In Vitro Modelling of the Formation of Inflammatory Platelet-Leukocyte Aggregates and their Adhesion on Endothelial Cells (an Early Event in Sepsis)

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Dedication

This thesis is dedicated to my great mother (Galia) who has been my true source of inspiration and strength throughout my life.

Abstract

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Azzah Alharbi

Sepsis, a severe systemic inflammatory response to an infection, is a global health problem with significant economic burden. Platelet leukocyte aggregates (PLAs) are extensively formed in sepsis, and correlate with severity of disease. Molecular interactions that lead to the formation and adherence of these cellular aggregates to endothelial cells might represent novel targets to use for therapy. Whole blood stimulation assays and flow cytometry are widely used to study the formation of PLA aggregates in an *in vitro* approach to understand the acute inflammatory reaction in the bloodstream to the presence of pathogen associated molecular patterns (PAMPs, most commonly LPS) during septicaemia. However, these assays are limited by the lack of robust and physiologically relevant conditions. Most importantly, the extent of spontaneous aggregate formation is unclear in most assays, as cells outside the body may aggregate, forming PLA. The aim of this work was to assess extent of spontaneous aggregate formation in whole blood stimulation assays and to compare the effects of endotoxin and of heat killed, clinically relevant, pathogens on aggregate formation and adhesion of complex aggregates to TNFα stimulated endothelial cells. Endotoxin from *E.coli* or *Salmonella enteritidis* was not a suitable stimulus to provoke sepsis relevant platelet leukocyte aggregates *in vitro,* as it did not further increase the extent of aggregates formed spontaneously in stasis of hirudin anticoagulated blood. By contrast, heat killed *Klebsiella pneumoniae* or *Staphylococcus aureus* produced significantly enhanced and complex cellular aggregates which adhered to TNFα stimulated endothelial cells. These were reliably captured by scanning electron microscopy. Adhesion of cellular aggregates could be blocked by incubation of endothelial cells with a commercial P-Selectin antibody and a novel angiopoietin-2 ligand trap. In conclusion, a method was developed that models the acute inflammatory reaction in whole blood in the presence of relevant pathogen surfaces.

Publications and presentations arising from this thesis:

Puplication:

- Alharbi, A., Thomas, R., Ali, M., Thompson, J. and Stover, C. (2016) 'Factors in Homo and Heterotypic Aggregate Formation in Sepsis', in SMGroup (ed.) sepsis. 1st edn. sigma e books, pp. 1-1-11.
  A full version of the article can be found at the following link:
  http://www.smgebooks.com/sepsis/chapters/SEP-16-08.pdf. (Apendix V)
- Alharbi, A., Stover, C. and Thompson, J. (2018) 'Formation of inflammatory platelet-leucocyte aggregates in vitro and their adhesion to inflamed endothelial cells', *British journal of anaesthesia*, 120(5), pp.
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- Alharbi, A.,Stover, C., and Thompson, J (2017). Modelling blood poisoning in the test tube; The problem of the baseline. Festival of postgraduate research 2017, University of Leicester. [Appendix VII]
- Alharbi, A.,Stover, C., and Thompson, J (2017). Comparative analysis of sepsis relevant stimuli on platelet leukocyte aggregate formation. East Midlands Doctoral Conference. Nottingham UK. September 2017.
- Alharbi, A.,Stover, C., and Thompson, J (2017). *In vitro* modelling of inflammatory platelet leukocyte aggregates in sepsis. Image of Research. University of Leicester UK. December 2017. Appendix VIII.
  https://www2.le.ac.uk/departments/gradschool/researchimages/2017-competition/iron-heart-of-the-crystal-2013-neutron-crystal-structure-of-ascorbate-peroxidase-compound-ii.
- Alharbi, A.,Stover, C., and Thompson, J (2018). *In vitro* model of the acute cellular interactions between platelet- leukocyte aggregates and endothelium, an early event in sepsis. Joint Respiratory Research Day. Leicester UK. May 2018. [Appendix X]

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List of Abbreviation

e.g: exempli gratia, means for example

i.e.: id est, means “in other words

RT: room temperature

°C: Degree Celsius

Sec: seconds

min: minute

%: Percent

LPS: lipopolysaccharides

PLAs: platelets leukocytes aggregates

PGAs: platelets granulocytes aggregates

BSA: Bovine serum albumin

cDNA: Complementary DNA

DNA: Deoxyribonucleic acid

RNA: Ribonucleic Acid

mRNA: Messenger Ribonucleic acid

PCR: Polymerase Chain Reaction

QPCR: Real-time Polymerase Chain Reaction

CD: Cluster of differentiation

FCS: Fetal calf serum

FFA: Free fatty acid

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

ICAM-1: Intercellular Adhesion Molecule

VCAM-1: Vasclular Adhesion Molecule

TF: Tissue factor

PSGL-1: p-selectin glycoprotein ligand-1.

IL: Interleukin

TNFα: tumour necrosis factor alpha

J774: Macrophage cell line

EAhy926: endoteheilial cell line

HUVEC: Human Umbilical Vein Endothelial Cells

NETs: neutrophil extracellular trap

OD: Optical Density

SEM: Scanning electron microscopy

TEM: transmission electron microscopy

PBS: Phosphate buffered saline

PKO: properdin deficient mice

PWT: Properdin wild type mice

RPMI: Roswell Park Memorial Institute medium

DMEM: Dulbecco's Modified Eagle Medium

ROS: reactive oxygen species

SDS: Sodium Dodecyl Sulphate

TBS: Tris buffered saline

TEMED: N, N, N', N'-tetramethyl ethylene diamine

TLR: Toll like Receptor

v/v: volume/volume

w/v: weight/volume

µl: Microliter

μm: Micrometer

μg: Microgram

VD: Vitamin D

ELISA: Enzyme linked immunosorbant assay

TLR: Toll-like receptor

PRR: pattern recognition receptors

DAMP: damage associated molecular pattern

PAMP: pathogen associated molecular pattern

vWF : Von Willebrand factor

μM: Micromolar

nM: nanomolar

g: gram/gravity

h: hour

mg: milligram

ml: millilitre

rpm: revolutions per minute

V: Volts

v/v: Volume/Volume

w/v: Weight/Volume

OA: Oleic acid

PA: palmitic acid

TLR: toll like receptor

Ang: angiopoietin

PAF: platelet activating factor

NO: Nitric Oxide

EDTA: Ethylene diaminetetraacetic acid

Chapter 1
General introduction

1.1 Vascular system

The vascular system, integral to the circulatory system, allows the blood to circulate constantly within an extensive network of blood vessels composed of arteries, arterioles, capillaries, venules and veins. It transports nutrients (such as amino acids, glucose, fatty acids, vitamins and electrolytes, proteins like albumin and antibodies, hormones, gases (namely Oxygen, O_2 and Carbon dioxide, $CO_{2)}$, and blood cells (platelets, red blood cells, leukocytes). It provides continuous optimal nutrient supply and defence against diseases, controls osmotic pressure and pH and maintains cellular homeostasis (Pugsley and Tabrizchi, 2000). Dysfunction of the circulatory system, blood vessels and blood elements, is involved in the pathogenesis of several diseases such as sepsis and atherosclerosis.

Structurally, most of the blood vasculature share common basic histologic features consisting of three distinct layers namely tunica intima, tunica media and tunica adventitia**.** The tunica intima is the innermost and thinnest layer of the blood vessels. It is composed of a monolayer of endothelial cells lied on the basement membrane, termed as basal lamina, supported further with subendothelial connective tissue layer and outlined with internal elastic lamina to ensure the endothelial cell strength as well as elasticity. Tunica media is the middle fibromuscular layer situated between internal and external elastic lamina. Smooth muscle cells are the main constituent of this layer with some elastic and collagen fibres. The outermost layer, tunica adventitia, is located directly outside the external elastic lamina and contains predominantly a mix of fibroblast and elastic fibres with vasa vasorum and nerve plexi, as a supply for blood vessels wall (Wang *et al*, 2017; Pugsley and Tabrizchi, 2000; Prasad, 2000). However, these layers vary in amount creating different histology to adapt to specific physiological functions. In large arteries and veins, for example, the fibroelastic component is more prominent than muscular elements to accommodate a large blood volume. While the vascular wall of capillaries consists of a very thin layer of endothelium without apparent fibroelastic elements to permit tissue perfusion and diapedesis of inflammatory leukocytes (Alberts *et al*, 2002; Tucker and Bhimji, 2017).

1.2 Vascular endothelial cell

The blood vessels of the circulatory system are lined with a unicellular monolayer of endothelial cell (EC), called endothelium. It provides a low friction surface for blood flow. They are mainly joined by junctional proteins that have significant roles in vascular permeability and leukocyte transmigration: adherens junctions, responsible for adhesion of adjacent cells, and tight junctions, sealing the paracellular space (Pfeiffer *et al*, 2008; Wallez and Huber, 2008). They are mounted directly on the basement membrane and supported by underlying subendothelial layer of fibroelastic tissue. They are surrounded as well with perivascular cells, pericyte, that provides the appropriate maintenance to endothelium (Pugsley and Tabrizchi, 2000; Lemos *et al*, 2016).

Their direct contact with the blood stresses the critical role of these cells in all aspects of vascular biology to assure an adequate internal steady state, so-called homeostasis. It mediates a wide range of complex functions that adapt locally to specific needs over a given time. Under physiological conditions, quiescent endothelial cells provide anti-thrombogenic surface to maintain the normal fluidity of blood using several antiplatelet and anticoagulant pathways. It regulates the expression and release of different molecules to the blood that bind and activate anticoagulant factors (Rajendran *et al*, 2013). For example, heparan sulfate synthesized by endothelium acts as a cofactor to activate antithrombin, a serpin protease, that subsequently inactivates several proteases such as factor IXa, factor Xa, and thrombin involved in the clotting cascade (Medeiros *et al*, 2012; Zhang *et al*, 2004). However, activated endothelial cells, due to diverse stimuli such as infection, stress or ischemia, display a completely different phenotype that tip the balance in favour of thrombus formation achieved by stimulation of pro coagulant activity and inhibition of anti-coagulant activity. Tumour necrosis factor, TNFα, for example, produced by several blood cells as well as endothelial cell in response to inflammation (Ranta *et al*, 1999), stimulates tissue factor expression, a significant procoagulant factor and inhibits throbomodulin production, an endothelial anticoagulant cofactor (Rajendran *et al*, 2013).

Endothelial cells also play an essential role in the inflammation, as a main target and regulator, in term of site, extent and duration to ensure a proper host inflammatory response and resolution (Kadl and Leitinger, 2005; Xiao, Liu and Wang, 2014).

Normally, it provides anti-inflammatory and non-adhesive surface to the blood flow. Thus, any changes in the vascular steady state which can be triggered with diverse stimuli, such as infection, minor trauma or different mediators produced during the inflammatory cascade, like complement and cytokines, are constantly sensed by the endothelium to provide immediate adaptive response converting them from a resting to activated state. Initial rapid and transient effects of their activation results in exocytosis of Weibel-Palade bodies and release of its components such as; P Selectin (Dole *et al*, 2005), vonWillebrand factor (vWF) (Valentijn *et al*, 2011) and angiopoietin 2 (Fiedler *et al*, 2004) which were shown to induce inflammation by triggering leukocyte and platelet adhesion respectively, and disturb the endothelial cell junctions (Xiao, Liu and Wang, 2014) to induce permeability. A more sustained response of activated endothelial cells stimulate gene transcription and *de novo* synthesis of different molecules contributing to many aspects of inflammation.

The inflamed endothelial lining is characterised by increased permeability to control the passage of plasma proteins and blood cells into the affected tissue, which are mainly controlled by the inter-endothelial junctional proteins involving catenins, cadherins and integrins (Sarelius and Glading, 2015). It also regulates leukocyte recruitment, adhesion and transmigration that begins by induced expression of adhesion molecules such as E Selectin. A summary of inflammatory cytokines expressed by activated endothelium is given in Table 1.1 (Pate *et al*, 2010).

Furthermore, endothelium contributes to the process of healing by induction of neoangiogenisis, the formation of new blood vessels, that is required for proper tissue repair. It also regulates the local vascular tone and tissue perfusion together with smooth muscle cells (van Hinsbergh, 2012).

Table 1.1: Inflammatory Mediators Expressed by Endothelial Cells.

Mediator	Examples
Interleukins	TNFα, IL-1, IL-6, IL-8, IL-11, IL-14, IL-15
Chemokines	CXC, CC, CX3C
Lipid mediators	Leukotrienes, prostanoids
Others	Nitric oxide

Recreated from (Pate *et al*, 2010)

1.3 The Blood

Whole blood is composed of two main components, cellular and plasma and both play a major role in homeostasis and immune defence. The cellular component includes red blood cell (known as erythrocytes, RBC), white blood cell (leukocyte, WBCs) as well as platelets which are suspended in plasma. Plasma consists of a mixture of solutes such as proteins, lipids, hormones, clotting factors, electrolytes, complement and others.

1.3.1 Leukocytes

They are considered as the main cellular mediators of innate immunity. They are classified into two broad categories based on their structural feature, presence or absence of cytoplasmic granules. The granulocyte includes eosinophil, basophil and neutrophil. These cells are also called polymorphonuclear cells based on their lobulated nucleus. In contrast, agranulocyte cells includes monocytes and lymphocyte and are called mononuclear cells. This description is rooted in the early light microscopic description of cells on a glass slide and their staining characteristics. Since then, surface markers have been characterised which distinguish further these basic subtypes. All

originate from a pluripotential hematopoietic stem cell in the bone marrow and fetal liver (Murphy and Weaver, 2016).

1.3.2 Platelet

Platelet is the smallest cells circulating in the blood. It is known as thrombocyte and characterised by lack of nucleus. It is derived from megakaryocyte in the bone marrow. Platelets play critical roles in the vascular homeostasis. For a long time, it has been thought to be contributed primarily to the coagulation and wound healing functions. Subsequent haematological studies have explored their significant roles in innate immunity and inflammation (Holinstat, 2017).

1.4 Inflammation

Inflammation is a protective response of tissue to harmful triggers both endogenous and exogenous, encountered in the form of damaged tissue and pathogens, respectively. The inflammatory response draws on multiple vascular and cellular events mediated by blood vessels, immune cells, and uses molecular mediators. Clinically, redness (rubor), heat (calor), pain (dolor), swelling (tumor) and decreased function are the five cardinal signs of inflammation. Dilatation of blood vessels around the affected site, vasodilation, increases the blood flow which manifests as focal redness and heat. Increased vascular permeability allows the leakage of fluid and plasma proteins into the affected tissue which accounts for swelling. Sensitivity to the pain is caused by several mediators produced by endothelium and leukocyte such as bradykinin and prostaglandin and activation of mast cells. Inflammation is ‘‘the local infiltration and activation of leukocytes,, (Xiao, Liu and Wang, 2014).The main goal of inflammation is to localise and eliminate the initial insult, remove the resulting tissue damage and initiate tissue repair.

1.4.1 Inflammatory cascade

The innate arm of immunity contributes significantly to the acute inflammatory reaction to pathogen infection and tissue injury (Takeuchi and Akira, 2010). Normally, the body is continuously protected against infection by innate immunity. This innate immune response is nonspecific and divided into two lines. The aim of the first line is to prevent the pathogen from entering the body by the presence of many physical and chemical and mechanical barrier. Physical barrier includes the specialized epithelial cells joined

tightly to form an impermeable layer that line the external surfaces of body such as skin and mucus membranes. Body secretions such as tears, stomach acidity, mucus, urine, enzymes and antimicrobial peptides such as β-defensins act as chemical barrier that helps to prevent adherence of pathogens. Ciliary, bowel movements and air flow are examples of mechanical barrier that prevent the settlement of pathogen and interfere with their adherence (Informed Health Online, 2010 Dec 7 [Updated 2016 Aug 4]; Janeway CA Jr, Travers P, Walport M, et a, 2001).

Defects in the first line of defence, due to damaged epithelium for example, allows penetration of the pathogen which in turn elicits a local acute inflammatory response as a second line of defence to block the growth and spread of infectious agents. Such response is initiated by locally recruited cells of innate immunity, mainly macrophages, fibroblasts, mast cells, innate lymphoid cells and dendritic cells (Newton and Dixit, 2012). Innate cells, whether confined locally or circulating, recognise and eliminate the invading (penetrating) pathogen as well as resulting tissue damage non-specifically without long lasting immunity (no memory). These cells express pathogen recognition receptors (PRRs) that recognise highly conserved molecular patterns expressed by pathogens, termed PAMPS or damage associated molecular pattern known as DAMPs expressed or produced by damaged cell as part of inflammation (Thompson *et al*, 2011; Newton and Dixit, 2012).

Four distinct types of PRRs have been described either associated with cell surface such as Toll-like receptors ,TLRs, and C-type lectin receptors ,CLRs, or found intracellular within cytoplasm like nucleotide binding oligomerization domain [NOD]-like receptors ,NLRs, and the Retinoic acid-inducible gene [RIG]-I-like receptors, RLRs (Takeuchi and Akira, 2010). Different classes of PRRs and their ligands are shown in detail in Table 1.2.

TLRs and NLRs are the main PRRs involved in innate immune defence against bacterial infection (Kumar *et al*, 2013). Transmembrane pattern recognition receptor, Toll like receptors, TLRs, have been extensively studied and well recognised (Campanholle *et al*, 2013). To date, eleven types of human TLRs has been discovered (Moresco, LaVine and Beutler, 2011; Mudaliar *et al*, 2014). Each of them is capable of recognising a small range of extracellular or intracellular molecular patterns. TLR2, for example, identifies lipoprotein component of gram positive bacteria and mycoplasma

while TLR 4 recognises LPS outer membrane component of gram negative bacteria (Takeuchi and Akira, 2010; Moresco, LaVine and Beutler, 2011). TLRs form heterodimers and thereby increase the binding range of infection relevant PAMPs. TLR4, of particular interest in this work, is associated with a co-receptor CD14 and, after binding of LPS (brought to the receptor after interaction with LPS binding protein), engages with MyD88 to begin the intracellular signalling. After kinase activation, transcription factors translocate to the nucleus to initiate transcription of pro inflammatory cytokines such as TNFα (Figure 1.1).

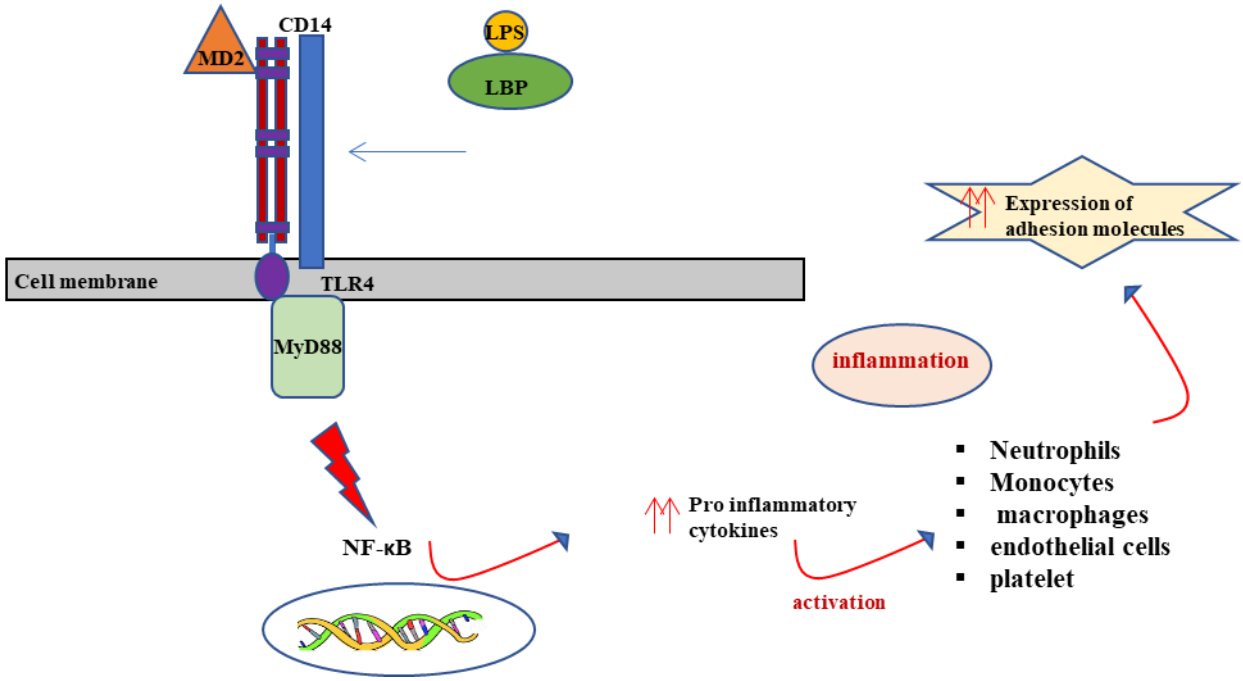


Figure 1.1: Schematic representation of innate immune response to LPS via TLR4. LPS binds to LPS binding protein (LBP) to facilitate its transfer to CD14. CD14, in turn, transfers LPS to the membrane anchored TLR4/MD-2 complex which then dimerizes and activates MyD88-NF-κB signalling pathways. Activation of such pathway results in augmented production and release of several inflammatory mediators that activate various blood immune cells.

Cytoplasmic PRR, nucleotide binding oligomerization domain [NOD]-like receptors, can recognise the PAMPs expressed on phagocytosed or cytosolic pathogens as well as many DAMPs which may be released intracellularly. Recently, 22 types of human NLRs are discovered (Kim, Shin and Nahm, 2016). Engagement of such PRRs to their ligands leads to downstream activation of different signalling cascades that brings about activation of transcription regulators such as NF-κB, AP-1 and IFR as well as activation of inflammasome and caspase-1. These factors contribute to production of a series of pro inflammatory mediators, cytokines and chemokines (Table 1.3) (Jin, Park and Jo, 2014) that regulate multiple, closely related, vascular and cellular events of inflammation within seconds.

Table 1.2: Pattern Recognition Receptors (PRRs) and Their Ligands.

PRRs	Localization	Ligand	Origin of the Ligand
TLR			
TLR1	Plasma membrane	Triacyl lipoprotein	Bacteria
TLR2	Plasma membrane	Lipoprotein	Bacteria, viruses, parasites, self
TLR3	Endolysosome	DsRNA	Virus
TLR4	Plasma membrane	LPS	Bacteria, viruses, self
TLR5	Plasma membrane	Flagellin	Bacteria
TLR6	Plasma membrane	Diacyl lipoprotein	Bacteria, viruses
TLR7 (human TLR8)	Endolysosome	ssRNA	Virus, bacteria, self

TLR9	Endolysosome	CpG-DNA	Virus, bacteria, protozoa, self
TLR10	Endolysosome	Unknown	Unknown
TLR11	Plasma membrane	Profilin-like molecule	Protozoa
RLR			
RIG-I	Cytoplasm	Short dsRNA, 5′triphosphate dsRNA	RNA viruses, DNA virus
MDA5	Cytoplasm	Long dsRNA	RNA viruses (Picornaviridae)
LGP2	Cytoplasm	Unknown	RNA viruses
NLR			
NOD1	Cytoplasm	iE-DAP	Bacteria
NOD2	Cytoplasm	MDP	Bacteria
CLR			
Dectin-1	Plasma membrane	β-Glucan	Fungi
Dectin-2	Plasma membrane	β-Glucan	Fungi
MINCLE	Plasma membrane	SAP130	Self, fungi

Recreated from (Jin, Park and Jo, 2014).

Table 1.3: Soluble Inflammatory Mediators.

Cell mediators	Main source	Function
IL-8	Macrophages	Neutrophil activation and chemotaxis.
TNFα and IL-1	Macrophages	Promote leukocyte chemotaxis and adhesion. Cause systemic effects; fever, tachycardia and loss of appetite.
IFN-γ	T and NK cells	Called as macrophage activating factor; antiviral, immunomodulatory
Leukotriene B4	Leukocytes	Promote leukocyte activation, adhesion to endothelium and transmigration. Induce ROS in neutrophils.
Nitric oxide	Macrophages and endothelial cells.	Vasodilator, cause smooth muscle relaxation and antimicrobial effect at high concentration.
Histamine	Mast cells, basophils, and platelets.	Couse vascular dilation and increases vascular permeability.
Prostaglandins	Mast cells and basophils	Fever, pain and vasodilatation.

Recreated from (Cruvinel *et al*, 2010)

1.5 Sepsis

Sepsis is a global health problem with increasing incidence and economic burden. It is defined as a severe systemic inflammatory response to an infection which can be bacterial, viral or fungal in origin. People at extremes of age, those with serious or chronic disease and immunocompromised patients are at higher risk of developing sepsis than others (Gauer, 2013). Early in sepsis, most cases present with symptoms of systemic inflammation which includes fever, chills and shivering, tachycardia and tachypnoea (Markiewski, DeAngelis and Lambris, 2008). Sepsis can progress to organ dysfunction, hypoperfusion or hypotension and septic shock, when adequate fluid resuscitation fail to manage sepsis induced hypotension (Matsuda *et al*, 2012).

In the UK, sepsis affects more than 100,000 people and accounts for 37,000 deaths per year (NHS, 2014). Current medical treatment such as various antimicrobial agents and fluid resuscitation contributes to only a modest improvement in outcome for septic patients (Gauer, 2013). More than 70% of cases are managed in critical care units and the treatment cost is estimated to exceed £2.5 billion annually in the UK (Carlson et al., 2014). Improvement of the medical care of septic patients requires a deeper understanding of the sepsis pathogenesis in order to identify novel targets or windows of opportunity to use for therapy.

1.5.1 Sepsis pathophysiology

Despite the clinical importance of sepsis and extensive studies, the pathophysiology of sepsis and its life threatening complications remains poorly understood (Bosmann and Ward, 2013; Ward, 2008; Soriano *et al*, 2005) For several years, it has been widely accepted that sepsis is the end result of an exaggerated inflammatory response to the microorganism (van der Poll, 2008). This assumption was concluded from different animal studies in which infusion of high doses of bacteria or bacterial products leads to sudden systemic release of a wide range of inflammatory mediators. Most of these mediators have been shown to be directly related to host death, including the pro inflammatory cytokines TNF- α and IL-1. However, almost all recent clinical trial using anti-inflammatory therapies, such as anti TNF-α and interleukin 1 receptor antagonists, have failed to improve the outcome of patients with sepsis (Soriano et al, 2005). Presumably because a relevant inflammatory response is an essential part of overcoming infection and the timing of the recorded lethal TNFα peak is unknown.

These results reflect the complexity of sepsis pathogenesis and the significant contribution of the host response to the sepsis associated mortality (Aird, 2003). Importantly, comorbidities influence susceptibility to developing sepsis and also acute and long-term survival after a septic event.

The pathogenesis of sepsis involves complex interactions between endothelium, platelets, leukocyte, coagulation system and multiple inflammatory mediators (Soriano *et al*, 2005; Koike *et al*, 2015). Interaction of neutrophils to activated platelets and endothelial cells plays a critical role in sepsis pathophysiology. Platelet neutrophil aggregate are extensively formed in sepsis and correlate with sepsis severity. There is growing evidence that adherence of neutrophils to activated platelets, forming heterotypic cellular aggregates circulating in blood or adhered to endothelium, significantly contribute to the development and aggravation of organ failure in patients with sepsis (Wang, Qin and Sun, 2014) Figure 2.

The cooperation of cells results in a stronger and faster response compared to the response caused by each of them independently and has important consequences on neutrophil function (Li *et al*, 2011). To explain, neutrophils activated by the interaction with platelets display an enhanced adhesive phenotype as well as phagocytic function and release of granular contents. Thus, these changes cause organ damage either directly by releasing proteolytic enzymes, oxygen free radicals and Neutrophil extracellular trap (NET) formation or indirectly through their enhanced adherence to the microvascular wall leading to occlusion, decrease in blood perfusion and hypoxia (Lewis *et al*, 2013). Kirschenbaum et al found that addition of activated platelets to the neutrophils stimulated with septic plasma significantly increased their adherence by 130% above that with plasma alone and decreased their rolling velocity and filtration (Kirschenbaum *et al*, 2004). The interaction of neutrophils with LPS stimulated platelets leads to the formation of NET within a few minutes, while this reaction takes around 2 to 4 hours of stimulation in the absence of activated platelets (Wang, Qin et al. 2014).

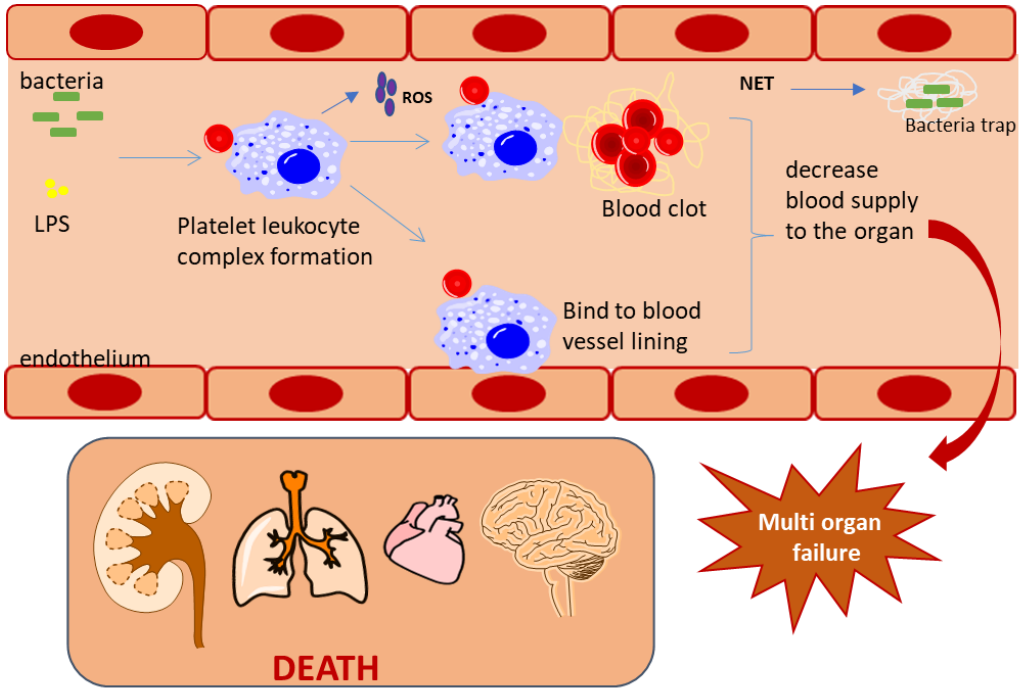


Figure 1.2: Platelet-leukocyte-endothelium interaction during bacterial sepsis. Profound Activation of platelets and leukocyte leads to the adherence of platelets to leukocytes via a selectin dependent process forming platelet leukocyte aggregate circulating in blood or adhered to activated endothelium. This interaction results in further cells activation leading to increase adhesion receptors, production of reactive oxygen species, thrombus formation and NET formation.

Early in sepsis, inflammatory leukocyte –endothelium -platelet complexes have been detected in several *in vivo* and *in vitro* models of sepsis (Koike *et al*, 2015). Contrasting with the above aggregating effect of cooperating cells on the extent of inflammation, interaction between LPS activated platelets with neutrophils adherent to endothelium in a model of sepsis has recently been characterised to have a beneficial role in bacterial trapping (Ma and Kubes, 2008). Although it could be seen as a defence mechanism for the host to confine the infection and limit its spreading, it might be strongly related to microvascular dysfunction as it leads to microvascular occlusion, slows the blood flow and activates the coagulation system preceding organ dysfunction and death (Souza,

Yuen and Star, 2015). Intravascular microscopic studies prove that platelet neutrophil aggregation on activated endothelium is an important determinant of microvascular occlusion during inflammation (Li *et al*, 2015). For example, it has been suggested that NET formation might be a trigger for formation of red blood cell rich thrombi leading to disseminated intravascular coagulation and organ failure (Wang, Qin and Sun, 2014; Stokes and Granger, 2012). Furthermore, activated endothelial cell, platelets and neutrophils express tissue factor and release micro particles which also express tissue factor, leading to activation of coagulation and aggravation of microvascular injury (Souza *et al*., 2015).

1.5.2 Pathogen recognition system in sepsis

The initial immune response following infection is triggered by recognition of conserved molecular structures of pathogens (secreted or wall bound) termed pathogen associated molecular patterns (PAMPs) by pathogen recognition receptors (PRRs). PRRs are expressed by innate immune cells (monocyte, macrophage and to some extent endothelial cell), and lead to activation of intracellular signalling cascades and production of inflammatory mediators such as TNFα, IL-1, IL-6, IL-12, and IL-8 (Sriskandan and Altmann, 2008; Matsuda *et al*, 2012) as described above. Prolonged activation of such signalling pathway leads to an exaggerated inflammatory response, which might result in tissue damage and release of damage–associated molecular patterns (DAMPs) (Matsuda *et al*, 2012; Bosmann and Ward, 2013; van der Poll, 2008). These DAMPs, such as hyaluronic acid and heat shock proteins, might be sensed by PRRs, expressed on endothelial cells, neutrophils and platelets, leading to amplification of the inflammatory response. LPS dependent TLR4 signalling is suggested as a key pathway in the pathogenesis of gram negative sepsis (Bosmann and Ward, 2013; Aziz *et al*, 2013). In murine studies using TLR4 lacking mice, administration of purified LPS failed to induce an immune response. Individuals with TLR4 polymorphisms might be more susceptible to meningococcal sepsis (Sriskandan and Altmann, 2008). Furthermore, it has recently been found that platelets also express TLRs that react with PAMPs and DAMPs leading to platelet activation, production of immunomodulatory agents resembling TNFα and promoting other cell activation involving neutrophils and endothelial cells (Wang, Qin and Sun, 2014; MA and Kubes, 2008). Activation of complement and release of antimicrobial peptides are significant in sepsis, pursuing roles of antimicrobial and cell instructive activities (Zimmer *et al*, 2015).

1.5.3 Cellular aggregate formation in sepsis

1.5.3.1 Receptors mediators

Neutrophils attach to activated platelets mainly through P-Selectin, a granular protein expressed on the platelet surface upon its activation and binds to PSGL-1 (P-Selectin Glycoprotein Ligand-1) present on the neutrophil surface (Wang, Qin et al. 2014), Figure 1.3. P-Selectin and PSGL-1 interaction is critical for tethering and rolling of neutrophil on platelet surface, as studies aimed at blocking one of these molecules with a monoclonal antibody resulted in complete inhibition of initial interaction between platelet and neutrophil (Evangelista, Smyth and Michelson, 2013; Zarbock, Polanowska-Grabowska and Ley, 2007). This interaction results in further neutrophil activation and upregulation of other adhesion molecules (integrins) such as macrophage-1 antigen (Mac-1 ,CD11b/CD18) also called complement receptor 3 (CR3) and lymphocyte function –associated antigen (LFA-1,CD11a/CD18) also called complement receptor 4 (CR4) that lead to firm adhesion (Evangelista, Smyth and Michelson, 2013). Mac-1 binds to GPIbα or junctional adhesion molecules-3 (JAM-3) presented on the platelet surface (Zarbock, Polanowska-Grabowska and Ley, 2007; Li *et al*, 2015). Additionally, binding of fibrinogen to Mac-1 on neutrophil and GPIIb/IIIa (αIIbβ3) on platelets stabilizes the platelet neutrophil adhesion (Evangelista, Smyth and Michelson, 2013; Zarbock, Polanowska-Grabowska and Ley, 2007; Kirschenbaum *et al*, 2004). Using several *in vivo* and *in vitro* studies of inflammation, blocking of Mac-1 and GPIbα molecules abolished the platelet neutrophil complex formation and improved the blood flow (Li *et al*, 2015). However, blocking of αIIbβ3 molecules does not decrease platelet neutrophil interactions. Thus, P-selectin and PSGL-1 and Mac-1 and GPIbα interactions are essential for platelet neutrophil interaction.

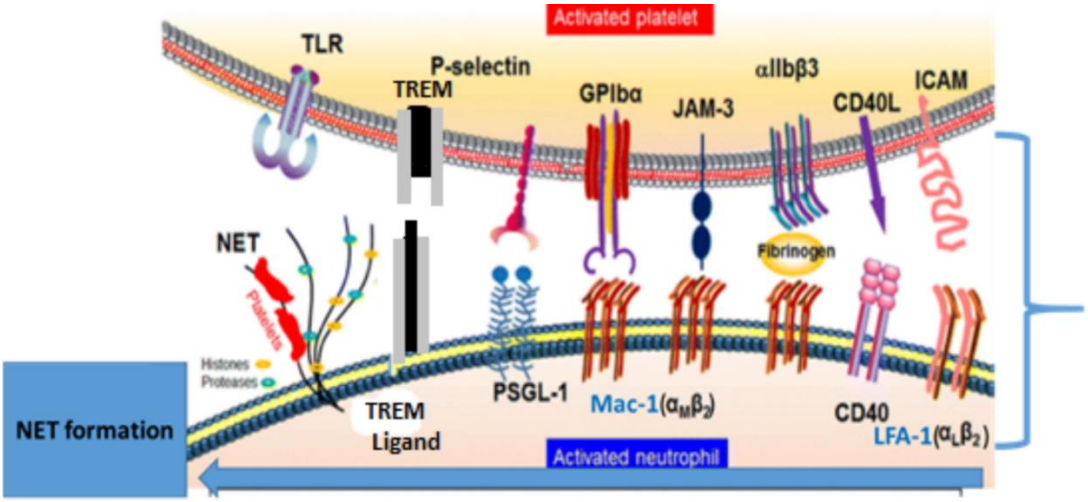


Figure 1.3: Main receptors involved in heterotypic neutrophil–platelet interactions. Heterotypic interactions are mainly mediated by the interactions of P-Selectin with PSGL-1 and αMβ2 integrin with GPIbα. Other molecules also contribute to heterotypic interactions, such as platelet JAM-3 binding to neutrophil αMβ2 integrin and triggering receptor expressed on myeloid cells (TREM-1) interact with its ligand on neutrophil. Platelet αIIbβ3 integrin can interact with neutrophil αMβ2 integrin through fibrinogen. Binding of LFA-1 to its legend on platelet leads to formation of NET. Recreated from (Li *et al*, 2015).

Furthermore, activated platelets express CD40 ligand (CD40-L) and shed this into circulation. Platelet derived-CD40L can bind to CD40 expressed on their surface leading to more platelet activation (Stokes and Granger, 2012) and to neutrophil CD40 resulting in its activation and production of reactive oxygen species, ROS (Rahman *et al*, 2012). It can also interact with endothelial CD40 leading to stimulation of endothelial cell to upregulate expression of various adhesion molecules, such as ICAM and VCAM, and to release the chemokine, CCL2, thereby promoting recruitment of neutrophils. Additionally, in sepsis, activated platelets can interact with neutrophils through triggering receptor expressed on myeloid cells (TREM1) that leads to further stimulation of neutrophils and increased expression of adhesion molecules (Brown *et al*, 2006) where, LFA-1 leads to NET formation (Wang, Qin and Sun, 2014).

Activated neutrophils bind to endothelial cells of post capillary venules through the expression of various adhesion molecules (Lewis *et al*, 2013). Selectins promote tethering and rolling on endothelium and the β2 integrin mediate firm adhesion. P-Selectin and E Selectin expressed on endothelium bind to specific glycoprotein ligands on neutrophil surface while L-Selectin expressed on neutrophil surface interacts with its ligand on endothelium (Lewis *et al*, 2013; Brown *et al*, 2006). Thus, neutrophils exposed to inflammatory stimuli, like Il-8 and platelet activating factor (PAF) which are expressed on the endothelial surface, become activated and upregulate β2 integrin that recognises intercellular adhesion molecules, ICAM-1 (CD54) and ICAM-2 (CD102) expressed on endothelium surface. It has been shown that neutrophils isolated from septic patients displayed a "supranormal adherence" to inflamed and resting endothelium and blocking of CD11b/CD18 (Mac-1) did not inhibit the neutrophil binding to endothelium. So, involvement of other adhesion molecules is strongly suggested. Lewis *et al* found that most of septic blood neutrophils express CD11c in contrast to only a small number of cells from healthy individual. Like CD11a and CD11b, CD11c was found to recognize endothelial surface receptor CD54 and trigger adhesion mediated cell activation. Other molecules such as CD64 and CD49d are also reported to be highly expressed on neutrophils during sepsis. Incubation of blood neutrophil from healthy subjects with septic plasma augment surface expression and adherence to inflamed endothelium which is antagonised by blocking antibodies such as anti CD49d and anti CD64 antibodies separately.

1.5.3.2 Soluble mediators

Early in sepsis, neutrophil- platelet aggregates formation on vascular endothelium might be mediated by a wide range of effector molecules, which include but are not limited to pro inflammatory cytokines, chemokines, secondary mediators for tissue injury such as Nitric Oxide (NO), coagulation and complement activation products. Local release of TNF-α and IL-1 lead to activation of vascular endothelium and increases expression of adhesion molecule such as P-Selectin which is essential for the neutrophil tethering process (Sriskandan and Altmann, 2008). While locally produced IL-8 plays a significant role in recruitment and activation of neutrophils, NO induces local vasodilation which results in slowing the blood flow rate and allowing neutrophil tethering to vascular wall.

The interactions presented so far work in the context of complement activation during sepsis. Complement activation occurs in response to invading microorganisms via three pathways, the classical, alternative, and lecitin pathway, engaging e.g. LPS of gram negative bacteria, complexes made of natural antibodies binding to pathogens, or bacterial, fungal or viral sugar moieties. Subsequently, convertase complexes are generated which result in the production of anaphylatoxins and a membrane attack complex. These end products of complement activation exert various biological effects to clear the infection and instruct cellular activities, alongside cytokine activities. In sepsis, disinhibited or uncontrolled complement activation might be a significant contributor to the pathogenesis of sepsis and results in exaggerated inflammatory response and host tissue damage. When neutrophils become activated, they increase expression of adhesion molecules, exocytose properdin (positive regulator of alternative complement pathway) and binding to activated platelets (Ruef *et al*, 2008). Properdin is a serum protein produced by stimulated neutrophils and endothelial cells (Kemper and Hourcade, 2008) and binds to activated platelets (Saggu *et al*, 2013). Properdin potentiates platelet leukocyte aggregation after Adenine di-Phosphate (ADP) pre-stimulation and increases CD11b expression (Ruef *et al*., 2008). Thus, it induces formation of platelet leukocyte aggregate through leukocyte activation. Properdin amplifies LPS-induced complement activation by stabilising C3 convertase through its binding to C3b found in the C3 convertase complex (C3bBb) attached to the cell membrane. C3 convertase generates C3b which is bound by the C3 convertase to generate the C5 convertase, but also binds to P-Selectin (Morigi *et al*, 2011). Properdin stabilises the C5 convertase to generate C5a, which leads to expression of procoagulant tissue factor in neutrophils (Kourtzelis *et al*, 2010). C5aR is expressed by microvascular endothelial cell (Laudes *et al*, 2002). Signalling through C5aR results in cell activation to generate ROS (O_2., H_2O_2, HO.) that are toxic to other cells, pathogens and connective tissue components (Ward, 2008). Moreover, tissue factor can be induced by membrane attack complex (C5b9) (Lupu *et al*, 2014). Tissue factor itself activates alternative pathway of complement, requiring properdin (Camous *et al*, 2011a). The function of complement and coagulation cascades are very closely interconnected (Jenny *et al*, 2015; Ritis *et al*, 2006; Krisinger *et al*, 2012; Amara *et al*, 2010).

Interactions between inflammatory and coagulation pathways are postulated to play a significant role in sepsis (Kirschenbaum *et al*, 2004). Increase expression of tissue factor in sepsis leads to activation of extrinsic clotting pathway and formation of thrombin and fibrinogen. Thrombin induces expression of endothelial cell adhesion molecules such as P and E Selectin, secretion of von Willebrand factor (VWF) and stimulates production of various soluble mediators such as Il-8 and platlet activating factor. While fibrinogen, in addition to its role in platelet neutrophil interaction, facilitates interaction between neutrophil and endothelial cell by binding to CD11b/CD18 on neutrophil and intracellular adhesion molecule -1 (ICAM-1) on endothelium.

1.6 Aims and objectives

In vitro formation of aggregates involving platelets and leukocytes has been widely studied to understand the acute inflammatory reaction in the bloodstream to the presence of pathogen associated molecular patterns (PAMPs, most commonly LPS) during bacterial septicaemia. However, there was a large variability in the reported levels of circulating PLAs and most of these studies are limited by the lack of robust and physiologically relevant conditions (Haselmayer *et al*, 2007). Thus, a reliable *in vitro* model to investigate the formation of inflammatory aggregates and their adhesion to endothelial cells is critically required to predict the effect of a novel blocker on the adhesion. To serve this purpose, this thesis aimed to develop a method to model the acute inflammatory reaction in the bloodstream to the presence of common bacterial PAMPs typically present during septicaemia, as well as to provide the basis for future studies on the mechanisms of cell interactions, including endothelial cells.

Objectives

1. To set up the protocol generating platelet leukocyte aggregates from whole blood.
2. To measure spontaneous aggregate formation in the whole blood assay.
3. To investigate whether LPS as single PAMP induces platelet leukocyte aggregates
4. To investigate the effect of PAMPs rich stimuli on the aggregate formation using organisms relevant to sepsis
5. To establish and characterise the platelet leukocyte aggregate adherence to endothelial cells.
6. To test the effect of a novel blocker, Ang2, on the inflammatory aggregate adhesion to endothelial cell under simulated clinical conditions.
7. To investigate the beneficial effect of Vitamin D on the inflammatory aggregate adhesion to endothelial cell under simulated clinical conditions

Chapter 2

Materials and Methods

2.1 Blood Sample collection

The project was approved by University of Leicester committee for Research Ethics (No. 5357). Whole blood was drawn carefully from antecubital vein under antiseptic conditions. The whole blood samples taken from a total of 10 healthy individuals (4 males and 6 females from different ethnicity, European and Middle Eastern) after informed, free and written consent. Most of them participated at least twice throughout the study. All individuals were non-smokers, with normal body mass index (BMI) and free of medication (in particular, non-steroidal anti-inflammatory drugs [NSAIDs] and aspirin) for at least 3 days. Initially the first 2 mL of blood were discarded before blood samples were drawn for analysis. But subsequently this step was omitted because of a lack of effect on the staining. Whole blood was collected into tubes containing 10 mM ethylenediaminetetraacetic acid (EDTA, pH 8), Hirudin (150 U/ml) (Merck Millipore, UK) or into heparin coated tubes (Sarstedt), inverted gently to ensure proper mixing of whole blood with anticoagulant and kept at room temperature for further processing.

- Preparation of 0.5 M EDTA (ethylenediaminetetraacetic acid) stock solution:

It was prepared by dissolving 186.12 g of EDTA.Na_2.$2H_2O$ (Molecular Weight 372.24) (Aldrich, UK) into 1000 ml of distilled water and the pH was adjusted at 8. It was autoclaved and kept at room temperature.

Table 2.1: blood samples with related results

	sex	Age range	Relevant figures (results)
1	Female	~ 30	3.2,3.3, 3.5, 3.7, 3.8, 3.11, 3.14 , 3.18,3.19, 3.20, 32.1, 4.2, 4.3, 4.5, 5.5, 5.7, 5.9, 5.14
2	Female	~ 45	3.2, 3.4, 3.6, 3.13.3.15,3.19, 3.22, 4.4, 4.7,4.8, 5.6, 5.11, 5.12, 5.13,5.15
3	Female	~ 40	3.5, 3.11, 3.15, 3.19, 4.4, 5.7, 5.13, 5.15
4	Female	~ 40	3.6, 3.13,3.15, 3.19,3.20, 3.21, 4.2, 4.3, 4.5, 5.2, 5.3,5.5,5.8, 5.9, 5.11, 5.12, 5.13, 5.15
5	Female	~ 30	3.7, 3.8, 3.15
6	Female	~ 55	3.11, 3.15, 3.18, 3.22, 4.4, 4.7, 5.2, 5.3, 5.14
7	Male	~ 35	3.6, 3.11, 3.20, 3.21, 4.7, 4.8,5.8
8	Male	~ 45	3.11, 3.14, 3.19, 5.6, 5.11, 5.12, 5.13, 5.15
9	Male	~ 32	3.3,3.4, 3.15, 3.20, 3.21, 4.5, 5.2, 5.3, 5.9
10	Male	~45	3.11, 3.18

2.2 Flow cytometry

Flow cytometry is a sensitive biophysical technique that is widely used nowadays for cellular immunophenotyping and cell sorting as well. It can analyse multiple parameters of each cell. As the cell pass through the laser beam, it illuminates, and the released light is captured by special detectors and the signal transformed into data file on computer for analysis. The interrogation point, known as analysis point, is the point where the flowing cell intersects and passes in front of laser beam. The released light is scattered in different directions and divided into forward scattered and side scattered light. Forward scatter is the emitted light in forward direction and reflects the size of the cell. While side scatter is released light at larger angle in lateral direction and reflects the cellular complexity and granularity.

Normally cells have autofluorescence, thus cell of interest should be stained with fluorescent dyes or fluorophores to overcome the interference of background and be ‘‘visible” to the cytometer. The fluorescent dyes can be conjugated to antibodies targeted against specific cell marker, so the fluorescence will be a readout for the number of cell to which the antibody has bound to.

2.2.1 Optimised protocol of detecting PLA by flow cytometry:

2.2.1.1 Sample preparation and immunolabeling:

Immunolabeling was performed within 10 minutes of collection of whole blood to minimise artefactual platelet activation that results from blood stasis or multiple centrifugation and washing steps. Aliquots of 100 µl of whole blood were incubated with specified monoclonal antibodies for 20 minutes on ice and well protected from light exposure. One aliquot was left unstained and incubated without addition of antibody.

