The Roles of Immunity in the Prevention and Evolution of Pulmonary Arterial Hypertension

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Physicians treating patients with pulmonary vascular diseases must account for a wide spectrum of etiologies and presentations that are associated with the syndrome, including HIV/AIDS, connective tissue diseases, chronic obstructive pulmonary disease, and eosinophilic granuloma, all of which have an inflammatory disease component. In addition, idiopathic pulmonary arterial hypertension (PAH) has long been recognized to overlap with collagen vascular disease–associated PAH (1). Patients with idiopathic PAH can present with autoimmune features including Raynaud's symptoms, anti-nuclear antibodies, and anti-fibroblast and anti-endothelial cell antibodies (2–4). It is now more than 20 years since the seminal publication by Marc Humbert and colleagues, which documented significantly elevated levels of IL-1 and IL-6 in the blood of patients with severe PAH (5). More recently both retrospective (6) and prospective (7) analyses have found that high levels of inflammatory mediators were predictive of a worse outcome. Against this backdrop, and in light of more recent reports and reviews (8, 9), the field now stands at the threshold of putting targeted immunotherapy to the test.

A National Institutes of Health (NIH) workshop document summarized the priorities in pulmonary vascular research in 2010 (10). Inflammation was acknowledged in the article as an area of emerging interest, with a notation about “the critical contributions of the immune system in terms

of immune surveillance of the vascular milieu.” However, relatively little has been done to move the field forward since then, possibly because of the ongoing question about whether inflammation is the cause of the pulmonary vascular disease or is merely the consequence of a biotoxic high-pressure microenvironment. Another consideration possibly limiting enthusiasm in the past was that, although the pathophysiology of PAH may begin with significant inflammation, by the time the condition comes to medical attention it has become refractory to immunotherapy; most diseases, after all, exhibit an inflammatory component. Understandable skepticism has affected this field of research, given the number of patients who fail to respond to broad immunosuppressive approaches, such as corticosteroids.

In this Perspective, we highlight a growing knowledge of immune pathways associated with PAH, which provides further argument about the usefulness of tailored immunotherapy for PAH. Our view can be integrated with other recent reviews in the *Journal*, which also cite emerging scientific discoveries to urge new therapeutic advancements (11–14). To begin to close the translational gap, which is considerable, it is important to carefully define these pathways more clearly, delineating unique signaling events for different forms of PAH. We already have examples for individualized antiinflammatory treatments. Patients with lupus erythematosus–associated PAH can respond to steroids (15), patients with HIV/AIDS-associated PAH to antiviral

therapy (16), and patients with the rare form of Castleman's lymphoma–associated PAH to rituximab (17). A randomized, placebo-controlled clinical trial of rituximab, for the treatment of systemic sclerosis–associated PAH, is anticipated to complete its enrollment in 2017–2018 (ClinicalTrials.gov identifier, NCT01086540). In addition, new trials are anticipated that should provide information about how immunotherapies should be administered depending on patient “endotypes.”

A Paradigm Shift That Is Based on New Concepts

Experimental pulmonary hypertension (PH) studies have dissected various inflammatory pathways and have identified a variety of mediators of inflammation (18–23). (By convention, preclinical models of PAH are referred to as “PH”). Hypoxic signaling likely also contributes to the inflammatory milieu of PH (24) and participates in the metabolic derangements noted in this disease. The metabolic theory proposes that the inhibition of glucose oxidation via mitochondrial suppression in pulmonary vascular cells can be observed in established disease and explains a number of features of the PAH vascular phenotype, including endothelial apoptosis resistance and proliferation (25). According to a Mayo Clinic study (26), about 13% of patients with idiopathic PAH display signs and symptoms of the metabolic syndrome, and another study

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found elevated blood levels of leptin in patients with severe PAH (20). One hypothesis is that adipose tissue is an important source of inflammatory mediators and angiogenesis promoters, which might either trigger the development of PAH or contribute to the progression and persistence of the pulmonary vascular disease. Distressed mitochondria, secreting mitokines, are likely an important source of inflammation in metabolically deranged tissues (25). Taken together, the metabolic and autoimmune theories of PAH pathogenesis provide a strong case to consider inflammation and metabolism in the design of new treatment targets.

