Dominant Role for Regulatory T Cells in Protecting Females Against Pulmonary Hypertension

Rasa Tamosiuniene, Olga Manouvakhova, Paul Mesange, Toshie Saito, Jin Qian, Mrinmoy Sanyal, Yu-Chun Lin, Linh P. Nguyen, Amir Luria, Allen B. Tu, Joshua M. Sante, Marlene Rabinovitch, Desmond J. Fitzgerald, Brian B. Graham, Aida Habtezion, Norbert F. Voelkel, Laure Aurelian, Mark R. Nicolls

Rationale: **Pulmonary arterial hypertension (PH) is a life-threatening condition associated with immune dysregulation and abnormal regulatory T cell (Treg) activity, but it is currently unknown whether and how abnormal Treg function differentially affects males and females.**

Objective: **To evaluate whether and how Treg deficiency differentially affects male and female rats in experimental PH.**

Methods and Results: **Male and female athymic *rnu/rnu* rats, lacking Tregs, were treated with the VEGFR2 (vascular endothelial growth factor receptor 2) inhibitor SU5416 or chronic hypoxia and evaluated for PH; some animals underwent Treg immune reconstitution before SU5416 administration. Plasma PGI_2 (prostacyclin) levels were measured. Lung and right ventricles were assessed for the expression of the vasoprotective proteins COX-2 (cyclooxygenase 2), PTGIS (prostacyclin synthase), PDL-1 (programmed death ligand 1), and HO-1 (heme oxygenase 1). Inhibitors of these pathways were administered to athymic rats undergoing Treg immune reconstitution. Finally, human cardiac microvascular endothelial cells cocultured with Tregs were evaluated for COX-2, PDL-1, HO-1, and ER (estrogen receptor) expression, and culture supernatants were assayed for PGI_2 and IL (interleukin)-10. SU5416-treatment and chronic hypoxia produced more severe PH in female than male athymic rats. Females were distinguished by greater pulmonary inflammation, augmented right ventricular fibrosis, lower plasma PGI_2 levels, decreased lung COX-2, PTGIS, HO-1, and PDL-1 expression and reduced right ventricular PDL-1 levels. In both sexes, Treg immune reconstitution protected against PH development and raised levels of plasma PGI_2 and cardiopulmonary COX-2, PTGIS, PDL-1, and HO-1. Inhibiting COX-2, HO-1, and PD-1 (programmed death 1)/PDL-1 pathways abrogated Treg protection. In vitro, human Tregs directly upregulated endothelial COX-2, PDL-1, HO-1, ERs and increased supernatant levels of PGI_2 and IL-10.**

Conclusions: **In 2 animal models of PH based on Treg deficiency, females developed more severe PH than males. The data suggest that females are especially reliant on the normal Treg function to counteract the effects of pulmonary vascular injury leading to PH. (*Circ Res*. 2018;122:1689-1702. DOI: 10.1161/CIRCRESAHA.117.312058.)**

Key Words: hypertension ■ prostacyclin ■ pulmonary sex ■ regulatory T cells

Pulmonary arterial hypertension (PH) is a lethal cardiopulmonary disorder characterized by a narrowing of the terminal pulmonary arterioles that causes right ventricular (RV) failure and death.[1] As with an increasing number of cardiovascular diseases,[2] abnormalities of regulatory T cell (Treg) number and function have also been reported in PH in association with clinical evidence of immune dysregulation.[3–8] Preclinical evidence shows that anti-inflammatory Tregs, generally defined as $CD4^+CD25^{high}$ FoxP3 (forkhead box P3)$^+$ cells, play a critical role in the resolution of the inflammatory process caused by putative PH disease triggers, such as viral infection or increased shear stress (reviewed in Tamosiuniene et al[9]). We and others have previously shown that T cell deficient *rnu/rnu* athymic male rats, which lack Tregs, are particularly susceptible to

Editorial, see p 1635
In This Issue, see p 1625
Meet the First Author, see p 1626

Original received September 12, 2017; revision received March 9, 2018; accepted March 13, 2018. In February 2018, the average time from submission to first decision for all original research papers submitted to *Circulation Research* was 12 days.

From the Stanford University School of Medicine, Department of Medicine, CA (R.T., P.M., T.S., J.Q., M.S., L.P.N., A.L., M.R., A.H., L.A., M.R.N.); VA Palo Alto Health Care System, CA (O.M., Y.-C.L., A.L., A.B.T., J.M.S., M.R.N.); University of Limerick, President, Ireland (D.J.F.); University of Colorado Denver, School of Medicine, Department of Medicine, Aurora (B.B.G.); Virginia Commonwealth University School of Medicine, Department of Internal Medicine, Richmond (N.F.V.); and University of Maryland School of Medicine, Baltimore (L.A.).

The online-only Data Supplement is available with this article at http://circres.ahajournals.org/lookup/suppl/doi:10.1161/CIRCRESAHA.117.312058/-/DC1.

Correspondence to Mark R. Nicolls, MD, The Stanford Professor of Pulmonary and Critical Care Medicine, Stanford University School of Medicine, 300 Pasteur Dr, Grant S140, Stanford, CA 94305. E-mail mnicolls@stanford.edu

Novelty and Significance

What Is Known?

- Pulmonary hypertension (PH) is a potentially fatal condition associated with abnormal regulatory T cells (Tregs) and dysregulated immunity.
- Abnormal Treg activity may predispose patients to PH.
- Replacing Tregs in athymic rats, which are Treg-deficient, prevents PH.

What New Information Does This Article Contribute?

- Female athymic rats that lack Tregs develop more severe PH than males.
- PH in Treg-deficient females is associated with greater inflammation and right ventricular fibrosis, lower plasma PGI_2 (prostacyclin), decreased lung COX-2 (cyclooxygenase 2), PTGIS (PGI_2 synthase), HO-1 (heme oxygenase 1), and PDL-1 (programmed death ligand 1) expression and reduced right ventricular PDL-1 levels.
- Tregs prevent experimental PH through several signaling pathways including COX-2, HO-1, and PDL-1.
- Tregs upregulate endothelial ERs (estrogen receptors) and PGI_2 production.

Dysregulated immunity contributes to the development of PH, but the effect of sex on vascular inflammation is unknown. Given the implications for clinical disease, we sought greater insight into how protective immunity works to maintain pulmonary vascular health. Treg immune reconstitution restores protective immunity in athymic rats and prevents PH, thus providing a platform to better understand these processes. In this study, male and female athymic rats were treated with the VEGFR2 (vascular endothelial growth factor receptor 2) inhibitor, SU5416, or chronic hypoxia to induce PH. Females developed more severe PH than males. Blood levels of the pulmonary vasodilator, PGI_2, were lower in females than males, possibly because of lower levels of the enzymes responsible for PGI_2 biosynthesis (COX-2, PTGIS). Females exhibited diminished pulmonary HO-1 and PDL-1 expression, but Treg immune reconstitution significantly upregulated these proteins, as well as COX-2, PTGIS, and systemic PGI_2. Inhibiting COX-2, HO-1, and PD-1 (programmed death 1)/PDL-1 pathways eliminated Treg protection from disease. Tregs directly upregulated endothelial COX-2, PDL-1, HO-1, ERs, and increased PGI_2 and IL-10 production. These experiments suggest that females are notably reliant on Tregs counteracting vascular injuries which cause PH. As cellular therapies are considered for PH, autologous Treg administration is a promising approach.

Nonstandard Abbreviations and Acronyms

CALP	calponin
COX-2	cyclooxygenase 2
ER	estrogen receptor
FoxP3	forkhead box P3
HMCEC	human microvascular cardiac endothelial cell
HO-1	heme oxygenase 1
IL	interleukin
IR	immune reconstitution
PD-1	programmed death 1
PDL-1	programmed death ligand 1
PGI_2	prostacyclin
PH	pulmonary arterial hypertension
PTGIS	prostacyclin synthase
RV	right ventricular (or right ventricle)
Treg	regulatory T cell
VEGFR2	vascular endothelial growth factor receptor 2

PH.[10–15] In these studies, vascular injury caused by treatment of male athymic rats with monocrotaline or the VEGFR2 (vascular endothelial growth factor receptor 2) antagonist, SU5416, resulted in exuberant perivascular inflammation and severe PH. Pathological changes in the lungs of T cell-deficient animals modeled the microangiopathy observed in the human condition, and this pulmonary vascular disease led to elevated right heart pressure, RV hypertrophy (RVH) and ultimately, death from PH. We showed that restoration of normal Treg activity through immune reconstitution (IR) of athymic rats with Tregs isolated from inbred, MHC-matched, littermate euthymic controls prevented PH in males through the induction of an active anti-inflammatory response.[14] However, whether Treg deficiency differentially impacts males and females is currently unknown.

Here, we report that female athymic rats treated with SU5416 or exposed to chronic hypoxia develop more significant PH than their male counterparts. Specifically, females lacking normal Treg activity exhibited greater inflammation, more dramatic RV microvascular dropout and increased periarteriolar fibrosis compared with males with PH. This phenotype was associated with decreased systemic levels of the pulmonary vasodilator, PGI_2 (prostacyclin). We subsequently investigated enzymes responsible for PGI_2 synthesis and discovered decreased lung COX-2 (cyclooxygenase 2) and PTGIS (prostacyclin synthase) expression. Two other proteins associated with vasoprotection and normal Treg function, HO-1 (heme oxygenase 1) and PDL-1 (programmed death ligand 1), were also reduced in female lung tissue. Extending this analysis to the heart showed that RV PDL-1 expression was also relatively diminished in Treg-deficient females with PH. Treg IR prevented PH development and was associated with significantly increased levels of plasma PGI_2, PTGIS, COX-2, HO-1, and PDL-1 in both sexes. Blocking COX-2, HO-1, and PD-1 (programmed death 1)/PDL-1 signaling abrogated Treg protection in both sexes and, in so doing, demonstrated that these pathways are important for Treg action after a pulmonary vascular insult. To evaluate how Tregs normally mediate protection of vascular endothelium, human Tregs were cocultured with cardiac endothelial cells and were found to specifically upregulate endothelial COX-2, HO-1, PDL-1, and cardioprotective ERs (estrogen receptors) as well as induce the biosynthesis of PGI_2 and the anti-inflammatory cytokine, IL (interleukin)-10. These data collectively confirm a special and dominant protective role for Tregs in females at risk for PH.

Methods

The data and methods that support the findings of this study are available from the corresponding author on reasonable request.

Animals

Inbred male and female WAG (RT1u) athymic nude rats (rnu/rnu) and euthymic (rnu/+) rats were obtained from Biomedical Research Models, Inc, (Worcester, MA) and bred in-house. Euthymic rats served as MHC-identical controls and as cell donors for IR experiments. To induce PH, 6- to 8-week-old male and female athymic animals were subcutaneously (SC) injected with a single dose of SU5416 (20 mg/kg) dissolved in dimethyl sulfoxide (DMSO; number D2650, Sigma Aldrich, St. Louis, MO) or DMSO alone as vehicle controls. Alternatively, athymic rats were exposed to hypoxia (10% O_2) in an airtight plexiglass hypoxia chamber with simulated oxygen controller (Biospherix; ProOx110, Parish, NY). Athymic rats maintained in normoxic conditions (21% O_2) served as controls for this experimental group. All animals were euthanized at 3-week post-treatment. The procedures and experimental protocols were approved by the Veterans Affairs Palo Alto Animal Care and Use Committee.

Antibodies and Reagents

Antibodies to CD3 (UCHT1) and CD28 (28.2; number 555725 and number 555329, respectively) were purchased from BD Pharmingen (San Jose, CA). Additional antibodies from BD Pharmingen were anti-CD25-PE antibody (OX39; number 554866) and anti-COX-2 PE (AS67; number 565125). β-actin antibody (number A5441) and in situ cell death detection kit (Roche number 11684795910) was from Sigma Aldrich (St. Louis, MO). The following antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): anti-PDL-1 (number sc-50298) and Donkey anti-goat horseradish peroxidase (HRP; number sc-2056) or from Abcam (Cambridge, MA): anti-PGIS (number ab23668), anti-Reca-1 (number ab9774), anti-CALP (calponin; number ab700), anti-α–smooth muscle actin (SMA; 1A4; number ab7817), and anti-HO-1 (number ab13243). Other antibodies were anti-PDL-1 (number 4059, ProSci, Poway, CA), anti-CD68 (ED-1; number MCA341R, Bio-Rad/ AbD Serotec, Hercules, CA), anti-CD274 (B7-H1/PDL-1) APC (MIH1; number 17-5983-42, eBioscience, San Diego, CA), anti-HO-1 FITC (HSP32; number NBP1-77460F, NovusBiologicals, Littleton, CO); anti-ER-alpha Alexa Flour488 (C542; number NBP1-19318AF488, NovusBiologicals); anti-Ki67 (number NB500-170, NovusBiologicals); anti-ER-beta PE (NR342; number IC7106P, R&D Systems, Minneapolis, MN). The following antibodies were from Cell Signaling Technology, (Beverly, MA): anti-COX-2 (D5H5), goat anti-mouse HRP (number 7076S) and donkey anti-rabbit HRP (number 7074S). Anti-PD-1 (RMP1-14), (number BE0146) and isotype control IgG (2A3), (number BE0089) were obtained from BioXCell, West Lebanon, NH. Alexa Fluor 488 donkey anti-mouse IgG (number 715-545-151), Alexa Flour 647 donkey anti-mouse IgG (number 715-605-150) and Cy3 donkey anti-rabbit IgG (number 711-165-152) were obtained from Jackson ImmunoResearch (West Grove, PA). VEGFR2 inhibitor SU5416 was synthesized utilizing a previously described modified protocol.[14] The HO-1 inhibitor, zinc (II) Protoporphyrin IX (number 691550) and selective COX-2 inhibitor NS-398, (number 70590) was purchased from Cayman Chemical (Ann Arbor, MI).

Tregs ($CD4^+CD25^{high}$) Isolation and IR

Rat Tregs were isolated from the inbred euthymic animals. Dissociated spleens were filtered through a 100 µm cell strainer (number 352360, Corning, NY) to obtain a single cell suspension, and the cells were washed with Dulbecco phosphate-buffered saline (DPBS, number 14190-144, Gibco, Grand Island, NY) supplemented with 2% fetal bovine serum (number 10438026, Gibco) by centrifugation. The $CD4^+CD25^{high}$Treg subset was isolated by a 2-step method using fully automated RoboSep (STEMCELL Technologies, Vancouver, Canada). Negative selection was performed using EasySep Rat $CD4^+$ T Cell Isolation Kit (number 19642, STEMCELL Technologies) followed by labeling $CD4^+$ T cells with anti-CD25-PE antibody; the $CD25^{high}$ cells subset was selected with the EasySep PE Selection Kit (number 18557, STEMCELL Technologies). This cell fraction was further purified by fluorescent-activated cell sorting (FACSAria II; BD Biosciences, San Jose, CA); the resulting $CD4^+CD25^{high}$ population was >98% pure. Purified rat Tregs ($CD4^+CD25^{high}$) cells (4–5×10^6 cells) were injected intravenously into AT male and female rats 7 days before SU5416 injection, and IR was confirmed 7-day post-SU5416 treatment by flow cytometry.

