Interleukin-6 Overexpression Induces Pulmonary Hypertension

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Abstract—Inflammatory cytokine interleukin (IL)-6 is elevated in the serum and lungs of patients with pulmonary artery hypertension (PAH). Several animal models of PAH cite the potential role of inflammatory mediators. We investigated role of IL-6 in the pathogenesis of pulmonary vascular disease. Indices of pulmonary vascular remodeling were measured in lung-specific IL-6–overexpressing transgenic mice (Tg⁺) and compared to wild-type (Tg⁻) controls in both normoxic and chronic hypoxic conditions. The Tg⁺ mice exhibited elevated right ventricular systolic pressures and right ventricular hypertrophy with corresponding pulmonary vasculopathic changes, all of which were exacerbated by chronic hypoxia. IL-6 overexpression increased muscularization of the proximal arterial tree, and hypoxia enhanced this effect. It also reproduced the muscularization and proliferative arteriopathy seen in the distal arteriolar vessels of PAH patients. The latter was characterized by the formation of occlusive neointimal angioproliferative lesions that worsened with hypoxia and were composed of endothelial cells and T-lymphocytes. IL-6–induced arteriopathic changes were accompanied by activation of proangiogenic factor, vascular endothelial growth factor, the proliferative kinase extracellular signal-regulated kinase, proliferative transcription factors c-MYC and MAX, and the antiapoptotic proteins survivin and Bcl-2 and downregulation of the growth inhibitor transforming growth factor-β and proapoptotic kinases JNK and p38. These findings suggest that IL-6 promotes the development and progression of pulmonary vascular remodeling and PAH through proangioproliferative and antiapoptotic mechanisms. (Circ Res. 2009;104:236-244.)

Key Words: interleukin-6 • pulmonary artery hypertension • proliferation

Pulmonary vascular remodeling is associated with increased pulmonary vascular resistance, pulmonary artery hypertension (PAH), and right heart failure. Advanced PAH is characterized by arteriopathy, which includes muscularization of distal pulmonary arterioles, concentric intimal thickening, and obstruction of the vascular lumens by proliferating endothelial cells to form plexiform lesions. Evidence suggests that PAH is associated with genetic perturbations favoring cellular growth, proliferation, and angiogenesis and inhibitors of apoptosis, previously thought to be only expressed in cancer cells, promoting a proliferative cellular phenotype, resulting in pulmonary vascular remodeling in PAH. The histopathologic features and known genetic susceptibilities of this condition have led to the hypothesis that PAH arises from hyperploproliferation of pulmonary artery smooth muscle cells (PASMCs) and endothelial cells (PAECs).

In addition to the formation of proliferative neointimal lesions and muscularization of the pulmonary vascular bed, perivascular inflammatory cell infiltrates are also present in advanced human cases of PAH. These infiltrates consist of T cells, B cells, and macrophages, suggesting that cytokines and growth factors associated with these inflammatory cells may be promoting PAEC and PASMC hyperproliferation. The proinflammatory cytokine interleukin (IL)-6 is consistently increased in the serum and lungs of patients with idiopathic PAH and in inflammatory diseases that are associated with PAH. In addition, Kaposi sarcoma–associated herpes virus, which may cause PAH in human immunodeficiency virus–negative Castleman’s disease, encodes a constitutively active form of IL-6, resulting in unregulated cell growth and escape from host antitumor defenses. Furthermore, unchecked production of IL-6 in tissues leading to chronic inflammation has exhibited a strong association with many cancers. Given mounting evidence for the role of inflammation and cancer-like mechanisms in the pathogenesis of PAH, we investigated whether IL-6 promotes the development of pulmonary arteriopathy and consequent PAH. We show that lung-specific overexpression of IL-6 in mice replicates the pathological lesions observed in advanced PAH, including both distal arteriolar muscularization and plexogenic arteriopathy, and leads to increased pulmonary vascular resistance (PVR) and PAH. At the cellular and
molecular level, these vasculopathic changes are associated with the activation of vascular endothelial growth factor (VEGF) and the mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase (ERK), with subsequent increases in protooncogene c-MYC/MAX transcription factor complex and the antiapoptotic proteins survivin and Bcl-2, with downregulation of the growth inhibitor transforming growth factor (TGF)-β and proapoptotic kinases JNK and p38. This suggests that IL-6 participates in the development of distal pulmonary proliferative arteriopathy and consequent elevation in PVR and development of PAH.

Materials and Methods
For details, see the expanded Materials and Methods section in the online data supplement, available at http://circres.ahajournals.org.

Indices of pulmonary vascular remodeling were measured in lung-specific IL-6–overexpressing transgenic mice (Tg+/H11001) and compared to wild-type (Tg-/H11002) controls in both normoxic and chronic hypoxic conditions.

Results
IL-6 Overexpression Increases Pulmonary Artery Pressure and Ventricular Hypertrophy
To test the hypothesis that increased IL-6 may cause increased PVR and PAH, we measured the right ventricular systolic pressure (RVSP) in IL-6 Tg+ and Tg- mice. Under normoxic conditions, Tg+ mice had elevated RVSP compared to Tg- mice (Figure 1a). In Tg+ mice, 3 weeks of exposure to 10% oxygen almost doubled RVSP compared to baseline, and this value was almost 2.6 times higher than the RVSP in hypoxic Tg- mice (Figure 1a).

Ventricular wall thickness increased in response to chronic pressure overload, which is a consequence of elevated resistance in the pulmonary artery. Right ventricular hypertrophy (RVH), as measured by right ventricle weight/(left ventricle weight + septum weight [RV/LV+S]) and absolute RV weight, were greater in Tg+ mice than in Tg- mice under normoxic conditions (Figure 1b and 1c). Hypoxia produced even greater RVH in Tg+ mice, whereas there was no change in ventricular wall thickness in Tg- mice (Figure 1b and 1c).
Figure 2. Pulmonary artery (PA) tree of IL-6 Tg− mice has increased muscularization that worsens with hypoxia. a through l, Representative photomicrographs of the elastic lamina of the PA vasculature of IL-6 Tg− and Tg+ mice in normoxic and hypoxic conditions. Main PA branches (Tg− [a and b] vs Tg+ [c and d]), PA at the level of the TBs (Tg− [e and f] vs Tg+ [g and h]); PA distal to TB (acinar) (Tg− [i and j] vs Tg+ [k and l]). Elastic tissue stain; magnification, ×400; scale bar=0.001 mm. m through p, Representative photomicrographs of smooth muscle hypertrophy of distal acinar arterioles of the PA vasculature of IL-6 Tg− in normoxic and hypoxic conditions (Tg− [m and n] vs Tg+ [o and p]). Immunohistochemistry with α-smooth muscle actin; magnification, ×400; scale bar=0.001 mm. q through t, Thickness of the medial wall is increased at all levels of the PA tree of IL-6 Tg− mice compared to Tg+ mice at baseline and worsens with hypoxia. q, Number of elastic lamina of main PA branches (‡P<0.05 vs normoxic Tg−, †P<0.05 vs normoxic Tg+). r, Percentage wall thickness of the main PA branches (‡P<0.05 vs hypoxic Tg−, †P<0.05 vs normoxic Tg+). s, Percentage wall thickness of the TB PA vessels (‡P<0.05 vs hypoxic Tg−, †P<0.05 vs normoxic Tg+). t, Percentage wall thickness of the acinar pulmonary arteriolar vessels (‡P<0.05 vs normoxic Tg−, †P<0.05 vs normoxic Tg+, †P<0.05 vs hypoxic Tg+, †P<0.05 vs normoxic Tg+).
The histological appearance of the hearts was consistent with RVH measurements, showing that right ventricular wall mass was greater in Tg\(^+\) mice in both normoxic and hypoxic conditions (Figure 1d). See the online data supplement for additional data.

