Supporting Information

Chen et al. 10.1073/pnas.1424236112

SI Materials and Methods

Animals. All experiments were conducted in accordance with the Guide for the Use and Care of Laboratory Animals and approved by the Harvard Medical School Standing Committee on Animals and by the Institutional Animal Care and Use Committee of Kaohsiung Chang Gung Memorial Hospital, Kaohsiung, Taiwan.

ST2$^{-/-}$ Mice, Floxed ST2, and Floxed IL33 Mice. ST2$^{-/-}$ mice were maintained on a 129 × C57BL/6 background as described previously (1–3). ST2 targeting vector (KOMP Project ID CSD35155) and IL33 targeting vector (KOMP Project ID CSD26735) were generated by the Knockout Mice Project (KOMP). Targeting vector sequences are provided in Dataset S1. For generation of ST2 conditional deletion mice, exons 4 and 5 of the ST2 gene (*IL1RL1*) were flanked by two directional Lox-p sites. Mouse embryonic stem cells with the correct homologous recombination were identified by Southern analysis and used for the production of chimeric mice. Germ-line transmission and the establishment of ST2 floxed mice (ST2$^{fl/fl}$) was confirmed by allele-specific genotyping and diagnostic restriction analysis of PCR products. ST2$^{fl/fl}$ mice were crossed with double transgenic MerCreMer-ZEG mice to generate ST2$^{fl/fl}$ /MCM/ZEG and ST2$^{fl/fl}$ /MCM mice or crossed with Tek-RFP-CreERT2 mice to generate ST2$^{fl/fl}$/Tek-RFP-CreERT2 mice. All genotypes were determined using differential PCR. We maintained all mice under a 12-h light:dark cycle with ad libitum access to regular food and water.

For generation of IL33 conditional deletion mice, exons 5, 6, and 7 of the IL33 gene were flanked by two directional Lox-p sites. Mouse embryonic stem cells with the correct homologous recombination were identified by Southern blot analysis and used for the production of chimeric mice. Germ-line transmission and the establishment of IL33 floxed mice (IL33$^{fl/fl}$) were confirmed by allele-specific genotyping and diagnostic restriction analysis of PCR products. IL33$^{fl/fl}$ mice were crossed with double transgenic MerCreMer mice to generate IL33$^{fl/fl}$/MCM mice or crossed with Tek-RFP-CreERT2 mice to generate IL33$^{fl/fl}$/Tek-RFP-CreERT2 mice. Genotyping was performed by PCR on tail DNA with the specific primers (Table S2).

MerCreMer-ZEG and Tek-RFP-CreERT2 Mice. Double transgenic MerCreMer-ZEG mice were generated by cross-breeding transgenic B6129-Tg (Myh6-cre/Esr1)1Jmk/J (MerCreMer) mice with the B6.Cg-Tg(ACTB-Bgeo/GFP)21Lbe/J reporter strain (ZEG, Jackson Laboratories) as previously described (4, 5). The C57BL/6-Tg (Tek-RFP-Cre/ERT2)27Narl (RMRC13162) mouse was generated and maintained by the National Laboratory Animal Center, Taiwan. Genotyping was performed by PCR on tail DNA with specific primers (Table S2). Maintenance of the colony and all experiments were conducted in accordance with the Guide for the Use and Care of Laboratory Animals and approved by the Harvard Medical School Standing Committee on Animals and by the Institutional Animal Care and Use Committee of Kaohsiung Chang Gung Memorial Hospital, Kaohsiung, Taiwan.

4-OH Tamoxifen Pulse. A previously validated protocol was used to induce Cre recombinase-mediated GFP expression and permanent deletion of β-galactosidase gene expression in the cardiomyocytes of MerCreMer-ZEG mice (4, 5). Tamoxifen (Sigma) was dissolved in peanut oil (Sigma) at a concentration of 5 mg/mL and injected intraperitoneally at a dose of 20 mg/kg/d for 5 d. Cre recombination in the cardiac tissue was analyzed by using immunochemistry of the ratio of GFP-positive cardiomyocytes in the heart from the targeted mice. Immunofluorescence staining for ST2 or IL-33 was performed to confirm conditional knockout efficiency of ST2 or IL-33 deletion in cardiomyocyte or ECs.

