genome GRCh38 using default
19 parameters in BWA-MEM version 0.7.17. Mitochondrial reads were removed using SAMtools version 1.9 (ref. 64).
20 PCR duplicates were removed using the “MarkDuplicates” function in Picard Tools version 2.19
21 (<http://broadinstitute.github.io/picard/>). Open chromatin was detected using MACS2 version 2.1.2 (ref. 65) with an
22 FDR < 0.01 . The number of reads in each peak was determined using featureCounts version 1.6.4 (ref. 66).
23 Differentially accessible peaks were identified using an FDR cut-off below 0.05 after normalization in DESeq2
24 version 1.20 (Bioconductor). Peaks were annotated using the “annotatePeaks.pl” function and scanned for motifs
25 using the “findMotifsGenome.pl” function in HOMER version 4.9.1.

26 **Single telomere length analysis.** DNA was extracted from 6,000 FACS-purified CD8⁺ T cells per subset
27 using a QIAamp DNA Micro Kit (Qiagen). Single telomere length analysis was carried out at the XpYp telomere as
28 described previously⁶⁷. Briefly, genomic DNA was eluted in 35 μ l of Tris (10 mM) containing 0.75 μ l of the

1 Telorette-2 linker (10 μ M). Multiple PCRs were then performed for each test DNA. Each reaction was set up in a
2 final volume of 10 μ l containing 1 μ l of DNA and 0.5 μ M of the telomere-adjacent and teltail primers in 75 mM
3 Tris-HCl pH 8.8, 20 mM (NH₄)₂SO₄, 0.01% Tween-20, and 1.5 mM MgCl₂, with 0.5 U of a 10:1 mixture of Taq
4 (Thermo Fisher Scientific) and Pwo polymerase (Roche Molecular Biochemicals). The reactions were processed in a
5 Tetrad2 Thermal Cycler (Bio-Rad). DNA fragments were resolved via 0.5% Tris-acetate-EDTA agarose
6 gel electrophoresis and identified via Southern hybridization with a random-primed anti-³²P-labeled (PerkinElmer)
7 TTAGGG repeat probe, together with probes specific for molecular weight markers at 1 kb (Stratagene) and 2.5 kb
8 (Bio-Rad). Hybridized fragments were detected using a Typhoon FLA 9500 Phosphorimager (GE Healthcare). The
9 molecular weights of the DNA fragments were calculated using a Phoretix 1D Quantifier (Nonlinear Dynamics).

10 **TCR-seq.** Total RNA was extracted separately from duplicate vials of 150,000 FACS-purified CD8⁺ T cells
11 per subset using an RNeasy Mini Kit (Qiagen). UMI-labeled 5' RACE TCR β sequencing libraries were prepared
12 using a Human TCR Profiling Kit (MiLaboratory LLC). Libraries were prepared in parallel using the same number
13 of PCR cycles and sequenced using a NextSeq 500/550 High-Output Kit with a NextSeq 500 Platform (Illumina).
14 Approximately 3×10^7 reads were obtained in total and assembled into 2.2×10^6 UMI-labeled cDNA molecules (up
15 to 10^5 per library). UMI extraction and consensus assembly were performed using MIGEC software version 1.2.9
16 (ref. 68) with a threshold of at least three reads per UMI. In-frame CDR3 β repertoires were extracted using MiXCR
17 software version 3.0.3 (ref. 69). Each library contained from 3,000 to 18,000 functional CDR3 β clonotypes.
18 Diversity metrics were calculated using VDJtools software version 1.2.1 (ref. 70) after normalization to 42,000
19 randomly selected UMIs per sample. D, R, and F2 metrics were calculated for the top 3,000 clones from each pair of
20 samples using VDJtools software version 1.2.1 (ref. 70).

21 **T cell transduction and culture conditions.** FACS-purified CD8⁺ T cell subsets were stimulated with a
22 MACS-GMP T Cell TransAct Cocktail (Miltenyi Biotec). Stimulated cells were transduced the following day with a
23 bidirectional lentiviral vector encoding a CD19-specific CAR with a CD28 costimulus in sense and the LNGFR
24 marker gene in antisense and then cultured for 13 d in TexMACS Medium (Miltenyi Biotec) supplemented with 3%
25 FBS, 1% penicillin/streptomycin, IL-7 (25 U/ml, Miltenyi Biotec), and IL-15 (50 U/ml, Miltenyi Biotec).
26 Magnetically purified CD3⁺ cells were processed similarly for control purposes.

27 **Mouse studies.** T_{STEM} and T_{PEX} cells were isolated from the PB of healthy donors based on differential
28 expression of PD-1. Eight-week-old female NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice (Charles River) were

1 infused retroorbitally with FACS-purified T_{STEM}, T_{PEX} or T_{EM} cells (1×10^6 per mouse) and autologous CD8⁻
2 PBMCs (6×10^6 per mouse) and sacrificed on day 28. To maximize recovery, spleen and lung cells were mixed
3 from the same experimental group, normalized in terms of the CD4 to CD8 ratio, and injected as above into
4 secondary NSG recipients (1×10^6 CD8⁺ T cells per mouse). Spleens were harvested on day 28 and processed to
5 single cell suspensions. Absolute numbers of T cells in blood were determined using CountBright Absolute
6 Counting Beads (Thermo Fisher Scientific). The frequencies of human CD4⁺ and CD8⁺ T cell subsets were
7 determined by flow cytometry. For tumor experiments, eight-week-old female NSG mice were injected
8 intravenously with 0.5×10^6 Lucia⁺/NGFR⁺/NALM-6 cells. After 4 d, mice were further injected with 3×10^6
9 CAR19-redirected T_{STEM}, T_{PEX}, or total CD3⁺ T cells. Untransduced CD3⁺ T cells were used as controls. Tumor
10 progression was monitored weekly via bioluminescence detection using QUANTI-Luc (InvivoGen) and expressed
11 as relative light units (RLUs).

12 **Statistics.** Statistical analyses were performed using Prism version 7.0c (GraphPad) or R software version
13 3.4.4. Significance was assigned at $P < 0.05$ unless stated otherwise. Specific tests are indicated in the relevant
14 figure legends.

15 **Reporting summary.** Further information on research design is available in the Nature Research Reporting
16 Summary linked to this article.

