BTS/BLF Young Investigators Prize Symposium

T1 PERTURBED TRANSFORMING GROWTH FACTOR β SIGNALLING IN FIBROBLASTS INCREASES THE SUSCEPTIBILITY OF ALVEOLAR EPITHELIAL CELLS TO INJURY IN A NOVEL MOUSE MODEL OF SYSTEMIC SCLEROSIS

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Background: Altered transforming growth factor β (TGFβ) bioactivity is implicated in the pathogenesis of systemic sclerosis (SSc). Transgenic mice with fibroblast-specific perturbation of TGFβ-signalling (TβRIIΔk-fib) develop cutaneous fibrosis, and sporadic lung fibrosis from 16 weeks, mimicking SSc. We postulate that increased susceptibility to alveolar epithelial cell (AEC) injury may underlie the development of lung fibrosis in the TβRIIΔk-fib model.
Methods: Wildtype (WT) and transgenic (TG) animals, aged 6 weeks, were injured with intratracheal saline (0.9%, pH 5.7) or bleomycin (0.03U); lungs were harvested after 3–21 days for biochemical, histological and electron microscopic analysis.
Results: There was ultrastructural evidence of baseline AEC abnormality in untreated TG with AEC crenellation; similar changes were seen in WT after injury, whereas bleomycin-treated TG (TG-B) showed severe epithelial damage. Increased lung cellularity, parenchymal connective tissue, and non-crosslinked collagen was demonstrated with H&E, Masson's trichrome and picrosirius red stains respectively. Compared with WT-B, TG-B lungs demonstrated increased TGFβ1 and PAI-1 expression, increased myofibroblast (α-SMA) persistence, reduced TTF-1 signal consistent with impaired type II AEC hyperplasia, and increased AEC II apoptosis (caspase-3). Saline treated TG (TG-S) and WT-B had phenotypic similarities, implying greater TG susceptibility to minor injury. These findings support the notion that TG animals exhibit an exaggerated fibroproliferative response to AEC injury, compounded by a reduced capacity for epithelial repair. Northern blotting revealed that collagen mRNA expression was elevated ($p<0.0001$) in TG compared with WT after saline or bleomycin. Colorimetric analysis showed that newly-synthesised collagen was increased in TG compared with WT after bleomycin ($p=0.002$) or saline ($p=0.0002$). Thus, RNA and protein analysis confirm increased matrix deposition in TG animals after injury. Despite exaggerated fibrosis, neutrophilic infiltration, quantified by myeloperoxidase activity, was attenuated in TG-B compared with WT-B ($p=0.008$).
Conclusions: Thus, our results suggest that in the context of systemic perturbation of TGFβ bioactivity in the TβRIIΔk-fib strain, even minor epithelial injury induces significant fibrosis. Our results support a model in which altered AEC structure and exaggerated response to injury, rather than neutrophilic inflammation, trigger lung fibrosis, and may be particularly relevant to SSc.

