Title: SUPPRESSED PRODUCTION OF SOLUBLE FMS-LIKE TYROSINE KINASE-1 CONTRIBUTES TO MYOCARDIAL REMODELING AND HEART FAILURE

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Short Title: sFlt-1 and Heart Failure

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SUPPLEMENTAL MATERIAL

Supplemental Methods

Immunohistology and Immunofluorescence

Cardiac tissue was fixed and frozen in OCT-embedding compound (Tissue Tek; Sakura Finetek, Tokyo, Japan) at -80° C. Then, 10-µm-thick sections were obtained and fixed with cold acetone. The following antibodies were used for immunohistology: rat anti-CD68 (dilution 1/10,000) (ab53444; Abcam, Cambridge, MA, USA) and mouse anti-MCP-1(dilution 1/2,000) (2D8; Nobus Biologicals, Littleton, CO, USA). Images were obtained by a fluorescent microscope (BZ-X700; KEYENCE, Osaka, Japan). The optional software (BZ-analysis; KEYENCE) was used for the analysis.

For immunofluorescent staining, 10-µm cryosections were fixed with cold acetone and blocked with 10% normal goat serum (Sigma-Aldrich, St. Louis, MO, USA). Sections were incubated for two hours at room temperature with primary antibodies. For analysis of microvessels and myocytes, rat anti-CD31 (dilution 1/100; BD Biosciences: 550274 and Wheat germ agglutinin Alexa Fluor 488 (WGA, dilution 1/1000; Invitrogen: W11261) were used. For analysis of macrophages, rat anti-CD68 and goat anti-CD206 (dilution 1/200) (AF2535; R&D Systems) were used. Secondary antibodies were as follows: Alexa Fluor 594 donkey anti-rat and Cy2 donkey anti-goat.

Macrophages, fibroblasts, endothelial cells, and myocytes were double stained with MCP-1 and cell-specific surface antigens in order to investigate localization of MCP-1 protein in the pressure-overloaded heart. Primary antibodies were as follows: mouse anti-MCP1 (dilution 1/500) (2D8; Nobus Biologicals), rat anti-CD68, rat anti-fibroblasts (dilution 1/400) (ER-TR7; Acris Antibodies GmbH, San Diego, CA, USA), rat anti-CD31 (dilution 1/100) (550274; BD Biosciences, Franklin Lakes, NJ, USA), and rabbit anti-Cardiac Troponin I (dilution 1/100) (ab47003; Abcam) followed by staining with secondary antibody: Alexa Fluor 488 anti-rat, Alexa Fluor 488 anti-rabbit, and Alexa Fluor 647 anti-mouse. Images of immunofluorescent staining were obtained by confocal microscopy (FLUOVIEW FV1000l; Olympus, Tokyo, Japan). The analyses for cardiomyocyte areas and the number of vessels-to-cardiomyocyte ratio were performed using Image J 1.46 software (https://imagej.nih.gov/ij).

Real-time Polymerase Chain Reaction

Cardiac mRNA was extracted from the left ventricle using Trizol reagent (Life Technologies, CA, USA). Expression levels of sFlt-1 and Flt-1 mRNA were measured as described previously.[1] Reverse transcription was performed using the QuantiTect Reverse Transcription Kit (One Step Real-time PCR Systems; Life Technologies, Grand Island, NY, USA). Levels of gene expression were quantified by real-time polymerase chain reaction using Taqman Gene Expression Assays (One Step Real-time PCR Systems; Life Technologies).

