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Interactions between *Streptococcus pyogenes* and the Human Immune System

Maria Weineisen

Institutionen för Laboratoriemedicin
Avdelningen för MIG
2006

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Abstract <p>Streptococcus pyogenes is a common human pathogen causing mild infections such as tonsillitis, but is also the cause of life-threatening conditions, such as necrotizing fasciitis. To be able to infect the human host, <i>S. pyogenes</i> express different virulence factors. The surface-expressed M protein is a major virulence factor of <i>S. pyogenes</i>, mediating resistance to killing by human neutrophils.</p> <p>Results presented in this thesis show that in the presence of plasma from non-immune individuals, <i>S. pyogenes</i> avoids killing by human neutrophils by M protein-mediated inhibition of adhesion to the neutrophils. By comparing a wild type strain and a strain deficient in M protein-expression, the interactions between <i>S. pyogenes</i> and human neutrophils were studied. The interactions between <i>S. pyogenes</i> and human neutrophils in the presence of non-immune plasma are mediated mainly by complement components deposited on the bacterial surface and CD11b/CD18 expressed on the neutrophil surface. This interaction leads to the effective ingestion and killing of the bacteria. The activation of CD11b/CD18 leads to the activation of tyrosine kinases and the subsequent activation of the small Rho GTPase Cdc42.</p> <p>In blood from immune humans, <i>S. pyogenes</i> are opsonized and killed. In this thesis, we show that killing is mediated by Abs directed against the N-terminal part of the M protein and that these Abs activate the complement system. In the presence of serum from immune individuals, the killing of <i>S. pyogenes</i> is mediated through activation of the complement system by M protein-specific Abs and activation of CD11b/CD18 and Cdc42 followed by phagocytosis.</p> <p>Almost all strains of <i>S. pyogenes</i> bind fibrinogen (Fg) and the Fg-binding is associated with members of the M protein family. We mapped the binding of Fg to the B repeats of the M1 and M5 proteins and demonstrated that the Fg-binding is important for these bacteria to resist killing when incubated in human blood.</p> <p>The conditions under which the interactions between <i>S. pyogenes</i> and human neutrophils were investigated in the studies described above resemble the situation in bacteremia (a low multiplicity of infection, MOI, i.e. bacteria: neutrophil ratio). However, at the initial stages of infection, occurring at skin or mucosal surfaces, the bacteria to neutrophil ratio is much higher. Using two different model systems to study the interactions of <i>S. pyogenes</i> and phagocytes at a higher MOI, we found that this interaction is mediated by fibronectin deposited on the bacterial surface and the integrins $\alpha 5\beta 1$ and $\alpha v\beta 3$ expressed on the phagocytes.</p>			
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Maria Weineisen

Institute of Laboratory Medicine
Section of MIG
2006



LUND UNIVERSITY
Faculty of Medicine

A doctoral thesis at a university in Sweden is produced either as a monograph or as a collection of papers. In the latter case, the introductory part constitutes the formal thesis, which summarizes the accompanying papers. These have already been published or are manuscripts at various stages (*in press, submitted, or in manuscript*).

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Till minne av min mormor

Abstract

Streptococcus pyogenes is a common human pathogen causing mild infections such as tonsillitis, but is also the cause of life-threatening conditions, such as necrotizing fasciitis. To be able to infect the human host, *S. pyogenes* express different virulence factors. The surface-expressed M protein is a major virulence factor of *S. pyogenes*, mediating resistance to killing by human neutrophils.

Results presented in this thesis show that in the presence of plasma from non-immune individuals, *S. pyogenes* avoids killing by human neutrophils by M protein-mediated inhibition of adhesion to the neutrophils. By comparing a wild type strain and a strain deficient in M protein-expression, the interactions between *S. pyogenes* and human neutrophils were studied. The interactions between *S. pyogenes* and human neutrophils in the presence of non-immune plasma are mediated mainly by complement components deposited on the bacterial surface and CD11b/CD18 expressed on the neutrophil surface. This interaction leads to the effective ingestion and killing of the bacteria. The activation of CD11b/CD18 leads to the activation of tyrosine kinases and the subsequent activation of the small Rho GTPase Cdc42.

In blood from immune humans, *S. pyogenes* are opsonized and killed. In this thesis, we show that killing is mediated by Abs directed against the N-terminal part of the M protein and that these Abs activate the complement system. In the presence of serum from immune individuals, the killing of *S. pyogenes* is mediated through activation of the complement system by M protein-specific Abs and activation of CD11b/CD18 and Cdc42 followed by phagocytosis.

Almost all strains of *S. pyogenes* bind fibrinogen (Fg) and the Fg-binding is associated with members of the M protein family. We mapped the binding of Fg to the B repeats of the M1 and M5 proteins and demonstrated that the Fg-binding is important for these bacteria to resist killing when incubated in human blood.

