The Pathological Roles of Neutrophil Extracellular Traps (NETs) in Sepsis

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By

Mohmad Alsabani

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Abstract

Neutrophils are amongst the first immune cells to defend against microbial infection and neutrophil extracellular traps (NETs) formation is an efficient microbicidal mechanism to prevent pathogen dissemination. However, NETs can also cause harm by promoting intravascular thrombosis and organ injury in animal models. Although NETs are recognised as targets for developing new therapeutic strategies, there is currently lack of robust and specific methods to quantify NETs, particularly in clinical settings.

To progress the translational relevance of NETs formation, this thesis establishes a novel assay for quantifying NETs formation and assesses its clinical relevance. To evaluate the pathogenic roles of NETs, complementary *in vivo* studies were carried out in two septic mouse models, without or with anti-NETs therapy.

The NETs formation assay was initially developed using plasma and sera from septic patients (n=54) admitted to the intensive care units (ICU) of Aintree and Royal Liverpool University Hospitals, or healthy volunteers (n=20). Differentiated neutrophil-like cell line (PLB-985) and isolated healthy (n=10) or patient (n=10) neutrophils were used to investigate the convenient source of neutrophils. Fluorescent staining of extracellular DNA demonstrated that e*x vivo* NETs formation was induced by directly incubating heterologous neutrophils with plasma or sera from ICU patients, but not normal plasma, unless supplemented with phorbol 12-myristate 13-acetate (PMA). Using this *ex vivo* NETs assay, critically ill patients can be stratified into 4 groups, absent, mild, moderate and strong NETs formation.

To determine the clinical potential of this assay, the NETs-forming capacity was measured in plasma from a consecutive cohort of prospectively recruited adult ICU patients (n= 341) admitted to the Royal Liverpool University hospital. Strong NETs-forming capacity was predominantly associated with critically ill patients diagnosed with sepsis. In addition, moderate to strong NETs formation was associated with higher sequential organ failure assessment (SOFA) scores on ICU admission and throughout the study duration (three days following admission). Multivariate regression analysis showed after adjusting for Acute Physiology and Chronic Health Evaluation (APACHE) II that measuring the degree of NETs formation in ICU admission could independently predict disseminated intravascular coagulation (DIC) and mortality whereas known NETs degradation markers, could not. High interleukin (IL)-8 levels were strongly associated with NETs-forming capacity of plasma and blocking IL-8 using either an anti-IL-8 monoclonal antibody or inhibitors of CXCR1/2 signaling (using reparixin) significantly attenuated NETs formation.

The pathogenic implications of NETs was determined in two mouse models of sepsis including cecal ligation and puncture (CLP) and intraperitoneal injection of *Escherichia coli*; sham operated animals or intraperitoneal injection of saline were used as controls. The roles of NETs in both models of sepsis were investigated by depleting neutrophils and treating with DNase I or reparixin.

Both the *ex vivo* assay and *in vivo* quantification of NETs showed dramatic increases, particularly in the lungs of septic mice. The agreement of both assays demonstrated that the *ex vivo* assay reflects *in vivo* NETs formation. The extent of NETs formation was strongly associated with fibrin deposition and lung injury, both of which were

dramatically reduced by neutrophil depletion and DNase I treatment. Macrophage inflammatory protein-2 (MIP-2), the CXCR1/2 ligand in mice, was significantly elevated and correlated to levels of NETs release. Inhibition of CXCR1/2 using reparixin decreased NETs formation, fibrin deposition and multiple organ injury without impairing bacterial clearance, and improved survival in septic mice. This study demonstrates that measuring *ex vivo* NETs formation induced by plasma, reflects *in vivo* NETs. Targeting NETs formation using inhibitors of IL-8 signaling may have therapeutic benefit in patients with sepsis.

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Publications and presentations

Publication

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Poster presentations

Poster presentation in the 32nd Annual Congress of the European Society of Intensive Care Medicine, 2019. Interleukin-8 is a major factor in inducing Neutrophil Extracellular Traps (NETs) in sepsis. Berlin, Germany.

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Flash poster presentation in Institute of Global Health and Infection, IGH Day, October 2018. Interleukin-8 is a major factor in inducing Neutrophil Extracellular Traps (NETs) in sepsis. Liverpool, UK.

Abbreviations

AF	Alexa Fluor
AKI	Acute kidney injury
ALI	Acute lung injury
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AML	Acute myeloid leukaemia
ANOVA	Analysis of variance
APACHE	Acute physiology and chronic health evaluation
APC	Activated protein C
ARDS	Acute respiratory distress syndrome
AST	Aspartate aminotransferase
ATRA	All trans retinoic acid
AU	Arbitrary unit
AUC	Area under the curve
BALF	Bronchoalveolar lavage fluid
bFGF	basic fibroblast growth factor
BSA	Bovine serum albumin
BUN	Blood urea nitrogen
CARS	Compensatory antagonistic response syndrome
CC	Cysteine – cysteine
CD11b	Cluster of differentiation molecule 11b
CFU	Colony-forming unit
cfDNA	Cell-free DNA
CGD	Chronic granulomatous disease
CI	Confidence interval
Cit-H3	Citrullinated histone H3
CLP	Cecal ligation and puncture
CNS	Central nervous system
COPD	Chronic obstructive pulmonary disease

C-X-C	Cysteine–X amino acid–cysteine
CXCL	C-X-C-motif chemokine ligand
CXCR	C-X-C-motif chemokine receptor
DAMP	Danger-associated molecular patterns
DAP	3,3'-diaminobenzidine
DCs	Dendritic cells
DIC	Disseminated intravascular coagulation
DMF	N,N-dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPI	Diphenyleneiodonium
DVT	Deep venous thrombosis
ECL	Enhanced chemiluminescence
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular receptor kinase
FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
F_iO_2	Fraction of the inspired oxygen
FITC	Fluorescein isothiocyanate
GCS	Glasgow Coma Scale
G-CSF	Granulocyte colony-stimulating
GM-CSF	Granulocyte-macrophage colony-stimulating
GMP	Granulocyte- macrophage progenitor
HCL	Hydrochloride
HIV	Human immunodeficiency virus
HMGB	High mobility group box
HRP	Horseradish peroxidase
HSC	Hematopoietic stem cells
ICU	Intensive care unit
IHC	Immunohistochemistry
IL	Interleukin

INF	Interferon
IP	Interferon-γ-inducible protein
IQR	Interquartile range
ISTH	International Society on Thrombosis and Haemostasis
JAAM	Japanese Association for Acute Medicine
KDIGO	Kidney Disease: Improving Global Outcomes
LIX	Lipopolysaccharide-induced chemokine
LOS	Length of stay
LPS	lipopolysaccharide
MAP	Mean arterial blood pressure
MCP	Monocyte chemoattractant protein
MEK	Mitogen-activated protein kinase
MEP	Megakaryocyte-erythrocyte progenitor
MIP	Macrophage inflammatory protein
MODS	Multiple organ dysfunction syndrome
MOF	Multiple organ failure
MPO	Myeloperoxidase
NADPH	Nicotinamide adenine dinucleotide phosphate
NE	Neutrophil elastase
NET	Neutrophil extracellular traps
NHS	National Health Service
OCI	Hypochlorite
OR	Odds ratio
PAD	Peptidyl Arginine Deiminase
PAMP	Pathogen-associated molecular pattern
P_aO_2	Partial pressure of oxygen in arterial blood
PAR	Protease-activated receptors
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
PE	Phycoerythrin

PFA	Paraformaldehyde
PI	Propidium Iodide
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear
PRR	Pattern recognition receptors
PT	Prothrombin time
aPTT	Activated partial thromboplastin time
PVDF	Polyvinylidene fluoride
RANTES	Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted
RBC	Red blood cell
RCD	Regulated cell death
REC	Research Ethics Committee
RLUH	Royal Liverpool University Hospital
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
ROS	Reactive oxygen species
RRT	Renal replacement therapy
SCCM	Society of Critical Care Medicine
SD	Standard deviation
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel
SEM	Standard error of mean
SIRS	Systemic inflammatory response syndrome
SOFA	Sequential organ failure assessment
TBS	Tris buffered saline
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TLR	Toll-like receptor
TMB	Tetramethyl-benzidine
TNF	Tumour necrosis factor
TRALI	Transfusion-related acute lung injury

VEGF	Vascular endothelial growth factor.
VWF	Von Willebrand factor
WBCs	White blood cells

Chapter 1: General introduction

1.1 Introduction of critical illness

The definition of critical illness is broad, but mainly focused on three major elements: disease complexity, severity of organ damage and risk of mortality (1). The mortality rate in unselected cohort of critically ill patients is 8-18%, but these measures, in fact, conceal the high mortality rate in acute illness such as in sepsis (35-45%) and acute lung injury (50-60%) (1-3). Long-term morbidity, such as physical morbidity (e.g. muscle loss and weakness) and non-physical morbidity (e.g. depression), is common among ICU survivors (1). Furthermore, long-term morbidity is associated with poor quality of life among ICU survivors compared to general population, which place a huge financial burden on the healthcare systems post-hospitalisation (4). Poor survival in critically ill patients is mostly driven by the complex series of physiological abnormalities that arise following admission to critical care and cause multiple organ dysfunction syndrome (MODS) (5).

1.2 Sepsis

1.2.1 Definitions

The modern concept of sepsis was introduced in 1991 by a north American consensus conference in which sepsis was described as systemic inflammatory response syndrome (SIRS) due to infection (6). The SIRS criteria is defined by the presence of two or more of the following criteria: temperature (<36°C or >38°C), heart rate (>90 beats per minute),respiratory rate (>20 breaths per minute), and white blood cell count(<4 $x10^9$/L or >9$x10^9$/L) (6).

The second meeting on consensus definitions for sepsis (sepsis-2) did not reach a more precise definition other than the SIRS criteria (7). The Third international meeting replaced SIRS criteria with end-organ injury classification systems, sequential organ

failure assessment (SOFA) (8, 9). Furthermore, sepsis was defined as dysregulated host response to infection that triggers the development of MODS. Whereas septic shock definition identifies sepsis patients at greater risk of increased mortality as a result of circulatory, cellular and metabolic abnormalities (9).

1.2.2 Disease burden

Sepsis is the most common cause of critical care admission with high morbidity and mortality due to MODS induced by host's response to infection (9, 10). The incidence and mortality related to sepsis are substantially variable according to geographical location (Figure 1.1) (11). It is estimated that sepsis causes more than 11 million deaths per year globally, contributing to approximately 20% of all deaths every year worldwide (11, 12). The incidence of sepsis is unknown but the estimated incidence is 49 million cases annually worldwide (12, 13). In the UK, a critical care bed costs £987 -£2082 per day (£2 billion annually), depending on the number of organs being supported, with septic patients likely to incur these higher costs, and the average ICU stay is 7.8 days with a mortality rate around 20% (14, 15).

Figure 1.1

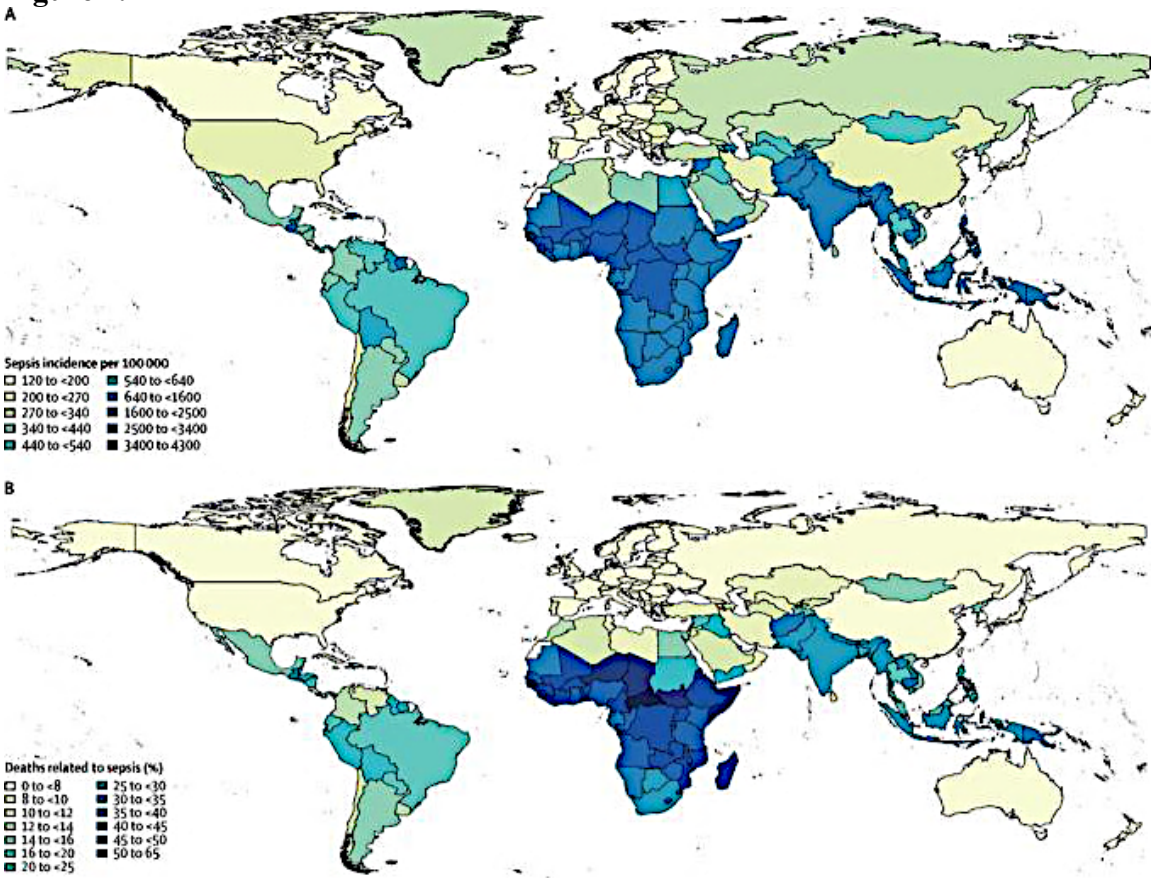


Figure 1.1 Sepsis-related incidences and deaths according to geographical location. (A) sepsis-related incidence per 100 000 population (B) sepsis-related deaths per 100 000 population, adapted from Rudd *et al* (11).

1.3 Multiple organ dysfunction syndrome (MODS)

1.3.1 Overview

Sepsis is defined as " *life-threatening organ dysfunction caused by a dysregulated host response to infection*" (9). Although any organ function can fail during sepsis, the term 'organ-organ crosstalk' implies that dysfunction of a single organ can lead to failure of another organ. In addition, mortality in sepsis correlates with the number of failing organs, with higher risk of death (40-100%) if ≥ 3 organs fail (16, 17). The extent of organ damage is determined using the SOFA scoring system (Table 1.1), which assesses the clinical manifestations of six major organ systems: respiratory, coagulation, liver, cardiovascular, neurological and renal systems.

Table 1.1 The sequential organ failure assessment (SOFA) scoring system, adapted from Vincent *et al* (8).

SOFA Score					
Organ system	**0**	**1**	**2**	**3**	**4**
Respiratory, P_aO_2/F_iO_2, mmHg	≥400	<400	<300	*≤200	*≤100
Coagulation, Platelets, $\times 10^3/\mu L$	≥150	≤150	≤100	≤50	≤20
Liver, Bilirubin, mg/dL	<1.2	1.2–1.9	2.0–5.9	6.0–11.9	>12.0
Cardiovascular	MAP ≥70 mmHg	MAP <70 mmHg	Dopamine ≤5 or dobutamine (any dose)	Dopamine 5.1–15 or epinephrine ≤0.1 or norepinephrine ≤0.1	Dopamine >15 or epinephrine >0.1 or norepinephrine >0.1
Central nervous system, GCS	15	13 – 14	10 – 12	6 – 9	<6
Renal, Creatinine, mg/dL. Urine output, mL/d.	<1.2	1.2 – 1.9	2.0 – 3.4	3.5 – 4.9 <500	>5.0 <200

SOFA, sequential organ failure assessment; PaO_2/FiO_2, ratio of partial pressure of arterial oxygen to fractional inspired oxygen; MAP, mean arterial blood pressure;GCS, Glasgow Coma Scale. * Values are with respiratory support.

1.3.2 Respiratory

The term '*acute respiratory distress syndrome'* was described five decades ago by Ashbaugh *et al.* as acute onset of clinically significant hypoxemia accompanied with diffuse pulmonary infiltrates manifested by reduced lung compliance, irresponsive to conventional respiratory therapy (18). The definition of ARDS has been reviewed and changed periodically and the latest Berlin definition of ARDS was proposed in 2012 which described ARDS as "*a type of acute diffuse, inflammatory lung injury, leading to increased pulmonary vascular permeability, increased lung weight, and loss of aerated lung tissue*" (19). The degree of ARDS is determined in the SOFA score using PaO2/FiO2 ratio, the partial pressure of oxygen in arterial blood (PaO2) to the fraction of the oxygen in the inspired air (FiO2).

Approximately 4% of all hospital admissions and 10% of ICU admissions are at risk of developing ARDS with the probability of in-hospital and ICU death increasing with ARDS severity (20, 21). ARDS develops in response to multiple underlying conditionssuch as, sepsis, acute pancreatitis, trauma and major surgery, with septic patients morelikely to progress to ARDS than other critical illnesses (19, 22). In an observational study of 778 critically ill patients diagnosed with severe sepsis, more than 6% fulfilledARDS criteria, which was associated with increased risk of death (23).

The current approaches to manage ARDS patients are: to adopt a ventilation strategy to maintain oxygen levels above 88%, maintain fluid balance, achieve haemodynamic stability and provide adequate nutritional support (24).

1.3.3 Coagulation

Overactivation of the coagulation system is invariably associated with sepsis (25).

Mild activation of the coagulation system is only detectable with highly sensitive assays, but a subtle fall in platelet count may indicate severe coagulation abnormalities. The critical role of thrombocytopenia typified by low platelet count (<150 $x10^3/\mu L$) in critical illness is highlighted by its inclusion in the SOFA score. Thrombocytopenia is common among critically ill patients, with an incidence rate between 35 – 44%. Approximately 50% of critically ill patients with sepsis develop thrombocytopenia, which often presents an indication for the development of disseminated intravascular coagulation (DIC) (26-29). DIC is a clinicopathological syndrome characterised by intravascular activation of coagulation which is elicited by both infectious and non-infectious aetiologies (30, 31). DIC is frequently associated with platelet consumption, prolongation of routine clotting assays, consumption of coagulation factors and increased levels of fibrin degradation products (25). The international society on thrombosis and haemostasis (ISTH) and the Japanese association for acute medicine (JAAM) demonstrated in two validation studies that about 30-51% of patients with sepsis developed DIC, with mortality rate 22-46% (32, 33). Furthermore, severe sepsis complicated by DIC can independently predict hospital and 28-days mortality (34).

Currently, there is no gold standard system to diagnose DIC, but the ISTH and JAAM suggested two objective measurements based on scoring algorithms using readily available global coagulation parameters. These include platelet count, prothrombin time (PT) or activated partial thromboplastin time (aPPT), fibrinogen and D-dimer. The ISTH scoring system proposed two terms, including overt (DIC score ≥ 5) and non-overt (DIC score < 5), to indicate haemostatic dysfunction with and without decompensation and clinical consequences, whereas the JAAM criteria has not reached the stage of decompensation.

The JAAM scoring system allows early diagnosis of DIC established by SIRS category incorporated within the scoring system, but the ISTH scoring system is only calculated after an indicative underlying disorder known to be associated with DIC is detected. Since DIC is a secondary disorder to another critical illness, the ISTH recommend vigorous but simultaneous treatment of the underlying disease and supportive therapies specifically targeting coagulation abnormalities e.g. anticoagulant treatment (35).

Since the clinical picture of coagulation abnormalities is variable depending on the aetiology, multimodal and personalised therapy are recommended (36).

1.3.4 Liver

Liver injury/failure during critical illness is a complex and multisystemic process that quickly progresses following hypoxic, toxic or inflammatory insults leading to systemic complications such as coagulopathy, increased risk of infection and encephalopathy (37). Liver injury is defined as an elevation in the most commonly measured hepatic enzyme, serum bilirubin (38). In addition, other hepatic enzymes such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP)are useful markers for liver injury (39).

Direct injury to hepatocytes may occur during the initial phase of septic shock due to hypoxic hepatitis complicated by inadequate oxygen delivery to the liver, such early dysfunction is reversible with supportive therapy (40). In sepsis-associated cholestasis, translocation of endotoxins to the liver portal circulation may initiate an inflammatory process activated by Kupffer cells (macrophages that reside in the liver) which are responsible for ingesting bacteria, removing microorganisms from the circulation and modulating the immune response (41).

Therapeutic approaches toward liver injury requires early diagnosis, identification of aetiology and screening measures such as microbiological testing and hepatic ultrasound followed by stopping hepatotoxic drugs and fluid resuscitation to maintain adequate perfusion to the liver (37).

1.3.5 Circulatory failure

Circulatory failure, or shock, is a serious consequence of critical illness, which affects about one third of ICU admissions (42). It manifests as tissue hypoperfusion and a rise in metabolic demands, as a result of hypotension and hyperlactatemia, an indication of abnormal cellular oxygen metabolism (43). The most common feature of circulatory failure is hypotension, manifested by low systolic blood pressure (< 90 mmHg), mean blood pressure (< 70 mmHg) or requirement for vasopressors, that is often irresponsive to fluid resuscitation and accompanied with acidaemia (43).

Apart from the clinical manifestations, myocardial injury can occur during circulatory failure, as evidenced by elevated circulating cardiac troponins (cTn) levels, including troponin complexes I and T, routinely measured biomarkers of myocardial injury (44). High cTn levels are detectable in approximately 60% of patients with sepsis and septic shock (45). Investigations among heterogenous cohorts of critically ill patients show increased mortality in patients with elevated troponin levels (45). Whereas studies on homogenous cohort of patients with sepsis and septic shock yielded that high concentrations of troponins are associated with disease severity and poor outcomes (46-49).

The management of sepsis-induced circulatory failure remains supportive, initiated with goal-directed fluid resuscitation guided by invasive haemodynamic monitoring, vasopressors and inotropic support (50).

1.3.6 Neurological

Coma or impaired consciousness, as measured by Glasgow Coma Score (GCS), is the most common feature of neurological dysfunction of MODS. Impaired consciousness can be due to iatrogenic effects of treatment such as, sedatives and analgesics or may be related to frequent complication of severe illness due to metabolic alterations, release of inflammatory mediators and reduced cerebral perfusion (5, 51).

1.3.7 Kidney

Acute kidney injury (AKI) has long been recognised as a major complication of critical illness, accounting for approximately 60% of ICU admissions (52, 53). Sepsis and septic shock represent the most common aetiologies of AKI (54, 55). AKI is defined as an abrupt loss of kidney function (within hours) and structural damage linked to pre-morbid or pre-renal risk factors for AKI (56-60). The SOFA score assigns serum creatinine (sCr) as a useful approach for quantitative assessment of AKI, which indeed constitute an element of the current operational definition for the diagnosis of AKI by the Kidney Disease: Improving Global Outcomes (KDIGO) (61).

Although prevention of sepsis-associated AKI remains difficult, supportive therapy such as fluids, vasoactive medications and diuretics are found effective to manage sepsis-associated AKI but should be monitored closely to avoid adverse outcomes such as oedema (62-66). Current data on renal replacement therapy (RRT) in sepsis-associated AKI informed by large multi-centre trials suggests that early administration of RRT may propagate organ failure and worsen outcomes with no significant difference on 60 days mortality when compared to delayed RRT administration (67, 68). Therefore, patients who developed or at risk of sepsis-associated AKI should be screened and monitored closely to enable better implementation of care plan (56).

1.4 Pathophysiology of multiple organ dysfunction

1.4.1 Overview

Development of organ dysfunction is the most critical event during the course of critical illness, as it directly relates to mortality and morbidity (69). Regardless of aetiology and risk factors, the mechanisms that underlies organ dysfunction are similar across all major organs (17). These include a combination of haemodynamic and cellular dysregulations that are initiated by external and/or internal factors from the host response to eliminate infection or damaged tissues (17).

The immune system is crucially involved in regulating this process to aid in restoring tissue homoeostasis, but collateral damage to the major organs might result from augmented immune response (70). The effects of this excessive immune response exceeds tissue damage and predominantly cause distant organ failure and poor disease outcomes (70).

1.4.2 Cell death

To date, cellular death/dysfunction is the subject of intense research. Diverse cell death pathways being tightly linked to the pathogenesis of organ dysfunction. Dying cells may be non-programmed such as in necrotic cell death or programmed such as in regulated cell death (RCD), the later phenomenon can be inhibited using therapeutic or genetic applications (71). Necrosis, a form of non-programmed cell death, is energy independent cell death pathway frequently observed following trauma or can be triggered by virulence factors released from pathogens (17, 72). Whereas RCD relies on certain molecular mechanisms that are either caspase-dependent or caspase-independent pathways (72). The types of RCD are shown in figure 1.2.

RCD are of particular importance to immune cells and can be lethal triggers of innate

immune system activation (Figure 1.3) (73, 74). The release of endogenous components from dying cells, also known as danger signals, can activate cells of the innate immune system – for example, neutrophils, dendritic cells (DCs) and macrophages, which can not only lead to cytokines secretion, but also cell death-upon activation (Figure 1.4) (71). Of interest, for example, the activation of neutrophils is often – but not always – associated with a process involving neutrophils dying, known as – NETosis (71). This form of cell death will be explored in detail later in this chapter.

Figure 1.2

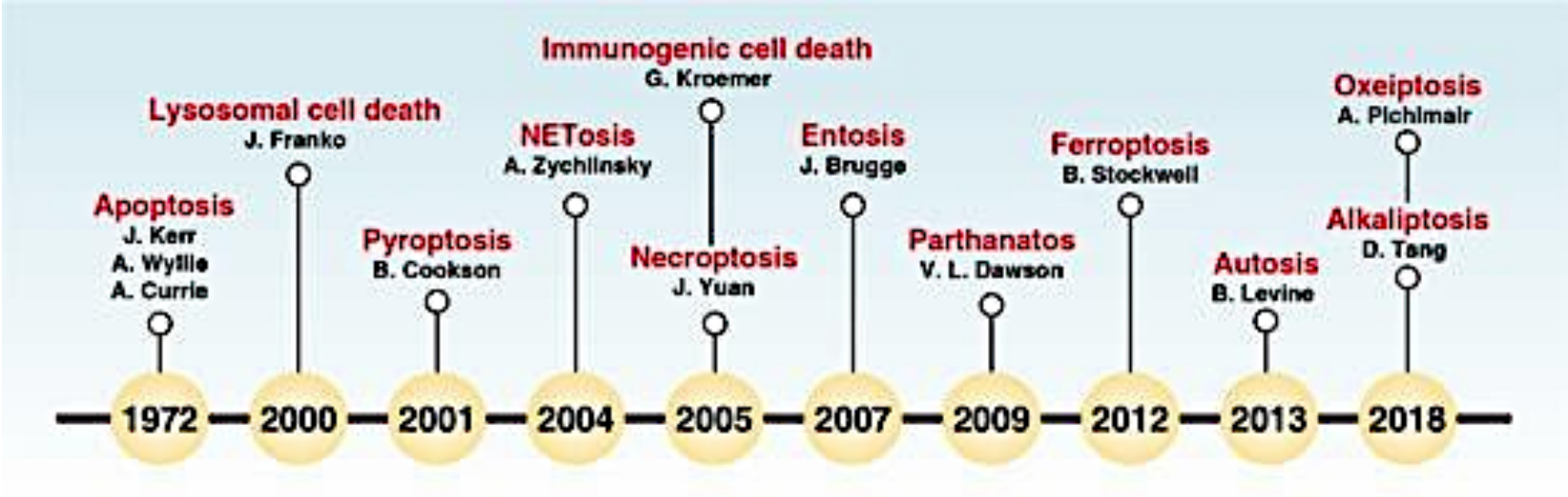


Figure 1.2 Timeline of regulated cell death (RCD) types. Apoptosis is the first form of RCD, which was discovered in 1972 (75). Apoptosis is defined as an active and morphologically distinct form of cell death that is characterised by apoptotic membrane blebbing and formation of apoptotic bodies. In 2000, lysosomal cell death was identified as another form of RCD which occurs after permeabilization of the lysosomal membrane (76). Lysosomal hydrolases (e.g. cathepsins) released following permeabilization are responsible for initiating several intracellular signalling cascades that lead to cell death. In 2001, pyroptosis was coined and characterised by the absence of DNA fragmentation and presence of nuclear condensation that will eventually lead to rupture of plasma membrane (77). Pyroptosis is mainly driven by the activation of inflammasome. In 2004, NETosis was identified as a unique form of neutrophil's death. NETosis is characterised by the ability of neutrophils to form web-like structures of DNA decorated with antimicrobial enzymes and proteins in response to various stimuli (78). In 2005, immunogenic cell death was explained as a form of RCD that drives an adaptive immune response in immunocompetent syngeneic hosts (79). Immunogenic cell death occurs in response to different stimuli such as, chemotherapy and radiotherapy. Necroptosis was initially described in 1996 but coined in 2005 and characterised as a programmed form of necrosis that mimic the features of both necrosis and apoptosis (80). Unlike apoptosis, the main feature of necroptosis is the extensive release of damage-associated molecular patterns (DAMPs). In 2007, Enotic cell death or Entosis was reported as a form of "cell cannibalism" in which a living cell engulfs and kill another cell to retrieve nutrients (81). In 2009, parthanatotic cell death or parthanatos was discovered and characterised by absence of apoptotic bodies, large-scale DNA fragmentation, loss of membrane integrity and absence of cell swelling (82). Few years later, ferroptosis was coined and described as iron-dependent cell death process that is accompanied with accumulation of large amounts of iron and oxidative degradation of lipids (83). More recently, autosis was discovered and reported as a form of cell death that can be induced by autophagy-inducing peptides, starvation and cerebral hypoxia–ischemia (84). Autotic cell death is regulated by Na+,K+-ATPase. Oxeiptosis and Alkaliptosis are the most recently discovered forms of RCD. Oxeiptosis is a reactive oxygen species (ROS)-dependent form of cell death that was observed upon exposure of mouse embryonic fibroblasts to hydrogen peroxide or ozone (85). Alkaliptosis is a pH-dependent cell death that was firstly identified in pancreatic ductal adenocarcinoma cancer in response to changes in pH (86)**,** adapted from Tang *et al* (87).

Figure 1.3

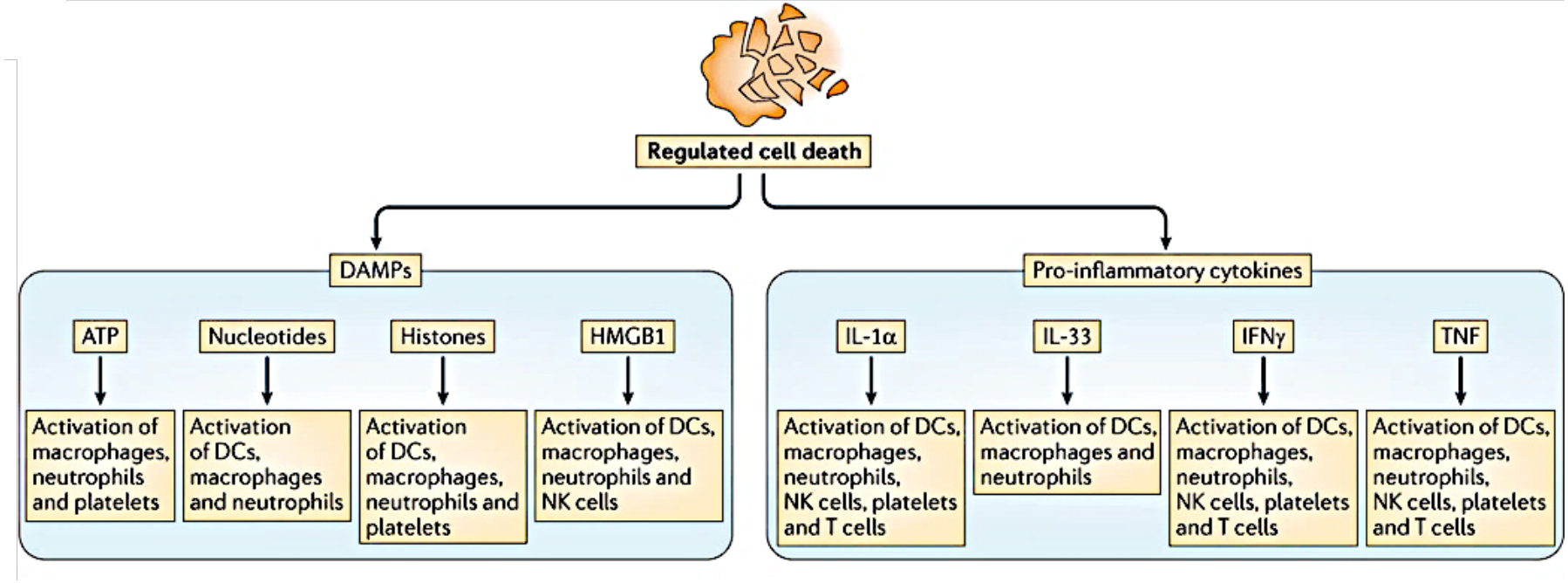


Figure 1.3 Immune cells activation by Regulated cell death (RCD) pathways. Activation of different immune cells can be promoted by the release of damage-associated molecular patterns(DAMPs) and pro-inflammatory cytokines triggered by RCD pathways, adapted Linkermann *et al* (72).

Figure 1.4

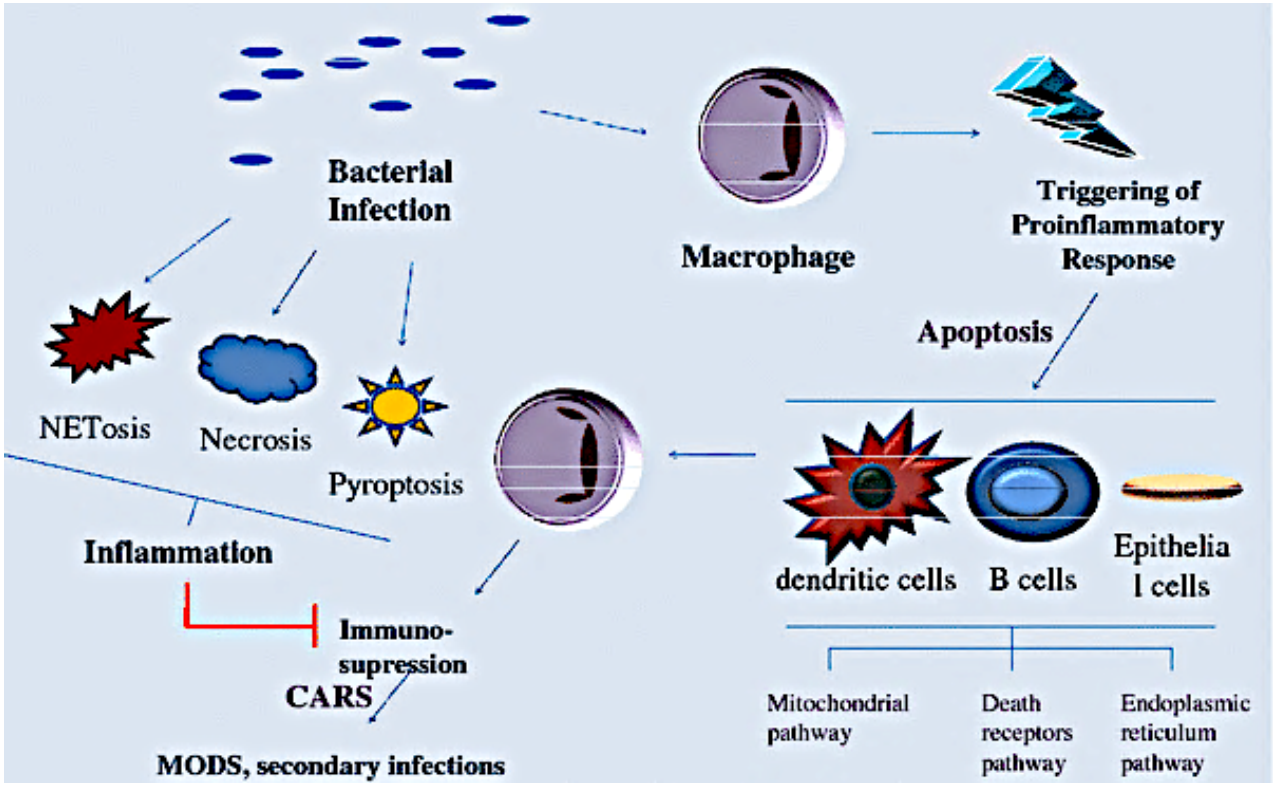


Figure 1.4 Cell death pathways triggered by bacterial infection. On one hand, bacterial infection can trigger macrophages to release proinflammatory cytokines, which causes apoptosis of dendritic cells(DCs), B cells and epithelial cells. On the other hand, bacterial infection could directly induce other types of cell death upon activation, including NETosis, necrosis and pyroptosis. Both mechanisms caninduce anti-inflammatory responses that may lead to compensatory antagonistic response syndrome (CARS) and multiple organ dysfunction syndrome (MODS), adapted from Da Silva *et al* (71).

1.4.3 PAMPs and DAMPs

In 1989, Janeway proposed a theoretical framework explaining how the innate immune system recognises and responds to inappropriate presence of microorganisms and microbial products to initiate inflammatory signalling cascades (88). These signals are molecularly distinct and capable of causing local inflammation and tissue damage via pathogen-associated molecular patterns (PAMPs). Multiple PAMPs have been described, including flagellin, peptidoglycans, viral RNAs and lipopolysaccharide (LPS) (89).

The PAMPs concept has led to the discovery of pattern recognition receptors (PRRs) present on the surface of immune and non-immune cells, responsible for the detection of PAMPs (90, 91). The activation of this broad class of receptors such as, toll-like receptors (TLRs) family can elicit proinflammation and microbial killing by neutrophils, monocyte/macrophages, and DCs (89, 92).

A complimentary model known as – the danger model was proposed in the mid 1990s, as the previous framework fails to explain how the inflammatory cascade is initiated in non-infectious (sterile) critical illnesses, such as trauma and burns (93). The hypothesis underpinning the danger model is that the local sterile inflammation and tissue damage are initiated when the innate immune system is triggered by endogenous molecules known as damage-associated molecular patterns (DAMPs) released from injured and necrotic cells (Figure 1.5). Similar to PAMPs, DAMPs can bind to PRRs and trigger innate immune system response (89). The most widely recognised DAMPS, include high mobility group box 1 (HMGB1) (94), histones (95), heat shock proteins (96), S100 proteins (97), mitochondrial and nuclear DNA (98, 99).

Sepsis is a life-threatening organ dysfunction syndrome, generally characterised by an

early proinflammatory phase and the subsequent immune suppressive/immune-paralysis phase (Figure 1.6) (100). The host's immune response in sepsis is initiated by a proinflammatory phase followed by a protective immunosuppressive phase (73). The initial phase is caused by the inappropriate presence of microorganisms and their products, which can be remarkably exaggerated by endogenous elements such as, extracellular DNA and histones. These elements contribute directly to the initiation and propagation of the inflammatory response (100). The later phase is characterized by failure to control primary infection and susceptibility to secondary infections. This phase is driven by multiple factors such as, release of anti-inflammatory cytokines and extensive cell death (73).

Figure 1.5

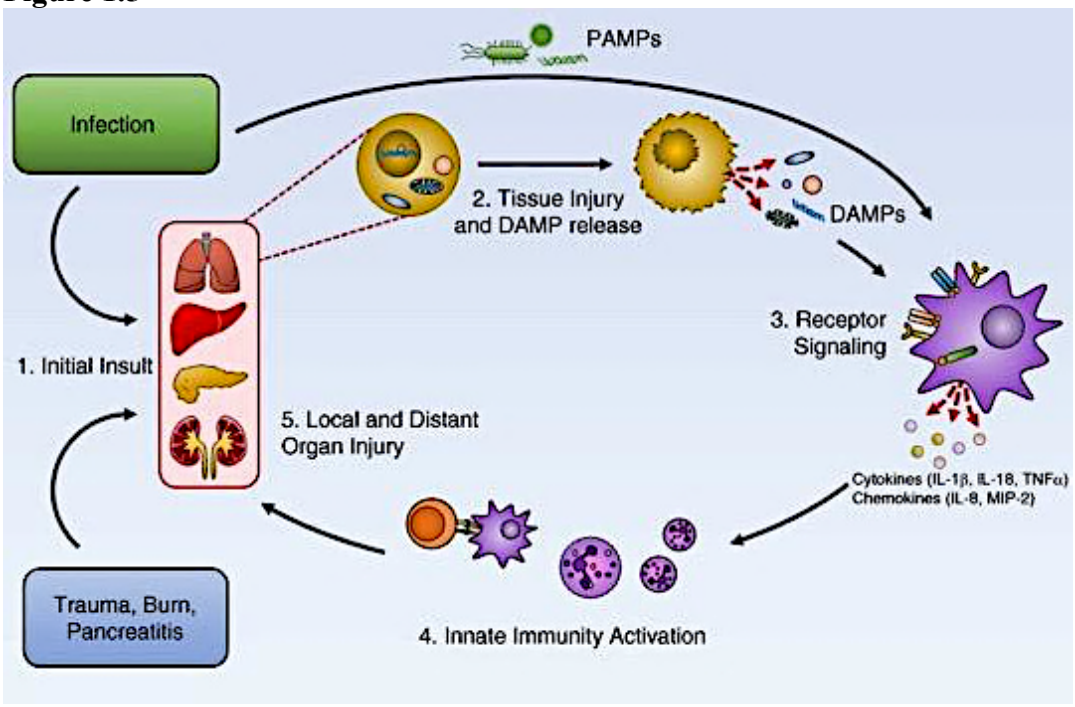


Figure 1.5 Schematic representation of the host response to infectious and non-infectious stimuli-mediated distal organ injury. (1) Insults during critical illness can be caused by either infectious agents (e.g. bacteria and viruses) or sterile insult (e.g. trauma and burn). (2) Pathogens releases pathogen-associated molecular patterns (PAMPs) and tissue injury causes damage-associated molecular patterns (DAMPs) release. (3) PAMPs and DAMPs bind to pathogen-recognition receptors and causes release of inflammatory mediators. (4) Activation of innate immune cells. (5) Subsequent organ injury, adapted from Ma *et al* (89).

