Monocyte and endothelial cell interaction during sepsis

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ABSTRACT

Sepsis complicated by coagulopathy is associated with a higher mortality rate. The cell surface expression of tissue factor (TF) may be important in its development. There at least three subsets of monocytes – classical, intermediate and non-classical – that vary in proportion during sepsis. This project used three approaches to investigate how the monocyte subsets and their endothelial interaction could contribute to sepsis-associated coagulopathy: the *in vitro* stimulation of healthy monocytes with lipopolysaccharide (LPS) and endothelial co-culture; the use of a human model of endotoxaemia; and the collection of blood samples from individuals with sepsis.

LPS stimulation of healthy monocytes demonstrated that the monocyte subsets express cell surface TF to different extents. Classical and intermediate monocytes express the highest proportion of TF in response to LPS. An *in vitro* monocyte-endothelial co-culture model demonstrated that monocytes could promote coagulation through the increased cell surface expression of TF, independent of LPS, and influence the endothelial fibrinolytic response.

Blood samples were taken at a range of time-points following an injection of LPS into a healthy volunteer. An increase in cell surface TF occurred within 90 minutes of exposure to LPS and was associated with an increase in markers of coagulation. Individuals varied in their response to LPS, with two groups identified: high and low responders. The response of individuals was consistent between the subsets.

Samples from individuals with sepsis expressed a higher level of cell surface TF compared to individuals who were critically ill but did not have sepsis. The surface expression of TF increased further when measured following recovery from sepsis.

This work demonstrates that the cell surface expression of TF varies between the monocyte subsets, that individuals may increase monocyte TF expression to different levels, and that individuals with sepsis express higher levels of TF both at the time of sepsis and following recovery.

Dedicated to Dr Patrick Kesteven

DECLARATION

I hereby declare that the material contained within this thesis is my own work unless otherwise stated within the text. The data contained within this thesis have not previously been submitted for the award of a higher degree at this or any other University.

Dr Kathryn Musgrave

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LIST OF ABBREVIATIONS

A1AT	alpha 1 antitrypsin
ANOVA	analysis of variance
AP-1	activator protein 1
APACHE	Acute Physiology and Chronic Health Evaluation
APC	allophycocyanin
BERK	Berkley
BV	Brilliant Violet
$CaCl_2$	calcium chloride
CD	cluster of differentiation
CHSFT	City Hospitals Sunderland NHS Foundation Trust
CLEC4D	C-Type Lectin Domain Family 4 Member D
CXRCR1	CX3C chemokine receptor 1
CO_2	Carbon dioxide
CT	computerised tomography
CXR	chest radiograph
Cy	Cyanine
DAPI	4′, 6-diamidino-2-phenylindole
dL	decilitre
DMSO	dimethyl sulphoxide
EDTA	Ethylenediaminetetraacetic acid
Egr-1	early growth response protein 1
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
FACS	fluorescence-activating cell sorting
FBS	foetal bovine serum
FDP	fibrin degradation product
FIX	factor IX
FIXa	activated factor IX
FITC	fluorescein isothiocyanate
FMO	fluorescence minus one
FV	factor V
FVa	activated factor V
FVII	factor VII

FVIIa	activated VIIa
FVIII	factor VIII
FVIIIa	activated factor VIIIa
FX	factor X
FXa	activated factor Xa
FXI	factor XI
FXIa	activated factor XI
FXII	factor XII
FXIIa	activated factor XII
FXIII	factor XIII
FXIIIa	activated factor XIII
Gr	glucocorticoid receptor
HBSS	Hank's Balanced Salt Solution
$HBSS^{+}$	Hank's Balanced Salt Solution with Mg^{2+} and Ca^{2+}
$HBSS^{-}$	Hank's Balanced Salt Solution without Mg^{2+} or Ca^{2+}
HLA	Human Leukocyte Antigen
ICU	intensive care unit
IL	interleukin
IMDM	Iscove's modified Dulbecco's medium
IN	inotropes
JNK	c-Jun amino terminal kinase
Kg	kilogram
LPS	lipopolysaccharide
Ly6C	Lymphocyte antigen 6 complex locus G6D
IV	invasive ventilation
MCP-1	monocyte chemoattractant protein-1
MFI	median fluorescent intensity
mg	milligram
min	minutes
mL	millilitre
mm	millimetre
mmHg	millimetres of mercury
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NIV	non-invasive ventilation
NuTH	Newcastle upon Tyne Hospitals NHS Foundation Trust

PAI-1	plasminogen activator inhibitor 1
PAMPs	pathogen-associated molecular patterns
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PE	phycoerythrin
PerCP	peridinin chlorophyll
PI	propidium iodide
PMVEC	human pulmonary microvascular endothelial cells
RBC	red blood cell
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
RRT	renal replacement therapy
SF	soluble fibrin
Siglec10	Sialic acid-binding Ig-like lectin 10
SP-1	specificity protein 1
TF	tissue factor
TLR	toll-like receptor
TNF	tumour necrosis factor
tPA	tissue plasminogen activator
TREM1	Triggering receptor expressed on myeloid cells 1
tSNE	t-distributed stochastic neighbour embedding
ULN	upper limit of normal
UV	ultra-violet

LIST OF UNITS

°C	degrees Celsius
%	percentage
g	gravity
hr	hours
dL	decilitre
kg	kilogram
L	litre
MFI	median fluorescent intensity
µg	microgram
µL	microlitre
µm	micrometre
mL	millilitre
mg	milligram
min	minutes
ng	nanogram
pg	picogram
pM	picomole

Chapter 1. Introduction

1.1 Overview

Sepsis is defined as life-threatening organ dysfunction caused by a disordered immune response to infection (Singer *et al.*, 2016). Despite significant advances in critical care medicine, it has an unacceptably high mortality rate (Angus *et al.*, 2001; Fleischmann *et al.*, 2016; Meyer *et al.*, 2018). Frequently, sepsis occurs concomitantly with a coagulopathy that further reduces an individual's chances of recovery. Multiple interventional studies have failed to find any therapies that can improve these outcomes (Warren *et al.*, 2001; Ranieri *et al.*, 2012; Vincent *et al.*, 2013). There is an urgent need to better understand the pathophysiology and to identify new therapeutic targets.

The role of intravascular tissue factor (TF), expressed on monocytes, and subsequent activation of the coagulation system is believed to be an important trigger for coagulopathy (Gando *et al.*, 2016). Monocytes can be divided into at least three functionally different subsets; however, there is a paucity of data regarding TF expression between these subsets (Stojkovic *et al.*, 2017). This work investigates the role of monocyte subsets and their TF expression during health, endotoxaemia and sepsis.

Monocytes are divided into subsets using the cell surface markers cluster of differentiation (CD)14, a co-receptor for LPS, and CD16, a weak Fc gamma receptor. The subsets are identified as 'classical' (CD14++CD16-), 'non-classical' (CD14+CD16++) and 'intermediate' (CD14++CD16+) (Ziegler-Heitbrock *et al.*, 2010a). Classical monocytes are mainly associated with bacterial killing, producing the most reactive oxygen species and being the most phagocytic; non-classical are believed to 'patrol' the endothelium and have a role in tissue repair; and intermediate monocytes have been associated with viral killing (Wong *et al.*, 2011).

During sepsis, there is an increase in circulating non-classical monocytes and a proportionate decrease in circulating classical monocytes (Fingerle *et al.*, 1993). This project investigates whether this change in circulating monocyte subsets may contribute to the coagulopathy of sepsis. Improved understanding of how TF expression varies between the monocytic subsets may allow for the identification of new therapeutic targets in the management of sepsis-associated coagulopathy.

This chapter will discuss the following in further detail: the poorer outcomes from sepsis-associated coagulopathy; possible causes of the coagulopathy; the role and function of TF; a

comparison of the monocyte subsets; and how best to model the immune response to inflammation to better understand the pathophysiology. Current literature will be reviewed, and a context established for the rest of this work.

1.2 Sepsis

1.2.1 Overview

This section discusses the incidence and mortality rates of sepsis over the last few decades and clarifies the changes in definition that have occurred.

1.2.2 The incidence and outcomes of sepsis

Sepsis is increasing in incidence. A review of over 750 million hospital admissions in the United States showed an increase in cases of sepsis, between 1979 and 2000, from 82.7 cases to 240.4 per 100,000 population (zieglerMartin *et al.*, 2003). The associated in-hospital mortality rate, however, has decreased over the last 30 to 40 years, from approximately 30 % to nearer 20 % (Angus *et al.*, 2001; Martin *et al.*, 2003; Brun-Buisson *et al.*, 2004; Fleischmann *et al.*, 2016; Meyer *et al.*, 2018). Most likely this reflects an improvement in critical care and evidence-based guidelines such as the Surviving Sepsis Campaign (Levy *et al.*, 2014). Despite this decrease, a significant proportion do not survive, and combined with the rising incidence there has been a three-fold increase in the total number of deaths (Martin *et al.*, 2003).

Most sepsis trials measure mortality at 28 days or over the 'in-hospital' period; the two rates have been shown to be similar (Stevenson *et al.*, 2014). Failure to consider the longer-term mortality rates, however, will likely underestimate the true mortality rate of sepsis. Several studies have demonstrated an increasing mortality rate in survivors of sepsis up to 5 years following the episode (Quartin *et al.*, 1997; Brun-Buisson *et al.*, 2004; Laupland *et al.*, 2005).

A significant proportion, approximately 20 %, of sepsis survivors are re-admitted to hospital within 30 days. This is higher than for other acute medical admissions (Jones *et al.*, 2015; Prescott *et al.*, 2015). Recent work has shown that although this percentage may be starting to drop, to some degree this has been offset by the number of attendances to the emergency department where patients received treatment but were not admitted. Due to the rising incidence, this has resulted in a three-fold increase in re-admissions (Meyer *et al.*, 2018). The most common reasons for re-admission are sepsis, congestive cardiac failure and pneumonia (Prescott *et al.*, 2015; Sun *et al.*, 2016).

Long-term morbidities associated with sepsis should also not be forgotten. Survivors develop more recurrent infections and are at higher risk for thromboembolic disease for at least 12 months following the episode of sepsis (Mejer *et al.*, 2015; Arens *et al.*, 2016; Ou *et al.*, 2016). The cause for these longer-term complications remains unclear. There is a trend towards higher inflammatory markers in survivors, whilst stimulation of ex-vivo blood samples of survivors showed a lower level of pro-inflammatory cytokine release (Arens *et al.*, 2016). To determine the longer-term impact on survivors of sepsis, further work is needed to understand the causes of these morbidities and increased mortality (Shankar-Hari and Rubenfeld, 2016).

1.2.3 Defining sepsis

In 1991, the American College of Chest Physicians (ACCP) and Society of Critical Care Medicine (SCCM) reached a consensus for defining sepsis that included the need for confirmation of infection and evidence of a host inflammatory response, i.e. the systemic inflammatory response syndrome (SIRS) (Bone *et al.*, 1992).

This was reviewed in 2001, when it was recognised that clinicians were unclear as to the definitions of sepsis and there was concern that SIRS criteria (extremes of core temperature, tachycardia, high respiratory rate, hypoxia, high or low white count or more than 10 % immature white cells in the peripheral blood) were not specific enough. Definitions were revisited at the 2001 International Sepsis Definitions Conference, which included the ACCP, SCCM, American Thoracic Society (ATS), European Society of Intensive Care Medicine (ESICM) and Surgical Infection Society (SIS). The diagnostic criteria for sepsis were expanded to include other physical and laboratory signs of SIRS that may occur due to infection (Levy *et al.*, 2003).

The poor specificity of the SIRS criteria remained. A large cohort study reviewed individuals with signs of infection and organ failure from 172 ICUs across Australia and New Zealand between 2000 and 2013. Approximately 10 % did not meet the conventional criteria for severe sepsis due to having ≤ 2 SIRS criteria; however, they had a similar mortality rate to those with > 2 (Kaukonen *et al.*, 2015).

As the pathophysiology of sepsis has become increasingly understood, the ESICM and SCCM again met throughout 2014 and into early 2015 to review the definitions. Sepsis was defined

as “life-threatening organ dysfunction caused by a dysregulated host response to infection” and septic shock was taken to refer to a subset of individuals who have “profound circulatory, cellular and metabolic abnormalities” (see Table 1.1). The new definitions do not include ‘severe sepsis’ and remove the need for SIRS criteria. They are based on the development of organ dysfunction, measured with the Sequential (Sepsis-related) Organ Failure Assessment (SOFA, see) score, and emphasise the need for close monitoring of all septic individuals (Seymour *et al.*, 2016). This work uses this latter definition.

Concept	Definitions
Sepsis	Life-threatening organ dysfunction caused by a dysregulated host response to infection
Organ dysfunction	Acute change of 2 or more points in the Sequential (Sepsis-related) Organ Failure Assessment (SOFA) score
Septic shock	Severely affected subset of sepsis, when circulatory, metabolic or cellular abnormalities are profound enough to increase mortality. Requires sepsis diagnosis with one of the below criteria.
Criteria for shock	*Vasopressors needed to maintain mean arterial blood pressure at greater than 65mmHg* *Lactate greater than 2mmol/L despite fluid resuscitation*

Table 1.1 The definition of sepsis

This table shows the 2015 definitions of sepsis and septic shock from "Sepsis-3: The Third International Consensus Definitions for Sepsis and Septic Shock", adapted from Singer et al. 2016 (Seymour et al., 2016; Singer et al., 2016).

SOFA score	1	2	3	4
Respiratory criteria				
PaO_2/FiO_2 (mmHg)	<400	<300	<220	<100
SaO_2/FiO_2	221-301	142-220	67-141	<67
Cardiovascular criteria				
Hypotension	MAP <70 mmHg	dopamine ≤5 or any dobutamine	dopamine >5 or norepinephrine ≤0.1	dopamine >15 or norepinephrine >0.1
Neurological criteria				
Glasgow coma score	13-14	10-12	6-9	<6
Renal criteria				
Creatinine (mg/dL)	1.2-1.9	2.0-3.4	3.5-4.9	>5.0
Urine output (mL/day)	-	-	<500	<200
Haematological criteria				
Platelets ($x10^3/mm^3$)	<150	<100	<50	<20
Hepatic criteria				
Bilirubin (mg/dL)	1.2-1.9	2.0-5.9	6.0-11.9	>12.0

Table 1.2 The Sequential (Sepsis-related) Organ Failure Assessment (SOFA) score

This table shows the Sequential (Sepsis-related) Organ Failure Assessment (SOFA) adapted from Jones et al. (Jones et al., 2009). Glasgow coma scale from Teasdale and Jennett (Teasdale and Jennett, 1974). Dopamine, noradrenaline and dobutamine to be administered for at least 1 hour, units - µg/kg/min.
(abbreviations: dL – decilitre, kg – kilogram, mg- milligram, min – minute, mL – millilitre, mm – millimetres, mmHg – millimetres of mercury).

1.2.4 Summary

Overall, there has been a rise in the incidence of sepsis, and it is associated with both a significant mortality rate and long-term morbidity. There is a need to better understand the underlying pathophysiology to help guide future studies. A continuing challenge to research is the difficulty in establishing a concise definition that is useful both clinically and in performing research studies. When reviewing literature regarding sepsis, it is important to bear in mind the recent changes in definition. A recent study prospectively scored cases of sepsis by the Sepsis-3 and earlier definitions, demonstrating that Sepsis-3 identified fewer cases but of a more severe phenotype than earlier definitions (Driessen *et al.*, 2018). This obviously has implications when considering studies of sepsis prior to the adoption of Sepsis-3.

1.3 Sepsis-associated coagulopathy

1.3.1 Overview

The association between coagulopathy and sepsis has been recognised for many decades, initially discussed in multiple case reports. Corrigan first systematically documented the effects of sepsis on coagulation in a series of 26 individuals. The cases were divided into two groups, normotensive and hypotensive, with the latter group displaying a more severe coagulopathy. The correlation between severity of disease and coagulopathy was highlighted (Corrigan *et al.*, 1968). This section will briefly outline the physiological processes of coagulation and fibrinolysis before discussing the causes and effects of sepsis-associated coagulopathy.

1.3.2 The coagulation system

The coagulation system was first described in the early 1960s, originally considered a cascade of reactions with three pathways, intrinsic, extrinsic and common (Davie and Ratnoff, 1964; Macfarlane, 1964). These pathways are composed of a series of reactions; inactive pro-enzymes of serine proteases are activated and then catalyse the next pro-enzyme in the cascade. This description of coagulation was based upon laboratory assays and was not a true reflection of the *in vivo* situation. The extrinsic pathway function is reflected by the prothrombin time (PT) measurement and the intrinsic pathway by the activated partial thromboplastin time (APTT).

Venous haemostasis is achieved by the formation of a stable clot composed of platelets and fibrin; however, the pro-thrombotic factors required to create this must also be contained and

localised to the site of injury. The cell-based model by Hoffman *et al.* proposed in the 1990s is a more accurate representation and involves three stages: initiation, propagation and amplification (Hoffman, 2003). During *initiation*, TF expressed on a cell surface binds and activates the pro-enzyme factor VII into the activated FVIIa (FVIIa); this complex then creates a small amount of activated factors X (FXa) and XI (FXIa) on the cell surface. This FXa remains on the cell surface and activates factor V (FVa). FXa and FVa then bind to form the prothrombinase complex (FXa:FVa), capable of cleaving prothrombin into thrombin. Although small amounts of thrombin are subsequently formed, this action is quickly inhibited by anti-thrombin (AT) and tissue factor pathway inhibitor (TFPI).

For a thrombus to form there must be an *amplification* step and this requires the proximity of an activated platelet. The small amounts of thrombin already formed serve to activate platelets, which release more Va. Factor VIII bound to its carrier protein von Willebrand factor (vWF) in the plasma then binds to the activated platelet. Thrombin cleaves the vWF and activated VIII (FVIIIa). By the completion of amplification, FVa and FVIIIa are bound to the activated platelet surface.

Both the prothrombinase and now the tenase (FIXa:FVIIIa) complexes form on the platelet surface, and *propagation* begins. The tenase complex greatly increases the formation of FXa, leading in turn to a much higher concentration of the prothrombinase complex and a greater production of thrombin. The cell-based model is further explained in Figure 1.1. Thrombin not only serves to cleave fibrinogen into fibrin but is also a potent aggregator of platelets.

Fibrin monomers polymerise to an insoluble fibrin network. This polymerisation is initially reversible but is stabilised by FXIII, activated by thrombin, that covalently crosslinks the fibrin strands (Weisel and Litvinov, 2017).

In vitro experiments have also given rise to the idea of a 'contact pathway'. This is composed of three proteins: factor XII (FXII), prekallikrein (PK) and high molecular weight kininogen (HK) that all become activated when on a negatively charged surface and can shorten *in vitro* clotting times. The *in vivo* activator has not been identified. FXII is known to auto-activate on negatively charged surfaces; it then activates PK to plasma kallikrein. Kallikrein itself can activate FXII. HK binds to the negatively charged surface and acts as a cofactor for both the activation of FXII and the production of kallikrein. The cleavage of HK by PK leads to the release of bradykinin, a proinflammatory mediator. C1NH is the main inhibitor of both FXIIa

and PK. The physiological role of this pathway in coagulation is yet to be elucidated and remains controversial. It is worth noting that deficiency in one of these three plasma proteins does predispose an individual to an increased risk of bleeding, as is seen with other coagulation factors (Schmaier, 2014; Wu, 2015; Maas and Renné, 2018).

Control of coagulation involves three anticoagulant pathways: the protein C system, TFPI and antithrombin. Thrombin binds to endothelially bound thrombomodulin and thereby activates protein C; this, along with its co-factor protein S, works to degrade FVIIIa and FVa, thereby inhibiting both the prothrombinase and the tenase complexes. TFPI is the main inhibitor of TF and works by binding to TF that is complexed to FVIIa. Antithrombin binds and forms a complex with both thrombin and FXa (Gando *et al.*, 2016). Alpha 1 antitrypsin (A1AT), a serine protease inhibitor, has been shown to bind strongly to a fibrin clot and may also play a role in the inhibition of coagulation, but this is still unclear (Talens *et al.*, 2013).

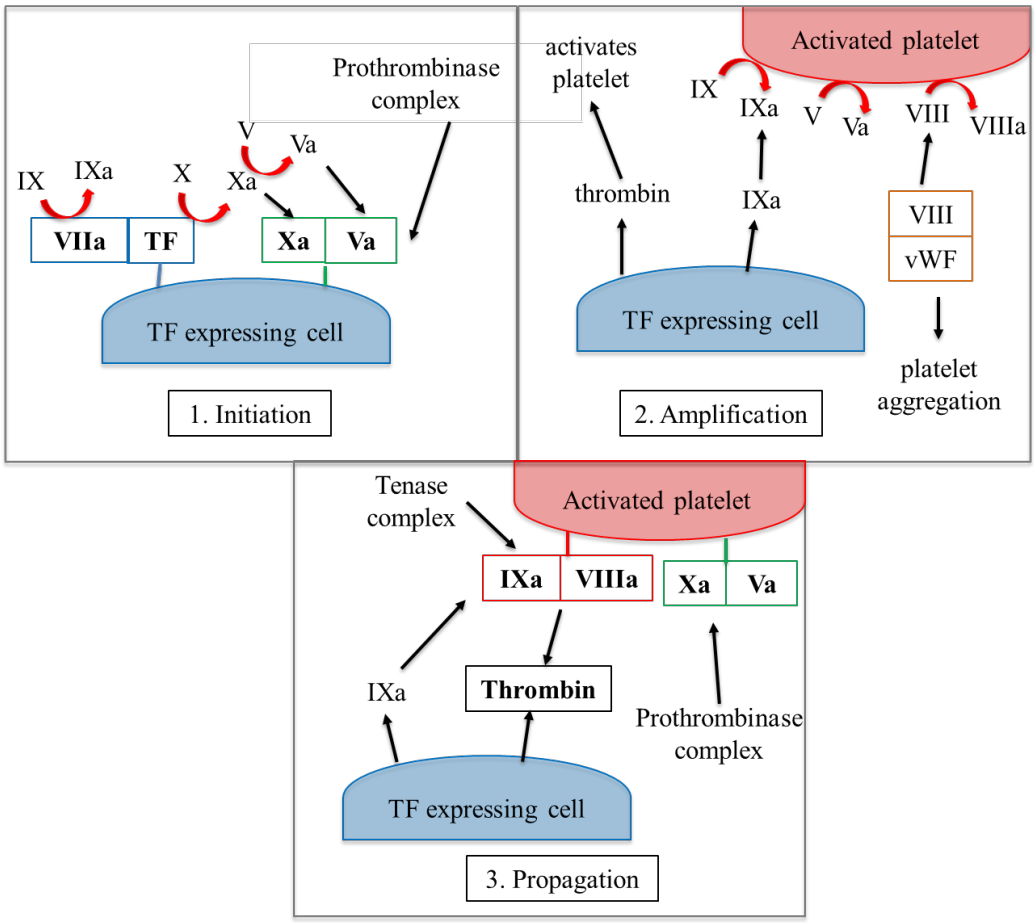


Figure 1.1 Cell-based model of the coagulation system.

Figure 1.1 demonstrates the cell-based model of coagulation that comprises initiation, amplification and propagation as described below.

Initiation

1.1. *TF on cells binds small amounts of factor VIIa in circulation and creates more VIIa from VII.*

1.2. *TF bound to VIIa activates factor X and IX to Xa and IXa respectively.*

1.3. *Factor Xa activates factor V (slowly) to Va.*

1.4. *Factors Xa and Va bind on the cell surface to create the prothrombinase complex that cleaves prothrombin into thrombin.*

1.5. *Thrombin can only be created in small quantities on the cell surface of the TF-expressing cell and is quickly mopped up away from the cell surface by circulating tissue factor pathway inhibitor (TFPI) and anti-thrombin (AT).*

Amplification

2.1. Occurs when the TF-expressing cell comes into contact with an activated platelet.

2.2. Factor IXa and small amounts of thrombin produced on the TF-expressing cell reach the activated platelet.

2.3. Factors VIII, IX and V are activated on the surface of the platelet to VIIIa, IXa and Va.

2.4. Factor VIII is thereby released from von Willebrand Factor (vWF), its carrier protein. The released vWF promotes platelet aggregation.

Propagation

3.1. Factors VIIIa and IXa bind together on the platelet surface to form the tenase complex that activates factor X into Xa.

3.2. Factors Xa and Va then bind on the platelet surface and form the prothrombinase complex.

3.3. The tenase complex on the platelet allows for the creation of thrombin from prothrombin on the cell surface.

3.4. Thrombin itself can then activate further platelets.

1.3.3 Overview of fibrinolysis

Fibrinolysis is the process of resolving a clot. Endothelial cells under normal conditions release tissue plasminogen activator (tPA) to maintain blood flow and prevent thrombus formation. tPA works to cleave plasminogen into plasmin, which is capable of degrading fibrin into fibrin degradation products (FDPs) (such as D-dimers). Monocytes also produce a plasminogen activator, urokinase plasminogen activator (uPA). Both activators have an inhibitor: plasminogen activator inhibitor (PAI) -1 and PAI-2 respectively. Monocytes can produce PAI-1 and PAI-2. Endothelial cells produce PAI-1 and tPA simultaneously, and it is the balance between them that determines whether fibrinolysis is promoted or suppressed (see Figure 1.2).

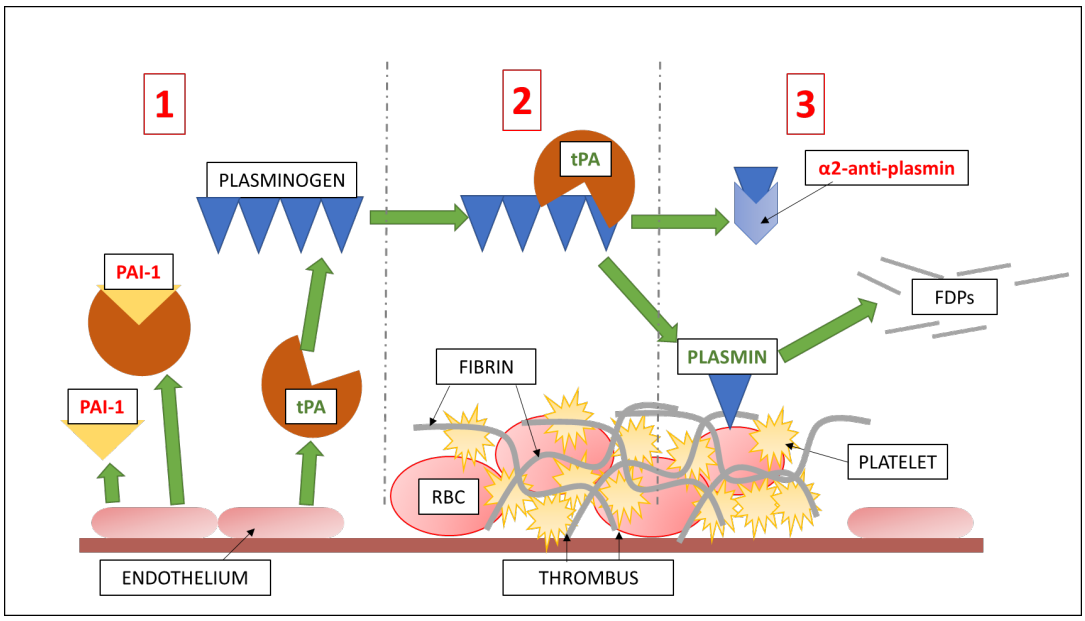


Figure 1.2 A summary of fibrinolysis.

Fibrinolysis is the breakdown of fibrin and is therefore essential for the resolution of thrombus. It is the process of activating plasmin to cleave fibrin into fibrin degradation products (FDPs). Step 1: tissue plasminogen activator (tPA) is released from the endothelium. tPA can be inhibited by plasminogen activator inhibitor (PAI-1). PAI-1 is primarily released by endothelium and a small amount of PAI-1 is released from monocytes. Step 2: tPA cleaves plasminogen into plasmin. Step 3: plasmin cleaves fibrin into fibrin degradation products. α2-antiplasmin works to inhibit plasmin. (RBC – red blood cell).

1.3.4 Thromboinflammation

Innate immunity and coagulation are not separate systems that work in isolation: increasingly, there is evidence of complex interaction between the two (Engelmann and Massberg, 2013). Invertebrates such as insects and crustaceans have not evolved two separate processes, but use a single system called haemolymph coagulation (Muta and Iwanaga, 1996; Dushay, 2009). Haemolymph of the horseshoe crab (*Limulus polyphemus*) contains haemocytes that are very sensitive to LPS; this is an endotoxin that forms part of the cell wall of Gram-negative bacteria and is a potent stimulus for inflammation. Once stimulated, haemocytes release their granular contents and trigger coagulation. Intravascular pathogens become trapped within the forming thrombus whilst anti-microbial substances also released from haemocytes destroy them (Muta and Iwanaga, 1996). How coagulation and immunity cooperate in humans is still to be elucidated.

Considering coagulation initially, thrombin, fibrinogen and fibrin have all been shown to recruit and activate innate immune cells. Thrombin induces the endothelial release of pro-inflammatory cytokines, including tumour necrosis factor (TNF)α, IL-1α and IL-6; this has been shown with *in vitro* and animal models to increase neutrophil migration (Drake *et al.*, 1992). Thrombin is also able to directly activate complement and can cleave both C3 and C5 (Huber-Lang *et al.*, 2006; Krisinger *et al.*, 2012).

Fibrinogen and fibrin have been shown repeatedly to have anti-microbial effects. Evidence from animal models suggests that fibrinogen and fibrin may limit the spread of pathogens intravascularly. Group A *Streptococcus* secretes the enzyme streptokinase, which is highly specific for human plasminogen. A transgenic murine model expressing human plasminogen has a higher mortality rate following subcutaneous group A streptococcal infection when compared to wild-type. This difference in mortality was not evident with intravenous infection. The authors hypothesise that fibrinogen works to prevent spread of pathogens through the vasculature and that streptokinase is a way for bacteria to circumvent this defence. The transgenic mice were shown to have a more widespread distribution of bacterial colonies when compared to wild-type following subcutaneous infection (Sun *et al.*, 2004). Fibrinogen and plasminogen also play a role in *Yersinia petis* infection. Either the creation of a plasminogen deficient murine model or the removal of the *Yersinia* plasminogen activator confers resistance to infection (Degen *et al.*, 2007).

Fibrinogen is able to directly activate innate immune cells. Through direct binding to complement receptor macrophage (MAC)-1 on neutrophils, fibrinogen induced degranulation, an increase in phagocytosis and delayed apoptosis (Rubel *et al.*, 2001). Stimulation of both murine and human monocytic cell lines with fibrinogen lead to an increase in monocyte chemokine secretion. This effect was not blocked by hirudin, a thrombin inhibitor, suggesting that it was fibrinogen rather than fibrin that was responsible (Smiley *et al.*, 2001).

It is challenging to identify the distinct anti-microbial roles of fibrinogen and fibrin. A fibrinogen resistant to cleavage by thrombin was added to a murine fibrinogen knockout model. Plasma from the model was unable to produce fibrin in response to thrombin, although the fibrinogen was still able to induce platelet aggregation. As with fibrinogen deficient mice, there was impaired clearance of *Streptococcus* following an intra-peritoneal injection. Model mice, however, had a lower early mortality rate when compared to the fibrinogen-deficient mice. This work suggests a role for both fibrinogen and fibrin. Potentially, fibrin may limit the spread of infection, but fibrinogen itself also exerts anti-microbial effects (Prasad *et al.*, 2015).

Platelets also appear to have an immune function. For many years it has been known that platelets will aggregate and become activated by the presence of LPS and bacteria (Clawson and White, 1971a; Clawson and White, 1971b; Clawson, 1973; Clawson *et al.*, 1975; Zhang *et al.*, 2009). They are also known to express the toll-like receptor (TLR)-2, TLR-4 and TLR-9 (Cognasse *et al.*, 2005). These receptors are important in the detection of pathogen-associated molecular patterns (PAMPs).

Platelets also have the potential to activate polymorphonuclear cells. Triggering receptor expressed on myeloid cells (TREM)-1 is expressed on neutrophils and monocytes. Following infection, TREM-1 is upregulated and promotes functions such as phagocytosis and IL-8 release (Bouchon *et al.*, 2000). Platelets have been shown to express a ligand for TREM-1. Whilst binding of TREM-1 did not induction platelet activation or aggregation, there was an enhancement in the neutrophil reactive oxygen species production following LPS incubation; this effect was blocked by the presence of either a TREM-1 specific Ab or excess of recombinant soluble TREM-1 (Haselmayer *et al.*, 2007).

Innate immune cells have also been shown to promote coagulation. Several innate immune cells have been shown capable of expressing TF on their cell surface (Giesen *et al.*, 1999;

Darbousset *et al.*, 2012). The role of TF on the monocyte surface will be discussed in more detail later (see section 1.4.4, page 28).

Intra-vital imaging during a mouse model of venous thrombus, induced by restriction of blood flow, demonstrated endothelial adhesion of neutrophils and monocytes prior to the formation of a thrombus. This was not seen in control mice that underwent a sham procedure. Repeating the experiment using P-selectin knockout models prevented not only the accumulation of the leucocytes but also the formation of thrombus. The development of thrombus in this model was TF-dependent. Repeating the experiment using a mouse that received a bone marrow transplant from a TF knockout donor prevented thrombus formation, suggesting it was the TF expressed on blood cells rather than the endothelium that initiated coagulation. Intra-vital microscopy also identified the presence of extracellular DNA 3 hrs following the flow restriction; the DNA was in close proximity to neutrophils. The authors propose that this may be due to release of neutrophil extracellular traps (NETs) and that they may also contribute to thrombus formation (Fuchs *et al.*, 2010; von Bruhl *et al.*, 2012). A knockout mouse deficient in the neutrophil serine proteases elastase and cathespin G shows reduced thrombus formation following carotid artery injury. Compared to wild-type mice, thrombi were smaller, more fragile and with a prolonged time to vessel occlusion (Massberg *et al.*, 2010).

Cooperation between the coagulation and innate immune systems appears to be necessary to fight infection. Potentially, a failure of thromboinflammation may predispose to both the coagulopathy and the immune dysregulation that occurs during sepsis.

1.3.5 Sepsis-associated coagulopathy and disseminated intravascular coagulation

Most cases of sepsis involve a degree of coagulopathy; it occurs in a spectrum from the very mild, detectable only by the most sensitive of laboratory assays, to the clinically evident, presenting with thrombosis and/or bleeding (Levi and van der Poll, 2017). Initially, a systemic activation of the coagulation process leads to the deposition of fibrin, particularly in vessels smaller than 1μm in diameter (micro-vessels). Later, a consumption of coagulation proteins and a reduction in platelet number can lead to haemorrhagic complications. The fibrinolytic system is also more active in sepsis, with an increase in fibrin degradation products such as d-dimer levels and a decrease in the circulating levels of tPA and uPA (Levi *et al.*, 1993).

Approximately 50-70 % of sepsis cases have associated coagulopathy, a third of which meet the criteria for disseminated intravascular coagulation (DIC), a syndrome where the activation of intravascular coagulation occurs in small and medium-sized vessels and is not localised to a specific site of injury. The widespread activation of coagulation occurs simultaneously with both an inhibition of fibrinolysis and a depletion in physiological anticoagulants, leading to intravascular thrombus which contributes to the organ dysfunction associated with sepsis (Gando *et al.*, 2016). Post-mortem studies have shown micro-thrombi to be a frequent presentation of DIC, with the kidney as the most commonly affected organ. Haemorrhage was present in only a minority of cases (Robboy *et al.*, 1972).

There is no specific therapy for DIC, other than to treat the underlying cause. As with sepsis, there had been a difficulty in defining DIC, with multiple definitions and guidelines available. In 2013, a scientific subcommittee of the International Society of Thrombosis and Haemostasis (ISTH) met to combine three guidelines from the British Committee for Standards in Haematology (BCSH), the Japanese Society of Thrombosis and Hemostasis (JSTH), and the Italian Society for Thrombosis and Hemostasis (SISET). Recommendations included the use of a DIC scoring system. Currently three scores are available: the ISTH overt score, which is most diagnostically specific; the Japanese Ministry of Health, Labor and Welfare (JMHLW) score; and the Japanese Association of Acute Medicine (JAAM) score, which is most diagnostically sensitive. All three scores can predict poorer outcomes (Takemitsu *et al.*, 2011; Wada *et al.*, 2013). A comparison of the three scores is given in Table 1.3.

Risk factors	ISTH	JAAM	JMHLW
Underlying disease capable of causing DIC	**0** (essential)	**0** (essential)	**1**
Clinical symptoms	0	**1** if SIRS score ≥3	**1** for bleeding; **1** for organ failure
Platelet count ($x10^9$/L)	**1** for 50-100; **2** for <50	**1** for 80-120 or >30% reduction; **3** for <80 or >50% reduction	**1** for 80-120; **2** for 50-80; **3** for <50
Fibrin-related marker	D-dimer, FDP or SF. **2** > ULN but <5X ULN; **3** > 5X ULN	FDP (μg/mL). **1** for 10-25; **3** for >25	FDP (μg/mL). **1** for 10-20; **2** for 20-40; **3** for >40
Fibrinogen (g/L)	**1** for <1	0	**1** for 1-1.5; **2** for <1
Prothrombin time	Prolonged PT (seconds above normal). **1** for 3-6; **2** for ≥6	PT ratio. **1** for >1.2	PT ratio. **1** for 1.25-1.67; **2** for >1.67
Score needed for diagnosis of DIC	**≥ 5**	**≥ 4**	**≥ 7**

Table 1.3 A comparison of three DIC scores

This table shows the three ISTH-recommended DIC scores; ISTH (Toh and Hoots, 2007), JAAM (Gando et al., 2013) and JMHLW (Wada et al., 2015). (SF – soluble fibrin, ULN – upper limit of normal).

1.3.6 Causes of coagulopathy

As mentioned above, thrombocytopenia is the most common manifestation of sepsis-associated coagulopathy. Causes include decreased production, consumption, splenic sequestration and possibly an increase in haemophagocytosis by mononuclear cells due to the increase in macrophage colony-stimulating factor (MCSF). In a study of fifty patients with sepsis and concurrent thrombocytopenia, haemophagocytosis was found in a bone marrow aspirate of 64 %. Although all individuals had high levels of MCSF, cases with haemophagocytosis had the highest levels (Francois *et al.*, 1997). Other co-existing conditions such as heparin-induced or drug-induced thrombocytopenia may also contribute to low platelet counts (Warkentin *et al.*, 2003; Levi and van der Poll, 2017).

TF is the most important activator of coagulation during sepsis. It is usually expressed only on extra-vascular tissues, but during sepsis it is upregulated on circulating monocytes (see section 1.4, page 27). There are various stimuli that increase the monocytic expression of TF, including TNFα , interleukin (IL)-1β and LPS (Rivers *et al.*, 1975; Conkling *et al.*, 1988; Schwager and Jungi, 1994). The upregulation of TF, and subsequent increase in intravascular thrombin, is believed to be an important cause of DIC. Human and animal models of endotoxaemia, where a low level of endotoxin is infused and then blood samples taken at intervals, demonstrate an initial increase in TNF, followed by a peak in IL-6 and IL-1 levels. These models demonstrate an upregulation of TF expression on the monocyte cell surface, a 125-fold increase in TF mRNA and an increase in thrombin production (van Deventer *et al.*, 1990; van der Poll *et al.*, 1994; Franco *et al.*, 2000; Gando *et al.*, 2016). Use of an antibody to inhibit TF during a primate model of endotoxaemia prevented both the development of coagulopathy and death (Taylor *et al.*, 1991; Levi *et al.*, 1994). The use of such models will be discussed in more detail in section 1.6. The *ex vivo* examination of monocytes from cases of meningococcal infection showed an increase in TF expression in approximately half, two-thirds of which had levels 60-300-fold higher than the rest of the cohort. All individuals with the highest levels died (Osterud and Flaegstad, 1983).

Sepsis-associated coagulopathy also involves an activation followed by an inhibition of fibrinolysis. Human models of endotoxaemia first demonstrated these biphasic changes, a rise in plasminogen activators (tPA and uPA) followed by a more sustained increase in plasminogen activator inhibitor (PAI)-1 (Suffredini *et al.*, 1989; van Deventer *et al.*, 1990). Pre-treatment with blocking antibodies to TNF and IL-6 in a chimpanzee endotoxaemia model greatly attenuated the increase in both plasminogen activators and PAI-1. The

sustained release of PAI-1 ultimately leads to an inhibition of fibrinolysis during DIC (Biemond *et al.*, 1995; Gando *et al.*, 2016). More recent work has also measured increased PAI-1 levels during cases of sepsis (Madoiwa *et al.*, 2006).

The dysregulation is compounded by disruption of the physiological anticoagulant pathways. Deficiencies in proteins C and S and antithrombin all occur during sepsis, due to either a decrease in production or loss from increased vascular permeability.

Antithrombin activity is potentiated up to a thousand-fold by the binding of heparin. The glycocalyx that covers the surface of the endothelium contains multiple heparin-like cofactors that behave in a similar way. During sepsis the production of endothelial glycosaminoglycans is reduced. Use of labelled sulphate molecules during the *in vitro* culture of porcine aortic endothelial cells showed that IL-1β or TNFα was able to reduce the production of heparin sulphate (Kobayashi *et al.*, 1990).

The anticoagulant protein C is activated by binding to endothelial-bound thrombomodulin. Skin biopsies from individuals with dermal purpura secondary to meningococcal sepsis showed a downregulation of both endothelial-bound thrombomodulin and the endothelial protein C receptor when compared to healthy controls (Faust *et al.*, 2001). This downregulation would limit the activity of protein C.

There is no consistent evidence to suggest that TFPI is expressed at a lower level during sepsis, but a baboon endotoxaemia model demonstrates a decrease in mortality by the addition of TFPI (Novotny *et al.*, 1991; Creasey *et al.*, 1993; Gando *et al.*, 2016).

1.3.7 Predicting outcomes from sepsis-associated coagulopathy

The development of coagulopathy is associated with a poorer outcome from sepsis (Fourrier *et al.*, 1992). Analysis of 840 individuals diagnosed with severe sepsis showed a correlation between 28-day mortality, higher SOFA scores and coagulopathy (Dhainaut *et al.*, 2005). Thrombocytopenia at diagnosis of sepsis has also been correlated with organ dysfunction as demonstrated with SIRS and SOFA scores, whilst lower anti-thrombin levels have also been correlated with a higher mortality rate (Fourrier *et al.*, 1992; Ogura *et al.*, 2007).

PAI-1 works to inhibit the breakdown of thrombus. Raised PAI-1 levels (>90ng/mL) at the diagnosis of DIC (using JMHLW scores) have been associated with a higher 28-day mortality

rate and correlated with increasing SOFA scores. Most non-septic cases in this study had an underlying malignancy, two-thirds of which were haematological. This suggests that a failure of the regulation of fibrinolysis plays a potential role in septic DIC (Madoiwa *et al.*, 2006). A recent meta-analysis of 19 studies confirmed this finding. PAI-1 levels appear to be useful in predicting disease severity and mortality in cases of sepsis (Tipoe *et al.*, 2018).

DIC associated with sepsis undoubtedly increases the mortality rate; however, there does appear to be an improvement in survival over the last few decades. Data from Japan recorded in 2010 and 2012 shows an increase in the incidence of DIC associated with sepsis (39.5 % vs 43.3 %) but a decrease in sepsis-associated DIC mortality at 14 days (20.4 % vs 17.9 %) and 28 days (31.1 % vs 27.7 %) (Murata *et al.*, 2014). This likely reflects a general improvement in the care of the critically ill patient (as discussed in section 1.2.2). As with sepsis as a whole, a significant rate of mortality remains.

1.3.8 Efforts to treat sepsis-associated coagulopathy

With regard to DIC management, the mainstay of treatment is to treat the underlying cause, i.e. the sepsis. There is little to no evidence to support replacing deficient coagulation factors or platelets with transfusion, unless there are bleeding complications or platelet counts are lower than 10-20x10^9/L (Wada *et al.*, 2013; Levi, 2016).

Multiple interventional studies, which will now be discussed, have been performed with the aim of improving the outcomes of individuals with sepsis-associated coagulopathy; unfortunately, there has been limited success. Heparin has been investigated not just as a potential anticoagulant but also for its possible role as an anti-inflammatory (Cornet *et al.*, 2007). Initial work using a baboon model of DIC, during which animals were given an infusion of thrombin, showed that pre-treatment with heparin improved both survival and coagulopathy. Treatment with heparin, two hours after the infusion of thrombin, did not correct coagulopathy but did reduce mortality rates to a lesser extent than pre-treatment (du Toit *et al.*, 1991). This model of DIC is significantly limited by the lack of fibrinolytic inhibition, reduction in physiological anticoagulants and possibility of increased bleeding due to the consumption of coagulation factors and platelets. A more recent study used a non-anticoagulant dose of heparin in a mouse model of sepsis (caecal ligation and puncture model, discussed further in section 1.6) and showed an improvement in inflammation and mortality (Wildhagen *et al.*, 2014). Evidence outside of animal models is lacking. A randomised

controlled trial of heparin as a treatment of sepsis-associated DIC is needed; however, this is always going to be hindered by the possibility of increasing a patient's risk of haemorrhage.

More work has been performed to investigate the efficacy of supplementing activated protein C (APC). Preclinical studies showed that recombinant APC prevented coagulopathy (measured by prevention of hypofibrinogenaemia) and reduced mortality in a baboon model of lethal *Escherichia coli* sepsis (Taylor *et al.*, 1987). A later phase 2 study of drotrecogin alfa activated (DrotAA), a recombinant form of APC, showed a dose-dependent reduction in d-dimer and IL-6 levels, suggesting it improved coagulopathy and inflammation (Hartman *et al.*, 1998). A phase 3 double-blinded placebo-controlled trial (PROWESS) followed and was stopped early due to a perceived benefit. It demonstrated a small reduction in any-cause mortality at 28 days (30.8 % vs. 24.7 %) but also an increased risk of bleeding (3.5 % vs. 2.0 %) during the infusion period. Those with the highest Acute Physiology and Chronic Health Evaluation (APACHE) II scores, i.e. the more severe disease, received the most benefit. As with the previous studies, D-dimer levels were significantly lower in the treated group compared to placebo (Bernard *et al.*, 2001). A retrospective review of the phase 3 data was performed using a modified ISTH DIC score. Participants with overt DIC derived a greater benefit from DrotAA (43 % untreated vs. 27 % treated 28-day mortality). Rates of serious bleeding (defined as intracranial bleeding, a need for more than three red cell transfusions or life-threatening bleeding), however, were worse in those with overt DIC (0.9 % untreated vs. 3.0 % treated) (Dhainaut *et al.*, 2004). As the original study was stopped early, some doubt remained regarding the benefit of DrotAA in treating certain populations, such as those with a lower severity of disease (Levi, 2008). A few subsequent studies also cast doubt on the benefit of DrotAA. Abraham *et al*. showed no benefit compared to placebo when it was used in those with less severe disease (APACHE II score <25 or single organ failure) (Abraham *et al.*, 2005). A further randomised placebo-controlled trial was performed (PROWESS-SHOCK); unfortunately, it failed to replicate the earlier results and no improvement was demonstrated in mortality rates at either 28 days or 90 days, but an increased risk of bleeding was shown (Ranieri *et al.*, 2012). Following these results, the use of activated protein C in sepsis in DIC was removed from the guidelines (Thachil *et al.*, 2012). DrotAA is no longer available as a therapeutic.

Another possible therapy is antithrombin (AT) concentrate. Multiple pre-clinical animal models have shown that AT can improve outcomes and coagulopathy, such as a rabbit endotoxaemia model that demonstrated less intravascular fibrin deposition with AT treatment

(Giebler *et al.*, 1999; Opal, 2000). Initially, a small phase 2 study of 35 cases of sepsis-associated DIC demonstrated that AT concentrate shortened the duration of coagulopathy and suggested that there might be a survival benefit from the treatment (Fourrier *et al.*, 1993). A much larger randomised placebo-controlled trial of over 2000 participants with sepsis compared three groups, placebo, AT and AT with heparin. No significant reduction in 28-day mortality was demonstrated in either treatment group, but an increase in bleeding was observed in those participants who received heparin and AT (Warren *et al.*, 2001). As with DrotAA, a retrospective analysis of the data showed a mortality benefit in the subset of participants with a diagnosis of DIC (again using a modified ISTH score) who received AT without concomitant heparin (Kinasewitz *et al.*, 2005). Later meta-analyses suggest a benefit for AT in DIC, but these results must be prospectively investigated before it can be considered as therapy (Wiedermann, 2018). In Japan, however, AT concentrate is recommended for the treatment of sepsis-associated DIC (Nishida *et al.*, 2018).

Despite no consistent evidence that TFPI is lowered during sepsis, there is some limited data to support the use of recombinant TFPI (rTFPI). A phase 2 study of 210 cases, comparing two doses of rTFPI, showed no significant reduction in mortality but suggested that the activation of coagulation was inhibited with a significant reduction in thrombin-antithrombin complexes. As with the previous interventions, there was a trend towards an increase in rates of bleeding, but in this case it was not significant (9 % in treated vs. 6 % untreated, p=0.39) (Abraham *et al.*, 2001). A larger phase 3 study showed no reduction in all-cause mortality at 28 days; however, there was improved survival in participants with an international normalised ratio (INR) of less than 1.2 (12.0 % in treated vs. 22.9 % in untreated). The use of rTFPI increased the risk of bleeding for all groups. Once again, the use of rTFPI did show lower levels of thrombin-antithrombin complex and prothrombin fragments 1+2, suggesting it did inhibit coagulation; this result was seen regardless of the baseline INR (Abraham *et al.*, 2003).

Thrombomodulin, which binds to active thrombin and acts as an important cofactor for APC when bound to endothelium, has also been investigated. A phase 3 double-blind trial compared the use of thrombomodulin to low-dose heparin in cases of DIC associated with either sepsis or haematological malignancy. Further analysis of the studies investigating APC, AT and TFPI suggests a possible improvement in mortality for those in the placebo group who received prophylactic dose heparin (Warren *et al.*, 2001; Abraham *et al.*, 2003). The use of soluble thrombomodulin, when compared to low dose heparin, reduced both the

duration of DIC and the risk of bleeding complications. In the small cohort of sepsis-associated DIC (99 cases), there appeared to be a trend towards reduced mortality with thrombomodulin (28.0 % vs. 34.6 %). In the group that received thrombomodulin, almost all markers of DIC were normalised, including thrombin:antithrombin, D-dimer and PAI-1 (Saito *et al.*, 2007). A further, placebo-controlled, phase 2 trial was performed to investigate the effects of thrombomodulin in cases of sepsis that met the ISTH criteria for DIC. Although thrombomodulin improved markers of DIC as before, there was no reduction in mortality or differences in either thrombosis or bleeding between the two groups. A sub-group analysis, however, did suggest a reduction in 28-day all-cause mortality in cases treated with thrombomodulin who had either cardiac or respiratory dysfunction, an INR higher than 1.4 and a platelet count between 30-150 $x10^9$/L (26.3 % in treated vs. 38.2 % untreated) (Vincent *et al.*, 2013). As with AT concentrate, thrombomodulin is used in Japan. A meta-analysis of all randomised trials and observational studies suggested a trend towards a decrease in mortality but, importantly, no significant increase in bleeding complications (Yamakawa *et al.*, 2019). Further phase 3 studies are needed.

A different approach has been to block the cytokines that are believed to contribute to both the inflammation and coagulopathy of sepsis. A phase 2 placebo-controlled study of a humanised monoclonal antibody against TNFα showed no difference in 28-day all-cause mortality. A serum-sickness like reaction was seen in 4 % of those treated. There was no analysis of coagulopathy specifically although a third of all cases met the criteria for DIC and were spread evenly between treatment and placebo groups (Abraham *et al.*, 1995). An earlier study of the chimpanzee endotoxaemia model showed that infusing a monoclonal antibody of IL-6 had no effect on the induced inflammation but did attenuate the activation of the coagulation system (lower thrombin:antithrombin, plasmin:antiplasmin and prothrombin 1+2 complexes) (van der Poll *et al.*, 1994). A later study of individuals with renal cancer showed that an infusion of recombinant IL-6 led to an activation of coagulation (Stouthard *et al.*, 1996). Infusion of an IL-1 antagonist, anakinra, has also been shown to reduce the markers of coagulopathy in cases of sepsis. Whereas IL-6 and TNFα peak within two hours of endotoxaemia, as shown by various models, IL-1β has appeared unchanged in some models of endotoxaemia and peaked at four hours in others (Andreasen *et al.*, 2008). This calls into question the role of IL-1β in the development of sepsis-associated coagulopathy (van Deventer *et al.*, 1990; Boermeester *et al.*, 1995).

Collectively, these trials suggest that perhaps a more targeted approach is needed. It is certainly ambitious to improve all-cause mortality in all degrees of sepsis-associated coagulopathy, but perhaps investigating specific groups, such as those at lower bleeding risk, or aiming to intervene prior to the full diagnosis of DIC, would be more fruitful. Recently, a retrospective analysis of over 400 cases of sepsis-associated DIC was published. Individuals with a baseline INR ≥1.57 and a SOFA score ≥13 were identified as at high risk of an early death; 83 % died within 3 days (Iba *et al.*, 2018). In the future using such a score may allow particular populations to be targeted.

1.3.9 Summary

There is an urgent need to understand the pathology of sepsis-associated coagulopathy in more detail, to diagnose it promptly and accurately, and to tailor therapies to improve the outcome for affected patients.

1.4 Tissue Factor

1.4.1 Overview

As already highlighted, TF plays an important role in activating the coagulation system and potentially the coagulopathy associated with sepsis. This section will discuss the roles of TF further.

1.4.2 The discovery of tissue factor

In the early 1900s, Morawitz proposed the 'classical theory of blood coagulation', describing how prothrombin, thrombin, calcium and fibrinogen are necessary for the formation of a blood clot. It was in 1905, however, that he published his seminal work *Die Chemie der Blutgerinnung* (The Chemistry of Blood Coagulation); for the first time, a substance in tissue was identified that activated clotting. Morawitz named it 'thrombokinase', but this would later be known as TF (Morawitz, 1905; Morawitz, 1958; Boulton, 2006). It would take almost another eighty years until TF was purified, initially from bovine and then from human tissue (Bach *et al.*, 1981; Broze *et al.*, 1985). This was swiftly followed by full sequencing of the TF gene, localisation to chromosome 1 and identification of the protein structure by several groups (Morrissey *et al.*, 1987; Scarpati *et al.*, 1987; Spicer *et al.*, 1987; Mackman *et al.*, 1989). TF has three domains: extracellular, transmembrane and intracellular. The extracellular domain, (the NH_2-terminal, residues 1–219) appears to fold in a similar way to the cytokine receptor superfamily, with seven β strands arranged as two β sheets; the primary protein sequence and structure appear similar to receptors for growth hormone or

erythropoietin. The transmembrane domain (residues 220–242) serves to anchor TF to the cell membrane, whereas the intracellular domain (the COOH-terminal, residues 243–263) has a role in cell signalling (Ruf and Edgington, 1994; Butenas, 2012).

1.4.3 Tissue factor: an activator of the coagulation system

TF is a 47kDa trans-membrane glycoprotein expressed predominantly by extra-vascular cells such as fibroblasts, creating what is frequently termed 'the haemostatic envelope' around the vasculature (Drake *et al.*, 1989). By binding to factor FVIIa on a cell surface, it serves to initiate coagulation where there is a vessel wall injury (ten Cate *et al.*, 1993). No other TF ligands, other than FVII and FVIIa, have been identified. TF, once bound to FVIIa, has been shown to activate factors X (FXa), FIX (FIXa) and FXI (FXIa) (Mackman, 2004). A full description of the cell-based coagulation model is given in Figure 1.1, but, briefly, the prothrombinase complex (a complex of activated factors V and X) works to cleave prothrombin to thrombin. This thrombin is quickly inhibited by antithrombin; to overcome this, more FXa is created by the tenase complex (activated factors IX and VIII). TF is therefore able to contribute to both the prothrombinase and tenase complexes and in doing so is integral to the activation of coagulation.

TF is inhibited primarily by TFPI, which serves to inhibit the binding of FVIIa to TF and the formation of the prothrombinase complex (see section 1.3.2, page 8). Two isoforms have been identified: TFPIα and TFPIβ. Both can inhibit the binding of TF to FVIIa, but only TFPIα also inhibits the prothrombinase complex (Wood *et al.*, 2013; Maroney and Mast, 2015). TFPIβ is predominantly expressed on the endothelial cell surface, whilst TFPIα is secreted from platelets (Maroney *et al.*, 2013). Platelets contain TFPIα and it is released when they are activated, such as by thrombin. The TFPIα in platelets appears to be important in controlling the formation of intravascular thrombus; TFPIα knockout mice showed an increase in thrombus volume following vascular injury when compared to wild-type mice (Maroney *et al.*, 2011).

1.4.4 Tissue factor expression on circulating monocytes and 'encryption'

Aside from contributing to the haemostatic envelope, it is now accepted that TF can be expressed on the surface of circulating monocytes; however, unless it is induced, this is at very low level (Osterud, 2012). The role of monocytic TF expression is poorly understood, but it may have a role in thromboinflammation (see section 1.3.4, page 16). The induction of TF expression on the monocyte surface has been shown with multiple agents including LPS,

C-reactive protein and P-selectin (Rivers *et al.*, 1975; Cermak *et al.*, 1993; Celi *et al.*, 1994). There is, however, great variability in TF expression between individuals, and so-termed 'hyper-responders', who express higher levels, have been associated with diseases that include a higher risk of thrombosis (Egorina *et al.*, 2005).

Maynard *et al*. first demonstrated that TF was present on cells within the circulation and appeared to be associated with the cell surface; however, they also noted the need to cause disruption of the membrane to increase activity (Maynard *et al.*, 1975). It is now accepted that the majority of cell-bound TF is supressed through the post-translational mechanism of 'encryption' within the cell membrane. Whilst encrypted TF is still bound to factor VIIa, and thereby demonstrates some procoagulant activity, this is much lower compared to 'decrypted' TF (Bach, 2006; Kothari *et al.*, 2013). The process of encryption is yet to be elucidated, but for many decades it has been recognised that phospholipids in the cell membrane affect the activity of TF and the initiation of coagulation (Studer, 1946; Nemerson, 1968; Jones *et al.*, 1985; Mann *et al.*, 1990). There are several theories to explain the possible role of lipids in encryption. The most widely accepted theory is that of phosphatidylserine (PS) asymmetry: usually this lipid remains in the inner cell membrane, but when it is externalised to the outer membrane it has been shown to increase the activity of TF. The organisation of TF within the cell membrane is likely also to be important. Other more recent work suggests a suppressive role for sphingomyelin in the cell membrane and the reduction/oxidation of a cysteine disulphide bond with the TF extracellular domain. All of these will now be discussed.

Phosphatidylserine (PS) is maintained by ATP transport on the inner cell membrane. *In vitro* work has shown that when PS becomes extracellular there is an increase in cell-bound TF activity (Forman and Nemerson, 1986). PS can be moved to the outer membrane in several ways *in vitro,* including by freezing and by increasing intracellular calcium. Several *in vivo* mechanisms have also been discovered, including the activation of platelets by thrombin leading to an increase in cytosolic calcium and possibly therefore to decryption (Bach and Moldow, 1997). There are three enzymes that are known to transport phospholipids in the cell membrane: flippase (which moves them inward), floppase (which moves them outward) and scramblase (which can rapidly break down the usual membrane asymmetry). Scramblase has been shown to increase the binding of Annexin V to the outer surface of activated platelets in a calcium-dependent manner (Bevers *et al.*, 1983; Zwaal *et al.*, 2005). Another mechanism, *in vivo*, can be seen in apoptotic cells, which are known to increase PS on the cell surface (Bach, 2006). Exactly how PS may decrypt TF is unclear, but a possibility is that PS

interrupts the 'self-association' of TF – the formation of homodimers in the presence of a ligand (Roy *et al.*, 1991; Aras *et al.*, 2004; Bach, 2006). Alternatively, perhaps, an increase in PS on the outer surface increases the binding of Xa (Rao *et al.*, 2012). Multiple diseases that have a thrombotic component have been associated with an increase in outer cell PS, including the red blood cells and platelets in diabetes mellitus and the 'sickled' red blood cells of sickle cell disease (Zwaal *et al.*, 2005).

Lipid rafts, cholesterol-rich microdomains within the outer cell membrane, have also been identified as potentially important in maintaining TF encryption. These rafts are composed primarily of sphingolipids and cholesterol in the outer cell membrane that connect with phospholipids and cholesterol within the inner membrane. They are organised structures that can move around the membrane, are more densely packed than surrounding regions and can cluster together (Simons and Ehehalt, 2002). Disruption of lipid rafts has been demonstrated to increase the activity of TF without a corresponding increase in the proportion of cell-bound TF or an increase in cell lysis (Dietzen *et al.*, 2004; Mandal *et al.*, 2005). Rafts have also been implicated in the management of intracellular calcium. Disruption of rafts by the removal of cholesterol has been shown to decrease the release of calcium from intracellular stores, increase the externalisation of PS and increase the procoagulant activity of affected cells (Kunzelmann-Marche *et al.*, 2002).

A more controversial theory involves the regulatory effect of protein disulphide isomerase (PDI) on thiol residues at the TF Cys186-Cys209 disulphide bond. There are two disulphide bonds within the extracellular domain of TF: one of these, Cys186-209, has been shown to be necessary for TF to demonstrate procoagulant activity (Rehemtulla *et al.*, 1991). It is hypothesised that unpaired thiol residues at Cys186 and 209 create an encrypted structure, whereas the creation of a disulphide bond decrypts the TF and increases activity. Chen *et al.* demonstrated *in vitro* that the addition of mercuric chloride, which oxidizes dithiols to disulphides, increased the activity of TF, whereas blocking unpaired cysteine thiols led to a decrease in TF activity. They suggest that PDI breaks the disulphide bond and may thereby control TF encryption and activate the TF procoagulant activity. The authors, however, were unable to demonstrate an excess of free cysteine thiols, which would be expected as the majority of cell-bound TF is believed to be encrypted (Chen *et al.*, 2006). This is supported by a murine model of vascular damage: microparticles expressing TF were injected into the damaged area, leading to an increase in fibrin. The addition of an anti-TF antibody prevented fibrin formation, as did the addition of an anti-PDI antibody. PDI was visualised around

activated platelets within the forming thrombus and vascular smooth muscle cells, suggesting its release following injury or platelet activation (Reinhart *et al.*, 2013). There is also some evidence for this mechanism in monocytes. It has long been recognised that the use of anti-thymocyte globulin (ATG) to deplete lymphocytes (such as prior to allogenic stem cell transplant) leads to activation of coagulation. ATG was shown to increase the TF activity of monocytes *in vitro*, but not through an increase in cell surface PS. Instead the increase in TF activity was prevented by blocking the number of free thiols (Langer *et al.*, 2013).

