Single-cell RNA-seq reveals TOX as a key regulator of CD8+ T cell persistence in chronic infection

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Progenitor-like CD8+ T cells mediate long-term immunity to chronic infection and cancer and respond potently to immune checkpoint blockade. These cells share transcriptional regulators with memory precursor cells, including T cell-specific transcription factor 1 (TCF1), but it is unclear whether they adopt distinct programs to adapt to the immunosuppressive environment. By comparing the single-cell transcriptomes and epigenetic profiles of CD8+ T cells responding to acute and chronic viral infections, we found that progenitor-like CD8+ T cells became distinct from memory precursor cells before the peak of the T cell response. We discovered a coexpression gene module containing Tox that exhibited higher transcriptional activity associated with more abundant active histone marks in progenitor-like cells than memory precursor cells. Moreover, thymocyte selection-associated high mobility group box protein TOX (TOX) promoted the persistence of antiviral CD8+ T cells and was required for the programming of progenitor-like CD8+ T cells. Thus, long-term CD8+ T cell immunity to chronic viral infection requires unique transcriptional and epigenetic programs associated with the transcription factor TOX.

Upon acute infection or vaccination, naïve T cells first differentiate into functional effector cells, a subset of which develop into memory cells and mediate immune protection. In contrast, during chronic viral infection and cancer, T cells become exhausted; this is characterized by progressive loss of T cell function and memory potential, upregulation of inhibitory receptors such as programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and reduced proliferation. In the past decade, checkpoint blockade immunotherapies directed against inhibitory receptors have achieved remarkable success in treating cancers. Recently, the hallmarks of T cell subsets with higher potential to respond to immunotherapies have become the focus of intensive study.

Effector CD8+ T cells in acute infection are heterogeneous, comprising short-lived effector cells and memory precursor cells. However, the heterogeneity of CD8+ T cells responding to chronic infection has only recently been explored. In mice chronically infected with lymphocytic choriomeningitis virus (LCMV) strain clone 13, PD-1+ CD8+ T cells, were selectively expanded after PD-1 blockade relative to the PD-1- subset. More recently, we and others identified a CD8+ subset during chronic LCMV infection and cancer that expresses the transcription factor T cell-specific transcription factor 1 (TCF1), which is encoded by Tcf7, and is required for long-term CD8+ T cell immunity and responses to checkpoint blockade. This progenitor-like CD8+ subset exhibits characteristics similar to stem cells, being capable of self-renewal and repopulating the terminally exhausted TCF1+ CD8+ subset. Differentiation of these progenitor-like CD8+ T cells is positively regulated by transcription factors TCF1, B cell lymphoma 6 protein homolog (BCL6) and E2A immunoglobulin enhancer binding factors E12/E47 (E2A), and suppressed by type I interferon (IFN), B lymphocyte-induced maturation protein 1 (Blimp-1), and interferon regulatory factor 4 (IRF4) (refs. 6-11).

Several known pathways that regulate progenitor-like CD8+ differentiation during chronic infection are also involved in memory T cell differentiation during acute infection. However, progenitor-like CD8+ T cells probably require additional transcriptional and epigenetic programs to specifically adapt to and persist in the immunosuppressive environment of chronic infection. Population-level messenger RNA profiling of virus-specific CD8+ T cells in acute versus chronic viral infections indicates that their transcriptomes gradually diverge after the first week post-infection. However, given the heterogeneity within exhausted CD8+ T cells, it is important to determine whether these differences reflect changes in the frequencies of subpopulations or distinct transcriptional programs at the single-cell level. A deeper understanding of these questions can help elucidate the establishment of regulatory mechanisms controlling T cell fate in chronic viral infections and cancer.
of the T cell exhaustion program and optimal treatment windows for immunotherapies.

Single-cell RNA sequencing (scRNA-seq) is a powerful tool used to identify new cell subsets and uncover transcriptional differences masked by averaging the gene expression of pooled cells\(^6\). In this study, we used scRNA-seq to determine the heterogeneity of virus-specific CD8\(^+\) T cells during acute and chronic LCMV infections. Our data revealed that the transcriptional programs of virus-specific CD8\(^+\) T cells during the two infections diverged before the peak of the CD8\(^+\) T cell response. Although both subsets expressed a Tcf7-associated gene module, progenitor-like CD8\(^+\) T cells in chronic infection distinguished themselves from memory precursor cells by their enrichment of a gene module containing Tox, encoding thymocyte selection-associated high mobility group box protein TOX (TOX), a member of the high-mobility group transcription factors\(^1\). Progenitor-like CD8\(^+\) T cells also showed distinct epigenomic features, exhibiting more abundant active histone marks at genes coexpressed with Tox. Moreover, TOX promoted the long-term persistence of virus-specific CD8\(^+\) T cells during chronic LCMV infection. Conversely, TOX deficiency led to loss of progenitor-like CD8\(^+\) T cells and impaired persistence of antiviral CD8\(^+\) T cells. Our results suggest that TOX endows CD8\(^+\) T cells with longevity that facilitates long-term antiviral CD8\(^+\) immunity during chronic infection.

**Results**

**Heterogeneity of antiviral CD8\(^+\) T cells in chronic infection.** In this study, we used scRNA-seq to perform an unbiased analysis of antiviral CD8\(^+\) T cells during chronic infection. We adoptively transferred naïve P14 CD8\(^+\) T cells, which express a transgene that encodes a T cell antigen receptor that recognizes H-2D\(^b\) presenting the GP33–41 epitope\(^2\), into C57BL/6 recipients. Mice were then infected with LCMV clone 13, which causes chronic infection\(^3\). On day 7 post-infection, splenic P14 CD8\(^+\) T cells were isolated (Supplementary Fig. 1a) and analyzed by scRNA-seq (10X Genomics). T-distributed stochastic neighbor embedding (t-SNE) and unsupervised graph-based clustering partitioned cells into four clusters based on their transcriptomes\(^4\) (Fig. 1a and Supplementary Fig. 1b). Among the top 126 genes upregulated in cluster 3 (Supplementary Table 1) were Tcf7, Id3 and Slamf6 (encoding lymphocyte antigen 108 (Lym108)), known markers of progenitor-like CD8\(^+\) T cells\(^5\) (Fig. 1b). In addition, cells in cluster 3 exhibited high expression of Tox, Ikkz2 and Hif1a, and low expression of Idd2, Gzma, Gzmz, Cxcr6 and Lgals3 (Fig. 1c,d and Supplementary Fig. 1c,d). Based on its transcriptional signature, cluster 3 most probably represents the progenitor-like CD8\(^+\) population. To determine how cells in cluster 3 overlap with progenitor-like cells at a single-cell transcriptomic level, we performed a single-cell gene enrichment analysis using 207 progenitor-like signature genes identified previously (Supplementary Table 2)\(^6\). Almost all cells in cluster 3 showed enrichment of progenitor-like signature genes, whereas few cells from other clusters showed enrichment (Fig. 1e). This conclusion was independently confirmed by using a published method (AUCell 1.2.4)\(^7\) (Supplementary Fig. 1e).

