MATERIALS AND METHODS

Data and code availability

Bulk and single-cell RNA-seq, and ATAC-seq data have been deposited at GEO under accession number GSE192728 and are publicly available as of the date of publication.

Mice

All mouse experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Brigham and Women's Hospital. Constitutive-Cas9-GFP mice (028555) were purchased from Jackson Laboratory and further crossed with SMARTA (Jackson Laboratory 030450) for *in vivo* screening. *Calca*$^{-/-}$ mice [1] were provided by Hiroki Kurihara (The University of Tokyo, Tokyo) and distributed by RIKEN BioResource Research Center. *Ramp1*$^{-/-}$ mice [2] were provided by Wade Kingery (Vet- erans Affairs Palo Alto Health Care System) with permission by Kazutake Tsujikawa (Osaka University, Osaka). *Ramp3*$^{-/-}$ mice [3] were provided by Kathleen Caron lab (the University of North Carolina at Chapel Hill). *Stat1*$^{flox/flox}$ mice were provided by John O'Shea lab (NIH, U.S.) and further crossed with CD4^{Cre} (Jackson Laboratory 022071).

LCMV infection and plaque assay

1 x 10^6 plaque-forming units (PFU) of Armstrong were injected intraperitoneally (I.P.) for acute LCMV infection. At day 8 of Armstrong infection, splenocytes were isolated and restimulated with LCMV peptide (GP $_{61-80}$ or GP $_{33-41}$) or PMA/ionomycine in the presence of GolgiPlug, Golgi-Stop for intracellular cytokine analyses. IA^b-GP$_{66-77}$ and H-2D^b-GP$_{33-41}$ tetramers (provided by NIH etramer Core Facility) were used for antigen-specific analyses. Vero cells were cultured on 6-well plates for 24 hours before doing viral load assay. Viral load was detected by incubating cultured Vero cells with diluted homogenized tissue samples for 1 hour followed by agarose overlay. After four days of culture, Vero cells were stained with neutral red, and plaques were quantified 14 hour later.

T cell isolation and *in vitro* differentiation

Naïve CD4$^+$ T cells were isolated from the spleen and peripheral lymph nodes (pLN) of WT or Cas9-GFP mice followed by flow cytometry sorting for CD4$^+$ CD25$^-$ CD44$^-$ CD62L$^+$ cells. Sorted

naïve $CD4^+$ T cells were activated with plate-bound anti-CD3 (1μg/ml, clone 145-2C11, Bio X Cell) and anti-CD28 (1μg/ml, clone PV-1, Bio X Cell) and further polarized with cytokines for Th1 (IL-12, 20ng/mL, R&D Systems) or Th2 (IL-4, 20ng/mL, R&D Systems). For Th1/Th2 dichotomous culture, medium was supplemented with both IL-12 (0.2ng/mL) and IL-4 (0.2ng/mL).

Bulk RNA-Seq time course

In vitro cultured T cells were collected at each time point into RLT-plus buffer, and RNA was extracted with RNeasy Plus Mini Kit (Qiagen). Extracted RNA was further used for cDNA amplification with the SMARTA-Seq2 protocol [4] for 12 cycles. cDNA was further sonicated, and sequencing libraries were prepared using a NEBNext Ultra II RNA Library Prep Kit for Illumina (NEB) and sequenced by HiSeq X with 2 x 150 paired-end reads.

Bulk RNA-seq data analyses

Sequenced reads were trimmed for adaptor sequences and amplification primers, and masked for low-complexity or low-quality sequence with Trimmomatic V0.30 [5]. Clean sequencing reads were mapped to the mouse genome version mm10 using bowtie2 v2.3.4.3 [6]. Fragments Per Kilobase of transcript per Million mapped reads (FPKM) were calculated using cufflinks V2.0.2 [7]. Differentially expressed genes (DEGs) were defined with DESeq2 [8] unless otherwise noted, , and false discovery rates (FDRs) were was computed using the Benjamini-Hochberg Procedure for multiple hypotheses testing correction. Gene sets were tested for enrichment in Gene Ontology terms using Enrichr [9-11]. Gene set enrichment scores and p-values were computed with GSEA [12,13].

T cell isolation after LCMV infection for scRNA-seq

At day 8 of Armstrong infection, splenocytes were isolated and sorted for activated $CD4^+$ T cells ($CD4^+$, $CD44^+$) and naïve $CD4^+$ T cells ($CD4^+$, $CD44^-$, $CD62L^{high}$) separately. Activated $CD4^+$ T cells and naïve $CD4^+$ T cells were mix with 10:1 ratio before doing single cell RNA-seq (10x Genomics).

Single cell RNA-seq

In vitro cultured T cells or *in vivo* isolated T cell populations were sorted and incubated with hashtag antibodies (BioLegend). 8 samples labeled with distinct hashtag antibodies were mixed

with equal cell numbers for each channel (10x Genomics). Libraries for *in vitro* samples were prepared using the Chromium Single Cell 5' Library & Gel Bead Kit (10x Genomics), and for *in vivo* samples using the Chromium Single Cell 3' Library & Gel Bead Kit (10x Genomics). All libraries were sequenced on a HiSeq X (Illumina) with paired-end reads of 28 cycles for read 1 and 91 cycles for read 2.