Figure 1.6

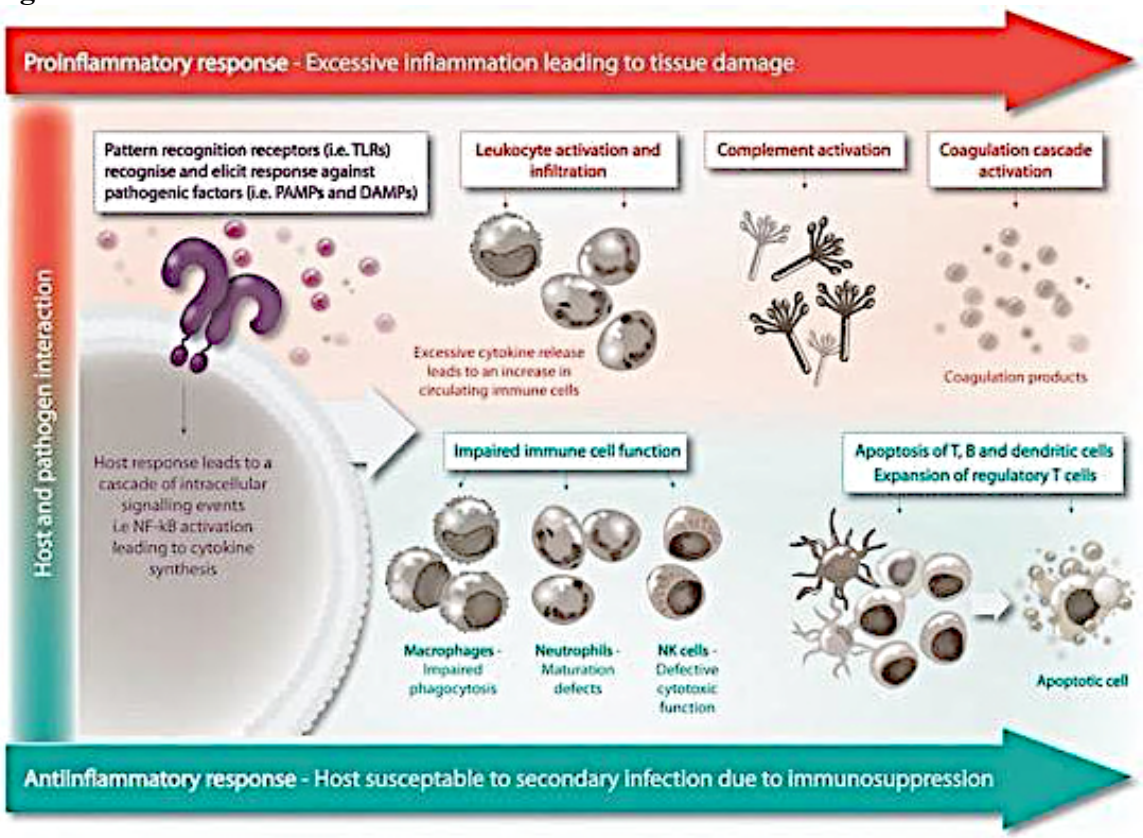


Figure 1.6 Proinflammatory and immunosuppressive phases of the host response during sepsis. The initial phase of the host response during sepsis is characterised by an overwhelming destructive inflammation followed by immunosuppressive phase that expose the host to secondary infections. Both phases are initiated after an insult occurs and causes the release of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). Following recognition of danger signals using pattern recognition receptors (PRRs), the initial host response causes inappropriate synthesis and release of cytokines. On one hand, this could lead to a proinflammatory phase in which multiple responses occur, including leukocyte activation and infiltration along with complement and coagulation activation. On the other hand, impaired immune cell function and cell death could occur during the immunosuppression phase, adapted from Nedeva *et al* (100).

1.4.4 Microvascular coagulopathy

Many patients with critical illness develop life-threatening coagulation abnormalities in response to overwhelming proinflammation caused by infection (101). Several haemostatic changes occur as a result, ranging from localised venous thrombosis formation to acute DIC, a state of widespread intravascular coagulation caused by excessive release of thrombin and rapid consumption of platelets and coagulation proteins (102, 103).

The elucidation of DIC as independent predictor of organ dysfunction and mortality in patients with sepsis has been supported by many histological studies of experimental sepsis, which proposed a role of excessive thrombi formation within the vascular system in the development of MODS (103, 104). Targeting DIC in experimental models aims at exploring therapeutics that can be either used to treat dysregulated coagulation or fibrinolysis to rescue from organ dysfunction and subsequent mortality (104, 105).

There are no specific experimental models for DIC, however in normal mouse, the classical method to induce DIC is known as the generalized Shwartzman reaction, in which two sublethal doses of LPS, spaced 24 hours apart, are injected in animals (106). Entrance of endotoxins to the blood circulation results in activation of immune and endothelial cells along with widespread release of cytokines, which eventually lead to coagulation activation (105). Moreover, other infectious agents such as bacteria and faecal contamination and procoagulant agents such as thrombin and tissue factor are used in animal studies to mimic human DIC or lead to certain activation of homeostasis (105).

The key events responsible for the activation of coagulation cascade are believed to be increased circulating inflammatory mediators along with invading pathogens and its derivatives (104). These factors can activate platelets which in turn lead to immunomodulatory effects on endothelial cells and leukocytes. Moreover, fibrin structures stabilize platelets aggregates to enhance thrombosis formation within the vasculature (107). The ensuing ischemia caused by excessive microvascular thrombosis and subsequent lack of oxygen delivery to the vital organ can lead to tissue injury and poor outcomes, including organ dysfunction (104).

Microorganisms and their products/PAMPs can upregulate the expression of tissue factor on monocytes and macrophages to promote coagulation activation during sepsis. Moreover, tissue factor induces thrombin generation via protease-activated receptors (PARs) to promote a proinflammatory response (108). In addition, other than PAMPs, the extracellular release of DAMPs from damaged and dead cells,such as HMGB1, can cause cell toxicity, proinflammation and coagulation activation (109).

Another significant aspect of the pathogenic roles played by coagulation factors in MODS is immunothrombosis, which describes an orchestrated interplay between the coagulation and innate immune systems. Immunothrombosis describes a protecting mechanism to the host whereby recognition, entrapment and disposal of pathogens is facilitated by innate immune cells and microvascular thrombi formation (110, 111). However, the host could develop DIC and even MODS and death when these reactions occur systematically over a long period of time (110).

Following the recognition of PAMPs and DAMPs via PRRs, immunothrombosis is triggered by activated monocytes and neutrophils. Activated monocytes and monocytes-derived microparticles initiate immunothrombosis via cleavage of tissue factor pathway inhibitor (TFPI) and enhanced expression of tissue factor (TF) on their surface (110). Increased expression of TF on microparticles is associated with procoagulant activity (110). Moreover, a recent driver of immunothrombosis is neutrophil extracellular traps (NETs) formation (112). To form NETs, neutrophils extrude DNA along with granular and cytoplasmic proteins to the extracellular space (78). Beside NETs being critical for killing pathogens, they have been observed to promote MODS through participation in clots formation (113). The procoagulant roles of NETs in MODS will be explored indetail later in this chapter.

1.5 The emerging role of NETs in critical illness

1.5.1 Production, recruitment and activation of primary neutrophils

Neutrophils or polymorphonuclear (PMN) leukocytes constitute a primary component of the innate immune system during infections (114). Neutrophils are produced in the bone morrow from hematopoietic stem cells (HSC) which differentiate into myeloid lineage progenitor cells (115) (Figure 1.7). The myeloid progenitors during the early phase differentiate into the granulocyte- macrophage progenitor (GMP) or megakaryocyte- erythrocyte progenitor (MEP) (116). The terminal differentiation and proliferation of neutrophils is tightly controlled by granulocyte colony-stimulating factor (G-CSF) to allow appropriate maturation of neutrophils before entering the blood stream (117). The promyelocyte, recognised by its round nucleus and relatively dark cytoplasm, is the first part of the maturation process in neutrophil-committed cells known as - mitotic pool in which cell proliferation continues and primary or azurophil granules are formed (115, 118). At the myelocyte stage, secondary or specific granules will form and the rounded nucleus will develop an initial dent and less dark cytoplasm (115, 118). Thereafter, the cells will enter the post-mitotic stage in which it will stop proliferating and become metamyelocyte, recognised by its kidney shape nucleus andclear cytoplasm (118). Banded neutrophil is the next phase of the post-mitotic neutrophils where the nucleus becomes horseshoe-like in shape and tertiary or gelatinase granule is formed (115, 118). Differentiated mature neutrophils will develop lobulated nucleus and secretory vesicles, with the ability to enter exchange state between bone marrow, bloodstream and tissues (118).

Neutrophil retention and release is orchestrated by chemokines that are produced by stromal cells in the bone marrow (117). In addition, neutrophil retention is controlled

by C-X-C-motif chemokine receptor (CXCR) 4 along with its ligand C-X-C-motif chemokine ligand (CXCL) 12, whereas neutrophil release from bone marrow to the blood is facilitated by CXCR2 along with its' ligands CXCL1, CXCL2, CXCL5 and CXCL8 (119-121).

Neutrophils are activated and rapidly recruited from the blood stream to tissues after apathogen has breached the epithelial barriers or in response to chemotactic factors released by pathogens or the host (122). Once at the site of infection and to fulfil the task of killing invading pathogens, neutrophils follow different major antimicrobial mechanisms, including reactive oxygen species (ROS) production, phagocytosis, degranulation and NETs release (78, 123). Neutrophil killing mechanisms during infection are activated by small molecule motifs found on and inside microorganisms, PAMPs which aredetected by the host's PRRs such TLRs (124).

Upon engaging a targeted microbe, neutrophils follow multiple antimicrobial processes one of which is known as degranulation where vesicles or granules fuse with plasma membrane to release its antimicrobial proteins into the extracellular space (125). The second mechanism in which neutrophils can engage a microbe is phagocytosis, where neutrophils engulf a microbe and carries out a potent arsenal using ROS in their granules to create a killing environment inside the phagosome (122).

Apart from the aforementioned-traditional antimicrobial defensive methods, neutrophils can extrude its DNA along with antimicrobial proteins and enzymes to the extracellular space to form NETs to facilitate microbial trapping and killing (78). The most common components of NETs are DNA, citrullinated histones-H3 (Cit-H3), myeloperoxidase (MPO), neutrophil elastase (NE), cathepsin G, leukocyte

proteinase- 3, lysozyme C and lactoferrin (126).

Figure 1.7

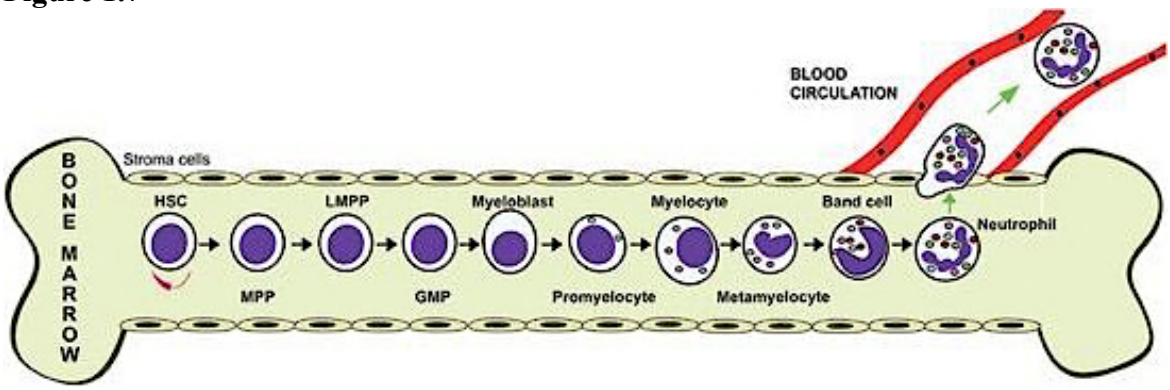


Figure 1.7 The maturation phases of neutrophils. The production of neutrophils occurs in the bone marrow. A self-renewing hematopoietic stem cell (HSC) differentiate into a multipotent progenitor (MPP) cell. From MPP cell, lymphoid-primed multipotent progenitors (LPMP) are formed, which differentiate into granulocyte-monocyte progenitors (GMP). Terminal neutrophil maturation begins with myeloblasts, which differentiate into the promyelocytes that commit to the neutrophilic cell line. The promyelocytes will then follow a maturation process including myelocyte, metamyelocyte, band cell and mature neutrophils, adapted from Rosales (115).

1.5.2 Neutrophil-like cells

Despite increasing interest in understanding the molecular mechanisms underlying the functions of primary neutrophils, their usage in laboratory investigations is challenging due to their short-life span (6-12 hours after isolation) (114). Recognising this, neutrophil-like cells are preferably used for convenience, currently their biological and clinical relevance is questionable. Different leukaemia cell lines have been investigated and characterised *in vitro* for their capacity to differentiate into neutrophil-like cells. In 1971, the first successful *invitro* cell differentiation was introduced when leukaemia cell line (murine erythroid leukaemia) were differentiated into mature phenotype (orthochromatic erythroblasts) using dimethyl sulfoxide (DMSO) (127). Following this, the capacity of different cell lines such as, HL-60 and PLB-985 to differentiate into mature neutrophil-like phenotypes have been established (128, 129). In addition, multiple chemical agents such as, DMSO (128), N, N-dimethylformamide (DMF) (130) and all trans retinoic acid (ATRA) (131, 132) have been identified and commonly used to induce leukaemia cell lines

differentiation into mature phenotypes .

The promyelocytic leukaemia cell line, PLB-985 cell line, was originally established from a 38-years old female with acute myeloid leukaemia (AML) but characterisation showed that the PLB-985 cell line a subclone from the AML cell line, HL-60 (133). PLB-985 cell line is bipotential and can be differentiated into either granulocyte or monocyte/macrophage, depending on the inducing agent (128, 130, 134, 135). This cell line has been widely used to study neutrophil processes including production of ROS and chemotaxis, as well as functional processes of neutrophils such as, phagocytosis and degranulation, but NETs release remain not fully elucidated (136-139). Thus, important outstanding question include to what degree differentiated PLB-985 replicate the capacity of primary neutrophils to produce NETs. Moreover, it remains elusive whether differentiated cell lines into neutrophil-like cells would be anideal model to study NETs formation.

1.5.3 Mechanisms of NETs release

NET release occurs primarily following a novel form of RCD termed NETosis. Signal transduction pathways leading to NETs formation can be activated using pathogenic and non-pathogenic stimuli (140). Although each transduction pathway has a specific molecular characteristics, the proteins and chromatin constituting NETs structures appear to be similar across all forms of NETs formation. Current literature investigating the mechanisms of NETs formation has relied on chemical inhibitors in laboratories and immunodeficiencies in humans, due to lack of cell lines that entirely mimic human or murine neutrophil behaviour (141).

There are two major signalling pathways of NETs formation, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase – dependent and NADPH oxidase–

independent pathways (Figure 1.8) (141, 142).

NETosis that leads to the activation of NADPH oxidase-dependent pathway can be induced *in vitro* using the pathogenic and non-pathogenic stimuli, such as phorbol 12-myristate 13-acetate (PMA), and is activated *in vivo* in infection models such as those mimicking sepsis. These stimuli cause ROS generation via NADPH- Oxidase that is initiated by triggering c-Raf, Mitogen-activated protein kinase (MEK), extracellular receptor kinase (ERK) and protein kinase C (PKC) (143, 144). NADPH-oxidase-dependent NETs formation pathway was first observed in neutrophils isolated from patients with chronic granulomatous disease (CGD) where it did not release NETs in response to mitogens and microbes due to mutations in NADPH-oxidase, therefore inability to generate ROS (145, 146).

The generation of ROS from neutrophils is initiated with the generation of superoxide anion from oxygen that is mediated by NADPH-oxidase. Superoxide is then converted to hydrogen peroxide (H_2O_2) which reacts with MPO to produce singlet oxygen (147-152). MPO is part of a protein complex stored in the azurophilic granules, MPO-deficient human neutrophils are unable to promote NETosis (153). MPO- H_2O_2 reaction will liberate neutrophil elastase (NE) and other serine proteases to translocate to the nucleus (153). Following this, the nucleus de-lobulates, nuclear membrane vesiculates and the cytoplasmic membrane ruptures to form NETs (141).

The role of ROS in NETs generation via the above pathway was extensively studied and showed that ROS generation via NADPH-oxidase pathway can contribute to NETs formation (147-152). Studies have also confirmed the role of ROS in NETs generation through treatment of neutrophils with inhibitors of ROS such as diphenyleneiodonium (DPI) and suggested that NETs formation was significantly abrogated (154).

Although NADPH Oxidase-independent pathway of NETs formation is poorly understood, Parker et al. highlighted that calcium ionophores such as ionomycin can still induce NETs formation even when oxidase activity is inhibited (155). Chromatin decondensation process is facilitated by citrullination or deiminiation of histones via Peptidyl Arginine Deiminase 4 (PAD4), a calcium-dependent nuclear enzyme (156). Histones citrullination is initiated by the conversion of arginine into citrulline resulting in loss of positive charge caused by PAD4 and thereby weakens the binding of histones to DNA and promoting the expulsion of DNA in the form of NETs (157). Cit-H3 is particularly well studied among other histone tails (H4 and H2b) and has been shown to be directly involved in the process of NETs release (158, 159).

Whilst there is a significant body of the literature emphasizing on the importance of PAD4 during NETs formation, Claushuis *et al* (160) suggested that PAD4 is not required for NETs formation in *Klebsiella pneumoniae*-induced sepsis. In their study, lung sections from PAD4 deficient mice infected with *Klebsiella pneumoniae* had low levels of Cit-H3, when compared to PAD4 positive mice. However, the authors also reported that scanning electron microscopy detected NETs-like structures in the lungs of PAD4 deficient mice. These observations were also supported by the presence of elevated levels of other NETs-related markers in bronchoalveolar lavage fluid (BALF) and plasma from both PAD4 positive and PAD4 deficient mice infected with *Klebsiella pneumoniae*.

Figure 1.8

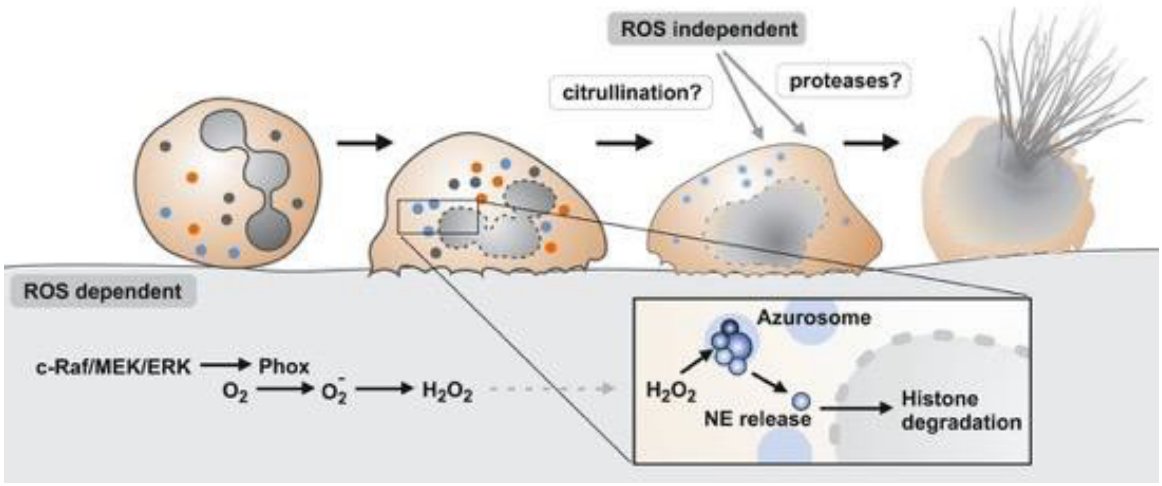


Figure 1.8 NADPH oxidase – dependent and NADPH oxidase–independent NET formation. Upon activation of neutrophil, neutrophil extracellular traps (NETs) formation often results from one of two common pathways. The first pathway is dependent on nicotinamide adenine dinucleotide phosphate (NADPH) Oxidase (Phox) and superoxide production. High amounts of superoxide (O_2-) are generated via the activation of the NADPH oxidase complex to dismutase superoxide into hydrogen peroxide (H_2O_2), which will be converted into halic acids to liberate neutrophil elastase (NE). Other serine protease will be also released to enter the nucleus and causes histone degradation and eventually lead to NETs formation. The second pathway does not involve ROS production but requires intracellular calcium influx to activate peptidylarginine deiminase-4 (PAD4) for histone citrullination, chromatin decondensation and subsequent NETs release, from Sollberger *et al* (141).

1.5.4 The dual function of NETosis

1.5.4.1 Overview

Neutrophils are functionally active and complex immune cells since they contribute effectively to the inflammatory cascade during infections. NETs formation was first identified as a novel killing mechanism through a well-orchestrated cell death program known as – NETosis, different from apoptosis and necrosis (146). Yet, later research identified NETs production as a major drawback to the host.

NETs are fragile and complex structures, approximately 15 nm in diameter, which form when the nucleus delobulates, granules disappear and cell membrane disintegrates (78). Chromatin condensation comprises the backbone of NETs structures, which allows contact between granular and cellular components before cytoplasmic membrane breakdown and NETs release (146, 161).

1.5.4.2 Trapping and capturing

NETs contribute to host defence by capturing, trapping and direct effects. Brinkmann *et al* (78) were the first to demonstrate using electron and immunofluorescence microscopies that exogenously added bacteria binds to extracellular DNA structures post-NETs formation, including *Staphylococcus aureus* (*S. aureus)*, *Salmonella typhimurium*, and *Shigella flexneri* (78). Later on, *in vivo* studies demonstrated that following lung infection with *Klebsiella pneumoniae* in mice, bacteria can adhere to the extracellular DNA structures of NETs (162). Although bacterial capturing via extracellular DNA is demonstrated by a substantial body of the literature, imaging evidence does not establish a definitive relationship on NETs trapping bacteria. Clark *et al* (163) showed that NETs capturing of *Escherichiacoli* (*E. coli*) is enhanced *in vitro* under flow conditions mimicking physiological shear forces within the circulation. Furthermore, systemic administration of DNase to breakdown NETs structures in mice infected with *S. aureus* resulted in rapid bacterialdissemination from the extracellular DNA structures, supporting the roles of NETs intrapping microorganisms (164).

Nucleases are secreted by various types of bacteria, including group A *Streptococcus*, which are implicated in different life-threatening diseases in humans such as, sepsis (165, 166). However, these bacterial nucleases do not only degrade NETs but can escape NETs trapping leading to increased bacterial invasiveness and dissemination (167).

Recurring fungal infections are common in immunocompromised neutropenic patients and in those with MPO-deficiencies, such as CGD (168). Interestingly, neutrophils can sense the size of the microbe in order to release NETs to capture and trap large pathogens such as, *Candida albicans* (*C. albicans*) because phagocytosis is inefficient

(169). Furthermore, NETs degradation was associated with fungal but not yeast dissemination in MPO-deficient mice (169). These observations indicate that yeast infection could be eliminated with phagocytosis, but due to impaired NETs formation fungal infection could not be eliminated.

The antiviral role of NETs has not been fully explored, although viruses can activate and induce NETs formation (170). Using a super-resolution structured illumination microscopy, human immunodeficiency virus (HIV)-1 virions was observed to bind to NETs structures. Interestingly, when HIV-1 vector was incubated with PMA-stimulated neutrophils, HIV-1 infectivity was reduced as a result of DNA released during NETs formation that mediated inactivation of HIV-1 (171).

1.5.4.3 Direct killing

Beyond capturing and trapping, there is much argument over NETs can kill microorganisms. Neutrophils can directly kill pathogens through phagocytosis, but when phagocytic function is compromised, neutrophils still retain other antimicrobial functions.

Certain antimicrobial molecules that are known to decorate NETs structures such as histones are recognised for decades to retain some microbicidal properties (172). Furthermore, NE has been shown to neutralize and inactivate extracellular bacterial virulence factors (173). Zinc chelation and opsonization of microbes is another antimicrobial mechanism by which NETs can stop microbial proliferation and facilitate killing via NETs-specific proteins such as calprotectin and pentraxin-3 (166, 174).

Disintegration of NETs structures using Deoxyribonuclease (DNase) I has shown to reduce killing and enhance proliferation and dissemination of *S. aureus*, despite

effective trapping and killing of bacteria in the absence of DNase I treatment (175).

1.5.4.4 Direct cytotoxicity

The vascular endothelium creates an active interface between blood and tissues with pivotal roles in regulating homeostasis, vascular permeability and leukocyte trafficking (176). The vascular endothelium can be considered as large as a football pitch (~3000-6000m^2) and its dysfunction could result in adverse clinical implications, in particular initiating MODS (177). In this context, following neutrophil activation and NETs formation, an amalgam of granular and cytoplasmic materials e.g. histones, MPO and NE are bound to DNA structures of NETs (78). Because of exposed toxic histones within NETs, collateral damage to endothelium may occur (163, 178) and initial evidence supporting this has reported that injury to the endothelium and surrounding tissue occurred following NETs formation caused by LPS-activated platelets (163). Later research showed that DNase I treatment only eliminated DNA, but not NETs-associated molecules, and partially reduced tissue injury (178). This implies that other NETs-related molecules such as, histone, are cytotoxic and in part involved in tissue injury (178). Both these studies have postulated the important and predominant cytotoxic role of histones derived from NET in vasculature injury and subsequent outcomes, such as MODS observed in sepsis.

Elastase is another abundant serine proteinase of NETs with critical implications in killing and clearing pathogens (179, 180). The detrimental roles played by elastase has been solidified more than 50 years ago when deficiency in an endogenous inhibitor ofNE was associated with chronic airflow obstruction and emphysema (181). Later research appreciated the destructive capacity of excessive production of NE in numerous critical illnesses, such as ARDS (182), sepsis (183, 184) and acute pancreatitis (185). Despite effective bactericidal activities of elastase,

proinflammatory cytokines production and increased permeability of alveolar capillaries during sepsis-induced ARDS are consequences of excessive NE production (186). It was reported that dismantling DNA structures resulted in increased elastase activities (187). However, in contrast to histones and MPO, Saffarzadeh *et al* (187) reported that NE bound to NETs did not exhibit cytotoxic effects to endothelial and alveolar epithelial cells. Consequent evidence suggested that NE is not required for neutrophils to produce NETs (188). Furthermore, elastase deficiency *in vivo* did not influence venous thrombosis formation in mice model of inferior vena cava stenosis (188).

Although NETs-derived components may be armed with antimicrobial machinery, it is now evident that NET-driven pathology contributes to bystander cells and surrounding tissue and therefore profound involvement in MODS. It is noteworthy that macrophages are involved in the removal of NETs, *in vitro* and *ex vivo* using isolated cells (189). However, excessive release and/or insufficient removal of NETs may justify why NETs are implicated in various pathologies (189).

1.5.5 NETs in acute organ injury

1.5.5.1 Overview

Whilst NETs formation is vital for trapping and killing pathogens, excessive NETs has been observed to cause collateral damage to host tissues. To date, there have been numerous studies of NETs formation being implicated in pathogenesis of acute of organ injury. Clark *et al* (163) was the first to demonstrate that NETs formation not only aids in bacterial trapping, but also causes damage to the endothelium and surrounding tissues (163).

1.5.5.2 Respiratory

The cascade of antimicrobial mechanism is routinely initiated by neutrophils via various killing machineries, including NETs release to combat pathogens at the affected sites (78, 123). This occurs more frequently in the lungs than in other major organs, with higher concentrations of neutrophils are readily exist in lungs vasculature than systemic circulation (190).

Acute lung injury (ALI) is initiated following direct (e.g. pneumonia) or indirect (e.g. sepsis) insults to the lungs. This may lead to increasing alveolar permeability, endothelial and epithelial injury and excessive neutrophilic infiltrates within pulmonary vasculature and alveolar space (191). Consequently, lung dysfunction could result from pulmonary microcirculatory dysfunction, leakage of protein-rich fluid, cell death and excessive inflammatory response (191). Using intravital microscopy Lefrançais *et al* (192) observed the degree of NETs formation within pulmonary microcirculation correlate with vascular permeability changes, which represents a key pathological feature of ALI (192). Similarly, excessive NETs formation was detected in transfusion-related acute lung injury (TRALI) withinthe pulmonary microcirculation of murine models and lung sections of fatal cases of TRALI (193). The authors suggested that NETs formation mediated by activated platelets led to increased pulmonary vascular permeability and oedema.

Large amounts of circulating NETs-breakdown products have been previously reported in various murine models of sepsis. However, the current literature on whether the lungs are susceptible to widespread NETs release during sepsis is controversial. Some groups have shown NETs are excessively produced with lungs vasculature following septic insults, such as cecal ligation and puncture (CLP), intraperitoneal (i.p.) injection of *E. coli* and LPS models (194-197), whilst others have

reported insignificant NETs levels within the lungs following septic insult, mainly in mice models injected with LPS (163, 198).

Patients with pneumonia showed significant increase in NETs formation as evident by NETs-breakdown products, which correlated with ARDS severity and mortality (192). Interestingly, pneumonia patients who developed ARDS displayed higher levels of NETs than those without ARDS, implying that the lungs are more susceptible to excessive NETs formation.

1.5.5.3 Coagulation

Fuchs *et al* (113) were the first to report a prothrombotic role of NETs (113). In this elegant study, NETs were shown to provide a scaffold for platelets adhesion as well as for thrombin-dependent fibrin formation. Furthermore, DNA structures of NETs can trap procoagulant molecules, such as von Willebrand factor (VWF), fibronectin, fibrinogen, factor XI I(FXII) and TF (113, 199). As such, the histone- DNA complex of NETs support fibrin localisation within thrombi (200). Indeed, thicker and rigid fibre structures are observed when fibrin binds to the histone-DNA complex of NETs (201).

Mechanistically, NETs formation induces thrombin generation through platelet-independent and dependent mechanisms. Increased thrombin generation in platelets-poor plasma requires the activation of coagulation factors XII and XI through DNA-dependent manner (202). Our group has recently demonstrated that histones directly induce thrombin generation by forming an alternative prothrombinase. Specifically, histones directly bind to prothrombin to facilitate FXa mediated prothrombin cleavage and thrombin generation (203). In addition, enhanced thrombin generation in platelets-rich plasma is observed after histones activates platelets via toll-like

receptors (TLR-2 and 4) (202). Apart from platelets, NETs can trap anticoagulants and allow degradation of TFPI by NE, which will eventually enhance fibrin formation (204).

In line with this, increased microvascular occlusion as a result of NETs-fibrin colocalization as well as thrombin generation within microvasculature were observed in a murine model of endotoxemia (205). Dismantling DNA structures bound to NETs or use of PAD4 knockout can reduce microvascular clot formation and thrombin generation as well as subsequent microvascular hypoperfusion and end organ damage (205).

1.5.5.4 Liver

Liver I/R injury commonly occurs after major insults, such as massive trauma and liver resection, in which the ischemic insult will lead to cellular damage and reperfusion will provoke the injury by augmenting the inflammatory response and immune responses (206, 207).

Following liver I/R injury, complex array of inflammatory mediators are released from necrotic or hypoxic hepatocytes and contribute to NETs formation (208). Huang *et al* (209) demonstrated that histones and HMGB1 mediate NETs formation in liver I/R injury mice models via TLR-9 or TLR-4 signalling (209). Tohme *et al* (210) also showed that NETs are involved in the inflammatory response during liver I/R injury, with IL-17A promoting NETs formation *in vitro* and *in vivo* (210).

1.5.5.5 Heart

Rapid restoration of blood flow is considered as a therapeutic approach in many serious cardiac pathologies, such as myocardial infraction to limit extended damage caused by lack of oxygen supply to the myocardium. However, collateral damage

following restoration of blood supply may result in I/R injury to the heart. I/R injury has been repeatedly shown to evoke adverse inflammatory state marked by leukocytes infiltration. Excessive neutrophil recruitment and NETs release aggregates has been observed within the myocardium and cause endothelium injury (208). On the other hand, Savchenko *et al* suggested that circulating NETs-breakdown products are excessively produced in myocardial I/R injury mouse model, which adversely contributed to the size of infarct and LV ejection fraction (211).

1.5.5.6 Kidney

Four major structures of the kidneys are affected following AKI, including tubules, glomeruli, interstitium and blood vessels (212). Uncontrolled immune response is frequently involved in the development of AKI (213). Leukocytes infiltration during ischemic AKI can impair renal function through improper capillary function, parenchymal cell injury and generation of inflammatory molecules such as ROS (212, 214).

In vitro, NETs have been visualised upon triggering by cytotoxic factors (e.g. histones) released from ischemic tubular cells following neutrophils incubation with media from necrotic human kidney cells (215). *In vivo*, excessive NETs release drives tubular necrosis, contributing adversely to post-ischemic AKI in mouse models of renal ischemia-reperfusion (I/R) injury (216, 217).

NETs formation can promote injury to the tubular epithelial cells and clotting within the peritubular capillaries, leading to hypoxia, tissue damage and eventually AKI (217). In addition, secretion of histones from ischemic tubular cells can prime neutrophils to release NETs *in vitro*, suggesting that histones-mediated NETs formation aggravates necroinflammation which in turn exacerbates kidney injury (215).

1.6 Targeting NETs in critical illness

As discussed above, there is expanding body of the literature describing the determinantal roles of NETs-associated organ injury in critical illness. Therefore, it isproposed that therapeutic strategies aiming at either prevention of NETs production orenhancement of NETs degradation are of particular interest in NET-associated organ injury.

PAD4 is an essential enzyme responsible for chromatin decondensation during NETosis via histone cleavage/citrullination (158, 218). Thus, one proposed strategy for targeting NETs formation is the inhibition of PAD4 activities via the administration of PAD4 inhibitors or knockout PAD4 in mice. In this context, using a murine model of pneumonia, Lefrançais *et al* (192) have shown that complete PAD4 knockout reduced NETs-induced lung injury but at the expense of bacterial clearance and local inflammation (192). In the same study, therapeutic inhibition of PAD4 reduced Cit-H3-DNA complex and inflammatory cytokines without affecting bacterial clearance; however, lung injury was not improved.

Conversely, Martinod *et al* (197) have demonstrate using a murine model of polymicrobial sepsis that complete knockout of PAD4 prevented Cit-H3 and reduced plasma DNA levels, without affecting bacterial clearance or impairing anti-inflammatory function (197).It is worth mentioning that PAD4 deficiency and subsequent NETs inhibition has been also shown to improve renal function as monitored by blood urea nitrogen (BUN) and creatinine following I/R injury to kidneys (216), as well as cardiac function after myocardial I/R injury (211).

The most widely used strategy to target pre-formed NETs is via nucleases, which are responsible for cleaving the phosphodiester bonds of DNA and RNA (219). There are

two major classes of nucleases has been identified: endonuclease and exonuclease (219). DNase I is an endonuclease enzyme that can digest bothsingle and double stranded DNA (219). DNase I has been extensively reported to dismantles DNA structures of NETs *in vitro* and in various pathological animal models.

In critical illness, DNase I treatment has been observed to partially reduce NETs and lung injury without impairing bacterial clearance in a murine model of severe bacterial pneumonia. In another study, treating mice with I/R-induced myocardial injury with acombination of DNase I and recombinant tissue-type plasminogen activator, an enzyme that helps to dissolve blood clots, significantly reduced NETs formation (211). In the same study, overall cardiac function demonstrated by LV function and infarct size were improved when therapeutic combination was used, compared to DNase I or recombinant tissue-type plasminogen activator alone.

Despite that DNase I can effectively digest DNA structures of NETs, DNase I does not inhibit nor reduce NETs-associated proteins and thereby could promote an inflammatory cascade (220). Mai *et al* (221) reported that following experimental sepsis model, inflammation and organ injury were provoked when DNase I administered early (221). One solution to this is to incorporate DNase treatment with other anti-inflammatory drugs (222).

Another important aspect concerning the production of NETs is the generation of ROS via NADPH oxidase or mitochondrial respiratory chain, which triggers neutrophils toform NETs (142). Experiments performed *in vitro* have shown significant reduction in NETs release when neutrophils were pre-treated with an antidiabetic drug called metformin, which is known to inhibits NADPH oxidase (223). The same research group also suggested that in a randomized placebo-controlled trial, prediabetes patients

after two months of metformin treatment had significantly lower NETs-related enzymes, such as Cit-H3, NE and proteinase-3. It is worth noting that metformin showed anti-inflammatory effects in diabetic patients, marked by reduced levels of IL-6 and tumour necrosis factor (TNFα) (224). However, the prognostic values of NETs after metformin therapy remain unclear, because the authors were not able to assess thrombotic events nor endothelial damage associated with NETs formation in diabetic patients (224).

Activated protein C (APC) is a natural multifunctional protease with antithrombotic, anti-inflammatory and cytoprotective functions (225). The antithrombotic effects of APC are derived from its circulating precursor molecule protein C and mediated by inactivation of certain blood clotting factors, including factor Va and VIIIa (226). Healy *et al* (227) demonstrated that activated platelets induce NETs formation but when neutrophils bind to APC, NETs and NETs-associated platelet adhesion can be prevented (227). However, observations from large scale clinical trials on humans suggested that APC has failed to improve survival of sepsis patients (228).

In summary, inhibiting NET formation may offer promising therapeutic option, however, NETs release under some circumstances is an essential antimicrobial strategy. Thereby, therapeutics aiming at targeting NETs formation should avoid disrupting favorable features of NETs e.g. bacterial clearance and target the harmful side of NETosis such as, NETs-degradation product.

1.7 NETs detection and quantification assays

As the adverse consequences of NETs release described in this chapter, it is now clear that detection and quantifying NETs in patients or animal models may inform clinical care and benefit scientists. The existing approaches for measuring NETs in blood are

based on enzyme-linked immunosorbent assay (ELISA) which quantifies various NETs components, such as nucleosomes, NE, MPO, MPO-DNA complexes and citrullinated histones (229, 230). However, nucleosomes, NE, and MPO measured via ELISA is not necessarily specific for NETs formation (231). In addition, standardisation of accurate measures of NETs degradation markers, such as MPO-DNA and Cit-H3 ELISAs have not reach a consensus yet (231).

Flow cytometric assays have also been proposed for rapid detection of NETs biomarkers on cells, particularly *in vivo* (232-234). Indeed, there has been increasing interest more recently to optimise NETs identification and quantification using flow cytometry analysis. This analysis involves the assessment of NETotic cells in a mixed cell population (232-234). However, this method lacks objectivity and specificity as the outcomes could be hampered by increased the risk of false positive, given the fact that DNA release also occurs following other forms of cell death (231, 235).

Imaging based applications are commonly utilised for the assessment and visualisation of NETs formation, including real-time imaging by intravital microscopy or live cell imaging under confocal microscopy (146, 178, 236), as well as high resolution imaging by scanning or transmission electron microscopy (78, 236). Conventional microscopy is another widely used application for the detection and visualisation of NETs release via immunodetection of granule proteins bound to DNA structures of NETs (231). One potential drawback to microscopy-based detection analyses, however, is that the release of extracellular DNA or granular proteins can occur due to other sources of cell death other than NETs formation (237-240), with exception to *in vitro* assessment of NETs release from isolated neutrophils (241).

Currently, there are no available standardised assay to accurately measure NETs formation, and cautions should be taken when interpreting current data on NETs,

particularly from patients. Indeed, while there is no doubt that our knowledge of NETs is continuing to grow, standardised methods to achieve deeper understanding of NETs formation is urgently required, especially in the clinical setting.

1.8 Thesis hypothesis and aims

Hypothesis: The ability of patient plasma to stimulate NETs formation will have biomarker and prognostic value.

