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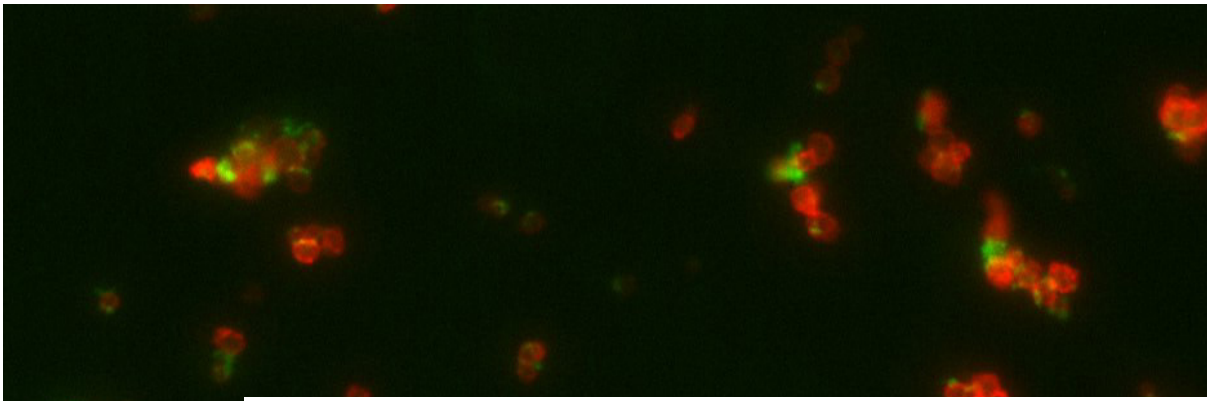

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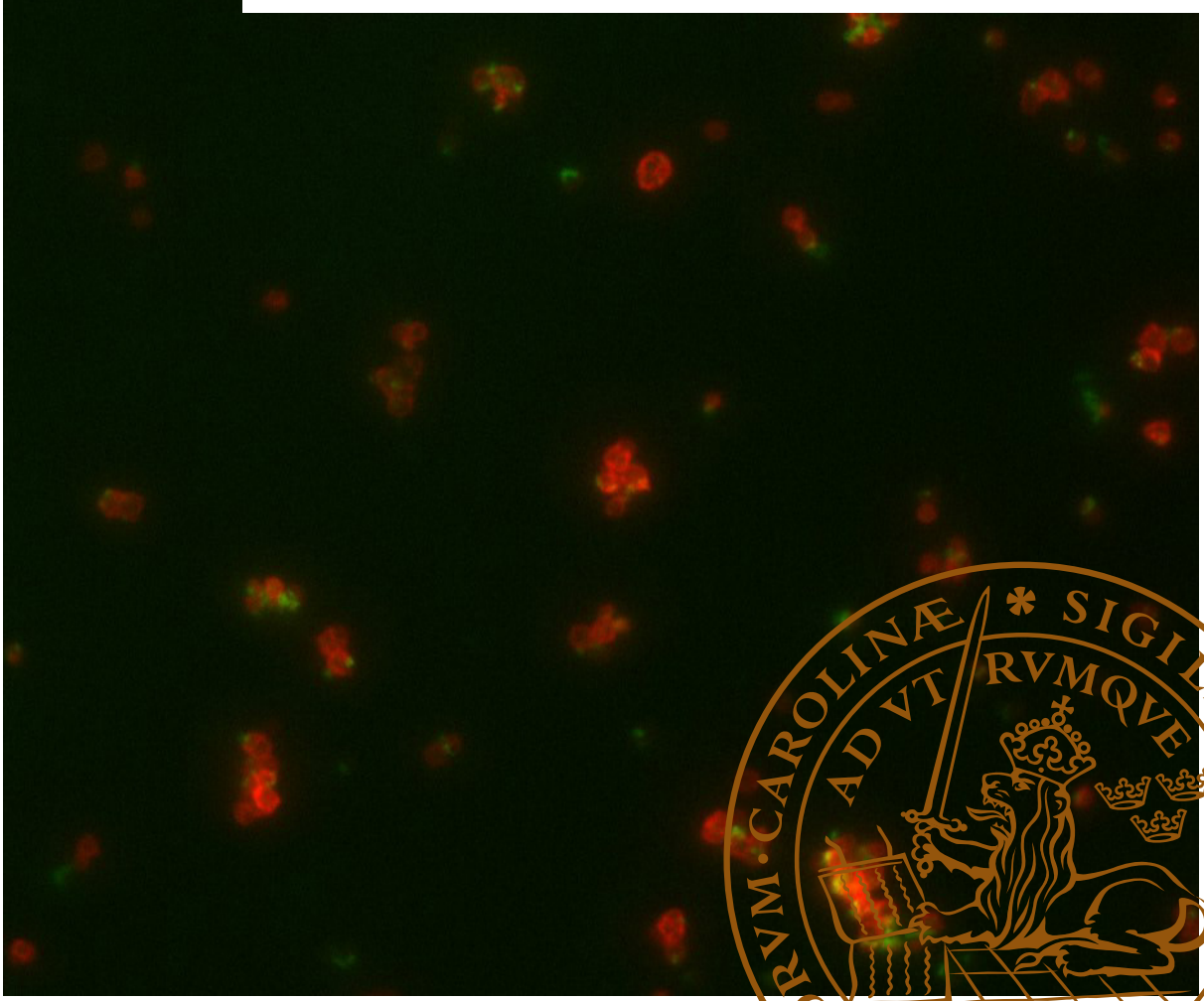

Novel aspects of pathogen-mediated platelet activation and the role of platelets in inflammation

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LUND
UNIVERSITY

DOCTORAL DISSERTATION

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Title and subtitle Novel aspects of pathogen-mediated platelet activation and the role of platelets in inflammation

Abstract Invasive bacterial infections and sepsis remain one of the major causes of death worldwide. Some of the hallmarks of sepsis are disturbed hemostasis and a dysregulated inflammatory state. The main regulators of hemostasis are platelets, and they also respond rapidly to inflammation and infection. *Streptococcus pyogenes* is a human-specific pathogen that can cause invasive disease and sepsis. One classical streptococcal virulence factor is the cell wall anchored M protein, which contributes to various aspects of bacterial pathogenesis such as evasion of phagocytosis and complement. The M protein is capable of forming protein-protein interactions with numerous plasma proteins such as fibrinogen and the Fc domain of IgG. There are more than 200 different serotypes of M protein and some, for example the *emm1* serotype, are more associated with invasive streptococcal disease than other serotypes. The M protein from the *emm1* serotype (M1 protein) can be released from the bacterial surface by host or bacterial proteases. The released M1 protein exhibits pro-inflammatory properties including activation of platelets, which is dependent on fibrinogen and specific IgG against the M1 protein. The overall aim of this thesis was to investigate the role of platelets during streptococcal sepsis further, mainly focusing on the interactions between the platelets and the streptococcal M protein. In *Paper I* we showed that C1q and downstream complement components were associated with the protein complexes that were formed by M1 protein in human plasma, and that the complement system was activated at the surface of M1 stimulated platelets, in an IgG- and fibrinogen-dependent manner. Furthermore, we demonstrated that platelet apoptosis and phagocytosis of platelets was increased after M1 stimulation. This revealed a novel mechanism of complement activation during streptococcal sepsis, which may contribute to the platelet consumption that occurs in sepsis. In *Paper II* the platelet-dependent pro-inflammatory effects of M protein serotypes associated with invasive infection (M1, M3, M5, M28, M49, and M89) were investigated. We showed that distinct M protein serotypes (M1, M3 and M5 protein) mediated fibrinogen- and IgG-dependent platelet activation and aggregation, and complex formation with neutrophils and monocytes. Neutrophil and monocyte activation was also mediated by M1, M3, and M5 protein serotypes, while M28, M49, and M89 proteins failed to mediate activation of platelets or leukocytes. This disclosed novel aspects of the immunomodulatory role of fibrinogen acquisition and platelet activation during streptococcal infections. In *Paper III* we isolated extracellular vesicles from platelets using acoustic trapping and characterized the protein cargo of vesicles from resting platelets, thrombin stimulated platelets, and M1 protein stimulated platelets. The vesicles from all three conditions contained platelet membrane proteins, granule proteins, coagulation factors and immune mediators. The vesicles from M1 protein stimulated platelets also contained increased levels of complement components and IgG3, as well as the M1 protein. The vesicles were functionally competent and mediated platelet activation, neutrophil activation, and cytokine release in whole blood. This highlights novel aspects of pathogen-mediated platelet activation that may contribute to the coagulation and immune dysfunction during invasive streptococcal disease. In *Paper IV* we established a model of *Streptococcus pyogenes* skin infection that progressed to an invasive infection over time. We observed bacterial dissemination to organs, a rapid increase in plasma cytokines, and finally organ damage. Platelets rapidly increased in the circulation post infection and platelet activation occurred, which was later followed by thrombocytopenia. Pathological changes in the organs were associated with intravascular clotting and accumulation of platelets in organs. This paper characterized platelet responses during infection and invasive disease. Collectively, this thesis enlightens new aspects of the immunomodulatory roles of platelets during invasive infections and highlights novel mechanisms of streptococcal pathogenesis.

Key words Platelets, *Streptococcus pyogenes*, M protein, sepsis, platelet-derived extracellular vesicles

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Coverphoto by Frida Palm: C1q (green) associated with platelets (red) activated by the streptococcal M1 protein

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Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we may fear less.

Marie Curie

Table of Contents

Abstract

Invasive bacterial infections and sepsis remain one of the major causes of death worldwide. Some of the hallmarks of sepsis are disturbed hemostasis and a dysregulated inflammatory state. The main regulators of hemostasis are platelets, and they also respond rapidly to inflammation and infection. *Streptococcus pyogenes* is a human-specific pathogen that can cause invasive disease and sepsis. One classical streptococcal virulence factor is the cell wall anchored M protein, which contributes to various aspects of bacterial pathogenesis such as evasion of phagocytosis and complement. The M protein is capable of forming protein-protein interactions with numerous plasma proteins such as fibrinogen and the Fc domain of IgG. There are more than 200 different serotypes of M protein and some, for example the *emm1* serotype, are more associated with invasive streptococcal disease than other serotypes. The M protein from the *emm1* serotype (M1 protein) can be released from the bacterial surface by host or bacterial proteases. The released M1 protein exhibits pro-inflammatory properties including activation of platelets, which is dependent on fibrinogen and specific IgG against the M1 protein.

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In *Paper IV* we established a model of *Streptococcus pyogenes* skin infection that progressed to an invasive infection over time. We observed bacterial dissemination to organs, a rapid increase in plasma cytokines, and finally organ damage. Platelets rapidly increased in the circulation post infection and platelet activation occurred, which was later followed by thrombocytopenia. Pathological changes in the organs were associated with intravascular clotting and accumulation of platelets in organs. This paper characterized platelet responses during infection and invasive disease.

Collectively, this thesis enlightens new aspects of the immunomodulatory roles of platelets during invasive infections and highlights novel mechanisms of streptococcal pathogenesis.

Original papers

Paper I

Complement Activation Occurs at the Surface of Platelets Activated by Streptococcal M1 Protein and This Results in Phagocytosis of Platelets

Frida Palm, Kristoffer Sjöholm, Johan Malmström and Oonagh Shannon

J Immunol 2019; 202:503-513

Paper II

Distinct Serotypes of Streptococcal M Proteins Mediate Fibrinogen-Dependent Platelet Activation and Proinflammatory Effects

Frida Palm, Sounak Chowdhury, Sara Wettemark, Johan Malmström, Lotta Happonen, Oonagh Shannon

Infection and Immunity 2022 Feb 17;90(2):e0046221

Paper III

Characterization of the protein cargo and pro-inflammatory effects of extracellular vesicles released from pathogen activated platelets.

Frida Palm*, Axel Broman*, Genevieve Marcoux, John W. Semple, Thomas L. Laurell, Johan Malmström, and Oonagh Shannon.

* These authors contributed equally

Manuscript

Paper IV

Platelet activation and accumulation in organs during invasive infection with *Streptococcus pyogenes*

Eleni Bratanis, **Frida Palm**, Christofer Karlsson, Alejandro Gomez Toledo, Gisela Hovold, Andrietta Grentzman, Johan Malmström, Oonagh Shannon

Manuscript

Additional papers not included in this thesis

Platelet activation and aggregation by the opportunistic pathogen Cutibacterium (Propionibacterium) acnes

Frida Petersson, Ola Kilsgård, Oonagh Shannon, Rolf Lood

PLoS One. 2018; 13(1): e0192051.

Abbreviations

ADCC – Antibody-dependent cell-mediated cytotoxicity

ADP – Adenosine diphosphate

APCs – Antigen presenting cells

C1-9 – Complement components 1-9

C4BP – Complement 4b binding protein

CARS – Compensatory anti-inflammatory response

CD40L – CD40 ligand

CTLs – Cytotoxic T-lymphocytes

DCs – Dendritic cells

DDA – Data-dependent acquisition

DIA – Data-independent acquisition

DIC – Disseminated intravascular coagulation

ECM – Extracellular matrix

E. coli – Escherichia coli

Efb – Extracellular fibrinogen-binding protein

ELISA – Enzyme-linked immunosorbent assay

EndoS – Endo-β-N-acetylglucosaminidase of streptococci

ESI – Electrospray ionisation

EVs – Extracellular vesicles

FcR – Fc receptor

FcγRIIA – Fc gamma receptor IIa

FDR – False discovery rate

Fg – Fibrinogen

FSC – Forward scatter

GP – Glycoprotein

HBP – Heparin binding protein

HCD – Higher energy collisional disassociation

HRP – Horseradish peroxidase

IdeS – IgG-degrading enzyme of *S. pyogenes*

Ig – Immunoglobulin

IL – Interleukin

IVIG – Intravenous immunoglobulin

ITAM – Immunoreceptor tyrosine-based activation motif

K. pneumoniae – Klebsiella pneumoniae

LC – Liquid chromatography

LC-MS/ MS – Liquid chromatography tandem mass spectrometry

LTA – Lipoteichoic acid

LPS – Lipopolysaccharide

MAC – Membrane attack complex

MBL – Mannose-binding lectins

MCP – Monocyte chemotactic protein

MHC – Major histocompatibility complex

MMP-9 – Matrix metalloproteinase-9

MS – Mass spectrometry

NK – Natural killer

NETs – Neutrophil extracellular traps

NO – Nitric oxide

OSC – Open canalicular system

PAMPs – Pathogen-associated molecular patterns

PAR – Protease-activated receptor

PEVs – Platelet derived extracellular vesicles

PDGF – Platelet-derived growth factor

PF4 – Platelet factor 4

PMC – Platelet-monocyte complex

PNC – Platelet-neutrophil complex

PRP – Platelet-rich plasma

PRRs – Pattern recognition receptors

PS – Phosphatidylserine

PSGL-1 – P-selectin glycoprotein ligand-1

RNA – Ribonucleic acid

ROS – Reactive oxygen species

S. aureus – Staphylococcus aureus

ScpA – Group A streptococcal C5a peptidase

SIC – Streptococcal inhibitor of complement-mediated lysis

SIRS – Systemic inflammatory response

SLE – Systemiclupus erythematosus

SLO – Streptolysin O

SLS – Streptolysin S

SOFA – Sequential organ failure assessment

SpeB – Streptococcal pyrogenic exotoxin B

S. pneumoniae – Streptococcus pneumoniae

SpyCEP – *S. pyogenes* cell envelope protease

S. pyogenes – Streptococcus pyogenes

SRM – Selected reaction monitoring

SSC – Side scatter

SSL5 – Staphylococcal superantigen-like protein 5

STSS – Streptococcal toxic shock syndrome

TF – Tissue factor

TFPI – Tissue factor pathway inhibitor

TGF-β – Transforming growth factor beta

TLRs – Toll-like receptors

TNF-α – Tumor-necrosis factor-α

TPO – Thrombopoietin

VEGF – Vascular endothelial growth factor

vWF – von Willebrand factor

Popular science summary

Infectious diseases are a major public health problem that cause about a third of all deaths (more than 15 million) in the world each year, especially in low-income countries. A common site of infection that can affect humans is the throat, which is most often caused by bacteria called group A streptococci. Group A streptococci also commonly cause skin infections, such as impetigo. Sore throat and impetigo are relatively mild infections that are easily cured with antibiotics, but group A streptococci can also spread and cause more serious infections, so-called invasive infections, that are more difficult to treat and in the worst case are life-threatening. A serious condition that can be caused by group A streptococci is sepsis.

