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Challenges and opportunities in treating inflammation associated with pulmonary hypertension

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Abstract

Inflammatory cells are present in the lungs from patients with many, if not all, forms of severe pulmonary hypertension. Historically the first inflammatory cell identified in the pulmonary vascular lesions was the mast cell. T and B lymphocytes, as well as macrophages, are present in and around the pulmonary arterioles and many patients have elevated blood levels of interleukin 1 and 6; some patients show elevated levels of leukotriene B₄. An overlap between collagen-vascular disease-associated pulmonary arterial hypertension (PAH) and idiopathic PAH exists, yet only a few studies have been designed that evaluate the effect of anti-inflammatory treatments. Here we review the pertinent data that connect PAH and inflammation/immune dysregulation and evaluate experimental models of severe PAH with an emphasis on the Sugen/athymic rat model of severe PAH. We postulate that there are more than one inflammatory phenotype and predict that there will be several anti-inflammatory treatment strategies for severe PAH.

1. Introduction

Pulmonary vascular disorders have in recent years moved from sidelined orphan diseases to emerge as a group of pulmonary hypertensive diseases that are being treated with ‘targeted therapies’. Following the introduction of prostacyclin, treatment for severe forms of pulmonary arterial hypertension (PAH) and the introduction of oral agents, clinicians worldwide have gained experience with these drugs and have recognized that the median survival of some groups of pulmonary hypertension (PH designates all forms of pulmonary hypertension including PAH) patients has improved (1–4), but also that patients treated with single agents or with combination therapy still die from right heart failure (1). The presently used drugs are vasodilators; they do not modify the pathobiology of severe PAH and a new generation of investigators are searching for new drugs to treat their patients.

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Whereas the pathophysiology of severe PAH is quite well understood: pulmonary vasoconstriction and high shear stress increase the resistance to blood flow and stimulate a remodeling process of the small pulmonary arterioles, modern mechanistic concepts of the *pathobiology* of the pulmonary vascular remodeling are based on endothelial cell apoptosis and the evolution of phenotypically altered and apoptosis-resistant vascular cells (1).

The vascular lesions and their grades of severity have been first described 60 years ago by Donald Heath and Jesse Edwards in 1958(5); this pathological Grade 1–6 severity of lung lesions characterization remains in use today. Heath and Edwards believed that there is an evolution of the vascular lesions from early muscularisation and intima fibrosis (Grade 1 and 2) to late angiomatous and vasculitic changes (Grade 5 and 6).

Although inflammatory cells in the pulmonary vascular lesions had been noted more than 40 years ago (6,7) and --to the best of our knowledge---the first report of autopsy findings regarding a patient with pulmonary vascular disease by a Viennese pathologist in 1865 were summarized with the diagnosis of “endarteritis pulmonalis obliterans” (8), PAH investigators have during the last two decades (Table 1, [9– 21]) more frequently called attention to inflammation in PAH. This topic of “Inflammation and Pulmonary Arterial Hypertension” has recently been reviewed (18,22,23). Here we will attempt to address the question: “are inflammation and autoimmunity cause or consequence of pulmonary vascular disease?”

This question is pertinent and highly relevant in the context of a translational medicine approach, and we believe that the lack of a clear answer to this question has paralyzed investigators and the Pulmonary Hypertension community at large. This paralysis continues to be reflected in the paucity of clinical trials which have been designed to treat the inflammatory component of PAH (24). Yet a large number of publications and workshop reports (18, 24–27) make it clear that our understanding of the complex interplay between pulmonary vascular remodeling and inflammation and immunity is incomplete.

In the following we will show that the answer to this question likely is: cause in some conditions and consequence in other conditions. While the nature of the inflammatory/immune response, the cells, cytokines and growth factors involved are likely different in different forms of pulmonary hypertension (“inflammation” is not “inflammation”), there may be some basic pathobiologically valid concepts that apply to all forms of severe and progressive PAH, for example: the concept of “wound healing gone awry” (1). If so, then inflammation in PAH can be understood as part of the biology of wound healing.

2. Inflammation and immune response as cause of PAH

If we tentatively define inflammation as a response to infections and cell injury, then some degree or form of inflammation is a component in the development of any form of PAH. Infection is the trigger in schistosomiasis -related PH and also in HIV/AIDS. In both instances there is a pulmonary vascular injury and an inflammatory pulmonary vascular response develops. In AIDS-associated PAH there is T lymphocyte immune insufficiency and treatment with antiviral drugs improves the PAH in some patients (28). In contrast, the schistosoma parasites lodge in the lung vessels and initially trigger a T helper 1 (TH1)

immune response (29) which then switches to a T helper 2 response which is associated with the development of lung and liver lesions.

Recent experiments in the mouse have shown that aspergillus antigen generates a T cell - dependent pulmonary vascular response culminating in a very impressive muscularisation of the pulmonary arterioles, however, surprisingly this muscularisation was not associated with lumen obliterating endothelial cell proliferation, (30). The muscular vessel wall changes were prevented in mice that were CD4 T cell depleted.