The specific aims that will be explored in this thesis are outlined below:

1. **Determine whether neutrophil-like cell line or primary neutrophils can produce NETs when incubated with plasma from septic patients.**

Monitoring NETs in clinical settings is limited to NETs-breakdown products that are not specific and susceptible to systemic degradation (231, 242), thus limiting its clinical potential. I will explore whether physiological inducers of NETs formation are present in patient plasma, and could trigger primary neutrophils or neutrophil-like cells (PLB-985) to form NETs. The use of neutrophil-like cells may offer a convenient source of neutrophils for potential clinical application. This will offer a robustly optimised and validated assay to studying NETs in the laboratory and clinical settings. If neutrophils form variable degrees of NETs in response to patients' plasma, a viable quantification method can be developed.

2. **Determine the translational relevance of NETs and NETs-driving factors in critically ill patients.**

NETs surrogate markers are elevated in critical illness, but lack of specificity and correlation with disease severity or outcomes are major shortcomings of these markers (243-245). If the plasma NETs-forming capacity assay show broader clinical application, this may provide a useful tool in assessing the pathological relevance and association with clinical outcomes. In addition, identifying and specifically targeting NETs-driving factors in patient samples may inform on key physiological components that contribute to NET release in individual patients.

3. **Investigate the roles and potential therapeutic targeting of NETosis in mouse models of sepsis.**

Identifying the pathological relevance of NETs and targeting the key factor driving the NETs-forming capacity may hold promise as a potential strategy for personalized therapeutic approach. This proof-of-concept study will help confirm the *in vivo* relevance of novel therapeutic approaches using mice models, prior to undergoing complementary future clinical trials in patients.

Chapter 2: Materials and Methods

2.1 Materials

Table 2.1 List of materials

Material	Company
RPMI-1640 medium	Sigma-Aldrich (Dorset, UK)
Foetal Bovine Serum	
Penicillin-Streptomycin	
Dimethyl sulfoxide	
All Trans-Retinoic Acid	
Dimethylformamide	
Phosphate-buffered saline	
Histopaque-1077	
Percoll	
Paraformaldehyde	
Propidium Iodide	
Tween-20	
DPX mountant for histology	
Recombinant IL-8	
Lithium carbonate	
Phorbol 12-myristate 13-acetate	Abcam (Cambridge, UK)
Mouse CXCL2 ELISA kit	
Anti-MPO antibody (ab 45977)	
Anti-Histone H3 antibody (citrulline R2 + R8 + R17) (ab 5103)	
Anti-Histone H3 antibody (ab 1791)	

Goat anti-Rabbit IgG-PE secondary antibody (ab 72465)	Abcam (Cambridge, UK)
Anti-fibrinogen (ab 34269)	
SYTOX green	Thermo Fisher Scientific (Massachusetts, USA)
Myeloperoxidase ELISA kit	
Molecular weight marker	
Red blood cells lysis buffer	
Alexa Fluor 700	
ECL Plus Western Blotting Detection System	
Triton x-100	Fisher Scientific (Loughborough, UK)
Methanol	
DAKO REAL EnVision Detection Systems	Dako Agilent Technologies (Santa Clara, USA)
PLB-985 cell line	ATCC (Manassas, USA)
Glass chamber slides	BD Biosciences (Oxford, UK)
Bovine serum albumin	Melford Laboratories Ltd. (Ipswich, UK)
Recombinant histone H3 protein	New England Biolabs (Ipswich, USA)
Haematoxylin	Merck (Hertfordshire, UK)
Xylene	
DNase I	
Recombinant CXCL2 protein	R&D systems (Minneapolis, USA)
Anti-IL-8 mAb	
Anti-NE antibody (sc-9521)	Santa Cruz Biotechnology (Texas, USA)
Anti-MPO antibody (sc-365436)	
Mouse anti-rabbit IgG-HRP secondary antibody (sc-2357)	

Donkey anti-goat IgG-FITC secondary antibody (sc-2024)	
Bio-Plex Pro human cytokine 27-plex assay	Bio-Rad (Hertfordshire, UK)
Hydromount	National diagnostics (Atlanta, USA)
Reparixin	Dompé (Milan, Italy)
Ly6G-specific monoclonal antibody 1A8	BioXcell (Lebanon, USA)
AZD5069	AstraZeneca (Cambridge, UK)
EasySep Mouse Neutrophil Enrichment Kit	StemCell Technologies (Grenoble, France)
CD11b conjugated anti-human antibody PE	BD Pharmingen (San Jose, USA)
Hydrogen peroxide	Acros (Geel, Belgium)
Cl-Amidine	Cambridge Biolabs (Cambridge, USA)
Anti-dsDNA antibody	Roche Diagnostics (Mannheim, Germany)

2.2 Assay development

Neutrophil extracellular traps (NETs) assay was developed using a cohort of septic patients (n=54) admitted to the intensive care unit (ICU) at Aintree University Hospital and the Royal Liverpool University Hospital (RLUH). Ethical approval was granted from the National Health Service (NHS) Research Ethics Committee REC (Reference Number: 13/WA/0353).

2.2.1 PLB-985 culture and differentiation

The human myeloid leukaemia cell line (PLB-985) was cultured in RPMI-1640- L-glutamine medium supplemented with 10% (v/v) Foetal bovine serum (FBS) and 1% (v/v) Penicillin-Streptomycin at 37^{o}C in a humidified incubator with 5% CO_2. PLB-985 cells were passaged three times a week into 1:3 ratio to maintain cell density

between $1x10^5$ and $1x10^6$/ml. PLB-985 cells were differentiated into neutrophil-like cells using two conditions. Cells at a density of ($1x10^6$) were cultured in RPMI-1640 medium containing either 1.25%Dimethyl sulfoxide (DMSO) or 2 μM All Trans-Retinoic Acid (ATRA) and 0.5% Dimethylformamide (DMF). Cells were then incubated at 37°C in a humidified incubator with 5% CO_2 for 6 days, and medium was renewed on day 3.

2.2.2 Evaluation of CD11b surface expression

Successful differentiation of PLB-985 progenitor cell lines into mature neutrophils was determined using flow cytometry using the neutrophil marker CD11b. Briefly, undifferentiated and differentiated PLB-985 (dPLB-985) cells ($5x10^5$cells) were suspended in buffer (200 μL total volume) containing phosphate-buffered saline (PBS) and 3% (w/v) bovine serum albumin (BSA) (180 μL) and 20 μL of the anti-human CD11b-PE conjugated antibody. Cells were then incubated at room temperature in the dark for one hour. After incubation, cells were centrifuged at 400g for 5 minutes, supernatant was discarded and pellet was washed with 1x PBS. Cells were then centrifuged at 400g for 5 minutes, supernatant was then discarded, and pellet was resuspended with 1x PBS and placed on ice in the dark until examination using BD Accuri C6 flow cytometer.

2.2.3 Blood samples

Plasma was isolated from citrated peripheral blood samples of all patients (n=54) or healthy volunteers (n=20) following centrifugation at 2600g for 20 minutes at 20°C. The resultant plasma was then aliquoted and stored at -80°C until further analysis. Matched plasma (n=10) and sera (n=10) were also isolated from whole blood samples and stored at -80°C.

2.2.4 Fluorescent plate reader assay

The usefulness of dPLB-985 as a model of neutrophils to study NETs was studied by continuous quantification of cell-free DNA (cfDNA) release using the plasma membrane-impermeable DNA-binding dye, SYTOX green. The capacity of dPLB-985 cells to release extracellular DNA was initially examined using phorbol 12-myristate 13-acetate (PMA), a well-known inducer of NETs. Using a 96-well microplate, dPLB-985 cells ($1x10^6$) were incubated in RPMI-1640 supplemented medium without or with various concentrations of PMA. In addition, the capacity of these cells to release extracellular DNA in response to platelet-poor plasma was tested by adding patient plasma or normal plasma (n=10) to dPLB-985 cells. SYTOX green was then added at a final concentration of 2 μM. Continuous fluorescence was performed for 4 hours and using a plater reader at excitation and emission wavelengths of 488 nm 523 nm, respectively.

2.2.5 Isolation of human neutrophils

Written informed consent was obtained from healthy volunteers after ethical approval from Liverpool University Interventional Ethical Committee (Reference: RETH000685). Neutrophil isolation follows a two-step gradient centrifugation using (1) a Histopaque-1077 gradient and (2) a Percoll gradient. In brief, citrated blood was transferred to a sterile 50 mL tube and mixed with 1x phosphate buffered solution (PBS) at 1:1 ratio. Mixed citrated blood was then carefully layered over Histopaque (density: 1.077 g/mL) (2:1 ratio). This was performed to sediment peripheral blood mononuclear cells (PBMC) following 30 minutes of centrifugation at 700 x g. Following centrifugation, the top layers containing plasma, PBMC and Histopaque were discarded. Neutrophils-rich layer containing red blood cells (RBCs) was washed with 1x PBS for 10 minutes at 400 x g. The supernatant was discarded, and the pellet

resuspended in PBS and carefully layered onto the top of five Percoll gradients (65%, 70%, 75%, 80% and 85%). This was followed by centrifugation for 20 minutes at 600 x g with slow acceleration and deceleration to avoid mixing. After centrifugation, neutrophils-rich layer appeared at the 70-80% Percoll interface. Neutrophils were pipetted gently to a new tube and washed using 1x PBS and further centrifuged for 10 minutes at 400 x g. The supernatant was then discarded and cell pellet was incubated with RBCs lysis buffer for 10 minutes before being centrifuged again for 10 minutes at 400 x g. Neutrophils with >90% purity were resuspended in PBS. Cell count was performed thereafter and media volume was adjusted for a final concentration of $2x10^5$ cells/well.

2.2.6 Microscopic assessment of NETs forming capacity assay

To assess the ability of patient plasma to induce NETs formation, patient or normal plasma (100 µL) was incubated in glass chamber slides for 4 hours at 37°C in 5% CO_2 with dPLB-985 cells or heterologous neutrophils from healthy volunteers ($2x10^5$ cells/well) (or patients' neutrophils, where indicated). Assay optimisation was also carried out to compare NETs formation of matched patient plasma/sera. Following incubation, cells were fixed with 2% (v/v) paraformaldehyde (PFA) for 10 minutes and staining of extracellular DNA was performed with Propidium Iodide (PI; 10 µg/mL). Slides were then mounted using Hydromount and glass cover-slips until visualisation using immunofluorescent microscopy (Nikon Eclipse 80i) with 20x magnification, unless otherwise stated.

2.2.7 Validation of NETs structures

Primary neutrophils were isolated from peripheral blood of healthy volunteers as described in section 2.2.5 and *ex vivo* NETs formation assay was performed (section 2.2.6). Thereafter, cells were fixed with 4% (v/v) PFA for 10 minutes then washed

with 1x PBS. Cells were blocked with 2% (w/v) BSA in 1x PBS for 30 minutes, prior to adding the primary antibodies, rabbit polyclonal antibody (pAb) to myeloperoxidase (MPO) and Goat pAb to neutrophil elastase (NE). All antibodies were diluted in 2% (w/v) BSA. Fixed cells were incubated overnight at 4°C. Following incubation, slides were washed then incubated with Fluorescein isothiocyanate (FITC) and Alexa Fluor (AF)-700 secondary antibodies for 45 minutes per antibody, with 2x washes between incubation. Finally, the extracellular DNA structures were stained with 10 μg/mL of PI.

Further validation was performed by blocking peptidyl arginine deiminase (PAD) 4, an essential enzyme responsible for NETs release, by pre-treatment of neutrophil with Cl-Amidine (10 μM) for 10 minutes prior to adding patient plasma and performing NETs assay (section 2.2.6).

2.2.8 Quantification of NETs formation

The levels of NETs formation were quantified using a semi- quantitative approach bythree double-blinded assessors of extracellular DNA release, where the average percentage was used for data analysis. The extent of NETs release was graded according to four categories: absent= no NETs release per microscopic field, mild= 1to 25% of neutrophils formed NETs, moderate= 26% to 50% and strong= more than 50% of neutrophils formed NETs, including large web-like structures.

2.3 Clinical translational relevance of NETs assay

A prospective cohort study of 341 critically ill adult patients admitted to the general ICU at the RLUH, United Kingdom, between June 2009 and June 2013 were evaluated for recruitment. The ethical approval for the study was granted by a National Research Ethics Service Committee North West – Greater Manchester West and Liverpool

Central (reference numbers: 07/H1009/64 and 13/NW/0089). Written informed consent was obtained for participation in the study. Critically ill patients meeting the following criteria were excluded: (1) transfers from other ICUs; (2) readmission to ICU within 30 days; (3) receiving intravenous heparin treatment; (4) insufficient plasma to carry out effective analysis.

2.3.1 Clinical samples

For assessment of translational relevance, peripheral blood samples from critically ill patients (n = 341) were collected within 24 hours of ICU admission as well as 24 hours, 48 hours and 72 hours post-ICU admission. Plasma was isolated from peripheral blood and stored at -80 until further analysis. Counts of neutrophils, platelets and white blood cells (WBC) from whole blood samples were measured using a Beckman Coulter DxH800. Low platelet counts (thrombocytopenia) was also confirmed via microscopic evaluation. Coagulation parameters including PT, activated partial thromboplastin time (aPTT), fibrinogen levels and D-dimers were measured using ACL TOP® 700 analyser (Werfen Ltd, UK) according to standard protocols in the coagulation laboratory of the RLUH.

2.3.2 Clinical Data collection

Demographics, laboratory, admission diagnosis, clinical scores and outcome measures data were recorded and kept in an electronic database according to local standard operating procedures. The admission diagnosis was recorded after being confirmed by two independent experienced clinicians. The clinical scores including the admission acute physiology and chronic health evaluation (APACHE) II as well as daily sequential organ failure assessment (SOFA) and modified SOFA (platelet count component removed) were calculated and recorded. The modified SOFA score was calculated to avoid bias that may be caused by thrombocytopenia. Outcome data

including days on mechanical ventilation, days on cardiovascular support, length of hospital stay (LOS) and 28-days mortality were recorded from ICU admission. Sepsis was defined according to the sepsis-2 definition (6), and all sepsis patients in this cohort met the revised sepsis-3 definition (9).

The International Society for Thrombosis and Haemostasis (ISTH) criteria for disseminated intravascular coagulation (DIC) were used to calculate daily scores of DIC (96 hours from ICU admission) (246). DIC scores are calculated from the following components: platelet ($\geq$100 x10^9/L=0; 50 to <100 x10^9/L=1; <50 x10^9/L=2), fibrinogen (0= $\geq$1.0 g/L; 1= <1 g/L), D-dimers (0= <1 µg/mL; 2= 1 and 2 µg/mL; 3= >2 µg/mL) and prolongation of prothrombin time (PT) (0= 3 seconds; 1= >3 but < 6 seconds; 2= >6 seconds) (246). The diagnosis of DIC is confirmed with a cumulative score of $\geq$5 (246).

2.3.3 Ex vivo NETs assay

NETs-forming capacity of patient plasma was performed and quantified for all patients on ICU admission (as described in sections 2.2.6 and 2.2.8) using heterologous neutrophils isolated from healthy volunteers (as described in section 2.2.5).

2.3.4 Measurement of cell-free DNA

The method of quantifying cell-free DNA (cfDNA) was according to a previous study (247). Briefly, patient plasma (25 µL) was diluted in 1x PBS to a final volume of 100 µL containing 2 µM final concentration of SYTOX green. Fluorescence was determined using a plater reader at excitation and emission wavelengths of 488 nm 523 nm, respectively. The final concentrations of cfDNA were calculated from known concentrations of genomic DNA as standards.

2.3.5 MPO ELISA

MPO levels in plasma were quantified using the human MPO instant sandwich enzyme-linked immunosorbent assay (ELISA) Kit. The 96-well plate is pre-coated by the manufacturer with capture anti-human MPO antibody, biotin-conjugated anti-human MPO antibody and Streptavidin-HRP detection enzyme. Distilled water (100 µL) was added to each blank, standard and sample wells, prior to adding 50 µL of the pre-diluted plasma (1:50). Plate was then incubated for the 3 hours at room temperature with gentle shaking. Following incubation, liquid was decanted and wells were washed 5 times via aspiration and gentle tapping at absorbent towel. Tetramethyl-benzidine (TMB) solution (100 µL) was added to all wells and incubated at room temperature in the dark for 10 minutes, while colour development was closely monitored. The enzymatic reaction was then stopped using stop solution (1M Phosphoric acid) and plate was read using a plate reader (Thermo Scientific Multiskan Spectrum) to immediately determine optical density values at a measurement wavelength of 450 nm and 620 nm as a reference. MPO concentrations were calculated using a standard curve.

2.3.6 MPO-DNA complex ELISA

The 96-well plate was coated with anti-MPO capture antibody and incubate overnight at 4°C. Following incubation, the solution was decanted and the plate was washed three times with 1x PBS with 0.5% Triton x-100 then blocked with 1% BSA (w/v) in PBS for 1 hour at room temperature. Thereafter, wells were washed and 100 µL of pre-diluted plasma (1:1 in 1x PBS) was added for overnight incubation at 4°C. On the next day, the plate was washed prior to the addition and incubation of anti-dsDNA antibody (100 µL) as a detector for 90 minutes at room temperature. The plate was washed and 50 µL of TMB solution was added to each well for 30 minutes, and

reaction was stopped using 2 N H2SO4. The absorbance was measured at 405 nm and 490 nm as a reference using a microplate reader (Thermo Scientific Multiskan Spectrum).

2.3.7 Circulating histones H3 by Western blot

Circulating histones H3 levels were detected via Western blot as described in previous studies (248-250). Briefly, plasma or recombinant histone H3 protein as standard were diluted in 2x sodium dodecyl sulphate (SDS) loading buffer (4% (v/v) SDS, 0.1 M dithiothreitol, 20% (v/v) glycerol 0.0625 M Tris-HCL and 0.004% (w/v) bromophenol blue) to a final volume of 20 μL and boiled for 10 minutes at 100°C. Samples were then loaded to 5% acrylamide stacking and 15% acrylamide running sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) gels. The gels were then subjected to electrophoresis at 30 mAMP/gel for 1 hours. The gel was transferred to a polyvinylidene fluoride (PVDF) membrane for protein transfer at 400 mAMP for 1 hour. Following transfer, each membrane was blocked for 1 hour. After washing 3x with tris buffered saline (TBS) containing 0.1% Tween-20 (TBST), membranes were incubated overnight at 4°C with primary antibody to histone-H3 (1:2000, Ab 1791) on a rocker. On the next day and following 3x washes in TBST, horseradish peroxidase (HRP)-conjugated secondary antibody (1:10000) was applied for 45 minutes. Bands were visualised using Enhanced chemiluminescence (ECL) Plus Western Blotting Detection System and the resultant bands were quantified using GeneSnap (software 7.05) from Syngene.

2.3.8 Circulating chemokines, cytokines and growth factors

A panel of 27 cytokines, chemokines and angiogenic factors (Table 2.2) were measured in the plasma of healthy volunteers and critically ill patients upon ICU admission by the Bio-Plex Pro human cytokine 27-plex assay (BioRad), according to

manufacturer's instructions. Residual storage solution was first removed from the beads by washing 2x in wash buffer (supplied by the manufacturer). Beads were then incubated in a 96 well plate with 50 µL of plasma and assay standards for 60 minutes at room temperature. The plate was subsequently washed and 25 µL of the detection secondary antibody was added and incubated for 30 minutes at room temperature, after which another washing step follow. Afterwards, 50 µL of the streptavidin-conjugated phycoerythrin reagent was added to the plate and incubated for 30 minutes. The plate was washed thereafter and 125 µL of assay buffer was pipetted to each well. Data was acquired using Bio-Plex 100 (BioRad).

Table 2.2 List of factors from Bio-Plex Pro human cytokine 27-plex assay

Cytokines	Interleukin (IL)-1β, IL-1ra, IL-2, TNFα, IL-6, IL-15
Chemokines	IL-8, IP-10, MCP-1, MIP-1a, MIP-1b, RANTES
T cell-related	IL-4, IL-5, IL-9, IL-10, IL-12 (p70), IL-13, IL-17, Eotaxin, INFγ
Bone marrow-derived	IL-7, granulocyte macrophage-colony stimulating factor (GM-CSF), G-CSF
Angiogenic factors and endothelial mitogens	bFGF, PDGF-bb, VEGF

IL, interleukin; TNF, tumour necrosis factor; IP, interferon-γ-inducible protein; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; RANTES, Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted; INF, Interferon; GM-CSF, granulocyte-macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; bFGF, basic fibroblast growth factor; PDGF-bb, platelet-derived growth factor; VEGF, vascular endothelial growth factor.

2.3.9 Inhibition of NETs formation

The role of IL-8 in NETs formation was firstly established by adding recombinant IL-8 at a pathological concentration (100 pg/mL) to normal plasma prior to incubation with healthy neutrophils for 4 hours. The remaining procedures was described in sections 2.2.5 and 2.2.6. To confirm the role of IL-8 in NETs formation, blocking IL-8 receptors (CXCR1/2) was performed by pre-treatment of neutrophils for 10 minutes using one of the following inhibitors, anti-IL-8 monoclonal antibody (mAb; Clone 6217) (1 µg/mL), reparixin (250 µg/mL)or AZD5069 (10 nM) prior to adding patient plasma.

2.4 Animal investigations

2.4.1 Mice

C57/BL6 male, 8 to 10 week-old mice were purchased from Beijing Vital River Laboratory Animal Technology and kept in specific-pathogen free conditions with free access to waterand food at the research centre of genetically modified mice (Southeast University, Nanjing, China). All animal experimental procedures were kindly performed by Dr. Zhenxing Cheng (License number 2151981, Jiangsu province) and were carried out according to State laws and the use of animals was monitored by local inspectors.

2.4.2 Animal experiments

Age and gender-matched mice were used in all animal experiments to minimize variations that may result from age and gender. In each experimental group, the sample size was determined from previous experience. Therefore,a sample size of 6 mice were used to analyse for differences between groups and 6-8 mice for survival analysis.

Two models of sepsis were generated via cecal ligation and puncture (CLP) or intraperitoneal (i.p) injection of live bacteria as described previously (251, 252). Briefly, severe sepsis was induced the CLP model via ligation of 75% of the cecum followed by 2x puncher with 21 gauge needle to achieve 100% lethality within 48 hours. The other less severe model of sepsis was induced via i.p of *Escherichia coli* (*E. coli*) K12 strain. Mock mice underwent sham CLP via open laparotomy but the cecum was neither ligated nor punctured. Mock control for the i.p injection of *E. coli* received saline instead. Mice were closely monitored every hour and euthanized at 4, 6 and 10 hours. No mouse died before euthanization.

The roles of neutrophils and NETs in sepsis were addressed by neutrophil depletion and DNase I treatment. Twenty-four hours prior to CLP, mice were injected i.p. with the Ly6G-specific monoclonal antibody 1A8 (1.0 mg/mouse) to selectively deplete neutrophils (253). As a control, mice were injected with control mouse IgG. Using a clinical setting based on flow cytometry, no significant changes were observed in circulating neutrophils of the control group (using a mouse isotype control antibody) compared to over 90% of neutrophils were depleted by 1A8 antibody. For NETs dismantlement, CLP mice received i.p injection of DNase I (50 mg/kg) at 1h and 5h after sepsis induction. All mice were euthanized at 10 hours. No mouse died before euthanization.

The value of anti-IL-8 therapy in NETs and sepsis pathology was investigated using reparixin, a small allosteric inhibitor of IL-8 receptors (CXCR1/2). Mice underwent CLP and *E. coli*-induced sepsis received subcutaneous injection of reparixin (20 mg/kg/dose) 1h before, and 2h, 4h after sepsis induction. Whereas mock controls received subcutaneous injection of saline at the same time points. Mice were euthanized at 4, 6 and 10 hours. For survival rate, mice underwent CLP surgery received either saline or reparixin as described above plus 12h, 24h or 48h doses and observed for a total of 72 hours. All mice were closely monitored every 2h and dying mice were euthanized. The mouse clinical assessment score for sepsis was used to enable the assessment of disease severity and identification of dying mice (254).

2.4.3 Sample collection

Blood: Samples were collected following euthanasia and processed immediately. Citrated blood was centrifuged at 2000g for 10 minutes at room temperature followed by collection of plasma, which was stored at -80°C ahead of further analysis.

Tissues: Major organs including, lungs, liver, kidneys and heart were dissected and

fixed directly in 4% (w/v) PFA for 24 hours followed by 70% (v/v) Ethanol until processing and paraffin embedding were performed.

2.4.4 Isolation of mice neutrophils

To obtain fully mature neutrophils, citrated blood (~1-1.5mL) was extracted from normal mice that were euthanized usingSchedule 1 procedure by a technician in the Animal Unit of University of Liverpool. Neutrophils were isolated from whole blood by immunomagnetic negative selection method using EasySep Mouse Neutrophil Enrichment Kit. In brief, RBCs from whole blood were lysed using 1x RBCs lysis buffer for 10 minutes followed by centrifugation at 300 x g for 6 minutes, and supernatant was discarded. Cell pellet was resuspended with RPMI-1640 medium supplemented with 10% FBS to the recommended cell concentration. Normal rat serum was mixed with cells (50 µL/mL of sample) to prevent non-specific antibody binding. In addition, a combinationof biotinylated monoclonal antibodies (EasySep mouse neutrophil enrichment cocktail) that label unwanted (non-neutrophils) cells was added to the mixture (50 µL/mL of sample). The cells were then incubated for 15 minutes at 2-8ºC. Following incubation, cells were washed by adding the recommended volume of media to the sample and then centrifuged at 300 x g for 10 minutes, and supernatant was discarded.Cell pellet was resuspended with media as well as a combination of two mouse IgG_1 monoclonal antibodies (50 µL/mL of sample) directed against biotin and dextran (EasySep Biotin Selection Cocktail). The cells were then incubated for 15 minutes at 2-8ºC. After incubation, magnetic particles, which bind to unwanted cells, were mixedwith the sample (150 µL/mL of cells) and incubated for further 10 minutes at 2-8ºC. Total volume was then adjusted according to manufacturer's instruction and sample tube was placed in magnet rack for 5 minutes prior to pouring neutrophil-rich cell suspension into a new tube. Cell

count was performed and media volume was adjustedto a final concentration of $2x10^5$ cells/well.

2.4.5 NETs-forming capacity assay in mice

The NETs forming capacity of mice plasma was tested using heterologous healthy mice neutrophils as previously described in sections 2.2.6. In mice, it is well established that the gene coding for IL-8 is lacking, and MIP-2 is the functional homologue of IL-8. However, recombinant human interleukin-8 (rhIL-8) is functional on mouse receptors (CXCR1/2). *In vitro,* both MIP-2 and rhIL-8 have been shown to induce strong and dose-dependent neutrophil migration and calcium flux (255). Similarly, injecting mice with rhIL-8 can induce neutrophils migration to the peritoneal cavity (256). Moreover, rhIL-8 has been used for disease modeling in mice. Singer *et al* (257) demonstrated that administration of rhIL-8 and invasive *Shigella* strain to the colonic epithelia surface in mice caused strong neutrophils recruitment and acute colitis. Collectively, CXCR2 in mouse species mediates neutrophils chemoattraction in response to rhIL-8, that is approximately similar to IL-8 homologues in mouse. Accordingly, the role of IL-8 in NETs formation was investigated in mice as follow: IL-8 (100 pg/mL) or CXCL2 (macrophage inflammatory protein (MIP)-2 (8000 pg/mL)) were mixed with plasma from healthy mice and incubated with healthy mouse neutrophils ($2x10^5$ cells/well). for 4 hours at 37 ^oC and 5% CO_2. PMA was used as a positive control (100 nM). The role of CXCR2 signaling in NETs formation was addressed as follow: healthy mouse neutrophils were pre-treated with reparixin (250 mg/mL) for 15 minutes and then incubated with plasma from septic mice for 4 hours at 37 ^oC and 5% CO_2. Cells were fixed and DNA structures were stained using 2% PFA and 10 µg/mL PI, respectively. Validation, visualisation and quantification of NETs formation were performed as

mentioned in sections 2.2.7 and 2.2.8.

2.4.6 Immunohistochemical (IHC) analysis

Mice organs were removed at specific time points (previously described in section 2.4.2) and prepared as previously mentioned in section 2.4.3. Immunostaining of tissue samples from mice was used to measure *in vivo* NETs formation and fibrin deposition. Tissue sections mounted to microscopic glass slides were deparaffinised using 100% xylene for 20 minutes followed by rehydration using graded series of ethanol until water is used. Antigen retrieval procedure was used next to reveal masked epitopes via heat induction at 96^{o}C in high-pH buffer using DAKO PT Link antigen retrieval machine (Agilent technologies). Tissue sections were washed afterwards using 1x PBS with 0.2% tween-20 for 10 minutes, then washed with distilled water for three times with 5 minutes each wash. Endogenous peroxidase activities were then blocked with 6% (v/v) hydrogen peroxide(H_2O_2) for 30 minutes. Tissue sections were then washed with distilled water followedby 1x PBS with 0.2% (v/v) tween-20 for 3 times with 5 minute each wash. Next, tissuesections were blocked using 5% BSA in 1x PBS with 0.2% (w/v) tween-20 for 1 hour.Thereafter, tissue sections were incubated overnight with primary antibodies, anti- Histone H3 antibody (citrulline R2 + R8 + R17) to detect NETs or anti-fibrinogen antibody to evaluate fibrin deposition. Following incubation, tissue sections were washed with 1x PBS with 0.2% tween-20 for 3 times with 5 minute each wash. Secondary antibody (DAKO EnVision DetectionSystems) was and incubated for 50 minutes. Slides were then washed as previously described. To observe 3,3'-diaminobenzidine (DAP) staining, which forms as brown precipitates, chromogen was diluted in substrate buffer according to manufacturer's instructions (DAKO EnVision Detection Systems kit). Tissue slides were washed with ddH_2O and then counterstained with Haematoxylin and lithium carbonate prior to

dehydration with series of graded ethanol and xylene. Slides were then mounted with DPX mountant and glass cover-slips for microscopic evaluation.

2.4.7 Visualisation and semi-quantification of IHC staining

Microscopic evaluations were carried out to visualise and semi-quantify stained tissue sections using an Olympus BH-2 light microscope with a Nikon digital camera DXM1200 (Olympus) to capture images. For each stained slide, 4 random fields were chosen from each organ section and scored in a semi-quantitative method that was adopted from a previous study (258). The stained slides were scored by 3 investigators who were double-blinded to mice treatment groups and IHC staining targeted protein and average scores were used. For each stained tissue tested, a basic scoring scheme was used to evaluate immunoreactivity (demonstrated by development of brown colour) of the targeted protein to obtain the final immunoreactivity score. The staining intensity was scored as follow: 0 (no tissue staining), 1 (weak or small amount of tissue staining), 2 (moderate amount of tissue staining) and 3 (strong or extensive amount of tissue staining).

2.4.8 Evaluation of organ injury in mice

Mice organs were removed then processed as previously described in section 2.4.3. Haematoxylin and Eosin (H&E) staining was performed and visualised using light microscope with 60x magnification, unless otherwise mentioned (Olympus). Four random field were chosen from lung sections of each stained slide to assess for acute lung injury (ALI) by 3 double-blinded investigators according to a scoring system (Table 2.3) from a previous study (259). The final ALI score was calculated from the following formula: score= [(20 x A)+(14 x B)+(7 x C)+(7 x D)+(2 x E)]/(number of fields x 100). Histopathological evaluation of remaining organs (heart, liver and kidneys) showed no obvious changes and therefore circulating injury markers were

used. Briefly, blood was collected into citrate-coated syringes following cardiac punctures and circulating injury markers were detected using an automated clinical analyser (Beckman Coulter, California, United States). Kidney, liver and heart injury were evaluated via blood urea nitrogen (BUN), alanine transaminase (ALT) and cardiac troponin I (cTnI), respectively (252).

Table 2.3 Lung injury scoring system

Parameter	Score per field		
	0	1	2
A. Neutrophils in the alveolar space	none	1-5	>5
B. Neutrophils in the interstitial space	none	1-5	>5
C. Hyaline membranes	none	1	>1
D. Proteinaceous debris filling the airspaces	none	1	>1
E. Alveolar septal thickening	<2x	2x-4x	>4x

2.4.9 Quantification of macrophage inflammatory protein (MIP)-2 by ELISA

MIP-2 (mouse CXCL2) levels were quantified in mice plasma samples using an ELISA kit according to the manufacturer's instructions. Briefly, each plasma sample was thawed at 37oC and gently mixed prior to 1:2 dilution with sample dilution buffer. Standards containing MIP-2 recombinant protein were serially diluted as per protocol instructions. Thereafter, 100 µL of diluted samples or standards were pipetted to 96-well plate coated with an antibody specific for mouse MIP-2. Plate was then covered and incubated for 2.5 hours at room temperature. Following incubation, solution was discarded and wells were washed five times with 300 µL of washing buffer. Next, 100 µL of pre-prepared biotinylated anti-mouse MIP2 antibody was added to each well and incubated for 1 hour at room temperature with gentle shaking. The solution from each well was decanted and washed as previously described to remove unbound

biotinylated antibody. After washing, 100 µL of HRP-conjugated streptavidin was pipetted to the wells and incubated for 45 minutes at room temperature with gentle shaking. The plate was washed and 100 µL of TMB substrate solution was added for 10 minutes at room temperature with shaking in the dark. Stop solution (50 µL) was added to stop colour change. The absorbance at 450 nm (against 620 nm as a reference) was measured immediately using a plate reader (Thermo Scientific Multiskan Spectrum). MIP-2 levels in pg/mL were generated from standard curve values.

2.4.10 Bacterial load

To determine the effect of reparixin on bacterial clearance, sepsis was induced for 10 hours via CLP without or with reparixin treatment as described in section 2.4.2 . The mice were euthanized thereafter by cervical dislocation and organs (lung, kidney, spleen, liver and heart) were collected and washed with saline. Each organ (200 mg) was homogenised afterwards in 1.8 mL of PBS using Automatic CryoMill (Shanghai Jingxin Ltd.) and the resultant tissue lysate and 50 uL citrated blood underwent 7 serial dilutions with PBS (1:9 each time). Using LB agar plates, 20 mL of diluted suspensions were incubated overnight at 37^{o}C. The number of bacteria was calculated as colony-forming units (CFU)/gram tissue from each organ.

2.5 Statistical analysis

Histograms, Q/Q plots and Shapiro-Wilk tests were used to assess the distribution of continuous clinical variables. Cell line data were presented as mean ± standard error of mean (SEM) obtained from 3 independent experiments. Patient data are presented as median and interquartile ranges [1st, 3rd quartiles] due to the non-parametric nature of clinical data. Findings from NETs forming-capacity assay were presented as continuous variables based on the percentage NETs per microscopic field, or categorical variables based on the degree of NETs release (absent, mild, moderate and strong). Findings from animal experiments were reported as mean ± standard deviation (SD) obtained from three independent experiments. Analysis of difference was performed by comparing medians using Mann Whitney U test or by comparing means using student t-test. Kruskall-Wallis or analysis of variance (ANOVA) tests were used for comparison of more than two groups. For cytokines and NETs-related marker analysis, the degree of NETs formation was compared to healthy controls. Demographical and clinical categorical variables were compared using Chi-squared test between either two or more groups. Correlation was assessed by Spearman rank's test. The value of NETs-forming capacity and NETs surrogate markers for independent prediction of DIC and mortality was assessed via multivariable analysis of crude and adjusted odds ratios, with patients adjusted for APACHE II scores. Receiver operating characteristic (ROC) curves were generated for NETs levels and other continuous variables on ICU in the prediction of DIC and mortality, with comparisons were examined using Delong's test performed in MedCalc statistical software. Kaplan-Meier plot was generated for survival analysis and log-rank test was performed to compare survival between two groups.

Chapter 3: Direct NETs formation induced by plasma from patients, assay development, optimisation and quantification

3.1 Introduction

Inflammation is one of the key elements involved in the pathophysiology of sepsis. The implications of excessive recruitment and activation of immune cells, including neutrophils, has been widely recognised in the pathogenesis of sepsis (100). Neutrophilsare integral components of the immune system and first-line defenders against intruding pathogens via effective protective mechanisms, including phagocytosis, degranulation and production of neutrophil extracellular traps (NETs) (260, 261). However, whilst activation of neutrophils is a normal physiological response to eliminate unwanted pathogens, excessive recruitment and prolonged activation of neutrophil may impose significant damage to the host tissues (262).

NETs release is a novel cell death process in which activated neutrophils extrude its DNA extracellularly in network-like structures along with granular proteins to entrap and kill pathogens. NETs can be induced by various microorganisms (263-267), bacterial toxins (78, 268), cytokines (78, 269-272), histones (249) and activated platelets (193, 273). Despite that the factors driving NETosis are not fully described, NETosis can be distinguished from other forms of cell death via its distinctive cellular changes and biochemical properties. However, the detection of NETs in clinical settings relies on invasive organ biopsy or surrogate markers such as circulating cell-free DNA (cfDNA), neutrophil elastase (NE), myeloperoxidase (MPO), Cit-H3 or MPO-DNA complexes (243-245). This detection has been argued recently over its clinical potential since some of these surrogate markers (MPO, NE, and cfDNA) may be the consequence of other types of cell death rather than NETosis (231). It is recognised that NETs formation occurs due to different biological processes, but it

is doubtful that the same capacity of DNA extrusion is found among various diseases orbe inhibited by targeting one specific driving factor. Hence, a new reliable assay is needed.

Given the increasingly recognised role of immune response in sepsis, overwhelmed immune system can cause uncontrolled release of inflammatory mediators such as, cytokines within the circulation (100). These factors may have determinantal implications at the cellular level as it has been previously reported that plasma from septic patients is cytotoxic and can directly induce apoptosis and necrosis to various human cell lines in vitro (274). However, it is still unknown whether patient plasma may impose any direct effects on the ability of primary neutrophil or neutrophil-like cells to produce NETs.

Therefore, the overall aim of this chapter is to develop a novel and reliable NETs assay utilising plasma or sera from patients with sepsis to investigate the ability of each individual patient's plasma/sera to promote NETs formation. In addition, the human myeloid leukaemia cell line (PLB-985) will be differentiated into neutrophil-like cells to assess their usefulness as a model to study NETs. Primary human neutrophils are ideal, but relatively challenging in clinical laboratories, unless industry engagement provides unlimited source of freshly isolated neutrophils. Cell lines have the potential to overcome these drawbacks due to their potential to terminally differentiate into granulocytes with the appropriate inducing agent, although may respond differently toprimary human neutrophils. Thus, the performance of neutrophil-like cells will be compared to primary healthy neutrophils to establish the most robust and reliable neutrophil model for assay development.

3.2 Results

3.2.1 Differentiation of PLB-985 cells toward neutrophil-like phenotype and generation of NETs formation

To test the potential of PLB-985, a myeloid leukaemia cell line, to differentiate into neutrophil-like cells, PLB-985 cells were cultured in medium without or with two different conditions including 1.25% Dimethyl sulfoxide (DMSO) or 2 µM All Trans-Retinoic Acid (ATRA) and 0.5% Dimethylformamide (DMF) (139, 275). Cells were then incubated for 6 days and fluorescence-activated cell sorting (FACS) was used to assess for the expression of CD11b surface marker, an early marker of granulocytes differentiation and maturation (276). Figure 3.1 shows that non-differentiated PLB-985 cells express low levels of surface CD11b. Differentiated PLB-985 (dPLB-985) cells from both conditions similarly induced positive expression of CD11b surface marker. The differentiation protocol using 1.25% DMSO was used for the subsequent dPLB-985 experiments.

Figure 3.1

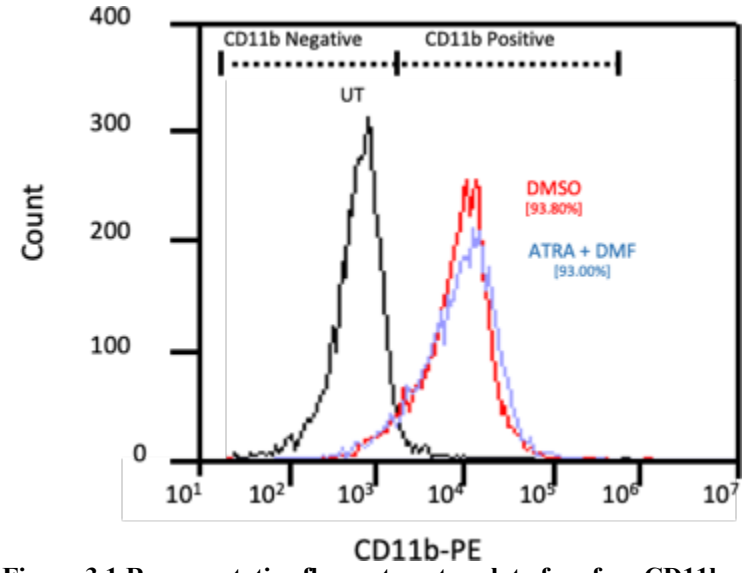


Figure 3.1 Representative flow cytometer plot of surface CD11b expression on non-differentiated and differentiated PLB-985 cells. Cells were cultured in conditioning media containing either 1.25% DMSO or 2 µM ATRA and 0.5% DMF for 6 days, with media renewed on day 3. Cells ($5x10^5$ cells/mL) were incubated in buffer (200 µL total volume) containing 1x phosphate buffered saline (PBS) with 3% BSA (180 µL) and the CD11b conjugated anti-human antibody PE (20 µL) for 1 hour. Cells were

analysed after washing with 1x PBS for the expression of CD11b using FACS. Uundifferentiated PLB-985 cells did not express CD11b on their surface (black histogram). Following differentiation using DMSO (red histogram), or ATRA + DMF (purple histogram), CD11b surface expression was increased (DMSO = 93.80%; ATRA + DMF = 93.00%).

