Evidence for the Involvement of Type I Interferon in Pulmonary Arterial Hypertension

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ABSTRACT

Rationale: Evidence is increasing of a link between interferon (IFN) and pulmonary arterial hypertension (PAH). Conditions with chronically elevated endogenous IFNs such as systemic sclerosis (SSc) are strongly associated with PAH. Furthermore, therapeutic use of type I IFN is associated with PAH. This was recognised at the 2013 World Symposium on Pulmonary Hypertension where the urgent need for research into this was highlighted.

Objective: To explore the role of type I IFN in PAH.

Methods and Results: Cells were cultured using standard approaches. Cytokines were measured by ELISA. Gene and protein expression were measured using RT-PCR, Western blotting and immunohistochemistry. The role of type I IFN in PAH in vivo was determined using type I IFN receptor knock out (IFNAR1−/−) mice. Human lung cells responded to types I and II but not III IFN correlating with relevant receptor expression. Type I, II and III IFN levels were elevated in serum of SSc-PAH patients. Serum IP10 and ET-1 were raised and strongly correlated together. IP10 correlated positively with pulmonary haemodynamics and serum brain natriuretic peptide and negatively with 6-minute walk test and cardiac index. Endothelial cells grown out of the blood of PAH patients were more sensitive to the effects of type I IFN than cells from healthy donors. PAH lung demonstrated increased IFNAR1 protein levels. IFNAR1−/− mice were protected from the effects of hypoxia on the right heart, vascular remodelling and raised serum ET-1 levels.

Conclusion: These data indicate that type I IFN, via an action of IFNAR1, mediates PAH.

Keywords: Pulmonary arterial hypertension, interferon, endothelin-1, IP10, inflammation, immunology

Nonstandard Abbreviations and Acronyms:

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<tr>
<th>Abbreviation</th>
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<tr>
<td>PAH</td>
<td>Pulmonary arterial hypertension</td>
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<td>SSc</td>
<td>Systemic sclerosis</td>
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<td>SSc-PAH</td>
<td>Pulmonary arterial hypertension associated with systemic sclerosis</td>
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<td>IFN</td>
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<td>ET-1</td>
<td>Endothelin-1</td>
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<td>IP10</td>
<td>Interferon gamma inducible protein 10</td>
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<td>DLCO</td>
<td>Diffusion capacity of lung for carbon monoxide</td>
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<td>HPASMCs</td>
<td>Human pulmonary artery smooth muscle cells</td>
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<td>HLFs</td>
<td>Human lung fibroblasts</td>
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<td>HMVECs</td>
<td>Human lung microvascular endothelial cells</td>
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<td>Human epithelial lung carcinoma cell line</td>
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<td>Beas2B</td>
<td>Bronchial epithelial cell line</td>
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<td>TT1</td>
<td>Type I alveolar epithelial cell line</td>
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<tr>
<td>BOECs</td>
<td>Blood outgrowth endothelial cells</td>
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<td>PVR</td>
<td>Pulmonary vascular resistance</td>
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<td>RVSP</td>
<td>Right ventricular systolic pressure</td>
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<td>RVH</td>
<td>Right ventricular hypertrophy</td>
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<td>mPAP</td>
<td>mean pulmonary artery pressure</td>
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<td>BNP</td>
<td>Brain natriuretic peptide</td>
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<td>RV/BW</td>
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INTRODUCTION

Pulmonary arterial hypertension (PAH) is a rare but devastating disease, which is defined as a mean pulmonary artery pressure of 25mmHg or greater with a normal pulmonary capillary wedge pressure. It is characterised by remodelling of the muscular, pre-capillary vessels, leading to an increase in pulmonary vascular resistance. The associated strain exerted upon the right heart ultimately results in right heart failure and premature death.\(^1,2\)

Autoimmunity has long been implicated in PAH\(^3\) and most recently evidence has emerged implicating interferon (IFN)\(^4\). IFN is central to the innate immune response to viral infection and three types have been identified; type I IFN (IFN\(\alpha\) and IFN\(\beta\)) which signals through a heterodimeric receptor consisting of IFNAR1 and IFNAR2, type II IFN (IFN\(\gamma\)) which signals through IFNGR1 and IFNGR2 and type III IFN (IFN\(\lambda\)) the receptor for which comprises IL10RB and IL28RA.

There is growing evidence that clinically proven PAH can be precipitated with type I IFN therapy\(^5-8\). Furthermore, in as many as 48% of patients receiving IFN\(\alpha\) treatment, lung function (diffusion capacity of lung for carbon monoxide (DLCO)) is reduced by at least 15%\(^9\), which may be due to undiagnosed pulmonary vascular pathology\(^10\). This is particularly relevant when considering that endothelin (ET)-1, a key mediator in the pathogenesis of PAH, is elevated in a sub-population of patients receiving IFN\(\alpha\) therapy for hepatitis C viral infection\(^11\) and that our group has pioneered the idea that ET-1 is an IFN inducible gene in vascular smooth muscle cells\(^12,13\). Concern surrounding IFN and PAH has reached such a level that at the recent World Symposium on Pulmonary Hypertension (Nice, February 2013), type I IFN was added to the list of drugs that may be associated with PAH.

It is well appreciated that patients with HIV, where IFNs are chronically elevated, have an increased incidence of PAH\(^14\). However, arguably the most compelling clinical case for endogenously produced IFN and PAH may be that associated with systemic sclerosis (SSc). SSc is a chronic autoimmune disease that affects up to 286 people per 1 million population\(^15\) and is associated with pulmonary vascular pathology in as many as half of patients\(^16\) manifesting as diagnosed PAH in 12-15%\(^17\). The mechanisms underpinning SSc associated PAH are still unclear. Patients with SSc have high levels of IFN\(^18,19\) and endothelin-1 (ET-1)\(^20\) but any link between IFN in SSc and PAH has not been fully addressed. Importantly, IFN induces a specific signature of genes, one of the most responsive being interferon gamma-induced protein 10 (IP10; CXCL10)\(^21\). IP10 is increasingly recognised as a potential mediator of inflammation, including that associated with the lung\(^22,23\).

The mounting evidence base for a link between IFN and PAH has led us to hypothesize that ‘activation of IFN pathways is central to the pathobiology of PAH and, as such, could represent an important contributing factor to SSc associated disease’. We have used in vitro and in vivo experimental techniques as well as clinical samples from patients with SSc-PAH to address this hypothesis.

METHODS

In vitro cell culture.

All cells were serum deprived for 24 hours and subsequently treated with IFNs in the presence and absence of TNF\(\alpha\) for 24 hours before supernatant was removed for analysis. IFN\(\alpha\) and IFN\(\gamma\) were used at 10ng/ml or 30ng/ml while IFN\(\lambda\) was used at 1000ng/ml to reflect its 100 fold lower specific activity. TNF\(\alpha\) was used at 10ng/ml. Cell culture supernatant levels of ET-1 and IP10 were measured by ELISA.
Isolation of blood outgrowth endothelial cells (BOECs).
Blood was collected from patients with PAH and healthy controls and BOECs were isolated as per previously published protocols 24, 25. BOECs were treated with IFNα for 24 hours and supernatant levels of IP10 and ET-1 were measured by ELISA. The number of colony forming units that emerged from PBMC cultures within 3 days was counted and proliferation of BOECs was measured over 24 hours using the alamarBlue® method.

Quantitative reverse transcriptase-PCR.
qRT-PCR for the type I IFN receptor (IFNAR1 and IFNAR2), the type II IFN receptor (IFNGR1 and IFNGR2) and the type III IFN receptor (IL10RB and IL28RA) was performed using TaqMan® gene expression assays.

Lung tissue immunohistochemistry.
IFNAR1, IFNGR1 and IL28RA expression was studied using immunohistochemistry performed on lung tissue from patients with SSc-PAH, idiopathic (I)PAH and healthy controls.