After incubation, 2 ml of 1x RBC lysis buffer was added to each tube and incubated in dark at room temperature for exactly 12 minutes. 1x RBC lysis buffer was prepared by diluting the 10X RBC lysis buffer (Biolegend, UK) into endotoxin free water (Sigma,UK) to minimise cell activation and adjusted at pH 7.4. Using endotoxin free water was determined essential because of previous *in vitro* observation using mouse macrophages. Here, even minimal content of LPS leads to formation of a foam cell

response (Appendix I). Then, all tubes were centrifuged at 800 xg for 5 minutes at room temperature. This centrifugation power was optimised to include the platelets in the pellet. The resulting supernatant was carefully discarded using fresh plastic pipette and 2ml of cell staining buffer (Biolegend, UK) was added to each tube and spun down at 800x g for 5 minutes at room temperature to wash the cells. After removing the supernatant, the pellet was gently dissolved in 500 µl of 2% paraformaldehyde –PBS fixation buffer. The tubes were protected from light and kept at 4oC for next day flow cytometric analysis.

2.2.1.2 Antibodies:

A titration of antibodies was done to determine the optimal antibody concentration that gives the brightest signal with the lowest background. The following murine monoclonal antibodies (Biolegend, UK) were used according to the manufacturer's instruction

Table 2.2: Antibodies used for flow cytometric analysis

For detection of	Antibody	Clone No	Volume /test
Platelet	PE-conjugated anti human CD42b. PE mouse IgG 1(isotype)	HIPI MOPC-12	5µl 5µl
Leukocyte (granulocyte)	FITC- conjugated anti human CD66b FITC mouse IgM (isotype) APC- conjugated anti human CD66b APC mouse IgM (isotype)	G10F5 MM-30 G10F5 MM-30	5µl 5µl 5µl 5µl

2.2.1.3 Endotoxin free phosphate buffered saline solution (PBS)

It was prepared by mixing 100 ml of endotoxin free water (Sigma, UK) with one tablet of PBS (OXOID, England) under sterile condition. Then, the solution was autoclaved and kept at room temperature.

2.2.1.4 2% (w/v) paraformaldehyde in PBS fixation buffer

It was prepared by cautious addition of 2g of paraformaldehyde into mixture of 5 µl of 10% (w/v) NaOH and 100 ml of distilled water. Then, one tablet of PBS was dissolved into the mixture and kept at 4oC for further use.

2.2.2 Flow cytometer instrument setup

Samples were analysed within 24h using FACS Aria II Flow Cytometer (BD Biocsiences, San Jose, USA) or BD FACS Canto A (BD Biocsiences, San Jose, USA) equipped with FACSDiva software Version 6.1.3 (BD Biocsiences, San Jose, USA). To ensure the sensitivity, reproducibility and standardization of the results, the flow cytometer was routinely calibrated using flow check beads of known fluorescence intensity and size. FITC (fluorescein) was excited by a 488 nm blue laser while APC (allophycocyanin) was excited by Red laser with wave length of 640 nm. PE was excited by a 561 nm Yellow-Green Laser. Their emissions were collected with specific filters. The common filter for fluorescein (530/30 filter) permits passage of light that lies between 515-545 nm while that of APC (660/20) allow the light between 650 and 670 nm to pass. The phycoerythrin (PE) filter (585/42) allows only passage of light between 564-606 nm. The overlap of the fluorescein or allophycocyanin into the PE detector result in measuring some of fluorescein or allophycocyanin molecule in the PE detector (Yip, Ignjatovic et al. 2013) (Figure 2.1)**.** This spectral overlap was compensated by using BD CompBeadsTM set Anti-mouse Ig,k (BD Bioscience,UK).

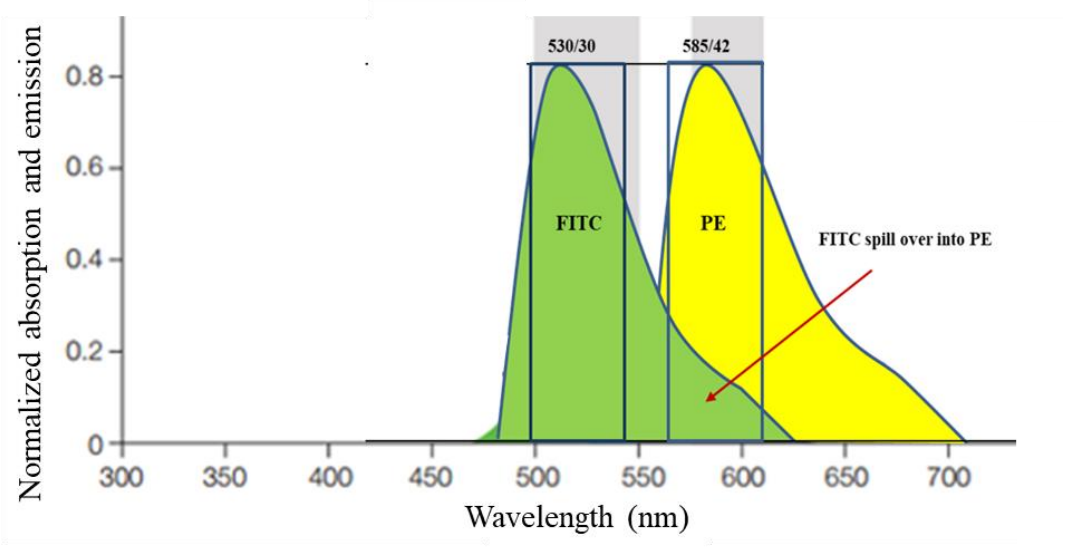


Figure 2.1: Excitation and emission curves of FITC and PE showing FITC spill over into PE channel**.** FITC (fluorescein) was excited by a 488 nm blue laser. PE was excited by a 561 nm Yellow-Green Laser. Their emissions were collected with specific filters: the fluorescein (FITC) (530/30 filter) permits the passage of light that lies between 515-545 nm. The phycoerythrin (PE) filter (585/42) allows only passage of light between 564-606 nm. The overlap of the fluorescein into the PE detector result in measuring some of fluorescein molecule in the PE detector.

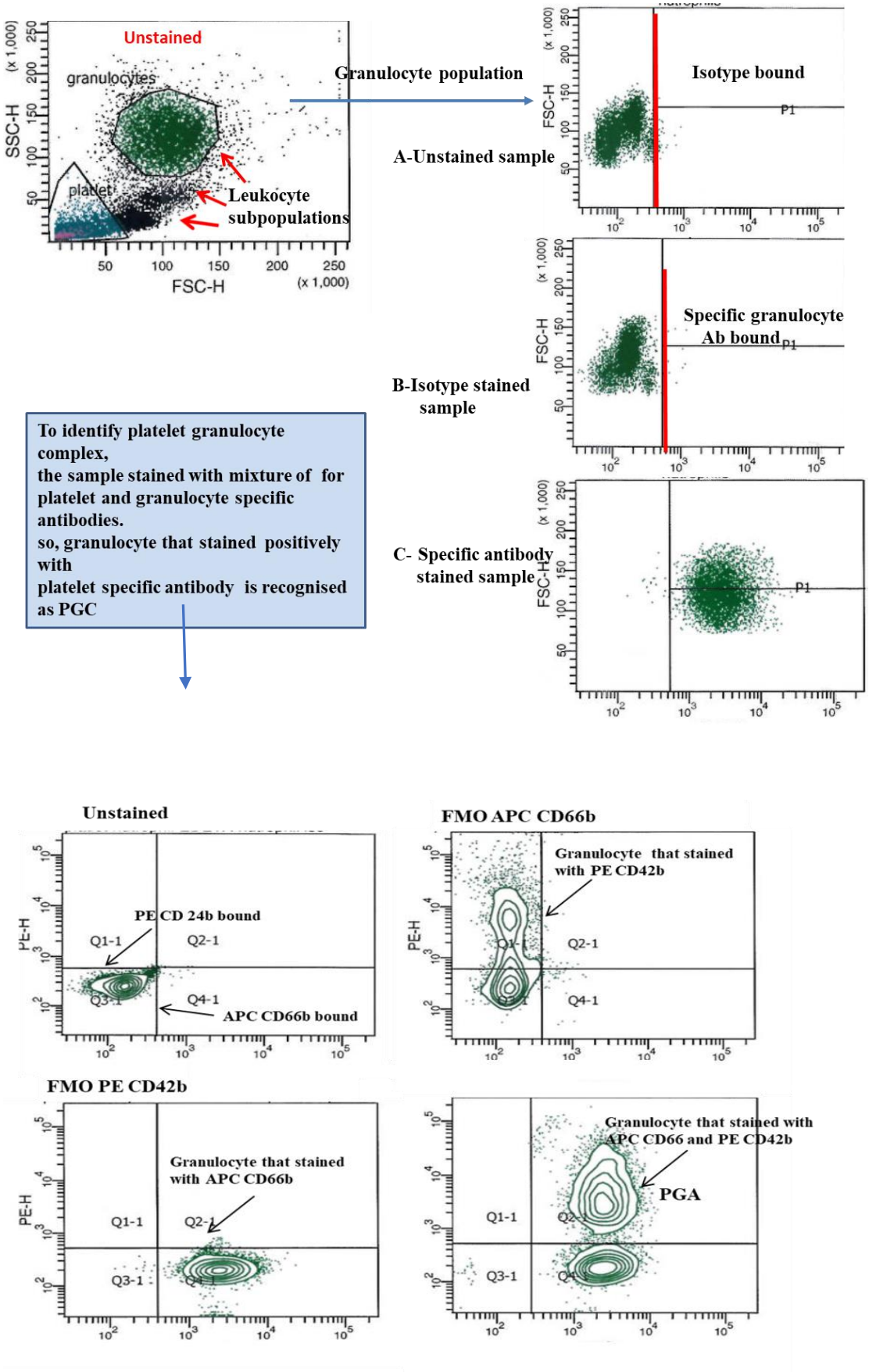


Figure 2.2: Detection of PGAs by flow cytometry. The granulocyte population was clearly identified in whole blood based on light scatter characteristic, size and

granularity. Granuolocytes stained with anti CD66b antibody while platlet stained with anti CD42b antibody. The detection limit of the positive staining adjusted by using different controls, unstained sample, isotype stained sample and flouroscence minus one control (FMO). Approximately 99% of the cells in granulocyte gate were positive for the granulocyte marker CD66 which express the specificity of marker staining and accuracy of cells identification according to their forward and side scatter characteristic feature. From this granulocyte gate, events that stained positively for both CD66 (APC or FITC conjugate) and platelet marker CD42b (PE conjugate) were identified as PGA.

2.2.3 Compensation

BD CompBeads (BD Bioscience, UK) consist of positive and negative stained population. For each fluoro chrome conjugated mouse Ig antibody, two tubes were prepared: one contained negative population without antibody as a negative reference point and other one contained equal amount of positive and negative population (one drop) incubated with the desired antibody (5 µl) for 20 minutes in dark at room temperature. After that, 2ml of cell staining buffer was added to each tube and spun down at 200 xg for 10 minutes. The supernatant was carefully discarded and the pellet re suspended in 500 µl of cell staining buffer. Finally, the tubes were analysed to set the compensation by using same instrument set up software.

2.3 PLA isolation

2.3.1 Cellular Sorting

To isolate the platelets granulocytes Aggregate (PGA) population by Fluorescence Activated Cell Sorting (FACS Aira), the whole blood aliquots were stained for both markers of platelet and granulocytes as described above. FMO controls were also prepared. Then, the samples were first recorded after applying the compensation to adjust the gate on the desired population for sorting. Then, the gated population were sorted into a new external collection tube containing PBS by Dr Jennifer Higgins, department of Genetics & Genome Biology, university of leicester. Post sorting analysis was performed to determine the purity of the sorted population.

2.3.2 Density gradient isolation using Histopaque

It is a common laboratory technique to isolate the viable granulocytes and mononuclear cells from whole blood. Firstly, a density gradient was created by adding histopaque -1077 on top of histopaque -1119 (both purchased from Sigma-Aldrich Company Ltd.

(Dorset, UK)) at equal volumes. Then, anticoagulated whole blood was added carefully on top of the histopaque at double volume. The tube was centrifuged at 700xg for 30 minutes at RT. Two distinct opaque layers was performed. After discarding upper layer, the second opaque layer (granulocyte) was transferred into new tube, washed with 10 ml of PBS by spinning at 200 xg for 10 minutes and then used for further analysis.

2.4 Cell culture methods

Generally, all cell culture works were performed and handled under sterile condition in a CAT2 cell culture hood and incubated at 37oC using a culture humidified incubator containing 5% CO_2.

2.4.1 Cell line

Two cell lines used throughout the project

2.4.1.1 EAhay 926 cell line

Immortalized human umbilical vein endothelial cells (EAhy 926) were kindly provided by Dr N. Abbassian, Department of Infection, Immunity and Inflammation, University of Leicester. These cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific, Loughborough, UK) with added 10% (v/v) heat-inactivated foetal bovine serum (FBS), penicillin (10^2 IU.ml^{-1}), streptomycin (100 µg.ml^{-1}), 2mM L-glutamine (Gln). EAhy926 cells were shown to be mycoplasma negative on a regular basis by real time qPCR.

2.4.1.2 J774 cell line

J774 cells mouse macrophage cell line (provided by Dr. B. Burke, Department of Infection, Immunity and Inflammation, University of Leicester) was maintained in a growth medium comprising RPMI (Roswell Park Memorial Institute medium) (Gibco) supplemented with 2mM L-glutamine, 10% (v/v) foetal calf serum (FCS), penicillin (10^2 IU.ml^{-1}), and streptomycin (100 µg.ml^{-1}).

2.4.2 Thawing cells

The cryovials containing the frozen cells were taken from –80 °C and defrosted within one minute by slight swirling in a water bath at 37oC. Once they become completely thawed, they were centrifuged at 1000 rpm for 5 minutes (Allegra X-22 centrifuge, Beckman Coulter, US). The supernatant was removed, and the pelleted cells were

washed one time by adding their growth media to neutralize the toxic effect induced by the remaining of freezing media. After centrifugation and discarding the supernatant, the cells were re suspended in 1 ml of media and placed into tissue culture flask containing growth media. The cells were checked for their attachment and viability using an inverted microscope on a daily basis and the media was changed every 3 days till reach the confluence.

2.4.3 Cell subculture (passage)

When the cells reached 70% confluence, they were split. Firstly, the culture medium was pipetted into a waste pot. The flask was washed with 10ml of PBS to remove the inhibitory effects of FCS during the process of trypsinization with 5 ml of trypsin EDTA (Sigma). Trypsin interrupts adhesion molecules involved in cell - cell and cell-matrix adhesion while EDTA removes Ca2+ and Mg2+ that inhibit trypsin. The flask was incubated for 5 minutes at 37°C, to allow the cells to detach from their monolayer and culture surface. During this step, they were closely monitored by inverted microscope till most of the cells observed floated off. After that, the detached cells suspension was transferred to culture tube and diluted with 10 ml of culture media containing serum to terminate the proteolytic reaction of trypsin and centrifuged at 200g for 5 minutes. Depending on the purpose of the experiment, the pellet was re suspended in appropriate amount of culture media and seeded at different densities specific to culture vessels (flask or plate) used after counting using haemocytometer (Neubauer counting chamber,Weber, England).

2.4.4 Cell counting

First, the counting chamber was set up by placing the cover slip on the chamber properly. Then, around 10 µl of well mixed cell suspension was added at the front of the gap between the chamber and the cover slip to be drifted in by the capillary force. Under microscopy, all cells defined within all four squares were counted and divided by 4 to obtain average of cell number per ml expressed as x104 /ml.

Sometime, cell counting by blue dye exclusion methods was used for counting as well as viability check. At this situation the, 100 µl of trypan blue solution 0.9 % (w/v) (Sigma) was added with 100 µl of well mixed cell suspension. Live cell with intact membrane appears unstained while dead cell stains completely with blue due to loss of their intact membrane (or energy dependent transport mechanisms)

Common formula

No. of cells (4 squares) / 4 = Average of cell number (x104 /ml).

When dye exclusion methods followed a modification of formula for counting

No. of cells (4 squares) x 2 (dilution factor) / 4 = Average of cell number (x104/ml).

Viability

% viable cells = [1.00 – (Number of blue cells ÷ Number of total cells)] × 100.

2.4.5 Freezing the cell

After counting, the cells were diluted to 1x106 /ml and centrifuged at 250g for 5 minutes. The supernatant was decanted, and the pellet resuspended in 1ml of freezing media (10% Dimethyl Sulfoxide (DMSO) in FCS and kept in a cryovial tube at -80 oC.

2.5 Microscopy methods

2.5.1 Light microscopy

2.5.1.1. Oil red O staining

The oil red o staining was used in this study to stain the intracellular fat droplet induced by incubating cells with LPS or free fatty acids. The cells were cultured on coverslips using 6 well- plates. After treatment, the supernatant was removed from each well avoiding carry over under sterile conditions. After that, wells were washed by adding around 2 ml of phosphate buffered saline (PBS) for 2 minutes. This step was repeated three times. After removing the washing buffer, the cells were fixed by adding around 2ml of normal buffered formalin (10%) into each well and incubated for 10 minutes at room temperature. The fixative solution was removed from each well. Each well was rinsed three times using distilled water. Then, mixture of 4ml propan2ol , 6ml of oil red o solution and 6ml of distilled water was prepared. Enough amount of this mixture was added to cover each well and incubated for 10 minutes at room temperature. The mixture was removed from the wells and washed three times using distilled water. About 1 ml of hematoxilin was added to each well and left for three minutes at room temperature. Then the stain solution was removed. The wells firstly rinsed by distilled water and then by running tap water for 5 minutes carefully. The slides were labelled

and left to dry. One drop of glycerol (glyec mex) was added on top of each slide. Carefully, each cover slip was picked by forceps, turned over and placed gently on the suitable slide. After drying, the slides can be examined using light microscope to visualise the red fat droplets.

- **Oil red O solution preparation**

It was prepared by adding 100 mg of powdered Oil red O stain (Sigma, UK) into 50 ml of propan2ol solution. It was mixed, filtered and kept at room temperature.

- **Normal buffered formalin (10%) preparation**

It was prepared by mixing 90 ml of PBS with 10 ml of 38% (w/v) formaldehyde, prepared in distilled water as stock.

2.5.1.2 Cytological staining (Leishman's stain and Wright's stain):

Leishman's stain and Wright's stain are commonly used stains to differentiate blood cell types. They were used in this study as a qualitative and quantitative method to analyse the PLAs adherence to endothelium. To do this, the cells were grown on 6 or 12 well plates containing coverslips and treated further for aggregate adhesion according to the purpose of each experiment. The supernatants were discarded, and the coverslips were washed with PBS at least three times to get rid of unbound blood cells. Each coverslip was covered completely with the stain and left for 2 minutes at room temperature. Then, approximately double the volume of distilled water containing 5µl of NaOH (10M) was added and mixed with already present stain to dilute (2:1) and left for 10 minutes, when Leishman's stain used, or 4 minutes when Wright's stain used. After that, the coverslips washed with distilled water, dried on filter paper and placed on a microscopic slide as described above to be ready for microscopic analysis.

- **Leishman's stain preparation**
  It was prepared by dissolving 0.2 g of Eosin-Methylene blue powder (Leishman's stain powder) in 100 ml methanol, then, wormed at 56oC for 10 to 15 minutes, filtered and stored at RT.

2.5.2 Fluorescence microscopy

Fluorescence imaging is a common and robust laboratory technique used to visualise the distribution of certain molecule within the sample by highly specific targeting with aid of a fluorescing agent in many ways. With this information, it was used in this project to generate a method that can analyse the effect of different conditions, stimuli as well as therapeutic agents, on PLAs adherence to the endothelium.

2.5.2.1 Direct immunofluorescence for platelet and leukocyte markers

In general, a monolayer of endothelial cells, which was cultured on coverslips and incubated with stimulated blood, was prepared under different conditions depending on the aim of experiment. Then, the coverslips were washed 3 times with PBS 5 minutes each to get rid of unattached cells. All coverslips were incubated initially with human Fc receptor blocking solution 5µl /each (TruStain FcX™, Biolegend, UK) for 20 minutes in the dark at room temperature. This step done to block Fc receptors that are expressed by endothelial cells and many circulating blood cells such as granulocytes, monocyte, B cells, NK cells and dendritic cells (Li and Kimberly, 2014; Holt, 2006; Ruiz *et al*, 1992). Thus, it minimises unwanted immunofluorescent staining resulting from the FcRs-mediated Ig Fc binding. After incubation, the cells were washed to remove any residual blocking solution and stained with or without (control) a mixture of 5 µl of each direct fluorescent Ab (PE-CD42b to detect platelet and FITC-CD66b to detect granulocytes) and incubated for 20 minutes in dark at 4oC. Coverslips incubated with a mixture of antibody isotypes (PE mouse IgG 1 and FITC mouse IgM) were also prepared as another control. Then, the samples were washed and fixed with 2% paraformaldehyde for 15 minutes. After the final wash to remove the fixative buffer, they were mounted by Fluoroshield Mounting Medium with DAPI and carefully inverted on a microscopic slide for florescence microscopy. Finally, all slides were analysed with the confocal microscope (OLYMPUS microscope, the software's name is FLUOVIEW).

2.5.2.2 Cellular structure labelling with Fluorescent Dyes for Imaging

2.5.2.2 .1 Staining cellular membrane of PLAs with Vybrant™ DiO Cell-Labeling Solution (Thermos Fisher, UK)

It is a lipophilic, green fluorescent dye that is characterised by stability and low cytotoxicity. It provides a uniform staining of cell membranes. With these features, it was selected to label the PLAs and track their adherence to the endothelium prepared during different experimental set up under static condition. Initially, after optimisation, 100 µl of whole blood stained with 5 µl of Vybrant™ DiO Cell-Labeling Solution for 30 minutes in dark at room temperature. Whole blood without dye was also prepared as a control. Then, the samples were processed for RBCs lysis as described previously. Then, they were washed with 1x PBS 3 times to remove excess of dye which might interfere with the analysis. The samples were incubated on inflamed (TNFα stimulated) endothelium for adhesion assay analysis, washed and fixed with 2% paraformaldehyde for 15 minutes. Finally, they were washed and mounted for fluorescent microscopic analysis.

2.5.2.2.2 Staining of nuclear DNA of endothelium or blood cells

Blue fluorescent DAPI, 4', 6' diamino-2-phenylindole 2HCl, is typically utilised to stain the nuclear DNA in multicolour fluorescence imaging technique as a counterstain. It was used in this study to contrast the green fluorescence of Vybrant™ DiO Cell-Labelling cell membrane dye in a whole blood or adhesion assay. In general, it is used last after other stain and fixation with 2 % paraformaldehyde. The coverslip was washed in PBS and covered completely with around 100 µl of DAPI (Thermo Fisher Scientific, Loughborough, UK) for 5 to 10 minutes at RT. Finally, it was rinsed with PBS, mounted and viewed with fluorescent microscopy.

2.5.3 Electron microscopy

Scanning electron microscopy (SEM) was done in this study to analyse the morphology of PLAs formed and their adherence to the endothelium under different condition while transmission electron microscopy (TEM) was done to analyse the physical association between the cells involved in the interaction under different condition as well. For whole blood stimulation assay, the samples were washed (800 g, 5 min) after erthrolysis and prepared on 24 well plates that contained glass cover slips (diameter 13mm). Where

for the adhesion assay, prepared cell culture on cover slips were washed after whole blood incubation. Then all samples, were processed as follows, fixed with 2.5 % glutaraldehyde in PBS for 30 minutes, washed in PBS buffer three times (10 minutes each), postfixed in 1% osmium tetroxide/0.1M in PBS for 45 min, followed by washing 3x for 10 min in double distilled water. The samples were next dehydrated in serial ethanol concentrations. For scanning electron microscopy (SEM), the samples were critical point-dried with CO_2, mounted onto aluminium stubs using carbon sticky tabs and sputter coated with a 300-Å layer of gold palladium for 90 sec 2.2 kV. Then, samples were examined with the Hitachi S3000H Scanning Electron Microscope with an accelerating voltage of 10 kV. For transmission electron microscopy (TEM), the samples were incubated through serial concentrations of low viscosity resin Agar in propylene oxide (25%, 50%, 75% and 100 %), embedded, and polymerised at 60 for 16 hours. Samples were sectioned using a Reichert Ultracut S Ultramicrotome followed by double staining with 2% Uranyl Acetate and Reynold's Lead citrate and viewed on the JEOL 1400 TEM with an accelerating voltage of 100kV. Images were captured using Mageview III digital camera with iTEM software.

2.6 Toxicity assay

2.6.1 MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

MTT mitochondrial activity assay was performed to assess cell metabolic activity. Cleavage of tetrazolium ring by dehydrogenase enzyme leads to conversion of MTT into insoluble purple formazan which can be solubilized and read by spectrophotometer. As only mitochondria of living cells produce dehydrogenases that also cause this reaction, it is used as a measure of cellular viability. Firstly, 50 µl of MTT stock solution (5mg/ml) was added to each well containing 100 µl of culture at concentration of 10x104 cell/ml using fresh tips. The cultures were incubated for 4 hours at 370C and 5% CO2. Then, the supernatant was carefully removed from each culture. The crystals were solubilized by adding 100 µl of acidic isopropanol (0.04-0.1 N HCl in absolute isopropanol, Sigma, UK). Absorbance of converted dye was measured by spectrophotometer at a wavelength of 570 nm (Thermo scientific, Multiskan FC).

- **MTT preparation**

It was prepared by dissolving 5 mg of MTT (Sigma,uk) in one ml of RPMI without phenol red. Then, the solution was filtered through a 0.2 μm filter and stored at 2-8 oC for further use.

2.6.2 Crystal violet assay (CV)

Crystal violet, also called hexamethyl pararosaniline chloride or Gentian violet, is a triphenylmethane dye that has the ability to bind the DNA of living cell (Feoktistova, Geserick and Leverkus, 2016). Thus, the crystal violet assay is commonly used to evaluate the cell viability. The cells were cultured in a 96 well plate and treated at different condition in triplicate according to the experiments aim. The supernatants were removed, and the cells were stained with 50μl per well crystal violet, which was prepared by dissolving 5g of dye powder in 20% (v/v) methanol in H_2O, for around 4 minutes at room temperature. After discarding the dye, the plate was washed 3x and dried by inverting the plate on a paper towel. Finally, the precipitated dye was solubilised by addition of 100μl /well of 20% (v/v) acetic acid in H_2O and the absorbance at 540nm measured by a plate reader (Thermo Scientific, MULTISKAN FC).

2.7 Protein methods

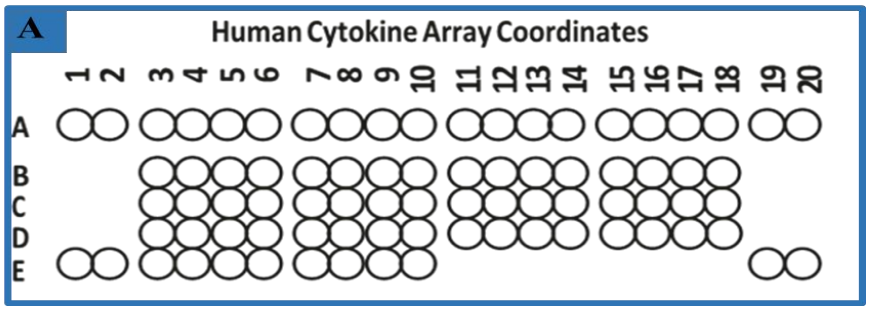
2.7.1 Proteome ProfileTM of human cytokine

The abundance of cytokines was analysed in different plasma samples (described in chapter 4) by Proteome profiler antibody array (R and D system) following manufacture's protocol. Human cytokine array kit had 4 nitrocellulose membranes. Each membrane had different capture antibodies in duplicate to specifically detect different human cytokine in the used sample (Figure 2.3). To detect these cytokines, first, each membrane was blocked by adding 2ml of array buffer 6 for one hour at room temperature. Meanwhile, 0.5 ml of array buffer 4 was added to each sample and then it adjusted with array buffer 6 to 1.5 ml final volume. 15 μL of detection antibody was added to each prepared sample and incubated for one hour at room temperature. Each sample antibody mixture was added into each membrane and incubated overnight at 2-8oC. After washing streptavidin-HRP was added to each membrane and incubated for 30 minutes at room temperature. After that, 1ml of chemi reagent mix was added to each membrane. Finally, the cytokine expression was detected by exposing the membrane to ultraviolet ray for 1 to 10 minutes using UV machine from BioRad.

2.7.2 Proteome profile Data Analysis

Each spot that developed on the membrane was analysed by Image J software to measure its pixel densities as readout of relative protein expression level. The pixel density of all the following spots was calculated in duplicate: each tested protein, negative control (PBS), reference spots (positive control) and background signal which corresponds to a clear area of the array where no antibody spotted. Then, the mean average of each related duplicate spots was calculated. Next, the average of the background density was subtracted from mean average of each spot that was normalised further with the reference spot. Finally, the graphs were plotted to compare the signals of the all proteins in the different samples.

A



B

Coordinate	Target/Control	Entrez Gene ID	Alternate Nomenclature
A1, A2	Reference Spots	N/A	_____
A3, A4	CCL1/I-309	6346	P500, SCYA1, SCYA2, TCA-3
A5, A6	CCL2/MCP-1	6347	MCAF
A7, A8	MIP-1α/MIP-1β	6348/6351	CCL3/CCL4
A9, A10	CCL5/RANTES	6352	_____
A11, A12	CD40 Ligand/TNFSF5	959	CD154, CD40LG, gp39, TRAP
A13, A14	Complement Component C5/C5a	727	C5/C5a
A15, A16	CXCL1/GROα	2919	CINC-1, KC
A17, A18	CXCL10/IP-10	3627	CRG-2
A19, A20	Reference Spots	N/A	_____
B3, B4	CXCL11/I-TAC	6373	β-R1, H174
B5, B6	CXCL12/SDF-1	6387	PBSF
B7, B8	G-CSF	1440	CSFβ, CSF-3
B9, B10	GM-CSF	1437	CSFα, CSF-2
B11, B12	ICAM-1/CD54	3383	_____
B13, B14	IFN-γ	3458	Type II IFN
B15, B16	IL-1α/IL-1F1	3552	_____
B17, B18	IL-1β/IL-1F2	3553	_____
C3, C4	IL-1ra/IL-1F3	3557	_____
C5, C6	IL-2	3558	TCGF
C7, C8	IL-4	3565	BCDF, BSF1
C9, C10	IL-5	3567	_____
C11, C12	IL-6	3569	BSF-2
C13, C14	IL-8	3576	CXCL8, GCP1, NAP1
C15, C16	IL-10	3586	CSIF
C17, C18	IL-12 p70	3592/3593	CLMF p35

Coordinate	Target/Control	Entrez Gene ID	Alternate Nomenclature
D3, D4	IL-13	3596	_____
D5, D6	IL-16	3603	LCF
D7, D8	IL-17A	3605	CTLA-8
D9, D10	IL-17E	64806	IL-25
D11, D12	IL-18/IL-1F4	3606	IGIF
D13, D14	IL-21	59067	_____
D15, D16	IL-27	246778	IL-27 A
D17, D18	IL-32α	9235	
E1, E2	Reference Spots	N/A	_____
E3, E4	MIF	4282	GIF, DER6
E5, E6	Serpin E1/PAI-1	5054	Nexin, PLANH1
E7, E8	TNF-α	7124	TNFSF1A
E9, E10	TREM-1	54210	CD354
E19, E20	Negative Control	N/A	_____

Figure 2.3: Proteome ProfileTM of human cytokine. (A) The Human cytokine Array coordinates. (B) The reference table of the 38 proteins.

2.7.3 Western blotting

Western blot or immunoblot is a common laboratory technique to detect the expression of protein of interest. It was used to analyse the expression of complement protein C5 and its cleavage product, C5a in whole blood assay as a read out of induced inflammatory condition, *in vitro*.

2.7.3.1 Preparation of samples

For whole blood stimulation assay, the whole blood was stimulated with different stimuli and the plasma extracted from theses sample by centrifugation at 200xg for 7 min. For adhesion assay, the samples prepared as follow. Endothelial cells were cultured on coverslip and incubated with whole blood at different conditions of whole blood and endothelial cell treatment. After incubation, the plasma was extracted from each condition and analysed for complement activation by western blot.

2.7.3.2 Protein assay

Pierce 660nm protein assay was originally used to determine the protein concentration of the sample (blood plasma or cell lysate) according to manufacture instructions (Thermo Scientific). Firstly, 10 point standard curve was prepared using 2 fold serial dilution of Bovine Serum Albumin (BSA) as protein source in lysis buffer. About 10 µL from each concentration of standard and samples were placed in each well of 96 well plate in triplicate. After that, 150 µL of protein assay reagent were added to each well. The plate was incubated for 5 minutes at room temperature and absorbance read by spectrophotometer at 650nm (Figure 2.4). All optical density values of samples were on the standard curve range.

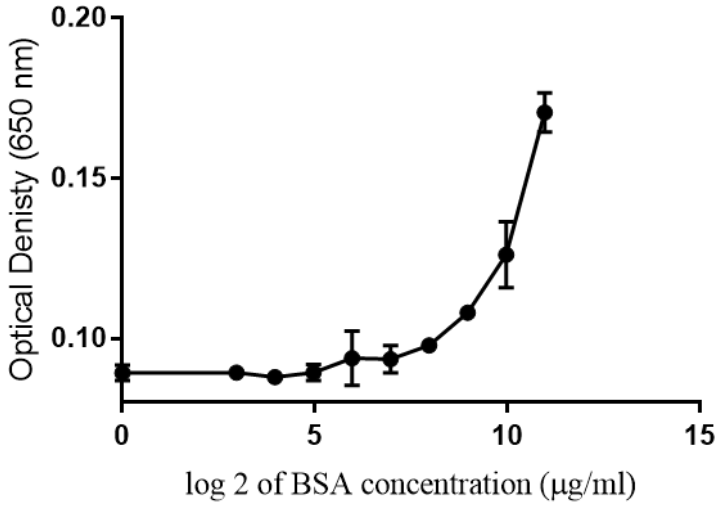


Figure 2.4: Bovine serum albumin standard curve. 10-points standard curve was generated from the known protein concentrations of Bovine Serum Albumin (BSA) prepared by 2 fold serial dilution and their absorbance values at 650nm.

2.7.3.3 Preparation of 10% SDS-PAGE gels

Initially, the plates and comb were cleaned with 70% Ethanol. The plates were assembled on the casting stand and the leakage was tested by using d.H2O. After mixing the resolving gel components, it was pipetted between the plates gently, to avoid the bubbles formation, up to certain level to allow space for stacking gel and comb. Then, it was left for 10 to 15 minutes to polymerize. After that, stacking gel mix was prepared and pipetted carefully between the plates on top of the polymerized resolving

gel until the edge of the short plate. The appropriate comb was carefully inserted and polymerisation awaited.

2.7.3.4 Gel preparation

A- Resolving gel:

It was prepared by mixing the following reagents:

- 40% Acrylamide/ bisacrylamide 1.25 ml
- Resolving buffer 1.25 ml
  (1.5M pH 8.8 Tris, 0.4% SDS)
- Distilled water 2.5 ml
- TEMED 4 µl
- 10% APS 50 µL
  Total 5 ml

B- Stacking gel:

It was prepared by mixing the following reagents

- 40% Acrylamide/ bisacrylamide 375 µl
- Stacking buffer 750 µl
  (1M Tris, pH 6.8, 0.4% SDS)
- Distilled water 1.875 ml
- TEMED 3 µl
- 10% APS 30 µL
  Total 3 ml

TMED and Ammonium persulfate (APS) were added immediately before use.

2.7.3.5 C5 and C5a western blot protocol:

The protein samples were firstly prepared for SDS-PAGE analysis by mixing about 20µg from each sample with 2x reducing loading buffer at 1:2 dilution into new reaction tubes. The samples heated at 95C˚ for 5 minutes to denature the protein to their primary structure by interrupting the disulphide bonds and immediately placed into ice. After that, the samples were spun down at 200 xg for 5 second and the supernatants were used to eliminate insoluble debris. Then, the protein samples loaded and run in 10% SDS-

PAGE gel in parallel with a 5µl protein marker (ThermoFisher scientific, UK) in 1x running buffer 60mA for 1-1.5 hour. Then, the separated proteins on the gel was electrophoretically transferred onto a cellulose membrane (Thermo Fisher,UK) by blotting at 250mA for 1 hour in transfer buffer. The membrane was incubated directly with 20ml of 5% (w/v) dried fat free milk in PBS for 1 hour at room temperature on shaker. Then, it was washed 3 times for 15 minutes each by PBS 0.05% Tween 20 with shaking at room temperature. After that, the membrane was probed with the primary antibody rabbit polyclonal anti-human C5a IgG (Gene Tex.com) diluted in 5% skimmed milk in PBS (w/v) at a final concentration of 1µg/ml overnight at 4C°.Then, it was washed as stated above and probed with a secondary antibody HRP conjugated Swine anti-rabbit (Dako REF P0399) which diluted in in 5% skimmed milk in PBS (w/v) at a final dilution of 1:30000 for 2 hours maximum with shaking as well at RT. Finally, the membrane was washed as described above and the face of the membrane exposed to equal amounts of Enhanced chemiluminescence (ECL) western blot substrate reagents (Amersham Biosciences) for 2 minutes following the manufacture instructions wrapped in cling film and exposed to ultraviolet ray (UV) using the UV machine from Bio-Rad to detect the protein bands.

Table 2.3: Preparation of buffers used in western blot.

Solution	**Preparation**
Non-reducing 2x Loading Buffer	3ml d H_2O, 1ml 0.5mM Tris –HCL pH 6.8, 800 µl Glycerol, 1.6 ml 10% Sodium dodecyl sulfate (SDS) (Fisher) (w/v) in H2O. For reducing condition, [200mM Dithiothreitol (DTT) (Sigma)] is added to above components.
10x Running buffer	30g Tris -Base, 144g Glycine (Fisher) and 10 g SDS in 1L d H_2O (for 1x Running buffer dilute 1:10 in d H_2O .
1x Blotting Buffer	5.9g Tris -Base, 2.9g Glycine, complete to 1L H_2O . 10% of 100% Methanol were added to this solution. This solution should be prepared fresh.

2.7.3.6 SDS-PAGE gel staining

Separated proteins in SDS-PAGE gel were identified by staining the gel with Coomassie brilliant blue R-250 which prepared by mixing the following ingredients: 3g stain in 100ml acetic acid, 450ml IMS, 450ml H_2O). Then, the excess of stain was eliminated with a de-staining solution (50ml IMS, 100ml acetic acid, 850ml H_2O) (Figure 2.5).

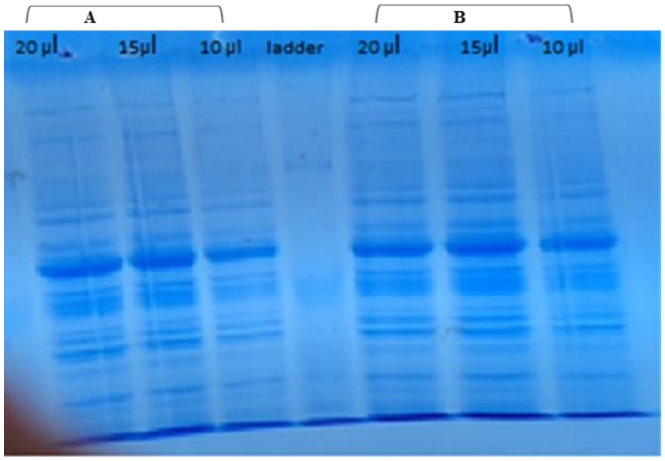


Figure 2.5: Example of plasma samples stained with Coomassie blue to visualize the protein bands on SDS-page gel. Different volume 20,15, 10 µl of plasma samples from two voulnteers, A and B were separated on 10% SDS-PAGE gel and visualized with Coomassie blue staining.

2.7.3.7 Ponceau S Staining of Western blots

To locate the protein bands on the blot quickly, the membrane was stained with Ponceau S Staining [0.5% (w/v) Ponceau S with 1% acetic acid] and the excess of staining was removed by washing with distilled water (Figure 2.6)

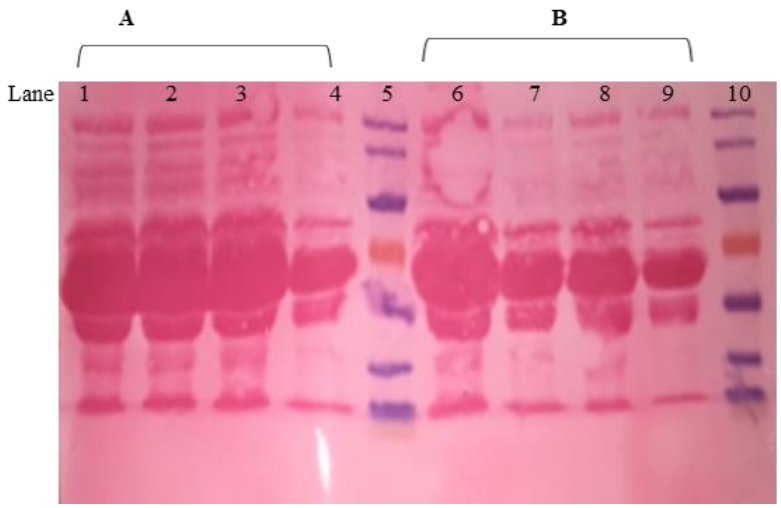


Figure 2.6: Example of plasma samples stained with Ponceau S to visualize the protein bands on nitrocellulose membrane. The plasma samples from two voulnteers, A and B were separated on 10% SDS-PAGE gel, blotted on nitrocellulose membrane and stained with Ponceau S stain to locate the protein bands and confirm its transfer on the memrane. Lane 5 and 10, protein markers; lane 1 and 6, HKK stimulated WB after 1 h incubation; lane 2 and 7, LPS stimulated WB after 1 h incubation; lane3 and 8, unstimulated WB after 1h incubation (spontinous aggregation); lane 4 and 9 unstimulated WB at zero time point.

2.8 Quantitative measurement by Enzyme linked Immunosorbent assay (ELISA)

2.8.1 Mouse TNF alpha ELISA (R and D system)

Following manufacturer's instructions, a 96 well plate (Nunc Maxisorp) was coated with 100µl /well of diluted (800ng/ml) capture antibodies overnight at room temperature. After that, the plate was washed 4 times by 300 µl of 0.05% (v/v) Tween-20 in PBS as wash buffer and blocked by adding Reagent Diluent to each well for 1hour incubation at room temperature. Different concentrations of Mouse TNF-α Standard were prepared by double serial dilution according to instruction (ranged from zero standard (0 ng/mL) which contain diluent only to (2 ng/ml) (Figure 2.7). Then, the plate was washed and incubated with 100 µl samples and slanderers for 2 hours at room temperature in triplicate. After washing, detection antibody was added to each well and incubated for 2 hours at room temperature (50ng/ml). The plate was washed again and

streptavidin-HRP was added to each well and left at room temperature for 20 minutes. After that, substrate solution was added to and incubated for 20 minutes at room temperature. Finally, the reaction was interrupted by adding stop solution and red spectrophotometrically at 450nm. All absorbance values of samples plotted within the determination range of standard curve.

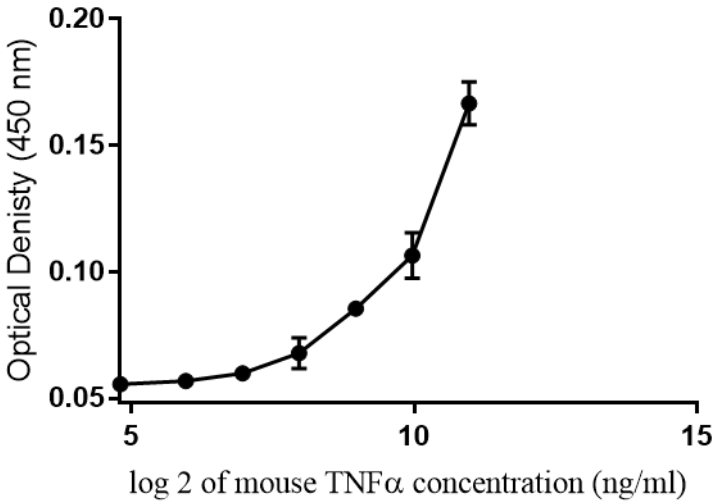


Figure 2.7: Mouse TNFα standard curve. 7-points standard curve was generated from the known concentrations of Mouse TNF-α Standard,prepared by double serial dilution according to instruction (ranged from zero standard (0 ng/mL) which contain diluent only to (2 ng/ml), and their absorbance values at 450nm.

2.8.2 Human TNF alpha ELISA (PeproTech, US)

ELISA 96 well microplates were coated with TNF alpha capture antibody diluted with PBS to a concentration of 1µg/ml. 100µl of the diluted capture antibody were added to each well. The plate was sealed and incubated overnight at RT. On the following day, the excess of antibody's fluid was removed, and the plate was washed four times with 300µl of washing buffer, 0.05% (v/v) Tween-20 in PBS. After that, the plate was inverted and blotted on paper towels to drain the remaining buffer. Then, it was blocked

with 300µl per well with 1% (w/v) BSA in PBS and kept for 2 h at RT. Then, the plate was washed again as described. 10 points of 2 fold serial dilution of Human TNF-α standard was prepared in diluent (0.05% (v/v) Tween-20, 0.1% BSA in PBS) starting from 4ng/ml to zero (Figure 2.8). Then 100µl of standard, and the diluted plasma samples were immediately added to each well in triplicate and kept at room temperature for 2 hours. Afterward, the plate was washed 4 time with washing buffer, and 100µl of detection antibody which was diluted with diluent to 0.5µg/ml, was added for each well and incubated at RT for 2 hours. After removing the fluid and washing the plate 4 times using a wash buffer. Streptavidin HRP conjugate was diluted to 1:2000 in diluent for a final volume of 10 ml, and 100µl were added per well, and kept for 30 minutes at room temperature. The plate was aspirated, and washed 4 times, and 100µl of substrate solution TMB 3,3',5,5'-Tetramethylbenzidine (Sigma) were added to each well, and incubated at room temperature for 20 minutes to allow colour development. The reaction was stopped by 100µl adding stopping solution (Sulfuric acid 6.6ml+ D.W 4.4ml) was added, and plate was read using an ELISA plate reader at 620 wavelength (680 Microplate Absorbance Reader).

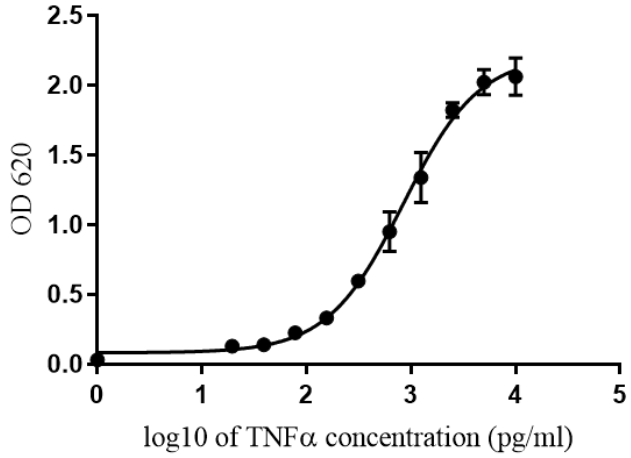


Figure 2.8: Human TNFα standard curve. 10-points standard curve was generated from the known concentrations of humanTNF-α Standard,prepared by double serial dilution according to instruction (starting from 4ng/ml to zero standard (0 ng/mL) which contain diluent only, and their absorbance values at 620nm.

2.8.3 Human interleukin 8 (Il-8) ELISA

Human IL-8 Mini TMB ELISA Development Kit from peprotech was used following the manufacturer's instruction. To prepare the 96 well microplates IL-8 capture antibody was first diluted with PBS to a concentration of 0.25µg/ml and added at volume of 100µl per well. The plate was sealed and incubated overnight at RT. Next day, the excess of antibody's fluid was removed, and the plate was washed four times with 300µl of washing buffer, 0.05% (v/v) Tween-20 in PBS. After that, the plate was inverted and blotted on paper towels to remove the residual buffer. Then, 300µl of blocking buffer compromising of 1% (w/v) BSA in PBS was added to each well to block nonspecific binding sites and the plate kept for 2h at RT. Then, the plate was washed again as described. Two-fold serial dilution of human IL-8 standard was prepared in diluent (0.05% (v/v) Tween-20, 0.1% BSA in PBS) starting from 1ng/ml to zero (Figure 2.9). Then 100µl of standard, and the diluted plasma samples were immediately added to each well in triplicate and kept at room temperature for 2 hours. Afterward, the plate was washed 4 time with washing buffer, and 100µl of detection antibody which was diluted with diluent to 0.5µg/ml, was added for each well and incubated at RT for 2 hours. After removing the remaining fluid and washing the plate 4 times using a wash buffer, Streptavidin HRP conjugate was diluted to 1:2000 in diluent for a final volume of 10 ml, and 100µl were added per well, and kept for 30 minutes at room temperature. The plate was aspirated, and washed 4 times, and 100µl of substrate solution TMB 3,3',5,5'-Tetramethylbenzidine (Sigma) were added to each well, and incubated at room temperature for 20 minutes for colour development. The reaction was stopped by 100µl adding stopping solution (Sulfuric acid 6.6ml+ D.W 4.4ml) was added, and plate was read using an ELISA plate reader at 620nm wavelength (680 Microplate Absorbance Reader).

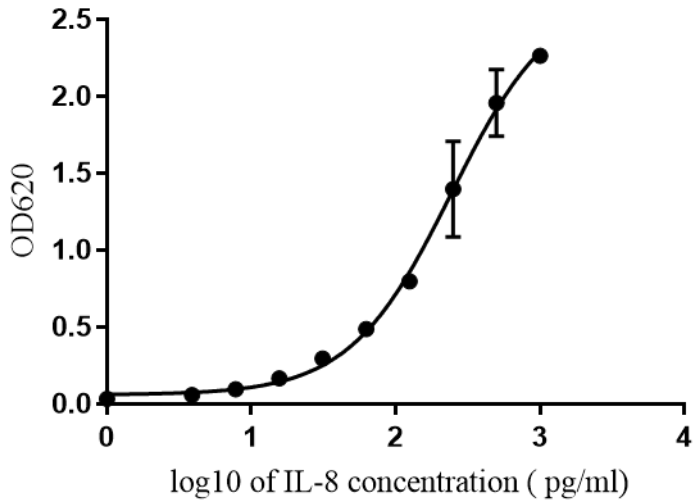


Figure 2.9: Human IL-8 standard curve.