17 **Data availability.** Publicly available scRNA-seq data were retrieved from the Gene Expression Omnibus via
18 accession code GSE120575 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE120575>). Microarray data
19 from YFV-17D-specific CD8⁺ T cells were retrieved from the Gene Expression Omnibus via accession code
20 GSE26347 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE26347>). Gene sets of interest were retrieved
21 from the Molecular Signatures Database (<http://www.broadinstitute.org/gsea/msigdb/index.jsp>). The ATAC-seq data
22 reported in this paper are available on request. The bulk RNA-seq and scRNA-seq data reported in this paper have
23 been deposited in the Gene Expression Omnibus under accession code GSE147398. The TCR-seq data reported in
24 this paper have been deposited at the European Bioinformatic Institute under accession code E-MTAB-8892. All
25 other data that support the findings of this study are available on request from the corresponding author.

26 **Code availability.** Scripts used to analyze the ATAC-seq data are available at
27 https://github.com/luglilab/SP018_CD8_Galletti_et_al. All other codes are available on request.

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1 **Supplementary Fig. 1. Strategy for the isolation of T cell subsets via FACS. a,** Flow
2 cytometric gating strategy for the isolation of CD8⁺ naive, T_{STEM}, T_{SCM} PD-1⁻ TIGIT⁻, T_{CM} PD-
3 1⁻ TIGIT⁻, T_{PEX}, and T_{EM} cells. **b,** Representative flow cytometric analysis of early differentiated
4 CD8⁺ memory T cells showing the expression of PD-1 and TIGIT.

5

1 **Supplementary Fig. 2. Transcriptomic comparison of T_{SCM} and T_{CM} cells after depletion of**
2 **T_{PEX} cells.** Heatmap showing DEGs (adjusted *P* value < 0.01) for the indicated CD8⁺ memory T
3 cell subsets (n = 3 donors for T_{EM}, n = 5 donors for T_{SCM}, T_{CM}, and T_{PEX}). Significance was
4 evaluated using edgeR analysis with glmQLFTest and Benjamini-Hochberg correction.

5

1 **Supplementary Fig. 3. Proliferation and self-renewal capabilities of CD8⁺ T cell subsets. a,**

2 Dot plot showing proliferation indices for the indicated FACS-purified CD8⁺ T cell subsets after

3 stimulation for 10 d with IL-15. Each dot represents one donor (n = 6 from four independent

4 experiments). Bars indicate mean \pm SEM. **b,** Dot plot showing the expression of selected

5 markers among the indicated CFSE^{dim} CD8⁺ T cell subsets after stimulation as in **a**. Each dot

6 represents one donor (n = 5 from four independent experiments for T_{EM}, n = 6 from four

7 independent experiments for all other subsets). Bars indicate mean \pm SEM. **P* < 0.05, ***P* <

8 0.01, ****P* < 0.001, *****P* < 0.0001 (two-way ANOVA). **c,** Dot plot showing median telomere

9 lengths for T_{STEM}, T_{PEX}, and T_{EM} cells. Each dot represents one donor (n = 6). Bars indicate mean

10 \pm SEM. ***P* < 0.01 (one-way repeated measures ANOVA). **d,** Bar graph summarizing the

11 expression of PD-1 and TIGIT among FACS-purified T_{STEM} and T_{PEX} cells after stimulation with

12 anti-CD3 plus CD28 for 4 d in the presence of IL-7 and IL-15 (n = 5 donors from three

13 independent experiments). Bars indicate mean \pm SEM. ****P* < 0.001 (two-tailed Mann-Whitney

14 U test).

15

1 **Supplementary Fig. 4. Epigenetic and transcriptomic comparison of activated T_{STEM} and**
2 **T_{PEX} cells.** FACS-purified T_{STEM} and T_{PEX} cells were stimulated with anti-CD3 plus CD28 for 4
3 d in the presence of IL-2 and IL-12. Cells were then processed for ATAC-seq (n = 3 donors) or
4 RNA-seq (n = 4 donors). **a**, Normalized enrichment score (NES) of selected gene sets obtained
5 from GSEA of the RNA-seq data in **Fig. 4h** (adjusted *P* value < 0.05 based on 1,000
6 permutations). **b**, Heatmap showing DARs related to the experiment in **Fig. 4i**. Labels highlight
7 accessible genes associated with memory or effector differentiation or exhaustion. **c**, TFBMs
8 enriched among the DARs identified between activated T_{STEM} and T_{PEX} cells in **Fig. 4i**.
9 Enrichment was assessed using a one-sided hypergeometric test in HOMER with correction for
10 FDR. Stim: stimulated.

11

1 **Supplementary Fig. 5. Antigen specificity and repertoire characteristics of T_{STEM} and T_{PEX}**
2 **cells. a**, UMAP plot showing the expression of selected markers as determined by CyTOF.
3 Similar data were obtained from other healthy donors (n = 4). **b**, UMAP plots showing the
4 distribution of T_{STEM} and T_{PEX} cells (top left) and antigen-specific CD8⁺ memory T cells as
5 determined by CyTOF. Similar data were obtained from other healthy donors (n = 4). **c**, Dot plot
6 showing the matched frequencies of all (wt) or high-avidity (KA) CMV NV9-specific CCR7⁺
7 CD8⁺ T cells expressing the T_{PEX} signature marker GZMK. Data were obtained using flow
8 cytometry. Each dot represents one donor (n = 7 from three independent experiments for wt, n =
9 4 from three independent experiments for KA). Bars indicate mean ± SEM. **d**, Dot plot showing
10 the Chao1 estimator of clonal diversity for TCRβ repertoires obtained from the T_{STEM}, T_{PEX}, and
11 T_{EM} subsets. Each dot represents one donor (n = 6). Bars indicate median values. **P* < 0.05, ***P*
12 < 0.01, ****P* < 0.001 (two-tailed paired t-test with Bonferroni correction). **e**, Dot plot showing
13 pairwise comparisons of weighted overlap (F2 metric) for the TCRβ repertoires obtained in **d**.
14 Each dot represents one donor (n = 6). Bars indicate median values. **P* < 0.05, ***P* < 0.01, ****P*
15 < 0.001 (two-tailed paired t-test with Bonferroni correction). **f**, Dot plot showing clonotype
16 frequency correlations (R metric) for the TCRβ repertoires obtained in **d**. Higher values indicate
17 stronger correlations. Each dot represents one donor (n = 6). Bars indicate median values. ****P*
18 = 0.0007 (two-tailed paired t-test with Bonferroni correction).

