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Divergent clonal differentiation trajectories of T cell exhaustion

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Abstract

Chronic antigen exposure during viral infection or cancer promotes an exhausted T cell (Tex) state with reduced effector function. However, whether all antigen-specific T cell clones follow the same Tex differentiation trajectory remains unclear. Here, we generate a single-cell multi-omic atlas of T cell exhaustion in murine chronic viral infection that redefines Tex phenotypic diversity, including two late-stage Tex subsets with either a terminal exhaustion (Tex^{term}) or a killer cell lectin-like receptor (KLR)-expressing cytotoxic phenotype (Tex^{KLR}). We use paired single-cell RNA and T cell receptor (TCR) sequencing to uncover clonal differentiation trajectories of Tex^{term}-biased, Tex^{KLR}-biased, or divergent clones that acquire both phenotypes. We show that high TCR signaling avidity correlates with Tex^{term}, while low avidity correlates with effector-

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like Tex^{KLR} fate. Finally, we identify similar clonal differentiation trajectories in human tumor-infiltrating lymphocytes. These findings reveal clonal heterogeneity in the T cell response to chronic antigen that influences Tex fates and persistence.

Chronic antigen exposure during viral infections and cancer leads to impaired CD8⁺ T cell responses characterized by reduced effector function, diminished proliferative capacity, and high expression of inhibitory receptors, including PD-1, LAG-3, and TIM3, termed T cell exhaustion^{1,2}. However, exhausted T cells (Tex) maintain some effector functions and persist long-term, suggesting that Tex may control pathogen burden while maintaining immune homeostasis^{3,4}. Recent studies have identified heterogeneity in Tex phenotypes characterized by distinct surface receptors, functionality, proliferative capacity, and tissue localization⁵⁻¹². Some studies support a linear differentiation model, whereby TCF1⁺CXCR5⁺PD-1⁺ progenitor Tex (Tex^{prog}) self-renew and maintain downstream Tex subsets, including CX3CR1⁺PD-1⁺ intermediate Tex (Tex^{int}) and PD-1⁺TIM3⁺ terminal Tex (Tex^{term})^{5-9,13-15}. These subpopulations exhibit distinct epigenetic states with TCF1 and BACH2 driving Tex^{prog} formation, while the high mobility group transcription factor (TF), TOX, orchestrates the exhaustion program in all Tex states^{4,7,16-21}. Finally, Tex subsets are further distinguished by their ability to respond to immune checkpoint blockade (ICB); Tex^{term} possess a stable epigenetic program and cannot be reinvigorated by ICB, while Tex^{prog} can proliferate in response to ICB and may be important for therapeutic response^{3,6,22}. Despite these advances, we lack a comprehensive view of Tex states, their clonal relationships, and the molecular programs underlying their differentiation, particularly in polyclonal T cell responses.

Here, we generate a Tex differentiation atlas using single-cell chromatin accessibility, transcriptome, and T cell receptor (TCR) sequencing of antigen-specific CD8⁺ T cells during chronic lymphocytic choriomeningitis virus (LCMV) infection. We discover new Tex subsets, including an early effector exhausted subset (Tex^{eff}) that initiates the molecular exhaustion program, and a killer cell lectin-like receptor-expressing Tex subset (Tex^{KLR}) which emerges as a late-stage phenotype concurrent with Tex^{term}. T cell clone tracing with scRNA/TCR-seq nominates diverse Tex differentiation trajectories, including Tex^{term}-biased, Tex^{KLR}-biased, or divergent fates, comprising both cell types. Tex clones traffic to multiple organ sites where their differentiation trajectories are conserved; however, Tex^{KLR}-biased clones are depleted in the liver, suggesting that Tex^{term} may be phenotypically adapted for specific tissue microenvironments. Finally, we show that clone trajectories correlate with TCR signaling avidity; high-avidity TCR clones are biased towards Tex^{term}, while low-avidity TCR clones are biased towards Tex^{KLR}. Overall, these results provide an in-depth view of the gene regulatory programs and clonal dynamics of Tex states during chronic infection.

A multi-omic atlas of T cell exhaustion

To profile CD8⁺ T cell exhaustion, we used mouse models of acute (LCMV Armstrong – Arm) or chronic (LCMV Clone 13 – C113) viral infection. These two viral strains share immunodominant epitopes, enabling direct comparison of antigen-specific T cell

responses²³. We generated paired single-cell RNA- and T cell receptor (TCR)-sequencing (scRNA/TCR-seq) and single-cell assay for transposase accessible chromatin with sequencing (scATAC-seq) data from LCMV glycoprotein 33–41 tetramer positive (gp33⁺) and tetramer negative (gp33⁻) splenic CD8⁺ T cells at Day 8 (D8) and Day 21 (D21) post-infection (Fig. 1a-d). At D21 of C113 infection, we also generated scRNA/TCR-seq of gp33⁺ and gp33⁻ populations from two additional organs (lung and liver; Fig. 1a,c and Extended Data Fig. 1a). Finally, we sorted D21 C113 splenic T cells using previously defined surface markers that identify Tex^{prog} (PD-1⁺SLAMF6⁺), Tex^{int} (PD-1⁺CX3CR1⁺), and Tex^{term} (PD-1⁺SLAMF6⁻CX3CR1⁻) phenotypes and performed scRNA/TCR- and scATAC-seq (Extended Data Fig. 1b)^{7-9,13}. In total, we obtained 96,750 scRNA-seq profiles that passed quality control and detected TCR alpha and beta sequences in 88,696 T cells (91.7%), comprising 5,197 expanded T cell clones (clones >1 cell; Fig. 1c and Extended Data Fig. 1c). In addition, we obtained 62,731 scATAC-seq profiles that passed quality control (Fig. 1d, Extended Data Fig. 1d,e).

We performed uniform manifold approximation and projection (UMAP) for dimensionality reduction and identified 11 scRNA-seq clusters, which were annotated based on differentially expressed genes (DEGs). In Arm infection, we observed naïve T cells (T^{naive}; *Ccr7*, *Sell*, *Lef1*), effector T cells (T^{eff}; *Klrg1*, *Ly6c2*), effector memory T cells (T^{em}; *Klrb1c*, *Klrd1*, *S1pr1*), and memory T cells (T^{mem}; *Il7r*, *Arl4c*, *Il18r1*; Fig. 1e, Supplementary Table 1). In C113 infection, we observed Tex^{prog} (*Tcf7*, *Slamf6*, *Id3*), Tex^{int} (*Lgals3*, *S100a4*, *Mki67*), and Tex^{term} (*Gzma*, *Cd101*, *Entpd1*), as expected (Fig. 1c,e). In addition, we also observed early effector exhausted cells (Tex^{eff}; *Xcl1*, *Top2a*, *Mif*, a predominant population at D8 of C13 infection), killer cell lectin-like receptor (KLR)-expressing exhausted cells (Tex^{KLR}; *S1pr5*, *Cx3cr1*, *Klrc1*; emerging late in C13 infection), lung terminal exhausted cells (Tex^{lung}; *Lag3*, *Ifng*, *Ccl4*), and interferon signature gene (ISG) exhausted T cells (Tex^{ISG}; *Isg15*, *Ifit1*, *Isg20*; Fig. 1c,e). We observed 8 analogous T cell populations in the scATAC-seq data based on integration with scRNA-seq data (Fig. 1d, Extended Data Fig. 1f). Since our goal was to analyze Tex epigenetic states, we did not perform scATAC-seq at D21 in Arm infection, or in lung or liver T cells in C113 infection; thus, scATAC-seq clusters did not include T^{em}, T^{mem}, or Tex^{lung} subsets. However, scATAC-seq clusters did reveal additional heterogeneity, consisting of two effector populations mainly derived from the Arm condition (T^{eff} and T^{eff2}) and an Tex^{eff} population from the C113 condition (Fig. 1d,f).

scATAC-seq profiles were analyzed at the level of: (1) *cis*-regulatory element chromatin accessibility (open chromatin regions; OCRs), (2) gene activity scores, computed from OCR accessibility, weighted by distance, and (3) TF activity, computed from TF binding site enrichment in OCRs or genome-wide^{24,25}. Cell type-specific OCR accessibility was correlated with gene expression at marker gene loci that define Tex subsets, including *Tcf7*, *Pdcd1*, and *Tox* (Fig. 1g,h, Supplementary Table 2). T^{naive}-specific OCRs were enriched for the TCF/LEF motifs, which were also enriched in Tex^{prog}, along with other known Tex^{prog} TFs (e.g., BATF, AP-1 and BACH)^{14,18,26,27}. Tex^{eff} showed NFAT motif enrichment, while KLF motifs were specifically enriched in the Tex^{int}, T^{eff}, and Tex^{KLR} populations. Finally, Tex^{term}-specific OCRs exhibited strong enrichment for NR4A, RUNX and NFAT TF motifs (Extended Data Fig. 1g, Supplementary Table 3)²⁸⁻³¹.

CX3CR1⁺ Tex comprise two functionally distinct subsets

We next examined heterogeneity within CX3CR1⁺ Tex cells, which have recently been described as a functional intermediate cell state between Tex^{prog} and Tex^{term}^{8,9,13}. scRNA-seq of sorted CX3CR1⁺ T cells from D21 of C113 infection revealed heterogeneity that primarily spanned two distinct phenotypes (Tex^{int} and Tex^{KLR}; Fig. 2a). To understand the transcriptional programs that distinguish Tex^{int} from Tex^{KLR}, we performed DEG analysis and found 206 Tex^{KLR}-biased genes and 384 Tex^{int}-biased genes (Fig. 2b, Supplementary Table 4). Pathway analysis revealed enrichment for T cell exhaustion-related biological terms in Tex^{int}, cell cycle related terms in both populations, and T cell activation/motility-related terms in Tex^{KLR} (Extended Data Fig. 2a). Notably, many markers of terminal effector and effector memory T cells, such as the killer cell lectin-like receptor (KLR) family members (e.g., *Klrp1*, *Klrk1*, *Klrcl1*, *Klre1* and *Klrg1*), the TF *Zeb2*, and its target gene, *Slpr5* (a marker of tissue emigrating T cells), showed a highly specific expression pattern in Tex^{KLR}^{32,33}. In contrast, Tex^{int} expressed canonical exhaustion markers (e.g., *Ctla4*, *Pdcd1*, *Lag3*) and TCR signaling genes (e.g., *Lck*, *Coro1a*) (Fig. 2b). Similar gene expression signatures were detected when comparing Tex^{KLR} and Tex^{term} with a more pronounced exhaustion signature in the latter (e.g., *Cxcr6*, *Cd101*, *Tigit*) (Fig. 2b, Supplementary Table 5). To support an intermediate exhaustion state in Tex^{int}, we analyzed CXCR6 surface expression in Tex^{prog} (CX3CR1⁻SLAMF6⁺KLRG⁻), Tex^{int} (CX3CR1⁺SLAMF6⁻KLRG⁻), and Tex^{term} (CX3CR1⁻SLAMF6⁻KLRG⁻) by flow cytometry, which showed intermediate CXCR6 expression in Tex^{int}, compared to Tex^{term} and Tex^{prog} (Extended Data Fig. 2b).

Next, we focused on Tex^{KLR} and assessed the gene signature of terminal effector memory T cells (T-T^{em}), a recently described T^{em} subset identified during acute LCMV infection which express effector T cell markers, including KLRs³⁴. The T-T^{em} gene signature was highly expressed in Tex^{KLR}, suggesting that this subset may represent a parallel differentiation path to T-T^{em} (Fig. 2c,d). Finally, we assessed the functionality and proliferative capacity of gp33-specific Tex^{term}, Tex^{int}, and Tex^{KLR} (CX3CR1⁺KLRG1⁺SLAMF6⁻) by measuring IFNG, LAMP-1 (cytotoxic degranulation marker), and MKI67 using flow cytometry. These analyses demonstrated superior functional and proliferative capacity of Tex^{KLR}, compared to Tex^{term} and Tex^{int} (Fig. 2e; Extended Data Fig. 2c). In summary, the CX3CR1⁺ T cell pool contains two Tex subsets with distinct surface and functional phenotypes.

Tex acquire organ-specific terminal exhaustion signatures

We re-clustered scRNA-seq profiles from gp33⁺ and gp33⁻ CD8⁺ T cells from the spleen, lung, and liver at D21 of C113 infection and examined Tex subset distribution across organs (Fig. 2f). Relative to splenic T cells, cells in the lung exhibited an alternative terminal exhausted phenotype (Tex^{lung}) and a reduced Tex^{prog} population, with similar Tex^{KLR} and Tex^{int} proportions (Fig. 2f,g). Strikingly, T cells in the liver almost exclusively adopted the Tex^{term} phenotype, as previously described (93.9% Tex^{term}; Fig. 2f,g)¹⁵. We further examined tissue-specific differences in the exhaustion signature by pairwise DEG analyses. Compared to splenic Tex^{term}, liver-derived Tex^{term} possessed a strong tissue-resident memory T cell signature (e.g., *Cd69*, *Cxcr6*, *Ccl3*) and a gene program linked to TNF-, and glucocorticoid signaling (Extended Data Fig. 2d, Supplementary Table 6). Similarly,

lung-derived Tex^{term} also exhibited typical markers of lung-resident memory T cells (e.g., *Cxcr6*, *Cd44*) and several integrin genes (e.g., *Itga4*, *Itgab7*, *Itgab1*; Extended Data Fig. 2d, Supplementary Table 7). Furthermore, both liver- and lung-derived Tex^{term} expressed higher levels of pro-survival genes (e.g., *Bcl2*, *Bcl2a1b*, *Bcl2a1d*) than splenic Tex^{term} (Extended Data Fig. 2d, Supplementary Table 8). These results suggest that Tex^{term} can obtain tissue residency programs and persist in tissues in the setting of chronic antigen.

Despite tissue-specific differences, we observed a common Tex^{term} gene signature across all organs. This signature ($n=35$ genes) contained previously described exhaustion-related genes, such as immune checkpoint inhibitory receptors, *Pdcd1* and *Tox* (Fig. 2h). Next, we ranked the severity of exhaustion among Tex^{term} from each organ using a previously defined exhaustion gene signature⁶. We observed that liver-derived Tex^{term} scored the highest, followed by splenic and lung-derived Tex^{term} (Fig. 2i). In line with these findings, flow cytometry analysis demonstrated increased frequencies of Tex^{term} in the liver (88% of $\text{PD1}^+\text{CD8}^+$ T cells), compared to the spleen (47%) and lung (51%; Fig. 2j,k,l). In contrast, we observed the highest frequency of Tex^{prog} in the spleen (18.3% of $\text{PD1}^+\text{CD8}^+$ T cells). Tex^{int} and Tex^{KLR} were increased in the spleen (Tex^{int} : 32.33%; Tex^{KLR} : 6.59% of $\text{PD1}^+\text{CD8}^+$ T cells) and lung (Tex^{int} : 44.77%; Tex^{KLR} : 10.01%), compared to the liver (Tex^{int} : 10.72%; Tex^{KLR} : 2.58%; Fig. 2j,k,l). We also scored Tex^{term} and Tex^{int} profiled by scRNA-seq based on cell cycle activity, which ranked liver-derived cells as the least proliferative, followed by the lung and spleen, inversely correlating with the severity of exhaustion, and we confirmed these observations with flow cytometry (Extended Data Fig. 2e,f). These results demonstrate that T cell exhaustion develops across multiple organs with a common gene expression signature but microenvironment-specific effects.

Clone tracing reveals divergent Tex differentiation fates

We next leveraged paired scRNA/TCR-seq data to analyze clonal trajectories of gp33^+ and gp33^- (other reactive clones and non-reactive T^{naive} cells) T cells during Arm and C113 (D8 and D21) infection in the spleen (Fig. 3a). We identified 212 and 280 expanded gp33^+ T cell clones (>1 cell; 53 and 40 expanded clones/1,000 cells sequenced) at D8 and D21 of Arm infection, respectively, and 134 and 338 expanded clones (95 and 36 expanded clones/1,000 cells sequenced) at D8 and D21 of C113 infection, respectively. At D8 of Arm infection, clonally expanded gp33^+ T cells were largely restricted to the T^{eff} pool, while at D21, clonally expanded gp33^+ T cells exhibited a balanced distribution between T^{em} and T^{mem} (Fig. 3b and Extended Data Fig. 3a). In contrast, clonal expansion in gp33^+ T cells at D8 in C113 infection occurred almost exclusively in Tex^{eff} (Fig. 3c). Importantly, the Tex^{prog} population showed weak clonal expansion at D8. However, at D21, gp33^+ specific expanded clones adopted all Tex phenotypes (Figure 3c,d and Extended Data Fig. 3b).