Presence of Inflammatory Cells in the Lungs of Patients with Severe PAH

The presence of numerous mast cells, macrophages, lymphocytes, and dendritic cells in and around hypertensive pulmonary vascular lesions is *prima facie* evidence that these cells may contribute to disease, but definitive evidence is more difficult to obtain and early trials will likely require an empiric approach. The cells that are lining the pulmonary vessels and are exposed to increased shear stress, that is, microvascular endothelial cells, also exhibit inflammatory biomarkers. These intimal cells express the 5-lipoxygenase enzyme (27) and the multifunctional proinflammatory protein macrophage migration inhibitory factor (28), both of which have been implicated to play a role in angiogenesis and cancer pathogenesis. Several interleukins expressed in pulmonary vascular endothelium (29, 30) also can up-regulate C-X-C chemokine receptor 4 (CXCR4) in a hypoxia-inducible factor-1 α -dependent fashion (31, 32). Bone morphogenetic protein receptor 2 (BMPR2) haploinsufficiency is associated with increased cytokine release in vascular smooth cells cultured from pulmonary hypertensive animals and patients (33), and the outer adventitial layer is proinflammatory and a key site of immune activation (34, 35). Even though only 20% of BMPR2 mutation carriers develop clinical disease (36), dysfunctional BMPR2 signaling due to other causes beyond genetic mutation has been implicated in the disease pathogenesis (37–39) and is

factored into our model of PAH pathogenesis (*see below*).

Inflammation is associated with pulmonary vascular insults, including viral infections, hypoxia, and shear stress. Although there is compelling evidence implicating infections, especially viral infections, as a nidus for immune attack, inflammation may not require a pathogen and so-called “sterile” inflammation may drive vascular injury. Sterile inflammation occurs when damage-associated molecular patterns (DAMPs) are released into the extracellular environment as a result of tissue injury. This form of inflammation can be observed when a cell undergoes necrotic, as opposed to apoptotic, cell death and has been implicated in the pathogenesis of experimental PH (40). Part of the inflammatory response is localized to the perivascular space harboring cells of the immune system, including macrophages and T and B lymphocytes. Although our understanding of the role of lymphocytes in pulmonary vascular remodeling is still in its infancy, one interpretation of experimental data obtained in mice (41) and rats (42) could be that cytotoxic T cells play a role in the process of pulmonary arterial muscularization (41), whereas regulatory T cells (Tregs) may protect against lumen-obliterating endothelial cell proliferation (42). Even less is known about the role of B lymphocytes; nevertheless, in a rat model where severe PH had been triggered by the combination of SU5416-induced vascular endothelial cell growth factor receptor inhibition and ovalbumin-induced helper T-cell type 2 (Th2) immune system activation, B-cell depletion protected against the development of angioproliferative PAH (43).

With this background, there appears to be a good rationale for greater study of the immune pathways that have been the subject of reviews (1, 9); here we provide only some of the foundation that supports the much larger structure of “inflammation and PAH.” In various forms of PAH, such as HIV-associated PAH (44), sickle cell anemia-associated PH (45), or interstitial lung disease-associated PH, with both pre- and postcapillary disease manifestations, various layers of etiology-dependent inflammatory signaling mechanisms may be involved. For example, an elegant study by the Lawrie team at Sheffield provides a coherent view of how one such pathway (tumor necrosis factor–related apoptosis-inducing

ligand, or TRAIL) can contribute to disease in several models of preclinical PH (46).

The Autoimmune Component

If inflammation is the normal response of the precapillary lung vessels to injury, the appearance of lymphocytes in and around the vessels and a potentially pathobiological role in vascular remodeling may depend on several additional factors. For example, injured lung vascular endothelial cells may express non-self-antigenic epitopes, and B lymphocytes may be involved in the generation of anti-endothelial cell antibodies (4). An abnormal Treg response (1, 47) may be a centrally important feature for this immune disequilibrium; these cells are essential for preventing exaggerated inflammatory responses. In T cell–deficient athymic nude rats prone to PH, an accumulation of B cells and macrophages is noted within 1 week of SU5416 administration (42). These findings were not seen in animals that underwent Treg immune reconstitution before SU5416 administration and suggest that the loss of self-tolerance in animals missing normal Treg populations is associated with the appearance of various autoantibodies and autoimmune disease (48, 49). With complete elimination of CD4⁺CD25^{hi} cells, systemic autoimmunity occurs and results in autoantibody production (50). In this way, 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 and autoimmune disease.

