

## Supplemental Material

### Detailed, Expanded Methods.

**Hemodynamic Measurements.** Rats were anesthetized with ketamine hydrochloride (70 mg/kg), and xylazine (10 mg/kg) injected intraperitoneally (i.p.) before right heart catheterization. Right ventricular systolic pressure (RVSP) measurements were obtained by insertion of a Micro Tip pressure transducer catheter (model SPR-671, 1.4F, Millar Instruments, Houston, TX) through the jugular vein into the right ventricle (RV). Signals were recorded continuously with a TC-510 pressure control unit (Millar Instruments, Houston, TX) coupled to a Bridge Amp. Data were collected with the Powerlab4/30 data acquisition system and analyzed with Chart Pro software (both from AD Instruments, Colorado Springs, CO). The RV was dissected from the left ventricle (LV) and the septum (S), and each was weighed. The ratio of RV/(LV+S) was calculated to determine the degree of right ventricular hypertrophy (RVH).

**Echocardiography.** Echocardiographic evaluation of right ventricular dimensions and pulmonary hemodynamics were performed with Vivid 7 Dimension Cardiovascular Imaging System (GE), equipped with a 14-MHz transducer. Rats were lightly sedated with isoflurane, volatized with compressed air (3% for induction, 1% for maintenance) for the duration of the procedure. Standard left ventricular measurements were made from 2dimensional-guided M-mode images at the level of the papillary muscles. Pulmonary artery and tricuspid valve Doppler tracings were obtained from separate parasternal short-axis views. The RV free wall and chamber were imaged from a modified parasternal long-axis view. All measurements were made in the expiratory phase of the respiratory cycle. The sonographer was blinded to the study groups during echo acquisition and subsequent analyses.

**Immunohistochemistry and morphometry.** For COX-2, PTGIS, HO-1, PDL-1, alpha SMA, the rat lung and heart paraffin sections were deparaffinized, rehydrated and then sections were permeabilized with 0.2% Triton (Sigma Aldrich). After antigen retrieval with steamer set (#IW-1102; IHC-Tek), slides were incubated with dual endogenous enzyme blocker (#S2003; Dako), blocked with 5% goat serum (Jackson ImmunoResearch), and then incubated for 1h at RT with primary antibodies, and sections were then processed with the LSAB2 System-HRP Research Kit (#K0609, #K0675; Dako), followed by 3,3-diaminobenzidine (Dako) and counterstained with hematoxylin (#7211; Richard-Allan Scientific). Quantification of the percentage of wall thickness of  $\alpha$ -SMA positive vessels was determined using the methodology described by Beppu et al. and was as follows:  $\% \text{ wall thickness} = (\text{WT1} + \text{WT2}) \times 100\% / (\text{external diameter of vessel})$  where WT1 and WT2 refer to wall thicknesses measured at two points diametrically opposite to each other. The endothelial component of the vessel wall was excluded from the measurements of wall thickness. Quantification was performed in a blinded fashion in randomly chosen lung tissue sections in each experimental group. (n=4 per group). 10 vessels per animal were counted and in total one hundred twenty small pulmonary vessels were analyzed using Image J (version 1.48) software. Microscopic analysis was performed with the Leica DMLB light microscope (Leica Microsystems, Wetzlar, Germany).

**RV histology and quantifications.** Paraffin-embedded RV samples were sectioned at 4  $\mu\text{m}$ , and subjected to Masson's trichrome staining. For RV *interstitial fibrosis* quantitative morphometric analysis was blindly assessed from five random sections of trichrome slides by determining the total tissue areas occupied by cardiomyocytes and collagen and excluding the lumen (i.e. empty spaces), as well as excluding annotations of perivascular fibrosis. The percentage/amount of fibrosis was analyzed using Image J (version 1.48) software from the RGB images with a macro described by Kennedy, et al. (n=4 per group). For RV the area of

*perivascular fibrosis* quantitative morphometric analysis was blindly assessed from five random sections of trichrome slides by determining the ratio of the fibrosis area surrounding the vessel to the total vessel area using Image J (version 1.48) software. In each RV heart tissue, ≈5-7 small arteries were examined (n=4 per group). *Quantification of macrophages (CD68) staining in rat RV.* CD68+ cells were blindly counted in ten, 400x fields in rat heart tissue sections/animal. (n=4 per group).

*Quantification of capillaries (Reca-1) staining:* The percentage of Reca-1 positive area of total RV tissue/slide area was blindly analyzed in ten, 400x fields in 4 random heart tissue sections/animal using Image J (version 1.48) software with a macro. (n=4 per group).

**Real time PCR.** Heart and lung tissue were homogenized in RLT buffer (Qiagen, Louisville, KY No. 74104), RNA extraction was performed using an RNeasy mini kit (Qiagen, Louisville, KY No. 74104). The extracted RNA was reverse transcribed into cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Vilnius, LT No. 4368814). The cDNA was normalized to 1 µg, and diluted 1:10. Quantitative real-time PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK No. 4368708) on a Applied Biosystems Abi Prism machine using a 10 µL reaction mixture consisting of 2 µL of the 1:10 cDNA dilution, 5 µL SYBR master mix, and .025 µM (1:10 dilution= 0.0025 µM) concentration of primer. Primers were either sourced from literature or designed using the Roche Universal ProbeLibrary Assay Design Center and are listed in table below. The amplification conditions consisted of one cycle at 50°C for 2 min, one cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The fold change was determined using cycle threshold (Ct) values, and normalized using the housekeeping gene GAPDH.