Sepsis is defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection. The body's own immune system overreacts to an infection. During invasive infections and sepsis, bacteria can enter the bloodstream and encounter several different cells, including cells in the immune system and platelets. Platelets play an important role in wound healing where they stick together to form a clot and stop bleeding. In sepsis, the function of the platelets in wound healing is disturbed, which can lead to both bleeding and blood clots throughout the body. It is believed to be caused by platelets overreacting to the infection and being consumed in blood clots, resulting in a shortage of platelets.

Previous research shows that platelets also play an important role in the immune system and can interact with several different bacteria and viruses. Platelets can also interact with other cells in the immune system and affect their defences against infections. In the research presented in this dissertation, we have further investigated the interaction between platelets and group A streptococci, in order to increase the understanding of the role of platelets in invasive infections. We have described new ways in which platelets interact with group A streptococci. We have also reported how platelets respond to the bacteria, and propose mechanisms that cause both blood clots and a shortage of platelets in invasive infections. Finally, we have shown new ways in which platelets interact with other cells in the immune system and modulate the immune cell defence against infection. We conclude that platelets play an important protective role in the immune system during invasive infections, but they can also contribute to the overreaction and subsequent organ damage that occurs in sepsis.

Populärvetenskaplig sammanfattning

Infektionssjukdomar är ett stort folkhälsoproblem som orsakar ungefär en tredjedel av alla dödsfall (mer än 15 miljoner) i världen varje år, framför allt i låginkomstländer. En vanlig infektion som kan drabba oss människor är halsfluss som oftast orsakas av bakterier som heter grupp A streptokocker. Grupp A streptokocker orsakar också svinkoppor, en vanlig hudinfektion bland barn. Halsfluss och svinkoppor är relativt milda infektioner som lätt botas med antibiotika men grupp A streptokocker kan också sprida sig och orsaka allvarligare sjukdomar, så kallade invasiva sjukdomar, som är svårare att behandla och som i värsta fall kan vara livshotande. Ett allvarligt tillstånd som kan orsakas av grupp A streptokocker är sepsis.

Sepsis definieras som livshotande organskada som orsakas av att kroppens egna immunförsvar överreagerar på en infektion. Vid invasiva infektioner och sepsis kan bakterier komma ut i blodet och stöta på flera olika celler, bland annat celler i immunförsvaret och blodplättar, som också kallas trombocyter. Trombocyter har en viktig roll i sårläkning där de klibbar ihop sig, eller koagulerar, för att bilda en propp och stoppa blödningen. Vid sepsis störs trombocyternas funktion i sårläkningen vilket kan leda till både blödningar och blodproppar i hela kroppen. Man tror att det beror på att trombocyterna överreagerar på infektionen och förbrukas i blodproppar, vilket leder till brist på trombocyter.

Tidigare forskning visar att trombocyter även har en viktig roll i immunförsvaret och kan interagera med flera olika bakterier och virus. Trombocyter kan också interagera med andra celler i immunförsvaret och påverka deras försvar mot infektioner. I forskningen som presenteras i den här avhandlingen har vi undersökt interaktionen mellan trombocyter och grupp A streptokocker vidare, för att öka förståelsen för trombocyternas roll vid invasiva infektioner. Vi har visat nya sätt som trombocyter interagerar med grupp A streptokocker. Vi har också beskrivit nya sätt som trombocyter reagerar på bakterierna, som kan orsaka både blodproppar och brist på trombocyter vid invasiva infektioner. Slutligen har vi demonstrerat nya sätt som trombocyter interagerar med andra celler i immunförsvaret och påverkar immuncellernas försvar mot infektion. Sammanfattningsvis har vi kommit fram till att trombocyter har en viktig skyddande roll i immunförsvaret vid invasiva infektioner, men att de också kan bidra till den överreaktion och följande organskada som uppstår vid sepsis.

Background

Introduction

A healthy human is colonized with the normal microbiota at a ratio of ten times more bacterial cells than human cells. These bacteria are found in the skin, mouth, nose, gastrointestinal tract, and vagina. The microbiota has a number of beneficial functions in the human body, such as digesting food, synthesising vitamins and preventing colonization by pathogens. Shifts in the microbiota can cause disease, such as bacterial vaginosis where inflammation is caused by changes in the vaginal microbiota (1, 2). Some members of the microbiota are so called opportunistic bacteria that can cause disease when the immune system is suppressed or when they enter a location where they normally don't reside. Other bacteria are known as pathogens that cause disease in humans. However, not all strains of a pathogen always cause disease, and not all individuals are always susceptible to a certain pathogen (3). The ability to cause disease is known as virulence, and factors contributing to the ability to cause disease are known as virulence factors. Thus, the loss of a virulence factor generally results in reduced ability to cause disease. However, the ability to cause disease is most often multifactorial, and the same factor can be a virulence factor for one bacterium but not for others, which makes virulence difficult to define and to study (3-5). Bacteria are classified into gram-positive bacteria, with a cell wall composed of a thick layer of peptidoglycan containing lipoteichoic acid (LTA), and gram-negative bacteria, with cell wall composed of a thin layer of peptidoglycan and an outer membrane containing lipopolysaccharide (LPS) (6).

The immune system

The first barrier

The first line of defence against invading microbes in the human body is the skin and mucosa. The epithelial cells in the skin are tightly attached to each other via tight junctions and prevent bacteria from penetrating the skin. To breach the skin, bacteria must take advantage of wounds or evolve strategies to damage or invade epithelial cells (3). Furthermore, the skin is dry, acidic, and constantly shed, which makes it an unfavourable environment for most bacteria (6). Present in the skin are also antimicrobial substances and commensal microbiota that occupy potential colonization sites. The respiratory tract, gastrointestinal tract and urogenital tract are directly exposed to the environment outside the human body. To prevent colonization these epithelial areas are covered with a layer of mucus that trap and shed bacteria (3). Mucus also contains antimicrobial peptides, that are cationic and can depolarise or insert into bacterial membranes and kill bacteria. Antimicrobial peptides are also found in the skin, mouth, vagina, lungs, and gastrointestinal tract (7).

The innate immune system

The invading pathogens that manage to breach the skin and mucosa are challenged by the second line of defence, the innate immune system, that is always present and ready to respond. The innate immune system is composed of phagocytic cells, such as neutrophils, monocytes, macrophages, and dendritic cells (DCs), that engulf the invading pathogen, natural killer (NK) cells, that kill infected host cells with intracellular pathogens, and proteins, such as cytokines and complement, that regulate and complement the activities of the innate immune cells. Dendritic cells migrate from the blood stream to tissue to be ready for invasion. The endothelial cells that line the blood vessels are not tightly attached to each other, which allows immune cells such as neutrophils to pass through the endothelium to reach an invading pathogen or injury. However, this may also allow the invading pathogens to pass through the endothelium and spread throughout the body. The invading pathogens express pathogen associated molecular patterns (PAMPs), such as flagella, bacterial DNA and ribonucleic acid (RNA), and the bacterial cell wall

components LPS and LTA. The innate immune cells recognise these PAMPs via pattern recognition receptors (PRRs), such as toll-like receptors (TLRs). Engagement of PRRs mediates activation of the innate immune cells and results in neutrophil and monocyte migration to the site of invasion. The monocytes differentiate into macrophages, that are efficient phagocytes and that release pro-inflammatory cytokines, such as tumor-necrosis factor-α (TNF-α) and interleukin (IL)-1β and chemokines that recruit additional innate immune cells to the site of invasion. The neutrophils increase their phagocytotic ability, release bactericidal nitric oxide (NO) and release neutrophil extracellular traps (NETs) to trap and kill invading pathogens (3, 9, 10). The DCs links the innate and adaptive immune systems and migrate to the lymphoid tissues where they present antigen to and stimulate the cells of the adaptive immune system.

The complement system

Another link between the innate and adaptive immune systems is the complement system, an important part of the human defence against invading pathogens. The complement system is a set of proteins, complement components, that circulate the blood stream as inactive precursors and that are activated in a cascade by proteolytic cleavage, called complement activation. Complement activation can be initiated in three ways: By mannose-binding lectins (MBL) that recognise mannose residues at the bacterial surface, by the alternative pathway where direct complement activation is mediated by bacterial surface molecules, such as LPS and LTA, and by the classical pathway where complement activation is mediated by specific antibodies, produced in the adaptive immune response, that form immune complexes with microbial antigens. Thus, the complement system links the innate and the adaptive immune systems. Complement components C1-C9 are activated in a proteolytic cleavage cascade, resulting in generation of opsonins (C3b), that bind to the bacterial surface and enhance phagocytosis, chemotactic molecules (C3a and C5a), that recruit phagocytes to the site, and the membrane attack complex (MAC), that forms pores in the bacterial membrane and kills the bacteria through lysis. Host cells are protected from the complement system by complement regulatory proteins, such as factor H that results the alternative pathway and complement 4b binding protein (C4BP) that regulates the classical and lectin pathway (3, 11, 12).

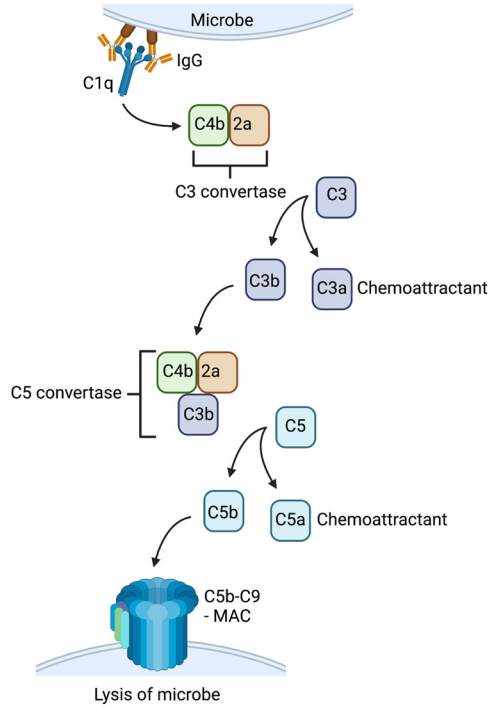

Figure 1. Schematic figure of the classical pathway of the complement system
Activation of the classical pathway of the complement system is mediated by specific IgG that form immune complexes with microbial antigens. Complement components C1-C9 are activated in a proteolytic cleavage cascade, resulting in generation of opsonins (C3b), that bind to the bacterial surface and enhance phagocytosis, chemotactic molecules, or chemoattractants (C3a and C5a), that recruit phagocytes to the site, and the membrane attack complex (MAC), that forms pores in the bacterial membrane and kills the bacteria through lysis. Figure 1 was created with Biorender.

The adaptive immune system

Many pathogens have developed mechanisms to evade the innate immune system, such as resistance against phagocytosis and complement activation. To battle these pathogens the human body has evolved a second defence system, the adaptive immune system. The adaptive immune system battle invading pathogens in a targeted fashion, using specific antibodies and antigen presenting receptors against a particular microbe. The adaptive immune response is significantly slower than the innate immune response, and it takes more than a week for specific antibodies to develop. However, upon additional encounters with the same pathogen, the response in faster and only takes a day or two (3, 13). As described above antigen presenting cells (APCs), such as DCs and macrophages, recognise invading pathogens and present antigen via major histocompatibility complexes (MHC) to cells of the

adaptive immune system, T-lymphocytes. Antigen presentation via MHC-I stimulates cytotoxic T-lymphocytes (CTLs) and antigen presentation via MHC-II stimulates helper T-lymphocytes (14, 15). Cytotoxic T-lymphocytes recognise specific epitopes presented by cells infected with intracellular pathogens and kills them by releasing apoptotic and cytolytic molecules. Helper T-lymphocytes stimulate B-lymphocytes to become plasma B-lymphocytes and produce antibodies. A fraction of activated T-lymphocytes become memory T-lymphocytes that circulate in small numbers and respond more rapidly upon additional encounters with the same microbe. In a similar fashion a small fraction of activated B-lymphocytes become memory B-cells (3, 16). Antibodies, or immunoglobulins (Ig), consist of a variable antigen binding Fab region that binds specifically to antigens foreign to the body, such as a microbial antigen, and a constant Fc region that binds to Fc receptors (FcRs) on immune cells and to complement component C1 to induce phagocytosis, antibody-dependent cell-mediated cytotoxicity (ADCC) and complement activation (3). Humans have four classes of immunoglobulins: IgG, IgM, IgA, and IgE. IgG is the most prevalent immunoglobulin in blood, and there are four subclasses of IgG: IgG1-4. IgG1 is the most prevalent subclass, and IgG1 and IgG3 are most efficient at opsonization and complement activation. IgM is a multimer that is most prevalent in the early antibody response, whereas IgG is most prevalent in the later stages of the antibody response or upon additional encounters with the same antigen. IgM levels are detected during early infection but disappears thereafter, whereas IgG levels remain in circulation after infection. Due to the multimeric structure, IgM can interact with multiple Fc receptors or C1 components and is the most efficient mediator of complement activation. Furthermore, IgM and all IgG subclasses bind to released toxins and to antigens at the microbial surface and prevent them from interacting with host proteins, called toxin and microbe neutralisation. IgA is the most prevalent antibody in mucosa and is important for the defence of mucosal surfaces, such as the gastrointestinal tract, pulmonary tract, and urogenital tract. sIgA is a dimer that binds to the microbial surface to trap microbes in mucus and prevent them from reaching and binding to the mucosal surface. IgE is present at low levels in plasma and is important during parasite infections and allergic responses (3, 17, 18).

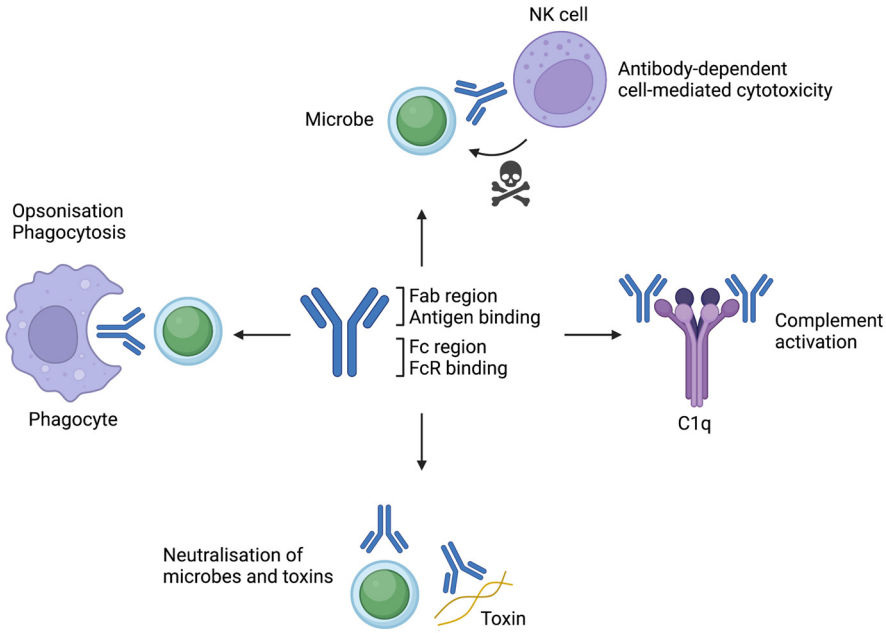

Figure 2. IgG-mediated immunity
IgG consists of a variable antigen binding Fab region that binds specifically to antigens foreign to the body, such as a microbial antigen, and a constant Fc region that binds to Fc receptors (FcRs) on immune cells and to complement component C1q to induce opsonization and phagocytosis, antibody-dependent cell-mediated cytotoxicity (ADCC) and complement activation. Furthermore, IgG binds to released toxins and to antigens at the microbial surface and prevent them from interacting with host proteins, called toxin and microbe neutralisation. Figure 2 was created with Biorender.