Western Blot Analysis

Western blot was performed as described previously.[14] Briefly, frozen rat lung and heart tissue (−80°C) were homogenized in Pierce protein extraction RIPA buffer (number 89901, Thermo Scientific, Rockford, IL) containing Halt protease inhibitor cocktail (number 78429, Thermo Scientific) at 4°C. The BCA protein assay kit (number 23225, Thermo Scientific) was used to measure protein concentration. Protein samples (30 µg) were loaded onto Bolt 4% to 12% Bis-Tris Plus precast gels (number NW04125BOX, Invitrogen, Carlsbad, CA) followed by electrophoresis and immunoblot analysis. Primary antibodies were anti-HO-1 (1:500), anti-PDL-1 (1:200), anti-COX-2 (1:500), anti-PGIS (1:200), and anti-β–actin (1:2000) antibodies. Immunoblots were imaged with molecular imager Chemidoc XRS+ (Bio-Rad, Hercules, CA) and data are expressed as densitometric units determined using ImageJ (version 1.48) software.

Immunofluorescence Imaging

Rat lungs were insufflated with a 1:1 mixture of optimal cutting temperature compound and 30% sucrose and embedded in Tissue Tek optimal cutting temperature compound (number 4583, Sakura, Torrance, CA) in subzero temperature. Rat heart tissue was frozen with optimal cutting temperature in dry ice. Cryosections (7 µm) of lung/heart tissue were placed on superfrost/plus slides (number 48311–703, VWR, Radnor, PA). For immunofluorescent staining, the slides were fixed in methanol/acetone (1:1), washed with PBS, and incubated in 0.2% Triton X-100 (number T9284, Sigma Aldrich). The sections were blocked with normal 10% donkey serum (Jackson ImmunoResearch, West Grove, PA), and then exposed for 1 hour or overnight to primary antibodies to CD68 (ED-1; 1:50), Reca1 (1:50), CALP (1:50), HO-1 (1:100), COX-2 (D5H5; 1:200), and anti-PDL-1 (1:200), Ki67(1:50), anti-α SMA (1:100) followed by Alexa Fluor 488 conjugated donkey anti-mouse IgG, Alexa Flour 647 conjugated donkey anti-mouse IgG, Cy3 conjugated donkey anti-rabbit IgG conjugated secondary antibodies (1:600) and Apoptosis detection kit. The sections were mounted with mounting medium and DAPI (number H-1200, Vector Laboratories, Burlingame, CA). Quantification of Ki67 and TUNEL positive vascular smooth muscle cell was performed in a blinded fashion in randomly chosen lung tissue sections in each experimental group (n=4 per group). Ten vessels per animal were counted and in total for each marker 240 small pulmonary vessels were analyzed using Image J (version 1.48) software. Microscopic analysis was performed with the LSM 710META confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany).

ELISA

ELISA kits were used to measure the levels of estradiol (number ES180S-100, Calbiotech, Spring Valley, CA), 6-keto-prostaglandin F1α (number 515211, Cayman Chemical, Ann Arbor, MI), and IL-10 (number 100764, Abcam, Cambridge, MA).

Statistics

GraphPad Prism_6.0 was used for statistical analysis. Differences between multiple groups were compared using 1-way ANOVA with Bonferroni multiple comparisons test and between 2 groups, a Student *t* test. For Western blot analyses, for Ki67 and TUNEL positive vascular smooth muscle cell quantification analyses, 2 analyses were performed (1) to compare male to female values with normal distribution, an unpaired *t* test with Welch correction was used and for nonparametric data, the Mann–Whitney test and (2) to compare same-sex vehicle and SU5416+Treg groups to the same-sex SU5416 group, the ANOVA with Bonferroni multiple comparisons test was used. A P value of <0.05 was considered significant. Data are expressed as mean±SEM.

Results

Female Athymic Rats Have Increased Disease Severity, Attenuated by Treg IR

We previously showed that athymic (T-cell deficient) male rats develop significant PH after treatment with the

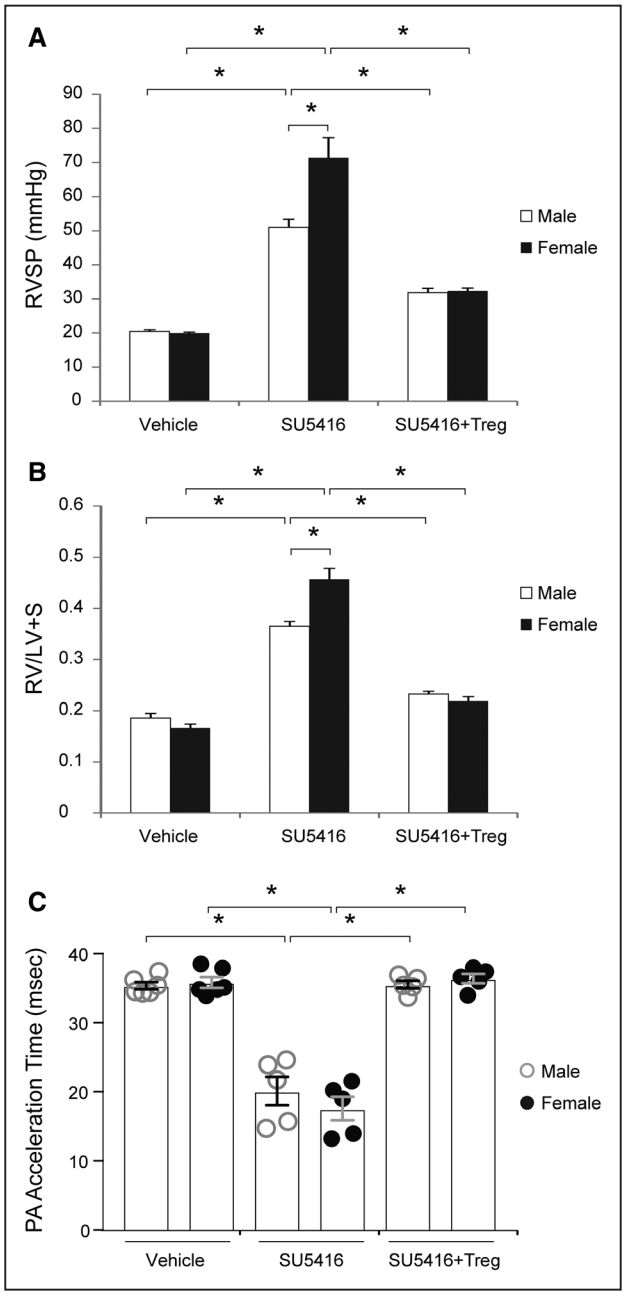


Figure 1. Severe pulmonary hypertension (PH) in athymic female rats. A, Right ventricular systolic pressure (RVSP) measurements assessed on day 21 posttreatment of athymic male and female rats with vehicle control, SC VEGFR2 (vascular endothelial growth factor receptor 2) inhibitor SU5416 (20 mg/kg) single dose, and SC SU5416 (20 mg/kg) with regulatory T cells (Tregs). **B**, RV hypertrophy (RVH) measurements determined by RV/(LV+S) ratio assessed on day 21 posttreatment of athymic male and female rats with vehicle control, SC VEGFR2 inhibitor SU5416 (20 mg/kg) single dose, and SC SU5416 (20 mg/kg) with Tregs. **C**, Pulmonary artery acceleration time (PAAT) by sequential echocardiography observed on day 21 in all athymic male and female animal groups before preterminal right heart catheterization. (n=5–7 per group). Data are shown as means with error bars representing SEM (*P<0.05).

VEGFR2 antagonist, SU5416 because of the absence of anti-inflammatory $CD4^+CD25^{high}$ $FoxP3^+$ Tregs.[11,13,14] To determine whether female athymic rats are similarly susceptible to PH, rats of both sexes were treated with SU5416 (20 mg/kg) or DMSO (vehicle) and evaluated 21-day posttreatment. PH severity was greater in female rats as determined by higher RV systolic pressures (Figure 1A) and a greater degree of RV hypertrophy (Figure 1B) and reduced pulmonary artery acceleration times (Figure 1C). Increased disease severity was also observed in female athymic rats housed in hypoxic conditions for 3 weeks (10% inspired O_2; Pao_2≈40 mm Hg; Online Figure I). SU5416-treated PH lungs exhibited a greater $CD68^+$ pulmonary macrophage infiltration in females (Online Figure II) and a larger decline in RV capillary density associated with greater RV perivascular and interstitial fibrosis (Online Figure III). Consistent with our previous findings in male rats,[14] IR with $CD4^+CD25^{high}$Tregs (3×10^6 IV 7 days before SU5416 treatment) prevented PH[14] and was equally protective in male and female animals with near-normal RV systolic pressures and RV/LV+S (right ventricle/left ventricle+septum) values in both sexes (Figure 1). Not surprisingly, the immunologically-pleiotropic and vasoprotective hormone, estradiol[16] was higher in female serum for all experimental groups evaluated (Online Figure IV); a result which suggested that any role for estradiol, if present, in PH was necessarily complex given that its expression is higher in both females with PH and in females protected from PH.

Increased Disease Severity in Female Rats Is Associated With Decreased PGI_2 Synthesis

To better understand the factors involved in the increased disease severity of athymic females and begin to elucidate the function of Tregs in PH prevention, we asked whether vasoprotective pathways are altered in a sex-specific fashion in athymic rats and whether there was a differential effect of Treg IR in males versus females. We first focused on PGI_2, a potent vasodilator that exerts multiple vasoprotective effects, and the enzymes most responsible for its synthesis.[17] PGI_2 has a half-life of 42 seconds and is broken down into 6-keto-prostaglandin F1α,[18] a metabolite that is used to estimate systemic levels. PGI_2 levels were assessed 21 days after treatment with vehicle or SU5416 with or without Treg IR. Plasma PGI_2 levels were decreased in females with PH suggesting that reduced PGI_2 synthesis may contribute to worse PH in Treg-deficient females (Figure 2A). A ≈4-fold increase in the PGI_2 values above the control baseline was observed in both sexes undergoing Treg IR with serum levels of ≈1300 pg/mL in males and females, showing here the special reliance females have for normal Treg function. To further evaluate factors responsible for PGI_2 biosynthesis, we evaluated COX-2 and PTGIS. Consistent with lower PGI_2 blood levels in females, Western blot analysis of male and female PH lungs analyzed at day 21 post-SU5416 treatment demonstrated relatively reduced COX-2 and PTGIS levels in female compared with male PH lungs (Figure 2B through 2D). Administration of the COX-2 inhibitor, NS-398, abrogated the IR-associated increase in PGI_2 levels. Thus, lower pulmonary COX-2 and possibly lower pulmonary PTGIS, in athymic females provides one explanation of why females have lower circulating PGI_2. Collectively, these results show that more severe PH in females, occurring in the absence of

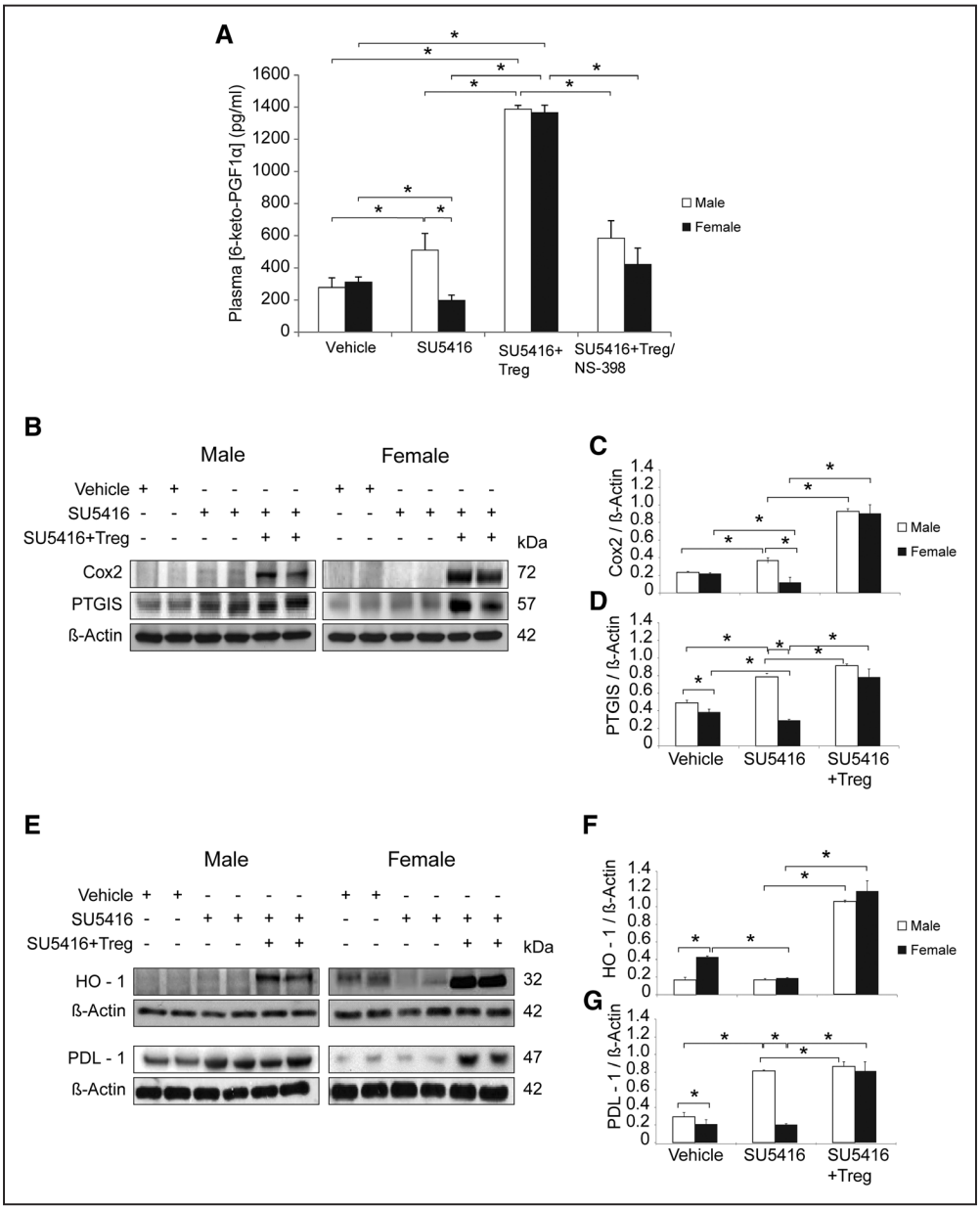


Figure 2. Athymic female rats with pulmonary hypertension (PH) express decreased systemic PGI2 (prostacyclin) and reduced pulmonary COX-2 (cyclooxygenase 2), PTGIS (prostacyclin synthase), HO-1 (heme oxygenase 1), and PDL-1 (programmed death ligand 1). A, Prostacyclin release in vivo was measured by 6-keto-prostaglandin F1α (6-ketoPGF1α) levels in plasma of athymic male and female rats treated with vehicle control, SU5416, or SU5416+ regulatory T cells (Tregs) and analyzed on day 21 (n=4 per group). **B–D**, Representative Western immunoblots and densitometric quantification of COX-2, PTGIS, relative to β-actin in lung lysates from vehicle-, SU5416-, and SU5416+Treg-treated athymic male and female rats assessed on day 21 (n=5 per group). **E–G**, Representative Western immunoblots and densitometric quantification of HO-1, PDL-1, relative to β-actin in lung lysates from athymic male and female rats treated with vehicle control, SU5416, SU5416+Treg, and assessed on day 21 (n=5 per group). Data are shown as means with error bars representing SEM (*P<0.05).

normal Treg activity, correlates with relatively reduced pulmonary COX-2 and PTGIS expression and a concomitant decrease in serum PGI_2.