COX-2 (NCBI GenBank Accension No: NM011198.1, expected PCR product size 583 bp) forward 5'-ACTCACTCAGTTTGTGAGTCATTC-3', reverse 5'-TTTGATTAGTACTGTAGGGTTAATG-3'.
COX-1 (NCBI GenBank Accension No: NM017043.1, expected PCR product size 450 bp) forward 5'-TGCATGTGGCTGTGGATGTCATCAA-3', reverse 5'-CACTAAGACAGACCCGTCATCTCCA-3'.
PDL-1 (NCBI GenBank Accension No: NM_001191954.1, expected PCR product size 90 bp) forward 5'- CAGGTGTCTACTGCTGCATGA -3', reverse 5'- GGTTGATTTTGC GG TATGGA -3'.
HMOX-1 (NCBI GenBank Accension No: NM_012580.2, expected PCR product size 72 bp) forward 5'- GTCAAGCACAGGGTGACAGA -3', reverse 5'- CTGCAGCTCCTCAAACAGC -3'.
PTGIS (NCBI GenBank Accension No: NM_031557.2, expected PCR product size 60 bp) forward 5'- ATGCCATCAACAGCATCAA -3', reverse 5'- GCTCCAGGTCGAAATGAGTC -3'.
TxS (NCBI GenBank Accension No: D31798.1, expected PCR product size 555 bp) forward 5'- ACCCAAGCTGATAGCAGACA -3', reverse 5'- GTGACCATGTCAAAGGCTTC -3'.

GAPDH (NCBI GenBank Accension No: AF106860.2, expected PCR product size 558 bp)

forward 5'- GTGAAGGTCGTGTCAACGGATTT -3',  
reverse 5'- CACAGTCTTCTGAGTGGCAGTGAT -3'.

**Human Treg isolation.** Human Tregs were obtained from fresh buffy coats collected from healthy male donors (Stanford Blood Bank). Peripheral blood mononuclear cells (PBMCs) were obtained by Lympholyte H (#CL5020, Cedarlane, Burlington, NC) density gradient centrifugation. CD4<sup>+</sup>CD25<sup>high</sup> cells were isolated from the PBMCs using the two-step method that includes negative selection with the Human CD4<sup>+</sup>CD25<sup>high</sup> T Cell Isolation Kit (#18062A, STEMCELL Technologies) and the fully automated RoboSep™. CD4<sup>+</sup>CD25<sup>high</sup> cells were then analyzed by FACS and shown to be 97.5%-99% pure.

**Cell co-culture experiments.** Human Cardiac Microvascular Endothelial Cells (hCMECs), (#cAP-0021), male donor were obtained from Angio-Proteomie (Boston, MA). They were cultured in endothelial cell basal medium (EBM), (Angio-Proteomie) supplemented with 5% FBS, growth supplements (ECGS), (#cAP-20, Angio-Proteomie), penicillin (100 IU/ml), (#15070063) and streptomycin (100 µg/ml), (#15070063, Gibco, Waltham, MA) in a humidified incubator (21% O<sub>2</sub>, 5% CO<sub>2</sub>) at 37°C. A suspension of 1 × 10<sup>6</sup> hCMECs for overnight culture was seeded onto gelatin-coated 6 well (#353046, Corning, NY) plates. Experiments were performed on hCMECs at passages 4-6.

**Coculture of human CD4<sup>+</sup> CD25<sup>hi</sup>Tregs and human cardiac microvascular endothelial cells (hCMECs).** Immediately after isolation human CD4<sup>+</sup> CD25<sup>high</sup>Tregs were stimulated by culture (48hr) with plate bound anti-human anti-CD3 mAb (UCHT1) at a 10 µg/mL concentration with soluble anti-human anti-CD28 (28.2) mAb at 2 µg/mL concentration. Culture medium was RPMI 1640, (#32404014, Gibco) supplemented with 10% FBS, L-glutamine (2nM), (# 25030081, Gibco), penicillin (100 U/mL), streptomycin (100 µg/mL). Cells were cultured at 37°C, 95% humidity, and 5% CO<sub>2</sub> in 24-well flat-bottom plates (#3524, Corning, NY). Activated human CD4<sup>+</sup>CD25<sup>high</sup> Tregs (2 × 10<sup>6</sup>) were co-cultured with previously-seeded hCMECs (1 × 10<sup>6</sup>) in RPMI 1640-based culture medium (overnight, 37°C), and the hCMECs were harvested and culture supernatants were collected for analysis.

**Flow cytometry.** After overnight co-culture with human CD4<sup>+</sup> CD25<sup>high</sup>Tregs, hCMECs were washed and resuspended in PBS and 1% FCS and incubated with combinations of the following anti-human mAbs: anti- CD274 (B7-H1/PDL-1) APC (MIH1), anti-COX-2 PE (AS67), anti-HO-1 FITC (HSP32), anti-ERalpha Alexa Flour488 (C542), anti-ERbeta PE (NR342). For analysis of surface markers, cells were incubated with the indicated mAb for 30 min at 4°C. For intracellular staining of mAb, the buffer set was used per the manufacturer's protocol. Flow cytometry was performed on a FACS LSRII (BD Biosciences, San Jose, CA), using *DIVA* (BD Biosciences, San Jose, CA), and FlowJo software (Tree Star, Ashland, OR).

