Detailed Materials and Methods for Hulin et al.

Mice

All mouse experiments conform to NIH guidelines (Guide for the Care and Use of Laboratory Animals) and were performed with protocols approved by the Institutional Animal Care and Use Committee at Cincinnati Children's Research Foundation and the Research Institute at Nationwide Children's Hospital. C57BL/6J mice were used for WT studies and were obtained from Jackson Laboratory. *PostnCre* and *Cx3cr1Cre* transgenic mice were previously described[1,2] and were obtained from Dr. Simon Conway (Indiana University) and from Dr. Tony DeFalco (Cincinnati Children's Medical Center), respectively. Cre activity in *PostnCre* and *Cx3cr1Cre* mice was monitored using *Rosa26 membrane Tomato/membrane EGFP* (*R26*^{mTmG}) reporter mice[3]. *CD45Cre* and *Gt(Rosa)26Sor* $^{tm9(CAG\text{-}dtTomato)Hze}$ reporter mice (*Ai9*) were a gift from Dr. Edwin Horwitz at Nationwide Children's Hospital and are previously described[4,5]. *Axin2*$^{LacZ/+}$ mice were previously described[6]. Mutant mice were maintained on a 129/B6 mixed background. Mice were sacrificed under isoflurane inhalation followed by cervical dislocation at the specified time point for each experiment. Male and female mice were used together for all analyses.

Human valve tissue

Mitral valve posterior leaflets (P2 segments) were harvested and processed as previously described[7]. Control mitral valves (n=6) were acquired from hearts rejected for transplantation. Myxomatous mitral valves (n=6) were obtained from patients undergoing elective valvuloplasty surgery to correct mitral regurgitation. The control group consisted of 6 individuals (4 males, 2 females) with an average age of 59.7 $\pm$ 9.8 years. The myxomatous group comprised of 6 individuals (males only) with an average age of 60.8 $\pm$ 8.2 years. Written consent was obtained from every patient in both groups. This study conforms to the principles outlined in the Declaration of Helsinki and was approved by the Ethics Committee of Liege University Hospital (B70720071262).

Histology and immunofluorescence labeling

Murine hearts were harvested from neonatal, juvenile, and adult mice at indicated time-points and processed for paraffin embedding. Hearts were fixed in 4% paraformaldehyde (PFA) for 2hr at room temperature, dehydrated through a graded ethanol series, cleared in xylenes, and embedded in paraffin. Murine hearts were sectioned at 7µm. For immunofluorescence studies of mouse and human tissue sections, all slides were pretreated for 5 min using citric acid antigen retrieval (Vector Laboratories) in a pressure cooker. For MHCII, CD206, and CD64 staining, slides were pre-treated with proteinase K (20µg/ml) for 3 min. The primary antibodies used were pHH3 (Millipore #06570, 1:300), CD45 (R&D Systems #AF114, 1:200), GFP (Abcam #AB290, 1:1000), GFP (Abcam #AB13970, 1:200), RFP (Rockland #600-401-379, 1:3000), Vimentin (Novus Bio #NB300-223, 1:200), CD31 (Abcam #AB28364, 1:50), CD11b (Biorad #MCA711G, 1:200), MHCII (eBioscience #145321-82, 1:500), MHCII (Abcam #ab157210, 1:1000), CD206 (R&D Systems #AF2535, 1:500), CD64 (Abcam #ab140779, 1:150), HAPB (Millipore #385911, 1:100), and IαI (Dako, 1:100). For fluorescent detection of antibody staining, Alexa Fluor-488 or 568 conjugated secondary

antibodies (Abcam, Life Technologies) were used at 1:500 dilution and Alexa Fluor-647 conjugated streptavidin-647 (ThermoFisher) was used at 1:200 to detect HABP. For TUNEL staining an in situ cell death detection kit was used following manufacturer's protocol (Roche-applied Science). Nuclei were counterstained with DAPI (4', 6-Diamidino- 2-Phenylindole) (Life Technologies, 1:10,000). Images were captured using a Nikon A1-R confocal system with NIS-Elements D 3.2 software. The percent of cells positive for the specified staining was determined as the number of positively-stained cells/total number of nuclei per valve leaflet section determined by DAPI staining. The percent of Iαl-stained area (positive pixels) was determined as the area of Iαl-stained/ HABP-stained area. A minimum of 4 mice per genotype was analyzed. Human mitral valves were stained using Movat's pentachrome according to manufacturer's (American MasterTech) protocols.

