Supplemental Experimental Procedures

Tissue lymphocyte isolation

For lymphocyte isolation from liver and lung, mice were perfused with PBS and tissues were digested with collagenase A (1mg/ml) and Dnase I (1 unit/ml) for 45 minutes at 37 degrees. For isolation of lamina propria lymphocytes, small and large intestines were first incubated with 1mM EDTA and 1mM DTT in PBS for 15 minutes at 37 degrees. Samples were washed and incubated with collagenase A and Dnase I for 15 min at 37 degrees for two rounds. Digested cell suspensions were filtered and lymphocyte-containing fractions were isolated using differential Percoll centrifugation.

Bone marrow chimeras

A 9:1 mix of T cell-depleted *Foxp3*$^{GFP\text{-}DTR}$ and *Foxp3*$^{GFP\text{-}Cre\text{-}ERT2}$ x *R26Y* bone marrow was injected intravenously (i.v.) into lethally irradiated (950 rad) *Foxp3*$^{GFP\text{-}DTR}$ recipients. Mice received neomycin (2mg/ml) in their drinking water for 3 weeks following transfer.

LCMV infection

Mice were infected by intraperitoneal injection with $2x10^5$ p.f.u LCMV Armstrong. I-A^b/GP66-77 (GP66) specific CD4 T cells were isolated on day 7 and day 75 p.i. For fate mapping of LCMV-experienced cells, infected *Foxp3*$^{GFP\text{-}DTR}$*CD4*^{CreERT2}R26^{tdTomato} mice were gavaged with tamoxifen on day 0 and day 1 p.i. To assess recall responses of fate-mapped LCMV-experienced cells, Tomato$^+$ effector or Treg cells were isolated on day 60 p.i. and mixed with resting naïve CD4 T cells or resting Treg cells, respectively. The cell mix was labeled with CellTrace^{TM} and $2x10^5$ cells were transferred into congenically marked recipients, infected with LCMV one day post-transfer. Mice were analyzed 11 days p.i.

Maintenance of Foxp3-independent activation signature

Activated GFP^{+} "Treg wannabe" cells expressing a *Foxp3* reporter null allele and lacking Foxp3 protein expression were isolated from CD45.1 *Foxp3^{GFPKO}* males. Activated Treg cells (aTr) were isolated from CD45.1 Foxp3$^{GFP\text{-}DTR}$ mice. Activated *Foxp3^{GFPKO}* or aTr cells were transferred into CD45.2 Foxp3$^{GFP\text{-}DTR}$ recipients that received 0.5ug DT i.p. to induce suboptimal transient Treg cell depletion two days prior to transfer to increase efficiency of engraftment. Cells were isolated one month post-transfer and analyzed by RNA sequencing.

RNA-sequencing

FACS-sorted cell populations were collected in Trizol. RNA was extracted and cDNA libraries were prepared using SMARTer amplification (Clontech). cDNA samples were processed for sequencing on Illumina Hiseq.

Chromatin immunoprecipitation and sequencing

FACS-sorted cell populations ($1x10^5$ cells) were crosslinked in 1% paraformaldehyde for 5 minutes at RT. The reaction was quenched by addition of 125mM glycine. Crosslinked cells were lysed and partially digested with micrococcal nuclease (12,000 units/ml) for 1 minute at 37 degrees. Digestion was stopped by addition of EDTA and digested nuclei were resuspended in nuclear lysis buffer containing 1% SDS. Following sonication, chromatin was incubated overnight with antibodies against H3K27me3, H3K27Ac, or H3K4me1 and immunocomplexes were precipitated with protein A Dynabeads. After washing, precipitated chromatin was decrosslinked overnight at 65 degrees in the presence of proteinase K and DNA fragments were isolated using Qiagen PCR-purification kit. Purified DNA was processed for sequencing on Illumina Hiseq.

ATAC-sequencing

ATAC-seq libraries were prepared as previously described (Buenrostro et al., 2013). FACS-sorted cell populations ($5x10^4$ cells) were lysed and accessible chromatin was transposed using Nextera Tn5 transposase. Transposed DNA fragments were isolated using Qiagen MinElute kit and amplified 5-10 cycles using Nextera PCR primers. ATAC-seq libraries were submitted for paired end sequencing on Illumina Hiseq.

Processing and analysis of ATAC-seq data

Starting from fastq files containing ATAC-seq paired-end reads, sequencing adaptors were removed using Trimmomatic (Bolger et al., 2014). Trimmed reads were mapped to the mm9 mouse genome using Bowtie2 (Langmead and Salzberg, 2012) allowing at most 1 seed mismatch and keeping only uniquely aligned reads. Duplicates were removed using Picard (http://picard.sourceforge.net). For peak-calling the read start sites were adjusted (reads aligning to the +/- strand were offset by +4bp/-5bp, respectively) to represent the center of the transposon binding-event, as described in (Buenrostro et al., 2013).

For each of the three Treg cell activation states (resting, activated, “memory”), ATAC-seq was run on two biological replicates. Peak calling was performed on each cell type individually by pooling reads from biological replicates, and using MACS2 (Zhang et al., 2008) with a permissive P-value threshold (-p 1e-2). To find a set of peaks that are reproducible across the two biological replicates of a given cell condition, we calculated the per-replicate P-value of each peak using only the read count at the peak from the individual replicate, and estimated the irreproducible discovery rate (IDR) (Li et al., 2011) from the two sets of P-values that the replicates produced. Only peaks with an IDR of 0.5% or less were kept for downstream analyses. This procedure generated a set of reproducible accessible sites for each cell condition. To create a single atlas of accessible sites for the three Treg conditions, we merged peaks from two conditions if their

overlap was 75% or more; if they overlapped by 25% or less, two peaks were kept separate by removing the overlapping region. In this way we created an atlas of 34,326 accessible sites (or peaks) that were reproducible in at least one cell condition. To link site accessibility to regulation of gene expression, we associated each peak to its nearest gene in the mouse genome.

