**Functional Asymmetry of Cardiac Macrophages in Left and Right Atria**

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**METHODS**

**Animals and nuclei isolation for single nucleus RNA sequencing**

Atrial tissue was acquired from 2 male and 2 female adult mice with Lats1/2flox/flox alleles that served as a wild type control in a separate study. Atria were minced in lysis buffer provided by the 10X Genomics Nuclei Isolation Kits. Samples were then dissociated with pestles and filtered with Nuclei Isolation Columns. The flowthroughs were resuspended with Debris Removal Buffer and spun down at 700 x g for 10 minutes. The supernatant was removed, and the nuclei pellet was resuspended and washed with PBS with RNase inhibitor before nuclei concentration measurement. Isolated nuclei were then incubated with transposase to preferentially fragment the open regions of the chromatins and add adapter sequence to the ends of the DNA fragments. Next, transposited nuclei were loaded onto the 10X Genomics Chromium Controller to obtain the gel beads in emulsion. The sequencing libraries were prepared according to the 10X genomics protocol for Chromium Single Cell Gene Expression Kits and libraries were sequenced on an Illumina NovaSeq6000 (Novogene Corporation Inc, Sacramento, CA). Mice with Lats1/2flox/flox; Cx3cr1GFP/+; Ccr2RFP/+ (Ccr2–/–, KO) and wild type controls were used for the Ccr2-KO scRNA-seq experiment. Whole hearts were harvested from adult mice (8 weeks old) under anesthesia, perfused with ice-cold saline containing potassium chloride, and then finely minced. The tissue was enzymatically digested with Type II collagenase (Worthington, LS004177) to generate a single-cell suspension, after which cardiomyocytes were removed using a 20‑μm filter and red blood cells were lysed with ACK buffer. The remaining cells were washed and incubated with Dead Cell Removal MicroBeads (Miltenyi Biotec, 130‑090‑101) before passing through a column to collect unlabeled (live) cells. These live cells were then labeled with CD45 MicroBeads (Miltenyi Biotec, 130‑052‑301), washed, and passed through a magnetic column, yielding CD45+ cells. For scRNA-seq, approximately 10,000 CD45+ cells per sample were processed using the 10X Genomics Chromium platform. cDNA libraries were prepared following the manufacturer’s protocol and sequenced on an Illumina platform. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Baylor College of Medicine.

**SnRNA-seq and scRNA-seq data processing**

Single-nucleus data generated on the 10X Genomics platforms were processed using CellRanger v7.1.0 with default settings. All reads were aligned to the mm10 mouse reference genome. To remove background signals from ambient transcripts, we further processed the raw UMI count matrices by CellBender version v0.1.0 18 (cellbender remove-background) with --total-droplets-included = 25000, --low-count-threshold = 15, and --epochs = 200. To minimize the loss of valid cell barcodes called by CellRanger, we also set --expected-cells at 1.5 times of CellRanger output nuclei number. The output matrices from CellBender were filtered to include only valid cell barcodes identified by CellRanger. This allowed for removing ambient RNA background molecules and random barcode swapping from (raw) scRNA-seq gene-by-cell count matrices. To minimize the loss of valid cell barcodes called by CellRanger, we also set --expected-cells for CellBender at 1.5 times of output nuclei number. Output matrices were filtered to only include valid cell barcodes also identified by CellRanger. Additional quality controls at the single nucleus level were performed for each library. We first identified low-quality nuclei based on fixed cut-offs of UMI count per nucleus > 500, gene count per nucleus > 200 and mitochondria gene-derived UMI < 5%. Then, a set of cut-offs based on per-library data distribution was calculated, which is essential to account for heterogeneity between samples. In brief, for each library, an upper and lower bound were set at the 75th percentile plus 1.5 times the interquartile range (IQR) and the 25th percentile minus 1.5 times IQR, respectively, for UMI count and gene count per nuclei. All nuclei that failed to meet these criteria were subsequently removed from the datasets. Dynamic cut-off values were calculated based on the per-library data distribution to account for inherent heterogeneity across samples. For each library, upper and lower bounds were established at the 75th percentile plus 1.5 times the IQR and the 25th percentile minus 1.5 times the IQR, respectively, for UMI count and gene count per nucleus. Nuclei outside of the upper and lower bounds were removed from the data sets. Next, the remaining nuclei were evaluated by the Scrublet tool to identify potential doublets, with parameters expected\_doublet\_rate = 0.1 and call\_doublets threshold = 0.25. Seurat Toolkit version v5.0 in R v4.3 was used to perform downstream analyses. The UMI count matrices of cells that passed previous filtering were log normalized. For each sequenced library, we calculated the top 3000 most variable features for dimensional reduction and graph-based clustering. For both snRNA-seq and scRNA-seq, we used the algorithm “FastMNN” to correct batch effect, which allows for accurate integration of single-cell sequencing data by projecting cells into a shared embedding by grouping cell types.

