

Supplemental Material

Online Methods

Echocardiography

Cardiac function was assessed before and after TAC surgery at indicated time points using Vevo 2100 ultrasound system equipped with MS550D probe (VisualSonics, Fujifilm). Mice were anesthetized with isoflurane at a concentration of 5% (induction phase) and 1-1.5% (maintenance phase). Each animal was placed on the heating table in a supine position with the extremities tied to the table through four electrocardiography leads. Chest fur was removed with a chemical hair remover, and ultrasound gel was applied to the thorax surface to improve the visibility of the cardiac chambers. LVDd (left ventricular diameter at end-diastole, mm), LVDs (left ventricular diameter at end-systole, mm) (PWTd, mm), and fractional shortening (FS, %) were measured from M-mode images obtained by short-axis view visualizing both papillary muscles. Measurements and analysis were performed by two individuals who were blinded to the identity of the experimental groups of mice using a Vevo Lab software (VisualSonics, Fujifilm).

Hematopoietic parameter measurements

Peripheral blood cells were obtained from retro-orbital vein and collected into K2EDTA-added BD microtainer[®] blood collection tubes (Cat# 365974, BD). Hematopoietic parameters were analyzed by Element HT5 Veterinary Hematology Analyzer (Heska).

The antibodies used for flow cytometric analysis are listed in **Online Table 1**. Bone

marrow: Bone marrow cells were flushed out from 1 femur and 1 tibia, and red blood cells were lysed with RBC lysis Buffer (BioLegend, Cat# 420301) for 20 seconds at room temperature. Dead cell staining was performed using Zombie Aqua™ Fixable Viability Kit (BioLegend, Cat# 423102) in PBS for 15 minutes at room temperature. Cells were labeled with biotin-conjugated anti-mouse antibodies, followed by incubation with BV650-Streptavidin (BioLegend Cat# 405231) together with fluorochrome-conjugated antibodies for 20 minutes at room temperature. Peripheral blood: Peripheral blood cells were obtained from retro-orbital vein. Red blood cells were lysed with eBioscience™ 1X RBC Lysis Buffer (Thermo Fisher, Cat# 00-4333-57) for 5 minutes. Incubation with antibodies were done for 20 minutes at room temperature in the dark. Dead cells were excluded from analysis by DAPI staining (Thermo Scientific, Cat# 62248). Heart tissue: Hearts were flushed with 15 ml of cold PBS from apex and excised. Right ventricles were removed and left ventricles were minced and digested in collagenase I (450 U/ml), collagenase XI (125 U/ml), Hyaluronidase (450 U/ml), and DNase I (60 U/ml) (Cat# C0130, C7657, H3506, and D4513, respectively, Sigma-Aldrich) at 900 rpm at 37°C for 50 minutes using ThermoMixer® C (Eppendorf). Hearts were subsequently homogenized through a Falcon™ Cell Strainers (Fisher Scientific, Cat# 352350). Incubation with antibodies were done for 20 minutes at room temperature in the dark. For the macrophage proliferation assay, BrdU was injected 2 hours before death. Detection of BrdU incorporation was performed by flow cytometry using APC BrdU Flow Kit (BD

Bioscience, Cat# 552598) as described previously ¹. Dead cells were excluded from analysis by DAPI staining. 123count eBeads™ Counting Beads (Thermo Fisher, Cat# 01-1234-42) was used for data counting cell numbers. BD LSRII Flow Cytometer or Fortessa (BD Bioscience) was used for data acquisition. Cells were defined as described in Figure 1a, Supplementary Figures 1, and 4a. Data were analyzed with FlowJo Software. Single cell suspensions of heart tissue were made as described above and were stained to identify indicated cell populations. Cells were sorted on a FACS Aria II cell sorter (BD Biosciences) directly into either RLT buffer for subsequent RNA isolation. 2×10^4 neutrophils, Ly-6C^{hi} monocytes, and macrophages were sorted from infarcted myocardium on day 3 and total RNA was extracted using the RNeasy Micro Kit (Qiagen, Cat# 74004) followed by cDNA transcription (Thermo Fisher, Cat# 43-874-06).

