Trauma: Damage recognition and response

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This thesis has been submitted to Queen Mary University, London in accordance with the requirements for the degree of Doctor of Philosophy

Author's declaration

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Citation

Trauma: Damage recognition and response. Joanna Manson, Queen Mary, University of London 2012.

ACKNOWLEDGEMENTS

I would like to thank Professor Karim Brohi for giving me the opportunity to pursue this PhD and for his supervision and coaching. In addition, I would like to thank Col Nigel Tai, Professor Daniel Pennington, Dr Mark Turner, Dr Ute Meier and Professor Christoph Thiemermann for their guidance. This work would not have been possible without the dedication of the trauma research team; thank you: Ross Davenport, Amy Coates, Claire Rourke, Henry De'Ath, Sirat Khan, Imran Raza, Catherine Spoors, Simon Glasgow, Zane Perkins, Karen Hoffman, Elaine Cole and Anita West for your hard work and friendship. Thank you to the patients and families who gave their consent to participate in this research. Thank you to my colleagues in Neuroscience, Dr Paul Vulliamy, Dr Gary Warnes and Professor Joan Morris who have all provided invaluable assistance. In addition, thank you to the Royal College of Surgeons for funding a year of my PhD through The Philip King Charitable Settlement Trust & RCS of England Research Fellowship 2010-2011.

I would like to thank my wonderful husband for his support, assistance and understanding. Finally, I would like to thank my parents and my sister, for everything they have done to help me achieve my goals and for teaching me that I can do anything with a little perseverance.

ABSTRACT

Introduction: Patient outcome after trauma is influenced by their immune response to injury. How trauma activates the immune system and why this affects recovery is unclear. This investigation examined three aspects of the immune response after trauma: cytokine production, alarmin release and the innate immune cell populations.

Methodology: Timed blood samples were drawn from trauma patients recruited to a prospective observational cohort study at a London Major Trauma Centre. The first sample was drawn at admission, within 2h of injury and prior to intervention in order to capture early inflammation events. Patients were observed until death or discharge for clinical outcomes.

Results: Inflammation after traumatic tissue damage can be described in isolation. If haemorrhagic shock is also present, the inflammatory effects cannot be separated using the seven cytokines examined in this investigation. Within 2h of injury, isolated tissue damage is associated with systemic release of intracellular nuclear molecules. Tissue damage combined with shock, is associated with release of different nuclear materials. Low numbers of lymphocytes at 48h from injury are associated with poor clinical outcome. Patients who develop infections and multiple organ dysfunction syndrome during recovery, have high numbers of cytotoxic lymphocytes in their peripheral blood at admission.

Conclusion: Inflammation is activated prior to arrival at hospital. Haemorrhagic shock augments the inflammatory response after isolated tissue damage. Tissue damage and blood loss may lead to the release of different alarmin substances. Lymphocytes are implicated in the pathogenesis of poor outcome. The molecular events which lead to poor clinical outcome are activated before hospital admission and prior to intervention. Greater understanding of the activation mechanism(s) may result in development of therapeutics for early delivery, in order to improve patient recovery.

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ABREVIATIONS

MTC	*Major Trauma Centre*
HEMS	*London Helicopter Emergency Service*
LAS	*London Ambulance Service*
RLH	*Royal London Hospital*
TTL	*Trauma Team Leader*
ED	*Emergency Department*
OR	*Operating Room*
RCSEng	*Royal College of Surgeons of England*
NHS	*National Health Service*
WHO	*World Health Organisation*
UK	*United Kingdom*
LTO	*London Trauma Office*
NICE	*National Institute for Clinical Excellence*
ACCP	*American College of Chest Physicians*
MRC	*Medical Research Council*
NCEPOD	*National Confidential Enquiry into Peri-operative Deaths*
ELISA	*Enzyme-linked immunosorbant assay*
PBS	*Phosphate buffered saline*
bpm	*Beats per minute*
IL-	*Interleukin - Number*
TNF	*Tumour necrosis factor*
IFNγ	*Interferon gamma*
γδ	*Gamma Delta T cells*
SIRS	*Systemic Inflammatory Response Syndrome*
CARS	*Compensatory Anti-Inflammatory Syndrome*
PAMPs	*Pathogen-associated molecular patterns*
DAMPs	*Damage-associated molecular patterns*
TCR	*T cell receptor*
ICU	*Intensive Care Unit*

Part 1: Introduction & Methods

CHAPTER 1: INTRODUCTION

1. TRAUMATIC INJURY IS A DISEASE

Trauma is the disease of physical injury (Trunkey et al, 1974, (De et al, 2003). Annually, trauma is responsible for 6 million deaths around the globe and this death toll is expected to rise over the next decade (WHO, 2009). Recent military conflict has raised public awareness about the concept of traumatic injury, but it has failed to convey a key message; that trauma is principally a disease of the civilian population.

In the England and Wales, around 10,000 civilian deaths every year are attributable to physical injury (ONS, 2011). Although penetrating injuries, such as stabbings and shootings, are often perceived to be the most frequent traumatic injuries, these only account for 10% of the annual trauma fatalities (ONS, 2011). Recently, concern has been raised about the rising number of young males dying as a result of penetrating trauma, particularly in lower socio-economic areas (Rooney and Davis, 1999). The most common mechanisms of injury in the UK civilian population are however falls from a height and road-related incidents (Troop, 2005).

Traumatic injury presents a huge socio-economic burden for most countries in terms of loss of earnings, loss of tax contributions and the cost of medical treatment (Polinder et al, 2005, (Council, 2007). In the UK, treatment of injuries is estimated to consume 7% of the National Health Service (NHS) budget; approximately £1.6 billion per year (DOH, 1999). For every death from traumatic injury, a number of people will sustain long-term disability (WHO, 2008). Accurate outcome data for trauma survivors, detailing quality of life, return to employment and normal social participation, are currently unavailable but long-term disability is associated with low economic status and low re-employment (Brohi et al, 2011, (Soberg et al, 2007).

Optimising functional outcomes for trauma patients is therefore a key goal of modern trauma care (Brohi et al, 2011).

2. MODERN TRAUMA SYSTEMS

Modern trauma care is best provided by trauma systems, which manage the patient through each phase of the trauma patient journey. The journey begins at scene with pre-hospital assessment, treatment of life threatening conditions and transfer to the emergency department (ED) for resuscitation. In the ED, resuscitation runs alongside surgical strategies and intervention for haemorrhage control and injury repair. Intensive care treatment may then be required for organ support. Physiological recovery, wound healing and rehabilitation hopefully follow, allowing patients to return home in a fully functional state. The quality of medical care provided at any one of these stages can influence patient survival or long-term outcome.

Trauma outcomes are therefore as dependent upon medical organisation as they are upon medical treatment. Infrastructure and man-power are essential. Survival from traumatic injury is not wholly dependent upon the time to surgery or the quality of the operation. Early recognition of life-threatening injuries, transfer from scene to an appropriate hospital, access to blood for transfusion, imaging and interventional radiology, operating theatres, porters, nurses, elevators and many other logistical components are also vital; hence the term trauma system. A trauma system, led by clinicians with trauma expertise, results in improved survival for severely injured casualties (Davenport et al, 2010, (LTO, 2011). In fact, with good organisational strategies and sufficient resources, the definition of an 'un-survivable injury' is rapidly changing. This has been demonstrated by the military medical teams in Afghanistan, where they have achieved the highest conflict casualty survival rate, whilst tackling some of the most severe injuries ever encountered (Allcock et al, 2011).

Twenty years ago in the United Kingdom (UK), a report highlighted unacceptably high mortality rates for civilian trauma patients, as a result of failings in organisation and clinical care (Yates et al, 1992). More recent analysis showed little improvement (NCEPOD, 2007). A national strategy for trauma was therefore developed, to re-structure trauma care in the UK (LTO, 2011, (NHS, 2008, (RCSEng, 2009). The UK's first trauma system was activated in London in April 2010 and involved complete re-organisation of hospital facilities and the processes of patient care. Even within the first year, an improvement in survival from serious injury was achieved (LTO, 2011). Further trauma systems will be activated across the UK in the next few years (NHS, 2008). Trauma patients should obtain gold standard management, regardless of their geographical location and regionalised provision of trauma care services should improve outcomes in the UK. Nonetheless, there remains a great need for development of infrastructure, collaboration between different medical specialities and dissemination of knowledge about best practice.

In global terms, provision of trauma care is extremely variable (Mock et al, 2004, (Mock et al, 2006). Most trauma research literature is generated by high volume centres, in high income countries, with good clinical care standards. Their results bear no resemblance to the quality of care or outcomes achieved by less specialised centres within their own country or those in low and middle income countries. Addressing the vast inequalities of trauma care provision is as great a need, as advancing our understanding of the pathology of traumatic injury.

3. THE INFLUENCE OF TRAUMA RESEARCH ON TRAUMA CARE

Over the last decade, the standard of care provided by specialist trauma hospitals is thought to have improved as a result of greater understanding of the pathophysiological changes precipitated by traumatic injury. These improvements have been driven by trauma research and have led to the development of evidence-based treatment strategies. Only one study has

demonstrated improved patient survival since 2000 (Glance et al, 2012). This study examined trauma outcome within the state of Pennsylvania and demonstrated a 51% reduction in mortality for severely injured patients. The results cannot be generalised to other regions within the USA, or to other countries and further evidence in other geographical locations would be desirable.

Perhaps the most important of the advances in trauma care has been in the management of blood loss. Around 40% of the early deaths after trauma are due to severe haemorrhage (Gruen et al, 2006, (Sauaia et al, 1995, (Kauvar et al, 2006). Blood loss is an obvious consequence of tissue injury and vessel disruption; however, in 2003 it was reported that a subset of trauma patients also develop an inherent clotting defect which exacerbates blood loss and increases mortality (Brohi et al, 2003). This condition, called Acute Traumatic Coagulopathy (ATC), is estimated to occur in 25% of severely injured patients (Brohi et al, 2003). The molecular mechanisms which lead to its development are incompletely understood but it appears to be related to activation of the natural anti-coagulant, Protein C (Brohi et al, 2007a, (Brohi et al, 2007b, (Hess et al, 2008).

Identification of ATC led to the realisation that patients with active haemorrhage require early delivery of blood and clotting products. This initiated a fundamental change in trauma resuscitation strategies and the concept of Damage-Control Resuscitation (DCR) (Brohi et al, 2003, (Duchesne et al, 2010, (Davenport et al, 2011, (Roberts et al, 2011). The name derives from Damage-Control Surgery (DCS); an established surgical strategy used in trauma, as patients are in a fragile physiological condition during the first few hours after injury (Hirshberg and Mattox, 1993). Modern trauma care combines these two philosophies and aims for simultaneous haemorrhage control and coagulopathy correction (Hirshberg and Mattox, 1993, (Loveland and Boffard, 2004, (Duchesne et al, 2010). Implementation of these strategies has been shown to increase survival following severe injury, but they rely upon

integrated team work from a number of health professionals, operating within a well-organised system (Davenport et al, 2010, (Brown et al, 2011b).

4. OUTCOMES FROM TRAUMATIC INJURY

Selecting appropriate outcome measures to reflect the quality of trauma care is difficult, as all have limitations. Outcomes related to long-term function would be ideal, but none are currently unavailable (Brohi et al, 2011). Numerous quantitative outcomes are used, with 28 day or 30 day mortality and length of hospital stay being traditionally reported. Variations such as the number of days in critical care or the number of ventilator-free days have also been applied. Parameters related to treatment strategies, such as the number of units of blood transfused, may also be appropriate. Comparison between centres using quantitative measures is often difficult because of the variability between injured populations and trauma systems; they also seldom reflect the quality of care provided.

For my investigation, I elected to use four principal outcomes: 28 day mortality, length of hospital stay, development of multiple organ dysfunction syndrome (MODS) and development of infection during recovery.

a) MORTALITY

Mortality after traumatic injury was initially described as tri-modal; 'immediate deaths' due to airway compromise and torrential haemorrhage, 'early deaths' due to haemorrhage and brain injury, 'late deaths' due to multiple organ failure and sepsis (Trunkey et al, 1974). Since then, advancements in trauma care have undoubtedly improved survival within each of these categories, although, good quality evidence to support this is limited. Mortality after traumatic injury is principally influenced by the type of injuries sustained and their severity. Severity of injury is widely described using The Injury Severity Score, devised in 1974, which

relates the anatomical injury to the likelihood of patient death (Baker et al, 1974). The anatomical location of a patient's injuries undoubtedly influences survival with injuries to the head and chest carrying a particularly high mortality (Bouillon et al, 1999). Injury to more than one region of the body is also more likely to be fatal (Hensler et al, 2002, (Baker et al, 1974). Nonetheless, in settings that provide modern trauma care, patients are able to recover from injuries with high injury severity scores suggesting that there is more to survival than just the anatomical damage sustained (Allcock et al, 2011, (Brown et al, 2011b, (Davenport et al, 2010).

b) LENGTH OF HOSPITAL STAY

Advances in trauma care and resuscitation have increased survival in the first few hours after injury, but survivors frequently develop critical illness which leads to a long hospital stay and late mortality (Brown et al, 2011b). In addition, some logistical aspects can extend hospital stay after traumatic injury. Social reasons such as homelessness, domestic abuse and lack of carers if poorly mobile, may extend hospital stay in patients who would otherwise be medically fit for discharge. Waiting lists for specialist units, particularly spinal or neuro-rehabilitation can also lead to protracted hospital stays. Although frequently used and therefore important to include, the limitations make it a weak outcome measure.

c) MULTIPLE ORGAN DYSFUNCTION SYNDROME (MODS)

Multiple Organ Dysfunction Syndrome, first described as Multiple Organ Failure (MOF) in the 1970's, frequently effects severely injured trauma patients (Baue, 1975). Respiratory dysfunction occurs in almost all post-traumatic MODS patients and this often precedes failure of other organs (Ciesla et al, 2005). MODS is by no means exclusive to traumatic injury and is a complication of many other severe clinical conditions (Baue, 2006). Post-traumatic MODS however, shows great variability between patients in terms of time course and outcome (Sauaia et al, 1998). There is no agreed standard for the diagnosis of MODS. Various scoring

systems have been developed including the Marshall Score, Denver Score and the Sequential Organ Failure Assessment Score (SOFA); the selection is centre dependent (Sauaia et al, 2009, (Vincent et al, 1996). The reported incidence of MODS ranges between 2%-61% of trauma patients, depending on the score used and the patient population (Minei et al, 2012, (Sauaia et al, 1995, (Dewar et al, 2009b). Old studies observed a bimodal pattern of MODS development where 'early' organ dysfunction was predominantly cardiovascular and 'delayed' organ dysfunction was principally hepatic (Moore et al, 1996). A recent large study of patients with blunt traumatic injury described an early, single episode of MODS, on median day 2 (interquartile range 2-4) and no second peak (Minei et al, 2012). This has been attributed to the change in resuscitation strategies over the last 10 years (Minei et al, 2012, (Duchesne et al, 2010).

Distinction is now made between the MODS seen in the immediate injury phase (first 48h), which is directly related to the injury and will likely resolve with resuscitation and true MODS which develops after 48h (Ciesla et al, 2004). Known risk factors for MODS development include: male gender, older age, greater injury severity, obesity, co-morbidity, ≥6 units of blood transfusion and greater depths of haemorrhagic shock (Minei et al, 2012, (Ciesla et al, 2006, (Sauaia et al, 1994). Treatment is supportive and mechanical ventilation, ionotropic agents, extracorporeal kidney dialysis, repeated blood transfusions and liver support may all be required. Prolonged or non-resolving MODS is associated with high mortality. Mortality rates vary between centres but exceed 50% (Partrick et al, 1999, (Jastrow et al, 2009).

d) DEVELOPMENT OF INFECTION

In addition to the development of MODS, trauma patients appear to be extremely susceptible to the development of infection. Frequently, this leads to one or more episodes of sepsis; a state of systemic inflammation precipitated by pathogenic invasion (Box 1.1). Patients with MODS, more commonly develop nosocomial (hospital acquired) infection; although

development of infection does not herald subsequent development of MODS (Minei et al, 2012). It remains unclear why trauma patients are susceptible to infection.

Box 1.1: Definitions of sepsis, severe sepsis and septic shock

Sepsis : A documented or suspected infection with one or more of the following:

General variables:
- Fever (core temperature >38.3°C)
- Hypothermia (core temperature <36°C)
- Heart rate >90 min–1 or >2 SD above the normal value for age
- Tachypnoea
- Altered mental status
- Significant oedema or positive fluid balance (>20 mL/kg over 24 hrs)
- Hyperglycaemia (plasma glucose >120 mg/dL) in the absence of diabetes

Inflammatory variables:
- Leukocytosis (WBC count >12,000 µL–1)
- Leukopenia (WBC count <4000 µL–1)
- Normal WBC count with >10% immature forms
- Plasma C-reactive protein >2 SD above the normal value
- Plasma procalcitonin >2 SD above the normal value

Specific measurements
- SvO2 >70%
- Cardiac index >3.5 L/min/m^2

Severe sepsis: sepsis associated with organ dysfunction, hypoperfusion or hypotension.

Organ dysfunction variables:
- Arterial hypoxemia (PaO2/FIO2 <300)
- Acute oliguria (urine output <0.5 mL·kg-1·hr-1 or 45 mmol/L for at least 2 hrs)
- Creatinine > 2.0 mg/dL
- Coagulation abnormalities (INR >1.5 or aPTT >60 secs)
- Thrombocytopenia (platelet count <100,000 µL–1)
- Hyperbilirubinemia (plasma total bilirubin > 2.0 mg/dL or 35 mmol/L)
- Tissue perfusion variables:
- Hyperlactatemia (>2 mmol/L)
- Hemodynamic variables:
- Arterial hypotension (SBP <90 mm Hg, MAP <70, or SBP decrease >40 mm Hg)

Septic shock: is acute circulatory failure unexplained by other causes.

Acute circulatory failure is defined as persistent arterial hypotension (SBP <90 mmHg, MAP<60, or a reduction in SBP >40 mm Hg from baseline despite adequate volume resuscitation).

Taken from The Surviving Sepsis Campaign. The Institute for Healthcare Improvement

5. TRAUMA OUTCOMES ARE RELATED TO INFLAMMATION

Over the last 20 years, it has become evident that outcome after trauma is influenced by the human body's immune response to injury. Clinicians first observed that traumatic injury could activate a systemic inflammatory response (SIRS) as reviewed by (Lenz et al, 2007, (Bone et al, 1992). They also noted that trauma patients with a SIRS at admission, had worse clinical outcomes in terms of mortality and length of stay (Napolitano et al, 2000). Extended hospital stay and late deaths after trauma were largely attributed to development of MODS and sepsis (Sauaia et al, 1995). It was shown that patients who develop multiple organ failure had higher levels of inflammatory mediators in the first few hours after injury, than those who did not (Jastrow et al, 2009).

Bacterial infection was initially believed to be the underlying cause of MODS development (Fry et al, 1980). The source of bacteria was proposed to be the gastrointestinal tract, but clinical evidence did not support this theory (Moore et al, 1991). Goris et al were the first to observe that MODS after traumatic injury did not always occur in conjunction with infection, leading them to conclude that the root cause of MODS might be "massive activation of inflammatory mediators.....resulting in systemic damage to vascular endothelia, permeability oedema and impaired oxygen availability to the mitochondria despite adequate arterial oxygen transport" (Goris et al, 1985). Recent evidence demonstrates that patients with severe blunt trauma develop MODS at a median of day 2 (interquartile range 2-4) and that development of infection follows a few days later (Minei et al, 2012).

The molecular events which lead to the development of MODS remain unclear. Some authors have concluded that traumatic injury precipitates a state of "uncontrolled and imbalanced inflammation" or "an excessive" or "dysfunctional inflammatory response" (Tsukamoto et al, (Kimura et al, 2010, (Dewar et al, 2009a, (Visser et al, 2008, (Bone et al, 1997). Others conclude that traumatic injury precipitates a state of "immune-suppression" which puts patients at risk

of pathogen invasion (Tschoeke and Ertel, 2007, (Kimura et al, 2010). MODS has also been considered to have a substantial iatrogenic component as a result of "increasingly aggressive medical and surgical interventions" (Marshall, 2010). Initially it was thought that trauma precipitated an initial SIRS state followed by a later Compensatory Anti-Inflammatory Syndrome (CARS) state; however, recent genomic evidence demonstrating simultaneous activation of pro-inflammatory and anti-inflammatory genes in the first few hours after trauma, has raised doubt over the existence of the CARS response (Xiao et al, 2011a, (Dewar et al, 2009a).

Traumatic injury activates a state of sterile inflammation which influences the pathogenesis of MODS and the clinical recovery after injury (Chen and Nunez, 2010, (Rock et al, 2010). The molecular events which lead to activation and propagation of the inflammatory state remain unclear. Clinicians involved in trauma care are therefore keen to achieve a greater understanding of the molecular events which activate the immune system.

6. THE IMMUNE SYSTEM

The aim of the immune system is to limit damage, protect from further harm and promote healing, see review by (Bone et al, 1997). The immune system is a network of cells and molecules which co-ordinate and action the human defence mechanism against harmful stimuli, an excellent review is provided by (Medzhitov and Janeway, 2000a). Traditionally, it has been considered to have an 'innate' arm and an 'adaptive' arm, each with a different functional role. Inflammation is the acute response to damage and is the product of the innate immune system. The innate immune system is a primitive defence mechanism which launches an immediate, non-specific response to harmful stimuli. It is a genetically determined response, conserved and developed during evolution; inherited by each generation.

The adaptive arm of the immune system, comprises of T(Thymus) and B(Bone marrow) lymphocytes, which are recruited by the innate system to produce antibodies to attack specific molecular targets. Fragments of harmful molecules, called antigens, are presented by 'Antigen-Presenting Cells (APCs) to T-lymphocyte cells. T-lymphocytes then transfer the antigens to corresponding B-lymphocyte cells, which undergo clonal expansion to produce suitable antibodies. Adaptive immunity is not inherited and must be reinvented by each individual (Medzhitov and Janeway, 2000b).

As our understanding of immunology has developed, the boundaries between the innate and adaptive arms have become less distinct. In particular two of the subsets of T-lymphocytes, have been shown to participate in the innate response (Bonneville et al, 2010, (Hedges et al, 2005, (Cooper et al, 2001).

a) CELLS OF THE INNATE IMMUNE SYSTEM

Structurally, the innate immune system involves a collection of leukocytes (white blood cells), each of which, upon activation, deploy a chemical arsenal. These mediators can act locally and systemically to orchestrate a rapid response to combat harmful stimuli. In states of health, immune cell populations in peripheral circulation are low. In disease states, the cell populations fluctuate as cells are mobilised from the bone marrow, spleen, lymph nodes and tissues. The innate cells are broadly divided into two categories: granulocytes and lymphocytes.

i. Granulocytes

The category of granulocytes includes neutrophils, monocytes (some of which differentiate into macrophages or dendritic cells), mast cells, eosinophils and basophils. Most of the granulocytes are able to phagocytose (ingest) foreign material they identify for destruction. In addition, each cell type possesses a range of unique functions.

a) Neutrophils

Elevated levels of neutrophils are commonly found in patients with bacterial infection. They are also elevated in the first few hours after trauma (Botha et al, 1995b, (Botha et al, 1995c, (Liu et al, 2009). Neutrophils project receptors on their surface (Fc receptors, complement receptors and Toll-like Receptors) which allow them to respond to a wide range of stimuli (Sengelov, 1995, (Akira and Takeda, 2004, (Hayashi et al, 2003). They produce numerous cytokines and have a unique ability to pass from circulating blood, through endothelium, into injured tissues; a process called 'diapedesis' (Metchnikoff, 1901). When activated they can envelop bacteria for lysosomal destruction or form neutrophil extracellular traps (NETs) (Brinkmann et al, 2004). They can also generate reactive oxygen species (ROS) for bacterial destruction via a process called the 'respiratory burst'.

b) Monocytes

Monocytes are circulating granulocyte cells which can develop different phenotypes after activation, as described in an excellent review by (Robbins and Swirski, 2010). Those which enter tissues differentiate into macrophages or dendritic cells and develop tissue-specific roles (Robbins and Swirski, 2010, (Hume, 2006). In addition to phagocytosis and the production of cytokines, these cells can display antigens for recruitment of the adaptive immune cells. The most important antigen-presenting cells (APC) are dendritic cells.

c) Eosinophils, basophils and mast cells

Eosinophils, basophils and mast cells initially circulate in blood then mature when they localise to tissues (Schroeder, 2011). They have granular contents which influence their function. Eosinophils are commonly increased in patients with parasitic infection and, once activated, can engulf parasites and digest them with intracellular enzymes (Kita, 2011). Basophils release histamine and heparin resulting in local vasodilation and anticoagulation; they are implicated

in the pathogenesis of allergy (Schroeder, 2011). Mast cells are similar to basophils and they also participate in allergy and histamine release.

ii. Lymphocytes

Lymphocytes were originally thought to be exclusive to the adaptive immune system and dependent upon the innate immune cells for activation. It is now known that some lymphocytes can respond to direct stimulation by pathogens and therefore participate in the innate response (Hedges et al, 2005, (Arase et al, 2002). Only natural killer cells and gamma delta T-cells are known to have these properties but the list may expand in the future.

a) Natural killer cells

Natural killer cells (NK Cells) have a direct cytolytic action and can produce intracellular enzymes to destroy target cells. In addition they can produce cytokines, typically interferon-gamma (IFN-γ), see review by (Cooper et al, 2001). Two phenotypes have been described depending on the density of a cell surface marker called CD56. $CD56^{dim}$ NK cells have a lower CD56 density and account for around 90% of the population, $CD56^{bright}$ NK cells account for the remaining 10% (Cooper et al, 2001). $CD56^{bright}$ NK Cells bear receptors for monocyte-derived cytokines such as interleukin-10 (IL-10), IL-12 and IL-1 and are primarily responsible for NK cell IFN-γ production. The $CD56^{Dim}$ NK cells are more granular and have potent cytotoxic activity (Cooper et al, 2001).

b) Gamma delta T lymphocytes

Gamma delta T lymphocytes (γδ T cells) are newly identified participants in the innate immune system and their precise role remains unclear. They principally reside in mucosal tissues and represent only a small percentage of the circulating lymphocytes in the blood. Nonetheless, they are thought to play a critical regulatory role in the innate immune response (Bonneville et al, 2010, (Born et al, 2006). This ability is afforded to them by their possession of a T-cell receptor (TCR) which is germ-line encoded (Hedges et al, 2005). Several of their ligands are

endogenous, so called “self-molecules”, leading to the conclusion that γδ T cells are likely to be activated by conditions involving “cellular stress”, such as hypoxia and ischaemia, see reviews by (Born et al, 2006, (Hayday, 2009). They produce cytokines, such as IFN-γ and appear to regulate other cells, including dendritic cells and macrophages.

b) EFFECTOR MOLECULES OF THE INNATE IMMUNE RESPONSE

The chemicals produced by the innate immune system are called cytokines and chemokines. These small proteins are released by cells for communication purposes. Cytokines act as signalling messengers for cell-cell or cell-tissue communication and immune response regulation, see review by (Stow et al, 2009). Cytokines can co-ordinate tissue destruction or repair, activate or deactivate immune cells and some are also cytotoxic (Stow et al, 2009). Chemokines have ‘chemoattractant’ properties which stimulate migration of immune cells. Cell migration is necessary in pathological situations to call immune cells to the site of injury, stimulate growth or blood vessel development and promote healing (Furie and Randolph, 1995). Chemokines also play a homeostatic role, including corralling immune cells in lymph nodes and co-ordinating leucocyte maturation and renewal (Baggiolini, 1998). All immune cells have the capability to produce and release cytokines and chemokines; however, each cell type tends to only release a small range of mediators. Some mediators can be produced by several different types of cell. IL-1, for instance, can originate from monocytes, macrophages, fibroblasts, epithelial cells, endothelium and astrocytes. The mechanics of cytokine release are complex. The process must accommodate the need for rapid release which is timed, sequential, directional and may involve more than one substance (Stow et al, 2009). Some cells only start to produce inflammatory mediators when the cell is activated (Meylan et al, 2006). Other cells store pre-made mediators in secretory granules for rapid release upon cell activation (Arvan and Castle, 1998). Upregulation of production pathways may then occur to maintain mediator demand (Lieu et al, 2008).

7. ACTIVATION OF THE INNATE IMMUNE RESPONSE

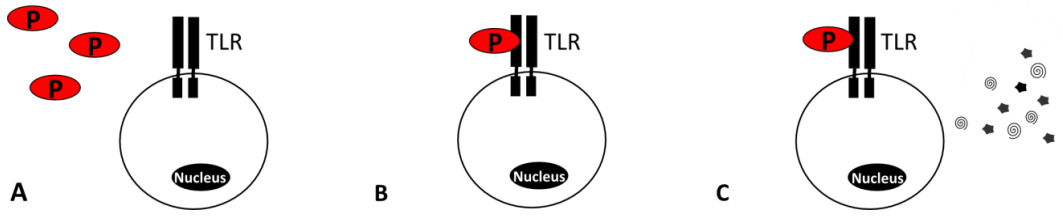
In order to produce rapid, non-specific defence against harmful stimuli, the innate immune system must be amenable to activation by a wide range of substances. It is not possible for innate immune cells to recognise every harmful stimulus individually, therefore they recognise common structural features (Meylan et al, 2006).

a) INNATE IMMUNE SYSTEM ACTIVATION IN SEPSIS

In the context of sepsis, these common features are molecules on bacteria cell surfaces. They are often essential to the pathogen's survival and are known as "pathogen-associated-molecular patterns" (PAMPs); examples include lipopolysaccharide (LPS) on gram negative bacteria and lipoteichoic acid (LTA) on gram positive bacteria, see review by (Medzhitov and Janeway, 2000b). Innate immune cells detect PAMPs using pattern-recognition receptors (PRRs) (Figure 1.1). Those on the cell surface detect extracellular PAMPs. Most of these are part of the family of Toll-Like Receptors (TLRs), reviews by (Takeda and Akira, 2004, (Akira and Takeda, 2004, (Meylan et al, 2006). In addition, there are intracellular PRRs which sense microbial and non-microbial "danger" signals produced inside cells. These include NOD-like Receptors (NLRs), the NALP3 inflammasome and RIG-like helicases (RLHs) (Meylan et al, 2006).

Once a PAMP ligand activates a PRR, there is immediate downstream activation of intra-cellular signalling. A sequence of intracellular proteins cascade the activation signal through the cytoplasm towards the nucleus resulting in activation of transcription factors, chiefly nuclear factor kappa-β (NF-kβ), and the production of effector protein molecules (Takeda and Akira, 2004). Once constructed, the effector molecules are released from the cell by a variety of mechanisms (Stow et al, 2009). This mechanism is an efficient production line, resulting in rapid release of inflammatory mediators (Medzhitov and Janeway, 2000b, (Meylan et al, 2006). Once released, cytokines activate other immune cells so production can be escalated in a state of emergency (Stow et al, 2009).

Figure 1.1:Activation of Toll-like receptors on immune cells produces inflammatory mediators



A: PAMPs (P) in the extracellular environment are detected by Toll-like Receptors (TLRs). B: TLRs are activated when the PAMPs bind to them. C: Activation of TLRs results in production of inflammatory mediators including cytokines and chemokines.

Of equal importance however, is the capacity to stop cytokine generation, see reviews by (Stow et al, 2009, (Nathan, 2002). Several mechanisms are thought to control acute inflammation. Production of anti-inflammatory cytokines can dampen pro-inflammatory effects of other mediators (Elenkov and Chrousos, 2002). Simultaneous production of pro-inflammatory and anti-inflammatory mediators can lead to a shift in action once a threshold concentration is reached. In response to environmental changes some mediators, such as tumour necrosis factor and interferon gamma, may switch from pro-inflammatory to anti-inflammatory actions (Nathan, 2002). To avoid accidental activation many immunological pathways require two signals for activation and, without that, will default to their resting status. In addition genetic pre-disposition almost certainly influences inflammatory responses (Nathan, 2002). Finally, acute inflammation is also subject to central-nervous system (CNS) modulation (Tracey, 2002).

b) INNATE IMMUNE ACTIVATION AFTER TRAUMATIC INJURY

The innate immunity can also be activated in the absence of bacteria to generate sterile inflammation, see review by (Chen and Nunez). Although many diseases involve sterile inflammation, the precise mechanism(s) of immune activation are unclear, see review by (Rock

et al, 2010). The pathway is thought to resemble the mechanism by which the immune system identifies and responds to pathogenic bacteria. In both sterile and septic inflammation, the immune system is not thought to be activated until tissue damage occurs (Matzinger, 2002, (Matzinger, 1994). In sterile inflammation, the molecules which activate the innate cells are believed to be endogenous, following release from damaged or dying cells (Matzinger, 2002, (Gallucci and Matzinger, 2001, (Nathan, 2002, (Scaffidi et al, 2002). These endogenous molecules are called alarmins (Harris and Raucci, 2006). Alarmins and PAMPs are collectively referred to as damage-associated-molecular-patterns (DAMPs) (Bianchi, 2007). A growing number of substances with Alarmin characteristics have now been reported, including bioactive peptides and intracellular molecules (Zedler and Faist, 2006, (Oppenheim and Yang, 2005). Alarmins and their clinical significance with respect to traumatic injury will be discussed in more detail, in Chapter 5.

8. LIMITATIONS OF CURRENT KNOWLEDGE IN TRAUMA

Traumatic injury activates a state of sterile inflammation and a systemic inflammatory response which is associated with higher mortality, longer length of stay and development of MODS (Lenz et al, 2007, (Jastrow et al, 2009, (Napolitano et al, 2000). Inflammation is thought to be activated by release of endogenous molecules from injured tissues but the precise mechanisms involved remain poorly understood. Greater understanding of how injury activates inflammation and how this influences outcome may lead to improvements in clinical management of trauma patients.

a) IDENTIFICATION OF PATIENTS AT RISK OF POOR OUTCOME

In current trauma care, patients at risk of poor outcome cannot be objectively and prospectively identified. Severe injuries often look very dramatic but the anatomical injury alone, does not dictate outcome. Injury severity is currently described using a variety of scores which reflect the anatomical injuries in isolation or in combination with the patient's physiology. Most scores are calculated retrospectively and do not influence patient care. The most widely used is the injury severity score (Baker et al, 1974). The anatomical injuries are graded according to the likelihood that they would lead to death. As death is not the only outcome measure of interest, modern trauma care would benefit from a more sensitive method of injury description.

b) QUANTITATIVE MEASUREMENT OF INJURY SEVERITY AT ADMISSION

Biomarkers are substances measured in biological fluids, which quantitatively measure the severity of a disease condition. They are often sought in asymptomatic disease states where early recognition and treatment may improve disease control and long-term patient outcome. In trauma, the burden of disease is often perfectly apparent but early identification of patients at risk of poor outcome may facilitate expedient treatment or early escalation of their care environment, prior to clinical deterioration.

c) TREATMENT MODIFICATION

Early stratification of patient injury burden would facilitate the development of clinical trials to test novel therapeutics or clinical treatments pathways. Traumatic injury can be considered as a disease with multiple components. Tissue injury and blood loss are the principle components, while the sequelae of blood loss and environmental factors such as hypothermia are secondary components. With greater understanding about how these components

influence the inflammatory response, we may be able to identify how medical management affects the response and also potentially modify our resuscitation techniques.

d) THERAPEUTICS TO IMPROVE OUTCOME

Although MODS and infection affect a significant proportion of trauma patients, treatment is simply supportive. Improved understanding about the activation mechanism(s) of the immune system may facilitate the development of anti-inflammatory therapeutics for traumatic injury. This is a controversial proposition in such a complex disease and the targets would have to be extremely specific. Steroids were previously administered to traumatic head injury patients, as part of the Medical Research Council's (MRC) Corticosteroid randomisation after significant head injury (CRASH-1) trial. A rise in all-cause mortality in the steroid cohort led to cessation of the trial in May 2004 (Edwards et al, 2005). The reasons for the increased mortality remain unclear but it suggests that therapeutics which globally dampen the inflammatory response are unlikely to be appropriate in trauma patients. Anti-cytokine therapies have demonstrated moderate success in other sterile inflammatory diseases, such as inflammatory bowel disease and rheumatoid arthritis (MacDonald et al, 2010). Opinion is divided about their efficacy in sepsis so, for the moment, they are only under consideration in trauma (Spruijt et al, 2010, (Reinhart and Karzai, 2001). Finally, anti-alarmin agents present an attractive therapeutic goal. High-mobility group box-1 (HMGB1) is a candidate alarmin molecule and anti-HMGB1 therapeutics are already under-development, see review by (Andersson and Tracey, 2011). With better understanding of the molecular pathways, targeted therapeutics may become a real possibility in trauma.

9. CONCLUSION

In conclusion, inflammation should be beneficial for healing and repair, but the immune response activated by traumatic injury appears to influence adverse patient outcome (Napolitano et al., 2000, Lenz et al., 2007 (Jastrow et al, 2009)). Our understanding of the molecular events which are involved in immune activation is extremely limited. A greater understanding of the mechanisms by which an immune response is generated after injury may influence management strategies and therapeutics aimed at improving patient outcome.

10. THE OBJECTIVES OF THIS PhD INVESTIGATION

This investigation was established to investigate the hyper-acute inflammatory response after traumatic injury. It focuses on patient samples taken within two hours of injury, immediately on arrival and prior to iatrogenic intervention. The investigation was devised to study the response from three principal aspects: cytokines, alarmins and lymphocytes.

a) STUDY AIMS

The first aim was to examine the innate immune response generated by traumatic injury, as evidenced by cytokine production. I began with a review of the literature to determine the current knowledge base. Cytokine quantification was then performed on plasma samples drawn from human trauma patients.

The second aim was to investigate activation of the innate immune response by examining potential trauma alarmins. I began with a review of the literature. I then sought to create a more precise definition of a trauma alarmin and used the literature to identify appropriate candidates. These candidates were then quantified in blood from human trauma patients.

The final aim was to investigate the effectors of innate immunity, by examining the white blood cell populations after traumatic injury. Once again, I began with a review of the literature to assimilate the current knowledge base in humans. Two separate studies were then performed. The first study examined the actual number of cells in peripheral blood of human trauma patients and their association with outcome. The second study examined the lymphocyte subsets in peripheral blood from human trauma patients at admission and the association with outcome.

This PhD work was conducted during a three year period of full time research. During this time, I was a fundamental part of the trauma research team. I contributed substantially to patient recruitment for ACIT2, an observational cohort study which I shall describe in detail during the next chapter. Research team participation involved consent, collection, processing and storage of patient samples, data entry, clinical follow-up and day-to-day organisation of the laboratory. By co-operating as a team, we were able to collect more samples than as individuals. The bio-bank which we established was then available for use by contributing members for their individual research projects.

CHAPTER TWO: METHODOLOGY

This project was conducted at The Royal London Hospital (RLH), one of London's Major Trauma Centres (MTCs). Three other MTCs have recently been established as part of the London Trauma System; however, the RLH has around 20 years of expertise in trauma care. Survival rates at the RLH are on a par with North American centres, which is far from standard in the UK (Davenport et al, 2010). At the current time, patients are three times more likely to survive serious injury if they receive treatment at the RLH compared with any other hospital in the UK (Davenport et al, 2010). The RAND corporation was recently commissioned, by the Department of Health, to assess the UK's capacity to conduct complex trauma research (Morgan Jones M, 2011). It concluded that the RLH is, by a considerable margin, the leading institution in this regard.

A prospective, observational cohort study of trauma patients was established at the RLH in 2008: The Activation of Coagulation and Inflammation in Trauma Study (ACIT2). The study has ethical approval to recruit up to 2800 patients and over 900 patients have been recruited to date. It is a multi-centre study with recruitment occurring in two other UK centres, Oxford and Newcastle, and additionally in Norway, Denmark, Switzerland and the USA. The RLH is therefore a fairly unique trauma facility at the current time. To understand the context in which this research has been conducted, it is essential to understand our patient population and pathway from scene to discharge.

1. THE STUDY SETTING AND PATIENT POPULATION

The trauma patient pathway involves several seamless phases; pre-hospital management, in-hospital assessment & resuscitation, Damage-Control Surgery (where required), stabilisation in the Intensive Care Unit (ICU), recovery and rehabilitation. The injury load will influence which of these phases are required and the length of time spent in each phase. In London, care is now provided by "Trauma Centres"(TCs) and "Major Trauma Centres"(MTCs) (LTO, 2011). Major trauma is defined as 'a traumatic injury which may cause death or severe disability' (RCSEng, 2009). Patients with major trauma should be taken directly to the MTC, while patients with less severe injuries should be taken to TCs. The pre-hospital team decide what level of care the patient requires. Pre-hospital care is provided by the London Helicopter Emergency Service (HEMS) and the London Ambulance Service (LAS).

a) THE ROYAL LONDON HOSPITAL: A MAJOR TRAUMA CENTRE

The RLH catchment area for trauma includes London (within the M25 motorway), Essex, Hertfordshire and Kent. All 999 requests for ambulance assistance within the M25 are received at the LAS Headquarters. A HEMS paramedic is stationed at LAS Headquarters 24h a day, to scan all 999 requests and determine those which require HEMS attendance. A pre-defined set of injuries trigger an automatic deployment for the HEMS team (Appendix 1). If serious injury is suspected, the paramedic can contact LAS crews at scene to enquire if HEMS assistance is required. The HEMS control room is at the RLH and the team deploy within 5 minutes of alert. In addition, traumatic injuries which occur in the immediate vicinity will be brought to the RLH Emergency Department (ED). HEMS services for Essex, Hertfordshire and Kent/Surrey & Sussex will transport patients to the RLH if the patient injuries require MTC admission. The hospital receives over 2200 patients a year who require full trauma team activation (See Appendix 2 for criteria); the RLH is currently estimated to be the busiest major trauma centre in Europe.

b) PRE-HOSPITAL PHASE

For both LAS and HEMS, the pre-hospital phase is focussed on airway control, optimising oxygenation, haemorrhage control and swift transfer to definitive care. The HEMS provides doctor-led, intensive care treatment to critically injured patients at the scene of injury. Early interventions, which are beyond the capacity of the LAS, can be life-saving. As far as possible, scene times are minimised. Pre-hospital interventions typically include intravenous access, bilateral thoracostomies (for tension pneumothorax), general anaesthetic (where appropriate) and placement of fracture splints and pelvic binders. Pre-hospital protocols use minimal crystalloid fluid administration. If severe haemorrhage is diagnosed, HEMS phone the RLH ED to declare a "Code Red". This alerts the hospital of the anticipated need for transfusion and rapid surgical intervention. Transfusions are ordered in advance and are available for administration when the patient arrives. Occasionally patients are administered blood units during the transfer between the helipad and the resuscitation room. Blood is now carried by HEMS for administration during flight; however, this study was completed prior to this change in management. On arrival at the ED, the majority of trauma patients have had only life-saving interventions, where necessary, short transfer times and minimal crystalloid fluid administration.

c) IN-HOSPITAL PHASE

The in–hospital resuscitation phase begins in the ED. The full trauma team is present from the outset and each member has a designated role. The Trauma Team Leader (TTL), an ED Consultant or Senior Registrar, co-ordinates the resuscitation phase. Assessment is conducted in two stages; a primary survey to identify and treat all life-threatening injuries and a secondary survey to identify and treat all injuries. A tertiary survey is then performed the following day to ensure all injuries are detected. Assessment is supported by radiological examinations such as the radiographs of the chest and pelvis, ultrasound of the torso (FAST

Scan), computed tomography (CT) and where necessary magnetic resonance imaging (MRI). Administration and delivery of blood and blood products including fresh frozen plasma (FFP), platelets and cryoprecipitate are guided by the "RLH code red protocol" to minimise delay in administration (Appendix 3). At any point in the resuscitation phase, the Trauma Surgeon(s) may decide that the patient requires surgical intervention. Limited surgery can be performed in the ED in emergency situations. Ideally, the need for surgery should be anticipated and patients transferred to the operating room (OR) expediently. Surgical intervention at this stage aims to stop bleeding and prevent death or limb loss; Damage-Control Surgery (DCS). DCS often requires temporary solutions such as strategic packing of the abdomen and stenting or ligation of vessels and bowel. Damage control resuscitation (DCR) continues simultaneously. At the end of the procedure, the patient may be transferred to the intensive care unit (ICU) for physiological stabilisation including warming, continued correction of coagulopathy and reversal of acidosis. If required, patients are returned to the theatre at 48h, for definitive repair.

d) Recovery and Rehabilitation Phase

The recovery phase is a very unpredictable time and is influenced by the severity of the injuries sustained and the host response to them. Once patients are physiologically stable, awake and obeying instructions their rehabilitation can begin. A variety of specialist therapists may be required depending on the injury profile and the patient need. Transfer to other medical centres may be necessary if patients require longer-term assistance. Alternatively some rehabilitation can be provided by community teams.

2. ACTIVATION OF COAGULATION AND INFLAMMATION IN TRAUMA (ACIT2)

ACIT2 is an on-going, prospective observational cohort study of trauma patients. It was established at The Royal London Hospital in January 2008 having received ethical approval from the East London and City Regional Ethics committee 1 on 13th November 2007 (07/Q0603/29). It was designed to facilitate investigation of the human response to traumatic injury. Timed blood samples are combined with daily follow-up for clinical outcomes. A team of medical researchers recruit trauma patients between 9:00-22:00h seven days a week.

a) PATIENT SELECTION

This investigation pertains only to patients who were recruited at the RLH. Throughout the whole research period, the number of patients available for study ranged from ACIT 001 to ACIT 638, but the investigations were staggered across three years. As such, the experiments were conducted with different numbers of patients. The number of patients and demographics of the study cohort are therefore described for each individual experiment. At the RLH, around 2200 admissions each year require full trauma team activation. More than 500 of these patients are categorised as 'severe' by an injury severity score (ISS) of >15 (Baker et al, 1974). There is a 20-30% 'over-triage' rate which means that not all patients require MTC level management. Within recruitment hours, trauma calls are attended by the on-call research team and patients are screened for eligibility. Any patient with an abnormality in the primary survey is eligible for recruitment to ACIT2. Pre-defined exclusion criteria for recruitment include:

- Age <16y
- Transfer from another hospital
- Arrival < 120 minutes from injury
- More than 2000ml crystalloid pre-hospital
- More than 5% burns
- Severe liver disease
- Known bleeding abnormality (including anticoagulant medication)
- Refused consent
- Vulnerable patients (in custody or in prison)

In addition, patients with no suspected abnormality are enrolled as a "control" population. Those who have an ISS between 0-4 at the completion of investigation and a normal base deficit are included in the control cohort during analysis.

At admission, it is usually impossible to determine whether a patient has severe liver disease, but the decision to recruit patients must be made rapidly and before a full past medical history can be elicited. Such patients may therefore, unknowingly, be enrolled into ACIT2. During admission, if liver disease is diagnosed or identified from the medical records, then these patients are retrospectively excluded. Patients later identified as taking regular anti-coagulation medications are also retrospectively excluded. Of the patients included in this investigation, retrospective exclusion for liver disease or anticoagulant medication was not required for any of the enrolled cohort. In addition, although not a defined exclusion criteria for ACIT2 recruitment, patients taking regular steroids or non-steroidal anti-inflammatory medications were excluded from my investigation due to their influence on immune function.

b) CONSENT

At admission, it is frequently impossible to obtain informed consent from the patient or their next of kin. Emergency research is permitted under these circumstances, using consent from a professional legally appointed representative (pLAR) in accordance with the Mental Health Act

2005 (Coats and Shakur, 2005, (Coats, 2006). At The RLH, trauma team leaders (TTLs) are allowed to act as a pLAR because they are responsible for the patient's care, independent of the research team and have no conflict of interest. With pLAR consent in place, we are permitted to collect and store blood samples for trauma research purposes. Written consent from the patient or next of kin is required to permit their use and long-term storage. Typically this consent is sought at 72 hours from admission. The emotional state of the patient, or their family, may influence consent timing and their ability to give informed consent. If consent is refused, samples are destroyed.

c) PROTOCOL FOR BLOOD SAMPLE COLLECTION

The ACIT2 study protocol involves three timed blood samples; the first immediately after admission (a median(IQR) of 10(6-15) min from arrival) and, as far as possible, prior to intervention, the second at 24h +/- 1h from admission and the third on the day which corresponds to 72h from admission. At each sample point the same blood samples are drawn:

- 2 x 4.5 ml citrated vacutainers for plasma collection (0.109M + buffered sodium citrate 3.2%, Becton Dickinson, Plymouth, UK)
- 1 x 2.7ml citrated vacutainer for ROTEM Analysis (0.109M + buffered sodium citrate 3.2%, Becton Dickinson, Plymouth, UK)
- 1 x 3ml hirudin vacutainer for Multiplate Analysis (>15ug/ml hirudin, Verum Diagnostica GmbH, Germany)
- 1 x 6.5ml Proteomic P100 for proteomics biobanking (Becton Dickinson, Plymouth, UK).
- 1 x 2.5ml RNA Paxgene for genetic analysis (PreAnalytix GmbH, Switzerland)
- A standard panel of blood tests for routine laboratory processing including; Full blood count, coagulation screen, fibrinogen and electrolytes
- An arterial blood gas (ABG) is also drawn as part of standard care at admission and at 24h or 72h if patients have an arterial line in-situ.

Multiplate, proteomics, ROTEM and RNA were not used during this investigation and therefore shall not be discussed further.

Daily follow-up is conducted by the research team until death or discharge. Clinical outcomes include: 28 day mortality, total length of stay, organ dysfunction and clinically relevant infection episodes. MODS is defined using the SOFA Score (Vincent et al, 1996). Infection was defined using the Centre for Disease Control and Prevention (CDC) criteria as a 'localised or systematic condition resulting from an adverse reaction to the presence of an infectious agent(s) or its toxin(s) occurring 48 hours post admission' (Horan et al, 2008). Infection is an important outcome measure, but one which is difficult to clinically diagnose with absolute certainty, particularly in critically unwell patients. Adverse clinical signs, such as pyrexia or pulmonary infiltrates on a chest x-ray, may indicate infection but are not specific to infection (Barie et al, 2004, (Marik, 2000). Optimal timing for administration of antibiotic therapy in intensive care unit patients therefore still remains controversial and cannot, in itself, be used as a surrogate indicator of infection (Hranjec et al, 2012). Direct observation of purulent exudates combined with positive culture results would be the gold standard but, this is not always possible. To aid consistency of infection data recording, results were correlated with those collected by my colleague (EC) who was compiling infection data for her own PhD.

When ACIT2 was first established, SOFA scores were only collected for intensive care patients. During my first year, I wished to analyse my cytokine measurements with SOFA scores and realised that patients on the ward, especially those in a high-dependency setting, often also had organ impairment which was not being captured. I therefore instigated a change in the study protocol to obtain prospective SOFA collection for all ACIT2 patients as part of the clinical follow-up. This was in operation from patient 400 onwards. Retrospective data were compiled for as many of the cytokine patients as possible. Poor quality of documented data limited this exercise and where evidence was inconclusive, the SOFA score was deemed

unobtainable. Although I was aware that the PaO^2/FiO^2 ratio for non-intubated patients with respiratory compromise would be inaccurate, I believed that it was an acceptable limitation and that it was better to include their organ impairment in my dataset than exclude it.

d) PROTOCOL FOR SAMPLE PROCESSING

i. Citrated plasma

The citrated vacutainers tubes were centrifuged at 3400rpm for 10 minutes in a Clinispin Horizon 853VES Laboratory Centrifuge (Woodley Equipment Company Ltd, Bolton, UK). Blood was separated into three layers; plasma, buffy coat and erythrocytes (red cells). The top two thirds of the plasma from each tube was placed into a clean 5ml polypropylene tube (Falcon Range, Becton Dickinson, Plymouth, UK) and centrifuged for a second time at 3400rpm. The double-spun, platelet-poor plasma was then transferred, in 500ul aliquots, to 3 x 0.6ml Cliklok microcentrifuge tubes (Simport, Jencons, UK). 6 microcentrifuge tubes in total. The lower third of the plasma from each tube was transferred, in 500ul aliquots, to a 0.6ml Cliklok microcentrifuge tube (Simport, Jencons, UK) for storage as single spun plasma. The erythrocytes were transferred in 500ul-1000ul aliquots to a 1.5 ml microcentrifuge tube (Fisher Scientific, UK). All samples were labelled and stored in catalogued cryoboxes at -80°C.

ii. Arterial Blood Gas

Arterial blood gas analysis was performed on a near patient testing device, ABL 500 Flex (Diamond Diagnostics, USA) within the ED department or the Intensive Care Unit.

3. SCORING SYSTEMS USED DURING ANALYSIS

A variety of scoring systems were used during this investigation. All are accepted and widely used in clinical trauma surgery and trauma research.

a) THE INJURY SEVERITY SCORE (ISS)

A number of scores have been developed to grade the severity of injuries and their relationship with mortality. The Injury Severity Score (ISS), the first of its kind, is the system used at the RLH to describe injury load (Baker et al, 1974). It is devised retrospectively once all injuries have been identified. Each anatomical region is graded between 0-5 to obtain an abbreviated injury score (AIS). Trained personnel use AIS scales to obtain consistent categorisation. The three highest AIS scores are squared and combined to obtain a total from a maximum of 75. Injuries scoring 9 are regarded as severe (not life threatening), 16 as severe (life threatening) and 25 as critical with uncertain survival (Box 2.1).

Box 2.1 : The Injury Severity Score

Anatomical Regions	**AIS Grading of severity**	**Injury severity Score**
Head & Neck Face Chest Abdominal or pelvic contents Extremities or pelvic girdle External / General	1 = Minor 2 = Moderate 3 = Severe (Not Life Threatening) 4 = Severe (Life Threatening but survival probable) 5 = Critical (survival uncertain)	Top three AIS Scores Square each one Add them together Obtain a score out of 75

b) THE SEQUENTIAL ORGAN FAILURE ASSESSMENT SCORE (SOFA)

There is no agreed standard for diagnosis of MODS. A number of scoring systems have been devised to describe the severity of organ failure consistently between patients but selection remains centre specific (Baue, 2006). The Sequential Organ Failure Assessment (SOFA), Marshall Score and Denver Score are three of the most widely used tools (Vincent et al, 1996, (Marshall et al, 1995, (Sauaia et al, 2009). Considerable variation still occurs in their application. For ACIT 2, we elected to use the SOFA Score (Box 2.2).

