SUPPLEMENTAL MATERIAL

Supplemental Methods

**Animals and physiological assessment**—The experiments described in this study were conducted using 8-14 week old female and male wild-type or Cx3cr1<sup>gfp/</sup>+ mice in the C57BL/6J background. Mice were purchased from The Jackson Laboratory (Bar Harbor ME, USA) or the Walter and Eliza Hall Institute (Melbourne, Australia). All experiments performed were approved by The Jackson Laboratory Institutional Animal Care and Use Committee (IACUC) or La Trobe University Animal Ethics Committee (AEC16-93).

Hypertension-induced cardiac hypertrophy and fibrosis experiments were achieved by subcutaneous implantation of osmotic pumps (Alzet, model 2002) loaded with angiotensin II or vehicle. Similar protocols were performed at the two sites, except AngII was administered at either 1.5 or 1.44 mg/kg/day at The Jackson Laboratory or La Trobe University, respectively. Surgical procedures conducted at the Jackson Laboratory were undertaken by The Jackson Laboratory Surgical Services platform. Echocardiography was performed on the Vevo3100 system at The Center for Biometric Analysis at The Jackson Laboratory. For analysis, B-mode and M-mode measurements were utilized in Vevo LAB software (VisualSonics). Conscious blood pressure detection was performed using a MC4000 tail cuff apparatus (Hatteras Instruments, NC, USA) at the La Trobe Animal Research and Training Facility (LARTF).

**Histology and immunostaining**—Hearts were fixed in formalin supplemented with 30 mM KCl (SigmaAldrich) and subsequently were paraffin-embedded (FFPE) then sectioned at 10 µm thickness for histological and immunohistochemical analyses. Masson’s trichrome staining was performed to visualize fibrosis following standard protocols. For fluorescence imaging, sections were stained with antibodies targeting thrombospondin IV (THBS4, 1:200, MAB7860SP, R&D Systems) and smooth muscle actin (ACTA2, 1:500, 55135-1-AP, Proteintech Group). Sections were additionally stained with Wheat Germ Agglutinin (WGA, 1:100, 29022-1, Biotium) and DAPI (1 µg/mL, D9542, Sigma Aldrich) before acquiring confocal micrographs. Brightfield or fluorescence micrographs were subsequently analyzed using Fiji software for quantification of tissue area and fibrosis (Masson trichrome images) and generation of composite fluorescence images.

**Cardiac tissue thick section immunostaining**—Cardiac thick sections were prepared and stained based on previously described protocols [40]. Briefly, hearts were fixed in 4% (w/v) formaldehyde and 150 µm sections prepared. After permeabilization (0.5% Triton X-100/PBS) and blocking, sections were stained with antibodies targeting mannose receptor C-type 1 (Mrc1;1:200, CO68C2, BioLegend) and GFP (1:200, ab13970, Abcam). Sections were additionally stained with Wheat Germ Agglutinin (WGA, 1:100, 29022-1, Biotium) and DAPI (1 µg/mL, D9542, Sigma Aldrich) before acquiring confocal micrographs. Micrographs were subsequently processed using Imaris software (Bitplane). Supplemental video 1 was prepared using Imaris and annotated using Inkscape (https://inkscape.org) and Blender (http://www.blender.org) software packages.
Simultaneous single cell and single nucleus isolation and sequencing—For simultaneous isolations of cardiomyocytes and non-myocytes, we adapted a protocol previously described for perfusion-based dissociation of the heart [8]. Recipes for all buffers used can be found in the aforementioned protocol with the following modifications, 2,3-butanedione monoxime (BDM) was replaced with 5mM blebbistatin (SigmaAldrich) and [0.1U/µL] murine RNase inhibitor (NEB) was also supplemented. One hour prior to tissue harvest, mice were administered an intraperitoneal heparin injection at 1 mg/kg bodyweight. Following euthanasia of mice by CO₂ asphyxiation, thoracic cavities were opened and inferior vena cava cut to allow exsanguination, before injecting 7 ml of EDTA buffer. Following perfusion, aortas were clamped using a hemostat and cut to extract hearts. The hemostat was subsequently placed on a 3D-printed platform (Supplemental Figure 27) enabling hearts to be suspended above a waste fluid collection reservoir, with apex of the heart pointing upwards. To perfuse fluid, a hypodermic needle, connected to a peristaltic pump through the 3D-printed platform (Supplemental Figure 27) which enabled it to be orientated above the heart, was inserted to the left ventricular chamber through the cardiac apex. Once the hearts were cannulated, EDTA buffer was pumped through using a peristaltic pump (~1 ml/heart/min) until blood cleared (~5 min) before perfusion with enzyme digest cocktail for 15 minutes, during which time cardiac tissue was basted with fresh digestion buffer to prevent desiccation of the tissue. All buffers were maintained at 37°C during perfusion.

Following tissue digestion, cardiac ventricles were isolated by removing aorta and atria, and the ventricular digestion was finished by gently pulling apart the tissue before a 5-minute incubation at 37°C followed by trituration with a 1000 µL pipette. The cell suspension was then passed through a 250 µM filter to remove large undigested debris and brought to 15 mL with cold perfusion buffer supplemented with 5% FBS in a conical tube. Three consecutive minute-long centrifugation sedimentations at 50g, 4°C were performed to separate cardiomyocytes and non-myocytes based on cell mass. The supernatant from each sedimentation step was collected and pooled in a 50 mL tube which was then centrifuged at 400 g, 4°C for 5 minutes to pellet the non-myocyte fraction of cells. Whole intact non-myocyte cells then underwent antibody and viability dye staining before flow cytometry or fluorescence-activated cell sorting (FACS), as described previously [4].

