Basic Science for Clinicians

Immune Dysregulation and Endothelial Dysfunction in Pulmonary Arterial Hypertension
A Complex Interplay

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Pulmonary arterial hypertension (PAH) corresponds to a heterogeneous group of severe clinical conditions characterized by precapillary pulmonary hypertension (PH) diagnosed when mean pulmonary artery pressure equals or exceeds 25 mm Hg at rest with normal pulmonary artery wedge pressure (≤15 mm Hg). According to the current clinical classification, PAH can be idiopathic (IPAH), heritable, drug or toxin induced, or associated with other diseases (eg, connective tissue diseases [CTDs], congenital heart diseases, HIV infection, and portal hypertension).1 PAH has a complex and multifactorial pathogenesis in which excessive migration and proliferation of pulmonary vascular cells (ie, endothelial cells [ECs] and smooth muscle cells [SMCs]) and dysregulated immune responses are critical contributors to the inappropriate pulmonary vascular remodeling. PAH is a fatal condition leading to right heart failure and death within 2 to 3 years after diagnosis if left untreated. During the last decade, therapeutic options for the treatment of this disease have improved exercise capacity, quality of life, and long-term outcomes. However, there is currently no cure available, and further insight into the disease pathophysiology is needed to advance drug development and to improve patient management.

It is now widely accepted that altered immune mechanisms play a significant role in PAH by recruiting inflammatory cells, remodeling the pulmonary vasculature, and promoting autoimmune responses.2-4 Inflammation is a general term for the local accumulation of fluid, plasma proteins, and white blood cells that is initiated by physical injury, infection, or a local immune response. These phenomena represent the innate immune response. The innate immune response contributes to the activation of adaptive immunity, which is the response of antigen-specific lymphocytes to antigen, including the development of immunologic memory. The unique features of adaptive immunity, based on clonal selection of lymphocytes bearing antigen-specific receptors, provide the ability to recognize all pathogens specifically and enhanced protection against reinfection. When adaptive immunity attacks the self, because of a breakdown of self-tolerance, an autoimmune response develops (ie, directed against self-antigens) that can give rise to an autoimmune disease. The pathophysiology of autoimmune diseases involves an aberrant interplay between the innate and adaptive immune systems, culminating in a loss of self-tolerance. Dysregulation of these processes results in T cell–mediated autoimmune responses and autoantibody formation. Normal inflammatory/immune responses proceed through initiation, effector, and resolution phases. In contrast to normal responses, there is sustained cellular activation in autoimmune diseases, resulting in chronic inflammation with concomitant tissue damage and remodeling.

It currently remains unclear how altered immune responses may contribute to PAH initiation, perpetuation, and worsening. This review highlights the central role of dysregulated immune mechanisms in PAH pathogenesis.

Clinical Evidence for Altered Immune Responses in PAH

Innate Responses

It is now well established that a wide array of inflammatory markers are increased in the serum and lungs of patients with PAH. Circulating levels of cytokines, including interleukin (IL)-1β and IL-6,5 and chemokines such as CC chemokine ligand 2/monocyte chemotactic protein-1,6 CC chemokine ligand 5/RANTES (regulated on activation, normal T cell expressed and secreted),7 and CX(3)C fractalkine8 are elevated in IPAH and CTD- and HIV-associated PAH patients.9 Increasing data indicate that inflammation is involved in PAH pathophysiology, and it has been shown that circulating levels of inflammatory mediators correlate with patient survival.10,11 These inflammatory mediators play a role in leukocyte recruitment and trafficking and are produced predominantly by inflammatory cells of the innate immune system, but they can also be produced by any of the cellular components of the vascular wall or adventitia.12 Classic arteritic PAH lesions


