Young Investigator Symposium

T1 CELL SIGNALLING AND PHENOTYPIC CHANGES IN CHRONIC HYPOXIC PULMONARY ADVENTITIAL FIBROBLASTS: POSITIVE EFFECTS OF FLUVASTASTIN ARE NOT SEEN WITH ESTABLISHED PULMONARY HYPERTENSION AGENTS

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Background: Hypoxic pulmonary hypertension (PH) is a common and important disease. Statins partially reverse experimental PH, but so do other PH therapies and we incompletely understand their utility. In hypoxic PH, pulmonary adventitial fibroblasts (PAFs) proliferate and release mitogens, causing fibroproliferative vascular remodelling. We recently identified a Rac1–p38 MAP kinase signalling pathway as the mediator of acute hypoxia-induced proliferation in PAFs, and showed that this can be inhibited by fluvastatin (Carlin *et al, AJRCMB*, 2007). It is, however, unknown if statins can influence the proliferative and mitogen-releasing PAF phenotype in chronic hypoxia. To evaluate hypoxic signalling in chronic hypoxic PAFs, further assess the therapeutic potential of statins and evaluate PAF mitogen release, we studied proliferative and MAP kinase signalling in chronic hypoxic PAFs, applied conditioned media from PAFs to pulmonary artery smooth muscle cells (PASMCs) and determined the effect of statins and other drugs.
Methods: Pulmonary hypertension was induced in rats using two weeks of hypobaric hypoxia. Cell proliferation was measured by [^{3}H] thymidine uptake. Kinase phosphorylation was determined by western blotting.
Results: Fluvastatin inhibited acute hypoxic proliferation in PAFs but sildenafil, UT-15c (a prostanoid) and bosentan had no effect. The hyperproliferative state of PAFs from chronic hypoxic rats was normalised by fluvastastin and a Rac1 inhibitor; reversal of constitutive p38 MAP kinase phosphorylation was also seen. Lobar PASMCs did not proliferate in acute hypoxia, or when conditioned media from normoxic PAFs was applied. Conditioned media from hypoxic PAFs caused PASMC proliferation. This was blocked by pre-treatment of the PAFs with fluvastastin but fluvastastin applied along with pre-conditioned media had no effect (implying an effect of fluvastatin on PAFs, rather than PASMCs). Sildenafil and UT-15c had no effect in conditioned media experiments but bosentan blocked the stimulant effect of hypoxic PAF media on PASMCs.
Conclusions: Phenotypic changes in hypoxia-stimulated PAFs are driven by a Rac1-p38 MAPK signalling pathway. PASMC mitogens released by hypoxic PAFs may include endothelin-1. Fluvastastin selectively inhibits acute and chronic hypoxia-initiated phenotypic changes in PAFs, an effect not seen with established PH drugs. These findings suggest that statin treatment may be beneficial in hypoxia-associated pulmonary hypertension.

T2 IL-33 IS SUFFICIENT TO INDUCE EOSINOPHILIC AIRWAY INFLAMMATION, AND EXACERBATES ESTABLISHED INFLAMMATION, THROUGH INCREASED ST2 DEPENDENT LOCAL TH2 CYTOKINE AND CHEMOKINE PRODUCTION

