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

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Author contributions

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., 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., 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., 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. wrote the manuscript. All authors contributed intellectually and approved the manuscript.

Competing Interests

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

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

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Abstract

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

Antigen recognition by $CD8^+$ naive T cells initiates a program of clonal expansion and effector differentiation that leads to the clearance of infected or malignant cells and the subsequent formation of heterogeneous memory populations that confer durable immunity[1]. These memory populations are thought to be organized in a developmental hierarchy, according to which stem cell memory T (T_{SCM}) cells self-renew and generate long-lived central memory T (T_{CM}) cells and short-lived effector memory T (T_{EM}) cells[2–6]. However, the mechanisms that underlie the enhanced multipotency of T_{SCM} cells relative to T_{CM} cells have not been clearly defined in molecular terms[7].

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Memory T cell differentiation can become corrupted under conditions of persistent antigenic stimulation, as observed during chronic viral infections and progressive malignancies, which promote a state of T cell exhaustion, characterized by an orderly loss of effector functions, impaired proliferation, and the upregulation of inhibitory receptors[8]. This dynamic process occurs over a period of weeks after the initial priming event[9,10] and involves the genome-wide accumulation of epigenetic modifications[11,12]. Recent studies have shown that exhausted T (T_{EX}) cell populations are developmentally and functionally heterogeneous, incorporating stem-like progenitors that express T cell factor 1 (TCF1) which give rise to highly differentiated T_{EX} cells that are constitutively dysfunctional and lack TCF1[13–16]. Importantly, the therapeutic benefits of immune checkpoint blockade in the context of chronic viral infections and various cancers are thought to operate via these $TCF1^+$ progenitors, which appear susceptible to interventions that specifically target the inhibitory receptor programmed death-1 (PD-1)[13,15,17–20].

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

Results

Two subsets of stem-like $CD8^+$ memory T cell progenitors exist in humans

We initially used single-cell RNA sequencing (scRNA-seq; 10X Genomics Platform) to characterize the full spectrum of human $CD8^+$ memory T cells in peripheral blood (PB) samples obtained from healthy donors (n = 4) (Supplementary Table 1). A total of 31,640 cells were isolated for this purpose via fluorescence-activated cell sorting (FACS) based on the expression of CD95 (Fig. 1a and Supplementary Fig. 1a), which identifies a vast majority of all memory T cells in humans[4]. Bioinformatic analysis of gene expression mapped in two dimensions via Uniform Manifold Approximation and Projection (UMAP)[21] identified 14 distinct clusters (denoted individually as C) (Fig. 1b). C1, C4, C5, and C7 were uniformly distant from the other cell populations and expressed high levels of *KLRB1*, which encodes CD161, and *IL7R*, which encodes the interleukin (IL)-7 receptor (IL-7R), also known as CD127. These clusters were therefore derived from mucosal associated invariant T (MAIT) cells[22]. An intermediate cluster, C9, which comprised less than 2% of all cells (Fig. 1c), overexpressed *TRDC* and *TRGC1*, which encode the constant regions of

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the T cell receptor (TCR) δ and γ chains, respectively, suggesting the expression of γδ rather than αβ TCRs.

scRNA-seq further identified seven different clusters related to conventional memory T cells (Fig. 1b), the most abundant of which were C0, C2, C3, C6, and C10 (Fig. 1c). C2, C6, and C10 expressed genes associated with early differentiated memory T cells, including *CCR7*, *LEF1*, *SELL*, which encodes L-selectin (CD62L), and *FOXP1* (Fig. 1b,d and Supplementary Table 2). In contrast, the highly abundant C0 overexpressed multiple effector transcripts, including *GZMK* and *GZMM*, which encode serine proteases termed granzymes, *IFNG*, which encodes interferon (IFN)-γ, the chemokine (C-C motif) ligands *CCL4* and *CCL5*, and genes encoding human leukocyte antigen (HLA) class II molecules, consistent with the identification of effector memory T (T_{EM}) cells (Fig. 1b,d and Supplementary Table 2). C3 displayed a gene expression profile reminiscent of terminal effector T (T_{TE}) cells, featuring high levels of *GZMB*, *GNLY, NKG7*, *ZEB2*, and *GZMA* (Fig. 1b,d and Supplementary Table 2). A lack of signature transcripts precluded the identification of C8 based on current knowledge of the T cell differentiation pathway (Supplementary Table 2).

To explore the heterogeneity of the *CCR7*$^+$ memory T cell pool, we focused on C2 and C6, because C10 comprised only ~1% of all sorted cells (Fig. 1c). We identified 160 differentially expressed genes (DEGs) between C2 and C6 (Supplementary Table 3). C2 expressed higher levels of effector molecules, including *CCL5*, *GZMK*, *GNLY*, *GZMA*, *JUN*, *GZMM*, *HOPX*, *IKZF3*, *RUNX3*, and *PRF1*, which encodes perforin, whereas C6 expressed higher levels of *IL6R*, *LTB*, *LEF1*, *NOSIP*, *GATA3*, and *SELL* (Fig. 1e and Supplementary Table 3). We then used anchor genes selected from the most prominent DEGs to compute transcriptional modules associated with memory differentiation (correlated with *CCR7* and *LEF1*), quiescence (correlated with *FOXP1*), or cytotoxicity and terminal effector differentiation (correlated with *GZMK*/*GZMB* and *ZEB2*, respectively) among the five conventional memory T cell clusters (C0, C2, C3, C6, and C10). Using this approach, we found that C2 and C10 were similar, exhibiting intermediate memory and effector scores, whereas C6 was skewed toward a high memory score and C0 and C3 were skewed toward high effector scores (Fig. 1f). scRNA-seq analysis therefore identified four major subsets of conventional CD8$^+$ memory T cells, namely early differentiated *CCR7*$^+$ *GZMK*$^-$ (C6) and *CCR7*$^+$ *GZMK*$^+$ (C2), T_{EM}-like *CCR7*$^-$ *GZMK*^{hi} (C0), and T_{TE}-like *GZMB*$^+$ (C3).

To confirm these findings at the protein level, we designed a high-dimensional flow cytometry panel based on the cluster signature markers CCR7, LEF1, CD161, GZMB, and GZMK (Fig. 1g). This panel was also equipped to detect memory and effector differentiation markers (CD27, CD28, CD45RO, CD127, and T-bet), activation markers (CD38 and HLA-DR), inhibitory receptors (PD-1 and TIGIT), and markers of tissue residency (CD69 and CD103) in CD8$^+$ T cells isolated from the peripheral blood and tissues (Supplementary Table 1). In line with the scRNA-seq data, UMAP analysis revealed that CD161^{hi} MAIT cells were largely distinct from other CD95$^+$ memory T cells (Fig. 1h). Among the effector subsets, CCR7$^-$ GZMK^{hi} cells expressed cytolytic molecules and generally lacked the memory markers LEF1, CD27, CD28, and CD127, whereas GZMB$^+$ cells also expressed granulysin (GNLY) and relatively high levels of T-bet (Fig. 1h). In contrast, the CCR7$^+$

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GMZK^{-} subset expressed relatively high levels of LEF1, CD27, CD28, and CD127 and lacked effector molecules, activation markers, and inhibitory receptors, whereas the CCR7^{+} GMZK^{+} subset expressed intermediate levels of LEF1 alongside PD-1 and TIGIT, which were not detected in the scRNA-seq analysis, together with relatively high levels of CD27, CD28, and CD127 (Fig. 1h). These subsets displayed variable expression of CD45RO (Fig. 1h). As expected, CD69^{+} CD103^{+} cells were only detected in tissues (Fig. 1h). A survey of different tissue sites revealed that CCR7^{+} GZMK^{-} PD-1^{-} TIGIT^{-} and CCR7^{+} GZMK^{+} PD-1^{+} TIGIT^{+} cells were relatively abundant in PB, lymph nodes (LNs), and bone marrow (BM), whereas CCR7^{-} GZMK^{hi} cells were ubiquitous, and GZMB^{+} cells predominated in PB and lung tissue (Fig. 1i).