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comprise pulmonary transmural inflammatory cell infiltrates with focal vessel wall necrosis and fibrinoid insudation, a histological pattern that has been etiologically linked with particularly severe forms of PAH. The histological “inflammatory mark,” which is much more frequent, corresponds to pulmonary perivascular inflammatory infiltrates, made up primarily of T and B lymphocytes, mast cells, dendritic cells (DCs), and macrophages.\textsuperscript{13–15} (Figure 1). Interestingly, it has recently been shown, in an analysis of 62 PAH explanted lungs, that marked perivascular inflammation is present in a high number of PAH lungs and correlates with intima and media remodeling.\textsuperscript{16} Despite these data, whether such inflammatory infiltrates are involved in the pathobiology of PAH or whether they are only epiphenomena linked to other pathological mechanisms leading to pulmonary vascular remodeling is still unclear. However, according to the experience gained in our national PH reference center, which gives us access to a large collection of pulmonary samples from severe PAH, inflammatory lesions seem more often to be associated with active and cellular arterial remodeling rather than cicatricial-like fibrotic lesions.\textsuperscript{12}

Innate responses and effectors such as natural killer (NK) cells may also play an important role in PAH pathogenesis. It has been shown in IPAH and heritable PAH, as well as in animal PH models, that NK cells display an altered and impaired phenotype.\textsuperscript{17} Furthermore, cytotoxic T, NK, and NK T cells may contribute to vascular remodeling in different physiological and pathological conditions. We have recently shown that immune regulation of cytotoxic, NK, and NK T cells could contribute differently to the pathophysiology of pulmonary veno-occlusive disease (PVOD) and PAH. We found that in PVOD there was a decrease in populations and subpopulations of cytotoxic and NK T cells but an increase in NK populations. We assessed their function through their capacity to produce granulysin and found that peripheral blood mononuclear cells and explanted lungs display lower levels of \textit{GNLY} demethylation in PVOD compared with PAH patients. Furthermore, despite the reduced granulysin-containing cells in patients with PVOD, granulysin serum levels were higher, suggesting that these cells were secreting their content.\textsuperscript{18} These results suggest that pulmonary vascular remodeling in PAH or PVOD might be linked to alterations of the circulating and pulmonary compartments of immune cells.

Although increased inflammatory mediators and cell infiltrates represent a common feature of various forms of PAH, only a small percentage of patients with established PAH respond to anti-inflammatory drugs. Complete reversibility of PAH is rare and appears mostly in the setting of active autoimmune diseases treated with corticosteroids or immunosuppressive agents. In a retrospective study, Sanchez and coworkers\textsuperscript{19} were able to distinguish a small subgroup of patients with

\textbf{Figure 1.} Inflammation in pulmonary arterial hypertension. \textbf{A}, Representative CD3 immunohistochemistry staining of a human distal remodeled pulmonary artery. \textbf{B}, Representative hematoxylin and eosin staining of a distal remodeled pulmonary artery associated with a lymphoid infiltration. \textbf{C} through \textbf{E}, Representative immunostaining of precapillary pulmonary arteries showing progressive occlusion of the arteries (\textalpha-smooth muscle actin–positive cells in white) associated with arterial infiltration/accumulation of CD11c\textsuperscript{+} dendritic cells (in red; \textbf{C}), lymphoid neogenesis characterized by periarterial lymphatic dilation (\textbf{D}), and tertiary lymphoid follicle formation (\textbf{E}) associated with c-kit\textsuperscript{+} cells (in green in \textbf{D} and \textbf{E}) possibly supplied by Lyve-1\textsuperscript{+} lymphatic vessels (in red in \textbf{D} and \textbf{E}). Cells were counterstained with DAPI.
CTD-associated PAH who responded to anti-inflammatory therapies with hemodynamic improvement (8 patients of 28 and none with systemic sclerosis [SSc]). Another study performed by our group showed that only 50% of CTD-associated PAH patients treated with immunosuppressants (cyclophosphamide and glucocorticoids) had significant improvement. Interestingly, this study highlighted that the patients who could benefit from immunosuppressive therapy could be those who have less severe disease at baseline. Furthermore, Montani et al reported that a patient displaying PAH associated with multicentric Castleman disease, HIV-1, and human herpesvirus-8 infections responded dramatically to anti-inflammatory treatment with complete reversibility of PAH, allowing weaning of continuous specific PAH therapy.