Cell culture

Mouse bone marrow-derived neutrophils were isolated as previously described ². 1 ml suspension of red blood cell-lysed bone marrow cells was overlaid onto the layers of 3 ml Histopaque[®]-1119 (Sigma-Aldrich, Cat# 11191) and 3ml of Histopaque[®]-1077 (Sigma-Aldrich, Cat# 10771) in a 15-ml conical centrifuge tube in room temperature. 30 min after centrifuge at 870 x g at room temperature without brake, neutrophils at the interface of the Histopaque[®]-1119 and Histopaque[®]-1077 layers were collected. The purity of neutrophil defined by (CD45⁺, CD11b⁺, Ly6G⁺) was confirmed to be more than 90% by flow cytometry analysis.

Transwell migration assay

Neutrophil transwell migration assay was performed as described previously ³. A total of 1×10^6 neutrophils from murine bone marrow was seeded in serum-free RPMI medium in the upper compartment of Transwell inserts (Falcon[™], Cat# 08-771-4). The indicated concentrations of recombinant Wnt5a (Stem RD, Cat# W5A-M-005) were added in the lower chambers and the cells were incubated for 2 hours at 37°C in 5% CO₂ chamber. Migrated cells were fixed with 4% PFA, labeled with DAPI (Thermo Scientific, Cat# 62248), and analyzed by fluorescent microscopy.

Histology

The heart tissues were harvested at indicated time points after TAC surgery. Heart

tissues were perfused with cold PBS from apex and fixed in 10% formalin at 4°C overnight. Samples were processed for paraffin embedding. 7- μ m-thick sections were de-paraffinized and rehydrated. Hematoxylin-eosin staining was performed using a standard method. Sections were incubated in filtered hematoxylin solution (Sigma, Cat# GHS316) for 2 minutes, washed in tap water, and differentiated in 1% acid alcohol. After dehydration in 70% ethanol, sections were incubated in eosin solution (Sigma, Cat# HT110116) for few seconds, and dehydrated in a series of ethanol (95% and 100%), cleared in xylene and mount in permanent mounting medium (Vector Laboratories, Cat# H-5000). Images were taken by a Keyence BZ-X710 microscope to show the global change of heart size. For cardiomyocyte cross-sectional area (CSA) analysis, heart sections were stained with Alexa Fluor 594 conjugated-WGA (Life Technologies, Cat# W11262). An operator who was blinded to mouse genotype quantified cardiomyocyte CSA by computer-assisted morphometric analysis of microscopy images acquired on a Keyence BZ-X710 microscope. The average CSA of randomly selected 80-100 round-shaped cardiomyocytes per section was used for analysis. For Mac2 immunohistochemistry staining, antigen unmasking was performed by boiling slides in citrate-based antigen unmasking solution (Vector Laboratories, Inc., Cat# H-3300). Endogenous peroxidase was inactivated by using 0.3% H₂O₂ for 30 min. Sections were blocked by 5% horse serum for 1 hour and then incubated with anti-rabbit Mac2 antibody (clone H-160, catalog #sc-20157, Santa Cruz) at 1:500 dilution at 4°C overnight. After washing slides, sections were incubated in anti-rabbit

IgG conjugated with peroxidase (Vector Laboratories, Inc., PI-1000) for 30 minutes and developed using DAB substrate kit (Vector Laboratories, Inc., Cat# SK-4100). Sections were counterstained with Harris hematoxylin. The number of Mac2 positive cell was counted in five high-magnification fields per section and per animal. For fibrosis staining, Masson's trichrome staining was performed according to the manufacturer's instructions (Sigma-Aldrich, Cat# HT15). For Picrosirius red staining, sections were incubated with freshly prepared staining buffer (1.2%/w picric acid in water, 0.1%/w Fast Green FCF and 0.1%/w Direct Red 80 solved in PBS) for 1 h at room temperature (Cat# 197378, Cat #F7252, Cat #365548, respectively. All products from Sigma-Aldrich). Sections were washed briefly in distilled H₂O and dehydrated. The slides were mounted by coverslip using Permanent mounting medium (Vector Laboratories, Cat# H-5000). The images were analyzed by Image J software (NIH) for quantification of fibrosis. Myocardial fibrosis size was expressed as a percentage of total LV area.