We visualized the distribution of phenotypes for the top 10 expanded gp33^+ T cell clones at D8 and D21 in each infection. At D8 of Arm infection, almost all cells acquired the T^{eff} phenotype. At D21, top expanded clones acquired both T^{em} and T^{mem} phenotypes, with clone sizes ranging from 77–321 (mean 153 cells, 3.8% of 4,030 total cells; Fig. 3e and Extended Data Fig. 3a). In contrast, the top expanded clones in C113 infection primarily acquired the Tex^{eff} phenotype at D8, with clone sizes ranging from 26–95 cells (mean 49,

3.5% of 1,414 total cells; Fig. 3e). Analysis of D21 of C113 infection identified substantially larger clones, ranging from 146–2,026 cells (mean 525, 5.6% of 9,343 total cells; Fig. 3e). Strikingly, these clones contained cells with multiple Tex phenotypes, although the frequency of each phenotype varied between individual clones. Namely, individual clones either preferentially acquired the Tex^{term} or Tex^{KLR} phenotypes or developed into both phenotypes (Fig. 3e and Extended Data Fig. 3b). We performed an analysis of the top 7 phenotypic distribution of clones (>3 cells), which revealed three main clonal differentiation patterns (clone behaviors): 1) Tex^{term}-biased clones, consisting of cells that predominantly acquired the Tex^{term} (46% of clones), 2) Tex^{KLR}-biased clones, consisting of T cells that predominantly acquired the Tex^{KLR} phenotype (18% of clones), and 3) divergent clones, consisting of cells that acquired Tex^{term} and Tex^{KLR} phenotypes (36% of clones; Fig. 3f). Divergent clones were the most clonally expanded and ranged from 4–2,026 cells (mean 145) per clone, while Tex^{term}-biased clones ranged from 4–111 cells (mean 17) per clone. Interestingly, Tex^{KLR}-biased clones were relatively small and ranged from 4–21 cells (mean 8) per clone (Fig. 3g,h). We also noted several larger clones (4–233 cells, mean 54) that skewed to the Tex^{KLR} phenotype (>50% of cells acquire the Tex^{KLR} phenotype), but had a small percentage of Tex^{term} (Extended Data Fig. 3c). To account for sampling bias, we randomized T cell phenotype and TCR clone assignment to generate a null distribution of clone patterns, which revealed a striking enrichment of Tex^{KLR}- and Tex^{term}-biased clone behavior over chance, whereas divergent clonal behavior was twice as likely to be detected by chance than observed in our data (Extended Data Fig. 3d). These results reveal novel divergent clonal Tex differentiation trajectories during chronic infection (Fig. 3i).

Directions of clonal Tex differentiation fates

To understand cell state transitions in Tex differentiation paths, we used dynamo to perform RNA velocity analyses^{35,36}. This analysis demonstrated that Tex^{eff} at D8 transition to either Tex^{int} or Tex^{prog} fates, and at D21, Tex^{int} showed a bifurcation to either Tex^{KLR} or Tex^{term} phenotypes (Fig. 4a). Furthermore, we applied dynamo to scRNA-seq profiles from individual TCR clones; divergent clones recapitulated bifurcating Tex differentiation trajectories from Tex^{int} to either Tex^{term} or Tex^{KLR}, accompanied by increased transcription of Tex^{term} or Tex^{KLR} genes (Fig. 4b,c). Additionally, RNA velocity pseudotime of a divergent clone revealed a gene expression map of Tex differentiation from Tex^{int} to either terminal Tex state (Extended Data Fig. 4a). In contrast, Tex^{term}-biased clones showed a linear RNA velocity path from Tex^{int} to Tex^{term}, while Tex^{KLR}-biased clones primarily comprised Tex^{KLR} (Fig. 4b,c). It is important to note that this analysis summarizes the direction and magnitude of dominant local trajectories in UMAP space, rather than nominating terminal differentiation states^{35,36}. For comparison, we performed velocity analyses in CD8⁺ T cells from Arm infection (Extended Data Fig. 4b).

We used scATAC-seq data to define changes in chromatin accessibility that accompany these fate transitions (Extended Data Fig. 4c,d). Comparisons of OCRs and TF motif enrichments between Tex subsets demonstrated a high degree of similarity between Tex^{eff} and Tex^{prog}. Similarly, Tex^{int} exhibited an intermediate chromatin state between Tex^{term} and Tex^{KLR} states, with very few unique OCRs (Extended Data Fig. 4d). We analyzed 15,809 variable OCRs for TF motif enrichments, as well as gene integration scores, across three

differentiation trajectories nominated by RNA velocity and clonal trajectories: (1) Tex^{prog} trajectory ($\text{Tex}^{\text{eff}} \rightarrow \text{Tex}^{\text{prec}}$ [precursor exhausted Tex, an early Tex^{prog} population present at D8 in C113 infection] $\rightarrow \text{Tex}^{\text{prog}}$), (2) Tex^{term} trajectory ($\text{Tex}^{\text{prog}} \rightarrow \text{Tex}^{\text{int}} \rightarrow \text{Tex}^{\text{term}}$), and (3) Tex^{KLR} trajectory ($\text{Tex}^{\text{prog}} \rightarrow \text{Tex}^{\text{int}} \rightarrow \text{Tex}^{\text{KLR}}$; Fig. 4d). The Tex^{prog} trajectory showed a loss of HOMEBOX TF motifs and enrichment of BATF, AP-1, BACH, NFkB, TCF and CTCF motifs. In contrast, in both Tex^{term} and Tex^{KLR} trajectories, we observed a loss of Tex^{prog} specific TF motifs in Tex^{int} , followed by enrichment of TF motifs that may guide the differentiation program of Tex^{KLR} (e.g., ZEB, KLF, ETS, TBX, RUNX) and Tex^{term} (e.g., RUNX, IRF, STAT, NR4A; Fig. 4d).

Finally, we assessed the *Tox* locus, and found a gradual increase in accessibility during the Tex^{eff} to Tex^{prog} transition. The Tex^{term} trajectory demonstrated a decrease in *Tox* accessibility during the Tex^{prog} to Tex^{int} transition and a subsequent increase in the Tex^{term} state (Fig. 4d). We annotated differentially accessible OCRs (compared to T^{naive}) in a $\pm 250\text{kb}$ window around the TSS and identified 88 OCRs. 16 and 8 OCRs were differentially accessible in Tex^{eff} or Tex^{prog} , respectively, which was also supported by high *Tox* expression in these subsets (relative to T^{naive}), indicating that TOX may initiate the molecular programming of Tex differentiation in these subsets (Extended Data Fig. 4e, Supplementary Table 9).

Early molecular programs of Tex differentiation

Next, we focused on this early stage of exhaustion by comparing D8 scATAC-seq phenotypes in Arm and C113 (Extended Data Fig. 5a). Memory precursor cells (T^{mp}) are present at D8 in Arm infection and cluster with an early Tex^{prog} population present at D8 in C113 infection that expresses *Tox* and *Tcf7* (Tex^{prec}), but these subsets were relatively infrequent compared to the effector populations in both infection models and at D5 of C113 infection (Extended Data Fig. 5b)^{14,37}. We compared the gene expression and chromatin state of Tex^{prec} and T^{mp} subsets at D8, which revealed strong exhaustion- and interferon-induced programs in Tex^{prec} (Extended Data Fig. 5c, Supplementary Table 10). Similarly, DEGs of effector populations revealed a strong Tex signature in the Tex^{eff} subset compared to T^{eff} ; T^{eff} showed a *bona fide* effector program (e.g., *Gzma*, *Klrd1*, *Ccr2*), while Tex^{eff} expressed higher levels of exhaustion marker genes (e.g., *Tnfrsf9*, *Lag3*, *Pdcd1*, *Havcr2*; Extended Data Fig. 5d, Supplementary Table 11). These observations were also supported by the chromatin state programs of these subsets (T^{eff} - 7,066 OCRs vs. Tex^{eff} - 5,211 OCRs) that were associated with T^{eff} -specific (ETS, RUNX) and Tex^{eff} -specific (NFAT, BATF) TF motifs (Extended Data Fig. 5d). These results support studies demonstrating the formation of Tex^{prec} early during chronic infection that exhibit molecular signatures of exhaustion, distinct from T^{mp} ^{14,37}. Moreover, we find that the exhaustion program, including *Tox* expression, is present in the Tex^{eff} stage, which might represent an early, heterogeneous effector pool with plasticity to seed Tex subsets. We tested this concept by adoptively transferring Tex^{eff} , which specifically expressed *Tnfrsf9* (encoding 4-1BB (Extended Data Fig. 5e). We transferred $\sim 400,000$ $\text{CD45.2}^+\text{TNFRSF9}^+\text{CX3CR1}^-\text{SLAMF6}^-\text{CD8}^+$ T cells from D5 of C113 infection to CD45.1^+ infection-matched hosts and analyzed the phenotypic distribution of the transferred cells. At D21, CD45.2^+ cells comprised $\sim 3.9\%$ Tex^{prog} , $\sim 2.5\%$ Tex^{KLR} , $\sim 17\%$ Tex^{int} , and

~76% Tex^{term} , supporting the concept that Tex^{eff} may represent an early stage of Tex commitment (Extended Data Fig. 5f).

Differentiation of Tex^{int} to Tex^{KLR} and Tex^{term}

Tex differentiation downstream of Tex^{prog} has been suggested to follow bifurcating differentiation paths, and the RNA velocity analysis suggested that Tex^{int} may represent the bifurcation point between Tex^{KLR} and Tex^{term} ^{3,9}. We nominated TFs specific for each Tex phenotype based on differential RNA expression (Extended Data Fig. 5g) and Tex^{term} - and Tex^{KLR} -specific TF motif enrichments (e.g., Tex^{term} – NFAT, STAT, NR4A, IRF; and Tex^{KLR} – TBX, KLF; Extended Data Fig. 5h). Notably, differential analysis of Tex^{term} and Tex^{KLR} relative to Tex^{int} identified only 4 shared OCRs, suggesting that these two cell states are epigenetically divergent. To test whether Tex^{int} could represent a bifurcation point between Tex^{KLR} and Tex^{term} , we adoptively transferred ~200,000 CD45.2^+ Tex^{int} cells ($\text{CX3CR1}^+\text{SLAMF6}^-\text{KLRG1}^-$) from C113 infection at D21 into CD45.1^+ infection-matched hosts. After 10 days, transferred Tex^{int} cells gave rise to both Tex^{term} and Tex^{KLR} , although relatively more Tex^{term} (Fig. 4e). To further test the potential for endogenous Tex^{int} to give rise to Tex^{term} , we performed genetic fate-mapping using *Cx3cr1^{CreER}Rosa26^{LSL-tdT}* mice that can trace the fate of *Cx3cr1*-expressing cells. Short-term fate mapping of *Cx3cr1*-expressing cells (which includes both Tex^{KLR} and Tex^{int} cells) at D22 in C113 demonstrated that by D25, 6.5% of Tex^{term} cells were labeled with tdTomato, supporting the concept that Tex^{int} cells can give rise to Tex^{term} (Extended Data Fig. 5i). These results support the concept that the Tex^{int} stage is a bifurcation point of Tex differentiation; however, it is important to note two caveats: (1) the genetic fate-mapping results do not inform the overall contribution of Tex^{int} to Tex^{KLR} , and it is possible that Tex^{KLR} also arise from a Tex^{int} -independent path, which is also supported by our TCR clone analysis, and (2) we cannot rule out the possibility that Tex^{term} are generated directly from Tex^{prog} in some settings.

Tex clone behaviors are shared across tissues

We next asked if clonal differentiation patterns are intrinsically programmed. We first determined whether expanded Tex clones could be found across organs (animal-matched) in C113 at D21 (Fig. 5a). In spleen-, liver-, and lung-derived scRNA/TCR-seq datasets, we detected expanded T cell clones across all three tissues, and the gp33^+ and gp33^- fractions showed minimal TCR overlap (Fig. 5b and Extended Data Fig. 6a). Importantly, there was significant TCR sharing across the different organs within both gp33^+ and gp33^- fractions (Extended Data Fig. 6a). We identified expanded organ-shared T cell clones that had at least 5 T cells, which consisted of at least 1 cell from each organ. This analysis identified 100 shared T cell clones among all organs, 37 clones shared between the lung and spleen, and 22 spleen-specific clones (Fig. 5c,d).

Next, we examined the phenotypic distribution of organ-shared clones (Fig. 5e,f and Extended Data Fig. 6b,c). First, we focused on comparisons between the spleen and lung and found highly conserved clone behaviors. Divergent clones in the spleen also maintained Tex^{term} and Tex^{KLR} phenotypes in the lung (although instead exhibiting the Tex^{lung}

phenotype; 35/48 divergent clones detected in both organs, Fig. 5e,f, and Extended Data Fig. 6b,c). Similarly, the majority of splenic Tex^{KLR} -biased clones (4/7 clones) and Tex^{term} -biased clones (11/14 clones) maintained their behavior in the lung (Fig. 5e and Extended Data Fig. 6c). In particular, we did not observe appreciable interconversion between Tex^{KLR} - and Tex^{term} -biased clones between these two organs (0/19 shared clones). Accordingly, quantification of Tex^{KLR} and Tex^{term} frequencies within individual clones showed a high concordance across organs (Fig. 5g). These results demonstrate that Tex clones are shared across organs and that clonal differentiation behavior is primarily intrinsically programmed.

Depletion of Tex^{KLR} clones in the liver microenvironment

We next analyzed clonal behavior in the liver, which showed an overall enrichment of Tex^{term} , compared to other organs (94% Tex^{term}). Thus, we expected an enrichment in clonal Tex^{term} frequency; however, this could be driven by a depletion of Tex^{KLR} -biased clones and/or interconversion of Tex^{KLR} -biased or divergent clones to Tex^{term} -biased clones. We first analyzed the Tex^{KLR} -biased clones from the spleen and found that only one of these clones was present in the liver (1/7 clones), suggesting that Tex^{KLR} -biased clones are depleted in the liver niche (Extended Data Fig 6c). Similarly, although divergent clones were largely detectable in the liver (52/58 clones shared between spleen and liver), we again observed a depletion of Tex^{KLR} cells, resulting in Tex^{term} -biased behavior in the majority of the cases (32/52 shared clones) (Extended Data Fig 6c). In contrast, the majority of Tex^{term} -biased clones remained Tex^{term} -biased in the liver, although they were heavily skewed towards Tex^{term} , with relative loss of Tex^{prog} and Tex^{int} (9/9 clones, Fig. 5e,f and Extended Data Fig 6c). Quantification of frequencies of Tex^{KLR} and Tex^{term} phenotypes of shared clones in the spleen and liver confirmed the depletion of Tex^{KLR} in the liver and a skewing of Tex^{term} -biased clones to the Tex^{term} fate (Fig. 5g,h).

To investigate differences in viral antigen levels in the three organ niches, we measured LCMV glycoprotein viral transcripts (*LCMV-gp*), which showed that *LCMV-gp* transcripts were higher in the lung and liver, compared to the spleen (Extended Data Fig. 6d). However, *LCMV-gp* transcripts were the highest in the lung, suggesting that additional microenvironment differences may underlie the depletion of Tex^{KLR} from the liver. Since IL-21 has been shown to be a critical cytokine supporting the maintenance of CX3CR1^+ Tex cells, we also measured *Il21* levels in each organ^{9,13}. We found significantly lower *Il21* levels in the liver, compared to the spleen and lung, suggesting that a lack of IL-21 may contribute to Tex^{KLR} depletion (Extended Data Fig. 6d).

TCR signaling avidity correlates with clone behavior

The difference in clonal expansion between Tex^{term} -biased clones and Tex^{KLR} -biased clones led us to examine whether Tex differentiation trajectories correlate with differences in TCR signaling avidity. We first used tetramer binding level as a proxy for TCR avidity and sorted gp33^- (n=8,914), gp33 -intermediate (gp33^{int} ; n=5,875), and gp33 -high ($\text{gp33}^{\text{high}}$; n=8,194) splenic CD8^+ T cells from C113-infected mice at D21 and performed scRNA/TCR-seq (Fig. 6a; Extended Data Fig. 7a). Analysis of TCR sequences identified 313 TCR clonotypes in $\text{gp33}^{\text{high}}$ cells, 1,576 in gp33^{int} cells, and 3,803 in gp33^- cells (Extended Data Fig. 7b). The

TCR repertoire showed a small overlap between gp33^{high} and gp33⁻ cells (13 shared TCRs), compared to the overlap between gp33^{high} and gp33^{int} cells (158 shared TCRs), or gp33^{int} and gp33⁻ cells (306 shared TCRs), and TCR repertoire similarity analysis demonstrated that gp33^{int} sorting captured a unique repertoire (Extended Data Fig. 7b,c).