Adaptive Responses

Fine targeted immune mechanisms are characterized by a specific response to an antigen; they are favored by an inflammatory background and refer to adaptive immunity. In the last decade, increasing evidence has highlighted the presence of dysregulated immune mechanisms in PAH patients. A role for autoimmune processes was first proposed in the context of CTD-associated PAH, in particular with patients with SSc. In the French PAH registry, CTDs (represented mainly by SSc) account for 15.3% of PAH cases, whereas the prevalence of PAH in SSc ranges between 7.8% and 12%. It is well known that PAH-SSc patients display circulating autoantibodies, but interestingly, it has been reported that a subset of patients with IPAH also display circulating immunoglobulin G–type autoantibodies directed against different components of the vascular wall, ECs, fibroblasts, and SMCs. A recent study has confirmed the prevalence of anti-EC autoantibodies (AECAs) that recognize cell surface components in patients with IPAH (62% prevalence) or associated PAH (78% prevalence). It is also estimated that 30% to 40% of patients with IPAH present antinuclear antibodies and that 10% to 15% of these patients have antiphospholipid antibodies. Interestingly, these antiphospholipid antibodies are found more frequently in chronic thromboembolic PH patients, a feature that could be ascribed to the thrombotic risk factor of the disease.

The main effectors of the adaptive immune response are the T and B lymphocytes, which are selected in the thymus and in the bone marrow, respectively, to react against the nonself and are activated only in the presence of foreign antigens from pathogens. The self-tolerance is controlled in the periphery by a particular population of T lymphocytes called regulatory T lymphocytes (Treg), which develops in the thymus and plays a role in the pathogenesis of several inflammatory and autoimmune diseases. Tregs are involved in the feedback control of the immune response and in the return to homeostasis. They are also known to dampen autoimmune responses and may delay the onset and progression of autoimmune disorders. Reduced Treg count or defective suppressor function has been observed in humans displaying several autoimmune diseases such as SSc. Little is known about the role of Tregs in pulmonary diseases, particularly PAH. Two studies showed an increase in Treg number in peripheral blood in PAH patients, whereas we recently reported, with more specific markers, that IPAH and PAH-SSc patients display a normal Treg cell count but with an altered function.

Another key cell type is represented by DCs. Their function is not primarily to destroy pathogen but to carry pathogen antigens to peripheral lymphoid organs and present them to T lymphocytes. DCs represent not only simple antigen-presenting cells responsible for the initiation of inflammatory responses but also key modulators of the whole immune process. Hence, DCs are highly specialized immune cells that induce both T-cell immunity and tolerance. It has recently been shown that these 2 seemingly opposite functions are played by 2 distinct DC activation stages. Mature DCs are considered to be immunogenic and potent inducers of T-cell immunity through their upregulation of major histocompatibility complex class II and costimulatory molecules. Mature DCs are indeed able to efficiently process and present antigens to T cells, leading to active effector T-cell responses. On the other hand, the immature subset of DCs express a small amount of major histocompatibility complex class II and costimulatory molecules and are able to maintain peripheral tolerance to self-antigens. It has also been shown that different stimuli can induce or inhibit the DC maturation level. Proinflammatory cytokines will promote DC maturation and induce T helper 1 responses, whereas anti-inflammatory molecules such as IL-10 will inhibit DC maturation, promoting T helper 2 responses or Treg activation. In IPAH and in PH animal models, we showed that immature DCs accumulate in remodeled pulmonary vessels and hence could be involved in the pathobiology of PAH.

In vascular lesions, all effectors of a local immune response are present around the remodeled vessels in patients with IPAH: macrophages, monocytes, mast cells, DCs, CD3+ T lymphocytes, CD8+ cytotoxic T cells, CD4+ T helper cells, and the few CD20+ B lymphocytes that could contribute to disease pathology. It is important to point out that patients with an associated immune disorder present pulmonary lesions that cannot be discriminated from those encountered in patients with IPAH.

We have recently shown that tertiary (ectopic) lymphoid tissues accumulate along the remodeled pulmonary vasculature of IPAH patients (Figure 1), suggesting the presence of local autoimmune responses in IPAH. Lymphoid neogenesis in the target organ is considered to be a major element of autoimmune diseases, and it has been suggested to correlate with local autoantibody production (Table). Despite increasing clinical evidence, it is still unclear how immune dysregulation could contribute to PAH pathogenesis. According to current knowledge, it is particularly difficult to assess whether altered immune responses represent a cause or an effect of the disease.