Western Blotting

The cardiac tissues were homogenized with lysis buffer (pH 7.6, 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 10 mM DTBA, 8M urea). The total 10-µg sample was loaded on 16% gel (TEFCO) and transferred to membrane. The membrane was blocked with Blocking One (Nacalai Tesque, Inc., Kyoto, Japan) for 20 minutes, and incubated overnight at 4° C with mouse anti-MCP-1 antibody (dilution 1/1,000) (2D8; Nobus Biologicals). Anti-mouse IgG, HRP-linked antibody (dilution 1/5,000) (#7076; Cell Signaling Technology, Danvers, MA, USA) was used as a secondary antibody for one hour at room temperature. The signals were detected by a SuperSignal West Dura chemiluminescent substrate (Fisher Scientific, Pittsburgh, PA, USA). The blots were also probed with a monoclonal GAPDH antibody (dilution 1/100,000) (Sigma-Aldrich) as a control.

References

1. Matsui M, Takeda Y, Uemura S, et al. Suppressed soluble Fms-like tyrosine kinase-1 production aggravates atherosclerosis in chronic kidney disease. *Kidney Int*. 2014;85:393–403.

Supplemental Table

Table S1. Blood pressure measurement.

Parameter	WT			sFlt-1$^{-/-}$		
	Sham (n = 4)	TAC (n = 3)	*P*	Sham (n = 4)	TAC (n = 3)	*P*
HR, /min	603.57±35.23	566.2±71.7	NS	533.35±45.09	682.13±70.94	NS,NS
SBP, mmHg	103.5±3.53	106.87±2.89	NS	103.15±4.77	106.3±8.50	NS,NS
MBP, mmHg	72.57±2.61	72.67±3.04	NS	64.23±4.42	72.57±3.35	NS,NS
DBP, mmHg	57.4±3.27	55.57±3.66	NS	44.975±5.73	55.8±7.23	NS,NS

There were no significant differences in heart rate or blood pressure between wild-type (WT) and sFlt-1$^{-/-}$ mice after sham or TAC operation. DBP indicates diastolic blood pressure; HR, heart rate; MBP, mean blood pressure; SBP, systolic blood pressure.

Table S2. Echocardiographic analysis for mice treated with control IgG or anti-PLGF neutralizing antibody (αPLGF)

Parameter	WT				sFlt-1$^{-/-}$			
	Sham		TAC		Sham		TAC	
	IgG	aPLGF	IgG	aPLGF	IgG	aPLGF	IgG	aPLGF
	(n = 6)	(n = 4)	(n = 9)	(n = 8)	(n = 6)	(n = 4)	(n = 7)	(n = 7)
IVSd, mm	0.60±0.02	0.63±0.01	0.80±0.01*	0.70±0.01‡	0.61±0.02	0.63±0.01	1.03±0.05*†	0.75±0.03*‡
PWd, mm	0.61±0.02	0.63±0.01	0.81±0.01*	0.73±0.02*‡	0.60±0.02	0.62±0.02	1.06±0.04*†	0.75±0.04*‡
LVDd, mm	2.67±0.11	2.72±0.10	2.52±0.13	2.91±0.15	2.64±0.10	2.80±0.06	2.43±0.25	2.67±0.13
EF, %	77.97±1.46	78.03±1.04	77.01±1.01	78.01±2.80‡	77.53±1.22	78.03±1.04	55.69±2.80*†	78.64±1.35‡

Treatment with αPLGF rescued cardiac hypertrophy and left ventricular systolic dysfunction after pressure overload in sFlt-1$^{-/-}$ mice. IgG indicates immunoglobulin G; IVSd, interventricular wall thickness dimensions; LVDd, left-ventricular end-diastolic dimensions; PLGF, placental growth factor; PWd, posterior wall thickness dimensions; sFlt-1, soluble Flt-1; TAC, transverse aortic constriction; WT, wild-type.
$^{*}P < 0.05$ vs. corresponding sham group; $^{†}P < 0.05$ vs. WT (TAC + IgG); $^{‡}P < 0.05$ vs. sFlt-1$^{-/-}$ (TAC + IgG). Data are mean ± SEM.