Nuclei extraction—Cardiomyocyte nuclei were isolated by swelling cardiomyocytes in a hypotonic solution (91 mM NaCl, 5.3 mM KCl, 0.5 mM MgCl₂, 0.293 mM CaCl₂, 10 mM glucose, 10 mM HEPES at pH 7.4) before trituration using a 30-gauge hypodermic needle to mechanically fragment cells. Nuclei were then isolated by filtration through a 20-µm cell strainer (pluriStrainer) to further fragment cells and exclude large debris. Finally, cell fragments were centrifuged at 1000g for 5 minutes before staining with DAPI, followed by FACS for isolation of nuclei. Alternatively, nuclei were analyzed using Amnis ImageStream® X (EMD Millipore) cytometer. For the proof-of-concept single-nucleus sequencing experiment (Supplemental Figure 1), nuclei from both groups were FACS-isolated independently after cardiomyocyte and non-myocyte isolation via centrifugation and subsequently pooled at specific proportions.
Single-cell transcriptomic library preparation and sequencing — FACS isolated intact non-myocyte cells and cardiomyocyte nuclei from each heart were manually counted using a hemocytometer and were mixed (mixing ratios for specific experiments indicated in Figure 1A and Supplemental Figure 1) and used for single cell transcriptomic library preparation on the Chromium controller (10X Genomics). Approximately 12,000 total cells/nuclei were loaded into each channel and processed using the Chromium Single Cell 3’ v2 reagent kit (10X Genomics). Following capture and lysis, we synthesized cDNA and amplified for 12 cycles as per manufacturer’s protocol (10X Genomics). The amplified cDNA was used to construct Illumina sequencing libraries that were each sequenced on one lane of an Illumina HiSeq 4000 to an approximate depth of 100,000 reads per cell. Isolation of single cells for the AngII experiment (Figure 1A) was performed in two batches on separate days, with samples from each treatment group split across days to mitigate batch effects.

Cardiac Tissue Assessment via Flow Cytometry — Flow cytometric analysis of cardiac non-myocyte cell proportion and abundance was performed using the distinct cardiac tissue mincing protocol published previously [1]. Two cell viability dyes were used, Sytox Green (ThermoFisher Scientific) and Calcein Blue (ThermoFisher Scientific). Antibodies used were MHCII (BD Biosciences clone clone:2G9, BUV395), Lyve1 (eBioscience clone:ALY7, eflour660), CD31 (BD Biosciences clone:390, BV605), CD45 (BioLegend clone:30-F11, APC-Cy7), CD64 (BioLegend clone:X54-5/7.1, PE-Cy7), Mcam (BD Biosciences clone:ME-9F1, BV711), CD3e (BD Biosciences clone:145-2C11, BV650), CD11b (BD Biosciences clone:m1/70, BUV737), and CD59a (BioLegend clone:mCD59.3, PE).

Analysis of single-cell RNA-Seq data — Cell Ranger version 2.1.1 (10X Genomics) was used to process raw sequencing data before subsequent analyses. This pipeline converted Illumina basecall files to fastq format, aligned sequencing reads to the mm10 transcriptome, and quantified the expression of transcripts in each cell. Analyses of processed scRNA-seq data were carried out in R version 3.4 or 3.6 [41] using the Seurat suite versions 2.3.4 and 3.0.2 [42] and Tidyverse packages. Single nuclei transcriptomes typically contain a higher fraction of reads mapping to introns than single cell transcriptomes. Although retaining intronic reads in transcriptomes from cardiomyocytes would more deeply profile these nuclei, we chose to analyze only exonic reads for both nuclei and whole cell transcriptomes in order to treat data from each cell type equally. This resulted in data from 29,682 cells that passed quality control steps implemented in Cell Ranger. As a further quality-control measure, cells meeting any of the following criteria were filtered out: <100 or >15,000 unique genes expressed, >50,000 UMIs, or >30% of reads mapping to mitochondria. These steps removed an additional 67 cells, resulting in a final dataset of 29,615 cells. Gene expression was quantified across 27,998 genes and a total of 17,170 genes expressed in at least ten cells in at least one of the eight samples were considered for further analysis.

Dimensionality reduction using PCA was undertaken to explore transcriptional heterogeneity and perform cell clustering. 30 PCs were selected that explained more variability than expected by chance using heuristics detailed in vignettes associated with the Seurat software.
PC loadings were used as input for a graph-based approach to cluster cells by cell type (clustering resolution 1.2), and as input for t-distributed stochastic neighbor embedding (t-SNE) or fast interpolation-based t-SNE (FiSt-SNE; [43]) for reduction to two dimensions for visualization purposes. For analyses of cells/nuclei isolated from untreated hearts, transcriptionally similar clusters were merged as previously described [4]. For analyses of the complete dataset containing 29,615 cells from 16 mice (AngII, sham, untreated), we removed 57 cells with high expression of hemoglobin genes that appeared to be erythrocytes (red blood cells; RBCs). For hexplot-style visualizations the scex package was used (https://github.com/SaskiaFreytag/schex).

Differential expression analysis—In order to identify differentially expressed (DE) genes, genes expressed in at least 10% of cells in at least one of the groups being compared were first identified. To test for differential expression the method MAST [44] was used including cellular detection rate as a covariate, which performed well in the single cell RNA-Seq differential expression benchmarking study of Soneson and Robinson [45]. A threshold of uncorrected \( p < 0.01 \) was used to define genes as statistically significantly and differentially expressed between groups.

Gene ontology analysis—Gene Ontology (GO) enrichment analysis for differentially expressed gene lists (uncorrected \( p < 0.01 \)) was performed using the enrichDAVID function from the clusterProfiler R package [46] which interacted with DAVID version 6.8 (https://david.ncifcrf.gov/). The over-representation of GO Biological Process terms was calculated using DAVID default population background gene list for *Mus musculus* with minimum and maximum gene set sizes as 2 and 500, respectively. The Benjamini-Hochberg adjusted \( p \)-value cut-off of 0.05 was used to determine statistically significant GO terms.