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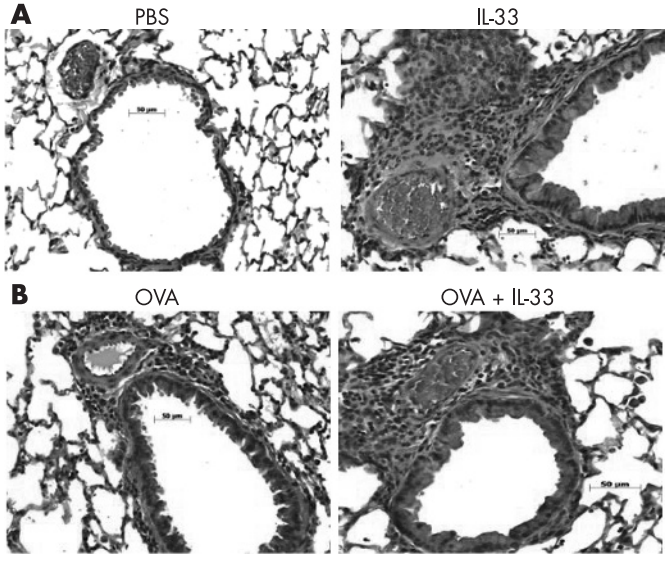
Introduction: The key pathological features of asthma are eosinophilic airway inflammation, mucus hypersecretion and airway remodelling, mediated by Th2 cytokines (IL-4, IL-5, IL-13). IL-4 or IL-13 administered directly to the airways are sufficient to induce these pathological features independent of antigen (Grunig G *et al*, *Science* 1998; Wills-Karp *et al*, *Science* 1998). IL-33 is a novel cytokine which binds to the orphan ST2 receptor and induces Th2 cytokine expression (Schmitz *et al*, *Immunity* 2005). We hypothesised that IL-33 would be sufficient to induce eosinophilic airway inflammation, and may exacerbate antigen-specific allergic airway inflammation.
Methods: The antigen-independent effect of IL-33 was assessed by administering PBS ± recombinant (r)IL-33 intranasally (i.n.) to BALB/c mice for 7 consecutive days. Bronchoalveolar lavage (BAL) was performed 24 h later. Differential cell counts were performed on cytopreps, and the concentration of BAL cytokines and chemokines determined by ELISA. Lungs were obtained for histological assessment. Allergic airway inflammation was induced in BALB/c mice by intraperitoneal (i.p.) sensitisation with ovalbumin (OVA) and alum on day 1, followed by i.n. challenge on days 9 and 11 with OVA ± rIL-33. Mice were analysed as above, and ST2 gene knockout mice were used to demonstrate the specificity of action of rIL-33.
Results: rIL-33 induced marked eosinophilic inflammation (fig A). BAL eosinophils (data not shown), cytokines (IL-5 and IL-13, but not IL-4) and chemokines (eotaxin-1, eotaxin-2 and TARC) were increased (table). Airway challenge with OVA following sensitisation induced eosinophilic inflammation, which was increased markedly by rIL-33 (fig B), as were BAL eosinophils, cytokines and chemokines (table). IL-33 had no effect in ST2KO mice in either model.
Conclusions: rIL-33 in the absence of antigen induced airway pathology very similar to that of experimental asthma. This was mediated by local induction of Th2 associated cytokines and chemokines, with the exception of IL-4, and was dependent on ST2 expression. In addition, IL-33 exacerbated allergic airway inflammation by a similar mechanism. Thus IL-33/ST2 represents an important mechanism of IL-4-independent airway inflammation, and represents a novel target for therapeutic intervention during both the initiation and maintenance phases of allergic asthma.



Abstract T2 Lung histology after (A) IL-33 alone and (B) OVA sensitisation and OVA ± IL-33 challenge.

T3 TOWARDS TARGETED TREATMENT FOR α_1-ANTITRYPSIN DEFICIENCY: RATIONALLY SELECTED SMALL MOLECULES ABOLISH THE POLYMERISATION OF Z α_1-ANTITRYPSIN IN VITRO AND IN A LIVER CELL MODEL OF DISEASE

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The common severe Z mutation (Glu342Lys) that causes α_1-antitrypsin deficiency changes the molecular structure of α_1-antitrypsin to a conformation termed M*. This structural change results in ordered self-association into chains, known as polymers, of α_1-antitrypsin. The intracellular retention of α_1-antitrypsin polymers within hepatocytes underlies the associated liver disease while lack of circulating protein compromises lung defence and predisposes to early-onset emphysema. We previously identified a hydrophobic cavity on the surface of α_1-antitrypsin that is a target to block polymerisation and so ameliorate the associated disease. In order to demonstrate the cavity's potential we prepared mutants attempting to fill it with bulky phenylalanine residues (Parfrey *et al*, *J Biol Chem* 2003;**278**:33060–6). The Thr114Phe mutation reduced the polymerisation of α_1-antitrypsin in vitro and increased the secretion of Z α_1-antitrypsin from *Xenopus* model of α_1-antitrypsin deficiency. We have now solved the crystal structure of the polymerisation-resistant mutant Thr114Phe α_1-antitrypsin. The structure (to 2.7Å resolution) demonstrates how blocking the hydrophobic cavity impedes the