These examples are instructive as they document that infections can initiate a pulmonary vascular response that leads to pulmonary hypertension. Interestingly, in the aspergillus antigen immunization experiments, the mice developed pulmonary vascular changes but not pulmonary hypertension (30).

Chronic hypoxia leads to pulmonary hypertension characterized by media thickening, but not by lumen obliteration. Within hours after the onset of exposure to chronic hypoxia there is an accumulation of inflammatory cells in the lung, as can be shown by a large increase in the amount of myeloperoxidase enzyme in the lung parenchyma. Chronic treatment of rats with a platelet activating factor (PAF) receptor blocker, that does not affect vascular tone, prevents pulmonary vascular remodeling and the development of PAH (31). These studies were the first that provided a clue that hypoxia caused lung inflammation and that prevention of the inflammatory response prevented the development of chronic PAH—at least in rats. In another popular model of chronic PH—the monocrotaline rat model it has been shown that the increased synthesis of eicosanoids in the lung preceded the onset of PH (32).

The discovery of the transcription factor hypoxia-induced factor 1 alpha (HIF-1alpha) opened new vistas and led to the understanding that hypoxia and inflammation are linked on a molecular and cellular level (33,34), see also below. The targeting of several inflammatory pathways and the resulting modulation of chronic PAH, based on such successful targeting (35 –37), all support the notion that inflammation plays an important role in the pathobiology of hypoxia-induced PAH. An important aspect of chronic hypoxic PAH models is that the PAH is reversible upon withdrawal of the hypoxic stimulus. It is intriguing to speculate that the resolution of the lung inflammation permits the de-remodeling of the lung vessels and the return to a near normal lung circulation. A rare form of severe PAH can occur in patients with Castleman lymphoma, a disease believed to be triggered by human herpes virus 8 (HHV8) infection. In this setting B lymphocyte depletion by the anti-CD20 antibody rituximab has resulted in reversal of the pulmonary hypertension (38).

In all of these examples there is an identifiable infectious agent, inflammation and/or a change in immunity that with some probability triggers the development of PAH, and the evidence is even stronger when anti-infectious or anti-inflammatory treatment strategies reverse established PAH.

Many patients with IPAH share symptoms and markers of autoimmune diseases with patients suffering from collagen vascular diseases. Some patients present with Raynaud's phenomenon, approximately 30 % of IPAH patients have elevated levels of antinuclear antibodies, about 15 % have antiphospholipid antibodies and anti-endothelial cell antibodies

have been found in patients with IPAH (16). Approximately 30 % of IPAH patients are hypothyroid (39), perhaps as a result of autoimmune thyroiditis. IPAH patients have elevated serum levels of the interleukins IL-1 and IL-6 (11), and recently it has been proposed that high levels of these cytokines predict a poor survival. (40). In this group of patients, it is presently unclear whether a smoldering inflammation harbored in the small lung vessels contributes to the lung vascular disease or whether the inflammation is an obligatory accompaniment of lung vascular injury. Steroid- or aspirin treatment have been without impact on PAH (24), and long-term treatment with prostacyclin, which has anti-inflammatory properties (36,41,42), does not reverse established PAH.

3. Innate immunity, adaptive immunity, the bone marrow

The innate immune system provides the cells and mechanisms that defend the host against invading organisms. The cells of the innate immune system recognize pathogens and respond in a generic way. Unlike the adaptive immune system, the innate immune response has traditionally not been considered to generate lasting immunity.

However, more recently long lasting memory in previously primed natural killer cells has been reported (for details see Sun JC et al [43]).

Adaptive or acquired immunity is triggered when a pathogen evades the innate immune system and generates “danger signals” for dendritic cells. Cardinal functions of the adaptive immune system are the recognition of specific ‘non-self’ antigens during the process of antigen presentation, generation of responses ‘designed’ to eliminate specific pathogens or pathogen-infected cells and to develop an immunological memory (44,45). The principle cells of the adaptive immunity are T and B lymphocytes. CD 8+ T cells are cytotoxic cells, CD4+ helper cells can after antigen presentation generate a T helper 1 (interferon gamma) response or a T helper 2 (IL-4, IL-5) response. Regulatory T cells (Treg) cells limit and suppress aberrant responses to self-antigens. Cytokines produced during innate immune responses are among those that activate adaptive immunity.

The first inflammatory/immune cells recognized to be present in the complex pulmonary vascular lesions in patients with primary pulmonary hypertension were mast cells (Table 1). At the time mast cells were associated with asthma and histamine release and it is of historical interest that histamine released from the pulmonary vessels was postulated to be the mediator of hypoxia-induced pulmonary vasoconstriction (46). Since then it is known that mast cells are also a source of the cysteinyl leukotriene C4 and of endothelin. More recently it has been observed that mast cells accumulate at the periphery of growing tumors perhaps playing a immunosuppressive role. We are tempted to speculate—applying the concept of a quasi-malignant pathobiology of severe PAH (47)—that the long appreciated mast cell hyperplasia of the plexiform lesions, may perhaps represent a dual mechanism of local vessel wall inflammation and local immune suppression (48). A concept of local pulmonary vascular immune suppression has so far not been experimentally addressed, specifically, whether IL-6 released by mast cells affects vascular Treg function and Th17 cell differentiation (49).

