The impact of trauma on neutrophil function

Running title: Neutrophils and trauma

Key Words: Damage associated molecular patterns (DAMPs), sepsis, neutrophil, post-traumatic complications.

April 19th 2014

The impact of trauma on neutrophil function

Jon Hazeldine1*, Peter Hampson1,2* and Janet. M. Lord1,3.

1NIHR Surgical Reconstruction and Microbiology Research Centre, Centre for Translational Inflammation Research, School of Immunity and Infection, Birmingham University Medical School, Birmingham, B15 2TT.

2Healing Foundation Centre for Burns Research, Queen Elizabeth Hospital, Birmingham, B15 2WB, UK.
Abstract

A well described consequence of traumatic injury is immune dysregulation, where an initial increase in immune activity is followed by a period of immune depression, the latter leaving hospitalised trauma patients at an increased risk of nosocomial infections. Here, we discuss the emerging role of the neutrophil, the most abundant leukocyte in human circulation and the first line of defence against microbial challenge, in the initiation and propagation of the inflammatory response to trauma. We review the findings of the most recent studies to have investigated the impact of trauma on neutrophil function and discuss how alterations in neutrophil biology are being investigated as potential biomarkers by which to predict the
outcome of hospitalised trauma patients. Furthermore, with trauma-induced changes in neutrophil biology linked to the development of such post-traumatic complications as multiple organ failure and acute respiratory distress syndrome, we highlight an area of research within the field of trauma immunology that is gaining considerable interest: the manipulation of neutrophil function as a means by which to potentially improve patient outcome.

Acknowledgements

Jon Hazeldine is funded by the National Institute for Health Research (NIHR) Surgical Reconstruction and Microbiology Research Centre. Peter Hampson is funded by the Healing Foundation.

1.0 Introduction

As the leading cause of morbidity and mortality in individuals aged 50 years and under, traumatic injury represents a major burden on the healthcare system, costing an estimated $27 billion dollars per year in the USA alone\(^1\). Recent advancements in treatment, notably those targeting blood loss and coagulopathy, have markedly reduced mortality rates that are directly attributable to initial trauma. However, secondary complications, such as acute respiratory distress syndrome (ARDS), multiple organ failure (MOF) and nosocomial infections remain a significant cause of death in hospitalised trauma patients\(^2\). Trauma-
induced changes in immune function are thought to be key drivers in the development of these conditions, with emerging evidence suggesting that alterations in the function of one particular cell type, the neutrophil, plays a decisive role.

Equipped with a range of microbicidal defensive strategies, which include reactive oxygen species (ROS) production, degranulation, phagocytosis and the recently described generation of extracellular traps, neutrophils are the first line of defence against rapidly dividing bacteria, fungi and yeast. Across a range of time-points following traumatic injury, ex vivo studies have reported significant alterations in a multitude of neutrophil functions, which are likely to contribute to the development of secondary complications. For instance, sequestration of hyperactive neutrophils in remote bystander organs is thought to underlie the development of both ARDS and MOF, whilst reduced neutrophil function may be one explanation for the increased susceptibility of hospitalised trauma patients to nosocomial infections.

In this review, we provide an update of the most recent studies to have investigated the impact that severe trauma has on neutrophil biology and summarise studies that have highlighted a pivotal role for neutrophils in the initiation and propagation of the inflammatory response to trauma. Furthermore, we discuss data that suggests alterations in neutrophil function may serve as a biomarker for predicting the outcome of hospitalised trauma patients and evaluate whether manipulating neutrophil function has potential as a future therapeutic strategy for the treatment of trauma patients.

1.1 Impact of trauma on neutrophil biology

Although it has been known for many years that traumatic injury leads to marked alterations in the phenotype, function and life-span of circulating neutrophils, novel findings continue to be reported. Examples of these include the recent description of trauma-induced
changes in the composition of the circulating neutrophil pool\textsuperscript{16} and the observation that trauma serves as a stimulus for the generation of neutrophil extracellular traps\textsuperscript{17}. Alongside these findings, it has become apparent through the use of proteomics and microRNA profiling that trauma has a significant impact upon neutrophil signalling\textsuperscript{18-20}. In this section, we review the findings of the most recent studies to have investigated trauma-induced changes in neutrophil biology.

1.1.1 Surface phenotype

Surface expression of L-selectin, a receptor that facilitates tethering of neutrophils to the endothelium, has been shown to be significantly reduced on circulating neutrophils following blunt chest injury\textsuperscript{21}, penetrative trauma\textsuperscript{22} and traumatic brain injury (TBI)\textsuperscript{6}. Recently, Mommsen and co-workers found that stimulating neutrophils \textit{in vitro} with tumour necrosis factor-alpha (TNF-\textalpha) resulted in shedding of L-selectin\textsuperscript{23}, suggesting that exposure to a pro-inflammatory environment may be one explanation for the reduced expression of L-selectin post trauma. Expression of CD11b, which forms part of the heterodimeric integrin Mac-1 that mediates firm adhesion to the endothelium, has been shown to be significantly increased following both TBI\textsuperscript{6} and thermal injury\textsuperscript{24}. Combined with the data for L-selectin, this increase in CD11b expression suggests that systemic activation of the circulating neutrophil pool occurs following trauma.

