

BTS/BLF/BALR Young investigators symposium

T1 REV-ERB α , A NOVEL ANTI-INFLAMMATORY TARGET, MODIFIES THE CIRCADIAN OSCILLATION OF PULMONARY INFLAMMATION

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Introduction Most inflammatory diseases demonstrate diurnal variation in severity, for example, chronic obstructive pulmonary disease (COPD) and asthma. We now show that the macrophage inflammatory response is regulated by the circadian cellular clock through Rev-erb α , a nuclear receptor. Additionally, a Rev-erb α ligand (GSK414112) regulates a distinct cytokine network in COPD alveolar macrophages.

Results Circadian oscillation of core clock genes BMAL1, Per2 and Rev-erb α in human macrophages (mdms) demonstrates a functional cellular clock. For the first time we reveal that the inflammatory response is dependent on clock phase, exemplified by altered IL-6 LPS stimulated expression ($p < 0.01$). The critical function of Rev-erb α was confirmed through inhibition of the exaggerated IL-6 response by GSK414112 ($p < 0.01$). Lentiviral knockdown of Rev-erb α expression enhanced the IL-6 LPS response ($p < 0.05$) and attenuated GSK414112's effect. It is known that GSK414112 recruits HDAC3 and NCOR onto Rev-erb α repressing transcription. To define whether Rev-erb α directly interacted with the IL-6 promoter, GSK414112's effect on a series of IL-6 reporter genes was investigated. The full length IL6-luc reporter was repressed by GSK414112 ($p < 0.01$) but mutations to the closely related C/EBP ($p < 0.01$) or 3'AP-1 binding sites ($p < 0.05$) inhibited this effect. ChIP studies confirmed direct interaction of Rev-erb α with the IL-6 promoter. Reporter genes expressing consensus sites for C/EBP and AP-1 showed regulation of these transcription factors by Rev-erb α . Novel Rev-erb α actions were identified through transcriptome profiling of human mdms. SERPINE2, IL-6, PTX3 and MMP-12, all implicated in COPD, are regulated by GSK414112. The analysis also revealed a novel mechanism of action, reverse cholesterol transport, previously implicated in pulmonary inflammation. Luminex analysis on mdms from healthy volunteers and COPD alveolar macrophages revealed that GSK414112 significantly repressed secretion of certain cytokines including IL-6, eotaxin, IL-10, IP-10, G-CSF whilst other cytokines, for example, IL-8, TNF α , MIP 1 α were unaffected.

Conclusion Through Rev-erb α an autonomous cellular clock modifies the macrophage LPS response. Ligands for this nuclear receptor exert anti-inflammatory effects through suppression of target gene transcription and up regulation of the cholesterol efflux pathway, employing a novel transcription factor cross-talk mechanism. This mechanism is effective in suppressing glucocorticoid resistant targets as well as targeting the temporal aspects of inflammation.

T2 BLOCKADE OF INTRAALVEOLAR P55 TNF-RECEPTOR SIGNALLING BY A DOMAIN ANTIBODY DECREASES INFLAMMATION AND OEDEMA IN AN *IN VIVO* MOUSE MODEL OF VENTILATOR-INDUCED LUNG INJURY

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Introduction and Objectives Tumour necrosis factor (TNF) alpha is transiently up-regulated within the alveolar space during ventilator-induced lung injury (VILI). We previously found that the two TNF

receptors play opposing roles during VILI in knock-out mice, with p55 promoting but p75 preventing pulmonary oedema. This suggests that specific blockade of p55 receptor signalling within the alveolar space may be beneficial in VILI. Domain antibodies (dAbs) are the smallest antigen-binding fragments of the IgG molecule, which may have advantages over complete antibodies due to their small size and monovalent binding (mAbs often have agonist activity due to receptor cross-linking). In this study we tested the effects of an intratracheally (i.t.) delivered dAb that binds to and inhibits the murine p55 receptor (Biopharmaceutical R&D, GlaxoSmithKline), on pulmonary oedema and inflammation in mouse models of VILI.

Methods C57BL6 mice were ventilated with a high-stretch protocol (standardised by plateau pressure at 12.5–13.5 cm H₂O; tidal volume 20–22 ml/kg, PEEP 3 cm H₂O, O₂ with 2–4% CO₂). Mice then received an i.t. bolus of either non-specific 'dummy' dAb or p55-specific dAb (25 μ g in 50 μ l), and were ventilated for up to 4 h (1-hit model). As a 2-hit model, 20 ng LPS were included in the dAb bolus. Development of lung injury was assessed by respiratory elastance and blood gases, and protein level in bronchoalveolar lavage fluid (BALF) at termination. In the 2-hit model, neutrophil infiltration into BALF, the activation state of alveolar macrophages, and neutrophil margination within lung vasculature were all assessed by flow cytometry.