Western Blot Analysis

Neutrophils were lysed with 50 mmol/L Tris·HCl, 150 mmol/L NaCl, 10 mmol/L EDTA, 1% Triton, Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktail (Thermo Scientific). The protein concentration was measured with Pierce™ BCA Protein Assay Kit (Thermo Scientific). Samples were incubated with Blue Loading Buffer (BioLabs) with 40 mmol/L DTT for 5 min at 95 °C. Samples containing equal amounts of protein were separated by SDS/PAGE. After blocking with 5% skim milk in PBS with Tween 20 (0.1%) for 1 h, the membranes were incubated with the specific antibodies overnight at 4 °C, followed by HRP-conjugated second antibody 1 h at room temperature. The following antibodies were used for immunoblotting: rabbit polyclonal anti-phospho-PI3K p85 (Tyr458), p55 (Tyr199), phospho-Erk1/2 (Thr202/Tyr204), phospho-JNK (Thr183/Tyr185), and β -actin (Cell Signaling Technology). Images were visualized using ECL™ Prime Western Blotting System (GE Healthcare).

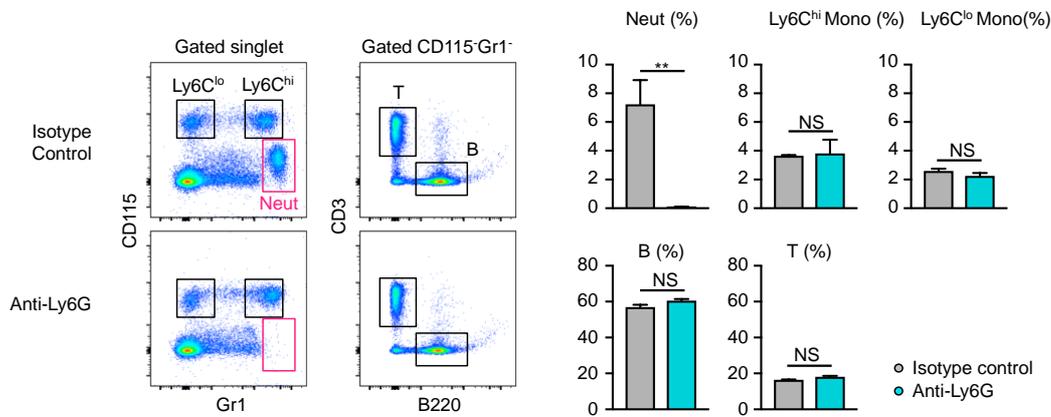
Gene expression analysis by qRT-PCR

Total RNA from tissue was isolated using QIAzol reagent (Qiagen, Cat# 79306), and was purified on the QIAcube. Total RNA from cultured cells was isolated using NucleoSpin RNA kit (Clontech, Cat# 740955.250). 1 μ g RNA was reverse transcribed with High-capacity RNA-to-cDNA Kit (Thermo Fisher Scientific, Cat# 4387406). qRT-PCR was performed with PowerUp SYBR® Green reagent (Thermo Fisher Scientific, Cat# 4367659) or Taqman gene expression master mix (Applied Biosystems, Cat#

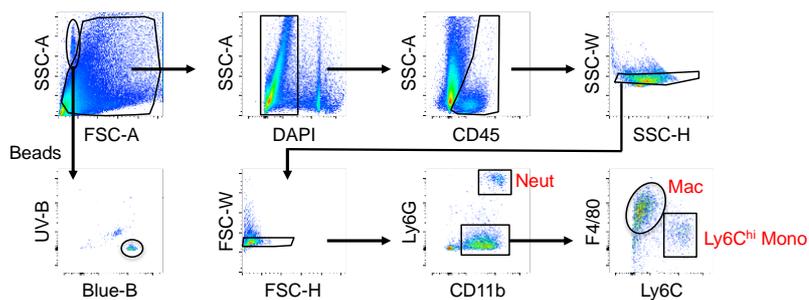
4369016) in a QuantStudio 6 Flex PCR system. Primers for mouse gene expression studies are shown in the Online Table 2. *36b4* was used as reference genes for normalization. For sorted cells, Quantitative Real-Time TaqMan PCR was performed using the following TaqMan primers (Applied Biosystems): *Wnt5a* (Mm00437347_m1) and housekeeping gene *Gapdh* (Mm99999915_g1). PCR was run on a 7500 thermal cycler (Applied Biosystems). Results were analyzed with the $\Delta\Delta\text{Ct}$ method.