In addition to changes in the expression of adhesion receptors, neutrophils from trauma patients display an altered phenotype with respect to chemokine receptors. Studies have reported reduced expression of the interleukin (IL)-8 receptors CXCR1 and CXCR2\textsuperscript{21,25} as well as the receptor for the complement component C5a, namely CD88\textsuperscript{21,26}. Of interest, Visser et al\textsuperscript{21} studied the surface density of CXCR2 and CD88 on neutrophils obtained from patients that had suffered isolated blunt chest injury and found a significant reduction in their
expression 3-hours post injury\textsuperscript{21}. By 24 hours, both CD88 and CXCR2 expression was comparable to that on neutrophils isolated from healthy volunteers, suggesting a transient activation of the circulating neutrophil pool\textsuperscript{21}.

Upon \textit{in vitro} stimulation with the bacterial tripeptide formyl-methionine-leucine-phenylalanine (fMLP), neutrophils from subjects that have suffered blunt, penetrative or head trauma fail to up-regulate the receptors CD11b and active FcγRII as efficiently as neutrophils from healthy controls\textsuperscript{21,27}. In the case of active FcγRII, fMLP-induced expression of this receptor correlated negatively with injury severity score\textsuperscript{27} and was significantly lower on the surface of neutrophils isolated from subjects who subsequently developed secondary complications\textsuperscript{22,27}. Thus, following trauma it appears that the responsiveness of circulating neutrophils to bacterial stimulation is suppressed.

In the studies described above, receptor expression was analysed in isolation. Recently, a small number of groups have performed multi-colour flow cytometry on patient samples and reported that traumatic injury leads to the emergence of distinct neutrophil subsets, culminating in a neutrophil pool that is heterogeneous in both phenotype and function\textsuperscript{16,21,28}. In severely injured patients, three distinct neutrophil subsets have been identified based on differential surface expression of the Fc receptor CD16 and L-selectin\textsuperscript{16}. Defined as CD16\textsuperscript{DIM} CD62L\textsuperscript{BRIGHT}, CD16\textsuperscript{BRIGHT} CD62L\textsuperscript{DIM} or CD16\textsuperscript{BRIGHT} CD62L\textsuperscript{BRIGHT} these subsets differ in their expression of adhesion receptors (e.g. CD11b, CD11c and CD54) and function, with CD16\textsuperscript{BRIGHT} CD62L\textsuperscript{DIM} neutrophils producing significantly larger amounts of reactive oxygen species (ROS) upon fMLP stimulation than both CD16\textsuperscript{DIM} neutrophils and neutrophils from healthy controls\textsuperscript{16}. Interestingly, through the robust generation of ROS, CD16\textsuperscript{BRIGHT} CD62L\textsuperscript{DIM} neutrophils were shown \textit{in vitro} to suppress both lymphocyte activation and proliferation, revealing an immunosuppressive function of this neutrophil subset that may contribute to the increased susceptibility of trauma patients to infection\textsuperscript{16}. 
Morphological assessment showed that circulating CD16$_{\text{DIM}}$ neutrophils possessed a “banded” nucleus, suggesting that trauma leads to the release of immature granulocytes from the bone marrow$^{16}$. This observation, which has since been confirmed in patients with severe systemic inflammatory response syndrome (SIRS)$^{28}$, supported a hypothesis made in an earlier study, where an influx of “young” neutrophils into the circulation was proposed to be responsible for the reduction in CD16 expression that was observed following blunt chest injury$^{21}$.

1.1.2 Chemotaxis

Recent advancements in experimental techniques mean it is now possible to study specific features of the chemotactic process such as speed (chemokinesis), velocity (chemotaxis) and persistence. Through the use of these methods, neutrophils from thermally-injured subjects have been shown to exhibit significantly reduced chemokinesis when compared to neutrophils from healthy volunteers$^{29}$. Interestingly, in this study, neutrophils from one thermally-injured patient demonstrated a level of chemokinesis that was comparable to that of neutrophils from healthy controls$^{29}$. Detailed examination of this patient revealed the presence of a bacterial infection at the time of blood sampling, leading to the suggestion that preservation of neutrophil chemokinesis post burn injury may signify an existing infection and the need to begin immediate antibiotic therapy$^{29}$.

In addition to speed, the directionality of neutrophil movement is altered following thermal injury. Kurihara and colleagues$^{9}$ demonstrated that when compared to those from sham controls, neutrophils from burn-injured rats exhibited defects in directionality during their migration towards fMLP$^{9}$. It was suggested that if replicated in vivo, this impairment would not only hamper the recruitment of neutrophils to the site of injury but would lead to
considerable bystander tissue damage as neutrophils would degranulate at sites unaffected by burn injury\textsuperscript{9}.

1.1.3 Microbicidal function

1.1.3.1 Phagocytosis and reactive oxygen species (ROS) generation

Compared to values obtained for neutrophils from healthy controls, a significant increase in spontaneous ROS production as well as ROS generation induced by fMLP and \textit{Escherichia coli} (\textit{E-coli}) stimulation has been reported for neutrophils isolated from TBI patients\textsuperscript{6,10} and individuals that have suffered spinal cord injury (SCI)\textsuperscript{7,30}. In the case of spontaneous ROS generation, this increase in oxidative activity was accompanied by a trauma-induced up-regulation of gp91\textsuperscript{PHOX}, a membrane residing subunit of the ROS generating enzyme nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase\textsuperscript{10,30}. Interestingly, both spontaneous ROS generation and gp91\textsuperscript{PHOX} expression by neutrophils isolated from TBI and SCI patients was significantly greater than that of neutrophils obtained from victims of blunt/penetrative trauma\textsuperscript{10,30}, suggesting that neurological trauma results in a more robust systemic inflammatory response. Mechanistically, this has been proposed to be the result of a loss of feedback control of immune function in TBI and SCI patients due to damage to the central nervous system\textsuperscript{10}.