19

1 **Supplementary Table 1. Samples used in this study.**

2
3 **Supplementary Table 2. Differentially expressed genes among the 14 clusters identified by**
4 **scRNA-seq (adjusted P value < 0.05, log2 fold change > 0.25, Wilcoxon rank sum test).**

5
6 **Supplementary Table 3. Differentially expressed genes between clusters C2 and C6**
7 **(adjusted P value < 0.05, log2 fold change > 0.20, Wilcoxon rank sum test).**

8
9 **Supplementary Table 4. Differentially expressed genes between T_{SCM} and T_{CM} cells**
10 **depleted of PD-1⁺ TIGIT⁺ cells (adjusted P value < 0.05, edgeR analysis with glmQLFTest**
11 **and Benjamini-Hochberg correction), differentially expressed genes between T_{PEX} cells and**
12 **pooled T_{SCM}/T_{CM} cells depleted of PD-1⁺ TIGIT⁺ cells (adjusted P value < 0.01, edgeR**
13 **analysis with glmQLFTest and Benjamini-Hochberg correction), and enrichment analysis**
14 **of G5 versus G10 signatures in T_{PEX} cells and pooled T_{SCM}/T_{CM} cells depleted of PD-1⁺**
15 **TIGIT⁺ cells (P value < 0.05, hypergeometric test).**

16
17 **Supplementary Table 5. Differentially expressed genes between activated T_{STEM} and T_{PEX}**
18 **cells (adjusted P value < 0.05, edgeR analysis with glmQLFTest and Benjamini-Hochberg**
19 **correction).**