Box 2.2 : The Sequential Organ Failure Assessment Score (SOFA) (Vincent et al, 1996)

Organ System	0	1	2	3	4
Respiration PaO_2/FiO_2 kPa	>53.3	40 - 53.3	< 39.9	<25.2	< 13.3
Haematological Platelets , x109/L	>150	101-150	51-100	21-50	0-20
Hepatic Bilirubin, µmol/l	0-19	20-32	33-101	102-204	>204
CNS Glasgow Coma Score	15	13-14	10-12	6-9	<6
Cardiovascular MAP, mmHg	>70	< 70 no support	Dopamine ≤5.0 or norepinephrine/ epinephrine ≤0.05 mcg/kg/min	Dopamine 5-14 or norepinephrine/ epinephrine ≤0.1 mcg/kg/min	Dopamine ≥15 or norepinephrine/ epinephrine > 0.1 mcg/kg/min
Renal Serum creatinine	<110	110-170	171-299	300-440 Or <500 mL/24h	>440 or dialysis Or <200 mL/24h

4. EXPERIMENTAL METHODOLOGY

a) ENZYME-LINKED IMMUNOSORBANT ASSAY (ELISA)

ELISA is a well-established technique which can be used to quantify the concentration of a chosen substance in a liquid sample. It exploits antigen-antibody affiliations and results in the creation of an antibody-antigen 'sandwich'. The last layer of the sandwich is a chromophore or flurophore whose emissions can be detected by a plate reader. The density of the signal mirrors the presence of the target substance, enabling the concentration of the substance to be calculated. In this investigation, the concentration of specific protein molecules in our patient plasma samples was determined using ELISA.

i. Cytokines

Plasma cytokine quantification was performed using a commercially available multiplex pro-inflammatory cytokine analysis platform which included TNFα, IL-1β, IL-6, IL-8, IL-10, IL-12p70 and IFNγ (Human Pro-inflammatory 7plex kit, Mesoscale Discovery, Maryland, USA, Cat K15008A-5). All patient samples were single-spun plasma at a 1:1 concentration; they were performed in duplicate and processed in accordance with the experimental protocol. 40 patient samples were performed per plate. A standard curve was prepared using the 'standard cytokine solution' provided, to achieve a range from 0.60 – 2500.00 pg/ml. The range of the standard curve was well within the detection limits of the apparatus.

The 96 well plates were pre-coated with capture antibodies for each of the 7 cytokines. Firstly, HSC assay diluent (25µL) was dispensed into each well and incubated for 30minutes at room temperature (RT). Standard solutions and human patient plasma (25µL) were then added to each well and incubated for 2h on a plate shaker at RT; one well per standard or patient sample, in accordance with a pre-defined scheme. The plate was then washed 3 times with phosphate buffered saline (PBS) and 0.05% Tween-20. Next detection antibody solution was added to each well (25µL) and incubated for 2h on a plate shaker at room temperature (RT).

The wash step was then repeated x3. Finally, Read buffer (150µL) was added to each well and the plate read on a SECTOR® Imager 2400 (Mesoscale Discovery, Maryland, USA). Unknown concentrations were obtained using the standard curve and the inbuilt Mesoscale software (Figure 2.1).

The cytokine concentration data was cleaned by subtracting the values obtained for 'blank' cells from the unknown sample concentrations on each plate. Data were then examined. Cytokine measurements below 0.6 pg/ml (the lowest standard), were quoted as their recorded value and included in the analysis. The sensitivity of the Mesoscale platform enables these values to be quoted, rather than excluded (verified by personal communication with Mesoscale). Concentrations below 0.6 pg/ml are also incredibly small and unlikely to be of clinical significance. No values were extrapolated beyond the highest point of the standard curve. Of the 456 samples measured, 6 samples (1.3%) were identified as outliers and omitted from analysis, after discussion with a statistician. Repetition and confirmation of these values would have been desirable, but was not practical.

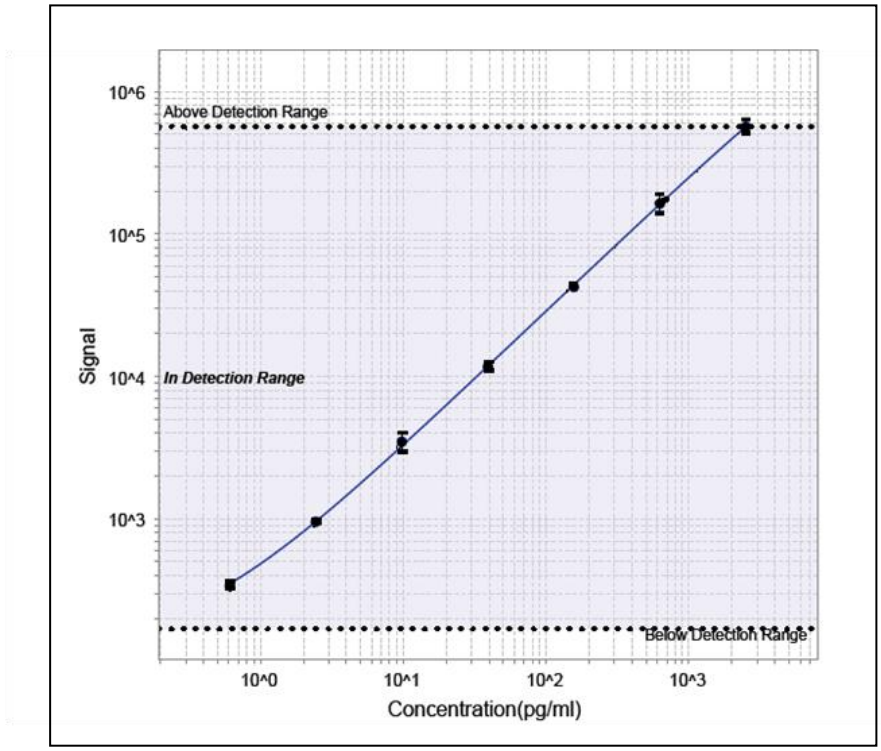
Box 2.3 Cytokine measurements excluded from analysis

Time point	No of patients in dataset	Cytokine	Value excluded	The range of remaining data
Hr 0	235	TNFα IL-10 IL-12 p70	394.7 1287.1 3632.4	0.0 - 47.7 0.0 - 572.3 0.0 - 930.98
Hr 24	123	IL-1β IL-12 p70	761.8 1200.2	0.0 - 93.0 0.0 - 405.2
Hr 72	98	IL-12 p70	741.5	0.0 - 71.5

There was nothing particularly remarkable about the demographics of these 6 patients or their outcome. In addition, the values obtained were well above those observed in other recent

studies (Hranjec et al, 2010, (Jastrow et al, 2009). It is possible that these patients represent an extreme phenotype and that by omitting their values I have weakened my conclusion, I am therefore mindful of this aspect for future investigations. On this occasion, however, I am satisfied that their exclusion from my analysis was a reasonable course of action.

Figure 2.1 : Typical standard curve of IL-6 on the Mesoscale SECTOR® Imager 2400



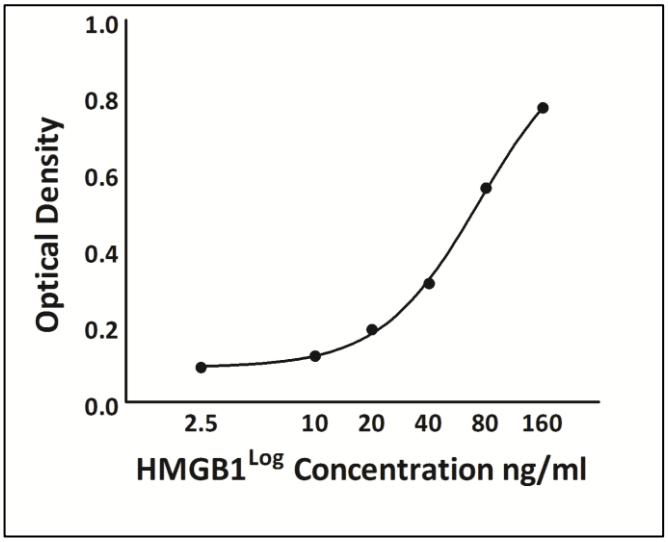
ii. High mobility group box 1 (HMGB1)

HMGB1 was quantified in the single-spun plasma, in duplicate, using ELISA (1x3400rpm). The HMGB1 ELISA (ST51011 Shino-Test Corporation via IBL International GMBH, Germany) was used according to the manufacturer's instructions. The 96 well plates were pre-coated with anti-HMGB1. Firstly, the standard solutions were reconstituted to enable construction of a standard curve with a range from 0-160 ng/mL. Diluent buffer (100µL) was then dispensed into each well. Standard solutions or the human plasma (10µL) were then added to each well

in a pre-defined scheme and incubated in a water bath at 37°C for 24h. The plates were washed x5 with the wash buffer. Enzyme conjugate (100µL) was added to each well and the plates were incubated for 2 hr at RT. The washing step was then repeated x5. Colour solution (100µL) was added to each well and incubated for 30 minutes. Stop substrate (100µL) was then added to stop the reaction. Plates were read at 450nm using the plate reader (SIAFR Synergy HT plate reader, BioTek, Winooski, USA).

Optical densities for each standard were plotted using Graph Pad and log transformed to create a standard curve. Unknown plasma HMGB1 concentrations were then obtained from the standard curve and transformed back into raw data (Figure 2.2). Unknown samples which lay outside the limits of the standard curves were discarded.

Figure 2.2: An example of a standard curve for an HMGB1 ELISA plate



For quality control purposes, the intra-assay coefficient of variation (%CV) was calculated and found to have an average(SD) of 3.9%(2.0-7.0). Duplicate values with great variability (%CV>15) were excluded from the analysis (9/320 = 2.8%). Although only a small percentage of the dataset, if I were to conduct this experiment again, I would advocate repeating these

measurements rather than excluding them. The mean(SD) inter-assay %CV was within acceptable range at 6.6(2.7). In addition, positive controls were used for each plate. These were found to be within the set limits for each plate (10-30ng/mL). The manufacturers recommended that an HMGB1 concentration above 1.4ng/mL should be considered outside of the normal range.

iii. Nucleosomes

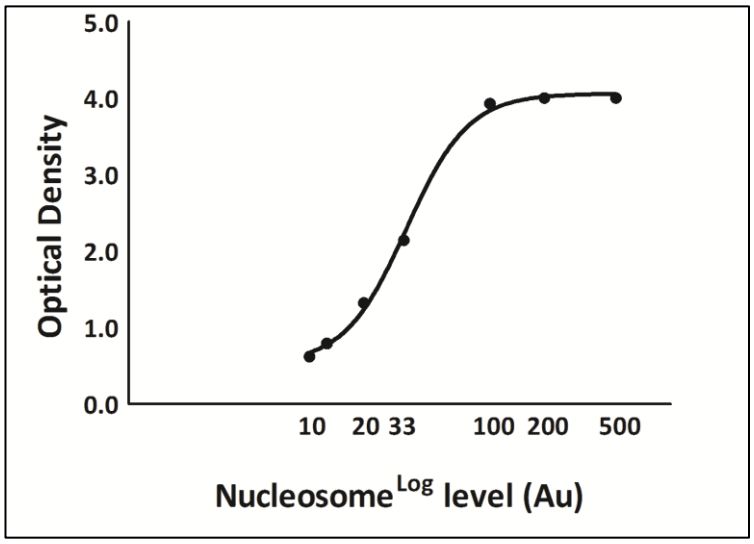
Nucleosomes were quantified using a 'Cell Death' ELISA (Cell Death ELISA plus, Roche Applied Science, UK) with a validated adaptation of the protocol as reported by Holdenrieder, using the purified nucleosome solution, provided in the kit, as a standard (Holdenrieder et al, 2001). Although this adaptation had many advantages from the original, it still posed a difficulty, as the reconstituted purified nucleosome solutions had varying concentrations which would prevent comparison of results between plates. To overcome this, the purified nucleosome preparations were pooled and this combined solution was used to construct the standard curve for all 8 plates. Dilutions of: 1:2, 1:5, 1:10, 1:30, 1:50, 1:80, 1:100 were used. As this standard solution was only viable for a few hours, all eight plates were processed simultaneously and I elected to perform them single-handedly.

The 96 well plate was pre-coated with streptavidin. To each well, 20µL of standard or sample was added in accordance with the pre-defined plate plan. An immunoreagent (80µL) containing a combination of anti-histone-biotin and anti-DNA-peroxidase was then added to each well and incubated for 2h at RT on a plate shaker. A wash step was performed x3 and then ABTS solution (100µL) was added to each well. This was allowed to incubate for 15minutes before ABTS Stop solution (100µL) was added. The plates were read at 405nm on the plate reader (SIAFR Synergy HT plate reader, BioTek, Winooski, USA).

Optical densities for each standard were plotted using Graph Pad and log transformed to create a standard curve. An arbitrary value of 1:1 = 1000Au was assigned. Unknown plasma

nucleosome concentrations were obtained from the standard curve and transformed back into raw figures (Figure 2.3). Unknown samples which lay outside of the limits for the standard curves were discarded.

Figure 2.3: Standard curve for a Cell-Death ELISA plate



For quality control purposes twelve results that were above the highest point of the standard curve were discarded rather than extrapolated. The intra-assay and inter-assay %CV were then calculated. Values greater than 15% were identified in 45/267 results (17%). The variation was probably due to pipetting error on my part, as a result of processing too many plates single-handedly in a limited time frame. Where one of the duplicates was obviously a blank 'missed well', the singlet value was included, CV% values up to 25% were accepted and the remaining values (n=13) were excluded. Eventually, 254 values were passed for analysis, the average (SD) intra-assay %CV was 17.5 (22) and the inter-assay %CV was 5.5(5.0) for standard 4 and 10.6 (16.3) for the 'blank'. At the time, I recognised that the omissions and variability of the dataset were undesirable and weakened the results, but I considered it necessary as the experiment

could not be repeated. I thought that the large numbers of patients included in the study would balance out the variability in the data values. On reflection, it would have been advisable to repeat the whole experiment with some assistance. It would not now be possible to perform this on the same patient cohort but confirming my findings in another group of patients would be desirable.

As a result of this experiment, however, I made a number of improvements in my technical approach to ELISAs. These included ensuring homogeneity by mixing thawed plasma with the vortex, prior to placement on the plate and then rapidly centrifuging samples to pellet debris if required. I also realise there is a limit to the number of plates that I can simultaneously process and, in future, would request help for such a task. The final consideration is the influence of freeze-thaw cycles. The limitations of freeze-thaw were recognised and, at times, prevented the measurement of some analytes, for example Annexin V which will degrade after one freeze-thaw cycle (communication with BioVendor R&D). In general, freeze-thaw was minimized throughout the study but it was, at times, also unavoidable. The order of the ELISAs was: cytokines, HMGB1 then nucleosomes and 225 samples were used for all three. ACIT2 samples are currently stored in 500ul aliquots. Storage in 60-100ul aliquots would enable single use of plasma samples and a change to our ACIT2 storage protocol is worth considering for the future.

b) QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION

I am very grateful to Mr Tolga Sursal MS, Mr Kiyoshi Itagaki, PhD and Dr Carl J. Hauser MD, FACS, FCCM from The Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston MA, 02215, USA who conducted the PCR for this investigation.

Quantitative real-time PCR (qPCR) is a technique which is used to amplify and quantify genetic molecules within a sample substrate. Cytochrome B is a molecule uniquely encoded in the mitochondrial deoxyribonucleic acid (MtDNA) genome. We wished to determine the concentration of Cytochrome B within the peripheral blood of our trauma patients using qPCR. As MtDNA is similar to bacterial DNA, bacterial 16S was also measured to identify bacterial contamination.

i. DNA isolation

Firstly 100µL of platelet-deplete plasma (double spun/2x 3400rpm) was centrifuged a third time at 5000xg to obtain a pellet and a supernatant. These were transferred to 2 separate microcentrifuge tubes and the pellet was re-suspended in 180µL of Buffer. Purified DNA was then isolated from both the pellet and supernatant using a QIAamp DNA blood mini-kit (USA QIAGEN Inc., Valencia, CA 91355). This technique has five phases; lysis, binding, washing x2 and elution. Lysis was achieved by the addition of 20µL of proteinase k. The solution was vortexed to mix and incubated for 1h at 56°C on a shaker plate. RNase A (4µL) was then added; the solution was vortexed to mix and incubated for 2 minutes at RT. Next, 200µL of Buffer AL was added, vortexed and incubated for 10 minutes at 70°C. Then ethanol 96.1% (200µL), was added, vortexed and incubated for 10 minutes at 70°C. The mixture was transferred to the spin column in a 2ml collection tube and centrifuged at 6000 xg for 1 minute. The contents of the spin column were then transferred to another 2ml collection tube and the filtrate discarded. This solution was centrifuged again at 6000 xg until all the solution had passed through the membrane. Buffer AW1 (500µL) was added, centrifuged at 6000 xg for 1 minute then transferred to another collection tube. Buffer AW2 (500µL) was added, centrifuged at

20,000xg for 3 minutes and then transferred into another collection tube. Finally, 80µL of DNase free water was added before centrifugation at 6000xg for 1 minute to elute the purified DNA. This process was performed separately for the pellet and the supernatant.

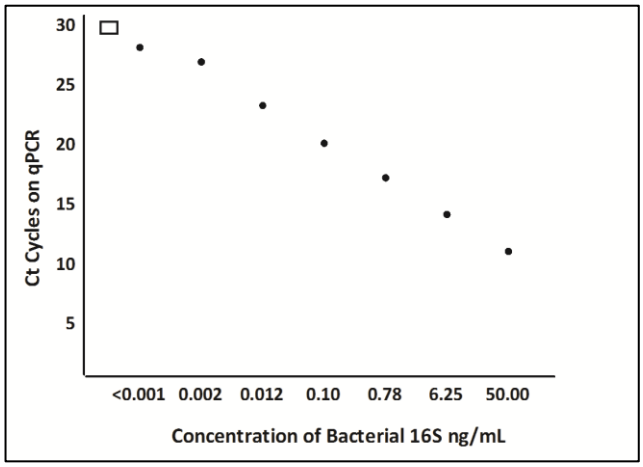
ii. Quantitative real-time Polymerase chain reaction (qPCR)

The purified DNA from the pellet and the supernatant were then analysed individually. Each patient sample was performed in triplicate. DNA from the pellet (5µl) and DNA from the supernatant (5µl) were individually placed into 96 well plates on ice. 2µL of a 10mM solution of forward and backward primers of bacterial 16S or human cytochrome B were added to the wells (Human cytochrome-B(CytB) primer, Invitrogen, Eugene, Oregon, USA: 5-ATGACCCCAATACGCAAAAT-3 (forward) and 5-CGAAGTTTCATCATGCGGAG-3 (reverse). Bacteria 16S ribosomal DNA primer, Invitrogen, Eugene, Oregon, USA: 59-CGTCAGCTCGTGTTGTGAAA-39(forward) and 59-GGCAGTCTCCTTGAGTTCC-39 (reverse). In addition, 12.5µL of SYBR Green Master Mix was added (Applied Biosystems, Foster City, CA 94404). Cycles of PCR were run using a Mastercycler EP Realplex (Eppendorf, Hauppauge, NY 11788). Standard curves, for each plate, were devised using purified preparations of Human Mitochondrial DNA or E.coli DNA (Invivogen, San Diego, CA 92121). Standard curves were plotted using log values in Excel (Microsoft, USA). Plasma concentrations of cytochrome B and bacterial 16S DNA for the patient samples were then calculated from their standard curves and transformed back into raw data (Figures 2.4 & 2.5). The ranges of detection were 0.2 – 50,000ng/ml for bacterial 16S and 0.4 – 109,600 ng/ml for cytochrome B. Primers were tested for specificity using purified solutions of bacteria(Figure 2.6). The methodology for the qPCR had already been validated in previous studies (Zhang et al, 2010a, (Zhang et al, 2010b).

For this study, 250 patient samples were sent for MtDNA & Bacterial 16S measurement in two batches. A methodology change between ACIT 001-100 meant that the first batch of results were no longer comparable with those from the next (n=150) cohort. In addition, 14 patients

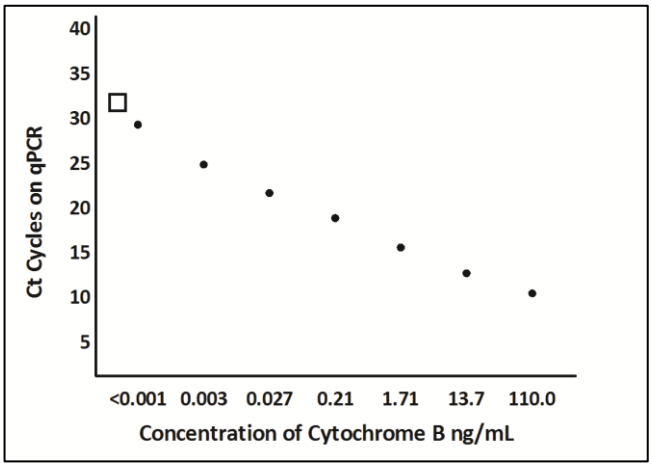
with penetrating traumatic injury had MtDNA measurements but were not included in the analysis. This resulted in 136 trauma patient samples being available for analysis. We determined that only the supernatant values would be used in the analysis, as these would describe the quantity of mtDNA circulating freely within patient plasma.

Figure 2.4: Standard curve for bacterial 16S



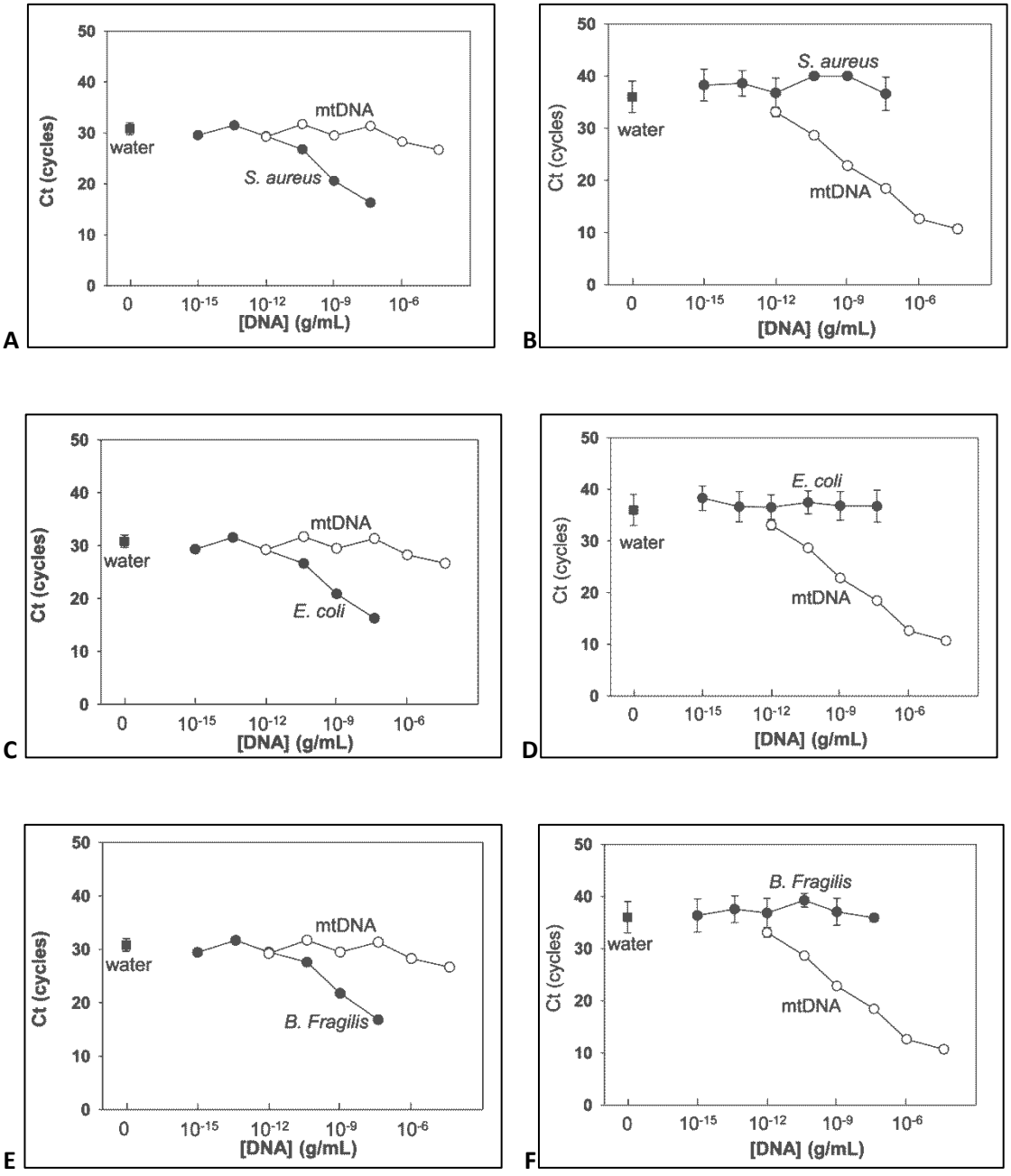
Varying concentrations of a purified bacterial 16S solution were used to create a standard curve for qPCR. High concentrations are more easily detected and therefore have fewer Ct Cycles. Water (□) was a negative control and was consistently below the lowest standard. Data are presented on a log(Base 2) scale.

Figure 2.5: Standard curve for Cytochrome B



Varying concentrations of a purified cytochrome B solution was used to create a standard curve for qPCR. Water (□) was a negative control and was consistently below the lowest standard. Data are presented on a log(Base 2) scale.

Figure 2.6: Primer specificity tests



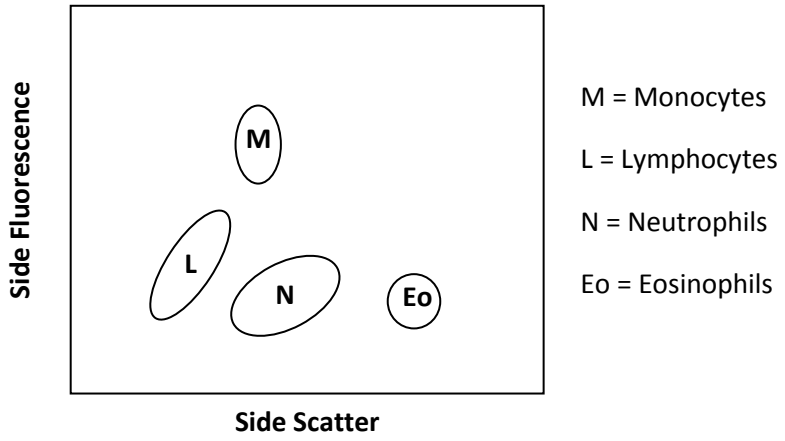
qPCR primers for the DNA of three specific species of bacteria and Mitochondrial DNA (MtDNA) were added to purified solutions of bacteria (A,C & E) and mitochondrial DNA (B, D & F). This experiment demonstrated that the primers used in the main investigation were specific for MtDNA.

c) DIFFERENTIAL WHITE BLOOD CELL ANALYSIS

White blood cell counts (WBC) and their differential breakdown are a routine investigation in trauma patients. The WBC count reports the total leucocyte count, neutrophil count, lymphocyte count, monocyte count, eosinophil count and basophil count (10^9 /L). Performed on arrival, almost always at the same time as the ACIT2 blood draw, these samples are processed by the hospital laboratory and used by the clinicians as part of patient treatment. In addition, a WBC count is performed (almost) daily as part of in-patient management. The blood samples were processed using a Sysmex SE2100 Analyser (Sysmex, Milton Keyes).

The Sysmex SE2100 Analyser is a fully automated analyser which uses flow cytometry principles to differentiate the main white blood cell subsets. Eighteen µl of blood is drawn through the white cell differential channel. A small volume (0.882ml) of reagent (Stromatolyser-4DL) is then added, at a dilution of 1:50. This results in lysis of the erythrocytes and platelets within 22 seconds. In addition, it also permeablises the membrane of the white blood cells. A polymethine dye within the reagent enters the WBCs and binds to nucleic acid and cytoplasmic organelles. Forty µl of the mixed sample passes through the flow cytometer, which uses a laser light of 633nm. The fluorescence intensity emitted by each cell is proportional to the quantity of dye bound within each cell. The different nucleic acid composition of each immune cell subset enables them to be differentiated. Basophils and neutrophils contain similar amounts of nucleic acid and therefore form a homogeneous population during this analysis. Basophils are measured by a similar process in a separate channel of the analyser. The neutrophil count is calculated by subtracting the basophil population from the neutrophil (N) population (Figure 2.8).

Figure 2.7: Illustration of the SE2100 display showing the position of the immune cell populations



d) FLOW CYTOMETRY

2-3ml of whole blood was drawn from a femoral artery, antecubital fossa vein or subclavian vein into an EDTA tube (Becton Dickinson, Plymouth, UK). The vacutainers were stored at 4°C until processing; a maximum of 2h from blood draw.

i. Sample processing

Lysis buffer (BD Pharm Lyse, Cat No: 555899, BD, Plymouth) was warmed to 37°C in a water bath. Two 15ml conical based falcon tubes were labelled 'control' and 'test'. Antibody tubes and the ETDA blood samples were placed on ice. Vacutainers were gently but repeatedly inverted to mix the blood sample. 500µl of whole blood was then transferred into each of the falcon tubes. 8mls of lysis buffer was added to each falcon tube in order to lyse the erythrocytes. Both tubes were agitated for 5-7 seconds using a bench top vortex device. If lysis was slow the tubes were returned briefly to the water bath. Lysis was completed within 5-7 minutes, as evidenced by a visual change; the red solution transforms from an opaque liquid to

a transparent liquid. Both tubes were then centrifuged at 200g for 5 minutes at 4°C (Multifuge 3SR+ Thermo Scientific, USA). From then onward, cells were kept on ice.

During centrifugation, the white blood cells (lymphocytes, granulocytes, monocytes etc) collect to form a pellet in the conical base of the Falcon tubes. The supernatant was removed, leaving 100-200µl above the pellet. 10µl of FCR blocking antibody (Human FcγR binding inhibitor 14-9161-73, eBioscience Ltd, UK) was added and the tube agitated gently to mix contents. The tubes were then incubated in the dark for 10 minutes at room temperature.

Six labelling antibodies were then added to both tubes:

- 2.5 µl Anti-human gamma delta TCR FITC (Cat No: 11-9959, eBioscience Ltd, UK)
- 2.5 µl Anti-human CD3 PE-Cy7 (Cat No: 25-0038, eBioscience Ltd, UK)
- 2.5 µl Anti-human CD4 eFluor®450 (Cat No: 48-0047, eBioscience Ltd, UK)
- 2.5 µl Anti-human CD56 (NCAM) APC (Cat No: 17-0567, eBioscience Ltd, UK)
- 2.5 µl Anti-human CD8a APC-eFluor®780 (Cat No: 47-0088, eBioscience Ltd, UK)
- 10 µl Anti-human CD45 PerCP-Cy5.5 (Cat No: 45-0459, eBioscience Ltd, UK)

A seventh antibody was added to the 'test' tube:

- 10 µl Anti-human CD69 PE (Cat No: 12-0699, eBioscience Ltd, UK)

Finally, to the 'control' tube a different seventh antibody was added:

- 2.5 µl Mouse IgG K Isotype Control CD69 PE (Cat No: 12-4714-42, eBioscience Ltd, UK)

Both tubes were gently agitated to mix the antibodies with the cells, then incubated in the dark at room temperature for 15 minutes.

The cells were washed using 2mls of PBS, agitated briefly and then centrifuged at 200g for 5 minutes. The supernatant was removed to leave 100µl remaining above the cell pellet. Antibodies were then fixed by adding 250µl of a fixing solution containing 4%

paraformaldehyde (BD Citofix™ Cat 554655, BD, Plymouth, UK). The tubes were agitated gently to mix the contents and then stored, in a dark box, at 4°C.

ii. Sample cytometry

Within 6-48h of fixation, the samples were transferred into 5ml tubes for cytometer analysis. The 'control' sample was vortexed for 3-4 seconds and transferred into a plain glass FACS tube. The 'test' sample was vortexed for 3-4 seconds and then transferred into a Trucount Tube (Cat 340334, BD Biosciences, San Jose, CA). Using a Canto II cytometer, 3 million events were counted for the 'control' sample. For the 'test' sample, the beads were set as a 'P1 event' and 20,000 events were recorded. Data were initially displayed in FACSDiva software version 6.1.03 (Becton Dickinson, UK) then exported to Flow Jo (Tree Star Inc., Oregon, USA) for analysis.

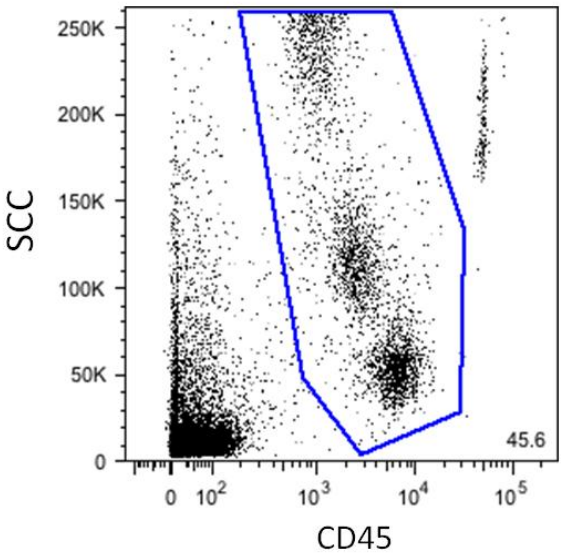
iii. Gating strategy

The gating strategy for the analysis enabled sequential analysis of specific, labelled lymphocyte subsets; namely T-helper cells (Th), Cytotoxic T cells (CTLs), Natural Killer Cells (NK Cells), Gamma Delta Cells (γδ Cells) and the 'unconventional' lymphocytes which include small sub-populations such as the Natural Killer T Cells (NKTs) (Figure 2.9). These unconventional cells were not specifically stained for individual examination, so can only be broadly discussed as a group. Box 2.2 outlines the gating strategy for cell identification. In addition, CD69 was examined as a marker of early T-lymphocyte activation.

Box 2.4: Flow cytometry antibodies and the gating strategy

Lymphocytes	CD45	CD3	CD4	CD8	CD56	γδTCR
All	+					
T Helper	+	+	+			
CTLs	+	+	-	+		
NK Cells	+	-	-	-	+	
γδ Cells	+	+	-	-	-	+
Unconventional T cells	+	+	-	-	-	-

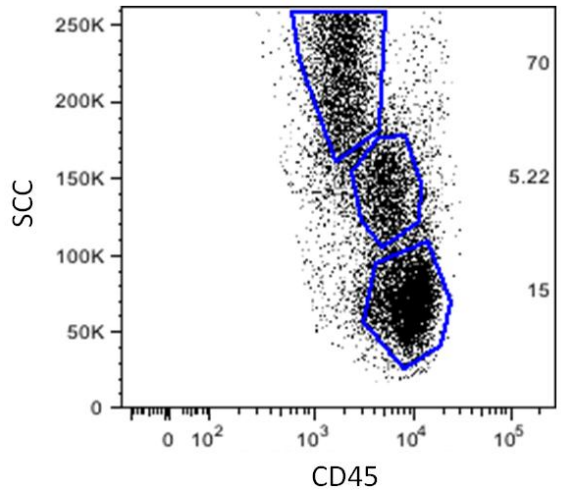
Figure 2.8 Gating strategy for Flow cytometry



A

A: CD45 vs SSC was used to defined the populations of lymphocytes.

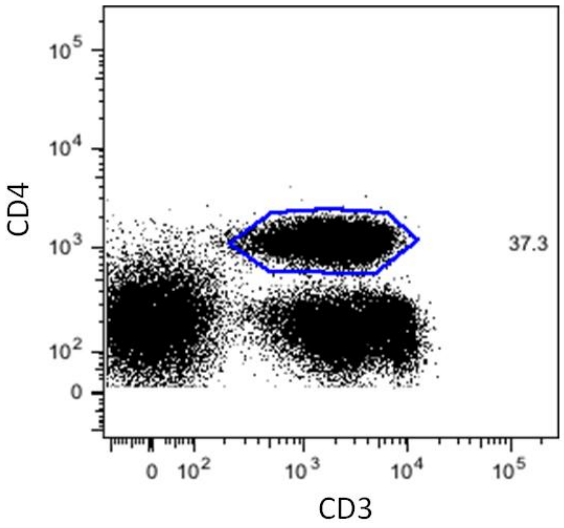
The cell populations were first gated to exclude the debris in the sample.



B

B: The cell populations were identified using side scatter and CD45

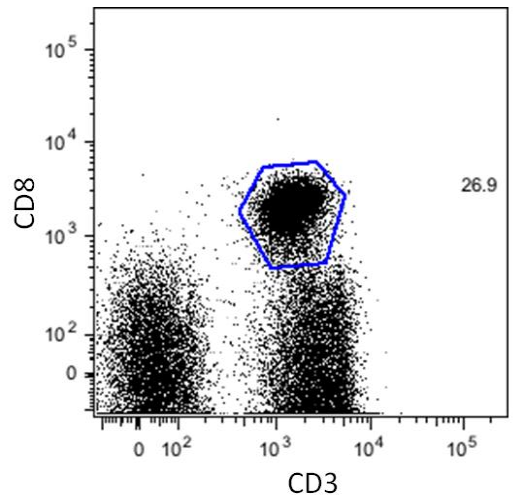
The lowest population (15%) included the lymphocyte population. The highest, most granular, population (7%) was interpreted to be mainly 'neutrophils'. The third population (5.22%), in the centre, was interpreted to be mainly 'monocytes'. Only the lymphocyte population were labelled to enable more detailed analysis.



C

C: The lymphocyte gated population was then subdivided.

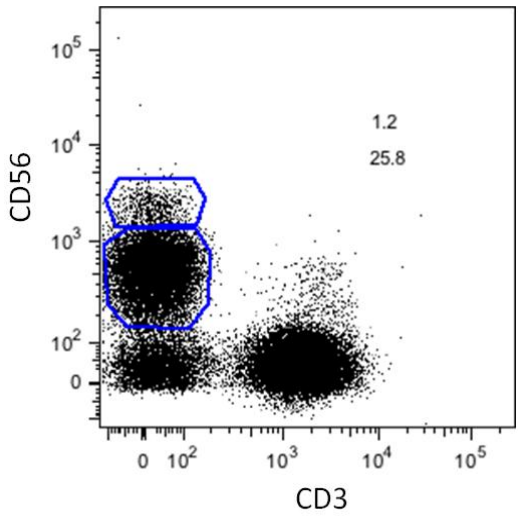
CD3 and CD4 revealed the T-helper cell population



D

D: The lymphocyte population was subdivided.

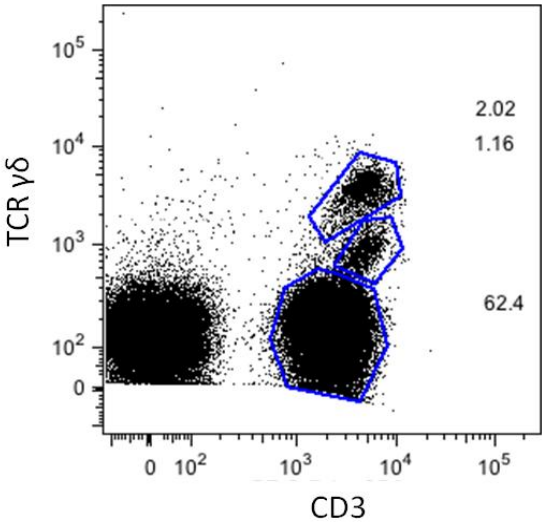
CD3 and CD8 revealed the Cytotoxic T cell population



E

E : The lymphocyte population was subdivided.

CD3 and CD56 revealed the Natural Killer Cell population. The higher population (1.2%) is the 'Bright' population. The lower population (25.8) is the 'Dim' population.

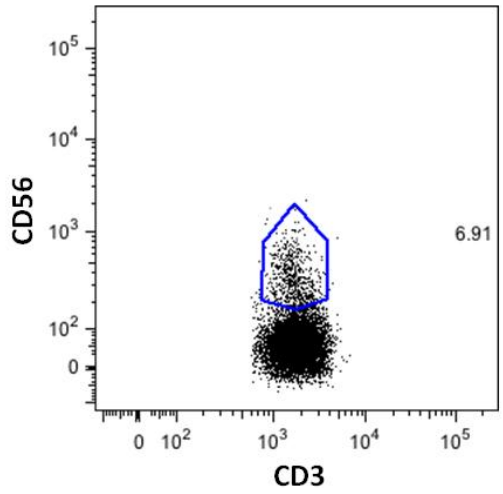


F

F: The lymphocyte population was subdivided.

CD3 and TCRγδ were used to reveal the gamma delta cell populations. The γδ cell 'high' population (2.02%) sits above the γδ cell 'low' population (1.16).

The lower circular population encompasses the 'unconventional' T cells. A small sub-population which includes, among others, the NKTs.



G

G: Finally the 'unconventional' lymphocytes were further separated.

The CD3+ TCR- population was further divided using CD3 and CD56 to identify the Natural Killer T-cells (NKTs) subset.

iv. Technique validation

As this was the first time that Flow cytometry had been used in our research group, this study was performed in collaboration with two Immunologists from The Blizard Institute: Professor Dan Pennington PhD, from the Centre for Immunology and Dr Ute Meier PhD, a post-doctoral researcher within the Neuroimmunology Group in the Centre for Trauma and Neuroscience. Each provided guidance on the protocol, antibody combination and analysis strategy for this study.

The ethics approval for ACIT2 permits draw of a maximum of 30ml of blood from each patient at each time point of the study. A large number of tests are already performed on that blood which meant that the volume available for this study was extremely limited. The protocol was therefore based on a technique Dr Meier had used in a paediatric study using small sample volumes. A handful of trauma studies had previously reported flow cytometry data using 100µl of blood (Yadav et al., 2009, Walsh et al., 2000). We were attempting to examine fairly rare cellular populations; therefore, the volume we elected to use in this study was 500µl. Use of lysis buffer, rather than Ficol separation, for removal of the erythrocytes did not require validation, as it is an accepted technique in human studies.

Samples were read using a Canto II FACS Flow Cytometer (BD, Plymouth, UK). This has three lasers; an argon laser (4 colours), Red HeNe (2 colours) and a violet laser (2 colours) – a total capability of detecting 8 fluorescent signals per sample. Prior to the study onset, calibration of the instrument was performed, using beads and individual antibodies, by Dr Gary Warnes PhD who manages the FACS Core Facility. This was repeated on two further occasions during the yearlong study to ensure consistency of measurements.

Antibody strengths were titrated at the beginning of the experiment, to enable efficient antibody use and optimal display of data, on samples from healthy subjects (n=4) and samples from trauma patients (n=2). Antibodies concentrations of 1:1, 1:2 and 1:4 dilutions were examined. Cell staining at a 1:2 dilution the manufacturers recommended dose was determined to be ideal. A number of samples from healthy subjects (n=4) were run to test the technique and staining protocol. The values obtained for each cellular population were consistent with other departmental work (performed by Dr Meier). The protocol was considered robust, reproducible and consistent between our patient samples. We concluded that the technique was feasible and appropriate therefore proceeded with the study.

5. MATERIALS AND REAGENTS

a) ELISA

Human Proinflammatory 7plex, Cat K15008A-5, Mesoscale Discovery, Maryland, USA

HMGB1, Ref ST51011 IBL International, Germany

Cell death Detection ELISA PLUS, Cat No 11 774 425 001, Roche Applied Science, UK

b) REAL-TIME PCR

QIAamp DNA Blood Mini Kit from Qiagen, Valencia, CA 91355, USA

Human cytochrome B (CytB) primer, Invitrogen, Eugene, Oregon, USA

5-ATGACCCCAATACGCAAAAT-3 (forward)

5-CGAAGTTTCATCATGCGGAG-3 (reverse)

Bacteria 16S ribosomal DNA primer, Invitrogen, Eugene, Oregon, USA

59-CGTCAGCTCGTGTTGTGAAA-39(forward)

59-GGCAGTCTCCTTGAGTTCC-39 (reverse)

c) FLOW CYTOMETRY

FACS antibodies

Human FcγR binding inhibitor (Cat No: 14-9161-73, eBioscience Ltd, UK

Anti-human gamma delta TCR FITC (Cat No: 11-9959, eBioscience Ltd, UK)

Anti-human CD3 PE-Cy7 (Cat No: 25-0038, eBioscience Ltd, UK)

Anti-human CD4 eFluor®450 (Cat No: 48-0047, eBioscience Ltd, UK)

Anti-human CD56 (NCAM) APC (Cat No: 17-0567, eBioscience Ltd, UK)

Anti-human CD8a APC-eFluor®780 (Cat No: 47-0088, eBioscience Ltd, UK)

Anti-human CD45 PerCP-Cy5.5 (Cat No: 45-0459, eBioscience Ltd, UK)

Anti-human CD69 PE (Cat No: 12-0699, eBioscience Ltd, UK)

Mouse IgG K Isotype Control CD69 PE (Cat No: 12-4714-42, eBioscience Ltd, UK)

Falcon tubes polypropylene 15mls, Cat No 352097, BD Biosciences, Oxford, UK

BD Pharm Lyse, Cat 555899, BD Biosciences, Oxford, UK

Trucount Tubes, Cat 340334, BD Biosciences, San Jose, CA

Falcon FACS tubes, Cat 352052, 5ml Polystyrene, BD Biosciences, Oxford, UK

BD Citofix™ Cat 554655, BD Biosciences, Oxford, UK

FACS Flow, BD Biosciences, San Jose, CA

6. INSTRUMENTS AND EQUIPMENT

a) BLOOD SAMPLING & STORAGE

BD vacutainers

- 0.109M + buffered sodium citrate 3.2%, 4.5ml, Becton Dickinson, Plymouth, UK
- K2EDTA 4ml, Becton Dickinson, Plymouth, UK

Clinispin Horizon 853VES Laboratory Centrifuge, Woodley Equipment Company Ltd, Bolton, UK

Cliklok microcentrifuge tubes, Simport, Jencons, UK

Arterial blood gases, ABL 500 Flex, Diamond Diagnostics, USA

Ultralow temperature freezer, New Brunswick Scientific, Enfield, CT, 06082-4444 USA

b) ELISAs

Lab dancer Vortex, 3365000, IKA-Werke, GmbH & Co, Germany

PMS-1000 Plate shaker, Grant-bio, Cambridgeshire, UK

ELx50 plate washer, BioTek, Winooski, USA

SIAFR Synergy HT plate reader, BioTek, Winooski, USA

SECTOR® Imager 2400, Mesoscale Discovery, Maryland, USA

c) REAL TIME PCT

Mastercycler EP Realplex, Eppendorf, Hauppauge NY 11788

d) FLOW CYTOMETRY & WHITE BLOOD CELL STUDIES

Canto II, BD Biosciences, San Jose, CA, USA

Julabo SW-20C Water bath, Jencons Scientific Ltd, Bedfordshire, UK

Multifuge 3SR+ , Thermo Scientific, USA

Sysmex SE2100 Analyser, Sysmex, Milton Keyes, UK

e) GENERAL

Cat II Hood, Medical Air Technology, UK

Pipittes & tips, Eppendorf UK Ltd, UK

Glassware, plastics and disposables provided by The Blizard Institute

f) COMPUTER SOFTWARE

Excel 2007, Microsoft, Redmond, USA

Prism 5.01, Graph Pad**,** CA, USA

Inkscape, Inkscape.org

KC4 v3.4, Biotek, Winooski, VT, USA

Part 2: Cytokines

CHAPTER THREE: A NARRATIVE REVIEW OF CYTOKINES IN TRAUMATIC INJURY IN HUMANS

1. INTRODUCTION

Traumatic injury activates the immune system and results in the production of pro-inflammatory and anti-inflammatory mediators, including cytokines and chemokines (Gebhard et al, 2000, (Chen and Nunez, 2010, (Jastrow et al, 2009). Cytokine and chemokine production is part of a normal, healthy response to harmful stimuli (Bone et al, 1997). Activated innate immune cells produce and release these mediators, but the mechanics of the response are complex and incompletely understood (Stow et al, 2009). Cytokine production often demonstrates huge variability between patients and between underlying diagnoses (Hranjec et al). Mediators have pleotropic effects which can be influenced by their environment (Bone et al, 1997).

After traumatic injury, patients who have a systemic inflammatory response syndrome (SIRS) at admission tend to have worse outcomes, in terms of length of stay and mortality (Napolitano et al, 2000). Outcomes can be very unpredictable, even between patients with similar injury load (Rixen and Siegel, 2000, (Jastrow et al, 2009). Whether cytokines are simply markers of inflammation or participants in the pathogenesis of inflammatory complications is unclear (Jastrow et al, 2009, (Oberholzer et al, 2000a, (Biffl et al, 1996b).

Cytokine concentration can be measured in peripheral blood samples. Their concentration reflects the magnitude of the inflammatory response generated. A wide range of inflammatory mediators and their receptors have been analysed in trauma patients. The range of analytes examined and the number of analytes examined per study have increased as technology has advanced. Most of the mediators examined in trauma patients demonstrate elevated concentrations but our understanding of cytokine function after trauma and how

cytokine concentration relates to clinical status, is still incomplete. This review was conducted to assimilate the available evidence about cytokine and chemokine production by trauma patients and determine our current level of understanding about their relationship to outcome.

2. METHODOLOGY

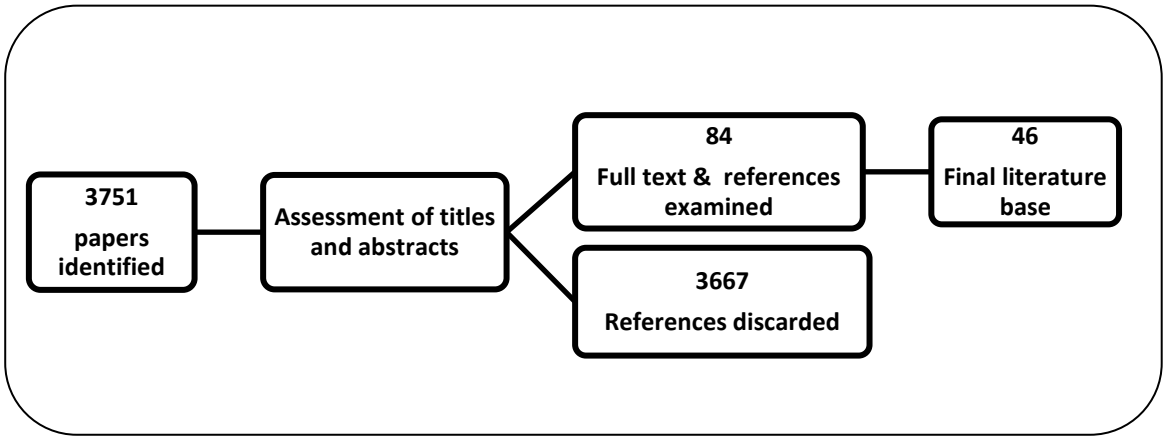
To evaluate the current evidence on human immune cell responses after traumatic injury, a literature search was conducted using Medline and Embase (Box 3.1). The search was limited to English publications. No time limit was applied. Studies were then hand-searched to include only those examining adult humans with blunt or penetrating traumatic injury; studies involving patients with burns or sepsis were excluded. References from those papers were also hand searched. A total of 46 studies formed the final literature base (Figure 3.1).

The quality of the manuscripts and experimental methodology was assessed, although not according to any set criteria, to ensure that only well conducted studies were included. Studies which investigated cytokine or chemokine concentrations, in blood samples obtained from trauma patients with polytrauma, were sought. Studies which exclusively examined patients with burns or traumatic brain injury were excluded. Studies examining tissues other than blood were also excluded.

Box 3.1: *Search strategy for cytokine review*

Adult >16 years Human English	**AND**	Traumatic injury/ trauma[ti]/ trauma[MESH]/ blunt/ penetrating/ polytrauma/ major trauma centre/	**NOT**	Burns/ thermal injury/ head injury/ brain injury/ neurology/ brain/ neurosurgery	**AND**	cytokines/ chemokines/ immune response / immune cells/ lymphocytes/ inflammation / SIRS/ inflammatory response/ immunity,innate

Figure 3.1 : Literature search



3. RESULTS

a) CYTOKINE RELEASE OCCURS EARLY AND CAN PERSIST

Elevated concentrations of cytokines occur almost immediately after trauma. The earliest measurements have been taken at the scene of injury with levels rising to a peak at 6h from hospital admission (Gebhard et al, 2000). Highest levels of cytokines are quite consistently found within the first few hours after injury (Gebhard et al, 2000, (Frink et al, 2007, (Jastrow et al, 2009). The response appears to be orchestrated rather than chaotic (Cinat et al, 1995). Reduction in concentration of these early pro-inflammatory cytokines, begins within the first 24h after injury; however, elevated levels of some cytokines can still be demonstrated in inpatients 14 days later (Frink et al, 2007).

b) CYTOKINE LEVEL REFLECTS INJURY LOAD

Blood concentrations of IL-2, IL-4, IL-6, IL-8, soluble TNF receptors and IL-1 receptors have been found to reflect severity of injury in at least one study (Tables 3.1 & 3.2 & 3.3). These findings are not consistently supported and there is some contradiction. IL-6 is the most widely studied cytokine and the mediator which most robustly shows correlation with injury severity score (ISS). It has been suggested that IL-6 is an "early marker of injury severity" (Gebhard et al, 2000). In one study, IL-2 and IL-8 also correlate with ISS (Yagmur et al, 2005). In another, IL-4 and IL-8 inversely correlate with TRISS (Trauma-related Injury Severity Score) (Hranjec et al, 2010). The region of injury appears to influence the cytokine profile as patients with isolated, severe head injury have lower cytokine levels than patients with polytrauma (Hensler et al, 2002).

c) ELEVATED CYTOKINE LEVELS PREDICT MORTALITY, MODS OR ARDS

A number of investigations consider the idea that the peripheral blood concentration of cytokines in trauma patients are associated with poor outcome in terms of the development of

Multiple Organ Dysfunction Syndrome (MODS), Adult Respiratory Distress Syndrome (ARDS) or mortality (Tables 3.1 & 3.2 & 3.3. Elevated circulating levels of IL-4, IL-6, IL-8, IL-10, TNFα, soluble TNF receptors and IL-1 receptors have all been correlated with the development of MODS, ARDS or mortality in at least one study; however, findings between studies are not consistently upheld (Tables 3.1 & 3.2 & 3.3. The largest volume of evidence has been accrued for IL-6, as it has been studied most frequently. Initially, an IL-6 level above 200pg/ml was found to be predictive of a Systemic Inflammatory Response Syndrome (SIRS) (Giannoudis et al, 2008). Later, an IL-6 level above 350pg/ml was shown to be associated with increased inflammatory sequelae after trauma and hailed as a useful biomarker for determining the subsequent development of MODS (Cuschieri et al, 2010). As our understanding progressed, investigators recognised that focussing on one mediator in a complex inflammatory soup, gave insufficient information about the inflammation generated by traumatic injury. A recent study assessed 27 mediators and used statistical modelling to demonstrate that early cytokine profile can be used to predict the patients who will develop MODS (Jastrow et al, 2009). In this study, cytokine biomarkers superseded traditional clinical predictors, such as age, ISS, admission haemoglobin and base deficit, which demonstrated no significant difference between organ failure and non-organ failure patients (Jastrow et al, 2009, (Sauaia et al, 1994). The strongest predictors of MODS in this study were two chemokines; Inducible protein-10 (IP-10) and Macrophage Inhibitory Protein (MIP-1β). Both have a range of identified functions, MIP-1β is known to modulate macrophage and T-cell responses but, their precise role in the development of MODS remains unclear. The most recent study, on this subject used statistical modelling to determine that elevated IL-4 concentration was associated with the highest relative risk of dying; although, IL-6, IL-8 and TNFα were also associated with mortality (Hranjec et al, 2010).

d) MEDIATORS OF POOR OUTCOME

Early evidence in sepsis suggests that patient outcome is influenced by the type of cytokines which are produced, inferring that cytokines participate in the pathogenesis of critical illness (Oberholzer et al, 2000b, (Bone et al, 1997). In trauma, however, opinion is divided between those who think that cytokines are simply markers of inflammation and those who believe that they participate in the process (Biffl et al, 1996b, (Jastrow et al, 2009). *In vitro* evidence demonstrates that cytokines IL-6 and IL-8 have immune stimulating capacity to directly activate neutrophils (Biffl et al, 1996a). Alveolar macrophages of ARDS patients have increased activation of Nuclear Factor Kappa-β (NF-kβ) implying that cytokine production alters the clinical pathology (Schwartz et al, 1996). Early cytokine levels are higher in trauma patients who develop MODS, compared with those who do not; however, these findings do not confirm pathogenic participation (Jastrow et al, 2009). Modulation of the post- traumatic cytokine response using recombinant cytokine receptors has been proposed, but did not progress to trial, perhaps as a result of their poor performance in sepsis studies (Partrick et al, 1999, (Fisher et al, 1994). The precise role of cytokines and chemokines in post-traumatic inflammation therefore remains unclear.

