Pulmonary arterial hypertension (PAH) is an incurable disease associated with viral infections and connective tissue diseases. The relationship between inflammation and disease pathogenesis in these disorders remains poorly understood.

**Rationale:** Pulmonary arterial hypertension (PAH) is an incurable disease associated with viral infections and connective tissue diseases. The relationship between inflammation and disease pathogenesis in these disorders remains poorly understood.

**Objective:** To determine whether immune dysregulation due to absent T-cell populations directly contributes to the development of PAH.

**Methods and Results:** Vascular endothelial growth factor receptor 2 (VEGFR2) blockade induced significant pulmonary endothelial apoptosis in T-cell-deficient rats but not in immune-reconstituted (IR) rats. T cell–lymphopenia in association with VEGFR2 blockade resulted in periarteriolar inflammation with macrophages, and B cells even prior to vascular remodeling and elevated pulmonary pressures. IR prevented early inflammation and attenuated PAH development. IR with either CD8 T cells alone or with CD4-depleted spleen cells was ineffective in preventing PAH, whereas CD4-depleting immunocompetent euthymic animals increased PAH susceptibility. IR with either CD4+CD25hl or CD4+CD25– T cell subsets prior to vascular injury attenuated the development of PAH. IR limited perivascular inflammation and endothelial apoptosis in rat lungs in association with increased FoxP3+, IL-10- and TGF-β-expressing CD4 cells, and upregulation of pulmonary bone morphogenetic protein receptor type 2 (BMPR2)–expressing cells, a receptor that activates endothelial cell survival pathways.

**Conclusions:** PAH may arise when regulatory T-cell (Treg) activity fails to control endothelial injury. These studies suggest that regulatory T cells normally function to limit vascular injury and may protect against the development of PAH. (Circ Res. 2011;109:867-879.)

**Key Words:** pulmonary arterial hypertension • inflammation • regulatory T cell • bone morphogenetic protein receptor type 2

Pulmonary arterial hypertension (PAH) is a frequently lethal disease of relatively mysterious origins. For over 50 years it has been recognized that autoimmune phenomena are associated with certain forms of PAH, but it has never been previously demonstrated that autoimmunity, itself, may be a root cause for PAH. Previous work by our group and others demonstrated that T-cell immunity is involved in PAH pathogenesis, but no study has yet sought to explain how immune dysregulation directly causes PAH. Human and rat lung PAH pathology demonstrate occlusive arterioles and immune cell aggregates comprised of macrophages, mast cells, B and T lymphocytes, and antiendothelial cell antibodies (AECAs) adherent to injured vasculature. Conditions commonly associated with PAH, such as systemic sclerosis and HIV, are associated with abnormalities in CD4 T-cell function and exhibit a similarly inflamed pulmonary vascular pathology. To date, it has not been clear whether inflammation surrounding the pulmonary vasculature is primarily the cause or the consequence of vascular remodeling. Given that athymic animals, which are congenitally T-cell deficient, are also particularly susceptible to the development of experimental PAH, we questioned whether inappropriately exuberant inflammation due to the lack of normal immune regulation could be a key predisposing factor for the development of this disease in these animals.

Injury resolution is an established function for a subset of T cells with strong regulatory activity, commonly referred to as Tregs. These cells typically express the coreceptor CD4+. 

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Our group and others have demonstrated that the athymic rat develops severe PAH following vascular injury induced by monocrotaline (MCT) or VEGFR2 blockade (SU5416).6–8 Prior studies utilizing athymic rats were limited by the availability of inbred animals, which precluded an evaluation of how immune dysregulation could account for the PAH diathesis.7 In this study, we administered SU5416 to genetically inbred athymic animals and performed immune reconstitution (IR) experiments with MHC-identical cells to assess, for the first time, the role of immune regulation in the development of PAH.

Methods

A detailed Methods section can be found as an online supplement available at http://circres.ahajournals.org.

Animal Model

The experimental protocol was approved by the Veterans Affairs Palo Alto Animal Care and Use Committee. Inbred WAG (RT1u) ATHYMIC nude rats (nu/nu) and euthymic (nu/+ ) rats were utilized for these studies (Biomedical Research Models, Inc., Worcester, MA). Euthymic rats served as MHC-identical controls and as cell donors for adoptive transfer experiments. Six to 8-week-old animals were injected s.c. with a single dose of VEGFR2 antagonist SU5416 (20 mg/kg) dissolved in either DMSO or DMSO (vehicle) alone. This dose was previously determined to be effective for inducing PAH in athymic rats.7 SU5416 was synthesized utilizing a previously described modified protocol.18 All animals were maintained in normoxic conditions.

Immune Reconstitution

We injected 20×10⁶ unfractionated spleen cells obtained from euthymic male rats into athymic rats i.p. 7 days prior to SU5416 administration except for the time-course experiment in which they were injected at d−7, d−5, d−3, d0, d3, d5, d7, and d10, with d0 being the day of SU5416 administration. For CD4⁺ and CD8⁺ T cell reconstitutions 20×10⁶ cells were administered i.v. to athymic rats, and for CD4⁺ CD25⁻ and CD4⁺ CD25⁺ cell reconstitutions, approximately 3×10⁶ cells were administered i.v. to athymic rats 7 days prior to SU5416 administration.

Cell Isolation and Purification

Isolation and Purification of CD4⁺ and CD8⁺ T Cells

Spleens from inbred euthymic rats were processed and filtered through a 100-µm cell strainer to obtain a single cell suspension, washed with DMEM, and then counted. CD4⁺ T cells were purified by labeling B cells, NK cells, CD8⁺ T cells, CD68⁺ cells, and mast cells with the following purified antibodies (all of which were mouse IgG1 isotype): anti-CD45RA (OX-33), anti-CD161a (10/78), anti-CD8a (OX8), antima-st (AR32AA4) (all BD Pharmingen, San Diego, CA), and anti-CD68 (ED-1) (AbD Serotec, Raleigh, NC). The labeled splenocytes were then incubated with rat antimonos IgG1 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s description. Live cells were recovered using the magnetic activated cell sorting (MACS) system with magnetic LS columns (Miltenyi Biotec). The collected negative fraction and enriched CD4⁺ T cells were further purified with a cell sorter (FACSaria) (BD Bioscience). The average purity was >98%. The same procedure was used to isolate CD8⁺ T cells except that instead of labeling the splenocytes with anti-CD8a prior to incubation with microbeads, they were labeled with purified anti-CD4 (OX-38) (BD Pharmingen) (average purity >98%). To obtain the CD4⁺ T cell depleted splenocytes, we incubated the splenocyte suspension with anti-CD4 (OX-38 and OX-35) antibodies (BD Pharmingen), and then the negative fraction was isolated with microbeads as described above.