2.8.4 MPO-DNA ELISA

To quantify the Neutrophil extracellular trap (NET) in human plasma extracted from whole blood that stimulated with different condition with HKK and Vitamin D and, then, incubated on treated endothelium with TNFα and Vitamin D, a capture ELISA was performed as described previously (Handono *et al*, 2016; Caudrillier *et al*, 2012; Yoo *et al*, 2014). Anti-myeloperoxidase capture antibody, 79µg/ml, (MPO mAb, Rabbit monoclonal, clone SP72, Sigma-Aldrich, UK) was diluted 1:100 and coated on coated onto 96-well plates (100µl each well) overnight at 4oC. On the following day, the excess of antibody's fluid was removed, and the plate was washed four times with 300µl of washing buffer, 0.05% (v/v) Tween-20 in PBS. After that, the plate was inverted and blotted on paper towels to remove the residual buffer. Then, 300µl of blocking buffer compromising of 1% (w/v) BSA in PBS was added to each well to block nonspecific binding sites and the plate kept for 2h at RT. Then, the plate was washed again as described. HRP-labeled anti-DNA mAb, 75µg/ml (Cambridge Bioscience Ltd, UK; Catalogue number: D5425-3-200) was diluted to 1:100 in diluent (0.05% (v/v) Tween-20, 0.1% BSA in PBS). 20 µl of human plasma with 80 µl of diluted HRP-labeled anti-DNA mAb was added to each well (total volume of 100µl /well) and incubated at RT for 2 h. 20 µl of diluent (not plasma) with 80µl HRP-anti DNA mAb was prepared as a lower detection limit for the assay.After washing, 100µl

of substrate solution TMB 3,3',5,5'-Tetramethylbenzidine (Sigma) were added to each well and incubated at room temperature for 20 minutes for colour development. Upper detection limit of the assay was prepared by adding 100µl of HRP-DNA mAb and 100µl of substrate solution TMB. The plate was read using an ELISA plate reader at 620nm wavelength (680 Microplate Absorbance Reader). Soluble NET formation level was expressed as increase in absorbance above the experimental control (OD).

2.9 DNA methods

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

2.9.1 Extraction of ribonucleic acids RNA from EAhy 926 cells

Confluent EAhy926 cells were incubated with whole blood under different condition in 25 cm_2 flask (details in chapter 4). After removing the blood, each flask washed with PBS and 1 ml of Trizol-reagent (Ambion® from Thermo Fisher Scientific, UK) was added for 5 minutes at room temperature to lyse cells. In order to isolate the RNA, the lysed cells from each sample were placed into 1.5ml new reaction tubes and incubated with 200 µl of chloroform (Fisher Chemical). Next, samples were shaken vigorously for about 15 seconds and, next, left at room temperature for 15 minutes. After centrifugation at 200xg for 15 minutes, three layers were formed in tube and just the uppermost colourless layer was gently transferred into new tube. After that, 500µL of Isopropanol (Fisher Chemical) was added to each sample and shaken well for 5seconds before incubation for 10 minutes at room temperature to precipitate the RNA. The samples were spun down at 200xg rpm for approximately 10 minutes to pellet the RNA, the supernatants were then removed and replaced with 1 ml of 75 (v/v) % ethanol in H_2O as a washing step spun down again at 200 xg for 5 minutes for washing. After that, the ethanol was discarded, and the pellet was left to air dry at room temperature. Thereafter, the pellet was re suspended in 25 µl DEPC (Diethyl pyrocarbonate, Sigma D-5758) 0.02 (v/v) % treated water. The concentration of RNA in each sample was measured (in ng/µl) by nanodrop machine equipped with a program RNA-40 (Invitrogen™ from Thermo Fisher Scientific, UK) at 260nm wavelength.

2.9.2 Synthesis of complementary Deoxyribonucleic Acid (cDNA)

The first strand of cDNA was formed from the extracted RNA by using the Thermo Scientific Revert Aid H Minus First Strand cDNA Synthesis Kit (K1639) according the

manufacturer's instruction. Firstly, 3µg of total RNA was added to 1µl of Oligo (dT) 18 primer and topped up with nucleases free water to a final volume of 12 µl for each reaction tube. To denature the total RNA, the samples were kept at 65 C for 5 minutes and then immediately chilled on ice. Thereafter, a mixture of 1µl of RiboLock RNase inhibitor (20 U/ µl), 1µL of RevertAid H Minus M-MuL Reverse Transcripase (200U/ µL), 2µl of dNTP Mix (10mM) and 4µl of 5xReaction buffer was added for a final volume of 20µl per each reaction. Afterward, the reaction mix was incubated for 60 min at 42C˚followed by heating at 70C˚ for 5min to terminate the reaction in the PCR machine (TECHNE, TC-521). Finally, the cDNA was stored at – 20oC for further use.

2.9.3 Reverse transcriptase polymerase chain reaction (RT-PCR)

RT-PCR is a very popular technique to amplify several target genes. The PCR reaction was set up by mixing the following reagents into a PCR tube: 3 µl from the complementary DNA that prepared as described above, 2.5 µl 10X Reaction buffer, 1.5 µl MgCl (25mM), 4 µl dNTPs (1.25mM), 0.2 µl Thermo Prime Enzyme (5 U/µl), 10.8 µl PCR grade water, 2 µl forward primer (5µM) and 2 µl reverse primer (5µM). The total volume was 25µl for each reaction. Next, the reaction was run by the following programme using PCR machine (TECHNE, TC-3000 from Bibby Scientific, Staffordshire, UK). It was initially heated at 94 for 2 minutes and then cycled up to 30 times. Each cycle consists of denaturation step at 94 oC for 1min followed by annealing step at 53-60 oC, according to the sequence of primer for 1 min, and elongation step at 72 oC for 1min, adjusted according to size of the expected product. After 30th cycle, the reaction was kept at 72oC for 10 min for final extension. Finally, the reaction stopped and maintained at 4 oC.

2.9.4 RT qPCR product analysis by Gel electrophoresis

1% agarose was prepared by dissolving 1 g of agarose powder with 100 ml of 1x TAE buffer. The Ethidium Bromide was added to the mixture at concentration of 0.5 µg/ml from a stock solution of 10mg/ml. Then, it was poured into appropriate gel tray with comb and left at room temperature to solidify. 2 µl of each DNA sample were mixed with 3 µl of 10x loading dye and 10 µl of DNA free water. The comb was removed, and gel tray was placed into gel electrophoresis chamber and the DNA samples were carefully loaded into the wells. 10 µl of 1Kb DNA ladder were loaded into first well of

agarose gel to determine the approximate size of DNA samples. Electrophoresis was accomplished by applying a stable electrical current at 95 V until the dye passed 75 - 80% of the gel. After that, the agarose gel was removed from the electrophoresis chamber and examined under ultraviolet light to visualize the DNA amplicons using the UV machine from Bio-Rad.

2.9.5 Real-Time Quantitative polymerase chain reaction (RT-qPCR)

It is highly sensitive method to quantify the specific gene expression in different samples. In principle, it uses SYBR Green I dye (SensiMixTM SYBR Kit, Cat.QT605) as a detector. SYBR Green I dye is a DNA binding dye that releases fluorescence only when it binds to the double strands DNA and can be measured at the end of each amplification cycle. Thus, the amount of amplified DNA is accurately measured on the basis of accumulated fluorescence. To do this, the RNA firstly was extracted from each sample to synthesize its own cDNA to be used as template for SYBR Green based qPCR. The cDNA of each sample was diluted in sterile nuclease free water at dilution of 1:4. Thereafter, the RT-qPCR master mix of 2µL reverse primer (5µM), 2µL forward primer (5µM), 10 µl of SYBR Green I dye (SensiMixTM SYBR Kit, Cat.QT605), and 3µl nuclease free H2O was mixed with 3µl of diluted cDNA. The final volume of each reaction is 20 µl. A non-template control was also prepared as a negative control by replacing cDNA with sterile nuclease free H2O. Each reaction was prepared in duplicate using strip of 4 tubes and 0.1 ml caps (QIAGEN, 981103). All samples and non-template control were run by the Corbett: Rotor-GeneTM 6000 machines and software. The reaction samples were heated for 10 minutes at 95°C and then subjected to 40 cycles of denaturation at 95°C for 15 second, annealing usually at 55 oC (should be determined individually according to sequences of primers) and extension at 72°C for 15 seconds then melt at 55-95°C for a second on the 1st step and 5 seconds on next steps (Table 2.3).

The amplification products of each target gene are tracked by detecting the accumulated fluorescence signals in real time. The cycle threshold (CT) ,which defined as the number of cycle at which the fluorescence signal cross the threshold, was calculated by the machine as the main output value for each sample. Generally, in real time qPCR, a CT value below CT 29 indicate a very strong positive reaction due to the abundance of

the target gene. While CT value between 30-37 reflects the presence of a moderate amounts of target gene. On the other hand, all CT value lie between 38 and 40 mean a very weak reaction due to a negligible amount of target that might be duo to the amplification environmental contaminants. In addition, all CT values under the threshold during all 40 cycles considered as negative results that indicate a very low or an undetectable expression of the target gene (Figure 2.10). The CT values of the samples were further analysed to determine the relative gene expression at different condition as described below.

The melting curve, another output created by the light cycler software, was used to confirm the amplification of a single product without any primer dimer or contaminants amplification products that expressed as a single peak (Tm) at around an 80-90oC (Figure 2.11).

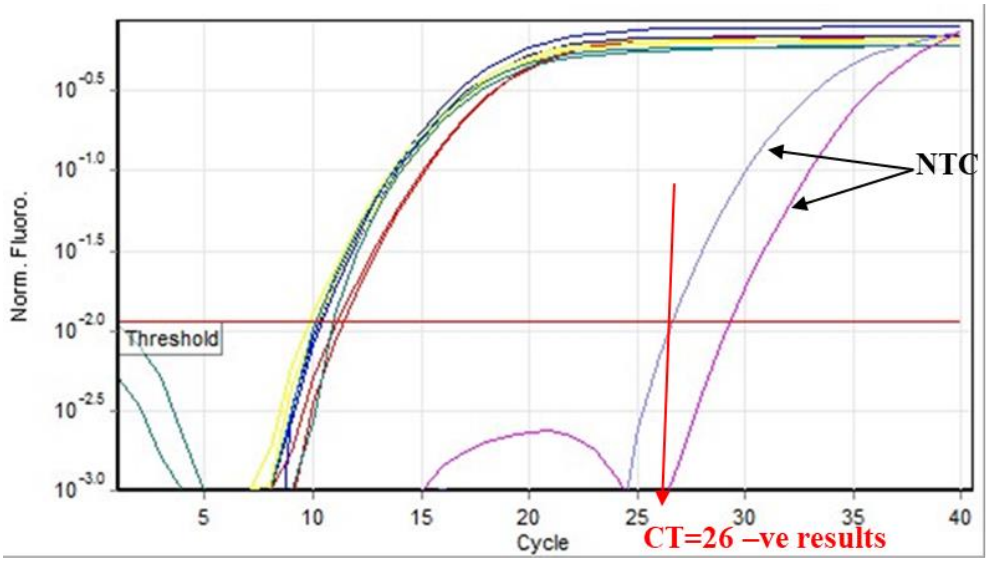


Figure 2.10: Amplification curve. The figure shows amplification curve of house keeping gene, GAPDH, from different cDNA samples; untreated EAhy926, EAhy926 +TNFα, EAhy926+TNF α+ HKK in medium and EAhy926+TNF α+ HKK-WB. Non-template control (NTC) indicated in arrow and shows that any CT value above 26 is considered as negative.

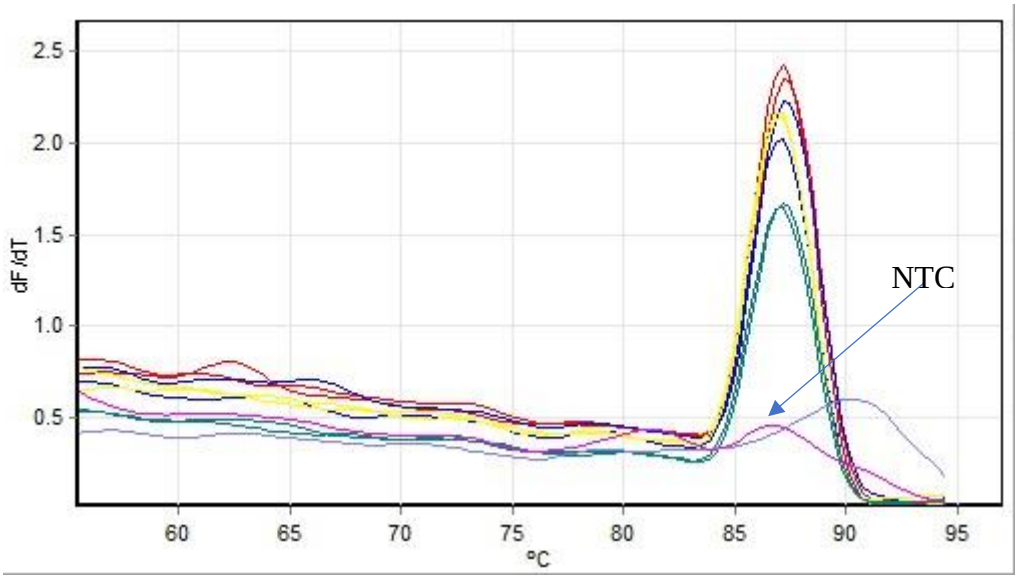


Figure 2.11: Melting curve analysis confirming specific amplification from qPCR using SYBR green. The figure shows example of house keeping gene, GAPDH, melting curves from different cDNA samples; untreated EAhy926, EAhy926 +TNFα, EAhy926+TNF α+ HKK in medium and EAhy926+TNF α+ HKK-WB. Non- template control (NTC) indicated in arrow.

Table 2.4: Sequences of primer pairs and their annealing temperatures.

	Forward Sequences 5'-3'	Reverse sequences 5'-3'	Annealing Tm	Product size
E Selectin	CCGAGCGAGGCTACATGAAT	GCATCGCATCTCACAGCTTC	60	178 bp
P Selectin	GCGGTGGCTTCTACGATAGG	TTCATGGGTGTTTATGGAAACCTTA	60	184 bp
VCAM1	TGTTTGCAGCTTCTCAAGCTTTT	GATGTGGTCCCCTCATTCGT	60	181 bp
ICAM1	CACCCTAGAGCCAAGGTGAC	GGGCCATACAGGACACGAAG	58	185 bp
CD40 ligand	AGCCAGTTTGAAGGCTTTGT	TTCAGCCCACTGTAACACAGAT	58	162 bp
Tissue Factor	AGTTCAGGAAAGAAAACAGCCA	CTGGCCCATACACTCTACCG	58	153 bp
GAPDH	CCTGGAGAAACCTGCCAAGTATG	AGAGTGGGAGTTGCTGTTGAAGTC	55	213 bp

2.9.6 RT-qPCR analysis and calculation

The results were analysed to measure the relative expression of target gene in each sample using (2-ΔΔCT) and Microsoft office Excel (2010). GAPDH was used as housekeeping gene due to its expression stability among treated samples and their related controls (untreated) (Gustin *et al*, 2008).

2^-ΔΔCT

ΔΔCT =ΔCT (test)-ΔCT (calibrator)

ΔCT (test) = CT (target gene) - CT (ref gene)

ΔCT (calibrator) = CT (target gene) - CT (ref gene)

CT: Cycle number at which detectable signal is achieved.

Calibrator: The control sample, meaning an un-treated sample.

Test: Test sample means treated.

Reference (ref): The reference gene is the gene that expressed at a constant level in all test and control samples without being affected by the experiment treatment.

2.10 Bacteriological methods

All bacterial works were performed under sterile condition in CAT2 lab.

2.10.1 Bacterial strains used in this study

Klebsiella pneumoniae (clinical isolate, KR3153) was obtained from the laboratory collection of Dr Kumar Rajakumar, Department of Infection, Immunity and Inflammation, University of Leicester). Community associated methicillin-resistant *Staphylococcus aureus* (MRSA) as a representative of *S. aureus* clinical isolates was provided by Dr J Morrissey, Dept. of Genetics, University of Leicester

2.10.2 Media preparation

The used media was prepared as stated in manufacture instructions and sterilized by autoclaving at 121oC for 15 minutes.

- **Brain Heart Infusion Medium (BHI)**

BHI broth was prepared by adding 6.14 g of powdered medium to 200 ml of distilled water. It was mixed very well, autoclaved and stored at room temperature.

- **Lauria Bertani medium (LB)**

LB broth was made by dissolving 2 g of NaCl, 1 g of yeast extract and 2 g of tryptone in 200 ml of distilled water. Then, it was sterilized by autoclaving and stored at room temperature.

- **Lauria Bertani agar (LA)**

LB agar was made by dissolving 2g of NaCl, 1g of yeast extract, 3 g agar and 2g of tryptone in 200 ml of distilled water. Then, it was autoclaved and left to be cooled to around 50 oC. 100µg/ml of ampicillin were added into medium, well mixed and poured into petri dishes at amount of 20 ml per dish.

2.10.3 Growth preparation

- **Growth condition**

Bacteria culture of both strains were prepared by inoculating single colony from LA plate aseptically into 10 ml of fresh LB broth in a 50ml tube. Then, it was placed into incubator at 37oC for approximately 14-16 hours overnight or until reach the desired optical density according to the purpose of experiment. For growth on solid media, each bacterial strain was streaked on LB agar plates incubated overnight at 37oC.

- **Glycerol stock preparation**

A -80oC stock of each strain was aseptically prepared by inoculating single colony of bacteria from the plate into 10 ml of LB broth. Then, the inoculated tubes were incubated at 37c until the culture reached the mid –log phase. 1 ml of each culture was used to measure the optical density at 600nm wavelength (OD600) by using the spectrophotometer till the OD600 reached 0.4 to 0.6. After that, the cultures were centrifuged at 3000 rpm for 15 minutes. For each culture, the supernatant was removed by plastic pipette and the pallet was suspended into 1ml of BHI broth containing 30%

sterilized glycerol. Each suspension was divided into stocks of 250 µl in sterilized Eppendorf tube and stored at -80.

2.10.4 Heat killing protocol

Each strain of bacteria were cultured in LB broth at 37oC and monitored by reading the optical density at 600 nm till reaching the late logarithmic phase, to ensure the maturity of the bacteria and maximal production of surface proteins prior t shedding (personal communication with Dr J Morrissey), harvested by centrifugation at 3000 rpm for 15 minutes, washed and re suspended in PBS. Then, after optimisation, *Klebsiella pneumoniae* suspension was heat inactivated at 60 °C for 30 minutes while *S. aureus* at 80 °C for 10 minutes. To check success of heat treatment, overnight incubation of a streaked suspension on LB agar was performed and did not show any bacterial growth. Gram stain of sample showed bacteria with intact surface. 200 µl aliquots of sample of each strain was stored at -80oC.

2.10.5 Colony forming unit assay

To determine the bacteria concentration in each phase, mid logarithmic phase or early stationary phase), 10-fold serial dilution was prepared, starting from 1/10 to 1/108, in PBS. 40µl of bacterial culture from each dilution were spotted on LA agar plates and incubated overnight at 37oC. Single colonies were manually counted, and the bacterial concentration of stock was calculated in colony forming unit per ml (CFU/ml) following this formula:

CFU/ml = (number of colonies x dilution factor) / volume of culture plate.

2.10.6 Gram stain

A thin film of heat killed bacterial suspension was prepared on microscopic slide for gram stain to check for the presence of intact surface. After fixing the smear by passing through flame three times, it was stained with crystal violet for 2 minutes at room temperature. Then, it was washed gently with tape water and covered with iodine solution for 2 minutes. After washing, it was decolorized with acetone for 5 seconds. Finally, it was washed, counter stained with safranin for 1 minute and washed again. As soon as it was completely dried, it was examined under light microscopy.

2.11 Statistical analysis

All results are given as mean ± standard error of the mean (SE). Significance of changes was assessed by Kruskal-Wallis test and post hoc testing by Dunn's multiple comparisons test or Mann-Whitney test. Flow cytometric data were analysed by ANOVA and Tukey's multiple comparisons test or pared t test as they follow Gaussian distribution. GraphPad Prism 7 software was used for all analyses. A value $p < 0.05$ was considered statistically significant in all cases. The linear and sigmoidal standard curves were plotted by Graph prism 7 software to calculate the unknown samples. Statistical advice was sought from Dr Maria Viskaduraki, B/BASH Biostatistician, University of Leicester.

Chapter 3

In vitro model of inflammatory platelet granulocyte aggregates (PGAs)

3.1 Setting up experimental protocol to study PGAs by flow cytometric analysis:

Flow cytometric measurement of platelet leukocyte aggregates has been widely used in the studies to investigate the effects of several *in vitro* blood stimulations, such as LPS, toxin, thrombin receptor activating protein (TRAP), adenosine diphosphate (ADP), platelet activating factor (PAF) or N-formyl-methionyl-leucyl-phenylalanine (fMLP) (Klinkhardt and Harder, 2005; Nkambule, Davison and Ipp, 2015a; Li, Goodall and Hjemdahl, 1997). This method is also used to quantify PLAs, platelet neutrophil (PNAs) and platelet monocyte aggregates (PMAs), in circulation where conditions such as acute coronary disease, acute lung injury and haemolytic uremic syndrome are present. Thus, it was selected in this project as a methodological tool for PGAs analysis. However, these studies follow different sample preparations and analysis protocols with no clear guideline emerging from the literature. Therefore, the aim of this part was to develop a precise and reproducible flow cytometric analysis strategy to measure the formation of PGAs under controlled condition *in vitro*. A subsequent analysis would deal with studying the effects of pathogen associated molecular patterns (PAMPs) on PGAs formation.

3.1.1 Protocol development

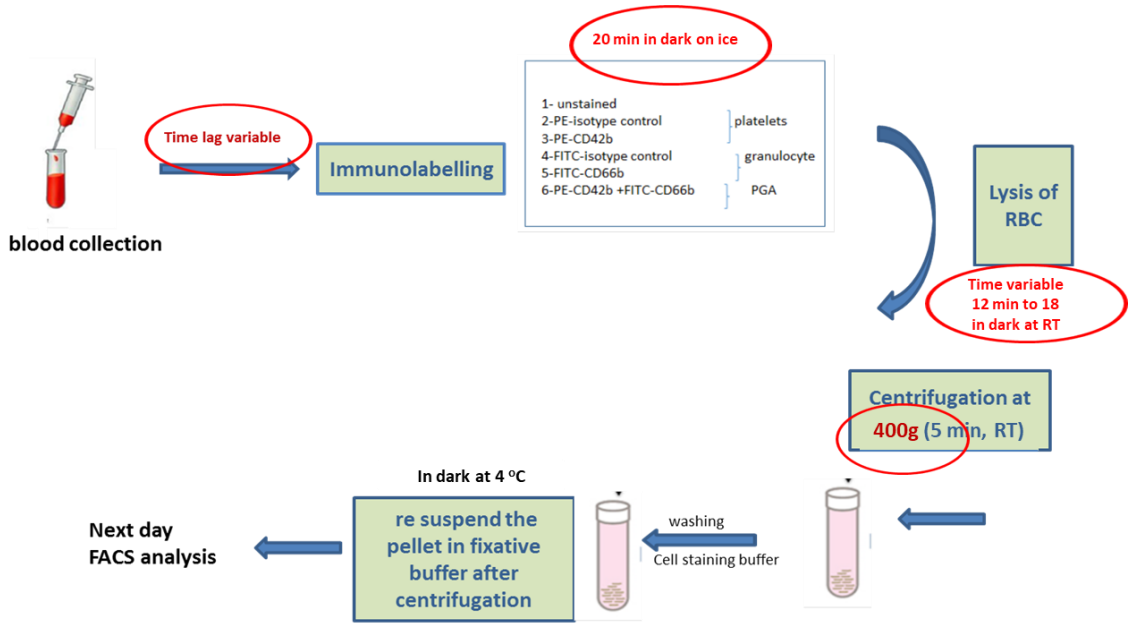


Figure 3.1: Experimental design to analyse PGAs by flow cytometry. Whole blood was collected with light tourniquet by peripheral venous puncture using G21 needle into tubes containing 10 mM ethylenediamine tetra acetic acid (EDTA, pH 8), or Hirudin

(150 U/ml inverted gently to ensure proper mixing of whole blood with anticoagulant, immunolabbeled strictly within 10 minutes of collection and incubated for 20 minutes in dark. After that, the RBCs were lysed by lysis buffer for exactly 12 minutes. Centrifugation force was adjusted at 800g to pallet most of the cells. Finally, the pellet was washed, suspended in fixative buffer, stored at 4 C^{o} and analysed by FACS Aira within 24 h.

As the main sources of cytometric data variability resulted from inconsistency of sample preparation, staining, FACS instrument setup and analysis, a standardized protocol of optimised blood sample collection, processing, staining, fixation and flow cytometric analysis methods was developed (Figure 3.1).

3.1.1.1 Effect of RBCs Lysis:

Whole blood samples from two healthy volunteers were collected into 10mM EDTA Then, aliquots of whole blood were stained with mouse antihuman CD42b: PE (to detect platelets) and mouse antihuman CD66b: FITC conjugated antibody (to detect granulocytes) simultaneously or PE mouse IgG 1 and FITC mouse IgM as isotype controls. CD42b and CD66b antigens are commonly used as markers to identify the platelets and granulocytes, respectively by flow cytometry. For RBCs lysis, the samples incubated with RBCs lysis buffer for 18 minutes at RT according to manufacture's instruction. Then, the samples were washed, fixed and processed for analysis as shown in figure 3.1.

Data was acquired using FACS Aira flow cytometer, a total of 20,000 events were collected including all type of leukocytes. Figure 3.2(A) demonstrates that lysis of live RBC for 18 minutes was not successful and might cause damage to the leukocytes as there appeared to be lots of debris, dead cells and the leukocyte populations can't be distinguished from each other.

Further experiments were done to optimise the RBC lysis methodology after adjusting the pH of lysis buffer at 7.4 and decreasing the time of incubation at 12 minutes, the minimum time range stated in the manufacture. Figure 2 B showed the effective lysis of RBCs without damaging leukocytes as their subpopulations can be clearly distinguished from each other based on the differences in the granularity and size. This was further confirmed using antibody against the granulocyte marker CD66b (Figure 3.2 b).

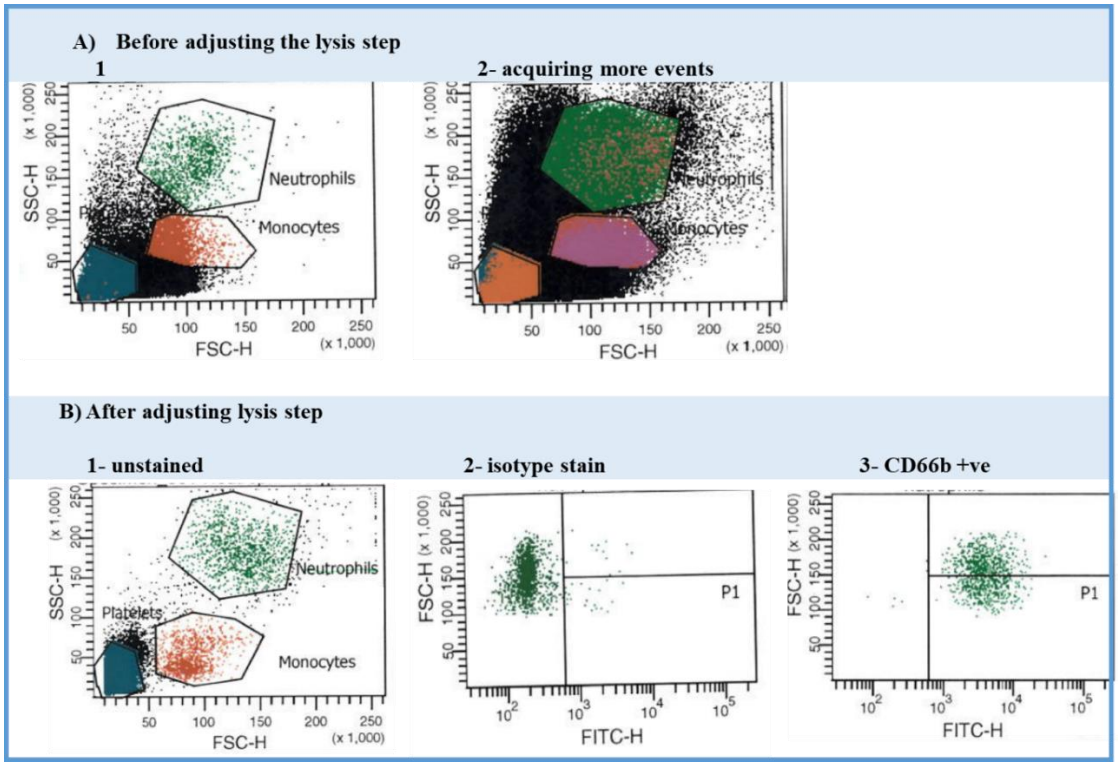


Figure 3.2: Effect of RBCs lysis. A1 and A2 both show live blood cells treated with lysis buffer for 18 minutes and demonstrate suboptimal cell lysis method, as there appears to be a lot of dead cells and debris resulted most probably from cell damage by lysis. These debris and dead cells interfered with leukocyte subpopulation identification and became more prominent when more events were acquired A2. B) shows blood where RBCs have been lysed with the same lysis buffer but after adjusting its ph at 7.4 and lysis time for exactly 12 minutes. The monocyte (red) and neutrophil (green) subpopulations can be clearly identified based on their size and granularity (B1). Optimal RBCs lysis was further confirmed by staining neutrophil population with their specific antibody CD66b (B2,B3) that showed nearly all neutrophil were CD66 positive and excluded any obvious damage to the leukocytes.

3.1.1.2 Optimizing brightness and gating strategy of granulocyte population:

After adjusting RBCs lysis, more experiments were set up. The results of FACS experiments which were obtained from labellings done on different days using whole blood samples from the different healthy individuals were inconsistent. They were variable in term of population density and pattern of population and required manual adjustment of the pre set gates for each sample within the same assay and between the two experiment while the same number of events (20,000.) was collected for each sample (figure 3.3). Additionally, there was a slipover as a result of spectral overlap which needed to be compensated.

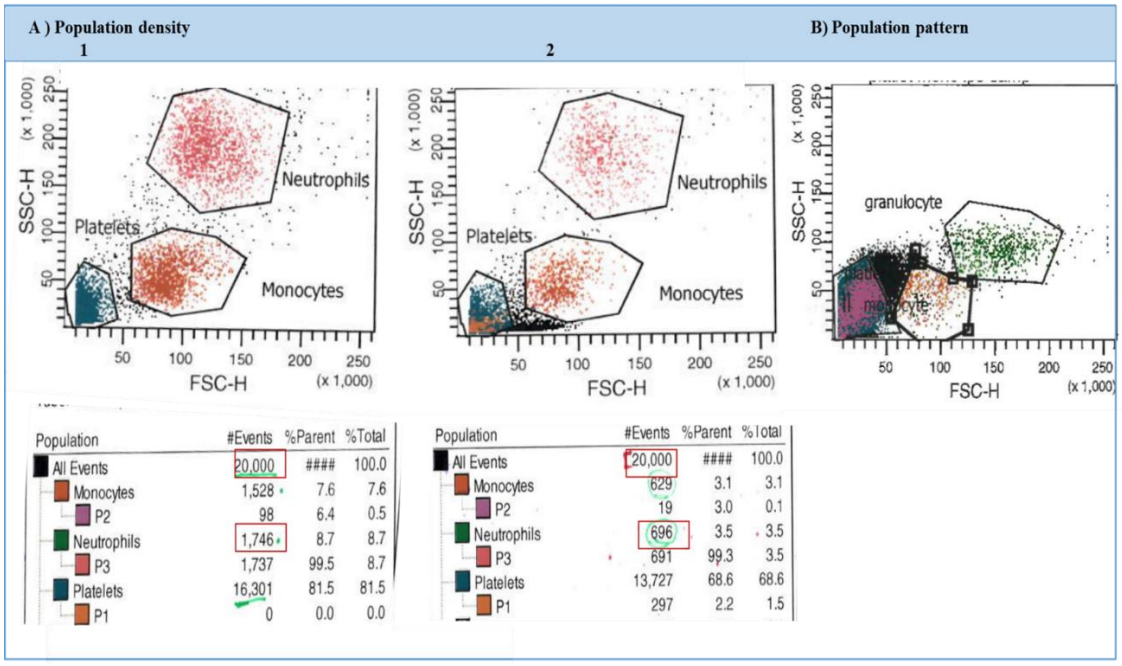


Figure 3.3: Shows the difference in population density and pattern between the sample 1 and 2. Although the same number of all events were collected for each sample (indicated in red box) significant variation of leukocyte subpopulations especially neutrophils (indicated in red box) was found. From a total of 20,000 events for each sample, around 1746 neutrophils were gated for sample 1 while only 696 neutrophils were detected for sample 2.

Further experiment was aimed at increasing the cell number per sample to increase the population density and applying the compensation by using blood samples from different healthy donor following a different protocol as agreed with supervisors (advice was sought from Dr Adam Wright, Prof Dave Cousins and Dr John McDonald). In this experiment, an aliquot of 300µl of whole blood was used (instead of 100 µl used in previous experiment) and lysed before staining, washed, the resulting pellet was re suspended into 100 µl of staining buffer and stained with specified antibodies. After incubation and washing, each pellet was re suspended and fixed in 500 µl of 2% paraformaldehyde. The FACS analysis result was superior to the previous results as it showed a large dominating population that interfered with the normal appearance of desired population that could be related to RBCs contamination (Figure 3.4).

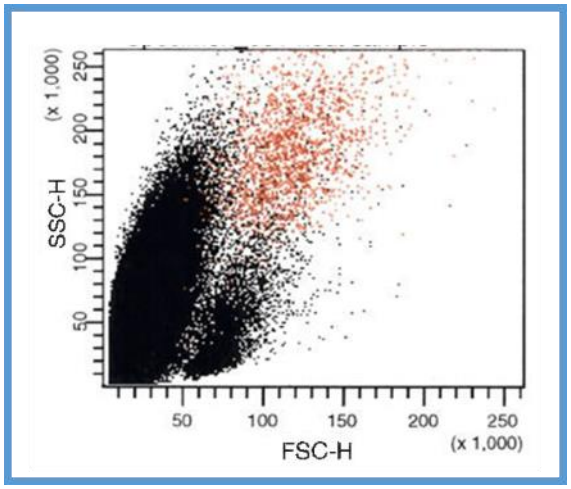


Figure 3.4: Ineffective RBCs lysis. Stainng large volume of blood after RBCs lysis did not improve the population density and showed a large dominating population (indicated in black) interfered with the normal appearance of desired population, granuolocyte (indicated n red. It could be related to an ineffective RBCs lysis.

Therefore, the difference observed in population density among individual, which could not be improved by using three times original volume of whole blood, might contribute to the normal variations of each blood cell type count between individual. For example, the leukocyte counts normally vary between individuals and ranging between 4.3 to 10.8 x 109 cells/L while the platelet count ranges between 150,000 to 450,000 cell/ µl. Such variation could be largely reflected as differences in population density when the same number of all events of blood cells (debris, platelets, leukocyte and remnants of

RBCs) was acquired. To address this issue, acquiring 5000 events of granulocytes instead of acquiring all events was targeted for more accurate analysis. Another variable that could affect the analysis is the centrifugation force. Because 400 RCF might not be enough to pellet all cells in the samples especially platelets. Thus, 800 RCF for 5 minutes (Starov, 2010) was chosen to pellet more cells and increase the population density. Additionally, the time lag between the sample collection and processing was adjusted to be within 10 minutes of blood collection. The speed in processing is important because blood stasis at room temperature has been shown to cause artefactual platelet activation (Maurer-Spurej *et al*, 2001).

Subsequent experiments thereafter were set up with a modification of the initial protocol (Figure 3.1). EDTA anticoagulated WB from different individuals was immediately aliquoted (100 µl), stained with specific antibodies as stated above and processed for flow cytometry analysis after RBCs lysis and fixation. Once these adjustments were made, the FACS analysis showed a consistent density of populations between all unstained WB samples (Figure 3.5, A). However, a difference in expression levels of granulocyte marker (CD66b) was observed. By using isotype matched control, the different percentage of different individuals, granulocyte population ranged from 66 to 99 % and were identified as CD66b positive (Figure 3.5, B). While the gene for CD66b (Carcinoembryonic antigen-related cell adhesion molecule 8) is not described to be polymorphic, the only obvious difference between donors was their age and ethnic background. Moreover, unstained eosinophils have strong intrinsic auto fluorescence that can be detected in FITC channel as false positive, CD66b positive (Weil and Chused, 1981) (Figure 3.5, C).

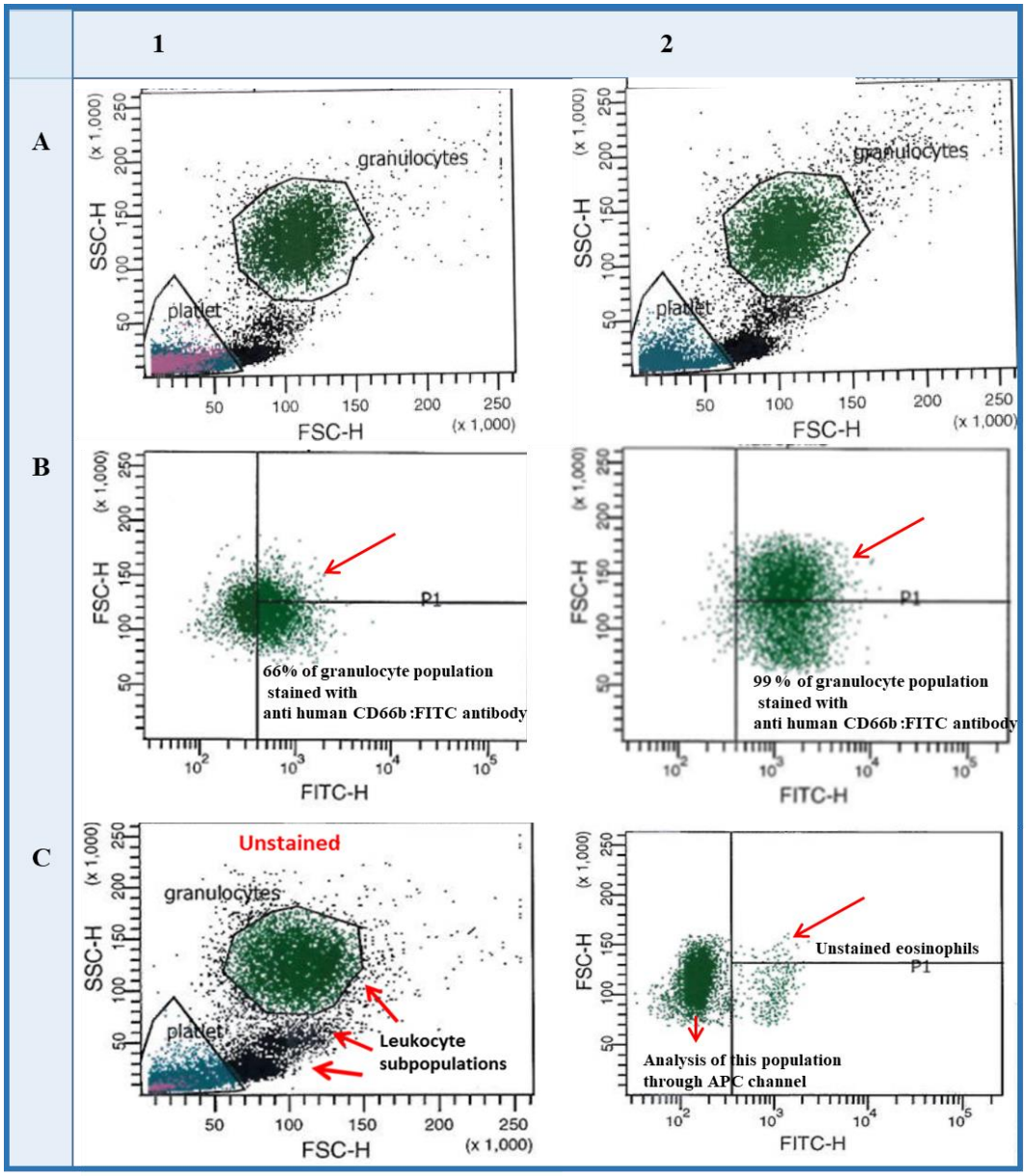


Figure 3.5: A shows a comparable populations density between unstained whole blood samples from individual 1 and 2. B shows that, in sample from individual 1, only 66% of granulocyte population are stained with CD66b marker, however, 99% of granulocyte from individual 2 samples are stained with CD66b marker. C shows the auto-fluorescence of eosinophil population.

Thus, further experiment was done using antihuman CD66b conjugated to APC (in particular, one of the brightest fluorochromes) instead of FITC to decrease the interference from eosinophil fluorescence and improve the brightness of antigen detection. Flow cytometric analysis showed a promising approach to improve the brightness of granulocyte marker CD66b as almost 98% of granulocyte population was stained with APC conjugated anti human CD66b. Furthermore, eosinophils were excluded from analysis by gating out through the FITC channel (Figure 3.6).

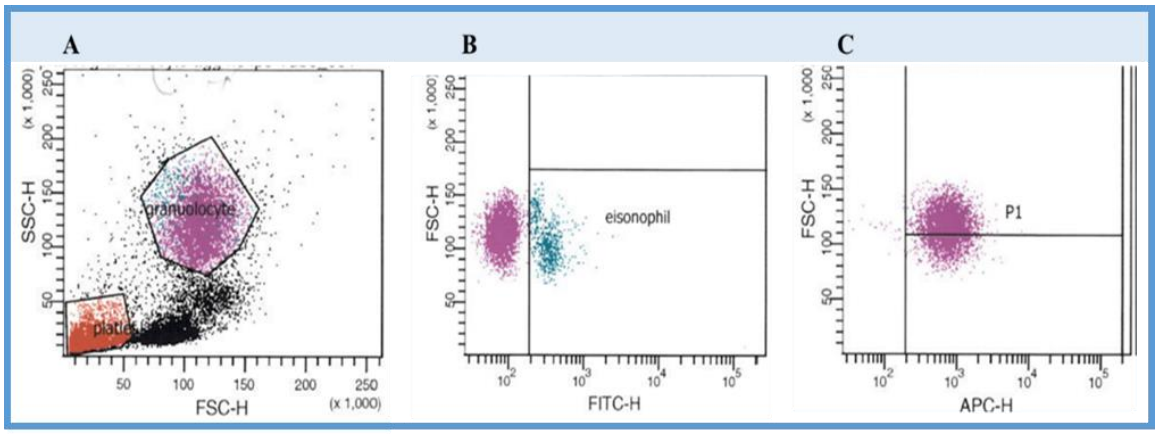


Figure 3.6: A shows granulocyte gate. B shows the unstained granulocyte analysis through FITC and eosinophils population auto fluorescence is shown in blue. C shows analysis of granulocyte which are stained with APC after excluding eosinophils.

3.1.2 Reproducibility of the developed methods:

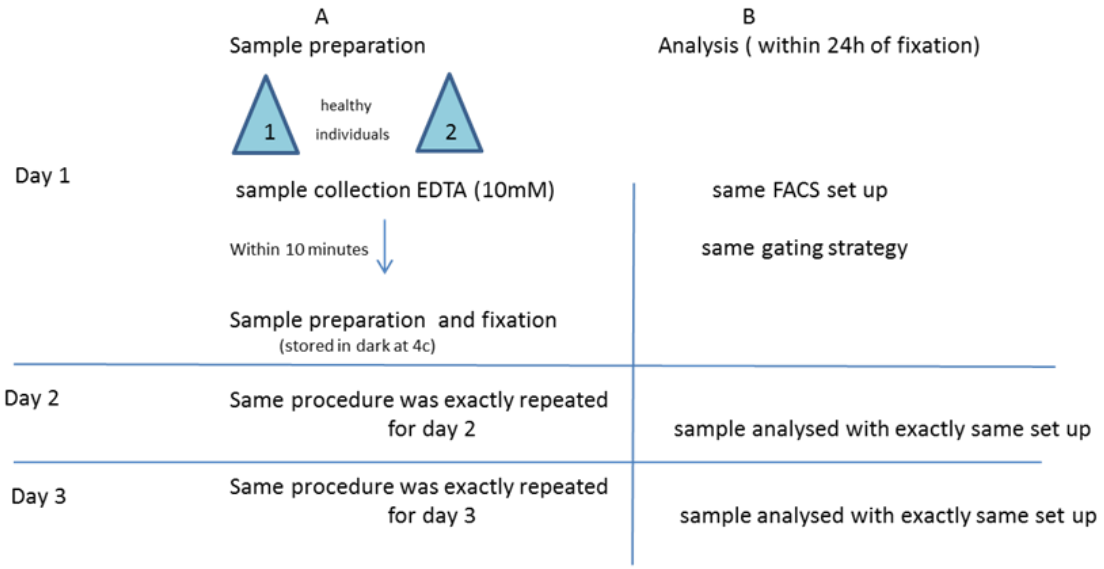


Figure 3.7: shows experimental design to examine the reproducibility of the developed assay. Whole blood samples were drawn from two healthy volunteers on three consecutive days. Each day, the samples were processed in parallel for flow cytometry.

These experiments were necessary to determine the reproducibility of flow cytometric data. Overview of strategy shown in figure 3.7. Freshly donated whole blood samples over 3 days (inter day validation) from two healthy individuals (inter person reproducibility) were collected in 10 mM EDTA. At each day, samples were immediately processed in parallel, to ensure that both samples were handled exactly in the same way so variability in only the staining could be accurately determined, within 10 minutes of blood collection. Then, aliquots (100µl) of whole blood were stained with mouse antihuman CD42b: PE (to detect platelets) and mouse antihuman CD66b: APC conjugated antibody (to detect granulocytes) simultaneously or PE mouse IgG 1 and APC mouse IgM as isotype controls. CD42b and CD66b antigens are commonly used as markers to identify the platelets and granulocytes, respectively, by flow cytometry.

A titration was performed for all fluorescent antibodies to determine the optimal antibody concentration that gives the brightest signal with the lowest background. Single stains were also prepared as florescence minus one control. The erythrolysis step was adjusted as described above to be exactly 12 minutes to minimise damage on WBC. Then, the samples were stored at 4 0C and analysed by flow cytometry within 24 h of fixation using FACS Aria II Flow Cytometer equipped with FACSDiva software Version 6.1.3. After compensation, using BDTM CompBeads set Anti-mouse Ig,k, samples were acquired with a medium flow rate to decrease the platelet leukocyte coincidence that gives false positive results of PGAs (Newby, 2007; Nkambule, Davison and Ipp, 2015b). Exactly the same flow cytometer experimental setup was used each time to ensure reporting of standardised results. A minimum of 5000 granulocyte events were acquired for analysis. A fixed template for gating on population was applied for all samples. Events that stained positively for both platelet and granulocyte markers were considered as PGA. The staining results were consistent between all samples in terms of population appearance, population intensity and percentage of platelet granulocyte aggregate. The third day staining results of individual 2 were excluded due to technical reasons. The coefficient variation (CV) for the assay for each individual was calculated for double positive population (PGA) to express the reproducibility of the staining results. The CV of assay 1 was 13.6 % while assay 2 was 18% (Figure 3.8), most likely related to biological variability.

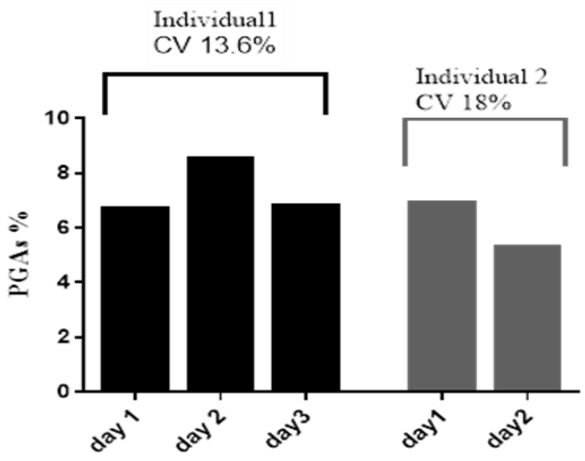


Figure 3.8: The reproducibility of platelet granulocyte complex flow cytometric analysis by inter-assay test and intra-assay test. Inter assay precision is expressed as coefficient of variation which is calculated by division of standard deviation by the mean and multiplied by 100, 13.6 % for individual 1 and 18% for individual 2. Intra assay precision is expressed as the average of the coefficient of variation, 15.8. CV, coefficient of variation.

3.2 Formation of inflammatory platelet granulocyte aggregates in a whole blood stimulation assay using a single PAMP:

3.2.1 Introduction:

In this project, a whole blood stimulation assay was used as *in vitro* approach to generate and analyse the formation of aggregates involving platelets and granulocytes. It was seen as a more accurate model to develop greater understanding of the acute inflammatory reaction in the bloodstream to the presence of PAMPs during septicaemia for the following reason: in contrast to stimulation of isolated peripheral blood cells or peripheral blood cell culture, it provides a more physiological environment that allows a broader assessment of relevant effector molecules in plasma. It also avoids as much as possible artefactual activation of cells of interest (Vollmar *et al*, 2003; Yang *et al*, 1999; Maes *et al*, 2007). Different types of LPS, purified from *E.coli* 0111:B4 and *Salmonella enteritidis*, were initially used to induce the formation of inflammatory aggregates. Endotoxin, a lipopolysaccharide found on the outer membrane of Gram negative

bacteria, is a dominant microbial stimulus implicated in the development of sepsis (Adamik, Smiechowicz and Kübler, 2016; Kamisoglu *et al*, 2015). Endotoxemia, namely a high level of circulating endotoxin, has been documented in most cases of sepsis (Ronco, Piccinni and Rosner, 2010). Moreover, administration of endotoxin to healthy volunteers was shown to induce clinical symptoms associated with septicaemia such as, fever, increased heart rate and hypotension (Lynn *et al*, 2003; Lamy and Thijs, 1992). Platelet granulocyte aggregates (PGA) are extensively formed in sepsis and their extent correlates with the severity of sepsis (Russwurm *et al*, 2002). The ability of LPS to induce the platelet leukocyte aggregate formation in blood was demonstrated *in vitro* (Nkambule, Davison and Ipp, 2015a; Ståhl *et al*, 2009; Willis and Whitfield, 2013). However, reviewing relevant literature describing LPS induced platelet-granulocyte aggregates in human peripheral venous blood, there is no consensus with regard to anticoagulants, buffers, sample handling and dilution, cell treatment, immune labelling protocols, and cytometer settings. Some of these studies isolate platelets and granulocytes from whole blood, mix them at a certain ratio and incubate the mixture in the presence or absence of LPS to study these aggregates, disregarding the possibly important effects of other plasma components, red blood cells and cell activation during lengthy sample handling on this interaction (Haselmayer *et al*, 2007). Most importantly, however, most of these studies compare the extent of LPS induced aggregates to conditions of the immediately stained, not incubated, unstimulated blood cell sample (Nkambule, Davison and Ipp, 2015b; Huang *et al*, 2009). We hypothesised that the experimental design of comparing the LPS stimulated blood sample stained after incubation with the immediately stained, unstimulated sample in favour of comparing the LPS stimulated blood sample with the unstimulated blood sample stained at the same time, was significant for the interpretation of results. Thus, we aimed to develop a whole blood stimulation protocol in order to investigate the extent of platelet-granulocyte aggregate formation when stimulated with LPS, allowing for spontaneous aggregate formation and to characterise these aggregates further.