Inflammation

Inflammation is an adaptive response triggered by a number of conditions, such as injury or infection. The inflammatory response during infection or injury is acute and transient, however inflammation can also become chronic during conditions such as cardiovascular disease. Inflammation is characterized by heat, pain, redness and swelling, due to increased vascular permeability and recruitment of immune cells to the site of injury, with the main purpose of providing protection against infection (19). Immune cells in the tissue, such as macrophages, recognise PAMPs with pattern-recognition receptors, as well as exposed tissue factor and collagen in the damaged tissue. When macrophages in the tissue recognise pathogens or injury, they become activated and start producing chemokines, cytokines, and eicosanoids, and thereby initiating the inflammatory response. Neutrophils are recruited to the site of injury, become activated and release inflammatory and antimicrobial factors, such as reactive oxygen species (ROS), from their granules. The ROS kills potential invaders, but also damages the host tissue, and when inflammation is dysregulated,

it can be detrimental to the host (20). Vascular leakage and increased permeability during inflammation results in plasma proteins and platelets gaining access to the extracellular matrix (ECM) and tissue. Factor XII is a plasma protein that becomes activated upon contact with collagen and other components of the ECM that are exposed during tissue damage. Factor XII initiates coagulation and activates the complement system. Factor XII also initiates fibrinolysis and clot dissolving (21). Platelets are also activated in response to the collagen, and release several inflammatory mediators, such as thromboxane and serotonin. Some inflammatory mediators circulate as inactive precursors, and their plasma concentration increase dramatically during the acute phase of inflammation, as a result of increased secretion by hepatocytes. One example of an acute-phase reactant and mediator of inflammation is fibrinogen (22). Fibrinogen interacts with leukocytes and facilitates leukocyte migration and activation. Furthermore, fibrinogen interacts with platelets and is an important co-factor for platelet activation (23-25). Several bacterial pathogens, including *Streptococcus pyogenes* (*S. pyogenes*), bind fibrinogen to subvert fibrinogen-mediated host antimicrobial function or facilitate invasion within the host (26-28). Other mediators are released or produced in response to inflammation inducers, such as TNF-α, IL-1, IL-6 from macrophages (19). After successful pathogen elimination, the inflammation transits into resolution and tissue-repair. This transit is mediated by a shift from pro-inflammatory cytokines and prostaglandins to anti-inflammatory lipoxins. Lipoxins inhibit the recruitment of neutrophils and promotes the recruitment of monocytes. The migrating monocytes will mature into macrophages in the tissue, that will remove dead cells and facilitate tissue repair (29).

Streptococcus pyogenes

Streptococcus pyogenes, or group A streptococcus, is a gram positive, beta hemolytic bacterium that can cause a wide range of infections exclusively in humans. *S. pyogenes* most commonly causes relatively mild throat and skin infections, such as pharyngitis and impetigo with around 600 and 111 million cases globally every year, respectively (30, 31). These mild *S. pyogenes* infections normally pass without further complications and are mostly easy to treat with antibiotics if necessary. However, *S. pyogenes* can also cause severe invasive infections, such as necrotizing fasciitis, streptococcal toxic shock syndrome (STSS), and sepsis, with around 663 000 cases globally each year, resulting in 163,000 deaths (30). Furthermore, *S. pyogenes* infection can trigger serious autoimmune conditions, such as acute glomerulonephritis, acute rheumatic fever, and rheumatic heart disease (30, 31). *S. pyogenes* infections markedly decreased in industrialised countries in the last century (32, 33) followed by a significant increase of severe *S. pyogenes* infections and outbreaks was observed during the last decades (34-37). *S. pyogenes* is sensitive to penicillin, but resistance to antibiotics such as macrolides, clindamycin, and lincosamides is emerging in some countries (38-40).

S. pyogenes infections are normally caused by spread from asymptomatic colonization in the nasopharyngeal mucosa and skin, or by transmission via close contact or respiratory droplets. There have also been several reports of outbreaks related to crowded conditions, contaminated food, and hospital-acquired disease (36, 41, 42). *S. pyogenes* outbreaks are also associated with emerging clones resulted by horizontal gene transfer (36, 43). The increase of severe *S. pyogenes* infections that was observed during the last decades is linked to the emergence of the M1T1 clone (35, 36, 43), that is the most common clinical isolate in the developed world. *S. pyogenes* can enter the blood stream directly, as a result of childbirth or injury, or transiently after colonization or infection of the throat or skin (36, 44). The presence of *S. pyogenes* in the bloodstream, bacteremia, is characterized by high fever and nausea caused by a strong inflammatory response. Streptococcal superantigens bind to T-cells, B-cells, monocytes and dendritic cells and mediate release of inflammatory markers such as IL-1β, IL-2, IL-6, IFN-γ and TNF-α, resulting in a dysregulated inflammatory response, tissue damage, organ failure and systemic shock, associated with a high mortality rate (30, 45, 46).

S. pyogenes has developed multiple mechanisms to colonize, disseminate, and evade the host immune system. Several adhesins are expressed at the bacterial surface that bind multiple host factors, facilitating colonization of various tissue niches (47). The M protein is a major streptococcal virulence factor that covers the bacterial surface and promotes adherence and colonization. The M protein mediates adherence and internalisation into epithelial cells and keratinocytes, and interacts with components of the ECM, such as fibronectin (48-50). The secreted bacterial protease streptococcal pyrogenic exotoxin B (SpeB) cleaves autophagy components, promoting intracellular survival and proliferation (51).

S. pyogenes has adapted to the human host and developed multiple virulence factors to evade the human immune system, resulting in resistance against the complement system, phagocytosis, opsonization, antimicrobial peptides and neutrophil-mediated killing. The M protein binds several complement inhibitory proteins, such as C4BP and factor H, resulting in abolished complement activation and decreased bacterial killing (52, 53). Furthermore, M protein binds fibrinogen and plasminogen, which prevents deposition of complement C3 convertase and the opsonin C3b respectively, both resulting in decreased phagocytosis (54, 55). Streptococcal inhibitor of complement-mediated lysis (SIC) is a secreted virulence factor expressed by distinct serotypes, that binds and inhibits formation of the of the MAC complex (56). Most serotypes are protected by a hyaluronic acid capsule that blocks antibody opsonization, complement deposition and phagocytosis (57). The capsule is upregulated in human blood and protects against NETs, promotes survival and dissemination in mouse models and is associated with invasive disease in humans (58-60). To prevent antibody opsonization, complement activation and Fc-mediated phagocytosis, the streptococcal M proteins, as well as the M-related proteins and M-like proteins, bind the Fc region of IgA and IgG (61). Furthermore, the IgG-degrading enzyme of *S. pyogenes* (IdeS) cleaves the lower Fc region of IgG, resulting in decreased phagocytosis and neutrophil activation (62). The secreted endoglycosidase endo-β-N-acetylglucosaminidase of streptococci (EndoS) hydrolyses the glycan on the heavy chain of IgG, resulting in decreased Fc receptor binding, complement activation and phagocytosis (63, 64). The broad-spectrum cysteine protease streptococcal pyrogenic exotoxin B is secreted by most *S. pyogenes* serotypes and degrades IgGA, IgGD, IgGE, IgG and IgM (64).

S. pyogenes secretes toxins that directly kill immune cells, such as streptolysin O (SLO) and streptolysin S (SLS). SLO and SLS contribute to beta-hemolysis on blood agar plates and form large pores in host cell membranes, resulting in neutrophil, monocyte and epithelial cell apoptosis, and decreased phagocytosis (65, 66). Furthermore, SLS mediate platelet lysis and SLO induces platelet-neutrophil complex formation, contributing to tissue damage during infection (65, 67). SLO mutants show reduced virulence, and the expression of both SLO and SLS increases virulence, in mouse models of invasive *S. pyogenes* infection (65, 68, 69).

S. pyogenes expresses several factors that cleave or inhibit antimicrobial peptides. Both SpeB and SIC inhibit human cathelicidin LL-37, by cleavage and binding respectively (70, 71). Furthermore, *S. pyogenes* expresses proteases that cleave chemotactic substances. IL-8 and C5a are cleaved by *S. pyogenes* cell envelope protease (SpyCEP) and group A streptococcal C5a peptidase (ScpA), respectively, resulting in decreased neutrophil recruitment and phagocytosis (72, 73), and increased virulence in a mouse model of invasive *S. pyogenes* infection (74). Serotype M1T1 also secretes Sda1, a DNase that degrades NETs and decreases neutrophil-mediated killing (75).

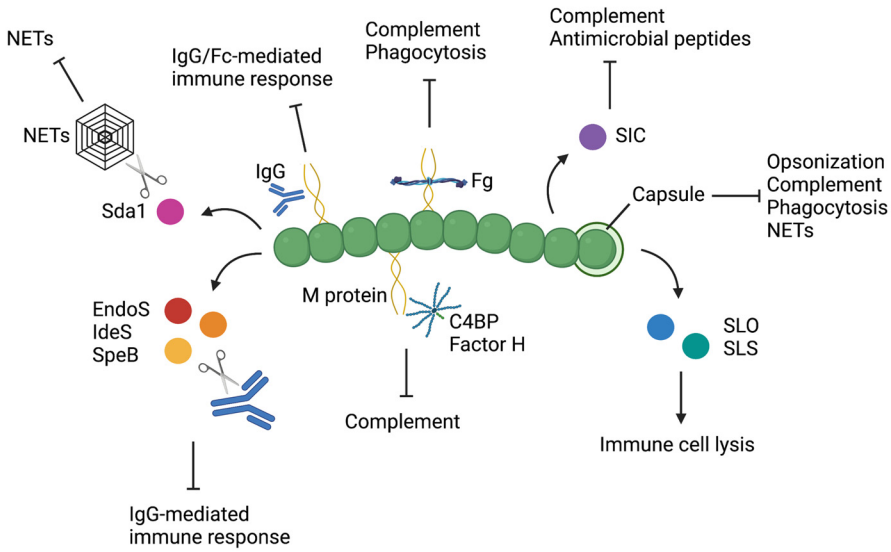

Figure 3. *S. pyogenes* immune evasion

S. pyogenes has adapted to the human host and developed multiple virulence factors to evade the human immune system, resulting in resistance against the complement system, phagocytosis, opsonization, antimicrobial peptides and neutrophil-mediated killing. The M protein binds several complement inhibitory proteins, such as C4BP and factor H, resulting in abolished complement activation. Furthermore, M protein binds fibrinogen, which prevents complement deposition and phagocytosis. Streptococcal inhibitor of complement-mediated lysis (SIC) is a secreted virulence factor expressed by distinct serotypes, that binds and inhibits formation of the of the MAC complex. Most serotypes are protected by a hyaluronic acid capsule that blocks antibody opsonization, complement deposition and phagocytosis. To prevent antibody opsonization, complement activation and Fc-mediated phagocytosis, many streptococcal M proteins bind the Fc region of IgG. Furthermore, the IgG-degrading enzyme of *S. pyogenes* (IdeS) cleaves the lower Fc region of IgG, resulting in decreased phagocytosis and neutrophil activation. The secreted endoglycosidase endo-β-N-acetylglucosaminidase of streptococci (EndoS) hydrolyses the glycan on the heavy chain of IgG, resulting in decreased Fc receptor binding, complement activation and phagocytosis. The broad-spectrum cysteine protease streptococcal pyrogenic exotoxin B is secreted by most *S. pyogenes* serotypes and degrades IgG. *S. pyogenes* secretes toxins that directly kill immune cells, such as streptolysin O (SLO) and streptolysin S (SLS), that form large pores in host cell membranes, resulting in neutrophil, monocyte and epithelial cell apoptosis. *S. pyogenes* also expresses several factors that cleave or inhibit antimicrobial peptides, and both SpeB and SIC inhibit human cathelicidin LL-37. Serotype M1T1 also secretes Sda1, a DNase that degrades NETs and decreases neutrophil-mediated killing. Figure 3 was created with Biorender.

Invasive *S. pyogenes* infection is characterized by dysregulated inflammation and coagulation, and the bacterium has developed multiple factors that interact with the inflammation, coagulation and clotting responses. *S. pyogenes* binds plasminogen, either via the M protein or other surface proteins, and secretes streptokinase, a plasminogen activator. Plasminogen binding and activation results in fibrinolysis, reduced clotting and release of trapped bacteria from fibrin clots (76). Plasminogen binding is associated with invasive disease in humans, and depletion of plasminogen binding reduces mortality in mouse models of invasive *S. pyogenes* infection (26, 77). Plasminogen is cleaved into plasmin that mediates fibrinolysis, which facilitates bacterial dissemination and escape from fibrin-rich clots (26). Plasmin also mediates increased vessel permeability, recruitment of immune cells and dysregulated inflammation and tissue damage (78). *S. pyogenes* also bind kininogen, which mediates release of bradykinin, resulting in vasodilation and inflammation (79). M protein can be cleaved off from the bacterial surface by SpeB, and the released M protein forms complexes with fibrinogen. The M protein/ fibrinogen complexes mediate neutrophil activation, degranulation and release of neutrophil heparin binding protein (HBP), resulting in increased vessel permeability, recruitment of immune cells and dysregulated inflammation and tissue damage (80, 81). M protein/ fibrinogen complexes have been found in tissue biopsies from patients with necrotising fasciitis and septic shock (80). M protein binding to fibrinogen and kininogen also activates the coagulation system, resulting in fibrin clot formation, generation of the pro-inflammatory factor bradykinin, vasodilation, and vascular leakage (79). This has been observed both in patients and mouse models of invasive *S. pyogenes* infection (82, 83). Furthermore, M protein mediates tissue factor upregulation on monocytes and endothelial cells, via TLR2 interaction, increasing their procoagulant activity (84, 85). M protein also mediates platelet activation and aggregation, further promoting a dysregulated coagulation and clot formation (86). Platelet-bacteria interaction might facilitate bacterial dissemination to organs and protect bacteria from the host immune system (87).

Collectively, this shows that inflammation and coagulation are linked during invasive *S. pyogenes* infection and that dysregulation of these systems are hallmarks of *S. pyogenes* pathogenesis. Treatment of invasive *S. pyogenes* infection includes antibiotics and hemodynamic stabilisation (31). Anti-inflammatory compounds and intravenous immunoglobulin (IVIG) are also potential treatments, but the effects of these drugs need to be evaluated further (88, 89). Since the human is the only natural *S. pyogenes* host, a vaccine against the bacteria has the potential to decrease transmission and ultimately eradicate the significant human pathogen. However, the development of a vaccine against *S. pyogenes* is hindered by serotype diversity, differences in geographical distribution of serotypes and the profound risk that antigens trigger autoimmune responses (90, 91). Future research is needed both to develop a *S. pyogenes* vaccine and to increase the understanding of, and develop treatments targeting, the inflammation and coagulation dysregulation during *S. pyogenes* infection.