Western blotting.
IFNAR1 expression in the lungs of patients with SSc-PAH, IPAH and healthy controls was studied by western blotting and Image-J Software was used to quantify the level of protein expression.

Chronic hypoxia-induced mouse model of pulmonary hypertension.
All studies were conducted in accordance with UK Home Office Animals (Scientific Procedures) Act 1986 and institutional guidelines. Male C57Bl/6J and IFNAR1–/– mice (all eight to ten weeks old; ≈20g) were housed in normal air or placed in a normobaric hypoxic chamber (FiO2 10%) for 14 days (n=8-15/group). Development of pulmonary hypertension was confirmed as previously described 26.

Mouse model of acute lipopolysaccharide (LPS) induced ET-1 release.
Male C57Bl/6J and IFNAR1–/– mice (eight to ten weeks old weighing ≈20g) were intraperitoneally injected with 8μl/g of either vehicle control (0.9% sterile saline) or LPS from Escherichia Coli 055:B5 (1.25mg/ml). At 4 hours, mice were humanely sacrificed. Blood was taken, serum obtained and IP10, ET-1, IFNs and KC levels were measured by ELISA.

Human and mouse tissue organ culture.
Segments of whole human pulmonary artery were freshly harvested from patients undergoing pulmonary resection. Pulmonary arteries were treated with IFNs for 24hrs before supernatants were removed and IP10 measured by ELISA.

Mouse aorta and lung tissue were obtained from C57Bl/6J mice after being humanely sacrificed. Segments of vessel or lung were treated with recombinant human IFNα2b, mouse IFNα A or human pegylated IFNα2b for 24 hours. Supernatant levels of IP10 were measured by ELISA.

Clinical samples.
63 patients with SSc (28 patients with SSc-PAH and 35 SSc patients without PAH) were recruited from 2 specialist centres – The Royal Free Hospital (RFH), London and Papworth Hospital Research Tissue Bank. Healthy controls were recruited from RFH and The Royal Brompton Hospital, London. Serum levels of ET-1, IP10, IFNs and related cytokines were measured by ELISA. Clinical data from each patient were collected. Patients with lung disease or left heart disease were excluded.

Statistical analysis.
Data is presented as mean ± the standard error of the mean (SEM). For all data the Kolmogorov-Smirnov test of normality was applied. All normally distributed data was analysed by one-way ANOVA followed

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RESULTS

Effect of IFNs on IP10 and ET-1 release by human lung cells in culture.

As we have shown previously \(^{27}\) type I IFN\(\alpha\) and type II IFN\(\gamma\) induced IP10 and ET-1 release from TNF\(\alpha\)-primed human pulmonary artery smooth muscle cells (HPASMCs) (Fig. 1A & B). Again, as we have seen before, type III IFN\(\lambda\) did not induce appreciable levels of IP10 or ET-1 release from HPASMCs. IP10 release in response to type I and type II IFNs was also seen in human lung microvascular endothelial cells (HMVECs) and in human lung fibroblasts (HLFs) (Fig. 1C & E). ET-1 release by HMVECs was predictably high \(^{28}\) and not increased by treatment with any of the IFNs studied (Fig. 1D). ET-1 release by HLFs was relatively low, and similar to that seen from HPASMCs, but unlike vascular smooth muscle, was not increased by IFN treatment (Fig. 1F). Based on full concentration response experiments (Online Figs. I-III), for type I and type II IFNs, threshold concentrations were in the low ng/ml range whilst type III IFN\(\lambda\) remained inactive at concentrations up to 1000ng/ml. Although TNF\(\alpha\) co-administration accentuated IFN-induced responses, it is not an absolute prerequisite for IFN sensing in some cells \(^{27}\), particularly with regard to the current study for IFN\(\alpha\) or IFN\(\gamma\) induced ET-1 release by HPASMCs (Online Figs. I B & D). As predicted by the strong cellular response to type I and II IFNs, HPASMCs, HMVECs and HLFs expressed both receptor subtypes for IFNAR and IFNGR (Online Fig. IV). By contrast, IFN\(\lambda\) was inactive in all three lung cell types tested. Using human hepatocytes, which respond well to IFN\(\lambda\) (Online Fig. V) as a reference, we found that whilst human lung cells expressed comparable levels of IL-10RB, they expressed very low levels of IL28RA (Fig. 1G & H).

Levels of IFN, IP10 and ET-1 in serum of patients with SSc with or without PAH.

Five (18\%) patients in the SSc-PAH group had ‘detectable’ levels of IFN\(\alpha\) whereas only 2 (5.7\%) had detectable levels in the SSc without PAH group. IFN\(\alpha\) was below the level of detection in serum samples of all control subjects. Similarly 6 (21\%) patients with SSc-PAH had detectable levels of IFN\(\gamma\) whereas this was the case in only 2 (5.7\%) patients with SSc and no PAH. IFN\(\gamma\) was not detectable in any controls. There was a clear but non-significant trend to increased levels of IFN\(\alpha\) in serum of SSc-PAH patients compared to those without PAH or controls (Fig. 2A-D). In line with these data we found that levels of our two key IFN stimulated gene products, IP10 and ET-1 were significantly increased in the serum of SSc-PAH patients as compared to controls and to patients with SSc without PAH (Fig. 2E & F). Patients in whom serum levels of one or more of the IFNs measured were detectable, so called “IFN positive patients” had significantly raised levels of serum brain natriuretic peptide (BNP) as compared to “IFN negative patients” (Fig. 3A). We hypothesised that levels of IP10 and ET-1 were linked since there was a strong correlation between them in the serum of SSc-PAH patients (r=0.44, p=0.02) (Fig 3B). In this patient group, there were positive correlations between IP10 and pulmonary vascular resistance (PVR) (r=0.44, p=0.02) (Fig. 4A), IP10 and mean pulmonary artery pressure (mPAP) (r=0.34, p=0.04) (Fig. 4B) and IP10 and serum levels of BNP (r=0.59, p=0.0049) (Fig. 4C) and strong negative correlations between IP10 and cardiac index (r=−0.61, p=0.0001) (Fig. 4D) and IP10 and 6 minute walk test (6MWT) (r=−0.33, p=0.047) (Fig. 4E). Importantly, there was no relationship between serum levels of IP10 and ET-1 in SSc patients without PAH suggesting that this axis has specific relevance to SSc-PAH (Online Fig. VI). As has previously been demonstrated in patients with IPAH \(^{29}\) and SSc-PAH \(^{30}\), we found increased levels of proinflammatory cytokines, such as IL-1\(\alpha\) and IL-8 and statistically significant
increases in levels of IL-6, IL-12p70 and TNFα (p<0.05), in the serum of SSc-PAH patients (Online Fig. VII).

**Immunohistochemistry, western blotting and response of blood outgrowth endothelial cells to IFNa.**

The biological consequences of increased IFN are not only governed by tissue and plasma concentrations, but also by the level of receptor expression. In order to explore further the relevance that IFNa may have in SSc-PAH, we looked at type I IFN receptor expression in the pulmonary vasculature of patients with SSc-PAH and compared it to expression in lung sections from control patients without PAH or SSc. We found a greatly increased level of IFNAR1 staining in the lung sections of patients with SSc-PAH (Fig. 5A & B) as compared to controls (Fig. 5G & H). There was increased IFNAR1 expression within the remodelled pulmonary arteries of lungs from SSc-PAH patients. This increased staining was most abundant within the smooth muscle layer, endothelium and vascular interstitium as well as within inflammatory intravascular cells. Interestingly this was also the case in patients with IPAH (Fig. 5D & E), which led us to consider that type I IFN may play a pathogenic role in other forms of PAH and not exclusively those associated with SSc.

