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Streptococcus pyogenes – a manipulator of human defences

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Abstract

Streptococcus pyogenes is a major human pathogen with more than 500 000 casualties annually of which at least 163 000 are due to invasive infections. The remainder is due to post-streptococcal complications with rheumatic heart disease constituting the majority. *S. pyogenes* also causes milder infections such as skin infections and pharyngitis with an estimation of more than 700 million cases each year. The mechanisms underlying the development of serious invasive infection are not yet fully understood.

S. pyogenes has developed multiple strategies to evade or manipulate the host defence systems thereby promoting its own survival. A major virulence determinant of *S. pyogenes* is the cell surface attached M protein. It has previously been shown that M proteins of certain serotypes form complexes with fibrinogen and that these can elicit a pathological response, which may contribute to the massive vascular leakage present in streptococcal toxic shock (STSS). This thesis demonstrates that preformed antibodies against certain epitopes on the M protein contribute to triggering the neutrophils to secrete heparin binding protein, a potent inducer of vascular leakage. This might explain the inter-individual susceptibility in the development of STSS.

Platelets and neutrophils can form complexes through a mutual interaction with each other. Such complexes have been implicated in the pathophysiology of many diseases (e.g. sepsis, acute lung injury, atherosclerosis) and it has previously been shown that neutrophils in such complexes are more active. In this thesis M protein was shown to generate platelet-neutrophil complexes. The formation of platelet-neutrophil complexes was dependent on specific IgG directed against the central domain of M protein. Such antibodies together with fibrinogen and M protein induce the formation of platelet-neutrophil complexes. This is another example of how M protein in the presence of specific antibodies elicits a more inflammatory phenotype.

The contribution of platelets to the pathogenesis of a bacterial infection was studied in a murine model of invasive *S. pyogenes*. *S. pyogenes* infection gave rise to a profound thrombocytopenia and concomitant rise in circulating platelet-neutrophil complexes. Reduction of the number of platelets with an antibody prior to infection resulted in diminished dissemination of bacteria to the spleen. The animals also decreased significantly less in weight and had a diminished acute phase response implying platelet participation in bacterial spreading and to the pathogenesis of this infection.

In clinical infections *S. pyogenes* seldom causes an abscess outside the pharyngeal tract. This thesis reports a rare case of an axillary abscess due to *S. pyogenes*. Since the patient already was on treatment with antibiotics cultures were negative. Nevertheless determination of the aetiological agent as well as typing could be achieved using molecular biology techniques from abscess material. Serology indicated that the bacterium had expressed procoagulant factors. The expression of such factors could have contributed to the development of venous thrombosis, which was a complication of abscess formation in this patient.

Taken together this thesis demonstrates how *S. pyogenes* promotes its own survival by manipulating different host defence systems.

Key words
Streptococcus pyogenes, M protein, neutrophils, platelets, platelet-neutrophil complexes, fibrinogen, IgG, abscess, venous thrombosis, heparin binding protein, thrombocytopenia

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Streptococcus pyogenes – a manipulator of human defences

LUNDS UNIVERSITET
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2012

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Cover image:
Identification of complexes of streptococcal M1 protein, IgG and fibrinogen in a tissue biopsy obtained from a patient with severe *Streptococcus pyogenes* infection. Specific IgG antibodies against the M1 protein were demonstrated to give rise to large complexes containing M1 protein (yellow), fibrinogen (magenta) and IgG (green).
Image credit: Anette Hofmann and Anna Norrby-Teglund, Karolinska Institutet.
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Antibodies against a surface protein of Streptococcus *pyogenes* promote a pathological inflammatory response
F. Kahn, M. Mörgelin, O. Shannon, A. Norrby-Teglund, H. Herwald, A. I. Olin and L. Björck
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Till minne av morfar

When Kepler found his long-cherished belief did not agree with the most precise observations, he accepted the uncomfortable fact. He preferred the hard truth to his dearest illusions; that is the heart of science.

Dr. Carl Sagan
(American astronomer, writer and scientist)

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I. **Kahn F**, Mörgelin M, Shannon O, Norrby-Teglund A, Herwald H, Olin A, Björck L.
Antibodies against a surface protein of *Streptococcus pyogenes* promote a pathological inflammatory response
PLoS Pathog. 2008; 4(9): e1000149.

II. **Kahn F**, Nordenfelt P, Björck L, Shannon O.
Platelet-neutrophil complex formation mediated by M1 protein from *Streptococcus pyogenes*
Manuscript.

III. **Kahn F**, Hurley S, Björck L, Shannon O.
Platelets promote bacterial dissemination in a mouse model of sepsis
Manuscript.

IV. **Kahn F**, Linder A, Petersson AC, Christensson B, Rasmussen M.
Axillary abscess complicated by venous thrombosis: identification of *Streptococcus pyogenes* by 16S PCR. J Clin Microbiol. 2010; 48(9): 3435-7.

Abbreviations

ADCC antibody dependent cellular cytotoxicity
AnxA1 annexin A1
ARF acute rheumatic fever
BPI bactericidal permeability-increasing protein
C5a complement factor 5a
CD cluster of differentiation
CGD chronic granulomatous disease
CR3 complement receptor 3
CR4 complement receptor 4
DAMP damage-associated molecular pattern molecules
FcγR Fcgamma-receptor
FcRn neonatal Fc-receptor
fMLF formyl-methionyl-leucyl phenylalanine
FPR formyl-peptide receptor
G-CSF granulocyte colony stimulating factor
GM-CSF granulocyte macrophage colony stimulating factor
GPIIb/IIIa glycoprotein IIb/IIIa
GPI glycosyl-phosphatidylinositol
IL interleukin
IVIG intravenous immunoglobulin
ITAM immunoreceptor tyrosine-based activation motif
ITIM immunoreceptor tyrosine-based inhibitory motif
LTB_4 leukotriene B_4
LXA_4 lipoxin A_4
MHC major histocompability complex
MPO myeloperoxidase
NET neutrophil extracellular traps
NF necrotising fasciitis
NGAL neutrophil gelatinase associated lipocalin
PAR proteinase-activated receptors
PMN polymorphonuclear leukocyte
PSGL-1 P-selectin glycoprotein ligand-1
RHD rheumatic heart disease
SAA serum amyloid A
SAP serum amyloid P component
Sda streptodornase D
SIC streptococcal inhibitor of complement
SIRS systemic inflammatory response syndrome
SGD specific granulae deficiency
SLO streptolysin O
SpeB streptococcal pyrogenic exotoxin B
SpyCEP *S. pyogenes* cell envelope protease
STSS streptococcal toxic shock syndrome
TAFI thrombin-activatable fibrinolysis inhibitor
TCR T-cell receptor
TLR Toll-like receptor
TNF tumour necrosis factor
vWF von Willebrand factor

Abstract

Streptococcus pyogenes is a major human pathogen with more than 500 000 casualties annually of which at least 163 000 are due to invasive infections. The remainder is due to post-streptococcal complications with rheumatic heart disease constituting the majority. *S. pyogenes* also causes milder infections such as skin infections and pharyngitis with an estimation of more than 700 million cases each year. The mechanisms underlying the development of serious invasive infection are not yet fully understood.

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Platelets and neutrophils can form complexes through a mutual interaction with each other. Such complexes have been implicated in the pathophysiology of many diseases (e.g. sepsis, acute lung injury, atherosclerosis) and it has previously been shown that neutrophils in such complexes are more active. In this thesis M protein was shown to generate platelet-neutrophil complexes. The formation of platelet-neutrophil complexes was dependent on specific IgG directed against the central domain of M protein. Such antibodies together with fibrinogen and M protein induce the formation of platelet-neutrophil complexes. This is another example of how M protein in the presence of specific antibodies elicits a more inflammatory phenotype.

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Taken together this thesis demonstrates how *S. pyogenes* promotes its own survival by manipulating different host defence systems.

Preface

My thesis is about *Streptococcus pyogene*s and its intriguing manipulation of the host's different defence systems. Most bacteria, whether commensals or pathogenic bacteria, have evolved mechanisms to circumvent the host's attempt to eradicate them. Commensals have learned to live more or less in harmony with its host. Pathogenic bacteria on the other hand, when infecting the host, most often give rise to a host response although some bacteria do its best to dampen it. To survive, multiply and flourish, pathogenic bacteria try to manipulate and sometimes hijack the defence systems whether it be to promote spreading, to hide itself from the immune system, or to get more nutrients et cetera. *S. pyogenes* have evolved an armada of different mechanisms to do so. The first chapter of this thesis is a very short overview of different host defence systems together with a short historical overview of their discovery. The following sections focus in depth on those systems that are particularly important for this thesis. The next section is about *S. pyogenes*. My first intent was to have a third section dealing with the interactions between different host defence systems and *S. pyogenes* but it turned out to be difficult to separate the description of the different virulence factors of *S. pyogenes* and simultaneously not describing their function and their interactions with host defence systems. Therefore these two subjects have been integrated in the section describing the pathogen *S. pyogenes*. The final section describes the present investigation and puts the findings from this thesis into a broader perspective. I have tried to write in a way that a biologist or a physician not familiar with the research in the covered areas will be given a background and also be up to date with current knowledge.

Lund, February 2012

Fredrik Kahn

Introduction

All organisms are constantly exposed to a hostile environment and among other factors in the environment are different microorganisms that are potentially harmful. Microorganisms can be divided in protozoa, fungi, bacteria and viruses, although prions and algae also may be included. The modern era of microbiology started in the 17th century when *Antoniee van Leeuwenhoek* identified bacteria, which he called animalcules. Although most observations were not fully consistent since there was no theory to structure the observations[1]. The prevalent idea was that microorganisms evolved spontaneously through what was called *generatio æquivoca* or *generatio spontanea* (spontaneous generation). By the end of the 18th century this was challenged by the Italian *Lazzaro Spallanzani* and finally falsified by *Louis Pasteur*. The microbiology evolved during the 19th century paradigms proposed by e.g. *Koch* (transmission of anthrax, *Mycobacterium tuberculosis* and *Vibrio cholerae*), *Ignaz Philipp Semmelweiss* (puerperal fever) and *Joseph Lister* (aseptic surgery) among others. In 1884 (modified in 1890) *Koch* published his postulates, which with some modifications are valid even today[2]. While living in this hostile environment of microbes all multicellular organisms have evolved some type of host defence systems.

The first line of defence that an invading microbe comes in contact with are the physical barriers lining the outer surface of the organisms, whether it be the skin, the respiratory epithelium or the epithelium of the gastrointestinal tract. The defence of the barrier is composed of mucus, cilia, peristalsis, resident microbial flora and antimicrobial peptides. The antimicrobial peptides (AMPs) are present in practically all forms of higher life from plants to humans[3]. Many of the mammalian antimicrobial peptides can be divided in two major classes: the defensins and the cathelicidins[4]. Not only the barriers of the organism express AMPs but also many other cells, such as neutrophils, eosinophils and platelets[4]. If the barriers of the organism are breached the next line of defence comes into play. Pathogens possess molecules that are vital for their ability to survive[5]. Such pathogen-associated-molecular–patterns (PAMPs) can be detected through pattern-recognition receptors (PRRs) on the host's immune cells. The terminology with PAMPs and PRRs has lately been questioned since it would be more accurate to state that it is indeed molecules that are recognised and not patterns and it is not only pathogens that are recognised but indeed all microbes[6]. PRRs include molecules such as Toll-like receptors (TLRs)[7-9], NOD-like receptors (NLRs)[10],retinoic acid inducible gene-I

(RIG-I)-like receptors (RLRs)[11] and C-lectin receptors (CLRs)[12]. By being recognised the invading pathogen triggers a response, which activates many cells and the synthesis of factors that help in killing the invading microorganisms. This part of the immune system is called the innate immune system and is present in both plants and animals underlining the antiquity of the system[13].

On the interface between the innate and the adaptive immune system, but belonging to the former, is complement. This system of proteins was first described in the late 1800s when it was discovered that fresh serum contained *alexin,* a heat-labile bactericidal component, as opposed to the later discovered heat-stable components (e.g. antibodies)[14]. But it would take half a century until 1941 when *Louis Pillemer* was able to purify and dissect different components of the complement system belonging to what is now part of the so called classical pathway[15]. In 1954 *Pillemer* published the discovery of properdin, a component of the alternative pathway[16]. In the late 1900s the third branch of the complement system, the lectin pathway, was discovered[17, 18]. The complement system with its three branches is activated by pathogens either directly (alternative and lectin pathway) or via bound IgG or IgM (classical pathway). Upon activation proteins are bound to the pathogen leading to facilitation of phagocytosis or lysis of the pathogen. Much of the activity of the innate immune system is carried out by cellular components such as neutrophils, macrophages, dendritic cells, natural killer cells (NK cells) and natural killer T cells (NKT cells)[19]. Neutrophils and macrophages are of myeloid lineage and mediate phagocytosis and exocytosis of bioactive molecules but also take part in orchestrating the adaptive immune system as well as being part of the effector cells of the adaptive immune system. A central theme for these cells is the capability of phagocytosis. Phagocytosis stems from the Greek words φαγειν (phagein – «to devour»), κυτος (kytos – ”cell”), and ωση (-osis –”a suffix meaning the process”). By eating up the intruder the process of killing it is separated from the environment and the toxic compounds used are sealed off, not harming the surrounding tissue. The concept of phagocytosis was discovered by Ilya Ilyich (Elie) Mechnikov in the 1880s and for his discoveries he received the Nobel Prize in 1908 (shared with Paul Ehrlich – se below)[20]. Belonging to the innate immune system but intimately connected to the adaptive immune system is the dendritic cell whose main function is to present antigens to T-lymphocytes[21]. For the discovery of these cells Ralph Steinman was awarded the 2011 Nobel Prize[22]. Also on the edge between the to systems and actually blurring the border is the NK cells. These cells are of lymphoid origin but lack antigen-specific surface receptors, such as the T-cell receptor or the B-cell receptor/antibody and have thus been considered a part of the innate immune system. NK cells patrol the body for virus-infected cells and tumours but can also kill or affect dendritic cells, macrophages and neutrophils thereby regulating the immune response. Lately there has been some evidence of memory; a feature until now only associated with

the adaptive immune system[23].

The other branch of the immune system, the adaptive immunity, only exists in vertebrates[13] although younger than the innate immune system it dates back 500 millions years to the development of vertebrates during the Cambrian explosion[24]. The adaptive immune system in mammals consists of B- and T-lymphocytes and their progeny and their effector molecules. The central themes for adaptive immunity are adaptation, specificity and memory. What most people associate with adaptive immunity are antibodies and vaccination. Already in the 15th century China dried powdered scabs which where inhaled as a prophylaxis for variola[25]. Inoculation with variola (variolisation) was practised in the 17th century in Turkey and was spread to England during the 18th century by *Robert Sutton* and *Daniel Sutton*[1]. In the year 1796 Edward Jenner performed the first vaccination and published this in 1798[26]. Almost hundred years later in the summer of 1880 Louis Pasteur by chance found a working vaccination against chicken cholera[27](which is now known to be *Pasteurella multocida*), which was followed by his discovery of a vaccine against anthrax in 1882. In the year 1885 Pasteur tried a vaccine against rabies on a boy that had been dog bitten and infected with rabies. The boy miraculously survived[28]. In 1888 *Emile Roux* and *Alexander Yersin* demonstrated the toxic effect of diphtheria serum even after the bacteria had been removed[29] and 1890 *Emil von Behring* and *Shibasaburo Kitasato* could induce an antitoxin in animals that had received the diphtheria toxin[30]. This antitoxin is what we now call antibodies. For his discovery von Behring was awarded the first Nobel Prize in medicine in 1901 and in his Nobel lecture he stated that *"Briefly expressed, serum therapy works through anti-bodies, iso-therapy through iso-bodies."*[31]. *Paul Ehrlich* continued these studies and did much work about the relationship between the antigen and the antibodies[32]. Due to their migration in electrophoresis of plasma, antibodies were called gammaglobulins and are now more accurate called immunoglobulins. There are five different classes of immunoglobulins: IgG, IgM, IgA, IgE and IgD. All immunoglobulins are synthesised by B-lymphocytes or plasma cells. As opposed to the innate immune system B-lymphocytes have their own unique molecular structure towards which their receptors are directed. This is accomplished by somatic recombination. The surface receptors of the B-lymphocytes are membrane bound antibodies. This branch of the adaptive immune system is called humoral immunity[33]. The other branch of the adaptive immunity is called cellular immunity with the T-lymphocytes as the effector cell. T-lymphocytes can not by routine staining or electron microscopy be distinguished from B-lymphocytes but have different surface antigens which can be employed to identify them. The T-lymphocytes can be divided into two different classes: cytotoxic T-cells (CD8-positive) and helper-T-cells (CD4-positive) (although there are other subpopulations)[34]. The cytotoxic cells main function is to patrol the body and kill altered cells (such as virus infected cells or cancer cells) while the helper-T-cells are the conductor of the adaptive

immunity orchestrating and controlling the cytotoxic cells as well as the B-cells. The T-lymphocytes gain their specificity through the T-cell receptor (TCR), which is composed of two proteins chains (α and β and on a few T-cells γ and δ instead). The T-cells recognise their antigen on special receptors called *major histocompability complex (MHC)* or in humans *human leukocyte antigen (HLA)*. There are two types of MHC molecules, MHC class I which are expressed by almost all cells and are the ligands of for the TCR of cytotoxic T-cells and MHC class II which are expressed by antigen presenting cells, mainly macrophages, dendritic cells and B-cells and are the ligands for the TCR on helper-T-cells[35]. Due to this arrangement, the need for helper-T-cell participation as a conductor, and the negative selection of self-identifying T-cells, the adaptive immune can be both almost complete in its repertoire against different epitopes and yet in most cases not reacting against self-antigens[36].

The coagulation system, the fibrinolysis system and the platelets are generally considered to maintain vascular integrity although growing evidence highlights the contribution of these systems to the host's defence[37-39]. The ability of the blood to form clots and some knowledge of the inheritance of haemophilia have been known since antiquity and was described about a thousand years ago by Abu al-Qasim Khalaf ibn 'Abbas al-Andalusi al-Zahrawi. But it was not until the middle of the 19th century that the first correct theories on blood coagulation evolved[40]. Through the works of Johannes Müller (discovery of fibrin), Rudolph Virchow (discovery of fibrinogen), Prosper Sylvain Denis (isolation of fibrinogen), Alexander Schmidt (discovery that the conversion of fibrinogen to fibrin is an enzymatic process), Nicolas Maurice Arthus (calcium is essential for coagulation), Paul Morawitz (discovery of tissue factor and formulation of a simple theory of coagulation) the first steps towards a theory of coagulation were made[41]. The complex system with serine proteases in the coagulation cascade was further elucidated during the 20th century and the aetiology of haemophilia A and B was described in the 1950s[42]. The other player in the coagulation process, the platelet, was described by Max Schultze in 1865 and its function recognized by Giuolo Bizzozero in 1882[43, 44]. Bizzozero gave a detailed description on the new blood cell:

"Untersucht man den Inhalt solcher Gefässe (gleichviel ob Venen oder Capillaren) mit einem Immersionsobjective, so gelangt man zu dem überraschenden Ergebnisse, dass wirklich neben den rothen und weissen Blutkörperchen noch morphologische Elemente einer dritten Art in den Gefässen circuliren (Fig. 2). Es sind dies äusserst dünne Plättchen in Gestalt von Scheiben mit parallelen Flächen oder seltener von linsenförmigen Gebilden, rund oder oval und von 2-3mal kleinerem Durchmesser als die rothen Blutkörperchen."[45]

and its functions:

"Nachdem man eine solche gefunden und dieselbe in die Mitte des Gesichtsfeldes des Mikroskops gebracht

hat, übt man mittelst einer feinen Nadel einen leichten Druck auf einen beschränkten Punkt der Arterienwand aus. Nach wenigen Augenblicken sieht man einen Thrombus entstehen. Die vom Blutstrome fortgerissenen Blutplättchen werden, sobald sie an die lädirte Stelle der Arterienwand gelangt sind, angehalten; zuerst sieht man deren nur 2-4-6; sehr bald steigt ihre Zahl auf Hunderte (Fig. 8). Gewöhnlich bleibt stehen. Nach und nach an Volumen zunehmend, erfüllt der Thrombus bald das Gefässlumen und behindert immer mehr den Blutstrom."[45].

