The Role of Mitochondrial DNA in the Post-Injury Inflammatory Response Following Major Trauma

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degree of Doctor of Philosophy of Medicine

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Declaration:

I hereby certify that to the best of my knowledge and belief this thesis is my own work and contains no material previously published or written by another person except where due references and acknowledgements are made. It contains no material which has been previously submitted by me for the award of any other degree or diploma in any university or other tertiary institution.

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List of Publications in the Order Included in This Thesis:

1. McIlroy DJ, Bigland M, White AE, Hardy BM, Lott N, Smith DW, Balogh ZJ**. Cell necrosis–independent sustained mitochondrial and nuclear DNA release following trauma surgery.** *J Trauma,* 2015;78(2):282

2. McIlroy, DJ, Jarnicki AG, Au GG, Lott N, Smith DW, Hansbro PM, Balogh ZJ, **Mitochondrial DNA neutrophil extracellular traps are formed after trauma and subsequent surgery.** *J Crit Care*, 2014; 29:1333e1 – 1333e5

3. McIlroy, DJ, Jarnicki AG, Au GG, Lott N, Briggs G, Smith DW, Hansbro PM, Balogh ZJ, **Mitochondrial DNA Induces Mitochondrial DNA Neutrophil Extracellular Trap Formation After Trauma and Subsequent Surgery.** *Frontiers in Immunology* (under review)

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5. Tuboly E, McIlroy D, Briggs G, Lott N, Balogh ZJ, **Clinical implications and pathological associations of circulating mitochondrial DNA.** *Front Biosci (Landmark Ed).* 2017; 22:1011-1022 **(*Background: Appendix 1*)**

6. Balogh ZJ, McIlroy DJ, Smith DW, Hansbro PM, **The origin and the role of mitochondrial DNA in postinjury inflammation.** *J Crit Care,* 2013 ;28(6):1099-100 ***(Hypothesis: Appendix 2)***

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List of Abbreviations:

AIF – Apoptosis Inducing Factor
ARDS – Acute Respiratory Distress Syndrome
AST – Aspartate Transferase
ATLS® - Advanced Trauma Life Support
ADP – Adenosine Di-Phosphate
ATP - Adenosine Tri-Phosphate
ACTH - Adrenocorticotropic hormone
bDNA – Bacterial DNA
C - Complement
CARS - Compensatory Anti-inflammatory Response Syndrome
CD18 – Integra beta-2 protein
Cf-DNA – Cell-free DNA
CK – Creatine Kinase
CNS – Central Nervous System
Cyp-D - Cyclophilin D
Cyt c – Cytochrome C
DAMP – Damage Associated Molecular Pattern
DNA – Deoxyribonucleic Acid
DNase – Deoxyribonuclease
Fas – Fas ligand / receptor
FP – Formyl Peptides
GM-CSF - Granulocyte-macrophage colony-stimulating factor
H_2O_2 – Hydrogen Peroxide
HIF – Hypoxia Inducible Factor
ICAM – Intracellular Adhesion Molecule
ICU – Intensive Care Unit
IKK - inhibitory kappa B Kinase
IL – Interleukin
IL1-ra - IL-1 receptor antagonist
IR – Ischaemia Reperfusion
ISS – Injury Severity Score
LDH – Lactate Dehydrogenase
LOS – Length of Stay
LPS – Bacterial Lipopolysaccharide
MOD – Multiple Organ Dysfunction
MODS – Multiple Organ Dysfunction Syndrome
MOF – Multiple Organ Failure
mtDNA – Mitochondrial DNA
nDNA – Nuclear DNA
NET – Neutrophil Extracellular Trap
NFκB – Nuclear Factor Kappa B
P - Protein

PAMP – Pathogen Associated Molecular Pattern
PaO_2 – Partial Oxygen Pressure
PARP – Poly-ADP-Ribose Polymerase
PCO_2 – Partial Carbon Dioxide Pressure
PICS - Persistent Inflammatory, immunosuppressed Catabolic Syndrome
PKC – Protein Kinase C
PMA - Phorbol Myrisate Acetate
PRR – Pathogen recognition receptor
RIPK - Receptor Interacting Protein Kinases
RNA - Ribonucleic acid
rRNA – Reporter Ribonucleic Acid
tRNA – Transfer Ribonucleic Acid
ROI – Reactive Oxygen Intermediate
SIRS – Systemic Inflammatory Response Syndrome
TLR – Toll-Like Receptor
TNFα – Tumour Necrosis Factor Alpha
VCAM – Vascular Cellular Adhesion Molecule

Abstract:

Introduction:

Trauma is the leading cause of death in the developed world in those aged under 45 years. The main potentially modifiable cause of late death after injury is post-injury multiple organ failure (MOF). Early MOF is characterised by a lethal combination of systemic inflammatory response syndrome (SIRS) which underpinned by neutrophil proliferation and "priming" as a result of the initial injury and haemorrhagic shock. If primed neutrophils are then exposed to "second hit" then dysregulated neutrophil driven inflammation can occur, resulting in end organ sequestration, parenchymal damage, MOF and ultimately death. Interest has increased in endogenous drivers of the innate immune system that exert a potent pro-inflammatory effect by activating pathogen recognition receptors (PRR's), which are designed to respond to pathogen associated molecular patterns (PAMPs) found in bacteria. Endogenous factors that can trigger this response in the absence of sepsis have been termed "alarmins" or damage associated molecular patterns (DAMPs). Mitochondrial DNA (mtDNA) is a potently pro-inflammatory DAMP, which has been found to be highly elevated in the post-injury state. Mitochondrial DAMPs have also been associated with neutrophil mediated end organ injury.