Contribution of Dysregulated Immunity to PAH Pathogenesis

Endothelial Dysfunction Leading to Vascular Remodeling as a Result of Altered Immunity

Recently, there has been increasing evidence for inflammatory involvement in the initiation of pulmonary vascular remodeling in PH animal models. Transgenic mice overexpressing
the proinflammatory cytokine IL-6 specifically in the lung spontaneously develop PH, whereas IL-6 knockout mice are protected against PH.\textsuperscript{55,56} In a rat model of PH induced by monocrotaline, a number of immunosuppressive and anti-inflammatory approaches have been successful in treating or preventing the development of the disease.\textsuperscript{57,58}

It has been shown that the pulmonary vasculature is sensitive to inflammation and can respond to inflammatory stimuli by abnormal proliferation or migration and apoptotic-resistant phenotype.\textsuperscript{4} These aberrant responses can lead to pulmonary vascular remodeling characterized by SMC hyperplasia, adventitial remodeling, and endothelial dysfunction. More generally, in systemic vessels, chronic arteritis causes intimal thickening, leading to stenosis or occlusion. For example, in giant cell arteritis, inflammatory infiltrates are located in all 3 tunics of the arterial wall, with giant cells forming granulomas in the media, particularly at the intima-media border (Figure 2). The arterial lumen can be partially or completely occluded as a result of intimal hyperplasia, fibrosis, stenosis, and thrombus formation (particularly in large vessels such as the aorta and its branches), which lead to ischemic complications commonly observed in giant cell arteritis. Inappropriate activation, maturation, and retention of DCs in the adventitia may constitute one of the earliest steps in the pathogenesis of giant cell arteritis. Subsequent events such as T-cell activation, cytokine secretion, and macrophage activation could be DC dependent. Thus, abnormal cross-talk between altered immunity and vascular wall dysfunction may lead to subsequent vessel remodeling.\textsuperscript{59} Besides these similarities, it is important to underline that giant cell arteritis can be treated by oral corticosteroid therapy, whereas only a small PAH patient subset responds to anti-inflammatory drugs, as discussed above,\textsuperscript{19–21} highlighting that PAH is characterized by complex and dysregulated immune responses.

**Table. Evidence of Lymphoid Neogenesis in Chronic Diseases**

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**Figure 2. Examples of inflammation in systemic arteritis.**

A. Representative hematoxylin and eosin staining of temporal giant cell arteritis or Horton disease showing the characteristic granulomatous arteritis with lymphocyte and giant cell infiltration. B. Representative hematoxylin and eosin staining of Takayasu arteritis showing the inflammatory pattern of the aorta.
It has been shown that pulmonary ECs and SMCs proliferate or migrate in vitro in response to proinflammatory cytokines/chemokines and growth factors such as serotonin, platelet-derived growth factor, epidural growth factor, vascular endothelial growth factor, and fibroblast growth factor-2. We recently showed that platelet-derived growth factor, fibroblast growth factor-2, and platelet-derived growth factor signaling pathways converge to p130cas, an adaptor protein modulating several signaling pathways that controls cell migration and proliferation and acts as an amplifier of downstream signals. We showed that p130cas is increased in IPAH (in serum, in human isolated pulmonary arteries, and in cultured pulmonary ECs and SMCs) and in experimental models of PH. We also reported that p130cas deficiency by RNA interference causes attenuated extracellular signal-regulated kinase 1/2 activation and normalized migration and proliferation of pulmonary SMCs and ECs derived from IPAH.

An important player in the innate immunity is represented by the complement system, constituted by 3 distinct pathways that all converge to C3 activation. Interestingly, it has been shown that the complement system, in particular C3, plays a role in the development of PH in a mouse model, promoting pulmonary vascular remodeling and a prothrombotic phenotype. Furthermore, increased C3 deposition was observed in human lung sections compared with controls. Because the complement system is a major component of the innate immunity and can be brought into action by the adaptive immune system, these results highlight the possible link between innate and adaptive altered responses in PH pathogenesis.