To model the gradual changes in transcriptomes during cell differentiation, we used pseudotime analysis to plot gene expression profiles along two principle components of variance\(^8\) (Fig. 1f, left). The value of principle component 2 positively correlated with Tcf7 expression (Supplementary Fig. 1f) and enrichment of a progenitor-like gene signature (Fig. 1f, right). This finding suggested the association of principle component 2 with the transition between progenitor-like CD8\(^+\) T cells and their terminally exhausted counterparts. Principle component 1 probably reflects cell cycle progression through the G1-S-G2M phases (Supplementary Fig. 1g). Whereas cells in clusters 0 and 3, both with high component 1 values, were mostly in the G1 phase, cells in cluster 1 were mostly in the G2M or S phase (Supplementary Fig. 1h). Thus, our results suggest that progenitor-like CD8\(^+\) T cells (cluster 3) might first undergo terminal differentiation and then commence proliferation.

**CD8\(^+\) T cell transcriptomes in acute and chronic infections diverge early.** Given the heterogeneity of antiviral CD8\(^+\) T cells responding to acute and chronic infections, we used scRNA-seq to compare their transcriptional programs and the timing when the bifurcation occurs. We collected P14 CD8\(^+\) T cells from days 4.5 and 7 after infection by an acute (Armstrong) or chronic (clone 13) LCMV strain (hereafter referred to as day 4.5 Arm, day 4.5 C13, day 7 Arm and day 7 C13) for both scRNA-seq and bulk RNA-seq. Day 4.5 and 7 represent the antiviral CD8\(^+\) responses at the mid-phase or near the peak of initial expansion, respectively. We performed the scRNA-seq experiments twice and observed strong correlations between replicates (Supplementary Fig. 2a,b). In addition, scRNA-seq data and bulk RNA-seq data from the same condition were highly correlated (Supplementary Fig. 2b). To avoid batch effects, scRNA-seq data from one experiment were pooled for downstream analyses. P14 cells were transcriptionally more active with higher numbers of detected mRNA molecules and genes on day 4.5 than day 7 after infection (Supplementary Fig. 2c).

Using t-SNE analysis, we found that whereas day 4.5 Arm and day 4.5 C13 CD8\(^+\) T cells commingled and generally occupied the same area, day 7 Arm and day 7 C13 CD8\(^+\) T cells were partitioned into distinct areas (Fig. 2a). Thus, transcriptional programs between CD8\(^+\) T cells responding to acute infection versus chronic infection diverged before the peak of initial expansion. Furthermore, the differences between day 7 Arm and day 7 C13 CD8\(^+\) T cells reflected distinct transcriptomes at a single-cell level, not merely changes in the frequencies of subpopulations. The expression patterns of top differentially expressed genes confirmed that differential programming of antiviral CD8\(^+\) T cells responding to acute and chronic viral infections began between day 4.5 and day 7 post-infection (Fig. 2b). Gene ontology analysis revealed that genes involved in translation were upregulated on day 7 C13 CD8\(^+\) T cells (Supplementary Fig. 2d). As expected, day 7 Arm CD8\(^+\) T cells upregulated genes related to T cell activation and killer cell cytotoxicity (Supplementary Fig. 2e).

We then used an unsupervised approach to assign virus-specific CD8\(^+\) T cells to different clusters based on their transcriptional profiles. We identified 11 clusters, including 6 (1, 3, 4, 5, 7, 9) from both day 4.5 samples, 4 (2, 6, 7, 8) from day 7 Arm cells and two (0, 10) unique to day 7 Cl13 cells (Fig. 2c). Cluster 7 included cells from day 4.5 Arm, day 4.5 C13 and day 7 Arm. Cells in this cluster exhibited elevated expression of type I and type II interferon gene signatures and a transcriptional signature of memory precursor cells, including upregulation of Bcl2, Ccr7, Id3, Sell and Tcf7, and downregulation of Ccr2, Gzmb, Id2, Klrk1 and Zeb2 (Fig. 2d–f and Supplementary Fig. 2f). These data suggest that the memory precursor cell fate was established in a subset of antiviral CD8\(^+\) T cells by day 4.5 post-infection. Upregulation of Bcl2, Id3 and Tcf7 was also observed in cluster 5 (day 4.5 Arm and day 4.5 C13) and cluster 10 (day 7 C13). In addition, high expression of Mti1 encoding metallothionein-1, which promotes T cell dysfunction during cancer\(^8\), was found in cluster 4 (day 4.5 Arm and day 4.5 C13) and cluster 0 (day 7 C13). It was not present in cluster 10 (day 7 C13), which is enriched for hypoxia response genes (Fig. 2e,f and Supplementary Fig. 2f). Of note, clusters 10 and 0 correspond to TCF1\(^b\) progenitor-like and TCF1\(^b\) terminally exhausted CD8\(^+\) T cells during chronic viral infection, respectively. Thus, single-cell transcriptomes of progenitor-like and memory precursor cells diverged by day 7 post-infection despite similar expression of signature genes such as Tcf7.

TOX distinguishes progenitor-like from memory precursor cells. Given their similarities, it is unclear whether the transcriptional program of progenitor-like CD8\(^+\) T cells is distinct from that of
Fig. 1 | Heterogeneity of virus-specific CD8+ T cells from chronic LCMV infection delineated by scRNA-seq. a–f, Naive P14 CD8+ T cell were transferred to C57BL/6 mice that were subsequently infected with LCMV clone 13. P14 cells were isolated on day 7 post-infection. N = 2,597 cells were used for the scRNA-seq analyses. a, The t-SNE projection of P14 cells, determined by Seurat v.2. Each dot corresponds to one individual cell. A total of four clusters (clusters 0–3) were identified and color-coded. 

The memory precursor gene signature (Supplementary Table 3) also 

memory precursor cells. Although the progenitor-like signature previously identified was enriched in most progenitor-like cells (cluster 10), it was also enriched in approximately 60% of cluster 7 cells, a subset with memory precursor cell characteristics, and in approximately 30% of cluster 5 cells (Fig. 3a and Supplementary Fig. 3a). The memory precursor gene signature (Supplementary Table 3) also
Fig. 2 | Single-cell transcriptomes of virus-specific CD8^+ T cells responding to acute and chronic viral infections diverged between day 4.5 and day 7 post-infection. a–f, Naïve P14 cells were transferred into C57BL/6 mice that were subsequently infected with either LCMV Armstrong or LCMV clone 13. P14 cells were collected on day 4.5 and day 7 post-infection. N = 16,042 cells were used for the scRNA-seq analyses. a, t-SNE plots of 16,042 P14 cells (day 4.5 Arm: n = 4,651 cells; day 4.5 Cl13: n = 4,116 cells; day 7 Arm: n = 4,678 cells; day 7 Cl13: n = 2,597 cells) determined by Seurat v.2. Each dot represents a single cell. Cells from different samples are color-coded. b, Heatmap of the top 15 genes expressed in each sample. The columns correspond to the cells; the rows correspond to the genes. Cells are grouped by samples. The color scale is based on a z-score distribution from −2 (purple) to 2 (yellow). c, Left panel: t-SNE plots of cells from all four samples determined by Seurat v.2. A total of 11 clusters (clusters 0–10) were identified and color-coded. Right panel: percentages of cells from each cluster in each sample. d, Heatmap of the top 10 genes expressed in each cluster. Cells are grouped by clusters. The color scale is based on a z-score distribution from −2 (purple) to 2 (yellow). e, Dot plots of gene ontology, determined by Metascape 3.0. Each column represents one cluster; each row represents a pathway. The enrichment scores are color-coded. The ranges of the log_{10} P values are represented by the diameter of the circles. f, Single-cell transcript levels of Tcf7, Ccr7, Gzmb and Mti illustrated in t-SNE plots. Transcript levels are color-coded: gray, not expressed; purple, expressed.
failed to distinguish progenitor-like cells from memory precursor cells (Supplementary Fig. 3b). Thus, we sought to identify genes that were differentially expressed between progenitor-like CD8+ T cells in chronic infection (cluster 10) and other CD8+ populations that expressed Tcf7 (clusters 5 and 7). Among the top genes upregulated in cluster 10 were the ribosomal genes, Cd200, Pdcd4 and Tox (Fig. 3b–d). Tox encodes a transcription factor that regulates the development of CD4+ T cells and innate lymphocytes.

