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Platelets and their immunomodulatory roles during *Streptococcus pyogenes* infection

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Sepsis and invasive bacterial infection is a major cause of human disease and death worldwide. *Streptococcus pyogenes* is one of the major pathogens responsible for bacterial infections, which range from mild to severe and life threatening conditions. *S. pyogenes* contains a cell wall anchored M protein, which is an important virulence factor that can interact with many cells of the immune system.

One of the smallest and most rapidly responding cells in our bloodstream is platelets. There are hundreds of millions of platelets in our blood system, which demonstrate multifaceted roles. They mend and seal our blood vessels at the site of vasculature damage and recruit, alert and activate other majorly important immune cells some of which have been investigated herein.

Collectively, this thesis has revealed multiple strategies for *S. pyogenes* to modulate the host immune response during invasive bacterial infection by interacting directly and indirectly with cells of our immune system: platelets, neutrophils, monocytes and endothelial cells. These interactions may have far reaching consequences during the pathogenesis of sepsis.

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Platelets and their immunomodulatory roles during *Streptococcus pyogenes* infection

Sinéad M. Hurley

2016

Doctoral Dissertation

With due permission of the Faculty of Medicine, Lund University, Sweden, this thesis will be defended on March 31st 2016 at 09:15 in Segerfalksalen, Biomedical Center, Sölvegatan 17, Lund, Sweden.

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Abstract

Sepsis and invasive bacterial infection is a major cause of human disease and death worldwide. *Streptococcus pyogenes* is one of the major pathogens responsible for bacterial infections, which range from mild to severe and life threatening conditions. *S. pyogenes* contains a cell wall anchored M protein, which is an important virulence factor that can interact with many cells of the immune system. One of the smallest and most rapidly responding cells in our bloodstream are platelets and they are a main focus for the studies presented in this thesis and have been investigated. The overall aim of this thesis was to delineate and understand the role of platelets, neutrophils and endothelial cells in response to bacterial infection and sepsis pathogenesis in both *in vitro* and *in vivo* model systems.

Platelets can bind to neutrophils, resulting in the formation of heterotypic complexes, however the function of neutrophils within these complexes has not been described. In *paper I,* we investigated platelet-neutrophil complexes (PNCs) generated in response to thrombin, a key factor involved in coagulation and compared these to complexes formed in response to *S. pyogenes* M1 protein. We determined that platelet dependent neutrophil activation occurs in response to thrombin, while *S. pyogenes* M1 protein compromised neutrophil functions by a platelet-dependent mechanism. In addition, this was dependent on donor specific IgG against M1 protein. This paper highlights the modulation of neutrophil function by platelets during inflammation and infection.
In *paper II,* platelet aggregation and platelet-leukocyte complex formation was investigated in whole blood in response to *S. pyogenes* bacteria. Platelet aggregation occurred and heterotypic complexes were formed between activated platelets and neutrophils and monocytes. Platelet dependent activation of these leukocytes was observed and bacteria were associated with the platelet and leukocyte complexes. The platelet aggregates remained stable over time, were viable and did not disaggregate. Taken together this study provides new insights into the role of platelets and heterotypic complex formation for bacterial survival in blood.
In *papers III and IV,* the contribution of platelets and the kinetics of platelet activation during invasive *S. pyogenes* infection were investigated *in vivo* in a mouse model. We demonstrated that during a bacterial infection thrombocytopenia, neutrophil activation and platelet-neutrophil complex formation occurs. The acute pro-inflammatory response to the infection and diminished bacterial dissemination in platelet-depleted animals indicated that platelets contribute to the immune response. In *paper IV* we determined that monitoring platelet activation, particularly PNC formation might provide prognostic information during the progression of sepsis. Platelet activation occurred and aggregated platelets accumulated in the liver at the late stages of sepsis.
In *paper V,* we investigated the effect of the *S. pyogenes* M1 protein on endothelial cells *in vitro* using two cell lines. We have demonstrated that M1 protein binds to endothelial cells, increases endothelial cell vascular permeably in a TLR-2 and Rho kinase dependent manner and generates limited cytokine release. This may reflect an innate immune recognition of the bacterial protein, however increased vascular permeability and vascular leakage has also been reported to contribute to the pathogenesis of *streptococcal* infection.

Collectively, this thesis has revealed multiple strategies for *S. pyogenes* to modulate the host immune response during invasive bacterial infection by interacting directly and indirectly with platelets, neutrophils, monocytes and endothelial cells.

Key words: Platelets, neutrophils, *streptococcus pyogenes*, M1 protein, endothelial cells

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Platelets and their immunomodulatory roles during *Streptococcus pyogenes* infection

Sinéad Hurley

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Sweden

The cover represents a drawing of a Platelet-Neutrophil complex containing S.pyogenes and its surface
associated M-protein and an image of platelets (red) in complex with neutrophils (blue), embedded in a
fibrinogen network (green) generated in response to M1 protein.

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For my family, myself and
precious ones =)

Sláinte

PREFACE

“ A book is not a book unless it is an experience”

This PhD for the last number of years has been a complex mix of challenge and commitment but it has also been rewarding and fulfilling. As scientists we always question “why”? We constantly search to explore, to invent, to change, to create and to inspire. It is our willingness to seek and strive, to rediscover the known and discover the unknown that earns us the title of “scientist”. My journey throughout these last years has certainly been a journey of discovery.

My interest in the human body and its ability to protect and defend us from all harm absolutely fascinates me and this interest has developed through working on the studies that are presented within this thesis today. When I initially embarked on this PhD one could say that I was assailed by the “flight or fight syndrome”, however with the endless support, enthusiasm, knowledge and friendship from those around me it has been a very fulfilling and rewarding Journey.

I have invested much time in conducting this research, realising that it was not a sprint but a marathon. Still, time spent doing what you desire is time well spent.

The starting point of all achievement is desire. Desire was the key to all my motivation but it was my unrelenting determination and commitment to the pursuit of my goals - a commitment to excellence - that enabled me to attain the success, which I sought and present herein. It has been a true learning experience.

Dear friends & scientists, here’s to you, I hope you enjoy reading it.

Beatha agus sláinte daoibh go léir

Sinéad M Hurley.

Sinéad M Hurley

Lund, 23th of February 2016

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ABSTRACT

Sinéad M. Hurley

Sepsis and invasive bacterial infection is a major cause of human disease and death worldwide. *Streptococcus pyogenes* is one of the major pathogens responsible for bacterial infections, which range from mild to severe and life threatening conditions. *S. pyogenes* contains a cell wall anchored M protein, which is an important virulence factor that can interact with many cells of the immune system. One of the smallest and most rapidly responding cells in our bloodstream are platelets and they are a main focus for the studies presented in this thesis and have been investigated.

The overall aim of this thesis was to delineate and understand the role of platelets, neutrophils and endothelial cells in response to bacterial infection and sepsis pathogenesis in both *in vitro* and *in vivo* model systems.

Platelets can bind to neutrophils, resulting in the formation of heterotypic complexes, however the function of neutrophils within these complexes has not been described. In *paper I,* we investigated platelet-neutrophil complexes (PNCs) generated in response to thrombin, a key factor involved in coagulation and compared these to complexes formed in response to *S. pyogenes* M1 protein. We determined that platelet dependent neutrophil activation occurs in response to thrombin, while *S. pyogenes* M1 protein compromised neutrophil functions by a platelet-dependent mechanism. In addition, this was dependent on donor specific IgG against M1 protein. This paper highlights the modulation of neutrophil function by platelets during inflammation and infection.

In *paper II,* platelet aggregation and platelet-leukocyte complex formation was investigated in whole blood in response to *S. pyogenes* bacteria. Platelet aggregation occurred and heterotypic complexes were formed between activated platelets and neutrophils and monocytes. Platelet dependent activation of these leukocytes was observed and bacteria were associated with the platelet and leukocyte complexes. The platelet aggregates remained stable over time, were viable and did not disaggregate. Taken together this study provides new insights into the role of platelets and heterotypic complex formation for bacterial survival in blood.

In *papers III and IV,* the contribution of platelets and the kinetics of platelet activation during invasive *S. pyogenes* infection were investigated *in vivo* in a mouse model. We demonstrated that during a bacterial infection thrombocytopenia, neutrophil activation and platelet-neutrophil complex formation occurs.

The acute pro-inflammatory response to the infection and diminished bacterial dissemination in platelet-depleted animals indicated that platelets contribute to the immune response. In *paper IV* we determined that monitoring platelet activation, particularly PNC formation might provide prognostic information during the progression of sepsis. Platelet activation occurred and aggregated platelets accumulated in the liver at the late stages of sepsis.

In *paper V,* we investigated the effect of the *S. pyogenes* M1 protein on endothelial cells *in vitro* using two cell lines. We have demonstrated that M1 protein binds to endothelial cells, increases endothelial cell vascular permeably in a TLR-2 and Rho kinase dependent manner and generates limited cytokine release. This may reflect an innate immune recognition of the bacterial protein, however increased vascular permeability and vascular leakage has also been reported to contribute to the pathogenesis of *streptococcal* infection.

Collectively, this thesis has revealed multiple strategies for *S. pyogenes* to modulate the host immune response during invasive bacterial infection by interacting directly and indirectly with platelets, neutrophils, monocytes and endothelial cells.

ORIGINAL PAPERS

Paper I

Platelet-dependent neutrophil function is dysregulated by M protein from *Streptococcus pyogenes*.
Sinéad M Hurley, Fredrik Kahn, Pontus Nordenfelt, Matthias Mörgelin, Ole E Sørensen, Oonagh Shannon.

Infection and Immunity. 2015 Sep; 83 (9): 3515-25.

Paper II

Streptococcus pyogenes induces platelet activation in human blood and modulates leukocyte function in a platelet dependent manner.
Lisbeth Svensson#, **Sinéad M Hurley#,** Oonagh Shannon.

Manuscript (# equal contribution).

Paper III

Platelets promote bacterial dissemination in a mouse model of streptococcal sepsis.
Fredrik Kahn, **Sinéad M Hurley**, Oonagh Shannon.

Microbes and Infection. 2013; 15 (10-11): 669-76.

Paper IV

The dynamics of platelet activation during the progression of streptococcal sepsis.
Sinéad M Hurley, Nataliya Lubuy, Bo Holmqvist, Oonagh Shannon.

Manuscript (submitted)

Paper V

M1-protein from *Streptococcus pyogenes* increases endothelial vascular permeability and mediates cytokine release.
Sinéad M Hurley, Jana Fisher, Pontus Nordenfelt, Oonagh Shannon.