To allow direct quantification of protein expression, we performed Western Blot experiments from total lung homogenate of patients with IPAH, SSc-PAH and controls. We found that IFNAR1 expression was significantly raised in all IPAH patients tested and in some but not all SSc-PAH patients (Fig. 5J & K). It is known that all patients with SSc-PAH display histological features of pulmonary arterial inflammation with varying degrees of pulmonary venous occlusive disease and that those patients with a greater propensity to venous disease are less responsive to specific PAH therapies 31, 32. Our interesting observation that there appeared to be a heterogeneity of IFNAR1 expression within SSc-PAH patients led us to hypothesise that those patients with high IFNAR1 gross lung levels might be those that morphologically resemble an IPAH pattern of arterial involvement as opposed to pulmonary venous disease. To some extent, all six of the SSc-PAH patients examined displayed important venous involvement. However, of these, the three patients with high gross lung IFNAR1 expression displayed profound arterial inflammation and concentric laminar fibrosis of the intima – both features consistent with true PAH and histomorphologically closest to IPAH (Fig. 5C & F). Patients with low IFNAR1 expression in total lung homogenates had a predominance of venous involvement with fibrotic occlusive remodelling and surrounding alveolar septa with capillary hemangiomatosis-like appearance; features frequently encountered in SSc-PAH (Fig. 5I).

As levels of IFNg and IFNλ were also raised in the serum of SSc-PAH patients, we performed further immunohistochemistry experiments for IFNGR1 and IL28RA staining. There was an increase in IFNGR1 staining in the pulmonary vasculature of SSc-PAH patients compared to controls (Online Fig. VIII A-C) and although far less profound, it followed similar morphological patterns to that of IFNAR1. Pulmonary vascular IL28RA staining was also increased in SSc-PAH patients but the pattern of receptor expression differed with staining predominantly seen within the epithelial cell layer (Online Fig. VIII D-F).

The finding that the type I IFN receptor is upregulated in the lungs of PAH patients is consistent with our hypothesis that endogenous type I IFNs may have a role in driving PAH and is highly relevant to our understanding of how therapeutic IFN preparations may be associated with pulmonary toxicity. In line with increased staining of IFNAR1 in the vasculature, we found that endothelial cells grown from blood of a heterogeneous group of patients with PAH (one IPAH, one connective tissue disease-PAH, one congenital heart disease-PAH, one sarcoid-PAH) were more sensitive to stimulation with IFNα even in the absence of TNFα (Fig. 6). As with other cell types used in this study, TNFα co-administration enhanced the sensitivity of BOECs to IFNα in all cells and this response was exaggerated in cells from PAH patients (Online Table I). Interestingly, there was a trend for BOECs from PAH patients to release higher levels of ET-1 than those from healthy individuals under basal conditions and in response to IFNα.
Online Fig. IX A & B). We went onto assess the angiogenic activity of these cells and found that whilst there was a trend to an increased number of colony forming units of BOECs grown from the blood of PAH patients as compared to controls this did not reach statistical significance and over a range of seeding densities, no difference in proliferation of these cells was noted (Online Fig. X A & B).

In vivo experiments and animal model of PAH.

While consistent with our hypothesis, the above data is observational and does not provide definitive proof for a direct association between IFN and PAH. As previously discussed, the type I IFN receptor consists of a heterodimeric IFNAR1-IFNAR2 complex. It has recently been demonstrated that these two subunits have different functions and that IFNAR1 (and not IFNAR2) via an IFNAR1-IFN complex can independently transduce pro-inflammatory signals under the control of type I IFN 33. Thus, in order to directly explore the effect of type I IFN signalling on the development of PAH, we investigated the response to chronic hypoxia of mice lacking functional IFNAR1 (IFNAR1−/−). Under hypoxic conditions, wild type (C57Bl/6J) mice developed elevations in RVSP (Fig. 7A) and an increase in the percentage of muscularized pulmonary vessels (Fig. 7B). Along with pulmonary vascular changes, C57Bl/6J mice exposed to hypoxia developed right ventricular hypertrophy (RVH) (Fig. 7C) and had higher circulating levels of ET-1 in their serum (Fig. 7D). In line with our hypothesis, IFNAR1−/− mice were protected from the effects of hypoxia with a significant reduction in RVSP, percentage of muscularized vessels and RV/BW ratio (Fig. 7A-C). Furthermore, consistent with the development of PAH, hypoxia induced raised serum levels of ET-1 in C57Bl/6J mice but not in IFNAR1−/− mice (Fig. 7D). Serum IP10, IFNα and IFNγ levels were undetectable in all mice. Serum IFNλ levels could be measured but were not influenced by the development of PAH (Online Fig. XI). There was no significant difference in body weight between the C57Bl/6J (27.6 ± 2.2 grams) and IFNAR1−/− mice (25.6 ± 2.7 grams) and IFNAR1−/− mice were also protected from developing RVH when calculated using Fulton’s index (RV/(LV+septum), (Online Fig. XII A). Representative images of pulmonary arteries from a C57Bl/6J mouse and an IFNAR1−/− mouse housed in hypoxia are shown (Online Figs. XII B&C respectively).

Importantly, under control conditions, IFNAR1−/− mice have previously been well characterised and are known to have similar cardiovascular physiology, systemic blood pressure and ventricular function to C57Bl/6J mice 34.

These data confirm our earlier findings implicating IFNs in the development of PAH and more specifically highlight a potential pathological role for type I IFN signalling in PAH which may be mediated by ET-1. In order to further explore the underlying mechanisms we attempted to create a model of PAH secondary to exogenous IFN administration. Given the short half-life of IFN (approximately 8 hours), for a 14-day chronic hypoxia study it would be necessary to use pegylated IFN. As pegylated forms of mouse IFN are not commercially available, in the first instance we undertook experiments using pegylated human IFNα and compared responses with authentic (unpegylated) human IFNα and with mouse IFNα. We found that human IFNα (both pegylated and native forms) are inactive in mouse tissue as compared to mouse IFN (Online Fig. XIII A & B). Consequently, we elected to utilise a different approach in which to provide further mechanistic evidence for the relevance of IFN in the generation of ET-1 and hence PAH. Systemic levels of ET-1 are increased experimentally in two animal models; (i) hypoxia driven PAH (demonstrated above) and (ii) LPS induced endotoxemia 35. LPS, via the pattern recognition TLR4 activates two adapter proteins which give rise to separate groups of genes. TLR4 activates TRIF, which is associated with expression of IFNs and IFN related genes, such as IP10. TLR4 also activates MyD88, which activates NFκB regulated genes including KC (the mouse homologue of IL-8 / CXCL8). In C57Bl/6J mice, LPS induced systemic release of IFNα, IFNγ, ET-1, IP10 and KC but not IFNλ (Fig. 8 A-F). Importantly, in IFNAR1−/− mice, LPS-induced release of IFNα, IFNγ, ET-1 and IP10 was significantly abrogated. By contrast, levels of KC induced by LPS were similar in both C57BL/6J and IFNAR1−/− mice (Fig. 8 A-F). This provides further mechanistic evidence that ET-1 production is mediated by type I IFN via the type I IFN receptor IFNAR1, but is independent of IFNλ.
Further study of in vitro roles of types I, II and III IFN.