20
21 **Supplementary Table 6. Flow cytometry reagents.**

22
23 **Supplementary Table 7. Mass cytometry reagents.**

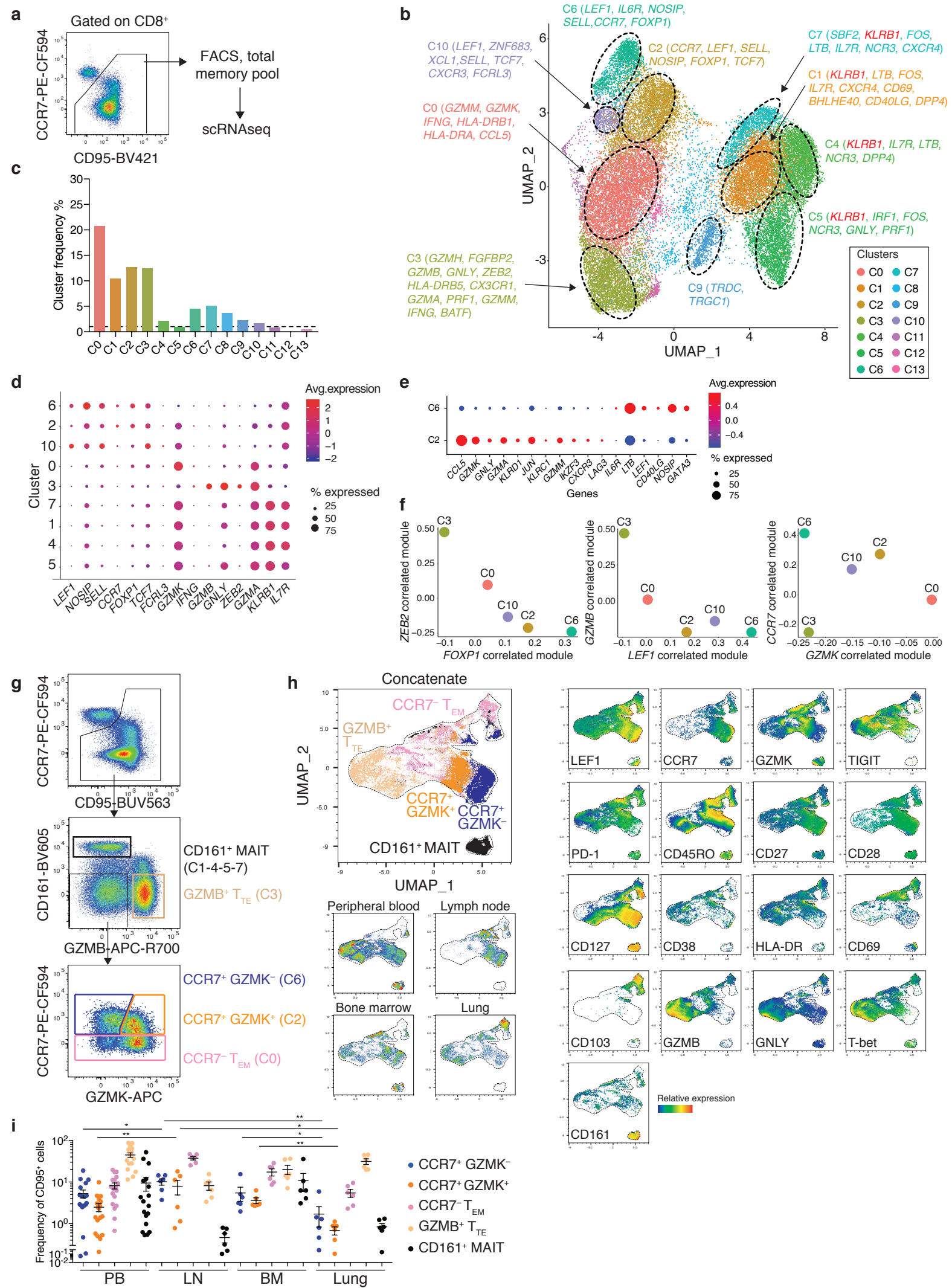
