

Contribution of inflammation to the pathology of idiopathic pulmonary arterial hypertension in children.

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Short Title: Inflammation in children with in IPAH and APAH

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ABSTRACT

Idiopathic Pulmonary Arterial Hypertension (IPAH) is an incurable disease of multifactorial origin. Inflammation is frequently observed in IPAH, but its role in the pathobiology is unclear. In this study we characterised and compared the distribution, nature and number of inflammatory cells in peri-arterial infiltrates in lungs of children with IPAH, pulmonary arterial hypertension associated with congenital heart disease (APAH), and in normal lung tissue, using immunohistochemistry. The influence of treatment with combined prostacyclin and endothelin receptor blockers was also studied. In children with IPAH, both treated and untreated, but not in APAH or normal children, extensive peri-arterial infiltrates were present comprising macrophages and T-lymphocytes with S100A4 and bone morphogenetic protein receptor type 2 (BMPR2) positive cells. Although rarely co-expressing macrophage specific antigens, BMPR2 positive cells were frequently closely associated with macrophages and lymphocytes. They were more abundant around peripheral arteries of children with IPAH than in APAH or normal lungs (respectively 15.1 ± 3.5 ; 2.3 ± 0.9 ; 2.3 ± 0.9 cells/mm external elastic lamina; $p < 0.01$ for IPAH versus APAH or normal lungs). Prostacyclin with endothelin receptor blockade resulted in a significant reduction in endothelial cell activation as indicated by HLA-DR expression (treated 17% versus untreated 100%, $p < 0.002$). This study shows that pulmonary inflammation is present in the lungs of children with IPAH. This may indicate a role for inflammation in the pathobiology of IPAH and provide the rationale for novel therapeutic intervention.

KEY WORDS

Bone morphogenetic protein receptor type II; endothelial activation; inflammation; pulmonary hypertension; treatment outcome.

INTRODUCTION

Idiopathic Pulmonary Arterial Hypertension is a rare, incurable disorder occurring in children with a normally formed heart. It is characterised by irreversible occlusion of small pulmonary arteries resulting in a sustained increase in pulmonary arterial pressure. Death results from right heart failure. Presentation is late and in the majority of children severe structural changes have occurred in the pulmonary arteries by the time of diagnosis. Treatment with prostacyclin, sildenafil and endothelin receptor antagonists aims to dilate and remodel the pulmonary arteries. These drugs have improved survival and quality of life but are not curative and the only therapeutic option for end-stage disease is lung transplantation.

IPAH is multifactorial in origin and genetic and environmental factors are thought to influence the development of this disease. Mutations of members of the TGF β family of regulatory proteins, bone morphogenetic protein type-2 receptor (BMP2), ALK-1 and endoglin are present in a large proportion of individuals with familial⁽¹⁾ and sporadic IPAH⁽²⁾, and in patients with the inherited disorder haemorrhagic telangiectasia sometimes associated with pulmonary arterial hypertension⁽³⁾. BMP2 expression has been reported in the macrophages of adults with IPAH⁽⁴⁾.

Endothelial dysfunction and inflammation are thought to play an important role in development of IPAH^(5,6). Endothelial dysfunction is associated with reduction in endothelium dependent vasodilatation and enhanced vasoconstriction. Adhesion and migration of circulating inflammatory cells occurs at sites of endothelial damage, but the cascade of signalling pathways resulting from such damage and the endothelial dysfunction associated with the inflammatory change are not well understood in IPAH. Normally the lung is maintained as an inflammation-free environment by a unique organisation of the immune system⁽⁷⁾ but in adults with IPAH proinflammatory cytokines⁽⁸⁾ and platelet aggregation is enhanced⁽⁹⁾. Circulating neutrophils from adult IPAH patients show an amplified release of inflammatory mediators when stimulated and this response is reduced by prostacyclin⁽¹⁰⁾.

Pulmonary hypertension is a common feature of congenital heart disease associated with high shear stress and endothelial dysfunction, but not with genetic and other factors known to contribute to the pathogenesis of IPAH. Endothelial damage occurs in children with congenital heart disease⁽¹¹⁾ but the contribution of an inflammatory response is unknown.