PDL-1 and HO-1 Is Decreased in Treg-Deficient Females With PH

The PD-1/PDL-1 negative costimulatory pathway is critical for Treg immunomodulatory function.[19] In immunoblotting assays, PDL-1 expression was reduced in female lungs compared with males (Figure 2E and 2G). HO-1, another enzyme which helps mediate Treg function[20] was strongly expressed in control female lungs but was notably decreased in PH; HO-1 was not significantly expressed in male lungs with or without PH (Figure 2E and 2F). The data suggested that reduced PDL-1 and HO-1 expression could contribute to increased disease severity in Treg-deficient females. Treg IR significantly increased the expression of both PDL-1 and HO-1 in both males and females, underscoring the apparent contribution of these pathways to Treg activity after vascular injury.

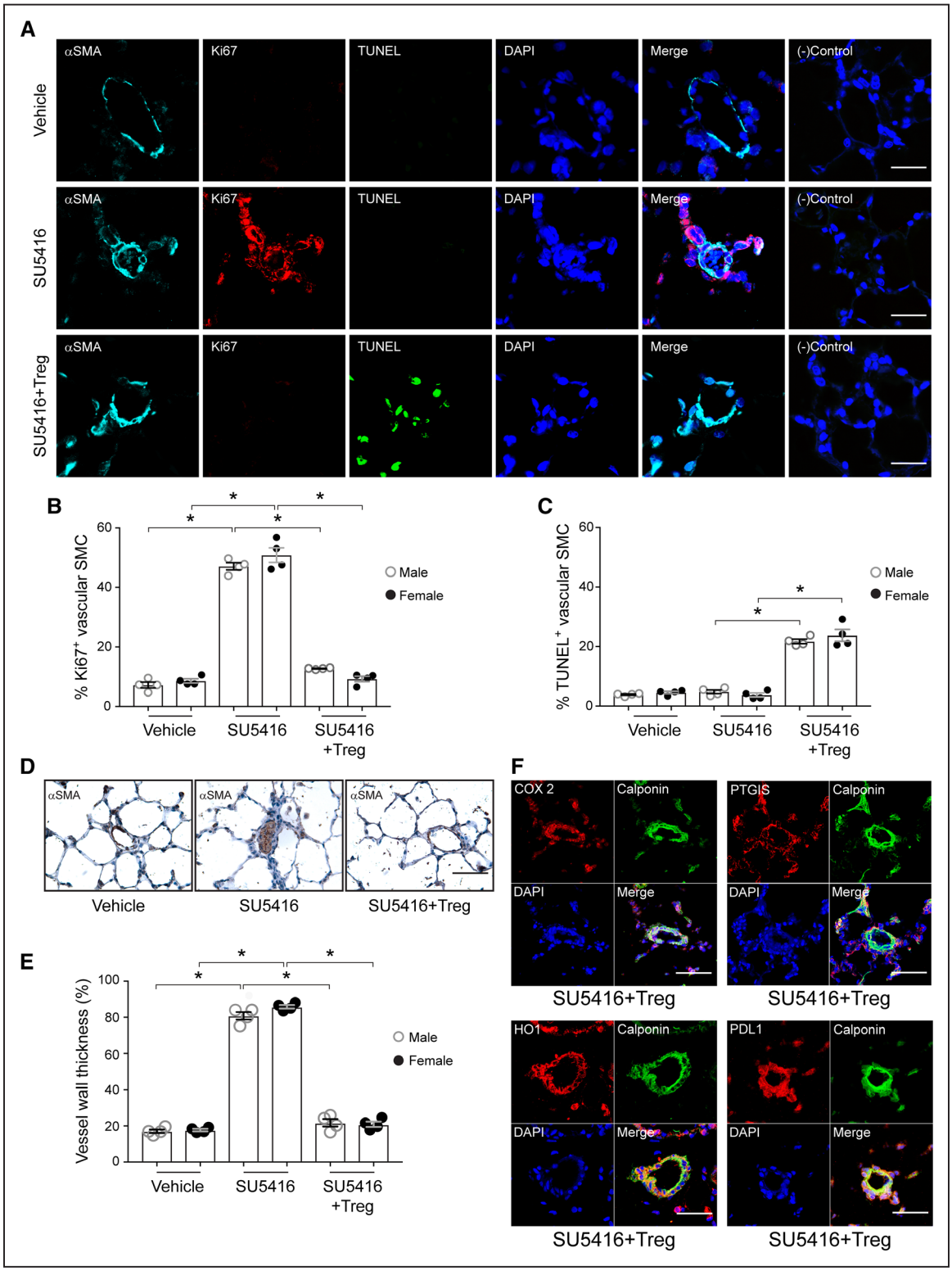


Figure 3. Immune reconstitution (IR) with regulatory T cells (Tregs) before vascular injury decreases pulmonary arteriolar remodeling with reduced vascular smooth muscle cell (SMC) proliferation and enhances apoptotic activity with increased COX-2 (cyclooxygenase 2), PTGIS (prostacyclin synthase), HO-1 (heme oxygenase 1), and PDL-1 (programmed death ligand 1) expression. **A**, Immunofluorescent images of lung tissue sections from female athymic rats treated with vehicle control, SU5416 and SU5416+IR with Tregs on day 21 and immunolabeled with α-smooth muscle actin (SMA; cyan), Ki67 (red), TUNEL (green). Nuclei stained with DAPI (blue). Negative control represents samples exposed only to conjugated secondary antibodies (n=4 per group). **B**, Percentage of Ki67 positive cells in vascular SMC in all groups of athymic male and female rat lungs. **C**, Percentage of TUNEL positive cells in vascular SMCs in all groups of athymic male and female rat lungs. **D**, Immunohistochemistry images of vessels stained with α-SMA (brown) in lung tissue at day 21 after treatment with vehicle control, SU5416 and SU5416+IR with Tregs (n=5 per group). **E**, Percentage of the wall thickness of α-SMA positive vessels <100 μm in external diameter in all groups of athymic male and female rat lungs. **F**, Immunofluorescent (*Continued*)

IR With Tregs Before Vascular Injury Decreases Pulmonary Arteriolar Remodeling With Reduced Vascular Smooth Muscle Cell Proliferation and Enhanced Apoptotic Activity With Increased COX-2, PTGIS, HO-1, and PDL-1 Expression

To assess vascular remodeling, male and female lungs were evaluated for smooth cellular proliferation by Ki67 staining and for apoptosis with TUNEL assay (Figure 3A through 3C). Vascular smooth muscle apoptosis was induced by Treg IR, similar to that observed with estradiol protection.[21] The occlusive arteriopathy athymic rats are characterized by α-SMA$^+$ cells obstructing the vascular lumen (Figure 3D) and enhanced vascular wall thickness (Figure 3E); both processes being prevented by Tregs. Having seen that Treg IR upregulates COX-2, PTGIS, HO-1, and PDL-1 protein expression, we wanted to better understand their tissue localization better in reconstituted animals protected from PH. Rat lungs, from animals that had undergone IR, were evaluated by immunohistochemistry at 21-day post-SU5416 administration (Figure 3F). Histologically, Treg-conferred protection was manifested by prevention of SU5416-induced occlusive vasculopathy and increased expression of all 4 proteins in the CALP$^+$ pulmonary arteriolar smooth muscle cells (Figure 3F). Similar results were observed in male and female rats. (Online Figure V).

Tregs Induce RV Expression of COX-2, PTGIS, HO-1, and PDL-1

RV failure is a sentinel event predicting rapid clinical decompensation in PH and is considered a maladaptive response to progressive pulmonary vascular disease.[22] In the athymic rat model, RV changes are not readily detectable by echocardiography before day 10 post SU5416 administration,[14] and it remains unknown to what extent RV changes are because of progressive pulmonary vascular resistance as opposed to processes that may be intrinsic to the RV alone. To examine whether males and females also differ in terms of RV expression of the enzymes involved in the PGI_2 synthesis, protein extracts from male and female RVs were blotted with antibodies to COX-2 PTGIS, as well as HO-1 and PDL-1. As with the male PH lungs, male RVs exhibited an increase in PDL-1 expression with the disease, but this was not the case in female RVs; Treg IR administered before SU5416 increased the RV expression of all 4 vasoprotective proteins (Figure 4A through 4E). Cardiac histology of athymic rats 21-day post-SU5416 administration confirmed COX-2, PTGIS, HO-1 and PDL-1 upregulation in vascular endothelial cells and myocardium from the male and female animals which had undergone Treg IR (Figure 5; Online Figure VI). Real-time PCR of lung and RV tissue for all experimental groups were consistent with the protein expression data (Online Figure VII). Collectively, data suggest that female RVs may be rendered more vulnerable to PH in the absence of normal Treg activity because of relatively less PDL-1 activity than males. However, Tregs protect RVs in both sexes, through COX-2, PTGIS, HO-1, and PDL-1 modulation, further suggesting that these cells play a more dominant role in females to prevent PH after vascular injury.

Blocking COX-2, HO-1, and PD-1/PDL-1 Signaling Pathways Abrogates Treg-Mediated Protection From PH

To confirm that the increased PH severity in Treg-deficient females is because of decreased COX-2, HO-1, and PDL-1 signaling, we examined whether blocking these pathways abrogates Treg-mediated protection. Athymic male and female rats treated with NS-398 (a selective COX-2 inhibitor) at the time of Treg IR were not protected against PH development as manifested by significantly higher RV systolic pressures and greater RV hypertrophy assessed 21 days after SU5416 (Figure 6A and 6B). Administration of the HO-1 inhibitor zinc (II) protoporphyrin IX, into animals that had undergone Treg IR, at the time of SU5416 treatment, also resulted in a loss of Treg-mediated protection in both sexes, as did treatment with an anti-PD-1 (PDL-1 ligand) antibody. Collectively, these findings support the conclusion that the systemic Treg protection against PH depends on intact COX-2, HO-1, and PD-1/PDL-1 pathways in both male and female rats.

Tregs Upregulate COX-2, HO-1, PDL-1, PGI_2, and ER Surface Expression in HMCECs and Increase Culture Supernatant Concentrations of PGI_2 and IL-10

To examine whether Tregs were sufficient to directly induced the upregulation of COX-2, PTGIS, HO-1, and PDL-1 in human microvascular cardiac endothelial cells (HMCECs) and simultaneously assessed the expression of the highly vasoprotective ERs.[23] Purified human CD4$^+$CD25^{high} Tregs were cocultured with HMCECs (12 hours; 37°C). HMCECs, cocultured with CD4$^+$CD25$^-$, T cells served as controls. The HMCECs were subsequently stained with antibodies to COX-2, HO-1, PDL-1, and the estradiol receptors, ER-α and ER-β, and analyzed by flow cytometry. Surface expression of COX-2, HO-1, and PDL-1 was significantly upregulated in cardiac endothelial cells cocultured with Tregs, as compared with those cocultured CD4$^+$CD25$^-$T cells (Figure 7A through 7C). Furthermore, a dramatic upregulation of both ERs was observed in the Treg-cocultured endothelial cells but not in CD4$^+$CD25$^-$T-cocultured endothelial cells (64-fold for ER-α and 22-fold for ER-β; Figure 7D and 7E).

Finally, because RV cardiac endothelial cells appeared to be a site for COX-2 localization, we wanted to know whether Tregs could act on vascular endothelium in concert with estradiol (through the ERs) to induce vasodilation through the release of prostanoids, including PGI_2.[23] HMCECs were cultured with Tregs (12 hours; 37°C) in the presence or absence of inhibitors for ERs (ICI 180, 780[24]), COX-2, and HO-1, and the culture supernatant was assayed for the PGI_2 metabolite 6-keto-prostaglandin F1α. Blocking ERs, COX-2, and HO-1 significantly reduced supernatant PGI_2 levels (Figure 7F). Further, because PGI_2 is known to stimulate a key anti-inflammatory Treg cytokine, IL-10, we also evaluated

Figure 3 Continued. images of lung tissue sections from athymic rats treated with SU5416+IR with Tregs on day 21 and immunolabeled with COX-2, PTGIS, HO-1, and PDL-1 in red and calponin (green) for vessel smooth muscle cells. Nuclei stained with DAPI (blue). Scale bars: (**A**) 25 μm. **D**, 50 μm. **F**, 25 μm. Data are shown as means with error bars representing SEM (*P<0.05).

its expression under similar conditions.[25] Compared with the Treg-endothelial control group, those cultures in which ER signaling, COX-2, and HO-1 were blocked demonstrated significantly reduced IL-10 (Figure 7G). Collectively, the data suggest that Tregs may directly mediate vasoprotection through upregulation of ERs, COX-2, HO-1, PGI_2, and IL-10 biosynthesis in cardiac, vascular endothelial cells.

