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

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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 are strongly associated with PAH. Furthermore, therapeutic use of type I IFN is associated with PAH. This was recognized 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 reverse transcriptase polymerase chain reaction, Western blotting, and immunohistochemistry. The role of type I IFN in PAH in vivo was determined using type I IFN receptor knockout (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 patients with systemic sclerosis associated PAH. Serum interferon γ inducible protein 10 (IP10; CXCL10) and endothelin 1 were raised and strongly correlated together. IP10 correlated positively with pulmonary hemodynamics 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 remodeling, and raised serum endothelin 1 levels.

Conclusions: These data indicate that type I IFN, via an action of IFNAR1, mediates PAH. (Circ Res. 2014;114:677-688.)

Key Words: chemokine CXCL10 ■ endothelin-1 ■ IFNAR1 subunit, interferon alpha-beta receptor ■ inflammation ■ interferon type 1 ■ pulmonary arterial hypertension ■ scleroderma, systemic

Pulmonary arterial hypertension (PAH) is a rare but devastating disease, which is defined as a mean pulmonary artery pressure of ≥25 mmHg with a normal pulmonary capillary wedge pressure. It is characterized by remodeling of the muscular, precapillary vessels, leading to an increase in pulmonary vascular resistance. The associated strain exerted on the right heart ultimately results in right heart failure and premature death.1,2

Autoimmunity has long been implicated in PAH3 and, most recently, evidence has emerged implicating interferon (IFN).4 IFN is central to the innate immune response to viral infection, and 3 types have been identified; type I IFN (IFNα and IFNβ) that signals through a heterodimeric receptor consisting of IFNAR1 and IFNAR2, type II IFN (IFNγ) that signals through IFNGR1 and IFNGR2, and type III IFN (IFNλ) the receptor for which comprises interleukin (IL)10RB and IL28RA.

In This Issue, see p 587

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677
There is growing evidence that clinically proven PAH can be precipitated with type I IFN therapy. Furthermore, in as many as 48% of patients receiving IFNα treatment, lung function (diffusion capacity of lung for carbon monoxide [DLCO]) is reduced by ≥15%, which may be due to undiagnosed pulmonary vascular pathology. This is particularly relevant when considering that endothelin 1 (ET-1), a key mediator in the pathogenesis of PAH, is elevated in a subpopulation of patients receiving IFNα therapy for hepatitis C viral infection, and that our group has pioneered the idea that ET-1 is an IFN inducible gene in vascular smooth muscle cells. 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.

Isolation of Blood Outgrowth Endothelial Cells
Blood was collected from patients with PAH, and healthy controls and blood outgrowth endothelial cells (BOECs) were isolated as per previously published protocols. 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 peripheral blood mononuclear cell cultures within 3 days was counted, and proliferation of BOECs was measured during 24 hours using the alamarBlue method.

Quantitative Reverse Transcriptase Polymerase Chain Reaction
Quantitative reverse transcriptase polymerase chain reaction for the type I IFN receptor (IFNAR1 and IFNAR2), the type II IFN receptor (IFNγR1 and IFNγR2), and the type III IFN receptor (IL10RB and IL28RA) was performed using TaqMan gene expression assays.

Lung Tissue Immunohistochemistry
IFNAR1, IFNγR1, and IL28RA expression was studied using immunohistochemistry performed on lung tissue from patients with SSc-PAH, idiopathic (IPAH), 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 ImageJ software was used to quantify the level of protein expression.

Chronic Hypoxia–Induced Mouse Model of Pulmonary Hypertension
All studies were performed in accordance with UK Home Office Animals (Scientific Procedures) Act 1986 and institutional guidelines. Male C57Bl/6J and IFNAR1−/− mice (all 8 to 10 weeks old; n=20 g) 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.

Mouse Model of Acute Lipopolysaccharide–Induced ET-1 Release
Male C57Bl/6J and IFNAR1−/− mice (8 to 10 weeks old, weighing 0–20 g) were intraperitoneally injected with 8 μg/g of either vehicle control (0.9% sterile saline) or lipopolysaccharide (LPS) from Escherichia Coli 055:B5 (1.25 mg/mL). At 4 hours, mice were humanely euthanized. Blood was taken; serum obtained; and IP10, ET-1, IFNs, and keratinocyte-derived chemokine (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 24 hours before supernatants were removed, and IP10 was measured by ELISA. Mouse aorta and lung tissue were obtained from C57Bl/6J mice after being humanely euthanized. Segments of vessel or lung were treated with recombinant human IFNα2b, mouse IFNα, or human pegylated IFNα2b for 24 hours. Supernatant levels of IP10 were measured by ELISA.