Isolation and Purification of Tregs (CD4⁺CD25⁺) and CD4⁺CD25⁻ Cells

Spleens were removed from inbred euthymic rats and prepared as a single cell suspension as described above. CD4⁺ T cells were

the transcription factor Forkhead box protein 3 (FoxP3), and highly express the IL-2 receptor-α chain (ie, CD25hi). In addition to these classical Treg populations, rats also exhibit inflammatory injuries such as burns and infection.13–15 In the comparison with other PAH models, which require chronic hypoxia or a surgical pneumonectomy, T-cell immunodeficiency renders these athymic animals particularly sensitive to the development of severe PAH under normoxic conditions. The athymic rat is the result of an autosomal recessive mutation on the rnu locus (rnu/rnu) of chromosome 10 leading to T-cell deficiency, thymic aplasia, and hairlessness.17 Following MCT or SU5416 administration, athymic rats of different genetic backgrounds develop significant RV remodeling, perivascular inflammation, and occlusive arteriolar lesions that are similar to lesion observed in severe clinical PAH.6–8 Prior studies utilizing athymic rats were limited by the availability of inbred animals, which precluded an evaluation of how immune dysregulation could account for the PAH diathesis.7 In this study, we administered SU5416 to genetically inbred athymic animals and performed immune reconstitution (IR) experiments with MHC-identical cells to assess, for the first time, the role of immune regulation in the development of PAH.

Non-standard Abbreviations and Acronyms

AECAs: anti-endothelial cell antibodies
ANP: atrial natriuretic peptide
AU: arbitrary units
beta-MHC: beta-myosin heavy chain
BMPR2: bone morphogenetic protein receptor type 2
BNP: brain natriuretic peptide
Ca2⁺: ATPase 2a
DIC: differential interference contrast
dP/dt: maximal rate of pressure increase
ESPVR: end systolic pulmonary pressure volume relations
EDPVR: end diastolic pulmonary pressure volume relations
FoxP3: Forkhead box protein 3
IL-6: interleukin 6
IR: immune reconstitution
LV: left ventricle
LVEDP: left ventricle end diastolic pressure
MCT: monocrotaline
mPAP: mean pulmonary artery pressure
PAAT: pulmonary artery acceleration time
PADia: pulmonary artery diameter
PAH: pulmonary arterial hypertension
RSVP: right ventricle systolic pressure
RV: right ventricle
RVEDP: right ventricle end diastolic pressure
RVH: right ventricular hypertrophy
RVU: relative volume units;
S: septum
SERCA2a: sarcoplasmic reticulum
SkAct: skeletal β-actin
TNF-α: tumor necrosis factor α
Treg: regulatory T cell
VEGFR2: VEGF receptor 2

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purified as described above. To obtain CD4+CD25hi and CD4+CD25lo subsets, pure CD4+ T cells were labeled with mouse anti-CD25-PE antibody (OX39) (BD Pharmingen), followed by incubation with anti-PE microbeads. LS columns (Miltenyi Biotec) were used with the MACS system to fractionate the 2 populations. Both cell fractions were then sorted with a fluorescent activated cell-sorting machine, FACS Aria (BD Biosciences). The average purity obtained was >97.5%. Expression of FoxP3 in both of these populations was assessed by using the FoxP3 staining set buffers (ebiScience, San Diego, CA) for intracellular staining with anti-FoxP3 antibody (150D) (Biolegend, San Diego, CA) according to the manufacturer’s protocol. These cells were costained with antibodies to CD4 (OX-38), CD8a (OX8), CD25 (OX39), IL-10 (A5–4), CD45RA (OX-33) (all BD Pharmingen), and TCR (R73) (ebiScience). Flow cytometry was performed on a FACS Calibur (BD Biosciences), and the data were analyzed with CellQuest software (BD Biosciences).

Hemodynamic Measurements
The rats were anesthetized with ketamine hydrochloride (70 mg/kg) and xylazine (10 mg/kg) injected i.p. before right and left 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 RV. Signals were recorded continuously with a TC-510 pressure control unit (Millar Instruments) coupled to a Bridge Amp (AD Instruments). Data were collected with the Powerlab4/30 data acquisition system (AD Instruments) and analyzed with Chart Pro software (AD Instruments). LV combined pressure–volume signals were recorded by insertion of a pressure–volume conductance (PV) catheter (model SPR-838, 2F) (Millar Instruments) through the carotid artery into the LV. Subsequently, a median sternotomy was performed, the pericardium was opened, and the PV catheter was introduced into the RV for measurements of RV pressure loops and pulmonary artery pressures. The PV catheter was connected to a MPVS Ultra single segment pressure volume system (Millar Instruments), and signals were recorded digitally. All data were acquired and analyzed with Chart Pro software (AD Instruments). 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 RVH.

Statistical Methods
GraphPad Prism® was used for statistical analysis. Differences between multiple groups were compared using 1-way analysis of variance with Bonferroni multiple-comparisons test for post hoc analyses. For datasets with nonparametric data, multiple groups were compared using the Kruskal–Wallis test, and Dunn’s multiple comparisons test was used for the post test analysis. For the cytokine, histological, and Western blot analyses, unpaired t tests assuming unequal variances were utilized, with the comparisons being negative for athymic control versus athymic SU and athymic SU versus euthymic IR-SU groups. All data are represented as means ± SEM, and probability value <0.05 is considered significant.

