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TNF Induces Obliterative Pulmonary Vascular Disease in a Novel Model of Connective Tissue Disease Associated Pulmonary Arterial Hypertension (CTD-PAH)

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Abstract

Objective: Connective tissue disease (CTD) associated pulmonary arterial hypertension (PAH) is the second most common etiology of PAH and carries a poor prognosis. Recently, it has been shown that female human TNF-transgenic (TNF-Tg) mice die by 6-months from cardiopulmonary disease. We aimed to characterize this pathophysiology and assess its potential as a novel model of CTD-PAH.

Methods: Histologic analysis was performed on TNF-Tg and wild type mice to characterize the pulmonary vascular and right ventricular pathology (n=40, 4–5 mice per group/time point). Mice underwent right heart catheterization (n=29) and micro-CT angiography (n=8) to assess vascular disease. Bone marrow chimeric mice (n=12), and anti-TNF vs. placebo treated mice (n=12) were assessed. RNA sequencing was performed on lung tissue (n=6).

Results: TNF-Tg mice display a pulmonary vasculopathy marked by collagen deposition (p<0.001) and vascular occlusion (p<0.001) with associated right ventricular hypertrophy (p<0.001) and severely increased right ventricular systolic pressures (RVSP = 75.1 ± 19.3 mmHg

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vs 26.7 ± 1.7 mmHg in WT animals, $p < 0.0001$). TNF-Tg mice had increased α SMA staining which corresponded to proliferation and loss of vWF⁺ endothelial cells ($p < 0.01$). There was an increase in α SMA⁺vWF⁺ cells ($p < 0.01$) implicating endothelial-mesenchymal transition. Bone marrow chimera experiments revealed that mesenchymal but not bone-marrow derived cells are necessary to drive this process. Treatment with anti-TNF therapy halted progression of disease. This pathology closely mimics human CTD-PAH, in which patient lungs demonstrate increased TNF signaling and significant similarities in genomic pathway dysregulation.

Conclusion: The TNF-Tg mouse represents a novel model of CTD-PAH, recapitulates key disease features, and can serve as a valuable tool for discovery and assessment of therapeutics.

Introduction

Pulmonary arterial hypertension (PAH) is a severe cardiopulmonary disease that is characterized by: 1) an obliterative vasculopathy and vascular remodeling affecting pulmonary arterioles, 2) progressive increase in pulmonary vascular resistance, 3) right ventricular hypertrophy and failure, and 4) premature death(1). The primary histopathological features of PAH include intima and media thickening, muscularization of distal pulmonary arteries, inflammatory cell infiltration, thrombosis, and vascular occlusion(2).

Amongst patients with PAH, the most common etiology is idiopathic PAH (IPAH), followed by connective tissue disease associated PAH (CTD-PAH)(3). Within CTD-PAH, systemic sclerosis is the most common form (75% of CTD-PAH and 12–15% of SSc patients), with lupus and mixed connective tissue disease also having increased incidence. In some published data sets, CTD-PAH patients, particularly those with SSc-PAH, fare worse than patients with pulmonary hypertension of other etiologies, and demonstrate poorer hemodynamic response to targeted PAH therapies(4, 5).

Although increased vascular tone is an important contributor to PAH physiology, emerging data suggests that PAH vascular remodeling is complex, and involves multiple cellular and molecular abnormalities including: 1) vascular fibrosis, 2) smooth muscle and fibroblast proliferation, 3) glycolytic cellular metabolism, and 4) abnormalities in apoptosis in pulmonary artery endothelial cells(6). In addition to these mesenchymal phenotypes, there is mounting evidence that immune dysregulation is prevalent in PAH, and that immune abnormalities may define unique subsets of PAH patients(7, 8). Despite improved understanding of the inflammatory and fibrotic features of SSc and other connective tissue diseases, the underlying molecular mechanisms responsible for development of CTD-PAH remain poorly understood. Moreover, progress has been hampered by a lack of human tissue biospecimens and suitable animal models that recapitulate the pathophysiology of severe CTD-PAH.