Manuscript

ADDITIONAL ARTICLES
not included in this thesis

Active but inoperable thrombin is accumulated in a plasma protein layer surrounding *Streptococcus pyogenes*.
Clement Naudin, **Sinéad M Hurley**, Erik Malmström, T Plug, Oonagh Shannon, J C M Meijers, Matthias Mörgelin, Lars Björck, Heiko Herwald.

Thromb Haemost. 2015 May 21; 114 (3)

ABBREVIATIONS

PNC, Platelet-neutrophil complex

S. pyogenes, Streptococcus pyogenes

PRR, Pattern recognition receptor

PAMP, Pathogen associated molecular pattern

DAMP, Damage associated molecular pattern

TLR, Toll-like receptor

fMLF, Formyl-methionyl-leucyl-phenylalanine

Fab, Fragment antigen binding

Fc, Fragment crystallizible

G-CSF, Granulocyte colony stimulating factor

Spe B, *Streptococcal* pyrogenic exotoxin B

SLO, Streptolysin O

SIC, *Streptococcal* inhibitor of complement

ECM, Extracellular matrix

MHC, Major histocompatibility complex

SIRS, Systemic inflammatory response syndrome

DIC, Disseminated intravascular coagulation

TNF-α, Tumor necrosis factor alpha

IL-Interleukin

HBP, Heparin binding protein

ICAM-1, Intercellular adhesion molecule-1

TPO, Thrombopoietin

TGFβ, Transforming growth factor β

PF4, Platelet factor 4

Groα, Growth-regulating oncogene-alpha

RANTES, Regulated on activation, normal T cell expressed and secreted

OCS, Open canalicular system

CAM, Cell adhesion molecule

ITAM, Immunoreceptor tyrosine-based activation motifs

ADP, Adenosine diphosphate

ATP, Adenosine triphosphate

vWF, von Willebrand Factor

PAR, Protease activated receptor

GP-, Glycoprotein

PSGL-1, P-selectin glycoprotein ligand-1

TF, Tissue factor

Mac-1, Macrophage-1 antigen

Ig, Immunoglobulin

SAg, Superantigen

LPS, Lipopolysaccharide

ClfA, Clumping factor A

HIV, Human immunodeficiency virus

ICAM-2, Intracellular adhesion molecule-2

JAM-3, Junctional adhesion molecule 3

CD40L, CD40 ligand

NETs, Neutrophil extracellular traps

MPO, Myeloperoxidase

NGAL, Neutrophil gelatinase associated lipocalin

GTP, Guanosine triphosphate

LAD, Leukocyte adhesion deficiency

PAF, Platelet activating factor

FPR, Formyl peptide receptor

CR3, Complement receptor 3

ROS, Reactive oxygen species

CGD, Chronic granulomatous disease

HSC, Hematopoietic stem cell

WPB, Weibel-Palade body.

MCP-1, Monocyte chemoattractant protein-1

LFA-1, Leukocyte-function associated antigen-1

PECAM-1, Platelet endothelial cell adhesion molecule-1

VE-cadherin, Vascular endothelial-cadherin

VCAM-1, Vascular cell adhesion molecule-1

IAP- Integrin associated protein

VLA-4, Very late antigen 4

IAP, Integrin associated protein

APC, Activated protein C

TFPI, Tissue factor pathway inhibitor

AT, Antithrombin

PAI-1, Plasminogen activator inhibitor-1

"Life can be much broader once you discover one simple fact, and that is;

- *Everything around you that you call life, was made up by people that were no smarter than you. And you can change it, you can influence it, you can build your own things that other people can use." Steve Jobs*

-

1. THE IMMUNE SYSTEM- OUR PROTECTOR AND DEFENDER

Host-Pathogen Interaction

The host is constantly exposed to bacteria on a daily basis and our bodies are continually being colonised by bacteria, including non-pathogenic commensals within the human micro-biome, such as those found on the skin and mucosal surfaces in particular the gastrointestinal tract. All individuals are unique in their micro-biome content (1). The human body possesses a very potent immune system to defend and protect from bacterial infection. However, despite this very efficient immune system, which is amply attempting to eradiate invading bacteria, pathogens have developed ways to overcome our defence systems and invade and damage the bodies' tissues. This thesis addresses aspects of host-pathogen interaction within the bloodstream.

The cells of our bloodstream- Transporters, Defenders, Preventers!

Erythrocytes or red blood cells (RBCs) are the most abundant cells in the blood, essential transporters that carry oxygen around the body and carbon dioxide away to be eliminated out from the body. The leukocytes in the blood consist of five main types; neutrophils, eosinophils, basophils, monocytes and lymphocytes which together account for between 4-11 x 10^9 cells/L of blood (2). They are defenders and protect us from harmful threats, including bacteria. Neutrophils, monocytes and macrophages are some of the key professional phagocytic cells that play central roles in preventing and resolving a bacterial invasion or inflammation. These cells originate from the myeloid progenitor cell in the bone marrow.

Platelets, the smallest of all the blood cells range from 150-400 x 10^9/L in healthy individuals (3). These cells have a small discoid shape. Together with the coagulation system platelets form blood clots and prevent blood loss during vascular damage allowing maintenance of vasculature integrity. These cells circulate in an inactive state in the blood but rapidly become activated in response to vessel damage and this activation is attributed to their very specialised structure.

The Innate & Adaptive immune response

The human body protects us from infection by two interlinked defence systems, the innate and the acquired immune system. The innate immune system is the first defence mechanism against an invading pathogen or in response to tissue injury or damage. This response is rapid and consists of physical barriers such as the skin and mucosal membranes and chemical barriers such as host derived chemicals and our innate immune cells. The innate immune system is comprised of the following; phagocytes such as neutrophils and macrophages, dendritic cells, the coagulation system, the complement system and other cells that participate in the innate immune response such as epithelial and endothelial cells.

The complement system forms part of the immune response and consists of three main pathways, the classical, the alternative and the lectin pathway. The final step results in the generation of the membrane attack complex (MAC) that may mediate cell lysis. The central step for all pathways is the generation of C3 convertase (4), which cleaves C3 into C3a and C3b and can attach to the surfaces of microbes, coating and opsonising the microbe.

Host pattern recognition receptors (PRRs) form the backbone of the innate immune response by distinguishing self from non-self by recognition of pathogenic associated features and patterns called pathogen associated molecular patterns (PAMPs) (5) (6). The innate immune system also recognises molecules released as a result of tissue damage, known as damage associated molecular patterns (DAMPs) (7,8). Different types of PRRs exist including, the Toll-like receptors (TLRs), Nod-like receptors, C-type lectin receptors and retinoic acid-induced gene (RIG)-1-like receptors (9). There is also the f-Met-Leu-Phe receptor (fMLP) (10). This receptor recognises bacterial peptides that are released when combating an infection.

Inflammation

Inflammation is a protective innate host response that is composed of four key components which are written in Latin as they were first described by the roman scholar Celsus; calor (heat), rubor (redness), dolor (pain) and tumor (swelling, oedema) (11). When the host receives signals, generated externally from pathogens or internally from tissue damage, an inflammatory response is initiated. An inappropriate host response that results in too little or too much inflammation will lead to progressive tissue damage.

The calor is derived from vasodilation, where a rise in blood volume results in heat and then redness. Initial responses involve changes in the vasculature, whereby blood flow is increased so that cells and plasma proteins can be transported to the local area and so the healing process can begin (Figure 1). The blood vessel walls become more permeable so that solutes can pass leading to fluid leakage (edema). Solutes can exudate to the tissues and within a few hours the process of extravasation takes place whereby granulocytes, particularly neutrophils can enter the tissue and facilitate the repair process. Leukocyte transmigration is a key event in the accumulation of effector cells at the site of damage (12).

The coagulation system plays a role in prevention of blood loss and consists of primary and secondary hemostasis (13). In primary hemostasis the goal is to prevent blood loss as a result of vascular damage. The coagulation system does this through platelet activation and platelet plug formation. In secondary hemostasis, clotting factors are initiated which results in fibrin deposition and further contribute to the cessation of blood loss (14). The coagulation system consists of a cascade of serine proteases, which become activated during the cascade process. The coagulation system is also involved in inflammation and results in fibrin deposition (15). Fibrinogen and fibronectin are deposited and dying cells contribute to pus formation during inflammation. Finally the process of resolution occurs and normal tissue architecture is resolved (16). In response to tissue damage, due to prolonged inflammation a more severe chronic cellular response occurs whereby macrophages and lymphocytes infiltrate the damaged area.

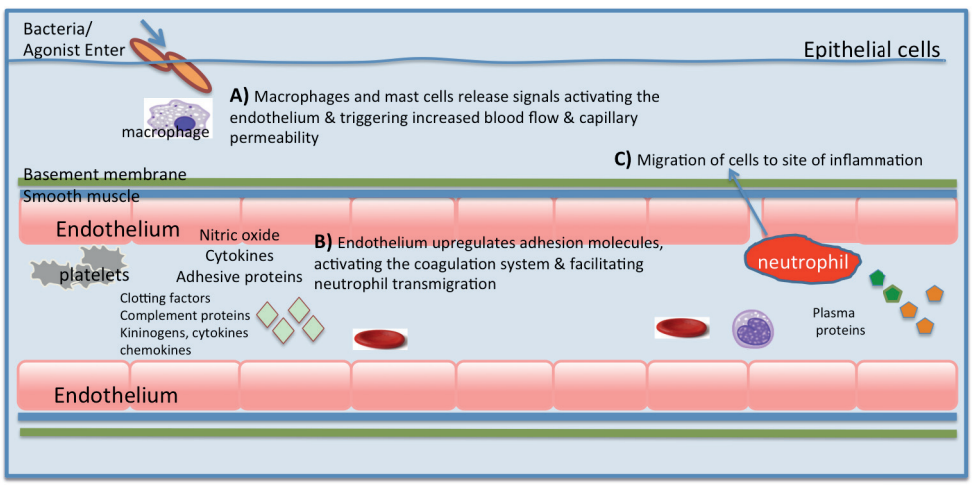

Figure 1: Stages of inflammation.

The innate immune system responds immediately to eliminate invading pathogens. In contrast, a second branch of our immune system, the adaptive immune system responds more slowly on first encountering a pathogen. However, this response is more specific and consists of an immunological memory, which means a more finely tuned and heightened pathogen specific response can be mobilised when the same pathogen is encountered again (17). The primary cells involved are the lymphocytes in communication with antigen-presenting cells. B- and T-lymphocytes respond to microbial invaders by production of antibodies or generation of cell-mediated responses, respectively (18).

