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Regulatory T cells and Pulmonary Hypertension

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Abstract

Pulmonary hypertension (PH) is a disease of high lethality arising from numerous causes. For a significant subset of PH patients, autoimmune biomarkers or frank autoimmune disease are simultaneously present, but the extent to which lung inflammation contributes to PH is currently unknown. However, emerging experimental and clinical evidence suggests that immune dysregulation may lead to the propagation of vascular injury and PH. A recent preclinical study has demonstrated that regulatory T cells (Tregs) are important mediators normally enlisted to control inflammation and that, if absent or dysfunctional, may predispose to the development of PH.

Introduction

For over 50 years it has been recognized that autoimmune phenomena are associated with certain forms of PH, but it has never been previously demonstrated that immune dysregulation, itself, may be a root cause for PH. To address this issue, our group recently demonstrated how immune dysregulation exacerbates vascular inflammation and results in severe PH (Tamosiuniene et al., 2011). In this experimental model, athymic (T-cell deficient) rats developed significant pulmonary vascular disease following treatment with SU5416, a vascular endothelial growth factor-2 (VEGFR-2) inhibitor. The main finding of this study was that PH may arise when the absence of normal anti-inflammatory Treg activity results in a failure to control inflammatory endothelial injury. Restoring Tregs to these animals, prior to vascular injury, prevents the development of PH. The relevant clinical parallel is that a number of diseases associated with PH are, like the athymic rat, associated with conditions having abnormal Treg numbers or activity (reviewed in (Nicolls et al., 2005)). The purposes of this review are to discuss the findings of this recent scientific study (Tamosiuniene et al., 2011), to put into context what is known about Treg activity in clinical PH and, finally, to discuss future research directions emerging from this work.

The Animal Model: Athymic Rats in PH Research

The athymic rat is the result of an autosomal recessive mutation on the *rnu* locus of chromosome 10 leading to T-cell deficiency, thymic aplasia and hairlessness (Rolstad, 2001). Our group and others have demonstrated that athymic rats on different genetic backgrounds subject to different types of vascular injury develop severe PH (Miyata et al., 2000; Ormiston et al., 2010; Tamosiuniene et al., 2011; Taraseviciene-Stewart et al., 2007). Both monocrotaline and SU5416 are effective in generating significant disease in these T-

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cell deficient animals. Additionally, lung disease in these animals is surprisingly inflammatory given the absence of putatively injurious cytotoxic T cells (Taraseviciene-Stewart et al., 2007). This latter finding suggested that the absence of Tregs was the dominant defect and could explain severe PH in these animals. In this experimental model, pulmonary arterioles become occluded with proliferating endothelial cells, and are surrounded by collections of macrophages, B cells, and mast cells. There is also evidence of anti-endothelial antibody deposition in the diseased pulmonary microcirculation (Tamosiuniene et al., 2011; Taraseviciene-Stewart et al., 2007). SU5416-treated athymic animals also demonstrate smooth cell hyperplasia, but this pathology is less prominent than in human disease. Right ventricular inflammation is evident and is associated with increased collagen type 1 deposition {Taraseviciene-Stewart, 2007 #3974}. Compared to other PH models, which may require chronic hypoxia or a surgical pneumonectomy to develop high pulmonary artery pressures, T cell immunodeficiency renders these athymic animals particularly sensitive to the development of severe PH even under normoxic conditions. In fact, most athymic rats simultaneously subjected to SU5416 and hypoxia together, die from PH within 3 weeks (Taraseviciene-Stewart et al., 2007).