## References:

1. Beppu H, Ichinose F, Kawai N, Jones RC, Yu PB, Zapol WM, Miyazono K, Li E, Bloch KD. BMPR-II heterozygous mice have mild pulmonary hypertension and an impaired pulmonary vascular remodeling response to prolonged hypoxia. *Am J Physiol Lung Cell Mol Physiol.* 2004; 287(6): L1241-7.
2. Kennedy DJ, Vetteth S, Periyasamy SM, Kanj M, Fedorova L, Khouri S, Kahaleh MB, Xie Z, Malhotra D, Kolodkin NI, Lakatta EG, Fedorova OV, Bagrov AY, Shapiro JI.

Central role for the cardiotonic steroid marinobufagenin in the pathogenesis of experimental uremic cardiomyopathy. *Hypertension*. 2006 Mar; 47(3):488-95.

## Online Figure Legends

**Online Figure I. More severe PH in athymic female rats exposed to chronic hypoxia.** A. Chronic hypoxia (10% O<sub>2</sub>) at d21 induced higher RVSP at d21 in athymic female than male rats. (n=7 per group). B. Chronic hypoxia (10% O<sub>2</sub>) induced RVH as measured by RV/LV+S ratio is higher at d21 in athymic female than male rats. (n=7 per group). Data are shown as means with error bars representing SEM (*\*P*<0.05).

**Online Figure II. Evidence of greater perivascular inflammation in athymic female rats with PH.** A. Immunofluorescent images of RV sections from athymic male and female SU5416 treated rats stained with CD68 (green) for macrophages at d21 after SU5416 administration. Nuclei were stained with DAPI (blue). Differential interference contrast (DIC) represents small RV vessel histology. Scale bars: 50µm. B. Morphometric analysis of macrophages (CD68+ cells) in RV sections at d21 in vehicle, after SU5416 administration and after SU5416 administration plus Treg IR in male and female athymic rats (n=4 per group). Data are shown as means with error bars representing SEM. (*\*P*<0.05)

**Online Figure III. Pronounced RV microvascular dropout and fibrosis in females with PH.** A. Immunofluorescent images of RV sections from athymic male and female vehicle and SU5416-treated animals stained with Recla1 (red) for capillaries at d21 after SU5416 administration. B. Morphometric analysis of capillaries (Recla1) density in RV sections at d21 after SU5416 administration (n=4 per group). C. Representative short-axis images of intramuscular arteries of RV of athymic female and male rats with perivascular fibrosis at d21 in vehicle-, SU5416- and SU5416 + Treg IR-treated groups stained with Masson trichrome stain. Blue color indicates collagen fibers. D. Bar graph shows quantified perivascular collagen content/ratio described in methods section. (n=4 per group). E. Representative short-axis images of RV myocardium of athymic female and male rats with interstitial fibrosis at d21 in vehicle-, SU5416- and SU5416 + Treg IR-treated groups stained with Masson trichrome stain. Blue color indicates collagen fibers. F. Bar graph shows quantified interstitial fibrotic areas (%) described in methods section. Data are shown as means with error bars representing SEM (*\*P*<0.05). Scale bars: (A, C, E) 50µm.

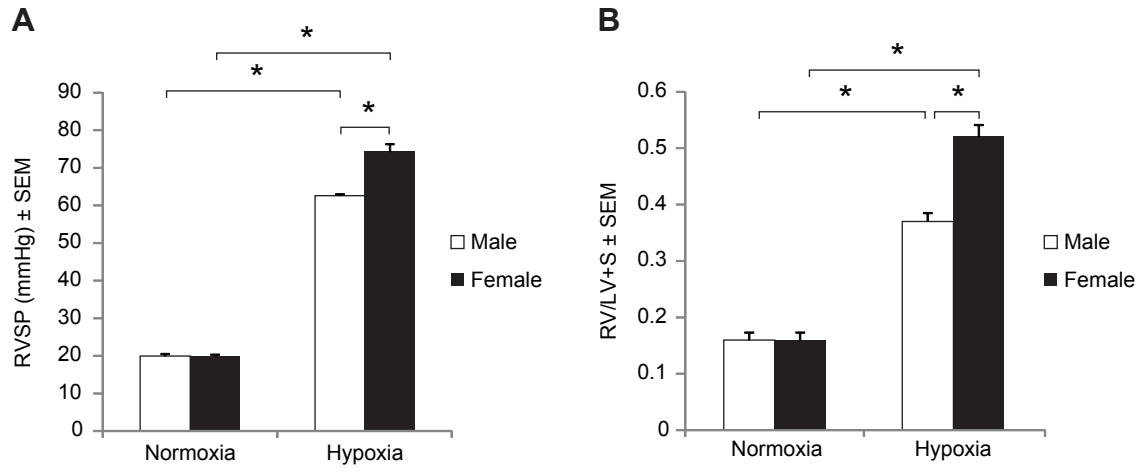
**Online Figure IV. Athymic female rats have higher plasma E<sub>2</sub> levels than male rats in the vehicle and treatment groups.** Circulating plasma E<sub>2</sub> content is measured by ELISA in vehicle, SU5416, SU5416+Treg, chronic hypoxia groups at d21 in athymic female and males rats. (n=4 per group). Data are shown as means with error bars representing SEM. (*\*P*<0.05).