In the following sections those host defence systems that I have particularly studied in regards to *Streptococcus pyogenes* will be further characterised.

Neutrophils

The neutrophil is the most abundant white blood cell in the human circulation. Under normal conditions there are between 1.7–7.5x10^9/L in healthy adults[46]. Mice have more neutrophils with 1.9-11.5x10^9/L when taken from the tail vein and lower when taken from the heart[47] Under stressful conditions, such as during an infection, the numbers rise both as a consequence of mobilisation of the so called marginating pool as well as increased synthesis and release from the bone marrow; the relative importance of the different mechanisms depends on the stimulus.[48]. The neutrophil is of myeloid lineage and its production is increased by granulocyte colony stimulating factor (G-CSF) and granulocyte macrophage colony stimulating factor (GM-CSF) among other factors. The neutrophil arises from the hematopoietic stem cell via multipotential progenitors, myeloid progenitors, granulocyte/macrophage progenitors, myeloblast, promyelocyte, myelocyte, metamyelocyte and, band cell to the fully mature neutrophil[49, 50]. During this development the cell, in a controlled fashion, acquires the characteristics of the mature neutrophil, such as expression of cell surface receptors, phagocytic ability, differentiation of granule contents and, the ability to produce reactive oxygen species[51, 52](Figure 1). The neutrophil is also called a polymorphonuclear leukocyte (PMN) due to its multilobulated nucleus. After the neutrophil has entered the blood stream it has generally been considered to survive for approximately 5-10 hours unless it is recruited to a site of infection[48]. Lately there have been some doubts about this and there are reports that the neutrophil actually survives for up to 5.4 days in the circulation[53]. However considering the rate that has been reported for neutrophil production by the bone marrow and in order to maintain the steady state of circulating neutrophils a half-life of 5 hours in the circulation is necessary. This implies that one of the results above must be revised[54]. Once recruited out in the tissues to a site of inflammation the lifespan of the neutrophil is prolonged[55].

Neutrophil functions

The neutrophils function as the effector cells of innate immunity as well as adaptive immunity (e.g. through phagocytosis of antibody-opsonised microbes). The importance of the neutrophil is evident from the pathological conditions suffered by cancer patients with drug-induced neutropenia as well as individuals suffering from severe congenital neutropenia (among other mutations Kostmann's syndrome, discovered by the Swedish physician Rolf Kostmann)[56]. Classically

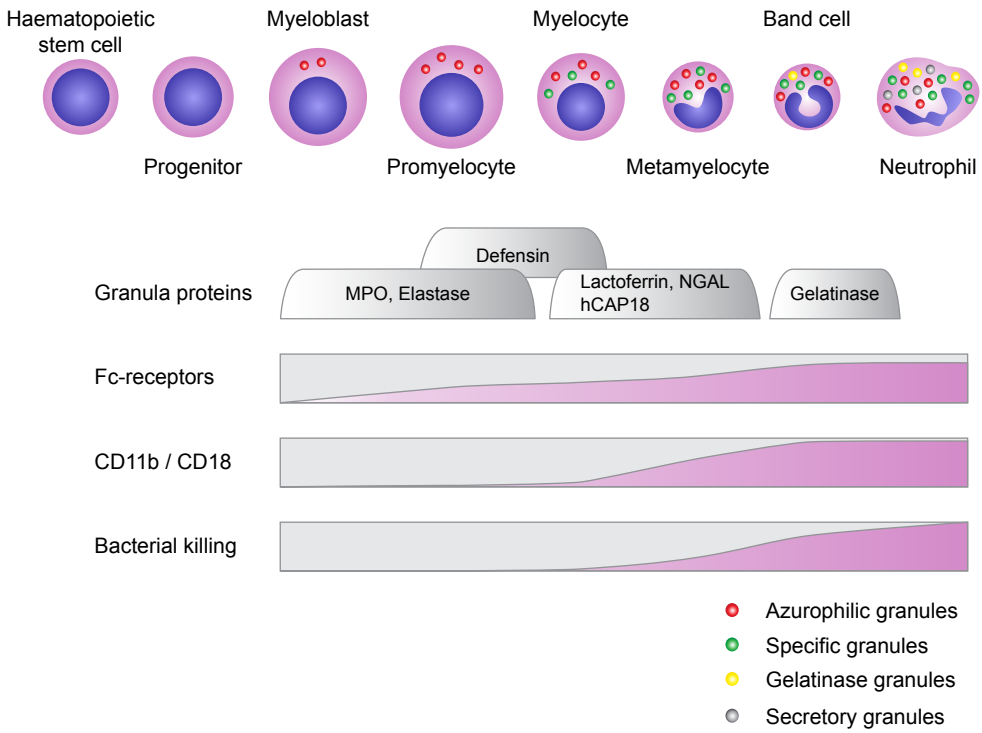


Figure 1
Development of the neutrophil

the neutrophil has been associated with phagocytic functions and bactericidal effects mediated by granulae-protein and reactive oxygen. The neutrophil has not been regarded as a cell that regulate the immune system by synthesising signalling molecules. The paucity of ribosomal material and mitochondria has been taken as evidence for this notion. Lately the much broader effector functions of the neutrophil have been appreciated. The neutrophil synthesises cytokines thereby regulating the immune system, cross-talking with other cells of both the innate and adaptive immune system as well as participating in the resolution of inflammation[57]. Neutrophils can also secrete so called alarmins, which are mediators recruiting and activating antigen-presenting cells. Proteins stored in the neutrophilic granulae (e.g. a-defensins, human cationic antimicrobial protein 18, lactoferrin) as well as present in the nucleus (e.g. high-mobility group box-1 protein) may function as alarmins[58]. In 2004 the novel concept of neutrophil extracellular traps (NETs) was discovered[59]. Neutrophils can, in addition to necrosis and apoptosis, undergo a controlled suicide, netosis, whereby the nuclei swell, the chromatin is dissolved and long strands of DNA with attached cytosolic and granular proteins are extruded from the cell forming a meshwork in which bacteria are trapped and killed. The attached proteins are mainly cationic

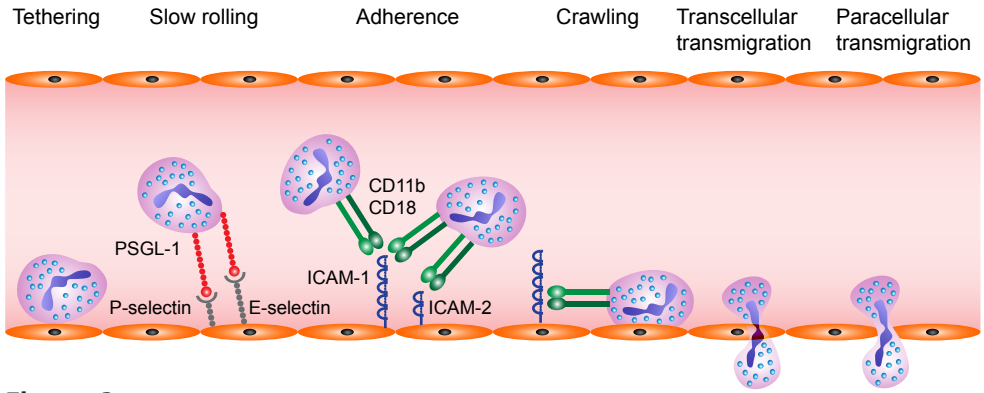


Figure 2
Neutrophil recruitment

and bactericidal[50]. Interestingly NET-formation is dependent on hydrogen peroxide and subsequently myeloperoxidase (MPO). Therefore patients with chronic granulomatous disease (CGD)[60] or MPO-deficiency[61] cannot form NETs. Neutrophils from new-borns are also devoid of a functional system for producing NETs, however the mechanism responsible remains unknown[62]. There have also been reports that the process of netosis may be initiated by platelets[63].

Recruitment to the site of infection

The "classical" task of the neutrophil is phagocytosis and killing of microbes. To do so the neutrophil is first recruited to the inflammatory site. This recruitment can be divided into different phases: tethering, slow rolling, integrin activation, adhesion, intravascular crawling and, transmigration[64] (Figure 2). Under normal circumstances the blood flow in the vessels is laminar with cells mainly flowing in the middle of the vessel. Due to an inflammatory stimulus various vasoactive substances are secreted (such as prostaglandins and NO). These are mainly synthesised by macrophages and in the case of NO also by endothelial cells that have been stimulated by interleukin-1β (IL-1β) or tumour necrosis factor (TNF) released from macrophages. This leads to a relaxation of the vessels and converts the laminar flow to a turbulent flow. In turbulent flow the cells are spread out over the whole cross-sectional area of the vessel and thus flow more slowly and closer to the vessel wall enabling interactions with the endothelial cells[65]. In the next step the neutrophils commence making contact with the endothelial cells – slow rolling. This process mainly takes place in the postcapillary venules, which is the site for the so-called marginating pool of neutrophils. The rolling process is mainly mediated by selectins expressed by the endothelial cells and their neutrophilic counter parts[64]. Upon stimulation with inflammatory mediators endothelial cells start expressing P-selectin and E-selectin[50]. P-selectin exists preformed in the Weibel-Palade bodies whereas E-selectin is synthesised *de novo*[66]. These selectins

bind to their neutrophilic counter part mainly P-selectin glycoprotein ligand-1 (PSGL-1). The affinity of selectins to their counterparts increases when force is exerted on it (called catch bond) and therefore when the blood flow stops the binding detaches giving rise to the characteristic bouncing of the neutrophil during this stage[67, 68]. The rolling process is followed by integrin activation. Due to stimulation by TNF and IL-1β but also to interferon-g (IFNγ), endothelial cells start expressing intracellular adhesion molecules-1 (ICAM-1)[65, 69]. ICAM-2 is constitutively expressed by the endothelial cell and therefore may take part in the initial anchoring of neutrophils[70]. These molecules bind to integrins on the neutrophilic surface. Integrins on the neutrophilic surface are also upregulated due to inflammatory stimuli[71]. At the same time neutrophils change from spherical cells to more flattened cells ready for cell-cell- interactions. This process of neutrophil recruitment is both eligible to pharmacological interventions as well as disturbed in some diseases. For example in the autosomal recessive disorder, leukocyte adhesion deficiency type 1 (LAD1), there is a mutation in CD18 (β_2) making the expression of functional neutrophilic integrins lower than normal thereby hindering the neutrophils to extravasate[19]. Monocytes, which extravasate through a β_1-dependent mechanism do so in a normal manner[65]. Corticosteroids are also known, among many other things, to downregulate the expression of E-selectin and ICAM-1 and this may be a part of their anti-inflammatory effects[72]. The neutrophil then starts crawling slowly across the endothelial cell in a CD11b/CD18 – ICAM-1-dependent manner[73]. The last step of recruitment is transmigration through the vessel wall. The combined actions of chemoattractants and binding to the endothelial cell layer facilitate neutrophil transmigration through the vessel and then further through the tissues to the site of infection. Neutrophil chemoattractants are among others IL-8 (CXCL-8), leukotriene B_4 (LTB_4), formyl-methionyl-leucyl phenylalanine (fMLF) and complement factor 5a (C5a). The chemotactic gradient guides the neutrophils towards their target[66]. A hierarchy has been proposed to exist in these chemoattractants with neutrophils favouring pathway end target attractants (such as fMLF and C5a) over intermediate targets (e.g. IL-8 and LTB_4)[74]. It is interesting to note that lipopolysaccharide (LPS) does not function as a chemoattractant but quite the opposite; it inhibits the activity of some the other chemoattractants[74]. Neutrophils can also be recruited to sterile sites through the actions of damage-associated molecular pattern molecules (DAMPs) such as hyaluronan, precipitated uric acid, high mobility group protein B1 (HMGB1) or ATP[66]. While crossing the vessel wall neutrophils can exploit both a paracellular way between the endothelial cells as well as a transcellular way[75].

Phagocytosis and bacterial killing

Neutrophils in the tissue are ready for phagocytosis and bacterial killing. To be phagocytised efficiently most microorganisms need to be opsonized, mainly through IgG or complement factors. However, there are exceptions such as

β-glucan on fungi, which binds directly to dectin-1[76]. The neutrophil expresses different Fc-receptors and complement receptors on its surface to which IgG and complement factors can bind. Their expression is regulated by different inflammatory mediators. Neutrophils in the tissue have shown to be more active phagocytes than neutrophils present in peripheral blood[77]. Neutrophils are very effective phagocytes and can phagocytose an IgG-opsonized target within 20 seconds[78]. The microbe is phagocytised into a phagosome, which then undergoes maturation. Phagosome maturation is a complex process that differs between neutrophils and macrophages. The exact events in this process have still not yet been completely understood. During maturation various proteases and bactericidal proteins are delivered to the phagosome, some of which originate from the neutrophilic granules[79]. The neutrophilic system of oxidative burst is also assembled giving rise to reactive oxygen species. This is accomplished through an NADPH-dependent process. Patients with granulomatous disease (CGD), which exists in both an autosomal recessive and an X-linked form, have mutations in this system of oxidases and are especially sensitive to catalse-postive bacteria such as *Staphylococcus aureus*, *Burkholdia cepacia* and *Serratia marscens* among others[80]. However, lately catalase production as a risk factor has been questioned[81, 82].

As the neutrophil oxidase burst system is assembled granular proteins are simultaneously delivered to the phagosome and participate in the killing of the microbe. Among others, myeloperoxidase (MPO) is delivered from azurophilic granules (see *Neutrophilic granulae and their contents*). MPO participates in the generation of hypochlorous acid, which is more potent as a bactericidal agent than hydrogen peroxide. Nevertheless patients with a deficiency of MPO do not show a clear phenotype[83]. Patients with glucose-6-phosphate dehydrogenase deficiency also have impaired production of NADPH through the pentose phosphate shunt but almost normal neutrophil function. These patients suffer from anaemia. This discrepancy is due to the fact that their enzyme is unstable, breaking down during the life span of the erythrocyte but exceeding the life span of the neutrophil[84].

The killing of the microbe is thought to be mediated by the combination of reactive oxygen species, hypochlorous acid and various bactericidal proteins, such as bactericidal permeability-increasing protein (BPI), cathelicidins, defensins, lactoferrin, lysozyme and neutrophil gelatinase associated lipocalin (NGAL)[85].

Neutrophilic granulae and their contents

During maturation the neutrophil acquires different granulae. As these are formed, proteins synthesised during their time of formation are incorporated in them. This “targeting of timing” hypothesis is thought to explain the different contents of the granulae[86]. (see also Figure 1). There are three main types of granulae in the neutrophil, formed in

the following order during neutrophil development: azurophilic (primary), specific (secondary) and, gelatinase (tertiary) granulae. In addition the neutrophil also contains secretory vesicles, which are formed out of endocytotic material[87, 88]. Secretory vesicles are completely mobilised after stimulation with fMLF[89] or after contact with an activated endothelium[90] and are thus exocytosed already in the blood vessel. Upon mobilisation they release their contents of soluble proteins and the neutrophilic receptors present fuse with the cell membrane thus mediating neutrophil contact and adherence. Gelatinase granulae are secreted during the transmigration in the tissue whereas specific granulae are mobilised at the site of inflammation when the neutrophil is in contact with the pathogen. Azurophilic granulae are mostly directed to the phagosome and are seldom completely mobilised to the exterior[52, 79]. This is in agreement with

Table 1
Contents of neutrophilic granulae

Functions	Primary (azurophilic)	Secondary	Gelatinase (tertiary)	Secretory vesicles
Attachment/ Adhesion		CD11b/CD18[95]	CD11b/CD18[95]	CD11b/CD18[95]
Receptors				Complement receptor 1 (CD35)[96] FcγRIII (CD16)[97] CD14[97] fMLF-receptor[98]
ECM degradation/ Proteases	Cathepsin G[99] Elastase[100] Proteinase3[101]	Collagenase[102] Gelatinase (MMP9)[103]	Gelatinase (MMP9)[104] Leukolysin(MMP25)[105]	Leukolysin(MMP25)[105]
Production of ROS		gp91^{phox} and p22^{phox}[106]	gp91^{phox} and p22^{phox}[107]	gp91^{phox} and p22^{phox}[108]
Antibacterial effects	Defensins[109] BPI[110] MPO[111] Lysozyme	hCAP18[112] NGAL[113] Lysozyme Lactoferrin[104]	Lysozyme	
Others	HBP[114]	SLPI[115] β_2-microglobulin[116]	β_2-microglobulin[117]	HBP[114] Plasma proteins[88]

Modified from Borregaard et al[118]

findings that stimulated neutrophils mobilise granulae in the opposite order as they were formed; secretory vesicles are most easily mobilised whereas azurophilic granulae are only partly mobilised and then only after intense stimulation[50, 91]. This is partly explained by the different calcium-sensitivity of the various granulae, with azurophilic granulae demanding the highest calcium concentration[92], although a recent study showed that fusion between the azurophilic granulae and the fully formed phagosome is calcium independent[93]. Table 1 shows the contents of selected proteins from the different granulae. The importance of granulae is demonstrated by individuals with specific granulae deficiency (SGD) (due to a mutation in a myeloid-specific transcription factor (C/EBP$_{\varepsilon}$)[94]) who suffer from repeated infections, especially on the skin and mucous membranes.

Heparin-binding protein

Heparin-binding protein (HBP) also referred to as azurocidin or cationic antimicrobial protein of 37kDa (CAP37) was isolated in 1984 from neutrophils and named CAP37 due to its charge and antimicrobial properties[119]. It was later isolated from azurophilic granulae with an apparent molecular weight of 29kDa and named azurocidin[120]. In 1991 *Flodgaard et al* isolated a protein from human as well as porcine neutrophils and due to its strong binding to heparin it was named heparin-binding protein[121]. The amino acid composition of CAP37 was determined by *Pohl et al*[122] and the genomic sequence was almost simultaneously solved by *Morgan et al* for the 37kDa protein[123] whereas *Almeida et al* reported the sequence for the 29kDa protein[124]. The two different proteins were then proven to be one and the same. Due to different levels of glycosylation the protein has a molecular weight of 29-37kDa[119, 120, 125]. HBP was later shown to be stored in the secretory vesicles (18%) as well as in the azurophilic granulae (74%) of neutrophils[114]. Recently HBP was reported also to be secreted by a monocytic cell line[126]. HBP is a member of the serprocidin family of serine proteases[120, 125] although it is thought to have lost its enzymatic activity due to replacement of serine and histidine in the catalytic triad[121, 127]. However, it was recently reported that HBP might serve as a protease, cleaving insulin-like growth factor binding protein-1, -2 and -4 (IGFB-1, -2 and -4)[128]. Other members of the serprocidin family (serine proteases with microbicidal activity) are elastase, cathepsin G and proteinase 3[129].