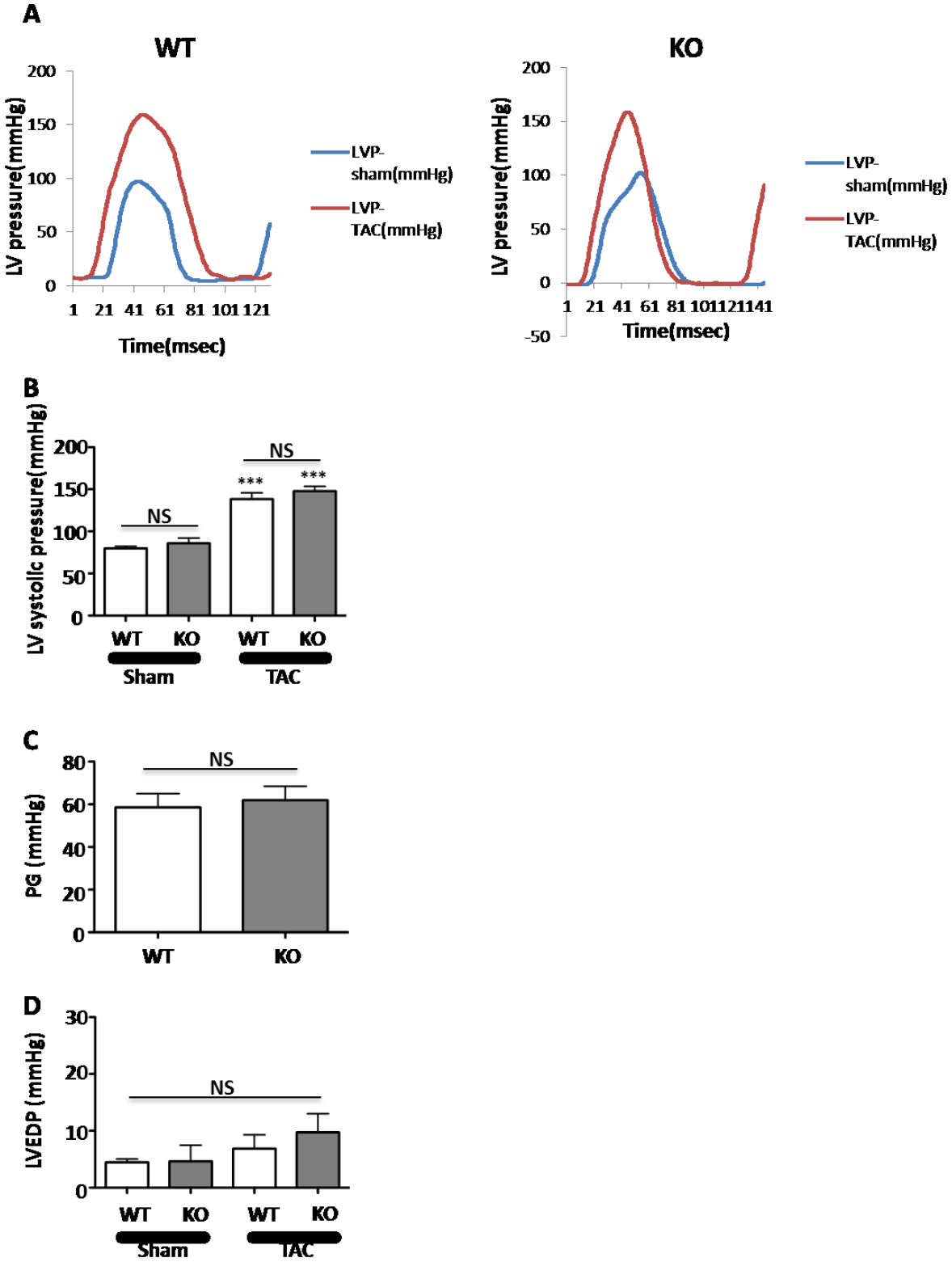
Table S3. Echocardiographic analysis for mice treated with control IgG or MCP-1 neutralizing antibody (MCP-1Ab)