The streptococcal M protein

The M protein is a major streptococcal virulence factor that covers the bacterial surface and is anchored to the peptidoglycan cell wall through an LPxTG motif (47). The protein is a dimer composed of two chains that form an α-helical coiled-coil structure (92). The protein consists of four repeat regions called A, B, C and D, a highly conserved C-terminal region, including the C and D regions, and a hypervariable N-terminal region, including the A region. Some *S. pyogenes* serotypes lack the semi-variable B region of the M protein (93). M protein is encoded by the *emm* gene and serotyping of *S. pyogenes* is based on *emm* amino acid sequence variations in the N-terminal region (47). Invasive disease is associated with distinct serotypes, and the *emm1* serotype in particular has dominated the epidemiology of invasive infections in the northern hemisphere since the 1990s (94). Furthermore, some serotypes are associated with throat infection, while others more commonly cause skin infections (95). The tissue preference is linked to structural patterns of the M proteins, and the bacteria can be divided into groups based on these patterns (90, 96). Pattern A-C serotypes consists of all four repeat regions and are associated with throat infection, pattern D serotypes lack the A repeat region and are associated with skin infection, and pattern E serotypes lack the B repeat region and are considered generalists causing both throat and skin infections to the same extent (97).

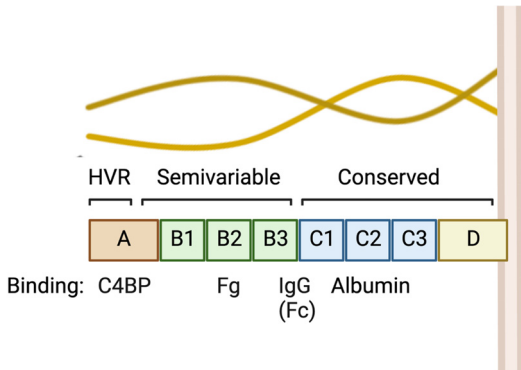

Figure 4. Schematic figure of the streptococcal M1 protein
The M1 protein consists of a four repeat regions called A, B, C and D, a highly conserved C-terminal region, including the C and D regions, a semivariable B region, and a hypervariable N-terminal region (HVR), including the A region. The N-terminal region contains the cell wall anchoring motif. The binding sites of complement 4b binding protein (C4BP) , fibrinogen (Fg), the Fc region of IgG and albumin are shown in the figure. Figure 4 was created with Biorender.

Figure 5. Schematic figure of the streptococcal groups that are based on structural patterns of the M proteins Pattern A-C serotypes, including M1, M3 and M5 protein, consists of all four repeat regions and are associated with throat infection, pattern D serotypes lack the A repeat region and are associated with skin infection, and pattern E serotypes, including M28, M49 and M89, lack the B repeat region and are considered generalists causing both throat and skin infections to the same extent. Figure 5 was created with Biorender.

M protein contributes to diverse aspects of bacterial pathogenesis: adhesion, invasion, and evasion of phagocytosis and complement (81). M proteins use distinct binding domains to interact with several host plasma proteins, and the different M protein patterns result in different binding repertoires of the M proteins. The binding repertoire of the *emm1* serotype M1 protein includes fibrinogen, albumin, the Fc domain of IgG, and the complement regulatory proteins factor H and C4BP, contributing to evasion of the complement system, opsonization and phagocytosis (98, 99). The M1 protein can be cleaved off from the bacterial surface by the streptococcal cysteine protease SpeB or by host-derived proteases (80, 100). The ability to shed a dominant surface protein during an infection may have important implications for the functional effects of this virulence factor during distinct phases of pathogenesis. The released M1 protein exhibits pro-inflammatory properties, including activation of neutrophils, monocytes, and T cells (80, 85, 101, 102). The released M1 protein can also mediate platelet activation, platelet/leukocyte complex formation, and modulation of the inflammatory response of neutrophils (86, 103). This might be a mechanism for the bacteria to cause distant thrombus formation and avoid bacterial entrapment in platelet aggregates. Platelet activation by M1 protein is dependent on binding of M1 protein together with plasma fibrinogen and specific anti-M1 IgG to the fibrinogen receptor and Fc receptor on the platelet surface (80). M protein-mediated platelet activation occurs in a donor-dependent fashion and correlates with the level of anti-M protein IgG present in the donor plasma (101). Some M proteins bind IgG1, IgG2 and IgG4, while others bind only IgG3, and only M22 and M28 have been shown to bind all four IgG subclasses (96). Mouse platelets lack the Fc gamma receptor IIa (FcγRIIA), which makes it difficult to study the Fc-dependent platelet activation in mouse models. However, FcγRIIA transgenic mice have been generated and could be a valuable tool to study this mechanism in-vivo (104).

Several M protein serotypes interact with fibrinogen via the B repeat region, resulting in evasion of phagocytosis and diminished complement activation at the bacterial surface, regardless of the presence of anti-M protein IgG (105, 106). The binding of fibrinogen and albumin to the surface bound M protein might result in sterical hindrance of IgG opsonization (107). The interaction with fibrinogen and the pro-inflammatory effects are dependent on the characteristic nonideal sequence of the M protein, that gives rise to specific irregularities in the coiled-coil structure. Furthermore, the specific irregularities of the M protein are similar to the structure of myosin and tropomyosin, which gives rise to cross-reactive antibodies in autoimmune conditions following *S. pyogenes* infection (108). The released M1 protein also interacts with fibrinogen via the B repeat region, and the M1 protein/ fibrinogen complex mediates neutrophil activation, degranulation and HBP release (80). HBP levels are elevated in sepsis patients and HBP release results in endothelial cell activation with subsequent vascular leakage, vasodilation, and bleeding (80, 109). The vascular leakage caused by the M1 protein/ fibrinogen complex is increased in the presence of anti-M1 protein IgG (101). M1 protein interacts with TLR2 on monocytes and mediates release of IL-6, IL-1β and TNF-α, as well as upregulation of tissue factor, resulting in a pro-inflammatory and procoagulant state (85). Collectively, the streptococcal M protein mediates a pro-inflammatory state and dysregulated coagulation and clot formation that contributes to the *S. pyogenes* pathogenesis during invasive infection.

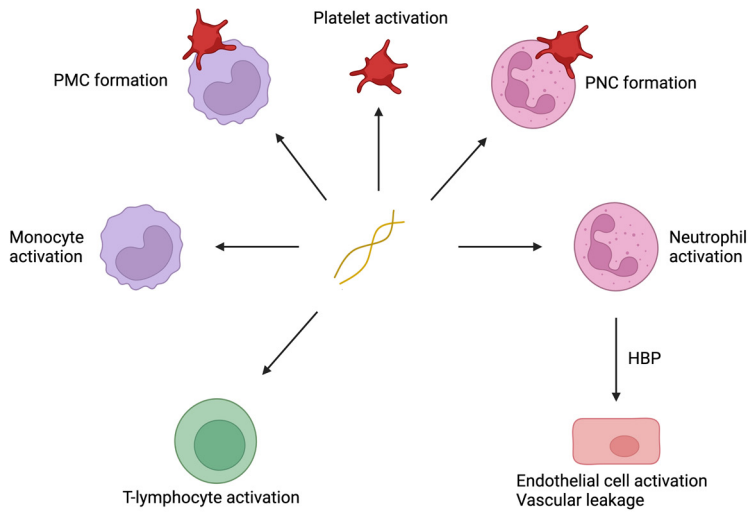

Figure 6. The pro-inflammatory effects of the streptococcal M1 protein

The M1 protein mediates platelet activation, platelet-neutrophil and platelet-monocyte complex (PNC and PMC) formation, and platelet-dependent modulation of the inflammatory response of neutrophils. The M1 protein also mediates activaton of neutrophils, resulting in degranulation and HBP release. The HBP release results in endothelial cell activation with subsequent vascular leakage, vasodilation, and bleeding. Furthermore, the M1 protein mediates activation of monocytes, resulting in pro-inflammatory cytokine release and procoagulant tissue factor upregulation. The M1 protein also mediates activation of T-lymphocytes. Figure 6 was created with Biorender.

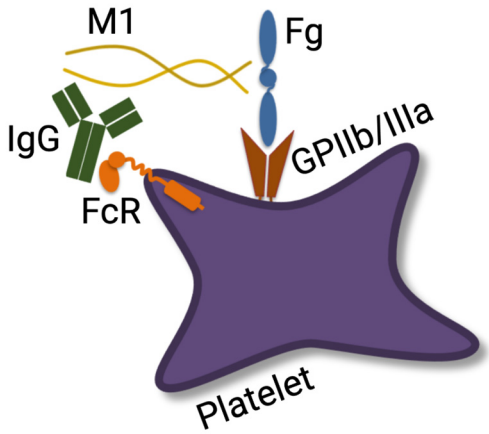

Figure 7. The mechanism of M1 protein-mediated platelet activation
Platelet activation by M1 protein is dependent on binding of M1 protein together with plasma fibrinogen (Fg) and specific anti-M1 IgG to the fibrinogen receptor GPIIb/IIIa and Fc receptor (FcR) FcγRIIA on the platelet surface.

Sepsis

Sepsis is a major global public health challenge, with 11 million sepsis-related deaths reported in 2017, representing approximately 20% of all deaths globally (110). In accordance with the Sepsis-3 definition, sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to bacterial, viral, or fungal infection (111), assessed using the Sequential Organ Failure Assessment (SOFA) score. Hallmarks of sepsis include dysregulated hemostasis and dysregulated inflammation.

Inflammation is normally controlled by a fine-tuned balance of pro- and anti-inflammatory signals, resulting in clearance of the invading pathogen, restored hemostasis, and tissue repair. The hallmarks of sepsis include disturbed hemostasis and a dysregulated systemic inflammatory state (112, 113). This is caused by an excessive systemic inflammatory response (SIRS), followed by the compensatory anti-inflammatory response (CARS). The initial pro-inflammatory cytokines in sepsis mediate prolonged and enhanced neutrophil and monocyte activation, which contributes to the dysregulated inflammation and to organ damage (111, 114). The activated neutrophils release ROS and NETs that contribute to organ damage. Furthermore, NETs are negatively charged and promote assembly and activation of the coagulation system, resulting in thrombin generation, platelet activation and thrombosis in response to inflammation and infection (115).

The excessive systemic inflammatory response mediates systemic activation of the endothelium, resulting in increased vascular permeability, vascular leakage, hypotension, and decreased oxygen supply to organs (116). The activated endothelium also mediates platelet adhesion, activation, and aggregation, resulting in thrombus formation throughout the body and disseminated intravascular coagulation (DIC) (117). DIC further contributes to the decreased oxygen supply to organs and organ damage (118). Tissue factor is upregulated on the activated endothelial cells and monocytes, further increasing the formation of stable fibrin-rich clots. The activated endothelium also releases vasoactive mediators, such as prostacyclin and nitric oxide, resulting in vasodilation and hypotension (119). The normal compensatory antidiuretic response, such as vasopressin release, is inhibited during sepsis. Furthermore, the systemic production of NO results in decreased response to vasoconstrictors in sepsis patients (119). Collectively, this results in hypotension, decreased oxygen supply to organs and organ damage. The

compensatory anti-inflammatory signals result in haltered clearance of the invading pathogen and increased susceptibility to secondary infections (120).

Sepsis diagnosis and treatment remains challenging due to the complexity and severity of the condition. Current sepsis treatment includes antibiotics, fluids, vasopressors, and ventilation. Drugs targeting inflammation, such as TNF-α blockers, have also been tested, however many are inefficient or fail clinical trials (121, 122). Therefore, there is a great need for further research focusing on both biomarkers and treatment of sepsis.

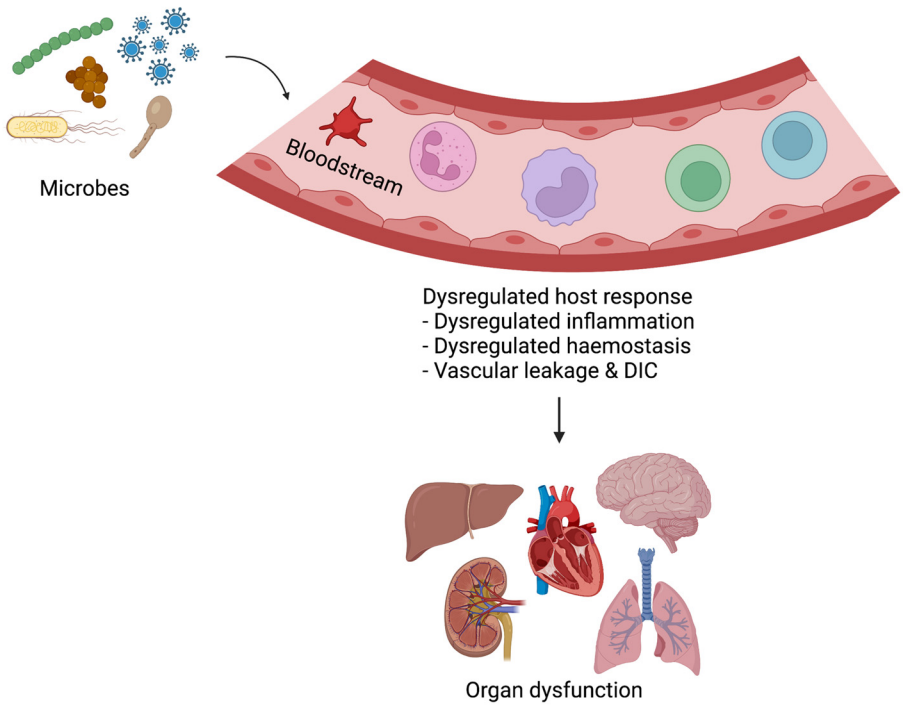

Figure 8. Schematic figure of the patophysiology of sepsis
Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to bacterial, viral, or fungal infection. The hallmarks of sepsis include disturbed hemostasis and a dysregulated systemic inflammatory state. This is caused by an excessive systemic inflammatory response (SIRS). The pro-inflammatory cytokines in sepsis mediate prolonged and enhanced neutrophil and monocyte activation, which contributes to the dysregulated inflammation and to organ damage. The activated neutrophils release ROS and NETs that contribute to organ damage. Furthermore, NETs are negatively charged and promote assembly and activation of the coagulation system, resulting in thrombin generation, platelet activation and thrombosis in response to inflammation and infection. SIRS mediates systemic activation of the endothelium, resulting in increased vascular permeability, vascular leakage, hypotension, and decreased oxygen supply to organs. The activated endothelium also mediates platelet adhesion, activation, and aggregation, resulting in thrombus formation throughout the body and disseminated intravascular coagulation (DIC). DIC further contributes to the decreased oxygen supply to organs and organ damage. The activated endothelium also releases vasoactive mediators, resulting in vasodilation and hypotension. Figure 8 was created with Biorender.

Platelets

Introducing platelets

Platelets are small (1-2 μm) cell fragments that circulate the blood stream in great numbers of 150 000 to 400 000 per μl of blood (123, 124). Platelets are produced by fragmentation of megakaryocytes, mainly in the bone marrow, and have a short life span of around 10 days before they are cleared in the liver and spleen (125). The main function of platelets is to maintain hemostasis by preventing bleeding in case of vascular injury. Under normal circumstances the platelets circulate in a resting state and do not interact with the vessel wall or with each other. However, in case of vascular injury platelets rapidly adhere with surface receptors to subendothelial ECM proteins, such as collagen and von Willebrand factor (vWF), that are exposed when the vessel is injured (126, 127). First, tethering occurs, followed by rolling, platelet activation, and finally firm platelet adhesion (128). Platelet activation results in increased cytosolic Ca^{2+} levels, cytoskeletal rearrangements, and granule release, which in turn results in recruitment and activation of more platelets (129, 130). The increased Ca^{2+} levels also result in translocation of phosphatidylserine (PS) from the inside to the outside of the plasma membrane, called membrane flip (131).