Our results that Tox and Tcf7 were coexpressed in progenitor-like CD8+ T cells but not memory precursor cells indicate that these two genes might be involved in two separate gene regulatory circuits. Thus, we used coexpression network analysis of our scRNA-seq data to identify genes that were differentially expressed between progenitor-like CD8+ T cells (Supplementary Fig. 3b). Thus, we sought to identify genes that were differentially expressed between progenitor-like CD8+ T cells in chronic infection (cluster 10) and other CD8+ populations that expressed Tcf7 (clusters 5 and 7). Among the top genes upregulated in cluster 10 were the ribosomal genes, Cd200, Pdcd4 and Tox (Fig. 3b–d). Tox encodes a transcription factor that regulates the development of CD4+ T cells and innate lymphocytes.

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data to identify modules of coexpressed genes. Forty-nine modules were identified and color-coded, including a module containing genes such as *Tcf7*, *Tnfsf8* and *Id3* (module 29, saddlerbrown) and a separate module containing *Tox* (module 12, tan) (Fig. 3e, Supplementary Fig. 3c and Supplementary Tables 4 and 5). Based on a previous study that identified TOX binding sites in effector CD8+ T cells using chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq)24, we identified 90 of the 200 genes in the *Tox* module as potential direct targets of TOX. Next, we determined the enrichment of each gene module in individual cells. Module 29 exhibited strong enrichment in cells from cluster 5, 7 and 10, similar to the *Tcf7* expression pattern (Fig. 3f and Supplementary Fig. 3d), whereas most cells with enrichment of module 12 fell in cluster 10 (Fig. 3g and Supplementary Fig. 3e). To confirm these findings, we isolated day 7 Cl13 progenitor-like (TIM3loBlimp-1lo), day 7 Cl13 terminally exhausted (TIM3hiBlimp-1hi), day 7 Cl13 memory precursor (KLRG1lo) and day 7 Cl13 short-lived effector (KLRG1hi) P14 CD8+ T cells collected from mice 7 d after infection with LCMV clone 13 or Armstrong. 

**Fig. 4** | Progenitor-like CD8+ T cells exhibit a H3K27ac profile distinct from that of memory precursor cells. a-d, H3K27ac ChIP-seq was performed with progenitor-like (TIM3loBlimp-1lo), terminally exhausted (TIM3hiBlimp-1hi), memory precursor (KLRG1lo) and short-lived effector (KLRG1hi) P14 CD8+ T cells. The number of peaks in each category is indicated. b, Pie charts demonstrating the distribution of common or variable H3K27ac peaks across the genome (3′ untranslated region (UTR), 5′ UTR, exon, intron, non-coding, promoter-TSS and transcription termination site (TTS)). c, Hierarchical clustering of progenitor-like, terminally exhausted, memory precursor and short-lived effector cells based on their H3K27ac profiles. d, Deposition of H3K27ac centered on variable peaks (±4 kb) in progenitor-like, terminally exhausted, memory precursor and short-lived effector CD8+ T cells. Each row represents a peak. Red represents higher signal intensity; blue represents lower signal intensity.

Progenitor-like CD8+ T cells display a distinct epigenome. Epigenomic programming is critical for the differentiation of T cells25-27. In this study, we used ChIP-seq to characterize the genome-wide profiles of histone H3 lysine 27 acetylation (H3K27ac), a marker of active enhancers28, in day 7 Cl13 progenitor-like, day 7 Cl13 terminally exhausted, day 7 Arm memory precursor and day 7 Arm short-lived effector P14 cells. Progenitor-like CD8+ T cells had the highest number of unique peaks among all four cell types (Fig. 4a and Supplementary Fig. 4a,b). Compared to common peaks, peaks not shared by all cell types (that is, variable peaks) were enriched in intergenic regions or introns and reduced in promoters (Fig. 4b and Supplementary Fig. 4c). This finding suggests that the differentiation of virus-specific CD8+ T cells is more likely to be impacted by differential activity of enhancers versus promoters. Hierarchical clustering analysis and a heatmap of these H3K27ac profiles showed that progenitor-like CD8+ T cells were most distant from the other three cell types (Fig. 4c,d). Hierarchical clustering of scRNA-seq data also placed progenitor-like CD8+ T cells (cluster 10) on the most distant branch (Supplementary Fig. 4d).
Next, we measured H3K27ac modification of genes coexpressed with Tox and found greater H3K27ac modification in progenitor-like CD8+ T cells than in memory precursor cells (Fig. 5a, left). In addition, genes with TOX binding sites\(^\text{14}\) exhibited greater H3K27ac in progenitor-like CD8+ T cells than in memory precursor cells (Fig. 5a, right). Moreover, progenitor-like and terminally exhausted signature genes (Supplementary Tables 2 and 6) exhibited more H3K27ac peaks at the upstream regulatory elements of Gzmb (Fig. 5c) and lacked an H3K27ac peak 3′ of Id2 (Supplementary Fig. 4g). In contrast, there were multiple intronic H3K27ac peaks at the Tox locus that were unique and/or significantly enriched (for example, +50, +122 and +133 kb) in progenitor-like CD8+ T cells (Fig. 5f).

Based on published ChIP-seq data, several potential transcriptional regulators of CD8+ T cells during chronic infection, including nuclear factor of activated T-cells, cytoplasmic 2 (NFAT1), IRF4, runt-related transcription factor 3 (RUNX3), forkhead box protein O1 (FOXO1), signal transducer and activator of transcription 3 (STAT3) and signal transducer and activator of transcription 5B (STAT5B)\(^\text{12,30–33}\), bind to the Tox locus (Supplementary Fig. 4h). To determine whether the calcineurin-NFAT pathway upregulates Tox in virus-specific CD8+ T cells during chronic infection, we treated mice that were infected with LCMV clone 13 with a calcineurin inhibitor FK506 (tacrolimus) starting from day 4 post-infection. On day 7 post-infection, blocking the calcineurin-NFAT pathway resulted in reduced TOX protein in virus-specific CD8+ T cells, confirming that the calcineurin-NFAT pathway positively regulates TOX expression (Supplementary Fig. 4i).