Next, we evaluated the clone size distribution of the sorted populations, which revealed an increase in the percentage of large clones (clones with 5–200 or >200 cells) as a function of higher tetramer binding (fluorescence), with an accompanying decrease in clonal diversity (Extended Data Fig. 7d). To link unique TCR clones to each gp33-tetramer fraction, we compared the overlap of clones between gp33 fractions and identified 592 unique gp33⁻ clones, 114 unique gp33^{int} clones, and 88 unique gp33^{high} clones (Fig. 6b,c). Importantly, we found phenotypic skewing in the unique clones (Fig. 6c; Extended Data Fig. 7e,f). Namely, gp33^{high} cells contained ~3.3 times more cells with Tex^{term} and Tex^{int} phenotypes, compared to either gp33⁻ or gp33^{int} cells (39% Tex^{term}, 19% Tex^{int} in gp33^{high}; 11% Tex^{term}, 6.7% Tex^{int} in gp33^{int}; 11% Tex^{term}, 7.0% Tex^{int} in gp33⁻). In contrast, gp33^{int} cells exhibited phenotypic skewing towards the Tex^{KLR} phenotype, compared to gp33^{high} and gp33⁻ cells (27% Tex^{KLR} in gp33^{int}; 7.9% Tex^{KLR} in gp33^{high}; 13% Tex^{KLR} in gp33⁻; Fig. 6d; Extended Data Fig. 7e). We validated differences in Tex phenotype distribution and function across gp33 fractions using flow cytometry (Extended Data Fig. 7g,h).

To analyze differentiation trajectories, we visualized the top 10 unique expanded clones in each gp33-tetramer fraction and assessed their phenotypic composition. We found that the top clones in the gp33⁻ and gp33^{high} fractions were biased towards Tex^{term} or divergent phenotypes (10/10 gp33⁻ clones and 10/10 gp33^{high} clones; expanded gp33⁻ clones likely represent other antigen specificities), while the largest clones in the gp33^{int} pool exhibited skewing towards the Tex^{KLR} phenotype (5/10 gp33^{int} clones; Fig. 6d). We analyzed the top 7 clone behaviors of the three gp33 fractions, which demonstrated that clones from the gp33⁻ fraction primarily exhibited Tex^{term}-biased and Tex^{KLR}-biased behaviors, clones from the gp33^{int} fraction were heavily enriched for Tex^{KLR}-biased differentiation, and clones from the gp33^{high} fraction were biased towards Tex^{term}-biased and divergent clone behaviors, and lacked Tex^{KLR}-biased clones (Fig. 6e).

Finally, we tested TCR signaling avidity of unique TCR clonotypes from gp33^{high} and gp33^{int} fractions (top 3 TCRs derived from divergent and Tex^{KLR}-biased clones). We computationally assembled full-length TCR α and TCR β sequences from scRNA/TCR-seq data (Extended Data Fig. 7i; Supplementary Table 12), cloned, and virally transduced into an NFAT-GFP reporter cell line. We also cloned the P14 TCR (positive control; high-affinity gp33-specific TCR) and an OT-1 TCR recognizing Ovalbumin (negative control). We co-cultured each T cell hybridoma TCR line with increasing concentrations of gp33 peptide-pulsed splenocytes and measured GFP expression as an indicator of downstream of TCR signaling. These studies supported two concepts: (1) TCRs from the gp33^{int} and gp33^{high} fractions induced GFP expression after co-culture with gp33 peptide, confirming gp33-reactivity, and (2) divergent TCRs from the gp33^{high} fraction exhibited higher signaling avidities (mean EC₅₀=8.4nM; positive control P14 – 50nM), compared to Tex^{KLR} clones from the gp33^{int} fraction (mean EC₅₀=3,200nM; Fig. 6f). These results indicate that

divergent/gp33^{high} TCRs exhibit ~400x higher signaling avidity than Tex^{KLR}/gp33^{int} TCRs, supporting a role for TCR signal strength in driving divergent Tex trajectories.

Human TILs show conserved Tex clone behavior

We analyzed tumor infiltrating lymphocytes (TIL) to investigate whether Tex clone behaviors are conserved in human cancer. We reanalyzed a dataset of 109,089 CD8⁺ T cells profiled by scRNA/TCR-seq from 47 patients spanning 10 cancer types, which were previously categorized as 7 subsets: 1) T^{naive}, 2) effector memory (T^{em}), 3) tissue-resident memory (T^{rm}); 4) *TCF7*⁻ exhausted (Tex^{TCF7+}); 5) *NME1*⁺ memory-like (Tm^{NME1+}), 6) terminally exhausted (Tex^{term}), and 7) *CX3CR1*⁺ terminally differentiated effector memory (Temra^{CX3CR1+}; Fig. 7a)³⁸. We scored each TIL subset for enrichment of gene signatures from Tex subsets in C113 infection and found a concordance between murine and human T cell types. Namely, the murine Tex^{term} gene signature was highly enriched in human Tex^{term}, the Tex^{KLR} gene signature was highly enriched in human Temra^{CX3CR1+}, the Tex^{int} and Tex^{eff} gene signatures were enriched in Tm^{NME1+}, and the Tex^{prog} gene signature was enriched in Tex^{TCF7+} (Fig. 7b-d).

Finally, we analyzed the phenotypic distribution of expanded TIL clones (n=729) and found that most clones exhibited Tex^{term}-biased behavior (n=483), which comprised Tex^{TCF7+}, T^{em}, T^{rm}, and Tex^{term} (Extended Data Fig. 8a,b). In addition, we detected Temra^{CX3CR1+}-biased clones (n=225), which primarily comprised cells with a Tex^{KLR}-like phenotype (Supplementary Fig. 8a,b). Finally, we also observed TIL clones with divergent behavior, comprising Tex^{term} and Temra^{CX3CR1+} (n=24 clones; Extended Data Fig. 8a,b). Analyzing the top 10 clones with each behavior demonstrated that apart from a few large Temra^{CX3CR1+}-biased clones, clone sizes were similar between Tex^{term} and Temra^{CX3CR1+} clones, and smaller in divergent clones (Fig. 7e). Altogether, these results demonstrate similarities in gene expression signatures of human CD8⁺ TIL and LCMV-reactive murine Tex subsets, and that human TIL clones can exhibit divergent, Tex^{KLR}-biased, and Tex^{term}-biased differentiation paths analogous to those observed in C113 infection.

Discussion

Here we report a single-cell multi-omic atlas of T cell exhaustion during chronic viral infection, which reveals new Tex subsets and their molecular programs, identifies multiple differentiation trajectories of Tex clones, and nominates TCR signal strength as a driver of clonal behavior. Prior studies have described multiple Tex subsets with distinct phenotypic and functional traits, including CX3CR1⁺ transitory exhausted cells^{8,9,13}. Here we show that this CX3CR1⁺ population encompasses two Tex subsets with distinct functionalities: 1) Tex^{int}, which represent a bifurcation point in Tex differentiation to either Tex^{term} or Tex^{KLR}, and 2) Tex^{KLR} with superior functionality and proliferative capacity, compared to Tex^{int} and Tex^{term}. This heterogeneity may help to explain prior discrepancies in understanding the lineage potential and differentiation path of CX3CR1⁺ Tex cells^{8,9,15}. Notably, the development of Tex^{KLR} occurs via two distinct clonal differentiation paths (Tex^{KLR}-biased and divergent clones), and future studies are needed to understand whether clonally unrelated Tex^{KLR} cells possess distinct functional characteristics.

Given the stable epigenetic state of Tex^{22,39}, it is important to understand the stage at which the Tex epigenetic program is initiated. Previous studies demonstrated that early TCF1⁺ Tex^{prec} cells possess the epigenetic signature of Tex and can seed additional Tex subsets^{14,37}. Here, we find that the Tex program is initiated at an earlier stage in Tex^{eff}. scATAC-seq analysis and adoptive transfer experiments suggest that this fate decision may be driven by NFAT and BATF, followed by subsequent activation of BACH2 and TCF-1 to give rise to Tex^{prec}/Tex^{prog}^{14,18}. These findings, coupled with early clonal expansion of Tex^{eff}, support a model in which the Tex^{prec} and Tex^{prog} pool originates from Tex^{eff}. However, it is important to note that our results do not exclude the possibility that Tex^{prog} can also differentiate to Tex^{eff}. Future lineage tracing experiments will further clarify the relationship between these early Tex cell types.

Downstream of Tex^{prog}, the differentiation trajectory of Tex has largely been thought to follow a linear path, although some studies have suggested a bifurcating path^{3,9}. Our data identifies two late-stage Tex types (Tex^{KLR} and Tex^{term}), and that individual clones can follow three differentiation trajectories resulting in Tex^{term}-biased, Tex^{KLR}-biased, or divergent fates, comprising both cell types. Furthermore, we find that the differentiation trajectory of Tex clones correlates with TCR signaling avidity and thus seems to be programmed by the TCR and conserved across different tissues. However, additional paths to induce TCR signal strength variation – for example, via inhibitory receptor signaling, access to antigen, cytokines, or other factors – may also promote the development of the Tex^{KLR} phenotype. Importantly, Tex^{KLR}-biased clones were depleted in the liver microenvironment, suggesting that these clones are unable to persist in the liver, while Tex^{term} were able to persist. Given the high viral load and inflammatory microenvironment of the liver during infection, these results suggest that the Tex^{term} phenotype may improve Tex persistence and preserve anti-viral effector function in specific organ systems⁴⁰.

Finally, these findings may have several implications for cancer. First, several ongoing therapeutic strategies aim to reverse exhaustion; however, our results suggest that Tex^{term} may be specifically adapted to survive in high antigen niches^{4,16,17,19–21,41}. Whether the pro-survival aspects of T cell exhaustion can be maintained while reinvigorating effector function will require further study. Second, our findings reinforce the notion that TCR signal strength can direct the fate of T cells^{42,43}. Thus, the generation of TCR-based cellular therapies should assess the phenotypic outcomes of TCR activation, in addition to antigen recognition. Finally, the observation that a polyclonal T cell response to chronic antigen balances persistence, effector, and potential memory functions via the development of two Tex states suggests that future cellular therapies may wish to establish divergent phenotypes⁴⁴. Indeed, a recent study identified a natural killer (NK) cell-like signature in exhausted human chimeric antigen receptor (CAR)-T cells, which resembles the Tex^{KLR} signature, suggesting that this cell type may be present in adoptive cell therapy settings as well⁴⁵. Manipulation of these features and gene regulatory programs of the Tex state may provide important avenues for improved T cell-based immunotherapies.

Methods:

Mice and infection

Male C57BL/6J (CD45.2) and B6.SJL-*Ptprca*^a *Pepcb*^b/BoyJ (CD45.1) mice were purchased from Charles River Laboratories or The Jackson Laboratories. All mice were housed in a specific pathogen-free facility and were used for infection at 8–12 week of age. Mice were housed in a 12 light/12dark cycle, and temperature was kept in between 65–75F with 50% humidity. LCMV infection was performed essentially as described previously⁴⁶. All experiments were performed according to protocols approved by Stanford University's (protocol number: 33814) and Washington University's (protocol number: 21–0244) Institutional Animal Care and Use Committee.

Tissue preparation

Single cell suspension of the different organs was prepared by manual dissociation. Organs were minced and gently pushed through a 40-micron strainer. Spleen single cell suspensions were spun, and red blood cells were lysed with ACK-lysis buffer by resuspending the cell pellet followed by 2 minutes incubation. Cells were then washed with ice-cold PBS and stained for sorting in FACS buffer (PBS, 0.1% BSA, 2mM EDTA, 5% FBS). For the lung and liver single-cell suspension, organs were cut into small pieces and gently pushed through a 40-micron diameter strainer. Single-cell suspensions were then layered on top of Ficoll-Paque Plus (Cytiva) and centrifuged according to the manufacturer's recommendations. The lymphocyte fraction was collected and washed with ice-cold PBS, and then stained for sorting.

RNA isolation and viral transcript determination

Approximately 10mg of spleen, liver and lung pieces were cut and tissues were flash frozen on dry-ice followed by the addition of 750ul Trizol and smashed with a plastic pestle, which was further homogenized with a 25-gauge needle. From this tissue homogenate, 375ul was use and further diluted with 125 ul Trizol. This mixture was mixed with 100ul chloroform, and were spun at maximum speed at 4 °C. RNA was precipitated from the aqueous phase with isopropanol, followed by centrifugation and 70% ethanol wash of the RNA pellet. 500ng RNA was used for reverse transcription (Qscript), and quantitative real-time PCR (SYBR green) was performed with the following primers: *LCMV-gp* forward primer: CATTACCTGGACTTTGTGACAGACTC; *LCMV-gp* reverse primer: GCAACTGCTGTGTTCCCGAAAC; *II21* forward primer: TCATCATTGACCTCGTGGCCC; *II21* reverse primer: ATCGTACTTCTCCACTTGCAATCCC. Gene expression was normalized to the expression of the *Hprt* housekeeping gene: *Hprt* forward primer: AGGTTGCAAGCTTGCTGGT; *Hprt* reverse primer: TGAAGTACTCATTATAGTCAAGGGCA.

Staining T cells for sorting and analysis

Single cell suspensions were stained with the following antibodies: CD8b (BioLegend Cat# 126610; conjugated with PerCP-Cy5.5, dilution – 1:800), CD4 (BioLegend Cat#100414; conjugated with APC-Cy7, dilution – 1:400), PD-1 (BioLegend Cat# 135216; conjugated

with PE-Cy7, dilution – 1:200), CX3CR1 (BioLegend Cat# 149008; conjugated with APC, dilution – 1:400), SLAMF6 (BD Biosciences Cat# 745250; conjugated with BV605, dilution – 1:100), CXCR6 (BioLegend Cat# 151119; conjugated with PE-Cy7 or APC-Cy7, dilution – 1:200), TNFRSF9 (BioLegend Cat# 106105; conjugated with PE, dilution – 1:200), KLRG1 (BioLegend Cat# 138413; conjugated with BV421, dilution – 1:200) and the class I tetramer, H-2Db LCMV gp33–41 (KAVYNFATC) (PE conjugated, dilution – 1:100). Cells were stained with the tetramer for 20 minutes at 4C followed by staining with the combination of the other antibodies for 20 minutes. Cells were washed in FACS buffer and stained with LIVE/DEAD Fixable Aqua dead cell stain for 20 minutes in PBS. Intracellular staining was performed with the Foxp3 intracellular staining kit (eBioscience) according to manufacturer's protocol with the following antibodies: MKI67 (BioLegend Cat# 652410; conjugated with FITC or PerCP-Cy5.5, dilution – 1:200), IFNG (BioLegend Cat# 505806; conjugated with FITC, dilution – 1:200), and LAMP-1 (BioLegend Cat# 121624; conjugated with PE-Dazzle 594, dilution – 1:200). Flow cytometry analysis and cell sorting were performed by BD FACSAria III (BD Biosciences) using BD FACSDiva Software 6.0 (BD Biosciences). Flow cytometry data were analyzed with FlowJo v10 (FlowJo; BD Biosciences).

Adoptive transfers

Adoptive transfer experiments were performed by transferring early effector exhausted T cells (Tex^{eff} , CD8^+ TNFRSF9^+ , SLAMF6^- , CX3CR1^-) T cells into infection-matched CD45.1 host. Approximately 4×10^5 cells were transferred from D5 following C113 infection into infection-matched hosts (n=3). Transferred cells (CD45.2^+) were analyzed at D21 following C113 infection. Due to this stringent sorting strategy that excludes Tex^{int} , Tex^{KLR} and Tex^{prog} from the transferred cell pool, the Tex^{eff} population comprised 8.7% of total CD8^+ CX3CR1^- SLAMF6^- fraction at D5, which represents a smaller fraction of total Tex^{eff} than would be expected from the scRNA-seq results (52.36% of the gp33+ fraction was Tex^{eff}). This may also be in part due to inaccuracies in scRNA-seq cluster-based cell classification. Intermediate exhausted T cells (Tex^{int} , CD8^+ , CX3CR1^+ , KLRG1^- , SLAMF6^-) were isolated from D21 following C113 infection and transferred into infection-matched CD45.1 host (n=5). Approximately 2×10^5 cells were transferred from D21 and transferred, CD45.2^+ Tex subsets were analyzed at D31. Tex subsets were identified by the following markers: Tex^{int} – CX3CR1^+ , SLAMF6^- , KLRG1^- ; Tex^{prog} – SLAMF6^+ , CX3CR1^- ; Tex^{KLR} – CX3CR1^+ , KLRG1^+ , SLAMF6^- , Tex^{term} – CX3CR1^- , SLAMF6^- . We would like to emphasize that while both Tex^{eff} and Tex^{term} were defined as CX3CR1^- SLAMF6^- , the double negative fraction at D21 post C113 infection contained ~1% Tex^{eff} cells based on scRNA-seq (Extended Data Fig. 1b – scRNA-seq UMAP) 1; therefore, additional gating for Tex^{term} cells was not performed in the Tex^{int} transfer experiment. Flow cytometry analysis and cell sorting were performed by BD FACSAria III (BD Biosciences) using BD FACSDiva Software 6.0 (BD Biosciences). Flow cytometry data were analyzed with FlowJo v10 (FlowJo; BD Biosciences).

scATAC-seq sample and library generation

Single cell ATAC-seq experiments were performed on the 10x Chromium platform as described earlier⁴⁷. Briefly, after sorting, T cells were washed with PBS + 0.04% BSA

and then subjected to nuclei isolation according to the protocol of the manufacturer. Nuclei were counted and on average ~10,000 nuclei were submitted for tagmentation. After tagmentation, nuclei were loaded for capture using the 10x Chromium controller. After Gel emulsion generation, linear amplification was performed, followed by DNA purification according to the manufacturer's protocol. The resulting DNA was used for library construction as described on the website of the manufacturer. Libraries were quantified by Agilent Bioanalyzer and were sequenced on an Illumina NovaSeq S4 sequencer, using the following setup: 50bp read 1N, 8bp i7 index, 16bp i5 index and 50bp read 2N. In this reaction, 1N and 2N refers to the DNA insert sequencing, while i5 and i7 sequencing identifies the individual barcodes of single cells.