Our group has recently shown that transgenic mice expressing chronic low levels of human tumor necrosis factor (TNF-Tg, 3647 line(9, 10)) display interstitial lung disease (ILD)(11) with disease which is more severe in female mice(12). These mice die prematurely, and we were intrigued to find that in addition to ILD, these animals also demonstrated perivascular lung inflammation and right heart disease(12). To further define whether these findings

might be consistent with a murine model of PAH, here we extensively phenotype the pulmonary vascular and cardiac phenotype of these animals. Excitingly, TNF-Tg mice develop a severe progressive obliterative and fibrotic pulmonary vascular lesion which closely resembles that seen in CTD-PAH; the pathology is associated with loss of endothelial cells, and vascular smooth muscle proliferation. Moreover, unlike other mouse models of PAH, TNF-Tg mice have progressive right ventricular (RV) pressures that closely recapitulate human disease, with dramatic and progressive right heart pathology. Adoptive transfer studies reveal that these effects are modulated by mesenchymal, and not hematopoietic derived TNF, and anti-TNF treatment halts the pulmonary vascular phenotype. As a clinical correlate, we show that human CTD-PAH patients demonstrate increased TNF expression, and transcriptome studies reveal that TNF-Tg mice have a strong overlap in gene expression profiles with patients having CTD-PAH. Taken together, these findings indicate that the TNF-Tg mouse displays the requisite features of a faithful animal model of CTD-PAH.

Materials and Methods.

These experiments utilized a line of TNF-transgenic (TNF-Tg) mice (line 3647) originally obtained from Dr. George Kollias(9, 10, 13) carrying a modified human TNF transgene in which the 3'-region of the TNF gene was replaced with that of the human β -globin gene and littermate controls. We used female TNF-Tg mice for all in vivo experiments(12). Data indicating histologic progression of disease over time were analyzed from 4 to 5 mice per group at months 3, 4, and 5.5 (n = 20 mice)(12), and additional cohorts of mice analyzed at 1.5, 2.5, 4, 5, and 5.5 months (n=40) were used both as a replication/validation of this data and to perform further biochemical, imaging, and physiologic characterization of the cardiac and pulmonary phenotype in addition to mice that were used in bone marrow chimera (n=12) and anti-TNF treatment (n=12) studies. All mouse procedures were conducted in accordance with approved guidelines of the University of Rochester Committee for Animal Resources. Detailed descriptions of the methods used for histology, micro-computed tomography (micro-CT), right heart catheterization, immunostaining, bone marrow adoptive transfers, anti-TNF therapy, RNA sequencing, and statistical analyses are all presented in the Supplementary Materials and Methods. For human lung tissue, paraffin embedded lung biopsy sections were examined from five female patients with CTD-PAH (4 SSc-PAH and 1 Behcet's disease-associated PAH) and two healthy female control lungs obtained from the pathology department at the University of Rochester Medical Center. Samples were de-identified and were obtained as part of an IRB approved protocol at the University of Rochester.

Results

TNF-Tg female mice have progressive pulmonary vessel occlusion as they age, leading to pulmonary arterial hypertension (PAH)

Histopathology evaluation of TNF-Tg lungs demonstrated a substantial and progressive pulmonary vasculopathy from 1.5 months to 5.5 months of age (Figure 1A). At 3 months, extensive adventitial remodeling was seen followed later by muscularization with collagen

deposition and finally extensive fibrosis, cellular infiltration, and vascular occlusion. Histomorphometry of total vessel and medial perimeter showed a significant increase with doubling in size between 1.5 and 5.5 months (Figure 1B,C). Analysis of vessel muscularization revealed progressive decreases of non-muscularized vessels and increases in muscularized vessels, while vessel muscularization, vessel area and max length (diameter) also increased in the total vessel, the vessel/lumen ratio, and the tunica media, and the lumen size progressively decreased (Supplemental Figure 1). By 4 months of age, over 80% of pulmonary vessels were partially occluded, and 50% were completely occluded by 5.5 months (Figure 1D). While male mice do develop increased vessel size relative to controls, female mice have much more impressive pathology, and only females develop occlusive disease (Supplemental figure 3). As this sexual dimorphism reflects human CTD-PAH in which 90% of patients are female, female mice were used for all subsequent studies. Impressive vascular collagen deposition was observed and also increased with age (Figure 1A, lower panels, 1E). While the TNF-Tg mice do develop a cellular predominant interstitial lung disease as they age (12, 14, 15), the interstitial disease was not fibrotic (Supplemental Figure 4). These animals display impairment in gas exchange (DFCO 0.43 in TNF-Tg vs 0.65 in WT, $p < 0.001$), which inversely correlated with pulmonary arterial perimeter (Spearman's $Rho = -0.85$, $p < 0.05$) which is likely reflective of both ILD and PAH pathology (Supplemental figure 5).