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

Exhausted-like CD8^{+} memory T cell progenitors express GZMK, PD-1, and TIGIT

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

On the basis of these results, we hypothesized that differential inclusion of the transcriptionally distinct CCR7^{+} GZMK^{+} PD-1^{+} TIGIT^{+} subset could explain some of the previously reported differences between T_{SCM} and T_{CM} cells. To test this possibility, we analyzed the transcriptomes of T_{SCM} and T_{CM} cells after depletion of the CCR7^{+} PD-1^{+} TIGIT^{+} (GZMK^{+}) population, hereafter termed T progenitor exhausted-like (T_{PEX}) (Supplementary Fig. 1a and Supplementary Fig. 2). In line with our hypothesis, T_{SCM} and T_{CM} cells depleted of T_{PEX} cells were very similar at the transcriptional level and could only be distinguished on the basis of eight DEGs (adjusted P value < 0.01) (Supplementary Fig. 2 and Supplementary Table 4). One of these DEGs was *HNRNPLL*, which encodes heterogeneous nuclear ribonucleoprotein L-like, a master regulator of alternative splicing responsible for the expression of CD45RO[24], which is commonly used as a phenotypic marker to differentiate between T_{SCM} and T_{CM} cells[25]. In contrast, T_{PEX} cells were largely distinct, featuring lower expression levels of *SATB1*, which encodes a negative regulator of PD-1 expression[26], *MYC*, *DPP4*, which encodes CD26, *IL6ST*, *LEF1*, *IL6R*, and *NT5E* and higher expression levels of transcription factor (TF) genes recently associated with T cell exhaustion, including *TOX*[27–32], *EOMES*[33], and *MAF*[34], and other genes associated with effector differentiation and cytolytic activity, including *ZEB2*, *GZMK*, *GZMA*, *TBX21*,

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PRF1, *IFNG*, and *NKG7* (Fig. 2c and Supplementary Table 4). As expected, *PDCD1*, which encodes PD-1, and *TIGIT* were also expressed at high levels, validating the integrity of cell isolation via FACS. Several other genes previously found to distinguish T_{SCM} from T_{CM} cells were also identified among these DEGs[4] (Fig. 2c and Supplementary Table 4). In line with the transcriptional data, T_{PEX} cells stimulated with anti-CD3 plus CD28 and a combination of effector (IL-2 plus IL-12) or homeostatic cytokines (IL-7 plus IL-15) proliferated less vigorously than PD-1^- $TIGIT^-$ T_{SCM} and T_{CM} cells under identical conditions (Fig. 2d). However, all three subsets proliferated similarly and remained phenotypically stable in response to IL-15, suggesting equivalent self-renewal capabilities (Supplementary Fig. 3a,b). Accordingly, T_{SCM} and T_{CM} cells were better defined by the $CCR7^+$ PD-1^- $TIGIT^-$ phenotype, hereafter termed stem-like T (T_{STEM}), whereas early differentiated memory cells with dysfunctional, exhausted-like traits were characterized by the $CCR7^+$ PD-1^+ $TIGIT^+$ phenotype (T_{PEX}). Of note, the gene expression profiles of T_{STEM} and T_{PEX} cells overlapped significantly with those of C6 and C2, respectively ($P < 0.01$ for each comparison using a hypergeometric test; not shown), confirming the shared identity of subsets analyzed via scRNA-seq and flow cytometry (Fig. 1e).

Flow cytometric analyses demonstrated that T_{STEM} cells expressed CD26 and LEF1 more commonly and CCR5 and Eomes less commonly than T_{PEX} cells (Fig. 2e). Gene set enrichment analysis (GSEA) further revealed that T_{STEM} cells were characterized by transcripts associated with the naive state, quiescence, oxidative phosphorylation, the Wnt[35] and Notch signaling pathways[36], and proteasome activity[37], whereas T_{PEX} cells were characterized by transcripts associated with the TGF-β signaling pathway[38], potassium regulation[39], and other mechanistic correlates of exhaustion, including the PD-1^{hi} state (Fig. 2f). Transcripts associated with the cell cycle and the TCR and mTOR signaling pathways, collectively suggesting a predisposition to antigen-driven proliferation and effector differentiation, were also upregulated in T_{STEM} versus T_{PEX} cells (Fig. 2f). Previous analyses have shown that progenitor exhausted $CD8^+$ T cells from tumors express stem-like genes along with *PDCD1*, *TIGIT* and *GZMK*[16], thereby suggesting a shared identity with T_{PEX} cells. Indeed, the transcriptional features of T_{PEX} cells aligned closely with those reported previously for progenitor exhausted-like (*CCR7*^{hi} *GZMK*^{hi}) but not memory-like $CD8^+$ T cells (*CCR7*^{hi} *GZMK*^{lo}) isolated from melanomas[18], whereas the opposite result was obtained in a parallel analysis of T_{STEM} cells ($P < 0.05$ for each comparison using a hypergeometric test) (Supplementary Table 4).

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

T_{STEM} cells are functionally superior to T_{PEX} cells

To validate our transcriptional and phenotypic data, we compared the functional properties of FACS-purified T_{STEM} and T_{PEX} cells. In response to TCR-dependent stimulation with Staphylococcal enterotoxin B (SEB), T_{STEM} cells upregulated CD25 and CD69 to a greater extent than T_{PEX} cells (Fig. 3a), and activated $CD25^+$ $CD69^+$ T_{STEM} cells expressed higher levels of T-bet than activated $CD25^+$ $CD69^+$ T_{PEX} cells (Fig. 3b). Likewise, T_{STEM} cells

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

To determine the in vivo relevance of these observations, we performed serial adoptive cell transfers (ACTs) in NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) humanized mice (Fig. 3h). T_{EM} cells failed to repopulate these mice efficiently after the first ACT (not shown), as reported previously[4]. Although the early memory subsets both expanded to similar numbers in primary hosts (not shown), T_{STEM} cells proliferated more rapidly in PB (Fig. 3i) and repopulated the spleen more efficiently in secondary hosts compared with T_{PEX} cells (Fig. 3j). Of note, the suboptimal proliferative capabilities of T_{PEX} cells observed in vitro and in vivo were not associated with differences in telomere length relative to T_{STEM} cells, whereas T_{EM} cells harbored shorter telomeres ex vivo compared with either T_{STEM} or T_{PEX} cells (Supplementary Fig. 3c).

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

T_{PEX} cells are committed to a terminally dysfunctional state

Epigenetic regulation plays a key role in T cell fate decisions[40]. We therefore employed the Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq) to compare the open chromatin landscapes of T_{STEM} and T_{PEX} cells in terms of differentially accessible regions (DARs). Naive and T_{EM} cells were analyzed in parallel as lineage controls. Principal component analysis (PCA) revealed that T_{STEM} and T_{PEX} cells were globally similar, although T_{STEM} cells mapped toward the naive subset, whereas T_{PEX} cells mapped toward the T_{EM} subset (Fig. 4a). However, we also identified a total of 13,414 DARs between T_{STEM} and T_{PEX} cells (Fig. 4b). Genes associated with T cell dysfunction (*e.g.*, *TOX*, *TOX2*, *TIGIT*, *PDCD1*, *NFATC2*, and *MAF*), terminal differentiation (*e.g.*, *ZEB2* and *BATF*), and other immune-related processes previously identified at the mRNA level (*e.g.*, *EOMES* and *GZMA*) were more accessible in T_{PEX} versus T_{STEM} cells (Fig. 4b,c). In contrast, genes associated with T cell memory (*e.g.*, *LEF1*, *SELL*, *CCR7*, *BACH2*, and *SATB1*) and effector functions (*e.g.*, *GZMB* and *RORA* [41]) were more accessible in T_{STEM} versus T_{PEX} cells (Fig. 4b,c). Computational analysis of these DARs further identified differentially accessible TF binding motifs (TFBMs). Motifs linked to TFs associated with thymocytes and naive and early memory cells (RUNX2, RUNX1, LEF1, and FOXP1), effector differentiation (RORA)[41], and cytokine signaling (STAT5, STAT4, and STAT1) were enriched in T_{STEM} versus T_{PEX} cells, whereas the TBX21 (T-bet), EOMES, and combined TBOX:SMAD motifs were enriched in T_{PEX} versus T_{STEM} cells (Fig. 4d).