We have demonstrated that pulmonary vascular cells respond strongly to types I and II IFN to release IP10 and ET-1 and that serum levels of these IFNs as well as IP10 and ET-1 are raised in patients with SSc-PAH. It was interesting therefore to observe that IFNλ was inactive in cells due to restricted receptor expression but raised in the serum of this patient group and we consequently investigated this phenomenon further. It is well established that type I IFN strongly induces IFNλ. Whilst this raises the possibility that IFNλ may represent a marker of type I IFN signalling, we wished to explore further its potential to play a pathological role in its own right. Having found IFNλ to retain activity only in hepatocyte cells in culture, we wished to exclude the possibility that primary cells in culture might display an altered phenotype and so assessed the activity of IFNs –α, –γ and –λ on segments of freshly harvested human pulmonary artery. In direct corroboration of our data with cultured pulmonary vascular cells, we found that only IFNs –α and –γ induced IP10 release from whole vessel and that IFNλ was inactive (Online Fig. XIV). Using immunohistochemistry, we then looked for the presence of the specific IFNλ receptor, IL28RA within the pulmonary vasculature of SSc-PAH and found that there was increased receptor expression predominantly within the lung epithelium (Online Fig. VIII D&E) which reflects what is known of the IFNλ receptor in that it is expressed solely on epithelial surfaces and hepatocytes. Based on these findings, we assessed for the presence of the two IFNλ receptor subunits in the human lung epithelial A549 cell line and found that while the IL10RB subunit appeared to be widely expressed, the IL28RA subunit was only expressed on hepatocytes and A549 cells (Online Fig. XV A&B). Interestingly we found that whilst these cells express relatively high levels of both IFNλ receptor subunits, they did not respond to IFNλ but responded strongly to IFNα and IFNγ releasing IP10 (Online Fig. XVI) and ET-1 (Online Fig. XVII) in the presence of TNFα. This was in contrast to hepatocytes which did respond to IFNλ to release IP10 (Online Fig. V A). We repeated these experiments in two further epithelial cell types and as with A549 cells, neither the TT1 cell line, an immortalised human type I alveolar epithelial cell line (Online Fig. XVIII) or the Beas2B cell line, a bronchial epithelial cell line (Online Fig. XIX) responded to IFNλ in the presence or absence of TNFα.

DISCUSSION

In the current study we have systematically addressed the role of IFN in pulmonary arterial hypertension. In order to demonstrate the sensing of IFNs by cells in vitro, the pathological relevance of the IFN system in patients with PAH and the underlying IFN pathways at play in an in vivo model of PAH, we have extensively explored the roles of IP10 and ET-1. Previous work from our group has shown that ET-1 is an IFN gene and it is well established that ET-1 is a critical mediator and therapeutic target in PAH. Furthermore, IP10 is one of the best characterised of all the IFN stimulated genes. It is the cognate ligand of CXCR3 and is an antiviral and chemoattractant that promotes the formation of lymphoid infiltrates commonly seen in viral infection and autoimmune disease by attracting T lymphocytes, monocytes and natural killer cells. IP10 which is secreted by endothelial cells (amongst other cell types) also potentiates the adhesion of T lymphocytes to the endothelium, promotes the migration of CXCR3+ cells to the lung and has been shown to play a pathogenic role in the development of interstitial lung disease. Indeed CXCR3 expression at both the gene and protein level has previously been demonstrated to be upregulated in patients with PAH and IP10 has been shown to mediate endothelial dysfunction by disrupting calcium homeostasis.

Our data suggest that human lung cells sense type I and type II IFNs readily, but are insensitive to type III IFN, and that this is explained by relative IFN receptor expression across these cell types. Taken together with our in vivo data, we suggest that while IFNλ is raised in the serum of SSc-PAH patients, it does not play a pathological role. Elevated systemic type I IFN induces the expression of many IFN stimulated genes including IFNλ. Some, such as ET-1 and potentially IP10, play an important role...
immunopathological role in PAH. However, many other IFN stimulated genes such as IFNλ are not involved in the disease process and we suggest that their upregulation may be a reflection of type I IFN activity driven by an underlying dysregulated innate immune system. Whilst it is not the subject of this study, our finding that by contrast to human hepatocytes, human lung cells are insensitive to IFNλ, is interesting and potentially clinically relevant since pegylated IFNλ is currently in Phase III clinical trials for the treatment of chronic Hepatitis C Virus. It is tempting to speculate that these drugs may spare the lungs thereby displaying an improved side effect profile as compared to existing IFNα preparations.

The data we present in this paper are consistent with the large body of work that now recognises the importance of inflammation in PAH. As others have previously demonstrated, the concentrations of IFN required to activate cells in vitro are much higher than levels detected in serum of patients with PAH. It is well established that serum IFN levels may not accurately represent cytokine activity at the tissue level. The half-life of IFNα is between 10 and 20 minutes and very low levels are found in the circulation as a result of dilution in bodily fluids, diffusion from plasma to extracellular fluid compartments, binding to cell surface receptors and rapid catabolism by the kidneys, liver, muscle and lungs. This means that only a small amount of IFNα is in steady state in the circulation. Detectable circulating levels reflect increased tissue activity and so serum levels can only provide a representative and relative readout of the dynamic on-going homeostatic balance between production and excretion.

The ability for extra-pulmonary vascular cells to sense IFNs is as yet not fully characterised. We know that endothelial cells and vascular smooth muscle cells from systemic vessels also sense IFNs and while these observations illustrate that IFNs are not selective for pulmonary vascular cells in ‘healthy’ individuals, it is known that vessels that are primed for proliferation and remodelling (such as is the case in PAH), will respond far more profoundly to inflammatory stimuli. Furthermore, the pulmonary vasculature is a low-pressure system and so small changes to vascular resistance carry far greater clinical significance than they would do systemically.

In this study, we found clear evidence that IFN, IFNAR1 and downstream mediators are increased in PAH and correlate strongly with disease severity and established biomarkers of disease. In line with these observations, we also found that endothelial cells grown from the blood (BOECs) of patients with PAH have an increased sensitivity to exogenous IFNα than cells from control donors. It was interesting to note that IPAH patients and SSc-PAH patients with a histological predominance of arterial disease express far higher amounts of lung IFNAR1 than SSc-PAH patients with a greater burden of venous disease and healthy controls. By discovering this heterogeneity of IFNAR1 protein expression, we may have identified specific groups of patients with PAH where targeting type I IFN in the form of novel therapies might be most successful. Whether or not raised pulmonary vascular IFNAR1 expression is mirrored by serum evidence of IFN activation now requires further investigation. If that proves to be the case, serum IP10, for example, could represent a circulating marker of arterial pathology in SSc-PAH and therefore be a predictor of increased likelihood of response to specific PAH therapies.

In order to demonstrate a causal relationship for type I IFN in PAH, we employed the use of genetically modified mice lacking a functional type I IFN receptor. We found that type I IFN signalling mediates the deleterious effects of hypoxia on the pulmonary vasculature, on the right ventricle and on increased serum ET-1 levels. As mentioned above, our group has pioneered the concept that ET-1 is an IFN driven gene and these data are fully supportive of this. However, in order to better understand whether the reduction in circulating ET-1 levels was principally due to the amelioration in pulmonary vascular pathology observed or could be ascribed to the specific effects of IFN signaling on ET-1 release, we performed additional experiments where mice were treated with LPS. Here, as predicted, IP10 but not KC release induced by LPS was mediated by IFNAR1. KC is the mouse homologue of human IL-8 and is released by LPS independently of IFN. Importantly LPS-induced ET-1 release, like IP10, was dependent on IFNAR1. In addition to supporting and validating our hypothesis linking type I IFN with PAH, this is the first in vivo demonstration of ET-1 as an IFN dependent gene.

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Thus, using a number of in vitro and in vivo experimental techniques as well as studies in patients, we have demonstrated for the first time that type I IFN is associated with human PAH and mechanistically linked to the development of PAH in mice. We have shown that this link is associated with IP10 and ET-1 and is regulated by the type I IFN receptor, IFNAR1. These findings contribute significantly to our understanding of PAH mechanisms and help to explain why IFN therapies can cause pulmonary vascular pathology, which in extreme cases may lead to PAH. Furthermore, we conclude that modulation of IFN pathways may represent a novel therapeutic target in the treatment of PAH.