T2 HAPLOTYPE ANALYSIS AND FUNCTIONAL STUDIES REVEAL A ROLE FOR F2R (PAR1) POLYMORPHISMS IN SARCOIDOSIS

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Background: Activation of Proteinase-Activated Receptor-1 (PAR_1) by coagulation proteinases such as thrombin contributes to lung inflammation and fibrosis following lung injury. F2R (PAR_1) gene expression is increased in fibroproliferative lung disease and F2R-deficient mice are protected in an experimental model of bleomycin-induced lung inflammation and fibrosis; however, the regulation of F2R, and the role of genetic influences, has not been characterised. We previously reported that the F2R promoter polymorphism, -506ins, associates with sarcoidosis in UK white and Afro-Caribbean subjects. We have now performed a haplotype analysis and report the first functional findings for the -506ins.
Methods: Five F2R polymorphisms were genotyped in a UK sarcoidosis population by PCR-based techniques. The function of the -506ins polymorphism was investigated using reporter gene assays in HeLa and A549 cell types.
Results: Haplotype analysis revealed that the -506ins conferred a greater risk of susceptibility to sarcoidosis when in the presence of other F2R polymorphisms. In reporter gene assays, both basally, and following exogenous TNF-α for 2 hours, the -506ins allele had significantly higher promoter activity compared with the wild type. For HeLa cells, this was 2.0 (0.22)-fold higher basally ($p<0.0001$), and 2.7 (0.04)-fold higher ($p<0.0001$). For A549 cells, this was 2.3 (0.03)-fold higher basally ($p<0.0001$), and 1.9 (0.27)-fold higher upon exposure to TNF-α ($p<0.0001$). Interestingly, in fibroblast cell lines derived from healthy lung tissue, only 2/9 (22%) carried the -506ins compared with 2/3 (67%) derived from fibrotic lung tissue.
Conclusion: The -506ins polymorphism appears functional, with the -506ins allele increasing promoter activity by approximately twofold. Furthermore, haplotype analysis suggests that other F2R polymorphisms may also be important, possibly functionally interacting with the -506ins. Elucidating the function of F2R polymorphisms may help ascertain the mechanism by which PAR_1 contributes to fibroproliferative lung disease.

T3 OSTEOPONTIN IS EXPRESSED IN HUMAN PERIPHERAL BLOOD EOSINOPHILS AND CONTRIBUTES TO THEIR PRO-ANGIOGENIC ACTIVITY

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Rationale: Eosinophils are key cells in allergic airway inflammation and play an active role in airway remodelling and angiogenesis. Osteopontin (OPN), also known as Eta-1 (early T-lymphocyte activation 1), is a novel Th1 cytokine with structural homology to matrix proteins. It is highly expressed protein in a range of lung diseases and has been shown to regulate aspects of inflammation, fibrosis, and angiogenesis. In this study we hypothesise that eosinophils are a source of OPN and exert their pro-angiogenic effects throught this novel cytokine.
Methods: OPN expression was characterised on human eosinophils purefied from peripheral blood of atopic donors (fluorescence microscopy and RT-PCR analysis) and in eosinophilic nasal polyp speciments (immunohistochemistry). The release of OPN from eosinophils treated with GM-CSF, IL5, IL3 or Eotaxin was measured in their supernatants (ELISA). The effect of OPN on eosinophil migration was also studied in vitro using ChemoTx System microplate wells and the involvement of VLA-4 integrin in OPN-induced eosinophil migration was investigated by pre-treating the cells with a selective compound for VLA-4 before performing the chemotaxis assay. To study the contribution of OPN in eosinophil-induced angiogenesis in vivo the eosinophils were incubated for 30 minutes with neutralising anti-OPN antibodies and their effect on vessel sprouting in chick embryo chorioallantoic membranes (CAM) was evaluated.
Results: Eosinophils constitutively expressed OPN at protein and messenger RNA levels. GM-CSF and IL-5, but not IL-3 and Eotaxin enhanced the OPN release by eosinophils in a time- and dose-dependent manner with the highest quantity observed after 15 hours of treatment at 2 ng/ml ($p<0.01$). Recombinant OPN significantly enhanced eosinophil chemotaxis in a dose-dependent manner with the highest effect at 500 ng/ml ($p<0.01$) and this effect was completely inhibited by pretreatment of the eosinophils with a selective antagonist for VLA-4. Neutralisation of eosinophil-derived OPN reduced significantly eosinophil-induced angiogenesis in the CAM assay ($p<0.01$).
Conclusions: OPN can be considered as a new eosinophil-derived cytokine/matrix protein with functional relevance in eosinophil-associated airway diseases, where angiogenesis takes place.

