A unique epigenomic landscape defines CD8+ tissue-resident memory T cells

Frank A. Buquicchio1,2,7, Raissa Fonseca3,7, Julia A. Belk1,4, Maximilien Evrard3, Andreas Obers3, Yanyan Qi1, Bence Daniel1, Kathryn E. Yost1, Ansuman T. Satpathy1,2,5,6,8,## and Laura K. Mackay3,8,##*

1Department of Pathology, Stanford University, Stanford, CA, USA. 2Program in Immunology, Stanford University, Stanford, CA, USA. 3Department of Microbiology and Immunology, The University of Melbourne at The Peter Doherty Institute for Infection and Immunity, Melbourne, VIC, Australia. 4Department of Computer Science, Stanford University, Stanford, CA, USA. 5Parker Institute for Cancer Immunotherapy, Stanford University, Stanford, CA, USA. 6Gladstone-UCSF Institute of Genomic Immunology, San Francisco, CA, USA. 7These authors contributed equally to this work. 8These authors jointly directed this work. #Co-corresponding authors

*Correspondence should be addressed to L.K.M. (lkmackay@unimelb.edu.au) or A.T.S. (satpathy@stanford.edu)
Graphical Abstract

**scATACseq**

- **T<sub>RM</sub> cells are an epigenetically unique T cell subset**
- **Divergent epigenetic trajectories of memory T cell subsets**
- **F<sub>CyRiIB</sub> identifies precursor cells enriched for T<sub>CIRC</sub> fate**

- **Peaks Shared Liver T<sub>RM</sub> Skin T<sub>RM</sub>**
  - Increased Accessibility **CISH, Gpr55**
  - Decreased Accessibility **Klf2, St1pr1**

- **T<sub>RM</sub> cells share a core epigenetic signature**
- **Microenvironment shapes epigenome and site-specific T<sub>RM</sub> development**
- **Distinct epigenetic features of T<sub>RM</sub> and T<sub>EX</sub> cells**

### Highlights

- scATAC atlas reveals the epigenetic variance of memory CD8<sup>+</sup> T cell subsets over the course of acute infection
- Early bifurcation of memory precursors leads to circulating versus tissue-resident cell fates
- Integrating transcriptional and epigenetic analyses identified organ-specific T<sub>RM</sub> cell regulators including HIC1 and BACH2
- Epigenetic distinction of T<sub>RM</sub> cells and T<sub>EX</sub> cell subsets
Summary

Memory T cells provide rapid and long-term protection against infection and tumors. The memory CD8+ T cell repertoire contains phenotypically and transcriptionally heterogeneous subsets with specialized functions and recirculation patterns. While these T cell populations have been well characterized in terms of differentiation potential and function, the epigenetic changes underlying memory T cell fate determination and tissue-residency remain largely unexplored. Here, we examined the single-cell chromatin landscape of CD8+ T cells over the course of acute viral infection. We reveal an early bifurcation of memory precursors displaying distinct chromatin accessibility and define epigenetic trajectories that lead to a circulating (T\textsubscript{CIRC}) or tissue-resident memory T (T\textsubscript{RM}) cell fate. While T\textsubscript{RM} cells displayed a conserved epigenetic signature across organs, we demonstrate that these cells exhibit tissue-specific signatures and identify transcription factors that regulate T\textsubscript{RM} cell populations in a site-specific manner. Moreover, we demonstrate that T\textsubscript{RM} cells and exhausted T (T\textsubscript{EX}) cells are distinct epigenetic lineages that are distinguishable early in their differentiation. Together, these findings show that T\textsubscript{RM} cell development is accompanied by dynamic alterations in chromatin accessibility that direct a unique transcriptional program resulting in a tissue-adapted and functionally distinct T cell state.

Key words
Tissue-resident memory T cells, peripheral immunity, single-cell genomics, epigenetic analysis
Introduction

CD8+ T cells are key mediators of protective immunity against infectious diseases and tumors. Following antigen encounter, activated T cells can infiltrate sites of infection where they mediate pathogen control. Disease resolution is followed by the generation of heterogeneous memory T cells with specialized functions and recirculation patterns. Whereas some memory T cells circulate throughout the blood and lymphatics (including central (T_{CM}) and effector (T_{EM}) memory T cells), others are permanently stationed in peripheral organs. These non-migratory tissue-resident memory T (T_{RM}) cells have been identified in virtually all tissues across species, and are critical for infection and cancer control (Masopust and Soerens, 2019; Okla et al., 2021).

Over recent years, high-throughput sequencing technologies have been used to define transcriptional and epigenetic changes associated with T cell differentiation in various settings (Joshi et al., 2007; Kallies et al., 2009; Mackay et al., 2016; Milner et al., 2017, 2020; Pauken et al., 2016; Roychoudhuri et al., 2016; Sen et al., 2016; Skon et al., 2013; Utzschneider et al., 2020; Zhou et al., 2010). Gene expression analyses and protein profiling have demonstrated that variations in tissue microenvironments, cytokine exposure, antigen persistence, and TCR signal strength poise T cells at different stages and shape the effector response, culminating in the development of heterogeneous circulating memory (T_{CIRC}) and T_{RM} cell subsets (Beura et al., 2018; Christo et al., 2021; Joshi et al., 2007; Kumar et al., 2017; Mackay et al., 2016; Masopust et al., 2010; Sarkar et al., 2008; Solouki et al., 2020; Wakim et al., 2010). These populations of memory T cells display distinct stemness and functional abilities, and together provide optimal immune protection and long-term immunity against pathogens and tumors (Behr et al., 2020; Christo et al., 2021; Fonseca et al., 2020; Jameson and Masopust, 2009; Kaech and Cui, 2012; Park et al., 2019; Sallusto et al., 1999). In certain contexts, such as chronic infections or persistent tumors, continuous T cell stimulation prompts terminal differentiation leading to the development of exhausted T (T_{EX}) cells that display increased expression of inhibitory receptors and reduced functional ability as compared to memory T cell populations (Im et al., 2016; Mackay et al., 2012a; McLane et al., 2019; Miller et al., 2019; Paley et al., 2012).
Accordingly, dynamic genome-wide changes in DNA methylation and chromatin accessibility observed during T\textsubscript{CIRC} development has demonstrated the complex determination of effector and memory T cell fates in both mice and humans (Akondy et al., 2017; Araki et al., 2009; Scharer et al., 2013). Most recently, epigenomic profiling of single cells has demonstrated changes in cis- and trans-regulatory elements associated with regulation of gene expression in individual cell types, allowing for reconstruction of trajectories associated with cellular differentiation (Lareau et al., 2019; Satpathy et al., 2019). While it is known that heterogeneous T\textsubscript{CIRC}, T\textsubscript{EX} and T\textsubscript{RM} cells exist and rely on distinct transcriptional circuitries, the epigenomic changes steering their ontogeny, as well as when their differentiation trajectories diverge is not known. Moreover, our understanding of the collective epigenetic variation of T\textsubscript{RM} cells across different tissues remains unexplored.

Here, we used the single-cell assay for transposase accessible chromatin with sequencing (scATAC-seq) to examine the dynamic genome-scale changes in chromatin accessibility that occur in CD8\textsuperscript{+} T cells over the course of viral infection. We found subset-specific variations in the epigenetic landscape of memory T cells that are generated in response to acute LCMV infection, with differences in cis-regulatory element accessibility being established early post-infection in memory precursor populations. Increased Fcgr2b locus accessibility reflected the dynamics of Fc\gammaRIIB expression in T cells \textit{in vivo}, allowing the selection of memory precursors with enhanced capacity to generate either T\textsubscript{CIRC} or T\textsubscript{RM} cells in the liver. Whereas T\textsubscript{RM} cells were epigenetically distinct from their circulating counterparts and exhibited conserved features between tissues, our analyses also revealed transcription factors that regulate T\textsubscript{RM} cell formation in a tissue-specific manner. Moreover, despite confirming considerable phenotypic similarities between T\textsubscript{RM} and T\textsubscript{EX} cells, we demonstrated that T\textsubscript{RM} cells display a distinct chromatin landscape that share relatively few features with T\textsubscript{EX} cell subsets. Together, our data indicate that distinct epigenetic landscapes accompany memory T cell differentiation and form the basis of the transcriptional and functional differences associated with unique T cell ontogenies. Additionally, we provide a \url{public genome browser} for interrogating the chromatin accessibility profiles of effector and memory CD8\textsuperscript{+} T cell populations, which will facilitate future investigations.
Results

**T<sub>RM</sub> cells are an epigenetically distinct subset of memory T cells**

To investigate the epigenetic landscape of CD8<sup>+</sup> memory T cell populations, we first utilized a model of acute LCMV infection (Armstrong strain) in combination with adoptive transfer of TCR transgenic CD8<sup>+</sup> T cells. To this end, we transferred naïve congenically labelled CD45.1<sup>+</sup> P14 cells specific for LCMVgp33-41 into C57BL/6 mice that were infected with LCMV to generate antigen-specific memory CD8<sup>+</sup> T cells across organs. At 30 d post infection (p.i.), T<sub>EM</sub> and T<sub>CM</sub> P14 cells were isolated from the spleen, and T<sub>EM</sub>, T<sub>CM</sub> and T<sub>RM</sub> P14 cells were isolated from the liver, alongside P14 cells from naive animals (Figure S1A). Naïve and memory P14 cell populations were then subjected to scATAC-seq (Satpathy et al., 2019) (Figure 1A). In total, 15,740 single cells passed quality control filters of at least 1,000 unique fragments per cell and a transcription start site (TSS) enrichment score greater than or equal to 5 (Figure S1B). To analyse memory T cell epigenetic profiles, we utilised ArchR (Granja et al., 2021): (1) for dimensionality reduction using Latent Sematic Indexing (LSI), Uniform Manifold Approximation and Projection (UMAP) embedding and cell clustering(Becht et al., 2019; Cusanovich et al., 2015); (2) to analyze changes in accessibility of individual regions in the genome (‘peaks’); (3) to calculate deviation in accessibility for transcription factor (TF) motifs; (4) to visualize aligned ATAC-seq reads; (5) to predict ‘marker peaks/genes’ or changes in accessibility that are specific to a given cluster; (6) to model differential gene expression using gene activity score (‘gene score’) and (7) to perform pseudo-time differentiation trajectory analysis to model epigenetic changes that occur over the course of a projected differentiation trajectory (Figure 1B).

T cell visualization by UMAP revealed four major clusters (Figure 1C). These projected clusters nearly exclusively aligned with sorted T cell subsets, suggesting that scATAC-seq accurately identifies the epigenetic heterogeneity within CD8<sup>+</sup> memory T cells (Figure S1C). Thus, cluster identity was assigned according to the sorted T cell population represented in each cluster (naïve T cells (C1), T<sub>EM</sub> (C2), T<sub>RM</sub> (C3) and T<sub>CM</sub> (C4) cells). Whereas our analyses revealed comparatively little distinction between the chromatin state of T<sub>CM</sub> and T<sub>EM</sub> cells regardless of tissue origin, T<sub>RM</sub> cells derived from the liver comprised a discrete cluster, positioned separately


from the T\textsubscript{CIRC} cell populations (Figure 1C). To identify epigenetic differences between T\textsubscript{CIRC} and T\textsubscript{RM} cells, we compared each memory T cell cluster to naïve T cells to identify differences in peak accessibility and compared these peak sets across the three memory clusters. This analysis showed that each cluster exhibited unique peak accessibility changes that were not observed in the other respective clusters, as well as a peak set that was shared across memory T cells. Importantly, the number of peaks exclusive to the T\textsubscript{RM} cell cluster (2,424 increased, 4,007 decreased) was similar in magnitude to the number of T\textsubscript{CM} (2,213, 1,276) and T\textsubscript{EM} (2,491, 7,552) cell-exclusive peaks, further confirming that T\textsubscript{RM} cells are an epigenetically distinct subset of memory T cells, while T\textsubscript{CM} and T\textsubscript{EM} cells shared proportionally higher number of peaks (2,120, 2,416), supporting the increased epigenetic similarity observed between these clusters (Figure 1D).