**Fig. 5 | Tox-associated genes are epigenetically active in progenitor-like CD8+ T cells.** a, Deposition of H3K27ac centered on the TSS of genes in module 12 (left) or on TOX binding peaks (right) in progenitor-like and memory precursor CD8+ T cells. b, Volcano plots of differentially modified H3K27ac peaks between progenitor-like and terminally exhausted CD8+ T cells (left) or between progenitor-like and memory precursor CD8+ T cells (right). The H3K27ac data include \(n=2\) biological replicates per group. Differential abundance of H3K27ac is shown as the log\(_2\) fold change and is plotted against −log\(_2\)(FDR). The horizontal dashed lines denote FDR = 0.1, whereas the vertical dashed lines denote a fold change of ±1.5. Each dot represents a peak. Peaks associated with significantly upregulated genes (fold change >2, FDR < 0.05) in progenitor-like (\(n=2\) biological replicates), terminally exhausted (\(n=2\) biological replicates) or memory precursor (\(n=3\) biological replicates) cells are color-coded. FDR was determined by edgeR v.20.9. c-f, Normalized H3K27ac profiles at Tcf7 (c), Klrg1 (d), Gzmb (e) and Tox (f) loci in progenitor-like (red), terminally exhausted (green), memory precursor (orange) and short-lived effector (blue) CD8+ T cells. Data are representative of 2 independent experiments.

**Fig. 4 | H3K27ac activity in the Tox locus in virus-specific T cells.** a, b, Distribution of H3K27ac peaks in short-lived effector (a) and terminally exhausted (b) virus-specific T cells. c, d, Heat maps of normalized H3K27ac enrichment at each 50-kb bin in short-lived effector (c) and terminally exhausted (d) virus-specific T cells. e, Normalized H3K27ac enrichment at each 50-kb bin in virus-specific T cells with longevity in multiple modules. f, Normalized H3K27ac enrichment at each 50-kb bin in virus-specific T cells with longevity in the Tox locus. g, Heat maps of normalized H3K27ac enrichment at each 50-kb bin in virus-specific T cells with longevity in all 14 modules. h, Normalized H3K27ac enrichment at each 50-kb bin in virus-specific T cells with longevity in the Tox locus.
Persistence of virus-specific CD8⁺ T cells during chronic infection, we overexpressed TOX in P14 CD8⁺ T cells by retroviral transduction (Supplementary Fig. 5a,b) and transferred transduced cells into C57BL/6 mice, which were immediately infected with LCMV clone 13. TOX overexpression greatly increased the number and frequency of splenic P14 cells on day 14 and day 28 post-infection (Fig. 6a,b) but did not affect the initial expansion of P14 cells (Fig. 6a,b) or the frequency of TCF1hi cells (Fig. 6c and Supplementary Fig. 5c). Thus, TOX overexpression enhanced the persistence of virus-specific CD8⁺ T cells without skewing their differentiation toward progenitor-like cells. Next, we sorted control and TOX-overexpressing TIM3hi (TCF1*terminally exhausted) P14 CD8⁺ T cells on day 7 post-infection (Supplementary Fig. 5d) and transferred them into infection-matched mice. On day 5 post-transfer, significantly more TOX-overexpressing P14 cells were present than control cells, although both groups remained committed to the TCF1hi lineage (Fig. 6d and Supplementary Fig. 5e). These data suggest that ectopic expression of TOX is sufficient to endow terminally exhausted CD8⁺ T cells with the ability to persist during chronic viral infection.

We profiled the transcriptomes of TIM3loLy108hi(TCF1⁺progenitor-like) and TIM3hiLy108hi (TCF1⁺ terminally exhausted) P14 cells (Supplementary Fig. 5f) from control and TOX overexpression samples. TOX overexpression led to upregulation of 266 genes and downregulation of 358 genes in progenitor-like P14 cells, and upregulation of 48 genes and downregulation of 57 genes in terminally exhausted P14 cells (fold change > 1.5, P < 0.05, FDR < 0.1) (Supplementary Tables 7 and 8). Among the differentially expressed genes, transcription factors (for example, Ifi27), inhibitory receptors (for example, Pdcd1) and effector molecules (for example, Tfy) (Fig. 6e). We confirmed that TOX overexpression moderately increased protein PD1 and T cell immunoreceptor with Ig and ITIM domains (TIGIT) protein on P14 cells on day 7 post-infection (Supplementary Fig. 5g,h). However, a slight decrease in leukocyte immunoglobulin-like receptor subfamily B member 4 (LILRB4) level was observed in TOX-overexpressing P14 cells (Supplementary Fig. 5i). In addition, we found representative production of IFN-γ, tumor necrosis factor (TNF) and interleukin 2 (IL-2) by TOX-overexpressing P14 cells after restimulation on day 7 post-infection (Supplementary Fig. 5j–m). However, in vitro cytotoxicity of CD8⁺ T cells was unaffected by TOX overexpression (Supplementary Fig. 5n).

Using gene set enrichment analysis (GSEA) to determine whether TOX governs the activity of the Tox gene module, we found that TOX overexpression upregulated the gene signature associated with this module in both progenitor-like and terminally exhausted P14 CD8⁺ T cells (Fig. 6f,g). In addition, both progenitor-like and terminally exhausted CD8⁺ T cells, TOX overexpression led to the upregulation of genes in the hypoxia pathway and downregulation of signatures such as oxidative phosphorylation, mTOR signaling, IFN-α response and DNA repair (Fig. 6h and Supplementary Fig. 5o–s). To determine the effects of TOX overexpression on the activity of the phosphatidylinositol 3-OH kinase (PI3K)/Akt/mTOR pathway, we quantified phospho-Akt (S473) and phospho-ribosome protein S6 (S235/S236) in control and TOX-overexpressing P14 cells on day 7 post-infection. Consistent with the GSEA results, TOX-overexpressing P14 cells exhibited lower PI3K/Akt/mTOR signaling than control cells (Fig. 6i,j). Taken together, TOX overexpression promotes long-term persistence of virus-specific CD8⁺ T cells during chronic infection but reduces PI3K/Akt/mTOR signaling and cytokine production.

Progenitor-like cells and persistent CD8⁺ immunity require TOX. To determine the cell-intrinsic role of TOX in the antiviral CD8⁺ T cell response during chronic infection, we generated mixed bone marrow chimeras. Reconstitution of lethally irradiated wild-type CD45.2 mice with mixed bone marrow either from wild-type CD45.1 and wild-type CD45.2 donors or from Tox⁻/⁻CD45.1 and wild-type CD45.2 donors enabled comparison of Tox⁻/⁻ and wild-type CD8⁺ T cells exposed to the same environment. Despite comparable frequencies of LCMV-specific cells between the wild-type and Tox⁻/⁻ CD8⁺ compartments on day 7 after LCMV clone 13 infection (Supplementary Fig. 6a), frequencies of TCF1hiTIM3hi cells were significantly lower in Tox⁻/⁻ LCMV-specific CD8⁺ T cells than in their wild-type counterparts in the same mice (Fig. 7a,b). This finding suggests that progenitor-like CD8⁺ T cells require cell-intrinsic TOX activity. To confirm these findings, we adoptively transferred CD45.1 Tox⁺/⁻CD45.2Cre (Tox conditional knockout) and Tox⁺/⁻Flp/Flp (wild-type) P14 cells into wild-type CD45.1⁺ recipients and analyzed their number and phenotype on day 7 post-infection. Consistently, Tox deficiency led to a substantial reduction in the frequency of TCF1hiTIM3hi virus-specific CD8⁺ T cells without impacting the magnitude of the initial expansion (Supplementary Fig. 6c).