The fact that PAH is more prevalent in women may be related to the autoimmune component of the disease which, in turn, may be associated with estrogen physiology. The “estrogen paradox” refers to a number of phenomena in PAH that suggest both protective and deleterious effects of this sex hormone (51); one general paradox is that estrogen, 17 β -estradiol (E₂), is generally considered cardiovascular-protective (52), and yet PAH is significantly more common in women. E₂ is a highly pleiotropic hormone with respect to immune function, being both proinflammatory and antiinflammatory under different conditions and with different cell types (53). High E₂ can actually promote Treg differentiation (54–57) while also enhancing Th2 responses (58) and B-cell/macrophage activation (59–61). We postulate that in PAH, the protective effects of E₂ may be offset by its immune-stimulatory effects, which

predominate in the absence of normal immune regulation. If autoimmunity is actually a fundamental cause of PAH and there is a strong female predilection in both processes, then the argument can be made that, in PAH, the cardiovascular-protective effects of E_2 are offset by the proinflammatory effects of E_2 . Certainly other X chromosome-related factors may be involved, but given the large amount of research establishing the importance of E_2 in both PAH and autoimmunity, an E_2 focus appears justified as a key player in both autoimmunity and pulmonary vascular disease pathogenesis.

The Translational Gap

Animal experiments demonstrate that pharmacological and cell-based therapies can prevent or reverse established severe PAH (19, 21–23, 62). New trials are beginning to test the efficacy of immunomodulatory treatments in various forms of severe PAH. (This work was summarized in a review by Rabinovitch and colleagues [9]). As these trials proceed, emerging data will facilitate an improved characterization of immune PAH phenotypes. For example, there may be subgroups of patients who present with high serum levels of leukotriene B_4 (LTB_4) (19, 34), and other patients may display high blood levels of IL-6 (5, 6). If these biomarkers are involved with disease propagation, they can help identify the best patients for particular therapies such as the leukotriene hydrolase inhibitor ubenimex (19), the anti-IL-6 antibody (tocilizumab [63]), or the IL-1 receptor antagonist anakinra (64). The antiinflammatory mechanisms of intact BMPR2 signaling may be exploited by using drugs that up-regulate BMPR2 expression (65, 66). Greater phenotyping insights may be gained by profiling the gene expression pattern of circulating peripheral blood monocytes, endothelial cell precursors, endothelial cells derived from induced pluripotent stem cells, and even endothelial cells sampled from the pulmonary arteries at the time of right heart catheterization. With some caution such expression profiles may aid in establishing a treatment-responder profile.

A major concern is how to close the translational gap more quickly given the great time required to bring discoveries forward, as well as to obtain appropriate regulatory approval and, just as challenging,