Following monocrotaline or SU5416 administration, athymic rats develop significant RV remodeling, perivascular inflammation, smooth muscle hypertrophy and occlusive arteriolar lesions that to some extent mimicks severe human pulmonary arterial hypertension PH (Miyata et al., 2000; Ormiston et al., 2010; Tamosiuniene et al., 2011; Taraseviciene-Stewart et al., 2007). We reasoned that athymic PH, occurring with different induction agents on different genetic backgrounds, was possibly attributable to the T-cell deficiency state, and that replacing T cells could remove this disease diathesis. A significant issue which needed to be addressed prior to answering this question required finding a source of inbred athymic rats. In order to prevent graft versus host disease, replacing T cells (also referred to as 'immune reconstitution') requires that donor T cells be identical at the major histocompatibility complex (MHC) locus to the MHC of recipient athymic animals. . All studies, prior to Tamosiuniene et al (Tamosiuniene et al., 2011) were limited by the availability of inbred animals; a fact which precluded an evaluation of how immune reconstitution could prevent the PH. However, working with inbred WAG strain euthymic (T cell replete) lymphocyte donors and athymic rats (available through Biomedical Research Models, Inc. Worcester, MA), it became possible to determine whether correcting T cell deficiency, through the adoptive transfer of MHC-identical lymphocytes, removed the predisposition for PH in athymic animals. More specifically, it became possible to test which T cell subset was responsible for conferring protection.

A controversy which sometimes arises in this line of research is the choice of rats versus mice to conduct studies. This issue has recently been thoroughly addressed in an article by Gomez-Arroya et al (Gomez-Arroya et al., 2012). Briefly, working in mouse models of PH allows for an elegant dissection of genetic mechanisms not currently possible in the rat, but the main limitations with mice are that hemodynamic pressures are frequently not severe nor does the pathology closely match clinical disease. As presented in Gomez-Arroya's perspective, athymic mice treated with SU5416 do not develop significant PH, and this negative finding was ultimately the main basis for choosing the rat model to address the question of Tregs in PH development described in detail below.

Immune Reconstitution of Athymic Rats Prevents PH

To test the general principle that T-cell deficiency was the reason why athymic rats develop severe PH relative to euthymic rats, we first investigated whether injecting unfractionated splenocytes (which contain T and B cells as well as other mononuclear cells) prevented PH. Spleen cell injections effectively attenuated disease induced by SU5416 if administered

prior to SU5416 administration. Immune reconstitution was most effective if performed at least seven days prior to vascular injury. PH in this model is robust with right ventricular systolic pressures in the 50–75 mm Hg range. The mean pulmonary artery pressures in non-reconstituted SU5416-treated athymic rats were 72 ± 12 mm Hg vs. 37 ± 7 mm Hg in reconstituted SU5416-treated animals (compared to untreated controls: 26 ± 10 mm Hg). Right ventricle pressure-volume loops in SU516-treated athymic rats were consistent with severe PH. Left ventricular end diastolic pressures were unaffected, and left ventricular disease did not contribute to PH.

Immune Reconstitution Exerts an Anti-Inflammatory Effect

A common question about pulmonary inflammation in PH is whether the inflammation itself causes PH or whether the inflammation is an epiphenomenon of the pulmonary vascular disease. Alternatively, inflammation could be occurring for both reasons. In the athymic rat treated with SU5416, inflammation was found to precede a detectable elevation in right-sided pressures by several days (and, therefore, it was deemed unlikely that PH was the cause of early pulmonary inflammation). Significant increases in pulmonary macrophages, mast cells and B cells are evident seven days after SU5416 administration. Concomitantly, elevated serum levels of TNF- α and IL-6 are also progressively evident. These inflammatory markers have previously been implicated in PH pathogenesis (Fujita et al., 2001; Steiner et al., 2009). Immune-reconstituted animals were remarkable for fewer pulmonary macrophages, mast cells and B cells. In immune-reconstituted animals there was significantly less vascular remodeling (with less luminal occlusion and smooth muscle cell hypertrophy). Additionally, SU5416-treated, immune-reconstituted athymic rats demonstrated a perivascular infiltration with CD4⁺ T cells expressing Forkhead box protein 3 (FoxP3), IL-10 and TGF- β .

Injury resolution is an established function for Tregs. These cells typically express the co-receptor CD4⁺, the transcription factor FoxP3, and highly express the IL-2 receptor- α chain (i.e. CD25^{hi}) (Sakaguchi, 2005). Tregs regulate immune responses following a variety of inflammatory injuries such as burns and infection (McKinley et al., 2006; Montagnoli et al., 2006; Murphy et al., 2005). Tregs control inflammation through a number of mechanisms including: 1) secretion of the anti-inflammatory cytokines TGF- β and IL-10, 2) killing of effector T cells, 3) disruption of the metabolic function of effector cells (for example, by serving as an IL-2 'sink' due to high Treg surface concentrations of the IL-2 receptor protein), 4) polarizing macrophages towards a M2 phenotype and 5) modification of dendritic cell function (such as through the induction of the immunosuppressive enzyme indoleamine 2,3-dioxygenase) (Liu et al., 2011; Vignali et al., 2008). In the absence of Tregs, inflammatory injury is less readily resolved, and serious disease may develop (Shih et al., 2004). Our study suggests that for immunologically intact individuals, Tregs may be important immune regulators preventing the propagation of vascular injury by limiting inflammation.