Next, we analyzed pooled scRNA-seq data from 4,409 wild-type and 5,296 Tox⁻/⁻ LCMV-specific CD8⁺ T cells from mixed bone marrow chimera on day 7 after LCMV clone 13 infection. Significant portions of wild-type and Tox⁻/⁻ cells occupied distinct spaces in the t-SNE plots, suggesting that Tox deficiency led to a substantial change in the differentiation program of virus-specific CD8⁺ T cells (Fig. 7c). Unsupervised clustering assigned cells into four subsets. Fewer cluster 2 cells, which expressed high levels of Tcf7, Id3 and Slamf6 and represent the progenitor-like population, were present among Tox⁻/⁻ cells (Fig. 7d,e). In addition, more cluster 0 cells,
which expressed signature genes typical of terminally exhausted
cells, were present in Tox−/− cells. However, Tox−/− cells in cluster 0
did not fully comingle with wild-type cells in the same cluster, sug-
gesting that Tox deficiency altered the differentiation of terminally
exhausted cells (Fig. 7c,d). Indeed, Tox−/− cells upregulated Klrk1,
which is typically associated with short-lived effectors during acute
viral infection (Fig. 7f). In addition, Tox−/− cells upregulated
Gzma and Gzmb, and downregulated Pdcd1, indicating a potential
increase in the effector function. However, Tox−/− cells also downregulated
Hif1a and Batf, both encoding transcription factors required for
sustaining antiviral CD8+ T cells and long-term CD8+ immunity during chronic
infection.

Moreover, the increased M11 expression in Tox−/− cells also suggests
that these cells are more dysfunctional.31

To determine whether cell-intrinsic Tox deficiency affects the
maintenance of virus-specific CD8+ T cells, we tracked CD8+ T
responses in these chimeras four weeks after infection. Notably,
there were significantly lower frequencies of LCMV-specific cells
within Tox−/− CD8+ T cells than within wild-type CD8+ T cells
in the same mice (Fig. 7g,h). Therefore, cell-intrinsic TOX activ-
ity is required both for the programming of progenitor-like
CD8+ T cells and long-term CD8+ immunity during chronic viral infection.
**Fig. 7** | TOX is required for progenitor-like CD8⁺ T cell differentiation and persistent antiviral CD8⁺ T cell responses. Mixed bone marrow chimeras that received wild-type CD45.1 and wild-type CD45.2 (wild-type + wild-type) or Tox⁻/⁻ CD45.1 and wild-type CD45.2 bone marrows (wild-type + Tox⁻/⁻) were infected with LCMV clone 13. 

- **a.** Representative FACS plots of TCF1 and TIM3 staining in splenic H-2Db GP33 tetramer⁺ CD45.1 or CD45.2 CD8⁺ T cells in chimeras on day 7.
- **b.** Frequencies of TCF1⁺TIM3⁺ cells within H-2Db GP33 tetramer⁺ CD45.2 wild-type (unfilled) and CD45.1 wild-type or Tox⁻/⁻ (filled) splenic CD8⁺ T cells on day 7. *n* = 5 mice in each group. NS, not significant.
- **c.** Heatmap of the top 10 genes expressed in each cluster as defined in Fig. 7d. The columns correspond to the cells; the rows correspond to the genes. Cells are grouped by clusters. The color scale is based on a z-score distribution from −2 (purple) to 2 (yellow).
- **d.** t-SNE plots of wild-type (*n* = 4,409 cells) and Tox⁻/⁻ (*n* = 5,296 cells) CD8⁺ T cells, determined by Seurat v.2.
- **e.** t-SNE plots of wild-type (*n* = 4,409 cells) and Tox⁻/⁻ (*n* = 5,296 cells) cells that were assigned to four clusters and color-coded based on the clusters.
- **f.** Violin plots of Batf, Ccr2, Gzma, Gzmb, Hif1a, Krgr1, Mt1 and Pdcd1 expression in wild-type (*n* = 4,409 cells) and Tox⁻/⁻ (*n* = 5,296 cells) cells. The violin represents the probability density at each value; each dot represents one cell.
- **g.** Representative FACS plots of H-2Db GP33 tetramer staining on CD45.1 or CD45.2 CD8⁺ T cells in the spleen of chimeras 4 weeks after infection.
- **h.** Frequencies of H-2Db GP33 tetramer⁺ cells within CD45.2 wild-type (unfilled) and CD45.1 wild-type or Tox⁻/⁻ (filled) splenic CD8⁺ T cells four weeks after infection. *n* = 7 mice in the wild-type + wild-type group; *n* = 9 mice in the wild-type + knockout group. Data in **a, b, g, h** are representative of 2 independent experiments. In **b, h,** statistical significance was determined with a two-sided, paired Student's *t*-test; the centers and error bars represent the mean and s.d. **P < 0.01,** ***P < 0.001.
Discussion
Progenitor-like CD8+ T cells found in chronic viral infection and cancer have attracted great attention as a potential target for adoptive cell therapies and checkpoint blockade therapies4,11,26,40. However, it is unclear whether progenitor-like CD8+ T cells require distinct transcriptional and epigenetic programs to persist in the immunosuppressive environment induced by chronic viral infection and cancer. In our study, we revealed distinct transcriptional and epigenetic programs of progenitor-like CD8+ T cells in chronic infection using scRNA-seq and H3K27ac ChIP-seq. Compared to memory precursor cells in acute infection, progenitor-like cells exhibited a greater transcriptional activity and higher H3K27ac in a gene module containing Tox, identified through our scRNA-seq analyses. We further demonstrated the critical role of TOX in the persistence of antiviral CD8+ T cells and differentiation of progenitor-like CD8+ T cells during chronic viral infection.

Progenitor-like CD8+ T cells are exposed to continuous antigen stimulation and immunosuppression during chronic infection4. Therefore, compared to memory precursor cells, progenitor-like CD8+ T cells probably exhibit differential activities in the pathways required for the adaptation to chronic viral infection. Previous studies mostly relied on bulk transcriptomic analysis of progenitor-like CD8+ T cells, which reflect the population average and are affected by the bias introduced by the markers selected to purify this population. Using single-cell transcriptomes of virus-specific CD8+ T cells from mice after acute and chronic viral infections, we found that Tcl7+ CD8+ T cells from day 7 acute and chronic LCMV infections partitioned in separate areas in the t-SNE plot. This finding suggests that memory precursor and progenitor-like cells have distinct transcriptional programs despite sharing common markers. For example, we found pronounced upregulation of gene signatures, such as translation and hypoxia, in progenitor-like CD8+ T cells relative to memory precursor cells. Progenitor-like CD8+ T and memory precursor cells also differed in their H3K27ac profiles, suggesting that their differentiation might be regulated by activation of different sets of enhancers. Nonetheless, while intriguing, the epigenomic data in this study suffer from similar limitations of population-level profiles as discussed earlier. Thus, future studies that employ single-cell epigenomics might help further elucidate the differential regulation of progenitor-like CD8+ T and memory precursor cells.

