BTS/BLF/BALR Early Career Investigator of the Year

T1 HYPOXIA-INDUCIBLE FACTOR 2α REGULATES NEUTROPHILIC INFLAMMATION IN HUMANS, MICE AND ZEBRAFISH

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Neutrophilic inflammation plays an important role in inflammatory lung diseases but therapeutic targeting of neutrophil (PMN) persistence is lacking. PMN lifespan and function is regulated by hypoxia, a characteristic feature of inflamed tissues, via the HIF/VHL/hydroxylase pathway, specifically hypoxia inducible factor-1α (HIF-1α) and prolyl hydroxylase-3 (PHD3). Targeting HIF-1α in myeloid cells impaired immune function, but PHD3 regulated PMN lifespan without affecting function. Given that PHD3 preferentially regulates HIF-2α, we investigated the role of HIF-2α in PMN-mediated inflammation.

Peripheral blood PMNs isolated from healthy volunteers and mice expressed HIF-2α and expression was enhanced by heat-killed bacteria. Using PMNs isolated from patients with active inflammatory arthritis (IA) we demonstrated significant upregulation of *HIF2A* mRNA (IA 92.9±30.3 vs. control 4.3±0.9 AU relative to *ACTB*, $P<0.05$) and protein (IA 0.26±0.05 vs. control 0.01±0.01 OD relative to P38, $P<0.01$) in circulating inflammatory PMNs. PMNs recruited to the airways of patients with COPD also displayed strong HIF-2α staining. The consequences of HIF-2α upregulation were examined using human PMNs from patients with gain-of-function mutations in the *HIF2A* gene. Neutrophils isolated from these patients had reduced rates of constitutive apoptosis. Recapitulation of the human *HIF2A* mutations in the orthologous *HIF2A* gene, *epas1a*, in zebrafish delayed resolution of inflammation in a tail injury model (24 hrs post injury, *epas1a* 12.7±1.4 vs. ctrl 5.2±0.5 PMNs, $p<0.001$) with an associated reduction in PMN apoptosis (*epas1a* 1.0% vs. ctrl 1.6%, $p<0.05$). Mice with myeloid-specific deletion of *Hif2a* had normal PMN survival in response to hypoxia and the cells showed no functional defect *in vitro*. Importantly, in a PMN-mediated acute lung injury model, myeloid-specific deficiency of HIF-2α markedly enhanced resolution of inflammation (BAL PMN count 48 hours following nebulised LPS, WT $2.13\times10^6\pm0.08$ vs. KO $1.39\times10^6\pm0.24$, $p<0.05$) and reduced lung injury (BAL IgM at 48 hours, WT 211±22.8 vs. KO 74.7±27.2 ng/ml), implicating HIF-2α in PMN persistence in inflamed lung tissue.

These data support a critical and selective role for HIF-2α in the resolution of inflammation through the maintenance of PMN survival, and provide a platform to dissect the therapeutic utility of targeting HIF-2α in chronic inflammatory diseases.

T2 MSCTRAIL AS A NOVEL CELLULAR THERAPY FOR MALIGNANT MESOTHELIOMA

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Background Malignant pleural mesothelioma (MPM) is an aggressive fatal cancer with no effective treatments. Mesenchymal stem cells (MSCs) migrate and incorporate into tumour stroma making them good vehicles for the delivery ofanti-cancer therapies. TNF-relatedapoptosis inducing ligand (TRAIL) selectively induces apoptosis in malignant cells without affecting healthy tissues. This study aimed to test whether MSCs modified to express TRAIL (MSCTRAIL) could be a successful cell therapy for MPM.

Methods Human MSCs were transduced with a lentiviral vector containing TRAIL IRES-GFP under the control of a tetracycline dependent promoter. Successful transduction was measured using flow cytometry and TRAIL expression was confirmed by immunoblotting and ELISA. The biological activity of MSCTRAIL was determined using co-culture experiments. DiI stained MPM cells were plated in a 1:1 ratio with MSCTRAIL cells. TRAIL production was activated and cells were treated for 48 hours. Both cells and supernatant were collected and stained with Annexin V and DAPI to detect apoptosis and death respectively on flow cytometry.