In and prominently around the pulmonary vascular lesions we find T- and B lymphocytes as well as dendritic cells (10, 17) and closely associated with complex vascular lesions there are tertiary lymphoid follicles which are organized structures containing many different immune cells around a germinal center. (50). Clearly we are just at the beginning of our understanding of these localized vascular immune responses.

One hypothesis is that these cells promote and perpetuate a local inflammatory response and suppress the function of vascular T regs. Tregs improve endothelial cell function, inhibit B cells and activation and migration of macrophages, they modulate lipid metabolism and suppress macrophage accumulation and release of pro-inflammatory cytokines from smooth muscle cells (51).

The athymic, nude rat model of severe PAH which develops after a single injection of the vascular endothelial growth factor (VEGF) receptor blocker Sugen 5416 has been established to probe experimentally how important immune disequilibrium is in the development of PAH (52). Our group originally developed this model to address the hypothesis that T cell-mediated inflammation contributed to PH development. Our expectation was that T-cell deficient rats would have *less* inflammation and therefore manifest attenuated disease; the discovered result was the opposite – these animals died from PH when put in hypoxic chambers and exhibited greater pulmonary inflammation than T- cell replete rats (53)

As with the study of numerous autoimmune and transplant models, it is possible to study the effects of immune cell populations like Tregs through the process of immune reconstitution (also called adoptive transfer). Mice and rats that have genetically-absent cell populations can be evaluated with or without these cells present to gauge the effect of different immune subsets on disease development or, conversely, health. Because T cell-deficient rats developed severe PH, there was a strong possibility that their absence could explain the proclivity for these animals to develop disease. This is a compelling avenue of research because PAH conditions are associated with Treg abnormalities (54,55) (19). With this possibly fundamental observation, PH as a disease could be reframed as a condition characterized by immune dysregulation evolving from impaired Treg activity occurring in the face of serious pulmonary vascular injury (56). Exactly how Tregs work to limit the develop vascular injury in healthy individuals is the subject of ongoing research.

We next explored which immune cell populations were activated in rats with deficient Treg activity and that were disease enhancing. We chose to focus on CD68⁺ macrophages which appeared to infiltrate athymic rat lungs following Sugen 5416 administration and were also a prominent feature of clinical PAH. Leukotrienes, long implicated in the development of PAH, were notably upregulated in the blood and lungs of these affected animals; leukotriene B4 (LTB₄) in particular was implicated as an important molecule in this model of autoimmune PAH (57). Macrophage-derived LTB₄ was found to induce PA endothelial cell apoptosis and PA smooth muscle cell proliferation and hypertrophy (20). A followup study demonstrated that LTB₄ also caused human pulmonary artery adventitial fibroblast proliferation, migration, and differentiation through its cognate G-protein-coupled receptor, leukotriene B4 receptor 1 (BLT1) (58).

This model is providing important new insights that may ultimately connect VEGF, leukotriene B4 (LTB4) and bone morphogenetic protein receptor 2 (BMP2) signaling (59). Intriguingly Sugen 5416 is immunosuppressive (60) and this raises the question whether intact VEGF receptor signaling is a requirement for the control of lung inflammation and proper immune cell function (both B cells and T cells express VEGF receptors [61]).

As in all models of PH, the athymic/Sugen model is characterized by a decreased expression of BMP2 (20); this decreased BMP2 expression promotes inflammation (62). Intact VEGFR signaling is necessary for efferocytosis (phagocytic removal of apoptotic cells) (63) and impaired efferocytosis promotes persistence of inflammation and autoimmunity (64). Taken together the athymic/Sugen model of severe PAH continues to provide great opportunities to dissect the mechanistic and causative roles of inflammatory pathways and immune cells in angioproliferative PAH. In addition, experiments that investigate lung microvascular EC and Tregs can be designed to model the vascular immune synapsis, i.e. the important interactions between activated and antigen-presenting microvascular EC and various types of T lymphocytes.

4. Cytokines, chemoattraction, activated lung microvascular endothelial cells

As mentioned, 20 years ago, Marc Humbert and his colleagues reported high interleukin 1 and 6 serum levels in patients with PAH (11), and prior to this report Voelkel et al had demonstrated that in the highly inflammatory monocrotaline rat model of PAH, treatment with anti-interleukin antibodies prevented the development of PAH (65). More recently IL-6 has attracted several groups of investigators to examine its role in PAH models, and it has now been shown that elevated levels of cytokines in PAH patients are strong predictors of poor survival (40). A recent publication by Hashimoto-Kataoka et al (66) localized IL-21 to pulmonary vascular lesions in the lungs from patients with severe PAH and showed that IL-21 played a role in the development of PH in chronically hypoxic mice. In this report the authors also define a role for IL-17 in the phenotype switch of alveolar macrophages.