Adenoviral Vector for Gene Transfer. Mouse ST2L and sST2 cDNA was amplified by using the same 5′-GCG ACT AGT ATG ATT GAC AGA CAG AGA ATG GGA-3′ sense primer including a SpeI restriction site. The antisense primers were 5′-CTC CTC GAG CTA TCA TCA AAA GTG TTT CAG GTC-3′ and 5′-CTC CTC GAG CTA TTA TCA AGC AAT GTG TGA GGG-3′ for ST2L and sST2, respectively, that included a XhoI restriction site. The PCR products were subcloned into the pShuttle-IRES-hrGFP-1 vector between the SpeI and XhoI sites and confirmed by sequencing. Adenoviral constructs expressing mouse ST2L (IL1RL1 isoform a, NM_001025602) and mouse sST2 (IL1RL1 isoform b, NM_010743) in tandem with constitutive GFP were constructed at the Harvard Gene Therapy Initiative. We also constructed an identical virus vector expressing GFP alone (Ad-GFP) as a control.

Adenoviral Gene Transfer, TAC-Induced Cardiac Hypertrophy, and Cardiomyocyte Size Analysis. Targeted ST2$^{-/-}$ mice and their WT littermates were used for the experiments. TAC was produced as described previously on 8- to 10-wk-old mice (1). All operative procedures were performed by a single operator with over 20 y of rodent cardiac surgery experience who was blinded to genotype and the randomized treatment assignment. Briefly, male ST2$^{-/-}$ and WT mice from the same litter (age 8–12 wk) were anesthetized with pentobarbital (~40 μg/g i.p.) and injected with adenovirus directly into the left ventricular free wall (5.4×10^6 pfu in 10 μL). After 7 d, these mice were anesthetized again and subjected to TAC. This is done by tying a 5–0 silk suture around the aorta against a blunted 27-gauge needle. After 7 d, all hearts were harvested and collected and fixed in 4% (wt/vol) paraformaldehyde. All surgeries were performed in a blinded and randomized manner with respect to treatment. All hearts were embedded in paraffin and cut into 5-μm sections. All sections were imaged with fluorescent microscopy to determine the CSA of each cardiomyocyte. Only myocytes that had both a visible nucleus in the center and an intact cellular membrane were included in our studies. The cellular borders were traced in ImageJ, allowing CSA to be calculated.

Immunohistochemistry and Immunofluorescence. Mouse hearts were fixed with 4% paraformaldehyde, paraffin embedded, sectioned, and stained with standard immunohistochemistry and fluorescent microscopy methods as previously described (1, 6). An antigen retrieval step was used in all experiments, by heating samples in a citrate-based buffer (Dako) to 95 °C for 20 min. Primary antibodies were used as follows: rabbit GFP-specific antibody 1:400 (Abcam), rabbit β-galactosidase–specific antibody 1:200 (Invitrogen), mouse troponin T-specific antibody 1:200 (Abcam), IB4 1:200 (Invitrogen), and mouse IL-33 polyclonal antibody 1:200 (AF3626, R&D Systems). Alexa-Fluor (Invitrogen) secondaries were used at 1:200 dilutions for all immunofluorescence. A biotinylated anti-rabbit secondary followed by ABC reagent (avidin and biotinylated horseradish peroxidase) and 3,3′-diaminobenzidine (DAB) (Vector Laboratories) was used for immunohistochemistry. Microvessels were stained by using anti-CD31 antibody (Abcam). Fibrosis areas were analyzed by Masson's Trichome Staining (6).

Real-Time PCR. For the quantitative RT-PCR (qRT-PCR) analysis of gene expression, cardiac tissues from mice were harvested and RNA was extracted. Total RNA was isolated using TRIzol re-

agent per the manufacturer's instructions (Invitrogen). cDNA was synthesized from 1 μg of total RNA and random hexamers using the TaqMan Reverse Transcription Kit (Applied Biosystems). We used 1 μL of diluted cDNA per 20 μL reaction. Real-time PCR was performed in a 7300 Realtime PCR system (Applied Biosystems), with 40 cycles of 95 °C × 15 s, 60 °C × 1 min. All reactions were performed in duplicate. Primers for qPCR are as follows: ANP [forward (F), 5′-GGACTAGGCTGCAACAGCTTC-3′; reverse (R), 5′-GTGACACACCACAAGGGCTTA-3′], BNP (F, 5′-CGTCAGTCGTTTGGGCTGTAA-3′; R, 5′-CACTTCAAAGGTGGTCCCAGAG-3′), β-MHC (F, 5′-ATGTGCCGGACCTTGGAA-3′; R, 5′-CCTCGGGTTAGCTGAGAGATCA-3′), GAPDH (F, 5′-TGTGTCCGTCGTGGATCTGA-3′; R, 5′-TTGCTGTTGAAGTCGCAGGAG-3′), mouse IL33 (F, 5′-GCTGCGTCTGTTGACACATT-3′; R, 5′-CACCTGGTCTTGCTCTTGGT-3′), and mouse sST2 (F, 5′-ACGCTCGACTTATCCTGTGG-3′; R, 5′-CAGGTCAATTGTTGGACACG-3). Taqman primer and probe sets were used for mouse Col1a2, Periostin, eNOS, VEGF, TNF-α, IL-6, IL-1β, IL13, and TGF-β1 (Applied Biosystems).