Table 1. Platelet receptors and their interacting ligands

Platelet receptor	Interacting ligand
FcγRIIA	IgG in immune complexes
GPIa/IIa	Collagen
GPIb	vWF
GPIb-IX-V complex	vWF, coagulation factors XI and XII
GPIIb/IIIa	vWF, fibrinogen
GPVI	Collagen
P2Y	ADP
PAR	Thrombin
P-selectin	PSGL-1, tissue factor

Platelet plug formation via fibrinogen bridges

The activated platelets adhere firmly to the matrix and to each other, via crosslinking of the platelet surface glycoprotein (GP) IIb/IIIa, also called integrin αIIbβ3, by fibrinogen. This crosslinking results in platelet aggregation and the formation of a platelet plug, or thrombus, which prevents bleeding at the site of injury (132-134). On resting platelets, GPIIb/IIIa has an inactive conformation that does not interact with fibrinogen. Upon platelet activation signals from within the platelets, so called inside-out signalling, results in activation of GPIIb/IIIa to an active conformation that binds fibrinogen with high affinity. Fibrinogen binding results in clustering of GPIIb/IIIa and signals into the platelet, so called outside-in signalling, that results in platelet aggregation (135). This platelet-driven hemostasis is called primary hemostasis. Vessel injury also exposes tissue factor (TF), which initiates a cascade of protease activation of coagulation factors that will result in thrombin generation (128). This coagulation cascade in called secondary hemostasis. Tissue factor is also expressed on monocytes, and the expression of tissue factor is upregulated upon monocyte activation, increasing their procoagulant activity (136). The primary and secondary hemostasis promote each other. Activated platelets expose phosphatidylserine on their surface that provides a negatively charged binding surface for coagulation factors, and coagulation factors in turn mediate further platelet activation via generation of the platelet agonist thrombin.

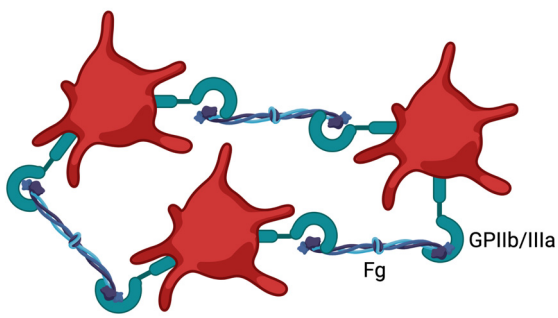

Figure 9. Illustration of a platelet plug
Platelet activation results in activation of the platelet surface glycoprotein (GP) IIb/IIIa to an active conformation that binds fibrinogen with high affinity. The activated platelets adhere firmly to the matrix and to each other, via crosslinking of GPIIb/IIIa by fibrinogen (Fg). This crossbinding results in platelet aggregation and the formation of a platelet plug, or thrombus. Figure 9 was created with Biorender.

The platelet agonist thrombin in hemostasis and inflammation

Secondary hemostasis results in generation of thrombin. Thrombin cleaves fibrinogen into fibrin, that will stabilise the thrombus (128). Thrombin is also a potent platelet agonist that binds to protease-activated receptors (PARs) on the platelet surface. Thrombin activates the receptor by proteolytic cleavage. Thrombin activation of platelets results in platelet aggregation and degranulation. Upon platelet activation membrane phospholipids, such as phosphatidylserine, are exposed on the platelet surface. The anionic phospholipids provide a negatively charged surface that binds coagulation factors, resulting in increased thrombin generation and an increased platelet procoagulant activity. Thrombin also binds to PARs on endothelial cells and mediates release of von vWF and chemokines, and expression of the endothelial and platelet surface molecule P-selectin, resulting in recruitment of platelets and leukocytes to the endothelium. Thrombin also mediates increased endothelial vascular permeability and endothelial-dependent vasodilatation, facilitating inflammation (136). Thus, thrombin signalling via PARs is important in both hemostasis and inflammation.

Regulating platelet plug formation

To prevent uncontrolled platelet thrombus formation there are inhibitory signals, such as nitric oxide, prostacyclin, and prostaglandins, that are produced by platelets and endothelial cells upon activation and limit platelet activation, platelet aggregation and thrombus formation in a negative feedback loop (137, 138). Furthermore, plasminogen will be cleaved into plasmin, that in turn will cleave fibrin and help degrade the thrombus (128). The generation of thrombin is also regulated, as thrombin activates the protein C system, a system that regulates the coagulation cascade, to terminate its own production (136). The coagulation system is also regulated by the tissue factor pathway inhibitor (TFPI).

Platelet granules

Platelet activation results in platelet granules fusing with the membrane and release of the granule content. The granules contain an array of molecules that mediate recruitment and activation of additional platelets, resulting in amplification of the platelet activation process (134). Platelet granules are divided into three types: α-granules, dense granules (δ-granules) and lysosomes (λ-granules). The α-granules are the most abundant of the granules, and contain various heterogenous cytokines,

such as IL-1, chemokines, such as platelet factor 4 (PF4) and monocyte chemotactic protein (MCP), and growth factors, such as and platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF-β). The α-granules also contain pro-inflammatory molecules, such as CD40 ligand (CD40L), prothrombotic molecules, such as vWF and fibrinogen (fg), and the platelet receptors GPIIb/IIIa, GPIb and CD62P, or P-selectin, that are upregulated at the platelet surface upon platelet activation and granule release (139-141). Dense granules contain fewer, more homogenous proteins, including Ca^{2+} ions, histamine, serotonin, and nucleotides, such as adenosine diphosphate (ADP), a major platelet agonist that generates a feedback amplification loop by activating additional platelets. Lysosomes contain various enzymes, such as serine peptidases, carbohydrases and phosphatases (142). Different granules of the same type contain different cargo, and can result in differential chemotactic, thrombotic, and coagulative effects depending on stimuli and cargo (140, 143, 144). For example, it has been shown that the proangiogenic protein vascular endothelial growth factor (VEGF) and the antiangiogenic protein endostatin are separated in distinct subpopulations of alpha-granules. The prothrombotic molecules vWF and fibrinogen have been shown to be stored both in separate and distinct alpha-granules (140, 143). Thus, selective cargo sorting can result in either enhanced or contradictory effect based on the granule subpopulation released.

Platelet cytoskeleton

The platelet cytoskeleton consists of cytoplasmic actin filament that mediate contractile events, and membrane skeleton that stabilises the plasma membrane. Platelet activation results in cytoskeletal reorganisation and a platelet shape change. Resting platelets have a discoid shape and a majority of the actin is monomeric. Platelet activation results in actin polymerisation and cytoskeletal reorganisation occurs, which results in a shape change of the platelets where pseudopodia extend from the platelet surface (145). The shape change increases the area of the external platelet surface, which facilitates interaction with matrix proteins and other platelets, and thereby increases platelet aggregation (128). The platelet membrane has unique invaginations that form an open canalicular system (OCS), that facilitates both transport of molecules into the platelets and platelet granule release (146).

Platelet derived extracellular vesicles

Extracellular vesicles (EVs) are small vesicular bodies (30 nm to 1 μm) that are derived from the cell membrane of various cells upon activation or injury (147). A majority (70-90%) of the circulating EVs in blood are derived from platelets (131). Platelet derived EVs (PEVs) contain a wide range of proteins and present platelet-specific surface antigens. PEVs are continuously produced and help to maintain hemostasis (148). The pro-coagulative nature of PEVs is due to exposed surface phosphatidylserine, a negatively charged molecule that is normally located on the intracellular surface of the platelet plasma membrane, that provides a binding surface for coagulation factors that generate thrombin through the coagulation cascade (149, 150). Furthermore, PEVs bind fibrinogen, collagen and von Willebrand factor and co-aggregate with platelets (148, 150). Some PEV components are selectively enriched compared to platelets. For example, PEVs contain high local concentrations of PS resulting in a PEV surface that is approximately 50- to 100-fold more procoagulant than the platelet surface. Many surface receptors, such as P-selectin, are also enriched on PEVs compared to platelets (131). PEVs function as signal mediators and contain several cytokines and chemokines, such as IL-1, thromboxane A2, surface receptors, such as CD40L, RNA, transcription factors and even mitochondria (151-153). Since PEVs readily circulate they can act as an efficient transfer system to target cells both locally and systemically. For example, PEVs can interact with the endothelium via P-selectin and interact with several immune cells via PF4, CD40L and IL-1β (131). The PEVs can be selectively sorted and packaged and the content of PEVs may differ depending upon the platelet agonist mediating platelet activation (154, 155). Agonists that stimulate a strong platelet activation, such as thrombin and collagen, result in PEVs that contain higher levels of proteins involved in platelet activation and degranulation than agonists that stimulate a weaker platelet activation, such as ADP (155). For example, the enrichment of P-selectin on PEVs is a result of agonist-induced platelet granule release upon activation, and PEVs generated in response to calcium ionophore A23187 contain α-granule proteins, such as platelet factor 4 and fibrinogen, whereas PEVs generated in response to dibucaine do not. Similarly, PEVs generated in response to thrombin and collagen express GPIIb/IIIa, whereas PEVs generated in response to the MAC complex do not (131). LPS stimulation of platelets via TLR4 generates PEVs that contain increased levels of IL-1β compared to PEVs from resting platelets. PEVs generated in response to LPS mediate increased VCAM-1 expression on endothelial cells, compared to PEVs from resting platelets (151). Staphylococcal superantigen-like protein 5 (SSL5) also activates platelets and generates PEVs that bind to monocytes, resulting in monocyte aggregation, migration and release of cytokines, such IL-1β, tumor necrosis factor-α (TNFα), and chemokines, such as MCP-1 and matrix metalloproteinase-9 (MMP-

9). The number of circulating PEVs is increased in several conditions, such as thrombosis, and levels corelate with disease progression (156).

Platelets and coagulation in the immune response

In addition to their classical role in maintaining hemostasis, platelets also have an emerging role in the immune response. This is a relatively new discovery, and most immunology books still don't mention platelets as immune cells. From an evolutionary perspective hemostasis and immune defence goes hand in hand, and invertebrates have cells called hematocytes that are responsible for both coagulation and immune response (157). Immunothrombosis is a new concept that links coagulation with the immune system, that refers to the role of thrombosis in the immune system. Immunothrombosis is characterized by the involvement of innate immune cells, mainly monocytes, neutrophils and DCs, in clot formation. The immune cells are recruited by pathogen-activated platelets and endothelial cells, and contribute to clot formation by mediating both further platelet activation and fibrin formation. Platelets interact with the recruited immune cells and mediate their immune functions. Monocyte activation mediated by pathogen or PAMPs results in increased expression of tissue factor at the monocyte surface, further promoting coagulation. Similarly, pathogen-mediated neutrophil activation results in NET formation. NETs trap and kill bacteria and provide negatively charged surfaces that bind coagulation factors. Fibrin clots also trap bacteria, and it has been shown that fibrin exhibits direct antimicrobial effects. Fibrin and fibrinogen also bind to and activate leukocytes. In conclusion, immunothrombosis contributes to recognition, entrapment and killing of pathogens. However, dysregulated immunothrombosis can result in pathological clot formation and vessel obstruction, such as myocardial infarction, stroke and DIC (158).

Platelet interactions with bacteria

Platelets express pattern recognition receptors and can interact both directly with many pathogens as well as with their released factors (159, 160). For example, platelets express TLR4, that binds LPS from gram negative bacteria such as *Escherichia coli* (*E. coli*) (159). Platelets also express Fc receptors and can recognise immune complexes and opsonized bacteria (161, 162). For example Fc receptors bind IgG opsonized dengue virus, mediating thrombocytopenia during dengue virus infection (160). The platelet fibrinogen receptor GPIIb/IIIa also interacts with bacteria, with fibrinogen as a bridging molecule (163, 164). For example, extracellular fibrinogen-binding protein (Efb) from *Staphylococcus*

aureus (*S. aureus*) binds fibrinogen that can bind GPIIb/IIIa with fibrinogen as a bridging molecule (165). Platelet interaction with bacteria and bacterial compounds results in platelet activation, degranulation, and aggregation (160, 165). Activated platelets can directly kill pathogens by trapping them and with anti-microbial peptides (166-169). It has been shown that platelets trap *E. coli, S. aureus, and S. pyogenes* in fibrin-rich clots, and thereby prevents bacterial dissemination (166, 170). The platelet-bacteria aggregates are then removed by resident immune cells of the spleen and liver (171). The platelets can kill trapped bacteria by FcγRIIA-dependent uptake of opsonized bacteria or with released antimicrobial peptides (169, 172). Platelets efficiently kill both *E. coli* and *S. aureus*, whereas *Streptococcus pneumoniae* (*S. pneumoniae*) and *S. pyogenes* are resistant to killing by platelet-derived antimicrobial peptides (170, 173).

The platelet FcγRIIA receptor

As mentioned above, many bacteria and bacterial compounds interact with platelets via FcγRIIA on the platelet surface. The interaction with FcγRIIA is dependent on specific IgG against bacterial antigens, and specific antibodies against bacterial proteins, such as clumping factor from *S. aureus* and M1 protein from *Streptococcus pyogenes*, have been identified in serum from healthy individuals (101, 174). FcγRIIA is unique to higher primates and is expressed on various human cells, such as monocytes, neutrophils, dendritic cells (DCs). FcγRIIA is the only Fc receptor expressed on human platelets and the structure of FcγRIIA is unique, as it is a single-chain receptor, compared to the multichain structure of other FcRs. Binding of IgG containing immune complexes to FcγRIIA results in receptor activation via phosphorylation of an immunoreceptor tyrosine-based activation motif (ITAM) in the cytoplasm, which is characteristic for immune receptors. Activation of FcγRIIA results in increases in cytosolic calcium levels, platelet activation, aggregation, and granule release. However, platelet aggregation and granule release in response to bacteria is dependent on GPIIb/IIIa co-stimulation. Additionally, FcγRIIA can amplify platelet activation in response to GPIIb/IIIa activation in an IgG-independent fashion. FcγRIIA can also be activated in response to autoimmune related immune complexes, for example in systemic lupus erythematosus (SLE), resulting in platelet activation that contributes to the autoimmune pathogenesis. Interestingly, FcγRIIA expression vary between individuals and can also vary within individuals during disease (161, 174).

Platelet interactions with immune cells and complement

Upon activation, platelets release factors that can mediate recruitment and activation of leukocytes. For example, platelet factor 4 can recruit monocytes and mediate monocyte differentiation. Similarly, platelet CXCL7 can recruit neutrophils and levels of CXCL7 has been shown to be elevated in mice suffering from SIRS and associated with platelet-mediated NET formation (175). Furthermore, platelets can interact directly with leukocytes, via P-selectin on the platelet surface binding to P-selectin glycoprotein ligand-1 (PSGL-1) on the leukocyte surface (175), and mediate formation of platelet-neutrophil and platelet-monocyte complex (PNC and PMC) formation. Platelet-leukocyte interaction result in increased phagocytosis, ROS production and NET formation, as well as increased surface expression of TF, pro-inflammatory cytokine synthesis and formation of EVs of monocytes (175, 176). Platelets also mediate cytokine and chemokine release by endothelial cells. For example, platelet CD40L mediates release of the chemokines IL-8 and MCP-1 by endothelial cells, resulting in recruitment of leukocytes, PNC, and PMC formation (175). Activated platelets also release complement components C8 and C9, which will mediate formation of the MAC complex. The MAC complex induces TF and wVF release from the endothelium, which initiates coagulation system activation and further platelet activation (177, 178).

Furthermore, factors released upon platelet degranulation recruit DCs, promote dendritic cell maturation and increase their antigen presentation capacity (179). For example, platelet CD40L mediates release of IL-6 from DCs and enhances DC maturation, resulting in increased killing of bacteria, such as *S. aureus* (175). Platelets also interact directly with DCs via P-selectin on the platelet surface binding to PSGL-1 on the DC surface, enhancing DC maturation, or via platelet CD40L binding to CD40 on the DC surface, increasing the phagocytosis by DCs. Furthermore, platelets express the antigen presentation molecule MHC class I on their surface, suggesting that platelets are involved directly in antigen presentation (180). Platelets interact with T-lymphocytes and B- lymphocytes, both directly and via released factors, and modulate their function (181). For example, platelet factor 4 can recruit T-lymphocytes and promote their activation (175). Activated platelets can bind the complement component C1q and mediate activation of the classical complement system (177). Collectively, this highlights the important role of platelets as bridging cells between coagulation and the immune system.