The chromatin accessibility data suggested that T_{PEX} cells were predisposed to the generation of dysfunctional progeny and susceptible to the inhibitory effects of TGF-β

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signaling via SMADs. Accordingly, T_{PEX} cells proliferated to a lesser extent and produced less GZMB than T_{STEM} cells in response to stimulation with anti-CD3 plus CD28 and IL-15 (Fig. 4e). The addition of TGF-β further inhibited these responses, especially the production of GZMB, in parallel cultures of T_{PEX} cells, whereas minimal effects were observed in parallel cultures of T_{STEM} cells (Fig. 4e). Importantly, most T_{PEX} cells retained a PD-1^+ $TIGIT^+$ phenotype after stimulation with anti-CD3 plus CD28 in the presence of the effector cytokines IL-2 and IL-12 (Fig. 4f,g) or the homeostatic cytokines IL-7 and IL-15 (Supplementary Fig. 3d). In contrast, T_{STEM} cells generated all possible combinations of phenotypes defined by PD-1 and TIGIT (Fig. 4f,g). We then used RNA-seq to profile the transcriptomes of T_{STEM} and T_{PEX} cells after stimulation with anti-CD3 plus CD28 in the presence of IL-2 and IL-12 (Fig. 4h). Activated T_{STEM} cells overexpressed the memory-related genes *BACH2*, *ID3*, *IL2*, and *SATB1* alongside the effector-related genes *IRF8*, *RORC*, *GNLY*, *XBP1*, *IL26*, and *IL23R*, whereas activated T_{PEX} cells overexpressed the dysfunction/exhaustion-related molecules *TOX*, *PDCD1*, *TIGIT*, *MAF*, and *CXCL13*[42], together with various chemokine genes, *IKZF3*, which encodes an inhibitor of IL-2 production, *SMAD3*, and genes associated with cytolytic activity, including *GZMK*, *GZMH*, and *GZMA* (Fig. 4h and Supplementary Table 5). Some of these genes were also differentially expressed between the corresponding subsets in ex vivo analyses (Fig. 2c). GSEA further demonstrated that activated T_{STEM} cells were preferentially enriched for gene sets associated with early differentiation and proliferation, whereas activated T_{PEX} cells were preferentially enriched for gene sets associated with the TGF-β and PD-1 signaling pathways and exhaustion in the tumor microenvironment[18] (Supplementary Fig. 4a). PCA of ATAC-seq data from paired ex vivo and activated samples revealed that stimulation profoundly altered the chromatin accessibility landscape in T_{STEM} and T_{PEX} cells (Fig. 4i). However, the major epigenetic differences between these subsets in the ex vivo state were maintained after stimulation (Fig. 4i), both at the level of specific genes (Supplementary Fig. 4b) and in terms of enrichment for particular TFBMs (Supplementary Fig. 4c).

To assess the in vivo relevance of these findings, we employed a stringent ACT protocol in which T_{STEM} and T_{PEX} cells were redirected using a chimeric antigen receptor (CAR) targeting CD19 and transferred in the absence of autologous $CD4^+$ T cells or exogenous cytokines into NSG mice previously injected with the acute lymphoblastic leukemia cell line NALM6. In line with the in vitro data, T_{STEM} cells displayed enhanced control of leukemic burden compared with T_{PEX} cells at multiple time points after ACT (Fig. 4j).

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

T_{PEX} cells are abundant in persistent infections and clonally distinct from T_{STEM} cells

$CD8^+$ T cell dysfunction and exhaustion develop in response to persistent antigenic stimulation via cognate TCRs[43]. We therefore reasoned that acute viral infections would preferentially generate antigen-specific T_{STEM} cells, whereas chronic viral infections would preferentially generate antigen-specific T_{PEX} cells. A single round of yellow fever virus (YFV) vaccination is known to induce long-lived memory cells with an early differentiated

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T_{SCM}-like $CCR7^+$ $CD45RA^+$ (or $CD45RO^-$) $CD95^+$ phenotype[44,45]. To determine the composition of these T_{SCM}-like populations in terms of $CCR7^+$ $PD\text{-}1^-$ $TIGIT^-$ T_{STEM} and $CCR7^+$ $PD\text{-}1^+$ $TIGIT^+$ T_{PEX} cells, we compared publicly available gene expression data from vaccinated individuals[45] with our RNA-seq data (Fig. 2c). In line with our hypothesis, we found that YFV-specific $CCR7^+$ $CD45RA^+$ $CD95^+$ cells analyzed years after vaccination were transcriptionally related to T_{STEM} but not T_{PEX} cells (Fig. 5a).

To extend these findings, we used peptide-HLA class I tetramers in conjunction with mass cytometry (CyTOF) to investigate the phenotypic characteristics of $CCR7^+$ $CD8^+$ memory T cells specific for acute (influenza virus or rotavirus) or chronic viruses (cytomegalovirus [CMV] or Epstein-Barr virus [EBV]) in healthy donors (n = 3) and HIV^+ patients (n = 2). Signature markers of T_{PEX} cells, namely PD-1, TIGIT, GZMK, GZMA, and CCR5, were expressed at higher levels among EBV-specific and, to a lesser extent, CMV-specific versus influenza virus-specific and rotavirus-specific $CD8^+$ T cells (Fig. 5b,c and Supplementary Fig. 5a,b). Chronic virus-specific $CD8^+$ T cells also overexpressed 2B4. Moreover, high-avidity CMV-specific $CD8^+$ T cell populations, selectively identified using a double point-mutated peptide-HLA class I tetramer[46], incorporated T_{PEX} cells at frequencies equivalent to those detected among the corresponding total CMV-specific $CD8^+$ T cell populations, suggesting that persistent antigenic drive rather than signal strength determined the acquisition and maintenance of dysfunctional, exhausted-like traits[43] (Supplementary Fig. 5c).

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

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

Discussion

In this study, we used an unbiased approach guided by scRNA-seq to capture the extensive heterogeneity that exists in the human $CD8^+$ memory T cell pool under physiological

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conditions. We identified two previously unrecognized subsets of stem-like $CD8^+$ memory T cells, neither of which corresponded with previous descriptions of early differentiated progenitors based on the expression of CCR7, CD45RA/RO, and CD95. These subsets were defined by core transcriptional signatures that could be distilled phenotypically into simple profiles, namely $CCR7^+$ $PD\text{-}1^-$ $TIGIT^-$ (T_{STEM} cells) and $CCR7^+$ $PD\text{-}1^+$ $TIGIT^+$ (T_{PEX} cells). Moreover, the distinct gene expression profiles of T_{SCM} and T_{CM} cells were mostly attributable to the differential inclusion of T_{PEX} cells, indicating a need for refined models to understand the process of human $CD8^+$ memory T cell differentiation.

T_{STEM} cells proliferated vigorously in response to activation and generated a diverse array of memory and effector progeny, collectively enabling functionally superior immunity in vivo. Of note, the ex vivo frequencies of T_{STEM} cells were sufficient to overcome current limitations associated with the relative paucity of T_{SCM} cells, potentially facilitating immunotherapies that rely on specificity redirection by providing an alternative source of progenitors with self-renewal capabilities and a propensity for effector differentiation. In contrast, T_{PEX} cells were committed to the generation of progeny with reduced functionality and proliferated less efficiently in response to activation, at least via the TCR. Importantly, T_{STEM} and T_{PEX} cells were also clonally, epigenetically, and transcriptionally distinct, suggesting a branching point in the early memory compartment associated with the initial antigen recognition event(s). This interpretation was supported by the observation that persistent antigenic stimulation was preferentially associated with the development of T_{PEX} cells. On the basis of these findings, we propose a revised model of T cell differentiation, according to which T_{PEX} cells become hardwired to a dysfunctional-like signature after immune activation and effector differentiation, compatible with the generation of a parallel lineage[47] defined by genome-wide epigenetic modifications[48,49], whereas T_{STEM} cells remain multipotent and relatively resistant to exhaustion, resulting in enhanced functionality and protective immunity in vivo. This model will likely need further refinement to accommodate a degree of plasticity within the T_{STEM} subset, given that initially functional $CD8^+$ memory T cells can become exhausted as a result of continuous exposure to high-dose antigen in mice chronically infected with LCMV[50].

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

In summary, we have identified two subsets of human stem-like $CD8^+$ memory T cell progenitors with distinct fate commitments and lineage relationships. Although further work is required to characterize the molecular mechanisms that underlie the early dichotomy

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between T_{STEM} and T_{PEX} cells, we anticipate that such efforts will reveal novel targets for therapeutic interventions designed to inhibit or reverse the process of exhaustion, with obvious implications for the treatment of persistent infections and various cancers.