ELISA. Serum levels of IL-33 (R&D Systems, DY3626) or sST2 (R&D Systems, DY1004) were analyzed by using an ELISA kit according to the manufacturer's instructions.

Echocardiography. Echocardiographic acquisition and analysis were performed by an echocardiographer blinded to the treatment group. Light anesthesia with spontaneous respiration was achieved with isoflurane (1.5–3%). All images were taken at a heart rate greater than 400 beats per minute to minimize effects of anesthesia, using a Sonos-4500 echocardiography system (Philips) and a 15-MHz transducer (Philips) (1). The average of three consecutive cardiac cycles was used. Left ventricular fractional shortening was calculated as a percentage as follows: (EDD – ESD) / EDD × 100, where EDD is end-diastolic dimension and ESD is end-systolic dimension. The observer was unaware of the genotype and treatments.

Cell Isolation. Hearts from two mice per group were pooled and minced with dissecting scissors into 3-mm pieces and digested with 2 mg/mL collagenase type IV (Worthington) and 1.2 units/mL dispase II (Worthington) and 2 mM $CaCl_2$ in PBS at 37 °C for 45 min with agitation every 15 min. After 45 min, enzymes were neutralized by adding twice the original volume of Ham's F-10 with L-glutamine (HyClone) and 15% FCS (HyClone), filtered through sterile 70-mm nylon mesh cell strainer, centrifuged at 300 × *g* for 5 min, and resuspendend in hemolytic buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA in H_2O) for 5 min at room temperature. The cells were then resuspended in PBS. The cells were then incubated with anti-CD31 antibody-conjugated Dynabeads (Invitrogen) to capture CD31+ ECs according to the manufacturer's instructions. The mixture was incubated for 20 min at room temperature in a nonadhering 15-mL tube with gentle agitation. The unbound cells were moved to a new 3-cm dish from the mixture after Dynabead-bound cells were immobilized on the tube wall using a magnet (Invitrogen). The unbound cells were incubated in 37 °C for 1.5 h. The unattached cells (cardiomyocytes) were removed. The attached cells (fibroblasts) were washed with PBS three times for mRNA preparation. The isolated CF population was confirmed by flow cytometry showing >95% purity with positive vimentin staining.

The Dynabead-bound cells were washed three times with 6 mL DMEM using the same magnet device. The CD31+ cells were directly lysed with TRIzol for mRNA preparation. Isolated cardiac ECs were confirmed by flow cytometry showing >95% purity with positive VE-Cadherin and CD31 staining.

Statistical Analysis. All data are presented as mean ± SD. Statistical analysis was performed with the one-way ANOVA or Mann–Whitney test between groups.

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2. Seki K, et al. (2009) Interleukin-33 prevents apoptosis and improves survival after experimental myocardial infarction through ST2 signaling. *Circ Heart Fail* 2(6):684–691.
3. Townsend MJ, Fallon PG, Matthews DJ, Jolin HE, McKenzie AN (2000) T1/ST2-deficient mice demonstrate the importance of T1/ST2 in developing primary T helper cell type 2 responses. *J Exp Med* 191(6):1069–1076.
4. Hsieh PC, Davis ME, Lisowski LK, Lee RT (2006) Endothelial-cardiomyocyte interactions in cardiac development and repair. *Annu Rev Physiol* 68:51–66.
5. Loffredo FS, Steinhauser ML, Gannon J, Lee RT (2011) Bone marrow-derived cell therapy stimulates endogenous cardiomyocyte progenitors and promotes cardiac repair. *Cell Stem Cell* 8(4):389–398.
6. Yoshioka J, et al. (2005) Cardiomyocyte hypertrophy and degradation of connexin43 through spatially restricted autocrine/paracrine heparin-binding EGF. *Proc Natl Acad Sci USA* 102(30):10622–10627.