To link differentially accessible ATAC-seq peaks to genes, we examined gene-level accessibility changes, both across the gene body and in linked distal sites, by gene score (Granja et al., 2021; Pliner et al., 2018). Direct comparison of gene scores between T\textsubscript{RM} and T\textsubscript{CM} and/or T\textsubscript{EM} cell clusters revealed the expected differential accessibility in peaks corresponding to Klf2, Ccr7, Sell, S1pr5, Zeb2 and Cx3cr1, genes all known to be downregulated in T\textsubscript{RM} cells as compared to circulating populations (Figure 1E). Importantly, the downregulation of KLF2 and its target S1PR1, as well as ZEB2 and S1PR5, are known to be critical to halt tissue egress in order for T\textsubscript{RM} cells to develop in organs (Evrard et al., 2022; Mackay et al., 2013; Skon et al., 2013). Our data also revealed the differential accessibility of genes with no known role in liver T\textsubscript{RM} cell formation such as Fcgr2b and Hic1, as well as increased accessibility of adhesion-related genes such as Chn2, Cdh1, Itga9, and Gpr55, a G-protein coupled receptor known to regulate intraepithelial lymphocyte (IEL) migration (Sumida et al., 2017) in T\textsubscript{RM} cells (Figure 1E and 1F). Diminished gene scores of Ccr7 and Sell were also observed in T\textsubscript{EM} relative to T\textsubscript{CM} clusters (Sell; L2fc = -.327, FDR = 2.45. Ccr7; L2fc = -.820, FDR = 24.20) as well as increased accessibility in S1pr5 and Zeb2 loci in T\textsubscript{EM} cells (Figure S1D and S1E) in line with the anticipated expression pattern of these molecules.

Reduced accessibility at the Klf2 and S1pr1 loci in T\textsubscript{RM} cells was accompanied by decreased KLF2 motif accessibility (Figure 1G and 1H). Conversely, while the Hic1 locus displayed increased accessibility in T\textsubscript{RM} cells, the HIC1 motif was significantly less accessible in this population,
consistent with its role as a transcriptional repressor (Pinte et al., 2004) (Figure 1F and 1H). Similarly, the transcriptional repressor BLIMP-1, encoded by Prdm1, displayed decreased motif accessibility in the TEM cell subset and increased Prdm1 gene score (Figure S1F-S1H), supporting previous findings that demonstrated the expression and major role for this TF in TEM development by promoting CD8+ T cell proliferative response and differentiation (Rutishauser et al., 2009). Moreover, while reduced expression of BLIMP-1 is observed in TRM cells, BLIMP-1 and HOBIT deficiency was shown to be detrimental for LCMV-specific TRM cell formation in the liver (Mackay et al., 2016). Together, our data demonstrate that TRM cells are an epigenetically distinct memory T cell subset and the ability of scATAC-seq to identify unique features of memory T cell subsets at the chromatin level.

**Memory T cell subsets display distinct epigenetic trajectories**

Recent evidence suggests that TRM cell fate is determined early during infection (Kok et al., 2020, 2021; Kurd et al., 2020; Mani et al., 2019; Milner et al., 2017). We sought to understand the progression of epigenetic changes during T cell differentiation and to determine whether progenitors that preferentially give rise to either TCIRC or TRM cell populations could be identified. For this, we focused our analyses on the liver, an organ comprising TRM, TEM and TCM cell population (Fernandez-Ruiz et al., 2016), and sorted P14 cells at 7, 14, and 30 d following LCMV infection, alongside TEM and TCM P14 cells from the spleen. At 14 d p.i., we sorted both CD69- and CD69+ cells from the liver to more finely define the CD69+ TRM cell-poised population (Figure S2A). As above, samples were subjected to scATAC-seq, analyzed, and visualized by UMAP alongside naïve P14 cells from non-infected mice (Figure 2A).

We found that early effector P14 cells isolated at 7 d p.i. were highly heterogenous and fell into three distinct clusters (C2, C3, C4). Whereas C2 was comprised almost solely of 7 d p.i. cells and denoted as effector T cells (TEFF) (by abundance at 7 d p.i. and by loss of accessibility at the Il7r and Il2 loci), other cells isolated from this timepoint clustered together with cells isolated at 14 d p.i. in C3 or C4 (Figure 2A and S2B) indicating the early divergence of TRM and TCIRC precursors following infection. We denoted C3 and C4 as memory precursor cells (TMP); CD69+ cells isolated at 14 d p.i. were nearly exclusively contained within C3 (TMP-1), indicating close relation to the
TRM-exclusive clusters, whereas CD69+ samples were distributed between C3 and C4 (TMP-1 and TMP-2, respectively; Figure S2B). We identified genes with cluster-specific accessibility increases in potential cis-regulatory elements, including Zeb2 in TEM cells, Ccr7 in TCM cells, and Fasl and P2rx7 in TRM cells (Figure S2B). Accordingly, correlation analysis of these marker peaks supported the epigenetic similarity of TMP-1 and TRM cells indicating an association between these populations (Figure 2B). Moreover, TMP-1 exhibited a higher gene score in the TRM cell-associated gene Cxcr6 (Fernandez-Ruiz et al., 2016; Wein et al., 2019) and decreased gene score in Slpr1 when compared to TMP-2, further suggesting that the TMP-1 cluster precedes TRM cell differentiation (Figure 2C).

Next, we constructed differentiation trajectories using the T_EFF cluster as a starting point to computationally order T cell clusters along a pseudotime axis. Here, single cells are aligned to the trajectory by calculating the nearest cell-to-trajectory distance, which allows cells to be ordered by their determined position in pseudotime (Granja et al., 2021). Our analysis thus far suggested that TMP-1 (C3) preceded TRM cell development, whereas TMP-2 (C4) bore closer similarity to T_CIRC (TEM and TCM) cell clusters. As such, our pseudotime trajectories navigated through these TMP clusters, from T_EFF → TMP-1 → TRM, or from T_EFF → TMP-2 → TCM and TEM cell populations (Figure 2D).

We observed several expected changes along these developmental trajectories, including a progressive loss in Slpr1 gene score along the T_EFF → TRM trajectory, coupled with increased Slpr1 gene score from T_EFF → TCM and T_EFF → TEM (Figure 2E). Further, Cxcr6 and Sell followed anticipated trends, with Cxcr6 gene score increasing in the T_EFF → TRM trajectory, while remaining relatively consistent in T_EFF → TEM. Sell accessibility decreased from T_EFF → TRM but remained accessible in the T_EFF → TCM trajectory (Figure 2C and 2D). Gene accessibility changes in Sell and Cxcr6 were reflective of protein expression changes observed by flow cytometry at the same time points (Figure S2E). Trajectory analysis demonstrated a decrease in the Tbx21 (encoding T-BET) gene score along the T_EFF → TRM trajectory (Figure 2F), aligning with previous work showing T-BET downregulation during TRM cell development (Laidlaw et al., 2014; Mackay et al., 2015). Additionally, we found an increase in the TCF7 motif accessibility along the T_EFF → TCM trajectory, corroborating the known role of this TF in promoting TCM differentiation (Gattinoni et al., 2009; Jeannet et al., 2010) (Figure 2G).
Global changes in gene score and motif accessibility across pseudotime from T\textsc{eff} to either T\textsc{cm} or T\textsc{rm} cells revealed several patterns between these developmental pathways. In the T\textsc{eff} → T\textsc{mp-2} → T\textsc{cm} trajectory, the accessibility of Id3, Tcf7, Lef1, and Sell progressively increased along this path, together with increased motif accessibility in differentiated T\textsc{cm} as anticipated (Figure 2H).

In the T\textsc{eff} → T\textsc{mp-1} → T\textsc{rm} trajectory, Cx3cr1 and KlrG1 accessibility was lost early in the T\textsc{eff} → T\textsc{mp-1} transition, consistent with evidence showing the inability of CX3CR1\textsuperscript{+}KLRG1\textsuperscript{+} effector T cells to give rise to T\textsc{rm} cells (Gerlach et al., 2016; Herndl-Brandstetter et al., 2018) (Figure 2I).

Similarly, KLF2 motif accessibility was lost along the T\textsc{rm} cell transition whereas BHLHE40 motif accessibility was gained during the T\textsc{mp-1} to T\textsc{rm} cell transition (Figure 2I), consistent with the observed role of BHLHE40 in this subset (Li et al., 2019). Consistent with changes observed in cells isolated from the liver at day 30 post infection, an increase in Hic1 gene score was observed in T\textsc{rm} cells over pseudotime (Figure 2J), followed by a reduction in motif accessibility of HIC1 sites in T\textsc{rm} cells, with opposite trends in T\textsc{eff} → T\textsc{cm} (Figure 2K), suggesting that HIC1 represses target gene accessibility early in T\textsc{rm} cell differentiation.

While HIC1 is known to regulate the IEL population in the small intestine (Burrows et al., 2017), whether this transcription factor is required for liver T\textsc{rm} cell development is not known. To investigate the functional relevance of HIC1 in liver T\textsc{rm} cell development, we used CRISPR-Cas9 to ablate HIC1 in P14 cells that were subsequently transferred into LCMV infected recipients (Figure 2L). Importantly, we found that the genetic deletion of HIC1 resulted in significant reduction of CD69\textsuperscript{+} T cells in the liver as early as 9d p.i. (Figure S2F-S2H). At 30d p.i., HIC1-deficient liver T\textsc{rm} cells were further depleted, with decreased effect of HIC deletion on T\textsc{circ} populations (Figure 2M-2N and S2I), suggesting that HIC1 is a critical regulator of T\textsc{rm} cell differentiation. Together, these data support previous findings (Kok et al., 2020; Kurd et al., 2020; Milner et al., 2020) and reinforce the notion that T\textsc{rm} cell fate may sealed early post T cell activation (Kok et al., 2021).

FcγRIIB expression identifies precursors enriched for the T\textsc{circ} cell fate
Our trajectory analyses indicated that a subset of T cells may be poised for T_{RM} cell differentiation at early stages following infection. To identify surface markers that would allow for the isolation of putative T_{RM} cell precursors, we investigated genes encoding cell surface markers with differential accessibility in T_{MP-1} relative to T_{MP-2} cell clusters (Figure 3A). To account for potential heterogeneity within the T_{MP} clusters, we also accounted for whether these genes were also differentially accessible in fully differentiated T_{RM} cells relative to T_{CIRC} cells. Among the genes observed, Cx3cr1 and S1pr5 showed increased gene scores in T_{MP-2} and T_{CIRC} relative to T_{MP-1} and T_{RM} cells, indicating the presence of an effector population poised for T_{CIRC} cell differentiation (Figure 3B). We also observed significantly decreased accessibility of Fcgr2b (FcγRIIB) in both T_{MP-2} and T_{RM} cells relative to T_{MP-1} and T_{CIRC} cells (Figure 3B and S3A). FcγRIIB is a low-affinity Fc receptor known to act as an inhibitory receptor in CD8^+ T cells and promote apoptosis (Morris et al., 2020; Starbeck-Miller et al., 2014), although its role in T_{RM} cell differentiation has not been explored.