References

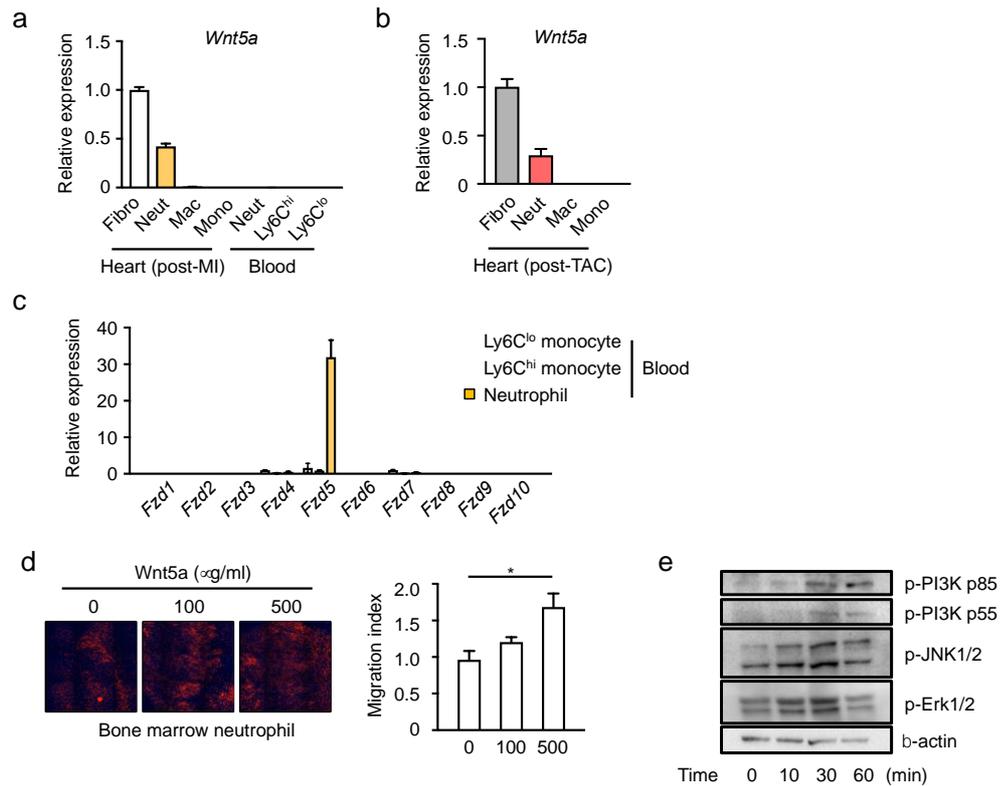
1. Anzai A, Choi JL, He S, Fenn AM, Nairz M, Rattik S, McAlpine CS, Mindur JE, Chan CT, Iwamoto Y, Tricot B, Wojtkiewicz GR, Weissleder R, Libby P, Nahrendorf M, Stone JR, Becher B and Swirski FK. The infarcted myocardium solicits GM-CSF for the detrimental oversupply of inflammatory leukocytes. *J Exp Med*. 2017;214:3293-3310.
2. Swamydas M, Luo Y, Dorf ME and Lionakis MS. Isolation of Mouse Neutrophils. *Curr Protoc Immunol*. 2015;110:3 20 1-3 20 15.
3. Yona S, Hayhoe R and Avraham-Davidi I. Monocyte and neutrophil isolation and migration assays. *Curr Protoc Immunol*. 2010;Chapter 14:Unit 14 15.



Supplemental Figure 1. Intraperitoneal administration of Ly6G antibody effectively depletes neutrophil. Representative flow cytometry data to show Ly6G antibody (1A8) administration (500 μ g/injection/mouse, 2 injections with 24h interval) depletes neutrophil in peripheral blood without affecting other immune cell populations. Neutrophil was defined as CD115⁻, Gr1⁺ cells. n=3 in isotype control group and n=4 in anti-Ly6G group. Statistical analyses were performed using two-tailed unpaired Student's *t* tests. NS=not significant, ***p*<0.01.