Discussion

Treg abnormalities are a clinical feature of PH conditions.[3–8] Although increased numbers of Tregs are observed in the blood of PH patients,[6] reduced Tregs are observed in the lungs of these individuals.[26] Idiopathic PH is associated with an expansion of peripheral blood Tregs that are nonsuppressive,[4] and PH associated with connective tissue disorders exhibit increased Th17/Treg ratios.[7] The Paris group, led by Marc Humbert, reported a recent clinical study of 62 patients with idiopathic, heritable, and connective tissue-associated PH discovered dysfunctional Tregs in all 3 subsets and implicated defective leptin-signaling as a possible Treg disturbance in idiopathic and connective tissue disease PH, but not heritable PH.[3] Tregs limit pulmonary vascular injury that can cause experimental PH.[14] However, their ability to differentially affect males versus females, and the involved mechanisms are unknown. We report that in the absence of protective Tregs, PH was more severe in female than male rats using both the SU5416 and chronic hypoxia model systems. SU5416-induced PH is a well-established model of PH[27] based on pulmonary artery endothelial injury in contrast to the hypoxia model which occurs as a consequence of sustained pulmonary

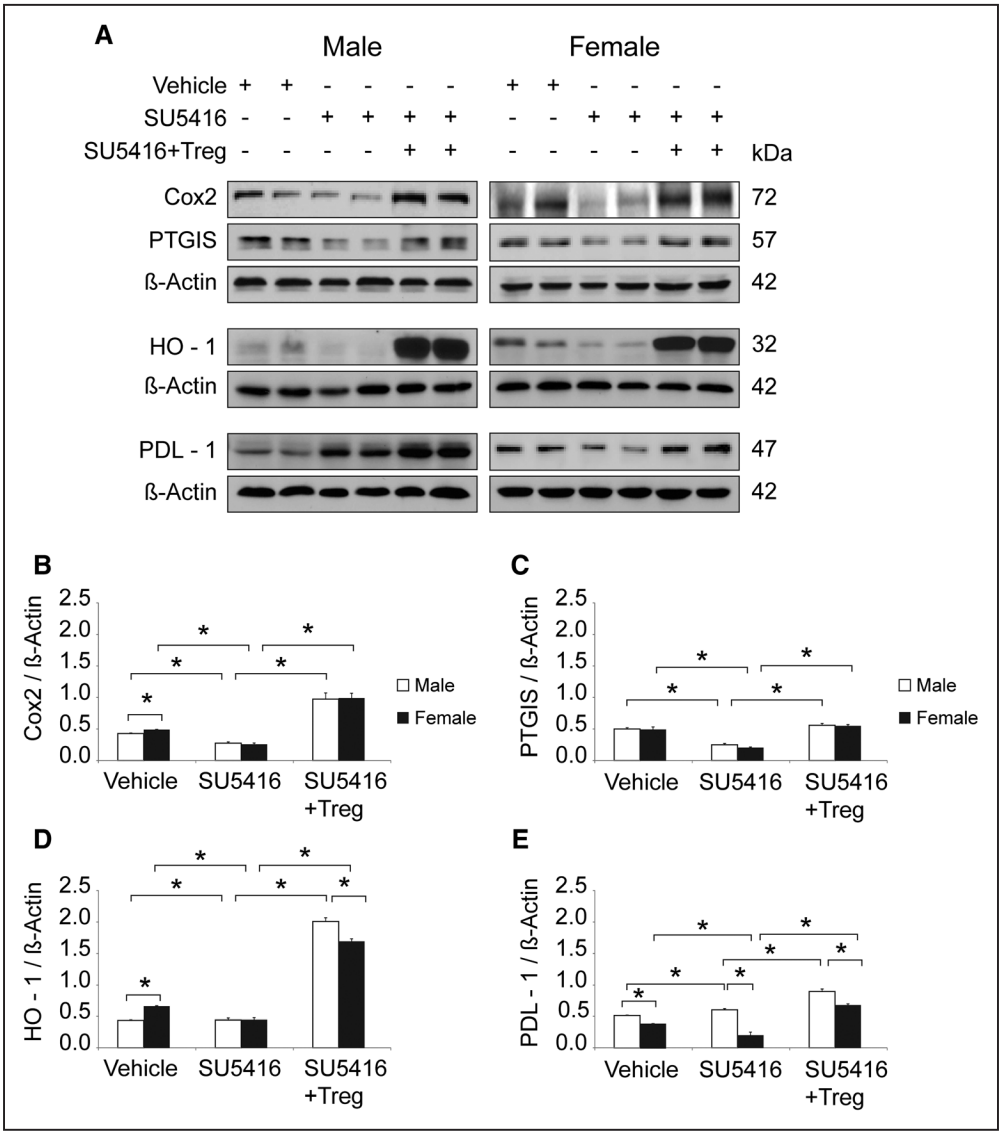


Figure 4. Immune reconstitution (IR) with regulatory T cells (Tregs) operates through activation of COX-2 (cyclooxygenase 2), PTGIS (prostacyclin synthase), HO-1 (heme oxygenase 1), PD-1 (programmed death 1)/PDL-1 (programmed death ligand 1) pathways in right ventricular (RV) tissue of both male and female athymic rats. A, Representative Western immunoblots of COX-2, PTGIS, HO-1, and PDL-1 relative to β-actin in RV lysates from untreated, SU5416-, and SU5416+Treg-treated athymic male and female rats; all groups analyzed on day 21 (n=5 per group). **B**–**E**, Representative densitometric quantification of COX-2, PTGIS, HO-1, PDL-1 relative to β-actin in RV lysates assessed in the vehicle, SU5416-, and SU5416+Treg-treated athymic male and female rats; all groups analyzed on day 21 (n=5 per group). Data are shown as means with error bars representing SEM (*P<0.05).

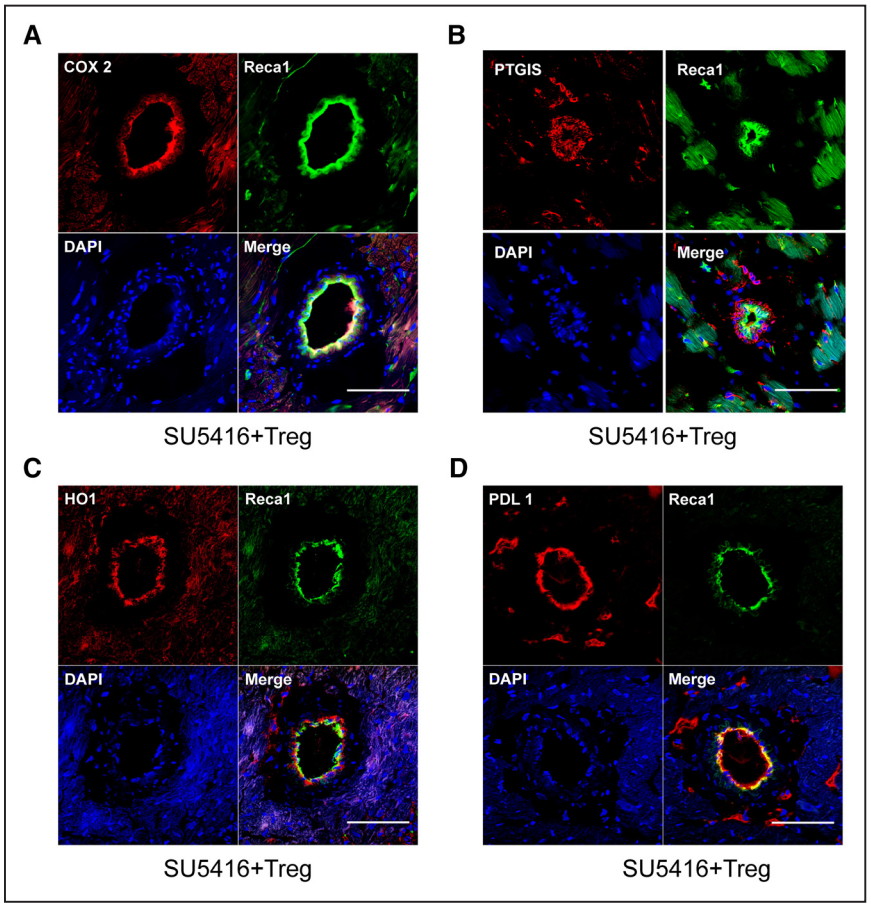


Figure 5. Immune reconstitution (IR) with regulatory T cells (Tregs) leads to increased COX-2 (cyclooxygenase 2), PTGIS (prostacyclin synthase), HO-1 (heme oxygenase 1), and PDL-1 (programmed death ligand 1) expression in endothelial cells of right ventricular (RV) intramyocardial coronary vessels. A–D, Representative immunofluorescent images of RV tissue sections of athymic rats after treatment with SU5416+IR with Tregs on day 21 and immunolabeled with COX-2 (**A**), PTGIS (**B**), HO-1 (**C**), and PDL-1 (**D**) in red and Reca1 (green) for RV vessel endothelial cells. Nuclei were stained with DAPI (blue). Scale bars: (**A–D**) 50 μm.

vasoconstriction. Histologically, female athymic rats with PH exhibited greater pulmonary inflammation, a more significant dropout in RV microvessels, more RV periarteriolar fibrosis with Tregs exerting a more dominant protective function in females compared with their male counterparts. This athymic rat model of PH is distinguished from some other disease models in which males have more significant disease. With the frequently-used monocrotaline and chronic hypoxia PH models, female rodents develop unreliable or mild PH compared with males.[28–33] These studies have identified estradiol (or its 16a-hydroxyestrone metabolite) as either a PH disease mediator or a protective agent.[34] A recent study using SU5416 and a different strain of athymic rats showed worse disease in males[35]; this study differed from the current one by using genetically-outbred rats, which constrains against IR experiments, and was relatively limited in scope. Variation in strain, even between colonies of the same strain, has a remarkable influence on the nature and severity of the response to SU5416, consistent with an important role for genetic modifiers of the PH phenotype.[36]

We started this evaluation by comparing systemic PGI_2 concentrations in both sexes after PH was established because of its important role as an endogenous pulmonary vasodilator and discovered relatively decreased concentrations in females. Two enzymes implicated in PGI_2 biosynthesis, COX-2, and PTGIS, were also diminished in female lungs. PGI_2 is an important endogenous vasoactive factor that acts through adenylyl cyclase and cAMP to cause vasodilation and limit pulmonary artery smooth muscle cell proliferation. PGI_2 synthesis is reduced in PH patients who also demonstrate lower pulmonary artery endothelial cell PTGIS.[37,38] It is possible that the lower PGI_2 levels observed in clinical PH is attributable to the results being obtained in a predominantly female and immune-dysregulated population. Estrogen, acting mainly through ER-α, is known to acutely activate PGI_2 synthesis in the vascular endothelium of females.[23,39,40] Yet, even though female athymic rats have higher estrogen levels than males in all experimental conditions, female PGI_2 systemic levels were relatively low in disease. This finding raises the possibility that, in the absence of Tregs, poor estrogen signaling is occurring in the cells mainly responsible for PGI_2 synthesis. With normal immunity, estrogens have been shown to increase the production of nitric oxide (NO) and PGI_2, and attenuate both vasoconstriction, vascular remodeling, and cardiac fibrosis.[39,41–44] Our in vitro findings suggest that Tregs dramatically upregulate ER isoform expression and provide one explanation of how, in the absence of normal Treg function, estrogen is rendered less effective for promoting PGI_2 synthesis.

To further examine why females developed more severe PH than males in the absence of Tregs, we evaluated 2 other vasoprotective proteins which also direct Treg immunomodulatory function, HO-1 and PDL-1. HO-1 is an anti-inflammatory molecule that also helps mediate Treg function[20] and may confer cardioprotection in females.[45] Decreased HO-1 activity has been established to exacerbate pulmonary inflammation and RV hypertrophy.[46,47] We found that pulmonary HO-1

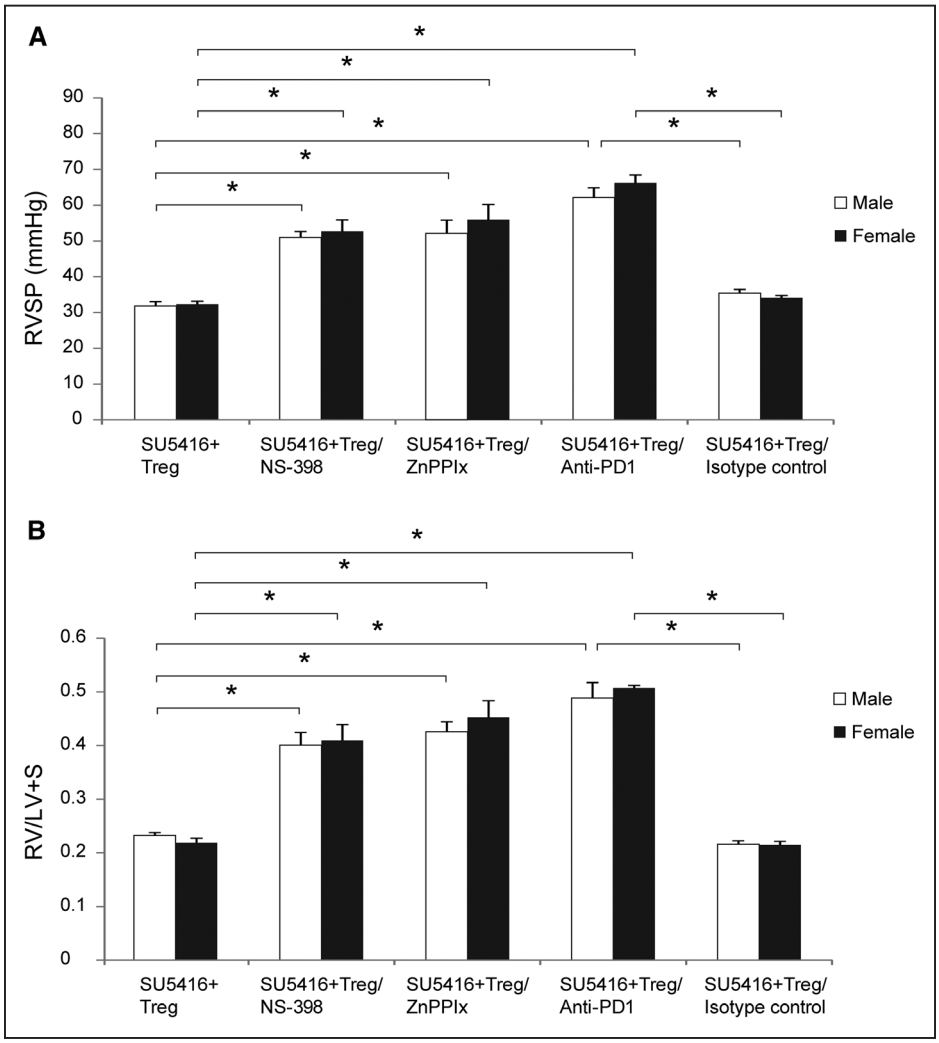


Figure 6. Blocking COX-2 (cyclooxygenase 2), HO-1 (heme oxygenase 1), and PD-1 (programmed death 1)/PDL-1 (programmed death ligand 1) signaling pathways abrogate regulatory T cell (Treg)-mediated protection from pulmonary hypertension (PH). **A**, Right ventricular systolic pressure (RVSP) measurements in athymic male and female rats treated with SU5416+Tregs at day 21 and injected IP with NS-398 (a selective COX-2 inhibitor; 10 mg/kg) daily from day 0 until day 21, injected IP with zinc (II) protoporphyrin IX (ZnPPIX)/HO-1 inhibitor, 40 mg/kg on days 0, 2, 3, 7, 14, and 18, injected IP with anti-PD-1 (PDL-1 ligand) antibody (600 mg/kg loading dose) on day 0 with 300 mg/kg every other day until day 21, injected with isotype control antibody (600 mg/kg loading dose) on day 0 and 300 mg/kg every other day until day 21 (n=4–5 per group). **B**, RV hypertrophy (RVH) measurements as assessed by RV/(LV+S) ratio in athymic male and female rats treated with SU5416+Tregs at day 21 and injected IP with NS-398 (a selective COX-2 inhibitor; 10 mg/kg) on day 0 and when daily until day 21, injected IP with zinc (II) protoporphyrin IX (ZnPPIX)/HO-1 inhibitor, 40 mg/kg on days 0, 2, 3, 7, 14, and 18, injected IP with anti-PD-1 (PDL-1 ligand) antibody (600 mg/kg loading dose) on day 0 with 300 mg/kg every other day until day 21, injected with isotype control antibody (600 mg/kg loading dose) on day 0 with 300 mg/kg every other day until day 21 (n=4–5 per group). Data are shown as means with error bars representing SEM (*P<0.05).

protein levels decreased in female PH lungs suggesting that HO-1 may also confer protection in females. Next, we evaluated PDL-1. PDL-1 and its ligand, PD-1, are both detected on Tregs and control the development, maintenance, and function of induced Tregs.[32] In the current study, PDL-1 was significantly reduced in the lung and RV tissues of females athymic rats with PH. Why males upregulate pulmonary PDL-1 to a greater degree than females in the absence of Tregs is not clear. However, it is relevant that, although most research on PD-1/PDL-1 signaling focuses on controlling T-cell immunity, this pathway is also involved in limiting inflammation caused by other cells, such as NK cells, and therefore reduced PDL-1 expression in females could enhance inflammation even in the absence of T cells.[19] Collectively, the reduced expression of these related vasoprotective proteins (PDL-1, HO-1, COX-2, and PTGIS), may help explain why females that lack normal Treg activity may develop more severe PH.