To test the effect of MSCTRAIL in vivo a bioluminescent tumour model was established. Plasmid containing fireflyluciferase and YFP was expanded and the correct sequences confirmed by restriction digest. Lentivirus was produced, viral titres were determined using flow cytometry and MPM cells were transduced (MPMLuc). A pure population was generated using hygromycin selection. 80,000 MPMLuc cells were injected into the pleural cavity of NOD/SCID mice and their growth was assessed using an IVIS Lumina system to detect bioluminescence. 1 million MSCTRAIL cells were delivered via tail vein injections on days 5, 9, 12, 15 and 18 post tumour inoculation and bioluminescence was measured twice weekly.

Results MSCs were successfully transduced with TRAIL with 96% efficiency and TRAIL production was confirmed by ELISA. Seven human MPM cell lines were tested with 6/7 (86%) being sensitive to MSCTRAIL. In vivo delivery of MSCTRAIL resulted in a significant reduction in MPM tumour growth.

Conclusions Delivery of TRAIL via MSCs causes a significant reduction in MPM tumour growth and is a potential novel cellular therapy for this currently incurable disease.

T3 ACUTE MUSCLE LOSS IN THE CRITICALLY ILL: FROM BEDSIDE TO BENCH

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Background Critical illness survivors demonstrate skeletal muscle wasting with associated functional impairment. We prospectively characterised this process, and defined the pathogenic roles of altered protein synthesis and degradation.

Methods Critically ill patients (n=63, 59% male, age 54.7±18.0 years, APACHE II score 23.5±6.5) were recruited <24 hours following Intensive Care Unit (ICU) admission. Muscle loss trajectory was determined through serial ultrasound measurement of rectus femoris cross-sectional area (RF_{CSA}) and, in a subset (n=28), quantification of myofibre area ($Fibre_{CSA}$) and protein/DNA ratio in vastus lateralis biopsies. Histopathological analysis was also performed. Muscle protein synthesis and breakdown rates were determined ([1,2$^{-13}C_2$]Leucine incorporation and D_5-Phenylalanine dilution, n=11), and respective signalling pathways examined (Luminex technology and Western Blotting, n=33).

Results (i) *RF_{CSA} decreased significantly*, (–17.7±12.1% [p<0.001]), but underestimated muscle loss determined by either $Fibre_{CSA}$ (–10.3±10.9% vs.–17.5±30.2%, p=0.31), or assessment of protein/DNA ratio (–10.3±10.9% vs. –29.5±41.5%, p=0.03). (ii) *Fall in RF_{CSA} was greater in multi- than single-organ failure* (–21.5±10.5% vs –7.2±9.7% respectively, p<0.0001), even by day 3 (–8.7±16.3% vs –1.8±9.6%, p<0.01). Those suffering ≥ 4 organ were worst affected (–26.3±12.0% vs –19.5±9.4% for 2–3 organ failure, p<0.01). (iii) *Histopathological myofibre necrosis* occurred in >50% (17/33) of subjects. (iv) *Protein synthesis was depressed,* to levels observed in fasted controls (0.035±0.018%/hr vs. 0.039±0.011%/hr respectively, p= 0.57). Synthesis rates increased by day 7 (0.076±0.066%/hr, p=0.07) to levels associated with healthy fed controls (0.065+0.018%/hr, p0.30). These effects were independent of nutritional calorie and protein load received. (v) *Protein breakdown remained elevated throughout* (8.5±5.7 to 10.6±5.7 mmol phe/min/IBW, p=0.4). (vi) *Principal component analysis of patterns of intracellular signalling suggested an orchestrated programme* of increased breakdown (r –0.83, p0.005) and depressed synthesis (r–0.69, p0.041).

Conclusions Skeletal muscle wasting (defined for the first time by three independent measures) (1) occurs early and rapidly in critical illness and (2) is greatest in those with multi-organ failure. Suppression of protein synthesis and increases in catabolism (isotope uptake and intracellular signalling data) were shown, for the first time, to underpin these changes. Importantly, these overall effects appear independent of feeding status, and also to be commonly associated with (previously unrecognised) myonecrosis.