Table 3.1: Cytokines measured in adult human trauma patients

Molecule	Raised early after trauma	Injury severity	SIRS	Sepsis	MODS	ARDS	Mortality	References
IL-6	✓	✓	✓ ^		✓	✓	✓*	(Biffl et al, 1996b, (Gebhard et al, 2000, (Giannoudis et al, 2008, (Hoch et al, 1993, (Svoboda et al, 1994, (Cuschieri et al, 2010, (Partrick et al, 1996, (Jiang et al, 1997, (Martin et al, 1997, (Giannoudis et al, 1998, (Seekamp et al, 1998, (Rixen and Siegel, 2000, (Oberholzer et al, 2000a, (Hensler et al, 2003, (Yagmur et al, 2005, (Tschoeke et al, 2007, (Maier et al, 2007, (Frink et al, 2007, (Lausevic et al, 2008, (Jastrow et al, 2009, (Billeter et al, 2009)
IL-8	✓	✓			✓		✓	(Hoch et al, 1993, (Partrick et al, 1996, (Jiang et al, 1997, (Rixen and Siegel, 2000, (Liener et al, 2002, (Yagmur et al, 2005, (Maier et al, 2007, (Frink et al, 2007, (Jastrow et al, 2009, (Hranjec et al)
IL-10	✓				✓			(Seekamp et al, 1998, (Oberholzer et al, 2000a, (Hensler et al, 2003, (Tschoeke et al, 2007, (Maier et al, 2007, (Frink et al, 2007, (Lausevic et al, 2008, (Heizmann et al, 2008, (Jastrow et al, 2009)
IL-1β								(Svoboda et al, 1994, (Yagmur et al, 2005, (Frink et al, 2007, (Jastrow et al, 2009)
IL-2	✓	✓						(Svoboda et al, 1994, (Yagmur et al, 2005)
sTNFRs	✓	✓			✓			(Ertel et al, 1995, (Cinat et al, 1995, (Partrick et al, 1999, (Hensler et al, 2003, (Maier et al, 2007)
IL-1Ra	✓	✓			✓			(Ertel et al, 1995, (Cinat et al, 1995, (Seekamp et al, 1998, (Partrick et al, 1999, (Jastrow et al, 2009)
ICAM-1	✓							(Partrick et al, 1996)
TNFα	✓				✓		✓	(Jiang et al, 1997, (Yagmur et al, 2005, (Frink et al, 2007, (Namas et al, 2009, (Jastrow et al, 2009)
sE-selectin	✓							(Giannoudis et al, 1998)
E-α1PI	✓							(Giannoudis et al, 1998)
PCT	✓			✓				(Oberholzer et al, 2000a, (Billeter et al, 2009)
MIP-1β	✓†				✓			(Liener et al, 2002)
PMN Elastatse	✓							(Hensler et al, 2003, (Jastrow et al, 2009)
IL-11	✓							(Schinkel et al, 1995, (Heizmann et al, 2008)
TREM	✓							(Tschoeke et al, 2007)
PLA-2-II	✓							(Lausevic et al, 2008)
IFNγ	✓				✓			(Heizmann et al, 2008, (Jastrow et al, 2009)
CRP	✓							(Lausevic et al, 2008)

Key: SIRS = Systemic inflammatory response, MODS = Multiple Organ Dysfunction Syndrome, ARDS = Adult Respiratory Distress Syndrome, * = IL-6 >350 pg/ml, ^ = IL-6 >200pg/ml, ICAM-1 = Intracellular adhesion molecule 1, PCT = Pro-calcitonin, MIP-1β = Macrophage inflammatory protein 1, TREM = Triggering Receptor Expressed on Myeloid Cells, PLA-2-II = Phospholipase A2 Group II

Table 3.2: Chemokines measured in adult human trauma patients

Molecule	Raised early after trauma	Injury severity	SIRS	Sepsis	MODS	ARDS	Mortality
IL-4	✓						(Heizmann et al, 2008, (Jastrow et al, 2009)
IL-12(p70)	✓						(Heizmann et al, 2008, (Jastrow et al, 2009)
IL-18	✓						(Heizmann et al, 2008, (Jastrow et al, 2009)
IP-10	✓†				✓		(Jastrow et al, 2009)
IL-5	✓†						(Jastrow et al, 2009)
IL-7	✓†				✓		(Jastrow et al, 2009)
IL-9	✓†				✓		(Jastrow et al, 2009)
IL-13	✓†				✓		(Jastrow et al, 2009)
IL-15	✓†						(Jastrow et al, 2009)
IL-17	✓†				✓		(Jastrow et al, 2009)

Table 3.3: Other inflammatory mediators measured in adult human trauma patients

Molecule	Raised early after trauma	Injury severity	SIRS	Sepsis	MODS	ARDS	Mortality
Eotaxin	✓†				✓		(Jastrow et al, 2009)
FGF Basic	✓†						(Jastrow et al, 2009)
GCSF	✓†				✓		(Jastrow et al, 2009)
MCP-1	✓†				✓		(Jastrow et al, 2009)
MIP-1α	✓†						(Jastrow et al, 2009)
PDGF	✓†				✓		(Jastrow et al, 2009)
RANTES	✓†				✓		(Jastrow et al, 2009)
VEGF	✓†						(Jastrow et al, 2009)

Key: SIRS = Systemic inflammatory response, MODS = Multiple Organ Dysfunction Syndrome, ARDS = Adult Respiratory Distress Syndrome, * = IL-6 >350 pg/ml, ^ = IL-6 >200pg/ml. † No control population for comparison. ICAM-1 = Intracellular adhesion molecule 1, PCT = Pro-calcitonin, MIP-1β = Macrophage inflammatory protein 1, TREM = Triggering Receptor Expressed on Myeloid Cells, PLA-2-II = Phospholipase A2 Group II, FGF Basic = Fibroblast Growth Factor, GCSF= Granulocyte Stimulating Factor, IP-10 = Inducible protein 10, MCP-1 = Monocyte chemotactic protein-1, PDGF = Platelet Derived Growth Factor, RANTES = Regulated upon Activation, Normal-T-Cell Expressed and Secreted , VEGF = Vascular Endothelial Growth Factor, E-α1PI = Elastase-alpha 1-Proteinase inhibitor complex

4. LIMITATIONS OF THE CURRENT LITERATURE

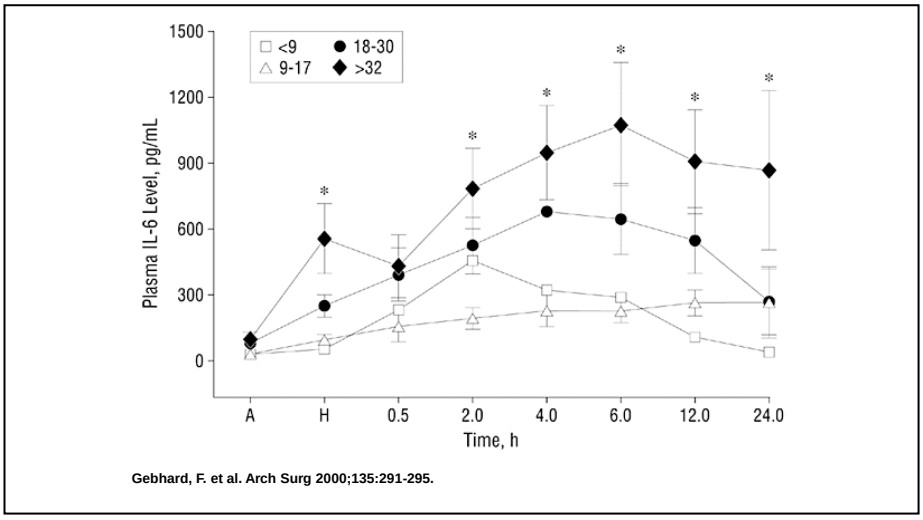
Although many studies have examined cytokine production after traumatic injury, there is little clear evidence to demonstrate their role and function. The principal reason for this is poor patient characterisation, resulting in poor comparability between studies. Cytokine levels are influenced by a wide range of factors. In trauma studies, this creates inconsistency which can be described within four main categories: timing, shock, iatrogenic factors and patient factors.

a) TIMING OF BLOOD SAMPLES

Gebhard's study in 2000, elegantly documented the changes in IL-6 concentration during the immediate post injury phase (Gebhard et al, 2000). The scene samples (A) were collected 'before colloid administration.... and before cardiopulmonary resuscitation, when appropriate' then processed in the helicopter during transfer. A rise in IL-6, proportional to ISS, was demonstrated as the time from scene increased. A maximum concentration was attained at 6h from hospital admission (Figure 3.2). This study demonstrates that in order to compare findings between studies, the timing of blood samples must be clearly defined. Pre-hospital care provision will influence the time of transfer from the scene to the hospital. The most accurate way to describe samples points is therefore from the time of injury, not the time from scene. Sample points at intervals from the time of injury were only reported in 11 of the identified studies. The first sample point ranged from ≤2h to <12h from injury (Hoch et al, 1993, (Ertel et al, 1995, (Cinat et al, 1995, (Keel et al, 1996, (Giannoudis et al, 1998, (Partrick et al, 1999, (Oberholzer et al, 2000a, (Hensler et al, 2002, (Namas et al, 2009, (Billeter et al, 2009, (Yagmur et al, 2005). The wide range of sample points makes it difficult to compare their findings. Six of the studies took blood samples within 4h of injury, but measured between 2 and 7 mediators and therefore drew a variety of unrelated conclusions (Yagmur et al, 2005,

(Ertel et al, 1995, (Hoch et al, 1993, (Keel et al, 1996, (Cinat et al, 1995, (Gebhard et al, 2000). The early cytokine response to traumatic injury has therefore not been clearly demonstrated.

Figure 3.2: Changes in IL-6 concentration in the immediate post-injury phase (Gebhard et al, 2000)



Plasma concentrations (mean ± SD) of interleukin 6 (IL-6) in 94 patients allotted to 4 groups according to the different trauma impact. The severity of injury was assessed using the Injury Severity Score (ISS): an ISS less than 9 indicates minor injury; an ISS of 9 to 17, medium injury; an ISS of 18 to 30, severe injury; and an ISS greater than 32, most severe injury. The more severely the patients were traumatized, the higher the IL-6 values throughout the observation period. After 4 hours following hospital admission, there is a clear distinction between the 4 groups, with lowest values in patients with minor injuries and maximum concentrations in patients with the most severe trauma. A indicates at the scene of the unintentional injury; H, at hospital arrival; and asterisk, p=0.03 for the most severe and severe injuries vs medium and minor injuries

b) THE INFLUENCE OF HAEMORRHAGE

Blood loss (haemorrhage), is a common feature of many trauma patients. Haemorrhage is known to activate an inflammatory response; in animal models, isolated haemorrhage has been associated with TNFα elevation (Ayala et al, 1990, (Ayala et al, 1991). Base deficit is an accepted clinical biomarker for haemorrhagic shock and cellular hypoperfusion (Davis et al, 1991, (Davis, 1994, (Davis et al, 1996). In human trauma patients with an ISS ≥16, a high base deficit has been associated with higher concentrations of IL-6 (Rixen and Siegel, 2000). When

measuring the cytokine levels in trauma patients, describing the degree of shock is therefore important. Only one study considered the influence of shock on cytokines and this examined IL-6 in isolation (Rixen and Siegel, 2000). The influence on other cytokines is therefore unclear.

c) IATROGENIC INTERVENTION

Circulating cytokine concentration is greatly influenced by iatrogenic intervention. Surgical operations, administration of blood transfusions and even minor procedures, such as placement of a urinary catheter, have been associated with elevated concentrations of cytokines, principally elevations of IL-6 (Tschoeke et al, 2007, (Hensler et al, 2003, (Ayala et al, 1990). Iatrogenic intervention, soon after hospital arrival, is usually essential to survival in trauma. Trauma patient blood therefore becomes more and more contaminated as the hours from admission pass. To avoid confounding influence, samples for cytokine analysis should therefore be taken before intervention.

In the studies identified, sample times ranged from 'at scene', to 'at admission', to '72h from hospital admission'. Pre-resuscitation samples were only obtained in four studies (Hoch et al, 1993, (Yagmur et al, 2005, (Gebhard et al, 2000, (Liener et al, 2002). Their findings supported a link between IL-6 and ISS and an association between early IL-6 and IL-8 levels and poor outcome; however none of these studies reported the degree of shock in their patient groups.

d) THE INFLUENCE OF PATIENT FACTORS

In addition to the injury characteristics, a number of patient factors also influence the cytokine concentration. Age, gender, obesity, genetics, exercise, adrenal impairment, catecholamine release, circadian rhythm and alcohol consumption have all been demonstrated to influence cytokine production (Gudewill et al, 1992, (Rodrick et al, 1986, (Li et al, 1993, (Donnikov et al, 2009, (Hoen et al, 2002, (Hildebrand et al, 2005, (Crews et al, 2006, (Wahle et al, 2005, (Covelli

et al, 1992). Trauma patients are typically, but not exclusively, young, male and without co-morbidity. Nonetheless, the influence of patient factors presents additional complexity to our interpretation and understanding of the cytokine response to trauma.

5. COMMENTS ON INFLAMMATION AFTER TRAUMA

The state of acute, sterile inflammation which is generated by traumatic injury is a complex phenomenon. Cytokine production is one of the end-products of immune cell activation. The type of cytokines produced and their concentration must, in some way, reflect the molecular machinery which is in motion. Interpreting the significance of the peripheral blood concentration is however not an easy task. Local cytokine release and systemic release may not be the same; a finding illustrated by comparison between blood and bronchoalveolar lavage samples in trauma patients (Keel et al, 1996).

Using ELISA, one can only measure 'free' cytokines not those bound to receptors and several have a short half-life in circulation (Malone et al, 2001). Complex technology, such as PCR (polymerase chain reaction) and Flow Cytometry, can be used as an alternative but they are more labour intensive and open to the same criticisms. Cytokine quantification in human samples is simple and cheap to perform on multiple patients. In clinical disease states, interpretation of findings requires compensation for confounding elements and careful patient characterisation. Cytokine concentration remains a valid method by which to obtain an indication of the magnitude of immune activation, if this can be achieved.

CHAPTER FOUR: CHARACTERISING THE HYPER-ACUTE IMMUNE RESPONSE TO TRAUMATIC INJURY

1. INTRODUCTION

Trauma activates a sterile systemic inflammatory response syndrome (SIRS) but the mechanism for this activation is unknown (Lenz et al, 2007). Tissue damage is believed to be the underlying stimulus for immune system activation (Matzinger, 2002). Trauma is a composite disease, principally characterised by physical tissue damage and blood loss. Whether these two aspects of injury activate the same inflammatory response is unclear.

Concentration of cytokines and chemokines can indicate the extent of an inflammatory response. A number of investigations have measured the levels of these inflammatory mediators after trauma but, as discussed in the previous chapter, their findings are unable to clearly describe the molecular events in the first few hours after injury. In addition, the relationship between mediator concentration and clinical outcome still requires clarification.

2. THE AIM OF THIS INVESTIGATION

This study was designed to examine the hyper-acute inflammatory response following traumatic injury. The primary objective was to measure the cytokine concentration generated by isolated tissue damage. The second objective was to describe the time course of the isolated tissue damage response, over the first 72h. The third objective was to determine whether isolated tissue damage can generate an inflammatory response sufficient to affect clinical outcome. Finally, I wished to establish whether tissue damage and blood loss generate different inflammatory profiles and whether they can be separately distinguished by the pattern or degree of cytokine release.

3. METHODS

To determine the cytokine generation by isolated tissue damage in trauma patients, I wished to examine blood samples from trauma patients without shock, prior to iatrogenic intervention or administration of significant volumes of intravenous fluid. To determine the influence of blood loss, I wished to examine blood samples from patients with injury and shock, prior to iatrogenic intervention or administration of significant volumes of intravenous fluid.

Between January 2008 and January 2010, 372 patients were enrolled and consented for ACIT2 participation. From this biobank, 235 patients were selected to form the study cohort for cytokine quantification. Patients were selected for inclusion on the basis of their injury profile (ISS, BD and injury description) to ensure a wide range of injury severity was represented. A total of 201 patients were included in the study analysis. Twelve patients, who had received blood transfusion between the helipad and the resuscitation room, were retrospectively excluded from analysis, as were 22 patients who had minimal tissue damage (ISS<4) but a base deficit >2 mmol/l. This patient cohort was perplexing as the injury profile was assessed and it was evident that the elevation in BD could not be attributed to haemorrhage. The median (IQR) BD was 3.3 (2.4-5.1) mmol/L, 32% (7/22) had sustained a penetrating injury and 68% (15/22) had sustained a blunt injury. The cohort had a median (IQR) age of 32 (20-42) with a systolic blood pressure of 127 (120-147), lactate of 2.4 (1.7-3.5) and the median (IQR) of crystalloid fluid administered was 0 (0-0). The precise cause of the elevated BD in these patients was unclear but may have been secondary to sudden, severe exertion incurred during fighting. I felt that their inclusion would introduce inaccuracy in to the dataset. With hindsight, it may have been beneficial to include these as a comparison to illustrate the inflammation generated by an isolated elevation in BD. For interest I therefore include their admission cytokine concentrations [mean (95% CI)] which are, in general, above the normal range but still at very low levels: IFNγ=0.28(0.11-0.71), IL-1β=0.03 (0.01-0.10), TNFα =3.03(2.55-3.61), IL-6=5.77(3.27-10.18), IL-8=(3.44(2.63-4.52), IL-10=3.79 (1.66-8.67), IL-12p70= 1.45(0.55-3.86)

pg/ml. These values were not statistically different to the control values used in the analysis except for IL-1β (p=0.03). In future work this may be something which I need to consider further, in order to achieve good patient characterisation. With regard to this investigation, this patient group have not sustained significant traumatic injury and are not at risk of poor outcome, therefore I consider that their exclusion from this analysis is reasonable.

a) PRE-DEFINED ANALYSIS PLAN

Although trauma is a heterogeneous disease, the nature of the urban trauma population for the RLH and the pre-hospital trauma system, actually result in an admission cohort with a relatively consistent demographic. The cohort (n=201) for this investigation consisted of patients who were 75% (363/483) male, 81% (390/483) had a blunt mechanism of injury and 74% (359/483) were less than 50 years old. Great variability however comes from the injury itself and the treatment administered. To avoid confounding influence from iatrogenic management, the admission blood samples were taken prior to in-hospital treatment. For analysis, patient groups were then defined by injury characteristics, specifically injury load and severity of blood loss. Injury load was described using the Injury Severity Score, which is a composite anatomical score (described in detail in Chapter 2) (ISS)(Baker et al., 1974). ISS has been repeatedly associated with the inflammatory response to traumatic injury (as discussed in Chapter 3) (Gebhard et al, 2000). Blood loss was described using the admission base deficit (BD), from the blood gas. The normal range for BD in our laboratory is -2 to +2 mmol/L. Isolated haemorrhagic shock has been associated with elevated cytokine levels in rabbits (Douzinas et al, 2008). No data for the inflammatory effect of isolated haemorrhage in humans is available. I elected to use BD because in traumatic injury research, BD is an accepted surrogate marker for the severity of haemorrhagic shock. (Davis et al., 1998, Davis and Kaups, 1998). Serum lactate could have been used as an alternative (Vandromme et al, 2010, (Davis, 1994, (Manikis et al, 1995). High BD and lactate, after trauma, have both been recognised as

independent predictors of outcome, even in the presence of acute alcohol or drug intoxication (Dunne et al, 2005). Finally, as administration of crystalloid fluid and blood products are known to influence the immune response, this was also considered during analysis (Hensler et al, 2003). Patients who receive more than 2L of CSL in the pre-hospital setting do not fulfil the criteria for ACIT2 enrolment; however 2L is still a large volume of crystalloid to administer and would be sufficient to influence the BD. As HEMS support a policy of minimal pre-hospital fluid administration, large volumes of fluid are not commonly observed, even in patients with severe injury and haemorrhage. Fluid administration prior to blood draw and throughout the first 24hs of admission was recorded and reported. This approach is consistent throughout the whole PhD investigation and where more specific criteria were applied, this is stated.

Using the ISS and admission BD, three patient groups were defined: 'Control', 'Tissue Damage' and 'Tissue Damage & Shock'. The 'Control' group included trauma patients who were found, at the end of assessment, to have no injury or only minor injuries (ISS score of ≤4) and a BD ≤2mmol/L. The 'Tissue Damage' cohort included patients with a range of injury from moderate to critical (ISS ≥5) but no evidence of hypoperfusion (BD ≤2 mmol/L). The 'Tissue Damage & Shock' cohort included a range of injury from moderate to critical (ISS ≥5) and an elevated BD (>2 mmol/L). Sub-group analysis was then required to control for the influence of injury load (ISS) and time from injury to blood sample. This is described in greater detail with the results.

The clinical outcome measures selected were 28-day mortality, length of hospital stay (LOS), ≥1 episode of infection and multiple organ dysfunction syndrome (MODS), as measured by the Sequential Organ Failure Assessment (SOFA) score (Vincent et al., 1996).

b) DATA AND STATISTICS

The first examination of the data used scatterplots to examine the distribution of the data. Due to the wide range of values, cytokine concentrations were transformed into their natural log (LN) for graphical display and analysis. The tabulated data therefore report the geometric means with 95% confidence intervals. Statistical analysis was conducted using Excel 2007 (Microsoft, Redmond, USA) and Prism 5.01 (Graph Pad, CA, USA) software packages. Significance was tested using ANOVA with Dunnett's post test, or Student's T test. Non-parametric data are reported as a median with inter-quartile range (IQR) and significance was tested using a Kruskal Wallis or Mann Whitney U test.

4. RESULTS

The study cohort consisted of 201 patients and represented a broad spectrum of injury severity; 74(45%) were critically injured (ISS ≥25). (Table 4.1). There were 39 patients in the 'Control' group, 77 in the 'Tissue Damage' (TD) cohort and 85 in the 'Tissue Damage & Shock' (TD&S) cohort. Admission (0h) samples were drawn within a median (IQR) of 10 (6-15) minutes from arrival and 90 (70-116) minutes from injury. More than half (53%) had no crystalloid fluid prior to the 0h sample and only 30% received more than 250 ml before blood draw. No patient received blood or blood products prior to the 0h blood draw. Cytokine measurements were performed on 201 x 0h, 105 x 24h samples and 86 x 72h samples. A full set of samples at each time-point could not be obtained if patients died, were discharged or declined further blood draw.

The results will be presented in two parts. Firstly, the inflammatory response to isolated tissue damage is considered, including the cytokine profile, time course of the response and the association with outcome. In the second part, the TD and TD&S groups with matched ISS are compared to examine the influence of co-existing blood loss.

a) ISOLATED TISSUE DAMAGE AND INFLAMMATION AFTER TRAUMATIC INJURY

To examine the effect of isolated tissue damage on the inflammatory response to trauma, all patients with haemorrhagic shock and cellular hypoperfusion were excluded. Two cohorts of patients were defined: a Tissue Damage (TD) cohort, defined as an ISS≥5 and a BD ≤2mmol/L (n=77) and a Control cohort (C), defined as an ISS 0-4 and a BD ≤2mmol/L (n=39). The cytokine concentrations between each cohort were compared.

Table 4.1: Demographics of the study cohort

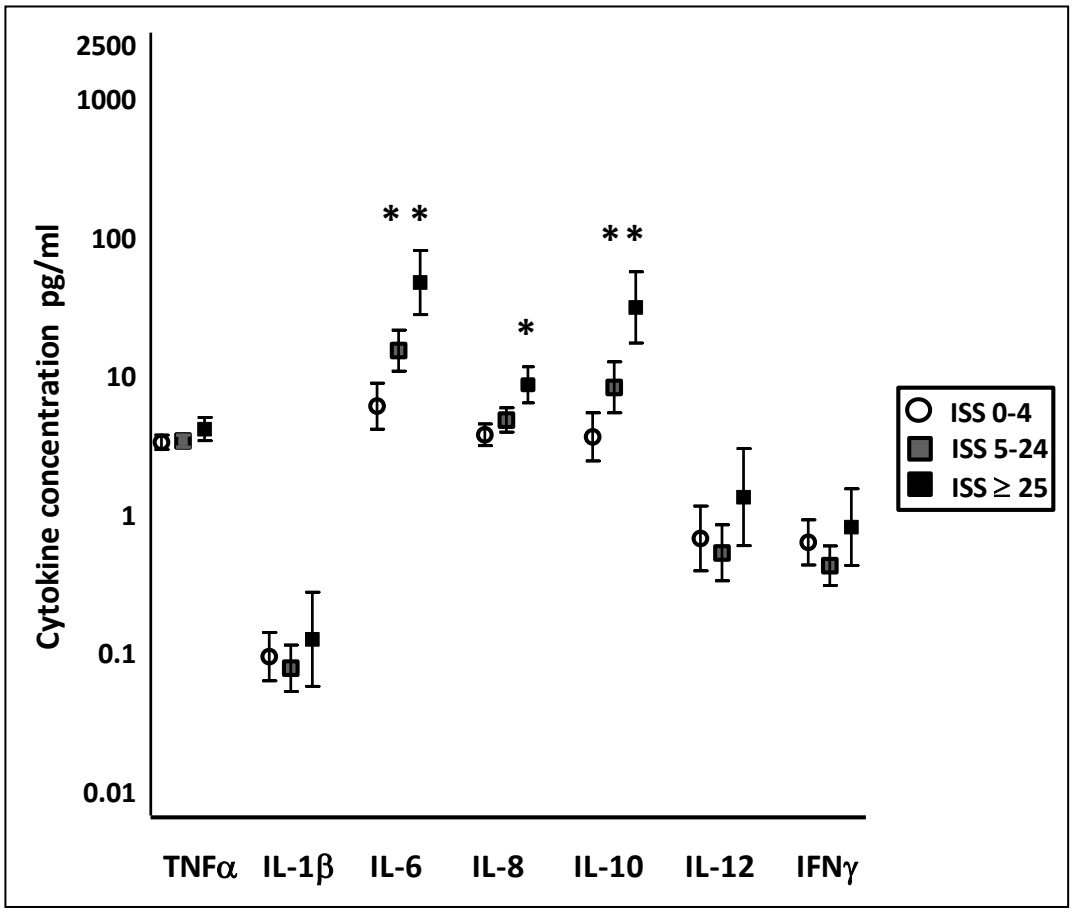
	All patients	Controls	Tissue Damage	P value
Demographics				
Number of patients	201	39	77	--
Age (years) ‡	33 (16-95)	32 (17 - 95)	30 (16 - 79)	0.90
Males (%)	84	82	86	0.60
No of samples at Hour 0	201	39	77	--
No of samples at Hour 24	105	12	46	--
No of samples at Hour 72	86	10	35	--
Injury severity				
Blunt (%)	81	82	82	1.00
ISS ‡	13 (8-27)	1 (1 - 4)	13 (5 - 36)	**<0.01**
ISS ≥ 15 (%)	45	0	40	**<0.001**
Injury to blood draw (mins)	89 (70-116)	73 (59 – 85)	85 (69 – 111)	**0.03**
CSL pre-draw (mls) ‡	0 (0-500)	0 (0)	0 (0 - 250)	**0.01**
% > 250mls pre-draw	30	8	22	0.07
Of those with >250mls	6-- (500-1000)	300 (400-500)	500 (500-600)	0.36
Admission Physiology				
SBP (mmHg) ‡	132 (111 – 148)	135 (125 - 148)	140 (125 - 156)	0.78
Base deficit ‡	1.7 (-3.4 – 21.9)	0.6 (-3.4 - 2.0)	0 (- 3.2 - 2.0)	0.28
Hour 0 †				
TNFα	3.98 (3.71-4.28)	3.32 (2.95-3.73)	3.62 (3.30-3.96)	0.28
IL-1β	0.16 (0.12-0.20)	0.09 (0.06-0.14)	0.09 (0.06-0.13)	0.36
IL-6	28.05 (21.95-35.84)	6.05 (4.18-8.77)	21.96 (16.25-29.66)	**<0.01**
IL-8	7.19 (6.19-8.34)	3.76 (3.16-4.47)	5.81 (4.89-6.89)	**0.01**
IL-10	14.44 (11.24-18.55)	3.63 (2.46-5.34)	12.72 (8.87-18.23)	**0.02**
IL-12 p70	1.03 (0.79-1.35)	0.67 (0.40-1.13)	0.71 (0.47-1.06)	0.77
IFNγ	0.72 (0.57-0.91)	0.63 (0.44-0.90)	0.53 (0.39-0.71)	0.89
Hour 24 †				
TNFα	9.99 (6.91-14.43)	1.99 (1.27-3.13)	12.29 (6.80-22.20)	**0.03**
IL-1β	0.20 (0.13-0.31)	0.05 (0.02-0.17)	0.09 (0.06-0.15)	0.78
IL-6	56.35 (40.51-78.37)	15.57 (5.96–40.68)	32.39 (21.73-48.28)	0.52
IL-8	7.01 (5.41-9.09)	2.19 (1.44-3.34)	4.73 (3.63-6.16)	0.24
IL-10	4.58 (3.31-6.36)	1.18 (0.48-2.90)	2.91 (1.88-4.50)	0.53
IL-12 p70	1.09 (0.57-2.09)	0.14 (0.04-0.55)	0.74 (0.26-2.12)	0.13
IFNγ	1.15 (0.71-1.86)	0.18 (0.07-0.51)	1.00 (0.50-1.98)	0.33
Hour 72 †				
TNFα	3.71 (3.19-4.32)	2.60 (1.88-3.61)	3.47 (2.81-4.30)	0.28
IL-1β	0.23 (0.15-0.35)	0.09 (0.04-0.19)	0.17 (0.10-0.30)	0.46
IL-6	24.28 (16.79-35.09)	5.07 (2.21-11.65)	18.30 (10.62-31.53)	0.21
IL-8	5.23 (4.06-6.74)	2.19 (1.47-3.28)	4.13 (2.93-5.81)	0.45
IL-10	2.81 (2.05-3.83)	1.16 (0.63-2.12)	2.26 (1.38-3.72)	0.18
IL-12 p70	1.06 (0.64-1.75)	0.33 (0.12-0.92)	0.69 (0.31-1.51)	0.26
IFNγ	0.74 (0.46-1.17)	0.27 (1.14-0.52)	0.62 (0.31-1.25)	0.39
Outcomes				
Mortality (%)	10	0	7	0.11
Length of stay (days) ‡	8 (2 - 18)	1 (0 - 6)	8 (3 - 16)	**<0.01**
Infections (%)	18	0	16	**0.01**
Worst SOFA score ‡	3 (1 - 7)	1 (0 - 2)	2 (1- 4)	0.18

p values describe significance between the tissue damage cohort and control cohort at that time-point.
†= mean (95% confidence interval), ‡ Median (IQR); ISS = Injury severity score, CSL = crystalloid intravenous fluid.
***** denotes significance between timed concentration and 0h levels. Data for SOFA score: All (n = 155), Control (n = 23), Tissue Damage (n = 60).

i. Isolated tissue damage and cytokine concentration

To determine whether isolated tissue damage is able to generate an inflammatory response, the admission (0h) cytokine concentration was stratified according to ISS (Figure 4.1). Elevated concentrations of IL-6, IL-8 and IL-10, were observed. The cytokine levels demonstrated a dose-dependent relationship with the ISS. No elevation of TNFα, IL-1β, IL-12p70 or IFNγ was demonstrated.

Figure 4.1: The cytokine profile for the patients with isolated tissue damage at admission

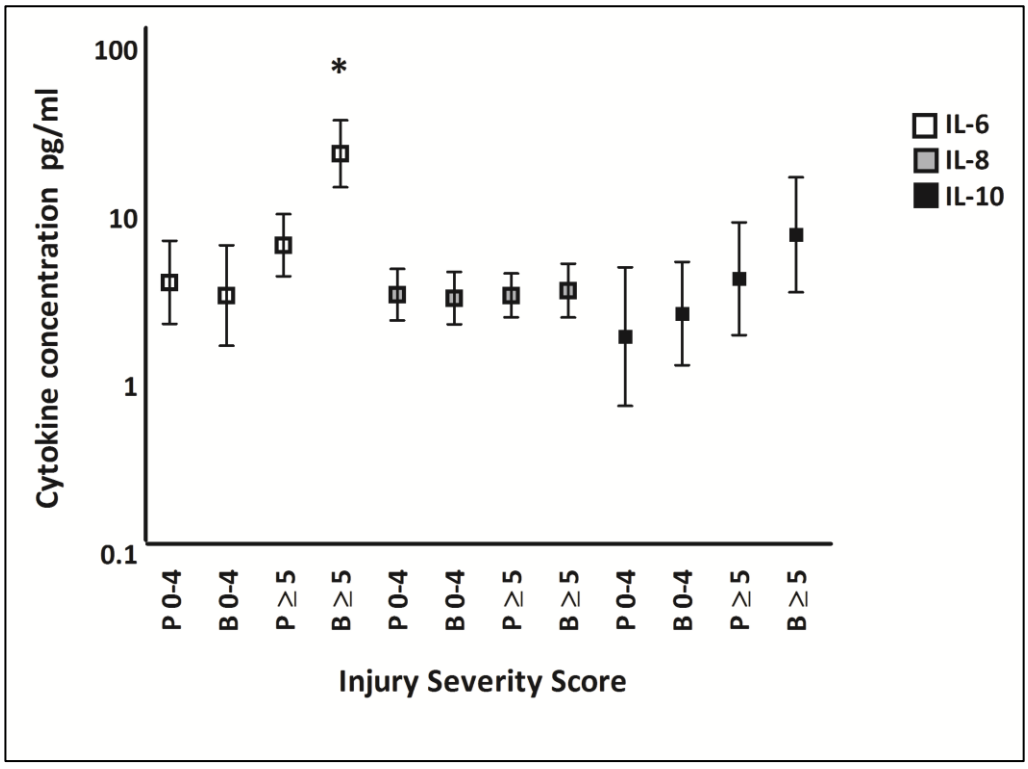


The inflammatory response generated by isolated tissue damage is characterised by an elevation of IL-6, IL-8 and IL-10 (n=77). A dose dependent rise, in cytokine concentration, was observed with increasing ISS. Values are reported, on a natural log scale, as mean with 95% confidence interval in pg/ml. * denotes $p < 0.05$ using ANOVA with Dunnett's post test.

To determine the effect of injury load on cytokine concentration, the isolated tissue damage cohort was analysed with respect to the mechanism of injury. In general terms, blunt injury

would be expected to result in more extensive tissue damage than penetrating injury. Whether this would be reflected by the cytokine concentration was unknown. Patients who sustained blunt injuries had a higher median ISS than patients who sustained penetrating injuries. A sub-group, with identical injury score (ISS), was therefore formed (n=26 in each group). Patients with blunt mechanisms of injury and an ISS of 5 or more, had concentrations of IL-6 which were significantly higher than those from patients with penetrating mechanisms of injury. IL-10 showed a trend towards elevation with blunt injury but did not reach significance (Figure 4.2).

Figure 4.2: Cytokine concentration is influenced by the extent of tissue damage



In trauma patients with matched ISS (n= 26 with penetrating injury and n=26 with blunt injury), admission (0h) concentrations of IL-6 were higher after blunt injury. Average(SD) ISS for the cohorts were 1(1) for the 0-4 cohorts and 11(5) for the 5+ cohort. **IL-6**: P 0-4= 4.13(2.49-6.85), B 0-4=3.46(1.88-6.36), P ≥5=6.88(4.67-10.13), B≥5=24.03(15.85-36.43). **IL-8**:P0-4=3.50(2.56-4.79), B0-4=3.33(2.42-4.58), P≥5=3.46(2.64-4.54), B≥5=3.70(2.65-5.16). **IL-10**:P0-4=1.97(0.85-4.58), B0-4=2.70(1.44-5.06), P≥5=4.35(2.16-8.75), B≥5=7.93(3.88-16.20). Values are reported, on a natural log scale, as mean with 95% confidence interval in pg/ml. * denotes p <0.05 with an unpaired t-test when blunt and penetrating with the same ISS were compared.

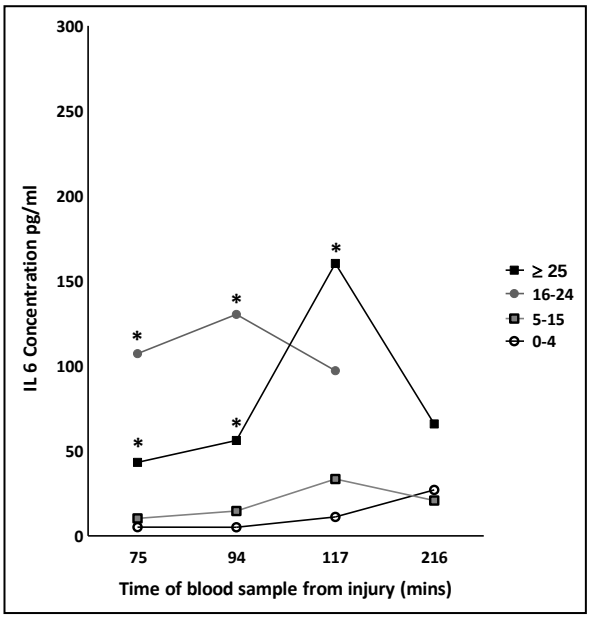
ii. Time course of the isolated tissue damage response

Having established that injury severity influences the cytokine concentration in patients with isolated tissue injury, I wished to determine the influence of time. Two aspects were considered, the time from injury and the time from admission. This analysis focussed on IL-6 to enable comparison with the analysis by Gebhard (Gebhard et al, 2000).

a) Inflammatory profile varies with time from injury

In patients with isolated tissue damage (n=116: TD + C), the mean concentration of IL-6 at admission (0h), was examined with regard to the time of the blood draw from injury (Figure 4.3). Patients were stratified by ISS. For all injury levels, concentration of IL-6 demonstrated a steady increase from the time of injury. A peak was observed at approximately 2h from injury.

Figure 4.3: IL-6 concentration varies with injury severity and time from injury



IL-6 concentration at 0h (admission) presented according to the time from injury (in quartiles) in trauma patients with isolated tissue damage and no shock (BD ≤2mmol/L) (n=77). IL-6 concentration increases with time from injury in patients of all injury levels. [ISS: 75, 94, 117, 216]. **ISS 0-4**: 5.2 (3.7-12.0), 5.1 (3.6-10.5), 11.2(10.8-1172.5), 27.1(26.3-46.3). **ISS 5-15:** 10.3(8.6-21.2), 14.7(13.3-21.2), 33.5(32.4-36.1), 20.8(19.7-27.8). **ISS 16-24:** 107.2(106.2-108.2), 130.3(129.3-131.3), 97.2(96.2-98.4), not available. **ISS ≥25:** 43.3(42.3-45.1), 56.2(55.1-63.5), 160.3(159.3-162.9), 65.9(64.8-68.3). Data are presented as geometric means (95% confidence intervals), CIs are omitted from the graph for clarity. * denotes $p<0.05$ when compared to the control values at the same time-point using ANOVA with Dunnetts Post Test.

b) Inflammatory profile varies with time from admission

To illustrate the time course of the cytokine response to isolated tissue damage after admission, the concentration of the TD cohort was compared with the controls. To compensate for the influence of injury load, this analysis used only those TD patients with critical injury, defined as an ISS ≥25 but IL-6, IL-8 and IL-10 were all examined (Table 4.2). In addition, all three time-point samples were available for these patients to avoid survivor bias.

Table 4.2: Demographics of critically injured Tissue Damage cohort.

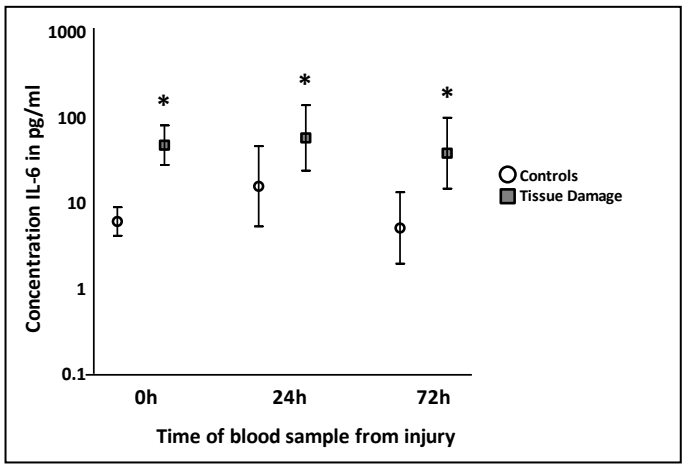
Demographics	**Tissue Damage**
Number of patients	25
Age (years) ‡	44 (16-79)
Males (%)	80
Injury severity	
Blunt (%)	96
ISS ‡	27 (25 – 33)
CSL pre-draw (mls) ‡	0 (0-500)
% > 250mls pre-draw	40
Admission Physiology	
SBP (mmHg) ‡	142 (115 – 157)
Base deficit ‡	0.8 (-1.6 – 2.0)
Outcomes	
Length of stay (days) ‡	14 (8-20)
Mortality (%)	20
Worst SOFA score ‡	6 (3-9)
Culture +ve sepsis (%)	40

p value refers to comparison between Tissue Damage and Tissue Damage & Shock cohorts, †= mean (95% Confidence interval), ‡ Median (IQR), ISS = Injury severity score, CSL = crystalloid intravenous fluid

The concentration of IL-6 was highest at admission (0h) and at 24h (Figure 4.4). There was no statistical difference between the two values: 47.05pg/ml (28.38-78.00) vs 57.17pg/ml (25.36-128.86), p=0.68. IL-6 remained elevated above Controls at 72h. The concentration of IL-8 followed the same trend as IL-6. By contrast, IL-10 was highest at admission and returned to control levels by 24h.

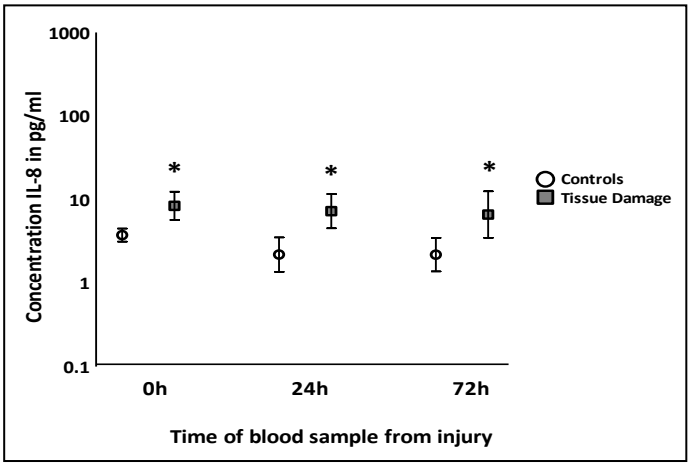
Figure 4.4: The time course of the cytokine response after admission

Cytokine concentrations for Controls (ISS 0-4, BD≤2 mmol/L) were compared with those of critically injured patients with isolated tissue damage at 0h, 24h and 72h from admission. Values are reported, on a natural log scale, as mean with 95% confidence interval in pg/ml. * denotes p <0.05 when compared against the 0h Control value, Student's T Test.



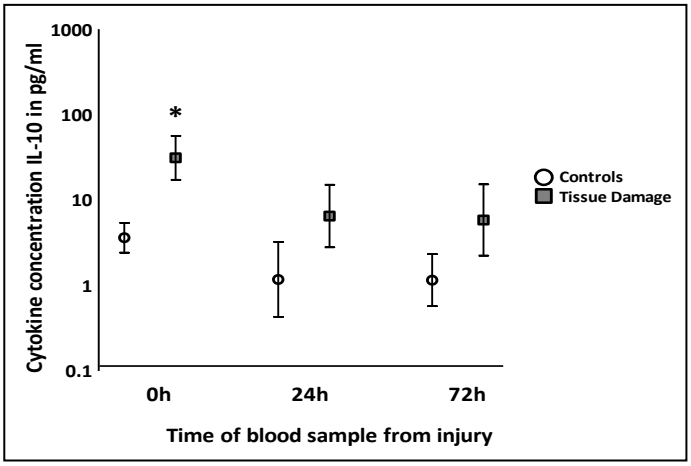
A

A: Time course of IL-6 from admission (n=25). At all three time-points IL-6 levels remain elevated above the 0h Control value. **0h:** C=6.05 (4.17-8.76), TD=47.05 (28.38-78.00), p<0.01. **24h:** C=15.57 (5.96-40.98), TD= 57.17 (25.36-128.86), p<0.01. **72h:** C=5.07 (2.21-11.65), TD=37.94 (15.80-91.40), p<0.01.



B

B: Time course of IL-8 from admission (n=25). The highest concentration of IL-8 was observed at admission. Levels remained high at 72h. **0h:** C=3.76 (3.16-4.47), TD= 8.40 (5.86-12.06), p<0.01. **24h:** C=2.19 (1.44-3.34), TD= 7.26 (4.69-11.22), p<0.01. **72h:** C=2.19 (1.47-3.27), TD=6.61 (3.67-11.90), p=0.02.



C

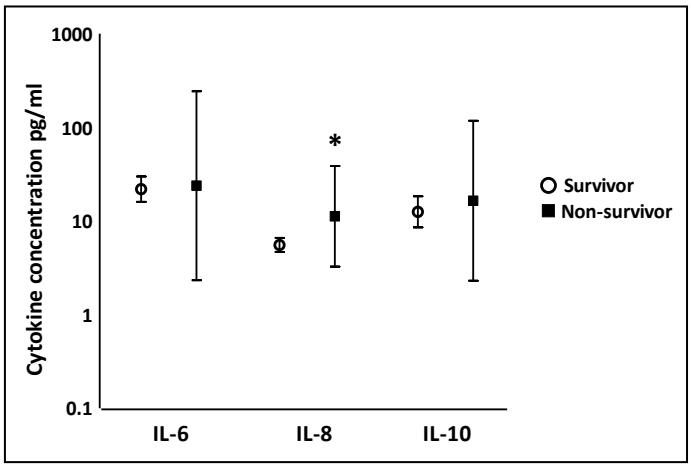
C: Time course of IL-10 from admission (n=25). The highest concentration of IL-10 was observed at admission. Concentration returned to Control levels by 24h. **0h:** C=3.62 (2.46-5.34), TD=31.18 (17.79-54.66), p<0.01. **24h:** C=1.18 (0.48-2.90), TD=6.50 (2.98-14.16), p=0.15. **72h:** C=1.16 (0.63-2.12), TD=5.85 (2.42-14.12), p=0.26.

iii. Damage induced inflammation is associated with poor clinical outcome

Clinical outcome measures were analysed for the whole 'Tissue Damage' cohort (n=77). Patients were divided into two groups depending on whether, or not, that outcome measure was observed. The admission (0h) cytokine concentrations, for the positive and negative group, were then compared for each outcome; 28 day mortality, ≥1 episode of infection and MODS (SOFA Score). In this study, a SOFA Score of ≥5 was used to define "multiple organ failure". High IL-8 at admission was weakly associated with 28-day mortality. High circulating concentrations of IL-6, IL-8 and IL-10, at admission, were associated with the development of one or more episodes of infection during admission. High levels of IL-6 and IL-10 were also associated with the development of organ failure (Figure 4.5).

Figure 4.5: Inflammation generated by isolated tissue trauma is associated with adverse clinical outcome

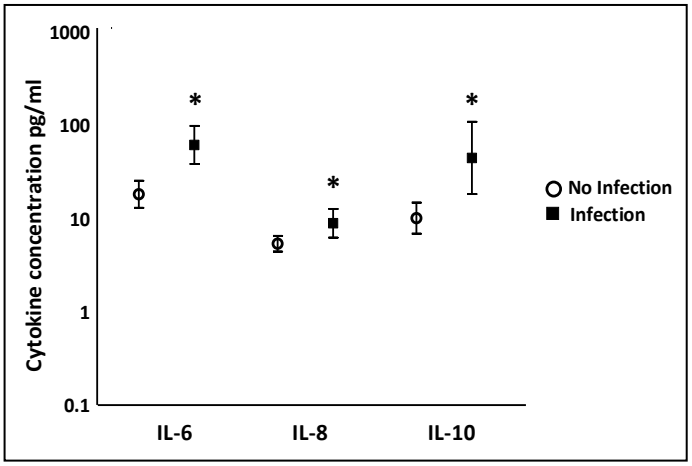
Clinical outcomes, for the isolated Tissue Damage cohort (n=77), were assessed and the admission cytokine concentrations (0h) compared. Values are reported, on a natural log scale, as mean with 95% confidence interval in pg/ml, * denotes $p < 0.05$ using a Student's T test.



A

A: 28 day mortality is weakly associated with tissue damage inflammation (n=77).

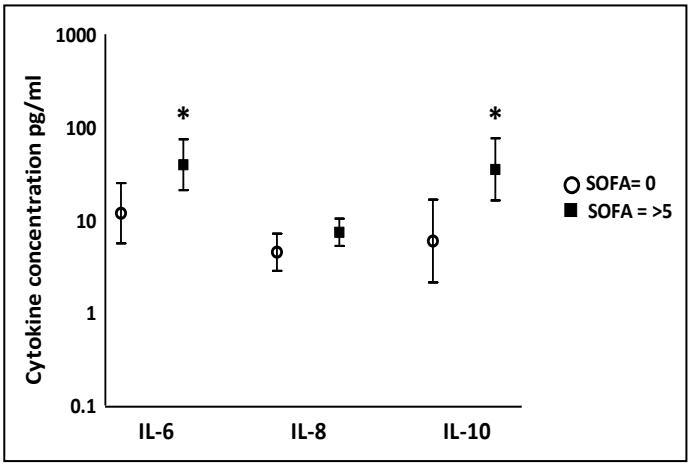
High IL-8 levels at admission were associated with 28 day mortality. **IL-6:** 21.84 (15.84-30.10), 23.74 (4.61-122.25), p=0.89. **IL-8:** 5.55 (4.68-6.58), 11.20 (4.68-26.82), p=0.048. **IL-10:** 12.49 (8.58-18.18), 16.42 (4.11-65.61), p=0.72.



B

B: Development of infection is associated with tissue damage inflammation (n=77).

High concentrations of IL-6, IL-8 and IL-10 at admission were associated with the development of culture positive sepsis. **IL-6**: 18.16 (13.08-25.22), 61.36 (40.45-93.07), p= <0.01. **IL-8:** 5.37 (4.44-6.49), 8.88 (6.47-12.19), p=0.04. **IL-10:** 10.09 (6.92-14.71), 44.49 (20.12-98.38), p<0.01.



C

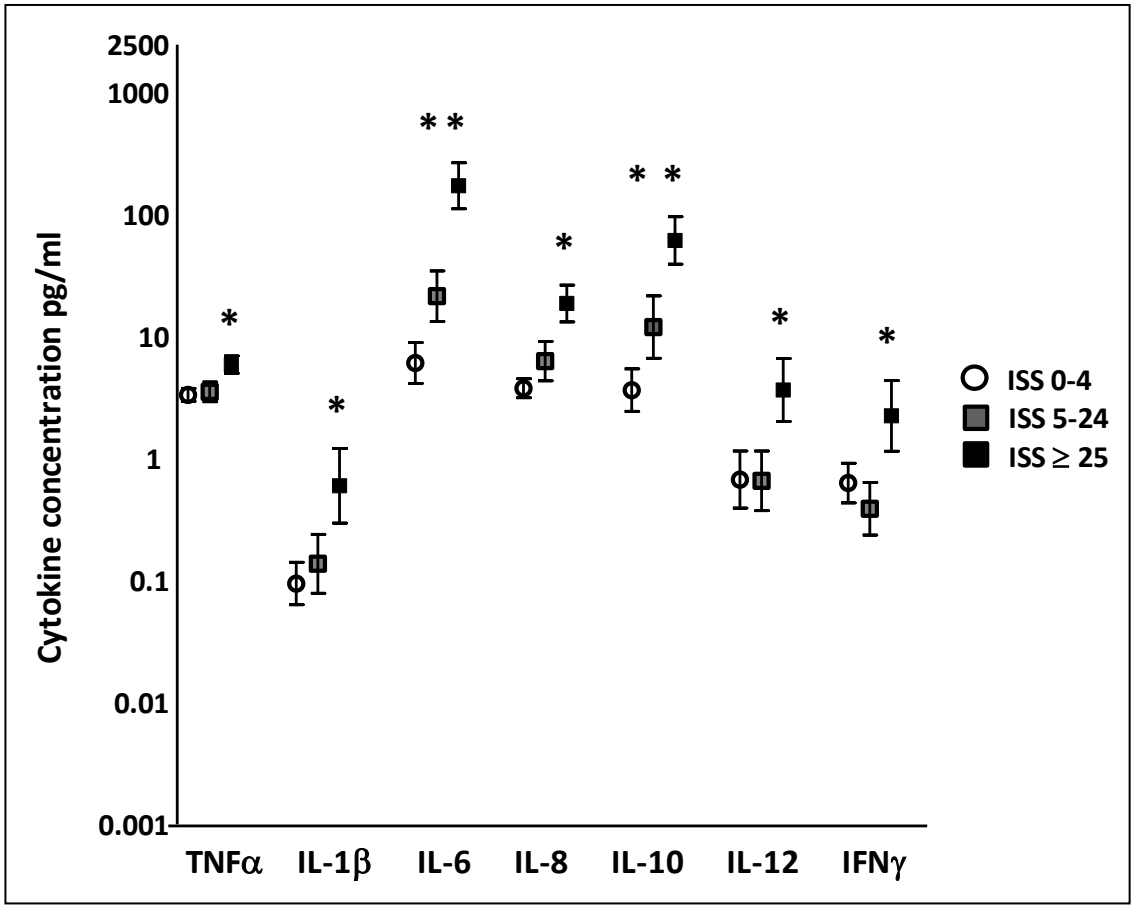
C: Development of Organ failure is associated with tissue damage inflammation (n=60).