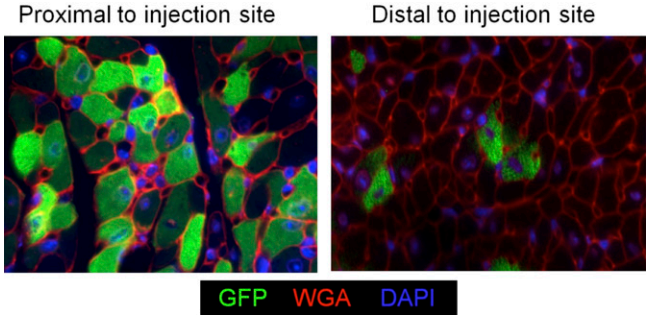

Fig. S1. Nonuniform gene transfer by adenoviral gene transfer. Adenovirus carrying GFP constructs were injected into the left ventricular free wall in mice. Seven days after adenovirus gene transfer, the mice were killed, and cardiac tissues were analyzed for GFP expression. Proximal region to virus injection site shows higher transduction efficiency, whereas the distal region to injection site shows lower transduction efficiency. Blue, DAPI staining for nucleus; green, GFP; red, WGA staining.

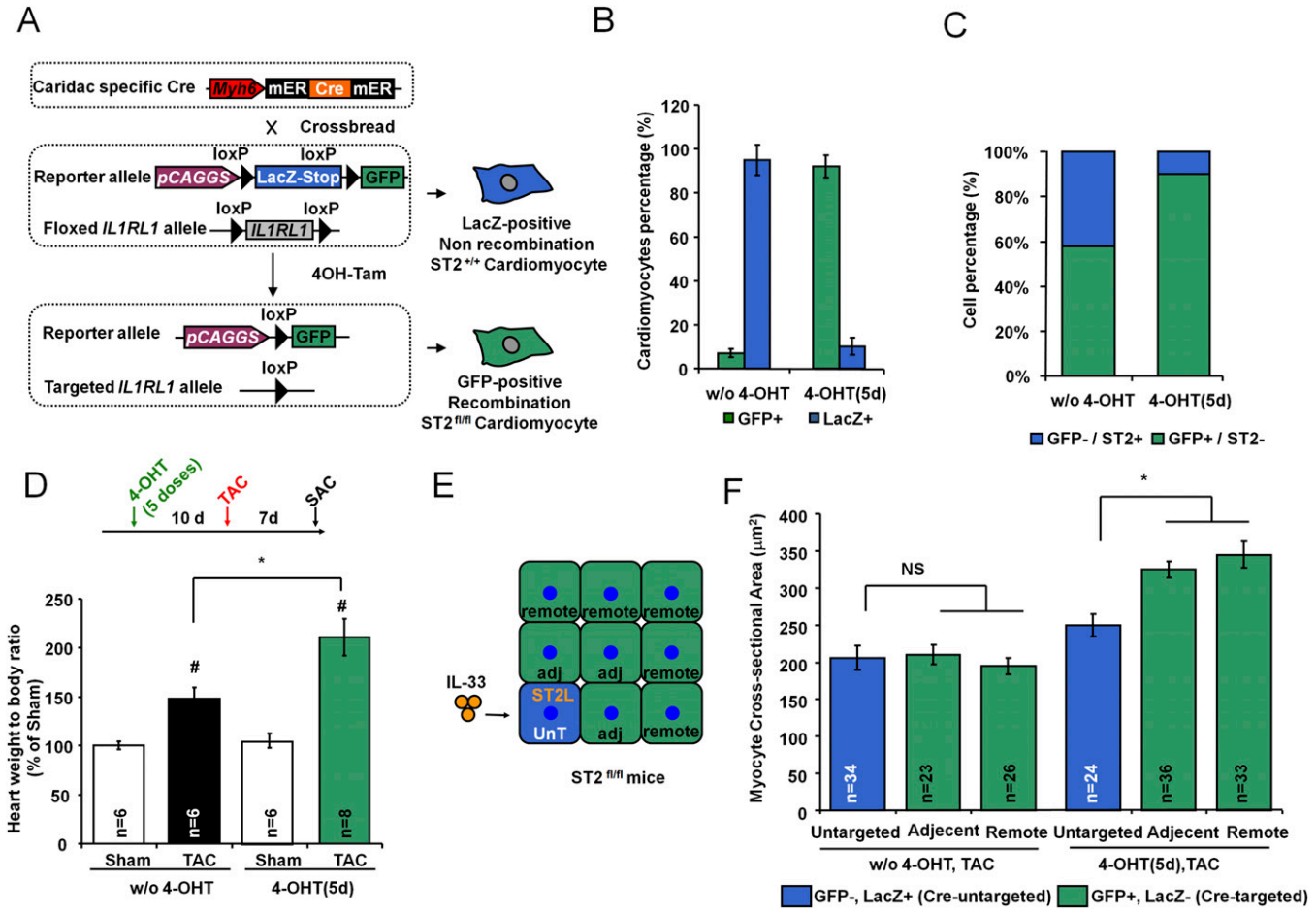

Fig. S2. Cardiomyocyte-specific ST2 deficiency exacerbates pressure overload-induced hypertrophy. (*A*) Mice carrying the *Myh6*-mER-Cre-mER (MCM) expression cassette were cross-bred to mice carrying the floxed *IL1RL1* allele and Z/EG reporter to obtain $ST2^{fl/fl}$/mER-Cre-mER/ZEG mice. Before 4-OH tamoxifen induction, cardiomyocytes express LacZ and ST2 genes. After 4-OH tamoxifen-induced Cre recombination, LacZ and *ST2* alleles were conditionally deleted and the targeted cells express GFP. (*B*) $ST2^{fl/fl}$ mice with MCM and ZEG transgenes were injected with or without 4-OH tamoxifen for 5 d. Cre-targeted cells expressing GFP increased after 4-OH tamoxifen injection. (*C*) Percentage of GFP and ST2 expression cells in the cardiac tissues. (*D*) Cardiomyocyte-specific ST2-deficient mice develop greater