SUPPLEMENTAL MATERIAL

Data Supplement to:

Tumor Necrosis Factor:

A Mechanistic Link between Angiotensin-II-induced Cardiac Inflammation and Fibrosis

Duerrschmid: TNF Signaling in Ang-II-induced Fibrosis

Clemens Duerrschmid, MS;[1] JoAnn Trial, PhD;[1] Yanlin Wang, MD, PhD;[2]

Mark L. Entman, MD;[1] Sandra B. Haudek, MS, PhD[1]

[1]Division of Cardiovascular Sciences

[2]Division of Nephrology

[1,2]Department of Medicine, Baylor College of Medicine, Houston, TX

1. METHODS

1.1 Animals, Ang-II Infusion, and Bone Marrow Transplantation

Eight-to-10 week old male and female C57BL/6-Tnfrsf1a^{tm1Imx}/J (TNFR1-KO mice) and C57BL/6J (WT mice) were purchased from Jackson Laboratory. Mice were randomly chosen to be infused with 1.5 µg/kg/min human Ang-II (Sigma-Aldrich) via subcutaneously implanted osmotic mini-pumps (Durect Corp.) for short (1 or 3 days) and longer (7 days) periods; control animals were implanted with sterile saline-filled pumps.[1, 2]

For bone marrow transplantation experiments, bone marrow from donor mice was isolated from femur and tibia and washed in ice cold DMEM. Recipient mice were irradiated with 100Gy (Cs-137, Gammacell®40 Extractor, MDS Nordion) and received ~10^6 cells in 150 µl PBS via tail vein injection. From 48 hours before and until 2 weeks after the procedure, mice received kanamycin-sulfate (Teknova) in drinking water (2 mg/ml) ad libitum. After a 6-7-week recovery period, animals were infused with Ang-II or saline as described above.

All mice were euthanized with 2% isofluorane followed by cervical dislocation. Hearts were either immediately used for cell isolation (flow cytometry), perfusion-fixed (immunostaining, histology), or snap frozen in liquid nitrogen (RNA isolation).

All mice were fed standard mouse chow and water ad libitum. The investigations conformed to the *Guide for the Care and Use of Laboratory Animals* published by the NIH. All animals were treated in accordance with the guidelines of the Baylor College of Medicine Animal Care and Research Advisory Committee.

1.2 Cell Isolation and Flow Cytometry

Hearts were rapidly excised, and cardiac cells were isolated by enzymatic digestion using collagenase (0.1 mg/ml Liberase TH Research Grade (Roche Applied Science), 10 mM Hepes, 20 U/ml DNaseI (Calbiochem) in 1x HBSS including calcium and magnesium (Invitrogen)) as described previously;[1-3] cardiomyocytes were not present in the resulting cell suspension, as they were disrupted during the isolation procedure. Briefly, hearts were dissected, washed in ice-cold base buffer (Krebs-Henseleit buffer pH 7.2 (Sigma-Aldrich) supplemented with 10 mM Hepes and 30 mM Taurine), transferred to 5 ml collagenase buffer, quickly minced into small 2x2 mm pieces, and incubated in a 37◦C water bath for 5 min under gentle shaking. The supernatant was then carefully removed and transferred through a 70 µm nylon cell strainer (BD Bioscience) into 15 ml of ice-cold stop buffer (base buffer plus 10% FBS and 20 U/ml DNaseI). 5 ml of fresh collagenase buffer was added to the remaining tissue fragments and incubation / separation was repeated 4-5 times until all tissue was dissolved. Isolated cells were combined, washed once in ice-cold stop buffer, and immediately used for flow cytometry.

For flow cytometry, approximately 1x10^5 freshly isolated cells were incubated with 50 nM calcein^{AM} (Invitrogen Molecular Probes) (which is converted to a green fluorescent product only by live cells) together with 0.5 µg PE-conjugated primary antibody (CD16/32, CD34, CD86 [BD Pharmingen]; CD150 [eBioscience]; CD206, CD301, TNFR1 [Biolegend]; and biotin-conjugated CD45 antibodies followed by PE/Cy-5-conjugated streptavidin (both from BD Pharmingen) or PE-conjugated secondary antibody (Jackson ImmunoResearch). Cells were washed in wash buffer (PBS, 0.1% gelatin, 0.1% NaN_3). Alternatively, calcein-incubated cells were exposed to

PE-labelled CD45 (BD Pharmingen), followed with biotinylated anti-TNFR1 (Biolegend) and PE/Cy-5 streptavidin. In separate setups, cells were incubated with external antibodies as listed above (but no calcein), then fixed and permeabilized (Cytofix/Cytoperm™ kit from BD Pharmingen), and incubated over night with either 1 µg anti-collagen-type-I (Rockland), 1 µg anti-TNF (Pierce), or 1 µg anti-αSMA (Sigma-Aldrich) antibody followed by a FITC-conjugated anti-rabbit or anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, Inc.). FITC/PE/Cy-5 fluorescence intensities were measured on an Epics XL-MCL using EXPO32 software or a Cell Lab Quanta SC with companion software (both Beckman Coulter). Suppl. Figures 1A and 1B outline our gating strategies using FlowJo X 10.0.7 (FlowJo LLC).

1.3 Perfusion Fixation, Immunofluorescence, and Histology

Mouse hearts were arrested in diastole by infusion with cardioplegic solution, perfusion fixed in Zinc-Tris buffer (0.1M Tris-HCl pH 7.4, 0.05% calcium acetate, 0.5% zinc acetate, 0.5% zinc chloride) for 4 hours, then gradually transferred into 70 % ethanol and embedded in paraffin. Three-5 µm thick sections close to the mid-papillary level were made and stained with hematoxylin / eosin for initial evaluation.[1-3]

For immunofluorescence, embedded heart sections were deparaffinized in xylene and rehydrated in decreasing ethanol concentrations. Rehydrated sections were permeabilized using 1% Triton X, washed in PBS containing 0.3% Tween-20, blocked with 1% BSA in PBS containing 0.3% Tween and stained with primary antibodies (CD86 [BD Pharmingen]; CD206, CD301, TNFR1 [Biolegend]; TNF [Pierce]; pro-collagen-type-I ([Santa Cruz Biotechnology]; iNOS [Abcam]; arginase [R&D Systems]) over night at 4°C, followed by PE- and/or FITC-conjugated secondary antibodies if needed (all from Jackson Immunoresearch) for 1 hour at room temperature. Slides were mounted using Fluoroshield™ with DAPI (GeneTex, Inc.) and analyzed on an Olympus AX70 upright microscope. Monochrome images for each fluorescence channel were acquired using QCapture Pro 6.0 software (QImaging). Composite images were created using ImageJ software (v1.46r, NIH).