Next, to identify the distribution of vessels involved in this process, we performed ex vivo imaging of barium perfused lungs by μ CT. This imaging demonstrated striking pruning of the pulmonary vascular tree in the TNF-Tg mice, with a significant reduction in the number of branches (Figure 1F–G). Quantification of vessels in the left lung revealed significant loss of small vessels (Figure 1H–I), which correlated with histologic vessel size (Figure 1J).

Right heart catheterization performed on female TNF-Tg mice demonstrated significantly elevated right ventricle systolic pressure (RVSP) with a mean of 75.1 ± 19.3 mmHg in 5 month old mice ($n=12$) and 44.5 ± 9.9 mmHg in 2.5 month old mice ($n=9$) vs. 25.7 ± 0.4 mmHg in 2.5 and 5 month old littermate control mice ($n=8$, $p < 0.001$) (Figure 2A–C). Hemodynamic assessments of maximum and minimum dP/dT , which are measures of cardiac contractility and diastolic function respectively, were also significantly altered (Figure 2D, Supplemental figure 6). Moreover, there was a strong correlation between RVSP and histologic vessel size (Figure 2E, Spearman's $Rho=0.96$, $p < 0.0001$).

To assess the effect of the pulmonary hypertension phenotype on the heart, we analyzed cardiac chambers, valves, and cell morphology. The right ventricle showed significant hypertrophy (Figure 2F), which increased over time. TNF-Tg RV were significantly enlarged by 4-months of age and more than doubled in size by 5.5-months (Figure 2G). We also assessed the left ventricle (Figure 2H and Supplemental Figure 7A), which did not demonstrate hypertrophy. Interestingly, while the left sided mitral and aortic valves demonstrated no abnormalities (Figure 3D now 2I, Supplementary Figure 7B), there was hypertrophy of the tricuspid valve (Figure 2F, bottom), and valve area was significantly increased compared to WT animals (Figure 2I, $p < 0.01$). Of note, this phenotype is in stark contrast to the TNF-Tg 197 line, which demonstrate hypertrophy of the left sided heart valves with no pathology of the tricuspid valve (16). Consistent with this observation, the

gross RV weight (Supplemental Figure 7C), and the Fulton index (ratio of mass of RV/LV +septum), were significantly increased in TNF-Tg mice (Figure 2J, $p<0.05$). RV thickening was further assessed by measuring myocyte area and interstitial collagen deposition all of which were elevated in the TNF-Tg mice (Supplemental figure 8A,B,D,E). To assess RV hypertrophy and dysfunction biochemically, qPCR of genes previously shown to correlate with RV failure in rats(17), was performed in right ventricles. TNF-Tg mice had significantly elevated RV gene expression levels of *B natriuretic peptide* (BNP) and *collagen 1A1*, and decreased levels of *angiopoietin-1* (Supplemental Figure 8C).

Vessels from other organs including the liver, kidney, and large vessels including the aorta were assessed for signs of systemic vasculopathy. We examined other organs including the kidney, liver, and aorta, and no organ other than the lung demonstrated a proliferative obliterative vasculopathy (Supplemental figure 9).

TNF-Tg lungs have decreased Vessel Number, Vascular Endothelial Loss, and Smooth Muscle Proliferation

To assess changes in pulmonary vessel numbers over time, TNF-Tg lungs were stained with von Willebrand Factor (vWF). Compared with WT animals, TNF-Tg mice had a marked decrease in numbers of vWF+ distal pulmonary arterioles at 3-months of age that decreased modestly over time afterward (Figure 3A–B). The most pronounced vWF+ vessel loss was seen amongst peripheral non-muscularized vessels, whereas centrally located and muscularized vessels largely remained stable over time.

To determine the activation and source of increased alpha-smooth muscle actin expressing (α SMA+) cells seen in TNF-Tg lungs, multicolor immunofluorescent histochemistry was performed to co-localize α SMA+ cells with either the proliferation marker Ki-67, or the vascular endothelial marker vWF. Increased proliferation was seen in TNF-Tg pulmonary vascular smooth muscle cells, as marked by co-localization of α SMA+ and Ki-67+ cells. A large number of α SMA+ smooth muscle cells expressed Ki-67, suggestive of increased proliferation, together with the growth of central vessels during aging (Fig 3C). In a small but significant number of muscularized arteries with evidence of vascular occlusion, α SMA+ cells co-localized with vWF+ cells, raising the possibility that these cells underwent endothelial-mesenchymal transition (Endo-MT). This process is known to occur in human and rat pulmonary hypertension(18), and the extent of this abnormality increased over time (Figure 3 D–E)(19).