The loss of self-tolerance in animals missing normal Treg populations is associated with the appearance of various disease-specific autoantibodies (Kim et al., 2007; Sakaguchi, 2004). With complete elimination of CD4⁺CD25^{hi} cells, systemic autoimmunity occurs as manifested by multiorgan inflammation and autoantibody production (Shih et al., 2004). 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 CD4⁺CD25^{hi} Tregs are not restricted to regulation of the adaptive immune system but also affects the activation and function of innate immune cells including monocytes, macrophages, dendritic cells and NK cells (Ghiringhelli et al., 2006; Liu et al., 2011; Mahajan et al., 2006; Taams et al., 2005; Tiemessen et al., 2007).

In Experimental PH, Regulatory T cell Activity is Localized in the CD4⁺ Subset

Immune reconstitution of athymic rats with unfractionated spleen cells successfully prevents the development of severe PH (Tamosiuniene et al., 2011). Because the specific immune deficit in athymic animals is T cell deficiency, we next sought to identify which T cell subset possessed regulatory activity. CD8⁺ T cells administered to animals prior to SU5416 administration were not protective and severe PH developed. By contrast both CD4⁺CD25^{hi} T cells and CD4⁺CD25⁻ cells exhibited regulatory activity when used as the reconstituting cell population, and PH was significantly attenuated. To rule out the possibility that a non-CD4⁺ T cell population in spleen cell reconstitution (e.g., c-kit⁺ stem-like cells, regulatory macrophages) was contributing to the observed protection, CD4⁺ T cell-depleted spleen cells were used to immune-reconstitute athymic rats and were found to be non-protective. Finally, because T-cell deficient animals may develop in unconsidered ways, we tested whether CD4⁺ T cell depletion would render euthymic rats, not normally susceptible to PH, susceptible to disease. CD4⁺ T cell-depleted animals developed PH (in contrast to non-depleted animals). Thus, regulatory activity in this animal model localized to the CD4⁺ T cell compartment.

Although classic Tregs are described as being CD4⁺CD25^{hi}FoxP3⁺ cells in mice, in rats, several studies have demonstrated regulatory activity in CD4⁺CD25⁻ cells (Aiello et al., 2007; Hillebrands et al., 2006; Mizobuchi et al., 2003). Further, we demonstrate evidence that some injected CD4⁺CD25⁻ are converted *in vivo* into CD4⁺CD25^{hi}FoxP3⁺ cells, a phenomenon that has been previously described (Chen et al., 2003). For this reason, we were unable to conclude whether it was the originally infused cells or a peripheral conversion of T cells into a classic Treg phenotype that was responsible for Treg-mediated protection against PH. Regardless, this important series of experiments provide good evidence that the reason athymic rats are predisposed to develop experimental PH is because they lack Tregs and don't appropriately control pulmonary vascular inflammation.

The clinical relevance of this study is that certain PH conditions are also associated with aberrant Treg numbers/activity or simply reduced CD4⁺ T cell numbers. Numerous examples of this association include HIV infection and autoimmune diseases such as systemic sclerosis, systemic lupus erythematosus, Hashimoto's thyroiditis, Sjögren's syndrome and the antiphospholipid antibody syndrome (reviewed in (Nicolls et al., 2005; Tamosiuniene et al., 2011)). It is possible that injury induced by a vasculopathic virus, shear stress or some other vasotoxic factor is normally resolved by appropriate CD4⁺ Treg activity. However, in individuals with abnormal CD4⁺ T cells, the capacity to endogenously mitigate inflammation through enhanced Treg activity, may be impaired, and unresolved vascular inflammation culminates in PH. Of note, several reports have described increased Tregs in the peripheral circulation of idiopathic PH patients (Austin et al., 2009; Ulrich et al., 2008) even as one report notes that there is more CD8⁺ T cell inflammation in idiopathic PH lungs (Austin et al., 2009). It is possible that in the case of idiopathic PH patients, Treg trafficking is impeded or that, in fact, Treg abnormalities may not contribute to the pathogenesis of the disease in certain patient groups.