Fig. 1

Fig. 2

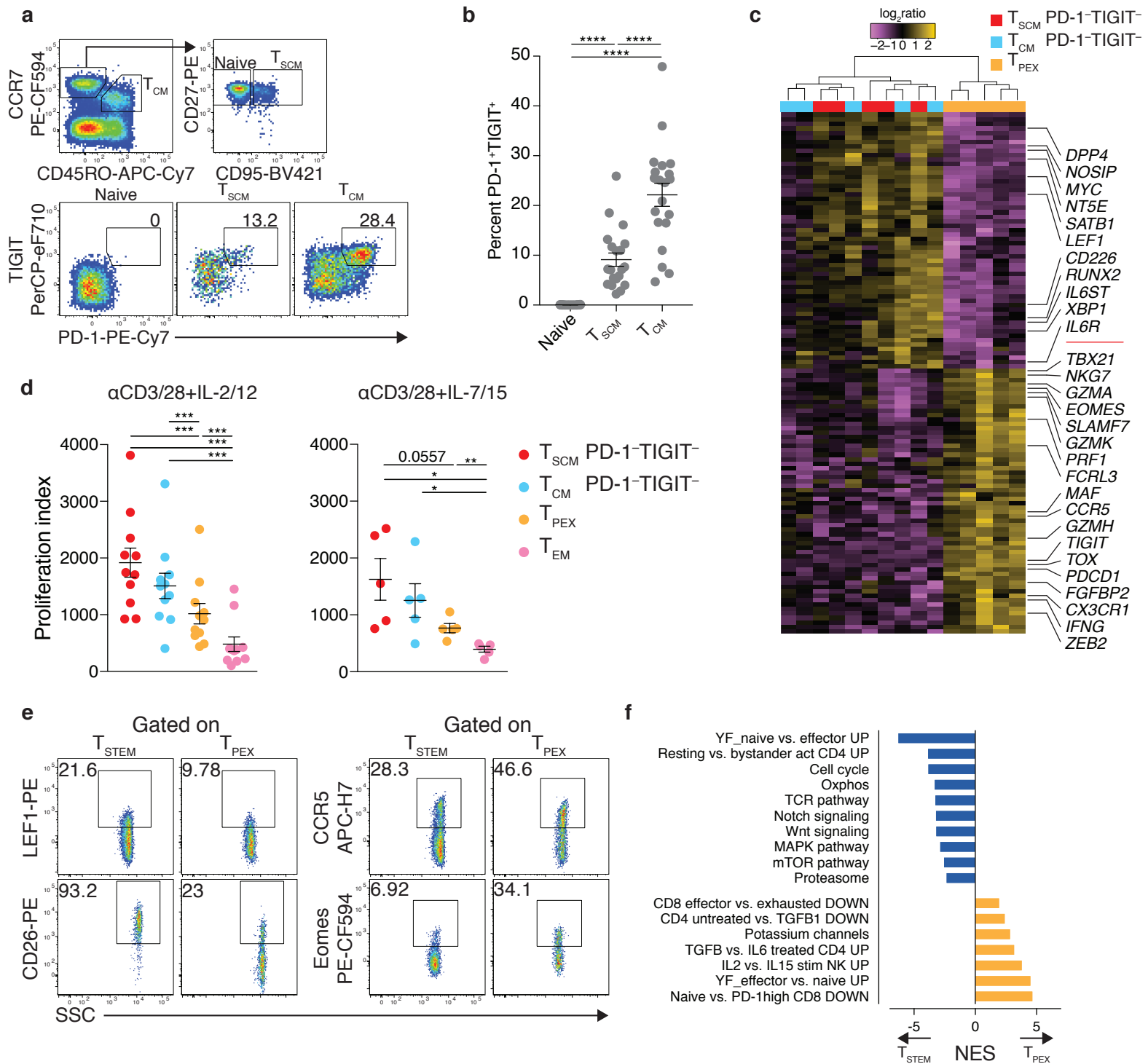


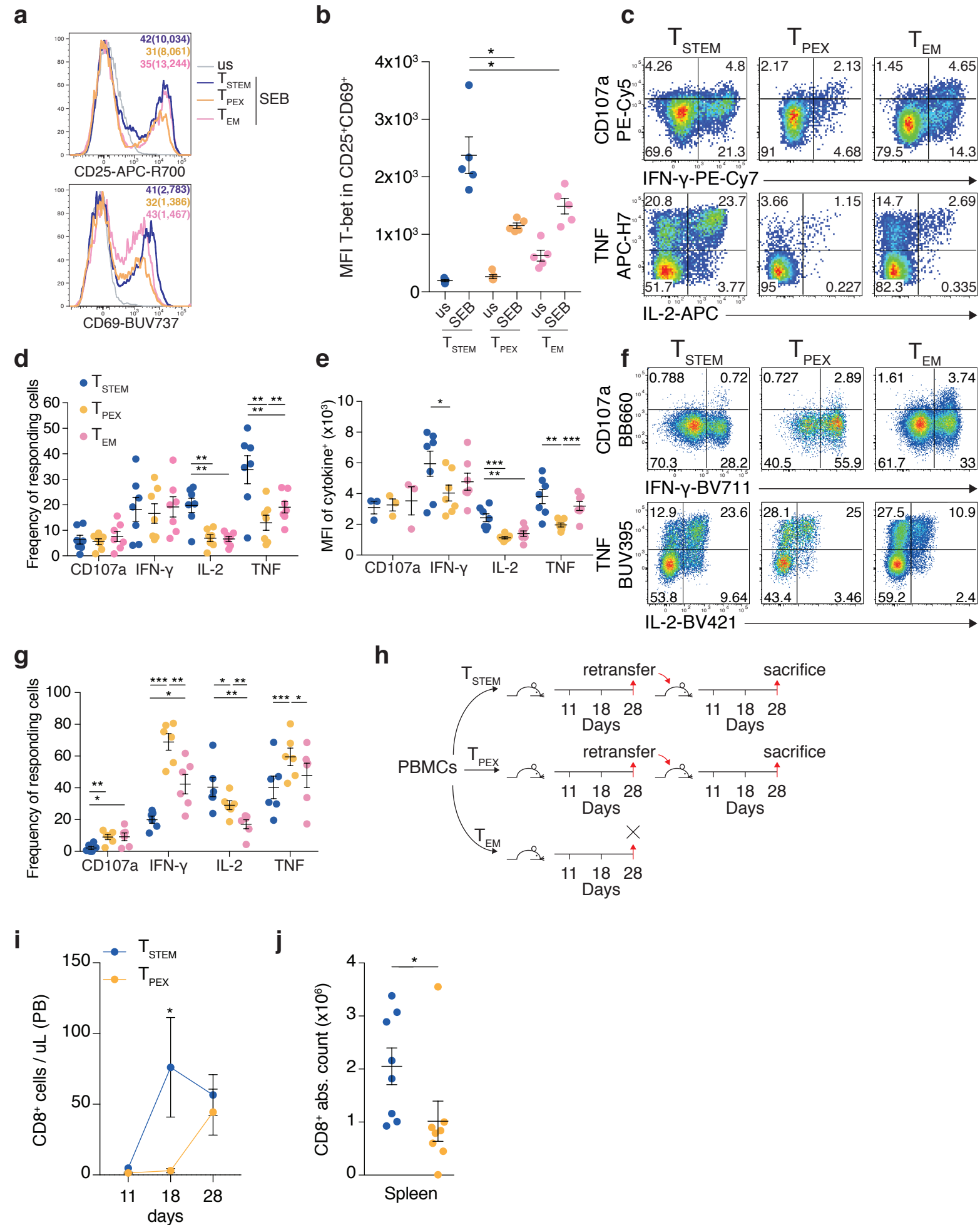
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Fig. 4