For histology, rehydrated sections were stained in 0.05% picrosirius red (Direct Red 80 in saturated picric acid; both from Sigma-Aldrich) for 1 hour, developed in acidified water (5 ml glacial acetic acid in 1 L distilled water), and quickly dehydrated in ethanol and xylene. Slides were mounted in Cytoseal™ XYL (Thermo Fisher Scientific). For each histological measurement, 4-6 sections per mouse were stained. Per section, at least 4 predetermined microscope fields within the anterior, lateral, and posterior left ventricle region were evaluated (thus a minimum of 16 microscopic fields per mouse were assessed). Bright field images were captured on an Olympus CKX41 inverse microscope using QCapture Pro 5.0 software (QImaging). Quantitative analysis of collagen stained areas were calculated using ImagePro Plus software 5.1 (Media Cybernetics Inc.) as percentages of the total myocardial area.

1.4 RNA Isolation and Quantitative PCR

Frozen hearts were ground in liquid nitrogen using mortar and pestle. Tissue was disrupted using a polytron homogenizer (Polytron Corp.) and whole RNA was isolated using Trizol reagent (Life Technologies). cDNA was synthesized from whole RNA using a Verso reverse transcription kit (Thermo Fisher Scientific). 1:10 dilutions of cDNA were used as PCR template and run on a CX1000 Touch™ Thermal Cycler with a CFX96™ Real-Time System

using SSOAdvanced™ SYBR® green mastermix (all from BioRad Laboratories). All primers were validated according to MIQE guidelines prior to their use.[4] Relative gene expression compared to control (WT saline levels = 1-fold) was calculated using the ΔΔCq method. See Suppl. Table 1 for specific primer sequences (all according to the corresponding mouse sequence).

1.5 Monocyte Isolation and in vitro Transendothelial Migration Assay (TEM)

Untreated mice were euthanized as described above and the spleen was harvested. Monocytes from spleen were isolated using an EasySep™ Mouse Monocyte Enrichment Kit (Stemcell Technologies) according to the manufacturer's instructions. In vitro TEM was performed as previously reported for human cells,[1, 3, 5] but was adapted for murine cells. Briefly, mouse cardiac endothelial cells (MCEC; CLU510, Cedarlane Labs) were seeded at 40,000 cells/insert (membrane pore size 8 µm; Corning). MCEC were allowed to attach and grow confluent for 3-4 days. Mouse spleen monocytes ($25x10^4$) in high glucose DMEM (Invitrogen) with 1% fetal bovine serum (FBS; HyClone), antibiotic/antimycotic (Gibco), and 10mM Hepes (Gibco) were added to the top (insert) well. Mouse MCP-1 (650 ng/ml; R&D Systems) in RPMI 1640 was added to the bottom well for chemoattraction. Some setups received both Ang-II (1 ng/ml, Sigma-Aldrich) and TNF (10 ng/ml, R&D Systems) in the top well. Cells were allowed to migrate for 4 days. Adherent cells in the bottom well were fixed in 4% paraformaldehyde and stained with Giemsa (Sigma). Fibroblasts were distinguished from other cells by their morphology (spindle-shaped, elongated cells [see [1, 5] for detailed cell identification]) and manually counted. Each setup was measured in triplicate. In separate setups, migrated cells after 1, 4, or 6 days were allowed to adhere on poly-d-lysine coated coverslips (Corning® BioCoat™). Cells were fixed in 4% paraformaldehyde and stained with fluorescent-labeled antibodies (CD86 [BD Pharmingen]; CD206, CD301, TNFR1 [Biolegend]; iNOS [Abcam]; pro-collagen-type-I [Santa Cruz Biotechnology]) as described earlier for human cells.[5]

1.6 Statistical Analysis:

Statistical analyses were performed with GraphPad InStat 3.06 software; a P value of <0.05 was considered statistically significant. Data are expressed as mean ± SEM (in text, and indicated by lanes ± error bars in dot plot graphics); box plots represent the first and third quartiles, the band inside the box the second quartile (median), and whiskers the minimum and maximum. We evaluated all data for a Gaussian distribution using a Kolmogorov-Smirnov test, as well for differences among the standard deviations (SDs) using a Bartlett's test. Depending on the outcome, we proceeded with the appropriate parametric or nonparametric test:

Figure 1: The percentages of positively gated cells were normally distributed, except for the TNFR1-KO 0-day control groups, in which we had too few samples to test for normality, and most mouse groups in panel D2. Bartlett's testing further revealed that most differences among the SDs were significant in all panels. Therefore, we analyzed these data using a nonparametric Kruskal-Wallis test followed by Dunn's Multiple Comparisons post-hoc testing.

Figure 2: All data passed the normality test, but some groups displayed differences in their SDs. Thus, we analyzed these data using a nonparametric Kruskal-Wallis test followed by Dunn's Multiple Comparisons post-hoc testing.

Figure 3: Because data for the control group (saline) were too few to pass normality and some groups had differences among their SDs, data were analyzed using a two-tailed nonparametric Mann-Whitney test.

Figure 4/A2: Data were either too few to pass normality and/or had differences among the SDs and were thus analyzed using a nonparametric Kruskal-Wallis test followed by Dunn's Multiple Comparisons post-hoc testing.

Figures 4B and 5: Data for the control group (TNFR1-KO/TNFR1-KO) as well as comparison group (WT/WT) were too few to pass normality and too few to calculate differences in SDs. We therefore used a nonparametric Kruskal-Wallis test followed by Dunn's Multiple Comparisons post-hoc testing to evaluate these data.

Figure 6: All groups passed normality tests and displayed no differences in SDs. Therefore, we used a one-way ANOVA followed by Tukey-Kramer Multiple Comparisons post-hoc testing for data evaluation.