The conditions under which the interactions between *S. pyogenes* and human neutrophils were investigated in the studies described above resemble the situation in bacteremia (a low multiplicity of infection, MOI, i.e. bacteria: neutrophil ratio). However, at the initial stages of infection, occurring at skin or mucosal surfaces, the bacteria to neutrophil ratio is much higher. Using two different model systems to study the interactions of *S. pyogenes* and phagocytes at a higher MOI, we found that this interaction is mediated by fibronectin deposited on the bacterial surface and the integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$ expressed on the phagocytes.

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Abbreviations

Ab	antibody
C4BP	C4b-binding protein
CD	cluster of differentiation
CR	complement receptor
DAG	diacylglycerol
ECM	extracellular matrix
FcR	Fc receptor
Fg	fibrinogen
FH	factor H
FHL-1	factor H-like protein 1
FI	factor I
fMLP	N-formyl-methionyl-leucyl-phenylalanine
Fn	fibronectin
GAP	GTPase activating protein
GDI	guanine nucleotide dissociation inhibitor
GEF	guanine nucleotide exchange factor
GPCR	G protein-coupled receptor
GPI	glycosyl phosphatidyl inositol
HBP	heparin-binding protein
ICAM	intercellular adhesion molecule
Ig	immunoglobulin
IL-8	interleukin-8
IP ₃	inositol-(1,4,5)-triphosphate
LPS	lipopolysaccharide
LTA	lipoteichoic acid
LTB ₄	leukotriene B ₄
MAC	membrane attack complex
MCP	membrane cofactor protein
MPO	myeloperoxidase
NADPH	nicotinamide adenine dinucleotide phosphate
OF	opacity factor
PAK	p21-activated kinase
PECAM-1	platelet endothelial adhesion molecule-1
PI3-K	phosphoinositide 3-kinase
PIP ₂	phosphatidylinositol-(4,5)-bisphosphate
PIP ₃	phosphatidylinositol-(3,4,5)-trisphosphate
PKC	protein kinase C
PLC	phospholipase C
PLD	phospholipase D
WHO	World Health Organization

Original Papers

This thesis is based on the following papers, which will be referred to in the text by their roman numerals.

- I **Weineisen, M., Sjöbring, U., Fällman, M. & Andersson T.** Streptococcal M5 protein prevents neutrophil phagocytosis by interfering with CD11b/CD18 receptor-mediated association and signaling. *J Immunol* (2004) **172**: 3798-807
- II **Nilsson, M., Wineisen, M., Andersson, T., Truedsson, L. & Sjöbring, U.** Critical role for complement receptor 3 (CD11b/CD18), but not for Fc receptors, in killing of *Streptococcus pyogenes* by neutrophils in human immune serum. *Eur J Immunol* (2005) **35** 1472-81
- III **Ringdahl, U., Svensson, H. G., Kotarsky, H., Gustafsson, M., Wineisen, M., & Sjöbring, U.** A role for the fibrinogen-binding regions of streptococcal M proteins in phagocytosis resistance. *Molec Microbiol* (2000) **37**: 1318-26
- IV **Frentzell, M. #, Wineisen, M. #, Ståhl, A., Sakai, T., Johansson, S., Herwald, H., Mörgelin, M., Andersson, T., Ringdahl, U., Berlikowski, M., Nilsson, M., Kotarsky, H., Truedsson, L., Fässler, R., Sjöbring, U.** Adhesion of *Streptococcus pyogenes* to phagocytic cells is achieved by two distinct mechanisms that are both blocked by M protein expression. *Submitted*

MF and MW contributed equally to this study

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Introduction

Every day, we encounter potentially harmful microorganisms. To prevent these microorganisms from causing infection, the body mounts a broad arsenal of defence mechanisms including physical barriers such as the skin and mucosa, complement, antibodies, and phagocytic cells.

Streptococcus pyogenes is a common human pathogen causing uncomplicated infections such as tonsillitis and impetigo. This bacterium can, however, also give rise to life-threatening infections such as necrotizing fasciitis and toxic shock. In addition, *S. pyogenes* can cause post-streptococcal complications, such as acute rheumatic fever and acute post-streptococcal glomerulonephritis. To be able to infect the host, the bacterium utilizes an array of virulence factors mediating adhesion, invasion, and avoidance of the hosts defence mechanisms.

Neutrophils are part of the body's first line of defence against invading microorganisms. These cells circulate with the blood and migrate into the tissue at the site of infection, where they bind, phagocytose, and kill invading microorganisms. To be able to execute their mission, the neutrophils express surface receptors mediating adhesion to the vessel wall, extravasation, migration to the site of infection, binding of foreign particles, and finally phagocytosis. The integrins comprise one group of receptors that are involved in these processes. These receptors bind different extracellular matrix proteins and complement components and are involved in phagocytosis.