Ligand-receptor intercellular communication network analysis—In order to represent potential intercellular communication between cardiac cell populations, we obtained mouse orthologs of human ligand-receptor pairs [47] from BioMart database version 86 using the biomaRt R package, as described previously [4]. A ligand/receptor with non-zero expression in more than 20% of cells in a particular cell population was deemed an “expressed” ligand/receptor. To construct the potential cell-cell communication network, we linked expressed ligands with their corresponding receptors between and within major cell populations. The signaling direction from ligand to receptor is indicated by arrows represented in the chord plot in Figure 6A. The total number of communication signals transmitted and received by a certain cell population is represented by the numbered bands in the circular visualization. The Circlize R package [48] was used to plot the putative intercellular communication network.

Sex Analysis—In order to examine sexually dimorphic gene expression patterns, male and female cells were separated based on expression of *Xist* and five Y chromosome genes (*Ddx3y, Eif2s3y, Gm29650, Kdm5d and Uty*). A cell with non-zero expression of *Xist* but zero expression of our Y chromosome genes was classified as a female cell, whereas a cell expressing one or more of the five Y chromosome genes with no expression of *Xist* was
classified as a male cell. Because female cells should not contain a Y chromosome but male cells could express Xist transcript, cells with low or moderate Xist expression and without low expression of Y genes were additionally classified as male. Specifically, cell library size-normalized read counts were considered and cells with summed Y expression >1 UMI per thousand (which corresponded to cells not in the lowest 10% of Y gene expression among male cells called above) and Xist expression <8 UMI per thousand (which corresponded to cells with less than median Xist expression among female cells called above) were classified as male. Cells that expressed neither Xist nor Y chromosome genes, or cells that did not meet the criteria defined above, were removed from our sex analysis. ~75% of cells from our initial cell yield were assigned a sex for the downstream sex dimorphism analysis. This analysis contained 15,181 female and 7,116 male cells (see Fig.7B). Sexual dimorphism in gene expression was examined in Control and AngII-treated animals separately using methods for differential gene expression analysis described above.

**RNA velocity and pseudotime analyses**—To explore dynamic patterns of transcriptional changes among fibroblasts in resting and hypertrophic hearts, 15,446 fibroblasts were isolated from our complete dataset. RNA velocity was examined using Velocyto version 0.17.17 [15] to count spliced and unspliced reads and scVelo version 0.1.21 [16] to compute velocity. scVelo was run with the arguments min_shared_counts=10 and n_top_genes=2000 to the function filter_and_normalize() and analyses carried out using 30 principal components. To identify genes that differed in velocity between Fibroblast-Cilp and Fibroblast-Thbs4 compared to the remaining fibroblasts, the function rank_velocity_genes() was used. In addition, two pseudotime inference algorithms were applied to all fibroblasts isolated from AngII-treated mice. Slingshot version 1.3.2 [49] was used with default arguments and cells grouped according to clusters defined in our Seurat analysis (above). monocle3 version 0.1.1 [50] was used, preprocessing with 15 principal components and regressing out percent mitochondrial reads as well as number of UMIs per cell.

**Analysis of Genotype Tissue Expression project (GTex) RNA-Seq data**—The Genotype-Tissue Expression (GTEx) project provides a resource for investigators to study gene expression in diverse human tissues and comprises samples isolated and systematically processed from post-mortem donors with variable demographic and health characteristics. GTEx version 7 data was downloaded from https://gtexportal.org/home/datasets (accessed 02/21/2019), including sample attributes, subject phenotypes, and gene TPMs from RNA-Seq. To examine gene expression in human hearts, subjects within the age windows 40-49, 50-59, and 60-69 were examined because these age windows contained greater than 25 samples per window. Samples with highest 20% NPPB expression (without regard to age or sex) were classified as putatively hypertrophic. Differences in gene expression between putative hypertrophy/non-hypertrophy cohorts were tested using linear models. Specifically, in these models the response variable was the logarithm of gene expression (TPM) and predictors included age class, sex, hypertrophy class, and sex×hypertrophy interaction, with the final term added because sexual dimorphism in response to hypertrophy was of particular interest. Results in the main text reporting differences in gene expression between hypertrophy classes were calculated with respect to the additive hypertrophy term with two-sided p-values.
Other statistical analyses—Statistical analyses not described above were performed using the ggpubr package in R (https://github.com/kassambara/ggpubr) or using Prism 8 for Windows software (GraphPad Software, Inc.).
Supplemental figure and table legends

Supplemental Figure 1. Isolation and analysis of cardiac cell nuclei for scRNA-seq.
A) Experimental schematic for evaluating scRNA-seq analysis of nuclei of cardiac non-myocytes and myocytes isolating by perfusion-mediated dissociation. B) Example images of isolated cardiomyocytes using the perfusion based cardiac single cell preparation method (see Supplemental Methods for more detail) (top, phase contrast micrograph scale bar indicates 100 µm) and isolated cardiomyocyte nuclei imaged using the Amnis ImageStream system (bottom) showing DAPI and phase images of single nuclei. C) tSNE projection displaying cardiomyocyte nuclei (indicated) and non-myocyte nuclei analyzed by single-nucleus RNA-seq, as proof of concept for single-nucleus sequencing from live cardiac tissue (see Supplemental Methods).

Supplemental Figure 2. Comparison of two cardiac cell isolation techniques on cellular composition and yield.
A) Flow cytometry gating strategy to identify relative proportions of endothelial cells (ECs), resident mesenchymal cells (RMCs) and leukocytes. Note: only non-myocytes are included in this analysis. B) Quantified proportions of three major cardiac cell types (% Calcein+ cells). Endothelial cell (EC) yield is significantly lower when performing the perfusion protocol, however significantly greater proportions of leukocytes are liberated by perfusion. Resident mesenchymal cell proportions are not altered by either protocol. Statistical analysis was conducted using an unpaired t-test. FSC-H = forward scatter height, FSC-W = forward scatter width, FSC-A = forward scatter area.