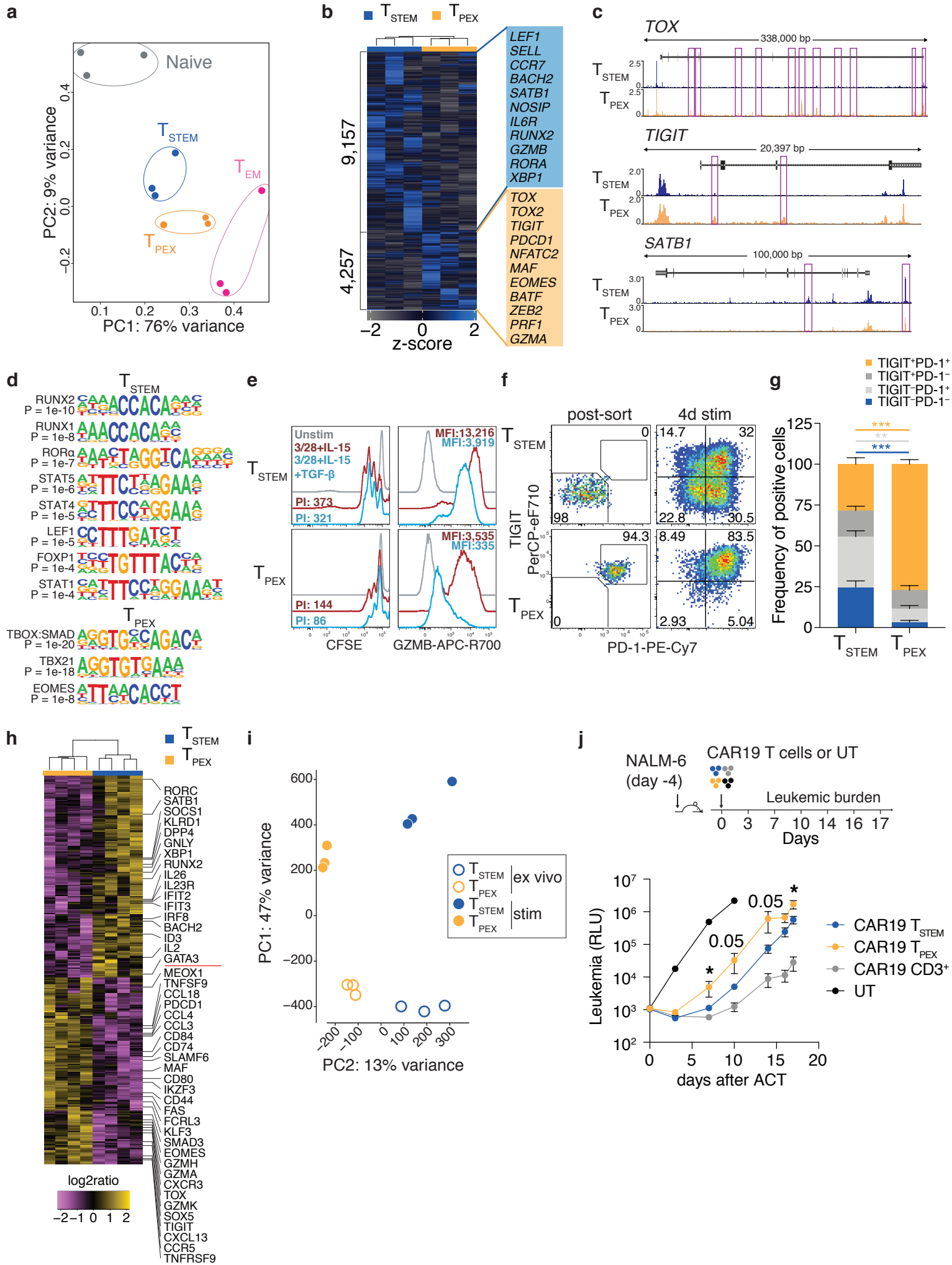
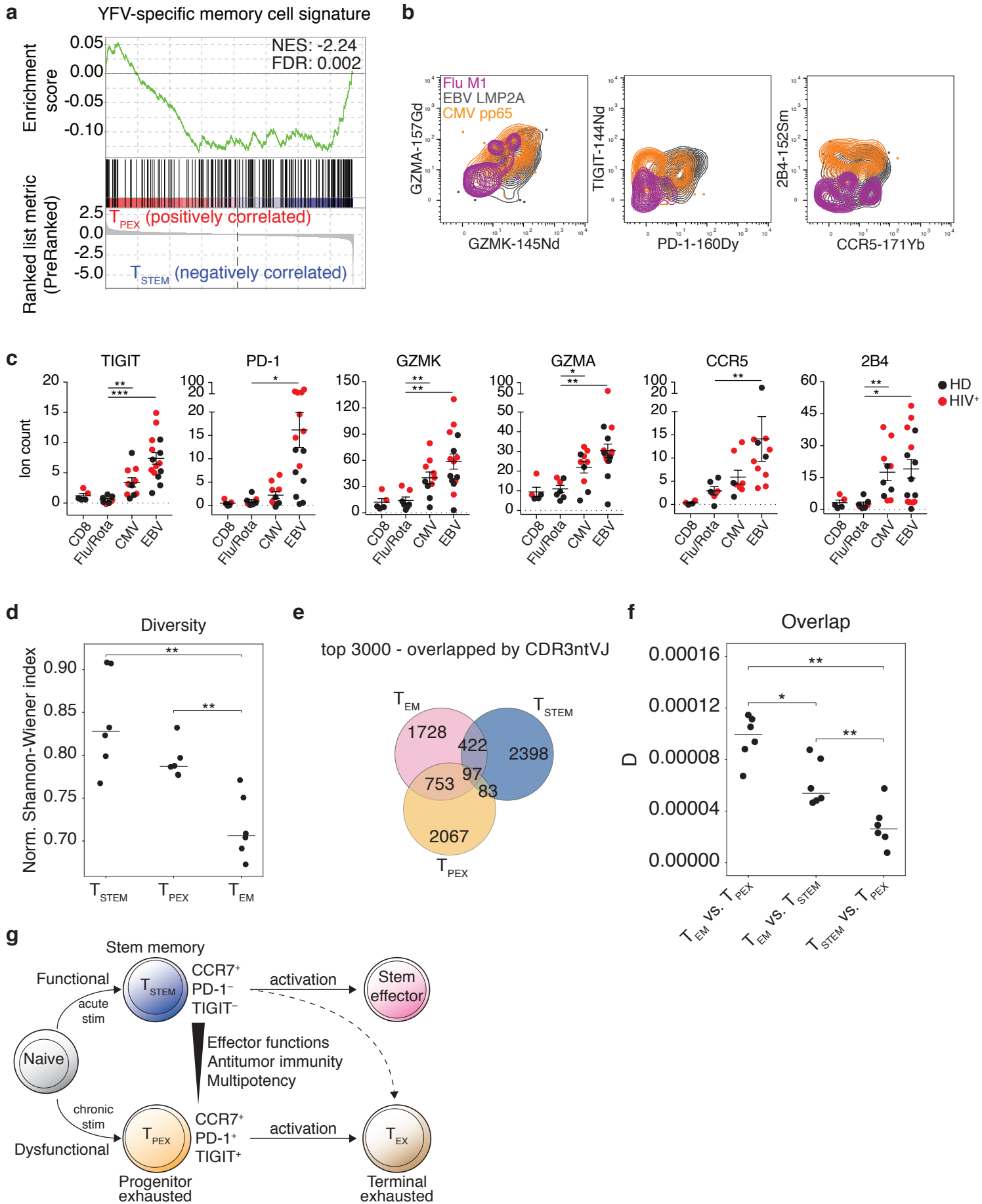
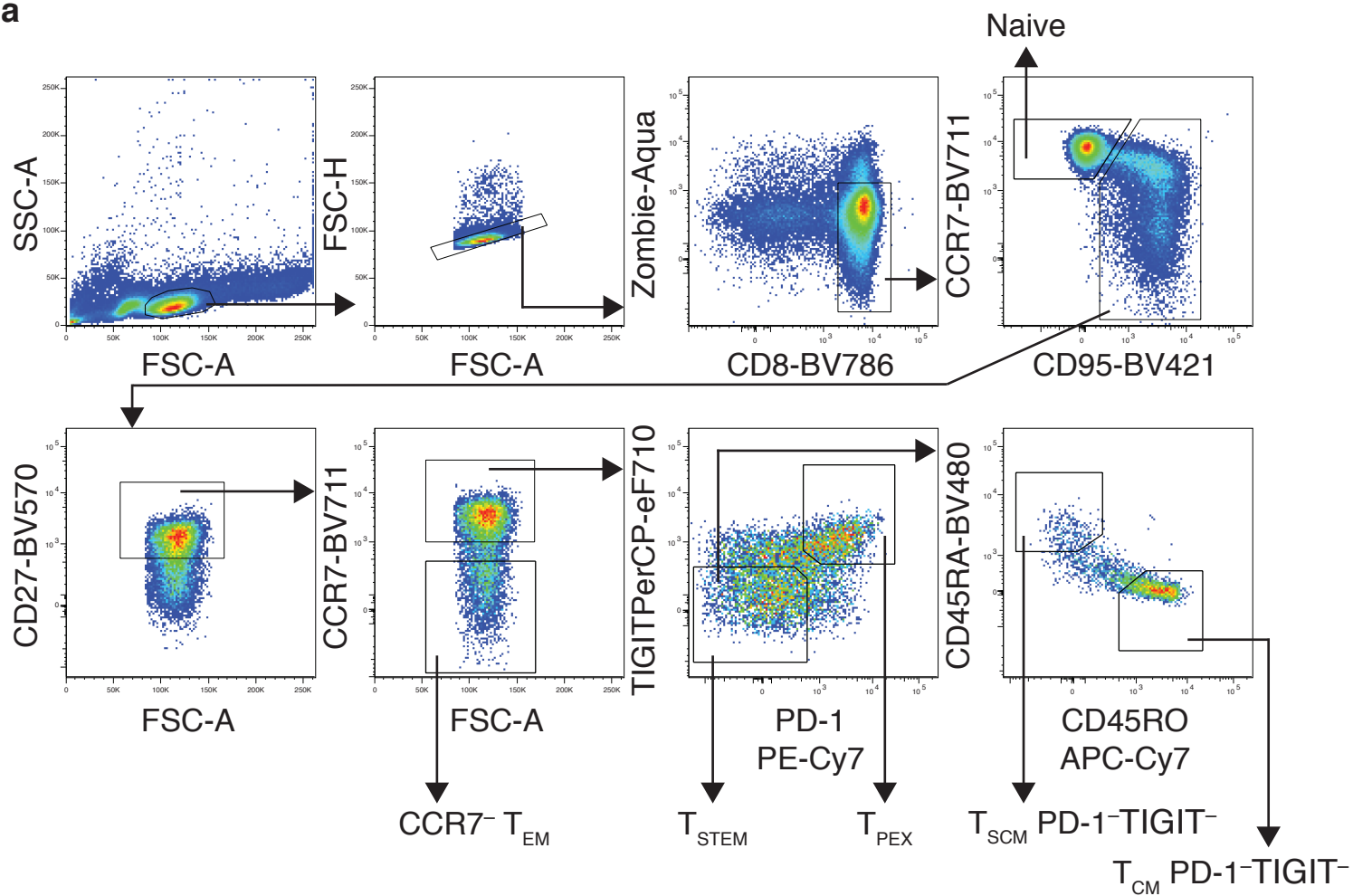