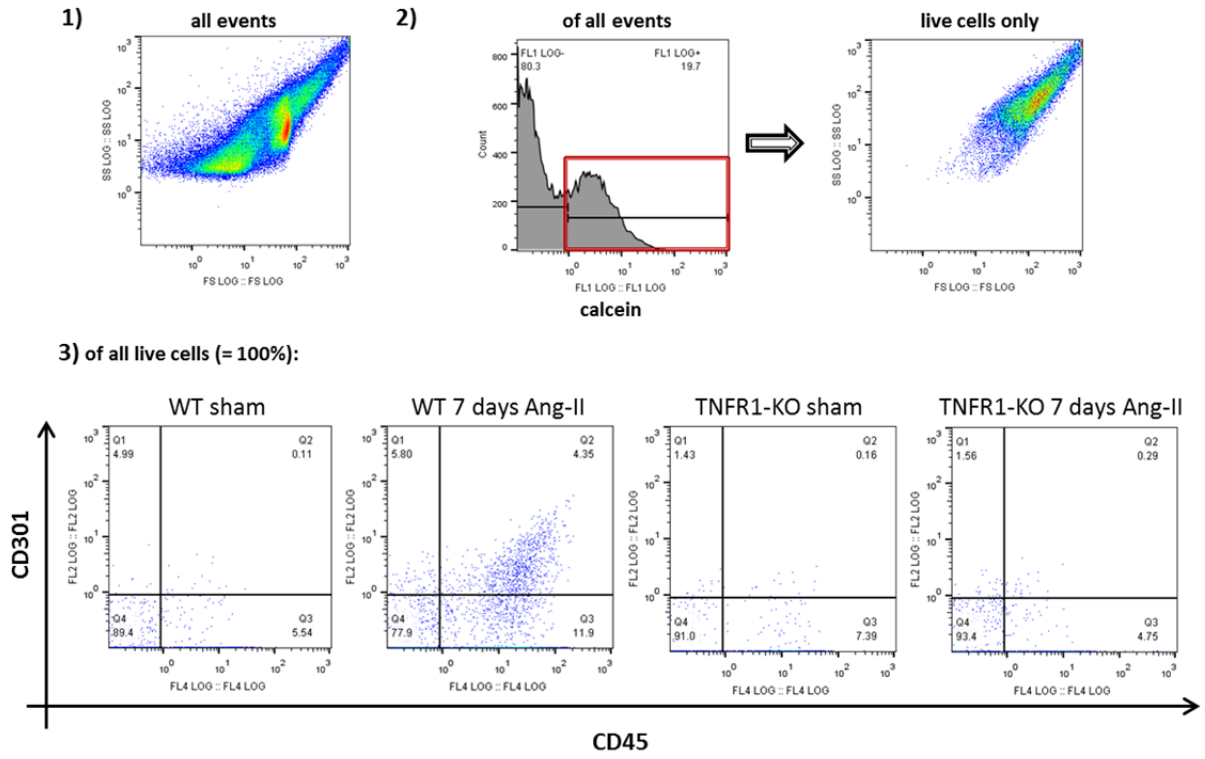
2. TABLES

Supplemental Table 1: Primer sequences for quantitative PCR (according to corresponding mouse sequences).

Gene	Forward primer	Reverse Primer
18s RNA	cggacaggattgacagattg	caaatcgctccaccaactaa
TNF	ccagtgtgggaagctgtctt	aagcaaaagaggaggcaaca
MCP-1	tccacaaccacctcaagcacttc	ggcatcacagtccgagtcacac
IL-1β	ttgacggaccccaaaagatgaaggg	tccacagccacaatgagtgatactg
IL-6	agttgccttcttgggactga	acaggtctgttgggagtggt
IL-13	tccaattgcaatgccatcta	tgagtccacagctgagatgc
IFN-γ	actggcaaaaggatggtgac	gacctgtgggttgttgacct
IL-4	cctcacagcaacgaagaaca	atcgaaaagcccgaaagagt
IL-13	gtgtctctccctctgaccct	ggggagtctggtcttgtgtg
TGF-β1	cactggagttgtacggcagtg	agagcagtgagcgctgaatc
TNFR1	gctgaccctctgctctacgaa	gccatccaccacagcataca
osteopontin	tgatgacgatgatgatgac	ctcagtccataagccaag
collagen-type-I	gtatgcttgatctgtatctg	cgactcctacatcttctg
collagen-type-III	gatgaggagccactagactg	gccatcaggaagcacagg

