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## Immune and Inflammatory Mechanisms in Pulmonary Arterial Hypertension

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### Abstract

Altered immunity and inflammation are increasingly recognized features of pulmonary arterial hypertension (PAH). This is suggested by infiltration of various inflammatory cells (e.g., macrophages, T and B lymphocytes), increased cytokine and growth factor (e.g., VEGF and PDGF) expression in remodeled pulmonary vessels, and the presence of circulating chemokines and cytokines. In certain diseases associated with PAH, increased expression of growth and transcriptional (e.g., Nuclear Factor of Activated T cells or NFAT) factors, and viral protein components (e.g., HIV-1 *Nef*), appear to contribute directly to recruitment of inflammatory cells in remodeled vessels, and may potentially serve as specific therapeutic targets. This section provides an overview of inflammatory pathways highlighting their potential role in pulmonary vascular remodeling in PAH and the possibility of future targeted therapy.

### A. Introduction

Pulmonary arterial hypertension (PAH) is Group 1 of the pulmonary hypertension (PH) classification and is defined by a mean pulmonary arterial pressure greater than 25 mm Hg with a capillary wedge pressure  $\leq$  15 mmHg. PAH includes a heterogeneous group of clinical entities sharing similar pathological changes that have been subcategorized as idiopathic PAH, familial PAH, pulmonary hypertension associated with other diseases such as connective tissue diseases (CTD, e.g., systemic sclerosis (SSc), lupus erythematosus, and mixed CTD), portal hypertension and pulmonary hypertension related to HIV infection, drugs and toxins<sup>1</sup>. PAH is characterized by increased pulmonary vascular resistance due to remodeling and occlusion of the pulmonary arterioles. Left untreated, PAH leads irreversibly to right ventricular (RV) hypertrophy and eventually failure from pressure and volume overload load resulting in death within 2–3 years<sup>2</sup>.

In the past two decades, novel therapies have been developed based on the observation that endothelial cell dysfunction and an imbalance between endothelial-derived vasoactive substances were central to the pathogenesis of severe PAH<sup>3</sup>, eventually leading to the use of

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agents targeting specifically three biological pathways, i.e., the endothelin, nitric oxide/cyclic GMP, and prostacyclin pathways<sup>4</sup>. These therapeutic drugs have had a clear but variable impact depending on the etiology of PAH and thus agents targeting alternate pathways in this disease are needed.

The role of altered immunity and inflammation has been increasingly recognized in various types of PH, such as IPAH and diseases associated with PAH (e.g., CTD and HIV infection) based on the observation of circulating autoantibodies, such as antinuclear antibodies, elevated levels of circulating proinflammatory cytokines interleukin-1 (IL-1) and IL-6<sup>7</sup>, as well as association of PAH with inflammatory conditions (e.g., CTD and HIV). Inflammation is also prominent in various experimental animal models of PH which will be reviewed here. Consequently, a better understanding of inflammatory pathways and their role in the pathogenesis of PAH (see Figure 1) may lead to targeted therapeutic approaches similar to those achieved from an improved understanding of endothelial dysfunction.

## B. The role of inflammation in human PAH

Inflammation appears to be a prominent pathologic feature in PAH as suggested by infiltration of inflammatory cells, including macrophages and T and B lymphocytes, and dendritic cells in pulmonary perivascular spaces and around the plexiform lesions in PAH. In addition, levels of macrophage inflammatory protein-1 alpha, IL-1 $\beta$  and IL-6 and P-selectin<sup>11</sup> (expressed on leukocytes and platelets and able to bind to their ligands on endothelial cells (EC)), are increased in severe IPAH.

Recruitment of inflammatory cells such as leukocytes, macrophages, and lymphocytes abound in the complex vascular lesions of IPAH as described by Tuderet al<sup>12</sup> and Dorfmueller et al<sup>13</sup> who suggested that the inflammatory response is not confined to plexiform lesions but also observed in other vascular lesions of PAH lungs.

### Chemokines in PAH

Interactions between EC and leukocytes are important in the inflammatory process, and involve successive events (e.g., rolling, adhesion, and extravasation of leukocytes) in response to a chemoattractant gradient related to release of chemokines. Thus the presence of such chemokines in PAH further support a role for inflammation in this syndrome.

The chemokine fractalkine or chemokine (C-X3-C motif) ligand 1 (CX3CL1) promotes the receptor, CX3CR1-expressing leukocyte recruitment and mediates the rapid capture, integrin-independent adhesion, and activation of circulating CX3CR1<sup>+</sup> leukocytes under high blood flow. CX3CR1 is upregulated in circulating CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes from PAH patients as compared with controls<sup>14</sup>, which accounts for increased sensitivity of these cells to soluble CX3CL1. This abnormal response of T lymphocytes to CX3CL1 appears specific to PAH since PAH patients have elevated soluble CX3CL1 plasma concentrations as compared with patients with other forms of PH (e.g., chronic thromboembolic PH) and normal controls. Increased CX3CL1 mRNA expression is also found in lung tissue samples from PAH patients as compared with controls, and pulmonary artery (PA) EC obtained from these patients express CX3CL1 protein<sup>14</sup>.