It has been proposed that antibodies directed to vascular endothelium could promote EC apoptosis and that endothelium aggression could initiate a dysfunction leading to uncontrolled proliferation. Using a proteomic approach, Dib and coworkers were recently able to identify target antigens for AECAs in IPAH, which include lamin A/C and tubulin β-chain, major components of the inner nuclear membrane and microtubules, respectively. These proteins identified as targets of AECAs are ubiquitous and play key roles in different cell types in that they are involved in cell metabolism, morphology, and protein folding, but the exact role of AECAs in patients with IPAH remains unclear. It is known that AECAs can activate ECs and induce apoptosis in SSC patients, but it needs further investigation in IPAH. Terrier et al also showed that the target antigens recognized by antifibroblast antibodies present in IPAH are ubiquitous proteins involved in the main cellular systems: regulation of cytoskeletal organization (vimentin, calumenin, and phosphatidylsinositol 3-kinase), cell contraction (tropomyosin 1), oxidative stress (heat shock protein-27 and -70 and glucose-6-phosphate dehydrogenase), and protein metabolism. Of note, although several potentially important target antigens have been identified, the technical approach used does not allow the identification of target antigens at the cell membrane, and such cell membrane targets may be of importance in the immunopathology of PAH. Nevertheless, the identification of target antigens suggests that they may contribute to EC apoptosis and fibroblast dysfunction in IPAH patients. Functional analyses are still needed to better define the potential role of AECAs and antifibroblast antibodies in IPAH. Recently, the presence of autoantibodies against vascular SMCs has also been demonstrated in IPAH. Interestingly, these antibodies can modulate SMC contraction, and they bind mainly to 2 target antigens involved in vascular remodeling: stress-induced phosphoprotein 1 and α-enolase. Although this is the first evidence of a clear functional role of autoantibodies in IPAH, further investigations are needed to identify the potential pathogenic contribution of autoantibodies to IPAH.

Among all the autoantibody targets identified, it remains difficult to define which ones are recognized by pathogenic antibodies that would influence vascular dysfunction or play a role in remodeling. One may hypothesize that autoantibodies directed against cytoplasmic or nuclear components could emerge after initial EC aggression, inducing EC apoptosis and neoantigen exposure. Thus, these autoantibodies argue in favor of humoral mechanisms in the pathogenesis of PAH. Taken together, these data suggest that altered immunity may initiate or contribute to endothelial dysfunction in PAH, leading to pulmonary vascular wall remodeling, the hallmark of the disease.

Endothelial Dysfunction Perpetuates Altered Immune Responses

It is also known that genetic predisposition and environmental factors such as hypoxia may trigger or contribute to endothelial aberrant immune responses. In particular, it has recently been shown that cellular microparticles contribute to the endothelial production of various proinflammatory cytokines and chemokines such as IL-1β, IL-6, and CC chemokine ligand 2 and to the expression of intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and E-selectin. Microparticles are plasma membrane vesicle fragments released from various cell types during activation by agonists or physical or chemical stress. Interestingly, their role is a function of the parent cell from which they stem, as well as the stimulus used for their generation. Furthermore, erythrocytes are responsible for pulmonary endothelial proinflammatory transcriptional responses to hypoxia such as endothelial nuclear factor-κB activation and hypoxia-inducible factor-1α stabilization, leading to upregulation of endothelial leukocyte adhesion receptors, E-selectin, and intercellular adhesion molecule-1. It is also known that this PAH proinflammatory environment, with increased production of IL-1 and IL-6, represents a condition favorable to activation, proliferation, and differentiation of B lymphocytes. Local autoimmunity is also suggested by the presence of mast cells in and around vascular lesions as a source of IL-4 needed for local B-cell expansion. This cytokine production represents another important link between innate and adaptive immune responses within the context of vascular lesions. Mast cells are bone marrow–derived cells that contain large granules rich in histamine and heparin and that are resident in many tissues. These cells are important in hypersensitivity reactions, wound healing, and defending against pathogens. Mast cell accumulation has been described in several types of PAH and an animal model. Our group has recently identified an increase in e-kit–positive cells (including mast cells) in remodeled vessels and mobilization of bone marrow–derived circulating progenitor cells. How mast cells may contribute to PAH pathophysiology is
not clear, but proposed mechanisms include direct vasoactive effects and stimulation of remodeling by increased production of matrix metalloproteinases.71