Long-term persistence is a cardinal hallmark of T cell stemness and is critical for the CD8+ T cell response against chronic infection and cancer27,41. Moreover, the longevity of therapeutic T cells directly affects the efficacy of adoptive cell therapies41. Thus, it is important to determine the molecular mechanisms underlying the longevity of progenitor-like CD8+ T cells. We found that Tox was among the most differentially expressed genes between progenitor-like CD8+ T and memory precursor cells and is in a gene module most highly expressed by progenitor-like CD8+ T cells. Consistent with our results, a previous study that used microarrays to compare the transcriptomes between CD8+ T cells responding to acute and chronic LCMV infections identified Tox as a hub gene in the difference network between acute and chronic infections13. In this study, we have shown that Tox deficiency led to defects in the long-term persistence of antiviral CD8+ T cells and a loss of progenitor-like CD8+ T cells, and that TOX overexpression enhanced the persistence of both progenitor-like and terminally exhausted virus-specific CD8+ T cells. In addition, our comparison of single-cell transcriptomes between wild-type and Tox-deficient antiviral CD8+ T cells underlines a critical role of TOX in dictating the transcriptional program of virus-specific CD8+ T cells responding to chronic infection. These findings suggest that TOX plays an essential role in promoting persistence of T cells in chronic infection and might promote the persistence of therapeutic T cells in immunotherapies.

Interestingly, a recent study has shown that TOX is required for the pathogenicity of autoreactive CD8+ T cells in the brain42. Similar to our observation in chronic viral infection, Tox-deficient autoreactive CD8+ T cells downregulated Tcl7 and upregulated short-lived effector markers such as Gzmb and Klrg1. These data suggest a potential overlap of the molecular circuit involving TOX between autoreactive and antiviral CD8+ T cells. Of note, although less prevalent, some terminally exhausted CD8+ T cells expressed Tox and/or were enriched with the Tox module. Moreover, Tox deficiency also altered the differentiation program of terminally exhausted CD8+ T cells, suggesting a role of TOX in these cells. Whether progenitor-like cells directly give rise to TOX-expressing terminally exhausted cells remains to be investigated.

In summary, through scRNA-seq and H3K27ac profiling, we found that progenitor-like CD8+ T cells responding to chronic viral infection are regulated by distinct transcriptional and epigenetic programs despite their similarities with memory precursor cells. Importantly, this includes upregulation of Tox, which promotes persistent antiviral CD8+ T cell response and is necessary for progenitor-like CD8+ T cell differentiation. Our study adds to our understanding of T cell stemness and may shed new light on the development of more effective immunotherapies.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at https://doi.org/10.1038/s41590-019-0403-4.

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Author contributions
T.W. conceived the study. T.W., C.Y., P.L.S. and J.J.O. designed the experiments. T.W., C.Y., P.L.S. and J.J.O. performed the experiments. O.K. and E.J.W. provided the P14 Toxlox\(^{loxP}\)/CD4-Cre mice. T.W., C.Y., P.L.S. and J.J.O. analyzed and interpreted the results. T.W., C.Y., P.L.S. and E.J.W. drafted the manuscript.

Competing interests
E.J.W. has consulting agreements with and/or is on the scientific advisory board for Merck, Roche, Pieris, Elstar and Surface Oncology. E.J.W. has a patent licensing agreement on the PD-1 pathway with Roche/Gentech. Y.J. has stock in Cellular Biomedicine Group.

Additional information
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Methods
Mice, infection, adoptive transfer, mixed bone marrow chimeras and FK056 treatment. C57BL/6 and wild-type CD45.1 (B6.SJL-Ptprca Pep8/BoyJ) mice were purchased from The Jackson Laboratory. P14 mice carry a transgenic T-cell antigen receptor that recognizes H-2Dd GP33–41 epitope on LCMV−1 (Blimp-1−, yellow fluorescent protein (YFP) reporter mice) purchased from The Jackson Laboratory, were bred to P14 to generate P14 Blimp-1− YFP mice. Tox-deficient mice were generated by deleting the first exon and promoter region using CRISPR-Cas9. Tox−/− mice have been described previously and were crossed to CD4−Cre and P14 mice. All mice were kept on a C57BL/6 background. Mice used in the experiments were age- and sex-matched. All animal husbandry and experiments were approved by the National Human Genome Research Institute (NHGRI) or the National Institute of Neurological Disorders and Stroke Animal Care and Use Committees (proocols G98-3 and 1295-12).

For acute viral infections, mice were injected intravenously with 2 × 106 plaque-forming units LCMV−1. For chronic viral infections, mice were injected intravenously with 2 × 106 plaque-forming units LCMV clone 13. For the adoptive transfer experiments, 5,000 naïve splenic P14 cells were adoptively transferred into mice before infection. To generate the mixed bone marrow chimeras, lethally irradiated (950 rad) wild-type mice were reconstituted with 1:1 mixed bone marrow chimeras, 2,000 CD4+ and 2,500 donors that were either wild-type or Tox-deficient. After at least two months from the date of reconstitution, chimeras were used for the experiments. For the FK056 treatment, 10 mg kg−1 FK056 were injected subcutaneously into mice daily from day 4 to day 7 post-infection, as defined previously.

Retroviral transduction. The TOX (UniProt ID: Q66W31–1) overexpression construct was made with a murine stem cell virus-internal ribosome entry site-green fluorescent protein (MSCV-IRES-GFP) backbone (pMIG) as described previously. Activated P14 CD8+ T cells were spinoculated with retroviruses carrying a control (pMIG) or a TOX overexpression construct for 90 min, cultured overnight with 2 μg ml−1 IL-7 and sorted by flow cytometry for GFP− transduced P14 cells. Five thousand GFP+ P14 cells were injected into each C57BL/6 recipient which was immediately infected with LCMV clone 13.

Antibodies, dyes, flow cytometry and cell sorting. Anti-CD90 (clone 53-6-7), anti-CD45.1 (clone A20), anti-CD45R (B220; clone RA3-6B2), anti-CD366, anti-CD8a (clone 53-6.7), APC-Cy anti-IL-2 (clone JES6-5H4), anti-Ly6G (clone 1A8) and PE anti-Ly108 (clone XMG1.2) antibodies and the LIVE/DEAD Fixable Aqua Dead Cell Stain Kit were purchased from Thermo Fisher Scientific; Alexa Fluor 488 anti-CD45.2 (clone 104), anti-KLGr1 (clone 2F1), Alexa Fluor 647 anti-CD72 (P1-1; clone RPM1-30), anti-LILRB4 (clone H1.1) and Alexa Fluor 488 anti-CD44 (clone IM7) antibodies were purchased from Biolegend; Alexa Fluor 647 anti-Atk (pS473) (clone 50E2), fluoroRocio anti-TNF (clone MPX-T22), APC-Cy anti-IL-2 (clone JES6-5H4), anti-CD44 (clone 1A8) and PE anti-Ly108 (clone 1G3) antibodies were purchased from BD Biosciences; anti-phospho-S6 ribosomal protein (Ser235/236; clone D57.2.2E) antibody was purchased from Cell Signaling Technology. The H-2Dd GP33–41 tetramer was obtained from the The Jackson Laboratory. P14 mice carry a transgenic T-cell receptor that recognizes H-2Dd GP33–41 epitope on LCMV−1 (Blimp-1−, yellow fluorescent protein (YFP) reporter mice) purchased from The Jackson Laboratory, were bred to P14 to generate P14 Blimp-1− YFP mice. Tox-deficient mice were generated by deleting the first exon and promoter region using CRISPR-Cas9.

mRNA-seq. For each biological replicate, cells from at least three mice were pooled; 5 × 106 cells were sorted and resuspended in QIAsol reagent (QIAGEN). Total RNA was extracted with the miRNeasy Mini Kit (QIAGEN). mRNA was selected using the NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs). RNA-seq libraries were generated with the NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs) and NEBNext Multiplex Oligos for Illumina (New England Biolabs) according to the manufacturer’s instructions. The libraries were sequenced on a HiSeq 3000 with 50 cycles of single reads.