Although not rigorously investigated, it is possible that a prominent source of the high serum levels of IL-1 and IL-6 is the lung microvascular endothelium (67). While IL-1 β is secreted, IL-1 α is not and IL-1 α may play in activated endothelial cells the role of a transcription factor (68). IL-1 β activates the bone marrow to release hematopoietic precursor cells (69).

Apoptotic endothelial cells contain the full-length IL-1 α precursor (70); IL-1 α appears to be a DNA damage sensor (71) and play a role in cellular senescence (72). Whether IL-1 α plays an important role in inflammation and autoimmunity in PAH remains to be investigated. Cytokines are pleiotrophic, have local and systemic effects and are likely targets for therapeutic interventions in different form of PAH. Little information is available in regards to a potential role of anti-inflammatory cytokines such as interleukin 10 (IL-10) in PAH(73). It will be both a challenge and an opportunity to examine the particular inflammation/autoimmunity patient phenotypes. Patterns of expressed blood biomarkers will be established and activated circulating cells can be isolated and studied *in vitro*. Interleukin

blocking antibodies are already undergoing clinical testing in a variety of human diseases, including rheumatoid arthritis, post-myocardial infarction syndromes and cancer (74–77).

Toll-like receptor (TL-R) activation by hypoxia, endotoxin, hyaluronic acid or heat shock proteins may contribute to hypoxic pulmonary hypertension in mice, because TL-R4-deficient mice were protected against hypoxia-induced PAH (78) and, of interest, hematopoietic stem cell differentiation is controlled by TL-Rs (79) and viral infection can increase TL-R expression in pulmonary vascular smooth muscle cells (80).

Without doubt, there are different signaling cascades, cell-cell interactions local and long-distance mechanisms involved in the complex pulmonary vascular wound healing process that involves chemotaxis, cell proliferation and cell phenotype shifts. A great challenge will be to identify those upstream events that control the sterile inflammation and can be safely modified without compromising antibacterial and antiviral defenses -- and without promoting malignant cell growth.

5. Modifiers of immune responses

Immune responses can actively contribute to the pathobiology or a weak or aberrant response can be permissive for the development of PAH. Observational studies over the last years have established that at least in the USA severe PAH is increasingly diagnosed in obese, menopausal or post-menopausal women (81,82). A large and growing literature backs up the concept of adiposity as an inflammatory state and a connection has been made between PAH and the metabolic syndrome (83,84). As adipose tissue expands, there is an increase in chronic smoldering inflammation due to infiltration of cytokine producing macrophages, T- and B lymphocytes and eosinophiles (85, 86). A recent analysis by Ussavarungsi et al from the Mayo Clinic found that 13% of patients with IPAH also carried the diagnosis of 'metabolic syndrome' (87) and it is of importance that Huertas et al (88), in search of mediators of PH in patients with PAH and the metabolic syndrome, found high circulating leptin levels. The investigators went on to show that endothelial cells from the lungs of PAH patients released high levels of leptin and that circulating Treg cells expressed leptin receptors and that leptin impaired Treg cell function. Furthermore, these authors showed that leptin KO mice were protected from developing hypoxia-induced PH (89). Women with the metabolic syndrome demonstrate elevated serum levels of myeloperoxidase, CRP, leptin and PAI-1 (90), thus obese women and women with the metabolic syndrome may develop PAH under the influence of adipokines and also the influence of estrogen, an upstream activator of VEGF gene expression. Leptin expression in skin wounds is associated with exuberant wound healing (91) and leptin expression in cancer tissues is associated with resistance to tumor therapy (92). In our opinion, it will be important -and perhaps productive, to examine whether obese women with or without the metabolic syndrome represent an inflammatory phenotype that sets them aside from PAH patients that have AIDS or scleroderma.

Another immune modulator is hypoxia (93). Hypoxia stabilizes the HIF-1 α protein which transcribes NF κ B (94) and HIF 1 α impacts on the differentiation of Treg cells (95), HIF-1 α KO in dendritic cells leads to a decreased production of Foxp3 Tregs and

mice lacking the inflammasome adaptor protein ASC are protected against chronic hypoxia induced pulmonary vessel muscularization (96).

Thus, in the aggregate, a picture emerges that is highly suggestive: inflammation and altered immunity have a strong impact on the lung vessels; it is reasonable to postulate that most forms of severe PAH—if not all forms of PAH—have an inflammatory component that either is sufficient (together with one or more co-conditions) to initiate the pathobiology of exuberant wound healing, or that contributes to the persistence and progression of the pulmonary vascular disease. If the first hit is a genetic susceptibility, then immune modifiers may weaken the normal homeostatic vascular defense mechanisms further permitting vascular remodeling to occur.