In contrast to ROS production, neutrophil phagocytosis is markedly impaired following neurological trauma, with the uptake of opsonised \textit{E-coli} by neutrophils from TBI\textsuperscript{10} and SCI\textsuperscript{7} patients significantly lower than that of neutrophils from healthy controls. This decline in phagocytosis has been suggested to represent a compensatory mechanism, where reduced phagocytosis offsets the deleterious effects that the abovementioned increase in ROS generation would have on bystander tissue\textsuperscript{10}. However, reduced phagocytosis may also be detrimental to the host given that neutrophils are the first-line of defence against invading
microbes. Aberrant phagocytosis may explain in part the high incidence of nosocomial infections reported amongst patients following neurological trauma\textsuperscript{31;32}.

1.1.3.2 Generation of neutrophil extracellular traps (NETs)

Consisting of a DNA backbone studded with histones and a multitude of granule-derived peptides and enzymes such as myeloperoxidase (MPO) and NE, neutrophil extracellular traps (NETs) are a recent addition to the defensive armamentarium of neutrophils\textsuperscript{3}. Studied primarily in models of infection, NETs are renowned for their ability to capture and disarm invading pathogens\textsuperscript{3;33;34}. However, in recent years it has become increasingly apparent that NETs are also generated at sites of sterile inflammation\textsuperscript{35;36}.

To date, only one study has categorically shown NET production to be a feature of the SIRS response that occurs after injury. Using DNA staining alongside antibodies specific for histone H1 and NE, Hamaguchi et al\textsuperscript{17} detected NETs in blood smear samples obtained from patients following thermal or traumatic injury. In other studies, the measurement of circulating cell-free DNA (cf-DNA) has been used as a surrogate marker of NET formation\textsuperscript{37;38}. When compared to corresponding samples from healthy controls, serum/plasma taken from non-infectious SIRS patients\textsuperscript{37} and subjects that have suffered blunt/penetrating trauma\textsuperscript{38} have been found to contain significantly greater amounts of cf-DNA. From these results, it has been inferred that NET formation occurs as part of the early inflammatory response to trauma\textsuperscript{38}. However, in the absence of direct evidence showing that the cf-DNA measured in these studies was derived from activated neutrophils, only the work of Hamaguchi and colleagues\textsuperscript{17} can support this claim.

Treating freshly isolated neutrophils from healthy volunteers with 20\% plasma from SIRS patients has been shown to lead to NET release \textit{in vitro}\textsuperscript{37}. Thus, circulating soluble factors are likely to be responsible at least in part for the induction of NET formation that occurs
following traumatic injury\textsuperscript{17,37}. Indeed, pre-treatment of SIRS plasma with antibodies against TNF-\textalpha, IL-1\textbeta and IL-8 was found to significantly reduce its ability to drive NET production\textsuperscript{37}. Alongside pro-inflammatory cytokines, the nuclear protein high-mobility group box protein 1 (HMGB1), whose circulating levels are elevated following trauma\textsuperscript{39}, may be another factor that triggers NET release. HMGB1 has been shown both \textit{in vitro} and \textit{in vivo} to induce NET formation by binding to the pathogen recognition receptor toll-like receptor 4 (TLR4) on the neutrophil surface\textsuperscript{40}.

NETs have been proposed to function as double-edged swords of the innate immune system\textsuperscript{41}. On the one hand, these structures are an effective form of anti-microbial defence via their ability to capture and neutralise invading microbes. However, under non-infectious conditions, the presence of histones and granule-derived peptides in the extracellular environment are likely to have detrimental effects for the host. \textit{In vitro}, NET-bound histones and MPO induce epithelial and endothelial cell death\textsuperscript{42}, whilst \textit{in vivo}, NETs appear to serve as a stimulus for thrombus formation\textsuperscript{43-45}. Thus, the release of NETs that occurs following traumatic injury\textsuperscript{17} may contribute not only to the onset of MOF but also the development of thrombosis, a secondary complication that has a high incidence amongst TBI and thermally-injured patients\textsuperscript{46-48}. Thus, targeting NETs either for degradation, via the administration of DNase, or neutralisation, by reducing the activity of the DNA-bound granule-derived proteins, may represent a novel therapy by which to protect trauma patients from NET-induced endothelial damage and thrombosis\textsuperscript{38,45}. However, given the potent anti-microbial properties of NETs, such strategies may increase the susceptibility of trauma patients to nosocomial infections and sepsis\textsuperscript{33}.

\textit{1.1.4 Apoptosis}
In the absence of stimulation, neutrophils are short-lived cells, surviving *in vitro* for around 8-12 hours before undergoing apoptosis. However, following traumatic injury, their life-span is markedly increased. In line with data published within the field of burns research, Junger et al and Paunel-Gorgulu and co-workers have recently shown the rate of apoptosis for neutrophils isolated from TBI patients and victims of blunt/penetrating trauma respectively is significantly lower following overnight culture than that of neutrophils from uninjured controls.