cardiac hypertrophy after TAC. (*E*) Schematic representation of Cre-untargeted cardiomyocyte expressing ST2L and responding to local IL-33. Adj, adjacent cells; remote, remote cells to the untargeted cardiomyocyte; UnT, untargeted cells. (*F*) Deficiency of ST2L signaling in Cre-targeted cardiomyocytes develops greater cell size compared with untargeted cells. CSA was measured in ≥10 distinct microscope fields for each slide. Three hearts from each group were analyzed.

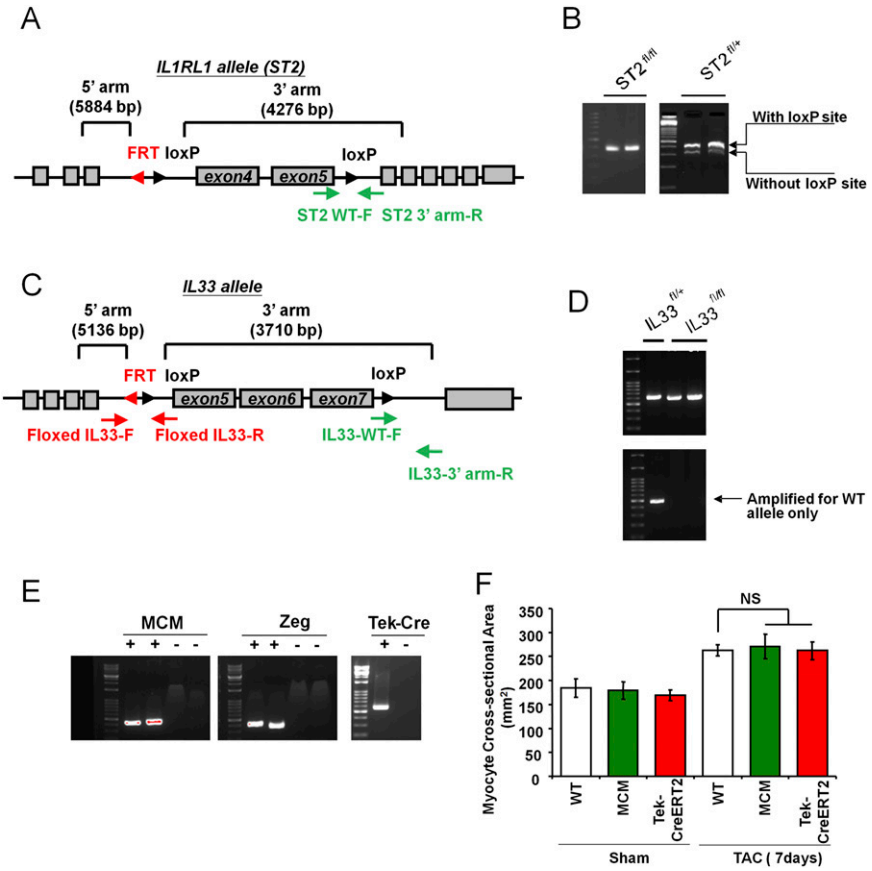

Fig. S3. Generation of mice with floxed ST2 and floxed IL33 alleles. (*A*) Schema of IL1RL1 (ST2) floxed and deleted loci. Exons 4 and 5 were flanked by two LoxP sites (arrowheads). (*B*) PCR genotyping of floxed alleles using the primer set against tail genome and deleted alleles from indicated mice. (*C*) Schema of IL33 floxed and deleted loci. Exons 5, 6, and 7 were flanked by two LoxP sites (arrowheads). (*D*) PCR genotyping of floxed alleles using the primer set against tail genome and deleted alleles from indicated mice. (*E*) Mice with myh6-Mer-Cre-Mer (MCM), ZEG, or Tek-CreERT2 (Tek-Cre) alleles were genotyped using the specific primers. (*F*) MCA analysis shows no difference between WT mice and mice with either MCM or Tek-Cre alleles, which indicates the transgenes only affect cardiac hypertrophy after TAC. Data are shown as mean ± SD. $*P < 0.05$. NS, no significance.