Chemokines produced from small PA of PAH patients appear to contribute to inflammatory cell recruitment and PA-SMC proliferation as demonstrated by Perros et al<sup>15</sup>. Examining the expression of CX3CL1 and CX3CR1 by immunohistochemistry and competitive reverse transcription polymerase chain reaction (RT-PCR) on laser-captured microdissected PA in monocrotaline-induced PH, these authors revealed that CX3CL1 was expressed by inflammatory cells surrounding PA lesions and that PA-SMC had increased CX3CR1

expression. Thus, fractalkine may act as a growth factor for PA-SMC. Further evidence of a chemokine participation in PA remodeling is suggested by significant increases in Chemokine (C-C motif) ligand 2 (CCL2, also known as monocyte chemoattractant protein-1 (MCP-1)) protein levels in plasma and lung tissue of IPAH patients compared to controls<sup>16</sup>. The cellular sources of CCL2 in IPAH appear to be EC and PA-SMC. Monocyte migration is markedly increased in the presence of pulmonary EC from patients with IPAH, which is significantly prevented by CCL2-blocking antibodies. Finally, CCL2 expression is markedly increased in PA-SMC from IPAH patients, likely explaining stronger migratory and proliferative responses of these cells to CCL2 as compared to cells obtained from controls<sup>16</sup>.

RANTES (Regulated upon Activation, Normal T cell expressed and secreted: CCL5), an important chemoattractant for monocytes and T-cells, plays a central role in several vascular inflammatory processes such as glomerulonephritis, Kawasaki disease, and Takayasu's arteritis. CCL5 may also play an indirect role in PAH through the induction of endothelin-converting enzyme-1 and endothelin-1, a potent endothelium-derived factor with strong vasoconstrictive and mitogenic action. Involvement of this potent mediator in the pathogenesis of PAH is suggested by increased CCL5 mRNA copies detected by RT-PCR in lung samples from PAH patients as compared to controls<sup>17</sup>, with EC being the major source of CCL5 as demonstrated by *in situ* hybridization and immunohistochemistry of lung samples<sup>17</sup>. The exact relevance of these findings to the pathophysiology of PAH requires further investigation.

### Cytokines in PAH

Recent studies suggest that cytokines are key players in the pathogenesis of PAH. IL-6, a pleiotropic cytokine, is significantly elevated in the serum and lungs of patients with PAH<sup>7</sup> and its level is independently associated with mortality in one study<sup>18</sup>. Similarly, patients with COPD with PH have elevated circulating IL-6, that correlates with mean pulmonary arterial pressure (mPAP) whereas patients without PH do not. Furthermore, elevations in mPAP are highest in patients with certain polymorphisms of the gene encoding IL-6 (i.e., IL-6 GG genotype as compared to CG or CC)<sup>19</sup>.

At this time, there is convincing experimental evidence linking IL-6 to excess cellular proliferation and pulmonary vascular remodeling. Injection of recombinant human IL-6 leads to PH and RV hypertrophy in mice<sup>20</sup>. Steiner et al reported that lung-specific overexpression of IL-6 in mice mimics the pathobiological lesions observed in advanced PAH (e.g., distal PA muscularization and formation of plexiform-like lesions) and increases RV systolic pressure, changes that were amplified in response to chronic hypoxia exposure<sup>21</sup>. The vascular proliferative lesions were composed essentially of EC and T lymphocytes. In addition, IL-6-induced vascular lesions were accompanied by activation of vascular endothelial growth factor (VEGF), proto-oncogene transcription factors c-MYC and MAX, and the anti-apoptotic proteins survivin and Bcl-2 while the pro-apoptotic kinases JNK and p38 were downregulated<sup>21</sup>.

In contrast, IL-6 deficiency attenuates hypoxia-induced PH (as assessed by increases in RV systolic pressure and pulmonary vascular remodeling) and lung macrophage accumulation in a mouse model of PH although increased expression of adhesion molecules (ICAM-1 and VACM-1) and cytokines (MCP-1) was unchanged<sup>22</sup>.

The source of IL-6 in the lung is unclear as several cells can express this cytokine including leukocytes, vascular cells such as EC and SMC, fibroblasts and myocytes. IL-6 induces migration of PA SMC *in vitro*, and while cultured human PA SMCs constitutively express IL-6, this expression is significantly increased in response to hypoxic exposure<sup>19</sup>. Taken

together, these findings suggest a preponderant role for this cytokine in SMC growth and vascular remodeling triggered by a hypoxic stimulus.