**IL-6 Overexpression Induced Musclezation Throughout the Entire Pulmonary Vascular Bed**

To determine the cause of increased PVR, we examined specific regions of the pulmonary vascular tree for remodeling. Examination of the proximal branches of the main PA revealed that the elastic lamina was increased in normoxic Tg\(^+\) mice compared to their Tg\(^-\) counterparts (Figure 2c versus 2a) and was quantitatively confirmed by counting the number of elastic lamina (Figure 2q). Following hypoxia, the number of elastic lamina in Tg\(^+\) mice more than tripled compared to baseline and exceeded the number in hypoxic Tg\(^-\) littermates by a factor of 5. Main PA branches in Tg\(^+\) mice exhibited an increase in not only the number of elastic lamina but also the percentage vessel medial wall thickness, relative to the Tg\(^-\) control, under both normoxic and hypoxic conditions (Figure 2r). The medial wall of the main PA branches in Tg\(^-\) mice more than doubled in thickness in response to hypoxia compared to their Tg\(^-\) normoxic controls, whereas PA medial wall thickness did not change in hypoxic Tg\(^+\) mice compared to normoxic controls.

The terminal bronchioles (TBs) and distal acinar arterioles were examined for evidence of muscularization. The most notable findings were that the distal acinar arterioles of Tg\(^+\) mice were muscularized at baseline and became more thickly muscularized in hypoxia unlike the Tg\(^-\) mice arterioles, as shown by elastic staining (Figure 2k and 2l [Tg\(^+\)] versus 2i and 2j [Tg\(^-\)]) and by immunohistochemistry with \(\alpha\)-smooth muscle actin (Figure 2o and 2p [Tg\(^+\)] versus 2m and 2n [Tg\(^-\)]). See the online data supplement for detailed results, together with the quantitative results of the medial wall thickness of both the TBs and acinar vessels.

**IL-6 Overexpression Induced Arteriolar Neointimal Occlusive Lesions**

We examined the vascular bed for occlusive neointimal lesions that, like arteriolar muscularization, may contribute to increased PVR. We found that the arterioles in Tg\(^+\) mice had thick intimal walls, with many of the arteriolar lumens being partially (27±5%) or completely occluded (4±2%). Hypoxia increased the number of partially (55±3%) or completely occluded arterioles (14±1%, Figure 3b, 3d, and 3e). In contrast, all arterioles in the lungs of Tg\(^-\) normoxic mice were patent, and the intima was not thickened. Only after exposure to hypoxia did the arteriolar lumens become partially occluded, with the number of these vessels reaching 3±2% (Figure 3a, 3c, and 3e).

**Hypoxia Induced Loss of Pulmonary Arterioles in IL-6-Overexpressing Mice**

See the online data supplement and supplemental Figure I for results.

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**Figure 3.** Arterioles of IL-6 Tg\(^+\) mice have neointimal occlusive lesions. a through d, Representative photomicrographs of the lung parenchyma of IL-6 Tg\(^+\) and Tg\(^-\) mice showing neointimal hyperplasia of acinar arterioles in IL-6 Tg\(^+\) mice in normoxic conditions (b) and occlusive arteriopathy in hypoxic conditions (d). No neointimal hyperplastic or occlusive lesions are seen in Tg\(^-\) mice (a and c) (hematoxylin/eosin staining; magnification, \(\times400\); scale bar=0.001 mm). Arrows indicate arterioles. e, Pie chart demonstrates that IL-6 Tg\(^+\) mice have a higher percentage (%) of partially (P.) occluded and closed luminal acinar arterioles at baseline that worsens with hypoxia compared with Tg\(^-\) mice. Hematoxylin/eosin stain included in key to demonstrate representative examples of an open (blue), partially occluded (red), and closed acinar arteriole (yellow); magnification, \(\times40\); scale bar=0.001 mm.
Figure 4. Angioproliferative lesions are present in Tg + mice distal arterioles. a through l, Representative photomicrographs showing the formation of thick occlusive neointimal lesions. Endothelial cells (factor VIII) are forming thick layers in the distal acinar arterioles of Tg + mice (c and d) and have increased expression of VEGFR2 (g and h), which was not seen in Tg - mice (a and b or e and f). There is increased cellular proliferation in the walls of the distal arterioles of IL-6 Tg + mice (PCNA) (k and l) in normoxic and hypoxic conditions, which was not seen in Tg - mice (i and j). Immunohistochemistry staining; magnification, ×400; scale bar=0.001 mm.

IL-6–Induced Distal Pulmonary Vascular Wall Endothelial Cellular Growth and Proliferation

To determine whether PAEC types contribute to intimal wall thickening in arterioles of Tg + mice, we performed immunohistochemical analysis of factor VIII, an endothelial cell marker. We found that the arteriole walls were thickened, in part, by multiple layers of PAECs. Under both normoxic and hypoxic conditions, the PAEC layers either formed smooth and thick concentric occlusive lesions or plexogenic-like occlusive lesions caused by a piling up of PAECs in a nonuniform fashion (Figure 4c and 4d), whereas arteriolar walls in Tg - mice had a normal appearance under both normoxic and hypoxic conditions (Figure 4a and 4b). At baseline and in hypoxic conditions, PAECs lining the arterioles of Tg + mice exhibited minimal expression of VEGF receptor (VEGFR)2 (Figure 4e and 4f), whereas PAECs had elevated VEGFR2 expression in Tg + mice (Figure 4g and 4h), suggesting that the growth potential of these cells was increased and confirming that the plexogenic-like lesions in Tg + mice consisted predominately of PAECs. In Tg + mice, increases in PAECs and VEGFR2 expression were associated with increased proliferation within the intimal wall of arterioles at baseline and in hypoxic conditions, as assessed by staining for the proliferation marker proliferating-cell nuclear antigen (PCNA) (Figure 4k and 4l). No change in PCNA levels were detected in the Tg - lungs (Figure 4i and 4j).

Characterization of Pulmonary Vasculopathy: Progrowth/Proproliferative Factors and Prosurvival/Antiapoptotic Mediators Are Activated in IL-6 Tg + Mice

We determined whether factors that stimulate growth, proliferation, and survival and inhibit apoptosis of PAECs and PASMCs were underlying the mechanism through which IL-6 promotes the characteristic pathophysiologic phenotype of PAH. This was preformed by investigating a number of key modulators that may be involved, at the level of the protein in immunoblot analysis of whole lung lysates (supplemental Figure II, a through e). See the online data supplement for the results.

