## SI MATERIALS AND METHODS

### Mice

Mice were maintained in our specific-pathogen-free facility at Harvard Medical School. All mice were maintained on the B6 genetic background.

### Cell isolation and flow cytometry

Mice were asphyxiated with $CO_2$ and perfused with 30ml of phosphate-buffered saline (PBS) containing 5mM ethylenediaminetetraacetic acid (EDTA). VAT was digested for 20 min with 1.5 mg/ml collagenase type II (Sigma) in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2% fetal calf serum (FCS); liver was digested in DMEM containing 0.5mg/ml collagenase IV (GIBCO) and 150 ug/ml DNase I (Sigma) for 30 min; skin was digested in DMEM containing 2mg/ml collagenase type II (Sigma), 150 ug/ml DNase I (Sigma), and 0.5mg/ml hyaluronidase (Sigma) for 1 hour. The digested materials were filtered through a 100um nylon cell strainer and centrifuged at 450g for 10 min. For liver, cells were resuspended in 36% isotonic percoll in PBS and centrifuged at 800g for 10min to separate lymphocytes from hepatocytes. After the supernatant was removed, the cells were resuspended in DMEM 2% FCS and centrifuged a second time for 500g for 5min. For liver and adipose, red blood cells were lysed with ACK buffer and samples were washed in DMEM 2% prior to being resuspended in FACS buffer for staining.

Cells were stained with anti-CD45.1 (A20), -CD45.2 (104), -CD3 (17A2), -CD4 (GK1.5), -KLRG1 (2F1/KLRG1) (all from Biolegend) or anti-ST2 (RMST2-2) (eBioscience)

mAbs. For Foxp3 staining, cells were fixed, permeabilized and intracellularly stained (FJK-16s, eBiosciences) according to the manufacturer's instructions. Data were analyzed using FlowJo software.

**Single-cell studies**

For coupled scRNA-seq and sc*Tcr*-seq: cells stained for multiplexed analysis using TotalSeq hashing antibodies (BioLegend). Encapsulation, RNA and hash library construction, and sequencing were performed by the Broad Institute Genomic Services.

**scRNA-seq analysis**

Cellranger was used to align and demultiplex the scRNA-seq data. Quality control measures were as follows: ~80,000 mean reads per cell and ~800 median genes expressed per cell for the initial 3' scRNA-seq data of splenic Treg populations; ~25,000 mean reads per cell and ~1,200 median genes expressed per cell for the subsequent 5' scRNA-seq data of nonlymphoid-tissue Tregs and splenic Tregs. Hashed samples were demultiplexed using custom python scripts by finding genes with high reads of their respective hashing barcode and removing cells with no hash or multiple hash reads. Cells were filtered for quality control to avoid doublets and dead cells using the following metrics: for the initial 3' dataset from splenic Treg populations, we analyzed cells that had a minimum of 250 and a maximum of 2500 expressed genes, and a maximum of 10% reads from mitochondrial genes; for the subsequent 5' dataset from splenic and tissue-Treg populations, we analyzed cells that had a minimum of 250 and a maximum of 2500 expressed genes, a minimum of 250 and maximum of 22,000 UMIs, and a maximum of

8% reads from mitochondrial genes. Counts were normalized using the scran package in R. Dimensionality reduction, visualization and clustering analysis were performed within the Seurat or scanpy package. After clustering, additional low-quality cells that clustered together and had higher percentages of mitochondrial genes and lower numbers of expressed genes were removed.

For sc*Tcr*-seq, the *Tcr*-seq library was analyzed with the cellranger vdj pipeline (v3.10) using default parameters. This pipeline was used for the assignment of V and J genes, CDR3 regions and sequences. Cells with a productive TCR that contained a full V-J spanning read were annotated with their distinct clonotype ID by cellranger. Quality-control measures were as follows: ~16,000 mean reads per cell, and ~5,000 cells with an identified productive V-J spanning read. Data were filtered to analyze cells only with both an alpha and beta chain identified. Cells were called clones and assigned unique clonotype IDs if they shared exactly the same alpha and beta chain nucleotide sequence. The cell barcode was then used to match the clonotype ID to the gene expression matrix generated by the scRNA-seq analysis described above. Details of the identified TCR sequences are listed in Dataset S2.

**Generation of tissue-Treg gene-expression signatures**

RNAseq from a previously published dataset was used to generate these signatures {10829}. Counts were normalized and differential expression analysis was performed with edgeR. Unique gene signatures were defined as those genes upregulated only in their respective tissue-Treg population over lymph node Tregs with a fold-change > 2 and an FDR < 0.1. Tissue scores were calculated using the score_genes function in

the scanpy package. Briefly, this function calculates a gene-expression score as the average expression of a set of genes after subtraction of the average expression of a reference set of genes. The reference set is randomly sampled from all the genes expressed in that cell, randomly choosing 50 genes in each bin for 25 binned expression values.