Antibodies produced by activated B cells bind microbes and facilitate their elimination via phagocytosis or complement activation. Antibodies exist in five different classes of which the IgG class is the most abundant throughout the body. The IgG antibody is composed of two regions, the Fab fragment (Fragment antigen binding), which recognises and binds to antigens and the Fc region (Fragment crystallizible) whereby the molecule binds receptors (Fc receptors) and interacts with cells of the immune system.

The Fc receptors expressed by phagocytes are responsible for binding to the IgG class of immunoglobulins are known as the Fcγ receptors (19). Fc receptors bind to antibodies at their Fc region, which may be coating the surface of microbes, and this process induces phagocytosis. An important mechanism of phagocytosis is FcγR- mediated phagocytosis. Human cells express six Fcγ receptors (Fcγ-RI, -RIIA, -RIIB, -RIIC, -RIIIA and FcγRIIIB). Neutrophils constitutively express the Fc receptors, FcγRIIa (CD32), FcγRIIIb (CD16) and FcγRIV (20,21) and they express FcγRI (CD64) upon stimulation with granulocyte colony stimulating factor (G-CSF) (22).

The ability of the host to distinguish self from nonself is an important phenomenon of our immune system. Generally, host PRRs do this flawlessly because they recognise a molecular pattern that is only produced by the pathogen and not by the host. In contrast, receptors of the adaptive immune system occasionally recognise self-antigens, which may lead to autoimmune disease. The immune system must respond adequately to protect us but it must not over-respond and result in over activation and inflammation, which would be deleterious to the host. *S.pyogenes* can cause mild infections but can also lead to severe infectious diseases such as sepsis. In sepsis our immune system responds in an uncontrolled manner and it is this over-activation of the host response itself that leads to the severity of the disease.

In the following sections I will discuss these host systems and host-pathogen interactions in greater detail.

2. *STREPTOCOCCUS PYOGENES*

Streptococcus comes from the Greek words (strɛptəʊˈkɒkə); streptos meaning "chain" and kokkos meaning "grain/berry". The name is derived from the fact that streptococci are linked together in cocci chains like a pearled necklace. The streptococcal bacterium was isolated in 1879 by Louis Pasteur, from blood cultures from a woman with puerperal sepsis. It wasn't until 1884 that Friedrich Rosenbach described the bacteria, which he isolated from wounds and skin infections, and coined the actual term *Streptococcus pyogenes* for the first time (23).

Approximately 10% of the adult population are asymptomatic carriers of this pathogen (24,25). *S. pyogenes* infections range from mild superficial skin infections such as pharyngitis and impetigo to life-threatening diseases such as necrotizing fasciitis, sepsis and streptococcal toxic shock syndrome. There are approximately 1.78 million new severe GAS infections worldwide each year for example due to rheumatic fever, rheumatic heart disease and invasive disease (26).

S. pyogenes is a Gram-positive facultative anaerobic bacterium which was classified as Group A streptococci by Rebecca Lancefield based on the carbohydrate composition of the antigens found on the bacterial cell wall (27). Another mechanism of classification was introduced in 1928 based on variations of M-protein on the surface of *S. pyogenes* (28). This forms the basis of serotyping of the bacteria, however nowadays this is based on sequencing of the emm gene that encodes for the M protein.

Group A *streptococcus* (GAS) stimulates the innate immune system, causing local inflammation and damage, resulting in the initiation of an immune response. *S. pyogenes* has developed many mechanisms that enable it to penetrate the constitutive host defence systems and in some cases cause invasive disease. The *S. pyogenes* genome has been sequenced and multiple genes encode for virulence factors (29). *S. pyogenes* produces a wide range of virulence factors which allow the pathogen to attach to host tissue, degrade tissue proteins, multiply and spread in the host (Figure 2). The bacteria can be surrounded by a carbohydrate capsule composed of hyaluronic acid, a peptidoglycan cell wall and proteins are embedded in the cell wall such as protein H, F and M protein that contribute to bacterial attachment and invasion of host cells (30,31). The capsule has been shown to prevent phagocytosis (32) and may alter the response of antibodies to bacterial products or the M protein. The bacteria can secrete proteases for example streptococcal pyrogenic exotoxin B (Spe B), streptokinase and streptolysins that facilitate tissue invasion (33) (34). Streptolysin O (SLO) is a pore forming toxin that can destroy red blood cells, leukocytes and other cell types.

Secreted IdeS cleaves IgG at the hinge reason thereby inactivating Fc effector functions (35) (36,37). *Streptococcal* inhibitor of complement (SIC) described in 1996 was initially reported to inhibit formation of the membrane attack complex during complement activation (38), however it has more recently been reported to bind to and neutralise antimicrobial peptides.

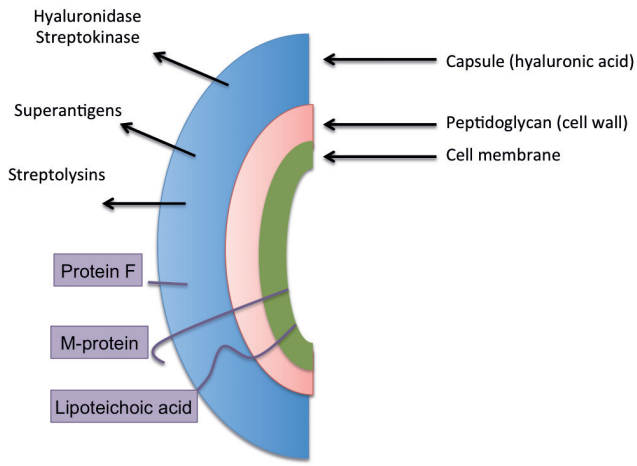

Figure 2: *Streptococcus pyogenes* stucture & virulance factors.

Plasminogen

Following activation, plasminogen is converted to plasmin, a protease that can breakdown fibrin clots, adhesion molecules, the extracellular matrix (ECM) and connective tissue (39,40). *S pyogenes* secretes streptokinase which binds and activates plasminogen (41). In addition to degrading fibrin, plasmin can also bind fibrinogen (42) and this increases the streptokinase induced plasminogen activation (43). This is reported to play a significant role in the pathogenesis of invasive *S. pyogenes* infection (44) and in enhancing the dissemination of the bacterium from the local infection.

Superantigens

There are five classes of superantigens (I-V). They are toxic substances that bind to major histocompatibility complex class II and rapidly trigger an uncontrolled proinflammatory response (45,46) and toxic shock syndrome (47). These antigens activate T cells non-specifically i.e. they do not undergo the normal mechanism of antigen processing and presentation but directly bind to the T cell receptor. These bacterial toxins can stimulate an inflammatory and cytokine storm and very low concentrations of bacterial SAgs (< 0.1 pg/ml) are sufficient to induce toxic levels of cytokines in the bloodstream and an uncontrolled adaptive immune response (48). *S. pyogenes* can release a number of different superantigens. In addition, the M protein that is released from the *S. pyogenes* surface exhibits superantigenic properties (49).

M Protein

M-protein is one of the main virulence factors that cover the surface of *S. pyogenes*. M-protein is normally anchored on the bacterial surface but can be released by the action of cysteine proteases secreted from the bacteria (50), neutrophil proteases (51) and small quantities of M protein can be shed from the bacteria during growth *in-vitro* (52). M protein has a α-helical fibrillar coiled-coil structure and each homodimer chain of M-protein is composed of four repeating domains (Figure 3 A-D), each with various host interacting partners and functions during infection. A structural model of M protein was first proposed by Fischetti in 1989 and 1991 (53) (54). The M protein was later confirmed to have irregularities within the coiled-coil structure that facilitated interaction with host proteins, including fibrinogen (55). M protein can facilitate attachment to host cells and tissues for example it can bind to extracellular matrix and cell surface glycosaminoglycans (56), it can bind directly to extracellular matrix components such as fibronectin (57) and indirectly these molecules bind to host cells and thereby act as bridges, promoting uptake of bacteria into these cells (58,59).

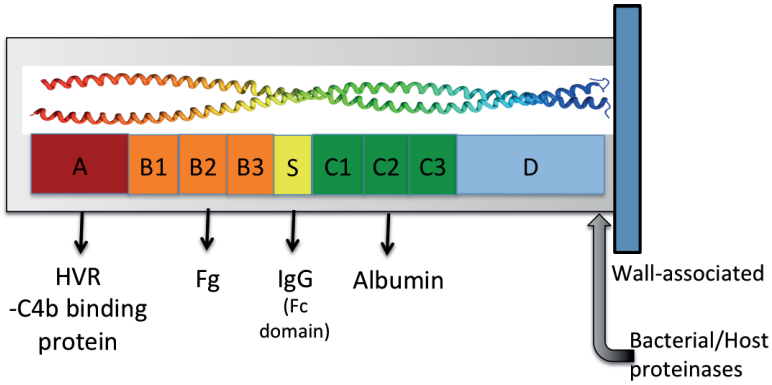

Figure 3: Each chain of the M protein includes different regions (A-D) that have different sizes and amino acid sequences. A - amino-terminal region or hypervariable region (HVR), B- Binds Fibrinogen, S- IgG binding region, C & D- Both the C and D region can bind factor H and serum albumin, D- Contains an LPxTG motif that attaches to the peptidoglycan which is required for correct anchoring of the protein in the cell wall.

The antigenic diversity at the N-terminal hypervariable region (HVR) is the basis of M-protein serotyping. Type specific antibodies are directed against this HVR and have been proposed to confer protection against subsequent infection with the same serotype (60). There are >100 serotypes of the M protein known (60) (61) but only a few are particularly associated with invasive disease where M1 and M3 serotypes in particular have dominated (62).

M protein is strongly anti-phagocytic because it can bind plasma proteins and the Fc region of IgG antibodies thereby preventing activation of complement. Complement regulatory components can bind to the HVR of M protein and limit complement activation on the bacterial surface, for example C4b-binding protein which degrades C3 convertase and decreases opsonisation (63-65). M protein of certain serotypes may bind factor H and prevent C3b binding to the bacteria. Another mechanism that prevents complement activation is the binding of the plasma protein, fibrinogen to M protein. Fibrinogen acquisition by *S. pyogenes* was first described by Kantor, however the importance of this interaction for bacterial pathogenesis was not clarified in this study (66). In 1985, *Whitnack et al* demonstrated that when fibrinogen binds to M protein the binding of C3b is inhibited and this prevents activation of the alternative complement pathway and phagocytosis (67). Fibrinogen binding has also been shown to prevent complement activation by blocking the classical complement pathway (68).