### Regulatory T cells

PAH is associated with a defect in the CD4<sup>+</sup>T-cell compartment reflected by an absolute deficiency of CD4<sup>+</sup>cells, a decreased CD4<sup>+</sup>/CD8<sup>+</sup>ratio and/or a diminished relative percentage of CD4<sup>+</sup>CD25<sup>+</sup>cells, the putative regulatory T-cell (Treg) subset<sup>9</sup>. The role of T-cells in the control of self-reactive T-cells and B-cells in the maintenance of self-tolerance is well known<sup>23</sup>. In fact the depletion of suppressor T-cells, and more specifically the CD4<sup>+</sup>CD25<sup>+</sup>Tregs, is a pivotal event in the development of autoimmunity<sup>24</sup> and is enough to break natural self-tolerance leading to chronic autoimmune diseases. Patients with IPAH have diminished circulating CD8<sup>+</sup> T cells compared with controls, as well as an elevated proportion of FoxP3<sup>+</sup> cells within the CD4<sup>+</sup> T cell population, presumably identifying an increase in circulating Tregs with suppressor activity<sup>25</sup>. In a recent study, Tamosiuniene et al demonstrated that vascular endothelial growth factor receptor 2 (VEGFR2) blockade induced significant pulmonary endothelial apoptosis in T-cell-deficient rats but not in immune-reconstituted (IR) rats. IR with either CD4<sup>+</sup>CD25<sup>hi</sup> or CD4<sup>+</sup>CD25<sup>-</sup> T cell subsets prior to vascular injury attenuated the development of PAH<sup>26</sup>. Taken together, these studies suggest that T regs normally function to limit vascular injury and may play a protective role against the development of PAH.

### C. Growth factors in PAH: tyrosine kinase signalling as a potential target in PAH

Several growth factors, including platelet-derived growth factor (PDGF), basic fibroblast growth factor (b-FGF)<sup>29</sup>, epidermal growth factor (EGF)<sup>30</sup> and VEGF<sup>31-33</sup>, have been implicated in the abnormal proliferation and migration of PA vascular cells. These growth factors form homo- or heterodimers, stimulate cell surface receptors which activate the major signaling transduction pathways like the Ras-mitogen activated protein kinase pathway, and act as potent mitogens and chemoattractants for SMCs, fibroblasts and EC. Responses to these growth factors include inflammation, cellular proliferation, migration and resistance to apoptosis.

#### Growth factors and inflammation

Cool *et al* demonstrated exuberant expression of the VEGF receptor KDR, coupled with a reduced expression of p27/kip1 (a cell cycle inhibitory protein) in the EC of plexiform lesions<sup>34</sup>. However, phenotypically distinct EC lined the plexiform lesions and the sites of neoangiogenesis, suggesting differential angiogenic activity within the plexiform lesion. Other growth factors, such as VEGF and hypoxia inducible factor (HIF-1) subunits  $\alpha$  and  $\beta$  are highly expressed in EC of plexiform lesions in severe PAH<sup>35</sup>. In addition, decreased expression of c-Src kinase<sup>35</sup>, a protein that mediates VEGF-induced production of prostacyclin and nitric oxide in the EC<sup>36</sup>, is decreased in PAH. Taken together, these findings suggest a central role in PAH for VEGF, a mediator of angiogenesis but also a factor involved in permeability and inflammatory processes of the vascular endothelium.

#### Platelet derived growth factor (PDGF)

Active PDGF is composed of polypeptides (A and B chain) that form homo- or heterodimers and stimulate  $\alpha$  and  $\beta$  cell surface receptors. Ligand binding induces receptor dimerization and autophosphorylation of tyrosine residues in the PDGF Receptor kinase domain followed by phosphorylation events involving sequential activation of mitogen-activated protein

kinase Ras-mitogen activated protein kinase cascades. These events play a role in inflammation, cell proliferation and differentiation.

PDGF, which is synthesized by many different cell types including SMC, EC, and macrophages, induces the proliferation and migration of SMC and fibroblasts, and is a key mediator in the progression of several fibroproliferative disorders such as atherosclerosis, lung fibrosis, and PH<sup>27</sup>. The pathogenic role of PDGF in human PAH is also demonstrated by increased expression of PDGF and its receptors by RT-PCR performed on laser capture microdissected PA from explanted lungs of patients with severe IPAH<sup>37</sup>. PDGF-A, PDGF-B, PDGF-R $\alpha$  and PDGF-R $\beta$  mRNA expression is increased in small PA from patients with severe IPAH as compared to controls with more pronounced PDGF-B and PDGF-R  $\beta$  than PDGF-A and PDGF-R $\alpha$  expression. In small PA, PDGF-B is mainly expressed in EC, SMC, and in some perivascular inflammatory cells, while PDGFR- $\beta$  is mainly expressed in SMC. Furthermore PDGF-BB-induced proliferation and migration of PA-SMC was found to be inhibited by imatinib treatment<sup>37</sup>. Recently, tyrosine kinase inhibitors targeting PDGF have been shown effective in experimental models of PH.