Inflammatory Cells Contribute to IL-6–Induced Neointimal Lesions in Arterioles

Given that lymphocytes form conglomerates near major airways in this mouse model,14 we examined the pulmonary vascular bed for evidence of a similar cellular inflammatory response at sites of arteriolar occlusive lesions. Under normoxic conditions, the number of periarteriolar lymphocytes (determined by the high nuclear to cytoplasmic ratio) was greater in Tg + mice than in Tg - mice (Figure 5c), with T cells, determined by immunohistochemistry (Figure 5g), but not B cells (Figure 5k) being increased within the pulmonary vascular bed of Tg + animals. Other inflammatory cells were not seen (Figure 5a through 5d). Following hypoxia, these T cells (Figure 5d) contributed to the obstruction of the arterial lumen. This was confirmed by immunoblot analysis of whole lung lysates (supplemental Figure III). Taken together, IL-6 mainly recruits lymphocytes, and the lymphocytes that are recruited to the pulmonary vascular bed are predominately T cells, not B cells. Further characterization of lymphocyte recruitment and function was determined in IL-6 Tg + mice. The data can be seen in the online data supplement, together with supplemental Figure III.

Discussion

Patients with severe PAH and animal models of PAH15–21 exhibit increases in inflammatory cells, growth factors, and cytokines. IL-6, a pleiotropic cytokine, is frequently elevated,
sugestng that PAH development is associated with IL-6--induced inflammation. Our results demonstrate that IL-6 lung-specific overexpression produces distal arteriolar-occlusive plexogenic lesions and arteriolar wall muscularization. These changes in the distal vascular bed are associated with and may lead to proximal pulmonary artery wall hypertrophy and RVH, as well as increased RVSP and PVR. Injection of recombinant human IL-6 (rhIL-6) also produces RVH in rats^{22} and mice.^{18} However, they lack the associated distal oblitative muscularized vascular lesions observed in the transgenic mice that constitutively overexpress IL-6. Importantly, however, IL-6 knockout mice exposed to hypoxia are resistant to the development of increased RVSP.^{23} The lack of correlation between pulmonary vascular remodeling and the presence of elevated pulmonary artery pressures in other murine models^{24–27} has slowed our understanding of the pathobiology of PAH. IL-6 Tg^{+} mice, in which the pathological and physiological changes observed in the pulmonary artery bed correlate with the severity of PAH, may enable a better understanding of PAH pathobiology, including the role of increased IL-6 in the development of PAH in humans.

A major finding in this study is that distal vascular remodeling in Tg^{+} mice is similar to that seen in patients with severe PAH,^{5} with (1) concentric intimal wall thickening, (2) plexogenic lesions, (3) recruitment of inflammatory cells, and (4) distal arteriolar wall muscularization. These features occurred de novo under normoxic conditions and worsened with hypoxia. In other rodent models,^{15–21} vascular remodeling is limited to either dysregulated PAECs or PASMCs, but not both. The Tg^{+} mouse, which exhibits all 4 main pathological features of PAH, is, to our knowledge, the only in vivo model that recapitulates the pathological features of PAH in humans. Therefore, this model may reveal how interactions between hyperproliferative PASMCs and PAECs and inflammatory cells contribute to PAH development.

Disorganized PAEC proliferation leading to the formation of neointimal obliterator lesions is described in many cases of idiopathic PAH^{5} or associated PAH and may be why the human form of severe PAH is difficult to treat with the present available drugs.^{28–30} This has led to the search for newer models of PAH in which a neointima is formed and occludes the vascular lumen. A number of 2-hit injurious murine models have been able to reproduce neointimal occlusive lesions,^{15,31–37} as well as a genetically altered model^{38}; however, less than 5% of these mice developed these lesions. Our study is of interest because we show that by solely overexpressing IL-6 without an additional stress, PAECs are stimulated to either form smooth concentric multilayers, leading to thickening of the intimal wall, or to pile up on top of one another, narrowing the distal arteriolar lumen and forming a plexiform lesion. Both features were present in all mice under normoxic conditions, when PAH is mild, and increased under hypoxic conditions, when RVSP is maximal and the disease is severe. This suggests that aberrant PAEC proliferation and lesion formation are pathologically relevant and useful markers of disease progression and that overexpression of IL-6, a single genetic perturbation, is able to reproduce the characteristic obliterator lesions seen in the aforementioned models and replicate that of human disease.

Distal extension of smooth muscle into small peripheral, normally nonmuscular, pulmonary arteries within the respiratory acinus is notable in all forms of PAH. The cellular processes underlying muscularization of this distal part of the pulmonary vascular bed are incompletely understood but are thought to result from the abnormal growth of PASMCs, which have impaired responses to antiproliferative proapoptotic stimuli such as bone morphogenic protein (BMP) and TGF-β.^{35–37} We show that lung-specific overexpression of IL-6 induces 3 forms of muscularization. First, IL-6 results in distal extension of smooth muscle into the small peripheral pulmonary arteries at the level of the acinus, and, with the added insult of hypoxia, the medial wall further hypertrophies. Secondly, IL-6 results in an increase in the medial wall thickness of the main and bronchial level pulmonary arteries, and, thirdly, there is an increase in the number of layers of elastic lamella, both of which increase further in hypoxic conditions. The combination of these striking changes in muscularization have not been observed in other PAH murine models. However, in the spontaneously hypertensive rat,^{38} increased arterial medial wall thickness is associated with increased number of lamina in major blood vessels, as well as increased wall thickness, although less striking than in hypoxic IL-6 Tg^{+} mice. Furthermore, the hypertrophic changes observed are augmented under increased pressure, suggesting that secondary structural adaptations become superimposed on primary genetic ones. In IL-6 Tg^{+} mice, where growth development is altered,^{34} as in the fawn-hooded rat,^{39} early genetic abnormalities in pulmonary vascular development may contribute to the progression of PAH in the adult Tg^{+} mice with and without a hypoxic injurious stimulus.

It is unclear what triggers PAECs and PASMCs to have a proproliferative phenotype while maintaining an insensitivity toward growth inhibitory stimuli in patients with PAH. In humans, plexiform lesions express angiogenic factors including VEGF and its receptor VEGFR2,

^{40} suggesting that VEGF may play a proangioproliferative role in the development of plexiform lesions, a growth factor shared by the plexiform lesions observed in IL-6 Tg^{+} mice, as well as other animal models with angioproliferative lesions.^{33} VEGF may also be an important survival factor for PASMCs in the presence of IL-6. IL-6 triggers cultured smooth muscle cell proliferation both directly, through upregulation of VEGFR2 expression and phosphorylation, and indirectly, through upregulation of matrix metalloproteinase-9.^{41} IL-6–induced VEGF expression may also indirectly increase the number of PASMCs by transforming PAECs into smooth muscle–like cells, as observed in cultured human PAECs.^{52} These findings, taken with our results, suggest that the presence of abnormal levels of IL-6 may activate, amplify, and maintain the growth and proliferation of PAECs and PASMCs by upregulating VEGF expression.

TGF-β/BMP signaling, a network of proteins that control cell growth, is impaired and the TGF-β receptor is absent in the PAECs in the core of plexiform lesions in PAH.^{43,44} This suggests that PAECs in plexiform lesions have lost their
check-and-balance system to control PAEC growth, giving rise to a hyperproliferative PAEC phenotype. In addition, PASMCs from patients with PAH are resistant to the anti-proliferative effects of TGF-β, suggesting that the failure of TGF-β to suppress PASMC growth in PAH may, in part, underlie the increased muscularization of normally nonmuscularized distal pulmonary arteries of patients with PAH. IL-6 has recently been found to negatively regulate the TGF-β/BMP signaling cascade. In the IL-6 Tg+ mouse model, in which both muscularization and angioproliferative lesions are abundant and PAH is present, we show that the expression of TGF-β is reduced, in a rich milieu of angioproliferative growth factor VEGF and its receptor. Taken together, the IL-6 Tg+ mouse model shares similar growth factor characteristics to that of patients with PAH, and thus this model may enable investigators to delineate the trigger behind the molecular imbalance that favors the increased expression of proliferative growth factors.