The M protein - fibrinogen complex has been show to protect against phagocytosis of group A streptococci (69). In addition the recruitment of the Fc region of IgG to the bacterial surface is also a mechanism to prevent complement activation and bacterial killing (70). Furthermore, M protein contributes to intracellular survival of *S. pyogenes* that has been phagocytosed by neutrophils (71).

The released M1 protein has potent pro-inflammatory properties that are associated with the formation of a complex between M1 protein and plasma fibrinogen. This complex mediates neutrophil activation and release of vasoactive substances (such as heparin binding protein) and has been shown to induce vascular leakage in animal models of disease (51,72,73). Furthermore, M1 protein stimulates neutrophil and mast extracellular formation and the bacteria can survive inside these extracellular traps (74). M1 protein activates platelets (75), monocytes (76) and epithelial cells (77) and initiate's an inflammatory response. In addition, the M protein can also interact with the coagulation system where it has been shown to cause tissue factor production from monocytes (78) and contact system activation at its surface with the release of bradykinin, a potent mediator of inflammation (79).

Severe bacterial infection: Sepsis

When *S. pyogenes* disseminates into the bloodstream, life-threatening disease and sepsis may result. Sepsis is the result of a systemic inflammatory immune response to a bacterium, characterised by a number of clinical parameters. The definition of sepsis that is in use today arose from the sepsis consensus conference held in the USA in 1991 led by Prof. R.C. Bone and colleagues (80). This was later refined at another sepsis consensus conference in 2001 to enhance the definition to contain additional added criteria (81). Sepsis is a complex and life-threatening condition that has a high mortality rate in those severely affected individuals and despite continuous research and continually emerging antibiotics, sepsis still remains a very complicated condition to detect and treat.

Each year in the US there are approximately 750,000 new cases of severe sepsis (82) and in 2001, sepsis resulted in approximately 215,000 deaths (83). The initial clinical symptoms for diagnosing sepsis include the systemic inflammatory response syndrome (SIRS) criteria; fever, high temperature, increased respiratory rate, elevated heart rate and an altered white blood cell count (Figure 4) (80).

There are three clinical stages of sepsis; sepsis, severe sepsis and septic shock and each have a different definition depending on the severity of the clinical symptoms (Figure 4) (84). One important cardiovascular change in these individuals is hypotension, low blood pressure and if this occurs rapidly, in addition to other symptoms and the patient are irresponsive to fluids, septic shock can be diagnosed which is the phase with the highest mortality rate (70-80%) (85,86).

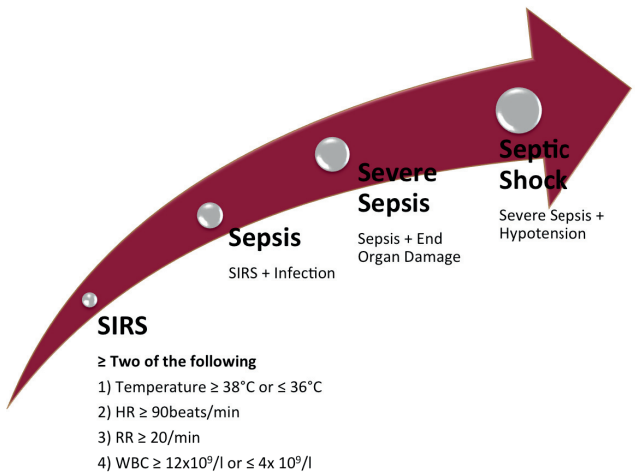

Figure 4: The classification of Sepsis. The initial clinical presentation is the systemic inflammatory response syndrome (SIRS) and then a progession to sepsis, severe sepsis and septic shock.

Sepsis involves a deregulation of both pro- and anti-inflammatory pathways. The inflammatory response generated during sepsis is very often followed by a period of immune suppression or immunoparalysis which can persist for days and involves bacterial endotoxins and superantigens (SAgs) (87,88).

During sepsis there may be a high or low neutrophil count (Figure 4) or the presence of greater than 10% immature blast cells and the neutrophils function is generally altered (89) and impaired for example an impaired neutrophil chemotactic ability was seen in patients with severe sepsis (90). The gene expression of the neutrophil may be increased during sepsis (91) and becomes altered as the condition progresses.

This leads to a gene suppression of proinflammatory molecules and a decreased production of neutrophil reactive oxygen species (ROS). The result may be an abnormal recruitment of neutrophils to sites of inflammation and a subsequent accumulation of these cells, which have deregulated responses.

Some different theories have been proposed about the immune response during sepsis for example according to *Hodchkiss et al.* one theory proposed that both pro- and anti-inflammatory responses occur during sepsis where patient deaths are due to more of an immunosuppression and a second theory proposed that deaths from sepsis are more due to a stronger innate immune response than the adaptive response (87). Sepsis is a very complicated phenomenon and a lot more work is still to be done to better understand the role of the immune system.

Coagulation and Sepsis

Sepsis and septic shock involve a massive dysregulation of the pro- and anti-inflammatory systems, but also the coagulation system. Significant crosstalk occurs between coagulation and inflammation. During sepsis the coagulation system is fully activated and thrombin is generated, which will result in the generation of a fibrin clot, activation of the endothelium and platelet activation. This will further escalate the coagulation system and in advanced sepsis disseminated intravascular coagulation (DIC) may occur. Furthermore, bacterial toxins can directly activate the coagulation system by upregulation of tissue factor (92) and indirectly activate the system through the activation of inflammatory molecules such as tumor necrosis factor alpha (TNF-α) and interleukins (93). The end result is a state of pro-coagulation and generation of fibrin clots. During DIC, systemic activation of the coagulation system occurs with fibrin deposition in small vessels, consumption of coagulation factors and regulators of coagulation. This can culminate in an increased bleeding risk in these patients. Platelets are affected during DIC. Thrombocytopenia occurs during invasive bacterial infection and is an independent marker of mortality (94,95). The activation and aggregation of platelets could be responsible for platelet consumption and the decreased platelet numbers observed in these individuals.

Sepsis Management

Sepsis diagnosis, management and treatment continues to remain a major health challenge due to the disease complexity, therefore identifying a reliable biomarker of sepsis is extremely difficult. Some of the current septic biomarkers include procalcitonin, cytokine biomarkers, such as IL-8 or IL6, mRNA expression in blood and in leukocytes, micro RNAs (miRNA) (96). Some cell receptors or adhesion molecules can also serve as biomarkers including soluble ICAM-1 or E-selectin. Heparin binding protein (HBP), also known as azurocidin is released from activated neutrophils during sepsis and is an inducer of vascular leakage (97). Plasma HBP was shown to be a prognostic marker of the severity of septic progression (98) and HBP was shown to be a possible marker of circulatory failure in septic patients (99).

Management and treatment for sepsis includes fluids, vasopressors, ventilation and antibiotic administration. Some studies are aimed at blocking inflammatory molecules for example TNF-α (100), however, targeting one specific molecule in sepsis usually is inefficient and many have failed in clinical trails (101). Due to the complex nature of sepsis finding a single and effective treatment is rare and a combination of treatments offer more potential.

3. THE POWERS OF PLATELETS

Platelets are small anucleate cells, 3µm in diameter and the human body has between 150-400 x 10^9 platelets per litre of blood, of which one hundred billion are produced daily from the bone marrow (102). Megakaryocytes are precursor cells produced from pluripotent stem cells in the bone marrow and James Homer Wright was the first to note that platelets are produced from megakaryocyte fragmentation in the bone marrow (103).

Within the bone marrow, maturation of the megakaryocyte takes place, which increase in size and content. The production of megakaryocytes in the bone marrow is mainly regulated by the platelet growth factor thrombopoietin (TPO) (104-107) which facilitates the in vitro culture and expansion of megakaryocytes and therefore advanced our understanding with regard to the platelet developmental process (108). Following maturation, the mature megakaryocytes then degrade their basement membrane and the cell cytoplasm is reorganised into beaded extensions called proplatelets (109-112). An intermediate before this step occurs in the sinusoidal blood vessel of the bone marrow and are now defined as preplatelets, which are larger than platelets and can convert into proplatelets (113). As proplatelets continue to develop and mature, platelet specific granules are taken along microtubule bundles to the proplatelets before finally releasing platelets into the blood vessel from their surface (114). Each megakaryocyte produces between 1000-3000 platelets and on average the platelet has a lifespan between 8-10 days in the circulation (115). Mouse platelets circulate in greater numbers than that of human platelets and have shorter lifespans (116,117).

Platelet Cytoskeleton

The small and discoid shape of the platelet allows them to be close to the vessel edge and therefore to quickly respond to vascular damage. Their unique shape is maintained by their cytoskeletal structure. Platelets have an open canalicular system (OCS) which serves as a conduct for granule secreted substances, to facilitate their transport to the platelet surface and to allow substances to be transported into the cell (118,119). Platelets contain small numbers of mitochondria which provide energy to the platelet for processes such as platelet activation and granule secretion (120). Glycolysis and oxidative phosphorylation are the main energy demanding process that take place during platelet activation and secretion (121).

Platelet Granules

Platelets have three main granules, α-granules, dense granules and lysosomes, of which the α-granules are the most abundant (122) (123). Platelet α-granules contain more than 300 proteins per platelet including adhesive proteins, coagulation clotting factors such as fibrinogen, fibrinolytic factors, cellular mitogens, proteases, growth factors and proteins (124). They contain cytokines and chemokines, for example transforming growth factor β (TGFβ) (125), platelet factor 4 (PF4), Growth-regulating oncogene-alpha (Groα) and regulated on activation, normal T cell expressed and secreted (RANTES). P-selectin is an important membrane protein stored in the α-granules of resting unactivated platelets. This cell adhesion molecule (CAM) becomes translocated from the platelet granules and upregulated to the platelet surface following platelet activation (126). Endothelial cells have also been shown (in 1989) to contain p-selectin stored in the Weibel-Palade bodies (WPBs) (127). In addition platelets release antimicrobial peptides from their α-granules for example after activation with thrombin, platelets release peptides called thrombocidins (128). Actin in the cytoskeleton plays a key role in the release of platelet α-granules by the action of actin polymerisation (129) (130). Microparticles are small vesicle structures that are shed from the plasma membrane of cells in the bloodstream. The release of microparticles involves actin and cytoskeleton detachment from the plasma membrane and primarily requires calcium (131).