Supplemental Figure 3. Abundance of heart cells within scRNA-seq datasets in this study from wild-type mice.
Cellular composition of the sampling of sequenced cells derived from mouse hearts. Cells from two samples of wild-type mice are shown in columns (batch). Each sample consisted of cells from female and male mice (see Supplemental Methods). Colors indicate cell populations and are identical to Figure 1B. Heights of individual boxes comprising each bar represent the proportion of cells classified as each cell population. Cellular composition shown here is not directly comparable to a homeostatic mouse heart due to the sorting strategy employed (see Figure 1A).

Supplemental Figure 4. Physiological assessment of cardiac function after AngII reveals sexual dimorphism in functional cardiac decline.
A-D) Echocardiographic assessment of experimental mice prior to AngII stimulus (Male/Female-pre) and after two weeks AngII treatment, or saline control. Echocardiographs were used to calculate (A) ejection fraction percentage, (B) fractional shortening percentage, (C) diastolic volume, and (D) systolic volume. Statistical analysis was conducted using an unpaired t-test, *P< 0.05, **P< 0.005. E) Conscious blood pressure assessment after AngII, or saline control, in males and females, ***P< 0.0005, ****P< 0.0001. F) Heart-weight to body-weight ratio after AngII, or saline control, in males and females. ***P< 0.0005.
Supplemental Figure 5. Fibrosis of hearts from saline or AngII-treated.
Representative bright-field micrographs of heart sections following Masson’s trichrome staining (detailed view of images shown in Figure 2B). Non-magnified and magnified views of saline-treated (A) or AngII-treated (B) heart sections are shown.

Supplemental Figure 6. Abundance of heart cells within scRNA-seq datasets in this study from mice with and without AngII treatment.
A) Cellular composition of sequenced cells derived from mouse hearts at homeostasis (‘WT’) without any surgical intervention, after administration of saline for two weeks (‘Sal’), and after administration of AngII for two weeks (‘AngII’). Cells from individual samples are shown in columns (batch), with two WT, two Sal and four Ang samples analyzed. Each sample consisted of cells from female and male mice (see Supplemental Methods). Colors indicate cell populations and sub/populations and are used throughout this study. Heights of individual boxes comprising each bar represent the proportion of cells classified as each cell population. Cellular composition shown here is not directly comparative to hearts isolated from treated and untreated mice due to the sorting strategy employed (see Figure 1A). B) Similar to (A), but showing individual points representing observed cell abundances (in units of cells per thousand sequenced) of each population. Individual AngII-treated or control (wild type or saline administration) samples mice are indicated by red and black dots, respectively. Light grey lines indicate binomial proportion confidence intervals and are calculated using Jeffreys interval.

Supplemental Figure 7. Sub-clustering of cardiac cell populations.
A) tSNE projections of cardiac cell populations with cell identity designations as shown in Figure 2D (left-side column of tSNE projections) or following re-clustering of the cell populations in isolation (right-side column of tSNE projections). Labelling on left indicates cell populations pooled for subclustering. The fibroblast pool includes proliferating mesenchymal cells (Prolif. mes. cells in Figure 2D). Point-range plots summarize cell abundances in cell populations from control or AngII-treated mouse hearts. The cluster each plot corresponds to is indicated by the number above each plot. P-values shown on plot are derived from Wilcoxon rank sum test for differences between the groups. B) tSNE projections show results of re-clustering granulocytes or epicardial cells. No additional clusters were discovered upon re-clustering.

Supplemental Figure 8. Sexual dimorphism in cardiac cellular composition changes in response to AngII detected by flow cytometry
A) Flow cytometry gating strategies to identify relative proportions of live, metabolically active (C+V+) cardiac cell types. B) Quantified proportions of each cell type normalized to the mean of each WT control (WT mean = 1). Lymphatic ECs were significantly elevated in AngII-treated male mice compared to AngII-treated females. Macrophages were also significantly increased in male AngII mice compared to male WT controls within the major cardiac cell compartment. No changes were observed within the resident mesenchymal cell compartment. However, in the leukocyte compartment male AngII mice exhibited significantly elevated myeloid cells compared to both male WT mice as well as female AngII mice. Consequently, these aforementioned increases in leukocyte proportions were observed in macrophages.
including their polarization sub-types MHCII$^{hi}$ and MHCII$^{lo}$. Statistical analysis was conducted using a two-way ANOVA (*P< 0.05, **P< 0.005). FSC-H = forward scatter height, FSC-W = forward scatter width, FSC-A = forward scatter area, SSC-H = side scatter height.

**Supplemental Figure 9. Cell cycle scoring to identify dividing cardiac cells from mice with and without AngII treatment.**
Cell cycle scores are determined by considering expression levels G2/M and S phase marker genes (see https://satijalab.org/seurat/v3.1/cell_cycle_vignette.html).

**Supplemental Figure 10. Relationship between number of differentially expressed genes identified and cell population size**
Scatter plot summarizing the relationship between number of differentially expressed (DE) genes identified in AngII-treated hearts compared to control (Y-axis) and cell population size from both control and AngII-treated hearts (X-axis).

**Supplemental Figure 11. Downregulated genes after induction of fibrosis.**
A) Dot plot summarizing the top 10 downregulated genes for each cell population. Duplicate entries of genes have been removed. Fold change in gene expression is indicated by circle size. Circle color indicates relative expression level in cells from control samples (expression level before reduction). Red dots indicate reduction in gene expression where $p<0.01$ (uncorrected; also see Table S3). B) Enrichment of GO terms in downregulated genes. Number of genes downregulated (circle size) and adjusted p-value (circle color) are indicated. GO terms are ordered by their frequency of significant enrichment in different cardiac cell populations (also see Table S4).

**Supplemental Figure 12. Total transcript corresponding to ECM-related GO terms.**
Dot plot showing expression of genes classified within the GO categories shown on Y-axis. Circle size represents the sum of transcripts for genes corresponding to each GO term within each cell population.