Methods

Study approval

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

Cells

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

Flow cytometry and cell sorting

High-dimensional flow cytometry was performed as described previously[54]. Dead cells were excluded from all analyses using Zombie Aqua (BioLegend). Fluorochrome-conjugated monoclonal antibodies were purchased from commercial vendors (Supplementary Table 6). All reagents were titrated prior to use to determine optimal concentrations. Cells were fixed/permeabilized for intracellular analyses using a Cytofix/Cytoperm Kit (BD Biosciences). Transcription factors and intranuclear molecules were measured in conjunction with a FoxP3 Transcription Factor Staining Buffer Set (eBioscience) or a Transcription Factor Buffer Set (BD Biosciences). Cell proliferation was determined by measuring the progressive dilution of CFSE (Thermo Fisher Scientific). The proliferation index (PI) was calculated as follows: (MFI of the non-proliferating fraction / MFI of the proliferating fraction) × (% cells with diluted CFSE)[16]. Samples were acquired using a FACSymphony A5 or an LSR Fortessa

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equipped with FACSDiva software version 8.0.1 (all from BD Biosciences). Electronic compensation was performed using single-stained controls prepared with antibody-capture beads (BD Biosciences)[54]. T cell subsets were sorted to purity using a FACSAria III (BD Biosciences) as shown in Supplementary Fig. 1a.

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

Peptide-HLA class I tetramers

Biotinylated wildtype and D227K/T228A (KA) HLA-A*0201 complexes refolded with CMV $pp65_{495–503}$ NLVPMVATV (NV9) were multimerized with streptavidin-PE (Sigma-Aldrich) as described previously[52]. Cells were stained with each tetramer at a concentration of 5 μg/ml for 15 min at 37°C.

Mass cytometry by time of flight (CyTOF)

Cryopreserved PBMCs from three healthy donors and two HIV^+ donors were obtained from the HVTN. Purified monoclonal antibodies were purchased from commercial vendors (Supplementary Table 7) and labeled according to the Maxpar Antibody Labeling Kit Protocol (Fluidigm). Streptavidin was produced and labeled as described previously[55]. Myc-tagged peptide-HLA class I monomers were synthesized and biotinylated as described previously (Supplementary Table 7)[56]. Peptide-HLA class I tetramers were generated via the addition of heavy metal-labeled streptavidins in a triple coding scheme and used to stain cells in a cocktail format as described previously[55]. All reagents were titrated prior to use to determine optimal concentrations. Antibody staining, live/dead discrimination, and DNA staining were performed as described previously[57].

Cell culture and stimulation conditions

Cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 1% penicillin/streptomycin, and 2 mM L-glutamine. To induce cytokine production, magnetically-enriched $CD8^+$ T cells were stimulated for 3 h with phorbol 12-myristate 13-acetate (PMA, 10 ng/ml) and ionomycin (500 ng/ml) (both from Sigma-Aldrich) in the presence of anti-CD107a (clone H4A3, BD Biosciences) and the protein transport inhibitors GolgiPlug (brefeldin A, 1 μl/ml, BD Biosciences) and GolgiStop (monensin, 0.67 μl/ml, BD Biosciences). Subsets were identified among bulk $CD8^+$ memory T cells by gating as specified in the legend to Fig. 3f. Alternatively, FACS-purified $CD8^+$ T cell subsets were stimulated for 12 h with anti-

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CD3 plus CD28 DynaBeads (bead-to-cell ratio 1:2, Thermo Fisher Scientific). To evaluate the expression of activation markers and T-bet, FACS-purified $CD8^+$ T cell subsets were mixed with CD8-depleted autologous PBMCs (cell-to-cell ratio 1:4) and stimulated for 24 h with SEB (1 μg/ml, Sigma-Aldrich). To evaluate differentiation and proliferation, FACS-purified $CD8^+$ T cell subsets were stimulated for 3 or 4 d with anti-CD3 plus CD28 DynaBeads (bead-to-cell ratio 1:2, Thermo Fisher Scientific) in combination with various cocktails of human cytokines, including TGF-β, IL-2, IL-7, IL-12, and IL-15 (each at 10 ng/ml, Peprotech). To evaluate self-renewal capacity, FACS-purified $CD8^+$ T cell subsets were stimulated for 10 d with IL-15 (25 ng/ml, Peprotech). Unstimulated samples were used as controls in all assays.

scRNA-seq

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

scRNA-seq data analysis

Sample demultiplexing, barcode processing, and unique molecular identifier (UMI) counting were performed using Cell Ranger version 2.1.1 (10X Genomics). Briefly, raw base call files were demultiplexed in FASTQ format using the "cellranger mkfastq" pipeline, and the "cellranger count" pipeline was run with "--transcriptome=refdata-cellranger-GRCh38-1.2.0" for each sample. Outputs from "cellranger count" were concatenated in a single matrix. Libraries were then normalized to an identical sequencing depth using the "cellranger aggr" pipeline. Pooled data were imported into R version 3.5.1 using Seurat version 3.0.1 (ref. 58). Genes detected in less than three cells or cells containing less than 200 features were excluded from the analysis. Cells with unique feature counts less than 200 or greater than 3,500 were also filtered out, along with cells containing mitochondrial counts above 10%. The resulting dataset was normalized using a global scaling method converted by a scale factor (10,000) and log-transformed using the "ScaleData" function in Seurat version 3.0.1. Data were then subjected to cluster analysis using standard package procedures and the "FindClusters" function in Seurat version 3.0.1. Parameters were set to the first 20 principal components and a resolution of 0.6. DEGs for each cluster were identified using the Wilcoxon rank sum test with default correction for multiple comparisons in Seurat version 3.0.1.

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Anchor gene analysis of memory cell clusters from scRNA-seq data

Six different gene modules were computed from the scRNA-seq dataset using selected genes as anchors. Transcriptional scores were built by calculating the mean expression profiles of the top 100 genes most correlated with the anchors (Pearson's correlation).

Bulk RNA-seq

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

Bulk RNA-seq data analysis

Raw sequence data were quality-controlled using FastQC version 0.11.8 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Single-end reads were aligned to the human genome (GENCODE Human Release 29, reference genome sequence GRCh38/hg38) using STAR version 2.5.1b (ref. 59). Alignments were performed using default parameters. Reads associated with annotated genes were counted using the STAR aligner option "-quantMode geneCounts". Differential gene expression was assessed using the edgeR package version 3.20.9. Benjamini-Hochberg correction was applied to estimate the false discovery rate (FDR). Paired-end reads were processed similarly after removing adapter sequences and poor-quality bases with Trimmomatic version 0.36.

Overrepresentation analysis

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

Enrichment analysis

Normalized scRNA-seq counts were downloaded from the Gene Expression Omnibus (GSE120575). Analysis was restricted to cells belonging to clusters G5 or G10 as defined in Sade-Feldman *et al.* [18]. DEGs with an adjusted P value < 0.01 in the pairwise comparisons

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of G5 versus G10 were identified using the "FindAllMarkers" function in Seurat version 3.0.1. Hypergeometric tests were used to compare the G5 or G10 signatures with the combined T_{SCM}/T_{CM} or T_{PEX} signatures in "phyper" R.

Microarray data analysis

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

ATAC-seq

Libraries were prepared using a protocol adapted from Buenrostro *et al.* [62]. Briefly, 50,000 FACS-purified $CD8^+$ T cells per subset were washed in $PBS^{-/-}$ and resuspended in 50 μl of lysis buffer (10 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 0.1% IPEGAL CA-630). Nuclei were pelleted by centrifugation for 10 min at 500 g and resuspended in a final reaction volume of 50 μl comprising 1 μl of Tn5 transposase (made in-house), 10 μl of 5x transposase buffer (50 mM Tris-HCl pH 8.4, 25 mM $MgCl_2$), and 39 μl of ultrapure water (Milli-Q). The reaction was incubated with mixing at 300 rpm for 30 min at 37°C, supplemented with 10 μl of clean-up buffer (900 mM NaCl, 30 mM EDTA), 5 μl of 20% SDS, 0.7 μl of ultrapure water (Milli-Q), and 4.3 μl of proteinase K (18.6 μg/μl, Thermo Fisher Scientific), and incubated for a further 30 min at 40°C. Tagmented DNA was isolated using 2x SPRI Beads (Beckman Coulter) and amplified via PCR. Fragments smaller than 600 bp were isolated via negative size selection using 0.65x SPRI Beads (Beckman Coulter) and purified using 1.8x SPRI Beads (Beckman Coulter). Quality control was performed using a 4200 TapeStation System (Agilent) in conjunction with a Qubit 2.0 Fluorometer (Thermo Fisher Scientific). Libraries were then multiplexed in an equimolar pool and sequenced using a NextSeq 500/550 Platform (Illumina). At least 20 million single-end 75 bp reads were generated per sample.