Although, all or much of the protease activity has been lost, HBP serves many important biological functions mainly related to host defence and inflammation. It possesses antimicrobial activity[119],binds to monocytes[130, 131] and is chemotactic for monocytes[127, 132]. Recently it was demonstrated that in a murine model HBP is predominantly chemotactic for monocytes of the inflammatory subtype and the chemotactic effect was shown to be at least partly mediated by the formyl peptide receptor[133]. It has also been demonstrated that HBP, in the presence of LPS, enhances monocyte cytokine release[134]. Furthermore HBP activates macrophages with upregula-

tion of FcγRII and FcγRI and enhanced phagocytosis of IgG opsonised bacteria, which could be inhibited by blocking CD18[135]. It has been suggested that HBP might opsonise *Staphylococcus aureus* and increase its phagocytosis by monocytes but not by neutrophils[136]. HBP has also been demonstrated to be chemotactic for fibroblasts[121] as well as for T-cells[137]. HBP also interacts with the endothelium enhancing expression of E-selectin, ICAM-1 and, VCAM-1 thus augmenting the recruitment of neutrophils and monocytes[138]. Neutrophil-secreted HBP has been shown to bind to the endothelial cell and thereby enhances monocyte binding to the endothelial cell as well as induces an upregulation of intracellular calcium in the monocyte[139], which could be blocked by inhibiting CD18[131].

The ability to increase vascular permeability is of fundamental importance in inflammation. *Wedmore and Williams* showed that neutrophils could control the vascular permeability[140] and it was later demonstrated that this was dependent on the participation of CD18 which facilitates neutrophil adherence to the endothelial cells[141]. Several factors have been suggested to be involved in the control of vascular integrity (e.g. TNF, CXCL3, CXCL8). However, these factors have not been shown to be stored pre-formed in large amounts by neutrophils adhering to the vascular wall thereby making their contribution *in vivo* controversial[142]. Many of the factors known to induce vascular leakage, such as LTB_4, fMLF and C5a do not exert an effect on the endothelium *per se*. In 2005 *Gautam et al* demonstrated that the neutrophil factor that induces vascular leakage is HBP and that HBP is secreted in a CD18-dependent manner[143]. It was further shown that neutrophils exposed to LTB_4 induce vascular leakage in an HBP-dependent manner through the BLT1 receptor on neutrophils[144].

HBP has been evaluated as a marker for infection-mediated vascular leakage. Infections span a continuum from mild to fulminant cases of septic chock and death. Sepsis is defined as an infection fulfilling the clinical and/or laboratory criteria of systemic inflammatory response syndrome (SIRS). When circulation is impaired sepsis has progressed to severe sepsis signified by hypotension. Vascular leakage is often seen in cases of severe sepsis. In a study by *Linder et al,* HBP was shown to be elevated in patients with severe sepsis and septic shock and in some cases even up to twelve hours before the development of severe sepsis[145]. Patients suffering from hypotension of other genesis did not show an elevated HBP level. A notable exception to this is patients with burns, who in a small study had elevated HBP levels[146]. HBP has also been shown to be elevated in cerebrospinal fluid from patients with acute bacterial meningitis[147]. In a study with patients admitted to the hospital, the discriminating capability of HBP for differentiating viral from bacterial infections was only exceeded by procalcitonin (PCT)[148]. However, in subsequent studies conducted on patients admitted to the Intensive Care Unit (ICU) there was no difference in HBP levels in patients with shock of septic or non-septic aetiology[149], and the levels of HBP did not correlate

with mortality[149, 150]. Thus the role for HBP as a marker for severe infectious diseases remains to be established.

Neutrophil surface receptors

Neutrophils have a plethora of surface receptors through which they communicate with their surroundings, mediate binding and effector functions such as phagocytosis. Human neutrophils express eight of the ten known toll-like receptors (TLRs), only lacking two of the four intracellular receptors (TLR3 and TLR7)[9, 151]. TLRs respond to several pathogen-associated molecular patterns (PAMPs), such as di/triacylated peptides, LPS and flagellin, as well as to damage-associated molecular patterns (DAMPs), such hyaluronan, heat-shock proteins (HSP) and high-mobility group protein B1 (HMGB1). The binding of ligands induces multiple effects with cytokine production, production of reactive oxygen species, receptor expression and phagocytosis[151].

Neutrophils also express different receptors for chemoattractants such as chemokines, anaphylatoxins (C5a and C3a), platelet activating factor, leukotrienes and formylated peptides[152]. Although the ligands are very different all chemoattractant receptors belong to the seven-membrane spanning G-protein coupled receptor family.

The formyl-peptide receptor (FPR) exists in humans in three variants, of which two are expressed by neutrophils; FPR1 (formerly named FPR) and FPR2 (formerly named FPRL-1). They are both G-protein coupled receptors that signal through G_i-protein coupling. They both recognise N-formyl-methionyl-phenylalanine (fMLF) although FPR1 has a sensitivity of >1nM whereas FPR2 recognises fMLF >10mM[153]. N-formylated peptides are formed during bacterial protein synthesis as well as from mitochondrial proteins (a reminiscence of their prokaryotic origin)[154]. Among other effects, fMLF upregulates the surface expression of CD11b/CD18 on neutrophils. In particular FPR2, also has additional ligands such as serum amyloid A (SAA), annexin A1 (AnxA1) and lipoxin A_4 (LXA_4). Activation of FPR1 has a strong chemotactic effect on neutrophils and overrides an opposing gradient of more indirect chemoattractants such as for example IL-8. Activation of the receptor induces desensitisation through uncoupling the receptor from the G-protein as well as agonist-induced receptor internalisation[153].

In the following sections I will describe, in more detail, two of the receptors that have been targeted in studies presented in this thesis.

Fc-receptors

The cells of the immune system express various IgG-receptors mediating functions such as phagocytosis, degranulation and antibody dependent cellular cytotoxicity (ADCC). These are collectively called Fcγ-receptors. The nomenclature of Fcγ-receptors (FcγR) can be quite confusing since the numbering and naming differ between mouse and man. In hu-

	Expression	Signalling	IgG binding (where relevant different allels)
FcγRI (CD64)	Monocyte, DC, macrophage ***Neutrophil***, eosinophils	Common γ-chain	$IgG_3>IgG_1>IgG_4>>IgG_2$
FcγRIIa (CD32a)	Monocyte,DC, macrophage Platelet (human not on murine) ***Neutrophil***	ITAM on receptor	H131: $IgG_3>IgG_1>IgG_2>>IgG_4$ R131: $IgG_3>IgG_1>>IgG_2$ and IgG_4
FcγRIIc (CD32c)	NK cell	ITAM on receptor	$IgG_3>IgG_1$ and IgG_4 No binding of IgG_2
FcγRIIIa (CD16a)	NK cell Monocyte,DC, macrophage	γ-chain	V158: $IgG_3>IgG_1>>>IgG_2$ and IgG_4 F158: $IgG_3>>>IgG_1$ No bindning of IgG_2 or IgG_4
FcγRIIIb (CD16b)	***Neutrophil***, mast cell, eosinophil	No signalling known GPI-anchored	$IgG_3>>IgG_1$ No binding of IgG_2 or IgG_4
Inhibiting receptor			
FcγRIIb (CD32b)	B cell, plasma cell Monocyte,DC, macrophage ***Neutrophil***, basophil, mast cell	ITIM on receptor	$IgG_3>IgG_1$ and IgG_4 No binding of IgG_2

Table 2
Fcγ-receptors

mans there are three main types of FcγRs (FcγRI, FcγRII and FcγRIII) whereas in mice there are four types (FcγRI-IV)[155]. The affinity for different subclasses of IgG and the expression on various cells also differs between the species. The rest of this section will focus on human receptors.

The different main types can be further divided into subtypes represented by minuscules, for example FcγRIIb. In addition there may also be various allelic variants. Fcγ-receptors are part of the IgG superfamily and the receptors extend out of the cell with two immunoglobulin-like domains (D1 and D2) except for the high affinity receptor FcγRI, which has three domains (D1-D3). These domains interact with the C_H2 domains of IgG. Three of the four activating FcγRs (FcγRI, FcγRIIa, FcγRIIc, FcγRIIIa)

are transmembrane receptors and are associated with an immunoreceptor tyrosine-based activation motif (ITAM) on the cytoplasmic side. The ITAM motif is either a part of the receptor itself (FcγRIIa, FcγRIIc) or associated with a separate molecule (the common γ-chain of Fc-receptors), which is the case for FcγRI and FcγRIIIa. The fourth activating FcγR (FcγRIIIb)is anchored to the cell membrane through a glycosyl-phosphatidylinositol (GPI) anchor and does not have a cytoplasmic domain. Activation of the different receptors occurs via crosslinking[156]. The inhibitory FcγR (FcγRIIb) has an immunoreceptor tyrosine-based inhibitory motif (ITIM) as a part of its cytoplasmic portion. ITAM and ITIM then subsequently phosphorylate downstream targets giving rise to the effector functions of the receptors. The downstream events of ITIM lead to subsequent dephosphorylation of the downstream targets of ITAM[157]. The signalling pathway for FcγRIIIb, which is devoid of a cytoplasmic domain, has not been completely clarified. It has been suggested that the receptor signals through CD11b/CD18 as is the case for FcγRIIa, or that the receptor is located to specialist signalling rafts in the membrane[158].

The distribution of the different receptors varies between different cell types. Neutrophils express three activating receptors (FcγRI, FcγRIIa, FcγRIIIb) as well as the inhibitory FcγRIIb. Of the activating Fc-receptors, FcγRIIa and FcγRIIIb are constitutively expressed (FcγRIIa approx. 30000 copies/cell and FcγRIII approx. 130000 copies/cell[159]) whereas FcγRI is not expressed by resting cells but upregulated is response to G-CSF and IFNγ[160, 161]. Studies have indicated that the expression of FcγRI on neutrophils might be a helpful tool in diagnosing sepsis although more studies are warranted[162].

The different FcγRs have different specificities for the target IgG. FcγRI is the high affinity receptor and the only one that binds monomeric IgG with an affinity in the nanomolar range[158]. This results in the receptor always being saturated with IgG in a plasma environment. The other receptors have low to medium affinity for IgG in the micromolar range and only bind IgG as part of immune complexes[156]. There are also different preferences among the receptors for various IgG subclasses. Recently the affinity of the receptors for IgG_3 was reconsidered since much of the previous experiments have been made by surface plasmon experiments and not on cell associated FcγRs[163](Table 2). The affinity for IgG is dependent on the glycosylation of IgG and IgG devoid of its glycan does not interact with FcγRs[164]. It was recently shown that IgG devoid of its glycan no longer supports antigen dependent enhancement (ADE) in a dengue virus infection model[165]. It has also been shown that the glycosylation status of the Fcγ-receptor itself affects the affinity for different IgG subclasses[158].

Furthermore, for some of the receptors different allelic variants exist with different affinity for IgG. An example of this is $FcγRIIa^{H131}$ and $FcγRIIa^{R131}$. Of the FcγRs it is only FcγRIIa that binds IgG_2 and $FcγRIIa^{H131}$ does so with much

higher affinity than FcγRIIa^{R131}. The importance of this is evident from studies of bacteria with a polysaccharide capsule (for example *Streptococcus pneumonia*, *Neisseria meningitides*, *Haemophilus influenzae*) towards which antibodies are mainly of IgG_2-class. Individuals with the low IgG_2-binding FcγRIIa^{H131} have exhibited higher susceptibility to invasive pneumococcal disease and higher risk for meningococcal septic shock[166-170]. FcγRIIIa also exists in different allelic variants with different affinities and the polymorphisms in FcγRIIa and FcγRIIIa may explain some of the differences in outcome when treating patients with anti-tumour antibodies such as rituximab[171-174]. Also FcγRIIIb, exists in different isoforms (NA1, NA2 and SH) with different properties with NA1 being more effective in inducing phagocytosis[175].

The ratio between activating and inhibiting signals (FcγRIIb) through the FcγRs is called the A/I ratio[176]. This ratio is affected by the IgG subclass as well as of the glycosylation status of both the IgG and the FcγR. Since different immune cells express different FcγRs the immune system can in this way fine-tune the response[156].

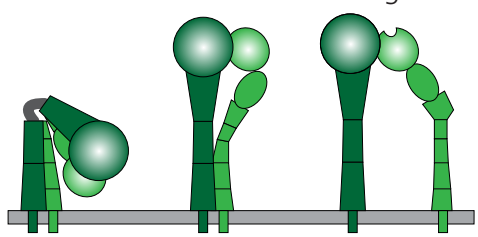
The inhibitory Fcγ-receptor FcγRIIb is important in downregulating and modifying immunological responses and exists in three different forms FcγRIIb1 (expressed on B cells and at low levels on monocytes), FcγRIIb2 (expressed on myeloid cells such as neutrophils) and, the soluble form FcγRIIb3[157]. FcγRIIb may serve as an example of the interactions between infectious diseases and autoimmunity. FcγRIIb exists in different variants, which affects the signalling ability of the receptor. Individuals with the low signalling variant have been shown to be more prone to develop systemic lupus erythematosos (SLE)[157]. On the other hand low signalling variants have been shown to confer protection against malaria and these alleles are more common in individuals of African origin than in Caucasians[177, 178]. This may be a part of the explanation as to why Africans have a higher incidence of SLE.

FcγRs can also bind members of the pentraxin family (among others CRP and serum amyloid P component (SAP))[179-181] and therefore potentially opsonise bacteria for phagocytosis via FcγRs, although this remains to be proven *in vivo*.

Integrins

Integrins are proteins expressed on cell surfaces and mediate contact between the cell and other cells or the extracellular matrix. They exist as non-covalently linked heterodimers formed by an α–unit and a β-unit. In vertebrates 8 α–units and 18 β-units exist, giving rise to 24 different integrins (not all α–units combine

Figure 3
Different confirmations of integrins



Name	Alternative names	Ligands
$\alpha_L\beta_2$	CD11a/CD18	ICAM-1-5
	LFA-1	Type I collagen
		Telencephalin
		Junctional adhesion molecule-1 (JAM-1)[188]
$\alpha_M\beta_2$	CD11b/CD18	***More than 30 ligands***
	Mac-1	***among others:***
	Mo-1	ICAM-1
	CR3 (complement receptor 3)	ICAM-2
		ICAM-4
		iC3b
		Fibrinogen
		Factor X
		Heparin
		Laminin
		LPS
		Zymosan
		Collagen
		Elastase
		Oligodeoxynucleotide
$\alpha_X\beta_2$	CD11c/CD18	iC3b
	p150/95	Fibrinogen
	CR4 (complement receptor 4)	Collagen
		Heparin
		LPS
		Denatured protein
		FcεRII

Modified from *Zhang et al*[189]

Table 3

Some of the integrins on the neutrophil and their ligands

with all β-units). These subunits extend as type I transmembrane glycoproteins with very short intracellular domains (with the exception of β_4). They can adopt three different conformations; low-affinity with their heads bent down, an intermediate-affinity binding state (extending with closed headpieces) and the high-affinity conformation with extended bodies and open head pieces[182] (Figure 3). The integrins transfer signals both from inside the cell to its surroundings as

well as receiving signals to the cell from the environment. The so called inside-out signalling is mediated by stimuli from other receptors via signalling pathways leading to conformational changes by the integrin, often transferring it to a high affinity state[183]. The conformational change of the integrins is mediated by a protein docked between the two legs of the αβ-units[184]. Inside-out signalling increases the affinity and also induces clustering of the integrins, which increases the avidity of the interaction[185]. Inside-out signalling is exemplified by neutrophils rolling on the endothelium. Whilst rolling and engaging PSGL-1, intracellular signals activate the integrins to adopt a high-affinity state that in turn brings the neutrophil to an arrest[186]. Outside-in signalling occurs when the integrin has bound to its ligand and then transfers a signal into the cell to change its behaviour. As mediators of contact between the cell and its surroundings, integrins have been implicated in participating in many important functions and various diseases such as infection, inflammatory diseases, coagulation, cancer and many others.

Neutrophils express mainly β_2 integrins, which associate with three different α–subunits forming $\alpha_L\beta_2$, $\alpha_M\beta_2$, and $\alpha_X\beta_2$ respectively. The nomenclature of neutrophil integrins can be confusing. Since the integrins mediate many different functions they have been named differently depending upon the circumstances they have been studied. Table 3 shows different names and the ligands for the β_2-integrins. Although CD11a/CD18 and CD11b/CD18 both bind ICAM-1 they recognize different domains. CD11a/CD18 binds to D1 whereas CD11b/CD18 binds to D3[71]. It is also interesting to notice that the Fab fragment of the monoclonal antibody abciximab (ReoPro®), used in clinical practice to inhibit platelet aggregation through blocking $\alpha_2\beta_3$, also have inhibitory effects on ligand binding to CD11b/CD18 on monocytes[187].

The so-called ouside-in signalling has lately been revised. β_2-integrins have been shown to signal through ITAM-containing adaptor molecules transferring the signal to the cell's interior[190-192]. The ITAM-containing adaptor molecules have for neutrophil β_2-integrins been suggested to be FcRg and DAP-12[190] whereas for platelets, signalling through GPIIb/IIIa ($\alpha_2\beta_3$), the adaptor protein has been suggested to be FcγRIIa[193]. Cross-talk between various receptors is today considered a common theme and for example Toll-like-receptors (TLRs) have also been shown to interact with ITAM-containing adaptor molecules[194]. These new insights highlight yet another link between innate and adaptive immunity. The vigorous response elicited by the dual stimuli in **Paper I** may be an example of such cross-talk and explain the violent response mediated by streptococcal M1 protein in immune individuals which triggers both a β-integrin pathway and an Fc-mediated pathway.