T4 ENDOSTATIN: THE LINK BETWEEN ABERRANT ANGIOGENESIS AND DYSREGULATED EPITHELIAL REPAIR IN IDIOPATHIC PULMONARY FIBROSIS

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Introduction: Pathological studies of IPF suggest aberrant angiogenesis with evidence of both epithelial apoptosis and defective re-epithelialisation of the alveolar barrier. The role of inflammation in this process is controversial. Endostatin is a 20 kda product of collagen XVII which is found in the basement membranes of alveolar capillaries. It has been implicated in the microvascular damage found in pre-eclampsia and used as a treatment for neo-angiogenesis of tumours. We hypothesised that endostatin levels may be elevated in the broncho-alveolar lavage fluid (BALF) and plasma of patients with IPF and that it may contribute to the dysfunction of the epithelial/endothelial barrier with subsequent loss of lung function.
Methods: Twenty seven patients with IPF, diagnosed by the ATS/ERS consensus statement, and 10 normal controls underwent BAL. Endostatin was measured in BALF and plasma by ELISA. BALF cytokines and VEGF were measured by ELISA. Primary distal lung epithelial cells (DLEC) were cultured to a monolayer and wounded with a 1 mm mechanical wound. The wounds were photographed at 0 and 18 hours and analysed with Scion image. The cells were incubated with 100 ng Endostatin, with and without anti-FASL (1 ng/ml) and the caspase inhibitor Z-DEVD-FMK (20 μM/ml). Apoptosis was assessed by flow cytometry and viability by Cell titre. The presence of endostatin fragments was confirmed with Western blotting.
Results: Endostatin was elevated in the BAL (mean 1.27 *v* 0.40 ng/ml $p=0.007$) and plasma (mean 259.2 *v* 94.7 $p=0.026$) of patients with IPF compared with normal controls. Western blotting confirmed the presence of multiple type XVIII collagen fragments in the BAL. Endostatin had a negative correlation with forced vital capacity, a marker of severity in IPF ($r=-0.538$, $p=0.007$). BAL Endostatin correlated with the pro-angiogenic factor VEGF ($r=0.532$, $p=0.001$) as well as the pro-inflammatory cytokines IL-6 ($r=0.449$, $p=0.028$) and IL-8 ($r=0.472$, $p=0.006$). Endostatin inhibited wound repair by 44% ($p=0.011$). It increased apoptosis of the DLEC by 9% ($p<0.001$) and reduced viability by 39% ($p<0.001$). This effect was partially FASL ($p=0.03$) and caspase ($p=0.003$) dependent.
Conclusions: This is the first study to demonstrate the presence of endostatin in the BAL and plasma of patients with IPF. Endostatin levels correlated with the degree of lung function impairment and levels of inflammation. In addition to its known inhibitory effects on endothelial cells, endostatin also impairs DLEC wound repair. These effects on DLEC appear to involve apoptosis, be partially FASL dependent and can be abrogated by caspase inhibition. These data suggest that endostatin may provide a molecular link between collagen degradation, aberrant angiogenesis and epithelial repair.