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MH reports personal fees from Actelion, Bayer, GSK, Novartis, Pfizer and United Therapeutics
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REFERENCES


10. George PM, Mitchell JA. Decline in pulmonary function during chronic hepatitis c virus therapy with modified interferon alfa and ribavirin therapy. *J Viral Hepat.* 2013;20:592


FIGURE LEGENDS

**Figure 1**: Response of primary pulmonary vascular cells to types I, II and III IFNs and type III IFN receptor expression in pulmonary vascular cells as compared to hepatocytes. Human pulmonary artery smooth muscle cells (HPASMCs) (A & B), human microvascular endothelial cells (HMVECs) (C & D) and human lung fibroblasts (HLFs) (E & F) were treated with IFNα (10ng/ml), IFNγ (10ng/ml) and IFNλ (1000ng/ml) in the presence of TNFα (10ng/ml) and assayed for IP10 (A,C,E) and ET-1 (B,D,F). Data are presented as mean ± standard error of the mean (SEM) from n=3-6 experiments performed in singlicate. Statistical significance (*p<0.05) compared to control was determined by one-way ANOVA with Dunnett’s multiple comparison post-test adjustment. IL-10RB (G) and IL-28RA (H) gene expressed as mean ± SEM fold difference compared to hepatocytes from n=3 experiments in the absence of TNFα. Statistical significance (*p<0.05) compared to hepatocytes was determined by one-way ANOVA with Dunnett’s multiple comparison post-test adjustment.

**Figure 2**: Serum IP10, ET-1 and IFN levels in patients with SSc-PAH, SSc without PAH and healthy controls. Serum levels of IFNα (A), IFNβ (B), IFNγ (C), IFNλ (D), IP10 (E) and ET-1 (F) were analysed from controls, n=9, Systemic sclerosis (SSc) patients without PAH, n=35 and SSc-PAH patients, n=28. Individual data points refer to each patient and means of all patients in the cohort are represented by horizontal lines. Statistical significance (*p<0.05) for all three groups compared to each other was determined by Kruskal-Wallis test followed by Dunn’s multiple comparison post-test for non-parametric data (A-D) and by one-way ANOVA followed by Bonferroni’s multiple comparison post-test for normally distributed data (E & F).

**Figure 3**: Serum BNP, IP10 and ET-1 levels measured in a cohort of SSc-PAH patients. Serum BNP levels in “IFN positive” and “IFN negative” patients (A). Data expressed as mean ± SEM. Statistical significance (*p<0.05) determined using a t-test. Serum IP10 and ET-1 levels measured in SSc-PAH patients, n=28 (B). Data points represent individual patient readouts. Correlation was determined using Pearson’s correlation coefficient and r and p values are shown.

**Figure 4**: Correlation of IP10 with clinical and haemodynamic parameters in SSc-PAH patients. Pearson’s correlation coefficient was determined between IP10 and pulmonary vascular resistance (PVR) (dynes), n=27 (A), mean pulmonary artery pressure (mPAP) (mmHg), n=27 (B), cardiac index, n=27 (D), 6-minute walk test (6MWT) (metres), n=27 (E) and Spearman’s rank correlation coefficient was determined between IP10 and serum brain natriuretic peptide (BNP) levels (pg/ml), n=18 (C). Data points represent individual patient readouts.

**Figure 5**: Immunohistochemistry and western blotting demonstrating IFNAR1 in PAH lung. IFNAR1 staining in lung sections from a patient with SSc-PAH (A&B), Idiopathic (I)PAH (D&E) and non-PAH controls (G&H). Figures shown are representative images from n=3 patients. Lung samples from SSc-PAH patients with high (C&F) and low (I) IFNAR1 expression; HE and HES staining. C: A small pulmonary artery displaying arteritis with transmural inflammatory infiltrate (arrows) and important intimal thickening. F: A pulmonary artery adjacent to a small bronchiole (Br), showing quasi occlusive fibrosis of the intima. I: Small pulmonary vein displaying important fibrotic occlusive remodelling, a feature frequently encountered in SSc-PAH; note the surrounding alveolar septa with capillary hemangiomatosis-like appearance (arrows). IFNAR1 expression (J);1-3, n=3 controls; 3-6, n=3 patients with IPAH; (separate blot) 7-9, n=3 SSc-PAH with predominantly venous disease and 9-12, n=3 SSc-PAH with predominantly arterial disease. Blot (J) quantified as expression relative to βActin (K). Statistical significance (* p<0.05) was determined by one-way ANOVA with Bonferroni’s multiple comparison post-test.
**Figure 6:** IP10 release from IFNα stimulated endothelial cells grown from the blood of patients with PAH and healthy controls. Endothelial cells grown from the blood of patients (BOECs) with PAH, n=4 and healthy controls, n=4 were treated with IFNα (30ng/ml) and assayed for IP10. Data are presented as mean ± standard error of the mean (SEM) and statistical significance (*p<0.05) was determined by two-way ANOVA with Bonferroni’s multiple comparison post-test.

**Figure 7:** Influence of type I IFN signalling on the development of PAH explored using the chronic hypoxic mouse model and mice lacking a functional type I IFN receptor. Mice lacking a functional type I IFN receptor (IFNAR1−/−) exposed to hypoxia (10% O2) or normoxia (room air) compared to wild type (C57Bl/6J) mice exposed to the same conditions. Data presented as mean ± standard error of the mean (SEM) from n=4-15 mice. Right ventricular systolic pressure (RVSP) (mmHg) (A), percentage of muscularized pulmonary vessels over total number of vessels (B), ratio of right ventricular (RV) mass to body weight (BW) (RV/BW) (mg/g) (C) and serum endothelin (ET)-1 levels (pg/ml) (D) were measured. Statistical significance determined by one-way ANOVA followed by Bonferroni’s multiple comparison post-test (**p<0.0001 and **p<0.005 for normoxic vs. hypoxic conditions) and (****p<0.0001, ***p<0.005 and *p<0.05 for IFNAR1−/− vs. C57Bl/6J mice).

**Figure 8:** Influence of type I IFN signalling on LPS-induced ET-1 generation explored using mice lacking a functional type I IFN receptor. Mice lacking a functional type I IFN receptor (IFNAR1−/−) injected with either LPS or vehicle control for 4 hours and compared to wild type (C57Bl/6J) mice. Data presented as mean ± standard error of the mean (SEM) from n=6 mice. Serum levels of ET-1 (A), IP10 (B), IFNα (C), IFNγ (D), IFNλ (E) and KC (F) were measured. Statistical significance determined by one-way ANOVA followed by Bonferroni’s multiple comparison post-test (****p<0.0001 for LPS vs. Vehicle) and (****p<0.0001 for IFNAR1−/− vs. C57Bl/6J mice).
Novelty and Significance

What Is Known?

- Type I Interferon (IFN) is an effective treatment but it causes adverse effects in the lung including, in rare cases, severe and sometimes irreversible pulmonary vascular inflammation.

- IFN induces a number of genes including IP10 (which is implicated in lung inflammation) and ET-1 (which is central to the pathogenesis of pulmonary arterial hypertension (PAH)).

- Autoimmune conditions where type I IFN is chronically overproduced such as Systemic Sclerosis (SSc) show a robust association with PAH but mechanisms are poorly understood.

What New Information Does This Article Contribute?

- IFNs activate human lung cells and along with associated downstream targets correlate with known biomarkers and clinical endpoints of PAH, particularly that associated with SSc.

- The type I IFN receptor (IFNAR1) mediates the pathological hallmarks of pulmonary hypertension induced by hypoxia in mice and the induction of ET-1 by LPS in vivo is mediated by type I IFN signalling.

- Type I IFN receptor expression is increased in defined cases of PAH and the link between type I IFN and PAH extends beyond that associated with SSc having relevance to other forms of the disease including idiopathic (I)PAH.