Platelets

Platelets are small anuclear cell fragments circulating in the blood stream for 7-10 days and have a diameter of approximate 2μm and a thickness of 0.5μm They participate in haemostasis but has also other functions. They are formed in the bone marrow by budding off from megakaryocytes and are finally cleared from the circulation by macrophages in the spleen and the liver. However, there have been reports that megakaryocytes also lodge in the lungs as well as mature platelets multiplying though fission despite their lack of a nucleus[195, 196]. Anucleate platelets are only found in mammals whereas lower vertebrates possess nucleated cells and invertebrates do not have specialised cells for haemostasis but in stead a cell called haemocyte (see below). Under normal conditions in humans the platelet count is 125-340 x 10^9/L[197]. Mice have much higher platelet counts with 900-1600 x 10^9/L[198]. The production of platelets can be increased as much as twentyfold upon increased demand or due to inflammation[199]. In their interior platelets have different granulae. The a-granulae, which are the most abundant with 40-80/platelet, contain more than 300 different proteins among others; adhesive proteins (e.g. von Willebrand factor (vWF), fibrinogen), clotting factors (e.g. factor V, factor XI), fibrinolytic factors, proteases, growth factors, chemokines (e.g. platelet factor 4 (PF4), IL-1β), antimicrobial proteins and membrane receptors (e.g. glycoprotein IIb/IIIa (GPIIb/IIIa), P-selectin)[200]. Recently it has been proposed that it exists two different classes of a-granulae exist; one containing fibrinogen and the other containing vWF[201]. Dense granulae (δ-bodies) are rarer and there are 1-2/platelet. They contain nucleotides and other second wave messengers (e.g. ADP, ATP, serotonin, pyrophosphate, calcium, magnesium). Platelets also contain a few lysosomes[202]. Although platelets are anuclear they do contain ribosomes and have recently been demonstrated to be capable of protein synthesis. As they bud off from megakaryocytes, mRNA is transferred to the platelet to-be, and this mRNA can after stimulation be translated[203]. The main function of platelets has classically been considered to participate in haemostasis. However the platelets' roll in regeneration, inflammation, defence against microbes, angiogenesis, the embryonic formation of a vascular system and the postnatal closing of ductus arteriosus is being increasingly recongnised. Platelets have also been implicated in the pathogenesis of rheumatoid arthritis, multiple sclerosis, cancer metastasis[200].

Haemostasis

Most platelets live their lives patrolling the blood vessels never going into action, but when an injury appears their swift contribution is essential. Haemostasis is classically divided into primary (i.e. platelets) and secondary (i.e. the coagulation cascade – *see Coagulation and fibrinolysis*). Primary haemostasis can be divided in an initiation phase (when a monolayer of platelets covers the exposed extracellular matrix), an extension phase (the recruitment of more platelets and platelets aggregation) and a stabilisation phase (when the aggregate formed is stabilised)[204]. The tight control of platelet activation and aggregation is needed to prevent the extension of the thrombus beyond the injury or initiation of improper activation. Below I will give a short overview of primary haemostasis.

Platelet have multiple mechanisms of activation which can be divided into categories of adhesion activation and soluble activators. When the endothelial lining is injured proteins of the extracellular matrix are uncovered e.g. vWF, collagen[205]. vWF is synthesised by endothelial cells and megakaryocytes and stored in Weibel-Palade bodies and a-granulae respectively. Endothelial cells also secrete vWF to the extracellular matrix as well as to the blood[206]. Upon uncovering collagen, vWF binds to collagen thus exposing the A1 domain for which the platelet receptor glycoprotein Iba (GPIba) has affinity. GPIba is part of a complex consisting of GPIba, GPIbb, GPV and GPIX in a 2:4:1:2 configuration with approximately 12500 copies of the complex/platelet[205].

Table 4 shows some of the major receptors mediating contact between the platelet and the extracellular matrix or other cells. The binding of vWF to the GPI-V-IX complex slows down the platelet making it possible for other ligands to bind, even if the binding also directly give rise to an outside-in signal[207]. The main collagen receptor on platelets, glycoprotein VI, then binds to collagen and via associated FcRg start transmitting signals to the platelets interior[208, 209]. The platelet is activated and the a- and δ-granulae fuse with the cell membrane, releasing endogenous platelet activators and giving rise to a feedback loop. ADP is released and binds to its two G-protein coupled receptors $P2Y_1$ and $P2Y_{12}$ further activating the platelet[207]. Thrombin, generated through the coagulation cascade, cleaves the N-terminal part of the two proteinase-activated receptors (PAR), PAR1 or PAR4 (PAR3 and PAR4 in mice), which also signals through G-protein coupled receptors[210, 211]. By cleaving off the N-terminal part from PAR, the receptor is activated by a so-called tethered ligand[212]. Due to the aforementioned activation steps, the platelet starts to synthesise thromboxane A_2, which also signals through a G-protein coupled receptor. In this way G-proteins of three different classes, G_{13}, G_q and G_i, are activated and in turn via second messengers (e.g. increased Ca^{2+} concentration, phosphorylation and diacylglycerol) activate the platelet, induce degranulation, change the shape of the platelet and transfer the integrins to a high-affinity state[213]. The increased Ca^{2+} concentration also activates special proteins that flip the phosphatidylserine from the inner leaflet of

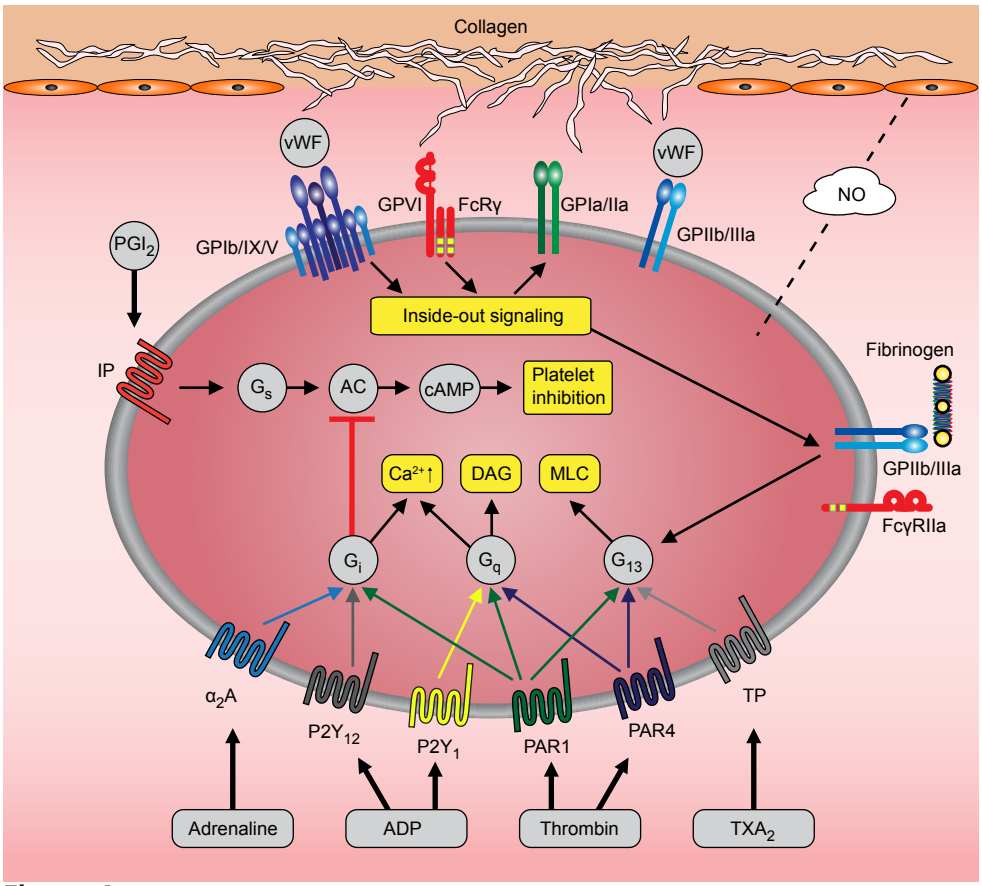


Figure 4
The platelet in haemostasis

the plasma membrane to the outer, generating a more negatively charged surface for activation of coagulation factors[214]. The activation of G_i also inhibits adenylate cyclase, which normally is stimulated by G_s downstream the receptor for prostacyclin. Thus the antithrombotic effects by prostacyclin are reversed in this way. Clopidogrel exerts its effects by blocking the $P2Y_{12}$ receptor upstream G_i[205] (Figure 4).

A central event in response to platelet activation is the propagation of the signals to the different integrins, shifting them from their low-affinity state to a high-affinity state (inside-out signalling) ready for interaction with their respective ligands. One of the most important integrins on the platelet is GPIIb/IIIa (CD41/CD61, $\alpha_2\beta_3$). This integrin, present in 40000 – 80000 copies/platelet, mediates binding to many proteins but most importantly to fibrinogen and vWF[215]. There is also an intracellular pool of GPIIb/IIIa in a-granulae released upon activation[216]. By binding to fibrinogen and vWF, the platelet anchors to other

	Alternative name	Molecules per platelet (on the surface)	Integrin	CD	Ligands	Main functions
GPIbα		25000		CD42b	vWF	Binding/
GPIbβ		50000		CD42c	P-selectin	Signalling
GPV		12500		CD42d	CD11b/CD18	
GPIX		25000		CD42a	FXI	
					FXII	
					Thrombin	
					Thrombospondin	
GPIIb/IIIa		40000-80000	α_2	CD41	Fibrinogen	Binding/
			β_3	CD61	vWF	Signalling
					CD40L	
					Fibronectin	
					Vitronectin	
					Thrombospondin	
GPVI				-	Collagen	Signalling
FcRγ(FcεRII)				CD23		
GPIa/IIa	VLA-2	2000-4000	α_2	CD49b	Collagen	Binding/
			β_1	CD29	Laminin	Signalling
Fibronectin-receptor	VLA-5		α_5	CD49e	Fibronectin	Binding
			β_1	CD29		
Laminin-receptor	VLA-6		α_6	CD49f	Laminin	Binding
			β_1	CD29		
Vitronectin-receptor		500?	α_V	CD51	Vitronectin	Binding
			β_3	CD61	Fibronectin	
					Osteopontin	
P-selectin	GMP-140			CD62P	PSGL-1	Binding
	PADGEM					

Table 4
Platelet surface receptors

platelets and aggregates thus stabilising the thrombus. Upon binding its ligands the receptor also signals to the interior, so called outside-in signalling leading to platelet spreading, further granulae secretion, adhesion and clot retraction[217]. This signalling was recently discovered to be mediated by G_{13}, making this the

first example of an integrin functioning as a non-canonical G-protein coupled receptor[218]. It was also recently shown that GPIIb/IIIa signalling is mediated through FcγRIIa demonstrating yet another cross-talk between integrins and adaptor proteins of the adaptive immune system[193].

Platelets and the immune system

Platelets do not only contribute to haemostasis but also to other important functions. The interactions, through direct contact or mediated by secreted molecules, between platelets and cells of the immune system, have recently been demonstrated or implicated. It is perhaps not surprising that platelets can mediate immune functions when considered from an evolutionary perspective. In arthropods a nucleated cell called the haemocyte circulates and mediates functions that in mammals are mediated by macrophages. In addition the haemocyte participates in wound healing and has the ability to coagulate the haemolymph in case of an exoskeletal breach. This cell might be the evolutionary ancestor of the platelets found in mammals[219].

Upon platelet activation P-selectin (CD62P, granule-membrane protein 140/GMP-140, platelet activation dependent granular-external membrane protein/PADGEM) is transferred from its intracellular store in a-granulae to the surface. P-selectin can then interact with P-selectin glycoprotein ligand-1 (PSGL-1) expressed on leukocytes *(see also Neutrophils – Recruitment to the site of infection)*[220]. Leukocytes have been shown to be able to roll on adherent platelets in a P-selectin-dependent manner and then transmigrate through them[216]. P-selectin has also been suggested to recruit tissue factor bearing microparticles via PSGL-1 to sites of thrombus formation and thus the tissue factor present during thrombus formation is not only from the subendothelial compartment but also recruited from the blood[220]. Tissue factor is the initiator of the extrinsic coagulation pathway (*see Coagulation and fibrinolysis*) Lately it has also been demonstrated that platelets also synthesise tissue factor themselves[221]. A further step in understanding the role of platelets in the immune system was taken when it was discovered that platelets contain precursor-mRNA for Il-1β and that thrombin stimulation could induce splicing and translation of this into IL-1β, a central mediator of the immune system[222]. Later it was realised that stimulation with LPS also induces IL-1β production and in fact does so with greater potential than thrombin[223]. During the last decade it has also become evident that platelets express Toll-like receptors (TLRs) and that at least some of them are fully functional[219]. For instance LPS from *E. coli* O157:H7 was found to bind and activate platelets. Bound LPS was found on platelets from patients, who had developed haemolytic uremic syndrome (HUS) but not on platelets from patients infected with *E. coli* O157:H7, who had not developed HUS[224].

Platelets in addition also express receptors and ligands known to bind to their partners on immune cells (e.g. CD40 ligand (CD40L, CD154), CD40, the ligand for Triggering Receptor Expressed on Myeloid cells 1 (TREM1-ligand)). CD40L can be recognised by endothelial cells and induce an inflammatory upregulation of adhesion molecules (e.g. ICAM-1) but also endothelial production of chemokines[225, 226]. CD40L derived from platelets has also been shown to induce isotype switching in B-cells as well as augment CD8+ T cell response to viral infections[227]. CD40L can also influence dendritic cell maturation thus forming a link between adaptive and innate immunity[227]. Patients with immune thrombocytopenia have been demonstrated to have deficiencies in regulatory T-cells, the function of which was restored upon treatments augmenting the platelet count[198]. CD40L may also promote CD11b/CD18 expression on neutrophils thus facilitating the formation of platelet-neutrophil complexes (see below)[228]. Furthermore, stimulated platelets secrete various chemokines and growth factors known to affect the immune system (e.g. platelet factor-4/CXCL4, RANTES/CCL5) as well as antimicrobial peptides (e.g. thrombicidin 1 and 2)[198].

Platelet-neutrophil complexes (PNC)

An important interaction between platelets and cells of the immune system is the formation of platelet-leukocyte complexes. Leukocytes may associate with immobilised platelets as well as with single flowing platelets. Leukocytes adhere to immobilised platelets though the interactions of P-selectin – PSGL-1 with concomitant expression of chemokines from the platelet (e.g. platelet factor 4/CXCL4, RANTES/CCL5). The dual signalling through PSGL-1 as well as through G-protein coupled receptors on the neutrophil induces an upregulation of CD11b/CD18. CD11b/CD18 then binds directly to GPIba or indirectly to the platelet via fibrinogen and GPIIb/IIIa thereby mediating firm adhesion and arrest of the leukocyte[216]. Adhesion through ICAM-2 on the platelet to CD11a/CD18 on the neutrophil has also been described[229] as well as adhesion between CD11a/CD18 and junctional adhesion molecule -3 (JAM-3)[230]. CD11c/CD18 has been implicated in platelet-induced activation of neutrophils but not to the adhesion of the to cells[231] although others claim that CD11c/CD18 is involved[232]. After arterial injuries it has been shown that GPIba is the main platelet receptor binding to CD11b/CD18[233].

Platelets also form complexes with monocytes although the propensity to form complexes differs. Platelets have easiest to form complexes with monocytes, followed by neutrophils and are least likely to form complexes with lymphocytes. Even among the lymphocytes the capability to form complexes differs; where large NK-cells are the cells that most easily associate with platelets[234].

Platelets associated with the different leukocytes have been demonstrated to alter the production of mediators by both cell types. Lymphocytes, which are not able to synthesise prostaglandins, can through

their interaction with platelets, be provided with the raw material for the production of thromboxane A_2. Complexes between platelets and neutrophils have been shown to synthesise lipoxin A_4 and lipoxin B_4 which neither cell type can synthesise alone[235]. Platelets activated by thrombin have been shown to adhere to monocytes with concomitant secretion of the chemokine RANTES (CCL5, **R**egulated upon **A**ctivation **N**ormal **T** cell **E**xpressed presumed **S**ecreted) and thereby induce monocyte chemokine secretion[236] Platelets associated to monocytes have also been shown to induce cyclooxygenase -2 expression in monocytes[237] and the eicosanoids derived can have negative effects during the pathogenesis of atherosclerosis[238].

Platelet-leukocyte complexes have been demonstrated in the circulating blood. Circulating neutrophils in PNCs have been shown to be more activated and more effective at phagocytosis and reactive oxygen species production[239]. Associated platelets also activate the leukocyte, making it more prone to interactions with the endothelium. In 2007 *Clark et al* demonstrated a novel mechanism of platelet-neutrophil interactions[63]. Platelets activated by LPS or by plasma from septic patients associated with neutrophils and triggered them into the formation of NETs. At least some of the platelet activation was dependent on TLR4 since this could be blocked by a TLR4-antagonist. In a murine model the platelet-neutrophil-induced NETs were shown to trap bacteria but also induced damage to the endothelium. This netosis was demonstrated to take place mainly in the liver and in the lungs. The LPS concentrations used in the study were very high and it has been speculated that this mechanism might be the body's last resort in combatting an infection. Interestingly some bacteria have DNases capable of degrading NETs. All virulent strains of *Streptococcus pyogenes* have been shown to possess DNase[240, 241].

Platelet-neutrophil complexes have also been studied *in vivo*. Patients with acute myocardial infarction have been demonstrated to possess increased numbers of PNCs. In some small studies, septic patients admitted to the ICU have been shown to have elevated PNCs although when progressing into multiorgan failure the ratio of PNCs diminished[242, 243]. These PNCs were dependent on P-selectin[244]. It has been proposed that the PNCs decline because PNCs are more activated and thus more prone to adhere to the endothelium and become sequestered in the capillaries, primarily in the lungs and in the liver, during multiorgan failure. The importance of platelet-neutrophil complexes has also been investigated in a murine model of acute lung injury. Acid-aspiration caused lung injury but blocking the formation of platelet-neutrophil complexes aborted the injuries. This indicates that the presence of P-selectin on platelets and the formation of PNCs is vital for the development of acute lung injury following acid aspiration[245]. PNCs have also been shown to be elevated in sickle cell disease and proposed to be responsible for the hypoxic/reoxygenation induced vascular injury[246].

Bacterial-platelet interactions

An increasing number of bacteria have been demonstrated to directly or indirectly interact with platelets preferentially among organisms known to cause infectious endocarditis. The classical example is *Staphylococcus aureus*, which through its clumping factor A, binds fibrinogen that in turn associates with GPIIb/IIIa on the platelet[247-249]. This type of plasma protein bridging to mediate aggregation has also been demonstrated for other Gram-positive bacteria (e.g. *Staphylococcus epidermidis*[250], *Streptococcus agalactiae*[251], *Streptococcus pyogenes*[252], *Aerococcus urinae*[253]) Direct bacterial-platelet adhesion has also been studied among other Gram-positive bacteria[254]. An example of this is Platelet Adhesion Binding protein A (PadA) on *S. gordonii*, which directly associate with GPIIb/IIIa[255]. In third mechanism, for example demonstrated by *Porphyromonas gingivalis*, is the secretion of a protease that activates platelets. Secreted cysteine proteases, gingpains, leads to cleavage of the PAR-1 in a manner analogous to thrombin thereby activating the platelet[256].

Platelets are also able to bind to and internalize bacteria although it is not clear whether the bacteria are killed or merely find an intracellular compartment to hide in. Platelets being only 2μm are not much bigger than the bacterium and may more be like a "covercyte".[257, 258].

M1 protein from *Streptococcus pyogenes* has been shown to activate and aggregate platelets and induce PNCs in an antibody-dependent manner[259]. Gram-positive bacteria isolated from patients induced PNC formation and platelet aggregation in the host from which they were isolated[260]. Lipoteichoic acid, a natural ligand for TLR2, has shown to exert different effects on platelet aggregation. It induced adhesion to *Staphylococcus epidermidis*[261]. Synthetic TLR2 ligands did not induce any effects in concentrations that activate neutrophils but ten times higher concentrations induced the formation of PNCs[262, 263]. Platelet aggregation have also been shown to be initiated by LTA on *Streptococcus pneumoniae*[264] whereas lipotechoic acid from *Staphylococcus aureus* inhibited platelet aggregation[265].