RNA-seq analysis. RNA-seq reads were mapped to the mouse genome (build mm10) with Bowtie v1.1.1 (ref. 15). Peaks were identified using model-based analysis for ChIP-seq (MACS v1.4.2; default P value threshold of 1 ×10−5) (ref. 16). Only peaks identified in both biological replicates were selected for downstream analysis. Peak annotation was performed with the Hypergeometric Optimization of Enrichment Peaks (HOMER) program v.4.9 (ref. 17). Peaks were visualized in the Integrative Genomics Viewer. To determine differentially modified H3K27ac peaks between different CD8+ subsets, reads per peak were calculated by HOMER. Statistically differentially modified peaks were then determined using edgeR v2.0.9, defined as those with an FDR (Benjamini–Hochberg) < 0.1 and a fold change > 1.5.

scRNA-seq. The scRNA-seq libraries were generated using Chromium Single Cell 3’ Library & Gel Bead Kit v2 (10x Genomics) according to the manufacturer’s protocol. Briefly, 1–1.5 × 104 live cells were FACs-sorted and used to generate single-cell gel-beads in emulsion. After reverse transcription, gel-beads in emulsion were disrupted. Barcoded complementary DNA was isolated and amplified by PCR (12 cycles). Following fragmentation, end repair and A-tailing, sample indexes were assigned and indexed PCR (8 cycles). The purified libraries were sequenced on a HiSeq 3000 (Illumina) with 26 cycles of read 1, 8 cycles of read 2 and 98 cycles of read 2.

FACS-based in vitro killing assay. EL4 cells were labeled with the CellTrace Violet Proliferation Kit (Thermo Fisher Scientific), pulsed with GP33 peptide at 1 μg ml−1 for 4 h at 37°C and used as target cells. In vitro activated control or TOX-overexpressing CD8+ T cells were added and incubated with peptide-pulsed EL4 cells at different ratios for 4 h at 37°C. The percentages of dead EL4 cells were measured with the LIVE/DEAD Fixable Aqua Dead Cell Stain Kit.

Statistical analysis. Statistical analyses were conducted with R v3.4.0 and R Core Team (2017). Unless otherwise stated, a two-tailed paired or unpaired Student’s t-test was used to determine statistical significance (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001). The mean and s.d. are presented in the figures. The error bars represent the s.d.

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

Data availability
All data generated during this study are available within the paper. The RNA-seq, ChIP-seq and scRNA-seq data have been deposited with the Gene Expression Omnibus (GEO), accession no. GSE119943. The TOX, NFAT1, IRF4, RUNX3, FOXO1, STAT3 and STAT5B ChIP-seq data are publicly available (GEO accession nos. GSE39953, GSE664407, GSE49938, GSE46943, GSE50128 and GSE102317).

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Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
  - Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
  - Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
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- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

BD FACSDIVA™ for flow cytometry, HCS 3.4.0 for HiSeq3000 system.

Data analysis

Flowjo9.9 was used for analyzing flow cytometric data. GraphPad Prism 6 and R3.4.0 were used for statistical test unless otherwise indicated. Cell Ranger v2.1.0, Seurat 2, AUCell, and Monocle 2 were used for analyzing single-cell RNA-seq data. Topological overlap matrix, hierarchical clustering, and gene modules were generated by Weighted Correlation Network Analysis (WGCNA). Bowtie v1.1.1 was used to map CHIP-seq reads to mouse genome. Peaks were identified by MACS v1.4.2. Peak annotation was performed with the Hypergeometric Optimization of Motif EnRichment program (HOMER) v4.9. Peaks were visualized in Integrative Genomics Viewer. edgeR v3.20.9 was used to identify differentially modified peaks. RNA-seq reads were mapped using TopHat v2.1.0. ANOVA analyses were performed with Partek Genomics Suite v6.6. RNA-seq reads were pseudo-aligned to ENSEMBL transcript sequences with kallisto v0.44.0. Metascape and clusterProfiler v3.8.1 were used for identifying enriched pathways. Details of data analyses were described in the Methods section.

For manuscripts utilizing custom algorithms or software that are not central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. Github). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

All data generated during this study are available within the paper. The RNA-seq (Fig. 4, 5 and Supplementary Fig. 2, 3, 5), CHIP-seq (Fig. 4 and Supplementary Fig. 4), and scRNA-seq (Fig. 1-3, 6 and Supplementary Fig. 1-4) data generated in this study have been deposited in the Gene Expression Omnibus (GEO accession...
Field-specific reporting

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

No statistical method was used to predetermine sample size. For infection experiments, more than 4 mice per group were used. Based on our previous studies and/or pilot experiments, these sample sizes allow for statistically valid comparisons. For single-cell RNA-seq experiments, more than 2000 cells were collected from each sample. For bulk RNA-seq experiments, 2-3 replicates were used for each group and each replicate was collected from a pool of more than 3 mice. For ChIP-seq experiments, two replicates were used for each group and each replicate was collected from a pool of more than 5 mice.

Data exclusions

No data was excluded from analysis.

Replication

Biological replicates were used to ensure the reproducibility of this study. All experiments were performed independently 2-3 times, and all results described in the study could be reproduced. When representative data were shown, the experimental findings were reproduced independently with similar results. The reproducibility of single-cell RNA-seq and bulk RNA-seq data were evaluated in supplementary Fig. 2a, b. All replicates in the scRNA-seq, bulk RNA-seq, and ChIP-seq experiments were collected from different cohorts of mice in separate experiments. For infection experiments, the number of replicates (n) equal the number of mice per group. For in vitro experiment, the number of replicates (n) equal the number of separate culture wells per group.

Randomization

Mice were sex- and age- matched to control for covariates. WT recipient mice were randomly assigned to different groups in adoptive transfer experiments. In the overexpression experiments, control- and overexpression- construct transduced cells were from the same donor. WT and KO mice were littermates.