In contrast, platelet dense granules are smaller and carry other activating factors such as adenosine triphosphate (ATP), adenosine diphosphate (ADP), serotonin and cations such as calcium and magnesium. Platelets have a few lysosomes, which store digestive and acid hydrolase enzymes including cathepsin, β-galactosidase and acid phosphatases. Platelets also have cytoplasm peroxisomes that store the enzyme, catalase.

Platelets express thousands of copies of receptors on their surface and these numbers are continuously expanding as research reveals new receptors with increasingly new functions. Among these, some receptors are present in platelet granules and are only expressed on the platelet surface upon activation. Platelets express TLR's, initially shown to express TLR1, TLR2 and TLR3 on both mouse and human platelets but further studies have demonstrated that platelets express TLR's 1-9 and some are functional (132-134).

Platelets express one receptor class for IgG, FcγRIIA (135). When Fcγ binds the Fc region of IgG a number of downstream signalling events occur including phosphorylation of tyrosine residues in the receptor within immunoreceptor tyrosine-based activation motifs (ITAMs) which begins with the Src family of tyrosine kinases (136). These signalling events have been shown to result in the activation of the platelet integrin GPIIbIIIa (137). Table 1 indicates some of the most important platelet receptors and their ligands.

Receptors	**Ligands**	**Family**	**References**
GPIIbIIIa	Fibrinogen, fibrin, vWF,fibronectin	Integrins	(138)
GPVI	Collagen	Ig Superfamily	
$P2Y_1$, $P2Y_{12}$	ADP	G protein-coupled receptors	(139)
PAR1, PAR4	Thrombin	G protein-coupled receptors	(139)
CD62P	PSGL-1, GPIb, TF	C-type lectin receptor family	
GPIb-IX-V complex	Thrombin, vWF, FXI, FXII, P-selectin, Mac-1	Leucine-rich repeat family	
GPIaIIa	Collagen	Integrin	
FcγR's	IgGs	Ig superfamily	
TPα	Thromboxane	G protein-coupled receptors	(139)

Table 1: Platelet receptors and their coresponding ligands.

Platelets & Haemostasis

Platelets patrol the vasculature in an inactive state, however following vessel injury or inflammation platelets are rapidly activated and responsive. Platelets are activated in response to agonists such as collagen, Adenosine diphosphate (ADP) and thrombin, which bind to their respective receptors on the platelet surface (Figure 5). ADP and thrombin bind to G-protein coupled receptors on the platelet and initiate a downstream signalling event, that results in a calcium influx which can result in further platelet granule release and platelet integrin conformational changes.

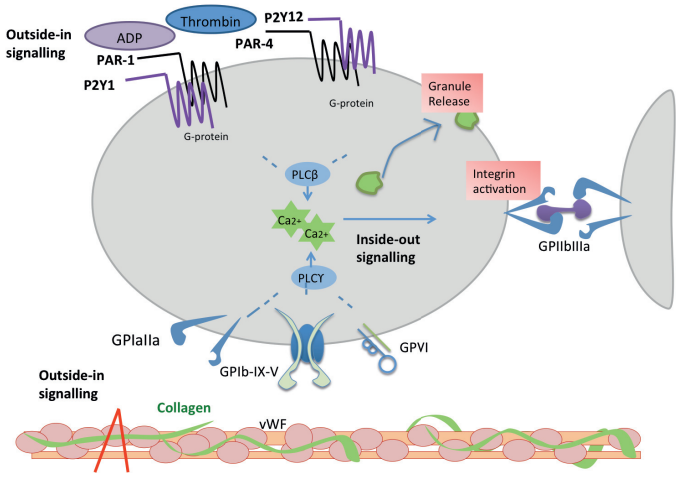

Figure 5: Platelets become activated and release their granules upon stimulation. Upon vascular damage collagen is exposed which can bind to platelet receptors and initiate a process of firm platelet attachment and endothelial transmigration.

During primary haemostasis platelets undergo a series of events that ultimately result in repair or cessation of blood flow (Figure 6). This is one of the primary functions of the platelet. Upon vessel injury, subendothelial extracellular matrix components are exposed such as collagen (140). One of the initial steps in platelet adhesion is the immobilisation of circulating von Willebrand Factor (vWF) on the collagen surface which exposes the vWF binding site and subsequently promotes platelet tethering and attachment (141,142). The A1 domain of collagen bound vWF interacts with GP-Ib-IX-V receptor complex on the platelet surface (143,144). Further interactions of vWF-GPIb are formed and platelet rolling occurs. Other more stable interactions occur that involve integrins including the collagen receptors GPIaIIa and GPVI and the GPIIbIIIa receptor.

The GPIIbIIIa receptor which is activated by inside-out signalling (145-147) binds fibrinogen which bridges adjacent platelets and recruits more circulating platelets to the site of damage resulting in further platelet-platelet interactions and formation of a platelet plug (148). The platelet plug is sufficient to cease blood loss in small vessels, however, if the injury is more extensive in the larger blood vessels, this in insufficient and secondary haemostasis occurs.

Secondary haemostasis is the stage where insoluble fibrin is generated (149), which stabilises the platelet plug. The process requires the regulated control of coagulation factors, cofactors and inhibitors to generate the protease thrombin, which initiates the formation of fibrin.

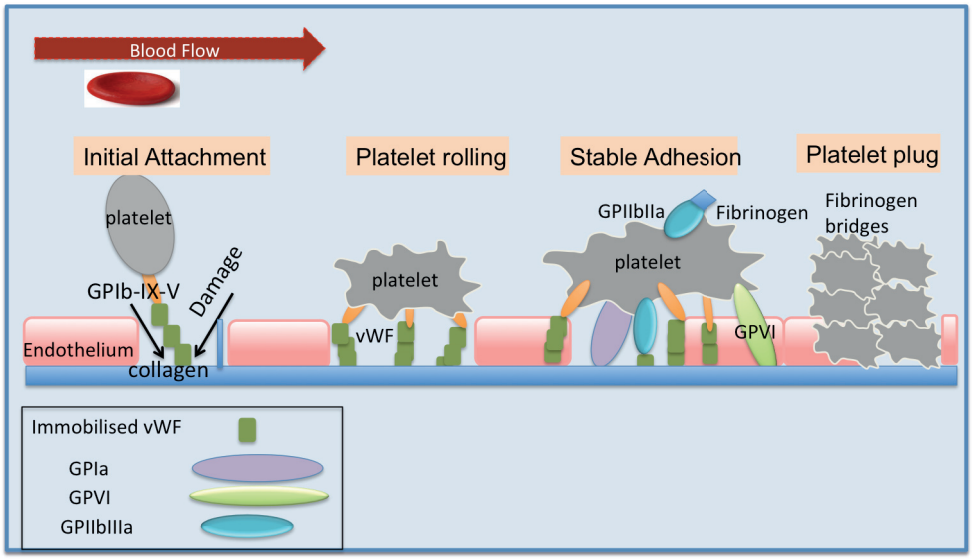

Figure 6: Schematic representation of the events involved in formation of a platelet plug, cessation of blood flow and repair of the damaged vessel.

Fibrinogen

Fibrinogen is a major plasma protein and coagulation factor in blood. The molecule has multifunctional roles due to its complex structure and functions. Fibrinogen has multiple binding sites which are open or which open as a result of proteolysis and/or conformational change. The fibrinogen molecule is a trinodular structure that consists of two sets of three different polypeptide chains Aα-, Bβ- and γ- chains (150).The chains are joined by disulphide bonds to form the N-terminal amino acid 'E' domain of the molecule and the two D domains are connected to this domain by a coiled-coil segment (Figure 7). Fibrinogen is involved in inflammation, repair, fibrinolysis and cellular and matrix interactions (151).

The interaction between fibrinogen and platelet GPIIbIIIa plays an important role in the generation of platelet-platelet aggregates and platelet thrombi. Fibrinogen can bind to the activated platelet integrin GPIIbIIIa receptor and that initiates an outside-in signalling event, which provides bridging between platelets.

Following activation of the plasma coagulation cascade fibrinogen is converted to fibrin by thrombin (Figure 7). The molecule has two recognition sites for thrombin in the E domain (EA and EB). Thrombin cleaves fibrinogen at these sites, first cleaving fibrinopeptide A (FPA) followed by fibrinopeptide B (FPB). The structure is stabilised by FXIII which crosslinks the fibrin molecules. Fibrin combines with other fibrin molecules to form long threads of fibrin polymers that intertwines with the forming thrombus and forms a scaffold i.e. a blood clot.

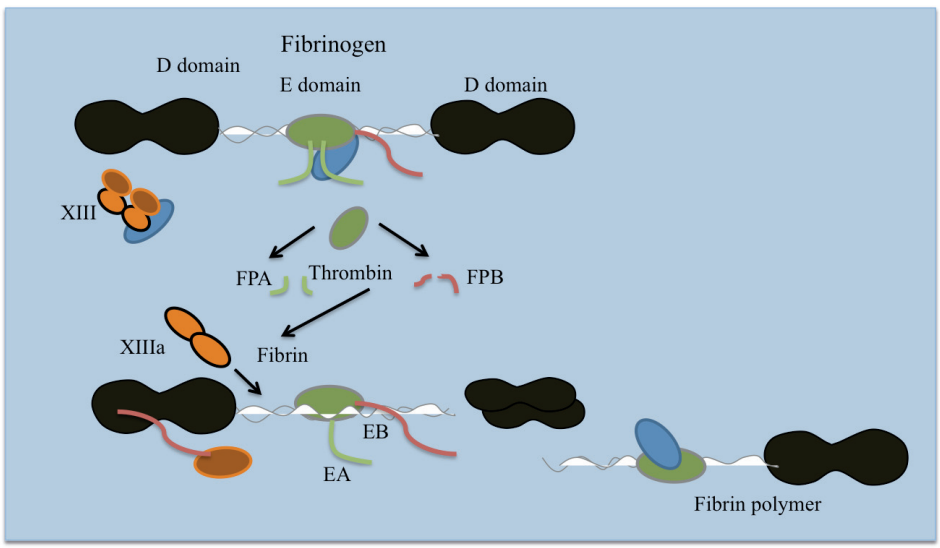

Figure 7: Schematic diagram of the fibrinogen structure and the thrombin cleavage sites.

Bacteria-Platelet interaction

During severe bacterial infection platelets are either directly or indirectly affected and thrombocytopenia can occur. Thrombocytopenia may be due to the increased consumption of clotting factors and the subsequent DIC. It may also be as a result of direct interaction of bacteria with platelets resulting in activation and aggregation. Many *in vitro* studies have shown that bacteria can interact with platelets, however less is know about the role of these interactions *in vivo*.