In the case of parasitic infection, platelets have been studied in the pathophysiology of malaria, where the platelet seems to have a dual role. On one hand platelets were shown to promote the sequestering of infected erythrocytes in the cerebral vasculature[266]. On the other hand platelets demonstrated the ability to kill erythrocytes infected with malaria trophozoites and mice treated with acetyl salicylic acid had higher mortality rates[267]. The role of the platelets in malaria is thus complex and multifaceted and many studies have been conducted[268].

Other diseases where platelets are implicated

Cardio-vascular diseases

Perhaps one of the first examples where the influence of platelets was evident was in the development of atherosclerosis, where platelets have been shown to bind to inflamed endothelium and thereby

altering the behaviour of other immune cells[269]. Upon binding, platelets express P-selectin thus recruiting monocytes. The platelet contribution of P-selectin has in atherosclerosis-prone mice shown to enhance the atherosclerotic development[270]. Platelets also shed large amounts of CD40L that contribute to atherosclerosis[219]. Platelets expressing CD40L have also been shown to inhibit the recruitment of regulatory T cells thus promoting atherogenesis[271]. Platelet-monocyte complexes are increased during acute coronary syndromes[272] and microparticles shed from activated neutrophils can through CD11b/CD18 associate with platelets via GPIba and activate them[273].

Transfusion-related acute lung injury (TRALI)

Transfusion-related acute lung injury (TRALI) is characterised by a non-cardiogenic pulmonary oedema in connection with blood transfusions and is a leading cause of transfusion-related deaths[274]. The mechanisms involved in TRALI are still not understood and many hypotheses have been proposed. Recently it was suggested that platelets released CD40L during storage and that this stimulated neutrophils and triggered TRALI[275].

Cancer

Malignancies are associated with an increased risk of thrombosis[276]. Platelet might also aid in the metastasis of cancer and thrombocytosis is associated with poor prognosis in brain, breast, colon endometrial, gastric, lung, ovarian, pancreatic and renal malignancies[277]. Platelets attached to tumour cells protect the tumour cells from being attacked by NK cells and platelet depletion reduced the metastatic potential of NK cell-sensitive tumours[278]. Furthermore, in a mouse model platelet depletion diminished the metastatic potential of tumours but concomitant depletion of NK cells eliminated this reduction[279]. Knock-out mice for PAR-4 also exhibited reduced metastasis of tumour cells[280]. The formation of tumour cell – platelet aggregate has been suggested to be mediated at least in part by selectins[281]. It is thus interesting to notice that heparin diminishes the metastatic capability; possibly through inhibition of thrombin but also by binding to the negatively charged mucins on the tumour cells impeding their binding to P-selectins on platelets[277]. In the next step of metastasis, extravasation from the blood stream, the participation of integrins as well as GPIb-IX-V has been implicated but for the latter not proven[277].

Thrombocytopenia

Thrombocytopenia, a lowered platelet count, can occur as part of immune thrombocytopenic purpura (ITP) caused by autoantibodies against platelets. It exists in both an acute and a chronic form. The platelets are consumed through Fc-mediated phagocytosis. It is recognised that ITP often occurs after a bacterial or viral infection. Interestingly, it was recently demonstrated that the phagocytosis of antibody opsonised platelets increased significantly in the presence of LPS[282].

Thrombocytopenia induced by bacterial infections is considered an ominous sign in sepsis and has been associated with

poor prognosis in ICU patients with nosocomial bloodstream infections[283, 284]. Thrombocytopenia can occur due to decreased production as well as to increased destruction. In a murine model it has been demonstrated that LPS induces thrombocytopenia in a TLR4-dependent manner. Furthermore platelets migrated into the lungs in a TLR4-dependent manner due to LPS treatment but this migration was preceded by neutrophils lodging in the lungs. The neutrophils were essential for the platelet migration whereas the reverse relationship did not occur. Moreover, P-selectin did not seem to be involved since LPS did not induce an upregulation of P-selectin[285]. Thrombocytopenia has also been shown to decrease the TNF production in a mouse model[286]. A potential mechanism for infection-associated thrombocytopenia was presented in 2008 when it was shown that the *Streptococcus pneumonia* through a sialidase (neuraminidase) desialyated von Willebrand factor and platelets. These desyalilated platelets were captured by the Ashwell receptor in the liver thus giving rise to thrombocytopenia. In a murine model this thrombocytopenia was associated with prolonged survival, which the authors attributed to reduced coagulopathy and reduced risk of disseminated intravascular coagulation[287].

Coagulation and fibrinolysis

Primary haemostasis by platelets works together with the secondary haemostasis to prevent blood loss in the case of an injury. Secondary haemostasis consists of several soluble serine proteases cleaving each other and culminates in the cleavage of fibrinogen by thrombin. Fibrinogen is cleaved into fibrin, which forms a network of fibres. Traditionally the coagulation cascade has been divided into the extrinsic pathway (activated by substances outside the blood and uncovered during injuries) and the intrinsic pathway (activated by substances present within the blood). Current knowledge focuses on an integrated view with the intrinsic pathway as an amplification loop[215]. The extrinsic and intrinsic pathways coalesce with the central mediator thrombin (Figure 5). Thrombin is also a serine protease and has many functions as well as an activator but also as an inhibitor of coagulation. Thrombin enhances coagulation by cleaving fibrinogen into fibrin, activating platelets through PAR1 and 4, and activating factor V, VIII and XI. On the other hand thrombin combined with thrombomodulin on endothelial cells activates protein C, which cleaves and inactivates the procoagulant factors Va and VIIIa. In addition thrombin also activates thrombin activatable fibrinolysis inhibitor (TAFI).

The formed clot can then be dissolved by the fibrinolytic system. Tissue-type plasminogen activator or urokinase-type plasminogen activator cleaves and activates plasminogen to plasmin. Plasmin then cleaves the fibrin in the clot thereby dissolving it. The regulation of coagulation and fibrinolysis is intricately related and will not be discussed further in this thesis. Below I will give a very short description of two components from these systems that have been investigated in this thesis.

Thrombin activatable fibrinolysis inhibitor (TAFI)

Thrombin activatable fibrinolysis inhibitor (TAFI) is a metallo-carboxypeptidase that is activated by thrombin. The potential of thrombin to activate it is rather low but enhanced more than a thousandfold when thrombin associates with thrombomodulin[288]. In addition TAFI can also be activated by plasmin. Activated TAFI cleaves off C-terminal lysines from the fibrin clot. These are important co-factors in the generation of plasmin from plasminogen. Thus TAFI abrogates the fibrinolytic capability[289]. The normal concentration of TAFI in the plasma is 4-15μg/ml but very little of this is in

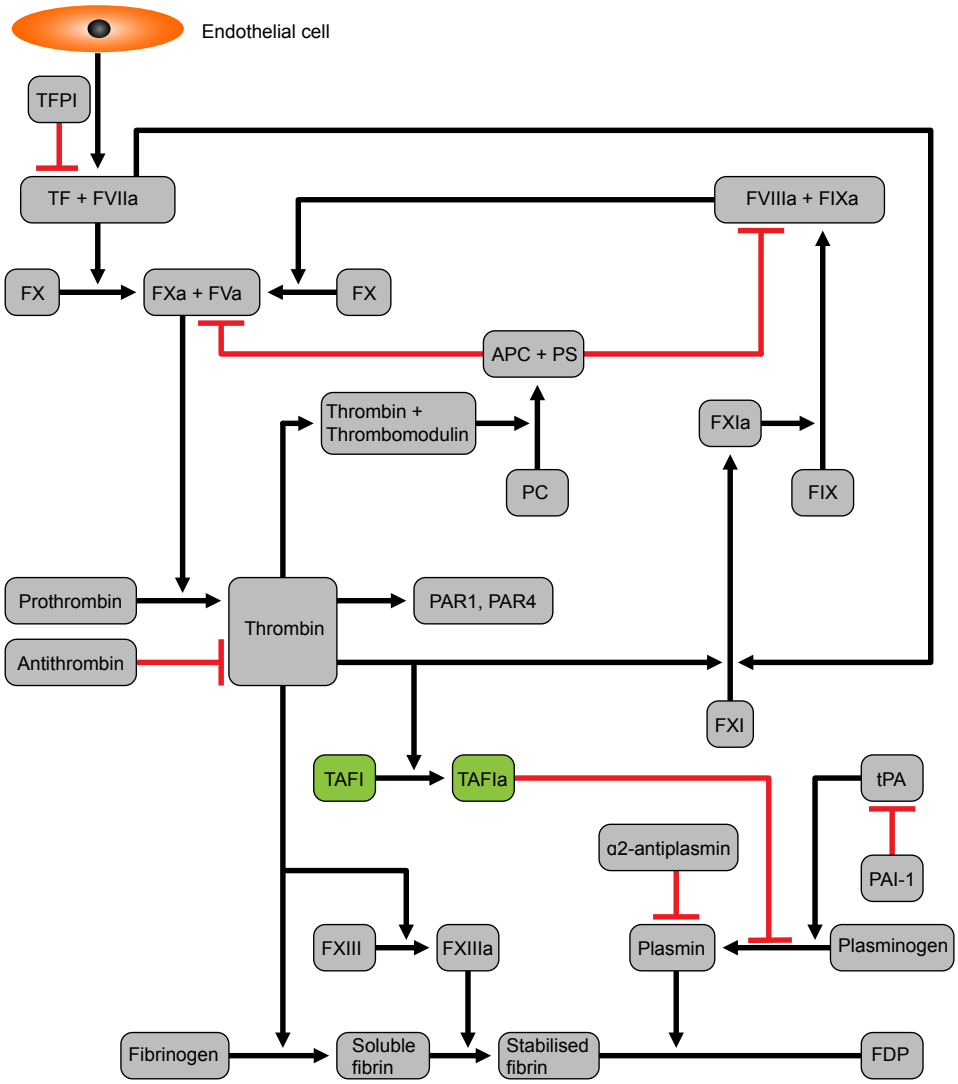


Figure 5
The coagulation cascade

its active form. Therefore only a slight increase in active TAFI may dramatically increase its function. Epidemiological studies indicated TAFI/TAFIa as risk factors for venous thrombosis[290, 291].

Fibrinogen

Fibrinogen is a plasma protein present in a concentration of 2-4g/L in plasma[197]. It is composed of 6 polypeptide chains, 2 copies each of the three chains Aa, Bb and g. All N-terminals are located in a

central domain, the E domain. From this domain the polypeptide chains stretch out in two directions in a coiled-coil formation. One pair of AaBβg makes up each coiled-coil that terminates in a globular domain, the D domain. The D domain contains the C-terminal ends of the Bb and g chains, whereas the Aa chain turns back towards the centre again[292]. Upon activation of the coagulation cascade thrombin is formed and cleaves off short peptides from the N-terminals in the central E domain. This allows the N-terminals to join to D-domains in another fibrinogen molecule forming a fibrin network. This network is then stabilised through the action of factor XIII. Cells can bind to fibrinogen through integrins. GPIIb/IIIa on platelets associates with fibrinogen through RGD-sequences and the leukocyte integrins CD11b/CD18 and CD11c/CD18 also have affinity for fibrinogen but also for other sequences[293-295]. In addition many bacterial proteins can associate with fibrinogen (e.g. M protein from *Streptococcus pyogenes – see S. pyogenes M protein*).

Immunoglobulins

Immunoglobulins (antibodies) are proteins that aid in the clearance and neutralisation of pathogens and antigens. Immunoglobulins are synthesised by the B-cell. There are five classes, called isotypes, of immunoglobulins: IgG, IgM, IgA, IgE, and IgD. The immunoglobulins consist of two identical heavy chains and two identical light chains (except some odd examples in lower animals)[296]. The light chains are linked to the heavy chains by disulphide bonds and disulphide bonds also link the two heavy chains to each other. The nature of the heavy chain defines the immunoglobulin class. Each of the chains is made up of immunoglobulin domains, of approximately 110 amino acids, bridged together by disulphide bonds. At the furthermost N-terminal is the V (variable) domain and the most N-terminal parts of the two V domains from the light and heavy chain make up the antigenic cleft to which the antigen binds. In addition to the V domain each light chain has a constant domain whereas the heavy chains have three to four more domains[297]. A vast discussion of immunoglobulins is beyond the scope of this thesis and I will just briefly discuss some features of immunoglobulin G.

Immunoglobulin G

Immunoglobulin G (IgG) is the most abundant isotype in blood and is present at 6.4-13.5 g/L and has a half-life of 23 days[297]. The molecular weight is 150 kDa; 50kDa for each heavy chain and 25kDa for each light chain. Of the total IgG-content of the body approximately 45 % is found in the blood and the rest is found in the extracellular fluid[298]. The heavy chains of IgG consist of one variable and three constant regions. The different regions of the antibody are named according to Figure 6. The Fc-region (c because it can be crystallised), is composed of the constant regions and mediates the binding to the IgG-receptors, the Fcg-receptors (*see – Neutrophil, Fc-receptors*) as well as the interaction with the protein C1q that initiates the complement cascade. The antigenic specificity is located to the N-terminal region, the Fab-region. The proteolytic enzyme papain cleaves IgG into two pieces, one Fc-fragment and one $F(ab')_2$-fragment. Pepsin on the other hand cleaves on the N-terminal side of the disulphide bonds, thus generating one Fc-fragment and two Fab-fragments. The streptococcal IgG-cleaving enzyme IdeS cleaves C-terminal compared to papain (i.e. on the C-terminal side of the disulphide bonds), hence generating one $F(ab')_2$-fragment and two separate chains of the Fc-part[299].

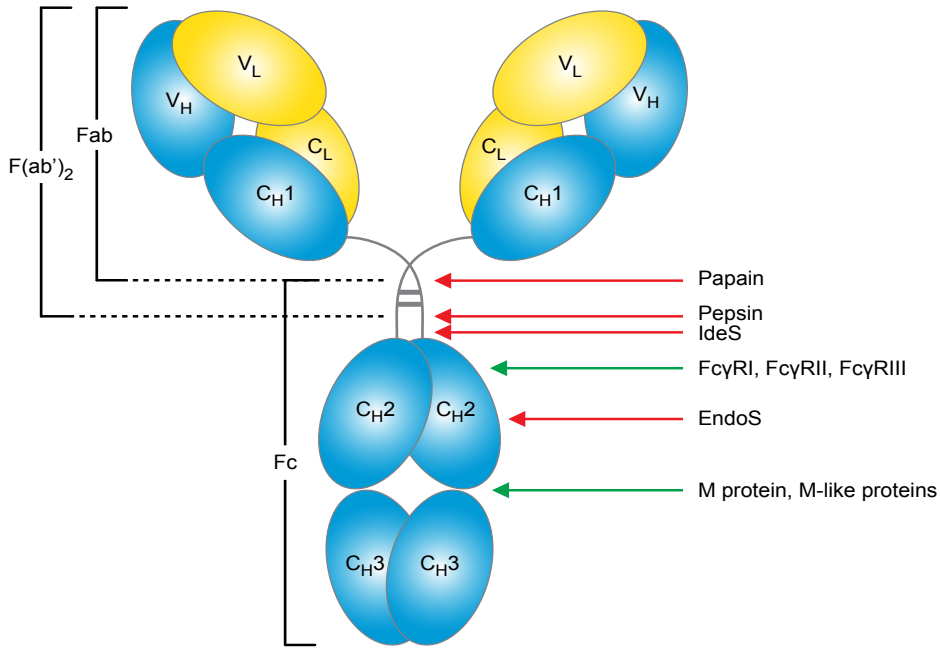


Figure 6
Immunoglobulin G

There are two different classes of light chains; κ and λ. Approximately 60% of the IgG consist of κ–chains and the rest of λ. The IgG can also be subdivided into four distinct subclass: IgG_1-IgG_4, which among other attributes have different affinities for the Fcg-receptors (*see – Neutrophil, Fc-receptors*). Antibodies against carbohydrate antigens tend to be of IgG_2-class and it is only IgG_1 and IgG_3 that can fix complement[297]. Like all antibodies, IgG is a glycoprotein. Removal of the glycosylation affects the binding to Fcg-receptors as well as the ability to bind C1q[300]. In the serum of healthy individuals several different variants of glycosylation exist[301] and serum from patients with autoimmune disorders have changed their glycosylation levels[156]. The streptococcal enzyme EndoS strips the IgG of its glycans, thus interfering with the ability of IgG to interact with its receptors[302].

In addition to the ordinary Fcg-receptors a neonatal Fc-receptor, FcRn also exists. The neonatal FcRn is not related to the other Fc-receptors but instead has homology with MHC- class I[303]. The FcRn transfers IgG from the mother to the foetus but it also exists in adults as a regulator of the half-life of IgG by saving IgG from degradation. Overloading this receptor is thought to be one effect of intravenous immunoglobulin (IVIG) treatment. The overloading generates an increased turnover of the host's own IgG that is replaced by the administered IgG[304].

Streptococcus pyogenes

Streptococcus pyogenes is a Gram-positive catalase-negative facultative anaerobic coccus with a diameter of 0.6-1.0μm. It demands rich media for optimal growth and is traditionally grown on blood agar, where its haemolytic activity is clearly visible. The haemolytic zone is were toxins produced by the bacteria have lysed the erythrocytes (haemolysis). When grown in the laboratory it forms long chains of coccoid bacterial cells , like pearls on a string, but *in vivo* the chains tend to be much shorter. *S. pyogenes* is exclusively a human pathogen and was in a study estimated to be among the ten pathogens causing most deaths globally[305]. Despite extensive treatment with penicillin it remains uniformly penicillin-sensitive. Although recently there has been a report on penicillin-resistant strains[306] but this has not been verified and the existence of such strains has been questioned[307].

The discovery of *S. pyogenes*

Although diseases caused by *S. pyogenes* (e.g. wound infections, puerperal fever) have been known since antiquity, *S. pyogenes* was initially described by *Theodor Billroth*. Billroth described the bacteria in wound and skin infections and used the term *streptococcus* to describe the growing form of what he called *Coccobacteria septica*[308]. Streptococcos comes from the Greek words; *Streptos (*στρεπτός) meaning "chain" and *kokko*s (κόκκος) meaning "grain or seed". In 1879 *Louis Pasteur* isolated the bacterium from the blood of a woman dying from puerperal fever and during a discussion in the French Medical Academy he proclaimed his findings:

"One day, in a discussion on puerperal fever at the Academy, one of his most weighty opponents was eloquently enlarging upon the causes of epidemics in lying-in hospital. Pasteur interrupted him: "None of those things cause the epidemic; it is the nursing and medical staff who carry the microbe from an infected woman to a healthy one." And as the orator replied that he feared that microbe would never be found, Pasteur went to the blackboard and drew a diagram of the chain-like organism, saying, "There, that is what it is like!"[309]

In 1883 *Friedrich Fehleisen* described the organism but did not call it *Streptococcus*[310]. Finally in 1884 *Friedrich Rosenbach* for the first time used the term *Streptococcus pyogenes* to describe bacteria isolated from wounds and skin infections[311].