Single-cell RNA-seq library preparation

Single-cell RNA-seq libraries were prepared using the 10X 5' Single Cell Immune Profiling Solution Kit (v1.1 Chemistry), according to the manufacturer's instructions. Briefly, FACS sorted cells were washed once with PBS + 0.04% BSA and on average 10,000 cells were submitted for capture using the 10x Chromium controller. Following reverse transcription and cell barcoding in droplets, emulsions were broken, and cDNA was purified using Dynabeads MyOne SILANE followed by PCR amplification (98°C for 45 sec; 14 cycles of 98°C for 20 sec, 67°C for 30 sec, 72°C for 1 min; 72°C for 1 min). For gene expression library construction, 50 ng of amplified cDNA was fragmented, end-repaired, and double-sided size selected with SPRIselect beads. Purified DNA was subjected to PCR amplification with sample indexing primers (98°C for 45 sec; 14 cycles of 98°C for 20 sec, 54°C for 30 sec, 72°C for 20 sec; 72°C for 1 min). Amplified DNA was double-sided size selected with SPRIselect beads and were quantified using Agilent Bioanalyzer. Single-cell RNA-seq libraries were sequenced on an Illumina NovaSeq S4 sequencer using the following read configuration 26bp Read1, 8bp i7 Index, 91bp Read2.

Single-cell TCR library generation

Single-cell TCR libraries were prepared with the 10x Chromium Single Cell V(D)J Enrichment Kit for mouse T cells (v1.1 Chemistry) following the manufacturer's protocol. Briefly, after cDNA amplification and clean up, 2ul of cDNA was used for target enrichment. First, target enrichment 1 was performed by specific primers followed by a SPRIselect bead clean-up. Second, target enrichment 2 was performed with specific primers followed by double-sided size selection with SPRIselect beads. After the two target enrichment steps, the quality of the product was assessed with Agilent Bioanalyzer. Amplified product was then subjected for fragmentation, followed by end repair and A-tailing. End repaired product was then subjected to adaptor ligation followed by SPRIselect bead purification. Product was amplified and barcoded with adaptor specific primers and the quality of the resulting libraries were determined by Agilent Bioanalyzer. Single-cell TCR-seq libraries were sequenced on an Illumina NovaSeq S4 sequencer using the following read configuration 26bp Read1, 8bp i7 Index, 91bp Read2.

In vitro assessment of TCR signaling avidity

Sequences of CDR3 variable regions and VDJ gene annotations of paired *Tcrα/Tcrβ* expressed in gp33^{high} and gp33^{int} cells were extracted from the single cell sequencing

data⁴⁸. Full length TCR sequences were assembled by www.imgt.org and confirmed using TRUST4. The P14-TCR sequence was obtained from GenBank (accession numbers: X06772 and X06771). Each Vb-TRBC2-(GSG linker)-T2A-Va sequence was synthesized with 5' linker (5'-AGGCGCCGGAATTAGATCTCTCGAGCCACC-3') and 3' linker (5'-GCAGAGGGTGCTGTCCTGAGACCGAGGATC-3') sequences (Twist Biosciences) and cloned into XhoI and BamHI sites of the MSCV-based retroviral plasmid MigCaRCh that contains a TRAC *IRE5* mCherry sequence downstream of the cloning sites (a gift from Chyi-Song Hsieh, Washington University) using an NEB HiFi assembly kit. Each paired *Tcr α* /*Tcr β* was retrovirally expressed in 58a^{-b-} NFAT-GFP reporter cells^{49,50} (Thy1.1⁺, a gift from Kenneth Murphy, Washington University) that had been engineered to express *Cd8a/b* and sorted based on expression of CD8a/b, mCherry and TCR β . 2.5×10^4 cells of each hybridoma were stimulated with 5×10^5 irradiated C57BL/6 splenocytes (3,000 rad) in the presence of varying concentrations of LCMV-gp33 peptide (Genscript) for 40-hours in RPMI 1640 media supplemented with 10% fetal bovine serum and 50 μ M of 2-mercaptoethanol, and frequencies of GFP⁺ cells in Thy1.1⁺ cells were measured by flow cytometry using an Attune flow cytometer.

Cx3cr1-creER-mediated fate mapping

Lineage tracing of *Cx3cr1*-expressing cells was performed as described previously¹³. Briefly, *Cx3cr1*-creER^{T2} mice were crossed to *Rosa26*-CAG-loxP-stop-loxP-tdTomato reporter mice and infected with LCMV-C113. Infected mice were treated with 1 mg of tamoxifen (Sigma) dissolved in corn oil (Sigma) by oral gavage on 22 dpi (days post infection), followed by analysis of tdTomato expression in LCMV-gp33-specific CD8 T cells on 25 dpi.

scATAC-seq data processing and analysis

scATAC-seq datasets were processed as described previously⁵¹. Briefly, reads were filtered, trimmed, and aligned to the mm10 reference genome using 10X Genomics' cellranger-atac count pipeline (version 1.2.0).

Processed fragment files were loaded into ArchR (version 1.0.1) for additional processing and analysis. All functions used default parameters unless otherwise specified. Cells were filtered during Arrow file generation using ArchR's createArrowFiles function to remove cells with an enrichment of Tn5 insertions in transcription start sites (TSS enrichment) of less than 4 or less than 1000 unique fragments. Doublets were identified using ArchR's addDoubletScores function and predicted doublets removed using ArchR's filterDoublets function. Dimensionality reduction was performed using Iterative Latent Semantic Indexing (LSI) using ArchR's addIterativeLSI function. After initial clustering and UMAP projection, we excluded a small cluster of non-T cells. Cell clustering was performed using ArchR's addClusters function on IterativeLSI reduced dimensions 1:10 and a resolution of 0.4 (reducedDims = "IterativeLSI", dimsToUse = 1:10, resolution = 0.4). The same dimensions were used for single cell embedding by Uniform Manifold Approximation and Projection (UMAP) using ArchR's addUMAP function using IterativeLSI reduced dimensions 1:10 and a minimum distance of 0.1 (reducedDims = "IterativeLSI", dimsToUse = 1:10, minDist = 0.1). Cell clustering and UMAP projection for Chronic LCMV (D8 and D14, Figure 3) and

Day 8 (Chronic and Acute, Figure S3B) subsets were performed as described above with the following modifications: `dimsToUse = NULL`, `resolution = 0.2`, and `minDist = 0.4`.

GeneScore matrices were computed by summing Tn5 insertions in the gene promoter and gene body during Arrow file generation using ArchR's `createArrowFiles` function⁵¹. Gene score imputation was performed with Magic using ArchR's `addImputeWeights` function⁵². After clustering the cells, peaks were called by MACS2 on pseudoreplicates sampled from each cluster to obtain a reproducible peak set retaining cell type specific peaks using ArchR's `addReproduciblePeakSet` function. Peak co-accessibility and Peak2Gene linkages were computed using ArchR's `addCoAccessibility` and `addPeak2GeneLinks` functions. Transcription factor (TF) motif deviations were computed with chromVar using ArchR's `addDeviationsMatrix` function²⁵. Pseudo-bulk tracks for indicated groups of cells were plotted using ArchR's `plotBrowserTrack` function with default normalization method based on reads in transcription start sites ("ReadsInTSS"). Differential peak testing was performed using ArchR's `getMarkerFeatures` function with `testMethod = "wilcoxon"` and `bias = c("TSSEnrichment", "log10(nFragments)")`. TF motif enrichment in differential peaks was performed using ArchR's `peakAnnoEnrichment` function. Trajectory analysis was performed using ArchR's `addTrajectory` and `plotTrajectory` functions. Identification of positive TF regulators was performed using ArchR's `correlateMatrices` function to examine the correlation between chromVar deviation z-scores of TF motifs ("MotifMatrix") and imputed gene expression ("GeneIntegrationMatrix") following cross-platform linkage with scRNA-seq data using ArchR's `addGeneIntegrationMatrix`.

scRNA-, TCR-seq computational methods

scRNA-seq reads were aligned to the mm10 reference genome and quantified using cellranger count (10x Genomics, version 3.1.0). Filtered gene-barcode matrices that contained only barcodes with unique molecular identifier (UMI) counts that passed the threshold for cell detection were used for further analysis. scTCR reads were aligned to the mm10 reference genome and consensus TCR annotation was performed using cellranger vdj (10x Genomics, version 3.1.0). TCR annotation was performed using the 10x cellranger vdj pipeline as described.

Additional analysis was performed in R (version 4.0.3) using Seurat (version 4.0.1) using default function parameters unless otherwise noted⁵³. Doublets were predicted using DoubletFinder (version 2.0.3)⁵⁴. Cell types were predicted using SingleR (version 1.4.1) based on mouse bulk RNA-seq reference data (MouseRNAseqData) from celldex (version 1.0.0)⁵⁵. Cells with less than 200 genes detected, greater than 5% mitochondrial RNA content, predicted doublets from DoubletFinder, and cells annotated as non-T and non-NK cells by SingleR were excluded from analysis. We predicted cell cycle phase based on previously defined gene sets using the CellCycleScoring function⁵⁶. We then split cells by experimental batch and cell cycle (non-cycling or G1 vs. cycling or G2M/S) into four datasets using Seurat's `SplitObject` and performed batch correction using Seurat's reciprocal PCA workflow. First, we normalized and identified variable features for each dataset independently using Seurat's `NormalizeData` and `FindVariableFeatures`. Then we selected variable features across datasets using Seurat's `SelectIntegrationFeatures`. We

excluded variable TCR (^Tr.v) genes, variable Ig (^Ig.v) genes, cell cycle genes (used for cell cycle scoring), and mitochondrial genes (^mt-) from integration features used for downstream analysis. We then scaled data and ran PCA on each dataset independently using these features using Seurat's ScaleData and RunPCA. We identified integration anchors using Seurat's FindIntegrationAnchors using non-cycling datasets as reference datasets and rpca for dimensionality reduction. We integrated all datasets using Seurat's IntegrateData using dims=1:50. Integrated data was used for data scaling with ScaleData and PCA dimensionality reduction with RunPCA. After initial clustering we noted three small clusters representing 7% of total cells which had low number of genes detected and high mitochondrial RNA content which were excluded from further analysis. Clusters were identified using shared nearest neighbor (SNN) based clustering based on the first 10 PCs with resolution = 0.45. The same principal components were used to generate the UMAP projections, which were generated with a minimum distance of 0.1. Cell clustering and UMAP projection for Chronic Day 21 T cells (all tissues, Figure 2 and Figure 5), spleen derived T cells (Chronic and Acute, Day 8 and Day 21, Figure 4 and Figure 6), Day 8 T cells (Spleen, Chronic and Acute, Supplemental Figure 5), Chronic including Day 5 (Spleen, Chronic, Days 5, 8, and 21, Supplemental Figure 5) were performed as described above with the following modifications:

Chronic Day 21 T cells: dims = 1:10, resolution = 0.25, min.dist = 0.1

Spleen derived T cells: dims = 1:8, k.param = 50, resolution = 0.45, min.dist = 0.1

Day 8 T cells: dims = 1:12, k.param = 40, resolution = 0.28, min.dist = 0.2

Chronic including Day 5: dims = 1:6, resolution = 0.22, min.dist = 0.01, spread = 1.5

Expression of selected genes was plotted using log normalized gene expression values based on original RNA count data prior to data integration. Marker genes were identified using Seurat's FindAllMarkers using a cutoff of $p_val_adj < 0.01$. Differential gene expression analysis was performed using Seurat's FindMarkers using a cutoff of $p_val_adj < 0.05$ and $abs(avg_log2FC) > 0.25$. Gene module scoring was performed using Seurat's AddModuleScore. TCR clone behaviors were visualized using UpSetR (version 1.4.0). To assess if tetramer staining leads to T cell activation, we used a gene module score from the results of the pathway analysis under the term, "T cell activation", containing the following genes: *Ccr5*, *Cd3e*, *Cd3g*, *Hla-a*, *Ii10ra*, *Irf1*, *Itgb2*, *Jak1*, *Maf*, *Nfatc1*, *Nfatc3*, *Runx3*, *Tgfb2*. Null distribution of TCR clone behaviors was determined by randomly shuffling TCR clonotype and scRNA phenotype and generating a distribution of TCR clone phenotype combinations (n=50 iterations). Morisita-Horn index for quantifying TCR overlap was calculated using the mh function from the R package divo (version 1.0.1).

scRNA velocity analysis

Spliced and unspliced transcript counts were computed using velocity's run10x (version) 0.17.17 on scRNA-seq cellranger outputs³⁵. The resulting loom files were used for subsequent RNA velocity analysis and visualization with dynamo (version 1.0.0). Preprocessing was performed using dynamo.pp.recipe_monocle. RNA velocity was computed using dynamo.tl.dynamics with model='stochastic'. Cell transition

probabilities were computed using `dynamo.tl.cell_velocities` with `method='pearson'` and `other_kernels_dict={'transform': 'sqrt'}`. RNA velocity was visualized using `dynamo.pl.streamline_plot`. Pseudotime was estimated using single cell potential (`umap_ddhodge_potential`) based on the vector field topology computed using `dynamo.vf.VectorField` with `basis='umap'` and `dynamo.ext.ddhodge` with `basis='umap'`. We note here that a caveat of these analyses is that highly distinct transcriptional cell states (e.g., Tex^{term} or Tex^{prog} ; similarly, in Arm infection T^{eff} , T^{em} , or T^{naive}), as well as local minima/maxima neighboring distinct cell states, can drive velocity signal, influencing 'start' or 'end' points of the analysis. Further, RNA velocity pseudotime is calculated from the potential of the RNA velocity field and summarizes the direction and magnitude of the dominant local trajectories in UMAP space, rather than nominating terminal differentiation states. Differentiation predictions from these studies should be validated using orthogonal lineage-tracing and adoptive transfer experiments.

Human scRNA-, TCR-seq analysis

Processed scRNA-, TCR-seq data from human tumor-infiltrating T cells were downloaded from zenodo³⁸. For further analysis we used scRNA-seq expression data from the R object `data/expression/CD8/integration/CD8.thisStudy_10X.seu.rds` and scTCR-seq data from the R object `data/tcr/byCell/tcr.zhangLab.comb.flt.rds`. Additional analysis was performed using Seurat (version 4.0.1). Phenotypic clusters were defined using the provided `meta.cluster.simplified` assignment. Enrichment of mouse LCMV gene signatures was calculated using Seurat's `AddModuleScore`. TCR clones were defined using the provided `cloneID` annotation. TCR clone behaviors for tumor-infiltrating T cells (`loc == 'T'`) were visualized using UpSetR (version 1.4.0). For UMAP visualization of single cells, provided normalized expression data was batch corrected using harmony's `RunHarmony` based on the 'patient' variable (version 1.0)⁵⁷. The first 10 harmonized embeddings were used to generate the UMAP projections, which were generated with a minimum distance of 0.1.

Statistics and Reproducibility

Statistical analyses were performed in R or GraphPad Prism. Gene expression measurements by qPCR were presented as means \pm SD. In the bar graphs, significant changes were determined by two tailed, unpaired t-test at $p < 0.05$. The exact replicate numbers are indicated in the figure legends for each experiment. Differential gene expression analysis was performed using a cutoff of adjusted p-value < 0.05 and absolute (average \log_2 fold change) > 0.25 . Statistical parameters are also reported in the figure legends. Data distribution was assumed to be normal but this was not formally tested. No statistical test was used to predetermine sample size. No data were excluded from the analysis. The experiments were not randomized. The Investigators were not blind to allocation during experiments and outcome assessment.