### **Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1)**

Abnormalities of signaling involving the TGF- $\beta$ 1 superfamily, and specifically one of its members the bone morphogenetic protein (BMP) receptor-2 are closely linked to the pathogenesis of PAH since germline mutations of the BMP receptor-2 gene are found in about 80% of patients with the familial form of PAH and between 10 and 40% of patients with the sporadic form<sup>40</sup>. There is also evidence for a general imbalance in TGF signaling in PAH; for instance, loss of function mutations of the TGF- $\beta$  receptor I, Alk1, has been linked with PAH related to the hereditary hemorrhagic telangiectasia<sup>41</sup>, and somatic microsatellite instability of the TGF- $\beta$  receptor II gene is found in the plexiform lesions of IPAH patients<sup>42</sup>. Increased TGF- $\beta$  expression and signaling smad (e.g., smad2) is found predominantly in EC lining remodeled vessels in IAPH patients. Finally, an imbalance in TGF- $\beta$  signaling has also been demonstrated in some animal models of PH with early enhancement of TGF- $\beta$ /Alk5 signaling in the rat monocrotaline model of PH. In this model, SD-208, an orally active small-molecule TGF- $\beta$  receptor I inhibitor, attenuates the development of PH and pulmonary vascular remodeling<sup>43</sup>. Further work is needed to elucidate the complexities of TGF signaling in PAH and how to target this important pathway.

### **Fibroblast growth factor (FGF)**

FGF-2 (previously known as basic FGF) is ubiquitously expressed and known to be upregulated in response to hypoxia and shear stress in PA-SMC and in lambs with increased pulmonary blood flow and PH<sup>29</sup>. However, due to a lack of selective inhibitors of the FGFR system, no functional data are currently available and the role of this growth factor in PAH remains to be further explored.

### **Epidermal growth factor (EGF)**

The EGF receptor (EGFR), has intrinsic tyrosine kinase activity, and is widely expressed on numerous cell types. Upon binding, the intrinsic kinase is activated and EGFR tyrosyl-phosphorylates itself and several intermediary effector molecules. This initiates a multitude of signaling pathways. The integrated biological responses to EGFR signaling include mitogenesis or apoptosis, enhanced cell motility, protein secretion, and differentiation or dedifferentiation. EGF-dependent proliferation and migration of SMC is dependent on the extracellular matrix component Tenascin C<sup>45</sup>. In addition, EGF colocalizes with Tenascin C in PAH lesions<sup>46</sup>, suggesting a direct role in disease progression. In this context, it is noteworthy that the EGF receptor inhibitor PKI166 reverses established monocrotaline-

induced PH in rats <sup>30</sup>, which further supports a role for EGF in pulmonary vascular remodeling.

### Vasoactive intestinal peptide (VIP) and PAH

VIP is a 28-amino-acid peptide that belongs to the glucagon-secretin family of peptides. The effects of VIP are mediated by G protein-coupled receptors. Two subtypes cloned from human tissues, VPAC1 and VPAC2, are widely distributed in the central nervous system, the heart, blood vessels, and other tissues. VIP has a high affinity for both receptor subtypes. Binding of VIP to its receptors results in activation of adenylyl cyclase and formation of cAMP, which is responsible for vasodilation and many other vascular effects of the peptide. Other actions may be mediated by inositol trisphosphate synthesis and calcium mobilization. Some of the actions of VIP include, but are not limited to, relaxation of vascular SMC <sup>47</sup>, inhibition of PA-SMC proliferation *in vitro*<sup>48</sup>, reduction of pulmonary vasoconstriction induced by endothelin<sup>49</sup>, and antithrombotic effects <sup>50</sup>.

Genetic deletion of VIP results in overexpression of genes that promote pulmonary vascular SMC proliferation, downregulation of antiproliferative genes, as well as upregulation of pro-inflammatory genes <sup>51</sup> suggesting a preponderant role of VIP in pulmonary vascular remodeling and inflammatory response in the setting of PAH. Said et al demonstrated that VIP knockout mice spontaneously develop PAH (i.e., in the absence of exposure to hypoxia) <sup>52</sup>. Histological examination of the lungs of mice lacking the VIP gene demonstrates pulmonary vascular smooth muscle and collagen proliferation, right ventricular (RV) hypertrophy and evidence of lung inflammation, all features relevant to human PAH. Furthermore, both the right ventricular hypertrophy and the vascular remodeling can be significantly decreased after VIP treatment of the mice <sup>52</sup>. In piglets with high output-induced PAH (achieved by a shunt from the left subclavian artery to the PA trunk), circulating VIP is significantly decreased together with an up-regulation of VPAC1 <sup>53</sup>, further supporting a role for VIP in the remodeling process.

Preliminary clinical experience suggests that PAH patients treated with the inhaled VIP analog Aviptadil have significant improvements in PA pressure, cardiac output, and peripheral vascular resistance (PVR) <sup>54</sup>. This suggests that local VIP administration exerts an immuno-regulatory role in humans, and that VIP inhalation might be an attractive therapeutic option for patients with PAH, although randomized clinical trials are needed to confirm these findings.