In 1919 *James Howard Brown* presented a system for grouping the different streptococci according to their haemolysis of blood agar[312]. Streptococci that give haemolysis and a clear zone around the colonies were named b-haemolytic. If the zone around the colonies was green the streptococci were called a-haemolytic and if no haemolysis occurred they were called g-haemolytic streptococci. Later in 1933 the pioneering work of *Rebecca Lancefield* gave a system to divide streptococci according to the carbohydrate antigens present on the surface[313]. In 1937 *James M Sherman* divided the genus into pyogenic, viridans, enterococcal and lactic streptococci[314]. It is important to realise that these different systems overlap and different strains from a certain species can belong to different Lancefield groups and have different haemolytic properties. All strains of *Streptococcus pyogenes* are b-haemolytic and express the group A antigen, therefore *S. pyogenes* are also known as b-haemolytic group A streptococcus (GAS). Some strains of *Streptococcus anginosus* can also be b-haemolytic and express the group A antigen but the colony morphology is different from that of *S. pyogenes*.

Rebecca Lancefield presented a grouping system for *S. pyogenes* which distinguished different serotypes based on one of their surface proteins, the M protein[315]. A different system was developed by Griffith, the so-called T protein agglutination reaction[316]. Both systems are used today in clinical settings, although the M system is preferred in scientific publications. Today the grouping is often done genomically by sequencing the *emm* gene and more than 200 sequence types have been described[317] (for a more detailed discussion on M protein – *see M protein*).

Clinical picture

Streptococcus pyogenes give rise to a variety of infections spanning from mild (e.g. impetigo and tonsillitis) to life-threatening and fatal infections (e.g. streptococcal toxic shock syndrome (STSS) and necrotising fasciitis (NF)).

Streptococcal pharyngitis/tonsillitis

Pharyngitis/tonsillitis due to streptococcal infection ("strep-throat") is a very common disease especially among children aged 5 to 15. Most of these infections are caused by *S. pyogen*es but also streptococci of serogroup C and G can cause pharyngitis. It has been estimated that 616 million cases of pharyngitis caused by *S. pyogenes* occur per year[305]. The disease is spread directly person-to-person most likely via droplets but both food borne and waterborne outbreaks have been documented. The magnitude of asymptomatic carriage has been discussed. The carriage rate among asymptomatic children has been estimated to 12% in a recent meta-analysis (range 3-26%) with lower prevalence in children under the age of five[318]. The same meta-analysis estimated that a 37% of children visiting a doctor for a sore throat were infected with *S. pyogenes*.

After an incubation period of 2-4 days

the patient turns ill with an abrupt onset of sore throat, fever, malaise and headache. Most cases are self-limiting but due to the risk of complications *(see Rheumatic fever*) all verified cases should be treated. Asymptomatic carriage on the other hand, does not seem to predispose for rheumatic fever and can thus be left untreated[319, 320]. Suppurative complications may occur with peritonsillar or retropharyngeal abscesses, peritonsillar cellulitis and cervical lymphadenitis[321].

Scarlet fever/Scarlatina

Scarlatina is characterised by a diffuse red blush with points of deeper red that blanch on pressure. The erythema often starts on the chest and then spreads. In skinfolds lines of deeper red can be seen (Pastia's lines). Increased capillary fragility can be present and evident from scattered petechiae. The increased fragility can be tested with Rumpel-Leeds test. The rash often spares the skin around the mouth so-called circumoral pallor. The erythema often fades after a few days and after a week desquamation occurs beginning in the face and progressing downwards. The tongue can be covered with a white coating, through which the papillae can be seen (white strawberry tongue). Eventually the coating disappears and the tongue now presents itself as thick and red (red strawberry tongue).

The appearance of scarlatina is often preceded by pharyngitis but any infection caused by *S. pyogenes* may be followed by scarlatina. Traditionally it was thought that scarlatina was due to the erythrogenic toxins SpeA, SpeB and SpeC. However it has now been demonstrated that SpeB is not erythrogenic and previous results were due to co-precipitation/contamination of SpeA or SpeC[322].

Impetigo

Impetigo or pyoderma is a mild skin infection either caused by *S. pyogenes* or *Staphylococcus aureus*. The predilection age is lower than for pharyngitis and children aged 2 to 5 years are mostly affected. The pathogen first colonises the skin and then, probably through small preexisting lesions, enters the skin and gives rise to impetigo[323]. Impetigo gives rise to multiple papules that evolve into vesicles surrounded by an erythema. If the lesion is deeply ulcerated it is called echtyma. Systemic involvement is usually absent and suppurative complications are rare. Interestingly rheumatic fever does not seem to be a complication of impetigo. On the other hand cutaneous infection with some strains often precedes poststreptococcal glomerulonephritis. However, there is no firm evidence that treatment of an individual infected with a nephritogenic strain prevents the development of acute poststreptococcal glomerulonephritis[324] although this may ease the community burden of such strains.

Erysipelas

Erysipelas is classically a streptococcal infection localised to the dermis. The lesions are typically raised above the

surrounding skin, light red and sharply demarcated from the surrounding tissue. The cutaneous involvement is often accompanied by systemic involvement with fever and chills[325]. In some cases a deeper involvement is present that more resembles a cellulitis. The infection then spreads in the subcutaneous tissue. This is not what is classically defined as erysipelas but sometimes referred to as erysipelas. These lesions are not raised and are deeper red and the demarcation line is not sharp.

Necrotising fasciitis and Streptococcal Toxic Shock Syndrome

Although the diseases described hitherto are usually not life-threating, *S. pyogenes* can cause very serious and life-threating infections with high mortality rates. In necrotising fasciitis the infection extends deeper in the subcutaneous tissue. It was first described by Meleny in 1924 who called it haemolytic streptococcal gangrene[326]. The infections described by Meleney seem to be less fulminant that present day cases. Furthermore, Meleney described mortality rates of 20%, as compared to mortality rates of 30-70% seen today in cases associated with streptococcal toxic shock (STSS)[327]. Necrotising fasciitis is signified by a deep infection in the subcutaneous tissues often accompanied by extreme pain. The pain is out of proportion to what is superficially. In cases of fever in combination with such pain necrotising fasciitis must be suspected.

Table 5
Case definition of streptococcal toxic shock syndrome

I. Isolation of *S. pyogenes*
   A. From a normal sterile site = definite case
   B. From a nonsterile site = probable case

II. Clinical signs of severity
   A. Hypotension; Systolic blood pressure <90mm Hg
   AND
   B. ≥2 of the following signs
      1. Renal impairment
      2. Coagulopathy
      3. Liver involvement
      4. Adult respiratory distress syndrome
      5. A generalised erythematous macular rash that may desquamate
      6. Soft tissue necrosis

Case definition for the Streptococcal Toxic Shock Syndrome

Cases must fulfill I + II. IA = definite case, IB = probable case

Modified from The Working Group on Severe Streptococcal Infections[335]

In the mid 1980s there was a sudden rise in invasive streptococcal infections and cases of streptococcal toxic shock (STSS) were reported[328]. Until then a decline in severe streptococcal infections had been seen. Alredady in the late 19th century before the introduction of antibiotics streptococcal infections tend to be less severe.[329]. The reappearance of this, for centuries feared bacterium, and its increased virulence has since then attracted much interest. Despite much effort the molecular mechanisms underlying this disease has not been fully elucidated.

Cone et al described two cases of streptococcal toxic shock in 1987. Owing to the similarities with toxic shock syndrome caused by *Staphylococcus aureus* they called it streptococcal toxic shock syndrome[330]. The case definition for streptococcal toxic shock was established in 1993 and is shown in table 5. The mortality is very high with estimated mortality rates of 30-70% in patients with STSS and 20-50% in necrotizing fasciitis[329]. In a study from Sweden carried out between 2002-2004 the incidence of invasive streptococcal infections was 3 per 100 000 inhabitants[331] which was higher than that reported during an outbreak of invasive M1T1 serotype between 1987-1989[332, 333]. In the study from 2002-2004 the overall case fatality rate was 14,5% and 39% among those suffering from STSS. Of patients presenting with NF, 51% also had STSS. During 2003 and 2004 a European study was conducted in eleven countries. The incidence of invasive *S. pyogenes* infection in the northern countries was 3 per 100 000 inhabitants with 8% of the patients having NF. Among those patients developing STSS the mortality was 44%[334]. Although many patients had predisposing factors, 21% lacked such factors. NF is often associated with STSS although STSS can arise due to any infection with *S. pyogenes*.

STSS can present itself like an influenza-like disease with fever, chills, myalgia, nausea, vomiting and diarrhoea[329]. Fever is present in 90% of the patients and many patients are also confused and the signs of shock and organ failure soon follow. Swift treatment is life saving. Source control (i.e. surgery to remove affected parts if necessary) is important, as is antibiotic treatment. Although *S. pyogenes* is uniformly penicillin-sensitive, penicillin should be combined with clindamycin. The rational for the addition of clindamycin stems from the fact that (1) animal models have shown an increased survival due to treatment with clindamycin[336], (2) penicillin-binding proteins are not expressed during the stationary phase of growth[337], (3) clindamycin supresses the production of important virulence factors such as exotoxin, superantigen, and M protein[338-341].

Intravenous immunoglobulin G (IVIG)

Antibodies that help in the fight against the bacteria are believed to be important as well as the capability of specific antibodies to neutralise toxins produced by the streptococcus. Several studies have addressed the titres of antibodies against

several different streptococcal antigens in patients with severe streptococcal infections and evaluated treatment with IVIG. In a study from Sweden, patients infected with M1T1 *S. pyogenes,* were investigated for antibody titres against M1. Low antibody titres were seen in patients with invasive or fatal cases whereas high titres were seen among patients with uncomplicated infections[333]. In another study of 33 patients with invasive M1T1 *S. pyogenes* infection (21 severe, 12 non-severe) all patients had lower anti-M1 titres than age-matched controls although there was no difference between those with severe and non-severe disease[342]. Low antibodies against superantigens and various other streptococcal virulence factors have also been demonstrated in patients with severe infections[343, 344]. In yet another study *S. pyogenes* was isolated from patients with invasive streptococcal infections. Overnight cultures contained superantigenic activity, which could be neutralised with plasma from patients treated with IVIG. Plasma taken before the IVIG-treatment had significantly less ability to neutralise the superantigenic activity[345]. Intravenous immunoglobulin G preparations (IVIG) have also been shown to contain antibodies against the M1 protein[346].

A Canadian study of IVIG as a treatment for patients with STSS demonstrated a decreased 30-day mortality in patients receiving IVIG, 33% as compared to 66% in the control group. However, the controls were historical and had received significantly less clindamycin and surgerical intervention[347]. A multicentre study conducted in Sweden and other European countries was terminated prematurely because of slow enrolment. Twenty-one patients were included in the study and in a group of ten who received IVIG the mortality was 10% while in a group of eleven who received placebo the mortality was 36%. This was however not significant due to the low number of patients enrolled in the study. The ability of the patients' plasma to neutralise superantigens increased significantly in the IVIG treated group[348].

In a murine model of IVIG treatment humanised mice were infected with *S. pyogenes* and treated with IVIG. When IVIG was given concomitantly with the bacteria it neutralised superantigens and reduced the inflammatory response as well as enhancing the systemic clearance of bacteria. However, when the treatment was delayed 24 hours and given together with penicillin and clindamycin, IVIG conferred no additional beneficial effect[349].

Puerperal fever

Puerperal fever was a feared complication to childbirth and was the leading cause of maternal mortality in the United Kingdom until the early 20th century[350]. Puerperal fever typically arises perinatal within two days of birth. Although rare in the industrialised world, cases still occur and *S. pyogenes* actually accounts for the majority of infection related puerperal deaths[351].

Others manifestations

S. pyogenes can also give rise to lymphangitis, pneumonia, endocarditis and meningitis as well as benign conditions such as perianal cellulitis and vulvovaginitis.

Nonsuppurative sequelae

Rheumatic fever

Acute rheumatic fever (ARF) is a sequela of streptococcal infection. It is likely an autoimmune disease but other theories such as toxic effects of streptolysin or serum sickness have been suggested[352]. It is characterised by carditis/valvulitis, arthritis (often polyarthritis), chorea, erythema marginatum, and subcutaneous nodules. It is often acute, febrile and mostly self-limiting. The most feared complication is rheumatic heart disease (RHD) which can lead to valvular fibrosis and valvular failure[353] It has been estimated that the prevalence of rheumatic heart disease is 15.6 million cases responsible for 230 000 deaths annually[305]. A more recent study pointed out the global difference in incidence with the highest incidence of 1% in sub-Saharan Africa[354].

The first report of autoimmunity and molecular mimicry was from *Kaplan et al* in 1962 when they reported cross-reactivity between antibodies against *S. pyogenes* cell wall proteins and human heart tissue[355]. In 1982 it was shown that antibodies against M protein cross-react with human heart tissue[356]. An alternative mechanism for ARF has also been proposed, whereby collagen binds to M3 protein and elicits antibody formation in a mouse model. High titres of anti-collagen IV were also found in patients with ARF[357]. Individuals with untreated tonsillitis are thought to have a risk of 0.4-3% of developing ARF[352]. Already in the 1950s it was demonstrated that penicillin-treatment of the antecedent streptococcal infection, even up to nine days after the debut, could prevent ARF[358]. Traditionally it has been considered that ARF only follows tonsillitis and not impetigo but some epidemiological evidence suggests that skin strains may play a role[353].

Poststreptococcal acute glomerulonephritis

After an infection with certain strains of S. *pyogenes* poststreptococcal acute glomerulonephritis may develop. Such strains are called nephritogenic. Certain M types seem to be more nephritogenic but not all strains of that M type are nephritogenic[352]. The mechanism of the disease has not yet been elucidated but is speculated to involve various nephritogenic antigens[359]. Poststreptococcal acute glomerulonephritis can follow both tonsillitis and skin infections and it is not thought that penicillin-treatment of the antecedent infection has any major preventive effect[324]

Streptococcal virulence factors

In order to cause infection *S. pyogenes* must first colonise the host which is often followed by a spreading of the bacterium. To do so *S. pyogenes* uses many different virulence factors to circumvent the defence mechanism of the host. Below I will discuss in detail those factors that are most relevant to the studies presented in this thesis. I will also mention briefly some of the other virulence factors of *S. pyogenes*.

M protein

M protein is a major virulence factor of *S. pyogenes* and has been attributed many diverse functions. M protein is a cell wall-anchored protein. It consists of an a-helical coil that dimerises to give rise to an a-helical coiled coil extending approximately 0.5µm from the surface of the streptococcus[360]. The M protein consists of several different domains and is encoded by the *emm* gene (Figure 7). The N-terminal contains a hypervariable domain (HVR), which underlies the basis of different serotypes of the M protein. Type-specific antibodies are directed against this domain and as already shown by Lancefield, these antibodies confer protection[315]. (Streptococci present in none immune blood survive but in the presence of protective antibodies the streptococci are killed.) Today, molecular typing is based on the 160 most N-terminal base pairs of the mature protein[361]. The hypervariable domain (A region) is followed by B repeats. The number of repeats differs

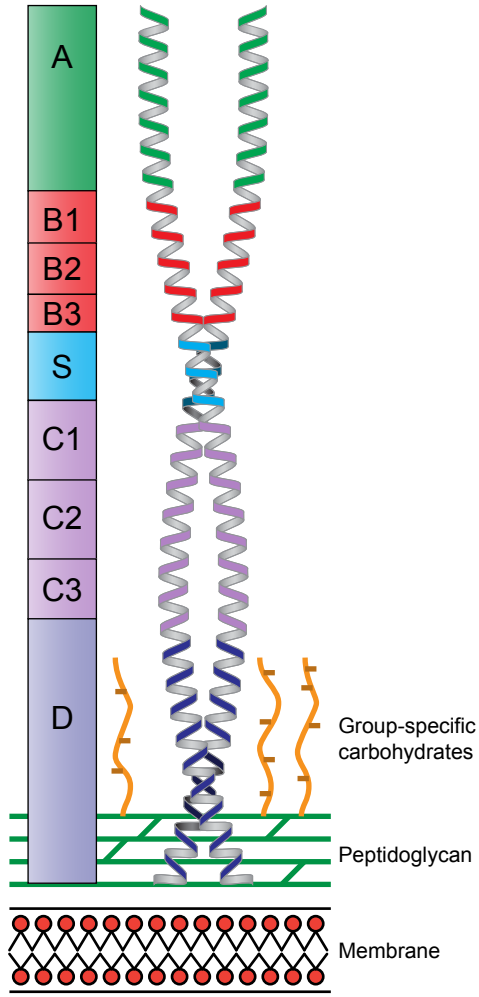


Figure 7
M1 protein from *S. pyogenes*

between various M serotypes[362]. In the M1 protein the B repeats are followed by a special domain, the S domain, which has been shown to exhibit binding to the Fc domain of IgG[363]. The B domains are followed by the C repeats and most of the C- terminal is the D domain. The wall-spanning region of the D domain contains an LPxTG motif that covalently attaches to the peptidoglycan[364, 365]. The

sequence variation between different M proteins follows a gradient along the M protein with C-terminal parts being more conserved than N-terminal[360]. M proteins can also be divided into two classes. Class I M proteins express C domains reacting with antibodies against the C domain whereas class II do not react with such antibodies[366]. The expression of class II M proteins is coupled to the expression of serum opacity factor which is a lipoproteinase that cleaves apoprotein A1 thereby leading to the opacification of serum[367-369]. The expression of M protein is under the regulation of the *mga* regulon (**m**ultiple **g**ene **r**egulator). Mga strongly upregulates the expression of proteins controlled by it (e.g. M protein, SclA, SpeB, C5a-peptidase) and is most active during the exponential phase of growth as well as stimulated by the presence of CO_2[370]. The exponential growth phase is seen when bacteria are cultivated *in vitro*. During a period the bacteria divide by fission and increases exponentially. After the bacteria have reached a certain concentration the growth velocity slows down and the bacteria enters the stationary phase. It is hard to fully translate this into the *in vivo* situation but the exponential phase is thought to occur after colonisation but before the supply of nutrients get scarce.

As stated above the hypervariable domain is the basis for serotyping *S. pyogenes* and antibodies directed against this domain confer protection[371-374]. It was recently been shown that this part of the M protein has weak antigenic properties and that in fact most antibodies against the M protein are directed more to the C-terminal[375]. This might be one way that the streptococcus protects itself.

The hypervariable region has also been shown to be of importance by conferring protection against the antimicrobial peptide LL-37[376]. The hypervariable region is also capable of interfering with complement binding to the streptococcus. Complement component C4b-binding protein (C4BP) binds to the HVR and by binding this protein the streptococcus can diminish complement activation on its surface and confer protection[377-381].

The binding of fibrinogen to M protein was first reported by *Kantor et al*[382] and later mapped to the N-terminal portion of the protein[383]. The binding of fibrinogen has been shown to be crucial for the protection against phagocytic killing[384] among other effects by interfering with the deposition of complement[385]. Fibrinogen has also been shown to be important for the recruitment of plasminogen to the bacterial surface, which is important for the transition from a localised to an invasive infection[386-389]. In addition to binding to fibrinogen, plasminogen can also be recruited directly to the streptococcal surface by binding directly to M-like proteins[390]. The fibrinogen binding and the Fc-mediated IgG binding to the S domain of M1 protein are mutually exclusive due to the vicinity of the binding sites[363]. The C-repeats have been shown to bind to albumin[391].