The National Pulmonary Hypertension Service for Children is based in Great Ormond Street Hospital for Children which is a pediatric cardiothoracic transplantation centre. In this study we have reviewed structural abnormalities in explanted lung tissue from children with IPAH treated with continuous intravenous prostacyclin and an endothelin receptor antagonist and compared the findings with those in archival lung tissue from untreated children. Findings in IPAH were also compared with those in APAH. We used immunohistochemistry and image analysis to characterise inflammatory infiltrates in and around the pulmonary arteries noting the influence of current drug therapies on the inflammatory environment in IPAH.

MATERIALS AND METHODS

Thirty-three cases were studied (Table 1), 15 IPAH cases, (8 samples taken at lung transplantation, 4 post-mortem samples and 3 diagnostic lung biopsies), 9 cases of congenital heart disease with pulmonary hypertension (2 explanted samples, 1 post-mortem sample and 6 diagnostic biopsies); and archival post-mortem samples from 8 children and 1 adult having no cardiovascular abnormality. All tissue samples were used with the permission of the Local Research Ethics Committee of Great Ormond Street Hospital for Children (REC 05/Q0508/49), and additionally, the explanted tissue was used with the explicit informed consent from the parents.

Diagnostic groups	Number of Cases	Age range	Median Age per Group	Origin of sample		
				Biopsy (treated/untreated)	Transplant (treated/untreated)	Postmortem (treated/untreated)
Idiopathic Pulmonary Arterial Hypertension	15	3yr-16yr	8yr	3(0/3)	8 (6/2)	4 (0/4)
Pulmonary Hypertensive Congenital Heart Disease	9	6m-16yr	7yr	6	2	1
Normal Pulmonary Vasculature ± infection*	9	3m-29yr	8yr			9

Table 1. Grouping of samples used for immunohistochemistry

Immunohistochemistry

Sections were incubated with primary antibodies, (Table 2) and Biotin conjugated secondary antibodies with avidin/streptavidin amplification and visualised with diaminobenzidine. Anti-BMP2 was a gift from Professor N Morrell (Addenbrook's NHS Foundation Trust, Cambridge UK) and anti-S100A4 a gift from Prof N Ambartsumian, Department of Molecular Cancer Biology, Copenhagen, Denmark). Specificity of staining was controlled with an inappropriate secondary antibody and/or by omission of the primary antibody. Slides were examined using a Leitz Dialux 20 microscope and images acquired using a Zeiss AxioCam with Axiovision software.

Antibody	Target Cell	Clone	Dilution	Supplier
Calprotectin	monocyte, neutrophil	MAC 387	1:100	Neomarkers
HLA-DR		LN3	1:50	Novocastra
CD68	tissue macrophage	CBL-260	1:100	Cymbus Biotech
CD3	T-lymphocyte	PS1	1:100	Novocastra

CD20	B-lymphocyte	L26	1:200	Novocastra
CD1A	dendritic cell	MTB1	1:25	Novocastra
CD31	endothelial cell	JC/70A	prediluted	DAKO
CD34	endothelial and haemopoietic stem cells	QBEnd10	1:50	DAKO
KI67	replicating cells	MIB-1	prediluted	Zymed
BMPR2		rabbit polyclonal	1:20	N Morrell
S100A4		rabbit polyclonal	1:2000	N Ambartsumian

Table 2. Antibodies used for immunohistochemistry

Analysis of HLA-DR and BMPR2 staining

Slides were anonymised to ensure blinding of scoring. The distribution of HLA-DR staining in the pulmonary vasculature was assessed semi-quantitatively in 13 IPAH, 8 APAH and 6 normal cases. All sections were examined using a x40 objective and scored for presence or absence of positively staining cells within the intima, media and adventitia of every artery and in the alveolar capillary bed.

Quantitative analysis of BMPR2 expressing cells around pre and intra-acinar arteries and airways was made on 13 IPAH, 5 APAH and 6 normal cases. Images of 6-8 arteries and accompanying airways were made with an x10 objective. For each artery the external perimeter of the media⁽¹²⁾ was measured (Openlab, Improvision) and the number of BMPR2 positive cells in the media and adventitia counted. Similarly the inner epithelial surface length of each airway was measured and BMPR2 positive cells in epithelial and submucosal layers counted. For each specimen results were expressed as total number of BMPR2 cells per mm of the pooled external arterial medial length or pooled inner airway epithelial length.