The ability of neutrophil-like dPLB-985 cells to form NETs was investigated using phorbol 12-myristate 13-acetate (PMA), a known inducer of NETs formation, and extracellular DNA release was stained with SYTOX green and fluorescence levels were quantified using fluorometric analysis. As shown in figure 3.2, dPLB-985 cells using 1.25% DMSO were activated by PMA to induce NETs marked by the release DNA to the extracellular medium, unlike dPLB-985 cells without PMA.

Figure 3.2

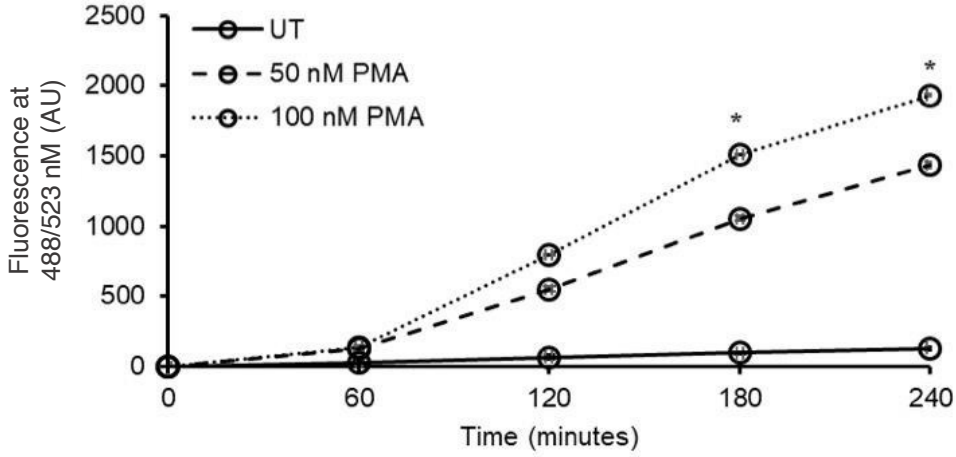


Figure 3.2 dPLB-985 form NETs in response to PMA. dPLB-985 into neutrophil-like cells were seeded in 96-well plate without (UT) or with PMA (50 and 100 nM) conditioning media containing SYTOX green (2 µM final concentration) for DNA staining with continuous quantification of extracellular DNA release using fluorometric assay for 4 hours. Data are presented as mean ±SEM from at least three independent experiments. AU= arbitrary unit. ANOVA test, $^{*}P< 0.05$ when compared with untreated.

3.2.2 Effects of patient plasma on neutrophil-like PLB-985 cells

It is known that neutrophils form NETs in response to various stimuli, but it is still unclear whether plasma or sera from patients would have the potential to trigger NETs release. Furthermore, the usefulness of dPLB-985 cells as a potential model of neutrophils to establish a novel NETs assay using patient plasma is not fully elucidated. Hence, dPLB-985 cells were incubated for 4 hours with platelet-poor

plasma from a cohort of septic patients (n=10). Upon continuous quantification of NETs release using fluorometric analysis, no significant difference was noted between dPLB-985 treated without or with patient's plasma (Figure 3.3).

Priming has been shown to modulate activation and enhance functional responsiveness of neutrophils in response to various stimuli such as, bacterial molecules and cytokines. Hence, the potential of primed dPLB-985 cells to form NETs was studied by using tumour necrosis factor (TNF)-α for 10 minutes as a priming factor prior to stimulation with patient plasma. However, as shown in figure 3.4, primed dPLB-985 did not trigger NETs release when stimulated with patient plasma.

Figure 3.3

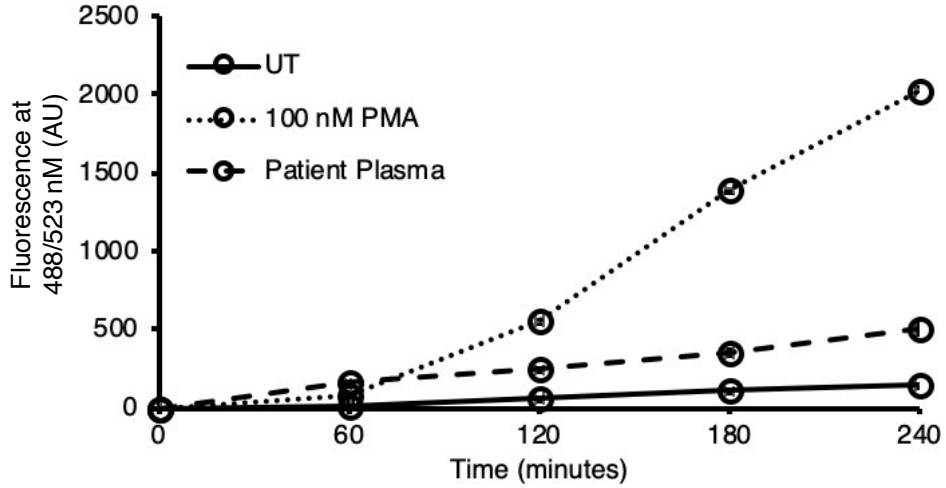


Figure 3.3 Effects of patients' plasma on dPLB-985 cells. dPLB-985 were seeded in 96-well plate and incubated with media alone (UT), PMA (100 nM PMA) or septic patient plasma (Patient Plasma) for 4 hours with SYTOX green (2 μM final concentration) for continuous quantification of extracellular DNA release using fluorometric plate reader. Data are presented as mean ±SEM from at least three independent experiments. AU= arbitrary unit. No significant difference was found on NETs levels between UT vs Patient Plasma.

Figure 3.4

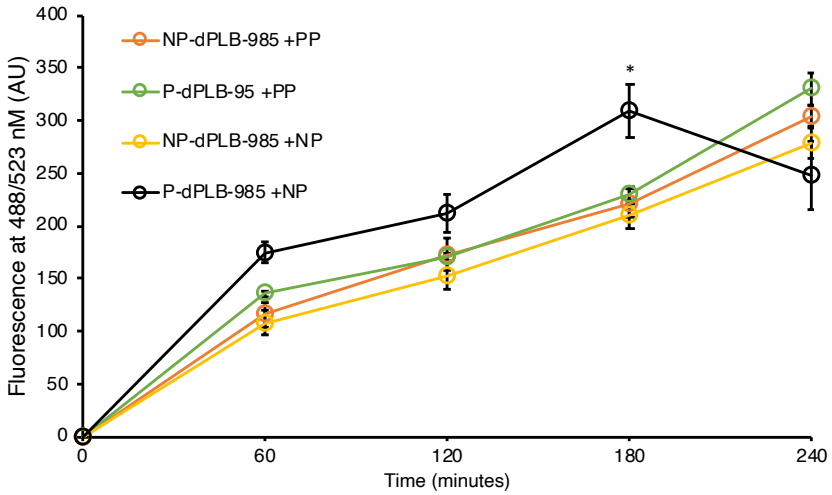


Figure 3.4 Effects of patients' plasma on primed dPLB-985 cells. dPLB-985 cells were primed with TNF- α (10 ng/ml) for 10 minutes. Primed (P-dPLB-985) and non-primed (NP-dPLB-985) cells were seeded in 96-well plate and incubated with patient plasma (+PP) or normal plasma (+NP) for 4 hours with SYTOX green (2 μM final concentration). Data are presented as mean ±SEM from at least three independent experiments. AU= arbitrary unit. ANOVA test, $^{*}P< 0.05$.

3.2.3 Development, validation and optimisation of novel ex vivo NETs formationassay using sera or plasma from septic patients

The capacity of dPLB-985 cells and heterologous healthy neutrophils to release NETs in response to septic patient platelet-poor plasma was assessed using immunofluorescence microscopy. Unlike dPLB-985 cells, primary healthy neutrophils released strong and aberrant NETs formation in response to patient plasma (Figure 3.5). By contrast, plasma or serum from healthy donors (n=20) did not stimulate NETs formation and neutrophils retained their normal polymorphic nuclear morphology, unless co-incubated with phorbol 12-myristate 13-acetate (PMA), a known chemical inducer of NETs formation (Figure 3.5).

In addition, different degrees of NETs production were observed, and was categorised into: Absent (No neutrophils forming NETs per microscopic field); Mild (1% to 25% of neutrophils released NETs per microscopic field); Moderate (26% to 50%) and

Strong (≥50%) (Figure 3.6). In this cohort of 54 septic patients: 38.89% (21/54) had absent NETs, 27.78% (15/54) patients induced mild NETs-forming capacity, 18.52% (10/54) patients induced moderate NETs-forming capacity and 14.81% (8/54) patients induced strong NETs-forming capacity similar to PMA-induced NETs in healthy samples.

Figure 3.5

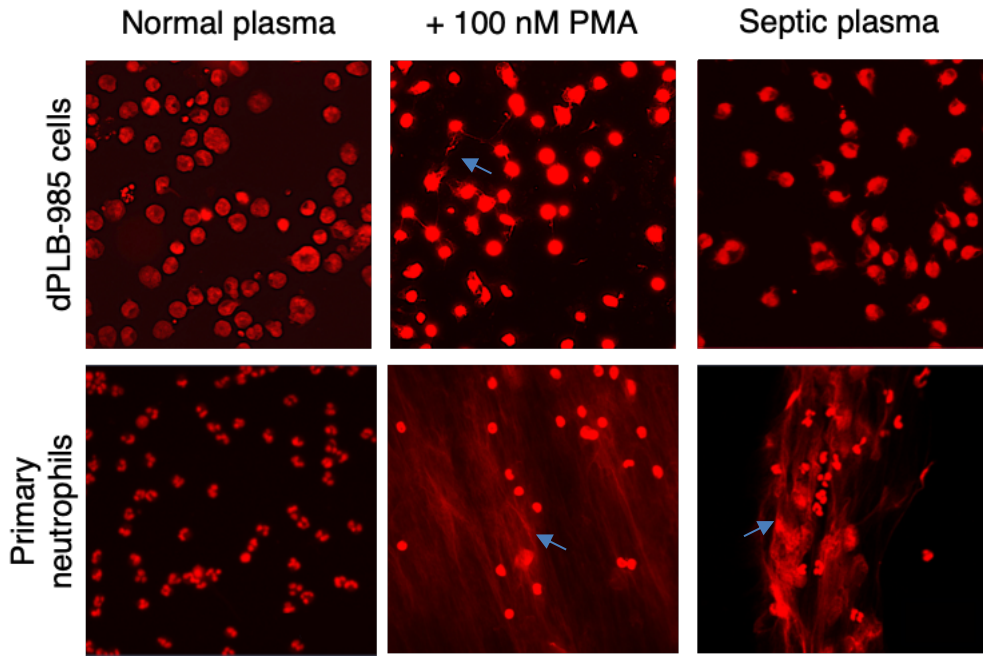


Figure 3.5 Stimulation of differentiated PLB-985 cells and normal neutrophils with PMA induces NETs formation. dPLB-985 cells using 1.25% DMSO **(Upper panel)** or normal neutrophils isolated from healthy volunteers **(Lower panel)** were incubated with either healthy volunteer plasma alone (Normal plasma) (n=20), normal plasma and PMA (+100 nM PMA) (n=20) or patient plasma (Septic plasma) (n=54) for 4 hours. Propidium Iodide (PI) was used to stain for extracellular DNA. Representative images are shown. Blue arrows indicate NETs.

Figure 3.6

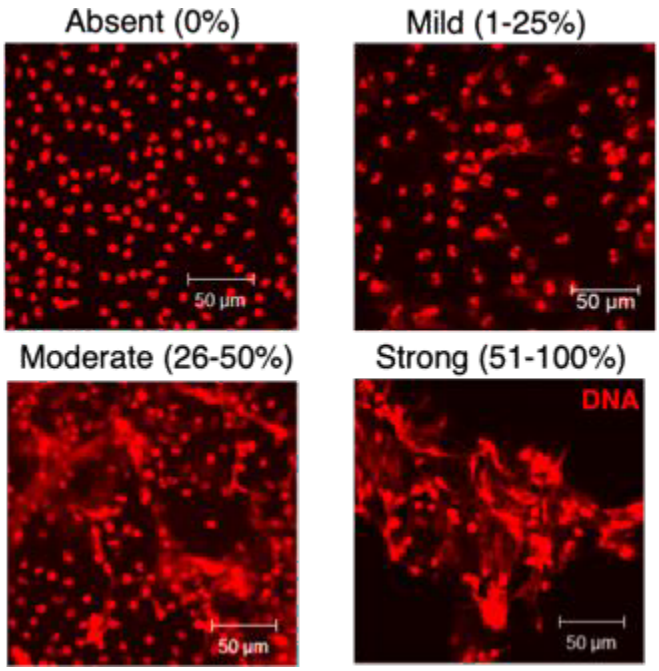


Figure 3.6 Categorisation of *ex vivo* NETs-forming capacity levels. The levels NETs formation induced by septic patients' plasma (n=54) using healthy neutrophil volunteers was categorised into 4 groups (absent, mild, moderate and strong) based on the percentage of neutrophils releasing NETs per microscopic field. Extracellular DNA was stained with PI and visualized using fluorescent microscopy. Typical images are presented. Scale bar 50 µM.

3.2.4 Validation and optimisation of the ex vivo NETs formation assay

To validate that the typical features of NETs existed following patient plasma-induced NETs, staining was performed with antibodies against neutrophil elastase (NE) and MPO and corresponding FITC and AF700-conjugated secondary antibodies. Analysis of NETs structures by confocal microscopy showed that NE (green stain) and MPO (blue stain) are decorated across DNA (red stain) structures, which represent a hallmark characteristic of NETs (Figure 3.7.A).

In addition, it is widely recognised that peptidyl arginine deiminase (PAD)-4 is an essential nuclear enzyme that is required for histone citrullination by ROS for chromatin condensation that precedes NETs release (277). It was therefore important to carry out an inhibitory study *ex vivo* to validate whether PAD4 inhibition can block

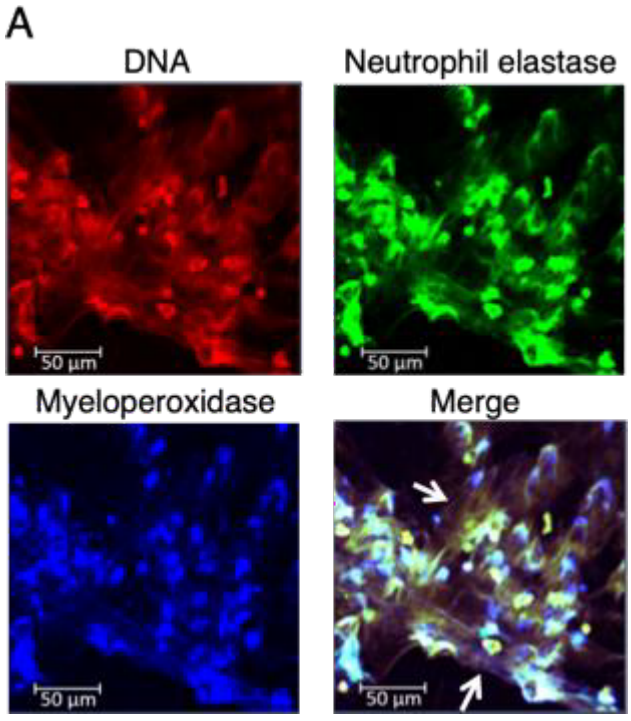
the capacity of plasma or serum to induce NETs. As shown in figure 3.7.B and C, Cl-amidine,an inhibitor of PAD4, significantly attenuated the levels of plasma or serum-induced NETs formation.

To further optimise this assay, patient-specific plasma incubated with their own neutrophils (n=10) or healthy donor neutrophils (n=10) were compared to determine if their NETs-forming capacity differ. As shown in figure 3.8, no significant difference in NETs formation was observed.

In addition, plasma and serum isolated from an individual patient (n=10) at the same time were compared to determine their functional capacity to induce NETs. Similar amounts of NETs were induced by either plasma or serum (Figure 3.9).

Following this extensive assay validation, these findings suggest that the assay is robust and reliable to assess and measure NETs formation. Furthermore, normal neutrophils and plasma will be used to assess the clinical and translational relevance of this assay in the later chapters of this thesis.

Figure 3.7



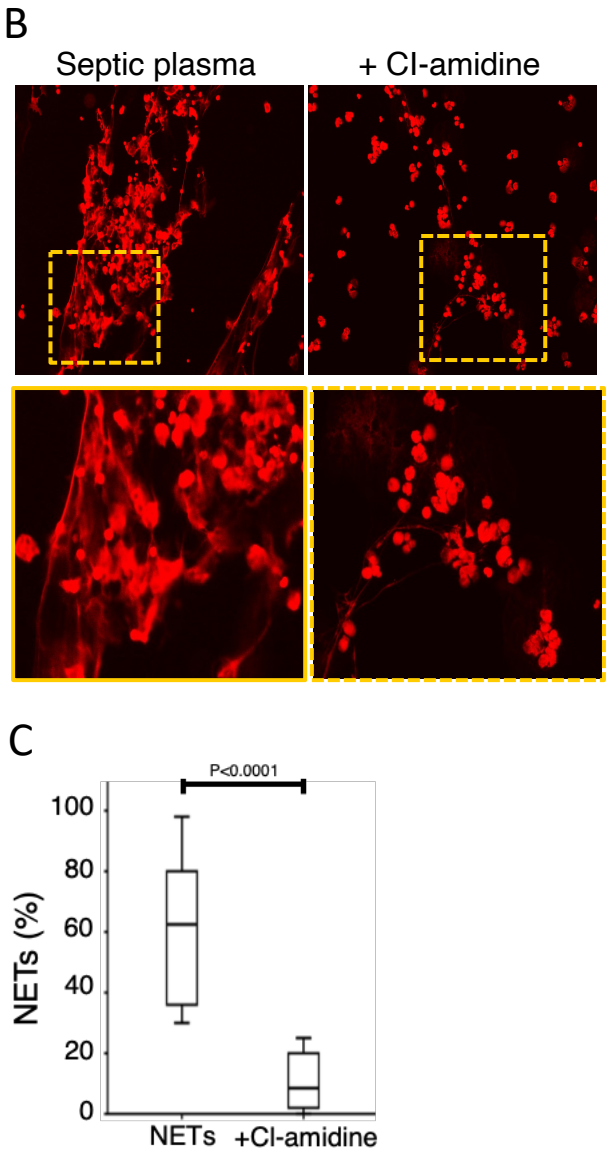


Figure 3.7 Primary neutrophils form typical NETs structures in response to patient plasma. Normal neutrophils isolated from healthy donors were incubated with plasma from critically ill patient. Extracellular DNA release was stained with PI (red), human NE (FITC; green) and human MPO (AF700; blue). Confocal microscopy was used to visualise NETs (arrows). Typical images are presented **(A)**. Scale bar 50 μM. Normal neutrophils pre-treated with PAD inhibitor known as Cl-amidine prior to incubation with plasma septic patient to block NETs formation (n=10). Typical images are presented **(B)**. The levels of NETs were quantified according to neutrophils forming NETs per microscopic field **(C)**. Number of neutrophils forming NETs/ total number of neutrophils x100%. Data are presented as mean ±SD. (ANOVA; P<.05).

Figure 3.8

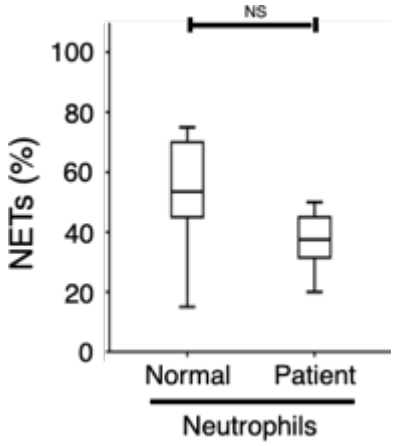


Figure 3.8 Comparable NETs levels are induced by normal or patient neutrophils. Neutrophils from healthy volunteers or patients with sepsis were incubated with matched septic patient plasma (n=10) for 4 hours. The levels of NETs were quantified according to neutrophils forming NETs per microscopic field. no significant difference was observed in their NETs-forming capacity. Data are presented as mean ±SD. (ANOVA; P<.05).

Figure 3.9

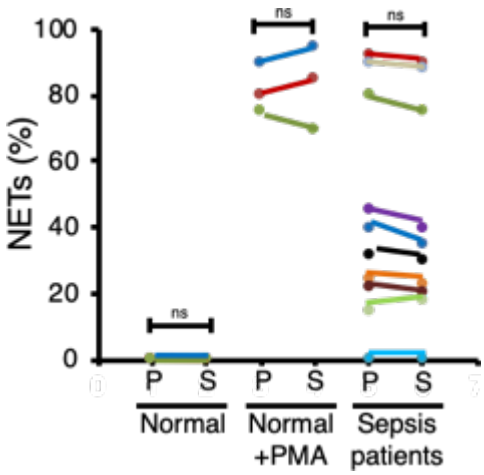


Figure 3.9 Plasma or sera can induce comparable NETs formation. NETs formation was not observed when normal healthy human neutrophils incubated with matched normal plasma (P) (n=20) or serum (S) (n=20), unless co-incubated with 100 nM PMA (n=3). Excessive NETs release was observed when incubating normal healthy human neutrophils with matched plasma (n=10) or serum (n=10) but no significant difference was observed in their NETs-forming capacity. The levels of NETs were quantified according to neutrophils forming NETs per microscopic field. Data are presented as mean ±SD. (ANOVA; P<.05). ns= not significant.

3.3 Discussion

The deleterious roles of NETs in diverse infectious and non-infectious pathologies demand reliable and consistent methods to assess, monitor and quantify NETs formation, particularly in the clinical setting. Currently, methods are limited to using enzyme-linked immunosorbent assay (ELISA) to assess and quantify NETs-related markers (278). Other studies use DNA quantification assays to measure the levels of extracellular DNA release in cell-free body-derived fluids such as, plasma and sera (278). Circulating cfDNA levels and NETs-related components are evidently increased in many critical illnesses (279, 280). However, it is not fully elucidated whether excessive cfDNA release reflects a state of NETosis or simply different forms of cell death. In addition, ELISA-based methods to assess NETs-related components do not specifically determine NETs formation (231). On one hand, some of these components such as, MPO can be produced due to neutrophil and/or macrophage activation (281). On the other hand, other ELISA methods targeting MPO-DNA complex and Cit-H3 lack standardisation (231). Thus, alternative methodologies for NETs assessment and quantification are greatly needed to circumvent the drawbacks associated with the current testing methodologies.

In this chapter, a novel assay was developed and optimised to offer a consistent and reliable approach for monitoring and quantification of NET release, mainly in clinical settings. The NETs-forming capacity of blood-derived fluids including plasma and sera causes different degrees of NETs formation upon incubation with primary heterologous neutrophils. This *ex vivo* NETs methodology offers a simple semi-quantitative approach to deliver quantifiable data on the NETs-forming capacity of individual patient plasma by using microscopic examinations of extracellular DNA release from NETotic neutrophils. Indeed, this NETs assay can be easily interpreted,

according to the standardised definition of NETosis, and categorised by into absent, mild, moderate and strong NETs release.

Despite realising the importance of measuring NETs formation in clinical settings, this novel assay provides a robust, simple and flexible approach to perform it in clinical laboratories. To clarify this, patient or healthy donor neutrophils showed concordant NETs levels when triggered with patient plasma. It is noteworthy that a wide range NETs release was induced by either plasma or serum. This may be attributed to the broad-ranging potency of circulating pathological/physiological inducers of NETosis in each individual patient. Integrating the NETs-forming assay into routine laboratory practice has a great potential since freshly isolated neutrophils can be obtained from blood banks in most large hospitals.

Nevertheless, one of the major challenges linked to neutrophil studies is the demand for careful isolation techniques to avoid neutrophil activation during handling. In light of this, a promyelocytic leukaemia cell line was successfully differentiated into neutrophil-like cells. However, these cells lack the ability to form comparable NETs when compared to primary neutrophils. This may be due to different receptor expression profiles or signal transduction pathways in the cell line compared to primary neutrophils. However, dissecting this question is beyond the scope of this thesis. Therefore, dPLB-985 cells were considered unreliable source of neutrophils to study the clinical implication of NETs using the NETs-forming capacity assay.

There was a limitation to this study. Impairment of neutrophil function during sepsis is probable. Although the present data suggested that the degree of NETs formation was comparable between healthy and patients' neutrophils in response to patient plasma, our understanding remains incomplete if NETs formation is affected in patients with impaired neutrophil function.

Chapter 4: Clinical relevance of the plasma NETs-forming capacity assay during critical illness

4.1 Introduction

Critically ill patients often require complex and invasive therapeutic approaches and, in some cases, replacing the function of multiple-organs with artificial organ support systems (282). As a result, morbidity and mortality remain high due to lack of understanding of the pathophysiology of these diseases (282). Increased mortality and morbidity in critical illness is often complicated by multiple organ dysfunction syndrome (MODS) that is triggered by infection and dysregulated host response (9). The pathophysiology underpinning this process remain inconclusive (283). The interplay between coagulation and innate immune system was recently identified in the pathogenesis of MODS (284). Dysregulation of this process may lead to 'immunothrombosis' and promote excessive formation of immunologically-mediatedthrombi (112).

Neutrophils play a pivotal role in the initial response against microbial infections, but their role in immunothrombosis is increasingly recognised (112). Neutrophils eliminate pathogens via various killing mechanisms, including phagocytosis, degranulation andgeneration of reactive oxygen species (ROS) (260). Neutrophils can also expel their nuclear chromatin decorated with histones and granular proteins to form neutrophil extracellular traps (NETs) to enhance their antimicrobial machinery (285). Current, but not conclusive, evidence have linked NETs to organ dysfunction. How the systemic inflammatory response triggers the innate immune system and the subsequent organs dysfunction remains elusive. However, complex interaction between innate immune system and the coagulation system is importantly acknowledged in this process.

It has been demonstrated that the inflammatory "cytokine storm" constitute an integral part of MODS (286). This progressive process of systemic inflammation and cytokines release can lead to abnormal activation of the coagulation system for reparative purposes but, in some cases, it could progress to disseminated intravascular coagulation (DIC) and MODS (287). In normal conditions, activation of the anticoagulant pathways such as, thrombomodulin and protein C serves as major systems for preventing thrombosis (288). However, overwhelmed anticoagulant systems can result in excessive formation of intravascular thrombosis and even consumptive coagulopathy or DIC, mainly driven by overconsumption of platelets (288). Excessive NETs formation has been reported to contribute to immunothrombosis and DIC (113, 202, 289-292), to impair microcirculation and contribute to organ dysfunction (193, 293, 294). This is because NETs directly enhance clot formation and promote DIC by forming a scaffold for trapping red blood cells (RBCs),platelets and fibrin (295).

Recently, NETs have been viewed as a promising therapeutic target in critical illness (193, 296, 297). Thus, real-time monitoring of NETs in clinical practice may benefit these patients. NETs-associated markers are unstable and susceptible to enzymatic degradation (113, 242, 249, 298). In clinical practice, high levels of NETs-related markers have been found in patients with critical illness (290, 297, 299), but do not correlate with disease severity (243-245).

The growing evidence surrounding the value of NETs in non-sterile organ injury has been outlined in detail (Chapter 1). In acute inflammation (i.e. sepsis), excessive NETs release may occur in response to pathogenic and host factors (i.e. cytokines) leading to tissue injury and microcirculatory dysfunction and subsequent organ dysfunction. However, it is difficult to obtain direct evidence of NETs formation in patients,

although there is no doubt that NETosis occurs and play pathogenic roles in various diseases including sepsis. Therefore, the NETs-forming capacity of blood-derived supernatant including plasma or sera has been investigated and determined as a consistent approach to study and quantify NETs (Chapter 3). The aims of this chapter were to: (1) examine the association between NETs-forming capacity of patient plasma and clinical parameters from a cohort of critically patients; (2) determine which host circulating inflammatory factor is principally associated with elevated levels of *ex vivo* NETs formation.

4.2 Results

4.2.1 Patient characteristics

Based on the previous assay development (Chapter 3), further work was carried out toexamine this novel assay in plasma taken from a larger cohort to determine the clinicalrelevance of NETosis in ICU patients (n=341). All analysis was performed on adult patients (≥18 years old) with ICU admission diagnosis were confirmed by two independent clinicians. The consort diagram for patient recruitment to this study is shown (Figure 4.1). Analysis was restricted to new ICU admissions without pre-existing causes of neutropenia (n=23) to minimise confounding bias associated with failure of neutropenic patients to produce NETs. Furthermore, all ICU patients on intravenous heparin treatment were excluded (n=14) due to that heparin treatment.

Table 4.1 summarises patient demographics and clinical parameters. The median age was 62 years with 167 (49.0%) males. The median disease severity score represented by acute physiology and chronic health evaluation (APACHE II) scoring system was (median [IQR]: 19.0 [14.0-25.0]). Of the 341 critically ill patients, 143 (41.9%) patients were diagnosed with sepsis, followed by 61 (17.9%) trauma patients and others 137 (53.5%). Of those with sepsis, 14.7% and 14.1% had sepsis of abdominal and pulmonary origins, respectively. At the point of ICU admission, median sequential organ failure assessment score (SOFA) score was 7.0 [4.0-9.0]. The median requirement for mechanical ventilation and cardiovascular support was 6.0 [0.0-10.5] days and 8.0 [5.0-11.0] days, respectively. The median length of hospital stay was 9 days and overall mortality rate was 19.6%.

Figure 4.1

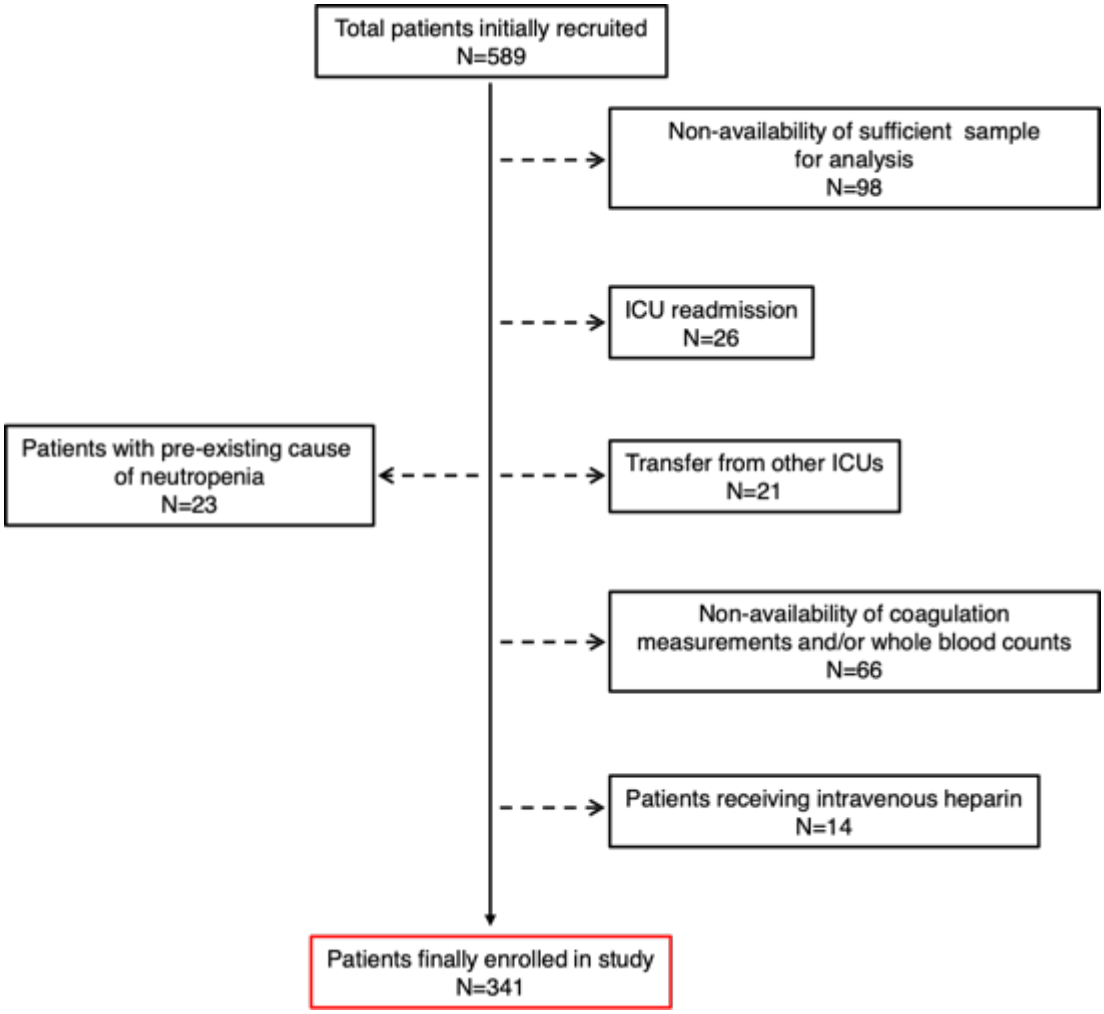


Figure 4.1 CONSORT diagram showing the breakdown of numbers of study subject.

Table 4.1Patient characteristics and clinical outcomes

Variable	Total patients
Total number (n)	341
Age (years), Median [IQR]	62.0 [48.0-72.0]
Male (n) [%]	167 [49.0%]
White ethnicity (n) [%]	298 [87.4%]
APACHE II score, Median [IQR]	19.0 [14.0-25.0]
Admission diagnosis (n) [%]	
Sepsis	143 [41.9%]
Respiratory sepsis	48 [14.1%]
Abdominal sepsis	50 [14.7%]
Urological sepsis	18 [5.3%]
Other septic location	25 [7.3%]
Trauma	61 [17.9%]
Cardiovascular	33 [9.7%]
Respiratory	48 [14.1%]
Gastrointestinal	35 [10.3%]
Renal	5 [14.7%]
Central nervous system	16 [4.7%]
SOFA score, Median [IQR]	
Admission	7.0 [4.0-9.0]
24 hrs post admission	7.0 [4.0-10.0]
48 hrs post admission	7.0 [4.0-10.0]
72 hrs post admission	6.0 [3.0-10.0]
Organ support (days), Median [IQR]	
Mechanical ventilation	6.0 [0.0-10.5]
Cardiovascular support	8.0 [5.0-11.0]
Length stay (days), Median [IQR]	9.0 [5.0-17.0]
Mortality (n) [%]	67 [19.6%]

IQR, interquartile range; APACHE II, Acute Physiology and Chronic Health Evaluation II; SOFA, sequential organ failure assessment.

4.2.2 Sepsis is the primary ICU diagnosis associated with NETs formation

Of 341 critically ill patients, it was found that in 78% (266/341) of the cohort, their plasma could trigger *ex vivo* NETs on admission, whereas 22% (75/341) patients, their plasma could not induce NETs**.** Patients were categorised according to the degree of NETs-forming capacity: 49.9% (170/341) mild, 14.4% (49/341) moderate and 13.8% (47/341) strong NETs formed. As shown in table 4.2, no association was observed between the degrees of *ex vivo* NETs formation and age (R= 0.276, P= 0.730), gender (P= 0.940) or ethnicity (P= 0.474). Conversely, strong association was found between the degrees of *ex vivo* NETs formation and primary diagnosis, in particular sepsis (P<0.0001) (Table 4.2, Figure 4.2). The majority of plasma that induced moderate and strong NETs were from septic patients (63.3% and 70.2%, respectively), whereas more than two-thirds of mild NETs was triggered by plasma from patients with other critical illness. Interestingly, the degree of NETs formation according to different aetiologies of sepsis is strongly associated with respiratory sepsis (P=0.0004).

Table 4.2 The correlation analysis of NETs degrees to various admission variables.

	Correlation (*R* value)	Absent NETs (n=75)	Mild NETs (n=170)	Moderate NETs (n=49)	Strong NETs (n=47)	P value*
NETs percentage, Median [IQR]	-	0.0 [0.0-0.0]	8.0 [4.0-19.0] †	45.0 [37.3-50.0] †,‡	85.5 [67.0-95.0] †,‡,§	**<.0001**
Age (years), Median [IQR]	.276	60.0 [47.0-69.0]	62.0 [49.0-73.0]	62.0 [44.0-76.0]	61.0 [52.0-71.0]	.730
Male (n) [%]	-	37 [49.3%]	85 [50.0%]	22 [44.9%]	23 [48.9%]	.940
Female (n) [%]	-	38 [50.7%]	85 [50.0%]	27 [55.1%]	24 [51.1%]	.940
White ethnicity (n) [%]	-	62 [82.7%]	150 [88.2%]	45 [91.8%]	41 [87.2%]	.474
Admission diagnosis (n) [%]						
Sepsis						
Respiratory sepsis	-	31 [41.3%]	48 [28.2%] †	31 [63.3%] †,‡	33 [70.2%] †,‡	**<.0001**
Abdominal sepsis	-	10 [13.3%]	16 [9.4%]	16 [32.7%] †,‡	6 [12.8%] §	**.0004**
Urological sepsis	-	12 [16.0%]	21 [12.4%]	4 [8.2%]	13 [27.7%] ‡,§	**.032**
Other septic location	-	6 [8.0%]	2 [1.2%] †	5 [10.2%] ‡	5 [10.6%] ‡	**.008**
Trauma	-	3 [4.0%]	9 [5.3%]	6 [12.2%]	7 [14.9%] †,‡	**.047**
Cardiovascular	-	13 [17.3%]	36 [21.2%]	6 [12.2%]	6 [12.8%]	.366
Respiratory	-	8 [10.7%]	21 [12.4%]	1 [2.0%]	3 [6.4%]	.149
Gastrointestinal	-	10 [13.3%]	29 [17.1%]	8 [16.3%]	1 [2.1%] †,‡	.071
Renal	-	9 [12.0%]	22 [12.9%]	2 [4.1%]	2 [4.3%]	.142
Central nervous system	-	1 [1.3%]	2 [1.2%]	1 [2.0%]	1 [2.1%]	
	-	3 [4.0%]	12 [7.1%]	0 [0.0%]	1 [2.1%]	

NETs, neutrophil extracellular traps; IQR, interquartile range. † Significant vs absent NETs patients. ‡ Significant vs mild NETs patients. § Significant vs moderate NETs patients. * indicates statistical differences between all groups obtained either from Kruskall-Wallis test for continuous variables or Chi-squared test for categorical variables. R value calculated from Spearman's rank test with percentage NETs. Bold font indicates significant P value of < 0.05.

Figure 4.2

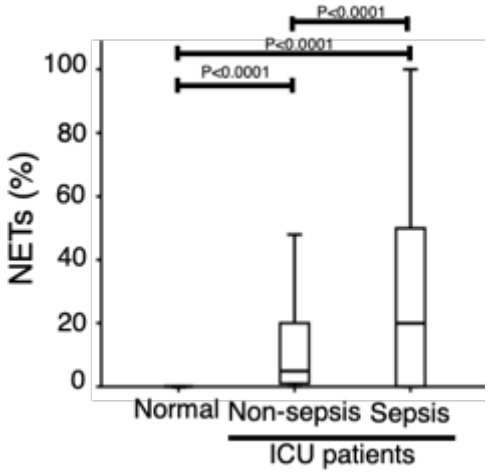


Figure 4.2 Increased NETs formation is associated with sepsis. Plasma from healthy volunteers (n=20) did not induce *ex vivo* NETs formation when incubated with normal neutrophils. ICU patients (n=341) were classified according to their diagnosis on ICU admission into non-septic (n=198) and septic (n=143). Plasma from septic patients induced significantly higher levels of NETs compared to those without sepsis. (ANOVA; P<0.05).

4.2.3 Levels of NETs formation are highly associated with DIC

The correlation analysis of the different degrees of NETs levels on admission with peripheral blood cell counts is presented in table 4.3. The levels of NETs from critically ill patients had significant association with thrombocytopenia (platelets <150 x10^9/L) (P<0.0001). Indeed, thrombocytopenia was found in more than 60% of patients with moderate to strong NETs-forming capacity compared to only 15.9% of the absent and mild NETspatients (P<0.001). However, levels of NETs formation were not significantly associated with white blood cell (WBC) (R=-0.336, P=0.062) and neutrophil counts (R=-0.309, P=0.114). Moreover, NETs-forming capacity levels of patients' plasma did not correlate with NETs surrogate markers (Table 4.4).

Considering the strong association between high levels of NET and thrombocytopenia, we endeavoured to examine NETs levels with other coagulation parameters (Table 4.5). Compared to mild or absent NETs formation, there were significant associations between moderate or strong NETs formation with DIC parameters including,

abnormality in prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen, as well as D-dimer ($P<0.05$) (Table 4.5). Indeed, strong and moderate NETs formation (39.4% and 26.6%, respectively) were significantly associated with DIC development compared to mild and absent NETs formation (1.2% and 4.2%, respectively) (χ^2 test, $P<0.0001$). Patients with DIC (median [IQR]: 50.0% [25.0%, 88.0%]) had significantly higher levels of NET formation compared to those without 5.0% [0.0%, 20.0%] ($P<0.0001$).