There are several problems with this theory, however. The addition of an oxidising agent such as mercuric chloride may increase the surface expression of anionic phospholipids such as PS; this could therefore explain the increase in TF activity without the creation of a disulphide bond. There has also been no conclusive evidence that PDI associates with TF, and human endothelial cells transfected with mutant TF that does not contain the Cys186-209 bond have a similar ability to activate coagulation *in vitro* (Pendurthi *et al.*, 2007; Kothari *et al.*, 2013). Finally, *in vitro* work with human endothelial cells demonstrates that inhibition of PDI can be procoagulant by increasing the cell surface expression of PS independent of calcium (Popescu *et al.*, 2010). Further work is needed, primarily to identify how PDI may associate with TF, but this remains an intriguing possibility.

Recent work has suggested that the presence of sphingomyelin in the outer cell membrane may work to 'dampen down' or encrypt TF. Sphingomyelin is a phospholipid comprising up to 50 % of the outer cell membrane (Wang *et al.*, 2017). Wang *et al.* have shown in macrophages that the hydrolysis of sphingomyelin through the action of sphingomyelinase increases TF activity. Through stimulation with adenosine triphosphate, acid-sphingomyelinase was shown to translocate to the outer cell membrane without an increase in the proportion of PS (Wang *et al.*, 2017). This suggests that perhaps sphingomyelin acts to suppress TF activity and maintain it in an encrypted state.

Whilst much remains to be understood in relation to encryption, it certainly appears to be an important physiological process that is necessary to control TF activity on circulating blood cells.

1.4.5 The role of monocyte surface TF expression in coagulation

As discussed, TF is expressed on cells other than monocytes including activated endothelium. Several pre-clinical studies have investigated the specific contribution of TF expressed on the

surface of monocytes. Lethally irradiated mice were transplanted with haematopoietic cells from mice that did not express TF. They were compared to wild-type mice transplanted with normal haematopoietic cells. Both groups were injected with intra-peritoneal LPS and had markers of coagulation measured at 3 hrs. Although both groups showed an increase in the thrombin-antithrombin (TAT) complex, the level was higher in the wild-type group that had an also demonstrated an increase in haematopoietic cell TF expression of (Schoenmakers *et al.*, 2004). A floxed mouse model that, with a specific myeloid promotor, deleted the expression of TF in myeloid cells also demonstrated a reduction in TAT following endotoxaemia. The group did not detect TF mRNA in the murine platelets (both unstimulated and stimulated), but did demonstrate it from blood leucocytes. Further work using a Tie-2 promoter created a mouse with a > 95 % reduction in TF on all cell types (including endothelial). By transplanting them with bone marrow from control mice, a model was created that did not express endothelial TF but did express haematopoietic TF. Selective deletion of TF expressed on the endothelium did not reduce the TAT levels following endotoxaemia. This work demonstrated that the TF expressed on leucocytes, and not the endothelium or platelets, that activates coagulation following endotoxaemia (Pawlinski *et al.*, 2010). The role of TF on platelets has been debated between groups. Although several groups have reportedly detected TF mRNA and protein in human platelets, this is disputed by other groups who have failed to replicate these results (Zillmann *et al.*, 2001; Butenas *et al.*, 2005; Østerud and Bjørklid, 2006; Pawlinski and Mackman, 2010).

Later work involved using the Berkley murine model of sickle cell disease (BERK), a model known to express higher levels of TF on leucocytes compared to controls. The leucocytes found to express TF on their surface were identified morphologically as monocytes. Transplanting the Tie-2 flox model with cells from the BERK mouse again created a model that expressed TF only on haematopoietic cells. Coagulation markers were higher in the BERK transplanted model and not reduced by removing the non-haematopoietic expression of TF (Chantrathammachart *et al.*, 2012).

These pre-clinical data support the idea that TF expressed on the monocytic surface is functionally active.

1.4.6 High and low responders to the LPS induction of monocytic TF cell surface expression

The monocytic cell surface expression of TF, following LPS stimulation, varies considerably between individuals. This phenomenon was first noted by Ossterud in 1995: whole blood

samples were taken from 135 healthy volunteers and stimulated *ex vivo* with 5 ng/mL LPS for 2 hours prior to the measurement of TF activity. Two groups of individuals were identified, those with a high response and those with a low response; the TF activity was shown to vary between individuals by as much as 50-fold (Ossterud, 1995). Later work by the same laboratory used flow cytometry to measure TF cell surface expression on a CD14 positive cell population and demonstrated a high and low responder group; once again, high and low responder groups were defined by the level of TF activity.

The cause of this difference in response is not fully understood. The presence of platelets seems to increase the LPS induction of TF. Ossterud *et al*. (1990) used blood preparations that contained 15 % of the original platelet count; adding platelets from a low responder to a high responder sample resulted in a lower TF activity when compared to the addition of autologous platelets (Osterud *et al.*, 1990). The same group also demonstrated that the monocytic TF activity induced with LPS was reduced by two thirds when the monocytes were suspended in platelet-poor plasma compared to platelet-rich plasma. Other work has suggested that granulocyte stimulation may increase monocytic TF activity; this effect was reduced by blocking CD15 (a p-selectin ligand). Potentially, an interaction between granulocytes, monocytes and platelets enhances the LPS induction of monocyte surface expression of TF (Osterud *et al.*, 1990; Halvorsen *et al.*, 1993; Ossterud, 1995).

More recent work has also demonstrated the ability of red blood cells to increase monocyte TF activity following LPS stimulation in a dose-dependent manner. Comparing the red blood cell counts of healthy human volunteers to their monocyte TF activity, however, failed to show a clear correlation (r=0.199, p=0.001 using Pearson's co-efficient). The group used a mouse model of endotoxaemia to investigate the monocytic cell surface expression of TF. Mice were given an intraperitoneal injection of LPS and blood was collected from the inferior vena cava five hours post injection. There was an increase in TF surface expression on murine monocytes at five hours following injection when compared to controls injected with phosphate-buffered saline. This effect was abolished by the use of Duffy anti-gen receptor for chemokines (DARC)-null mice (Østerud *et al.*, 2015). DARC is a red blood cell receptor that varies in expression between populations; it is present in a high proportion of white Caucasians but a much smaller proportion of sub-Saharan African (Meny, 2010). DARC is a receptor for a range of pro-inflammatory chemokines including monocyte chemoattractant protein (MCP)-1 and IL-8 (Gardner *et al.*, 2004; Meny, 2010).

Whether a high versus a low response affects clinical outcomes or predisposes to thrombotic disease is not yet known. A small group of 54 individuals with a family history, but not a personal history, of myocardial infarction were investigated. Only a small proportion of individuals with high cholesterol were considered high responders (10 %) whereas those with normal cholesterol levels had a much higher proportion of high responders (55 %). There was no control group without a family history of myocardial infarction (Østerud *et al.*, 2002). Further work is needed before any conclusions can be drawn.

1.4.7 The pathway of lipopolysaccharide-induced tissue factor expression

The induction of TF expression on monocytes by LPS has long been recognised (Drake *et al.*, 1989; Gregory *et al.*, 1989); however, the pathway(s) by which LPS induces TF gene expression has not fully been elucidated, and particularly not in relation to monocyte subsets. LPS is known to bind to the lipopolysaccharide-binding protein and then to CD14 and TLR-4. Nuclear factor (NF)–κB, extracellular signal–regulated kinase (ERK) and c-Jun amino terminal kinase (JNK) pathways are all activated by LPS and have all been associated with TF transcription (Dokter *et al.*, 1993; Groupp and Donovan-Peluso, 1996; O'Connell *et al.*, 1998; Guha *et al.*, 2001; Bode and Mackman, 2014). Studies in THP-1 cells, a human monocytic cell line, have demonstrated that LPS is able to increase TF mRNA and increase the cell surface expression of TF (Bode and Mackman, 2014).

Multiple regions within the TF promoter have been identified that are suitable for transcription factors to bind (see Figure 1.3). Proximally there are two Sp-1 sites and three overlapping Sp-1/Egr-1 sites that appear important for basal TF expression (Mackman, 1995). Egr-1 is also required for maximal TF expression; inhibition of the Egr-1 binding site within the TF promoter led to a 72 % reduction in TF expression (Guha *et al.*, 2001). Distally, a 56-base pair LPS responsive element (LRE) has been identified through the work of Mackman *et al.*, using the THP-1 cell line. The LRE comprises two AP-1 sites and a NF-κB binding site, both of which are needed for full LPS-induced transcription (Mackman *et al.*, 1991; Mackman, 1995). The NF-κB site, interestingly, does not match the κB DNA consensus sequence and binds a c-Rel/p65 heterodimer. There is also a suggestion that an interaction between this heterodimer and AP-1 is required for TF to be fully transcribed following LPS stimulation. Increasing the distance between the NF-κB and AP-1 binding sites prevented LPS-induced TF transcription (Glover and Harrison, 1995; Parry and Mackman, 1995; Oeth *et al.*, 1997).

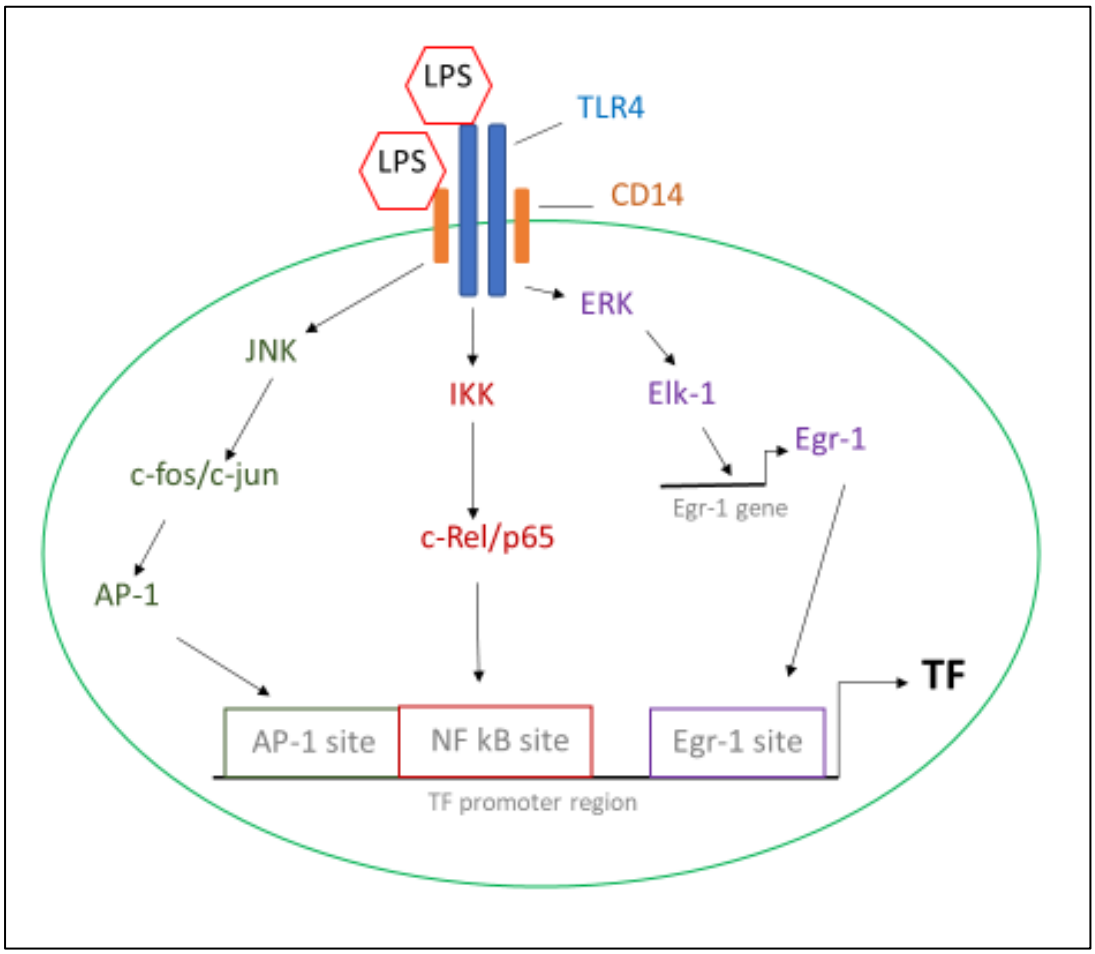


Figure 1.3 LPS-induced TF expression.

Figure 1.3 demonstrates the three transcription pathways associated with the lipopolysaccharide (LPS) induction of TF on monocytes. The AP-1 and NF-κB binding sites make up the lipopolysaccharide-responsive element. The Egr-1 site appears to be important in the maximal expression of TF.

1.4.8 Innate immune training and TF

Over the last decade there has been increasing evidence to support the idea of the innate immune system having a memory, with the potential for innate cells to be 'trained' (Kurtz, 2004). Initial evidence came from the study of invertebrates that do not have an adaptive immune system. Recovery from infection in a crustacean was shown to relate to previously encountered pathogens. If there had been previous infection with a microbe with similar molecular patterns the crustacean was more likely to recover from the infection (Kurtz and Franz, 2003). Witteveldt and colleagues also demonstrated that oral vaccination against a virus was possible in shrimp; a reduction in mortality was demonstrated, although the effect was significantly reduced at 21 days (Witteveldt *et al.*, 2004). The mechanisms underpinning this 'memory' are still unclear, although it has been hypothesised that it may be due to long-lasting upregulation of molecules that recognise the pathogen (Kurtz, 2004). Murine models have demonstrated the ability of PAMPs to induce immune training in monocytes. Mice that lacked both B and T lymphocytes were shown to have protection from a lethal dose of *Candida albicans* following prior infection with a sub-lethal dose. This protection did not occur in monocyte deficient mice (Quintin *et al.*, 2012).

Several human studies have also supported the concept of innate training. Following Bacillus Calmette-Guérin (BCG) vaccination in healthy volunteers, isolated monocytes stimulated with LPS demonstrated an upregulation in PRRs and an increase in pro-inflammatory cytokines for up to 12 months (Kleinnijenhuis *et al.*, 2014). Epigenetic study of the isolated human monocyte that had been 'trained' with β-glucan (part of the fungal cell wall) showed changes in histone H3 acetylation. Looking at the transcriptome revealed an upregulation of genes associated with macrophages (Saeed *et al.*, 2014). Later work demonstrated that LPS could induce macrophage epigenetic modification of histone H3 via cyclic AMP-dependent transcription factor (ATF)-7, which conferred resistance to *Staphylococcus aureus* infection (Yoshida *et al.*, 2015).

Potentially, the monocytic surface expression of TF could be a consequence of innate immune training.

1.4.9 TF expression and its role in sepsis

For several decades, TF has been associated with the development of coagulopathy in sepsis (Osterud and Flaegstad, 1983). Several animal models have demonstrated an association between TF and DIC. The injection of an anti-TF antibody to a rabbit model of

endotoxaemia, prior to the injection of LPS and TF, prevented the formation of coagulopathy seen in those not given the antibody (via a decrease in fibrinogen and factors V and VIII) (Warr *et al.*, 1990). A similar model in baboons showed a decrease in mortality following endotoxaemia in animals given an antibody to block TF (Taylor *et al.*, 1987). This work was then extended by the same group using an injection of TFPI prior to a lethal infusion of *Escherichia coli* in a baboon model; in this case the TFPI prevented death from sepsis in comparison to the control group, which did not receive TFPI and had 100% mortality (Taylor *et al.*, 1991). Later work blocking the active site of FVIIa reduced coagulopathy but only prevented death in half of the cases. TFPI prevents the formation of both the TF:FVIIa complex and also the prothrombinase complex. This suggests that it is the complex of TF:FVIIa that is most important and that it may play a role in not just coagulation but also the inflammatory response to sepsis (Taylor *et al.*, 1998).

TFPI has been shown to improve survival in other models, including a rabbit model of Gram-negative peritonitis in which recombinant TFPI was administered four hours after the induction of peritonitis, and several murine models of superantigen-induced shock and polymicrobial intra-abdominal sepsis (Camerota *et al.*, 1998; Opal *et al.*, 2001). As previously discussed, however, the use of recombinant TFPI had no effect on mortality when trialled in humans (see section 1.3.9, page 25) (Abraham *et al.*, 2003).

As mentioned earlier, there has been very little investigation of the high and low responder phenomenon in thrombotic illnesses and none looking particularly at sepsis. Potentially a high response during sepsis could predispose to the development of sepsis-associated coagulopathy, allowing for the identification of at-risk individuals and a stratified approach to management. Such a stratification has been used in other aspects of critical illness. An excellent example is that shown by the work of Calfee *et al.* (2015), who have identified biomarkers that can be used to distinguish direct (due to pneumonia or aspiration) and indirect (e.g. non-pulmonary sepsis) forms of acute respiratory distress syndrome (ARDS) (Calfee *et al.*, 2015). Each form of ARDS has been associated with varying clinical outcomes and mortality rates (Luo *et al.*, 2017). Future clinical trials can therefore investigate the efficacy of new therapies in each subtype. Hinds *et al.* have taken a similar approach to sepsis, identifying four endotypes using the expression of 140 genes. One of the four endotypes was associated with both 28-day and 1-year mortality (Scicluna *et al.*, 2017).

1.4.10 Summary

TF undoubtedly plays a role in the development of sepsis-associated coagulopathy and in inflammatory responses. Further work is needed to fully understand its effects with the hope that future interventions may improve the outcome of individuals with sepsis.

1.5 Monocyte subsets

1.5.1 Overview

Over the last few decades, monocytes have been divided into subsets that are believed to be functionally different. The development of subset classification, the role of each subset and the implications this has during sepsis will now be discussed.

1.5.2 Classification of the three subsets

Monocytes vary by morphology and function and there have been many attempts to divide them into sub-populations. Initially this was done by size and shape using elutriation. Two populations were defined: small and large. They differed in reactive oxygen species production, how they responded to stimuli such as LPS, and their degree of antigen presentation (Yasaka *et al.*, 1981; Esa *et al.*, 1986; Turpin *et al.*, 1986; Shiotsuki *et al.*, 1987). As well as using morphology, adhesion to fibronectin was used to separate monocytes into subpopulations (Owen *et al.*, 1992). These methods were fraught with difficulty: monocytes needed to be isolated prior to subset identification, which might alter their function; it was not possible to ensure there was no contamination with smaller cell populations such as dendritic cells; and these methods varied by the skill of the operator (Ziegler-Heitbrock, 1996b).

An alternative method for dividing monocytes into subsets is the use of cell markers. Three populations were first identified by Passlick *et al*. using the markers CD14 (an LPS co-receptor) and CD16 (a low affinity Fc gamma immunoglobulin receptor, FcγRIII) (Passlick *et al.*, 1989). The use of markers reduces the risk of contamination with non-monocytes and does not require cell isolation prior to identification (Ziegler-Heitbrock, 1996b; Ziegler-Heitbrock *et al.*, 2010a). In 2010, nomenclature for monocyte subsets was internationally agreed and approved by the Nomenclature Committee of the International Union of Immunological Societies (see Table 1.4). Three human subsets were identified: classical (CD14++, CD16-), the major subset, accounting for approximately 85 % of circulating monocytes; intermediate (CD14++, CD16+), accounting for 5 %; and non-classical (CD14+, CD16++), accounting for 10 % (see Figure 1.4). This nomenclature is useful to help compare

studies and avoids problematic descriptions such as ‘inflammatory monocytes’ (Ziegler-Heitbrock *et al.*, 2010a; Wong *et al.*, 2011). Most of the work performed from this time has considered only two monocyte subsets, classical and non-classical; there is great variability between authors regarding how the intermediate subset was determined. It was either ignored completely or incorporated within the non-classical population. For clarity, all further discussion of subsets will be based on the internationally agreed nomenclature unless otherwise stated.

	Human subsets			Murine subsets		
	Classical	Intermediate	Non-classical	Classical	Intermediate	Non-classical
Markers included in international classification	CD14++ CD16-	CD14++ CD16+	CD14+ CD16++	Ly6C++ CD43+	Ly6C++ CD43++	Ly6C+ CD43++
Other markers	CCR2+ CXCR1+ CXCR2+ CLEC4D+ IL-13Rα1+	HLA-ABC+ HLA-DR+ CD40+	CD115+ CD294+ Siglec10+	CX3CR1+ Gr1++		CX3CR1++ Gr1+

Table 1.4 Markers of human and murine monocytes used to identify subsets

This table lists markers that differ between the subsets, based on the international standard and others. + means this marker is expressed at a level at least 10-fold higher than isotype control, whereas ++ means an expression at least 100-fold higher. (CLEC4D - C-Type Lectin Domain Family 4 Member D, CXRCR1 - CX3C chemokine receptor 1, Gr - glucocorticoid receptor, HLA – human leukocyte antigen, Ly6C - Lymphocyte antigen 6 complex locus G6D, Siglec10 - Sialic acid-binding Ig-like lectin 10)

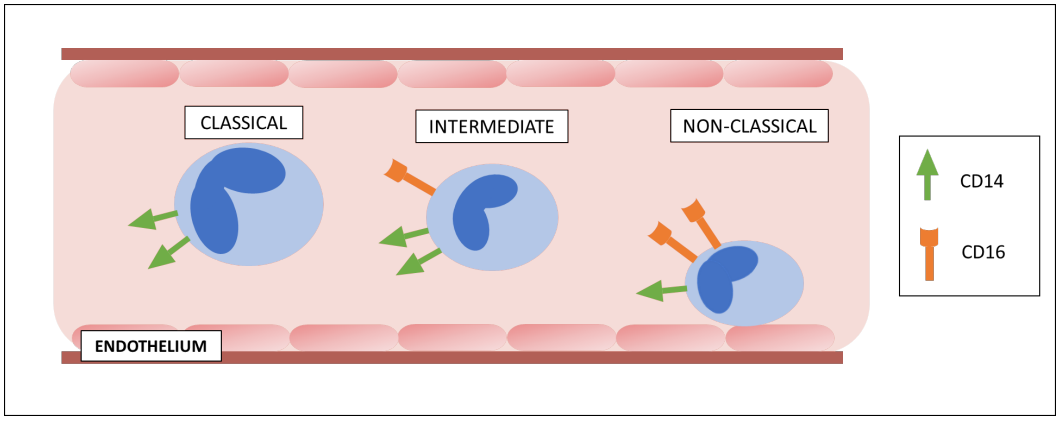


Figure 1.4 Human monocyte subsets.

This figure shows the internationally accepted nomenclature for monocyte subsets as agreed in 2010 (Ziegler-Heitbrock et al., 2010a). Non-classical monocytes are morphologically smaller than classical. Non-classical monocytes are known to 'patrol' the endothelium (Auffray, 2007; Cros et al., 2010).

Some significant limitations to this classification remain. Firstly, they refer to monocytes in 'a steady state', whereas CD markers may change during conditions such as inflammation or potentially through the method of monocyte isolation (Zhou *et al.*, 2012; Mukherjee *et al.*, 2015). Secondly, there is increasing evidence that monocytes can transition between subsets (classical to intermediate to non-classical); this can make the distinction between subsets challenging, particularly as the gating of flow cytometry is operator-dependent and it is challenging to separate the intermediate and non-classical subsets (Yona *et al.*, 2013). To some extent this can be improved through use of analysis software such as t-distributed stochastic neighbour embedding (tSNE) where similar cells are clustered together (Maaten and Hinton, 2008).

Following the definition of the three subsets, there have been additional markers proposed to further define the CD16+ subsets. This include TIE2 (angiopoietin receptor) and Slan (a carbohydrate modification of PSGL-1). Studies of tissue repair following limb ischaemia suggest a majority group of non-classical monocytes that express TIE2 at times of critical ischaemia in humans and decrease again following recovery. The expression of the Slan marker has been shown to be decreased on the intermediate subset in conditions such as sarcoidosis (Patel, 2013; Hofer *et al.*, 2015). This suggests that these markers may be useful to define the subsets more accurately, and they may have clinical implications.

1.5.3 Mouse monocyte subsets

In 2001, Palframan *et al.* first discussed the possibility of mouse monocyte subsets, during an investigation of monocyte recruitment to lymph nodes (Palframan *et al.*, 2001). Since then, mouse monocyte subsets have been shown to be homologous in some respects to human subsets. The use of murine models has been very helpful for investigating monocyte subsets. Nomenclature for mouse monocyte subsets was also suggested in 2010, using the markers Ly6C (lymphocyte antigen 6C, a haematopoietic differentiation antigen) and CD43 (cell surface sialoglycoprotein, a member of the surface mucin family). The subsets are defined as classical (Ly6C++, CD43+), intermediate (Ly6C++, CD43++) and non-classical (Ly6C+, CD43++) (Ziegler-Heitbrock *et al.*, 2010a). Unlike human monocytes, there are equal numbers of all subsets (Geissmann *et al.*, 2003; Sunderkotter *et al.*, 2004). As with human monocyte subsets, mouse classical monocytes are recruited through CCR2 and non-classical through CX3CR1 (Nahrendorf *et al.*, 2007).

A comparison of human and monocyte subset genetic profiles revealed 269 human and 561 mouse genes that were differentially expressed with a greater than two-fold difference between subsets. An algorithm to rank genes between species for similarity was used to identify genetic conservation between species. Both classical and non-classical subsets showed similar genetic profiles between human and mouse monocytes. Staining for cell surface markers appeared to confirm that these similarities were also present at the protein level (Ingersoll *et al.*, 2010).

A model commonly used to visualise mouse monocytes uses $CX3CR1^{GFP/+}$ mice. The use of green fluorescent protein (GFP) associated with CX3CR1 allows the two subsets (CX3CR1++/Ly6C+, non-classical and CX3CR1+/Ly6C+, classical) to be visualised using intra-vital microscopy. This method was used during a murine model of skeletal muscle injury and showed again that classical monocytes were recruited first to the site of injury but several days later non-classical monocytes predominated. The classical monocytes were shown to produce TNF-α & IL-1β when compared to the non-classical, which produced more IL-10 and TGF-β1 (Arnold *et al.*, 2007). A murine model of intra-peritoneal infection using *Toxoplasma gondii* once again demonstrated an influx of classical monocytes; repeat with a $CCR2^{-/-}$ knockout mouse model abrogated the recruitment of classical monocytes to the site of infection and led to increased mortality (Robben *et al.*, 2005). These studies suggest that the classical subset is important in fighting infection.

In a murine model of myocardial ischaemia, different and complementary functions were identified. Classical monocytes in the peripheral blood were predominant for the first five days, following coronary artery ligation, whilst non-classical predominated from day five and remained at higher than normal levels until day sixteen. In the tissue, classical monocytes – but not non-classical – showed a high proteinase activity and expressed TNF-α. Non-classical monocytes expressed a high level of vascular endothelial growth factor (VEGF) compared to classical. This strongly suggests different functional roles for each subset. The authors suggest that, potentially, the classical monocytes assist in phagocytosing damaged tissue, whereas the non-classical may be needed for tissue repair (Nahrendorf *et al.*, 2007). There may be a role for classical monocytes in the development of atherosclerosis. Apolipoprotein $(apoE)^{-/-}$ mice fed a high-fat diet, a murine atherosclerotic model, showed a monocytosis with a proportionate expansion of classical monocytes that infiltrated developing plaques. Non-classical monocytes were also shown to be capable of infiltrating plaques, but, interestingly,

without the need for CX3CR1, which was instead used by classical monocytes along with CCL2 and CCR5 (Tacke *et al.*, 2007).

More recent work also suggests a role of non-classical monocytes in tissue repair. Using a model of skeletal muscle injury in a $CX3CR1^{GFP/+}$ mouse, both monocyte subsets were recruited to the site of injury, reaching a plateau level at 7 days. Increasing the proportion of non-classical monocytes promoted muscle fibre regeneration and decreased fibrosis (San Emeterio *et al.*, 2017).

Auffray *et al.* investigated the movement of the subsets further. Using the $CX3CR1^{GFP/high}$ mouse, they showed that $CX3CR1^{high}/Gr1^{-}$ monocytes 'crawled' along the endothelium. This movement was not dependent on blood flow and these monocytes were confined to a particular area, hence the author's description of this crawling as 'patrolling'. This movement was abrogated in CX3CR1-deficient mice and in mice where either CD11b or CD18 were blocked. In response to tissue damage, the $CX3CR1^{high}/Gr1^{low}$ subset extravasated rapidly and prior to PMNs (Auffray, 2007). A limitation of this study, and others, is the use of the Gr-1 epitope on Ly6C. This should ideally be avoided when identifying monocyte subsets, as it occurs on both Ly6C and Ly6G, but monocytes only express the former (Geissmann *et al.*, 2003).

Mice injected with *Listeria monocytogenes* experience a monocytopenia in the peripheral blood followed by a monocytosis, primarily formed of classical monocytes, 72 hours later. This suggests that murine monocytes are released from the bone marrow as classical and later differentiate into other subsets (Sunderkotter *et al.*, 2004). Murine classical monocytes have been shown to enter sites of inflammation rapidly, and over time appear to differentiate into non-classical monocytes (Jutila *et al.*, 1988; Geissmann *et al.*, 2003). *In vitro* studies have shown that murine classical monocytes lose Ly6C expression after several days of culture (Jutila *et al.*, 1988). Dichloromethylene-bisphosphonate-loaded liposomes have been used to eliminate monocytes in a murine model; the *in vivo* monocyte recovery was then investigated. The initial monocytes to return to the circulation were classical (Ly6C++), later followed by non-classical (Ly6C+) (Sunderkotter *et al.*, 2004).

Later work by Yona *et al* supports the differentiation of classical to non-classical monocytes. Once again, classical monocytes were isolated from $CX3CR1^{GFP/+}$ mice. The monocytes were then adoptively transferred into wild-type mice. One day following transplant all GFP-

positive monocytes were classical; however, by day three, GFP-positive non-classical monocytes were detectable. Elimination of classical monocytes reduced the levels of non-classical and, interestingly, appeared to extend the half-life of the Ly6C^{low} monocytes that were already present (Yona *et al.*, 2013).

1.5.4 Human monocyte subsets

Human monocyte subsets vary in their cell surface expression of markers other than CD14 and CD16. Classical monocytes express the majority of CCR2, CXCR1, CXCR2, CLEC4D and IL-13Rα1; intermediate monocytes highly express HLA-ABC, HLA-DR and CD40; non-classical monocytes can be distinguished by the expression of CD115, CD294 and Siglec10 (Wong *et al.*, 2011). As with the murine subsets, the terms classical, intermediate and non-classical as defined in Table 1.4 will be used; if monocytes were identified in a different way, this will be stated. In studies prior to the agreement on nomenclature, the classical subset was defined in the same way, but the non-classical subset may have included intermediate monocytes depending on the author's flow cytometry gating methods.

Although it is challenging to reliably separate the monocyte subsets, as with mice, there is evidence that the three subsets have distinct roles. Examination of the transcriptome of each subset has provided further information regarding roles. A comparison of monocyte transcriptomes and proteomes performed in 2009, prior to the agreement on identifying the three subsets, compared CD16+ and CD16- monocytes. Classical monocytes had an upregulation of genes associated with anti-microbial actions such as *myeloperoxidase* and *IL-8*. With regard to phagocytosis, there are conflicting reports, with one group suggesting that classical monocytes express most genes associated with phagocytosis, whilst another described a different pattern (Mobley *et al.*, 2007; Zhao *et al.*, 2009).

The pattern of cytokine secretion by each subset is controversial and varies by the methods used to measure it. In general, it is accepted that classical monocytes release the widest range of cytokines, but there is debate over the specific cytokines released by each subset (Wong *et al.*, 2011). Frankenberger *et al.* performed initial work in 1996: a Ficoll density isolation was used and then peripheral blood mononuclear cells (PBMCs) were sorted into CD14++/CD16- and CD14+/CD16+ subsets using FACS. These subsets were then incubated with 1μg/mL of LPS for 4 hours. Reverse transcriptase polymerase chain reaction (RT-PCR) was used to identify transcripts. TNF levels were the same in both subsets, whereas IL-10 levels were higher in CD14++/CD16- (classical) monocytes (Frankenberger *et al.*, 1996). More recently,

whole blood was stimulated with LPS (1μg/mL for between 1 and 4 hours) for various time-points, after which brefeldin A was added to prevent secretion and subsets were stained for intracellular cytokines. This showed that classical monocytes produce the most IL-1β and TNF-α; contradictory to previous studies, intermediate (and not non-classical) monocytes produced the most IL-10 (Mukherjee *et al.*, 2015). Other groups have isolated monocyte subsets using FACS; cells were then cultured in the presence of LPS (0-100ng/mL for up to 18 hours) and cytokine levels measured from supernatants. These groups found that it was the non-classical monocytes that produced the majority of IL-1β and TNF-α, whereas the classical subset produced the most IL-10 (Cros *et al.*, 2010; Wong *et al.*, 2011). This variability may be due to the dose, the length of incubation and the changes in subset definitions.

Most studies suggest that classical monocytes are effective at fighting bacterial infection. They seem to display the most phagocytic activity. Mukherjee *et al*. used whole blood analysis to investigate cell surface marker expression and, in their hands, classical monocytes were shown to express the most cell markers associated with phagocytosis (CD36 and CD163) and to demonstrate the most phagocytic activity *in vitro* through the ingestion of latex beads and GFP-stained *E. coli* (Cros *et al.*, 2010; Mukherjee *et al.*, 2015). Classical monocytes have also been shown to release the most reactive oxygen species (ROS) in response to LPS (Cros *et al.*, 2010). *Aspergillus fumigatus* is a source of life-threatening infections in patients following allogeneic stem cell transplantation. Monocyte subsets were isolated from allogeneic stem cell transplant donors who had received granulocyte colony-stimulating factor (GCSF). Two subsets were identified, CD14+/CD16- and CD14+/CD16+. Their proportions in the sample were similar to those in healthy donors who had not received GCSF, and they remained viable following the procedure of leukapheresis. Both subsets were shown to phagocytose Aspergillus spores, but only the classical monocytes were able to prevent germination (Serbina *et al.*, 2009).

As mentioned earlier, Auffray *et al.* demonstrated that $Gr1^-$ mouse monocytes patrol the endothelium. Cros *et al.* investigated the ability of non-classical monocytes to act in a similar way. Human monocyte subsets were isolated using FACS into classical, intermediate and non-classical and labelled with fluorescent probes. Each subset was then injected into a murine model and their behaviour visualised using intra-vital microscopy. Most non-classical monocytes were seen to adhere rapidly to the endothelium and crawl for a prolonged period, similar to the $Gr1^-$ subset in mice (Auffray, 2007; Cros *et al.*, 2010). Analysis of the non-

classical transcriptome agrees with this finding: most genes differentially expressed in this subset related to migration and trans-endothelial motility, as well as MHC class I functions (Hofer *et al.*, 2015).

Non-classical monocytes display less phagocytic activity than the other subsets and express very little reactive oxygen species. They were shown to produce TNF-α and IL-1β in response to the measles and herpes simplex virus (HSV)-1. To a lesser extent, a similar response was seen in the intermediate subset (Cros *et al.*, 2010).

Generally, the role of intermediate monocytes is less defined, in part because monocytes were identified initially in two subsets (CD16+ and CD16-) but also because of the challenges in reliably distinguishing them from the non-classical subset. Genetic profiling and cell surface expression suggests they express major histocompatibility (MHC) class II receptor and are involved in antigen presentation (Mukherjee *et al.*, 2015). Zawada *et al*. focussed on transcriptome differences between the intermediate and non-classical subsets. They identified more genes associated with phagocytosis, production of ROS, and MHC class II antigen processing and presentation in the intermediate compared to the non-classical subset (Zawada *et al.*, 2011).

A higher proportion of intermediate monocytes has been reported in patients with coronary artery disease (Schlitt *et al.*, 2004). An increase in the proportion of circulating intermediate monocytes has been associated with a higher rate of cardiovascular events and death in patients receiving long-term dialysis (Heine *et al.*, 2008; Rogacev *et al.*, 2011). This suggests a possible role for these cells in cardiovascular disease.

Increasingly, it is accepted that the intermediate monocyte, as well as having an individual functional role, is also a transitional cell from classical to non-classical. Following the introduction of the 2010 nomenclature defining the three subsets, Wong *et al.* examined the transcriptome of all monocyte subsets from healthy individuals. The number of differentially expressed genes between classical and non-classical subsets was higher than between either intermediate and non-classical, or intermediate and non-classical subsets (1456 compared to 249 and 942 respectively). The intermediate subset was shown to express 87 % of genes at a level between that of the classical and non-classical subsets; a similar result was found when they examined cell surface markers, with intermediate monocytes expressing them at levels between those of the other two subsets (Wong *et al.*, 2011). Ancuta *et al.*, prior to the

definition of the three subsets, compared the transcriptome of monocyte subsets from healthy volunteers, Comparing CD16+ to CD16- monocytes showed that 13,569 genes were similarly expressed, whilst 250 genes were down-regulated and 228 up-regulated with at least a 2-fold difference in expression. CD16+ monocytes had upregulation of markers also present in dendritic cells and macrophages. CD16- monocytes had high levels of granulocyte and myeloid markers (Ancuta *et al.*, 2009). The work of both groups supports the idea of monocyte subsets having a common progenitor and transition from the classical subset through to intermediate and then on to non-classical.

Several mechanisms have been proposed for differentiation between subsets. Macrophage colony-stimulating factor (MCSF) is released by many cells, including endothelial cells and monocytes (Stanley *et al.*, 1978; Vellenga *et al.*, 1988). Two studies in particular suggest a role for MCSF in monocyte subset differentiation. Firstly, a study of monocyte subsets was carried out in patients with cancer, a proportion of whom were given recombinant MCSF. Initial results showed that patients with gastrointestinal cancer already had an expansion of CD16+ monocytes prior to receiving MCSF. Following the administration of recombinant MCSF there was a significant increase in the number of monocytes but also a 20-fold increase in the proportion of circulating CD16+ monocytes. The authors noted that the majority of these CD16+ cells were also low in their CD14 expression, but this work was over a decade before the identification of the intermediate subtype (Saleh *et al.*, 1995a). Later work investigated the use of an antibody to MCSF in a patient with rheumatoid arthritis. A week following administration of the antibody there was a complete loss of non-classical monocytes, which returned to normal four weeks after the infusion (Korkosz *et al.*, 2012).

Accurate understanding of the distinct functions of monocyte subsets is challenging. As discussed above, there are multiple contradictory reports relating to monocyte subset function, cell surface expression and cytokine secretion. In part this is due to the relatively recent agreement on defining the subsets, with work performed prior to 2010 tending to consider intermediate and non-classical monocytes together or to ignore the intermediate subset completely. Methods of examining the subsets are also imprecise; flow cytometry gating is very operator-dependent and the nomenclature for the subsets applies only in ‘steady state’ conditions. There has also been some work to suggest that the isolation of monocytes from whole blood can itself affect the proportion of subsets (Zhou *et al.*, 2012; Mukherjee *et al.*, 2015). Single cell sequencing of whole blood enriched for HLA DR+ cells identified four monocyte subsets. Using principal component analysis, an unbiased classification was

performed. Four monocyte clusters were identified. The two largest clusters (Mono1 and Mono2) contained the classical and non-classical monocytes respectively. Intermediate monocytes however did not form a homogenous group but were divided between Mono1 and Mono2 as well as the two remaining smaller clusters (Mono3 and Mono4) (Villani *et al.*, 2017).

1.5.5 Monocyte subsets during sepsis

Expansion of different monocyte subsets has been associated with various disease states; as mentioned previously, caution is needed when identifying subsets using methods designed for the 'steady state' (Ziegler-Heitbrock *et al.*, 2010a). With regard to sepsis, there is also the added complication of a changing definition (see section 1.2.3, page 4).

During sepsis, several studies have demonstrated an increase in CD16+ monocyte numbers (Fingerle *et al.*, 1993; Herra *et al.*, 1996; Skrzeczynska *et al.*, 2002; Ziegler-Heitbrock, 2007). Most studies suggest that this expansion is in non-classical monocytes, although others report an expansion of both intermediate and non-classical or intermediate alone (Skrzeczynska *et al.*, 2002; Poehlmann *et al.*, 2009; Mukherjee *et al.*, 2015). This is likely due to the heterogenous population of individuals with sepsis, the rapidly changing status of patients with sepsis, the range of treatments for such patients, the variations in classifying monocyte subsets and the difficulties in separating intermediate and non-classical subsets using flow cytometry.

Serial measurements of monocyte subtypes throughout the course of sepsis reveal a transient increase in non-classical monocytes for approximately 3 days. No increase in total monocyte numbers was shown, simply a change in proportion (Fingerle *et al.*, 1993; Fingerle-Rowson *et al.*, 1998).

HLA DR is a major histocompatibility complex class II molecule. Its expression has been known to decrease in sepsis and critical illness; this has been associated with the development of innate immunosuppression, which can lead to secondary infection in sepsis. A prolonged decrease in expression has been associated with worsened outcomes (Conway Morris *et al.*, 2013). All subsets have been shown to decrease their expression of HLA DR during sepsis (Poehlmann *et al.*, 2009). Later work has demonstrated that during systemic inflammation (post-abdominal aorta surgery) the expression of HLA DR varies between the subsets, with expression being lower in CD14++ versus CD14+ subsets (Kim *et al.*, 2010). Further work is

needed to enable a better understanding of the control of HLA DR expression between subsets.

As previously discussed, MCSF is believed to be involved in the differentiation of classical to non-classical monocytes. MCSF levels increase during sepsis, and this has been shown to occur particularly in individuals who have an associated thrombocytopenia, potentially due to haemophagocytosis (Francois *et al.*, 1997; Oren *et al.*, 2001). Perhaps the changes in MCSF levels may explain the variations between monocyte subsets during sepsis.

Although the expression of TF on circulating monocytes has been investigated, very little work has considered TF expression between subsets. The induction of TF following IL-33 stimulation demonstrated that TF expression can vary between monocyte subsets (Stojkovic *et al.*, 2017). The possible role of TF expression on monocyte subsets during sepsis has yet to be considered.

1.5.6 Summary

The current system of classifying monocyte subsets is still arbitrary and may require the addition of more markers in future, so that we can adequately separate functionally different cells. Problems remain with the operator-dependent nature of flow gating, although clustering algorithms can help. There do appear to be distinct differences, however, between the current classical, intermediate and non-classical subsets. Future work investigating the mechanisms underpinning sepsis should consider monocyte subsets and their individual contributions.

1.6 Modelling the immune response to infection

1.6.1 Overview

This section discusses the use of models to investigate the pathology of sepsis, with a focus on the human endotoxaemia model.

1.6.2 Why do we need a model?

To comprehend sepsis and its associated coagulopathy better, we need to understand the pathophysiological changes that occur prior to an individual's presentation to healthcare services. Sepsis itself is heterogeneous; with a range of aetiologies, presentations and outcomes, cases are difficult to standardise or to control for. The use of models is therefore an important part of studying sepsis.

1.6.3 Difficulties with animal models

There are three main animal models used in sepsis research: the addition of a toxin or virulence factor such as lipopolysaccharide (LPS); the addition of a viable pathogen such as bacteria administrated intravenously or intra-peritoneally; and the use of host-barrier dysfunction such as caecal ligation and puncture (CLP). The use of animal models in sepsis has led to several possibilities for improving outcomes and coagulopathy that have unfortunately, as discussed in section 1.3.7, not been translatable into humans (Warren *et al.*, 2001; Abraham *et al.*, 2003; Ranieri *et al.*, 2012; Vincent *et al.*, 2013; Fink, 2014).

There are differences between the response to endotoxaemia in animals compared to humans. Mice and baboons are much more resistant to LPS than humans; rabbits are more sensitive but show a very variable response. Agents such as D-galactosamine (LPS is metabolised by the liver, but this agent is hepatotoxic) have been used in the past to further sensitise animal models to LPS (Galanos *et al.*, 1979). Endotoxaemia models are based on a single insult, whereas the development of sepsis is believed to involve multiple insults. Endotoxaemia itself is not a model of sepsis but of inflammation; it is not able to replicate the response seen to viable pathogens (Buras *et al.*, 2005).

Animal models have been designed to produce sepsis within hours to days without therapeutic intervention. Once sepsis is identified in a patient, they are treated with antimicrobials and supportive therapies, but such measures are usually not used in animal models. This may lead to a therapy appearing more effective in the animal model compared to the patient who has ongoing care (Buras *et al.*, 2005).

Finally, a major concern is the lack of standardisation between animal models, although there have been recent efforts to rectify this. A group of international experts met at a conference held in 2017 to discuss a literature review of the 260 most cited papers on pre-clinical models of sepsis and to write guidelines for the standardisation of such models, published as "*Minimum Quality Threshold in Pre-Clinical Sepsis Studies*". Recommendations include that the model accurately reflects the clinical situation (for example by employing appropriate supportive and antimicrobial therapies), that clear methodologies are included in publications to allow for accurate replication by different research groups, that LPS should not be used as a model of sepsis in animals, and that humane treatment is universally applied (Osuchowski *et al.*, 2018).

1.6.4 The human endotoxaemia model

The limitations of animal models can to some extent be addressed using human pre-clinical models; the most common and long-standing is the administration of LPS to cause endotoxaemia in humans. LPS can be administered in several ways, for example intravenously, intradermally and via the inhaled route. As mentioned earlier this is not a model of sepsis but does lead to activation of coagulation, fibrinolysis and inflammation (van Deventer *et al.*, 1990). The main benefit of the model is that it can allow investigators to understand the early physiological changes that occur during systemic inflammation and potentially provide new therapeutic targets to be further studied (Andreasen *et al.*, 2008).

LPS is a component of the Gram-negative bacterial outer membrane and has been used as an experimental model of inflammation in animals for over 200 years. In the late 1800s it was used as a treatment for cancer patients; probably any effects were due to the increase in TNFα (Andreasen *et al.*, 2008). It was first clearly identified in 1935 by Boivin and Mesrobeanu and its biochemistry determined in 1952 by Westphal and Luderitz (Boivin and Mesrobeanu, 1936; Westphal *et al.*, 1952). LPS works to form a hydrophobic barrier around the bacterium, conferring protection against host defences such as complement and some anti-microbials (Donaldson *et al.*, 1974; Nikaido and Nakae, 1980; Taylor, 1983). Endotoxin strictly refers to the complex of LPS, phosphates and proteins when bound to the bacterial cell wall, although most literature when discussing models of endotoxaemia refers to the use of purified LPS (Andreasen *et al.*, 2008). LPS binds to TLR-4, with the CD14 receptor acting as a co-receptor. The binding leads to the activation of NF-κB transcription factors that lead to production of pro-inflammatory cytokines including TNFα and IL-1β (Lu *et al.*, 2008).

Intravenous LPS studies in humans have used a range of doses up to 4ng/kg, but a dose of 2ng/kg has been shown to stimulate inflammation, coagulation and fibrinolysis (van Deventer *et al.*, 1990). The source of LPS used in these studies is important when choosing dose. The standardised source of *E. coli* group O 113:H10:K negative, used for models between the 1970s and 1990s, for example, was depleted and multiple investigators noted it became less potent over time (Lowry and Fong, 1996) Another reference endotoxin was produced, again from *E. coli* 0 113, by the American National Institute of Health Clinical Center. A comparison of the two endotoxin sources demonstrated the dose-responsive nature of LPS and confirmed that the former batch had lost potency (Suffredini *et al.*, 1999).

Unlike in animal models, human participants show a very stereotypical response. As discussed in section 1.3, infusion of LPS into healthy human participants leads to an initial activation of the coagulation system in the first few hours, and there is a 125-fold increase in TF mRNA. The TF peaks at 3 hours post-infusion and is followed by an activation of coagulation at 4 hours as demonstrated by an increase in thrombin:antithrombin levels and prothrombin fragments 1 and 2 (Franco *et al.*, 2000). After the activation of coagulation there is a biphasic fibrinolytic response, with an initial activation of fibrinolysis for several hours followed by a more sustained inhibition with high levels of PAI-1 (Suffredini *et al.*, 1989; van Deventer *et al.*, 1990; Franco *et al.*, 2000; Andreasen *et al.*, 2008). This is comparable to what is expected during sepsis (Levi *et al.*, 1993). Endotoxaemia models were primarily responsible for revealing the important role TF plays in causing sepsis-associated coagulopathy and DIC. An important difference with regard to sepsis-associated coagulopathy, however, is that the physiological anticoagulants change very little or not at all following an infusion of LPS (Krabbe *et al.*, 2006).

A striking feature of the human endotoxaemia model is an early monocytopenia that reaches its nadir between 1 and 3 hours and resolves over the following 24 hours. The monocyte count drops to <5% of pre-LPS levels. Examining the recovery of monocytes following LPS shows that classical monocytes recover first at about 8 hours, followed by intermediate and then non-classical subsets after 24 hours (Tak *et al.*, 2017). This provides further support for the hypothesis that classical monocytes differentiate through intermediate into non-classical (see section 1.5.4, page 45). The level of activation between subsets also appears to vary. Intermediate and non-classical monocytes have been shown to produce the highest levels of IL-6 and IL-8 following LPS infusion. CD11b (important for leucocyte adhesion and migration) is a marker of monocyte activation and was expressed most highly by the intermediate subset (Thaler *et al.*, 2016). This suggests that each monocyte subset may have a different role in systemic inflammation.

1.6.5 Strengths and limitations

The human endotoxaemia model has multiple strengths: it is reproducible; it has a strong safety record; it allows the ability to measure temporal changes following endotoxaemia; and it measures responses in humans prior to clinical trials (Andreasen *et al.*, 2008).

As previously stated, the human endotoxic model is not a model of sepsis. Obviously, it would be unethical to cause organ dysfunction in human participants. As with animal models,

it measures the response to a single insult, unlike sepsis which has multiple causes and can be a response to repeated exposures. LPS itself is only present in the circulation for approximately 15 minutes before it is cleared (van Deventer *et al.*, 1990). Endotoxaemia causes a rapid increase in cytokine levels that then quickly return to normal, while individuals with sepsis have a much more sustained exposure to endotoxin that can last several days (Guidet *et al.*, 1994; Andreasen *et al.*, 2008).

LPS is present on the cell wall of Gram-negative bacteria, but a significant proportion of sepsis is caused by Gram-positive, fungal and viral pathogens. However, most intracellular pathways and the induction of inflammation are similar with other causes (Andreasen *et al.*, 2008).

1.6.6 Summary

Endotoxaemia, although not a true model of sepsis, is a good way of measuring systemic inflammation in a human model. More work is needed to understand the effects on monocyte subsets, particularly in relation to TF expression.

1.7 Overall summary

Despite a great deal of research, sepsis continues to have a high mortality rate that is highest in those with sepsis-associated DIC. There is an urgent need to develop a better understanding of the pathophysiology that predisposes to the development of coagulopathy during sepsis, so it can be recognised earlier, and new therapeutic targets identified.

The cell surface expression of TF on monocytes is crucial to the development of coagulopathy but there is little to no work comparing the expression between monocyte subsets. This project will compare the cell surface expression and activity of TF between the monocyte subsets. Monocyte TF will be investigated during health, endotoxaemia and sepsis to investigate the role of monocyte subset TF in the development of sepsis-associated coagulopathy.

1.8 Hypotheses

1. The classical monocyte subset predominates in the circulation during health. This subset expresses a low level of cell surface TF, which shows little activity.
2. Following infection, there is an increase in the cell surface expression of TF and an increase in its activity.
3. Interaction between monocytes and the endothelium allows a local low-level activation of coagulation.
4. During sepsis, there is a decrease in the proportion of circulating classical monocytes and an increase in the proportion of non-classical. Non-classical monocytes, when compared to the other monocyte subsets, express higher levels of TF, leading to a more pro-coagulant response and predisposing to sepsis-associated coagulopathy.

1.9 Aims & Objectives

These hypotheses will be tested in three conditions: health, endotoxaemia and sepsis.

Monocytes will be isolated from healthy volunteers and investigated in culture with commercially-sourced human primary microvascular endothelial cells (PMVEC). These experiments will be discussed in chapter three and aim:

- to investigate the cell surface expression and activity of TF between monocyte subsets with and without LPS stimulation
- to create an *in vitro* model of monocyte-endothelial interactions during sepsis
- to understand the pathway through which LPS induces TF expression on the cell surface monocyte subsets.

A human model of endotoxaemia will be used to simulate the initiation of sepsis. This involves the injection of LPS (2ng/kg) to a healthy volunteer followed by venepuncture at the following time points: pre-injection, 90 minutes, 4, 6, 10, 24 hours and 7 days following the injection. These experiments will be discussed in chapter four, and aim:

- to identify the changes in the proportion of circulating monocyte subsets following LPS exposure
- to investigate the effect of endotoxaemia on the cell surface expression of TF between the monocyte subsets

Blood samples will be taken from individuals with sepsis on a critical care unit. Sepsis will be defined by the requirement of organ support and the presence of an infection (as shown by either a positive blood culture, bronchoalveolar lavage or microbiological evidence of a deep-seated infection such as peritonitis). A further blood sample will be retrieved on discharge from the critical care unit in those individuals who recover and consent to a second sample. These experiments will be discussed in chapter five and aim:

- to identify changes in the proportion of circulating monocyte subsets during sepsis and on recovery
- to investigate the cell surface expression of TF between monocyte subsets during sepsis and on recovery
- to investigate the monocyte-endothelial interactions during sepsis using the same *in vitro* model as used with healthy monocytes

Chapter 2. Materials and Methods

2.1 Overview

This chapter will outline all methods used to produce the data discussed later. All materials, reagents and equipment used are listed under the relevant providers. Details of (a) studies used to obtain blood samples from healthy volunteers, (b) the human endotoxaemia model and (c) studies in patients with sepsis will be included, with protocols, information sheets and consent forms attached as appendices.

2.2 Lists of materials and reagents

Abcam (Cambridge, UK)

- Human PAI-1 enzyme-linked immunosorbent assay (ELISA) Kit
- Human tissue-type plasminogen activator ELISA Kit
- Tissue Factor Activity Assay Kit (Human, colorimetric).

Agar Scientific (Stanstead, UK)

- Paraformaldehyde 16 % Solution (methanol-free) - 10x10 mL ampoules

BD Biosciences (Wokingham, UK)

- Alexa Fluor® 647 anti-human cluster of differentiation (CD) 354 (TREM-1), mouse IgG1, (clone 193015)
- BD™ CompBead anti-mouse Ig, κ/negative control compensation particles set
- BD FACS™ lysing solution 10x concentrate
- BD Horizon™ brilliant stain buffer
- BD Trucount™ Absolute Counting Tubes
- Brilliant Violet (BV) 421 anti-human TLR4 (CD284), mouse IgG1, κ (clone TF901)
- Fluorescein isothiocyanate (FITC) anti-human CD45, mouse IgG1, κ (clone HI30)
- Phycoerythrin (PE) anti-human tissue factor (CD142) mouse IgG1, κ (clone HTF-1)
- Violet (V) 500 anti-human CD16, mouse IgG1, κ (clone 3G8)

Biolegend (London, UK)

- Allophycocyanin (APC)/Cyanine7 anti-human CD19 antibody, mouse IgG1, κ (clone SJ25C1)
- APC/Cy7 anti-human CD16 antibody, mouse IgG2b, κ (clone 3G8)
- APC/Cy7 anti-human CD20 antibody, mouse IgG2b, κ (clone 257)

- Brilliant Violet 605™ anti-human CD3 antibody, mouse IgG2a, κ (clone OKT3)
- Brilliant Violet 785™ anti-human HLA-DR antibody, mouse IgG2a, κ (clone L243)
- PE anti-human CD1c antibody, mouse IgG1, κ (clone L161)
- PE anti-human CD3 antibody, mouse IgG2a, κ (clone HIT3a)
- PE anti-human CD7 antibody, mouse IgG2a, κ (clone CD7-6B7)
- PE anti-human CD19 antibody, mouse IgG1, κ (clone 4G7)
- PE anti-human CD20 antibody, mouse IgG2b, κ (clone 2H7)
- PE anti-human CD34 antibody, mouse IgG1, κ (clone 581)
- PE anti-human CD56 antibody, mouse IgG1, κ (clone HCD56)
- PE anti-human CD66b antibody, mouse IgM, κ (clone G10F5)
- PE anti-human CD123 antibody, mouse IgG1, κ (clone 6H6)
- PE/Dazzle™ 594 anti-human CD14 antibody, mouse IgG1, κ (clone HCD14)
- Peridinin chlorophyll/Cyanine (PerCP/Cy) 5.5 anti-human CD14 antibody, mouse IgG1, κ (clone HCD14)
- Zombie UV™ Fixable Viability Kit.

Fisher Chemical (Loughborough, UK)

- Isopropanolol.

Greiner Bio-One (Stonehouse, UK)

- VACUETTE® TUBE 4.5 mL 9NC Coagulation sodium citrate 3.8 % blue cap-black ring
- VACUETTE® TUBE 5 mL CAT Serum Separator Clot Activator gold cap-gold ring
- VACUETTE® TUBE 9 mL K3E K3EDTA lavender cap-black ring, non-ridged
- VACUETTE® TUBE 4 mL K3E K3EDTA lavender cap-black ring, non-ridged.

Lonza (Slough, UK)

- Iscove's Modified Dulbecco's Medium (IMDM)
- Roswell Park Memorial Institute medium (RPMI) 1640 with glutamine.

Miltenyi Biotec (Surrey, UK)

- Sterile pre-separation filters, 30 μm.

Promocell (Lutterworth, UK)

- Endothelial cell growth basal medium MV 2

- Endothelial cell growth medium MV 2 supplement mix
- Human pulmonary microvascular endothelial cells isolated from the lung of a single donor (C-12281).

R&D Systems (Abingdon, UK)

- IT 901, NF-κB c-Rel sub-unit inhibitor
- SR 11302, activator protein (AP)-1 inhibitor
- FR180204, selective ERK 1 and ERK 2 inhibitor
- Human M-CSF DuoSet ELISA

Sarstedt (Leicester, UK)

- Cell culture flasks with ventilation cap (T25 and T75)
- Cell culture plates (24- and 96-well)
- Falcon tubes (15 mL and 50 mL)
- Pipette tips (10 µL, 20 µL, 200 µL, 1000 µL, 5 mL and 10 mL).

Sigma Aldrich Ltd. (Gillingham, UK)

- 4′, 6-diamidino-2-phenylindole (DAPI)
- Bovine serum albumin (BSA)
- Dimethyl sulphoxide (DMSO)
- Citrate concentrated solution 4 %
- Giemsa stain solution
- Lipopolysaccharide from *Escherichia coli* 026:B6
- Nalgene® Mr. Frosty, freezing container
- Phosphate-buffered saline (PBS)
- Propidium iodide (PI)
- Sodium azide
- Triton 10 %
- Trypsin
- Trypan blue solution 0.4 %
- TWEEN® 20.

STARLAB (Milton Keynes, UK)

- Cryovial with Internal Thread, Silicone Seal Cap, 1.8mL, Skirted (Sterile).

2.3 Equipment

- BD FACS Aria III (BD Biosciences, Wokingham, UK)
- BD FACS Fusion (BD Biosciences, Wokingham, UK)
- BD Symphony A5 (BD Biosciences, Wokingham, UK)
- FLUOstar Omega spectrophotometer microplate reader (Aylesbury, UK)
- MoFLO Astrios (Beckman Coulter, High Wycombe, UK)
- ThermoScientific Shandon Cytospin 3 centrifuge (Fisher Scientific UK Ltd, Paisley, UK).

2.4 Software

- Flow cytometry was performed using FACS Diva software (version 8.01) for acquisition and FCS Express (version 6.06.002) for analysis.
- Omega (version 5.10) acquisition and MARS data analysis software (version 3.02) were used for the multi-plate reader.

2.5 Monocyte isolation and preservation

2.5.1 Isolation of peripheral blood mononuclear cells from whole blood

Peripheral blood mononuclear cells (PBMCs) were isolated using a Ficoll-Paque™ density gradient separation (density = 1.077 g/mL). In brief, 30 mL of citrated (final concentration of 1 % sodium citrate) whole blood was centrifuged at 200 *g* for 10 min at 21 ºC. The plasma layer was removed (see Figure 2.1A). A proportion of plasma was aliquoted, and serum obtained by adding 1M $CaCl_2$ (220 µL per 10 mL of plasma) in a glass tube followed by incubation in a water bath at 21 °C until the platelets were aggregated. The serum was then removed and either used in experiments that day or frozen at -80 ºC. The volume of plasma removed was replaced with warmed 0.9 % saline solution and the cell pellet re-suspended. This was layered over 15 mL of Ficoll-Paque™ and the solution centrifuged at 400 *g* for 30 min at 21 ºC to create a layer of PBMCs (see Figure 2.1B & C). The PBMC layer was removed and washed twice using Hanks' Balanced Salt Solution without Ca^{2+} or Mg^{2+} ions ($HBSS^-$). A 200 µL aliquot was taken. Of this, 100 µL was stained with 5 µL 0.4 % Trypan blue, which stains positive if the cell membrane is disrupted (thereby identifying dead cells), and a cell count of live cells performed using a haemocytometer. The remaining 100 µL was used to perform a cytospin to ensure the purity of the sample. Cytospin slides were fixed in acetone for 10 min and then stained in Giemsa for 10 min. Only PBMC preparations with > 95 % purity and viability were used. Typically, 30 million PBMCs are isolated from 30 mL

of whole blood. The PBMCs are then re-suspended in either Hanks' Balanced Salt Solution with Ca^{2+} and Mg^{2+} ($HBSS^{+}$) or IIMDM with phenol red, depending upon the assay to be performed.

2.5.2 Isolation of monocytes using adherence

PBMCs were isolated as detailed above (see section 2.5.1, page 61) and re-suspended in 37 °C IMDM with 10 % autologous serum at a concentration of 1 million per mL. Cells were cultured in a 24-well plate for one hour, with 500,000 cells added per well, in an incubator at 37 °C and 5 % CO_2. Following this, wells were washed twice with $HBSS^{-}$ to remove all non-adherent cells. 500 µL of medium (IMDM with 10% autologous serum at 37 °C) was added to each well. Light microscopy was used to ensure that > 90 % remaining cells were monocytes.