3.2.2 Experimental design

Two different types of LPS were used in this study because they differed in their stimulatory effect for complement activation and we wanted to include analysis of the effect of complement on the aggregate formation. A study aimed at investigating the effect of *E.coli* (O111:B4) LPS on complement activation found that incubation of lepirudin anticoagulated blood with *E.coli* LPS at lower than 1000 ng/ml did not activate complement (Brekke *et al*, 2013). Ongoing mouse work in our laboratory, aimed at studying the activity of complement in response to LPS, showed that incubating mouse serum with *Salmonella* LPS for 1 hour induced complement activation as was described previously (Kotimaa *et al*, 2014). EDTA (divalent cation chelator) was used as a control based on its inhibitory role in complement activation, while hirudin (a lepirudin which binds active thrombin) does not affect complement activity. In fact, it is the most suitable anticoagulant to use to allow studies of complement activities (Bexborn *et al*, 2009a; Mollnes *et al*, 2007). Furthermore, latest work in our laboratory conducted by Stover and collaborators (University of Sheffield) aimed at studying the intra vital dynamics of formation and adherence of cellular micro aggregate (granulocyte /platelets) to experimentally inflamed endothelium which proceeds to thrombohaemorrhage in capillary end beds of experimental mice. In this study, the model was developed by following the principle of Shwartzman reaction in which two subsequent exposures to inflammatory stimuli (intrascrotal injection of TNFα and intraperitoneal LPS injection) produce inflamed endothelium of the microvasculature and systemic cellular activation, subsequently. Intra-vital microscopic analysis of inflamed post capillary venules revealed that formation of granulocyte – platelets aggregate in the wild type mouse contrasted significantly with the phenotype displayed by properdin deficient mouse in which the aggregate formation was scanty and dispersed. Therefore, these results were related to the role of properdin as it not only amplifies the alternative complement pathway but also increases expression of tissue factor which activates the alternative complement pathway. Thus, these two contributions in the type result in abundance of C3b which is diminished in properdin knockout mice (Figure 3.9).

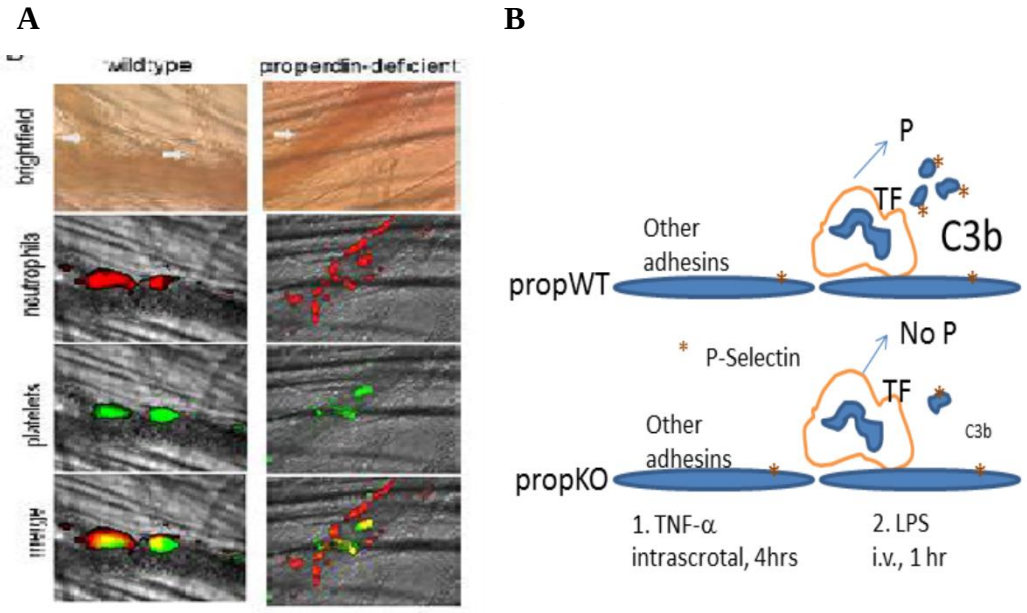


Figure 3.9: Properdin enhances platelet leukocyte aggregate adherence to the inflamed endotheilium. A is intravital microscopic analysis of PLA adherence to the inflamed endotheilium in proerdin wild type, Prop WT, and properdin knok out mice, propKO. B shows the proposed mechanism of enhancing PLA adherence to the endotheiilum by properdin.After TNFα induced activation, endothelial cells upregulate expression of factors such as P-Selectin (Pan et al., 1998), which tether neutrophils to their surface. They in turn activate and exocytose properdin (Kemper and Hourcade, 2008), which amplifies LPS-induced complement activation locally. They also provide a procoagulant surface (TF, tissue factor), which itself activates the alternative pathway (Camous et al., 2011; Kourtzelis et al., 2010). Taken together, these two contributors provide in the properdin wildtype microvasculature (propWT) abundance of C3b. C3b binds to P-Selectin on the endothelial cells and activated platelets (Morigi et al., 2011). Generation of C3b is diminished in the absence of properdin (propKO). Image and schematic diagram by C. Stover.

Briefly, whole blood was collected into hirudin (150 U/ml) or EDTA (10mM) and processed within 10 minutes of collection. To analyse the spontaneous PGAs at zero time point, aliquots of the whole blood sample were immune labelled and processed for flow cytometric analysis immediately. Meanwhile, to analyse the effect of LPS stimulation, whole blood was immediately incubated with and without (control) *LPS* from *E.coli 0111:B4* and from *Salmonella enteritidis* at 1 µg/ml for 1 h at 37oC and then stained and processed for flow cytometry. All materials used such as tubes, tips and solutions were endotoxin free to avoid artefactual introduction of endotoxin.

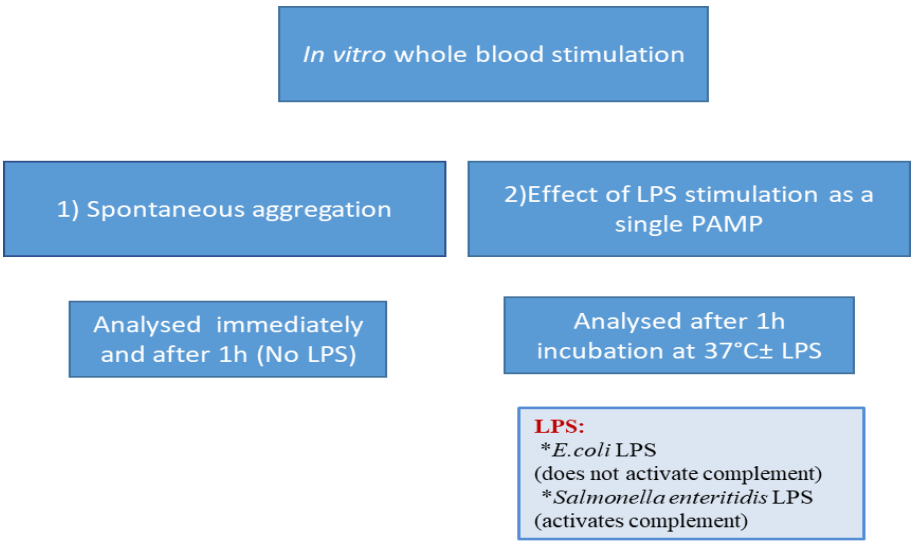


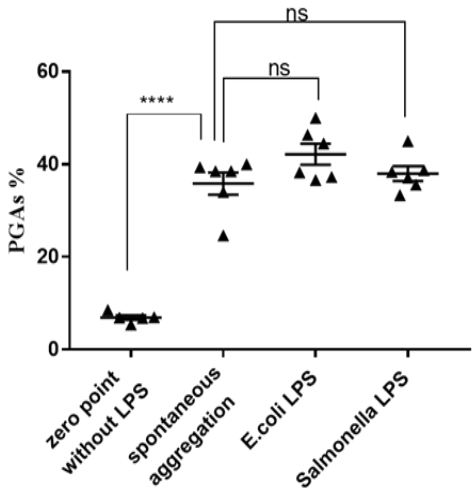
Figure 3.10: Experimental design to analyse PGAs at different conditions; 1) spontaneous aggregation at zero time point, 2) PGAs in response to LPS stimulation.

3.2.4 Results:

3.2.3.1 The effect of whole blood LPS stimulation on PGA formation:

As shown in figure 3.11, incubation of unstimulated whole blood at 37°C for 1 h induced a significant increase in platelet granulocyte aggregate formation compared to baseline at zero point. Unexpectedly, incubation of whole blood with *E.coli* LPS 0111:B4 or *Salmonella enteritidis* LPS for 1h for both EDTA and hirudin anticoagulated samples did not induce a superior increase in platelet neutrophil aggregate levels compared to that of unstimulated whole blood incubated for1h at 37°C. The contribution of spontaneous cellular aggregation to the intended readout was unexpected and more experiments were set up to confirm this observation.

A



B

		PGAs %			
Samples	Type of anticoagulant	Zero time point	Spontaneous aggregation	*E.Coli* LPS	*Salmonella LPS*
A	EDTA	6.8	34	37.3	38.4
B	EDTA	8.6	24.7	38.3	45
C	EDTA	6.9	38.5	36.6	35.6
D	Hirudin	5.4	39.4	50	37.12
E	Hirudin	7	38.5	46.5	33.4
F	Hirudin	-	40	44.5	38.7

Figure 3.11: Platelet granulocyte aggregate induced by incubation with and without LPS (1000 ng/ml) determined by flow cytometry. The granulocyte population was clearly identified in whole blood based on their light scatter characteristic, size and granularity. Approximately 99% of the cells in granulocyte gate were positive for the granulocyte marker CD66:APC which express the specificity of marker staining and accuracy of cells identification according to their forward and side scatter characteristic feature. In granulocyte gate, cells that stained positively for both CD66:APC and CD42b:PE (platelet marker) were regarded as PGA. A shows the percentages of PGA at different conditions. B shows a table of analysed samples with type of anticoagulant Data are presented as mean ± standard Error of mean (SEM) of at least 5 to 6 independent experiments. Significance of changes was assessed by analysis of variance (ANOVA) and Tukey's multiple comparisons test. Changes were considered significant if p-value was <0.05.

3.2.3.6 Effect of whole blood LPS stimulation at different concentrations and incubation time on PGA level:

While most of published in *vitro* whole blood LPS stimulation studies used LPS at 1 µg/ml for 1h, some used LPS at various doses ranging from 0.5 to 10 µg/ml for different times 1, 4 and 16 h (Nkambule, Davison and Ipp, 2015a; Ståhl *et al*, 2009). After finding no significant increase in PGA level over spontaneous aggregation when different type of LPS at1 µg/ml for 1h and different anticoagulants were used, we wanted to find out if different concentrations of LPS at different time points of incubation could lead to a significant increase in these aggregates. To do so, aliquots of hirudin anticoagulated whole blood (1ml) were incubated with different concentrations of *Salmonella* LPS (0 [control], 0.5, 1.0 and 10.0 µg/mL) for 1h and 4 h. The samples were immune labelled and analysed by flow cytometry as described previously. As shown in table 3.1, different doses of *Salmonella* LPS did not increase the number of platelet granulocyte aggregates significantly over the spontaneous aggregation observed at baseline at 1 and 4 hours' stimulation.

Table 3.1: PGAs % in hirudin anticogulated whole blood that is stimulated with different doses of *Salmonella* LPS for 1 and 4 h.

Salmonella LPS	No LPS	0.5µg/ml	1µg/ml	10 µg/ml
PGA% (1h at 37°C)	**43.6**	**34.9**	**32.7**	**45.4**
PGA% (4h at 37°C)	**43.6**	**39.8**	**37.0**	**35.7**

3.2.3.7 *In vitro* investigation of LPS-induced inflammatory effects in a cell culture model:

Having demonstrated, in previous sections, that various doses of different type of LPS were not able to induce the inflammatory aggregate formation in whole blood, we now wanted to assess the ability of LPS to induce an inflammatory cellular response in terms of lipid inclusion and increased TNF α level using J744

macrophage cell line which have been shown to express TLR4 mRNA in our lab (Kheder, Hobkirk and Stover, 2016).

J774 cells were cultured and incubated with different concentrations of LPS (no LPS as a control, 100ng/ml and 1000ng/ml) for 24 h at 37°C, in a humidified atmosphere containing 5% CO_2. Each condition was done in triplicate. Light microscopic examination revealed that in comparison to untreated cells, LPS treated cells had changed to increase their size, developed intracellular vacuoles and membrane projections and these morphological changes were more prominent in cells stimulated with 1000ng/ml LPS (data not shown). Microscopic examination of Oil Red O staining of J744 cells treated with different concentrations of LPS showed that high dose of LPS (1000ng/ml) stimulated more macrophages to accumulate lipid droplets as compared with 100ng/ml LPS stimulation and control condition (Figure 3.12). Consistently, around 25% of foam cells, lipid laden macrophages, were previously reported as an inflammatory response to LPS stimulation (Lee *et al*, 2009).

Analysis of TNFα level in culture supernatants of J774 macrophages in response to high dose (1000ng/ml) LPS stimulation for 4 hours (acute effect) and 24 hours (prolonged effect) showed that LPS induced a significant increase in TNFα level at both incubation periods compared to the unstimulated samples (Figure 3.12). While 4 hours TNFα measurements reflect preformed TNFα, 24 hours captures novel synthesis and secretion.

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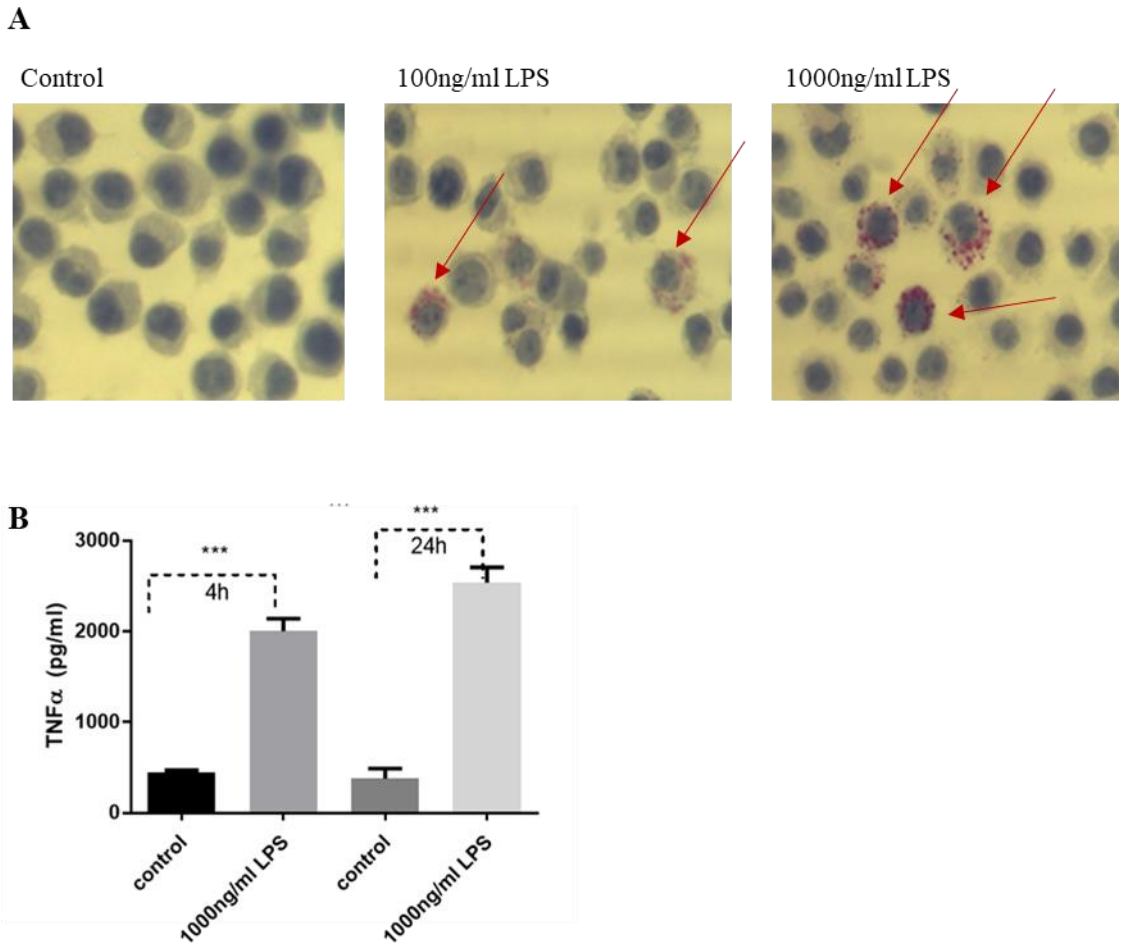


Figure 3.12: shows ability of *E.coli* LPS, 1000 ng/ml, to induce inflammatory response in J774 mouse macrophages cell line expressed in induction of intracellular inclusions which were stained with Oil Red O and TNF α level detected in culture supernatants. Significance of changes was assessed by Mann-Whitney test, D, n=3. Changes were considered significant if p-value was <0.05.

3.2.3.8 Characterisation of platelet granulocyte aggregates in response to LPS using scanning electron microscopy:

Having determined that quantitative analysis, by flow cytometry, of PGA formation in response to LPS did not detect any significant increase over spontaneous aggregation in addition to the ability of LPS to induce inflammatory response in cell culture model, we wanted to investigate the possibility of LPS to induce PGAs that might be structurally different from spontaneously forming aggregates, which would make these difficult to

be analysed by flow cytometry. SEM was used to visualise the morphology and the composition of inflammatory aggregate induced by LPS.

3.2.3.8.1 Preparing the samples for SEM analysis:

The flow cytometry associated cell sorter technique was initially selected for isolating and sorting the double positive events (PGAs) as well as platelets and leukocyte cells (as control to identify the cell clearly) from each blood preparation to be further processed by Scanning electron microscopy (SEM). Firstly, hirudin anticoagulated blood sample was immediately stimulated with and without LPS for 1h and, then, it was aliquoted, immunolabeled and analysed by flow cytometry using exactly the same gating strategy as described above to sort the desired population from each condition using FACS Aira sorter. Unfortunately, sorting of pure double positive events, PGAs, was not successful. Because not only the concentration of the sorted population was very low, (1464 PGA /ml) but also the post sort analysis of this population showed a very low purity due to the detected contamination from other quadrants, platelets as well as leukocytes (Figure 3.13). This means that the actual concentration of sorted PGAs is far lower than the detected fraction and would further limit a clear SEM analysis. Microscopic examination of stained smear prepared from sorted PGAs population further confirmed this observation as it showed some platelets and leukocytes with no PGAs (data not shown).

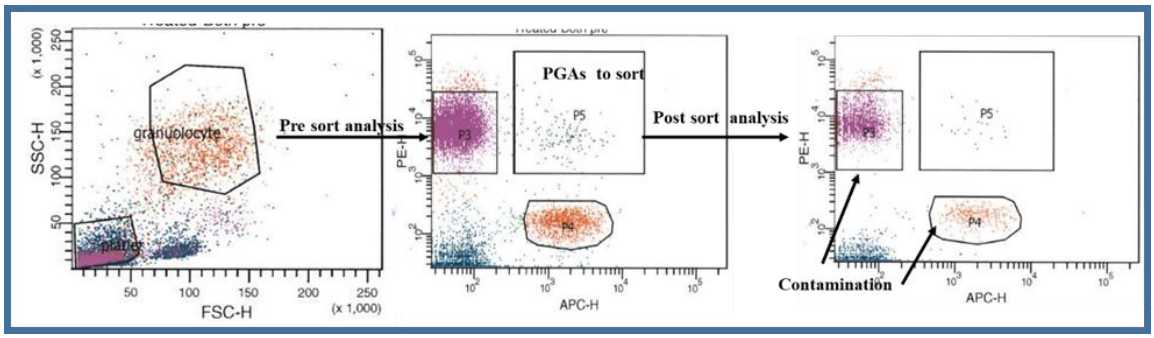


Figure 3.13: PGAs sorting approach. It shows pre sort analysis of granulocytes population to gate on the PGAs, which is stained with both markers of platelet and granulocytes. Then, the PGAs were sorted. Finally, post sort analysis of PGAs was performed to determine the purity of population and shows contamination from other populations, platelets and granulocytes.

Another attempt was made to isolate these aggregates for SEM with density gradient media, Histopaque-1119 and Histopaque-1077. This technique allows isolation of granulocytes as a layer from whole blood, thus attached platelets might be isolated within the granulocytes layer. However, this method was not successful in isolating PGAs efficiently from whole blood which was demonstrated by prepared stained smear.

The concentration of RBCs is around 1000 times greater than that of leukocytes (Li *et al*, 2014). Thus, in order to properly analyse the PGAs within whole blood by SEM, RBCs were lysed as described above and the pellet re suspended in 100 µl PBS. The success of this method was further evaluated by preparing a smear of lysed blood after stimulation with LPS to directly visualize PGAs as shown in (Figure 3.14).

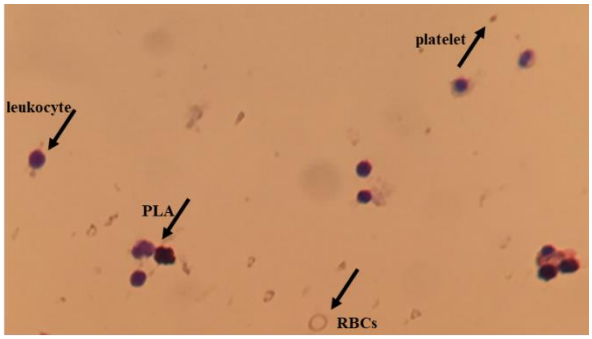


Figure 3.14: Blood smear after RBCs lysis shows the platelets leukocytes aggregates (PLA), leukocytes, platelets and remaining RBCs indicated in arrow. X40.

3.2.3.8.2 SEM results:

Hirudin anticoagulated whole blood samples taken from four male and female healthy volunteers on different days were processed immediately as following: each individual's sample was incubated with *Salmonella* LPS (1000ng/ml) or left without stimulus as a control for1h at 37oC and processed in parallel for SEM. Briefly, the

RBCs were lysed, and the cells were pelleted at 800 rcf for 5 minutes. After washing, the pellet was re suspended in PBS, adsorbed on superfrosted glass, fixed with 2.5% glutaraldehyde in PBS (v/v) and processed as described in methodology section. For analysis of these aggregates, representative SEM images (n = 64 images from n = 4 independent isolations per treatment) were captured blinded to the conditions.

In SEM images, the leukocytes were identified based on their surface morphological features including folds, ruffles, projections and microvilli as well as their size, which ranged from 6 to 14 μm (Yang *et al*, 1999). Although each WBCs subtype (lymphocytes, monocytes, and granulocytes) under normal condition had different surface features recognisable by SEM, there is overlap in the criteria, making the distinct identification of each subtype by SEM alone quite difficult (Polliack, 1981; Yang *et al*, 1999). In addition, one has to take into account that inflammatory stimulation of the cells of interest is likely to induce variability in their surface morphology that contributes in its own right to the difficulty of identifying leukocyte subtypes by SEM. That is why the PGA will be named as PLA in following sections. Platelets were reliably identified on the basis of the previous observations of their surface morphological appearance which existed in two forms: an inactivated form with discoid shape and smooth surface (around 2μm in diameter) and an activated disc or spherical shape with irregularly distributed long pseudopodia and protrusions (Yang, 1989; Zilla *et al*, 1987). Some remaining red blood cells were identified by their general biconcave shape or as crenated RBC, spherical with spicules, due to the effects of osmotic pressure changes during RBC lysis and SEM preparation (Rooney and Woodhouse, 2014).

In the unstimulated sample, most of the cellular aggregates were formed of one or two leukocytes and were found similar to the form of dominating aggregates induced by whole blood stimulation with LPS (Figure 3.20,A 1and 2).

To further assess the effect of LPS on the aggregate formation, the surface area of the aggregates was measured using image J software. As shown in figure 3.20 (B), the calculated surface area of cellular aggregates was found similar at unstimulated conditions with a mean =117.678 μm2 and 140.973 μm2 after LPS stimulation.

3.2.3.9 *In vitro* investigation of LPS inflammatory effects in a whole blood stimulation model:

An investigation of the ability of LPS to induce an inflammatory response in the whole blood assay was critically required at this step in order to understand why LPS was able to induce an inflammatory cellular response in a cell culture model while no effect was observed on PGA formation by flow cytometric and SEM analysis. Different donors' whole blood samples anticoagulated with hirudin or EDTA and incubated with or without (control) *Salmonella* or *E.coli* LPS at 1000ng/ml for 1 h at 37oC were used to prepare the plasma samples. Samples at zero time point incubation were also prepared.

Il-8 is a key pro inflammatory cytokine that is rapidly released by different cell types in response to inflammatory stimuli (Hirao *et al*, 2000; Atta-ur-Rahman, Harvey and Siddiqui, 1999). So, IL-8 levels in response to different stimulations were measured by ELISA from at least 3 individuals.

No significant increase in inflammatory marker (IL-8) was observed in both EDTA (data not shown) and hirudin anti-coagulated blood stimulated with two different types of LPS independently at 1000ng/ml (Figure 3.15, A). These results were in agreement with the range of IL-8 concentrations detected in plasma samples of unstimulated and *Salmonella* stimulated whole blood which were incubated on EAhy 926 endothelial cell line for 1h at 37 °C (described in chapter 4). The significantly lower IL-8 level detected in plasma sample at zero time point compared to the unstimulated or LPS stimulated samples (one hour incubation) further confirmed the effect of incubation time and condition but did not support the presence of LPS induced effects.

TNFα levels of these samples were also measured and reveal no significant difference between unstimulated and LPS stimulated samples (Figure 3.15, B).

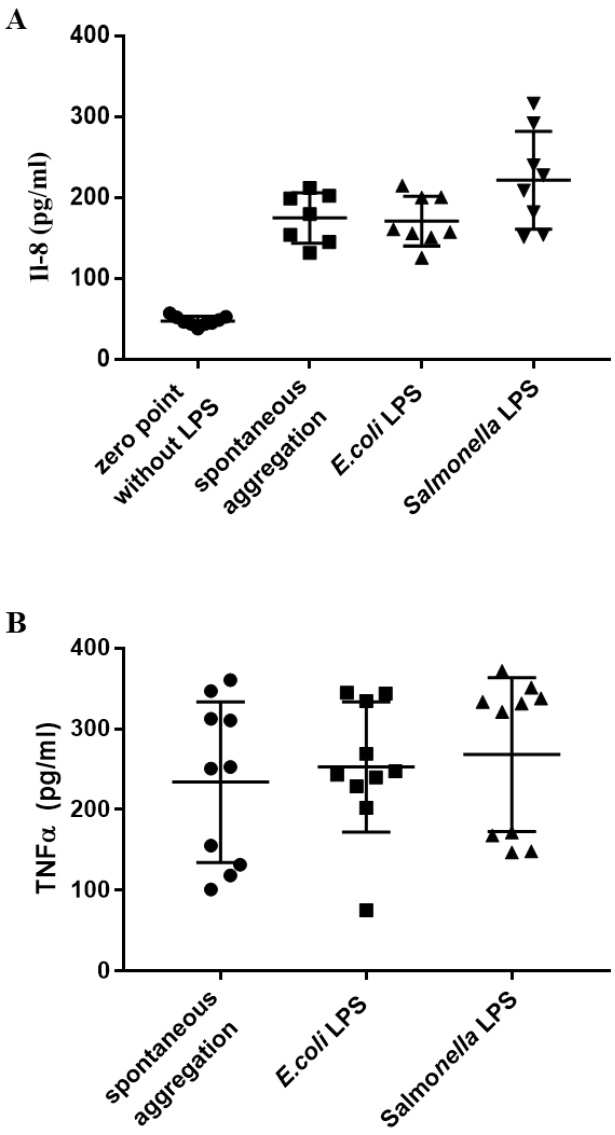


Figure 3.15: Analysis of inflammatory markers IL-8 (A) and TNFα (B) at different conditions of whole blood stimulation. Significance of changes was assessed by Kruskal-Wallis test and Dunn's multiple comparisons test C, n=3 at least. Changes were considered significant if p-value was <0.05. TNF alpha level detected in sample at zero point was below the lower detection limit of standard curve and is therefore depicted here.

3.3 Formation of inflammatory aggregates in a whole blood stimulation assay using PAMP rich stimuli:

3.3.1 Introduction:

In the previous section (3.2), I established that LPS stimulation of whole blood did not lead to a significant increase in PLAs formation. Therefore, two clinical isolates were alternatively used because they provide a wide range of sepsis-relevant PAMPs. For the purpose of the model, they were heat killed so as not to compromise eukaryotic cell viability. In this way we aimed to produce a model of acute cellular interactions in the fluid phase which could be found in the initial phase of sepsis.

A) *Klebsiella pneumoniae*:

Klebsiella pneumoniae, a gram negative encapsulated bacterium, is the second most common isolated pathogen in patients with gram negative sepsis (Wiersinga *et al*, 2014; Achouiti *et al*, 2016). In addition to the LPS component, its capsular polysaccharides are considered the most significant virulence factors in causing sepsis (Cortes *et al*, 2002).

B) *Staphylococcus aureus*:

Staphylococcus aureus, a gram positive bacterium, is one of the most frequently isolated microorganism in gram positive sepsis (Ramachandran, 2014; Powers and Wardenburg, 2014). Various surface bound proteins and cell wall components of *S. aureus* are pro inflammatory and induce a host response in a similar way to gram negative lipopolysaccharide (Powers and Wardenburg, 2014).

The aim of this part of project was to prepare non-viable bacteria with intact surface receptors (PAMPs), such as LPS, capsular polysaccharides and proteins, that can be sensed by inflammatory blood cells, especially granulocytes and platelets as part of the innate cellular response. And, then, they were used further to establish a whole blood stimulation model (Figure 3.16). The lab investigating phagocytosis of live bacteria by monocytes in whole blood had previously shown that bacteria proliferate in the nutrient rich environment. This is why heat killed bacteria were used for this work.

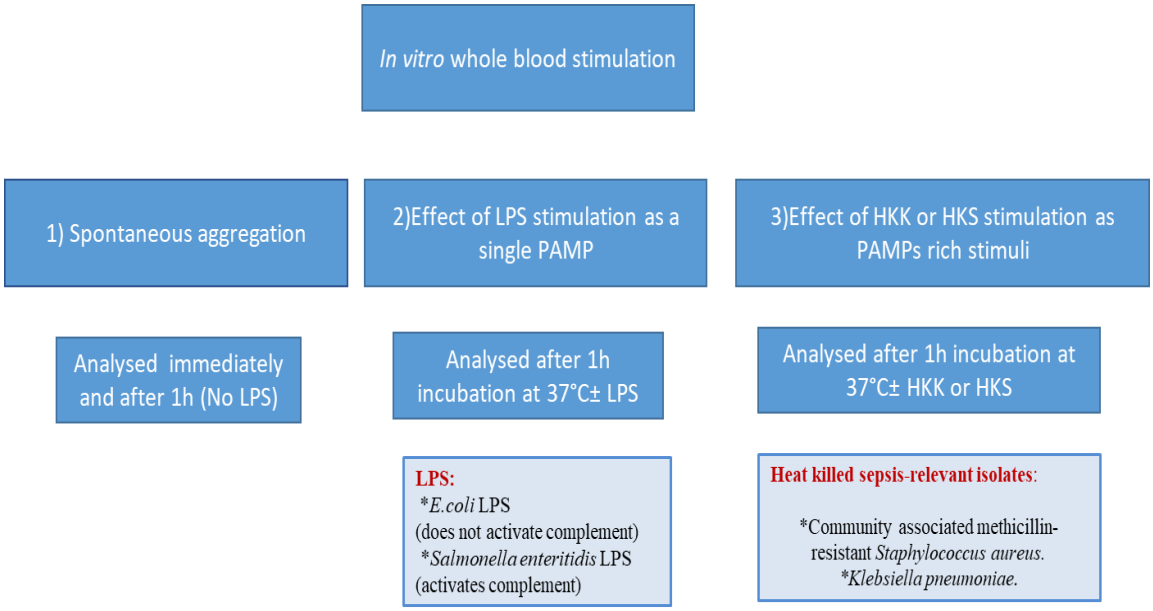


Figure 3.16: Experimental design to analyse PGAs at different conditions; 1) spontaneous aggregation at zero time point and after 1 hour incubation, 2) PGAs in response to LPS stimulation, 3) PGAs in response to sepsis-relevant, heat killed clinical isolate stimulation.

3.3.2 Results:

3.3.2.1 Preparation of heat killed *Klebsiella pneumoniae* and *Staphylococcus aureus* stocks:

The overnight culture of each type was spun down and the pellets were washed and re suspended in PBS. Small volume of each bacterial suspension was used to determine the bacterial concentration of their stock by viable counting (CFU) before heat killing. The remaining volume of *K. pneumoniae* suspension was immediately heat inactivated at 60 °C for 30 minutes while that of *S. aureus* was heat inactivated at 80 °C for 10 minutes. This step was critical, as the aim was to stop the growth of microorganism without causing bacterial lysis that would affect the integrity of its surface. To check bacterial viability, overnight incubation of LA agar plates that were streaked with HK bacterial suspensions, separately did not show any bacterial growth. Gram stain was

used to assess intactness of heat killed bacteria (Figure 3.17). Bacterial stocks were aliquoted and frozen at -80°C.

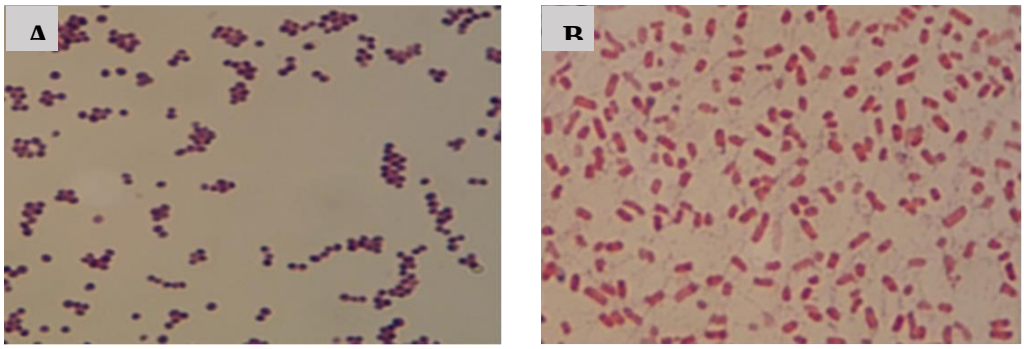


Figure 3.17: Gram stain of heat killed bacteria, *Staphylococcus aureus* (A) and *Klebsiella pneumoniae* (B) shows intactness of most bacteria.

3.3.2.3 **Whole blood stimulation with Heat killed bacteria:**

EDTA anticoagulated whole blood was stimulated immediately with or without (control) heat killed *K. a pneumoniae* (HKK) at 106 CFU/ml. To our knowledge, this was the first study to investigate the effect of the HK bacteria on the formation of platelet leukocyte aggregates, so there was no reference paper for the dose to choose. So, we chose the dose based on studies in which *in vitro* whole blood stimulation of heat killed bacteria was used to investigate the inflammatory response in terms of TNFα and IL-6 production (Schultz *et al*, 1998). The stimulated samples were incubated at 37oC for 1 hour. Then, aliquots of whole blood were stained with platelet and granulocyte specific markers simultaneously. Unstained, isotype reactive, and FMO controls were also prepared. Flow cytometric analysis of all samples obtained from male and female probands showed a significant increase in platelet granulocyte aggregate level in stimulated samples compared to unstimulated samples, respectively (Figure 3.18).

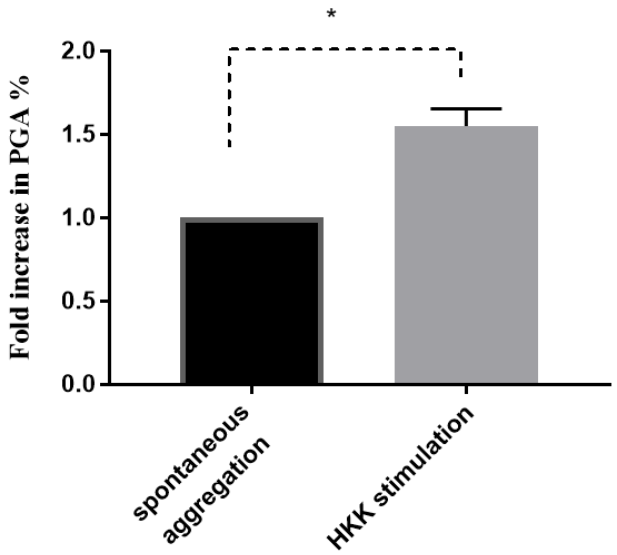


Figure 3.18: PGAs induced by incubation of whole blood with and without heat killed bacteria**.** PGAs were determined by flow cytometry as CD66+CD42b+ events, and expressed as a fold increase in percentages of PGAs from total granulocytes population at different conditions. Stimulation of EDTA anticoagulated whole blood with HKK induce a significant increase in PGAs over spontaneous aggregation. Data are presented as mean ± SEM. Significance of changes was assessed by paired t test ($p < 0.0117$) analysis. n=3. Changes were considered significant if p-value was <0.05.

Having demonstrated that stimulation of EDTA anticoagulated whole blood with HKK led to a significant increase in PLA formation, we now wanted to find out if stimulation of whole blood with HKK and HKS as well -but using hirudin as anticoagulant, that provides a more physiological state (Bexborn *et al*, 2009b)- would also lead to an increase in PLA.

Multiple whole blood samples were collected into hirudin at different days from healthy volunteers of different ages, sex and ethnicity. Each sample was divided into three aliquots and incubated immediately without any stimulus as a control or with HKK and HKS (106 CFU/ml) separately at 37°C for 1 hour. Then, the samples were stained and processed as described previously for flow cytometric analysis. Flow cytometric analysis result of HKS stimulated sample of donor 5 was excluded due to technical error. As shown in figure 3.19, in comparison to the percentages of platelet granulocyte

aggregates detected in unstimulated samples, there was no significant increase in PGAs % towards heat killed *K. pneumoniae* or heat killed *S. aureus*.

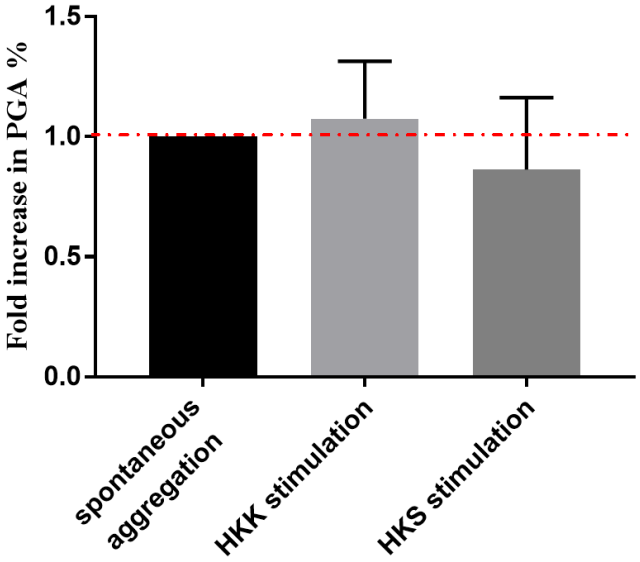


Figure 3.19: Stimulation of hirudin anticoagulated whole blood with heat killed *K.pneumoniae* (HKK) or heat killed *S.aureus* (HKS) did not induce a significant increase in PGAs over spontaneous aggregation. Data are expressed as fold increase in percentages of PGAs from total granulocytes population at different conditions. Data are presented as mean ± SEM. Significance of changes was assessed by analysis of variance (ANOVA) and Tukey's multiple comparisons test. n=5. Changes were considered significant if p-value was <0.05.

3.3.2.3 Analysis of platelet granulocyte aggregates in response to different stimuli using scanning electron microscopy:

Scanning electron microscopy was used to visualize the morphology and the composition of inflammatory aggregates induced by different stimuli in a hirudin anticoagulated whole blood stimulation model. Hirudin anticoagulated whole blood samples were taken from four male and female healthy volunteers on different days and were processed immediately as follows: each individual's sample was incubated with HKK, HKS or left without stimulus as a control for 1h at 37 °C and processed in parallel for SEM. The samples were processed as described in previous section. For analysis of these aggregates representative SEM images (n = 64 images from n = 4 independent isolations per treatment) were captured.

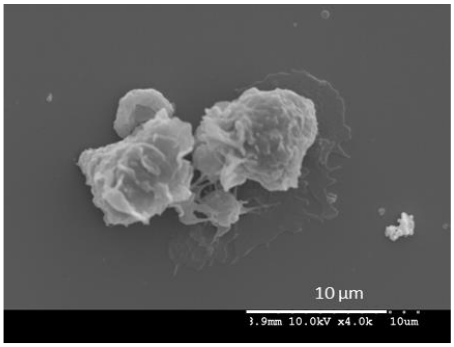
In SEM images, the leukocytes, platelets and RBC were identified based on their surface morphological features as described earlier.

In the unstimulated samples, most of the cellular aggregates were formed of one or two leukocytes surrounded by a few platelets and RBC (Figure 3.20, A1). However, when whole blood was stimulated with HKK or HKS, the majority of the cellular aggregates exhibited morphology and composition that was significantly different from that of aggregates formed in unstimulated conditions. They are more complex and composed of many leukocytes, activated platelets, crenated RBCs and plenty of tiny vesicles with a size less than 1μm, which might be micro particles released by activated platelets, leukocytes and RBCs (Tissot *et al*, 2013) (Figure 3.20, A 3, 4).

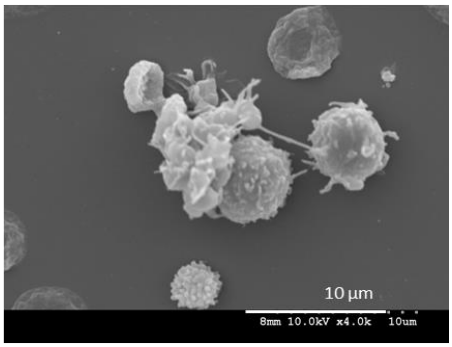
To quantify the impression of more complex and larger size aggregates, the surface area of the aggregates was measured by image j software. As shown in figure 3.20 B, the calculated surface area of cellular aggregate at unstimulated conditions was a mean =117.678 μm2. On the other hand, HKK and HKS stimulation lead to a significant increase of cellular aggregates' surface area to a mean surface area of 470.611 μm2 and 518.399 μm2, respectively ($p < 0.0001$ vs. unstimulated conditions, Kruskal-Wallis test, followed Dunn's multiple comparisons test).

Furthermore, heat killed bacteria stimulation of whole blood induce a significant increase in plasma level of both IL-8 and TNFα over their measured levels observed in spontaneous as well as LPS stimulated whole blood (Figure 3.21).

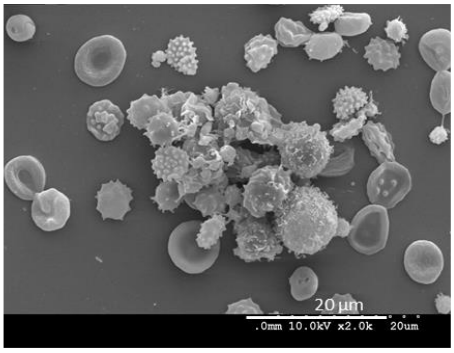
A



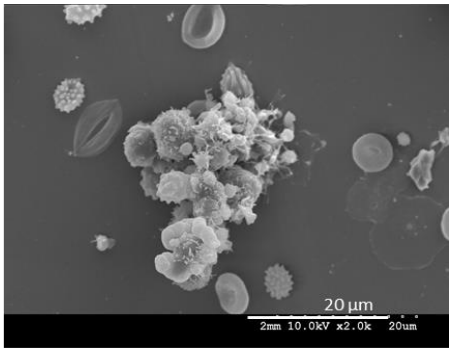
1- spontaneous aggregation



2- *Salmonella* LPS



3- HKK 10^6 CFU/ml



4- HKS 10^6 CFU/ml

B

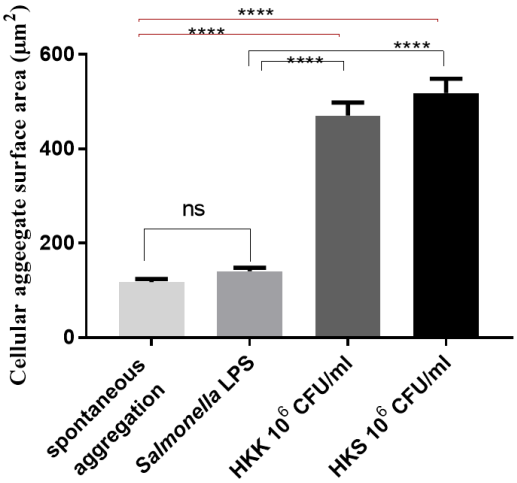


Figure 3.20: Scanning EM analysis of cellular aggregate morphology and composition. Panel A shows representative images of cellular aggregates from unstimulated sample (control) (1), after salmonella LPS stimulation at 1000ng/ml (2) after heat killed *K. pneumoniae* stimulation (HKK) at 106 CFU/ml (3) and after heat killed *S. aureus* at 106 CFU/ml (4). Semi-quantitative analysis (B) was performed to assess the extent of cellular aggregate in term of its surface area (µm) using Image J software. Data are expressed as means ± SEM and were analysed by means of Kruskal-Wallis test,

followed Dunn's multiple comparisons test between groups. Changes were considered significant if p-value was <0.05. Error bar represent SEM. Scale represents 10 (A1 and 2) or 20 μm (A3 and 4). n = 64 images from n = 4 independent isolations per condition.

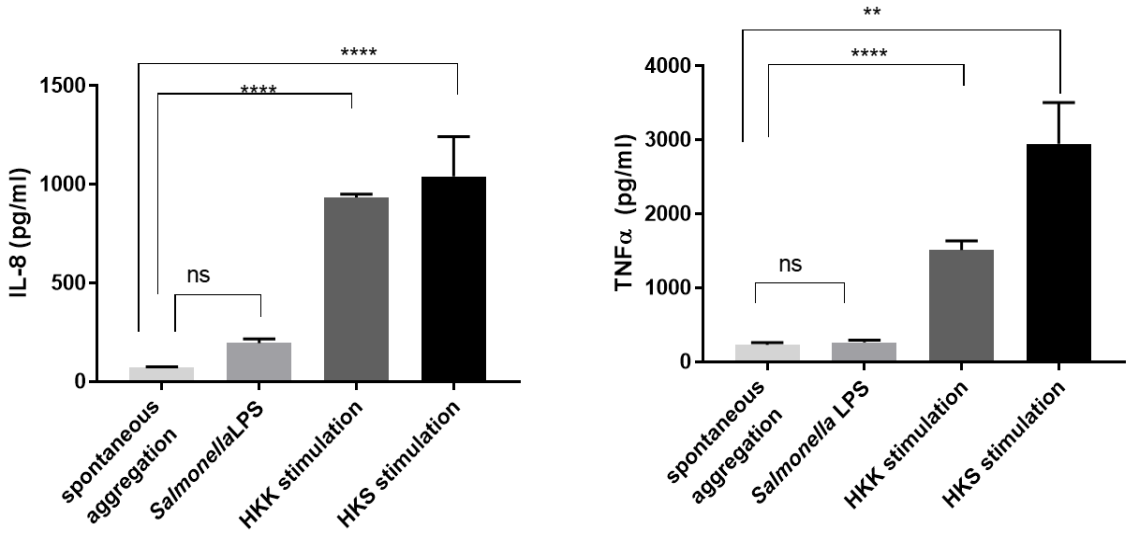


Figure 3.21: shows IL-8 and TNFα levels in plasma prepared from whole blood samples at different conditions. Data are expressed as means ± SEM and were analysed by means of Kruskal-Wallis test, followed Dunn's multiple comparisons test between groups. Changes were considered significant if p-value was <0.05. Error bar represent SEM. n=4.

Complement activation was used as another inflammatory marker to further investigate the whole blood inflammatory response induced by different conditions of stimulation *in vitro*. Western blot analysis of C5 cleavage product C5a as a low molecular weight marker of complement activation by any or all of the three pathways was performed in order to compare the efficiency of enzymatic activation of blood samples at different conditions; whole blood at zero time points, unstimulated whole blood after 1h incubation, LPS (*Salmonella*) stimulated whole blood after 1h incubation and HKK stimulated whole blood after 1h incubation. After separation of fragments by 10% SDS-PAGE, the blots were probed with Rabbit anti human C5a Antibody (1:250). As expected, an additional dense band at the predicted molecular mass of C5a was found in HKK stimulated blood but not in other conditions. The Western blot bands of C5

cleavage products of whole blood sample stimulated with or without LPS for 1h were similar and contrasted with whole blood sample at zero time point where nearly no small C5 cleavage product can be found (Figure 3.22). This means that LPS could not induce further cleavage of C5 (complement activation) over the spontaneous C5 cleavage.