Table 4.3 The correlation analysis of NETs levels with peripheral blood cell count

Variable	Total (n=341)	Correlation (*R* value)	Absent NETs (n=75)	Mild NETs (n=170)	Moderate NETs (n=49)	Strong NETs (n=47)	P value*
WBC (x10^9/L), Median [IQR]							
Admission	12.0 [7.8-18.0]	-.336	12.1 [6.8-17.5]	12.4 [8.5-18.1]	12.3 [7.0-18.9]	9.6 [5.0-18.4] ‡	.145
24 hrs post admission	11.9 [8.0-17.4]	-.289	11.6 [7.9-17.7]	12.1 [8.7-17.2]	11.2 [7.7-17.4]	10.5 [5.0-18.0]	.835
48 hrs post admission	12.0 [8.5-16.4]	-.276	12.7 [8.8-15.0]	12.0 [9.2-16.4]	10.8 [7.9-18.3]	12.0 [6.0-17.0]	.523
72 hrs post admission	11.5 [8.0-16.0]	-.114	11.4 [8.0-15.0]	11.4 [9.0, 16.2]	10.4 [8.0-15.3]	12.0 [6.9-20.1]	.862
Neutrophils (x10^9/L), Median [IQR]							
Admission	10.1 [6.6-15.4]	-.309	9.8 [6.4-15.5]	10.4 [7.3-15.4]	10.2 [6.3-14.4]	8.3 [3.9-17.7]	.469
24 hrs post admission	9.5 [6.8-15.0]	-.311	9.5 [6.5-15.1]	10.5 [7.4-15.2]	8.5 [6.3-16.2]	8.2 [4.9-13.9]	.399
48 hrs post admission	9.6 [6.4-13.9]	-.348	10.2 [7.0-14.0]	9.7 [7.1-14.0]	9.0 [6.1-13.8]	8.3 [4.1-14.0]	.430
72 hrs post admission	9.5 [6.7-13.1]	-.329	10.1 [7.2-12.8]	9.4 [7.0-13.6]	8.7 [5.9-14.9]	7.6 [4.3-14.4]	.484
Platelets (x10^9/L), Median [IQR]							
Admission	203.0 [136.5-299.0]	-.648	241.0 [173.0-320.0]	217.5 [175.5-331.0]	135.0 [72.0-222.0] †,‡	106.0 [61.0-166.0] †,‡	**<.0001**
24 hrs post admission	203.5 [120.0-277.8]	-.643	218.5 [173.3-303.3]	223.0 [163.0-306.8]	136.0 [64.0-233.3] †,‡	91.5 [53.0-150.8] †,‡	**<.0001**
48 hrs post admission	193.0 [102.5-267.0]	-.677	236.0 [170.0-287.0]	214.0 [163.0-301.0]	113.5 [48.8-203.3] †,‡	69.0 [39.5-131.0] †,‡	**<.0001**
72 hrs post admission	193.0 [102.0-285.5]	-.639	222.0 [165.5-303.8]	225.0 [166.3-309.5]	99.0 [42.0-205.8] †,‡	82.0 [42.0-118.0] †,‡	**<.0001**

NETs, neutrophil extracellular traps; WBC, white blood cells; IQR, interquartile range. † Significant vs absent NETs patients. ‡ Significant vs mild NETs patients. § Significant vs moderate NETs patients. * indicates statistical differences between all groups obtained from Kruskall-Wallis test. R value calculated from Spearman's rank test with percentage NETs. Bold font indicates significant P value of < 0.05.

Table 4.4 The correlation analysis of NETs-forming capacity levels with NETs-related markers

NETs-related markers	Normal	Correlation (R value)	Absent NETs (n=75)	Mild NETs (n=170)	Moderate NETs (n=49)	Strong NETs (n=47)	P value*
cfDNA (ng/ml), Median [IQR]	245.70 [154.63-443.21]	-.134	617.9 [378.8-971.3] †	521.8 [237.6-1015.8]	530.3 [367.9-990.5]	496.0 [316.8-1237.4]	.864
MPO (ng/ml), Median [IQR]	12.40 [4.55-35.39]	.327	97.4 [36.8-180.8] †	65.5 [39.4-96.1] †	154.4 [51.7-312.2] †, §	101.1 [33.1-192.2] ‡	.204
DNA-MPO (AU), Median [IQR]	0.97 [0.89-1.11]	.158	0.89 [0.83-1.16]	0.96 [0.84-1.19]	0.94 [0.84-1.08]	0.92 [0.82-1.10]	.982

NETs, neutrophil extracellular traps; cfDNA, cell-free DNA; MPO, myeloperoxidase. † Significant vs absent NETs patients. ‡ Significant vs mild NETs patients. § Significant vs moderate NETs patients. * indicates statistical differences between all groups obtained from Kruskall-Wallis test. R correlation with percentage NETs was performed using Spearman's rank test.

Table 4.5 The correlation of different NETs-forming capacity groups and coagulation parameters

	Total (n=341)	Correlation (*R* value)	Absent NETs (n=75)	Mild NETs (n=170)	Moderate NETs (n=49)	Strong NETs (n=47)	P value*
PT (seconds), Median [IQR]							
Admission	15.0 [13.2-18.1]	.435	14.8 [13.6-16.8]	14.6 [12.9-16.6]	15.4 [13.3-19.2]	17.7 [13.3-21.3] †,‡	**.014**
24 hrs post admission	14.6 [13.0-17.2]	.399	14.5 [13.2-16.8]	14.4 [12.9-16.5]	14.7 [13.3-18.5]	16.4 [12.8-20.4]	.332
48 hrs post admission	13.9 [12.5-16.0]	.292	14.0 [12.8-15.7]	13.8 [12.3-15.7]	13.9 [12.1-18.1]	14.7 [12.1-17.1]	.706
72 hrs post admission	13.7 [12.3-15.2]	-.126	13.9 [12.8-16.2]	13.6 [12.2-14.9]	13.9 [11.7-16.9]	13.7 [12.5-15.4]	.446
aPTT (seconds), Median [IQR]							
Admission	32.3 [28.6-38.4]	.621	30.3 [27.3-35.5]	31.4 [28.0-37.0]	35.8 [30.2-44.2] †,‡	40.2 [32.8-51.4] †,‡	**<.0001**
24 hrs post admission	33.3 [29.0-39.6]	.553	31.9 [28.2-35.9]	32.8 [28.9-38.6]	35.7 [30.6-43.0] †	39.4 [31.8-45.5] †,‡	**<.0001**
48 hrs post admission	32.1 [28.6-38.3]	.581	30.6 [28.4-35.5]	31.5 [28.4-37.3]	34.5 [30.5-42.6] †,‡	37.5 [30.4-43.9] †,‡	**<.0001**
72 hrs post admission	31.5 [28.4-36.5]	.550	30.5 [28.3-34.0]	31.0 [28.3-35.8]	34.4 [29.8-41.6] †,‡	34.7 [29.0-39.5] †,‡	**.004**
Fibrinogen (g/L), Median [IQR]							
Admission	3.8 [2.5-5.0]	-.565	4.5 [3.0-5.4]	4.0 [3.0-5.1]	3.4 [2.1-4.9] †	2.3 [1.5-3.5] †,‡,§	**<.0001**
24 hrs post admission	4.1 [2.9-5.2]	-.568	4.5 [3.2-5.5]	4.3 [3.3-5.4]	3.7 [2.6-5.2] †,‡	2.7 [1.8-3.8] †,‡,§	**<.0001**
48 hrs post admission	4.4 [3.4-5.4]	-.560	4.6 [4.0-5.8]	4.6 [3.5-5.7]	4.1 [2.1-5.1] †,‡	3.1 [2.3-4.4] †,‡	**<.0001**
72 hrs post admission	4.6 [3.5-5.6]	-.531	4.8 [3.9-6.0]	4.8 [3.8-5.9]	3.6 [2.2-5.2] †,‡	3.7 [2.5-5.0] †,‡	**<.0001**
D-dimer (ng/ml), Median [IQR]							
Admission	4073.5 [2054.8-7759.4]	.377	3788.0 [1925.3-6335.0]	3756.2 [1865.0-6284.8]	5549.3 [1877.6-12276.0]	6261.0 [2464.4-15527.6] †,‡	.143
24 hrs post admission	4485.0 [2147.0-8737.0]	.214	4687.5 [2941.0-10879.3]	4975.8 [1227.0-7251.2]	5064.0 [2015.0-7022.0]	5791.0 [3809.0-15167.0]	.494
48 hrs post admission	5044.1 [2175.9-7394.8]	.333	4145.0 [2783.0-10341.0]	5150.1 [1803.3-7252.7]	4504.5 [2252.3-9001.2]	4729.0 [2784.0-14336.9]	.888
72 hrs post admission	4931.0 [2386.5-7762.7]	.319	4407.0 [2211.0, 9089.0]	4743.0 [2952.0-6919.0]	5275.9 [2733.2-14048.1]	5437.0 [1563.5-19292.0]	.983
Total DIC (n) [%]	58 [17.0%]	-	8 [10.7%]	7 [6.7%]	16 [32.7%] †,‡	27 [57.4%] †,‡,§	**<.0001**
Time to develop DIC (n) [%]							
Admission	28 [8.2%]	-	5 [6.7%]	5 [2.9%]	4 [8.2%]	14 [30.0%] †,‡,§	**<.0001**
24 hrs post admission	13 [3.8%]	-	0 [0.0%]	1 [0.6%]	5 [10.2%] †,‡	7 [14.9%] †,‡	**<.0001**
48 hrs post admission	10 [2.9%]	-	3 [4.0%]	1 [0.6%]	2 [4.1%]	4 [8.5%] ‡	**.001**
72 hrs post admission	7 [2.1%]	-	0 [0.0%]	0 [0.0%]	5 [10.2%] †	2 [4.3%]	**<.0001**
Developed DIC ≥24 hrs post admission, (n) [%]	30 [8.8%]	-	3 [4.2%]	2 [1.2%]	12 [26.6%] †,‡	13 [39.4%] †,‡	**<.0001**

NETs, neutrophil extracellular traps; PT, prothrombin time; aPTT, activated partial thromboplastin time; DIC, disseminated intravascular coagulation; IQR, interquartile range. † Significant vs absent NETs patients. ‡ Significant vs mild NETs patients. § Significant vs moderate NETs patients. * indicates statistical differences between all groups obtained from Kruskall-Wallis test for continuous variables or Chi-squared test for categorical variables. R value calculated from Spearman's rank test with percentage NETs. Bold font indicates significant P value of < 0.05.

4.2.4 Levels of NETs are significantly correlated with multiple organ failure, disease severity and mortality

SOFA and APACHE II scoring systems are commonly used to assess for disease severity and monitor multiple organ failure (MOF) in ICU settings. Findings from correlation analysis from Spearman's rank test are demonstrated in table 4.6. Analysis of SOFA scores from ICU admission up to 72 hours post-admission showed that the degree of *ex vivo* NETs were significantly associated with MOF (Table 4.6). Patients with their plasma that induced moderate to strong NETs formation had higher SOFA score on ICU admission (median [IQR]: 7 [4-11] and 9 [7-12]) compared to those with absent to mild NETs formation (6 [4-9] and 6 [3-8]); $P<0.001$). These observations persisted up to 96 hours (study duration). For further validation, a modified SOFA score was used with platelet counts removed to avoid confounding bias that might be caused by thrombocytopenia. Similarly, patients with strong NETs formation had higher modified SOFA scores on ICU admission 8.0 [5.0-11.0] compared to mild and absent 6.0 [3.0-8.0] groups ($P=0.002$). Modified SOFA scores remained significantly higher 72 hours post-admission. Correlation analysis exhibited significant positive correlation between NETs levels and SOFA or modified SOFA scores on admission and up to 72 hours post-admission (R values are presented in table 4.5). Furthermore, patients with strong NETs production had higher APACHE II scores (median [IQR]: 23.0 [17.0-29.0]) compare to mild 19.0 [14.0-24.0] and absent 19.0 [13.0-23.0] groups ($P=0.013$). Spearman correlation analyses revealed significant positive correlation ($R=.442$). The mortality rate of patients with moderate (30.6%) and strong (34.0%) NETs-forming capacity was also higher than that of the absent (12.0%) and (15.9%) mild groups (χ^2 test, $P<0.003$).

Table 4.6 The correlation analysis of NETs degrees to various clinical parameters and outcomes.

Variable	Total patients (n=341)	Correlation (*R* value)	Absent NETs (n=75)	Mild NETs (n=170)	Moderate NETs (n=49)	Strong NETs (n=47)	P value*
APACHE II score, Median [IQR]	19.0 [14.0-25.0]	.442	19.0 [13.0-23.0]	19.0 [14.0-24.0]	20.0 [15.0-27.0]	23.0 [17.0-29.0] †,‡	**.013**
SOFA score, Median [IQR]							
Admission	7.0 [4.0-9.0]	.521	6.0 [3.8-9.0]	6.0 [3.0-8.0]	7.0 [4.0-11.0] ‡	9.0 [7.0-12.0] †,‡,§	**<.0001**
24 hrs post admission	7.0 [4.0-10.0]	.567	7.0 [4.0-8.0]	6.0 [4.0-9.0]	8.0 [5.0-12.0] †,‡	10.0 [7.0-13.0] †,‡	**<.0001**
48 hrs post admission	7.0 [4.0-10.0]	.597	6.0 [4.0-8.0]	6.0 [3.0-8.0]	9.0 [4.0-11.0] †,‡	11.0 [7.0-13.0] †,‡,§	**<.0001**
72 hrs post admission	6.0 [3.0-10.0]	.605	6.0 [3.0-9.0]	5.0 [3.0-7.0]	9.0 [4.0-12.0] †,‡	10.5 [8.0-14.0] †,‡	**<.0001**
Modified SOFA score, Median [IQR]							
Admission	6.0 [3.0-8.0]	.465	6.0 [3.0-8.0]	6.0 [3.0-8.0]	6.0 [3.0-10.0]	8.0 [5.0-11.0] †,‡	**.002**
24 hrs post admission	7.0 [4.0-9.0]	.483	7.0 [4.0-8.0]	6.0 [4.0-8.3]	7.0 [4.0-11.0]	8.0 [6.0-11.0] †,‡	**.001**
48 hrs post admission	6.0 [4.0-9.0]	.506	5.0 [4.0-8.0]	5.5 [3.0-7.3]	7.0 [3.0-10.0]	8.5 [6.0-11.0] †,‡,§	**<.0001**
72 hrs post admission	6.0 [3.0-8.0]	.522	6.0 [3.0-8.0]	5.0 [3.0-7.0]	7.0 [3.3-9.0] ‡	8.5 [6.0-11.0] †,‡	**<.0001**
Organ support (days), Median [IQR]							
Mechanical ventilation	6.0 [0.0-10.5]	.322	6.0 [0.0-10.5]	4.0 [1.0-10.0]	2.0 [0.0-16.0]	8.0 [3.0-14.0] ‡	.194
Cardiovascular support	8.0 [5.0-11.0]	.356	8.0 [5.0-11.0]	7.0 [3.0-14.0]	8.0 [4.0-19.5]	10.0 [7.0-17.0] †,‡	.050
Length stay (days), Median [IQR]	9.0 [5.0-17.0]	.321	8.0 [4.0-15.0]	9.0 [5.0-16.3]	7.0 [4.0-20.5]	11.0 [7.0-19.0] ‡	.144
Mortality (n) [%]	67 [19.6%]	-	9 [12.0%]	27 [15.9%]	15 [30.6%] †,‡	16 [34.0%] †,‡	**.003**

NETs, neutrophil extracellular traps; IQR, interquartile range; APACHE II, Acute Physiology and Chronic Health Evaluation II; SOFA, Sequential Organ Failure Assessment. † Significant vs absent NETs patients. ‡ Significant vs mild NETs patients. § Significant vs moderate NETs patients. * indicates statistical differences between all groups obtained from Kruskall-Wallis test for continuous variables or Chi-squared test for categorical variables. R value calculated from Spearman's rank test with percentage NETs. Bold font indicates significant P value of < 0.05.

4.2.5 Univariate and multivariate logistic regression analysis

Prior to constructing multivariate models, we used univariate analyses to test if clinical factors, *ex vivo* NETs formation levels and comparator NETs assays are associated with DIC and mortality in critically ill patients. Results of the univariate analysis are displayed in tables 4.7 and 4.8.

In the univariate analysis, multiple variables were associated with DIC including, initial diagnosis of sepsis due to respiratory illness and trauma, APACHE II, bacteraemia, all source of infections and interleukin (IL)-8 (Table 4.7). Furthermore, admission diagnosis of trauma, APACHE II, bacteraemia, respiratory-related and other infections were significantly associated with mortality (Table 4.8).

Further analysis was carried out to determine if NETs formation on ICU admission was associated with DIC development following ICU admission. After excluding patients who had DIC on ICU admission (n=20), univariate analysis was performed for both continuous and categorical data of NETs. As shown in tables 4.7 and 4.8, NETs levels as continuous variables showed an odds ratio (OR) for DIC was 1.06 [95% confidence interval (CI) 1.04-1.08] and mortality 1.02 [1.01-1.03] (P<0.0001). Similar observations were also obtained from categorical data for (DIC: OR= 14.52, 95% CI 3.76-56.06, P<0.0001) and (mortality: OR=3.785, 95% CI 1.51-9.51, P=.005) for strong and (DIC: OR= 8.12 95% CI 2.14-30.77, P=.002) and (mortality: OR= 3.235, 95% CI 1.28-8.15, P=.013) for moderate NETs formation groups. As for other NETs-related assays, only DNA-MPO complex exhibited a significant association with DIC.

The multivariate model was constructed based on fitting parameters with P< 0.1 from univariate analysis followed by stepwise regression approach by sequentially

removing variables with P > 0.1. Results of the final multivariate analysis are displayed in table 4.9. After adjustment for APACHE II, subsequent multivariate analysis showed independent association between NETs-forming capacity and both DIC and mortality (Table 4.9, Figure 4.3).

4.2.6 Ex vivo NETs formation has strong predictive values for DIC and mortality

The value of measuring *ex vivo* NETs formation in predicting clinical data was determined and compared to ICU scoring systems and NETs-related biomarkers. The NETs-forming capacity assay was strong in predicting DIC (area under the curve (AUC)=0.851; P<0.001) (Figure 4.4.A) and mortality (AUC=0.656; P<0.001) (Figure 4.4.B, Table 4.9). The assay was comparable to the ICU scoring systems, APACHE II(AUC= 0.683; P<0.001) (Delong's test vs NETs; P= 0.440) and SOFA (AUC= 0.604; P= 0.009) (Delong's test vs NETs; P= 0.381) (Table 4.9). Other NETs-related biomarkers including MPO, cfDNA and MPO-DNA complex did not show significant association with DIC and mortality (Table 4.9).

Table 4.7 Univariate analysis of clinical variables for the prediction of DIC.

	Crude Odds ratio [95% CI]	P value*
DIC		
Clinical variables		
Age	0.988 [0.967-1.009]	.255
Gender	0.538 [0.249-1.165]	.116
Initial diagnosis		
Sepsis	REF	
Cardiovascular	0.000 [0.000-0.000]	.998
CNS	0.000 [0.000-0.000]	.998
Gastro	0.258 [0.058-1.151]	.076
Renal	0.000 [0.000-0.000]	.999
Respiratory	0.087 [0.019-0.661]	**.018**
Trauma	0.222 [0.064-0.770]	**.018**
Hypotension	2.220 [1.001-4.923]	.050
ARDS (P/F)	1.000 [0.996-1.003]	.945
APACHEII	1.144 [1.084-1.208]	**<.0001**
SOFA score	1.435 [1.274-1.616]	**<.0001**
Bacteraemia	7.625 [3.027-19.205]	**<.0001**
Source of infection		
No infection	REF	
Respiratory	7.843 [2.625-23.431]	**<.0001**
Abdomen	5.337 [1.702-16.736]	**.004**
Neuro	11.091 [2.724-45.150]	**.001**
Other	10.893 [2.953-40.183]	**<.0001**
Laboratory variables		
IL-1β	0.993 [0.923-1.068]	.845
IL-6	1.000 [1.000-1.000]	.113
TNFα	0.999 [0.994-1.003]	.501
IL-8	1.000 [1.000-1.000]	**.049**
Absent NETs	REF	
Mild NETs	0.274 [0.045-1.677]	.161
Moderate NETs	8.121 [2.143-30.770]	**.002**
Strong NETs	14.517 [3.759-56.057]	**<.0001**
NETs [%]	1.059 [1.041-1.078]	**<.0001**
cfDNA	1.001 [1.000-1.001]	060
MPO	1.001 [0.997-1.004]	.664
DNA-MPO	17.428 [1.976-153.679]	**.010**

CI, confidence interval; DIC, disseminated intravascular coagulation; CNS, central nervous system; ARDS, acute respiratory distress syndrome; APACHE II, Acute Physiology and Chronic Health Evaluation II; SOFA, Sequential Organ Failure Assessment; IL, interleukin; TNF, tumour necrosis factor; NETs, neutrophil extracellular traps; cfDNA, cell-free deoxyribonucleic acid; MPO, myeloperoxidase. * P value for crude odds ratio to predict DIC. NETs were analysed either as continuous NET (%) or categorical variables (absent, mild, moderate and strong) as defined in materials and methods. Univariate binary logistic regression analysis for association between DIC (categorical) and continuous variables. Bold indicates P<0.05.

Table 4.8 Univariate analysis of clinical variables for the prediction of mortality

	Crude Odds ratio [95% CI]	P value*
Mortality		
Clinical variables		
Age	1.014 [0.997-1.031]	.102
Gender	0.850 [0.498-1.451]	.551
Initial diagnosis		
Sepsis	REF	
Cardiovascular	1.036 [0.442-2.427]	.935
CNS	0.184 [0.024-1.442]	.107
Gastro	0.461 [0.167-1.273]	.135
Renal	0.000 [0.000-0.000]	.999
Respiratory	0.395 [0.155-1.003]	.051
Trauma	0.417 [0.182-0.957]	**.039**
Hypotension	1.113 [0.641-1.933]	.704
ARDS (P/F)	0.998 [0.996-1.001]	**.204**
APACHEII	1.087 [1.047-1.128]	**<.0001**
SOFA score	1.087 [1.017-1.162]	**.014**
Bacteraemia	2.109 [1.228-3.623]	**.007**
Source of infection		
No infection	REF	
Respiratory	2.914 [1.421-5.973]	**.004**
Abdomen	1.457 [0.651-3.233]	.355
Neuro	1.166 [0.317-4.280]	.817
Other	2.914 [1.194-7.109]	**.019**
Laboratory variables		
IL-1β	1.008 [.0981-1.035]	.570
IL-6	1.000 [1.000-1.000]	.907
TNFα	0.999 [0.997-1.002]	.671
IL-8	1.000 [1.000-1.000]	.380
Absent NETs	REF	
Mild NETs	1.385 [0.617-3.109]	.430
Moderate NETs	3.235 [1.284-8.152]	**.013**
Strong NETs	3.785 [1.506-9.511]	**.005**
NETs [%]	1.020 [1.010-1.030]	**<.0001**
cfDNA	1.000 [1.000-1.000]	.232
MPO	0.998 [.0994-1.002]	.353
DNA-MPO	2.005 [0.430-9.359]	.376

CI, confidence interval; CNS, central nervous system; ARDS, acute respiratory distress syndrome; APACHE II, Acute Physiology and Chronic Health Evaluation II; SOFA, Sequential Organ Failure Assessment; IL-, Interleukin; TNF, tumour necrosis factor; NETs, neutrophil extracellular traps; cfDNA, cell-free deoxyribonucleic acid; MPO, myeloperoxidase * P value for crude odds ratio to predict DIC and mortality. NETs were analysed either as continuous NET (%) or as categorical variables (absent, mild, moderate and strong) as defined in materials and methods. Univariate binary logistic regression analysis for association between mortality (categorical) vs continuous variables. Bold indicates P<0.05.

Table 4.9 Multivariate logistic regression analysis of independent predictors for DIC and mortality

	Crude Odds ratio [95%CI]	P value*	Adjusted odds ratio [APACHEII]	P value†	AUC	P value‡
DIC						
NETs-forming capacity assay						
Absent NETs	REF		REF			
Mild NETs	0.274 [0.045-1.677]	.161	0.248 [0.039-1.560]	.137	-	-
Moderate NETs	8.121 [2.143-30.770]	**.002**	7.176 [1.765-29.177]	**.006**	-	-
Strong NETs	14.517 [3.759-56.057]	**<.0001**	13.035 [3.157-53.829]	**<.0001**	-	-
NETs [%]	1.059 [1.041-1.078]	**<.0001**	1.058 [1.039-1.078]	**<.0001**	0.851	**<.0001**
NETs-related biomarkers						
cfDNA	1.001 [1.000-1.001]	.060	1.001 [1.000-1.001]	.118	0.607	.324
MPO	1.001 [0.997-1.004]	.664	1.001 [0.997-1.005]	.689	0.609	.236
DNA-MPO	17.428 [1.976-153.679]	**.010**	9.780 [0.972-98.424]	.053	0.713	**.013**
Cytokines						
IL-1β	0.993 [0.923-1.068]	.845	0.999 [0.920-1.084]	.973	0.588	.272
IL-6	1.000 [1.000-1.000]	.113	1.000 [1.000-1.000]	.139	0.546	.499
TNFα	0.999 [0.994-1.003]	.501	0.999 [0.995-1.003]	.593	0.658	**.044**
IL-8	1.000 [1.000-1.000]	**.049**	1.000 [1.000-1.001]	.083	0.666	**.002**
Scoring systems						
APACHEII	1.144 [1.084-1.208]	**<.0001**	-	-	0.753	**<.0001**
SOFA score	1.435 [1.274-1.616]	**<.0001**	-	-	0.837	**<.0001**
Mortality						
NETs-forming capacity assay						
Absent NETs	REF		REF			
Mild NETs	1.385 [0.617-3.109]	.430	1.370 [0.601-3.125]	.454	-	-
Moderate NETs	3.235 [1.284-8.152]	**.013**	2.889 [1.114-7.494]	**.029**	-	-
Strong NETs	3.785 [1.506-9.511]	**.005**	2.995 [1.162-7.720]	**.023**	-	-
NETs [%]	1.020 [1.010-1.030]	**<.0001**	1.016 [1.006-1.026]	**.002**	0.851	**<.0001**
NETs-related biomarkers						
cfDNA	1.000 [1.000-1.000]	.232	1.000 [1.000-1.000]	.532	0.607	.324

MPO	0.998 [.0994-1.002]	.353	0.998 [0.993-1.002]	.261	0.609	.236
DNA-MPO	2.005 [0.430-9.359]	.376	1.432 [0.286-7.161]	.662	0.713	**.013**
Cytokines						
IL-1β	1.008 [.0981-1.035]	.570	1.003 [1.011-1.119]	.804	0.501	.984
IL-6	1.000 [1.000-1.000]	.907	1.000 [1.000-1.000]	.904	0.596	.064
TNFα	0.999 [0.997-1.002]	.671	0.999 [0.996-1.002]	.598	0.511	.846
IL-8	1.000 [1.000-1.000]	.380	1.000 [1.000-1.000]	.563	0.574	.141
Scoring systems						
APACHEII	1.087 [1.047-1.128]	**<.0001**	-	**-**	**0.683**	**<.0001**
SOFA score	1.087 [1.017-1.162]	**.014**	-	**-**	0.604	**.009**

CI, confidence interval; APACHE II, Acute Physiology and Chronic Health Evaluation II; AUC, area under the curve; NETs, neutrophil extracellular traps; cfDNA, cell-free deoxyribonucleic acid; MPO, myeloperoxidase; IL, Interleukin; TNF, tumour necrosis factor; SOFA, Sequential Organ Failure Assessment. * P value for crude odds ratio to predict DIC and mortality. † P value for adjusted odds ratios to predict DIC and mortality in a multivariable analysis (with patients adjusted for APACHE II scores). ‡ P value for ROC analysis to predict DIC and mortality. Bold font indicates significant P value of < 0.05.

Figure 4.3

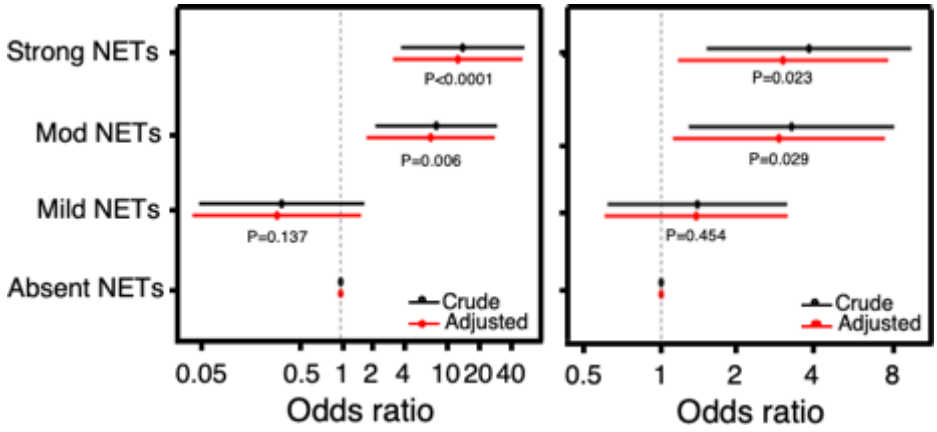


Figure 4.3 NETs-forming capacity independently predicts DIC and mortality. Multivariable analysis of crude and adjusted odds ratios demonstrating that the NETs-forming capacity assay can independently predict DIC development (left panel) and mortality (right panel) (n=341). Odds ratios were adjusted for APACHE II scores.

Figure 4.4

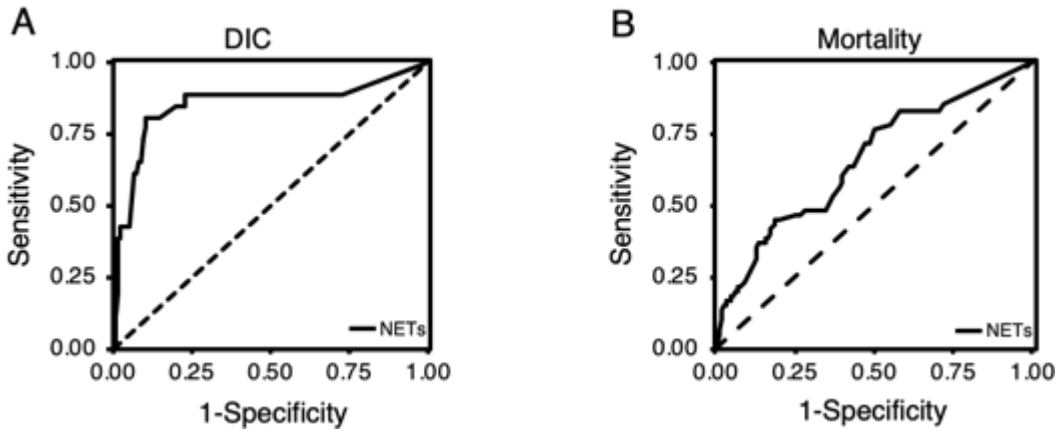


Figure 4.4 ROC curves of DIC and mortality predication. Receiver Operating Characteristic (ROC) analysis of NETs-forming capacity for predicting DIC development (**A**) and mortality (**B**) on ICU admission in critically ill patients (n=341). Dash line indicate ROC reference line.

4.2.7 Circulating levels of IL-8 are significantly correlated with NETs formation

A variety of NETs-inducing factors have been widely studied, such as IL-1β, IL-6, IL-8, tumour necrosis factor alpha (TNFα) and extracellular histones (78, 269-272). The cytokine profile was therefore analysed in the same cohort to explore potential association with *ex vivo* NETs formation levels. IL-8 was the only cytokine that showed positive and significant association with NETs formation, whilst IL-5, IL-9, IL-12, IL-13, IL-17, basic fibroblast growth factor (bFGF), granulocyte-macrophage colony-stimulating factor (GM-CSF) and regulated on activated normal T-cell expressed (RANTES) negatively correlated with NETs formation (Table 4.10, Figure 4.5). Patients with their plasma induced NETs formation had significantly higher circulating IL-8 levels compared to those without (P=0.036), whereas other cytokines known to induce NETs formation did not demonstrate significant association.

4.2.8 Blocking IL-8 partially attenuated ex vivo NETs production

To investigate the functional relevance of IL-8 in driving NETs, IL-8 at pathologically relevant concentration (100 pg/ml) was added to normal plasma, which was then incubated with healthy neutrophil. Normal plasma supplemented with IL-8 triggered significantly higher NETs levels compared to non-supplemented plasma (P=0.008) (Figure 4.6.A). To confirm if IL-8 was in part responsible for the generation of NETs-forming capacity of patient's plasma, healthy neutrophil were pre-treated with anti-IL-8 monoclonal antibody (mAb) or clinically trialled IL-8 receptor antagonists, Reparixin or AZD5069 and then stimulated with plasma from patients with sepsis (n=10). NETs formation levels from patients with sepsis (median [IQR]: 57.5 [47.5, 78.8]) were significantly reduced following IL-8 blocking with anti-IL-8 mAb 19 [10.0, 22.5] (P<0.001), Reparixin 7.0 [3.5, 41.3] (P<0.001) and AZD5069 18.5 [10.0, 28.8] (P<0.001) (Figure 4.6.B).

Table 4.10 Circulating levels of cytokines according to different NETs-forming capacity degrees on ICU admission

	Normal	Correlation (R value)	Absent NETs (n=75)	Mild NETs (n=170)	Moderate NETs (n=49)	Strong NETs (n=47)	P value*
General activation							
IL-1β (pg/ml), Median [IQR]	5.65 [4.65-6.34]	-.293	5.86 [3.61-8.98]	5.51 [3.33-7.88]	4.95 [3.72-9.37]	4.51 [2.88-8.62]	.773
IL-1ra (pg/ml), Median [IQR]	177.58 [153.24-247.02]	-.286	1098.86 [318.56-6046.29] †	1025.10 [270.46-5705.21] †	443.08 [190.69-2598.12] †	522.78 [170.83-5580.57] †	.485
IL-2 (pg/ml), Median [IQR]	0.00 [0.00-3.38]	-.297	0.00 [0.00-7.78]	0.00 [0.00-3.53]	0.00 [0.00-0.00] §	0.00 [0.00-4.23]	.176
TNFα (pg/ml), Median [IQR]	96.50 [61.56-125.53]	-.265	113.80 [60.41-158.11]	70.51 [51.83-138.10]	94.47 [43.99-150.35]	100.57 [40.56-171.85]	.663
IL-6 (pg/ml), Median [IQR]	15.15 [12.36-19.52]	.265	161.99 [69.65-1123.54]	445.30 [61.09-1216.67] †	146.80 [40.43-608.98] †	343.86 [85.97-2449.15] †, ‖	.177
IL-15 (pg/ml), Median [IQR]	0.00 [0.00-12.59]	-.241	12.53 [0.00-79.01]	20.91 [0.00-45.54] †	0.00 [0.00-32.41]	24.55 [0.00-55.59] †	.279
Chemokines							
IL-8 (pg/ml), Median [IQR]	29.31 [26.74-55.93]	.529	63.27 [39.28-143.31] †	128.89 [48.47-255.64] †	114.36 [52.76-314.29] †	127.53 [73.50-331.65] †, ‡	.069
IP-10 (pg/ml), Median [IQR]	540.68 [379.22-656.62]	.221	1118.65 [668.75-6877.97] †	1037.15 [527.40-1914.35] †	1632.67 [494.98-7669.59] †	1077.73 [683.36-2954.90] †	.529
MCP-1 (pg/ml), Median [IQR]	4.90 [0.00-19.81]	.235	58.00 [22.67-167.01] †	87.17 [27.21-200.48] †	31.54 [2.35-181.35] §	112.28 [33.96-237.29] †	.172
MIP-1a (pg/ml), Median [IQR]	5.93 [4.14-7.62]	-.230	5.14 [2.98-7.81]	4.37 [2.98-6.09]	4.92 [3.33-7.61]	4.45 [2.36-6.47]	.768
MIP-1b (pg/ml), Median [IQR]	23.64 [14.72-34.15]	-.307	92.75 [71.93-240.96] †	100.55 [61.86-172.69] †	96.33 [68.23-164.86] †	91.12 [60.66-139.34] †	.649
RANTES (pg/ml), Median [IQR]	3142.25 [1278.65-3558.20]	-.560	4690.15 [2372.52-6221.61]	5085.97 [3566.83-7098.21] †	3164.76 [1716.22-4301.35] §	2905.09 [14445.22-4311.39] ‡, §	**<.0001**
T cell-related							
IL-4 (pg/ml), Median [IQR]	6.19 [5.74-7.23]	-.463	6.36 [4.65-8.42]	6.90 [5.30-8.08]	5.86 [4.12-7.94]	5.00 [3.19-7.60] §	.126
IL-5 (pg/ml), Median [IQR]	20.18 [8.99-21.52]	-.608	11.36 [4.79-16.28] †	10.55 [4.79-15.18] †	9.08 [1.90-15.29] †	1.63 [0.00-6.40] †, ‡, §, ‖	**.001**
IL-9 (pg/ml), Median [IQR]	17.54 [14.05-44.76]	-.476	56.71 [23.00-94.63] †	47.95 [26.42-84.40] †	30.53 [21.40-49.81] §	28.25 [18.08-59.16] ‡, §	**.043**
IL-10 (pg/ml), Median [IQR]	17.81 [12.79-26.34]	-.266	31.99 [17.63-136.96]	33.80 [20.01-43.01] †	21.20 [13.85-74.45]	37.54 [16.94-79.03]	.432
IL-12 (pg/ml), Median [IQR]	8.88 [2.73-26.86]	-.576	16.05 [7.46-21.45]	13.95 [9.04-18.96]	11.09 [6.10-19.36]	7.74 [3.10-13.60] ‡, §	**.004**
IL-13 (pg/ml), Median [IQR]	8.62 [3.77-13.82]	-.528	5.31 [2.14-7.81]	5.15 [3.91-9.85]	5.31 [2.19-8.98]	2.71 [0.11-4.39] †, §, ‖	**.009**
IL-17 (pg/ml), Median [IQR]	29.13 [7.53-33.46]	-.512	39.07 [26.70-71.62]	44.29 [21.38-71.00] †	25.41 [15.91-60.50]	21.74 [5.07-39.31] ‡, §	**.007**
Eotaxin (pg/ml), Median [IQR]	87.97 [62.29-113.57]	-.200	95.45 [58.38-127.69]	93.91 [65.52-123.15]	81.49 [58.91-133.11]	91.30 [61.74-119.68]	.960
INFγ (pg/ml), Median [IQR]	162.57 [124.31-201.07]	-.369	162.97 [119.33-269.09]	146.16 [122.53-205.00]	163.95 [119.55-241.74]	134.46 [79.72-223.60]	.415
Bone marrow-derived							
IL-7 (pg/ml), Median [IQR]	22.05 [15.07-29.12]	-.223	17.72 [8.73-22.44]	14.91 [8.57-23.56]	16.36 [7.48-23.51]	14.38 [5.43-30.81]	.973
GM-CSF (pg/ml), Median [IQR]	0.00 [0.00-0.00]	-.365	90.08 [0.00-177.10] †	66.70 [31.51-143.91] †	0.00 [0.00-55.59] ‡, §	51.78 [0.00-143.58] †, ‖	**.006**
G-CSF (pg/ml), Median [IQR]	112.28 [108.85-139.09]	-.077	152.70 [112.77-318.01]	226.19 [90.74-821.29]	117.67 [79.94-364.42]	178.59 [76.70-738.95]	**.**509
Angiogenic factors and endothelial mitogens							
bFGF (pg/ml), Median [IQR]	32.85 [10.92-153.67]	-.529	64.80 [50.95-92.73]	77.01 [51.64-99.15]	41.55 [18.20-62.48] ‡, §	48.86 [22.89-76.03] ‡, §	**<.0001**
PDGF-bb (pg/ml), Median [IQR]	636.75 [152.18-863.18]	-.458	528.68 [147.68-1308.47]	662.11 [284.66-888.55]	455.37 [138.82-825.18]	324.95 [122.72-653.54] §	.196
VEGF (pg/ml), Median [IQR]	11.99 [2.21-62.04]	-.464	78.93 [29.29-112.58] †	65.28 [35.64-107.89] †	54.48 [20.10-98.32] †	49.91 [14.05-82.45] §	.105

NETs, neutrophil extracellular traps; IQR, interquartile range; IL, interleukin; TNF, tumour necrosis factor; IP, interferon-γ-inducible protein; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; RANTES, Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted; INF, Interferon; GM-CSF, granulocyte-macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; bFGF, basic fibroblast growth factor; PDGF-bb, platelet-derived growth factor; VEGF, vascular endothelial growth factor.* P value for comparisons of absent vs mild vs moderate vs strong NETs-formation in ICU patients. † Significant vs Normal controls. ‡ Significant vs absent NETs patients. § Significant vs mild NETs patients. ‖ Significant vs moderate NETs patients. * P indicates comparison among five groups. Kruskall-Wallis test for continuous variables.

R value calculated from Spearman's rank test with percentage NETs. Bold font indicates significant P value of < 0.05.