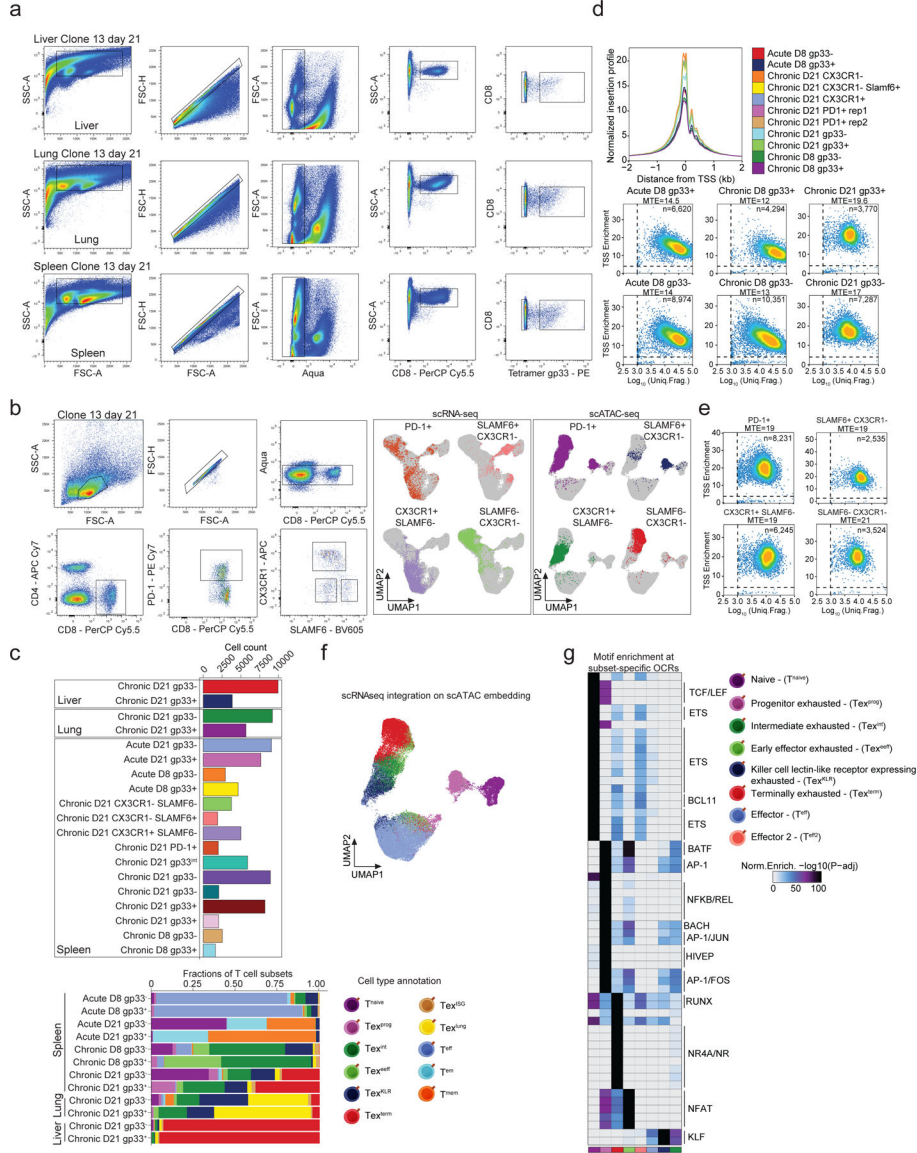
Data Availability:

All sequencing data generated in this study is available under GEO accession: GSE188670.

Code Availability:

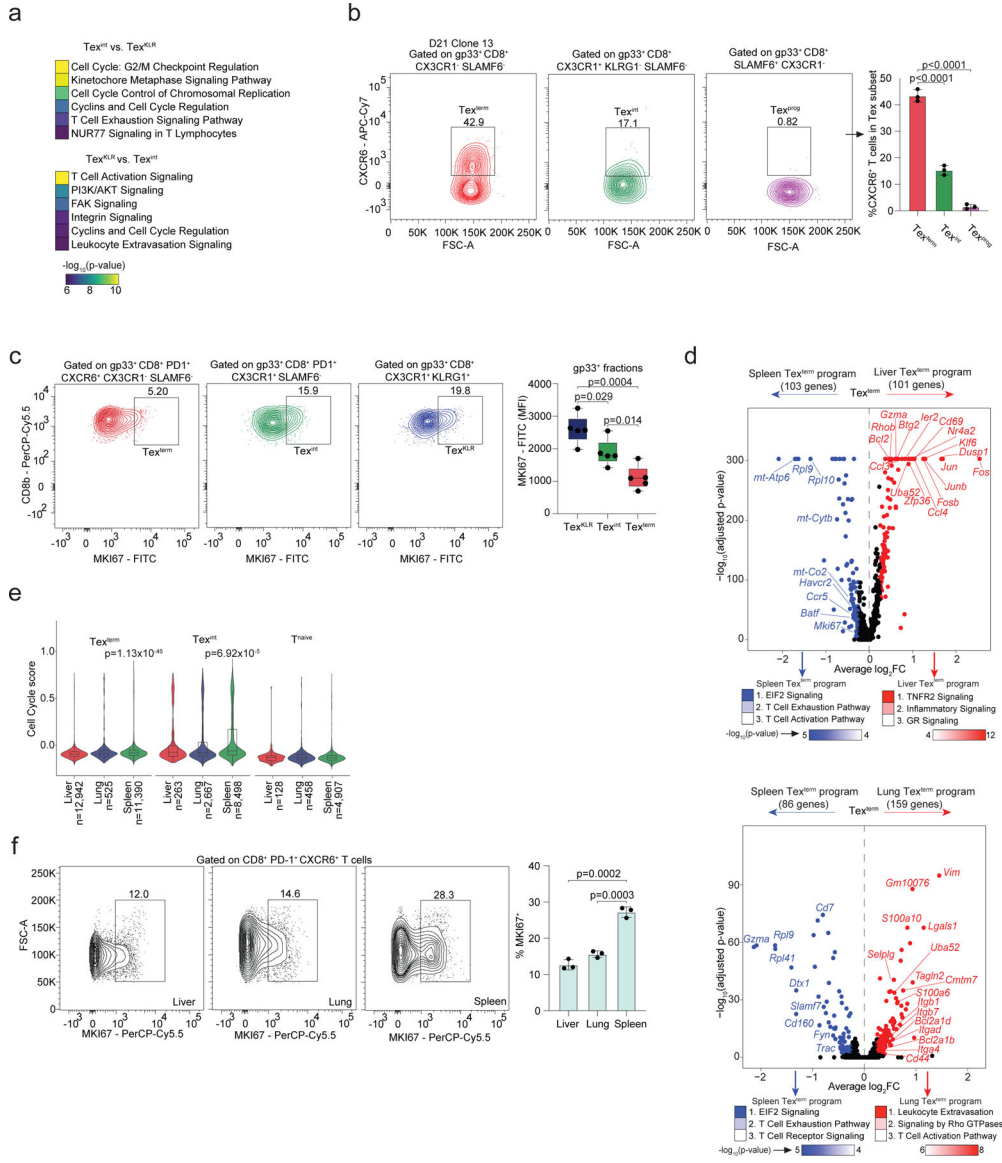
Custom code used in this study is available at <https://github.com/katieyost/LCMV-code-2022>.

Extended Data



Extended Data Figure 1. Sorting strategy and quality controls for scATAC-seq data. (a) Sorting strategy to obtain antigen specific gp33⁺ and gp33⁻ CD8⁺ T cells from different organs. (b) Sorting strategy to obtain the main Tex subsets (left). UMAPs of scRNA-seq and scATAC-seq results, originating from the indicated Tex subsets. (c) Bar plot of cell counts from the scRNA-seq samples (top). Stacked bar plot of the phenotypic composition of the indicated scRNA-seq samples (bottom). (d) Quality control of scATAC-seq data. Histogram shows normalized read enrichment on the transcription start sites (TSS) of genes from the

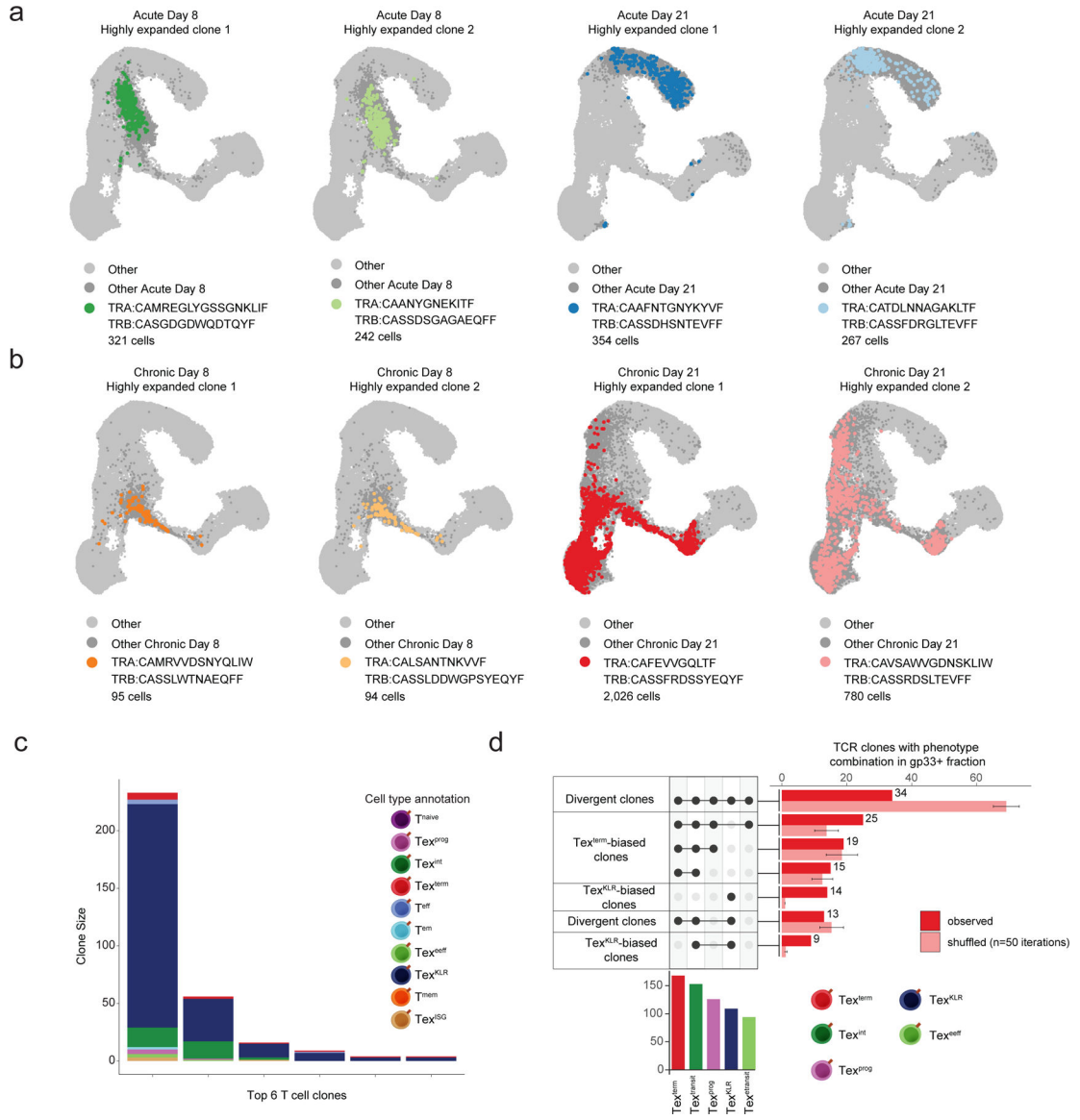
indicated samples (top). Density plots depict the cells that passed the TSS enrichment and Log_{10} unique fragment count threshold. Median TSS enrichment (MTE) is also indicated. **(e)** Density plots of scATAC-seq data from the main Tex populations depicting the same quality controls as panel (c). **(f)** UMAP of scATAC-seq data colored by integrated scRNA-seq cluster labels. **(g)** Heat map of TF motif enrichment at the specific open chromatin regions (OCRs) of the annotated T cell populations (p-values determined by hypergeometric enrichment and adjusted using the Bonferroni correction method).



Extended Data Figure 2. Characterization of Tex^{int} and Tex^{KLR} subsets and organ-specific exhaustion signatures.

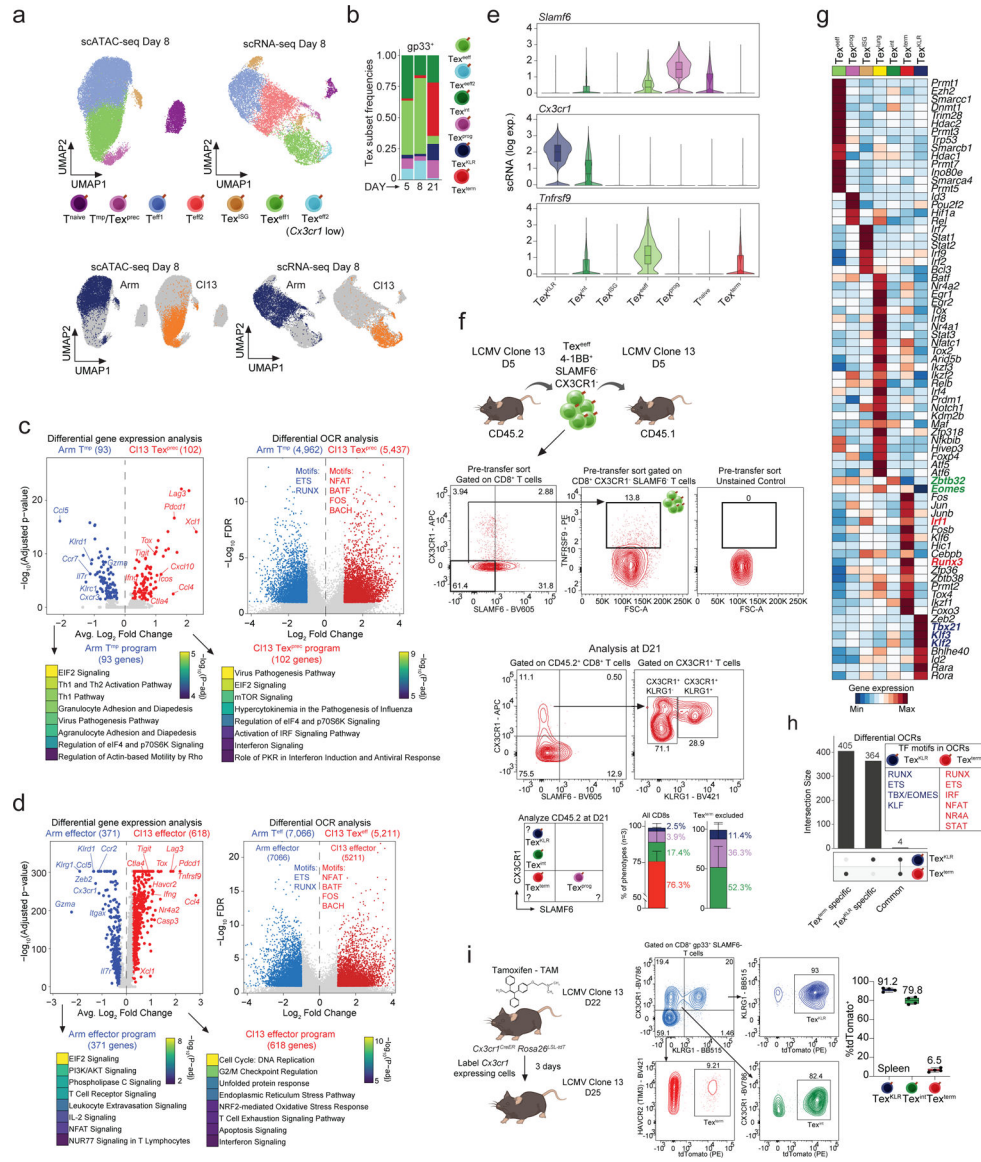
(a) Ingenuity pathway analyses of the differentially expressed genes identifying enriched biological pathways between the two subsets (Tex^{int} versus Tex^{KLR}). Top 6 hits are shown. **(b)** Representative flow cytometry plots that quantify CXCR6 expression in Tex^{term} , Tex^{int} and Tex^{prog} . Barplots summarize the quantification across three biological replicates.