Blinding

Experiments were not performed in a blinded manner, because subjective measurement was not involved. We chose objective readouts as measurements of our experiments. Therefore, the data were not prone to subjective evaluation.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ☒   | Antibodies            |
| ☒   | Eukaryotic cell lines |
|     | Palaeontology         |
|     | Animals and other organisms |
|     | Human research participants |
|     | Clinical data         |

Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☒   | ChIP-seq              |
|     | Flow cytometry        |
|     | MRI-based neuroimaging |

Antibodies

The following monoclonal antibodies, tetramers and dyes were used for flow cytometry experiments:

- anti-CD8a-PerCP-Cyanine5.5 (Catalog#45-0081-82, Lot#1941169, Clone#53-6.7, Dilution 1:100),
- anti-CD45.1-APC (Catalog#17-0453-82, Lot#4395672, Clone#A20, Dilution 1:100),
- anti-CD45.2-BV605 (Catalog#1098641, Lot#2130658, Clone#104, Dilution 1:100),
- anti-B220-eFl780 (Catalog#47-0452-82, Lot#1936932, Clone#RA3-622, Dilution 1:100),
- anti-Tim3-PE/Cy7 (Catalog# 25-5870-82, Lot#4342910, Clone#RMT3-23, Dilution 1:50),
- anti-TOX-PE/Catalog#12-6502-82, Lot#4272023, Clone#NXRX10, Dilution 1:100),
- anti-TIGIT-PE/Cy7 (Catalog#25-9501-82, Lot#4302469, Clone#G1G7, Dilution 1:20),
- anti-IFNg PE/Cy7 (Catalog#25-7311-82, Lot#07672-1634, Clone#XMG1.2, Dilution 1:100),
- and LIVE/DEAD™ Fixable Aqua (Catalog#L34966, Dilution 1:100) werefrom Thermo Fisher Scientific.
- anti-CD45.1-Bright Violet 421 (Catalog#110732, Lot#B739765, Clone#A20, Dilution 1:100),
- anti-CD45.2-BV605 (Catalog#109841, Lot#19411696259214, Clone#104, Dilution 1:100),
anti-KLRG1-PE [Catalog#138408, Lot#B177871, Clone#2F1, Dilution 1:100],
anti-PD1-BV421 [Catalog#109121, Lot#B255055, Clone#RMPI-30, Dilution 1:100],
anti-ULRB4-PE [Catalog#144904, Lot#B252437, Clone#H1.1, Dilution 1:50],
and anti-CD44-BV421 [Catalog# 103040, Lot#B252144, Clone#WM7, Dilution 1:100] were from Biogend.
anti-pAKT(S473)-BV421 [Catalog#562599, Lot#T1998801, Clone#M85-61, Dilution 1:200],
anti-TNFα-APC [Catalog#506308, Lot#B255367, Clone#MPE-X727, Dilution 1:200],
anti-I-2-PE [Catalog#503808, Lot#B173935, Clone#IS6.5n4, Dilution 1:100],
anti-lygG-APC/Cy7 [Catalog#560500, Lot#B277987, Clone#IAB, Dilution 1:100],
anti-Ly108-PE [Catalog#561540, Lot#T1257999, Clone#L13G3, Dilution 1:100] were from BD Biosciences.
Anti-p53(S253/Y256) PE/Cy7 [Catalog#344115, Lot#2], Clone#D57.2.26, Dilution 1:200 was from Cell Signaling Technology.
H-2Db GP33-41 tetramer conjugated with APC was obtained from NIAID Tetramer Core Facility, and was used at 1:100 dilution.
TCF1 antibody [Catalog# 22035, Lot#7, Clone# C6309] was purchased from Cell Signaling Technology.
The following antibody was used for ChiP-seq: Anti-Histone H3 acetyl K27 antibody was purchased from Abcam [Catalog# ab4729, Lot# GR211895-1].

Validation
All antibodies used in this study are commercially available. All antibodies have been validated by previous studies from other groups and our laboratory. Representative flow panels were shown in Fig 6a,6d, 6i, 6j, 7a, 7g and supplementary Fig 1a, 3f, 3g, 3i, 4i, 5c), 6a, 6c.

Eukaryotic cell lines
Policy information about cell lines

Cell line source(s)  HEK 293T and EL4 cells were purchased from ATCC
Authentication  EL4 was validated by morphology. HEK 293T cells were validated by cell morphology and ability to produce retroviral particles.
Mycoplasma contamination  HEK293T Cells were used for packaging retroviruses. EL4 cells were used for in vitro killing assay. No mycoplasma contamination test was performed. Cells used for experiments had been passed less than 10 passages.
Commonly misidentified lines (See ICLAC register)  Not listed in ICLAC

Animals and other organisms
Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

Laboratory animals  C57BL/6 and WT CD45.1 (B6.SJL-Ptprca Pepcb/Bjy) were purchased from the Jackson Laboratory. P14 mice carry a transgenic TCR that recognizes H2Db GP33-41 epitope on LCMV. Blimp1-YFP reporter mice were purchased from the Jackson Laboratory, bred to P14 to generate P14; Blimp1-YFP mice. Tcx deficient mice were generated by deleting the first exon and promoter region. Toddylox/pox mice were provided by Jonathan Kaye and were crossed to CD4-Cre and P14. All mice were kept on a C57BL/6 background. Female and male mice between 8 and 16 weeks of age were used for experiments. Mice used in each experiment were age- and sex-matched.

Wild animals  No wild animal was used in this study.
Field-collected samples  No field-collected sample was used in this study.
Ethics oversight  All animal husbandry and experiments were approved by the National Human Genome Research Institute (NHGRI) or the National Institute of Neurological Disorders and Stroke (NINDS) Animal Care and Use Committees (protocols G98-3 and 1295-12).

Note that full information on the approval of the study protocol must also be provided in the manuscript.

ChiP-seq
Data deposition
☐ Confirm that both raw and final processed data have been deposited in a public database such as GEO.
☐ Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

Data access links
May remain private before publication.  https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE119943
Secure token (auxnweqvumhup)

Files in database submission  Fastq files are available for the following samples:
- Chipseq_H3K27Ac_P14_Arm_D7_ShortLivedEffector_1
- Chipseq_H3K27Ac_P14_Arm_D7_ShortLivedEffector_2
- Chipseq_H3K27Ac_P14_Arm_D7_MemoryPrecursor_1
- Chipseq_H3K27Ac_P14_Arm_D7_MemoryPrecursor_2
- Chipseq_H3K27Ac_P14_CI13_D7_TerminallyExhausted_1
- Chipseq_H3K27Ac_P14_CI13_D7_TerminallyExhausted_2
- Chipseq_H3K27Ac_P14_CI13_D7_ProgenitorLike_1
Flow Cytometry

Plots

Confirm that:

☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
☒ All plots are contour plots with outliers or pseudocolor plots.
☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation: Cells from spleen were obtained by mechanical disruption. After red blood cell lysis, splenocytes were passaged through a cell...
| Sample preparation | Strainer to obtain single-cell suspension. Isolated cells were stained with antibodies and/or tetramers, as described in the Methods section. |
|-------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Instrument        | BD LSRII and BD FACSAria IIIu cell sorter.                                                                                                                                                        |
| Software          | BD FACSDIVA™ was used for data acquisition. FlowJo 9.9 software (TreeStar) was used to analyze flow cytometry data.                                                                             |
| Cell population abundance | Post-sort fractions were > 98% pure, verified through flow cytometry analysis on the sorter used for FACS.                                                                                           |
| Gating strategy   | Initial gating strategy: Dead cells positive for LIVE/DEAD Fixable Aqua were excluded. FSC-A/SSC-A was used to gate on live lymphocytes. Doublets were excluded through FSC-A/FSC-W and SSC-A/SSC-W. CD8 T cells were gated as CD8a positive and B220/Ly6G negative. These were followed by gating strategy described in the figures and figure legends. |

- Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.