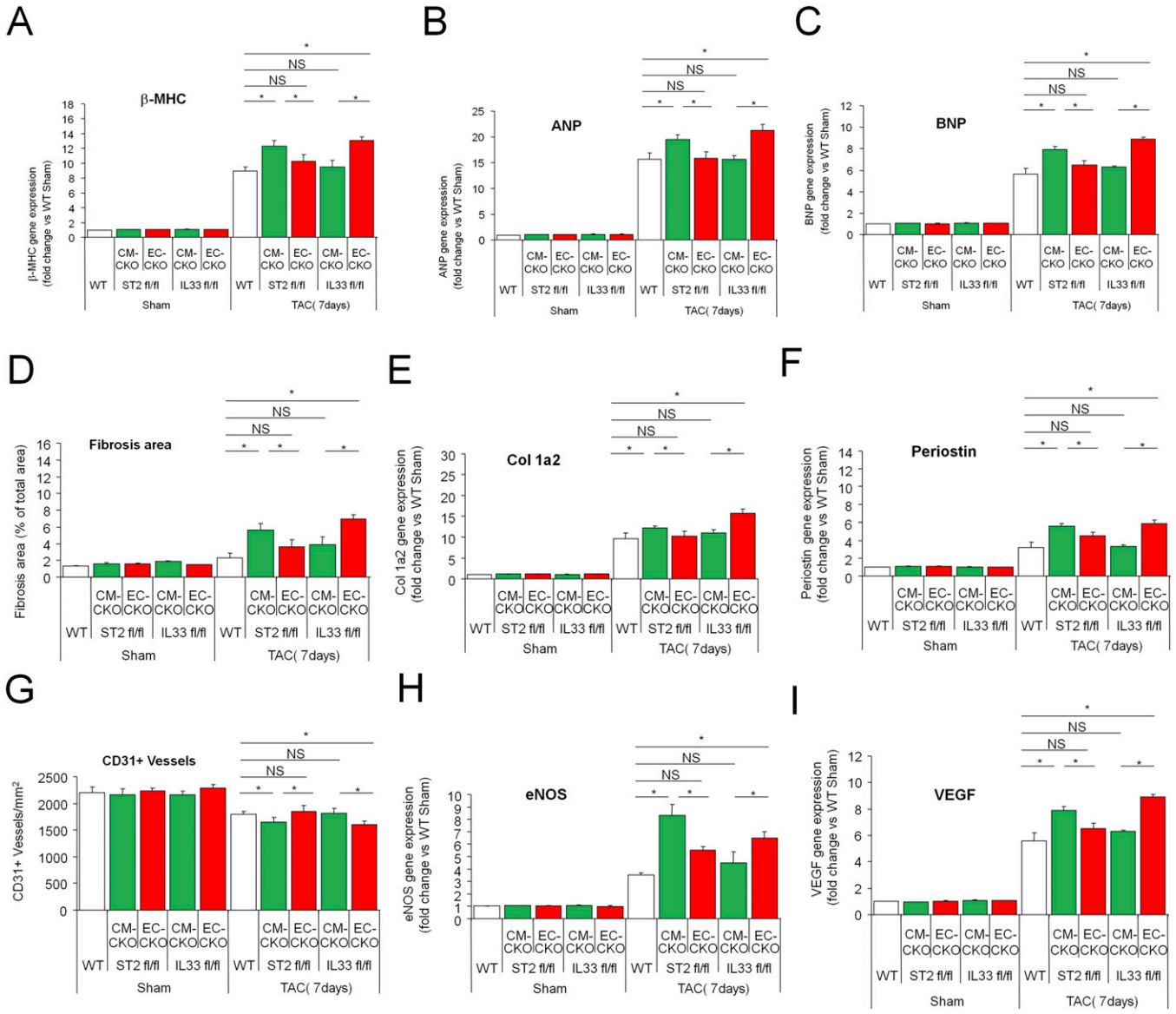

Fig. S4. Cardiac-specific deletion of ST2 or endothelial-specific deletion of IL33 enhanced TAC-induced hypertrophic, fibrotic, and angiogenic gene expressions. Expression of (*A*) β-MHC, (*B*) ANP, and (*C*) BNP was quantified in left ventricular tissues by qRT-PCR. (*D*) Percentage of fibrosis areas was analyzed by using Masson's trichrome staining. Expression of (*E*) Collagen 1a2 and (*F*) Periostin was quantified in left ventricular tissues by qRT-PCR. (*G*) Number of CD31-positive vessels was analyzed by using CD31 staining. Expression of (*H*) eNOS and (*I*) VEGF were quantified in left ventricular tissues by qRT-PCR. Relative mRNA expression of target genes was normalized with endogenous GAPDH and represented as fold change versus WT Sham. Five hearts from each group were analyzed. Data are shown as mean ± SD. $*P < 0.05$. NS, no significance.

