Necrosome Complex Release and Necroinflammation in Hyper-Inflammatory ARDS

R. Handslip[1], S. Mumby[2], C. S. Calfee[3], C. M. O'Kane[4], K. Delucchi[1], D. F. McAuley[5], M. Takata[6], B. V. Patel[7]; [1]Anaesthesia, Pain medicine and Intensive care, Dept. Surgery and Cancer, Imperial College London, London, United Kingdom, [2]Imperial College London, London, United Kingdom, [3]Univ of California At San Francisco, San Francisco, CA, United States, [4]Imperial College, London W12 0NN, United Kingdom, [5]Centre for Experimental Medicine, Queen's University Belfast, Belfast, United Kingdom, [6]Anaesthesia, Pain medicine and Intensive care, Dept. Surgery and Cancer, Imperial College London,, London SW10 9NH, United Kingdom, [7]Anaesthetics, Pain Medicine and Intensive Care, Imperial College London, London, United Kingdom.

Introduction The role of different programmed cell death pathways during the progression of ARDS remains unknown. Necroptosis requires formation of necrosome complexes, made of phosphorylated receptor interactive serine/threonine kinase-3 (RIPK3) and mixed lineage kinase like domain (MLKL), and their subsequent insertion into the cell membrane induces cell lysis. We hypothesized that when substantive necroptosis occurs within ARDS lungs, detectable levels of necrosome complex are released extracellularly from lysing cells which enter the circulation, and these circulating necrosome complex levels may reflect the degree of necroinflammation during ARDS Methods Primary human pulmonary microvascular endothelial cells (HPMEC) were stimulated with TNF-α, with TNFR1 signalling pathways modulated using a combination of inhibitors of cell survival pathway (SMAC mimetic, BV6), caspase-dependent apoptosis pathway (ZVAD), and necroptosis pathway (necrosulfonamide, NSA). Cell death (estimated by Draq7 dye), caspase-8 activation (measured by FLICA assay), and endothelial activation (ICAM-1 expression) were measured by flow cytometry. Co-immunoprecipitation (Co-IP) of necromsome RIPk3/pMLKL complexes was performed in cellular and media fractions. Necroptosis signalling molecules (RIPK3) were measured by ELISA in 30 ARDS patients plasma from the HARP-2 ARDS study[1], 15 identified as hyper and 15 as hypo-inflammatory endotypes. Co-IP of RIPL3/pMLKL was also performed in 6 patient samples. Results Treatment of TNF+BV6+ZVAD showed enhanced caspase-independent cell death suggesting necroptosis, with ICAM-1 upregulation ($p< 0.01$). This was associated with increased intracellular necrosome complex formation (2.9-fold vs control), as well as release into the supernatant (5.6-fold vs control). Treatment of TNF+BV6+ZVAD with necroptosis inhibitor (NSA) abrogates cell death, ICAM-1 upregulation, and induces significant caspase-8 activation ($p< 0.01$) whilst reducing intracellular necrosome formation In comparison to hypo-inflammatory ARDS, hyper-inflammatory ARDS plasma contains significantly increased TNF-α, TNFR-1 and angiopoetin-2 at baseline, persisting to 14 days ($p< 0.01$). Hyper-inflammatory ARDS plasma demonstrated higher circulating RIPk3 from baseline to 14 days ($p< 0.0001$). Using Co-IP, we were able to confirm that this RIPk3 was associated with MLKL in the form of circulating necrosome complexes.

	% Cell death	Endothelial activation	Apoptosis
	Draq 7	ICAM-1 MFI	Caspase 8 MFI
Control	4.0%± 2.3%	65.6 ±11.5	52.13±21.3
TNF	5.9%± 5.3%	3716±1002 **	87.1±97.3
TBZ	28.1%± 5.1% **	2664 ±181.4 **	79.41±55.5
TBZ + NSA	4.9% ± 1.4% **	1190±296.1 **	1191±511.2 **

Table. 1 ± Standard deviation mean. 2-way ANOVA. * $p < 0.05$, ** $p< 0.01$. Treated with TBZ (TNF, BV6, ZVAD) and NSA (Necrosulfonamide) for 16 hours.

Conclusion Using an in vitro model of human pulmonary endothelial cell necroptosis with various cell death pathway inhibitors, we demonstrated that a substantive amount of necrosome complex (RIPK3-pMLKL) is released extracellularly during the process of necroptotic cell death. Early and persistent release of necrosome complexes into the circulation of hyper-

inflammatory ARDS patients suggests release from cells undergoing programmed necrosis and lysis. This offers the opportunity for early detection and pharmacological inhibition of necroptosis to promote a switch from a necroinflammatory to a pro-resolving apoptotic phenotype

This abstract is funded by: European Society of Intensive care medicine Basic research award.