T5 NEW TUBERCULOSIS VACCINES CAN DIMINISH AS WELL AS ENHANCE BCG INDUCED PROTECTION: RESULTS FROM A MURINE MODEL

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Introduction: There is an urgent need to improve the efficacy of BCG. TH1 CD4 T cells are essential to protection, but CD8 T cells are also important in preventing *M tuberculosis* (*M tb*) reactivation. Heterologous prime-boost vaccine strategies, using BCG (B) as a prime are being developed. Immunodominant mycobacterial antigens, such as antigen 85A (Ag85A), delivered using viral vectors, are being investigated as boosts. Pox viruses, such as MVA, are good at inducing CD4 T cells, whereas adenoviruses are known to induce CD8 T cells. We hypothesised that combining these viral vectors would enhance protection against *M tb* challenge.
Method: Mice were vaccinated with BCG (B) followed by different combinations of Ag 85A containing recombinant viral vectors. Ag 85A specific effector and regulatory responses were determined in the lungs and spleens prior to aerosol *M tb* challenge. Numbers of CD4 and CD8 IFNγ producing cells were determined by ex vivo IFNγ Elispot and phenotyped by FACs. Quantification of TH2, inflammatory and regulatory cytokines was assessed by RT-PCR and cytometric bead assay, and T regulatory cells (T_{Reg}), by FACs. Protective efficacy was determined, 4 weeks after *M tb* challenge, by counting CFU of homogenised organs plated on Middlebrook plates and by histopathological scoring of inflammation.
Results: The bacterial load in the lungs was reduced from 6.5 in naïve mice to 4.7 $\log_{10}$ CFU in B vaccinated mice. Mice boosted twice with MVA85A (BMM) further reduced the bacterial load to 4.0 $\log_{10}$ CFU ($p<0.01$), whereas boosting with Adeno85A followed by M (BAM) increased the bacterial load to 5.1 $\log_{10}$ CFU ($p<0.05$). There was a direct association between CFU counts and severity of pathology. The number of IFNγ producing CD4 T cells was similar between regimens pre-challenge, whereas there were significantly more CD8+ T cells in the BAM regimen ($p<0.05$). 90% of antigen specific CD4 T cells were of an effector phenotype, irrespective of regimen. There were phenotypic differences in CD8 T cells, with greater CD43+ expression in BAM vaccinated mice ($p<0.05$). Induction of inflammatory cytokines such as TNFα and IL6 was also greater in the BAM regimen. Immunoregulation was similar between regimens, with the same number of T_{Reg}, and concentration of IL10, TGFβ and TH2 cytokines. Using RT-PCR, the pattern of IFNγ responses induced by antigen 85A stimulated cells pre-challenge was mirrored by those of unstimulated lung homogenates 6 days post TB challenge.
Conclusions: BMM enhanced B induced protection in a pre-exposure model, yet the induction of more IFNγ producing CD8 cells and more inflammatory cytokines by BAM was associated with diminished protection. This emphasises the requirement of new TB vaccines to induce a balanced immune response. It would be interesting to assess efficacy of the same regimens 6 months after vaccination.

T6 COGNITIVE DEFICITS IN PATIENTS WITH MODERATE TO SEVERE OBSTRUCTIVE SLEEP APNOEA

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Background: Obstructive sleep apnoea (OSA) is characterised by intermittent hypoxia and daytime somnolence. We hypothesised that OSA patients would show cognitive deficits commensurate with the amount of intermittent hypoxia.
Methods: Forty two patients (median (range) age 51 (30–69) years; 5 female) and 31 controls (age 48 (33–69); 13 female) free from comorbidities were recruited from sleep clinics and the general population, respectively. Attention, semantic and episodic memories were tested using a battery of neuropsychological tests. OSA was measured by polysomnography. Subjective (Epworth Sleepiness Scale; ESS) and objective (OSLER test) measurements of sleepiness were also made. Patients were grouped according to the amount of intermittent hypoxia (oxygen desaturation index (ODI); mild <15 events/h, moderate-severe >15 events/h) and outcomes were compared with Mann-Whitney U tests.
Results: Thirteen mild (mean (SD) ODI: 9.7 (2.6) events/hr) and 29 moderate-severe (ODI: 44.8 (32.0) events/h) patients and 31 controls (ODI: 1.8 (1.5) events/h) were studied. Compared to controls, patient groups did not differ in age ($p=0.17$), objective sleepiness (missed OSLER responses, standardised for time on task; $p=0.67$) subjective sleepiness (ESS; $p=0.67$), or years of education ($p=0.19$). Moderate-severe patients had impaired semantic memory compared to controls (median (range) category fluency test: controls 117 (51–165), mild 105 (64–156), and moderate-severe 90 (54–181); $p=0.05$). Verbal episodic memory was also impaired in the moderate-severe patients compared to controls (Logical memory immediate recall test: controls 43 (16–64), mild 38 (28–69), moderate-severe 35 (21–54) $p=0.01$; Logical memory delayed recall test, controls 27 (7–41), mild 23 (15–42) and moderate-severe 22 (14–36) $p=0.01$). Mild patients had no impairment in semantic ($p=0.58$) or episodic memory (Logical memory immediate recall test, $p=0.25$; Logical memory delayed recall, $p=0.06$) compared to controls.
Conclusions: Patients with moderate-severe intermittent hypoxia show deficits in semantic and verbal episodic memory in the absence of significant sleepiness.

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