Given the differences in responses of athymic male and females, we were interested in determining whether differences between the sexes could also be detected when they were protected from PH. Treg IR was equally effective in male and female rats in preventing SU5416-induced PH, limiting pulmonary inflammation, diminishing RV fibrosis, upregulating systemic PGI_2 biosynthesis and enhancing cardiopulmonary expression of COX-2, PTGIS, HO-1, and PDL-1. The importance of these vasoprotective pathways in mediating Treg protection against PH was demonstrated when selective inhibition of COX-2, HO-1, and PD-1 abrogated the conferred benefit of

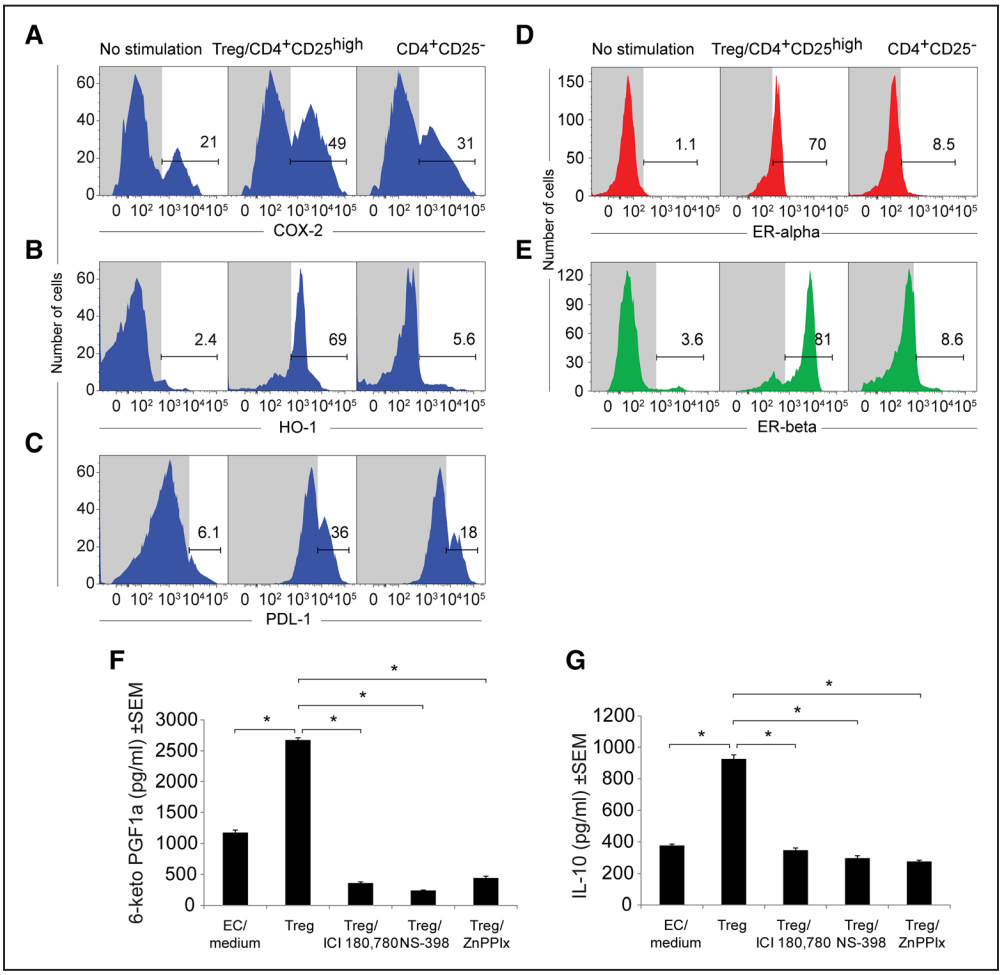


Figure 7. Regulatory T cells (Tregs) upregulate COX-2 (cyclooxygenase 2), HO-1 (heme oxygenase 1), PDL-1 (programmed death ligand 1), and ER (estrogen receptor) expression in human cardiac microvascular endothelial cells (HCMECs) and increase culture supernatant concentrations of PGI_2 (prostacyclin) and IL (interleukin)-10. A–E, Fluorescence histogram from flow cytometric analysis of COX-2, HO-1, PDL-1, ER-α, ER-β expression in human cardiac microvascular cells (HCMECs; male donor) after 24 h coculture with human (male donor) Tregs. **F** and **G**, Prostacyclin release was measured by 6-keto-prostaglandin F1α (6-ketoPGF1α) and IL-10 levels from ELISA of culture supernatants after HCMECs (male donor) 24 h coculture with human (male donor) Tregs, treated with ICI180, 780 (ER inhibitor), (10 μmol/L), NS-398 (a selective COX-2 inhibitor), 10 μmol/L, zinc (II) protoporphyrin IX (ZnPPIX)/HO-1 inhibitor, 10 μmol/L, n=4 per group. Data are shown as means with error bars representing SEM (*P<0.05).

IR. The mechanism by which Tregs induce PGI_2 synthesis is unknown with both direct and indirect interactions between Tregs and PGI_2-producing endothelial cells being potentially involved. This possibility is supported by the finding that vascular COX-2 is a major contributor to systemic PGI_2 and a promoter of Treg function.[48–51] We found that Treg-induced COX-2 expression in SU5416-treated athymic rats as well as in endothelial cells in vitro and augmented PGI_2 production. These findings collectively demonstrate that Treg deficiency more profoundly reduces the vasoreparative upregulation of vascular COX-2 in females compared with males and thus confirm a dominant function that Tregs play in females when the disease is prevented.

Lung-specific HO-1 upregulation prevents the development of hypoxia-induced PH and inhibits pulmonary vascular remodeling and pulmonary inflammation.[52,53] Cardiac HO-1 helps regulate myocardial cell energetics to limit cell death, pathological remodeling, and fibrosis and may confer this cardioprotection by decreasing oxidant projection and limiting endothelial cell damage. Although HO-1 can help mediate Treg immunomodulatory activity,[20] we found that Tregs induce endothelial and myocardial HO-1 surface expression in vitro and in vivo with cardiopulmonary HO-1 expression being significantly elevated by Treg IR in both male and female athymic rats. The PD-1/PDL-1 pathway delivers inhibitory signals for controlling immune responses and helps maintain central and peripheral tolerance.[54] Estrogen normally induces the expression of PD-1[55,56] and may serve to enhance the anti-inflammatory action of normally-functioning Tregs. The vulnerability of athymic females to more severe PH may, in part, relate to the absence of Treg-induced HO-1 and PDL-1 expression in the vasculature and myocardium.

Our in vitro studies in Treg-HCMEC coculture studies indicate that $CD4^+CD25^{hi}FoxP3^+$Tregs, but not $CD4^+CD25^-$ T cells, induce PGI_2 biosynthesis in an ER-, COX-2– and HO-1–dependent manner. This is particularly interesting because estrogen promotes the expansion and frequency of Treg cells and upregulates the expression of FoxP3, PD-1, and CTLA-4

via ER-α signaling.[55,57] Indeed, it has been suggested that the protective effects of estrogen in autoimmune conditions may be because of estrogen-mediated Treg expansion and activation.[57] However, the effect of sex hormones on immune cells in health may differ from that occurring during vascular injury and disease.[55,58] Conversely, Treg-mediated upregulation of ERs on HCMECs could enhance the cardioprotective effect of estradiol after vascular injury. Although the direct mechanism of this contribution is still unclear, our data suggest that, in animals lacking Tregs, the potentially beneficial role played by estradiol in limiting vascular inflammation may be significantly restricted. The relationship between Tregs, vascular endothelium, PGI_2, COX-2, HO-1, PD-1/PDL-1, and ER signaling is schematically modeled in Figure 8.

The increased severity of female PH in the current athymic rat study, using inbred WAG animals, either exposed to SU5416 or chronic hypoxia, indicates that Treg deficiency as the basis of PH pathogenesis (relative to other models not relying on immune dysregulation). Beyond Treg anomalies, other forms of immune dysregulation are also likely important in PH pathogenesis, such as those attributable to abnormal regulatory natural killer cells. There are several caveats to the current studies. Clinical PH is more prevalent in women but more severe in men,[59] and thus our animal model can only draw inferences about the relative importance of Tregs as one of many components (eg, BMPR2 mutation status, hormonal differences, and environmental factors) in disease pathogenesis in both sexes. Our experiments are costly and time-consuming spanning >5 years for the current study, with 3 to 4 Treg donors being required per athymic recipient; not surprisingly, several key questions remain unaddressed. Although female RVs demonstrated a higher microvascular density in health, and correspondingly, the apparent vascular dropout with PH development was more profound in females, there is a potential bias in the capillary density measurements because of the anisotropic orientation of the RV vasculature. Without using a proper stereologic methodology, which evaluates the entire volume of the ventricle, it's not possible to say with certainty whether there was an actual dropout of vessels in PH. The mechanism by which Tregs induce endothelial PGI_2 biosynthesis is currently unknown. We also don't know why females differ from males in the expression of vasoprotective proteins in PH; a question that could be addressed by evaluating the specific impact on estradiol in males and females through hormonal supplementation, antagonism, and oophorectomies. In the studies involving Treg-HCMEC cocultures, the media were supplemented with fetal calf serum containing picomolar levels of estradiol, and it will be informative to understand how titrating concentrations of estradiol would affect PGI_2 levels. Finally, because of the many possible Treg interactions, the current study was unable to evaluate every cell–cell interaction nor evaluate Treg migration in vivo. In light of the current findings, it will be interesting to determine how Tregs directly influence PASMCs and pulmonary artery adventitial fibroblasts and track their movements to various in vivo compartments. Determining the differential effects of

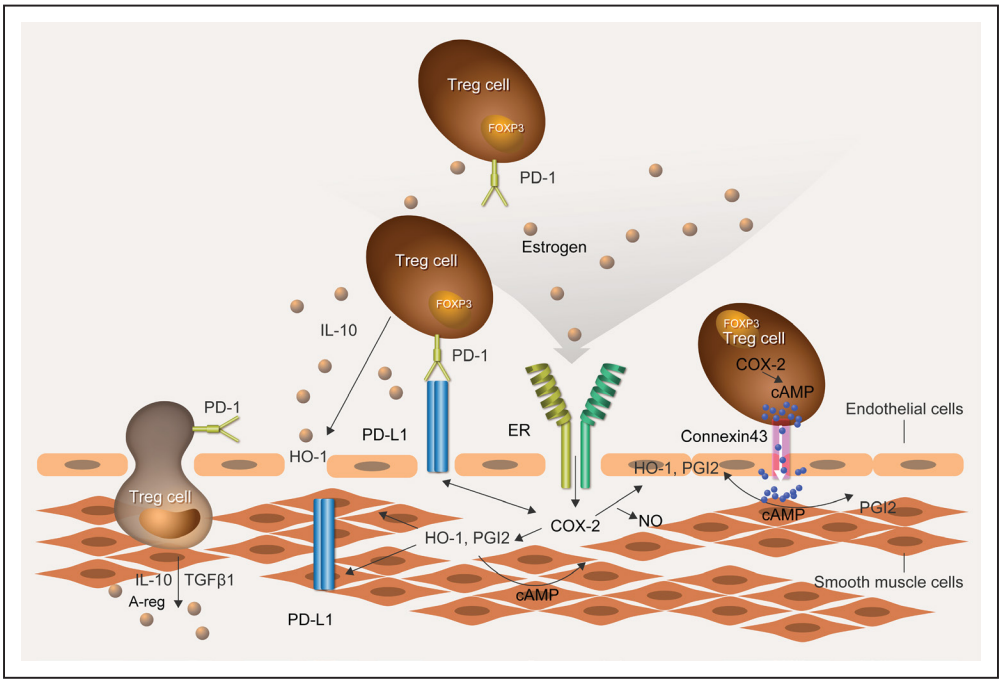


Figure 8. Speculative model of regulatory T cell (Treg) signaling in the vascular wall. Tregs exert paracrine and direct effects on vascular endothelial and smooth muscle cells. Treg PD-1 (programmed death 1)/PDL-1 (programmed death ligand 1) interactions help quell inflammation in concert with estrogen interacting with ERs (estrogen receptors). Tregs act on the vascular intima and smooth muscle layer to promote COX-2 (cyclooxygenase 2) expression and PGI2 (prostacyclin) biosynthesis. Tregs similarly enhance vascular HO-1 (heme oxygenase 1), IL (interleukin)-10, Areg (amphiregulin), and TGF (transforming growth factor)-β concentrations which have pronounced anti-inflammatory and tissue repair properties. Treg produces and respond to PGE_2 (prostaglandin E2), which increases intracellular cAMP that, in turn, upregulates FoxP3 (forkhead box P3) expression. Immunosuppressive activity may be mediated by intercellular transfer of cAMP from Treg to endothelial cells via gap junctions presumably formed by Cx43, which is the connexin found in T cells. These cumulative actions limit vascular injury that would otherwise lead to pulmonary vascular remodeling.

PGI_2 therapy in males versus females is also warranted in light of the current findings. Ongoing studies are designed to address these questions and will evaluate animals after longer periods of observation to ascertain the durability of the differences between the sexes observed at 3 weeks.