Results
IR of Athymic Rats With MHC-Identical Splenocytes Attenuates the Development of PAH When Injected Prior to SU5416 Administration
We first investigated whether inbred athymic rats exhibited more profound PAH than did euthymic rats on a WAG strain (RT1u) genetic background. The heterozygous mru/+ euthymic rats used in this study are T cell replete and are MHC identical to the athymic rats; a characteristic that makes them suitable as T-cell donors for the athymic rat. Right ventricle systolic pressures (RVSPs) were used to estimate PAH. SU5416-treated athymic rats developed severe PAH and right ventricular hypertrophy (RVH) in comparison with immune-replete SU-5416-treated euthymic rats, and vehicle (DMSO)–treated athymic controls. Athymic rats underwent IR with 20×106 spleen cells 7 days prior to SU5416 administration to test whether IR with splenocytes from euthymic donors was protective against the development of PAH; IR was later confirmed by flow cytometry of peripheral blood 7 days after cell injection. IR prevented the development of PAH (Figure 1A and 1B). PAH was associated with histologically evident pulmonary vascular remodeling, which was prevented by IR (Online Figure I). IR led to a larger percentage of vessels being open in comparison with athymic SU5416 animals (Online Figure II). Additionally, pressure–volume studies were performed; the RVSP and dp/dt max data from these studies supported the hypothesis that athymic SU5416 animals develop significant PAH and that the PAH is minimal in IR-athymic SU5416 animals (Online Figure III; Online Tables I and II).

Animals from the 3 athymic treatment groups were compared to confirm PAH by echocardiography. SU5416-treated athymic rats had significantly faster pulmonary artery acceleration times (PAAT) (Figure 1C), and an expanded pulmonary artery diameter (PADia) (Figure 1D), parameters all indicative of worsening PAH. No significant echocardiographic left ventricle differences were noted between the 3 groups (data not shown). Athymic-SU5416-treated rats underwent IR at multiple time points in relation to SU5416 administration to address whether IR protection from PAH required that the reconstituted cells be present close to the time of SU5416-mediated vascular injury; they were evaluated at d72 (Figure 2). IR became progressively less effective the later it was performed in relation to vascular injury. These results suggested that the protection conferred by IR involves controlling pathological events soon after the induction of vascular injury by SU5416.

Early Pulmonary Inflammation Precedes Vascular Remodeling and PAH
To assess the possibility that regulating early inflammation was responsible for the protective effect of IR, we evaluated animals d7 after SU5416 administration. Inflammation with macrophages and B cells was pronounced by d7 (Figure 3A through 3D). Notably, PAH was not detected by right heart catheterization or echocardiography prior to 10 days, nor was there evidence of histological evidence of vascular remodeling at this early time points. To determine whether there was evidence of systemic inflammation, we next evaluated 2 key cytokines that have been previously implicated in the development of PAH: IL-6 and TNF-α. Serum was obtained from animals 7 and 21 days after SU5416 or vehicle treatment and evaluated by ELISA. PAH was associated with high-serum IL-6 and TNF-α levels, and was suppressed by IR (Figure 3E and 3F).

Early Migration of Putative Tregs to Lungs in IR Animals
Given that the systemic anti-inflammatory effect that IR had on SU5416-treated animals was detected within 7 days of SU5416 administration, we next investigated whether anti-inflammatory Tregs could be detected in the lungs and blood of reconstituted animals at the time when the anti-inflammatory effect was noted. Putative Treg cells (CD4+CD25+FoxP3+; CD4+IL-10+) were readily detected in periarteriolar areas and peripheral blood of IR
athymic rats 7 days after IR (Figure 4A through 4D; Online Figure V). Because TGF-β is an important cofactor expressed by Tregs to maintain self-tolerance following vascular injury,21,22 we subsequently investigated the expression of TGF-β as well as phosphorylated Smad2 and Smad3 (pSmad2/3), which are cytosolic proteins involved in TGF-β signaling. Lungs of IR-athymic rats demonstrated CD4+ TGF-β pSmad2/3 cells, and Western blot analysis confirmed increased pSmad2 levels in IR athymic animals (Figure 4E through 4I). This early accumulation of putative Treg cells expressing the anti-inflammatory cytokines, TGF-β and IL-10, correlated with the suppression of pulmonary and systemic inflammation (Figure 3) observed in reconstituted rats.

**CD4+ CD25+ and CD4+CD25− Subsets Are Individually Sufficient to Confer Protection Against the Development of PAH**

We next sought to determine which cellular subset of spleen cells used for IR was essential for the prevention of vascular injury and PAH. Although the athymic rat lacks T cells, it has a normal complement of bone-marrow-derived B cells. Although T-dependent responses such as transplant rejection and hypersensitivity reactions are impaired in these rats, the innate immune response is intact, including mast cells, NK cells, macrophages, and neutrophils.17 Therefore, the assumption was that a missing T cell subset (absent in athymic animals) was responsible for these rats’ predisposition for disease. We hypothesized that a putative CD4+ Treg population was the essential T-cell subset. To rule out that CD8 T cells conferred protection against the development of PAH, we next immune-reconstituted animals with $20 \times 10^6$ fractionated CD8+ T cells 7 days prior to SU5416 administration and assessed the rats at d21. IR was confirmed by flow cytometry of blood at 7 days post SU5416. CD8+ T cell reconstitution was nonprotective (Figure 5A and 5B). Next, to rule out the possibility that other spleen cell populations besides CD4+ T cells were conferring protection, we performed IR with CD4-depleted spleen cells. As with CD8+ T cell reconstitutions, depletion of CD4+ cells from the spleen cell inoculums prior to IR eliminated the ability of adoptively transferred splenocytes to block PAH and further confirmed that the protective subset of cells was CD4+ (Figure 5C and 5D).