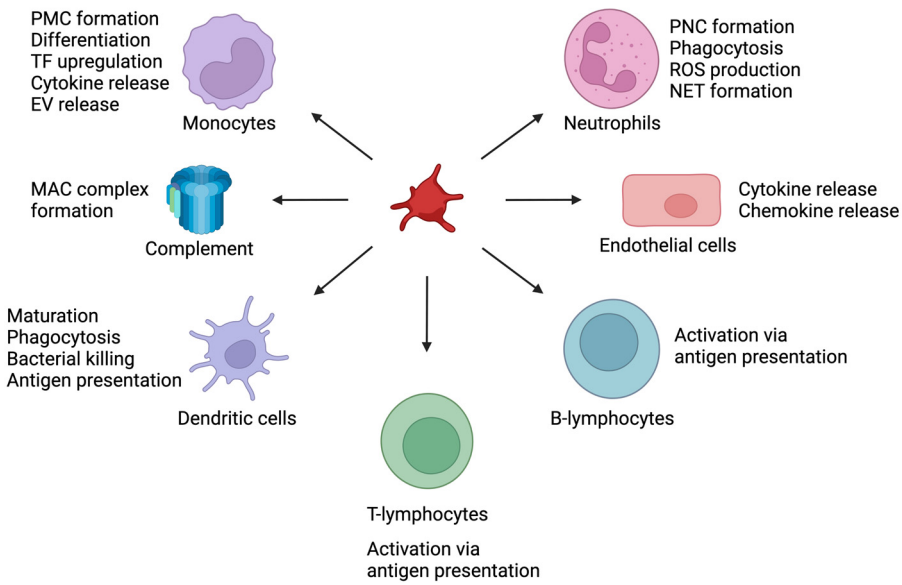

Figure 10. Platelet immunomodulation
Platelets interact with neutrophils and monocytes, directly in platelet-neutrophil and platelet-monocyte complexes (PNC and PMC) or via released factors, and mediate neutrophil extracellular trap (NET) formation, phagocytosis and reactive oxygen species (ROS) production, as well as differentiation, upregulation of tissue factor (TF), pro-inflammatory cytokine release and formation of extracellular vesicles (EVs) of monocytes. Platelets also interact with endothelial cells, directly or via released factors, and mediate cytokine and chemokine release by endothelial cells. Furthermore, platelets release complement components C8 and C9, which will mediate formation of the MAC complex, and activated platelets bind the complement component C1q and mediate activation of the classical complement system. Platelets also interact with dentritic cells (DCs), directly or via released factors, and promote dendritic cell maturation, increased antigen presentation capacity, increased killing of bacteria, and increased phagocytosis by DCs. Platelets express the antigen presentation molecule MHC class I on their surface, participate in antigen presentation and promote the activation of T-lymphocytes and B-lymphocytes. Figure 10 was created with Biorender.

Platelets in sepsis

Platelets link coagulation, inflammation, and infection and play an important role in the dysregulated inflammation and coagulation observed during sepsis. Platelet activation is increased in sepsis, and platelet aggregation to ex-vivo stimulation is decreased in sepsis patients, suggesting that platelet activation has occurred in-vivo (182, 183). Endothelial activation and vascular leakage are hallmarks of sepsis, resulting in platelet activation and aggregation. Platelet adhesion to the activated endothelium results in fibrin clot formation, contributing to DIC and subsequent organ damage (184). Upon activation, platelets release CD40L, which mediates further endothelial activation, creating a viscous circle (185). Platelets also release serotonin, that can cause vasodilation and hypotension, resulting in systemic shock

(186). Furthermore, consumption of platelets and coagulation factors eventually results in an increased risk of bleeding in sepsis patients (118).

Platelet activation also mediates leukocyte recruitment, platelet-leukocyte complex formation, and modulation of neutrophil and monocyte function (175). In sepsis models, platelet-leukocyte complexes accumulate in organs, such as the lungs and the liver, and PNC formation corelate with organ damage (187, 188). Neutrophils in PNCs are activated in a platelet-dependent manner, and exhibit NET formation, which results in trapping and killing of bacteria, such as *E. coli* and *S. aureus* (189, 190). However, NETs also mediate further platelet activation, thrombosis, and organ damage (191, 192). The M1 protein from *S. pyogenes* also mediates PNC formation in a fibrinogen-dependent manner, however the neutrophils in these complexes are functionally impaired and don't produce NETs (103). PMC formation is also observed in sepsis, and the monocytes in PMCs show increased endothelial adhesion, increased procoagulant activity, such as TF production, and increased cytokine release (193, 194). Platelet activation mediates production of both pro- and anti-inflammatory cytokines, which contributes to the cytokine storm and dysregulated inflammation observed during sepsis (195).

Thrombocytopenia is common (20–60%) in sepsis patients and correlates with a poor prognosis. Thrombocytopenia is included as a measure of hematological failure in the SOFA score (196, 197). The causes of thrombocytopenia in sepsis are not fully understood, but decreased platelet production and increased platelet activation and consumption may contribute to thrombocytopenia (198). Activated platelets are rapidly cleared, and therefore platelet activation decreases the life span of platelets (125). Some virus and bacteria can cause platelet apoptosis, which could also contribute to thrombocytopenia (199, 200). It has also been suggested that apoptosis generally follows platelet activation, and that platelet apoptosis results in increased phagocytosis of platelets by monocytes, activation of the coagulation system, thrombin production and thrombosis (201-203). Anti-platelet antibodies, such as anti-PF4, are detected in sepsis patients and could result in immune complex-mediated platelet destruction and clearance (204, 205). In sepsis, platelets are accumulated in organs and in fibrin-rich thrombi, which could also contribute to the thrombocytopenia observed in sepsis patients. Recent studies show an increase in immature platelets in patients with severe sepsis, which indicates that platelet production is not decreased but rather increased during sepsis (206). Furthermore, the number of megakaryocytes in the bone marrow remains normal, and the level of thrombopoietin (TPO) increases, during sepsis (207, 208). Collectively, these findings suggest that the thrombocytopenia observed in sepsis patients is due to increased platelet activation, consumption, and clearance, and not due to decreased platelet production.

Platelets play a protective role in host defence during sepsis but can also contribute to organ damage and dysfunction. Sepsis models with platelet depletion prior to infection has been carried out to investigate the role of platelets is sepsis further. In

a sepsis model with *Klebsiella pneumoniae* (*K. pneumoniae*) infection platelet depletion resulted in increased bacterial load and increased host mortality, suggesting a protective platelet role in sepsis (209). However, in a model with *S. pyogenes* infection platelet depletion resulted in decreased bacterial survival and dissemination, suggesting a detrimental effect of platelets in sepsis (87). Platelet activation and platelet-leukocyte complex formation is higher in patients with sepsis caused by gram-positive bacteria, as compared to gram-negative bacteria, suggesting that the platelet response differs depending on pathogen (210).

PEVs exhibit immunomodulatory effects by transfer of bioactive cargo to target cells, such as leukocytes and endothelial cells, resulting in target cell activation (211). PEVs recruit leukocytes to the site of infection and mediate leukocyte interaction and cytokine release (212, 213). PEVs also contribute to innate and adaptive immunity through antigen presentation via MHC-1 molecules, and have access to the lymphoid organs and to the bone marrow (214). The number of circulating EVs increase in a number of pathological states, such as inflammation and sepsis (156, 215). Interestingly, PEVs seem to have a protective role in sepsis, and low levels of circulating PEVs corelate with both thrombocytopenia and sepsis mortality (216, 217).

General methodology

Mass spectrometry

Mass spectrometry (MS) is used to measure the proteome, i.e., the whole protein set expressed from the genome, or at least parts of the proteome at a given time. The proteome can be measured as intact proteins, called top-down proteomics, or as peptides from digested proteins, called bottom-up proteomics (218, 219). The mass spectrometer performs better at measuring smaller units, and therefore bottom-up proteomics is more common. Trypsin is the enzyme most used to digest proteins into peptides in mass spectrometry samples, as it generates peptides that are easily ionised and that are of ideal length for the mass spectrometer that measures the mass-to-charge (m/z) ratio of ions (220). Before entering the mass spectrometer the peptides are separated, most commonly based on their hydrophobic properties with a liquid chromatography (LC) system directly connected to the mass spectrometer (221). Importantly, the mass spectrometer struggles to analyse large numbers of peptides at the same time, and the separation allows the peptides to enter the mass spectrometer at different times. Furthermore, this retention time will facilitate the identification of the peptide. The separated peptides are ionised, most commonly using electrospray ionisation (ESI), a technique that resulted in the Nobel prize in chemistry in 2002 (222). Finally, the ionised peptides enter the mass spectrometer and data acquisition commences.

Data-dependent acquisition (DDA), or Shotgun MS, aims at identifying as many peptides as possible, and m/z ratios and peptide abundances are measured in MS1 scans. Each peak in the MS1 spectrum could result from multiple combinations of amino acids in the peptide sequence, giving rise to the low sensitivity and poor reproducibility of DDA. To improve this, the ionised peptides with the highest intensities, called precursor-ions, are fragmented by collision with an inert gas, most commonly using higher energy collisional disassociation (HCD) (223). The fragmented ions are measured again, generating a MS2 spectrum that is used for peptide identification and abundance. In data-independent acquisition (DIA), repeated cycles of MS1 scans result in the generation of multiple MS2 spectra that are used to identify a complete set of fragment ions. Thus, DIA aims at constructing a near-to-complete digital archive of the whole ionisable peptide content of the sample and circumvents the limitations of DDA where only the most intense ions are measured. The peptide annotation in DDA is done by comparison to a sequenced

genome, theoretically digested by the enzyme used to predict the peptides present in the sample (224). Since the peptide annotation is based on comparing experimental spectra with predicted spectra, the false discovery rate (FDR) is determined to validate the data statistically. A decoy dataset is generated by reversing the sequence of all predicted peptides. The experimental spectra is searched against the decoy dataset, and an FDR score is calculated based on the random matches with the decoy dataset (225). The peptide annotation in DIA is based on spectral libraries that are commonly generated through several DDA analyses preceding the DIA analysis (226). Therefore, only peptides identified in the DDA analyses and in the concomitant spectral libraries will be included in the final DIA analysis, and multiple DDA analyses are performed to increase the included peptides. In DIA the peptide quantification is based on fragmented ions at MS2 level, which results in a more precise quantification than in DDA, where the quantification is based on ionised peptides at MS1 level. Even more precise peptide quantification, better reproducibility and consistency can be achieved with targeted mass spectrometry, such as selected reaction monitoring (SRM). In SRM, pre-selected peptides are measured and compared to reference peptides within the sample, so called label-free quantification, or to reference peptides that are added to the sample. The reference peptides that are added to the sample are often labelled with heavy isotope tags, and the intensities of heavy and light peptides are measured and compared to quantify the target peptides (227).

Finally, the peptides are mapped to proteins and some peptides, so called non-proteotypic peptides, could belong to multiple proteins. The non-proteotypic peptides are normally excluded from the analysis, since it is difficult to determine which protein they originate from, and whether one or several of those proteins are present in the sample. Thus, the non-proteotypic peptides were excluded from the analysis in this thesis and only peptides that could be uniquely mapped to a single protein, so called proteotypic peptides, were included in the analysis.

Bottom-up proteomics and liquid chromatography tandem mass spectrometry (LC-MS/ MS) was used throughout this thesis. DDA followed by SRM was performed to identify and quantify the plasma proteins that interacted with the M1 protein in paper I. DDA followed by DIA was performed to identify and quantify the proteins that interacted with the M proteins in plasma in paper II, were associated with platelet-derived extracellular vesicles in paper III and were present in the organs in paper IV.

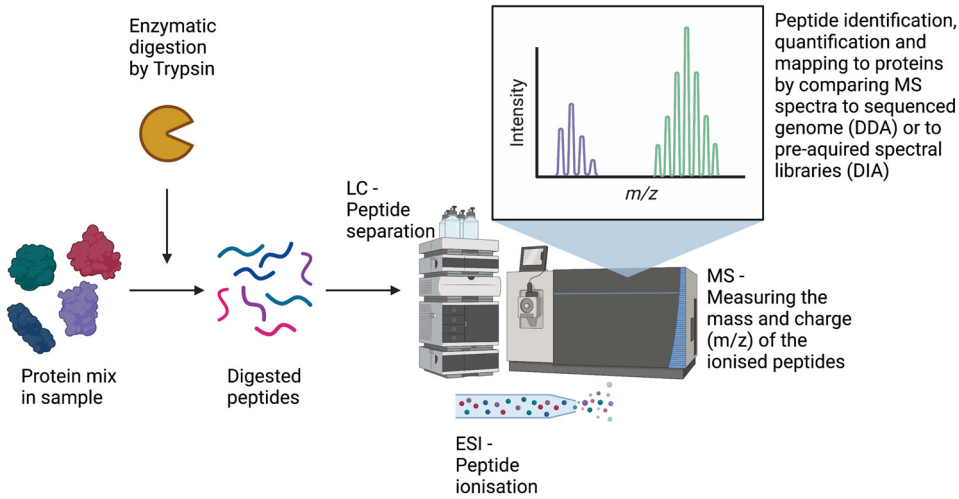

Figure 11. Bottom-up proteomics with liquid chromatography tandem mass spectrometry (LC-MS/ MS) work flow

Proteins in the sample are digested into peptides by an enzyme, most commonlyTrypsin. Before entering the mass spectrometer the peptides are separated, most commonly based on their hydrophobic properties with a liquid chromatography (LC) system directly connected to the mass spectrometer. The separated peptides are ionised, most commonly using electrospray ionisation (ESI), and finally the ionised peptides enter the mass spectrometer. The mass spectrometer measures the mass-to-charge (m/z) ratio of ions and the resulting MS spectra is compared to a sequenced genome in data-dependent acquisition (DDA) or to pre-aquired spectral libraries in data-independent acquisition (DIA), for peptide identification, quantification and mapping to proteins. Figure 11 was created with Biorender.

The extracellular vesicles that were characterized in paper III were isolated from platelets in platelet-rich plasma (PRP). Preliminary work aimed to characterize the proteins that were associated with extracellular vesicles derived from isolated washed platelets to avoid a high background signal from plasma. Therefore, a protocol for washing platelets and stimulating washed platelets with M1 protein was successfully established. Platelet activation was detected as CD62P upregulation using flow cytometry. Furthermore, vesicles derived from M1 stimulated washed platelets were isolated and investigated using DDA and DIA mass spectrometry. However, M1 protein-mediated platelet activation is dependent on fibrinogen and IgG, which was therefore added to the washed platelets prior to M1 protein stimulation. Unfortunately, the intense fibrinogen and IgG signals masked the signals from the less abundant proteins that were associated with the platelet derived extracellular vesicles. As a result of recent development of a novel method for isolating vesicles from more complex samples such as plasma, extracellular vesicles were isolated from platelets in platelet-rich plasma instead.

Flow cytometry

In this thesis flow cytometry was used to study platelet activation, immunomodulation, and extracellular vesicle production. Flow cytometry is used to analyse single cells in a solution that flow past lasers. As the cells flow past the lasers they give rise to visible light scatters in the forward direction, so called forward scatter (FSC), and at 90°, so called side scatter (SSC), that indicates the relative cell size and granularity, respectively. Furthermore, the cells can be labelled with fluorescent antibodies, dyes or proteins that emit fluorescent light. Dichroic filters steer fluorescent light at specific wavelengths to detectors (228). When combining different fluorescence labels, the fluorescent light at different labels is detected by different filters, or channels. There is often some spillover, or spectral overlap, between the different channels, especially between channels that detect light at wavelengths close to each other. The spectral overlap can be adjusted for by correcting the fluorescent signal in one channel based on the signal in the other channels. This is called compensation and can either be done manually or, more common nowadays, automatically (229). The fluorescent signals are converted into electronic signals that can be computationally analysed. The different cell populations can be analysed based on their light scatter and fluorescent signals. The cell populations are first gated based on their light scatter and/ or fluorescent signals, and other parameters are investigated within the gates. Additionally, the cell count of the different populations can be determined, either without an internal reference in an instrument calibrated to generate volumetric counts or by comparing to fluorescent beads of a known concentration that are added to the sample (228).