Pulmonary vasculopathy is driven by stromal TNF production and is attenuated with anti-TNF therapy

To assess the role of stromal versus bone-marrow-derived cells, we performed bone marrow chimera experiments. TNF-Tg mice reconstituted with WT bone marrow displayed pulmonary artery (Figure 4A) and right ventricular (Figure 4B) phenotypes similar to TNF-Tg mice reconstituted with TNF-Tg bone marrow. In contrast, WT mice reconstituted with TNF-Tg bone marrow did not demonstrate lung pathology, and were similar to WT mice reconstituted with WT bone marrow. Quantification of PA vessel size (Figure 4C), vascular occlusion (Figure 4D), lumen/vessel ratio (Supplemental figure 2B), RV area (Figure 4E)

and RV myocyte area (Figure 4F) were all significantly increased in WT->TNF compared to the TNF->WT mice.

To determine if blockade of TNF could reverse this pathology, 3-month old-female TNF-Tg mice were treated with either anti-TNF monoclonal antibody CNTO12 or placebo for 6 weeks. While placebo treated animals developed progressive pulmonary arterial thickening, fibrosis and occlusion (Figure 4G, I, J; Supplemental Figure 2C), this process was halted in anti-TNF treated animals whose pulmonary arteries at 4.5 months resembled those of 3 month old animals from the longitudinal study (Figure 1A). Cardiac remodeling was prevented in the anti-TNF treated animals (Figure 4H, K, L).

Human CTD-PAH lungs show TNF- α protein expression and significant overlap in regulatory gene networks with TNF-Tg lungs

Human lung tissue from either healthy regions adjacent to tumor excision (n=2), or affected CTD-PAH (n=5), were assessed for α -SMA and TNF- α . Immunostaining demonstrated expression of TNF- α in perivascular infiltrates adjacent to the pulmonary arteries/arterioles from affected CTD-PAH lungs, while no significant expression of TNF- α was seen in control lung arterioles (Figure 5A, Supplemental Figure 10).

We next analyzed gene expression for the KEGG TNF- α pathway in mice using RNA-seq of WT and TNF-Tg lungs at 4-months of age, as well as publicly available data derived from human lung explants from a study of donor and recipient lungs collected at the time of lung transplantation with samples from healthy donor lungs and patients with PAH (GSE113439). Both TNF-Tg mice and human PAH patients demonstrated significant differential regulation of genes in the TNF pathway, which was sufficient to distinguish them from control individuals using unsupervised hierarchical clustering (Figure 5B), and using a condensed TNF pathway score (Figure 5C)(20).

Next, to determine what type of human lung disease the TNF-Tg mice most closely recapitulate at a molecular level, we assessed the 100 most differentially expressed genes between diseased and healthy lungs from 6 different clinical lung diseases derived from five different GEO data sets in the RNA-seq data from the TNF-Tg and WT mice. We found that the gene expression pattern of the TNF-Tg mice closely resembled the CTD-PAH and scleroderma associated lung disease (SSc-ILD), while they did not show significant similarity with idiopathic pulmonary hypertension or idiopathic pulmonary fibrosis (Fig 8A, green). To assess the specificity of our model compared to other PAH models, we performed the same analysis of using other models of PAH (rats treated with monocrotaline and mice treated with SU5416 and hypoxia) using GEO data and found that these models more had significant overlap with idiopathic pulmonary arterial hypertension (IPAH) but not CTD-PAH (Figure 6A).

To demonstrate the similarity between human CTD-PAH and murine TNF-Tg lung disease, we used the 100 most differentially regulated CTD-PAH genes to generate a heatmap (Figure 6B, left), which demonstrates the striking distinction between TNF-Tg and WT mice using this gene list. We also generated a reciprocal heatmap (Figure 6B, right), in which we

found that the top 100 most differentially expressed genes from the TNF-Tg mouse were also able to distinguish human CTD-PAH patients from controls.

To assess whether pathways that have been implicated in the pathogenesis of PAH are altered in TNF-Tg mice, we performed a literature search to identify pathways that have a high degree of evidence for their contribution to disease(2). We identified 27 literature-defined PAH molecular pathways, of which we were able to map 15 to defined KEGG pathways. We assessed all genes in each of these 15 pathways (Figure 6C) in the RNA-Seq data from TNF-Tg and WT mice, and calculated pathway scores and p-values for each. All 15 pathways were significantly dysregulated (Supplemental Figure 11), and 14 out of 15 remained significant ($p < 0.05$) after correction for multiple hypothesis testing.