It is widely accepted that drugs can in susceptible individuals trigger the development of severe PAH (97), however, chronic cigarette smoking is surprisingly not considered a risk factor for the development of clinically significant PAH. This is surprising in view of the fact that eosinophilic granuloma-associated severe PH is a disease of smokers and also that there is a subgroup of COPD patients (chronic smokers) that develop severe PAH (98). In addition, Trip et al reported on a group of patients with IPAH that were characterized by a surprisingly low diffusing capacity; these patients had a worse prognosis and they were cigarette smokers (99). Although extensive lung tissue destruction can easily be cited as an explanation for the development of PAH in these patient groups, it is necessary to also consider the impact of chronic smoking on immune responses. The smoking-induced chronic, smoldering inflammation in the lung leads to a tolerance break and altered immunity. Kearley et al recently focused on a smoking-induced IL-33-dependent proinflammatory response (100), Yuan F. et al (101) showed that cigarette smoke extract causes macrophage M2 polarization and Heijink IH et al (102) reported that cigarette smoke promotes neutrophil necrosis. In our opinion there is a need to investigate the pathobiology of cigarette smoke-inhalation—related pulmonary vascular diseases and to consider in addition to direct toxic endothelial cell effects also aberrant immune responses.

6. Does targeted therapy for PAH modify inflammation?

If smoldering inflammation is involved in many forms of severe PAH, then it makes sense to introduce drugs into the treatment plan that modify inflammation and perhaps autoimmunity. One question is whether presently used drugs have anti-inflammatory properties. With the exception of prostacyclin there is little specific information in regards to this question. Prostacyclin via the IP receptor elevates intracellular cAMP and suppresses Th-2 mediated inflammatory responses and it has been suggested that prostacyclin enhances the production of the anti-inflammatory cytokine IL-10 (103). Prostacyclin inhibits platelet activating factor release and the adhesion of lymphocytes to endothelial cells (104). Whether prostacyclin has also anti-inflammatory properties in the stressed right ventricle of patients with severe PAH is presently unknown.

The endothelin receptor blocker bosentan has become a drug of choice for the treatment of patients with severe PAH. There are several studies that show that bosentan inhibits endothelin 1-induced IFN γ release from CD4 $^{+}$ cells (105) or endotoxin-stimulated

cytokine release from pulmonary arterial smooth muscle cells (106), however, Brun et al assessed the systemic inflammatory response in congenital heart disease -associated PAH patients and found no effect of bosentan treatment inflammation (107).

Sildenafil and Tadalafil are phosphodiesterase V inhibitors frequently used in the therapy of PAH patients. Tsai BM et al (108) reported that hypoxia-induced IL-1 β expression in pulmonary vascular smooth muscle cells was suppressed by tadalafil, Pifarre P. et al described an increase in the numbers of Tregs induced by sildenafil (109) and Califano JA et al (110) the reversal of tumor specific immune suppression by tadalafil in patients with head and neck cancer. A cardioprotective action –decrease of inflammation and of apoptosis has been reported by Westermann et al, (111).

Overall prospective studies designed to examine potential anti-inflammatory activities of presently used PAH therapies are lacking.

7. Scleroderma-associated PAH: A Paradigm for Inflammation and PAH

In 2016, the NIH trial evaluating B cell depletion for the treatment of systemic sclerosis-associated PAH (SSc-PAH) is scheduled to be completed; this is presumably the first of a number of trials designed to test the potency of adjunctive immunomodulation layered onto standard-of-care vasodilators. SSc-PAH presents in three basic forms: 1) severe SSc-PAH accompanying limited cutaneous SSc, 2) SSc-PAH accompanying (secondary to) interstitial lung disease, and 3) a more indolent form of SSc-PAH which reflects vascular pathology of SSc (112). Although there appear to be different subtypes of SSc-PAH, it is not known if and how the pathogenesis differs with each form. In all patients with SSc, SSc-PAH significantly worsens survival and is the leading cause of mortality in these patients. Treatment for this disease is currently limited to the same vasodilator therapy employed in all forms of PAH. An evaluation of 91 patients with PAH treated with prostanoids revealed that SSc-PAH patients had the worst survival of all subgroups analyzed (113); a finding later confirmed by the REVEAL registry studies (114,115). Lungs from patients with SSc-PAH exhibit a characteristic vascular pathology, the plexiform lesion, which is structurally similar to plexiform lesions found in other PAH conditions such as idiopathic PAH and HIV-associated PAH (116). In SSc-PAH, both plexiform and concentric obliterative lesions stain positively for factor VIII-related antigen consistent with abnormal endothelial cell proliferation. Macrophages, T and B cells are noted clustering in and around the vascular lesions. Interestingly, the primary proliferative abnormalities within the pulmonary arterial walls are similar to those found in SSc digital arteries. Both digital and pulmonary arteries have medial and adventitial fibrosis that lead to structural luminal narrowing.