actually finance a clinical trial. We are encouraged by our experience with ubenimex and the LIBERTY (A Phase 2, Randomized, Double-Blind, Placebo-Controlled Study of Ubenimex in Patients with Pulmonary Arterial Hypertension) trial (Eiger BioPharmaceuticals, Palo Alto, CA), which took only 3 years from the publication of the preclinical science in 2013 (19) to the phase 2 clinical trial in 2016. This was facilitated by use of a repurposed drug with a well-established safety profile occurring in a biomedical community that actively fosters academic-industry partnerships. In contrast, the opportunity to test rituximab in systemic sclerosis PAH (ASC01) was made possible for our group through a grant from the Autoimmunity Centers of Excellence (Division of Allergy, Immunology, and Transplantation [DAIT], National Institute for Allergy and Infectious Diseases, [NIAID], NIH), and, certainly, NIH partnerships are needed when a tractable business model for a potential PAH cure is lacking. One quality of PAH that lends itself to new therapeutics is how well the historically profitable vasodilator drugs have fared in the marketplace, which makes it easier to attract the attention of potential investors for new drugs. As an orphan disease without cure, it is less challenging to obtain U.S. Food and Drug Administration approval than for more common conditions. Finally, for PAH, there appears to be greater willingness to “dip one’s toe in the water” and test therapies in relatively small clinical trials such as the LARIAT (A Dose-Ranging Study of the Efficacy and Safety of Bardoxolone Methyl in Patients with Pulmonary Hypertension) trial testing bardoxolone; this study used only 24 patients and demonstrated a modest treatment effect before moving on to a phase 3 trial. But challenges still loom. In 2016, adjunctive therapies face the additional hurdle of having to demonstrate efficacy exceeding that of standard-of-care vasodilator therapies. Further, we suspect that precision medicine will evolve to show that different PAH phenotypes are best treated by different vasodilator combinations with different adjunctive therapies. Given that PAH is a rare disease, now more than ever, group efforts to test these new therapies in a step-wise fashion will be needed to provide the best-tailored therapy.

Figure 1 presents a speculative model of how a specific endothelial injury could lead to different outcomes depending

on the effectiveness of the endogenous antiinflammatory mechanisms. Here, three likely PAH-relevant cell types are compared in two different contexts: T cells, macrophages, and pulmonary arterial endothelial cells in the settings of ineffective and effective immune regulation. Several putative immune checkpoints are identified (IL-6, PD-1 [programmed death 1]/PDL1 [programmed death ligand 1], and IFN- γ) that may influence whether injury culminates in endothelial-mesenchymal transition and obliterative pulmonary vascular disease or in endothelial restoration and health. Evidence suggests that Treg activity is abnormal in PAH (reviewed in Reference 47) and that IL-6 is an important biomarker and participant in the pathogenesis. IL-6 promotes Th17 inflammation, inhibits Treg differentiation, and fosters Treg conversion into Th17 cells (reviewed in Reference 67). It is also possible that ineffective Treg action may simply be due to overwhelming Th17-mediated inflammation. PD-1/PDL1 interactions are critical for Treg functionality, and blocking PD-1 enhances immunity (68). In transplantation immune tolerance (69–73), IFN- γ exhibits pleiotropic effects that could similarly influence differential immune regulation in early PAH development. IFN- γ up-regulates neopterin (74), which can be released by macrophages, dendritic cells, and endothelial cells (75); neopterin is a general marker of systemic inflammation, including autoimmunity. IFN- γ also induces indoleamine 2,3-dioxygenase (IDO), a key immunomodulating molecule produced by antiinflammatory macrophages, T cells, and endothelial cells. Up-regulating endothelial IDO is protective against the development of PH (76). Unlike significant roles demonstrated for type I interferons in PH, there is currently no known role for IFN- γ in established disease. Experience drawn from transplantation science suggests that this cytokine may be an especially important mediator for maintaining vascular health because of its role in promoting IDO.

By catalyzing the oxidative cleavage of the indole ring of tryptophan, IDO removes a key nutrient required by T cells to become activated and proliferate. In addition, IDO activates Tregs and blocks their conversion into Th17 cells (77). As noted previously, up-regulation of endothelial IDO is sufficient to prevent hypoxia-associated PH. This effect may also relate to the functional similarity of IDO to nitric oxide synthase (NOS) and its vasorelaxing properties (78). A more recent

Figure 1. A speculative model of how a specific endothelial injury could lead to different outcomes depending on the effectiveness of the endogenous antiinflammatory mechanisms. BMPR2 = bone morphogenetic protein receptor 2; IDO = indoleamine 2,3-dioxygenase; *Oct4* = octamer-binding transcription factor 4; PD-1/PDL1 = programmed death 1/programmed death ligand 1; *Sox2* = sex-determining region y-box 2; Th17 = helper T-cell type 17; Treg = regulatory T cell.