**RNA-velocity analysis**

Briefly, the spliced and unspliced counts were calculated from the original sequencing data using the RNA velocity package. The spliced and unspliced expression matrices were then integrated into the single-cell data matrix, and scvelo was used to calculate the velocity vectors using the dynamical model of transcriptional dynamics and splicing kinetics. The calculated velocities were then projected onto the tSNE embedding calculated during the scRNA-seq analysis.

**Lineage-inference analysis**

The lineage inference analysis {11476} was performed using the CellRank package in python. Briefly, the single-cell RNA velocity analysis was used in CellRank to find the initial and terminal states in the data automatically. The transition probabilities into these terminal states were calculated for each individual cell. Finally, a latent time (pseudotime) was inferred using scvelo from the initial and terminal states identified by CellRank, which is used to draw a lineage trajectory based on the transition probabilities and plotted using a PAGA plot in the original tSNE embedding.

## SI REFERENCES

## SI FIGURE LEGENDS

**Figure S1: Expression of additional egress-associated transcripts by the splenic PPARγ$^{lo}$ Treg population.**

Violin plots of up-regulated (A) and down-regulated (B) transcripts

**Figure S2: Expression of the pan-tissue Treg signature in splenic Treg cell clusters.**

Heatmap showing expression of genes in the pan-tissue-Treg up-signature. Color-code to the right.

**Figure S3: Sharing of TCR sequences between the PPARγ$^{lo}$ putative precursor populations in the spleen**

Pie charts depicting cell clones shared between the two putative precursor tissue-Treg populations in the spleen. Offset pie slices and texts below the pies indicate the number and frequency of splenic Tregs in precursor Y (*left*) and precursor X (*right*) clusters that share CDR3-encoding *Tcra* and *Tcrb* sequence with the opposite precursor population. Shared clones are shown in individual colors; non-shared clones are colored in grey. Colors shared across the splenic subpopulations do not connote shared sequences

**Figure S4: Adoptive transfer of PPARγ$^{lo}$ Tregs – an independent repeat**

5x10$^4$ PPARγ$^{lo}$ Tregs from spleen and lymph nodes of 6- to 8-week-old CD45.1$^-$2$^+$ *Pparg-Tdt.Foxp3-Gfp* mice were sorted and transferred into 10-week-old CD45.1$^+$2$^+$ *Foxp3-Dtr*

recipients treated with DT on the day before, of and after the transfer. Summary plot showing frequencies of donor-derived cells among Tregs in the indicated organs. Mean +/- SD.

Fig. S1

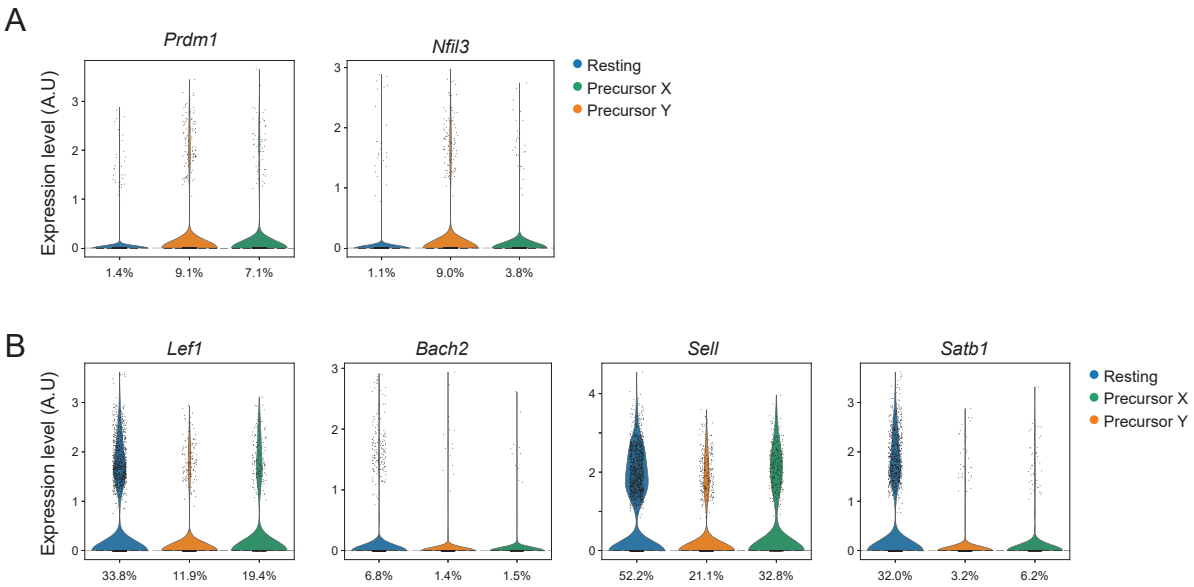
A
Prdm1
Nfil3
Expression level (A.U)
Resting
Precursor X
Precursor Y
1.4%
9.1%
7.1%
1.1%
9.0%
3.8%
B
Lef1
Bach2
Sell
Satb1
33.8%
11.9%
19.4%
6.8%
1.4%
1.5%
52.2%
21.1%
32.8%
32.0%
3.2%
6.2%