Given the concomitant lack of Fcgr2b accessibility in T_{MP-2} and T_{RM} cells, we hypothesized that we may be able to identify T_{RM}-poised precursors based on the surface expression of this marker. To observe FcγRIIB expression dynamics in vivo, we transferred P14 cells into LCMV infected mice and analysed FcγRIIB expression on P14 cells at various times p.i. Notably, the dynamics of Fcgr2b gene score in our predicted pseudotime trajectories closely reflected FcγRIIB expression by flow cytometry in the putative T_{RM}, T_{CM}, and T_{EM} cell populations over the course of infection (Figure 3C and 3D). Additionally, the comparison of FcγRIIB expression in memory T cell populations demonstrated increased expression in T_{CIRC} cells over time, with highest expression detected in the liver T_{EM} cell subset (Figure S3B and S3C).

We next asked whether the lack of FcγRIIB expression marked a population of precursor cells that might preferentially give rise to T_{RM} cells. For this, P14 cells were isolated from the spleen 7 d after LCMV infection and sort-purified populations of FcγRIIB^- or FcγRIIB^+ P14 cells were transferred into infection-matched recipients (Figure 3E). At 30 d post-transfer, we observed a global reduction of FcγRIIB^+ T cells (Figure 3F) consistent with their increased apoptotic potential (Morris et al., 2020), while FcγRIIB expression remained consistent as per the transferred population (Figure S3D). Interestingly, we found that the progeny of FcγRIIB^+ cells displayed an
increased proportion of T_{CM} and T_{EM} cells and an impaired conversion to the CD69^{-} T_{RM} cell phenotype (Figure 3G-3I). Accordingly, FcγRIIB\(^{+}\) showed an increased propensity to form T_{RM} cells at the detriment of either T_{CIRC} cell population in the liver. Together, these data indicate that differential FcγRIIB expression allows the identification of effector cells that appear to be differentially poised to navigate distinct memory T cell trajectories.

T_{RM} cells share a core epigenetic signature across tissues

Our data thus far demonstrates that liver T_{RM} cells display a distinct epigenetic signature compared to their circulating counterparts derived from both the liver and spleen. Despite considerable variation of T_{RM} cells between organs, these cells exhibit a core transcriptional signature that is shared across tissues and species (Kumar et al., 2017; Mackay et al., 2016; Milner et al., 2017). Therefore, we sought to understand the extent of epigenetic similarity between CD8\(^{+}\) T_{RM} cells in different tissues and for this we compared liver T_{RM} cells to those derived from the skin. These populations represent extremes in T_{RM} cell-associated phenotypic and transcriptional variation, as driven by differential responsiveness to the cytokine TGF-\(\beta\). Beyond upregulation of the integrin CD103, TGF-\(\beta\) governs a suite of transcriptional changes in the TGF-\(\beta\)-responsive skin T_{RM} cell population, in addition to restraining their functional capacity (Christo et al., 2021).

To define epigenetic features that are conserved between liver and skin T_{RM} cells, we utilized a model of HSV skin infection to induce a skin T_{RM} cell population. To this end, CD45.1\(^{+}\) gBT-I T cells specific for HSV gB\(^{498-505}\) were transferred into C57BL/6 mice that were infected with HSV on the skin flank. At 14 and 30 d post HSV infection, gBT-I T_{RM} cells were isolated from the skin as defined by the expression of CD69 and CD103, alongside circulating gBT-I T cells (T_{CIRC}) derived from the skin-draining lymph node (dLN) (Figure S4A). Sort-purified cells were subjected to scATAC-seq and data was subsequently integrated with the aforementioned LCMV T cell dataset comprising spleen naïve and memory T cell subsets, and liver effector and memory T cell subsets (Figure 4A). As expected, liver and skin T_{RM} cells clustered separately, reflective of the major differences in their tissue microenvironment and phenotype. Differences were also observed in the clustering of T_{CIRC} cell populations, with clusters being defined by the model of infection (Figure S4B). Heatmap visualization of gene scores highlighted genes that were uniquely
regulated in each cluster, as well as genes that were shared between skin (C2) and liver (C3) T<sub>RM</sub> cells, including Xcl1, Cdhl, Acrv1b, and Tnfsf11 (Rankl) (Figure 4B). As expected, skin T<sub>RM</sub> cells had increased accessibility in genes related to TGF-β signalling (Tgfbr2, Tgfbr3), skin homing and adhesion molecules (Ccr4, Ccr8, Itgae) and genes previously shown to be preferentially expressed in skin T<sub>RM</sub> cells (Cish, Litaf, Pdcd1, Haver2). The loss in accessibility in genes that antagonize T<sub>RM</sub> cell development (Klf2, S1pr1) could be observed within both T<sub>RM</sub> cell clusters, together with an increased gene score in Xcl1 and Chn2, genes identified as part of the core T<sub>RM</sub> gene signature that is shared across organs (Mackay et al., 2016) (Figure 4C and S4C-S4D).

We next sought to determine if T<sub>RM</sub> cells residing different organs had conserved accessibility in <i>cis</i>-regulatory elements. We identified significant changes in peaks relative to naïve T cells (C1) for all clusters and determined the extent to which sets of peaks were common across different clusters. Similar to the conservation of peaks in LCMV-induced memory subsets (Figure 1D), there was a strong conservation of <i>cis</i>-regulatory elements that exhibited changes in accessibility in all memory clusters relative to naïve (1,800 increased, 5,089 decreased) (Figure 4D). Across all peaks with significant changes in peak accessibility, 462 were increased and 1,082 decreased exclusively in both liver and skin T<sub>RM</sub> cells. Mapping those 1,544 peaks back to genes revealed the genes with the most numerous changes in <i>cis</i>-regulatory element accessibility; expectedly, Klf2 and S1pr1 exhibited the most losses in peaks accessibility in T<sub>RM</sub> cells. Among the genes with the most gains in <i>cis</i>-regulatory element accessibility were Gpr55, a G protein-coupled receptor that negatively regulates IEL T cell migration (Sumida et al., 2017) and Cish (Figure 4E and 4F), a negative regulator of TCR signalling (Palmer et al., 2015). Interestingly, we also observed conserved gains in peak accessibility at the Tgfbr1 locus in both skin and liver T<sub>RM</sub> cells (Figure 4E), which is intriguing given the opposing effect of TGFβ signalling on regulating T<sub>RM</sub> cell development in these tissues (Christo et al., 2021).

To further understand gene-level accessibility changes unique to T<sub>RM</sub> cells, we identified genes with significant changes in gene score relative to naïve T cells that did not appear in any other memory cluster (Figure S4E). In total, there were 64 genes with differential accessibility exclusively observed in both skin and liver T<sub>RM</sub> cells. The 41 genes with increased accessibility in T<sub>RM</sub> cells included the residency-associated chemokine Xcl1, cytokines Il22 and Tnfsf10, and genes
associated with cell adhesion (Cd93, Gpr55) or modulation of cytokine and TCR signalling (Cish, Socs2, Tnfsf9, Rgs1). We also analyzed whether T\(_{RM}\) cell-exclusive cis-regulatory elements shared transcription factor motifs that may control gene expression associated with changes in accessibility (Figure 4G). We observed a broad enrichment of motifs belonging to transcription factors from the bZIP family, with AP-1 motifs contributing to 30-40% of the 462 peaks with increased accessibility. In contrast, a reduction in KLF motifs was the most significantly enriched within the 1,062 peaks with decreased accessibility, in line with the role of KLF2 in antagonizing T\(_{RM}\) cell development (Skon et al., 2013). Together, these data indicate that a conserved epigenetic signature defines the T\(_{RM}\) cell population.

**Local microenvironment shapes the epigenome and promotes site-specific T\(_{RM}\) cell development**

While T\(_{RM}\) cells shared an epigenetic signature across organs, it is well known that T\(_{RM}\) cells in different tissues exhibit discordant phenotypes and are regulated by distinct molecular cues (Christo et al., 2021; Fonseca et al., 2020; Frizzell et al., 2020; Kumar et al., 2017). Using our scATAC-seq data, we sought to determine the changes in chromatin landscape between T\(_{RM}\) cells from different organs and identify unique transcriptional regulators that may account for such differences. First, to determine the extent to which the chromatin state of skin and liver T\(_{RM}\) cells diverge, we compared with differential peak accessibility and gene scores between skin and liver T\(_{RM}\) cells (relative to T\(_{CIRC}\)) (Figure S5A). In line with increased P2\(_{rx}\)7 mRNA levels (Mackay et al., 2016) (Figure S5B) and its known requirement for liver T\(_{RM}\) cell development (Stark et al., 2018), the P2\(_{rx}\)7 locus displayed increased accessibility in liver T\(_{RM}\) cells. In addition, we observed increased Ahr and Ccr8 gene scores in skin T\(_{RM}\) cells, with a similar trend observed at the mRNA level (Figure S5A and S5B), fitting with the known roles for AHR and CCR8 in skin T\(_{RM}\) cell development (McCully et al., 2018; Zaid et al., 2014). Accordingly, molecular signature analysis of enriched motifs (MSigDB) in skin versus liver T\(_{RM}\) cells indicated participation of TGF-β signalling in skin T\(_{RM}\) cells, while liver T\(_{RM}\) cell motifs displayed enrichment in pathways related to IFN signalling (Figure S5C), confirming previous findings on the dependency of these respective cytokines for T\(_{RM}\) cell formation (Christo et al., 2021; Hirai et al., 2020; Holz et al., 2020; Mackay et al., 2013).
Next, we sought to identify putative T_{RM} cell regulators by combining transcriptional data from GSE70813 and epigenetic data to reveal transcription factors with both increased RNA expression and motif accessibility in a tissue-specific manner (Figure 5A). Compared to liver T_{RM} cells, skin T_{RM} cells had increased expression and accessibility in AP-1 family members, including JUN, JUNB, JUND, FOS, FOSB, FOSL1, and FOSL2, suggesting that one or more of these factors may specifically influence skin T_{RM} cell development. Of note, these comparisons also uncovered certain motifs with increased accessibility in skin compared to liver T_{RM} cells, but did not exhibit observable differential gene expression between these two T_{RM} subsets, such as BACH2. Accordingly, gene expression analysis comparing T_{RM} and T_{CIRC} cell subsets demonstrated that skin T_{RM} cells showed increased gene expression for Fos, Fosb, Fosl1 and Fosl2, in addition to a small reduction in Bach2 expression when compared to other T cell subsets, including liver T_{RM} cells (Figure S5D). This indicated a potential role for these transcription factors in skin T_{RM} cells that is not reflected in transcriptional data. As a confirmatory approach, we generated the list of peaks with increased accessibility exclusively in skin T_{RM} cells when compared to liver T_{RM} cells (relative to respective T_{CIRC} cell populations) and used the HOMER motif analysis to determine motif enrichment within skin T_{RM}-exclusive peaks (Figure 5B). Here, AP-1 motifs were found in around 40% of peaks and the BACH2 motif was also amongst the top 10 enriched in the 2,663 peaks evaluated. When observed across memory T cell subsets, our data revealed that skin T_{RM} cells display the highest motif accessibility for FOS, FOSB, FOSL1, and BACH2 relative to all memory populations sequenced, indicating a putative role for these transcription factors in skin T_{RM} cell development (Figure 5C and S5E-S5F).