Clinical Samples
Sixty-three patients with SSc (28 patients with SSc-PAH and 35 SSc patients without PAH) were recruited from 2 specialist centers: Royal Free Hospital, London, and Papworth Hospital Research Tissue Bank. Healthy controls were recruited from Royal Free Hospital and 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 are presented as means±SEM. For all data, the Kolmogorov–Smirnov test of normality was applied. All normally distributed data were analyzed by 1-way ANOVA followed by Bonferroni post-test.
adjustment for multiple comparisons and for correlations with clinical parameters, the Pearson correlation test was applied. Nonparametric data were analyzed by the Kruskal–Wallis test followed by Dunn multiple comparison test and, for correlations, the Spearman rank test was used. Graphpad Prism was used for all statistical analysis.

An expanded Methods is available in the Online Data Supplement.

Results

Effect of IFNs on IP10 and ET-1 Release by Human Lung Cells in Culture

As we have shown previously,27 type I IFNα and type II IFNγ induced IP10 and ET-1 release from TNFα-primed human pulmonary artery smooth muscle cells (HPASMCs) (Figure 1A and 1B). Again, as we have seen before, type III IFNλ 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 and in human lung fibroblasts (Figure 1C and 1E). ET-1 release by human lung microvascular endothelial cells was predictably high28 and not increased by treatment with any of the IFNs studied (Figure 1D). ET-1 release by human lung fibroblasts was relatively low and similar to that seen from HPASMCs; however, unlike vascular smooth muscle, it was not increased by IFN treatment (Figure 1F). Based on full concentration response
experiments (Online Figures I–III), for type I and type II IFNs, threshold concentrations were in the low ng/mL range, whereas type III IFNλ remained inactive at concentrations up to 1000 ng/mL. Although TNFα coadministration 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 IFNα or IFNγ-induced ET-1 release by HPASMCs (Online Figures IB and ID). As predicted by the strong cellular response to type I and II IFNs, HPASMCs, human lung microvascular endothelial cells, and human lung fibroblasts expressed both receptor subtypes for IFNAR and IFNGR (Online Figure IV). By contrast, IFNλ was inactive in all 3 lung cell types tested. Using human hepatocytes, which respond well to IFNλ (Online Figure V) as a reference, we found that although human lung cells expressed comparable levels of IL10RB, they expressed low levels of IL28RA (Figure 1G and 1H).

Levels of IFNs, IP10, and ET-1 in Serum of SSc Patients With or Without PAH

Five (18%) patients in the SSc-PAH group had detectable levels of IFNα, whereas only 2 (5.7%) patients had detectable levels in the SSc without PAH group. IFNα was below the level of detection in serum samples of all control subjects. Similarly 6 (21%) patients with SSc-PAH had detectable levels of IFNγ, whereas this was the case in only 2 (5.7%) patients with SSc and no PAH. IFNγ was not detectable in any controls. While IFNβ levels were similar in all 3 groups, there was a clear but non significant trend to increased levels of IFNλ in serum of patients with SSc-PAH compared with those without PAH or controls (Figure 2A–2D). In line with these data, we found that levels of our 2 key IFN-stimulated gene products, IP10 and ET-1, were significantly increased in the serum of patients with SSc-PAH as compared to controls and to SSc patients without PAH (Figure 2E and 2F). Patients in whom serum levels of ≥1 of the IFNs measured were detectable, so-called IFN-positive patients, had significantly raised levels of serum brain natriuretic peptide as compared with IFN-negative patients (Figure 3A). We hypothesized that levels of IP10 and ET-1 were linked because there was a strong correlation between them in the serum of patients with SSc-PAH (r=0.44; P=0.02; Figure 3B). In this patient group, there were positive correlations between IP10 and

Figure 2. Serum interferon (IFN) γ-inducible protein 10 (IP10), endothelin 1 (ET-1), and IFN levels in patients with systemic sclerosis (SSc)-pulmonary arterial hypertension (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 analyzed from controls, n=9, patients with SSc without PAH, n=35, and patients with SSc-PAH, 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 3 groups compared with each other was determined by Kruskal–Wallis test followed by Dunn multiple comparison post-test for nonparametric data (A–D) and by 1-way ANOVA followed by Bonferroni multiple comparison post-test for normally distributed data (E and F).
pulmonary arteries of lungs from patients with SSc-PAH. 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 (Figure 5D and 5E), 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 patients with IPAH tested and some, but not all, patients with SSc-PAH (Figure 5J and 5K). 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. Our interesting observation that there seemed to be a heterogeneity of IFNAR1 expression within patients with SSc-PAH led us to hypothesize 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 6 of the patients with SSc-PAH examined displayed important venous involvement. However, of these, the 3 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 (Figure 5C and 5F). Patients with low IFNAR1 expression in total lung homogenates had a predominance of venous involvement with fibrotic occlusive remodeling and surrounding alveolar septa with capillary hemangiomatosis-like appearance, features frequently encountered in SSc-PAH (Figure 5I).