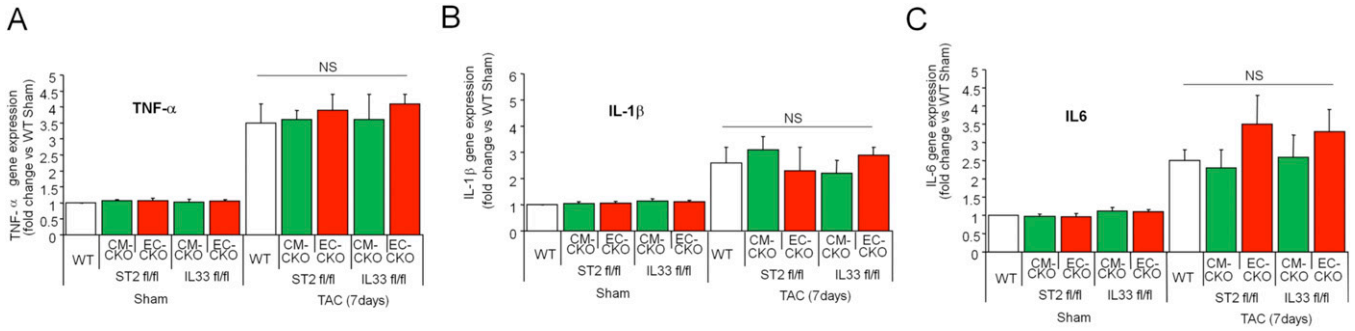

Fig. S5. Cardiac-specific deletion of ST2 or endothelial-specific deletion of IL33 did not alter selected TAC-induced inflammatory gene expression. Expression of (*A*) TNF-α, (*B*) IL-1β, and (*C*) IL-6 was quantified in left ventricular tissues by qRT-PCR. Relative mRNA expression of target genes was normalized with endogenous GAPDH and represented as fold change versus WT Sham. Five hearts from each group were analyzed. Data are shown as mean ± SD. **P* < 0.05. NS, no significance.

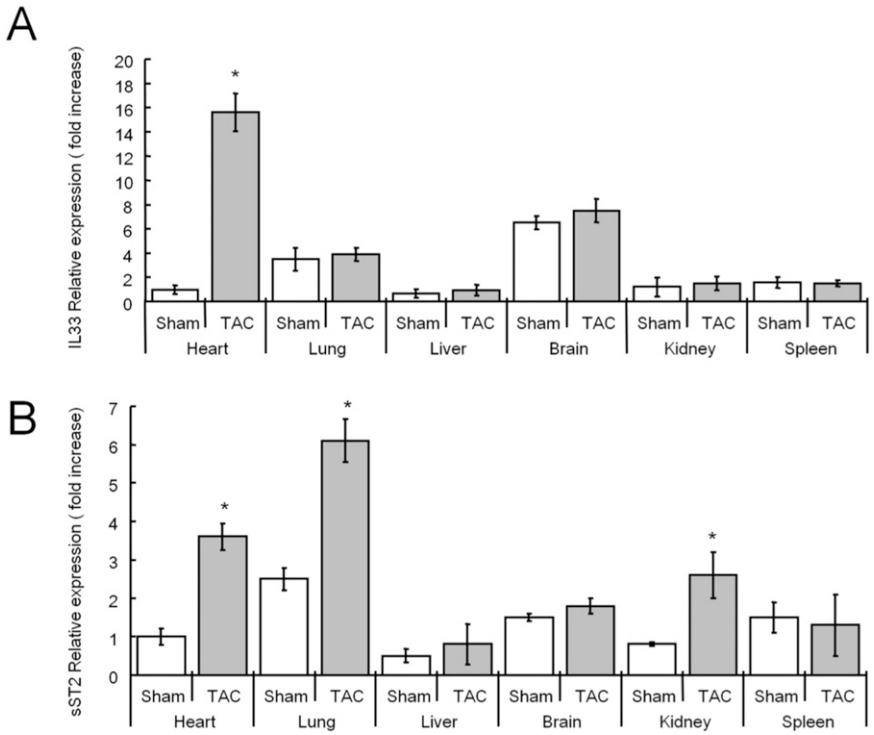

Fig. S6. Expression of IL33 and sST2 mRNA transcripts in tissues from mice after TAC. Expression of (*A*) IL33 and (*B*) sST2 genes in tissues from Sham and TAC-operated mice (7 d) was quantified by qRT-PCR. Relative mRNA expression of target genes was normalized with endogenous GAPDH and is presented as fold change versus heart expression of Sham group. Organs from three mice from each group were analyzed. Data are shown as mean ± SD. **P* < 0.05 compared with Sham control of each organ. NS, no significance.