Supplemental Figure 2. Flow cytometry gating strategy of cardiac immune cells. Myeloid cell analysis of cardiac tissue (7 days after TAC) is shown. Cells are defined as: (i) total white blood cells (CD45⁺), (ii) neutrophils (CD11b⁺, Ly6G⁺), (iii) Ly6Chi monocytes (CD11b⁺, Ly6G⁻, Ly6C^{hi}, F4/80^{lo}), (iv) macrophages (CD11b⁺, Ly6G⁻, Ly6C^{hi}, F4/80^{hi}). Neut= neutrophil, Mono= monocyte, Mac= macrophage.



Supplemental Figure 3. *Wnt5a* accelerates neutrophil migration in vitro.

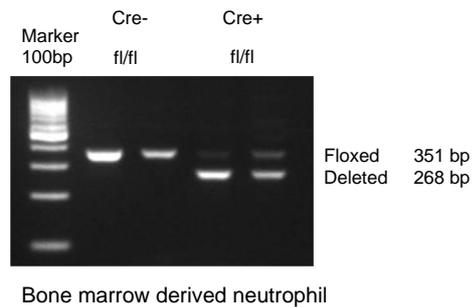
a. Analysis of transcript expression of *Wnt5a* in fibroblast, neutrophil, macrophage and monocyte sorted from ischemic heart (n=3 in each group) and in neutrophil, Ly6C^{hi} monocyte and Ly6C^{lo} monocyte isolated from peripheral blood (n= 3 in each group). *Gapdh* was used as a reference for normalization.

b. Analysis of transcript expression of *Wnt5a* in fibroblast, neutrophil, macrophage and monocyte sorted from heart at 3 days post-TAC (n=3 in each group). *Gapdh* was used as a reference for normalization.

c. Analysis of transcript expression of *Frizzled* receptors in Ly6C^{hi} monocytes, Ly6C^{lo} monocytes, and neutrophils sorted from peripheral blood. *36b4* was used as a reference for normalization (n=3 in each group).

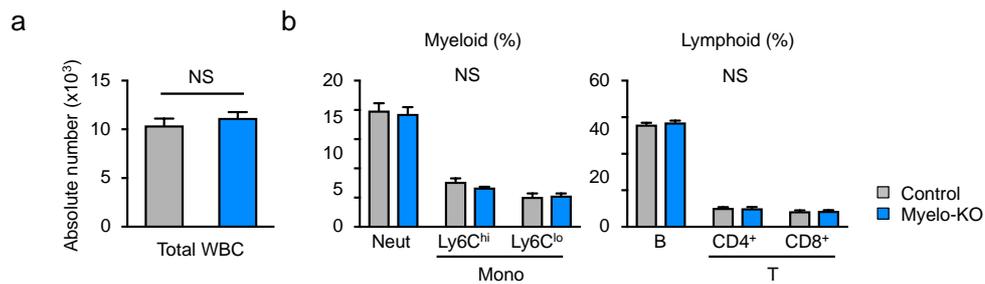
d. Representative image and analysis of neutrophil migration assay of bone marrow neutrophils in response to a recombinant *Wnt5a* gradient (n=3 in each group). Statistical analysis was performed using Kruskal-Wallis test with post-hoc Dunn's multiple comparison tests.

e. Representative immunoblot of PI3K, JNK1/2, Erk1/2 phosphorylation in bone marrow isolated neutrophil treated with 400 ng/mL recombinant *Wnt5a*. **p*<0.05.

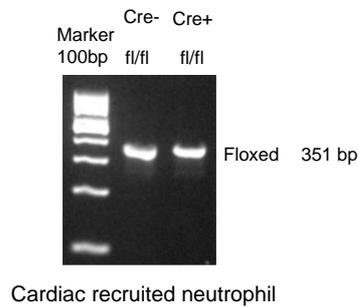


condF: 5'- TCTCGGAAGGTAAGTCTATCTCCTACC -3'
condR: 5'- GGCTCCCTCAGTCAGTGTTTTTCAT -3'
5'NeoF: 5'- GACTCAACAGCATTGAGACATGTTTGT -3'

Supplemental Figure 4. PCR analysis of Wnt5a gene recombination in bone marrow derived neutrophil. Wnt5a gene recombination has been evaluated in genomic DNA of neutrophil isolated from bone marrow from wild type and Wnt5a-KO mice.