Fig. S2

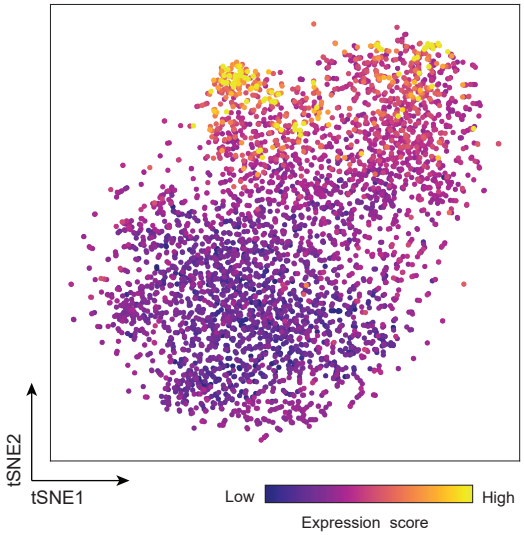

Fig S3

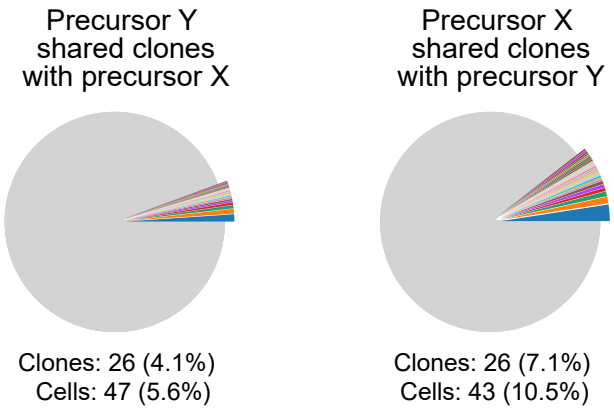
Precursor Y
shared clones
with precursor X
Clones: 26 (4.1%)
Cells: 47 (5.6%)
Precursor X
shared clones
with precursor Y
Clones: 26 (7.1%)
Cells: 43 (10.5%)

Fig S4

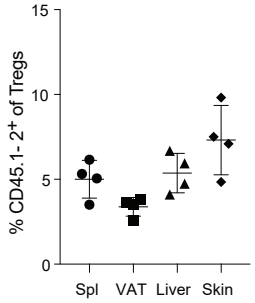

**Table S1**

| | Precursor Y vs Resting | Precursor X vs Resting |
|---|---|---|
| **VAT Treg Signature** | 3.44E-148 | 8.37E-83 |
| **Skin Treg Signature** | 2.43E-189 | 1.81E-90 |
| **Liver Treg Signature** | 1.45E-44 | 1.05E-52 |
| **Pan-tissue Treg Signature** | 0.00E+00 | 3.88E-250 |

**Table S1: p-values for the comparison of tissue-Treg scores between splenic Treg populations.**

p-values for the comparison between Resting Tregs and the two putative precursor populations in the spleen. Wilcoxon rank-sum test.

## DATASET LEGENDS

**Dataset S1: List of tissue-Treg gene signatures.**

Excel spreadsheet containing the list of genes for the VAT, liver, skin, and pan-tissue Treg signatures, derived as described in the Materials and Methods.

**Dataset S2: TCR sequences for Treg clones.** Excel spreadsheet containing a list of all identified clones in the sc*Tcr*-seq dataset. Dataset contains the clone name, the number of cells sharing the same sequence for each tissue Treg and precursor compartment, the V and J genes, CDR3 length, amino acid sequence and nucleotide sequence of the identified alpha and beta chains. Further details on the TCR analysis can be found in the SI Materials and Methods.