# SUPPLEMENTAL MATERIAL

## SUPPLEMANTAL MATERIALS AND METHODS

### DETAILED METHODS

**Minimally Invasive Transverse Aortic Constriction.** Minimally invasive transverse aortic constriction (TAC) was performed on wild-type (WT) mice and IL-6 knockout (*IL-6*$^{-/-}$) mice. Mice were anesthetized using 2% isoflurane mixed with 100% oxygen. Mice received sustained release buprenorphine (0.3-1.0 mg/kg SQ) prior to surgery. All surgeries were performed under aseptic protocol and pre- and postsurgical gentamicin (5mg/kg i.m.) was administered. Limited median sternotomy was performed from the suprasternal notch to the second rib using angled scissors under a surgical microscope. The aorta was freed by blunt dissection and a blunt 27-gauge needle was placed along the aortic arch. A 7-0 nylon suture was placed around the aorta and the needle between the innominate and the left common carotid artery. Next, the suture was tied and the blunt needle was promptly removed thereafter. The sham surgery was identical to the above description except that the knot was not tied around the aorta. The chest wall was closed using a purse-string suture and the surgical wound was closed with interrupted sutures. Mice were allowed to recover under intensive monitoring.

In order to measure pressure gradient post-TAC in both groups of mice, right and left carotid arteries were cannulated with flame stretched PE 50 tubing. Catheters were connected to pressure transducers accepting a 23-gauge needle bore at the spout, which was connected to the catheter. The transducer was connected to PowerLab 16/30 data acquisition system (AD Instruments, Colorado Springs, CO) to record arterial blood pressures. After the pressure monitoring system was ready, TAC procedure was conducted. Before aortic constriction, systolic pressures in both WT and IL-6$^{-/-}$ mice were similar, there was no difference in the pressure gradients between the left carotid (distal to the suture) and right carotid (proximal to the suture) which remained constant throughout the subsequent 60-min period in both groups after TAC.

**Tissue Harvest for Morphometric and Histological Studies.** Each mouse was anesthetized and injected with 0.5 ml of 5% KCl (i.v.) to ensure the heart arrests in diastole. The heart was rapidly excised and the aorta was cannulated with a 20-gauge needle. This set-up was then mounted in a Langendorff apparatus to wash out blood using calcium and magnesium-free PBS and maintain diastolic fixation by formalin perfusion. Each heart was transversely cut into two halves near the mid-papillary region and the apical half was processed and embedded in paraffin. Tissue was harvested for analysis at several different pre-specified time-points after the TAC procedure.

**Histology.** The paraffin embedded-apex was sectioned at 4 μm intervals, and stained with Masson's trichrome stain (Sigma Aldrich, St. Louis, MO). The mid-section was used to measure LV diameter.[1, 2] These anatomical parameters were corrected to a uniform sarcomere length

(2.1μm).[1] For determination of myocyte cross-sectional area, cardiac myocytes with a circular profile and a central nucleus were analyzed.[2] For assessment of fibrosis, tissue sections were stained with picrosirius red (Sigma Aldrich, St. Louis, MO). To measure interstitial collagen percentage, 9-18 fields (without any blood vessels) of the left ventricle (excluding papillary muscles) were randomly imaged. Perivascular collagen ratio was calculated from 9-12 randomly chosen fields containing vasculature and it was expressed as the ratio of fibrotic area immediately surrounding the vessel to cross-sectional area of the vessel.[3] All measurements were made using ImagePro Plus software version 7.0 (Media Cybernetics, Rockville, MD). All morphometric analyses were performed in a blinded fashion.

**Tissue Harvest for Gene Profiling and Protein Analysis.** Each heart was excised and weighed after the removal of great vessels and atria. The right ventricular free wall was removed to weigh the left ventricle, which was then cut into two parts longitudinally and snap frozen to be stored at -80$^{o}$C for mRNA and protein assays.

**Echocardiography.** Echocardiograms were obtained using a Vevo 2100 Ultrasound System (VisualSonics, Toronto, Canada) equipped with a high-frequency (30 MHz) linear array transducer. Echocardiography was performed prior to TAC, and 1, 2, 4, and 6 weeks following surgery. The mice were anesthetized with isoflurane (3% for induction and 1-1.5% for maintenance) mixed in 1 L/min $O_2$ via a facemask. Hair was removed from the anterior chest using a chemical hair remover. Using a rectal temperature probe, body temperature was carefully maintained close to 37.0°C with a heating pad throughout the study. Modified parasternal long-axis and parasternal short-axis views were used to obtain two-dimensional (2-D), M-mode, and spectral Doppler images.[1, 2] Systolic and diastolic anatomic parameters were obtained from M-mode tracings at the mid-papillary level. LV mass was estimated by the area-length method. All views were digitally stored in cine loops consisting of >300 frames. Subsequent analysis was performed off-line on a workstation installed with Vevo LAB software (version 1.7.1) (VisualSonics, Toronto, Canada) by an experienced operator who was blind to the treatment allocation and was unaware of data from other modalities.