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ATAC-seq data analysis

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

Single telomere length analysis

DNA was extracted from 6,000 FACS-purified $CD8^+$ T cells per subset using a QIAamp DNA Micro Kit (Qiagen). Single telomere length analysis was carried out at the XpYp telomere as described previously[67]. Briefly, genomic DNA was eluted in 35 µl of Tris (10 mM) containing 0.75 µl of the Telorette-2 linker (10 µM). Multiple PCRs were then performed for each test DNA. Each reaction was set up in a final volume of 10 µl containing 1 µl of DNA and 0.5 µM of the telomere-adjacent and teltail primers in 75 mM Tris-HCl pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01% Tween-20, and 1.5 mM $MgCl_2$, with 0.5 U of a 10:1 mixture of Taq (Thermo Fisher Scientific) and Pwo polymerase (Roche Molecular Biochemicals). The reactions were processed in a Tetrad2 Thermal Cycler (Bio-Rad). DNA fragments were resolved via 0.5% Tris-acetate-EDTA agarose gel electrophoresis and identified via Southern hybridization with a random-primed anti-^{32}P-labeled (PerkinElmer) TTAGGG repeat probe, together with probes specific for molecular weight markers at 1 kb (Stratagene) and 2.5 kb (Bio-Rad). Hybridized fragments were detected using a Typhoon FLA 9500 Phosphorimager (GE Healthcare). The molecular weights of the DNA fragments were calculated using a Phoretix 1D Quantifier (Nonlinear Dynamics).

TCR-seq

Total RNA was extracted separately from duplicate vials of 150,000 FACS-purified $CD8^+$ T cells per subset using an RNeasy Mini Kit (Qiagen). UMI-labeled 5′ RACE TCRβ sequencing libraries were prepared using a Human TCR Profiling Kit (MiLaboratory LLC). Libraries were prepared in parallel using the same number of PCR cycles and sequenced using a NextSeq 500/550 High-Output Kit with a NextSeq 500 Platform (Illumina). Approximately 3×10^7 reads were obtained in total and assembled into 2.2×10^6 UMI-labeled cDNA molecules (up to 10^5 per library). UMI extraction and consensus assembly were performed using MIGEC software version 1.2.9 (ref. 68) with a threshold of at least three reads per UMI. In-frame CDR3β repertoires were extracted using MiXCR software version 3.0.3 (ref. 69). Each library contained from 3,000 to 18,000 functional CDR3β clonotypes. Diversity metrics were calculated using VDJtools software version 1.2.1 (ref. 70) after normalization to 42,000 randomly selected UMIs per sample. D, R, and F2 metrics

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were calculated for the top 3,000 clones from each pair of samples using VDJtools software version 1.2.1 (ref. 70).

T cell transduction and culture conditions

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

Mouse studies

T_{STEM} and T_{PEX} cells were isolated from the PB of healthy donors based on differential expression of PD-1. Eight-week-old female NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice (Charles River) were infused retroorbitally with FACS-purified T_{STEM}, T_{PEX} or T_{EM} cells (1×10^6 per mouse) and autologous $CD8^-$ PBMCs (6×10^6 per mouse) and sacrificed on day 28. To maximize recovery, spleen and lung cells were mixed from the same experimental group, normalized in terms of the CD4 to CD8 ratio, and injected as above into secondary NSG recipients (1×10^6 $CD8^+$ T cells per mouse). Spleens were harvested on day 28 and processed to single cell suspensions. Absolute numbers of T cells in blood were determined using CountBright Absolute Counting Beads (Thermo Fisher Scientific). The frequencies of human $CD4^+$ and $CD8^+$ T cell subsets were determined by flow cytometry. For tumor experiments, eight-week-old female NSG mice were injected intravenously with 0.5×10^6 $Lucia^+/NGFR^+$/NALM-6 cells. After 4 d, mice were further injected with 3×10^6 CAR19-redirected T_{STEM}, T_{PEX}, or total $CD3^+$ T cells. Untransduced $CD3^+$ T cells were used as controls. Tumor progression was monitored weekly via bioluminescence detection using QUANTI-Luc (InvivoGen) and expressed as relative light units (RLUs).

Statistics

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability

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

Code availability

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

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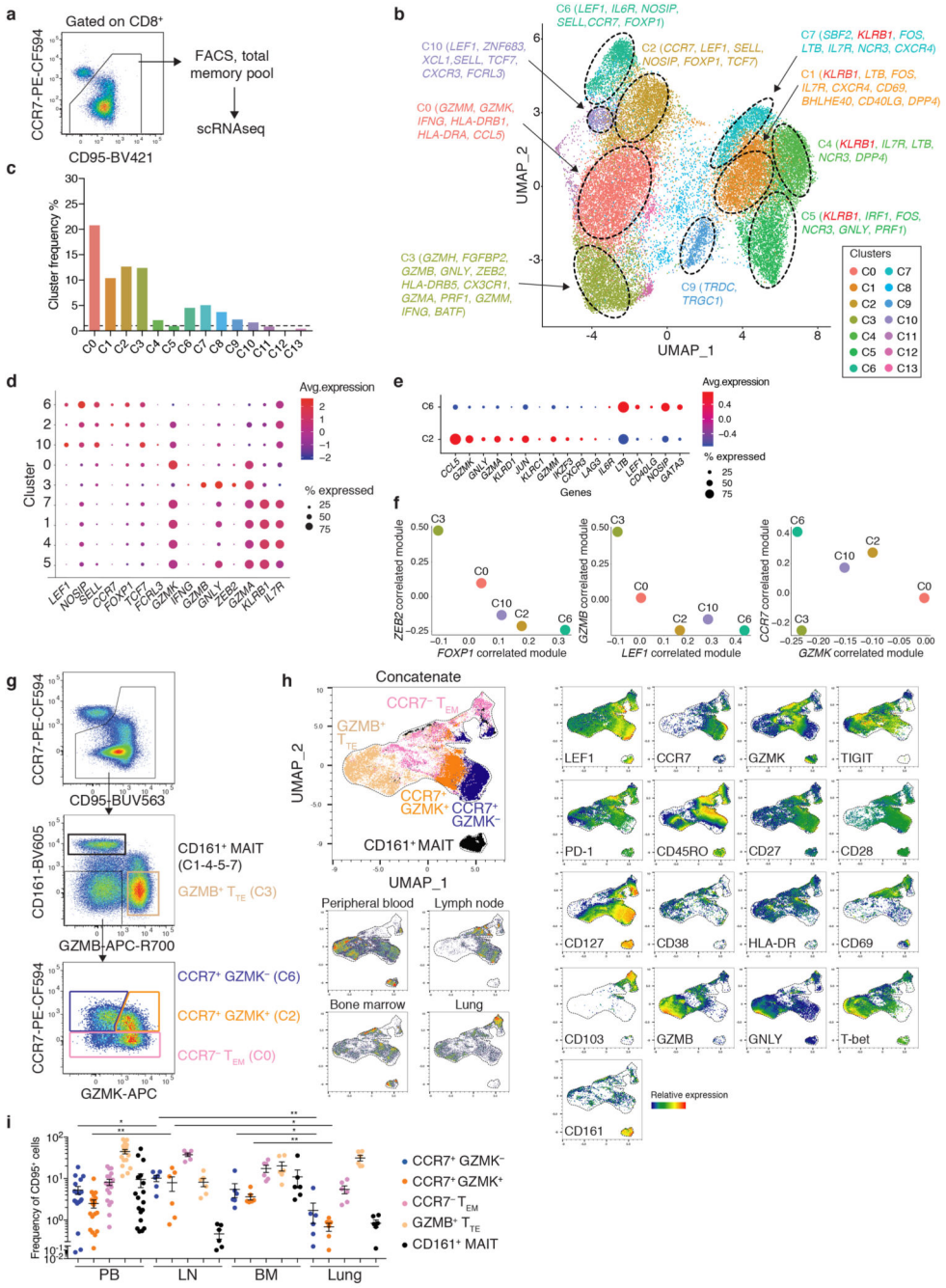

Fig. 1. Heterogeneity of the human CD8$^+$ memory T cell pool.