Results High stretch ventilation produced deteriorations in elastance and PO₂ and high BALF protein in both models. Treatment with the p55-specific dAb substantially attenuated all of these changes in the 1-hit model (Abstract T2 Table 1). In the 2-hit model, p55 blockade prevented deteriorations in elastance and oxygenation, and significantly decreased neutrophil margination, intraalveolar neutrophil infiltration and ICAM-1 expression on alveolar macrophages.

Abstract T2 Table 1

	dummy dAb		p55-dAb	
	After instillation	End	After instillation	End
1-hit model				
Elastance (ml/cmH ₂ O/kg)	0.91 \pm 0.13	1.07 \pm 0.21*	0.91 \pm 0.16	0.93 \pm 0.18
PO ₂ (mm Hg)	444 \pm 72	287 \pm 180*	450 \pm 26	415 \pm 86
BALF protein (mg/ml)		2.9 \pm 1.7		1.4 \pm 0.4†
2-hit model				
Elastance (ml/cmH ₂ O/kg)	0.94 \pm 0.11	1.08 \pm 0.2*	0.88 \pm 0.08	0.9 \pm 0.11
PO ₂ (mm Hg)	486 \pm 21	305 \pm 168*	497 \pm 16	434 \pm 114
BALF protein (mg/ml)		3.0 \pm 1.6		2.1 \pm 1.5
Lung neutrophils		2.30 \pm 0.57 $\times 10^6$		1.25 \pm 0.34 $\times 10^6$ †
BALF neutrophils/ml		1.92 \pm 1.69 $\times 10^5$		0.47 \pm 0.41 $\times 10^5$ †
ALveolar macrophage ICAM-1 (MFI)		80 \pm 20		56 \pm 13†

Mean \pm SD, N=8–10.* $p < 0.05$ vs after instillation.† $p < 0.05$ vs dummy.

Conclusions Use of dAbs to selectively inhibit intra-alveolar p55 TNF receptor signalling may open new therapeutic approaches for ventilated patients with acute lung injury. This study was supported by Biopharmaceutical R&D, GlaxoSmithKline.

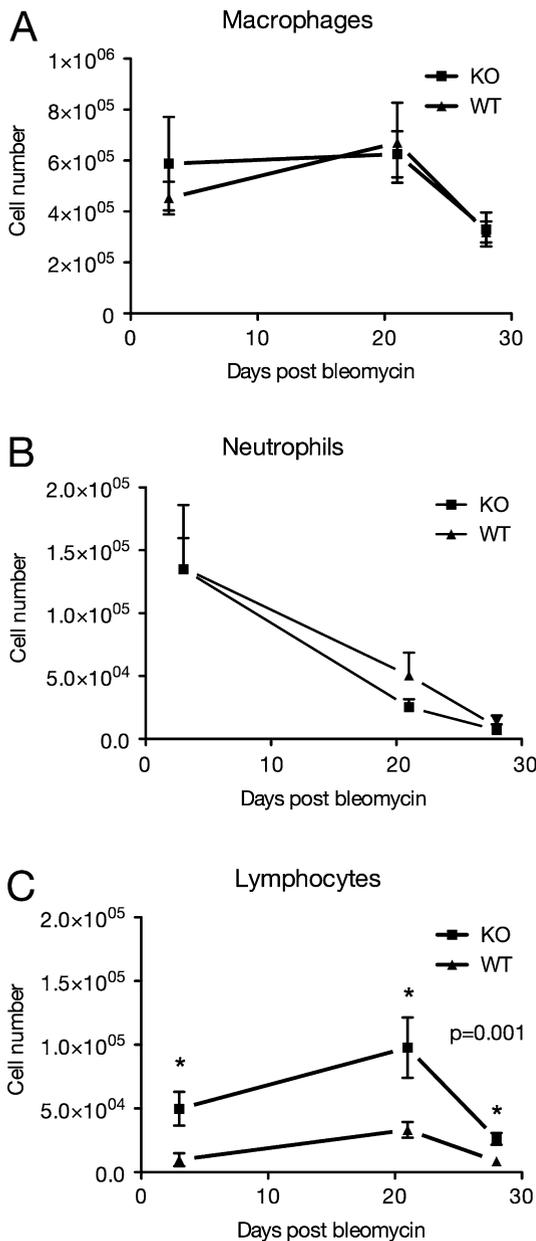
T3 TISSUE INHIBITOR OF METALLOPROTEINASE-3 (TIMP3) PROTECTS AGAINST INFLAMMATORY PROCESSES IN INTERSTITIAL LUNG DISEASE (ILD)