Based on these data, we hypothesized that FOS family members, specifically FOS, FOSB and FOSL1, may uniquely regulate skin T_{RM} cells, in addition to their established role in controlling TCR-induced genes and T cell expansion (Roychoudhuri et al., 2016). To test this, we used CRISPR-Cas9 to ablate either Fos, Fosb, Fosl1 or Fosl2 in effector P14 cells and then cells were co-transferred together with cells edited with a control guide, into LCMV-infected mice (Figure 5D). To induce skin T_{RM} cells following LCMV infection, mice were treated with 2,4-dinitrofluorobenzene (DNFB) on the skin as previously described (Frizzell et al., 2020). Ablation of Fosb led to a general decrease in memory P14 cell formation in comparison to respective controls 30 d p.i. (Figure S5G). To observe location-specific defects in memory T cell formation,
we compared the number of Fosb-deleted and control-edited cells in the skin and liver to normalized splenic cell numbers, and found a dominant defect in skin TRM cell formation (Figure 5E). Similarly, the deletion of Fos and Fosl1 (Figure 5F and 5G) revealed a loss of the skin TRM cells population, whereas the deletion of Fosl2 did not impact memory T cell formation (Figure S5H), consistent with the lack of changes in motif accessibility observed in TRM cells.

To next investigate the role of the transcriptional repressor BACH2 in skin TRM cell formation, we ablated this transcription factor in effector P14 cells via CRISPR-Cas9 and co-transferred edited cells and control cells into LCMV-infected DNFB-treated recipient mice (Figure 5H). Akin to our findings above, we observed a pronounced defect in BACH2-deleted T cells specifically in the skin, as compared to those isolated from the spleen or liver (Figure 5H and S5I). Together, this demonstrates the utility of integrating transcriptional and epigenetic analysis to identify major regulators of tissue-specific T cell development. Further, scATAC-seq enabled the identification of differential BACH2 activity where RNA-seq could not, highlighting the utility of scATAC-seq in nominating novel transcriptional regulators of cell state.

TRM and TEM cells are epigenetically distinct

A canonical feature of TRM cells is their elevated expression of inhibitory receptors as compared to circulating memory T cells. This is particularly striking for skin TRM cells, which share several phenotypic characteristics with TEM cells generated in response to chronic viral infection, presenting similar reduced capacity for cytokine production (Christo et al., 2021). To directly compare TEM and memory T cell subsets, we transferred congenically marked naïve CD8+ P14 cells into C57Bl/6 recipient mice infected with LCMV Armstrong or LCMV Clone-13 (Cl-13) infection, a widely used model for inducing CD8+ T cell exhaustion, or gBT-I cells after HSV infection (Figure 6A). Based on exhaustion and memory T cell markers (Figure S6A), UMAP visualization revealed four major clusters (Figure 6A), with skin gBT-I TRM cells exhibiting similar expression of the checkpoint molecules PD1 and TIM3 as splenic P14 cells isolated from mice infected with LCMV Clone-13 (Cl-13) (Figure 6B). In contrast, liver TRM cells generated in response to acute LCMV do not express PD1 or TIM3 to the same extent (Figure 6A and 6B). Further, tumor-infiltrating lymphocytes (TIL) have also been reported to have a TRM cell-like...
transcriptional profile (Djenidi et al., 2015; Malik et al., 2017; Milner et al., 2017; Nizard et al., 2017; Park et al., 2019; Savas et al., 2018). The seemingly convergent phenotypic profiles of TRM and TEx cells has led to speculation that TEx and TRM cell lineages are related (Blank et al., 2019). It is unclear however, the extent to which the chromatin state of TRM and TEx cells overlap, or when these subsets diverge during T cell differentiation.

To assess epigenetic differences between these subsets, we analyzed scATAC-seq data from gp33 tetramer+ CD8+ T cells from LCMV Cl-13 infected mice at 8 and 21 d p.i. from GSE188670 (Daniel et al., 2021) together with our scATAC-seq data of liver and skin TRM cells, as generated by LCMV Armstrong and HSV infections, respectively (Figure 6C). These integrated datasets revealed that TEx cells separated into 5 distinct clusters; cells isolated at 8 d p.i. were primarily classified as C4 or C5, and cells isolated at 21 d p.i. mostly inhabited C3, C10, and C11 (Figure S6B). To separate stem-like TEx cells, intermediate TEx cells, and terminal TEx cells (Raju et al., 2021), we used Haver2, Cx3cr1, Pdcd1, and Tcf7 gene scores, identifying C3, C11 and C10 respectively (Figure 6D). Notably, LCMV Cl-13-induced TEx cells clustered separately from cells isolated from LCMV Armstrong infected hosts at all stages of differentiation, highlighting distinct chromatin states for TRM and TEx cells (Figure 6E).

Next, we sought to compare gene and motif accessibility for transcription factors commonly associated with T cell exhaustion in TEx and TRM cells. We found that Tox gene scores in both skin and liver TRM cells were reduced in comparison to all TEx subsets (Figure 6F), supporting low Tox expression in TRM cells (Figure S6C). Additionally, similar to the expression at the protein level, increased Tcf7 gene score was observed in stem-like TEx cells and liver TRM cells (Figure 6G), consistent with the increased stemness observed in these populations that maintain increased differentiation capacity. Motif deviation for several TEx-associated transcription factors, TCF1, IRF4, and EOMES, were highest in TEx clusters (Figure S6D). RUNX3, however, exhibited the highest motif deviation in the skin TRM cell population, consistent with its required role for residency in several tissues (Milner and Goldrath, 2018) (Figure S6D).

To more broadly understand the shared epigenetic regulation of TRM and TEx cells, we compared peaks with significantly increased or decreased accessibility in each T cell subset relative to TEff
cells generated after LCMV Armstrong infection. First, we compared shared T\textsubscript{RM} peaks (significant peaks present in both liver and skin T\textsubscript{RM}) to shared T\textsubscript{EX} cell peaks (significant peaks present in all three T\textsubscript{EX} subsets isolated 21 d p.i.), i.e. T\textsubscript{RM} and T\textsubscript{EX} programs, respectively. This analysis showed that among the 1,510 peaks that have increased accessibility in both T\textsubscript{RM} subsets, only 177 were shared with the T\textsubscript{EX} program (2,640 peaks) (Figure 6H). We assigned these 177 peaks to the nearest gene and ordered the genes with the most assigned peaks; Slc24a5, a cation exchanger, and Sesn3, a stress-sensing protein that can promote NKR recognition in CD8\textsuperscript{+} T cells (Pereira et al., 2020), were the most commonly assigned genes with 3 peaks each (Figure S6E).

Comparing the T\textsubscript{EX} program to individual skin or liver T\textsubscript{RM} cell peaks sets yielded similar results (272 shared of 4,579 total peaks in skin T\textsubscript{RM} cells; 1,263 shared of 19,385 total peaks in liver T\textsubscript{RM} cells) with skin T\textsubscript{RM} and liver T\textsubscript{RM} cells sharing 5.94% and 6.51% peaks with T\textsubscript{EX} cells, respectively (Figure 6I). Interestingly, 2 of the 272 peaks that skin T\textsubscript{RM} cells share with T\textsubscript{EX} are nearest to the Pdcd1 gene; one peak ~23kb from the TSS of Pdcd1 is a known T\textsubscript{EX} enhancer that mediates sustained PD1 expression, previously thought to be specific to T\textsubscript{EX} cells (Pauken et al., 2016; Sen et al., 2016) (Figure 6J). The presence of this peak in both skin T\textsubscript{RM} and gp33-specific CD8\textsuperscript{+} T\textsubscript{EX} cells could potentially explain the constitutive expression of PD1 in both T cell subsets.

Finally, we then compared the peak set of skin and liver T\textsubscript{RM} clusters with each individual T\textsubscript{EX} cluster to determine which T\textsubscript{EX} cell population most closely shared the epigenetic features of T\textsubscript{RM} cells. Interestingly, skin T\textsubscript{RM} cells exhibited similar amounts of significant increased or decreased peaks with the three T\textsubscript{EX} subsets, while liver T\textsubscript{RM} cells shared mostly peaks with stem-like T\textsubscript{EX} cells (Figure 6K and L), potentially due to stem-like T\textsubscript{EX} cells being the most “memory-like” of the T\textsubscript{EX} cell subsets. Altogether, our results indicate that even though T\textsubscript{RM} cells may share phenotypic similarities with exhausted T cells, epigenetic analyses demonstrate major differences in gene accessibility changes throughout development that define specific memory or exhausted T cell subsets.

Discussion
Here, we utilized scATAC-seq to examine epigenetic changes that occur over the course of the T cell response against acute, local and systemic, or chronic viral infections. Our data defines the epigenetic variation between individual T cell subsets at various stages of infection and reveals an early divergence of memory precursors destined for a circulating or tissue-resident cell fate. We demonstrate that T_{RM} cells are an epigenetically distinct T cell subset that share a conserved epigenetic signature across organs, as well as tissue-specific epigenetic variation. Together, our findings highlight the dramatic changes in chromatin landscape that underlie cellular differentiation, as well as the resolution of scATAC-seq to finely distinguish individual populations within the CD8^{+} T cell pool (Lareau et al., 2019; Satpathy et al., 2019). Differences in chromatin accessibility across effector and memory T cells can be further interrogated in our public genome browser, allowing visualization of scATAC-seq reads in specific gene loci to investigate T cell biology.

Our data supports a model by which memory T cell fate is determined early post infection, supporting previous findings that demonstrate early fate decision for T_{RM} cell generation in the skin and gut (Kok et al., 2020; Milner et al., 2020). Using trajectory analyses, we modelled gene and transcription factor motif accessibility over time in effector, memory precursors and memory T cells, providing a genome-wide view of changes in the epigenome over the course of memory T cell differentiation. Importantly, by looking at genes with increased accessibility in the T_{RM}-poised memory precursor cluster we identified major epigenetic divergences defining early effector commitment to the T_{RM} and T_{CIRC} cell populations. Specifically, we uncovered the differential expression of FcγRIIB between T_{RM} and T_{CIRC} cells that is retained from their respective precursors. These findings allowed the selection of an effector population with enhanced capacity for generating each of those subsets, adding to the previous characterization of the role of FcγRIIB in triggering apoptosis to limit T cell mediated immunity (Morris et al., 2020).

We identified a common epigenetic signature conserved between T_{RM} cells from different organs, consisting of key gene regulatory networks that contribute to T cell retention, in addition to reduced ability to traffic in the blood and secondary lymphoid organs. In addition to this conserved T_{RM} cell program (Kumar et al., 2017; Mackay et al., 2016; Milner et al., 2017), cells residing in different organs exhibit divergent phenotypes and functional capacities as shaped by extrinsic cues.
in their distinct tissue microenvironments (Fonseca et al., 2020; Frizzell et al., 2020). We leveraged our chromatin accessibility data to identify potential transcriptional regulators that support tissue-specific TRM cell formation and validated the role of several transcription factors including HIC1, as well as AP-1 factors, FOSB, BACH2, FOS, and FOSL1, adding to their previously established role in T cell memory by regulating the availability of AP-1 motifs to limit the expression of TCR-driven genes during T cell effector responses (Roychoudhuri et al., 2016; Yukawa et al., 2020). Together, these results support the concept that epigenetic differences can underlie tissue-specific modulation of gene expression and that interrogation of these changes can reveal novel, tissue-specific transcriptional regulation.

TRM cells generated in response to acute infection and T\textsubscript{EX} cells in tumors and in chronic infection share considerable phenotypic overlap (Christo et al., 2021; Milner et al., 2017, 2020). Skin TRM cells, in particular, display restricted functional capacity and increased inhibitory receptor expression, characteristics that are typically associated with the T\textsubscript{EX} cell lineage (Christo et al., 2021; Park et al., 2019). A longstanding question has been whether the phenotypic and functional overlap between skin TRM cells and T\textsubscript{EX} cells is indicative of a shared epigenetic state or a convergence of cell types. Here, epigenetic analysis of TRM and T\textsubscript{EX} subsets demonstrated that despite expression similarities in the expression of certain co-inhibitory receptors such as PD-1, the epigenetic state of TRM, T\textsubscript{CIRC} and T\textsubscript{EX} subsets are ultimately distinct. Our detailed analysis demonstrated that despite similarities in the accessibility of certain genes, the majority of the epigenetic changes occurring in T\textsubscript{EX} cells are not present in skin or liver TRM cells.