Endothelial cell apoptosis may be the first event in the pathogenesis (117). Anti-endothelial cell antibodies (AECA) are found in the circulation of SSc patients and have been posited to play a role in the development of vascular disease found in SSc, including PAH (118). An instigating injury to endothelial cells in SSc that may trigger such autoantibody formation may be a viral infection (119). A number of investigators have found evidence for viral infections, such as Epstein-Barr virus, parvovirus B19 and hepatitis C, E, and G in patients with SSc (120–125). Although the role of cytomegalovirus (CMV) in the pathogenesis of

SSc is debated (126), indirect evidence for a role of CMV-specific antibodies in the development of this disease has also been presented (127,128). Not only are absolute lymphocyte counts reduced in SSc (129,130), but SSc patients also have relatively fewer CD4⁺CD25⁺ cells in the peripheral circulation compared to healthy controls (131). In this setting of diminished regulatory T cells, a dysregulation of B cells is also observed (132). Of note, CD19 expression is increased by $\approx 20\%$ on B cells in patients with SSc, while other B cell markers CD20, CD22 and CD40 are normally expressed (133). Several different autoantibodies have been implicated in the pathogenesis; anti-topoisomerase I (anti-Scl-70) and anticentromere antibodies, for example, are relatively specific for SSc (134), whereas antibodies to Cu/Zn superoxide dismutase and anti-topoisomerase II have been associated with localized scleroderma (135,136). In summary, SSc is an autoimmune disorder that has been associated with viral infection, endothelial damage, diminished regulatory T cells, dysregulated B cells, abundant mast cells and serum autoantibodies.

Very little is known about the pathogenesis of SSc-PAH, but it appears to be a complex and multifactorial process. Unlike familial PAH, SSc-PAH is not associated with a mutation of bone morphogenetic protein receptor type 2 (BMPR2), a member of the TGF- β superfamily of receptors (137). However, it has been recognized that altered expression of TGF- β superfamily receptors, interacting proteins, or downstream signaling molecules occurs in SSc (138–140). Defects in the balance of vasoconstrictors and vasodilators have been another research focus in SSc-PAH with a notable elevation of endothelin-1 (ET-1), a potent vasoconstrictor (141).

In addition to the interest in TGF- β superfamily alterations and abnormal vascular tone, there is significant work focusing on dysregulated immunity in the pathogenesis of SSc and SSc-PAH. Plexiform lesions found in the arterial walls of SSc PAH patients include an inflammatory infiltrate (142) consisting of macrophages, T cells, B cells and mast cells (143,144).

Endothelial alterations may affect stimulatory changes that involve many cells, including T cells, macrophages, mast cells and fibroblasts. Once activated, these cells secrete a variety of substances, including enzymes and their inhibitors and cytokines and their soluble receptors. These substances lead to changes in the extracellular matrix proteins, including fibronectin, proteoglycans, and collagen types I, III, V, and VII which result in fibroproliferative changes (145). Activation of the immune system appears to be of paramount importance in the pathogenesis of SSc. Antigen-activated T cells infiltrate the skin and produce the profibrotic cytokine IL-4. B cells may contribute to fibrosis, as deficiency of CD19, a B-cell transduction molecule, results in decreased fibrosis in animal models as described below.

CD19, a critical cell-surface signal transduction molecule of B cells, is the most positive response regulator of B cells and has been implicated in SSc. Transgenic mice which overexpress CD19 lose self-tolerance and generate autoantibodies spontaneously (146). CD19 overexpression in the tight-skin mouse model of SSc results in increased autoantibody production (147) whereas CD19 deficiency significantly decreases skin fibrosis (148). B cell depletion in the tight-skin mouse model was recently noted to be effective in reducing skin fibrosis and autoimmunity during the initiation of disease, but was not effective in

established disease (149). It has been hypothesized that chronic B cell activation due to augmented CD19 signaling leads to skin fibrosis and enhanced autoimmunity in SSc (150,151).

In the athymic model of autoimmune PAH, T-cell deficient animals with PAH exhibit an accumulation of B cells and macrophages within one week of SU5416 administration. These findings were not observed in athymic nude rats that underwent immune reconstitution prior to SU5416 administration. These results are concordant with the loss of self-tolerance in animals missing normal Treg populations and the appearance of various autoantibodies and autoimmune disease (152,153). With complete elimination of CD4⁺CD25^{hi} cells, systemic autoimmunity occurs as manifested by multiorgan inflammation and autoantibody production (154). 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. This latter principle suggested that a therapy which addressed autoreactive B cells and possibly self-directed antibodies could be effective in PAH associated with immune dysregulation. As noted, this finding is relevant in SSc-PAH patients who likely have abnormally functioning Treg cells. Dysregulated B cell immunity, as a direct consequence of diminished regulatory T cell control in the T-cell deficient athymic rat model, is important because presently B cells can be more easily targeted with a therapeutic intervention than abnormal Treg activity.