Neutrophil apoptosis occurs through one of two independent pathways, namely intrinsic or extrinsic. Alterations in the expression levels of molecules involved in the induction of both pathways have been observed following trauma, highlighting a potential mechanism for the abovementioned extension in neutrophil life-span. Mediated through the mitochondria, the intrinsic pathway of apoptosis is regulated in part by the Bcl-2 protein family. Anti or pro-apoptotic in nature, Bcl-2 family proteins regulate mitochondrial membrane potential. Thus, the balance between pro and anti-apoptotic proteins is a critical factor in the induction of apoptosis by the intrinsic pathway. Recently, neutrophils isolated from multiply injured patients (Injury severity score [ISS] >16) were found to express significantly greater amounts of the anti-apoptotic protein myeloid cell leukemia 1 (Mcl-1) and significantly lower levels of the pro-apoptotic protein Bax, a balance that would favour stabilisation of mitochondrial membrane potential and cell survival. These findings were in line with those of an earlier study, where in a rodent model of thermal injury, increased expression of the anti-apoptotic protein Bcl-xL was found in neutrophils isolated from burnt rats when compared to those from uninjured controls. The extrinsic pathway of apoptosis is initiated by ligation of surface expressed death receptors, an example of which is the TNF-α superfamily member Fas. Increased levels of soluble Fas (sFas) have been reported in serum samples taken from patients with multiple blunt and penetrating injuries. Interestingly, culturing neutrophils
from healthy donors *in vitro* with sera from critically-injured patients has been shown to markedly reduce apoptosis induced by the agonistic anti-Fas antibody CH11, demonstrating that sFas protects against the induction of the extrinsic pathway of apoptosis\(^4^9\). Indeed, a twofold increase in cell death was reported for CH-11 stimulated neutrophils that had been pre-treated with patient sera depleted of sFas\(^4^9\). Thus, based on these observations, it was proposed that the trauma-induced delay in neutrophil life-span is attributable in part to reduced activation of the extrinsic pathway of apoptosis\(^4^9\).

Culturing neutrophils isolated from healthy volunteers overnight in the presence of serum from trauma patients has been shown to significantly prolong their life-span *in vitro*\(^5^0;^5^2\). This suggests that circulating soluble factors mediate at least in part the delay in neutrophil apoptosis that occurs following injury. Indeed, Paunel-Gorgulu et al showed that treating serum from critically-injured patients with neutralising antibodies against the pro-inflammatory cytokine granulocyte macrophage-colony stimulating factor (GM-CSF) significantly reduced its ability to delay neutrophil apoptosis *in vitro*\(^5^2\), an affect that appeared to occur through inhibition of the intrinsic pathway of apoptosis. Compared to those treated with sera from healthy controls, neutrophils exposed to serum obtained from trauma patients have been found to express significantly greater amounts of the anti-apoptotic protein Mcl-1, an elevation that was abolished when patient sera was pre-treated with neutralising antibodies against GM-CSF\(^5^2\). In addition to GM-CSF, IL-18 has been proposed to contribute to the delay in neutrophil apoptosis post trauma. In a rodent model of burn injury, Akhtar and colleagues found that administration of an anti-IL-18 antibody to thermally-injured rats reversed the burn-induced delay in neutrophil apoptosis that was observed in untreated controls\(^5^4\).

1.1.5 MicroRNA and proteomic profiling
To ascertain whether alterations to signalling pathways may underlie trauma-induced changes in neutrophil biology, comparative proteomics and microRNA (miRNA) profiling have been performed on neutrophils isolated from healthy controls and injured subjects\textsuperscript{18-20}. MicroRNAs (miRNAs) are evolutionary conserved, small non-coding RNA molecules that assist in post transcriptional regulation\textsuperscript{55}. In the first study of its kind in humans, Yang and colleagues\textsuperscript{18} recently described a unique miRNA signature in neutrophils following traumatic injury. Comparison of neutrophils from five control subjects and five severely injured patients (ISS $\geq 17$) revealed that eight MiRNAs were significantly up-regulated and five MiRNAs were significantly down-regulated post trauma\textsuperscript{18}. However, validation by qRT-PCR revealed only four miRNAs were significantly altered in neutrophils from patients with major trauma, namely hsa-miR-3945 and hsa-miR-125a-5p, which were both up-regulated and hsa-miR-363-3p and hsa-miR-150-5p, which were both down-regulated\textsuperscript{18}. Signalling pathways and genes associated with these altered MiRNAs included those involved in cell adhesion and ubiquitin-dependent protein catabolism\textsuperscript{18}. A trauma-induced increase in neutrophil MiRNA expression had previously been reported in a rodent model of SCI, where at 12-hours post injury, a significant up-regulation in miR-223 levels was observed in neutrophils\textsuperscript{56}. In this setting, an up-regulation in miR-223 expression may be a mechanism aimed at reducing neutrophil-mediated secondary damage to the spinal cord\textsuperscript{56}.

Two independent groups have recently investigated the impact of trauma on the neutrophil proteome\textsuperscript{19,20}. Of these, Zhou et al compared the proteomic profile of neutrophils from five healthy donors and five severely injured subjects and reported a trauma-induced alteration in the abundance of 197 proteins, 144 of which were up-regulated and 53 down-regulated\textsuperscript{19}. Analysis revealed proteins that were significantly up-regulated included those involved in interleukin signalling, chemokine signalling and pattern recognition\textsuperscript{19}, which would be predicted to result in increased neutrophil reactivity. In addition, an up-regulation of proteins
with potential anti-apoptotic properties (e.g. caspase-1, cyclin-dependent kinase 2 and protein kinase B) was observed and accompanied by a significant down-regulation of proteins with potential pro-apoptotic properties\textsuperscript{19}. Such changes would promote neutrophil survival and thus may contribute to the extended half-life of neutrophils following trauma\textsuperscript{6,49,50}.