**Online Figure V. IR with Tregs leads to increased COX-2, PGIS, HO-1 and PDL1 expression in lung tissue of both male and female athymic rats.** (A-D) Representative images of lung tissue sections of male and female athymic rats in vehicle and after treatment with SU5416 plus IR with Tregs on d21 and immunolabeled with COX-2 (A), PTGIS (B), HO-1 (C) and PDL1 (D). Scale bars: (A-D) 50µm

**Online Figure VI. Immune reconstitution with Tregs leads to increased COX-2, PGIS, HO-1 and PDL1 expression in RV tissue of both male and female athymic rats.** (A-D) Representative images of RV tissue sections of male and female athymic rats in vehicle and after treatment with SU5416 plus IR with Tregs on d21 and immunolabeled with COX-2 (A), PTGIS (B), HO-1 (C) and PDL1 (D). Scale bars: (A-D) 50µm

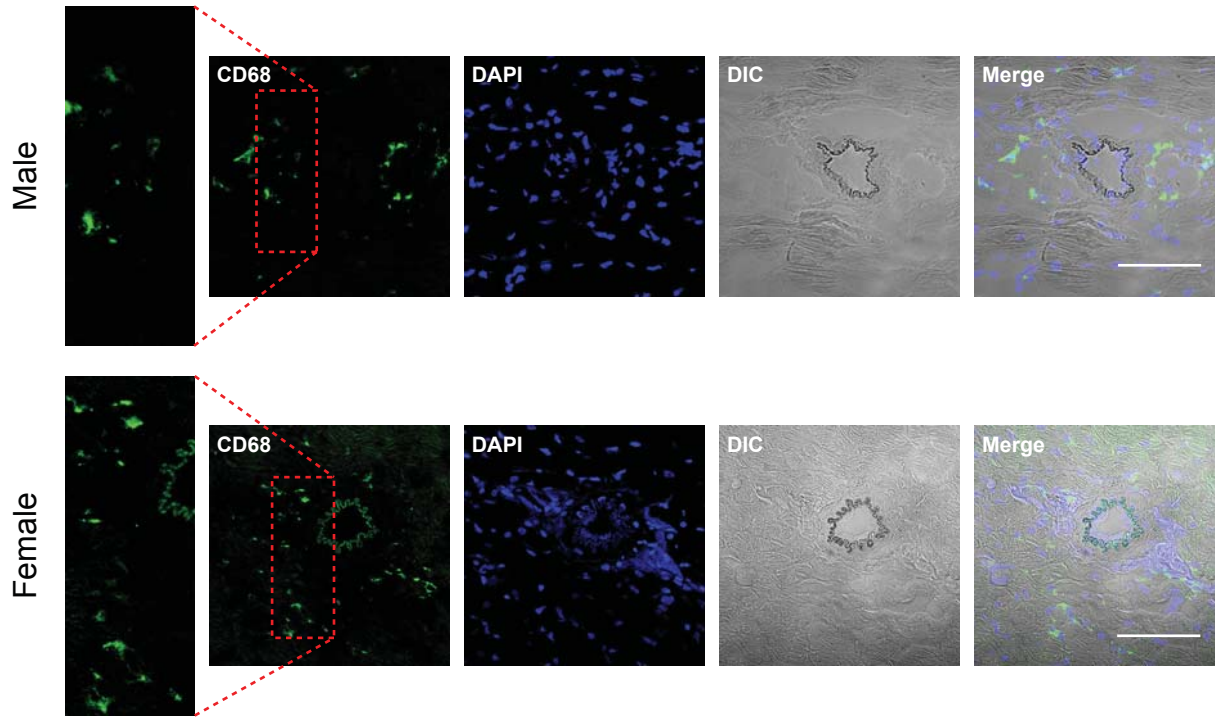
**Online Figure VII. IR with Tregs of athymic rats significantly increase expression of COX-2, PTGIS, HO-1 and PD-L1 mRNA in lungs and RV of athymic female and male rats.** (A-B)

Real-time PCR analysis of athymic female and male lung and RV myocardial tissues in the vehicle, SU5416 and SU5416+Treg groups for expression of COX-2, PTGIS, HO-1 and PD-L1 genes at d21. Results are expressed as a fold change after normalization with GAPDH. (n=3-4 per group). Data are shown as means with error bars representing SEM. qPCR data-multiple t test was done and taken female vehicle as control=1. \* $P<0.05$  comparing to control, # $P<0.05$  comparing female vs male.

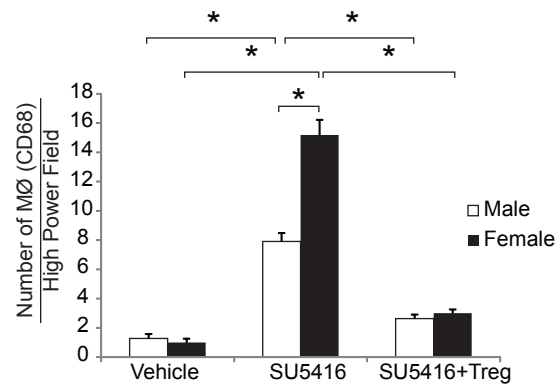


Online Figure I.