High admission levels of IL-6 and IL-10 were associated with organ dysfunction. **IL-6:** 11.79 (9.31-14.94), 39.11 (21.99-69.56), p=0.02. **IL-8:** 4.49 (3.03-6.65), 7.36 (5.40-10.04), p=0.07. **IL-10:** 5.92 (2.48-14.16), 34.92 (17.28-70.56), p<0.01.

b) THE INFLUENCE OF BLOOD LOSS IN COMBINATION WITH TISSUE DAMAGE ON THE INFLAMMATORY RESPONSE TO TRAUMATIC INJURY

The second part of this investigation, considered whether the inflammatory profile generated by traumatic injury is influenced by the addition of blood loss to tissue damage. Ideally this analysis should have been performed using a cohort of patients with severe haemorrhagic shock and minimal ISS however, such a cohort was unavailable. This is not surprising, since extensive tissue damage will usually accompany life threatening blood loss after severe injury, particularly when 80% of the study population has a blunt mechanism of injury. Two cohorts of patients were therefore included for comparison, an isolated 'Tissue Damage' (TD) cohort and a 'Tissue Damage and Shock' (TD&S) cohort. In addition, a control population was also used.

Figure 4.6: The cytokine profile for combined tissue damage and shock cohort at admission



Admission cytokine concentration was stratified according to ISS, for trauma patients (n=85) with tissue damage and evidence of hypoperfusion (BD>2mmol/L). Patients with no injury or minor injury (ISS 0-4) and a normal BD represented a control population (n=39). IL-6, IL-8 and IL-10 demonstrated a dose dependent rise with ISS but of greater magnitude than for tissue damage alone. In addition, IL-1β, TNF-α, IL-12 and IFNγ were elevated in patients with 'critical' injury load (ISS≥25). The combination of shock with tissue damage appears to augment the inflammatory response. Values are reported, on a natural log scale, as mean with 95% confidence interval in pg/ml. * denotes p <0.05 using ANOVA with Dunnett's post-test.

i. Tissue damage combined with blood loss alters the admission cytokine profile

On first examination, the profile of cytokines produced by the TD&S cohort appeared very different from that of the isolated tissue damage cohort (Figure 4.6). Principally, this was because all seven cytokines were elevated. Secondly, the concentrations of IL-6, IL-8 and IL-10 were of greater magnitude. The elevations of all seven cytokines demonstrated a dose-dependent association with ISS.

The Injury Severity Scores (ISS) for each cohort were compared; this demonstrated that the TD&S cohort was more severely injured (Median ISS (IQR): TD=13(9-25), TD&S=25(13-34), $p<0.01$). To enable further comparison between the TD and TD&S cohorts, two groups of patients with matched injury severity were required. The ISS was used to form two identical sub-groups of patients from the original cohorts (n=47 in each sub-group). The demographics and outcomes for those sub-groups are detailed in Table 4.3.

The admission (0h) cytokine values for the TD and TD&S sub-groups were then compared (Table 4.4). No statistical difference was demonstrated between shocked and non-shocked patients with injuries of moderate and severe intensity. For patients with critical injury loads, higher concentrations of TNFα and IL-6 were found in the TD&S group.

Table 4.3: Demographics for the 'Tissue Damage' and 'Tissue Damage & Shock' sub-groups, with matched injury severity

	Tissue Damage	Tissue Damage & Shock	p value
Demographics			
Number of patients	47	47	-
Age (years)‡	31 (24-47)	35 (25-55)	0.21
Male (%)	83	81	1.00
Injury severity			
Blunt (%)	81	74	-
ISS ‡	16 (10-27)	16 (10-27)	1.00
Injury to blood draw (mins)	84 (74-122)	93 (77-116)	0.56
CSL prior to blood draw	0 (0-250)	200 (0-500)	0.09
% >250ml CSL	24	36	0.26
Of those with >250mls	500 (500-800)	750 (500-1000)	0.37
Admission physiology			
SBP (mmHg) ‡	139 (125-152)	125 (101-138)	**0.03**
Base deficit (mmol/L) ‡	0.6 (-0.6-1.3)	4.0 (2.5-7.7)	**<0.01**
Outcomes			
Mortality (%)	9	13	0.74
Length of stay (days) ‡	9 (6-16)	8 (3-28)	0.10
Culture +ve infection (%)	19	21	1.00
MODS (%) *	50	45	0.72
Acute Lung Injury (%) *	42	29	0.39

‡ median (IQR), CSL = crystalloid fluid, ISS= Injury severity score, MODS = Multiple organ dysfunction syndrome defined as a SOFA score ≥5 more than 48h after admission. Acute Lung Injury was defined as a PaO2/FiO2 ratio <40kPA more than 48h after admission. *Data only available for 26/46 TD patients and 31/46 TD&S patients.

Table 4.4: Admission cytokine concentrations in the TD and TD&S groups

Cytokine	ISS	Tissue Damage	Tissue Damage & Shock	p value
TNFα				
	5-24	3.38 (2.99-3.81)	3.14 (2.60-3.79)	0.53
	≥ 25	3.96 (3.24-4.84)	5.98 (4.62-7.73)	**0.02**
IL-1β				
	5-24	0.08 (0.05-0.13)	0.11 (0.06-0.19)	0.45
	≥ 25	0.13 (0.06-0.29)	0.51 (0.18-1.45)	0.05
IL-6				
	5-24	16.12 (10.50-24.74)	17.73 (10.81-29.07)	0.78
	≥ 25	42.88 (23.66-77.69)	115 (60.44-218.90)	**0.03**
IL-8				
	5-24	5.14 (4.10-6.46)	5.23 (3.63-7.55)	0.94
	≥ 25	8.25 (5.83-11.68)	14.20 (9.14-22.06)	0.07
IL-10				
	5-24	8.16 (4.56-14.62)	8.61 (4.61-16.08)	0.90
	≥ 25	27.52 (13.87-54.64)	43.22 (20.39-91.63)	0.39
IL-12				
	5-24	0.62 (0.33-1.16)	0.50 (0.29-0.86)	0.62
	≥ 25	1.07 (0.48-2.40)	3.26 (1.26-8.45)	0.09
IFNγ				
	5-24	0.55 (0.42-0.73)	0.38 (0.23-0.62)	0.20
	≥ 25	0.77 (0.44-1.33)	2.36 (0.86-6.50)	0.06

Admission cytokine concentrations were analysed according to ISS and BD categories, 26 patients were included in the ISS 5-24 category and 20 were included in the ISS ≥25 category.

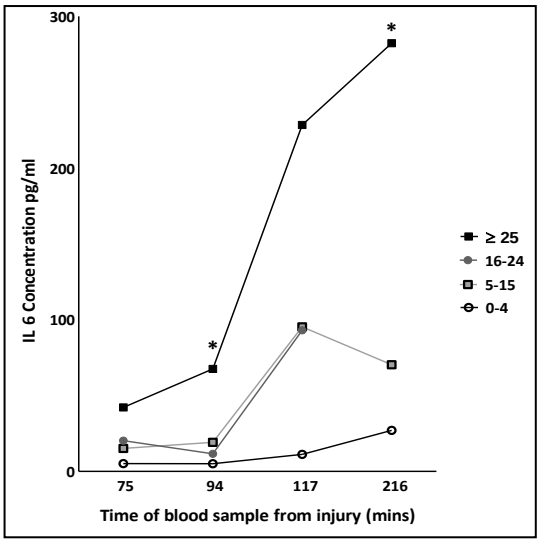
ii. Blood loss and tissue damage effect the time course of the inflammatory response

The influence of time on the inflammatory response was examined in the TD&S cohort, using the same method as the TD cohort; the time from injury to blood draw and the time from admission.

a) Cytokine concentration and time from injury in the TD&S cohort

The admission cytokine concentrations for the TD&S cohort were examined in terms of their ISS and time from injury; exactly the same analysis as the TD cohort. The number of patients available in the TD and TD&S matched ISS sub-groups, were too small to adequately represent every injury level. As an alternative, for this first analysis, the whole TD&S cohort was examined (n=85). The time from injury was described in quartiles of the whole dataset.

Figure 4.7: Mean IL-6 concentration at admission in terms of the time from injury to blood draw



IL-6 concentration at 0h (admission) presented according to the time from injury in trauma patients with tissue damage and shock (BD>2mmol/L). IL-6 concentration increases with time from injury in patients of all injury levels. In the critical injury group, concentrations rose more steeply than the isolated tissue injury group. Data are presented as geometric means. * denotes $p<0.05$ when compared to the control values at the same time-point, ANOVA with Dunnetts Post Test.

A very high spike in IL-6 concentration was observed between 117-216 minutes from injury (Figure 4.7). To examine whether this was due to the influence of blood loss, the TD and TD&S matched ISS subgroups were compared. The IL-6 concentration was higher in patients with combined shock and tissue damage (TD=60.06 (42.59-84.70), TD&S=273.23 (170.76-437.20), p<0.01). This finding was not true for the previous quartile, 94-117 minutes (TD=213.22 (92.08-493.74), TD&S=82.19 (18.53-364.60), p=0.52). Insufficient numbers in these sub-groups (n=47) prevented further breakdown. As an alternative, IL-6 levels from patients with blunt injury from the full TD and TD&S cohorts were compared Table 4.5. Patients with critical injury load demonstrated a difference in IL-6 concentration between shocked and non-shocked patients, although the numbers of patients included in this analysis was small.

Table 4.5: IL-6 concentration varies with time from injury and injury severity in patients with blunt injury

Time from injury (mins)	ISS	n= TD/TDS	Tissue Damage	Tissue Damage & Shock	P value
< 75	Controls	9/ -	5.2 (3.7-12.0)	-	
	5-15	14/4	10.3 (8.6-21.2)	15.2 (14.2-19.5)	0.53
	16-24	1/2	107.2 (106.2-108.2) *~	20.3 (20.0-1123.2)	n/a
	≥25	3/2	43.3 (42.3-45.1) *	42.2 (41.8-424.3)	0.99
76 - 94	Controls	10/-	5.1 (3.6-10.5)	-	
	5-15	7/4	14.7 (13.3-21.2)	19.2 (18.1-27.9)	0.70
	16-24	2/3	130.3 (129.3-131.3) * ^	11.6 (10.7-18.9)	**0.04**
	≥25	5/8	56.2 (55.1-63.5) *	67.6 (66.2-75.1) *	0.78
95 - 117	Controls	2/-	11.2 (10.8-1172.5)	-	
	5-15	5/3	33.5 (32.4-36.1)	95.3 (94.4-103.0)	0.08
	16-24	3/1	97.2 (96.2-98.4) ~	93.0 (92.0-94.0) ~	n/a
	≥25	4/14	160.3 (159.3-162.9) *	228.4 (226.0-244.8) ~	0.71
118 - 216	Controls	3/-	27.1 (26.3-46.3)	-	
	5-15	5/4	20.8 (19.7-27.8)	70.3 (69.3-76.5)	0.11
	16-24	0/-	n/a	n/a	n/a
	≥25	8/16	65.9 (64.8-68.3)	282.3 (280.8-285.7) * ^	**<0.001**

ISS = Injury severity score, * denotes statistically different from Control value using ANOVA with Dunnetts Post test p<0.05, ^ denotes statistical difference between non-shocked and shocked patients with matched ISS. ~ denotes n=1 therefore no statistical test can be applied

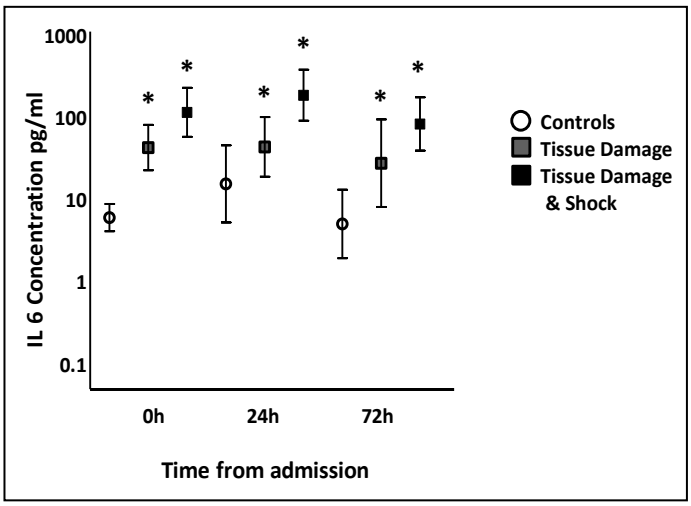
b) Cytokine concentration and time from admission in the TD&S cohort

The change in cytokine concentration from the time of admission was then examined. In order to adjust for injury severity, only patients with an ISS ≥25, from the TD and TD&S sub-groups, were used. This selection focused on the population with critical injury severity and those patients most at risk of poor outcome, but it also significantly reduced the number of patients available for study and the statistical power of the results. A larger analysis would have been desirable. The number of patients included in this analysis was 20 x 0h, 12 x 24h, 12 x 72h.

Patients with critical levels of injury, had concentrations of IL-6 which were elevated above controls at admission, 24h and at 72h (Figure 4.8). IL-8 elevation persisted to 72h in patients with shock but had returned to control concentrations in non-shocked patients by 24h. IL-10 concentrations were highest at admission in both shocked and non-shocked patients with critical injury load. Elevated IL-10 levels were very short-lived in the non-shocked patients and returned to control values by 24h. By contrast, IL-10 in the shocked patients remained elevated at 24h.

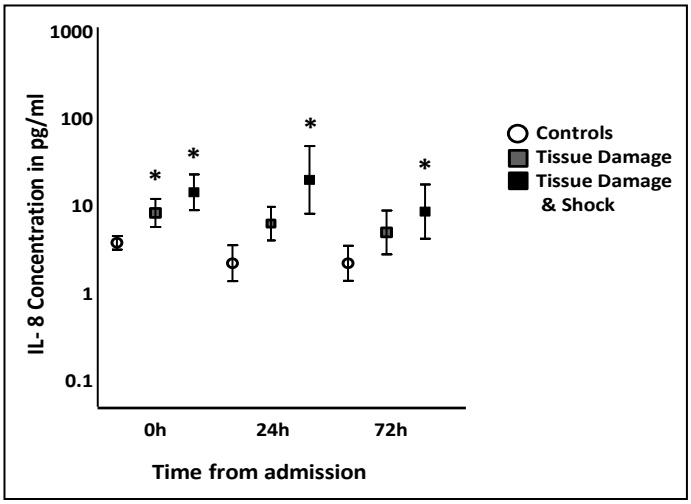
Figure 4.8: Cytokine concentration varies with time from admission in patients with and without shock

Concentrations of IL-6, IL-8 and IL-10 at each time-point from admission were examined (20 x 0h, 12 x 24h, 12 x 72h). The values between the TD sub-group and TD&S sub-group were compared against the 0h control and each other using ANOVA with Dunnetts post test. * denotes p<0.05



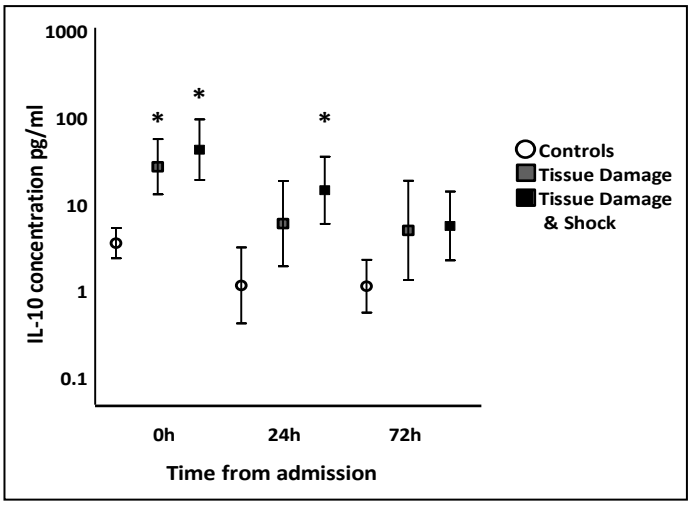
A: Timing of IL-6 activation.

IL-6 levels were elevated above 0h control values for 72 hours in both groups. **0h:** C=6.05 (4.17-8.76), TD=42.88 (23.66-77.69), TD&S=115.02 (60.44-218.90), p<0.01. **24h:** C=15.57 (5.96-40.68), TD=43.72 (20.76-92.09), TD&S=185.63 (98.37-350.28), p<0.01. **72h:** C=5.07 (2.21-11.65), TD=27.72 (9.44-81.45), TD&S=82.93 (42.69-161.10), p<0.01.



B: Timing of IL-8 activation.

IL-8 remained elevated at 72h in shocked patients. In TD patients, levels were down at control values by 24h. **0h:** C=3.76 (3.16-4.47), TD=8.25 (5.83-11.68), TD&S=14.20 (9.14-22.06), p<0.01. **24h:** C=2.19 (1.44-3.34), TD=6.23 (4.19-9.25), TD&S=19.70 (8.93-43.43), p<0.01. **72h:** C=2.19 (1.47-3.27), TD=4.93 (2.97-8.20), TD&S=8.54 (4.52-16.14), p<0.01.



C: Timing of IL-10 activation.

IL-10 levels were highest at admission and fell rapidly. Levels were at control values by 24h in the non-shocked TD group and 72h in the TD&S group. 0h: C=3.62 (2.46-5.34), TD= 27.52 (13.87-54.64), TD&S= 43.22 (20.39-91.63), p<0.01. 24h: C=1.18 (0.48-2.90), TD=6.08(2.22-16.67), TD&S= 14.75 (6.66-32.65), p=0.01. 72h: C=1.16 (0.63-2.12), TD=5.08 (1.59-16.20), TD&S= 5.72 (2.54-12.88), p=0.56.

iii. The co-existence of blood loss with tissue damage does not alter clinical outcome

The relationship between admission cytokine concentration and clinical outcome was then examined. Clinical outcome measures were compared between patients in the TD and TD&S groups, matched for ISS. Mortality at 28 days, length of stay, development infection, MODS and Acute lung injury during recovery were examined (Table 4.3). No difference in outcome was demonstrated between the two groups. As SOFA scores were not collected prospectively at this point in the study, they were only contemporaneously recorded for ITU patients. Where documentation was sufficient, SOFAs for ward based patients were calculated retrospectively. The data must therefore be interpreted with some caution and ideally, analysis should be repeated with more accurate data. The data presented suggest that although patients with tissue damage and shock have higher levels of inflammation, this does not influence clinical outcome.

iv. Can the effect of blood loss be separated from the effect of tissue damage?

Finally, to investigate whether tissue damage and shock have separate influences over the inflammatory response to traumatic injury, multiple regression was performed. Advice was sought from a medical statistician. Using the natural log values of the 0h cytokines, exponential coefficients were calculated to describe the relationship between cytokine concentration and a single point increase in the ISS or BD (Table 4.6). The analysis demonstrated that IL-6 and IL-8 concentrations are considerably influenced by both ISS and BD. IL-1β and TNFα are also influenced by both parameters, but IL-1β has a stronger association with BD. IL-10, IFNγ and IL-12p70 demonstrated an association with ISS and not BD. The mathematical results contradict some of the observations seen in the earlier analysis; however, they demonstrate that cytokine concentrations are influenced by both ISS and BD. Interpreting cytokines values in a clinical disease context like trauma is therefore very difficult.

Table 4.6: Determining the influence of tissue damage and blood loss on the admission cytokine concentration

Cytokine	Trauma Component	Proportional Increase (%)
TNF	ISS	1.01 (1.01 - 1.02)
	BD	1.02 (1.00 - 1.04)
IL1	ISS	1.02 (1.00 - 1.04)
	BD	1.17 (1.08 - 1.26)
IL6	ISS	1.08 (1.07 - 1.10)
	BD	1.11 (1.06 - 1.17)
IL8	ISS	1.04 (1.03 - 1.05)
	BD	1.07 (1.03 - 1.11)
IL10	ISS	1.08 (1.06 - 1.10)
	BD	*1.03 (0.96 - 1.10)*
IL12	ISS	1.05 (1.02 - 1.07)
	BD	*1.04 (0.96 - 1.13)*
IFN	ISS	1.03 (1.01 - 1.05)
	BD	*1.07 (0.99 - 1.15)*

The relationship between admission cytokine concentration and injury severity (ISS) or blood loss (BD) was investigated using regression analysis (n=195). Exponential coefficients with 95% confidence intervals (CI) were calculated using a natural log of the cytokine values. The proportional increase in cytokine level for each point of the ISS or BD is reported.

5. DISCUSSION

This single-centre study was designed to examine the hyper-acute human inflammatory response generated by traumatic injury. It demonstrated that isolated tissue damage results in elevated concentrations of IL-6, IL-8 and IL-10 at admission to the ED and prior to intervention. The concentrations of these cytokines reflected the severity of injury load sustained and were associated with poor clinical outcome. The co-existence of haemorrhagic shock augmented release of IL-6 and IL-8. It also raised concentrations of other cytokines, such as TNFα and IFNγ, which were not observed after isolated tissue injury. Cytokine concentrations appear to be influenced by ISS (tissue damage) and BD (blood loss) therefore meaningful interpretation of the precise value in this clinical disease state is very difficult. Cytokine concentrations demonstrated a fairly transient rise after isolated tissue damage but when combined with shock, the rise was more protracted. This raises the possibility that when blood loss is present there is evolution of the inflammatory response.

The methodology of the ACIT2 study facilitates robust collection of a large number of trauma patient blood samples, within 2 hours of injury and prior to resuscitation. Early blood draw, immediately on admission, ensures that the samples avoid confounding influences from iatrogenic intervention. In addition, pre-hospital resuscitation protocols at our institution support minimal use of crystalloid fluid; the 0h blood sample therefore capture the patient's response to injury. The ISS and BD at admission were used to characterise trauma patient injury. This characterisation is robust and reproducible and could be used by other trauma researchers when reporting results to optimise data comparison between different trauma centres. It also enables the effects of tissue damage to be considered as a separate entity from shock.

Although several studies have demonstrated an association between IL-6 and ISS, this is the first to isolate the relationship between inflammation and tissue damage. It is important to

understand which aspects of traumatic injury are inflammatory stimuli if we are to deepen our understanding of the mechanism of immune activation. The findings of this study are supported by animal work which demonstrates a rise of IL-6 with tissue injury and TNFα with haemorrhage (Ayala et al, 1991). Cytokine levels have been linked to poor outcomes but whether they participate in the pathogenesis remains unclear (Jastrow et al, 2009). This study demonstrates that although poor outcomes are associated with the admission concentrations of IL-6, IL-8 and IL-10, the concentrations of these particular cytokines are decreasing by 24 or 72h from injury. It may be that poor outcomes follow the production of related mediators later down the clinical pathway, as part of an orchestrated response (Cinat et al, 1995). This concept requires further study.

Cytokines are biological molecules which hold promise as biomarker candidates in trauma. Existing technology would enable cytokine measurement to become a routine part of initial trauma patient assessment, if interpretation of the results made a meaningful contribution to patient management (Jastrow et al, 2009). IL-6 has previously been proposed as "an early indicator of injury severity", with levels of IL-6 "above 200pg/ml (said to) indicate SIRS" and "IL-6 above 350pg/ml associated with a risk of MODS" (Gebhard et al, 2000, (Giannoudis et al, 2008, (Cuschieri et al, 2010). This investigation demonstrates that while they may reflect the extent of the immune response activated by trauma, none of the seven cytokines measured, can be used to reliably indicate the extent of injury severity in trauma patients. IL-6 demonstrated the strongest association with tissue damage but a strong influence from shock limits the use of IL-6 as a clinical biomarker of injury. IL-10 rose with ISS and was independent of BD but could not distinguish between blunt and penetrating tissue injury.

Release of cytokines into the systemic circulation is thought to indicate that local defences are struggling and adaptive cells are required to assist (Bone et al, 1997). IL-6, IL-10 and TNFα can all be produced by a range of immune cells, including macrophages, monocytes, neutrophils

and T lymphocytes and nerve tissue, as discussed in a review by (de Oliveira et al, 2011). A wide range of pleiotrophic actions are attributed to these seven cytokines and other inflammatory mediators, including the generation of the SIRS symptoms, production of pain and activation of coagulation (de Oliveira et al, 2011). In addition, TNFα is implicated in the progression of autophagy and apoptosis (Harris, 2011). The precise roles which these molecules play, after traumatic injury, are likely to be extensive. Although, anti-cytokine therapeutics are used in other sterile inflammatory diseases, such as Rheumatoid Arthritis, the use of specific anti-cytokine agents in trauma is unlikely to yield clinical benefit in the near future (Mima and Nishimoto, 2009).

This is one of the largest studies of traumatic injury inflammation undertaken but a number of limitations are still acknowledged. The most important, is that the multiplex panel of 7 cytokines selected for this study, only describes a very small component of the inflammatory response. A broader assessment may identify substances which have stronger association with tissue damage or shock. This study would also have benefitted from a systems modelling approach to adjust for known confounding influences and this may be applied to future work. Use of the ISS to describe injury load is controversial as it is an anatomical scoring system, which is mortality weighted and does not reflect mechanical tissue damage. In the absence of relevant biomarkers for tissue damage, scoring systems are the only practical surrogate. By excluding patients with a raised BD I was able to describe the response to ISS in isolation. Systolic blood pressure was used to describe the clinical status of the patients but, measurement of mean arterial blood pressure may have been a better choice. As blood pressure is known to be an unreliable indicator of haemorrhage, no analysis was conducted using blood pressure as a defining characteristic (Kortbeek et al, 2008). Base deficit was used as a surrogate marker of shock. Lactate could have been used as an alternative and inclusion of both would have, perhaps, been ideal. Both measurements have limitations. A normal BD at admission does not guarantee the absence of haemorrhage, it simply means that at the time

of 0h blood draw there was no cellular hypoperfusion. Interventions after admission will influence cytokine values at 24h and 72h and should be interpreted with caution. Later time-point samples are influenced by participation bias; patients who are discharged, die or refuse blood draw are excluded. Finally, the time-point profiles used only IL-6 to allow for comparison with Gebhard's work but inclusion of IL-8 and IL-10 would have been desirable.

6. CONCLUSION

This study demonstrated that isolated tissue damage is associated with an inflammatory response that is proportional to the severity of injury sustained. In addition, it demonstrated that even after isolated tissue damage, high concentrations of cytokines at admission are associated with adverse clinical outcomes, specifically 28-day mortality, SOFA>5 and development of infection. In patients with high ISS and elevated BD, there was an augmented inflammatory response. The response had significant overlap with that of isolated tissue damage, based on the cytokines examined in this study. A cytokine response specific to tissue damage was therefore not robustly identified. The study findings do however, provide some evidence to suggest that tissue damage and blood loss may provoke different inflammatory responses. The limitations of ISS, BD and study size prevented causality from being determined but, the concept warrants further investigation.

The investigation supports the 'danger model' theory of immune activation in the context of traumatic injury (Matzinger, 1994, (Matzinger, 2002). Isolated tissue damage is associated with an immune response within the first few hours of injury. Shock and tissue injury probably should be considered as separate activators of inflammation when searching for clinically relevant trauma alarmins. If poor outcome is dictated from the outset, then a window of opportunity for therapeutic intervention may exist if suitable targets can be identified.

Part 3: Alarmins

CHAPTER FIVE: TRAUMA ALARMINS – A NARRATIVE REVIEW

This review was published in the British Journal of Surgery Trauma Supplement. BJS Jan 2012;99(S1):12-20

1. INTRODUCTION

A systemic inflammatory response syndrome (SIRS) commonly follows traumatic injury in humans and is the result of innate immune system activation (Chow et al, 2005, (Lenz et al, 2007, (Tsukamoto et al). The primary initiators of post-traumatic SIRS are unknown, but the clinical features are similar to those observed during sepsis (Lenz et al, 2007, (Tsukamoto et al, (Faist et al, 1983, (Jastrow et al, 2009). Although there have been attempts to demonstrate a microbial cause, it is now generally accepted that the inflammatory response following trauma is sterile (Moore et al, 1991, (Deitch et al, 1990, (Magnotti et al, 1998, (Matzinger, 2002).

The activation of innate immunity is thought to result from tissue damage (Matzinger, 2002). Injured tissues putatively release endogenous molecules, called alarmins, which interact with immune cells to initiate the inflammatory response (Harris and Raucci, 2006, (Matzinger, 2002, (Oppenheim and Yang, 2005). Several potential alarmins have been described, but the precise role of these molecules in humans requires further elucidation (Bianchi, 2007, (Zedler and Faist, 2006, (Oppenheim and Yang, 2005).

This field provides an exciting platform for potential advances in trauma care. Modulation of the immune system after traumatic injury is a complex and controversial proposition, but if the mechanisms of activation can be clarified and manipulated, then survival rates may be improved. Trauma represents perhaps the most extreme form of human tissue damage; however, many other surgical diseases involve tissue damage and blood loss. As understanding develops, the findings from this research may become relevant to other surgical specialties.

This narrative review examines the evidence for alarmin-induced inflammation after traumatic injury in preclinical and clinical studies.

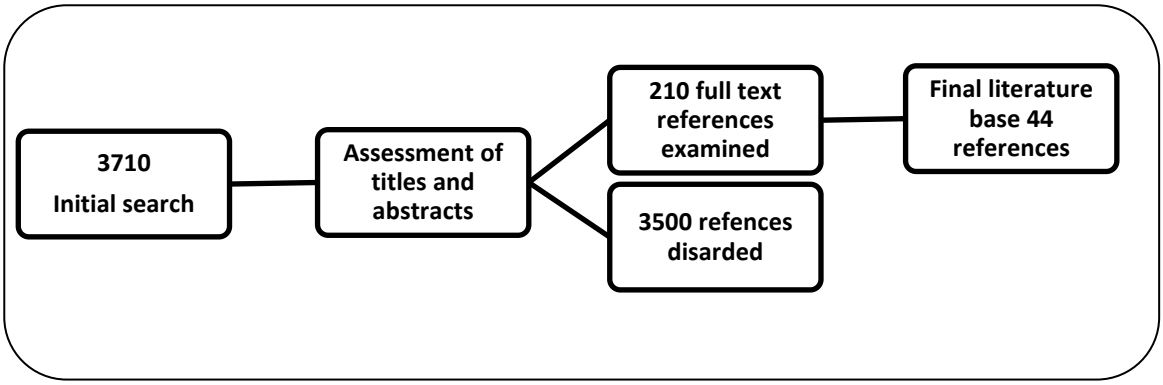
2. METHODOLOGY

A literature search was conducted using Medline and EMBASE using the following search terms (Box 5.1 & figure 5.1). No time limit was placed upon the search strategy but it was limited to English publications.

Box 5.1: Search strategy for alarmins review

Adult >16 years Human English	**AND**	Traumatic injury/ trauma[ti]/ trauma[MESH]/ blunt/ penetrating/ polytrauma/ major trauma centre/	**NOT**	Burns/ thermal injury/ head injury/ brain injury/ neurology/ brain/ neurosurgery	**AND**	alarmins/ DAMPs/ PAMPs/ Danger/ Sterile inflammation/ immunity,innate		

Figure 5.1 Search strategy for alarmins review



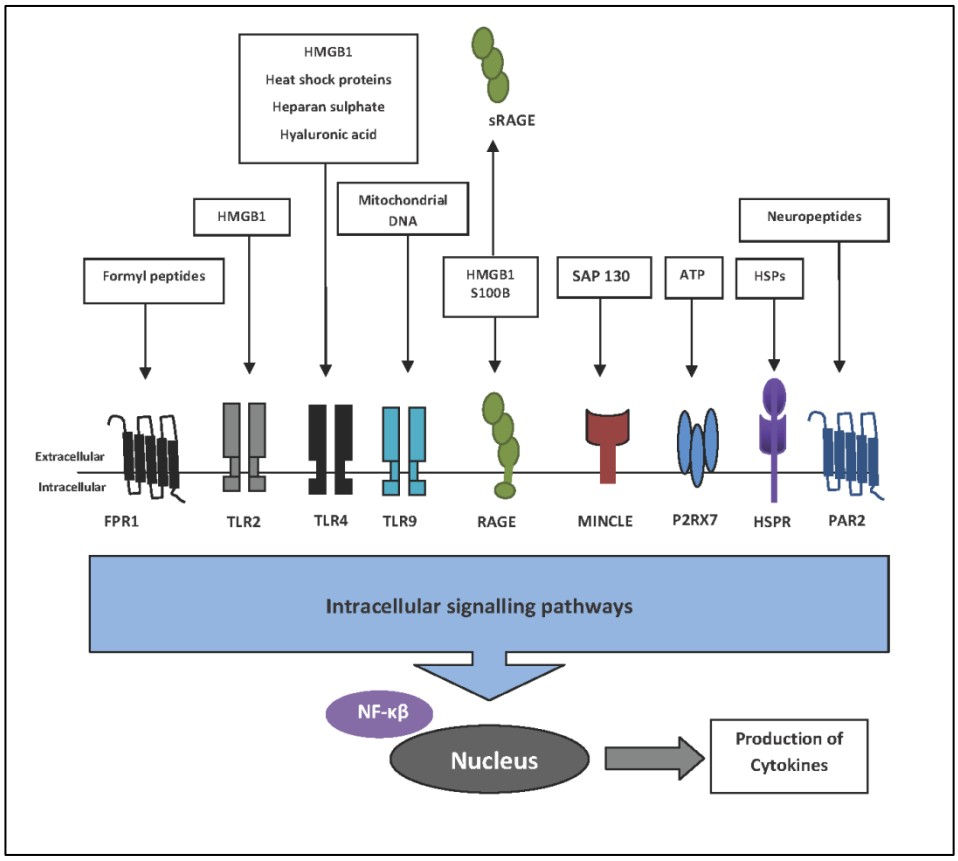
3. MECHANISMS OF INFLAMMATORY ACTIVATION

Innate immunity is no longer thought to be activated by the simple presence of foreign non-self material (Janeway and Medzhitov, 2002). Immune activation actually follows the development of tissue damage and detection of harm to the organism (Matzinger, 2002). The subsequent response may even be tissue-specific (Matzinger and Kamala, 2011). For bacterial sepsis, the mechanism of immune activation is fairly well established (Brunn and Platt, 2006, (Cohen, 2002). Structural proteins from the bacterial surface interact with receptors on innate immune cells (Cohen, 2002). These danger signals are called pathogen-associated molecular patterns (PAMPs), and several have been identified including lipopolysaccharide (LPS, from Gram-negative bacteria) (Xiang and Fan), lipoteichoic acid (Gram-positive bacteria) (Xiang and Fan), flagellin (bacterial flagellum) (Matzinger and Kamala, 2011) and bacterial DNA (Lipford et al, 1998). Interaction between the PAMP and an immune cell receptor triggers cytokine production (Cohen, 2002). Cytokine cascades propagate the innate and adaptive immune response, and sepsis occurs when the level of immune activation is overwhelming (Cohen, 2002). The efficacy of antibiotics stems from their ability to reduce immune activation as much as from their ability to remove the bacterial activity.

The mechanism of immune activation following sterile tissue damage is less well understood. Endogenous alarmin molecules reputedly interact with innate immune cell receptors to trigger cytokine production (Matzinger, 2002) (Figure 5.2). As any intracellular molecule suddenly expelled into the extracellular environment could potentially be an alarmin, the key is to identify those that are clinically relevant (Matzinger, 2002). In addition, alarmins may interact with one or more types of receptor, which can be located on the cell surface or within the cytoplasm (Zedler and Faist, 2006, (Yang et al, (Meylan et al, 2006, (Kaczorowski et al, 2008). Many of the receptors demonstrate promiscuous tendencies to bind multiple ligands, and some ligands can initiate variable responses depending on the environmental conditions (Matzinger and Kamala, 2011, (Matzinger, 2002, (Xiang and Fan). Several substances have

been described as having alarmin properties, but confusion exists regarding their classification (Gallucci and Matzinger, 2001, (Oppenheim and Yang, 2005, (Tewary et al, 2010, (Yang et al, (Bianchi, 2007, (Zedler and Faist, 2006). The term requires further clarification because it is used to describe any endogenous 'danger' signalling substance, whether intracellular, extracellular or secreted, irrespective of where the molecule participates in the pathway of immune activation (Gallucci and Matzinger, 2001, (Matzinger, 2002, (Tewary et al, 2010, (Oppenheim and Yang, 2005).

Figure 5.2: Trauma alarmins and their receptors for pro-inflammatory immune activation



Trauma alarmins and their receptors for proinflammatory immune activation. TLRs =Toll-like receptors, PARs =Proteinase-activated receptors, HSPRs =Heat shock protein receptors, RAGE =Receptor for glycation end products, sRAGE =Secretory RAGE, Mincle =Macrophage inducible C-type lectin, P2RX7=Purinergic receptor, FPRs=Formyl peptide receptors, HMGB1=High mobility group box 1, P2RX= purinergic receptor.

4. TRAUMA ALARMINS

The defining criteria for alarmin molecules were agreed by consensus in 2006 (Harris and Raucci, 2006). The original definition incorporated a structurally diverse group of intracellular proteins that were produced by unconventional secretory mechanisms. Their key features were release by necrosis but not apoptosis and a capacity to induce innate and adaptive immunity by activating inflammation-related pathways (Harris and Raucci, 2006). Since then, it has been suggested that stricter criteria should be applied, to include only substances that are immunologically active in their pure form, at biological concentrations, and where the mechanism of activation can be confirmed by blockade at receptor level (Kono and Rock, 2008). Most substances postulated to be alarmins do not meet these criteria using the available evidence (Table 5.1 & 5.2).

From a clinical perspective, the goal is to identify the alarmin(s) released immediately after traumatic tissue damage – the molecules that activate the first immune cells. The extracellular concentration of the alarmin should reflect the severity of the trauma load. The endogenous downstream mediators that propagate the response can be deciphered subsequently. Trauma is a complex clinical condition incorporating a range of pathophysiological conditions. Trauma always involves tissue injury, but haemorrhage, shock, acidosis, hypoxia and ischaemia–reperfusion coexist in some patients. Whether the same inflammatory pathways are activated in all circumstances remains to be established.

Trauma alarmin candidates are likely to originate from two sources, necrotic cells and physiologically 'stressed' cells. For the purposes of this review, the evidence for each alarmin candidate was assessed according to the following seven criteria: it has direct pro-inflammatory activity in cultured cells; the mechanism of activation has been established in cell culture; plasma or serum levels are increased in animal models of trauma; it can provoke a direct SIRS response in animals; the mechanism of activation has been validated using

knockout animal models; human plasma or serum levels are raised after traumatic injury; the plasma or serum concentration correlates with the extent of human immune activation.

a) ALARMINS RELEASED PASSIVELY BY DAMAGE-INDUCED (NON-PROGRAMMED) CELL DEATH

Mechanical cell rupture inevitably leads to sudden expulsion of intracellular contents into the extracellular environment. The extent of cell necrosis that occurs after trauma is unknown and varies with mechanism of injury. Several intracellular substances have been demonstrated to undergo passive release from necrotic cells *in vitro* (*Table 1*). Direct pro-inflammatory action has also been established, in cell culture, for the majority of these candidates. This supports their alarmin status, although the ability of some candidates to independently activate immunity has been questioned (Tsan, 2011). High-mobility group box (HMGB) 1, a nuclear-binding protein found in the nucleus of all eukaryotic cells, and intracellular heat-shock proteins (HSP) may require prior binding with PAMPs such as LPS in order to activate immune cells (Harris and Raucci, 2006, (Bianchi, 2009, (Rouhiainen et al, 2007). Similarly, mitochondrial *N*-formyl peptides are thought to require a co-factor, mitochondrial transcription factor, which is the mitochondrial equivalent of HMGB1 (Crouser et al, 2009). The precise mechanics of the activation pathway therefore remain unclear. A variety of receptor interactions have been identified *in vitro*[33,] (Levy et al, 2006, (Park et al, 2004, (Yamasaki et al, 2008). In animal trauma models, a Toll-like receptor (TLR) 4 mechanism has been convincingly established for HMGB1 (Levy et al, 2007). In a clinical setting, it is not yet clear whether the receptors work in isolation or in concert.

Table 5.1: Summary of current evidence about alarmins in pre-clinical and clinical studies- Part I

	Cellular evidence				Animal Model				Human Trauma	Ref
Substance	**Location**	**Inflammation**	**Receptor**	**Mechanism**	**Raised**	**Inflammation**	**Receptor**	**Alarmin**	**Raised**	
Passive release into extracellular environment following non-programmed cell death										
HMGB1	Nucleus	✓*	TLR2, TLR4, RAGE	✓	✓	✓*	TLR4	✓	✓	(Scaffidi et al, 2002, (Bianchi, 2007, (Bianchi, 2009, (Rouhiainen et al, 2007, (Park et al, 2004, (Levy et al, 2007), (Kim et al, 2005, (Yang et al, 2006), (Cohen et al, 2009)
Nucleosomes	Nucleus									(Kono and Rock, 2008)
Histones	Nucleus	✓				✓		✓		(Xu et al, 2009)
Mammalian DNA	Nucleus								✓	(Lo et al, 2000)
Nucleophosmin	Nucleus	✓								(Nawa et al, 2009)
SAP 130	Nucleus	✓	Mincle	✓						(Yamasaki et al, 2008)
HDGF	Neurons									(Bianchi, 2007)
Heat shock proteins	Nucleus Cytoplasm Mitochondria	✓	TLR2, TLR4 HSPRs		✓				✓	(Pespeni et al, 2005, (Basu et al, 2000, (Osterloh and Breloer, 2008, (Cox and Kalns, 2010)
Mitochondrial DNA	Mitochondria	✓	TLR9			✓		✓	✓	(Zhang et al, (Zhang et al, 2010a, (Krysko et al, 2011)
N-Formyl peptides	Mitochondria	✓#	FRPs							(Zhang et al, (Krysko et al, 2011, (Carp, 1982)
ATP	Mitochondria	✓	P2RX7	✓						(Krysko et al, 2011, (Yang et al, 2010, (la Sala et al, 2003, (Di Virgilio, 2007, (Ghiringhelli et al, 2009, (Willart and Lambrecht, 2009)
Formyl peptides	Cytoplasm	✓								(Xiao et al)
Uric acid	Cytoplasm	✓			✓	✓		✓		(Kono and Rock, (Shi et al, 2003)

* may act as a co-factor with PAMPs, # required presence of TFAM for cytokine stimulation

Table 5.2: Summary of current evidence about alarmins in pre-clinical and clinical studies- Part II

	Cellular evidence				Animal Model				Human Trauma	Ref
Substance	**Location**	**Inflammation**	**Receptor**	**Mechanism**	**Raised**	**Inflammation**	**Receptor**	**Alarmin**	**Raised**	
Active or passive release in to the extracellular environment by "stressed" non-immune cells										
HMGB1	Nucleus	✓*			✓	✓	TLR4	✓	✓	(Tsung et al, 2005, (Yang et al, 2006, (Kim et al, 2005, (Cohen et al, 2009)
S100	Cytoplasm		TLR, RAGE						✓	(Leclerc et al, 2009)
Cytochrome C	Mitochondria	✓				✓				(Krysko et al, 2011, (Ott et al, 2007, (Adachi et al, 2004)
ROS	Mitochondria	✓								(Ott et al, 2007, (Rutault et al, 1999)
Hyaluronan	Membranes	✓*	TLR4			✓	TLR4			(Yang et al, 2010, (Kaczorowski et al, 2008)
Heparan sulfate	Membranes	✓	TLR4			✓	TLR4			(Kaczorowski et al, 2008, (Wrenshall et al, 1995, (Wrenshall et al, 1991)
Upregulated or Over expressed in "stressed" non-immune cells										
HMGB1	Nucleus	✓*								(Tsung et al, 2005)
Heat shock proteins	Intracellular	✓*			✓					(Cox and Kalns, 2010)
CD-40-L	Activated platelets	✓								(Gallucci and Matzinger, 2001, (Henn et al, 1998)
Unlikely trauma alarmins										
Fibrinogen	Extravascular	✓	TLR4							(Smiley et al, 2001)
Granulysin	Lymphocytes	✓								(Yang et al, 2010)
Defensins	Membranes	✓								(Oppenheim and Yang, 2005)
Cathelicidins	Immune cells	✓								(Oppenheim and Yang, 2005)
EDN †	Eosinophils	✓								(Oppenheim and Yang, 2005)
Cytochrome C	Mitochondria	✓				✓				(Adachi et al, 2004)
Plasmin	Blood									(Li et al, 2007)
sRAGE	Extracellular								✓	(Cohen et al, 2010)
Histamine	Mast Cells	✓								(Steinhoff et al, 2000)
Neopterin	Macrophages								✓	(Roumen et al, 1995, (Fuchs et al, 1992)
Neuropeptides	Neurons	✓	PAR2							(Nathan, 2002, (Steinhoff et al, 2000)

† Eosinophil-derived neurotoxin

In animal models of trauma, raised levels of circulating HMGB1 and HSP have been demonstrated (Kim et al, 2005, (Levy et al, 2007, (Cox and Kalns, 2010). In injured patients, increased levels of HMGB1, mitochondrial DNA (mtDNA), eukaryotic DNA and HSP have been reported (Cohen et al, 2009, (Zhang et al, (Lo et al, 2000, (Pespeni et al, 2005). Within a mean(+/- s.d.) of 32(6) min after injury, HMGB1 levels correlated with the Injury Severity Score and base deficit, and were associated with levels of inflammatory mediators and complement activation (Cohen et al, 2009). To date, no other alarmin candidates have been shown to correlate with the degree of human immune activation. Mean (s.e.m.) plasma mtDNA concentrations have been shown to be 2.7(0.9) μg/ml, thousands of fold higher in injured patients than in healthy controls (Zhang et al). Whether levels of mtDNA correlate with injury severity has not yet been established. Levels of circulating eukaryotic DNA reflect the severity of injury and correlate with outcome (Lo et al, 2000). Insufficient evidence exists to determine whether the other passively released substances play an alarmin role after human traumatic tissue damage.

b) ALARMINS ORIGINATING FROM 'STRESSED' NON-IMMUNE CELLS

i. Alarmins actively released from 'stressed' non-immune cells

Hypoxia, acidosis and haemorrhagic shock are well recognized causes of cell stress *in vitro*. Although they frequently accompany traumatic injury, SIRS can also be generated in their absence. Preclinical data have provided evidence of active alarmin release during some of these cell stress conditions but, in clinical practice, which cell stresses occur and how they influence the clinical state remains unclear.

Hypoxia in hepatocytes has been reported to cause time-dependent extracellular release of HMGB1, with no reduction in cell viability (Tsung et al, 2005). By contrast, hypoxia in cardiomyocytes leads to cell necrosis and passive HMGB1 release (Andrassy et al, 2008). Hepatic and cardiac ischaemia–reperfusion lead to upregulation of HMGB1 expression (Tsung

et al, 2005, (Andrassy et al, 2008). The resultant necrosis and inflammation was reduced by administration of anti-HMGB1 antibodies, suggesting that HMGB1 plays an important role in the clinical sequelae of ischaemia–reperfusion (Tsung et al, 2005, (Andrassy et al, 2008). It remains unclear whether the release during ischaemia–reperfusion is active or passive. S100 proteins are calcium-binding proteins that have tissue-specific distributions. S100B is found within oligodendrocytes, astrocytes and Schwann cells (Leclerc et al, 2009). High plasma levels of S100B have been identified following traumatic brain injury (Hayakata et al, 2004, (Vos et al, 2010). S100B proteins are ligands for the trans-membrane receptor for advanced glycation endproducts (RAGE) and have direct immunological activity (Leclerc et al, 2009). They can be actively secreted into the extracellular environment following mechanical (Ellis et al, 2007) or physiological (Gerlach et al, 2006) stress. It is unclear whether cell necrosis leads to passive release of S100B. Reactive oxygen species are generated by mitochondria during apoptosis and released in conditions of cellular stress. They are known to have inflammatory capacity, but along with several other mitochondrial components their role as a trauma alarmin has not yet been examined (see review (Krysko et al, 2011)). Finally, the cell surface saccharides, heparan sulphate and hyluronan, have inflammatory capabilities through TLR4 (Brunn and Platt, 2006, (Johnson et al, 2004) and can instigate a SIRS response in rodents (Johnson et al, 2004). Their release from the cell surface requires active proteolytic cleavage (Platt et al, 1990). They are biologically active and participate in coagulation, but their role in SIRS after injury has not been considered.

ii. Alarmins that are upregulated or overexpressed by 'stressed' non-immune cells

Upregulation of proteins to the cell surface presents a red flag signal to the immune system. This process may well result in early alarmin signals for the SIRS response after traumatic injury. Upregulation of HMGB1 and HSP to the cell surface has been demonstrated in animal trauma models (Tsung et al, 2005, (Cox and Kalns, 2010). Although this may have mechanistic significance, the effect of these upregulated molecules on inflammation is unknown. Activated

platelets upregulate CD40-L to their surface within seconds of exposure to thrombin *in vitro* and to damaged endothelium (Henn et al, 2001, (Ware and Heistad, 1993). This has been reported to generate a direct inflammatory response in the vessel wall and could potentially also activate innate immune cells (Gallucci and Matzinger, 2001, (Henn et al, 1998). This finding presents an interesting cross-talk between the inflammation and coagulation, and requires more investigation. The diagnostic value of upregulated proteins is probably limited, since it is unlikely that they will be quantifiable unless they are released into the circulation.

c) MOLECULES THAT DO NOT MEET THE CRITERIA FOR TRAUMA ALARMINS

Since they were first defined, many endogenous inflammatory mediators have been called alarmins. To be of clinical value, alarmins require a more specific description. A true trauma alarmin should be a molecule released from damaged tissue that initiates the inflammatory response to injury. The endogenous molecules with immunological properties that participate in downstream immune signalling may be better classified as secondary danger signals. This category includes stored antimicrobial substances such as granulysin, defensins, cathelicidins and eosinophil-derived toxins that are actively released from immune cells following stimulation by PAMPs or inflammatory mediators (Yang et al, 2004, (Oppenheim and Yang, 2005, (Tewary et al, 2010). It may also include neopterin, which is released from activated macrophages and monocytes in response to cytokine stimuli such as interferon γ (Fuchs et al, 1992). Extracellular secretory RAGE (sRAGE or RAGE_v1) could also be included in this category. RAGE has 20 known isoforms that are tissue-specific, and sRAGE is expressed in brain and endothelial cells. Extracellular release of sRAGE is an active process that occurs after proteolytic cleavage of the full-length RAGE molecule (Galichet et al, 2008). It is not known whether necrosis leads to passive release of sRAGE. High levels of sRAGE have been demonstrated in the plasma of injured patients within 30 min of injury, associated with coagulopathy and complement activation (Cohen et al, 2010). Finally, extracellular fibrinogen has also been suggested to have alarmin characteristics owing to its proinflammatory actions

in extravascular tissues (Smiley et al, 2001, (Kono and Rock, 2008). As increased vessel permeability is a consequence of inflammation, a previous inflammatory stimulus must initiate the leakage of fibrinogen to the extracellular environment. These endogenous molecules have immune properties but should not be termed alarmins because they cannot be the first danger signal released following tissue injury.

5. DISCUSSION

Traumatic injury is a complex disease process. Tissue damage is often complicated by the additional presence of haemorrhagic shock and necessity for blood transfusion, clotting products and surgical intervention. Identifying the important immune triggers for traumatic disease is not easy. Immune activation, however, occurs early after injury and is associated with poor clinical outcome (Gebhard et al, 2000, (Lenz et al, 2007). If outcome is dictated from the outset then, in principle, a window of opportunity for intervention exists. According to the danger model, tissue damage is the key stimulus for the immune response (Matzinger, 1994, (Matzinger, 2002). In traumatic injury, inflammation has been attributed to many components, including surgery and blood transfusion, but not to isolated tissue damage (Sihler and Napolitano, (Tschoeke et al, 2007). Separating the tissue damage response from the haemorrhagic shock response, in both humans and animal models, is difficult but necessary in order to identify the clinically relevant alarmins.

Since the publication of the danger model in 1994, many endogenous molecules have been shown to have inflammatory capacity and have earned the name of alarmin (Matzinger, 1994, (Matzinger, 2002). Within the context of traumatic injury, more work is required to identify the clinically relevant alarmins and their mechanisms of action. HMGB1 is currently the only alarmin molecule with robust preclinical evidence for its release after injury and with a direct link to inflammation. It has also been implicated in the development of clinical sequelae in the

form of gut, lung and liver injury and therapeutic agents for blockade of HMGB1–receptor interactions are currently under development (Levy et al, 2007, (Yang et al, 2006, (Kim et al, 2005, (Tsung et al, 2005, (Andersson and Tracey, 2011). Although it is highly unlikely that a single molecule will control the inflammatory pathway, this is a big step towards new advancements in trauma care. Evidence for mtDNA as an alarmin also shows promise (Zhang et al). Mitochondria are believed to originate from bacteria and share several structural features. These features resemble PAMPs and may directly activate innate immune receptors (Gray et al, 1999). Extracellular release of mtDNA after trauma has been demonstrated, but the correlation with injury load, clinical outcome and inflammation is yet to be determined (Zhang et al, (Zhang et al, 2010a).