Type I IFN treatment for hepatitis C causes detectable changes in the lung in about 48% of patients. In some individuals this can manifest as severe and irreversible pulmonary hypertension. Conditions such as SSc, which are strongly associated with PAH, are typified by chronically increased endogenous type I IFN production. However the mechanisms linking IFN to PAH are poorly understood. Here, we demonstrate the involvement of type I IFN in the pathogenesis of PAH. This may be mediated by specific IFN stimulated genes, including IP10 and ET-1, via the type I IFN receptor IFNAR1. We found that the relationship between IFN and PAH may extend beyond SSc associated disease and may have broader relevance to other aetiologies including IPAH. Our findings significantly advance the scientific understanding of the relationship between dysregulated innate immunity and pulmonary vascular pathology and suggest that modulation of type I IFN pathways could be a novel strategy for the treatment of PAH.
Figure 2

Panel A: IFNα (pg/ml)

Panel B: IFNβ (pg/ml)

Panel C: IFNλ (pg/ml)

Panel D: Control SSc without PAH SSc-PAH

Panel E: IP10 (pg/ml)

Panel F: ET-1 (pg/ml)

* indicates a significant difference between the groups.
Figure 3

A

![Bar graph showing BNP levels in IFN negative and IFN positive groups.]

- IFN negative
- IFN positive

B

![Scatter plot showing correlation between ET-1 and IP10 levels.]

- $r = 0.44$
- $p = 0.02$
Figure 4

A) PVR (dynes) vs. IP10 (pg/ml) with r=0.44 and p=0.02.
B) mPAP (mmHg) vs. IP10 (pg/ml) with r=0.34 and p=0.04.
C) BNP (pg/ml) vs. IP10 (pg/ml) with r=0.59 and p=0.0049.
D) Cardiac Index vs. IP10 (pg/ml) with r=-0.61 and p=0.0001.
E) 6MWT (metres) vs. IP10 (pg/ml) with r=-0.33 and p=0.0047.
Figure 5
Figure 6

IP10 (pg/ml)

Healthy
PAH

Control
IFNα
Control
IFNα

*
Figure 7

A

RVSP (mmHg)

<table>
<thead>
<tr>
<th>C57BL/6J IFNAR1 /-</th>
<th>C57BL/6J IFNAR1 /-</th>
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<tr>
<td>Normoxia</td>
<td>2 weeks Hypoxia - 10%O₂</td>
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B

% Muscularized vessels (over total number)

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<tr>
<th>C57BL/6J IFNAR1 /-</th>
<th>C57BL/6J IFNAR1 /-</th>
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<td>Normoxia</td>
<td>2 weeks Hypoxia - 10%O₂</td>
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C

RV/BW (mg/g)

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<th>C57BL/6J IFNAR1 /-</th>
<th>C57BL/6J IFNAR1 /-</th>
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<tr>
<td>Normoxia</td>
<td>2 weeks Hypoxia - 10%O₂</td>
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D

ET-1 (pg/ml)

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<tr>
<th>C57BL/6J IFNAR1 /-</th>
<th>C57BL/6J IFNAR1 /-</th>
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<tbody>
<tr>
<td>Normoxia</td>
<td>2 weeks Hypoxia - 10%O₂</td>
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</table>
Figure 8

**A**

ET-1 (pg/ml)

- C57/BL6
- IFNAR1 -/-
- Vehicle
- LPS

**B**

IP10 (pg/ml)

- C57Bl/6J
- IFNAR1 -/-
- Vehicle
- LPS

**C**

IFNα (pg/ml)

- C57/Bl/6J
- IFNAR1 -/-
- Vehicle
- LPS

**D**

IFNγ (pg/ml)

- C57Bl/6J
- IFNAR1 -/-
- Vehicle
- LPS

**E**

IFNλ (pg/ml)

- C57Bl/6J
- IFNAR1 -/-
- Vehicle
- LPS

**F**

KC (pg/ml)

- C57Bl/6J
- IFNAR1 -/-
- Vehicle
- LPS
Evidence for the Involvement of Type I Interferon in Pulmonary Arterial Hypertension

Peter M George, Eduardo Oliver, Peter Dorfmüller, Olivier D Dubois, Daniel M Reed, Nicholas S Kirkby, Nura A Mohamed, Frederic Perros, Fabrice Antigny, Elie Fadel, Benjamin Emmanuel Schreiber, Alan Holmes, Mark Southwood, Guy Hagan, Stephen J Wort, Nathan Bartlett, Nicholas W Morrell, Gerry Coghlan, Marc Humbert, Lan Zhao and Jane A Mitchell

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Supplemental Material

Expanded Methods:

**In vitro cell culture:**

Human pulmonary arteries from healthy segments of lung were isolated from patients undergoing pulmonary resection at the Royal Brompton Hospital (RBH), London, UK (Research Ethics Committee study number 02-081, sub-amendment 3). Human pulmonary artery smooth muscle cells (HPASMCs) and human lung fibroblasts (HLFs) were cultured as previously described. Human lung microvascular endothelial cells (HMVECs) isolated from arterioles, venules and capillaries were purchased (Lonza, UK), grown and maintained as per manufacturer’s instructions and as previously described. The human epithelial A549 lung carcinoma cell line and the type I alveolar epithelial TT1 cell line were purchased from Life technologies, UK and maintained as per manufacturer’s instructions. All cells were serum deprived for 24 hours and subsequently treated with IFNs and or TNFα (R&D Systems, Abingdon, UK) for 24 hours before supernatant was removed for analysis. IFNα and IFNγ were used at 10ng/ml or 30ng/ml while IFNλ was used at 1000ng/ml to reflect its 100 fold lower specific activity. TNFα was used at 10ng/ml. Cell culture supernatant levels of ET-1 and IP10 were measured by ELISA (R&D, Abingdon, UK) according to manufacturer instructions.

**Isolation of blood outgrowth endothelial cells (BOECs):**

Blood was collected from healthy controls and patients with PAH (one patient with congenital heart disease, one with connective tissue disease, one with IPAH and one with sarcoidosis) at the RBH (ethics code 10/H0504/9) and written consent was obtained from all participants. BOECs were isolated as per previously published protocols. The number of colony forming units that emerged from PBMC cultures within 3 days of the first colony forming were noted using light microscopy at 10X magnification. Proliferation of BOECs plated at densities of 1000, 3000 and 10000 cells per well was measured over 24 hours using the alamarBlue® method.

**RNA isolation and quantitative reverse transcriptase PCR:**

RNA was extracted from cells using a silica-column based kit (Qiagen, Crawley, UK) and converted to cDNA using SuperScript II reverse transcriptase (Life Technologies, UK) and oligo(dT) primers (Life Technologies, UK). cDNA was amplified and quantitated by qPCR using a RotorGeneQ instrument (Corbett Robotics, UK) and TaqMan gene expression assays (Life Technologies, UK) for IFNAR1 (Hs01066116 m1), IFNAR2 (Hs00174198 m1), IFNGR1 (Hs00988304 m1), IFNGR2 (Hs00194264 m1), IL10RB (Hs00175123 m1) and IL28RA (Hs00417120 m1). Data were analysed using the comparative Ct method by comparison to the expression of the endogenous control GAPDH (Hs02758991_g1).