Mechanism of bacterial-platelet interactions

Bacteria can bind either directly or indirectly to platelet receptors. They can bind directly via bacterial surface proteins or they can associate indirectly via acquisition of plasma proteins including fibrinogen, fibronectin, IgG and the complement component C1q that can bind to both the bacteria and the platelet. In addition bacteria can also release products such as bacterial toxins that can interact with and activate platelets for example α-toxin, lipopolysaccharide (LPS) and *streptococcal* M1-protein (75,152,153).

In the case of *S. pyogenes* and *S. aureus* indirect binding to platelets can occur via bridging molecules that connect the bacteria to the platelet surface (154-156). For example *S. aureus* clumping factor A (ClfA) binds to fibrinogen and interacts with the platelet through the GPIIbIIIa receptor (157-159). These studies of *S. aureus* aggregation are IgG dependent. A similar mechanism of fibrinogen and IgG dependent platelet aggregation has been described for *S. pyogenes* bacteria (160). Furthermore, *S. pyogenes* can release M protein from the bacterial surface, which forms a complex with plasma fibrinogen and engages with the platelet fibrinogen receptor (Figure 8) and in the presence of specific IgG against M1 protein bound to the platelet FcγRIIa receptor, platelet activation can occur (75). In the absence of this IgG, M1 protein binding still occurs but the platelets do not become activated.

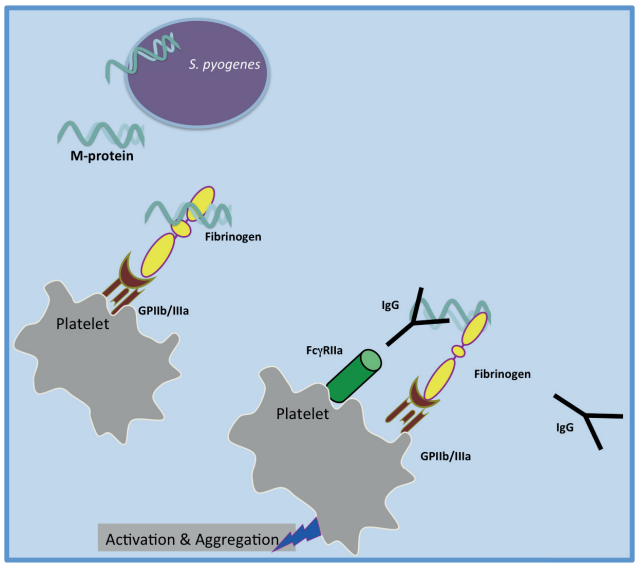

Figure 8: Schematic image of platelet activation and aggregation in response to *S. pyogenes* M1 protein.

Bacteria play a role in infective endocarditis where platelet thrombin adhere and accumulate on the heart valve and in septicaemia where a decrease in the platelet count is seen as an indicator of illness (161). Bacteria have developed resistance to platelet antimicrobial peptides that are aimed at killing the bacteria (162). Although demonstrated by few studies, platelets have also been shown to bind and internalise bacteria for example *S. aureus* (163-165). Platelets have been shown to engulf *S. aureus* and human immunodeficiency virus (HIV) in a subcellular component and the bacteria appeared to be associated with platelet granule secretory products (166). This work also demonstrated that platelets, once activated, could increase their ability to internalise bacteria, therefore it was considered to be important that platelet activation had previously occurred in order for the process to take place more effectively.

Platelet-Neutrophil Complexes

Platelets can form complexes and communicate with leukocytes by the formation of platelet-leukocyte complexes (PLC) during vessel injury and inflammation (167). Platelets can interact and form complexes at different levels with the respective cell types. They form complexes most easily with monocytes {Michelson: 2001ep}, followed by neutrophils. Furthermore, platelets bind monocytes stronger than neutrophils under vascular shear (168). PLC interactions are observed in diseases including, inflammatory diseases for example inflammatory lung disease such as cystic fibrosis (169), allergy (170), atherosclerosis (171), autoimmune diseases for example rheumatoid arthritis (172) and infection for example in sepsis progression and septic shock (173,174).

Platelets interact with neutrophils through different receptors, mainly selectins and integrins (Table 2). The most well studied is platelet P-selectin (CD62P) interacting with the PSGL-1 (P-selectin glycoprotein ligand-1, CD162) receptor on the neutrophil (175).The signalling through PSGL-1 results in the up regulation of the β2-integrin Mac-1 or CD11b/CD18 complex on neutrophils (176). CD11b/CD18 may bind directly to the platelet GPIb receptor or indirectly bind to the plasma protein fibrinogen which then associates with the platelet GPIIb/IIIa receptor (167). Blocking of the platelet GPIb receptor has been shown to prevent PNC formation at the endothelium (177).

At the endothelium neutrophil rolling can occur on platelets adhered to the endothelium via PNC formation (178,179). Activated platelets release chemokines, adhere to the endothelium and may subsequently recruit neutrophils to the endothelium and enhance their transmigration (180,181).

Other adhesion partners between platelets and leukocytes have been reported, although these are not as well studied. Intracellular adhesion molecule-2 (ICAM-2) is the main β2-integrin ligand present on activated platelets and can interact with neutrophil CD11b/CD18 or CD11a/Cd18, promoting neutrophil adhesion (182). In addition, neutrophil CD11a/Cd18 can interact with the platelet receptor junctional adhesion molecule 3 (JAM-3) (183). Activated platelets express CD40 ligand (CD40L) (184) which can interact with neutrophil CD40 (185). The table below (Table 2) outlines some of the most well studied interactions that occur between platelets and neutrophils.

Platelet	Neutrophil
P-selectin	PSGL-1
ICAM-2	CD11a/CD18
JAM-3	CD11a/CD18
CD40L	CD40
GPIIbIIIa	CD11b/CD18
GPIbα	CD11b/CD18

Table 2: The table demonstrates the most well studied mechanisms of interactions between platelets and neutrophils of which platelet p-selectin interacting with its coresponding receptor PSGL-1 on neutrophils is the most well known.

Platelets in complex with neutrophils have been shown to have enhanced functions while combined. Platelet binding can induce neutrophils to release neutrophil extracellular traps (NETs) which can entrap bacteria (186,187). PNC formation facilitates transcellular metabolism of arachidonic acid metabolites, which enhances the synthesis of proinflammatory and vasoconstrictive compounds amplifying the levels of neutrophil eicosanoids such as lipoxins and leukotrienes (188) (189).

PNCs have been reported to occur during sepsis and in some patient studies PNCs are speculated to contribute to the development of multiorgan failure (190) (173,186). It is however unclear whether PNC formation is directly mediated by the bacteria or is an indirect consequence of the overwhelming immune dysregulation observed in sepsis. Bacterial LPS has been shown to mediate PNC formation (186). Bacteria isolated from patients with bacteraemia generated PNCs *ex-vivo* in blood samples from the same patient from which the bacteria were isolated, indicating that PNC formation may occur in direct response to pathogeneic bacteria (191).

4. NEUTROPHILS-TRAVELLERS AND ENGULFERS

In the nineteenth century, the German researcher Paul Ehrlich was the first to distinguish the different leukocytes on the basis of their nuclear morphology and granule content by utilising special cell staining techniques. The neutrophil is the most abundant leukocyte in the blood stream and has an important immune role. They are also designated as polymorphonuclear leukocytes (PMN) since they have a multilobed nucleus and contain large numbers of intracellular granules and vesicles (192). The exciting, yet relatively short-lived life of a neutrophil begins in the bone marrow during granulopoiesis when a hematopoietic stem cell differentiates first into the common myeloid progenitor cells and then into polymorphonuclear leukocytes (193). Each day 10^{11} neutrophils are produced from the bone marrow from the hematopoietic stem cell (194). They have been considered to have relatively short lifespans with a half-life of approximately 8 h in humans (195) and approximately 1.5 hours in mice in the blood circulation, but more recent studies suggest they have longer lifespans (196). The neutrophil is an exceptionally efficient phagocyte displaying an immense ability to engulf and destroy microbes. If they do not encounter an infectious agent, neutrophils enter the reticuloendothelial organs or return to the bone marrow to undergo programmed cell death. Maintaining neutrophil homeostasis is very important and is regulated by the rate of differentiation and proliferation of the neutrophil precursors in the bone marrow.

Neutrophil Granules

Neutrophil granules can be subdivided into three main subsets. The utilisation of multiple techniques over the last number of years, including fractionation techniques, immune electron microscopy and flow cytometry have shown that neutrophils possess three main granule types; Azurophilic (primary), specific (secondary) and gelatinase (tertiary) subsets (197). Neutrophils also contain secretory vesicles whose origin may differ from the other granule types (198,199). The granules contain about 300 proteins (Figure 9) which include receptors which can become part of the cell membrane, proteolytic and bactericidal proteins and pro-inflammatory molecules (200) (201) which can all be rapidly transported to the cell surface when necessary.

During the transition from myeloblast to promyelocyte the granules begin to appear. Neutrophil granules are released in an hierarchical order and this is the opposite to that of which they were formed (202). The azurophilic granules are the largest (203) and are only fully mobilised after a strong stimulation that release molecules such as HBP and myeloperoxidase (MPO). The secondary (specific) granules are smaller than the azurophilic, contain many plasma molecules and play a role in oxidative burst (204). These are rich in antimicrobial substances such as neutrophil gelatinase associated lipocalin (N-GAL), lactoferrin, lysozyme among others and these granules contribute to the contents of the phagosome during bacterial clearance. The secretory vesicles and tertiary granules are more easily mobilised and play a role in neutrophil transmigration (205). Granules are not released until a signal transduction event is initiated whereby signals are sent to the cytoplasm to activate their movement to the membrane for cell degranulation and granule secretion. Neutrophil degranulation requires calcium, guanosine triphosphate (GTP) and ATP hydrolysis.