Figure 4.5

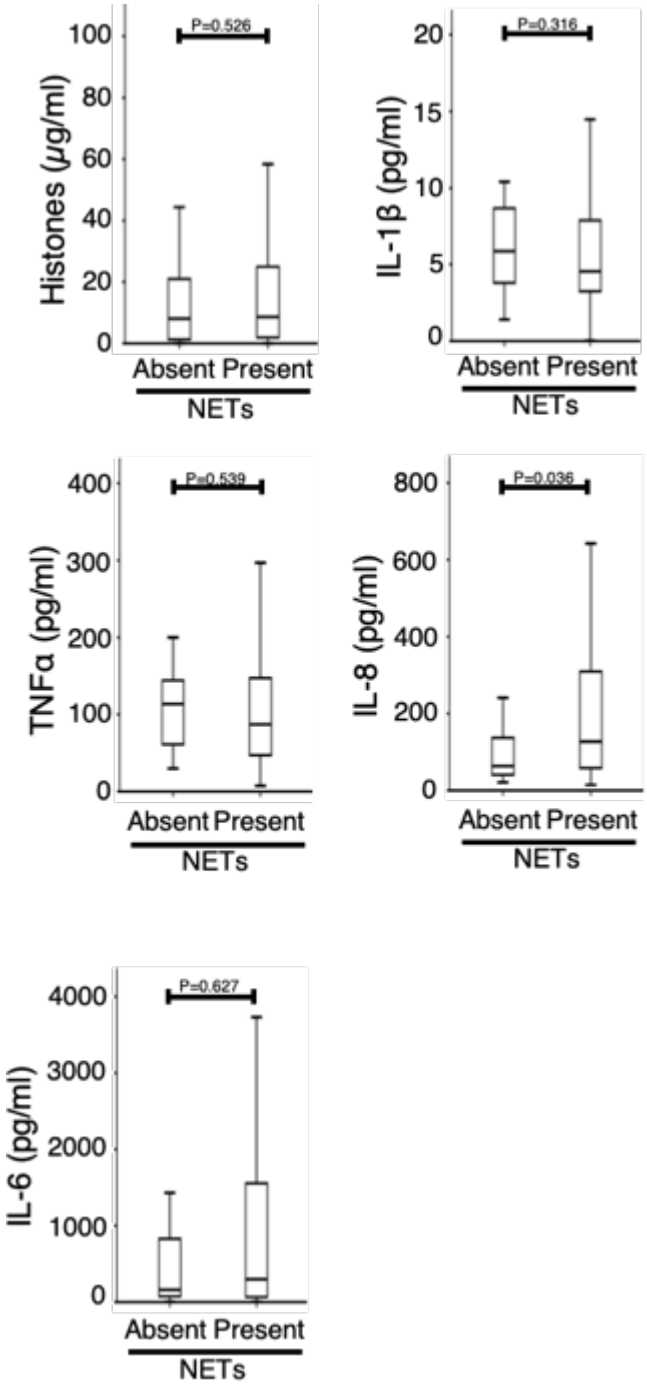


Figure 4.5 IL-8 levels are associated with *ex vivo* NETs levels. Histones, IL-1β, IL-6, IL-8 and TNFα were quantified in the circulation of critically ill patients (n=341), exhibited no significant association with NETs-induced by patient's plasma except for IL-8 (ANOVA, $P < 0.05$) (n=341).

Figure 4.6

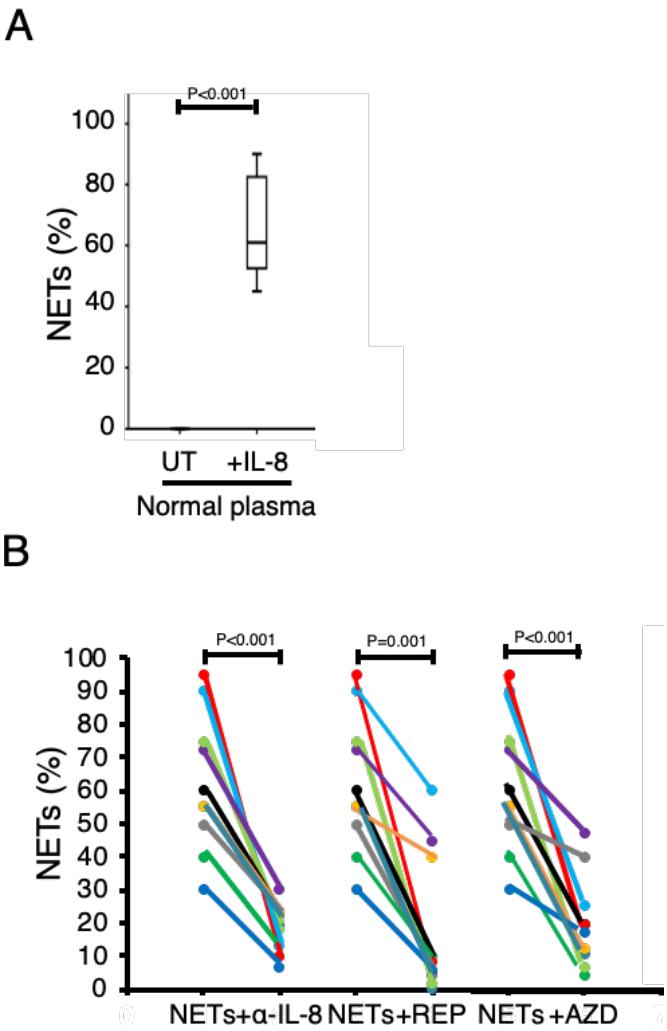


Figure 4.6 Blocking IL-8 attenuates NETs-forming capacity of patient plasma. (A) Stimulation of normal healthy neutrophils with normal plasma supplemented with 100 pg/ml IL-8 for 4 hourscompared to normal plasma alone (n=10), demonstrated higher levels of NETs formation (ANOVA; P<.05). (B) Normal healthy neutrophils pre-treated with anti-IL-8 mAb (α-IL-8) (n=10), Reparixin (REP) (n=10), AZD5069 (n=10) and then incubated with septic patient plasma for 4 hours showed partial inhibition of NETs formation (ANOVA, P< 0.05).

4.3 Discussion

In chapter 3, we endeavoured to develop a NETs assay that can overcome limitations associated with current methods to quantify NETs in clinical settings. In this chapter, the clinical relevance of the *ex vivo* NETs-forming capacity assay was investigated in a cohort of critically ill patients. The extent of NETs formation was significantly associated with sepsis, clinical scores of disease severity and MOF, coagulation complications and mortality in the ICU. The multivariate logistic regression analysis reveals that our NETs-forming capacity assay,but not indirect measures of NETs or other factors, was independently associated withDIC and mortality. Moreover, levels of NETs formation are capable of predicting DIC and 28-day mortality on ICU admission, comparable to the routinely measuredAPACHE II and SOFA scoring systems. The finding that IL-8 is a key factor drivingthe NETs formation in our cohort suggest possible usefulness as a new candidate for targeting NETs in critical illness.

NETosis has been shown to prevent translocation and dissemination of infection through microbial trapping and killing in blood and tissue samples (78, 163, 300, 301). In this chapter, patient plasma induced NETs release is strongly correlated with consumptive coagulopathy and MOF. These findings suggest that excessive release of intravascular NETs can enable platelet aggregation and thrombosis formation leading to diminished perfusion to the distant organs and subsequently organ injury. The complex interplay between NETosis, coagulation and organ injury supporting our theory has been elaborated by McDonald *et al* (205) where they demonstrated that NETs are critical contributors to intravascular coagulation, microvascular occlusion and resultant organ dysfunction in experimental models of sepsis. They also reported lack of association between histones and both platelet trapping within NETs structures and release of NETs. This is consistent with findings from this study in which histones

levels are not significantly associated with NETs formation. The release of extracellular histones due to extensive cell death of different types of cells during critical illness may explain this lack of association.

The role of NETs to be critical inflammatory mediators during lung injury has been increasingly acknowledged (302). NETs levels in patients with pneumonia-induced ARDS were associated with alveolar damage and high bronchoalveolar lavage fluid (BALF) concentrations of IL-8 (303). To elaborate these findings, moderate to strong NETs-forming capacity is associated with respiratory-related sepsis. These findings are indicative that NETs formation during sepsis could be more pronounced within the lungs than other major organs.

A key strength that adds value to the *ex vivo* NETs assay is the illustration of how clinical data links into discovering putative therapeutic approaches for blocking NETs formation. Consistent with the findings from this chapter, Yang *et al* (304) showed that NETs formation was more likely to be induced by plasma from septic than non-septic patients. However, no correlation to patient outcomes was reported in their study. Yet, the small sample size of 52 septic patients may explain the lack of correlation. In this chapter, the NETs formation assay was compared to other NETs-related assays (245, 247, 305-307) and showed that cfDNA, MPO and MPO-DNA complexes did not correlate with complications and outcomes. This may be due to that indirect measurements of NETs are affected by NETs degradation enzymes or contamination from other forms of cell death, whereas the novel assay is not being affected as it could directly measure the NETs-forming capacity of patient plasma (Figure 4.7).

Cytokines release is a distinctive characteristic of the inflammatory response during

critical illness (286), with their ability to drive NETs release has been intensively studied recently (78, 269-272). In addition, multiple potent activators of neutrophils trafficking to the site of infection such as, TNFα, IL-1β and IL-8 have been also shown to promote NETs formation (308). Herein, only IL-8 was positively associated with *ex vivo* NETs formation assay. Furthermore, blocking IL-8 using mAb and therapeutic antagonists, NETs formation was inhibited in these patients plasma. However, no independent predictive values were observed for IL-8 to DIC and mortality, along with other known inducers of NETs formation (IL-1β, IL-6 and TNFα). This could be due to lack of specificity, as cytokines are not uniformly elevated during the inflammatory cascade. These findings collectively provide an additional advantage to the assay and support its ability to inform on NETs driving factors in individual patient plasma, enhancing its potential to guide clinical management as part of personalised medicine.

An interesting finding of this chapter is that the levels of T-cell related cytokines were negatively associated with the degree of NETs formation in critically ill patients. Uncontrolled apoptosis of T-cells is associated with lower cytokines production and mediates immunosuppression during sepsis (309). In our patient cohort, the degree of NETs formation was higher in patients with DIC than those without DIC. Therefore, it is possible that immunosuppression, commonly seen in DIC, is likely to play a role in this negative association.

There was a limitation to this work. The relative contribution of IL-8-CXCR1/2 signaling pathway to NETs formation has not been fully elucidated. However, the strong and positive association between NETs and IL-8 levels presented in this chapter provided underlying evidence. Yet, *in vivo* studies are needed to provide supporting evidence of our understanding on the ability of IL-8 to drive NETs formation during critical illness.

In summary, this chapter presents data of the significant clinical importance and translational relevance of the NETs-forming potential in the circulation to identify critically ill patients at risk of developing DIC following ICU admission and poor outcomes. As such measuring NETs-forming capacity may be candidate for use as a stratification tool to enhance the early and accurate stratification of severely ill ICU patients, to enable early administration of supportive therapy. As NETs formation is acontributing factor in intravascular coagulation through platelet activation (288), the findings obtained in this chapter provide a rational for using NETs as a therapeutic target in sepsis and consumptive coagulopathy. Showing that IL-8 is a factor driving NETs formation in many patients of our cohort provide evidence for the potential use of our novel assay system in precision therapeutic targeting of NETs.

Figure 4.7

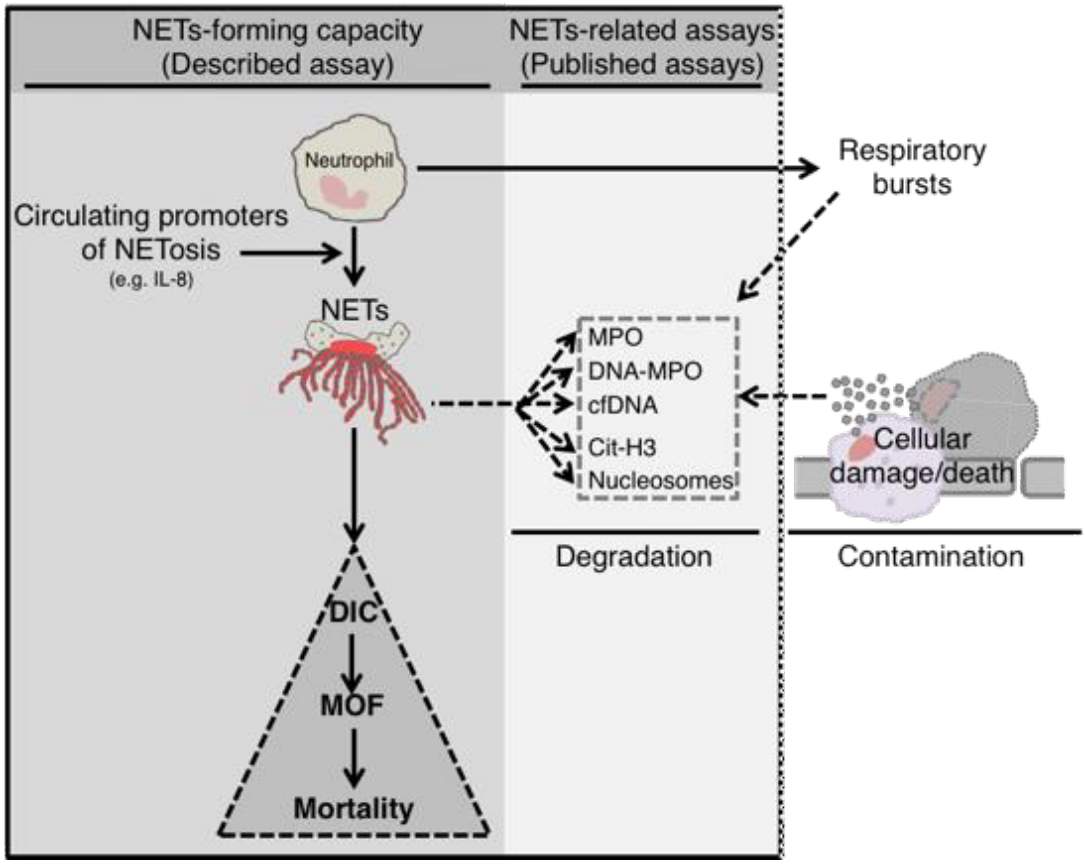


Figure 4.7 Schematic representation of the performance of NETs-forming capacity compared to other NETs-related assays.

Chapter 5: Ex vivo NETs-forming capacity reflect in vivo NETs formation in animal models of sepsis

5.1 Introduction

Sepsis is a progressive, injurious and inflammatory disease, caused by a dysregulated host response to infection (9). Despite recent advances in understanding the pathophysiology of sepsis, the inflammatory response during sepsis can adversely progresses to organ dysfunction (9). A major inflammatory feature of sepsis is the robust release of chemoattractant to recruit leukocytes including neutrophils, to prevent bacterial spreading (310). However, there is compelling evidence suggesting that sepsis substantially results in deleterious accumulation of neutrophils to distal organs leading to multiple organ dysfunction syndrome (MODS) (311).

Experimental animal models of different species are amongst the most effective tools currently available to address disease progression, pathophysiology and therapeutic trialling for sepsis (312, 313). In general, animal models of sepsis can be generated using non-invasive and invasive techniques (314). The most widely used non-invasive methods to generate sepsis in mice are performed via injection of endotoxins such as bacterial Lipopolysaccharides (LPS) and infusion of live bacteria. Live bacterial infusion has been recognised as causative organisms to mimic peritonitis-sepsis in animals, with *Escherichia coli* (*E. coli*) remains the most commonly used pathogen to replicate gram-negative bacterial sepsis (315, 316). The magnitude of disease severityis dose-dependent and high doses of bacterial infection are usually required to overcome host defence (317, 318).

On the other hand, the invasive models of sepsis require surgical induction such as cecal ligation and puncture (CLP) and bowel perforation (314). The CLP model has been considered as the gold standard approach to study polymicrobial sepsis in animal

due its simplicity and reproducibility (319-321). The cecum is ligated and punctured using a needle, in which the number of punctures and the size of the needle's gauge manipulate the severity of sepsis (322, 323). The principle of CLP models is the peritonitis induced by host-derived flora, mimic many human diseases, which is of better clinical relevance (314).

Neutrophil extracellular traps (NETs) formation is highlighted in highly vascularized organs such as the lungs and liver (163, 194-196, 198). Yet, it is debated which organ is mainly susceptible for excessive NETs deposition. Lee et al. and Luo et al. observed extensive NETs formation within the lungs of CLP mouse model (194, 195). McDonald et al. suggested that the lungs are more prone for excessive NETs formation in septic mouse model induced by intraperitoneal injection (i.p) injection of LPS (196). Still, widespread NETs deposition is observed within the lungs and liver following challenge with bacterial LPS (163, 198).

Despite the efficient role of the immune system in eliminating infections, over exuberant innate immune response is implicated in systemic inflammation and organ injury in sepsis (100). The aggregation of activated neutrophils within the vasculature of different organs is one mechanism by which organ dysfunction is augmented in sepsis (40, 324). In recent years, NETs have emerged as a fundamental link between innate immune system and coagulation in sepsis (325). NETs were found to potentiate microvascular occlusion by enhancing fibrin formation *in vitro* (113), and providing a scaffold for fibrin deposition *in vivo*, where they contribute to platelets and red bloodcells (RBCs) entrapment (193, 294). Nevertheless, it is difficult to monitor NETs within the organs in humans, unless through invasive specimens or indirectly using NETs degradation biomarkers e.g. myeloperoxidase (MPO) and cell-free DNA (cfDNA) (243-245). As a result, improved monitoring of NETs formation in patients

is urgently needed tobetter define those at risk of developing MODS and to identify potential treatments, but the situation is complicated by some fundamental knowledge lacking on how *in vivo* NETs formation correspond with individual capacity to form NETs.

In chapter 3, the functional effect that plasma/sera from septic patients may have on heterologous neutrophils from healthy individuals was elucidated. In chapter 4, the clinical relevance of the NETs-forming capacity assay in critically ill patients was determined. The levels of NETs formation is associated with sequential organ failure assessment (SOFA) scores and could independently predict disseminated intravascular coagulation (DIC) on intensive care unit (ICU) admission. However, the translational relevance of the *ex vivo* NET-forming capacity assay in animal models remain unknown.

Therefore, the aims of this chapter were to (1) measure the NETs-forming capacity ofplasma from two mouse models of sepsis at various time points; (2) identify which organ is at risk of enhanced NETs release; (3) determine whether *ex vivo* NETs levels correlate with *in vivo* NETs formation within the organs; (4) measure the association between NETs-fibrin deposition and organ injury.

5.2 Results

5.2.1 Ex vivo NETs formation can be induced from plasma of both CLP and *E.coli* infusion mice models of sepsis

Plasma from patients were found to activate neutrophils and subsequently cause *ex vivo* NETs formation as demonstrated in chapters 3 and 4. Nevertheless, the translational relevance of the *ex vivo* NETs formation assay in animal models of sepsis remain uncertain. In this study, two mouse models of sepsis were subjected to either CLP or intraperitoneal (i.p) injection of *E. coli* along with mock CLP and i.p injection of saline and blood was collected 4, 6 and 10 hours post sepsis induction.

To determine the capacity of plasma-induced NETs formation, heterologous mice neutrophils were isolated from blood and incubated with mice plasma for 4 hours at 37^{o}C and 5% CO2. Following staining of DNA structures with propidium iodide (PI), we identified typical NETs structures, but not by plasma from control mice (Figure 5.1.A and Figure 5.2.A). There was significant NETs formation following the induction of sepsis in both models, which remained during the model duration (10 hours) (Figure 5.1.B and Figure 5.2.B). For further validation of NETs structures (defined by the presences of extracellular DNA and granule proteins), staining was carried out using anti-neutrophil elastase and anti-myeloperoxidase using specific primary antibodies and corresponding Fluorescein isothiocyanate (FITC) and Phycoerythrin (PE) conjugated secondary antibodies (Figures 5.3 and 5.4). Typical NETs structures were observed as demonstrated by NETs-defining markers. There was no significant difference in NETs formation between both models (p=0.122). Altogether, these data suggest that the newly established *ex vivo* NETs formation assay is a valid method for determining NETs-forming capacity in sepsis.

Figure 5.1

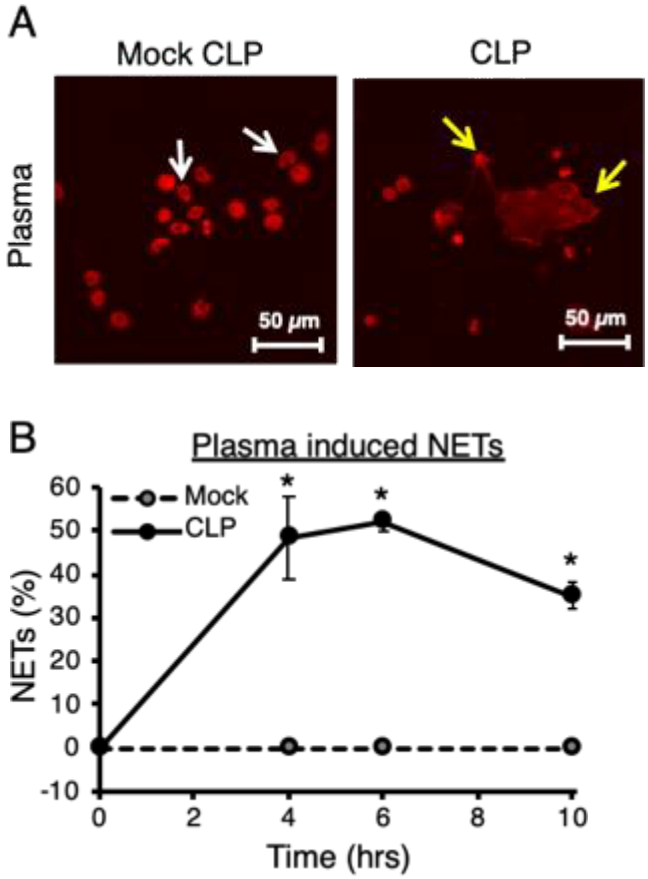


Figure 5.1 Generation of Ex vivo NETs formation using plasma from CLP-induced sepsis model. C57BL/6 male mice were anesthetized and sepsis was induced by CLP. Blood was collected at 4, 6 and 10 hours following sepsis induction along with mock CLP as controls (3 mice per time point per group). *Ex vivo* NETs formation assay was performed using neutrophils isolated from whole blood of normal mice and incubated with plasma from the CLP mice and controls followed by fixation with 2% PFA and staining of DNA structures with 10μg/ml of PI. Typical images of NETs formation are presented **(A)**, white arrows indicate normal neutrophils, yellow indicates NETs. Scale bar, 50 μM. NETs formation was quantified as NETs percentage/field and means ± SD are presented **(B)**. ANOVA test, *P<0.05 increase NETs formation compared to mock controls. n=3 mice per timepoint per group.

Figure 5.2

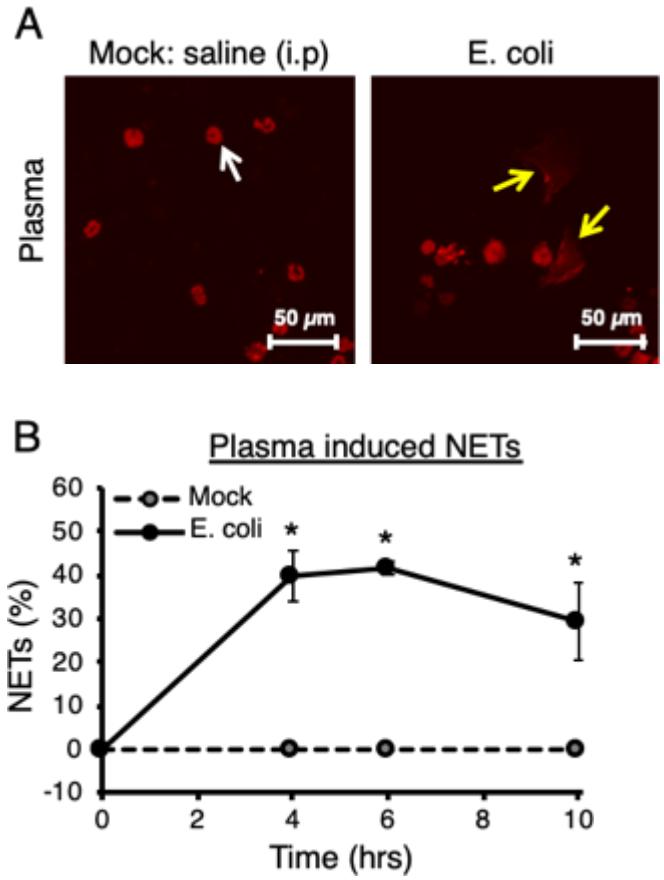


Figure 5.2 Plasma from *E. coli*-induced sepsis can stimulate NETs formation. C57BL/6 male mice were anesthetized and of *E. coli* was administered i.p to induce sepsis. Mice were euthanised and blood was collected at different timepoints (4, 6 and 10h) following sepsis induction. Mock controls were injected with i.p saline (3 mice per time point per group). *Ex vivo* NETs formation assay was performed by incubating neutrophils with plasma from the i.p *E. coli* mice or mock controls. Typical images of NETs formation are presented **(A)**, white arrows indicate healthy neutrophils, yellow indicates NETs formation. Scale bar, 50 µM. NETs formation was quantified as NETs percentage/field and means ± SD are presented **(B)**. ANOVA test, *P<0.05 increase NETs formation compared to mock controls. n=3 mice per timepoint per group.

Figure 5.3

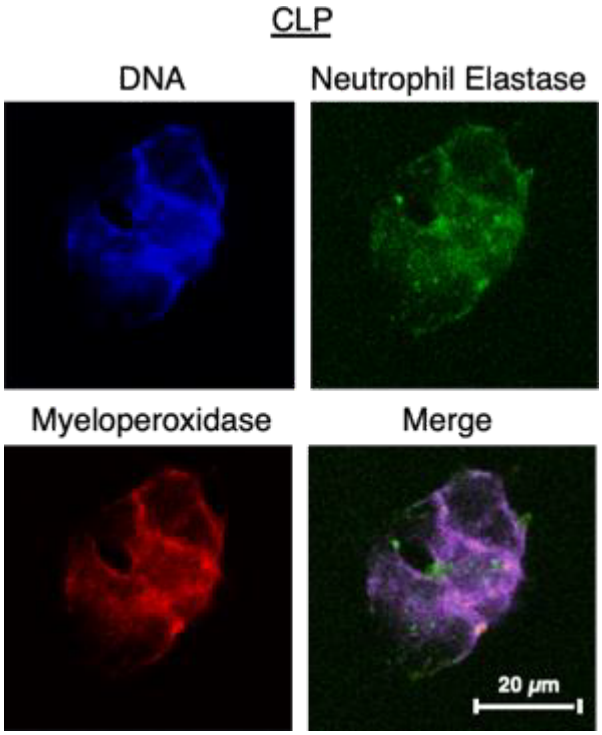


Figure 5.3 Typical NETs structures induced by plasma from CLP mouse model. Plasma from CLP mice were incubated with normal mice neutrophils for 4 hours. Following fixation with 2% PFA, NETs were stained overnight with goat anti-neutrophil elastase and rabbit anti-myeloperoxidase primary antibodies. NETs were visualised by confocal microscopy following staining with anti-goat FITC and anti-rabbit- PE conjugated secondary antibodies, along with propidium iodide staining of DNA. Typical images are presented. Scale bar, 20 μm.

Figure 5.4

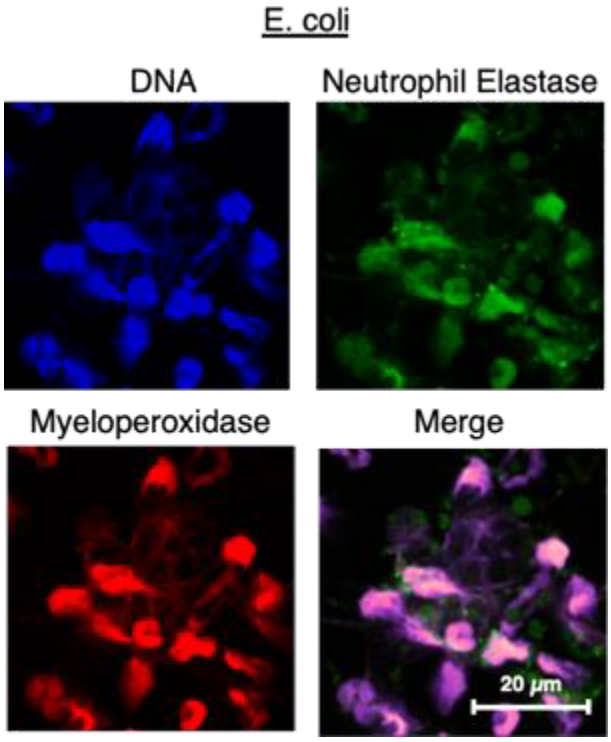


Figure 5.4 Plasma from *E. coli*-induced sepsis model generates typical NETs structures. NETs formation was induced by incubating plasma from i.p *E. coli* mice with normal mice neutrophils for 4 hours. Following fixation with 2% PFA, NETs were stained overnight with goat anti-neutrophil elastase and rabbit anti-myeloperoxidase primary antibodies. NETs were visualised by confocal microscopy following staining with anti-goat FITC and anti-rabbit- PE conjugated secondary antibodies, along with propidium iodide staining of DNA. Typical images of NETs structures are presented. Scale bar, 20 μm.

5.2.2 In vivo NETs form mainly within the lungs and correlate with ex vivo NETs formation in septic mice models

In experimental sepsis, aberrant NETs deposition is observed *in vivo* (163, 194-196, 198). Having shown that plasma from CLP and *E. coli* models induced NETs formation *ex vivo*, the possibility that the *ex vivo* NETs formation assay reflect NETs deposition *in vivo* arises. Using immunohistochemical (IHC) staining of mouse organ sections for citrullinated histone H3 (Cit-H3), a marker of NETs (144), systematic examinations of major organs were carried out to observe NETs formation within the lungs, liver, kidneys and heart after CLP and *E. coli*-induced sepsis (Figures 5.5 and 5.6). Significant *in vivo* NETs formation particularly within the lungs, was observed 4 hoursafter sepsis induction which remained elevated throughout the 10 hours study

duration(Figure 5.7 and Figure 5.8). No significant difference between two sepsis mouse models was found.

A correlation analysis was performed to examine the relationship between the levels of *ex vivo* NETs assay and the extent of *in vivo* NETs formation over the time course following sepsis induction (4 to 10 hours) in both mouse models. Strong positive correlation ($r=0.869$, $p<0.001$ in CLP, $R=0.781$, $P<0.001$ in *E. coli* injection model) was found between *in vivo* and *ex vivo* NETs formation. Collectively, these findings demonstrate that NETs are uniquely identified within the lungs of both models of sepsis. Moreover, the NETs forming capacity demonstrated by*ex vivo* NETs assay can reliably reflect *in vivo* NETs deposition.

Figure 5.5

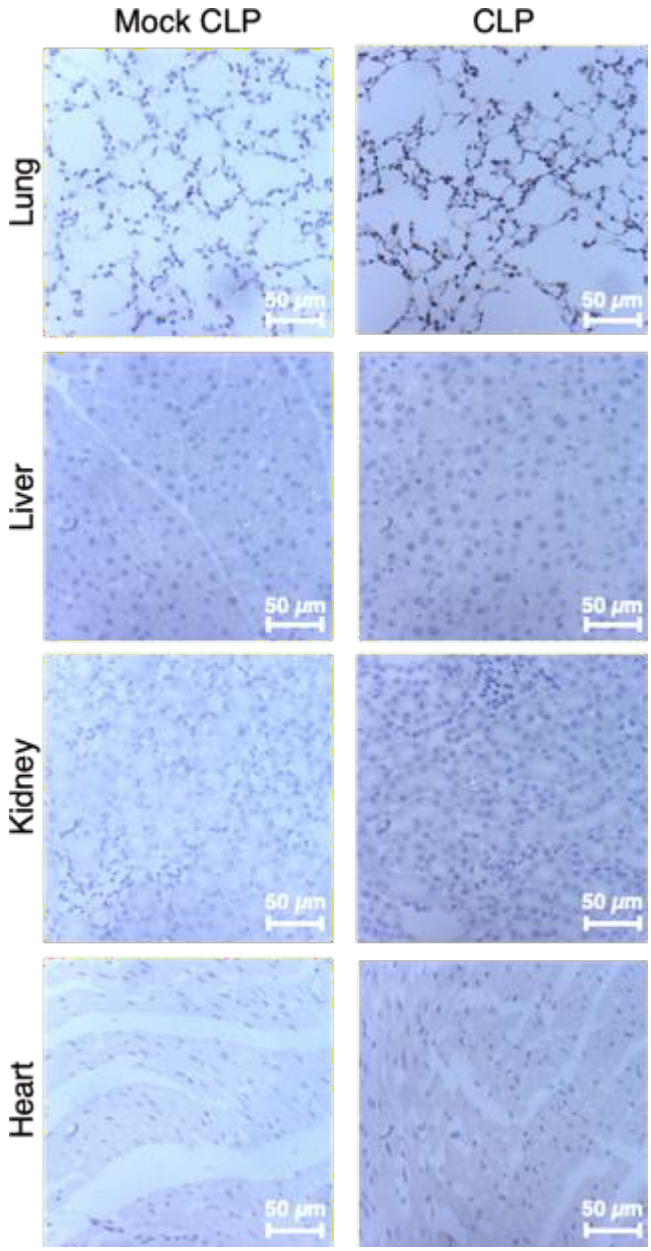


Figure 5.5 *In vivo* NETs are uniquely formed within the lungs of CLP-induced sepsis model. Tissue sections of the lungs, liver, kidney and heart from mouse model of CLP-induced sepsis along with mock control were collected at 4, 6 and 10 hours following sepsis induction. IHC staining with anti –Cit-H3 antibody was used to detect NETs and performed as previously described in material andmethods. Typical images of anti-Cit-H3 staining are presented. Scale bar, 50 µm.

Figure 5.6

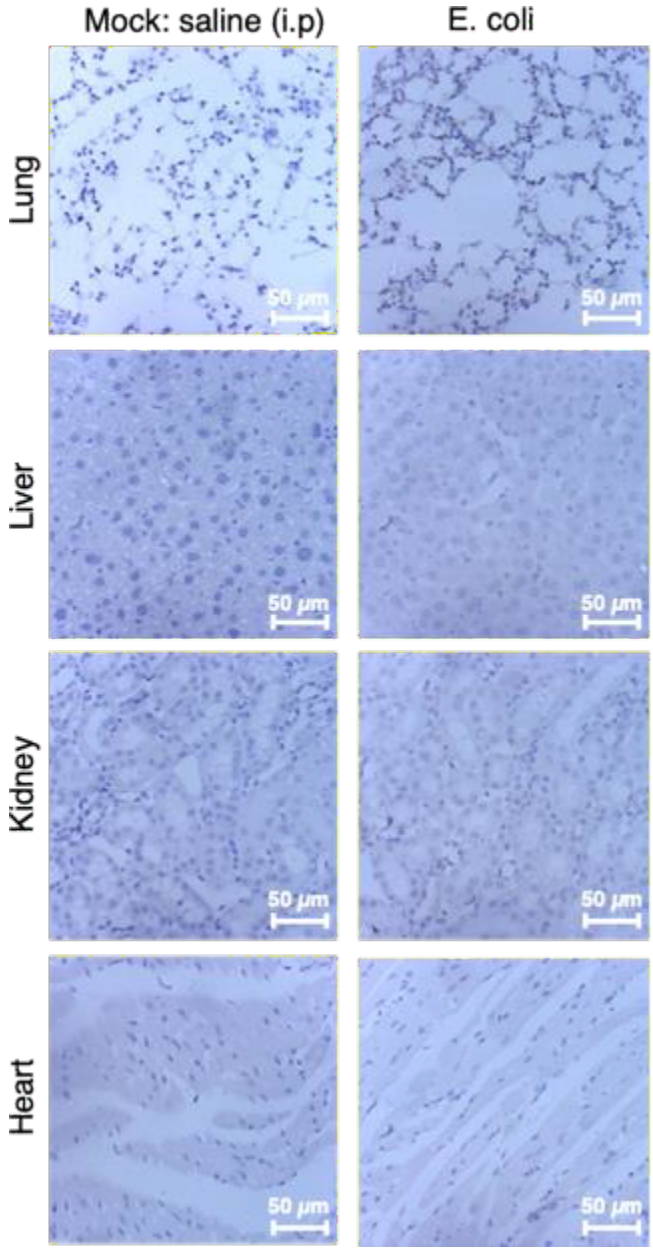


Figure 5.6 NETs formation within the lungs of *E. coli*-induced sepsis model. Following sepsis induction using i.p *E. coli* or saline for mock controls, major organs (lung, liver, kidneys and heart) were collected at 4, 6 and 10 hours. NETs formation was detected using anti-Cit-H3 antibody via IHC. Typical images are presented. Scale bar, 50 µm.

Figure 5.7

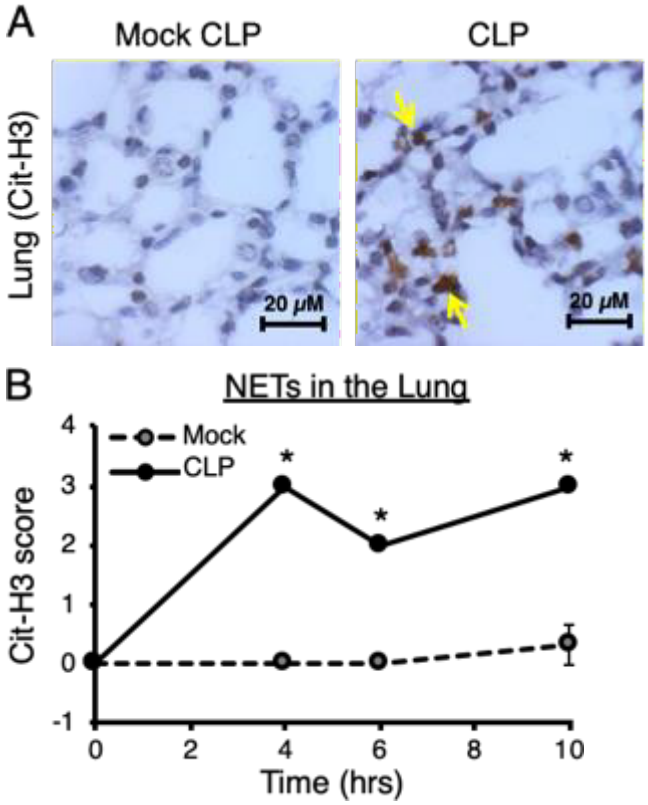


Figure 5.7 Quantification of NETs levels within the lungs of CLP mouse model. Representative images of IHC-stained lung sections with positive staining to anti-Cit-H3 antibody is demonstrated (yellow arrows). Scale bar, 20 μm **(A)**. Cit-H3 staining intensity was quantified at 4,6 and 10h and means ± SD are presented **(B)**. ANOVA test, *P<0.05 increase NETs formation compared to mock CLP control.

Figure 5.8

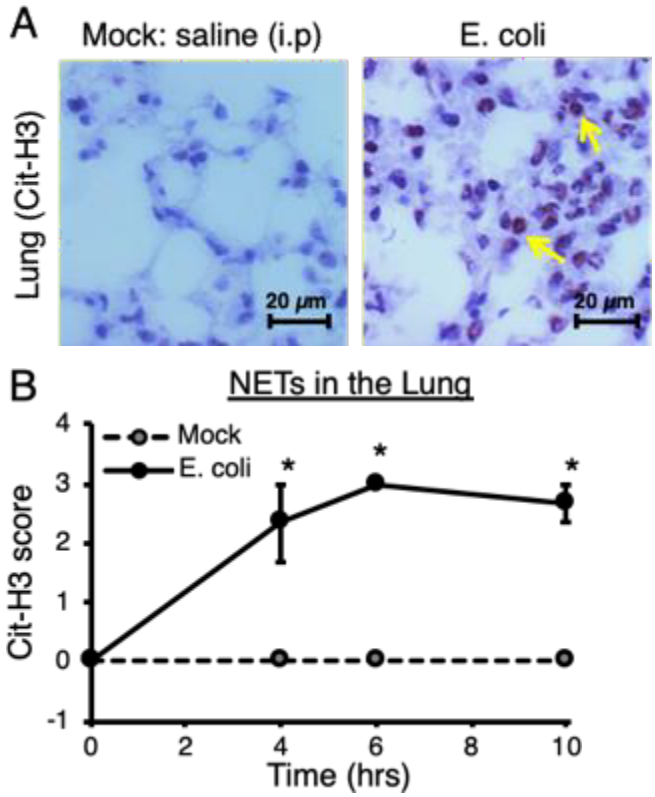


Figure 5.8 Quantification of NETs levels within the lungs of *E. coli* mouse model. (A) Lung sections were stained with anti-Cit-H3 antibody using IHC. Positive staining to anti-Cit-H3 antibody indicates NETs formation (yellow arrows)**.** Scale bar, 20 μm. **(B)** NETs formation was quantified at 4,6 and 10h and means ± SD are presented based on Cit-H3 staining intensity. ANOVA test, *P<0.05 increase NETs formation compared to i.p saline control.