In Ssc patients, even partial reversal of the PAH has the potential to be life-saving. In this respect, some patients have shown clinical improvement in their PAH with rituximab. In one published case, a patient with severe SSc-PAH who was refractory to a number of therapies, responded very well to several rounds of rituximab therapy (155). In several unpublished cases (e.g. a case of Waldenstrom's macroglobulinemia-associated PAH in Denver, Colorado), patients have enjoyed significant clinical improvement following rituximab therapy. The following is an example of one such case from Stanford University Medical Center: A 30-year-old patient with SLE presented with Class IV heart failure symptoms, along with necrotizing digital ulcers. An echocardiogram revealed an RVEF of 10% and an LVEF of 35% along with evidence of myocarditis. While the patient's left ventricular function improved with diuresis and steroids, the patient's right heart systolic pressures were systemic. The patient did not respond to cyclophosphamide and was subsequently treated with several doses of rituximab. Within weeks, the patient's symptoms improved dramatically with a fall in the pulmonary artery pressures from the 80s-90s to 25 mmHg and Class II heart failure symptoms (Dr. Roham Zamanian; personal communication).

Another illustrative case at Stanford involved a woman with PAH secondary to the antiphospholipid antibody syndrome. The patient had suffered thromboembolic disease, and despite having undergone thrombendarterectomy twice, continued to have worsening symptoms of PAH. The patient had been anticoagulated and treated with prostacyclin. After the patient was treated with rituximab this patient enjoyed a significant improvement allowing a downgrading to Class II symptoms. This improvement, attributed to rituximab, was credited with the clinical change and the conversion from intravenous prostacyclin to oral therapy. In summary, while there has been relatively little published to date on the use of rituximab in SSc, there appears to be a strong scientific and clinical basis for using this

therapy, which is well studied in other diseases, in SSc patients who otherwise do not have much hope for long-term survival.

The NIH trial, that is concluding in 2016, is a multicenter, randomized, double-blinded and placebo-controlled trial. A number of mechanistic studies including Treg evaluations, multiplex cytokine assessments, autoantibody panels, microbiome/virome studies will be evaluated at baseline and over time after B cell depletion. We expect that these results will be highly informative in regards to the phenotypes of SSc-PAH patients as well as the endotypes if certain SSc-PAH patients (a highly heterogeneous group to start with) exhibit responsiveness to rituximab therapy and others consistently do not. Both positive and negative endotype information will inform future immunotherapy trials in PAH.

8. Right Heart Failure and Inflammation

Right ventricular dysfunction has now been recognized as a rate-limiting factor in patients with severe PAH; in fact, the quality of life and the outcome depend on the right ventricular functional reserve (156, 157). Experimentally, over-expression of various cytokines has been demonstrated in heart failure models. While data derived from heart tissue of PAH patients are presently lacking, it is not farfetched to postulate that effective treatment of patients with severe PAH decreases the activity of inflammatory cells in the myocardium and the activation of cardiomyocytes and fibroblasts (36, 158). Alternatively, a reduction of the degree of systemic inflammation may also reduce the inflammatory response in the stressed heart. Thus, it is important in future clinical trials that examine the effects of anti-inflammatory drugs and strategies—including exercise programs—to consider mechanisms that lead to improved cardiac performance.

9. Expert Commentary

There have been a great number of studies which have established that markers of inflammation are detectable in peripheral blood samples from patients with a variety of forms of PAH—and an even greater number of experimental studies and preclinical trials that shed light on the complex association of severe PAH, inflammation and disturbed immunity. In fact, over the last decades the evidence for such associations has become overwhelming. Yet, in spite of such evidence, the translational efforts and the conduct of clinical trials designed to explore anti-inflammatory treatments or strategies directed towards immune system response modification have been few.

One such recent trial tested the hypothesis that thromboxane plays a significant role in PAH and the effect of low-dose aspirin treatment was tested in patients with severe PAH; no clinical effect could be demonstrated (24). However, the importance of this endeavor was that a specific hypothesis has been tested. What is now needed is that further clinical trials are designed which test specific hypotheses. For example, if there is a strong case for a critically important role for IL-1 or IL-6 in the pathogenesis of severe PAH, anti IL-1 or anti IL-6 antibodies should be used for treatment of PAH patients that highly express these proteins in their blood.

As we have alluded to: it is not likely that the identical inflammatory pathways and immune disturbances play out in each and every PAH patient, or even within a particular subgroup of patients; instead inflammation phenotyping will be required to distinguish between patients and to make predictions about mechanistically important targets, and whether or not a particular treatment strategy has an acceptable risk/benefit ratio. The treatment targets then are the inflammatory cells and the resident vascular cells that have been activated to produce damaging molecules—both in the lung circulation and in the heart.

Many challenges and opportunities are lying ahead; here just a few of these: to identify and characterize treatment responders, to examine right heart function as an outcome parameter and to investigate whether patients in the early phases of PAH respond to treatments and whether the treatment of a smoldering inflammation in patients with established disease and treated for many years with presently available drugs changes outcome.