Because levels of IFNγ and IFNγ, were also raised in the serum of patients with SSc-PAH, we performed further immunohistochemistry experiments for IFNGR1 and IL28RA staining. There was an increase in IFNGR1 staining in the pulmonary vasculature of patients with SSc-PAH compared with controls (Online Figure VIIIIA–C) and, although far less profound, it followed similar morphological patterns to that of IFNAR1. Pulmonary vascular IL28RA staining was also increased in patients with SSc-PAH; however, the pattern of receptor expression differed with staining predominantly seen within the epithelial cell layer (Online Figure VIIID–F).

The finding that the type I IFN receptor is upregulated in the lungs of patients with PAH 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 (1 IPAH, 1 connective tissue disease-PAH, 1 congenital heart disease-PAH, 1 sarcoid-PAH) were more sensitive to stimulation with IFNγ even in the absence of TNFα (Figure 6). As with other cell types used in this study, TNFα coadministration enhanced the sensitivity of BOECs to IFNγ in all cells, and this
response was exaggerated in cells from patients with PAH (Online Table I). Interestingly, there was a trend for BOECs from patients with PAH to release higher levels of ET-1 than those from healthy individuals under basal conditions and in response to IFNα (Online Figure IXA and IXB). We went on to assess the angiogenic activity of these cells and found that although there was a trend to an increased number of colony forming units of BOECs grown from the blood of patients with PAH as compared with controls, this did not reach statistical significance and, over a range of seeding densities, no difference in proliferation of these cells was noted (Online Figure XA and XB).

In Vivo Experiments and Animal Model of PAH

Although consistent with our hypothesis, the above data are observational and do 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 2 subunits have different functions, and IFNAR1−/− mice have previously been well characterized and are known to have an increase in right ventricular (RV) systolic pressure (Figure 7B). Along with pulmonary vascular resistance (PVR; dynes), n=27 (A); and mean pulmonary artery pressure (mPAP; mmHg), n=27 (B); Spearman rank correlation coefficient was determined between IP10 and serum brain natriuretic peptide (BNP) levels (pg/mL), n=18 (C); cardiac index, n=27 (D); and 6-minute walk test (6MWT; meters), n=27 (E). Data points represent individual patient readouts.

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 signaling in PAH, which may be mediated by ET-1. 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 (≈8 hours), for a 14-day chronic hypoxia study, it would be necessary to use pegylated IFN. Because 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 with mouse IFN (Online Figures XIIA and XIIIB). Consequently, we elected to use a different approach in which to provide further mechanistic
George et al. Interferon and Pulmonary Arterial Hypertension

Evidence for the relevance of IFN in the generation of ET-1 and hence PAH. Systemic levels of ET-1 are increased experimentally in 2 animal models: (1) hypoxia-driven PAH (demonstrated above) and (2) LPS-induced endotoxemia. LPS, via Toll like receptor 4 (TLR4), activates 2 adapter proteins, which give rise to separate groups of genes. Toll like receptor 4 activates TIR domain-containing adaptor inducing IFN-beta (TRIF), which is associated with expression of IFNs and IFN-related genes, such as IP10. Toll like receptor 4 also activates myeloid differentiation primary response gene 88 (MyD88), which activates nuclear factor κB–regulated genes including KC (the mouse homolog of IL-8/CXCL8).

In C57Bl/6J mice, LPS induced systemic release of IFNα, IFNγ, ET-1, IP10, and KC but not IFNλ (Figure 8A–8F). 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 (Figure 8A–8F). 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 because of 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. Although this raises the possibility that IFNλ may represent a marker of type I IFN signaling, we wished to explore further its potential to play a pathological role in its own right. Having found IFNλ to be inactive in cells because of 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. Although this raises the possibility that IFNλ may represent a marker of type I IFN signaling, 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 therefore 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 Figure XIV). Using immunohistochemistry, we then looked for the presence of the specific IFNλ receptor, IL28RA, within the pulmonary vasculature of patients with SSc-PAH and found that there was increased receptor expression predominantly within the lung epithelium (Online Figure VIIID and VIIIE), 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 2 IFNλ receptor subunits in the human epithelial lung carcinoma cell line (A549) cell line and found that although the IL10RB subunit seemed to be widely expressed, the IL28RA subunit was only expressed on hepatocytes and A549 cells (Online Figure XV A and XVB). Interestingly, we found that although 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 Figure XVI) and ET-1 (Online Figure XVII) in the presence of TNFα. This was in contrast to hepatocytes that did respond to IFNα, to release IP10 (Online Figure VA). We repeated these experiments in 2 further epithelial cell types and as with A549 cells, we found that neither the immortalized human type I alveolar epithelial (TT1) cell line (Online Figure XVIII), nor the bronchial epithelial (Beas2B) cell line (Online Figure 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 PAH. 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 characterized 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 (among 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 although IFNλ is raised in the serum of patients with SSc-PAH, 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 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. Although 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 because 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 with existing IFNα preparations.