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).

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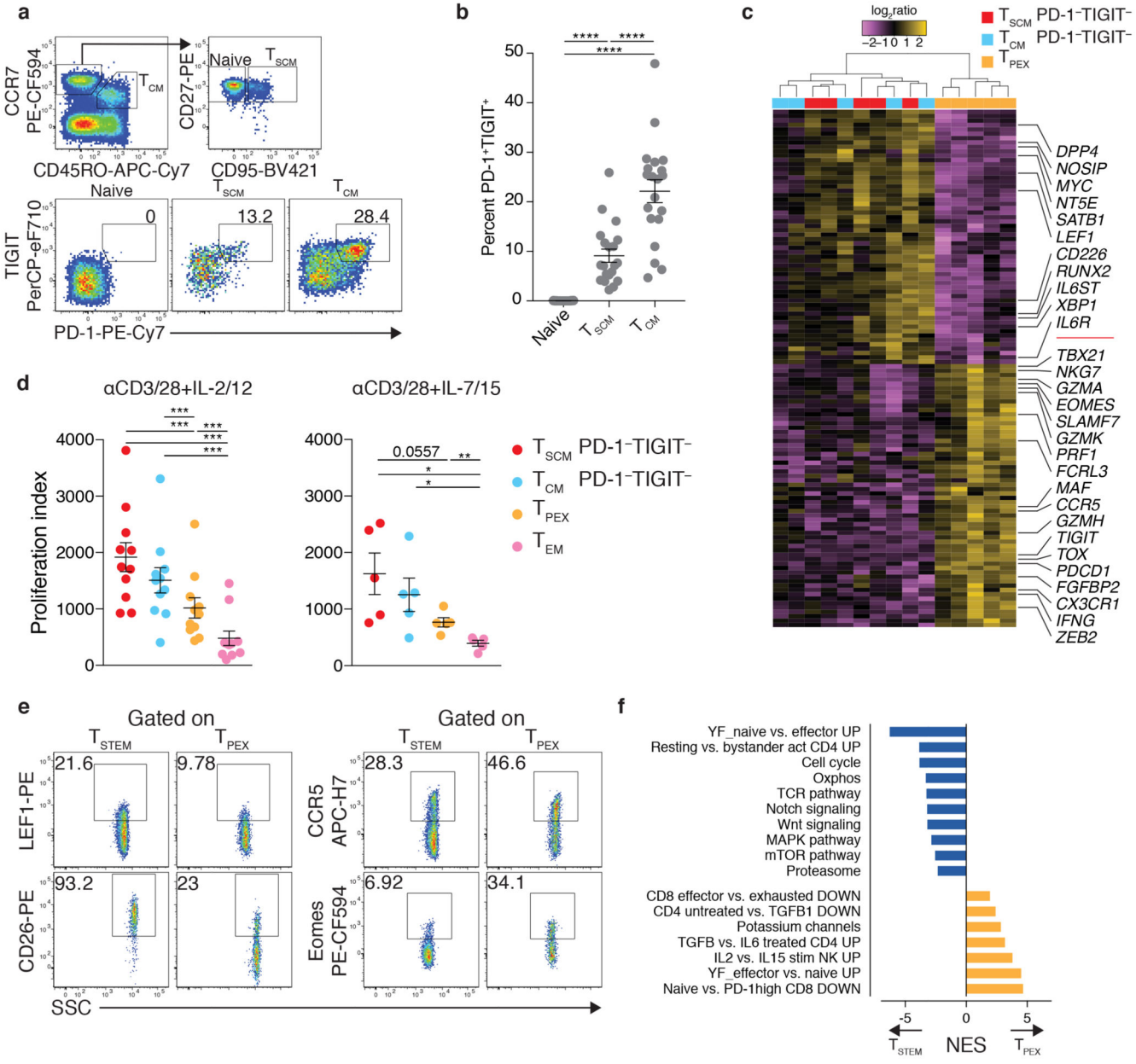

Fig. 2. Identification of stem-like CD8$^+$ memory T cell progenitors with differential expression of GZMK, PD-1, and TIGIT.

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 dot represents one donor (n = 20 from two independent experiments). Bars indicate mean ± SEM. ****$P < 0.0001$ (one-way repeated measures ANOVA). **c**, Heatmap showing DEGs (adjusted P value < 0.01) for the indicated CD8$^+$ memory T cell subsets (n = 5 donors). Labels highlight genes associated with memory or effector differentiation or exhaustion. Significance was evaluated using edgeR analysis with glmQLFTest and Benjamini-Hochberg correction. **d**, Proliferation of

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the indicated $CD8^+$ memory T cell subsets in response to stimulation with anti-CD3 plus CD28 for 4 d in the presence of IL-2 and IL-12 (n = 11 donors from six independent experiments) or IL-7 and IL-15 (n = 5 donors from three independent experiments). Index calculations were based on the dilution of carboxyfluorescein succinimidyl ester (CFSE). Each dot represents one donor. Bars indicate mean ± SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (two-tailed Wilcoxon signed rank test for each population versus T_{EM} in the presence of IL-2 and IL-12, two-tailed paired t-test for all other comparisons). **e**, Representative flow cytometric analysis of T_{STEM} ($CCR7^+$ $PD\text{-}1^-$ $TIGIT^-$) and T_{PEX} cells ($CCR7^+$ $PD\text{-}1^+$ $TIGIT^+$) showing the expression of markers selected from the DEGs identified in **c**. Numbers indicate percentages in the drawn gates. Similar data were obtained from other donors (n = 5 for LEF1 and CCR5, n = 4 for CD26 and Eomes). **f**, GSEA based on 1,000 permutations showing manually curated signatures that differed significantly (adjusted *P* value < 0.05) between T_{STEM} and T_{PEX} cells. NES, normalized enrichment score.

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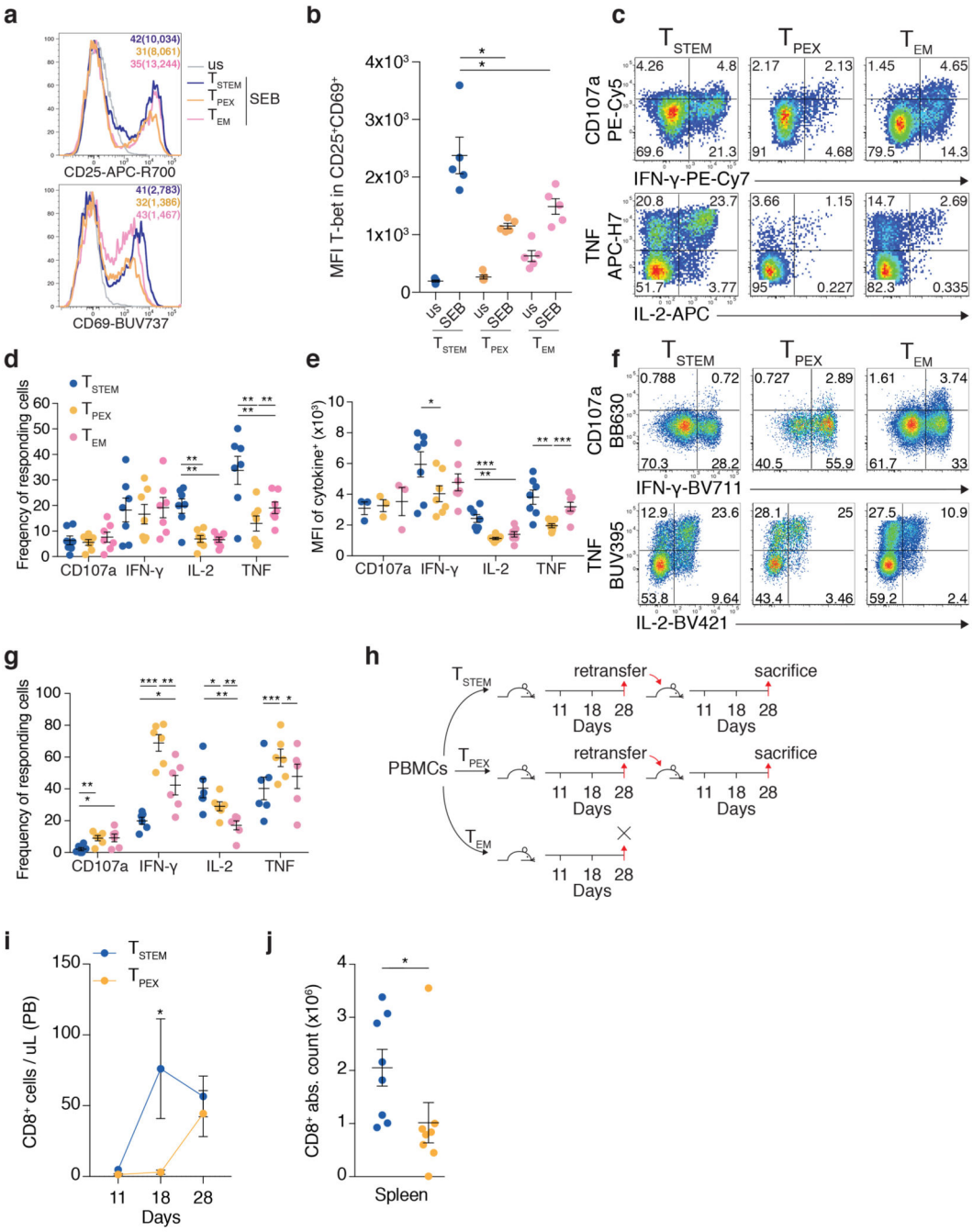