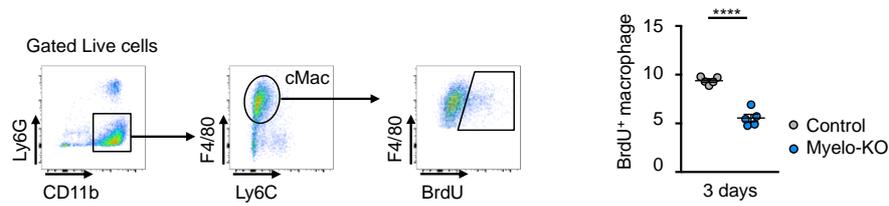


Supplemental Figure 5. Myeloid-specific *Wnt5a* deficiency does not affect peripheral blood profiles. **a.** Absolute number of peripheral blood of WBC from Myelo-KO mice (n=6) and control mice (n=6). Statistical analysis was evaluated by two-tailed unpaired Student's *t* tests. **b.** The result of flow cytometry analysis of peripheral blood from Myelo-KO mice (n=6) and at sham state (n=6), showing no obvious difference in myeloid and lymphoid populations. Statistical analysis was performed using multiple *t* tests. NS= not significant. Myelo-TG= myeloid-specific *wnt5a* overexpression mice, WBC= white blood cell, Neut= neutrophil, Mono= monocyte, B= B cell, T= T cell.

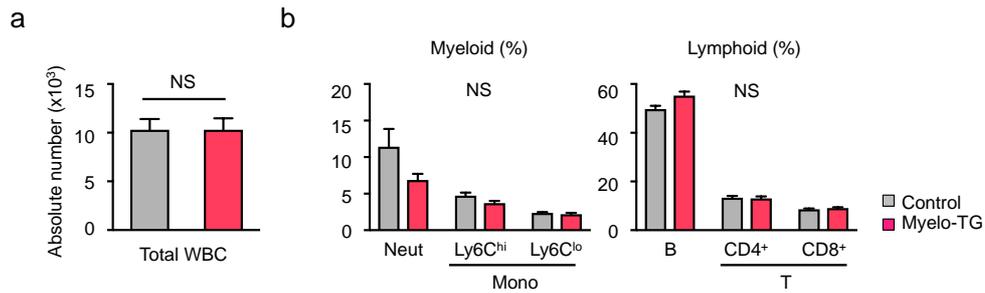


condF: 5'- TCTCGGAAGGTACTGCTATCTCCTACC -3'
condR: 5'- GGCTCCCTCAGTCAGTGTTCAT -3'
5'NeoF: 5'- GACTCAACAGCATTGAGACATGTTTGT -3'

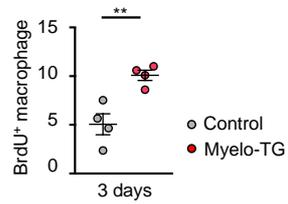
Supplemental Figure 6. PCR analysis of Wnt5a gene recombination in cardiac recruited neutrophil. Wnt5a gene recombination has been evaluated in genomic DNA of neutrophil isolated from heart 3 days after TAC from wild type and Wnt5a-KO mice.



Supplemental Figure 7. Effect of Wnt5a manipulation on proliferation of cardiac macrophages in TAC-treated mice. Flow cytometry analysis of BrdU incorporation into cardiac macrophages from Myelo-KO ($Wnt5a^{fl/fl}$, $LysM^{Cre/+}$) versus littermate control ($Wnt5a^{fl/fl}$, $LysM^{+/+}$) mice ($n=5$ in each group). BrdU was injected 2 hours before death and 3 days after TAC. Statistical analysis was performed using two-tailed unpaired Student's t tests. **** $p<0.0001$.