**Supplemental Figure 13. Detection of THBS4+ cells in AngII-treated and untreated mouse hearts.**
A) Confocal micrographs of two consecutive sections from an AngII-treated mouse heart with and without addition of anti-THBS4 antibody. Sections also stained with DAPI (for nuclei), anti-ACTA2 antibody, and wheat germ agglutinin (WGA). AF647 indicates reagent used for detecting antigen-bound THBS4 antibody. B) Confocal micrograph of saline-treated control mouse heart stained with DAPI, anti-ACTA2 antibody, WGA, and anti-THBS4. Figure insert (B’) indicates two rare THBS4+ cells.

**Supplemental Figure 14. Markers of matrifibrocytes and Fibroblast-Cilp and Fibroblast-Thbs4.**
Figures show FIt-SNE projections for cardiac cell populations (as shown in Figure 2D) with relative gene expression indicated by color (red=high, gray=low).
Supplemental Figure 15. Shifts in fibroblast cell state in response to chronic stress imposed by AngII treatment.

**Left:** Pseudotime trajectories of fibroblasts isolated from AngII-treated mice inferred using monocle3. Cells are visualized using UMAP dimensionality reduction and colored according to fibroblast cluster. Gray line indicates inferred pseudotime trajectory. **Right:** Pseudotime trajectories of fibroblasts isolated from AngII-treated mice inferred using slingshot. Cells are visualized using UMAP dimensionality reduction and colored according to fibroblast cluster. Black line indicates inferred pseudotime trajectory.

Supplemental Figure 16. Transmitted and received signals by cardiac cells with and without AngII treatment inferred by scRNA-seq.

**A)** Number of signals transmitted as determined by ligand encoding genes which are expressed by cells which have cognate receptors expressed by other cardiac cell populations. **B)** Number of signals received as determined by receptor encoding genes expressed by cells which have cognate ligands expressed by other cardiac cell populations.

Supplemental Figure 17. GO terms corresponding to downregulated ligand and receptor genes.

**A)** GO terms enriched in downregulated ligand genes. **B)** GO terms enriched in downregulated receptor genes. Number of downregulated ligand or receptor genes associated with each GO term and adjusted p-value are indicated by circle size and color, respectively (also see Table S9).

Supplemental Figure 18. Summed transcript levels of genes annotated as responsive to estradiol that are upregulated in cardiac cells following AngII treatment

A bar plot which summarizes the total transcript abundance for genes that are upregulated in cardiac cells following AngII treatment and included in the ‘response to estradiol’ GO term.

Supplemental Figure 19. Distribution of males and female cells based on expression of Y chromosome-related genes and Xist gene.

Blue and red dots indicate male and female cells, respectively. Grey dots represent cells which could not be discriminated. Genes considered are outside of the pseudoautosomal regions of the X and Y chromosomes (see Supplemental Methods).

Supplemental Figure 20. Sex hormone receptors in cardiac cells from mice with and without AngII treatment.

Violin plots generated with Seurat showing the expression of various hormone receptors across all cardiac cell types. Androgen receptor expression (Ar) is greatest in cardiac fibroblasts. Estrogen receptor α and estrogen receptor β (Esr1, Esr2, respectively) are both lowly expressed, but most highly detected in cardiac fibroblasts. The membrane bound G protein-coupled estrogen receptor 1 (Gper1) is also lowly expressed, but greatest in endothelial cells and pericytes.
Supplemental Figure 21. Relationship between number of sexually dimorphic genes discovered and cell population size.
Top panel summarizes the relationship in cardiac cell populations from control animals. Bottom panel summarizes the relationship in cardiac cell populations from AngII-treated animals.

Supplemental Figure 22. Strategy for determining sexually dimorphic genes regulation following AngII treatment (related to figure 7D-F).
For each cell population, female and male cells were considered in isolation and genes upregulated or downregulated following AngII treatment determined. For dimorphisms in upregulated genes (results shown Figure 7D-F), a list of genes upregulated in either females or males was generated and sexual dimorphisms in gene expression was examined. For dimorphisms in downregulated genes (results shown Supplemental Figure 23), a list of genes downregulated in either sex was generated and dimorphism in expression was again examined.

Supplemental Figure 23. Sexual dimorphism in gene expression amongst genes that are downregulated in cardiac cells following AngII treatment.
The number of genes which are downregulated in response to AngII as well as exhibiting sexually dimorphic gene expression, presented for each cardiac cell type (see Table S11-S12). In this context upregulated refers to a gene that is greater in either sex, but overall remains lower after AngII. Strategy to determine sexually dimorphisms is outlined in Supplemental Figure 22.

Supplemental Figure 24. Sexually dimorphic genes in cell populations from control mouse hearts.
A) A dot plot showing genes differentially expressed at 2-fold or greater between males and females. Red and blue circles represent genes which are more highly expressed in females or males, respectively (see Table S10). Circle size represents the difference in the percentage of cells expressing the gene between sexes. Solid black dots indicate a statistically significant difference in expression with $p < 0.01$ (uncorrected). B) GO terms enriched in sexually dimorphic gene sets in female and male cardiac cell populations. Red and blue text indicate GO terms enriched in sets of genes that are upregulated in females and males, respectively.

Supplemental Figure 25. Abundances of endothelial cells in female and male hearts determined by flow cytometry
A) Flow cytometry contour plots outlining the gating strategy used to determine frequency of endothelial cells. B) Absolute number of endothelial cells normalized to heart tissue mass. C) Proportion of endothelial cells relative to total non-myocytes. P-values shown on plot are derived from the Wilcoxon rank sum test for differences between the groups; n = 8 per group.