IL-6 overexpression may predispose to proliferative cellular phenotypes and exaggerated PAH as a result of unopposed MAPK intracellular signaling, normally countered by anti-proliferative TGF-β-mediated signaling. Both p38MAPK and ERK are noted to be unopposed in PASMCs from patients with mutations in the TGF-β/BMP pathway, resulting in a proliferative apoptotic resistant phenotype. The IL-6 Tg+ mice share a similar biology to PASMCs of the patient, whereby ERK activity also is upregulated in an unopposed environment, which is, in part, attributable to the lack of TGF-β and, in part, attributable to the lack of proapoptotic MAPKs p38 and JNK. These findings are also consistent with in vitro work, in which IL-6-stimulated human endothelial cells also have reduced p38 and pJNK. In other cell systems, IL-6 activates the MAPK signaling pathway via ERK and, in turn, blocks the TGF-β/BMP pathway by preventing the nuclear translocation of Smad, a downstream BMP signaling protein, resulting in cellular proliferation. Given that this mouse model and the vasculature of PAH patients are deficient in growth controlling TGF-β/BMP proteins in the setting of elevated IL-6 levels and that both share ERK activation, further investigation of unopposed IL-6/ERK signaling may uncover the mechanism by which vascular cells switch from a balanced growth controlled state to an excessively proproliferative one.

IL-6 overexpression may predispose to exaggerated PAH as a result of coordinating a number of downstream proproliferative, prosurvival, and antiapoptotic signaling pathways (Figure 6). We found that c-myc and its obligatory binding partner, MAX, may be key in the downstream proliferative signal cascade of IL-6, promoting cellular proliferative phenotypes in PAH. However, it is also clear from our work that IL-6 selectively blocks apoptosis within the pulmonary vascular bed by downregulating TGF-β and proapoptotic MAPKs, pJNK, and p38, while upregulating prosurvival factors survivin and Bcl-2. Taken together, these complex obliterator vascular lesions are likely forming because IL-6 is favoring a proproliferative antiapoptotic cell state, as observed in angioproliferative lesions of patients with PAH. See the online data supplement for an expanded discussion regarding IL-6 and its role in regulating proliferative and antiapoptotic pathways.

Elevation of IL-6 in the serum and inflammatory cellular infiltrate in pleiform lesions in PAH patients and now IL-6 Tg+ mice with replicative pathophysiological changes of PAH suggest that cellular immunity may play an active role in the dysregulation of PAECs and PASMCs and the development of PAH. Further discussion regarding our findings and controversies of the role inflammation may play in this disease is highlighted in the online data supplement.

The importance of the loss of small pulmonary arteriolar vessels, which may contribute to the development of PVR, is controversial; for further discussion regarding our data, see the online data supplement.

In summary, our work supports the hypothesis that IL-6 directly promotes a proproliferative apoptotic resistant milieu within the PA wall, resulting in a vasculopathy mirroring that seen in patients with severe PAH. Unlike other murine models, this transgenic mouse model replicates the pathophysiology of human PAH, with muscularization and arteriolar-occlusive changes occurring in the distal vascular bed, leading to elevated PVR and secondary chronic pressure
overload occurring in the main PA and right ventricle. Our work demonstrates that, in the pulmonary vascular bed, regulation of inflammatory cytokine IL-6 is tightly linked with cellular growth and proliferation through a number of proliferative apoptotic resistant downstream pathways. Future work to establish the underlying mechanism of these complicated signaling systems in the lung-specific IL-6 Tg mice, to determine several of the key molecules necessary to inhibit the excessive proliferative cellular state, will improve our understanding of the disease and thereby help in developing new therapies that target the angioproliferative lesions in patients with refractory PAH. The development of PAH in mice overexpressing IL-6 in the lung, together with the presence of increased IL-6 in PAH patients, suggests that IL-6 is integral to the development and progression of pulmonary vascular remodeling, PVR, and PAH.

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Disclosures

None.

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Materials and Methods

Animals  The subcommittee on research for animal care at the Massachusetts General Hospital approved the experimental protocol. CC10-IL-6 transgenic mice (Tg(+) were bred on a C57/BL6 (Tg(-)) background. In these mice, the Clara cell 10-kD promoter (CC10) was used to constitutively drive lung-specific expression of IL-6. Tg(-) littermates served as controls in all experiments. Five Tg(-) and five Tg(+) mice, age- and sex-matched, were exposed to room air (21% FiO2) and then another five Tg(-) and five Tg(+) mice age- and sex-matched, were exposed to hypoxia (10% FiO2) at sea level. All animals were 3 months old and weighed 22.5 ± 2.0 grams.

For hypoxia, animals were placed in a sealed chamber. The O2 concentration in the chamber was maintained at 10% by controlling the inflow rate of compressed air and N2. Gas was circulated in the chamber with a fan. The CO2 concentration was maintained at < 0.4% with a CO2 absorbent. Gas samples were tested twice per day during the entire experimental period to monitor O2 and CO2 tension. The chamber was unsealed for less than 30 minutes twice per week to replenish the food, replace CO2 absorbent and clean the cages.

Measurement of right ventricular systolic pressure (RVSP)  All mice were administered ketamine (80 mg/kg) and diazepam (5 mg/kg), which produced anesthesia with spontaneous breathing. A midline sternal skin incision was made from the second intercostal space to the xiphoid process. A 25-gauge needle was attached to a male/male
luer slip connector, which was joined, in a successive order, to an 18-gauge blunt needle, polyethylene 190 tubing (ID 1.19 mm; OD 1.70 mm), 18-gauge blunt needle connected to a physiological transducer (Becton Dickinson DTXPlusDT-XX) via a two-way plastic stopcock. The stopcock facilitated flushing of the needle and in situ measurement of atmospheric pressure without introducing air bubbles. The entire system was flushed with sterile saline to eliminate bubbles. The transducer was positioned 1.0 cm above the level of the midaxillary line. The needle was then inserted into the right ventricle by following a 45º trajectory between the right second and third intercostal space above the xiphoid process. Pressures were recorded on a Gould chart recorder (Model RS3400) with an embedded Gould transducer amplifier (Model 13-4615-50) at paper speeds ranging from 2.5 to 200 mm/s. Peak RVSPs were obtained, as previously described.² Placement of the needle into the right ventricle was confirmed by postmortem examination.