**A**

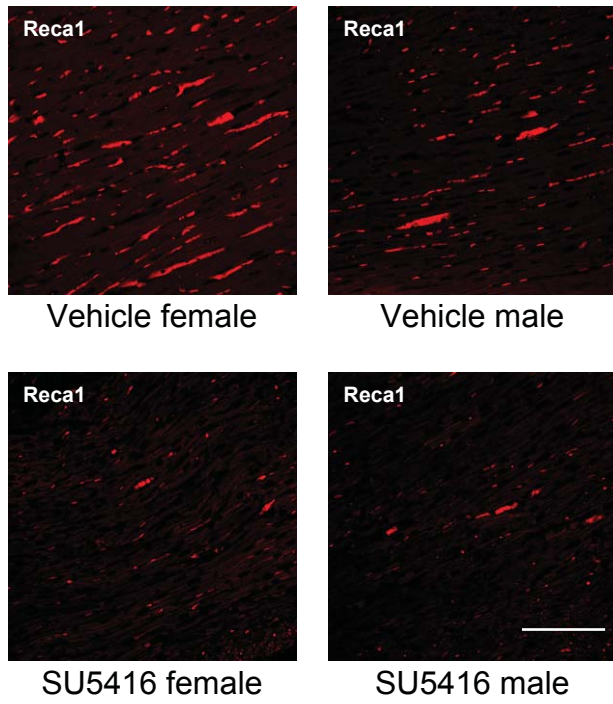
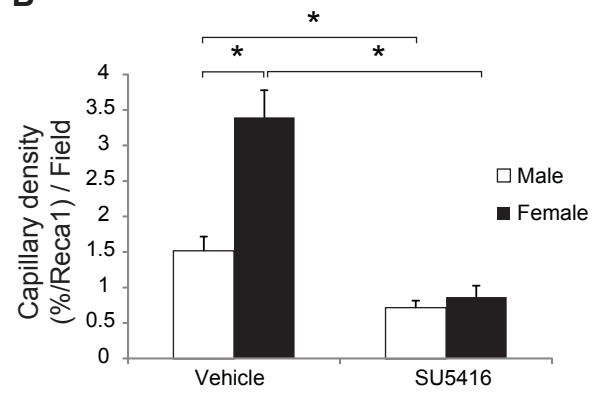
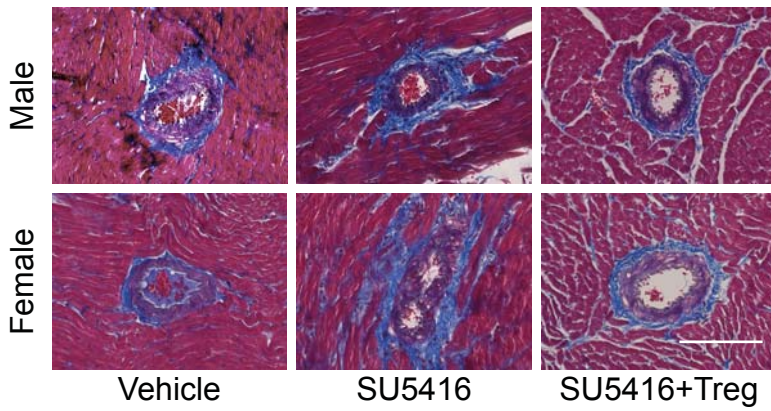
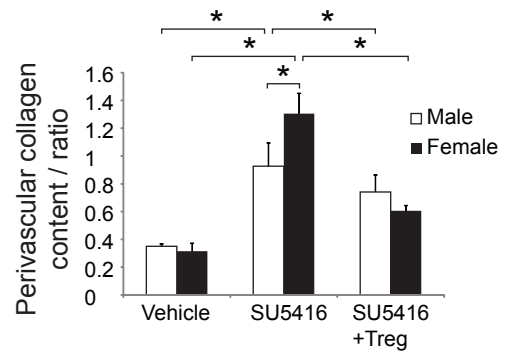
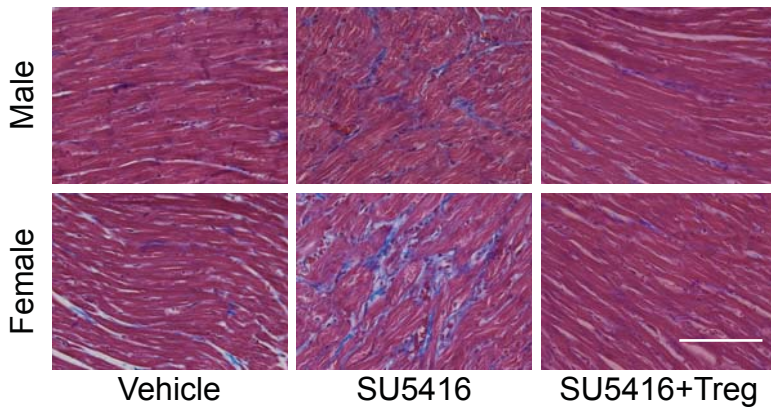
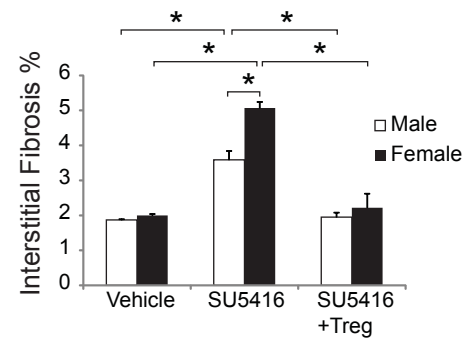


**B**

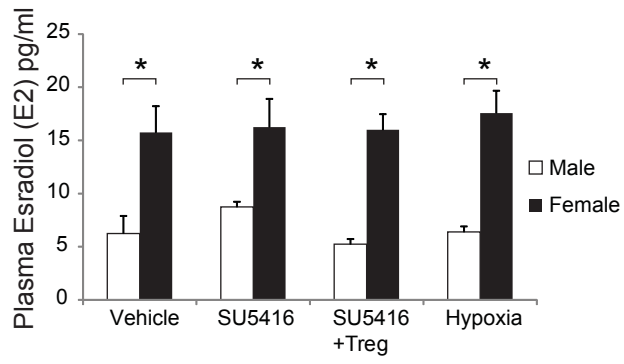


Online Figure II.