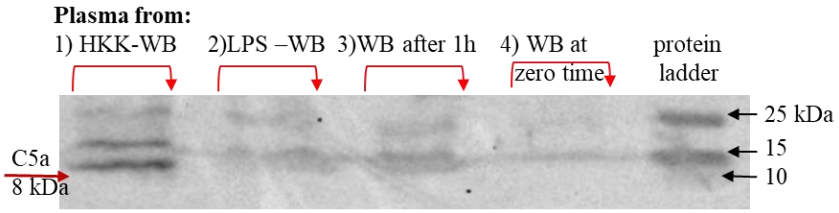


Figure 3.22: Western blot of Complement C5 cleavage product (C5a) in whole blood at different stimulation conditions.

3.4 Discussion:

3.4.1 Setting up experimental protocol to study PGAs with flow cytometric assay

In sepsis, there is extensive formation of inflammatory platelet granulocyte aggregates (PGAs) that circulate and adhere to inflamed vascular endothelium. PGAs correlate with severity of disease (Russwurm *et al*, 2002). Several published studies analysed these aggregates by flow cytometry. However, there was no consistent and clear protocol to follow in term of handling, staining, RBC lysis, centrifugation, fixation and gating. Additionally, these studies used either whole blood or more commonly isolated peripheral blood cells.

The whole blood approach, which is employed in this project, is an appropriate test system because it not only reduces the artefacts related to cells isolation but also maintains almost all circulating blood components as well as different cell types which they may interact.

The results of the study reported here demonstrated important issues for the development of a suitable and sensitive assay to carry out the objectives of this project. Firstly, the optimised blood sample collection, processing, staining, RBCs lysis and

fixation used in conjunction with optimised flow cytometric instrumental set up and gating strategy provide a significant improvement in the assay performance for more accurate results. Processing the sample within first 10 minutes of collection is important to avoid confounding effects resulted from blood stasis. To analyse the PGAs from the whole blood, it is also very important to lyse RBCs without damaging leukocytes. Effective RBCs lysis was performed at 7.4 pH for exactly 12 minutes and further confirmed by a consistent pattern of a clearly identified leukocytes subpopulations gates on flow cytometry dot plot. Furthermore, with this approach, the possible effects of RBCs on aggregate formation were excluded as they were lysed after the aggregates have been formed. Additionally, using CD66b antibody conjugated to bright fluorophore such as APC was significant because it improved the brightness of CD66b (granulocyte marker) and its antibody specificity to 99 % among individuals. Method development experiment using the same antigen, CD66b, conjugated to FITC (dim fluorophore) showed 66% to 99% of antibody specificity between individuals which underestimated the cell population. APC conjugated CD66b also decreased interference from eosinophil that underestimated positivity further when FITC was used as a fluorophore to detect CD66b. Eosinophils (unstained) have strong auto fluorescence through FITC channel which interferes with adjusting the exact cut-off point applied to mark the boundary for the positive population. Therefore, most of truly positive stained granulocyte population will be regarded as a negative, background, staining level and excluded from analysis due to false cut-off point. Since interindividuals' variations in the number of blood cells and relative percentages in their subtypes are preserved, acquiring 5000 events of granulocytes instead of all events improved the consistency of the population intensity among individuals.

Secondly, by employing a carefully standardized assay, involving instrumental set up, I have established a significant assay reproducibility. Flow cytometric data was examined using freshly donated whole blood samples over 3 days (inter-day validation) from two healthy individuals (inter-person reproducibility) stained and analysed in parallel. The CV of assay 1 was 13.6 % while assay 2 was 18%. This variation was accepted because it met the general criteria for flow cytometry assay precision with CV% less than 20% (Carey, McCoy and Keren, 2007) and could be contributed to the biological variability resulting from freshly drown blood over the several days.

In conclusion, the strategies taken to improve this assay methodology provided a more robust and reproducible approach to analyse the effect of PAMPs on PGAs in whole blood samples.

3.4.2 Formation of inflammatory platelet granulocyte aggregates in a whole blood stimulation assay using a single PAMP:

An important issue arose from our observations using unstimulated whole blood regarding the true level of circulating leukocytes that are positive for platelet markers. Present results of the whole blood collected (by venepuncture) into hirudin or EDTA and immunolabelled immediately at zero time point prior to any treatment and further processing showed on average ~11% +/- 5% PGAs in the total granulocyte population (PGA/G) by flow cytometry. This is consistent with data from other laboratories (Li, Goodall and Hjemdahl, 1997). These authors showed a baseline (i.e. zero time point) of ~ 15.3 % leukocyte forming aggregates with platelets in untreated blood from 36 healthy volunteers. They concluded that these aggregates were already preformed *in vivo* because blocking antibodies did not induce a significant decrease in theses aggregate levels in the absence of *in vitro* stimulation (Li, Goodall and Hjemdahl, 1997). A study done by Elalamy et al, 2008 reported nearly similar baseline of circulating PLAs (7+/-4% PLA /L) among healthy controls (Elalamy *et al*, 2008). Thus, the detected baseline can be used to reflect the *in vivo* state and enables further experiments to investigate the underlining mechanisms involved in their formation.

However, this baseline was significantly increased to ~37% +/-10 PGA/G when untreated blood was incubated at 37°C for up to 60 minutes. The unexpected large proportion of aggregate formation at baseline between granulocytes and platelets observed in this study under static condition has not clearly been described in previous studies. One study observed that fixation of the blood or lysis of erythrocytes associated with multiple centrifugation and washing result in artefactual increase in PLAs to 3-5-fold of PLAs detected in unfixed blood (Li, Goodall and Hjemdahl, 1997). The reported PLAs fold increase agrees with that observed in the present study after 1 h incubation at 37oC. The discrepancies between the data could be attributed to the differences in sample processing and measurement. And, most importantly, it indicates the sensitive behaviour of the whole blood system *ex vivo*. Another two studies conducted by the same principle author incubated the whole blood with fibrin polymerising inhibiting

peptide such as GPRP and RGDS to reduce the aggregate formation (Stahl, Sartz and Karpman, 2011) Ståhl et al, 2009). The spontaneous aggregate formation observed in our conditions (hirudin or EDTA), however, could not be blocked by addition of GPRP at the recommended dose of 5mM. These results were associated with a significant increase in both IL-8 and TNF α after 1h incubation at 37C in unstimulated blood sample. This is a significant discovery, namely the spontaneous aggregate condition that might confound the analysis of whole blood LPS stimulation.

After treatment with LPS, no significant increase in percentage PGA could be detected over time. These results are inconsistent with most of current papers as they compare the effect of stimulation, requiring incubation condition, on PGA % with unstimulated sample stained immediately at zero time points (Nkambule, Davison and Ipp, 2015b; Huang *et al*, 2009). However, only one study demonstrated no significant increase in platelet leukocyte aggregate level after stimulation of whole blood with *E.coli* LPS at various concentrations with a range of 0.1, 1.0 and 10 μg/ml (Mirlashari, Hagberg and Lyberg, 2002). Similar to the present study, the latter study used a control incubated at the same condition of LPS stimulated sample,thus, allowed an accurate analysis of the effect of LPS stimulation.

The same results were observed after stimulation with higher doses of LPS for 1 and 4 h incubation. In addition, these results were comparable with SEM results, which showed almost no significant differences in ultrastructural morphology of aggregate formation with similar calculated surface area, allowing it to be accurately analysed by flow cytometry.

However, the LPS types used to induce the PGA formation were able to stimulate an inflammatory cell response in a mouse macrophage cell line in the presence of 10% fetal calf serum in terms of TNFα production and lipid inclusions. These results are at variance with the ability of LPS to induce an inflammatory response in a whole blood assay that showed no significant difference in IL-8 concentration or TNFα when EDTA or hirudin anticoagulated whole blood were stimulated with *Salmonella* or *E.coli* LPS.

These data demonstrated that whole blood stimulation with LPS preparations from *E.coli* 0111:B4 and from *Salmonella enteritidis* did not increase the number of PGA significantly over the spontaneous aggregation observed at the same time of incubation without LPS. This observation was further confirmed by qualitative SEM analysis. The

fact that no significant effect of LPS on PGA formation in a whole blood stimulation assay was detected by quantitative (flow cytometry) and qualitative methods (SEM) could be due to the unavailability of an appropriate dose of LPS to induce inflammation, resulting from the ability of the plasma compartment to basically neutralise its effect. For example, plasma lipoproteins, high density lipoprotein, low density lipoprotein, very low density lipoprotein and chylomicron remnants, have been shown to neutralise the endotoxin effect by binding LPS (Parker *et al*, 1995; Kitchens *et al*, 2001; Harris, Gosnell and Kumwenda, 2000). Additionally, several studies demonstrated the ability of antimicrobial peptides and many biological molecules with poly cationic structure such as procalcitonin, a precursor of calcitonin hormone, to neutralise the LPS effect (Matera et al, 2012). Various plasma factors and membrane receptors are found to bind LPS and neutralise its effect. Lactoferrin, apolipoprotein A-1, apolipoprotein B, soluble CD14 (sCD14) and receptors expressed on macrophages, scavenger receptors, CD11-CD18 receptors are involved in LPS detoxification (Van Leeuwen *et al*, 2005).

The finding that incubation of the LPS unstimulated sample for the same time as parallel samples that are stimulated with LPS (1h) led to considerable aggregate formation means that the baseline with which the LPS stimulated samples need to be compared was significantly elevated over the immediately stained whole blood sample. This observation significantly impacts on the conclusions drawn from this *in vitro* model of whole blood stimulation.

It should be mentioned that properdin knockout mice could not be analysed in the study. This was because properdin sharpens the cellular response, so we could not have been able to dissect out the humoral vs cellular effects of properdin presence or absence. Also, there were no mouse endothelial cells and we could not acquire a properdin-blocking antibody.

To conclude, a whole blood stimulation assay was developed as a mode to study inflammatory PGAs *in vitro.* We have demonstrated that endotoxin is not a suitable stimulus to provoke sepsis relevant platelet granulocyte aggregates *in vitro.* We hypothesised that our method could be applied to model the acute inflammatory reaction in the bloodstream to the presence of a variety of PAMPs typically present during septicaemia, as well as provide the basis for future studies on the mechanisms of cell interactions.

3.4.3 Formation of inflammatory aggregates in a whole blood stimulation assay using PAMP rich stimuli

Flow cytometric analysis of EDTA anticoagulated whole blood from two male and female volunteers of different ages and ethnicity showed a significant increase in PGA%, representing 1.5-2 fold increase above the background level of PGA in controls, after being treated with HKK. This is in good agreement with other studies which have shown nearly the same amount of PLA increase that resulted from treatment of EDTA anticoagulated whole blood with thrombin receptor activating peptide (TRAP at 0.3 μM) (Kornerup *et al*, 2010) or citrated whole blood with shiga toxin (Ståhl et al, 2009).

It was, however, surprising that no significant increase in PGA% could be detected by flow cytometry when hirudin anticoagulated whole blood treated with HKK and HKS at the same dose of 106 CFU/ml and processed exactly in the same way. This might be related to effects of different anticoagulants used in this study. Previous whole blood studies analysing the PLA formation have mostly been performed using several anticoagulants that may greatly affect the blood physiology and cellular response as well, such as EDTA with sub physiological calcium and magnesium concentration, citrate that chelate calcium or heparin that binds to anti thrombin (Li *et al*, 2000). They also interfere with complement activity. Therefore, these results might not reflect the true physiological condition and not be representative of the condition we tried to model *in vitro*, namely sepsis. Thus, hirudin was subsequently used as an anticoagulant alternative to EDTA to provide a more physiological environment with minimal effect on cellular interactions (Bexborn *et al*, 2009a). In contrast to other anticoagulants, EDTA, citrate and heparin, it allows a more accurate investigation of platelet leukocyte interaction without interference with complement activity or calcium and magnesium ions depletion as it binds to and inhibits activated thrombin (Bates and Weitz, 2000).

No significant increase in PGA formation being detected in hirudin whole blood stimulation model compared with EDTA could be due to the effects of blood calcium and magnesium levels and complement activity on extent of this cellular interaction. In addition to several adhesion molecules which contribute to this interaction (described in introduction section), heterotypic platelet leukocyte aggregate formation is mainly mediated by calcium dependent interactions of P-Selectin, expressed on platelets, with PSGL-1, expressed on leukocyte such as neutrophil and monocyte (Li *et al*, 2000;

Wang, Qin and Sun, 2014; Bournazos *et al*, 2008). P Selectin blocking studies have demonstrated that P- selectin plays a critical role in platelet neutrophil aggregate formation both *in vivo* and *in vitro* (Mauler *et al*, 2016). Magnesium dependent integrin interaction is another important player that contributes to the aggregate formation as described previously (Bournazos et al, 2008). Complement activation by the three pathways, classical, alternative and lectin pathway, also accounts for an essential part of aggregate formation. The end products of complement activation, anaphylatoxins and a membrane attack complex, exert various biological effects that results in amplification and propagation of this interaction (PLA). Importantly, it is not only its activation that leads to platelets activation (Colman, 2006; Hamad *et al*, 2010; Del Conde *et al*, 2005; Williams, 2011) but also activated platelets result in complement activation (Saggu *et al*, 2013; Del Conde *et al*, 2005). It was shown that various complement activation products such as C3 and C5-C9 potentiate platelet activation and aggregation induced by thrombin. Also, it was found that the anaphylatoxin C3a induced the platelet activation (Del Conde et al, 2005). The reverse, whereby the platelet could activate the complement system is also documented. Del Conde et al (2005) demonstrated capability of platelet to activate the complement system. As binding of C3b to the P- Selectin (expressed on platelet) was identified and shown to initiate the complement activation in this study. Additionally, release of properdin (positive regulator of alternative complement pathway) by activated neutrophil contributes to complement system activation by activated platelets (Saggu et al, 2013). It binds to activated platelet and recruits C3b to form C3bBb convertase. Activated neutrophil as well leads to activation and amplification of complement system by production of properdin and complement activation in turn leads to further neutrophil stimulation mainly by C5a and C5-C9 complex deposition on its surface (Camous *et al*, 2011b). Properdin potentiates platelet leukocyte aggregation after Adenine di-Phosphate (ADP) pre-stimulation and increases CD11b expression (Ruef *et al*, 2008).

This provides a clear link for the involvement of all these factors, calcium, magnesium and complement system, in the platelet leukocyte aggregate formation and leads us to believe that the extent of aggregate formation in terms of ultrastructural morphology when hirudin is used could be more complexed thereby making it difficult to be analysed properly using flow cytometry. Another important point to stress on is that the reliance on forward and side scatter gate as a way to recognise PLAs from the strictly

gated granulocyte population might underestimate the results. To explain, aggregated cells are larger than single cells (granulocyte population) and located beyond the granulocyte population. Thus, it can be missed if there is a tight forward vs side scatter gate. Although they were very rare events appeared on the right side of the flow cytometry plot, it might affect the result. It should be mentioned that the gating strategies used in this project depends on how theses cells were commonly identified on the literature.

Thus, further qualitative analysis by scanning electron microscopy, of the aggregate formation in hirudin anticoagulated whole blood incubated with different stimuli, *Salmonella* LPS as single PAMP, HKK and HKS as PAMPs rich stimuli at the same doses used for previous whole blood samples were analysed by flow cytometry and showed significant differences in the cellular aggregate morphology, complexity and surface area in HKK and HKS stimulated samples compared to the unstimulated sample.

Our data imply that, in contrast to the qualitative analysis by SEM, the quantitative analysis using flow cytometry may not be accurate to assess PLAs formation in response to inflammatory stimulation of a whole blood assay for several reasons. First of all, in this study, the size of inflammatory aggregate was found to affect the accuracy of flow cytometric analysis. SEM results showed that most of the aggregates formed in response to HKK or HKS stimulation of hirudin anticoagulated whole blood were too large (with a mean surface area of 470.611 μm2 and 518.399 μm2, respectively) to be detected by flow cytometry, because the internal diameter of flow cell being used by modern flow cytometers usually ranges from 50 to 250 μm2 (Givan, 2013). Therefore, the large particles with sizes more than 250 μm2 are not analysable by flow cytometry. Thus, detecting a significant increase in aggregate formation after HKK stimulation when EDTA anticoagulated whole blood is used in contrast to hirudin when no increase was detected appears to be strongly related to the effect of EDTA on the cellular interaction. This is because a considerable component of divalent cation platelet leukocyte dependent interaction and complement related effects on propagation of this interaction is excluded that leads to underestimation of the actual aggregate with less complexity allowing them to be analysed using flow cytometry. Another important factor that affects accuracy of flow cytometric analysis of inflammatory aggregate formation is the morphology and the relative composition of the aggregate. In SEM

analysis, different forms of aggregate, which can be analysed by flow cytometry in regard to their size, were found. Aggregates composed of two or more leukocytes (which could be any mix of granulocyte, monocyte or lymphocyte) and plenty of platelets at different ratios which are interpreted by flow cytometry as a single event as one platelet with one granulocyte. Some globular structure with corresponding size of granulocyte was completely covered by platelets which could mask the surface expression of granulocyte marker and be interpreted as single platelet by flow cytometry. The coincidence events, non-adherent but coincident leukocytes and platelets that pass through the interrogation point of laser near each other and considered as PLA events, are another confounding parameter, which contributed to 30% of the detected PLAs, demonstrating a relative inaccuracy of flow cytometric analysis (Hui *et al*, 2017).

To sum up, the findings presented here clearly indicate the importance of analysing the platelet leukocyte aggregate formation under simulated physiological conditions i.e., in whole blood with normal extracellular divalent cation levels and intact complement system. In the present study, most importantly, the quantitative analysis of inflammatory aggregate formation by flow cytometry should be reevaluated due to the lack of precision. Thus, particular attention is required when interpreting and comparing results of inflammatory aggregate formation from studies that used different anticoagulants that alter the physiological state significantly and relied on flow cytometry in their analysis.

Chapter 4

Establishing and characterising an *in vitro* model of platelet leukocyte aggregate adhesion to endothelial cells under pro-inflammatory conditions, an early event in sepsis

4.1 Introduction:

Platelets leukocytes aggregates adhesion to the endothelium have been analysed in *in vivo* models of sepsis (Koike *et al*, 2015) and of acute inflammation (He *et al*, 2006). Koike et al used a lipopolysaccharide-induced mouse model of sepsis (*E. coli* serotype 0128:B12, 10mg/kg) to investigate dynamic real time formation of ‘‘leukocyte-endothelium-platelet aggregation’’ *in vivo* with using two-photon laser-scanning microscopy (TPLSM) technique. Study by He *et al* investigated the composition of adhered PLAs and its relation to the microvascular permeability. He and colleagues utilised a combination approach of systemic TNFα (3.5 μg/kg) stimulation with local microvascular stimulation with platelet activating factor (1nM) to mimic the acute inflammation and then analysed the platelet leukocyte aggregates adhesion to the endothelium by fluorescent staining and confocal microscopic imaging (He *et al*, 2006).

However, no *in vitro* study can be found to investigate the platelet leukocyte aggregate adhesion to endothelium in a condition that mimics sepsis. The underlying mechanisms of platelet leukocyte endothelial interaction are still not fully understood despite of their significance in sepsis pathophysiology (as described in section 1.5.1). More studies, that are amenable to more standard laboratories, are required to address these.

Under normal circumstances, any changes that occur in the extravascular environment, such as transient bacterial infection or minor trauma, are constantly sensed by endothelium to provide an immediate adaptive response. However, in sepsis, the extent of endothelial cell activation crosses the threshold from the adaptive to maladaptive response where there is a persistent and progressive systemic endothelial activation (Aird, 2003; Skibsted *et al*, 2013). This phenotype of endothelium activation is either induced directly, via pathogen invasion or endothelial cell recognition of PAMPSs by its surface receptors (PRRs), or indirectly via host derived factors such as cytokine, chemokine, complement and activated platelets and leukocytes (Aird, 2003). Endothelial cell activation leads to increased expression of adhesion molecules such as P and E selectin, VCAM and ICAM that mediate EC-leukocyte-platelet interactions (Wilhelmsen, Farrar and Hellman, 2013) and of cytokines and chemokines (Baronas-Lowell, Lauer-Fields and Fields, 2004).

Extensive *in vitro* studies conducted to understand the mechanism of platelet leukocyte or leukocyte–endothelial interactions under inflammatory condition revealed the

involvement of a wide range of adhesion molecules and soluble mediators (described in section 1.5.3). However, the majority of this work does not take into account the complexity of this interaction that are driven by the effects of other blood components. For example, there was augmented surface expression and adherence of blood neutrophils isolated from healthy subjects to inflamed endothelium when incubated with plasma from patients with sepsis (Lewis *et al*, 2013).

A review of the literature conducted as part of this thesis underlined the importance of studying the interaction under simulated physiological condition, using whole blood. In this chapter, PLAs adhesion to inflamed endothelium that is formed in response to different stimuli and their associated inflammatory response with regards to inflammatory marker and adhesion molecule expression were investigated *in vitro*.

4.3 **Experimental design of platelet leukocyte aggregate (PLAs) adhesion to endothelial cells**

The setup to study this interaction was designed to use a freshly donated whole blood sample, which was stimulated with sepsis relevant PAMPs rich stimuli or endotoxin for 1h at 37 oC as described in the previous chapter. Inflammatory PLAs will be formed and these will be incubated for 1h at 37oC on endothelial cell monolayers pre-stimulated with TNF α under static condition. This step allows the adhesion to take place. In principle, this setup was consistent with the general guideline of many previously described adhesion assays used to investigate leukocyte adhesion to endothelial cells (Wilhelmsen, Farrar and Hellman, 2013).

Human umbilical vascular endothelial cell line (HUVEC) is commonly used to understand the cell interaction especially in sepsis (Wang *et al*, 2015). EAhy 926 cells, an endothelial hybrid cell line, was chosen as an *in vitro* model for endothelium to study the interaction between PLAs and endothelium because the cell is characterized by high expression of endothelial marker proteins and has functional characteristics of human vascular endothelium (Edgell, McDonald and Graham, 1983; Brown *et al*, 1993). EAhay926 have a similar response to the primary human umbilical vein endothelial cells in terms of leukocyte adhesiveness and expression of adhesion molecules (E-selectin, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1)) when stimulated with TNFα (Thornhill, Li and Haskard, 1993).

Afterwards, the samples and supernatants (cell culture supernatant or plasma) are processed with several methodologies according to the purpose of experiment as shown in figure 4.1.

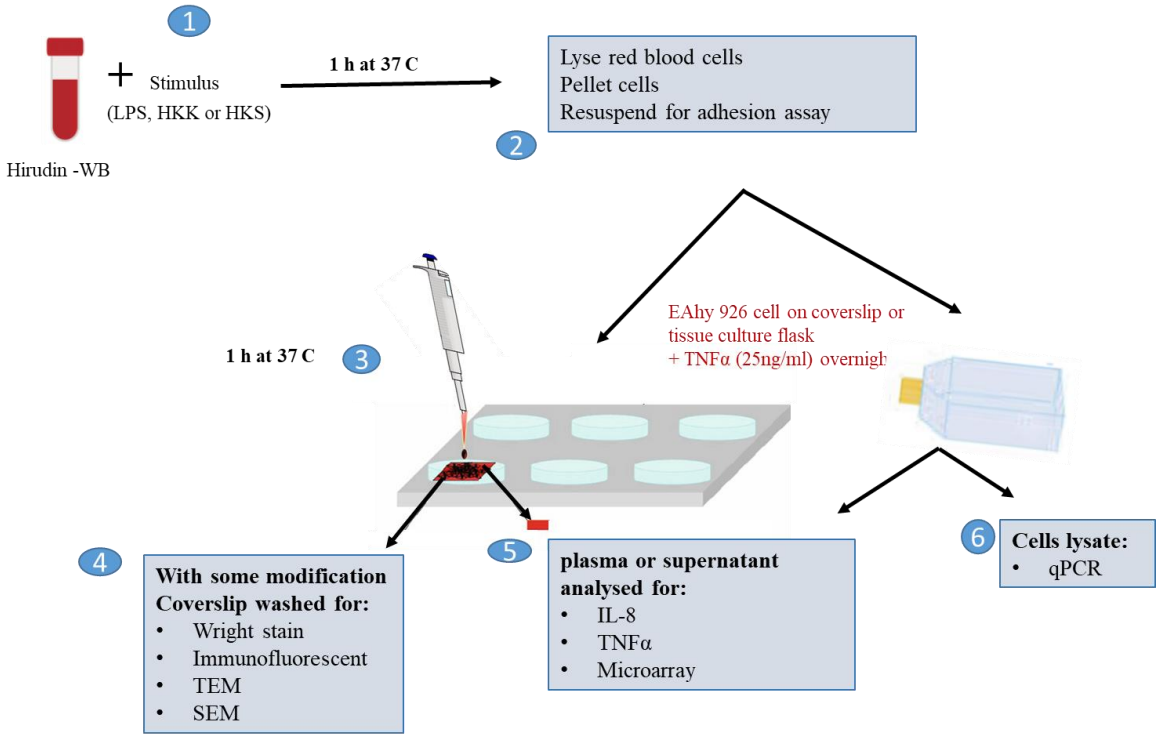


Figure 4.1: An overview of the experimental steps taken and analytical flow.

4.4 **Results:**

4.3.1 analysis of platelet leukocyte aggregate (PLAs) adhesion to endothelial cells:

4.3.1.1 Immunofluorescent microscopic analysis of PLAs adhesion to endothelium:

To test the functionality of this setup, an initial study was performed in order to visualize and quantify the extent of HKK induced PGA adhesion to the inflamed endothelial cells by evaluating the overlap of two immune fluorescent probes specific to platelets and leukocytes. Immortalized human umbilical vein endothelial cells (EAhay 926) were cultured on coverslips till confluency and stimulated with 25ng/ml of TNF α overnight. The next day, hirudin anticoagulated whole blood was stimulated with HKK (106 CFU/ml) for 1 h at 37oC to induce formation of PLAs. Cells culture were washed with PBS containing Ca2+ and Mg2+ (as subsequent cell adhesion is dependent on calcium ions) and incubated with RBCs lysed - HKK stimulated whole blood for 1h at 37oC. After washing to get rid of unbound cells, the cultured cover slips were stained with or without (control) a mixture of 5 µl of each direct fluorescent Ab (PE-CD42b to detect platelets and FITC-CD66b to detect granulocytes) and incubated for 20 minutes in the dark at 4oC. Then, the samples were fixed with 2% paraformaldehyde for 15 minutes and mounted for fluorescence microscopy.

As shown in figure 4.2, staining of platelets (in red) and granulocytes (in green) reflected that the majority of the platelets and granulocyte adhere to the endothelial cells and its binding was strong enough to resist multiple manual washing steps. Co-localization of FITC and PE can be apparent in structures that appear yellow, because of the combined contributions of green and red fluorescence, respectively. Unexpectedly, merging platelets and granulocyte immunofluorescences was not successful as only a few yellow dots appeared (indicated by an arrow). Therefore, this method did not appear sensitive enough to detect the adherence of PGAs to the endothelium monolayer. This might be explained by binding of direct immunofluorescence antibodies at their targets which are located far away from each other due to the difference in cell sizes and therefore difficult to be overlapped and detected by the merged image. The morphological complexity, detected by SEM in the previous chapter, might mask the antigenic marker and is another possible factor in under-estimating the cellular aggregate adhesion and, thereby, affects the sensitivity of this method.

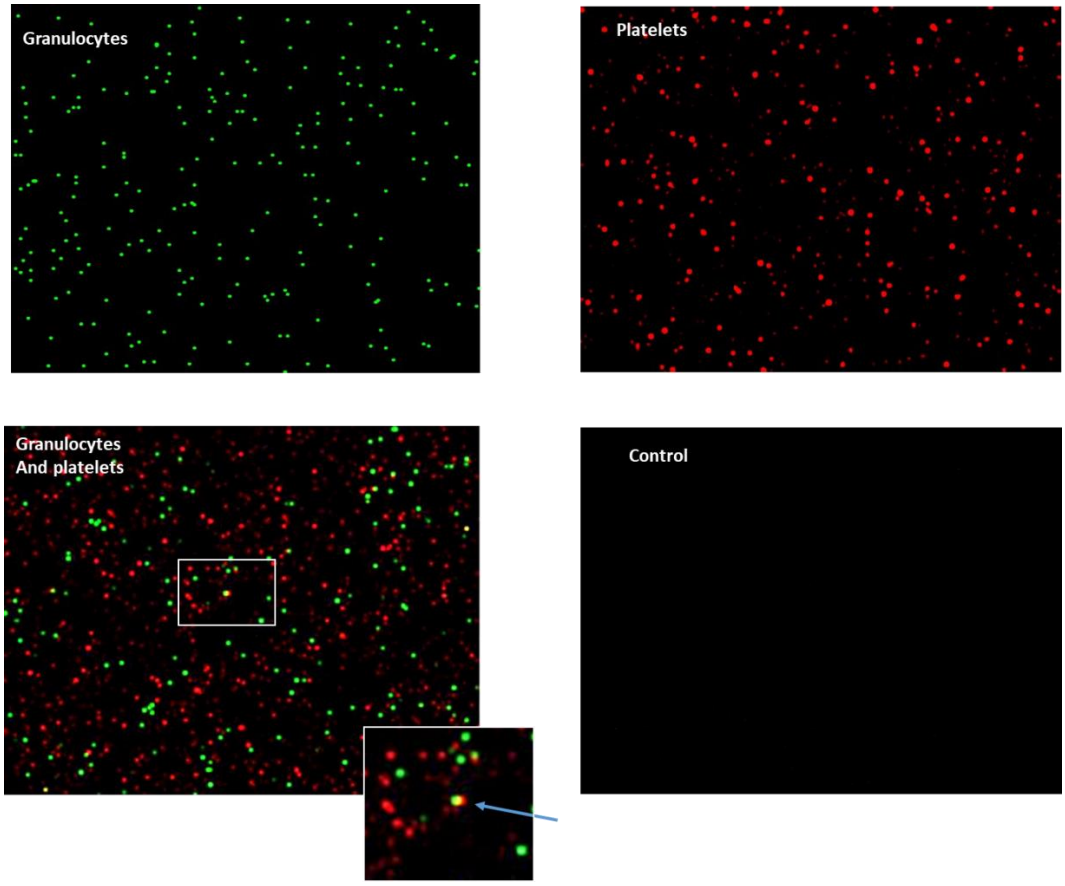


Figure 4.2: Immunofluorescence microscopy of PLAs adhesion to endothelium. TNF α stimulated HUVEC were incubated with HKK stimulated whole blood for 45m and then stained for CD42b (PE conjugated primary antibody) and CD66b (FITC-conjugated primary antibody) by direct immunofluorescence to detect the adherent platelets and leukocytes. Immunofluorescent images at magnification of (10 ×) shows that most leukocytes (in green, A) and platelets (in red, B) adhere to the endothelial cell. (C) is the overlap between PE and FITC probes were used to identify the adhered PLAs on endothelium as yellow structure. The merged image shows only a few yellow dots and how they are partially overlap indicated in arrow. IgG with an irrelevant specificity (isotype control) did not give staining of these cells, D.

Wright stain of the above coverslips was performed to determine the presence of PGAs on endothelium using light microscopy. As shown in figure 4.3, heterotypic aggregates of platelets and neutrophil were indeed seen on or adjacent to the endothelial cell layer which had previously been activated (A, B, F, E). Homotypic aggregates of platelets or neutrophils were also observed either on top of or adjacent to the endothelium (B). In some areas, neutrophils bound to one or more platelets on their surface, where a destruction of endothelial cell layer was observed which was likely to be a result of sample processing (removing mountant from coverslip) (C). Other constituents of blood such as eosinophil, monocyte and lymphocyte, were also seen (E, F).

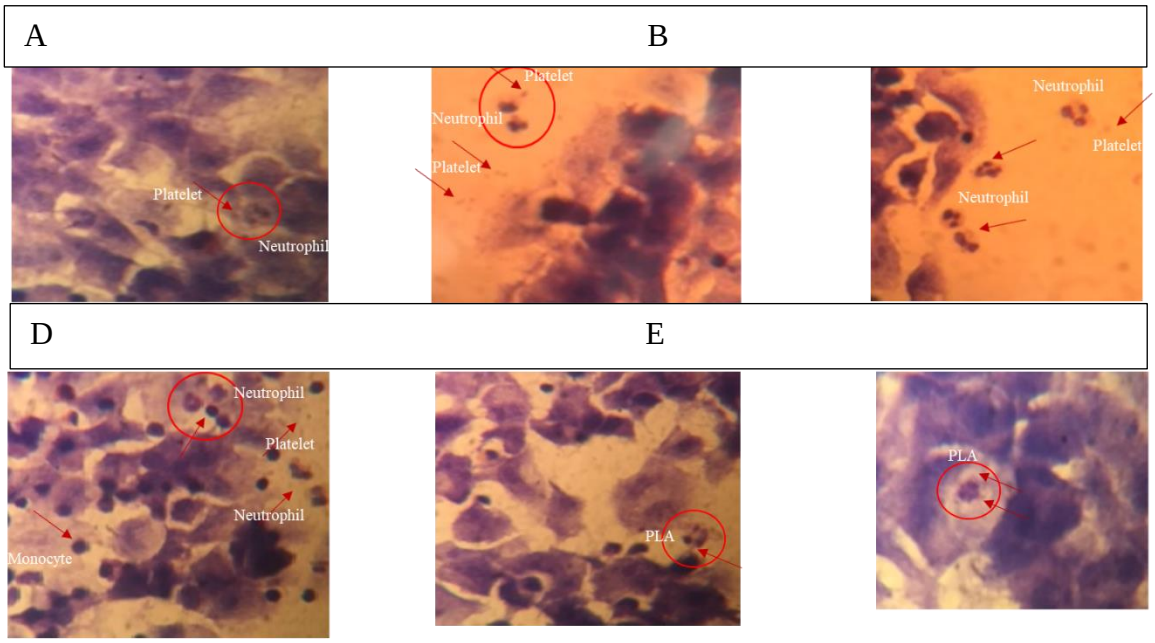


Figure 4.3: Wright's stained preparation of EDTA HKK–treated whole blood samples incubated on inflamed endothelium from previous experiment demonstrating platelet leukocyte complex adherence to endothelium. Arrows in each image point at neutrophils, monocyte, platelets or PLAs.

4.3.1.2 Light microscopic analysis of PLAs adhesion to endothelium

Based on the promising identification of blood cells and platelets adhering to the endothelial cells, the experiment was repeated for analysis by Wright stain using stimulated whole blood.

First, inflamed endothelial cells culture were prepared on coverslips as described earlier. To induce the aggregate formation, aliquots of hirudin anticoagulated whole blood were stimulated with *Salmonella* LPS, HKK or HKS separately as described in the previous chapter. One aliquot was left without stimulus (control). After RBCs lysis, TNFα inflamed endothelial cell culture was washed with PBS containing Ca2+ and Mg2+ and incubated with unstimulated (control), LPS, HKK or HKS stimulated blood separately for 60 minutes at 37oC. Then the well plate was washed and stained with Wright stain. "Inflamed endothelium" on coverslip and HKK stimulated blood smear after lysis were prepared for Wright stain as well as controls. Images of several fields at different positions (e.g., 10 fields) for each condition were acquired and analysed using light microscope with a 40X or 10X objective. Different cell types were identified according to their morphological features. HUVECs, stained with light purple, were flat and polygonal-shaped, arranged in short spindles or demonstrating cobblestone morphology (Figure 4.4). Leukocytes with different sizes found as individual cells in a random manner and in clusters, stained with dark purple, while individual platelet, stained in light red, appeared as small dots near or attached to clusters. Some red blood cells were also found.

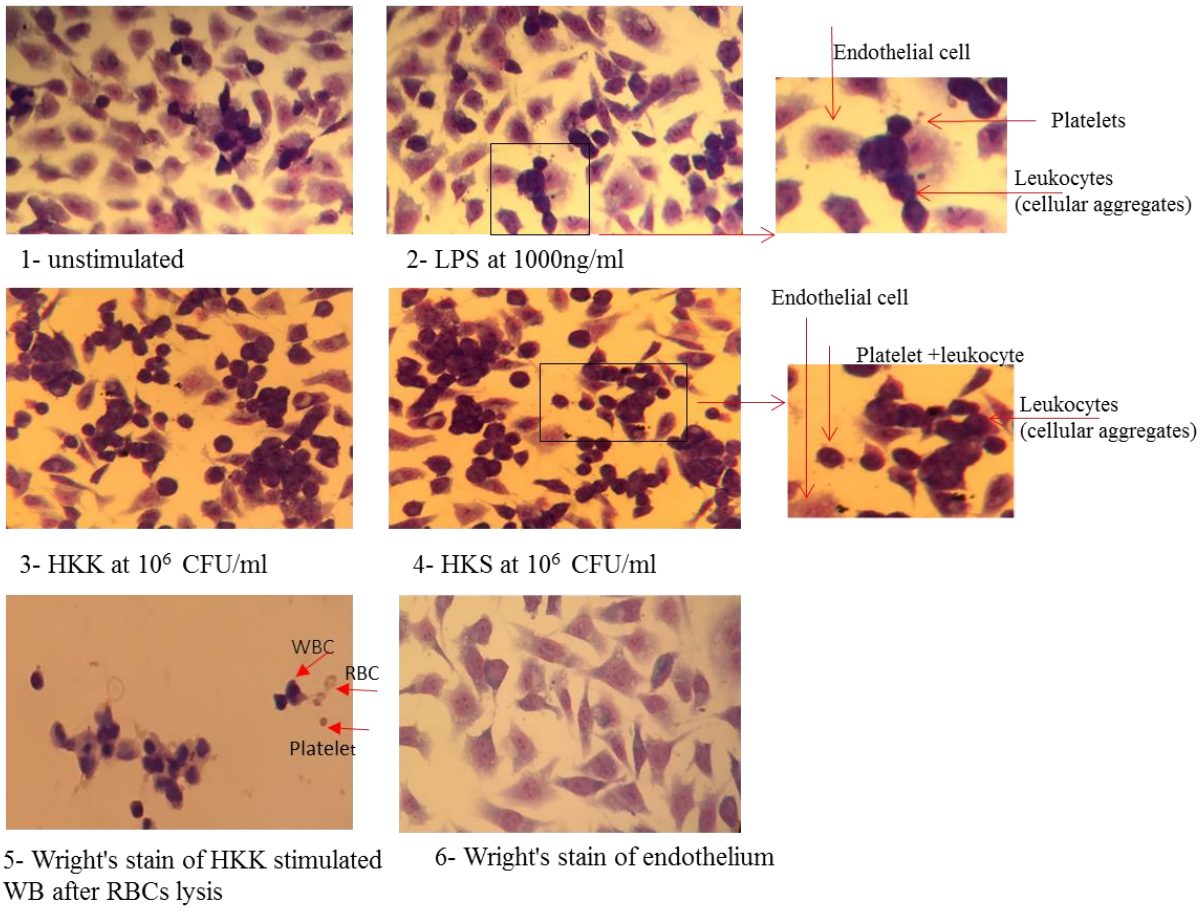
In figure 4.4, A1 as expected, a small number of cellular aggregates, formed spontaneously in unstimulated whole blood sample, adhered in a sporadic manner to endothelial cell and no obvious changes in this phenomenon was seen when LPS stimulated whole blood sample was used which showed a nearly similar pattern of cellular aggregate adhesion (Figure 4.4,A2). In contrast, this pattern of adhesion was particularly obvious when whole blood stimulated with heat killed bacteria were used in which lots of large and complexed cellular aggregate were frequently found adhered on endothelial layer (Figure 4.4 A3 and 4). Further quantitative analyses, shown in figure 4.4 (B), were performed by counting the number of cellular aggregate (indicated in dark purple) per mm2 of endothelial layer and the number of platelets and leukocytes

involved in each cellular aggregate. Similar results were found when another observer analysed the same experiment.

Similar numbers of cellular aggregates/mm2 were detected when the blood incubated on the endothelium layer was left unstimulated or stimulated with LPS. The adhered aggregates at both conditions (-/+ LPS) were composed of nearly the same numbers of leukocytes. However, the number of platelets involved in the aggregate composition analysed by light microscopy is nearly two-fold that of unstimulated sample and similar to that of HKK and HKS stimulated sample. This could point to an important activity of platelets to initiate aggregate formation. In inflammation, platelets play a key role in several steps of leukocyte recruitment, activation and adhesion to endothelial cell (Rossaint and Zarbock, 2015; Ed Rainger *et al*, 2015).

On the contrary, a nearly two- fold increase in the two parameters was observed when heat killed bacteria, HKK or HKS, were used as a stimulus to the whole blood. These results, consistent with the previous chapter, further confirm that a PAMP rich stimulus is superior to single PAMP such as (LPS) in a whole blood inflammatory model.

A



B

Stimulus	No of cellular aggregate /mm² (mean of n=5)	No of platelets/leukocytes in aggregates
none	16/mm²	1.6 leukocytes with 3.8 platelets (Mean of n=5)
LPS	15/mm²	1.9 leukocytes with 7.3 platelets (Mean of n=9)
HKK	34/mm²	2.75 leukocytes with 7.75 platelets (Mean of n=4)
HKS	27/mm²	3.1 leukocytes with 7.5 platelets (Mean of n=5)

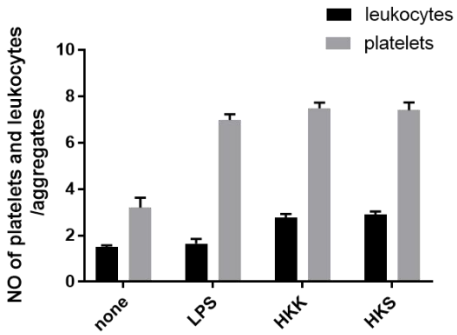


Figure 4.4. Light microscopic analysis of PLAs adherence to endothelium in response to different stimuli. Whole blood was stimulated with none (control), LPS (1000 ng/ml), HKK (106 CFU/ml) or HKS (106 CFU/ml) and co incubated with inflamed endothelium to prepare the samples for wrights stain analysis under light microscopy. 1and 2 are representative images of the adhesion model using unstimulated whole blood(1) or LPS (2) showing the sporadic attachment of aggregate and its complexity under 40 magnification while 3 and 4 are representative images of the model using HKK (3) and HKS (4) show the abundance and complexity of aggregates adhesion. For proper analysis, HKK stimulated whole blood smear after lysis (5) showing the

aggregate morphology before its incubation with endothelium while 6 is Wright stain of TNF alpha inflamed endothelium without blood. Table b shows the Amount of cellular aggregate (dark purple indicated in) per mm2 and the amount of the platelet and leukocyte involved in aggregate composition under each condition.

4.3.1.3 Transmission electron microscopic analysis of PLAs adhesion to endothelium.

Although the effect of different stimuli on the pattern of cellular aggregate adhesion to inflamed HUVEC is readily observable under light microscopy, transmission electron microscopy was performed to specifically provide an even clearer image of the adhesive interaction's ultrastructural morphology. Briefly, hirudin anticoagulated blood was stimulated with or without (negative control) LPS, HKK, and HKS separately and incubated on inflamed endothelium as described earlier. Inflamed endothelium monolayer without blood incubation was also prepared as a control (to identify cells clearly). All samples fixed and processed for TEM. Relevant training was received, and acquisitions controlled by electron microscopy staff for quality.

Representative images for each condition was chosen from a total number of 200 images. Under TEM examination, in this study, leukocyte and platelet can be identified based on their size and different cellular ultra-structures. Platelets were determined by their size (2-5 μm in diameter), absence of nuclei, glycogen store, open canalicular system and formation of filipodia (Neumüller, Ellinger and Wagner, 2015), while leukocytes were mainly recognised by their diameter, which range from 8 to 10 μm, and nuclei (Schmid-Schonbein, Shih and Chien, 1980).

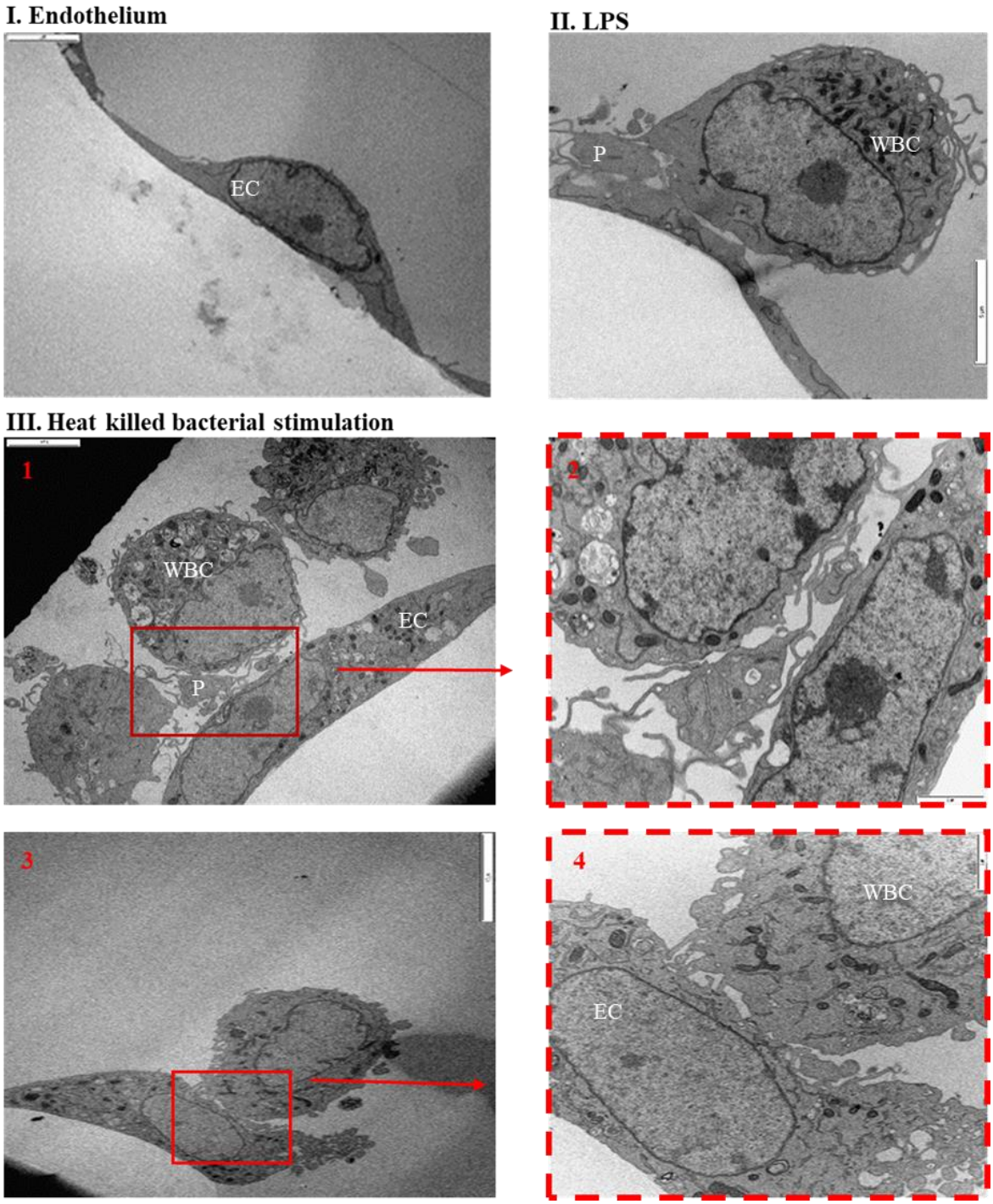


Figure 4.5: PLA aggregate adhesion to the endothelium in response to different stimuli. The transmission electron microscope micrograph shows a human endothelial monolayer treated with proinflammatory stimuli (TNFα) and incubated with human peripheral blood stimulated with different stimuli (LPS, HKK and HKS). I is a monolayer of endothelium. II is a representative image of adhered aggregate, pre-formed in response to whole blood-LPS stimulation, while III are the representative image of attached aggregates formed in response to heat killed bacteria. Scale represents 5 and 2 μm.WBC(whight blood cells), EC (Endotheilium), P (platelet).

Figure 4.5 shows ultrastructural evidence of cellular aggregate adhesion to endothelium monolayer and further confirm the complexity of adhered cellular aggregate. Similar to adhesion pattern observed by light microscopy, more complexed PLAs that involve three or more cells was attached to endothelium at frequent manner when heat killed bacteria stimulated whole blood was used as shown in. However, the aggregate of unstimulated blood or LPS stimulated blood adhered to the endothelium in a similar manner and characterised by less structural complexity as shown. Furthermore, both platelet as well as leukocyte bind directly to the endothelium and act as bridge, figure 4.5 (2,4), respectively.

4.3.2 Inflammatory marker and adhesion molecule expression generated by PLAs adhesion to endothelium.

The purpose of this test was to evaluate and quantify the inflammatory response in our novel heat killed bacterial stimulated whole blood and EAhy 926 model.

4.3.2.1 Adhesion molecule expression:

Several biomarkers involved in adhesion events between leukocyte, platelet and activated endothelium under inflammation as described earlier (introduction section) such as P and E Selectin, ICAM-1, CD40L and TF were analysed for their mRNA expression level by qPCR. These might give an indication of their corresponding proteins depending on the correlations between the mRNA expression and proteins level (Gry *et al*, 2009). EC cells pre-treated with TNFα were incubated with or without (only medium) previously prepared, HKK stimulated whole blood as described earlier, at 37oC for 1h. In addition, pre-treated EC incubated with HKK in medium (no human serum) was prepared to see the effect of HKK stimulation on endothelium. Untreated EC incubated with medium was prepared as a baseline. After incubation, EAhy 926 monolayer samples were gently washed and used for expression analysis of the adhesion molecules by RT-qPCR. The supernatants were kept for pro inflammatory cytokine analysis as another read out of the inflammatory reaction namely, IL-8 and TNFα. (Figure 4.9)

For q-PCR analysis, total mRNA from inflamed endothelial cell (+/- HKK stimulated WB) cells and other 2 conditions stated above, (see methodology) was extracted, then cDNA was prepared from (5μg) mRNA and analysed by RT-qPCR. GAPDH was previously used as a house keeping gene for pentraxin-3 gene in EAhy926 cells (Gustin *et al*, 2008). RT-qPCR products of GAPDH from all cDNA were run on gel electrophoresis, and the results showed comparable mRNA expressions (Figure 4.6).