Supplemental Figure 8. Myeloid-specific Wnt5a overexpression does not affect peripheral blood profiles. **a.** Absolute number of peripheral blood of WBC from Myelo-TG (*Rosa-Wnt5a*, *LysM^{Cre/+}*) versus littermate control (*LysM^{Cre/+}*) mice ($n=5$ in each group). Statistical analysis was evaluated by two-tailed unpaired Student's *t* tests. **b.** The result of flow cytometry analysis of peripheral blood from Myelo-TG mice ($n=5$) and littermate control mice ($n=5$), showing no obvious difference in myeloid and lymphoid populations. Statistical analysis was performed using multiple *t* tests. NS= not significant. Myelo-TG= myeloid-specific *wnt5a* knockout mice, WBC= white blood cell, Neut= neutrophil, Mono= monocyte, B= B cell, T= T cell, Myelo-TG= myeloid-specific *wnt5a* overexpression mice, *Wnt5a*-Myelo-KO= myeloid-specific *wnt5a* knockout mice.



Supplemental Figure 9. Effect of Wnt5a manipulation on proliferation of cardiac macrophages in TAC-treated mice. Flow cytometry analysis of BrdU incorporation into cardiac macrophages from Myelo-TG (Rosa-Wnt5a, LysM^{Cre/+}) versus littermate control (LysM^{Cre/+}) mice (n=4 in each group). BrdU was injected 2 hours before death and 3 days after TAC. Statistical analysis was performed using two-tailed unpaired Student's *t* tests. ***p*<0.01.

Supplemental Table 1. Antibodies used for flow cytometry analyses.

BONE MARROW				
ANTIBODIES	FLUOROPHORE	CLONE	SOURCE	IDENTIFIER
Anti-CD11b	(Biotin)	M1/70	BioLegend	Cat# 101204; RRID: AB_312787
Anti-Ly6G/Ly6C (Gr-1)	(Biotin)	RB6-8C5	BioLegend	Cat# 108404; RRID: AB_313369
Anti-TER-119	(Biotin)	TER-119	BioLegend	Cat# 116204; RRID: AB_313705
Anti-CD45/B220	(Biotin)	RA3-6B2	BioLegend	Cat# 103204; RRID: AB_312989
Anti-CD3e	(Biotin)	145-2C11	BioLegend	Cat# 100304; RRID: AB_312669
Anti-CD127 (IL-7Ra)	(Biotin)	A7R34	BioLegend	Cat# 135006; RRID: AB_2126118
Anti-CD117(c-kit)	APC	2B8	BioLegend	Cat# 105812; RRID: AB_313221
Anti-Ly-6A/E (Sca-1)	PE/Cy7	D7	BioLegend	Cat# 108114; RRID: AB_493596
Anti-CD16/32	APC/Cy7	93	BioLegend	Cat# 101328; RRID: AB_2104158
Anti-CD34	FITC	RAM34	Thermo Fisher	Cat# 11-0341-81; RRID: AB_465020
PERIPHERAL BLOOD				
ANTIBODIES	FLUOROPHORE	CLONE	SOURCE	IDENTIFIER
Anti-CD45.2	eFluor450	104	Thermo Fisher	Cat# 48-0454-80; RRID: AB_11039533
Anti-CD115	PE	AFS98	Thermo Fisher	Cat# 12-1152-81; RRID: AB_465807
Anti-CD3e	PE-eFluor610	145-2C11	Thermo Fisher	Cat# 61-0031-80; RRID: AB_2574513
Anti-CD4	FITC	RM4-5	Thermo Fisher	Cat# 11-0042-81; RRID: AB_464895
Anti-CD8a	BV510	53-6.7	BioLegend	Cat# 100752; RRID: AB_2563057
Anti-Ly6C	APC	AL-21	BD Biosciences	Cat# 560595
Anti-CD45R	APC-Cy7	RA3-6B2	BD Biosciences	Cat# 552094
Anti-Ly6G	PerCP-Cy5.5	1A8	BD Biosciences	Cat# 560602
Anti-CD43	BUV737	S7	BD Biosciences	Cat# 564398
Anti-Gr-1	BV605	RB6-8C5	Biolegend	Cat# 108439
HEART				
ANTIBODIES	FLUOROPHORE	CLONE	SOURCE	IDENTIFIER
Anti-CD45	Pacific Blue	30-F11	BioLegend	Cat# 103126; RRID: AB_493535
Anti-CD11b	APC-Cy7	M1/70	BioLegend	Cat# 101226; RRID: AB_830642
Anti-Ly6G	PE	1A8	BioLegend	Cat# 127602; RRID: AB_1089180
Anti-Ly6C	FITC	HK1.4	BioLegend	Cat# 128006; RRID: AB_1186135
Anti-F4/80	PE-Cy7	BM8	BioLegend	Cat# 123116; RRID: AB_893481

Supplemental Table 2. Real-time PCR primers used in this study.