Immunofluorescent co-localisation

Co-localisation of BMPR2 with cellular markers (Table 2) was carried out using the method of Cattoretti

(<http://icg.cpmc.columbia.edu/cattoretti/Protocol/immunohistochemistry/DoubleIHC.html>).

Sections were examined using a fluorescent microscope (Leica Dialux) and images acquired sequentially with 494nm and 547nm wavelength illumination and x40 objectives. Images were merged using Openlab (Axiovision, UK). In some samples co-localisation was confirmed by confocal microscopy (BioRad Radiance).

Statistical analysis

Values obtained in arteries and airways from children with IPAH, APAH and normal children were compared using the 2-sample test of proportion, and analysis of variance (ANOVA) with Tukey's pairwise comparisons (for normally distributed data). Kruskal Wallis and Mann-Whitney tests were used to compare non-normally distributed data.

RESULTS

Histological appearance of the peripheral pulmonary arteries

In normal children small pre- and intra-acinar pulmonary arteries were thin walled whereas in children with IPAH many were partially or completely obstructed by intimal proliferation. All IPAH sections had at least 1 plexiform lesion in terminal, respiratory bronchiolar or alveolar duct arteries⁽¹³⁾. The morphology of the plexiform lesions in vasodilator treated and untreated IPAH patients was similar but lesions were significantly larger in treated than in untreated children (treated: mean \pm standard error of mean $227 \pm 7.9 \mu\text{m}$, untreated: $113.5 \pm 7.9\mu\text{m}$, $p<.001$). A single plexiform lesion was found in 1/9 APAH cases.

Inflammation of the lung periphery:

Normal: 7/9 cases showed no endothelial activation as assessed by HLA-DR (Figure 1A) and S100A4 expression (supplemental data). One child with a chest infection and one with asthma displayed patchy endothelial expression of HLA-DR and S100A4 .

There was little evidence of macrophage infiltration identified by expression of either MAC387 (calprotectin) or CD68. No MAC387 labelled monocytes were found within the pulmonary artery intima or media (Figure 1B). Small aggregates of B and T lymphocytes were present in the submucosa of small pre-acinar airways and bronchi but few were present in the parenchyma (supplemental data). No CD1A positive dendritic cells were found in the intima, media or adventitia of peripheral pulmonary arteries but they were present in the mucosa of small airways and in lymphoid aggregates associated with proximal airways.

IPAH: In contrast to healthy children, endothelial activation was frequent in IPAH. Endothelial expression of HLA-DR was observed in all IPAH cases who had not received epoprostenol and bosentan treatment (Table 3). It was present in patches of the alveolar capillary bed (8/8 cases, Figure 1A) and in small intra-acinar arteries (5/8 cases, Figure 1A). All children, treated and untreated, expressed S100A4 on endothelial cells lining muscular arteries (15/15 cases supplemental data), on the alveolar walls, the arterial media and intimal proliferative regions of plexiform lesions (supplemental data).

	n	arterial endothelium	regions of intimal proliferation	capillary endothelium
Normal	6	0	0	1
IPAH untreated	8	4	6	8
IPAH epoprostenol and bosentan	6	1	2	1
APAH	9	0	1	1

Table 3. Number of cases with HLA-DR expression in the arterial wall

In all cases, treated and untreated, S100A4 was expressed by an extensive mixed infiltration of inflammatory cells. These included alveolar macrophages and a subset of circulating and migrating

inflammatory cells in pre-and intra-acinar arteries, in sub-endothelial regions of plexiform lesions and in the parenchyma (supplemental data).

In all treated and untreated cases MAC387 positive monocytes were abundant in alveolar capillaries and MAC387 and CD68 positive cells were found in intimal proliferative areas and the adventitia of obstructed arteries and plexiform lesions (Figure 1B). In 2/8 untreated cases MAC387 was expressed by a thin layer of endothelial cells in some vessels within plexiform lesions (Figure 1B).

In all cases, treated and untreated, frequent lymphoid aggregates contained CD3 and CD20 expressing T and B lymphocytes. Few B-lymphocytes were found around peripheral pulmonary arteries but T-lymphocytes were frequently seen within obstructed and dilated peripheral arteries and the parenchyma (supplemental data). The distribution of CD1A positive dendritic cells was similar to that in control tissue.