study (79) evokes another function of this enzyme, which is to promote tryptophan metabolism and alter the activity of the kynurenine pathway. In this clinical evaluation of 26 patients with PAH, kynurenine concentrations and the kynurenine-to-tryptophan ratio correlated positively with the inducible Treg/Th17 and inversely with Th17 subsets. Curiously, elevated kynurenine levels were associated with an adverse clinical course. Again, this finding is reminiscent of two prior studies reporting increased Tregs in the blood of patients with PAH (80, 81). Of note, there appear to be reduced Tregs in the lungs of patients with PAH (82). Cumulatively, these findings are consistent with (but do not prove) the notion that increased Tregs are generated in response to pulmonary inflammation but do not traffic effectively in patients with the disease. The enhanced tryptophan metabolism observed in PAH is

also relevant because it results in increased serotonin, which is a potent vasoconstrictor also implicated in PAH pathogenesis. IL-6 is another potent inducer of IDO (83), and serotonin, in turn, is an inducer of IL-6 in vascular smooth muscle cells (84). In this context, it is possible that increased tryptophan metabolism, mediated by increased IDO, could yield greater inflammation and vasoconstriction rather than modulation of inflammation by promotion of Treg activity, especially if there were fewer pulmonary Tregs present (85).

We have demonstrated that immune reconstitution with $CD4^+CD25^{hi}FoxP3^+$ T cells, if given before SU5416, effectively prevents PH and results in a pulmonary influx of Tregs expressing IL-10 and a reduction of activated macrophages (expressing inducible NOS [iNOS] and Ser-271-phosphorylated 5-lipoxygenase [p5-LO]) (19, 42). Little is presently known

about the role of IL-10 in PH. Our model of PH development posits that after vascular injury, normal Treg activity skews macrophage responses to promote injury resolution, but that in the absence of normal regulation, macrophages become activated toward, for example, increased production of leukotriene (LTB_4), an eicosanoid capable of inducing endothelial apoptosis in addition to pulmonary artery smooth muscle and fibroblast pathology (19, 34); other macrophage-derived vasotoxic inflammatory mediators seem likely. Although Treg control of macrophage activation has been described *in vitro* (86, 87), little work has been done *in vivo* to test Treg effects on macrophages (88). Macrophages are heterogeneous cell populations, and distinct subsets, which can be proinflammatory or antiinflammatory, are generated in response to injury (89, 90). For example, “M1” macrophages are “classically” activated after induction with a variety of proinflammatory agents that produce inflammatory cytokines such as tumor necrosis factor- α , IFN- γ , IL-6, and IL-12 (91–93), whereas “Mox” macrophages are an important antiinflammatory subset producing heme oxygenase-1, sulfiredoxin-1, and thioredoxin reductase (94). These latter macrophage populations exert antiinflammatory actions on the vasculature *in vivo* through the sequestration of iron, the production of bilirubin, and carbon monoxide; molecules that may protect endothelial cells and smooth muscle cells from oxidative stress and, through carbon monoxide, promote vasodilation. In reality, macrophage classification schemes belie a bewildering array of cell types that have diverse functions and phenotypes. Although a more detailed classification of antiinflammatory, alternatively activated macrophages based on gene expression profiles or differentiation status will facilitate target identification for therapeutic interventions in various pathological conditions (95, 96), the fact remains that macrophages are so highly mutable that any classification should be built with an understanding of their evanescent natures. This being said, we hypothesize that Tregs skew macrophages toward antiinflammatory functionality.

BMPR2 signaling may further contribute to either ineffective or effective immune regulation after endothelial injury. BMPR2 mutations are strongly associated with the development of familial PAH (36), and promoting BMP signaling is a promising