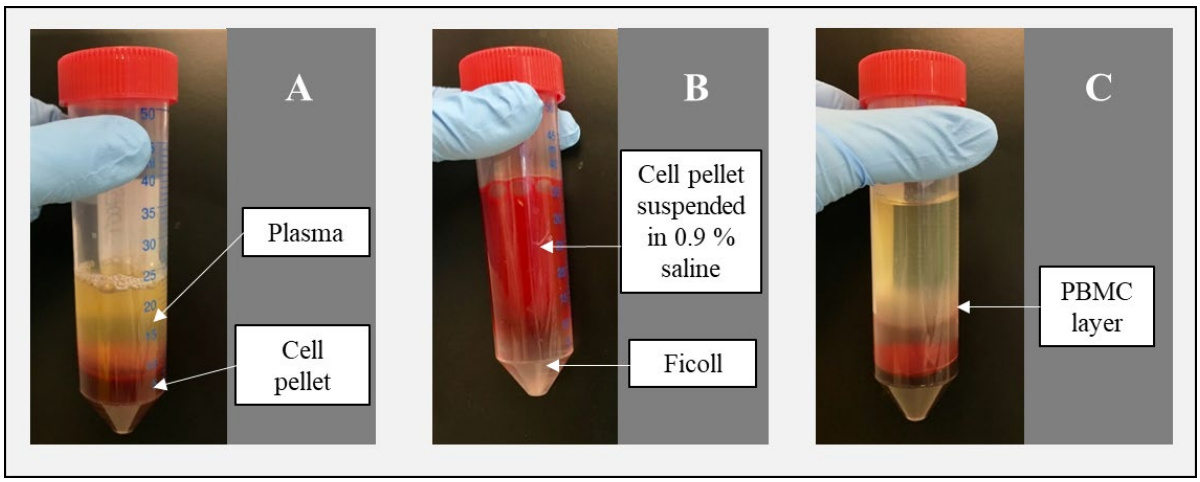


Figure 2.1 Isolation of peripheral blood mononuclear cells from whole blood isolation

This figure depicts the stages of isolating mononuclear cells from whole blood using a Ficoll-Paque™ density gradient separation. ***A*** *– The whole blood sample is centrifuged to pellet the cells and separate the plasma.* ***B –*** *The cell pellet is re-suspended in warm 0.9 % saline and layered over 15 mL of Ficoll-Paque™.* ***C*** *– The sample is centrifuged at 400* g *for 30 min and a layer of PBMCs isolated.*

2.5.3 Negative selection of monocytes using fluorescence-activated cell sorting

PBMCs were isolated as described above (section 2.5.1, page 61). They were centrifuged at 300 *g* for 5 minutes at 4 ºC and re-suspended in flow buffer at a concentration of 10 million cells per 100 µL. Flow buffer was prepared using PBS with 0.2 % bovine serum albumin and 0.1 % sodium azide; it was stored at 4 °C. The antibody mix detailed in table 2.1 was added; samples were vortexed and incubated at 4 °C in the dark for 30 min. Following incubation, cells were washed by the addition of 500 µL flow buffer at 4 °C to each sample, and centrifugation at 200 *g* for 5 min at 4 ºC. The supernatant was removed and the pellet re-suspended in flow buffer at 4 °C at a concentration of 20 million cells per 1 mL of buffer. The sample was filtered using a sterile filter with a pore size of 30 µm.

The Newcastle University Flow Cytometry Core Facility assisted in negatively selecting monocytes using fluorescence-activated cell sorting (FACS). Sorting was performed using a 70 µm nozzle on a BD FACS Fusion cell sorter.

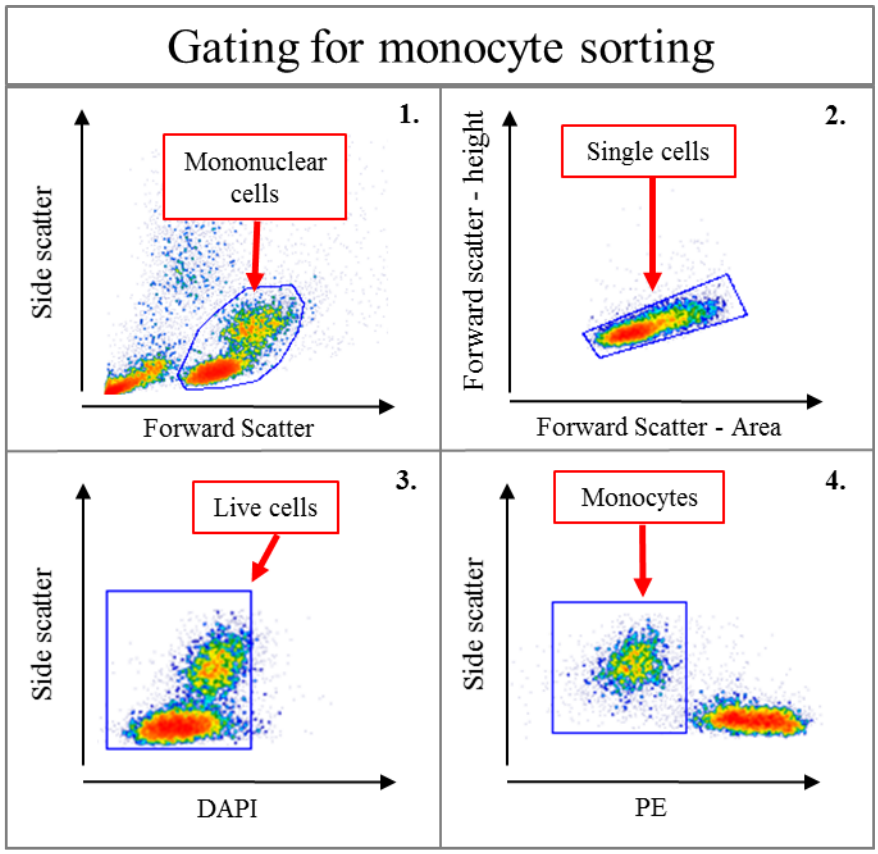
Mononuclear cells were identified using forward and side scatter. Of these, the live cells were selected and all PE-positive cells discarded (see Figure 2.2). The sample was maintained at 4 °C. Monocytes were sorted into autologous serum, obtained as described earlier (section 2.5.1), and used immediately. The purity of the monocytes was tested using flow cytometry for CD14 and CD16 using the panel detailed in Table 2.2.

Both fluorochrome panels were compensated using BD™ anti-mouse compensation beads. Each fluorochrome was incubated, at the same concentration used for experimental samples, with mouse beads (positive and negative together) for 30 min at 4 °C in the dark. The samples were then washed with the addition of 500 µL of flow buffer. They were centrifuged at 300 *g* for 5 min at 4 ºC and the supernatant removed. The sample was re-suspended in flow buffer and stored at 4 °C in the dark until used. The beads were used to identify the positive and negative populations. FACS Diva software was used to calculate compensation between fluorochromes.

Antigen	Primary cells to be identified	Fluorochrome	Wavelength	Volume to be used
CD3	T cells	PE	561 586/15	5 µL of each per 10 million cells
CD7	T cells, NK cells			
CD20	B cells			
CD19	B cells			
CD123	Haematopoietic progenitors, Basophils, Mast cells, Dendritic cells			
CD34	Haematopoietic progenitors			
CD66b	Neutrophils, Eosinophils			
CD56	NK cells			
DAPI	To test cell viability		405 450/50	5 µL per 10 million cells (0.1 µg/mL)

Table 2.1 Flow cytometry panel for the negative selection of monocytes from PBMCs

This table details the volume of each antibody used per 10 million PBMCs as well as the amount of DAPI used to assess viability. The cells excluded by each CD marker are included.


Gating for monocyte sorting
1.
Mononuclear cells
Side scatter
Forward Scatter
2.
Single cells
Forward scatter - height
Forward Scatter - Area
3.
Live cells
Side scatter
DAPI
4.
Monocytes
Side scatter
PE

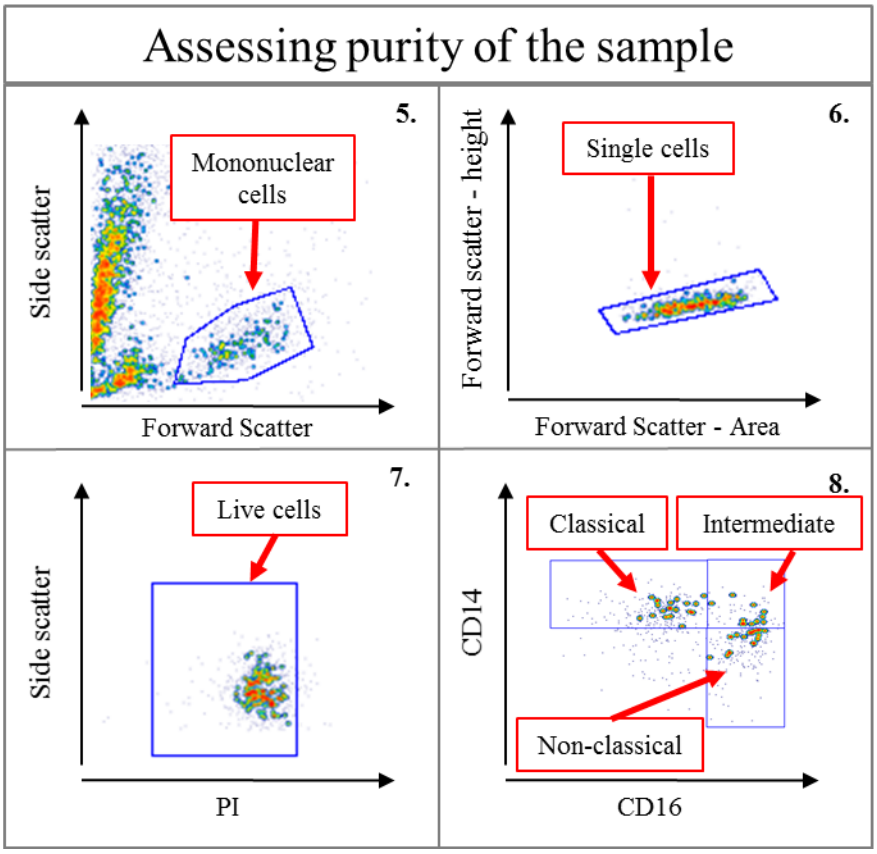

Assessing purity of the sample
5.
Mononuclear cells
Side scatter
Forward Scatter
6.
Single cells
Forward scatter - height
Forward Scatter - Area
7.
Live cells
Side scatter
PI
8.
Classical
Intermediate
Non-classical
CD14
CD16

Figure 2.2 Gating used to negatively sort monocytes from PBMCs using FACS

This figure shows an example of healthy volunteer negatively-sorted monocytes and a check of sample purity post-sort.

***1** – Mononuclear cells are gated using forward and side scatter. **2** – Forward scatter height and area are used to select single cells. **3** – Live cells are gated by using DAPI-negative events. **4** – Monocytes are selected using the PE-negative population, excluding lymphocytes, natural killer (NK) cells, neutrophils, basophils and eosinophils.*

***5** – Mononuclear cells are gated using forward and side scatter variables. **6** – Single cells are gated using forward scatter area and height variables. **7** – Live cells are gated using PI staining. **8** - Monocyte subsets are sorted using CD14 and CD16 positivity.*

Antigen	Primary cells to be identified	Fluorochrome	Wavelength	Volume to be used
CD14	Classical and Intermediate subsets	PerCP/Cy5.5	488 695/40	1.25 µL per 2 million cells
CD16	Intermediate and Non-classical subsets	APC/Cy7	640 780/60	1.25 µL per 2 million cells
PI	To test cell viability		561 610/20	1.5 µL of 0.5 mg/mL

Table 2.2 Flow cytometry panel used to assess the purity of the negatively sorted monocytes

This table details the volume of each antibody used per 2 million negatively sorted monocytes, as well as the amount of PI to be used to assess viability.

2.5.4 Positive selection of monocyte subsets using fluorescence-activated cell sorting

PBMCs were isolated and re-suspended in flow buffer at 4 °C as described earlier (section 2.5.3, page 64). The antibody mix detailed in table 2.3 was added and the samples incubated at 4 °C in the dark for 30 min. Following incubation, samples were washed with the addition of 500 µL of flow buffer per 10 million cells at 4°C and centrifugation at 200 *g* for 5 min at 4 ºC. The supernatant was discarded and the pellet re-suspended in flow buffer at a concentration of 20 million cells per mL of buffer. Prior to sorting, the sample was filtered using a sterile filter with a pore size of 30 microns.

The Newcastle University Flow Cytometry Core Facility assisted in positively selecting monocyte subsets using FACS. Sorting was performed using a 70 µm nozzle on a BD FACS Fusion cell sorter. Mononuclear cells were selected using forward and side scatter variables. The live cells were selected and BV421 positive cells excluded. The monocytes were sorted into classical, intermediate and non-classical using CD14 and CD16 positivity.

The sample was maintained at 4 °C. Monocytes were sorted into IMDM medium and used immediately.

Antigen	Primary cells to be identified	Fluorochrome	Wavelength	Volume to be used
CD3 CD20 CD19 CD56	T cells B cells B cells NK cells	BV421	405 450/50	5 µL of each per 10 million cells
CD14	Classical and Intermediate monocytes	PE dazzle	561 610/20	5 µL per 10 million cells
CD16	Intermediate and Non-classical monocytes	V500	405 525/50	5 µL per 10 million cells
DAPI	To test cell viability	UV	405 450/50	5 µL per 10 million cells (0.1 µg/mL)

Table 2.3 Flow cytometry panel for the positive selection of monocyte subsets from PBMCs

This table details the volume of each antibody used per 10 million PBMCs, as well as the amount of DAPI used to assess viability. The cells identified by each CD marker are included.

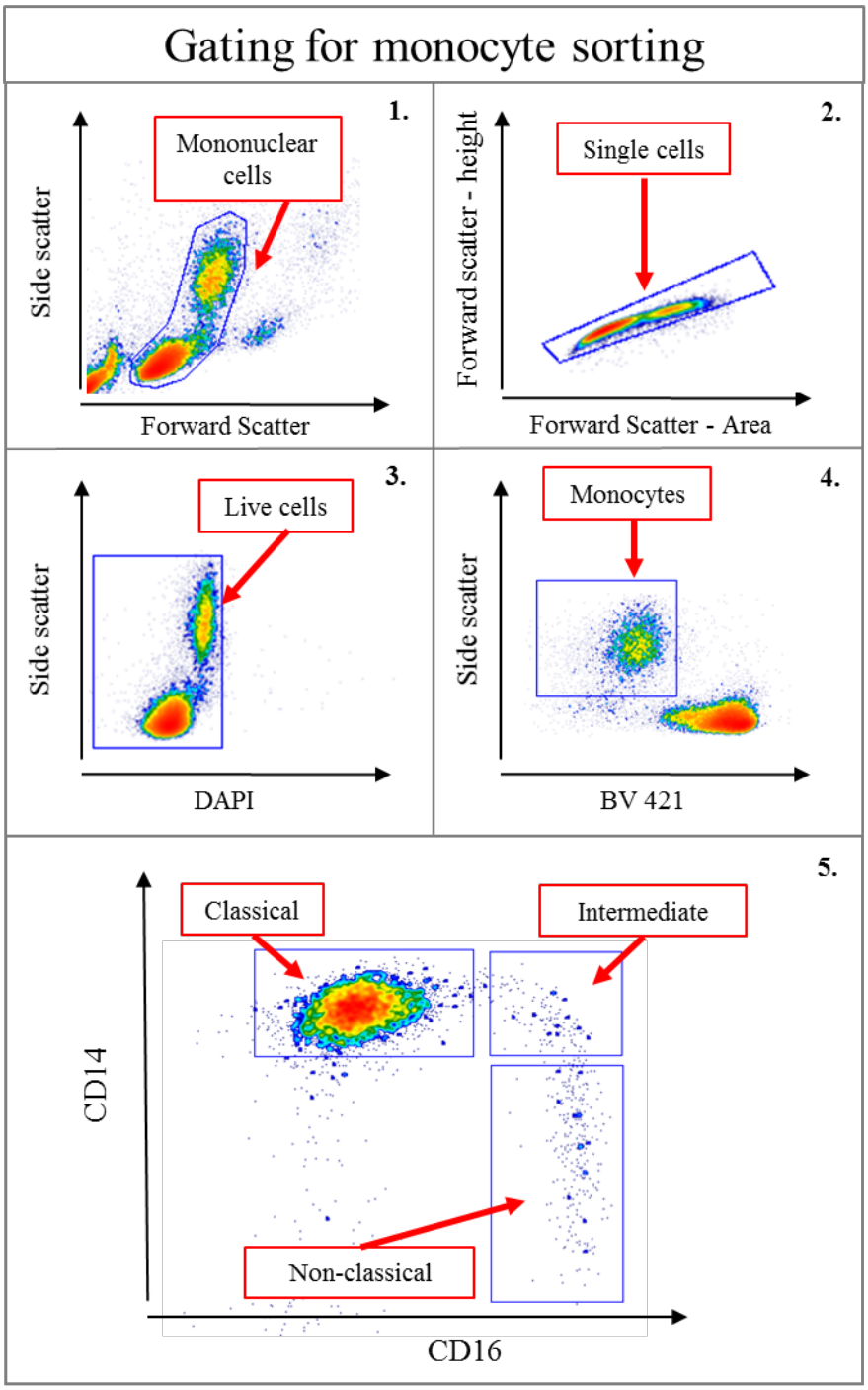


Figure 2.3 Example of the gating used to positively sort monocytes from a healthy volunteer into subsets using FACS

1 – Mononuclear cells are gated using forward and side scatter. 2 – Forward scatter height and area are used to select single cells. 3 – Live cells are gated using DAPI-negative events. 4 – Monocytes are selected using the BV421 negative population, thereby excluding CD3, CD19, CD20 and CD56. 5 – Monocyte subsets are sorted using CD14 and CD16 positivity.

2.5.5 Cryopreservation of peripheral blood mononuclear cells

Freezing medium was prepared using 10 mL foetal bovine serum (FBS), 10 mL of sterile RPMI-1640 and 5 mL of DMSO. PBMCs, isolated as described above (section 2.5.1, page 61), were re-suspended in complete (c)RPMI medium at a concentration of 20 million per mL. The volume of the cell suspension was then doubled using the freezing medium, creating a final concentration of 10 million PBMCs per mL. PBMCs were stored in aliquots of 1 mL in cryovials and placed in a Mr Frosty freezing container filled with 70 % isopropanol. The container was stored at -80°C for 24 hours before the samples were transferred into liquid nitrogen.

Samples were thawed on the day they were to be used. Cryovials were removed from the liquid nitrogen and their caps loosened to allow any liquid nitrogen that might have seeped into the vial to escape. Samples were then thawed quickly in a water bath. Once only a small amount of ice crystal remained in the sample, the cryovials were quickly cleaned with 1 % Distel High Level Laboratory Disinfectant, followed by 70 % ethanol. The PBMCs were diluted in 10 mL of HBSS and then centrifuged for 5 mins at 300 *g* at 21 ºC. PBMCs were then re-suspended in the medium appropriate for the experiment to be performed. An aliquot was taken to perform a viable cell count and cytospin (see section 2.5.1, page 61).

2.6 Flow cytometry to determine monocyte subsets and tissue factor expression

Flow cytometry was used to identify the monocyte subsets and their TF expression. Flow cytometry was run using the BD Symphony machine with FACS Diva software. Analysis was performed using the FCS express software.

2.6.1 Flow cytometry for whole blood samples

A panel of antibodies was used to identify monocytes and exclude other cells (see Table 2.4). Initially mononuclear cells were gated using CD45 positivity. Single cells and live cells (Zombie UV positive) were then chosen. B cells were excluded using markers for CD19 and CD20. T cells were excluded with CD3. Size and HLA-DR were used to distinguish monocytes from NK cells. This left a population of monocytes that was gated into subsets using CD14 and CD16 positivity (see Figure 2.4).

To calculate the absolute numbers of monocyte subsets in each whole blood sample, BD Trucount™ absolute counting tubes were used. These tubes contain a known number of

beads; flow cytometry was run until 20,000 bead events had been measured. This was then used to identify the absolute number of monocytes using the calculation:

$$\text{Absolute cell count} = \frac{\text{number of positive cell events}}{\text{number of bead events}} \times \frac{\text{total number of beads}}{\text{volume of blood tested}}$$

100 µL of whole blood (anticoagulated with 1 % sodium citrate) was added via reverse pipetting to the mix of antibodies listed in table 2.4, within the Trucount™ tubes. To reduce non-specific antibody binding, 50 µL of flow buffer at 4 °C was added to each sample. Tubes were then vortexed to suspend the bead pellet and incubated at 4 °C in the dark for 30 min. BD FACS™ lysing solution was used to lyse the red cells and fix the antibody staining. Each sample was incubated with 1.5 mL of the solution in the dark at room temperature for 20 min.

2.6.2 Flow cytometry for samples containing isolated monocytes

For samples using isolated cells (either PBMCs or monocytes) the same antibody mix was used (see Table 2.4). The cells were pelleted by centrifugation at 200 *g* for 5 min at 4 ºC, and the supernatant was removed and stored at –80 ºC before re-suspension in 100 µL flow buffer at 4 ºC (see section 2.5.1, page 61). The mix of antibodies was added and left to incubate for 30 min at 4 ºC in the dark. Following incubation, a further 500 µL of flow buffer at 4 ºC was added to wash unbound antibody. Samples were centrifuged for 5 min at 200 *g* and the supernatant discarded. The pellet was re-suspended in 300 µL of flow buffer and stored in the dark at 4 ºC until flow cytometry.

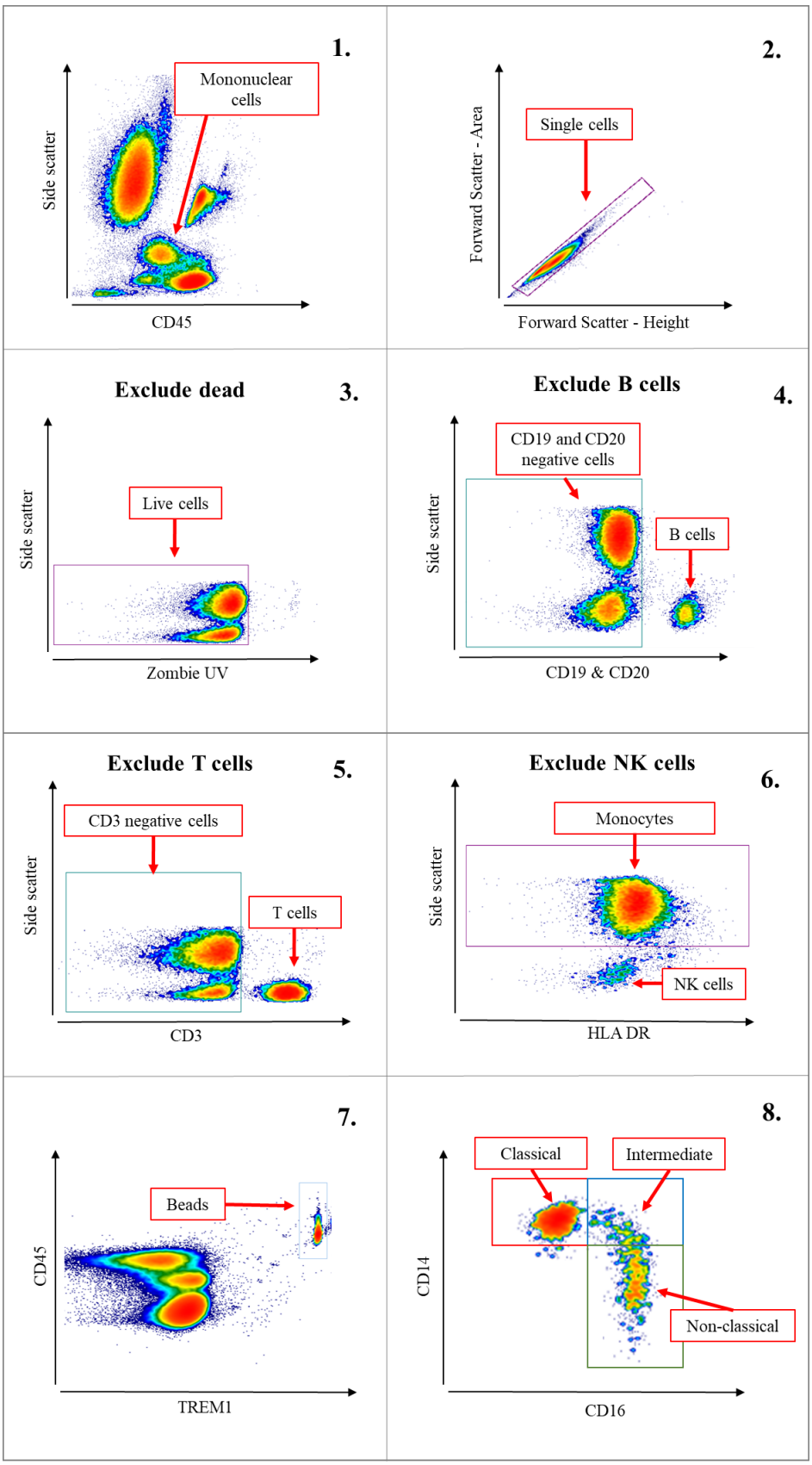

1.
Mononuclear cells
Side scatter
CD45
2.
Single cells
Forward Scatter - Area
Forward Scatter - Height
Exclude dead
3.
Live cells
Side scatter
Zombie UV
Exclude B cells
4.
CD19 and CD20 negative cells
B cells
Side scatter
CD19 & CD20
Exclude T cells
5.
CD3 negative cells
T cells
Side scatter
CD3
Exclude NK cells
6.
Monocytes
NK cells
Side scatter
HLA DR
7.
Beads
CD45
TREM1
8.
Classical
Intermediate
Non-classical
CD14
CD16

Figure 2.4 Example (using blood from a healthy volunteer) of the monocyte gating strategy for whole blood samples.

1 *– Mononuclear cells were gated using CD45 positivity and side scatter.* ***2*** *– Forward scatter height and area were used to select single cells.* ***3*** *– Live cells were gated using Zombie UV-negative events.* ***4*** *– B lymphocytes were removed using the CD19 and CD20 negative population.* ***5*** *– T lymphocytes were removed using the CD3 negative population. 6 – NK cells were excluded using HLA-DR and side scatter variables. 7 – Beads were gated using two fluorochromes.* ***8 -*** *Monocyte subsets were sorted using CD14 and CD16 positivity. (CD – cluster of differentiation, NK – natural killer, UV – ultra-violet).*

Antigen	Description	Fluorochrome	Wavelength	Volume to be used per 100 µL of whole blood (µL)
Zombie UV	Viability marker	UV	355 379/28	5
TLR4	LPS receptor	BV421	405 450/50	5
CD16	Intermediate and Non-classical monocyte marker	V500	405 525/50	5
CD3	T cell marker	BV605	405 610/20	5
HLA-DR	MHC class II cell surface receptor	BV785	405 780/60	5
CD45	Leucocyte marker	FITC	488 530/30	20
CD142	Tissue factor	PE	561 586/15	20
CD14	Classical and Intermediate monocyte marker	PE Dazzle	561 610/20	5
TREM1	Stimulator of monocyte and neutrophil inflammatory response	AF647	640 670/30	5
CD19	B cell marker	APC Cy7	640 780/60	5
CD20	B cell marker	APC Cy7	640 780/60	5

Table 2.4 Antibody panel for whole blood flow cytometry

This table details the panel used to identify monocyte subsets from whole blood as well as their cell surface expression of TLR4, HLA-DR, TREM1 and TF.
(HLA-DR - Human Leukocyte Antigen DR isotype, TF – tissue factor, TLR4 – Toll-like receptor 4, TREM1 – Triggering receptor expressed on myeloid cells 1)

2.6.3 Optimising the flow cytometry panel

As previously described, the antibody panel was compensated for use on the BD Symphony flow cytometer. Compensation was performed using beads and cells. All antibodies were titrated to ensure that the optimal concentrations were used to detect all positive events. The Zombie UV antibody is affected by the level of protein in samples; for this reason, 5 µL was used per 100 µL in whole blood samples and 1 µL per 100,000 isolated cells. Both conditions were titrated.

2.6.4 Flow cytometry controls

Flow gating was based on fluorescence-minus-one (FMO) controls and non-specific binding was identified using isotype controls.

FMOs were performed for: CD14, CD16, CD142 (tissue factor), HLA-DR, TREM1 and TLR4. Gating of classical, intermediate and non-classical monocytes was guided by the use of a flow sample minus both CD14 and CD16 (see Figure 2.5). This was later refined for endotoxic and septic samples, where FMOs were used for CD14 and CD16 (see Figure 2.6). A more detailed description of these antigens is shown in Table 2.4. For each, a sample was prepared as previously described (see methodology sections 2.6.1, page 72 and 2.6.2, page 73) but the antibody of interest was omitted. This allows for accurate identification of positive events and accounts for cellular auto-fluorescence. Isotype controls were performed for all antibodies to identify non-specific binding. These controls were prepared in the same way as for the usual samples for flow cytometry, with the exception that the antibody of interest was replaced with the same concentration of isotype control (see methodology sections 2.6.1, page 72 and 2.6.2, page 73).

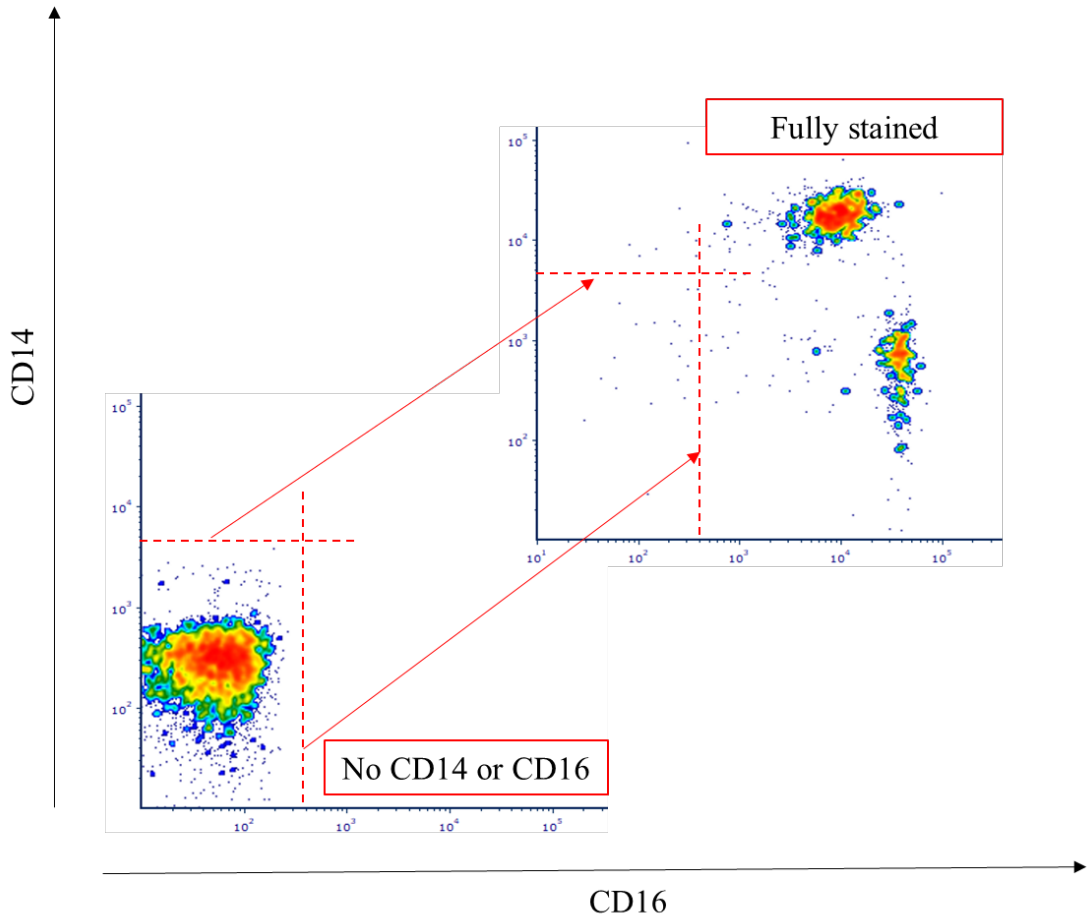


Figure 2.5 The gating of monocyte subsets using the flow panel for sorted monocytes

An example of monocyte subset gating, using monocytes sorted using FACS from a healthy volunteer sample. Flow plots are shown for sample using all the whole flow panel (fully stained), the second sample without the CD14 and CD16 antibodies (No CD14 or CD16). The red dashed lines delineate the negative population of cells not positive for the markers.

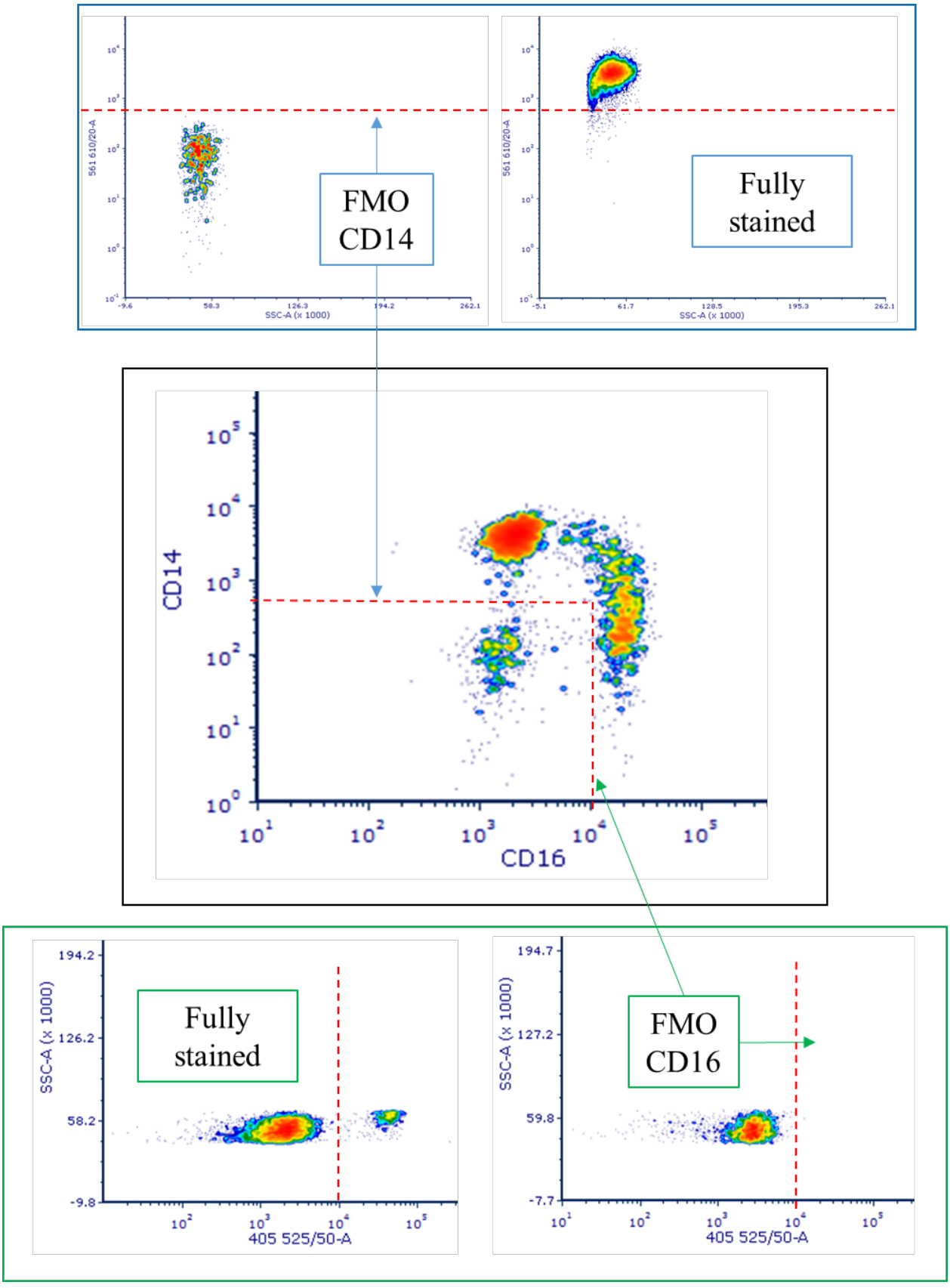

FMO
CD14
Fully
stained
CD14
CD16
Fully
stained
FMO
CD16
SSC-A (x 1000)
405 525/50-A

Figure 2.6 Using FMO to guide the gating of monocyte subsets with the flow panel for whole blood

An example of FMOs using a healthy volunteer sample with the whole blood flow panel. Flow plots are shown for sample using all the whole flow panel (fully stained), the second sample without the CD14 antibody (FMO CD14) and a third sample without the CD16 antibody (FMO CD16). The red dashed lines delineate the negative population of cells that are not positive for the relevant marker.

2.7 Endothelial cell culture

All tissue culture was performed using a Class II biological safety cabinet. Primary human pulmonary microvascular endothelial cells (PMVEC) were grown with endothelial cell basal medium MV 2 plus supplement mix (all from Promocell). The incubator was set at 37°C and with 5 % carbon dioxide (CO_2).

2.7.1 Culture of primary pulmonary microvascular endothelial cells

Cells were cryopreserved in liquid nitrogen with 10% DMSO, thawed rapidly using a water bath at 37 °C and added into 5 mL of endothelial cell medium at 37 °C. Cells were then washed and centrifuged at 200 *g* for 10 min at 21 ºC. The supernatant was discarded and the pellet re-suspended in endothelial medium at 37 °C.

Cells were grown in a 75 cm^2 flask at a density of 500,000 cells in 10 mL of the prepared medium. Once seeded, cells were incubated for 24 hr before the medium was changed. Following this initial change, medium was changed every 48 to 72 hr. The medium was warmed for 15 min in a water bath at 37 °C before being added to cells.

Primary PMVEC were used at a passage number between 2 and 8, at a confluence of approximately 90 %. Flow cytometry was used to ensure they expressed the expected endothelial cell markers (CD31).

Once confluence was reached, cells were removed from the 75 cm^2 flask and split between another 75 cm^2 'stock' flask and 24-well plates for the co-culture experiments described below (see methodology section 2.7.2, page 82). The cells were washed with PBS and then incubated with 2.5 mL of 0.05 % trypsin for 5 min at 21 °C. Cells were detached by a sharp tap on the side of the flask and, if necessary, with the use of a cell scraper. Trypsin was de-activated with the addition of 7.5 mL of warmed endothelial medium. The detached cells were centrifuged at 200 *g* for 5 min at 21 ºC, the supernatant discarded, and the pellet re-suspended in warmed endothelial medium. Each stock flask was seeded with 500,000 cells in 10 mL medium whilst each well was seeded with 50,000 cells in 500 µL medium.

2.7.2 Co-culture experiments

Monocyte-endothelial co-cultures were set up as shown in Figure 2.7. PMVEC cells were grown to 90 % confluence in a 24-well plate in endothelial cell medium. Monocytes from healthy volunteers were sorted by FACS into autologous serum and added to the PMVEC, 50,000 monocytes were added to each well.

To investigate the effects of co-culture on activated endothelium, a proportion of PMVECs were stimulated with LPS at a final concentration of 100 ng/mL for 1 hour at 37 °C. To allow the LPS to be functional, autologous serum at a final concentration of 10 % was added to the wells. Following this stimulation, the LPS was thoroughly washed off with two washes using warmed endothelial cell medium.

Controls included: PMVEC alone, monocytes alone, PMVEC pre-stimulated with LPS and monocytes stimulated by LPS. Monocytes were stimulated with 10 ng/mL of LPS and the LPS was not washed off to avoid losing monocytes through washing.

Co-cultures performed using samples from individuals with sepsis did not use FACS sorted monocytes but instead used PBMCs. Due to the smaller volume of blood obtained from individuals with sepsis insufficient monocytes could be sorted for co-culture. PBMCs were isolated using a Ficoll-Paque™ density gradient separation and 100,000 PBMCs were added to each well. As with the healthy volunteer monocytes, the same concentration of LPS was used to stimulate the PBMCs, and once again it was not washed off but remained for the whole co-culture.

Prepared plates were incubated at 37°C in 5 % CO_2 for 24 hr, following which adherent cells were scraped from the wells and, with the surrounding medium, were centrifuged at 200 *g* for 10 min. Supernatants were frozen in aliquots of 500 mL at -80°C. Cell pellets were prepared for flow cytometry (see methodology section 2.6.2, page 73).

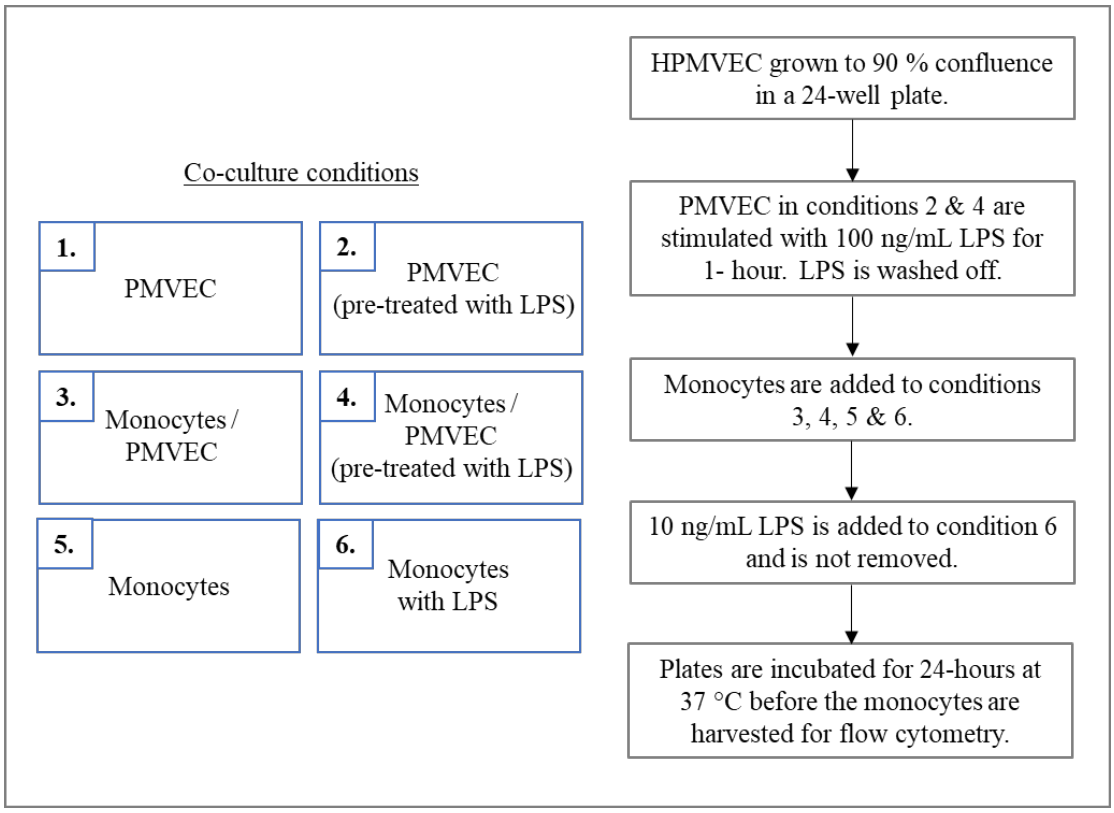


Figure 2.7 The co-culture conditions and assembling the co-culture experiment

This figure shows the six co-culture conditions used. The flow chart describes how the co-culture experiment was assembled.

2.7.3 ELISA

Sandwich ELISA kits were used to measure the MCSF, PAI-1 and tPA in the supernatants stored following monocyte-endothelial co-culture experiments. The ELISAs were performed according the manufacturer's instructions. Sandwich ELISAs were also performed to measure levels of coagulation complexes by our collaborators in Maastricht. These included: TAT, FIXa-AT, FXa-AT, FXIa-AT and FXIa-a1AT.

Briefly, standards were prepared using serial dilution from a stock standard. Either standards or samples were added in triplicate to 96-well plates pre-coated with primary antibody. Three wells served as a 'blank' and contained only assay buffer, antibodies and substrate. Wells were coated overnight by the addition of 100 µL of capture antibody prior to washing with wash buffer (0.5 % BSA and 0.1 % Tween 20 dissolved in PBS). In the case of PAI-1 and tPA the plates were pre-coated.

The plate was blocked with 200 µL blocking buffer (1 % BSA in PBS) added to each well for 1 hour at room temperature. The primary incubation of 100 µL of either sample or standard in each well occurred at room temperature on the shaker at 300 rpm. The plate was then washed five times with wash buffer. Secondary incubation with 100 µL per well of biotinylated detection antibody then took place at room temperature on a shaker at 300 rpm. This was followed by five washes with wash buffer. Streptavidin-horseradish peroxidase (HRP) was added (100 µL per well) to bind the biotin, for 30 minutes at room temperature on the shaker at 300 rpm, followed by a further five washes with wash buffer. Following this, 100 µL of a 1:1 mixture of 3,3',5,5'-Tetramethylbenzidine (TMB) solution and hydrogen peroxide (H_2O_2) was added to each well and left for 30 minutes in the dark. The reaction was stopped with 100 µL of 2 N sulphuric acid.

Absorbance was read at the appropriate wavelength using the FLUOstar Omega spectrophotometer microplate reader. Omega analysis software was used to calculate a curve using a 4-parameter fit. Only r^2 values higher than 95 % were accepted for the standard curve.

2.8 Investigating monocytic TF expression

2.8.1 LPS stimulation of monocytic TF expression

PBMCs and sorted monocytes were cultured with 500,000 cells per well in 450 µL of IMDM and 50 µL of serum in 24-well plates. Where available, autologous serum was used (obtained

as described in section 2.5.1), otherwise it was substituted with FBS. PBMCs were incubated with a range of LPS concentrations (0, 1, 10, 100, 1000 ng/mL) for 24 hr. The incubator was set at 37 °C and with 5 % CO_2. All work was performed in a Class II biological safety cabinet.

Following incubation, the samples were removed from the wells with scraping and centrifuged for 5 min at 200 *g*. The supernatants were frozen at -80 °C. Cell pellets were re-suspended in 100 µL flow buffer (see section 2.5.1, page 6162) and flow cytometry antibodies added in quantities detailed in table 2.4. All panels were compensated and FMOs with CD14 and CD16 were used to gate the subsets (see sections 2.6.3, page 77 and 2.6.4, page 77). The proportion of each subset was measured using BD Diva software.

2.8.2 Measuring TF activity

Monocyte subsets were isolated using FACS (see section 2.5.3, page 64) and collected in IMDM without serum. A 96-well plate was used to incubate each subset at 30,000 cells per 100 µL of IMDM. LPS was added at a concentration of 1 ng/mL with 1 µL of autologous serum to each well, making a final concentration of 1 % serum. Cells were incubated at 37 °C with 5 % CO_2 for 24 hr. A well containing 99 µL IMDM and 1 µL of autologous serum was used to calculate the amount of TF activity in cell-free serum; this value was then subtracted from the values recorded from the subsets.

Following incubation, the cells were lysed with the addition of 0.1 % Triton for 10 min at 4 °C. Each well was scraped and mixed.

A TF activity was measured using a human TF activity colorimetric assay kit according to the manufacturer's instructions. Briefly, eight TF activity standards were reconstituted using serial dilutions and one blank. An assay mix containing 50 µL assay diluent, 10 µL of FX and 10 µL of FVII were added to each well, followed by 10 µL of either a standard or sample. The plate was incubated at 37 °C for 30 min. In the presence of TF, FX will become FXa. Each well had 20 µL of FXa substrate added; in the presence of FXa a yellow para-nitroaniline (pNA) chromophore is released.

Absorbance was measured at 405 nm every 5 min for 25 min on a FLUOstar Omega spectrophotometer microplate reader. The Omega analysis software was used to create a standard curve using a 4-parameter fit; only curves with r^2 values higher than 0.95 were

accepted. Concentrations were measured during the time frame when the TF standards recorded a steady level. The TF activity was calculated and expressed as pM per minute.

2.8.3 Inhibition of LPS induction of TF cell surface expression

Monocytes were isolated as described earlier (section 2.5.2, page 62). A total of ten wells in a 24-well plate were filled with 500,000 PBMCs, with a presumption that approximately 100,000 monocytes would adhere.

Three inhibitors were used to investigate the role of ERK, cRel and NFκB signalling in the LPS induction of TF transcription. Each inhibitor had been previously shown to be effective (Fanjul *et al.*, 1994; Shiohara *et al.*, 1999; Ohori *et al.*, 2005; Perrett *et al.*, 2013; Shono *et al.*, 2016). All were titrated to ensure optimum inhibition whilst maintaining cell viability above 90 % (see section 3.3.3, page 115). FR180204, an inhibitor of ERK1 and ERK2, was used at a concentration of 15 μM. IT901, an inhibitor of the NFκB subunit cRel, was used at a concentration of 1.0 μM. SR11302, an inhibitor of AP-1 was titrated and used at a concentration of 1.0 μM. All were reconstituted as per the manufacturer's instructions using DMSO; further dilutions were performed using IMDM.

The inhibitors were added at titrated concentrations to monocytes isolated by adherence and the cells were left to incubate for 30 min. Following incubation, LPS was added at a concentration of 1 ng/mL and the cells incubated for 24 hr. Flow cytometry was performed on cells as described earlier (see section 2.6.2, page 73).

2.9 Healthy volunteer samples

2.9.1 Recruitment

Healthy volunteers were recruited into the study 'The role of inflammation of human immunity' from the students and staff of Newcastle University (Research Ethics Committee number 12/NE/0121). Participants were screened and excluded if they had a history of infection, autoimmune disease or significant illness, or if were taking regular medication (excluding the oral contraceptive pill). All participants received a participant information sheet and were given an opportunity to discuss the study with a researcher before providing written, informed consent (see appendix A). Venepuncture was performed with a 21-gauge butterfly needle and blood collected into a 50 mL Falcon tube containing a final concentration of 1 % sodium citrate.

2.9.2 Processing of samples

Samples were stored at room temperature and processed within 30 minutes of venepuncture. PBMCs were isolated using Ficoll-Paque™ as described earlier (see methodology section 2.5.1, page 61). Once isolated, PBMCs were stored on ice and used within 1 hour.

2.10 Human endotoxic samples

2.10.1 Recruitment

Healthy volunteers were recruited through electronic mail advertisement to the study 'Does the DNA of our cells' batteries influence our response to bacteria?' (Research Ethics Committee number 17/YH/0021). This study was performed in the integrated critical care unit (ICCU) at City Hospitals Sunderland NHS Foundation Trusts (CHSFT), in a dedicated research bed space by a Consultant Intensivist. Interested volunteers were sent a participant information sheet and given time to consider the information.

Screening of possible participants occurred in the out-patients department at CHSFT and was performed by a Consultant Intensivist. This involved a short medical history, physical examination, a blood sample (for a full blood count, coagulation screen, liver and renal function), an electrocardiogram and a pregnancy test in female participants. Exclusion criteria included: age outside the range 18-40 years; known history of respiratory, cardiac or infectious diseases; pregnancy; current medication except the oral contraceptive pill in female participants; and laboratory blood results outside of the reference range.

Following screening, written informed consent was taken and the participant's general practitioner informed of their involvement. Female participants were advised to use contraception for 48 hr following the study. Copies of the participant information sheet, consent forms and protocol can be found in appendix B.

2.10.2 Protocol

The study involved three visits. The initial visit involved a stay of 10 hr on the ICU. A bolus injection of 2 ng/kg of U.S. reference *Escherichia coli*-derived endotoxin was given to induce a state of systemic inflammation. Vital signs (pulse rate, blood pressure, blood oxygen saturations, respiratory rate and temperature) were measured at 30 min intervals throughout the ICCU stay. An intravenous cannula was placed into each arm; one was used to give an infusion of Hartmann's solution and the other to take blood samples at baseline, 90 min, 4 hr, 6 hr and 10 hr. Blood was collected into three types of vacutainers, containing either

ethylenediaminetetraacetic acid (EDTA), sodium citrate or serum-separating silica (see table 2.5).

Two further study visits were performed at 24 hr and 7 days following the injection of endotoxin. The participant was reviewed at the out-patient department in CHSFT, where a short history, examination and blood sampling was performed.

Time-point	Volume in each sample tube (mL)		
	EDTA anticoagulant	Sodium Citrate anticoagulant	Serum separating tube
Baseline (pre-endotoxin)	84	4.5	10
90 min	84	4.5	10
4 hr	4	4.5	10
6 hr	84	4.5	10
10 hr	84	4.5	10
24 hr	4	4.5	10
7 days	4	4.5	10

Table 2.5 Blood samples taken following endotoxaemia

This table details the samples taken at each time point.

2.10.3 Processing of samples

At each time point, a 1 mL sample of EDTA anti-coagulated blood was processed according to the flow cytometry protocol as outlined in section 2.6.1 (page 72).

At baseline, 90min, 6 hr and 10 hr time-points, nine extra 9 mL EDTA samples were taken and used to isolate PBMCs that were then frozen as described earlier (see sections 2.5.1, page 61 and 2.5.3, page 64).

The remaining 6 mL EDTA and citrate samples were centrifuged at 300 *g* for 10 min 21 ºC. Sodium heparin tubes were centrifuged at 1500 *g* for 10 min at 21 ºC to separate the serum. Plasma and serum were removed and stored in 200-400 µL aliquots at -80 °C.

2.11 Critically ill and septic samples

2.11.1 Recruitment

Participants from the intensive care units (ICUs) at Newcastle upon Tyne Hospitals NHS Foundation Trust (NuTH) and CHSFT were recruited into the study 'The effects of critical illness on innate immunity' (Research Ethics Committee number 18/NE/0036). Samples for this work were taken from June 2017 until March 2019. The study involved taking a 20 mL blood sample, and collecting medical data, whilst patients were in ICU. In those participants who recovered, a second blood sample was taken within one working day of discharge from the ICU. Inclusion and exclusion criteria for the study are shown in Table 2.6.

Inclusion Criteria	Exclusion criteria
Expected to remain in the ICU for longer than 24 hr	<16 years of age
Expected to survive for longer than 24 hr	Pregnancy
Provision of written consent (or written consultee declaration)	Known infection with human immunodeficiency virus
Requires one or more of the following organ supports: inotropes, invasive ventilation, non-invasive ventilation, haemofiltration or dialysis.	Use of immunosuppressants or corticosteroids at a dosage higher than the equivalent of prednisolone 10 mg/day
	Haematological malignancy

Table 2.6 Inclusion and exclusion criteria for the study 'The effects of critical illness on innate immunity'

This table lists inclusion and exclusion criteria for the study used to obtain samples from critically ill participants.

2.11.2 Consent

Individuals were identified and assessed for capacity to consent to the study by a Consultant Intensivist in charge of their care. If all eligibility criteria were met and the individual had capacity to consent, they were provided with a participant information sheet and given as much time as they needed to read and understand the information. Research nurses and myself were available to discuss the study with the individual and their relatives. If they wished to proceed with the study, they were asked to provide written informed consent.

Frequently, individuals staying on ICU did not have the capacity to give informed consent. In this setting, the next of kin was approached to act as a personal consultee and given a copy of a personal consultee consent form. Again, the research team were available to answer any questions. If the personal consultee believed the individual would agree to the study, they were asked to sign a personal consultee declaration form. If there was no next of kin, a Consultant Intensivist, who was independent of the research study, was asked to act as a professional consultee. The relevant consultant was given a professional consultee information sheet and asked to sign a professional consultee declaration form.

If those participants recruited using a consultee later regained capacity, they were given a recovered capacity information sheet and the study was explained. The participant would then be asked to sign a recovered capacity consent form if they wished to remain in the study. Individuals who did not wish to remain in the study were asked if samples and data already gathered could be kept; if they did not agree, samples were destroyed.

It was clearly explained to all participants, their families and the clinical teams caring for them that they could withdraw from the study at any time.

Copies of the study protocol, all participant information sheets and consent and declaration forms are included in appendix C.

2.11.3 Processing of samples

A single sample of 20 mL of whole blood was collected into a 50 mL Falcon tube with a final concentration of 1 % sodium citrate on recruitment into the study and then, if appropriate, within one working day of discharge from ICU.

A 1 mL aliquot of whole blood was taken to perform flow cytometry as described earlier (methodology section 2.6.1, page 72).

The whole blood sample was initially centrifuged for 5 minutes at 300 *g* at 21 ºC to separate cells from plasma. The plasma was removed and stored at -80 °C in aliquots of 200 µL. The volume removed was then replaced with warmed 0.9 % saline. This was layered over Ficoll-Paque™ and PBMCs were isolated as previously described (methodology section 2.5.1, page 61).

Serum samples were also obtained from plasma as previously described (section 2.5.1, page 61) using 1 mL of plasma. Serum was then stored in aliquots of 200 µL at -80 °C.

Recovery samples were processed in the same way.

2.11.4 Data collection

Clinical data were collected at the time of the first sample. This included clinical, radiological and microbiological evidence of infection; types of organ support used; information to calculate the Acute Physiology and Chronic Health Evaluation (APACHE) II and Sequential Organ Failure Assessment (SOFA) scores; co-morbidities; concurrent medication; and recent full blood count and coagulation screen. A copy of the data collection form is shown in appendix C.

2.12 Statistical analysis

Statistical analysis was performed using Graphpad Prism (version 7.01).

Data were tested for normality using the D'Agostino & Pearson normality test. The significance level for all analysis was set at $p<0.05$.

Data assuming a normal distribution were analysed using a paired *t*-test if the data sets were dependent, or an unpaired *t*-test if they were not. If more than two variables were compared, either a one-way or repeated measures ANOVA was used.

Data not assuming a normal distribution were analysed using the Wilcoxon signed-rank test when they were dependent or the Mann-Whitney U test if they were not. Where more than

two measurements were compared, significance was analysed using Friedman's test with Dunn's multiple comparisons test (if repeated measures were compared) or the Kruskal-Wallis test (when there was no matching between variables).

Fisher's exact test was used to compare the data of contingency tables.

Chapter 3. Investigation of Tissue Factor Expression across Monocyte Subsets

3.1 Overview

Monocytes are known to express TF on their cell surface; however, there are very limited data comparing this expression between monocyte subsets (Rivers *et al.*, 1975; Stojkovic *et al.*, 2017). There is no published work investigating the effects of LPS on TF induction, TF activity or transcription of TF between the subsets.

TF is believed to be important in the pathogenesis of disseminated intravascular coagulation (DIC), a widespread activation of coagulation that occurs in small blood vessels and increases mortality from sepsis (Warr *et al.*, 1990). Multiple groups report a change in monocyte subsets during sepsis, with a decrease in classical and an expansion of either intermediate or non-classical monocytes (Fingerle *et al.*, 1993; Herra *et al.*, 1996; Mukherjee *et al.*, 2015). Potentially, relative TF expression on these subsets could contribute to the coagulopathy of sepsis.

This chapter will focus on the cell surface expression of TF between the monocyte subsets and investigate the interactions between monocyte subsets and microvascular endothelium.

3.2 Research aims

- to investigate the cell surface expression and activity of TF between monocyte subsets with and without LPS stimulation
- to create an *in vitro* model of monocyte-endothelial interactions during sepsis
- to understand the pathway through which LPS induces TF expression on the cell surface monocyte subsets.

3.3 Investigating changes in monocyte subsets and TF expression following LPS stimulation

To investigate the expression of monocyte TF, monocytes were cultured with LPS, a component of the Gram-negative bacterial cell wall. Monocytic co-culture PMVECs, with and without LPS stimulation, was used to investigate changes in TF cell surface expression in the subsets.

PBMCs were isolated from healthy volunteer whole blood using Ficoll-Paque™ density gradient separation. FACS was then used to sort monocytes as a whole or each subset individually (see methodology section 2.5.3 and 2.5.4, pages 64 to 67).

3.3.1 Effect of LPS stimulation on monocyte subsets

Monocytes (not sorted into individual subsets) were obtained using FACS and were cultured for 24 hours with LPS in IMDM and 10 % autologous serum. Into each well 50,000 monocytes were added. Following this the proportions of monocyte subsets were assessed using flow cytometry. Figure 3.1 shows the percentage of sorted monocytes that were recovered following *in vitro* culture with LPS. The majority of monocytes were not recovered following culture. Propidium iodide was used to assess cell viability. The LPS concentrations used (1 ng/mL to 1000 ng/mL) did not impact cell viability (Figure 3.2).

There was a dose-dependent increase in the proportion of classical monocytes following LPS stimulation (Figure 3.3). This was accompanied by a relative reduction in the intermediate and non-classical subsets. Further analysis of the flow data, using the same samples, showed a decrease in the CD16 median fluorescent intensity (MFI), whilst the CD14 MFI was unchanged (Figure 3.4).

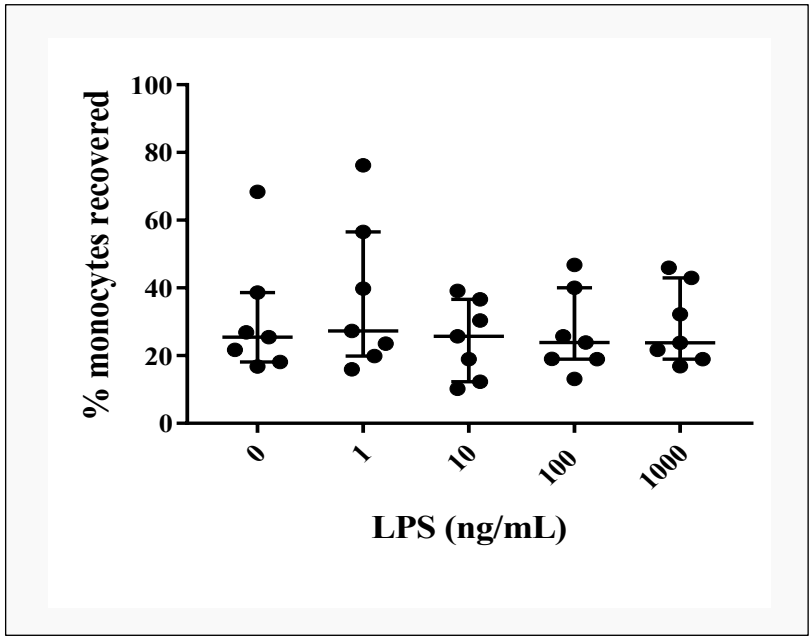


Figure 3.1 Percentage of sorted monocytes recovered following culture with LPS

Monocytes, negatively sorted using FACS, were incubated with varying concentrations of LPS in IMDM and 10% autologous serum. Following a 24-hour incubation, flow cytometry was performed to measure the proportions of monocyte subsets and TF surface expression. This figure shows the percentage of monocytes recovered from each sample following the in vitro culture with increasing levels of LPS. The results of 7 independent experiments are displayed. Black dots show the median values whilst error bars show the interquartile range. Significance was calculated using the Friedman test with Dunn's multiple comparisons test. There was no statistically significant difference between the time-points.

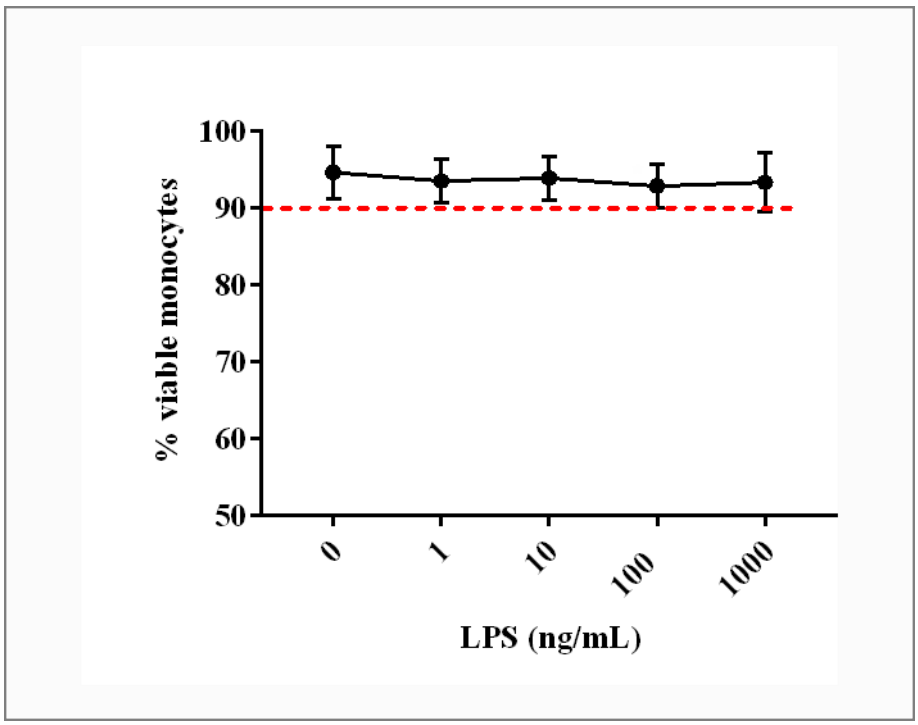


Figure 3.2 Viability of recovered monocytes

Monocytes, negatively sorted using FACS, were incubated with varying concentrations of LPS in IMDM and 10% autologous serum. Following a 24-hour incubation, flow cytometry was used to measure cell viability using propidium iodide. This figure shows the percentage of live cells in each sample with increasing levels of LPS. The results of 7 independent experiments are displayed. Black dots show the median values whilst error bars show the interquartile range. The red dashed line shows 90% cell viability. Significance was calculated using the Friedman test with Dunn's multiple comparisons test. There was no statistically significant difference between the time-points.