Although we established that it is possible to uniquely generate PAH in rats under normoxic conditions that have a genetic absence of T cells, we thought that the relevance of the general model would be strengthened if an immunologically normal rat exposed to simultaneous vascular injury and CD4 depletion would also develop PAH; the immune system of animals devoid of all T cells from birth may develop in unconsidered ways. For this reason, we investigated immunologically intact euthymic rats, which are normally refractory to developing significant PAH with SU5416 therapy in normoxic conditions (Figure 1). Administration of a CD4-depleting monoclonal antibody, which eliminated $\geq 98\%$ of circulating CD4+ T cells prior to SU5416 administration, rendered these animals susceptible to the development of PAH (Figure 5E and 5F; Online Figure VI). CD4-depleted animals developing PAH demonstrated increased perivascular inflammation with macrophages and B cells (Online Figure VIA and VIB). These studies...
cumulatively demonstrated that a population of CD4$^+$ T cells was required to prevent PAH.

To determine whether CD4$^+$ cell subsets were differentially protective, we reconstituted athymic rats with either CD4$^+$CD25hi cells or CD4$^+$CD25lo cells. Cell subset purity following isolation was $\approx98\%$. CD4$^+$CD25$^+$FoxP3$^+$ cells were detected in the blood 21 days after SU5416 (Online Figure VII) in both CD4$^+$CD25hi-reconstituted athymic rats (4.8% of circulating CD4$^+$ cells) and CD4$^+$CD25lo-reconstituted animals (1.5% of circulating CD4$^+$ cells), the latter finding being consistent with a peripheral conversion of CD4$^+$CD25lo cells into CD4$^+$CD25hi cells.23 Both CD4 T cell subsets attenuated the development of PAH (Figure 5G and 5H). Therefore, in this rat model, both CD4$^+$CD25hi cells and CD4$^+$CD25lo cells exhibited regulatory activity.

**IR Prevents Vascular Apoptosis**

Because PAH induced by SU5416 has been attributed to the death (by apoptosis) of vascular endothelial cells,24 we evaluated whether IR also attenuated this process. The presence of apoptotic cleaved caspase 3$^+$CD31$^+$ cells was first noted in SU5416-treated animals 7 days after SU5416 administration (Figure 6A). Vascular apoptotic cells was more marked by d21 when CD31 staining is minimal, presumably due to prior endothelial cell death (Figure 6B). In established PAH, apoptotic cells within the vascular wall were notably CD31$^+$. Evidence of widespread pulmonary apoptosis was also demonstrated by increased lung cleaved caspase 3 protein levels in nonreconstituted SU5416 animals on day 7 (Figure 6C and 6D) and day 21 (Figure 6E and 6F). A morphometric assessment of cleaved caspase 3$^+$ cells per vessel demonstrated relatively fewer apoptotic cells per vessel in IR-athymic rats (Figure 6G).

**IR Results in Upregulation of Pulmonary BMPR2**

To further evaluate why IR was protective against endothelial apoptosis, we next investigated BMPR2 levels in the 3 treatment groups. Given that BMPR2 is a protein characterized as vascular-protective in health and vascular-defective in PAH,25 we tested whether IR impacted the expression of this receptor in lung tissue. Notably, all 3 groups exhibited rare BMPR2-expressing macrophages (Figure 7A). Global lung tissue BMPR2 levels appeared elevated in athymic IR-SU by immunohistochemistry (Figure 7B). BMPR2-expressing cells were frequently noted adjacent to CD4$^+$ cells in immune-reconstituted animals (Figure 7C). Vascular BMPR2 expression in athymic IR-SU was notably elevated (Figure 7D and
7E). By Western blot analysis of whole lysates, BMPR2 expression was significantly increased in athymic IR-SU animals (Figure 7F and 7G). Thus, the protection conferred by immune reconstitution in this PAH model was strongly associated with upregulation of pulmonary BMPR2.

Discussion

The observations that T-cell-deficient athymic rats develop particularly exaggerated PAH6–8 and that immunodeficiency itself promotes autoimmunity26 suggested that restoring a missing lymphocyte population could prevent PAH by limiting inflammation. Here we demonstrate for the first time that IR attenuates early inflammation following vascular injury induced with a VEGFR2 inhibitor and is protective against the subsequent development of PAH. IR of athymic animals was effective only if administered prior to SU5416 administration. This finding is consistent with the idea that in normal, immunocompetent animals, the early promotion of injury resolution by Tregs suppresses vascular inflammation and prevents PAH. In PAH development in the SU5416 model, ongoing pulmonary vascular endothelial injury and apoptosis is followed by a progressive proliferative phase of phenotyp-
ically abnormal luminal cells; coupled with vascular smooth muscle hypertrophy and vasoconstriction, these pathological changes significantly increase the pulmonary vascular resistance and cause PAH.27 This process is modeled in Figure 8.

Inflammation in T-cell-deficient animals was notable for an accumulation of B cells and macrophages within 1 week of SU5416 administration. This finding is concordant with the fact that the loss of self-tolerance in animals missing normal Treg
tory T cells are not restricted to regulation of the adaptive B-cell tolerance. Moreover, recent data demonstrate that regulatory activity in the CD4 T cell subset, CD4<sup>+</sup>/CD25<sup>+</sup> cells, systemic autoimmunity occurs as mediated by high levels of these cytokines. Thus a loss of Treg-mediated self-tolerance leads not only to a loss of T-cell tolerance but also to a breakdown in B-cell tolerance. Moreover, recent data demonstrate that regulatory T cells are not restricted to regulation of the adaptive immune system but also affect the activation and function of innate immune cells including monocytes, macrophages, dendritic cells, and NK cells. Exuberant inflammation following vascular injury in our model can therefore be explained by a loss of self-tolerance in the absence of normal immune regulation. We determined that both CD4<sup>+</sup>/CD25<sup>+</sup> and CD4<sup>+</sup>/CD25<sup>+</sup> populations were sufficient for IR-mediated protection from PAH. Although CD4<sup>+</sup>/CD25<sup>+</sup> are well described as the Tregs in mice, rats exhibit additional Treg populations in comparison with mice. In rats, coexpression of CD25 on CD4 T cells does not consistently identify all CD4 cells with regulatory function. Several studies demonstrate regulatory activity in the CD4<sup>+</sup>/CD25<sup>+</sup> population in rats, and this population also appears to have regulatory activity in the current model. We also present evidence that immune reconstituted CD4<sup>+</sup>/CD25<sup>+</sup> cells may have been converted to CD4<sup>+</sup>/CD25<sup>+</sup> Tregs, as has been previously described. Whether it is this conversion in the periphery or the fundamental nature of rat CD4<sup>+</sup>/CD25<sup>+</sup> subset, these cells clearly demonstrated regulatory activity. The identification of Tregs as a crucial component of self-tolerance in other diseases has led to major advances in the understanding of how immune injury in autoimmunity and transplantation can be initiated when either insufficient numbers of Tregs or compromised Treg function occurs.