Parameter	WT				sFlt-1$^{-/-}$			
	Sham		TAC		Sham		TAC	
	IgG (n = 4)	MCP-1Ab (n = 4)	IgG (n = 7)	MCP-1Ab (n = 8)	IgG (n = 4)	MCP-1Ab (n = 4)	IgG (n = 7)	MCP-1Ab (n = 9)
IVSd, mm	0.61±0.02	0.58±0.02	0.78±0.01*	0.76±0.0*‡	0.58±0.02	0.62±0.01	1.03±0.04*†	0.78±0.01*‡
PWd, mm	0.61±0.02	0.63±0.01	0.81±0.01*	0.76±0.02*‡	0.62±0.01	0.62±0.01	1.06±0.05*†	0.77±0.02*‡
LVDd, mm	2.65±0.04	2.48±0.03	2.93±0.16	2.84±0.14	2.43±0.03	2.48±0.03	2.61±0.13	2.67±0.13*‡
EF, %	78.18±0.93	77.63±1.29	75.67±2.23	77.23±2.21‡	78.98±0.92	77.90±1.29	59.41±2.81*†	72.76±1.42‡

Treatment with MCP-1Ab prevented pressure-overloaded cardiac hypertrophy and dysfunction in sFlt-1$^{-/-}$ mice. IgG indicates immunoglobulin G; IVSd, interventricular wall thickness dimensions; LVDd, left-ventricular end-diastolic dimensions; MCP-1Ab, monocyte chemoattractant protein-1 antibody; PWd, posterior wall thickness dimensions; sFlt-1, soluble Flt-1; TAC, transverse aortic constriction; WT, wild-type.

$^{*}P < 0.05$ vs. corresponding sham group; $^{†}P < 0.05$ vs. WT (TAC + IgG); $^{‡}P < 0.05$, $^{‡}P < 0.05$ vs. sFlt-1$^{-/-}$ (TAC + IgG). Data are mean ± SEM.