avenue for PAH therapeutics (66, 97). BMPR2 is a member of the transforming growth factor- β receptor family and is a ligand for bone morphogenetic proteins (BMPs) 2, 4, 6, and 7 (98). BMP2 and BMP4 have roles in the development, growth potential, and apoptosis of T and B cells. BMP2 and BMP4 (and likely its receptor, BMPR2) are also essential for differentiation of thymocytes, which are T-cell precursors (99–101). In addition, BMP2 mediates growth arrest and apoptosis of B-lineage cells (102, 103). BMP4 is also essential for the generation of B-cell and natural killer progenitors (104). As a receptor for these BMPs, BMPR2 may be significantly involved in these immune effects, and this possibility has already been suggested for the BMP effects on thymic maturation (99). BMPR2 is also expressed on immune cells, and a number of studies have demonstrated that BMPs, specifically BMP-6, can regulate the proliferation of B cells, T cells, and macrophages, through BMPR2 (105–108). Thus, it is possible and perhaps likely that BMPR2 mutations or down-regulation of BMPR2 signaling could deleteriously affect the normal development, maturation, growth arrest, and death of lymphocytes and in so doing, influence the development of PAH. In a manner analogous to patients developing PAH because of autoimmune regulator gene mutations (109), germline BMPR2 mutations may, in part, result in PAH because they lead to a fundamental defect in immune tolerance (e.g., inability to delete autoreactive B cells). Alternatively, Th17 immunity itself could conceivably affect normal BMP signaling in individuals without germline mutations and abrogate self-tolerance. Other PAH-relevant cells link BMP signaling with enhanced immunity. Reduced BMPR2 expression induces granulocyte macrophage colony-stimulating factor translation and macrophage recruitment to exacerbate PH (110). In addition, BMPR2 loss in fibroblasts can cause increased inflammation (111).

In a healthy response to pulmonary vascular injury, effective immune regulation is strongly associated with increased BMPR2 signaling and vasoprotection (42). Our prior study showed that immune reconstitution with Tregs in athymic T cell-deficient rats treated with SU5416 effectively prevented the development of PH (42). These reconstituted animals suffered less endothelial apoptosis and expressed higher pulmonary muscle cell BMPR2 than negative or diseased control rats. Interestingly, BMPR2 was noted to be up-regulated in nonvascular cells in the lungs of the athymic rats receiving Tregs; these cells were poorly characterized mononuclear cells sometimes observed adjacent to CD4⁺ T cells. Of possible relevance, a close association between BMPR2-expressing cells and T cells was also demonstrated in idiopathic PH in children (112). In this situation, the local milieu around injured blood vessels may favor the replacement of damaged endothelium with local populations of stem-like cells with the subsequent return of normal endothelium. By contrast, in the face of harmful, poorly controlled inflammation, increasing lines of evidence suggest that widespread endothelial apoptosis will lead to cellular transformation and endothelial-mesenchymal transition (reviewed in Reference 113).

Conclusions

There can be no doubt that elements of inflammation and an immune dysequilibrium characterize pulmonary hypertensive syndromes. Because right heart failure determines the survival of patients with severe PAH and because levels of circulating cytokines are correlated with outcome (6, 7), it is tempting to postulate that inflammation in PAH also impairs heart function. Both right and left ventricular

blood flow reserve is compromised in severe PAH (114), and we wonder to what extent systemic and myocardial inflammation (115), and not only the pressure-overloaded right ventricle, limits heart function. The “sick lung circulation” (115) is one source of inflammatory mediators, and it is possible that ischemic or otherwise stressed myocardium produces its own performance-limiting factors (116). There is thus a strong rationale to explore, in various forms of PH, antiinflammatory and immune-modulating treatment options, very much in analogy to the options reviewed by Dinarello in the article “An Expanding Role for Interleukin-1 Blockade from Gout to Cancer” (64). In 1799, a rock stele was discovered in the town of Rosetta, northeast of Alexandria in Egypt. Into the rock was chiseled the text of a decree. Notably, below the hieroglyphs was placed a Greek translation of the decree, which, after the work of 23 years, led to the decipherment of this ancient language. It may take many more years before the “language of inflammation” can be easily read. While we wait for the discovery of our “PAH Rosetta stone,” we may be well advised to test the existing new therapeutic options.

To summarize, injury to the small lung vessel endothelium evokes an inflammatory response that initially may serve a homeostatic purpose. Ineffective immune regulation (immune dysequilibrium) is associated with, and may contribute to, the pathobiology of angio-obliterative PAH. “Bad humors” (products of inflammation) affect the heart; and, finally, inflammation in PAH is important because it is likely treatable. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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