Understanding the regulation and differentiation of TRM cells is critical to informing the design of therapies that aim to modulate tissue immunity. Our data demonstrates that TRM cells are an epigenetically distinct T cell subset that arise from epigenetically poised precursors generated early after infection, as well as revealing novel regulators of TRM cell differentiation. Altogether, our results provide critical insights into TRM cell differentiation and phenotype and will act as resource for further investigation into events that precede TRM, T\textsubscript{CIRC} and T\textsubscript{EX} cell differentiation, and epigenetic regulators that contribute to TRM cell maintenance and function.
Acknowledgements. We thank the Flow Cytometry Unit and Bioresources Facility at Peter Doherty Institute (University of Melbourne) for technical assistance and the Stanford Functional Genomics Facility for sequencing support. This work was supported by an Institutional Training Grant 5T32AI007290 (F.A.B.), a Howard Hughes Medical Institute and Bill & Melinda Gates International Research Scholarship OPP1175796 (L.K.M.), National Health and Medical Research Council (NHMRC) AP1113293 (L.K.M.), the National Institutes of Health (NIH) K08CA230188, U01CA260852, and UM1HG012076 (A.T.S.), the Parker Institute for Cancer Immunotherapy (A.T.S.), a Career Award for Medical Scientists from the Burroughs Wellcome Fund (A.T.S), and a Pew-Stewart Scholars for Cancer Research Award (A.T.S). L.K.M is a Senior Medical Research Fellow supported by the Sylvia and Charles Viertel Charitable Foundation.


Declaration of interests. A.T.S. is a scientific founder of Immunai and founder of Cartography Biosciences and receives research funding from Merck Research Laboratories, 10x Genomics, and Allogene Therapeutics.
Figure legends

Figure 1. T_{RM} cells display a unique epigenetic landscape amidst memory T cell subsets. (A-H) Congenically marked naïve CD8^+ P14 cells were transferred into C57Bl/6 naïve recipient mice followed by LCMV Armstrong infection. T_{CM} (CD62L^+ CD69^-), T_{EM} (CD62L^- CD69^-) and T_{RM} (CD62L^- CD69^+) cells were flow sorted from the spleen and liver 30 d p.i. and scATACseq was performed. (A) Experimental schematics of scATACseq droplets and representative flow plots of sorted populations from spleen and liver. (B) Analyses performed in single-cell chromatin accessibility data using ArchR. (C) UMAP projection of memory T cells. (D) Venn diagram of differential peaks in identified clusters individually compared to naive cluster (log2 FC > 1, FDR > 10). (E) Gene score volcano plots identifying genes with significantly different accessibility (log2 FC > 1, FDR > 10) between T_{RM} and T_{EM} or T_{CM} memory clusters; notable genes annotated manually. (F) UMAP depicting relative gene accessibility (gene score) across clusters. (G) Klf2 and Slpr1 genome tracks (height normalized) and (H) KLF2 and HIC1 motif deviation in indicated clusters.

Figure 2. Distinct epigenetic trajectories define T_{CIRC} and T_{RM} cell development. (A-K) Congenically marked naïve CD8^+ P14 cells were transferred into C57Bl/6 naïve recipient mice followed by LCMV Armstrong infection. P14 cells were sorted from the liver at 7 d (total P14 cells), 14 d (CD69^- and CD69^+), alongside T_{RM} (CD62L^- CD69^+), T_{CM} (CD62L^+ CD69^-) and T_{EM} (CD62L^- CD69^-) from the liver and spleen at 30 d p.i. and scATACseq was performed. (A) UMAP projection of scATAC profiles of flow sorted populations. (B) Marker peak heatmap identifying cis-regulatory elements uniquely active in individual clusters; peaks in or linked to notable genes are annotated and colored by cluster. (C) Cxcr6 and Slpr1 gene scores in individual clusters. (D) Predicted differentiation trajectories of identified memory clusters. (E) Slpr1 and (F) Tbx21 gene score over pseudotime for individual trajectories. (G) Motif accessibility of TCF7 over pseudotime during T_{CM} and T_{RM} cell epigenetic trajectories. (H) Heatmaps with dynamic gene score (left) and (I) motif accessibility (right) over pseudotime during T_{CM} and T_{RM} differentiation trajectories; notable genes and motifs that appear in the trajectory are annotated near their approximate position in pseudotime. (J) Hic1 gene score and (K) motif accessibility over pseudotime. (L-N) Control (sgCtrl) or Hic1 (sgHic1) ablation was performed using CRISPR-Cas9 in distinct congenically
marked naïve CD8+ P14 cells. Cells were then transferred into LCMV infected recipients and isolated from the spleen and liver 30 d.p.i. (L) Experimental schematics. (M) Log2 fold change of sgHic1 and sgCtrl indicated cell subsets normalized to the spleen and (N) Representative flow plots of transferred cells for the indicated subsets in liver. Data is representative from (L-N) 2 independent experiments with n=10 mice each. In (M) symbols represent individual mice. Box plots show the median, interquartile range and minimum/maximum whiskers. *** p≤0.001, One-way ANOVA with Bonferroni post-test.

Figure 3. FcγRIIB expression identify memory precursors with T_{CIRC} cell differentiation bias. (A-C) Congenically marked naïve CD8+ P14 cells were transferred into C57Bl/6 naïve recipient mice followed by LCMV Armstrong infection. P14 cells were flow sorted: from the liver at 7 d.p.i., CD69- and CD69+ at 14 d.p.i. and T_{CM}, T_{EM} and T_{RM} from the spleen and liver at 30 d.p.i. and scATACseq was performed. (A) UMAP projection of scATACseq profiles of flow sorted populations with memory T cell precursor clusters (T_{MP}-1 and T_{MP}-2) highlighted. (B) Gene score volcano plots identifying genes with significantly different accessibility (log2 fold change > 1, FDR > 10) between T_{CIRC} and T_{RM} clusters with similar changes in T_{MP}-2 and T_{MP}-1 clusters; notable genes were annotated manually. (C) Fcgr2b gene score over pseudotime for individual trajectories and (D) flow cytometry analysis of FcγRIIB expression at indicated time points. (E-I) Congenically marked naïve CD8+ P14 cells were transferred into C57Bl/6 naïve recipient mice followed by LCMV Armstrong infection. FcγRIIB- and FcγRIIB+ effector P14 cells were flow sorted from the spleen at 7 d.p.i. and transferred into infection matched recipients. Transferred cells were isolated from the spleen and liver 30 d.p.i. (E) Experimental schematics. (F) Total number of T_{CM}, T_{EM} and T_{RM} P14 cells generated from FcγRIIB- and FcγRIIB+ precursors and (G) Representative flow plots of CD69 and CD62L expression. (H) CD69 expression of FcγRIIB- and FcγRIIB+ T cell progeny in the liver 30 d.p.i. (I) Proportion of T_{CM} or T_{EM} and T_{RM} cells formed by FcγRIIB- and FcγRIIB+ transferred cells. Data is pooled from 2 independent experiments with n=5 mice each. In (D) symbols represent mean. In (F, H, I) symbols represent individual mice. Bars represent mean. * p≤0.05, ** p≤ 0.01, *** p≤0.001, two-tailed Student’s t test.

Figure 4. T_{RM} cells share a common epigenetic signature across tissues. (A-G) Congenally marked naïve CD8+ P14 cells were transferred into C57Bl/6 naïve recipient mice followed by
LCMV Armstrong infection. P14 cells were flow sorted: from the liver at 7 d.p.i., CD69- and CD69+ at 14 d.p.i. and TCM, TEM and TRM from the spleen and liver at 30 d.p.i. Congenically marked naïve CD8+ gBT-I cells were transferred into C57Bl/6 naïve recipient mice followed by HSV infection. gBT-I cells were flow sorted: from the axillary LN and from the skin (CD69+CD103+) at 14 and 30 d.p.i., and scATACseq was performed. (A) UMAP projection and (B) Marker gene heatmap identifying genes that are uniquely accessible in each cluster; notable marker genes for each cluster annotated manually. (C) Histogram distribution of cluster gene scores for Klf2, Slpr1, Xcl1 and Cnh2. (D) UpSet plot of shared peak sets in memory cells compared to naïve cells. (E) Common genes with most peak changes in TRM cells relative to naïve cells. (F) Genome track of Cish in cluster aggregated scATAC-seq data (height normalized). (G) HOMER motif enrichment analysis of shared TRM cluster peaks.

**Figure 5. Tissue-specific epigenetic signatures depicts transcriptional regulators of TRM cell development. (A-C)** Congenically marked naïve CD8+ P14 cells were transferred into C57Bl/6 naïve recipient mice followed by LCMV Armstrong infection. P14 cells were flow sorted: from the liver at 7 d.p.i., CD69- and CD69+ at 14 d.p.i. and TCM, TEM and TRM from the spleen and liver at 30 d.p.i. Congenically marked naïve CD8+ gBT-I cells were transferred into C57Bl/6 naïve recipient mice followed by HSV infection. gBT-I cells were flow sorted: from the axillary LN and from the skin (CD69+CD103+) at 14 and 30 d.p.i., and scATACseq was performed. (A) Transcription factors enriched in significant TRM cell motif deviations were selected and paired with DEGs between skin and liver TRM cells normalized to gene expression in TCM cells from GSE70813. (B) HOMER motif enrichment analysis of skin TRM exclusive peaks. (C) FOSB, FOS, FOSL1 and BACH2 motif deviations in indicated populations of memory T cells. (D-H) Distinct congenically marked naïve CD8+ P14 cells were in vitro activated, and ablation of specific targets was performed using CRISPR-Cas9. Cells were then transferred into LCMV infected recipients that were treated with DNFB on the skin. Transferred cells were isolated from the spleen, liver and skin 30 d.p.i. (D) Experimental schematics. Log2 FC of cells edited with (E) sgFosb, (F) sgFos, (G) sgFosl1 and (H) sgBach2 in the indicated tissues relative to sgCtrl normalized to the spleen. Data is pooled from (D-H) 2 independent experiments with n=5-6 mice each. In (E-H) symbols represent individual mice. Box plots show the median, interquartile range and minimum/maximum
whiskers. * p≤ 0.05, ** p≤ 0.01, *** p≤ 0.001, ns p>0.05, One-way ANOVA with Bonferroni post-test.