**Lung tissue Immunohistochemistry:**

Immunohistochemistry was performed on 3 um-thick sections of paraffin embedded tissue. One slide was assessed per patient – these were histologically selected on HES staining from 15-19 paraffin blocks where characteristic PAH lesions were present. After routine preparation and pressure cooker unmasking at pH8 (Electron microscopy science, Hatfield, USA) and blocking with horse serum, slides were processed with rabbit anti-IFNAR1 antibody (1μg/ml, HPA018015; Sigma-Aldrich, Lyon, France). According to manufacturer’s recommendations, biotinylated horse anti-rabbit, streptavidin-alkaline phosphatase conjugate and Vector Red Substrate (Vector laboratories, USA) were used for primary antibody detection. Controls used for this antibody included omission of the primary antibody and substitution of the primary antibody by rabbit IgG. IFNAR1, IFNGR1 and IL28RA expression was studied in the lungs of patients with SSc-PAH, IPAH and healthy controls.
**Western blotting:**

Human lung samples obtained from healthy controls and patients with IPAH or SSc-PAH were prepared in lysis buffer containing 1% Igepal, 20 mM Tris HCl, 137 mM NaCl, 10% Glycerol, 2 mM EDTA, 1 mM Na3VO4, leupeptine 10 µg/µl, lepstatine 10 µg/µl, aprotinine 10 µg/µl and protease inhibitor cocktail (aprotine, leupeptine, and PefaBloc (Roche, Meylan, France)). Total Protein lysates (40 µg) were separated on SDS-PAGE and transferred to nitrocellulose membrane. After blocking, membranes were incubated in T-TBS and 5% nonfat milk overnight at 4°C with primary antibodies: rabbit anti-IFNAR1 polyclonal antibody (1:500, LifeSpan BioSciences) and mouse monoclonal antibody against βActin (Sigma) 1:2000. Blots were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse diluted 1:10000 (Cell Signaling) or with HRP-conjugated goat anti-rabbit diluted 1:5000 (Cell Signaling), respectively. Antibodies were revealed using ECL reagents (Perkin Elmer). ImageJ Software was used to quantify the level of protein expression.

**Chronic hypoxia-induced mouse model of pulmonary hypertension:**

Male C57Bl/6J mice (Charles River, Margate, UK) and male mice lacking a functional type I IFN receptor (IFNAR1−/−, on a C57Bl/6J background) 8, aged 8 – 10 weeks old and weighing ≈20g were placed in a normobaric hypoxic chamber (FiO2 10%) for 14 days (n=8-15/group) to induce pulmonary hypertension. Controls were housed in normal air for the same period. At day 14, animals were weighed and anesthetized (fentanyl/fluanisone 0.25 ml/kg; midazolam 25 mg/kg IP), and right ventricular systolic pressure (RVSP) was measured via direct cardiac puncture using a closed-chest technique in the spontaneously breathing, anesthetized animal, to verify development of pulmonary hypertension as previously described 9. Blood was taken, and serum obtained for analysis. Right ventricular hypertrophy was assessed as the ratio of the weight of the right ventricle (RV) to body weight (BW). Lungs were fixed by inflation with 10% formalin, embedded in paraffin, and sectioned for histology. Transverse lung sections were stained with elastic Van Gieson and alpha smooth muscle-actin antibody (Sigma, UK). Vascular muscularisation was defined as the proportion of alpha smooth muscle actin positive vessels (<50 µm diameter) of the total number of vessels stained with elastin. Two separate sections from each animal were quantified, and counting was performed by two investigators blinded to genotypes. All studies were conducted in accordance with UK Home Office Animals (Scientific Procedures) Act 1986 and institutional guidelines.

**Mouse model of acute lipopolysaccharide (LPS) induced ET-1 release:**

Male C57Bl/6J mice (Charles River, Margate, UK) and male mice lacking a functional type I IFN receptor (IFNAR1−/−, on a C57Bl/6J background) 8, aged 8 – 10 weeks old and weighing ≈20g were intraperitoneally injected with 8 µL per gram of either vehicle control (0.9% sterile saline) or LPS from *Escherichia Coli* 055:B5 (1.25 mg/ml, L6529; Sigma-Aldrich, Poole, UK). At 4 hours, mice were humanely sacrificed. Blood was taken, serum obtained and IP10, ET-1, IFNs and KC levels were measured by ELISA (R&D systems, Abingdon, UK).

**Human and mouse tissue organ culture:**

Whole human pulmonary artery (PA) was harvested from healthy segments of lung from patients undergoing pulmonary resection at RBH (RBH and Harefield local ethics committee, number 09/H0708/72). Segments of PA were dissected clean and placed into individual wells of 96 well plates as previously described 3. Segments of PA were left to equilibrate for 24 hours and were then treated with IFNs with supernatant removed after a further 24 hours. IP10 levels were measured by ELISA (R&D Systems, Abingdon, UK).

Mouse aorta and lung tissue were obtained from C57Bl/6J mice after being humanely sacrificed. Segments of vessel or lung were plated in individual wells of a 96 well plate and treated with recombinant human IFNa2b, mouse IFNa A (both R&D, Abingdon, UK) or
human pegylated IFNa2b (ViraferonPeg®, Schering-Plough, UK) for 24 hours before supernatant was removed. IP10 levels were measured by ELISA (R&D Systems, Abingdon, UK).

**Clinical samples:**

63 patients with SSc (28 patients with SSc-PAH and 35 SSc patients without PAH) were recruited from 2 specialist centres – the Royal Free Hospital, London (RFH) (REC ref 6398) and Papworth Hospital Research Tissue Bank (REC ref 08/H0304/56). Healthy controls were recruited from RFH and RBH. Serum was analysed for ET-1 and IP10 (Quantikine ELISA, R&D Systems, Abingdon, UK). Serum IFNs and related cytokines were measured by cytokine array (tebu-bio sciences, France). Clinical data from each patient were collected. Patients with lung disease or left heart disease were excluded.

**Supplemental References:**

**Online Table I**: IP10 release from Blood Outgrowth Endothelial Cells grown from PAH patients

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>IFNα</th>
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<tr>
<td><strong>No TNFα</strong></td>
<td>292.97 ± 81.01pg/ml</td>
<td>818.78 ± 576.29pg/ml</td>
</tr>
<tr>
<td><strong>+ TNFα</strong></td>
<td>5643.15 ± 2490pg/ml</td>
<td>23071.52 ± 6875.86pg/ml *</td>
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</table>

IP10 release from BOECs grown from PAH patients (1 congenital heart disease, 1 IPAH, 1 connective tissue disease, 1 sarcoid). Data displayed as mean ± SEM. Statistical significance determined by two-way ANOVA followed by Bonferroni's multiple comparison post-test comparison (* p<0.05).
Online Figure I: Response of pulmonary artery smooth muscle cells to types I, II and III IFN with and without TNFα

Human pulmonary artery smooth muscle cells treated with IFNα (A&B), IFNγ (C&D), IFNλ (E&F) in the presence and absence of TNFα (10ng/ml) and assayed for IP10 (A, C, E) and ET-1 (B, D, F). Data are presented as mean ± SEM from n=4 experiments. Statistical significance determined by one way ANOVA followed by Dunnett’s post-test comparison for increasing concentrations of IFN (*p<0.05) and two-way ANOVA followed by Bonferroni’s multiple comparison post-test comparison for effect of TNFα (#p<0.05). NS = Not Significant.
Online Figure II: Response of human lung microvascular endothelial cells to types I, II and III IFN with and without TNFα

Human lung microvascular endothelial cells treated with IFNα (A&B), IFNγ (C&D) and IFNλ (E&F) in the presence and absence of TNFα (10ng/ml) and assayed for IP10 (A, C, E) and ET-1 (B, D, F). Data are presented as mean ± SEM from n=4 experiments. Statistical significance determined by one way ANOVA followed by Dunnett’s post-test comparison for increasing concentrations of IFN (*p<0.05) and two-way ANOVA followed by Bonferroni’s multiple comparison post-test comparison for effect of TNFα (#p<0.05). NS = Not Significant.
Online Figure III: Response of human lung fibroblasts to types I, II and III IFN with and without TNFα