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Introduction TIMP3 expression in the lung increases with age and in ILD. TIMP3 binds extracellular matrix (ECM) where it influences

cellular behaviour both via inhibition of MMPs and unique functions including inhibition of ADAM17 mediated release of membrane-bound TNF α . TIMP3 gene polymorphisms protect against hypersensitivity pneumonitis (HP) and TIMP3^{-/-} mice show spontaneous pulmonary airspace enlargement, increased inflammatory responses in models of hepatic injury and arthritis and increased neoangiogenesis. We hypothesise that TIMP3 and TIMP3 gene polymorphisms contribute to inflammatory processes in ILD. This has been investigated by TIMP3 gene SNP discovery and a case-control SNP association study in sarcoidosis. SNP function has been investigated in primary human cells from sarcoidosis patients and controls of known genotype. Finally the response of TIMP3^{-/-} mice to lung injury with bleomycin has been examined. **Methods** *SNP discovery*: the TIMP3 gene was PCR amplified and sequenced in 22 subjects. *Association studies*: 175 UK AfroCaribbean sarcoidosis patients and 284 controls were genotyped using TaqMan



Abstract T3 Figure 1 Leukocyte populations in BAL fluid of wild type and TIMP3^{-/-} animals at day 3, 21 and 28 post-bleomycin treatment, showing A. Total macrophage numbers, B. Total neutrophil numbers, C. Total lymphocyte numbers. A significant lymphocytosis was seen in TIMP3^{-/-} mice ($p=0.001$, two-way ANOVA). $n=9$ in both genotypes at all time points.

assays. *Functional studies*: alveolar macrophages (AM) were isolated from bronchoalveolar lavage (BAL), $n=18$, and monocyte-derived macrophages (MDM) from venous blood, $n=14$, with real-time PCR of TIMP3 mRNA via TaqMan assay. *Animal studies*: TIMP3^{-/-} mice and controls were treated with oropharyngeal bleomycin (2 mg/kg) and lungs assessed at 3, 21 and 28 days for BAL, histology, RNA and protein analysis.

Results The TIMP3 gene is conserved with 2 promoter SNPs and 2 synonymous SNPs in exon 3 identified. Carriage of at least one SNP showed a protective effect against sarcoidosis, odds ratio (OR) 0.68 ($p=0.019$) driven by patients <35 years, OR 0.56 ($p=0.016$). The protective haplotype associates with increased TIMP3 gene expression in AM and MDM ($p=0.021$). TIMP3^{-/-} mice show increased lymphocytosis in BAL at days 21 and 28 ($p=0.001$) (Abstract T3 Figure 1) more diffuse injury and increased neoangiogenesis within lesions on histological assessment.

Conclusions TIMP3 promoter SNPs may increase gene expression and appear protective against granulomatous lung disease. Absence of TIMP3 leads to increased lymphocytic inflammation in the bleomycin model. Mechanisms for this are being investigated. Together these findings suggest that TIMP3 restricts lymphocytic inflammation in the lung.

T4 INCREASED RETICULAR BASEMENT MEMBRANE THICKNESS BUT NOT AIRWAY SMOOTH MUSCLE IN ENDOBRONCHIAL BIOPSIES OF SEVERE PRESCHOOL WHEEZERS

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Introduction About a third of all children wheeze (Martinez, NEJM 1995), yet only half will develop asthma (Morgan, AJRCCM 2005). We know that increased reticular basement membrane (RBM) thickness, a feature of airway remodelling in older children with asthma, develops at 2–3 years of age in preschool severe recurrent wheezers (Saglani, AJRCCM 2007) but unlike older children (Regamey, AJRCCM 2008) nothing is known about any changes in airway smooth muscle (ASM) at pre-school age.

Hypothesis There is increased ASM and ASM infiltration by mast cells in preschool children.

Methods Endobronchial biopsies (EBx) were obtained from preschool children undergoing clinically indicated bronchoscopy from 2002 to 2005; severe, recurrent wheezers ($n=47$, median age 21.5 months) and non-wheezers ($n=21$, median age 19 months). Up to 12 ($5 \mu\text{m}$) sections were cut and stained with haematoxylin and eosin. ASM volume fraction was measured using point and line intersection counting (Regamey, AJRCCM 2008). A subgroup of children ($n=33$) had sections stained for mast cell tryptase. Area of subepithelium and smooth muscle were calculated using image analysis. Mast cells were expressed per area of subepithelium and ASM (Brightling, NEJM 2002).

Results ASM was present in EBx of 50 (73.5%) children, 17 controls (median age 17 months) and 33 wheezers (median age 21 months), with an average of 1.9 (range 1–5) sections with ASM per child. There was no difference in volume fraction of ASM, either indexed to volume of subepithelial tissue ($p=0.52$) or surface area of RBM ($p=0.14$), between wheezers and controls. There was no correlation between age and ASM in wheezers or controls. Submucosal mast cells were similar in wheezers ($n=25$, $139/\text{mm}^2$) and controls ($n=8$, $121/\text{mm}^2$) ($p=0.5$). No difference was found between wheezers and controls in the number of mast cells within ASM ($p=0.17$).

Conclusion Severe preschool wheezers have evidence of increased RBM thickness, but not increased ASM compared to age-matched non-wheezing controls. Since both are features of airway