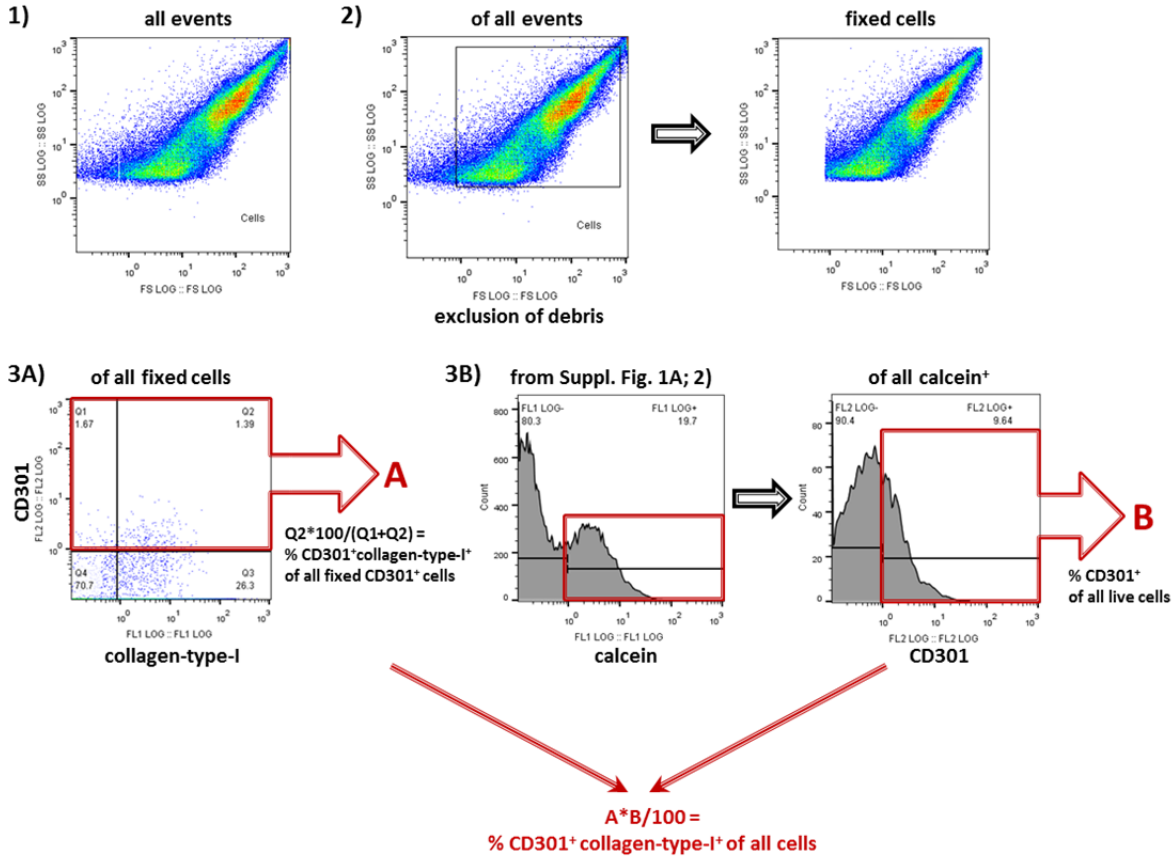
3. FIGURES

Supplemental Figure 1A:



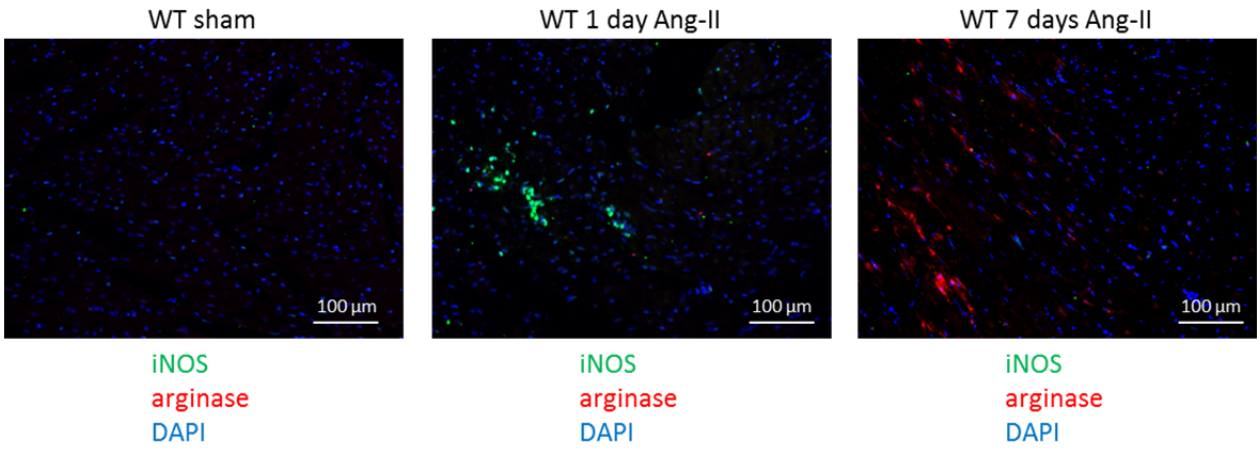
Flow cytometry gating strategy for external marker measurements (representative example for "*% of all live cells*" using calcein, anti-CD301, and anti-CD45): Hearts were removed and cells isolated. Immediately, cells were incubated with calcein and antibodies against external markers of interest. **1)** Ungated events, x-axis: LOG forward scatter (FS), y-axis: LOG side scatter (SS). **2)** Of all measured events, only cells that were positive for green-fluorescent calcein (FL-1, x-axis) were selected (only live cells are able to metabolize calcein into its green fluorescent form). **3)** Of all live cells (= 100%), the percentages of PE/Cy5-labeled $CD45^+$ cells (FL-4, x-axis) and PE-labeled $CD301^+$ cells (FL-2, y-axis) were determined. Representative diagrams for sham-treated WT and TNFR1-KO and 7-day Ang-II-treated WT and TNR1-KO hearts are shown. Q1: $CD301^+CD45^-$ cells, Q2: $CD301^+CD45^+$cells, Q3: $CD301^-CD45^+$cells, Q4: $CD301^-CD45^-$ cells. Values for Q2 were used to generate graphs in Figs. 1D-F1, 3E-H, 5A-C1, and Suppl. Figs. 3A-B1, 5, and 6A.

Supplemental Figure 1B:



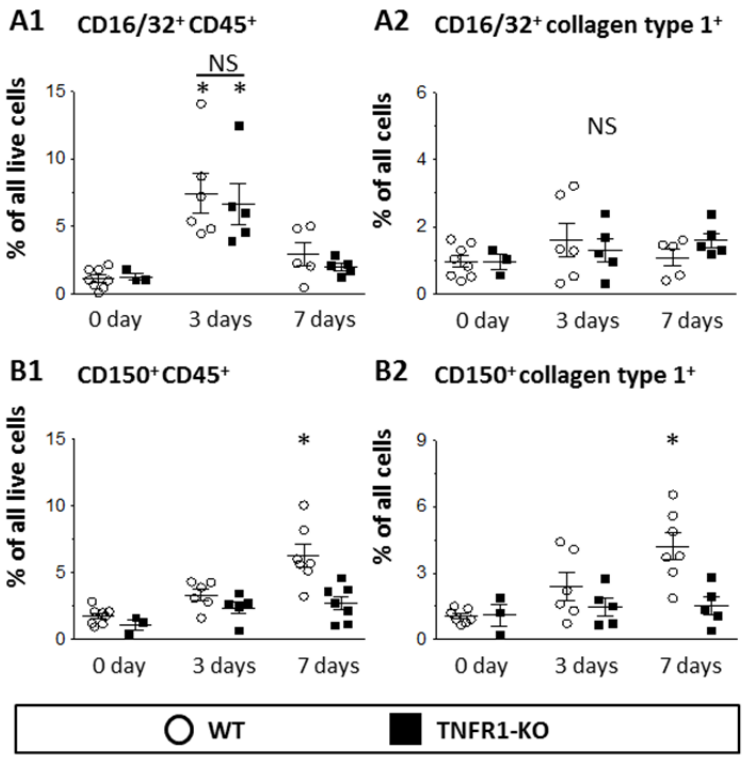
Flow cytometry gating strategy for external and internal marker measurements (representative example for *"% of all cells"* using anti-CD301, anti-collagen-type-I and calcein): Hearts were removed and cells isolated. Cells were first incubated with antibodies against external markers of interest, then were permeabilized, fixed, and incubated with antibodies against internal markers of interest **1)** Ungated events, x-axis: LOG forward scatter (FS), y-axis: LOG side scatter (SS). **2)** Of all measured events, cell debris was excluded by size and granularity. **3A)** Of all fixed cells, the percentages of FITC-labeled collagen-type-I$^+$ cells (FL-1, x-axis) and PE-labeled CD301$^+$ cells (FL-2, y-axis) were determined. A representative diagram for a 7-day Ang-II-treated WT heart is shown. Q1: CD301$^+$collagen-type-I$^-$ cells, Q2: CD301$^+$collagen-type-I$^+$ cells, Q3: CD301$^-$collagen-type-I$^+$ cells, Q4: CD301$^-$collagen-type-I$^-$ cells. Values in Q1 and Q2 were used to calculate the % CD301$^+$collagen-type-I $^+$ cells of all fixed CD301$^+$ cells (= value A). **3B)** In parallel setups, cells isolated from the same heart were incubated with calcein and PE-labelled anti-CD301 as described in Suppl. Fig. 1A and the % CD301$^+$ cells of all live cells was calculated (= value B). **3A+B)** Values of fixed cells were normalized to live cell content to give the % CD301$^+$collagen-type-I$^+$ of all cells. Final numbers were used to generate graphs in Figs. 1D-F2, 5A-C2, and Suppl. Figs. 3A-B2, and 6B (in Figs. 3A-D2, and Suppl. Fig. 4 and 10A, values for Q2 (from 3A) are given, because the number of % calcein$^+$ cells was not measured in these cell isolations).

Supplemental Figure 2:



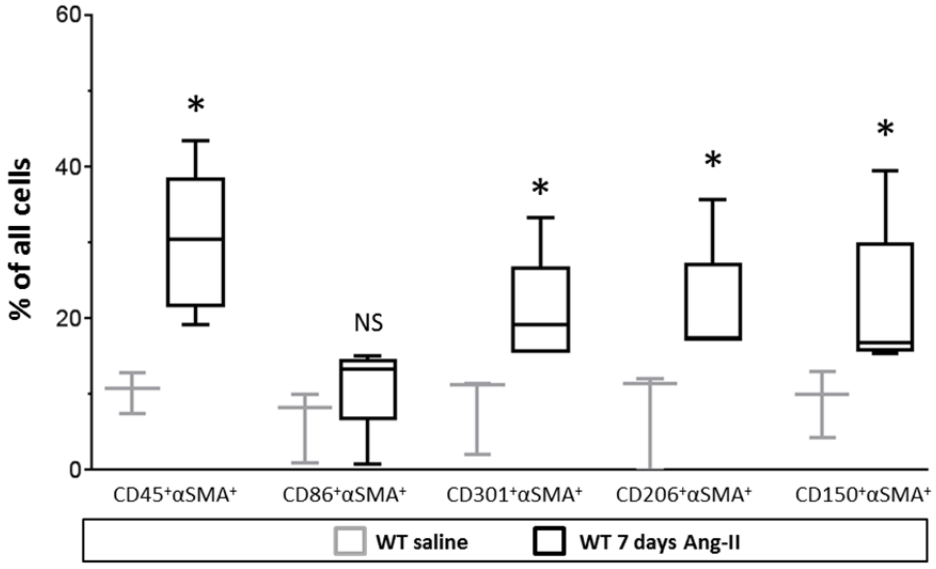
Early appearance of inducible nitric oxide synthase (iNOS, M1 marker) and late appearance of arginase (M2 marker) positive cells in the heart after Ang-II infusion: After 0 (sham), 1, and 7 days of Ang-II infusion, perfusion-fixed WT heart sections were stained with FITC-labeled anti-iNOS (green) and PE-labeled anti-arginase (red); (blue = nuclear DAPI). Representative images are shown; they indicate an early appearance of M1-cells at 1 day ($iNOS^+$, middle image) that were absent after 7 days (right image), and a later presence of M2-cells at 7 days ($arginase^+$, right image) that were only few in number at 1 day (middle image). Both were absent in sham-treated hearts (left image).

Supplemental Figure 3:



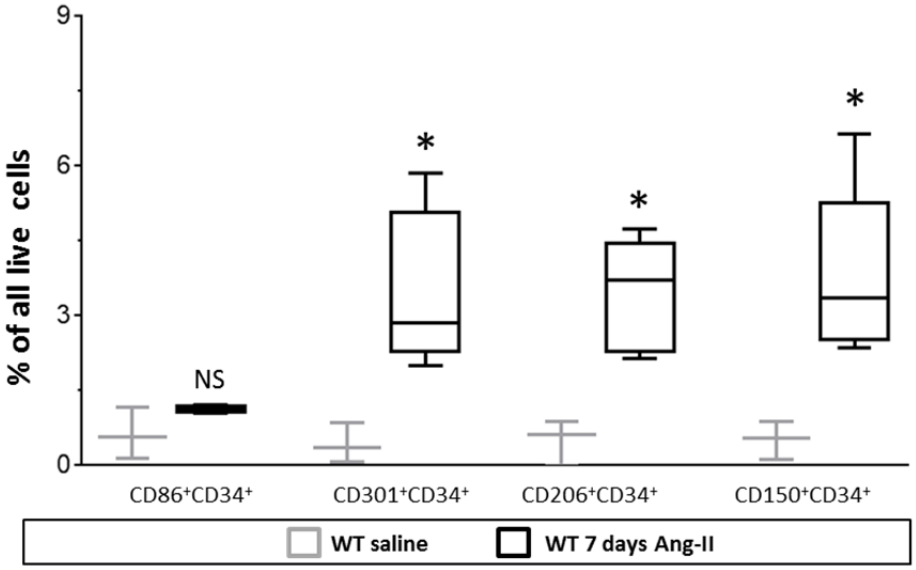
Early appearance of M1-cells and late appearance of M2-cells in the heart after Ang-II infusion: Similar to Fig.1, isolated cells from WT and TNFR1-KO hearts were subjected to flow cytometry. "Live" cells were identified via calcein uptake that was metabolized to a green-fluorescent product. **A1, B1:** In WT hearts, hematopoietic M1-cells ($CD16/32^+CD45^+$) appeared maximally at 3 days, whereas M2-cells ($CD150^+CD45^+$) increased maximally by 7 days. In TNFR1-KO mice, M1-cells were equally upregulated, but M2-cells were fewer than in WT hearts. **A2, B2:** $CD16/32^+$ cells were collagen-type-I negative, whereas $CD150^+$ cells were collagen-type-I positive. **Statistics:** The percentages of positively gated cells were normally distributed, except for the TNFR1-KO 0-day control groups (too few samples to test for normality). Bartlett's testing revealed that most differences among the SDs were significant for all panels. Therefore, we analyzed these data using a nonparametric Kruskal-Wallis test followed by Dunn's Multiple Comparisons post-hoc testing. (*) indicates a statistically significant difference between saline-treated (WT: n=8; TNFR1-KO: n=3) and 3-day (WT: n=6; TNFR1-KO: n=5) or 7-day (WT: n=5-7; TNFR1-KO: n=5-7) Ang-II-infused mice within the same genetic background. NS: not significant.

Supplemental Figure 4:



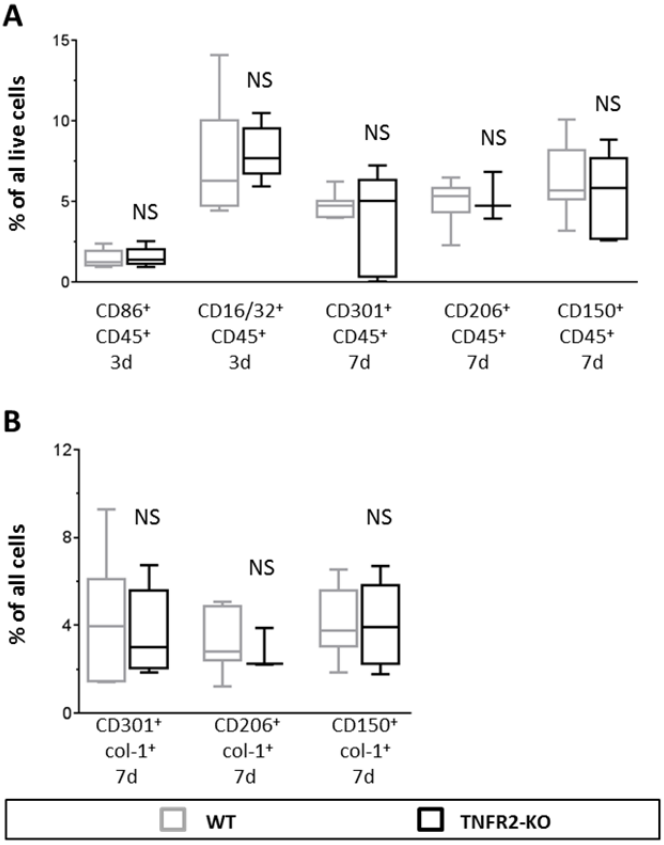
Ang-II induced the appearance of M2-related α-smooth muscle actin (αSMA) positive cells in the WT heart: Isolated cells from saline (n=3) and 7-day Ang-II (n=5) stimulated WT hearts were subjected to flow cytometry. Ang-II increased the number of hematopoietic ($CD45^+$) cells that were also positive for αSMA. In addition, we also found increased numbers of M2-cells ($CD301^+$, $CD206^+$, and $CD150^+$) that expressed αSMA. By contrast, the number of M1-cells ($CD86^+$) positive for αSMA did not change by Ang-II stimulation. **Statistics:** Because the number of samples in the saline-treated group was too few to test for normality, we used a two-tailed nonparametric Mann-Whitney test for data analysis. Bartlett's testing revealed no differences among the SDs for all groups. (*) indicates a statistically significant difference ($p< 0.05$) between saline-treated and 7-day Ang-II-infused WT mice. NS: not significant.

Supplemental Figure 5:



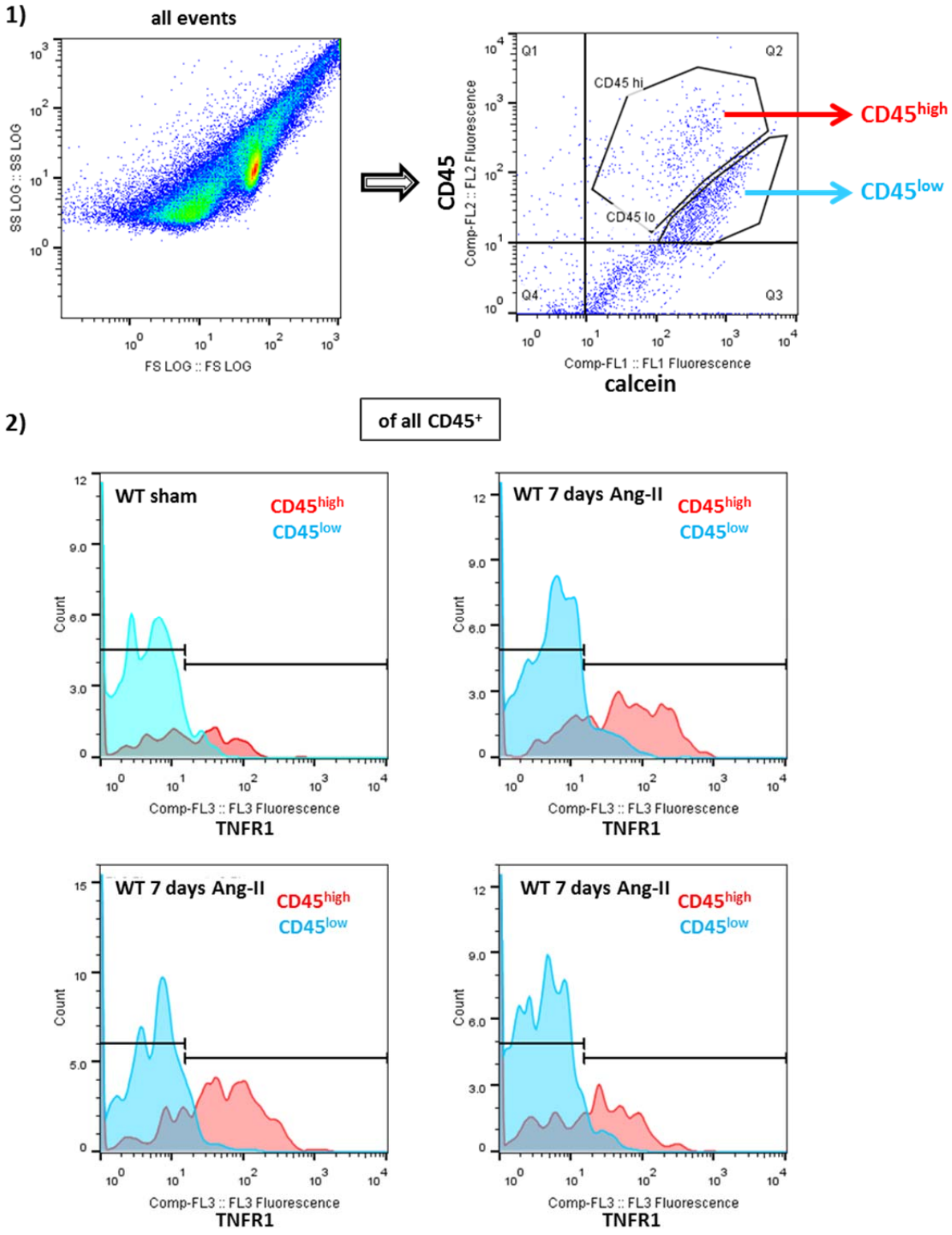
Ang-II-induced M2-cells expressed CD34: Isolated cells from saline (n=3) and 7-day Ang-II (n=5) stimulated WT hearts were subjected to flow cytometry. Ang-II increased the number of live (calcein^{+}) M2-cells (CD301^{+}, CD206^{+}, CD150^{+}) that were also positive for CD34. By contrast, M1-cells (CD86^{+}) were negative for CD34 expression. **Statistics:** Because the number of samples in the saline-treated group was too few to test for normality, we used a two-tailed nonparametric Mann-Whitney test for data analysis. Bartlett's testing revealed no differences among the SDs for all groups. (*) indicates a statistically significant difference ($p< 0.05$) between saline-treated and 7-day Ang-II-infused WT mice. NS: not significant.

Supplemental Figure 6:



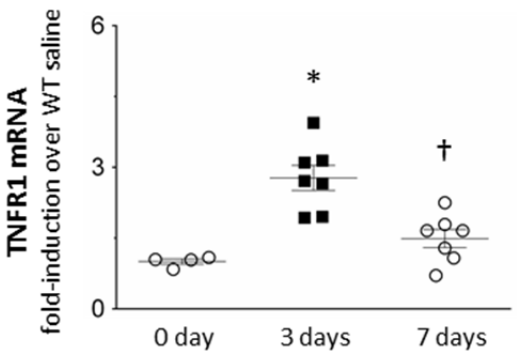
In TNFR2-KO hearts, Ang-II-induced the early uptake of M1-cells and later uptake of M2-cells to similar extents as in WT hearts: Isolated cells from WT and TNFR2-KO hearts were subjected to cytometry. **A:** "Live" cells were identified via calcein uptake that was metabolized to a green-fluorescent product. In WT and TNFR2-KO hearts, hematopoietic M1-cells ($CD86^+CD45^+$, $CD16/32^+CD45^+$) appeared maximally at 3 days, whereas M2-cells ($CD301^+CD45^+$, $CD206^+CD45^+$, $CD150^+CD45^+$) increased maximally by 7 days. **B:** The amount of M2-cells expressing collagen-type-I (col-I) was not different between WT and TNFR2-KO mouse hearts. **Statistics:** Data for CD301 and CD206 were either too few to pass normality and/or had differences among the SDs and were thus analyzed using a two-tailed nonparametric Mann-Whitney test. All other groups were analyzed with an unpaired two-tailed Student's t test. WT: n=6 and TNFR2-KO: n=5 for 3-day Ang-II-infusion, WT: n=7 and TNFR2-KO: n=3-6 for 7-day Ang-II-infusion. There were no statistically significant differences ($p< 0.05$) between groups. NS: not significant.

Supplemental Figure 7:



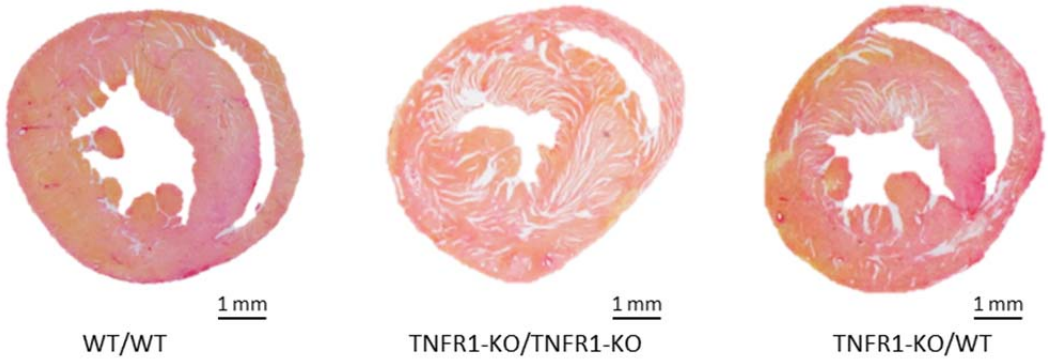
Ang-II-induced TNFR1^{+} cells express high levels of CD45: 1) Gating strategy: Isolated cells were incubated with calcein (to identify live cells), PE-labelled anti-CD45, and PE/Cy-5-labeled anti-TNFR1 and subjected to flow cytometry similar to Suppl. Fig. 1A. Live (calcein^{+}) cells expressing high levels of CD45 (CD45^{high}, red) were distinguished from those expressing low levels (CD45^{low}, blue). Each group was then examined for TNFR1 expression. **2)** Representative images for 1x WT sham and 3x WT 7day Ang-II-treated hearts are shown: Of all CD45 positive cells, overlapping curves for CD45^{high} (red) and CD45^{low} (blue) expression that also expressed TNFR1 (FL3, x-axis).The data indicate that most Ang-II-induced TNFR1^{+} cells also expressed CD45^{high}, whereas most CD45^{low} cells were negative for TNFR1 expression.