1.2 Neutrophils in the initiation of the SIRS response to trauma

The danger theory first proposed by Polly Matzinger in 1994 challenged the notion that the immune system functions simply by discriminating self from non-self by suggesting that "the immune system is more concerned with damage than foreignness, and is called into action by alarm signals from injured tissues rather than by the recognition of self"\textsuperscript{57}. Since then, it has become increasingly recognised that tissue damage leads to the release of endogenous damage-associated molecular patterns (DAMPs). DAMPs can include substances which are: (i) secreted as a result of cellular activation such as HMGB-1, (ii) intracellular components such as mitochondrial DNA (mtDNA), which are released as a result of cellular necrosis, or (iii) extracellular matrix (ECM) components that are released upon tissue damage such as hyaluronic acid. DAMPs can be derived from a number of cellular compartments including the cytoplasm, mitochondria, nucleus and endoplasmic reticulum (ER). Like conserved pathogen-associated molecular patterns (PAMPs), which are expressed by invading pathogens, DAMPs can activate cells of the immune system via a set of germ-line encoded pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs), many of which are expressed by neutrophils. It has therefore been postulated that the activation of neutrophils by DAMPs, via their binding to PRRs, may play a role in the initiation of the SIRS response (Figure 1). This section will review the literature in support of this theory focussing on DAMPs that have been shown to be elevated in the circulation of trauma patients.
1.2.1 The expression of pattern recognition receptors on neutrophils

Human neutrophils constitutively express all of the currently identified TLRs with the exception of TLR3\textsuperscript{58}. TLR1, TLR2, TLR4, TLR5 and TLR6 are primarily expressed on the cell surface, whereas TLR7, TLR8 and TLR9 are expressed intracellularly on endolysosomal membranes\textsuperscript{58}. Whilst TLRs are renowned for their ability to bind PAMPs, some of these receptors also bind endogenous DAMPs leading to cellular activation (see section 1.2.2). In addition to the TLRs, neutrophils express other PRRs capable of binding DAMPs. These include, the formyl peptide receptors FPR1 and FPR2\textsuperscript{59}, which bind mitochondria-derived DAMPs released following trauma, and the cytoplasmic nucleotide-binding oligomerization domain (NOD)-like receptor (NLR), NLRP3, which forms part of the inflammasome that binds a number of DAMPs\textsuperscript{60;61}.

1.2.2 The effect of DAMPs on neutrophil function

High-mobility group box protein 1 (HMGB1)

Originally described for its role in gene expression and nucleosome remodelling\textsuperscript{62}, HMGB1 is one of the best characterised DAMPs, having a well-defined role in the response to tissue damage. This DNA-binding nuclear protein can be actively secreted in response to both pro-inflammatory cytokines and cellular stress\textsuperscript{63;64}. In a mouse model of endotoxemia, serum levels of HMGB1 were found to be elevated 8–32 hours following endotoxin exposure, sometime after the release of the pro-inflammatory cytokines TNF\textalpha{} and IL-1\beta{}\textsuperscript{65}. Consequently, it is thought that HMGB1 acts as a late mediator of endotoxin driven inflammation. Furthermore, in patients with sepsis, those individuals who survived had significantly lower serum levels of HMGB1 when compared to non-survivors\textsuperscript{65}. 
In addition to its active release, HMGB1 can be passively released from cells as a result of necrosis. Whilst HMGB1 is retained in the cell during apoptosis, as a result of irreversible binding to chromatin, it has been shown to be released from necrotic cells. In 2006, Yang and colleagues demonstrated that trauma patients with hemorrhagic shock sampled within 6 hours of injury displayed higher serum HMGB1 levels than controls. More recently, HMGB1 plasma levels were shown to be significantly elevated following mechanical trauma (ISS ≥ 15), being 30 times greater than those in controls 1 hour after injury, and peaking 2-6 hours post injury. Moreover, high levels of HMGB1 have been shown to be associated with injury severity score and survival. The release of HMGB1 into the circulation following trauma has since been linked to the subsequent inflammatory response. In an animal model of trauma, the blockade of HMGB1 using antagonistic antibodies prevented increases in pulmonary levels of the pro-inflammatory cytokines, IL-6, and IL-1β. Consequently, HMGB1 is now accepted as an early and key mediator of sterile inflammation.

Once released into the extracellular milieu, HMGB1 can activate immune cells via its binding to several receptors including TLR2, TLR4 and receptor for the advanced glycation end products (RAGE) all of which are expressed by neutrophils. Indeed, it has been demonstrated that HMGB1 can lead to the up-regulation of pro-inflammatory cytokine expression (IL-1β, TNFα and IL-8) in human neutrophils; an effect that was dependent on the activation of nuclear factor kappa beta (NFκB) and the p38 and extracellular signal-regulated kinase 1/2 (ERK1/2) mitogen-activated protein kinase (MAPK) signalling pathways. Similarly, a study by Silva et al showed that stimulation of neutrophils from sepsis-induced acute lung injury patients with HMGB1 up-regulated the gene expression of cytokines, chemokines and coagulation related genes, an effect that involved p38 and NFκB activation. More recently, HMGB1 administration in mice has been shown to lead to the formation of neutrophil extracellular traps (NETs) via TLR4. In addition, intraperitoneal injection of
HMGB1 has been shown to promote mac-1 dependent neutrophil recruitment, an effect that was dependent on the expression of RAGE by neutrophils. However, Tadie and colleagues found that incubation of mouse neutrophils with HMGB1 reduced neutrophil bacterial killing both \textit{in vitro} and \textit{in vivo}. Based on this observation, the authors postulated that this may be a novel mechanism by which HMGB1 can potentiate sepsis-associated organ dysfunction. A recent publication has demonstrated that the effect of HMGB1 on neutrophils can depend upon its concentration. Berthelot and colleagues showed, using whole blood, that at low concentrations (50-100 ng/ml), HMGB1 was able to reduce neutrophil migration towards IL-8 whereas at higher concentrations (5000 ng/ml), HMGB1 had a chemoattractant affect via IL-8 production from an unidentified source.