Figure 6. **TRM cells are epigenetically distinct from T_{EX} cells.** (A, B) Congenically marked naïve CD8\(^+\) P14 cells were transferred into C57Bl/6 recipient mice followed by LCMV Armstrong or LCMV Clone-13 infection. Congenically marked naïve gBT-I cells were transferred into C57Bl/6 recipient mice followed by HSV infection. Spleen, liver and skin of infected mice were harvested at indicated timepoints for flow cytometry. (A) Experimental schematics and UMAP projection of the indicated T cell populations in tissues based on flow cytometric analysis. (B) UMAP depicting expression of indicated markers across clusters. (C-L) Congenically marked naïve CD8\(^+\) P14 or gBT-I cells were transferred into C57Bl/6 recipient mice followed by LCMV Armstrong or HSV infection respectively. A separate group of naïve C57Bl/6 mice were infected with LCMV Clone-13. Total P14 cells were flow sorted from the liver at 7 d p.i., CD69\(^+\) at 14 d p.i. and TRM cells at 30 d p.i. gBT-I cells were flow sorted from the skin (CD69\(^+\)CD103\(^+\)) at 30 d p.i. Endogenous gp33\(^+\) cells were flow sorted from the spleen of Clone-13 infected mice at 8 and 21 d p.i. scATACseq was performed in isolated populations. (C) Experimental schematic and UMAP projection based on scATACseq analysis. (D) UMAP depicting relative gene accessibility (gene score) across clusters. (E) Heatmap identifying peaks that are uniquely accessible in each cluster relative to all clusters. (F) Tox and (G) Tcf7 gene scores in TRM and T_{EX} clusters. (H) Venn diagram depicting similar peaks with increased or decreased accessibility in TRM and T_{EX} cells and (I) specific peaks shared with liver or skin TRM and T_{EX} cells. (J) Pdcd1 genome tracks of cluster aggregated scATAC-seq data (height normalized); peaks with qualitative height differences highlighted. (K) UpSet plot of skin or (L) liver TRM cluster shared peak sets with exhausted T cell subsets compared to effector T cells.
**Supplementary Figure legends**

**Figure S1 (Related to Figure 1). Epigenetic variation of memory T cell subsets following LCMV infection.** (A-H) Congenically marked naïve CD8+ P14 cells were transferred into naïve recipient mice followed by LCMV Armstrong infection. T<sub>CM</sub> (CD62L<sup>+</sup> CD69<sup>-</sup>), T<sub>EM</sub> (CD62L<sup>-</sup> CD69<sup>-</sup>) and T<sub>RM</sub> (CD62L<sup>-</sup>CD69<sup>+</sup>) cells were flow sorted from the spleen and liver 30 d p.i. and scATACseq was performed. (A) Summary of antigen expression among CD8<sup>+</sup> T cell subsets for each marker contained in the cell sort antibody panel. (B) Representative quality control plots. (C) Cluster composition by sample identity based on CD8<sup>+</sup> T cell subsets sorted. (D) Gene score volcano plots identifying genes with different accessibility (log2 FC > 1, FDR > 10) between T<sub>EM</sub> and T<sub>CM</sub> memory clusters; notable genes annotated manually. (E) Genome tracks of Slpr5 and Zeb2 (height normalized). (F) BLIMP-1 motif deviation, (G) UMAP depicting relative gene accessibility (gene score) across clusters and (H) cluster aggregated genome track of scATAC-seq data (height normalized).

**Figure S2 (Related to Figure 2). Epigenetic and phenotypic variations drive development of distinct memory T cell subsets.** (A-D) Congenically marked naïve CD8<sup>+</sup> P14 cells were transferred into naïve recipient mice followed by LCMV Armstrong infection. P14 cells were flow sorted: from the liver at 7 d p.i., CD69- and CD69+ at 14 d p.i. and T<sub>CM</sub>, T<sub>EM</sub> and T<sub>RM</sub> from the spleen and liver at 30 d p.i. and scATACseq was performed. (A) Experimental schematics and representative flow plots of sorted populations from the liver 14 d p.i. (B) Cluster composition by sample identity. (C) Genome tracks of Cxcr6 and Sell (height normalized). (D) Cxcr6 and Sell gene score over pseudotime for individual trajectories. (E) Flow cytometry analysis of CXCR6 and CD62L expression by CD8<sup>+</sup> P14 cells after LCMV Armstrong infection at indicated time points. (F-I) Control (sgCtrl) or Hic1 (sgHic1) ablation was performed using CRISPR-Cas9 in distinct congenically marked naïve CD8<sup>+</sup> P14 cells. Cells were then transferred into LCMV infected recipients and isolated from the spleen and liver 9 and 30 d p.i. (F) Experimental schematics and (G) Log2 FC of sgHic1 and sgCtrl indicated cell subsets normalized to the spleen at 9 d p.i. (H-I) Representative flow plots of transferred cells (H) in the spleen and liver 9 d p.i. and (I) in the indicated subsets in the spleen 30 d p.i. Data is representative of (E) 2 independent experiments with n=5 mice each and (F-I) 2 independent experiments with n=6-10 mice each. In
(G) symbols represent individual mice. Box plots show the median, interquartile range and minimum/maximum whiskers. *** p≤0.001, One-way ANOVA with Bonferroni post-test.

Figure S3 (Related to Figure 3). FcγRIIB expression reflects gene accessibility in T cell subsets. (A) Congenically marked naïve CD8\(^+\) P14 cells were transferred into naïve recipient mice followed by LCMV Armstrong infection. P14 cells were flow sorted: from the liver at 7 d p.i., CD69\(^-\) and CD69\(^+\) at 14 d p.i. and T\(_{CM}\), T\(_{EM}\) and T\(_{RM}\) from the spleen and liver at 30 d p.i. and scATACseq was performed. Genome tracks of Fcgr2b (height normalized). (B, C) Congenically marked naïve CD8\(^+\) P14 cells were transferred into naïve recipient mice followed by LCMV Armstrong infection and isolated 30 days later from the spleen and liver. (B) Experimental schematics and representative flow plots of CD69 and FcγRIIB expression in the spleen and liver. (C) FcγRIIB expression by T\(_{CM}\), T\(_{EM}\) and T\(_{RM}\) subsets. (D) Congenically marked naïve CD8\(^+\) P14 cells were transferred into naïve recipient mice followed by LCMV Armstrong infection. FcγRIIB\(^-\) and FcγRIIB\(^+\) effector P14 cells were flow sorted from the spleen at 7 d p.i. and transferred into infection matched recipients. Transferred cells were isolated from the liver 30 d p.i. Experimental schematics and FcγRIIB expression in isolated progeny. Data is pooled from 2 independent experiments with n=5 mice each. In (C, D) symbols represent individual mice. Bars represent mean. ** p≤0.01, *** p≤0.001, One-way ANOVA with Bonferroni post-test.

Figure S4 (Related to Figure 4). T\(_{RM}\) cells display a conserved epigenetic profile across tissues. (A-E) Congenically marked naïve CD8\(^+\) P14 T cells were transferred into naïve recipient mice followed by LCMV Armstrong infection. P14 T cells were flow sorted: from the liver at 7 d p.i., CD69\(^-\) and CD69\(^+\) at 14 d p.i. and T\(_{CM}\), T\(_{EM}\) and T\(_{RM}\) from the spleen and liver at 30 d p.i. Congenically marked naïve CD8\(^+\) gBT-I T cells were transferred into naïve recipient mice followed by HSV infection. gBT-I T cells were flow sorted: from the skin-draining (axillary) LN and from the skin (CD69\(^+\)CD103\(^+\)) at 14 and 30 d p.i., and scATACseq was performed. (A) Experimental schematic and representative flow plots of sorted populations from skin and skin-draining LN 14 and 30 d p.i. (B) Cluster composition by sample identity. (C) Dot plot depicting liver and skin T\(_{RM}\) exclusive gene scores relative to naïve cells. (D) Cish, Tnfrsf10 and Bach2 gene scores in individual indicated clusters. (E) Genes with significant gene score differences exclusively in skin and liver T\(_{RM}\) cells relative to naïve.
Figure S5 (Related to Figure 5). Epigenetic profile reveals tissue-exclusive pathways and requirements for T<sub>RM</sub> cell development. (A, C, E, F) Congenically marked naïve CD8<sup>+</sup> P14 T cells or gBT-I T cells were transferred into naïve recipient mice followed by LCMV Armstrong or HSV infection, respectively. P14 T<sub>RM</sub> cells were flow sorted from the liver and gBT-I T<sub>RM</sub> cells were flow sorted from the skin at 30 d p.i. scATACseq was performed in isolated populations. Volcano plot depicting differences between skin and liver T<sub>RM</sub> cells exclusive gene peaks and gene scores. (A) Volcano plot depicting differences between skin and liver T<sub>RM</sub> cells exclusive peaks and gene scores. (B) Indicated genes log counts per million (logCPM) in skin and liver T<sub>RM</sub> cells from GSE70813. (C) Pathway analysis of liver and skin T<sub>RM</sub> cell exclusive motifs enriched in the 2020 Molecular Signature Database (MSigDB). (D) Indicated genes log counts per million (logCPM) in spleen T<sub>CM</sub> and T<sub>EM</sub> cells, liver T<sub>RM</sub> cells and skin T<sub>RM</sub> cells from GSE70813. (E) Volcano plot depicting skin and liver T<sub>RM</sub> cell exclusive motifs. (F) FOSL2 motif deviation in indicated populations of memory T cells. (G-I) Distinct congenically marked naïve CD8<sup>+</sup> P14 cells were in vitro activated, and ablation of specific targets was performed using CRISPR-Cas9. Cells were then transferred into LCMV infected recipients that were treated with DNFB on the skin. Transferred cells were isolated from the spleen, liver and skin 30 d p.i. (G) Representative flow plots of sgFosb and sgCtrl transferred cells. (H) Log2 FC of sgFosl2 and sgCtrl cells. (I) Representative flow plots of sgBach2 and sgCtrl transferred cells. Data is pooled from (G-I) 2 independent experiments with n=5-6 mice each. In (G-I) symbols represent individual mice. Box plots show the median, interquartile range and minimum/maximum whiskers. * p≤ 0.05, ** p≤ 0.01, *** p≤0.001, One-way ANOVA with Bonferroni post-test.

Figure S6 (Related to Figure 6). T<sub>RM</sub> and T<sub>EX</sub> cell subsets are epigenetically distinct. (A, C) Congenically marked naïve CD8<sup>+</sup> P14 cells were transferred into recipient mice followed by LCMV Armstrong or LCMV Clone-13 infection. Congenically marked naïve gBT-I cells were transferred into recipient mice followed by HSV infection. Spleen, liver and skin of infected mice were harvested at indicated timepoints for flow cytometry. (A) Summary of the antibody panel utilized for clustering CD8<sup>+</sup> T cell subsets in Fig. 6a. (B, D, E) Congenically marked naïve CD8<sup>+</sup> P14 cells were transferred into recipient mice followed by LCMV Armstrong. Congenically marked naïve gBT-I cells were transferred into recipient mice followed by HSV infection. Naïve
mice were infected with LCMV Clone-13. Endogenous gp33+ cells were flow sorted from the spleen at 8 and 21 d p.i. scATACseq was performed in isolated populations. (B) Cluster composition by sample identity. (C) Shown is TOX expression in T cell clusters. (D) Tcf7, Irf4, Runx3 and Eomes motif deviation in indicated T cell clusters. (E) Gene assignment of peaks with increased accessibility in both T_{RM} and T_{EX} cell subsets relative to Arm T_{EFF} cells.
Material and Methods

Mice. C57BL/6, gBT-1.CD45.1, P14.CD45.1 and P14.CD45.1.2 mice were bred in the Department of Microbiology and Immunology at the University of Melbourne. Six- to eight-week-old C56BL/6 were used for experiments. All experiments were approved by the University of Melbourne Animal Ethics Committee.

Adoptive cell transfers, infections and DNFB treatment. For naïve transgenic T cell transfers, cells were isolated from lymph nodes and spleen and transferred intravenously (i.v.) to C57BL/6 mice at 5×10^4 cells per recipient. Skin infections were done by skin scarification with 1×10^6 plaque-forming units (PFU) of HSV-1 KOS as described. LCMV Armstrong infections were done by intraperitoneal injection of 2×10^5 PFU to establish acute infections. LCMV Clone-13 experiments were done by i.v. injection of 1×10^6 PFU to establish chronic infections. For treatment with 1-Fluoro-2,4-dinitrobenzene (DNFB), mice were shaved and depilated before treatment with 15 μl of DNFB (Sigma-Aldrich) diluted at 0.25% in acetone:oil (4:1) on the skin as described (Mackay et al., 2012b) or on the ears 3 d after LCMV infection.