In this thesis the platelets were gated based on their forward scatter and side scatter, and/ or fluorescent signals of platelet-specific markers. Other parameters, such as the platelet count, were investigated within this gate. Fluorescent antibodies were used to detect receptors and proteins at the platelet surface, to investigate platelet activation, complement activation, platelet apoptosis and phagocytosis of platelets. Furthermore, neutrophils and monocytes were gated and counted based on their forward scatter and side scatter, and/ or fluorescent signals of neutrophil or monocyte-specific markers, respectively. Fluorescent antibodies were used to investigate neutrophil and monocyte activation as well as platelet positive neutrophils and monocytes in platelet-neutrophil and platelet-monocyte complexes. Flow cytometers with enhanced sensitivity can detect smaller particles, such as extracellular vesicles (228). In this thesis, a flow cytometer with enhanced sensitivity was used to detect and count platelet-derived extracellular vesicles based on their forward scatter and fluorescent signal of a platelet marker.

Fluorescence microscopy

In fluorescence microscopy, particles, cells, or tissue on microscopy slides are detected with fluorescent antibodies, proteins, or dyes. The fluorochromes are excited with excitation light and emits fluorescence light at a specific wavelength, emission light. Since the emitted fluorescent light is weaker than the excitation light, filters are used to block the bright excitation light. The absorbed excitation light is redirected to the specimen by a dichroic mirror and blocked by excitation filters. Emission filters direct emitted fluorescent light at specific wavelengths to the detector. The most common type of fluorescence microscopy is wide-field microscopy, where the whole specimen is illuminated simultaneously to excite the fluorophores in the specimen to emit fluorescent light. In wide-field microscopy the emitted fluorescent light is detected by a camera. Wide-field microscopy is ideal for thin specimens, such as single cell layers. The image quality is decreased if the specimen contains fluorescent signals above or below the plane of focus, which can be the case in thick specimens such as tissue (230). To include the fluorescent signals outside the plane of focus, and thereby improve image quality, deconvolution can be utilised. In deconvolution, a series of images along the z-axis are recorded and analysed together computationally. Thus, the information outside the plane of focus is included and incorporated in the final image (231).

In this thesis wide-field fluorescence microscopy was used to visualize complement activation at the platelet surface and phagocytosis of platelets by monocytes in paper I. Fluorescent antibodies were used to detect platelet-specific markers and complement components at the platelet surface. A fluorescent DNA-binding dye was used to detect the monocyte nuclei and multiple fluorescent antibodies, added before and after phagocytosis, were used to detect intracellular and extracellular platelets, respectively. Furthermore, the technique was used to detect, visualize, and count platelets in organs in paper IV. In paper IV, platelets were detected in tissue, which resulted in fluorescent signal outside the plane of focus and decreased image quality. To include the fluorescent signals outside the plane of focus, and thereby improve image quality, deconvolution was applied in paper IV.

Platelet function assays

In addition to using flow cytometry to measure platelet activation, platelet aggregation and granule release was investigated to determine the platelet function. Platelet aggregation was investigated ex vivo, using an aggregometer. This technique used was light transmission aggregometry that monitors platelet aggregation of platelets in platelet-rich plasma upon exposure to platelet agonists, such as collagen, thrombin, ADP, and/ or thromboxane A2, while stirring (232). The

aggregometer measures the light transmission, and platelet aggregation in platelet rich plasma results in increased light transmission through the solution. The platelet aggregation can be quantified as percentage platelet aggregation and lag time, which is the time to initiated platelet aggregation. Platelet aggregation is used both experimentally and clinically, since it can be used to detect platelet abnormalities in certain medical conditions, such as autoimmune disorders, genetic bleeding disorders, medication side effects, leukemia and kidney disease (232). In paper II of this thesis, platelet aggregation in platelet-rich plasma was monitored in response to collagen and streptococcal M proteins, using platelet aggregometry.

In the same paper, platelet granule release in response to streptococcal M proteins was measured using enzyme-linked immunosorbent assay (ELISA). ELISA generally refers to any assay that uses an enzyme-linked conjugate and enzyme substrate to generate a color change to show antigen-antibody interaction. The method is used to detect and measure antibodies that are specific for a selected antigen or an antigen that is specific for a selected antibody. The selected antigen or antibody is absorbed to a surface, such as to the bottom of wells in a microplate, and is used to capture the corresponding antigen or antibody in a sample. The captured antigen or antibody can either be directly enzyme tagged or detected with a secondary enzyme tagged antibody. Washing is applied to remove unbound antigens or antibodies. Finally, a substrate is added to generate a colour change, indicating antigen-antibody interaction. The results are read on a spectrophotometer at a specific wavelength that corresponds to the conjugate characteristics (233). In paper I and paper II of this thesis, an in-house ELISA was performed to measure levels of M protein-specific IgG in plasma. A microplate was coated with the M proteins and was incubated with plasma from healthy donors. The M protein-specific IgG in the plasma was then detected by a secondary antibody that was tagged with the enzyme horseradish peroxidase (HRP). Subsequent incubation with a substrate resulted in a colour change, and the absorbance was measured with a spectrophotometer. In paper I, the plates were precoated with fibrinogen to inhibit IgG binding with the Fc region to the M1 protein. In paper II, the IgG cleaving enzyme IdeS was used to cleave IgG, and only the Fab bound fragments were detecting using an anti-Fab antibody. Commercial ELISA kits were used in paper II to measure platelet granule release and in paper IV to measure levels of organ damage markers. The commercial ELISA kits were sandwich ELISAs, which refers to that a capture antibody was used to capture the antigens in our samples and an enzyme-tagged detection antibody was used to detect the antigens. Thus, the antigen is placed between two antibodies and resembles a sandwich. The sandwich approach has been shown to increase the sensitivity, compared to other ELISAs (233). ELISA is also used clinically, and can be used to measure anti-platelet antibodies, platelet surface markers and platelet granule release. This information can aid diagnosis of several medical conditions (233).

Acoustic trapping

In this thesis, acoustic trapping was used to isolate platelet-derived extracellular vesicles. The most common way to isolate extracellular vesicles is by ultracentrifugation, where large sample volumes are centrifuged at high speed for an extended amount of time. Thus, ultracentrifugation requires large sample volumes, is time consuming and exposes the samples to large forces that may result in vesicle damage, fusion and aggregation, and inclusion of unspecific protein complexes (234). Furthermore, ultracentrifugation in inconsistent between studies due to the use of different protocols and rotors (235, 236). Acoustic trapping is a novel, gentle and contact-free method for isolating vesicles, that enables small sample volumes. Acoustic trapping captures particles by generating a standing ultrasonic wave inside a microfluidic channel. The ultrasonic wave creates a pressure node that captures particles down to a few microns and enables isolation, washing, enrichment, and buffer exchange (237, 238). To capture submicron particles, the trap can be pre-loaded with seed particles. Nanoparticles, such as EVs, can then also be captured in the trap through scattered sound interactions with the seed particles. (239, 240). In paper IV we use acoustic trapping to isolate platelet-derived extracellular vesicles. We used a novel acoustic trapping devise with a large microfluidic channel and multinode resonance, instead of the standard single-node resonance, generating nine trapping pressure nodes instead of one. This novel acoustic trapping device allows higher flow rates and an increased number of trapped particles, resulting in a gentle, rapid method for isolating extracellular vesicles with high capacity (241).

Present investigations

Introduction

Streptococcus pyogenes is a strictly human pathogen that normally causes local throat and skin infections, such as pharyngitis and impetigo, but also invasive life-threatening conditions, such as necrotizing fasciitis, STSS and sepsis. *S. pyogenes* has developed multiple strategies to colonize, spread and evade the immune system. One important streptococcal virulence factor is the cell wall anchored M protein, which contributes to bacterial adhesion, invasion, and evasion of phagocytosis and complement. The M protein interacts with several plasma proteins, such as fibrinogen, the Fc domain of IgG and complement regulatory proteins. Serotyping of *S. pyogenes* is based on variations in the *emm* gene that encodes for the M protein. There are more than 200 different M protein serotypes and around ten serotypes are commonly associated with invasive infections, with the *emm1* serotype being the most prevalent. The M protein from the *emm1* serotype, the M1 protein, can be released from the bacterial surface by host and bacterial proteases. The released M1 protein exhibits pro-inflammatory properties and mediates activation of neutrophils and monocytes. M1 protein can also activate platelets and stimulate platelet-leukocyte complex formation. M1 protein-mediated platelet activation is dependent on fibrinogen and specific anti-M1 protein IgG engaging with both the Fc and the fibrinogen receptors on the platelet surface. Platelets are the main regulators of hemostasis but also play an important role in the immune system. Some of the hallmarks of sepsis are disturbed thrombosis and a dysregulated inflammatory state, and platelets contribute to the pathophysiology of sepsis. The overall aim of this thesis was to investigate the role of platelets during streptococcal sepsis further, mainly focusing on the interactions between the platelets and the streptococcal M protein, using both in vitro and in vivo systems.

Paper I

Background

The complement system is a key mediator in the immune response to infection. The system can be activated by three pathways, classical, alternative and lectin, which recognise microbial motifs directly or via immune complex formation. Complement activation generates opsonins (C3b) that facilitate phagocytosis, pro-inflammatory and chemotactic mediators (C5a, C3a), and, finally, a terminal membrane attack complex that can mediate bacterial cell lysis. Human cells are protected from complement activation by acquisition of complement regulatory proteins; factor H regulates the alternative pathway and C4BP regulates the classical and lectin pathway. Many bacteria have developed strategies to evade the complement system. The cell wall bound streptococcal M protein can bind C4BP and factor H and prevent complement activation at the bacterial surface. Furthermore, M protein can bind fibrinogen and the Fc domain of IgG and thereby evade phagocytosis. The M protein from the *emm1* serotype (M1 protein) can be cleaved off from the bacterial surface by host or bacterial proteases. The released M1 protein binds fibrinogen and the Fab region of specific anti-M1 IgG. This protein complex exhibits pro-inflammatory properties, including activation of platelets. Platelet activation by M1 protein is dependent on binding of M1 protein together with plasma fibrinogen and specific anti-M1 IgG to the fibrinogen receptor GPIIb/IIIa and the Fc receptor FcγRIIa on the platelet surface. The consequences of this immune-mediated platelet activation in sepsis are not fully understood, in particular with regard to interactions with the complement system.

Aim

The aim of this study was to characterize the protein complex formed on addition of M1 protein to human plasma and investigate the consequences of pathogen-mediated activation for platelet function.

Results

Quantitative mass spectrometry showed that IgG3, C1q and complement components were associated with protein complexes that are formed by M1 protein in human plasma. C1q was also associated with the surface of M1 stimulated platelets, and the complement system was activated, as demonstrated using flow cytometry. The C1q association and the complement activation were observed exclusively in individuals with specific IgG against the M1 protein. Furthermore, using flow cytometry and microscopy, we showed that platelet apoptosis and phagocytosis of platelets was increased after M1 stimulation.

Conclusion

In conclusion, we demonstrated that C1q was acquired to the surface of M1-activated platelets in an IgG immune complex-mediated fashion. This resulted in activation of the complement system and increased apoptosis and phagocytosis of the platelets in susceptible individuals. This reveals novel consequences for pathogen-mediated platelet activation, and a novel mechanism of complement activation during streptococcal sepsis, which may contribute to platelet consumption in sepsis.

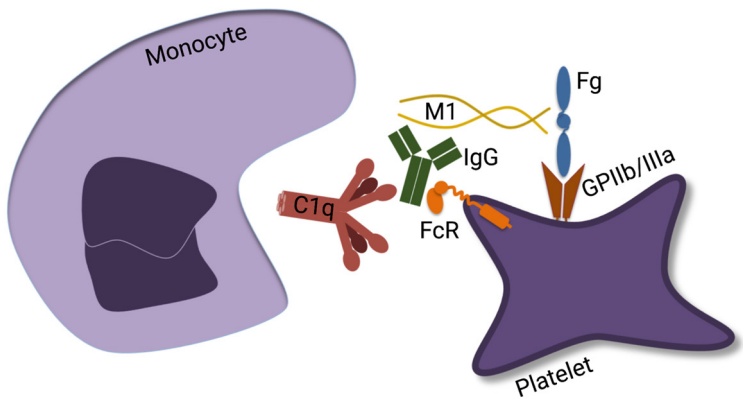

Figure 12. Conclusion of paper I
M1 protein mediates platelet activation that is dependent on binding of M1 protein together with plasma fibrinogen (Fg) and specific anti-M1 IgG to the fibrinogen receptor GPIIb/IIIa and Fc receptor (FcR) FcγRIIA on the platelet surface. C1q is acquired to the surface of M1-activated platelets in an IgG immune complex-mediated fashion. This results in activation of the complement system and increased apoptosis and phagocytosis of the platelets in susceptible individuals.

Paper II

Background

M protein is encoded by the *emm* gene, and serotyping of *S. pyogenes* is based on *emm* amino acid sequence variations in the N-terminal region. Another classification system, which is based on A, B, C, and D domain arrangements of the M proteins, can be used to assign serotypes into groups; patterns A-C, D, and E, which are associated with relatively distinct tissue tropisms of skin and throat or as generalists that can occupy both niches. The *emm* pattern A-C contains all A, B, C, and D domains, the *emm* pattern D contains B, C, and D domains, and the *emm* pattern E only contains the C and D domains. There are more than 200 different *emm* serotypes; however, fewer than 10 serotypes are predominant in clinically significant invasive streptococcal infections. The four serotypes *emm1, emm28, emm3, and emm89* account for about 50 to 70% of all invasive *S. pyogenes* infections in Europe and North America, with the *emm1* serotype being the most prevalent. These four serotypes represent the distinct patterns of A-C or E, respectively, implying that distinct tissue tropisms may not be associated with invasive infection. We have previously shown that platelet activation and neutrophil activation mediated by M1 protein is dependent on fibrinogen and specific anti-M1 protein IgG engaging with both the Fc and the fibrinogen receptors on the platelet surface. Fibrinogen is a predominant blood protein that responds to infection as an acute-phase reactant and mediator of inflammation, interacting with leukocytes and platelets. Some bacterial pathogens, including *S. pyogenes*, bind fibrinogen to subvert fibrinogen-mediated host antimicrobial function or facilitate invasion within the host. The ability of the M proteins to acquire plasma fibrinogen is not conserved for all serotypes and may be associated with distinct serotype patterns.

Aim

In this study, we assess the importance of fibrinogen acquisition for platelet and platelet-dependent neutrophil and monocyte activation mediated by invasive serotypes of M protein from two serotype patterns, the M1, M3, and M5 proteins from pattern A-C and the M28, M49, and M89 proteins from pattern E.

Results

The platelet-dependent pro-inflammatory effects of serotypes of M protein associated with invasive infection (M1, M3, M5, M28, M49, and M89) were investigated using a combination of multiparameter flow cytometry, ELISA, aggregometry, and quantitative mass spectrometry. Distinct M protein serotypes (M1, M3 and M5 protein) bound fibrinogen in plasma and mediated fibrinogen- and IgG-dependent platelet activation and aggregation, release of granule proteins, upregulation of CD62P to the platelet surface, and complex formation with neutrophils and monocytes. Neutrophil and monocyte activation was also mediated

by M1, M3, and M5 protein serotypes, while M28, M49, and M89 proteins failed to mediate activation of platelets or leukocytes.