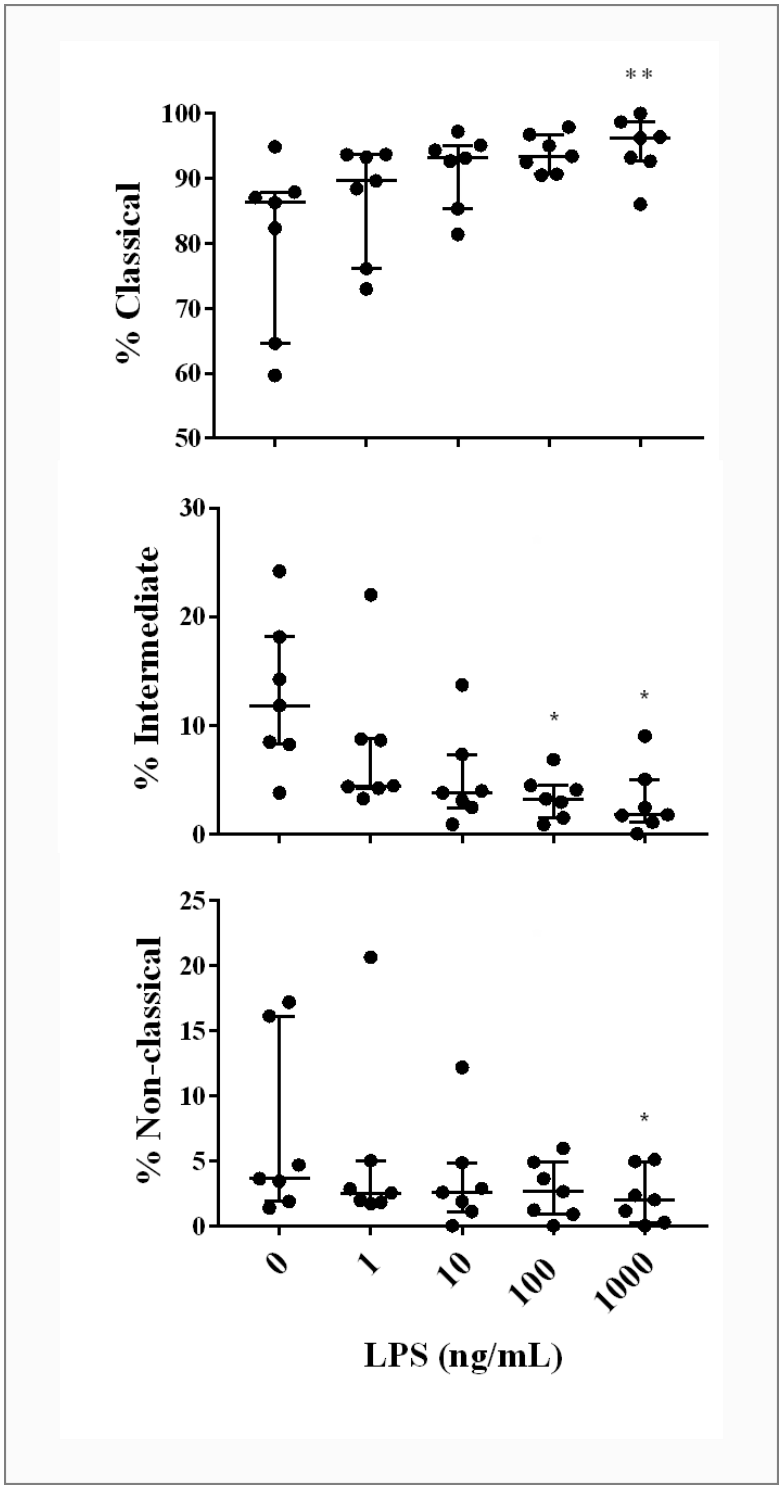

% Classical
100
90
80
70
60
50
**
% Intermediate
30
20
10
0
*
*
% Non-classical
25
20
15
10
5
0
*
0
1
10
100
1000
LPS (ng/mL)

Figure 3.3 Incubation with LPS increases the proportion of classical subset monocytes

*Monocytes, negatively sorted using FACS, were incubated with increasing concentrations of LPS in IMDM and 10% autologous serum. Following a 24-hour incubation, flow cytometry was used to measure the proportions of monocyte subsets. This figure shows the results of 7 independent experiments. Error bars represent median values and the interquartile range. The classical subset increased in proportion ($p=0.006$ by Friedman test, ** - $p<0.001$ between 1000 ng/mL LPS and no LPS using Dunn's post hoc test). The intermediate subset decreased following incubation with LPS ($p=0.006$ by Friedman test, *- $p<0.05$ between 100 ng/mL LPS and 1000 ng/mL LPS compared to no LPS using Dunn's post hoc test). The non-classical subset decreased in proportion ($p=0.06$ by Friedman test, * -$p<0.05$ between 1000 ng/mL LPS and no LPS using Dunn's post hoc test).*

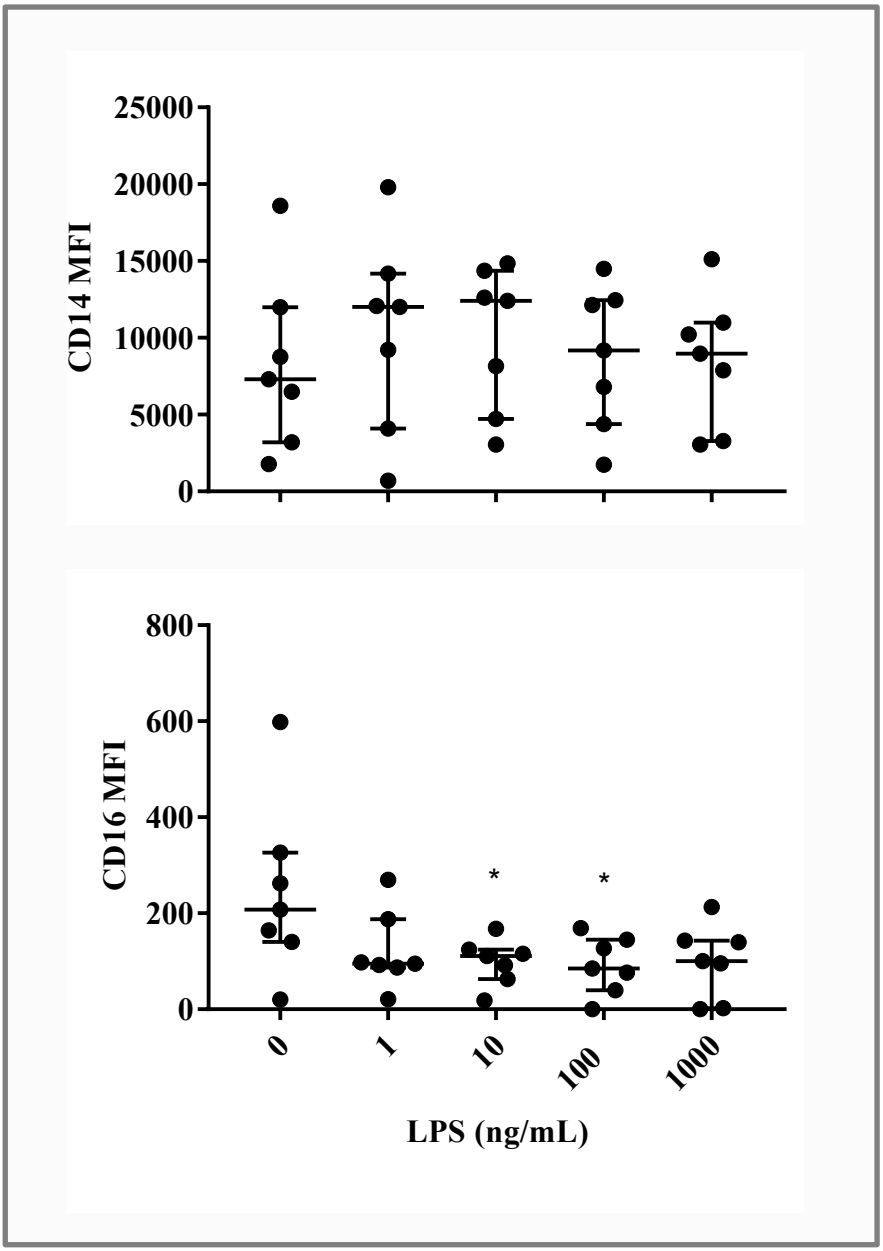


Figure 3.4 Incubation with LPS reduces the expression of CD16

*Monocytes, negatively sorted using FACS, were incubated with increasing concentrations of LPS in IMDM and 10 % autologous serum. Following a 24-hour incubation, flow cytometry was used to measure the total monocyte median fluorescence for CD14 and CD16. This figure shows the results of 7 independent experiments. Error bars represent median values and the interquartile range. There was no statistically significant difference in the CD14 MFI between the LPS concentrations (p = 0.53 by Friedman test). The CD16 MFI decreased with LPS incubation (p=0.02 by Friedman test, *- p<0.05 when the 10 ng/mL and 100 ng/mL LPS were both compared to no LPS by Dunn's post hoc test).*

3.3.2 Changes in TF expression following LPS incubation

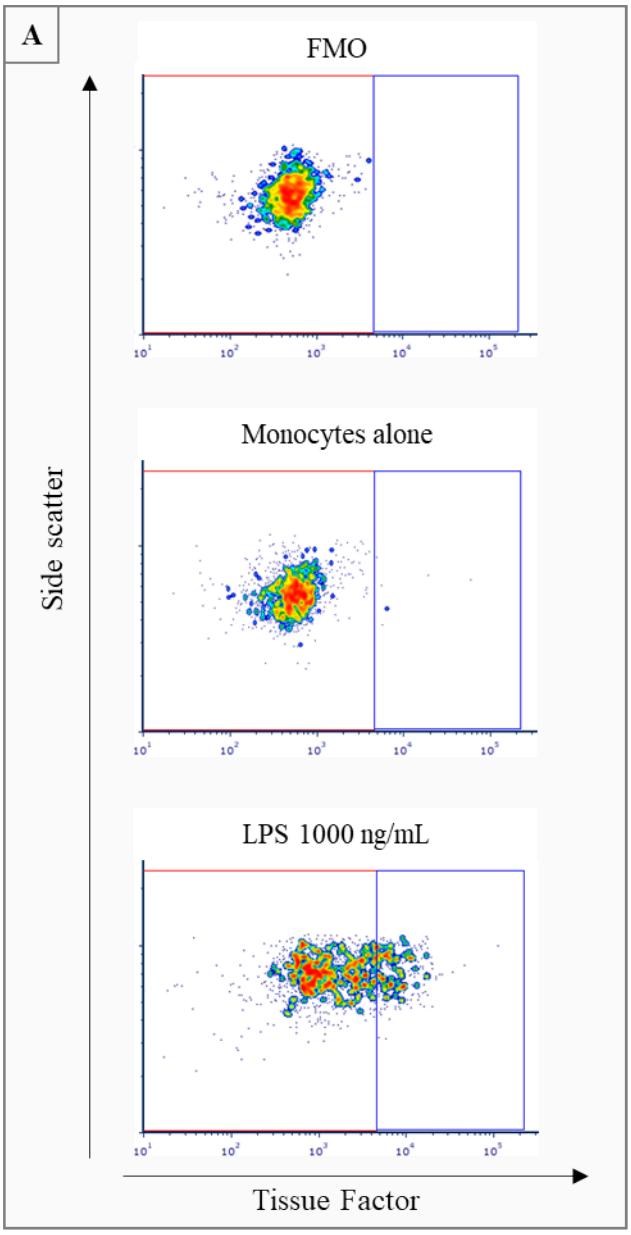
Using the same 7 donors as described in figures 3.3 and 3.4 the cell surface expression of TF was measured on monocyte subsets using flow cytometry. As before, this followed incubation in IMDM and 10 % autologous serum with increasing concentrations of LPS. Initially, the total monocyte population was examined. This showed a dose-dependent increase in TF in response to LPS stimulation (Figure 3.5).

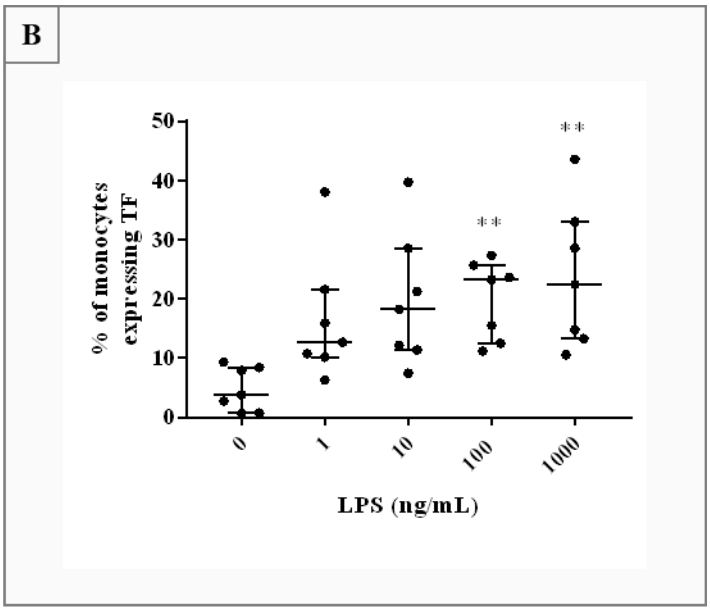
A comparison between the monocyte subsets demonstrated a variation in TF expression. All monocytes subsets increased TF expression with LPS stimulation. Classical and intermediate monocytes expressed similar amounts, whilst non-classical had both fewer cells expressing TF and a lower MFI (Figure 3.6).

To investigate whether the expressed TF was functionally active, the ability of TF to activate FX was compared between the subsets. PBMCs from 5 different healthy volunteers were sorted into monocyte subsets using FACS (see methodology section 2.5.4, page 69). Each subset was incubated at a concentration of 10,000 cells per 500 µL, with and without 1 ng/mL LPS for 24-hours. A TF colorimetric assay was used to measure the amount of FXa created from FX. LPS requires LPS-binding protein to be effective; this is found in autologous serum, and for this reason 5 % serum was added to each culture. Autologous serum was used for each culture and the level of TF measured in serum alone at the same dilution. The serum value of TF was subtracted from each monocyte sample and the activity expressed as pM per 10,000 cells.

TF activity varied by subset. Classical monocytes had the highest level of total activity per 10,000 cells, whereas intermediate and non-classical monocytes showed similar total activity levels. However, as shown in Figure 3.6, compared to the classical and intermediate subsets, fewer non-classical monocytes increase TF cell surface expression in response to LPS. Earlier experiments have shown a median of 15 % of both the classical and intermediate subsets express TF on the cell surface following incubation with 1 ng/mL LPS. This is higher than the 10 % of non-classical monocytes that express TF on the cell surface at the same LPS concentration. Assuming these percentages are representative of the monocytic TF from the 5 different healthy volunteers used to investigate TF activity, the classical and non-classical monocytes show a similar TF activity (Figure 3.7).

Although incubation with LPS increases the cell surface expression of TF, incubation with LPS does not increase the activity of TF. Using the same five donors as the earlier TF activity work, a comparison between monocyte subsets cultured alone and those incubated with LPS showed that TF activity did not increase in response to LPS (Figure 3.8).


A
FMO
Monocytes alone
LPS 1000 ng/mL
Side scatter
Tissue Factor


B
% of monocytes expressing TF
50
40
30
20
10
0
**
**
0
1
10
100
1000
LPS (ng/mL)

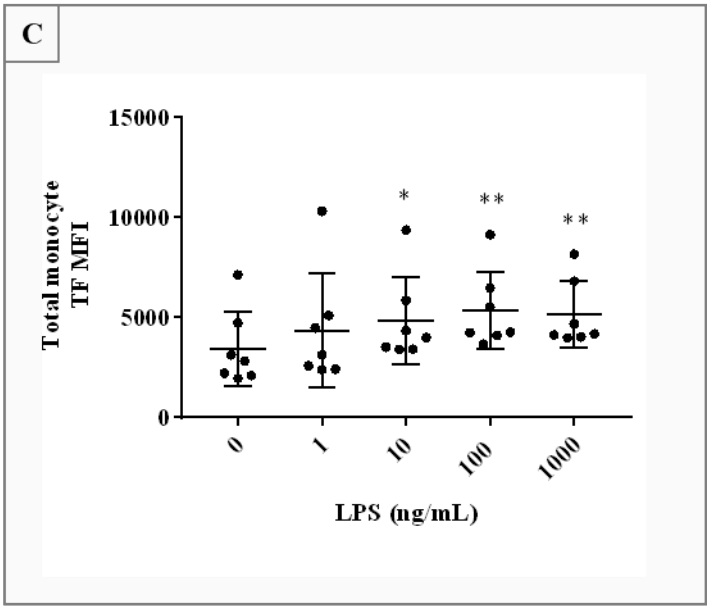

C
Total monocyte TF MFI
15000
10000
5000
0
*
**
**
0
1
10
100
1000
LPS (ng/mL)

Figure 3.5 Incubation with LPS increases the monocytic cell surface expression of TF

Monocytes, negatively sorted using FACS, were incubated with varying concentrations of LPS in IMDM and 10 % autologous serum. Following a 24-hour incubation, flow cytometry was used to measure the percentage of monocytes expressing TF. Error bars in figures ***B*** *and* ***C*** *represent the median values and interquartile range. The results of 7 independent experiments are shown. Both the 1 and 10 ng/mL concentrations of LPS were statistically compared to no LPS.* ***A*** *demonstrates the TF gating and is a representative example of monocytes incubated without LPS, with 1000 ng/mL of LPS and monocytes not stained for TF. The red gate shows the negative population as set by the FMO, whilst the blue line gates the TF positive population.* ***B*** *shows the percentage of monocytes expressing TF, as measured by flow cytometry using an FMO control. The percentage of monocytes expressing TF on their surface increases following incubation with LPS (p=0.04 by Friedman's test, **- p<0.01 for both 100 and 1000 ng/mL of LPS using Dunn's post hoc test).* ***C*** *shows the change in TF MFI. The TF MFI increases with LPS incubation (p=0.003 by Friedman's test, *- p<0.05 for 10 ng/mL of LPS, **-p<0.01 for 100 and 1000 ng/mL of LPS using Dunn's post hoc test).*

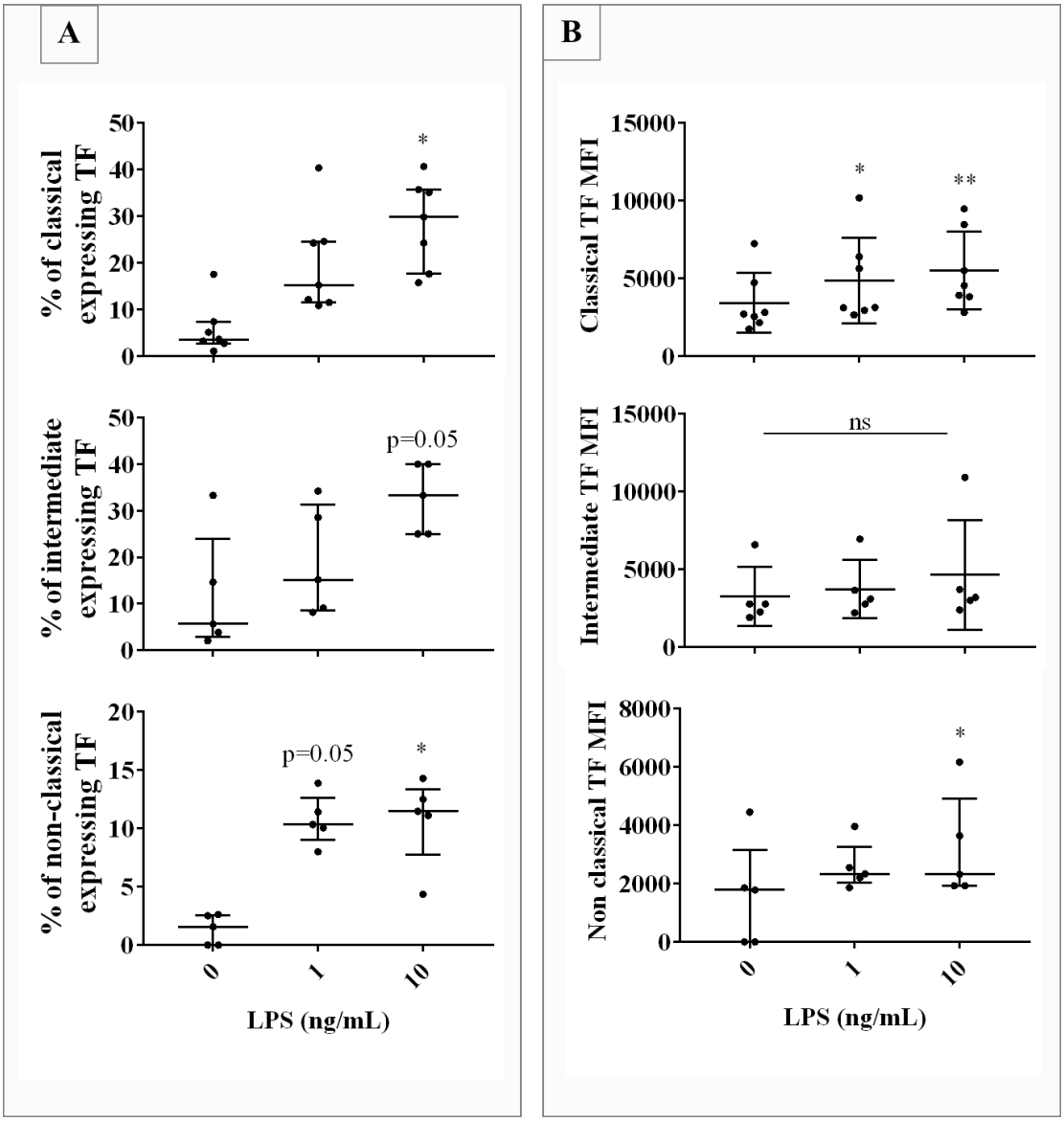
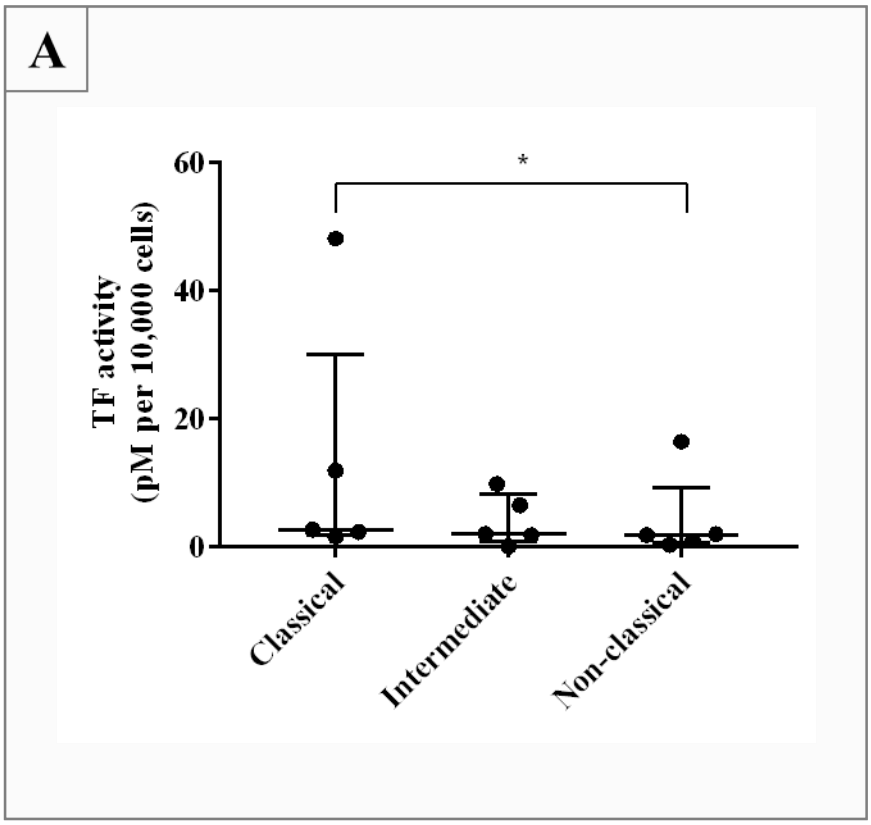

A
% of classical expressing TF
50
40
30
20
10
0
*
% of intermediate expressing TF
50
40
30
20
10
0
p=0.05
% of non-classical expressing TF
20
15
10
5
0
p=0.05
*
0
1
10
LPS (ng/mL)
B
Classical TF MFI
15000
10000
5000
0
*
**
Intermediate TF MFI
15000
10000
5000
0
ns
Non classical TF MFI
8000
6000
4000
2000
0
*
0
1
10
LPS (ng/mL)

Figure 3.6 LPS-induced TF expression differs between monocyte subsets

Monocytes, negatively sorted using FACS, were incubated with and without 1 ng/mL of LPS in IMDM and 10% autologous serum. Following a 24-hour incubation, flow cytometry was used to measure the percentage of monocyte subsets and the monocytic expression TF. This figure shows the results of 7 independent experiments (the intermediate and non-classical subset incudes 5 experiments as there were too few of these monocytes to allow accurate gating). Error bars represent median values and the interquartile range. Both the 1 and 10 ng/mL concentrations of LPS were statistically compared to no LPS. ***A*** *shows that all monocyte subsets increased the cell surface expression of TF following incubation with LPS (classical TF %: p=0.04 by Friedman's test, * - p<0.05 for 10 ng/mL LPS using Dunn's post hoc test; intermediate TF %: p=0.04 by Friedman's test, p=0.05 for 10 ng/mL of LPS using Dunn's post hoc test; non-classical TF %: p=0.02 by Friedman's test, * - p<0.05 for 10 ng/mL of LPS, p=0.05 for 1 ng/mL using Dunn's post hoc test).* ***B*** *shows that the classical and non-classical subsets increased TF MFI following incubation with LPS (classical TF MFI: p=0.003, *-p<0.05 for 1 ng/mL LPS, **-p<0.01 for 10 ng/mL LPS by Dunn's post hoc test; non-classical TF MFI: p=0.04 by Friedman's test, p=0.05 for 10 ng/mL LPS by Dunn's post hoc test). There was no statistically significant difference between the intermediate subset at different concentrations (p=0.52 by Friedman's test).*


A
*
60
40
20
0
TF activity
(pM per 10,000 cells)
Classical
Intermediate
Non-classical

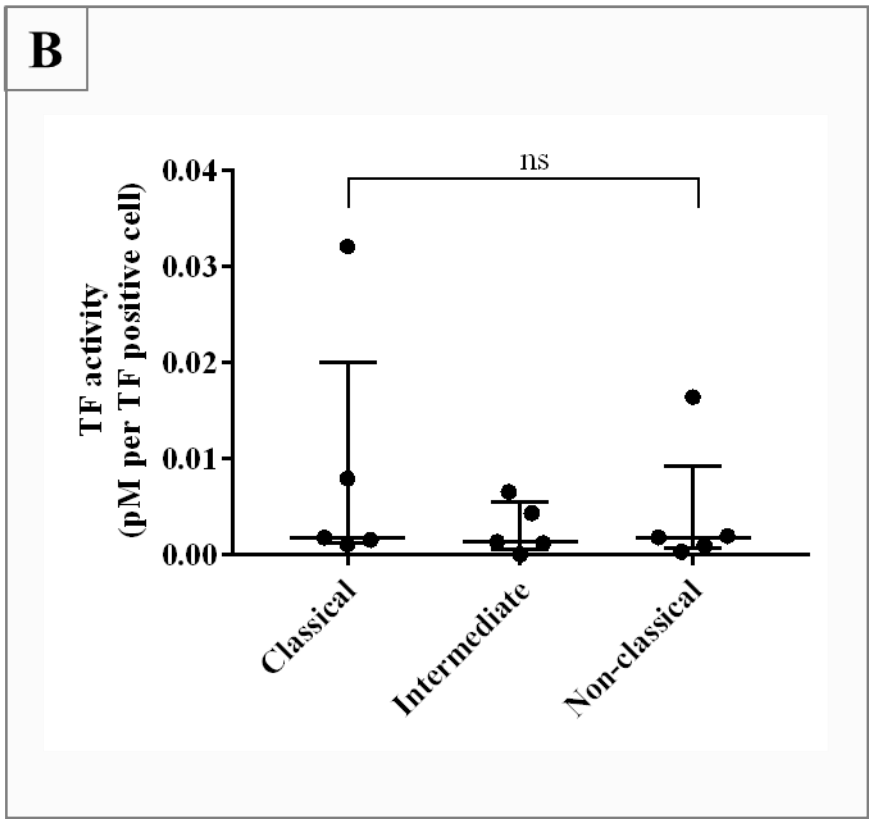

B
ns
0.04
0.03
0.02
0.01
0.00
TF activity
(pM per TF positive cell)
Classical
Intermediate
Non-classical

Figure 3.7 Monocyte subset TF activity

Monocytes were positively sorted into subsets using FACS. Each subset was incubated at a concentration of 50,000 cells per 500 µL with 1 ng/mL of LPS for 24-hours. Following incubation, the TF activity of each subset was measured using a TF colorimetric assay that quantified the activated FX. Both figures show the results of 5 independent experiments. ***A*** *shows the TF activity measured as pM per 10,000 cells. The classical subset displays the highest TF activity (p=0.03 by Friedman's test, * - p<0.05 compares the non-classical to the classical subsets by Dunn's post hoc test).* ***B*** *shows TF activity per TF positive cell. Assuming the classical and intermediate subsets have 15 % of cells expressing TF on the cell surface, whilst the non-classical subset has 10 % of cells. This is based on earlier work shown in Figure 3.5. There is no statistical difference between the subsets (p=0.09 by Friedman's test).*

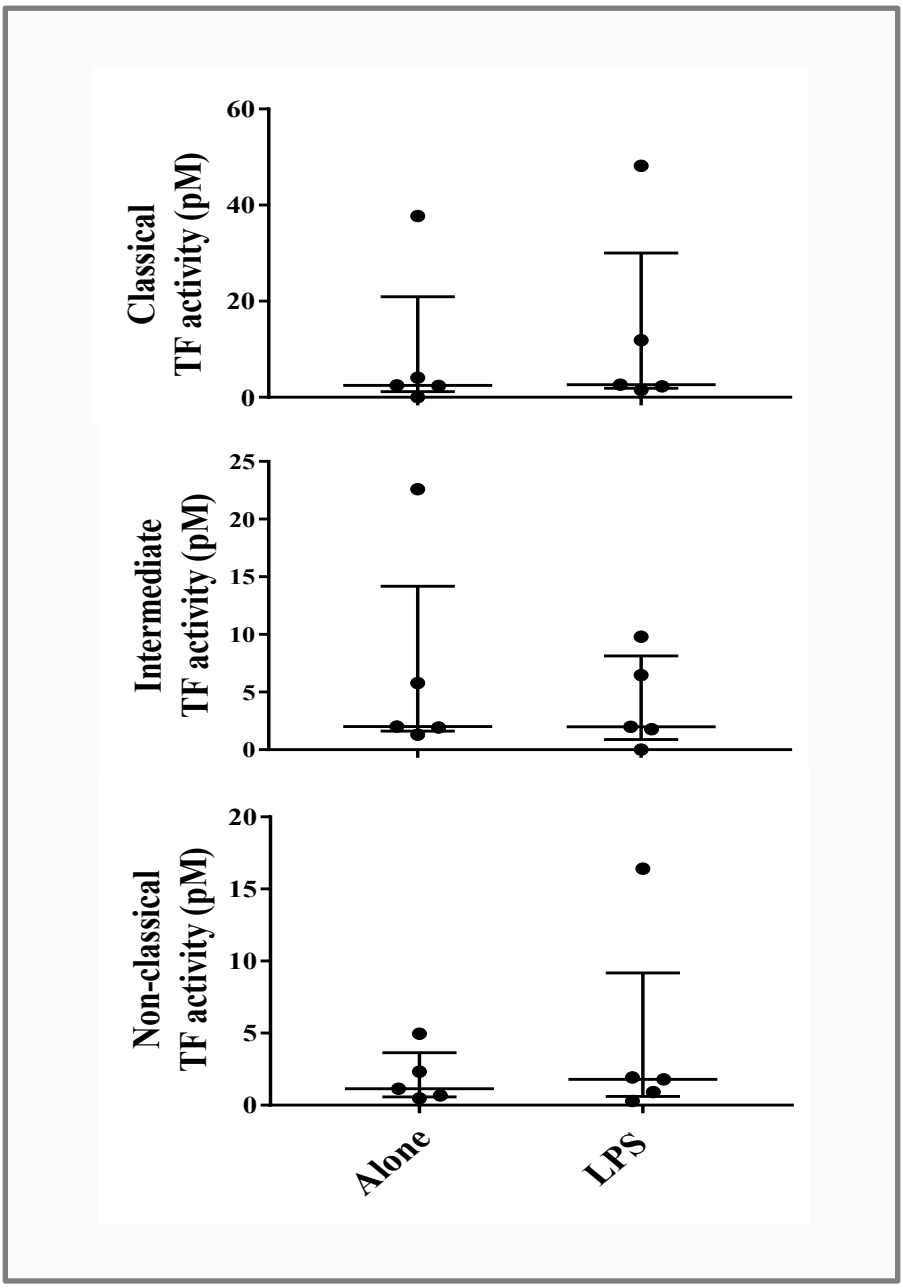


Figure 3.8 Incubation with LPS does not seem to increase TF activity

Monocytes were positively sorted in subsets using FACS. Each subset was incubated with and without 1 ng/mL of LPS for 24 hours. Following incubation, the TF activity of each subset was measured using a TF colorimetric assay that measured the amount of activated FX. Values represent the TF activity per 10,000 cells. Error bars represent median values and the interquartile range. This figure shows the results of 5 independent experiments. There was no statistically significant difference between each subset cultured alone and each subset cultured with LPS (classical- p=0.82, intermediate- p=0.63, non-classical- p=0.44 by the Wilcoxon test).

3.3.3 Inhibiting the LPS induction of monocytic cell surface expression of TF

The ERK, NFκB and JNK transcription pathways have all been shown to be important in monocytic TF expression (Bode and Mackman, 2014). To investigate whether the pathways were important in all subsets, PBMCs were obtained from 6 different healthy volunteers using Ficoll-Paque™ density gradient separation. Monocytes were isolated using the adherence method and were incubated with inhibitors prior to application of LPS at 1 ng/mL in a 24 well plate (see Figure 3.9 for the plate set-up). The LPS remained in the culture for the duration of culture. TF cell surface expression was once again measured using flow cytometry (see methodology section 2.6.2, page 73).

Three inhibitors were used to investigate the role of ERK, cRel and NFκB signalling in the LPS induction of TF transcription. All were titrated to ensure optimum inhibition whilst maintaining cell viability above 90 % (see Figure 3.10). FR180204, an inhibitor of ERK1 and ERK2, was used at a concentration of 15 μM (Ohori *et al.*, 2005; Perrett *et al.*, 2013). IT901, an inhibitor of the NFκB subunit cRel, was used at a concentration of 1.0 μM (Shono *et al.*, 2016). SR11302, an inhibitor of AP-1 was titrated and used at a concentration of 1.0 μM (Fanjul *et al.*, 1994; Shiohara *et al.*, 1999). All were reconstituted as per the manufacturer's instructions using DMSO; further dilutions were performed using IMDM.

The cell surface expression of TF was lower than in earlier experiments. No results were statistically significant, although there was a trend for TF expression to decrease in response to inhibitors in the total monocyte population, without a drop in cell viability (see Figure 3.11). A similar trend was seen in the classical and intermediate monocyte subsets but not in the non-classical subsets (see Figure 3.12).

	1	2	3	4	5	6
A	M	M	M ERK	M cRel	M AP-1	
B	M + L	M + L	M + L ERK	M + L cRel	M + L AP-1	

Abbreviation	**Meaning**
M	Monocyte
L	Lipopolysaccharide (LPS)
ERK	ERK inhibitor, FR180204
cRel	cRel inhibitor, IT901
AP-1	AP-1 inhibitor, SR11302

Figure 3.9 Set-up of TF cell surface expression inhibitor plate

This figure demonstrates the set-up of a 24-well plate used to investigate the LPS induction of monocytic cell surface TF expression.

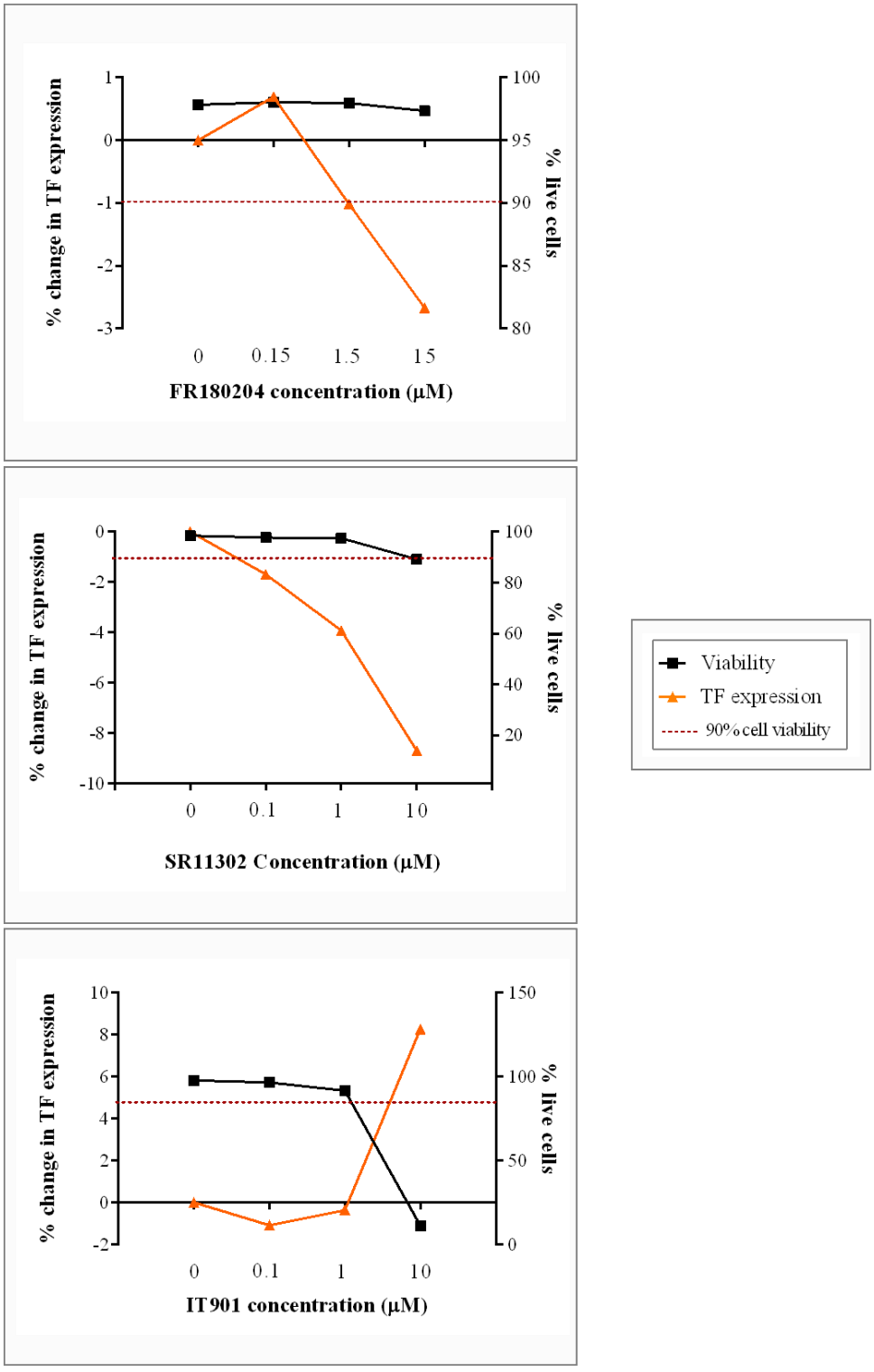


Figure 3.10 Dose-response of TF transcription inhibitors

This figure details the change in TF expression on monocytes following incubation with various inhibitors. The inhibitors were titrated to identify the concentration associated with a clear decrease in TF expression whilst maintaining cell viability above 90 %.

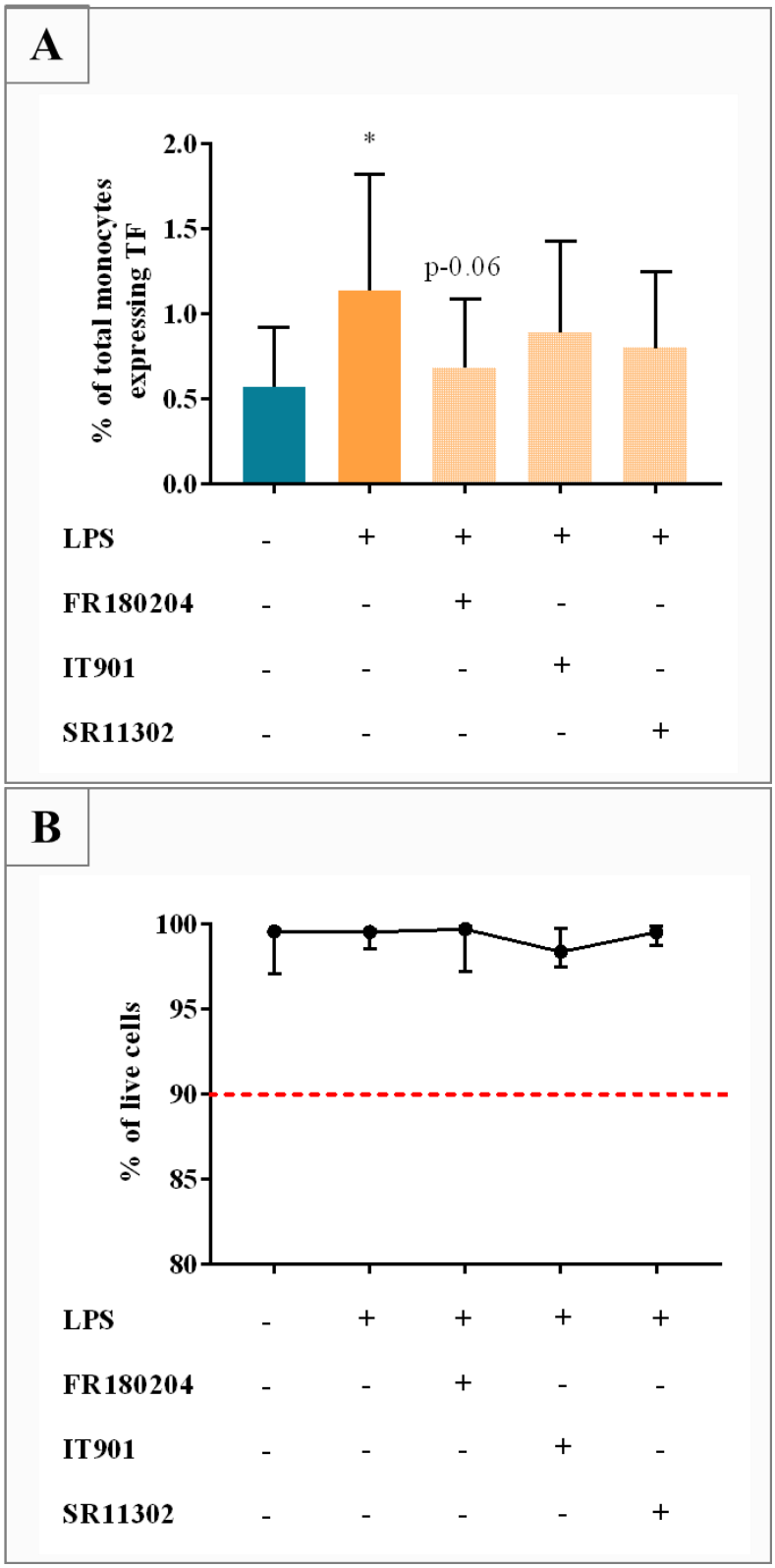

A
% of total monocytes expressing TF
2.0
1.5
1.0
0.5
0.0
*
p-0.06
LPS - + + + +
FR180204 - - + - -
IT901 - - - + -
SR11302 - - - - +
B
% of live cells
100
95
90
85
80
LPS - + + + +
FR180204 - - + - -
IT901 - - - + -
SR11302 - - - - +

Figure 3.11 Effects of transcription factors on LPS-induced TF cell surface expression

PBMCs were incubated in IMDM in 24-well plates for 1 hour and then washed to leave only adherent monocytes. Monocytes were incubated with and without 1 ng/mL of LPS in IMDM and 10% autologous serum. In some wells, inhibitors to ERK1 & 2 (FR180204, 15 μM), AP-1 (SR11302, 1 μM) and c-Rel (IT901, 1 μM) were added 30 minutes prior to the LPS. Following a 24-hour incubation, flow cytometry was used to measure the percentage of monocytes expressing TF. The results of 6 independent experiments are shown. ***A*** *shows the effects of the inhibitors of the cell surface TF expression of all monocytes. The blue bar represents monocytes alone, dark for orange for monocytes with LPS and light orange when an inhibitor is added. Error bars show the upper quartile and bar height the median values. The inhibitors did not significantly decrease the LPS-induced TF expression ($p=0.03$ by Friedman's test,* ****** *- $p<0.05$ when monocytes incubated with LPS are compared to those incubated alone, $p=0.06$ when monocytes incubated with LPS and FR180204 are compared to monocytes incubated with LPS alone using Dunn's post hoc test).* ***B*** *shows the cell viability for each condition. Black dots show the median value and the error bars the interquartile range. Viability remained above 90 % for all conditions. There were no statistically significant differences ($p=0.19$ by Friedman's test).*

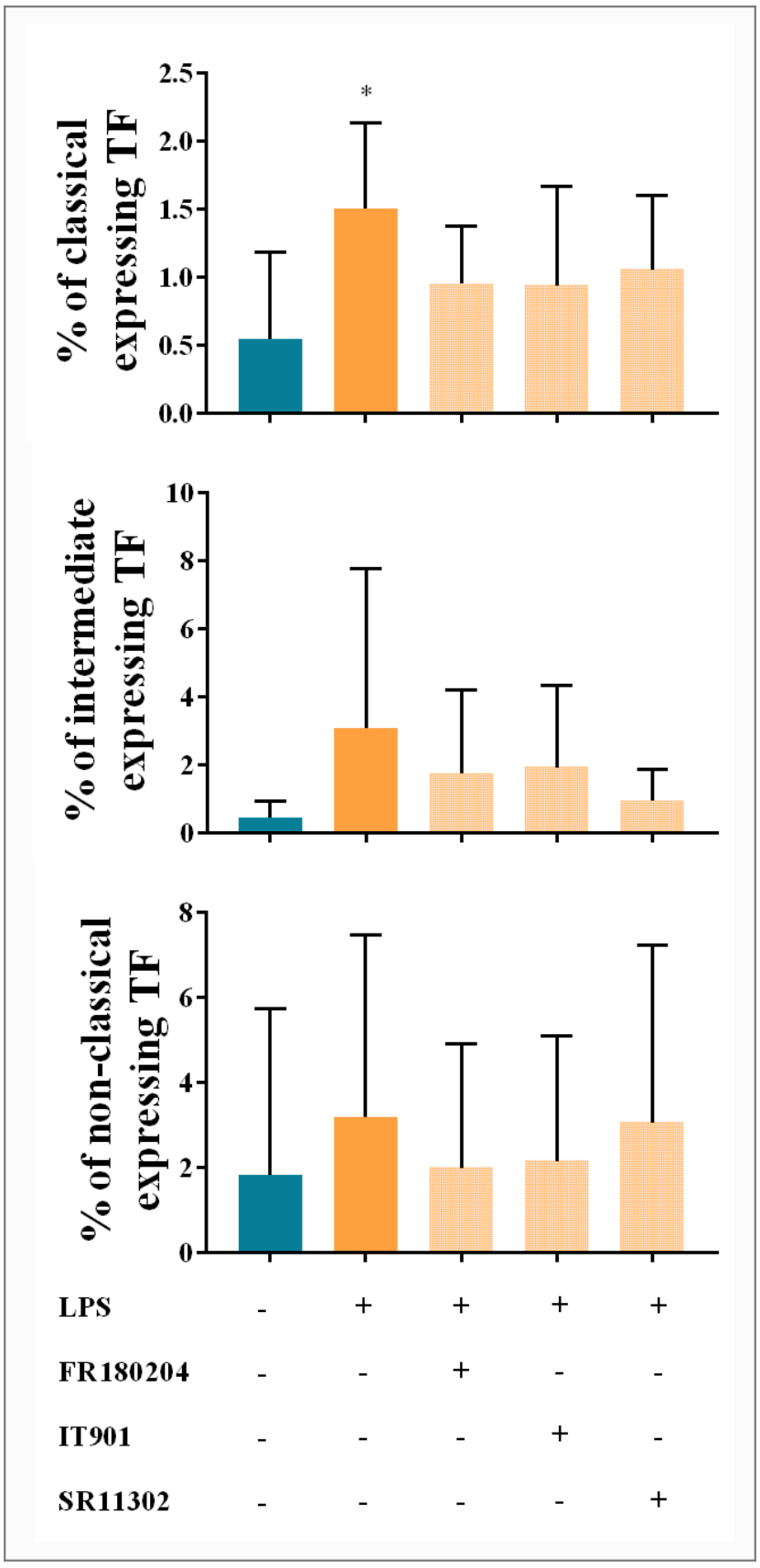

% of classical expressing TF
2.5
2.0
1.5
1.0
0.5
0.0
*
% of intermediate expressing TF
10
8
6
4
2
0
% of non-classical expressing TF
8
6
4
2
0
LPS - + + + +
FR180204 - - + - -
IT901 - - - + -
SR11302 - - - - +

Figure 3.12 Effects of transcription factors on LPS-induced TF cell surface expression across the monocyte subsets

*PBMCs were incubated in IMDM in 24-well plates for 1 hour and then washed to leave only adherent monocytes. Monocytes were incubated with and without 1 ng/mL of LPS in IMDM and 10% autologous serum. In some wells, inhibitors to ERK1 & 2 (FR180204, 15 μM), AP-1 (SR11302, 1 μM) and c-Rel (IT901, 1 μM) were added 30 minutes prior to the LPS. Following a 24-hour incubation, flow cytometry was used to measure the percentage of monocytes expressing TF. The results of 6 independent experiments are shown. The blue bar represents monocytes alone, dark for orange for monocytes with LPS and light orange when an inhibitor is added. Error bars show the upper quartile and bar height the median values. The inhibitors had no statistically significant effect on the LPS-induction of cell surface TF expression. (Classical: p=0.05 by Friedman's test, *-p<0.05 for monocytes with LPS compared to monocytes alone by Dunn's post hoc test; intermediate: p=0.17 by Friedman's test; non-classical: p=0.05 by Friedman's test).*

3.4 The effects of monocyte-endothelial interactions on TF cell surface expression

An *in vitro* model of monocyte-endothelial cell interaction was used to investigate the effects on TF and monocyte subsets. Monocytes were sorted with FACS from PBMCs using 5 healthy donors. They were cultured for 24 hours with endothelial cells in PMVEC medium. Some PMVEC were pre-treated with LPS (100 ng/mL) for one hour; the LPS was then washed off and monocytes were added to the culture.

Some monocytes were stimulated by the addition of LPS (10 ng/mL); this LPS was not washed off (see methodology section 2.7.2, page 82). These conditions were set-up in a 24-well plate as shown in Figure 3.13.

The co-culture of monocytes with unstimulated PMVEC increased in the proportion of the classical subset. Endothelial stimulation with LPS prior to the addition of monocytes does not appear to alter this effect (Figure 3.14).

Monocyte-endothelial co-cultures were also used to investigate the effect on TF expression. TF expression was shown to increase in both the classical and intermediate subsets. This effect was once again independent of LPS. There were no statistically significant changes in the non-classical subset (Figure 3.15).

To investigate whether contact was needed for the observed monocyte-endothelial cell interaction, co-cultures were repeated, using 5 different donors, with transwells to prevent cellular contact (see methodology section 2.7.2, page 82). The yield of monocytes from transwells was too poor to accurately identify monocyte subsets; however, considering the flow data for all monocytes, contact did not affect monocyte TF expression (Figure 3.16).

As discussed earlier, MCSF has been shown to alter monocyte subsets by promoting the differentiation of classical, through intermediate, to non-classical (Saleh *et al.*, 1995b; Korkosz *et al.*, 2012). Measurement of MCSF in the co-culture supernatants showed no significant difference in levels between the conditions (Figure 3.17).

The endothelial response to co-culture was measured using the supernatants. There was no significant change in tPA levels. PAI-1 levels increased following co-culture with monocytes and PMVECs pre-treated with LPS (figures 3.18 and 3.19).

	1	2	3	4
A	M	M	M + L	M + L
B	PM	PM	PM+L	PM+L
C	M/PM	M/PM	M/PM+L	M/PM+L

Abbreviation	**Meaning**
M	Monocyte
PM	Human pulmonary microvascular endothelial cell (PMVEC)
L	Lipopolysaccharide (LPS)

Figure 3.13 Co-culture plate set-up

This figure depicts the set-up of a 24-well plate for a co-culture experiment of PMVEC and monocytes. LPS (100 ng/mL) was added to PMVEC, and washed off after 1 hour, prior to the addition of monocytes. LPS (10 ng/mL) was added to monocytes and not removed. Co-culture were incubated at 37 ºC and 5 % CO_2 for 24 hours. Following this, monocyte were harvested, and flow cytometry used to measure the monocytes subset proportions and the percentage of TF-positive monocytes.