Supplemental Figure 8:



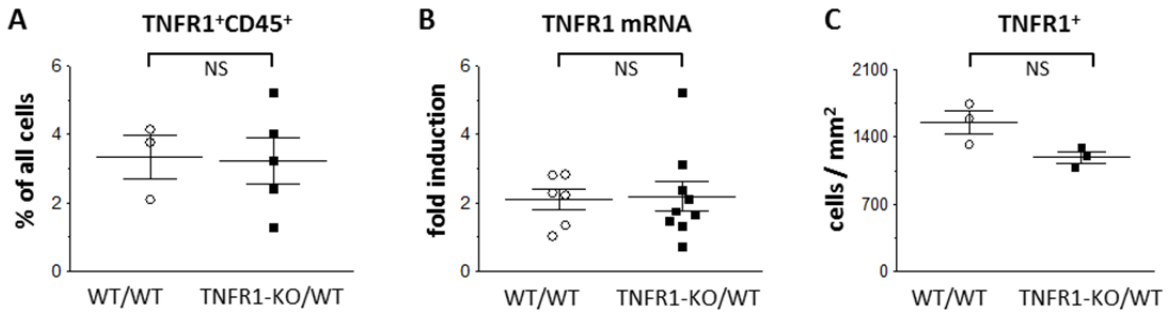
Transcriptional activation of TNFR1 mRNA expression: WT mice were infused with Ang-II for 0 (sham), 1, or 7 days. RNA from whole hearts was subjected to quantitative PCR using specific primers for TNFR1 (see Suppl. Table 1). Data are represented as fold-increase over WT saline levels (=1-fold). **Statistics:** Data were either too few to pass normality and/or had differences among the SDs and were thus analyzed using a nonparametric Kruskal-Wallis test followed by Dunn's Multiple Comparisons post-hoc testing. Saline group n=4, each treated group: n=7. (*) indicates a statistically significant difference between saline-treated and Ang-II-infused mice; (†) indicates a statistically significant difference between 1- and 7- day Ang-II-infused mice.

Supplemental Figure 9:



Ang-II induced the development of cardiac fibrosis in chimeric TNFR1-KO mice with WT bone marrow: Mice (WT mice with WT bone marrow [WT/WT], TNFR1-KO mice with TNFR1-KO bone marrow [TNFR1-KO/TNFR1-KO], and TNFR1-KO mice with WT bone marrow [TNFR1-KO/WT]) were subjected to 7-day Ang-II exposure. Perfusion-fixed tissue was stained with picrosirius red for collagen detection; representative images of whole heart sections are shown (see Fig.4 for higher magnification images and quantitative analysis). In response to Ang-II, chimeric TNFR1-KO/WT mice developed interstitial cardiac fibrosis similar to WT mice and significantly more than TNFR1-KO mice.

Supplemental Figure 10:



After 7 days of Ang-II exposure, the amount of TNFR1 expressing cells in chimeric TNFR1-KO/WT hearts was similar to WT/WT hearts: A: Flow cytometry analysis of isolated cells (WT/WT: n=3, TNFR1-KO/WT: n=5). **B:** Transcriptional expression of TNFR1 mRNA by quantitative PCR analysis over saline levels (WT saline = 1-fold; WT/WT: n=6, TNFR1-KO/WT: n=9). **C:** Perfusion-fixed tissue was stained for TNFR1 and positive cells were counted (WT/WT: n=3, TNFR1-KO/WT: n=3). **Statistics:** A and C: Because the number of samples in the control group (WT/WT) was too few to test for normality, we used a two-tailed nonparametric Mann-Whitney test for data analysis. Bartlett's testing revealed no differences among the SDs for all groups. B: Both groups passed normality tests and displayed no differences in SDs; thus we used an unpaired two-tailed Student's t test for data evaluation. There were no statistically significant differences ($p < 0.05$) between groups. NS = not significant.

4. REFERENCES

(1) Duerrschmid C, Crawford JR, Reineke E, Taffet GE, Trial J, Entman ML, Haudek SB. TNF receptor 1 signaling is critically involved in mediating angiotensin-II-induced cardiac fibrosis. *J Mol Cell Cardiol.* 2013; 18;57C:59-67.

(2) Haudek SB, Cheng J, Du J, Wang Y, Hermosillo-Rodriguez J, Trial J, Taffet GE, Entman ML. Monocytic fibroblast precursors mediate fibrosis in angiotensin-II-induced cardiac hypertrophy. *J Mol Cell Cardiol.* 2010;49:499-507.

(3) Haudek SB, Xia Y, Huebener P, Lee JM, Carlson S, Crawford JR, Pilling D, Gomer RH, Trial J, Frangogiannis NG, Entman ML. Bone marrow-derived fibroblast precursors mediate ischemic cardiomyopathy in mice. *Proc Natl Acad Sci USA.* 2006;103:18284-9.

(4) Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem.* 2009;55:611-22.

(5) Trial J, Cieslik KA, Haudek SB, Duerrschmid C, Entman ML. Th1/M1 conversion to th2/m2 responses in models of inflammation lacking cell death stimulates maturation of monocyte precursors to fibroblasts. *Front Immunol.* 2013;4:287.