Conclusion

Only M protein released from distinct streptococcal serotypes (M1, M3, and M5 proteins) bound plasma fibrinogen and Fab-bound IgG to mediate rapid platelet activation, complex formation with neutrophils and monocytes, and activation of these classical drivers of inflammation. Platelet-neutrophil and platelet-monocyte complex formation was dependent on platelet activation since the platelet-specific agonist thrombin resulted in platelet-neutrophil and platelet-monocyte complex formation, in the absence of leukocyte activation. The M28, M49, and M89 proteins failed to engage fibrinogen or mediate platelet-dependent inflammation. Our findings show that platelets activated by bacterial proteins can release immunomodulatory granule proteins and engage with and activate leukocytes, further highlighting the pro-inflammatory role of platelets during infection and sepsis. This reveals novel aspects of the immunomodulatory role of fibrinogen acquisition and platelet activation during streptococcal infections.

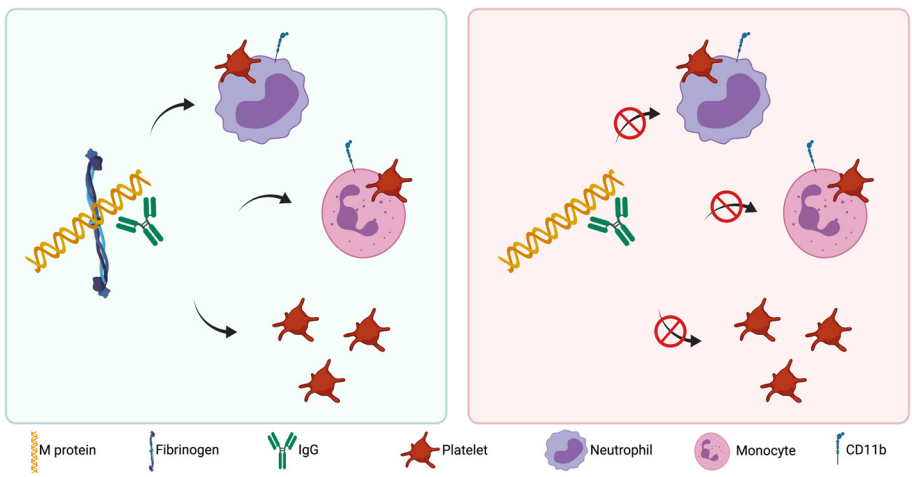

Figure 13. Conclusion of paper II
Only M protein released from distinct streptococcal serotypes (M1, M3, and M5 proteins) bind plasma fibrinogen and Fab-bound IgG to mediate rapid platelet activation, complex formation with neutrophils and monocytes, and activation of these classical drivers of inflammation (green square). The M28, M49, and M89 proteins failed to engage fibrinogen or mediate platelet-dependent inflammation (red square). Figure 13 was created with Biorender.

Paper III

Background

Extracellular vesicles are derived from the membrane of cells upon activation or injury. Most of the circulating EVs in blood are derived from platelets. Just like their parent cell, platelet derived EVs are attributed an important role in hemostasis and exhibit immunomodulatory effects by transfer of bioactive cargo to target cells, such as leukocytes and endothelial cells, resulting in target cell activation. PEVs recruit leukocytes to the site of infection and mediate leukocyte interaction and cytokine release. Platelet activation and release of circulating platelet derived EVs increase in several pathological inflammatory diseases, such as sepsis. The content of PEVs may differ depending upon the platelet agonist mediating platelet activation. The physiological platelet agonist thrombin interacts with PARs at the platelet surface to mediate platelet activation and release of PEVs. We have previously shown that the streptococcal M1 protein mediates platelet activation that is dependent on fibrinogen and specific anti-M1 protein IgG engaging with both the Fc and the fibrinogen receptors on the platelet surface. However, the release of PEVs in response to the M1 protein and the pro-inflammatory effects of EVs derived from M1 protein stimulated platelets have not been previously investigated. Acoustic trapping is a novel method for extracellular vesicle isolation, washing and enrichment. Acoustic trapping captures particles by generating an ultrasonic wave inside a microfluidic channel, and offers a gentle and rapid method for isolating extracellular vesicles with high capacity.

Aim

Investigating EVs from activated platelets in comparison to EVs from resting platelets, by characterizing the differences in the protein cargo and the pro-inflammatory effects of EVs isolated from resting platelets, physiologically stimulated (thrombin) platelets, and immune complex (M1 protein) stimulated platelets.

Results

We isolated extracellular vesicles from activated platelets using acoustic trapping and applied quantitative mass spectrometry-based proteomics to characterize the differences in the protein cargo of extracellular vesicles isolated from resting platelets, physiologically stimulated (thrombin) platelets, and immune complex (M1 protein) stimulated platelets. We determined that M1 protein-mediated platelet activation and generated platelet derived extracellular vesicles, into which the M1 protein itself was incorporated. The isolated extracellular vesicles derived from all three conditions contained a protein cargo of platelet membrane proteins, granule proteins and cytoskeletal proteins, in combination with coagulation factors and immune mediators. We showed an increased level of blood coagulation proteins

after stimulation with thrombin and M1 protein, as compared with resting platelets. The extracellular vesicles isolated from M1 protein stimulated platelets also contained increased levels of complement proteins and IgG3. The isolated extracellular vesicles derived from all three conditions exhibited pro-inflammatory effects on addition to blood, including platelet-neutrophil complex formation, neutrophil activation, and cytokine release.

Conclusion

Collectively, our findings showed that blood coagulation proteins, complement components and IgG were enriched in EVs derived from platelets after stimulation with thrombin or M1 protein, as compared with resting platelets. A specific increase in complement components and IgG3 after immune complex-mediated platelet activation with M1 protein was observed. M1 protein was transported with platelet derived EVs after stimulation and pro-inflammatory responses were generated in blood cells exposed to isolated EVs. Pro-coagulant EVs may contribute to the coagulopathy in sepsis, which may be an important attribute for M1 protein during invasive streptococcal disease. This paper reveals novel immunomodulatory aspects of pathogen-mediated platelet activation that may contribute to the coagulation and immune dysfunction during invasive streptococcal infection, and that may be used to identify biomarkers of pathogen-mediated platelet activation.

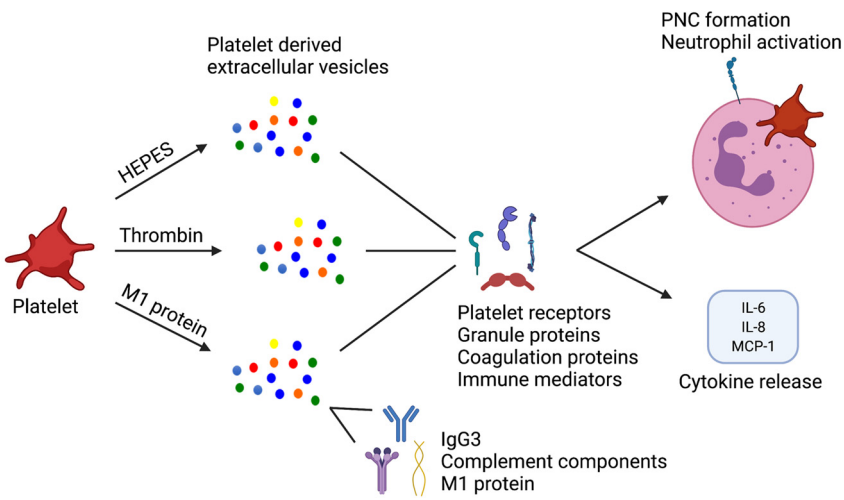

Figure 14. Conclusion of paper III
Isolated extracellular vesicles (EVs) derived from resting platelets, platelets activated with thrombin and platelets activated with M1 protein all contain a protein cargo of platelet receptors, granule proteins and cytoskeletal proteins, in combination with coagulation factors and immune mediators. We showed an increased level of blood coagulation proteins after stimulation with thrombin and M1 protein, as compared with resting platelets. The extracellular vesicles isolated from M1 protein stimulated platelets also contained increased levels of complement proteins and IgG3. The isolated extracellular vesicles derived from all three conditions exhibited pro-inflammatory effects on addition to blood, including platelet-neutrophil complex formation, neutrophil activation, and cytokine release. Figure 14 was created with Biorender.

Paper IV

Background

In accordance with the Sepsis-3 definition, sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to bacterial, viral, or fungal infection, assessed using the SOFA. Hallmarks of sepsis include dysregulated hemostasis and dysregulated inflammation. Platelets play important roles in coagulation, inflammation, and infection, and are involved in the sepsis pathology. The crosstalk between platelets and the endothelium is dysregulated in sepsis, resulting in platelet activation and fibrin clot formation that contributes to DIC and subsequent organ damage. Activated platelets also release factors that mediate inflammation, vasodilation, and hypotension, resulting in systemic shock.

Consumption of platelets and coagulation factors eventually results in an increased risk of bleeding in sepsis patients. Thrombocytopenia is associated with sepsis mortality and included as a measure of haematological failure in the SOFA score. However, the causality of thrombocytopenia and the role of platelets in host defence or organ damage in sepsis is not fully understood. Intense research has resulted in increased knowledge of sepsis pathophysiology. However, the increased knowledge has not translated in improved sepsis care and treatment. One reason is the difficult translation from mouse models to human trials.

Aim

To establish a mouse model of local streptococcal skin infection that progresses to sepsis and investigate the role of platelets in sepsis pathophysiology.

Results

The cellular and molecular responses to infection were followed over time using a combination of flow cytometry, ELISA, histopathology, immunofluorescence microscopy, and quantitative mass spectrometry. Bacterial dissemination was associated with increased cytokine levels, and an initial leucocytosis and thrombocytosis that progressed into leukopenia and thrombocytopenia over time. Thrombocytopenia was preceded by platelet activation and coincided with accumulation of platelets in the organs and with intravascular clotting, which was mainly observed in the liver. We observed organ damage that progressed over time, as characterized by increased levels of organ damage plasma markers of, histopathological changes, and an altered proteome in the damaged organs. Furthermore, *S. pyogenes* was shown to bind to and activate platelets ex-vivo, using flow cytometry. Since platelet activation in vivo preceded both thrombocytopenia and organ damage, we investigated the platelet-specific proteome in the damaged organs. We showed that platelets mobilised to the local site of skin infection but also accumulated in the liver and kidney at the same time as bacteria disseminated to these organs.

Conclusion

We conclude that bacteria-mediated platelet activation may contribute to the pathogenesis of streptococcal sepsis which progresses from thrombocytosis to platelet activation and finally thrombocytopenia. Platelet aggregates accumulate in organs and may contribute to the organ dysfunction observed. This paper reveals novel aspects of the role of platelets in infection and sepsis pathophysiology.

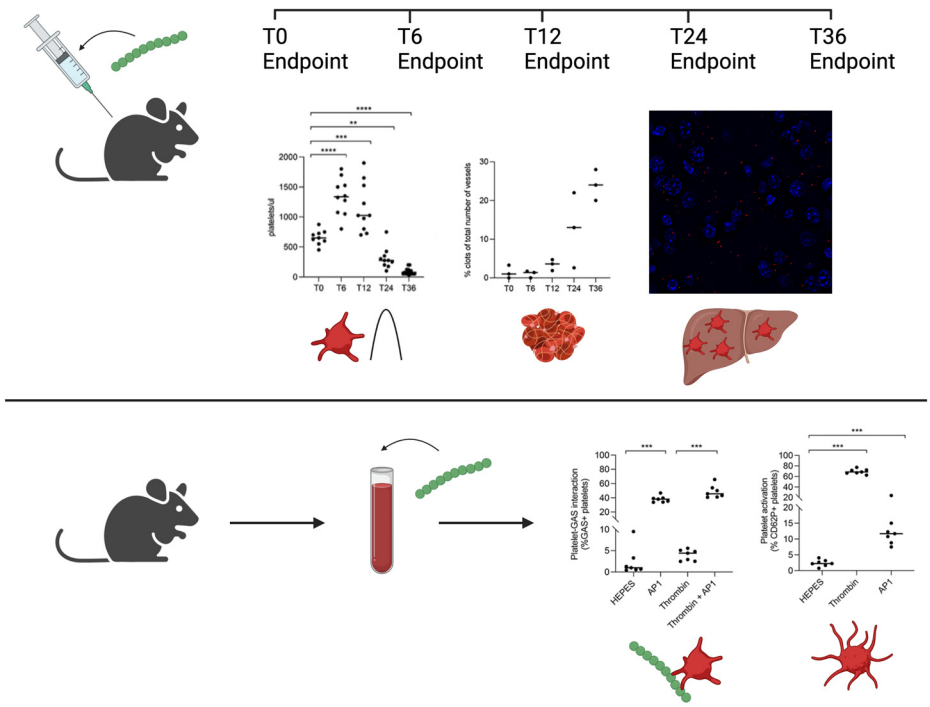

Figure 15. Conclusion of paper IV

We established a mouse model of local streptococcal skin infection that progresses to sepsis. Bacterial dissemination was associated with increased cytokine levels, and an initial leukocytosis and thrombocytosis that progressed into leukopenia and thrombocytopenia over time. Thrombocytopenia was preceded by platelet activation and coincided with accumulation of platelets in the organs and with intravascular clotting, which was mainly observed in the liver. We observed organ damage that progressed over time, as characterized by increased levels of organ damage plasma markers of, histopathological changes, and an altered proteome in the damaged organs. Furthermore, *S. pyogenes* was shown to bind to and activate platelets ex-vivo, using flow cytometry. Since platelet activation in vivo preceded both thrombocytopenia and organ damage, we investigated the platelet-specific proteome in the damaged organs. We showed that platelets mobilised to the local site of skin infection but also accumulated in the liver and kidney at the same time as bacteria disseminated to these organs. We conclude that bacteria-mediated platelet activation may contribute to the pathogenesis of streptococcal sepsis which progresses from thrombocytosis to platelet activation and finally thrombocytopenia. Platelet aggregates accumulate in organs and may contribute to the organ dysfunction observed. Figure 15 was created with Biorender.

Summary

This thesis reveals novel aspects and consequences for pathogen-mediated platelet activation during invasive streptococcal infection. We present a novel mechanism of complement activation, which may contribute to platelet consumption in sepsis, and new aspects of the immunomodulatory role of fibrinogen acquisition and platelet activation during streptococcal infections. Furthermore, new aspects of pathogen-mediated platelet activation that may contribute to the coagulation and immune dysfunction during invasive streptococcal infection, and that may be used to identify biomarkers of pathogen-mediated platelet activation are presented. Finally, this thesis reveals novel aspects of the role of platelets in infection and sepsis pathophysiology. Collectively, this thesis enlightens new aspects of the immunomodulatory roles of platelets during invasive infections and illuminates novel mechanisms of streptococcal pathology.

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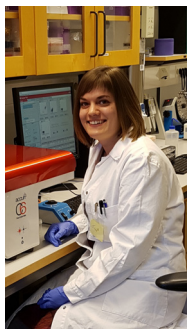
Novel aspects of pathogen-mediated platelet activation and the role of platelets in inflammation



Invasive bacterial infections and sepsis remain one of the major causes of death worldwide. Some of the hallmarks of sepsis are disturbed hemostasis and a dysregulated inflammatory state. The main regulators of hemostasis are platelets, and they also respond rapidly to inflammation and infection. *Streptococcus pyogenes* is a human-specific pathogen that can cause invasive disease and sepsis. One classical streptococcal virulence factor is the cell wall anchored M protein, which contributes to various aspects of bacterial pathogenesis such as evasion of the immune system. The M protein exhibits pro-inflammatory properties, including activation of platelets. The overall aim of this thesis was to investigate the interactions between the platelets and the streptococcal M protein further. Collectively, this thesis enlightens new aspects of the immunomodulatory roles of platelets during invasive infections and highlights novel mechanisms of streptococcal pathogenesis.

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