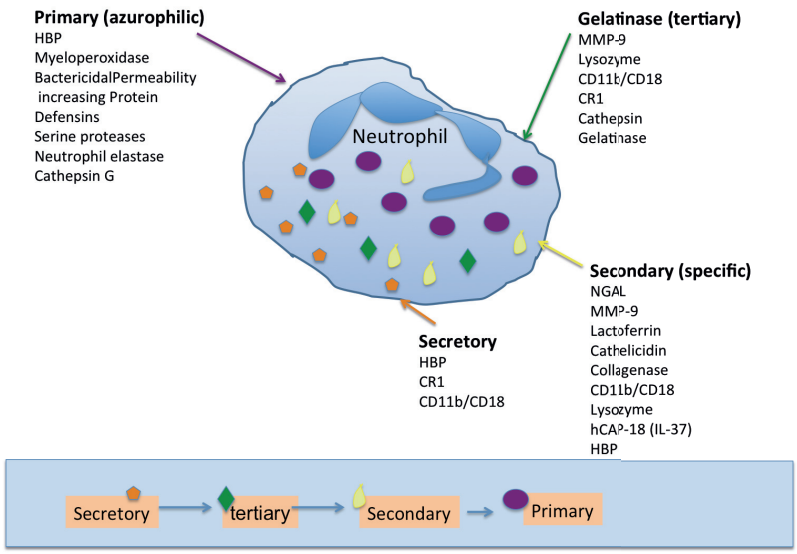

Figure 9. Neutrophil granule subsets.

Neutrophil Function

Neutrophils are one of the first responders at the site of inflammation, adhering and migrating rapidly to the necessary sites and are crucial for bacterial clearance. This is one of the neutrophils primary functions – extravasation to the site of inflammation and/or damage. This process is discussed in more detail later in this thesis. It is complex and involves a number of processes including neutrophil rolling, arrest, spreading, crawling and transmigration (206). The neutrophil granules facilitate transmigration across the endothelium, particularly the secretory and gelatinase (tertiary) granules. Secretory granules can incorporate molecules into their membrane such as selectins which then play a role in the initial steps of transmigration (207). Tertiary granules contain membrane and matrix degrading proteins such as collagenase that degrades collagen (208).

The importance of neutrophil function is demonstrated in patients with leukocyte adhesion deficiency (LAD), a rare yet life-threatening genetic condition. There is a deficiency of CD18, an adhesive glycoprotein on the surface of leukocytes that facilitates cellular interactions such as adhesion and subsequent leukocyte transmigration (209). Leukocytes are unable to transmigrate to the site of infection and thus are unable to clear pathogens. This results in these individuals being severely immunocompromised and unable to clear bacterial infection, therefore they experience recurrent necrotic soft tissue infections and impaired wound healing.

Neutrophils express a vast array of receptors on their surfaces that play important roles in pathogen recognition and phagocytosis of microbes. They express pathogen recognition receptors such as TLRs which are essential for PAMP recognition or DAMP recognition and generation of antimicrobial responses (210) but also opsonic receptors including the Fc and complement receptors.

Neutrophils migrate in a directional manner in a process called chemotaxis, which was first described by Leber in 1888. They move towards a chemical gradient in response to chemotactic factors or chemoattractants such as platelet activating factor (PAF), formyl-methionyl-leucylphenylalanine (fMLF), complement factor 5a (C5a) and chemokines such as interleukin 8 (IL-8). When the orientation of the chemoattractant changes the neutrophil cytoskeleton reorganises and it moves in the direction of that chemoattractant.

The neutrophil may use this method when migrating towards its target (see review (211)). fMLF is a proteinogenic amino acid found in bacteria and is a potent chemotactic factor when the peptides are released during infection. The formyl peptide receptor (FPR) receptor on the neutrophil recognise these peptides and initiates a cell signalling event. The neutrophil express two formyl peptide receptors, known as FPR1 and FPR2 and are both G-protein coupled receptors (212,213).

Pathogen Elimination

Phagocytosis is an active receptor mediated process that is actin driven and assists in the clearance of bacteria. The process was originally shown in 1977 to be IgG dependent (214), but later studies revealed a role for other receptors, including the complement receptors (215). There are a number of key steps involved in the process of phagocytosis, which involves receptor mediated particle recognition by the phagocyte, particle uptake into a vesicle, formation of a phagolysosome and finally clearance of the pathogen digested particles (216). Initially, the phagocyte must recognise many types of pathogen targets for which it has evolved many receptors on its membrane surface. PRRs on the neutrophil recognise PAMPs (5) or the phagocyte Fcγ receptors and complement receptor 3 (CR3, CD11b/CD18, αMβ2 integrin) (217) recognise pathogens that are opsonised by antibodies or complement components bound to their surface (218). When signalling networks are initiated and cytoskeletal actin rearrangements occur the plasma membrane surrounds the microbe and engulfs it, forming the phagosome. The microbe, once within the membrane bound phagosome is entrapped and fuses with the lysosome to form a phagolysosome (219). The phagolysosome is an acidic compartment in which the pathogen is broken down and processed for antigen presentation (220).

Neutrophils also have an oxidative (respiratory) burst mechanism. Neutrophils generate reactive oxygen species (ROS), which essentially are oxygen-derived molecules that are oxidising agents or converted to radicals. The process of ROS production requires nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) (221). Chronic granulomatous disease (CGD) is an inherited disorder, which occurs due to a defect or deficiency in the subunits, which make up the NADPH oxidase enzyme system (222). The disease is characterised by recurrent bacterial infections due to the inability of phagocytes, including neutrophils, to perform one of their functions in releasing reactive oxygen species.

Another mechanism by which neutrophils can entrap and clear bacteria is through the release of NETs (223). NET formation occurs when neutrophils undergo a special type of controlled cell death called NETosis where the nuclear chromatin is dissolved and extracellular strands of "sticky" threads of DNA are released. These strands of DNA also contain antimicrobial peptides. Platelets may have a role in NET formation in response to LPS and *Escherichia coli* (186).

5. THE ENDOTHELIUM - MORE THAN JUST A BARRIER BREACHED!

The endothelium consists of a single monolayer of specialised cells that provide the inner lining to blood vessels and as such is a major interphase for circulating immune cells in their passage from blood to tissues. In this way the endothelium can be considered an important barrier between blood and underlying tissues. Endothelial cells arise from the precursor hemangioblast, which can either differentiate into hematopoietic stem cells (HSCs) or into angioblasts (224). The hematopoietic and angiogenic lineage is derived from the mesodermal stem cells during embryonic development. The endothelium has the ability to secrete cytokines, chemokines and express adhesion molecules and is therefore viewed as an important host immunological organ (225). Endothelial cells maintain normal homeostasis by maintaining an antithrombotic state, regulating secretion of vasodilator and vasoconstrictor molecules (prostacyclin and nitric oxide), control vascular tone and blood pressure (226) and control leukocyte migration and fluid permeability across the vessel wall (227).

Activation of the Endothelium

Endothelium cell activation occurs in response to “danger” signals from pathogens and/or inflammatory mediators. Activating agonists such as thrombin, TNFα and bradykinin induce endothelial signalling events and vascular leakage (228). Endothelial cells express functional PRRs, which include the TLRs- TLR 1,2,4,5 and 6 that recognise bacterial PAMPs (229) such as bacterial lipoproteins, peptidoglycan, carbohydrates and endotoxins for example LPS released from gram negative bacteria (230). PRRs also recognise DAMPs, which are generated in response to tissue damage. The *S. pyogenes* M protein has recently been shown to be recognised as a PAMP by keratinocytes and is involved in the upregulation of many signalling partners (77).

Leukocyte Cell Migration

The initial events in leukocyte transmigration involve leukocyte capture, rolling and adhesion. Mediators of acute inflammation stimulate molecules such as selectins to be translocated from endothelial cells and facilitate the initial rolling process. There are three selectins involved -E, -P and -L selectins. Endothelial p-selectin, found in WPBs of endothelial cells is the primary selectin involved in leukocyte capture and rolling, which interacts with selectin ligands on leukocytes, particularily PSGL-1 and this facilitates rapid leukocyte adhesion (231). Once the leukocyte is in contact with the endothelium and the rolling process has occurred, further endothelial adhesion molecules are upregulated and chemokines such as IL-8, GROα and monocyte chemoattractant protein-1 (MCP-1) are secreted by the endothelium (232,233). The contact between chemokines and leukocyte chemokine receptors leads to the activation of leukocyte integrins.

Leukocytes express β2 integrins, and when upregulated, they tightly adhere to the endothelium (234). This stage of firm leukocyte arrest involves leukocyte-function associated antigen-1 (LFA-1/ (CD11a/CD18) and Mac-1 (CD11b/CD18) that can bind to ICAM-1 and ICAM-2 on endothelial cells promoting firm leukocyte arrest (235). Once leukocytes are firmly attached the process of leukocyte transmigration takes place. Adhesion molecules involved with transmigration include platelet endothelial cell adhesion molecule-1 (PECAM-1), vascular endothelial (VE) -cadherin, vascular cell adhesion molecule-1 (VCAM-1), integrin associated protein (IAP,CD47) and very late antigen 4 (VLA-4, α4β1) (236).

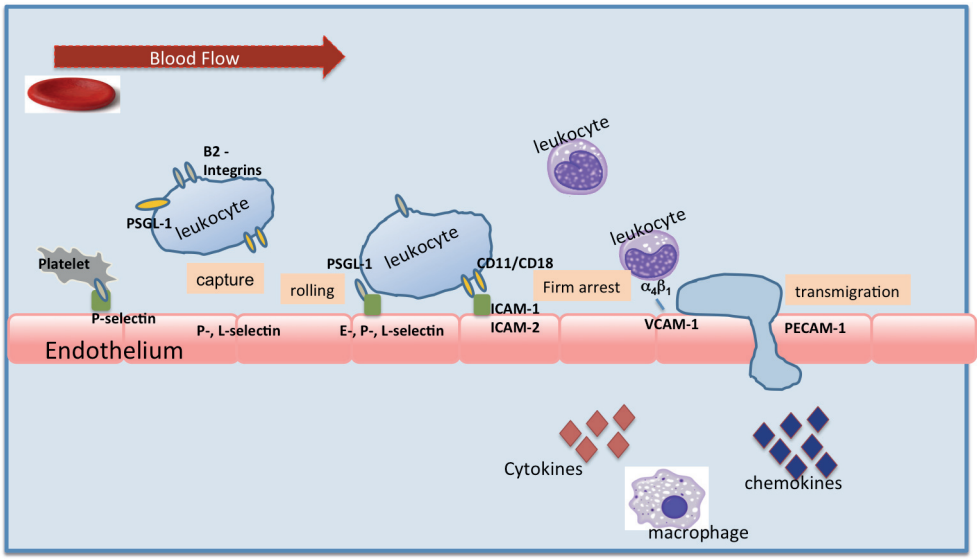

Figure 10: The diagram indicates the main events and interacting patners involved in leukocyte transmigration.