### Serotonin and the serotonin transporter

Serotonin (5-HT) exerts vasoactive and mitogenic effects on PA-SMCs. In contrast to the constricting action of 5-HT on SMC, which is mainly mediated by 5-HT receptors (5-HT 1B/D, 2A, and 2B) <sup>55</sup>, the 5-HT transporter (5-HTT) is required for the mitogenic and co-mitogenic effects of 5-HT. Serotonin is synthesized by EC in the normal lung as a result of tryptophan hydroxylase-1 enzyme activity, and appears to be the main growth factor produced by ECs, acting on PA-SMCs in a paracrine fashion. Such cross-talk between ECs and SMCs mediated by 5-HT appears unique to the pulmonary circulation, since 5-HT is not a potent mitogenic factor for SMCs from systemic vessels <sup>58</sup>. Also, the uptake of indoleamine in this process is central. Accordingly, drugs that competitively inhibit 5-HTT also block the mitogenic effects of 5-HT on SMC.

5-HTT is abundantly expressed in the lung, where it is predominantly located in PA-SMCs. 5-HTT is required as a mediator of the mitogenic activity of 5-HT as suggested by studies demonstrating that mice with targeted 5-HTT gene disruption developed less severe hypoxic PH than wild-type controls <sup>61</sup>, and that selective 5-HTT inhibitors attenuated hypoxia- and

monocrotaline (MCT)-induced PH<sup>62</sup>. It is noteworthy that 5-HTT-deficient mice have altered expression of genes responsible for stress response and immune regulation, genes related to muscle structure, vasoreactivity, or actin dynamics, and genes involved in cell cycle and apoptosis, energy metabolism, and matrix formation<sup>63</sup>. Conversely, transgenic mice with selective overexpression of 5-HTT in SMC spontaneously develop PH<sup>64</sup> which is exacerbated after exposure to hypoxia<sup>65</sup>. Even without any change in the bioavailability of 5-HT, PH appears to develop in these mice as a result of the increased expression of the 5-HTT protein in SMC. Taken together, these observations suggest a close correlation between 5-HTT expression and/or activity and the extent of pulmonary vascular remodeling in experimental PH.

There is also abundant evidence that 5-HTT plays an important role in the pathogenesis of human IPAH. 5-HTT expression is increased in circulating platelets and in lungs from patients with IPAH, where it predominates in the media of thickened PA<sup>60</sup>. Moreover, PA-SMC obtained from patients with IPAH exhibit faster growth compared to cells obtained from controls when stimulated with serotonin, due to increased 5-HTT expression<sup>60</sup>, an effect abrogated in the presence of 5-HTT inhibitors. In conclusion, PA-SMC hyperplasia in IPAH appears to result from the overexpression of tryptophan hydroxylase-1 enzyme and 5-HTT, causing an abnormal 5-HT production by ECs and an enhanced PA-SMC proliferative response<sup>66</sup>.

## D. Transcriptional factors and their role in PAH

### The NFAT in inflammation and vascular remodeling

The nuclear factor of activated T cells (NFAT) increases the transcription of multiple inflammatory mediators, such as interleukins and tumor necrosis factor. NFAT also activates T and B cells<sup>67</sup>. An increase in  $[Ca^{2+}]_i$ , in response to hypoxia or other stimulus, activates calcineurin, which dephosphorylates cytoplasmic NFAT, allowing its entry to the nucleus where it forms complexes with other important transcription factors (e.g., activator protein-1) regulating gene transcription<sup>67</sup>.

NFAT activation causes downregulation of Kv1.5<sup>68</sup>, a channel with a preponderant role in pulmonary vasoconstriction and development of PAH. ET can activate NFAT, which increases B-cell lymphoma-2 expression, contributing to the antiapoptotic effects of ET in the heart<sup>69</sup>. In addition, NFAT regulates the transcription of several genes that affect mitochondrial function (e.g., pyruvate decarboxylase and the electron transport chain enzyme cytochrome C oxidase)<sup>70</sup>. Importantly, NFAT is upregulated and activated in circulating inflammatory cells in patients with PAH, including IPAH and scleroderma-associated PAH<sup>71</sup>. CD3-positive cells with activated NFAT are present in remodeled PAs. However, NFAT is also activated in the PA-SMCs of remodeled arteries.

Directly relevant to the pathogenesis of PAH, PA-SMCs isolated from lungs of PAH patients, but not from normal lungs, have a unique phenotype (downregulated Kv1.5, upregulated B-cell lymphoma-2 bc, hyperpolarized mitochondria), which is associated with activated NFAT and resistance to apoptosis. Experimental inhibition of NFATc2 (which is the predominant NFAT isotype in PAH) by VIVIT (a competitive peptide that inhibits the docking of NFAT to calcineurin) or cyclosporine (an inhibitor of calcineurin), restores Kv1.5 expression and current and decreases  $[Ca^{2+}]_i$ ,  $[K^+]_i$ , bcl-2, and mitochondrial membrane potential, leading to increased apoptosis in vitro<sup>71</sup>. In the rat, cyclosporine treatment decreases established MCT-induced PAH<sup>71</sup>. A role for NFAT in the pathogenesis of PH is further suggested by the observation that exposure of PA-SMCs to chronic hypoxia leads to NFAT activation, hyperpolarization of mitochondria, and downregulation of Kv1.5, similar to the SMC phenotype observed in PAH. Finally, inhibition with VIVIT or

cyclosporine reverses this phenotype, normalizing the mitochondrial membrane potential and level/function of Kv1.5 in these cells.