Fig. 3. Functional properties of T_{STEM} and T_{PEX} cells.

a, Representative flow cytometric analysis of FACS-purified T_{STEM}, T_{PEX}, and T_{EM} cells showing the expression of CD25 and CD69 before (us, unstimulated) and after stimulation with SEB for 24 h. Similar data were obtained from other donors (n = 4). Numbers colored to match each subset indicate percent marker$^+$ cells with the corresponding median fluorescence intensity (MFI) in brackets. **b**, Dot plot showing the expression of T-bet among CD25$^+$ CD69$^+$ T_{STEM}, T_{PEX}, and T_{EM} cells before (us) and after stimulation as in **a**. Data are shown in terms of MFI. Each dot represents one donor (n = 5 from two independent

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experiments). Bars indicate mean ± SEM. *$P < 0.05$ (two-tailed paired t-test). **c**, Representative flow cytometric analysis showing the expression of CD107a, IFN-γ, IL-2, and TNF among T_{STEM}, T_{PEX}, and T_{EM} cells stimulated with anti-CD3 plus CD28 for 12 h. Numbers indicate percentages in the drawn gates. **d**, Dot plot summarizing the data obtained as in **c**. Each dot represents one donor (n = 7 from four independent experiments). Bars indicate mean ± SEM. **$P < 0.01$ (two-tailed paired t-test). **e**, Dot plot showing the function $^{+}$ populations identified in **d** in terms of MFI. Each dot represents one donor (n = 3 from four independent experiments for CD107a, n = 7 from four independent experiments for IFN-γ, IL-2, and TNF). Bars indicate mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (two-tailed paired t-test). **f**, Representative flow cytometric analysis showing the expression of CD107a, IFN-γ, IL-2, and TNF among T_{STEM}, T_{PEX}, and T_{EM} cells after stimulation of magnetically-enriched $CD8^+$ T cells with PMA and ionomycin for 3 h. Numbers indicate percentages in the drawn gates. Subsets were gated as $CCR7^+$ $GZMK^-$ (T_{STEM}), $CCR7^+$ $GZMK^+$ (T_{PEX}), or $CCR7^-$ $CD45RO^+$ $CD95^+$ (T_{EM}). **g**, Dot plot summarizing the data obtained as in **f**. Each dot represents one donor (n = 6 from three independent experiments). Bars indicate mean ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (two-tailed paired t-test). **h**, Schematic layout of the serial transfer experiments. **i**, Line chart showing the absolute numbers of $CD8^+$ T cells in PB on days 11, 18, and 28 after transfer of T_{STEM} or T_{PEX} cells into secondary NSG recipients. Data were pooled from two independent experiments (total n = 8 mice). Bars indicate mean ± SEM. *$P = 0.0117$ (two-way ANOVA). **j**, Dot plot showing the absolute numbers of $CD8^+$ T cells in spleen on day 28 after transfer of T_{STEM} or T_{PEX} cells into secondary NSG recipients. Each dot represents one mouse (n = 8 from two independent experiments). Bars indicate mean ± SEM. *$P = 0.0148$ (two-tailed Mann-Whitney U test).

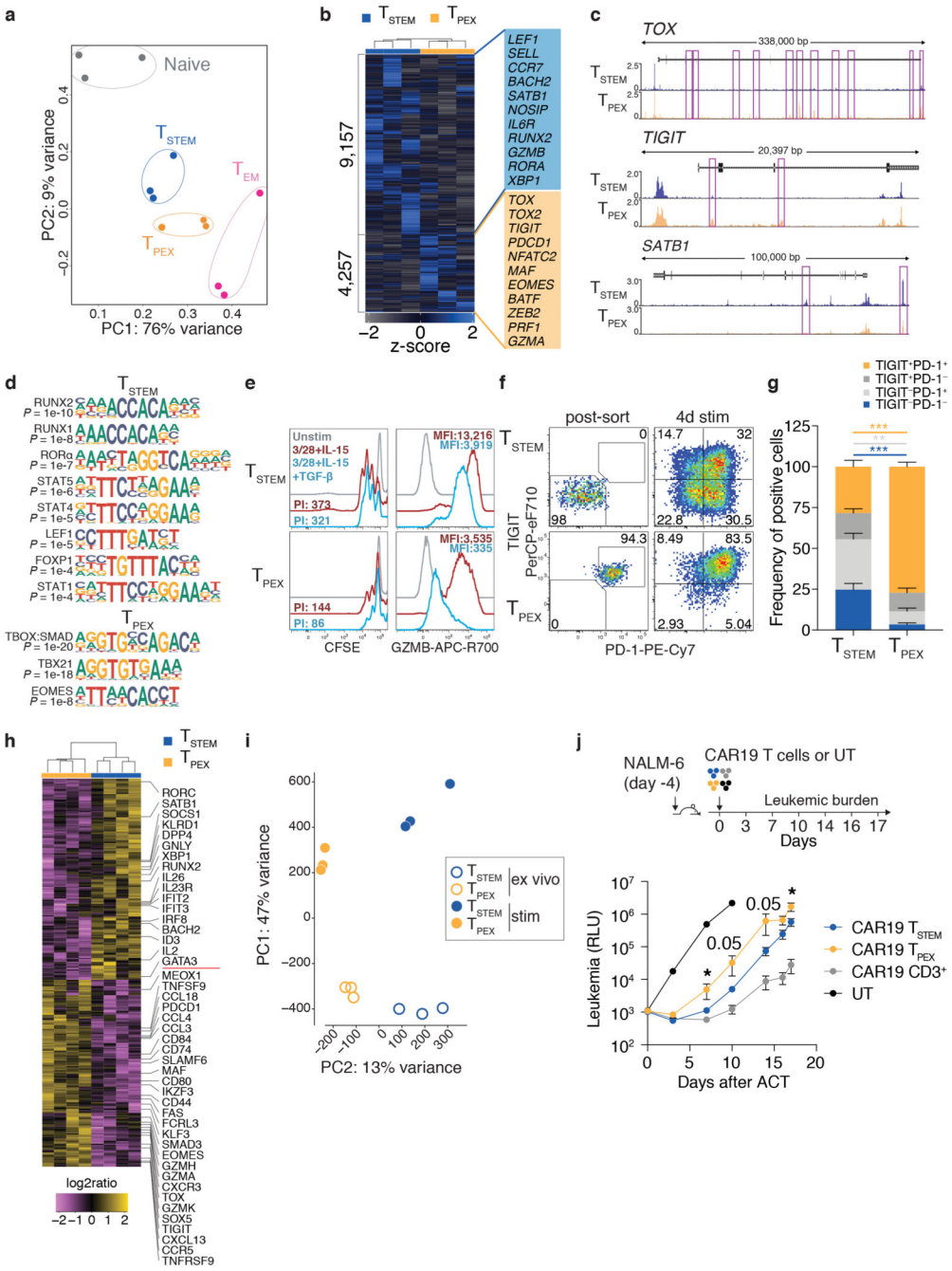

Fig. 4. Fate commitments of T_{STEM} and T_{PEX} cells.