**Heart weight** The right ventricular free wall was detached and removed under a dissecting microscope. The left ventricle and septum were weighed separately from the right ventricle, with measurements being taken after drying at 90ºC for 24 h and 48 h. If the difference between the two readings was greater than ± 0.5 mg, the specimens were dried for another 24 h.²

**Histology of pulmonary vasculature and heart** The heart and lungs were flushed with normal saline, removed and fixed in formalin for 72 h. The aorta was removed under a dissecting microscope to access the pulmonary trunk. Two 3-cm silicone tubes (0.03 cm
× 0.05 cm; SMI, Saginaw, MI) were inserted into the pulmonary trunk through a small opening that was made in the lateral wall to maintain vessel wall patency during fixation. One tube was advanced through the main artery into the right pulmonary artery while another tube was inserted into the left pulmonary artery until resistance was encountered at their respective hila. Both main branches of the PA were lifted away from the heart and lungs, using the tubing as an aid. The tubing was removed before tissue embedding. Both the formalin-inflated left lung and the whole heart were sliced into 5 μm-thick sections. Slices of the lung, heart, main pulmonary branches, and pulmonary trunk were dehydrated and embedded in paraffin. Sections were then stained with hematoxylin & eosin, elastic, and giemsa stains.

Assessment of pulmonary remodeling Pulmonary remodeling was assessed by the percent wall thickness of the main PA branches (central) and parenchymal PA vessels indexed to terminal bronchioles and acini (i.e., vessels indexed to respiratory bronchioles or alveolar ducts). Wall thickness was measured with an ocular micrometer and expressed as the medial wall thickness (the distance between the internal and external lamina) divided by the diameter of the vessel (the distance between the external lamina) × 100 (% wall thickness; %WT). For vessels with a single elastic lamina, the distance between the elastica and endothelial basement membrane was measured. The total number of peripheral arteries was expressed as the number of arteries per every 100 alveoli in each field and was verified using sections in which the vessels were stained with elastin. A quantitative analysis of luminal obstruction was performed by counting at least 50 small pulmonary arterioles (outer diameter < 50 μm) in every hematoxylin-
eosin stained lung section. These arterioles were assessed for occlusive lesions and scored as follows: (1) no evidence of lumen occlusion (open), (2) partial (< 50%) luminal occlusion, and (3) full luminal occlusion (closed). All morphometric analyses were performed by one blinded observer.

**Antibodies** Antibodies used for immunohistochemistry included rat anti-mouse B220 antigen (1:100; Clone RA3-6B2, BD Biosciences, San Diego, CA), rabbit polyclonal anti-human CD3 (1:400), polyclonal antibody to factor VIII-related antigen (1:250, Dakocytomation, Carpinteria, CA), and biotinylated goat anti-rabbit IgG (1:200; Vector laboratories, Inc., Burlingame, CA). Mouse monoclonal FLK-1 (VEGF-R2, Clone A3, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used at a dilution of 1:200 for immunoblot and 1:50 for immunohistochemistry. Anti-actin smooth muscle specific (AB-2) mouse mAb(1A4) (1:750; EMD Chemicals, Inc., Gibbstown, NJ). Antibodies used for immunoblot include mouse monoclonal VEGF antibody (1:1000; Abcam, Cambridge, MA), TGF-β antibody (rabbit polyclonal antibody; 1:1000), TNF-α (rabbit polyclonal antibody; 1:200), goat anti-rabbit IgG -HRP(1:2000), p38 (rabbit monoclonal antibody total 1:500 and phosphorylated 1:200), JNK (rabbit polyclonal antibody total 1:500 and phosphorylated JNK 1:50), c-MYC (rabbit polyclonal antibody 1:200), β-actin (rabbit polyclonal antibody, 1:2000), caspase 3 (rabbit polyclonal antibody 1:200), cleaved caspase 3 (rabbit monoclonal antibody 1:200), survivin (rabbit polyclonal antibody 1:400), Bcl-2 (rabbit polyclonal antibody 1:200) and BAX (rabbit polyclonal antibody 1:400) from Cell Signaling Technology, Inc; Danvers, MA), PDGF-B (rabbit polyclonal antibody 1:100), MCP-1 (rabbit polyclonal antibody 1:100), ERK1/2 (rabbit polyclonal antibody 1:1000), and ERK2 (rabbit polyclonal antibody 1:2000).
polyclonal, 1:500; Clone 14), and MAX (rabbit polyclonal antibody; 1:50) from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. Goat anti-mouse IgG (1:2000; Jackson Immunoresearch Laboratories, Philadelphia, PA), anti-fractalkine (0.1 μg/ml; cleaved c-CX3CL1 [30 kDa] and total CX3CL1, R & D Systems, Inc., Minneapolis, MN). Purified mouse anti-NF-ATc3 monoclonal antibody (1:200; BD Biosciences, Franklin Lakes, NJ).

Immunohistochemistry Formalin-fixed, paraffin-embedded tissue sections were baked for 10 min at 60ºC, deparaffinized, and rehydrated. For antigen retrieval, tissues were rinsed in water and heated in a Decloaker pressure cooker in 0.01M Na-citrate (pH 6.0) for 3 min. The Na-citrate bath was returned to room temperature (RT) before the sections were rinsed in water. Endogenous peroxidase activity was quenched by treating sections with 3% H2O2 for 5 min. Sections were then washed in phosphate-buffered saline (PBS) and successively blocked for 15 min at RT with appropriate serum, avidin, and biotin. Tissue sections were incubated with primary antibodies overnight at 4ºC, washed with PBS, and if necessary (if primary was not biotinylated), incubated with secondary biotinylated antibody for 1 h at RT. Bound antibodies were detected by treating sections with streptavidin-horseradish peroxidase complex for 30 min. Peroxidase activity was visualized with 3,3’ diaminobenzidine, and sections were counterstained with hematoxylin. Primary antibody was omitted from staining reactions as a negative control. Thymus and spleen tissue served as positive controls for leukocyte staining.

Immunoblotting Frozen lung tissue was homogenized for 30 min on ice in RIPA buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1%
SDS; Boston BioProducts, Worcester, MA) supplemented with phosphatase inhibitor 
(Calbiochem, La Jolla, CA) and protease inhibitor (Calbiochem). Homogenates were 
centrifuged at 2000 rpm at 4°C for 30 min, and centrifugation was repeated on the 
supernatants. The protein concentration of the resulting supernatant was determined 
using Bradford reagent (Bio-Rad Laboratories, Hercules, CA). Proteins (70 μg) were 
electrophoresed on 4 – 20% gradient Tris-Hepes-SDS gels (Pierce, Rockford IL) and 
transferred to Immobilon PVDF membranes (Millipore Corporation, Bedford, MA). 
Prestained molecular mass marker proteins (Bio-Rad Laboratories) were used as 
standards for the Tris-Hepes-SDS gel electrophoresis. The PVDF membranes were 
blocked with 5% non-fat dry milk in Tris-Buffered Saline containing 0.1% tween-20 
(TBST) for 1 h at RT, washed with TBST, and probed with primary antibody diluted in 
blocking buffer for 2 h at RT. The membrane was subsequently washed, incubated with 
secondary antibody diluted in blocking buffer for 1 h at RT. Antibody labeling was 
visualized using chemiluminescence reagent (Cell Signaling Technology).

TdT-mediated dUTP nick end labeling staining. End labeling of exposed 3’-OH ends 
of DNA fragments was undertaken with the TdT-mediated dUTP nick end labeling 
(TUNEL) in situ cell death detection kit (FragEL, Calbiochem, La Jolla, CA).

Statistical analysis. Data are expressed as mean ± s.e.m. Multiple comparisons were 
made using the non-parametric Kruskal-Wallis test, and statistical significance (p ≤ 0.05) 
between two groups was confirmed using the non-parametric Wilcoxon-Mann-Whitney 
test. Statistics were performed using Statview 4.5 (Abacus Concepts, Inc., Berkeley,
In all experiments, n is equal to 5 replicates apart from immunoblots where the n is equal to 4 replicates.