TNF-α and IL-6 were elevated in the serum of rats developing PAH. TNF-α overexpression augments PAH. IL-6, a proinflammatory and vasodilator cytokine, has been shown to have a significant association with mortality in patients with PAH. Increased serum and plexiform lesion levels of IL-6 have been found in PAH patients as well as in IL-6 Tg mice with replicative pathophysiological changes of PAH. IL-6 has also recently been found to negatively regulate TGF-β/BMP signaling cascade. The current study demonstrates that Tregs limit the systemic liberation of IL-6 and TNF-α, a finding that lends credence to the concept that Tregs have therapeutic potential in immune-mediated inflammatory diseases characterized by high levels of these cytokines.

Although prior studies have described TGF-β in relation to the vascular endothelium in PAH (eg, Zaiman et al), the current study highlights how active TGF-β may also be an important cofactor expressed by Tregs to maintain self-tolerance following vascular injury. The proposed mechanisms by which Tregs control inflammation are numerous and involve several basic mechanisms including (1) secretion of anti-inflammatory cytokines (most notably TGF-β and IL-10), (2) cytolsis of effector T cells, (3) metabolic disruption of effector cells (for example, by serving as an IL-2 “sink” due to high Treg surface concentrations of the IL-2 receptor protein), and (4) modification of dendritic cell function (such as through the induction of the immunosuppressive enzyme indoleamine 2,3-dioxygenase). A striking accumulation of putative Tregs on day 7, including CD4<sup>+</sup>/FoxP3<sup>+</sup> and CD4<sup>+</sup>/TGFβ<sub>pSMAD2/3</sub>, CD4<sup>+</sup>/IL-10<sup>+</sup> cells, were noted in only the IR-SU5416 lungs and were concentrated around the arterioles. Whole-lung lysate levels of pSMAD2 levels were evaluated and were increased in the IR group on d7. Over time, the diseased athymic-SU5416-treated animals developed increasing pSmad2 levels as well, indicating that increased TGFβ signaling, in isolation, cannot explain protection from disease but should be considered in the...
context of which cells are involved. Tregs noted around the pulmonary arterioles in IR animals also expressed IL-10. IL-10–mediated Treg effects have been particularly evident in antigen-specific responses and represent a normal compensatory mechanism antagonizing the inflammatory response. IL-10 is a potent immunomodulator that inhibits the secretion of various proinflammatory cytokines, including IL-6 and IL-8. Adenoviral expression of IL-10 protects against monocrotaline-induced pulmonary hypertension in rats. Thus, IL-10 and TGF-β expression by Tregs offer plausible mechanisms explaining how these cells limit inflammatory injury and possibly accelerate pulmonary recovery.

IR resulted in decreased vascular apoptosis following SU5416 administration. Because endothelial apoptosis is the chief mech-
Figure 7. IR of athymic rats leads to increased BMPR2 expression in the lung. A, Immunofluorescent images show colocalization of BMPR2 expressing cells and CD68 in all 3 animal groups. B, Immunohistochemistry and quantitation of BMPR2 expressing cells in lungs sections of athymic control, athymic SU, and athymic IR-SU rats. C, Immunofluorescent image of lung tissue shows BMPR2 expressing cells (red) are adjacent to CD4+ cells (green) in athymic IR-SU group. D, Immunofluorescent images of lung section shows that vWF colocalizes with BMPR2 (arrow) in vascular endothelial cells at d21 in athymic IR-SU rats (n=4 per group). E, The vWF-BMPR2 colocalization coefficient at d21. F and G, Western blot analysis of BMPR2 protein levels at d21 from whole lung lysates (n=8 per group). Data are shown as means with error bars representing SEM. *P<0.05. Scale bar: (A and C) 25 μm; (B and D) 50 μm.
anism by which SU5416 induces PAH, we sought factors associated with IR that could have protected endothelium and found that IR induced vascular BMPR2 expression. BMPR2 mutations are strongly associated with familial PAH, and decreased pulmonary vascular BMPR2 expression is associated with idiopathic PAH. In experimental models, hypoxia-induced PAH is associated with down-regulation of pulmonary vascular BMPR2, and genetic ablation of pulmonary vascular BMPR2 favors PAH development. In the current study, BMPR2-expressing cells in athymic control and athymic SU5416 groups were confined to rare cells that we were not able to characterize, except that they were sometimes CD68/macrophages. By contrast, IR athymic SU5416 animals exhibited large numbers of BMPR2-expressing cells often adjacent to CD4+ cells. Additionally, we demonstrate increased BMPR2-expressing pulmonary vascular cells only in the IR group. The mechanism by which IR could result in such BMPR2 up-regulation is not known, nor is it understood whether there is a direct causal relationship between immune competency and BMPR2 expression. Of interest, a close (physical) association between BMPR2 expressing cells and T cells was recently observed in idiopathic PAH, and future studies will investigate by what mechanism T cells might directly induce endothelial BMPR2.