Endothelial cells play an important role in vascular healing. Upon vascular damage platelets become activated and adhere to subendothelial components, initially to vWF, which is bound to collagen at the subendothelium. Platelets interact with adhesion molecules such as P-selectin, PECAM-1 and integrins (237). Once adhered platelets aggregate together forming a platelet plug that seals vessel damage.

The endothelium normally maintains an antithrombotic state (238) with molecules such as activated protein C (APC), of which thrombomodulin is a cofactor for thrombin-induced activation of APC and the anticoagulant proteins, tissue factor pathway inhibitor (TFPI) and antithrombin (AT). These molecules primarily prevent blood from clotting by inhibiting the activation of clotting factors on a normal endothelial cell surface. The inhibitor, plasminogen activator inhibitor-1 (PAI-1) (239) plays a role in the fibrinolytic system. When endothelial cells become activated, the endothelium changes to a prothrombotic state and the clotting factor thrombin generated by the coagulation system can bind to receptors (PARs) on endothelial cells and platelets, which stimulates endothelial cytokine and chemokine production. TF is a potent activator of the coagulation cascade and is constitutively expressed by cells surrounding blood vessels (240) and is induced by cytokines from endothelial cells. TF is also present on monocytes and macrophages (241) and platelets and granulocytes play a role in its generation (242). In addition both the *S. pyogenes* M1 and M3 serotypes have been shown to stimulate procoagulant activity in endothelial cells via TF upregulation (243).

PRESENT INVESTIGATIONS

When *S. pyogenes* disseminates into the bloodstream, life-threatening disease such as sepsis can result. Upon interacting with our immune system this pathogen or its bacterial surface M protein has multiple effects and can activate several cells including platelets, neutrophils and endothelial cells which are investigated in this thesis. Platelets are a key focus throughout the studies of this thesis and we have investigated their function in both *in vitro* and *in vivo* model systems.

PAPER I

Background: Platelets can interact and form complexes with other immune cells. In this paper we investigate the formation of platelet- neutrophil complexes (PNCs). We focused on the relative contribution of each cell type involved in PNC formation and the functional effect these complexes have on neutrophils. During severe bacterial infection both the coagulation and inflammatory systems are activated (244) and we investigated these systems by comparing different agonists throughout this study. These included ADP, fMLF, the coagulation factor thrombin and the *S. pyogenes* virulence factor, M1 protein. It has previously been shown *in vitro* that in order for platelets to become activated in response to M1 protein, fibrinogen binding and the presence of anti-M1 IgG antibodies are required in those individuals (75). Therefore the effects of this protein were investigated in this study in different donors that could then be categorised as "responders" and "non-responders". PNC formation and neutrophil activation was investigated in a whole blood environment, thus resembling normal physiological conditions.

The main aims were to investigate the effects of distinct agonists on neutrophil function in PNCs and to distinguish any differences between the agonists.

Results & Conclusions: The agonist thrombin gave rise to platelet dependent PNC formation in all donors investigated and the neutrophils in response to this agonist demonstrated enhanced phagocytic and killing abilities, while chemotaxis was unaffected. In comparison, M1 protein only demonstrated PNC formation in certain donors that contained specific IgG against M1 protein and a direct correlation was made between donors with anti-M1 IgG antibodies and their level of PNC formation.

Neutrophil functional readouts differed in PNCs generated in response to M1 protein as compared to thrombin or ADP in combination with fMLF. Those PNCs generated in response to the M1 protein diminished the phagocytic and chemotactic functions of the neutrophils. In addition immunofluorescent and scanning electron microscopy revealed that PNCs in response to M1 protein were entrapped in a platelet and fibrinogen network.

PAPER II

Background: *S. pyogenes* has been shown to aggregate and subsequently disaggregate platelets over time in platelet rich plasma (245) and this bacteria has the ability to evade the immune detection systems and escape neutrophil phagocytosis (246) (63,68,71). The aim of the study was to investigate the role of platelet activation and aggregation in response to *S. pyogenes* for leukocyte function in blood. To this end we investigated the interactions between washed bacteria, platelets and leucocytes in whole blood, with a particular focus on bacterial survival.

Results & Conclusions: We demonstrate for the first time that platelet aggregation, platelet-neutrophil complex formation and platelet- monocyte complex formation occurs in direct response to *S. pyogenes* in whole blood. This results in platelet dependent neutrophil and monocyte activation, tissue factor up-regulation and clot formation. Furthermore, bacteria were directly associated with all of these homotypic and heterotypic cell aggregates, as visualised with immunofluorescent microscopy. Bacteria were entrapped within these cell aggregates over time, however bacterial killing was not observed and viable bacteria could be removed by sonication of the samples after one hour. We propose that bacterial entrapment in platelets or platelet leukocyte complexes might contribute to bacterial survival in human blood.

PAPER III and IV

Background: Platelets have been reported to play a role during invasive bacterial infections and the inflammatory response that follows and their numbers decrease dramatically during the progression of sepsis. In these two papers we assessed the role of platelets during the pathophysiology of sepsis in an animal model of *S. pyogenes*, infection. There have been many studies of platelet-bacteria interactions *in vitro* and *ex vivo* but animal models of platelet function during infection have been lacking. Thrombocytopenia occurs during severe and invasive bacterial infection and there is a correlation between the platelet count and the severity of sepsis (94,95,247). It is not clear whether platelets play a protective role in the prevention of sepsis or whether platelet activation contributes to the pathogenesis of the syndrome, therefore in paper III we investigated how platelets contribute to the acute response to bacterial infection using a mouse model of *S. pyogenes* bloodstream infection in platelet depleted mice as compared with healthy controls. Platelet activation was observed in paper III, therefore paper IV was initiated as a follow up study to investigate the kinetics of platelet activation during the progression of sepsis in the same animal model. A particular focus of this study was to determine which platelet activation assays could provide prognostic information on the progression of *S. pyogenes* sepsis and whether platelet activation was associated with organ damage.

Results & Conclusions: Collectively, the results of paper III and IV demonstrate an important role for platelets during the pathophysiology of *S. pyogenes* infection. In paper III platelets are reported to contribute to bacterial dissemination from the blood to the organs of animals, infected with *S. pyogenes*. The bacterial load in the blood, spleen and lungs was significantly decreased in platelet-depleted animals. These animals also exhibited reduced plasma IL6 levels and weight loss during the infection, suggesting that the pathogenesis of infection was decreased in these animals at this time point. We also observed that there was a significant increase in PNC formation during *S. pyogenes* infection as compared with uninfected controls, therefore PNC formation may be important during bacterial infection.

Paper IV was initiated to investigate the kinetics of platelet activation and in particular PNC formation throughout the progression of *S. pyogenes* sepsis and the results suggest that PNC formation may be a more robust biomarker to demonstrate platelet activation during disease progression. In our model neutrophil activation occurred early and remained high during the course of infection but platelet activation and PNC formation was more discriminatory between different time points.

The platelet count progressively decreased in blood during the infection while PNC formation was first increased and then decreased during the later stages of infection. The PNCs do not correlate to neutrophil activation, which implies that PNCs in this model are platelet dependent events, as we have previously demonstrated *in vitro*.

As *S. pyogenes* infection progressed to a late stage of sepsis, organ damage was observed at the same time point as PNCs were decreased in the blood of the animals and thrombocytopenia was most profound. Platelet aggregates were detected in the liver therefore suggesting that platelet activation and PNC formation precedes thrombocytopenia and platelets may contribute to the pathogenesis of organ damage during sepsis.

Very little work has been carried out on the therapeutic potential of targeting PNCs, as most studies are still at the investigation and mechanistic phases but further studies should aim to better understand their functional role and their future potential use as diagnostic or therapeutic tools.

PAPER V

Background: The endothelium constitutes the inner lining of blood vessels with a single layer of cells and is a semi-permeable barrier that plays an important role in regulating the movement of fluids, proteins and the transmigration of cells from blood to the tissue. Endothelial permeability and endothelial responses are altered in the presence of activating substances, such as TNFα and thrombin and when bacteria are present in the bloodstream. In this paper we investigated the effects of the Gram-positive bacterial protein, M1 protein on endothelial cells. Previously, M1 protein in complex with a plasma protein has been shown to induce the release of HBP from neutrophils (51) and HBP can increase endothelial cell permeability. In addition M1 protein activates platelets (75) and within platelet-neutrophil complexes we have shown that the neutrophils are dysfunctional (248). The aim of this paper was to determine whether M1 protein released from *S. pyogenes* binds to and activates endothelial cells, and the consequences of this for endothelial cell permeability. We investigated endothelial cell function either with M1 protein alone or in the presence of a plasma cofactor, fibrinogen.

Results & Conclusions: M1 protein increased endothelial vascular permeability in a Rho kinase and TLR-2 dependent manner, at levels equivalent to the positive control, thrombin. M1 protein has previously been shown to bind human monocytes by TLR-2 engagement and increase cytokine production from these cells (76). It has also been shown that M1 protein induces vascular nitric oxide production via TLR-2 (249). The results of these studies are in agreement with our TLR-2 findings and we demonstrate M1 protein can bind to both human and immortalised endothelial cell lines.

M1 protein may contribute to endothelial dysfunction and vascular leakage during infection. M1 protein has been shown to induce a proinflammatory cytokine response in epithelial cells (77). This may suggest that M1 protein induces an activation of the innate immune response, however in our studies there is a relative lack of cytokine production from the immortalised cell line and weak cytokine release from the primary human endothelial cells in response to M1 protein, indicating that the protein alone induces a weak response. However, in the presence of the plasma protein fibrinogen binding of M1 protein to endothelial cells and cytokine production were increased. It could be speculated that M1 protein requires another bridging molecule in addition to TLR-2 for an enhanced endothelial response. In addition, M1 protein has been shown to lose its specialised structure at 37°C (250) and perhaps the plasma protein herein is playing a role in stabilising M1 protein.

All together the findings of this thesis provide new evidence for the role of platelets, neutrophils, monocytes and endothelial cells during the pro-inflammatory response that is initiated during the pathogenesis of infection. The combination of pro-inflammatory stimuli, *S. pyogenes* and its virulence factor M1 protein, used throughout these studies contribute and affect these cells which are stimulated and play central roles in the immune response. M1 protein directly binds endothelial cells and effects vascular function, directly activates platelets, which in turn can interact with leukocytes whereby the neutrophils are dysfunctional and entrapped in aggregates which may accumulate in damaged organs and ultimately contribute to the outcome of sepsis.

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