Abnormal Treg function is a clinical feature of PH and may be responsible for the immune dysregulation observed in the disease.[60,61] Sexual differences are present in various PH manifestations, and the study of how regulatory immunity differentially impacts men and women with this condition is a highly promising new avenue of scientific investigation. The experimental findings of the current study suggest that Tregs play a dominant role quelling vascular injury in females. In IR groups, a notable pulmonary infiltration of Tregs occurs, and by this action, PH is prevented through distinct anti-inflammatory and vasoprotective immunity.[14] This finding has led to an interest in using autologous, conditioned Tregs as a basis for treating patients. Treg immunotherapy has been criticized for its requirement of potentially billions of cells.[62] Further, in the rat model of PH, Treg immunotherapy (albeit with far fewer cells) loses efficacy if administered after SU5416 dosing.[14] Clinical efforts are underway to overcome these problems by expanding Tregs ex vivo to optimize Treg dose, improve antigen specificity, and use adjunct therapies to potentiate Treg therapeutic effects.[63] As Treg therapy for a variety of immunologic disorders begins to enter the clinic,[63,64] vulnerable PH patients represent a particularly promising target population for this and other immunomodulatory therapies.

Acknowledgments

We thank Davis McKinley for help with managing the inbred athymic rat breeding colony and Dr Lusijah Sutherland for assistance with cell sorting. We would also like to acknowledge Biomedical Research Models, Inc (Worcester, MA) which generously provided the inbred athymic rats used to populate a breeding colony in Palo Alto.

Sources of Funding

This study was supported by National Heart, Lung, and Blood Institute HL122887 (M.R. Nicolls), HL014985 (M.R. Nicolls), HL125739 (M.R. Nicolls), HL120001 (M.R. Nicolls), HL138473 (M.R. Nicolls), and 1T32HL098049 (J. Qian).

Disclosures

None.

References

1. Voelkel NF, Gomez-Arroyo J, Abbate A, Bogaard HJ, Nicolls MR. Pathobiology of pulmonary arterial hypertension and right ventricular failure. *Eur Respir J*. 2012;40:1555–1565. doi: 10.1183/09031936.00046612.
2. Meng X, Yang J, Dong M, Zhang K, Tu E, Gao Q, Chen W, Zhang C, Zhang Y. Regulatory T cells in cardiovascular diseases. *Nat Rev Cardiol*. 2016;13:167–179. doi: 10.1038/nrcardio.2015.169.
3. Huertas A, Phan C, Bordenave J, Tu L, Thuillet R, Le Hiress M, Avouac J, Tamura Y, Allanore Y, Jovan R, Sitbon O, Guignabert C, Humbert M. Regulatory T Cell dysfunction in idiopathic, heritable and connective tissue-associated pulmonary arterial hypertension. *Chest*. 2016;149:1482–1493. doi: 10.1016/j.chest.2016.01.004.
4. Sada Y, Dohi Y, Uga S, Higashi A, Kinoshita H, Kihara Y. Non-suppressive regulatory T cell subset expansion in pulmonary arterial hypertension. *Heart Vessels*. 2016;31:1319–1326. doi: 10.1007/s00380-015-0727-4.
5. Nicolls MR, Taraseviciene-Stewart L, Rai PR, Badesch DB, Voelkel NF. Autoimmunity and pulmonary hypertension: a perspective. *Eur Respir J*. 2005;26:1110–1118. doi: 10.1183/09031936.05.00045705.
6. Ulrich S, Nicolls MR, Taraseviciene L, Speich R, Voelkel N. Increased regulatory and decreased CD8+ cytotoxic T cells in the blood of patients with idiopathic pulmonary arterial hypertension. *Respiration*. 2008;75:272–280. doi: 10.1159/000111548.
7. Gaowa S, Zhou W, Yu L, Zhou X, Liao K, Yang K, Lu Z, Jiang H, Chen X. Effect of Th17 and Treg axis disorder on outcomes of pulmonary arterial hypertension in connective tissue diseases. *Mediators Inflamm*. 2014;2014:247372. doi: 10.1155/2014/247372.
8. Jasiewicz M, Moniuszko M, Pawlak D, Knapp M, Rusak M, Kazimierczyk R, Musial WJ, Dabrowska M, Kaminski KA. Activity of the kynurenine pathway and its interplay with immunity in patients with pulmonary arterial hypertension. *Heart*. 2016;102:230–237. doi: 10.1136/heartjnl-2015-308581.
9. Tamosiuniene R, Nicolls MR. Regulatory T cells and pulmonary hypertension. *Trends Cardiovasc Med*. 2011;21:166–171. doi: 10.1016/j.tcm.2012.05.004.
10. Miyata M, Sakuma F, Ito M, Ohira H, Sato Y, Kasukawa R. Athymic nude rats develop severe pulmonary hypertension following monocrotaline administration. *Int Arch Allergy Immunol*. 2000;121:246–252. doi: 10.1159/000024324.
11. Tian W, Jiang X, Tamosiuniene R, et al. Blocking macrophage leukotriene b4 prevents endothelial injury and reverses pulmonary hypertension. *Sci Transl Med*. 2013;5:200ra117. doi: 10.1126/scitranslmed.3006674.
12. Qian J, Tian W, Jiang X, et al. Leukotriene B4 activates pulmonary artery adventitial fibroblasts in pulmonary hypertension. *Hypertension*. 2015;66:1227–1239. doi: 10.1161/HYPERTENSIONAHA.115.06370.
13. Taraseviciene-Stewart L, Nicolls MR, Kraskauskas D, Scerbavicius R, Burns N, Cool C, Wood K, Parr JE, Boackle SA, Voelkel NF. Absence of T cells confers increased pulmonary arterial hypertension and vascular remodeling. *Am J Respir Crit Care Med*. 2007;175:1280–1289. doi: 10.1164/rccm.200608-1189OC.
14. Tamosiuniene R, Tian W, Dhillon G, Wang L, Sung YK, Gera L, Patterson AJ, Agrawal R, Rabinovitch M, Ambler K, Long CS, Voelkel NF, Nicolls MR. Regulatory T cells limit vascular endothelial injury and prevent pulmonary hypertension. *Circ Res*. 2011;109:867–879. doi: 10.1161/CIRCRESAHA.110.236927.
15. Ormiston ML, Deng Y, Stewart DJ, Courtman DW. Innate immunity in the therapeutic actions of endothelial progenitor cells in pulmonary hypertension. *Am J Respir Cell Mol Biol*. 2010;43:546–554. doi: 10.1165/rcmb.2009-0152OC.
16. Straub RH. The complex role of estrogens in inflammation. *Endocr Rev*. 2007;28:521–574. doi: 10.1210/er.2007-0001.
17. Ricciotti E, Yu Y, Grosser T, Fitzgerald GA. COX-2, the dominant source of prostacyclin. *Proc Natl Acad Sci USA*. 2013;110:E183. doi: 10.1073/pnas.1219073110.
18. Cawello W, Schweer H, Müller R, Bonn R, Seyberth HW. Metabolism and pharmacokinetics of prostaglandin E1 administered by intravenous infusion in human subjects. *Eur J Clin Pharmacol*. 1994;46:275–277.
19. Francisco LM, Sage PT, Sharpe AH. The PD-1 pathway in tolerance and autoimmunity. *Immunol Rev*. 2010;236:219–242. doi: 10.1111/j.1600-065X.2010.00923.x.
20. Xia ZW, Zhong WW, Xu LQ, Sun JL, Shen QX, Wang JG, Shao J, Li YZ, Yu SC. Heme oxygenase-1-mediated CD4+CD25high regulatory T cells suppress allergic airway inflammation. *J Immunol*. 2006;177:5936–5945.
21. Yuan P, Wu WH, Gao L, Zheng ZQ, Liu D, Mei HY, Zhang ZL, Jing ZC. Oestradiol ameliorates monocrotaline pulmonary hypertension via NO, prostacyclin and endothelin-1 pathways. *Eur Respir J*. 2013;41:1116–1125. doi: 10.1183/09031936.00044112.
22. Ryan JJ, Huston J, Kutty S, Hatton ND, Bowman L, Tian L, Herr JE, Johri AM, Archer SL. Right ventricular adaptation and failure in pulmonary arterial hypertension. *Can J Cardiol*. 2015;31:391–406. doi: 10.1016/j.cjca.2015.01.023.
23. Sobrino A, Oviedo PJ, Novella S, Laguna-Fernandez A, Bueno C, García-Pérez MA, Tarín JJ, Cano A, Hermenegildo C. Estradiol selectively stimulates endothelial prostacyclin production through estrogen receptor-{alpha}. *J Mol Endocrinol*. 2010;44:237–246. doi: 10.1677/JME-09-0112.
24. Osborne CK, Wakeling A, Nicholson RI. Fulvestrant: an oestrogen receptor antagonist with a novel mechanism of action. *Br J Cancer*. 2004;90(suppl 1):S2–S6. doi: 10.1038/sj.bjc.6601629.
25. Jaffar Z, Wan KS, Roberts K. A key role for prostaglandin I2 in limiting lung mucosal Th2, but not Th1, responses to inhaled allergen. *J Immunol*. 2002;169:5997–6004.
26. Savai R, Pullamsetti SS, Kolbe J, et al. Immune and inflammatory cell involvement in the pathology of idiopathic pulmonary

arterial hypertension. *Am J Respir Crit Care Med*. 2012;186:897–908. doi: 10.1164/rccm.201202-0335OC.
27. Nicolls MR, Mizuno S, Taraseviciene-Stewart L, Farkas L, Drake JI, Al Husseini A, Gomez-Arroyo JG, Voelkel NF, Bogaard HJ. New models of pulmonary hypertension based on VEGF receptor blockade-induced endothelial cell apoptosis. *Pulm Circ*. 2012;2:434–442. doi: 10.4103/2045-8932.105031.
28. McMurtry IF, Frith CH, Will DH. Cardiopulmonary responses of male and female swine to simulated high altitude. *J Appl Physiol*. 1973;35:459–462. doi: 10.1152/jappl.1973.35.4.459.
29. Rabinovitch M, Gamble WJ, Miettinen OS, Reid L. Age and sex influence on pulmonary hypertension of chronic hypoxia and on recovery. *Am J Physiol*. 1981;240:H62–H72. doi: 10.1152/ajpheart.1981.240.1.H62.
30. Burton RR, Besch EL, Smith AH. Effect of chronic hypoxia on the pulmonary arterial blood pressure of the chicken. *Am J Physiol*. 1968;214:1438–1442. doi: 10.1152/ajplegacy.1968.214.6.1438.
31. Smith P, Moosavi H, Winson M, Heath D. The influence of age and sex on the response of the right ventricle, pulmonary vasculature and carotid bodies to hypoxia in rats. *J Pathol*. 1974;112:11–18. doi: 10.1002/path.1711120104.
32. Farhat MY, Chen MF, Bhatti T, Iqbal A, Cathapermal S, Ramwell PW. Protection by oestradiol against the development of cardiovascular changes associated with monocrotaline pulmonary hypertension in rats. *Br J Pharmacol*. 1993;110:719–723.
33. Parker TA, Ivy DD, Galan HL, Grover TR, Kinsella JP, Abman SH. Estradiol improves pulmonary hemodynamics and vascular remodeling in perinatal pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol*. 2000;278:L374–L381. doi: 10.1152/ajplung.2000.278.2.L374.
34. Lahm T, Tuder RM, Petrache I. Progress in solving the sex hormone paradox in pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol*. 2014;307:L7–L26. doi: 10.1152/ajplung.00337.2013.
35. Guihaire J, Deuse T, Wang D, Fadel E, Reichenspurner H, Schrepfer S. Sex differences in immunology: more severe development of experimental pulmonary hypertension in male rats exposed to vascular endothelial growth factor receptor blockade. *Biomed Res Int*. 2015;2015:765292. doi: 10.1155/2015/765292.
36. Jiang B, Deng Y, Suen C, Taha M, Chaudhary KR, Courtman DW, Stewart DJ. Marked strain-specific differences in the SU5416 rat model of severe pulmonary arterial hypertension. *Am J Respir Cell Mol Biol*. 2016;54:461–468. doi: 10.1165/rcmb.2014-0488OC.
37. Christman BW, McPherson CD, Newman JH, King GA, Bernard GR, Groves BM, Loyd JE. An imbalance between the excretion of thromboxane and prostacyclin metabolites in pulmonary hypertension. *N Engl J Med*. 1992;327:70–75. doi: 10.1056/NEJM199207093270202.
38. Tuder RM, Cool CD, Geraci MW, Wang J, Abman SH, Wright L, Badesch D, Voelkel NF. Prostacyclin synthase expression is decreased in lungs from patients with severe pulmonary hypertension. *Am J Respir Crit Care Med*. 1999;159:1925–1932. doi: 10.1164/ajrccm.159.6.9804054.
39. Sherman TS, Chambliss KL, Gibson LL, Pace MC, Mendelsohn ME, Pfister SL, Shaul PW. Estrogen acutely activates prostacyclin synthesis in ovine fetal pulmonary artery endothelium. *Am J Respir Cell Mol Biol*. 2002;26:610–616. doi: 10.1165/ajrcmb.26.5.4528.
40. Egan KM, Lawson JA, Fries S, Koller B, Rader DJ, Smyth EM, Fitzgerald GA. COX-2-derived prostacyclin confers atheroprotection on female mice. *Science*. 2004;306:1954–1957. doi: 10.1126/science.1103333.
41. Lahm T, Albrecht M, Fisher AJ, Selej M, Patel NG, Brown JA, Justice MJ, Brown MB, Van Demark M, Trulock KM, Dieudonne D, Reddy JG, Presson RG, Petrache I. 17β-Estradiol attenuates hypoxic pulmonary hypertension via estrogen receptor-mediated effects. *Am J Respir Crit Care Med*. 2012;185:965–980. doi: 10.1164/rccm.201107-1293OC.
42. Lahm T, Crisostomo PR, Markel TA, Wang M, Weil BR, Novotny NM, Meldrum DR. The effects of estrogen on pulmonary artery vasoreactivity and hypoxic pulmonary vasoconstriction: potential new clinical implications for an old hormone. *Crit Care Med*. 2008;36:2174–2183. doi: 10.1097/CCM.0b013e31817d1a92.
43. Hara A, Yuhki K, Fujino T, Yamada T, Takayama K, Kuriyama S, Takahata O, Karibe H, Okada Y, Xiao CY, Ma H, Narumiya S, Ushikubi F. Augmented cardiac hypertrophy in response to pressure overload in mice lacking the prostaglandin I2 receptor. *Circulation*. 2005;112:84–92. doi: 10.1161/CIRCULATIONAHA.104.527077.
44. Kaneshige T, Saida Y, Tanaka R, Soda A, Fukushima A, Ida N, Takenaka M, Yamane Y. Effect of long-term administration of a prostacyclin analogue (beraprost sodium) on myocardial fibrosis in Dahl rats. *J Vet Med Sci*. 2007;69:1271–1276.
45. Pósa A, Kupai K, Ménesi R, Szalai Z, Szabó R, Pintér Z, Pálfi G, Gyöngyösi M, Berkó A, Pávó I, Varga C. Sexual dimorphism of cardiovascular ischemia susceptibility is mediated by heme oxygenase. *Oxid Med Cell Longev*. 2013;2013:521563. doi: 10.1155/2013/521563.
46. Goto J, Ishikawa K, Kawamura K, Watanabe Y, Matumoto H, Sugawara D, Maruyama Y. Heme oxygenase-1 reduces murine monocrotaline-induced pulmonary inflammatory responses and resultant right ventricular overload. *Antioxid Redox Signal*. 2002;4:563–568. doi: 10.1089/15230860260220058.
47. Issan Y, Kornowski R, Aravot D, Shainberg A, Laniado-Schwartzman M, Sodhi K, Abraham NG, Hochhauser E. Heme oxygenase-1 induction improves cardiac function following myocardial ischemia by reducing oxidative stress. *PLoS One*. 2014;9:e92246. doi: 10.1371/journal.pone.0092246.
48. Yu Y, Ricciotti E, Scalia R, Tang SY, Grant G, Yu Z, Landesberg G, Crichton I, Wu W, Puré E, Funk CD, FitzGerald GA. Vascular COX-2 modulates blood pressure and thrombosis in mice. *Sci Transl Med*. 2012;4:132ra54. doi: 10.1126/scitranslmed.3003787.
49. Sharma S, Zhu L, Yang SC, Zhang L, Lin J, Hillinger S, Gardner B, Reckamp K, Strieter RM, Huang M, Batra RK, Dubinett SM. Cyclooxygenase 2 inhibition promotes IFN-gamma-dependent enhancement of antitumor responses. *J Immunol*. 2005;175:813–819.
50. Ha TY. The role of regulatory T cells in cancer. *Immune Netw*. 2009;9:209–235. doi: 10.4110/in.2009.9.6.209.
51. Pidgeon GP, Tamosiuniene R, Chen G, Leonard I, Belton O, Bradford A, Fitzgerald DJ. Intravascular thrombosis after hypoxia-induced pulmonary hypertension: regulation by cyclooxygenase-2. *Circulation*. 2004;110:2701–2707. doi: 10.1161/01.CIR.0000145613.01188.0B.
52. Minamino T, Christou H, Hsieh CM, Liu Y, Dhawan V, Abraham NG, Perrella MA, Mitsialis SA, Kourembanas S. Targeted expression of heme oxygenase-1 prevents the pulmonary inflammatory and vascular responses to hypoxia. *Proc Natl Acad Sci USA*. 2001;98:8798–8803. doi: 10.1073/pnas.161272598.
53. Christou H, Morita T, Hsieh CM, Koike H, Arkonac B, Perrella MA, Kourembanas S. Prevention of hypoxia-induced pulmonary hypertension by enhancement of endogenous heme oxygenase-1 in the rat. *Circ Res*. 2000;86:1224–1229.
54. Keir ME, Liang SC, Guleria I, Latchman YE, Qipo A, Albacker LA, Koulmanda M, Freeman GJ, Sayegh MH, Sharpe AH. Tissue expression of PD-L1 mediates peripheral T cell tolerance. *J Exp Med*. 2006;203:883–895. doi: 10.1084/jem.20051776.
55. Polanczyk MJ, Carson BD, Subramanian S, Afentoulis M, Vandenbark AA, Ziegler SF, Offner H. Cutting edge: estrogen drives expansion of the CD4+CD25+ regulatory T cell compartment. *J Immunol*. 2004;173:2227–2230.
56. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. *Annu Rev Immunol*. 2008;26:677–704. doi: 10.1146/annurev.immunol.26.021607.090331.
57. Offner H, Polanczyk M. A potential role for estrogen in experimental autoimmune encephalomyelitis and multiple sclerosis. *Ann N Y Acad Sci*. 2006;1089:343–372. doi: 10.1196/annals.1386.021.
58. Khan D, Ansar Ahmed S. The Immune system is a natural target for estrogen action: opposing effects of estrogen in two prototypical autoimmune diseases. *Front Immunol*. 2015;6:635. doi: 10.3389/fimmu.2015.00635.
59. Ventetuolo CE, Praestgaard A, Palevsky HI, Klinger JR, Halpern SD, Kawut SM. Sex and haemodynamics in pulmonary arterial hypertension. *Eur Respir J*. 2014;43:523–530. doi: 10.1183/09031936.00027613.
60. Nicolls MR, Voelkel NF. The Roles of immunity in the prevention and evolution of pulmonary arterial hypertension. *Am J Respir Crit Care Med*. 2017;195:1292–1299. doi: 10.1164/rccm.201608-1630PP.
61. Rabinovitch M, Guignabert C, Humbert M, Nicolls MR. Inflammation and immunity in the pathogenesis of pulmonary arterial hypertension. *Circ Res*. 2014;115:165–175. doi: 10.1161/CIRCRESAHA.113.301141.
62. Riley JL, June CH, Blazar BR. Human T regulatory cell therapy: take a billion or so and call me in the morning. *Immunity*. 2009;30:656–665. doi: 10.1016/j.immuni.2009.04.006.
63. Tang Q, Bluestone JA. Regulatory t-cell therapy in transplantation: moving to the clinic. *Cold Spring Harb Perspect Med*. 2013;3:1–15. doi: 10.1101/cshperspect.a015552.
64. Singer BD, King LS, D'Alessio FR. Regulatory T cells as immunotherapy. *Front Immunol*. 2014;5:46. doi: 10.3389/fimmu.2014.00046.