\textit{Mitochondrial DAMPs}

Recently, it has been demonstrated that danger signals derived from mitochondria, so called mitochondrial DAMPs (mtDAMPs), are released into the circulation following trauma and are thus involved in the immune response to cellular damage. Interestingly, mitochondria are proposed to originate from \textit{α}-Protobacteria which lived as intracellular symbionts in eukaryotic cells. As a result of this, mitochondria still display characteristics of their bacterial ancestors including methylated CpG DNA repeats and formylated peptides. A full list of mitochondrial derived DAMPs is listed in Table 1.

Zhang and colleagues have demonstrated that mtDNA is released into the circulation following trauma, where circulating levels amongst 15 trauma patients was 2.7\(\mu\)g/ml, which was thousands of fold higher than the levels detected in controls. In addition, the same group were able to detect even higher levels of mtDNA in femur fracture reamings collected during clinical fracture repair. It is thus well established that mtDAMPs are released into the circulation following tissue damage. It has also been demonstrated that mtDNA is
positively correlated with injury severity in a cohort of trauma patients\textsuperscript{81}. In addition, a recent study has shown that circulating plasma mtDNA levels, measured within hours of admission to hospital, can act as an independent predictor of the occurrence of post-traumatic SIRS\textsuperscript{82}. Furthermore, it has also been suggested that comparing plasma mtDNA levels to plasma bacterial DNA levels may help distinguish sterile systemic inflammation from bacterial sepsis\textsuperscript{83}.

It has been suggested that mtDAMPs released as a result of tissue damage may play a role in the initiation of the SIRS response via the activation of the immune system, ultimately resulting in the associated problems of MOF, ARDS and sepsis. Indeed, rats given an intravenous injection of mtDAMPs exhibited marked lung injury within hours of injection\textsuperscript{77}. Moreover, this was shown to be via neutrophil activation as evidenced by the presence of neutrophils in the bronchoalveolar lavage as well as the accumulation of elastase in the lung\textsuperscript{77}. \textit{In vitro}, stimulation of human neutrophils with mtDAMPs has been shown to not only lead to the activation of both p38 and p42/44 MAPK\textsuperscript{77,80,84} but also induce the release of matrix metalloproteinases (MMP)-8 and MMP-9 along with interleukin (IL)-8\textsuperscript{77,80,84}.

MtDAMPs have been shown to cause increased permeability of endothelial cells \textit{in vitro}, in both a neutrophil-dependent and independent manner. mtDAMPs applied directly to endothelial cells in culture caused a significant increase in endothelial cell permeability, an effect abrogated by the addition of protease, suggesting a mitochondrial-protein dependent mechanism\textsuperscript{85}. This effect was likely via the activation of the p38 and ERK MAPK pathways as both proteins were phosphorylated in response to the addition of mtDAMPs\textsuperscript{85}. The same study also demonstrated that exposure to mtDNA increased the adherence of neutrophils to an endothelial cell layer via the up regulation of intercellular adhesion molecule 1 (ICAM-1) and E-selectin on the endothelial cells as well as CD18 and L-selectin on the neutrophil
Together, these data provide evidence that mtDAMPs released from sites of injury may contribute towards the increased endothelial permeability seen during SIRS.

As well as leading to the increased adherence of neutrophils to endothelial cells, the work of Paul Kubes has elegantly shown that mtDAMPs play a central role in the recruitment of neutrophils to sites of focal hepatic necrosis in a mouse model of sterile inflammation. In this model, it was demonstrated that neutrophils were able to adhere to the endothelium and migrate into the liver sinusoids within 30 to 60 minutes following injury. However, following administration of apyrase (an exogenous ATPase), there was a significant reduction in the number of neutrophils recruited to the liver in response to injury. Interestingly, apyrase did not affect the chemotaxis of the few cells that were recruited. The effect of ATP was shown to be through the P2X7 receptor, which led to IL-1β and caspase-1 processing via the Nlrp3 inflammasome. This data shows therefore that ATP activates pathways that initiate neutrophil adhesion, but does not itself act as a chemotactic signal. In addition, the same study showed that mitochondrial derived formyl peptides released from necrotic tissue were responsible for providing the “necrotactic” signal necessary for the neutrophils to migrate the final 150µm to the site of necrosis. This was demonstrated by the fact that in formyl-peptide receptor 1 knockout mice (Fpr1−/−), neutrophils migrated to within 150µm of the necrotic site (via CXCR2 mediated signalling) but failed to migrate to the area of injury. Interestingly, it has recently been shown that blunt/penetrating injury and severe TBI leads to the up-regulation of the A3 adenosine receptor on the surface of circulating human neutrophils, and that the level of expression of this receptor correlated positively with patient injury severity score. Since ATP is released upon tissue damage, the up-regulation of A3 receptor expression may be one mechanism underlying the heightened activation status of circulating neutrophils from trauma patients.