a, PCA plot showing the top 1,000 hypervariable peaks obtained from ex vivo ATAC-seq analysis (adjusted *P* value < 0.01) of T_{STEM}, T_{PEX}, and T_{EM} cells. Each dot represents one donor (n = 3). **b**, Heatmap showing DARs. Labels highlight accessible genes associated with memory or effector differentiation or exhaustion. **c**, Representative genomic regions showing the ATAC-seq profiles of *TOX*, *TIGIT*, and *SATB1* in T_{STEM} and T_{PEX} cells. DARs are highlighted in purple. **d**, TFBMs enriched among the DARs shown in **b**. Enrichment was assessed using a one-sided hypergeometric test in HOMER with correction for FDR. **e**,

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Representative overlay histograms showing CFSE dilution (left) and GZMB expression profiles (right) for T_{STEM} and T_{PEX} cells stimulated with anti-CD3 plus CD28 and IL-15 for 3 d in the absence or presence of TGF-β. Unstimulated controls are shown for comparison. Similar data were obtained from other donors in the absence (n = 8 from four independent experiments) or presence of TGF-β (n = 6 from four independent experiments). PI, proliferation index. **f**, Representative flow cytometric analysis of T_{STEM} and T_{PEX} cells showing the expression of PD-1 and TIGIT after stimulation with anti-CD3 plus CD28 for 4 d in the presence of IL-2 and IL-12. Numbers indicate percentages in the drawn gates. **g**, Bar graph summarizing the data obtained as in **f** (n = 5 donors from three independent experiments). Bars indicate mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$ (two-tailed Mann-Whitney U test). **h**, Heatmap showing selected DEGs (adjusted P value < 0.05) for T_{STEM} and T_{PEX} cells stimulated as in **f** (n = 4 donors). Significance was evaluated using edgeR analysis with glmQLFTest and Benjamini-Hochberg correction. **i**, PCA plot as in **a** comparing T_{STEM} and T_{PEX} cells before and after stimulation as in **f**. **j**, Top: schematic layout of the adoptive transfer experiment. Bottom: time series plot showing the growth of NALM6 cells in NSG mice (n = 5/group) adoptively transferred with T_{STEM} or T_{PEX} cells expressing a CAR specific for CD19 (CAR19). RLU, relative light unit; UT, untransduced $CD3^+$ cells. Follow-up was stopped when RLU values ≥ 10^6 were observed in more than 75% of mice in one of the treated groups. Bars indicate mean ± SEM. $^{*}P < 0.05$ (two-tailed unpaired t-test for CAR19 T_{STEM} versus CAR19 T_{PEX} on day 17, two-tailed Mann-Whitney U test for all other comparisons).

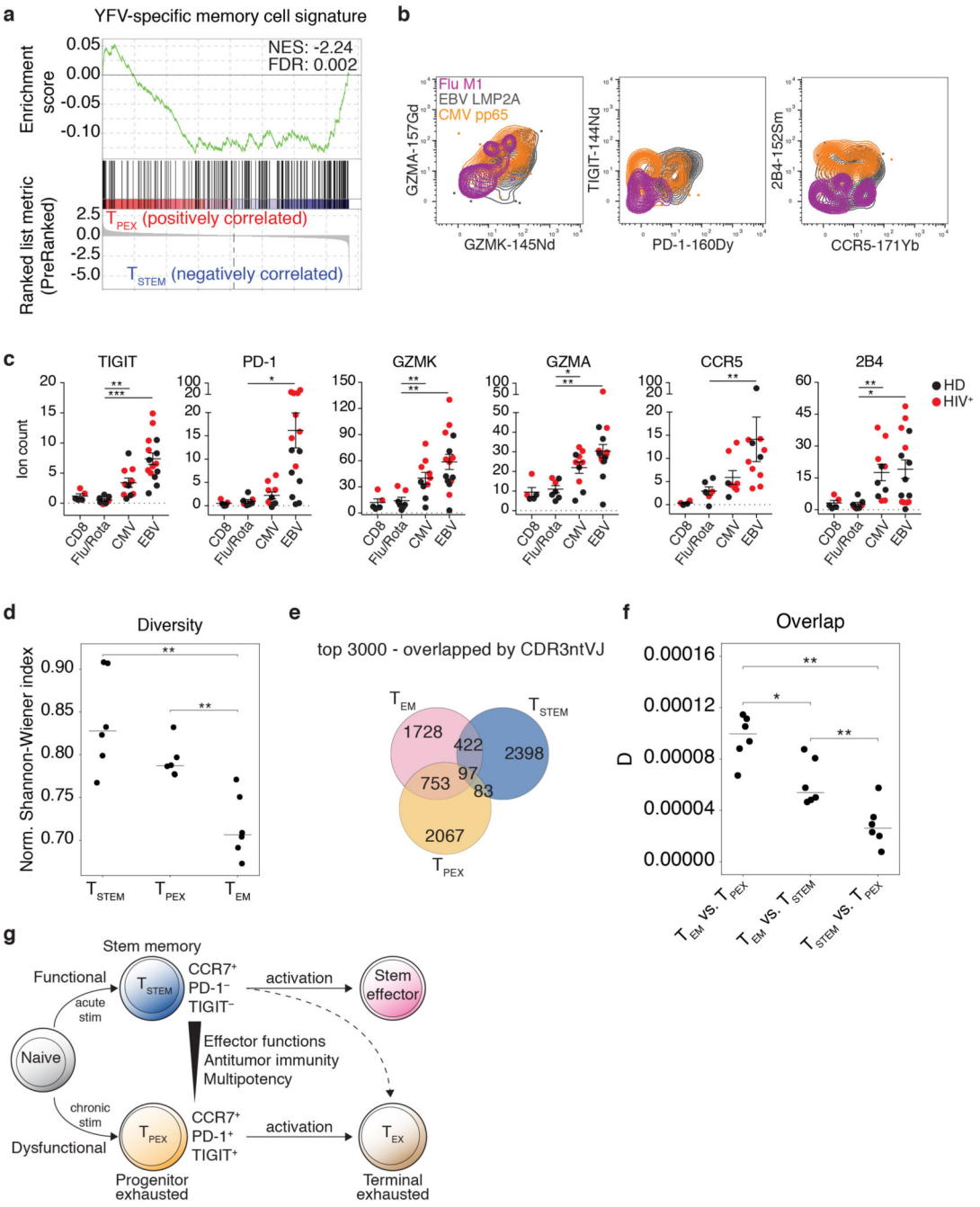

Fig. 5. Antigen specificity and clonal identity of T_{STEM} and T_{PEX} cells.

a, GSEA of the YFV-specific $CD8^+$ memory T cell signature[45] in T_{STEM} versus T_{PEX} cells. **b**, Representative CyTOF analysis showing the expression of GZMK, GZMA, PD-1, TIGIT, CCR5, and 2B4 among $CCR7^+$ virus-specific $CD8^+$ T cell populations from healthy (n = 3) and HIV^+ donors (n = 2). **c**, Dot plots summarizing the data obtained as in **b**. Epitopes derived from influenza virus (n = 6) and rotavirus (n = 1) were pooled for simplicity. Comparative data are shown for the corresponding total $CD8^+$ T cell populations. Each dot represents one specificity in one donor (n = 3 healthy donors, n = 2 HIV^+ donors). Bars

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indicate mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (two-tailed Mann-Whitney U test for CCR5 comparisons, two-tailed unpaired t-test for all other marker comparisons). HD, healthy donor. **d**, Dot plot showing the normalized Shannon-Wiener index for TCRβ repertoires obtained from the T_{STEM}, T_{PEX}, and T_{EM} subsets. Each dot represents one donor (n = 6). Bars indicate median values. **$P < 0.01$ (two-tailed paired t-test with Bonferroni correction). **e**, Venn diagram showing the numbers of shared and unique clonotypes among T_{STEM}, T_{PEX}, and T_{EM} cells from a representative donor. Similar data were obtained from other donors (n = 5). Analysis was restricted to the top 3,000 clonotypes. **f**, Dot plot summarizing the pairwise comparisons among subsets illustrated in **e**. D metric in VDJtools. Each dot represents one donor (n = 6). Bars indicate median values. *$P < 0.05$, **$P < 0.01$ (two-tailed paired t-test with Bonferroni correction). **g**, Proposed model showing the origins and differentiation trajectories of T_{STEM} and T_{PEX} cells.

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