**Hemodynamic Studies.** Invasive hemodynamic measurements were performed at the 35-day follow-up before sacrifice. The mice were anesthetized using isoflurane (3% for induction and 1–1.5% for maintenance) and ventilated using a rodent ventilator. Heparinized saline (1ml) was injected intraperitoneally before the procedure to fluid load animals. Core body temperature was maintained at 37.5$^{o}$C. The LV apex was exposed through an incision between the seventh and eighth ribs, and a 1.0 Fr PV conductance catheter (PVR-1045; Millar Instruments, Houston, TX) was advanced through the apex to lie along the longitudinal axis. Absolute volume was calibrated, and pressure–volume (PV) loop data were obtained from the 10–15 successive cardiac cycles at steady state and during transient reduction of venous return by occluding the inferior vena cava with a 6-0 silk snare suture using PowerLab 16/30 data acquisition system (AD instruments, Colorado Springs, CO) and analyzed with LabChart® 7 Pro (AD instruments, Colorado Springs, CO). Parallel conductance corrections were performed in duplicate for each animal by injecting 2μL of 30% NaCl solution into the jugular vein.

**Terminal dUTP Nick End-labeling (TUNEL) Assay.** Myocardial sections were deparaffinized, rehydrated and incubated with recombinant proteinase K (PCR grade, Roche, Basel, Switzerland) for 10 min at room temperature. After rinsing with PBS, the positive controls were incubated with recombinant DNase1 (Roche, Basel, Switzerland). Sections were then blocked with 10% donkey serum (Jackson ImmunoResearch, West Grove, PA). Apoptotic cells were detected using *in situ* cell death detection kit, fluorescein (Roche, Basel, Switzerland). All myocytes were stained with monoclonal anti-α-sarcomeric actin (Sigma Aldrich, St. Louis, MO) and mounted with Vectashield H-1500 (Vector Laboratory, Burlingame, CA) containing DAPI to counterstain the nuclei. While counting the apoptotic nuclei, only nucleated myocytes in the left ventricle were chosen excluding the papillary muscles and outer edges of tissue. The number of TUNEL-positive nuclei was expressed as a percentage of total cardiomyocytes in left ventricle.

**Adult Cardiomyocyte Isolation and Culture.** Each heart was digested using a buffer containing 168 U/ml collagenase type 2 (Worthington, Lakewood, NJ), 160 U/ml collagenase type 4 (Worthington, Lakewood, NJ), 0.02 mg/ml proteinase XIV (Sigma Aldrich, St. Louis, MO) and 40μM $CaCl_2$ (Sigma Aldrich, St. Louis, MO). The great vessels, atria, anulus, and right ventricular free wall were removed from the heart, and LV tissue was minced with fine scissors in the buffer and gently pipetted to cause individual cell separation. Myocytes were filtered through 150-μm nylon mesh, counted, and exposed to increasing doses of $CaCl_2$. Each heart yielded approximately $1x10^6$ myocytes, more than 70% of which were rod-shaped. Myocytes were plated on laminin-coated dishes. Myocytes were either measured for size or cultured overnight for molecular studies.

**Cardiomyocyte Size.** Cardiomyocytes were isolated from WT and IL-$6^{-/-}$ mice six weeks after sham and TAC procedures. They were imaged using an Olympus IX 71 microscope (Olympus, Tokyo, Japan) fitted with a DP 72 digital camera (Olympus, Tokyo, Japan) under a 20X objective after 4 hours of plating. Only binuclear cardiomyocytes were assessed after exclusion of misshapen myocytes. Total surface areas, lengths and widths were calculated for a minimum of 100 representative cardiomyocytes per mouse and 6 mice for each group, using cellSens Standard software (Olympus, Tokyo, Japan) for analysis.

**H9c2 Myoblast Culture.** H9c2 myoblasts were purchased from the American Tissue Culture Collection (Manasas, VA, USA). Cells were cultured in 1x Dulbecco's modified Eagle's medium (DMEM; Cambrex Bio Science, Walkersville, MD, USA) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Rockville, MD, USA) and antibiotics (25U/ml penicillin and 25U/ml streptomycin) at 37°C in a humidified atmosphere containing 5% $CO_2$. Cells were plated and cultured for 24h in the medium. Prior to treatment, the cells were serum starved for 12 hours.