The repeats in M protein are imperfect and the elucidation of the crystal structure of M protein has provided new insights into the importance of this. This crystal structure showed a substantial number of structural irregularities in the coiled coil[392]. These irregularities separate the B repeats from each other. The importance of these irregularities is evident from the fact that idealisation of these aborts the fibrinogen binding properties of the M protein[392]. The instability of the structure may also explain the instability of M protein at 37°C[393-395].

M protein also confers protection from activation of the complement system. In addition to the above-mentioned interaction with complement C4-binding protein, M proteins of certain serotypes may interact with factor H or factor H-like protein thereby regulating the activity of the complement cascade[396, 397]. Subsequent studies have questioned the importance of the interaction with factor H and factor H-like protein[398]. Quite surprisingly, it was demonstrated that even under non-immune conditions the classical pathway of complement seems to predominate and that complement C4-binding protein or IgA binding to M protein conferred protection[381]. The fibrinogen-binding properties of M protein are also important[399-401] and fibrinogen may block the activity of the classical pathway, which was active even under non-immune conditions[385].

It has been demonstrated that the phagocytosis of *S. pyogenes* both under immune and non-immune conditions is mediated by complement receptors and not Fc-receptors[402, 403]. However, it has also been reported that the M protein does not act primarily by inhibiting phagocytosis but by promoting intracellular survival, possibly by affecting the phagosome maturation or by escaping from the phagosome[404-407]. Living intracellular *S. pyogenes* has also been found *in vivo* in macrophages during a soft tissue infection[408].

M protein in addation has been shown to have many other important functions for the survival of *S. pyogenes*. It has been shown to be involved in adhesion of *S. pyogenes* to host cells[409-411] although only a few M types so far has been shown to bind directly to fibronectin[412, 413]. Moreover M1 protein has also been shown to bind to collagen I and collagen IV[414, 415]. M protein has also been implicated in the formation of bacterial aggregates[416, 417] as well as in the formation of biofilm[418]. There have also been reports of M protein binding to glucosaminoglycans[419]. M protein is also involved in the invasion of host cells[420-423]. Internalisation has been seen in patients with recurrent tonsillitis[424, 425]. Soluble M1 protein has also been shown to increase the expression of the chemokine MIG/CXCL9 in epithelial cells[426].

Moreover M protein can also interact with the coagulation system altering clot morphology[427]. It can also assemble components of the intrinsic pathway of coagulation to the bacterial surface where it is activated and bradykinin produced[428, 429].

Activation of the contact system has been shown in murine models of *S. pyogenes* infection[430, 431] and the presence of bradykinin has been demonstrated in biopsies from patients suffering from erysipelas[432].

M protein may also be released from the bacterial surface both due to the action of neutrophil proteases[433] as well as the bacterial protease. streptococcal cysteine protease B (SpeB)[434]

Furthermore superantigenic properties have been proposed for M proteins[435]. The superantigenic properties of M proteins have been questioned in subsequent studies, which failed to replicate these properties[436, 437]. Recently soluble M1 protein has shown superantigenic properties[438]. It has been speculated that the differences in effects might be due to the different experimental conditions used to purify M protein that affect the three dimensional structure[439].

M protein has also been reported to interact with monocytes. By acting in a TLR2-dependent manner M1 triggers the release of proinflammatory cytokines, which is augmented in the presence of HBP[131]. M1 protein has also been shown to upregulate tissue factor expression on monocytes[259, 440, 441].

The interactions of M protein with platelets have also been studied and M protein has been shown to aggregate and activate platelets and this is dependent on the presence of specific IgG towards the M protein[252, 259, 442, 443].

M1 protein in complex with fibrinogen can also trigger the release of HBP, a potent inducer of vascular leakage, from neutrophils[433] and induce lung damage in mice[444]. It has subsequently been shown that the presence of antibodies directed to the central domain of M1 (presumably the S domain) augmented the release of HBP from human neutrophils (**Paper I**). M1 protein has also been shown to induce the formation of neutrophil extracellular traps (NETs)[445]. M1 protein has also been shown to induce the secretion of resistin from neutrophils, presumably in a fibrinogen-dependent manner, and high levels of resistin have been found in patients with STSS or NF[446].

The distribution of *emm* types has been investigated in several studies. One of the most prevalent M types is M1 (*emm1*), although there is a global diversity in the prevalence of M types[447]. In a surveillance study of severe invasive streptococcal infections conducted in eleven European countries from 2003-2004, M1 (*emm1*) was the most prevalent type, followed by *emm28* and *emm3*[448]. STSS and NF were also particularly associated with *emm1* as well as with *emm3*. M1 was also the type that affected the highest proportion of individuals without any risk factors. In a simultaneous study in Sweden between 2002-2004 the most prevalent types were *emm* 89, 81, 28, and 1. However *emm1* was the most prevalent strain in patients with STSS or NF[331]. More recently *emm1* has been isolated from an outbreak of invasive streptococcal infections in Uppsala[449]. The rise in the prevalence of severe invasive *S. pyogenes* infections in the 1980s also coincided with the rise of

a special clone of the M1T1 serotype[450]. This clone has bacteriophage-encoded virulence factors such as exotoxin A

(SpeA) and extracellular streptodornase D (Sda1) and an increased production of streptolysin O (SLO)[451].

Streptococcal collagen-like surface protein

Streptococcal collagen-like surface protein A and B (SclA and B), also known as Scl1 and Scl2, are two cell wall-anchored collagen-like proteins extending from the bacterial surface. They both contain a collagen-like structure with GXY and a variable N-terminus but are differently regulated (SclA positively by *mga* and SclB negatively by *mga*)[452-456]. SclA has been implicated in adhesion to epithelial cells and binding to fibronectin, laminin and integrins as well as to low-density lipoproteins, whereas SclB has been shown to mediate binding to fibroblasts[453, 454, 457-459]. More recently both SclA and SclB have been shown to bind TAFI (thrombin activatable fibrinolysis inhibitor) thereby recruiting it to the bacterial surface where it can be activated and of the strains tested AP41 was the strain that bound TAFI most avidly[460]. After TAFI is activated it detaches from the bacterial surface and can act on substrates elsewhere[461]. TAFI recruited to the bacterial surface has also been shown to be able to convert bradykinin to desArg^9-bradykinin. This peptide is a ligand for the bradykinin-1-receptor, which is upregulated during inflammation and when stimulated gives a sustained inflammatory response[462]. Moreover, Scl from some strains, but not from M41, have been shown to bind factor H and factor H-related proteins[463].

Superantigens

S. pyogenes produces eleven known superantigens (speA, speC, speG, speH, speI, speJ, speK, speL, speM, ssa and smeZ)[464]. In addition, M1 has also been claimed to pocess superantigenic properties (*see - M protein*)[438]. Superantigens bypass the usual restriction of MHC class II presentation to only the matching T-cell receptor (TCR). They bind, without any previous processing, directly to both MHC class II and the TCR at sites normally not used, often the Vb region on the TCR. This unspecific interaction induces the expansion of approximately 20-30% of the T-cell population as opposed to the usual 0.01%[465]. This leads to an increased cytokine production. Most of the superantigens are found in prophage sequences[465]. Certain haplotypes of MHC class II are associated with decreased cytokine production in invasive *S. pyogenes* infections and individuals with these haplotypes are less likely to develop severe systemic infections[466]. It has also been shown that these low-risk haplotypes give a cytokine response of a more anti-inflammatory type[467].

Streptococcal cysteine protease B (SpeB)

SpeB (streptococcal pyrogenic exotoxin B, streptococcal cysteine proteinase, streptopain) is a broad-spectrum secreted cysteine proteinase present in almost all strains of *S. pyogenes*[468]. SpeB is maximally expressed during the late exponential growth phase to the stationary phase[469, 470]. First discovered in the 1940s, it has been attributed many biological functions, some of which seem conflicting[471, 472]. It has been shown to degrade a plethora of host and bacterial proteins (e.g. immunoglobulins of different classes, complement, chemokines, fibrinogen, plasminogen, EndoS, streptokinase, protein F1)[472]. An interesting mechanism is how SpeB can cleave α2-macroglobulin associated to the streptococcal surface through the cell wall-attached protein GRAB (**p**rotein **G**-**r**elated α_2-macroglobulin-**b**inding protein)[473]. By cleaving α_2-macroglobulin, SpeB is trapped inside α_2-macroglobulin (like in a cage). However, SpeB retains its activity and small antimicrobial peptides can diffuse into the cage and be inactivated by SpeB[474]. SpeB has also been shown to cleave M protein from the bacterial surface[434, 471]. SpeB production has been associated with a more severe clinical picture in some studies[475] although others have failed to show this[476]. Decreased SpeB activity has also been shown to lead to increased cell attached surface protease activity and subsequent dissemination *in vivo*[388].

Other virulence factors

S. pyogenes has the ability to modulate IgG with specific enzymes and this can confer protection to the bacterium. The bacterium secretes the IgG-cleaving enzyme IdeS (Streptococcal **I**gG-**d**egrading **e**nzyme of ***S***. *pyogenes*), which is a cysteine proteinase and cleaves IgG with a unique specificity[299, 477]. IgG can also be modulated by cleaving of the glycans from the Fc-part of IgG thereby rendering IgG incapable of interacting with Fc-receptors[478]. This is accomplished by the secreted enzyme EndoS, which has a unique specificity for IgG[302].

Streptococcal inhibitor of complement (SIC) was first isolated by Åkesson et al and was shown to interfere with the membrane attack complex of complement[479]. Sic is only present in the M1 serotype and a few other serotypes[479]. Later it was appreciated that it has the ability to inactivate antimicrobial peptides[480-483] as well as interfere with the contact system and the complement system[484, 485].

Streptolysin O is a cytolysin that by oligomerisation forms large pores in host cell membranes by binding to cholesterol[486]. SLO has been implicated to participate in the induction of apoptosis of keratinocytes[487] and macrophages[488], in escaping from the lysosome of epithelial cells[489]. There have also been reports of SLO mediating the formation of platelet-neutrophils complexes[490] as well as triggering release of neutrophil granulae[491].

S. pyogenes can escape from neutrophil extracellular traps (NETs) with the help of its DNase[241]. The upregulation of its production may serve as a selective advantage for an invasive clone.

The interleukin-8-protease is a cell wall-anchored protease that can cleave and inactivate IL-8 as well as other CXC chemokines[492]. It has been demonstrated that *S. pyogenes* isolated from blood cleaved the neutrophil recruiting chemokine IL-8 more effectively than isolates from throats and the IL-8 cleaving ability could be attributed to SpyCEP[493]. This could possibly account for the paucity of neutrophils seen in severe cases of necrotising fasciitis.

The streptococcal capsule is composed of glucoronic-b-1,3-N-acetylglucosamine and is synthesised by the hasABC hyaluronan synthase operon[494]. The capsule has been shown to provide resistance to phagocytosis although the importance may differ between different *emm* types[495, 496].

The many virulence factors of *S. pyogenes* cooperate in mediating survival and proliferation of the bacterium. It is most likely that the importance of the different factors varies depending upon different conditions but also depending on the host and the defence mounted by the host.

Present investigations

As outlined above *S. pyogenes* has multiple factors for manipulating or evading the host defence systems thereby promoting its own survival. The bacterium has evolved different mechanisms for promoting adhesion/colonisation of the host and in some cases subsequent invasion and dissemination. After a decline in severe invasive streptococcal infections during the 20th century there was a resurgence in the 1980s and this still persists[331, 497]. One of the most feared conditions is streptococcal toxic shock syndrome (STSS), which has a very high mortality[334]. Patients with STSS develop shock and have a very potent inflammatory response. Although much work has been done investigating the pathophysiology of severe streptococcal infections many issues have not yet been elucidated. To further investigate the pathophysiology is the focus of this thesis.

Paper I

Streptococcal toxic shock syndrome is characterised by a potent inflammatory response and patients often develop shock during the first 24 hours and require several litres of fluid to maintain the circulation and prevent multi organ failure. This is due to a massive vascular leakage. Previous works have highlighted the role of superantigens and the host's propensity for a massive cytokine response in the pathogenesis of STSS[466, 498]. Many cases of STSS are associated with the M1 serotype and recent work from our laboratory has shown that M1 protein together with fibrinogen form complexes that stimulate neutrophils to secrete heparin-binding protein (HBP)[433]. HBP is a potent inducer of vascular permeability[143] and was recently shown to be elevated in a prospective study of patients with severe sepsis or septic shock[145]. While studying the response to M1 protein we noticed a marked inter-individual response in the secretion of HBP. Our first hypothesis was that antibodies towards the M1 protein neutralised the HBP-releasing effects but when we investigated this we found, to our surprise, that individuals with high antibody titres against M1 protein were more prone to a massive release of HBP. This was a rather unexpected finding since antibodies usually confer protection.

To investigate this unexpected finding further, blood from non-responders was supplemented with serum or plasma from responders. This addition transformed the non-responder to a responder. Since both serum and plasma had this capability we ruled out any proteins of the coagulation cascade as possible me-

diators of the effect; especially different variants of fibrinogen which could have explained the difference. IgG was then a very plausible candidate. IgG purified from responder plasma could also confer HBP-release in a non-responder whereas serum depleted from IgG failed to do so.

To further characterise the findings, serum or purified IgG from a responder was treated with IdeS, an enzyme known to cleave IgG to one $F(ab')_2$ and two half Fc-fragments. Adding IdeS-treated serum or IdeS-treated IgG together with M1 to a non-responder did not yield any HBP-release. M1 protein in addition to its affinity for fibrinogen also has affinity for the Fc-part of IgG in a non-immune manner. This interaction is rather weak, approximately 3000 times weaker than the M1-fibrinogen interaction. We studied the M1-fibrinogen-IgG-compelex with surface plasmon resonance technology. We demonstrated that IgG from responders but not from non-responders associated with the complex formed by M1 and fibrinogen. In scanning electron microscopy protein aggregates containing neutrophils could be visualised in responder blood, whereas neutrophils were absent in complexes formed in non-responder blood.
Since immune complexes have been described to activate neutrophils, we investigated if the presence of fibrinogen in the complex was necessary for its HBP-releasing ability. We confirmed that the presence of fibrinogen was vital for the HBP-releasing capability, thus underlining the necessity for the trimetric complex of M1-fibrinogen-IgG.

The M1-fibrinogen complex had previously been reported to act through β_2-integrins on the neutrophil surface[433]. The question then arose whether the IgG interacted with another receptor on the neutrophil surface. EndoS is an enzyme that specifically strips IgG of its glycan rendering it incapable of interacting with Fc-receptors. Treating either the purified responder IgG or the responder serum with EndoS abolished the ability to stimulate HBP-release. This indicates that Fcg-receptors are involved. To further investigate this we selectively blocked the different Fcg-receptors and by doing so the response could be mapped to the FcγRII-receptor.

The epitope specificity of the IgG antibodies was also investigated. Antibodies against the N-terminal part of the M protein have long been known to confer protection against a streptococcal infection of the same serotype. The survival of *S. pyogenes* of the M1 serotype in blood from different individuals was investigated in parallel with HBP-release in blood from the same individual, and demonstrated that these abilities were not linked. This indicates that the IgG responsible for HBP-release is not directed towards the hypervariable most N-terminal parts of the M protein. We then used fragments of the M1 protein to further map the binding site for the IgG and located it to or near the central S domain. Transmission electron microscopy revealed IgG attaching to the central parts of the M1 protein in responder serum but not in non-responder serum.

It had previously been shown that M1 protein injected into mice leads to a lung injury[433]. By pre-treating the mice with responder and non-responder serum respectively we could show that mice treated with responder serum had more severe lung damage than mice treated with non-responder serum.

Finally we investigated the presence of M1, fibrinogen and IgG in a biopsy from a patient suffering from STSS. Importantly, M1 was found not only associated with bacteria but also dispersed in the tissue. All three components of the trimeric complexes were also found to co-localise in some parts of the biopsy.

In summary this study demonstrated that the presence of specific IgG towards the central part of the M1 protein enhances the HBP-release triggered by M1-fibrinogen. A later study has solved the structure for the M1-fibrinogen-complex demonstrating four fibrinogen molecules attaching perpendicular to the dimerised M1 proteins[499]. Interestingly, in this structure the S domain points out of the complex and is freely available for interaction with IgG.
It is interesting to notice that in studies of patients that have developed STSS low antibody titres against the M protein are found. However, the antibodies measured are directed against the whole M protein and not towards the central domain and the sera are taken after the patients have become ill. The mechanism outlined above could already have absorbed the "malicious" antibodies into the complex of M1-fibrinogen-IgG. The use of IVIG in the treatment of STSS has in studies

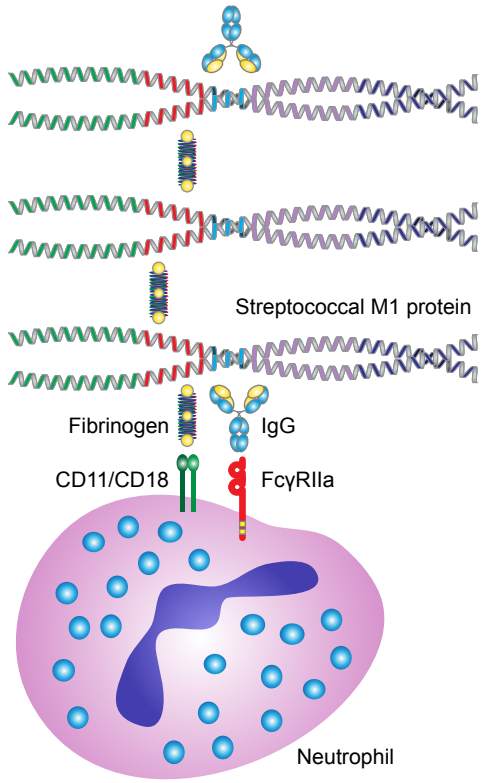


Figure 8
The M1-fibrinogen-IgG-complex stimulating the neutrophil

indicated but not confirmed a beneficial effect. This is by no means contradictory to our findings. IVIG in addition to supplementing antibodies, is known to be immunomodulatory and is used as such to treat many diseases. It is speculated that IVIG affects the half-life of IgG but also blocks activating Fcg-receptors as well as upregulating the inhibitory Fcg-receptors[500]. These effects would be protective against the inflammatory mechanism outlined in our study. The malicious effects of antibodies is a new concept regarding neutrophil function

during acute bacterial infections. However, in a study of *S. aureus* induced synovitis in chickens it was noticed that treating the chickens with sera from animals immunised with *S. aureus* lead to a more severe disease. The molecular mechanisms for this finding were not explained in the study[501]. In some viral infections, such as dengue fever, it is well known that re-infection induces a more severe disease and this involves pre-formed antibodies. This effect is called anti-body dependent enhancement (ADE)[502]. The mechanisms underlying this effect are very different from the mechanisms outlined here but nevertheless demonstrate the possible double-edged effect of IgG, also presented in our study.

Paper II

Platelets have been shown to adhere to and form complexes with neutrophils upon stimulation. Furthermore, neutrophils in such complexes have been found to be more activated[239]. In addition to our findings, that M1 protein, together with fibrinogen in the presence of IgG directed towards the central domains of M1, lead to HBP release, a previous study by *Shannon et al*[259] demonstrated the activation of platelets upon M1 stimulation in individuals with type specific anti-M1 antibodies. Considering these findings we addressed the question of potential interactions between platelets and neutrophils.