Lastly, to utilize an unbiased approach to determine which pathways in TNF-Tg and CTD-PAH lungs were dysregulated, we performed pathway analysis of all differentially regulated genes ($p < 0.05$) in each species, and then examined overlapping genes and pathways using the PANTHER database (Figure 6D)(21). TNF-Tg lungs were found to have dysregulation of a number of pathways including integrin signaling, inflammation, angiogenesis, endothelin, apoptosis, VEGF, and B cell signaling. When we looked at overlapping genes, 2,656 common genes were differentially regulated between lungs in TNF-Tg mice and CTD-PAH patients (significantly more than would be expected by chance, $p < 0.0001$), and the pathway abnormalities represented by this group of genes included angiogenesis, Notch signaling, apoptosis, and integrin signaling, findings consistent with other models of pulmonary hypertension (22).

Discussion

We find that female TNF-transgenic mice develop a severe form of pulmonary hypertension which closely models human pulmonary arterial hypertension associated with connective tissue disease (CTD-PAH). These mice develop a progressive pulmonary arterial thickening, vascular fibrosis, and luminal occlusion which correlates with RV hypertrophy, myocyte hypertrophy, RV fibrosis, and finally results in death by 6 months of age(12). These mice have significantly elevated right ventricular pressures (RVSP mean = 48.4 ± 3.7 at 2.5 months of age and RVSP mean = 88.1 ± 10.4 mmHg at 4 months of age), which are among the highest pressures recorded in a murine model of pulmonary hypertension(23). This dysregulated lung phenotype is driven by loss of endothelial cells (vWF staining), proliferation of smooth muscle cells, and endothelial-mesenchymal transition; it is not significantly driven by bone marrow-derived cells. TNF- α is a pivotal effector in the vascular disease, as blocking it halts progression. Additionally, the TNF- α gene pathway and protein expression are increased in pulmonary vessels from patients with CTD-PAH. Gene expression profiling reveals significant similarities in the global lung transcriptome between TNF-Tg mice and patients with CTD-PAH, which provides content validity for this model.

The TNF-Tg 3467 mouse line, which harbors an insertion of a single copy of the human TNF-alpha transgene, has been described as a model of inflammatory-erosive arthritis for over twenty years(10). A “pulmonary vasculitis” was described in these mice in 2016(24),

and our group recently identified a sexually dimorphic lung pathology with functional and histologic features consistent with restrictive lung disease and right ventricular involvement(11, 12). Building on this work, the current study comprehensively assessed the cardiopulmonary phenotype in these mice and has now established this TNF-Tg line as a bona fide mouse model of CTD-PAH.

Previous reviews of animal models of pulmonary hypertension have identified four key features that would be required for an ideal animal model of clinical PAH: 1) markedly increased RVSP (>50 mmHg), 2) pulmonary artery lumen obliteration, 3) formation of proliferative, occlusive lesions, and 4) severe RV hypertrophy, RV chamber dilation, and RV failure(23, 25, 26). Female TNF-Tg mice exhibit all of these features, with clear elevations in RVSP and pulmonary arterial luminal obliteration, proliferative occlusive lesions (supplemental figure 12), and presence of RV hypertrophy and chamber dilation.

Some of the most important similarities between human CTD-PAH and murine TNF-Tg disease include the presence of autoimmunity(24), female sex predominance(12), severe progressive obliterative/fibrotic lesion, presence of ILD overlap, and the presence of other inflammatory systemic disease, particularly arthritis. The only other mouse model purported to represent a model of CTD-PAH is the Fra-2 transgenic mouse(27, 28). While this model does share many features with scleroderma, these animals have a severe lung fibrosis and inflammation(29) with associated pulmonary hypertension more akin to WHO group III pulmonary hypertension. Moreover, Fra-2 transgenic mice have a much lower RVSP elevation (RVSP ~30 mmHg)(30) than the TNF-Tg mice (RVSP 50–100 mmHg). Moreover, unlike other animal models (monocrotaline treated rats and the SU5416/hypoxia treated mice) which share gene expression profiles with idiopathic pulmonary arterial hypertension (IPAH) but not CTD-PAH, the TNF-Tg mice have genomic overlap with CTD-PAH and not IPAH (Figure 8a) suggesting the specificity of this model.