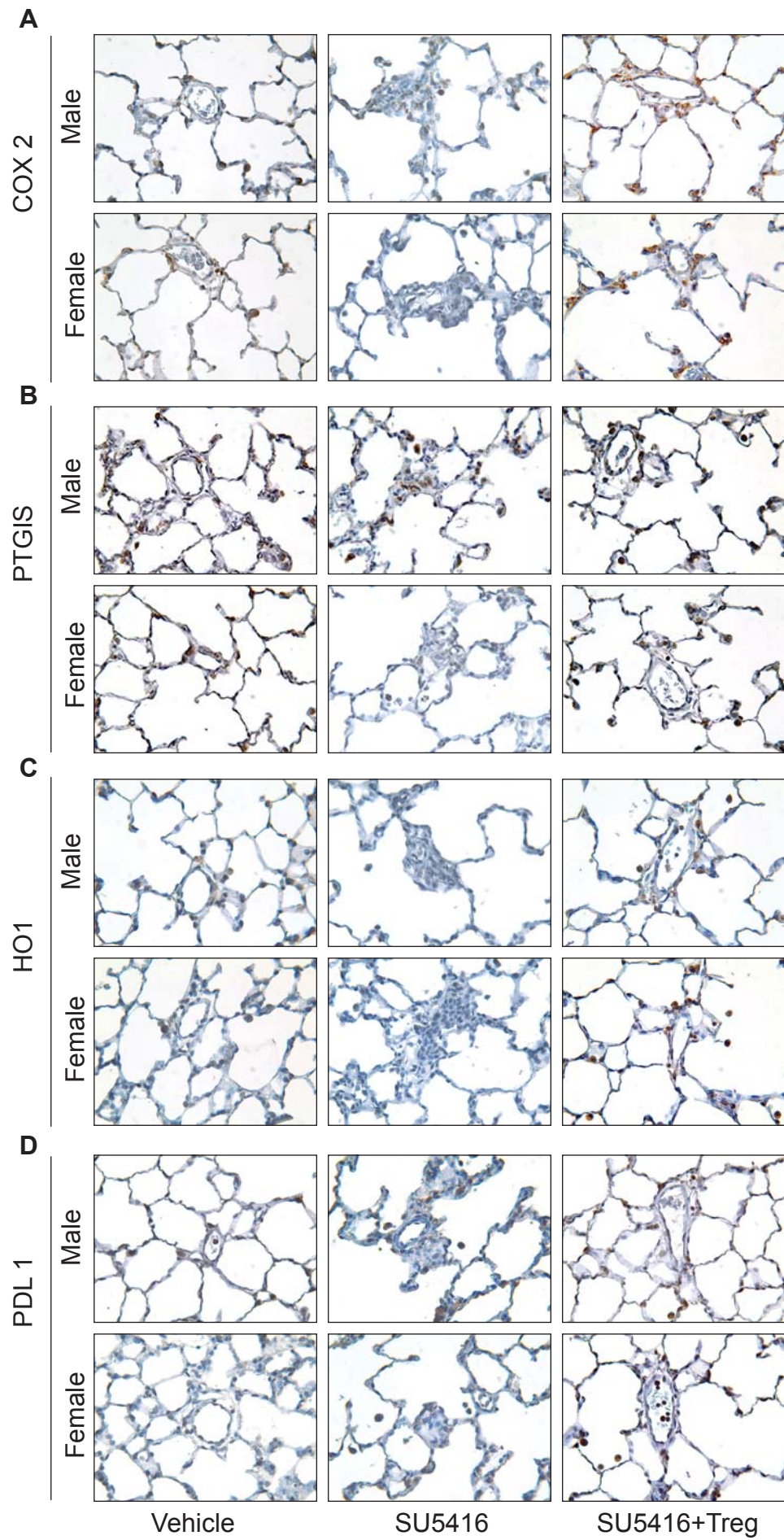


**A****B****C****D****E****F**