Significant changes were determined by two tailed, unpaired t-test at $p < 0.05$ ($n=3$). Shown are means with SDs. **(c)** Representative flow cytometry plots show the MKI67⁺ fractions of the indicated Tex subsets. Boxplot depicts the quantification of MKI67⁺ Tex subsets. Significant changes were determined by two tailed, unpaired t-test at $p < 0.05$ ($n=5$ biologically independent animals). Box center line, mean; limits, upper and lower quartiles; whiskers, minimum and maximum values. **(d)** Volcano plots of differentially expressed genes comparing Tex^{term} populations from different organs (\log_2 FC > 0.25, Bonferroni adjusted p-value < 0.01, p-values determined by two-sided Wilcoxon Rank Sum test). Ingenuity pathway analysis results on the differentially expressed gene groups (bottom). Top 3 hits are shown. **(e)** Violin plots of the Cell Cycle score of the indicated T cell populations across organs (n = number of scRNA-seq profiles, box center line, median; box limits, upper and lower quartiles; box whiskers, 1.5 \times interquartile range). P-values determined by two-sided Wilcoxon Rank Sum test relative to overall distribution of single cells from the indicated Tex subsets across all organs. **(f)** Representative flow cytometry of the MKI67⁺ fraction of Tex^{term} subsets in the indicated organs. Bar plot summarizes MKI67⁺ fractions across organs. Significant changes were determined by two tailed, unpaired t-test at $p < 0.05$ ($n=3$ biologically independent animals). Shown are means with SDs.



Extended Data Figure 3. Analysis of highly-expanded T cell clones in Arm and CI13 infection. (a) UMAPs of highly expanded clones from the Arm infection model at the indicated time points. (b) UMAPs of highly expanded clones of the CI13 infection model at the indicated time points. (c) Stacked bar plot of the phenotypic composition of individual T cell clones with a bias towards the Tex^{KLR} fate that also contain some cells with the Tex^{term} phenotype. Top 6 clones are shown. (d) Upset plot of the phenotype combinations of the observed and shuffled TCR clones. Bar represents the mean and error bars represent standard deviation for 50 randomized TCR shuffling iterations performed to obtain the shuffled distribution.

Benjamini & Hochberg procedure to obtain FDRs). Heat map of motif enrichment results at the unique OCR sets of the annotated T cell subsets (right, p-values determined by hypergeometric enrichment and adjusted using the Bonferroni correction method). **(e)** Upset plot of differentially accessible OCRs relative to T^{naive} at the *Tox* gene locus and their overlap among the different *Tex* subsets ($\log_2 FC > 1$, FDR < 0.01, p-values determined by two-sided Wilcoxon Rank Sum test and adjusted using the Benjamini & Hochberg procedure to obtain FDRs). Violin plot shows the gene expression level of *Tox* in the identified *Tex* subsets. Box center line, median; box limits, upper and lower quartiles; box whiskers, 1.5× interquartile range.

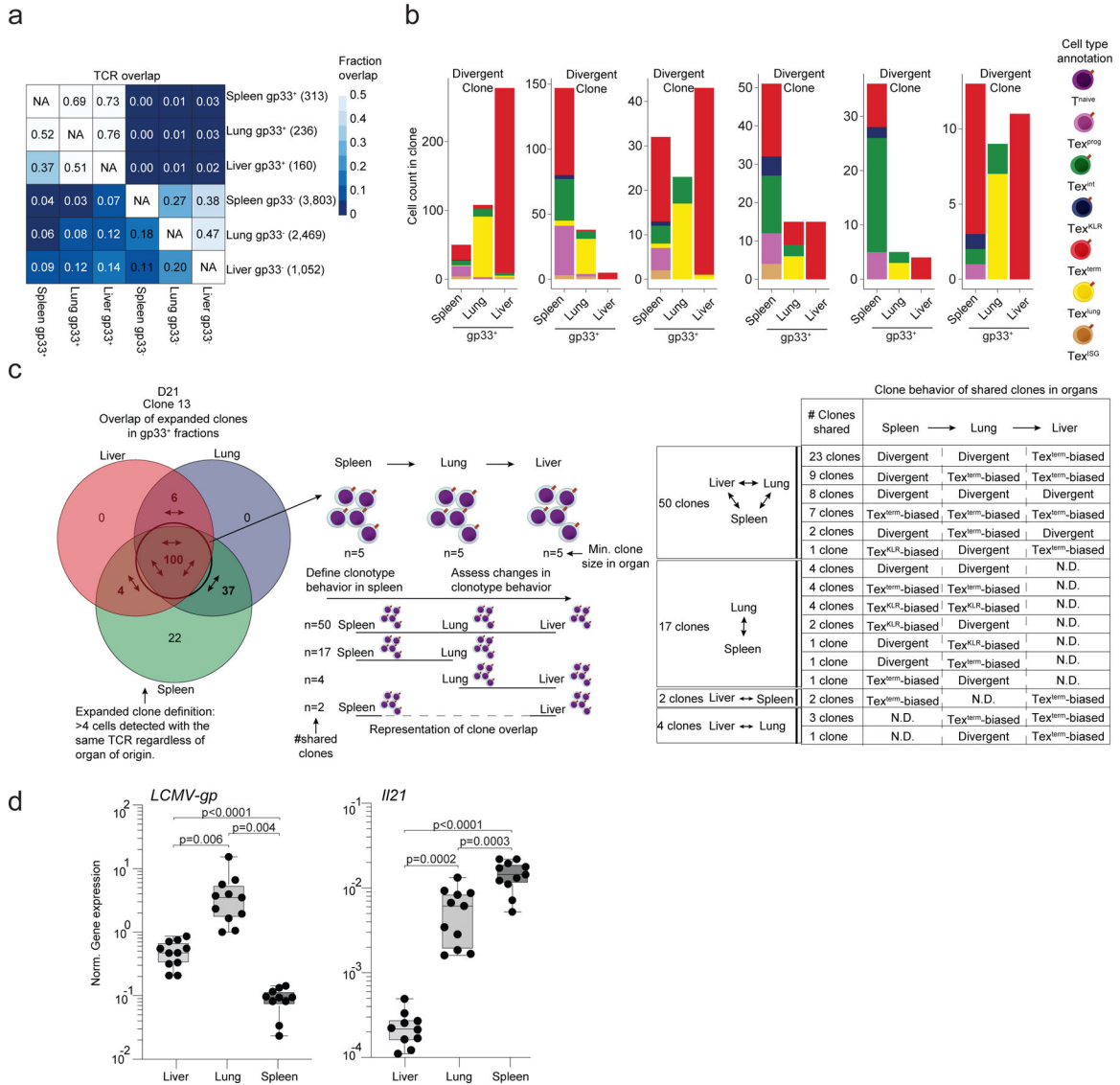


Extended Data Figure 5. Molecular programs of early effector- and progenitor-exhausted T cells and fate mapping experiments.

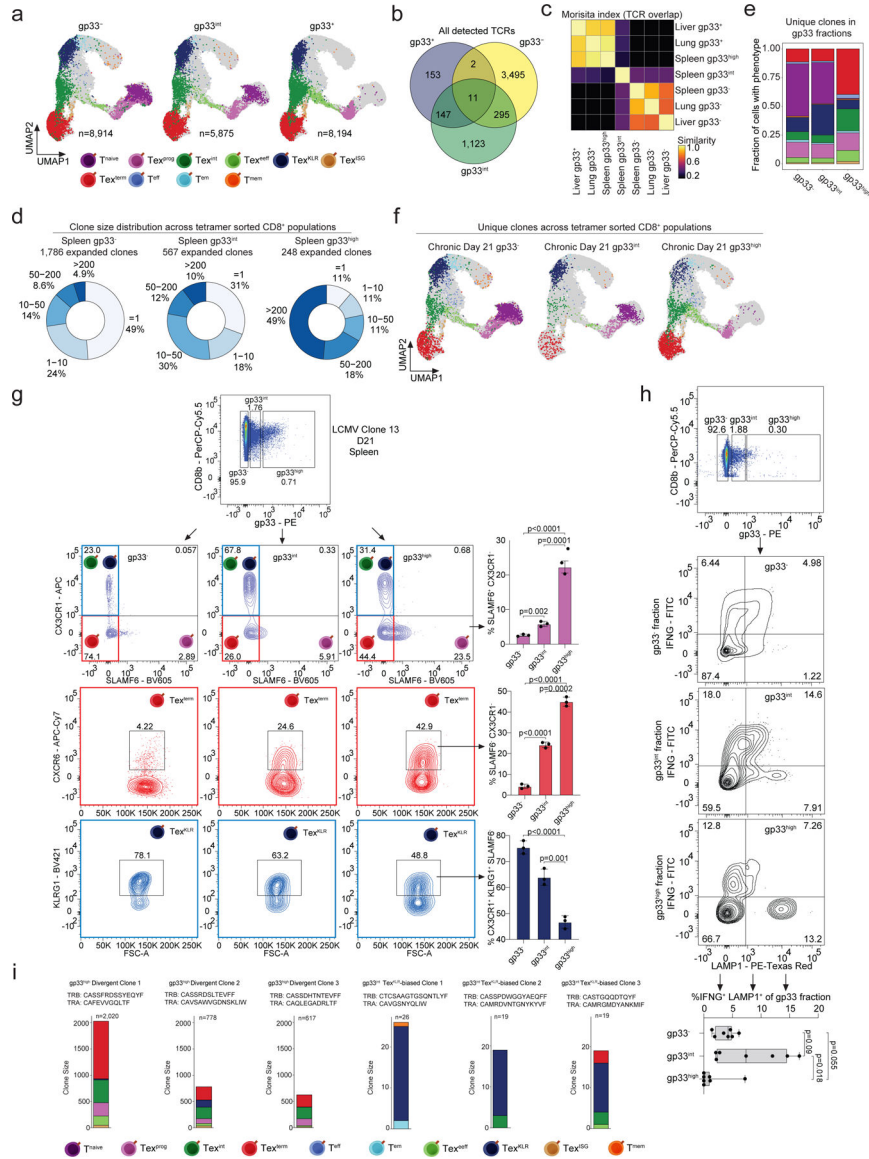
(a) UMAPs of scATAC-seq (left) and scRNA-seq (right) results from the infection models. Small UMAPs are colored by sample of origin (bottom). **(b)** Stacked bar plot depicts

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the phenotypic distribution of gp33⁺ CD8 T cells from scRNA-seq. **(c)** Volcano plot of differentially expressed genes (DEGs) between memory precursor T cells (T^{mp}) from Arm and the precursor exhausted T cells (Tex^{prec}) from C113 infections (top left). Differential gene expression analyses were performed as follows: \log_2 FC > 0.25, Bonferroni adjusted p-value < 0.01, p-values determined by two-sided Wilcoxon Rank Sum test. Ingenuity pathway analyses of the T^{mp} and Tex^{prog} specific gene sets. Volcano plot of the differential open chromatin regions (OCRs) of the T^{mp} and Tex^{prec} populations (top right). Differential OCR analyses were performed as follows: \log_2 FC > 1, FDR < 0.1, p-values determined by two-sided Wilcoxon Rank Sum test and adjusted using the Benjamini & Hochberg procedure to obtain FDRs. Enriched transcription factor (TF) motifs in specific OCRs of T^{mp} and Tex^{prog} subsets are shown (p-values determined by hypergeometric enrichment and adjusted using the Bonferroni correction method). **(d)** DEGs between the D8 effector T cells (T^{eff}) from Arm and early effector Tex cells (Tex^{eff}) from C113 infections (top left). Same statistical approach was used as in (c). Ingenuity pathway analyses of the T^{eff} and Tex^{eff} specific gene sets (bottom left). Volcano plot depicts the differentially accessible OCRs of T^{eff} and Tex^{eff} populations (right). Same statistical approach was used as in (c). Enriched TF motifs in T^{eff} and Tex^{eff} specific OCR sets (p-values determined by hypergeometric enrichment and adjusted using the Bonferroni correction method). **(e)** Expression of the indicated genes profiled by scRNA-seq. Box center line, mean; limits, upper and lower quartiles; whiskers, minimum and maximum values. **(f)** Schematic of the Tex^{eff} adoptive transfer experiment with pre-transfer sorting strategy (top). Representative flow cytometry plots show the analysis of the phenotypic content of recovered T cells. Stacked bar plots show the phenotypic distribution from 3 biologically independent animals (mean % of each subset is shown with SDs). **(g)** Heat map of differentially expressed TFs in Tex subsets. **(h)** Upset plot of differentially accessible OCRs of Tex^{KLR} and Tex^{term} relative to Tex^{int} (\log_2 FC > 1, FDR < 0.01, p-values determined by two-sided Wilcoxon Rank Sum test and adjusted using the Benjamini & Hochberg procedure to obtain FDRs) and their overlap. **(i)** Schematic of lineage tracing experiment (left). Gating strategy to analyze tdTomato⁺ fractions of Tex subsets. Boxplot of the % of tdTomato⁺ Tex subsets are shown. Box center line, mean; limits, upper and lower quartiles; whiskers, minimum and maximum values (n = 4 biologically independent animals).



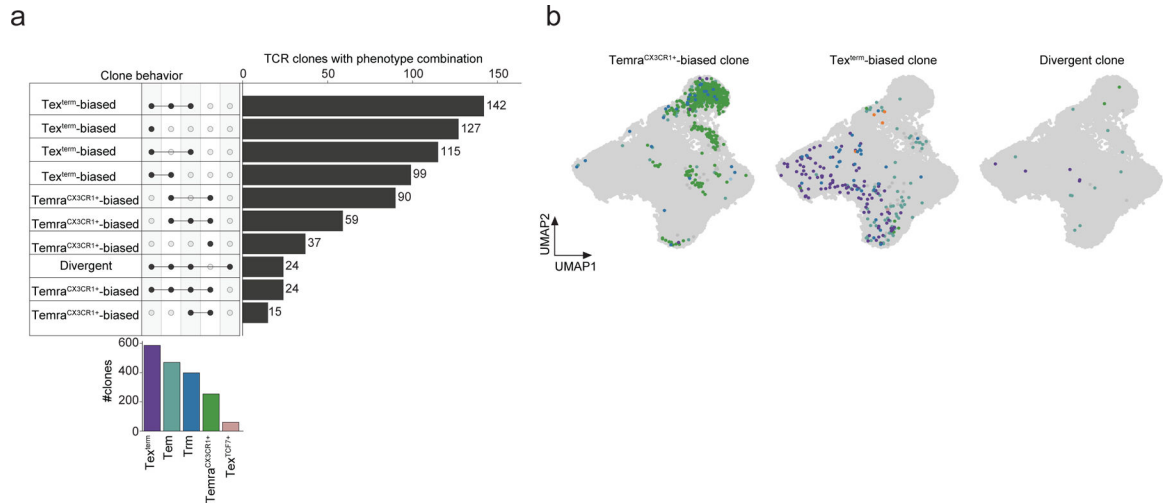
Extended Data Figure 6. scRNA/TCR-seq reveals T cell clone behaviors in different organs. **(a)** Heat map of the fraction overlap between the TCR repertoires of the indicated gp33⁺ and gp33⁻ CD8⁺ T cell subsets from different organs. **(b)** Stacked bar plot of the phenotypic composition of individual clones with divergent behavior across organs. **(c)** Schematic of the definition of an expanded, organ-shared T cell clone for clone behavior analysis. Only clones that had at least 5 T cells present in each organ were considered. Shared clone numbers across the organs are indicated (left). Table depicting the number of expanded clones that are shared across tissues and their clone behaviors (right). **(d)** Gene expression analysis of *LCMV-gp* transcript (left) and *Ii21* transcript (right) in indicated organs at D22 following CH13 infection. Significant changes were determined by two tailed, unpaired t-test (n=10 biologically independent animals). Box center line, mean; limits, upper and lower quartiles; whiskers, minimum and maximum values.



Extended Data Figure 7. scRNA-seq reveals the phenotypic composition of gp33⁻, gp33^{int}, and gp33^{high} T cell subsets.

(a) UMAPs of cell profiled by scRNA-seq in the three gp33 T cell fractions colored by the annotated T cell subsets. (b) Venn diagram shows the overlap of all detected TCR clones among the three gp33 T cell fractions. (c) Heat map depicting TCR repertoire overlap (Morisita index) among the different gp33 fractions from the indicated samples. (d) Pie chart representation of the fraction of the detected clone sizes in the three gp33 T cell fractions. (e) Stacked bar plot of the phenotypic distribution of the unique clones from the three gp33 T cell fractions. (f) UMAPs of unique TCR clones determined by scRNA/TCR-seq and colored by the phenotypic distribution of the three gp33 fractions of T cells. (g) Representative flow cytometry plots depict the gating strategy to analyze the fractions of Tex subsets in gp33⁻, gp33^{int}, and gp33^{high} CD8⁺ T cells. Bar plots quantify the frequencies of the indicated Tex subsets in each gp33 fraction. Significant changes were determined by two tailed, unpaired t-test at p<0.05 (n=3 biologically independent animals). Shown

are means with SDs. **(h)** Representative flow cytometry plots depict the gating strategy to analyze the functionality (IFNG/LAMP1 double positive CD8⁺ T cells) of gp33, gp33^{int}, and gp33^{high} CD8⁺ T cells. Boxplot quantifies the double positive fractions of T cells in each gp33 fraction. Significant changes were determined by two tailed, unpaired t-test at $p < 0.05$ ($n = 7$ biologically independent animals). Box center line, mean; limits, upper and lower quartiles; whiskers, minimum and maximum values. **(i)** Stacked bar plots show the phenotypic content of TCR clones that were tested for TCR signaling avidity. CDR3 amino acid sequences are indicated.