TNF is a pleiotropic cytokine with multiple downstream signaling pathways and cell-type specific effects(31). Thus, the mechanisms that underlie how constitutive systemic TNF over-expression drives pulmonary vascular disease is an enigma of great interest. Our bone marrow chimera experiments suggest that non-hematopoietic derived TNF promotes the PAH. While this finding was not unexpected, given that arthritis and other pathologies in TNF-Tg mice also requires a TNF-Tg stroma(32, 33), it raises the question of which stromal cells are critical in perpetrating the pathology. To better address this question, we performed staining of endothelial cells and smooth muscle cells to look for shifts in these two critical populations known to be central in the pathogenesis of PAH. We found that endothelial cell loss begins early in TNF-Tg lungs, which have few centrally located vessels that remain after 3 months. This process is likely driven specifically by TNF, which is well described to induce endothelial cell apoptosis in vitro(34). Beyond endothelial cell loss, however, we also found that cells expressing endothelial cell markers began to change morphology and express α SMA. The presence of double positive staining for both vWF and α SMA suggests that these endothelial cells undergo Endo-MT (19), and take on a more myofibroblastic phenotype. This process could explain the significant increase in collagen deposition and vascular fibrosis seen in the TNF-Tg vessels and has been previously seen in PAH patients and rat models(18, 19). The progressive increase in pulmonary vessel size seen over time in

these mice, however, cannot be explained solely by Endo-MT. In addition, compared to WT animals where there was rare co-expression of α SMA and Ki-67, TNF-Tg lungs display a progressive increase in smooth muscle cell proliferation (Figure 3D). These findings indicate that TNF drives opposing phenotypes (death and proliferation) in different cell types, which is consistent with previous studies looking at the effects of TNF in cultured cells(35–37).

TNF, through its receptors TNFR1 and TNFR2, signals through multiple different downstream pathways including the FADD/TRADD/caspase mediated apoptosis, RIP3K mediated necroptosis, p38/ERK, NF- κ B, Jak/STAT, MAP3K/Jnk, and PI3 kinase/Akt pathways(38). In addition to global TNF dysregulation (Figure 5B), we found that pathways involving apoptosis, PI3 kinase, and p38 were all substantially dysregulated in TNF-Tg mice and CTD-PAH lungs (Figure 6D) suggesting that increased TNF signaling alters these specific pathways and that they are likely related to the PAH phenotype. TNF's mesenchymal effects are frequently cell-type specific (36) and we speculate based on the degree of endothelial cell death and smooth muscle cell proliferation that are seen in the mouse, that increased TNF signaling through TNFR1 induces endothelial apoptosis, likely through the FADD/caspase pathway, while TNFR2 signaling in pericytes, smooth muscle cells, and/or fibroblasts may lead to activation of the p38 and PI3K/ERK pathways which may promote these cells' proliferation and induce vascular fibrosis and vessel occlusion.

Interestingly, previous work described TNF specifically over-expressed in epithelial cells (pneumocytes), by inducing expression with a surfactant (SpC) promoter in transgenic mice(39). While these mice did not spontaneously develop pulmonary hypertension, they were more susceptible to pulmonary hypertension (RVSP ~ 50 mmHg) when challenged with high altitude(39), but did not develop a proliferative vascular or an interstitial lung disease phenotype like that seen in the present study. The TNF-Tg 3467 line is a constitutive over-expression model which is not cell-type specific. Given this fact, we cannot localize the relative degree of cellular contributions to the PAH phenotype beyond the requirement for non-hematopoietic derived cells. In order to understand how TNF causes this pathology, cell-specific transgenic mice harboring this or similar TNF transgenes in relevant populations, such as endothelial cells, will need to be assessed.

The extent of cardiac disease, and particularly the presence of right ventricular dysfunction and absence of left ventricular pathology, is suggestive that the RV remodeling seen in the TNF-Tg mice is a pressure overload phenomenon. The presence of tricuspid valvular disease is particularly interesting in that other TNF-transgenic mouse models have recently shown to have mitral and aortic valvular disease(16, 40), but not tricuspid pathology. This suggests that elevated RV pressure and RV hypertrophy lead to tricuspid regurgitation, which then in turn leads to findings consistent with valvular leaflet hypertrophy.