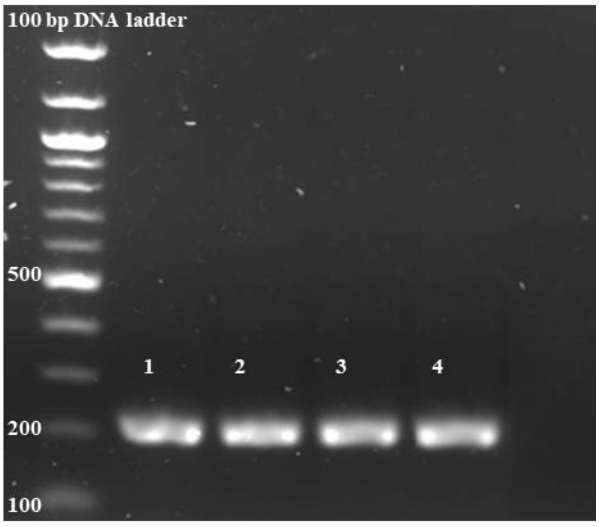


Figure 4.6: Gel electrophoresis analysis of RT-PCR products of GAPDH (size=213 bp) from different conditions; (1) untreated EAhy926 " control", (2) EAhy926+TNFa, (3) EAhy926 +TNFa+ HKK in media and (4) EAhy926+TNFa +HKK in WB. It is expressed in a comparable manner.

unexpecditly, in comparison to the negative control (NTC), the qPCR results clearly showed the negative mRNA expression of all tested adhesion markeres, P and E selectin, ICAM-1, VCAM, CD40L and tissue factor (TF). This experiment would be improved by primer efficiency determination on serial DNA dilution. However due to the technical limitation such as need of high blood volume that require a large amount of an expensive anticoagulant, alternative cost-effective methods to mesure the inflammatory effect at protein level were selected (IL-8, TNFα and protome profile).

The level of pro-inflammatory markers, IL-8 and TNFα, in the supernatants prepared from different conditions as stated in the above experiments were also measured to investigate further the inflammatory response. As expected, the level of both IL-8 and TNFα were higher in the condition where HKK stimulated whole blood was incubated with inflamed endothelium compared to other conditions (Figure 4.7).

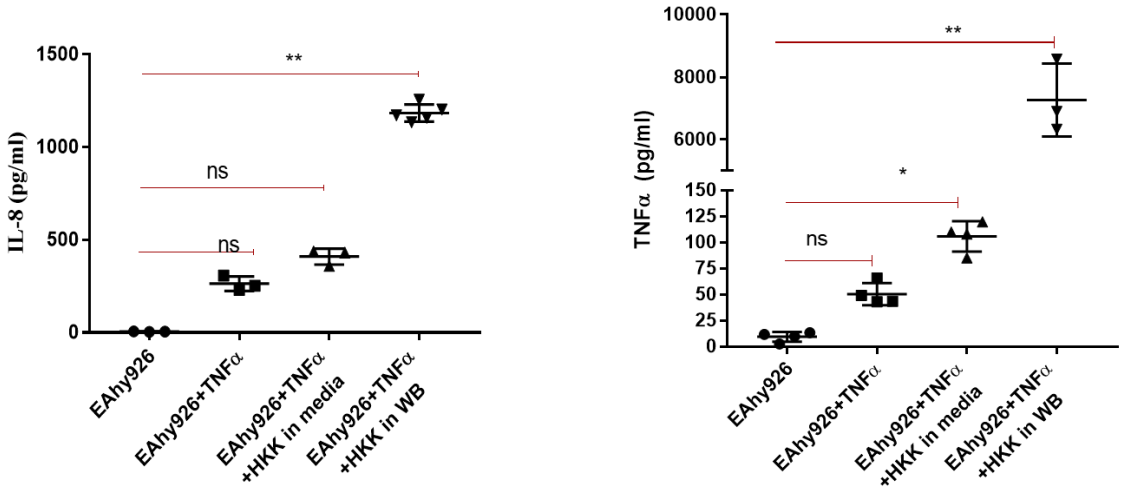


Figure 4.7: Inflammatory markers analysis under different conditions. TNFα stimulated EAhy926 that incubated with HKK-pre stimulated whole blood has a higher levels of IL-8 and TNFα compared to other conditions; untreated EAhy926, EAhy926+TNFa, and EAhy926 +TNFa+ HKK in media. Data were analysed by means of Kruskal-Wallis test, followed Dunn's multiple comparisons test between groups. Changes were considered significant if p-value was <0.05. n= 3. Errors bar is SEM.

4.3.2.2 Stimulation dependent cytokine analysis using proteome profile:

In order to characterise the inflammatory response of our novel model at the protein level, the expression of 36 different types of human cytokines, known to play a critical role in several biological processes such as inflammation and immunity, were scanned using the proteome profiler array kit (material and methods).

EAhy926 cells were sub-cultured in 6 well plates (1x 10^6 /well) and stimulated with TNFα 25 ng/ml overnight. Un-treated EAhy926 cells were prepared as control. The next day, they were incubated with whole blood stimulated with HKK or HKS. EAhy926 cells (+/- TNFα) incubated with unstimulated whole blood were used as controls. After 1h incubation, 200 µl of extracted plasma from each sample was analysed as described previously (Materials and Methods). The integrated pixel density for each spot was measured by Image J software and subtracted from background pixel density. Each biomarker was analysed in duplicate and its readings presented as mean pixel density. Negative (PBS) and positive (reference spots) controls were also examined and analysed for data accuracy. The biomarker results that did not show any noticeable changes between experimental condition are not presented here and only the ones that present remarkable differences are described.

Macrophage migration inhibitory factor (MIF) is a pro inflammatory cytokine that is released by various immune cells in response to sepsis. It appears to be strongly related to the systemic inflammatory response and the development of multiple organ failure (Larson and Horak, 2006). The plasma extracted from HKK stimulated whole blood incubated on inflamed endothelium showed a significant elevation of MIF compared with the control. By contrast, plasma extracted from HKS condition showed a slight increase in MIF which was above that of controls (Figure 4.8).

TNF α, macrophage inflammatory protein (MIP-1α and MIP-1β, known as CCL3 and CCL4, respectively), IL-1 and CCL5 (known as RANTES) are proinflammatory mediators that are elevated in sepsis (Chaudhry *et al*, 2013; Demoule *et al*, 2009). As shown in figure 4.8, in comparison to control conditions, a high expression in these mediators was observed in plasma of HKK stimulated blood incubated with TNFα stimulated EAhy926 cells. Although HKS stimulation induced a slight increase in most of these markers, their expression levels were lower than that of HKK stimulation. This difference might be related to either the microbial agent itself (such as the presence or

absence of capsules in *K. pneumonia* and *S. aureus*, respectively or to the host cellular response towards the pathogens.

Bacterial sepsis induces activation of the complement system and results in a subsequent production of C5a, potent proinflammatory anaphylatoxin, that contributed significantly to SIR and organ failure (Keshari *et al*, 2014). HKK and HKS stimulation increased production of C5 and its cleavage products, C5a compared to control. The increase in C5/C5a is also higher in HKK stimulation compared to HKS stimulation (Figure 4.8). This finding was analysed further in chapter 3.

The detection of ICAM-1 protein in plasma but not mRNA could mean that all mRNA is translated to their related protein. The plasma of unstimulated, HKK or HKS stimulated whole blood that was co-incubated with pro inflamed endothelium showed less expression in ICAM-1 protein compared to its expression in plasma of unstimulated whole blood incubated with unstimulated endothelium (Figure 4.8). These results might be related to the balance between the membrane anchored and circulating levels.

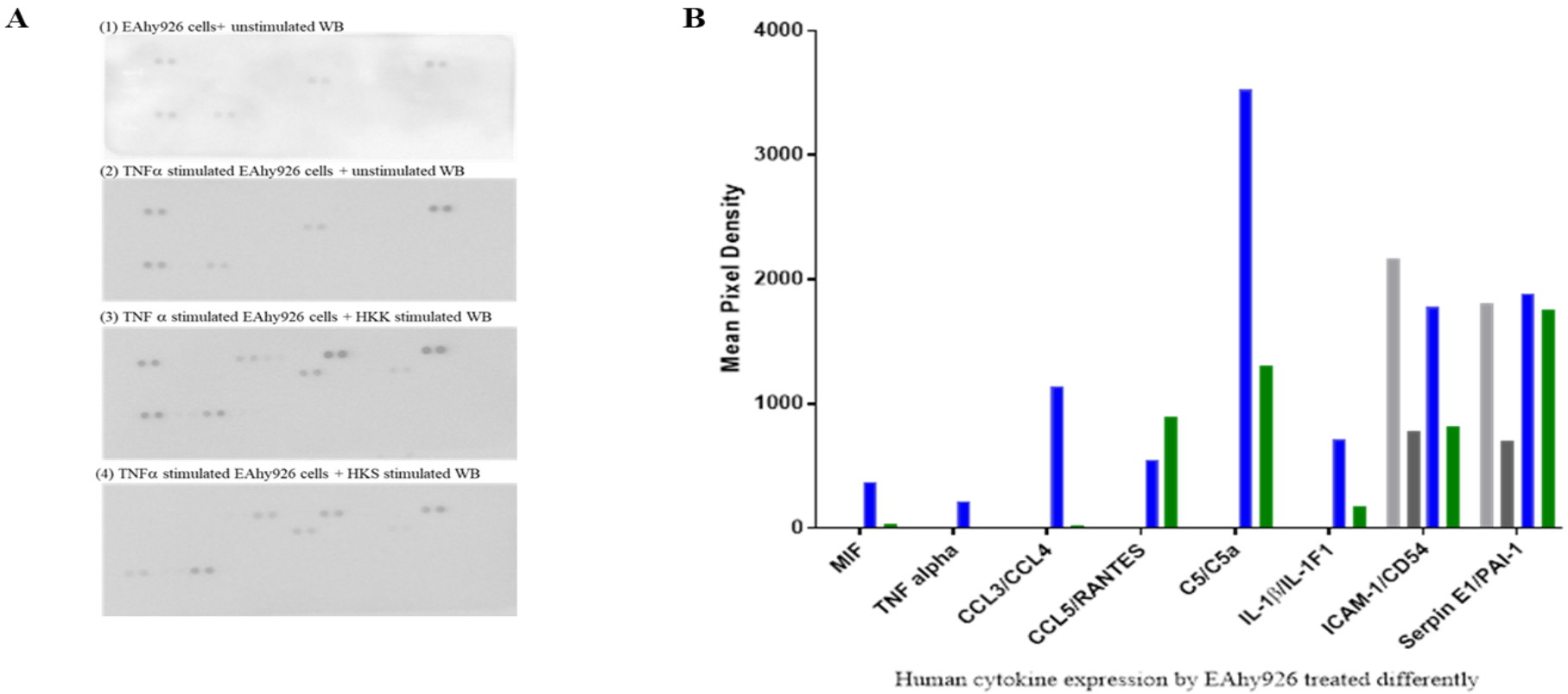


Figure 4.8: Proteome profiler of human cytokines detected under different conditions showed that TNF α stimulated EAhy926 cells incubated with whole blood that pre stimulated with heat killed bacteria, HKK and HKS have higher levels of MIF, TNF α, CCL3, CCL4, CCL5, C5a,C5a, IL-1β and IL-1F1 (B). (A) is the array membranes before analysis by image J. (B) is data blot after analysis. Data expressed as mean pixel density of two samples.

Next, TNFα and IL-8 levels were measured in plasma extracted from above stated condition. The TNFα and IL-8 ELISA results of 4 independent experiments using blood from different volunteer each time (2 males and 2 female) showed qualitatively the same pattern of secretion as from human cytokine array results, in particular MIF, TNFα, CCL3, CCL4, CCL5, C5a,C5a, IL-1β and IL-1F1,(Figure 4.9). HKK and HKS stimulation showed a significant production of both TNFα and IL-8 compared to their levels in unstimulated WB after incubation with endothelium whether inflamed or not. Inflamed endothelium induced a slight but not significant increase in TNFα and IL-8 compared to uninflamed endothelium.

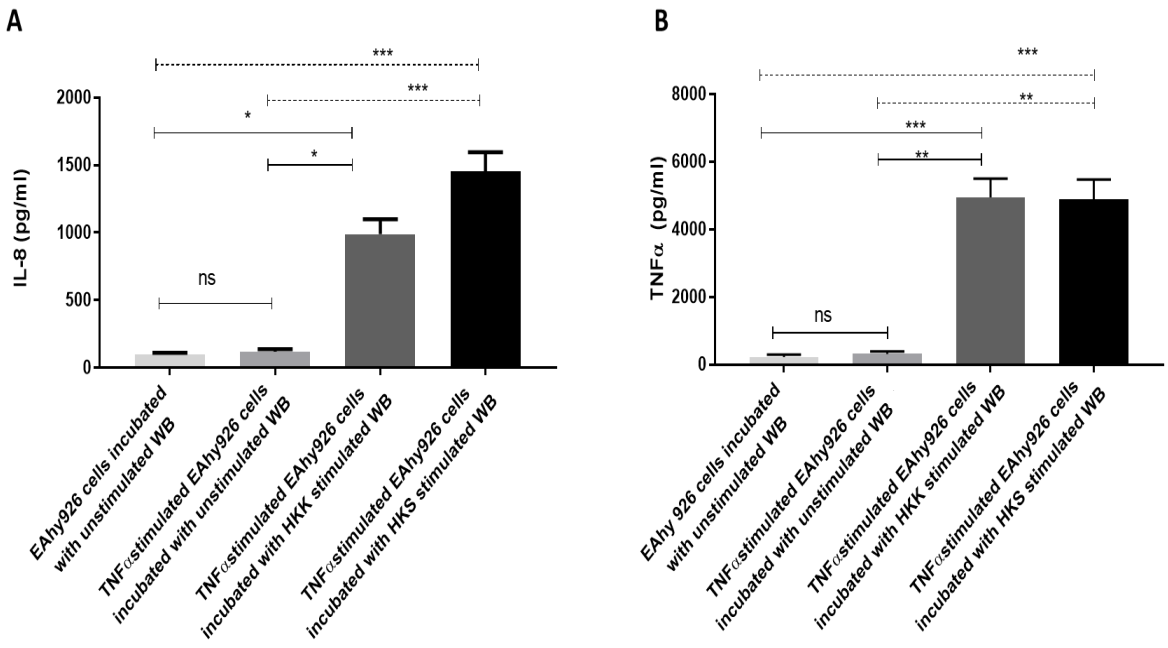


Figure 4.9: Analysis of inflammatory markers IL-8 (A) and TNFα, under different conditions involving activated endothelial cells. Data are expressed as means ± SEM and were analysed by means of Kruskal-Wallis test, followed Dunn's multiple comparisons test between groups. Changes were considered significant if p-value was <0.05. n= 4

4.4 Discussion

As was pointed out in the introduction to this thesis, platelet leukocyte endothelium interaction may result in vascular destabilisation that contributes to the pathology of many underlying diseases, thereby providing an explanation of long term mortality in patients with sepsis. Surprisingly, little is known about the mechanism of action and consequences of platelet leukocyte endothelium interaction. Only one *in vivo* study was conducted to investigate the association between platelet leukocyte endothelial aggregation and vascular permeability while the rest focused on either platelet-endothelial or leukocyte-endothelial interaction. No previous *in vitro* study has investigated platelet- leukocyte- endothelial cell inflammatory interaction in conditions modelling the humoral phase of sepsis. Here, a novel *ex vivo* model of inflammatory PLAs adhesion on the endothelial cells mimicking an early phase of sepsis was established.

Human umbilical vascular endothelial cell line (HUVEC) have been used frequently in various *in vitro* adhesion assays to investigate the leukocyte endothelial interaction (Burns and DePaola, 2005; Marino *et al*, 2017; Pu *et al*, 2002). It was shown that permanent human umbilical endothelial cell line, EAhy926, retain the similar characteristic features of primary human vascular endothelium cell, especially with regard to expression of integrins and their adhesive profile (BARANSKA *et al*, 2005). These properties make them ideally suited to study the adhesion interaction with whole blood cells under inflammation.

Principally, in this model, the endothelial cell line was stimulated with TNFα prior to incubation with stimulated whole blood. Indeed, this step was employed by most of previously described adhesion assay under static as well as dynamic condition to evaluate adhesion of isolated leukocytes (Vaporciyan, Jones and Ward, 1993; Zahr *et al*, 2016) or platelets (Xu *et al*, 2010) to the endothelium (HUVEC). TNFα upregulates adhesion molecules expression on endothelium, E selectin, ICAM and VCAM (Mackay *et al*, 1993; Chen *et al*, 2001).

It should be mentioned that the inflammatory aggregate adhesion to endothelial cells was modelled under static condition. Although it is obviously not an optimal experimental design with regard to stasis of blood, which was shown to cause artefactual activation of blood cells, triggering inflammation and aggregate formation

(Kawasaki *et al*, 2001). But still in contrast to other published adhesion assays under both static and dynamic condition, we have a system that utilized whole blood that was processed immediately and incubated on inflamed endothelium at 37oC for no more than 1h with minimal (and standardised) manipulation. Other adhesion assays as referred to above used isolated cells that require more manipulation, and lead to more artefactual stimulation of cells and, equally importantly, lack the effects of other blood components. Generally, the static system is preferred over the dynamic system in the analysis of the receptors involved in the adhesion for greater ease to set up and benefit a higher output (Butler, McGettrick and Nash, 2009).

More optimal conditions would include setting up the experimental design under flow system by circulating laminar flow with normal shear stress, however, this was not possible in this model. Because it is essential to flow controls as well as experimental condition prepared from the same blood samples in parallel at the same time to enable a sensitive evaluation, the available in house system for this project was unfortunately not suitable.

Additionally, analysis of the presented model here by different modalities, light microscopy, TEM, proteome profile, TNFα and IL-8 levels, produced compatible and reproducible results; ability of heat killed bacteria, over LPS or unstimulated condition, to stimulate the inflammatory aggregate formation and adhesion to the inflamed endothelium and to induce release of TNFα and IL-8. This is consistent with results of previous chapter and verifies the stability of this model.

In this project, the enhanced adherence of platelets, leukocytes and the aggregate were clearly determined by many methods, immunofluorescence, light microscopy, TEM which confirm the abundance of the adhesion molecules (proteins).

Thus, this model can be applied to determine the mechanism involved in blood cell adherence to activated endothelial cells and to analyse adhesion molecules under distinct conditions of activation. Furthermore, the model may be used to examine the effects of pharmacological agents on inflammatory conditions.

Chapter 5

Application of the *In Vitro* model that captures PLAs adherence to endothelium to a clinically significant problem, Obesity and sepsis

5.1 Sepsis and obesity:

Obesity is defined by body mass index (BMI) greater than 30 and is a major public health problem (WHO). It has attained epidemic proportions in many developed and developing countries over the last few decades. Current estimates suggest that approximately 24.9% of population in the United Kingdom are obese (NHS). Consequently, a considerable proportion of patients hospitalized in the intensive care unit (ICU) is obese. The coexistence of both conditions, obesity and sepsis, are frequent in clinical practice (Ball, Neto and Pelosi, 2017; Trivedi, Bavishi and Jean, 2015; RW.ERROR - Unable to find reference:119). In the general population, obesity is associated with higher rates of mortality (all-cause mortality and that driven by co morbidity such as hypertension, diabetes mellitus and cancer) and morbidity (RW.ERROR - Unable to find reference:111; Abdelaal, le Roux and Docherty, 2017; Trivedi, Bavishi and Jean, 2015). However, in sepsis, it is still uncertain whether obesity affects morbidity and mortality rates. This is because very little is known about the association between obesity and sepsis. But because sepsis is considered a major cause of death in critically ill patients, it could be that some, if not most, of the reported data from the studies investigating the potential effect of obesity on the outcome of critically ill patients are attributable to sepsis (Vachharajani, 2008).

To date, several observational clinical studies investigated the link between obesity and the outcome by means of mortality and morbidity rates among critically ill patients and showed contradictory or mixed results (Papadimitriou-Olivgeris *et al*, 2016a). Many studies revealed a higher mortality rate (Papadimitriou-Olivgeris *et al*, 2016b), increased demand of ventilatory support and prolonged hospitalisation (Tafelski *et al*, 2016; Akinnusi, Pineda and El Solh, 2008) in obese compared to normal weight critically ill patients while the opposite or null association was also reported (Oliveros and Villamor, 2008; Ball, Neto and Pelosi, 2017; Wardell *et al*, 2015). Thus, data interpretation and drawing a clear association regarding mortality from these studies is difficult and limited by many factors such as missing adjustment of confounders, different classification of obesity and clinical management-related factors (obese and lean patients might respond to the treatment modality differently) and lack of data homogeneity (Ball, Neto and Pelosi, 2017; Vachharajani, 2008). The conflict observed in a clinical setting (increased and decreased in the mortality) appears to be not sufficient to answer the question about the potential negative impact of obesity on the

outcome of septic patients, properly. Much more clinical research needs to be conducted to further clarify the association between the obesity and sepsis in particular. It is well established that obesity generates a low grade chronic inflammation. Adipose tissue secretes a wide range of inflammatory modulators, hormones and bioactive peptides, known as adipokines, such as IL-1, IL-6, IL-8, IFNγ, TNFα, leptin and resistin which act locally and systemically through autocrine, paracrine and endocrine functions that induce a chronic phenotype of inflammation. Other mechanisms such as increased production of cytokines, oxidative stress and activation of platelet, leukocyte and endothelial cells have been shown to induce this state of inflammation in obesity (Vachharajani, 2008; Castro, Macedo-de la Concha and Pantoja-Meléndez, 2017). These factors seem to create a sensitive environment that makes exposure to additional inflammatory stimuli in the obese produce an exaggerated inflammatory response and lead to sustained tissue damage. Consequently, the question raised is whether the underlying obesity causes exaggeration in inflammatory and prothrombogenic response in septic patients. The present study draws justification from one particular clinical study (Kolyva *et al*, 2014). It showed an increase in the proinflammatory cytokine production by obese septic patients compared to that of the non-obese. Studies carried out on septic animals prove that obesity enhances the inflammatory and coagulatory response (Vachharajani and Vital, 2006; Mittwede *et al*, 2016). Studies carried out on septic mice have demonstrated that obese septic mice are at higher risk of death (Kumar *et al*, 2013).

Currently, the available studies in this area have largely suggested a potential correlation between sepsis and obesity with regard to an increased inflammatory status. Further laboratory studies are required to address this question under a clinical setting. To the best of our knowledge this is the first *in vitro* study attempting to analyse the effect of elevated non-esterified fatty acids (found in obesity) on inflammatory and procoagulant condition associated with sepsis.

5.2 Modelling sepsis event during obesity *in vitro*

To mimic the clinical scenario of obese response to sepsis experimentally, EAhy926 were jointly stimulated with free fatty acids and TNFα prior to incubation with heat killed bacteria stimulated blood samples. It was designed as a closest and possible clinical scenario. Palmitic (PA, saturated) and Oleic acids (OA, monounsaturated) were

selected in this study because they are most abundant FFA found in the plasma of obese (Masi *et al*, 2011). Firstly, multiple experiments were set up to determine the effective FFA dose that induces intracellular lipid inclusion in the absence of a toxicity effect on the endothelium. For this purpose, EAhy926 cells at 70% confluence were co-incubated with TNFα (25 ng/ml) and different doses of FFAs (oleic acid/palmitic acid 2:1) overnight. FFAs complex (OA and PA) at a final concentration of 500 μM was initially selected as positive control based on a previous study that showed a 70% reduction in EAhy 926 cell viability after 24 h treatment with palmitic acid at 500 μM (Khan *et al*, 2012). Whereas FFAs at 25 μM was selected as a lower limit of the FFA concentration range because it did not show any toxic effect on J774 macrophage cell line (our lab work). Each condition was done at least 5 times. Cell viability as well as lipid body inclusions for each condition were measured in separate experiments.

Surprisingly, cells treated with TNFα and FFA at different concentrations had no significant impairment compared to the negative control (unstimulated cell) (Figure 5.1(A)). The unexpected results of 500 μM FFA might be related to the mix with oleic acid that is shown to attenuate the palmitic acid toxicity (Ricchi *et al*, 2009). Moreover, 24 h treatment of EAhy926 with oleic acid at 500 μM didn't affect their viability (Khan *et al*, 2012).

As indicated in figure 5.1 (B), among the tested range of FFA concentration, the maximum Oil red O staining was detected in the cells that were treated with TNFα and 500 μM FFA compared to unstimulated cells. Thus, 500 μM FFA (oleic and palmitic, 2:1) was selected for this study.

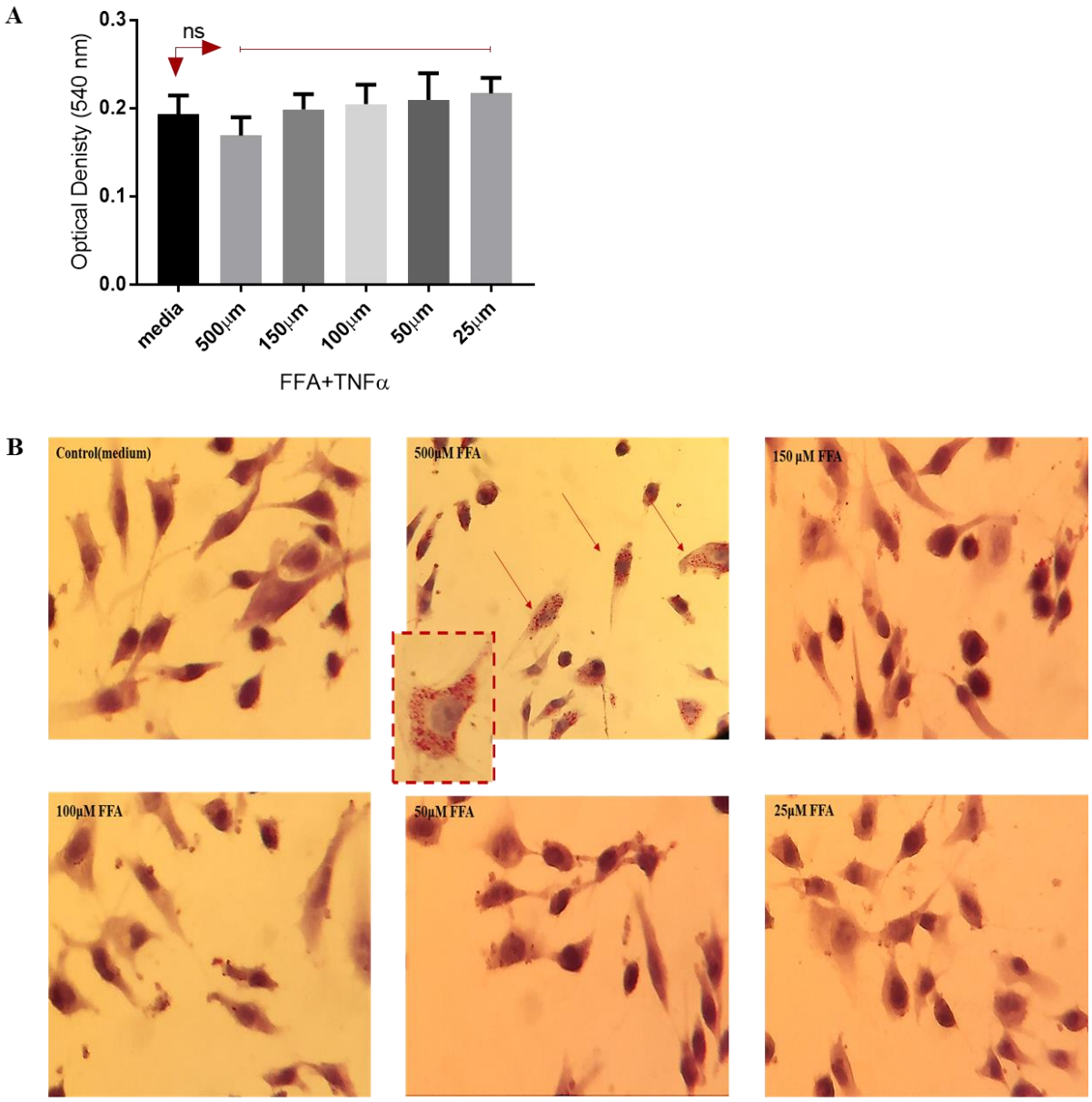


Figure 5.1: Effects of different FFA concentrations on EAhy926 cells. 70 % confluent EAhy 926 cells were co stimulated with TNFα (25 ng/ml) and FFA (oleic and palmitic, 2:1) at different concentrations to determine the optimal FFA dose that induce lipid inclusion without significant effect on cells viability. (A) Crystal Violet and (B) Oil red O staining showed that FFA at 500µM induces denser intracellular lipid inclusions (dotted box) with no significant toxic effects compared to the other conditions. The data are represented as means of duplicate ± SD (n = 5) (Kruskal-Wallis test and Dunn's multiple comparisons test). X 40.

5.3 Effect of FFA on adhesiveness of endothelial cells to inflammatory PLAs:

After determining the FFA concentration that induces intracellular lipid accumulation without significant interference on cellular viability, the model was conducted by incubating the EAhy926 (that pre-conditioned with TNFα and FFA overnight and, then, washed adequately) with HKK stimulated whole blood for 1h. The aim was to investigate to what extent FFA enhanced the inflammatory response and adhesive pattern of PLAs to endothelium. Incubating TNFα stimulated EAhy926 cells with HKK stimulated whole blood was prepared as a control. IL-8 and TNFα plasma levels were measured. It should be mentioned here that the inflamed endothelial cells were washed thoroughly prior the whole blood incubation and, thereby, the TNFα from the step of the overnight endothelial cells stimulation does not seem to interfere with TNFα plasma levels at the later step. Expectedly, a significant increase in IL-8 plasma level was induced by additional FFA incubation compared to control. However, plasma under this condition did not show an additional increase in TNF α level (Figure 5.2). This could be attributed to the maximum release of preformed TNFα at the time point of initial stimulation when additional stimuli were not sufficient to lead to TNF α production similar to IL-8. Chemokine production, IL-8, is more sensitive than cytokine production, TNFα, and found to be released early and continuously to recruit the polymorphonuclear cells to the site of infection (DeForge and Remick, 1991). Comparative analysis between inflammatory chemokines (IL-8 and MIP-1α) and cytokines (TNF α, IL-1 β, IL-1ra and IL-10) release in whole blood stimulation assay also showed an early and continuous production of chemokines in parallel to an absent significant increase in cytokine release (Foca *et al*, 1998).

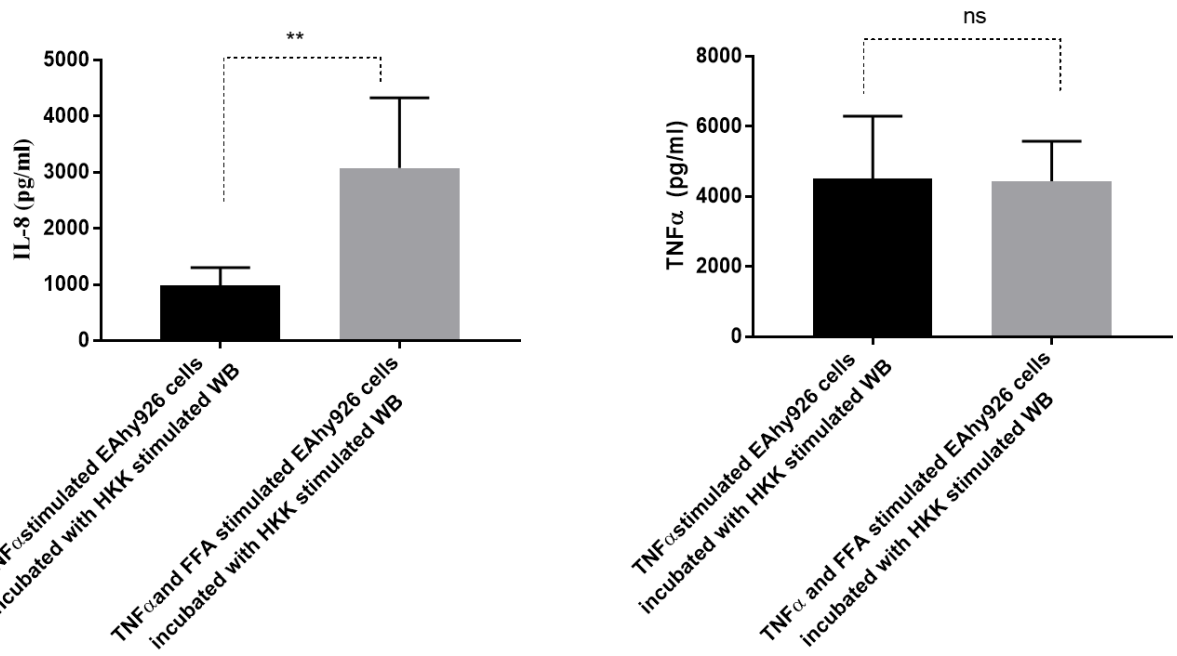
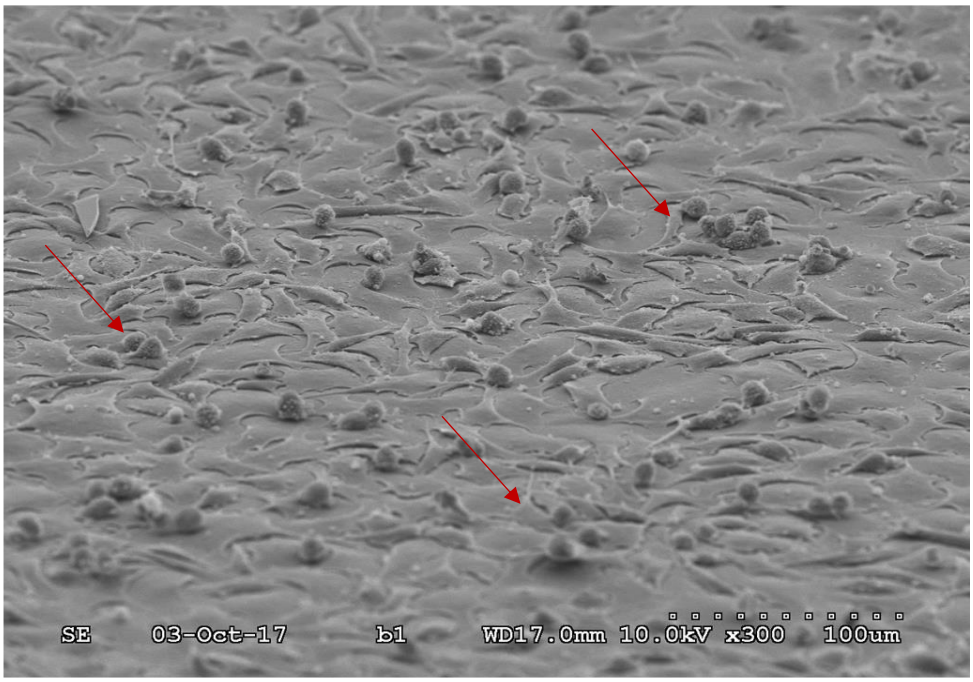


Figure 5.2: Inflammatory markers analysis in response FFA stimulation. EAhy926 cells were co stimulated with TNFα (25 ng/ml) and with or without FFA (500 μM, oleic and palmitic, 2:1), overnight. Then, they were incubated with HKK stimulated whole blood for 1 h at 37 oC. The plasma level of IL-8 and TNFα were measured. Data expressed as mean + SEM and analysed by Mann Whitney test n =3.

Subsequent qualitative assessment by SEM showed adherence of more complexed inflammatory aggregates to endothelium cells that were pre-treated with both TNFα and FFA, compared to control (Figure 5.3)

A (HKK+WB)+(TNFα endothelium)



B (HKK+WB)+(TNFα +FFA endothelium)

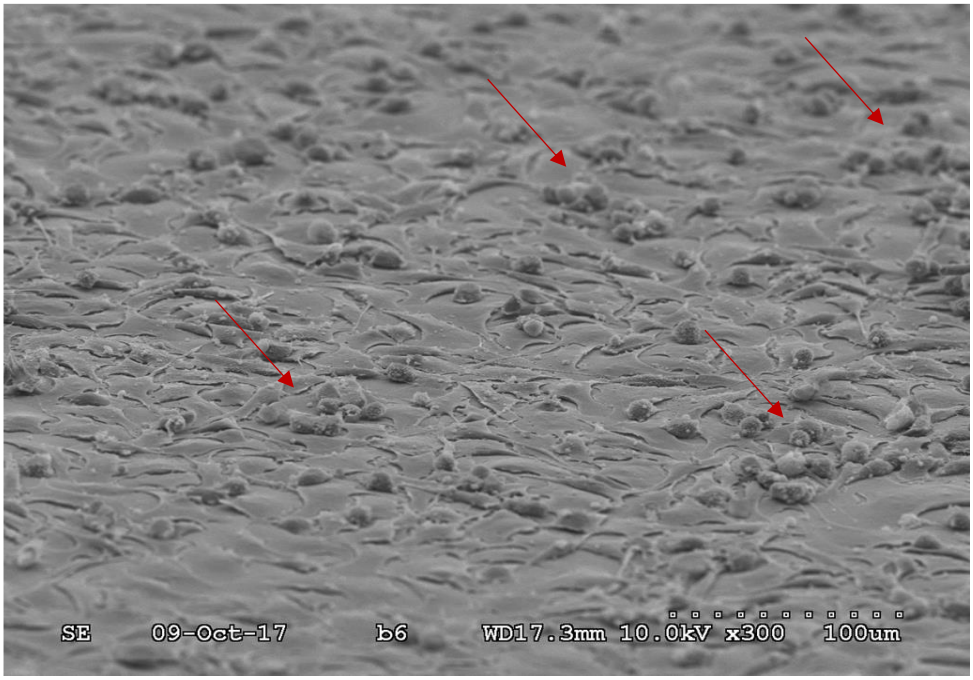


Figure 5.3: Electron micrograph showing adherence of more complexed inflammatory aggregates to EAhy926 cells that were pre-treated with both TNFα and FFA (B) compared to control, TNFα stimulated EAhy926 cells (A). Representative images are shown.

5.4 Effect of angiopoietin 2 ligand trap and P-Selectin blocking antibody on the adherence of inflammatory aggregates to inflamed endothelium:

In normal condition, quiescent endothelial cells secrete angiopoietin 1, named as Ang-1, that binds to tyrosine kinase Tie 2 receptor expressed on their surface. Ang-1/ Tie2 signalling pathway functions to keep the endothelium stability and inhibit gene expression of inflammatory and coagulatory system (Mikacenic *et al*, 2015a; Scharpfenecker *et al*, 2005). On the contrary, at the sites of inflammation, activated endothelial cells rapidly exocytose the preformed angiopoietin 2 (Ang-2) from their store, so-called Weibel- Palade bodies. Furthermore, TNFα induces gene overexpression of Ang 2 in human umbilical vein endothelial cell (Kim *et al*, 2000). Ang 2 competitively binds to Tie 2 receptor and thereby antagonises Ang-1 effects (David *et al*, 2013a; Hegen *et al*, 2004). Ang 2, in turn, over activates the endothelium and enhances the inflammatory response (El-Banawy *et al*, 2012). In addition to its ability to stimulate the endothelial cell directly by an autocrine mechanism (Hakanpaa *et al*, 2015), it also synergistically enhances the effects of other inflammatory mediators such as sensitising the endothelial cell to the TNFα stimulus and, thus, amplifies its effects (Graham *et al*, 2013; Fiedler *et al*, 2006). Moreover, it stimulates the neutrophils directly towards the proinflammatory response (Mussap *et al*, 2013).

Vascular endothelial growth factor (VEGF) induces white blood cell adherence to the endothelium. Normally, this response is supressed by Angiopoietin-1 that downregulates gene expression of E selectin, VCAM-1 and ICAM-1(Kim *et al*, 2001; Ismail *et al*, 2012). In inflammation, Ang 2 blocks this inhibitory effect of Ang1 and, then, intensifies the leukocytes adherence to endothelium. These findings in combination with the additive inflammatory effects induced by Ang2, as described above, suggests the potential role of Ang 2 in endothelial cell adhesiveness state.

In healthy individuals, high levels of Ang 1 and low or undetectable levels of Ang 2 are usually found in their plasma (Graham *et al*, 2013). In sepsis, however, their serum levels differ significantly (Mussap *et al*, 2013). High level of Ang2 and low level of Ang1 are consistently reported in several clinical and experimental studies (Zonneveld *et al*, 2017; Ricciuto *et al*, 2011; Fang *et al*, 2015a). Early in sepsis, high ratio of Ang-2/Ang-1 is associated with worse clinical outcome and mortality rates (Fang *et al*, 2015b; Mikacenic *et al*, 2015b). Elevated Ang-2 level in the plasma of septic patient is

corelated positively with markers of endothelial cells inflammation (David *et al*, 2013b), augmented complement activity, abnormal coagulation (Ganter *et al*, 2008) and stage of organ failure (Davis *et al*, 2010). Taken together, the data suggest that angiopoietin 2 could promote blood cell adherence to the vascular endothelial cell, which might contribute to the microvascular dysfunction associated with sepsis (Figure 5.4). Regardless of whether aggregation is protective or detrimental, it is imperative to investigate, as we aimed to, the effect of Ang 2 blockage on the adherence of inflammatory aggregate to the endothelial cells under simulated condition of sepsis and obesity.

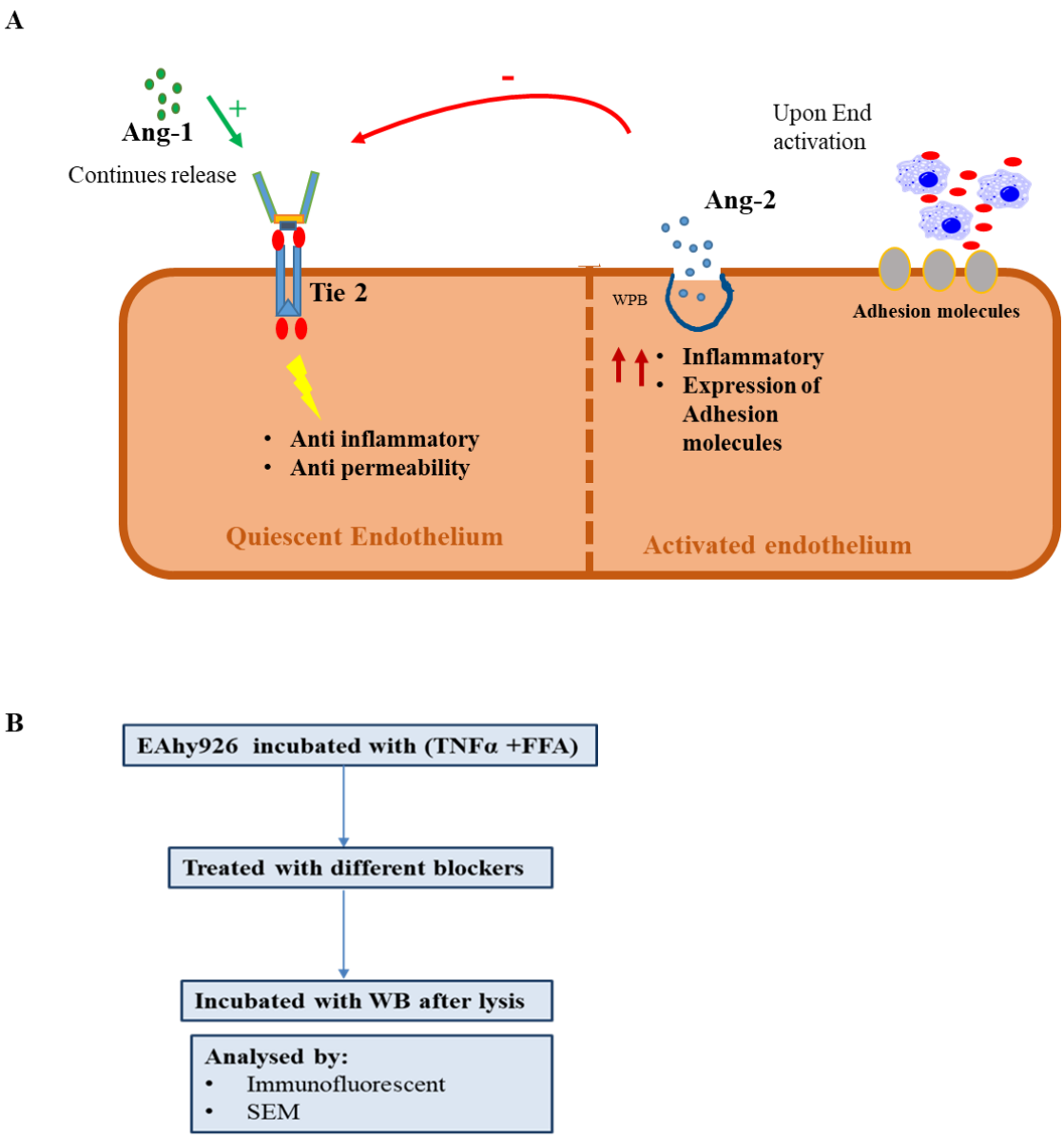


Figure 5.4: A is a proposed model of action of angiopoietin 2 on endothelial cells adhesiveness. Normally, angiopoietin 1 inhibits the inflammatory cascades by continuous signalling through tie 2 receptor. Activated endothelium rapidly releases angiopoietin 2 that competitively inhibits the Ang1/tie 2 signalling and induces the inflammation and expression of adhesion molecules on endothelial cells. B shows experimental design to analyse the effects of Ang2 blocker on the endothelial cells adhesiveness. This work benefited from ongoing collaboration with prof. N. Brindle, UOL.

5.4.1 Developing a suitable method to measure the effect of targeted intervention on inflammatory aggregate adhesion:

An initial experiment was done to analyse the effect of endothelial cell directed P Selectin blocker on the aggregate adhesion using immunofluorescence. Immunofluorescence intensity was selected as a readout to easily determine the effect of the blocker by expressing the percentage of the decrease in immunofluorescence intensity below the control, where no blocker was used. Having determined that the two fluorophores conjugated to the specific markers of platelets and leukocytes failed to be overlapped and quantified (as explained in detail in chapter 2), labelling the cellular membrane of the cells complex forming the inflammatory aggregate with fluorescent dye was chosen as an alternative. Using this approach, not only the fluorescence intensity can be measured but also the complexity of the aggregate can be figured out by tracking and measuring the surface area of the outermost fluorescence for each adhered cellular cluster.

To do this, EAhy926 cells were co-incubated with TNFα and FFAs overnight and pre-treated before addition of HKK stimulated and fluorescent labelled blood as follows: with culture medium (control), anti P Selectin isotype, anti P Selectin antibody (clone #11711, 9E1 respectively, R&D Systems, Abingdon, UK), (at 10 µg /ml, 30 minutes, after washing). HKK stimulated whole blood was labelled with Vybrant™ DiO Cell-Labelling Solution, Thermo Fisher Scientific, Loughborough, UK) for 30 minutes before RBC lysis and incubated with P Selectin-blocked endothelium for 1h at 37 oC. EAhy926 without blood and EAhy926 with unstained blood were prepared as controls. The highly lipophilic nature of this dye leads to a uniform labelling of the cellular membrane. Its properties of low cytotoxicity and stability for long duration with high resistance to the intracellular diffusion makes it optimally suited to label cells (manufacturer's data sheet).

Images at different fields of each sample were captured using phase contrast and fluorescent microscopies, separately. As expected, both analysing strategies showed that treatment of inflamed endothelial cell with anti P selectin prior to incubation with HKK stimulated whole blood resulted in a remarkable reduction in the inflammatory aggregate adhesion compared to untreated background profile (Figure 5.5). The small

and bright spherical cells visible under phase contrast are indeed blood cells in clusters or singly (indicated in an arrow).