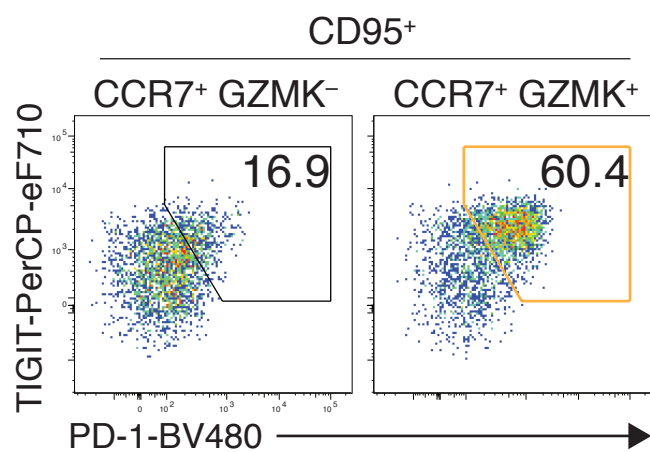
Fig. 5

Supplementary Fig. 1

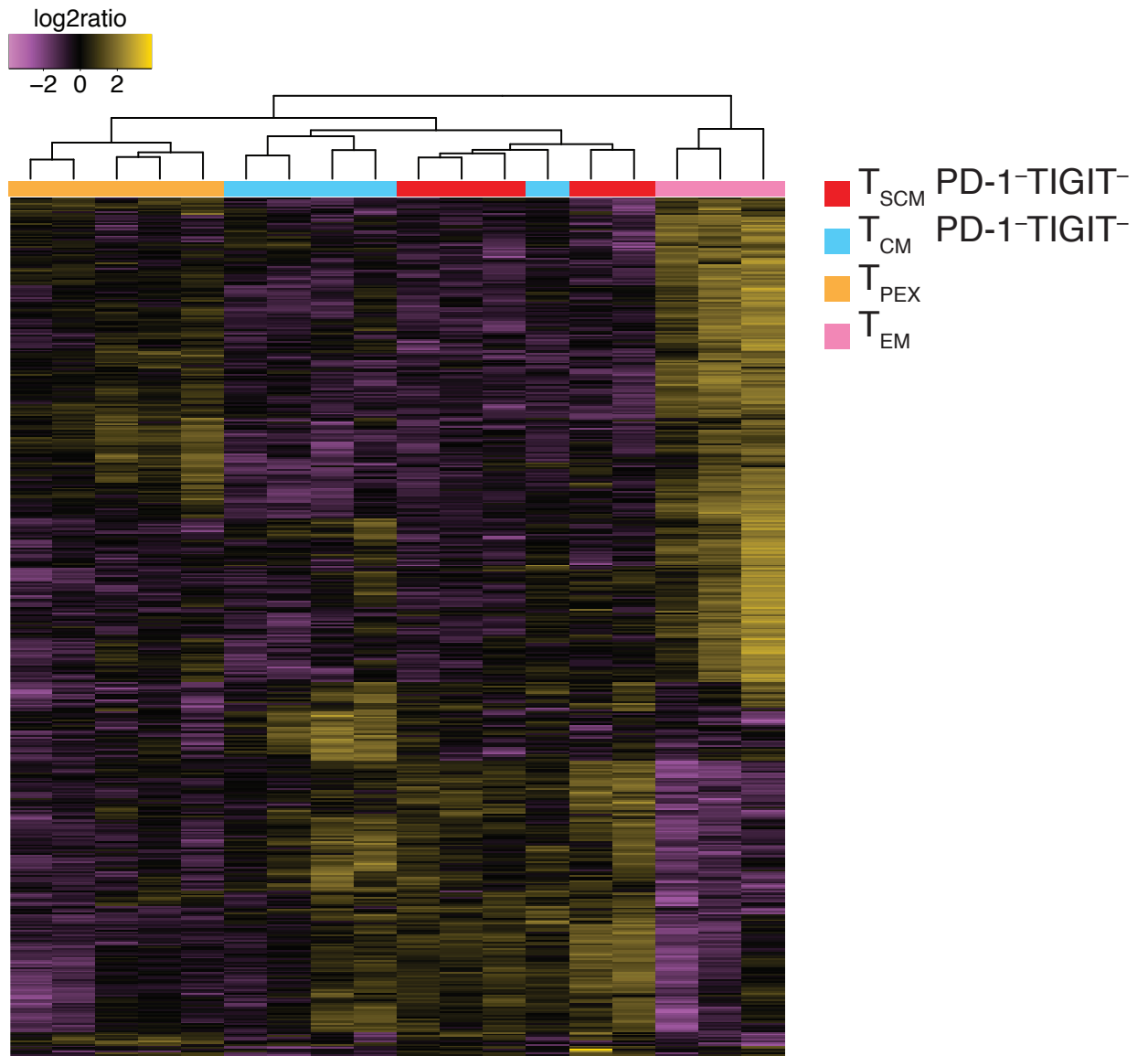
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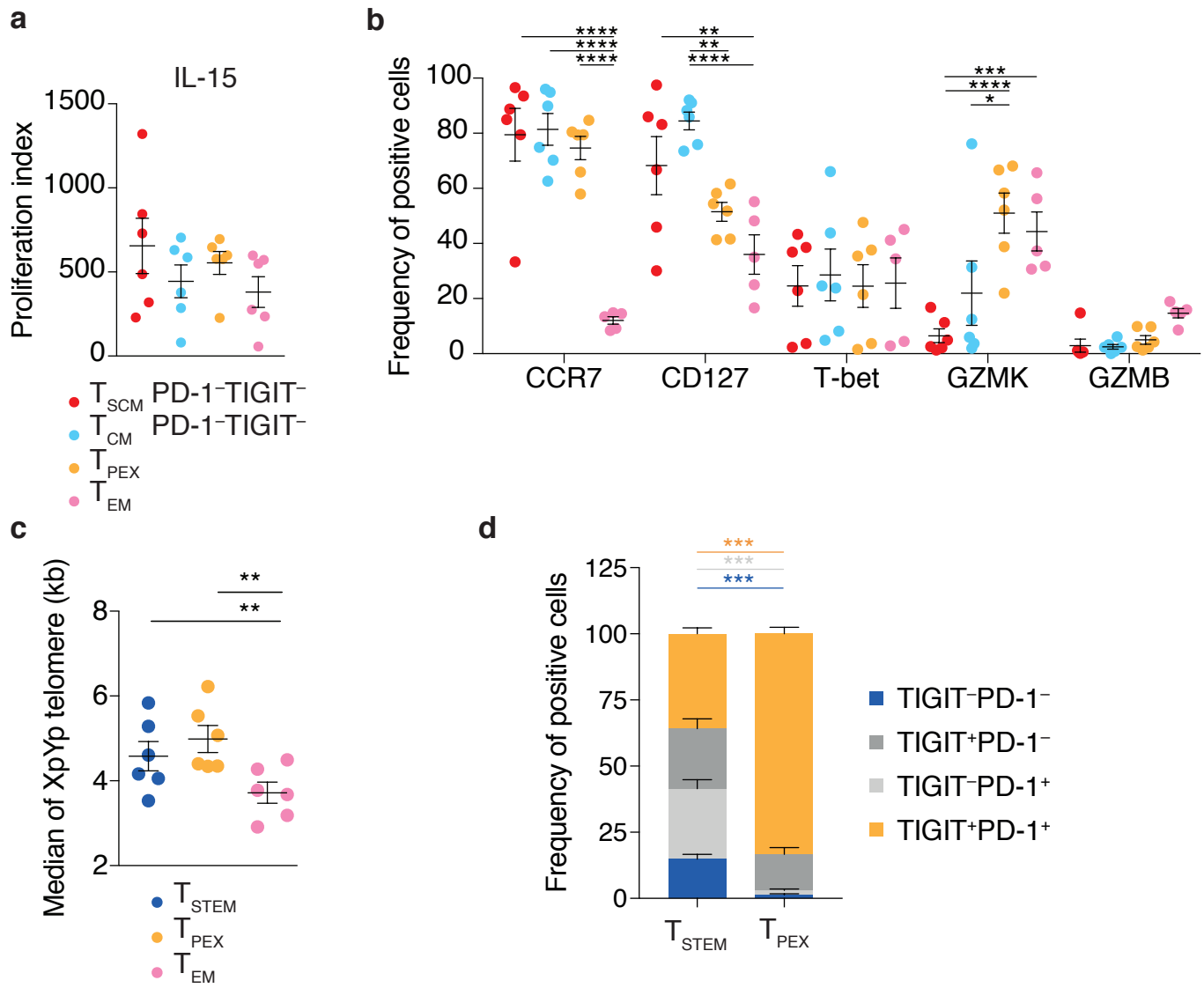