1.3 Neutrophil function as a predictor of outcome in traumatically-injured patients
In cohorts of critically-ill septic patients, neutrophil expression of the high affinity immunoglobulin receptor CD64 has been identified as a promising biomarker for the early diagnosis of bacterial infection, exhibiting a high sensitivity and specificity for predicting infection severity and patient mortality. Surprisingly, despite the vast and marked alterations that occur in neutrophil biology post trauma, only two studies have investigated whether any of these changes could serve as biomarkers to predict the outcome of severely-injured subjects, with both studies focusing upon NET generation. In a cohort of forty-five patients with multiple trauma, Margraf et al demonstrated that neutrophil-derived circulating free DNA (cf-DNA/NETs) could predict with high accuracy the posttraumatic development of sepsis in intensive care unit patients. Following on from this study, Altrichter and colleagues have recently shown that an early measurement of cf-DNA/NETs has both a high sensitivity and specificity for predicting mortality in patients with severe burn injury. Whilst the authors themselves stress that definite proof that the circulating cell-free DNA is derived from activated neutrophils is still needed, both these studies suggest that an early measurement of neutrophil function has potential as a prognostic marker for predicting patient outcome post trauma.

1.4 Manipulating neutrophil function and life-span: a potential therapeutic strategy for the treatment of trauma patients?

As trauma-induced changes in neutrophil biology have been linked to the development of such common post-traumatic complications as MOF and ARDS, investigating whether patient outcome can be improved via the manipulation of neutrophil function or life-span is an emerging theme in trauma research.
1.4.1 Animal studies

To date, rodent models of traumatic injury and inflammation have been used to examine whether: (i) the removal of neutrophils from the circulation has potential as a form of treatment for the management of TBI\textsuperscript{97}, (ii) reducing neutrophil infiltration into inflammatory lesions can improve outcome following SCI\textsuperscript{98}, (iii) inflammatory lung injury can be prevented by limiting granulopoiesis\textsuperscript{99} and (iv) restoring neutrophil function following thermal injury can improve survival\textsuperscript{8,9}. Regarding this latter strategy, Kinoshita et al\textsuperscript{8} demonstrated that IL-18 treatment could significantly improve the survival rates of burn-injured mice challenged with methicillin-resistant \textit{Staphylococcus aureus} (MRSA)\textsuperscript{8}. As well as increasing neutrophil counts, IL-18 treatment reversed the burn-induced depression in neutrophil phagocytosis that was observed in untreated burn-injured mice and also enhanced ROS production\textsuperscript{8}. Thus, neutrophils from IL-18-treated mice exhibited MRSA-killing activity that was significantly greater than that of neutrophils from untreated burn-injured mice and comparable to that of neutrophils from sham mice\textsuperscript{8}. Based on these observations amongst others, IL-18 treatment was proposed to be a potential therapeutic tool by which to fight the various kinds of bacterial infections encountered by hospitalised burns patients\textsuperscript{8}. Interestingly, whilst not measured in this study, exposure to IL-18 had previously been shown in a model of alcohol intoxication and burn injury to prolong the life span of circulating neutrophils\textsuperscript{54}. Together, these results suggest that IL-18 therapy would result in a longer-lived and more active neutrophil pool. Whilst these neutrophils would be beneficial in the context of fighting infection, a downside to IL-18 therapy would potentially be an increase in neutrophil-mediated tissue damage and organ failure\textsuperscript{54}.

1.4.2 Human studies
On the back of animal studies revealing that resuscitation with hypertonic fluids post trauma could reduce organ damage100, Junger and colleagues5,6 recently investigated whether by modulating the activation status and life span of circulating neutrophils, such a treatment regimen would be beneficial for human trauma patients. In the settings of TBI6 and hemorrhagic shock5, resuscitation with hypertonic saline (HS) was found to prevent trauma-induced activation of neutrophils as assessed by alterations in receptor expression (e.g. CD11b and L-selectin) and their ability to generate ROS ex vivo5,6. Furthermore, in the setting of TBI, treatment with hypertonic saline partially prevented the delay in apoptosis that was observed in vitro for neutrophils isolated from subjects that had received normal saline or HS containing dextran6. However, despite these immune-modulatory properties, in neither setting was pre-treatment with HS associated with any improvement in patient outcome5,6.

As traumatic injury results in a heterogeneous pool of circulating neutrophils16;21;28, could the removal of individual neutrophil subsets represent a novel therapeutic approach for the treatment of severely-injured patients? Such a strategy is currently being considered as a potential future treatment for patients with sepsis101. Ex vivo filtration of blood from patients with septic shock using a Polymyxin-B filtration system has been found to preferentially remove neutrophils with an activated phenotype (CD11b_{high}, CD64_{high} and CXCR1/2_{low}), whom in vitro caused considerable damage to endothelial monolayers102. This observation coupled with in vivo studies reporting that leucodepletion improves the outcome of patients with sepsis and septic shock103;104 has led to the suggestion that in cases of severe sepsis, removing activated neutrophils from the circulation could lead to patient benefit101. Currently, very little is known regarding whether such a strategy would be successful in severely injured subjects. Of note, one study reported that leucodepletion, which resulted in a forty percent reduction in circulating neutrophil numbers, improved the outcome of patients who developed SIRS following cardiopulmonary bypass105. Thus, it would be of interest if
future studies examined in cohorts of more severely injured subjects whether such an approach would also be beneficial. Furthermore, given the recent identification of a suppressive neutrophil subset in the circulation of traumatically-injured patients\textsuperscript{16}, removing these cells via filtration may reduce the degree of immune suppression that occurs post trauma, a consequence of which could be a reduced risk of nosocomial infection.