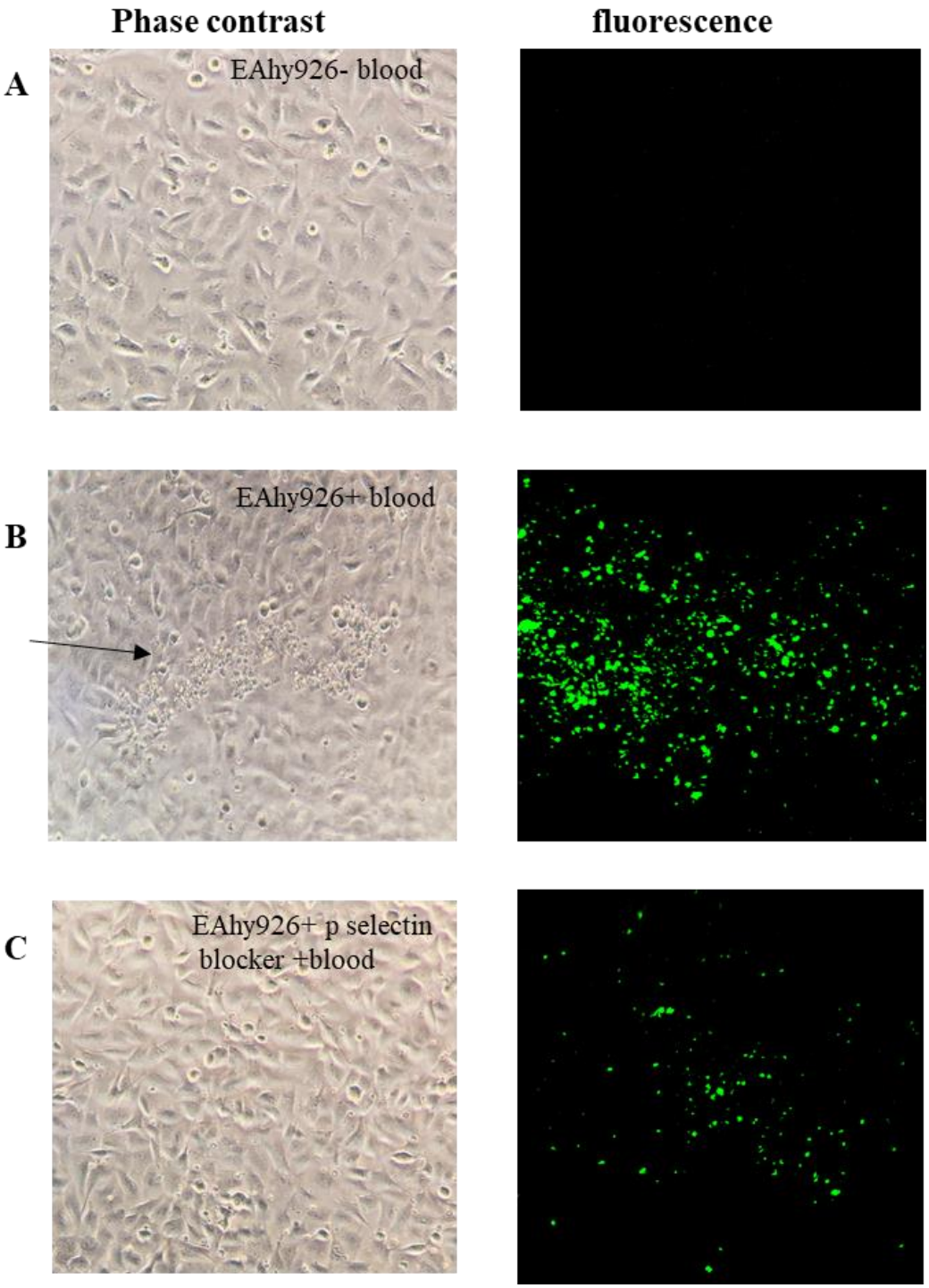


Figure 5.5: Phase contrast and immunofluorescence micrographs of cellular aggregate adhesion to monolayer of endothelial. Whole blood was stimulated with HKK to induce the formation of inflammatory aggregate, immunoassayed with cell membrane labelling

dye and then, incubated on inflamed EAhy 926 Cells, pre-treated with or without P Selectin blocker. A shows inflamed endothelium without blood as a control b) maximal adhesion of blood cells to EAhy926 cell was observed in the absence of p selectin blocker. C. Cell adhesion to EAhy926 could be maximal blocked by 10μg/ml. The small and bright spherical cells visible under phase contrast are indeed blood cells in cluster or individually (indicated in a arrow). Note: EAhy926 + blood without any labelling (as dye control) and endothelium pre-treated with p selectin blocker isotype + fluroscent labelled -HKK stimulated blood were used as control data not shown. Images immunofluorescence X10. Phase contrast X40.

Due to the promising results of P Selectin blocker visualised by membrane labelling dye, we wanted now to modify the approach further using a more specialised microscope, Olympus confocal immunofluorescent microscopy FV1000, and two fluorescent dyes, DiO to stain WB and DAPI to stain endothelial cell nuclei, for more accuracy of observation. With this system, bright field and multi-colour imaging can be acquired for the same area at the same time. Thus, the endothelial layer as well as adhered blood cells, found individually or in clusters can be visualised by merging. It not only provides information about the surface coverage of the dye but also gives data about the depth of the dye by 3D-*Z* stack function. Therefore, the complexity and the extent of the aggregate can be analysed by calculating the fluorescence intensities within three-dimensional shape of these aggregate. It also allows visualising images at different magnification power.

EAhy926 were treated as described and stained with nuclear stain (DAPI) prior to incubation with HKK stimulated blood that was pre-stained with cell membrane labelling dye (DIO) as described above. The samples were then mounted for microscopic analysis using Olympus confocal immunofluorescent microscopy. Unexpectedly, at DIO fields, the results showed staining of only a few cells with corresponding size of platelets but not WBCs. There was also a profound DIO staining of the endothelial cell which is further confirmed by DAPI staining of their nuclei (Figure 5.6). This observation was thought to be a confounding factor for the first approach and might be related to the excess of dye that could diffuse to stain endothelial cells.

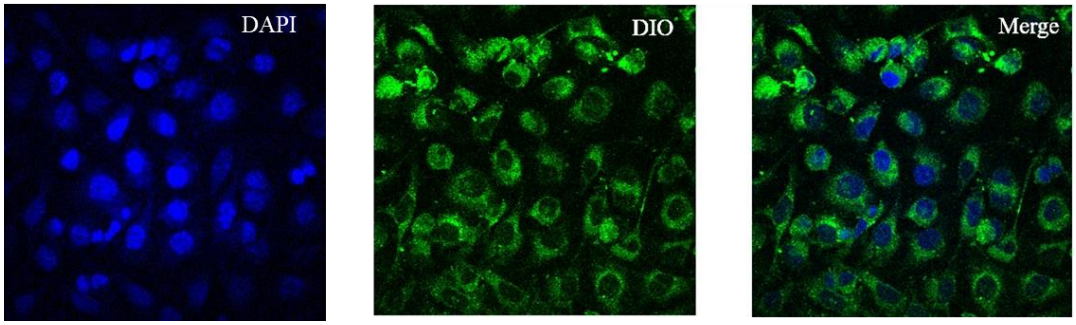


Figure 5.6: Immunofluorescent images of cellular aggregate adhesion to the endothelial cells monolayer. HKK stimulated whole blood was stained with cell membrane labelling dye (geen) prior incubation on endothelium. Endothelial cells were counterstained with DAPI (blue). Fluorescent microscopic analysis showed diffusion of cell membrane labelling dye (green) to the endothelial cells and absence of stained blood cells (in green). X40.

A subsequent experiment was set up to improve the sensitivity of the experimental protocol by diluting the DIO dye and increasing the washing to get rid of the excess dye. As can be seen in figure 5.7, there is no staining of endothelial cell membrane with DIO which is often associated with intense staining of the endothelial cell nuclei with DAPI. However, extremely few cells appeared to be stained brightly with DIO that do not seem to be related to the inflammatory aggregate. These cells fit with the size range of RBCs (red arrow) or platelets (yellow arrow). The unexpected results of not finding any inflammatory aggregate clumps with this method could be attributed to several reasons such as dye interference for their adherence, most of them are washed away or not stained. To exclude the first two possibilities, Leishman stain of the same fluorescent mounted samples was done and clearly showed the presence of adhered blood cellular clumps as well as lots of single blood cells (red arrow) even after removing the cover slip from the sticky mounting material to be stained.

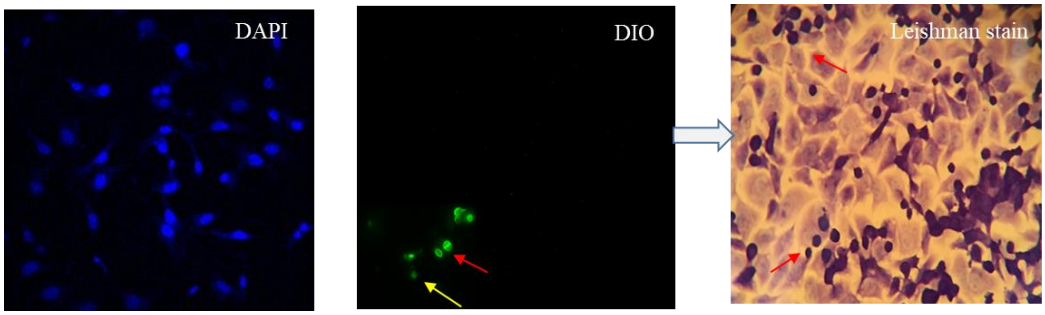


Figure 5.7: In ability to detect thee adhered inflammatory aggregates by Immunofluorescent. Immunofluorescent micrograph showed a successful staining of endothelial cells without membrane dye diffusion, bright staining of the remaining RBCs after lysis (indicated in red arrows) and week staining of others (yellow arrow) . Leishman staining of the same slides showed the presence of lots WBCs and platelets adhered, in cluster or sporadic, to endothelial monolayer.

Subsequently, these findings highly suggested the ability of dye to stain the remaining RBCs after lysis brightly but neither WBCs nor platelets, thus, inflammatory aggregate could not be detected. To examine this hypothesis, the whole blood was incubated with DIO to stain the cell membrane of all blood cells and then, washed and counter stained with DAPI to label the nuclei of blood cells. The stained blood was smeared and mounted for analysis. In principle, nucleated cells are stained with both DAPI and DIO while unnucleated cells stained only with DIO. Thus, WBCs, RBCs, and platelets can be differentiated straight away after merging. Examination of DIO field revealed the presence of cells cluster composed of many cells with different sizes and fluorescent intensities. The small cells appeared brighter than the large cells which were very faint and most of them lost their fluorescent signals during analysis. After merging the images to identify the cell type with some certainty, the bright cells were the RBCs (-ve DAPI) while very weakly stained cells were WBCs (+ve DAPI) (Figure 5.8). This was further confirmed by measuring their diameter and the RBCs auto fluorescence properties which excited at 280 nm and emitted at 335 nm (Zhurova *et al*, 2014). These findings might be related to the interaction of mixed cell types, within the sample, with the labelling dye in different ways that might be largely reflected in gaining different fluorescent intensities. Again, in this project, dealing with whole blood (mix of different

cells) instead of isolated cell types appeared to pose an obstacle for certain analytical approaches. Another dye such as phalloidin, an actin labelling dye, that is characterised by bright signals and stability making it highly preferred in most application was considered. But because of its cytotoxicity and the need for permeabilization with methanol which would activate the blood cells ultimately and lead to artificial formation of the inflammatory aggregate (Dhakal, Black and Mohanty, 2014), it was, then excluded from the study.

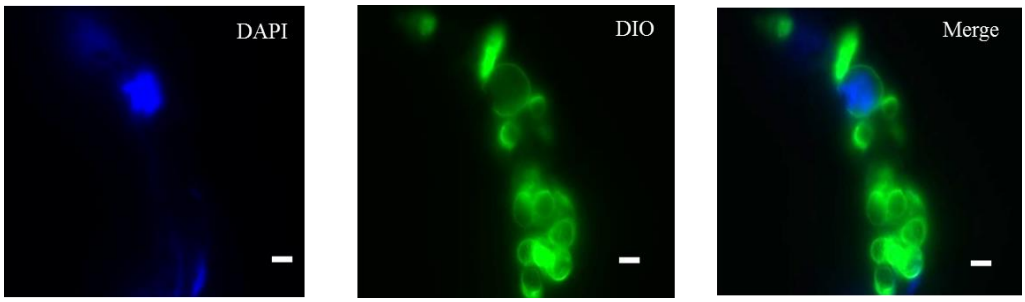


Figure 5.8: In ability of membrane labelling dye to stain WBCs. WBCs (nucleated cells, DAPI positive) were weakly stained with membrane labelling dye (DIO). However, RBCs (unnucleated cells, DAPI negative) were strongly stained with DIO. Scale is 5μm.

5.4.2 Angiopoietin 2 ligand trap and P-Selectin blocking antibody reduce inflammatory aggregates adherence to inflamed endothelium:

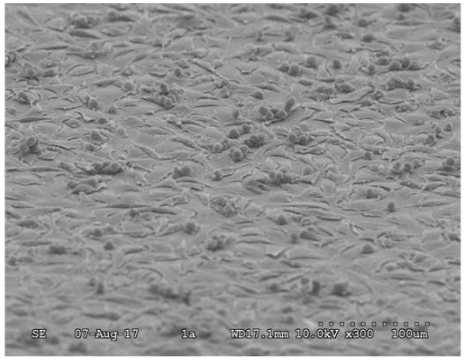
Having found that several immunofluorescence methods were not suitable for reliably detecting the effects of the employed blocker, another approach using SEM was used. SEM delivers significant advantages over cell membrane labelling because it does not interfere with the adhesion interaction. i.e. all sample processing steps are carried out after the stimulated blood cells (PLAs) have adhered and were fixed. Due to the ability to determine the effect of FFAs on the adhesiveness of the endothelium qualitatively, the effect of blockers could be also analysed by qualitative and especially semi quantitative way. i.e. counting the number of aggregates per electron micrographs, surface area taken from different fields of individual samples.

To do this, inflamed endothelial cells were pre-treated before addition of HKK stimulated whole blood as follows: with culture medium (negative control for the blocker), anti P selectin isotype, anti P selectin antibody (positive control for blocker) at 10 µg /ml, 30 minutes after TNFα and FFA stimulation or angiopoietin 2 ligand trap, R3 (10 µg/ml, overnight together with TNFα and FFA stimulation), then processed for SEM as described earlier.

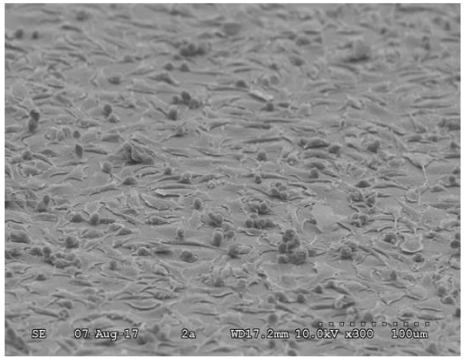
A so-called evolved Tie ectodomain with specificity for angiopoietin 2, termed R3 described previously (Brindle *et al*, 2013) was kindly provided by Prof Nicholas P Brindle, Department of Cardiovascular Sciences, University of Leicester, United Kingdom.

Treatment of inflamed endothelial cell with R3 at or anti P selectin prior to incubation with HKK stimulated whole blood similarly reduced the inflammatory aggregate adhesion significantly compared to untreated background profile (Figure 5.9).

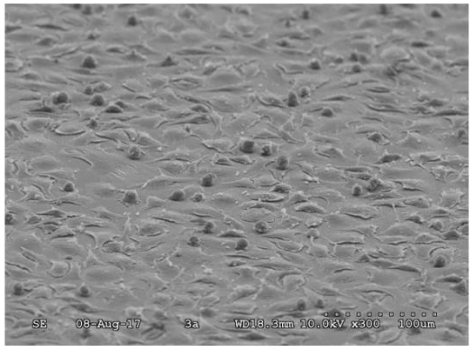
A



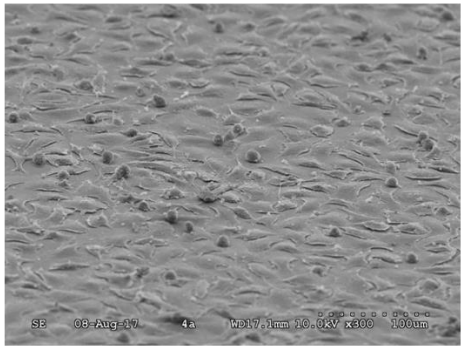
1- (HKK-WB)+(TNFα +FFA endothelium)
+no blocker



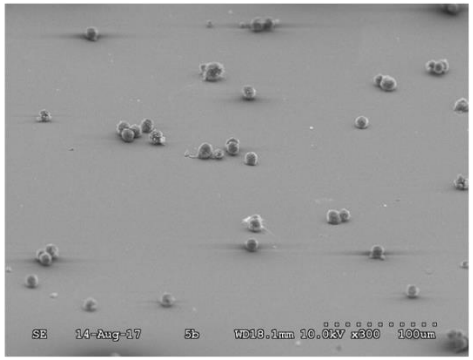
2- (HKK-WB)+(TNFα +FFA endothelium)
+p selectin isotype



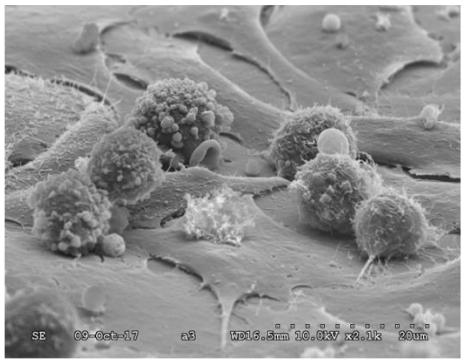
3- (HKK-WB)+(TNFα +FFA endothelium)
+p selectin blocker



4-(HKK-WB)+ (TNFα+FFA endothelium)+R3



5- HKK stimulated WB – endothelium



6- Aggregate adhesion

B

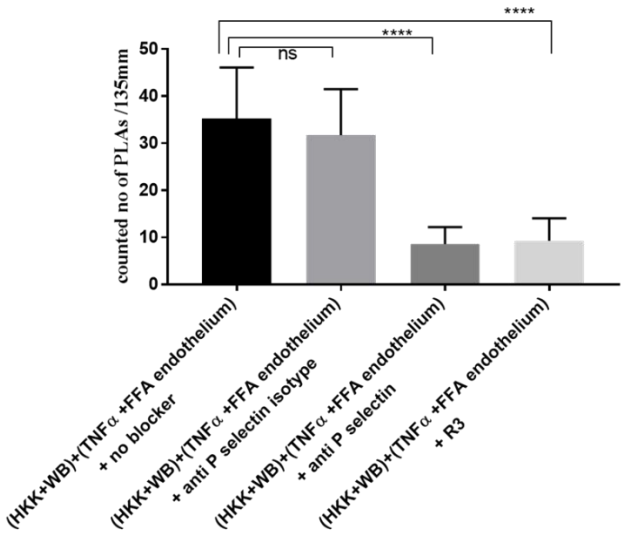


Figure 5..9: Blocking of inflammatory aggregates adherence to the inflamed endothelium by incubation with angiopoeitin 2 ligand trap (R3) and P-Selectin blocking antibody. A, Tilted scanning electron microscope micrograph at 75 shows inflamed endothelial monolayer pre-treated with (1) culture medium (control), (2) anti P selectin isotype, (3) anti P selectin antibody (3) and R3 (4) and incubated with human peripheral blood stimulated with HKK. 5 is SEM of HKK stimulated blood shown as control. 6 shows SEM of aggregate adhesion at higher magnification(2.1K). B shows semi quantitative analysis by counting the number of aggregate adhered to endothelium layer per 135 mm to assess the blocking effect using Image J software. Data are expressed as means ± SEM and were analysed by means of Kruskal-Wallis test, followed Dunn's multiple comparisons test between groups. Changes were considered significant if p-value was <0.05. Scale represents 100 µm (1, 2, 3, 4, 5) and 20 µm (6). SEM n = 30 images from n = 3 independent isolations per condition.

5.5 To analyse the effect of Vitamin D on the pro adhesive and inflammatory phenotype of the whole model.

5.5.1 Vitamin D in sepsis.

Vitamin D, a fat-soluble steroid hormone known as cholecalciferol, has an emerging immune modulatory effect as well as an important role in regulating the inflammatory response and chemokine production (Youssef *et al*, 2011a). VDR is expressed by almost all cell types involved in the inflammation and immune system such as lymphocytes, neutrophils, macrophages, dendritic cells and vascular endothelial cells which are all implicated in sepsis pathogenesis as described previously. Vitamin D enhances innate immunity locally to increase production of antimicrobial peptides (AMPs) such as cathelicidin and its activated form, LL-37. These AMP exert a wide range of antimicrobial effects through chemotaxis, cytokine production, phagocytosis, apoptosis, and others. There are AMP-independent effects of VD on innate immunity. VD also upregulates adaptive immune response via several effects on lymphocytes (T and B cells) including induction of proliferation and differentiation, autophagy and IFN-γ release (Hariri Ahari and Pishbin, 2014; Upala, Sanguankeo and Permpalung, 2015; Youssef *et al*, 2011a; Kempker *et al*, 2012). Furthermore, signalling of vitamin D (1,25(OH)2D) through its receptor VDR present in endothelial cells was found to limit vascular endothelial cell activation and inflammation (Bozic *et al*, 2015).

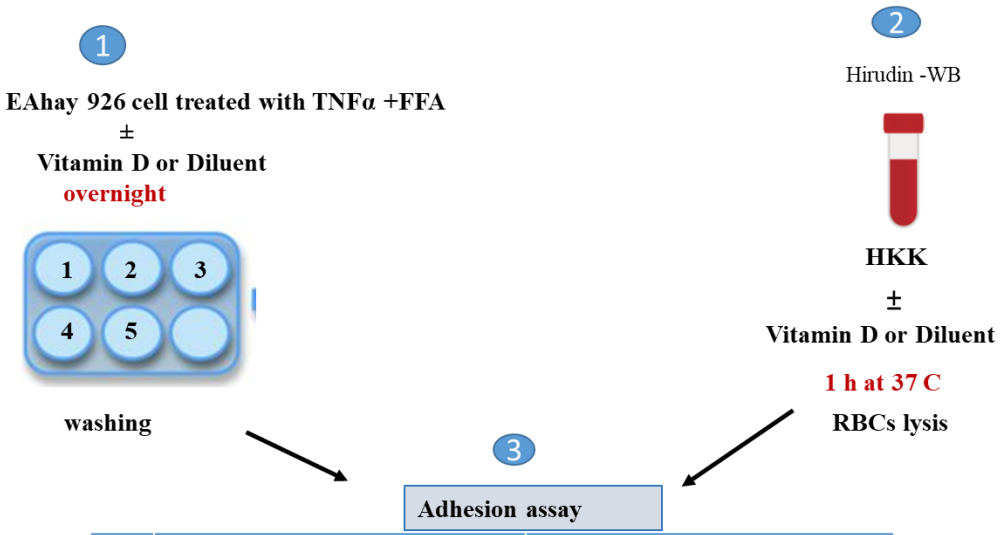
Clinical studies showed that 79% to 98% of intensive care unit (ICU) patients, including cases with sepsis, have insufficient Vitamin D levels (Kempker *et al*, 2012). Some studies have shown the positive association between the Vitamin D deficiency and the risk of sepsis as well as mortality rate, hospital stay and organ dysfunction (Kempker *et al*, 2012; de Haan *et al*, 2014). In support to this, improvement in Vitamin D level among septic patients with low levels has led to an improvement in the disease outcome in term of decrease in organ dysfunction severity (Alves *et al*, 2015). In addition, the incidence of sepsis and its associated mortality rate are highest during the winter season where low levels of serum Vitamin D levels are observed (Upala, Sanguankeo and Permpalung, 2015), although other seasonally linked factors play a role as well (Fares, 2013). Recently, Vitamin D has been given as a supplement to patients with sepsis. It therefore appears that maintaining the optimal concentration of

vitamin D influences outcome of sepsis. These data raised interest in this steroid and highlight the importance of conducting further experimental studies to uncover the possible relation between the acute phase in sepsis and Vitamin D.

Vitamin D has stepped into the limelight of antibacterial response as a nutritionally available modulator with measurable therapeutic effects at doses that replete a deficiency or are administered at higher, nontoxic doses in conditions of high demand. An intracrine effect for Vitamin D has been described which in part is exerted by its upregulation of antimicrobial peptides within the innate immune defence, but also impacts on the level of activation of cells operating in the adaptive immune response (Hewison, 2011; Gombart, 2009; Youssef *et al*, 2011b). No study yet has examined the cause and effect relationship between optimal levels of Vitamin D and the platelet-leukocyte-endothelial inflammatory interaction. The rationale for doing so comes from early studies demonstrating a direct effect of Vitamin D on the expressions of thrombomodulin and tissue factor on leukocytes, producing an antithrombotic phenotype (Koyama *et al*, 1998). Recently, it was shown that pre-treatment of blood immune cells isolated from healthy volunteers with active form of Vitamin D, 1,25(OH)2D3, (100nM) for 4 h, decreased the level of many pro-inflammatory cytokines, TNF-α, IL-1β, and IFN-γ, as well as the chemokine IL-8 53-fold following their stimulation with bacterial ligands such as heat-killed pneumococcal serotype 19F (HK19F) for 24h (Hoe *et al*, 2016). The mode of action to induce its anti-inflammatory effect was mediated by several mechanisms, by down regulating the expression of Toll-like receptor-2 and Toll-like receptor-4 proteins ; decreasing the phosphorylated p38 and p42/42 levels ; inhbiting the expression of phosphorylated signal transducer and activator of transcription 5 and reducing production of reactive oxygen species (Calton *et al*, 2015a).

The aim of this part therefore was to assess the role of basal levels of Vitamin D on the proinflammatory and pro adhesive phenotype of our model. The Vitamin D used in this study corresponds to 1, 25 (OH)2 D3.

5.5.2 Experimental design:



	Endothelium treatment	Whole blood treatment
1	TNF alpha +FFA	HKK
2	TNF alpha +FFA +Diluent	HKK+Diluent
3	TNF alpha +FFA	HKK+ Vitamin D
4	TNF alpha +FFA+ Vitamin D	HKK+ Vitamin D
5	TNF alpha +FFA+ Vitamin D	HKK

Figure 5.10: Experimental approach to study the effects of Vitamin D on the pro inflammatory and pro adhesive phenotype of the model.

EAhy 926 endothelial cells were cultured till reaching 70% confluence and co treated with TNF alpha (25ng/ml), FFA (oleic and palmitic, 2:1, 500 µM) and with or without Vitamin D (10nM) overnight as shown in figure 5.10. Because there are no previous published studies investigating the effect of vitamin D on endothelium, the dose of vitamin D was selected based on its anti-inflammatory effect on several immune derived cell lines where 10nM was the most commonly used dose to induce the anti-inflammatory effect (Calton *et al*, 2015b). The next day, freshly taken hirudin anticoagulated blood was co stimulated with HKK (106 CFU) with or without Vitamin D (100nM) and incubated for 1h at 37oC. Again due to unavailability of published *in vitro* data that analyse the effect of Vitamin D on the inflammatory status of whole blood, this dose was chosen based on clinical studies that conclude the importance of

reaching as high as > 100nM serum 25(OH)D to achieve its anti-inflammatory benefit (Calton *et al*, 2015a). More than 250 nM is considered harmful and toxic. After that, cell culture was washed and incubated again with lysed whole blood for 1h at 370C as indicated in figure. Addition of diluent instead of VD were also prepared in parallel as controls. Then, according to the purpose of the experiment, the sample was processed for microscopic analysis, scanning electron microscopy and light microscopy, or inflammatory marker study.

5.5.3 Treatment EAhy926 cells with Vitamin D attenuates their adhesiveness for the inflammatory aggregates:

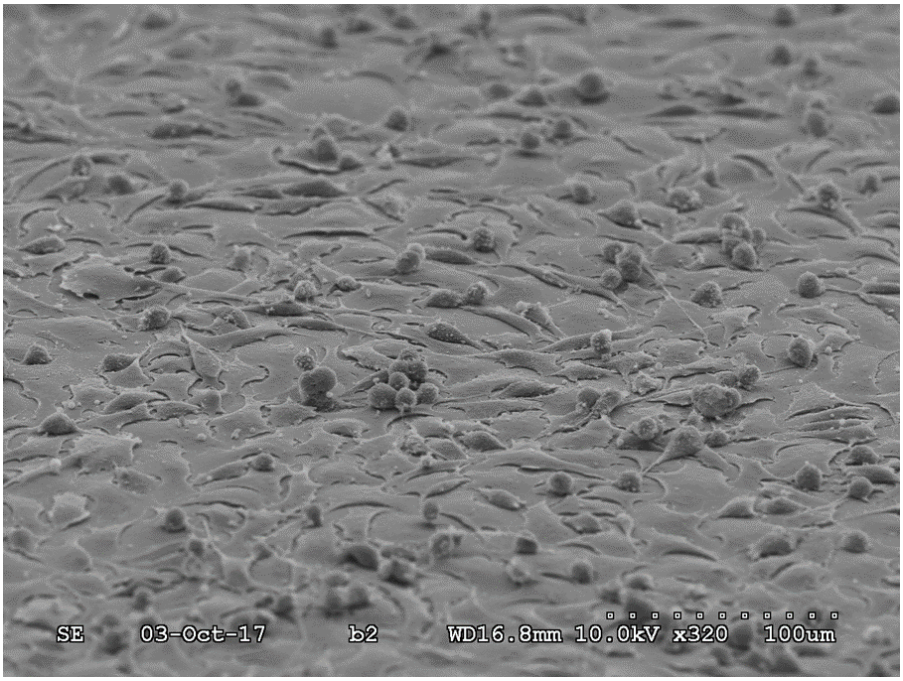
At 300X, as can be seen in figure 5.11 (C,D), preconditioning EAhy926 with vitamin D at 10 nM overnight together with TNFα and FFA stimulation before addition of HKK stimulated blood decreased the extent of inflammatory aggregate adherence to their surface compared to control [Figure 5.11(A)]. Moreover, most of the adhered aggregate at these conditions did not have the same complexity that adhered aggregates in control conditions had. Thus, vitamin D could either prevent the adherence of circulating aggregate or interfere with their formation on top of pre-conditioned endothelial monolayer. Similarly, analysis of the adhered aggregate at higher magnification (1500 to 2000 X) shows less complexed inflammatory aggregate [figure 5.12 (C,D)] compared to the aggregate complexity seen at control [Figure 5.12 (A)]. These findings fit well with its proposed anti thrombogenic effects. However, surprisingly, much more complexed inflammatory aggregate adhered to the large extent to the endothelium when vitamin D at 100 nM , together with HKK stimulation, was added to the whole blood but not endothelium, as seen with 300X and 2000X magnification [Figure 5.11 and 5.12 (B)]. Analysis of adhered inflammatory aggregate at this condition (B) with higher magnification shows the appearance of large netlike structures and fibres released from some of their cell components as shown in figure 5.13(A). This observation was not apparent at other conditions where no Vitamin D added to whole blood. This phenomenon could be attributable to what is called NETosis as it represents the typical characteristic features of NETs, neutrophil extracellular traps. NETosis defined as the extracellular release of the nuclear material, that is saturated with antimicrobial

proteins, from neutrophils upon their activation to trap and kill pathogens. It is commonly described as a large network of extracellular DNA fibres (Aulik *et al*, 2010).

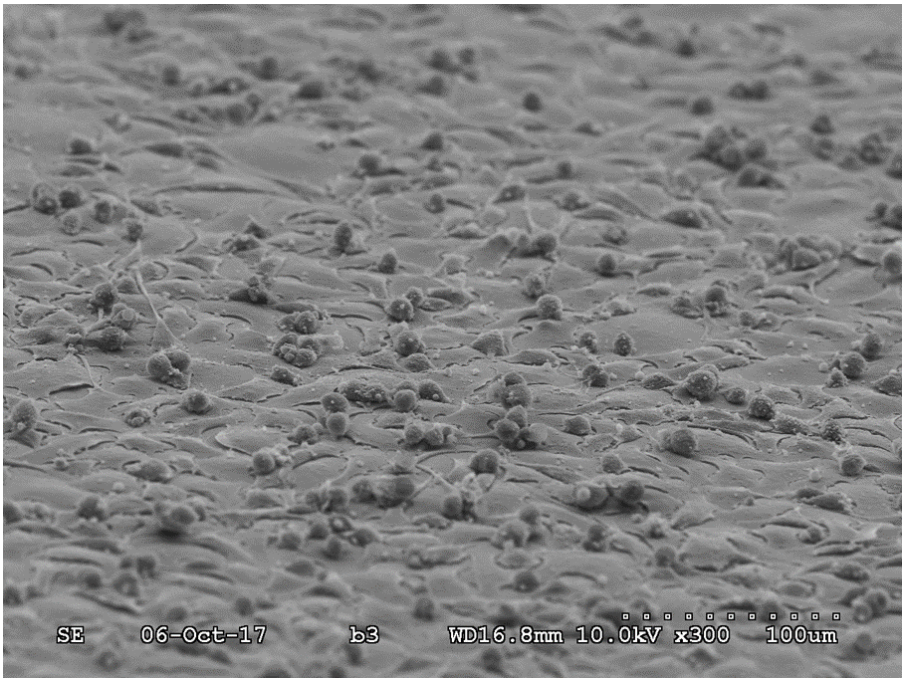
To investigate whether whole blood incubation of Vitamin D induced the NET formation, the amount of NETs at all of the experimental conditions was determined by ELISA as previously described (Handono *et al*, 2016; Caudrillier *et al*, 2012). As shown in figure 5.13 (B), compared to other conditions, there was no significant increase in neutrophil extracellular trap level when vitamin D was added to the HKK stimulated whole blood However, this assay is limited to measure the extracellular trap released by neutrophil only. In fact, the extracellular trap (ETs) are released by many cell types includes; mast cells, eosinophils, macrophages, monocytes (Goldmann and Medina, 2013). The extracellular trap, captured by SEM but not by ELISA, could be attributed to its production by other types of leukocytes.

Taken together, these finding could be still seen as a beneficial immune effect of vitamin D in stimulating more leukocyte to clear the pathogen with ETs, most probably.

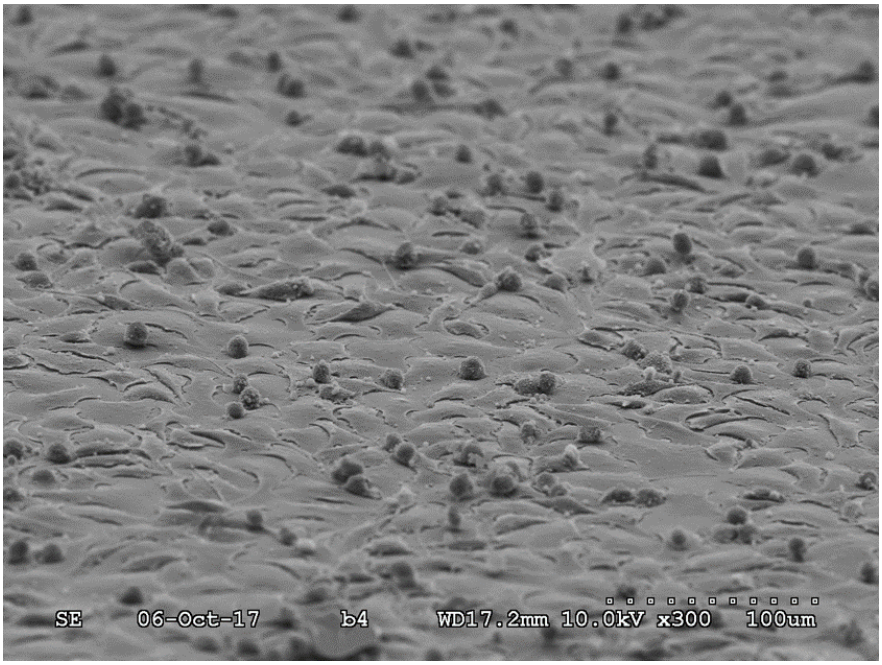
A: (TNFα+FFA +End +diluent) + (HKK+WB+ diluent)



B: (TNFα+FFA + End) + (HKK+WB+ VD)



C: (TNFα+FFA + End+VD) + (HKK+WB+ VD)



D: (TNFα+FFA + End+VD) + (HKK+WB)

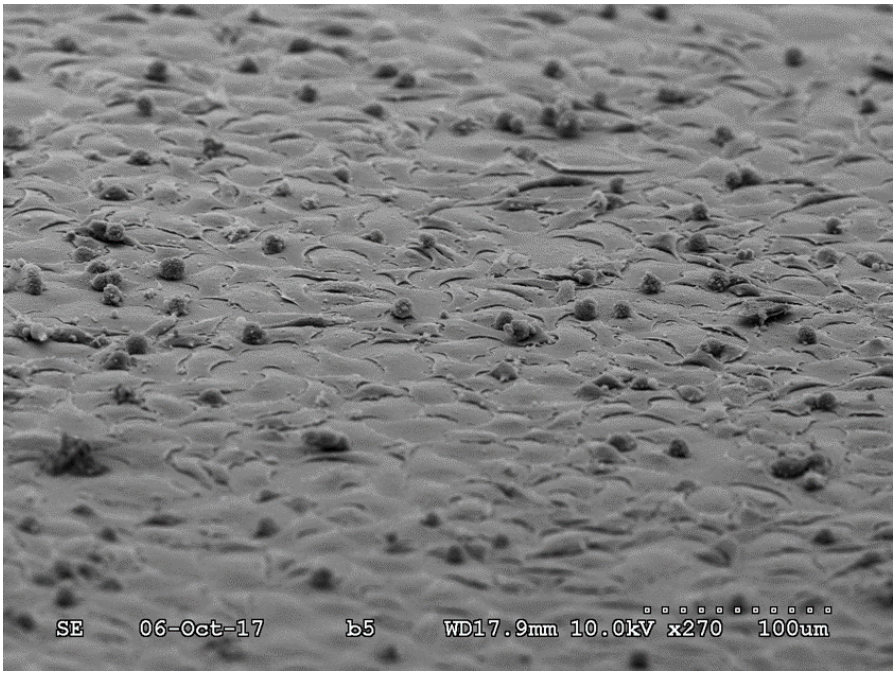


Figure 5.11: Effect of vitamin D on the adhesiveness of the endothelial cells. 24 h vitamin D (10 nM) pre-treatment of EAhy926 cells decreases their adhesiveness to the inflammatory aggregates.

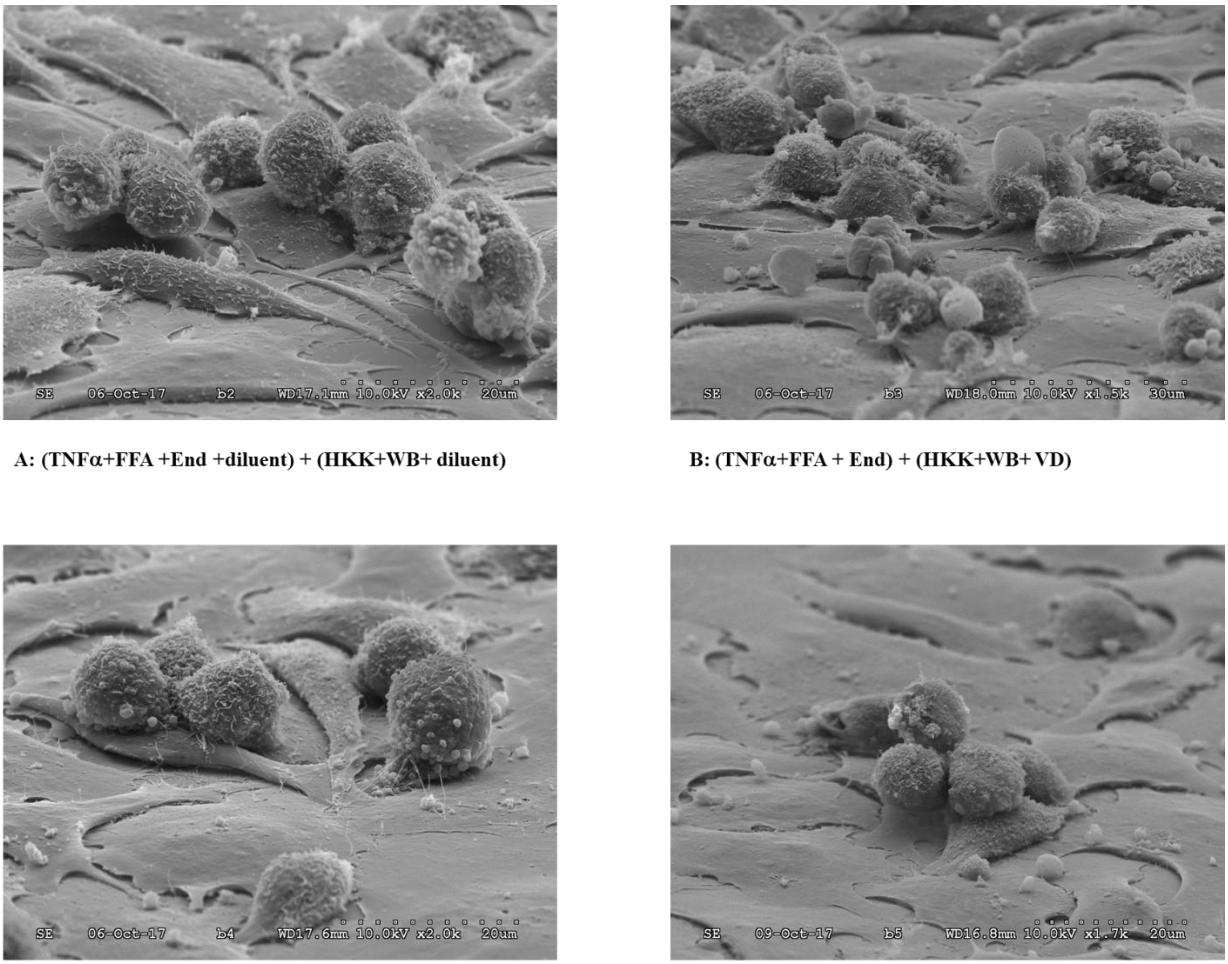


Figure 5.12: Effect of vitamin D on the complexity of the adhered cellular aggregates. overnight pre treatment of EAhy926 cells with Vitamin D associated with adherence of less complexed aggregates (C, D). Treatment of HKK stimulated whole blood with vitamin D (100nM) induced adherence of more complexed inflammatory aggregates (B).

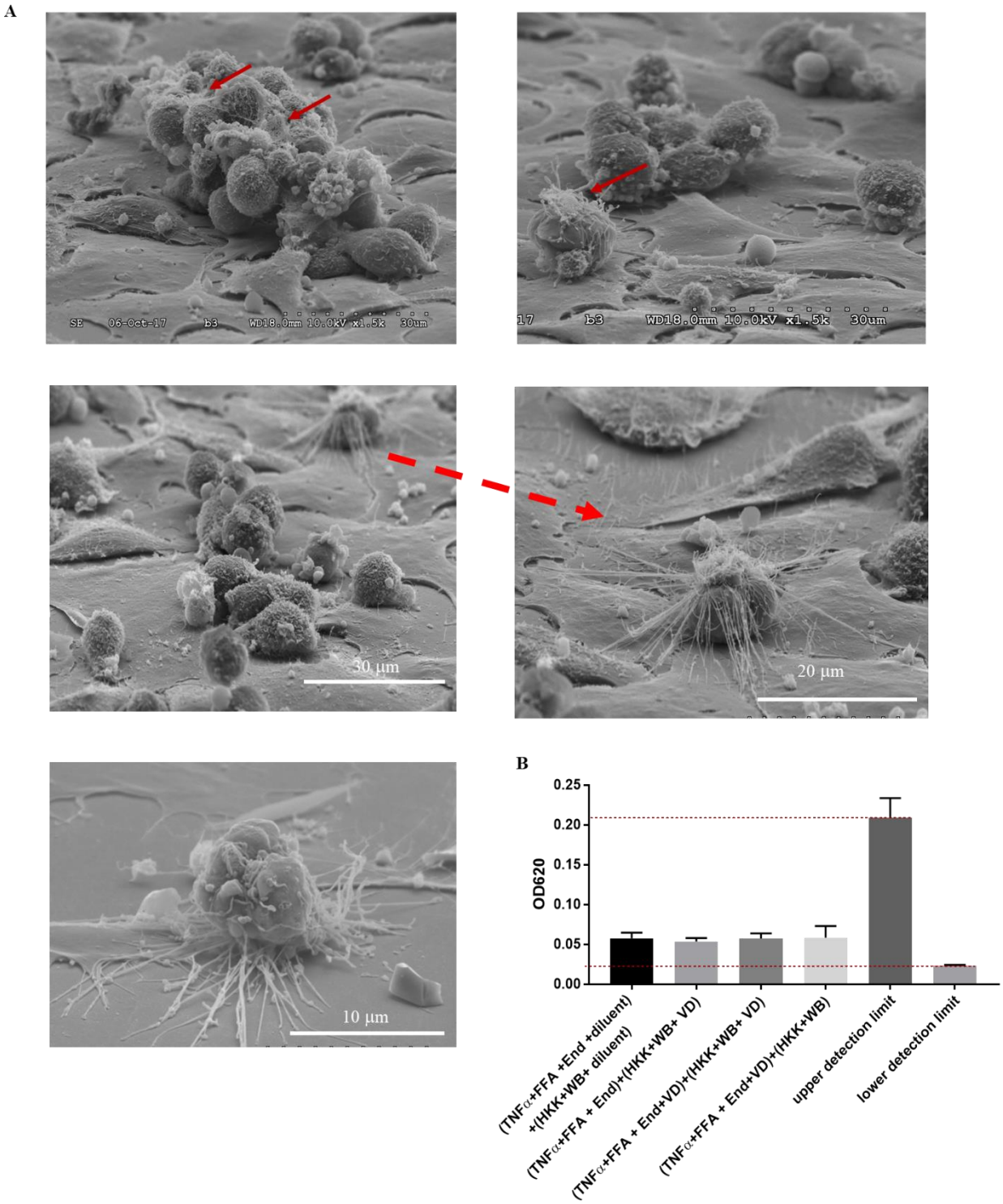
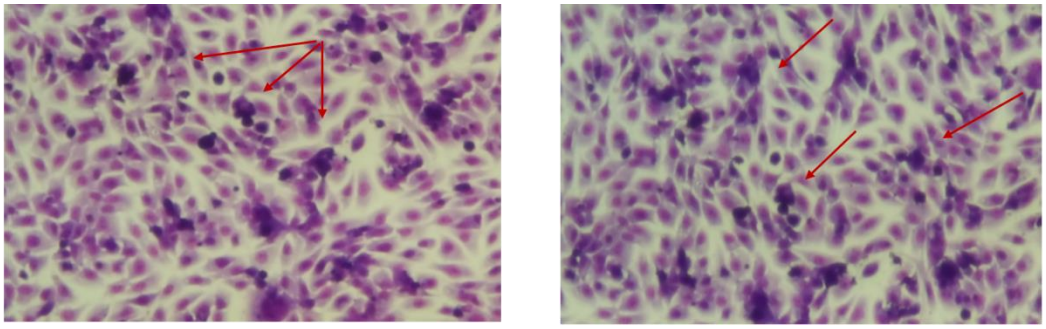


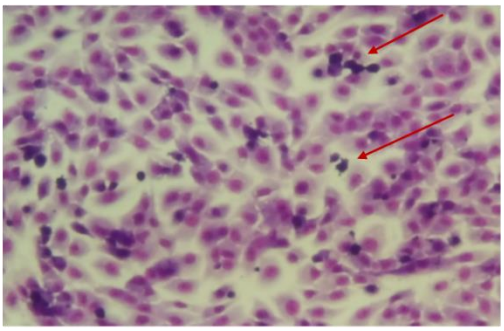
Figure 5 13: Vitamin D induces extracellular trap. A showed production of extracellular trap by blood cells. B showed the level of neutrophil extracellular trap (NETs) at different condition by ELISA. n= 4(B)

Subsequent experiments using blood taken freshly from two different volunteers were set up following exactly the same experimental design but analysed with different assessment tool, Leishman stain. It is an affordable and easy to set up tool to validate the previous results further (Figure 5.14) and demonstrates the light microscopic analysis of stained sample with Leishman stain at different condition. Consistent with previous results obtained by SEM analysis, preconditioning endothelial cells with vitamin D along with TNFα and FFA (C,D) reduced the adherence of inflammatory aggregate obviously compared to control (A). By contrast, treatment of whole blood with Vitamin D and HKK could not decrease the aggregate adherence to the endothelium (B). Quantitative analysis by counting the number of adhered aggregate per mm2 at each condition revealed nearly the similar results (Figure 5.14).

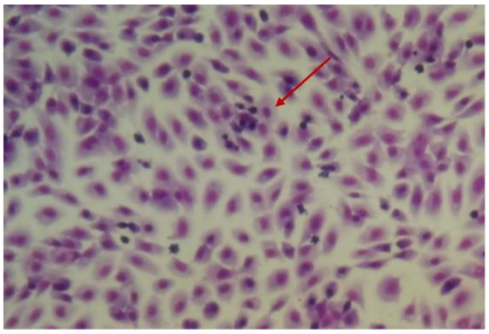


A: (TNFα+FFA +End +diluent) + (HKK+WB+ diluent)

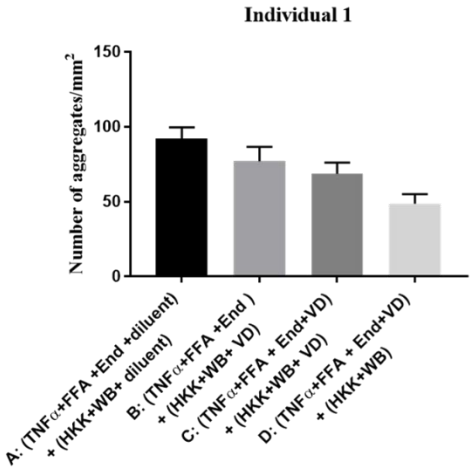
B: (TNFα+FFA + End) + (HKK+WB+ VD)



C: (TNFα+FFA + End+VD) + (HKK+WB+ VD)



D: (TNFα+FFA + End+VD) + (HKK+WB)



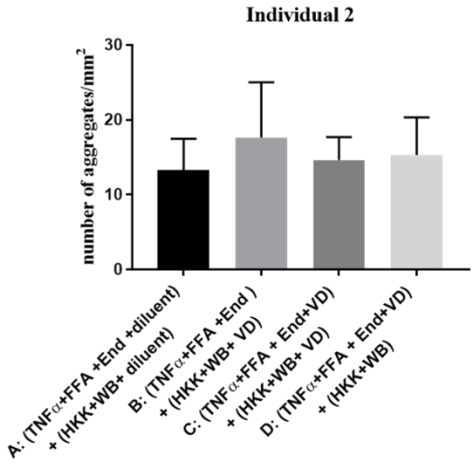


Figure 5.14: light microscopic analysis of vitamin D effects on the adhesiveness and complexity of the inflammatory cellular aggregates to the endothelial cells at different condition of stimulations. n= 2 X10.

5.5.4 Inflammatory markers analysis:

To investigate whether Vitamin D induced anti-inflammatory effects, IL-8 and TNFα levels in the plasma extracted from these different experimental conditions were measured by ELISA. A basal inflammatory condition was detected.

Unexpectedly, there was no significant differences in IL-8 and TNFα among these conditions. This means that vitamin D could not induce the expected decrease in the inflammatory markers profile. It could be attributed to the incubation time frame with blood, which was 1h.