Abstract T2 BAL cytokines and chemokines after administration of rIL-33 alone or in airway inflammation

	IL-33 alone (pg/ml (SEM))			IL-33 and OVA airway inflammation (pg/ml (SEM))		
Mediator	PBS (pg/ml)	IL-33 (pg/ml)	p Value	OVA (pg/ml)	OVA + IL-33 (pg/ml)	p Value
IL-4	4 (0.8)	8 (0.6)	NS	62 (17)	95 (30)	NS
IL-5	11 (4)	2022 (426)	<0.05	172 (58)	1268 (231)	<0.001
IL-13	1 (0.6)	447 (131)	<0.05	5 (4)	362 (71)	<0.001
Eotaxin-1	48 (9)	335 (26)	<0.001	60 (10)	134 (19)	<0.001
Eotaxin-2	0.3 (0.3)	1213 (90)	<0.001	82 (20)	725 (143)	<0.001
TARC	0.7 (0.7)	826 (76)	<0.001	72 (14)	478 (134)	<0.01

initial pathogenic conformational change to reduce polymerisation. We have used the cavity as a target to screen approximately 1.2 million drug-like small molecules in silico, seeking those with the potential to bind and block polymerisation in a similar way. 68 promising structures or ''hits'' have been identified, of which 6 appear to block polymerisation when tested in vitro. The most dramatic effects are seen with a compound we have labelled ''Covent Garden'' (CG). We have gone on to test 10 CG analogues: the best of these are able to block polymerisation down to a molar excess of just 2.5-fold. Moreover, treatment of a hepatic cell model of α_1-antitrypsin deficiency with CG resulted in a 70% increase in clearance of intracellular Z α_1-antitrypsin. Taken together these data demonstrate that small molecules can bind to Z α_1-antitrypsin and so block the polymerisation underlying the associated liver disease in vitro and in a liver cell model of α_1-antitrypsin deficiency. The compounds now need to be tested in vivo but this work represents an important advance in the search for effective therapies to treat α_1-antitrypsin deficiency.

T4 VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR LIGATION DIFFERENTIALLY REGULATES PRIMARY HUMAN ALVEOLAR TYPE II CELL REPAIR RESPONSES AND IS ANTI-APOPTOTIC FOR SFASL BUT NOT 0.03% H_2O_2 INDUCED CELL DEATH