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T4 MUTATIONS IN BMPR-II PROMOTE INFLAMMATION VIA ALTERED SUPEROXIDE SIGNALLING: INSIGHTS INTO THE MECHANISMS UNDERLYING PULMONARY ARTERIAL HYPERTENSION

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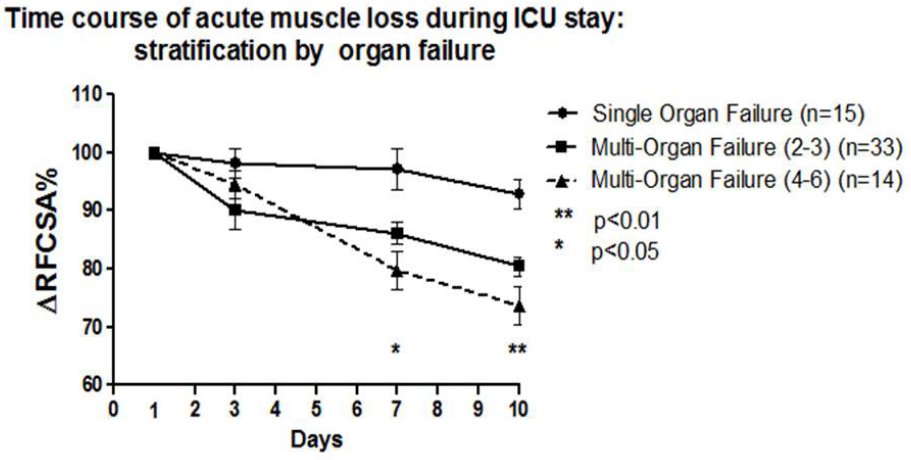
Introduction Although the presence of inflammation is well recognised in pulmonary arterial hypertension (PAH), the underlying mechanisms remain poorly understood. Mutations in the bone morphogenetic protein type II receptor gene, *BMPR2*, underlie the majority of heritable and a significant fraction of idiopathic PAH Here, we sought to determine whether loss of *BMPR2* was directly associated with inflammation, the underlying mechanisms, and possible therapeutic interventions.

Methods The initial approach employed human and mouse pulmonary arterial smooth muscle cells (PASMCs) from controls and subjects with a mutation in *BMPR2*. Cells were studied at baseline and in response to lipopolysaccharide (LPS), in the presence or absence of pharmacological inhibitors of known pro-inflammatory pathways. Cytokines were measured by ELISA Reactive oxygen species were measured using dihydroethidium staining. For *in vivo* studies, we employed the heterozygous null *bmpr2* mouse (*bmpr2*+/–) and wild-type littermates.

Results Chronic low dose LPS resulted in a marked elevation of right ventricular systolic pressure to ~40 mmHg in *bmpr2*+/– mice, but not in wild-type mice (Fig.1). *In vitro* studies showed that mouse *bmpr2*+/– and human *BMPR2mut* PASMCs produced more IL-6 and IL-8 at baseline and after stimulation with LPS For example, *bmpr2*+/– PASMCs produced 1533±280 pg/ml of IL-6 (*versus* 341±10 pg/ml in controls, *P*<0.01) after LPS stimulation. Reduced *BMPR2* was also associated with increased phospho-STAT3 levels and an abnormal pro-proliferative response to IL-6. Acute administration of LPS to the *bmpr2*+/– mouse reproduced these results *in vivo,* with increased lung and serum IL-6 and IL-8. The link between reduced *BMPR2* and increased cytokine expression was found to be associated with increased superoxide generation and loss of extracellular superoxide dismutase (SOD3). Treatment of *BMPR2* deficient PASMCs with the superoxide dismutase mimetic, Tempol, normalised the exaggerated secretion of IL-6 and IL-8. Co-administration of Tempol prevented the PAH phenotype in *bmpr2*+/– mice exposed to low dose LPS (Fig. 1).

Conclusions We have determined a novel link between *BMPR2* loss and increased expression of pro-inflammatory cytokines, which is dependent on mishandling of superoxide. Our results suggest that pro-inflammatory stimuli may represent disease-provoking triggers for patients with *BMPR2* deficiency. Promotion of SOD3 activity may represent a novel therapeutic approach in PAH.



*ΔRFCSA% = change in rectus femoris cross sectional area, Organ failure defined by cumulative scores of >2 per system in the Sequential Organ Failure Assessment (SOFA). Statistical analysis done by repeated measures 2 way analysis of variance, * denotes significant change from Day 1 in patients in Multi-Organ Failure, and in comparison to Single-Organ Failure*

Abstract T3 Figure 1