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


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 PGI2 (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 PGI2, 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 PGI2 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 PGI2 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 PGI2 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.3-5 Preclinical evidence shows that anti-inflammatory Tregs, generally defined as CD4+CD25high 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 al6). We and others have previously shown that T cell deficient rnu/rnu athymic male rats, which lack Tregs, are particularly susceptible to

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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 PGI₂ (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 PGI₂ 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, PGI₂, were lower in females than males, possibly because of lower levels of the enzymes responsible for PGI₂ 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 PGI₂. Inhibiting COX-2, HO-1, and PDL-1/PD-1 pathways eliminated Treg protection from disease. Tregs directly upregulated endothelial COX-2, PDL-1, HO-1, ERs, and increased PGI₂ 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

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>CALP</td>
<td>calponin</td>
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<tr>
<td>COX-2</td>
<td>cyclooxygenase 2</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
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<tr>
<td>FoxP3</td>
<td>forkhead box P3</td>
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<tr>
<td>HMVEC</td>
<td>human microvascular cardiac endothelial cell</td>
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<tr>
<td>HO-1</td>
<td>heme oxygenase 1</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>IR</td>
<td>immune reconstitution</td>
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<tr>
<td>PD-1</td>
<td>programmed death 1</td>
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<tr>
<td>PDL-1</td>
<td>programmed death ligand 1</td>
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<tr>
<td>PGI₂</td>
<td>prostacyclin</td>
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<tr>
<td>PH</td>
<td>pulmonary arterial hypertension</td>
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<tr>
<td>PTGIS</td>
<td>prostacyclin synthase</td>
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<tr>
<td>RV</td>
<td>right ventricular (or right ventricle)</td>
</tr>
<tr>
<td>Treg</td>
<td>regulatory T cell</td>
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<tr>
<td>VEGFR2</td>
<td>vascular endothelial growth factor receptor 2</td>
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PH.14–15 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, PGI₂ (prostacyclin). We subsequently investigated enzymes responsible for PGI₂ 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 PGI₂, 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 PGI₂ 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.
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Animals
Inbred male and female WAG (RT1u) athymic nude rats (nu/nu) and euthyemic (nu/+ rats were obtained from Biomedical Research Models, Inc, (Worcester, MA) and bred in-house. Euthyemic 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% O2) in an air tight plexiglass hypoxia chamber with simulated oxygen controller (Biospherix; ProOx110, Parish, NY). Athymic animals maintained in normoxic conditions (21% O2) 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 Diego, 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-20560) 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; I4A; number ab8718), 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 (MH12; number 17-5983-42, eBioscience, San Diego, CA), anti–HO-1 FITC (HSP32; number NBPI-77460F, NovusBiologicals, Littleton, CO); anti–ER-alpha and Alexa Fluor 488 donkey anti-mouse IgG, Cy3 conjugated donkey anti-rabbit IgG conjugated secondary antibodies were anti-HO-1 (1:200), anti-PDL-1 (1:200), anti–α-smooth muscle actin (SMA; 1:100) followed by Alexa Fluor 488 conjugated donkey anti-mouse IgG, Alexa Fluor 647 conjugated donkey anti-mouse IgG, Cy3 conjugated donkey anti-rabbit IgG conjugated secondary antibodies (1:600) and Apoptosis detection kit. 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 (DSH5: 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 Fluor 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 F1x (number 515211, Cayman Chemical, Ann Arbor, MI), and IL-10 (number 100764, Abcam, Cambridge, MA).

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 XR+ (Bio-Rad, Hercules, CA) and data are expressed as densitometric units determined using ImageJ (version 1.48) software.

Immunofluorescence Imaging
Rat lungs were inflamed with a 1:1 mixture of optimal cutting temperature compound and 30% sucrose and embedded in Tissue Tek optimal cutting temperature compound and 30% sucrose and embedded in Tissue Tek optimal cutting temperature compound and 30% sucrose and embedded in Tissue Tek optimal cutting temperature compound and 30% sucrose and embedded in Tissue Tek optimal cutting temperature compound and 30% sucrose and embedded in Tissue Tek optimal cutting temperature compound and 30% sucrose and embedded in Tissue Tek 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 (DSH5: 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 Fluor 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).