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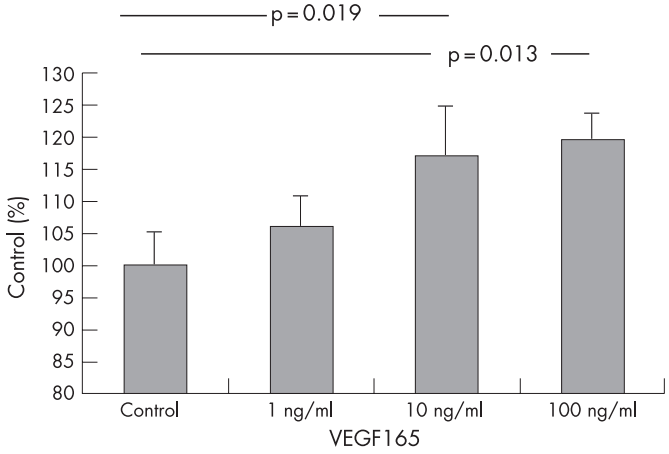
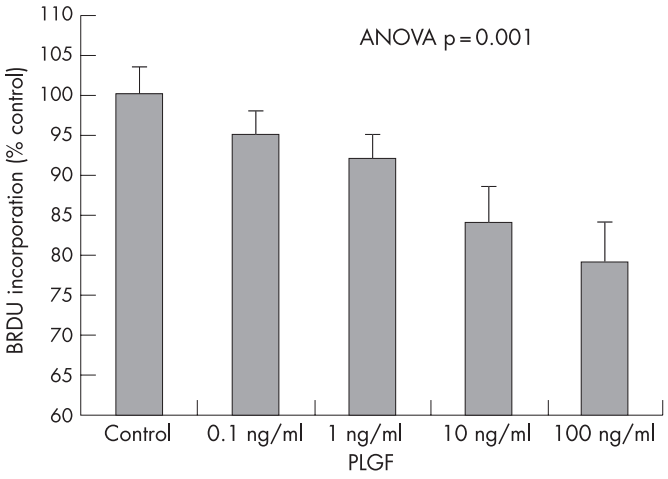
Introduction: Vascular endothelial growth factor (VEGF) signals predominantly through two receptors associated with tyrosine kinases, VEGFR-1 and VEGFR-2. Soluble VEGF isoforms VEGF165 and VEGF121 stimulate receptors 1 and 2 whereas placental growth factor (PLGF) is a specific VEGFR-1 ligand. The effects of VEGF upon primary human alveolar epithelial type II (ATII) cells are unknown. We aimed to determine the effect of VEGF on primary ATII cell proliferation, repair and apoptosis.
Methods: Primary human ATII cells were extracted from human lung resection tissue from patients with normal lung function (n = 18, mean FEV_1% predicted 98%) undergoing resection for lung cancer, using the method of Witherden and Tetley. Expression of surfactant protein C,



Abstract T4 Figure 1 ATII BRDU incorporation is stimulated by vascular endothelial growth factor.



Abstract T4 Figure 2 Placental growth factor inhibits ATII BRDU incorporation.

VEGFR-1 and VEGFR-2 was confirmed by PCR. Proliferation of ATII cells was assessed using a BRDU assay. The effects of VEGF were assessed using an in vitro scratch model. Recombinant sFASL and 0.03% H_2O_2 were used to stimulate submaximal apoptosis.
Results: Human ATII cells express mRNA for both VEGFR-1 and VEGFR-2. VEGF 165 caused a dose dependent increase in proliferation (fig 1). Thus, VEGF165 stimulated wound repair above control (+20.5% (SE 5), p = 0.045) and % total wound repair (37% vs 31%, p = 0.04). In contrast, PLGF (the VEGFR-1 ligand) caused a dose dependent reduction in BRDU incorporation; this was 79% (SE 5.12) of control (p = 0.001) following 100 ng/ml PLGF (fig 2). Unstimulated human ATII cells are resistant to exogenous sFASL protein (5–500 ng/ml). Pre-incubation of ATII cells with 1 ng/ml TNF-α for 6 h sensitised ATII cells to the effects of sFasL, causing a dose dependent reduction in viability (p = 0.001). Treatment of ATII cells with 10 ng/ml VEGF121 abrogated this effect (p = 0.005). VEGF did not protect ATII cells against the oxidative effect of 0.03% H_2O_2.
Conclusions: VEGF induces proliferation and wound repair of ATII cells. Since PLGF inhibits ATII proliferation, these effects appear mediated by VEGFR-2. VEGF protected against sFasL induced death; as VEGF did not protect against the effects of 0.03% H_2O_2 its protective effects appear dependent upon the injurious stimulus. These results suggest that VEGF may have potential as a rescue therapy for diseases such as ARDS which are associated with reduced VEGF bioactivity.