5.2.3 NETs formation is required for enhanced fibrin deposition in sepsis

Neutrophil infiltration is a major pathological hallmark of sepsis (326). Unregulated recruitment of neutrophils is increasingly implicated in the pathogenesis of organ dysfunction in sepsis (311). NET- fibrin deposition within the vasculature is reported to promote thrombus formation (327). These pathological structures may promote vascular occlusion and cause organ damage (328). Having shown that excessive NETs release is observed in vivo, particularly within the lungs, we sought to examine the pathological effects produced by NETs during sepsis, particularly fibrin deposition.

To clarify whether excessive neutrophil infiltration and NETs generation are requiredfor fibrin deposition during sepsis, the effects of neutrophils, including NETosis, were eliminated via neutrophil depletion using Ly6G-specific monoclonal antibody 24 hours prior to CLP induction. Moreover, DNase I was injected 1h and 5h after CLP induction to confirm the direct role of NETs in providing a scaffold for fibrindeposition. As shown in figures 5.9 and 5.10, obvious NETs and fibrin deposition in the CLP model were observed. However, in the CLP mice with neutrophil depletion or DNase I treatment no obvious NETs or fibrin deposition were found.

Figure 5.9

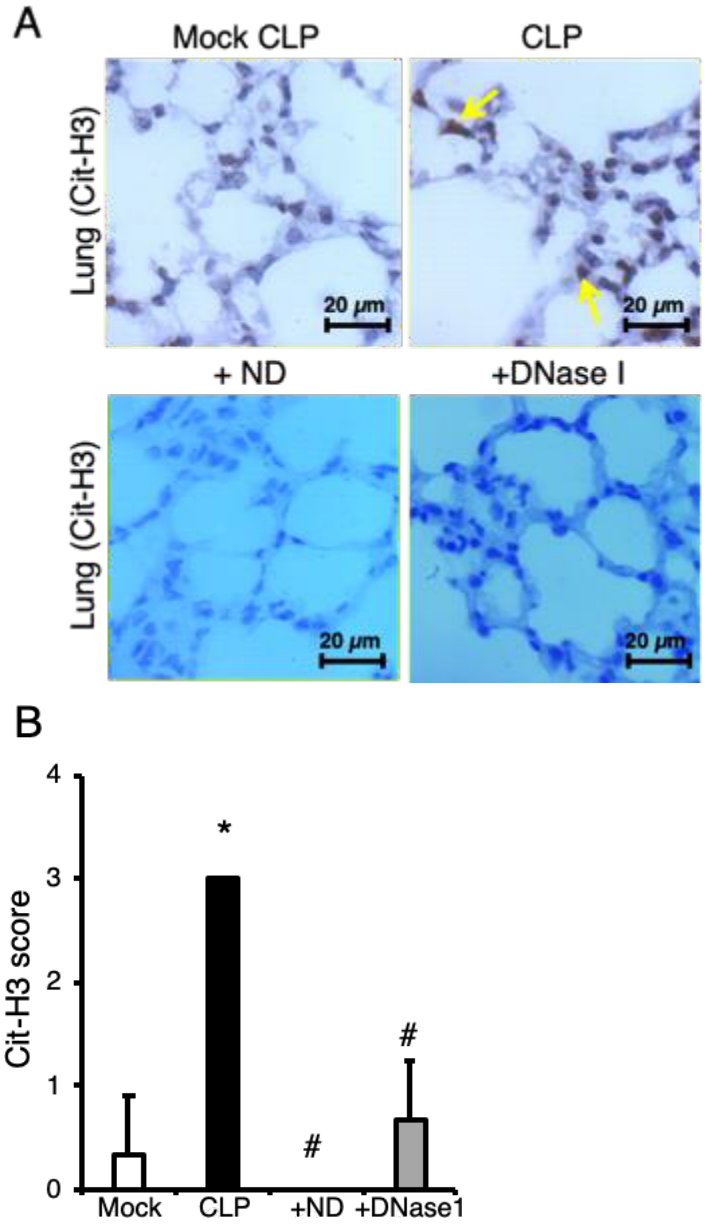


Figure 5.9 Excessive NETs can be eliminated by neutrophil depletion and DNase I treatment. (A) Representative images of lung sections stained with anti-Cit-H3 antibody (yellow arrows). Scale bar, 20 μM. **(B)** The levels of NETs release according to staining intensity are presented as means ± SD (n=3-6 per group). ND= neutrophil depletion. ANOVA test, *P<0.05 compared to mock group; $^{\#}$P<0.05 compared to CLP mice.

Figure 5.10

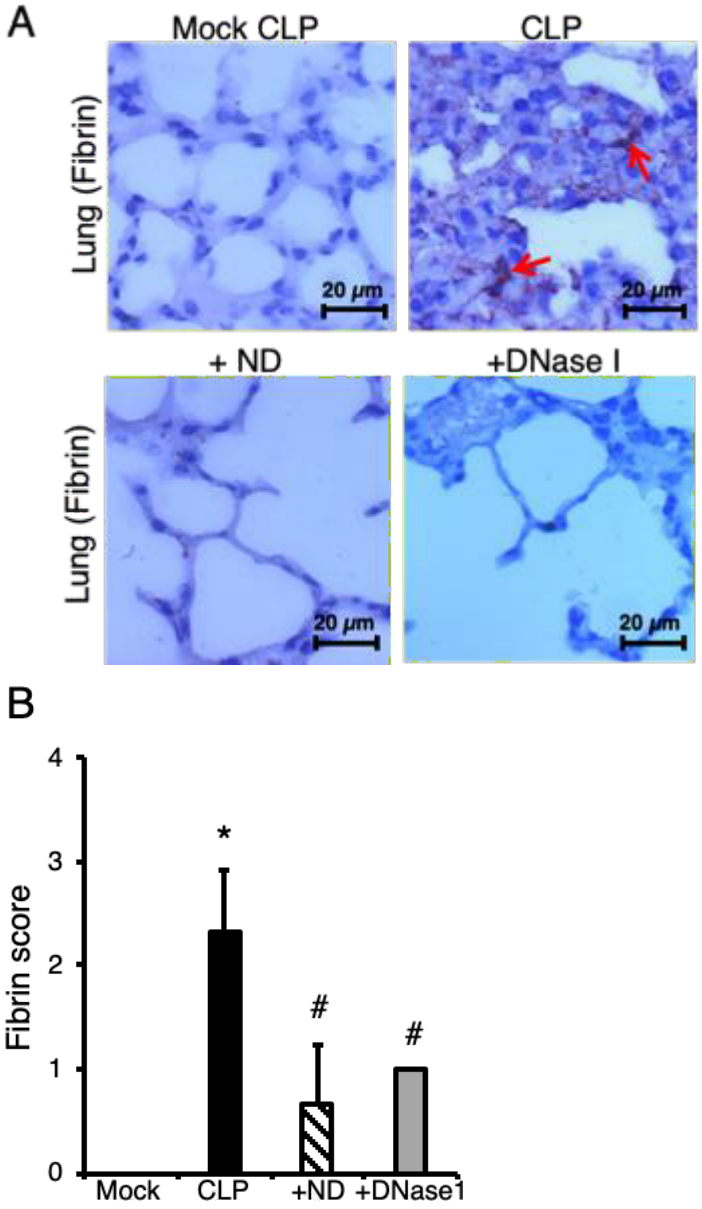


Figure 5.10 Fibrin deposition is decreased after digestion of NETs structures. (A) Lung sections stained with anti-fibrin antibody using IHC. Typical images are presented. Red arrows indicate positive anti-fibrin antibody staining. Scale bar, 20 μM. **(B)** The levels of fibrin deposition according to intensity of anti-fibrin antibody staining are presented as means ± SD (n=3-6 per group). ANOVA test, *P<0.05 compared to mock group; #P<0.05 compared to CLP mice.

5.2.4 NETs-fibrin deposition can promote organ injury in sepsis

NET-fibrin pathological structures can promote vascular occlusion and cause organ damage (328). Lung injury scoring was histologically examined and quantified as previously described (259). Briefly, lung injury scoring is typified by neutrophils recruitment to the alveolar and interstitial spaces, formation of hyaline membranes, proteinaceous debris in alveolar space and thickening of the alveolar wall. Typical histopathological changes and quantification of lung injury are shown in figure 5.11. CLP mice showed obvious histopathological changes that indicated the presence of lung injury, whereas sham CLP did not cause any discernible histopathological changes (Figure 5.11.A). In the neutrophil depletion and DNase I treatment groups, obvious improvements to Haematoxylin and Eosin (H&E) stained lung sections was observed (Figure 5.11.A), with sepsis-induced lung injury scores significantly reduced (Figure 5.11.B).

Upon examination of H&E-stained sections of the other major organs, including heart, liver and kidneys, no obvious pathological changes were observed (Figure 5.12). Alternatively, circulating injury markers were examined including, cardiac troponin (cTn) I for heart, alanine aminotransferase (ALT) for liver and blood urea nitrogen (BUN) for kidneys in the CLP model with neutrophil depletion. As shown in figure 5.13, liver and kidney injury showed no significant improvement, but cardiac injury, as indicated by cTnI levels, was significantly reduced by approximately 60%.

Neutrophil infiltration within the alveolar compartment and interstitial space constitute an integral component of the lung injury scoring system (259). Next, a modified lung injury score was used with the neutrophil component removed to confirm a true reduction in lung injury scores. Figure 5.14 demonstrates a significant reduction in sepsis-induced lung injury, even with removal of neutrophils from the lung injury

scoring. Based on these observations, neutrophils appear to be contributing directly to lung injury and indirectly to cardiac injury.

Figure 5.11

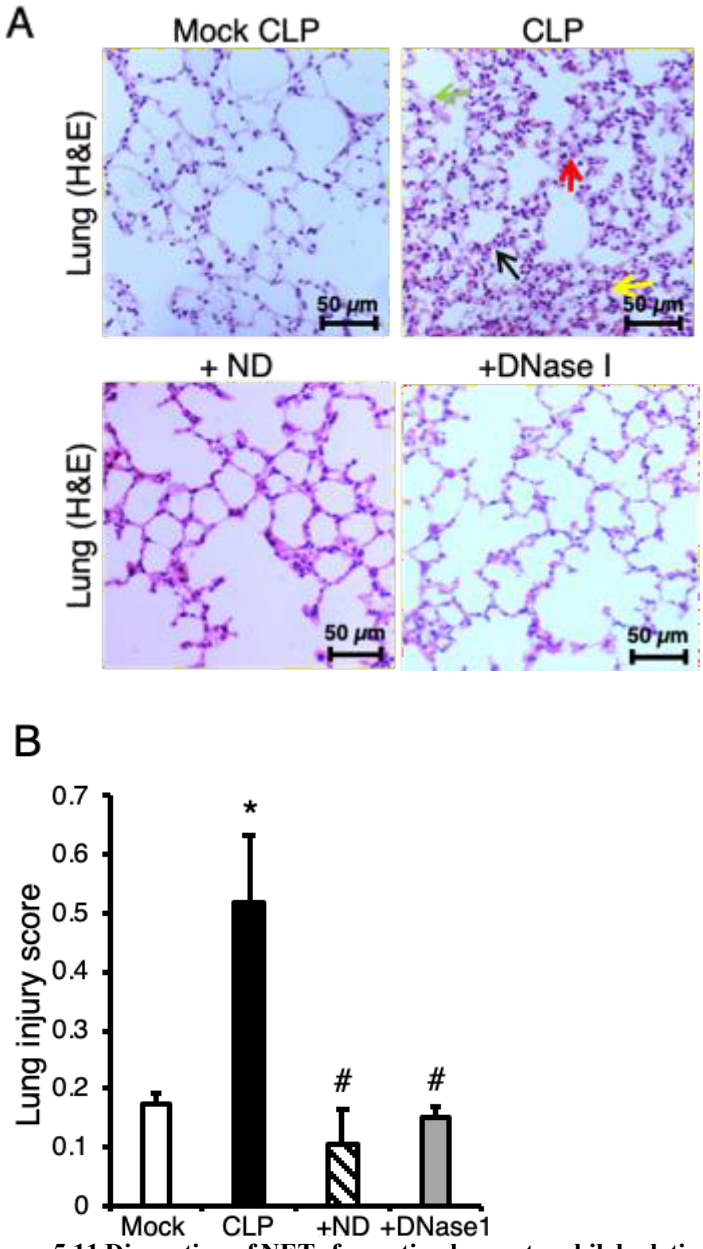


Figure 5.11 Disruption of NETs formation by neutrophil depletion and DNase I treatment directly attenuate lung injury in mouse model of sepsis. CLP model was induced in C57BL/6 malemice without (n=6) or with neutrophil depletion (n=6) by Ly6G-specific monoclonal antibody or with DNase I (n=3). Mock CLP group (n=6) served as control. **(A)** Typical images of H&E-stained lungs sections are presented. Red arrows indicate interstitial neutrophil, black arrows indicate membrane thickness, green arrows indicate proteinaceous debris, yellow arrows indicate hyaline membrane **(upper right panel)**. Scale bar, 50 μM. **(B)** Lung injury scores are presented as means ± SD, *P<0.05 comparedto mock group; #P<0.05 compared to CLP mice.

Figure 5.12

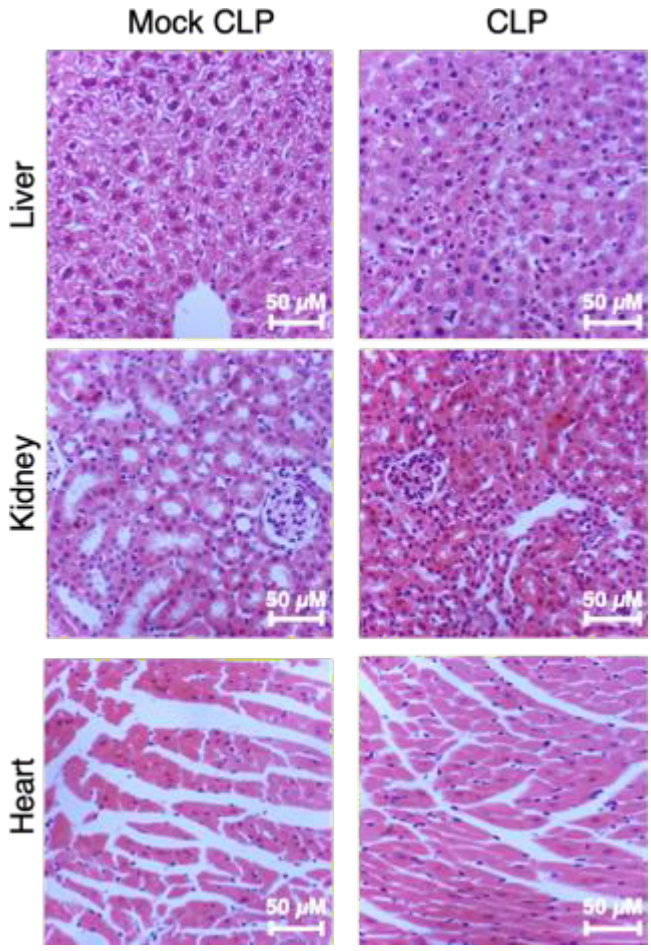


Figure 5.12 Histopathological examination of liver, kidney and heart tissues following sepsis and mock sepsis in mice. Tissue sections of CLP sepsis models along with mock controls were stained using H&E. Pathological changes to tissue morphology were not obvious in the liver, kidney and heart of the septic model compared to mock controls. Scale bar, 50 µm.

Figure 5.13

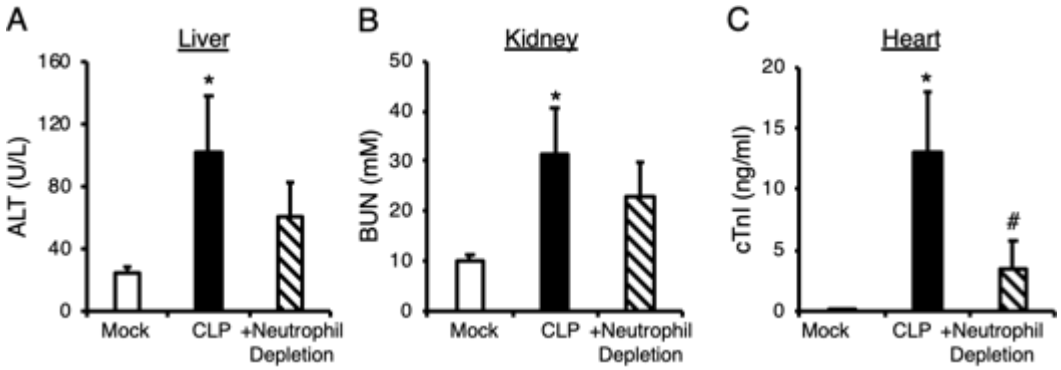


Figure 5.13 Neutrophil depletion reduced cardiac injury in septic mice. Disruption of NETs formation by neutrophil depletion indirectly causes significant protection against cardiac injury. Ten hours after sepsis induction by CLP, circulating markers of liver [ALT] **(A)**, kidney [BUN] **(B)** and cardiac [cTnI] **(C)** damage were quantified in plasma. Means ± SD are presented, *P<0.05 compared to mock group; $^{\#}$P<0.05 compared to CLP mice.

Figure 5.14

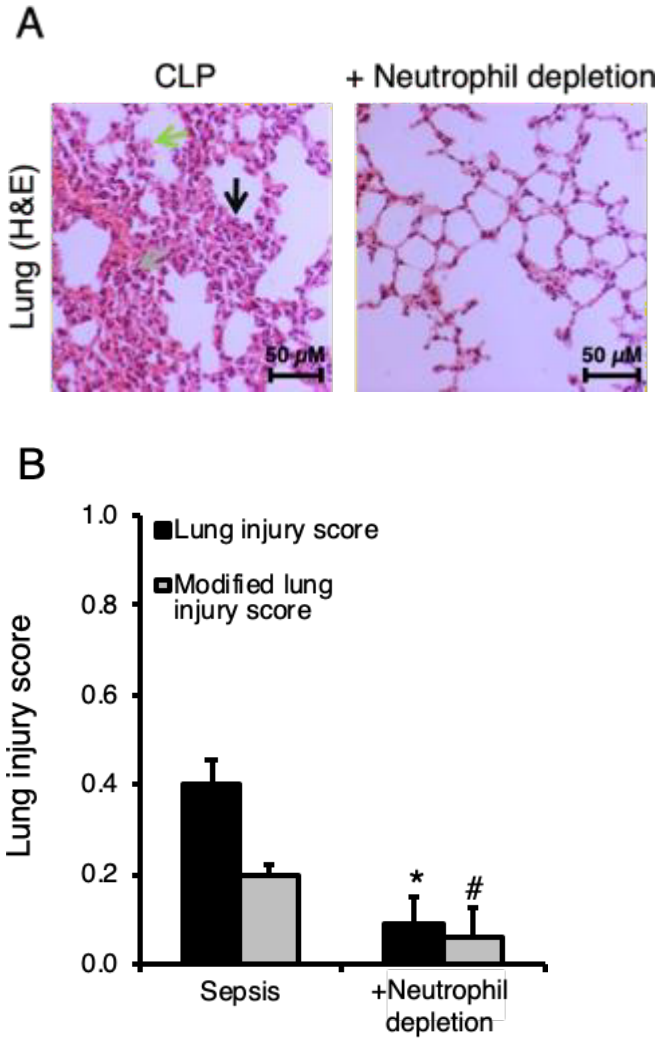


Figure 5.14 Neutrophil are integral components to mediate lung injury in sepsis. (A) Representative images of H&E-stained lung sections of CLP-induced sepsis without or with neutrophil depletion. There is increased cellularity predominated by neutrophils **(left panel)**. Arrows: red= interstitial neutrophil, black= membrane thickness, green= proteinaceous debris. Scale bar, 50 μm. **(B)** A significant increase in lung injury scoring, which was reduced after neutrophil depletion with Ly6G-specific monoclonal antibody 1A8. The modified lung injury scoring excluded only assessed the following criteria; formation of hyaline membranes, proteinaceous debris and alveolar wall thickening. Modified lung injury scoring was reduced in sepsis without and with neutrophil depletion. Means ± SD are presented. *P<0.05 compared to original lung injury score in sepsis group; $^{\#}$P<0.05 compared to compared to modified lung injury score in sepsis group.

5.3 Discussion

In the previous chapters, it is shown that NETs formation can be induced *ex vivo* using plasma or sera from patients with sepsis, but it was not feasible to determine if this assay directly reflect the extent of NETs formation within the organs. In this chapter, it was demonstrated that the degree of *ex vivo* NETs formation correlate with the level of NETs formation *in vivo*, particularly within the lungs of two mouse models of sepsis. A significant association between the levels of NETs formation and fibrin deposition within the lungs was also reported in this study, with injury to the lungs, heart, liver and kidneys, is markedly increased following CLP-induced sepsis. The levels of NETs and fibrin deposition within the lungs are significantly reduced after neutrophil depletion and DNase I treatment. This was accompanied with reduced injury to the lungs and heart after neutrophil depletion.

Earlier studies investigating NETs in clinical settings have used cfDNA and neutrophil-derived proteins such as MPO to quantitatively analyse NETs formation in patients with sepsis (329-331). However, the reliability of these methodological approaches are questionable (243-245), as cfDNA from other types of cell death (e.g. genomic and mitochondrial DNA) and NETs degradation enzymes (e.g. DNase I) are factors involved in altering the stability of NETs-derived products in fluid samples such as plasm and serum (332).

In this context, the *ex vivo* NETs formation assay directly measures the NETs-forming capacity of patients (chapter 3 and 4) and mice plasma led us to speculate that this assay may also reflect NETs formation within the tissues of septic mice, considering factors presenting in plasma may also be present in the highly vascularised lungs. In the correlation analysis, we found that NETs formation induced *ex vivo* were strongly correlated to *in vivo* NETs formation within the lungs of septic mice. This is suggestive

of the potential value of the *ex vivo* NETs formation assay in its ability to inform the degree of NETs release within the vasculature.

In sepsis, excessive neutrophil accumulation within the vasculature is linked to the inflammatory response in sepsis, with the release of NETs and NETs-derived proteins within the organs are implicated in experimental sepsis (311). Current literature reported that NETs formation is mainly observed within highly vascularised organs (163, 194-196, 198). Similarly, this study provides evidence of NETs release, particularly within the lungs, which was validated in two mice models, CLP and i.p *E. coli*-induced sepsis. Still, there are reports that widespread NETs formation is observed within the liver, mainly in bacterial LPS models (163, 196, 198).

This inconsistence may be due to different aetiologies. Models of LPS challenge are of scientific controversy over its relevance to human sepsis as it has inherent limitations (314, 333). In fact, LPS challenge is regarded as an endotoxic shock model but not sepsis, as it lacks the source of microbial infection and neglects the host-pathogen interaction of polymicrobial sepsis (314, 333, 334). Furthermore, the dose-dependent spectrum is often recognised in murine models of LPS challenge (314, 333). In this respect, it has previously been shown that LPS dose-dependently induce NETs release *in vitro* (0.1-25 μg/ml) (335), whereas in the *in vivo* studies, the dose of LPS ismarkedly varying (25μg/kg–20 mg/kg) (163, 196, 198). Although many species including mice are less sensitive to bacterial LPS, the use of high dose of LPS (1-25mg/kg) in non-human primates is argued (336). Compared with human, this dose is1000- fold to 10 000-fold greater than the dose that is measured in critical illness, and1000 000 times greater than the dose (2– 4 ng/kg) measured during mild illness such as fever (336). This inconsistency could be also related to the time in which blood andorgans were collected, which represent a major factor in disease progression. In

this regard, Tanaka *et al*. showed that NETs were generated 24 hours following LPS challenge, whereas the maximum time point in our study was 10 hours in both models of sepsis (198). Perhaps these explanations accentuate my findings that *in vivo* NETs are found mainly within the lung, which are also supported by prior experimental studies of CLP and *E. coli*-induced sepsis (194-196), as well as previous findings (chapter 4) that septic patients with respiratory origin had the highest level of NETs.

NETs-fibrin interaction can aggravate organ injury in sepsis by providing a scaffold for platelets entrapment leading to blood clots formation and microvascular occlusion (327). In this chapter, the pathological role of NETs in organ injury was studied by comparing CLP model to CLP model with neutrophil depleted mice using Ly6G-specific monoclonal antibody. Increased *in vivo* NETs and procoagulant responses are associated with lung and cardiac injury. Septic mice with high levels of *in vivo* NETs formation have significant fibrin deposition in the lungs, which was significantly reduced after neutrophil depletion and DNase I treatment. The association established from this translational study was in favour of neutrophils infiltration was the major sources of excessive NETs release that mediated lung and cardiac injury. These observations are supported by previous findings (chapter 4) that NETs were associated with organ injury in critically ill patients, demonstrated by significant association between *ex vivo* NETs and sequential organ failure assessment (SOFA) scores. Together, NETs-fibrin deposition are critical contributors to microvascular impairment in sepsis.

Chapter 6: CXCL2 is a major driving factor of NETs formation in animal models of sepsis

6.1 Introduction

Sepsis remains a substantial cause of mortality in critical care patients (10, 13). As is known, the initial, appropriate host immune responses to infection exceed containment and becomes substantially altered (337, 338). The innate immune response during the early phase of sepsis is elicited by pathogen-associated molecular pattern molecules (PAMPs) and damage-associated molecular patterns (DAMPs) that bind to pattern recognition receptors (PRRs) on specific immune cells, facilitating an acute inflammatory response that is mainly driven by inflammatory cytokines (92, 339).

Cytokines are the general term used to describe small signaling molecules (8 – 12 kDa), which include interleukins (IL), tumour necrosis factor (TNF) and interferons (IFN) (340). Chemokines are a family of cytokines which play a pivotal role in attracting immune cells in a process known as chemotaxis (340). In general, the chemokine family comprises of large number of ligands that bind to functional signaling receptors, which are distributed on the cell surface (341). Cellular activation and recruitment are mainly driven by chemokine ligands via chemokine receptors. There two major structural groups of the chemokine receptors including, cysteine – cysteine (CC) and cysteine–X amino acid–cysteine (C-X-C) (340).

The inflammatory cascade in sepsis is dysregulated due to intrinsic mechanisms associated with disease pathology (100). An excess of pro-inflammatory cytokines co-exist with a significant escalation in the innate immune response (342). The proinflammatory chemokine IL-8, also known as CXCL8, is a potent C-X-C chemoattractant produced by various tissues and blood cells, with distinct target specificity for neutrophils (343). IL-8 binds with high affinity to two 7-

transmembrane–domain of the G protein–coupled receptors, CXCR1 and CXCR2 (344, 345).

The CXC chemokine actions in septic response have traditionally been explained in relation to their direct chemotactic effects on leukocytes. Still, much less is known about how the specific G protein-coupled receptors that bind to chemokines exert effects on neutrophil function during sepsis. Neutrophils can be activated to release neutrophil extracellular traps (NETs) in response to chemicals, pathogens, host factors e.g. histones and cytokines (78, 140, 249). Previous data has shown that CXCR2, but not CXCR1, mediates NETs formation *in vitro* following stimulation of healthy neutrophils with IL-8 (346). Recently, interesting observations have emerged from clinical studies suggesting that CXCR2 regulate NETs formation in chronic obstructive pulmonary disease (COPD) and cancer patients (346, 347).

The CXC chemokines CXCL1/keratinocyte-derived chemokine (KC), CXCL2/macrophage inflammatory protein-2 (MIP-2) and CXCL5-6/Lipopolysaccharide (LPS)-induced chemokine (LIX) are regarded as functional homologues of IL-8 in mice (348). They can activate neutrophils by binding to their surface CXCR2 only (348). Furthermore, these chemokines are similarly elevated during infection in murine models (349, 350). CXCR2 is critically involved in immunomodulation of cecal ligation and puncture (CLP)-induced sepsis by enhancing neutrophil recruitment to the site of infection, which can be delayed by inhibiting CXCR2 function (351). Recent observations from CLP murine model suggest excessive NETs formation had the capacity to stimulate CXCL1-2 release in plasma and lungs (195). However, the impact of CXCL2-CXCR2 loss on NETs formation has not been previously exploredin sepsis.

Neutrophils also have an essential role in the interplay between immune system and thrombosis, also called immunothrombosis (352), which is implicated as independent defense mechanism through coagulation activation and recruitment of inflammatory cells to eliminate pathogens. However, high risk thrombosis formation is commonly observed in sepsis due to over-activation of coagulation cascade, which in severe cases may result in disseminated intravascular coagulation (DIC) and subsequently organ injury and death. Existing evidence has showed that thrombosis formation is highly dependent on leukocytes, with the level of immunothrombosis response, demonstrated by platelet-neutrophil aggregation, correlating with the degree of organ injury (353, 354). This process is mediated by CXCR1/2 signaling, which has the capacity to promote leukocyte migration through thrombi (355). Neutrophils also appear to mediate thrombotic events through NETosis (113, 295). In experimental models of deepvenous thrombosis (DVT), CXCR2 deficiency resulted in decreased levels of NETs inthrombi, which led to reduced frequency and size of thrombi (356).

Many potential treatments for sepsis have not fared well over the past few decades, with more than one-hundred clinical trials have examined putative treatments, but eventually failed to improve survival in sepsis (100). Therapeutic approaches currently aim to provide supportive interventions (e.g. fluid resuscitation and mechanicalventilation) at each step during disease progression (357). The lack of fundamental knowledge on the complex pathophysiology of sepsis revolves around the difficulty of designing effective drugs to treat sepsis and reduce mortality.

Many recent therapeutic inhibitors have been developed to target IL-8-CXCR1/2 such as, reparixin, AZD5069, SCH527123 and SB225002, in an attempt to reduce dysregulated inflammatory response and the associated pathology in various diseases (358-361). Reparixin, a small allosteric antagonist, which mainly prevents

downstream signal transduction to inhibit neutrophil recruitment by blocking the transmembrane domain of CXCR1/2 (358). The application of reparixin has been widely explored in animal models of peritonitis (358), LPS and acid-induced lung injury (362), ischemia-reperfusion injury (363, 364), breast cancer (365), neuropathic pain (366) and transplantation studies (367-369).

In the previous chapter, inhibiting neutrophils function by depleting neutrophils or digesting NETs structures could significantly reduce *in vivo* NETs formation. In addition, lung and cardiac injury were protected in a murine model of sepsis with neutrophil depletion. However, neutrophil depletion is not a viable option in the clinical setting. Thus, we planned to follow a more logical and clinically relevant context based on: (1) the fact that preclinical studies on sepsis should be investigated with pathogens presented to mimic clinical sepsis; (2) previous observation (chapter 4) that IL-8 couldbe a potential driving factor of NETs formation.

In chapter 4, strong and positive association has been established between the levels of NETs and the proinflammatory cascade, specifically IL-8, in critically ill patients. In chapter 5, excessive neutrophil recruitment was significantly associated with NETs formation and subsequent injury to the lungs and heart in experimental sepsis. Taken into consideration that the *ex vivo* NETs-forming capacity of critically ill patients was reduced (Chapter 4) after blocking the specific receptors of IL-8 (CXCR1/2), we hypothesized that CXCR2 signaling is important in driving NETs formation and subsequent pathological changes in experimental sepsis. Accordingly, in this chapter the effects of the CXCR1/2 inhibitor – reparixin were examined in two mouse models of sepsis including CLP and intraperitoneal (i.p) *Escherichia coli* (*E. coli*).

6.2 Results

6.2.1 CLP and E. coli- induced sepsis causes dramatic increase in CXCL2 levelsthat is sufficient to trigger NETs formation

In this study, two murine models of sepsis were investigated over time, CLP (n= 3 per timepoint; 4,6 and 10 hours) and *E. coli* (n=3 per timepoint; 4,6 and 10 hours), along with mock controls (n=3 per model per timepoint; 4,6 and 10 hours) to include sham CLP surgical procedure and i.p injection of saline, respectively.

As clarified in chapter 4, the major mediator of NETs formation in patients with sepsis is IL-8. Since CXCL2 is the major murine homologue of human IL-8 (348), the levels of CXCL2 were measured in the plasma from both sepsis mouse models by enzyme-linked immunosorbent assay (ELISA). CXCL2 levels were dramatically peaked at 4 hours post CLP-induced (Mean ±SD: 6219.97 ±1513.43 pg/ml) and *E. coli*-induced (8378.04 ±276.20 pg/ml) sepsis (Figure 6.1). A similar elevation was also observed at6 and 10 hours in both models. These levels were significantly higher compared withthose of the controls at 4 hours, mock CLP (21.40 ±18.89, P=0.015) and i.p injection with saline (7.92 ±4.64 pg/ml, P<0.001), indicating that the sham surgical procedure and i.p injection of saline did not trigger a pro-inflammatory response in both mock models.

Next, we tested whether the pathological levels of mouse CXCL2 could induce *in vitro* NETs formation of mouse neutrophils. To mimic physiological conditions, normal mouse plasma was supplemented with pathologically relevant concentrations of CXCL2/MIP-2 (8000 pg/ml) (Figure 6.2A and B) and clinically relevant IL-8 levels (100 pg/ml), as shown previously in chapter 3. Typical NETs structures were induced by both CXCL2 and IL-8 (Figure 6.2C and D).

Figure 6.1

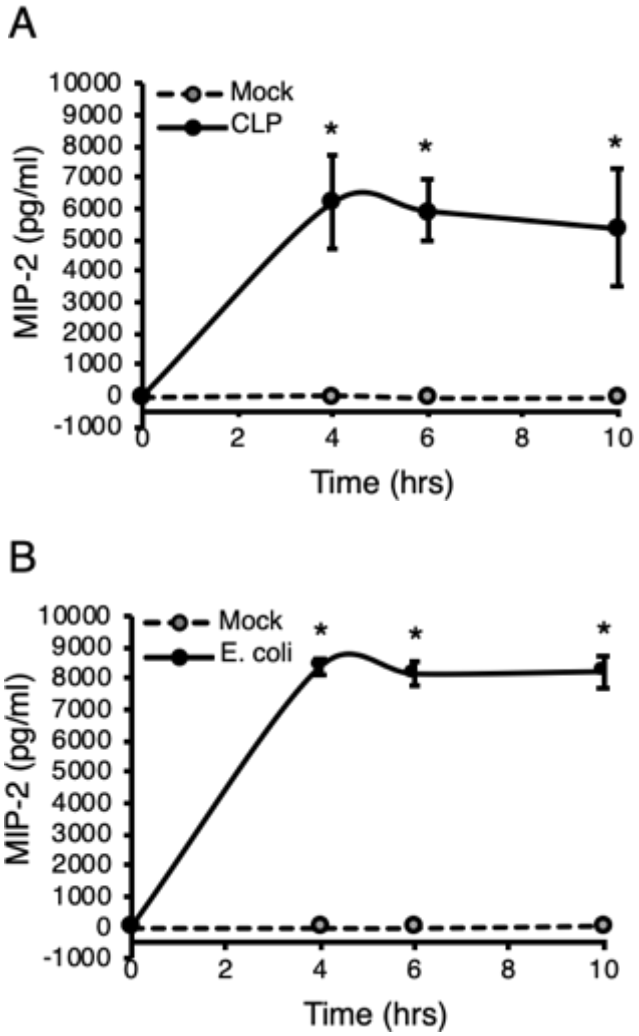


Figure 6.1 Levels of MIP-2 are significantly elevated following sepsis induction in mice. C57BL/6 male mice were anesthetized and sepsis was induced by either CLP or i.p *E. coil*. Mock CLP and i.p saline were used as controls. Blood was collected at 4, 6 and 10 hours. MIP-2 levels were quantified using ELISA in the mice circulation of both CLP **(A)** (n=9) *E. coli* **(B)** (n=9) sepsis and compared to mock controls (Mock CLP; n=9, Mock *E. coli*; n=9). Means ± SD are presented over the time course of the experiment. *P<0.05 increase in circulating MIP-2 compared to mock controls.

Figure 6.2

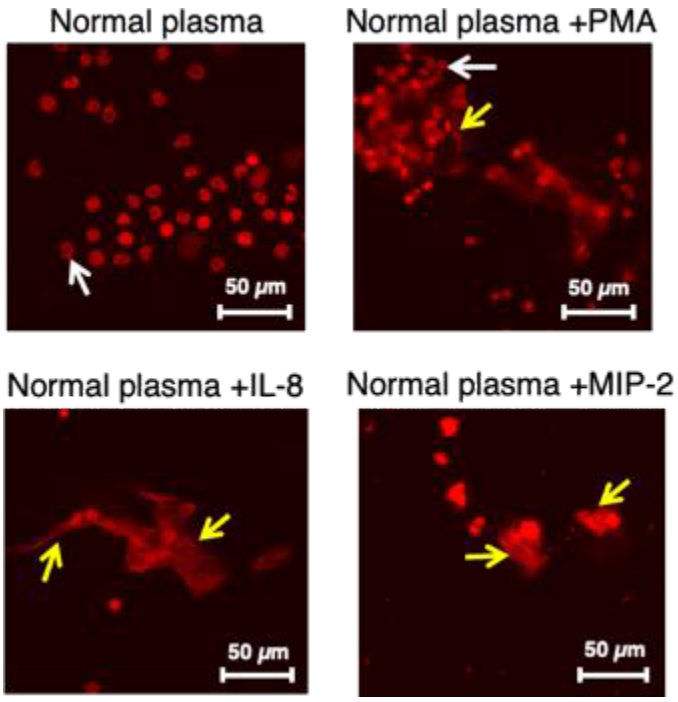


Figure 6.2 NETs formation can be induced by IL-8 and MIP-2. Representative fluorescence microscopy images showing NETs release after normal mice neutrophils were incubated for 4 hours with either, normal mice plasma alone, or supplemented with phorbol 12-myristate 13-acetate (PMA) (100nM), IL-8 (100 pg/ml) or MIP-2(8000pg/ml). Cells were then fixed with 2% paraformaldehyde (PFA) and extracellular DNA release was stained with 10µg/ml Propidium Iodide (PI). Scale bar, 50 µM.

6.2.2 Ex vivo NETs formation correlates with CXCL2 levels in septic mice

In chapter 4, *ex vivo* NETs formation correlates positively with the levels of IL-8 in critically ill patients. Furthermore, widespread *ex vivo* NETs release was observed following CLP and *E. coli*-induced sepsis at 4 hours (Chapter 5), which remained elevated throughout the study duration (10 hours). Herein, CXCL2 levels also demonstrated positive correlation with the levels of *ex vivo* NETs formation in both CLP-induced sepsis ($n=18$, $R= 0.902$; $P<0.001$) and *E. coli*-induced sepsis ($n=18$, $R= 0.904$; $P<0.001$).

6.2.3 Targeting CXCL2-CXCR2 using reparixin attenuates NETs-fibrin formation in septic mice

To demonstrate the role of IL-8 in neutrophil activation, and in particular on NETs formation, reparixin, an allosteric antagonist, was used to specifically block CXCR1/2 on neutrophils. As shown in figure 6.3, plasma from CLP and *E. coli*-induced sepsis was incubated with healthy mice neutrophils, which could directly induce NETs formation. However, when this plasma was spiked with reparixin (250ug/ml) NETs formation was clearly reduced. These observations indicate that CXCR2 signalling is essential to trigger NETs release in sepsis.

To investigate whether the inhibition of CXCR2 could abrogate NETs formation in CLP and *E. coli* mouse models of sepsis, mice were treated without and with 20/mg/kg/dose of reparixin at 1h before, and 2h, 4h after sepsis induction. Septic mouse treated with reparixin had decreased levelsof *ex vivo* NETs formation (Figure 6.4). Consistently, mice injected with reparixin significantly attenuated neutrophils capacity to produce NETs in both models. Similareffects were observed in *in vivo* NETs formation (Figure 6.5A and B). To establish that the reduced staining intensity is indeed due to specific inhibition of NETsformation, the proportion of NETosis formed cells was calculated from total neutrophils. I found that the percentage of NETs forming neutrophils was significantly reduced within the lungs by reparixin treatment (Figure 6.6).

As demonstrate in chapter 5, NETs and fibrin deposition are uniquely identified within the lungs of septic mice. These structures were reduced after neutrophil depletion and DNase I treatment. To further address whether neutrophil CXCR2 signaling is required in this process, the impact of reparixin on fibrin formation was

assessed in both models of sepsis. After 10 hours of induction, the septic mice treated with saline showed abnormal formation of fibrin within the lung vasculature, which was abrogatedin septic mice treated with reparixin (Figure 6.7A and B).