APAH: In contrast to children with IPAH, those with APAH showed little evidence of endothelial activation (Figure 1A). HLA-DR expressing alveolar capillaries were seen in only 1/9 children with APAH. Similarly fewer S100A4 expressing cells were seen (supplemental data). In APAH MAC387 positive cells were abundant in the parenchyma but not in intimal proliferative areas of obstructed arteries (Figure 1B). No MAC387 labelling of endothelial cells was observed. Lymphocytes and CD1A positive cells had a similar distribution to that of normal children.

BMPR2 expression

Periarterial BMPR2 staining cells are increased in IPAH

In all samples examined from normal, IPAH and APAH children cells intensely staining for BMPR2 were found in the lung parenchyma and around airways and arteries (Figure 1C). These cells resembled monocytes being rounded, $11.7 \pm 1.7 \mu\text{m}$ in diameter, and having large round nuclei. Similar cells were present in the peripheral mesenchyme of the developing lung of a normal fetus (Figure 1C inset).

Normal children and those with APAH had similar numbers of BMPR2 cells associated with arteries (mean 1.16 ± 0.19 and 3.0 ± 1.15 cells/mm respectively) (Figure 2) but there were significantly more BMPR2 cells associated with arteries in children with IPAH (mean 8.8 ± 0.9 cells/mm, $p < .01$ for both comparisons). The density of BMPR2 cells was similar in treated and untreated children with IPAH (8.9 ± 1.58 and 8.7 ± 1.1 respectively). The number of BMPR2 expressing cells around airways was similar in normal, IPAH and APAH groups (Figure 2).

BMPR2 was detectable in the endothelium and media of peripheral arteries of most normal and pulmonary hypertensive children (IPAH and APAH).

Characterisation of BMPR2 expressing cells

Immunofluorescent co-localisation studies on 5 IPAH (3 untreated, 2 treated) and 2 normal cases showed that BMPR2 cells did not express CD31, usually found on endothelial cells and platelets, or CD34, expressed by haemopoietic stem cells and endothelial cells (supplemental data). In IPAH BMPR2 cells were frequently found in close proximity to HLA-DR positive cells in the obstructing arterial intima and regions of inflammation within the airway sub-mucosa. Expression of BMPR2 by HLA-DR positive cells was rare, but infrequently weak BMPR2 expression was observed (supplemental data).

There was no co-expression of BMPR2 and MAC387 in macrophages within peripheral arteries, airways or alveolar walls (supplemental data). Although the distribution of CD68 expressing

macrophages in the peripheral arteries, airways and parenchyma was similar to that of BMP2 cells the latter were present in greater numbers. In all IPAH specimens, treated and untreated, a small number of BMP2 cells co-expressed CD68 however these stained less intensely for both proteins than adjacent cells expressing only a single protein (Figure 3A).

There was no co-localisation of BMP2 with CD3 or CD20 in IPAH or normal samples, but T-lymphocytes were frequently closely associated with BMP2 expressing cells (Figure 3B).

Influence of prostacyclin therapy and endothelin receptor blockade on inflammation

In this study we consistently found large numbers of inflammatory and BMP2 expressing cells in peripheral arteries of all IPAH patients who had received treatment (Figure 1C) and in all those who had not (Figures 1A,3). There was, however, less evidence of endothelial activation in treated than untreated cases. HLA-DR expression was found in the capillary endothelium of only 1/6 of the treated IPAH cases, but was present in the capillary endothelium in all 8 untreated cases (p=0.002, Figure 1A).

DISCUSSION

Our study is the first to compare the immunopathology of pulmonary arteries from children with Idiopathic Pulmonary Arterial Hypertension who have and have not received treatment with combined Bosentan and Epoprostenol, hypothesised to stimulate arterial remodelling and repair. Studies with this combination of vasodilators have yet to be reported in adults. A study on lungs of untreated and prostacyclin treated adults with IPAH showed an absence of remodelling with treatment, no significant change in morphology, but an increase in alveolar edema and in inflammation⁽¹⁴⁾. Here we confirm that in children receiving dual endothelin receptor blockade and prostacyclin therapy improvement in survival is not accompanied by reparative structural remodelling of the peripheral pulmonary vasculature. In contrast to adults treated with prostacyclin alone⁽¹⁴⁾ we observed a significant increase in the size of arterial lesions but a reduction in inflammation. We did not detect pulmonary edema in treated or untreated children.