The data we present in this article are consistent with the large body of work that now recognizes 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 low levels are found in the circulation as a result of dilution in bodily fluids, diffusion from plasma

![Figure 8. Influence of type I interferon (IFN) signaling on lipopolysaccharide (LPS)-induced endothelin 1 (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 with wild-type (C57Bl/6J) mice. Data presented as means±SEM from n=6 mice. Serum levels of ET-1 (A), interferon γ inducible protein 10 (IP10) (B), IFNα (C), IFNγ (D), IFNλ (E), and keratinocyte-derived chemokine (KC) (F) were measured. Statistical significance determined by 1-way ANOVA followed by Bonferroni multiple comparison post-test (***P<0.0001 for LPS vs Vehicle) and (**P<0.001 for IFNAR1−− vs C57Bl/6J mice).](http://circres.ahajournals.org/Download.jpg)
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, therefore, serum levels can only provide a representative and relative readout of the dynamic ongoing homeostatic balance between production and excretion.

The ability for extrapulmonary vascular cells to sense IFNs is as yet not fully characterized. We know that endothelial cells and vascular smooth muscle cells from systemic vessels also sense IFNs and, although 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 remodeling (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, therefore, 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 patients with IPAH and patients with SSc-PAH 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. 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.

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 signaling mediates the deleterious effects of hypoxia on the pulmonary vasculature, on the RV 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, to better understand whether the reduction in circulating ET-1 levels was principally because of 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 homolog 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.

Thus, using several 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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What Is Known?

- Type I interferon (IFN) is an effective treatment; however, it causes adverse effects in the lung including, in rare cases, severe and sometimes irreversible pulmonary vascular inflammation.
- IFN induces several genes including interferon γ inducible protein 10 (IP10) (which is implicated in lung inflammation) and endothelin 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 endothelin 1 by lipopolysaccharide in vivo is mediated by type I IFN signaling.
- 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 (IPAH).

Type I IFN treatment for hepatitis C causes detectable changes in the lung in ≈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 interferon γ inducible protein 10 (IP10) and endothelin 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 etiologies 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.
Evidence for the Involvement of Type I Interferon in Pulmonary Arterial Hypertension

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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 \(^1, 2\). 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 \(^3\). The human epithelial A549 lung carcinoma cell line and the type I alveolar epithelial TT1 cell line \(^4\) and the bronchial epithelial Beas2B cell line were maintained and cultured as previously described \(^5\). Human hepatocytes were purchased from Lifetechnologies\(^TM\), UK and maintained as per manufacturer’s instructions. All cells were serum deprived for 24 hours and subsequently treated with IFNs and or TNF\(\alpha\) (R&D Systems, Abingdon, UK) 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 (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 \(^6, 7\). 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\(^\text{®}\) 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), IFN\(\gamma\)R1 (Hs00988304_m1), IFN\(\gamma\)R2 (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 \(\mu\)m-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 (1\(\mu\)g/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, IFN\(\gamma\)R1 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, 20mM Tris HCl, 137 mM NaCl, 10% Glycerol, 2mM EDTA, 1mM Na3VO4, leupeptine 10µg/µl, lepstatine 10µg/µl, aprotinine 10µg/µl and protease inhibitor cocktail (aprotine, leupeptine, and Pefabloc (Roche, Meylan, France)). Total Protein lysates (40µg) 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 β-Actin (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 8µL per gram of either vehicle control (0.9% sterile saline) or LPS from Escherichia Coli 055:B5 (1.25mg/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 IFNα2b (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>
</tr>
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<tbody>
<tr>
<td>No TNFα</td>
<td>292.97 ± 81.01pg/ml</td>
<td>818.78 ± 576.29pg/ml</td>
</tr>
<tr>
<td>+ TNFα</td>
<td>5643.15 ± 2490pg/ml</td>
<td>23071.52 ± 6875.86pg/ml</td>
</tr>
</tbody>
</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 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 Dunnnett’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

**A** IFNAR1

**B** IFNAR2

**C** IFNGR1

**D** IFNGR2

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−/− mice 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

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).

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.
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).
Online Figure XIX: Effect of IFNs on Beas2B epithelial cells

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).