**Cell Treatment.** For isolated adult cardiomyocytes, which were treated with recombinant IL-6 (rIL-6) (R&D Systems, Minneapolis, MN) 50ng/ml for 24 hours, angiotensin II (Ang II) (Calbiochem, Darmstadt, Germany) $1x10^{-7}$ mol/L for 48 hours and phenylephrine (PE) (Sigma Aldrich, St. Louis, MO) 10μmol/L for 24 hours, respectively. For studies of myocyte hypertrophy, H9c2 cells were treated with rIL-6 (50ng/ml) in presence or absence of CaMKII inhibitor KN-62

(10μmol/L) (Millipore, Darmstadt, Germany) and STAT3 inhibitor WP1066 (6μmol/L) (Millipore, Darmstadt, Germany) for 24 h. To evaluate the phosphorylation of STAT3 and CaMKII induced by rIL-6, H9c2 cells were treated with rIL-6 (50ng/ml) for several time periods up to 12 h. To determine the relationship between CaMKII and STAT3, H9c2 cells were pretreated with KN-62 (5μmol/L or 10μmol/L) for 2 h, followed by treatment with rIL-6 (50ng/ml) for 30 min.

**Crystal Violet Staining.** After washing with PBS, H9c2 cells were fixed with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) for 15 min and stained with 1% crystal violet (Thermo Fisher Scientific, Lenexa, KS) for 2 h at room temperature to assess cell surface area. Images were obtained using a DP 72 digital camera (Olympus, Tokyo, Japan) on an Olympus IX 71 microscope (Olympus, Tokyo, Japan). Ten random photographs were taken from each group, and at least 200 individual cells were examined in each group. Cell surface area was analyzed using cellSens standard software (Olympus, Tokyo, Japan).

**Transfection.** H9c2 cells were plated in culture medium on the day before transfection and were 80% confluent at the time of transfection. Using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), cells were transiently transfected with plasmid encoding with CaMKII (Addgene, Cambridge, MA) in a dose-dependent manner while empty vector was used as control. Cells were incubated for 18-24 hours in 5% $CO_2$ at 37°C. The cells were then placed in fresh culture medium for another 24-30 hours prior to harvest.

**Quantitative PCR.** Total RNA was extracted from frozen tissues and cultured cells using Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. cDNA synthesis from 1μg RNA was carried out using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Real-time quantitative PCR (qPCR) was done using a SYBR® green PCR master mix kit (Applied Biosystems, Foster City, CA) on a ViiA™ 7 system (Applied Biosystems, Foster City, CA). GAPDH was used as control and the $2^{\Delta\Delta CT}$ method was used for data analysis. Primers used for qPCR are listed in Online Table I. The results from three independent experiments, each sample were performed duplicated in each experiment.

**Protein Extraction and Western Blot Analysis.** Cardiac tissue, cultured H9c2 myoblasts and adult cardiomyocytes were lysed in RIPA lysis buffer (Millipore, Darmstadt, Germany) with protease and phosphatase cocktail inhibitors (Roche, Basel, Swizerland) to yield total protein. Cytoplasmic and nuclear protein fractions were isolated using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific, Lenexa, KS) using the manufacturer's protocol. Protein concentration was determined using Pierce® BCA protein assay kit (Thermo Fisher Scientific, Lenexa, KS). Proteins were separated using 8%-14% SDS-polyacrylamide gel electrophoresis and transferred to an Immobilon-FL membrane (Millipore, Darmstadt, Germany). After blocking with Odyssey® blocking buffer (LI-COR, Lincoln, NE), membranes were hybridized with anti-p-CaMKII, anti-CaMKII, anti-p-STAT3 (Try705), anti-p-ERK1/2, anti-ERK1/2, anti-p-JNK, anti-JNK, anti-p-Akt, anti-Akt, anti-p-GSK3β, anti-GSK3β, anti-Bcl-2 (Cell Signaling Technology, Danvers, MA), anti-ANP, anti-STAT3, anti-CaMKIIδ, anti-Bax, anti-MMP9, anti-periostin, anti-GAPDH, anti-laminA (Santa Cruz, Dallas, TX) overnight at 4°C. Secondary antibodies labelled with fluorescent dye (IRDye) (LI-COR, Lincoln, NE) were used for 1 hour at

room temperature and immunoblots were scanned using ODYSSEY® CLx infrared imaging system (LI-COR, Lincoln, NE). GAPDH is total protein loading control; LaminA is nuclear protein loading control. Arbitrary optical densities were measured by Image Studio™ Lite software version 4.0 (LI-COR, Lincoln, NE).