T5 AIRWAY NEOVASCULARISATION BY AIRWAY SMOOTH MUSCLE IN ASTHMA: NEW CONDUITS TO INFLAMMATION AND REMODELLING

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Introduction: Airway neovascularisation, seen as increases in the size and number of bronchial blood vessels with angiogenic sprouts, and accumulation of airway smooth muscle (ASM) are prominent features of airway remodelling in asthma.[1] Bronchial vessels are conduits for the inflammatory process, where vascular engorgement, vasodilatation and vascular leakage lead to airflow obstruction and airway

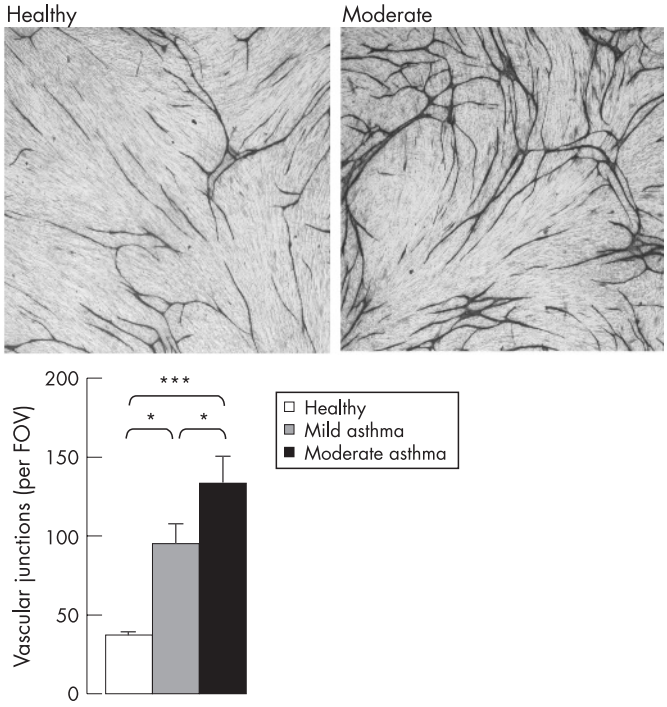
hyper-responsiveness. Recent studies suggest an imbalance between protective antiangiogenic and proangiogenic factors in asthmatic airways,[2 3] which leads to induction of vascular tubules.[3] Moreover, bronchial vessels and ASM bundles are in close anatomical proximity, and accumulating evidence indicates that ASM has important synthetic functions in asthma including the production of the angiogenic growth factor, vascular endothelial growth factor (VEGF).

Objectives: To characterise (i) the profile of angiogenic growth factors expressed by ASM, (ii) to examine the capacity of ASM to induce angiogenesis and (iii) to determine if this varies with presence and severity of asthma.

Methods: Healthy, non-atopic (n = 9), mild (n = 12) steroid naive and moderate (n = 8), atopic asthmatic volunteers underwent bronchoscopy and deep endobronchial biopsy. ASM explant cultures were established from microdisected biopsy tissue. Cell-conditioned medium (CM) was collected from unstimulated ASM, and angiogenic growth factor release profiles were obtained using antibody arrays and by sandwich ELISA. Proangiogenic activity in CM was investigated using an in vitro angiogenesis assay comprising co-cultures of human umbilical vein endothelial cells with human dermal fibroblasts. CM was added before and after selective protein-A agarose immunodepletion of either VEGF, angiopoietin [Ang]-1 or angiogenin. Vascularity was visualised following anti-CD31 labelling and numbers of vascular junctions, tubules and vascular length were quantified by image analysis. To investigate angiogenic growth factors in an airway context ex vivo model, whole endobronchial biopsies from healthy subjects were incubated for 72 h in the absence or presence of exogenous VEGF/Ang-1/angiogenin (all 50 ng/ml). Microvessels in sequential histology sections were labelled with anti-CD31 and counterstained with haematoxylin. Vessel numbers/mm^2 and percentage vascular area were determined by image analysis.

Results: Induction of CD31-labelled tubule formation was increased by CM from ASM cells cultured from subjects with mild asthma (3.0-fold) and moderate asthma (4.2-fold), compared with healthy ($p<0.05$, n = 6–11, fig). Levels of the multiple proangiogenic growth factors were similarly elevated in CM from asthmatic ASM compared with healthy controls ($p<0.05$, n = 4): VEGF (2.5-fold), angiogenin (4.4-fold), EGF (3.9-fold), IGF-1 (1.8-fold), IFNγ (2.5-fold), Ang-1 (2.3-fold), whereas antiangiogenic factors (endostatin and Ang-2) were unchanged. Selective VEGF immunodepletion abolished enhanced tubule formation ($p<0.001$, n = 4) by CM from asthmatic ASM ($p<0.01$), but depletion of Ang-1 or angiogenin had no effect. Ex vivo treatment of whole endobronchial biopsies from healthy subjects with VEGF/Ang-1/angiogenin increased the number of submucosal CD31-labelled microvessels (1.8-fold) and overall vascular area (1.9-fold) in histological sections, compared with controls ($p<0.01$, n = 3).