Primary aims:

The primary aim of this thesis was to characterise the effect of post-injury non-life saving orthopaedic surgery on circulating mtDNA levels. No study had looked at the effect of surgical intervention on levels of mtDNA after initial injury and possible sources of mtDNA release. Initially a pilot study of 35 trauma patients who subsequently underwent orthopaedic surgery was performed primarily measuring cell-free mtDNA and nuclear DNA (nDNA) with sequential plasma measurements over a 5-day perioperative period with comparison to 20 healthy control subjects. MtDNA levels continued to rise over the 5-day observation period following surgery and had no correlation to markers of cell-necrosis either in the form of direct musculoskeletal injury, or secondary inflammatory end organ injury. Whilst nDNA levels were elevated when compared to healthy controls no increase was observed in the 5-day observation period. Elevated mtDNA perioperative levels were directly correlated with the magnitude and early timing of surgical intervention. MtDNA levels were inversely proportional to the volume of crystalloid infused indicating a possible role for adequate resuscitation in modulating circulating mtDNA levels. A positive trend between mtDNA levels and incidence of post-

injury SIRS and MOF was observed but this failed to reach statistical significance. This lead to the genesis of the hypothesis that the persistently elevated mtDNA levels may have a primary inflammatory source.

Secondary aims:

The secondary aims of this thesis were threefold. Firstly, to determine whether there was a primary inflammatory source of mtDNA, namely focussing on possible neutrophil extracellular trap (NET) formation or "NETosis". Secondly, to determine what factors may propagate and influence mtDNA release. Finally, to investigate mechanisms for modulating circulating mtDNA levels following injury and subsequent surgery by looking at DNase activity. NETosis is characterised by the release of chromatin in conformational net-like structures in response to sepsis, however some authors had shown that under certain conditions NETs could be composed of mtDNA (mtDNA-NETs). The next study performed focussed on demonstrating whether NETs were formed after injury and subsequent surgery and what type of DNA they were composed of. The presence of NETs had been postulated after traumatic injury by one group based on observed high concentrations of cell-free DNA but they failed to define any microscopic evidence of NET formation. In our next paper we definitively demonstrated that NETs were formed after injury and subsequent surgery and also in response to elective orthopaedic hip replacement surgery. This was achieved microscopically using fluorescent DNA avid dyes to demonstrate the presence of conformational DNA-NET structures. Molecular genetic analysis of the NETs formed in response to injury and subsequent surgery or in response to elective surgery alone revealed that the NETs were mtDNA-NETs. Due to molecular similarities between mtDNA and bacterial DNA (bDNA) we hypothesised that mtDNA might trigger NETosis through a PRR mediated pathway. In the next paper we studied the effect of exposing healthy neutrophils and post-injury perioperative neutrophils to physiological concentrations of mtDNA we had measured in our initial pilot over the 5-day observation period. We then conducted a series of positive control experiments using phorbol myrisate acetate (PMA), a known potent stimulator of NETosis. NETs were triggered after trauma and healthy neutrophils were exposed to mtDNA. Notably the NETs formed in response to mtDNA were mtDNA-NETs in both trauma and healthy neutrophils, however trauma neutrophils were less responsive compared to healthy control neutrophils. This observation was thought to be possibly due to the exposure of trauma neutrophils to high levels of mtDNA after injury and surgery causing prior mtDNA-NET production. NETs formed in response to PMA

exposure were composed almost exclusively of nDNA (nDNA-NETs). Finally, we studied the plasma activity of DNase alongside mtDNA and nDNA concentrations in a much larger cohort of trauma patients (n=103) compared to our initial pilot (n=35). Circulating DNase isotypes are responsible for the digestion of extracellular DNA whether mtDNA or nDNA and also digest NETs. DNase activity was significantly reduced compared to that measured in healthy controls. This greater powered study did reveal a statistically significant positive correlation between perioperative mtDNA levels and SIRS but not MOF, despite a strong trend.

Conclusion / Clinical Relevance:

Our data suggests that after traumatic injury, the timing/magnitude of surgery and adequacy of resuscitation influence the levels of circulating mtDNA. Neutrophils contribute a significant amount of mtDNA through mtDNA-NET formation in the post-injury and perioperative period. MtDNA can essentially drive its own release through a positive feedback loop. This occurs through circulating mtDNA triggering further mtDNA-NET release, resulting in a vicious cycle of dysregulated inflammation and associated SIRS with a likely link to post-injury MOF. Most excitingly the finding of reduced DNase levels in the face of high mtDNA levels. This may offer up a novel therapeutic target for modulation of aggressive post-injury SIRS, through the potential administration of exogenous DNase in the post-injury and peri-operative recovery period.