The primary aim of the immune system is to limit tissue damage. The response moves from non-specific to specific, and is in part essential and in part destructive, resulting in the clinical complications seen after severe injury (Matzinger and Kamala, 2011, (Tsukamoto et al, (Chow et al, 2005). Identification of trauma alarmins and the molecular pathways that generate the immune response may provide an opportunity for the development of therapeutic agents able to modulate the response and improve outcomes. Binding of multiple ligands, dual roles for receptors, and genetic polymorphisms for cytokine production make devising immune therapeutics for trauma extremely challenging (Xiang and Fan, (Gearing, 2007, (Sutherland and Walley, 2009, (Krysko et al, 2011). Nonetheless, evidence from preclinical studies supports the idea that molecular blockade reduces adverse clinical sequelae and therapeutic agents are under development (Levy et al, 2007). There is growing evidence that immunological status also influences patient outcome in the elective setting (Marik and Zaloga, (Roxburgh and McMillan, (Anderson, 1997). The discovery and manipulation of alarmins may therefore have implications for other surgical conditions. Within the context of sepsis, targeted treatment strategies have gone some way to reduce in-hospital mortality (Daniels, 2011, (Dellinger et al, 2008). With time, there is no reason why that vision cannot also be achieved for trauma.

CHAPTER SIX: EVALUATION OF POTENTIAL ALARMINS IN A TRAUMA PATIENT POPULATION

1. INTRODUCTION

Based on current evidence, Mitochondrial DNA (MtDNA), HMGB1 and histones are three of the leading candidates for alarmins with clinical relevance. Compelling pre-clinical evidence suggests that these intracellular molecules play significant roles in the generation of inflammation and end organ damage after trauma or sepsis (Scaffidi et al, 2002, (Manson et al, 2012, (Kim et al, 2005, (Levy et al, 2007, (Xu et al, 2009). Elevated plasma levels of HMGB1 and MtDNA have been demonstrated after traumatic injury in humans (Zhang et al, 2010b, (Cohen et al, 2009). In a single clinical study, HMGB1 levels were associated with inflammation and adverse outcome (Cohen et al, 2009). A relationship between peripheral blood concentration of histones or mtDNA and inflammation or clinical outcome has yet to be demonstrated in trauma patients.

The candidate alarmins used for this investigation were selected on the basis of recent evidence, which indicates that they may play a role in the activation of innate immunity. Cytochrome B is a DNA fragment from the mitochondrial genome. As such it is hypomethylated and rich in CpG repeats (Cytosine-Guanine bases joined by a phosphodiester bond). Mitochondria evolved from bacteria and their shared structural features are thought to directly activate human Toll-like receptors (TLRs) (Krysko et al, 2011, (Gray et al, 1999). Previous reports have demonstrated that trauma patients have high levels of Cytochrome B in their peripheral blood although the circumstances leading to its release were unclear (Zhang et al, 2010b). HMGB1 is the archetypal alarmin and robust pre-clinical evidence indicates an important role for HMGB1 in the activation of inflammation and the generation of end-organ damage (Bianchi, 2007, (Harris and Raucci, 2006, (Levy et al, 2007, (Kim et al, 2005, (Tsung et

al, 2005, (Yang et al, 2007). Elevated levels of HMGB1 have been reported within half an hour of injury (Cohen et al, 2009) although in that heterogeneous patient group it was unclear whether release was a result of mechanical tissue damage or blood loss and shock. Extracellular histone release has not been examined in trauma patients, but strong evidence supports their involvement in inflammatory end-organ damage after sepsis (Xu et al, 2009).

2. THE AIM OF THIS INVESTIGATION

This study was devised to investigate the significance of peripheral blood concentration of three candidate alarmin substances within the context of the inflammatory response to traumatic injury. The primary aim was to determine whether circulating concentrations of MtDNA, HMGB1 and Histones reflected the degree of tissue damage sustained. Secondly, I wished to examine the relationship between circulating alarmin levels and immune activation after isolated tissue damage. The third aim was to establish whether the co-existence of haemorrhagic shock influences the peripheral blood concentration of these potential alarmins. Finally, I wished to determine whether the alarmin concentration at admission is associated with adverse clinical outcome as defined by 28 day mortality, development of infection and acute lung injury (ALI).

3. METHODS

Quantification of the three potential alarmin substances was performed on plasma from trauma patients recruited to the ACIT2 study. The concentration of MtDNA was measured using quantitative real-time polymerase chain reaction (qPCR). Quantification of HMGB1 was performed using an ELISA technique specific for HMGB1. Histone detection in plasma is known to be unreliable due to rapid degradation in peripheral blood; therefore, histones were

measured indirectly by quantifying histone-DNA complexes called Nucleosomes (Holdenrieder et al., 2001). Nucleosomes were quantified using a 'Cell Death' ELISA (Cell Death ELISA plus, Roche Applied Science, UK) with a validated adaptation of the protocol devised by Holdenrieder (Holdenrieder et al., 2001). The methodology for these experiments is described in detail in Chapter 2.

a) DATA ANALYSIS AND STATISTICS

In order to distinguish the effects of mechanical tissue injury from that of blood loss, patients were categorised into groups using their injury severity score (ISS) and admission base deficit (BD). ISS was used as a surrogate marker for tissue damage in a patient cohort without haemorrhagic shock. BD is an accepted biomarker for the severity of shock and hypoperfusion (Baker et al, 1974, (Davis et al, 1998, (Davis and Kaups, 1998).

By excluding all patients with a raised BD, defined as BD≤2 mmol/L, a cohort of patients with isolated tissue damage (TD) was defined. A control population (C) was also defined as trauma patients who were enrolled to ACIT2, but found to have no injury or only minor injuries and a normal BD (ISS 0-4, BD≤2 mmol/L). To assess whether shock influences alarmin release, patients with critical injury load as defined by an ISS ≥ 25, were stratified according to their admission BD: ≤2, 2.1-6, 6.1-10 and >10 mmol/L. Finally, to examine the relationship between alarmin concentration and clinical outcome, the full cohort of patients was examined.

Statistical analysis was conducted using Excel 2007 (Microsoft, Redmond, USA) and Prism 5.01 (Graph Pad, CA, USA) software packages. Cytokine and alarmin concentrations were transformed into natural log values for graphical display and analysis. Tabulated data report the geometric mean (95% CI). Unless stated, ANOVA with Dunnett's post-test or Student's t test were selected and a value of $p<0.05$ chosen to represent significance.

4. RESULTS

All patients enrolled into ACIT2, between January 2008 and December 2010, were available for this study. Plasma samples for each experiment were obtained from a bio-bank of 483 patients to represent a wide spectrum of injury severity. No patient received blood or blood products prior to blood draw and the prior administration of use of crystalloid was also minimal (Table 6.1).

Table 6.1: Clinical details for the whole patient cohort

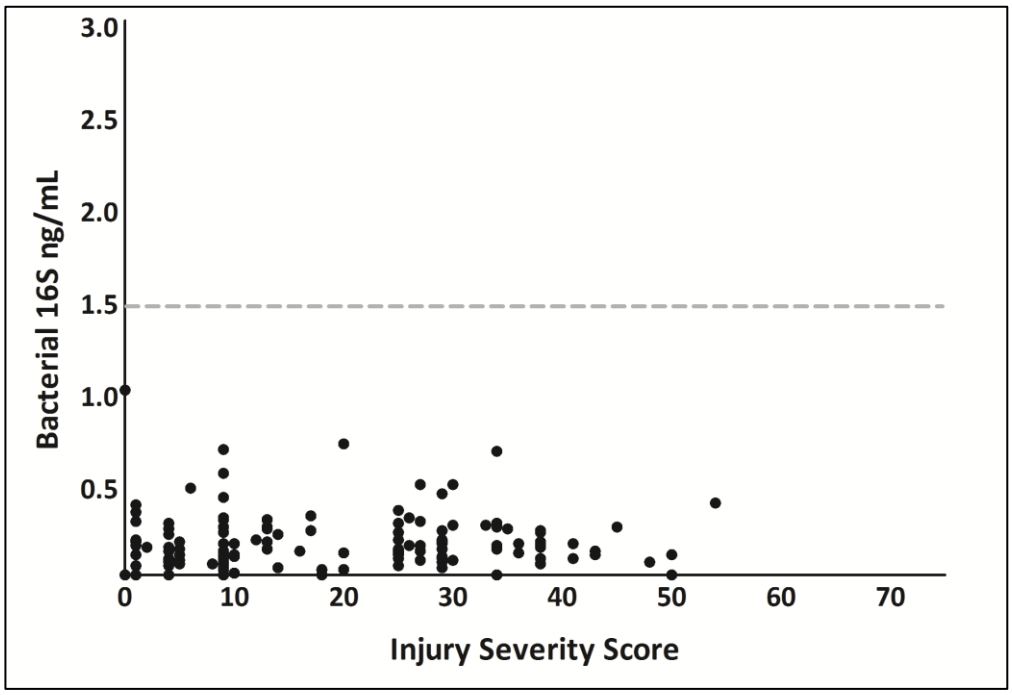
	Cytochrome B	HMGB1	Nucleosomes	p value
Demographics				
Number of patients	136	276	242	-
Age (years) ‡	32 (24-51)	34 (24-50)	34 (23-50)	0.88
Males (%)	82	82	81	0.95
Injury severity				
Blunt (%)	81	80	80	0.90
ISS ‡	13 (6-29)	13 (5-27)	14 (5-28)	0.83
ISS ≥ 25 (%)	40	36		0.66
Injury to blood draw (mins)	92 (72-115)	91 (69-116)	89 (70-115)	0.93
CSL pre-draw (mls) ‡	0 (0-500)	0 (0-500)	0 (0-500)	0.98
% > 250mls pre-draw	34	31	30	0.93
Of patients with >250mls ǂ (ml)	850 (500-1250)	600 (500-1000)	750 (500-1000)	0.85
Admission Physiology				
SBP (mmHg) ‡	135 (111-152)	132 (111-149)	132 (111-149)	0.92
Base deficit ‡	2.0 (0.2-4.7)	2.0 (0.4-4.1)	2.1 (0.7-3.9)	0.85
Outcomes				
28 day Mortality (%)	11	11	11	0.98
Length of stay (days) ‡	8 (2-18)	7 (2-19)	8 (2-19)	0.96
Culture +ve sepsis (%)	18	22	22	0.45

† Mean 95% CI, ‡ Median (IQR), Statistics use ANOVA / Kruskal-Wallis or Chi-Squared.

Primer specificity was tested prior to the investigation and results are reported in the Methodology chapter. Bacterial 16S was examined to determine the presence of bacteria in the sample. Bacteria could not be demonstrated in the patient samples, including those with open fractures (Figure 6.1). This investigation provides definitive evidence traumatic injury is a

state of sterile inflammation. It also confirms that the Cytochrome B detected in the plasma samples has been released from human mitochondria.

Figure 6.1: Bacterial 16S concentration for the trauma patient cohort



Bacterial 16S concentration was determined for all patient samples using qPCR technique. The dotted line represents the concentration of the second lowest standard on the standard curve (1.5ng/ml). There was no discernible difference in PCR cycles between the last two standards (lowest = 0.2ng/ml) and at 1.5ng/ml the curve became a plateau. No bacterial 16S could be demonstrated in the trauma patient plasma.

a) ALARMIN CONCENTRATION AND ISOLATED TISSUE INJURY

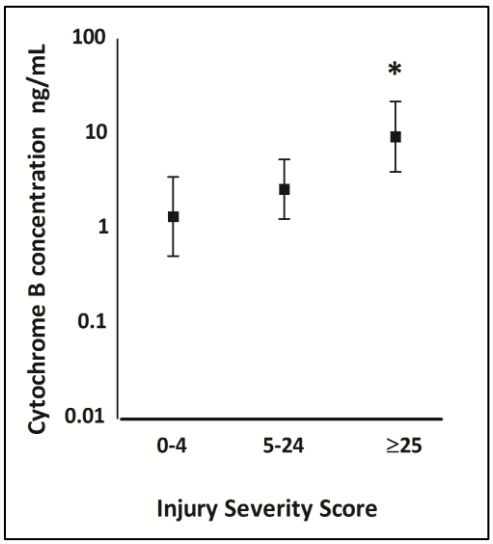
The association between isolated tissue damage and alarmin release was investigated using patients with isolated tissue injury (TD). The demographics of the cohorts are reported in Table 6.2 to demonstrate that although each alarmin candidate was measured in a different number of patients, the injury characteristics between the patient cohorts remained constant. A Control (C) population with no shock and no/minimal injury (ISS 0-4) and normal BD was used as a comparison. Patients were stratified according to their ISS and the admission (0h) alarmin concentrations examined (Figures 6.2).

Table 6.2: Patients with isolated tissue damage after traumatic injury

	Cytochrome B	HMGB1	Nucleosomes	p value
Demographics				
% of total patient cohort	68	139	116	-
Age (years) ‡	30 (23-46)	35 (23-48)	35 (23-48)	0.86
Males (%)	85	83	82	0.84
Injury severity				
Blunt (%)	81	80	78	0.17
ISS ‡	9 (5-20)	9 (4-22)	9 (4-22)	0.99
ISS ≥ 25 (%)	24	23	23	0.98
Injury to blood draw (mins) ǂ	80 (67-102)	84 (66-106)	85 (69-105)	0.86
CSL pre-draw (mls) ‡	0 (0-113)	0 (0-200)	0 (0-175)	0.72
% > 250mls pre-draw	16	19	20	0.70
Of the patients >250mls (mls) ǂ	500 (450-500)	500 (450-500)	500 (450-500)	0.99
Admission Physiology				
SBP (mmHg) ‡	141 (125-156)	138 (124-154)	139 (123-155)	0.91
Base deficit ‡	0.2 (-0.6-1.1)	0.4 (-0.7-1.4)	0.7 (-0.6-1.4)	0.42
Outcomes				
Mortality (%)	4	5	5	0.97
Length of stay (days) ‡	7 (2-13)	6 (1-12)	6 (2-13)	0.98
Culture +ve sepsis (%)	12	15	15	0.80

† Mean 95% CI, ‡ Median (IQR), Statistics use ANOVA / Kruskal-Wallis or Chi-Squared.

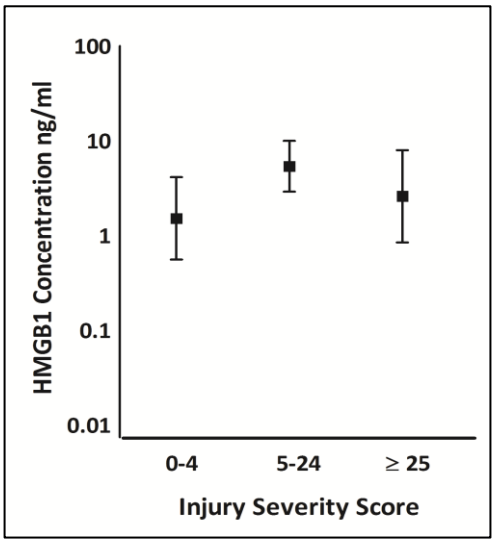
Figure 6.2: Isolated tissue injury and alarmin concentration at admission



A

A: Cytochrome B concentration at admission.

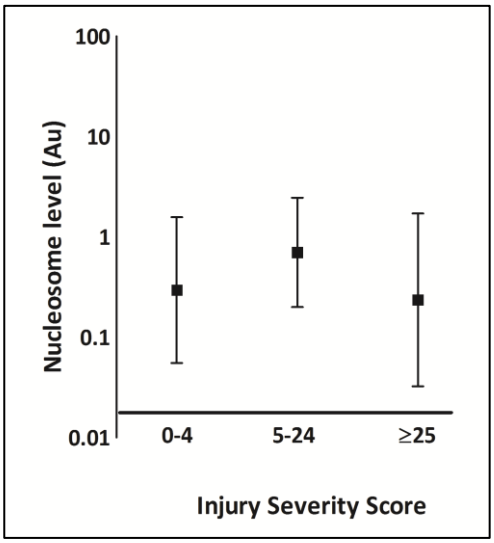
Cytochrome B demonstrated a dose-dependent relationship with ISS. Cytochrome B (n=68): 1.4(0.6-3.3), 2.6 (1.3-5.3), 9.4 (4.3-20.7), p=0.02. Data are displayed on a natural log scale as geometric means with 95% CI. Significance was tested using ANOVA with Dunnetts post-test allowing comparison of each group against the control. (* denotes p<0.05)



B

B: HMGB1 concentration at admission

HMGB1 (n=139): 1.5(0.6-4.0), 5.4(2.9-9.8), 2.6(0.9-7.6), p=0.08. Data are displayed on a natural log scale as geometric means with 95% CI. Significance was tested using ANOVA with Dunnetts post-test allowing comparison of each group against the control. (* denotes p<0.05)



C

C: Nucleosome concentration at admission.

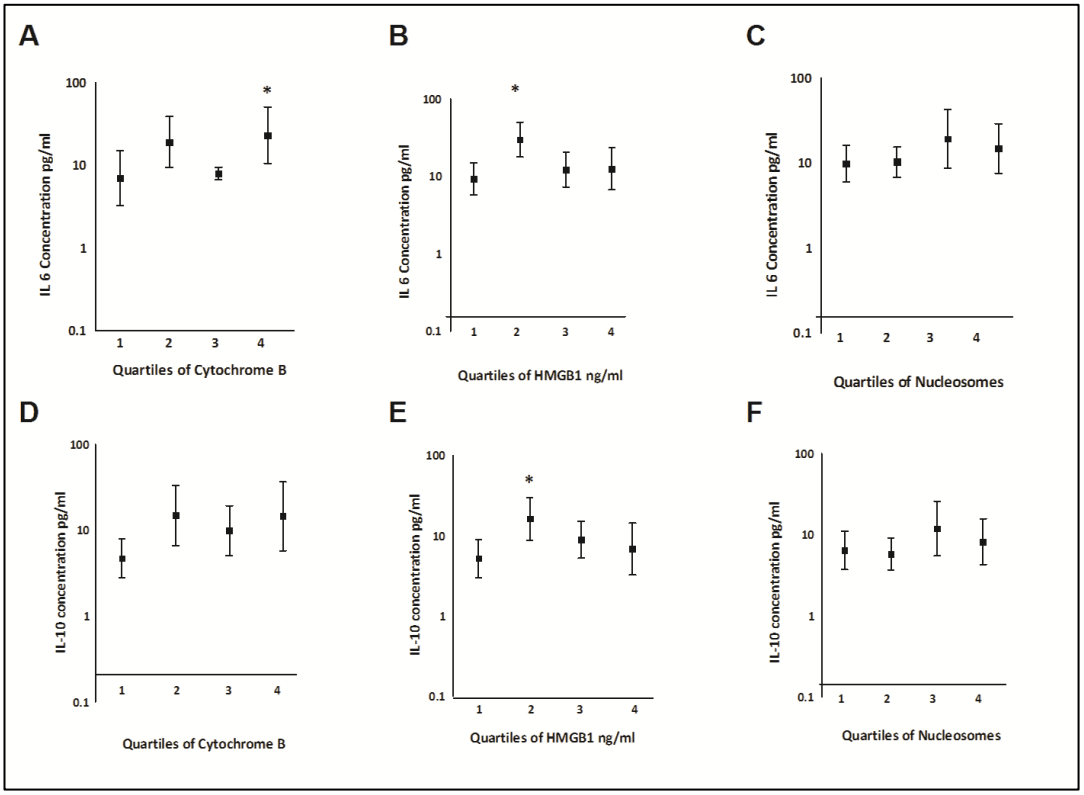
Nucleosomes (n=116): 0.3 (0.1-1.5), 0.7(0.2-2.4), 0.2 (0.0-1.6), p=0.55. Data are displayed on a natural log scale as geometric means with 95% CI. Significance was tested using ANOVA with Dunnetts post-test allowing comparison of each group against the control. (* denotes p<0.05)

The Cytochrome B concentration rose as ISS increased. Patients with an ISS ≥25 had circulating concentrations of Cytochrome B that were significantly raised above the control population. HMGB1 concentration was elevated in patients with moderate injury but not severe injury. A dose dependant relationship between injury severity and HMGB1 concentration was therefore not observed. The concentration of nucleosomes did not rise above control values, in patients with moderate or severe injury. Of the three alarmin candidates, only the circulating concentration of Cytochrome B rose as tissue damage increased.

b) ALARMIN CONCENTRATION AND INFLAMMATORY ACTIVATION AT ADMISSION

To examine the relationship between alarmin concentration and inflammatory activation, the concentrations of IL-6 and IL-10 were analysed in patients with isolated tissue injury (Figure 6.3). Elevated admission levels of both cytokines had previously been demonstrated in patients with isolated tissue injury (Chapter 4). High concentrations of Cytochrome B were associated with high IL-6 levels but the association with HMGB1 was irregular. No association between nucleosome level and cytokine concentration was observed. No robust relationship was demonstrated between the extracellular concentration of any of these alarmin candidates and the degree of inflammatory activation.

Figure 6.3: Alarmin concentration and inflammation at admission

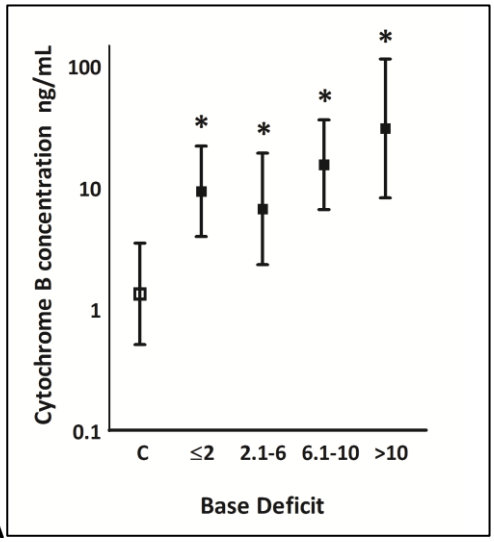


Admission cytokine concentration and admission alarmin concentration in patients with isolated tissue damage. No clear relationship between concentration of each candidate alarmin and the levels of IL-6 or IL-10 was observed. **A-C: Admission IL-6 concentration.** Alarmin: Quartile 1,2,3,4. Cytochrome B (n=68): 7.0(3.5-14.2), 19.0(9.9-36.6), 7.9(6.8-9.3), 23.0(11.2-47.6), p=0.01. HMGB1 (n=115): 9.3(5.9-14.8), 29.6(18.1-48.6), 12.2(7.5-20.0), 12.5(7.0-22.5), p<0.01. Nucleosomes (n-120): 9.8(6.1-15.8), 10.3(7.0-15.3), 19.2(9.2-40.2), 14.8(7.9-27.9), p=0.31. **D-F: Admission IL-10 concentration.** Cytochrome B (n=68): 4.7(2.9-7.6), 14.8(7.0-31.4), 9.9(5.3-18.4), 14.5(6.2-34.0), p=0.08. HMGB1 (n=115): 5.2(3.0-8.8), 16.2(9.1-28.9), 8.9(5.4-14.9), 6.9(3.4-13.8), p=0.03. Nucleosomes (n=120): 6.4(3.8-10.7), 5.7(3.7-9.0), 11.8(5.7-24.5), 8.2(4.5-15.0), p=0.33. Data are displayed on a natural log scale as geometric means (95% CI). Significance was tested using ANOVA with Dunnetts multiple comparison test allowing comparison of each group against the first quartile. (* denotes p<0.05)

c) ALARMIN CONCENTRATION AND THE INFLUENCE OF HAEMORRHAGIC SHOCK

To examine the influence of haemorrhagic shock on alarmin release, patients with critical injury levels (ISS≥25) were stratified in accordance with their BD (Figure 6.4). Concentration of cytochrome B was elevated above the control population for all critically injured patients, but levels did not rise further as shock became more pronounced. In contrast, a rise in HMGB1 levels was only observed once the BD was above the normal range and nucleosome concentration was only elevated once severe shock was present, as defined by BD >6mmol/L. cytochrome B was not augmented by shock, in contrast HMGB1 and nucleosome release did not occur in the absence of shock.

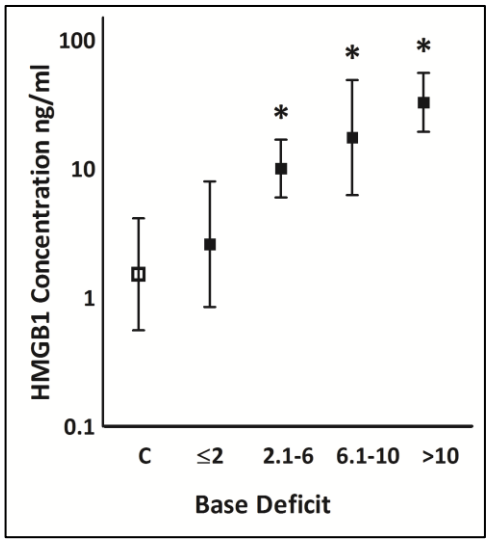
Figure 6.4: Alarmin concentration and the influence of haemorrhagic shock



A

A: Cytochrome B and base deficit in patients with critical injury load (ISS ≥25).

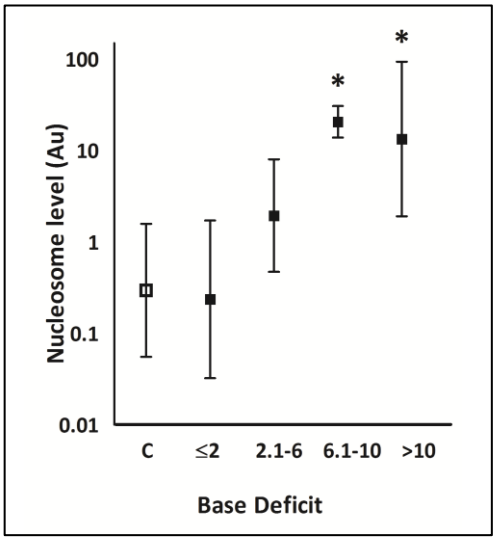
Levels of cytochrome B were elevated in all patients with severe injury. The co-existence of haemorrhagic shock did not raise levels further. Control population was defined as ISS 0-4, BD ≤2mmol/L. **C, ≤2, 2.1-6, 6.1-10, >10**: **n= 17,16, 16,21,9,7**: 1.4(0.6-3.3), 9.4(4.3-20.7), 6.8(2.5-18.2), 15.6(7.6-32.1), 31.0(10.8-88.9), p=<0.01. Data are displayed on a natural log scale as geometric mean (95% CI). Significance was tested using ANOVA with Dunnetts post-test which enables comparison of each group against the control.(* denotes p<0.01)



B

B: HMGB1 and base deficit in patients with critical injury load (ISS ≥25).

Severe injury without haemorrhagic shock was not associated with elevated levels of HMGB1. Once BD rose above the normal range, HMGB1 concentrations were significantly elevated, above that of control patients and those with isolated tissue damage. **C, ≤2, 2.1-6, 6.1-10, >10: n=24,64,32,34,18,12:** 1.5(0.6-4.0), 2.6(0.9-7.6), 10.0(6.1-16.4), 17.1(6.6-44.3), 32.5(20.3-52.1), p=<0.01.Data are displayed on a natural log scale as geometric mean (95% CI). Significance was tested using ANOVA with Dunnetts post-test which enables comparison of each group against the control.(* denotes p<0.01)



C

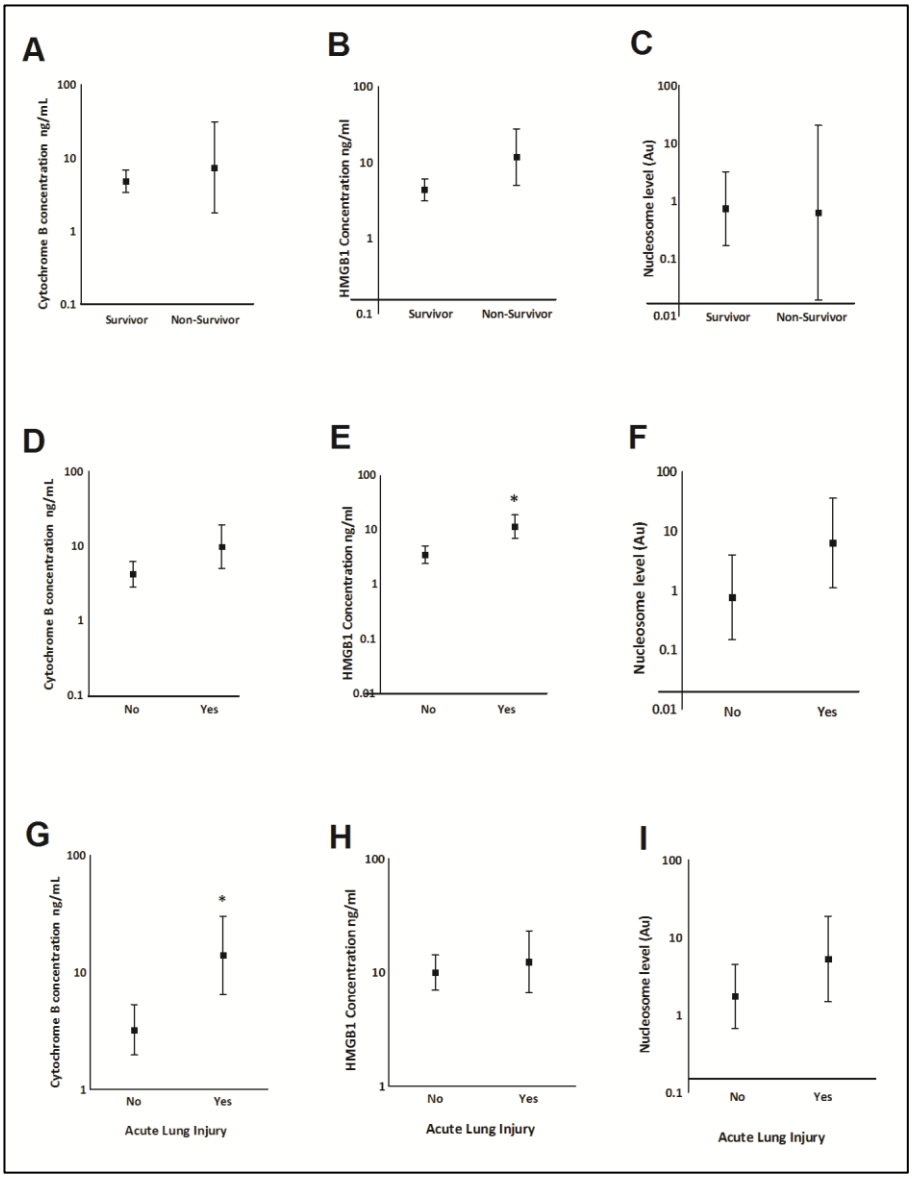
C Nucleosomes and base deficit in patients with critical injury load (ISS ≥25).

Severe injury without haemorrhagic shock was not associated with elevated levels of nucleosomes. Only once the BD rose above 6mmol/L did nucleosome levels appear to rise in the peripheral blood. **C, ≤2, 2.1-6, 6.1-10, >10**: **n=20, 64, 27,38,14,12.** 0.3 (0.1-1.5), 0.2(0.0-1.6), 1.9(0.5-7.6), 20.5(14.3-29.3), 13.3(2.4-75.1), p<0.01. Data are displayed on a natural log scale as geometric mean(95% CI). Significance was tested using ANOVA with Dunnetts post-test which enables comparison of each group against the control. (*denotes p<0.01)

d) ALARMIN CONCENTRATION AND CLINICAL OUTCOME

To determine whether the concentration of the candidate alarmins at admission could be used to predict adverse clinical outcome, all patients were assessed with respect to three adverse clinical outcomes; 28 day mortality, clinically relevant episodes of infection and development of Acute Lung Injury (as defined by a Pa02/Fi02 <40kPa/300mmHg). No alarmin candidates demonstrated an association between admission concentration and 28 day mortality, but this may be explained by the small number of deaths within the cohort (26/242, 11%). Patients who developed infections during their recovery had higher concentrations of HMGB1 in their blood at admission. Patients who developed Acute Lung Injury during their recovery had higher concentrations of Cytochrome B in their peripheral blood at admission (Figure 6.5). Retrospective SOFA score data was insufficient to determine the association with MODS analysis in this investigation. Nonetheless, peripheral blood concentration of the candidate alarmins, at admission, did demonstrate an association with some weak clinical outcome measures.

Figures 6.5: Alarmins and clinical outcomes



Admission levels of each alarmin candidate were examined against adverse clinical outcome. **A-C: 28 day mortality.** No association between admission alarmin levels and survival was identified. Alarmin: Survivor, Non-Survivor. Cytochrome B: 3.1(1.8-5.1), 2.0(0.7-5.7), p=0.45. HMGB1: 3.2(2.0-6.2), 1.7(0.1-0.2), p=0.05. Nucleosomes: 0.5(0.2-1.1), 0.0(0.0-1.8), p=0.92.**D-F: Development of infection during recovery.** Patients who developed clinically relevant episodes of infection during their recovery, had higher HMGB1 concentrations at admission. Alarmin: No Infection, Yes Infection. Cytochrome B: 2.9(1.7-5.0), 3.7(1.5-9.4), p=0.06. HMGB1: 2.5(1.5-3.7), 8.8(3.9-34.8), p<0.01. Nucleosomes: 0.3(0.1-0.9), 1.4(0.2-10.6), p=0.11. G**-I: Acute lung injury**. Patients who developed Acute Lung Injury during recovery, had higher Cytochrome B levels at admission. Alarmin: Yes ALI, No ALI. Cytochrome B: 3.2(2.0-5.2), 13.9(6.8-28.6), p<0.01. HMGB1: 10.0(7.0-14.1), 12.3(6.9-22.1), p=0.54. Nucleosomes: 1.7(0.7-4.3), 5.3(1.6-17.2), p=0.16. Data are displayed, on a natural log scale, as geometric mean with 95% CI. Significance was tested using a Students T Test. (* denotes p<0.05)

5. DISCUSSION

This study was designed to investigate the involvement of three intracellular molecules, with known inflammatory effects in-vitro, on innate immune activation after injury in humans. Our findings demonstrate that cytochrome B enters the peripheral blood after isolated tissue damage. Levels reflect the extent of injury severity and parallel admission (0h) IL-6 generation, although IL-6 concentration was not dose-dependent. The findings suggest that cytochrome B release may be a marker for tissue injury in patients without shock. Levels did not rise further in the cohort with combined blood loss and critical injury. This suggests that cytochrome B may be more dependent on tissue damage than tissue ischemia. Alternatively, cytochrome B release may already be maximal due to the severity of injury and unable to increase further.

Plasma concentrations of HMGB1 and nucleosomes were not seen to rise after isolated tissue damage but in the setting of major injury their extracellular concentrations increased in parallel with BD. Interpretation of the results is limited by the fact that the patients with haemorrhagic shock also had a higher ISS and the elevation in HMGB1 and nuclesomes may simply reflect this. Nonetheless, the findings still suggest that hypoperfusion may influence their release to a greater degree than tissue injury. HMGB1 concentrations increased as soon as the BD rose outside of the normal range, suggesting that ischaemic or hypoxic cells may release HMGB1 actively as an early danger signal. Nucleosome concentrations did not rise until shock was severe (BD >6mmol/L). These results suggest that cytochrome B may undergo passive release from mechanically damaged cells, whereas HMGB1 may be actively released by hypoxic 'stressed' cells. The finding that nucleosomes are not released until shock becomes severe suggests that there is cell death during severe shock. In addition, admission concentrations of these alarmins were associated with adverse clinical outcome in terms of development of infection and acute lung injury. The reasons for this are unclear but lend support to the concept that injured tissues release endogenous molecules which participate in the immune response.

In trauma, identifying the events which activate inflammation and participate in the transition to critical illness, is a difficult, but crucial process. This study has directly interrogated the release of intracellular alarmins after traumatic injury and correlated their release with downstream clinical inflammation and outcomes. Our sampling protocol was designed to capture the hyperacute biological events precipitated directly by injury, avoiding confounding influence from iatrogenic intervention. Trauma is a composite disease process which combines tissue injury with varying degrees of blood loss and its sequelae. It is very likely that these separate disease processes release different endogenous molecules and trigger different inflammatory responses. Many trauma patients present with isolated tissue injury and without haemorrhage or shock, yet still develop critical illness and MODS. Using the ISS and the BD, I was able to robustly categorise trauma patients into groups for analysis and investigation. Tissue damage and shock should be viewed as two separate pathological entities and patient characterisation with respect to those pathological events is essential.

The study findings, in part, contradict previous pre-clinical work; particularly, with regard to the role of HMGB1 as an alarmin in this setting. Passive release of HMGB1 has been reported during necrosis but in our study, isolated tissue damage did not result in a dose-dependent HMGB1 rise. In part, this may reflect the limitations of pre-clinical models where necrosis is induced by chemicals or freeze-thaw cycles (Scaffidi et al, 2002). Mechanical cell rupture may not lead to the same cellular events. The relationship between release of nucleosomes and severe shock is also important. Neutrophil activation may contribute to plasma nucleosome levels but the presence of neutophil NETs in trauma has not actually been demonstrated (Brinkmann et al, 2004). Nucleosomes are huge molecules that should not be able to cross biological membranes. During apoptosis, nuclear material is degraded in the cell cytoplasm (Th'ng, 2001). Extreme oxidative cell stress can however precipitate cell necrosis and rapid expulsion of cell contents (Bonfoco et al, 1995). The findings of this study suggest that severe shock may lead to cellular necrosis and expulsion of nuclear material into the extracellular

environment. This is particularly significant, given that the samples have been drawn before iatrogenic intervention and within 2h of injury.

A number of limitations must be taken into account during the interpretation of these results. Firstly, to facilitate robust patient characterisation, we used ISS and BD. In the absence of a tissue damage biomarker, scoring systems like ISS are the only available option. By subdividing patients according to their BD we can accommodate some of the confounding influence from shock. The bias of ISS toward mortality will inevitably result in some inaccuracy when used to describe tissue damage. Large numbers of patients were included in this study to ensure that these errors were minimised. In addition, a normal BD at admission does not preclude the presence of haemorrhage or the development of hypoperfusion during the resuscitation phase. BD does however demonstrate the presence of cellular hypoperfusion and so reflects cellular health at the time of blood draw. Nucleosomes were selected as a marker of histone release but are unlikely to effect the same cellular changes that unbound histones have demonstrated (Xu et al, 2009). Further investigation of histone release in this setting may be informative. Use of systems modelling would have been of benefit in this investigation to evaluate the concentration of the alarmin candidates when adjusted for ISS, BD, age, gender and time from injury. Larger patient numbers would have also been desirable.

6. CONCLUSION

Intracellular proteins are released in to peripheral blood after traumatic injury and their levels are influenced by the severity of tissue damage and blood loss sustained. On the basis of my findings, none of the three candidates measured could be recommended as a trauma biomarker, for use in the clinical setting. Nonetheless, the general concept warrants further investigation and mitochondrial proteins in particular, present a promising avenue for future study.

Mechanical tissue damage results in extracellular Cytochrome B release. Cytochrome B may have potential as a biomarker of tissue injury if larger patient numbers support our findings. The release was not dose-dependent at low injury levels and alternative mitochondrial proteins should also be considered. Extracellular HMGB1 release was related to hypoperfusion rather than tissue injury, suggesting release during hypoxia and not mechanical damage. This suggests that haemorrhagic shock may trigger danger signalling by ischaemic cells. The late appearance of extracellular nucleosomes in severe shock, suggests that necrosis may occur when hypoperfusion is extreme. This study supports the concept that tissue damage and shock lead to release of different intracellular molecules which may have mechanistic significance for subsequent biological events.

Part 4: Circulating leucocytes

CHAPTER SEVEN: HUMAN IMMUNE CELL RESPONSES AFTER TRAUMATIC INJURY

1. INTRODUCTION

Inflammation is the body's defence mechanism; it is a normal and necessary response to injury. The innate immune response delivers a rapid, non-specific, first-line defence against harm characterised by an arsenal of cytokines and chemokines and activated innate cells. The slower adaptive immune response then produces antibodies to destroy specific molecular targets (Cohen, 2002). For traumatic injury patients, the harmful stimulus is endogenous and inflammation is implicated in the pathogenesis of poor outcome; specifically, mortality, susceptibility to hospital acquired infection and development of multiple organ failure (MODS) (Sauaia et al, 1995, (Matzinger, 2002, (Zhang et al, 2010b, (Napolitano et al, 2000, (Jastrow et al, 2009, (Hranjec et al, 2010, (Sauaia et al, 1998, (Minei et al, 2012, (Chen and Nunez, 2010).

The post-traumatic inflammatory state is a condition of contradiction. In the early post injury phase, both pro-inflammatory and anti-inflammatory cytokines are produced (Jastrow et al, 2009, (Hranjec et al, 2010, (Gebhard et al, 2000). Recent genomic analysis has demonstrated that there is simultaneous activation of innate immunity and suppression of adaptive immunity within the first 12h of injury (Xiao et al, 2011b). In addition, hyper-activation of neutrophils has been described alongside reduced responses of T-cells (T-cell anergy) and monocytes (Walsh et al, 2000, (Kasten et al, 2010, (De et al, 1997, (Pellegrini et al, 2000). Although cytokines are known to be produced by immune cells, the behaviour of these immune cells and their precise role in the response to traumatic injury remains unclear. The main obstacle to the study of immune cells in humans after traumatic injury is that they are captured from the circulation, but are thought to function within the tissues. The significance of the circulating numbers is therefore hard to interpret.

Clinicians have previously concluded that adverse outcomes for trauma patients are the result of “excessive” or “persistent” state of inflammation (Giannoudis, 2003, (Tschoeke and Ertel, 2007). Others state that there is a “deregulated” or “dysfunctional” state of inflammation which leads to “immunoparalysis” (Marshall, 2001, (Tschoeke and Ertel, 2007, (Dewar et al, 2009a, (Kimura et al, 2010). A less convenient explanation may be that our understanding of the inflammatory processes after traumatic injury remains incomplete. This review was conducted to assimilate the current literature on cellular responses after traumatic injury in human subjects.

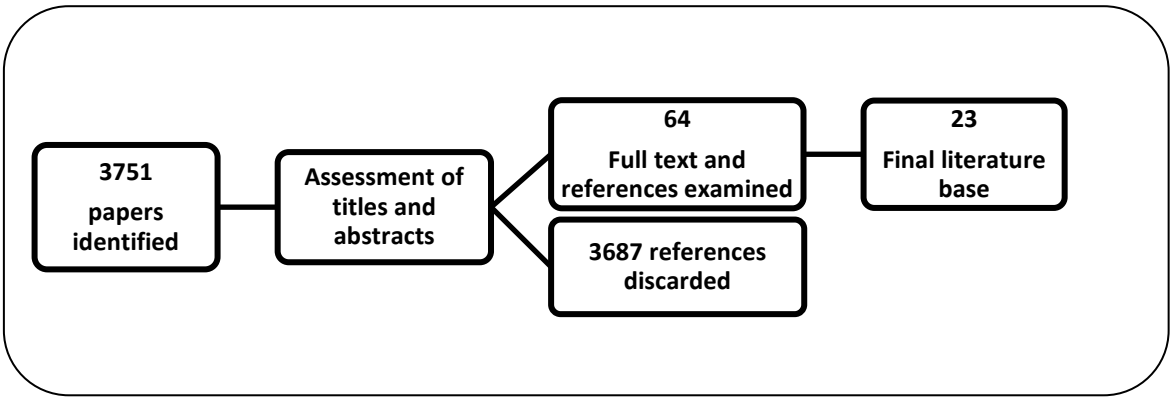
2. METHODOLOGY

To evaluate the current evidence on human immune cell responses after traumatic injury, a literature search was conducted using Medline and Embase (Box 7.1). The search was limited to English publications. No time limit was applied. Studies were then hand-searched to include only those examining traumatic injury; studies involving patients with burns or sepsis were excluded, although references from those papers were also hand searched. A total of 23 studies formed the final literature base (Figure 7.1).

Box 7.1 Search terms

Adult >16 years Human English	**AND**	Traumatic injury/ trauma[ti]/ trauma[MESH]/ blunt/ penetrating/ polytrauma/ major trauma centre/	**NOT**	Burns/ thermal injury/ head injury/ brain injury/ neurology/ brain/ neurosurgery	**AND**	immune response / immune cells/ inflammation / SIRS/ inflammatory response/ immunity,innate / lymphocytes/ neutrophils /monocytes /macrophages /T-cells /T-lymphocytes/ Natural Killer Cells/ NKTs/ Gamma Delta cells

Figure 7.1: Search strategy for review on human cellular responses



3. CELLULAR REPONSES AFTER TRAUMATIC INJURY IN HUMANS

The evidence pertaining to the cellular responses in the post-injury phase was considered according to cell type (Table 7.1).

a) NEUTROPHILS

It is well recognised that the number of circulating neutrophils in the peripheral circulation increases during the early post-injury phase. This is due to an influx of young neutrophils, identified by a lower expression of a receptor called FcγRIII (Visser et al, 2011). The elevation appears to be transient with a peak at 3h post injury and a fall by 6h (Botha et al, 1995c). Neutrophils have four phenotypes; quiescent, primed, activated and unresponsive, which can influence the type of response they generate (Fung and Silliman, 2009, (Visser et al, 2012, (Botha et al, 1995a). In trauma patients, it has been observed that a large number of neutrophils are activated and have an increase in their oxidative burst functionality (Kasten et al, 2010). In addition, large numbers of the neutrophils are primed; as evidenced by location of CD11b within lipid rafts and superoxide release (Botha et al, 1995b, (Kasten et al, 2010). High concentrations of pro-inflammatory cytokines, particularly IL-6, are thought to be responsible for neutrophil priming (Kasten et al, 2010). Similar activation occurs after sepsis and traumatic injury, suggesting that all DAMP molecules (PAMPs and alarmins) trigger similar down-stream inflammatory pathways (Visser et al, 2012).

Neutrophils have been implicated in the pathogenesis of end organ damage, particularly Acute Lung Injury (ALI) (Abraham, 2003). After injury, neutrophils migrate into tissues and are believed to cause local damage by production of cytokines, release of neutrophil elastase and the production of superoxide radicals via a respiratory burst (Tsukamoto et al). The degree of neutrophil activation has been associated with the severity of blood loss, although these findings should be interpreted with caution (van Deventer and Pajkrt, 1997, (Botha et al, 1997). Neutrophils are thought to play a significant role in the inflammatory response to

trauma and the development of MODS. Evidence to demonstrate their role is extremely limited.

b) MONOCYTES AND MACROPHAGES

The number of circulating monocytes has been shown to rise in the first 40 minutes from injury; few studies have examined beyond that point, but individual variation has been observed (Rainer et al, 1999). Most studies have focussed on monocyte function. Functional impairment of trauma patient monocytes has been characterised by an early and sustained decrease in cytokine production (Moore et al, 1990, (Spolarics et al, 2003) and reduced antigen presentation as evidenced by HLA-DR expression or mixed leucocyte reaction (MLR) (De et al, 2003). In a study of patients with critical injury (ISS≥25), those who recovered their HLA-DR expression recovered uneventfully, while those who had persistent reduction in HLA-DR expression developed infections (Cheron et al, 2010). HLA-DR expression at day 3 and 4 predicted development of sepsis with greater accuracy than traditional clinical parameters (ISS, transfusion requirements and critical illness scores) (Cheron et al, 2010). Monocyte dysfunction affects only a subset of trauma patients and is unrelated to ISS, but frequently co-exists with T-cell anergy and those affected have worse organ failure (De et al, 1997, (De et al, 2003)

The mechanisms underlying the reduced antigen presentation capacity (APC) are not fully understood but a 'differentiation defect' may play a part (De et al, 2003). Monocytes differentiate into macrophages or dendritic cells depending on environmental stimuli; macrophages have lower APC capabilities. One study demonstrated that in 34% of the trauma patients examined, monocytes preferentially developed into macrophages rather than dendritic cells. This was attributed to the influence of inflammatory mediators, specifically IL-6 which favour macrophage development (Chomarat P, 2000). The susceptibility was not dependent upon total IL-6 levels but the propensity of the monocyte to decrease CD126

expression (De et al, 2003). Animal models have demonstrated that monocytes play a fundamental role in the development of end-organ damage and the development of MODS (Seitz et al, 2010). Human clinical investigations also clearly demonstrate the importance of functional monocytes in the recovery after traumatic injury (Cheron et al, 2010). The factors which govern the phenotype and functional responses of these important cells in the post-injury phase remain unclear.

Table 7.1: Summary of the literature base regarding cellular responses in human trauma patients

Cell Type	Number	Function	1st sample	Related to adverse outcome	References
Neutrophils	↑	↑	3h post injury to D4	Yes	(Kasten et al, 2010, (Botha et al, 1995c, (Abraham, 2003, (Visser et al, 2011)
Monocytes	↓	↓	Admission-D7	Yes	(Cheron et al, 2010, (De et al, 2003, (Yadav et al, 2009)
Lymphocytes (all)	↓	-	Admission - <24h	Yes	(Cheadle et al, 1993, (Walsh et al, 2000, (Ditschkowski et al, 1999)
T-helper cells (CD4)	↓	↓	< 5h from injury	Yes	(Yadav et al, 2009, (Cheadle et al, 1993, (Kasten et al, 2010, (Walsh et al, 2000)
Cytotoxic T Cells (CD8)	↓	↓	Admission - D4	No	(Mrakovcic-Sutic et al, 2010, (Kasten et al, 2010, (Walsh et al, 2000)
Regulatory T Cells)	↔ ↓**(TBI)**	-	D1-D7	No	(Yadav et al, 2009, (Mrakovcic-Sutic et al, 2010)
NK T Cells	↓	-	D1-D4	No	(Mrakovcic-Sutic et al, 2010)
Gamma Delta T Cells (GD)	↓	-	Admission	No	(Walsh et al, 2000)
Natural Killer Cells (CD56)	↓	-	Admission	No	(Walsh et al, 2000, (Ditschkowski et al, 1999)
B Cells	↑	↓**HLA DR**	< 5h from injury	No	(Yadav et al, 2009, (Walsh et al, 2000, (Ditschkowski et al, 1999)

c) T- LYMPHOCYTES

Reduced numbers of T-lymphocytes, after traumatic injury, have been reported in several studies. Principally, this has referred to reduced numbers of naive CD4 and CD8 T-cells. The most significant finding from T-cell studies after trauma has been the discovery of impaired T-cell function or T-cell anergy (De et al, 2000). This state of functional impairment is defined by reduced IFNγ or IL-2 production after mitogen stimulation and reduced T-cell receptor (TCR) mediated signalling (Kasten et al, 2010, (De et al, 1997). In one study of severely injured patients, 46 with mechanical injury and 15 with >30% burns, T-cell anergy was identified in 69% of the cohort (Bandyopadhyay et al, 2007). In another mixed cohort, 52 trauma patients and 18 patients with more than 30% burns, anergy was identified in 34% of the cohort (Laudanski et al, 2006). These studies suggest that there is a subset of trauma patients who develop T-cell anergy after injury (De et al, 1997). The true incidence of T-cell anergy, how to identify patients with anergy and how it influences recovery after traumatic injury are unclear.

Massive naive T-cell apoptosis has been postulated to be the root cause of the anergic T cell response, because reduced numbers of T-cells impair the effectiveness of the adaptive immune response (Kasten et al, 2010). Changes within the individual T-cells may also hinder their reaction during the post-traumatic injury phase. Up-regulation of co-repressor molecules such as CD47, PD-1 and CD152 has been observed, suggesting that negative intracellular signalling may lead to T-cell anergy (Bandyopadhyay et al, 2007). Aberrant monocyte function has been shown to co-exist with T-cell anergy (Laudanski et al, 2006). Impaired monocyte-induced IL-13 production by T-cells, suggests a synergistic relationship between the two cell types (Laudanski et al, 2006).

T-cell anergy is independent of injury severity score (Kasten et al, 2010). A relationship between low lymphocyte count after traumatic injury and poor clinical outcome has been reported, but remains to be clearly demonstrated (Cheadle et al, 1993, (Heffernan et al, 2012). Lymphocyte function and phenotype have not been studied in sufficient detail to find association with clinical outcome (Bandyopadhyay et al, 2007, (Walsh et al, 2000). Simultaneous reduction in monocyte and T-cell function has been associated with worse organ failure scores (Laudanski et al, 2006). Impaired number or function of lymphocytes appears to influence trauma patient recovery, but a greater understanding of these complex mechanisms is required.

i. T helper cell subsets

Very few trauma studies have examined the responses of T-lymphocyte subsets. Naive T cells (Th0) are able to differentiate into one of two known T helper subsets, Th1 and Th2. Differentiation is influenced by exposure to cytokines in the environment. IL-12 and IFNγ favour Th1 lineage development, while IL-4 favours Th2 lineage. Together these two cell groups balance the cellular and humoral immune responses (Miller et al, 2007). Some investigators have suggested that traumatic injury predisposes patients to a Th2 response which renders them at risk for MODS and sepsis (O'Sullivan et al, 1995). Subsequent work has suggested that the boundaries between these responses are far less distinct (Heizmann et al, 2008). T-helper subsets have been observed to fall in number after injury, with a trough on day 3 which was associated with poor outcome (Cheadle et al, 1993). The precise role of T-helper cells in the response to traumatic injury remains unclear.

ii. Regulatory T cell subsets

The role of regulatory T-cells (Tregs), defined by a CD4+ CD25+ phenotype in traumatic injury has been considered. Treg (CD4+ CD25+) populations have been reported to remain at control levels immediately after injury and subsequently elevate by day seven (MacConmara et al, 2006). The clinical significance of this observation is unknown.

iii. Cytotoxic T cell subsets

In a study of trauma patients with severe traumatic brain injury, diminished numbers of cells with a cytotoxic phenotype (CD8, NK & NKT cells) were observed. The authors concluded that this may be responsible for the high number of extra-cranial infections to which the patients succumbed (Mrakovcic-Sutic et al, 2010). A reduction in intracellular expression of the cytotoxic protein perforin was also demonstrated in these lymphocytes; this may impair their cytotoxic function (Sotosek Tokmadzic et al, 2010).

iv. Gamma Delta T cell subsets

Gamma-delta T cells (γδ T cell) have been examined in humans with traumatic injury, in a small study involving 8 human subjects sampled at 67±18h after injury (Tschop et al, 2008). This study demonstrated a fall in absolute number of γδ T cells by 47% compared to healthy controls, which was attributed to apoptosis. In the same investigation, murine models then demonstrated that γδ T cells recruit neutrophils, regulate IFNγ production and mediate increased survival after sepsis (caecal ligation and puncture). Although they form only a small subset of the lymphocyte population, γδ T cells may well play important roles in critical illness and more work is required to understand their function.

v. Natural Killer (NKT) T cells sub populations

This newly discovered subset of T cells was only described in 1987. They are characterised by their display of both a TCR receptor and a natural killer cell marker; CD56 in humans and NK1.1 in mice hence the nomenclature (Godfrey et al, 2000). They are estimated to form less than 0.2% of the lymphocyte population, but along with gamma delta T cells, they are thought to have regulatory influence over the innate immune response in critical illness (Venet et al, 2008). No studies have examined their role in the inflammatory response to traumatic injury to date.

d) NATURAL KILLER CELLS

The behaviour of Natural Killer (NK) cells ($CD3^-$ $CD56^+$) after traumatic injury has only been examined by a handful of investigations. Most of these studies were conducted over ten years ago. Some report that the number of circulating NK cells decreases after injury; another that the numbers remain unchanged (Walsh et al, 2000, (Grob et al, 1988, (Joshi et al, 1998). Impairment of NK Cell function has been described in terms of reduced cytotoxicity (Hauser et al, 1997). The reason for this observation was postulated to involve aberrant interaction between NK cells and other immune cells or inflammatory mediators, but the precise mechanism and the implication for clinical recovery from trauma is unknown (Joshi et al, 1998).

e) EOSINOPHILS, MAST CELLS AND BASOPHILS

Eosinophils, Mast cells and Basophils have a phagocytic function. In addition mast cells degranulate upon activation, releasing histamine and other vasoactive products. Elevated histamine concentrations have been reported in blood samples from septic patients (Neugebauer et al, 1996). Their role in traumatic injury has not been studied.