Circulation Research
JOURNAL OF THE AMERICAN HEART ASSOCIATION

Dominant Role for Regulatory T Cells in Protecting Females Against Pulmonary Hypertension

Rasa Tamosiuniene, Olga Manouvakhova, Paul Mesange, Toshie Saito, Jin Qian, Mrinmoy Sanyal, Yu-Chun Lin, Linh P. Nguyen, Amir Luria, Allen B. Tu, Joshua M. Sante, Marlene Rabinovitch, Desmond J. Fitzgerald, Brian B. Graham, Aida Habtezion, Norbert F. Voelkel, Laure Aurelian and Mark R. Nicolls

Circ Res. 2018;122:1689-1702; originally published online March 15, 2018;
doi: 10.1161/CIRCRESAHA.117.312058
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0009-7330. Online ISSN: 1524-4571

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 α-SMA positive vessels was determined using the methodology described by Beppu et al. and was as follows: % wall thickness =(WT1+WT2) x100% /(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 μm, 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 □M (1:10 dilution= 0.0025 □M) 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′-ACTCACTCAGTTTGTTGAGTCATTC-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′- GGTTGATTTTGCGGTATGGA -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′- ATGCCATCAACAGCATCAAA -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 Accession 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. $CD4^+CD25^{high}$ cells were isolated from the PBMCs using the two-step method that includes negative selection with the Human $CD4^+CD25^{high}$ T Cell Isolation Kit (#18062A, STEMCELL Technologies*)* and the fully automated RoboSep™. $CD4^+CD25^{high}$ 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_2, 5% CO_2) at 37°C. A suspension of 1×10^6 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 $CD4^+$ $CD25^{hi}$Tregs and human cardiac microvascular endothelial cells (hCMECs)*.* Immediately after isolation human $CD4^+$ $CD25^{high}$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_2 in 24-well flat-bottom plates (#3524, Corning, NY). Activated human $CD4^+CD25^{high}$ Tregs (2×10^6) were co-cultured with previously-seeded hCMECs (1×10^6) 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 $CD4^+$ $CD25^{high}$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% O2) at d21 induced higher RVSP at d21 in athymic female than male rats. (n=7 per group). B. Chronic hypoxia (10% O2) 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 Reca1 (red) for capillaries at d21 after SU5416 administration. B. Morphometric analysis of capilliaries (Reca1) 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.

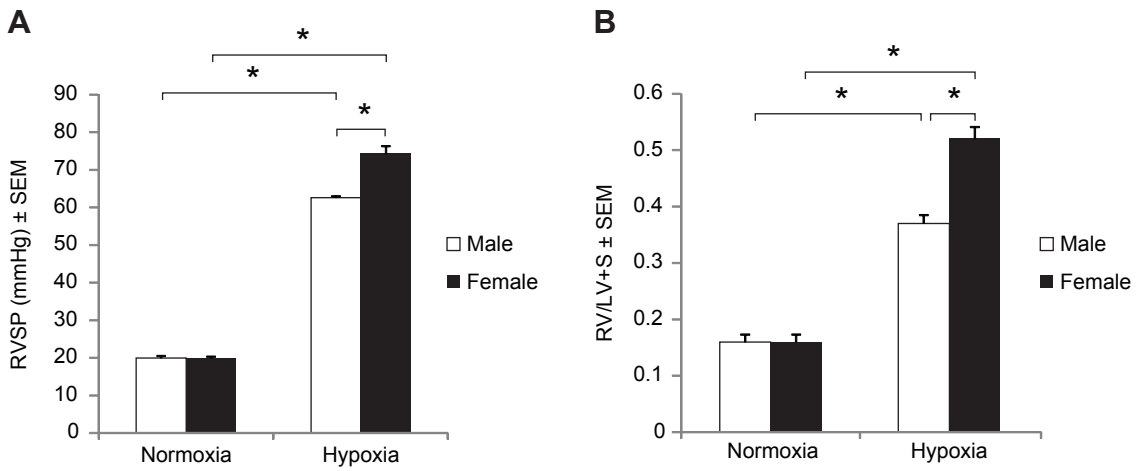
Online Figure IV. Athymic female rats have higher plasma E_2 levels than male rats in the vehicle and treatment groups. Circulating plasma E_2 content is measured by ELISA in vehicle, SU5416, SU5416+Treg, chronic hypoxia groups at d21in athymic female and males rats. (n=4 per group). Data are shown as 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 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

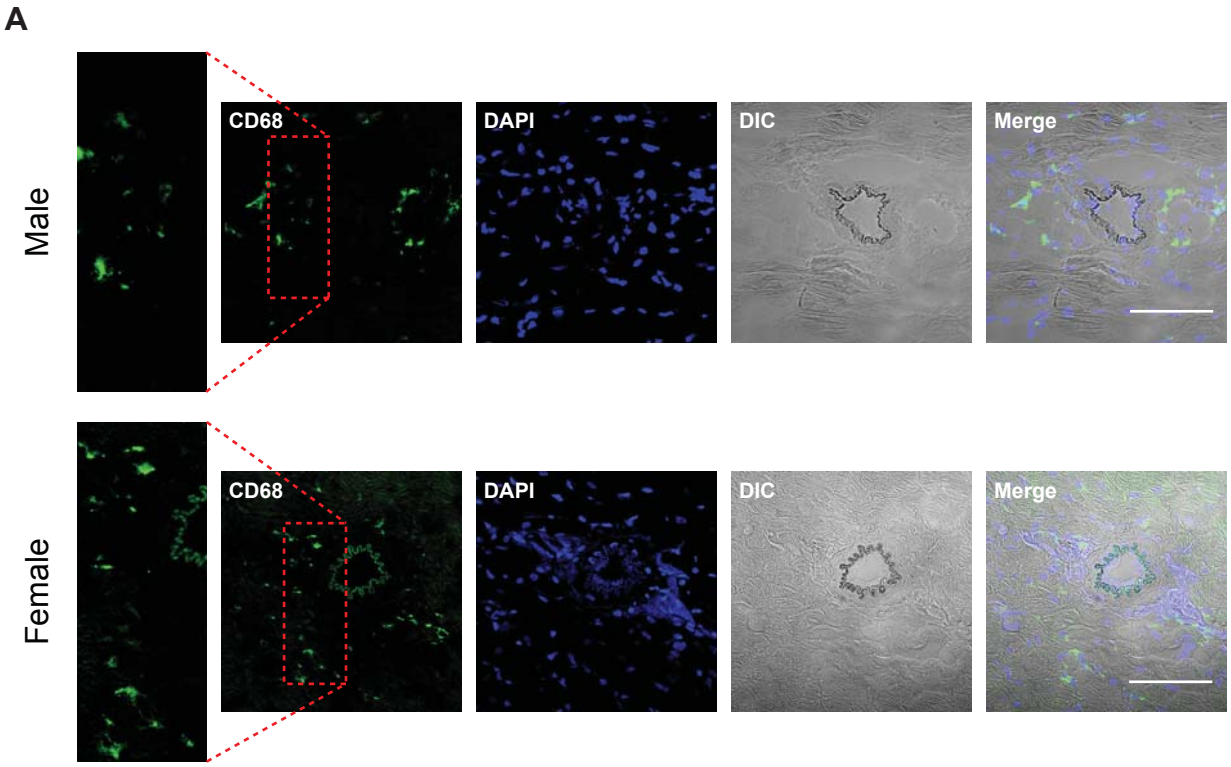
Online Figure VI. Immune reconstitution with Tregs leads to increased COX-2, PGIS, HO-1 and PDL1 expression in in RV tissue of both male and female athymic rats. (A-D) Representative images of RV tissue sections of male and female athymic rats in vehicle and after treatment with SU5416 plus IR with Tregs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

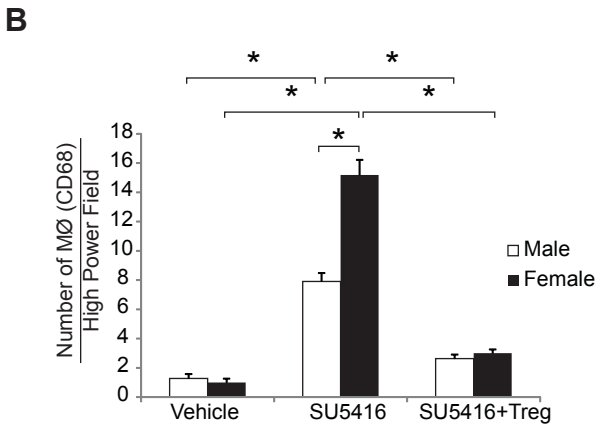
Online Figure VII. IR with Tregs of athymic rats significantly increase expression of COX-2, PTGIS, HO-1 and PD-L1 mRNA in lungs and RV of athymic female and male rats. (A-B)

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.