Results

IL-6 overexpression increases pulmonary artery pressure and ventricular hypertrophy (expanded)

To test the hypothesis that increased IL-6 may cause increased PVR and PAH, we measured the right ventricular systolic pressure (RVSP) by direct right ventricular puncture, in transgenic mice over expressing IL-6 (Tg(+)). Under normoxic conditions, Tg(+) mice had elevated RVSP compared to Tg(-) mice (34 ± 2 mmHg vs. 21 ± 1 mmHg, p<0.05, Figure 1a). In Tg(+) mice, 3 weeks of exposure to 10% oxygen almost doubled RVSP to 64 ± 7 mmHg (p<0.05 vs. normoxic), and this value was almost 2.6 times higher than the RVSP in hypoxic Tg(-) mice (24 ± 1 mmHg, p<0.05, Figure 1a). As in C57B1/6 mice,7 Tg(-) mice did not exhibit any significant increase in RVSP in response to hypoxia (compared to normoxic controls).

Ventricular wall thickness increased in response to chronic pressure overload, which is a consequence of elevated resistance in the pulmonary artery (PA). Right ventricular hypertrophy (RVH), as measured by right ventricle weight / (left ventricle weight + septum weight; RV/LV+S) and absolute right ventricle weight (RV), were greater in Tg(+) mice than in Tg(-) mice under normoxic conditions (RV/LV+S: 0.4 ± 0.02 vs. 0.3 ± 0.02, p<0.05, Figure 1b and RV: 0.007 ± 0.0001 gm vs. 0.005 ± 0.00001 gm, p<0.05, Figure1 b, c). Hypoxia produced even greater RVH in Tg(+) mice (RV/LV+S: 0.7 ± 0.1, and RV: 0.01 ± 0.001 gm, p<0.05 vs. normoxic control) while there was no change in ventricular wall thickness in Tg(-) mice (Figure 1c). The absolute
left ventricle weight remained unchanged between groups (data not shown). The histological appearance of the hearts was consistent with RVH measurements, showing that, in both normoxic and hypoxic conditions, right ventricular wall mass was greater in Tg(+) mice (Figure 1d).

**IL-6 overexpression induced muscularization throughout the entire pulmonary vascular bed (expanded)**

To determine the cause of increased PVR, we examined specific regions of the pulmonary vascular tree for remodeling. Examination of the proximal branches of the main PA revealed that the elastic lamina was increased in normoxic Tg(+) mice compared to their Tg(-) counterparts (Figure 2c vs. a) and was quantitatively confirmed by counting the number of elastic lamina (3 ± 0.3 vs. 2 ± 0.0, p<0.05; Figure 2q). Following hypoxia, the number of elastic lamina in Tg(+) mice was increased to 10 ± 1 (figure 2d), which exceeded the number in normoxic Tg(+) mice (p<0.05) and hypoxic Tg(-) littermates (2 ± 0.3, p<0.05; Figure 2q). No differences in the number of elastic lamina between normoxic and hypoxic Tg(-) mice were observed. Main PA branches in Tg(+) mice exhibited an increase in not only the number of elastic lamina, but also the percent vessel medial wall thickness, relative to the Tg(-) control, under both normoxic (7 ± 1% vs. 3 ± 1%, p<0.05) and hypoxic conditions (15 ± 1% vs. 6 ± 1%, p<0.05; Figure 2r). The medial wall of the main PA branches in Tg(+) mice more than doubled in thickness in response to hypoxia compared to their Tg(+) normoxic controls (15± 1% vs. 7 ± 1%, p<0.05), while PA medial wall thickness did not change in hypoxic Tg(-) mice compared to normoxic controls.
The terminal bronchioles (TB) and distal acinar arterioles were examined for evidence of muscularization. The most notable findings were that the distal acinar arterioles of Tg(+) mice were muscularized at baseline and became more thickly muscularized in hypoxia unlike the Tg(-) mice arterioles as shown by elastic staining (Figure 2 Tg(+) k, l vs. Tg(-) i, j) and by immunohistochemistry with α-smooth muscle actin (Figure 2 Tg(+) o, p vs. Tg(-) m, n).

Quantitatively, we found that TB medial wall thickness was greater in normoxic Tg(+) mice than normoxic Tg(-) mice (29 ± 2% vs. 24 ± 2%, p<0.05, Figure 2s). The same was true of the acinar medial walls (26 ± 1% vs. 21 ± 1%, p<0.05, Figure 2t). Under hypoxic conditions, medial wall thickness for Tg(+) mice was greater than that for Tg(-) mice in both the TBs (49 ± 4% vs. 30 ± 3%, p<0.05, Figure 2s) and acini (49 ± 1% vs. 32 ± 2%, p<0.05, Figure 2t). In Tg(+) mice, hypoxia nearly doubled medial wall thickness for both the TB vessels (49 ± 4% vs. 29 ± 2% for normoxic control, p<0.05, Figure 2s) and acinar vessels (49 ± 1% vs. 26 ± 1% for normoxic control, p<0.05, Figure 2t). In Tg(-) mice, hypoxia induced only a 1 – 1.5-fold change in medial wall thickness for TB vessels (30 ± 3% vs. 24 ± 2% for normoxic control, p<0.05) and acinar vessels (32 ± 2% vs. 21 ± 1% for normoxic control, p<0.05).

**Hypoxia induced loss of pulmonary arterioles in IL-6 overexpressing mice.**

To test whether a reduction in arterioles may contribute to the increase PVR in Tg(+) mice, we compared the number of arterioles (per every 100 alveoli) in the alveolar bed of Tg(+) and Tg(-) mice. Vessel number did not significantly differ between these mice under normoxic conditions. Hypoxia reduced the number of countable arterioles at the alveolar level in both Tg(+) mice (7 ± 1 vs. 12 ± 1 for normoxic control, p<0.05) and
Tg(-) mice (11 ± 1 vs. 14 ± 1 for normoxic control, p = 0.05), and the relative arteriolar
reduction was similar for both groups (Online Figure I). However, Tg(+) mice had a
lower number of arterioles compared to their Tg(-) littermates (7 ± 1 vs. 11 ± 1, p<0.05).
Therefore, the hypoxia-induced reduction in the number of distal pulmonary vessels is
similar for Tg(+) and Tg(-) mice, but the absolute reduction is greater in Tg(+) mice.

Characterization of Pulmonary Vasculopathy in IL-6 Tg (+) Mice: Growth Factors.

To determine the factors that stimulate growth of PAEC and PASMC, immunoblot
analysis of whole lung lysates (Online Figure IIa) was performed. We first confirmed
that both VEGF and VEGFR2 levels were higher in Tg(+) mice than Tg(-) mice under
normoxic conditions and hypoxic conditions. Transforming growth factor (TGF)-β, a
pro-apoptotic anti-proliferative protein, was reduced in Tg(+) mice compared to Tg(-)
mice under normoxic conditions and hypoxic conditions, while platelet derived growth
factor (PDGF) was similarly increased in all groups. This suggests that IL-6 is promoting
cellular growth by up-regulating growth factor VEGF and preventing inhibition of
cellular growth by down-regulating TGF-β.