Treg IR studies in rats are costly, are time consuming, and have limited reagents available in comparison with mice, but rat models benefit from more robust disease that more closely resembles human PAH pathology. Whereas pulmonary inflammation in PAH is a well-established phenomenon, it has been unclear whether the inflammation is itself pathogenic or is a secondary immune response to preexisting pulmonary vascular disease. The current study strongly implicates inflammation associated with CD4 immunodeficiency as a primary predisposing factor to experimental PAH development. The clinical relevance of this finding is that certain PAH-associated conditions are characterized by abnormalities in CD4+ T cell number and function. Examples of this association include HIV infection and autoimmune diseases such as systemic sclerosis, systemic lupus erythematosus, polymyositis, Hashimoto’s thyroiditis, Sjögren’s Syndrome, and the antiphospholipid antibody syndrome. The clinical relevance of this issue is that these CD4+ T cell irregularities may render patients susceptible to exaggerated vascular inflammation and subsequent vascular remodeling following vascular injury. By distinction, idiopathic PAH has been characterized by increased Tregs in peripheral blood accompanied by a 4-fold elevation of lung CD8+ cells. Therefore, in the case of idiopathic PAH, if immune dysregulation contributes to the lung inflammation observed in this condition, current evidence does not link pulmonary injury to abnormal CD4+ T cell populations detectable in the periphery. Correcting immune dysregulation with Treg therapy is an
exciting application in development, which could prove useful for some PAH conditions. Greater recognition that PAH patients have a condition that may begin with impaired resolution of vascular injury could have a significant impact on how this condition is both understood and treated.

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Disclosures
None.

References


Novelty and Significance

What Is Known?
- Pulmonary arterial hypertension (PAH) is a potentially fatal condition associated with connective tissue diseases and viral infections.
- Perivascular inflammation is strongly associated with PAH, but whether lung immunity contributes to disease pathogenesis is controversial.
- Regulatory T lymphocytes (Tregs) are important cells that control inflammation and prevent autoimmune injury.

What New Information Does This Article Contribute?
- Correcting immune deficiency prevents experimental PAH.
- The protective effect of IR was localized to Treg populations that prevented the early accumulation of macrophages and B cells, limited pulmonary endothelial apoptosis, and upregulated vascular bone morphogenetic protein receptor-II (BMPR2) expression.

Clinical conditions commonly associated with PAH, such as systemic sclerosis and HIV infection, are characterized by abnormal CD4 T lymphocyte function and number, T-cell abnormalities that are likely responsible for some of the dysregulated immunity observed in these conditions. Preclinical studies have shown that T-cell-deficient rats develop lung inflammation and exaggerated PAH following vascular injury. The purpose of the current study was to determine whether restoring T lymphocyte function and number abrogates exuberant inflammatory responses and prevents PAH. We found that lung injury resolution, in the presence of Tregs, is associated with significantly less inflammation and endothelial apoptosis. Inflammation was not merely a consequence of PAH because inflammation and apoptotic cell death occurred prior to the development of vascular remodeling and elevated pulmonary artery pressures. Unexpectedly, the restoration of T cells also led to upregulated vascular BMPR2 in protected rats. This finding is of interest because BMPR2 mutations have been implicated as a cause of familial PAH, and decreased pulmonary vascular BMPR2 expression has been observed in other forms of PAH. These findings suggest that anti-inflammatory Tregs, in association with inducible BMPR2, provide innate protection against PAH following pulmonary vascular injury.
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ONLINE DATA SUPPLEMENT

Regulatory T cells limit vascular endothelial injury and prevent pulmonary hypertension.

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METHODS

**Echocardiography.** Echocardiographic evaluation of right ventricular dimensions and pulmonary hemodynamics were performed using the Vevo 770 high resolution ultrasound imaging system (VisualSonics, Inc., Toronto, Ontario, Canada) equipped with 25 MHz and 35 MHz transducers. Rats were lightly sedated with isoflurane, volatized with compressed air (3% for induction, 1% for maintenance) for the duration of the procedure. The rats were laid supine on a warmed handling platform and their paws were lightly taped to electrode pads for ECG and respiratory cycle monitoring. Standard left ventricular measurements were made from 2-dimensional-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 Immunofluorescence imaging.** The left rat lungs were inflated with a 1:1 mixture of OCT and 30% sucrose and were embedded in Tissue Tek OCT (Sakura). A cryostat (HM550) (Microm) was used to cut 7 μm cryosections of lung and the sections were placed on superfrost/plus slides (Fisher Scientific). For immunofluorescent staining, the slides were fixed in methanol/acetone (1:1), washed with PBS, incubated in 0.2% Triton (Sigma), and then washed again in PBS. Next, the sections were blocked with normal 10% goat serum and then incubated for 1 h with anti-CD68 (ED-1) (AbD Serotec), anti-CD45RA (OX33) (AbD Serotec), anti-CD31 (TLD-3A12) (AbD Serotec), anti-rat mast cells (AR32AA4) (BD Pharmingen), anti-BMPR2 (G-17) (Santa Cruz Biotechnology), anti-cleaved caspase 3 (Asp175) (Cell Signaling), anti-IL-10 (R&D Systems), anti-FoxP3 (150D/E4) (eBioscience), anti-pSmad2/3 (Ser423/425) (Santa Cruz Biotechnology), anti-TGF-β1 (V) (Santa Cruz Biotechnology), anti-
CD4 (T4/Leu-3) (Abbiotec), anti-CD4 (OX-38) (BD Pharmingen), anti-alpha smooth muscle actin (ab5694) (Abcam) and anti-von Willebrand Factor (ab6994) (Abcam) antibodies. Isotype specific antibodies (BD Pharmingen) were used as negative controls. The slides were washed with PBS and then incubated with Alexa Fluor 488 goat anti-mouse IgG, Alexa Fluor 546 donkey anti-goat, Alexa Fluor 647 donkey anti-rabbit, Alexa Fluor 546 donkey anti-rabbit (all Invitrogen), or DyLight 488 donkey anti-mouse (Jackson) conjugated secondary antibodies. TO-PRO-3 (Invitrogen) was used for nuclear staining. The sections were mounted with Vectashield mounting medium (Vector Laboratories). Microscopic analysis was performed with the LSM 510META confocal laser scanning microscope (Carl Zeiss). Integrated fluorescence intensity was used to perform morphometric quantification of the degree of colocalization, expressed as fractional colocalization (value of 1.0 =100% colocalization) measured with ImageJ software.