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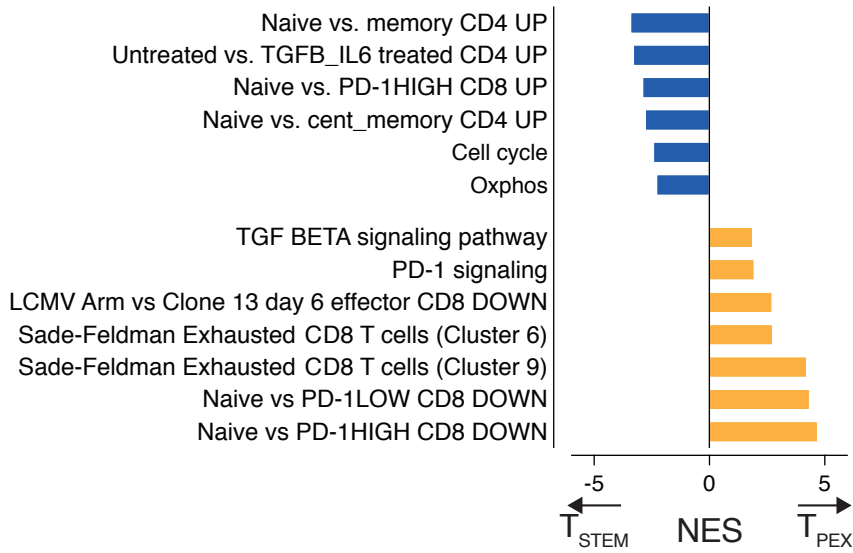
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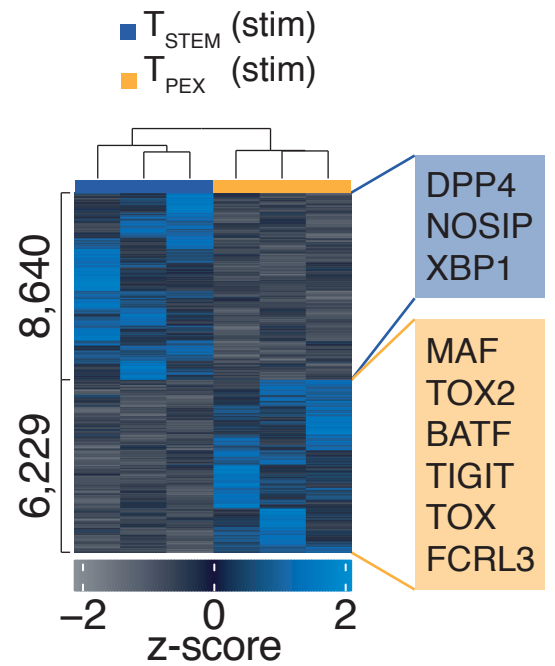


Supplementary Fig. 4

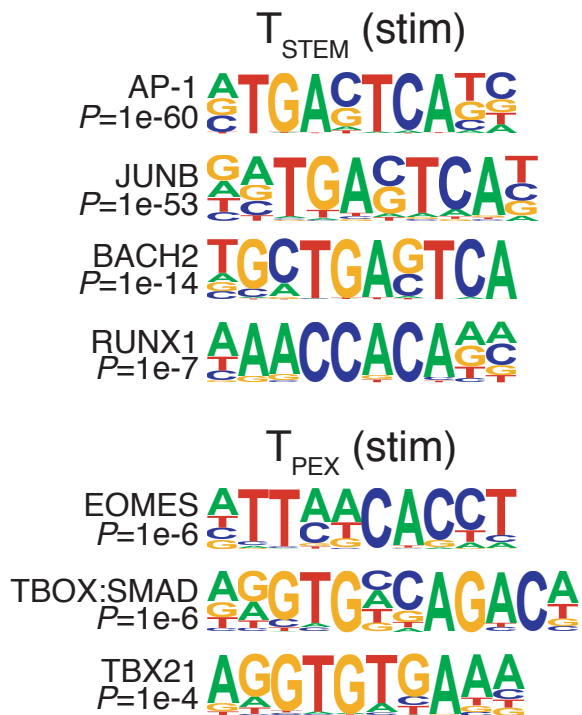
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c



Supplementary Fig. 5