Inflammatory cells in the lung parenchyma and in small pre-and intra-acinar arteries usually comprised macrophages and T-cells. These were frequently found close to endothelial cells expressing HLA-DR. Endothelial cell expression of MHC class II is inducible by cytokines, particularly IFN- γ , and is consistent with active T-cell and monocyte migration into areas of inflammation⁽¹⁵⁾. BMPR2 expressing cells were present in the same region as the T-cells and macrophages. However in spite of extensive immunohistochemical analysis we failed to define the origin of these cells since they did not co-express marker proteins for undifferentiated monocytes, lymphocytes, dendritic cells or haemopoietic stem cells. Co-expression with the macrophage marker CD68 and HLA-DR was observed in only a minority of these cells.

BMPR2 polymorphisms have a role in the pathogenesis of IPAH⁽¹⁶⁾, but its expression within the inflammatory infiltrate has not previously been explored. The functional significance of these cells in the lung is unknown but in mice similar BMPR-2 expressing cells were found in early experimental rheumatoid arthritis⁽¹⁷⁾ and other autoimmune diseases. When challenged with VEGF, athymic nude mice show an abnormally high susceptibility to the development of pulmonary hypertension associated with enhanced endothelial replication, peri-vascular inflammation and obstruction of peripheral arterioles⁽¹⁸⁾. The BMP2/4 pathway is important within the thymus in T-cell maturation where it is regulated⁽¹⁹⁾ via BMPRII expressed in stromal cells⁽²⁰⁾. The close association seen here between T-cells and BMPR2 expressing cells would be compatible with the latter playing an active role in the development or maintenance of the inflammatory process within the lung. Although the mechanisms responsible for the influx of inflammatory cells is unknown, an increase in the release of the chemokine CCL2 by endothelial cells has been reported in IPAH patients⁽⁶⁾ and which may result in enhanced monocyte migration and inflammation.

It is currently unclear how the aetiology of IPAH in childhood differs from that in adults. Whilst the genetic basis of IPAH in some patients may be common, the age of onset is earlier and disease progression faster in children⁽²¹⁾. Previous studies in adult humans and in mouse models have shown evidence of inflammation in IPAH^(4;22;23) which has been linked to the pathogenesis of the condition^(22;24). The role of inflammation in paediatric disease and its role in IPAH pathogenesis remain unknown. Unlike in adults with IPAH the early onset of an inflammatory process in childhood is likely to have an impact on lung development. This could have ramifications for treatment modalities in childhood IPAH.

In cases of pulmonary arterial hypertension associated with diseases of the immune system (e.g. HIV) and chronic inflammatory conditions anti-inflammatory treatment can ameliorate and perhaps even reverse pulmonary vascular disease⁽²⁵⁾. A subgroup of adult patients with IPAH have circulating autoantibodies⁽²⁶⁾ suggesting a link between IPAH and connective tissue and autoimmune diseases^(22;24). Pulmonary hypertension associated with anti-neutrophil cytoplasmic

antibody (ANCA) vasculitis can be improved with successful treatment of the vasculitis⁽²⁷⁾. This has been our own experience of children with pulmonary hypertension associated with vasculitis.

In the murine monocrotaline model of pulmonary hypertension chemokine expression by periarterial inflammatory cells has been linked to localised medial hypertrophy⁽²⁸⁾. We examined the expression of S100A4, a calcium binding protein which regulates a large range of cellular processes including proliferation⁽²³⁾ and overexpression of which in mice results in development of pulmonary hypertension and plexiform lesions⁽²³⁾. Our observation of enhanced S100A4 expression in endothelial, medial and intimal cells of arteries in children with IPAH reflect previous observations on children with APAH⁽²³⁾. Intracellular expression of S100A4 is associated with mitogenesis and enhanced cell motility whilst stromal localisation is associated with angiogenesis^(29;30). Both these pathways are associated with the pathology of IPAH.

The cause of inflammatory infiltration around peripheral arteries is unknown. It is unclear if this is a primary instigating insult in the development of IPAH or reflects a repair mechanism secondary to primary endothelial damage. Impaired endothelial regeneration has been observed in a murine model of IPAH⁽³¹⁾. Alternatively, intercurrent infection cannot be excluded as an explanation of the inflammatory response but seems unlikely as it affected predominantly the children with IPAH not those with APAH, and none had clinical evidence of a respiratory tract infection to account for our observations.