Characterization of Pulmonary Vasculopathy in IL-6 Tg(+) Mice: Mitogen
Activated Protein Kinases.

We evaluated for evidence of signal transduction pathways that may be linking pro-
growth factor responses to intracellular downstream pro-proliferative transcription
factors. We found that phosphorylated extracellular regulated kinase (ERK), a pro-
 proliferative growth mitogen activated protein kinase (MAPK), was elevated in Tg(+)
lung lysates compared to Tg(-) lysates in both normoxic and hypoxic conditions (Online Figure IIb). Pro-apoptotic MAPKinases such as p38 and pJNK were not increased in Tg(+) lung lysates compared to Tg(-) lysates in both normoxic and hypoxic conditions. Taken together this would suggest that IL-6 is specifically activating pro-proliferative growth kinase ERK while preventing the activation of kinases that promote apoptosis.

Characterization of Pulmonary Vasculopathy in IL-6 Tg(+) Mice: Pro-Proliferative and Anti-Apoptotic Targets.

Given that cellular proliferation is increased within the walls of the pulmonary vasculature of the IL-6 Tg(+) mice (Figure 4 k, l, and o) and proliferative MAPK ERK is increased (Online Figure IIb), we evaluated for evidence of downstream proliferative transcription factors. We found that c-myc, a basic-helix-loop-helix/leucine zipper transcription factor that controls the G1-S cell cycle promoting cellular growth and proliferation,8 was elevated in Tg(+) mice relative to their Tg(-) counterparts under normoxic and hypoxic conditions (Online Figure IIc). In addition, c-Myc’s obligate binding partner, MAX, was also exclusively elevated in Tg(+) mice relative to their Tg(-) counterparts under normoxic and hypoxic conditions. This suggests that IL-6 may be inducing cellular growth and proliferation and subsequent pulmonary vascular wall remodeling by up-regulating pro-proliferative oncogenic transcription proteins.

Given that the growth inhibitor TGF-β, and two MAPKinases that stimulate downstream apoptotic signals, phosphorylated 38 and JNK, are reduced, we investigated if
downstream pro-apoptotic caspase 3 is modulated by IL-6 in these mice. Cleaved caspase 3 activity was increased in IL-6 Tg(+) mice at baseline compared to Tg(-) mice. However in hypoxia cleaved caspase 3 activity was reduced in Tg(+) mice compared with baseline room air conditions (Online Figure IId). In order to determine if programmed cell death is occurring in the distal arteriolar lesions, we looked for DNA fragmentation, a marker of apoptosis, and observed that in the IL-6 Tg(+) mice, there was DNA fragmentation in epithelial cells and possibly in histiocytes of alveolar units but not in the walls of the distal arterioles (Online Figure IIe). Taken together, IL-6 overexpression may be selectively preventing programmed cell death by apoptosis within the pulmonary vascular bed.

To determine which anti-apoptotic proteins may be involved in inhibiting caspase 3 and preventing subsequent cell death within the vascular bed, we probed for two important anti-apoptotic proteins, Bcl-2 and survivin. We found that Bcl-2 was elevated in Tg(+) mice relative to their Tg(-) counterparts under normoxic and hypoxic conditions (Online Figure IId) while pro-apoptotic protein BAX levels were similar in Tg(-) and Tg(+) mice and remained unchanged in hypoxic conditions. We found that survivin is markedly elevated in Tg(+) mice compared to Tg(-) mice at baseline and in hypoxia (Online Figure IId). This suggests that IL-6 may be inducing a pro-survival cellular state by modulating several anti-apoptotic proteins.

Characterization of Lymphocyte Recruitment and Function.
To determine the signals that IL-6 employs to control the recruitment of lymphocytes, we probed for chemokines that are known to be involved in lymphocyte trafficking. We found that Tg (+) mice had elevated levels of activated fractalkine (c-CX₃CL1), a PAEC-derived chemokine that recruits T cells and mediates T cell adhesion to endothelial cells, at baseline and in hypoxic conditions (Online Figure III). Monocyte chemoattractant protein, MCP-1, a chemokine that recruits monocytes and lymphocytes and may have a role in smooth muscle cell proliferation was increased in IL-6 Tg (+) mice at room air but was reduced in hypoxia (Online Figure III). Tumor necrosis factor (TNF)-alpha was reduced in both normoxic and hypoxic conditions in the IL-6 Tg (+) mice compared to Tg (-) mice. Nuclear factor of activated T-cells, cytoplasmic, calcineurin-3 (NF-ATc3), which may have a role in promoting a state of proliferation and suppress mitochondrial–dependent apoptosis⁹, was slightly increased in IL-6 Tg (+) mice whole lung lysates (Online Figure III). Taken together, this data supports the concept that overexpression of IL-6 stimulates the recruitment of T cells through up-regulation of specific chemokines depending on the presence or absence of hypoxia and stimulates T cells to home to distal pulmonary arteriole vessels. In turn, T cells may be enhancing IL-6’s pro-survival apoptotic resistant cellular phenotypic effects.

Discussion

**IL-6 and cMyc/MAX Expanded**

IL-6 overexpression may predispose to exaggerated PAH as a result of coordinating a number of downstream pro-proliferative, pro-survival, and anti-apoptotic signaling pathways (Figure 6). We found that c-myc, and its obligate binding partner, MAX may
be key in IL-6’s downstream proliferative signal cascade, promoting cellular proliferative phenotypes in PAH. Consistent with our findings, endothelin stimulated rat aortic smooth muscle cells are shifted into a pro-proliferative state in the presence of ERK induced c-myc activation. It also has been shown that pulmonary arteries from rats exposed to hypoxia have elevated levels of c-myc mRNA. In addition, YY1 growth regulator of vascular smooth muscle cells targets c-myc downstream leading to increased smooth muscle specific gene expression, proliferation and neonatal piglet pulmonary hypertension. Furthermore, in the presence of TGF-β/BMP proteins, c-myc expression is attenuated contributing to PASMC apoptosis. How the c-myc/max complex may be altering its target cell cycle genes and whether the additional stress of hypoxia may alter its effects on downstream targets is unclear at present. However, given the number of angioproliferative lesions at baseline, which likely results in localized hypoxia, and the increase in the number of lesions in hypoxia, we hypothesize, based on extensive work in tumor hypoxia deprivation biology, that hypoxia inducible factors may be facilitating the formation of c-myc-max complex and thereby augmenting the expression of cell cycle genes and exaggerating the hyper-proliferative cellular phenotypes in this transgene mouse. Further investigation with this IL-6 Tg(+) mouse will allow us to evaluate the role of IL-6 in the presence of hypoxia and associated physiologic changes thus imposed by hypoxic vasoconstriction.