Supplemental Figure 26. GTEx bulk RNA-seq analysis from human hearts.
A) Expression of NPPB (in units of transcripts per million; tpm) in 257 female and male human hearts as a function of age and sex. Dotted red horizontal line indicates threshold used for classifying samples as putatively hypertrophic (highest 20% NPPB expression without regard
to age or sex). **B)** Expression of markers of pathological cardiac remodeling in human hearts. Boxplots show identical human samples to (A) classified as positive or negative for putative hypertrophy. To facilitate comparison of patterns for genes that have different absolute expression levels, gene expression levels (in units of tpm) were rescaled to set a maximum of one and minimum of zero. *P*-values provided above each gene are derived from linear models for the effect of hypertrophy classification while controlling for age and sex. **C)** Summed expression of gene transcripts encoded by mitochondrial DNA for identical human samples to (A) classified as positive or negative for putative hypertrophy. *P*-value provided below the boxplots is derived from a linear model for the effect of hypertrophy classification while controlling for age and sex. **D)** Expression of human orthologs to markers of Fibroblast-*Thbs4* in human hearts. Boxplots show identical human samples to (A) classified as positive or negative for putative hypertrophy. To facilitate comparison of patterns for genes that have different absolute expression levels, gene expression levels (in units of tpm) were rescaled to set a maximum of one and minimum of zero. *P*-values provided above each gene are derived from linear models for the effect of hypertrophy classification while controlling for age and sex.

**Supplemental Figure 27.** Cardiac tissue perfusion assembly for parallel perfusion-based dissociation of cardiac cells from multiple hearts.  
**A)** A close-up view of a heart being perfused through the apex by digestion enzymes. **B)** Equipment layout for simultaneous perfusion of multiple hearts.

**Supplemental Video 1.** Spatial distribution of Fibroblast-*Thbs4* cells in AngII-treated mouse hearts.  
3D projection video of a fluorescence micrograph from an AngII-treated mouse heart. The tissue is stained with anti-THBS4 antibody (red), wheat-germ-agglutinin (blue), anti-ACTA2 antibody (green) and DAPI (white). Red, blue, green and white represent Fibroblast-*Thbs4*, cell boundaries, ACTA2+ cells (SMCs and myofibroblasts) and cell nuclei, respectively.

**Supplemental Table 1.** Population markers related to Figure 1D  
**Supplemental Table 2.** Population and subpopulation markers related to Figure 2D  
**Supplemental Table 3.** Differentially expressed genes related to Figure 3A-B  
**Supplemental Table 4.** GO terms related to Figure 3C  
**Supplemental Table 5.** Differentially expressed genes related to Figure 4A  
**Supplemental Table 6.** GO terms related to figure 4A-D  
**Supplemental Table 7.** Ligands and receptors related to Figure 6A and 6D  
**Supplemental Table 8.** Changes in ligands and receptors related to Figure 6D  
**Supplemental Table 9.** GO terms related to Figure 6E-F  
**Supplemental Table 10.** Sexually dimorphic genes related to Figure 7D
Supplemental Table 11. Sexually dimorphic AngII response genes related to Figure 7E and Figure XXIII in the Supplement

Supplemental Table 12. Common AngII response genes related to Figure 7E and Figure XXIII in the Supplement

Supplemental Table 13. Common GO terms related to Figure 7E
Supplemental Figure I. Isolation and analysis of cardiac cell nuclei for scRNA-seq.
A) Experimental schematic for evaluating scRNA-seq analysis of nuclei of cardiac non-myocytes and myocytes isolating by perfusion-mediated dissociation. B) Example images of isolated cardiomyocytes using the perfusion based cardiac single cell preparation method (see Supplemental Material for more detail) (top, phase contrast micrograph scale bar indicates 100 µm) and isolated cardiomyocyte nuclei imaged using the Amnis ImageStream system (bottom) showing DAPI and phase images of single nuclei. C) tSNE projection displaying cardiomyocyte nuclei (indicated) and non-myocyte nuclei analyzed by single-nucleus RNA-seq, as proof of concept for single-nucleus sequencing from live cardiac tissue (see Supplemental Material).
**Supplemental Figure II.** Comparison of two cardiac cell isolation techniques on cellular composition and yield. 

A) Flow cytometry gating strategy to identify relative proportions of endothelial cells (ECs), resident mesenchymal cells (RMCs) and leukocytes. Note: only non-myocytes are included in this analysis. B) Quantified proportions of three major cardiac cell types (% Calcein+ cells). Endothelial cell (EC) yield is significantly lower when performing the perfusion protocol, however significantly greater proportions of leukocytes are liberated by perfusion. Resident mesenchymal cell proportions are not altered by either protocol. Statistical analysis was conducted using an unpaired t-test. FSC-H = forward scatter height, FSC-W = forward scatter width, FSC-A = forward scatter area.
Supplemental Figure III. Abundance of heart cells within scRNA-seq datasets in this study from wild-type mice. Cellular composition of the sampling of sequenced cells derived from mouse hearts. Cells from two samples of wild-type mice are shown in columns (batch). Each sample consisted of cells from female and male mice (see Supplemental Material). Colors indicate cell populations and are identical to Figure 1B. Heights of individual boxes comprising each bar represent the proportion of cells classified as each cell population. Cellular composition shown here is not directly comparable to a homeostatic mouse heart due to the sorting strategy employed (see Figure 1A).
Supplemental Figure IV. Physiological assessment of cardiac function after AngII reveals sexual dimorphism in functional cardiac decline.

A-D) Echocardiographic assessment of experimental mice prior to AngII stimulus (Male/Female-pre) and after two weeks AngII treatment, or saline control. Echocardiographs were used to calculate (A) ejection fraction percentage, (B) fractional shortening percentage, (C) diastolic volume, and (D) systolic volume. Statistical analysis was conducted using an unpaired t-test, *P< 0.05, **P< 0.005. E) Conscious blood pressure assessment after AngII, or saline control, in males and females. ***P< 0.0005, ****P< 0.0001. F) Heart-weight to body-weight ratio after AngII, or saline control, in males and females. ***P< 0.0005.
Supplemental Figure V. Fibrosis of hearts from saline or AngII-treated.
Representative bright-field micrographs of heart sections following Masson’s trichrome staining (detailed view of images shown in Figure 2B). Non-magnified and magnified views of saline-treated (A) or AngII-treated (B) heart sections are shown.
Supplemental Figure VI. Abundance of heart cells within scRNA-seq datasets in this study from mice with and without AngII treatment.