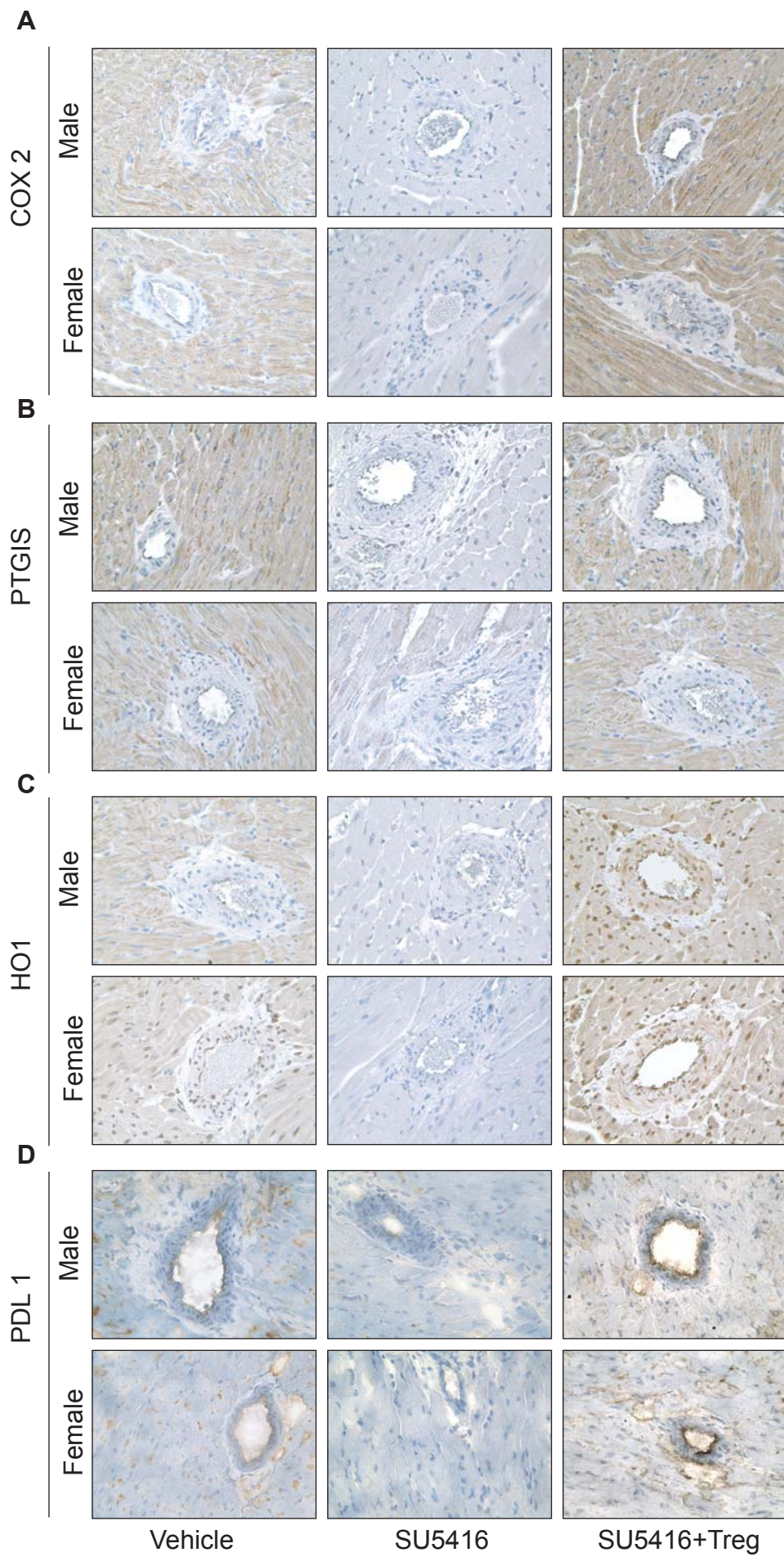
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Online Figure IV.