Flow cytometric analyses

Murine aortic and mitral valve leaflets were isolated and pooled from 3 to 5 animals at indicated time-points prior to flow cytometry. Tissues were digested using digestion buffer (2 mg/mL collagenase type IV, Worthington Biochemical Corporation and 1.2 U/mL dispase II (Sigma-Aldrich) in HBSS). Leaflets were incubated at 37°C for 3 x 20 minutes with gentle rocking. Supernatant was collected after each incubation and fresh digestion buffer was added to tissue pellets. Cell suspensions were filtered using a 35-µm cell strainer and centrifuged at 400*g* for 10 minutes. Cell pellets were resuspended and washed three times with 2% FBS/HBSS solution before staining with various antibodies and reagents for flow cytometry or FACS, after FC receptor blocking with CD16/CD32 antibody (BD Biosciences). BD Biosciences antibodies used were CD45 APC (#559864, 1:200), CD11b BV711 (#101241, 1:200), F4/80 PE (#123110, 1:200), MHCII PE/Dazzle 594 (#107647, 1:200), CD206 Alexa-488 (#141709, 1:400), CCR2 PE/CY7 (#150611, 1:200), CD11c APC-CY7 (#117323, 1:200). Dye Zombie UV (#423107, 1:100) was used to assess viability. Flow cytometry was conducted using a LSR II Fortessa Flow Cytometer (BD Biosciences). For compensation of fluorescence spectral overlap, UltraComp eBeads (eBioscience, Inc.) and GFP calibration beads (Takara) were used following the manufacturer's protocols. FCS 3.0 files were processed using FlowJo Software (Tree Star, Ashland, USA). For flow cytometric analyses, cell doublets were gated out using FSC-A vs FSC-H and SSC-A vs SSC-H followed by ZUV staining exclusion to identify live cells on viable (ZUV-) single cells. Then viable single cells were gated on CD45 expression. Flow cytometric analyses were performed on at least 4 different heart valve samples pooled from 3-5 mice per genotype or time-point.

Fluorescence activated cell sorting (FACS), RNA isolation, and real-time quantitative PCR (qPCR)

AoV and MV leaflets pooled from 4 mice were digested as described above and viable single cells were gated and sorted on CD45+, CD31+ or CD45-CD31- for leukocytes, endothelial or valvular interstitial cells, respectively using antibodies CD45 Alexa-488 (#103121, 1:400) and CD31 APC (#102409, 1:200). Total RNA was then purified using NucleoSpin RNA XS kit (Macherey-Nagel) following manufacturer's instructions. Reverse transcriptions were performed using SuperScript III First-Strand Synthesis Kit

(Invitrogen) according to manufacturer's instructions. Quantitative real-time PCR analyses were performed using Taqman probes (Applied Biosystems*), B2M* (*beta-2-microglobulin*) (Mm00437762_m1), *Col1a1* (Mm00801666_m1), *Col3a1* (Mm01254476_m1), *Sox9* (Mm00448840_m1), *Vcan* (Mm01283963_m1) and *Postn* (Mm00450111_m1). Level changes were calculated using $\Delta\Delta C_T$ method[8] normalized to B2M. The average of gene expression in CD31-CD45- cells was then set to 1 for each gene for calculations of fold-change. FACS was performed on 3 different pooled WT heart valve samples.

Statistics

One-way ANOVA and Mann-Whitney U tests were used to determine the significance of observed differences between the means or medians, respectively, using PRISM7 software package (GraphPad). Data are reported as means ± SEM or as medians with interquartile ranges. A p-value < 0.05 was considered significant.

References

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