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 means±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
VEGFR2 antagonist, SU5416 because of the absence of anti-inflammatory CD4<sup>+</sup>CD25<sup>high</sup> FoxP3<sup>+</sup> Tregs. 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<sub>2</sub>; P<sub>ao</sub>≈40 mm Hg; Online Figure I). SU5416-treated PH lungs exhibited a greater CD68<sup>+</sup> 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, IR with CD4<sup>+</sup>CD25<sup>high</sup>Tregs (3×10<sup>6</sup> IV 7 days before SU5416 treatment) prevented PH 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<sup>16</sup> 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<sub>2</sub> 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<sub>2</sub>, a potent vasodilator that exerts multiple vasoprotective effects, and the enzymes most responsible for its synthesis.<sup>17</sup> PGI<sub>2</sub> has a half-life of 42 seconds and is broken down into 6-keto-prostaglandin F1α, a metabolite that is used to estimate systemic levels. PGI<sub>2</sub> levels were assessed 21 days after treatment with vehicle or SU5416 with or without Treg IR. Plasma PGI<sub>2</sub> levels were decreased in females with PH suggesting that reduced PGI<sub>2</sub> synthesis may contribute to worse PH in Treg-deficient females (Figure 2A). A 4-fold increase in the PGI<sub>2</sub> 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<sub>2</sub> biosynthesis, we evaluated COX-2 and PTGIS. Consistent with lower PGI<sub>2</sub> blood levels in females, Western blot analysis of male and female PH lungs analyzed at day 21 post-SU5416 treatment demonstrated relatively decreased 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<sub>2</sub> levels. Thus, lower pulmonary COX-2 and possibly lower pulmonary PTGIS, in athymic females provides one explanation of why females have lower circulating PGI<sub>2</sub>. Collectively, these results show that more severe PH in females, occurring in the absence of...
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. 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, 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.
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. 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-mediated 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. In the athymic rat model, RV changes are not readily detectable by echocardiography before day 10 post SU5416 administration, 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 PGI2 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, PGI2, and ER Surface Expression in HMCECs and Increase Culture Supernatant Concentrations of PGI2 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. Purified human CD4+CD25high Tregs were cocultured with HMCECs (12 hours; 37°C). HMCECs, co-cultured 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 prostanoioids, including PGI2. HMCECs were cultured with Tregs (12 hours; 37°C) in the presence or absence of inhibitors for ERs (ICI 180, 78024), COX-2, and HO-1, and the culture supernatant was assayed for the PGI2 metabolite 6-keto-prostaglandin F1α. Blocking ERs, COX-2, and HO-1 significantly reduced supernatant PGI2 levels (Figure 7F). Further, because PGI2 is known to stimulate a key anti-inflammatory Treg cytokine, IL-10, we also evaluated
its expression under similar conditions.\textsuperscript{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\textsubscript{2}, and IL-10 biosynthesis in cardiac, vascular endothelial cells.

**Discussion**

Treg abnormalities are a clinical feature of PH conditions.\textsuperscript{3-8} Although increased numbers of Tregs are observed in the blood of PH patients,\textsuperscript{6} reduced Tregs are observed in the lungs of these individuals.\textsuperscript{26} Idiopathic PH is associated with an expansion of peripheral blood Tregs that are nonsuppressive,\textsuperscript{4} and PH associated with connective tissue disorders exhibit increased Th17/Treg ratios.\textsuperscript{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.\textsuperscript{7} Tregs limit pulmonary vascular injury that can cause experimental PH.\textsuperscript{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\textsuperscript{27} based on pulmonary artery endothelial injury in contrast to the hypoxia model which occurs as a consequence of sustained pulmonary

![Figure 4](http://circres.ahajournals.org/)

**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).
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 males35; 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. 23,39,40 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 directly affect Treg immunomodulatory function, HO-1 and PDL-1. HO-1 is an anti-inflammatory molecule that also helps mediate Treg function20 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
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. 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. 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 PGI2 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 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 (ll) 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 when daily 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 (ll) 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).
IR. The mechanism by which Tregs induce PGI2 synthesis is unknown with both direct and indirect interactions between Tregs and PGI2-producing endothelial cells being potentially involved. This possibility is supported by the finding that vascular COX-2 is a major contributor to systemic PGI2 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 PGI2 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-155,56 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+FoxP3+Tregs, but not CD4+CD25+ T cells, induce PGI2 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.

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 PGI2 (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).
via ER-α signaling. Indeed, it has been suggested that the protective effects of estrogen in autoimmune conditions may be because of estrogen-mediated Treg expansion and activation. However, the effect of sex hormones on immune cells in health may differ from that occurring during vascular injury and disease. Conversely, Treg-mediated upregulation of ERs on HCMCs 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, PGI2, 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, 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 PGI2 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 PGI2 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...
PGI2 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 disregulation 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 models, a notable pulmonary infiltration of Tregs occurs, and 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,64 vulnerable PH patients represent a particularly promising target population for this and other immunomodulatory therapies.

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Disclosures
None.

References


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


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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 RNaseasy 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.

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**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 lymphocyte 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% O₂, 5% CO₂) 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^hi^ 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% CO₂ in 24-well flat-bottom plates (#3524, Corning, NY). Activated human CD4^+^CD25^hi^ 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^hi^ 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:**

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 capillaries (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 E2 levels than male rats in the vehicle and treatment groups. Circulating plasma E2 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 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 VI.
Online Figure VII.

Fold-change of PTGIS mRNA expression

Fold-change of COX-2 mRNA expression

Fold-change of PDL-1 mRNA expression

Fold-change of HO-1 mRNA expression

LUNG

RV