Human lung microvascular endothelial cells treated with IFNα (A&B), IFNγ (C&D) and IFNλ (E&F) in the in the presence and absence of TNFα (10ng/ml) and assayed for IP10 (A, C, E) and ET-1 (B, D, F). Data are presented as mean ± SEM from n=3 experiments. Statistical significance determined by one way ANOVA followed by Dunnett’s post-test comparison for increasing concentrations of IFN (*p<0.05) and two-way ANOVA followed by Bonferroni’s multiple comparison post-test comparison for effect of TNFα (#p<0.05). NS = Not Significant.
Online Figure IV: Type I and II IFN receptor gene expression assessed in pulmonary vascular cells and compared to a standard reference point - human hepatocytes

Type I IFN receptor expression (IFNAR1 and IFNAR2) (A&B) in hepatocytes, human pulmonary artery smooth muscle cells (HPASMCs), human microvascular endothelial cells (HMVECs) and human lung fibroblasts (HLFs) and type II IFN receptor expression (IFNGR1 and IFNGR2) (C&D) in the same cell types. Data are presented as mean ± SEM from n=3 separate experiments and are plotted as fold difference vs hepatocytes (=1).
Online Figure V: Response of hepatocytes to types I, II and III IFNs

Human hepatocytes were treated with IFNα (10ng/ml), IFNγ (10ng/ml) and IFNλ (1000ng/ml) in the presence of TNFα (10ng/ml) and assayed for IP10 (A) and ET-1 (B). Data are presented as mean ± SEM from n=3 experiments. Statistical significance (*p<0.05) was determined by one-way ANOVA with Dunnett’s multiple comparison post-test.
Online Figure VI: Correlation between IP10 and ET-1 in patients with SSc but no PAH

Serum IP10 and ET-1 levels measured in patients with SSc and no PAH, n=35. Data points represent individual patient readouts. Correlation was determined using Pearson’s correlation coefficient and r and p values are shown.
Online Figure VII: Serum cytokine levels in patients with SSc-PAH, patients with SSc without PAH and healthy controls

Serum IL-1α (A), IL-4 (B), IL-5 (C), IL-6 (D), IL-8 (E), IL-10 (F), IL-12p70 (G) and TNFα (H) were measured in controls, n=9, SSc patients without PAH, n=35 and SSc-PAH patients, n=28. Data are presented as means and statistical significance (*p<0.05) was determined by Kruskal-Wallis test followed by Dunn’s multiple comparison post-test adjustment.
Online Figure VIII: Immunohistochemistry for type II and type III IFN receptor expression in SSc-PAH lung

IFNGR1 staining in a patient with SSc-PAH (A&B) and a healthy control (C) and IL28RA staining in a patient with SSc-PAH (D&E) and respective healthy control (F)
Online Figure IX: ET-1 release from BOECs grown from PAH patients and healthy controls

ET-1 release from BOECs grown out from healthy individuals (n=4) and patients with PAH (n=4) under basal conditions (A) and after treatment with IFNα (30ng/ml). Data are presented as mean ± SEM and analysed using a t-test (non-significant).
Online Figure X: Angiogenic functions of BOECs from healthy individuals and patients with PAH

Colony forming units from BOECs grown from patients with PAH, n=11 and healthy controls, n=11 displayed as mean ± SEM (A). Proliferation as measured by alamarBlue assay® with absorbance measured at 570-620nm at a range of seeding densities of BOECs from PAH patients, n=3 and healthy controls, n=5 (B).
Online Figure XI: Serum IFNλ levels in hypoxic mouse model

Serum IFNλ levels from C57Bl/6J and IFNAR1^-/- mice housed in normoxia or exposed to chronic hypoxia for 14 days. Data expressed as mean ± SEM from n=3-6 mice.
Online Figure XII: Further characterisation of hypoxic mouse model of PAH

Right ventricular hypertrophy as measured by Fulton’s Index (RV/(LV+Septum)) in C57Bl/6J and IFNAR1⁻/⁻ exposed to chronic hypoxia for 14 days (A). Data expressed as mean ± SEM from n=6-8 mice. Statistical significance was determined by t-test (*p<0.05). Histological example of a remodelled pulmonary artery taken from a C57Bl/6J mouse exposed to hypoxia (B) and of a normal pulmonary artery taken from an IFNAR1⁻/⁻ mouse exposed to chronic hypoxia (C)
Online Figure XIII: Response of mouse tissue to human and mouse IFNα

**A**

IP10 release from mouse aorta after treatment with Human IFNα2 (10ng/ml), mouse IFNαA (10ng/ml) and Human Pegylated IFNα2b (Viraferon™) (10ng/ml) (A). Data are presented as mean ± SEM from n=5 mice run in duplicate. Statistical significance was determined using one way ANOVA followed by Dunnett’s post-test comparison (*p<0.05).

**B**

IP10 release from mouse lung after treatment with Human IFNα2, Mouse IFNαA and Human Pegylated IFNα2b (Viraferon™) (B). Data are presented as mean ± SEM from n=5 mice run in duplicate. Statistical significance was determined using two way ANOVA followed by Bonferroni’s post-test comparison (*p<0.05).
Online Figure XIV: Human pulmonary artery organ culture

IP10 release from freshly harvested human pulmonary artery treated with IFNα (10ng/ml), IFNγ (10ng/ml) and IFNλ (1000ng/ml). Data are presented as mean ± SEM from n=4 experiments (separate donors). Statistical significance determined by one way ANOVA followed by Dunnett’s post-test comparison (*p<0.05).
Online Figure XV: IFNλ receptor gene expression assessed in pulmonary vascular and epithelial cells and compared to a standard reference point - human hepatocytes

IFNλ receptor expression (IL10RB (A) and IL28RA (B)) in hepatocytes, A549 epithelial cells, human pulmonary artery smooth muscle cells (HPASMCs), human microvascular endothelial cells (HMVECs) and human lung fibroblasts (HLFs). Data are presented as mean ± SEM from n=3 separate experiments and are plotted as fold difference vs hepatocytes (=1). Statistical significance was determined by one way ANOVA followed by Bonferroni’s post-test comparison (* p<0.05).
Online Figure XVI: Response of A549 epithelial cells to IFNs – IP10 release

IP10 release from A549 cells treated with IFNα (A), IFNγ (B), IFNλ (C) in the presence and absence of TNFα (10ng/ml). Data are presented as mean ± SEM from n=3 experiments. Statistical significance determined by one way ANOVA followed by Dunnett’s post-test comparison for increasing concentrations of IFN (*p<0.05) and two-way ANOVA followed by Bonferroni’s multiple comparison post-test comparison for effect of TNFα (#p<0.05). NS = Not Significant.
Online Figure XVII: Response of A549 epithelial cells to IFNs – ET-1 release

ET-1 release from A549 cells treated with IFNα, IFNγ or IFNλ in the absence (A) and presence (B) of TNFα (10ng/ml). Data are presented as mean ± SEM from n=3 experiments. Statistical significance determined by one way ANOVA followed by Dunnett’s post-test comparison.
Online Figure XVIII: Effect of IFNs on Alveolar type I epithelial cells

Human alveolar type I epithelial cells were treated with IFNα (10ng/ml), IFNγ (10ng/ml) or IFNλ (1000ng/ml) in the absence (A&B) and presence (C&D) of TNFα (10ng/ml) and release of IP10 (A&C) and ET-1 (B&D) were measured. Data are presented as mean ± SEM from n=3 experiments. Statistical significance was determined using one way ANOVA followed by Dunnett’s post-test comparison (*p<0.05).
Beas2B epithelial cells were treated with IFNα (10ng/ml), IFNγ (10ng/ml) or IFNλ (1000ng/ml) in the absence (A&B) and presence (C&D) of TNFα (10ng/ml) and release of IP10 (A&C) and ET-1 (B&D) were measured. Data are presented as mean ± SEM from n=3 experiments. Statistical significance was determined using one way ANOVA followed by Dunnett’s post-test comparison (*p<0.05).