Immune Reconstitution Limits Endothelial Cell Apoptosis and Upregulates Vascular BMPR2

The general study of Treg biology has mainly centered on inflammatory processes such as autoimmunity and transplantation rejection. Thus, the study of Treg activity in athymic rats was somewhat unique because of its focus on pulmonary vascular disease and right

ventricular deterioration. We questioned if other protective manifestations, beyond the limiting of pulmonary inflammation, could be similarly attributed to immune reconstitution and provide additional explanations about why the presence of an intact immune system normally prevents PH. We discovered that immune-reconstituted athymic rats treated with SU5416 had relatively less endothelial apoptosis and upregulated vascular bone morphogenetic protein receptor type 2 (BMPR2) compared with unreconstituted SU5416-treated rats. BMPR2 is a protein characterized as vascular-protective in health and vascular-defective in disease (Hong et al., 2008). BMPR2 mutations are strongly associated with familial PH and decreased pulmonary vascular BMPR2 expression is associated with idiopathic PH (Atkinson et al., 2002; Newman et al., 2001).

BMPR2 was also noted to be increased in nonvascular cells in the lungs of immune-reconstituted animals in poorly-characterized mononuclear cells sometimes seen adjacent to CD4⁺ T cells. Of interest, a close association between BMPR2-expressing cells and T cells was recently demonstrated in idiopathic PH in children (Hall et al., 2009). These experimental and clinical findings suggest that the role of BMPR2 signaling in PH development, or alternatively, in PH prevention, may extend beyond its currently understood activity in pulmonary arteriolar endothelial and smooth muscle cells. Further it remains unclear why immune reconstitution upregulates vascular BMPR2 and by what mechanism endothelial injury is attenuated. The relationship between normal immune regulation and the resolution of vascular injury remains poorly understood. Figure 1 explores putative mechanisms by which Tregs help to prevent the development of vascular injury.

Modulation of pulmonary vascular injury by Tregs

There are a number of intriguing avenues of research concerning how Tregs may normally protect vascular integrity; research which may provide additional and, possibly, more sophisticated explanations to the results described in (Tamosiuniene et al., 2011). As suggested in Figure 1 and described in (Tamosiuniene et al., 2011), pulmonary endothelial injury coupled with decreased Treg activity can lead to injurious vascular inflammation. The endothelium stands as a physical barrier to leukocytes and regulates their extravasation from the circulation into the surrounding tissue. The extravasation of Tregs is mediated by intercellular adhesion molecule 1 (ICAM1) and vascular cell adhesion molecule 1 (VCAM1). The common lymphatic endothelial and vascular endothelial receptor (CLEVER-1; also known as FEEL-1 and stabilin-1) is an intracellular trafficking receptor with multifunctional properties that is implicated in the recruitment of Tregs into inflamed tissue (Shetty et al., 2011). After contact with ECs, Tregs upregulate the surface expression of programmed death-1 (PD-1) receptor (another suppressive molecule) as well as secretion of IL-10 and TGF- β . Treg-secreted IL-10 is, itself, a vasorelaxing factor; it also rescues eNOS phosphorylation and reduces oxidative stress through the inhibition of NADPH oxidase activity. These properties suggest that IL-10 is an important element in the regulation of vascular endothelial function.

Dendritic cells (DCs) also appear to be a target of Treg-mediated immune suppression while simultaneously playing a crucial role in the maintenance of self-tolerance by presenting self-antigen, inducing unresponsiveness of T cells and by promoting Treg expansion (Onishi et al., 2008). Treg activation may result from encounters with activated plasmacytoid DCs, exposure to damage-associated molecular pattern molecules (DAMPs) or inflammatory mediators. The prolonged binding of Tregs with DCs leads to upregulation of effector molecules such as IL-10 and TGF- β 1. Cell contact through CTLA4 and CD80/86 binding to DCs causes decreased costimulation and decreased antigen presentation. Similarly, lymphocyte activating gene-3 (LAG3) or CD22 binding to MHCII molecules can lead to the prevention of DC maturation and the reduction in antigen presentation capabilities

(Workman et al., 2004). Finally, another intriguing Treg surface molecule, galectin-1, can bind to effector T cells and DCs and cause cell cycle arrest and/or apoptosis (Garin et al., 2007).