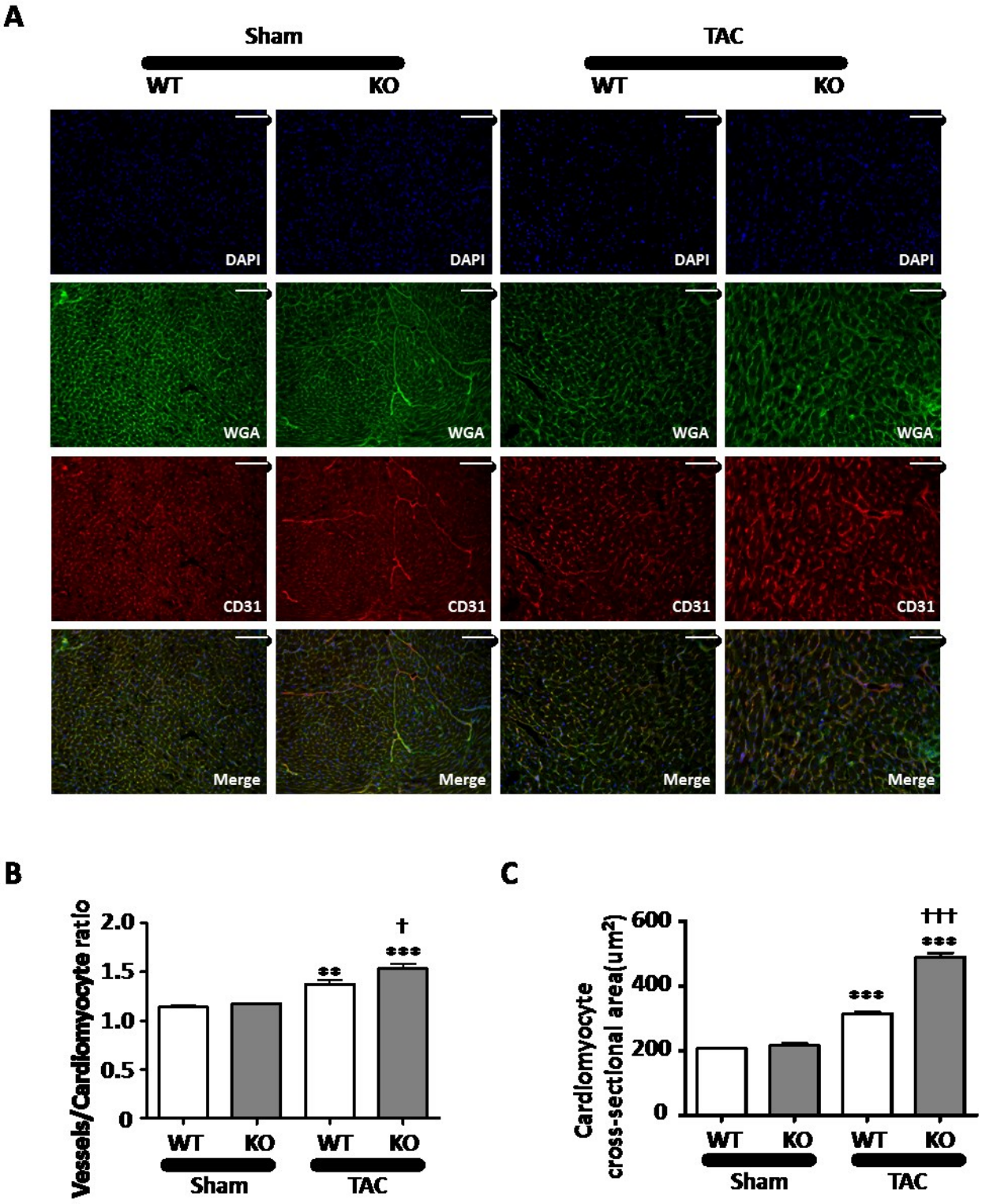
Figure S1.



Invasive hemodynamic measurement demonstrated that there was no difference in the pressure gradient, systolic pressure, and end-diastolic pressure of left ventricle after transverse aortic constriction (TAC) between wild-type (WT) and sFlt-1$^{-/-}$ mice (KO). **A**, representative

recordings of left ventricular pressure in sham- or TAC-operated mice. **B**, left ventricular systolic pressure. **C**, left ventricular pressure gradient. **D**, left ventricular end-diastolic pressure. $^{***}P < 0.001$ vs. sham. Data are mean ±SEM.