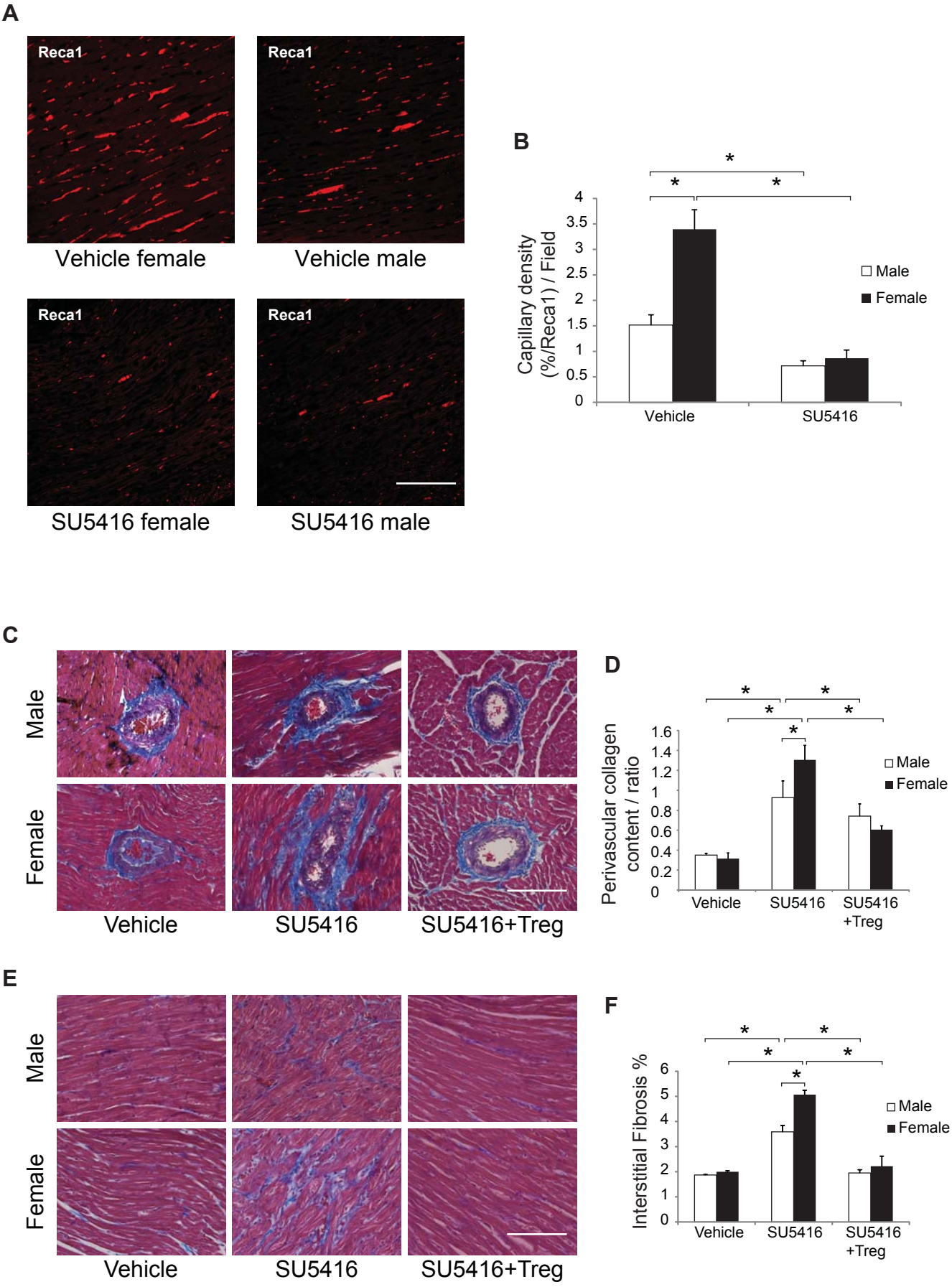


Online Figure I.

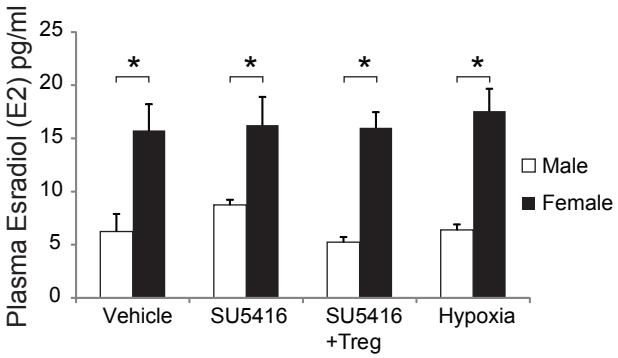




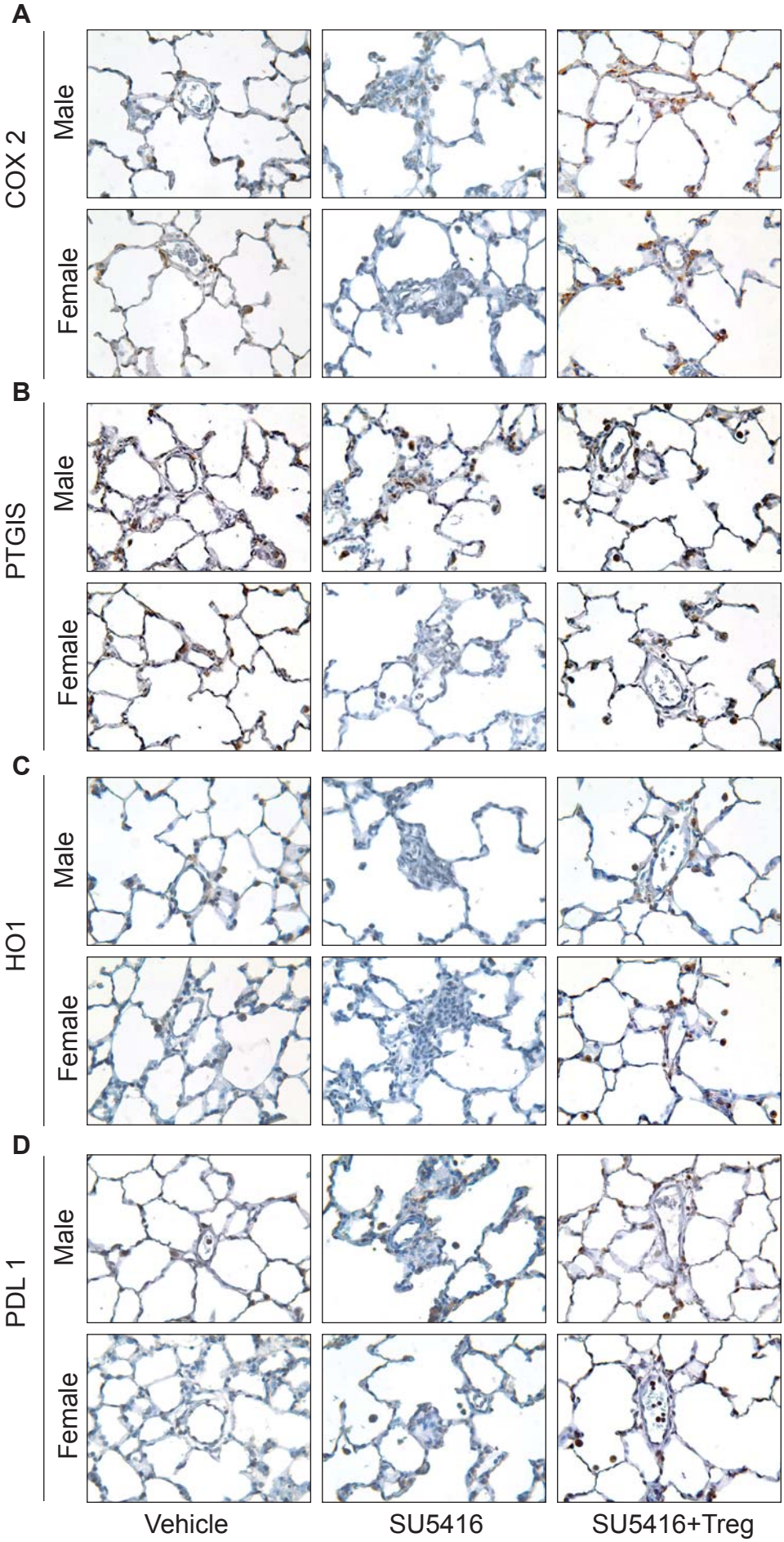
Online Figure II.



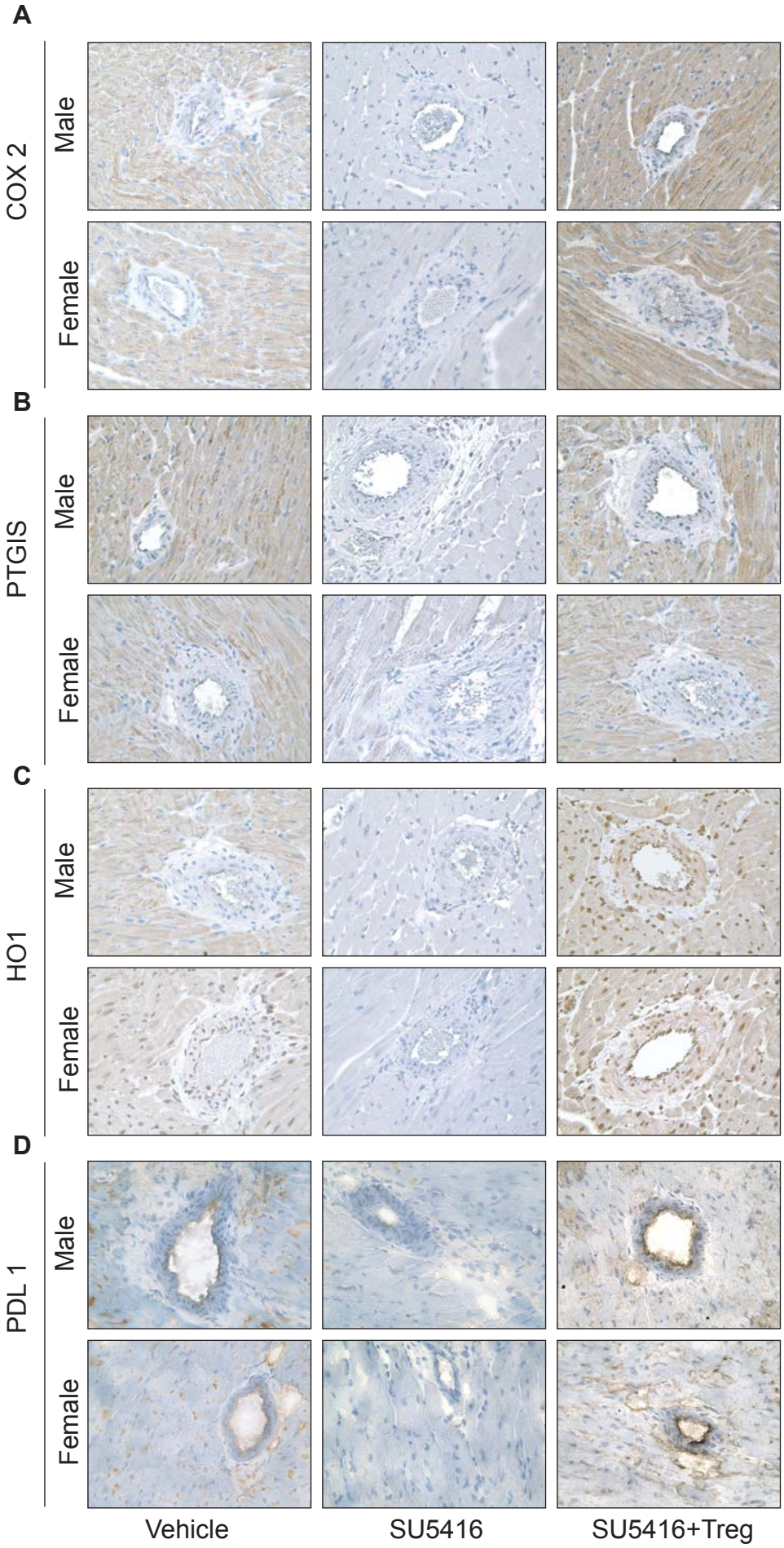
Online Figure III.



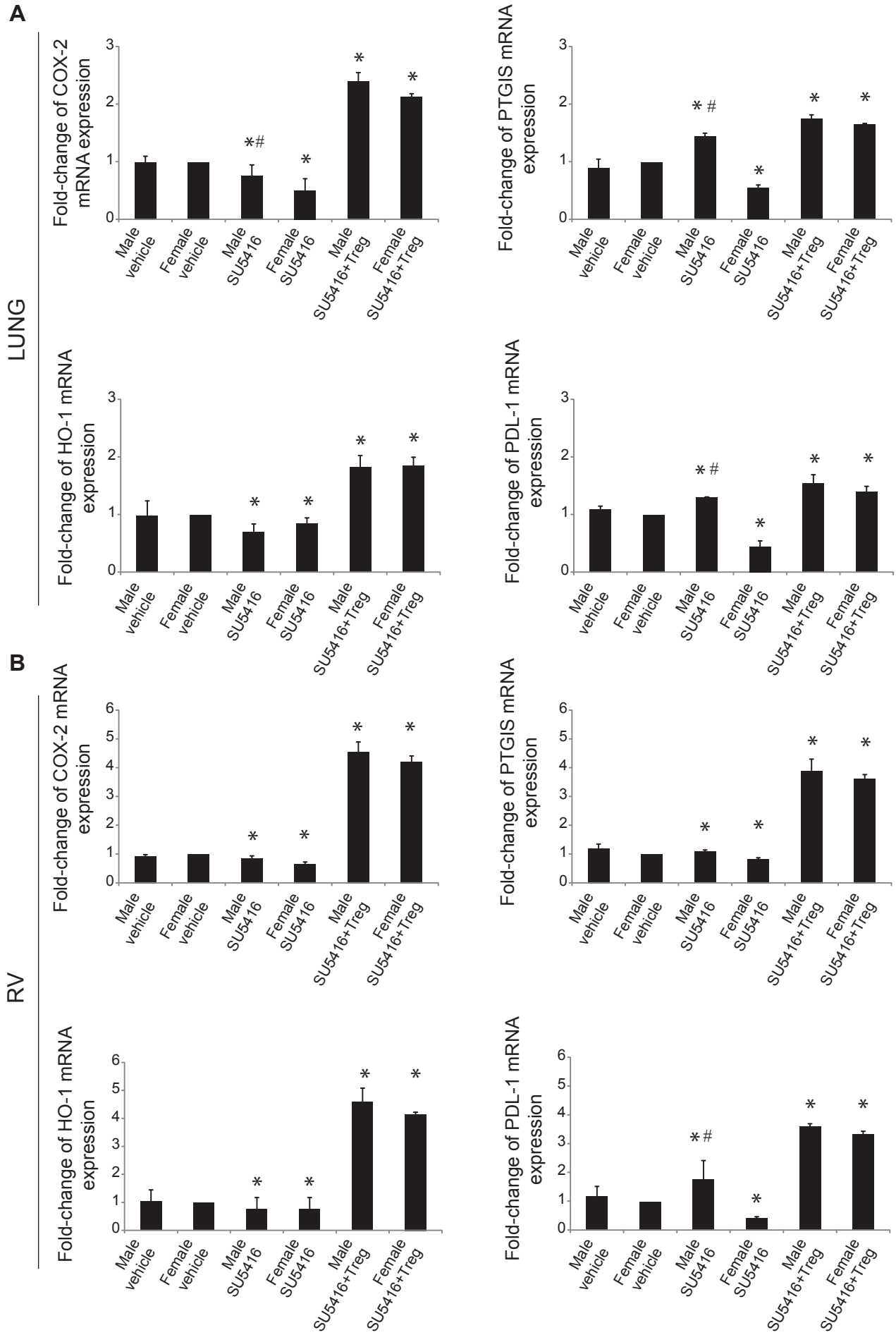
Online Figure IV.



Online Figure V.



Online Figure VI.



Online Figure VII.