Following Treg interaction with DCs, the release of indoleamine 2,3-dioxygenase (IDO) and heme oxygenase-1 (HO-1) controls the abundance of environmental tryptophan and carbon monoxide (CO), respectively. (HO-1 also exhibits protective effects due to its anti-oxidative potential relevant to PH (Dorfmüller et al., 2011; Raval and Lee, 2010)). Tryptophan metabolism to kynurenine via the action of IDO by antigen-presenting cells regulates T cell activation and, also leads to vascular relaxation and the regulation of blood pressure in systemic inflammation (Wang et al., 2010). In addition, induction of HO-1 has a potent anti-inflammatory effect that may be mediated through Treg activity, and HO-1 is protective against the development of PH (Vergadi et al., 2011).

During increased interaction with DCs, activated Tregs can acquire neuropilin-1 (NRP1), a ligand-binding receptor for class III semaphorin and co-receptor for VEGFA, through a process known as trogocytosis. (VEGFA normally acts through the tyrosine kinase receptors, VEGFR1 and VEGFR2 predominantly expressed on the surface of vascular ECs). Trogocytosis enables T cells to bind to DC-secreted VEGFA, potentially converting Tregs into VEGFA-shuttling cells and also promoting prolonged interactions between Treg cells and DCs that result in decreased antigen presentation and increased tolerance promotion. In addition, semaphorin 3A (Sema3A; ligand to NRP1) which is expressed on DCs, plays a role in adhesion and clustering between DCs and Treg and results in DCs transmigration (Bourbie-Vaudaine et al., 2006; Sarris et al., 2008; Takamatsu et al., 2010). NRP1 acts as a co-receptor in the TGF- β 1 pathway through TGF- β R2 and TGF- β R1 receptors. NRP-1 can control Smad1/5 and Smad2/3 signaling by interacting with TGF- β R2. In the presence of TGF- β 1/ NRP-1, the Smad1/5/8 pathway promotes ECs proliferation, migration and survival (Cao et al., 2010). In summary, the newly characterized role for VEGFA in Treg and DC signaling suggests that this mechanism of tolerance may be especially germane to endothelial biology.

Finally, studies in rodents and humans suggest a curious link between immune tolerance and the process of angiogenesis which is relevant to PH, often characterized as a disease of dysregulated angiogenesis. CD4⁺CD25⁺ Treg cells secrete higher amounts of VEGFA than CD4⁺CD25⁻ T cells both at steady state and under hypoxic conditions and promote endothelial cell proliferation *in vitro* and *in vivo*. (Facciabene et al., 2011). Tregs promote angiogenesis through the secretion of VEGFA which supports endothelial cell recruitment; Treg-derived VEGFA also promotes the expansion of other tolerogenic leukocyte populations such as plasmacytoid DCs (Clark et al., 2007).

Conclusion

Our recent study investigating how Treg activity normally limits endothelial injury and prevents PH puts new focus on immune dysregulation as an important risk factor for disease development (Tamosiuniene et al., 2011). This work builds on prior foundational studies demonstrating that inflammation contributes to the pathogenesis of PH (e.g. (Price et al., 2011)). The interplay between Tregs and the pulmonary vasculature is likely to be more complex than simply down-modulation of cellular inflammation. For example, the fact that the mere presence of Tregs significantly upregulates both local (i.e., vascular) as well as global (i.e., lung) BMPR2 expression suggests that the importance of BMPR2 signaling may extend beyond the gene's impact on endothelial cell survival and smooth muscle cell proliferation. Understanding these complex pathways will strengthen ongoing efforts to refine and personalize therapies for this otherwise progressive and life-threatening condition.

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Figure 1.
Modulation of Pulmonary Vascular Injury by Tregs.