ChIP-seq analysis

Histone modification ChIP-seq datasets for H3K4me1, H327ac and H3K27me3 were generated in the three Treg conditions. These signals were used to quantify chromatin dynamics at ATAC-seq peaks in the atlas and also at genes. Quality controls and signal processing were implemented as described elsewhere (Gonzalez et al., 2015). Briefly, strand cross-correlation was used to assess signal quality and to estimate average fragment length in each library. Histone modification read counts at peaks and genes were calculated after shifting reads (in a strand-dependent manner) by half the estimated fragment length.

For analysis of H3K27me3 deposition at Foxp3 bound sites, we first called high-confidence H3K27me3 peaks for each cell type using an IDR cutoff of 0.1. The number of HK27me3 reads mapped to each peak was calculated per replicate and the distance from it to the nearest Foxp3 peak was found. The scatterplots of HK27me3 fold change were drawn for the peaks no farther than 40 kb from a a site highly bound by Foxp3 (normalized read count >50). To draw figures, the function smoothScatter in the graphics library of R was used, using two-dimensional kernel smoothing with 256 equally sized bins and the default transformation function of x^0.25. Dotted red vertical lines were drawn by loess smoothing using the R function loess.smooth with default parameters.

Analysis of RNA-seq data

Adaptor-trimmed reads were mapped to the mm9 mouse genome using STAR (Dobin et al., 2013). Uniquely aligned reads were counted at all exons of a gene with the htseq-count function of the HTSeq Python package (Anders et al., 2015),

and count values were transformed to reads per kilobase per million (RPKM). For each sample in consideration, a gene was considered present in the sample if it had 1 RPKM or more. Only genes present in at least one sample were kept for further analyses. Differential gene expression between conditions was quantified with DESeq2 (Love et al., 2014).

Classification of stable and transient gene and peak groups

We defined groups of genes according to the dynamics of their expression levels in three conditions (resting, activated, "memory"). For that, we evaluated the three possible contrasts: resting vs. activated; resting vs. "memory"; and activated vs. "memory." After fitting the RNA-seq counts to a generalized linear model (GLM) using DESeq2, genes were assigned to a group when the GLM assigned differential expression with statistical significance (FDR ≤ 5%) to at least two of the three contrasts. For genes with two statistically significant comparisons, the criteria were defined as shown below:

Transient Up: resting to activated, FDR ≤ 5%, logFC > 0 AND activated to "memory," FDR ≤ 5%, logFC < 0
Transient Down: resting to activated, FDR ≤ 5%, logFC < 0 AND activated to "memory," FDR ≤ 5%, logFC > 0
Stable Up: resting to activated, FDR ≤ 5%, logFC > 0 AND resting to "memory," FDR ≤ 5%, logFC > 0
Stable Down: resting to activated, FDR ≤ 5%, logFC < 0 AND resting to "memory," FDR ≤ 5%, logFC < 0

For genes with three statistically significant comparisons, the average expression value per condition was calculated from the expression level normalized by the library size. To determine which two were placed closer together than the third, the geometric mean of the minimum and the maximum was compared with the median.

Transient Up: activated is the maximum, geometric mean > median
Transient Down: activated is the minimum, geometric mean < median
Stable Up: resting is the minimum, geometric mean < median
Stable Down: resting is the maximum, geometric mean > median

Genes selectively up- or downregulated in "memory" cells were rare and these groups were not analyzed further. A similar procedure was followed to find groups of ATAC-seq peaks and to classify gene expression changes in cells responding to LCMV infection. Enrichment of biological process gene ontology (GO) terms were calculated using the Bioconductor GOstats package (Falcon and Gentleman, 2007), with all the present genes used as background.

Supplemental References

Anders, S., Pyl, P.T., and Huber, W. (2015). HTSeq--a Python framework to work with high-throughput sequencing data. Bioinformatics *31*, 166-169.

Bolger, A.M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics *30*, 2114-2120.

Buenrostro, J.D., Giresi, P.G., Zaba, L.C., Chang, H.Y., and Greenleaf, W.J. (2013). Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. Nat Methods *10*, 1213-1218.

Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., and Gingeras, T.R. (2013). STAR: ultrafast universal RNA-seq aligner. Bioinformatics *29*, 15-21.

Falcon, S., and Gentleman, R. (2007). Using GOstats to test gene lists for GO term association. Bioinformatics *23*, 257-258.

Gonzalez, A.J., Setty, M., and Leslie, C.S. (2015). Early enhancer establishment and regulatory locus complexity shape transcriptional programs in hematopoietic differentiation. Nat Genet.

Langmead, B., and Salzberg, S.L. (2012). Fast gapped-read alignment with Bowtie 2. Nat Methods *9*, 357-359.

Li, Q., Brown, J.B., Huang, H., and Bickel, P.J. (2011). Measuring reproducibility of high-throughput experiments. The Annals of Applied Statistics.

Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol *15*, 550.

Zhang, Y., Liu, T., Meyer, C.A., Eeckhoute, J., Johnson, D.S., Bernstein, B.E., Nusbaum, C., Myers, R.M., Brown, M., Li, W.*, et al.* (2008). Model-based analysis of ChIP-Seq (MACS). Genome Biol *9*, R137.