4. LIMITATIONS OF THE CURRENT EVIDENCE

Many of the limitations discussed in the cytokine review also hold true for the studies investigating immune cell function in traumatic injury patients.

a) TIMING OF BLOOD SAMPLES

The principal limitation is the timing of the blood samples for investigation. The first blood samples were drawn anywhere between 'arrival', '<3h from injury' and 'within the first week of admission' (Botha et al, 1997, (Chen et al, 2009). Most samples were drawn in the first 24h but in some studies, samples were deliberately drawn on the second day of admission to avoid confounding from resuscitation (Bandyopadhyay et al, 2007, (Laudanski et al, 2006). Only four studies examined the inflammatory response at admission and only one of those highlighted that the blood was taken before intervention (Kirchhoff et al, 2009, (Yadav et al, 2009, (Walsh et al, 2000, (Botha et al, 1997). It has already been demonstrated that the time from injury influences the inflammatory response and the variation between these studies makes it difficult to draw firm conclusions from their results.

b) SMALL NUMBER OF STUDIES

The number of studies which have examined immune cell responses after traumatic injury in the last ten years is small. Clinical trauma care practices, particularly with regard to resuscitation, have changed so much that investigations conducted before 2004 are very unlikely to reflect the patient illness profile that we see now. Several of the investigations discuss mechanical trauma and thermal burns interchangeably. We now recognise these as two very different pathological processes (Polk et al, 1986, (Miller-Graziano et al, 1995, (Laudanski et al, 2006). This results in a small total of publications, concerning lymphocytes, neutrophils or monocytes and their roles in the immune response to traumatic injury.

5. FINAL COMMENTS

Evaluation of the current literature concerning the immune cell responses to traumatic injury demonstrates that the volume of literature pertaining to this subject is small and that the responses are complex and still incompletely understood. A number of questions about the human immune response to injury therefore remain unanswered. It remains unclear whether it is possible to mount an 'ideal' immunological response to traumatic injury or conversely, a 'bad' one. Investigation of the cellular responses requires careful study design, standardised timing of samples and good clinical outcome data but, if that can be achieved, we may be able to advance our understanding of the immune response to traumatic injury.

CHAPTER EIGHT: LEUCOCYTE RESPONSES TO TRAUMATIC INJURY

I am very grateful to Dr Paul Vulliamy MBBS BSc MRCS for obtaining the white cell differential counts for the ACIT2 patients from our electronic hospital records system and compiling the database for this analysis.

1. INTRODUCTION

The outcome for an individual after traumatic injury is influenced by their immunological response (Napolitano et al, 2000). How that immunological response is activated and why it affects trauma recovery is unknown. A number of cell types co-ordinate to action the immune response. The relationship between these cells and clinical outcome after trauma is unclear.

Immune cell responses can be considered in terms of number in circulation or function, either in circulation or in tissues. Previous research has highlighted a raised number of circulating neutrophils after injury and implied a role for neutrophils in the pathogenesis of organ damage (Botha et al, 1995b, (Botha et al, 1995c). In one study, reduced numbers of lymphocytes in the post-injury phase have been observed in association with development of infection and higher mortality; their functional role is unknown (Cheadle et al, 1993). Monocytes increase in number after trauma but decrease in function (Moore et al, 1990, (Rainer et al, 1999). Finally, a subgroup of trauma patients appears to develop lymphocyte anergy, sometimes in combination with reduced monocyte function (De et al, 1997, (Kasten et al, 2010). Improving our understanding of the cellular responses to traumatic injury and the factors which control them is essential.

2. THE AIM OF THIS INVESTIGATION

This was an exploratory study designed to examine the relationship between differential white cell count and clinical outcomes after traumatic injury. The primary aim was to demonstrate whether the number of immune cells circulating in peripheral blood after injury is influenced by injury severity. The second aim was to examine the relationship between early immune cell count and clinical outcome.

3. METHODOLOGY

The investigation was conducted using 300 patients recruited to the ACIT2 cohort study who had SOFA scores prospectively collected during daily follow-up. Differential white blood cell (WBC) counts from the day of admission until day 7 of the inpatient stay were retrieved from our electronic patient records system. As the need for blood draw was determined by the clinical team, not all patients were bled daily. Conversely, if patients had several blood tests during a day, only one value was recorded per day. In these cases the values selected were as near to routine phlebotomy time as possible, usually between 4-8am. Since episodes of clinical deterioration in hospital almost always result blood draw for analysis, the clinical condition of the patient may have some confounding influence over blood sample timing. The differential count included: total white cell population, neutrophils, monocytes, lymphocytes and eosinophils. The method by which these results are obtained is explained in the Methodology (Chapter 2).

a) DATA ANALYSIS

In keeping with the previous investigations, injury characteristics were described using the injury severity score (ISS), base deficit (BD), time from injury to blood draw, gender and age. Immune cell counts were correlated with a number of different clinical outcome measures: 28-

day mortality, length of hospital stay (LOS), development of infection and development of Multiple Organ Dysfunction Syndrome (MODS). A control group was formed from patients with no injury/minor injury (ISS 0-4) and a BD <2mmol/L.

For comparison of LOS, two groups were defined. The whole cohort was divided into quartiles according to their LOS. Those patients whose LOS was above the 75th percentile were defined as 'extended' LOS; in this case admission >20 days. Those patients whose LOS was ≤20 days were defined as 'normal' LOS. This is an accepted definition for large population studies (Almoudaris et al, 2012). Infection was defined using the Centre for Disease Control and Prevention (CDC) criteria as a 'localised or systematic condition resulting from an adverse reaction to the presence of an infectious agent(s) or its toxin(s) occurring 48 hours post admission' (Horan et al, 2008). MODS was defined as a SOFA Score of ≥5, on two or more consecutive days, at least 48h after admission. These conditions were specified because a) single organ failure can score a maximum of 4 points on SOFA and b) organ injury seen within the first 48h is frequently related to the injury and resuscitation process and can quickly resolve (Minei et al, 2012). Acute lung Injury (ALI) was also examined as a component of MODS and defined using standard criteria of a Pa02/Fi02 ratio <40kPa (Bernard et al, 1994). ALI was only confirmed if a ratio <40kPa was present for two or more consecutive days, more than 48h from admission.

Data analysis and construction of graphs were performed using Excel 2007 (Microsoft, CA) and Prism 5.01 (Graph Pad, CA). Data were tested for normality, using a histogram and Z-scores, and proven to be normally distributed. Data are reported as a mean with 95% confidence intervals (CI) and tested using Students Test. $p<0.05$ was chosen to represent significance.

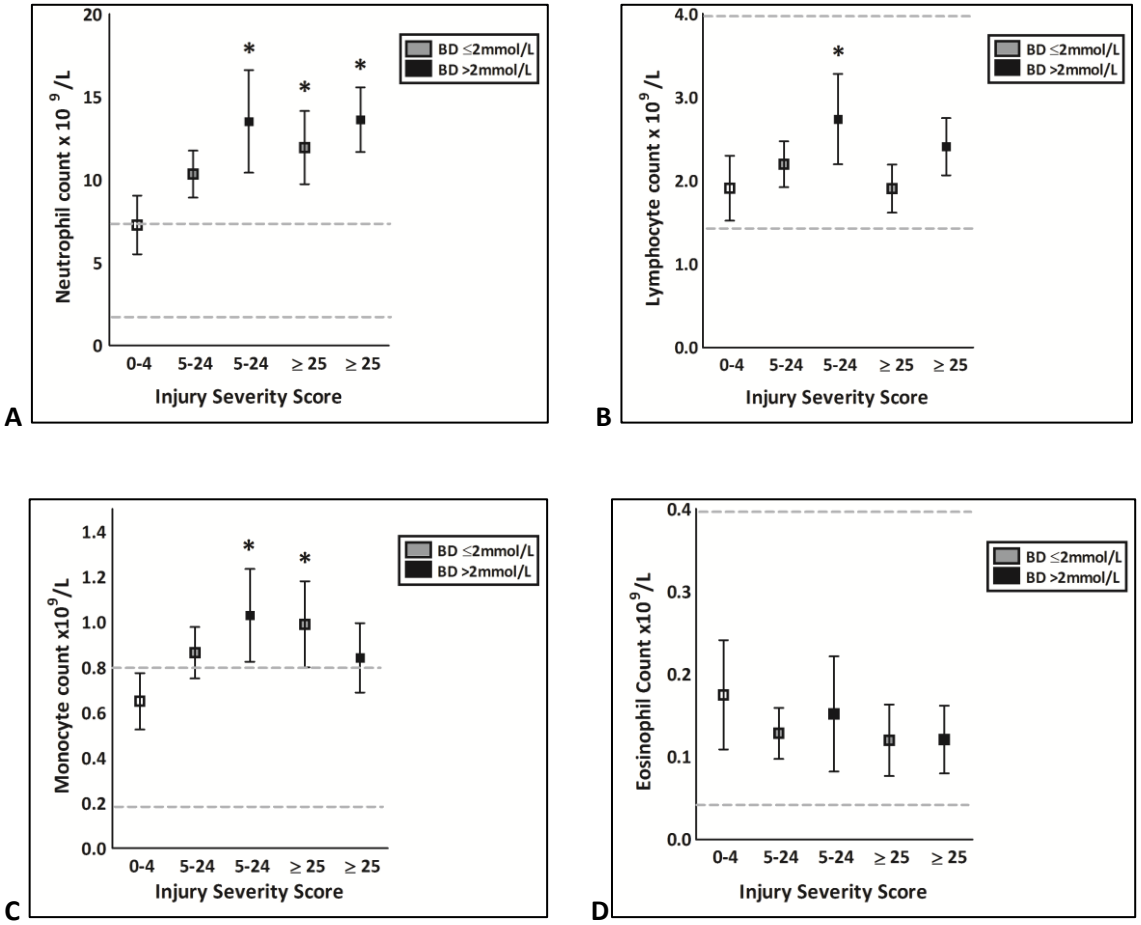
4. RESULTS

From the study cohort of 300 ACIT2 patients, those who had >2L of crystalloid or blood transfusion products prior to blood draw were retrospectively excluded to reduce confounding influence from iatrogenic intervention. A total of 245 patients were included in this investigation. A wide range of cell counts for neutrophils, lymphocytes and monocytes was observed at admission and throughout the first seven days. To determine the clinical significance of this variation, the relationship with injury severity and clinical outcome was examined.

a) IMMUNE CELL COUNT AND INJURY SEVERITY

Patients were stratified according to their ISS and admission BD (Table 8.1). Cell counts, at admission, for each of the four immune cell types were then examined. All injured patients demonstrated neutrophil counts above the upper limit of normal. Neutrophil counts rose with increasing injury severity (Figure 8.1). Patients with haemorrhagic shock, as described by a BD>2mmol/L, had slightly higher neutrophil levels than patients with a normal BD, but this did not reach significance. All patients, at admission, had lymphocyte and eosinophil counts within the normal range. Almost all injured patients had a monocyte count above the normal range. Monocyte count also rose as severity of injury increased; however, the most severely injured patients (ISS≥25 and BD>2mmol/L) had lower monocyte counts than the moderately injured patients. These findings demonstrate that both neutrophils and monocytes are mobilised into the peripheral circulation early after traumatic injury. They also suggest that monocyte mobilisation into circulation may be adversely influenced by critical levels of injury (ISS ≥25) or the addition of haemorrhagic shock.

Figure 8.1: Admission white cell count and injury severity



Patients were categorised by ISS and their admission white cell differential counts examined. ISS categories: 0-4 (n=24), 5-24 No Shock (n=60), 5-24 raised BD (n=27), ≥25 No Shock (n=30), ≥25 raised BD (n=48). **A-Neutrophils**: 7.3 (5.6-9.0), 10.3(8.9-11.7), 13.5 (10.6-16.5), 11.9 (9.8-14.1), 13.6 (11.7-15.5), p<0.01. **B-Lymphocytes:** 1.9 (1.5-2.3), 2.2 (1.9-2.5), 2.7 (2.2-3.3), 1.9 (1.6-2.2), 2.4 (2.1-2.7), p 0.02. **C-Monocytes:** 0.7 (0.5-0.8), 0.9 (0.8-1.0), 1.0 (0.8-1.2), 1.0 (0.8-1.2), 1.0 (0.7-1.0), p=0.04. **D-Eosinophils:** 0.2 (0.1-0.2), 0.1 (0.1-0.2), 0.2(0.1-0.2), 0.1 (0.1-0.2), 0.1 (0.1-0.2), p=0.50. Data are presented as mean (95%CI) and * denotes p<0.05 using ANOVA with Dunnetts Post Test allowing comparison between each group and the control. Grey dotted lines demonstrate the normal range.

Table 8.1: Demographics of the analysis cohorts for injury severity and immune cell count

ISS category	n	% male	% blunt	Age	ISS	BD	Time (mins)	LOS	Infections (%)	Mortality (%)
0-4	26	92	69	36 (27-45)	1 (1-2)	-0.7 (-1.9- -0.03)	67 (60-82)	2 (1-7)	0	0
5-24 NS	60	80	82	37 (20-50)	10 (9-17)	-0.7 (-1.6 - 0.8)	75 (57-88)	8 (2-20)	30	3
5-24 S	27	85	63	32 (24-50)	13 (9-18)	4.3 (3.3-5.9)	85 (64-97)	13 (8-19)	33	4
≥ 25 NS	30	90	97	37 (29-53)	29 (26-36)	1.1 (-0.2-1.6)	79 (66-93)	11 (6-38)	57	10
≥ 25 S	48	81	94	40 (24-52)	34 (29-41)	5.7 (3.3-9.5)	91 (68-109)	19 (7-38)	54	38

n = number of patients, Time refers to the time between injury and blood sample, LOS = Length of stay, NS = non-shocked Base deficit≤ 2mmol/L, S=Shocked=base deficit >2mmol/L

b) IMMUNE CELL COUNT AND CLINICAL OUTCOME

The relationship between four selected clinical outcomes and the peripheral blood immune cell count during the first seven days of admission was examined.

i. 28 day mortality

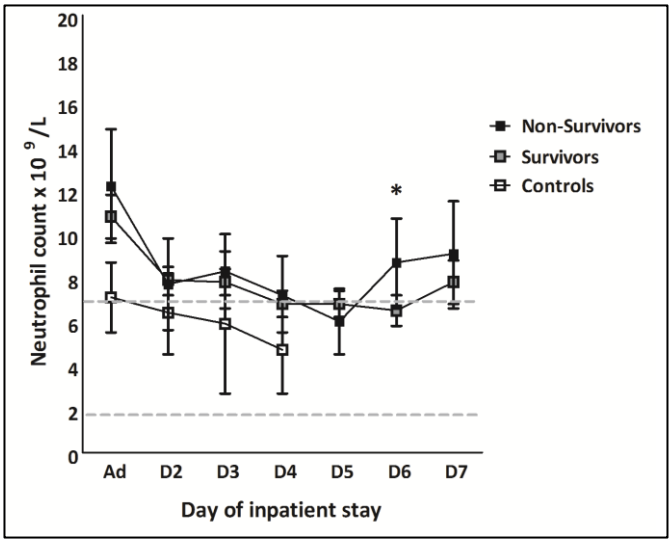
No difference in neutrophil count was demonstrable between survivors and non-survivors, until day 6, when elevated neutrophil levels were observed in the non-survivors cohort. In contrast, lymphocyte and monocyte count demonstrated significant differences between survivors and non-survivors on day 3, roughly 48h after admission. Lymphocyte count and monocyte count were both significantly lower on day 3 and day 4 in trauma patients who died before 28 days. Eosinophil levels were observed to fall to negligible levels at 24h from admission. This was significantly different when compared to the control patients but no difference between the two clinical outcome groups was observed (Table 8.2 & Figure 8.2).

Table 8.2: Demographics of the cohorts for 28 day mortality analysis

Demographics	**Controls (ISS 0-4)**	**Survivors**	**Non-survivors**	**p value**
Number of patients	26	166	26	-
% Male	92	81	69	0.07
Age ‡	36 (27-45)	36 (24-50)	45 (24-66)	0.07
Injury Severity ‡				
ISS	1 (1-2)	18 (9-29)	34 (26-41)	**<0.01**
% Blunt	69	77	96	**<0.01**
SBP	137 (124-150)	131 (114-149)	104 (98-151)	0.38
Base Deficit	-0.7 (-1.9 - -0.03)	1.2 (0.8-3.9)	6.3 (2.5-15.8)	**<0.01**
Time from injury	67 (60-82)	80 (62-95)	92 (66-101)	0.98
CSL prior to blood draw	0 (0-0)	0 (0-363)	250 (0-688)	**<0.01**
No (%) pts >250 ml pre-draw	0	44 (27)	13 (50)	**<0.01**
Of the pts with >250mls	0 (0-0)	500 (500-750)	750 (500-1000)	0.07
AIS Head & Neck	0 (0-0)	0 (0-3)	4 (3-5)	**<0.01**
AIS Face	0 (0-0)	0 (0-0)	0 (0-2)	0.1
AIS Thorax	0 (0-0)	3 (0-4)	4 (2-4)	0.07
AIS Abdo & Pelvis	0 (0-0)	0 (0-2)	0 (0-2)	0.43
AIS Extremity & Pelvis	0 (0-0)	2 (0-3)	0 (0-3)	0.49
Outcomes				
Length of Stay ‡	2 (1-7)	13 (6-30)	3 (1-11)	**<0.01**
MODS (%)	0	16	44	**<0.01**
Acute Lung Injury (%)	0	30	44	0.06
Infections (%)	0	38	44	0.47
Neutrophils †				
Admission	7.1 (5.4-8.7)	10.8 (9.8-11.8)	12.2 (9.6-14.8)	0.31
D2	6.9 (5.0-8.8)	7.9 (7.2-8.5)	7.7 (5.6-9.8)	0.83
D3	5.2 (2.8-7.6)	7.8 (7.2-8.4)	8.3 (6.6-10.0)	0.54
D4	4.9 (3.5-6.3)	6.8 (6.2-7.4)	7.2 (5.5-9.0)	0.65
D5	-	6.8 (6.2-7.4)	6.0 (4.5-7.5)	0.3
D6	-	6.5 (5.8-7.2)	8.7 (6.6-10.7)	0.03
D7	-	7.8 (6.8-8.8)	9.1 (6.6-11.5)	0.33
Lymphocytes †				
Admission	2.0 (1.6-2.3)	2.4 (2.2-2.6)	2.4 (1.9-2.9)	0.99
D2	1.7 (1.4-2.0)	1.2 (1.1-1.3)	1.1 (0.6-1.6)	0.65
D3	1.9 (1.6-2.2)	1.1 (1.0-1.2)	0.7 (0.5-0.8)	**<0.01**
D4	1.5 (1.3-1.7)	1.1 (1.0-1.2)	0.6 (0.4-0.7)	**<0.01**
D5	-	1.0 (0.9-1.1)	0.7 (0.6-0.9)	0.09
D6	-	1.1 (1.0-1.3)	0.9 (0.8-1.1)	0.28
D7	-	1.2 (1.1-1.3)	1.0 (0.7-1.3)	0.23
Monocytes †				
Admission	0.6 (0.5-0.8)	0.9 (0.8-1.0)	0.8 (0.6-1.0)	0.37
D2	0.8 (0.6-1.1)	0.9 (0.8-1.0)	0.7 (0.4-0.9)	0.11
D3	0.9 (0.5-1.4)	0.8 (0.7-0.9)	0.5 (0.3-0.6)	**<0.01**
D4	0.8 (0.5-1.1)	0.7 (0.6-0.8)	0.4 (0.3-0.6)	0.02
D5	-	0.8 (0.7-0.9)	0.6 (0.4-0.8)	0.17
D6	-	0.9 (0.8-1.0)	0.9 (0.6-1.2)	0.87
D7	-	1.0 (0.9-1.2)	0.9 (0.7-1.2)	0.52
Eosinophils †				
Admission	0.17 (0.11-0.23)	0.2 (0.1-0.2)	0.1 (0.1-0.1)	0.18
D2	0.14 (0.02-0.26)	0.0 (0.0-0.1)	0.1 (0.0-0.1)	0.15
D3	0.08 (0.01-0.15)	0.1 (0.1-0.1)	0.1 (0.0-0.1)	0.5
D4	0.20 (-0.01-0.41)	0.1 (0.1-0.2)	0.1 (0.0-0.1)	0.4
D5	-	0.2 (0.2-0.3)	0.1 (0.0-0.2)	0.17
D6	-	0.2 (0.2-0.3)	0.1 (0.0-0.2)	0.1
D7	-	0.2 (0.2-0.3)	0.1 (0.0-0.3)	0.15

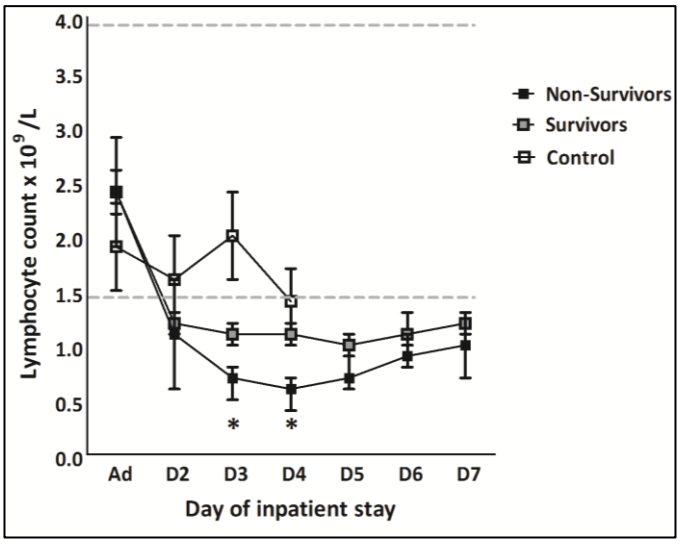
‡Median (IQR), †Mean (95% CI), CSL = Crystalloid, AIS = Abbreviated Injury severity score
Bold denotes p<0.01 on t-test between survivor and non-survivor

Figure 8.2: Immune cell count and 28 day mortality



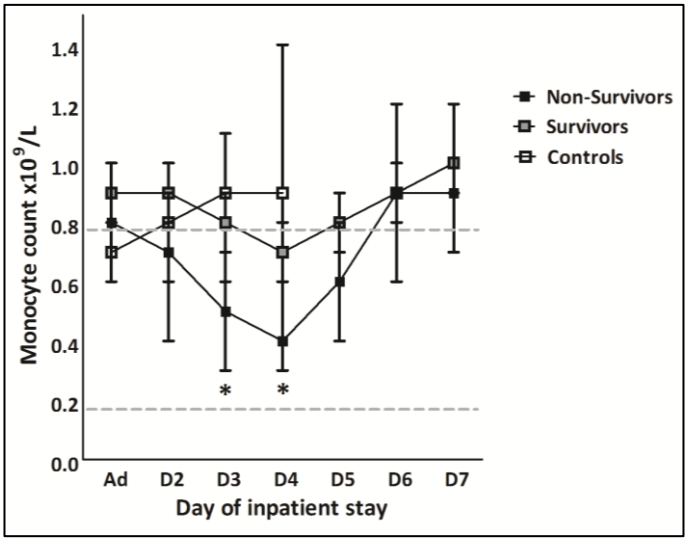
A: Seven day trend of neutrophil count according to 28 day mortality

Neutrophil count on day 6 demonstrated a significant difference between survivors(n=154) and non-survivors (n=25). Dotted line demonstrates the normal range. D6: S=6.5 (5.8-7.2), NS=8.7 (6.6-10.7), 0.03. Data are presented as mean (95% CI). *denotes p<0.05 when survivors are compared to non-survivors, Students T Test.



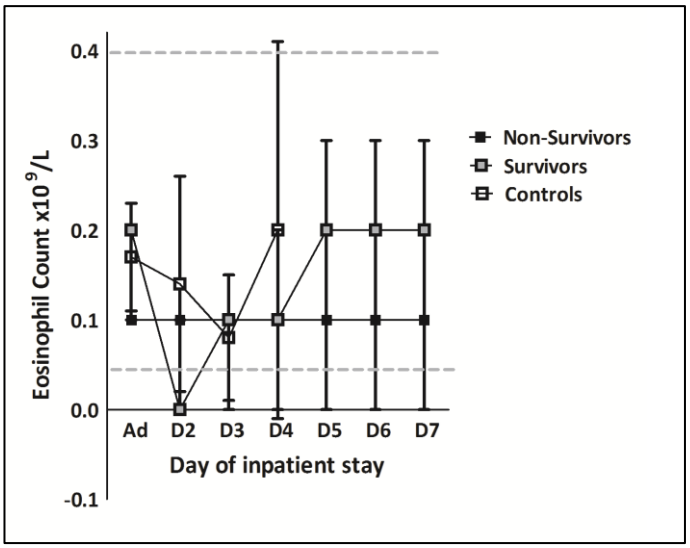
B: Seven day trend of lymphocyte count according to 28 day mortality

A fall in lymphocyte count at D2 (24h from injury) was observed in virtually all injured patients. On day 3 and day 4, the lymphocyte count in non-survivors (n=25) was significantly lower than survivors (n=154). Dotted line demonstrates the normal range. D3: S=1.1 (1.0-1.2), NS=0.7 (0.5-0.8), p<0.01. D4: S=1.1 (1.0-1.2), NS=0.6 (0.4-0.7), p=0.02. Data are presented as mean (95% CI). * denotes p<0.05 when survivors are compared to non-survivors, Students T Test.



C: Seven day trend of monocyte count according to 28 day mortality

Monocyte count on D3 and D4 demonstrated a significant difference between survivors (n=154) and non-survivors (n=25). Dotted line demonstrates the normal range. D3: S=0.8 (0.7-0.9), NS=0.5 (0.3-0.6), p<0.01. D4: S=0.7 (0.6-0.8), NS=0.4 (0.3-0.6), p=0.02. Data are presented as mean (95% CI). * denotes p<0.05 when survivors are compared to non-survivors, Students T Test.



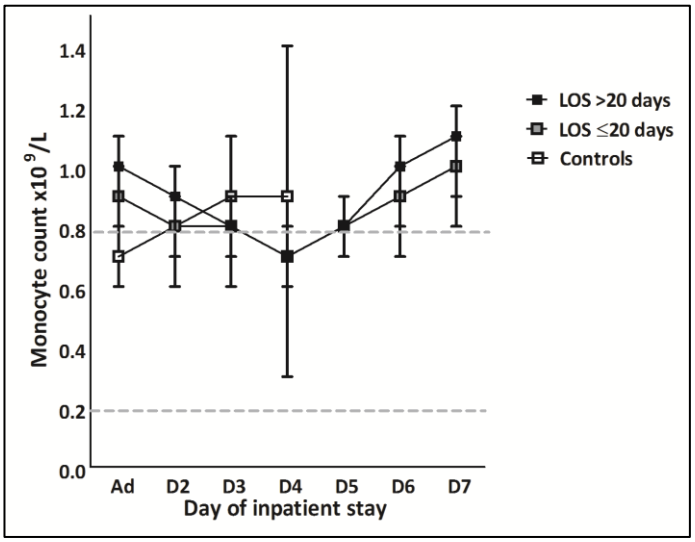
D: Seven day trend of eosinophil count according to 28 day mortality

Eosinophil count fell on D2 in injured patients. No difference between the two clinical outcome cohorts was observed throughout the seven day study period. Dotted line demonstrates the normal range. Data are presented as mean (95% CI).

ii. Length of hospital stay

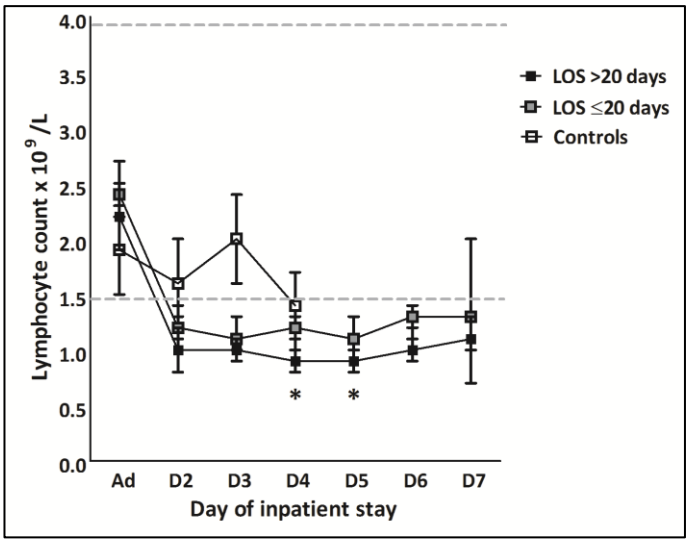
Two cohorts of patients were defined according to their length of hospital stay (LOS). Patients whose hospital stay was 20 days or less (n=100), were compared with those whose stay was >20 days and defined as 'extended' (n=50). Although some variation in immune cell count was observed, neither the neutrophil count nor the monocyte count demonstrated any difference between the two groups over the first 7 days of admission. The eosinophil count dropped to negligible levels at 24h from injury in injured patient but no difference between the two groups was observed. The lymphocyte count, however, did demonstrate clear differences between the two groups. On day 4 and day 5, the lymphocyte count was significantly lower in patients who had an extended LOS (Table 8.3 & Figure 8.3).

Figure 8.3: Immune cell count and length of stay



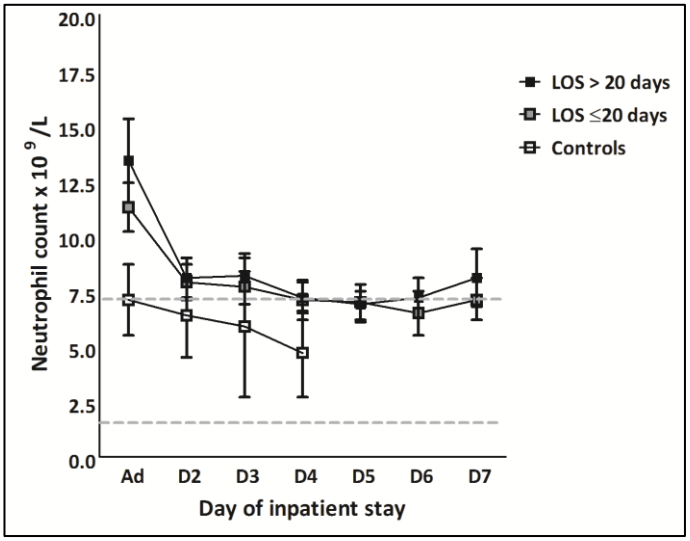
A: Seven day trend of neutrophil count in trauma patients according to length of hospital stay (LOS).

Extended stay was defined as greater than 20 days; the 75th percentile and above (n=50). LOS up to 20 days was defined as normal (n=100). No difference in neutrophil count was identified between the two patient cohorts. Data are presented as mean (95% CI). The dotted line marks the normal range. * denotes $p<0.05$, Students T test



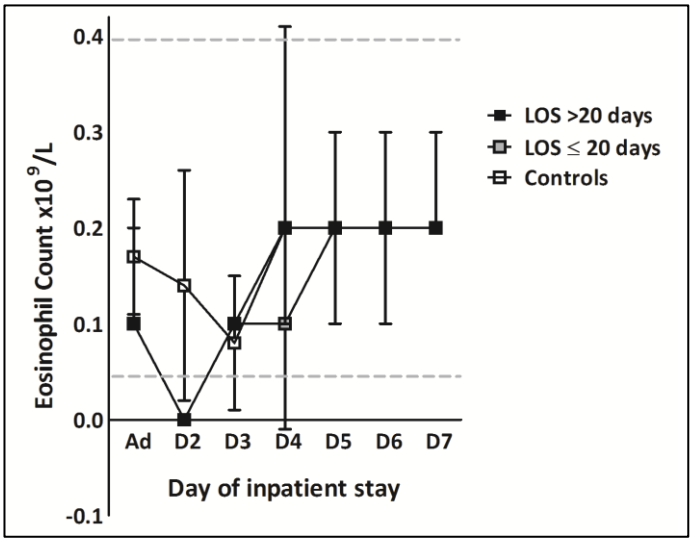
B: Seven day trend of lymphocyte count in trauma patients according to length of hospital stay (LOS).

Extended stay (75th percentile) was defined as greater than 20 days (n=50). Patients who had an extended hospital stay had lymphocyte counts which were significantly lower, on D4 and D5, than patients with a stay of ≤20days (n=100). D4: ≤20 = 1.2 (1.0-1.3), >20=0.9 (0.8-1.0), p=0.01. D5: ≤20 = 1.1 (1.0-1.3), >20=0.9 (0.8-1.0), p<0.01. Data are presented as mean (95% CI). * denotes p<0.05, Students T test



C: Seven day trend of monocyte count in trauma patients according to length of hospital stay (LOS).

Extended stay (75th percentile) was defined as greater than 20 days (n=50). Normal LOS ≤20days (n=100). No difference in monocyte count was identified between the two patient cohorts. Data are presented as mean (95% CI). * denotes p<0.05, Students T test



D: Seven day trend of eosinophil count in trauma patients according to length of hospital stay (LOS).

Extended stay (75th percentile) was defined as greater than 20 days (n=50). Normal LOS ≤20days (n=100). No difference in eosinophil count was identified between the two patient cohorts. Data are presented as mean (95% CI).

Table 8.3: Demographics of the cohorts for length of stay analysis

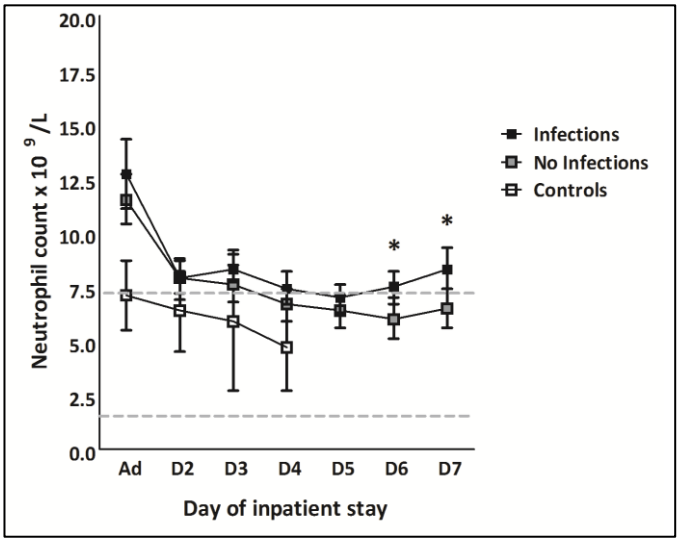
Demographics	Controls (ISS 0-4)	Normal LOS	Extended LOS	p value
Number of patients	26	104	56	-
% Male	92	85	86	1.00
Age ‡	36 (27-45)	32 (23-45)	48 (35-55)	**<0.01**
Injury Severity ‡				
ISS	1 (1-2)	15 (9-25)	26 (18-35)	**<0.01**
% Blunt	69	73	95	**<0.01**
SBP	137 (124-150)	132 (115-152)	130 (110-147)	0.41
Base Deficit	-0.7 (-1.9 - -0.03)	1.1 (-1.1-3.0)	2.4 (1.2-5.6)	**<0.01**
Time from injury	67 (60-82)	76 (55-89)	86 (75-102)	**0.02**
CSL prior to blood draw	0 (0-0)	0 (0-100)	250 (0-500)	**<0.01**
Of pts with >250ml CSL	0 (0-0)	500 (500-750)	500 (500-750)	0.51
AIS Head & Neck	0 (0-0)	0 (0-1)	1 (1-4)	**0.01**
AIS Face	0 (0-0)	0 (0-1)	0 (0-0)	0.84
AIS Thorax	0 (0-0)	3 (0-3)	3 (3-4)	**0.02**
AIS Abdo & Pelvis	0 (0-0)	0 (0-2)	0 (0-2)	0.34
AIS Extremity & Pelvis	0 (0-0)	1 (0-3)	3 (3-3)	**<0.01**
Outcomes				
Length of Stay ‡	2 (1-7)	8 (3-12)	39 (30-47)	**<0.01**
MODS (%)	0	8	36	**<0.01**
Acute Lung Injury (%)	0	20	52	**<0.01**
Infections (%)	0	19	79	**<0.01**
Neutrophils †				
Admission	7.1 (5.4-8.7)	11.3 (10.2-12.4)	13.4 (11.5-15.3)	0.06
D2	6.9 (5.0-8.8)	7.9 (7.1-8.7)	8.1 (7.2-9.0)	0.77
D3	5.2 (2.8-7.6)	7.7 (6.9-8.4)	8.2 (7.5-9.0)	0.32
D4	4.9 (3.5-6.3)	7.1 (6.2-8.0)	7.2 (6.5-7.9)	0.85
D5	-	7.0 (6.1-7.8)	6.9 (6.2-7.5)	0.88
D6	-	6.5 (5.5-7.5)	7.2 (6.3-8.1)	0.32
D7	-	7.1 (6.2-8.1)	8.1 (6.8-9.4)	0.25
Lymphocytes †				
Admission	2.0 (1.6-2.3)	2.4 (2.2-2.7)	2.2 (1.9-2.5)	0.37
D2	1.7 (1.4-2.0)	1.2 (1.1-1.4)	1.0 (0.8-1.2)	0.06
D3	1.9 (1.6-2.2)	1.1 (1.0-1.3)	1.0 (0.9-1.1)	0.25
D4	1.5 (1.3-1.7)	1.2 (1.0-1.3)	0.9 (0.8-1.0)	0.01
D5	-	1.1 (1.0-1.3)	0.9 (0.8-1.0)	**<0.01**
D6	-	1.3 (1.1-1.4)	1.0 (0.9-1.2)	0.06
D7	-	1.3 (0.7-2.0)	1.1 (1.0-1.3)	0.11
Monocytes †				
Admission	0.6 (0.5-0.8)	0.9 (0.8-1.0)	1.0 (0.8-1.1)	0.41
D2	0.8 (0.6-1.1)	0.8 (0.7-0.9)	0.9 (0.7-1.0)	0.62
D3	0.9 (0.5-1.4)	0.8 (0.7-0.9)	0.8 (0.7-0.9)	0.78
D4	0.8 (0.5-1.1)	0.7 (0.6-0.8)	0.7 (0.6-0.8)	0.51
D5	-	0.8 (0.7-0.9)	0.8 (0.7-0.9)	0.49
D6	-	0.9 (0.7-1.0)	1.0 (0.8-1.1)	0.39
D7	-	1.0 (0.8-1.1)	1.1 (0.9-1.2)	0.55
Eosinophils †				
Admission	0.17 (0.11-0.23)	0.1 (0.1-0.2)	0.1 (0.1-0.2)	0.63
D2	0.14 (0.02-0.26)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.66
D3	0.08 (0.01-0.15)	0.1 (0.1-0.1)	0.1 (0.1-0.1)	0.54
D4	0.20 (-0.01-0.41)	0.1 (0.1-0.2)	0.2 (0.1-0.2)	0.69
D5	-	0.2 (0.2-0.3)	0.2 (0.1-0.2)	0.28
D6	-	0.2 (0.1-0.3)	0.2 (0.2-0.3)	0.59
D7	-	0.2 (0.2-0.3)	0.2 (0.2-0.3)	0.92

‡Median (IQR), †Mean (95% CI), CSL = Crystalloid, AIS = Abbreviated Injury severity score, p values compare extended LOS and normal LOS with a t-test. Bold denotes p<0.01

iii. Development of infection

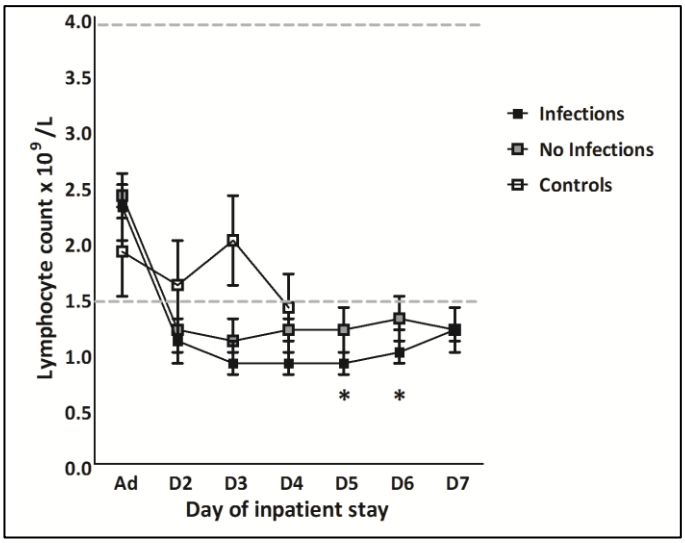
Immune cell counts in patients who developed clinically relevant infections during admission (n=73) were compared with those of patients who did not (n=103). Patients from both groups initially had high neutrophil counts which fell to nearly normal range by 24h (D2). No difference was observed between the two groups until D6 and D7 when the cohort who developed infections during admission, demonstrated higher neutrophil counts than those who did not. From normal range values at admission, lymphocyte count fell for patients in both groups by 24h (D2). No difference between the two groups was observed until D5 and D6. At this point the lymphocyte count was significantly lower in patients who developed infections. No difference in monocyte count or eosinophil count was observed between these two groups in this analysis (Table 8.4 & Figure 8.4).

Figure 8.4: Immune cell response and development of infection during admission



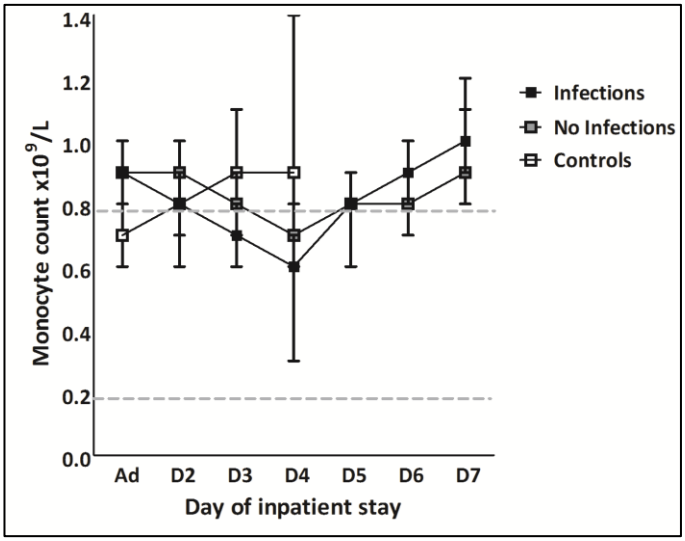
A: Seven day trend of neutrophil count in trauma patients who developed clinically relevant infections (n=73) compared with those who did not (n=103).

No difference between the two patient cohorts was identified until day 6. On day 6 and day 7, those patients who developed infections had higher neutrophil counts. D6: No Infection= 6.0 (5.1-7.0), Infection= 7.5 (6.7-8.2), p=0.03. D7: No Infection=6.5(5.6-7.4), Infection=8.3(7.4-9.3), p=0.02. Data are presented as mean (95% CI). * denotes p<0.05, Students t- test



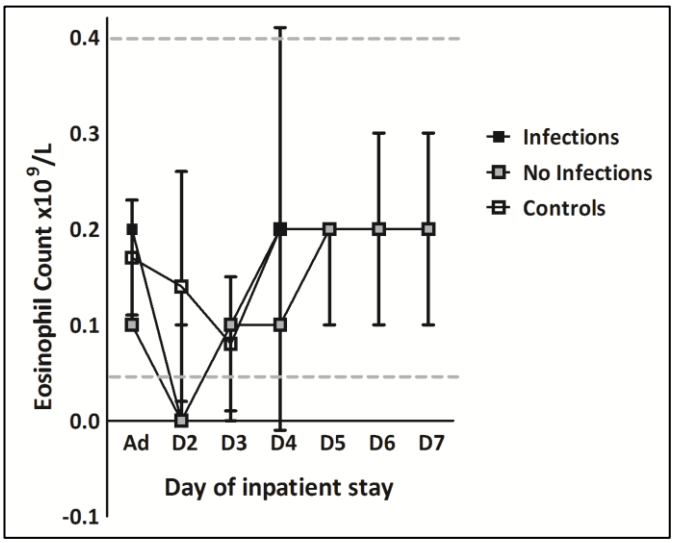
B: Seven day trend of lymphocyte count in trauma patients who developed clinically relevant infections (n=73) compared with those who did not (n=103).

On day 5 and day 6, patients who developed infections had lower lymphocyte counts than those who did not. D5: No Infection=1.2 (1.0-1.4), Infection=0.9 (0.8-1.0), p<0.01. D6:No Infection=1.3 (1.1-1.5), Infection=1.0 (0.9-1.2), p=0.02. Data are presented as mean (95% CI). * denotes p<0.05, Students t test



C: Seven day trend of monocyte count in trauma patients who developed clinically relevant infections (n=73) compared with those who did not (n=103).

No difference between the two patient cohorts was identified throughout the first 7 days of admission. Data are presented as mean (95% CI). * denotes p<0.05, Students t test



D: Seven day trend of eosinophil count in trauma patients who developed clinically relevant infections (n=73) compared with those who did not (n=103).

No difference between the two patient cohorts was identified throughout the first 7 days of admission. Data are presented as mean (95% CI).

Table 8.4: Demographics of the cohorts for infection analysis

Demographics	Controls (ISS 0-4)	No Infection	Infection	p value
Number of patients	26	117	77	-
% Male	92	78	83	0.48
Age ‡	36 (27-45)	32 (22-50)	41 (31-53)	**0.03**
Injury Severity ‡				
ISS	1 (1-2)	17 (9-27)	25 (18-34)	**<0.01**
% Blunt	69	72	92	**0.02**
SBP	137 (124-150)	131 (113-148)	130 (107-151)	0.90
Base Deficit	-0.7 (-1.9 - -0.03)	1.5 (-0.7-4.1)	2.3 (0.8-5.6)	**0.04**
Time from injury	67 (60-82)	78 (62-94)	85 (67-100)	0.10
CSL prior to blood draw	0 (0-0)	0 (0-250)	200 (0-500)	0.05
Of pts with >250ml CSL	0 (0-0)	625 (500-1000)	500 (500-750)	0.21
AIS Head & Neck	0 (0-0)	0 (0-3)	1 (0-4)	0.08
AIS Face	0 (0-0)	0 (0-1)	0 (0-1)	0.77
AIS Thorax	0 (0-0)	3 (0-4)	3 (0-4)	0.05
AIS Abdo & Pelvis	0 (0-0)	0 (0-2)	0 (0-3)	**0.01**
AIS Extremity & Pelvis	0 (0-0)	1 (0-3)	2 (0-3)	0.09
Outcomes				
Length of Stay ‡	2 (1-7)	6 (2-12)	29 (14-44)	**<0.01**
MODS (%)	0	4	44	**<0.01**
Acute Lung Injury (%)	0	15	60	**<0.01**
Infections (%)	0	0	100	**<0.01**
Neutrophils †				
Admission	7.1 (5.4-8.7)	11.5 (10.4-12.7)	12.7 (11.1-14.3)	0.25
D2	6.9 (5.0-8.8)	7.9 (7.2-8.7)	7.9 (6.9-8.8)	0.93
D3	5.2 (2.8-7.6)	7.6 (6.8-8.5)	8.3 (7.7-9.0)	0.21
D4	4.9 (3.5-6.3)	6.7 (5.9-7.4)	7.4 (6.7-8.2)	0.20
D5	-	6.4 (5.6-7.1)	7.0 (6.3-7.6)	0.25
D6	-	6.0 (5.1-7.0)	7.5 (6.7-8.2)	0.03
D7	-	6.5 (5.6-7.4)	8.3 (7.4-9.3)	0.02
Lymphocytes †				
Admission	2.0 (1.6-2.3)	2.4 (2.2-2.6)	2.3 (2.0-2.5)	0.45
D2	1.7 (1.4-2.0)	1.2 (1.0-1.3)	1.1 (0.9-1.3)	0.57
D3	1.9 (1.6-2.2)	1.1 (1.0-1.3)	0.9 (0.8-1.1)	0.05
D4	1.5 (1.3-1.7)	1.2 (1.0-1.3)	0.9 (0.8-1.0)	0.02
D5	-	1.2 (1.0-1.4)	0.9 (0.8-1.0)	**<0.01**
D6	-	1.3 (1.1-1.5)	1.0 (0.9-1.2)	0.02
D7	-	1.2 (1.0-1.4)	1.2 (1.1-1.4)	0.85
Monocytes †				
Admission	0.6 (0.5-0.8)	0.9 (0.8-1.0)	0.9 (0.8-1.0)	0.84
D2	0.8 (0.6-1.1)	0.9 (0.8-1.0)	0.8 (0.7-0.9)	0.30
D3	0.9 (0.5-1.4)	0.8 (0.7-0.9)	0.7 (0.6-0.8)	0.23
D4	0.8 (0.5-1.1)	0.7 (0.6-0.8)	0.6 (0.6-0.7)	0.20
D5	-	0.8 (0.6-0.9)	0.8 (0.6-0.9)	0.91
D6	-	0.8 (0.7-1.0)	0.9 (0.8-1.0)	0.31
D7	-	0.9 (0.8-1.1)	1.0 (0.9-1.2)	0.29
Eosinophils †				
Admission	0.17 (0.11-0.23)	0.1 (0.1-0.1)	0.2 (0.1-0.2)	0.11
D2	0.14 (0.02-0.26)	0.0 (0.0-0.0)	0.0 (0.0-0.1)	0.30
D3	0.08 (0.01-0.15)	0.1 (0.0-0.1)	0.1 (0.1-0.1)	0.34
D4	0.20 (-0.01-0.41)	0.1 (0.1-0.1)	0.2 (0.1-0.2)	0.23
D5	-	0.2 (0.1-0.2)	0.2 (0.1-0.2)	0.90
D6	-	0.2 (0.1-0.2)	0.2 (0.2-0.3)	0.23
D7	-	0.2 (0.1-0.3)	0.2 (0.2-0.3)	0.99

‡Median (IQR), †Mean (95% CI), CSL = Crystalloid, AIS = Abbreviated Injury severity score. P value compares infection and no infection with students t test. Bold denotes p<0.01.

iv. Development of multiple organ failure

Patients were divided into two groups, MODS and No MODS, as previously described. The immune cell counts for each group over the first 7 days were then compared. The neutrophil count at admission was highly elevated in injured patients from both groups. By 24h (D2), the neutrophil count had fallen back into normal range. From day 3 onwards, the No MODS cohort displayed neutrophil counts which were largely within normal range. The MODS cohort demonstrated consistently higher neutrophil levels, which breached the upper limit of normal by day 6. No significant difference between the two cohorts could be demonstrated (Table 8.5 & Figure 8.5).

For lymphocyte counts, admission levels were within the lower half of the normal range for all patients. No difference between MODS groups was identified. On day 2, the MODS and No MODS groups had lymphocyte counts which were significantly lower than the controls, demonstrating a rapid fall in the number of circulating lymphocytes after severe injury. From this point onwards, however, the No MODS group steadily increased their lymphocyte count; achieving a count comparable with the Controls by day 5. The lymphocyte count in the MODS group, by contrast, continued to fall and was significantly lower than the No MODS groups between day 3 and day 7.

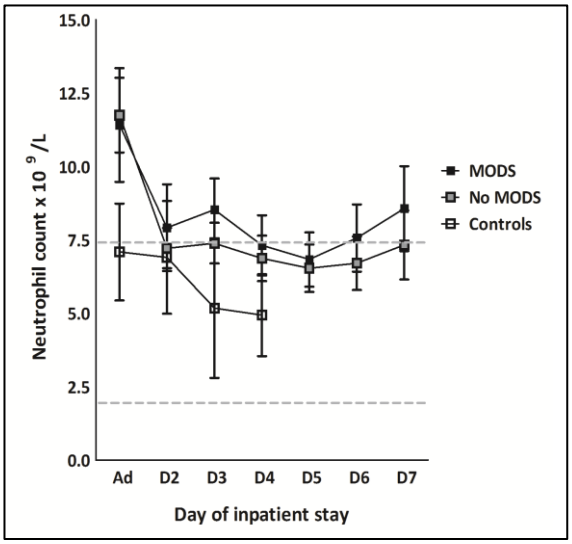
Injured patients from both groups had an elevated monocyte count at admission. Both MODS and No MODS patients then demonstrated falling monocyte counts to D4, although these largely remained within normal range. On D3 the monocyte counts of the MODS cohort were significantly lower than that of the No MODS cohort. On days 6 and 7, the monocyte count increased again, outside of the normal range, but no significant difference between the two groups was observed. Finally, apart from the fall in eosinophil count on D2, there was nothing remarkable about the eosinophil count between the two groups.