We found that treatment with epoprostenol and bosentan was associated with a reduction in endothelial HLA-DR expression, a potentially important observation. The stable prostacyclin analogue Treponistil inhibits the secretion of pro-inflammatory cytokines by alveolar macrophages⁽³²⁾ and endothelin receptor blockade reduces endothelial leakage and alveolar edema in experimental endotoxic shock⁽³³⁾. The reduction in endothelial HLA-DR in response to these drugs may indicate that they play a role in modulating lung inflammation in addition to their known effects on the vasculature.

The principal finding of this study was the high density of inflammatory cells in the lung periphery in both treated and untreated IPAH which was not observed in normal lungs, and which was greater than that observed in APAH. This observation could have important relevance for our understanding of the pathobiology of IPAH and the effect of treatment which results in enhanced survival but not in reparative remodelling. Whilst we do not understand the mechanisms, the present observations indicate an avenue for further investigation and possible therapeutic intervention. Certainly the findings indicate that any intercurrent respiratory tract infection should be treated vigorously in children with IPAH. Future studies addressing the cause and significance of lung inflammation in IPAH and elucidation of inflammatory modulation should be pursued as a means of ameliorating some of the pathogenic features of this fatal disease.

COMPETING INTERESTS

Professor Haworth has acted as a consultant and received unrestricted educational grants from Actelion Ltd, Encysive Pharmaceuticals, GlaxoSmithKline and Pfizer.
Dr Hall, Dr Brogan and Professor Klein have no conflicts of interest.

EXCLUSIVE LICENCE STATEMENT

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FOOTNOTES

Additional figures showing expression of CD3 and S100 and of colocalisation of BMPR2 with inflammatory markers are published online.

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FIGURE LEGENDS

Figure 1. Immunohistochemical staining for (A) HLA-DR, (B) MAC387 and (C) BMPR2 in peripheral lung tissue from normal children, from those with IPAH, untreated and treated, and from children with APAH. (A) In children with a normal pulmonary vasculature HLA-DR is not expressed by endothelium in alveolar capillaries or intra-acinar arteries (arrowed, L=lumen). In untreated children with IPAH HLA-DR is expressed by alveolar wall capillary endothelium and in small intra-acinar arteries. By contrast in children treated with epoprostenol and bosentan no HLA-DR is detectable in the alveolar capillaries or intra-acinar arteries. In children with APAH (A) HLA-DR expression is not visible in the alveolar capillary endothelium (expressed by alveolar macrophages and circulating inflammatory cells, arrowed). (B) MAC387 expressing monocytes were present in the submucosa of normal children but no endothelial expression can be detected. In untreated IPAH large numbers of circulating MAC387, calprotectin are observed in the wall of a plexiform lesion and MAC387 expressing cells line some vessels (arrowed) in the intimal proliferative areas this artery whilst unlabelled vessels are also present in the same area. In IPAH treated with epoprostenol and bosentan no MAC387 expression by endothelial cells was observed. Nor was it seen in children with APAH. (C) BMPR2 expressing cells were present in the submucosa of respiratory bronchiole of normal children, untreated and treated children with IPAH and APAH and in the mesenchyme of a human fetus at 119 days gestation (inset). In all children with IPAH, both treated and untreated, BMPR2 expressing cells were also present within the media and adventitia of small partially obstructed intra-acinar arteries (arrowheads). Few were seen in arteries of normal children or those with APAH. (Scale bar represents 50µm for all pictures)

Figure 2. Dot plots showing an increase in the density of BMPR2 expressing cells around pulmonary arteries of children with IPAH compared to normal children and those with APAH (ANOVA: normal v IPAH * $p < 0.01$, Mann Whitney APH v IPAH * $p < 0.01$). There is no difference in the density of these cells around airways of IPAH and normal children but significantly fewer cells in children with APAH (Mann Whitney normal v APAH, IPAH v APAH** $p < 0.05$).

Figure 3. Characterisation of BMPR2 expressing cells. Immunofluorescent labelling shows (A) co-expression of BMPR2 and CD68 in cells that have only weak BMPR2 expression (arrowed). (B) BMPR2 expressing cells are often seen in close association with CD3 expressing T-lymphocytes. In A clumps of erythrocytes autofluorescing at both 494nm and 547nm wavelength excitation (*) are visible within the artery lumen. (Scale bar represents 20µm).