10. Five-year View

Going into 2016, the future is bright and exciting for disease-modifying PAH treatments that target inflammation; there is an expectation that the next five years will see a number of adjunctive therapies being tested in clinical trials. Our expectation is that different modalities will affect different PAH conditions uniquely and that the timing of intervention will be important. Whereas inappropriate vasoconstriction is a feature of all forms of PAH, a reasonable expectation is that these newer therapies will be more targeted. For example, Eiger Pharmaceuticals will likely conduct a Phase II trial in 2016 to test the efficacy of Ubenimex, an inhibitor of leukotriene B₄ (LTB₄) biosynthesis. Our group described how LTB₄ secreted by CD68⁺ macrophages induces PAEC apoptosis, PASMC growth and adventitial fibroblast proliferation, migration, and differentiation (20,57). Blocking LTB₄ reversed advanced PH in two experimental models and LTB₄ is elevated in the blood of PAH patients. A different approach adopted by Spiekerkoetter and colleagues at Stanford was to screen FDA-approved compounds and to search for drugs that promote protective bone morphogenetic protein (BMP) signaling (159). They discovered that the calcineurin inhibitor, FK506, effectively upregulated BMP pathways at very low doses and reversed experimental PH. On the basis of these findings, Dr. Spiekerkoetter and her team initiated a randomized, double-blind, placebo-controlled phase IIa trial to evaluate the safety and tolerability of FK506 in PAH patients; this pilot study has now been completed with analysis pending. Outside of this trial, the Stanford group also published the results of treating patients with advanced disease (160). Here, low-dose FK506 was used compassionately in three patients with end-stage PAH who did not qualify for the Phase II trial because of the severity of their illness, and efficacy was clearly suggested. Finally, a third Stanford-based approach emanating from the basic science work of Marlene Rabinovitch has suggested that targeting neutrophil elastase with the endogenous inhibitor elafin may be yet another promising approach (161). Phase I studies with elafin are planned for the latter half of 2016.

In the decade ahead, as PAH patients are better phenotyped and endotyped, it is conceivable that combination therapies will emerge (e.g., rituximab plus low-dose FK506). Finally, as Treg therapy is being tried in a number of autoimmunity and transplantation trials, there is

an emerging rationale to consider this approach for PAH. This burgeoning armamentarium of therapeutics will surely foster precision medicine for PAH in the years ahead.

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11. Key issues

- It is well established that inflammatory cells are present in and around the remodeled pulmonary arterioles in many, if not all, forms of severe pulmonary hypertension, yet treatments targeting the inflammatory component in pulmonary vascular diseases have not been systematically evaluated.
- One source of circulating mediators of inflammation in patients with severe PH is the “sick lung circulation”
- Plasma levels of interleukins, leukotrienes and leptin can be measured and support phenotyping of patients with severe PH.
- Steroids have been shown to be effective in patients with systemic lupus erythematoses-associated PH and in patients with the POEMS syndrome.
- Ubenimex (bestatin), an inhibitor of the leukotriene B4 synthesis will be tested in a phase II pilot study, and the results of a controlled clinical trial examining the effect of the anti CD-20 antibody rituximab in patients with scleroderma-associated PH are expected at the end of 2016.
- Because most patients with PH die from right heart failure, one clinical endpoint of trials should become the right ventricular ejection fraction (RVEF) measured by cardiac MRI. The inflammation present in PH is likely also to determine myocardial function.
- It is perhaps intuitive that early treatment of inflammatory components in PH patients may be more successful than treatment in patients with long-standing disease.

Table 1

Inflammation in pulmonary arterial hypertension; a time line.

1865		1958	1967	1969	1983
			Mast cells in the lungs of crotalaria-treated rats, Kay	Mast cells in the lungs of patients with mitral stenosis, Heath	Leukotrienes in neonatal PAH, Stenmark et al.
		Vasculitic changes in severe PAH, Heath & Edwards			
Endarteritis pulmonalis deformans, Klob					
1994		1995	1998	2000	2001
Endothelial cells and inflammatory cells	IL-1 & IL-6 in patients		PAH in AIDS/HIV, Mehta et al.		
Present In PAH lung lesions, Tudor et al.	Humbert et al.		5 lipoxygenase and FLAP, Wright et al.		Angiogenesis factor Inflammatory cells expressed in vasc. lesions, Tudor et al.
2005			PAH in the POEMS syndrome, Lesprit et al.		
Anti-endothelial antibodies in PAH, Tamby et al.			2007		2009
					WHO workshop on PAH & inflammation report, Hassoun et al.
			Dendritic cells in PAH vascular lesions, Perros et al.		
2011		2013	2015		
Tregs in experiment. PAH, Tamosiuniene et al.	Elevated LTB4 levels in patients with PAH, Tian et al.		Inflammatory signature of PAH lung endothelial cells, Hires et al.		

IL: Interleukin; PAH: pulmonary arterial hypertension; FLAP 5-lipoxygenase activating protein; Treg: regulatory T cells.