A) Cellular composition of sequenced cells derived from mouse hearts at homeostasis (‘WT’) without any surgical intervention, after administration of saline for two weeks (‘Sal’), and after administration of AngII for two weeks (‘AngII’). Cells from individual samples are shown in columns (batch), with two WT, two Sal and four Ang samples analyzed. Each sample consisted of cells from female and male mice (see Supplemental Material). Colors indicate cell populations and sub-populations and are used throughout this study. Heights of individual boxes comprising each bar represent the proportion of cells classified as each cell population. Cellular composition shown here is not directly comparative to hearts isolated from treated and untreated mice due to the sorting strategy employed (see Figure 1A). B) Similar to (A), but showing individual points representing observed cell abundances (in units of cells per thousand sequenced) of each population. Individual AngII-treated or control (wild type or saline administration) samples mice are indicated by red and black dots, respectively. Light grey lines indicate binomial proportion confidence intervals and are calculated using Jeffreys interval.
Supplemental Figure VII. Sub-clustering of cardiac cell populations.

A) ISNE projections of cardiac cell populations with cell identity designations as shown in Figure 2D (left-side column of ISNE projections) or following re-clustering of the cell populations in isolation (right-side column of ISNE projections). Labelling on left indicates cell populations pooled for subclustering. The fibroblast pool includes proliferating mesenchymal cells (Prolif. mes. cells in Figure 2D). Point-range plots summarize cell abundances in cell populations from control or AngII-treated mouse hearts. The cluster each plot corresponds to is indicated by the number above each plot. P-values shown on plot are derived from Wilcoxon rank sum test for differences between the groups.

B) ISNE projections show results of re-clustering granulocytes or epicardial cells. No additional clusters were discovered upon re-clustering.
Supplemental Figure VIII. Sexual dimorphism in cardiac cellular composition changes in response to AngII detected by flow cytometry

A) Flow cytometry gating strategies to identify relative proportions of live, metabolically active (CD+CD+), cardiac cell types. B) Quantified proportions of each cell type normalized to the mean of each WT control (WT mean = 1). Lymphatic ECs were significantly elevated in AngII-treated male mice compared to AngIII-treated females. Macrophages were also significantly increased in male AngII mice compared to male WT controls within the major cardiac cell compartment. No changes were observed within the resident mesenchymal cell compartment. However, in the leukocyte compartment male AngII mice exhibited significantly elevated myeloid cells compared to both male WT mice as well as female AngII mice. Consequently, these aforementioned increases in leukocyte proportions were observed in macrophages including their polarization sub-types MHCIIhi and MHCIIlo. Statistical analysis was conducted using a two-way ANOVA (*P< 0.05, **P< 0.005). FSC-H = forward scatter height, FSC-W = forward scatter width, FSC-A = forward scatter area, SSC-H = side scatter height.
Supplemental Figure IX. Cell cycle scoring to identify dividing cardiac cells from mice with and without AngII treatment.

Cell cycle scores are determined by considering expression levels G2/M and S phase marker genes (see https://satijalab.org/seurat/v3.1/cell_cycle_vignette.html).
**Supplemental Figure X. Relationship between number of differentially expressed genes identified and cell population size**

Scatter plot summarizing the relationship between number of differentially expressed (DE) genes identified in AngII-treated hearts compared to control (Y-axis) and cell population size from both control and AngII-treated hearts (X-axis).
Supplemental Figure XI. Downregulated genes after induction of fibrosis.
A) Dot plot summarizing the top 10 downregulated genes for each cell population. Duplicate entries of genes have been removed. Fold change in gene expression is indicated by circle size. Circle color indicates relative expression level in cells from control samples (expression level before reduction). Red dots indicate reduction in gene expression where p<0.01 (uncorrected; also see Table S3). B) Enrichment of GO terms in downregulated genes. Number of genes downregulated (circle size) and adjusted p-value (circle color) are indicated. GO terms are ordered by their frequency of significant enrichment in different cardiac cell populations (also see Table S4).
Supplemental Figure XII. Total transcript corresponding to ECM-related GO terms.
Dot plot showing expression of genes classified within the GO categories shown on Y-axis. Circle size represents the sum of transcripts for genes corresponding to each GO term within each cell population.
Supplemental Figure XIII. Detection of THBS4+ cells in AngII-treated and untreated mouse hearts.
A) Confocal micrographs of two consecutive sections from an AngII-treated mouse heart with and without addition of anti-THBS4 antibody. Sections also stained with DAPI (for nuclei), anti-ACTA2 antibody, and wheat germ agglutinin (WGA). AF647 indicates reagent used for detecting antigen-bound THBS4 antibody. B) Confocal micrograph of saline-treated control mouse heart stained with DAPI, anti-ACTA2 antibody, WGA, and anti-THBS4. Figure insert (B') indicates two rare THBS4+ cells.
Supplemental Figure XIV. Markers of matrifibrocytes and Fibroblast-Cilp and Fibroblast-Thbs4. Figures show Flt-SNE projections for cardiac cell populations (as shown in Figure 2D) with relative gene expression indicated by color (red=high, gray=low).
Supplemental Figure XV. Shifts in fibroblast cell state in response to chronic stress imposed by AngII treatment.