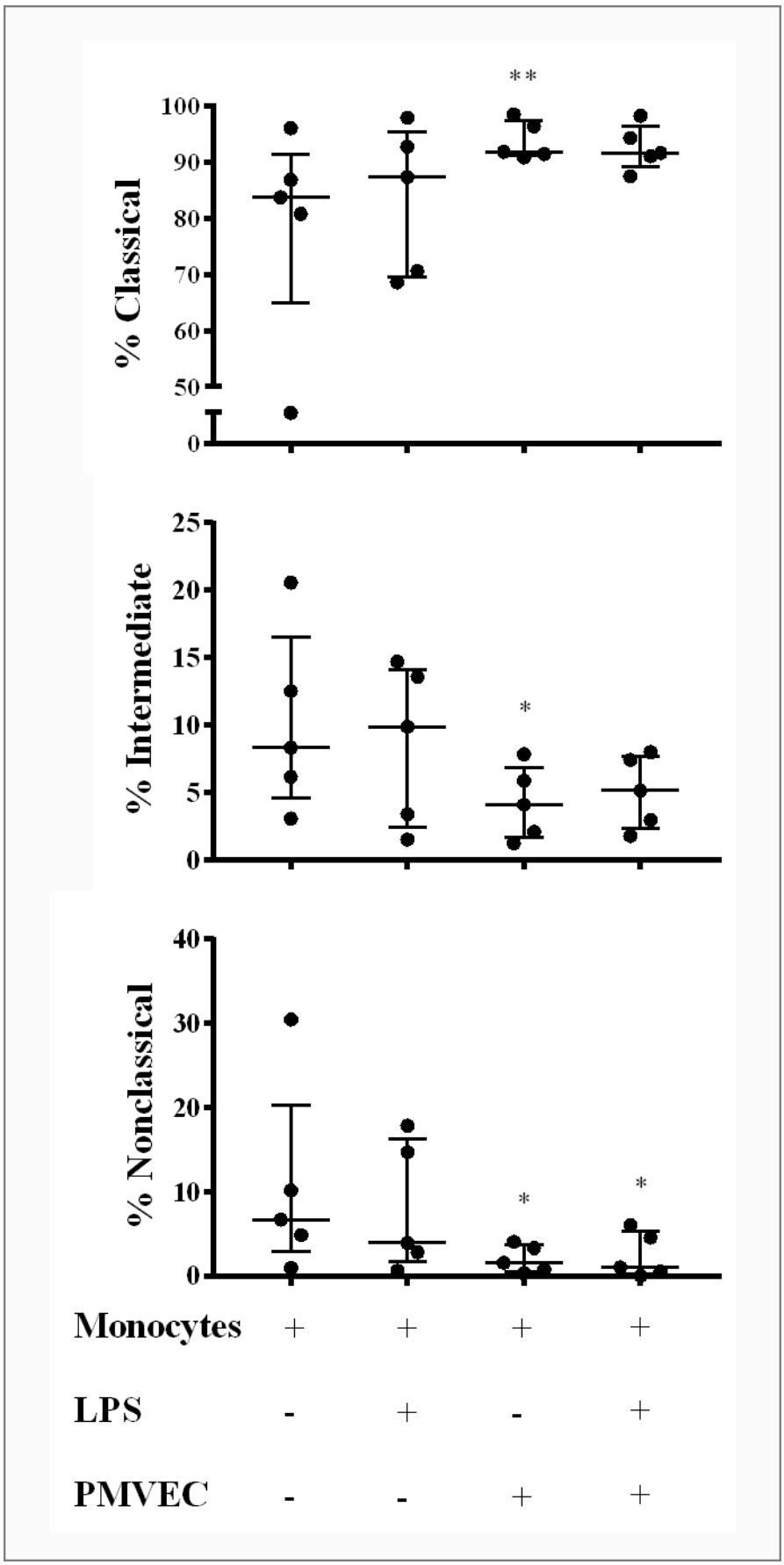

% Classical
100
90
80
70
60
50
0
**
% Intermediate
25
20
15
10
5
0
*
% Nonclassical
40
30
20
10
0
*
*
Monocytes + + + +
LPS - + - +
PMVEC - - + +

Figure 3.14 The proportion of classical monocytes are increased by PMVEC co-culture

*Monocytes were negatively sorted using FACS and incubated with and without PMVEC. A proportion of PMVEC were stimulated with 100 ng/mL of LPS for 1 hour, and the LPS was washed off prior to the addition of the monocytes. Some monocytes were cultured alone with 10 ng/mL LPS; unlike with the PMVEC stimulation, the monocyte LPS was not removed but remained there for the duration of culture. Following a 24-hour incubation, the proportions of monocyte subsets were measured using flow cytometry. This figure shows the results of 5 independent experiments. Error bars represent median values and the interquartile range. The proportion of classical monocytes increased following culture with PMVEC and LPS stimulated PMVEC ($p=0.01$ by Friedman's test, **-$p<0.01$ for monocytes cultured with unstimulated PMVEC compared to monocytes alone by Dunn's post hoc test). The intermediate subset decreases in response to PMVEC co-culture ($p=0.02$ by Friedman's test, *-$p<0.05$ for monocytes cultured with PMVEC compared to monocytes alone by Dunn's post hoc test). The non-classical subset decreases in response to PMVEC co-culture ($p=0.02$ by Friedman's test, *-$p<0.05$ for monocytes cultured with PMVEC and with LPS-stimulated PMVEC when compared to monocytes alone by Dunn's post hoc test).*

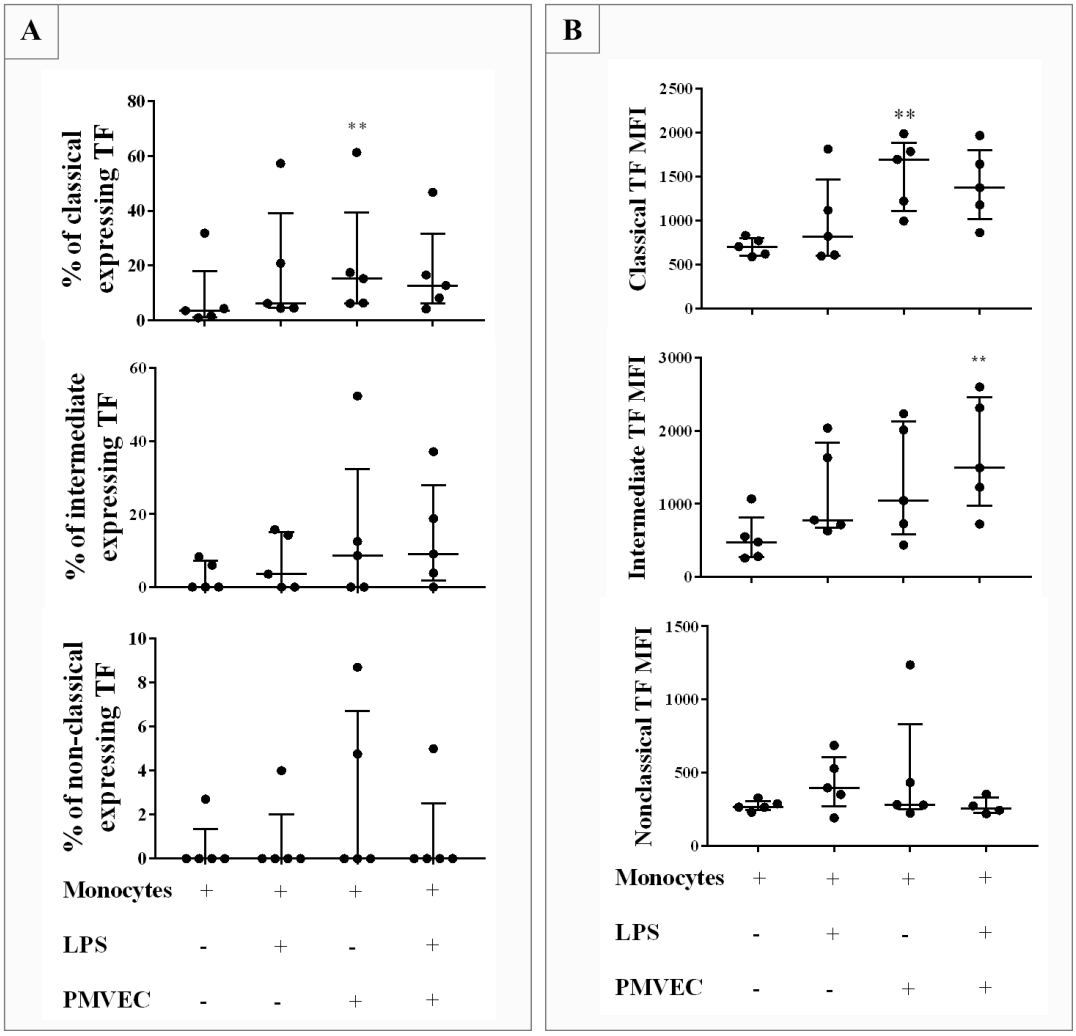

A
% of classical expressing TF
80
60
40
20
0
**
% of intermediate expressing TF
60
40
20
0
% of non-classical expressing TF
10
8
6
4
2
0
Monocytes + + + +
LPS - + - +
PMVEC - - + +
B
Classical TF MFI
2500
2000
1500
1000
500
0
**
Intermediate TF MFI
3000
2000
1000
0
**
Nonclassical TF MFI
1500
1000
500
0
Monocytes + + + +
LPS - + - +
PMVEC - - + +

Figure 3.15 Classical and intermediate monocytes increase TF expression after co-culture with PMVECs

Monocytes were negatively sorted using FACS and incubated with and without PMVEC. A proportion of PMVEC were stimulated with 100 ng/mL of LPS for 1 hour, the LPS was washed off prior to the addition of the monocytes. Some monocytes cultured without PMVEC were stimulated with 10 ng/mL LPS that was not removed but remained there for the duration of culture. Following a 24-hour incubation, the subset TF expression was measuring using flow cytometry. This figure shows the results of 5 independent experiments. Error bars represent median values and the interquartile range. ***A*** *shows the percentage of TF-positive cells for each subset following co-culture. Classical monocytes increase the cell surface expression of TF following PMVEC co-culture (p=0.02 by Friedman's test, **- p<0.01 for monocytes cultured with unstimulated PMVEC compared to monocytes alone by Dunn's post hoc test). Intermediate (p=0.31 by Friedman's test) and non-classical monocytes (p=0.88 by Friedman's test) do not increase the percentage of cells expressing cell surface TF.* ***B*** *shows the TF MFI of each subset following co-culture. Classical monocytes increase TF MFI following PMVEC co-culture (p=0.02 by Friedman's test, **- p<0.01 for monocytes cultured with unstimulated PMVEC compared to monocytes alone by Dunn's post hoc test). The intermediate subset increases TF MFI following co-culture with unstimulated and LPS-stimulated PMVEC (p=0.001 by Friedman's test, **- p<0.01 for monocytes cultured with LPS-stimulated PMVEC compared to monocytes alone by Dunn's post hoc test). There was no statistically significant change in the non-classical subset (p=0.80 by Friedman's test).*

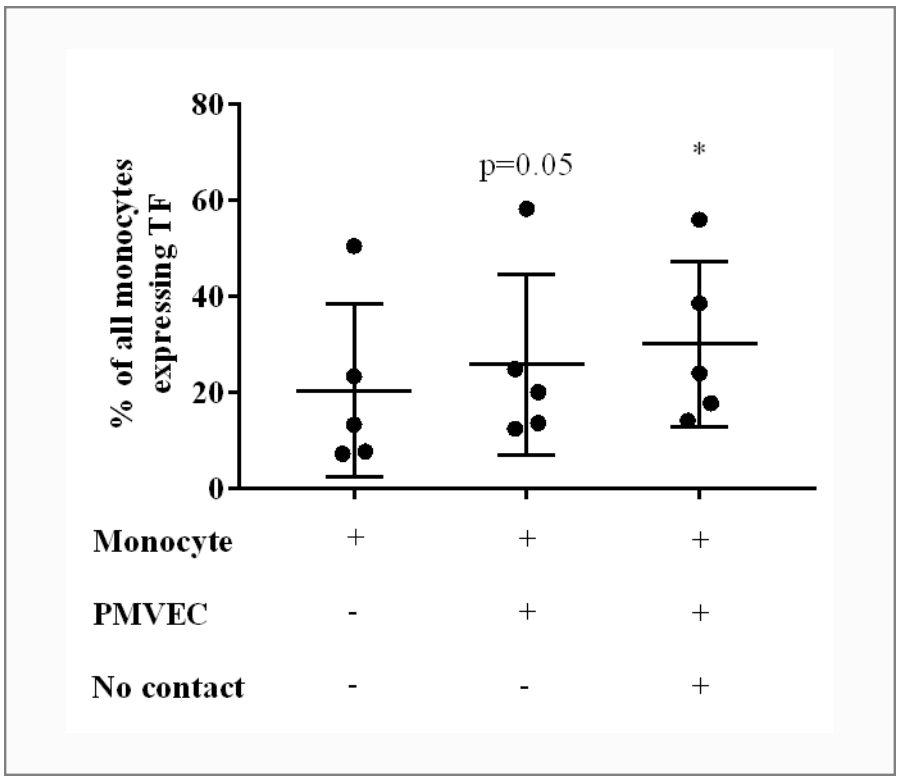


Figure 3.16 TF expression does not require contact between monocytes and PMVEC

*Monocytes were negatively sorted using FACS and incubated with and without PMVEC. A proportion of monocytes were cultured in transwells above PMVEC. Following a 24-hour incubation, the percentage of monocytes expressing TF was measuring using flow cytometry. This figure shows the results of 5 independent experiments. Error bars represent median values and the interquartile range. 'No contact' refers to the use of a transwell to prevent cellular contact between monocytes and PMVEC. Monocyte culture with PMVEC increased the cell surface expression of TF; this was not affected by the use of the transwell (p=0.02 by Friedman's test, *- p<0.05 for monocytes and PMVEC cultured with a transwell compared to monocytes alone, p=0.05 for monocytes and PMVEC cultured in contact compared to monocytes alone using Dunn's post hoc test).*

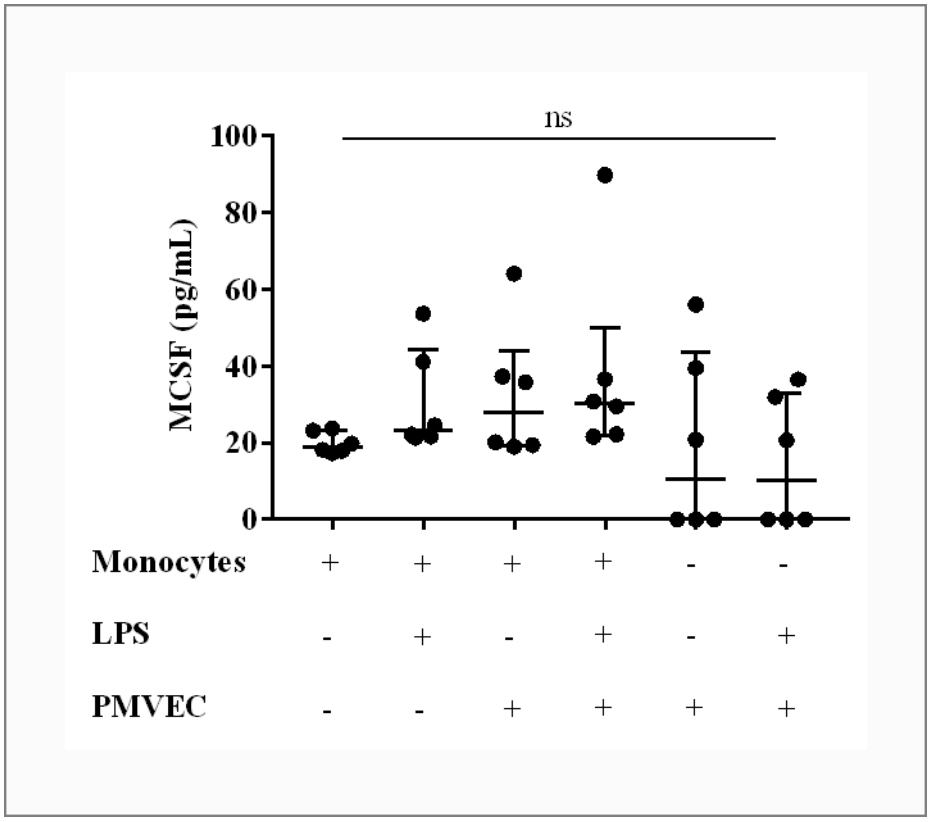


Figure 3.17 Co-culture of monocytes with PMVECs pre-treated with LPS does not affect MCSF levels

Monocytes were negatively sorted using FACS and incubated with and without PMVEC. A proportion of PMVEC were stimulated with 100 ng/mL of LPS for 1 hour, and the LPS was washed off prior to the addition of the monocytes. Some monocytes cultured without PMVEC were stimulated with 10 ng/mL LPS that was not removed but remained there for the duration of culture. This figure shows the quantity of MCSF in supernatants harvested following 24 hours of culture. MCSF was measured by ELISA. The figure shows data from 6 independent experiments. Error bars represent median values and the interquartile range. There was no significant difference between all the conditions (p=0.21 by Friedman's test, ns – non significant).

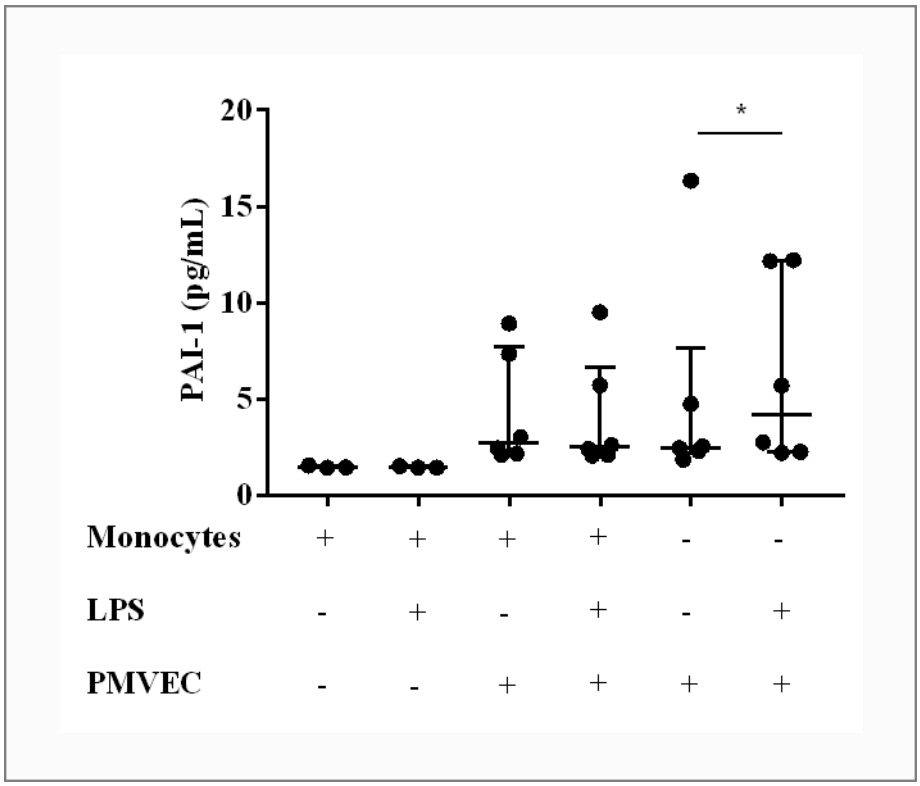


Figure 3.18 Co-culture of monocytes with LPS-pre-treated PMVECs increases PAI-1 levels

*Monocytes were negatively sorted using FACS and incubated with and without PMVEC. A proportion of PMVEC were stimulated with 100 ng/mL of LPS for 1 hour, and the LPS was washed off prior to the addition of the monocytes. Some monocytes cultured without PMVEC were stimulated with 10 ng/mL LPS that was not removed but remained there for the duration of culture. This figure shows the concentration of PAI-1 in supernatants harvested following 24 hours of culture. PAI-1 was measured using an ELISA. The figure shows data from 6 independent experiments. Error bars represent median values and the interquartile range. There was an increase in PAI-1 levels when monocytes were cultured with LPS-stimulated PMVEC compared to LPS-stimulated PMVEC cultured alone ($p=0.07$ by Friedman's test, *- $p<0.05$ by Dunn's post hoc test).*

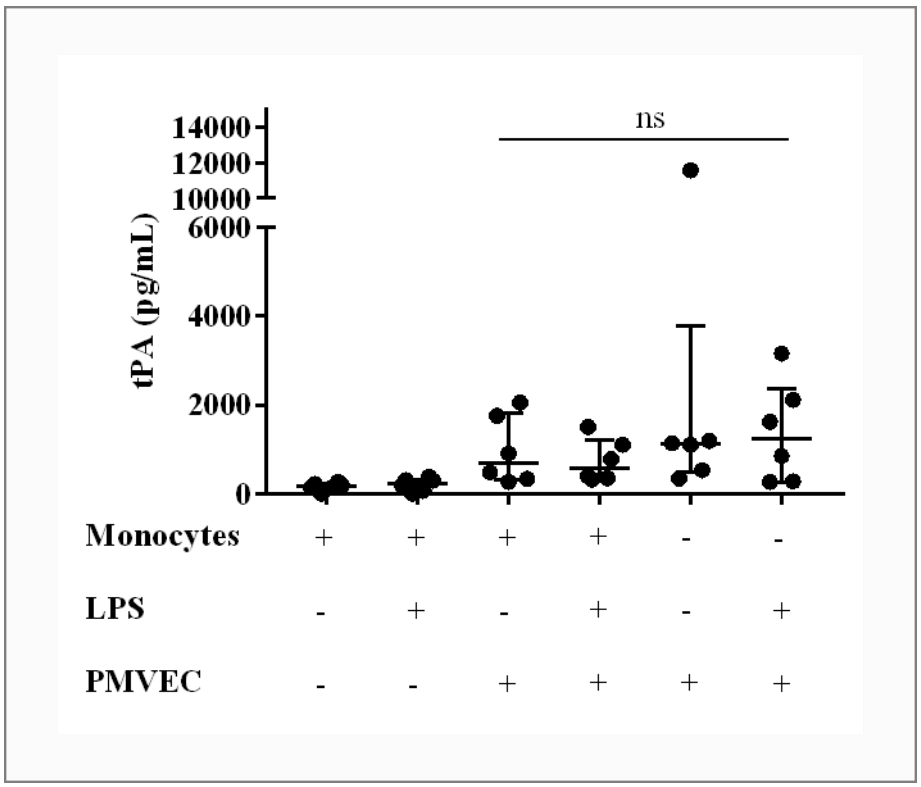


Figure 3.19 Co-culture of monocytes with LPS pre-treated PMVECs does not affect tPA levels

Monocytes were negatively sorted using FACS and incubated with and without PMVEC. A proportion of PMVEC were stimulated with 100 ng/mL of LPS for 1 hour, and the LPS was washed off prior to the addition of the monocytes. Some monocytes cultured without PMVEC were stimulated with 10 ng/mL LPS that was not removed but remained there for the duration of culture. This figure shows the concentration of tPA in supernatants harvested following 24 hours of culture. tPA was measured using an ELISA. The figure shows data from 6 independent experiments. Error bars represent median values and the interquartile range. There was no significant difference between the conditions involving PMVEC (p=0.027 by Friedman's test, ns – non-significant between the conditions containing PMVEC).

3.5 Discussion

3.5.1 Change in monocyte subsets following LPS incubation

The proportion of classical monocytes was increased by culture with LPS. This was associated with a lower expression of CD16, the marker used to distinguish intermediate and non-classical subsets. Interestingly, contrary to these results, multiple reports have suggested that LPS increases the expression of CD14. Marchant *et al*. showed an increase in CD14 expression within 30 minutes and 3 hours of incubation with LPS, an effect that did not require protein synthesis (Marchant *et al.*, 1992). Landmann *et al.* demonstrated that after a few hours' incubation, LPS appeared to decrease CD14 mRNA; this was followed, however, by an increase after 2 days. The authors also demonstrated an increase in the protein surface expression, with CD14 MFI on both PBMCs and purified monocytes increasing following incubation with LPS at doses higher than 1 ng/mL (Landmann *et al.*, 1996).

The cultures described in this chapter were incubated for 24 hours. It may be that this was too early to detect an increase in CD14 and supports a change in CD16 expression as a cause for the rise in proportion of the classical subset. There is some evidence to suggest that LPS stimulation of monocytes can result in the internalisation of CD16. Both the intermediate and non-classical subsets have been shown to be capable of internalising their CD16 receptor *in vivo* (Paniagua *et al.*; Picozza *et al.*, 2013).

It is important to note, however, that a change in monocyte subset does not necessarily translate into a change in monocyte function. As previously mentioned, the nomenclature for monocyte subsets applies during health (Ziegler-Heitbrock *et al.*, 2010b). Further work would need to be performed to investigate a change in function following stimulation of monocyte subsets with LPS.

Finally, this data is limited by the measurement of proportions. It is possible that during the experiment there was a disproportionate loss of non-classical and intermediate monocytes, rather than an increase in classical monocytes. As demonstrated, only a minority proportion of monocytes were recovered following *in vitro* culture. There were several parts of the protocol during which cells could be lost: these include harvesting monocytes that have adhered to the plastic wells, cell washing following antibody staining, and transfer to the flow cytometry tubes. Although there was no significant decrease in yield with increasing levels of LPS, it is possible that increasing levels of LPS may increase the adhesiveness of some subsets to plastic. Further work is needed to confirm whether this data represents a genuine

increase in the proportion of classical monocytes or in fact a disproportionate loss of the intermediate and non-classical subsets.

3.5.2 Expression of TF by monocyte subsets

All monocyte subsets were shown to increase their TF expression following LPS stimulation. The non-classical subset, contrary to my hypothesis, was shown to express the lowest levels of TF. To my knowledge there is no published work comparing the TF expression of monocyte subsets following LPS stimulation. Stojkovic *et al*. used IL-33 as a stimulus and showed an increase in the percentage of TF expressing intermediate monocytes, with a trend to increase demonstrated in the non-classical subset and no change noted in the classical subset. TF activity following IL-33 stimulation was not investigated in their study (Stojkovic 2017).

There is no published work comparing the activity of TF expressed on monocyte subsets. Accounting for the lower proportion of non-classical monocytes that express TF, there was no significant difference in TF activity across the monocyte subsets. Whilst LPS consistently increases the proportion of TF expressed by all monocytes, there was no evidence that it increased TF activity. The discrepancy between the level of cell surface expression and activity may be explained by the process of encryption, a post-translation modification of TF on the monocytic cell surface to control its activity (see introduction section 1.4.4, page 28) (Bach, 2006). Previous groups have suggested that the discrepancy between LPS-induced TF expression and its activity may be explained by an increase in cell death. The process of cell death leads to an externalisation of phosphatidylserine (PS) and thereby a 'decryption' of TF expressed by the cell (Henriksson *et al.*, 2007). The work described in this chapter, however, consistently showed a cell viability of greater than 90 % at the levels of LPS stimulation used, as measured by propidium iodide, thereby excluding late apoptosis and necrosis (Figure 3.1). It is still possible that the cells are entering early apoptosis and future work to identify the exposure of PS and signs of apoptosis would be useful. Potentially, this could involve the use of fluorescently-labelled Annexin V to assess the PS on the cell surface and DAPI to search for chromatin condensation (Cummings and Schnellmann, 2004). The low yield of recovered monocytes may also mean that many dead cells were lost during the experiment. This may have kept the viability artificially high.

3.5.3 Mechanisms involved in LPS-induced TF up-regulation on monocytes

As discussed earlier, the pathway(s) through which LPS induces transcription of TF are unclear. Three pathways have been implicated, with key roles for NFκB, ERK and JNK (Bode and Mackman, 2014). There is no published work considering the pathways in different monocyte subsets.

No statistically significant results were seen during this project, although there appeared to be a trend for inhibitors of all pathways to reduce TF surface expression, in total monocytes and in the classical and intermediate subset. No such trend was seen in the non-classical subsets. The TF expression during the inhibitor experiments was lower than that measuring during earlier experiments. This may be due to the use of adherence rather than FACS to isolate the monocytes. To fully understand the up-regulation of surface TF expression, future work should investigate the monocyte transcriptome and proteome post LPS activation.

3.5.4 The effect of monocyte-endothelial cell interaction on subset proportions and TF expression

Co-culture of monocytes and PMVECs led to an increase in the proportion of classical monocytes, which did not appear to be dependent on the presence of LPS. As discussed earlier (see introduction 1.5.5, page 49), MCSF can affect monocyte subset differentiation from classical to intermediate to non-classical (Saleh *et al.*, 1995a; Korkosz *et al.*, 2012). Measurement of the MCSF levels in supernatants following co-culture, however, showed no changes in MCSF levels between endothelial cells alone and those in contact with monocytes.

Another explanation may be the ability of each subset to transmigrate through an endothelial monolayer. Chimen *et al*. used monocyte and human umbilical vein endothelial cell (HUVEC) co-cultures to describe how all the subsets transmigrated from the apical to the basal side of an endothelial layer. Interestingly, the classical subset then underwent a reverse transmigration by 90 minutes such that they returned to the apical surface and remained on there. Intermediate and non-classical monocytes remained in a basal polarity (Chimen *et al.*, 2017). Human and murine monocyte subsets, as previously described (see introduction section 1.5), have been shown to move differently across the endothelium, which may explain this behaviour; in both cases, the non-classical subset was shown to adhere more strongly to endothelium (Auffray, 2007; Cros *et al.*, 2010). Future work investigating the transmigration and adhesion abilities of human subsets in endothelial co-cultures are needed to better understand this.

Endothelial co-culture led to an increase in TF expression on classical and intermediate monocytes. Again, this effect appeared to be independent of endothelial LPS stimulation. Lo *et al*. have previously demonstrated that direct contact between monocytes and endothelium led to an increase in endothelial TF expression, although monocytic TF expression was not investigated (Lo *et al.*, 1995). The work discussed in this chapter did not demonstrate a need for cellular contact between monocytes and endothelium to increase monocyte TF expression.

There are several potential mediators that may explain the induction of monocytic TF without the presence of contact or LPS. The release of homocysteine from endothelium at physiological levels has been shown to increase monocytic TF (Khajuria and Houston, 2000). P-selectin, bound to the endothelial surface, has also been shown to induce monocytic TF, but whether the release of soluble P-selectin could have a similar effect has so far not been investigated (Celi *et al.*, 1994; Semenov *et al.*, 1999). Interestingly, both membrane-bound and soluble P-selectin have been shown to increase the proportion of PS in the monocytic outer cell membrane. Potentially, this could support decryption of membrane-bound TF (del Conde *et al.*, 2005).

3.5.5 The endothelial response to co-culture

Sepsis-associated coagulopathy is associated with an inhibition of the endothelial fibrinolytic response (Suffredini *et al.*, 1989). In my experiments co-culture of monocytes and endothelial cells resulted in no difference in tPA released, but there was an increase in PAI-1 concentration when monocytes were co-cultured with PMVEC pre-treated with LPS. Monocytes are known to release a small proportion of PAI-1, but the increase in levels is higher than the amount of PAI-1 measured from monocytes cultured alone. Multiple authors have also noted an activation of endothelium following co-culture with monocytes (Rainger *et al.*, 1996; Tsouknos *et al.*, 2003). The increase in PAI-1 and unchanged level of tPA, following monocyte-endothelial co-culture has been noted previously by Funayama *et al*. They noted the effect occurred by 6 hours and was blocked by antibodies to IL-1β and TNF-α (Funayama, 1997). As previously shown, there appeared to be a trend towards an increase in IL-1β and TNF-α following co-culture in this project, however more work is needed to investigate a possible mechanism.

3.6 Summary of key findings

TF expression and activity has been shown to vary between monocyte subsets. Interestingly, a discrepancy was noted between the TF expression and activity in the subsets. This does not appear to be explained by cell death and warrants further investigation to consider the encryption of TF on each subset.

My initial hypothesis, that the non-classical subset would express the highest level of TF has been refuted. Although there is variation between the monocyte subsets, the classical and intermediate monocytes express the highest levels of TF, whilst there was no difference in TF activity.

The *in vitro* model of sepsis using endothelial co-culture has demonstrated an induction of monocyte TF that appears independent of LPS, as well as an increase in PAI-1 production. This suggests two potential ways in which monocytes may contribute to the coagulopathy of sepsis.

Chapter 4. Investigation of Monocyte Subsets following Endotoxaemia in Healthy Volunteers

4.1 Overview

Chapter 3 looked at the effects of LPS *in vitro*. To investigate how the TF on monocytes responds to LPS *in vivo*, a model of human endotoxaemia was used. Although not a model of sepsis, this allows for the temporal investigation of LPS activation of both the immune and coagulation systems (van Deventer *et al.*, 1990).

This chapter will discuss the impact of endotoxaemia on monocyte subsets and TF cell surface expression in healthy volunteers. Previous work has demonstrated a profound monocytopenia within 90 minutes of exposure of LPS. This is followed by the recovery of classical monocytes followed by intermediate and then non-classical subsets (Tak *et al.*, 2017). A review of the literature revealed no previous investigation of changes in monocytic TF expression.

4.2 Research aims

- to identify the changes in the proportion of circulating monocyte subsets following LPS exposure
- to investigate the effect of endotoxaemia on the cell surface expression of TF between the monocyte subsets

4.3 The human endotoxaemia model

The human endotoxaemia model involves the injection of LPS (2 ng/kg) to a healthy volunteer followed by venepuncture at the following time points: pre-injection, 90 minutes, 4, 6, 10 and 24 hours, and 7 days following the injection. The participant is monitored throughout the first 10 hours in a dedicated research space within a critical care unit. The experiment is performed by a critical care physician. A small proportion of the blood taken is processed by the hospital's laboratory to obtain full blood cell count and coagulation measurements. The remaining blood is processed by the research team, including whole blood flow cytometry.

Thirteen participants were recruited between July 2018 and February 2019. Their median age was 24.5 years (range 18-37 years) and 8 (62%) were male.

Most participants experienced a leucocytosis following the LPS injection. Peak levels were reached at 10 hours and all white blood counts had returned to within the reference range 7 days following the experiment. The predominant circulating leucocytes were neutrophils, with levels following the trend of the total white blood count. All participants developed a monocytopenia at 90 minutes and a lymphopenia at 4 hours (Figure 4.1).

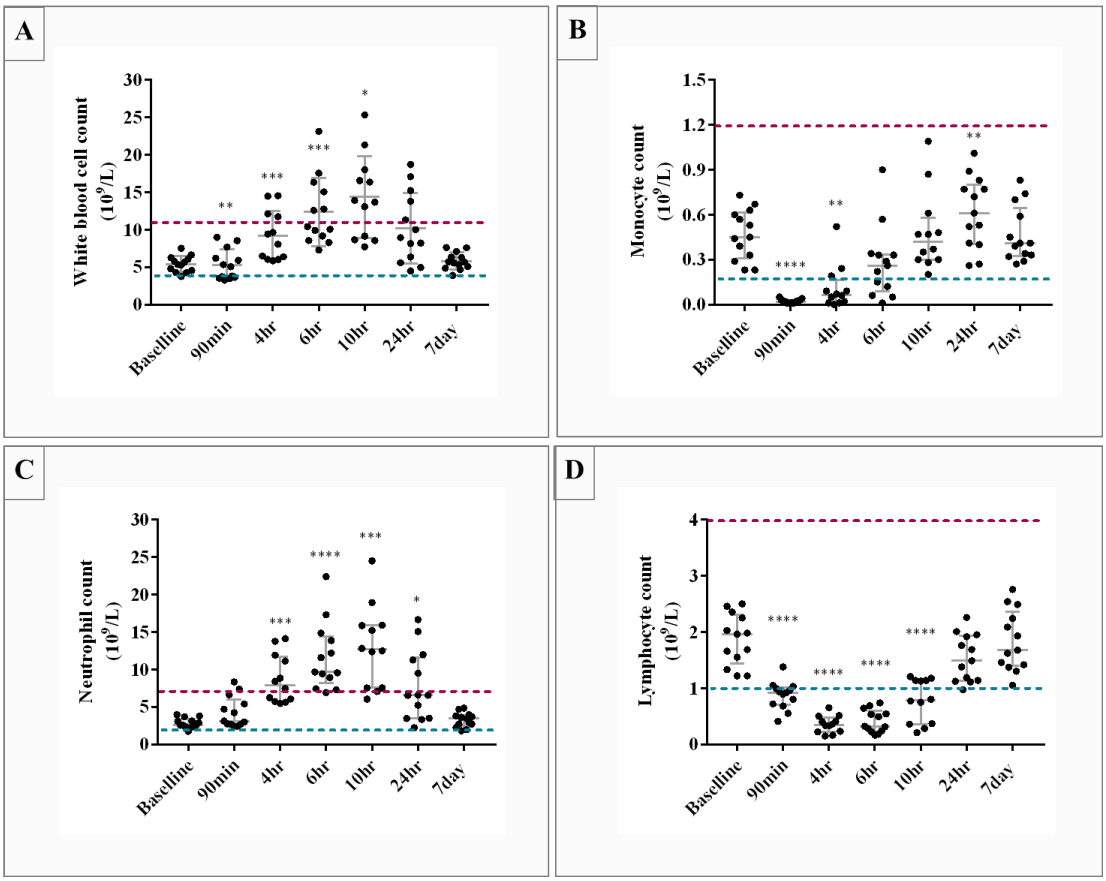


Figure 4.1 White cell counts of healthy controls following endotoxaemia

This figure shows the total white blood cell counts at each time-point following an injection of 2 ng/kg LPS in healthy volunteers (n=13). Cell counts were measured by flow cytometry in the hospital's laboratory using the Sysmex XN-3000™ analyser (Sysmex, Milton Keynes, UK). Most participants had a white blood count and white cell differential measured at each time-point, but two had a single missing sample; the 4 hr and 10 hr timepoints therefore show the results of 12 participants. The red dashed line marks the upper limit of the reference range, whilst the blue dashed line marks the lower limit. ***A*** *– white blood cell count,* ***B*** *– monocyte count,* ***C*** *– neutrophil count,* ***D*** *– lymphocyte count. Significance was calculated using the repeated-measures one-way ANOVA test (****A****,* ***B****,* ***C*** *and* ***D****: $p<0.0001$)) with Dunnett's multiple comparisons test to compare each time-point to baseline (* – $p<0.05$, ** – $p<0.01$, *** – $p<0.001$, **** – $p<0.0001$).*

4.4 Monocyte subsets following endotoxaemia

100 μL of whole blood from each time-point was used for flow cytometry in a BD Trucount™ tube to allow the calculation of absolute values (see methodology section 2.6.1, page 72).

As seen in the full blood count sample, there was a profound monocytopenia. This occurred in all participants and was most pronounced at the 90 min time-point following the LPS injection (Figure 4.2).

The recovery begins at 4 hours but is not complete until 24 hours. Reviewing the subsets individually shows a loss of virtually all intermediate and non-classical monocytes at early time points, with only a small population of classical remaining. The classical subset begins to recover at 4 hours, but the intermediate and non-classical subsets do not return until 24 hours (figures 4.3 and 4.4).

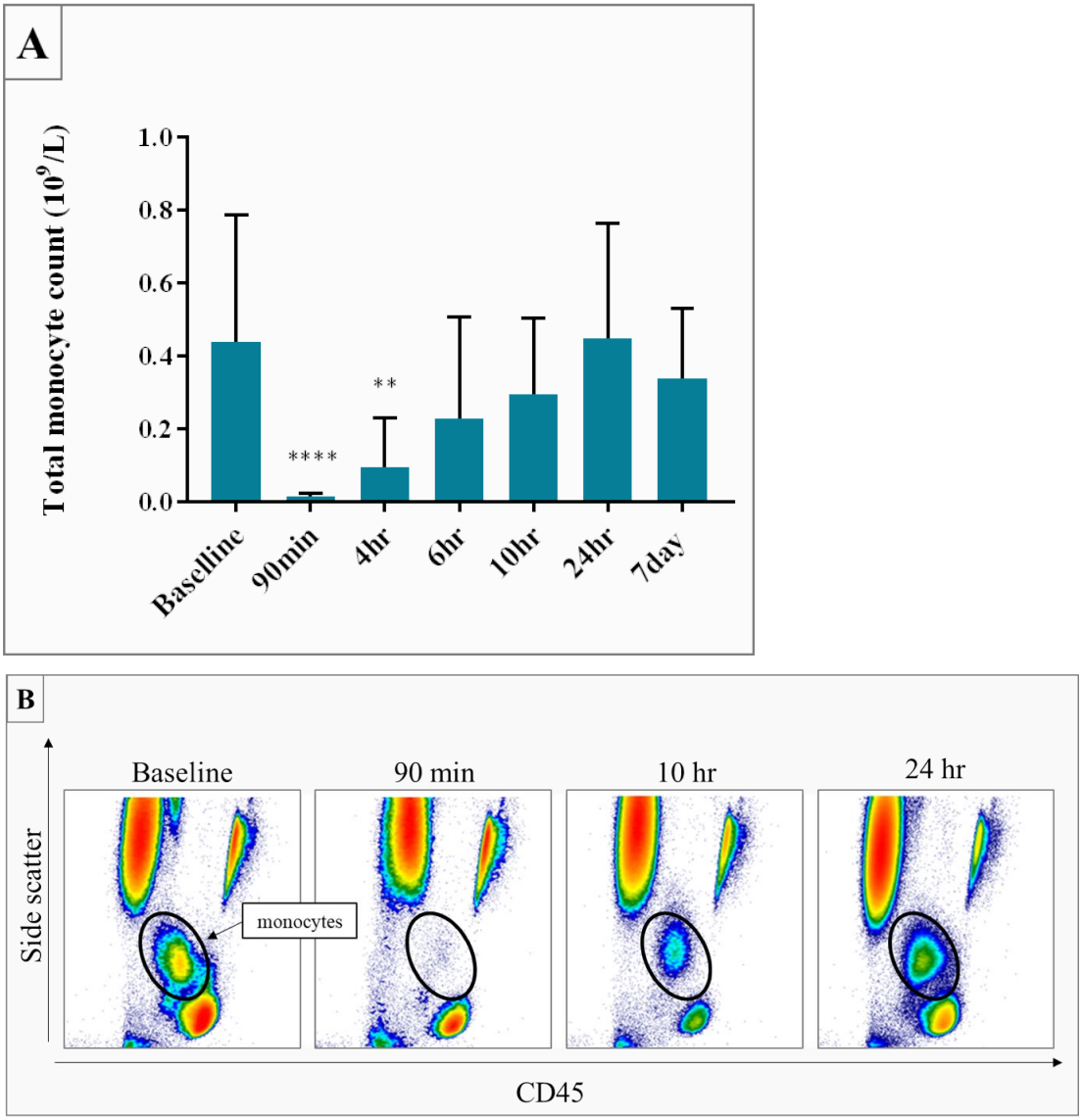


Figure 4.2 Endotoxaemia induces a profound monocytopenia

The monocyte count was measured with flow cytometry using 100 µL of whole blood in a BD Trucount™ tube for all 13 participants. ***A*** *– Endotoxaemia is followed by a profound monocytopenia. The bars represent median values and the error bar the upper quartile. The decrease in monocyte count was statistically significant ($p<0.0001$ by Friedman's test, ** – $p<0.01$ comparing 4 hr to baseline and **** – $p<0.0001$ comparing 90 min to baseline by Dunn's post hoc test).* ***B*** *– A representative example of one participant's flow cytometry at four time-points.*

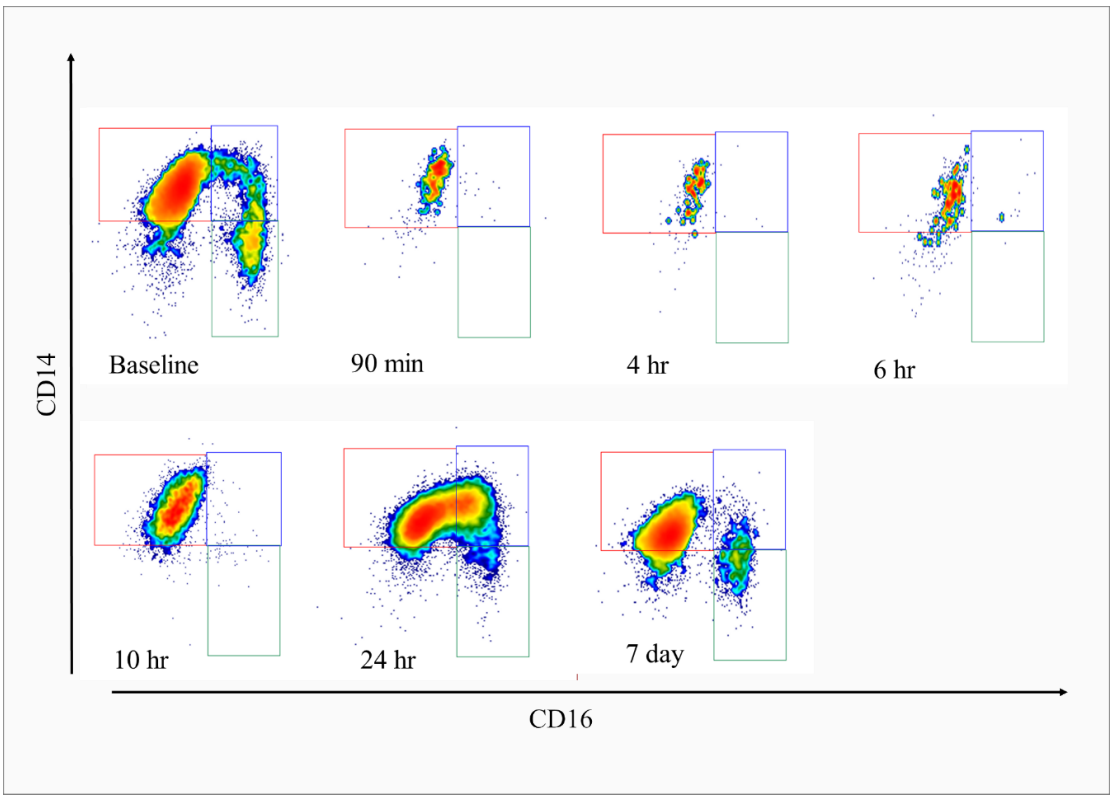


Figure 4.3 Monocyte subsets following endotoxaemia

This figure shows the changes in monocyte subsets, as gated by CD14 and C16 positivity, at each time-point following an injection of 2 ng/kg LPS in a single participant. The classical population is gated in the red gate, the intermediate in blue and the non-classical in green.

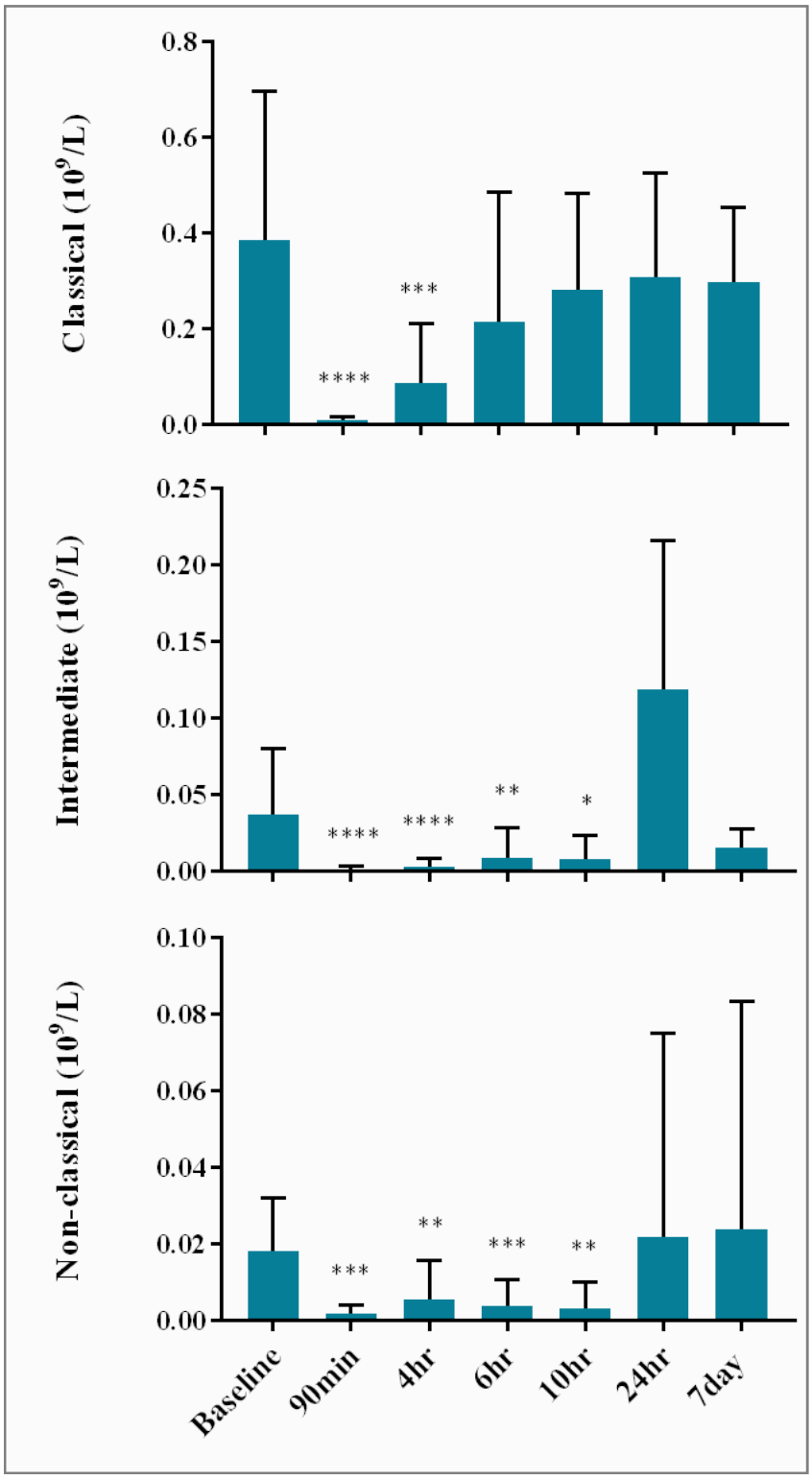

Classical (10^9/L)
0.8
0.6
0.4
0.2
0.0
****
***
Intermediate (10^9/L)
0.25
0.20
0.15
0.10
0.05
0.00
****
****
**
*
Non-classical (10^9/L)
0.10
0.08
0.06
0.04
0.02
0.00
***
**
***
**
Baseline
90min
4hr
6hr
10hr
24hr
7day

Figure 4.4 The classical subset recovers before the others following endotoxaemia-induced monocytopenia

*This figure shows the absolute monocyte subset counts at each time-point following an injection of 2 ng/kg LPS in healthy volunteers (n=13). Counts were measured with flow cytometry using 100 µL of whole blood in a BD Trucount™ tube. The bars represent median values and the error bars the upper quartile. Classical monocytes decrease at 90 mins and recover by 6 hr ($p<0.0001$ by Friedman's test, **** – $p<0.0001$ comparing 90 min to baseline and *** – $p<0.001$ comparing 4 hr to baseline by Dunn's post hoc test). Intermediate monocytes decrease at 90 mins and recover at 24 hr ($p<0.0001$ by Friedman's test, **** – $p<0.0001$ comparing 90 min and 4 hr to baseline, ** – $p<0.01$ comparing 6 hr to baseline and * – $p<0.05$ compares 6 hr to baseline by Dunn's post hoc test). Non-classical monocytes decrease at 90 mins and recover at 24 hr ($p<0.0001$ by Friedman's test, *** – $p<0.001$ comparing 90 min and 6 hr to baseline, ** – $p<0.01$ comparing 4 hr and 10hr to baseline by Dunn's post hoc test).*

4.5 TF expression following endotoxaemia

As part of the whole blood flow cytometry, the proportion of TF-expressing monocytes was measured. Only a very small proportion of circulating monocytes express TF. The percentage of cells expressing TF was much lower than reported with LPS *in vitro* stimulation as described in chapter 3 (see section 3.3.2, page 105).

The TF cell surface expression of each monocyte subset was investigated. When considering all 13 participants there appeared to be two broad categories of response: in 8 participants the proportion of TF-expressing monocytes increased after LPS administration (high responders), while in the remaining 5, monocytes expressed a very low level of TF that did not change in any subset following LPS (low responders). Figure 4.5 shows the data for all participants together as well as separately for each of these categories. The level of 0.5 % of cells expressing TF was used to distinguish the two populations. This level was chosen since the highest baseline level of TF noted in all participants was 0.48 %.

There appeared to be two peaks in TF expression, at 90 minutes and then at 6 hours. When the TF expression on the monocyte subsets from the two categories was investigated, the pattern in each subset mirrored the pattern observed for total monocytes (Figure 4.6). The intermediate subset had the highest levels of TF expression (Figure 4.6). This may be explained by the smaller number of cells present between 90 min and 6 hr, particularly in the intermediate and non-classical subsets (Figure 4.4).

There was no difference between the ages, sex or white blood count of these the high and low responders (see table 4.1). C-reactive protein (CRP) levels were measured, as a marker of inflammation, at baseline, 10 hr, 24 hr and 7 days in the hospital's laboratory using chemoluminescence with the cobas® 8000 analyser. The CRP increased for all participants at 10 and 24 hr before returning to normal at 7 days. There was no difference between the high and low responder groups. Although the changes seen in the low responder group were not statistically significant, this is likely due to the small number of participants, particularly as one participant did not have CRP measured at 10 and 24 hr (see Figure 4.7).

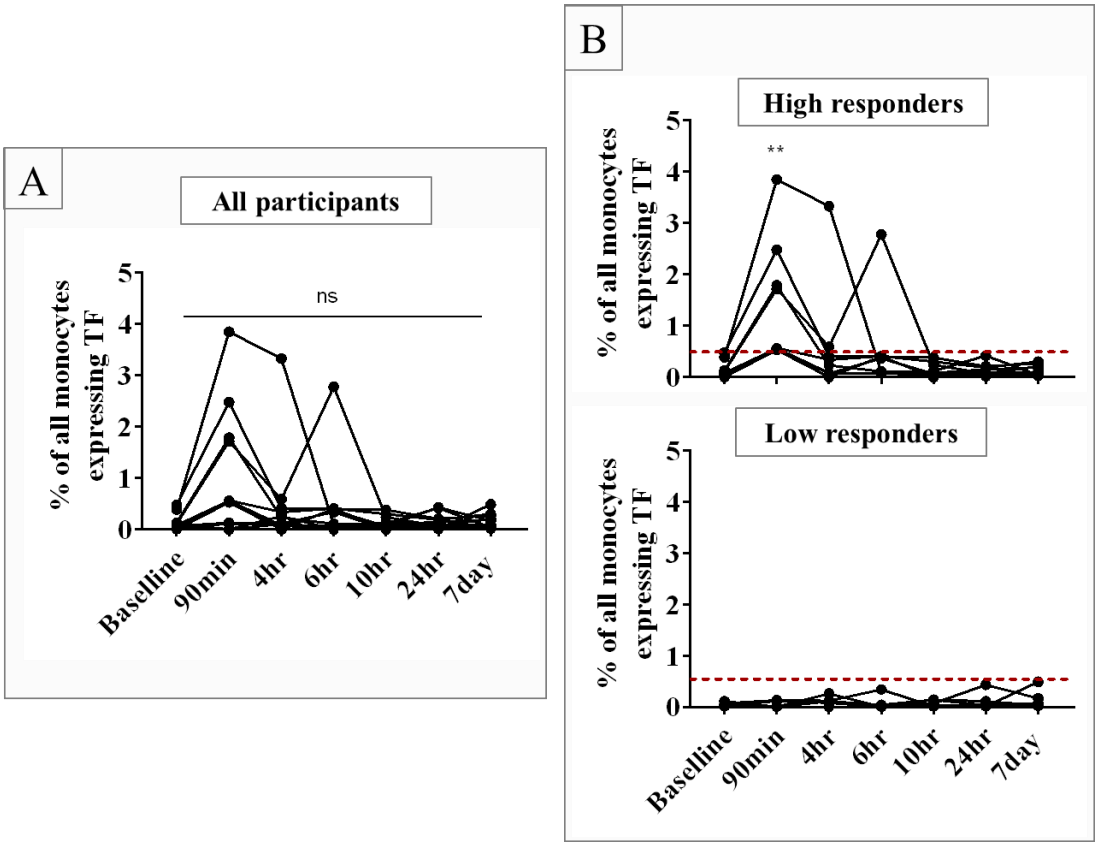


Figure 4.5 In a sub-population of participants monocyte TF expression is enhanced following endotoxaemia

This figure shows the percentage of monocytes that express TF at each time-point following an injection of 2 ng/kg LPS in healthy volunteers (n=13). TF expressing cells were measured with flow cytometry using FMO to gate the negative the population. ***A*** *– This figure includes all participant data (n=13). There was no statistically significant difference in the percentage of monocytes expressing TF (p=0.11 by Friedman's test).* ***B*** *– compares 'high responders' (participants who increased the percentage of cells expressing TF on their surface above 0.5%, n=8) and 'low responders' (participants who did not increase the percentage of cells above 0.5%, n=5). The red dashed line marks 0.5 % of cells expressing TF. There was an increase in the percentage of monocytes expressing TF at 90 min following LPS injection in the high-responder group (p=0.0005 by Friedman's test, * – p<0.05 comparing 90 min to baseline by Dunn's post hoc test). The percentage of monocytes expressing TF did not change in non-responder group (p=0.78 by Friedman's test).*

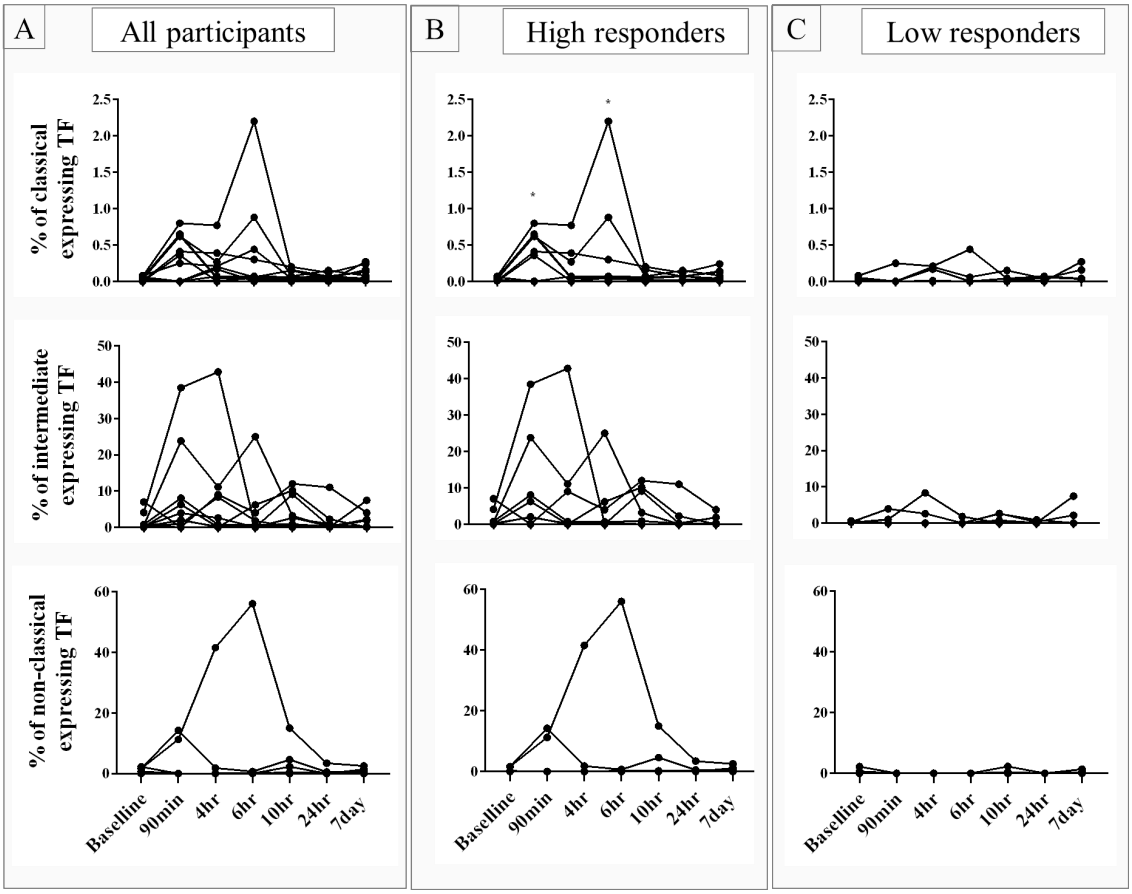


Figure 4.6 All monocyte subsets in the high responder population show an increase TF expression in response to endotoxaemia

This figure shows the percentage of each monocyte subset that expresses TF at each time-point following an injection of 2 ng/kg LPS in healthy volunteers (n=13). TF-expressing cells were measured with flow cytometry using FMO to gate the negative population. Frequently in the low responder groups and the non-classical subset, there were no cells expressing TF on their cell surface. ***A*** *– These figures include all participant data (n=13). The percentage of monocytes expressing TF did not change (classical: p=0.08, intermediate: p=0.34, non-classical: p=0.65 by Friedman's test).* ***B*** *– includes data for 'high responders' (increased TF expression to >0.5 % of the total circulating monocyte population, n=8). The classical monocyte subset increased the percentage of cells expressing TF (p=0.02 by Friedman's test, * – p<0.05 comparing 90 min and 6 hr time-points to baseline by Dunn's post hoc test).* ***C*** *– includes data for 'low responders' (did not increase their TF expression to >0.5 % of the total circulating monocyte population, n=5). There were no significant changes in the percentage of monocytes expressing TF in the low responders (classical: p=0.49, intermediate: p=0.56, non-classical: p=0.10 by Friedman's test).*

		High responders	Low responders
Number of participants		8	5
Median age (years)		25 (range 18 to 37)	24 (range 22 to 32)
Male		5 (62%)	3 (60%)
White blood count	Baseline	5.80	5.30
	90 min	3.73	6.18
	4 hr	8.07	11.92
	6 hr	10.05	12.75
	10 hr	13.70	14.82
	24 hr	8.21	11.32
	7 day	5.70	5.56

Table 4.1 Demographic comparison of responders and non-responders

This table compares the age, sex and white blood cell count of both populations. There was no statistically significant difference found. Cell counts and age of participants were compared using an unpaired t-test; the proportion of male participants in each group was compared using Fisher's exact test.

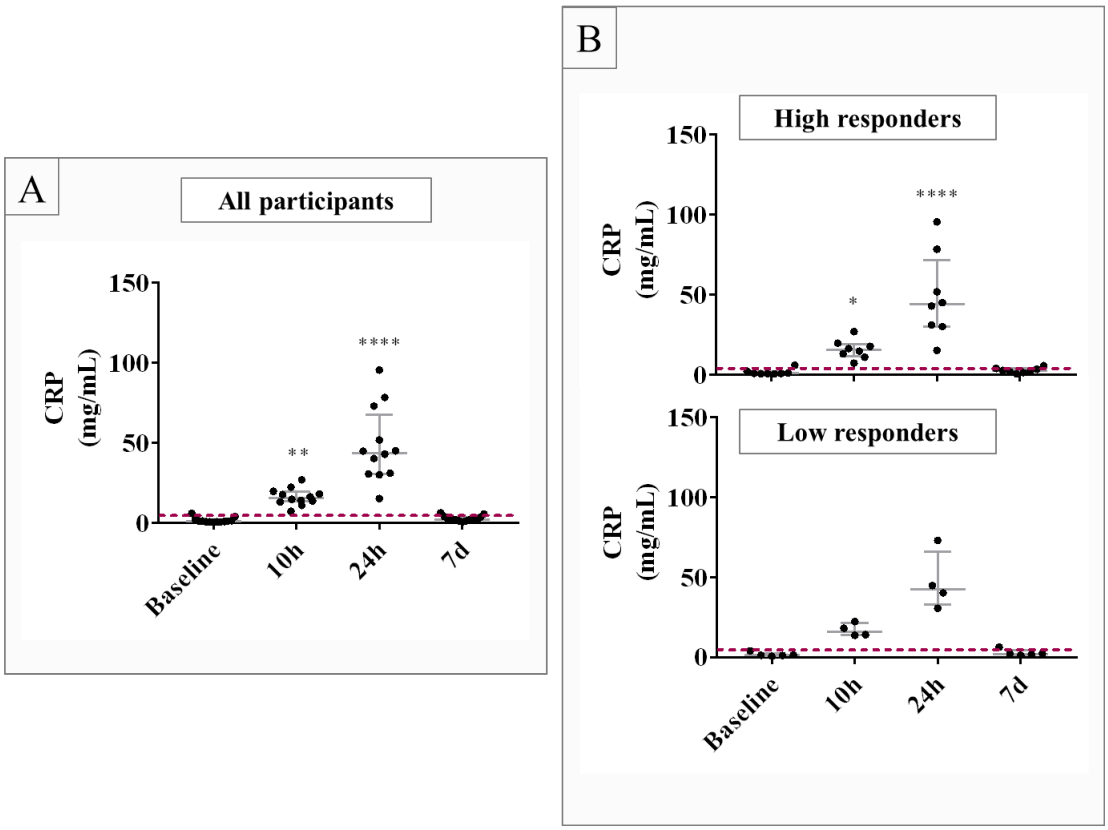


Figure 4.7 CRP levels following human endotoxaemia between the responder groups

This figure shows the level of CRP at baseline, 10 hr, 24 hr and 7 days following an injection of 2 ng/kg LPS in healthy volunteers (n=13). One participant missed two CRP measurements at the 10 and 24 hr timepoints; they were in the low responder group. The red dashed line marks the upper limit of the CRP reference range. ***A*** *– shows the CRP level for all participants. The CRP level increased at 10 and 24 hr ($p<0.0001$ by Friedman's test, ** – $p<0.01$ comparing 10 hr to baseline, **** – $p<0.0001$ comparing 24 hr to baseline using Dunn's post hoc test).* ***B*** *– The CRP levels for high and low responders are displayed. Both show an increase at 10 and 24 hr, although only the high responder group are statistically significant (high responders: $p<0.0001$ by Friedman's test, * – $p<0.05$ comparing 10 hr to baseline, **** – $p<0.0001$ comparing 24 hr to baseline using Dunn's post hoc test; low responders: $p=0.0009$ by Friedman's test, $p=0.08$ comparing 24 hr to baseline using Dunn's post hoc test).*

4.6 Markers of coagulation following endotoxaemia

As discussed earlier, TF, through binding to VIIa, activates the pro-enzyme clotting factors and creates FXa, FIXa and FXIa. This allows the formation of both the tenase and prothrombinase complexes, leading to the propagation phase of coagulation and an increased production of thrombin. Each factor has an inhibitor that binds to control coagulation: antithrombin (AT) binds to FIXa, FXa, FXIa and thrombin. FXIa is also inhibited by α-1-antitrypsin (FXIa-a1AT) (for a more detailed summary see introduction section 1.3.2, page 8).

The down-stream effects of TF were investigated by measuring TAT, FIXa-AT, FXa-AT, FXIa-AT and FXIa-a1AT. To investigate a role for the contact pathway during endotoxaemia, levels of FXIIa-C1NH were also measured. All levels were obtained using ELISA by our collaborators at Maastricht University, The Netherlands (see methodology 2.7.3, page 85).

There was an increase in TAT, FIXa-AT, FXa-AT and FXIa-AT at 4 hr following LPS injection. No change was seen in the FIXa-a1AT levels. At 6 hr there was a decrease in the FXIIa-C1NH levels (see Figure 4.8).
The high and low responder groups were compared but there was no consistent difference demonstrated (see figures 4.9 and 4.10).