Organ processing, flow cytometry, and cell sorting. Spleens were processed through metal meshes into single-cell suspensions followed by red blood cell lysis. Skin samples were excised and incubated at 37°C for 90 min in dispase (2.5 mg/ml; Roche) or in liberase (0.25 mg/ml; Sigma) followed by separation of epidermis and dermis. Chopped samples were incubated at 37°C for 30 min in collagenase III (3 mg/ml; Worthington). Liver samples were excised and meshed into single-cell suspensions through 70 μm meshes. Leukocytes were isolated using Percoll (35%; Sigma Aldrich). Single cell suspensions were stained with conjugated antibodies for flow cytometry or cell sorting. For intracellular staining of cytokines and transcription factors, cells were fixed and permeabilized using the Foxp3 Transcription factor Staining buffer set (Invitrogen) as per manufacturer’s instructions. The following antibodies from BD Biosciences, Biolegend, Cell signalling or Thermo Fisher Scientific were used: anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD8α (53-6.7), anti-CD8β (YTS1 56.7.7), anti-CD3 (500A2), anti-Vα2 (B20.1), anti-CD44 (IM7), anti-CD127 (A7R34), anti-CXCR6 (SA051D1), anti-CX3CR1 (SA011F11), anti-CD62L (MEL-14), anti-CD69 (H1.2F3), anti-CD103 (2E7), anti-PD-1 (29F.1A12), anti-Ly6C (HK1.4),...
anti-TCRβ (H57-597), anti-TIM-3 (RMT3-23), anti-CD43 (1B11), anti-Ly108 (330-AJ), anti-CD38 (90), anti-CD49a (HMa1), anti-CD32b (AT130-2), anti-gp33 tetramer, anti-TOX (TXRX10), anti-TCF7 (C63D9), anti-MHC-II (M5/114.15.2), anti-CD73 (TY/11.8), anti-LAG3 (C9B7W), anti-KLRG1 (2F1). Cell viability was determined using Ghost Dye Red 780 (Tonbo Biosciences). Flow cytometry was performed on a LSRFortessa (BD Biosciences) or an Aurora (Cytek) and analyzed with FlowJo software (TreeStar) or Omiq. For cell sorting experiments, P14 and gBT-I cells were isolated from the spleen, liver and skin as indicated and sorted using a FACSaria III (BD Biosciences).

CRISPR-Cas9 Gene Editing of CD8+ T cells. Single guide RNA (sgRNA) targeting: CD19 (5’-AAUGUCUCAGACCAUAUGGG-3’), Hic1 (5’-AGUGUGCCGAAGCGCGGAG-3’, 5’-CUUGUGCGACGUAUAC-3’), Fos (5’-TGTCACCGTGGGGATAAAAGTTGG-3’, 5’-GGTCTGCGATGGGCCAGGAGG-3’), Fosb (5’-AGACACGTACTGAGAAGGCCGG-3’, 5’-GTTGACCCCTTAGACATGCCGCCGG-3’), Fosl1 (5’-GGAACCGGGACGCAGTCGCCGGG-3’, 5’-GCTGCCCGGCGCCGGATTCCG-3’), Fosl2 (5’-GACCGAGGTCTAAAGTTCGCGG-3’, 5’-GGACATGGAGGTGATCACTG-3’), Bach2 (5’-TGCGCAGGAACTCAGCACAGCC-3’, 5’-GATGTTGGCACAGTGGACTGTGG-3’). sgRNA/Cas9 RNPs were formed by incubating 0.3nmol of sgRNA with 0.6 ml Alt-R S.p. Cas9 nuclease V3 (10 mg/ml; Integrated DNA Technologies) for 10 min at room temperature. P14 cells were in vitro activated with anti-CD3 and anti-CD28 (5 μg/ml) for 24 hours. In vitro activated or naïve P14 cells were resuspended in 20 μl of P3 (P3 Primary Cell 4D-Nucleofector X Kit; Lonza), mixed with sgRNA/Cas9 RNP and electroporated using a Lonza 4D-Nucleofector system (CM137) as previously described (Nüssing et al., 2020). Cells were expanded for 72 hours in the presence of IL-2 (25U/ml; Peprotech). Naïve and in vitro activated edited cells were mixed at a 1:1 ratio and 5×10^5 cells were transferred i.v. into LCMV-infected recipients.

scATAC-seq library preparation and sequencing. Sorted T cell populations were thawed, then subjected to the 10x Chromium scATAC protocol (https://support.10xgenomics.com/single-cell-atac). In short, nuclei were isolated and partitioned into gel-bead emulsions that allow barcoded transposition to happen at single cell scale. Following transposition, the emulsions were broken,
the product was cleaned and libraries were prepared for Illumina sequencing. Libraries were
sequenced on the Illumina HiSeq 4000.

**scATAC-seq computational analysis.** Fastq files were trimmed, aligned to the mm10 reference
genome, and deduplicated using the 10X genomics cellranger-atac count pipeline. Fragments files
for each sample, containing the unique aligned reads passing filter for each cell barcode, were then
loaded into ArchR for downstream analysis (Granja et al., 2021). Doublet identification and
removal, cell calling, clustering, peak calling, and motif analysis was performed using the default
ArchR workflow. After initial clustering, there was often a small cluster of contaminating non-T
cells -- these were manually identified, removed, and the remaining cells were reclustered
following the same procedure. Marker features were identified using Archr `getMarkerFeatures`.
GeneScore visualizations were performed using the ArchR implementation of Magic imputation.
Motifs enriched in specific peak sets were analyzed using the HOMER (Heinz et al., 2010)
`findMotifsGenome.pl` utility. For these analyses, the indicated peak set was compared to a
background peak set consisting of the ArchR union peak set for that group of samples. Therefore,
the motifs identified represent motifs enriched relative to the rest of the peaks in these samples,
rather than motifs enriched in, for example, T cells in general.

**RNA-Seq Analysis.** For TF expression analysis, previously generated RNA-seq count matrixes
were analyzed using DESeq2 (Love et al., 2014) using the default parameters. TrM samples were
compared to their respective TcRc populations using the ‘results’ function with α=0.05.

**Statistical analysis.** Statistical analyses were performed by one- or two-way analysis of variance
(ANOVA) test followed by Bonferroni’s post-test or by two-tailed Student’s t test using Prism 9
(GraphPad) as indicated in figure legends. P values were represented by * p < 0.05; ** p < 0.01;
*** p < 0.001; **** p < 0.0001; ns (not significant) p > 0.05. Results represent means ± SEM.

**Data availability.** All original data is available from the corresponding author upon reasonable
request. Sequencing data is available in the Gene Expression Omnibus database under accession
code GSE199799. Source Data are provided in the online version of the manuscript. Code
available upon request.
References

Akondy, R.S., Fitch, M., Edupuganti, S., Yang, S., Kissick, H.T., Li, K.W., Youngblood, B.A.,

Araki, K., Turner, A.P., Shaffer, V.O., Gangappa, S., Keller, S.A., Bachmann, M.F., Larsen,
108–112.

Becht, E., McInnes, L., Healy, J., Dutertre, C.A., Kwok, I.W.H., Ng, L.G., Ginhoux, F., and
Nat. Biotechnol. 37, 38–47.

Behr, F.M., Parga-Vidal, L., Kragten, N.A.M., van Dam, T.J.P., Wesselink, T.H., Sheridan, B.S.,

Beura, L.K., Wijeyesinghe, S., Thompson, E.A., Macchietto, M.G., Rosato, P.C., Pierson, M.J.,

Blank, C.U., Haining, W.N., Held, W., Hogan, P.G., Kallies, A., Lugli, E., Lynn, R.C., Philip,
665–674.

Burrows, K., Antignano, F., Bramhall, M., Chenery, A., Scheer, S., Korinek, V., Underhill,

instruct diversity in resident memory T cell function and plasticity. Nat. Immunol. 22.

Cusanovich, D.A., Daza, R., Adey, A., Pliner, H.A., Christiansen, L., Gunderson, K.L.,

Daniel, B., Yost, K.E., Sandor, K., Xia, Y., Qi, Y., Hiam-Galvez, K.J., Meier, S.L., Belk, J.A.,
Giles, J.R., Wherry, E.J., et al. (2021). Divergent clonal differentiation trajectories of T cell


Herndler-Brandstetter, D., Ishigame, H., Shinnakasu, R., Plajer, V., Stecher, C., Zhao, J.,
CD8+ T Cells Lose KLRG1, Differentiate into All Memory T Cell Lineages, and Convey

Hirai, T., Yang, Y., Zenke, Y., Li, H., Chaudhri, V.K., De La Cruz Diaz, J.S., Zhou, P.Y.,
Cytokine Allows for Selective Retention of Antigen-Specific Tissue-Resident Memory T Cells

Holz, L.E., Chua, Y.C., de Menezes, M.N., Anderson, R.J., Draper, S.L., Compton, B.J., Chan,
liver-resident memory CD8+ T cells that protect against rodent malaria. Sci. Immunol. 5, 1–14.

Im, S.J., Hashimoto, M., Gerner, M.Y., Lee, J., Kissick, H.T., Burger, M.C., Shan, Q., Hale, J.S.,
Lee, J., Nasti, T.H., et al. (2016). Defining CD8+ T cells that provide the proliferative burst after


role of the Wnt pathway effector Tcf-1 for the establishment of functional CD8 T cell memory.

Joshi, N.S., Cui, W., Chandele, A., Lee, H.K., Urso, D.R., Hagman, J., Gapin, L., and Kaech,
S.M. (2007). Inflammation Directs Memory Precursor and Short-Lived Effector CD8+ T Cell


Kok, L., Dijkgraaf, F.E., Urbanus, J., Bresser, K., Vredevoogd, D.W., Cardoso, R.F., Perié, L.,
precursor within the circulating CD8+ effector T cell pool. J. Exp. Med. 217.

Kok, L., Masopust, D., and Schumacher, T.N. (2021). The precursors of CD8+ tissue resident
Kumar, B. V., Ma, W., Miron, M., Granot, T., Guyer, R.S., Carpenter, D.J., Senda, T., Sun, X.,
Ho, S.H., Lerner, H., et al. (2017). Human Tissue-Resident Memory T Cells Are Defined by
Core Transcriptional and Functional Signatures in Lymphoid and Mucosal Sites. Cell Rep. 20,
2921–2934.
Kurd, N.S., He, Z., Louis, T.L., Milner, J.J., Omilusik, K.D., Jin, W., Tsai, M.S., Widjaja, C.E.,
Kanbar, J.N., Olvera, J.G., et al. (2020). Early precursors and molecular determinants of tissue-
resident memory CD8+ T lymphocytes revealed by single-cell RNA sequencing. Sci. Immunol.
5, eaaz6894.
Memory CD8+ T Cells during Influenza Viral Infection. Immunity 41, 633–645.
Lareau, C.A., Duarte, F.M., Chew, J.G., Kartha, V.K., Burkett, Z.D., Kohlway, A.S., Pokholok,
Li, C., Zhu, B., Son, Y.M., Wang, Z., Jiang, L., Xiang, M., Ye, Z., Beckermann, K.E., Wu, Y.,
Jenkins, J.W., et al. (2019). The Transcription Factor Bhlhe40 Programs Mitochondrial
Regulation of Resident CD8+ T Cell Fitness and Functionality. Immunity 51, 491-507.e7.
Mackay, L.K., Wakim, L., van Vliet, C.J., Jones, C.M., Mueller, S.N., Bannard, O., Fearon,
Chronic Antigen Stimulation and Repeated Reactivation for a Latent Virus Infection. J.
Immunol. 188, 2173–2178.


induced by co-stimulation is required for chromatin opening during T cell activation. J. Exp. Med. 217.


Figure 1. TRM cells display a unique epigenetic landscape amidst memory T cell subsets.