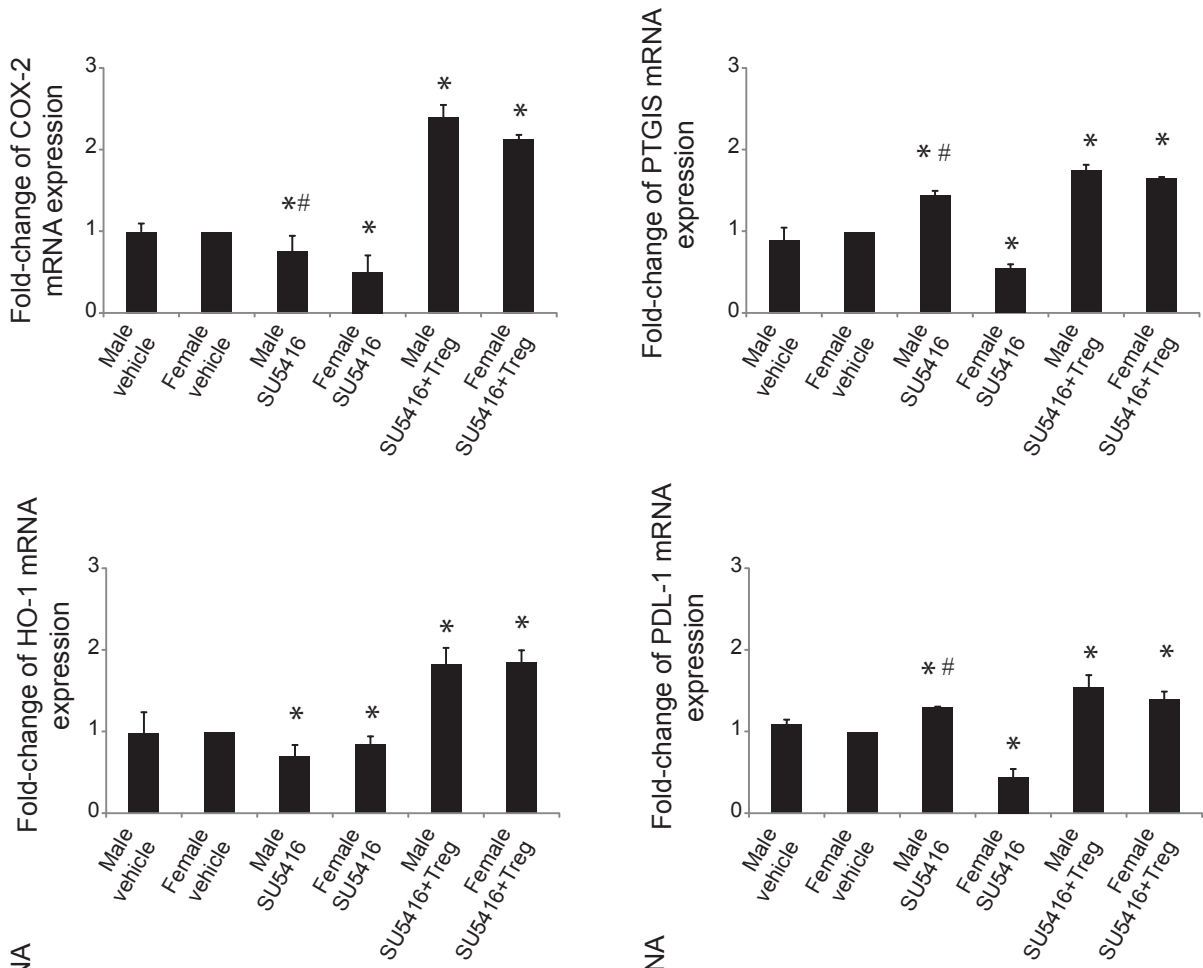
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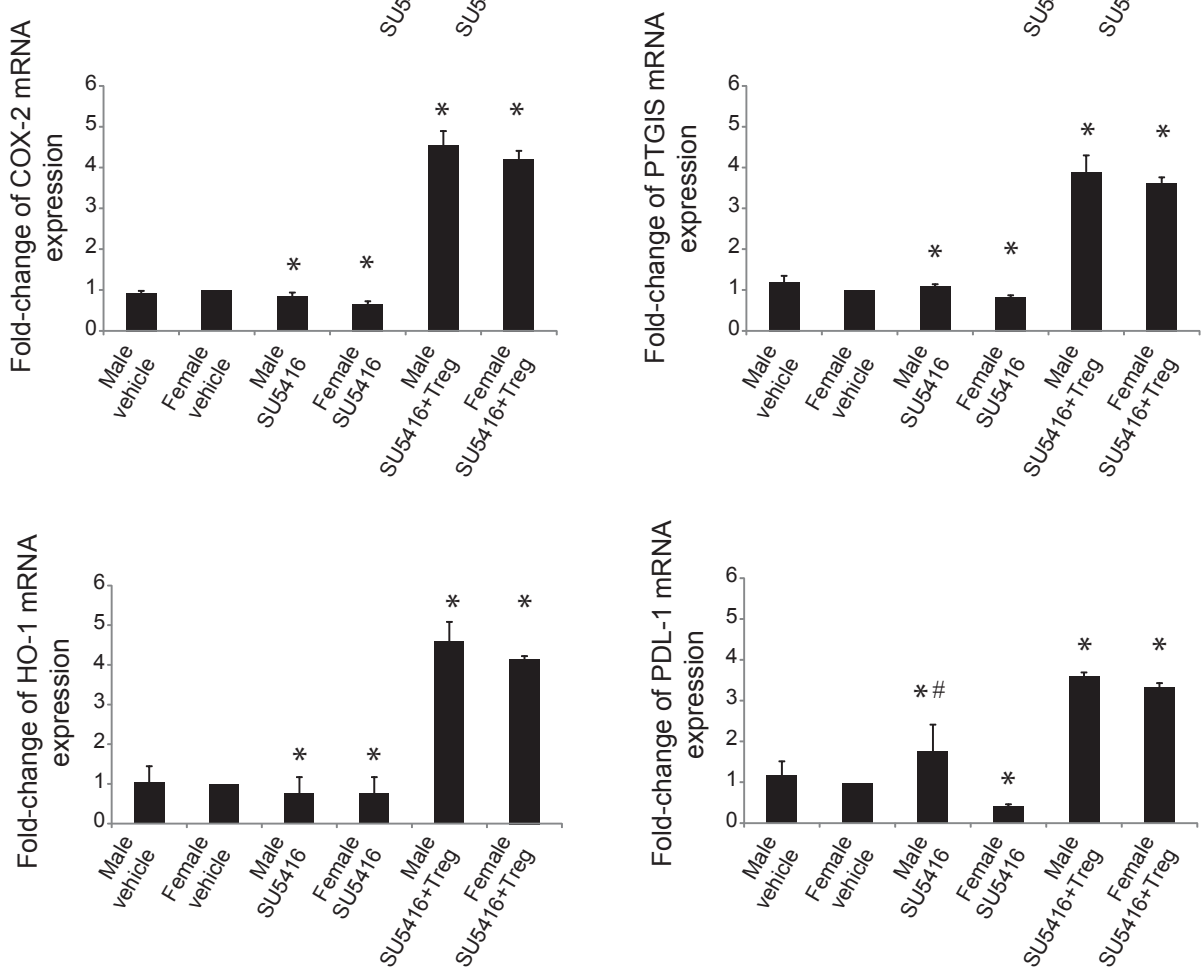
Online Figure VI.

**A**

LUNG

**B**

RV



Online Figure VII.