## REFERENCES

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## ONLINE FIGURE LEGENDS

**Online Figure I. Confirmation of *IL-6* deletion in *IL-6*$^{-/-}$ mice.** Ladder is Promega 100bp (G2101), bright band is 500bp. One band (174bp) is wild type (WT); two bands (174 and 380bp) are IL-6 heterozygous allele (*IL-6*$^{+/-}$); one band (380bp) is IL-6 homozygous mutated allele (*IL-6*$^{-/-}$).

**Online Figure II. Experimental protocol.**

**Online Figure III. Attenuation of pressure overload-induced activation of MAPK and Akt signaling pathways in the absence of IL-6. A-C,** representative Western immunoblots (**A**) and quantitative data for p-ERK1/2 (**B**) and p-JNK (**C**) in WT and *IL-6*$^{-/-}$ mouse hearts at 6 weeks after surgery (n=6 per group). **D-E,** representative Western immunoblots (**D**) and quantitative data for p-Akt (**E**) and p-GSK3β (**F**) in WT and *IL-6*$^{-/-}$ mouse hearts at 6 weeks after surgery (n=6 per group). Data represent means ± SEM. **P*<0.05 vs. Sham WT mice; # *P*<0.05 vs. TAC WT mice; § *P*<0.05 vs. Sham *IL-6*$^{-/-}$ mice.

**Online Figure IV. CaMKII contributed to STAT3 activation in IL-6-inudced cardiomyocyte hypertrophy. A-C,** isolated cardiomyocytes were treated with IL-6 with or without CaMKII inhibitor KN-62. Representative Western blots (**A**) and quantitative data for p-CaMKII (**B**) and pTyr-STAT3 (**C**). Data represent means ± SEM from three independent experiments. **P*<0.05 vs control; # *P*<0.05 vs IL-6 treatment only.

**Online Table I. Primers used for quantitative polymerase chain reaction**

| Primers | | Sequence 5'-3' | Species |
|---|---|---|---|
| *anp* | Forward | atctgatggatttcaagaacc | rat |
| | Reverse | ctctgagacgggttgacttc | |
| *bnp* | Forward | acaatccacgatgcagaagct | rat |
| | Reverse | gggccttggtcctttgaga | |
| *gapdh* | Forward | atgggaagctggtcatcaac | rat |
| | Reverse | gtggttcacacccatcacaa | |
| *anp* | Forward | cctaagcccttgtggtgtgt | mouse |
| | Reverse | cagagtgggagaggcaagac | |
| *bnp* | Forward | ctgaaggtgctgtcccagat | mouse |
| | Reverse | ccttggtccttcaagagctg | |
| *myh-7* | Forward | atcaatgcaaccctggagac | mouse |
| | Reverse | cgaacatgtggtggttgaag | |
| *αSK-actin* | Forward | gcgcaagtactcagtgtgga | mouse |
| | Reverse | tcgtcctgaggagagagagc | |
| *gata4* | Forward | tctccaaatgggatctctgg | mouse |
| | Reverse | tctccaaatgggatctctgg | |
| *periostin* | Forward | aaccaaggacctgaaacacg | mouse |
| | Reverse | tgtgtcaggacacggtcaat | |
| *Col1A1* | Forward | tctccaaatgggatctctgg | mouse |
| | Reverse | tctccaaatgggatctctgg | |
| *Col3A1* | Forward | gcacagcagtccaacgtaga | mouse |
| | Reverse | tctccaaatgggatctctgg | |
| *IL-6R* | Forward | ctcccggtggcccagtacca | mouse |
| | Reverse | tgcactggggcgaggacact | |
| *gapdh* | Forward | aggtcggtgtgaacggatttg | mouse |
| | Reverse | tgtagaccatgtagttgaggtca | |

**Online Table II. Reasons for excluding mice from studies in vivo**

| Groups | Sham WT | Sham IL-6 $^{-/-}$ | TAC WT | TAC IL-6 $^{-/-}$ | Total |
|---|---|---|---|---|---|
| Death | | | 3 | 3 | 6 |
| Technical problems | 1 | 1 | 2 | 1 | 5 |
| Cardiovascular abnormalities | | | 1 | | 1 |
| Heart weight < 120 mg* | | | 1 | 2 | 3 |
| Mice enrolled | 34 | 34 | 44 | 41 | 153 |
| Mice excluded | 1 | 1 | 7 | 6 | 15 |
| Mice included in study | 33 | 33 | 37 | 35 | 138 |
| Mice excluded from study (%) | 3.0 | 3.0 | 15.9 | 14.6 | 9.8 |
| Mortality (%) | | | 11.4 | 9.8 | 7.2 |

*Heart weight was less than 120 mg at 6 weeks of post TAC; Death, naturally dead after TAC; Technical problem, aortic rupture or pneumothorax.