A) Spleen Liver

B) 1. Clustering & cell type identification

2. Peak Accessibility (individual peak changes)

C) C1 C2 C3 C4

D) Peaks Accessibility (Rel. to Naive)

E) Up in TEM (n = 118) Up in TRM (n = 137)

F) S1pr1 Klf2 Sell Ccr7 Hic1 Fcgr2b

G) 5kb 10kb Naive T EM T RM T CM Klf2> Refseq

H) Motif Deviation

- Log10 FDR
- Log2 Fold Change

CC-BY-NC-ND 4.0 International license available under a CC-BY-NC-ND 4.0 International license.
Figure 2. Distinct epigenetic trajectories define TCIRC and TRM cell development.

Figure 3. FcγRIIB expression identify memory precursors with T cell differentiation bias.

A. UMAP plot showing the differentiation of T cells.

B. Gene score showing the fold change and log10 FDR for genes.

C. Heatmap showing the expression of FcγRIIB in TRM, TCM, and TEM.

D. Graph showing the percentage of FcγRIIB+ cells over time.

E. Diagram showing the experimental setup with FcγRIIB+ and FcγRIIB- cells sorted from P14+ LCMV.

F. Graph showing the total T cells in different tissue types.

G. Heatmap showing the CD69 and CD62L expression in the liver.

H. Graph showing the percentage of CD69+ cells.

I. Graph showing the distribution of FcγRIIB+ and FcγRIIB- cells in different tissue types.
Figure 4. TRM cells share a common epigenetic signature across tissues.

**Gene Score**

<table>
<thead>
<tr>
<th>Gene Score</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accessibility</td>
<td>0.2</td>
<td>0.4</td>
<td>0.6</td>
<td>0.8</td>
<td>1.0</td>
</tr>
</tbody>
</table>

**Genes with most peak changes**

- Naive
  - Klf2
  - S1pr1
  - Xcd1
  - Chm2
- Liver
  - C6
  - C7
  - C8
  - C9
  - C10
- Skin
  - C1
  - C2
  - C3

**TRM Exclusive Peaks in CISH**

- 10k
- 20k
- 30k
- 40k
- 50k

**HOMER Motif Enrichment Analysis**

**Known Up Peaks** (n = 426)

<table>
<thead>
<tr>
<th>Motif</th>
<th>Name</th>
<th>GSE</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCCTGTTTA</td>
<td>Cish</td>
<td>21512</td>
<td>1e-20</td>
</tr>
<tr>
<td>ATGCTTTTTGAGGG</td>
<td>Ap-1 (bZIP)</td>
<td>33912</td>
<td>1e-17</td>
</tr>
<tr>
<td>ATGCTTTTTGAGGG</td>
<td>Acr3 (bZIP)</td>
<td>33912</td>
<td>1e-17</td>
</tr>
<tr>
<td>ATGCTTTTTGAGGG</td>
<td>Gadd45a</td>
<td>46166</td>
<td>1e-16</td>
</tr>
<tr>
<td>TGGTACCTCCTG</td>
<td>Fosl1 (bZIP)</td>
<td>46166</td>
<td>1e-16</td>
</tr>
</tbody>
</table>

**Known Down Peaks** (n = 1086)

<table>
<thead>
<tr>
<th>Motif</th>
<th>Name</th>
<th>GSE</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATGCCAGCTTTG</td>
<td>Klf4 (Zf)</td>
<td>11431</td>
<td>1e-13</td>
</tr>
<tr>
<td>ATGCCAGCTTTG</td>
<td>Klf1 (Zf)</td>
<td>20478</td>
<td>1e-13</td>
</tr>
<tr>
<td>ATGCCAGCTTTG</td>
<td>Klf6 (Zf)</td>
<td>64557</td>
<td>1e-11</td>
</tr>
</tbody>
</table>
**Figure 5. Tissue-specific epigenetic signatures depict transcriptional regulators of T cell development.**

**A** Skin vs. Liver TRM RNA-seq DEGs

Transcription factors

<table>
<thead>
<tr>
<th>Motif Deviation (Skin - Liver) Higher in</th>
<th>SKIN TRM</th>
<th>LIVER TRM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fosl2</td>
<td>0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>Fosb</td>
<td>0.03</td>
<td>0.00</td>
</tr>
<tr>
<td>Jund</td>
<td>0.01</td>
<td>0.00</td>
</tr>
<tr>
<td>Bach2</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Hic1</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Lef1</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Fos</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Fosl1</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Prdm1</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

**B** HOMER Skin TRM Exclusive Peaks (n = 2,663)

<table>
<thead>
<tr>
<th>Rank</th>
<th>Motif</th>
<th>Name</th>
<th>GSE</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fos</td>
<td>110950</td>
<td>1e-268</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Fos2</td>
<td>43439</td>
<td>1e-267</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Fos1</td>
<td>46166</td>
<td>1e-264</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Bach2</td>
<td>44420</td>
<td>1e-86</td>
<td></td>
</tr>
</tbody>
</table>

**C** Motif Deviation

<table>
<thead>
<tr>
<th>Motif</th>
<th>Liver + Spleen</th>
<th>Skin Liver</th>
<th>Liver + LN</th>
<th>Skin Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fosl2</td>
<td>0.02</td>
<td>0.01</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Fosb</td>
<td>0.03</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Jund</td>
<td>0.01</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Bach2</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Hic1</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Lef1</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Fos</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Fosl1</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Prdm1</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

**D** sgTarget + sgCtrl + LCMV + DNFB

**E** sgFosb/sgCrl

<table>
<thead>
<tr>
<th>sgFosb/sgCrl</th>
<th>Skin Liver + LN</th>
</tr>
</thead>
<tbody>
<tr>
<td>-2.0</td>
<td>0.0</td>
</tr>
<tr>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>2.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

**F** sgFos/sgCrl

<table>
<thead>
<tr>
<th>sgFos/sgCrl</th>
<th>Skin Liver + LN</th>
</tr>
</thead>
<tbody>
<tr>
<td>-2.0</td>
<td>0.0</td>
</tr>
<tr>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>2.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

**G** sgFosl1/sgCrl

<table>
<thead>
<tr>
<th>sgFosl1/sgCrl</th>
<th>Skin Liver + LN</th>
</tr>
</thead>
<tbody>
<tr>
<td>-2.0</td>
<td>0.0</td>
</tr>
<tr>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>2.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

**H** sgBach2/sgCrl

<table>
<thead>
<tr>
<th>sgBach2/sgCrl</th>
<th>Skin Liver + LN</th>
</tr>
</thead>
<tbody>
<tr>
<td>-2.0</td>
<td>0.0</td>
</tr>
<tr>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>2.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>
Figure 6. TRM cells are epigenetically distinct from TEx cells.
Figure S1 (Related to Figure 1). Epigenetic variation of memory T cell subsets following LCMV infection.

A) Sort Panel

<table>
<thead>
<tr>
<th></th>
<th>Naive</th>
<th>TCM</th>
<th>TEM</th>
<th>TRM</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45.1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD8a</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Vn2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CD44</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD127</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD69</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CXCR6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

B) Liver TEM

- Cells Pass Filter: 4853
- Median Frags: 7151
- Median TSS Enrichment: 18.86

C) Liver TCM

- Cells Pass Filter: 714
- Median Frags: 13218.5
- Median TSS Enrichment: 18.25

D) Up in TEM (n = 45)

- Log2 Fold Change
- -log10 FDR

D) Up in TCM (n = 361)

- Log2 Fold Change
- -log10 FDR

E) 5kb

- <Keap1
- <S1pr5

F) Motif Deviation

- BLIMP-1
- Prdm1

G) UMAP1

- Log2 Fold Change

H) UMAP2

- Log2 Fold Change
- -log10 FDR

- <Prdm1
- Refseq
Figure S2 (Related to Figure 2). Epigenetic and phenotypic variations drive development of distinct memory T cell subsets.

A) P14 cells + LCMV → Liver d14

B) Cluster Composition

CD62L CXCR6 d7 d14 d30

C) Gene Score

Naive T eff T eff-1 T eff-2 T cm T cm-1 T cm-2 T mem-1 T mem-2

D) Pseudotime

Spleen Liver

E) Pseudotime

F) sgCD19

G) sgHic1

H) Spleen

I) Liver
Figure S3 (Related to Figure 3). FcγRIIB expression reflects gene accessibility in T cell subsets.

A

Naive
T Eff
TEM-1
TEM-2
TEV-1
TEV-2
TCM-1
TCM-2
TRM-1
TRM-2

**Ccgr2b**

B

Day 30 pi

Spleen
Liver

CD8

FcγRIIB

C

Spleen
Liver

T CM
T EM
T RM

D

P14 + LCMV FcγRIIB−
Sort FcγRIIB+

P14 + LCMV

CC-BY-NC-ND 4.0 International license available under a was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a
Figure S4 (Related to Figure 4). TRM cells display a conserved epigenetic profile across tissues.

Gene Score

TRM Exclusive (rel. to Naive)

A

B

C

D

Figure S4. TRM cells display a conserved epigenetic profile across tissues.
Figure S5 (Related to Figure 5). Epigenetic profile reveals tissue-exclusive pathways and requirements for TRM cell development.

A. **Peak Matrix**

- **Gene Score**
  - Up in Liver TRM (n = 6611)
  - Up in Skin TRM (n = 3116)

B. **Gene Score**

- y-axis: Log2 Fold Change
- x-axis: Log2 Fold Change
- **Gene Name**
  - Fyn
  - Itga4
  - Gcnt1
  - Agap1
  - $rW2a−ps$
  - P2rx7
  - Sema6d
  - Ahr
  - Ccr8
  - Il13

C. **Motif Matrix**

- **Motif Deviation**
- **Gene Name**
  - Tip60 (n = 1,314)
  - H3K4me1 (n = 1,314)
  - H3K4me2 (n = 1,314)
  - H3K4me3 (n = 1,314)

D. **Fos/Zip2/Zip4/Zip8**

- LogCPM
- **Gene Name**
  - Fos
  - Fosb
  - Fosl1

E. **Motif Matrix**

- **Gene Name**
  - Bach2
  - Jund
  - Irf3
  - Irf4
  - Irf2
  - Cebpe
  - Tbx21
  - Stat1

F. **Motif Deviation**

- **Gene Name**
  - Bach2
  - Jund
  - Irf3
  - Fosb

G. **Spleen, Liver, Skin**

- **Gene Name**
  - sgCtrl
  - sgFosl2

H. **sgCtrl + sgFosl2**

- **Gene Name**
  - P14
  - LCMV
  - DNFB

I. **Spleen, Liver, Skin**

- **Gene Name**
  - sgCtrl
  - sgBach2
  - sgFosl2

**Note:** The images show various gene expression profiles and statistical analyses related to tissue-specific responses and TRM cell development. The figures illustrate the differences in gene expression patterns across different tissues and conditions, highlighting the unique requirements for tissue-resident memory (TRM) cell development.
Figure S6 (Related to Figure 6). T_{RM} and T_{EX} cell subsets are epigenetically distinct.

Flow cytometry clustering panel

<table>
<thead>
<tr>
<th>Sorted Sample</th>
<th>LCMV Arm</th>
<th>LCMV Cl-13</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV d30 Skin T_{RM}</td>
<td>d14 Liver CD69</td>
<td>d21 Spleen PD1+</td>
</tr>
<tr>
<td>d21 Spleen PD1+</td>
<td>d7 Liver</td>
<td>d8 Spleen PD1+</td>
</tr>
<tr>
<td>d30 Liver T_{RM}</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Cell proportion

Figure S6 (Related to Figure 6). T_{RM} and T_{EX} cell subsets are epigenetically distinct.