We used four complementary bioinformatic approaches to demonstrate the specificity of the TNF-Tg mouse as a pre-clinical correlate to CTD-PAH. First, we compared the genomic profile of TNF-Tg mice and other established rodent models of PAH to a variety of human lung diseases. Intriguingly, while both the monocrotaline and Sugen/hypoxia PAH models showed genomic similarity only to idiopathic pulmonary arterial hypertension (IPAH), the TNF-Tg model showed similarity to CTD-PAH but not to IPAH thus demonstrating that the

TNF-Tg model has specificity to CTD related pathology. By performing a cross-species analysis using CTD-PAH differentially expressed genes (including *SPOCK2*, *ICAM2*, *PLOD2*, *VPS50*, *SUSD2*, *NEMF*, and *DLL4*), we were able to establish that these genes almost perfectly distinguished TNF-Tg mice from wild type animals (Figure 6B) suggesting a close similarity in pathophysiology between the human disease and the murine model. By evaluating gene profiles of TNF-Tg mice in literature-defined human PAH pathways, we were able to identify that 15/16 established PAH pathways were significantly altered in this model. The similarity across species was further confirmed by the dramatic overlap in differentially expressed transcripts between the TNF-Tg mouse and CTD-PAH with over 30% of TNF-Tg differentially expressed genes (DEGs) overlapping with human CTD-PAH DEGs. While many of the pathways shown in this analysis simply describe the overwhelming vascular phenotype (angiogenesis, VEGF, and Notch signaling), the over-representation of TNF signal transduction paths including apoptosis, PI3 kinase, and p38/MAPK pathways suggest that these signals downstream of TNF receptors may play a key role in CTD-PAH.

While the progressive and lethal course of the PAH phenotype in TNF-Tg 3647 mice provides face validation of human disease, to be applicable as a pre-clinical model for genetic and pharmacologic manipulation, the phenotype must be modifiable. Our experiments with anti-TNF treatment demonstrate pathologic resolution and yield additional important insights. In addition to validating previous studies which have shown that TNF inhibition can ameliorate ILD in these mice(41) and PAH in different species(42–44), our finding that anti-TNF treatment both halts the evolution of pulmonary vascular disease and prevents right ventricular remodeling even when started at 3 months of age demonstrates that this model represents a dynamic and modifiable phenotype which can likely be used both to show prevention and treatment of pulmonary hypertension under different conditions. This dramatic improvement also suggests that anti-TNF therapy may be valuable as a treatment in CTD-PAH especially given the high degree of overlap seen between gene expression in the TNF-Tg mice and CTD-PAH patient lungs. While there has been a small open label study looking at anti-TNF therapy for fibrotic manifestations of systemic sclerosis(45), there have not been human studies using these agents for PAH or vascular disease in connective tissue diseases.

There are limitations of this model which should be noted. For example, these animals have a severe inflammatory-erosive arthritis that is most reminiscent of rheumatoid arthritis (9, 24), but the PAH lung disease seen is more akin to that seen in scleroderma rather than RA, and yet the TNF-Tg mice do not have the fibrosis or generalized vasculopathy seen in scleroderma. Moreover, the highly abnormal immune phenotype seen in these animals is driven by over-expression of a single human cytokine (TNF), rather than the more complicated pathology underlying connective tissue diseases. This suggests that any immune abnormalities, or responses to immunomodulatory therapy seen in this model, need to be contextualized for more complex human diseases.

Another fascinating feature of this model is the sexual dimorphism in which disease is accelerated and more severe in females(12). This sex difference is consistent with human disease in which PAH is predominant in women(46); and CTD-PAH occurs with a 5–10:1

female:male ratio, and cohorts of CTD-PAH show close to a 10:1 female:male ratio(4, 46, 47). The sexual dimorphism in TNF-Tg mice is not isolated to the pulmonary vasculature(12), and the exact mechanism still remains unknown. It is likely driven at least in part by estrogen, which can be modulated by TNF(48), and TNF-Tg lungs have an altered estrogen signature (Supplemental Figure 11).

Conclusion

The TNF-Tg 3467 mouse line, an established model of inflammatory-erosive arthritis(9, 13), represents a valuable new murine model of PAH, and specifically CTD-PAH. Given the severity of hemodynamic PAH, reproducible obliterative pulmonary vascular lesion, and associated RV dysfunction, this murine model serves as an important tool to better understand the pathophysiology of CTD-PAH and assess novel PAH therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. TNF-Tg female mice develop a progressive obliterative pulmonary vasculopathy.

In representative H&E (A, upper) and Masson Trichrome (A, lower) images of pulmonary vessels adjacent to medium sized bronchi, a progressive vascular lesion is observed developing between 3 and 4 months of age, with severe occlusion at 5.5 months of age. Importantly, TNF-Tg vessels at 1.5 month of age are unaffected and look similar to WT vessels at 5.5 months. Quantification of these histologic features reveal consistent and progressive increase of the vessel perimeter and medial perimeter from 1.5 months to 5.5 months of age (B and C). When categorized by percent occluded, over 50% of the vessels are partially occluded by 3 months of age, and by 5.5 months over 50% are completely occluded (D). This is supported by the increasing percentage of vessel trichrome staining over time. Ex vivo μ CT of barium perfused WT and TNF-Tg lungs (F) revealed significant pruning of the small vessels with significantly less branches of their vascular tree (H), a 2.5 fold decrease in vessels <0.08 mm in diameter but not larger vessels (I) and a significant correlation between vessel number on μ CT and histology (G,J). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

Figure 2. TNF-Tg female mice have hemodynamic pulmonary arterial hypertension and histologic evidence of associated right ventricular pathology.