Although an interesting area of investigation, it should be said that attempting to manipulate neutrophil function in order to improve patient outcome is not without risk. Whilst enhancing the microbicidal capacity of neutrophils in order to protect against infection is on the one hand appealing, unregulated hyperactive neutrophils would cause considerable bystander tissue damage that underlies the development of ARDS and MOF. Conversely, attempting to dampen down the neutrophil inflammatory response in the immediate aftermath of a traumatic insult as a means by which to reduce immune-mediated tissue damage would leave the patient vulnerable to microbial infections. Developing strategies that will strike a balance between these two issues should be the focus of future studies that aim to modify the immune response in order to assist in patient recovery.

1.5 Future directions

Whilst ROS production and phagocytosis are the classic defensive strategies of neutrophils, these are not the only anti-microbial mechanisms utilised by these cells at sites of inflammation. Mesri et al were the first to demonstrate that neutrophils produce microparticles, small (50-100nm) vesicles released into the extracellular environment from multivesicular bodies\textsuperscript{106}. Recently, these neutrophil derived microparticles have been shown to possess both anti-bacterial\textsuperscript{107} and immunomodulatory properties\textsuperscript{108}. Interestingly, a recent study in trauma patients with ongoing sepsis demonstrated the presence of neutrophil-derived microparticles in the inflamed lungs and abdomen\textsuperscript{108}. Whilst the exact immunomodulatory
role of neutrophil derived microparticles remains to be elucidated, such studies suggest that they may play a role in regulating the inflammatory response to trauma and thus provide a future target by which to modulate this response.

In addition to their role in innate immunity, it is now recognised that neutrophils perform a number of other functions. For example, neutrophils interact with other cells of the immune system including dendritic cells (DCs) natural killer (NK) cells, B-cells and numerous subsets of T-cells (reviewed in reference 109). In particular, a recent study has demonstrated a reciprocal cross-talk between neutrophils and Th17 cells\textsuperscript{110}, which may have a role in amplifying neutrophil anti-bacterial function\textsuperscript{109}. Given that IL-17 is released into the circulation following trauma\textsuperscript{111}, this interaction may provide a future therapeutic target.

**Concluding Remarks**

Tissue damage as a result of traumatic injury leads to the release of endogenous DAMPs. These DAMPs are thought to be involved in the inflammatory response to sterile tissue injury ultimately leading to downstream complications such as MOF and sepsis. In addition, trauma has been shown to lead to an alteration in neutrophil function. Recent data has established a link between the release of DAMPs and altered neutrophil function in trauma. In particular, HMGB1 and mtDAMPs have been shown to activate neutrophils via their binding to PRRs expressed by the cells. Targeting DAMPs and their subsequent interaction with neutrophils via PRRs may therefore be a promising approach by which to reduce the initial SIRS response and the subsequent development of MOF and sepsis in trauma patients.
References


Figure Legend

Figure 1. Damage Associated Molecular Patterns (DAMPs) and the neutrophil response to trauma. In response to traumatic injury, endogenous DAMPs are released into the circulation as a result of tissue damage, either from necrotic cells or through active secretion. Via interaction with surface expressed pattern recognition receptors, mitochondrial and nuclear derived DAMPs cause neutrophil activation triggering a multitude of functional responses that are thought to contribute to the initiation and propagation of the inflammatory response to trauma. ATP, adenosine triphosphate; FPR, formyl peptide receptor; HMGB1, high-mobility group box-1; mtDNA, mitochondrial DNA; NET, neutrophil extracellular trap; RAGE, receptor for advanced glycation end products; TLR, toll-like receptor.
Table 1. A list of mitochondrial damage-associated molecular patterns (DAMPs), their receptors and effect on neutrophil function.

<table>
<thead>
<tr>
<th>Mitochondrial DAMP</th>
<th>Receptor</th>
<th>Effect on neutrophils</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>P₂X₇</td>
<td>Initiates adhesion</td>
<td>[86]</td>
</tr>
<tr>
<td>Cytochrome-C</td>
<td>?</td>
<td>Promotes neutrophil driven inflammation</td>
<td>[112]</td>
</tr>
<tr>
<td>mtDNA</td>
<td>TLR9</td>
<td>IL-8 release</td>
<td>[77;84]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adherence to endothelial cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Increased L-selectin, CD18 expression</td>
<td></td>
</tr>
<tr>
<td>Formyl Peptides</td>
<td>FPR1, FPR2</td>
<td>Chemotactic factor, Ca²⁺ influx, Ca²⁺ store depletion, IL-8 release, MMP-8 release, MMP-9 release</td>
<td>[77,80,84]</td>
</tr>
</tbody>
</table>

**Abbreviations:** ATP, Adenosine triphosphate; FPR, Formyl-peptide receptor; IL-8, Interleukin-8; MtDNA, mitochondrial DNA; MMP, matrix metalloproteinase; TLR, Toll-like receptor.