Supplemental figures

Figure S1 Immunohistochemical staining for (A) S100A4 and (B) CD3 in peripheral lung tissue from normal children, from those with IPAH, untreated and treated, and from children with APAH .

(A) In a normal child no S100A4 expression is seen in the endothelium of a muscular artery (lumen *). In untreated IPAH it is detectable in inflammatory cells in a plexiform lesion by sub-endothelial and intimal proliferative cells as well as by circulating inflammatory cells (vessel lumen *). In treated IPAH S100A4 is expressed by the endothelium (arrowed) lining a partially obstructed small muscular artery. In APAH (B) S100A4 expressing cells are less frequent in intimal regions of partially obstructed arteries (arrowed).

(B) Few CD3 T-lymphocytes are found in the alveolar walls of normal children. Scale bars represent 50µm in A, C and D and 25µm in B. In children with untreated and treated IPAH T-lymphocytes are found in and around partially obstructed peripheral arteries (lumen *) and in alveolar capillaries (arrowed). In children with APAH T-cell number and distribution is similar to normal. Scale bars represent A 25µm and B 50µm.

Figure S2. Immunofluorescent labelling shows (A) no co-localisation of BMPR2 (green) with CD34 (red). (B) BMPR2 and HLA-DR shows co-localisation only in cells weakly expressing BMPR2 (arrows), none in intensely BMPR2 expressing cells (arrowheads). (C) BMPR2 and MAC387 (red) showing no co-localisation. (D) co-expression of BMPR2 and CD68 in cells that have only weak BMPR2 expression (arrowed). (E) BMPR2 expressing cells are often seen in close association with CD3 expressing T-lymphocytes. In A clumps of erythrocytes autofluorescing at both 494nm and 547nm wavelength excitation (*) are visible within the artery lumen. (Scale bar represents 25µm).

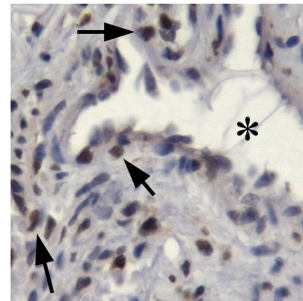
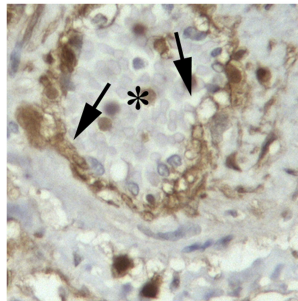
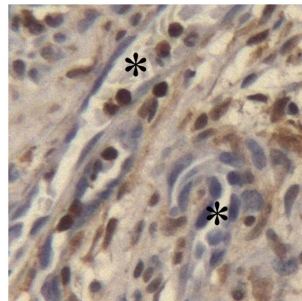
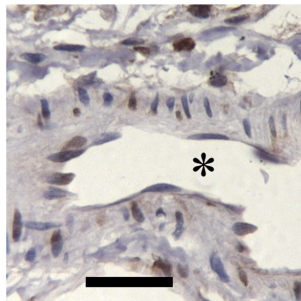
normal

IPAH untreated

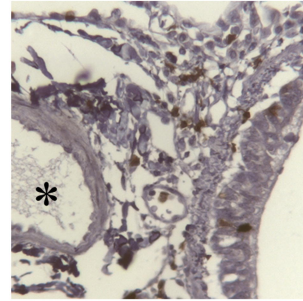
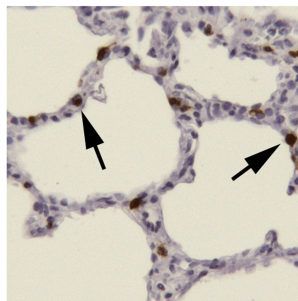
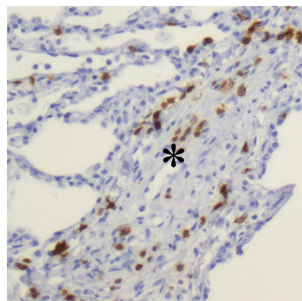
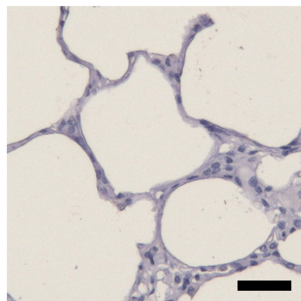
IPAH treated

APAH

A
S100A4



B
CD3



A

B

C

D

E