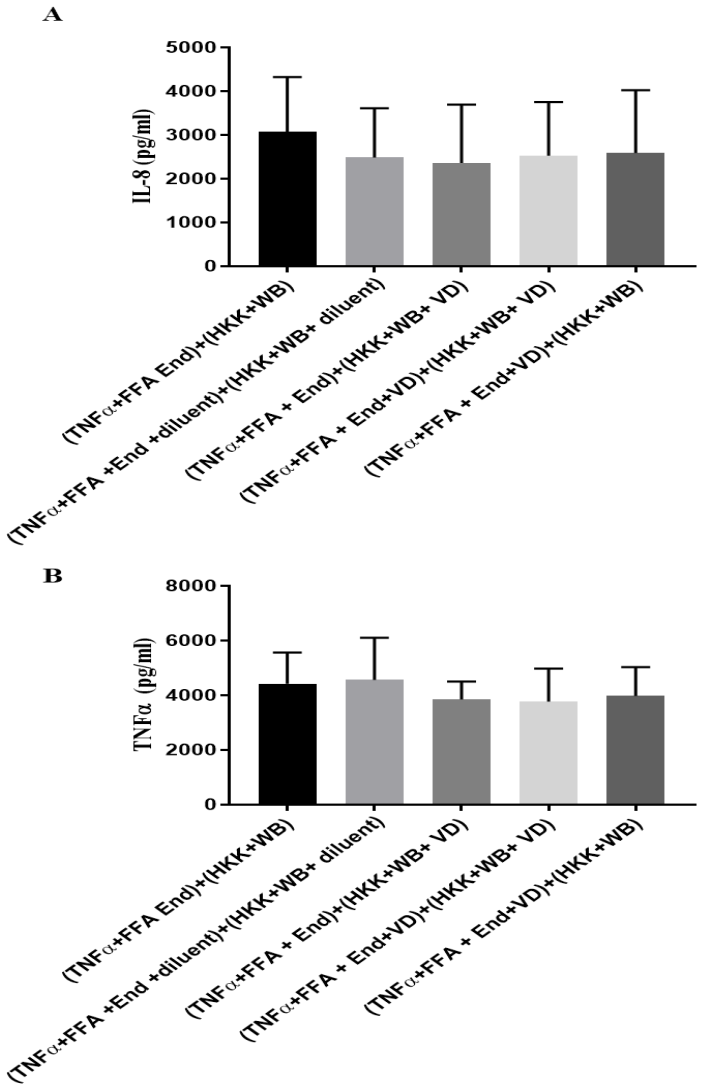


Figure 5.15: Acute effects of Vitamin D on the inflammatory markers, IL-8 and TNFα.TNFα and FFA stimulated EAhy926 were co-incubated with or without Vitamin D overnight. On the following day, EAhy926 were washed and incubated with whole blood that pre-treated with a combination of HKK and Vitamin D or HKK alone for 1hour. After incubation, the plasma was extracted and used to mesure the inflammatory markers level. Data ae analysed by Dunn's multiple comparisons test and showed no significant differences between the experimental conditions. n=4 at least.

5.6 Discussion:

Obesity is associated with low grade chronic inflammation (Li *et al*, 2017). In obesity, plasma level of FFA is always high due to the decrease in its clearance and increase in its release by accumulated fat tissue (Boden, 2008). FFA acts as an innate alarmin that is recognised by TLR4 and TLR2 to stimulate the inflammatory signalling cascades (Shi *et al*, 2006; Huang *et al*, 2012).This condition of chronic inflammation makes the milieu sensitive to an additional hit by inflammatory stimuli. This could lead to an exaggerated response that may negatively affect the ultimate outcome of sepsis. Due to the need for the exploration of the obese response to sepsis, a modification of a previously established, in chapter 2, novel model of *in vitro* platelet leukocyte aggregate adhesion to endothelial cells, representing a significant pathology in sepsis, was carried out. Thus, EAhy926 were stimulated overnight with a mix of non-esterified free fatty acids, which is most commonly found in obese plasma, together with TNFα stimulation. It was shown that TNFα stimulation enhances palmitic acid transcytosis into endothelial cells (Li *et al*, 2017). Palmitic acid induces expression of pro inflammatory cytokine in several endothelial cell line such as HUVECs, microvascular endothelial cells, and human arterial endothelial cell (Zhao *et al*, 2016). In principle, incubation of endothelial cell line with FFA such as palmitate has been widely adopted in order to understand the pathology of obesity associated endothelial dysfunction in several disease such as cardiovascular disease, diabetes and atherosclerosis(Liu *et al*, 2012; Lee *et al*, 2014)..

In the present study, chronic elevation of circulating FFA was modelled only partially by exposing endothelium alone without direct addition of these acids to the whole blood for 24h. This duration could not be modelled using whole blood because of the high sensitivity of whole blood outside the body when incubated for long period i.e. 24h (Dagur and McCoy, 2015). Thus, the possible adverse effects related to the whole blood treatment with FFA are not included.

The superiority of 500 μM over other concentrations were based on measuring cell viability and amount of lipid vacuoles. Results in section 5.2 showed that 500 μM is an effective dose in inducing more intracellular lipid vacuoles (i.e. more inflammatory reaction) without significant toxic effect on cell viability. Thereafter, the effect of pre-stimulating the endothelium with high concentration of FFA (500 μM) and TNFα prior

to their incubation with HKK stimulated whole blood on inflammatory and procoagulant state was analysed to understand their adverse effects on sepsis pathogenesis in terms of inflammatory markers and inflammatory cellular aggregate adhesiveness pattern. To the best of our knowledge, no publications can be found currently in the literature that address the additive FFA impact on inflammatory and aggregability of whole blood cells to the endothelium. However, in general, our results are consistent with most recent studies that prove a similar trend of an enhancement effect of FFA on LPS-induced inflammatory reaction in J774 macrophage cells where the same mix of free fatty acids (FFA, oleic acid/palmitic acid 2:1) was used (Kheder, Hobkirk and Stover, 2016). Most recent work in our lab showed the enhancement effect of FFA on the inflammatory cytokine release from osteoclasts (Zeyad Saeed, PhD).

Results in section 5.3 showed a significant consistent enhancing effect of FFA on IL-8 level as well as adhesion of more inflammatory aggregate when FFA were added. That means that addition of free fatty acids (FFA, oleic acid/palmitic acid 2:1) led to the aggravation of the inflammatory and adhesive reaction associated with the present model.

Similarly, pre-treatment of human coronary artery endothelial cells (HCAEC) with palmitic acid has been shown to enhance the cytokine -induced expression of adhesion molecules involved in the endothelial leukocyte interaction, ICAM-1,VCAM-1, and E-selectin expression (Reißig *et al*, 2003).

Thus, our results of increased inflammatory response (high IL-8) and aggregate adhesion when FFA were added could be explained by the ability of FFA to activate the endothelium further as an additional inflammatory stimulus, DAMP (Nogueira *et al*, 2008).

Moreover, activated endothelial cells release angiopoietin 2 that inhibits anti-inflammatory effect of angiopoietin-1 and exacerbates the inflammatory response as described above. Elevated markers of endothelial activation, dysregulated coagulation and advanced stage of organ failure are strongly associated with high Angiopoietin 2 levels in septic patients (Mikacenic *et al*, 2015c). Thus, angiopoietin 2 might be an attractive target for therapy. Blockade of angiopoiten 2 was performed by use of an evolved Tie ectodomain with specificity for angiopoietin 2, termed R3 (Brindle et al., 2013). R3 binds Ang2 and inhibits its effects on endothelial cells without any

interference with Ang1 activity. Thereby, it shifts signalling towards greater activity of endothelial protective angiopoietin 1, an alternative ligand for endothelial cell expressed tyrosine kinase Tie (Singh *et al*, 2012; Hansen *et al*, 2010; Alawo *et al*, 2017). Up to date, the effect of Ang 2 blocker on endothelial cell adhesiveness to blood cells has not yet been investigated. A well- controlled experimental design is crucial to analyse the effect of the blocker accurately and, thus, a solid foundation to the subsequent work or analyses can ensue. Because the aggregate formation is dependent to a large extent on the engagement of P-Selectin expressed on both platelets and endothelium and PSGL-1 on leukocytes (Langer and Chavakis, 2009; Granger and Senchenkova, 2010; Rumbaut and Thiagarajan, 2010), P Selectin blocker was analysed as a control in parallel to R3.

The results in the section 5.4. reveal that the ability of R3 or a functional P-Selectin antibody to block the adherence of inflammatory aggregate, formed in response to HKK stimulation, to endothelial cells incubated with TNFα and FFA was more accurately evaluated using SEM rather than immunofluorescence, which bore technical limitations (e.g. interference of immunolabeling with aggregation phenotype, cell specific variation in cell membrane labelling using Vybrant™ DiO Cell-Labeling Solution). The results in section 5.4.2 demonstrate the successful blockade of adhesion and formation of aggregates, respectively.

Therefore, our results further support the assumption of Ang2 contribution to the microvascular dysfunction and multiorgan failure in sepsis (David *et al*, 2012) which are mediated in part by blood cells adhesion to the endothelium. Importantly, it shows that our design provides a suitable, pathophysiologically relevant, model, with which to study *ex vivo* cell interactions in the acute phase of septic inflammation.

Thereafter, the model was used to investigate for the first time the relationship between optimal level of Vitamin D and platelet-leukocyte-endothelial inflammatory interaction. Our results showed that 24 h endothelium treatment with Vitamin D3 plays a significant role in decreasing the complexity and the extent of aggregate adherence that was confirmed by two methods. That was contrasted completely when whole blood was incubated with Vitamin D together with HKK prior addition to endothelium for 1h. More complexity and adherence of these aggregates was observed at this condition. These findings might result from acute effects of VD that aimed at clearing the stimuli

(HKK). Although, there was no significant effect in the inflammatory markers, IL-8 and TNFα between experimental condition, we are not able to tell whether the addition of VD might not have anti-inflammatory effect for the following reason. Their level was measured in the plasma extracted from whole blood after their incubation with vitamin D and HKK first and afterwards to the endothelial cell at different condition (illustrated in fig) for 1h each step i.e 2 h maximum incubation. This time might be not sufficient to induce an effect by vitamin D. While most of the studies investigating the anti-inflammatory effect of vitamin D, incubated vitamin d with experimental condition for at least 24 h (Calton *et al*, 2015a; Kheder *et al*, 2017).The inhibitory effect of vitamin D on the aggregate adhesion by 24 h endothelial vitamin D treatment support the explanation on the incubation time, further.

Chapter 6

Conclusion

6.1 Conclusion:

This PhD project has made a significant contribution to the field of study. Analysis of the literature at the beginning of the study demonstrated the need to develop a model of *in vitro* stimulation that addressed perceived shortcomings of methods so far. In particular the choice of the appropriate baseline for comparison of the effects to stimuli was found to be critical importance. Furthermore, conducting the *in vitro* stimulation in whole blood, thereby allowing cellular interaction in the presence of relevant plasma components, was important for conclusions which were meaningful for the *in vivo* situation. Sepsis is a complex condition which needs to be broken down to overseeable pathologically relevant units in order to advance knowledge, understanding and application of therapeutic approach. Our model of inflammatory PLAs formation and adhesion on the endothelial cells is a novel *ex vivo*, reliable, affordable, standardisable, and easily adaptable and applicable model. It was designed to mimic an early phase of sepsis and adapted to cover the obese response to sepsis. Consistent results obtained with different qualitative and quantitative analytical tools (SEM, light microscopy, TEM, C5a western blot, IL-8, TNFα, microarray analysis) point on the robustness of the model.

The study results revealed that:

- There is spontaneous PLA formation (after 1 hour) which is hardly detected at zero time point.
- Endotoxin from gram negative bacteria is not a suitable stimulus to provoke sepsis relevant platelet leukocytes aggregates in vitro.
- Heat killed bacteria are preferred in the studies of whole blood inflammatory cellular aggregates under simulated physiological conditions i.e., at the time point of stimulation, presence of divalent cations levels and intact complement activity.
- In my hands, SEM was superior to other, tested methods in this study; such as flow cytometry, and immunofluorescence, for analysing PLAs formation and adhesion to endothelial cell.
- By SEM, R3 (Ang-2 blocker) appears to block the aggregate adherence to endothelial cell to a nearly similar extent as anti- p selectin.

- Vitamin D appears to decrease the aggregate adherence to endothelial cells and induce the extracellular trap.

6.2 Future work:

This model can reliably provide insight in the acute inflammatory reaction in the blood vessels to to the presence of common bacterial PAMPs present during septicaemia via evaluating both, cell aggregates formation and their adherence to the endothelial cells as well as inflammatory markers. It can provide the basis for future studies on the mechanisms of cell interactions, including endothelial cells. Our model can be applied for *in vitro* analysis as illustrated, to examine the effects of pharmacological agents and nutraceuticals, a dietary supplement with benefits to health, on inflammatory conditions.

Presently, there is growing interest in the effect of genetic element on the sepsis susceptibility and outcome. The wide variability in the sepsis clinical course, seen in clinical practice, could be attributed to the genetic variability between individuals (Dahmer *et al*, 2005; Namath and Patterson, 2011). Based on that, this model can be adapted to examine the effect of different genetic polymorphism on aggregates formation and adherence to endothelium and production of inflammatory markers by using blood from individuals with known polymorphisms such as TLR4 (Asp299Gly/Thr399Ile, resulted in reduction of TLR4 expression (Dahmer *et al*, 2005)) and healthy volunteers. This polymorphism might explain the unexpected depressed inflammatory response of whole blood drawn from two healthy volunteers in contrast to others throughout the study.

Chapter 7

Appendix

7.1 Quality assessment of experimental condition:

To determine the effects of different concentration of LPS on foam cell formation, Firstly, J744 cells were aseptically cultured on coverslips using 6 well- plate at 37oC, 5% CO_2 till confluent stage. Cells, then, treated with culture medium (as negative control) or LPS at two different concentration (100ng/ml and 1000ng/ml) for 24h. Then, macrophage lipid inclusions were visualized with Oil Red O staining. As shown in (Appendix I, A), surprisingly,the number of lipid laden macrophages of control samples was approximately similar to that of 100 ng/ml LPS treated sample. Thus, the presence of another endotoxin source was highly suggested. However, high dose of LPS (1000ng/ml) stimulate more macrophages to accumulate lipid droplets as compared with 100ng/ml LPS stimulation condition.

So, further experiment was done using endotoxin free PBS to exclude the influence of endotoxin contamination of water used in preparation of PBS previously. J744 cells were cultured on coverslip with medium, 100ng/ml or 1000ng/ml of LPS for 24 hours. Then, the Oil Red O staining was carried out to analyse the lipid droplet formation. As expected, high concentration of LPS (1000ng/ml) result in presence of macrophages containing lipid droplets in their cytoplasm compared to medium treated cells. The control cells, which were incubated with culture medium, only, did not show lipid accumulation in macrophages (Appendix I,B).

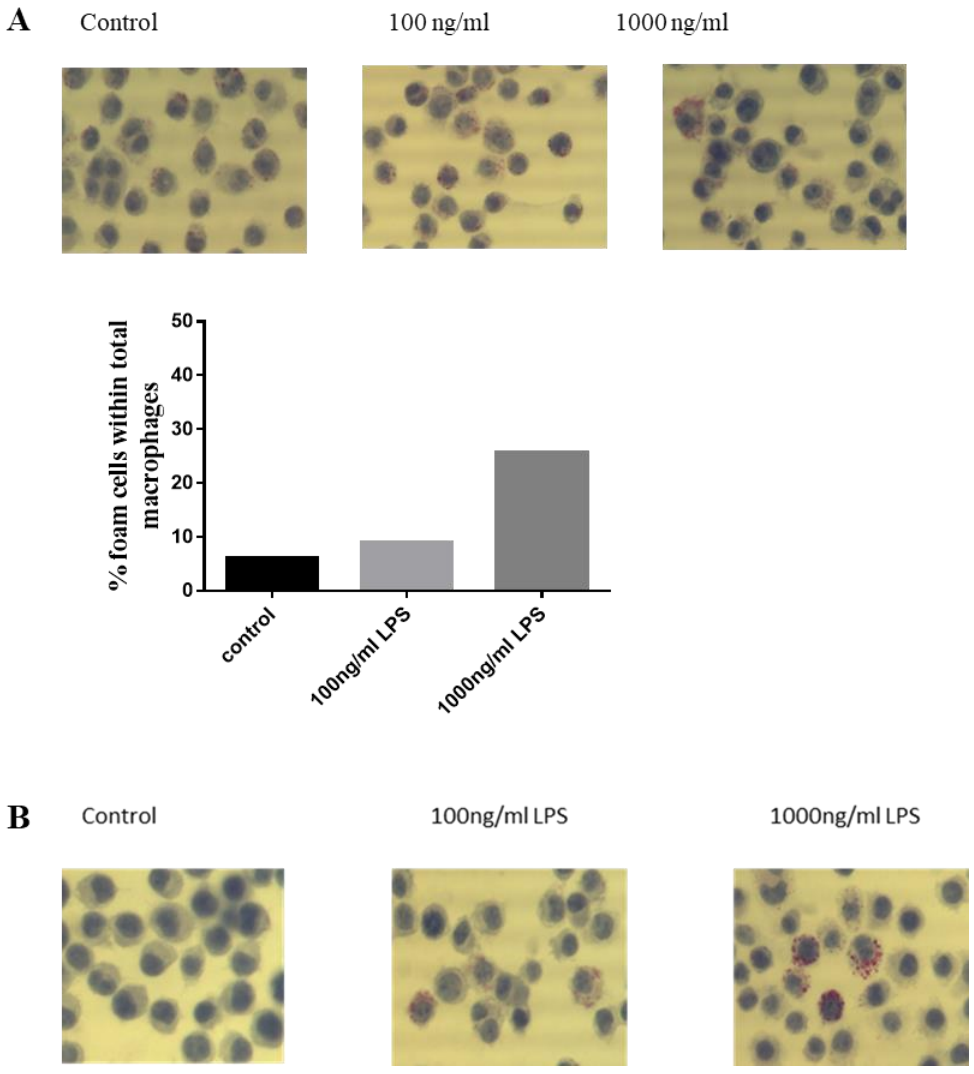


Figure (Appendix I): Analysis of macrophages' lipid inclusions at different conditions of LPS stimulation.

7.2: Effects of high phosphate level on the inflammatory aggregate formation and adherence to the endothelial cells:

Hyperphosphatemia is associated with endothelial dysfunction and high levels of circulating endothelial microparticles that ultimately lead to a prothrombogenic state (Abbasian *et al*, 2015). As a pilot experiment, we aimed to investigate whether the high phosphate levels in PBS (10mM) used throughout the experiments had a significant induction effect on the aggregate formation and their adherence to the endothelial cells. Phosphate buffered saline with a physiological level of phosphate (1mM) was prepared

in NaCl (pH:7.4). Two parallel adhesion assays using HKK – stimulated whole blood and TNFα–FFA stimulated EAhy926 that handled thorough all experimental steps with 1mM or 10mM PBS. The cells were stained with Leishman's stain and plasma was used to measure the level of inflammatory markers, IL-8 and TNFα. As shown below, there is no induction effects of high phosphate levels on the extent of the inflammatory aggregate formation and their adherence to endothelial cells and no significant difference in the level of inflammatory markers at both conditions, 1 or 10 mM PBS.

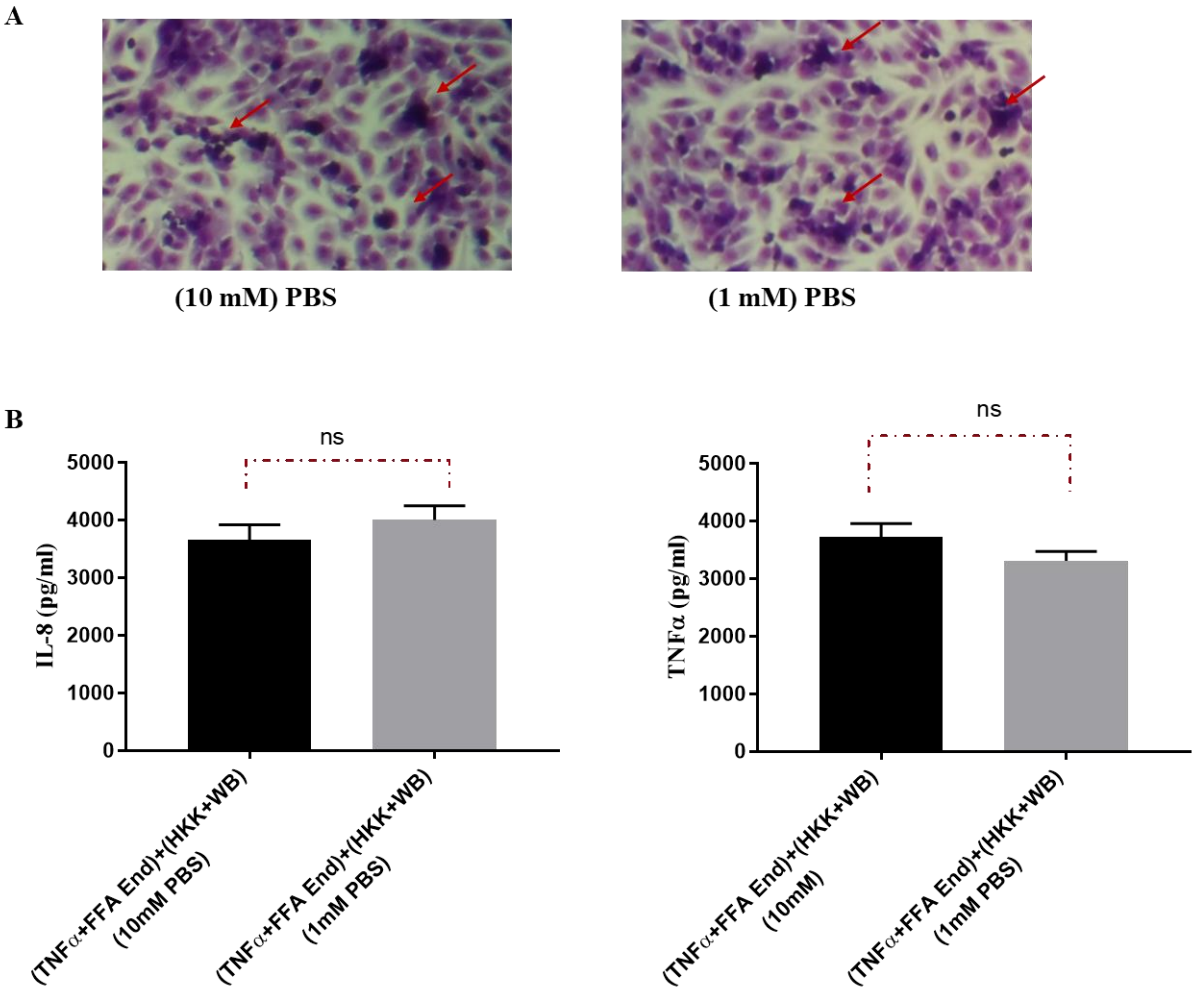


Figure (Appendix II): Effects of different concentration of phosphate on the inflammatory interaction of platelet -leukocyte -endothelium. (B) Data expressed as mean +/- SEM and analysed by Mann-Whitney test. n=2.

Fluorochrome	% fluorochrome	Spectral overlap
APC	PE	0.01
APC	FITC	0.05
FITC	PE	0.28
FITC	APC	0.02
PE	FITC	0.07
PE	APC	0.01

Figure (Appendix III): Spectral overlap between different fluorochrome that were compensetaed.

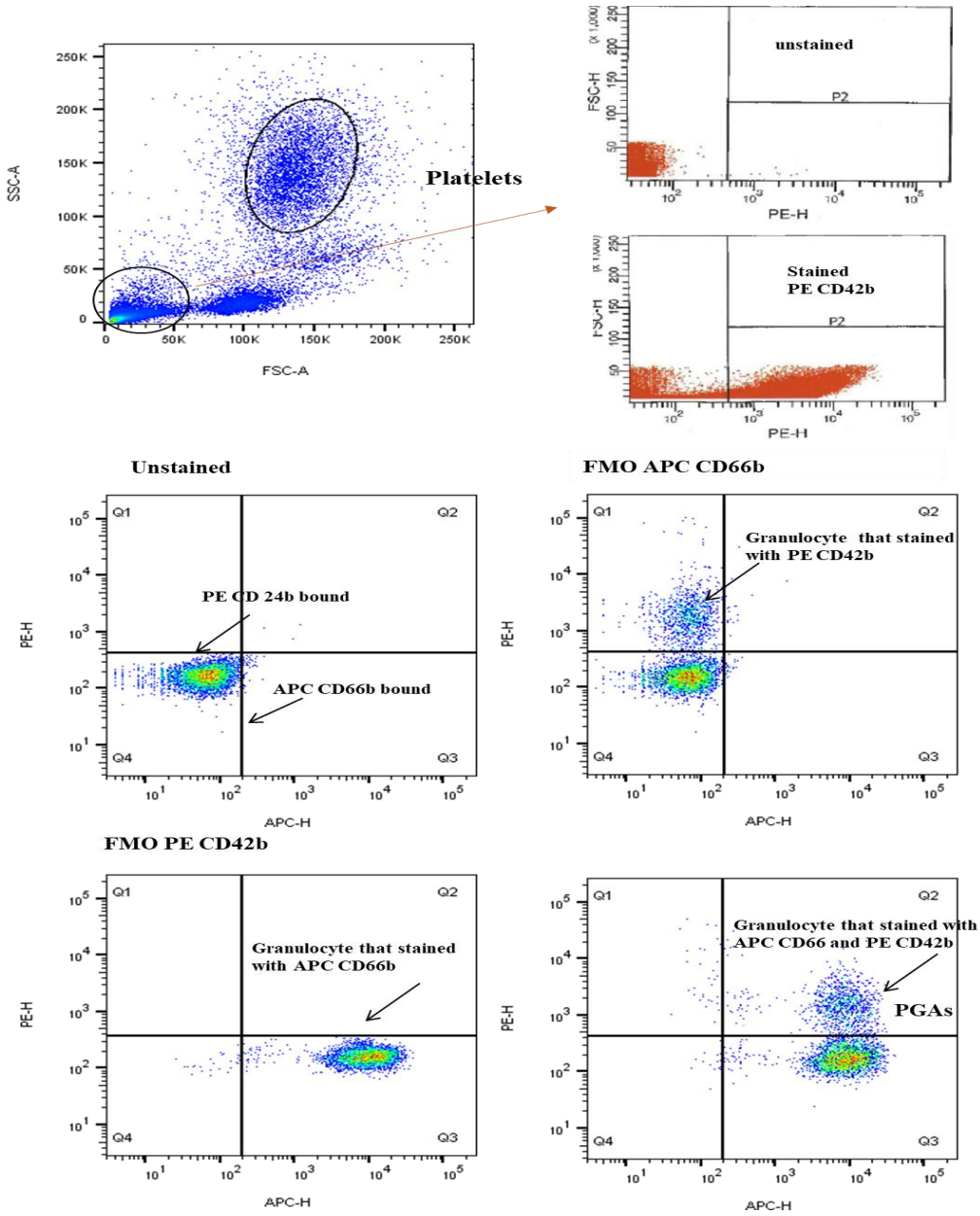
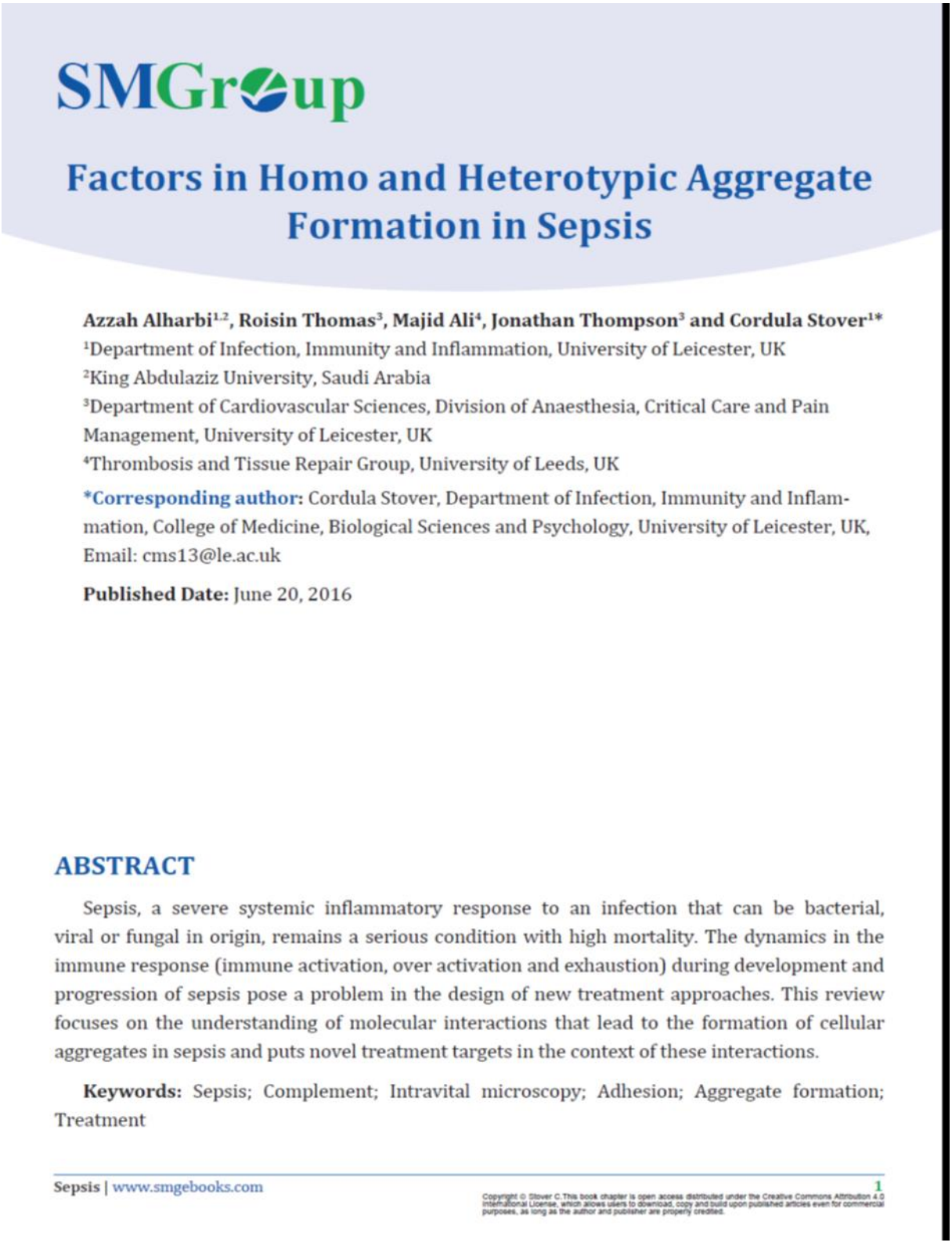


Figure (Appendix IV): A, detection of PGA by flow cytometry. The granulocyte and platelet populations were clearly identified in whole blood based on light scatter characteristic, size and granularity. PE CD42b bound (platlet marker) was adjusted on the basis of unstained samples and fixed. Thus, PGAs can be accurately determined. From this granulocyte gate, events that stained positively for both CD66 (APC conjugate) and platelet marker CD42b (PE conjugate) were identified as PGA.



SMGroup

Factors in Homo and Heterotypic Aggregate Formation in Sepsis

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ABSTRACT

Sepsis, a severe systemic inflammatory response to an infection that can be bacterial, viral or fungal in origin, remains a serious condition with high mortality. The dynamics in the immune response (immune activation, over activation and exhaustion) during development and progression of sepsis pose a problem in the design of new treatment approaches. This review focuses on the understanding of molecular interactions that lead to the formation of cellular aggregates in sepsis and puts novel treatment targets in the context of these interactions.

Keywords: Sepsis; Complement; Intravital microscopy; Adhesion; Aggregate formation; Treatment

Figure (Appendix V): Book chapter 'Factors in Homo and Heterotypic Aggregate Formation in Sepsis', published at sigma e books, pp. 1-1-11.

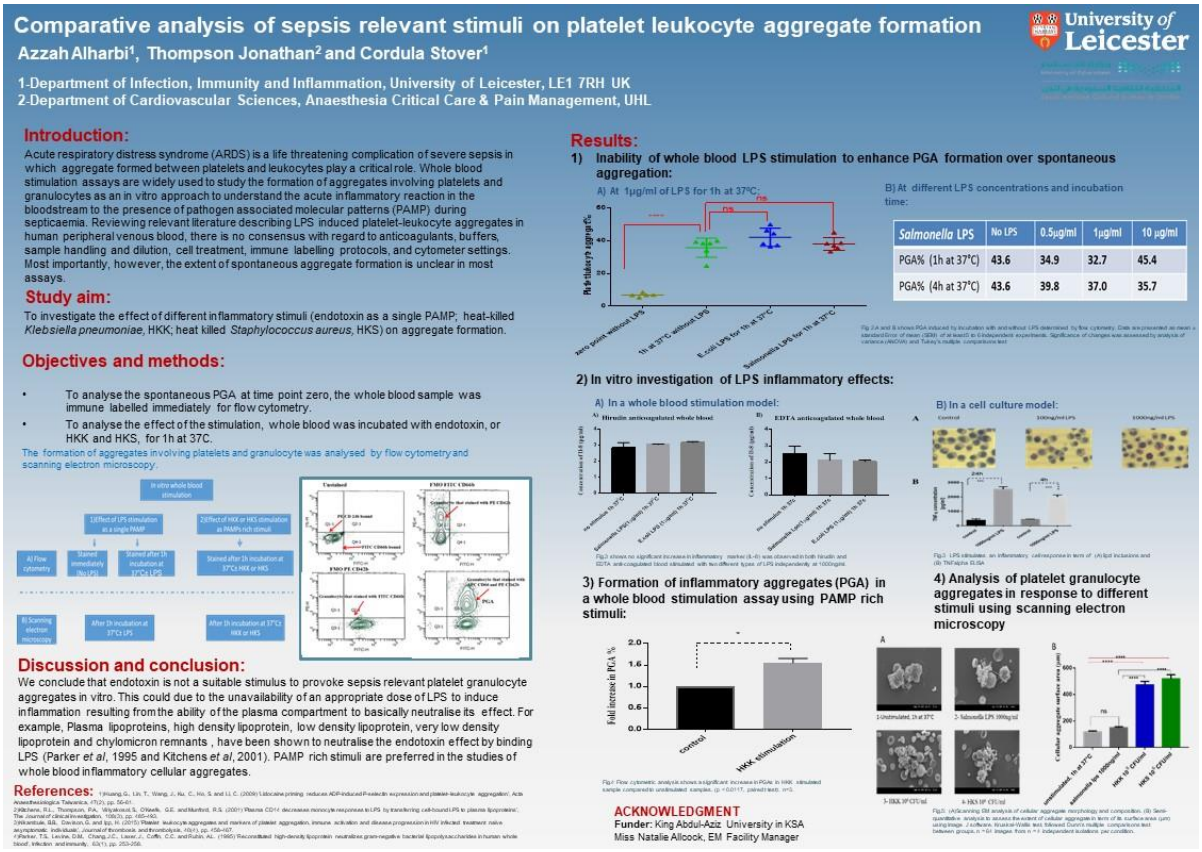


Figure (Appendix VI): Poster in Joint Respiratory Research Day. Nottingham UK. May 2017 and East Midlands Doctoral Conference. Nottingham UK. September 2017.

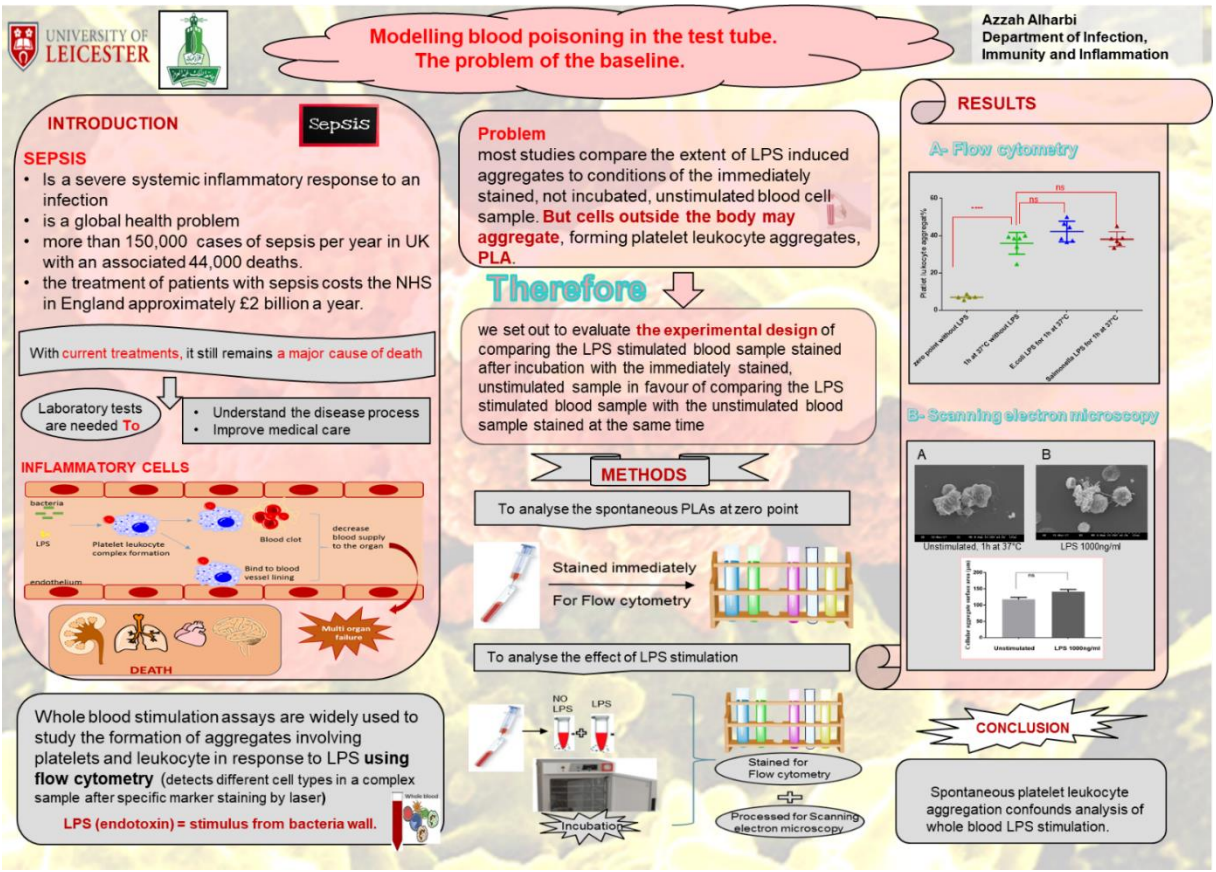
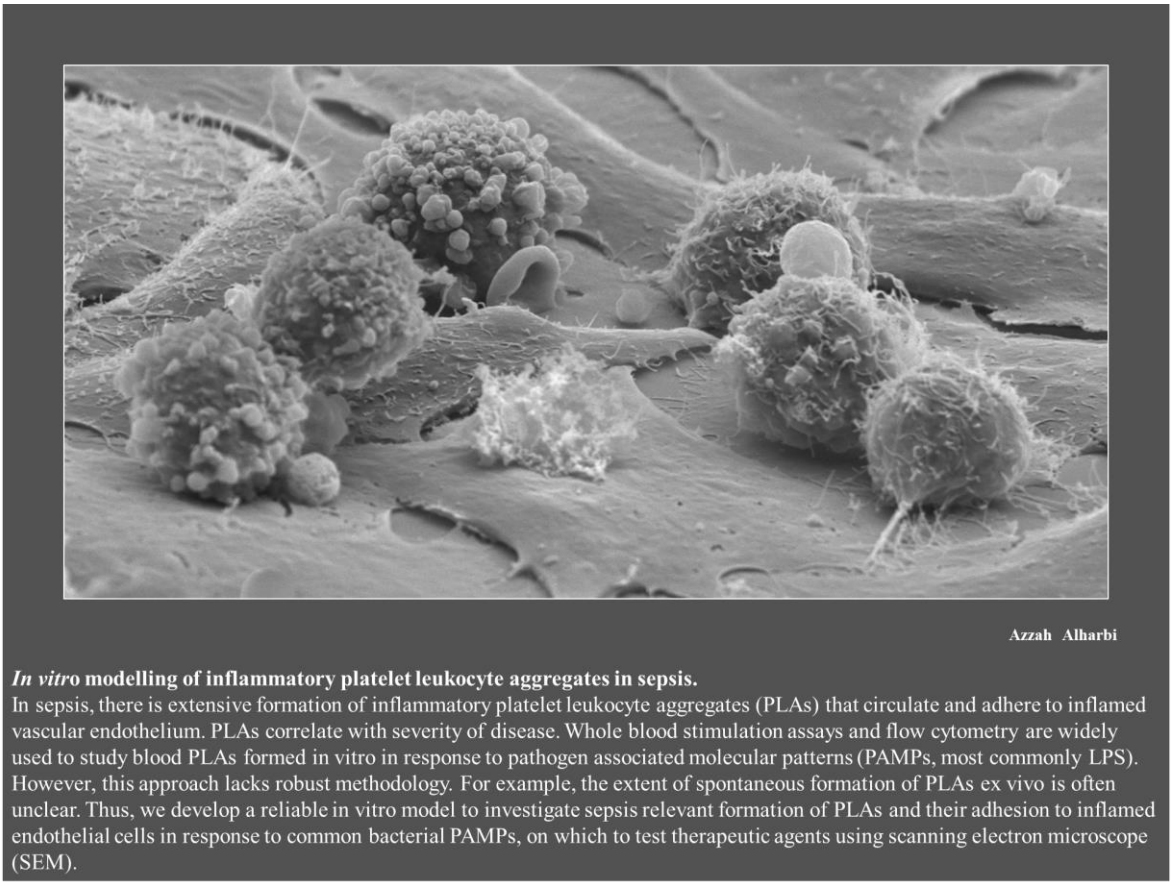


Figure (Appendix VII): Poster presented at Postgraduate Research Festival. I was chosen as one of the 50 most promising researchers among 1700 postgraduates at the University, University of Leicester, Leicester, UK, June 2017.



Azzah Alharbi

***In vitro* modelling of inflammatory platelet leukocyte aggregates in sepsis.**
In sepsis, there is extensive formation of inflammatory platelet leukocyte aggregates (PLAs) that circulate and adhere to inflamed vascular endothelium. PLAs correlate with severity of disease. Whole blood stimulation assays and flow cytometry are widely used to study blood PLAs formed in vitro in response to pathogen associated molecular patterns (PAMPs, most commonly LPS). However, this approach lacks robust methodology. For example, the extent of spontaneous formation of PLAs ex vivo is often unclear. Thus, we develop a reliable in vitro model to investigate sepsis relevant formation of PLAs and their adhesion to inflamed endothelial cells in response to common bacterial PAMPs, on which to test therapeutic agents using scanning electron microscope (SEM).

Figure (Appendix VIII): Image presented in Image of research that was chosen as one of the most promising researches among PGRs through to Principal Investigators and Research Leaders at the University, University of Leicester, Leicester, UK, December 2017.

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ABSTRACTS

ABSTRACTS

Abstracts from the *BJA* Research Forum Leicester, UK, November 23–24, 2017

The name of the person presenting the paper is shown in bold type. All authors have certified that, these appropriate, studies have been conducted with the approval of the relevant Human Ethics Committee or Animal Experimental Review Committee.

Formation of inflammatory platelet-leucocyte aggregates in vitro and their adhesion to inflamed endothelial cells

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In sepsis, there is extensive formation of inflammatory platelet leucocyte aggregates (PLAs) that circulate and adhere to inflamed vascular endothelium. PLAs correlate with severity of disease. Whole blood stimulation assays and flow cytometry are widely used to study blood PLAs formed in vitro in response to pathogen associated molecular patterns (PAMPs, most commonly LPS). However, this approach lacks robust methodology. For example, the extent of spontaneous formation of PLAs ex vivo is often unclear. Thus, a reliable in vitro model to investigate sepsis relevant formation of PLAs and their adhesion to inflamed endothelial cells is required. We aimed to develop a model of the acute inflammatory reaction to common bacterial PAMPs, on which to test therapeutic agents.

Whole blood from healthy volunteers was drawn and immediately immunolabelled with anti CD66b-APC and anti CD42b-PE to assess the extent of spontaneous PLA. Further, whole blood samples were incubated with either endotoxin (E. coli 0111: B4 or Salmonella enteritidis, Sigma), as single PAMP, or heat killed bacteria (Klebsiella pneumoniae or Staphylococcus aureus, departmental archive), for 1h at 37°C. PLAs were identified by flow cytometry as CD66b and CD42b positive events. Ultrastructural analysis of these aggregates was performed by scanning electron microscopy (Scanning EM). Whole blood was stimulated with LPS (1000 ng/ml), heat-killed K. pneumoniae or heat killed S. aureus (106 CFU/ml) or left unstimulated (control), then co-incubated with TNFα stimulated EAhy.926, to investigate PLA adherence to inflamed endothelial cells by transmission electron microscopy (TEM) and light microscopy. Secreted IL-8 was measured by ELISA. Data were analysed using the Kruskal-Wallis test, followed by Dunn's test for multiple comparisons between groups.

Twelve individual samples were tested from eight volunteers. There was significant spontaneous aggregation of platelets and leucocytes, which was not enhanced by LPS from E. coli or S. enteritidis. Scanning EM revealed similar PLA surface area between unstimulated and endotoxin-stimulated samples but a significant increase after whole blood stimulation with heat killed bacteria ($p<0.0001$). Light microscopy and TEM (Fig. 1) showed adherence of more complexed cellular aggregates on the endothelial layer after stimulation with heat killed bacteria, in conjunction with a significant increase in IL-8.

Unexpectedly, LPS was not a suitable stimulus to develop an in vitro model of acute cellular interactions between PLAs and inflamed endothelium. Rather, the combination of heat killed bacteria and Scanning EM successfully modelled formation of complex PLAs and their adhesion to endothelial cells.

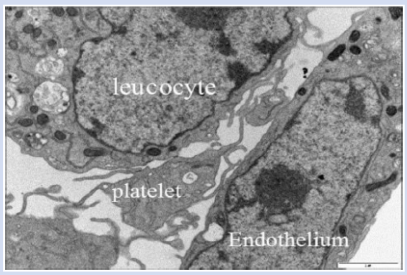

Fig 1. Transmission electron micrograph of platelet and leucocyte engaging with endothelial cell as indicated.

Figure (Appendix IX): Alharbi, A., Stover, C. and Thompson, J. (2018) 'Formation of inflammatory platelet-leucocyte aggregates in vitro and their adhesion to inflamed endothelial cells', *British journal of anaesthesia*, 120(5), pp. e5.

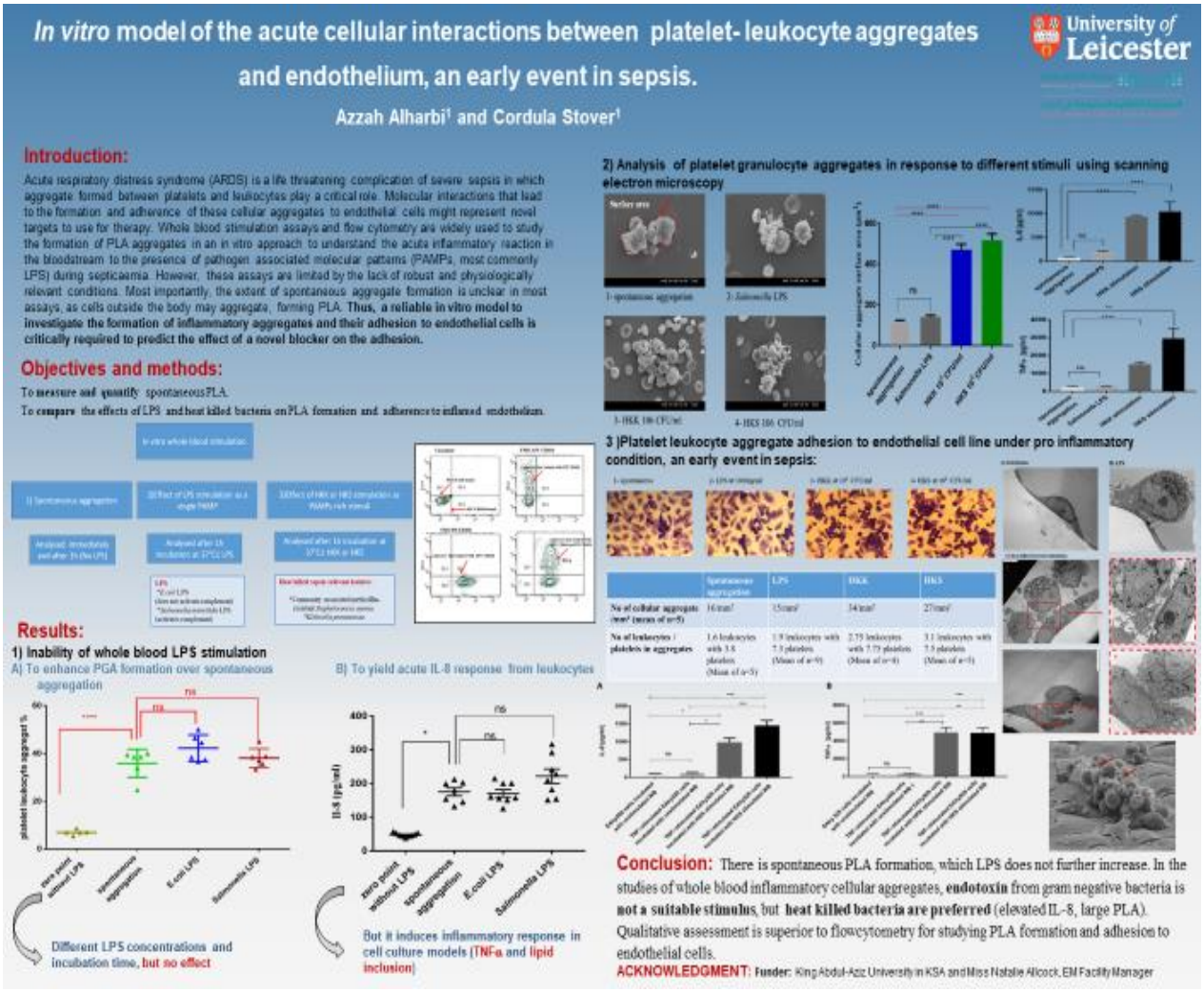

Figure (Appendix X): Poster in Joint Respiratory Research Day. Leicester UK. May 2018

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