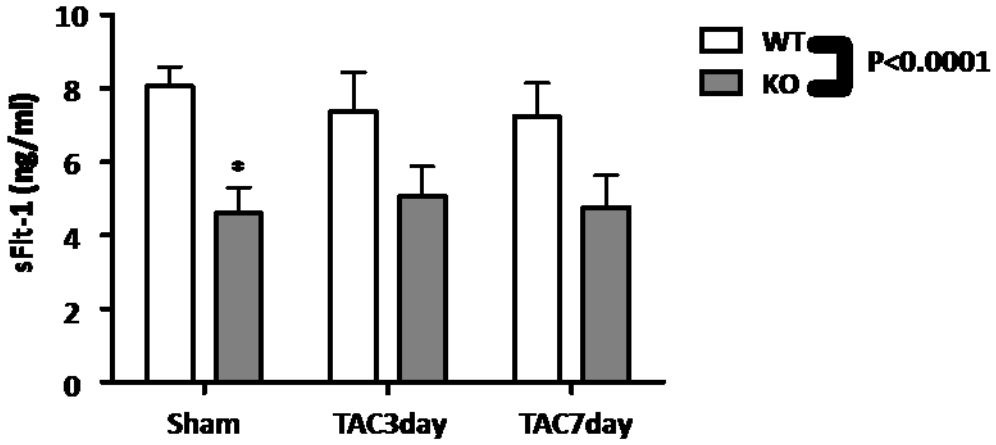
Figure S2



Cardiomyocyte areas and the number of vessels to cardiomyocyte in the myocardium were increased in sFlt-1$^{-/-}$ mice in response to TAC. **A,** Representative double staining by immunofluorescence of cardiac sections with wheat germ agglutinin (*green*) and CD31 (*red*), and DAPI (*blue*) in wild-type (WT) and sFlt-1$^{-/-}$ mice (KO) seven days after sham or transverse

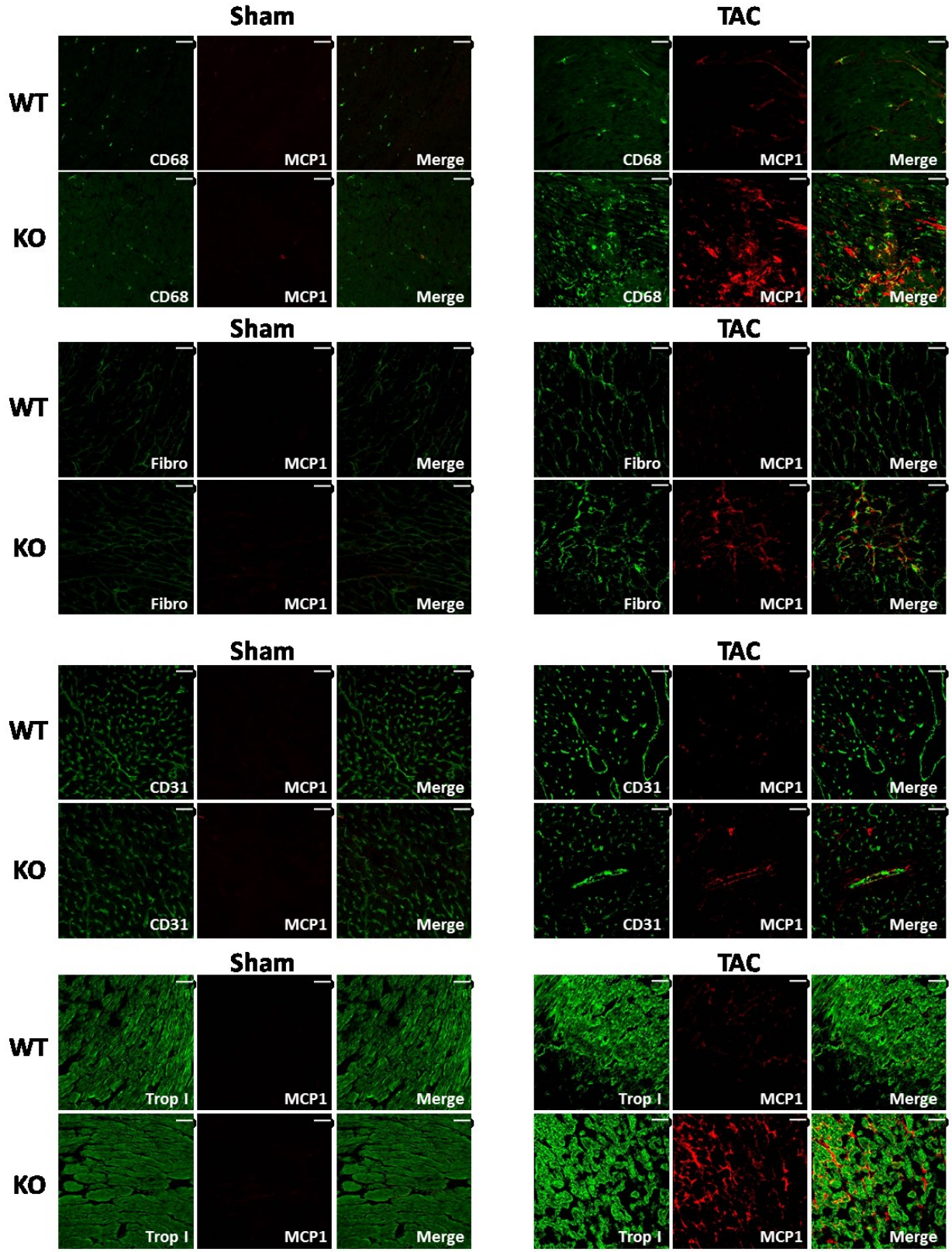
aortic constriction (TAC) operation. Scale bar, 100 μm. Magnification, × 200. **B,** Quantification of the number of vessels to cardiomyocyte ratio. **C,** Quantification of cardiac myocyte cross- sectional area. n = 4 for sham-operated mice (sham), n = 5-6 for TAC-operated mice (TAC). **P<0.01. ***P<0.001 vs sham, $^{\dagger}P<0.05$, $^{\dagger\dagger\dagger}P < 0.001$ vs. WT TAC. Data are mean ±SEM.