**Online Table III. Age, BW, HW, and TL analysis**

| Groups | n | Age weeks | Pre-OPBW g | Post-OPBW g | HW mg | TL mm | HW/BW mg/g | HW/TL mg/mm |
|---|---|---|---|---|---|---|---|---|
| Sham WT | 10 | 11.1±0.05 | 24.7±0.75 | 26.9±0.81 | 101.2±4.23 | 16.9±0.17 | 3.8±0.06 | 6.0±0.21 |
| Sham *IL-6* $^{-/-}$ | 10 | 11.0±0.04 | 24.6±0.41 | 26.6±0.28 | 100.7±1.97 | 16.9±0.09 | 3.8±0.06 | 6.0±0.14 |
| TAC WT | 12 | 11.3±0.23 | 24.5±0.33 | 28.5±0.42 | 174.4±7.40* | 17.2±0.09 | 6.1±0.24* | 10.1±0.44* |
| TAC *IL-6* $^{-/-}$ | 12 | 11.1±0.24 | 24.5±0.38 | 27.5±0.36 | 150.6±4.85§# | 17.1±0.05 | 5.5±0.13§# | 8.8±0.29§# |

Pre-OPBW, pre-operative body weight; Post-OPBW, post-operative body weight at 6 weeks; HW, heart weight; TL, tibia length. *$P$<0.05 vs. Sham WT mice; § $P$ <0.05 vs. Sham *IL-6* $^{-/-}$ mice; #$P$ <0.05 vs. TAC WT mice.

**Online Table IV. Morphometric measurement for transverse myocyte cell surface**

| Group | n | Cardiomyocyte size ($\mu m^2$) | Nuclear area ($\mu m^2$) | Nuc-cyto ratio (%) |
|---|---|---|---|---|
| Sham WT | 8 | 167.4±6.7 | 10.62±0.6 | 6.5±0.4 |
| Sham *IL-6*$^{-/-}$ | 8 | 135.1±13.2* | 8.6±0.9* | 6.8±0.5 |
| TAC WT | 12 | 256.4±17.7* | 13.56±0.9* | 5.5±0.2* |
| TAC *IL-6*$^{-/-}$ | 12 | 173.4±12.3# | 11.13±0.5# | 6.5±0.4 |

Data are means ± SEM. **P*<0.05 vs. Sham WT mice; #*P* <0.05 vs. TAC WT mice.

**Online Table V. Hemodynamics Analysis after 6 weeks of surgery**

| Groups | n | HR (bpm) | LVP (mmHg) | LVMP (mmHg) | LVESP (mmHg) | LVEDP (mmHg) | dP/dt max (mmHg/s) | dP/dt min (mmHg/s) | Tau (ms) |
|---|---|---|---|---|---|---|---|---|---|
| Sham WT | 12 | 511±6 | 100±1 | 41±1 | 99±1 | 5.7±0.3 | 8515±142 | -9115±148 | 6.5±0.1 |
| Sham *IL-6*$^{-/-}$ | 12 | 500±27 | 105±3 | 41±2 | 100±4 | 5.3±0.9 | 9310±532 | -9452±338 | 6.0±0.2 |
| TAC WT | 12 | 498±7 | 141±2* | 55±1* | 138±2* | 8.7±0.3* | 6747±95* | -7715±119* | 8.7±0.1* |
| TAC *IL-6*$^{-/-}$ | 11 | 574±6§# | 144±2§ | 54±1§ | 145±3§ | 5.9±0.1# | 9034±197# | -9332±203# | 6.7±0.2# |

HR, heart rate; LVP, left ventricular pressure; LVMP, LV mean pressure; LVESP, LV end-systolic pressure; LVEDP, LV end-diastolic pressure; dP/dt $_{max}$, LV maximum value of dP/dt; dP/dt $_{min}$, minimum value of dP/dt; Tau, LV relaxation constant. *$P$<0.05 vs. Sham WT mice; § $P$ <0.05 vs. Sham *IL-6*$^{-/-}$ mice; #$P$ <0.05 vs. TAC WT mice.