Single cell RNA-seq data preprocessing

Single cell RNA-seq reads were demultiplexed and aligned to the mm10 mouse reference genome using 10x Genomics Cell Ranger V2.2.0 [14] (10x Genomics). 5,774 single cells were detected for LCMV infection $CD4^+$ T cell dataset, and 4,403 high quality cells were retained after quality control (at least 1200 and no more than 3000 genes were detected in a cell (UMI count > 0). LCMV Armstrong infection data were normalized using log normalization (log1p counts per 10,000), and top 2000 highly variable genes were identified using the FindVariableFeatures function in Seurat V4.0.2 [15]. The RunPCA function was applied to the z-normalized expression of the highly variable genes, and the top 20 PCs were used for downstream analyses.

Single cell RNA-seq clustering and differential expression analysis

Cell clusters were identified using the FindNeighbors and the FindClusters (resolution = 0.5) functions in Seurat, which constructed a *k*-nearest neighbor (*k*-NN; *k*=30) graph for cells in the PC space and then optimized the modularity function to identify clusters with the smart local moving (SLM) community detection algorithm [16]. A Uniform Manifold Approximation and Projection (UMAP) embedding was computed using the RunUMAP function with the top 20 PCs. Specifically, min.dist and n.neighbors were set to 0.5 and 50 respectively to better preserve the global structure. Differentially expressed genes for each cluster were identified using the FindAllMarkers function in Seurat, using Wilcox rank-sum test comparing cells in each cluster to cells in all other clusters, and false discovery rates (FDRs) were computed using the Benjamini-Hochberg Procedure for multiple hypotheses testing correction (**Table S2**). Default parameters were used in the analyses above unless otherwise noted.

Pseudotime and trajectory analysis

Trajectory and pseudotime for cells of interest (clusters 0-4) were inferred by Slingshot [17] with the top 2 UMAP dimensions (results were very similar to using top 20 PCs; data not shown). Slingshot identifies global cell fate structure by constructing a minimum spanning tree on cluster centers, and then fitting simultaneous principal curves to obtain smooth trajectory curves and inferring pseudotime.

Signature scores in single cell profiles

Signature scores were computed using the AddModuleScore function in Seurat and cell-cycle scoring was performed using the CellCycleScoring function in Seurat. Cell-cycle genes provided by Seurat in the cc.genes object were used.

sgRNA library construction

3 sgRNAs targeting each selected candidate gene were designed using the online design tool (https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design). sgRNA library for pooled screening were generated and cloned into lentiviral vector at the Brie library from the Genetic Perturbation Platform at the Broad Institute. As a quality control, a deep sequencing analysis was performed showing a good representation of all sgRNAs in the pooled plasmids (data not shown). For lentivirus production, HEK293T cells were transfected with sgRNA library together with pMD2.G (Addgene 12259#) and psPAX2 (Addgene 12260#) using PolyJet (SignaGen Laboratories). At 48-hour post-transfection, cell culture supernatant was collected and frozen down at -80°C for following transduction.

***In vitro* screening**

On day 0, naïve T cells were isolated from Cas9 mice and activated with plate-bound anti-CD3 (1µg/ml, Bio X Cell) and anti-CD28 (1µg/ml, Bio X Cell) in the absence of polarization cytokines for 24 hours. On day1, spin transduction was conducted at 1,000g for 1 hour at 37°C with lentivirus-sgRNA library. After transduction, cells were rested with IL-2 (10ng/ml, Miltenyi Biotec) for 2days before restimulation with cytokines for another 2 days to get Th1 (IL-12^{+}) or Th2 (IL-4^{+}) cells. On day5, intracellular cytokine staining (IFNγ, clone XMG1.2, Biolegend; IL-13, clone eBio13A, eBioscience) was performed using the BD Fixation/Permeabilization Solution Kit (BD Biosciences) after stimulating cells for 4 hours with the PMA/Ionomycin (Sigma-Aldrich)

plus protein transport inhibitors (BD Biosciences). According to the expression of IFNγ or IL-13, four distinct cell populations (negative, low, intermediate (IM), or high) were sorted from the transduced cells (Vex^+) for sgRNA abundance sequencing. As a control sample without phenotypic selection, all Vex^+ cells were sorted for comparison.