Right heart catheterization tracings revealed that when compared with age matched WT animals (A), TNF-Tg mice (B), had significantly increased RV systolic pressure (C) and dP/dT max (D); representative tracings from 4 month old mice shown. There was a significant, strong positive correlation between RVSP and vessel perimeter (E). Representative coronal H&E images of WT and TNF-Tg hearts demonstrates the enlarged right ventricular walls (F, upper, 5x) and, interestingly, an enlarged tricuspid valve (TV) indicative of regurgitation (F, lower, 20x). Histomorphometry demonstrates a progressively enlarging RV wall area (G) and an unchanged LV wall area (H) with a dramatically increased tricuspid valve area without changes in the mitral or aortic valves (I). Further, the Fulton index from freshly dissected hearts was increased in TNF-Tg mice (J). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

Figure 3. Immunostaining of TNF-Tg Lungs Demonstrates Loss of Endothelial Cells and Smooth Muscle Cell Proliferation.

Immunostaining for von Willebrand Factor (vWF) demonstrates loss of endothelial cells in TNF-Tg mice by 3 months of age (A and B). Co-staining of α-SMA and proliferation marker Ki-67 shows a progressive increase in co-staining indicative of smooth muscle cell proliferation which is associated with the increased vessel size and αSMA positivity (C). Co-staining of αSMA and vWF show a progressively increasing number of TNF-Tg vessels over time have evidence of double-positive vWF+αSMA+ cells, suggesting endothelial-mesenchymal transition (EndoMT) (D and E). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Figure 5. Human CTD-PAH patient lungs express increased TNF.

Compared to healthy lung (left), CTD-PAH patients display a proliferative vasculopathy similar to that seen in TNF-Tg mice (A, top, H&E, 20x). Immunofluorescent staining for α SMA (A, green, 2nd row) and TNF (A, red, 3rd row) demonstrate that pulmonary vessels from CTD-PAH patients have proliferation of α -SMA positive cells and that these vessels are surrounded by a TNF positive cellular infiltrate (A, bottom, merge). Gene expression analysis using RNA-sequencing from WT and TNF-Tg mice (n=3) and human lungs from either controls (n=11) or PAH (n=15) demonstrate significant differences in expression of genes from the KEGG TNF pathway which were able to differentiate healthy from disease in both human and mouse using unsupervised hierarchical clustering (B). When these expression differences were condensed into a pathway score, this was significant in both species (C). ****p<0.0001

Gene expression profiles derived from 6 different human lung diseases were queried in TNF-Tg mice (green), monocrotaline (MCT) treated rats (blue), and SU5416 and hypoxia (SU5416/Hyp) treated mice (red). TNF-Tg mice had significant similarity to CTD-PAH and scleroderma lung disease and less similarity with other diseases including idiopathic pulmonary hypertension and idiopathic pulmonary fibrosis while MCT rats and SU/5416 mice had similarity to IPAH but not CTD-PAH (A). Analysis of the top 100 DEGs from human CTD-PAH was able to distinguish TNF-Tg from WT and the top 100 TNF-Tg DEGs was able to distinguish 4 CTD-PAH patients and one control from the other 10 control lungs using unsupervised hierarchical cluster analysis(B). 15 KEGG pathways identified as relevant to PAH from the literature were queried in TNF-Tg lungs and all demonstrated alterations in TNF-Tg vs WT mice (C). All differentially expressed genes ($p < 0.05$) were assessed for pathway enrichment using the PANTHER database (D). TNF-Tg mice demonstrated abnormalities in integrin, chemokine, angiogenesis, endothelin, apoptosis, VEGF, and B-cell activation signaling (D, upper left) while CTD-PAH patients had abnormalities in angiogenesis, purine biosynthesis, Notch, and MAPK signaling (D, upper right). Over one third of DEGs ($n=2656$) were shared across species (D, middle, $p < 0.0001$ compared to random chance). Differentially expressed pathways in the overlap set include known PAH pathways including angiogenesis, Notch, apoptosis, PI3 kinase, and VEGF signaling.