Conclusions: Consistent with the imbalance hypothesis in asthma,[2] ASM from asthmatics expresses elevated proangiogenic but not antiangiogenic factors, and promotes induction of CD31+ vascular structures in both an in vitro and ex vivo model of vascularity. The increased proangiogenic capacity of ASM from asthmatics was severity-related and resided in enhanced release of VEGF. Proangiogenic factors released by ASM could represent a critical pathway for airway neovascularisation in asthma. Defining these processes has potential importance for development of new asthma therapies that target neovascularisation to limit blood flow and delivery of inflammatory cells to remodelled airways. These findings have wider applicability to other chronic lung diseases characterised by increased vascularity.



Abstract T5 Induction of vascular tubules by unstimulated airway smooth muscle cells cultured from healthy subjects or patients with mild or moderate asthma. *$p<0.05$, ***$p<0.001$ (n = 6–11).

1. **Li X**, Wilson JW. *Am J Respir Crit Care Med* 1997;**156**:229–33.
2. **Asai K**, Kanazawa, et al. *J Allergy Clin Immunol* 2002;**110**:571–5.
3. **Simcock DE**, Kanabar V, *et al. Am J Respir Crit Care Med* 2007;**176**:146–53.

T6 REGULATION OF ID GENE EXPRESSION BY MAPK AND SMAD1/5 PATHWAYS IN PULMONARY ARTERY SMOOTH MUSCLE CELLS

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Background: Heterozygous germline mutations in the gene encoding the bone morphogenetic protein type II receptor (BMPR-II) have been identified in over 70% of patients with familial pulmonary arterial hypertension (FPAH). The impact of BMPR-II mutation on BMP signalling and regulation of gene expression in pulmonary vascular cells remains unclear. Since inhibitors of DNA binding (Id) genes are key targets of BMP signalling involved in cell cycle and development, we studied the regulation of Id gene expression in control pulmonary artery smooth muscle cells (PASMCs) and cells harbouring mutations in BMPR-II.

Methods and Results: BMP4 and 6 increased phosphorylation of Smad1/5 and extracellular signal-related kinase 1/2 (ERK1/2) in normal PASMCs. In mutant cells, delayed activation of Ras and ERK1/2 was observed, in addition to reduced Smad1/5 phosphorylation. Furthermore, we demonstrate that activation of ERK1/2 phosphorylates the linker region (serine 206) of Smad1, limiting c-terminal serine 463/465 phosphorylation and inhibiting Smad nuclear translocation. To establish the functional significance of these observations we determined the regulation of Id gene expression by BMPs. BMP4 markedly induced Id1 gene expression which was further enhanced by ERK1/2 inhibition. Conversely, activation of ERK1/2 by PDGF-BB inhibited BMP4 stimulated Id1 gene expression. Id1 and Id2 were shown to contribute to the antiproliferative effects of BMP4 in PASMCs, since knockdown of either gene by siRNA prevented growth inhibition by BMP4. Moreover, Id1 and 2 expression was significantly reduced within obliterative vascular lesions in lung sections from patients with familial pulmonary arterial hypertension (FPAH).

Conclusion: Taken together these findings indicate an important interaction between ERK1/2 and Smad1/5 in the regulation of BMP responsive genes in PASMCs, and demonstrate how important pathways implicated in vascular remodelling can critically reduce BMP signalling. Our studies further define Id1 and Id2 as a critical growth regulatory gene that are differentially regulated in mutant PASMCs and may contribute to the pathogenesis of FPAH.