Table 8.5: Demographics of the MODS and No MODS cohorts

Demographics	Controls	No MODS	MODS	p value
Number of patients	26	85	42	-
% Male	92	77	74	0.74
Age	36 (27-45)	35 (23-48)	42 (28-53)	**0.04**
Injury Severity				
ISS	1 (1-2)	15 (9-25)	32 (25-38)	**<0.01**
% Blunt	69	76	95	**<0.01**
SBP	137 (124-150)	125 (110-141)	125 (101-152)	0.30
Base Deficit	-0.65 (-1.88-0.03)	1.1 (-1.1-2.4)	3,7 (2.0-7.1)	**<0.01**
Time from injury	67 (60-82)	76 (57-90)	90 (72-100)	0.05
CSL prior to blood draw	0 (0-0)	0 (0-250)	225 (0-500)	**<0.01**
Of pts >250mls CSL	0 (0-0)	500 (500-675)	500 (500-750)	0.23
AIS Head & Neck	0 (0-0)	0 (0-1)	3 (2-4)	**<0.01**
AIS Face	0 (0-0)	0 (0-0)	0 (0-1)	0.08
AIS Thorax	0 (0-0)	2 (0-3)	4 (3-4)	**<0.01**
AIS Abdo & Pelvis	0 (0-0)	0 (0-2)	0 (0-2)	0.75
AIS Extremity & Pelvis	0 (0-0)	2 (0-3)	2 (0-3)	0.55
Outcomes				
Length of Stay	2 (1-7)	10 (5-20)	22 (13-43)	**0.01**
Mortality (%)	0	0	64	**<0.01**
Acute Lung Injury	0	20	88	**<0.01**
Infections (%)	0	27	81	**<0.01**
Neutrophils				
Admission	7.09 (5.44-8.74)	11.75 (10.48-13.02) ^	11.42 (9.48-13.35) ^	0.78
D2	6.91 (4.99-8.83)	7.22 (6.54-7.91)	7.92 (6.44-9.40)	0.35
D3	5.18 (2.80-7.56)	7.39 (6.70-8.09)	8.53 (7.47-9.60) ^	0.07
D4	4.94(3.54-6.33)	6.87 (6.10-7.65)	7.32 (6.29-8.34)	0.49
D5	-	6.54 (5.73-7.35)	6.84 (5.91-7.76)	0.64
D6	-	6.71 (5.80-7.62)	7.57 (6.42-8.71) ^	0.25
D7	-	7.33 (6.15-8.51)	8.57 (7.12-10.01) ^	0.19
Lymphocytes				
Admission	1.96 (1.59-2.32)	2.46 (2.19-2.73) ^	2.16 (1.81-2.52) ^	0.21
D2	1.72 (1.40-2.04)	1.11 (0.98-1.24) ^	1.00 (0.77-1.22) ^	0.35
D3	1.92 (1.62-2.22)	1.14 (0.98-1.29)	0.84 (0.68-1.00) ^	**<0.01**
D4	1.53 (1.35-1.70)	1.17 (0.98-1.36)	0.73 (0.62-0.84) ^	**<0.01**
D5	-	1.05 (0.91-1.19) ^	0.76 (0.65-0.88) ^	**<0.01**
D6	-	1.29 (1.12-1.46)	0.91 (0.78-1.03) ^	**<0.01**
D7	-	1.33 (1.14-1.51)	1.02 (0.85-1.20) ^	0.03
Monocytes				
Admission	0.64(0.53-0.75)	0.95 (0.85-1.05) ^	0.77 (0.61-0.93)	0.05
D2	0.84 (0.58-1.10)	0.83 (0.73-0.92)	0.80 (0.61-0.99)	0.74
D3	0.92 (0.45-1.39)	0.81 (0.69-0.92)	0.63 (0.51-0.76)	0.04
D4	0.80 (0.46-1.14)	0.71 (0.62-0.81)	0.57 (0.47-0.67)	0.05
D5	-	0.79 (0.65-0.92)	0.63 (0.52-0.75)	0.09
D6	-	0.93 (0.79-1.06)	0.86 (0.73-0.99)	0.50
D7	-	1.05 (0.88-1.22)	0.97 (0.82-1.11)	0.48
Eosinophils				
Admission	0.17 (0.11-0.23)	0.1 (0.1-0.2)	0.2 (0.1-0.2)	0.67
D2	0.14 (0.02-0.26)	0.0 (0.0-0.0)	0.0 (0.0-0.1)	0.29
D3	0.08 (0.01-0.15)	0.1 (0.1-0.1)	0.1 (0.1-0.1)	0.21
D4	0.20 (-0.01-0.41)	0.1 (0.1-0.2)	0.2 (0.1-0.2)	0.16
D5	-	0.2 (0.1-0.2)	0.2 (0.1-0.3)	0.69
D6	-	0.2 (0.1-0.3)	0.2 (0.2-0.3)	0.43
D7	-	0.2 (0.1-0.3)	0.2 (0.1-0.3)	0.56

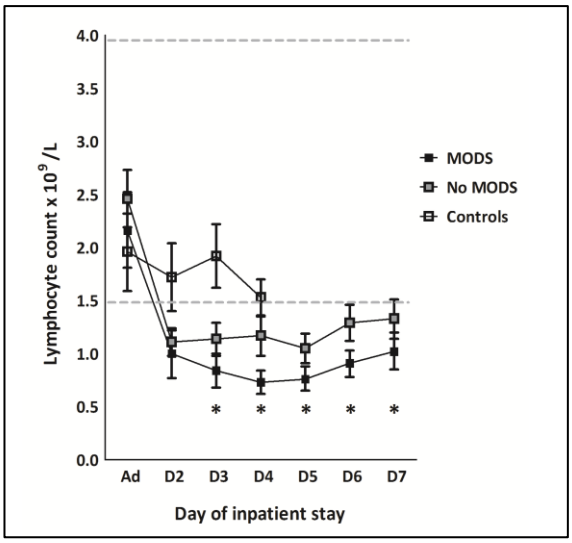
^ denotes p<0.05 when value compared to control , Categorical Tests =Fishers/Chi Squared, p value compares MODS and No MODS groups. Bold denotes p<0.01

Figures 8.5: Immune Cell count and multiple organ dysfunction syndrome



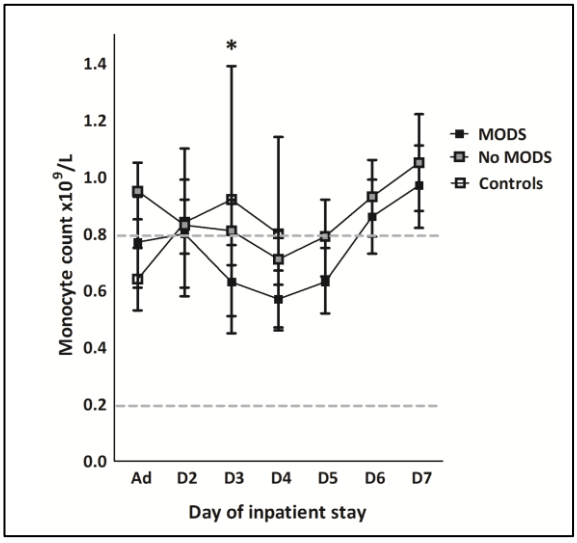
A: Seven-day trend of neutrophil count in patients with, and without, Multiple Organ Dysfunction Syndrome (MODS).

Neutrophil count cannot distinguish between these patients, No MODS (n=85), MODS (n=42). Dotted line marks the limits of the normal range. Data are presented as mean (95% CI). MODS and No MODS groups were compared using a Students t test.



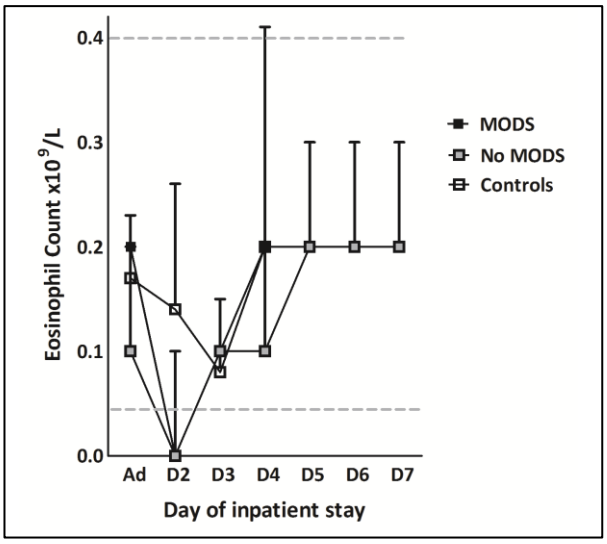
B: Seven-day trend of lymphocyte count in patients with, and without, Multiple Organ Dysfunction Syndrome (MODS).

Trauma patients who develop MODS during their admission, (n=42) have a lymphocyte count, between day 3 (48h) and day 7, which is significantly lower than those patients who do not develop MODS (n=85). Day: No MODS, MODS, p value. D3: 1.1(1.0-1.3), 0.8(0.7-1.0), p=0.01. D4: 1.2(1.0-1.4), 0.7(0.6-0.8), p<0.01. D5: 1.1 (0.9-1.2), 0.8(0.7-0.9), p<0.01. D6:1.3(1.1-1.5), 0.9(0.8-1.0), p<0.01. D7:1.3(1.1-1.5), 1.0(0.8-1.2), p=0.03. Dotted line marks the limits of the normal range. Data are presented as mean (95% CI). * Denotes p<0.05 when MODS and No MODS were compared using Students t test.



C: The seven-day trend of monocyte count in patients with, and without, Multiple Organ Dysfunction Syndrome (MODS).

Monocyte count does not differ between patients who develop MODS during admission (n=42) and patients who do not (n=85). Dotted line marks the limits of the normal range. Day: No MODS, MODS, p value. D3: 0.8 (0.7-0.9), 0.6 (0.5-0.8), p=0.04. Data are presented as mean (95% CI),



D: The seven-day trend of eosinophil count in patients with, and without, Multiple Organ Dysfunction Syndrome (MODS).

Eosinophil count fell sharply on D2 in all injured patients. In the first 7 days of admission, eosinophil count did not differ between patients who develop MODS during admission (n=42) and patients who did not (n=85). Dotted line marks the limits of the normal range. Data are presented as mean (95% CI),

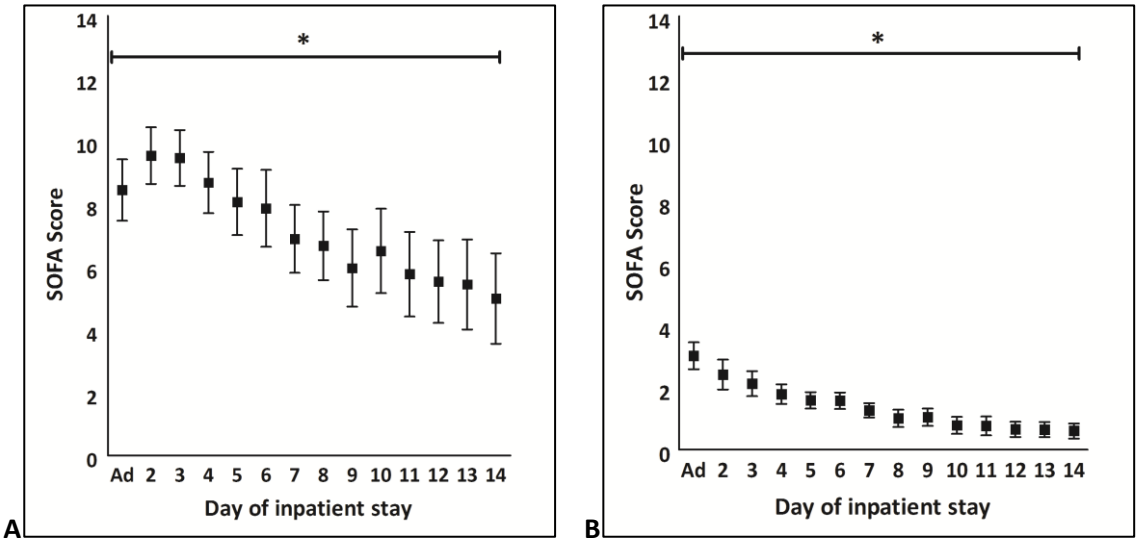
c) FURTHER ANALYSIS OF THE MODS COHORT

Although not a conventional outcome measure, development of MODS can be a terminal event for severely injured trauma patients. Over the first seven days of admission, significantly lower lymphocyte counts were demonstrated in the patients subsequently diagnosed with MODS. The MODS and No MODS cohort therefore underwent further analysis to establish whether the study findings may have mechanistic significance for the pathogenesis of MODS.

i. Time course of MODS development

The daily mean SOFA scores of the two groups were assessed to determine the time course for MODS development (Figure 8.6). MODS was noted to be an early clinical feature which occurred in 42/153 (27%) of the patients included in this analysis. It gradually resolved over the 14 days with no second peak. A degree of organ dysfunction was also observed in the No MODS patients but this was insufficiently severe to fulfil the criteria for MODS diagnosis and quickly resolved.

Figure 8.6 : Mean daily SOFA Scores for the patient cohort



A: Patients who developed MODS during admission (n=42). Defined as a SOFA Score of 5 or more, on 2 or more consecutive days at least 48h after admission (ie: from D4 onwards). In this cohort, MODS occurred early after admission and resolved over 14 days**. B: Patients who do not develop MODS during admission (n=85).** These patients do display some organ dysfunction, but not of sufficient severity to fulfil the MODS definition. The organ impairment is mild and quickly resolves. Data are median (IQR), * denotes p<0.05, Kruskal Wallis.

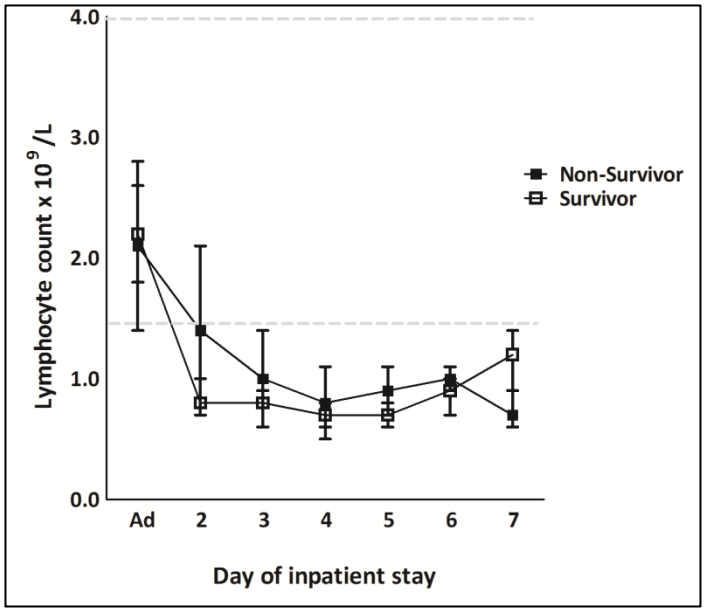
ii. Demographics of the MODS cohort

Examination of the demographics and injury characteristics revealed some interesting clinical differences between the two groups of patients (Table 8.5). Patients who developed MODS were older and had higher injury severity scores. Almost all of the MODS patients had a blunt mechanism of injury and evidence of co-existing blood loss; reflected by a raised base deficit, median (IQR) 3.7mmol/L (2.0-7.1). As expected, development of MODS was associated with higher mortality, a longer length of hospital stay and a higher proportion of patients who developed infections during their admission. The differences between the two cohorts, limit the strength of the conclusions which can be drawn from comparison between them.

iii. Survival of the MODS cohort

The lymphocyte count in the MODS cohort was then examined with regard to survival. These patients had matched injury severity (ISS scores) and matched admission BD concentration. In patients who developed MODS during admission, the lymphocyte count on D3 and D7 was significantly different between those patients who survived and those who died before 28 days. It was surprising to observe that in this MODS cohort, patients who survived had lower lymphocyte counts on D3 (48h). A higher proportion of the non-survivors were female; which was also an unexpected finding. Those who survived had longer lengths of stay and a higher proportion of ALI (Table 8.6).

Figure 8.7: Lymphocyte count in MODS patients and 28 day survival



Patients who fulfilled the criteria for MODS were categorised according to 28 day survival outcome (Survivors n=26, Non-survivors n=11). At admission, lymphocyte count was within the normal range and no difference, between the groups, was observed. Throughout, survivors of this cohort had lymphocyte counts which were lower than non-survivors but this did not reach significance. Data are displayed as mean (95% CI) and tested with Students t test. Significance was set at $p<0.01$ to accommodate for multiple comparisons. 5 patients excluded due to insufficient data. (The values are reported in table 8.6).

Table 8.6: Demographics of the MODS cohort and 28 day survival

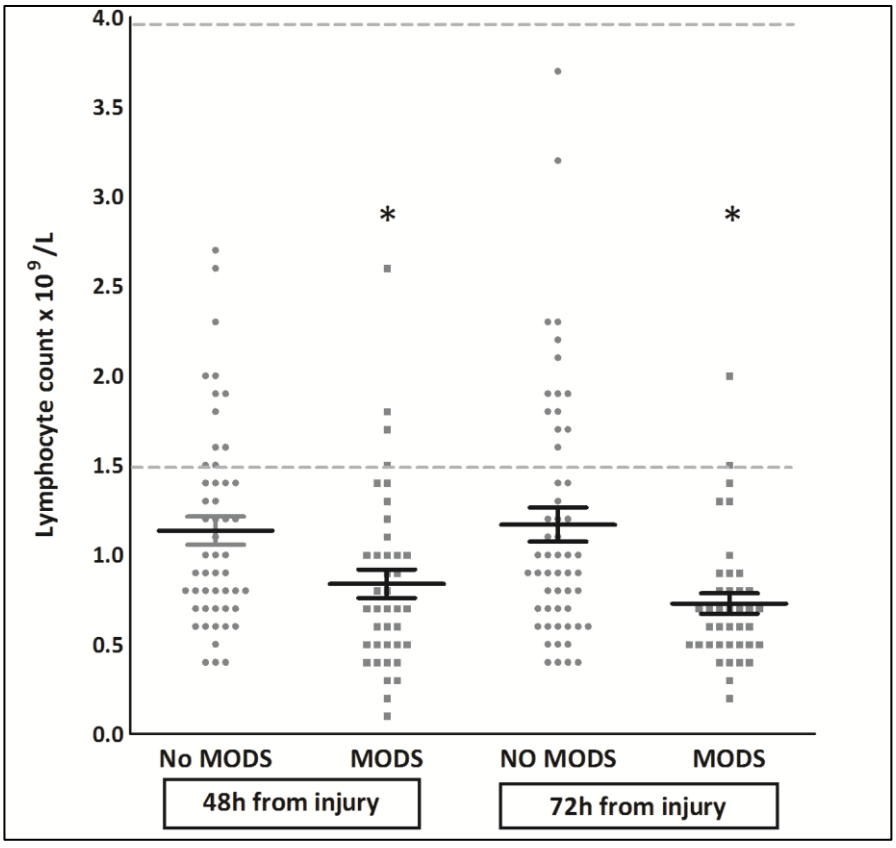
	Survivor	Non-Survivor	p value
Demographics			
Number of patients	26	11	-
% Male	88	55	**<0.01**
Age ‡	44 (28-53)	49 (35-68)	0.39
Injury Severity ‡			
ISS	33 (25-40)	34 (28-36)	0.71
% Blunt	96	100	0.12
SBP	122 (83-138)	100 (63-159)	0.90
Base Deficit	3.8 (2.0-6.7)	3.5 (2.3-16.2)	0.40
Time from injury	88 (80-100)	92 (76-103)	0.79
CSL prior to blood draw	325 (100-650)	200 (0-500)	0.42
AIS Head & Neck	3 (2-4)	3 (3-5)	0.74
AIS Face	0 (0-1)	0 (0-2)	0.32
AIS Thorax	4 (3-4)	4 (3-4)	0.37
AIS Abdo & Pelvis	0 (0-2)	2 (0-2)	0.28
AIS Extremity & Pelvis	2 (0-3)	2 (0-2)	0.83
Outcomes			
Length of Stay ‡	39 (21-47)	11 (8-15)	**<0.01**
Infections (%)	92	82	0.06
Acute Lung Injury (%)	100	88	**<0.01**
Lymphocytes †			
Admission	2.2 (1.8-2.6)	2.1 (1.4-2.8)	0.79
D2	0.8 (0.7-1.0)	1.4 (0.7-2.1)	0.03
D3	0.8 (0.6-0.9)	1.0 (0.6-1.4)	0.25
D4	0.7 (0.6-0.8)	0.8 (0.5-1.1)	0.74
D5	0.7 (0.6-0.8)	0.9 (0.7-1.1)	0.06
D6	0.9 (0.7-1.0)	1.0 (0.9-1.1)	0.27
D7	1.2 (0.9-1.4)	0.7 (0.6-0.9)	0.02

‡Median (IQR), †Mean (95% CI), CSL = Crystalloid, AIS = Abbreviated Injury severity score

d) THE SIGNIFICANCE OF THE LYMPHOCYTE COUNT

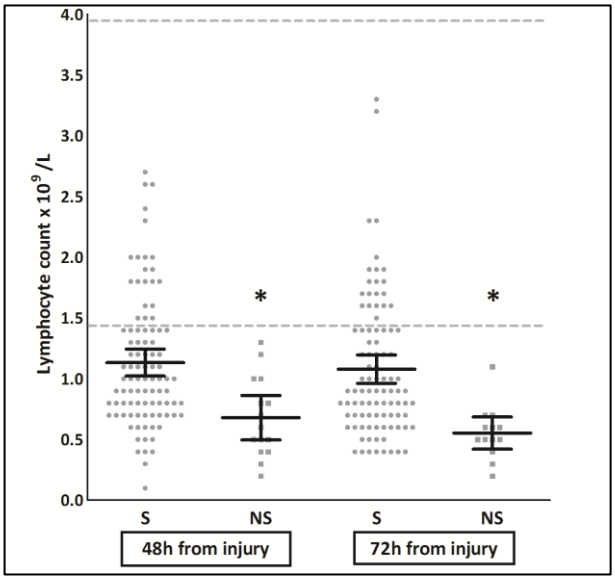
When examined as a whole cohort, a low lymphocyte count on day 3 and day 4 was associated with two substantial clinical outcomes; 28 day mortality and development of MODS. Values for D3 and D4 in both of these analyses were therefore examined in greater detail. Scatterplots demonstrate considerable overlap; nevertheless, a low lymphocyte count on D3 and D4 was clearly associated with poor outcome (Figure 8.8 & 8.9).

Figure 8.8: Lymphocyte count at 48h from injury in No MODS and MODS patients



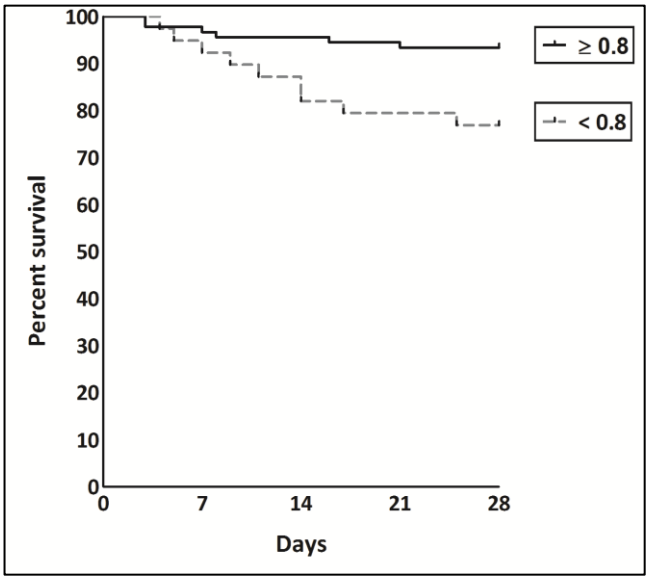
Patients with MODS at 48h(D3) and 72h(D4) have significantly lower lymphocyte counts than patients who do not develop MODS. 48h: No MODS=1.1(1.0-1.3), MODS=0.8 (0.7-1.0), p=0.01. 72h: No MODS=1.2 (1.0-1.4), MODS=0.7(0.6-0.8), p<0.01. Each patient measurement is represented by a dot and the mean (95% CI) is marked. Significance was determined using a Students t-test comparing the MODS and No MODS cohorts.

Figure 8.9: Lymphocyte count at 48h from injury for survivors and non-survivors



Non survivors(NS) at 48h (D3) and 72h (D4) from injury have significantly lower lymphocyte counts than survivors(S). 48h: S=1.1(1.2-1.0), NS=0.7 (0.8-0.5), p<0.01. 72h: S=1.1 (1.2-1.0), NS=0.6(0.7-0.4), p<0.01. Each patient measurement is represented by a dot and the mean (95% CI) is marked. Significance was determined using a Students t-test comparing the survivors with the non-survivors.

Figure 8.10: Kaplan-Meier survival curve for patients according to the 48h lymphocyte count



All patients in the analysis with a D3 lymphocyte count (n=130) were divided into two groups according to their lymphocyte count; less than 0.8x10 ^{9}/L or ≥ 0.8x10 ^{9}/L. The 28 day mortality in each group was assessed using a Kaplan-Meier estimate. D3 lymphocyte count below 0.8 x10^{9} /L had a significantly higher mortality rate: 9/39 (23%), 6/91 (7%), p<0.01 using Log-rank (Mantel-Cox) Test

To examine the importance of the lymphocyte count at D3 a survival analysis was performed using a Kaplan-Meier estimate (Figure 8.10). Patients were divided into two groups (n=130); those with a D3 lymphocyte count below 0.8 $x10^9$ /L (n=39) and those with a D3 lymphocyte count ≥ 0.8 $x10^9$ /L (n=91). The figure of 0.8 $x10^9$ /L was selected because the mean of the non-survivor cohort was 0.7 $x10^9$ /L. Follow-up to 28 days was assessed and the mortality within that time period plotted for both groups. The patients with a D3 lymphocyte count below 0.8 $x10^9$ /L had a significantly higher mortality rate: 9/39 (23%), 6/91 (7%), $p<0.01$ using Log-rank (Mantel-Cox) Test to compare the curves. Assessment of the whole cohort demonstrated that 31% of the patients had a D3 lymphocyte count of less than 0.8x109/L and of those 23% died, 50% developed MODS, 27% had an extended LOS and 61% developed infections. Lymphocyte count on D4 was also assessed (n=140). Those patients with a D4 lymphocyte count below 0.8 $x10^9$ /L had a mortality rate of 22% (12/55). Patients with a lymphocyte count below 0.8 $x10^9$ /L on both D3 and D4 had a mortality rate of 27% (9/33).

The influence of monocyte count was then considered. Of the 39 patients who had a D3 lymphocyte count below 0.8 $x10^9$ /L, 9 of them died before 28 days. Of those 9 patients, 8 also had a low monocyte count of <0.6 $x10^9$ /L. Assessment of the whole cohort of patients (n=142), demonstrated that 33% (47/142) had a monocyte count of <0.6 $x10^9$ /L on D3. Of these, 19% (9/47) died before 28 days. These data demonstrate that a low monocyte count and low lymphocyte count may co-exist. They also suggest that a low D3 and/or D4 lymphocyte count may influence survival.

5. DISCUSSION

This investigation used the differential white blood cell count, to describe circulating populations of immune cells in 245 trauma patient blood samples and examined their relationship to injury severity and clinical outcome. The most striking study finding was that a low lymphocyte count at 48h (D3) was associated with all four selected adverse clinical outcomes. More specifically, although the lymphocyte count was within normal range for almost all patients at admission, levels of lymphocytes then fell by 24h. Injured patients who died before 28 days, those who survived but had extended LOS, those who developed infections and those who developed MODS, had significantly lower lymphocyte counts at 48h (D3) and 72h (D4) after injury than injured patients who did not. Patients with a D3 lymphocyte count lower than $0.8x10^9$/L had a 23% mortality rate compared to 6% for those who had a D3 lymphocyte count of ≥0.8x109/L. It remains unclear whether the low lymphocyte count is responsible for poor outcome or is simply indicative of the patient's response. Nonetheless, the results suggest that lymphocytes may play a role in recovery from traumatic injury and this warrants further investigation.

The study also demonstrated that neutrophils and monocytes are rapidly mobilised into the peripheral circulation after injury; the count of both cell types at admission broadly reflects the severity of injury. Neutrophil counts over the first seven days were no different in patients with poor outcome, except on D6 and D7 in patients who developed infections. The rise in neutrophils at this point may be the result of infection, rather than the cause. Monocyte counts on D3 were significantly lower in patients who died within 28 days and those who developed MODS. The association was less convincing than that for lymphocytes, but these findings imply that monocytes may also influence recovery after traumatic injury; perhaps in concert with lymphocytes. Eosinophil counts were normal during the first few hours, but by 24h from injury, their peripheral blood populations became temporarily negligible in almost all

injured patients. No difference in outcome was associated with the eosinophil counts but the clinical reason for the fall at 24h is unclear and intriguing.

Although the low number of circulating lymphocytes was able to discriminate between patients with poor outcome and those who recovered, the actual number of cells in the peripheral circulation at 48hs (D3) is unlikely to hold the key to the human response to traumatic injury. Firstly, not all patients with a low D3 lymphocyte count had poor outcomes. Secondly, the number of cells in the peripheral circulation on D3 does not necessarily reflect lymphocyte function. The study does however raise the question of why the lymphocyte count should be low after traumatic injury, why it does not recover in some patients and why these events influence clinical outcome.

A fall in the peripheral blood lymphocyte count after traumatic injury has been previously reported in studies with samples taken between 0-96h after admission (Walsh et al, 2000, (Polk et al, 1986, (Kasten et al, 2010, (Cheadle et al, 1993, (Heffernan et al, 2012). Explanations for this fall in lymphocyte number have included migration into tissues due to the effects of catecholamines and wide spread apoptosis of naïve T cells (Moorhead and Claman, 1972, (Spry, 1972, (Yu et al, 1974, (Kasten et al, 2010). I would argue that there is no evidence to suggest that there is widespread apoptosis after traumatic injury, as the cited studies were designed to investigate septic patients (Hotchkiss et al, 1999a, (Hotchkiss et al, 1999b). Furthermore, trauma patients requiring splenectomy (3-8h after injury) formed a control population for a further study by the same group, which demonstrated that apoptosis occurred in only 4% of splenic lymphocytes compared to 12% for septic patients (Hotchkiss et al, 2001). In addition studies which demonstrate that trauma patient T-cells, when stimulated, more readily undergo apoptosis than healthy volunteer cells, do not provide evidence that apoptosis occurs in-vivo during the first 24h after injury (Pellegrini et al, 2000). How lymphocyte count relates to the T-cell 'anergy', which has been described in various traumatic

injury patients, remains unclear (Pellegrini et al, 2000, (De et al, 1997). The influence of trauma on lymphocyte number and lymphocyte function may lead to advances in understanding of the immunological response to injury, but much work is still required to develop our understanding.

A number of limitations must be considered when interpreting the findings of this study. The greatest of which is that the patients who succumbed to worse clinical outcomes had higher levels of injury and had more severe blood loss than those with better outcomes. In addition, there is a survivor bias. These aspects inevitably limit the strength of the conclusions that can be drawn from this data. Nonetheless this study robustly demonstrates immune cell counts at specified time intervals and examines their relationship with good clinical outcome data. Further investigation with matched injury groups and larger numbers would be desirable; however, this study provides the greatest evidence to date for the involvement of lymphocytes in the recovery from traumatic injury.

In conclusion, this study has examined the differential white blood cell count in peripheral blood, over the first seven days of admission, in a cohort of 245 trauma patients with mixed injury severity. This is the first study to examine such a large cohort of patients in such detail. The results demonstrate that a low lymphocyte count, of less than 0.8 $x10^9$/L, at 48h from injury is associated with poor outcome in terms of mortality, extended length of stay, development of infection and development of MODS. The data suggest that the lymphocyte population may play a role in the pathogenesis of poor outcome after traumatic injury. Whether this is whole body loss of lymphocytes or transient loss from circulation is unclear. How this influences lymphocyte function is also unknown and both require further investigation.

CHAPTER NINE: INNATE LYMPHOCYTE RESPONSES TO TRAUMATIC INJURY

1. INTRODUCTION

The previous chapter provided substantial evidence for the importance of lymphocytes in the immune response to traumatic injury. The results imply that early lymphocyte response to injury may play a role in the pathogenesis of poor clinical outcome. I therefore wished to conduct another exploratory study to examine the behavior of the lymphocyte population in greater detail using flow cytometry.

2. THE AIM OF THIS INVESTIGATION

The first aim of this investigation was to determine whether traumatic injury induces a change in the innate lymphocyte populations within 2h of injury. Population changes were assessed in terms of number of cells and percentage population. The second aim was to examine the relationship between the lymphocyte responses and clinical outcome. The third aim was to examine the activation status of the lymphocyte subsets.

3. METHODOLOGY

This study was performed as part of the ACIT 2 study. Patients were screened and enrolled as previously described in chapter 2. The rest of the methodology is unique to this investigation.

Between the 1st of December 2010 and the 31st of December 2011, patients who were enrolled into ACIT were also eligible for flow cytometry analysis. Patients were included if they met the inclusion criteria for ACIT2. Additional absolute exclusion criteria essential for this study

included: >2h from injury, administration of blood or blood products prior to blood draw, significant co-morbidity or anti-inflammatory medication. Patient inclusion was influenced by logistics such as availability of staff and the laboratory equipment.

a) DATA ANALYSIS

Two sets of data were obtained for each cell type: the percentage population (%) as a proportion of the total lymphocyte population in the sample and the precise number of cells. Both the percentage population (%) and the total cell number were then correlated with the clinical outcome measures. The outcomes selected included; 28 day mortality, length of hospital stay, development of infection and development of multiple organ dysfunction syndrome (MODS). The definitions for these outcomes were consistent with the previous analysis. The presence of Multiple Organ Dysfunction Syndrome (MODS) was defined as a SOFA Score ≥5, on two or more consecutive days at least 48h from admission. Outcome groups were compared against each other but also against control samples; defined as an ISS 0-4 and a BD ≤2mmol/L.

Data analysis and graph construction were performed using Excel 2007 (Microsoft, CA) and Prism 5.01 (Graph Pad, CA). Data were assessed for distribution and were found to be normally distributed. Data are therefore presented as mean with 95% confidence interval (95% CI) and tested using ANOVA or Student's t-test, as appropriate.

4. RESULTS

Between December 2010 and December 2011, 40 patients were enrolled into this investigation. The median age of the cohort was 35 (IQR 26-45). All blood samples were taken within 2h from injury. No in-hospital intervention occurred before blood draw. Pre-hospital interventions were life-saving only and fluid administration was minimal. The cohort comprised 8 control patients (ISS 0-4 and BD ≤2mmol/L) and 32 injured patients (ISS ≥5) with a wide range of co-existing haemorrhagic shock (as evidenced by the base deficit) on arrival (median 1.6mmol/L, range -3.2 - 25.9mmol/L).

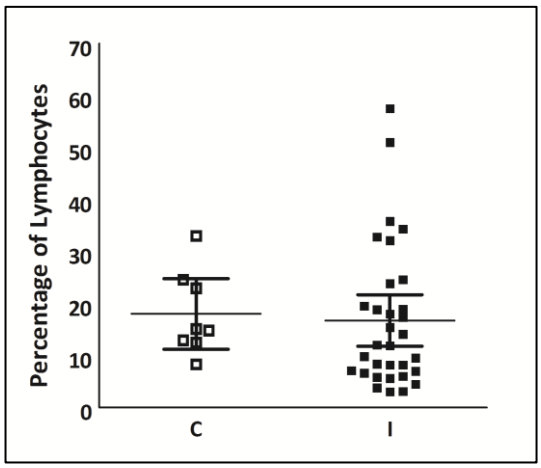
Comparison between the control population and the injured population demonstrated differences in the populations of lymphocytes. In addition, two other significant populations of cells were identified and gated on the flow cytometry plot. These two populations were not antibody labelled therefore cannot be discussed in any detail. Their position on the plots and characteristics during analysis strongly suggested that they represented neutrophils and monocytes. For ease, those names will be used to describe the populations during this chapter (Figure 9.1).

In injured patients (ISS ≥5), a wide range of percentage populations was observed for all three major leucocyte groups, but particularly lymphocytes and neutrophils. Examination of the 'monocyte' population demonstrated two clusters. This suggested that there is a subgroup of injured patients who have an elevated proportion of monocytes in their circulation, although this did not reach significance when compared to the control. Without specific staining, further comment on these cell populations is impossible.

Figure 9.1: The major populations of white blood cells examined using flow cytometry

Comparison between the 'control' (ISS0-4, BD ≤2mmol/L) and injured (ISS≥5 with a range of BD) populations. A wide range of cell percentage populations were observed in the injured cohort. No difference was identified between the control and injured populations when examined as a whole.

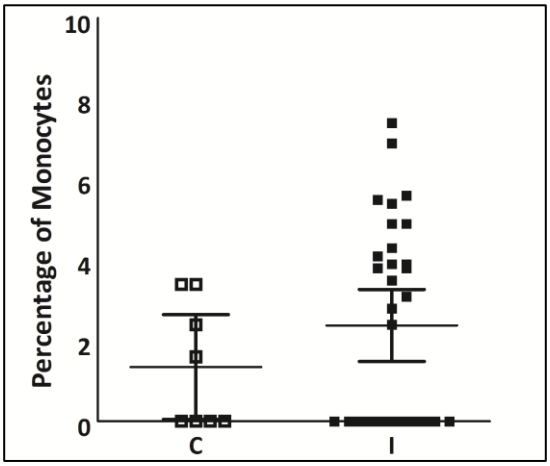
A



A: Lymphocytes.

C=18.0 (12.4-23.6), I=16.7 (11.9-21.5), p=0.80.

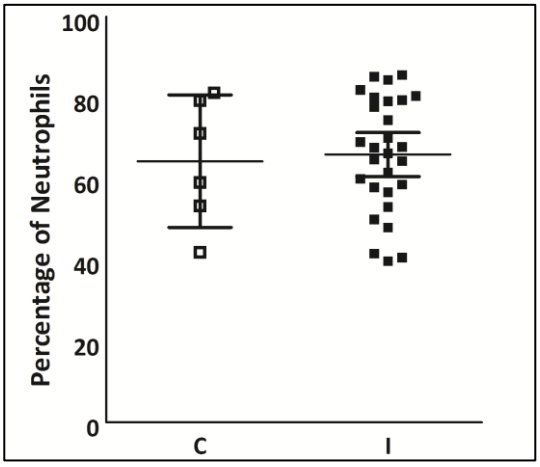
B



B: 'Monocytes'.

C=1.4 (0.3-2.4), I =2.4(1.5-3.2), p=0.27.

C



C: 'Neutrophils'.

C=64.4(51.9-76.9), I=66.0 (61.2-70.9), p=0.80. Data are presented as mean (95% CI), * denotes p<0.05 using Students t-test.

Six lymphocyte subsets were labelled and examined; T-helper cells (Th), Cytotoxic T Cells (CTLs), Natural Killer Cells Bright (NK Bright), Natural Killer Cells Dim (NK Dim), Gamma Delta T Cells High (γδ High), Gamma Delta T Cells Low (γδ Low).

a) CLINICAL OUTCOMES

Four clinical parameters were pre-defined as outcome measures for this study; 28 day mortality, Length of stay, development of clinically relevant infection and development of MODS.

i. 28 day mortality

In the study cohort of 40 patients, 4 died within 28 days (10%). Two deaths occurred on the day of admission, one occurred within 24h of admission and one occurred on day 8. The cause of death for the patients who died within 24h was principally traumatic brain injury. The death at day 8 was due to MODS. Unsurprisingly, patients who died had significantly higher injury severity and a greater degree of haemorrhagic shock. They also had higher volumes of crystalloid in the pre-hospital phase in keeping with their blood loss and need for volume support. The number of patients in the study population who died is small, but the proportion is consistent with our patient population. No differences in lymphocyte populations were observed, in terms of percentage population or absolute cell count, between survivors and non-survivors. (Table 9.1)

ii. Length of stay

In the study population, length of stay (LOS) ranged from 0-56 days. Extended LOS was defined in the previous chapter as above 20 days in duration. The same figure was used for this study as I considered this would be more meaningful than recalculating the 75th percentile in a small cohort of patients. Twenty-two patients had a 'normal' LOS (0-20 days) and 10 had an extended LOS (>20 days). No difference in terms of injury characteristics was observed

between these two cohorts except time from injury to blood draw (77(70-94), 95(82-128), p=0.02). No difference in lymphocyte populations (% or cell count) was observed between the two cohorts (Table 9.1)

iii. Development of infection

Thirteen patients developed infections during their admission (33%). The location of infection varied but included chest, wound, blood and urine. Patients who developed infections were more injured and more shocked at admission, as evidenced by their ISS and BD.

The lymphocyte populations between the two groups were examined. Higher circulating levels of Natural Killer cells were observed at admission in patients who subsequently developed infections. The total population of NK cells was shown to be elevated, in terms of both percentage and absolute count. Examination of the NK subsets revealed that this was due to an increase in the percentage and number of NK Dim cells. No difference in the population of NK Bright cells was identified (Table 9.2 & Figure 9.2)

Table 9.1: Cell counts and population percentages for lymphocyte subsets – Mortality and Length of stay

	Controls (ISS 0-4)	Survivors	Non-survivors	p value	Normal LOS	Extended LOS	p value
Demographics							
Number of patients	8.00	28.00	4.00	-	22.00	10.00	-
ISS ‡	2 (1-2)	18 (9-29)	47 (37-52)	**0.01**	17 (9-25)	31 (20-45)	0.09
BD ‡	-1.9 (-2.4- -0.6)	1.3 (-1.0-2.6)	13.1 (6.5-20.9)	**<0.01**	1.3 (-1.2 - 2.5)	2.7 (0.1-4.3)	0.12
% Male	100.00	72.00	75.00	0.75	82.00	80.00	0.86
CSL prior to blood draw ‡	0 (0-0)	0 (0-275)	500 (375-563)	**0.03**	0 (0-300)	200 (0-500)	0.27
Time from injury to blood draw (mins) ‡	82 (78-91)	78 (71-94)	102 (99-107)	0.13	77 (70-94)	95 (82-128)	**0.02**
Percentage †							
Lymphocytes	18.0 (12.4-23.6)	16.5 (12.2-20.9)	17.9 (-8.0-43.8)	0.85	18.1 (12.1-24.1)	13.6 (5.9-21.2)	0.39
T Helpers	31.9 (25.5-38.3)	27.5 (23.6-31.5)	26.2 (8.1-44.3)	0.83	27.3 (22.9-31.8)	27.4 (18.8-36.0)	0.98
Cytotoxic T Cells	22.5 (18.2-26.9)	24.3 (20.5-28.1)	22.1 (16.4-27.9)	0.69	26.1 (21.5-30.8)	19.3 (17.4-21.2)	0.07
Gamma Delta High	0.7 (0.4-1.0)	0.8 (0.4-1.2)	0.7 (0.1-1.2)	0.74	0.9 (0.5-1.3)	0.6 (-0.1-1.3)	0.40
Gamma Delta Low	2.1 (1.2-2.9)	2.2 (1.3-3.1)	1.5 (0.5-2.4)	0.54	2.6 (1.5-3.6)	1.1 (0.1-2.2)	0.11
Total NK Cells	15.6 (7.2-24.0)	18.6 (14.9-22.3)	28.1 (15.0-41.1)	0.10	17.7 (12.9-22.6)	24.3 (19.7-28.9)	0.11
NK Bright	0.6 (0.2-1.0)	0.8 (0.5-1.1)	0.3 (0.1-0.4)	0.19	0.8 (0.4-1.1)	0.7 (0.3-1.0)	0.73
NK Dim	15.0 (6.8-23.2)	17.8 (14.1-21.5)	27.8 (14.7-41.0)	0.08	17.0 (12.1-21.8)	23.7 (18.9-28.4)	0.11
Unconventional T cells	10.1 (1.3-18.9)	4.4 (2.2-6.5)	1.5 (0.6-2.4)	0.33	4.6 (2.0-7.2)	2.4 (0.7-4.2)	0.37
Cell Counts †							
Lymphocytes x 10^4	20.1 (12.6-32.0)	24.8 (20.2-30.5)	25.4 (11.5-55.7)	0.95	26.4 (20.8-33.5)	22.2 (16.5-29.8)	0.42
T Helpers x 10^4	5.5 (4.0-7.6)	5.9 (4.8-7.2)	6.0 (4.2-8.6)	0.94	6.6 (5.5-7.9)	4.7 (3.3-6.8)	0.10
Cytotoxic T Cells x10^4	4.3 (2.5-7.3)	5.6 (4.2-7.5)	4.9 (2.5-9.7)	0.75	6.2 (4.5-8.6)	4.4 (3.2-6.2)	0.22
Gamma Delta High x10^3	1.1 (0.5-2.6)	0.7 (0.3-1.4)	0.8 (0.1-9.3)	0.88	0.8 (0.4-1.9)	0.5 (0.2-1.4)	0.56
Gamma Delta Low x10^3	2.7 (0.9-8.0)	3.0 (1.9-4.9)	2.2 (0.4-10.8)	0.67	3.7 (2.2-6.3)	1.9 (1.0-3.7)	0.18
Total NK Cells x10^3	21.2 (7.3-61.4)	35.6 (23.2-54.5)	55.5 (10.7-287.7)	0.53	30.9 (18.0-53.3)	52.9 (36.7-76.3)	0.23
NK Bright x10^3	0.6 (0.2-2.3)	1.1 (0.8-1.6)	0.6 (0.2-1.8)	0.33	1.1 (0.7-1.6)	1.0 (0.5-2.0)	0.97
NK Dim x10^3	20.0 (6.8-59.2)	34.0 (22.1-52.3)	54.5 (10.4-286.4)	0.51	29.5 (17.1-51.1)	51.2 (35.2-74.4)	0.22
Unconventional T cells x10^3	8.6 (2.9-25.3)	3.8 (2.1-6.9)	2.1 (0.6-8.0)	0.25	3.7 (1.8-7.4)	2.4 (1.1-5.6)	0.48

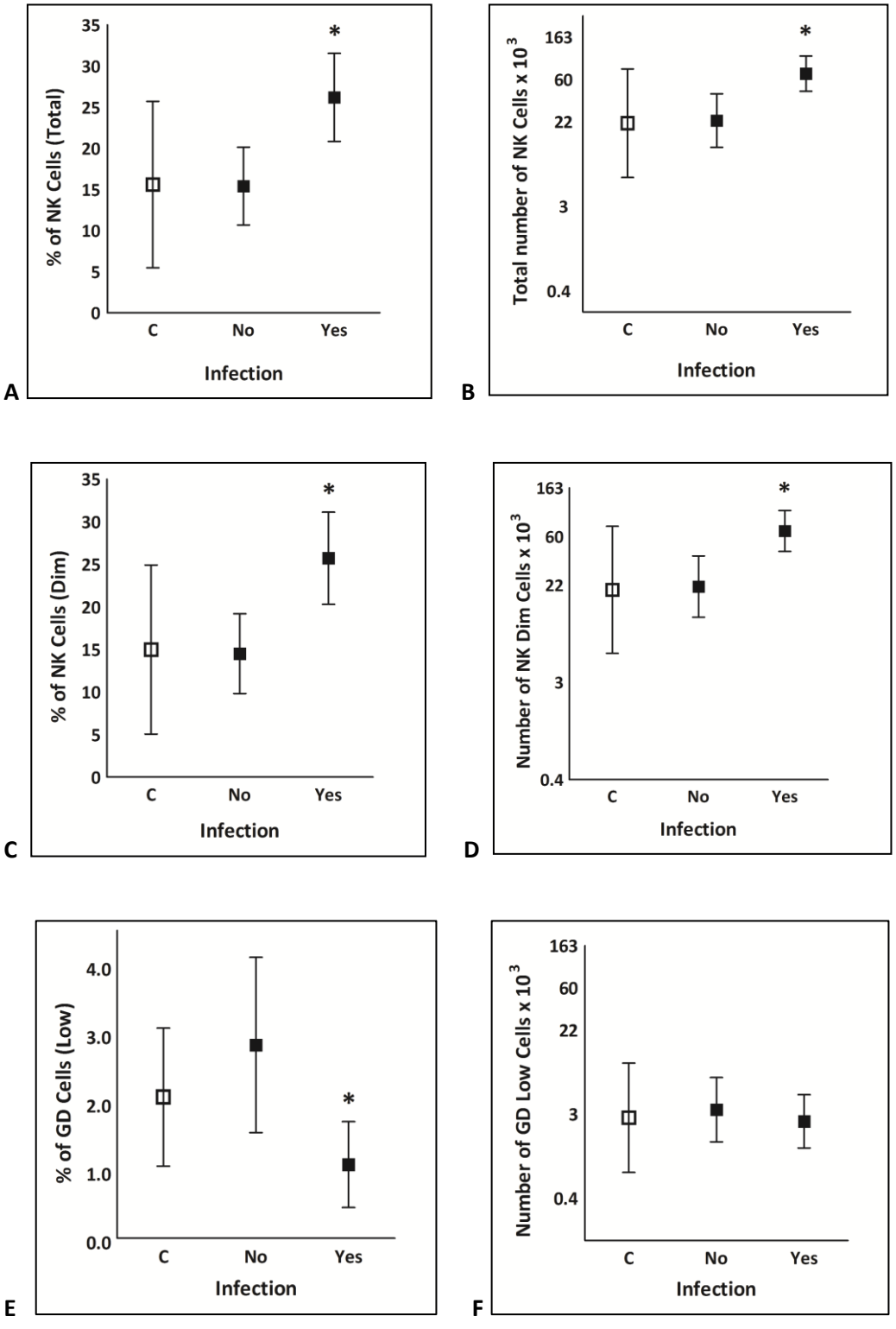
‡ Median (IQR), † Mean (95% Confidence Interval), LOS = Length of stay, CSL = Crystalloid, p values compare the Survivor vs Non-survivor or Normal LOS vs Extended LOS patients with a Student's t-test

Table 9.2: Cell counts and population percentages for lymphocyte subsets – Infection and Multiple Organ Dysfunction Syndrome

Demographics	Controls (ISS 0-4)	No Infection	Infection	p value	No MODS	MODS	p value
Number of patients	8.00	19	13	-	19	11	-
ISS ‡	2 (1-2)	10 (9-19)	33 (25-50)	**<0.01**	10 (9-18)	33 (27-46)	**<0.01**
BD ‡	-1.9 (-2.4- -0.6)	1.2 (-1.3 - 2.5)	2.2 (0.8-7.0)	**0.04**	-0.5 (-1.3 - 1.5)	4 (2-13)	**<0.01**
% Male	100.00	84	77	0.28	84	73	0.08
CSL prior to blood draw ‡	0 (0-0)	0 (0-250)	150 (0-500)	0.18	0 (0-0)	200 (0-500)	**0.03**
Time from injury to blood draw (mins) ‡	82 (78-91)	78 (71-96)	90 (76-102)	0.22	75 (65-87)	92 (78-101)	0.06
Percentage †							
Lymphocytes	18.0 (12.4-23.6)	15.7 (10.5-20.9)	18.2 (9.1-27.3)	0.61	17.7 (12.1-23.3)	17.3 (7.6-27.0)	0.95
T Helpers	31.9 (25.5-38.3)	28.5 (23.6-33.4)	25.7 (18.9-32.6)	0.51	28.4 (24.0-32.7)	23.9 (16.6-31.3)	0.28
Cytotoxic T Cells	22.5 (18.2-26.9)	26.7 (21.6-31.9)	20.0 (17.5-22.6)	0.06	25.8 (20.9-30.7)	20.5 (16.0-25.0)	0.16
Gamma Delta High	0.7 (0.4-1.0)	0.8 (0.4-1.3)	0.8 (0.2-1.4)	0.99	0.9 (0.4-1.3)	0.8 (0.1-1.5)	0.86
Gamma Delta Low	2.1 (1.2-2.9)	2.8 (1.6-4.0)	1.1 (0.5-1.6)	**0.03**	2.9 (1.7-4.1)	0.9 (0.5-1.3)	**0.02**
Total NK Cells	15.6 (7.2-24.0)	15.4 (11.0-19.8)	26.2 (21.4-31.0)	**<0.01**	16.2 (12.2-20.1)	25.8 (19.2-32.4)	**0.01**
NK Bright	0.6 (0.2-1.0)	0.9 (0.5-1.3)	0.5 (0.3-0.6)	0.12	0.9 (0.5-1.4)	0.5 (0.3-0.7)	0.12
NK Dim	15.0 (6.8-23.2)	14.5 (10.1-18.9)	25.7 (20.9-30.6)	**<0.01**	15.2 (11.3-19.1)	25.3 (18.7-32.0)	**<0.01**
Unconventional T cells	10.1 (1.3-18.9)	4.8 (1.9-7.7)	2.6 (1.5-3.6)	0.31	4.9 (2.0-7.8)	2.6 (1.5-3.7)	0.32
Cell Counts †							
Lymphocytes x 10^4	20.1 (12.6-32.0)	23.2 (17.8-30.3)	27.0 (20.1-36.3)	0.46	26.0 (20.7-32.8)	24.7 (17.2-35.5)	0.80
T Helpers x 10^4	5.5 (4.0-7.6)	6.0 (4.9-7.4)	5.7 (4.1-7.9)	0.75	6.7 (5.6-8.0)	4.7 (3.3-6.9)	0.08
Cytotoxic T Cells x10^4	4.3 (2.5-7.3)	5.6 (3.8-8.3)	5.5 (4.0-7.6)	0.95	6.2 (4.5-8.5)	5.0 (3.2-7.9)	0.46
Gamma Delta High x10^3	1.1 (0.5-2.6)	0.5 (0.2-1.3)	1.0 (0.4-2.6)	0.32	0.7 (0.3-1.7)	0.9 (0.3-2.5)	0.73
Gamma Delta Low x10^3	2.7 (0.9-8.0)	3.3 (1.7-6.5)	2.5 (1.4-4.4)	0.56	3.8 (2.0-7.3)	2.3 (1.3-3.9)	0.28
Total NK Cells x10^3	21.2 (7.3-61.4)	22.7 (12.7-40.5)	68.6 (47.1-99.8)	**<0.01**	30.2 (17.0-53.6)	57.8 (34.3-97.2)	0.14
NK Bright x10^3	0.6 (0.2-2.3)	1.2 (0.7-2.0)	0.9 (0.6-1.5)	0.45	1.3 (0.8-2.1)	0.8 (0.5-1.4)	0.20
NK Dim x10^3	20.0 (6.8-59.2)	21.4 (12.0-38.1)	67.1 (45.9-98.1)	**<0.01**	28.6 (16.0-50.9)	56.5 (33.5-95.5)	0.12
Unconventional T cells x10^3	8.6 (2.9-25.3)	3.3 (1.5-7.1)	4.3 (2.2-8.2)	0.67	3.7 (1.7-8.0)	4.3 (2.2-8.5)	0.75

‡ Median (IQR), † Mean (95% Confidence Interval), LOS = Length of stay, CSL = Crystalloid, p values compare the No infection vs Infection or No MODS vs MODS patients with a Student's t-test

Figure 9.2: Patients who developed infection during their inpatient stay



Percentage populations of: A-Total NK, C-NK Dim, E-GD Low. Number of NK cells: B-Total NK, D-NK Dim, F-GD Low. Natural Killer cells and Gamma delta T cells, were examined in trauma patients on admission using Flow Cytometry (n=40). Patients (n=13) who developed infections during admission (Yes) demonstrated higher levels of NK Dim cells, both in percentage and number, than patients (n=19) who did not develop infections (No) or the control population (C). These patients also demonstrated lower percentage populations of GD Low cells. Values are displayed in table 9.2. Data are mean (95% CI). * denotes $p<0.05$ when No and Yes groups are compared with Students t test.