### HIF-1

PASMC mitochondria normally produce reactive O<sub>2</sub> species (ROS), which are converted by superoxide dismutase 2 to diffusible H<sub>2</sub>O<sub>2</sub>. This serves as a redox-signaling mechanism, regulating pulmonary vascular tone and structure through effects on Kv1.5 and transcription factors. Thus, O<sub>2</sub> sensing is mediated by this mitochondria-ROS-HIF-1a-Kv1.5 pathway. In PAH, mitochondrial metabolism and redox signaling are reversibly disordered, creating a pseudohypoxic state characterized by normoxic decreases in ROS, a shift from oxidative to glycolytic metabolism and HIF-1a activation, which appears to decrease expression of Kv1.5<sup>74</sup>.

Bonnet et al demonstrated that dichloroacetate, the pyruvate dehydrogenase kinase inhibitor, restores oxidative metabolism in fawn-hooded rats (FHR) (which spontaneously develop PAH<sup>75</sup>) PASMCs. The metabolic changes induced reverse the hypoxic phenotype of FHR, restoring the relatively depolarized PASMCs and increasing ROS production to normoxic levels. This reverses HIF-1a activation and restores Kv1.5 expression, reducing the severity and mortality of FHR/PAH<sup>74</sup>.

### The Signal Transducers and Activators of Transcription STAT

STAT lung expression is enhanced in a model of hypoxic pulmonary hypertension suggesting a potential role for this signaling pathway in the pathogenesis of the syndrome<sup>76</sup>. STAT3 in particular has been recognized to play a critical role in cell growth stimulation and has significant anti-apoptotic effects<sup>77</sup>. Normally, bone morphogenetic proteins (BMPs) induce growth arrest through intracellular signaling pathways of the Smad proteins<sup>78</sup>, which act in part through interaction with, and inactivation of, STAT3. Thus, loss of the inhibitory BMP pathway is hypothesized to lead to amplified activation of STAT3. It is therefore intriguing that PA-EC of IPAH patients have increased arginase II, a STAT1/STAT3-regulated gene, essential for endothelial cell proliferation. Taken together, these data suggest that increased proliferation and survival of EC in IPAH may be dependent on persistent STAT3 activation<sup>81</sup>.

### E. Human immunodeficiency virus and PAH

PAH associated with HIV infection is classified in WHO Group 1 along with IPAH and CTD-associated PAH. Perhaps even more so than for other syndromes in this group (with the exception of CTD-PAH), there is strong evidence for underlying inflammatory mechanisms in the pathogenesis of the pulmonary vascular remodeling in HIV-PAH.

Although the exact mechanisms of pathogenesis of HIV-PAH are not well understood, several facts have been established, including the lack of evidence of a direct role for the HIV virus in the development of PAH. While the virus is present in macrophages and other inflammatory cells in the lungs, which may provide a potential reservoir for the transmission of the virus to circulating T-cells and a source for localized viral proteins such as *Nef*, *Tat* and *gp120*, it has not been detected in EC of patients with HIV-PAH. Rather, the pathophysiology of HIV-PAH has been linked to indirect mechanisms involving mediators such as cytokines, growth factors, and vasoconstrictors<sup>83</sup>.

The vasoconstrictor Endothelin 1 (ET-1) which is significantly elevated in a variety of PAH etiologies, is also elevated in HIV-PAH. The glycoprotein 120, which is crucial for the binding of the virus to CD4<sup>+</sup> cells, stimulates the secretion of ET-1 as well as cytokines, including TNF from macrophage<sup>84</sup>. Ehrenreich et al also demonstrated that circulating



monocytes in those infected with HIV show persistent activation of the ET-1 gene. Like in IPAH, PDGF is another key mediator in HIV-PAH. PDGF-A chain mRNA is significantly increased in both IPAH and HIV associated PAH when compared to controls <sup>27</sup>.