Gene Name	Species	Forward	Reverse
<i>Nppa</i>	<i>Mus Musculus</i>	5'- AAGAACCTGCTAGACCACCTG -3'	5'- TGCTTCCTCAGTCTGCTCAC -3'
<i>aMHC</i>	<i>Mus Musculus</i>	5'- GCAGCAGCCCAGTACCTC -3'	5'- GTCATCAGGCACGAAGCA -3'
<i>bMHC</i>	<i>Mus Musculus</i>	5'- CCTCACATCTTCTCCATCTCTG -3'	5'- TTGGATGACCCTCTTAGTGTTG -3'
<i>Il1b</i>	<i>Mus Musculus</i>	5'- TGACAGTGATGAGAATGACCTGTTC -3'	5'- TTGGAAGCAGCCCTTCATCT -3'
<i>Il6</i>	<i>Mus Musculus</i>	5'- GCTACCAAACCTGGATATAATCAGGA -3'	5'- CCAGGTAGCTATGGTACTCCAGAA -3'
<i>Cxcl1</i>	<i>Mus Musculus</i>	5'- CCGAAGTCATAGCCACACTCAA -3'	5'- CAAGGGAGCTTCAGGGTCAA -3'
<i>Cxcl2</i>	<i>Mus Musculus</i>	5'- TGACTTCAAGAACATCCAGAGCTT -3'	5'- CTTGAGAGTGGCTATGACTTCTGTCT -3'
<i>Cxcl5</i>	<i>Mus Musculus</i>	5'- GCCGCTGGCATTCTGTGTT -3'	5'- GGGCAGCTTCAGCTAGATGCT -3'
<i>Ccl2</i>	<i>Mus Musculus</i>	5'- CAGCCAGATGCAGTTAACGC -3'	5'- GCCTACTCATTGGGATCATCTTG -3'
<i>Frizzled1</i>	<i>Mus Musculus</i>	5'- GCGACGTACTGAGCGGAGTG -3'	5'- TGATGGTGCGGATGCGGAAG -3'
<i>Frizzled2</i>	<i>Mus Musculus</i>	5'- CTC AAGGTGCCGTCCTATCTCAG -3'	5'- GCAGCACAACACCGACCATG -3'
<i>Frizzled3</i>	<i>Mus Musculus</i>	5'- GGTGTCCCGTGGCCTGAAG -3'	5'- ACGTGCAAGAAAGGAATAGCCAAG -3'
<i>Frizzled4</i>	<i>Mus Musculus</i>	5'- GACAACCTTTCACGCCGCTCATC -3'	5'- CCAGGCAAACCCAAATTCTCTCAG -3'
<i>Frizzled5</i>	<i>Mus Musculus</i>	5'- CTGGCAGGCTTCGTGTCA -3'	5'- TGCCACCCTGCTTGATGA -3'
<i>Frizzled6</i>	<i>Mus Musculus</i>	5'- TGTTGGTATCTCTGCCGTCCTCTG -3'	5'- CTCGGCGGCTCTCACTGATG -3'
<i>Frizzled7</i>	<i>Mus Musculus</i>	5'- ATATCGCCTACAACCAGACCATCC -3'	5'- AAGGAACGGCAGGAGGAATG -3'
<i>Frizzled8</i>	<i>Mus Musculus</i>	5'- GTTCAGTCATCAAGCAGCAAGGAG -3'	5'- AAGGCAGGCGACAACGACG -3'
<i>Frizzled9</i>	<i>Mus Musculus</i>	5'- ATGAAGACGGGAGGCACCAATAC -3'	5'- TAGCAGACAATGACGCAGGTGG -3'
<i>Frizzled10</i>	<i>Mus Musculus</i>	5'- ATCGGCACTTCCTTCATCCTGTC -3'	5'- TCTTCCAGTAGTCCATGTTGAG -3'
<i>36b4</i>	<i>Mus Musculus</i>	5'- GCTCCAAGCAGATGCAGCA -3'	5'- CCGGATGTGAGGCAGCAG -3'