For immunohistochemistry, of CD68, CD45RA, CD31, IL-10, FoxP3, BMPR2, CD4, and cleaved caspase 3, the lung sections were fixed, incubated with blocking serum, and then the primary antibodies listed above. Sections were then either incubated with biotinylated donkey anti-goat or anti-rabbit antibodies (Santa Cruz Biotechnology) or processed with the Animal Research Kit (Dako), followed by streptavidin HRP and diaminobenzidine (Dako). Cells were counted in 10, 400x fields in 4 random sections/animal. Quantification of activated cleaved caspase 3 positive endothelial cells was performed in 4 randomly chosen sections in each experimental group and sixty small pulmonary vessels were analyzed. The percentage of wall thickness was determined using the methodology described by Beppu et al. (2004) and was as follows: % wall thickness = (WT1+WT2)/(external diameter of vessel)x100%, 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.

ELISA Analysis. Commercially available ELISA kits were used to determine serum levels of tumor necrosis factor (TNF) (eBioscience) and interleukin (IL)-6 (Thermo Scientific Pierce).

Real-time PCR. For real-time PCR analysis of myocardial gene expression, cardiac tissue was stored in RNA Later (Ambion) until RNA was purified with TRIzol reagent (Invitrogen) per product instructions. The RNA concentration was determined using the NanoDrop Spectrophotometer (Thermo Scientific). RNA (1 μg) was reversed transcribed with the SuperScript III First-Strand Synthesis System to generate cDNA for RT-PCR (Invitrogen). Real-time PCR was performed using the Taqman Gene Expression Assay (Applied Biosystems) and rat cardiac specific primers for each gene (Online Table III). The following genes were assessed: α- and β-myosin heavy chain (MHC), sarco(endo)plasmic reticulum Ca2+ ATPase 2a (SERCA2a), atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and skeletal α-actin (SkAct). Each sample was assayed in triplicate (10 ng cDNA/well) and 18S ribosomal RNA was used as an internal control in each well.

Western Blot Analysis. Frozen rat lung tissue was homogenized in protein extraction buffer and protease inhibitors (Complete Mini EDTA-free) (Roche) at 4°C. The BCA protein assay (Thermo Scientific Pierce) was used to measure protein concentration. 50 μg protein samples were loaded onto Tris-HCl precast gels with a Mini Protean apparatus (Bio-Rad). Transfer of proteins from the gel to nitrocellulose (Whatman) was performed with the mini transfer apparatus (Bio-Rad). The membranes were blocked for 1 h at room temperature in Odyssey blocking buffer (Li-COR), then incubated overnight at 4°C with anti-BMPR2 (1:500, BD Transduction Laboratories), anti–cleaved caspase 3 (Asp175) (1:1000, Cell Signaling), anti-pSmad2 (Ser465/467) (1:1000, Cell Signaling), anti-smad2 (1:1000, Cell Signaling) and anti-β actin (1:5000, AC-15) (Sigma) antibodies. The membranes were washed, incubated with fluorescent IRDye 800CW goat anti-mouse, donkey anti-goat or donkey anti-rabbit secondary antibodies (1:2000, Li-COR) for 1 h, washed again, and then imaged with the Odyssey system.
software (Li-COR). For quantification, densitometry of the protein band of interest was divided by the densitometry of the corresponding β actin band using ImageJ software. Reported values were normalized to control group values, which were arbitrarily assigned the value of 1.

**CD4⁺ T cell Depletion.** Monoclonal anti-rat-CD4 antibody, Medical Research Council Oxford (MRC OX) 38 was produced from a hybridoma (Gift of Prof. Bruce Hall, University of New South Wales, Australia). For depletion of CD4⁺ T cells, euthymic WAG rats were given i.p injections of anti-CD4 monoclonal antibody (OX-38) at 10 mg/kg on days −1, 0, +1, +2 and weekly thereafter for 3 weeks. As a negative control, rat isotype-matched antibody (mouse IgG2a) (Bio-X-Cell) was injected i.p. in euthymic WAG rats at 10 mg/kg at the same time points.

**SUPPLEMENT RESULTS**

Because pathological left ventricular hypertrophy is characterized by the expression of a signature gene profile which recapitulates embryonic development ¹, we questioned whether the fetal gene program was activated in the RV of the treatment groups. Athymic rat RVs were assessed by real-time PCR 21d after SU5416 or vehicle control administration (Online Figure IV). Fetal genes associated with left ventricular stress (β-MHC, skACT, BNP and ANP) were upregulated with PAH whereas adult cardiac muscle-specific genes (α-MHC and SERCA2a), were decreased with disease. The prevention of PAH by IR also diminished activation of the pathologic fetal gene program. No differential expression of fetal genes was observed in respective left ventricles of these animals (data not shown).

**SUPPLEMENTAL REFERENCE**

Online Figure I. IR attenuates medial thickening of precapillary pulmonary vessels. (A) Immunofluorescent images of vessels stained with α-smooth muscle actin in lung tissue at d21 (n = 6/group). (B) Percentage of wall thickness of α-smooth muscle actin positive vessels < 100 µm in external diameter. Scale bar = 50 µm.
**Online Figure II.** IR attenuates occlusion of precapillary pulmonary vessels. (A, B) Hematoxylin and eosin staining of lung sections in athymic SU and athymic IR-SU lung shows totally occluded and partially occluded vessels in lung tissue at d21 (n=4/group). p< 0.05 by chi-square test. Scale bar = 50 μm.
Online Figure III. Representative RV pressure-volume loops for athymic controls and athymic SU rats at d21. The red line indicates the RV end systolic pressure-volume relationship (ESPVR) while the straight blue line shows the RV end diastolic pressure-volume relationship (EDPVR). Data were obtained during occlusion of the vena cava. RVU - relative volume units.

Online Table I. RV hemodynamic parameters in athymic control, athymic SU and athymic IR-SU rats at d21.