***Ex vivo* and *in vivo* screens**

LSK cells from Cas9-SMARTA mice were isolated, transduced with sgRNA library lentivirus and then transferred to irradiated recipient mice as previously described [18]. After 8 weeks of immune reconstitution, Naïve $CD4^+$ T cells expressing both Cas9 and the sgRNA Vex reporter (Vex^+) were isolated from chimeric mice for *ex vivo* and *in vivo* cell differentiation. For *ex vivo* screens, sorted naïve $CD4^+$ T cells were differentiated *in vitro* for 3 days with plate-bound anti-CD3 / anti-CD28 in the presence of IL-12 (20ng/mL, R&D Systems) for Th1 or IL-4 (20ng/mL, Miltenyi Biotec) for Th2. Intracellular cytokine (IFNγ or IL-4) staining was performed on day3 with well differentiated Th1 / Th2 cells for sorting distinct populations (negative, low, intermediate (IM), or high). For *in vivo* screens, sorted Naïve $CD4^+$ T cells were transferred to WT recipient mice on day-1 followed by LCMV Armstrong infection at day0. Activated transferred T cells ($CD4^+$, $CD44^+$, $Cas9^+$ and Vex^+) were isolated from the spleen and sorted for distinct cell populations (negative, low, intermediate (IM), or high) of Th1 ($IFN\gamma^+$) and Tfh ($PD\text{-}1^+$ & $CXCR5^+$) cells at day8 post infection.

sgRNA cassette sequencing and data analysis

To evaluate the sgRNA abundance in each sorted cell population, the genomic DNA was extracted from each sample with QIAamp DNA FFPE kit (QIAGEN). The sgRNA cassette was amplified and sequenced with help from the Genetic Perturbation Platform at the Broad Institute. Sequencing reads were analyzed with PoolQ (Version 3.3.2) to quantify read counts for each sgRNA from the pooled library in a sorted cell population. Raw read counts were further normalized by the number of total sequencing reads in each sample, as follows: $\log_2$(Counts_for_each_sgRNA / Total_counts_in_PCR_well * 10^6 + 1). To evaluate the sgRNA depletion / enrichment, normalized sequencing depth for each sgRNA was further compared with the control sample (all Vex^+ cells) to calculate a Log_2 fold change (LFC) score. To compare the sgRNA abundance across distinct

sorted cell populations (IFNγ^{high}, IFNγ^{IM}, IFNγ^{low}, IFNγ^{neg}), a delta LFC value (LFC^{high} + LFC^{IM} - LFC^{low} – LFC^{neg}) was further calculated for each guide RNA.

ATAC-seq

ATAC-seq was performed following the Omni-ATAC protocol [19]. 10,000 cells were sorted for each ATAC-seq sample preparation. Sequencing libraries were barcoded using Ad1 and Ad2 primers and were sequenced on HiSeq X (Illumina) with 150bp paired-end reads.

Ad1: AATGATACGGCGACCACCGAGATCTACACTCGTCGGCAGCGTCAGATGTG

Ad2: CAAGCAGAAGACGGCATACGAGATxxxxxxxxGTCTCGTGGGCTCGGAGATGT

Sequenced reads were trimmed for adaptor sequence and low-quality sequence with Trimmomatic V0.30 [5], and mapped to reference mouse genome (version mm10) using bowtie2 v2.3.4.3 [6]. Samtools [20] were used to remove PCR duplicates, sort and index the bam files.. Peak calling was performed with MACS2 [21] and peaks were visualized using Integrative Genomics Viewer (IGV) [22].

Integrative analysis of ATAC- and RNA-seq data

Paired bulk ATAC- and RNA-seq for 4-hour *in vitro* cell differentiation (Th1 or Th2 condition) with or without CGRP treatment were analyzed together. Differential chromatin accessibility was determined using the csaw package [23], generating 50-bp sliding windows along the whole genome, and counting the number of reads overlapping the windows using the windowCounts function. The windows were then filtered using the local enrichment method: for each window, reads were counted in its 1,000bp surrounding neighborhood (+/-500bp from the window center) using the regionCounts function, the increase in read abundance in the 50bp window *vs*. the 1,000 bp neighborhood was calculated using the filterWindowsLocal function, and windows were excluded from downstream analysis if the increase is less than 3-fold. Specifically, read counts (both windowCounts and regionCounts function) were computed using paired-end reads with read-pair fragment length not exceeding 650bp, discarding alignments in the ENCODE Blacklist regions [24] or with mapping quality scores lower than 10. Count data were then normalized across samples using the offset normalization method, and tested for differential accessibility using edgeR (glmQLFit and glmQLFTest) [25]. Window level differential accessibility results for consecutive windows were combined using the mergeResults function. Merged regions were annotated with

ChIPseeker [26] using the TxDb.Mmusculus.UCSC.mm10.knownGene [27] and org.Mm.eg.db [28] databases. Differentially accessible regions (**Figure 6H**) were clustered using hierarchical clustering (Euclidean distance on row-scaled normalized accessibility, Wald ("ward.D" in the hclust function in the R stats v4.1.2 package) agglomeration method). Samples were clustered in the same way. Default parameters were used unless otherwise specified.

Differential expression analysis was performed using edgeR (glmQLFit and glmQLFTest). Only genes detected (>=10 reads) in all samples of at least one group (Th1/Th2 and Vehicle/CGRP combination) were used. Default parameters were used unless otherwise specified.

Quantification and statistical analysis

Statistical analysis of non-sequencing data was performed with the GraphPad Prism software (GraphPad, version 9.2.0). Unless otherwise specified, data are presented as the mean with ± S.D. and statistical significance was determined using unpaired two-tailed t test. P values < 0.05 are considered significant (ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

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