Extended Data Figure 8. Human expanded TILs exhibit divergent, Tex^{term}- and Tex^{KLR}-biased clone behaviors.

(a) Upset plot depicting the combination of phenotypes (clone behaviors) for expanded TIL clones. For clarity, the top 10 most common clone behaviors are shown. Bar plot shows the number of clones with the indicated phenotypes. **(b)** UMAPs of representative expanded TIL clones with the indicated clone behaviors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Competing Interest Statement:

A.T.S. is a founder of Immunai and Cartography Biosciences and receives research funding from Allogene Therapeutics and Merck Research Laboratories. H.Y.C. is a co-founder of Accent Therapeutics, Boundless Bio and Cartography Biosciences, and an advisor to 10x Genomics, Arsenal Biosciences, and Spring Discovery. K.E.Y. is a consultant for Cartography Biosciences. J.A.B. is a consultant for Immunai. E.J.W. is an advisor for Merck, Marengo, Janssen, Related Sciences, Synthekine, and Surface Oncology. E.J.W. is a founder of Surface Oncology, Danger Bio, and Arsenal Biosciences. E.J.W. has a patent on the PD1 pathway. The remaining authors declare no competing interests.

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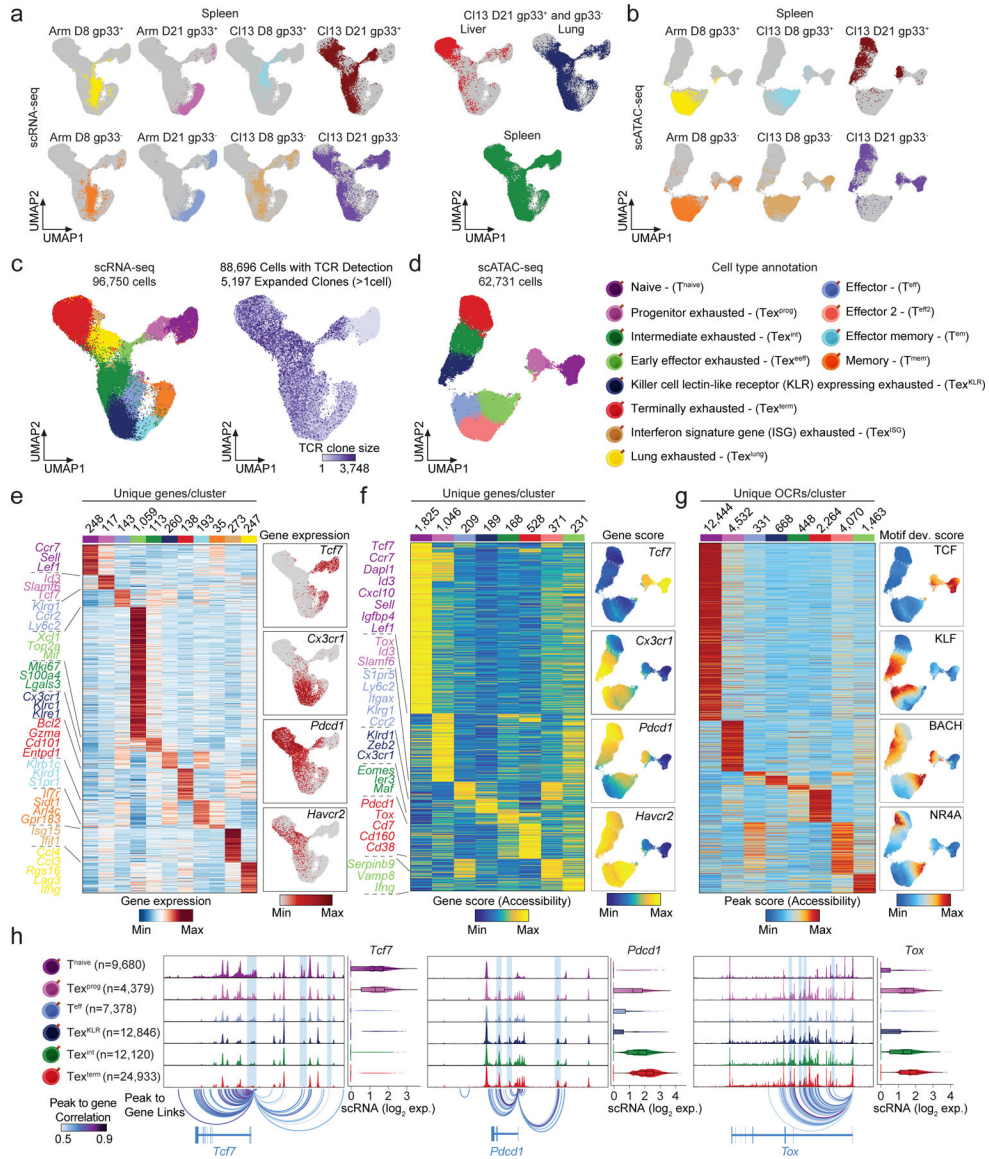


Figure 1. Single-cell genomic atlas of T cell exhaustion during LCMV infection. (a) scRNA-seq UMAPs colored by sample. (b) scATAC-seq UMAPs colored by sample. (c) scRNA-seq UMAP colored by annotated T cell subsets (left) or T cell clone size assigned by scTCR-seq (right). (d) scATAC-seq UMAP colored by annotated T cell subsets. (e) Heat map of subset specific marker genes determined by scRNA-seq (left, log₂ FC >0.25, Bonferroni adjusted p-value <0.01, two-sided Wilcoxon Rank Sum test). Feature plots of T cell subset specific gene markers (right). (f) Heat map of Gene score values (weighted accessibility at gene locus) determined by scATAC-seq (left, log₂ FC >0.5, FDR <0.01, two-sided Wilcoxon Rank Sum test adjusted using Benjamini & Hochberg procedure). Feature plots of T cell subset specific Gene score values (right). (g) Heat map of Peak score values at unique open chromatin regions (OCRs) of T cell subsets determined by scATAC-seq (left, log₂ FC >1, FDR <0.05, two-sided Wilcoxon Rank Sum test adjusted using Benjamini & Hochberg procedure). Feature plots of chromVAR deviation scores for T cell subset specific

motifs (right). **(h)** Genome accessibility tracks of indicated gene loci in Tex subsets. Violin plots of gene expression determined by scRNA-seq (n = number of scRNA-seq profiles; box center line, median; limits, upper and lower quartiles; whiskers, 1.5× interquartile range).

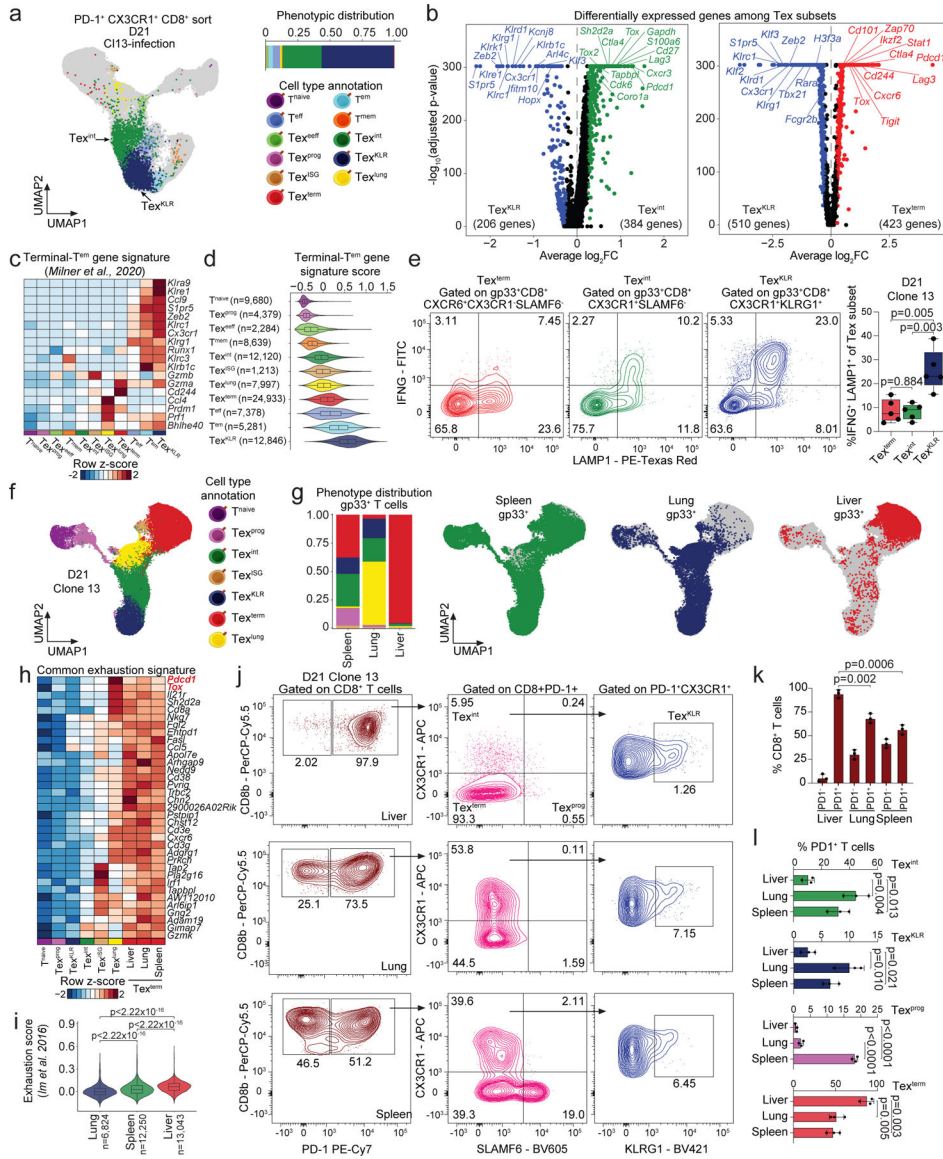


Figure 2. Identification of intermediate, KLR-expressing, and organ-specific Tex subsets. (a) scRNA-seq UMAP colored by T cell subset from sorted PD-1⁺CX3CR1⁺CD8⁺ T cells (left). Stacked bar plot of the sorted population phenotypic distribution (right) (b) Volcano plots of differentially expressed genes between indicated Tex populations (\log_2 FC >0.25, Bonferroni adjusted p-value <0.01, two-sided Wilcoxon Rank Sum test). (c) Heat map of scaled marker gene expression of terminal effector memory (T-T^{em}) cells (Milner *et al.* 2020) in indicated T cell subsets. (d) Violin plot of T-T^{em} gene signature score in indicated T cell subsets (n = number of scRNA-seq profiles, box center line, median; limits, upper and lower quartiles; whiskers, 1.5 \times interquartile range). (e) Representative IFNG/LAMP-1 flow cytometry plots of indicated Tex subsets (left). Boxplot quantifying IFNG⁺LAMP1⁺ percentage for indicated Tex subsets. Significance determined by two tailed, unpaired t-test (n = 5 biologically independent animals). Box center line, mean; limits, upper and lower quartiles; whiskers, minimum and maximum values. (f) scRNA-seq UMAP from three

organs at D21 following C113 infection colored by annotated T cell subsets **(g)** Stacked bar plot of gp33⁺ phenotypic distribution of annotated T cell subsets in three organs (left). UMAPs colored by organ (right). **(h)** Heat map of scaled gene expression values of common exhaustion gene signature among Tex^{term} from three organs. **(i)** Violin plot of Tex^{term} exhaustion scores from the three organs (Im *et al.* 2016; n = number of scRNA-seq profiles; box center line, median; limits, upper and lower quartiles; whiskers, 1.5× interquartile range). P-values determined by two-sided Wilcoxon Rank Sum test. **(j)** Representative flow cytometry plots of Tex subsets detected in organs. **(k)** Quantification of the PD-1⁺ and PD-1⁻ fractions of CD8⁺ T cells across organs. Significance determined by two tailed, unpaired t-test (n = 3 biologically independent animals). Shown are means with SDs. **(l)** Quantification of fractions of indicated Tex subsets across organs. Significance determined by two tailed, unpaired t-test (n = 3 biologically independent animals). Shown are means with SDs.

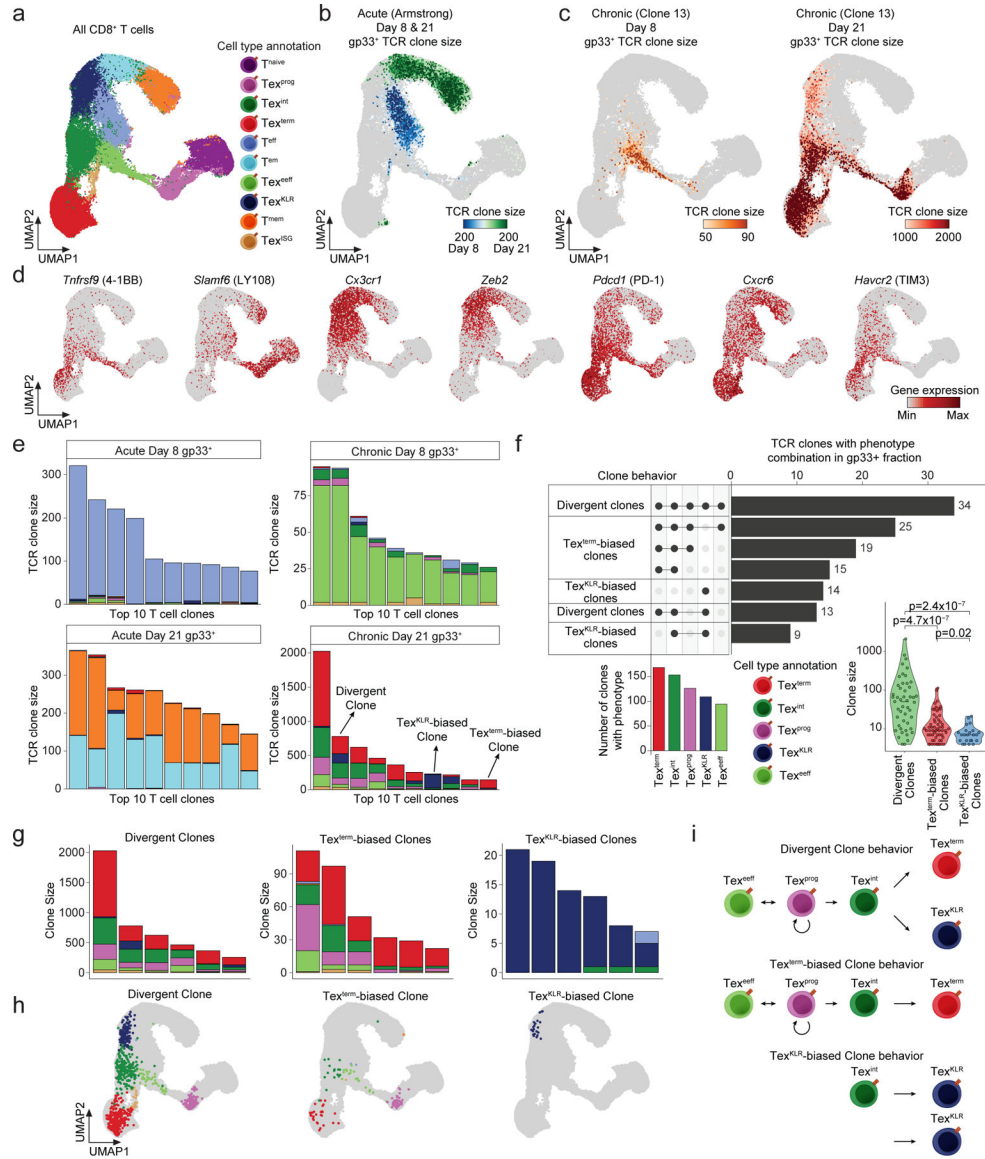


Figure 3. TCR-based lineage tracing reveals divergent Tex clonal trajectories.
(a) UMAP of scRNA-seq results from the gp33⁺ and gp33⁻ T cell fractions in the spleen at D8 and D21 of Arm and C113 infection. UMAP is colored by the annotated T cell subsets.
(b) UMAP of scRNA-seq results of gp33⁺ T cells colored by the size of the detected TCR clones at D8 and D21 in the Arm infection model. **(c)** UMAP of scRNA-seq results of gp33⁺ T cells colored by the size of the detected TCR clones at D8 in the C113 infection model (left). Same UMAP of gp33⁺ T cells colored by the TCR clone size at the D21 time point in the C113 infection model (right). **(d)** UMAPs colored by the expression of the indicated gene transcripts by scRNA-seq. **(e)** Stacked bar plot of the phenotypic distribution of the top 10 expanded clones in the gp33⁺ fraction at D8 and D21 of Arm infection (left) and C113 infection (right). **(f)** Upset plot depicting the number of expanded clones with specific phenotype combinations (clone behaviors). For clarity the top 7 most common clone behaviors are shown. Barplot of the number of clones with the indicated phenotypes

(bottom left). Violin plot of the clone size distribution of the detected clone behaviors (bottom right, p-values determined by two-sided Wilcoxon Rank Sum test). **(g)** Stacked bar plots show the top 6 expanded clones with the indicated clone behaviors. **(h)** UMAPs show representative examples of T cell clones with the detected clone behaviors. **(i)** Schematic of the phenotypic composition and the potential differentiation trajectories of the identified clone behaviors.