Marecki et al. have recently demonstrated the pathogenic role of the HIV *nef* gene in the development of HIV-PAH <sup>85</sup>. The Nef protein plays a key role in the pathogenesis of simian immunodeficiency virus infection <sup>86</sup>. HIV-1 Nef, an accessory protein made early in HIV infection, down-regulates CD4 <sup>87</sup> and blocks major histocompatibility antigen I trafficking to the membrane <sup>88</sup>, thus allowing cells with HIV to evade immune surveillance <sup>89</sup>. In human monocyte-derived macrophages, Nef activates several inflammatory pathways, such as the STAT1 pathway, and secretion of MIP-1, IL-6, and TNF- $\alpha$  <sup>90</sup>. In macaques infected with either the chimeric SHIV-*nef* virions (simian immunodeficiency virus containing a cloned HIV-1 *nef* gene) or with parental SIV strains containing the native SIV *nef* allele, vascular changes in the lungs characteristic of PAH were observed in the first group of animals, but were absent in animals infected with parental SIV strains. These experiments strongly suggest that *nef* is in part responsible for vascular remodeling in HIV-PAH. In addition, *nef* expression has been shown by immunohistochemistry in the lungs of HIV PAH patients <sup>85</sup>.

## F. Autoantibodies in PAH

Several autoantibodies have been reported in PAH, in particular IPAH and SSc-PAH. For example, PAH-SSc patients have been found to have antifibrillar antibodies <sup>91</sup>, and the poorly characterized anti-EC antibodies which correlate with digital infarcts <sup>92</sup>. Nicolls et al suggested that anti-EC antibodies can activate EC, induce the expression of adhesion molecules, and trigger apoptosis, thus contributing to the pathogenesis of PAH <sup>9</sup>. Other antibodies reported in PAH-SSc include antibodies to fibrin-bound tissue plasminogen activator (in patients with limited cutaneous SSc <sup>93</sup> and in IPAH patients with HLA-DQ7 antigen <sup>94</sup>), and anti-topoisomerase II- $\alpha$  antibodies, particularly in association with HLA-B35 antigen <sup>95</sup>. *In vitro*, autoantibodies from patients with CTD (antifibrillar antibodies and anti-dsDNA) can upregulate adhesion molecules (*e.g.*, endothelial leukocyte adhesion molecule-1) and histocompatibility complex class II molecules on human pulmonary arterial EC <sup>96</sup>, suggesting an inflammatory process can lead to proliferation and remodeling of the pulmonary vasculature.

The detection of anti-fibroblast antibodies in the serum of PAH-SSc and IPAH patients has significant pathogenic importance since fibroblasts are necessary components of the pulmonary vascular wall remodeling in PAH, and can be found in the remodeled neointimal layer in both SSc-PAH and IPAH. These antibodies can activate fibroblasts, in turn inducing collagen synthesis, which contributes potentially directly to the remodeling process. Antibodies from sera of patients with SSc induce a pro-adhesive and pro-inflammatory response in normal fibroblasts <sup>98</sup>. Patients with IPAH and PAH-SSc have IgG antifibroblast antibodies present in their sera which have distinct reactivity profiles in these two conditions <sup>99</sup>. A two-dimension immunoblotting technique showed that fibroblasts antigens which are recognized by serum IgG from IPAH and SSc-PAH patients included proteins involved in regulation of cytoskeletal function, cell contraction, cell and oxidative stress, cell energy metabolism and other pathways that play key roles in cell biology and maintenance of homeostasis <sup>100</sup>. Although the specific membrane antigen targeted by these auto-antibodies has not yet been determined, it is likely that the antibodies react to membrane components since they typically bind to unpermeabilized fibroblasts mediating the release of cytokines and growth factors which in turn might contribute to the pathogenesis of vascular remodeling in PAH <sup>99</sup>.

## G. Conclusion

Inflammatory processes appear to play an important role in the vascular remodeling characteristic of PAH and might be important targets for PAH therapy. While preclinical trials targeting specific inflammatory pathways have shown promising results in animal models, translating these findings to human PAH remains largely unexplored except in the case of the tyrosine kinase pathway where clinical trials are on-going. Recognizing the complexity of specific inflammatory pathways in animal models and human PAH, some of which may be predominant in certain forms of PAH but not in others, is important. However, deciphering their exact mechanisms of action in the process of pulmonary vascular remodeling will provide a wide spectrum of potentially novel targets that are desired considering the currently limited array of modern PAH therapies.

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## Abbreviation List

<b>BMP</b>	Bone morphogenic protein
<b>EC</b>	Endothelial cells
<b>CTD</b>	Connective tissue disease
<b>CX3CL1</b>	chemokine (C-X3-C motif) ligand 1
<b>CX3CR1</b>	Receptor to CX3CL1
<b>CCL2</b>	(C-C motif) ligand 2
<b>FGF</b>	Fibroblast growth factor
<b>EGF</b>	Epidermal growth factor
<b>ET-1</b>	Endothelin-1
<b>HIV</b>	Human immunodeficiency virus
<b>5HT</b>	5-hydroxytryptophane (serotonin)
<b>5-HTT</b>	serotonin transporter
<b>MAPK</b>	Mitogen activated protein kinase
<b>MCT</b>	Monocrotaline
<b>mPAP</b>	Mean pulmonary arterial pressure
<b>NFAT</b>	nuclear factor of activated T cells
<b>PAH</b>	Pulmonary Arterial Hypertension
<b>PDGF</b>	Platelet derived growth factor
<b>RV</b>	Right ventricle
<b>SMC</b>	Smooth muscle cell
<b>SSc</b>	Scleroderma
<b>STAT</b>	The Signal Transducers and Activators of Transcription
<b>TGF</b>	Transforming growth factor
<b>Tregs</b>	Regulatory T cells