**Gene set expression scoring**

We used AddModuleScore function provided by the Seurat suite to score each cell in snRNA-seq data for the expression of genes in each gene set. AddModuleScore is an implementation of a scoring approach developed by Tirosh et al. In each cell or spot, this function computes a score equal to the mean expression of genes in the given gene set, subtracted by the average expression of genes in a background gene set. For each gene, the method randomly selects 100 background genes with similar average expressions (computed over all cells or spots).

**Spatial transcriptomics**

A heart from a Lats1/2flox/flox control mice was collected for the spatial profiling using 10X Genomics Visium platform. Heart samples were perfused with PBS containing 30 mM KCl, fixed in formalin, and embedded in paraffin. Heart sections were sectioned at 7-µm thickness and placed onto Visium slides (10X Genomics). Mouse whole transcriptome probe (Visium Spatial Gene Expression for FFPE reagent kits, mouse transcriptome) panels were added to the permeabilized tissue. Probes were bonded to spatial barcodes before adding sample indexes and generating libraries. Libraries were then sequenced on an Illumina NovaSeq 6000 with 150 cycle paired-end setting (read 1 = 28 cycles, read 2 = 50 cycles, index 1 = 10 cycles, index 2 = 10 cycles). Preparation and sequencing were performed according to the manufacturer’s protocol (10X Genomics). ST data were integrated using the Seurat workflow as described above. Seurat toolkit version v5.0 in R v4.3 was used to perform integration, quality control, and downstream analysis. Only spots that overlayed on tissue were retained for downstream analyses. The spots were normalized by using the Seurat function SCTransform.

**Reanalysis of available human data**

We reanalyzed human single-nucleus RNA sequencing (snRNA-seq) data, specifically focusing on cardiac macrophages. The reference data used for this analysis were obtained from two sources: Litviňuková et al. in their publication "Cells of the adult human heart" 2. We obtained the data from the Human Cell Atlas data portal (https://www.heartcellatlas.org).

**Pacing induced atrial fibrillation**

Programmed intracardiac stimulation was performed to test AF incidence. Mice were performed to the same atrial burst-pacing protocols three times. If AF episode lasting more than 1 second was evoked by burst pacing in at least two out of three attempts, the mouse was identified as AF positive. The incidence of inducible AF was measured as the percentage of AF-positive mice divided by the total number of mice tested. Atrial fibrillation duration was capped at 5 min for any episodes lasting longer. The operator was blinded to the group information (different genotype or treatment, up to your experiment design) of mice.

**Statistical testing and data presentation**

Statistical analyses of all pacing experiments were performed using Graphpad Prism9 and bioinformatics analyses performed in R. If the data for all groups passed the the Shapiro-Wilk normality test, we used an unpaired two-tailed t-test to test for significance. If the data from any groups did not pass the normality test, the two-sided Wilcoxon rank-sum test was used. Differentially expressed genes from snRNA-seq were filtered using log2 fold-change > 0.25 and Benjamini-Hochberg adjusted P value < 0.05 cut-offs, using the default Wilcoxon rank-sum test. Center lines in all box plots show mean values, and whiskers extend to a maximum of 1.5× interquartile range beyond the boxes. \*P<0.5 and \*\*\*P<0.00001.