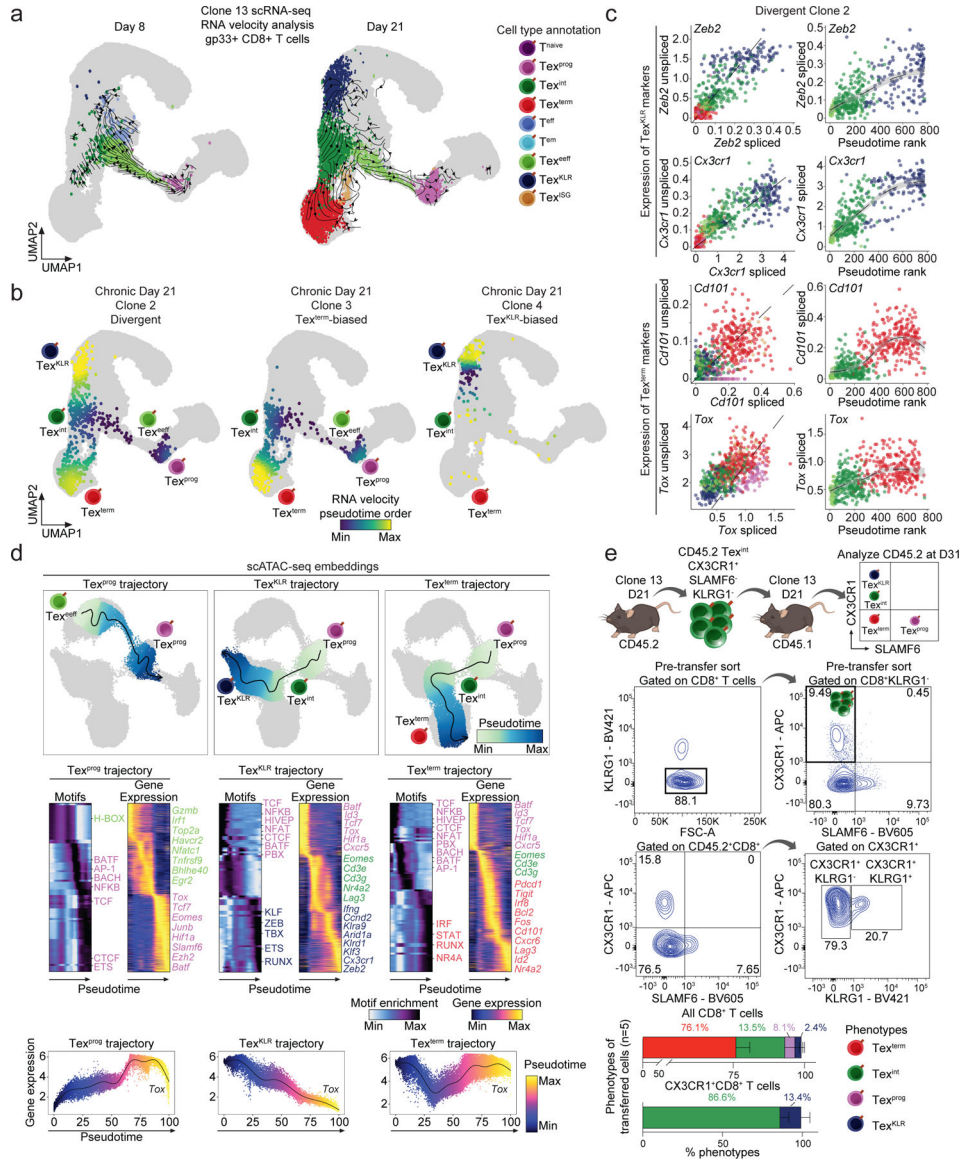


Figure 4. Tex^{int} represent a bifurcation point of exhausted T cell fate differentiation. (a) UMAPs of scRNA-seq results of D8 and D21 gp33⁺ T cells from the C113 infection model. UMAP is colored by the annotated T cell subsets and arrows represent Tex differentiation fate directions predicted by RNA velocity analysis. (b) UMAPs of individual Tex clones with the indicated clone behavior. Color gradient (RNA velocity pseudotime order) indicates directions of Tex differentiation fates determined by RNA velocity analysis. (c) Scatter plots of the expression level and spliced:unspliced ratio of the indicated gene transcripts in T cells of a divergent clone over pseudotime as determined by RNA velocity analysis. Expression of spliced and unspliced transcripts is derived from mean expression (first moment) of each gene calculated using k-nearest neighbors to alleviate dropout. (d) Pseudotime trajectory analyses of three potential Tex differentiation paths in scATAC-seq space (top). Heat maps of transcription factor motif deviation scores and gene integration scores (integrated scRNA-seq expression values) over the pseudotime trajectories (middle).

Gene integration scores for *Tox* expression in the three pseudotime trajectories (bottom). **(e)** Schematic of adoptive transfer experiments of Tex^{int} subset (top). Pre-transfer enrichment strategy for Tex^{int} cells (middle). Stacked bar plot of the phenotypic composition of transferred T cells with all *Tex* subsets shown or only the CX3CR1^+ subsets (bottom, $n = 5$ biologically independent animals). Mean % of each subset is indicated.

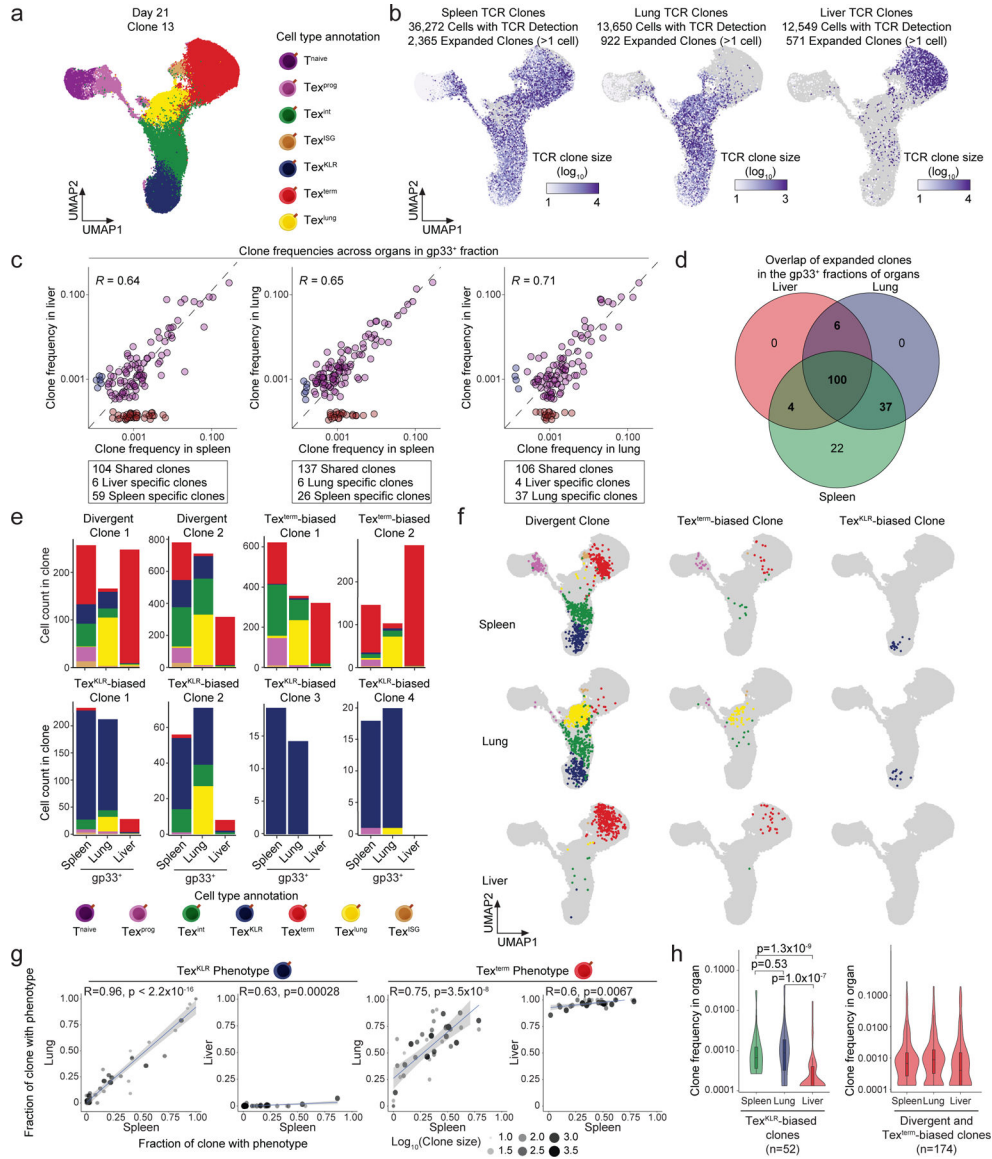


Figure 5. Conserved clonal T cell trajectories across organs and depletion of Tex^{KLR} in the liver microenvironment.

(a) UMAP of organ-derived T cells at D21 in C113 infection colored by the annotated T cell subsets. (b) UMAs colored by the detected TCR clone sizes in the different organs. (c) Scatterplots comparing the frequencies of expanded T cell clones from the indicated organs. The correlation coefficient (Pearson's R), and specific and shared clone numbers are indicated for each comparison. (d) Venn diagram depicting the overlap of expanded T cell clones in the gp33^+ fraction of the indicated organs. (e) Stacked bar plot of the phenotypic composition of individual clones across organs. (f) UMAs of individual clones with specific clone behaviors across organs. (g) Scatter plots comparing the fraction of cells in individual clones with Tex^{KLR} and Tex^{term} phenotypes between the indicated organs. Correlation coefficient calculated using Pearson's R and p-value determined using a two-sided t-distribution with $n-2$ degrees of freedom. Shaded area represents 95% confidence interval of linear model. (h) Violin plot of Tex^{KLR} -biased clone frequencies across the

organs, defined as clones with >50% Tex^{KLR} phenotype in the spleen (left). Violin plot of Tex^{term}-biased and divergent clone frequencies across the organs (right; n = number of scRNA-seq profiles; box center line, median; box limits, upper and lower quartiles; box whiskers, 1.5× interquartile range). P-values determined by two-sided Wilcoxon Rank Sum test.

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on % of GFP⁺ cells (\log_{10} EC50 values). Clone size and phenotype distribution of each clone is shown as a stacked bar plot. Bar plot shows normalized (to TCR expression) % of GFP⁺ cells (Norm. reporter activity) for all TCRs stimulated with a constant peptide concentration (100nM, bottom left). P14 LCMV-specific TCR was used as a positive control and OT-1 TCR (ovalbumin peptide specific) as a negative control. The mean of two technical replicates are shown of a representative experiment out of three independent experiments which all showed the same results.

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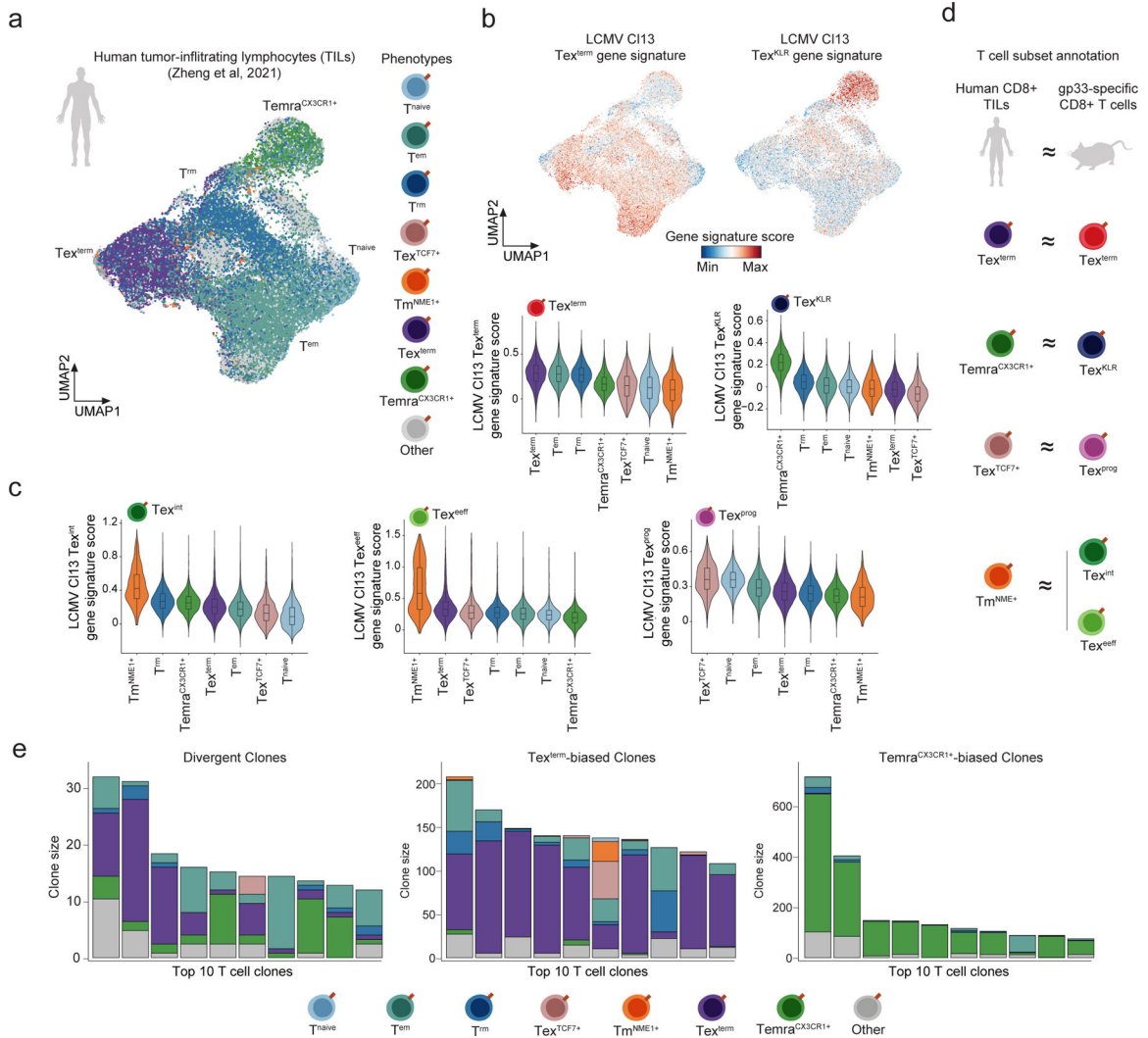


Figure 7. Human TILs exhibit shared Tex transcriptional programs and divergent clonal differentiation trajectories.

(a) UMAP of TILs profiled by scRNA-seq from Zheng *et al.*, 2021, colored by T cell phenotypes. (b) UMAPs colored by the indicated LCMV C113 Tex gene signature scores (top). Violin plots of LCMV C113 Tex gene signature scores across human TIL clusters (bottom, box center line, median; box limits, upper and lower quartiles; box whiskers, 1.5× interquartile range). For each violin plot, n = number of scRNA-seq profiles: T^{naive} = 6,879; T^{em} = 37,057; T^{rm} = 18,348; Tex^{TCF7+} = 757; T^{NME1+} = 593; Tex^{term} = 13,024; Temra^{CX3CR1+} = 11,702. (c) Violin plots of additional LCMV C113 Tex gene signature scores across human TIL clusters (box center line, median; box limits, upper and lower quartiles; box whiskers, 1.5× interquartile range). Human TIL clusters are ranked in decreasing order with cluster with highest enrichment of LCMV C113 Tex gene signature score on the left. For each violin plot, cell numbers are same as (b). (d) Schematic of the relationships found between human TIL subsets and mouse LCMV-reactive (gp33⁺) Tex subsets. (e) Stacked bar plots of the phenotype distribution of top 10 human expanded TIL

clones for the indicated clone behaviors that were detected among LCMV-reactive mouse T cells.

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