More proof of altered immune responses in PAH comes from the potential role of T lymphocytes, particularly Tregs, in PAH pathogenesis. Taraseviciene-Stewart and coauthors72 showed that, in contrast to vascular endothelial growth factor receptor blocker SU5416–treated euthymic rats that develop severe PH only in combination with chronic hypoxia, athymic nude rats developed severe PH and pulmonary vascular remodeling at normoxic conditions. It is therefore legitimate to hypothesize that altered immune responses may indeed play a role in PAH. Interestingly, Tamosiuniene and coworkers73 have shown a link between immune dysregulation, resulting from T-cell absence, and vascular injury. They demonstrated that immune reconstitution, performed by intravenously injecting T cells in athymic rats, attenuates early inflammation induced by vascular endothelial growth factor receptor 2 inhibitor–dependent vascular injury. These data suggest that in normal conditions, T cells play an important role in protecting against vascular inflammation secondary to a vascular injury and possibly in preventing the development of PAH.

Thus, identifying the functional status of Treg cells, key players in the autoimmunity onset, could help us better understand the potential mechanisms leading to the development and progression of PAH. As described above, we recently showed that IPAH patients display normal Treg cell count but with an altered function and demonstrated that Treg cell function is inhibited in a leptin-dependent manner in IPAH.35 Interestingly, the increased levels of circulating leptin in IPAH are endothelium derived. Importantly, the observed high levels of leptinemia and leptin receptor expression in IPAH patients are independent of the patient’s body mass index.35 Therefore, obesity should not be considered a risk factor for IPAH development. These results not only suggest a possible role for leptin and its receptor in PAH immunopathogenesis but also...
highlight the existing link between immune dysregulation and endothelial dysfunction in IPAH.

Conclusions
There is strong evidence that immune dysregulation plays an important role in the pathogenesis and progression of PAH, promoting inflammatory cell recruitment, stimulating autoantibody production, and triggering vascular wall remodeling, leading to inappropriate interplay between the immune system and the pulmonary endothelium (Figure 3). However, the exact mechanisms are still unclear, and several questions remain unanswered. Whether immune dysregulation represents a cause or an effect of PAH onset is still unknown. Although the pathogenesis of PAH shares several characteristics with systemic autoimmune diseases, why PAH represents a lung-specific disease has not yet been elucidated. This review clearly shows that altered immune responses in PAH represent an important contribution to its pathogenesis. Further studies are clearly needed to assess the inadequate cross-talk between immune mediators and the components of the pulmonary vascular wall. Identification of these factors could lead to novel therapeutic targets. The current PAH therapies are essentially focused on decreasing pulmonary vascular resistance by stimulating pulmonary vasodilation (prostacyclin analogs, phosphodiesterase type 5 inhibitors, and endothelin receptor antagonists). These agents have some antiremodeling properties, but there is no current antiremodeling strategy approved for PAH. Of note, some hemodynamic and clinical effects of the tyrosine kinase inhibitor imatinib have been reported in severe PAH, but at the expense of severe side effects. Because survival remains poor in the modern management era, new treatments targeting other PAH pathomechanisms would be useful to slow, stop, or even reverse disease progression.

Therefore, more data are needed on novel agents with anti-inflammatory or immunomodulatory properties. In this context, a randomized, clinical trial testing the safety and efficacy of the monoclonal antibody anti-CD20, a B lymphocyte protein, is currently in phase II in patients with SSc-PAH. Unfortunately, such a therapeutic strategy is not yet applicable in IPAH. Additional efforts should be made to further our understanding of the complex interplay between immune dysregulation and endothelial dysfunction in IPAH to develop new and more powerful drugs that could restore immune responses and Treg function.

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