Figure 6.3

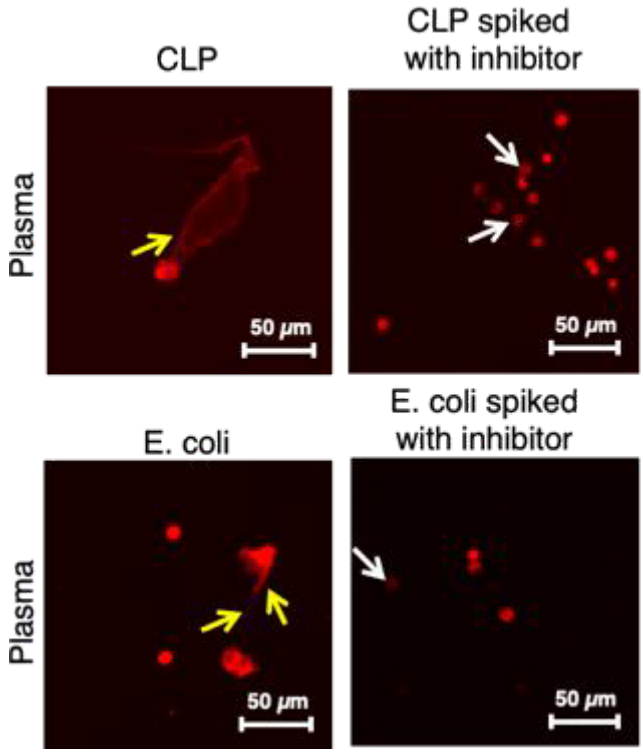


Figure 6.3 Supplementation of reparixin reduces septic mice plasma induced NETs formation. Normal mouse neutrophils were isolated from whole blood and incubated with septic mouse plasma without or with 250µg/ml reparixin (spiked with reparixin). Typical images are shown. Arrows: normal neutrophils= white, NETs= yellow. Scale bars, 50 µM.

Figure 6.4

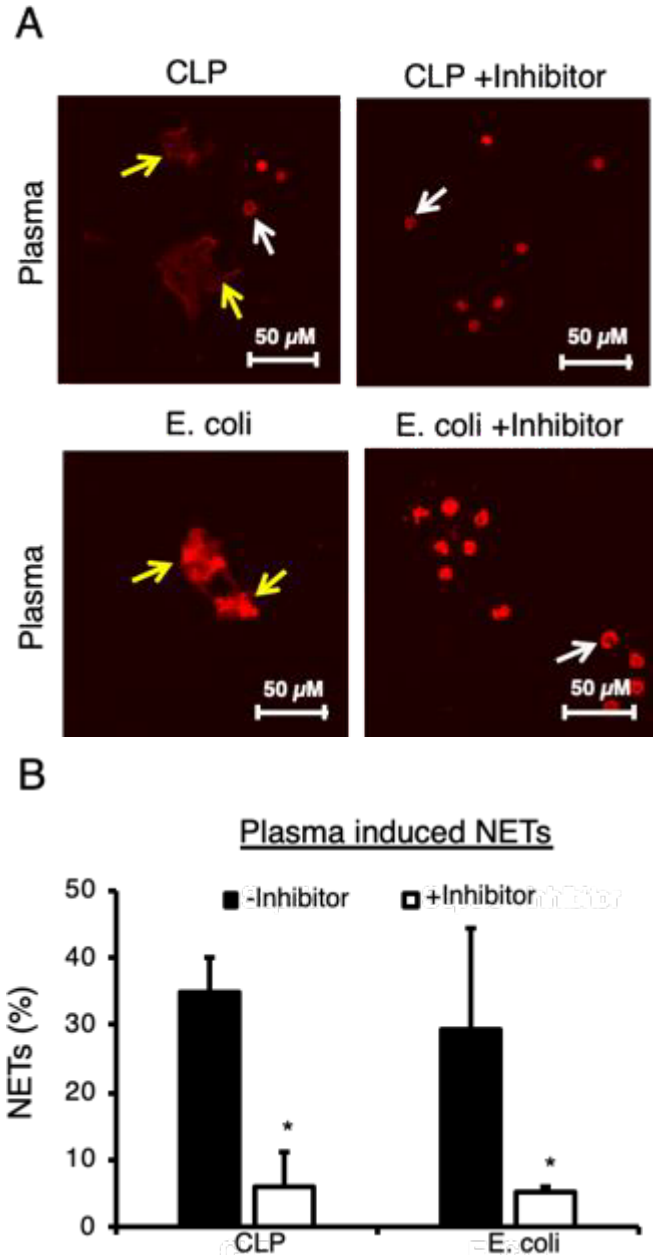


Figure 6.4 Blocking CXCL2-CXCR2 in septic mice significantly reduced *ex vivo* NETs-forming capacity. Sepsis was induced in C57BL/6 male mice and treated with reparixin or saline as positive control (6 mice per group). Blood was collected from healthy mice and neutrophils were isolated and treated with individual mouse plasma for 4 hours. **(A)** Typical images of DNA structures for quantification of *ex vivo* NETs formation is presented. Yellow arrows indicate NETs; white arrows indicate normal neutrophils. Scale bar, 50 μM. **(B)** quantification of *ex vivo* NETs formation is presented. *P<0.05 Mann-Whitney test, compared septic model without (-inhibitor) to with reparixin treatment (+inhibitor).

Figure 6.5

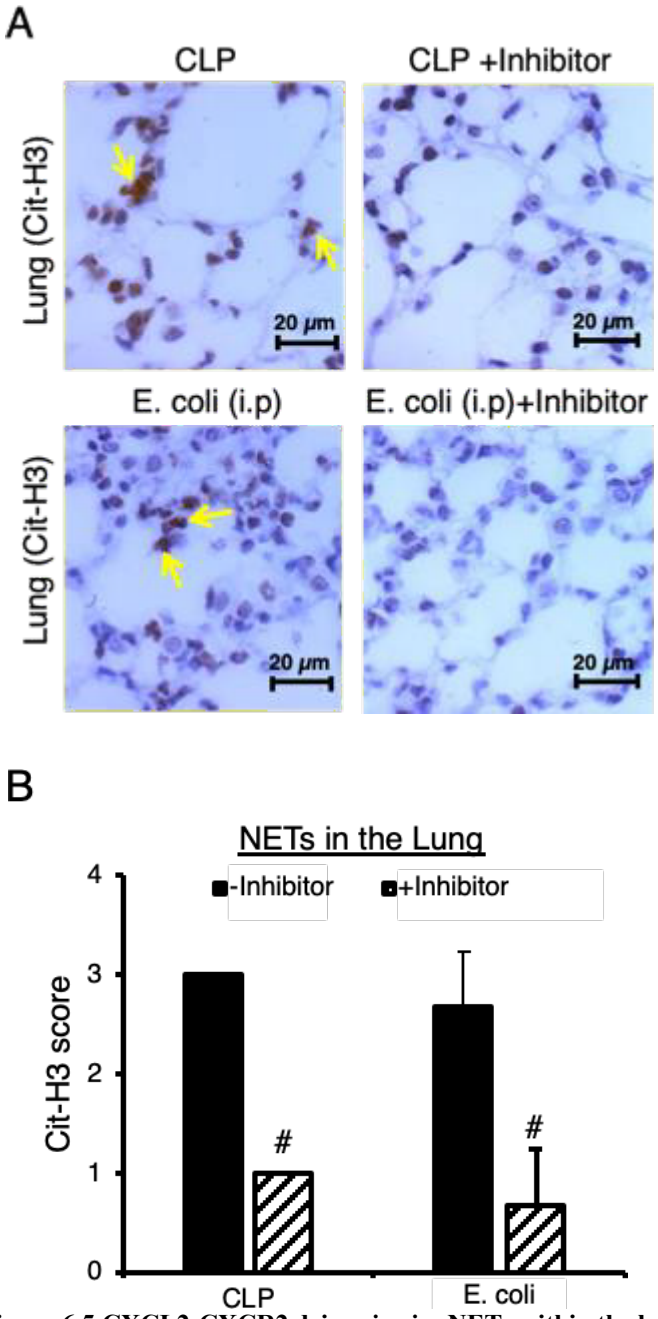


Figure 6.5 CXCL2-CXCR2 drives *in vivo* NETs within the lungs of septic mice. Sepsis was induced in C57BL/6 male mice and treated with reparixin or saline as positive control (6 mice per group). Tissues were collected 10 hours following sepsis induction. Tissue sections were stained using IHC as previously described in materials and methods **(A)** typical images of anti-Cit-H3 staining of lung sections for quantification of *in vivo* NETs formation are presented. Yellow arrows indicate NETs. Scale bar, 20 µM. **(B)** quantification of *in vivo* NETs formation. $^{\#}P<0.05$ Mann-Whitney test, compared septic model without (-inhibitor) to with reparixin treatment (+inhibitor).

Figure 6.6

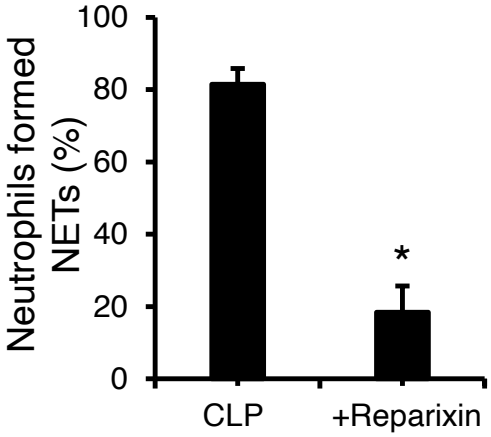


Figure 6.6 Reparixin treatment significantly reduced the percentage of neutrophils that formed NETs within the lungs of septic mice. The tissue sections from 10 hours CLP mice without and with reparixin were stained with anti-Cit-H3 antibody. The total number of neutrophils positive and negative to Cit-H3 staining was counted per microscopic field. Neutrophils formed NETs were calculated as a proportion of total neutrophil counts. Data are presented as means ±SD. *P< 0.05 vs. CLP, n = 3 per group.

Figure 6.7

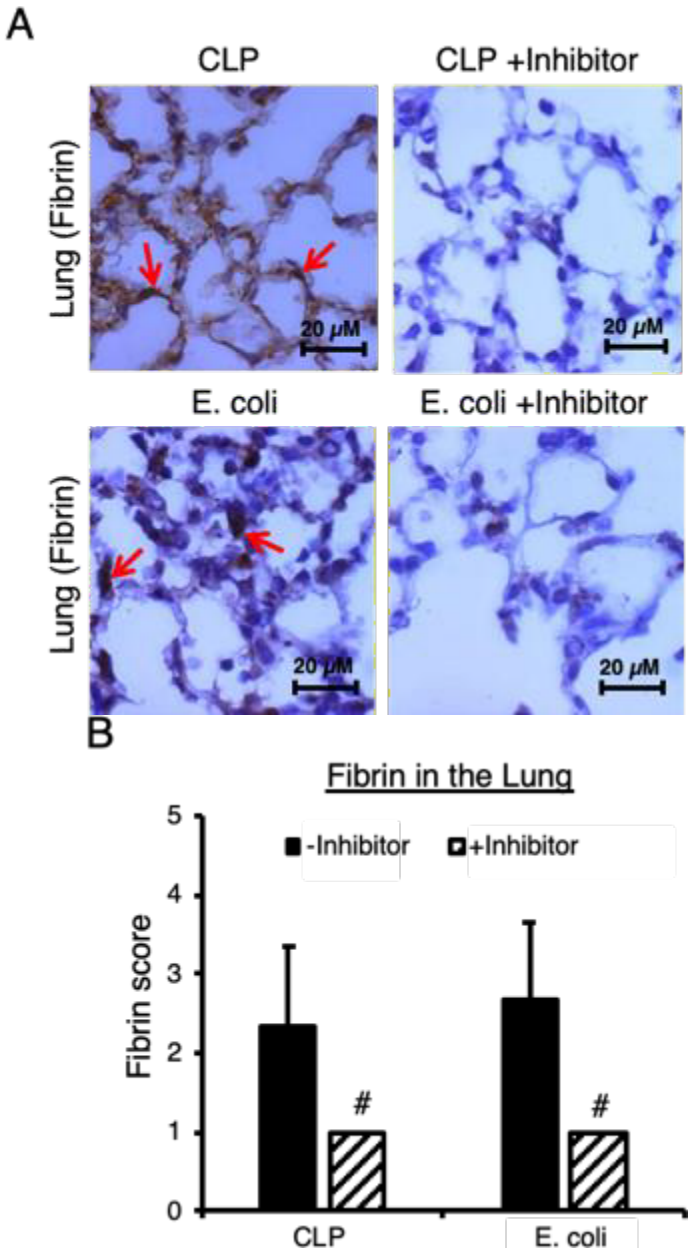


Figure 6.7 CXCL2-CXCR2 signalling drives *in vivo* fibrin deposition within the lungs of septic mice. (A) typical images anti-fibrin staining of lung sections for quantification are for quantification of *in vivo* fibrin formation are presented. Red arrows indicate positive fibrin formation. Scale bar, 20 μM. **(B)** quantification of *in vivo* fibrin formation. IHC scoring was based on intensity of positive staining to anti-fibrin antibody. $^{\#}P<0.05$ Mann-Whitney test, compared septic model without (-Inhibitor) and with reparixin treatment (+inhibitor).

6.2.4 Blocking CXCR1/2 signalling attenuates organ injury in septic mice

In chapter 4, plasma from septic patients with respiratory origin demonstrated tendency toward Moderate to strong NETs-forming capacity. Consistently, the formation of NETosis *in vivo* mostly within the lung's vasculature results in providing a scaffold for fibrin deposition, which directly augments lung injury (Chapter 5). Thus, neutrophils are seen as important elements in initiating this process.

In CLP model, the lungs became dark red colour and the appearances were much improved following reparixin treatment (Figure 6.8). Lung injury scores were significantly reduced in both CLP (from 0.52 ± 0.12 to 0.32 ±0.02) and similar findings were observed from *E. coli*-induced sepsis (from 0.50 ±0.15 to 0.22 ±0.05) ($P<0.05$, Figure 6.9). Furthermore, reparixin significantly reduced the levels of other major organ injury markers: alanine aminotransferase (ALT) in CLP (from 101.83 ±37.84 to 52.33 ±12.78units) and *E. coli* (from 176.50 ±25.33 to 121.60 ±24.47); blood urea nitrogen (BUN)in CLP (from 31.46 ±9.27 to 15.95 ±5.69) and *E. coli* (from 41.19 ±6.58 to 25.88 ±4.64); cardiac troponin (cTn) I in CLP (from 13.05 ±4.96 to 1.96 ±1.34) and *E. coli* (from 18.44 ±4.63 to 7.69 ±4.41) (Figures 6.10 and 6.11, $P<0.05$).

Figure 6.8

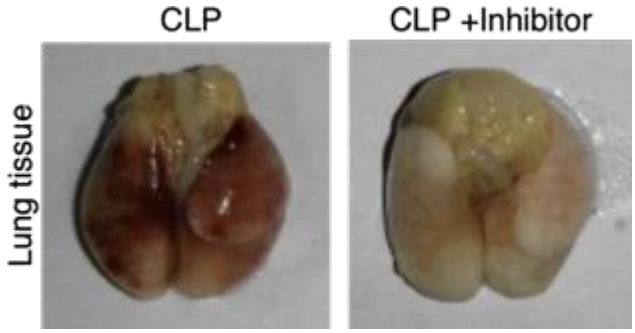


Figure 6.8 Improved overall lung appearance following reparixin treatment. Lung appearance 10 hours after CLP (CLP) and CLP treated with reparixin (CLP +Inhibitor).

Figure 6.9

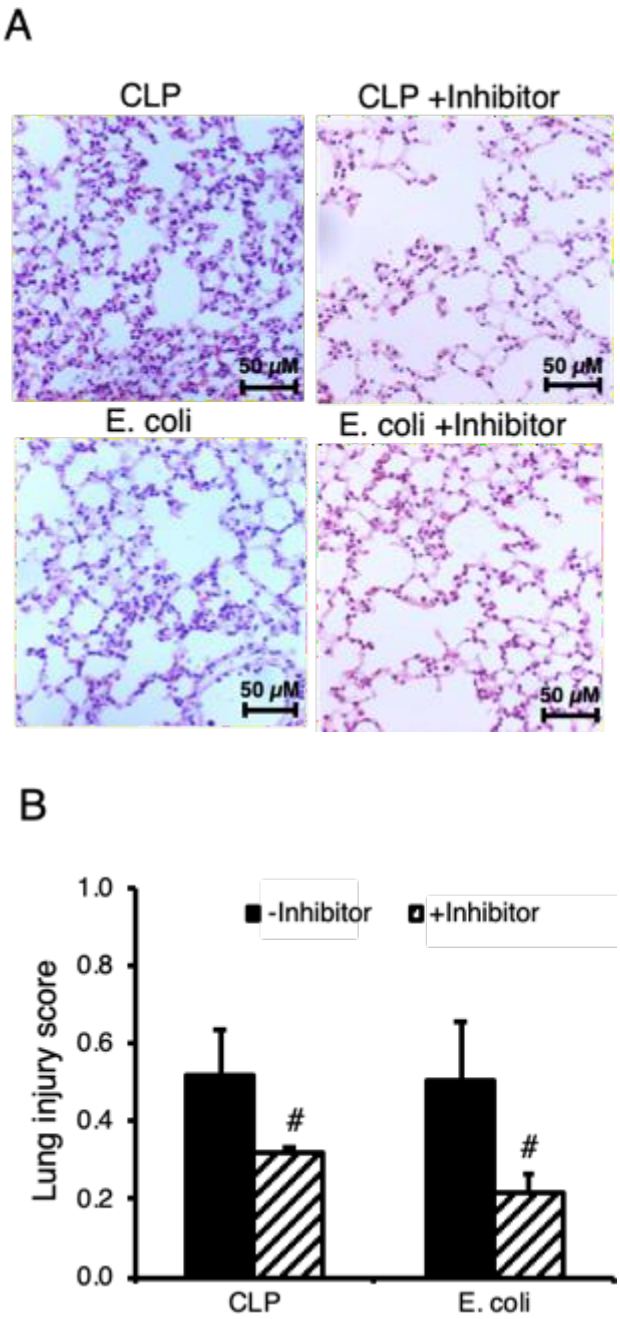


Figure 6.9 Blocking CXCR2 reduced lung injury in CLP and *E. coli*-induced sepsis. (A) Typical images of H&E-stained lung sections 10 hours after sepsis and sepsis with reparixin treatment (+Inhibitor). Scale bar, 50 μM. **(B)** quantification of lung injury scores of mock sepsis and septic mice treated with saline or reparixin. Lung injury scores were calculated as previously described in material methods. Data are presented as means ± SD. $^{\#}P< 0.05$ vs. CLP, n = 3 per group.

Figure 6.10

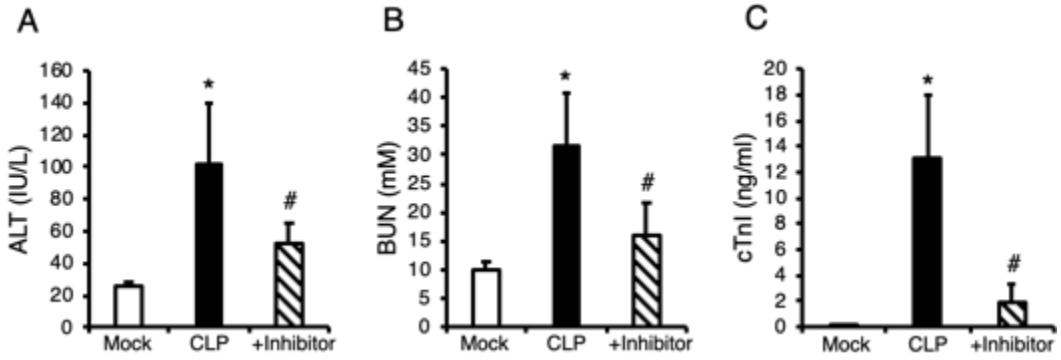


Figure 6.10 Protective effects of reparixin from multiple organ injury in CLP-induced sepsis. Organ injury markers of liver, kidney and heart of CLP-induced sepsis. Levels of ALT **(A)**, BUN **(B)** and cTnI **(C)** in the plasma were determined 10 hours following CLP induction. Mice underwent CLP or the similar surgical operation but without ligation and puncture of the cecum (Mock). CLP mice were treated with i.p injections of the reparixin (Inhibitor). Data are presented as means ± SD. *P< 0.05 vs. Mock and #P< 0.05 vs. CLP, n=6 per group.

Figure 6.11

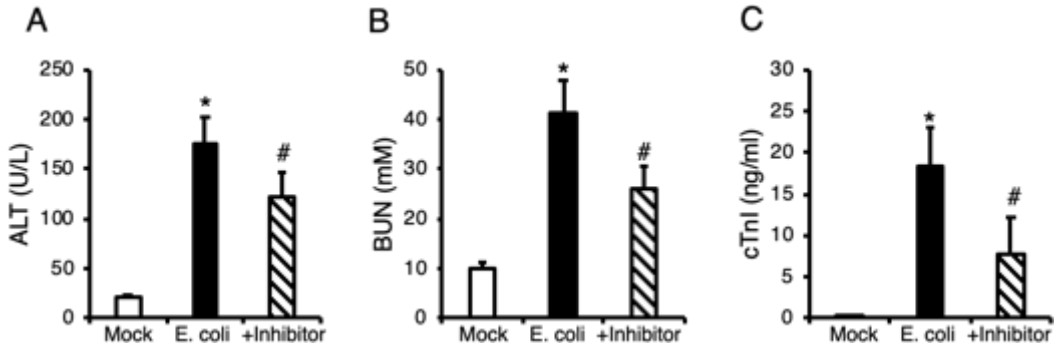


Figure 6.11 Organ injury markers in *E. coli*-induced sepsis are attenuated after blocking CXCR2. Mice received i.p. injection of saline (Mock) or *E. coli* without or with intraperitoneal injections of the reparixin (Inhibitor). Plasma levels of ALT for liver injury **(A)**, BUN for kidney injury **(B)** and cTnI for cardiac injury **(C)** were determined 10 hours after *E. coli* i.p. injection or saline (Mock). Data are presented as means ± SD. *P< 0.05 vs. Mock and #P< 0.05 vs. *E. coli*, n=6 per group.

6.2.5 Bacterial clearance is not affected by blocking CXCR2

As is known, neutrophils are fundamentally involved in the acute phase of inflammation when they actively eliminate pathogens, and even beyond that by modifying the overall immune response (114). Therefore, any interference with their role may lead to dysregulation of other neutrophil functions. Despite the detrimental role that NETs play in mediating inflammation, increase in NETs production appears to promote bacterial clearance *in vitro* and *in vivo* (78, 162, 164, 370).

In this context, bacterial cultures were used to determine whether bacterial burden in major organs (heart, liver, spleen, lungs, kidneys) is affected after reparixin treatment in septic mouse model. The colony forming units (CFUs) in septic mice treated with reparixin were not increased when compared to septic mice without treatment, with no significant difference found between reparixin treated and untreated septic mice (Figure 6.12).

These observations suggest that reparixin inhibited NETs formation but neither impaired nor improved the bacterial burden in both mouse models of sepsis.

Figure 6.12

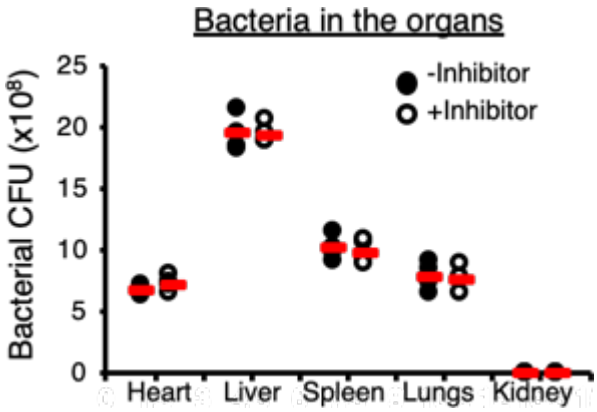


Figure 6.12 Blocking CXCR2 did not affect bacterial load following experimental sepsis. Bacterial counts were quantified in heart, liver, spleen, lungs and kidney from mice 10 hours following CLP-induced sepsis treated without (-Inhibitor) or with reparixin (20 mg/kg/dose) (+Inhibitor). Data are presented in means. No statistical difference was found.

6.2.6 CXCR2 signalling blockade prolong survival in septic mice

To evaluate the role of IL-8 in experimental sepsis, CXCR2 was blocked with reparixin 1 hour before CLP was generated, 2 and 4 hours thereafter. As shown in Figure. 6.13 blockade of CXCR2 significantly protected CLP mice against mortality (log rank test, P=0.036). When that CXCR2 inhibitor was administered, about 2/3 of mice survived over 24h (4/6) but in the severe CLP mouse model, no mouse survived over 24h. The level of protection was maintained until 72 hours, with two of the reparixin-treated CLP mice were still alive. These findings imply that blocking CXCR2 could have considerable role in improving survival from sepsis.

Figure 6.13

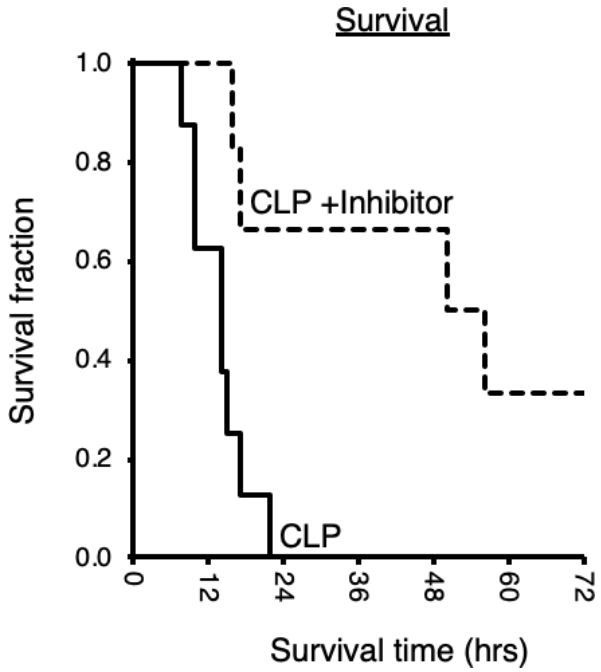


Figure 6.13 Reparixin improved survival in septic mice. Mice were subjected to CLP surgery and monitored for 72 hours without or with early reparixin treatment 1 hours pre-CLP and at 4 and 6 hours post-CLP. The number of hours from CLP induction to death in each group were recorded. Kaplan-Meier survival curve show that Reparixin (n=6) increases survival time of CLP sepsis mice (n=8).

6.3 Discussion

Sepsis is fundamentally an inflammatory disorder, which has caused huge burden to healthcare industry for a long time. Immunopathology is the primary pathological feature of sepsis and mainly causes organ damage and death (371). Constant and excessive neutrophil recruitment and NETs release are critical to mediate these outcomes (326, 330). Thus, balance between neutrophil migration and activation must be struck to prevent microbial dissemination and organ damage. In chapter 5, organ damage in experimental sepsis was mediated by the deposition of excessive levels of NETs and fibrin. In this chapter, the relationship between IL-8 and NETs formation was investigated in septic models by blocking CXCR2 of CXCL2 (MIP-2) in murine models of sepsis using a clinically relevant therapeutic drug reparixin. The main findings in this chapter can be summarised as follow: (i) CXCL2 was significantly elevated in both models of sepsis and is positively associated with the levels of *ex vivo* NETs formation; (ii) CXCR2 signaling is a major pathway to drive NETosis in sepsis; (iii) the blockade of CXCR2 using reparixin significantly reduced fibrin deposition within the lungs and attenuated organ injury in both models; (iv) bacterial burden is not affected after blocking CXCR2; (v) dramatic improvements in the general state of the lungs and survival time of septic mice after reparixin treatment.

Human IL-8, a potent chemoattractant of neutrophils during infection (343), induces neutrophil recruitment and activation by binding to CXCR1/2 on neutrophil's surface (344, 345). CXCL2 (MIP-2) is the mouse homologue of IL-8 and the major ligand of CXCR2 (348). IL-8 and CXCL2 have been identified as physiological inducers of NETs formation *in vitro* (78, 308, 372, 373). In this chapter, circulating CXCL2 levels were significantly increased in experimental sepsis and high levels of *ex vivo* NETs formation were strongly associated with circulating CXCL2. Furthermore, MIP-

2 increased 300-1000 fold and the pathological levels of MIP-2 can induce NETs formation both *in vitro* and *ex vivo*. In line with our data, neutrophils derived from COPD patients can induce *ex vivo* NETs when incubated with individual sputum, which also showed increased levels of IL-8 in culture supernatant (347). These NETs structures could be abrogated following pre-treatment of neutrophils with the CXCR2 inhibitor AZD5069.

Through NETosis, the systemic inflammatory response during sepsis promote activation and accumulation of neutrophils within the microvascular system, causing vascular occlusion and tissue injury and eventually resulting in organ dysfunction (205, 374). NETs formed predominantly in the lungs and were associated with fibrin deposition, causing direct damage to the lungs of septic mice (Chapter 5). Recruitment of neutrophils to the lungs is multistep process that is coordinated by CXC chemokines, such as CXCL1 and CXCL2 in mice and CXCL8 in humans (343, 348). In our mice models of sepsis, blocking the intracellular signal transduction events activated by CXCL2 attenuated *in vivo* NETs and fibrin formation within the lungs. This notion is supported by observations from CLP-induced sepsis showing that NETs depletion via DNase decreased the levels of CXCL1-2 in plasma and lungs, suggestion a major interplay between NETs and CXC chemokines in sepsis (195). Knowing that pulmonary NETs formation is significantly diminished following the blockade of CXCR2, it remains unclear whether this process is implicated in organ injury. In this context, subsequent injury to the lungs and other vital organs including liver, heart and kidney was significantly attenuated following the blockade of CXCR2 in both models of sepsis. In support of our data regarding CXCL2/CXCR2 mediated organ injury, Kaneider et al. clarified that the lethal sequelae of sepsis, including DIC and organ injury could be reversed after blocking CXCR1/2 following CLP-induced sepsis using

cell-penetrating lipopeptides (375). This chapter provides additional evidence that extensive NETs formation and coagulation activation in the lungs are driven by CXCL2-CXCR2 signaling, which play a major pathological role in murine sepsis models. In support of my findings, Zarbock *et al* (362) reported in ALI mouse models that reparixin reduced vascular permeability and partially inhibited neutrophils recruitment to the interstitial space and alveolar compartments by 50% and 60%, respectively. The present data in this chapter provide additional evidence on the capacity of reparixin to partially inhibit NETs during sepsis. This observation was confirmed by calculating the percentage of neutrophils that underwent NETosis within the lungs of reparixin treated mice.

Neutrophils are the first respondents during bacterial infection and their potent machinery is recognised in controlling these pathogens. We and others have demonstrated in experimental models of sepsis that organ injury was dependent on the presence of neutrophils in the tissues (311). Neutrophil depletion in mice infected with various pathogens has rarely induced lethality, but rather delayed pathogen clearance (376, 377). Interestingly, the systemic blockade of CXCR2 with reparixin in septic mice did not interfere with bacterial clearance. These data are in line with a previous study (351), suggests that the CXCR2 small molecule antagonists did not interfere with antimicrobial defence mechanism.

To summarize, when CXCR2 is blocked using a therapeutic small allosteric inhibitor, mice were significantly protected from the organ injury and mortality that follow experimental sepsis. Surprisingly, the blockade of CXCR2 did not affect bacterial clearance in organs. In addition, the blockade CXCR2 appeared to inhibit the production of NETs and fibrin deposition, that is significant because these pathological structures are important but unfavourable mediators in the septic response. Together,

the present study described how NETs aggravate sepsis and the molecular mechanism involved in this process with a potential target for the development of a new therapeutic approach. In this context, inhibiting NETs formation might be a more useful therapeutic strategy than targeting the general inflammatory response in sepsis.

Chapter 7: General discussion, future work and conclusion

7.1 Discussion

The process of neutrophil extracellular traps (NETs) formation following initial stimulation of neutrophils terminates with the externalisation of DNA scaffold coated with histones and granule proteins to the extracellular environment (78). NETosis was initially described as an active microbicidal mechanism of neutrophils (78), but later research described NETs as pathological mediator of poor outcome. Excessive NETs formation was reported as a major driver in the pathophysiology of sepsis models (163, 194-196, 198). Clinical studies have confirmed increased levels of NETs biomarkers in critical illnesses (290, 297, 299).

Despite research focusing on NETs in different pathologies, there are no currently standardised approaches to monitor and quantify NETs formation, primarily in the clinical settings. Early studies applied limited commercial and non-commercial approaches aiming at identifying and quantifying NETs-related biomarkers such as, DNA quantification and enzyme-linked immunosorbent assay (ELISA) such as, neutrophil elastase (NE) and myeloperoxidase (MPO) (208, 245, 247, 305-307). However, these approaches have been criticised for their lack of specificity as well aslack of association with clinically relevant information such as disease severity, complications and overall outcomes (243-245). Thus, establishing and investigating the NETs-forming capacity of plasma from critically ill patients and translating its findings into animal models are interesting directions of research presented in this thesis.

In our cohort of critically ill patients, various degrees of NETs formation were observed when human neutrophils were incubated with individual patient plasma/sera, with strong association to sepsis diagnosis, disease severity and multiple organ failure

(MOF). In addition, patients diagnosed with sepsis secondary to respiratory illness had moderate to strong NETs-forming capacity, which may inform an extensive role of NETs in the lungs during sepsis. These findings support studies documenting excessive NETs formation in sepsis (329-331), but most importantly opened up a new avenue of study linking NETs to clinical data.

In order to address the translational relevance of the *ex vivo* NETs formation assay, my study has also clarified its utility using two mice models of sepsis induced by caecal ligation and puncture (CLP) and intraperitoneal (i.p) injection of *Escherichia coli* (*E. coli*). Upon stimulation of normal mice neutrophils with septic mouse plasma, moderate to strong NETs formation was observed. I have demonstrated that NETs were also elevated within the lungs of both sepsis models, but not other major organs (heart, liver and kidneys). Importantly, NETs levels within the lungs correlated with *ex vivo* NETs-forming capacity of mice plasma. These findings further extend from previous findings in our cohort of critically ill patients, where septic patients with respiratory origin have moderate to strong increased NETs levels. Moreover, these observations support findings from other studies that reported similar *in vivo* observations in septic mouse models (194, 195). One reason why *ex vivo* NETs formation reflects *in vivo* NETs formation is that the factors driving NETs in plasma may also be present within the lung vasculature. However, further investigations are required to clarify these observations and provide supporting evidence to our interpretations.

When released to the extracellular space, DNA structures of NETs serve as scaffold for trapping activated platelets, red blood cells (RBCs) and fibrin to enhance thrombosis and promote extensive damage to the host (193, 293, 294). The contribution of NETs to the coagulation system has been extensively studied in the

literature. However, it remains not fully understood whether NET surrogate markers versus NETs-forming capacity of patient plasma is truly reflective of coagulation abnormalities within critically ill patients. To clarify this issue, this thesis directly compared the capacity of plasma to induce NETs formation to surrogate NETs markers in predicting the development of disseminated intravascular coagulation (DIC).

In the clinical observational study, the predictive value of NETs on intensive care unit (ICU) admission was examined for the development DIC post ICU admission. After adjustment for the clinical severity score acute physiology and chronic health evaluation (APACHE) II, levels of *ex vivo* NETs formation were independently associated with DIC after ICU admission. Moreover, NETs formation levels had strong predictive value for DIC, which was comparable to current clinical scoring systems. However, other indirect measures, including cell-free DNA (cfDNA) and MPO did not exhibit independent association nor predicative value for DIC. These findings provide for the first evidence on the ability of NETs-forming capacity assay, but not NETs components, to inform clinical utility on identifying critically ill patients at risk of developing major coagulation complications.

In addition, the interplay between NETs-biomarkers and fibrin deposition is well documented in animal models of deep venous thrombosis (DVT) (113). Fibrin is an essential large polymer that is required for thrombus stability (378). My findings that NETs are associated with fibrin deposition within the lungs of septic mice models may provide further insights to the current literature, particularly in sepsis. The specific inhibitory effects of global neutrophil function via neutrophil depletion as well as NETs formation via DNase I infusion post-treatment not only resulted in abrogation of NETs within the lungs but also reduced fibrin formation in the lungs of sepsis mice.

Thrombosis mediated by NETs-fibrin localisation may form a harmful pathophysiological mechanism that enhances tissue damage via microvascular occlusion to provoke organ injury. Since neutrophil depletion and DNase I treatment during sepsis showed similar findings on NETs-fibrin deposition, neutrophil depletion was investigated to further characterise the roles of NETs in organ injury in sepsis. Inmice subjected to CLP, neutrophil depletion significantly protected the lungs and heart. It is possible that NETs mediate direct injury to the lungs via enhanced fibrin deposition, increased thrombosis formation and microvasculature occlusion.

Despite the effectiveness of neutrophil depletion to abrogate NETs and improve organ injury, one major limitation to be noted is that removing the entire function of neutrophils is less relevant clinically. Neutrophils are important in the innate immune system and their presence is critical for the resolution of infection (376, 377). In addition, other concerns that may apply to DNase I treatment, with current reports link DNase I treatment to increased inflammation in animal models of sepsis (201). Thus, a more clinically relevant approach is required with limited side-effects to the host's innate immune function.

In this context, this work has identified interleukin (IL)-8 as a major factor driving *ex vivo* NETs formation in critically ill patients with sepsis. In addition, the role of IL-8 in mediating NETs release was confirmed through pre-treatment of neutrophils with anti-IL-8 inhibitors (reparixin, AZD5069**)** before adding plasma from sepsis patients. Of note, the potential of IL-8 to induce *in vitro* NETs formation is a matter of debate in the literature. This discrepancy was clarified in my study using physiological conditions, whereby pathological levels of IL-8 triggered NETs formation in the presence of normal plasma. This was not investigated in any of the previous literature (151, 379, 380). However, the responsiveness of patients' neutrophils to

IL-8 needs to be further studied.

Since inhibiting IL-8 reduced *ex vivo* NETs formation, we hypothesise that IL-8 may interact directly with neutrophils *in vivo* through cellular binding via their receptors (CXCR1/2), to activate neutrophils and enhance their ability to generate NETs. To clarify whether sepsis drives the release of IL-8, I initially quantified the levels of macrophage inflammatory protein (MIP)-2, the IL-8 homolog in septic mice. I found that MIP-2 levels were significantly elevated in septic mice by approximately 6000 times, compared to mock controls. With evidence that NETs form *ex vivo* and within lung tissues of septic mice, my study illustrated that blocking the IL-8 receptors (CXCR1/2) on neutrophils via reparixin have inhibitory effect on *ex vivo* and *in vivo* NETs formation. In addition, fibrin deposition within lung tissues was decreased.

Targeting NET-fibrin deposition within tissues using anti-IL-8 therapy may form a protective strategy to reduce microvascular thrombosis formation and organ injury. As demonstrated in chapter 5, both murine models of sepsis sustained marked injury to the major organs 10 hours following sepsis induction. More importantly, blocking CXCR2 (Chapter 6) reduced lung injury in addition to circulating damage markers ofother major organs in both models of sepsis. These findings support previous studies (351, 362), yet adds a layer of evidence on IL-8 in driving NETs-fibrin localisation inmediating organ injury during sepsis.

It is important to consider the potential adverse outcomes associated with complete inhibition of NETs formation, by specifically blocking peptidyl arginine deiminase (PAD)-4 activity or digesting NETs DNA structures using DNase I treatment. The complete elimination of NETs formation as a therapeutic approach has been appraised in the literature due to reports suggesting that it can promote bacterial dissemination

and increased mortality in sepsis models (218, 221). In this context, reparixin treatment to partially inhibit NETs formation induced by CXCR2 signaling did not alter bacterial burden within the major organs of sepsis mice and improved survival. These data provided evidence that NETs formation should be targeted in a carefully regulated way to maintain the beneficial function of NETs in preventing bacterial dissemination but inhibit the harmful effects through driving coagulation activation and end organ damage.

7.2 Limitations

The limitations to the work presented in this thesis are: (1) the principle behind *ex vivo* NETs reflect *in vivo* NETs is difficult to examine clinically due to the fact that organ tissue samples are invasive and difficult to obtain from humans; (2) reparixin is not currently a clinically approved medicine in sepsis.

7.3 Conclusions

- E*x vivo* NETs formation assay is a direct approach to measure neutrophils capacity to form NETs in response to circulating factors of both critically ill patients and animal models of sepsis.

- The ability of *ex vivo* NETs formation assay to independently predict DIC and mortality in critically ill patients informs prognostic utility and allows stratification of high-risk patients to initiate early supportive treatments.

- The *ex vivo* NETs formation assay has been invaluable tool to reflect on and assess *in vivo* NETs release that uniquely formed within the lungs of animal models of sepsis.

- Excessive NETs are likely to promote fibrin deposition and work together toward a pathogenic role that could be used as a therapeutic target to attenuate organ dysfunction and improve survival in sepsis.

- Targeting IL-8 may have therapeutic promise in both reducing NETs and fibrin formation as well as treating organ dysfunction without impacting bacterial clearance in sepsis, where efficient immune defence is required.

7.4 Future directions

- Studying neutrophils derived from other sources such as, stem cells and their ability to form NETs using the *ex vivo* NETs formation assay, to enable further simplification of the assay.

- Using animal models of other critical illnesses such as, acute pancreatitis and trauma to examine potential circulatory triggers of NETs formation associated with *ex vivo* NETs formation levels.

- Clinical investigations to assess the correlation between NETs within tissues and *ex vivo* NETs formation, possibly using post-mortem organ biopsies.

- Clinical studies to explore how to benefit septic patients from the potential effects of safe and approved anti-IL-8 therapies in blocking NETs formation as well as toresolve organ injury and improve mortality.

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