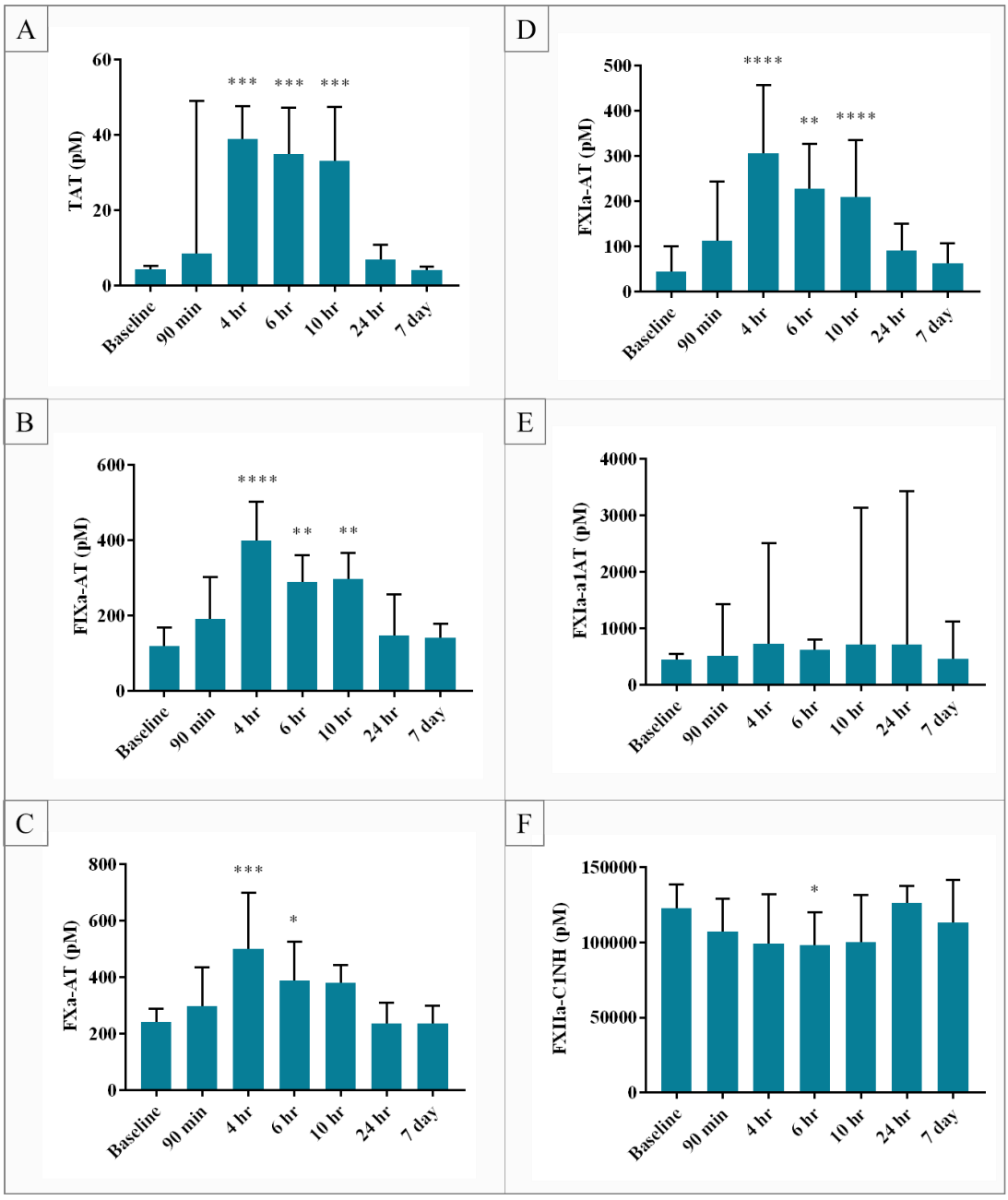

A
TAT (pM)
60
40
20
0
***
***
***
Baseline
90 min
4 hr
6 hr
10 hr
24 hr
7 day
D
FXIa-AT (pM)
500
400
300
200
100
0
****
**
****
Baseline
90 min
4 hr
6 hr
10 hr
24 hr
7 day
B
FIXa-AT (pM)
600
400
200
0
****
**
**
Baseline
90 min
4 hr
6 hr
10 hr
24 hr
7 day
E
FXIa-a1AT (pM)
4000
3000
2000
1000
0
Baseline
90 min
4 hr
6 hr
10 hr
24 hr
7 day
C
FXa-AT (pM)
800
600
400
200
0
***
*
Baseline
90 min
4 hr
6 hr
10 hr
24 hr
7 day
F
FXIIa-C1NH (pM)
150000
100000
50000
0
*
Baseline
90 min
4 hr
6 hr
10 hr
24 hr
7 day

Figure 4.8 Coagulation markers following endotoxaemia

This figure shows the changes in markers of coagulation at each time-point following an injection of 2 ng/kg LPS in healthy volunteers. Markers of coagulation were measured using an ELISA. This work was performed by our collaborators in Maastricht University, The Netherlands. Markers were measured in 12 participants; the 13th participant was unavailable at the time of processing. Bar height represents median values and error bars the upper quartile. ***A*** *– TAT levels were increased between 4 and 6 hr ($p<0.0001$ by Friedman's test, *** – $p<0.001$ comparing 4, 6 and 10 hr with baseline using Dunn's post hoc test).* ***B*** *– FIXa-AT levels were increased between 4 and 10 hrs ($p<0.0001$ by Friedman's test, **** – $p<0.0001$ comparing 4 hr to baseline, ** – $p<0.01$ comparing 4 and 6hr to baseline using Dunn's post hoc test).* ***C*** *– FXa-AT levels were increased at 4 and 6 hr ($p<0.0001$ by Friedman's test, *** – $p<0.001$ comparing 4 hr to baseline, * – $p<0.05$ comparing 6 hr to baseline using Dunn's post hoc test).* ***D*** *– FXIa-AT levels increased between 4 and 10 hr ($p<0.0001$ by Friedman's test, **** – $p<0.0001$ comparing 4 and 10hr to baseline, ** – $p<0.01$ comparing 6 hr and baseline using Dunn's post hoc test).* ***E*** *– FXIa-AT levels were unchanged following LPS injection ($p=0.44$ by Friedman's test).* ***F****– FXIIa-C1NH levels increased at 6hr ($p=0.03$ by Friedman's test, * – $p<0.05$ comparing 6 hr to baseline using Dunn's test).*

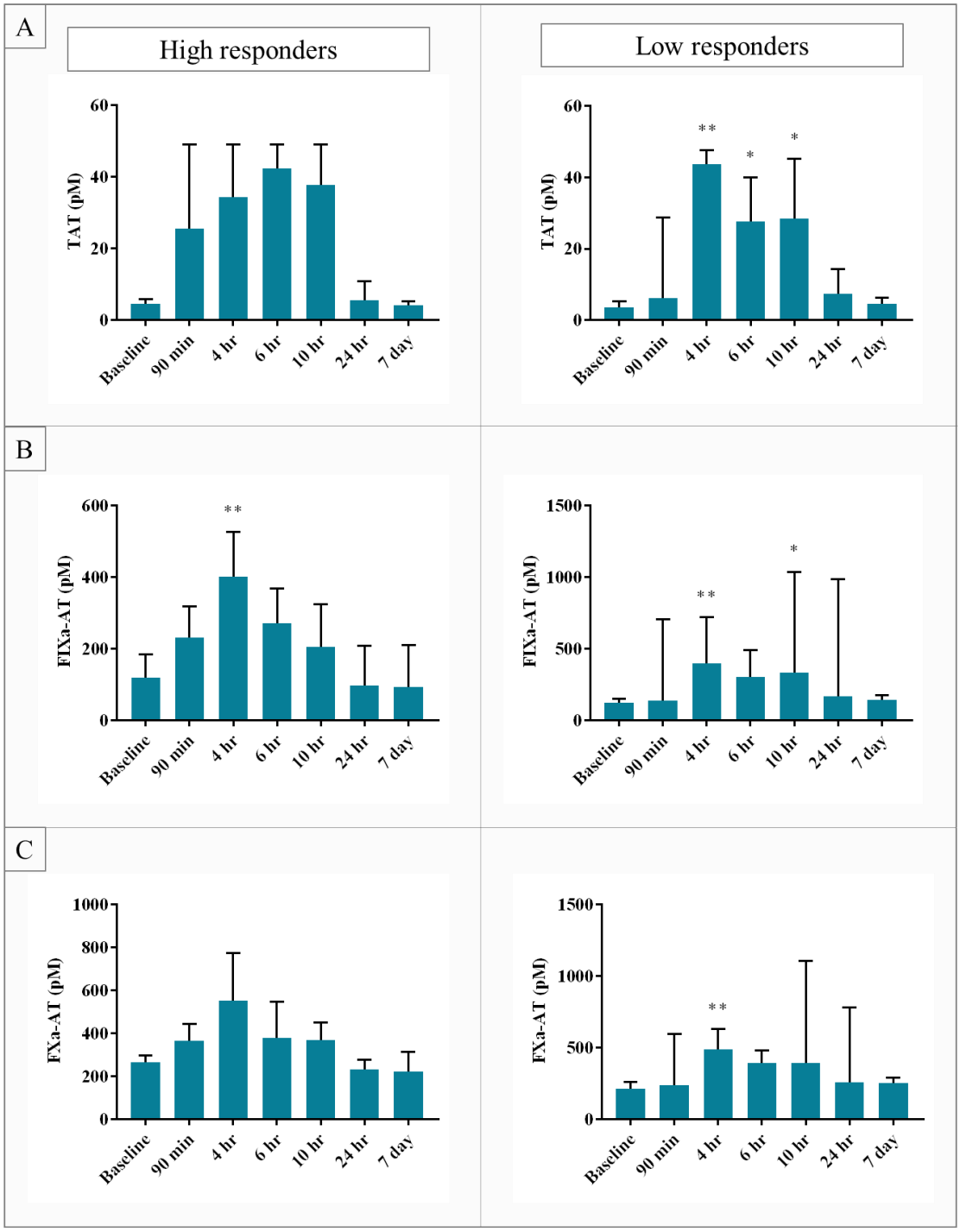

A
High responders
Low responders
TAT (pM)
60
40
20
0
**
*
*
Baseline
90 min
4 hr
6 hr
10 hr
24 hr
7 day
B
FIXa-AT (pM)
600
400
200
0
1500
1000
500
**
*
C
FXa-AT (pM)
1000
800
600
400
200
0
1500
1000
500
**

Figure 4.9 Comparison of TAT, FIXa-AT and FXa-AT between high and low responders

This figure compares the changes in coagulation markers at each time-point following a 2 ng/mL injection of LPS between high and low responders. Markers of coagulation were measured using an ELISA. This work was performed by our collaborators in Maastricht University, The Netherlands. Markers were measured in 12 participants; the 13th participant was unavailable at the time of processing. Bar height represents median values and error bars the upper quartile. There was no consistent difference between the two groups. ***A*** *– TAT levels were increased between 4 and 6 hr; this change was statistically significant in the low responder group (high responder: $p<0.0001$ by Friedman's test, $p=0.06$ comparing 4 hr with baseline using Dunn's post hoc test; low responder: $p=0.0007$ by Friedman's test, ** – $p<0.01$ comparing 4 hr to baseline, * – $p<0.05$ comparing 6 and 10 hr to baseline using Dunn's post hoc test).* ***B*** *– FIXa-AT levels were increased at 4 hrs in each group and at 10 hr in the low responder group (high responder: $p<0.0001$ by Friedman's test, ** – $p<0.001$ comparing 4 hr to baseline using Dunn's post hoc test; low responder: $p=0.007$ by Friedman's test, ** – $p<0.01$ comparing 4 hr to baseline, * – $p<0.05$ comparing 10 hr to baseline using Dunn's post hoc test).* ***C*** *– FXa-AT levels were increased at 4 hr; this was statistically significant only in the low responder group (high responder: $p<0.0001$ by Friedman's test, $p=0.08$ comparing 4 hr to baseline using Dunn's post hoc test; low responder: $p=0.008$ by Friedman's test, ** – $p<0.01$ comparing 4 hr to baseline using Dunn's post hoc test).*

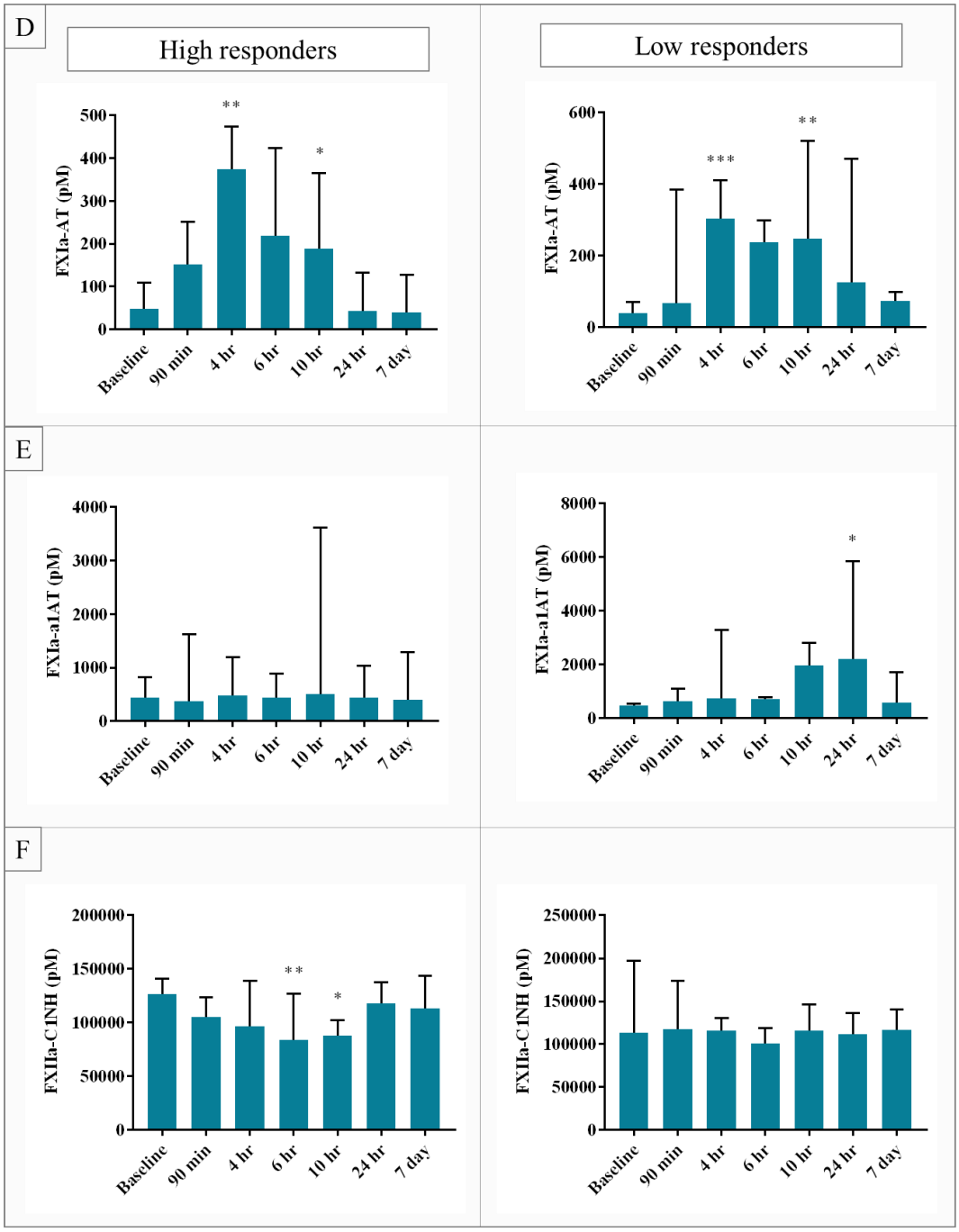

D
High responders
Low responders
FXIa-AT (pM)
E
FXIa-a1AT (pM)
F
FXIIa-C1INH (pM)
Baseline
90 min
4 hr
6 hr
10 hr
24 hr
7 day

Figure 4.10 Comparison of FXIa-AT, FXIa-a1AT and FXIIa-C1NH between high and low responders

This figure compares the changes in coagulation markers at each time-point following a 2 ng/mL injection of LPS between high and low responders. Markers of coagulation were measured using an ELISA. This work was performed by our collaborators in Maastricht University, The Netherlands. Markers were measured in 12 participants; the 13th participant was unavailable at the time of processing. Bar height represents median values and error bars the upper quartile. There was no consistent difference between the two groups. ***D*** *– FXIa-AT levels were increased between 4 and 10 hr; this change was statistically significant in the low responder group (high responder: p=0.0004 by Friedman's test, ** – p<0.01 comparing 4 hr with baseline, * – p<0.05 comparing 10hr to baseline using Dunn's post hoc test; low responder: p=0.002 by Friedman's test, *** – p <0.001 comparing 4 hr to baseline, ** – p<0.01 comparing 10 hr to baseline using Dunn's post hoc test).* ***E*** *– FXIa-a1AT levels were increased at 24 hrs in the low responder group, there was no statistically significant difference in the high responder group (high responder: p=0.94 by Friedman's test; low responder: p=0.05 by Friedman's test, * – p<0.05 comparing 24 hr to baseline using Dunn's post hoc test).* ***F*** *– FXIIa-C1NH levels were decreased between 4 and 6 hr in the high responder group (high responder: p<0.002 by Friedman's test, ** – p<0.01 comparing 6 hr to baseline, * – p<0.05 comparing 10 hr to baseline using Dunn's post hoc test; low responder: p=0.10 by Friedman's test).*

4.7 Discussion

4.7.1 Changes in blood counts and monocyte subsets

The human endotoxaemia model leads to a stereotypical response. All participants experienced a neutrophilia, lymphopenia and monocytopenia. The cause of such a profound monocytopenia is not clearly understood. Potentially, the monocytes may be marginating and adhering to the endothelium, which may have become activated following the administration of LPS. Several studies have described the ability of monocytes to 'roll' or 'crawl' along the endothelium (Auffray, 2007; Cros *et al.*, 2010). Blood samples that measure circulating monocytes would therefore fail to collect the marginated cells.

As shown by Tak *et al.,* following the monocytopenia, the subsets recover differentially (Tak *et al.*, 2017). Initially the classical monocytes recover at 6 hours, followed by intermediate and non-classical at 24 hours. This group also identified an increase in intermediate monocytes at 24 hours. Although no statistically significant difference was seen in this project, there did appear to be a trend in that direction (Tak *et al.*, 2017).

It is likely that monocytes are repopulated from the bone marrow. There is increasing evidence that monocytes can transition through the subsets, from classical to intermediate to non-classical. Murine classical monocytes have been shown to differentiate into the intermediate subset over several days (Yona *et al.*, 2013). Single-cell RNA sequencing data support the concept of separate classical and non-classical monocyte subsets, but suggest that the intermediate subset is not homogeneous and support the idea that it is a transitional population (Villani *et al.*, 2017). Patel *et al*. injected healthy volunteers with deuterium-labelled glucose and demonstrated that deuterium-labelled classical monocytes appeared first from the bone marrow and intermediate monocytes were seen with a lower level of deuterium, while labelled non-classical monocytes did not appear for 7 days. The group also injected deuterium 20 hours prior to performing the human endotoxaemia model. Their previous experiments had shown that at this time-point no circulating monocytes would be labelled. Deuterium-labelled classical monocytes were shown to repopulate the circulating pool from the bone marrow from 4 hours after LPS administration, whereas the intermediate and non-classical subsets did not appear until 24 hours (Patel *et al.*, 2017). All these experiments support the idea of monocytes repopulating the circulation from the bone marrow and then differentiating into intermediate and non-classical monocytes. What is not explained is the recovery in non-classical monocytes at 24 hours following LPS injection. Although the differentiation of intermediate cells from classical monocytes is feasible over 24 hours, the

non-classical population takes longer to appear (Patel *et al.*, 2017). Potentially, the non-classical subset is released from the endothelium at 24 hours. Further work is needed to clarify this as well as to investigate the cause of the initial monocytopenia.

4.7.2 Monocytic TF expression

The percentage of monocytes expressing TF was lower than reported in chapter 3. The cause of this is unclear but may relate to activation of monocytes following isolation (Bennett and Breit, 1994). Potentially the monocytes expressing most TF may not be circulating but be marginated. Sampling the circulating blood may not accurately reflect the TF surface expression on monocytes (Auffray, 2007; Cros *et al.*, 2010).

All participants had a low percentage of monocytes that expressed TF prior to injection but an increase occurred from 90 minutes and continued until 6 hours. Considering the percentage of TF positive cells, there appear to be two peaks of TF. TF mRNA has been previously shown to increase several hours after LPS injection. Increased transcription may account for the increase in TF expression at 6 hours but does not account for the earlier response at 90 minutes (Franco *et al.*, 2000).

Egorina *et al*. stimulated whole blood with LPS and measured the time course of TF expression. They also noted an increase within the first few hours. Immunostaining for TF revealed an intracellular pool near to the membrane. This is a plausible source of the initial peak in TF expression (Egorina *et al.*, 2005).

4.7.3 High and low responders

This work supports the phenomenon of high and low responders following LPS stimulation (Ossterud, 1995). Although TF transcription has been shown to increase throughout endotoxaemia, there has been no previous work comparing high and low responders (Franco *et al.*, 2000). Immunostaining has revealed that low responders produce monocytic TF and indeed have a higher proportion intracellularly than high responders. Potentially, the difference occurs either in the cell's response to LPS or in externalising the TF onto the cell membrane (Egorina *et al.*, 2005).

High responders have been associated with a higher risk of myocardial infarction (Osterud 2002). No work has been performed so far to investigate the role of a high response in the development of sepsis-associated coagulopathy. As previously discussed, there is evidence

that monocytic TF expression contributes to disseminated intravascular coagulation (DIC) (Warr *et al.*, 1990; Taylor *et al.*, 1991).

The difference in response may potentially help elucidate what leads an individual with sepsis to develop coagulopathy and allow for a personalised approach to management. As discussed earlier, the use of biomarkers to stratify individuals with sepsis into different endotypes can predict mortality. Scicluna *et al*. classified individuals with sepsis into four endotypes using the results from 38 previous studies that measured genetic expression in such individuals. One endotype was associated with an increased 28-day mortality. A combination of genetic and clinical criteria as well as suggested potential biomarkers for the different genetic profiles were identified (Scicluna *et al.*, 2017).

4.7.4 Coagulation markers following endotoxaemia

Three of the four markers of coagulation used to identify an increase in TF activity were raised between four and six hours. This coincides with the increase in monocyte cell surface TF expression. Previous work by Franco *et al.* has demonstrated a similar increase in TAT at 4 hours (Franco *et al.*, 2000). A review of the literature has not revealed any instances of measurement of the other complexes during human endotoxaemia, although previous work has associated increased levels with thrombotic conditions (Hobbelt *et al.*, 2017; Posma *et al.*, 2018). Earlier work has associated an increase in levels of FIX, FX and FXI following endotoxaemia. Whilst FIX and FX were shown to peak around the 4 hour timepoint, FXI levels were shown to peak later at 24 hours (Reitsma *et al.*, 2003).

As previously described (see introduction 1.3.2, page 8), the contact pathway can activate FXI and thereby promote thrombin production. Work in this chapter has shown a decrease in FXIIa-C1NH at 6 hours following LPS injection. The *in vivo* activator of the FXII is yet to be elucidated, but it can be inhibited by both AT and C1NH. An increase in FXIIa-AT and corresponding drop in FXIIa-C1NH have been associated with prothrombotic states such as individuals with systemic lupus erythematosus and a history of previous thromboembolic events. The pathology behind this is still unclear; it has been proposed that the inhibition of FXII may depend on the site and mechanism of initial activation. Incubation of FXII, plasma and fibrin *in vitro* shows an increase in FXIIa-AT but no detectable level of FXIIa-C1NH (Bäck *et al.*, 2013).

No differences between these coagulation markers were detected between the high and low responder groups, although this may be due to the small sample size.

A limitation of this work is the lack of direct measurement of monocyte TF activity; this was not possible due to the small numbers of monocytes available during the timepoints 90 minutes and 10 hours. All measurements performed are downstream effects and therefore affected by the rest of the complex coagulation system. The human endotoxaemia model is known to affect much more than just TF: it alters the levels of physiological anticoagulants, the contact pathway and fibrinolysis (Suffredini *et al.*, 1989; van Deventer *et al.*, 1990; Franco *et al.*, 2000; Krabbe *et al.*, 2006).

4.8 Summary of key findings

This chapter confirms the previous work by Tak *et al.* (2017), demonstrating the differential return of subsets following the profound monocytopenia post-endotoxaemia.

There are two peaks in TF expression: an early peak at 90 minutes, presumably due to pre-formed TF being externalised to the cell membrane, and a later one at 6 hours that may relate to an increase in TF transcription. An increase in the coagulation markers known to be activated by TF was demonstrated at a timepoint that coincided with this increase in cell surface expression.

For the first time, the TF cell surface expression on each monocyte subset has been documented following human endotoxaemia. There is clear distinction between high and low responders, and the response of each group is consistent within monocyte subsets.

Chapter 5. Investigation of Monocyte Subsets during Sepsis on the Intensive Care Unit

5.1 Overview

Sepsis-associated coagulopathy is believed to occur, in part, due to dysregulated monocyte TF expression (Warr *et al.*, 1990; Taylor *et al.*, 1991). During sepsis, multiple studies have shown a change in the proportion of circulating monocyte subsets with a reduction in classical monocytes (Herra *et al.*, 1996; Skrzeczynska *et al.*, 2002; Mukherjee *et al.*, 2015). The changes in TF cell surface expression and activity between these subsets remain uncharacterised.

This chapter will describe blood drawn from individuals on the ICU with a diagnosis of sepsis and compare features with further samples taken from those that recover. The changes in monocyte subsets and their TF expression will be investigated.

5.2 Research aims

- to identify changes in the proportion of circulating monocyte subsets during sepsis and on recovery
- to investigate the cell surface expression of TF between monocyte subsets during sepsis and on recovery
- to investigate the monocyte-endothelial interactions during sepsis using the same *in vitro* model as used with healthy monocytes

5.3 Demographics and clinical features of participants with sepsis

Between June 2018 and March 2019, 28 individuals were recruited from the ICUs at NUTH and CHSFT (see methods section 2.11, page 91). These included 18 with a diagnosis of sepsis and 10 who were critically ill without sepsis. A 20 mL blood sample was taken on recruitment to the study and, in 10 participants with sepsis who recovered and consented, a further 20 mL blood sample was taken following recovery and on discharge from intensive care. Clinical details were taken at the time of the first blood sample, including cause of admission to ICU, evidence for infection, co-morbidities, medication history, results of clinically indicated blood tests and medical observations.

Sepsis was diagnosed in 18 individuals with a need for organ support and evidence of infection. This evidence included pathogens grown from blood cultures, bronchoalveolar lavage or evidence of deep-seated infection such as microbiological samples from an abdominal abscess or a surgical wound site. The evidence for diagnosing sepsis, for each participant, is summarised in table 5.1.

The remaining 10 critically ill individuals fit the same criteria for organ support but did not have a diagnosis of sepsis. Most had no evidence microbiological evidence of infection. Participant 8 was found to have *Haemophilus influenzae* on sputum culture but not on bronchoalveolar lavage (BAL). Participant 9 had *E. coli* grown in a urine culture that was believed to be a contaminant and was not treated. The cause of ICU admission and the organ support required by this group are detailed in table 5.2.
Both the critically ill cohort and the cohort with a diagnosis of sepsis were evenly matched. There was no statistically significant in age, sex, length of ICU stays prior recruitment to study, type or organ support required, APACHE II or SOFA score and survival to ICU discharge (see table 5.3).

White blood cell counts, coagulation markers and platelet counts were similar between both cohorts (see Figure 5.1). Twelve (67 %) individuals in the sepsis cohort and six (60 %) in the critically ill had a high white cell count, composed primarily of neutrophils. Coagulopathy was defined as a platelet count below 150 X 10^9/L, prothrombin time (PT) above 15 sec (reference range 10-15 sec), activated partial thromboplastin time (APTT) above 35 sec (reference range 25-35 sec), or fibrinogen below 1.5 g/L (reference range 2.1-4.8 g/L). Coagulopathy was evident in 7 individuals (39 %) from the sepsis cohort and 7 (70 %)

individuals from the critically ill cohort (this was not statistically significant using Fisher's exact test, p=0.24).

Prognostic scores were calculated for both cohorts, APACHE II at the time of admission to ICU and SOFA at the time the first study blood sample was taken (see Figure 5.2). The median APACHE II score for this cohort was 27, which has previously been associated with a mortality rate of 55 % in medical patients (Knaus *et al.*, 1985). More recent work has demonstrated that this mortality rate is now likely to be lower due to advances in the care of critically ill patients (Sadaka *et al.*, 2017).
The median SOFA score, measured at the time the first blood sample was taken, was 9, which was previously associated with an in-hospital mortality of 33 % (Vincent *et al.*, 1996). More recent work has shown the SOFA score remains a useful predictor (Innocenti *et al.*, 2018).

Most individuals with sepsis survived to leave the ICU (n=14, 78%); of these, 10 consented to have further blood taken following their discharge from critical care, the other 4 declined a further blood test.

No.	Source of sepsis	Microbiological evidence	Radiological evidence	Organ support			
				IV	NIV	IN	RRT
1	Ischaemic bowel	*Klebsiella pneumoniae* and *Enterobacter cloacae* from wound	Ischaemic bowel on CT abdomen	y		y	
2	Respiratory	*Influenza A* PCR positive	Right-sided consolidation seen on CXR	y			y
3	Intra-abdominal abscess	*Enterococcus faecium* from blood culture	CT abdomen showed multiple liver abscesses	y		y	
4	Necrotising pancreatitis with abdominal collections	*Escherichia coli* from abdominal drain fluid	Multiple abdominal collections on CT abdomen	y			
5	Perforated bowel	*Enterococcus faecium* from drain fluid	CT abdomen showed proximal transverse colon perforation	y			y
6	Perforated gastric conduit	*Escherichia coli* from abdominal drain fluid	none	y			y
7	Respiratory	*Escherichia coli* from blood and bronchoalveolar lavage	Multi-focal consolidation on CT thorax	y		y	y
8	Post-operative debridement for cellulitis	*Proteus mirabilis* from debrided tissue	none		y		
9	Spontaneous bacterial peritonitis	Gram-negative rods from ascitic fluid, failed to grow on subculture	none	y		y	y
10	Renal	*Escherichia coli* from blood	CT abdomen showed pyelonephritis			y	
11	Cellulitis	*Group A Streptococcus* and *Staphylococcus aureus* from wound	none	y			
12	Ruptured oesophagus	*Lactobacillus gasseri, Lactobacillus fermentum, Streptococcus salivarius and Streptococcus parasanguinis* from sputum	Loculated pleural effusion seen on CT thorax	y			
13	Ruptured oesophagus	*Haemophilus influenzae* from sputum	CT thorax showed consolidation and collection	y		y	
14	Respiratory	*Escherichia coli* from bronchoalveolar lavage	Bibasal consolidation on CT thorax	y			
15	Respiratory	*Influenza A* PCR positive	CXR showed bilateral infiltrates and consolidation	y		y	y
16	Respiratory	*Escherichia coli* from blood and bronchoalveolar lavage, *Candida albicans* from bronchoalveolar lavage	Right-sided consolidation on CXR				y
17	Respiratory	*Influenza A* PCR positive	Extensive bilateral consolidation on CXR	y		y	
18	Respiratory	*Serratia marcescens* from blood and *Proteus mirabilis* from sputum	Consolidation and atelectasis on CXR	y			

Table 5.1 Diagnosis of sepsis

This table details the source and evidence of infection as well as the organ support required by all individuals with sepsis (n=18). Microbiological and radiological data were gathered at recruitment to the study. (CT – computerised tomography, CXR – chest radiograph, IV – invasive ventilation, NIV – non-invasive ventilation, IN – required inotropes or vasopressor medication, RRT – renal replacement therapy).

No.	Cause of ICU admission	Microbiological evidence	Radiological evidence	Organ support			
				IV	NIV	IN	RRT
1	Post craniotomy for brain tumour	nil	nil	y			
2	Head and bowel trauma	nil	CT showed free fluid and air in bowel wall	y			
3	Type I respiratory failure	nil	Bilateral consolidation on chest CXR		y		
4	Acute hepatic failure	nil	nil	y		y	
5	Cardiac arrest post abdominal aortic aneurysm repair	nil	nil	y		y	y
6	Type I respiratory failure secondary to worsening interstitial lung disease	nil	Bilateral consolidation on chest CXR	y		y	y
7	Intra-abdominal haemorrhage post gastrojejunostomy insertion	nil	nil	y		y	
8	Overdose of fluoxetine and oramorph on a background of cardiac failure	*Haemophilus influenzae* in sputum	Bilateral consolidation on chest CXR	y			
9	Alcohol withdrawal and type I respiratory failure	*E. coli* in urine culture	CXR showed lobar collapse	y			
10	Type I respiratory failure secondary to cardiac failure	nil	Bilateral consolidation on chest CXR				y

Table 5.2 Clinical details of critically ill cohort

This table details the source and evidence of infection as well as the organ support required by all individuals with critical illness who did not meet the criteria for sepsis (n=10). Microbiological and radiological data were gathered at recruitment to the study.

	Sepsis	Critically ill
Age (years)	66 (38-77)	58.5 (30-70)
Male	12 (67 %)	7 (70 %)
Length of stay (days)	7.5 (1-31)	5.5 (1-17)
Invasive ventilation	15 (83 %)	8 (80 %)
Non-invasive ventilation	1 (5 %)	1 (10 %)
Use of inotropes or vasopressors	8 (44 %)	4 (40 %)
Renal replacement therapy	7 (39 %)	3 (30 %)
APACHE II score	27 (15-45)	26.5 (9-40)
SOFA score	9 (3-16)	7 (4-14)
Survived until ICU discharge	14 (78 %)	4 (40 %)

Table 5.3 Comparison of critically ill cohort to the cohort with a diagnosis of sepsis

This table compares the demographics, length of ICU stays (prior to recruitment into the study), organ support requirements, prognostic scores and survival of both cohorts. SOFA scores were calculated at the time of the first blood sample; one critically ill individual and seven individuals with sepsis did not have a SOFA score calculated due to a lack of bilirubin measurement on that occasion. There were no statistically significant differences. Fisher's exact test was used to compare male sex, invasive ventilation, non-invasive ventilation, use of inotropes or vasopressors, renal replacement therapy and survival. The unpaired t*-test was used to compare age, length of stay, APACHE II score and SOFA score.*

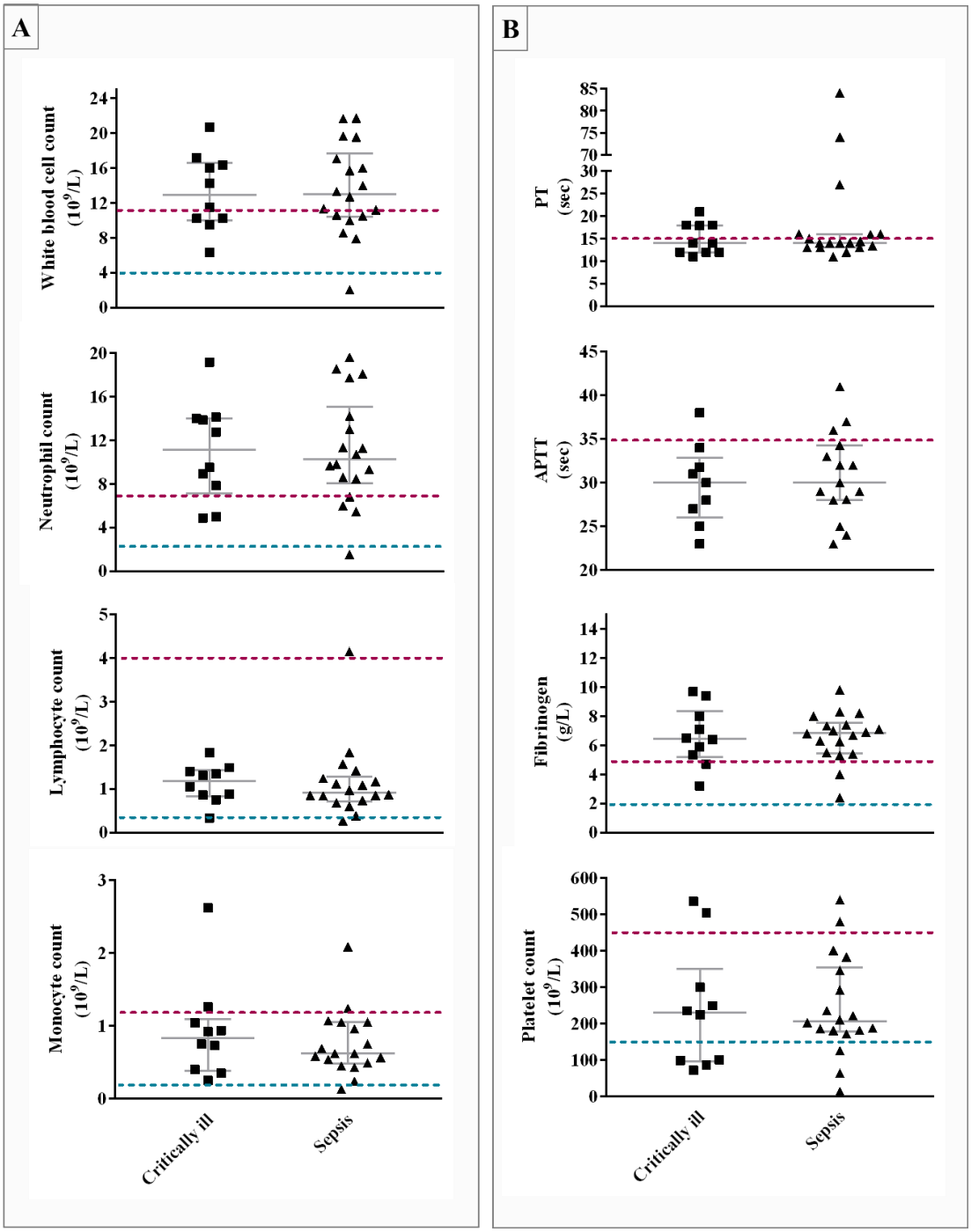
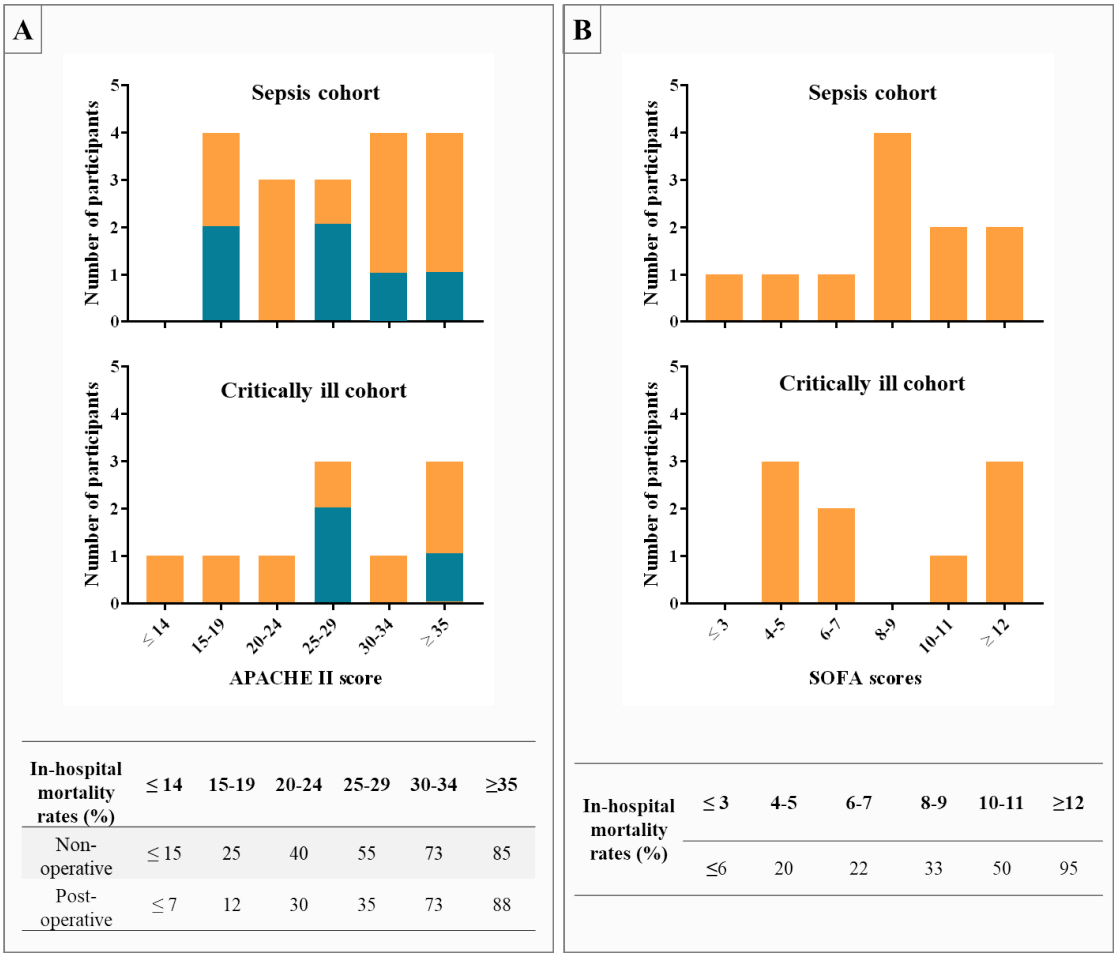

A
White blood cell count (10^9/L)
Neutrophil count (10^9/L)
Lymphocyte count (10^9/L)
Monocyte count (10^9/L)
Critically ill
Sepsis
B
PT (sec)
APTT (sec)
Fibrinogen (g/L)
Platelet count (10^9/L)
Critically ill
Sepsis

Figure 5.1 White cell counts and coagulation markers of the septic and the critically ill cohorts

White blood cell counts (WBC), platelet count, PT, APTT and derived fibrinogen levels were measured in the hospital laboratories using a Sysmex XN-9000™ (Sysmex, Milton Keynes, UK) and ACL Top® 700 (Beckman Coulter, High Wycombe, UK) analysers. All levels were taken from clinically indicated samples obtained on the day of recruitment to the study. ***A*** *shows the total white cell count and the individual neutrophil, monocyte and lymphocyte counts for the critically ill (n=10) and septic cohorts (n=18).* ***B*** *shows the PT, APTT, derived fibrinogen and platelet count in the critically ill cohort (n=10; one participant was excluded from the APTT figure as they were receiving a heparin infusion at the time of sampling) and septic cohort (n=18; 3 values were excluded from the APTT figure because these individuals were receiving therapeutic level heparin at the time of sampling). The red dashed line marks the upper limit of the reference range of that marker, whilst the blue dashed line marks the lower limit. Error bars represent the median values and interquartile ranges. There were no statistically significant differences between the cohorts for any marker. Significance was calculated using the Mann-Whitney U test (WBC: p=0.91, neutrophils: p=0.91, lymphocytes: p=0.45, monocytes: p=0.63, PT: p=0.68, APTT: p=0.59, fibrinogen: p=0.83, platelet count: p=0.99).*



In-hospital mortality rates (%)	≤14	15-19	20-24	25-29	30-34	≥35
Non-operative	≤15	25	40	55	73	85
Post-operative	≤7	12	30	35	73	88

In-hospital mortality rates (%)	≤3	4-5	6-7	8-9	10-11	≥12
	≤6	20	22	33	50	95

Figure 5.2 Prognostic scores for the septic and the critically ill cohorts

APACHE II score was based on information from the first 24 hours of ICU admission, SOFA score was calculated using information at the time of the first blood sample. ***A*** *shows the APACHE II scores for the individuals with sepsis (n=18) and critical illness (n=10). The blue bar represents the participants that were post-operative. Percentages denote the mortality rate associated with each APACHE II score (Knaus et al., 1985).* ***B*** *shows the SOFA score for the sepsis and critically ill cohorts. Eight participants were excluded from the SOFA figure as a score could not be calculated (a bilirubin level had not been measured on the day of the first study blood sample), seven were from the sepsis cohort and one from the critically ill cohort. The percentages denote the mortality rates associated with that level of SOFA score (Vincent et al., 1996). There was no statistical difference between the cohorts (APACHE II score: p=0.98, SOFA score: p=0.81 using the unpaired t-test).*

5.4 Monocyte subsets during sepsis, compared to those in health and those in the recovery after sepsis

As described previously, 100 μL of whole blood was taken from healthy controls (n=13), individuals with sepsis (n=18) and the subset of individuals who recovered from sepsis and consented to a further blood sample (n=10). Flow cytometry was performed using a BD Trucount™ tube to allow the calculation of absolute values (see methodology section 2.6.1, page 72).

There was no statistically significant difference between the absolute monocyte count of the critically ill cohort, the sepsis cohort and the recovery samples of individuals with sepsis (Figure 5.3).

Flow cytometry was used to identify the proportions of monocyte subsets during sepsis, and these were compared against the critically ill cohort or blood samples taken on recovery (figures 5.4 and 5.5). Both the proportion of subsets and the absolute monocyte counts were compared. No statistically significant differences in monocyte subsets were noted.

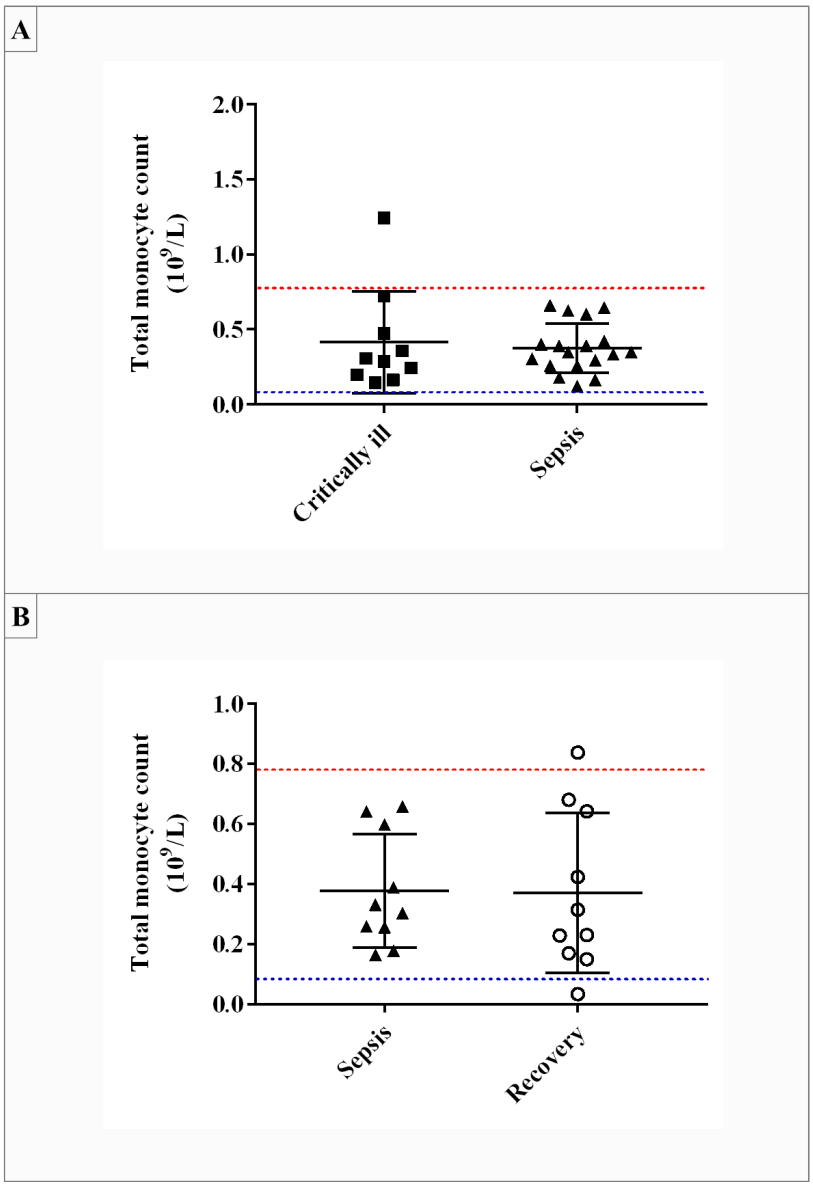
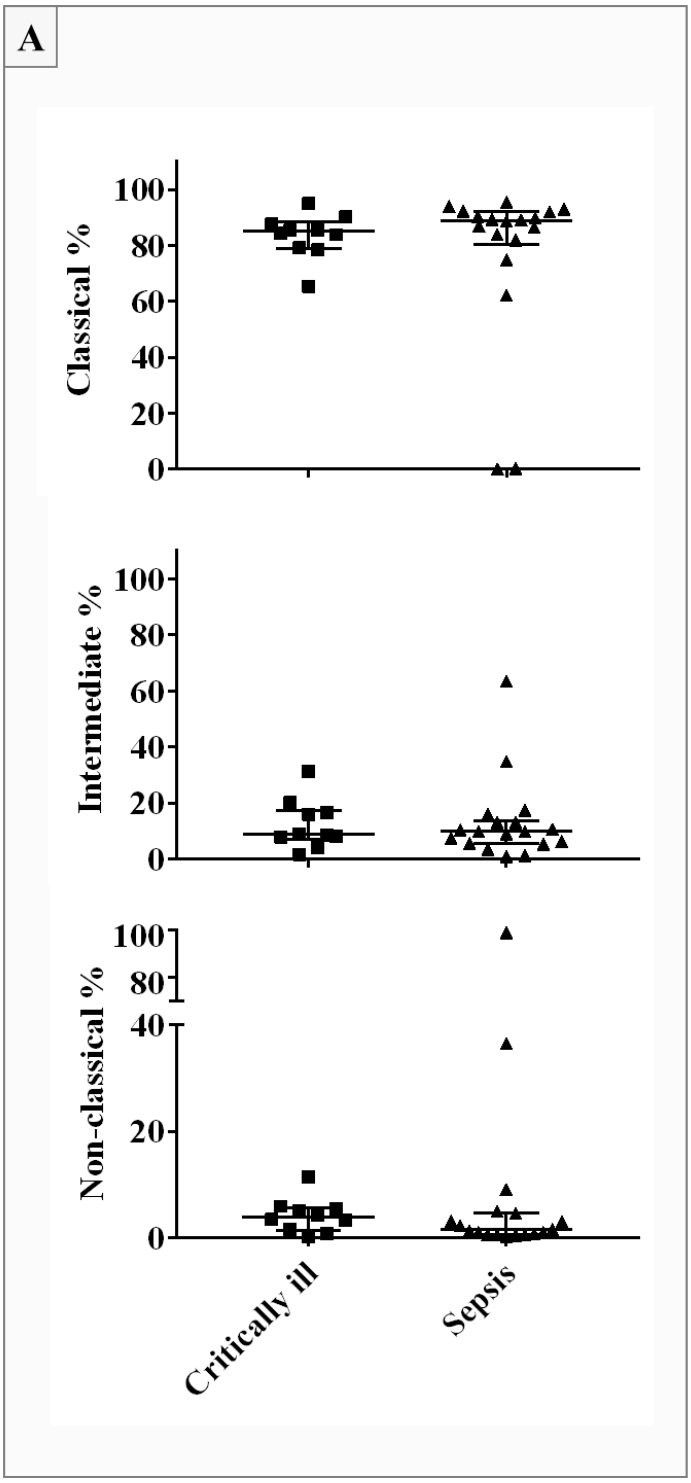


Figure 5.3 Total monocyte counts of the critically ill, sepsis and recovery cohorts

Whole blood flow cytometry and Trucount™ absolute counting tubes were used to measure the absolute monocyte counts in whole blood. Error bars represent median values and interquartile ranges. ***A*** *compares the total number of monocytes between the critically ill cohort (n=10) and individuals with sepsis (n=18). There was no statistically significant difference between the cohorts (p=0.65 by the Mann-Whitney U test).* ***B*** *compares the absolute monocyte counts between blood samples taken during sepsis with a sample following recovery (n=10). There was no statistically significant difference between individuals with sepsis and then on recovery (p=0.77 by the Wilcoxon matched-pairs signed rank test). The red dashed line marks the upper limit of the reference range of that marker, whilst the blue dashed line marks the lower limit. The reference range is based on 13 independent healthy controls and defined as two standard deviations either side of the mean.*


A
Classical %
100
80
60
40
20
0
Intermediate %
100
80
60
40
20
0
Non-classical %
100
80
40
20
0
Critically ill
Sepsis

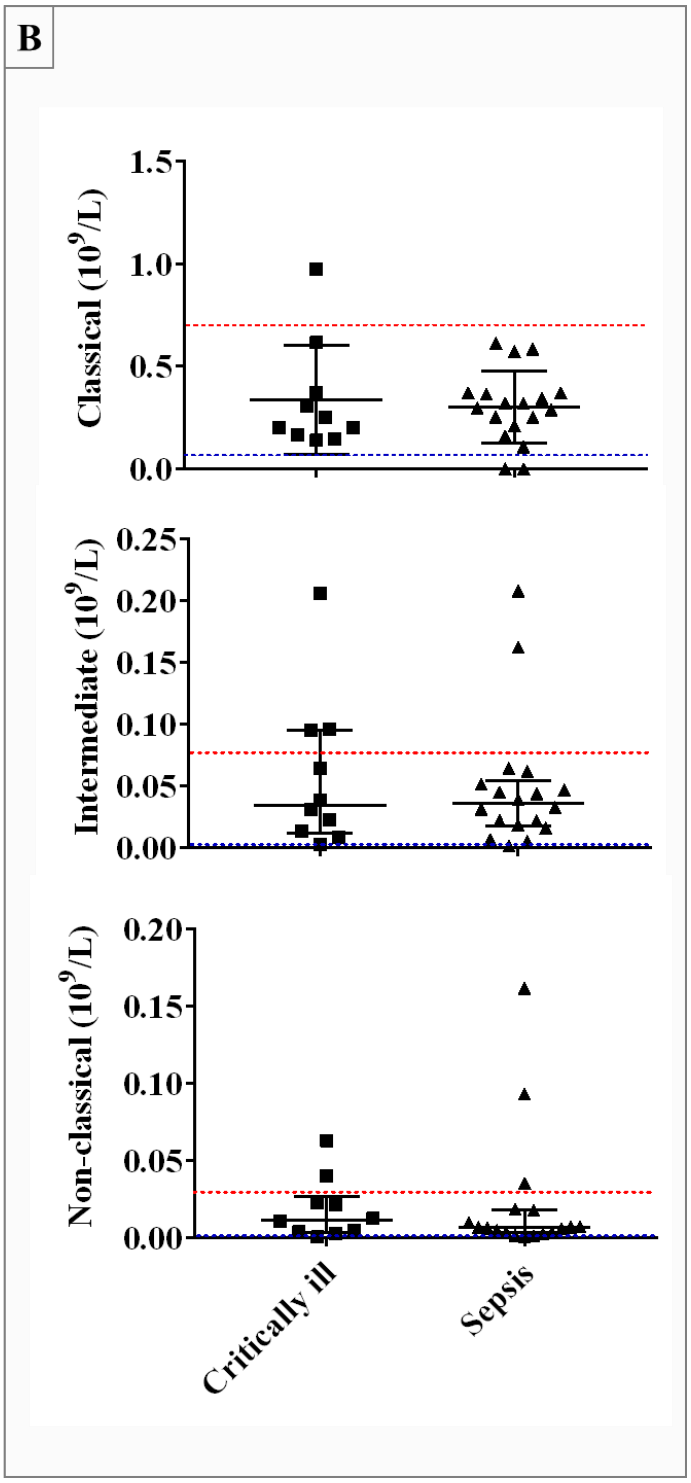

B
Classical (10^9/L)
1.5
1.0
0.5
0.0
Intermediate (10^9/L)
0.25
0.20
0.15
0.10
0.05
0.00
Non-classical (10^9/L)
0.20
0.15
0.10
0.05
0.00
Critically ill
Sepsis

Figure 5.4 Monocyte subsets in the critically ill and sepsis cohorts

Whole blood flow cytometry with Trucount™ absolute counting tubes were used to identify the counts of classical, intermediate and non-classical monocytes. The error bars represent the median value and inter-quartile range. ***A*** *compares the percentage of monocyte subsets between the critically ill cohort (n=10) and the sepsis cohort (n=18). There were no statistically significant differences between the cohorts (classical: p=0.38, intermediate: p=0.87, non-classical: p=0.27 by the Mann-Whitney test).* ***B*** *compares the absolute monocyte subset counts between the critically ill cohort (n=10) and the sepsis cohort (n=18). There were no statistically significant differences between the cohorts (classical: p=0.76, intermediate: p=0.87, non-classical: p=0.49 by the Mann-Whitney test). The red dashed line marks the upper limit of the reference range of that marker, whilst the blue dashed line marks the lower limit. The reference range is based on 13 independent healthy controls and defined as two standard deviations either side of the mean.*

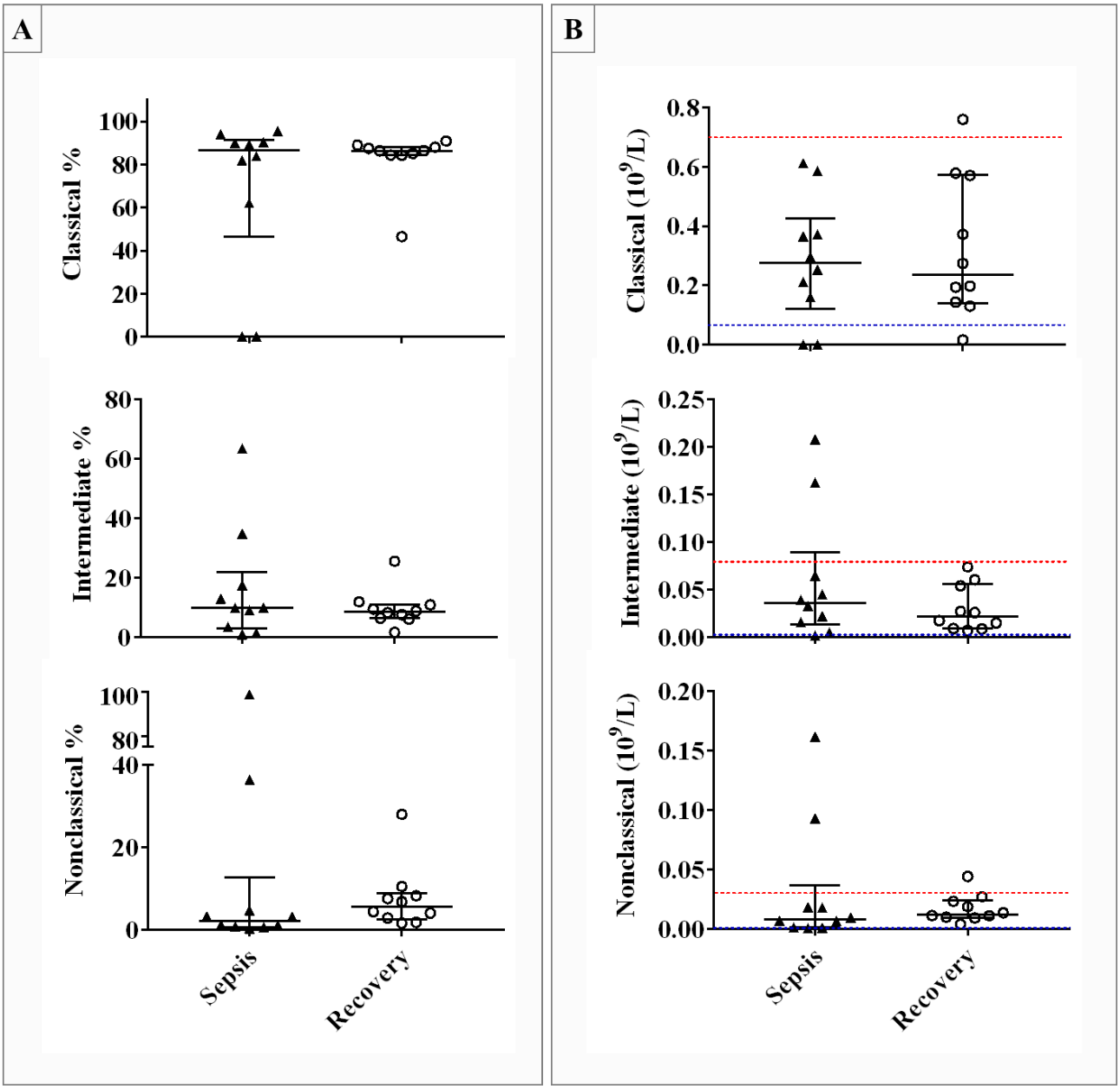

A
Classical %
100
80
60
40
20
0
Intermediate %
80
60
40
20
0
Nonclassical %
100
80
40
20
0
Sepsis
Recovery
B
Classical (10^9/L)
0.8
0.6
0.4
0.2
0.0
Intermediate (10^9/L)
0.25
0.20
0.15
0.10
0.05
0.00
Nonclassical (10^9/L)
0.20
0.15
0.10
0.05
0.00
Sepsis
Recovery

Figure 5.5 The proportions of monocyte subsets during sepsis and following recovery

Whole blood flow cytometry with Trucount™ absolute counting tubes were used to identify the absolute counts of classical, intermediate and non-classical monocytes. The error bars represent the median value and inter-quartile range. ***A*** *compares the percentage of monocyte subsets between paired samples of individuals with sepsis and the same individuals on recovery and discharge from ICU (n=10). There were no statistically significant differences between the cohorts (classical: p=0.56, intermediate: p=0.43, non-classical: p=0.70 by the Wilcoxon matched-pairs signed rank test).* ***B*** *compares the absolute monocyte subset counts between paired samples of individuals with sepsis and the same individuals on recovery and discharge from ICU (n=10). There were no statistically significant differences between the cohorts (classical: p=0.70, intermediate: p=0.32, non-classical: p=0.92 by the Wilcoxon matched-pairs signed rank test).*

5.5 TF expression non-septic critical illness, sepsis and recovery from sepsis

Whole blood flow cytometry, as previously described, was performed to identify monocytic TF expression. The gating of TF-positive events is demonstrated in Figure 5.6 and used fluorescence minus one (FMO) controls.

There was no statistically significant difference between the percentages of monocytes expressing TF between the critically ill and sepsis cohorts. Considering the patients who had sepsis, there was an increase in the proportion of TF-expressing monocytes in recovery samples (Figure 5.6).

TF expression was also considered on monocyte subsets. Individuals with sepsis had a higher percentage of TF-positive intermediate and non-classical monocytes when compared to critically ill individuals without sepsis. Classical monocytes were shown to have a higher percentage of TF-expressing cells following recovery from sepsis. There were no differences in the proportion of monocytes expressing TF in either the intermediate or non-classical subsets (Figure 5.7).

There was no difference between total monocyte TF expression between individuals with sepsis and evidence for coagulopathy, and those with sepsis but no coagulopathy (Figure 5.8).