Figure S3.



Circulating sFlt-1 levels were measured by ELISA three and seven days after TAC or sham operation in both WT and sFlt-$1^{-/-}$ mice. *P<0.05 vs WT, 2-way ANOVA, Bonferroni posttest. P<0.0001 between WT and KO groups by 2-way ANOVA. n = 6-7 per group.

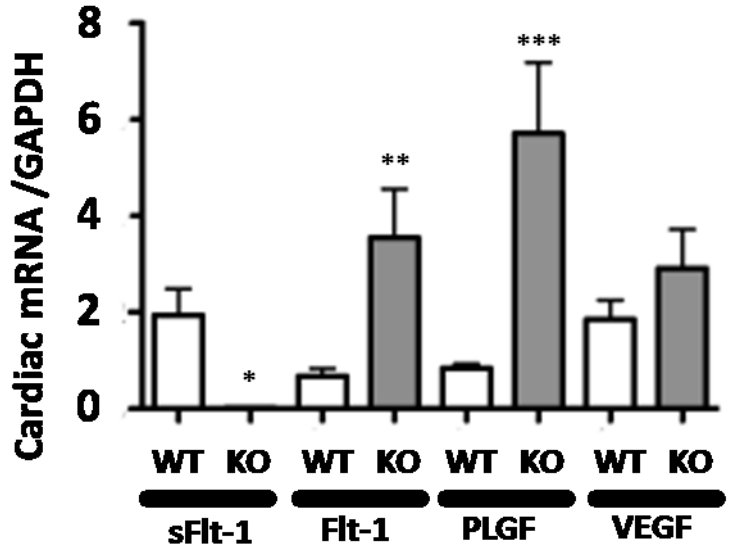
Figure S4.



Representative double staining by immunofluorescence of cardiac sections with MCP-1 (*red*) and CD68, fibroblast, CD31, troponin I (*green*) in wild-type (WT) and sFlt-1$^{-/-}$ mice (KO) seven

days after sham or transverse aortic constriction (TAC) operation. Monocyte chemoattractant protein-1 (MCP-1) was mainly expressed in macrophages, but was expressed in endothelial cells, interstitial cells, and cardiomyocytes in sFlt-$1^{-/-}$ mice (KO) during pressure overload. Scale bar, 40 μm. Magnification, × 400.

Figure S5.



Cardiac mRNA expression of sFlt-1, Flt-1, placental growth factor (PLGF), and vascular endothelial growth factor (VEGF) in wild-type (WT) mice and sFlt-$1^{-/-}$ mice (KO). n = 8 for WT, n = 7 for sFlt-$1^{-/-}$ mice (KO).

$^{*}P < 0.05$, $^{**}P < 0.01$ $^{***}P < 0.001$ vs. WT.