<table>
<thead>
<tr>
<th></th>
<th>Athymic control (n=10)</th>
<th>Athymic SU (n=8)</th>
<th>Athymic IR-SU (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RV-systolic pressure (mmHg)</td>
<td>25.78 ± 9.52</td>
<td>71.66 ± 11.69*</td>
<td>36.63 ± 6.81</td>
</tr>
<tr>
<td>dP/dt max (mmHg/s)</td>
<td>945.5 ± 92.5</td>
<td>3089 ± 1000*</td>
<td>1583 ± 576.7</td>
</tr>
</tbody>
</table>

dP/dt max, maximal rate of pressure increase.
Values are means ± SEM. *p<0.05 vs athymic controls&athymic SU, vs athymic SU&athymic IR-SU.

Online Table II. RV and LV hemodynamic parameters in athymic control and athymic SU rats at d21.

<table>
<thead>
<tr>
<th></th>
<th>Athymic control (n=12)</th>
<th>Athymic SU (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mPAP (mmHg)</td>
<td>20.52 ± 11.82</td>
<td>65.31 ± 7.7*</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>7.15 ± 4.71</td>
<td>7.37 ± 6.43</td>
</tr>
<tr>
<td>RVEDP (mmHg)</td>
<td>4.8 ± 2.81</td>
<td>9.10 ± 4.39*</td>
</tr>
<tr>
<td>RV ESPVR (mmHg/RVU)</td>
<td>2.63 ± 1.77</td>
<td>5.23 ± 3.38</td>
</tr>
<tr>
<td>RV EDPVR (mmHg/RVU)</td>
<td>0.51 ± 0.51</td>
<td>0.98 ± 1.39</td>
</tr>
</tbody>
</table>

mPAP, mean pulmonary artery pressure; L(R)VEDP, left (right) ventricular end-diastolic pressure, RV ESP(D)PVR, right ventricular end-systolic (diastolic) pressure volume relations.
Values are means ± SEM. *p<0.05 vs athymic controls.
Online Figure IV. IR of athymic rats prevents activation of the pathological fetal gene program. (A-F) Real-time PCR of RV myocardial tissue for detection of non-pathologic α-MHC and SERCA2a fetal genes along with pathologic β-MHC, SkAct, BNP and ANP fetal genes at d21. Results are expressed as arbitrary units (AU) and fold change after normalization for 18S. The AU was set as the average value of the control group (n=4/group). Data are shown as means with error bars representing SEM. * p<0.05.

Online Table III. Sequence of the primers used for the RT-PCR reaction.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>a-MHC F</td>
<td>CCTGTCACGCAAGAGAC</td>
</tr>
<tr>
<td>a-MHC R</td>
<td>CAGGCAAGTCAAGCATTATTTATGG</td>
</tr>
<tr>
<td>18S F</td>
<td>GCCGCTAGAGTGAAATCTTG</td>
</tr>
<tr>
<td>18S R</td>
<td>CTTTCGCTCTGGTCCGTCTT</td>
</tr>
<tr>
<td>BNP F</td>
<td>GGTGCTGCCAGAGATGAT</td>
</tr>
<tr>
<td>BNP R</td>
<td>CTGGAGACTGGCTAGAATTCC</td>
</tr>
<tr>
<td>Serca 2a F</td>
<td>GCCGAGATCGGCCTACA</td>
</tr>
<tr>
<td>Serca 2a R</td>
<td>GGCCAATTAGAGAGAGGTTT</td>
</tr>
<tr>
<td>SkAct F</td>
<td>CCACCTATAACAGCATCATGAAGT</td>
</tr>
<tr>
<td>SkAct R</td>
<td>GACATGCCTGTGTTCGCTACA</td>
</tr>
<tr>
<td>beta-MHC F</td>
<td>CGCTCACTGTGCGCTGGA</td>
</tr>
<tr>
<td>beta-MHC R</td>
<td>GCCCAAAAATGCAGCAT</td>
</tr>
<tr>
<td>ANP F</td>
<td>GGGAGCTCAAGGTGCTTT</td>
</tr>
<tr>
<td>ANP R</td>
<td>CTGGGCTCATTCTGTCAAT</td>
</tr>
</tbody>
</table>

All primers are presented in a 5’-3’ orientation.
Online Figure V. IR of athymic rats leads to increased infiltration of CD4+, FoxP3+, IL-10+ cells in the perivascular regions of lungs. (A, B, C) Immunohistochemistry and quantification for expression of CD4+, FoxP3+, IL-10+ cells (arrows) in lungs on d21 of athymic control, athymic SU and athymic IR-SU animals (n = 4/group). Data are shown as means with SEM. * p<0.05. Scale bar = 50 μm.
Online Figure VI. Evidence of perivascular inflammation and occlusion of small pulmonary vessels in lungs of euthymic SU rats after CD4 depletion. (A, B) Green immunofluorescent images for macrophages (arrows) stained with CD68 and B cells (arrows) stained with CD45RA in euthymic rats at d21 after anti-CD4 administration. Differential interference contrast (DIC) histology demonstrates occluded vessels (arrow) in lungs of euthymic SU animals after CD4 depletion (n=4/group). (C) Fluorescence histogram from flow cytometry for CD4 (OX-38) detection on peripheral blood of euthymic SU isotype control (red line) and euthymic SU rats after CD4 depletion (black line) at d21 after anti-CD4 administration (n=4/group). Scale bars=50 μm.
Online Figure VII. Confirmation of putative Tregs (CD4^+CD25^{hi}FoxP3^+) cells by flow cytometry in the peripheral blood within 21 days after IR. (A) Flow cytometry data of peripheral blood for TCRα/β and CD45RA detection on d7 in athymic controls, athymic SU, and athymic IR-SU. (n = 4/group). (B) Flow cytometry data for Tregs (CD4^+CD25^{hi}FoxP3^+) detection in the peripheral blood in athymic control, athymic SU, athymic IR-SU (CD4^+CD25^-) and athymic IR-SU (CD4^+CD25^+) on d21 (n = 8/group).