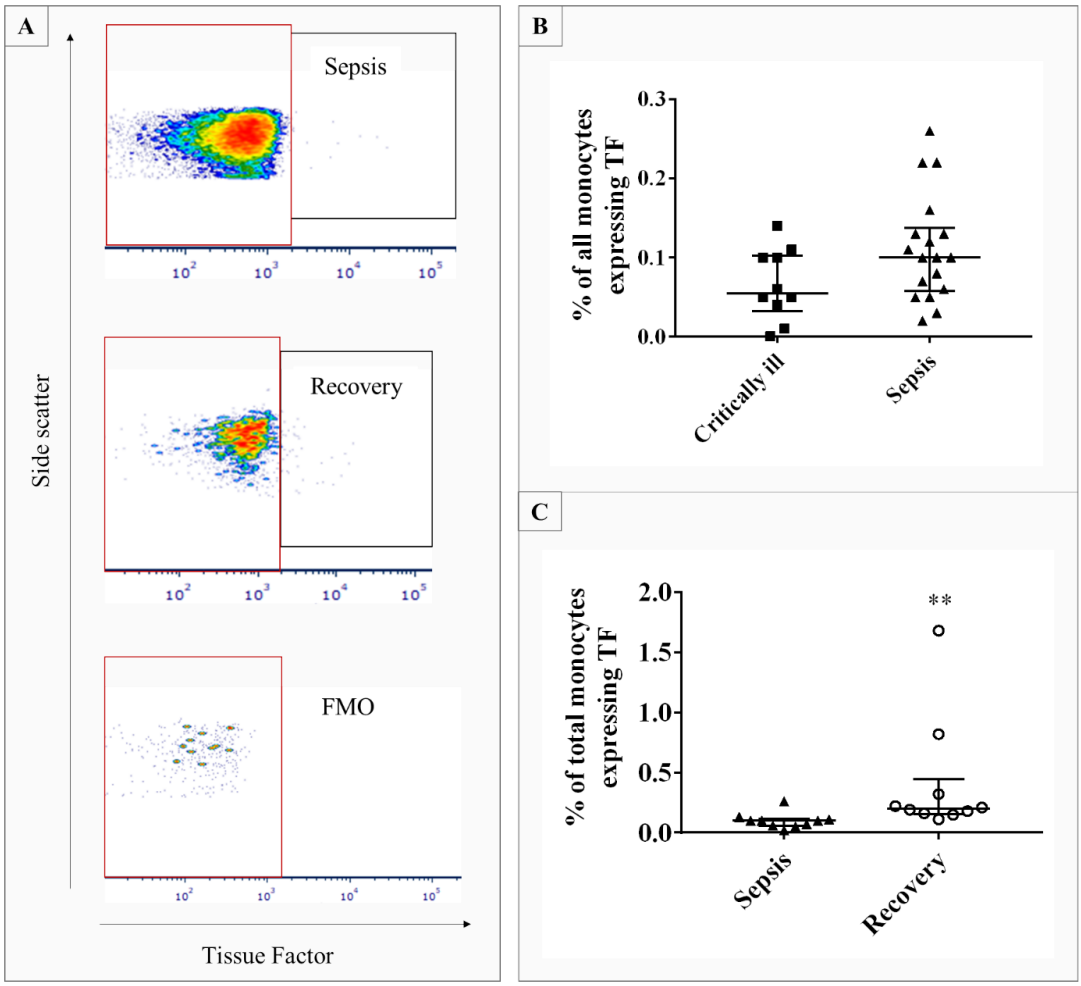


Figure 5.6 Monocyte tissue factor expression increases following recovery from sepsis

Whole blood flow cytometry was used to identify the percentage of total monocytes that expressed cell surface TF. Error bars represent median values and the interquartile ranges. ***A*** *shows a representative example of the flow gating used to identify TF-positive monocytes. The black gate shows positive events, whereas the red gate shows negative events, as demonstrated with the TF FMO.* ***B*** *compares the percentage of TF-positive monocytes in the critically ill (n=10) and sepsis cohorts (n=18). There was no statistical difference (p=0.09 using Mann-Whitney test).* ***C*** *compares the percentage of TF-positive monocytes between individuals with sepsis and the same individuals following recovery discharge from ICU (n=10). The percentage of TF-positive monocytes increased following recovery from sepsis (p<0.01 using the Wilcoxon matched-pairs signed rank test).*

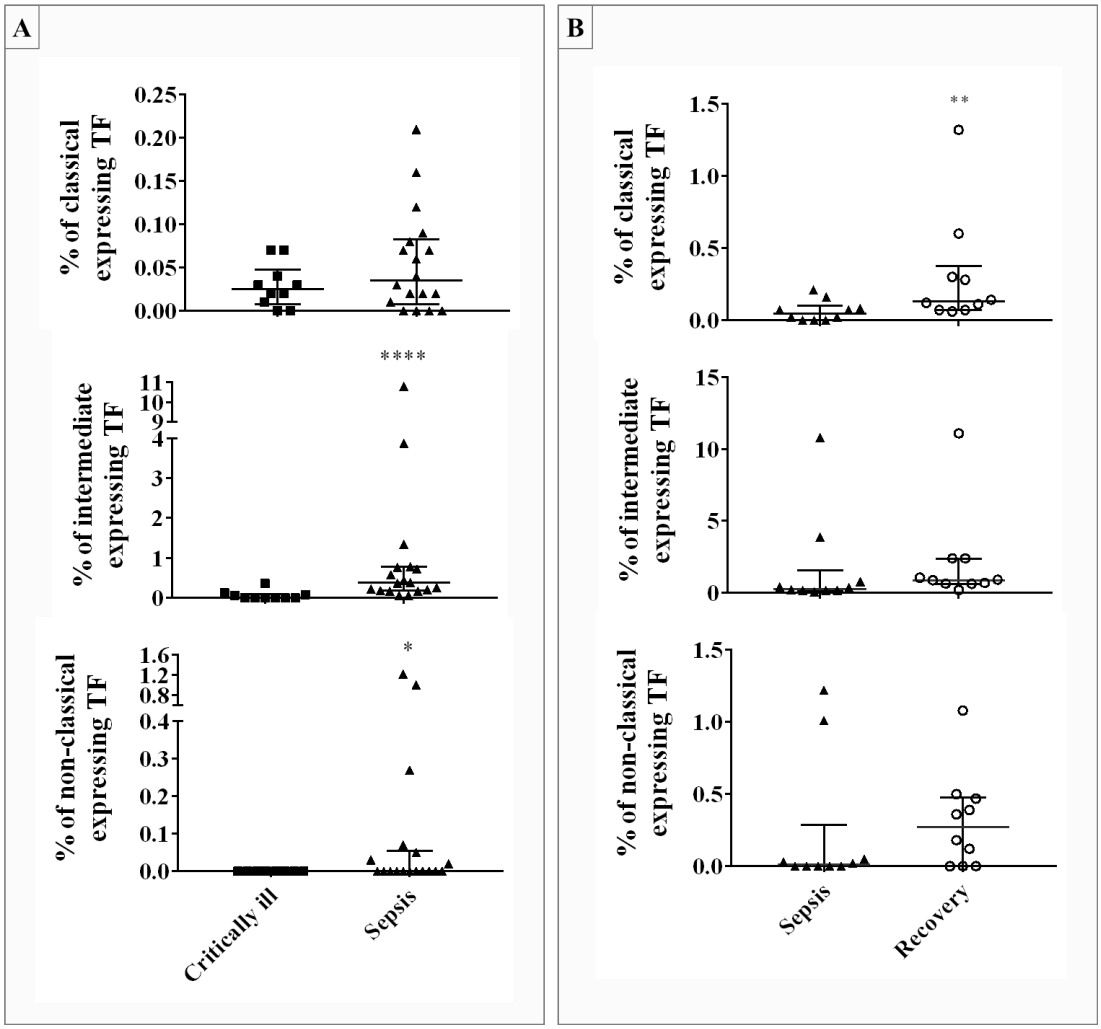


Figure 5.7 Classical monocyte TF expression increases with recovery from sepsis

Whole blood flow cytometry was used to identify the percentage of total monocytes that expressed cell surface TF. Error bars represent median values and the interquartile ranges. ***A*** *compares the percentage of TF-positive monocyte subsets of the critically ill (n=10) and sepsis cohorts (n=18). There was no statistically significant difference between the classical subset, but the intermediate and non-classical subset showed higher percentages of TF in the sepsis cohort (classical: p=0.40, intermediate: **** – p<0.0001, non-classical: p<0.05 using the Mann-Whitney test).* ***B*** *compares the percentage of TF-positive monocytes in individuals with sepsis and following their recovery (n=10). The classical subset showed an increased percentage of TF-positive cells on recovery; there were no significant differences in the intermediate and non-classical subsets (classical: ** – p<0.01, intermediate: p=0.11, non-classical: p=0.64 using the Wilcoxon matched-pairs signed rank test).*

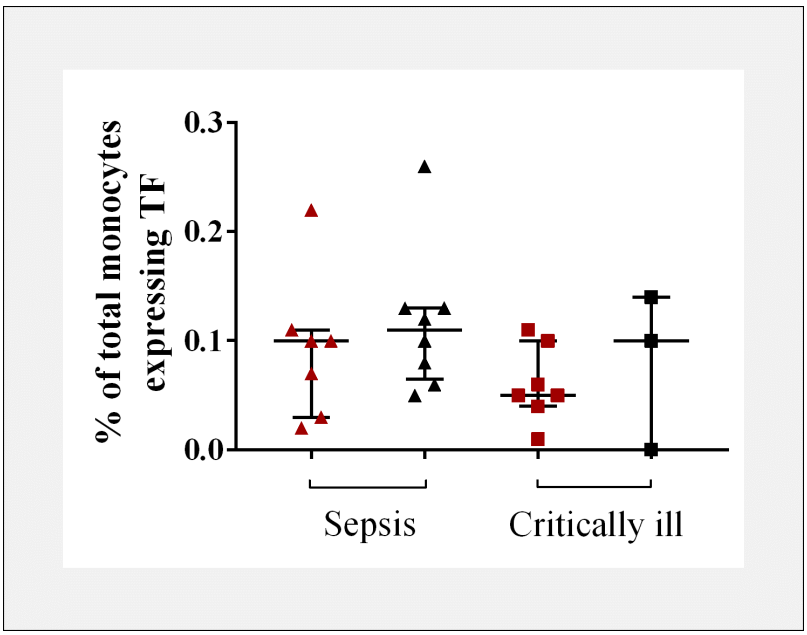


Figure 5.8 No change in the percentage of TF-positive monocytes between individuals with coagulopathy and individuals without coagulopathy

This figure shows the percentage of TF-positive monocytes in individuals with sepsis and evidence of coagulopathy,those with sepsis but no coagulopathy (n=8), individuals without sepsis and coagulopathy (n=7) and those without sepsis and no coagulopathy (n=3). Three individuals with sepsis were excluded from this analysis because they received therapeutic levels of anticoagulation. Coagulopathy was defined as platelet count below 150 x 10^9/L, PT above 15 sec, APTT above 35 sec or fibrinogen below 1.5 g/L. The error bars represent the median value and interquartile range. Red points show data from coagulopathic samples whilst black points show non-coagulopathic. There was no significant difference between the cohorts (p=0.38 using the Mann-Whitney test).

5.6 Monocyte-endothelial cell co-cultures

The *in vitro* model of monocyte-endothelial cell interactions described in chapter 3 (see section 3.4, page 122) was repeated with PBMCs from healthy volunteers and individuals with sepsis. Using Ficoll-Paque™ density gradient separation, PBMCs were isolated from whole blood samples. Due to the smaller volume of blood received from individuals with sepsis compared to that available from healthy volunteers (20 mL compared to 80 mL) it was not possible to conduct FACS on sufficient monocytes for co-culture. PBMCs were therefore used to repeat the co-culture experiments.

They were cultured for 24-hours with PMVEC cells in PMVEC medium. Some PMVECs were pre-treated with LPS (100 ng/mL) for one hour; the LPS was then washed off and PBMCs were added to the culture. Some PBMCs were stimulated by the addition of LPS (10 ng/mL); this LPS was not washed off (see methodology section 2.7.2, page 82). These conditions were set up in a 24-well plate as shown in Figure 5.9.

PBMCs from healthy controls were isolated, again using Ficoll-Paque™ density gradient separation, on each day a co-culture was set up. This allowed for a comparison of PBMCs from healthy controls and individuals with sepsis.

This experiment was performed only four times due to the availability of PMVECs and PBMCs from a septic sample. There were no statistically significant changes. A trend towards an increase in the proportion of classical monocytes (with a corresponding decrease in the intermediate and nonclassical monocyte subsets) was seen in co-cultures using PBMCs from healthy volunteers (Figure 5.10). This is comparable to the co-cultures described with healthy blood samples in Chapter 3 (see figure 3.14).

When PBMCs from individuals with sepsis were used, no clear trend for the classical subset to increase with co-culture was seen (figure 5.10). The intermediate subset from individuals with sepsis showed a statistically non-significant increase with co-culture, in contrast to the pattern observed using PBMC samples from healthy volunteers. Although the non-classical subset appeared to change in a similar way between both healthy and sepsis samples, the classical and intermediate subsets appear to vary between the two groups (figure 5.10). However, this work would need to be extended before any conclusions could be drawn. In particular the proportion of monocytes recovered from culture would need increased.

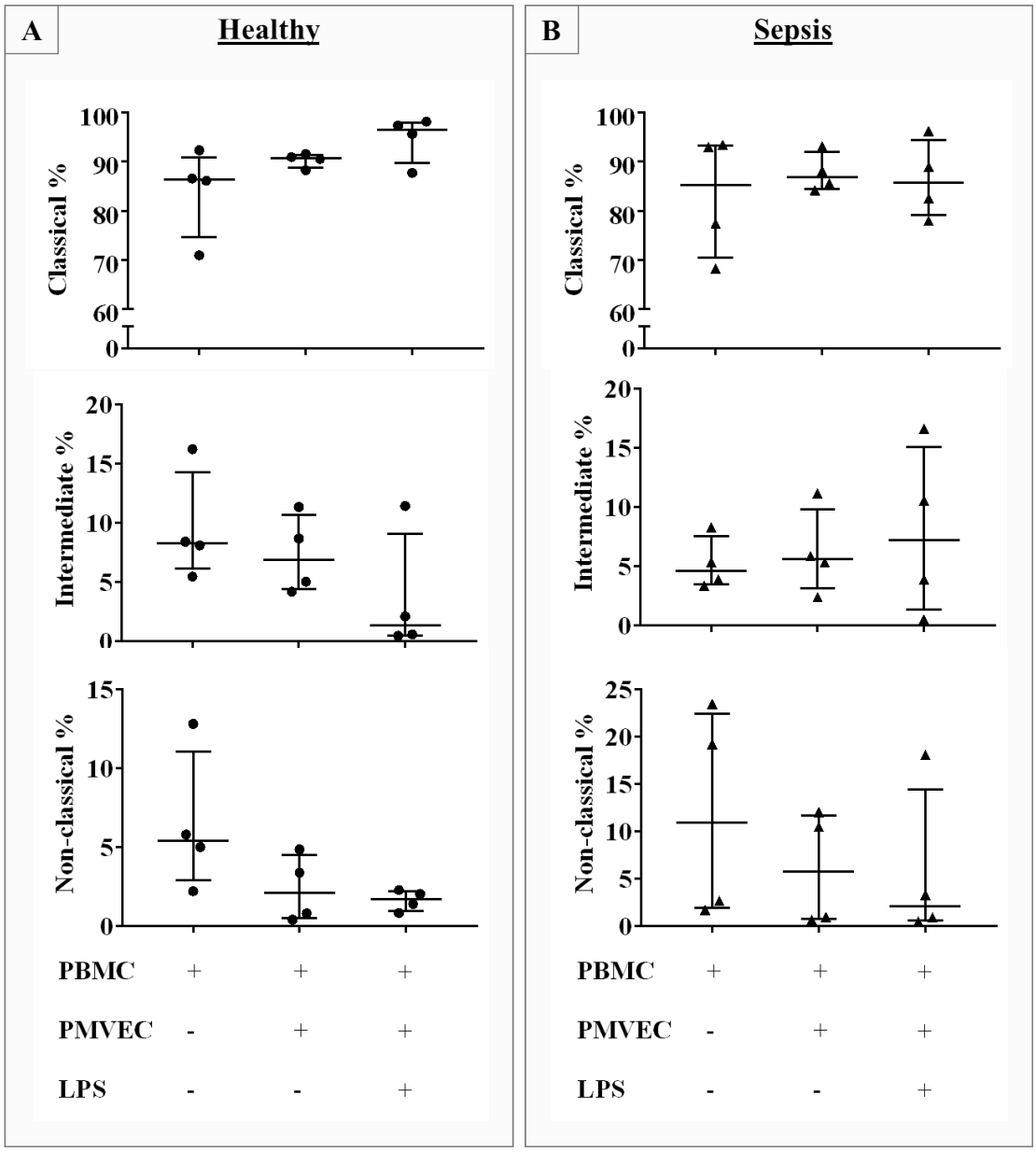
Although the healthy PBMCs tended towards an increase in the classical subset with co-culture (as previously shown; see section 3.4, page 122), this was not seen when using PBMCs from patients with sepsis (figure 5.10). No change in TF expression was seen when comparing isolated monocyte subsets from individuals with sepsis or healthy controls, after PBMCs had been co-cultured with PMVECs (figure 5.11).

	1	2	3	4
A	PB_{HV}	PB_{HV}	PB_{HV} + L	PB_{HV} + L
B	PB_{HV} /PM	PB_{HV} /PM	PB_{HV} /PM+L	PB_{HV} /PM+L
C	PM	PM	PM+L	PM+L
D	PB_S	PB_S	PB_S + L	PB_S + L
E	PB_S /PM	PB_S /PM	PB_S /PM+L	PB_S /PM+L

Abbreviation	**Meaning**
PB_{HV}	PBMC from healthy volunteer
PB_S	PBMC from individual with sepsis
PM	Human pulmonary microvascular endothelial cell (PMVEC)
L	Lipopolysaccharide (LPS)

Figure 5.9 Co-culture plate set-up

This figure depicts the set-up of a 24-well plate for a co-culture experiment of PMVECs and PBMCs. LPS (100 ng/mL) was added to PMVECs and washed off after 1 hour, prior to the addition of PBMCs. LPS (10 ng/mL) was added to PBMCs and not removed. Co-culture were incubated at 37 ºC and 5 % CO_2 for 24 hours. Following this, PBMCs were harvested, and flow cytometry used to measure the monocyte subset proportions and the percentage of TF-positive monocytes.


A
Healthy
Classical %
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Intermediate %
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15
10
5
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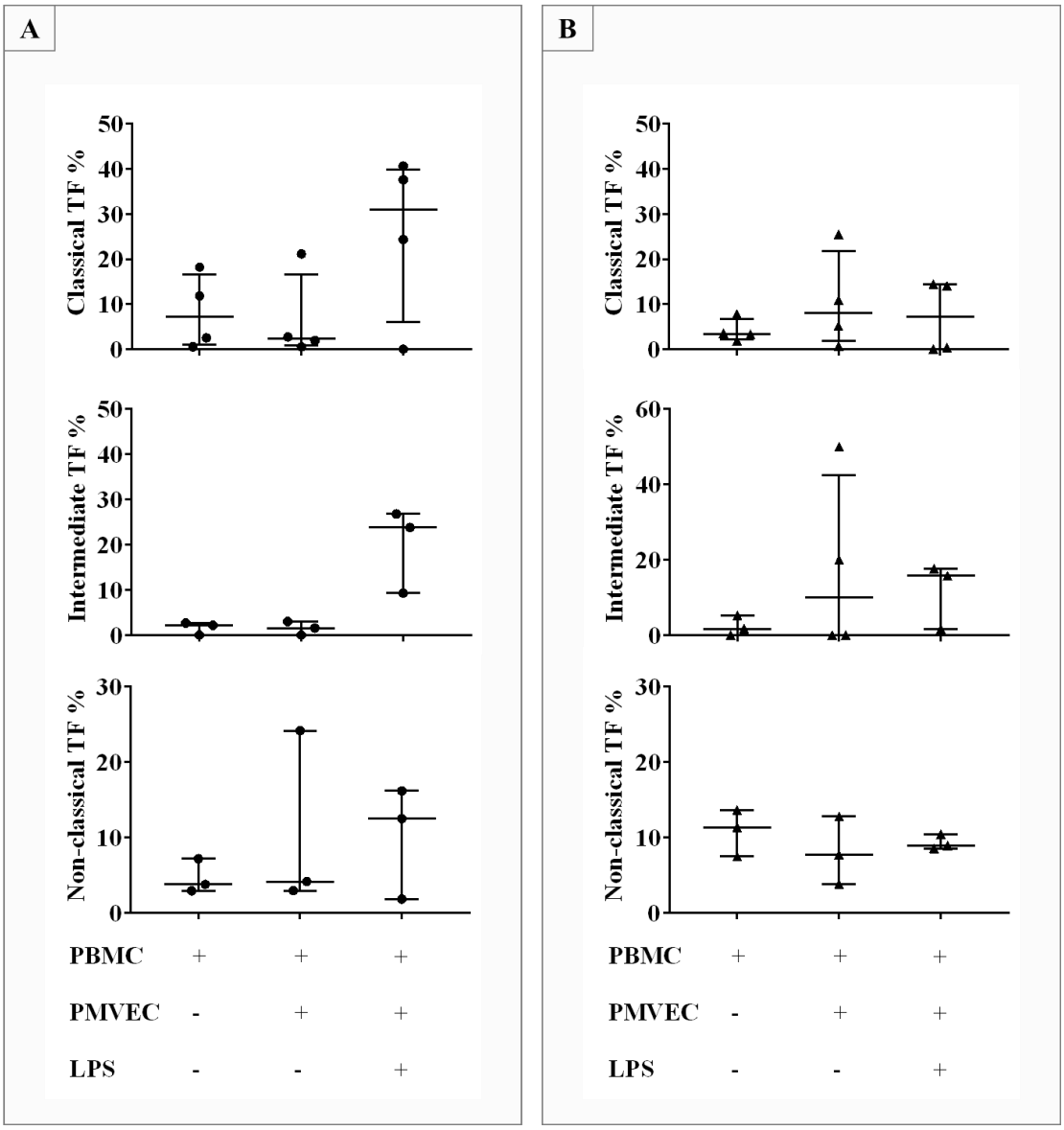
Non-classical %
15
10
5
0
PBMC + + +
PMVEC - + +
LPS - - +
B
Sepsis
Classical %
100
90
80
70
60
0
Intermediate %
20
15
10
5
0
Non-classical %
25
20
15
10
5
0
PBMC + + +
PMVEC - + +
LPS - - +

Figure 5.10 Comparing monocyte subsets following endothelial cell co-culture using PBMCs from healthy controls and sepsis

PBMCs were isolated using Ficoll-Paque™ density gradient separation using samples from healthy volunteers and individuals with sepsis. A proportion of PMVECs were stimulated with 100 ng/mL of LPS for 1 hour; the LPS was washed off prior to the addition of the PBMCs. Some PBMCs were cultured alone with 10 ng/mL LPS: in contrast to the PMVEC stimulation, the LPS was not removed but remained there for the duration of culture. Following a 24-hour incubation, the proportions of monocyte subsets were measured using flow cytometry. This figure shows the results of 4 independent experiments. Error bars represent median values and the interquartile range. There were no statistically significant differences between the coculture conditions using Friedman's test (classical healthy: p=0.13, intermediate healthy: p=0.13, non-classical healthy: p=0.07, classical sepsis: p=0.93, intermediate sepsis: p>0.99, non-classical sepsis: p=0.07).


A
Classical TF %
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Intermediate TF %
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0
Non-classical TF %
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20
10
0
PBMC + + +
PMVEC - + +
LPS - - +
B
Classical TF %
50
40
30
20
10
0
Intermediate TF %
60
40
20
0
Non-classical TF %
30
20
10
0
PBMC + + +
PMVEC - + +
LPS - - +

Figure 5.11 TF expression in monocyte subsets following PMVEC co-culture of PBMCs from healthy volunteers and patients with sepsis

PBMCs were isolated using Ficoll-Paque™ density gradient separation using samples from healthy volunteers and individuals with sepsis. A proportion of PMVECs were stimulated with 100 ng/mL of LPS for 1 hour; the LPS was washed off prior to the addition of the PBMCs. Some PBMCs were cultured alone with 10 ng/mL LPS: in contrast to the PMVEC stimulation, the LPS was not removed but remained there for the duration of culture. Following a 24-hour incubation, the percentage of TF-positive monocytes were measured using flow cytometry. This figure shows the results of 4 independent experiments: one sample was excluded from the intermediate and non-classical subset as there was too few monocytes to accurately gate the TF-positive events. Bars represent median values and error bars the upper quartile. There were no statistically significant differences between the co-culture conditions using Friedman's test (classical healthy: p=0.43, intermediate healthy: p=0.19, non-classical healthy: p=0.94, classical sepsis: p=0.93, intermediate sepsis: p=0.19, non-classical sepsis: p=0.94).

5.7 Discussion

5.7.1 The population of individuals with sepsis is similar to previously described cohorts.

The cohort of individuals with sepsis were severely ill as demonstrated by a median APACHE II score of 27 (calculated using information from their first 24 hours in ICU) and a median SOFA score of 9 (on the day of the first blood sample following recruitment to the study). The use of these is scores is limited by the change in ICU care that has occurred since they were first designed. Recent work shows that although the predicted mortality rates may not be as accurate, comparing APACHE II predictions and actual hospital mortality rates demonstrate that the score is still a good discriminator of disease severity and risk of mortality. This was particularly true in regard to individuals with sepsis (Godinjak *et al.*, 2016; Sadaka *et al.*, 2017).

Similar work has also demonstrated that the SOFA score remains a good predictor of mortality following sepsis (Innocenti *et al.*, 2018). A limitation of this study was the measurement of the SOFA score on a single occasion. A retrospective review of more than 180,000 adults on an ICU with an infection has shown that an increase in SOFA of more than two points is a good predictor of mortality (Raith *et al.*, 2017). The SOFA score was initially created to be performed daily, not as a predictor of mortality but as a way of monitoring organ failure over time (Vincent *et al.*, 1996). With regard to this work, a higher score is related to a more severe degree of organ failure, and it remains a useful way of describing the critically ill and sepsis cohorts.

Of this cohort, 22% did not survive to be discharged from the ICU. This is similar to other larger scale studies (Fleischmann *et al.*, 2016; Meyer *et al.*, 2018).

To ensure a diagnosis of sepsis, all patients had evidence of infection on microbiological culture and a need for organ support. This is based on the most recent international definition of sepsis (Singer *et al.*, 2016).

Coagulopathy was present in 39% of this cohort (7 individuals). This is in keeping with previous studies that have documented coagulopathy in between 30 and 75% of sepsis cases (Gando *et al.*, 2016).

5.7.2 Change in monocyte subset during sepsis

Multiple studies have shown an increase in either intermediate or non-classical monocytes associated with sepsis (Herra *et al.*, 1996; Skrzeczynska *et al.*, 2002; Mukherjee *et al.*, 2015). The work presented here, however, showed no statistically significant difference, although there was perhaps a trend towards an increase in the non-classical subset. Reasons for this difference seem likely to be explained, at least in part, by the heterogeneous nature of sepsis and the variation in flow cytometry gating between users. Mukherjee *et al*., for example, selected only HLA-DR positive cells; previous work has shown a decrease in monocytic HLA-DR expression is seen in some individuals with sepsis (Drewry *et al.*, 2018).

5.7.3 TF expression during sepsis and recovery

Previous work has demonstrated an increase in TF activity on isolated monocytes from 16 individuals with meningococcal infection with higher levels associated with a worse prognosis (Osterud and Flaegstad, 1983). The samples, however, were frozen and thawed prior to testing. Work performed by Maynard et al. demonstrated that the freeze-thaw cycle can de-crypt TF on monocytes and increase activity. It is therefore difficult to draw any definite conclusions about TF activity and sepsis (Maynard *et al.*, 1975; Osterud and Flaegstad, 1983).

Animal models have provided some further insight, showing improved survival in a septic model if a monoclonal Ab to TF was given. Monocytic TF was not investigated (Warr *et al.*, 1990; Taylor *et al.*, 1991).

Measurement of TF in plasma from individuals with sepsis has also been shown to be raised and to be particularly high in those meeting the criteria for DIC (Gando *et al.*, 1998).

Vickers *et al*. have previously investigated the proportion of monocytes expressing TF during sepsis. They showed an increase in the TF expression of CD14 positive monocytes in individuals with a diagnosis of sepsis compared to those who were critically ill but did not have a sepsis diagnosis. Individuals on the critical care unit were screened for signs of sepsis according to the criteria at that time. Participants were recruited to the study as soon as they reached the criteria for sepsis, and blood samples were taken within 12 hours of recruitment (Bone *et al.*, 1992; Vickers *et al.*, 1998). The work presented in this chapter supports these findings, with an increase in TF expression in those with sepsis when compared to critically ill individuals without sepsis. These data have also shown an increase in the proportion of

classical monocytes expressing TF following recovery from sepsis. This has not previously been reported and warrants further investigation.

Potentially, this prolonged change in monocytic TF expression could be an example of monocyte innate training (see section 1.4.8, page 36). Previous work has demonstrated a change in monocyte phenotype to up to 12 months following infection (Kleinnijenhuis *et al.*, 2014). Although there has been measurement of monocyte TF expression following innate training, there is evidence to suggest that it can promote atherosclerosis. The Apolipoprotein E knockout murine model is used to study atherosclerosis. Murine monocytes previously stimulated with low-dose LPS were adoptively transferred to the knockout model; using monocyte controls incubated with PBS, the LPS-trained monocytes were associated with an increase in atherosclerotic plaques (Geng *et al.*, 2016).

The percentage of monocytes expressing TF is similar to that shown following the human endotoxaemia model but again it is lower than that seen with *in vitro* work. As discussed earlier, this may be related to the margination of monocytes or activation of monocytes during the *in vitro* experiments.

5.7.4 Co-cultures of PMVECs and PBMCs from individuals with sepsis

A repeat of the PMVEC co-cultures with PBMCs from healthy controls or patients with sepsis showed no statistically significant changes between monocyte behaviour between the two groups. PBMCs isolated from healthy volunteers, however, did appear to follow the same trend as previously described in chapter 4, with an increase in the classical monocyte subset following co-culture with and without LPS (see section 3.4, page 122). PBMCs isolated from individuals with sepsis did not follow this trend. Interestingly, the intermediate monocyte subset appeared to increase following co-culture with PBMCs from an individual with sepsis and LPS-pre-treated PMVECs.

With regard to monocytic TF expression following co-culture, again no statistically significant changes were seen. Again, the PBMCs from healthy controls appeared to follow the trend seen previously, with TF expression increasing following co-culture. No such trend was seen in the co-culture of PBMCs from individuals with sepsis.

Before any firm conclusions can be drawn, however, further co-cultures would need to be performed.

5.8 Summary

The percentage of monocytes expressing TF within each monocyte subset varies across critical illness, sepsis and recovery from sepsis. To understand the implications this could have on the development of sepsis-associated coagulopathy, further work is needed to measure monocyte TF expression and activity sequentially from the diagnosis of sepsis and beyond initial recovery.

Chapter 6. Discussion

6.1 Overview

Sepsis-associated coagulopathy complicates a significant proportion of sepsis cases and is associated with worse outcomes (Gando *et al.*, 2016; Levi and van der Poll, 2017). To improve the situation, a better understanding of the pathophysiology is needed. TF is the most important *in vivo* initiator of coagulation. This work aimed to review the monocytic expression of TF and investigate its potential to contribute to the coagulopathy of sepsis.

Monocyte subsets have been shown to vary with sepsis. Multiple groups have demonstrated an increase in either the intermediate or non-classical monocyte subsets during sepsis (Herra *et al.*, 1996; Zielger-Heitbrock, 1996; Skrzeczynska *et al.*, 2002; Mukherjee *et al.*, 2015). There are a paucity of data examining monocytic TF expression during sepsis, particularly with regard to the monocyte subsets (Vickers *et al.*, 1998). Comparison of monocyte subsets following an IL-33 stimulus demonstrated a variable ability to express TF (Stojkovic *et al.*, 2017). No work had previously been performed to investigate the TF response on monocyte subsets to LPS or during the model of human endotoxaemia.

This project hypothesised:

1. The classical monocyte subset predominates in the circulation during health. This subset expresses a low level of cell surface TF, which shows little activity.

2. Following infection, there is an increase in the cell surface expression of TF and an increase in its activity.

3. Interaction between monocytes and the endothelium allows a local low-level activation of coagulation.

4. During sepsis, there is a decrease in the proportion of circulating classical monocytes and an increase in the proportion of non-classical. Non-classical monocytes, when compared to the other monocyte subsets, express higher levels of TF, leading to a more pro-coagulant response and predisposing to sepsis-associated coagulopathy.

6.2 The cell surface expression of TF varies between monocyte subsets

The initial results chapter examined the effect of an LPS stimulus on human monocyte subsets *in vitro*. For the first time, the monocyte subsets have been shown to express different levels of TF in response to LPS. My starting hypothesis was refuted, with classical and

intermediate subsets expressing the most TF and the no clear difference in TF activity between the subsets.

The majority of monocyte TF is known to be inactive or 'encrypted'. How it becomes 'decrypted' is yet to be elucidated. The discrepancy between TF surface expression and activity of the subsets suggests that there may be different levels of encryption between the subsets.

The mechanism by which monocytic surface expression of TF is not fully understood. Future analysis of both the transcriptome and proteome following LPS stimulation is needed.

To address the third part of the hypothesis, co-cultures of monocytes and PMVECs were used. Monocyte-endothelial interactions do appear to promote coagulation. Monocytes were induced to express TF when cultured with endothelium, even without the presence of LPS. This effect was not dependent on cellular contact. Further work is needed to investigate what could be mediating this increase in TF. The use of monocyte-endothelial co-cultures limits the ability to identify fully the activation of coagulation. Although an increase in cell surface TF may lead to an increase in coagulation, the creation of fibrin could not be measured in this model.

Co-culture was shown to alter the endothelial fibrinolytic response. Although tPA was unchanged, there was an increase in the expression of PAI-1, an inhibitor of fibrinolysis, following culture.

Together this work suggests that monocytes can influence not just the initiation of coagulation but the fibrinolytic response. Increasingly, the innate immune and coagulation systems have been shown to work cooperatively to help fight infection (Engelmann and Massberg, 2013). This work suggests that monocytes could be central to such cooperation.

6.3 Monocytic TF expression occurs early and transiently following exposure to endotoxaemia

To examine the temporal nature of monocytic TF expression, the human endotoxaemia model was used. Monocytes were shown to increase expression of TF between 90 minutes and 6 hours. Previous studies of the endotoxaemia model have shown an increase in TF mRNA from 4 hours following LPS, but the earlier response seen in this project suggests that there is

preformed TF intracellularly capable of externalising to the cell membrane (Franco *et al.*, 2000).

The human endotoxaemia model causes a transient increase in monocyte TF expression. As mentioned previously, endotoxaemia is a model of systemic inflammation and does not recapitulate all the features of sepsis. Sepsis is not caused by a single insult or virulence factor. Further work is needed to examine whether monocyte TF expression is indeed transient during the development of sepsis.

This project supports work from other groups who identified two phenotypes for monocyte TF expression after administration of LPS, those with a high and those with a low response. Very little is understood about this response or what effect it could have functionally, although high responders have been previously associated with an increased risk of myocardial infarction (Ossterud, 1995). Previously this difference in response has been examined on the monocyte population as a whole. My work has demonstrated that the change in response is consistent across all monocyte subsets. Future work should investigate whether those with a high response are at a higher risk of thrombosis or the development of sepsis-associated coagulopathy. The possibility of identifying those at higher risk of sepsis-associated coagulopathy could allow for more tailored treatments.

6.4 Monocytic TF expression varies during sepsis and recovery

To understand monocytic TF during sepsis better, blood samples were taken from individuals on the critical care unit with a diagnosis of sepsis and, where available, a repeat sample was taken when they recovered sufficiently to leave the critical care. Finding an appropriate control for participants who are critically ill with a diagnosis of sepsis is challenging because of the heterogeneous aetiologies of sepsis. This problem is compounded by the known variation between individuals, with regard to monocytic TF expression. For this reason, the use of recovery samples was a useful control.

Confirming previous work, monocytes were shown to express higher levels of monocyte TF during sepsis when compared to individuals who were critically ill and did not meet the criteria for sepsis (Vickers *et al.*, 1998). This work has demonstrated that this increase in the cell surface expression of TF occurs within the intermediate and non-classical subsets. Both subsets have been shown to be increased during sepsis (Fingerle *et al.*, 1993; Herra *et al.*,

1996; Skrzeczynska *et al.*, 2002; Ziegler-Heitbrock, 2007). Potentially this could contribute to the coagulopathy associated with sepsis and certainly warrants further investigation.

Another intriguing finding of this project was the increase in classical monocyte TF following recovery from sepsis which again has not previously been documented. Most sepsis studies have considered morbidity and mortality only for the first 30 days following diagnosis. Increasingly there is evidence that the risk of poor outcomes continues for much longer in survivors of sepsis. A systematic review of studies investigating the long-term effects showed an increase in mortality for up two years following the sepsis episode (Winters *et al.*, 2010). The ongoing thrombotic risk following an episode of sepsis has not been measured. Investigations into monocyte innate training have demonstrated an increase in the release of pro-inflammatory cytokines up to 12 months following infection (Kleinnijenhuis *et al.*, 2014), see section 1.4.8, page 36). Further work should investigate how long this increase in classical monocyte TF expression continues and its possible consequences for the individual. As discussed earlier (see section 1.3.4, page 16), there is a complex interplay between coagulation and innate immunity. It is hypothesised that sepsis-associated coagulopathy is a consequence of dysregulated thromboinflammation, predisposing individuals with sepsis to both DIC and secondary infections (Levi *et al.*, 1993; Levi and van der Poll, 2010; Levi and van der Poll, 2017). Changes in the monocyte phenotype are able to affect not only the immune response but also the coagulation system. This could explain some of the longer-term morbidity and mortality associated with survivors of sepsis.

6.5 Summary of monocytic TF expression

This work has increased our knowledge of monocytic TF. It has shown that TF varies between monocyte subsets in terms of both expression and activity. Following systemic inflammation, exemplified by the human endotoxaemia model, TF expression increases in all monocyte subsets and does so early, supporting the idea that it is preformed intracellularly and moves to the membrane. There is a change in TF expression on monocytes during sepsis, with the highest levels being found on the intermediate and non-classical subsets. Monocytic cell surface TF expression has been shown to be higher during critical illness with sepsis than in critical illness without sepsis, and to increase on the classical monocyte subset upon recovery from sepsis. These changes in expression are summarised in Figure 6.1.

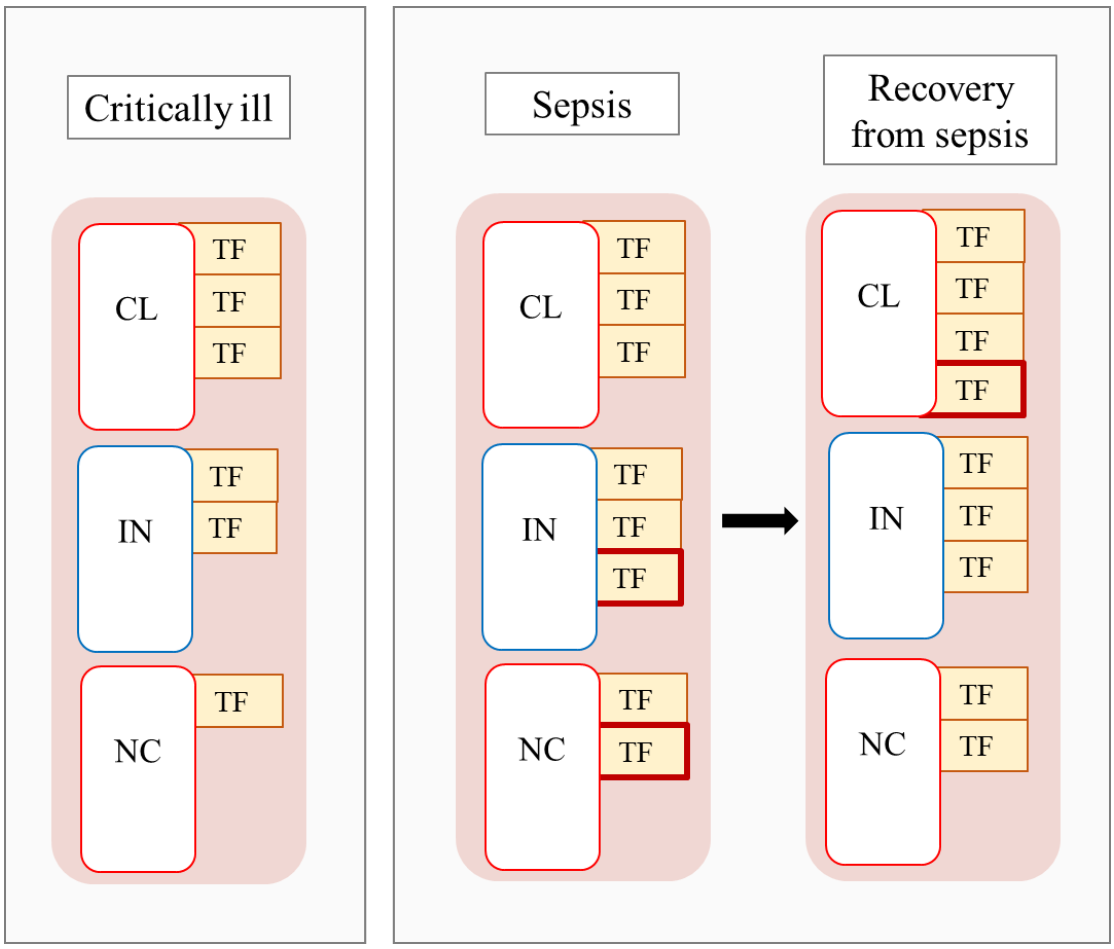

Figure 6.1 The changes in monocyte subset TF expression in different conditions

This figure summarises the changes in TF expression on monocyte subsets. The critically ill population, without a diagnosis of sepsis, show a relative decrease in TF expression on the intermediate and non-classical subsets. During sepsis, TF expression on the intermediate and non-classical monocyte subsets is higher than during critical illness without sepsis. Following recovery from sepsis, TF on classical monocytes is raised above levels seen during health. The thicker red outline represents the increase in TF. (CL – classical, IN – intermediate, NC – nonclassical).

6.5 Strengths

This work has led to many new insights. Prior to this work there had been no comparison of monocyte subset TF expression following LPS stimulation, although there is significant evidence to support a change in the circulating subsets during sepsis (Herra *et al.*, 1996; Skrzeczynska *et al.*, 2002; Mukherjee *et al.*, 2015).

The use of animal models to understand the pathophysiology of sepsis has failed to translate into benefit for patients with sepsis (Fink, 2014). This work has avoided the use of such models by using human cells, samples from individuals with a diagnosis of sepsis and the human endotoxaemia model (which allowed for the temporal investigation of monocyte TF expression). In particular, this allowed an assessment of the high and low monocyte TF responder phenotype over time (Ossterud, 1995).

The use of these three approaches has also allowed for a direct comparison of monocyte TF expression using the same flow cytometry panel run by a single investigator. Inter-user and inter-panel variability were therefore controlled for, minimising problems frequently encountered with regard to flow cytometry studies as a whole, but particularly in measuring TF cell surface expression.

As discussed earlier, choosing a suitable control for individuals with sepsis is challenging. A strength of this work was to use not just samples from the same individuals following recovery from sepsis. This would allow for the variation in response between individuals.

6.6 Limitations

There are also some important limitations of this work. Firstly, TF is only a small part of a much larger and complex process of coagulation that leads to the production of fibrin. This project is focussed on only a small part of this system. The initial response to TF is tempered by multiple downstream effects, especially physiological anticoagulants.

TF itself is also expressed on cells other than monocytes, such as the endothelium. Previous work demonstrates that even a small increase in whole TF can alter the coagulation, the effect of TF expressed on other cell types cannot be discounted (Butenas *et al.*, 2005).

TF expression on monocytes appears to be variable between time-points, disease states, individuals and subsets; it is hard to control completely for such variation. To fully understand monocytic TF expression, further work is needed to measure it at different time-points. The samples from individuals with sepsis were taken at range of time-points following admission to the critical care unit (a range of 1 to 31 days). Future work would benefit from a stricter time limit on recruitment.

Another limitation of this work is that although the TF activity was measured in different monocyte subsets in healthy volunteers, the activity during sepsis was not measured and not measured directly during endotoxaemia. The monocyte surface expression of TF during sepsis has not been demonstrated to be active. TF encryption may also vary significantly during different disease states and it should be examined in the future.

6.7 Future work

To better understand monocyte TF expression during sepsis, I would suggest a case-controlled study performed on the critical care unit comparing critically ill individuals with and without a diagnosis of sepsis, as well a second group of age- and sex-matched healthy controls. Similar to the study of Vickers et al, individuals on the critical care unit should be screened daily to look for signs of sepsis (Vickers *et al.*, 1998). Once a diagnosis of sepsis was made, they could be recruited, and the first sample taken within 12 hours. Later samples could be taken within the first few days and then perhaps at 30 days and 60 days. This would allow for the sequential measurement of monocytic TF expression, TF activity and other markers of coagulation such as prothrombin fragments 1+2 and thrombin-antithrombin complex.

To examine the consequences of the high and low responder phenomenon further, monocytes from individuals who have survived sepsis could be stimulated with LPS. Examination of medical records and blood samples would allow for identification of coagulopathy during their episode of sepsis. A comparison could then be made between their level of response and their risk of sepsis-associated coagulopathy. Samples could also be used to investigate possible mechanisms of high and low monocyte TF response.

Further work is also needed to better understand the mechanisms by which the subsets differ in both TF expression and activity. There is a possibility that the differences between expression and activity are related to different methods or levels of encryption between the

subsets. Work to investigate the lipid profile of each subset would be an interesting way to start this investigation. This project also suggests that the LPS induction of TF transcription may vary between the monocyte subsets. If further work was to prove that to be the case, then this could be potentially modified during conditions such as sepsis.

6.8 Final conclusions

Sepsis-associated coagulopathy is associated with high mortality, and the mechanisms underpinning it are poorly understood. The ability of monocytes to express TF, the main initiator of coagulation *in vivo*, and their interaction with endothelium that increases PAI-1 means they have an important role in thromboinflammation and may contribute to sepsis-associated coagulopathy.

TF expression varies between monocyte subsets. Classical and intermediate monocytes express the highest levels of TF on their cell surface. TF activity between monocyte subsets does not seem to vary. Interestingly, although LPS stimulation was shown to increase TF cell surface expression it did not increase the activity of the cell surface TF. The encryption of TF between monocyte subsets during health and sepsis should be investigated to better understand the contribution of monocyte TF surface expression to sepsis-associated coagulopathy.

The human endotoxaemia model has demonstrated that monocytes increase their cell surface expression of TF early following exposure to LPS. The model also showed a variation in response between individuals to the LPS induction of TF expression on the monocyte surface. This has been demonstrated for the first time across all the monocyte subsets. Future work should investigate whether this may allow for the stratification of individuals with sepsis and correlate with the risk of sepsis-associated coagulopathy.

Different monocyte subsets have been shown to predominate in the circulation throughout sepsis, and this may potentially contribute to the development of sepsis-associated coagulopathy. The cell surface expression of TF has been shown to increase in the intermediate and non-classical subsets when compared to a critically ill individuals without sepsis.

In conclusion, monocyte subsets vary in the cell surface expression of TF and this may contribute to sepsis-associated coagulopathy.

APPENDIX A.

The protocol, participant information sheet and consent form used to collect healthy volunteer blood samples.

PROTOCOL

The role of inflammation in human immunity

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E. Recording and Storage of Data/Samples	4

SECTION A: INVESTIGATORS

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- Dr Thomas Hellyer
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- Dr Fai Ng
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- Mr Jonathan Scott
- Prof Neil Sheerin
- Dr Peter Thelwall
- Dr Christopher Ward
- Dr Jolanta Weaver
- Dr John Widdrington
- Dr Sarah Wiscombe
- Prof Matthew Wright

SECTION B: BACKGROUND

Inflammation is implicated in a wide range of pathological processes including autoimmune disease, fibrosis, cancer, chronic viral infections, and cardiovascular disease. Deficient inflammatory processes are heavily implicated in pre-disposition to infection and in failure of resolution of inflammation. It has long been recognised that the initiation, maintenance, regulation and resolution of inflammation is a complex process, dependent on critical interactions. For example, during an inflammatory reaction neutrophils must interact with: soluble mediators in blood; vascular endothelium; interstitial cells, host-tissue epithelium; soluble mediators at the inflammatory site; other blood-derived cells at the site of inflammation (eg other neutrophils, platelets and mononuclear cells); foreign material (eg bacteria); and any drugs the patient may be taking. Despite recognition of this complexity, there is still a tendency for the human inflammation literature to focus on individual elements of the system. This project is based on the assumption that improved understanding of mechanisms underlying human inflammation can only develop through testing of the interaction between critical components of the system.

Therefore, while the focus is predominantly on the regulation of neutrophil function (ie mechanisms by which bacteria and host factors impair neutrophil function and how these can be restored pharmacologically), the project provides the capacity to dissect interactions between the critical circulating cells and mediators driving inflammation, ie elements of the liquid phase of blood (serum/plasma) and the cellular components of blood (red cells, platelets, neutrophils, monocytes, eosinophils, lymphocytes, dendritic cells).

The investigators have a range of experience providing the optimal skill mix to ensure maximally efficient design of experiments studying interactions between critical components of human inflammation.

The study focuses entirely on the study of the normal elements of human blood participating in inflammatory processes. Therefore research participants for the this study will be healthy adult volunteers.

SECTION C: THE VOLUNTEER'S VISIT – CONSENT AND SAMPLING

Volunteers will be identified through placement of adverts in Newcastle University notice boards, websites and email lists.

Volunteers who express an interest will be sent a Participant Information Sheet and Consent Form, and asked to contact the team again if they wish to take part. The volume of blood requested on a given day may range from 30-240 ml. If a request for a donation of >160 ml is being made, the volunteer will be asked to attend for one screening visit, in order to donate a sample of blood (up to 5 ml) which will be sent to the local laboratory for testing haemoglobin concentration. Such volunteers will be excluded from further study if the haemoglobin concentration is below the normal range.

In addition to low haemoglobin levels (in those donating >160 ml), exclusion criteria will consist of

- blood donation (eg to the Blood Transfusion Service or to research studies) in the previous 90 days.
- donation of >1 litre of blood in the previous year (this equates to around 3 donations to the Blood Transfusion Service in the past year).
- a history of anaemia in the past year.
- age under 18 years.
- any regular, prescribed medication (the oral contraceptive pill is permissible in female participants).

If a volunteer wishes to proceed, Prof Simpson or his personal assistant will check study records to determine if the volunteer has donated blood in the last 3 months (see Section E below). If

not, he/she will be invited to come to the Clinical Research Facility, Royal Victoria Infirmary at a mutually convenient date and time (in almost all cases this will be a weekday morning). It is expected that the intended volume of blood collection will be indicated to the volunteer at this stage.

On the study day a medically qualified member of the study team, or a registered nurse from the study team/Clinical Research Facility will inform the volunteer of the preferred size of blood donation, and will ask for witnessed, written, informed consent from the volunteer. If this is provided, the original consent form will be given to the volunteer to keep, and a copy will be made for retention by the study team (see Section E below). The volunteer should be asked for the name/address of his/her GP.

Blood sampling will be performed in the Clinical Research Facility, Royal Victoria Infirmary. The position most comfortable for the volunteer should be decided, but wherever possible phlebotomy should be performed in a semi-recumbent position, on a couch that has a self-reclining function. Upon completion of phlebotomy, pressure should be applied to the site until haemostasis is secured. If the volunteer has no known allergy to sticking plaster, one should be offered. The volunteer should be offered a carton of fruit juice and/or a drink of water. If the volunteer feels well he/she should be allowed to leave. A book token should be offered to the participant (for the time and inconvenience incurred by the study).

Vasovagal symptoms occasionally accompany phlebotomy. If the participant feels unwell during or after the procedure, phlebotomy should be discontinued, and the couch should be positioned so that the volunteer is supine. Management should proceed on an individual basis, but in the majority of instances the vasovagal symptoms are expected to be self-limiting.

After the volunteer has left, and assuming the appropriate consent has been given regarding ‘information for the GP’, a letter must be sent to the GP informing him/her of the volunteer’s participation. If the procedure has had to be abandoned because of a vasovagal episode or other reason, the GP should be informed of this in the letter.

SECTION D: SAMPLE PROCESSING

Blood samples will be processed in the Institute of Cellular Medicine. Samples will be separated into fluid phase and cellular components. Standard techniques will be involved – typically dextran sedimentation and density gradient centrifugation of anticoagulated whole blood will

be used for this process, yielding red cells, plasma, granulocytes and mononuclear cell fractions. Variations and extra steps will be used as indicated, for example: to prepare serum (eg adding $CaCl_2$ to plasma); to prepare platelets (eg centrifugation of platelet rich plasma); or to isolate specific populations of leukocytes (eg by positive or negative selection using antibody-loaded columns, or by flow sorting). DNA or RNA will be prepared from aliquots using standard DNA or RNA isolation kits.

DNA/RNA will not be used to test for any genes known to provide diagnostic information.

Cells will be used fresh. Liquid phase samples will be stored frozen. Cytospins may be prepared yielding glass slides with cells.

SECTION E: RECORDING AND STORAGE OF DATA AND SAMPLES

A logbook of participants will be kept in a locked drawer and will be accessible only to Prof Simpson (chief investigator), his personal assistant, and regulatory authorities with access to study documents.

The logbook will keep a record of all volunteers expressing an interest in donating blood for the study. The logbook will record any donation made by that individual, along with

- the volunteer's contact details and date of birth
- the name and address of the volunteer's GP (if known and if the volunteer agrees for information to be sent to the GP)
- the date of donation,
- the volume of donation and
- any comments relating to whether the procedure was well tolerated or not.

The logbook will also record the unique study number for each individual's donation. The first 6 letters indicate the study, the next 3 digits the individual's unique number, the last letter will indicate whether it is the individual's first donation or not.

eg ICM-BLD-001.A would be volunteer number 1's first donation

ICM-BLD-001.B would be volunteer number 1's second donation

ICM-BLD-074.D would be volunteer number 74's fourth donation etc.

The unique number will be used to label all samples arising.

Samples will be stored in freezers in the Institute of Cellular Medicine.

Any data held on University computers may only use the unique identifier number, ie no personal data may be used to label samples.

The only information linking the volunteer's identity to his/her samples will be held in the logbook. As described above, this will be held in a locked drawer, geographically distinct from the location of the freezers or university computers on which results are held.

Team investigators will be notified in advance of dates when donations are to be made. They will be invited to contact the researcher performing the blood prep, who will prepare aliquots of plasma / serum / neutrophils / eosinophils/ lymphocytes / monocytes / platelets etc, as appropriate.

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PARTICIPANT INFORMATION SHEET

(for volunteers providing ≤160 ml blood)

PART 1

The role of inflammation in human immunity

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish.

- Part 1 tells you the purpose of this study and what will happen to you if you take part.
- Part 2 gives you more detailed information about the conduct of the study.

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of the study?

Inflammation is the body's way of protecting organs from damage from insults such as infections. However under-active inflammation leaves patients prone to developing infection and over-active inflammation may actually damage the host's organs. Inflammation is now recognised to be a key factor in diseases as diverse as arthritis, cancer, fibrosis (scarring),

strokes, as well as infection. We require a far greater understanding of the processes controlling initiation, regulation and resolution of inflammation if new effective treatments are to be discovered. This project seeks to study the processes of initiation, regulation and resolution of inflammation.

Why have you been chosen?

You have been identified as a healthy individual who may be willing to take part.

Do you have to take part?

No. It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive in the NHS at any time.

How long can you take to decide?

You should take as much time as you wish.

What will happen to you if you take part?

You will visit the Clinical Research Facility at the Royal Victoria Infirmary, where a single blood sample will be taken by a doctor or nurse. Afterwards you will rest for a few minutes, and pressure will be applied to the vein. You will be given water or a carton of juice to drink. This is very similar to advice issued by the Blood Transfusion Service (www.blood.co.uk/giving-blood/what-happens/) which states "Once you have given blood, you should have a short rest before being given some refreshments usually a drink and biscuits." This ends your participation on the day. Your sample will be processed in the lab, dividing it into its constituent parts (various blood cells and the liquid part of blood). These will be used to study the behaviour and function of blood cells.

Are there situations in which you should not take part?

In healthy individuals the bone marrow rapidly replaces blood cells if any bleeding takes place. However we impose limits on when you can provide blood for us. These are any from

- if you have donated blood (eg to the Blood Transfusion Service or to research studies) in the previous 90 days.
- if you have donated >1 litre of blood in the previous year (this equates to around 3 donations to the Blood Transfusion Service in the past year).

- if you have been told by a doctor that you have anaemia in the past year.
- if you are under 18 years of age.
- if you are on any regular, prescribed medication (the oral contraceptive pill is permissible in female participants).

How much blood is taken as part of this study?

You must be aware that the amount of blood we request is more than the usual small samples of blood that you might have previously given at your GP surgery, but less than the size of a donation to the Blood Transfusion Service. Depending on the precise population of blood cells to be studied that day we may take between 30 and 160ml of blood. To place these figures in context, a tablespoon holds approximately 15ml, a typical clinical sample at the GP surgery is about 10ml, a typical 'unit' of blood that people provide for the Blood Transfusion Service is 320ml.

What are the possible disadvantages and risks of taking part?

As with all blood samples, the blood test may leave a small bruise. There is a low risk (<5%) of fainting when blood is taken. The risk is higher if you have a history of fainting in the past and we ask you to let us know in advance of taking blood if this is the case. We have taken steps to minimise any problems associated with the unlikely event of fainting, in that you will be on a reclining chair/bed in a medically supported environment.

What are the potential benefits of taking part?

There is no direct benefit to you.

Is there any reimbursement for taking part?

You will be offered a £5 book token, which is intended to take account of the time and inconvenience of the trip to hospital.

What if there is a problem?

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The detailed information on this is given in part 2.

Will your taking part in this study be kept confidential?

Yes. All the information about your participation in this study will be kept confidential. The details are included in Part 2.

Contact details

Should you wish further information please contact
John Simpson,
Professor of Respiratory Medicine,
Newcastle University
E-mail: j.simpson@ncl.ac.uk
Phone: 0191 222 7770

This completes Part 1 of the Information Sheet. If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making your decision.

PART 2

What will happen if you do not wish to carry on with the study?

You may withdraw from the study at any time. If you choose not to participate in the study now, or at any stage, this will not in any way influence the care you receive from the NHS at any stage in the future.

What if there is a problem?

If you have a concern about your treatment by members of staff during the study, you should ask to speak with the researchers who will do their best to answer your concerns (a contact number for Prof Simpson is at the end of Part 1 of the Information Sheet). If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from your hospital. In addition, the local Patient and Liaison Service (PALS) can provide very useful information if you have any concerns. The PALS website www.pals.nhs.uk/ will provide you with up to date details, but at the time of writing the local contact is

Ms Angie Brown
Patient Advice and Liaison Service
Newcastle Hospitals NHS Foundation Trust
New Victoria Wing
Royal Victoria Infirmary
Queen Victoria Road
Newcastle upon Tyne NE1 4LP
northoftynepals@nhct.nhs.uk
0800 032 0202

In the unlikely event that something goes wrong and you are harmed during the study there are no special compensation arrangements. If you are harmed and this is due to someone's negligence then you may have grounds for a legal action for compensation against the NHS/Newcastle University but you may have to pay for your legal costs. The normal NHS complaints mechanisms will still be available to you.

Will your taking part in this study be kept confidential?

All information that is collected about you during the course of the research will be kept strictly confidential.

Involvement of the GP

With your permission, we shall inform your GP by letter that you have taken part in our study.

Will any genetic tests be done?

We shall retain a sample from which DNA can be prepared. Our study protocol specifically states that **we must not test** for genes known to provide diagnostic information (eg the cystic fibrosis gene, muscular dystrophy genes, or certain cancer genes). Instead we are limited solely to studying the make up of genes that are involved in inflammation or in the way that the body handles certain drugs that interfere with inflammation. None of these genes are used diagnostically.

Will any information and material be stored?

Yes, but only with your permission. Your personal information (your name, date of birth, contact details and your consent form) will be kept in a locked drawer. A unique identifier number allocated to you will be kept with this information in the locked drawer. In this way, your blood samples and any research information held on computers ONLY carry the unique identifier number and not your details. As such, the only people who could potentially identify you from blood samples etc are Professor Simpson or his personal assistant.

Using this system we shall keep information about your health and your medicines on a password protected computer, ie the data will only be linked to your unique identifier number. We propose to store a sample of your blood but this will only be labelled with a study number. In line with Trust policy we propose to keep your information and samples for up to 15 years after completion of the study, at which point we propose to destroy the samples. As medicine advances and new information becomes available, we occasionally find good reasons to perform additional tests on stored samples in the future. Should this situation arise we may use your samples again, but this would be on the strict condition that you could not be identified from the sample except by our research team, and that we obtain fresh and separate permission from an ethics committee.

Can you take part in this study more than once?

Yes, but only under strict condition that you do not breach any of the 'exclusion' criteria (see bullet points in Part A). For example, if we take a blood sample of 80mls or less we may contact you again after 30 days to ask if you would give a further sample. If the volume of blood taken is between 81mls and 160mls we would contact you after 3 months (90 days). This is the *most often* we would ask you to give samples and may be much longer depending on volunteers and your availability. If we took the maximum allowed at each visit this is still less than 1 litre of blood over a year (equivalent to less than 3 blood donations).

What will happen to the results of the research study?

We intend for the results to be published in medical/scientific journals and presented at medical/scientific meetings. All information in the public domain will be anonymous, ie you cannot be identified from these publications/presentations.

Who is funding and organising the research?

The research is funded by Newcastle University.

Who has reviewed the study?

The study has also been reviewed and approved by a local Research Ethics Committee and the regional Research and Development Office.

Is there an independent doctor you can approach for further information?

If you would like to discuss any aspect of this research with an experienced researcher who is not linked in any way to this study, please feel free to contact

Dr Ian Forrest

Consultant Respiratory Physician

Royal Victoria Infirmary

Queen Victoria Road

Newcastle upon Tyne NE1 4LP

You will be given a copy of this Information Sheet and a signed consent form to keep.

Thank you for taking time to read this sheet and for considering taking part.

CONSENT FORM

(for volunteers providing ≤160 ml blood)

Title of Project: The role of inflammation in human immunity

Lead Investigator: Prof J Simpson, Institute of Cellular Medicine

Please initial box

1. I confirm that I have read and understood the information sheet dated ....5/6/13..... (version ....3.....) for the above study. I have had the opportunity to consider the information, ask questions and have had these questions answered satisfactorily. ☐

2. I have informed the study team of any blood donations I have provided (eg to the Blood Transfusion Service) in the last year and of any previous history of anaemia. ☐

3. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

4. I agree that samples prepared from my blood may be stored for up to 15 years after completion of the study. ☐

5. I agree that my samples may be used in future studies on condition that I cannot be identified from those samples and that ethical approval is granted for such studies. ☐

6. I agree that the research team may send a letter informing my general practitioner of my participation in this study. ☐

7. I understand that relevant sections of my records collected during the study may be looked at by individuals from regulatory authorities or from the NHS Trust, where this is relevant to my taking part in this research. ☐

_____	_____	_____
Name of Participant	Date	Signature
_____	_____	_____
Name of Person Taking Consent	Date	Signature
_____	_____	_____
Name of Phlebotomist	Date	Signature

1 copy of this form to be given to participant; 1 for researcher (to be filed in central file)

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APPENDIX B.

The protocol, participant information sheet and consent form used to obtain blood samples from the human endotoxaemia model.

Study Protocol

Does mitochondrial haplogroup predict the inflammatory response in a model of human endotoxaemia? (Does the DNA of our cell's batteries influence our response to bacteria?)

Chief Investigator	Prof John Simpson
Investigators	Dr Anthony Rostron Dr Alistair Roy Prof Muzlifah Haniffa Dr John Widdrington Dr Gavin Hudson Dr Angela Pyle Mr Jonathan Scott Dr Marie-Helene Ruchaud-Sparagano Dr Kathryn Musgrave Julie Furneval Ashley Allan Dr Sarah Wiscombe Dr Patrick Kesteven Prof Patrick Chinnery Prof Paul Corris
Funder	Newcastle University
Sponsor	Newcastle upon Tyne Hospitals NHS Foundation Trust
Protocol	Human endotoxaemia model v1.0 (29/07/2016)

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Dr Alistair Roy, Consultant in Anaesthesia and Intensive Care Medicine, City Hospitals Sunderland Foundation Trust, Sunderland

Prof Muzlifah Haniffa, Wellcome Trust Senior Research Fellow in Clinical Science, Lister Institute Prize Fellow, Senior Lecturer and Honorary Consultant Dermatologist, Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne

Dr John Widdrington, Clinical Research Fellow, Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne

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Dr Angela Pyle, Senior Research Associate, Institute of Genetic Medicine, Newcastle University, International Centre for Life, Newcastle upon Tyne

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Ashley Allan, Band 6 Research Nurse, Integrated Critical Care Unit, City Hospitals Sunderland

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Dr Patrick Kesteven, Consultant Haematologist, Newcastle upon Tyne Hospitals Foundation Trust

Prof Patrick Chinnery, Professor of Neurology and Head of the Department of Clinical Neurosciences, University of Cambridge, Cambridge

Prof Paul Corris, Professor of Thoracic Medicine, Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne

Introduction

Although sepsis is one of the oldest syndromes recognised within medicine, understanding of its biology remains incomplete. The healthcare burden from infection remains substantial. Severe sepsis is found in approximately 2% of hospital admissions in the USA, half of which require treatment in the intensive care unit (ICU)(1). As many as 30% of patients have sepsis on admission or during their ICU stay. The mortality associated with sepsis seems to be substantially higher than that of other syndromes leading to ICU admission (2) and it is the most common cause of death in non-surgical ICUs (3).

Risk factors for the development of severe sepsis are premorbid health status and physiological reserve of the host, causative organism, timeliness of intervention and host genetic characteristics. Gram-negative infections account for a greater proportion of severe sepsis than Gram-positive infections (4) and they are also associated with a worse outcome (5). Studies exploring genetic factors have concentrated on polymorphisms in nuclear genes involved in the innate inflammatory response (6), coagulation and fibrinolysis (1).

Impaired cellular respiration is important in the development of multi-organ dysfunction in severe sepsis (7). Natural variation in mitochondrial DNA (mtDNA) is an understudied area

likely to yield insights into how the host genome may influence outcome in severe sepsis. Human mtDNA is maternally inherited and codes for 13 essential protein components of the mitochondrial respiratory chain. Individuals can be divided into mtDNA haplogroups on the basis of specific single nucleotide polymorphisms (SNPs) in their mitochondrial genome. The most common subdivision of mtDNA in Europe, haplogroup H, is associated with enhanced oxidative phosphorylation and is a strong independent predictor of survival after admission to the ICU with severe infection (8). The frequency of haplogroup H is approximately 40% in North East England (9).