<b>VEGF</b>	Vascular endothelial growth factor
<b>VIP</b>	Vasoactive intestinal peptide

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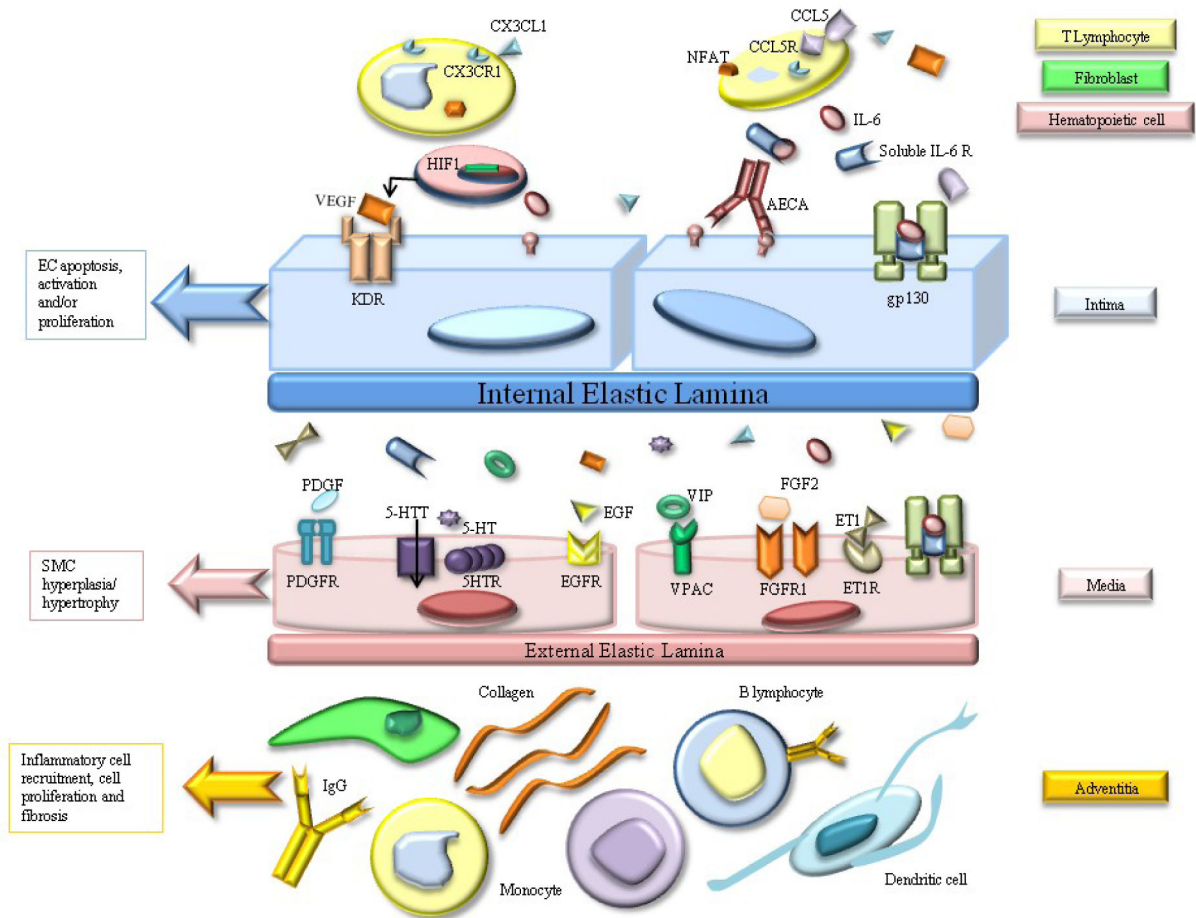
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**Figure 1.** Main inflammatory mediators in pulmonary vascular remodeling in PAH. CX3CL1 (Fractalkine) causes upregulation of its receptor CX3CR1 on CD4<sup>+</sup> and CD8<sup>+</sup>T lymphocytes. CCL5 (RANTES) and Nuclear factor of activated T cells (NFAT) attract T and B lymphocytes. IL-6 (Interleukin-6) binds to its soluble receptor (soluble IL-6 Receptor) and the complex then binds to gp130. Vascular Endothelial Growth Factor (VEGF) acts through its receptor KDR, a receptor tyrosine kinase. Other growth factors involved in vascular remodeling include platelet derived Growth Factor (PDGF), epithelial Growth Factor (EGF), and Fibroblast Growth Factor (FGF-2). The receptor (5-HTR) and transporter (5-HTT) of serotonin (5-HT), and VPAC (the receptors of Vasoactive intestinal peptide (VIP)) are all coupled to G-proteins. Endothelin1 (ET1) is a potent vasoconstrictor and mitogen.