Left: Pseudotime trajectories of fibroblasts isolated from AngII-treated mice inferred using monocle3. Cells are visualized using UMAP dimensionality reduction and colored according to fibroblast cluster. Gray line indicates inferred pseudotime trajectory. Right: Pseudotime trajectories of fibroblasts isolated from AngII-treated mice inferred using slingshot. Cells are visualized using UMAP dimensionality reduction and colored according to fibroblast cluster. Black line indicates inferred pseudotime trajectory.
Supplemental Figure XVI. Transmitted and received signals by cardiac cells with and without AngII treatment inferred by scRNA-seq.
A) Number of signals transmitted as determined by ligand encoding genes which are expressed by cells which have cognate receptors expressed by other cardiac cell populations. B) Number of signals received as determined by receptor encoding genes expressed by cells which have cognate ligands expressed by other cardiac cell populations.
Supplemental Figure XVII. GO terms corresponding to downregulated ligand and receptor genes. A) GO terms enriched in downregulated ligand genes. B) GO terms enriched in downregulated receptor genes. Number of downregulated ligand or receptor genes associated with each GO term and adjusted p-value are indicated by circle size and color, respectively (also see Table S9).
Supplemental Figure XVIII. Summed transcript levels of genes annotated as responsive to estradiol that are upregulated in cardiac cells following AngII treatment

A bar plot which summarizes the total transcript abundance for genes that are upregulated in cardiac cells following AngII treatment and included in the ‘response to estradiol’ GO term.
Supplemental Figure XIX. Distribution of males and female cells based on expression of Y chromosome-related genes and Xist gene. Blue and red dots indicate male and female cells, respectively. Grey dots represent cells which could not be discriminated. Genes considered are outside of the pseudoautosomal regions of the X and Y chromosomes (see Supplemental Material).
Supplemental Figure XX. Sex hormone receptors in cardiac cells from mice with and without AngII treatment. Violin plots generated with Seurat showing the expression of various hormone receptors across all cardiac cell types. Androgen receptor expression (Ar) is greatest in cardiac fibroblasts. Estrogen receptor α and estrogen receptor β (Esr1, Esr2, respectively) are both lowly expressed, but most highly detected in cardiac fibroblasts. The membrane bound G protein-coupled estrogen receptor 1 (Gper1) is also lowly expressed, but greatest in endothelial cells and pericytes.
Supplemental Figure XXI. Relationship between number of sexually dimorphic genes discovered and cell population size.
Top panel summarizes the relationship in cardiac cell populations from control animals. Bottom panel summarizes the relationship in cardiac cell populations from AngII-treated animals.
Supplemental Figure XXII. Strategy for determining sexually dimorphic genes regulation following AngII treatment (related to figure 7D-F).

For each cell population, female and male cells were considered in isolation and genes upregulated or downregulated following AngII treatment determined. For dimorphisms in upregulated genes (results shown Figure 7D-F), a list of genes upregulated in either females or males was generated and sexual dimorphisms in gene expression was examined. For dimorphisms in downregulated genes (results shown Supplemental Figure XXIII), a list of genes downregulated in either sex was generated and dimorphism in expression was again examined.
Supplemental Figure XXIII. Sexual dimorphism in gene expression amongst genes that are downregulated in cardiac cells following AngII treatment.

The number of genes which are downregulated in response to AngII as well as exhibiting sexually dimorphic gene expression, presented for each cardiac cell type (see Table S11-S12). In this context upregulated refers to a gene that is greater in either sex, but overall remains lower after AngII. Strategy to determine sexually dimorphisms is outlined in Supplemental Figure XXII.
Supplemental Figure XXIV. Sexually dimorphic genes in cell populations from control mouse hearts.

A) A dot plot showing genes differentially expressed at 2-fold or greater between males and females. Red and blue circles represent genes which are more highly expressed in females or males, respectively (see Table S10). Circle size represents the difference in the percentage of cells expressing the gene between sexes. Solid black dots indicate a statistically significant difference in expression with p < 0.01 (uncorrected). B) GO terms enriched in sexually dimorphic gene sets in female and male cardiac cell populations. Red and blue text indicate GO terms enriched in sets of genes that are upregulated in females and males, respectively.
Supplemental Figure XXV. Abundances of endothelial cells in female and male hearts
determined by flow cytometry
A) Flow cytometry contour plots outlining the gating strategy used to determine frequency of
endothelial cells. B) Absolute number of endothelial cells normalized to heart tissue mass. C) Proportion of endothelial cells relative to total non-myocytes. P-values shown on plot are derived
from the Wilcoxon rank sum test for differences between the groups; n = 8 per group.
Supplemental Figure XXVI. GTEx bulk RNA-seq analysis from human hearts.
A) Expression of NPPB (in units of transcripts per million; tpm) in 257 female and male human hearts as a function of age and sex. Dotted red horizontal line indicates threshold used for classifying samples as putatively hypertrophic (highest 20% NPPB expression without regard to age or sex). B) Expression of markers of pathological cardiac remodeling in human hearts. Boxplots show identical human samples to (A) classified as positive or negative for putative hypertrophy. To facilitate comparison of patterns for genes that have different absolute expression levels, gene expression levels (in units of tpm) were rescaled to set a maximum of one and minimum of zero. P-values provided above each gene are derived from linear models for the effect of hypertrophy classification while controlling for age and sex. C) Summed expression of gene transcripts encoded by mitochondrial DNA for identical human samples to (A) classified as positive or negative for putative hypertrophy. P-value provided below the boxplots is derived from a linear model for the effect of hypertrophy classification while controlling for age and sex. D) Expression of human orthologs to markers of Fibroblast-Thbs4 in human hearts. Boxplots show identical human samples to (A) classified as positive or negative for putative hypertrophy. To facilitate comparison of patterns for genes that have different absolute expression levels, gene expression levels (in units of tpm) were rescaled to set a maximum of one and minimum of zero. P-values provided above each gene are derived from linear models for the effect of hypertrophy classification while controlling for age and sex.
Supplemental Figure XXVII. Cardiac tissue perfusion assembly for parallel perfusion-based dissociation of cardiac cells from multiple hearts.
A) A close-up view of a heart being perfused through the apex by digestion enzymes. B) Equipment layout for simultaneous perfusion of multiple hearts.