**IL-6 and Apoptosis Expanded**

While IL-6 appears to be driving a number of proteins that promote cellular proliferation, it is also clear from our work that IL-6 is also affecting apoptosis by down regulating
TGF-β and pro-apoptotic MAPKs, pJNK and p38 (Figure 6). As with hyperoxic injury in Tg(+) mice\(^\text{(14)}\) and \(\text{H}_2\text{O}_2\) injury in IL-6 stimulated endothelial cells,\(^\text{(15)}\) caspase 3, the main gate keeper to programmed cell death, appears to be activated at baseline and is subsequently down-regulated when under the stress of hypoxia. However, evidence for DNA fragmentation, a marker of apoptosis, is observed only in epithelial cells and possibly histiocytes lining the alveoli, not in the cells of the obliterative lesions of the distal pulmonary vasculature at baseline or in hypoxia. This would suggest that IL-6’s role in preventing cell death by apoptosis is PA wall specific at baseline and persists in hypoxia. Dysregulation of mediators of apoptosis in the PA wall of patients with PAH favoring suppression of apoptosis has been shown in gene microarray studies.\(^\text{(16)}\) Anti-apoptotic proteins Bcl-2 and survivin have been found to be up-regulated in patients with PAH\(^\text{(16,17)}\) where they are thought to impair the activity of Kv channels\(^\text{(17,18)}\) and subsequent mitochondria-dependent apoptosis.\(^\text{(19)}\) Similar to patients with PAH, IL-6 Tg(+) mice also share a similar anti-apoptotic biology with increased expression of both bcl-2 and survivin. At present, it remains unclear what triggers induced by IL-6 modulate the expression of these two anti-apoptotic proteins. However it is clear that DNA fragmentation within the complex obliterative vascular lesions of L-6 Tg(+) mice is absent favoring a pro-proliferative cell state, as observed in angioproliferative lesions of patients with PAH.

**IL-6 and Inflammation Expanded**

Elevation of IL-6 in PAH patients suggests that cellular immunity may play an active role in the dysregulation of PAEC and PASMC and the development of PAH. Accordingly,
plexigenic and concentric lesions in patients with severe PAH contain inflammatory cells, consisting of T cells, macrophages, and to a lesser extent, B cells\textsuperscript{20,21}. Similarly, the obliterative complex distal arterial lesions in Tg(+) mice also contain an abundance of T cells and to a lesser extent, B cells, while B cells appear to surround the large and mid sized airways.\textsuperscript{22} The mechanisms underlying inflammatory cell recruitment and transendothelial migration into pulmonary artery vessel walls during PAH are unclear, although the chemokines RANTES\textsuperscript{23} and fractalkine\textsuperscript{24} may participate in inflammatory cell trafficking.\textsuperscript{25} Hyper-proliferative PAEC may mediate the adhesion of the T lymphocytes by over-expressing fractalkine, as the cleaved activated form of fractalkine is up-regulated in the lungs of IL-6 Tg(+) mice as well as in plexigenic lesions of patients.\textsuperscript{24} Further work examining T lymphocyte trafficking in Tg(+) mice will be instrumental in understanding their role in the vascular changes seen in patients with PAH.

There has been much controversy regarding the role of T cells in PAH, where T cell-mediated immunity has been found to be protective against PAH in VEGFR blocker treated nude rats\textsuperscript{5} while in antigen challenged mice the Th2 immune response is associated with vascular muscularization.\textsuperscript{26} In the IL-6 Tg(+) mouse, IL-6 and T cells may have a trans-signaling relationship, whereby T cell activation driven by IL-6 contributes to the perpetuation of IL-6’s pro-inflammatory pro-proliferative anti-apoptotic effects on neighboring PASM and PAEC (Figure 6). Such a relationship has been thought to have been an important mechanism underlying other chronic inflammatory diseases,\textsuperscript{27} and may be one of the many important IL-6 mechanisms involved in the pathogenesis of PAH. NFAT proteins, produced and carried to PA
vessels in lymphocytes have recently been noted to be activated in human PAH and may be contributing to the resistance to apoptosis within the PA wall. In IL-6 Tg(+) mice, NF-ATc3 was only slightly increased in the IL-6 Tg(+) mice compared with Tg(-) mice at baseline and in hypoxia, and therefore may or may not be associated with PAEC and PASMC insensitivity to apoptosis. Its significance may be under-represented by the lack of cell specificity as it was tested in whole lung lysates. Taken together, T cells in an IL-6 rich milieu are prominently situated within the distal arteriolar vessels, and further work to evaluate their role, whether protective or stimulatory, will be key to understanding their presence in human PAH disease.

**IL-6 and Loss of PA vessels Expanded**

Whether the loss of small pulmonary arteriolar vessels contributes to the development of PVR is controversial. The importance of this loss may be negligible since chronic hypoxic experimental PAH models have considerable angiogenesis and Rho kinase inhibitors normalize pulmonary pressures during chronic hypoxia via vasodilation. However, vessel loss is induced by many other experimental PAH stimuli, including injection of monocrotaline, monocrotaline combined with pneumonectomy, chronic hyperoxia, and creation of aortopulmonary shunts. Clinically, artery loss occurs in idiopathic PAH, as well as in conditions associated with PAH, including congenital heart disease and lung developmental abnormalities. Here, hypoxia induced a significant loss of distal arterioles in Tg(+) mice, which are known to have abnormal alveoli with enlarged terminal air sacs. This altered anatomy, which may result in a significant decrease in the number of arterioles, could significantly contribute to
increased PVR. We found no significant differences in vessel number between Tg(-) and Tg(+) mice under normoxic conditions and the hypoxia-induced arteriolar reduction was similar between groups, despite a significant increase in PVR in Tg(+) hypoxic mice. Thus, arterial vessel loss alone may not account for increased PVR in IL-6 Tg(+) mice.

References:


Legend (Supplement)

Online Figure I The number of arteries per 100 alveoli in IL-6 Tg(+) and Tg(-) mice in normoxic conditions are similar at baseline. Under hypoxic conditions, the number of arteries per 100 alveoli are reduced in both Tg(+) and Tg(-) mice (‡ vs. normoxic Tg(+); p<0.05, † vs. normoxic Tg(-); p<0.05) with IL-6 Tg(+) mice having the highest reduction (‡ vs. hypoxic Tg(-); p<0.05).

Online Figure II (a) Representative immunoblot protein expression of TGF-β, PDGF, VEGF, and VEGFR2 of lung lysates from IL-6 Tg(+) and Tg(-) mice exposed to normoxia and 3 weeks of hypoxia. (b) Representative immunoblot protein expression of phosphorylated ERK 42, ERK 42/44, phosphorylated p38, p38, phosphorylated JNK-1 and JNK2/3, and JNK. (c) Representative immunoblot protein expression of PCNA, c-myc, and MAX. (d) Representative immunoblot protein expression of survivin, Bcl-2, BAX, cleaved caspase-3, and caspase 3. β-actin used as control. (e) Representative photomicrographs of distal acinar arterioles of the PA vasculature of IL-6 Tg(+) and Tg(-) mice in normoxic and hypoxic conditions exposed to TdT-mediated dUTP nick end labeling (TUNEL) for DNA fragmentation evaluation. The distal arterioles (arrow with dark circle) and specifically the occlusive lesions in Tg(+) mice at baseline and in hypoxia have no evidence of DNA fragmentation, while epithelial cells and possibly histiocytes lining the alveolar walls are TUNEL positive at baseline (arrow, see inset). TUNEL staining, magnification x400, bar=0.001mm and inset, magnification x1000, bar=0.0005mm. β-actin used as a control.
Online Figure III Representative immunoblot protein expression of TNF-α, MCP-1, cleaved chemokine fractalkine (c-CX3CL1), total CX3CL1, NF-ATc3, T cells (CD3) and B cells (CD19) in whole lung lysates of IL-6 Tg (+) and Tg (-) mice exposed to normoxia and 3 weeks of hypoxia. β-actin used as a control.