Human survival from infection requires an appropriate inflammatory response: an unbalanced, hyperinflammatory response predisposes patients to overwhelming inflammation, while protracted immunosuppression is associated with organ dysfunction and heightened risk of nosocomial and opportunistic infections (10). An observational study performed in Newcastle suggested that patients with haplogroup H had a survival advantage, and higher fevers, when compared with non- H haplogroups (8). Recent data from our laboratory (manuscript in preparation) suggest that mitochondrial genes regulate the expression of TLR-4 and triggering receptor expressed on myeloid cells type 1 (TREM1) on human monocytes through interferon gamma-dependent pathways, which in turn influences the early inflammatory response to lipopolysaccharide (LPS).

Previous research on sepsis has focused on isolated mediators using a reductionist approach often derived from animal models (11). A genome wide study has questioned the validity of using murine models (12) whilst experimental human endotoxaemia shows much greater correlation with transcriptomic changes due to inflammatory stresses in human disease (13). Injection of LPS, a non-infectious Gram-negative bacterial cell-wall product, is a well-recognised, safe, investigational technique that has been used experimentally for over 50 years(14). The dose used is adjusted to body weight to ensure a standardised reaction in all participants. While we recognise that administration of low dose endotoxin is not a clinical model of sepsis, it does represent a significant improvement in experimental modeling over animal studies. Human endotoxaemia reproduces the earliest features of the pathogenesis of sepsis (which are almost impossible to study in the clinical setting), paving the way to define mechanisms of pathogenesis, and “drugable” targets.

The prevailing frequency of haplogroup H in the general population and the human endotoxaemia model together provide a unique opportunity to begin to explore the contribution of mitochondrial function to the early inflammatory response in sepsis. The aim of this seed application is therefore to test the hypothesis that ***mitochondrial haplogroup H leads to a more pronounced pro- inflammatory response to intravenous lipopolysaccharide in a model of human endotoxaemia.***

Experimental Plan using the Human Endotoxaemia Model

The human intravenous endotoxaemia model has been used for more than 50 years as a model of acute systemic inflammation, encountered in conditions such as sepsis and trauma. Low dose purified lipopolysaccharide (LPS, also referred to as endotoxin) from the cell membrane of *Escherichia coli* (*E.coli*) is administered to healthy volunteers resulting in transient, flu-like symptoms, and an acute systemic inflammatory response, which, at least partially, mimics the inflammatory response of early sepsis. The doses needed in humans to mimic the clinical entity of severe sepsis are ethically unacceptable.

The effects of Good Manufacturing Practice (GMP) grade LPS from *E.coli* are highly reproducible (15). Within an hour of the intravenous administration of LPS, volunteers experience varying degrees of flu-like symptoms e.g. chills, headache, myalgia , nausea, photophobia and sleepiness. In general the response is dose-dependent. Most subjects only experience symptoms for about two to six hours. The core temperature increases within one hour of administration and peaks at three to five hours and meets in the SIRS criterion for fever in most participants. Any drop in blood pressure is prevented by the administration of intravenous fluid, but an increase in heart rate of 20bpm within the first four hours is consistently seen. No severe cardiovascular complications resulting from endotoxin administration have been reported (15). Whilst, in some subjects the respiratory rate and minute ventilation increases with administration of high doses of endotoxin (4ng/kg), this is less often seen using lower doses (14). The change in white blood cell count is a little more delayed, but in most participants the peak is seen by nine hours and fulfilling the SIRS leukocytosis criterion.

Our group has considerable previous (16) and on-going experience with LPS challenge studies. Our close liaison with van der Poll's group in Amsterdam and Gilroy's group in

London give us confidence that we can safely extend our investigations to an intravenous model of human endotoxaemia using GMP grade endotoxin from the NIH endotoxin repository.

Following ethical approval, we shall recruit 30 healthy, non-smoking, volunteers aged 18 - 40. The sample size is pragmatic (given the novelty of this experimental design), informed by a previous observational, cohort study and by the distribution of haplogroups within the population (8). We shall dichotomise the study cohort into H and non-H haplogroups for analysis. We will attempt to recruit as many volunteers as possible up to a maximum of 30, but believe that analysis of data from 12 volunteers will be informative.

We will advertise the study at Newcastle University using mechanisms that have served us well in current and previous inhaled LPS studies. Participants providing informed, written consent will undergo an initial health screen and will be required to have no significant medical history or recent febrile illness, as well as normal physical examination, electrocardiography, oxygen saturation, full blood count, urea and electrolytes, and liver function tests. Blood will be drawn for determination of mitochondrial haplogroup, but we will be blinded to the results until study completion.

Volunteers will be asked to refrain from caffeine or alcohol for 24 hours before the study and will spend a full day (10 hours) in the Intensive Care Unit at City Hospitals Sunderland. Vital signs (pulse rate, blood pressure, temperature, respiratory rate and oxygen level) will be monitored at baseline and every 30 minutes throughout the day. A venous line will be inserted into each arm. Systemic inflammation will be induced by intravenous injection of a bolus of 2ng/kg of U.S reference *E. coli* endotoxin made available by the National Institute of Health (Bethesda, USA). Doses of up to 4ng/kg have been used but the inflammatory response appears to be similar whether 2ng/kg or 4ng/kg is used (15), Therefore in an attempt to reduce any unpleasant symptoms for volunteers the lower dose will be used. Participants can eat and drink at any point following injection of endotoxin. 20ml of blood will be drawn at baseline and at 90 minutes, 4, 6 and 10 hours. Volunteers will attend for blood sampling and follow-up the day after the study and then 7 days following the study.

Objectives

Primary Objective

The primary objective is to test the hypothesis that mitochondrial haplogroup predicts the inflammatory response in a model of human endotoxaemia, the primary outcome measure used will be the TNFα level 90 minutes after endotoxin administration.

Secondary Objectives

(i) To determine the expression of HLA-DR, TLR4 and TREM-1 by monocyte subsets in different haplogroups.
(ii) To determine the release of reactive oxygen species in blood in different haplogroups.
(iii) To determine the profile of additional proinflammatory and anti-inflammatory mediators in plasma in different haplogroups.
(iv) To determine mitochondrial DNA (mtDNA) copy number in plasma in different haplogroups.
(v) To determine the changes in leukocyte dynamics and function in response to intravenous LPS.
(vi) To measure the clinical response (temperature, pulse rate, blood pressure, respiratory rate and oxygen saturation) to endotoxin in different haplogroups.

Study Design

Participant Enrolment and Selection

An advert will be placed on Newcastle University email lists and notice boards. Potential participants will be asked to make contact with the research team only if they consider themselves to be healthy. Interested individuals will be invited to contact the research team who will send out information on the study (participant information sheets and screening consent form). Participants sent such information will be invited to contact the research team to arrange a screening visit (see below) or to decline participation. If no reply is received after 2 weeks, the research team will telephone the volunteer as a reminder.

Screening Visit

Healthy volunteers will be recruited from within Newcastle University. The screening visit will take the form of:

- a short history
- vital signs measurement (temperature, pulse rate, blood pressure)
- measurement of oxygen saturation breathing room air
- cardiorespiratory examination
- electrocardiogram
- blood sample for full blood count
- blood sample for urea & electrolytes assay, liver function tests and C-reactive protein
- urinary pregnancy test in women

Eligibility will be based on the results of this and inclusion and exclusion criteria (see inclusion/exclusion criteria).

Setting

All volunteers will be recruited through advertisement within Newcastle University. Volunteers will be screened at the preassessment clinic at City Hospitals Sunderland Foundation Trust. If found eligible for the study, they will be given time to consider if they wish to continue to study entry (minimum 24 hours) and invited to re-attend on a set day. Participants will be asked to attend the integrated critical care unit (ICCU, City Hospitals Sunderland Foundation Trust) for LPS administration. Downstream preparation and analysis of samples generated by the study will be performed in the Simpson lab, in the flow cytometry facilities (Centre for Life and Institute of Cellular Medicine), in the Wellcome Trust Centre for Mitochondrial Research (Centre for Life, eg for assessment of mitochondrial DNA), all Newcastle University. Processing of screening blood tests and routine clinical tests following administration of LPS will take place in the clinical laboratories at City Hospitals Sunderland.

Study Population

30 healthy participants will be recruited.

Inclusion criteria

- Healthy adult volunteers aged between 18 and 40 years of age
- Able to give informed consent

Exclusion criteria

A volunteer will not be eligible for inclusion in the study if any of the following criteria apply at entry:

1. Age <18 or >40 years.
2. Needle phobia.
3. Current participation in a clinical trial.
4. Known history of mitochondrial disease.
5. Past history of chronic respiratory disease.
6. Past or current history of conditions known to affect immunity or cardiac function (e.g. diabetes, ischaemic heart disease, congenital heart disease, valvular heart disease, cirrhosis, chronic renal impairment, recurrent urinary tract infection).
7. Known history of immunodeficiency.
8. Known history of hepatitis B/C or HIV.
9. History of an acute intercurrent cardiorespiratory illness.
10. Pregnant or breastfeeding.
11. Any current medication (except oral contraceptive pill).
12. Current history of smoking.
13. Reported alcohol intake >21 units per week.
14. Abnormal physical signs detected at cardiorespiratory examination.
15. Temperature >37.3 degrees celsius.
16. Oxygen saturation <95% breathing room air.
17. Haemoglobin outside the laboratory reference range.
18. Platelet count less than 100 x 10∧9/l or greater than 650 x 10∧9/l.
19. Total white cell count outside the laboratory reference range.
20. Any deviation of greater than 20% from normal in the differential white cell count.
21. Serum sodium, potassium, creatinine outside the laboratory reference range.

22. Blood urea greater than 10mg/dl.
23. Bilirubin greater than 30micromol/l.
24. Alanine transferase greater than twice the upper limit of the laboratory reference range.
25. Allergy to the any of the constituents of Hartmann's solution.
26. Currently participating in a clinical trial that the chief investigator feels would interfere with the analysis carried out as a result of this study.

Consent

All eligible volunteers will be given written and verbal information regarding study participation. All will be asked to give written consent with a minimum 24 hours to consider entering the study. Consent will be taken by Dr Rostron or Dr Roy. Pseudoanonymised data (linked by a unique study code) will be entered on an excel database.

Ineligible and non-recruited participants

For volunteers found to be ineligible at screening, or eligible but not subsequently entered into the study, the reason for ineligibility or non-recruitment will be entered on the excel database. Only anonymised data will be entered on to the database and this will include gender, age, “ineligible” or “non-recruitment” and the associated reason. If found ineligible, permission will be sought from participants to contact their GP with the results of screening tests and any further action required.

Study visit 1 (day 0)

Volunteers satisfying study criteria, wishing to proceed with the study, and providing written informed consent will attend the integrated critical care unit (ICCU), City Hospitals Sunderland on an agreed day. Volunteers will be asked to refrain from caffeine or alcohol for 24 hours before the study and during study visit 1. Volunteers will spend a full day (10 hours) in the Intensive Care Unit at City Hospitals Sunderland. A brief history (regarding symptoms of any acute illness) will be sought. In female participants, a repeat urinary pregnancy test will be performed and will be reviewed by a medical practitioner who will offer appropriate

advice. Only those participants who have a negative test will be allowed to proceed with the study. Vital signs (pulse rate, blood pressure, temperature, respiratory rate and oxygen level) will be monitored at baseline and every 30 minutes throughout the day. A venous cannula will be inserted into each arm. Intravenous fluid (Hartmann's solution, a solution with concentration of salts similar to that of blood) will be administered via one of the intravenous cannulae. Systemic inflammation will be induced by intravenous injection of a bolus of 2ng/kg of U.S reference E. coli endotoxin into the other intravenous cannula. Participants can eat and drink at any point following injection of endotoxin, but will be asked to refrain from caffeine and alcohol during the day of study visit 1. 80ml will be drawn at baseline, 90 minutes, 6 and 10 hours. 20ml of blood will be drawn at 4 hours following injection of endotoxin. Volunteers are advised to use a suitable method of contraception for 48 hours following injection of LPS.

Study visit 2 (day 1)

Volunteers will be asked to attend the ICCU at City Hospitals Sunderland. All will undergo a brief history, examination and blood sampling (20mls).

Study visit 3 (day 7)

Volunteers will be asked to attend the ICCU at City Hospitals Sunderland. All will undergo a brief history, examination and blood sampling (20mls).

Study Assessments: processing and analysis of blood samples

Screening visit blood samples will be sent to the hospital laboratories at City Hospitals Sunderland for assessment of full blood count, urea and electrolytes, liver function tests and C-reactive protein (CRP) (8mls). A sample will also be taken for mitochondrial haplogroup typing (2mls) which will be performed at the Centre for Life, Newcastle University

Blood samples will be taken at baseline, 90 minutes, 4, 6 and 10 hours after administration of intravenous LPS. Blood sample volume that will be drawn will be approximately 80mls will be drawn at baseline, 90 minutes, 6 and 10 hours.

20mls at 4 hours. Full blood count analysis and measurement of serum CRP and procalcitonin (PCT) will be done in the hospital laboratories at City Hospitals Sunderland NHS Foundation Trust. All other sampling will be performed at Newcastle University. Assessment of free mitochondrial DNA (mtDNA) will performed at the Centre for Life, all other scientific processing and analysis will be undertaken in the Medical School, Newcastle University.

Sampling at baseline, 90 minutes, 6 and 10 hours:

- Full blood count (2mls)
- Serum (9 mls) for CRP, PCT, cytokines and free mtDNA
- Flow cytometry (4 mls)
- Prothrombotic markers (5mls)
- Transcriptome (RNA sequencing), epigenome (ATAC sequencing) and proteome analysis (mass cytometry) of peripheral blood leukocytes (60mls) isolated by flow cytometry.

Sampling at 4 hour, 24 hour and 7 day time points:

- Full blood count (2mls)
- Serum (9 mls) for CRP, PCT, cytokines and free mtDNA
- Flow cytometry (4 mls)
- Prothrombotic markers (5mls)

The total volume of blood sampled over the study period (390ml) will be similar to the volume of blood drawn for the donation of blood. Scientific samples will be stored in a locked freezer, in swipe-card protected premises in the Institute of Cellular Medicine. Freezers are accessible to the research team, and to individuals who maintain university research freezers and their governance. Samples will be destroyed after five years, unless we believe there is advantage to keeping them, in which case we will take advice from sponsor as how to proceed.

Data Analysis

Data from analysis of blood samples will be compared between haplogroup H and non-H haplogroups for all time-points along with changes from baseline.

Risk Assessments

Blood sampling

Blood sampling can be accompanied by discomfort or by vasovagal symptoms. Risks are minimised through all samples being taken in a fully supported medical facility. Blood will be drawn whilst the volunteer is positioned on a bed or in a self-reclining chair. Volunteers feeling syncopal will be positioned supine and venepuncture will be discontinued. The total volume of blood to be sampled over the entire study is less than the volume required for a standard blood donation.

Intravenous administration of LPS

LPS will be administered to volunteers whilst they are monitored in the integrated critical care unit (ICCU) at City Hospitals Sunderland. Participants will be observed for 10 hours following LPS administration.

Expected symptoms include chills, headache, photophobia (aversion to bright lights), myalgia (muscle aches), arthralgia (joint pains), nausea (feeling sick), and rarely, vomiting. Peak symptom intensity occurs around 1 - 2 hr post-injection, abating afterwards to baseline by 6 - 8 hr. No severe or sustained adverse
effects secondary to endotoxin at this dose have been reported. Less than 5% of volunteers feel the need to treat these symptoms with medicines such as paracetamol/acetaminophen or non-steroidal agents by mouth or IV (e.g., aspirin, ibuprofen). Such agents may alter the inflammatory response and their use should be recorded. Other adverse signs include fever, increase or decrease in heart rate and decrease in blood pressure (hypotension), medications required to treat adverse effects will be readily available (e.g. intravenous fluid in the event of a significant episode of hypotension or paracetamol in the event of significant symptoms of fever). Significant hypotension only occurs rarely (less than 1 in 1000 volunteers) and is rapidly treated by the administration of an intravenous fluid bolus.

Vital signs will be measured at baseline, then every 30 minutes until six hours following LPS administration and then hourly thereafter until participants are allowed home. Symptoms scores for nausea, muscle aches, headache and chills will be recorded.

3. Burden of time

Volunteers wil be required to attend for a screening visit, a ten hour study day for induction of inflammation, observation and sampling, followed by two further study visits for follow-up and sampling. Volunteers will required to attend the Integrated Critical Care Unit of City Hospitals Sunderland for study visits. The host institution and follow-up study visits will facilitate safety monitoring for volunteers. In recognition of the burden on the volunteer in terms of time, volunteers will be offered £250 on study completion.

Adverse Events

Our research group has experience with the LPS challenge models. We have successfully administered 60µg of inhaled LPS to young, healthy participants with no significant adverse events recorded (16).

Intravenous LPS has been administered to healthy volunteers for over 40 years. There is a substantial body of evidence to support the safe administration of higher doses (4ng/kg) of intravenous LPS than we intend to use in this study.
Nevertheless, we feel that we must remain vigilant in detecting and recording any adverse events as a result of exposure to LPS or other procedures undertaken. Whilst recognising that this current work is not a clinical trial, our groups' previous work (which included a clinical trial) benefited from classifying and monitoring adverse events in the manner described below, and we have elected to continue using this terminology in this research.

Definitions
An ***adverse event*** (AE) is any untoward medical occurrence in a study participant.
A ***serious adverse event*** (SAE) is any untoward medical occurrence in a study participant or effect that:

- results in death
- is life threatening (i.e. the subject was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe)
- requires admission to hospital as an in-patient beyond one calendar day or requires the volunteer to stay longer than 12 hours following injection of lipopolysaccharide
- results in persistent or significant disability or incapacity
- is a congenital anomaly or birth defect

If an adverse event is detected, a member of the research team will make an assessment of seriousness as defined by the above definitions. If the event is deemed to be serious (SAEs) a member of the research team will then consider if the event was:

- **Related** – that is it resulted from administration of research procedures and/or
- **Unexpected –** that is a type of event that is not identified as an expected occurrence

Detecting and reporting AE and SAEs

All AEs and SAEs will be recorded from the time a participant consents to join the study until 24 hours after completing the final study assessments. A medically qualified member of the research team will ask about the occurrence of AEs/SAEs during the study. Information to be collected includes type of event, onset date, researcher assessment of implications, if any, for safety of participants and how these will be addressed, date of resolution as well as treatment required, investigations needed and outcome. All information will be recorded in the participants study file.

An AE/SAE may necessitate discontinuation of a given part of the study (but progression through the remainder of the study) or complete and immediate discontinuation of any further participation. All participants will maintain the right to discontinue or completely withdraw from the study at any time for any reason, or without stating a reason. The reason and circumstances for premature discontinuation (where known) will be documented in the participant's study file.

If a SAE has occurred, the research team must report the information to Newcastle upon Tyne Hospitals R&D within 24 hours. The SAE form must be completed as thoroughly as possible with all available details of the event, signed by the Investigator or designee. The SAE form should be transmitted by fax or by hand to the office.

NUTH R&D is responsible for reporting SAEs that are considered to be related and unexpected as described above to the Research Ethics Committee (REC) that approved the study (main REC) within 15 days of becoming aware of the event using the NRES Reporting of SAE Form. The Co-ordinator of the main REC should acknowledge receipt of related, unexpected safety report within 30 days.

Discharge criteria

The attending clinician will ensure that the participant's symptoms have settled and that their observations are trending to normal (all altered parameters, e.g., elevated heart rate and temperature, demonstrating consistent reduction toward baseline values) prior to sanctioning the end of observation and subsequent discharge. After bolus injection of 2ng/kg LPS symptoms normally fully abate by 6 - 8 hr. Individual observations follow overlapping but discrete time-courses. These have normally returned to baseline by 10hr. All monitoring and venous cannulae will be removed and haemostasis will be ensured. It will be confirmed that the participant is happy to be discharged home and has the contact details of the research team in case of any concern.

End of study

The study will be completed on day 7 of the final volunteer. Scientific analysis of samples in accordance with the experimental plan may be performed after this.

Process if new information is available

If any further information becomes available that leads to further studies then consent will be obtained from volunteers and ethic approval will be sought.

Criteria for terminating the project

In the event of a serious adverse event, the project will be terminated.

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Does the DNA of our cell's batteries influence our response to bacteria?

PARTICIPANT INFORMATION SHEET

HELPING YOU DECIDE WHETHER TO ENTER THIS STUDY

INTRODUCTION

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish.

- Part 1 tells you the purpose of this study, outlines what will happen to you if you take part, and informs you about the conduct of the study.

- Part 2 gives you more detailed information about what will happen to you if you take part.

Please ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

PART 1

What is the purpose of the study?

Sepsis (previously known as septicaemia or blood poisoning) is a common medical emergency. It is severe, life-threatening infection. Internationally, it claims the lives of eight million people every year, 44000 of which are in the United Kingdom. Sepsis arises when the body's response to an infection, the inflammatory response, injures its own tissues and organs. It can lead to shock, failure of the body's organ and death especially if not recognized early and treated promptly. The proportion of people who die when they have sepsis is greater than that of other causes of admission to critical care. One of the major difficulties is that we have limited understanding of the body's early response to infection.

In order to function, the body's organs are dependent on cellular energy production. A major source of energy is the cell's mitochondria, they are the batteries for the body's organs. The efficiency of these batteries is determined by the DNA (mitochondrial DNA) that codes for the mitochondrial proteins that make up the energy producing apparatus.

This study seeks to determine if an individual's mitochondrial DNA also has an effect on the inflammatory response to a purified bacterial cell wall component that stimulates a mild, transient inflammatory response.

All studies will take place in the Integrated Critical Care Unit at City Hospitals Sunderland, using established methods.

Why have you been chosen?

You have been chosen because you are a healthy individual who has responded to the e-mail we sent out to Newcastle University mailing lists or to adverts placed on university notice boards.

What will happen to you if you take part?

You will attend for a screening visit. If the screening visit is satisfactory and you want to proceed to the study you will then be asked to return for 3 separate study visits, over a week. The procedures we would like you to have are:

- Blood samples. Some of the samples will be used to measure the numbers of cells in the blood and the levels of markers of inflammation. They will be analysed in the clinical laboratories in City Hospitals Sunderland. The remainder and the majority of the blood

samples will be transferred by the research team to Newcastle University. They will be used to assess mitochondrial DNA in the blood (this will occur at the Centre for Life, Newcastle University) and which populations of the cells of the immune system change and how they change in response to LPS (this will occur at the Medical School, Newcastle University)

- Intravenous injection of lipopolysaccharide (LPS). LPS is a product made by certain bacteria and is just one small component of the bacterium – i.e. you would not be injected with actual bacteria that may cause an infection but a small, non-infectious component of the bacterium. The aim is to cause a mild, transient inflammatory response in the blood, which will result in temporary flu-like symptoms (typically lasting up to six hours).

Greater detail about each of these is found in Part 2.

Do you have to take part?

No. It is up to you to decide whether or not to take part.

You are free to withdraw from the study at any time and without giving a reason. A decision not to take part, or withdraw at any time, will not affect the health care you receive at any stage, now or in the future.

How long can you take to decide?

You should take as much time as you wish.

Do you have to complete all of the tests described?

While we obviously prefer to obtain all of the samples described, you are under no obligation to have any of the tests. You can complete all of the tests, or you can decline as many of the tests as you wish.

Involvement of your GP

With your permission, we shall inform your GP by letter that you are taking part in our study. With your permission, should any abnormal clinical results emerge during your tests, we would inform you and your GP.

Will any genetic tests be done?

Yes. Part of our research aims to determine how 'mitochondrial DNA' behaves in response to inflammation. Mitochondria are the 'batteries of cells', providing the cell with energy. Some research studies have suggested that mitochondrial DNA in white blood cells may respond differently during inflammation. We seek to test this in more detail in the current study. We shall not, at any point, be testing DNA for genes associated with specific medical conditions.

What are the potential benefits of taking part?

There is no direct benefit to you.

Is there any reimbursement for taking part?

Yes. We shall reimburse any travel costs. There is no reimbursement for attending the screening visit, but we will offer a payment of £250 for completing the subsequent study days. You are not obliged to take this if you do not wish to.

Can you access the results of the research?

Yes. We will be happy to send you a summary of the overall results of the study after its completion, should you so wish. If you would like us to do this, please contact Professor Simpson at the address shown below.

What will happen to the results of the research study?

We intend for the results to be published in medical/scientific journals and presented at medical/scientific meetings. All information in the public domain will be anonymous (i.e. you cannot be identified from these publications/presentations). We intend that the results of the study will inform the design of future studies and treatments for people with sepsis.

What if there is a problem?

If you have a concern about your treatment by members of staff during the study, you should ask to speak with the researchers who will do their best to answer your concerns (see contact details below for Professor Simpson). If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from your hospital.

In the unlikely event that something goes wrong and you are harmed during the study there are no special compensation arrangements. If you are harmed and this is due to someone's negligence then you may have grounds for a legal action for compensation against the NHS/Newcastle University but you may have to pay for your legal costs. The normal NHS complaints mechanisms will still be available to you.

Will any material be stored?

Yes, but only with your permission. We propose to store the liquid portion from blood samples (plasma/serum), some of the cells and their products and the mitochondrial DNA. These will be kept in an anonymised form (i.e. you cannot be identified from the samples). We propose to keep your samples for up to 5 years after completion of the study.
At 5 years we propose to destroy the samples and images. However, as medicine advances and new information becomes available, we occasionally find good reasons to perform additional tests on stored samples in the future. Should this situation arise we may ask to use your samples again, but this would be on the strict condition that we explain the nature of any further research we intend to carry out, the type of tests we wish to perform and that you give permission for this, that the information would be anonymous (i.e. you could not be identified from them) and that we obtain new and separate permission from an ethics committee.

Will your taking part in this study be kept confidential?

Yes. Your information and samples will be given a study identity number, and all the information would remain strictly confidential.
All information that is collected about you during the course of the research will be kept strictly confidential. We will keep a record that you have taken part in the study (name, date of birth, email address) but will not keep any other personal information about you save that required in the medical screening. All documents, samples and information about you will be pseudo-anonymized with a study code known only to the research team. Professional standards of confidentiality will be adhered to and the handling, processing, storage and destruction of data will be conducted in accordance with the Data Protection Act (1998). Ultimately the information we acquire may be published in journals or presented at conferences but it will not be possible to identify you or any other participant from these publications.

Who is organising and funding the research?

The research team is made up of clinicians and staff from Newcastle University, City Hospitals Sunderland NHS Foundation Trust and Newcastle Upon Tyne Teaching Hospitals NHS Trust. The research is funded by Newcastle University.

Who has reviewed the study?

The scientific basis of the study was independently reviewed by doctors/scientists at Newcastle University. The study has also been independently reviewed and approved by a local Research Ethics Committee and the regional Research and Development Office.

Is there an independent doctor you can approach for further information?

If you would like to discuss any aspect of this research with an experienced researcher who is not linked in any way to this study, please feel free to contact:

Dr Stephen Wright
Consultant in Anaesthesia and Intensive Care Medicine
Freeman Hospital
Freeman Road
NE7 7DN
Tel: 0191
stephen.wright@nuth.nhs.uk

This completes Part 1 of the Participant Information

If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making your decision.

THANK YOU FOR TAKING THE TIME TO READ THIS INFORMATION AND FOR CONSIDERING TAKING PART

Does the DNA of our cell's batteries influence our response to bacteria?

PARTICIPANT INFORMATION SHEET

PART 2

What will happen to you if you take part?

You will attend for a ***screening visit*** to see if you satisfy all the necessary conditions for the study. If you continue to take part you would come back at a later date for 3 separate ***study visits.***

Screening visit

On a day suitable to you, you would come to the Pre-assessment clinic at City Hospitals Sunderland. The purpose of this visit is to check that you do not have any features which might exclude you from our study, and to answer any questions you may have. Please also note that you should not be taking part in any other medical research studies at the time.

A doctor from the research team will take a short medical history and examine your heart and lungs.

You would also have:

- Simple measurements of heart rate, blood pressure and temperature
- Measurement of the oxygen level in the blood (this involves placing a probe on your finger and does not involve needles)
- A blood sample taken to check your 'full blood count' (which tells us about your white blood cells) and blood biochemistry. The volume of blood needed is equivalent to two teaspoons.
- For female participants a urine test to exclude pregnancy. The result will be reviewed by a medical practitioner and appropriate advice will be given.

The information obtained will be kept confidential by the research team. We shall inform you if we find any unexpected abnormalities and, with your permission, inform your general practitioner. If we are satisfied that there are no reasons to prevent you taking part, and if you still wish to participate in the study, you will be asked to return for the actual study.

Study visits in Summary

Study Visit 1:

Venue: Integrated Critical Care Unit (ICCU) City Hospitals Sunderland

Time: 11 hours

You will be asked to refrain from alcohol and caffeine for 24 hours before this visit. You will also be asked to fast from midnight before injection.

- Confirmation of consent
- Pregnancy test if female to confirm that the volunteer is not pregnant. The test will be reviewed by a medical practitioner and appropriate advice will be given.
- Brief medical history and examination of heart and lungs
- Insertion of intravenous cannulae, one in each arm
- Baseline blood tests
- Administration of intravenous fluid
- Administration of lipopolysaccharide into a vein through a cannula
- You will be allowed to eat and drink following lipopolysaccharide injection but asked to refrain from caffeine and alcohol during study visit 1. You will be allowed to bring your own food with you, alternatively sandwiches will be provided at meal times.
- Observation over the following 10 hours, within ICCU, City Hospitals Sunderland (including measurement of blood pressure, heart rate, temperature and oxygen level)
- Blood tests taken at 90 minutes, 4 hours, 6 hours and 10 hours after administration of LPS (blood tests will be taken from a cannula placed in your vein at the baseline blood test, so such that we can take blood more than once from a single use of a needle).

Study visit 2: the day after visit 1

Venue: Integrated Critical Care Unit (ICCU) City Hospitals Sunderland

Time: Approximately 30 minutes

- Brief medical history and examination of heart and lungs
- Blood test

Study visit 3: 7 days after visit 1

Venue: Integrated Critical Care Unit (ICCU) City Hospitals Sunderland

Time: Approximately 30 minutes

- Brief medical history and examination of heart and lungs
- Blood test

At the end of this visit, your involvement in the study will be complete.
A more detailed explanation and potential symptoms associated with each procedure are described in the next section.

Blood sampling

At the screening visit we shall take a single blood sample using a needle. The amount of blood on the screening day will be approximately 10mls.

On Study visit 1, you will have a cannula placed in each arm. One will be for administration of intravenous LPS, the other will be for administration of intravenous fluid and multiple blood samples. Blood will be taken at baseline, then at 90 minutes, 4 hours, 6 hours and 10 hours after the administration of intravenous LPS. The volume of blood we require at baseline, 90 minutes, 6 hours and 10 hours will be 80mls (about one third of a cup full) and the volume we require at 4 hours will be 20ml (about a tablespoon full) The total volume that we require during study visit 1 will be approximately 340ml

On study visit 2, we shall take a single blood sample using a needle. The amount of blood on the screening day will be approximately 20mls (about a tablespoon full).

On study visit 3, we shall take a single blood sample using a needle. The amount of blood on the screening day will be approximately 20mls (about a tablespoon full).

Therefore, the maximum amount of blood that a volunteer can be asked to provide over the course of this entire study (including screening visit) is approximately 390mls. To place this in context, this is similar to a standard blood donation to the Blood Transfusion Service..

Intravenous injection of LPS

LPS is a product made by certain bacteria and is just one small component of the bacterium – i.e., you would not be injected with actual bacteria that may cause an infection but a small, non-infectious component of the bacterium. The dose of LPS we use is 2 nanograms per kg bodyweight. (A nanogram is 1 x 10^{-9}g and is the mass of an average human cell, and the human body is made up of trillions of human cells). This dose has proved to be extremely

informative and safe in previous studies. We would be pleased to supply you with further literature from such studies if you wish. Your pulse, blood pressure, temperature and oxygen levels will be recorded after the injection of LPS..

The LPS is used to cause a mild inflammatory response in the blood for a few hours only. Volunteers may feel slightly tired or have a warm, flushed or 'flu-like' feeling a few hours afterwards. They may also feel like their heart rate has increased or decreased. Some but not all of volunteers may feel sick or have a headache, but this only lasts for a few hours. About 1 in 20 volunteers may feel the need to take medicines such as paracetamol or ibuprofen for symptom relief.

Rarely (less than 1 in 1000) volunteers may experience a drop in blood pressure, this will be corrected by intravenous fluid.

After the intravenous LPS you will rest in the integrated critical care unit for approximately 10 hours. During this period you will have blood samples (as described above) and intermittent recording of your pulse, blood pressure, temperature and oxygen levels.

You will be provided with a 24-hour telephone number to contact in the unlikely event that you feel unwell.

Contact Details

Should you wish any further information, please contact:

Professor John Simpson
Professor of Respiratory Medicine
Institute of Cellular Medicine
3rd Floor, William Leech Building
Medical School, Newcastle University
Framlington Place
NE2 4HH

Tel: 0191 222 7770
Email: j.simpson@newcastle.ac.uk

This completes Part 2 of the Participant Information

Does the DNA of our cell's batteries influence our response to bacteria?

CONSENT FORM

PARTICIPANT NUMBER					

TO BE COMPLETED BY THE PARTICIPANT (please initial each box):

1. I Confirm that I have read and understood the Participant information sheet, dated ……. version ……. for the above study. I have had the opportunity to consider information, ask questions and have had those questions answered fully. [ ]

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, and without my medical care or legal rights being affected. [ ]

3. I understand that information gathered for the purpose of the study will be kept in a secure confidential file. I agree that this file may be looked at by researchers involved in this study or, where relevant, by regulatory authorities overseeing the research. I understand that my personal data will be processed and stored in compliance with the 1998 Data Protection Act. [ ]

4. I agree to a clinical history and examination at each study visit and if clinically indicated [ ]

5. I agree to the injection of lipopolysaccharide (LPS) into a vein through a cannula [ ]

6. I agree to have blood tests, as outlined in the participant information sheet, and I am aware that while this includes some DNA analysis none of that DNA testing is about specific medical conditions [ ]

7. I agree that my blood may be stored for up to 5 years after completion of the study and that these will be stored in a secured, confidential way [ ]

8. I agree that my samples may be used in future studies, on condition that I understand the nature of any further research and the types of tests that will be done, that I cannot be identified from my samples, and that new ethical approval is granted for those studies [ ]

9. I agree that the research team may inform my general practitioner of any abnormal screening results or any adverse events during the study [ ]

10. I understand that relevant sections of my medical notes and data collected during the study, may be looked at by individuals from the research team, from regulatory authorities or from City Hospitals Sunderland NHS Foundation Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. [ ]

11. I agree that the research team may inform my general practitioner of my participation in the study [ ]

12. I agree to take part in this study [ ]

13. 7. I can confirm that I have retained a copy of the consent form and another copy has been retained by the research team and will be filed in the investigator site file [ ]

_____ _____ _____

Participant's Name Signature Date

_____ _____ _____

Researcher's Name Signature Date

APPENDIX C.

The protocol, participant information sheets, consent forms and data collection sheet used to obtain blood samples from individuals who were critically ill.

STUDY PROTOCOL

Investigating the effects of critical illness on the innate immune system

Chief Investigator	Prof John Simpson
Investigators	Dr Anthony Rostron Dr Alistair Roy Mr Jonathan Scott Dr Marie-Hélène Ruchaud-Sparagano Dr Kathryn Musgrave Dr Stephen Wright Dr Joy Allen Mr Jonathan Scott
Funder	Newcastle University
Sponsor	NUTH
Protocol	Innate immunity in Critical Illness version 1.0, 14/11/2017
Study dates	08/01/2018 – 31/07/2019

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Dr Alistair Roy, Consultant in Anaesthesia and Intensive Care Medicine, City Hospitals Sunderland Foundation Trust, Sunderland

Mr Jonathan Scott, Research Technician, Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne

Dr Marie-Hélène Ruchaud-Sparagano, Research Associate, Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne

Dr Kathryn Musgrave, Clinical Research Fellow, Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne

Dr Stephen Wright, Consultant in Anaesthesia and Intensive Care Medicine, Newcastle upon Tyne Hospitals Foundation Trust

Dr Joy Allen, Senior Research Associate-Methodologist, NIHR Newcastle In Vitro Diagnostics Co-operative, Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne

INTRODUCTION

Infections acquired during a stay within an intensive care unit (ICU) are common, affecting approximately 20% of patients (Vincent *et al.*, 1995). Large-scale international studies have demonstrated that the risk of developing a hospital-acquired infection increases with the duration of ICU stay. Those individuals who develop an infection have twice the ICU mortality rate (25% vs .11%, $p<0.001$) and an overall longer duration of stay when compared to individuals who do not develop an infection (Vincent *et al.*, 2009; Lambert *et al.*, 2011).

There are two reasons for this susceptibility to infection. Firstly, the use of necessary interventions such as mechanical ventilation and central venous lines increases the risk. Secondly, however there is mounting evidence that these patients have a dysfunction of their innate immune response. Dysfunction in several types of white blood cell within the ICU has previously been demonstrated and associated with an increased risk of developing an infection with the ICU. Complement mediated neutrophil dysfunction, a deactivation of monocytes (loss of HLA DR expression) and an increase in regulatory T cells that act to counter the inflammatory response have all be demonstrated in critically ill patients on the ICU. All were associated with an increased risk of developing a hospital-acquired infection (Conway Morris *et al.*, 2013).

Empirical antibiotics are often used in ICUs to ensure adequate coverage of critically ill patients and are often continued even after a specific pathogen is identified (Garnacho-Montero *et al.*, 2014). Understandably critical care clinicians will often prioritise the health of their patient over the need to limit antibiotic use because of the rise of bacterial strains resistant to antibiotics (Martin-Loeches *et al.*, 2017). There is an urgent need to identify alternative treatments that are not antibiotics, but to do this the basic mechanisms underpinning the susceptibility of critically ill patients to infection need to be further understood.

Monocytes, one of the white blood cells involved in the innate immune response, are now divided into at least three subclasses that have different functional properties: classical, intermediate and non-classical. Classical are the most involved in bacterial killing, they display the most phagocytic activity, release the widest range of reactive oxygen species and the most cytokines. Intermediate monocytes have been shown to express the most HLA class II molecules whilst non-classical monocytes have been shown to ‘patrol’ the endothelium and may have a role in tissue repair (Ziegler-Heitbrock, 1996a; Auffray, 2007; Ziegler-Heitbrock *et al.*, 2010a; Wong, 2011).

Although classical monocytes are best suited to bacterial killing, previous work demonstrates that critically ill patients have a reduction in this subclass and instead have an expansion in

the proportion of non-classical monocytes (Fingerle, 1993; Fingerle-Rowson, 1998). The cause of this change in monocyte subclass is not currently understood.

Human studies show that recombinant macrophage-colony stimulating factor (MCSF) can increase the proportion of non-classical monocytes in vivo. MCSF is present at high levels during sepsis and may explain the increase in the non-classical (Saleh, 1995).

This study will investigate the mechanisms that lead to an increased susceptibility to infection in critically ill patients; with the aim of identifying therapeutic targets for improving their innate immune response and reducing the frequency of infections.

ORIGINAL HYPOTHESIS

We hypothesize that individuals who are critically ill have an increased susceptibility to infection due to a defect in their innate immunity. In particular the reduction in classical monocytes and concurrent predominance of circulating non-classical monocyte contributes to the impaired immune response. We believe that the increase in MCSF is the cause of the switch in monocyte subset and could potentially offer a novel therapeutic target.

STUDY DESIGN

Participant Enrolment and Selection

Individuals who have been admitted to the intensive care unit (ICU) and would be suitable for the study will be identified by their clinical care team or research nurse. The individual, or their next of kin, will be given a copy of the study's participant information sheet and consent form. If they wish to be included in the study they will be recruited following written informed consent.

Setting

The intensive care units (ICU) at City Hospitals Sunderland NHS Trust (CHSFT) and The Newcastle upon Tyne Hospitals NHS Foundation Trust (NUTH).

Inclusion criteria

- Expected to remain in the ICU for longer than 24 hours
- Expected to survive in the ICU for longer than 24 hours
- Provision of written informed consent (either from the participant or next of kin)
- Requires organ support (either inotropes, ventilator, non-invasive ventilator, haemofiltration)
- They or their relatives/legal representative will be provided written, informed consent

Exclusion criteria

- <16 years of age
- Pregnancy

- Known infection with human immunodeficiency virus
- Haematological malignancy
- Concurrent use of immunosuppressant medication other than corticosteroids (allowed up to prednisolone 10mg/kg or equivalent)

Consent
Individuals eligible for the study will be identified by the clinical care team or research nurses and will be given verbal and written information regarding the study. All individuals who are approached will be given time to consider the information received prior to consent. All study subjects will be enrolled after giving signed informed consent. In those individuals where it is not possible to for them to give consent, the next of kin or appropriate legal representative will be approached. Wherever possible retrospective consent will be sought when an individual recovers. Consent will also include storage of blood for further testing, which will relate to this investigation.

Management of ineligible participants
For individuals found to be ineligible for the trial the reason for ineligibility or non-recruitment will be recorded. Only anonymised data will be entered on to the database and this will include gender, age, "ineligible" or "non-recruitment" and the associated reason.

Sampling
Study participation will involve two visits, each of less than 1 hour.

Study visit 1

This will occur following recruitment and include:

1. Data collection & preparation
    a. Details will be obtained from the subject through a medical interview and/or review of the medical notes; which will include but will not necessarily be limited to: age, sex, ethnicity, cause for the ICU admission, co-morbidities and concurrent medications.
2. Blood sampling
    a. Blood will be sampled following initial recruitment. 20 mL of blood will be collected. The most likely risk is that blood sampling may contribute to the development of anaemia in the participant. This risk has been minimised by limiting the amount of blood taken to 20 mL. This is similar to what is taken in a routine clinical blood test.
    b. Blood sampling can be accompanied by discomfort or by vasovagal symptoms. To minimise these risks samples will be taken wherever possible from an

indwelling line (an arterial line will be used preferentially if present, if not then a venous line). For the majority of this population the participant will be semi-recumbent in a hospital bed and sedated. In those few individuals who are not, samples will be taken with them in a bed or self-reclining chair. Participants who feel syncopal will be positioned supine and venepuncture will be discontinued.

c. Plasma will be removed and frozen (at -80^{o}C) for future testing
d. The cell fraction will used to isolate white blood cells for future testing.
e. Stored blood samples will be labelled with a unique anonymous identifier

3. Post procedure
    a. Subjects will be monitored by an ICU nurse

Study visit 2:

Where applicable this visit will occur within the next working day of discharge from the ICU between usual working hours. It will include:

1. Blood sampling – 20 ml of blood will be taken and processed as detailed above.
2. Post procedure - subjects will be monitored as following usual venepuncture with regular observations.

Adverse Events

Our research group has experience with taken blood samples from patients on the intensive care unit. Nevertheless, we feel that we must remain vigilant in detecting and recording any adverse events as a result of any procedures undertaken.

Whilst recognising that this current work is not a clinical trial, our groups' previous work (which included a clinical trial) benefited from classifying and monitoring adverse events in the manner described below, and we have elected to continue using this terminology in this research.

Definitions

An adverse event (AE) is any untoward medical occurrence in a study participant.

A serious adverse event (SAE) is any untoward medical occurrence in a study participant or effect that:

- results in death
- is life threatening (i.e. the subject was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe)
- results in persistent or significant disability or incapacity

If an adverse event is detected, a member of the research team will make an assessment of seriousness as defined by the above definitions. If the event is deemed to be serious (SAEs) a member of the research team will then consider if the event was:

- Related – that is it resulted from research procedures and/or
- Unexpected – that is a type of event that is not identified as an expected occurrence

Detecting and reporting AE and SAEs

All AEs and SAEs will be recorded from the time a participant consents to join the study and for up to an hour following venepuncture. Research nurses or members of clinical care team will inform the research team of any concerns that may constitute an AE or SAE. Information to be collected includes type of event, onset date, researcher assessment of implications, if any, for safety of participants and how these will be addressed, date of resolution as well as treatment required, investigations needed and outcome. All information will be recorded in the participants study file.

An AE/SAE may necessitate discontinuation of a given part of the study (but progression through the remainder of the study) or complete and immediate discontinuation of any further participation. Such an event may include, but not be limited to, a vasovagal response to venepuncture. All participants will maintain the right to discontinue or completely withdraw from the study at any time for any reason, or without stating a reason. The reason and circumstances for premature discontinuation (where known) will be documented in the participant's study file.

If a SAE has occurred, the research team must report the information to Newcastle upon Tyne Hospitals R&D within 24 hours. The SAE form must be completed as thoroughly as possible with all available details of the event, signed by the Investigator or designee. The SAE form should be transmitted by fax or by hand to the office.

NUTH R&D is responsible for reporting SAEs that are considered to be related and unexpected as described above to the Research Ethics Committee (REC) that approved the study (main REC) within 15 days of becoming aware of the event using the NRES Reporting of SAE Form. The Co-ordinator of the main REC should acknowledge receipt of related, unexpected safety report within 30 days.

Each AE should be clinically assessed for causality based on the information available, i.e. the relationship of the AE to the study should be established. All adverse events judged as having a reasonable suspected causal relationship to the study (i.e. definitely, probably or possibly related) are considered to be adverse reactions. If any doubt about the causality exists, the

local Principal Investigator should consult the Chief Investigator. In the case of discrepant views on causality between the Principal Investigator and others, the main REC and other bodies will be informed of both points of view.

Data analysis

Statistical analysis

Measurements of biomarker blood levels and proportions of monocyte subclass will be expressed within 95% confidence limits. Comparisons will be made between the two dependent timepoints using a paired t test and statistical significance identified by a nominal *p value* of <0.05. Statistical analysis will be performed using standard University software. We intend to write a prospective statistical analysis plan ahead of embarking on experiments with statistical help from the university (Dr Joy Allen, Senior Research Associate-Methodologist, Newcastle University).

Power Calculations

This is an exploratory pilot analysis.

Outcome Variables

The primary outcome variable will be the measurement of monocyte subclasses during critical illness and on recovery. As well as the measurement of MCSF at both timepoints. Secondary outcomes will include the effects of critical illness on the function of immune cells and their interactions with the coagulation system and the endothelium (blood vessel lining).

Research team and project management

The proposed project will be conducted by Professor John Simpson, Dr Anthony Rostron, Dr Marie-Hélène Ruchaud-Sparagano, Mr Jonathan Scott and Dr Kathryn Musgrave. Statistical work will be assisted by statisticians from the University.

EXPECTED VALUE OF RESULTS

To increase our understanding of the basic mechanisms under-pinning the susceptibility of critically ill individuals to infection.

To identify novel therapeutic targets to improve the innate immune response in the critically ill.

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PARTICIPANT INFORMATION SHEET: The Effects of Critical Illness on the Innate Immune System

Researchers: Dr Kathryn Musgrave, Dr Anthony Rostron and Prof John Simpson

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Ask us if there is anything that is not clear or if you would like more information.

Thank you for reading.

PART ONE

What is the background and purpose of the study?

Patients in an intensive care unit are more likely to develop an infection compared to other patients in a hospital. We believe that this is, in part, because of a problem with how their body fights infection. To treat infection we give antibiotic medicines but some bacteria are becoming resistant to these medicines. It is therefore important that we find alternative treatments.

White blood cells are essential to detect and fight infection. Monocytes are a type white blood cells, they help to fight infection but it is not yet fully understood how monocytes interact with other cells in the body to trigger the body's defence mechanisms. We will take samples of blood to allow us to analyse the ways in which monocytes - and other types of white cells - react to infection.

This study aims to better understand why patients staying in an intensive care unit are more likely to develop infections. We hope that this will allow us to identify ways in which the system could be helped to stop the increased number of infections in these patients.

Why have you been invited to take part?

You have been asked to take part because you are currently a patient on an intensive care unit and have been identified by the doctors and nurses looking after you.

Do you have to take part?

It is up to you to decide whether to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care received by you now or at any stage in the future.

How long can you take to decide?

You should take as much time as you wish.

What will happen to you if you take part?

If you decide to take part in the study, you will be asked to provide written consent.

What do I have to do?

The study will involve two visits from a researcher. At the first visit there will be discussion about your medical history and a blood sample will be taken. At the second and final visit a further blood sample will be taken.

What are the possible disadvantages and risks of taking part?
The taking of blood samples and may cause mild discomfort and bruising but wherever possible a tube already present in a blood vessel will be used.

What are the possible benefits of taking part?
There will be no direct benefits to you.

Is there any reimbursement for taking part?
No.

What will happen when the study ends?
You will continue with your usual care at the hospital.

What if there is a problem?
Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed.

Will your taking part in this study be kept confidential?
Yes. All the information about your participation in this study will be kept confidential.

Contact details
Should you wish further information Dr Kathryn Musgrave (Haematology Registrar and Clinical Research Associate) or Prof John Simpson (Professor of Respiratory Medicine) will be happy to answer your questions. The best contact number is 0191 222 7770.

This completes Part 1 of the Information Sheet. If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making your decision. Thank you.

PART TWO

What will happen if I wish to take part in the study?

There will be two visits from the researcher.

First visit (occurs once consent is given)

During this visit there will be:

4. Medical interview and review of medical records
    a. Discussion with a doctor where details are collected relating to but limited by: age; sex; ethnicity; cause for the ICU admission; other health problems; concurrent medications.
5. Blood test
    a. A blood sample of 20ml (about a tablespoon) with be taken, this is a small amount that is similar to the amount of blood taken during a routine blood test.
    b. Wherever possible blood will be taken from a tube that is already in place in a blood vessel
    c. We will process the blood sample to isolate the white blood cells from other parts of the blood, the other part will be stored for subsequent analysis.
    d. Stored blood samples will be labelled with a unique anonymous identifier
    e. The blood will be taken by a medically qualified professional (either a nurse or a doctor).

Second visit (occurs the next working day following discharge from the intensive care unit)

During this visit there will be:

1. Blood test
    a. A further blood sample of 20ml will be taken.
    b. This second sample allows us to compare how the white blood cells behave once an individual has recovered from illness

What will happen if I don't want to carry on with the study?

Participation in this study is completely voluntary and you can decide to withdraw from the study at any time.

Withdrawing from the study will not affect the care that you get from the NHS at any stage in the future.

What if there is a problem?

If you have a concern about your treatment by members of staff during the study, you should ask to speak with the researchers who will do their best to answer your concerns (a contact number is at the end of Part 1 of the Information Sheet). If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from your hospital.

In the unlikely event that something goes wrong and you are harmed during the study there are no special compensation arrangements. If you are harmed and this is due to someone's negligence then you may have grounds for a legal action for compensation against the NHS/Newcastle University but you may have to pay for your legal costs. The normal NHS complaints mechanisms will still be available to you.

Will your taking part in the study be kept confidential?

All information that is collected about you during the research will be kept strictly confidential.

Will your GP be informed that you are taking part in the study?

No.

Will any genetic tests be done?

Yes. The genetic tests will help us understand how blood cells respond to illness. We shall not, at any point, be testing for genes associated with specific medical conditions.

Will any information and material be stored?

Yes, but only with your permission. Information about you will be collected and entered onto a secure database. Access to this database will be password protected and only available to your doctors and the research staff. All data stored on computers will not use your name – you will be given a unique study number under which all data and test results will be entered.

Any blood that we obtain as part of the research will be processed in Professor Simpson's research laboratory at Newcastle University. We shall store the liquid component of blood (called serum or plasma) in freezers. The samples will only be labelled with your unique study number (i.e. your name will not appear).

It is possible that in the future new tests will become available that will help to predict the development of blood clots or infection. Should this situation arise we may use your samples again, but this would be on condition that you agree to this, that you could not be identified from the sample except by our research team, and that we obtain fresh and separate permission from a Research Ethics Committee. The Ethics Committee is completely independent from this study.

We may share samples with other investigators or commercial organisations in the UK or internationally, to help further understanding of severe infection. If this is happens, the samples shared would be anonymous and external investigators or organisations would not be able to identify you. The anonymised data collected as part of the study may also be used to understand the sample analyses.

This study is being overseen by Newcastle upon Tyne Hospitals NHS Foundation Trust. Authorised persons from the Trust or from other legally authorised regulatory bodies may look at some parts of your medical records and the data collected for the study. This is to ensure the quality of the work being carried out. All will have a duty of confidentiality to you as a research participant and nothing that could reveal your identity will be disclosed outside the research site.

What will happen to the results of the research study?

We intend for the results of this study to be published in medical/scientific journals and presented at medical/scientific meetings. All information in the public domain will be anonymous and it will not be possible to identify you from these publications/presentations.

Who is organising and funding the research?

This study has been funded by Professor Simpson's research group through an educational grant and will be overseen by the Newcastle Upon Tyne Hospitals NHS Foundation Trust and Newcastle University.

Who has reviewed the study?

This study has been reviewed by the Newcastle and North Tyneside Research Ethics Committee.

What if something goes wrong?

If you have any concerns about any aspect of this study, you should contact the local Principal Investigator (Prof John Simpson, telephone number 0191 222 7770), who will do their best to answer your questions. If you remain unhappy and wish to complain formally, you can do this through the normal NHS Complaints Procedure.

If something does go wrong and you are harmed due to someone's negligence, then they you may have grounds for a legal action against their NHS Trust, but you may have to pay legal costs.

Is there an independent doctor you can approach for further information?

If you would like to discuss any aspect of this research with an experienced researcher who is not linked in any way to this study, please feel free to contact Dr Ian Forrest, Consultant Respiratory Physician, Newcastle upon Tyne Hospitals NHS Foundation Trust.

Alternatively, you may prefer to raise your concerns through the Patient Advise and Liaison Service (PALS). This service is confidential and can be contacted on Freephone: 0800 032 0202

Alternatively, if you wish to make a formal complaint you can contact the Patient Relations Department through any of the details below:

Telephone: 0191 223 1382 or 0191 223 1454
Email: patient.relations@nuth.nhs.uk
Address: Patient Relations Department
The Newcastle upon Tyne Hospitals NHS Foundation Trust
The Freeman Hospital
Newcastle upon Tyne
NE7 7DN

Thank you for taking the time to read this information.

If you agree to take part in the study you will be given a copy of this information to keep, along with a copy of your signed consent form.

PARTICIPANT CONSENT FORM: Investigating the effects of critical illness on the innate immune system

Participant Number:_____

Researchers: Dr Kathryn Musgrave, Dr Anthony Rostron and Prof John Simpson

	Please initial in the box
I confirm that I have read and understood the information sheet dated 30/11//2017 (version 1.0) for the above study. I have had the opportunity to consider the information, ask questions, and have had these answered satisfactorily.	☐
I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	☐
I understand that relevant sections of any of my medical notes and data collected during the study may be looked at by responsible individuals from regulatory authorities or from the NHS Trust. I give permission for these individuals to have access to my records and understand that all information will be treated as confidential and in a way compliant with the Data Protection Act.	☐
I agree to a 20ml blood sample being taken on two occasions	☐
I agree to storage, in an anonymised fashion, of my blood samples for 5 years	☐
I agree to storage of information about my medical condition for 5 years	☐
I agree that my samples may be used in future studies, on condition that I understand the nature of any further research and the types of tests that will be done, that I cannot be identified from my samples, and that new ethical approval is granted for those studies.	☐
If I become more unwell and I am no longer able to understand that the study is continuing, I agree that all the information and samples already collected can still be used and I agree that the second blood sample can be taken.	☐

Name of Patient	Signature	Date
Name of Person taking consent	Signature	Date

1 for participant; 1 for researcher; 1 for case notes

PERSONAL CONSULTEE DECLARATION FORM:
Investigating the effects of critical illness on the innate immune system

Participant Number:_____

Researchers: Dr Kathryn Musgrave, Dr Anthony Rostron and Prof John Simpson

Please initial in the box

1. I (_____) have been consulted about (_____)'s participation in this research project. I confirm that I have read and understand the information sheet dated 30/11/17 (Version 1.0) for the above study. I have had the opportunity to consider the information, ask questions and have had these questions answered satisfactorily. ☐

2. I understand that his/her participation is voluntary and that I am free to withdraw them at any time, without giving any reason, without their medical care or legal rights being affected. ☐

3. I understand that relevant sections of any of his/her medical notes and data collected during the study may be looked at by responsible individuals from regulatory authorities or from the NHS Trust. I give permission for these individuals to have access to their records and understand that all information will be treated as confidential and in a way compliant with the Data Protection Act. ☐

4. I agree to a 20ml blood sample being taken on two occasions ☐

5. I agree to storage, in an anonymised fashion, of his/her blood samples for 5 years ☐

6. I agree to storage of information about his/her medical condition for 5 years ☐

7. I agree that these samples may be used in future studies, on condition that he/she understand the nature of any further research and the types of tests that will be done, that his/her cannot be identified from the samples, and that new ethical approval is granted for those studies. ☐

_____ _____ _____
Name of Consultee Signature Date

Relationship to participant: _____

_____ _____ _____
Name of Person taking consent Signature Date

1 for consultee; 1 for researcher; 1 for case notes

PROFESSIONAL CONSULTEE DECLARATION FORM:
Investigating the effects of critical illness on the innate immune system

Participant Number:_____

Researchers: Dr Kathryn Musgrave, Dr Anthony Rostron and Prof John Simpson

	Please initial in the box
1. I (_____) have been consulted about (_____)'s participation in this research project. I confirm that I have read and understand the information sheet dated 30/11/17 (Version 1.0) for the above study. I have had the opportunity to consider the information, ask questions and have had these questions answered satisfactorily. I am independent of this study.	☐
2. I understand that his/her participation is voluntary and that I am free to withdraw them at any time, without giving any reason, without their medical care or legal rights being affected.	☐
3. I understand that relevant sections of any of his/her medical notes and data collected during the study may be looked at by responsible individuals from regulatory authorities or from the NHS Trust. I give permission for these individuals to have access to their records and understand that all information will be treated as confidential and in a way compliant with the Data Protection Act.	☐
4. I agree to a 20ml blood sample being taken on two occasions	☐
5. I agree to storage, in an anonymised fashion, of his/her blood samples for 5 years	☐
6. I agree to storage of information about his/her medical condition for 5 years	☐
7. I agree that these samples may be used in future studies, on condition that he/she understand the nature of any further research and the types of tests that will be done, that his/her cannot be identified from the samples, and that new ethical approval is granted for those studies.	☐

_____	_____	_____
Name of Consultee	Signature	Date

Professional title: _____

_____	_____	_____
Name of Person taking consent	Signature	Date

1 for consultee; 1 for researcher; 1 for case notes

RECOVERED CAPACITY CONSENT FORM:
Investigating the effects of critical illness on the innate immune system

Participant Number:_____

Researchers: Dr Kathryn Musgrave, Dr Anthony Rostron and Prof John Simpson

	Please initial in the box
I confirm that I have read and understood the information sheet dated 30/11//2017 (version 1.0) for the above study. I have had the opportunity to consider the information, ask questions, and have had these answered satisfactorily.	☐
I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	☐
I understand that relevant sections of any of my medical notes and data collected during the study may be looked at by responsible individuals from regulatory authorities or from the NHS Trust. I give permission for these individuals to have access to my records and understand that all information will be treated as confidential and in a way compliant with the Data Protection Act.	☐
I agree to a 20ml blood sample being taken on two occasions	☐
I agree to storage, in an anonymised fashion, of my blood samples for 5 years	☐
I agree to storage of information about my medical condition for 5 years	☐
I agree that my samples may be used in future studies, on condition that I understand the nature of any further research and the types of tests that will be done, that I cannot be identified from my samples, and that new ethical approval is granted for those studies.	☐
If I become more unwell and I am no longer able to understand that the study is continuing, I agree that all the information and samples already collected can still be used and I agree that the second blood sample can be taken.	☐

Name of Patient	Signature	Date
Name of Person taking consent	Signature	Date

1 for participant; 1 for researcher; 1 for case notes

DATA COLLECTION SHEET **Participant**

DEMOGRAPHICS

Number:_____

Age (years): _____

Sex (delete as appropriate): MALE/FEMALE

Ethnicity (tick as appropriate):

White _____

Black or Black British _____

Asian or Asian British _____

Mixed _____

Other (please specify) _____

Does not wish to disclose _____

ADMISSION DETAILS

Cause of admission to the Critical Care Unit: _____

Co-morbidities:

Current Medications:

Are they currently requiring: (tick all that apply)

Invasive ventilation ☐ Non-invasive ventilation ☐

Inotropic support ☐ Haemofiltration/dialysis ☐

DIAGNOSIS OF SEPSIS

Do they have a diagnosis of sepsis? (delete as appropriate): YES/NO

<u>If yes, please complete the below section:</u>

Presumed focus of infection_____

Was the focus of infection found through (complete all relevant parts)**:**

Clinical presentation ☐

Please give details:_____

_____

Radiological evidence ☐

Please give details:_____

_____

Microbiological evidence ☐

Please give details:_____

_____

PROGNOSTIC SCORE

Complete the following table using data recorded now and during the first 24 hours of admission (choose the worst recorded levels).

	Worst recorded in first 24hrs of ICU admission	**At first study visit**
Clinical condition		
Age		
Severe organ dysfunction or immunocompromise prior to admission (see below)*		
Acute renal failure?		
Post elective surgery?		
Post emergency surgery?		
Observations		
Glasgow Coma Score (GCS)		
Temperature		
Mean arterial pressure (MAP)		

Heart rate		
Respiratory rate		
FiO2		
PaO2		
PaCO2		
Urine output (mL per day)		
Blood tests		
arterial pH		
Haematocrit		
white blood count		
Platelets		
Serum sodium		
Serum potassium		
Creatine		
Bilirubin		
Inotropes (tick all that apply):		
Dobutamine		
Dopamine (≤5µg/kg/min)		
Dopamine (>5µg/kg/min)		
Epinephrine (≤0.1µg/kg/min)		
Ephinephrine (>0.1µg/kg/min)		
Norepinephrine (≤0.1µg/kg/min)		
Norephinephrine (>0.1µg/kg/min)		

* **Definitions of immunocompromise and organ dysfunction** (tick all that apply)**:**

All must have occurred prior to this admission

	Tick all that apply
Cardiovascular dysfunction	
NYHA class IV (Severe limitations. Experiences symptoms even while at rest.)	
Renal dysfunction	
need for chronic dialysis	
Liver dysfunction	
biopsy proven cirrhosis	
portal hypertension	
previous upper GI bleed secondary to portal hypertension	
previous coma secondary to hepatic failure	
Respiratory dysfunction	
severe exercise restriction due to chronic restrictive, obstructive or vascular disease	
chronic hypoxia or hypercapnia	
secondary polycythaemia	
severe pulmonary hypertension	
respirator dependent	
Immunocompromise	
received radiation therapy	
long-term steroid use	
disease that affects the immune system	

Please note: high dose steroid therapy (>equivalent prednisolone 10mg/kg), haematological malignancy and HIV infection are exclusion criteria for this study

END

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