Shannon et al have previously demonstrated that upon M1 stimulation platelet-neutrophil complexes (PNCs) are formed and are dependent on the presence of IgG as well as interaction with the GPIIb/IIIa receptor on the platelets. To dissect the mechanisms involved in PNC formation further, we studied the formation of these complexes in whole blood from different individuals. Dual stimulation of platelets and neutrophils with ADP and fMLF resulted in PNCs in all individuals, while M1 stimulation only resulted in PNCs in some individuals. The formation of PNCs correlated with antibodies against the M1 protein. It is also notable that M1 protein seems to give rise to more complexes in responders than stimulation with ADP and fMLF in the same individual. Furthermore, the expression of the activation marker CD11b on the neutrophil was only increased in responders. Platelet-neutrophil complexes were also studied with immunofluorescence microscopy and this demonstrated that several platelets associated with each neutrophil. M1 protein led to a higher number of platelets per neutrophil than ADP and fMLF. These findings were also confirmed by FACS-analysis.

Most PNCs rely on binding between P-selectin on the platelet and PSGL-1 on the neutrophil. Since M1-induced PNCs have been shown to be dependent on the participation of GPIIb/IIIa we wanted to investigate the necessity of the participation of P-selectin. Blocking of P-selectin abolished the formation of PNCs. The formation of PNCs was also diminished by the addition a peptide (GPRP) mimicking the binding site of fibrinogen to β_2-integrins on the neutrophil.

Importantly, the blocking of P-selectin did not significantly affect the activation of the neutrophils, as measured by the expression of CD11b, indicating that the physical contact with the platelet did not mediate significant neutrophil activation and neutrophils could be activated on their own.

Various recombinant proteins spanning different parts of the M1 protein were constructed. With the help of these it was shown that the presence of the fibrinogen-binding B domains as well as the central S domain were crucial for inducing PNCs. These findings are in agreement with the mechanism proposed in **Paper I**.

Platelet-neutrophil complexes have been proposed to play a role in the pathogenesis of many different diseases. A few small studies have shown raised numbers of PNCs in septic patients but when progressing to multiorgan failure the ratio of PNCs diminished[242, 243]. The proposed explanation has been that these highly activated complexes are trapped in the microvasculature. Previously it has been shown that streptolysin O may also induce platelet-neutrophil complexes and intramuscular injection of exotoxins from *S. pyogenes* resulted in a dose-dependent decrease in perfusion of the local tissue[490]. The formation of PNCs on M1 stimulation may in part explain the vascular dysfunction and organ damage associated with STSS. The findings that these complexes formed only in blood of individuals that possess antibodies towards certain domains of the M1 protein are interesting and may be part of the explanation as

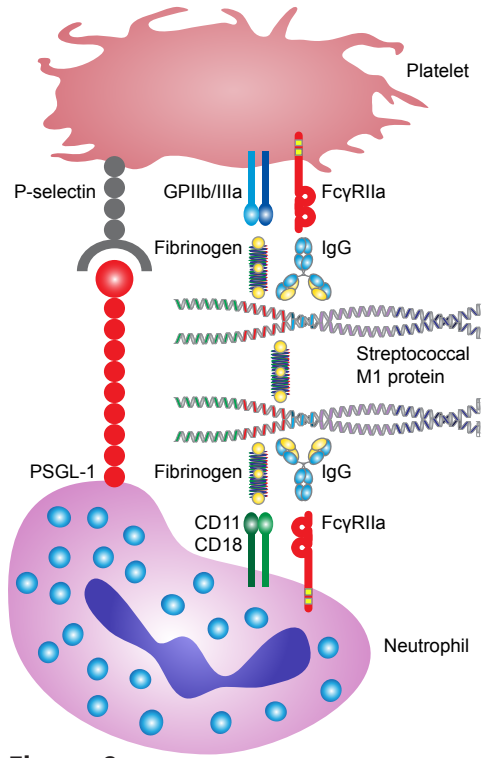


Figure 9
M1-induced platelet-neutrophil complex

to why only certain individuals are affected by serious complications of streptococcal infection. If this mechanism is verified in clinical studies, new treatment concepts and prognostic markers could be used. Further studies are warranted to elucidate the potential mechanisms and pathophysiological consequences of M1-induced PNC-formation.

Paper III

The role of platelets as an important cell for the immune system is being increasingly recognised. Thrombocytopenia

often occurs during severe infections and has been correlated with the severity of sepsis[243, 283, 503]. Many different models have investigated platelet-bacterial interactions *in vitro* and *ex vivo*[254]. There have also been a limited number of experiments *in vivo*. Animals have been platelet-depleted and the response has been examined. Many of these models have either used the injection of LPS or the use of a model where the colon is punctured and colonic contents and bacteria leak out into the abdomen. Gram-positive bacteria are major causes of sepsis and studies using LPS cannot be extrapolated to Gram-positive bacteria. Puncture of the colon results in an indefinable amount of bacteria leaking out and few studies have investigated the spreading of bacteria in thrombocytopenic animals. To study the effects of platelets in a Gram-positive infection we used *S. pyogenes* as a model pathogen.

Balb/c mice received an antibody directed against GPIba thereby decreasing their platelet-count to approximately 15% of normal. After 24 hours, platelet-depleted and control mice were infected with *S. pyogenes* by intraperitoneal injection. The mice were sacrificed 16-18 hours post infection and blood samples and organs were harvested for further analysis. Control mice exhibited a decrease in platelets due to infection, which mimicked the thrombocytopenia seen in severely ill patients. Control mice but not platelet-depleted mice demonstrated an increase in circulating platelet-neutrophil complexes as well as increased expression of the neutrophil marker CD11b. This difference was not due to decreased neutrophil counts in platelet-depleted mice, since the neutrophils were not significantly different nor was it due to an inability to form PNCs, since platelet-depleted mice *in vitro* formed PNCs upon thrombin stimulation. This suggests that either PNC formation contributes to neutrophil activation, or that another neutrophil activating stimulus is missing in the platelet-depleted animals.

Platelets have previously been shown to contribute to neutrophil extravasation to the lungs and lung damage in animal models of sepsis with bacterial products or mixed bacterial infections[245, 285, 504] but there have also been studies describing the platelet-independent recruitment of neutrophils[505]. In this study we could not detect any difference in neutrophil recruitment to the lung as measured by myeloperoxidase accumulation in the lungs. Platelet-depleted mice had significantly less bacteria both in the spleen, blood, and lungs. This was also mirrored by a decreased inflammatory reaction as measured by plasma interleukin-6. The platelet-depleted mice also exhibited significantly less weight loss during the acute infection. A survival study was also performed but due to the very high mortality among both control mice and platelet-depleted mice we could not detect any difference in mortality.

Taken together this study demonstrates that platelets contribute to the dissemination of bacteria from the blood to target organs. This is also reflected by the fact that platelet-depleted mice lost less weight, had lower IL-6 levels, had fewer PNCs and less activated neutrophils.

The concept of platelets contributing to spreading is well recognised in malignancies were platelets are thought to help in the metastasis of tumour cells[277].

Very recently *Vershoor et al* published a paper stating that *Listeria monocytogenes* uses platelets for homing to the spleen where it infects dendritic cells[506]. The findings presented by *Verschoor* et al are in accordance with the findings presented here although there are several differences. *Listeria monocytogenes* is an intracellular pathogen and as such can behave differently. *S. pyogenes* is mainly an extracellular pathogen although evidence exists that under certain conditions it can survive intracellularly. The study by *Vershoor et al* did not address spreading of the bacteria to other organs. In this study we could see enhanced spreading of bacteria to all organs tested in platelet-normal control mice, as well as an increased inflammatory response in these mice. Taken together this implicates the participation of platelets in bacterial dissemination and the acute inflammatory response during *S. pyogenes* infection.

Paper IV

This paper reports an abscess complicated by venous thrombosis caused by *Streptococcus pyogenes*. Although *S. pyogenes* is a common pathogen it is very seldom associated with abscess formation outside the oro-pharyngeal tract and few cases have been described in the literature. The case describes a previously healthy woman who developed an axillary abscess, which was complicated by a venous thrombosis. Since empirical antibiotic treatment had been instituted before puncture of the abscess all cultures were negative. The aetiological cause could yet be determined by means of PCR. Moreover, we were able to *emm* type the bacterium from DNA extracted from abscess material. This demonstrates that it is likely that the presence of, for example, resistance genes also can be determined without culturable bacteria, a situation not seldom encountered in the clinic.

The patient developed a venous thrombosis. The vicinity of the abscess to the vessels is probably the cause for this complication. But it is noteworthy that the strain of *S. pyogenes* isolated is a strong binder of TAFI (thrombin-activatable fibrinolysis inhibitor)[460]. TAFI inhibits fibrinolysis and has shown to be activated on the surface of the bacterium and then diffuse away mediating its functions[462]. We had the unique opportunity of having access to serum collected from the patient on two different occasions before, two times during, and finally after the infection. By measuring the antibody response we could determine that the bacteria had expressed SclB, which binds TAFI, thereby possibly establishing a procoagulant state. We can only speculate if this might have contributed to the unusual complication with venous thrombosis.

Conclusions

- IgG directed towards the central domains of the M1 protein forms complexes with M1 protein and fibrinogen thereby enhancing the HBP-releasing ability of the complex.

- Platelet-neutrophil complexes are formed in blood when stimulated with M1 protein only in the presence of IgG directed towards the M1 protein.

- M1 protein-induced platelet-neutrophil complexes do not only rely on the interaction between GPIIb/IIIa, fibrinogen and β_2-integrin but P-selectin is also essential.

- Platelets contribute to the dissemination of S. *pyogenes* in a murine model of invasive S. *pyogenes* infection.

- *S. pyogenes* rarely give rise to abscesses outside the oro-pharyngeal tract but can do so as presented here. Material for characterisation of the bacterium can be obtained from abscess material.

Populärvetenskaplig sammanfattning

Streptococcus pyogenes (en grupp A streptokock) är en vanlig bakterie som oftast orsakar mindre allvarliga infektioner såsom halsfluss, svinkoppor och rosfeber. Grupp A streptokocker är också orsaken till scharlakansfeber. Bakterierna kan dock ibland orsaka betydligt allvarligare infektioner. Under tidigare århundraden var barnsängsfeber, som orsakas av grupp A streptokocker, ett stort gissel tillsammans med allvarliga sårinfektioner. Även om mödradödligheten i västvärlden idag är mycket låg är fortfarande streptokockinfektion den vanligaste infektionsrelaterade dödsorsaken hos nyförlösta kvinnor i England.
Under 1900-talet verkade den tidigare så fruktade streptokocken bli mindre aggressiv och med hjälp av penicillin kunde man bota allt fler. Men så plötsligt, i mitten av 1980-talet, dök en ny sjukdomstyp upp som man inte sett tidigare. Helt friska personer kunde plötsligt drabbas av väldigt aggressiva invasiva streptokockinfektioner. I en hel del av fallen angrep bakterien vävnaden runt musklerna och orsakad vävnadssönderfall. På grund av detta fick den i pressen namnet ”köttätande mördarbakterie”. Medicinskt kallas detta nekrotiserande fasciit. Under dessa allvarliga infektioner utvecklar individerna ofta tecken på chock, det vill säga oförmåga att upprätthålla ett normalt blodtryck. Detta gav man namnet streptococcal toxic shock syndrome (STSS). Trots all modern antibiotika och intensivvård är dödligheten fortfarande runt 45 % i STSS. Förloppet kan vara väldigt snabbt och därför hinner inte alltid den antibiotika som ges verka, trots att bakterien är känslig för den. Ofta måste man operera och ibland även amputera den infekterade kroppsdelen hos den som drabbas av nekrotiserande fasciit för att få stopp på infektionen. Trots denna aggressiva behandling kan man inte alltid rädda personen. I studier har man sett att vissa som drabbas verkar ha låga nivåer av antikroppar mot streptokocken och de gifter den utsöndrar. Man har därför testat att ge antikroppskoncentrat som tillägg till behandlingen, dock utan att säkert ha kunnat bevisa de positiva effekter man tyckt sig se.
Streptokockinfektioner kan även leda till allvarliga följdsjukdomar. En obehandlad halsfluss kan i några procent av fallen leda till efterföljande reumatisk feber som i sin tur innebär en hög risk för utveckling av reumatisk hjärtsjukdom. Även om detta är ytterst ovanligt i Sverige är det tyvärr ett vanligt scenario

ute i världen och framför allt i fattiga länder. En undersökning som gjordes 2005 skattade att det finns 15,6 miljoner människor som lever med reumatisk hjärtsjukdom och 223 000 som dör årligen. Denna siffra är nog snarast underskattad. Årligen beräknas mer än en halv miljon människor drabbas av allvarliga invasiva streptokockinfektioner och tillsammans med de allvarliga följdsjukdomar bakterien kan ge placerar den sig på en nionde plats på listan över de infektioner som dödar flest människor i världen.

Trots att mycket ny kunskap har tillkommit så vet vi fortfarande inte helt säkert varför dessa allvarliga infektioner uppkommer. Många sjukdomsframkallande egenskaper hos streptokocken har identifierats. Bland annat så har den så kallade superantigener. Superantigener är proteiner som lurar immunförsvaret att överaktiveras istället för att ge ett lagom immunsvar. Denna förmåga att koppla sig förbi reglersystemen i immunförsvaret och ge en överaktivering har visats bero på vissa gener och därför skulle det kunna vara så att vissa personer löper större risk att drabbas av en allvarlig infektion om de infekteras med streptokocken. Streptokocker i sig är också olika och bär på olika sjukdomsalstrande proteiner och gener vilket i sin tur också har betydelse för hur sjuk en infekterad individ blir.

I tidigare undersökningar från vårt laboratorium har man visat att ett av streptokockens ytproteiner (M protein) bildar komplex med ett protein i blodet (fibrinogen). Detta komplex leder till en massiv aktivering av en typ av vita blodkroppar, neutrofiler. Neutrofiler är kroppens försvarsceller och har som huvuduppgift att vara först på plats och äta upp skadliga bakterier. Förutom denna uppgift kan neutrofilen signalera med olika substanser för att tillkalla mer hjälp från immunförsvaret; till exempel heparin-bindande protein (HBP) som gör att blodkärlen börjar läcka och underlättar för andra vita blodkroppar att ta sig ut. Samtidigt försvinner vätska ur blodbanan och detta leder till sänkt blodtryck och chock vilket är utmärkande symptom vid STSS.

I mitt första avhandlingsarbete fann jag att frisättningen av HBP inte förekommer hos alla människor om man stimulerar deras neutrofiler med M protein. Detta är högst intressant eftersom det skulle kunna vara en förklaring till varför endast vissa drabbas av STSS. Vi kunde visa att skillnaden beror på att vissa individer har antikroppar mot specifika delar av M proteinet. Normalt används antikroppar för att neutralisera skadliga substanser samt att märka bakterier som ska bekämpas. I detta fall ledde antikropparna, tillsammans med den specifika föreningen av M protein och fibrinogen, till att neutrofilen reagerade mycket kraftigt. Denna kunskap kan få betydelse för vår förmåga att förutsäga vem som riskerar att drabbas av en allvarlig infektion. Dessutom skulle de skadliga antikropparna från den insjuknade personen, men även från de antikroppskoncentrat som ges i behandlande syfte, kunna tas bort.

I mitt andra arbete fann jag att neutrofilerna slår sig samman (aggregerar) med blodplättar och bildar komplex då de stimuleras med M protein. Blodplättarna deltar normalt i kroppens koagulationssystem. Ett sådant komplex gör att båda cellerna blir mer aktiverade än normalt. Aggregeringen mellan neutrofiler och blodplättar beror i detta fall även den av komplexet mellan M protein och fibrinogen i närvaro av antikroppar. Den individ som saknar antikroppar mot M protein bildar således inte några komplex när man undersöker deras blod i provrör. Vid nekrotiserande fasciit är blodflödet till vävnaderna påverkat och man har tidigare sett att aggregat mellan neutrofiler och blodplättar skulle kunna vara en del av förklaringen eftersom de täpper till kärlen. Det är därför intressant att sådana aggregat bildas hos vissa personer som svar på streptokockproteiner.

I mitt tredje arbete har jag undersökt hur streptokocken kan spridas i kroppen och fann att hos möss medverkar blodplättar i denna spridning. För bakterier är detta ny kunskap medan man för cancerceller har starka indikationer på att blodplättar kan hjälpa cancerceller att metastasera. Om antalet blodplättar hos mössen minskades experimentellt så spreds bakterien mindre och inflammationen som bakterien orsakade blev också mindre omfattande.

I mitt sista arbete beskriver jag ett mycket ovanligt fall av streptokockinfektion. Streptokocker bildar vanligen inte bölder annat än möjligen i anslutning till svalget. Jag beskriver en person som drabbades av en streptokockböld i armhålan och som dessutom samtidigt fick en blodpropp i samma arm. Några bakterier kunde inte odlas fram men med undersökning av DNA-spår kunde vi bestämma bakterieart och undertyp. Att på detta sätt kunna identifiera gener hos döda bakterier gör att man även skulle kunna ta reda på vilken antibiotikaresistens de har. Den ovanliga typ av streptokock som beskrivs i fallet har en förmåga att via speciella proteiner binda till sig koagulationsfaktorer och skapa en blodproppsfrämjande miljö och vi kunde visa att just denna bakterie hade uttryckt dessa proteiner.

Sammanfattningsvis visar jag med mitt avhandlingsarbete några av de många sätt som streptokocken lurar kroppens försvarssystem och därigenom främjar sin egen överlevnad.

Tack

Först och främst vill jag rikta ett stort tack till min huvudhandledare **Lars Björck**. Lasse, du skapar med dina kunskaper och din entusiasm för forskning en fantastisk forskningsmiljö. Du är alltid intresserad av forskningen och blir minst lika upphetsad som jag över nya spännande resultat. Det är också skönt att veta att din dörr alltid står öppen för nya diskussioner. Den atmosfär som du skapar, där man får arbeta självständigt men där du alltid finns tillhands för nya diskussioner och råd har passat mig perfekt. Din förmåga att från data som ser misslyckade ut se det nya och spännande är avundsvärd. Jag kan inte tänka mig ett bättre ställe att få starta min vetenskapliga bana än hos dig. Tack!

Oonagh, min bihandledare och laborationspartner under många kvällar och tidiga morgnar. Du har bidragit och lärt mig väldigt mycket och, när saker inte gått som vi vill, funnits där för diskussion och nya planer. Vi har båda fått arbeta mycket det senaste halvåret och det känns som jag eller mina försök stört dig nästan varje helg. Du har varit en fantastisk handledare att bolla texter med och när jag tyckt att jag stört dig bara sagt att du tycker det är roligt. Jag har också dig att tacka för många av de tekniker med FACS och djurförsök som du lärt mig. Jag vill också passa på och tacka Daniel och Molly för att jag fått låna Oonagh även på helger och kvällar.

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To follow knowledge like a sinking star,
Beyond the utmost bound of human thought.

To strive, to seek, to find, and not to yield.

Alfred Lord Tennyson