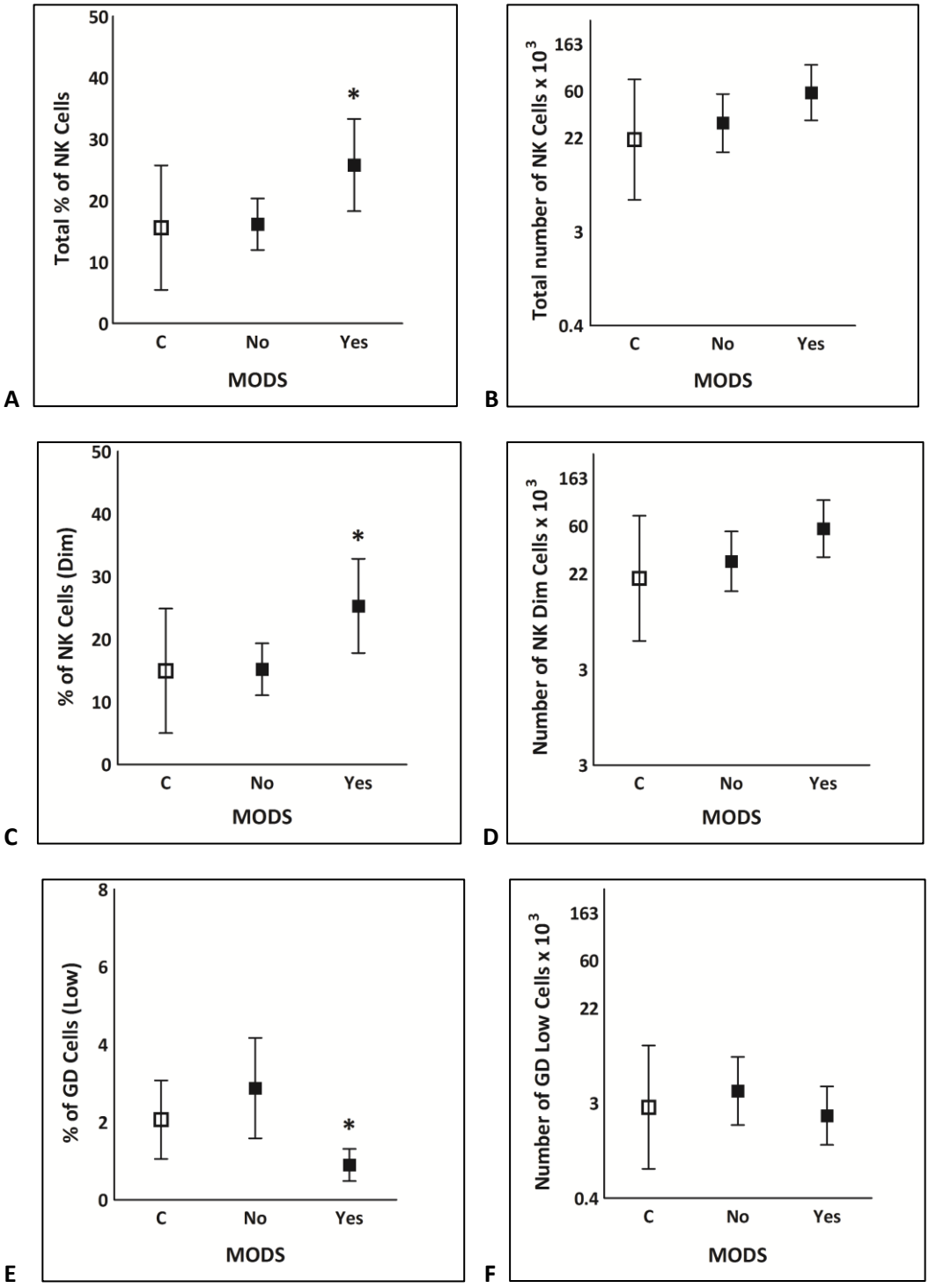
iv. Development of Multiple Organ Dysfunction Syndrome (MODS)

In the study population, eleven patients (28%) fulfilled the criteria for MODS; SOFA Score of ≥5 on two or more consecutive days after 48h from injury. Two patients were excluded from this analysis because they died before 48h and therefore did not fulfil the criteria for MODS development. Of the patients in the MODS cohort, only 2 died (18%). The cause of death was multiple organ dysfunction. Patients who developed MODS had higher injury severity scores, more severe shock as evidenced by higher BD and a larger volume of pre-hospital crystalloid reflecting their need for intravascular volume support.

The lymphocyte profile demonstrated interesting results. Patients who developed MODS during their inpatient stay had higher circulating levels of NK cells at admission (Figure 9.3). This increase was shown to be an elevation in the percentage population of NK cells rather than an absolute increase in cell numbers. As seen in the patients who developed infections, the increase was due to elevation of the NK Dim cell subset and not the NK Bright cells. In addition, a decrease in the percentage population of Gamma Delta (GD) Low cells was also observed in patients who developed MODS during their inpatient stay (Table 9.2).

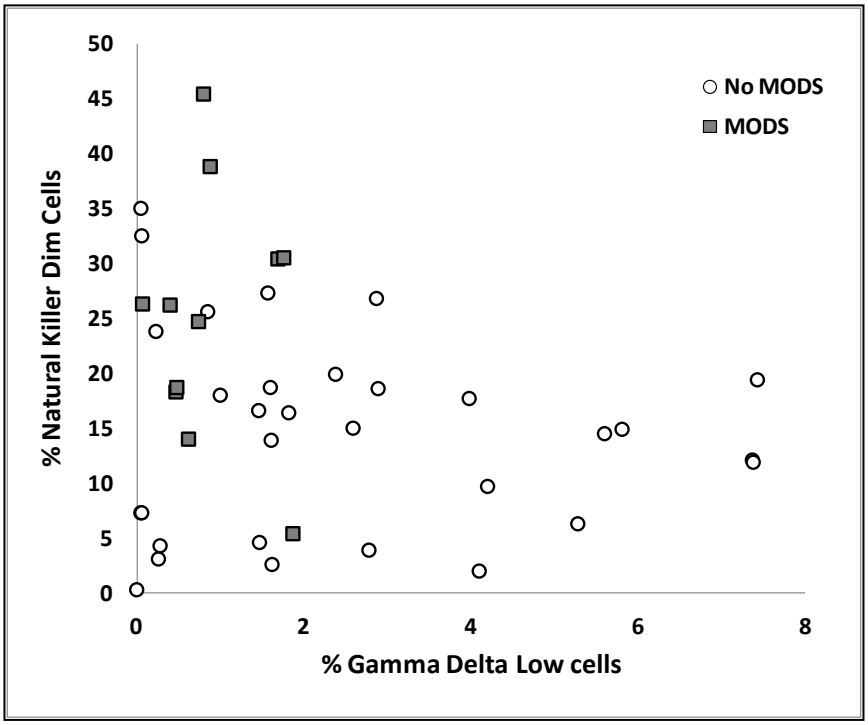
To examine the relationship between these two cell populations, a ratio of NK Dim divided by γδ low T cells was calculated. Patients who developed MODS had a higher ratio than patients who did not develop MODS (Figure 9.4 & 9.5). Finally to examine the relevance of these early findings to the cell responses during recovery, lymphocyte counts for the No MODS and MODS patients, from the FACS cohort, were examined (n=40). This demonstrated that all patients had lymphocyte counts within normal range at admission, including the control patients. At 48h from admission the MODS patients had lymphocyte counts which were significantly lower than those of the patients who did not develop MODS (Figure 9.6). The results provide more evidence for a relationship between lymphocyte responses at admission and lymphocyte responses during recovery. How and why lymphocytes influence outcome remains unclear.

Figure 9.3: Patients who developed MODS during their inpatient stay



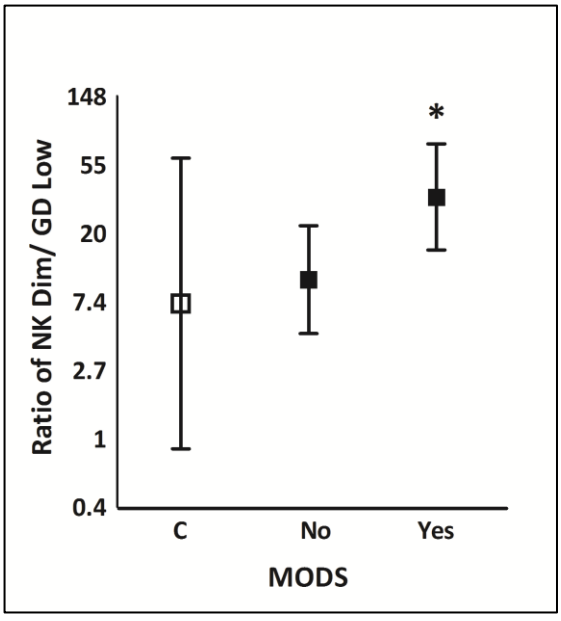
Percentage population: A-Total NK Cells, C-NK Dim Cells, E-GD Low Cells. Number of cells: B-Total NK Cells, D-NK Dim Cells, F-GD Low Cells. Natural Killer cells and their two subsets, Bright and Dim, were examined in trauma patients on admission using Flow Cytometry (n=40). Patients (n=11) who developed MODS during admission had a higher percentage population of NK Cells due to an increase in the circulating proportion of NK Dim cells. In addition, the same patients had low percentage populations of GD Low cells. Values are displayed in table 9.2. Data are mean (95% CI). * denotes $p<0.05$ when No MODS and MODS groups are compared with Students T Test.

Figure 9.4: The relationship between Gamma delta Low cells and Natural Killer Dim cells



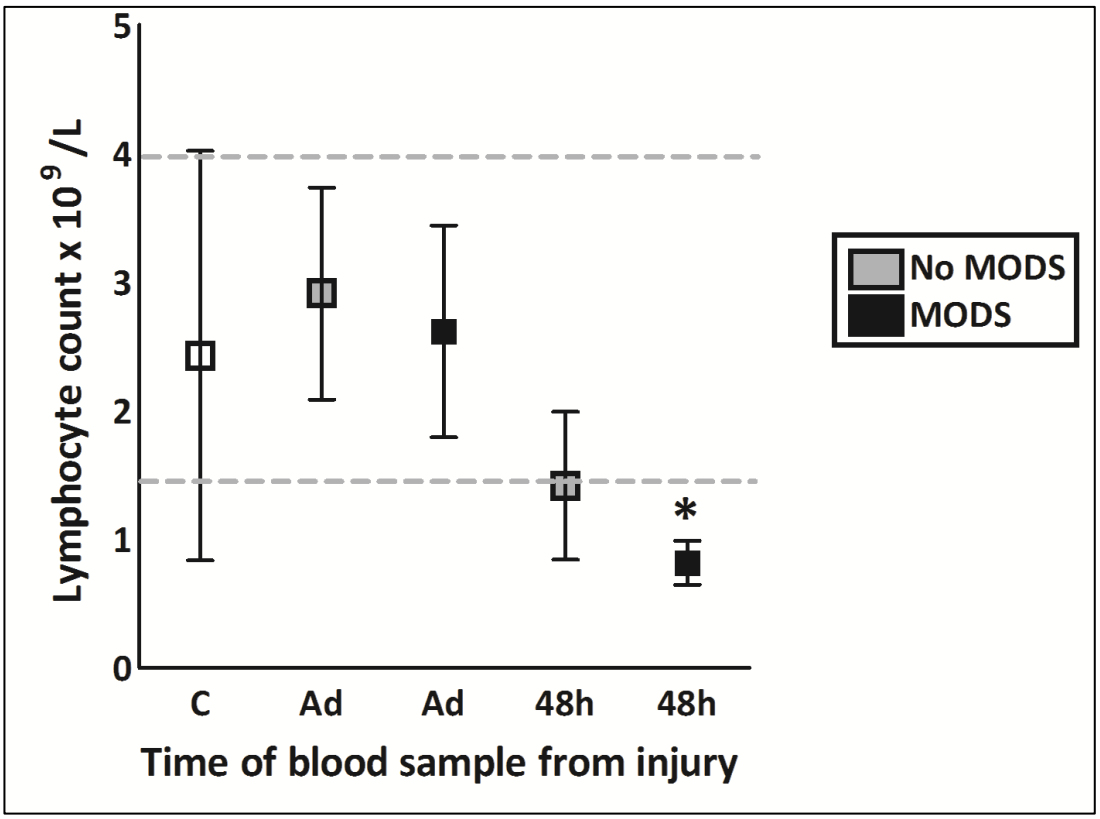
Trauma patients with injury (n=30) were categorised into two groups: No MODS (n=19) during admission or developed MODS (n=11) during admission. The population of NK Dim and GD Low cells in these patients was examined. All patients who developed MODS had low percentage population of GD Low cells. Most had high levels of NK Dim cells.

Figure 9.5: Ratio of NK Dim to GD Low cells



Ratio of the number of NK Dim cells divided by the number of GD Low cells in patients who did and did not develop MODS (the 'No' cohort n=19, the 'Yes' cohort n=11). Controls ('C') were patients with ISS 0-4 and normal BD. C: 109.3 (-82.0-300.5), No: 52.5 (-6.1-111.2), Yes: 64.2 (4.31-124.2), p=0.04 Data are displayed on a natural log scale as mean with 95% CI and values reported as geometric means (95% CI). * represents $p<0.05$ when No and Yes groups were compared with a Students' t test.

Figure 9.6: Relationship between status at admission and lymphocyte count during recovery



Lymphocyte counts were obtained from the full blood count samples, processed by the hospital laboratory, for the patients included in the FACS study (n=40). Lymphocyte counts, at admission and at 48h, were examined in the No MODS (n=19) and MODS (n=11) groups. At admission, all patients had lymphocyte counts within the normal range, including the controls (C). At 48h from admission, patients who developed MODS during their admission had a lower lymphocyte count than those who did not. Admission: Controls (C): 2.4 (1.2-3.7) $x10^9$, 2.9 (2.1-3.7) $x10^9$, 2.6 (1.9-3.3) $x10^9$, p=0.62. At 48h: No MODS=1.4 (1.0-1.9) $x10^9$, MODS=0.8 (0.7-1.0) $x10^9$, p=0.01. Data are presented as mean (95% CI) and * denotes $p<0.05$ when No MODS and MODS were compared with Students t test.

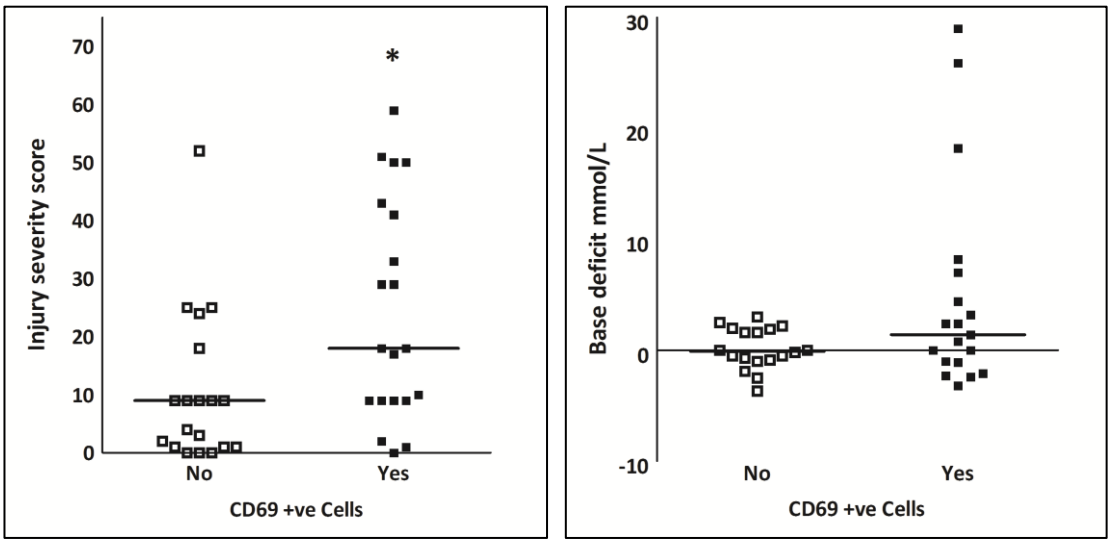
b) ACTIVATION STATUS

Early activation status of the lymphocytes was assessed by measuring CD69 expression. The CD69 expression of the cells in the 'Control' sample (containing the negative isotype contol antibody) were subtracted from those in the 'Test' sample for each patient, to identify the percentage of cells which were CD69 positive. Analysis was limited to the NK Dim and GD Low cells due to their association with clinical outcome (Table 9.3).

i. Natural Killer Dim Cells

Just over half of the patients examined (57%), expressed CD69 to a greater or lesser degree. Within this group, only a very small proportion of cells were CD69 positive when compared to the isotype control; all patients had levels below 5%. Patients with CD69-positive and patients without CD69-positive cells were compared. Those with CD69-positive NK Dim cells had higher injury severity scores but no difference was observed for base deficit (Figure 9.7).

Figure 9.7: CD69 expression in NK Dim cells

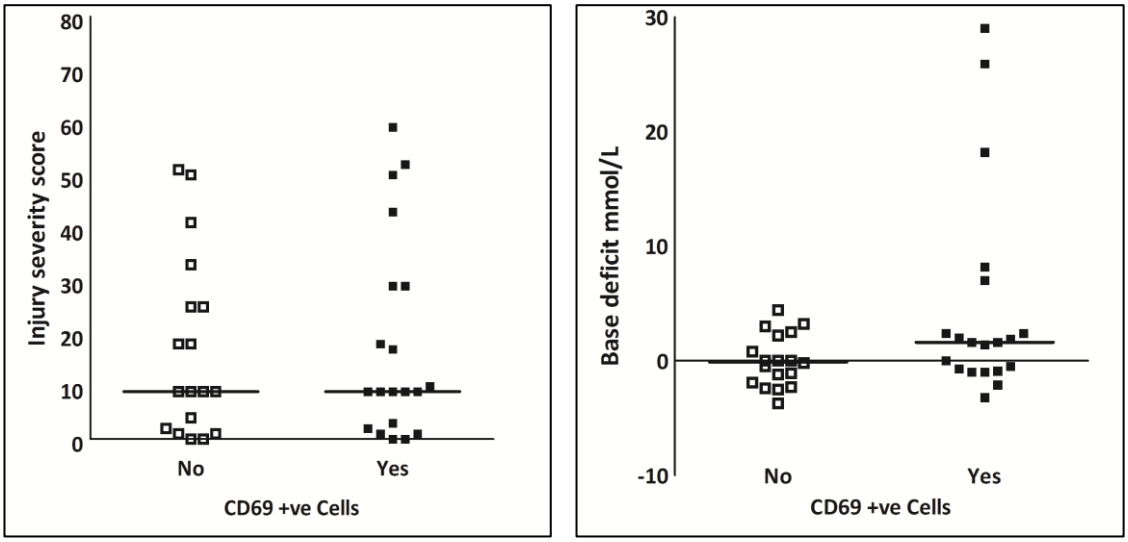


Patients with CD69 positive NK Dim cells (Yes) were compared with patients who did not have any CD69 positive NK Dim cells (No). Injury severity score: No = 9 (1-14), Yes = 18 (9-42), $p<0.05$. Base deficit: No = -0.1 (-1.7 – 1.9), Yes = 1.6 (-0.8-3.6), $p=0.12$. Data are presented as median(IQR) and Mann Whitney-U test was applied. * denotes $p<0.05$.

ii. Gamma Delta Low Cells

Patients with CD69 positive GD Low cells were compared with patients who did not have any CD69 expression on their GD Low cells. No difference in ISS or BD was observed between the two groups (Figure 9.8).

Figure 9.8: CD69 expression in GD Low cells



Patients with CD69 positive GD Low cells (Yes) were compared with patients who did not have any CD69 positive GD Low cells (No). Injury severity score: No = 9 (3-25), Yes = 9 (3-29), p=0.89. Base deficit: No = -0.1 (-0.9 – 1.8), Yes = 1.4 (-1.1-5.7), p=0.20. Data are presented as median (IQR) and Mann Whitney-U test was applied. * denotes p<0.05.

This study demonstrates that early activation of NK Dim cells occurs within 2h of injury. Patients with CD69 activation had higher injury severity scores but showed no difference in BD. This analysis is limited by the small number of patients and the fact that the most severely injured, frequently also had co-existing blood loss, making it difficult to isolate a clinical cause for these observations. Nonetheless, the findings raise the possibility that mechanical tissue damage may activate a cytotoxic cellular response and this requires further investigation.

Table 9.3: Cell activation status - % of CD69 positive cells

Patient	NK Dim	GD Low	ISS	BD
1	n/a	n/a	16	-2.1
2	n/a	n/a	10	-1.4
3	n/a	n/a	20	7.9
4	n/a	n/a	45	3.8
5	1.0	0.0	18	-0.7
6	0.0	0.3	3	N/D
7	n/a	n/a	9	1.2
8	0.0	0.0	1	-3.7
9	0.3	0.8	10	1.4
10	1.0	0.2	17	-2.1
11	0.0	0.0	25	2.2
12	0.4	0.0	51	1.6
13	0.0	0.1	52	4.4
14	0.0	0.0	9	-0.5
15	0.6	0.0	2	1.9
16	0.0	1.2	1	-2.4
17	0.0	0.4	9	0.8
18	0.0	0.0	18	-2.5
19	0.3	0.0	41	1.6
20	0.0	0.5	2	-2.3
21	0.0	0.0	25	2.5
22	0.0	0.0	1	-1.9
23	0.0	n/a	24	-1.2
24	1.6	4.4	43	18.2
25	0.6	2.4	29	7.0
26	0.0	0.0	9	3.0
27	0.3	0.0	9	-0.5
28	0.0	2.8	9	-1.1
29	0.1	0.7	59	2.4
30	1.2	0.0	33	2.0
31	0.2	1.6	9	2.4
32	1.7	0.0	9	-1.0
33	4.7	1.1	1	-1.0
34	4.0	1.0	18	-3.2
35	0.6	0.0	50	-0.9
36	1.2	2.5	50	29.0
37	0.0	0.0	9.0	3.2
38	0.1	0.6	29	25.9
39	0.1	2.5	9.0	8.2
40	0.0	0.0	4.0	-0.2

5. DISCUSSION

This study demonstrates that within 2h of injury, the subset of trauma patients who develop infections or MODS during recovery, have demonstrably different populations of circulating lymphocytes when compared to controls or patients who recover uneventfully. An elevation in the percentage population and absolute number of circulating NK Dim cells was associated with the development of clinically relevant infection episodes. The development of MODS was associated with elevated percentage populations of NK Dim cells and, in addition, a reduced percentage population of γδ Low cells. These findings demonstrate that trauma patients with poor clinical outcomes already have a different immunological response at the time of admission to hospital and before significant iatrogenic intervention.

This is the first study to investigate lymphocyte responses at such an early time point after trauma. In addition, many of the lymphocyte subsets, namely γδ cell subsets and NK cell subsets, have not previously been examined in human trauma patients. The findings suggest that a lymphocyte profile may enable early identification of patients at risk of poor outcome. They also raise the possibility that lymphocytes participate in the pathogenesis of poor outcome after traumatic injury.

Patients who developed infections and MODS had a high percentage of circulating NK Dim cells, within two hours of injury. NK Dim ($CD56^{Dim}$) cells, differ from $CD56^{Bright}$ cells by their expression of surface receptors including the Fcγ receptor III, Killer cell Ig-like receptor (KIR), CD16, chemokine receptors and adhesion molecules, which likely influence their functional phenotype as potent cytotoxic cells (Cooper et al, 2001). The mechanism of their cytotoxicity involves exocytosis of granules, containing enzymes such as perforin and granzyme, which can occur via direct synapse with target cells or remotely via membrane remodelling (Trinchieri, 1989, (Brown et al, 2011a). NK Dim cells, circulate in blood and their function is regulated via a balance between surface receptor inhibition and activation (Luci and Tomasello, 2008). NK

Cells are known to be activated in conditions of cellular 'stress', via their NKG2D receptors (also known as NK receptors); NKG2D ligands include molecules such as MIC-A (MHC-Class 1 chain-related A), MIC-B, ULBP (UL-16 binding protein) and BAT 3 (HLA-B-associated transcript 3) (Luci and Tomasello, 2008). Upregulation of these molecules has been demonstrated *in vitro*, in circumstances including cancer transformation, heat shock and viral infection, although with some inconsistency (see reviews by (Vivier et al, 2002, (Luci and Tomasello, 2008). The mechanism remains incompletely understood, since more recent work has demonstrated that the principle stimulus for NKG2D ligand upregulation is DNA damage (genotoxic stress), and not cancer transformation per se, in isolation or in combination with hypoxia, heat shock, acidosis or cytokine exposure (Gasser et al, 2005, (Gasser and Raulet, 2006). In cell culture and murine models, NK cell receptor activation by PAMPs such as LPS and flagelin has also been demonstrated (Arase et al, 2002, (Tsujimoto et al, 2005). The clinical significance of these cells has been demonstrated in a murine model of LPS sepsis, where NK cell depletion was associated with greater survival (Heremans et al, 1994). Within the context of traumatic injury, only one study, examining the role of NK cells could be identified. In this study, a murine model of trauma haemorrhage and induced 'second hit' sepsis (via caecal ligation and puncture), depletion of NK cells was associated with improved survival (Barkhausen et al, 2008). The findings of this study imply that NK Dim cells play a role in the pathogenesis of MODS and the development of infection.

The 'γδ Low' cell population consists mostly of Vγ2 cells and the γδ High cells, mostly of Vγ1 cells (Hayday, 2000). The Vγ2 cells are thought to be principally located within lymph nodes and peripheral blood; the Vγ1 cells principally within tissues. Tissue localization and specific functions of each subset are incompletely understood; however, δγ cells in general are known to play a significant role in the detection of cellular 'stress' and regulation of the subsequent effector cell response (Hayday, 2009). Similar to NK cells, ligands for the γδ TCR receptor include 'stress' signal molecules like MIC-A and endogenous pyrophosphates and

phosphoantigen molecules such as IPP (isopentenyl pyrophosphate) (Hayday, 2009, (Hayday, 2000, (Bonneville et al, 2010). In addition, γδ cells can be directly activated by pathogens, including e-coli (LPS) via conserved surface motifs (PAMPs) and mycobacteria via metabolites such as HMBPP ((E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate) (Hedges et al, 2005, (Bonneville et al, 2010). Activation results in rapid production of cytokines such as IFNγ, which likely originate from a tissue based CD27+ subset (Ribot et al, 2009). The effector functions of γδ cells include the destruction of infected or transformed cells via 'death-receptors', cytotoxic enzyme release and production of cytokines (such as IFNγ, TNFα, and IL-10) which play an immune-regulatory role (see review by (Bonneville et al, 2010). This study demonstrated that patients who developed MODS, after 48h from injury, had reduced percentage populations of γδ Low cells at admission. As the exact function of this subset is unclear and no previous study has investigated γδ cells in this clinical setting, the findings are difficult to interpret. In the absence of evidence, we are left to speculate whether alarmin release within injured tissues influences resident γδ cells. Whether low circulating levels of γδ Low cells occur due to sequestration in tissues, apoptosis, increases in other cell populations or other molecular events, as yet remains unclear. The study findings, however, provide initial evidence to support a role for γδ cells in the immediate immune response to traumatic injury.

Despite robust sample recruitment and standardised methodology, several limitations of this study must be taken into account. Of greatest importance is the fact that the patients who developed MODS and infection during admission were significantly different from the patients who recovered uneventfully, in terms of injury severity and the extent of BD. The reasons behind the different immune responses cannot therefore be determined. Although a study group of 40 patients is an acceptable number for an exploratory study, it is a small cohort and the heterogeneous nature of the injuries limits the ability to draw strong associations between the findings and particular injury characteristics or outcomes. Recruitment of patients was influenced, in part, by convenience sampling due to availability of trained staff and laboratory

equipment. Finally, no functional assessment was conducted during this study and cell numbers or percentage populations may not accurately reflect the pathophysiological function for these cells during the immediate post-injury phase.

In conclusion, this is the first study to examine the early lymphocyte response to traumatic injury in such detail. In a small group of trauma patients, this study demonstrated an association between the phenotype of circulating blood lymphocytes within 2h of injury and adverse clinical outcome. The phenotype, with increased NK^{Dim} cells and reduced GD^{Low} cells, was observed in a population of patients with more severe injuries. Whether this characteristic forms part of the standard response to severe injury or only occurs in the sub-group who develop poor outcomes, therefore remains unclear. The findings suggest that poor outcome may be related to immunological events that are already in play at the time of admission to hospital, before significant intervention. This study opens up new lines for future investigation to determine the relationship between lymphocytes and the pathogenesis of MODS or susceptibility to infection after injury.

Part 5: Discussion

CHAPTER TEN: DISCUSSION

1. INTRODUCTION

This thesis reports the findings from an investigation of the hyper-acute immune response to traumatic injury. This is the first study to investigate the immune response with a specific focus on blood samples taken within two hours of injury and prior to in-hospital iatrogenic intervention. The immune response was examined from three perspectives. The first was inflammation generation, as evidenced by cytokine production; the second, considered inflammation activation and trauma 'alarmin' molecules. Finally, the inflammation response was assessed by examining the leucocyte populations, with a focus on lymphocytes.

2. WHAT NEW KNOWLEDGE HAS THIS WORK DEMONSTRATED?

As this is the first study to focus on such an early time point after injury, a number of novel findings have been demonstrated. By examining injured patients, without evidence of hypoperfusion from blood loss, I was able to demonstrate that isolated tissue damage results in elevated levels of IL-6, IL-8 and IL-10, within 2h of injury. The levels were proportional to the severity of the injuries sustained and showed association with clinical outcome measures. The results suggest that tissue damage alone can activate an inflammatory response of sufficient magnitude to result in poor outcome, although a causal relationship was not demonstrated. Injured patients with co-existing haemorrhagic shock had greatly augmented cytokine concentrations, but I was unable to distinguish the separate influences of tissue damage and blood loss. The immune response after injury is dynamic and knowledge of the time between injury and blood draw is therefore essential for interpretation of the cytokine

concentration. Although they remain a valid measure of immune activation, the utility of IL-6, IL-8 or IL-10 as biomarkers of injury is questionable. The number of patient factors which influence their peripheral blood concentration are so numerous that clinical interpretation is very difficult. The mechanism(s) of cytokine production after injury also remain unclear.

The second part of this study, demonstrated that intracellular proteins can be measured in peripheral blood within 2h of injury. After isolated tissue damage, circulating levels of Cytochrome B, a mitochondrial protein, are proportional to the severity of the injury sustained. Injury and co-existing haemorrhage were associated with a rise in HMGB1 and nucleosome levels. The presence of nucleosomes in the circulation implies that cellular necrosis occurs in patients with severe shock. The identity of the cells which become necrotic is unclear. There was only a weak association between circulating concentrations of the measured alarmins and cytokines. The findings largely support the concept of the Danger Theory which suggests that release of endogenous intracellular proteins activates an immune response (Matzinger, 2002). The clinical relevance of these particular molecules is not clear and their use as biomarkers of injury severity cannot currently be supported. The exact molecules responsible for immune activation therefore, remain elusive and further investigation on this subject is warranted.

The final section, examined the white blood cell response to injury. The study demonstrated a relationship between the lymphocyte population and adverse clinical outcome after traumatic injury. At admission, lymphocytes are within normal range for all patients, regardless of injury severity. Circulating lymphocyte populations then fall dramatically in injured patients. The study demonstrates that those patients who continue to have low lymphocyte populations in their peripheral blood have worse clinical outcome. Whether this is cause or effect is unknown. In addition, the patients have higher levels of injury and blood loss than patients who recover uneventfully, therefore the reasons for this response remain unclear. Lymphocyte

subsets, examined at admission, reveal larger populations of cytotoxic NK Dim cells in patients who developed infections and/or MODS. In addition, patients who developed MODS also demonstrated low percentages of γδ Low cells, which are thought to be cyto-protective. The findings imply that lymphocyte phenotype at admission, prior to intervention, may influence patient recovery but this requires further study in a larger group of patients.

This study provides further evidence for the relationship between immune system activation and poor clinical outcome. The most significant novel finding was an association between adverse clinical outcome and a particular lymphocyte phenotype. Those with poor outcome had higher injury levels but it suggests, for the first time, that adverse outcome after traumatic injury may be related to immune system events initiated before hospital admission and prior to intervention. Although these findings require substantial further investigation, they raise some important new concepts. Firstly, they suggest that blood lymphocyte phenotype may potentially be used for early identification of patients at risk of poor outcome. Secondly, that therapeutic intervention may need to focus on the pre-hospital phase, if improvements in clinical outcome are to be achieved. If further studies support my findings, this would be a significant jump forward in our current understanding of the pathogenesis of MODS and susceptibility to infection after traumatic injury.

3. STRENGTHS OF THIS INVESTIGATION

The major strength of this investigation is its location, experimental design and recruitment protocol. The Royal London Hospital (RLH) is recognised as the UK leader in terms of traumatic injury research (Morgan Jones M, 2011). The experimental work was conducted in a laboratory facility with appropriate supervision to ensure technical success. When the appropriate expertise was not available within the Blizard Institute, collaboration with specialist units was achieved.

Although, the ACIT2 study has national and international recruitment partners, this investigation only utilised samples from the RLH. They are recruited within a single centre, with standardised pre-hospital care and resuscitation protocols. This optimises the comparability of the patients enrolled into the ACIT2 study. The use of a pLAR deferred consent process is now well established within the RLH and facilitates early blood sampling prior to intervention. It is a process which is supported by our colleagues, patients and relatives. Only a small number of patients or relatives refused participation when later approached for written consent. Using samples prior to intervention enabled me to work with samples 'uncontaminated' by iatrogenic intervention. Conducting an investigation of this nature is hard work as recruitment of patients and rapid processing of samples is very labour intensive. One of the most important qualities of the study is the strength of the clinical follow up, conducted by the trauma research team doctors. Daily assessment ensured that outcomes are collected prospectively and are clinically relevant. The second is the capture of patient samples at admission and prior to in-hospital intervention, which is the really unique aspect of this investigation.

The general demographic of the study population was 75% (363/483) male, of which 81% (390/483) had a blunt mechanism of injury and 74% (359/483) were less than 50 years old. Large numbers of trauma patients were included in this investigation but good patient characterisation is essential in human research, to enable reasonable comparisons to be made. Using ISS for injury and BD for haemorrhagic shock, robust and reproducible patient groups were defined. The use of a 'no injury or minor injury' group as a control population was also beneficial. Both of these methods have limitations, but I believe hold great benefit. Characterisation was also optimised by using pre-defined time-points for patient samples. This investigation therefore significantly improves upon previous work in this field.

Obtaining blood samples at admission is a key strength of this investigation. The ACIT2 sampling protocol, robustly captures the patient's immune response to traumatic injury while avoiding, as far as possible, confounding influences from injury management and surgical procedures. As a result of doctor led pre-hospital care, some intervention inevitably does occur in the pre-hospital setting, particularly for the most severely injured patients. Obtaining blood samples at scene would be the only way to avoid all intervention, but even within the London Trauma System, this is impractical at the current time. The interventions which may occur prior to blood draw include surgical procedures and administration of blood products and/or crystalloid fluids. Only a very small group of patients require surgical intervention, usually a clam-shell thoracotomy, for life-threatening conditions at the scene. If surgery precedes blood draw, the patients can be identified and, if necessary, retrospectively excluded from analysis. No patients with surgery prior to blood draw were included in this investigation. Secondly, blood product administration is documented fastidiously and, when necessary, patients with transfusion prior to blood draw were excluded. Finally, because of the influence on acute traumatic coagulopathy, administration of crystalloid fluid is actively minimised. Although the maximal allowance for ACIT2 eligibility is 2L, less than 50% of the study cohort receive >250mls and, of those administered >250mls, the median(IQR) volume was 500(500-1000) mls. While the effects of crystalloid fluids on inflammation, haemo-dilution and base deficit are acknowledged, the volumes administered are considered to be unavoidable. It is unlikely that the volumes of fluid administered had a significant impact upon the quality of the blood samples drawn on arrival to hospital, but more sensitive analysis than that conducted in this study would be necessary to determine whether this is true.

4. WEAKNESSES OF THIS INVESTIGATION

The limitations of each individual investigation have been discussed in the relevant chapters; however, there are some global aspects which are also worthy of mention. From the perspective of patient recruitment, we inevitably failed to recruit some of the most severely injured patients. Blood draw for research is a secondary priority when patients have arrested, or are peri-arrest and intravenous access for blood transfusion is an urgent requirement. We recruited as many critically injured patients as feasibly possible for ACIT2, to ensure that patients with life-threatening injury were represented in the investigations. The experiments for this investigation were conducted over the course of three years. In an ideal world, all of the experiments detailed in this thesis would have been performed on the same patient samples; however, as the numbers of patients recruited to ACIT2 increased over time, the later experiments have larger cohorts. Similarly, the early patient samples have limited SOFA score data and later patients have more reliable prospective follow-up. These elements reinforce the importance of study design in scientific investigations but while it is important to acknowledge limitations, these aspects do not detract from the key findings.

The tissue damage analysis is limited by the reliance upon ISS to define injury severity. Although well accepted and universally used, this anatomical score is unable to describe the extent of tissue damage accurately. This was counteracted by excluding all patients with shock, defined by a BD >2mmol/L, but does lead to the exclusion of some of the most severe patients. In the absence of a more suitable defining parameter, it is the only surrogate option. As a result, difficulties arise in this investigation when attempting to distinguish between the influence of shock and the influence of tissue damage on inflammation. Analysis with matched ISS and BD severity was used in the cytokine analysis; but, this is not ideal as patients have to be semi-selected. The root cause of the inflammation is therefore difficult to determine. Until biomarkers for tissue injury are identified, this situation will remain. Larger numbers of

patients may enable stronger comparison of non-shocked and shocked cohorts of matched levels of injury in future work.

The lymphocyte investigation is limited by the absence of mechanistic work or functional assessment, as changes in cell number do not necessarily reflect cell function. Lymphocytes are currently thought to play an active role within tissues and not peripheral blood; however, this makes the study findings all the more intriguing. More work is required to understand the cellular events in play during this early post injury phase.

Good patient characterisation is key to deciphering causal relationships and understanding the events in the early immune response. The key characteristics include age, gender, ISS, BD, time from injury, fluid administration, blood product administration and surgical intervention. Although study results could not always be adjusted with respect to these paramaters, they were always reported with each experiment. Some characteristics, which are known to influence the inflammatory response, were not recorded for the ACIT2 study. These include: race, height, weight, body mass index, temperature and sickle cell status. A relationship between race, inflammation and Duffy blood group status has been reported. Duffy negative patients, who are principally of African descent, have demonstrated reduced cytokine production after LPS administration (Mayr et al, 2008). No studies have examined the significance of Duffy antigen in trauma. Future studies may benefit from including these parameters. Examining markers of bone marrow activation may have also been of benefit.

Establishing causal relationships for the findings of this investigation would have required more sensitive statistical analysis than those performed. Where multiple comparisons were made, the accepted p-value was reduced to <0.01 but Bonferroni corrections were not calculated. As our appreciation for the complexity of biological systems increases, a reductionist approach cannot always be applied. There is increasing need for the use of computer modelling to help determine the significance of changes within protein networks

and cellular interactions. Computer modelling was not employed in this investigation but is likely to be of benefit to future work.

5. FUTURE WORK

Advances in resuscitation and initial management have improved survival in the early phases of trauma. Improving outcomes for the critically unwell is the next goal. Future research is required to decipher the molecular events after traumatic injury and determine whether we can intervene to improve patient outcome.

a) ALARMINS

Intracellular alarmin proteins remain potential candidates for injury biomarkers and therapeutic targets. The current study examined only a handful of likely candidates. Broad screening of trauma patient plasma, with mass spectroscopy, may enable a wider search for alarmin candidates. This has already been attempted, but a wide variety of sample time points and patient characteristics limit interpretation of the findings (Liu et al, 2006). It is likely that shock and mechanical tissue damage release different alarmin substances and may activate the immune system via different mechanisms. Good patient characterisation is therefore essential. With large patient numbers, proteomic studies may also be of benefit.

The identification of free circulating nucleosomes in patient plasma implies that there is cellular necrosis in patients who have a base deficit above 6mmol/L at admission, as a result of severe haemorrhagic shock. Necrosis itself can activate an immune response. The cells which undergo necrosis have not been identified. Necrosis of lymphocytes has been proposed; however, the evidence for this is unconvincing (Kasten et al, 2010, (Hotchkiss et al, 1999a). Confirming the presence of cell necrosis during shock and the identity of the cells which undergo necrosis may be a step forward in the search for trauma alarmins. Capturing a

dynamic process may be difficult but in principle, flow cytometric analysis of timed blood samples, using Annexin V and other necrosis markers, may be successful.

b) LYMPHOCYTES

The NK Dim cell and γδ T cell populations require future study in human trauma patients. Initially, this should entail an examination of their phenotype and behaviour during admission. An assessment of their functional status at this time point would also be important. Recruitment of large patient numbers may enable comparison of patients with matched injury severity to examine the influences of tissue damage and shock on their behaviour; although, accepting the limitations of this approach. The current study implicates admission lymphocyte behaviour in the pathogenesis of poor outcome. Future work should also consider examination of the cell populations at later time points, to determine whether lymphocyte phenotype alters during patient recovery and how phenotype relates to outcome.

Lymphocytes are thought to exert their functional roles within tissues. To understand why the admission lymphocyte profile is related to outcome, their function must be assessed. This investigation would require use of animal models of traumatic injury. It is unclear why the peripheral lymphocyte population falls by 24h after injury. Animal models may be able to demonstrate that the cells move from circulation into tissues using histology. The behaviour of each lymphocyte subset should be examined. Examination for apoptosis or necrosis is also required. Finally, specific examination of NK Dim cells and γδ T cell function within tissues and peripheral blood may advance our understanding about why their circulating populations are associated with adverse clinical outcome.

I have not investigated anergy during this project, but this phenomenon also requires further investigation to understand its clinical significance. Examination of function in all cell subsets at

different time points would be laborious but useful to determine the incidence and implications of this condition. Functional assessments would be required immediately after blood draw and after set intervals to determine whether cytokine production can recover with time.

6. GLOBAL LANGUAGE OF TRAUMA RESEARCH

Only a small number of centres worldwide undertake traumatic injury research. Those with an interest in inflammation are few. Universal adoption of descriptive patient characteristics and sampling time points would be helpful to optimise comparison between studies. Description of the extent of shock using base deficit or lactate is an essential inclusion, which is often overlooked. Trauma research is also hampered by a lack of agreement about the definitions for appropriate outcome measures. In other specialities, agreement has been achieved by consensus and trauma research may benefit from a similar process in due course.

7. FINAL COMMENT

The immune response is a dynamic process. Despite all we know about immunity, it is still unclear what a “normal” or “healthy” immune system looks like (Hayday and Peakman, 2008). It is also unclear whether patients are able to have a ‘good’ or ‘bad’ response to injury. The findings of this study demonstrate that the inflammatory response to trauma is already in full swing at 2h from injury. It also demonstrates that even after isolated tissue damage, there is inflammation of sufficient ferocity to be associated with mortality, MODS and infection. Endogenous intracellular molecules are detectable in peripheral blood by 2h from injury, but precisely which molecules activate the immune system in this clinical setting remains unclear. The most novel finding however, is that patients with poor clinical outcome, had a different lymphocyte response at 2h from injury than those who did not. This concept requires further study in patients of comparable injury severity, but it also opens up new lines for investigation. If proven to be correct, it may change our understanding of the pathogenesis of MODS and susceptibility to infection after traumatic injury.

A future of personalised medicine within traumatic injury is not simply a fanciful idea but one which holds real promise for clinical outcome improvement. Although no biomarker measurement will ever replace the need for urgent restoration of the airway, breathing and circulation, bedside testing may enable delivery of personalised therapeutic adjuncts to patients which improve their response to injury. I remain hopeful that, in time, we shall be able to interpret the inflammatory parameters of trauma patients, much like a blood gas analysis or laboratory blood test, and implement appropriate action to improve their outcome.

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Appendix 1

LONDON'S AIR AMBULANCE
roadside intensive care

DISPATCH OF THE PRE-HOSPITAL TEAM

REVIEW:	September 2010
APPROVAL/ ADOPTED:	PHC Policy Board
DISTRIBUTION:	PHC Doctors, PHC Paramedics, PHC Fire Crew, PHC Pilots
THIS DOCUMENT REFERS TO:	PHC Clinical Practice, Non-clinical Practice, Operational Procedure

Aims

Describe the procedure for interrogating calls

Identify the correct categorisation of calls for audit purposes

Describe the procedure for dispatching the aircraft

Describe the procedure for dispatching DA77 (HEMS car)

Background

The dispatch process is pivotal in the success of the London HEMS system. Identifying the clinically vulnerable patient at an early stage and dispatching an appropriate resource is essential to ensure good outcome. Appropriate dispatch avoids unnecessary cost through inappropriate aircraft and vehicle movements.

HEMS dispatches are defined in three ways.

1. Immediate dispatch
2. Interrogated dispatch
3. Crew Request

Immediate dispatch criteria are those where little further information could be obtained; the Mechanism of injury predicts where there is a need for HEMS intervention; or that high injury levels are common. Requests for HEMS from other emergency services are treated as immediate dispatches.

Interrogated calls are those that the flight paramedic in EOC identifies (through questioning the 999 caller) as requiring the extended skills of the Physician/Paramedic team.

Crew requests are those that the Flight Paramedic has decided are not for HEMS (or that they have missed), that an ambulance crew have decided would warrant extended intervention.

Policy

1. Immediate dispatch criteria and procedure

- Fall from greater than 2 floors (>20 feet)
- 'One unders' (fall or jumped in front of a train)
- Ejected from vehicle
- Death of a same vehicle occupant
- Amputation above wrist or ankle
- Trapped under vehicle (not motorcycle)
- Request from any other emergency service (including the RNLI/Coastguard)

As soon as any of the above criteria become apparent, either on the call taking screen or through monitoring the call, HEMS or DA77 are dispatched. Further interrogation may be appropriate if the car has a great distance to run or there is evidence of hoax or misinformation.

The relevant dispatch criteria box is ticked on the Tasking Data Sheet and the reason noted in the free text box. The target for immediate dispatch is 3 minutes. If this time is exceeded, reasons should be noted on the tasking data sheet.

2. Interrogated dispatch criteria and procedure

- Shooting
- Stabbing
- Explosions
- Road Traffic Collisions

- Industrial accidents/incidents
- Hanging
- Drowning
- Entrapments
- Amputations
- Burns/scalds
- Building site accidents
- Falls from height less than 2 floors
- Impaled on an object

The above list is not exclusive and anything that appears serious should be interrogated.

Interrogation can be in two forms. The passive or 'silent' interrogation is when a decision to dispatch is reached through monitoring the call in progress because the information obtained is sufficient for the medical team to be dispatched. Active interrogation is when the 999 caller is re-contacted or the call is passed to the flight paramedic from the call taker. In this instance, the paramedic will ask a series of questions in order to get a clear picture of whether HEMS resources are required. The interrogation structure is deliberately left 'loose' in order that the paramedic can adapt their questions appropriately. The skill is to be able to obtain a mental picture of the scene through piecing together all the information that is available. However, there a number of suggested questions that can guide staff or are used to identify the potential quality of the informant.

The Initial Questions

When you connect with the caller, introduce yourself:

- 'Hello, you are talking to a paramedic in the control room. I need to ask you a few more questions and I am not delaying help, which is on its way'
- Are you still on scene?
- Can you see the patient?

If the answer to these is negative and the caller cannot quickly reach the patient and there are other calls for the same incident, then quit the call and ring the next caller.

- Can you quickly tell me what happened?
- How many casualties are there?
- Where is the patient now? Have they been moved from scene (quite common with young children).

Incident specific mechanism of injury questions

- How far have they fallen?
- What surface has the patient fallen on
- Are they trapped (physically or relatively)
- How fast were they going?
- What has the vehicle hit?
- Was the pedestrian thrown through the air?
- How far is the pedestrian/ejected occupant from the vehicle
- Has the patient gone under the vehicle?
- Has the pedestrian caused any damage to the car – in the windscreen intact?
- Is the shooting/stabbing victim's assailant still on scene?
- How were they burnt / scalded?

Patient condition questions

- Is the patient awake – are they moving?
- Is the patient breathing normally? Is it noisy?
- Is the patient talking?
- If they are talking - do they make sense?
- Is there much bleeding?
- Does the patient know where they are?
- Is the patient still where the impact/injury occurred or have they moved?
- Does the patient obey commands – ask the caller to get the patient to squeeze their hand, touch their nose or similar
- Are there any obvious injuries?
- Has the patient moved all four limbs?
- Has the motorcyclist removed his crash helmet?
- Where in the body has the victim been shot /stabbed?
- Is the shooting/stabbing victim standing up or lying down?
- How much of the body has been burnt (nearly always overestimated by caller)
- Where are the burns?

When interrogating callers, consideration should be given to the fact that they may be distressed, confused, angry or frightened. Reassurance must be given that help is on the way and that further questions will not delay this. The interrogation style will need to be adapted to be the most appropriate for each occasion.

If a 999 caller happens to have medical or first aid training, the questioning style should be adapted appropriately – asking about GCS, signs of shock, respiratory function etc.

Interrogated dispatches are recorded in the relevant box on the tasking data sheet with the code number for the reason also noted. Interrogated dispatch criteria are found on the bottom of the tasking data sheet. Reasons for dispatch should also be noted in the call log

The dispatch target for interrogated calls is 7 minutes. Any breech of this target should be noted in the free text box on the tasking data sheet and on call log

3. Crew Requests

A crew request is simply an incident that an ambulance crew or FRU require the medical team's attendance. For auditing purposes, it is important to define what constitutes a crew request:

If the flight paramedic is interrogating a call and there is insufficient information (single caller, language barrier, too distressed, mobile phone switched off), then the paramedic can ask for a report from the first crew on scene. If they confirm the need for HEMS attendance, then the call is recorded as an interrogate. Likewise, if the first crew arrive whilst interrogation is taking place and they request HEMS presence, then that is an interrogated call.

If the Flight paramedic has interrogated a call, decides there is no reason to activate the team and an ambulance resource then asks for HEMS; that is recorded as a crew request. If the Flight Paramedic is not aware of a call (busy with other calls, out of the room, little or no information on the call log to indicate it is a potentially HEMS call) and a crew asks for HEMS, then it is recorded as a crew request.

4. Crew Reports

Crew reports (either ambulance or Fast Response Unit) can be a useful source of information for the EOC paramedic for either dispatching the team or cancelling where necessary. It is far better to speak to the ambulance crew direct rather than through the sector desk that controls that resource. Therefore, it is important to ask for a report on the HEMS channel (PD36 or PD09). The request for a report can be done using the electronic log for that specific call.

The FRU staff have mobile phones assigned to each vehicle. It is worth ringing an FRU for a report once they are on scene.

It is worth noting that a crew report should not be seen as a method of avoiding interrogation or as a 'safety net' to catch any calls that may have relevance to HEMS. There is a danger that the act of asking for a report may prompt an ambulance crew to request for the HEMS team when they would not have normally considered this. Anecdotally, it has been noticed that there is an increase of 'crew requests' for incidents that historically, would not have generated them.

Therefore, the paramedic should only ask for a report if, through interrogation, there is a specific lack of information that is needed to reach a dispatch decision.

5. Dispatching the Aircraft

When a suitable call has been identified, the activation button is pressed. This automatically dials the activation phone in HEMS operations. If the call is either a stabbing or shooting, then this must be conveyed in the opening conversation to enable the team to don the ballistic vests whilst the aircraft is starting. The following information is then passed:

- Map reference (small scale reference if there are more than one)
- Location (road names, junctions, landmarks etc)
- Nature of call (brief – 5 or 6 words)
- Dispatch criteria – immediate, interrogation or crew request.
- Time of origin of the call
- Time call passed to HEMS operations.

If the location of an incident is in that area close to the Royal London Hospital, ensure that location is not within the car response map, located on the HEMS desk. If it is, dispatch DA77 following the procedure below. All information must be added to the EOC incident log.

6. Informing the Police

The police must be informed every time the aircraft is dispatched. To contact Scotland Yard, press 'speed dial' marked police. If the call has come from the police, or there is a police CAD number already assigned to this call, use that to identify the incident. Otherwise, pass the details including the Ambulance CAD. Ensure that a police CAD number is obtained and noted, along with the time in the relevant box on the AS1.

7. Dispatching DA77 or DA99

DA77 is dispatched on the same criteria as the aircraft. However, due to the greater running times of this resource, there is a slightly different approach taken. The threshold to initially dispatch the car is deliberately lower than for the aircraft and as soon as a possible incident is identified, then the car is started on the call. As soon as the details are passed, the Flight Paramedic in EOC then commences interrogation of that call. If the incident does not warrant the team's involvement, then the call is cancelled. It is imperative that the EOC paramedic takes in to account the distance to be covered, the likely traffic conditions, and the type of incident and makes an informed decision as to whether the risk/benefit balance is acceptable. When the call is passed to the Doctor, the location is entered in to the satellite navigation unit and an ETA is worked out. This must be given to the EOC paramedic to enable them to inform the ambulance crew and place on the incident log. Communications to the car must be kept to a minimum. Clinical updates and requests for ETA's are not to be made and only those concerned with safety or changes in location should be passed.

8. Emergency Call Takers

It is of the utmost importance that the working relationship between the HEMS paramedic and the call takers is a strong one. Call takers will pass calls to the HEMS desk and these are often the best sources of information available. Ensure that feedback is given to them and encourage staff to continue this process. When calling an informant and there are multiple calls, use mobile phone numbers before land-lines as the caller is more likely to be near to the incident, or at least mobile enough to get to the patient.

Appendix 2

ROYAL LONDON HOSPITAL CRITERIA FOR ACTIVATION OF A FULL TRAUMA TEAM:

1. Vital Sign abnormality: GCS <14 / RR <10 or >29 / SBP <90
2. Person hit by a train
3. Occupant ejected from a motorised vehicle
4. Fatality in the same vehicle
5. Person trapped at scene
6. Fall >2m
7. Penetrating torso trauma (from neck to groin, proximal to the elbow or knee)
8. Chest trauma with altered physiology
9. Amputation proximal to the wrist or ankle
10. Spinal trauma with altered neurology
11. Suspected pelvic fracture
12. Suspected open or depressed skull fracture
13. Polytrauma with burns
14. At the request of the pre-hospital medical team

Appendix 3

CODE RED PROTOCOL