Table S1. Echocardiography measurements after TAC

	Sham					TAC, 7 d				
Parameters	WT, $n = 6$	$ST2^{fl/fl}$ CM-CKO, $n = 5$	$ST2^{fl/fl}$ EC-CKO, $n = 5$	$IL33^{fl/fl}$ CM-CKO, $n = 5$	$IL33^{fl/fl}$ EC-CKO, $n = 5$	WT, $n = 12$	$ST2^{fl/fl}$ CM-CKO, $n = 12$	$ST2^{fl/fl}$ EC-CKO, $n = 15$	$IL33^{fl/fl}$ CM-CKO, $n = 13$	$IL33^{fl/fl}$ EC-CKO, $n = 15$
BW, g	22.5 ± 0.3	22.7 ± 0.5	23.1 ± 0.4	22.8 ± 0.6	23.8 ± 0.5	23.4 ± 0.5	24.2 ± 0.7	24.1 ± 0.5	24.9 ± 1.1	24.7 ± 0.7
HR, bpm	525 ± 26	529 ± 25	531 ± 33	543 ± 36	549 ± 38	550 ± 49	545 ± 30*,#	553 ± 39	529 ± 32	561 ± 41
LVEDD, mm	3.1 ± 0.1	3.2 ± 0.1	3.1 ± 0.1	3.2 ± 0.1	3.3 ± 0.1	3.6 ± 0.2	3.8 ± 0.2*,#	3.7 ± 0.1	3.6 ± 0.1	3.9 ± 0.2*,#
LVESD, mm	1.8 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	1.8 ± 0.2	2.6 ± 0.3	2.8 ± 0.2*,#	2.6 ± 0.3	2.7 ± 0.2	2.8 ± 0.3*,#
PWTd, mm	0.6 ± 0.01	0.6 ± 0.01	0.6 ± 0.01	0.6 ± 0.01	0.6 ± 0.01	0.9 ± 0.06	1.0 ± 0.07*,#	0.9 ± 0.05	0.8 ± 0.06	1.0 ± 0.08*,#
IVSd, mm	0.6 ± 0.01	0.6 ± 0.01	0.6 ± 0.01	0.6 ± 0.01	0.6 ± 0.01	0.7 ± 0.04	0.8 ± 0.03*,#	0.8 ± 0.05	0.8 ± 0.07	0.9 ± 0.04*,#
FS, %	44 ± 1	45 ± 2	43 ± 2	44 ± 1	44 ± 2	32 ± 5	30 ± 4	32 ± 5	32 ± 5	29 ± 4

BW, body weight; FS, fractional shorting; HR, heart rate; IVSd, interventricular septum thickness in diastole; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; PWTd, posterior wall thickness in diastole. *$P < 0.05$ versus same genotype Sham controls. P values were calculated by Mann–Whitney analysis. #$P < 0.05$ compare with WT control after TAC. Data are shown mean ± SEM.

Table S2. Primers for genotyping

Target gene	WT allele	Gene name	Sense, 5′-3′	Antisense, 5′-3′
593 bp	—	floxIL33	AACCTCCTGGTCAATATTCAGT	CCGCCTACTGCGACTATAGA
—	482 bp	IL33-WT	CAAGTCTGGTCTCCAGCAAC	AGCAAGAACGGACCAGATGTA
339 bp	—	Z/EG	TGCCCATCCTGGTCGAGCT	GCTCGATGCGGTTCACCAG
405 bp	—	MCM	GTCTGACTAGGTGTCCTTCT	CGTCCTCCTGCTGGTATAG
410 bp	—	LacZ	TTTCCGTGACGTCTCGTTGCTGCATAAACC	TTCAGCAGCAGCAGACCATTTTCAATCCGC
145 bp	—	floxST2	CCA TACTGTGAGATGGCGC	GAAGGAGGAAATTCAGACTGGG
408 bp	296 bp	ST2-WT	GGCCACCAGATCATTCACAGTTGAAGG	GAAGGAGGAAATTCAGACTGGG

Other Supporting Information Files

Dataset S1 (PDF)