In this thesis, the interactions between *S. pyogenes* and human neutrophils are investigated with respect to binding, signalling events mediated by the β_2 integrin CD11b/CD18, and phagocytosis.

Streptococcus pyogenes

1

Streptococci are spherical Gram-positive bacteria growing in chains. They were first described 1874 by Billroth who gave them their name from two greek words; *streptos* (chain) and *kokhos* (berry). In the 1930ies Rebecca Lancefield classified streptococci based on group-specific polysaccharides¹. So far 13 different serological groups have been identified, of which group A, B, C, G and *Streptococcus pneumoniae* are the most important. Group A streptococci (GAS or *Streptococcus pyogenes*) is an exclusively human pathogen, causing a wide range of diseases. Group B streptococci (*S. agalactiae*) cause severe disease in the newborn. Groups C and G streptococci are common animal pathogens, but are increasing in importance as human pathogens.

S. pyogenes can cause infections ranging from mild conditions to life-threatening diseases. WHO estimates that over 500 000 deaths are caused each year by severe GAS infections.

1.1 Disease caused by *Streptococcus pyogenes*

The most common disease caused by *S. pyogenes* is tonsillitis, with over 600 million cases world wide each year² and although a relatively mild disease, the total costs for society are high. Streptococcal throat infections are most common in school aged children, and children are often asymptomatic carriers of *S. pyogenes*, thereby being a reservoir for the bacterium and being able to spread it to other humans. Throat infections caused by *S. pyogenes* are sometimes accompanied by scarlet fever, a reaction of the skin caused by a bacteriophage-encoded toxin (SpeA) secreted by some strains of *S. pyogenes*. *S. pyogenes* can also cause skin infections such as impetigo, erysipelas, and cellulitis. In addition, *S. pyogenes* can cause severe life-threatening invasive conditions such as necrotizing fasciitis, myositis, and toxic shock. These conditions have a rapid progression, often causing death within a few days. There are more than 600 000 new cases of invasive *S. pyogenes* disease every year, leading to more than 160 000 deaths, an overall death rate of approximately 30%².

Untreated pharyngitis caused by *S. pyogenes* is sometimes followed by acute rheumatic fever (ARF). Organs affected in ARF are the joints, brain, heart (carditis), and the skin. The carditis can be progressive and lead to severe injuries of the heart valves, hence ARF is an important cause of cardiovascular disease in developing countries³. Rheumatic heart disease affects

more than 15 million people throughout the world, with 280 000 new cases and 230 000 deaths annually². Another complication after infections caused by *S. pyogenes*, although not as common as ARF, is acute post-streptococcal glomerulonephritis (APSGN). While ARF is exclusively associated with throat infections, APSGN can follow both skin and throat infections.

1.2 Virulence factors

1.2.1 The M protein

The M protein (Figure 1.1) is a surface-associated protein protruding from the bacterial surface. This protein confers *S. pyogenes* with the property to resist killing in human blood. The M proteins occur in pairs, twisted around each other in a coiled coil structure⁴ – a structure rarely seen in bacteria, but common in human proteins. Antibodies (Abs) directed against the conserved parts of the M protein (in the coiled coil) have been shown to cross-react with human myosin and tropomyosin and are thought to be of importance in the pathogenesis of ARF⁵. Except for the most N-terminal part, the M protein has an α -helical structure. Each M protein has a short intracellular tail, and a conserved sequence LPXTG (leucine, proline, arbitrary aa, threonine, and glycine) which is important for the attachment of the protein to the bacterial cell wall⁶. The region N-terminal of the membrane anchor is believed to stabilize the protein in the cell wall by weaving between the cross-links in the peptidoglycan. The different regions of the M protein consist of several repeating blocks and this repetitive structure can be a location of recombination events, giving antigenic variation of the M protein. The M protein is highly conserved close to the cell wall, and variability increases towards the N-terminal. The N-terminal region of the M protein is hypervariable and only Abs directed against this part of the protein confer protective immunity. It is differences in the N-terminal that forms the basis for the typing of *S. pyogenes* into different serotypes. There are more than 100 different types of M proteins and Abs against one serotype only protect from reinfection with that specific serotype, and not against other serotypes.

The M protein belongs to the family of M-like proteins. This family includes the Emm, Enn, and Mrp proteins expressed by *S. pyogenes*, and M proteins from the group C and G streptococci. Some strains of *S. pyogenes* express one M-like protein, whereas other strains express two or even three M-like proteins. The genes encoding the M-like proteins are located adjacently on the chromosome and they belong to the the *mga* regulon (multiple gene regulator of group A streptococci). Other genes belonging to this regulon code for the C5a peptidase⁷, the opacity factor (OF)⁸, a collagen-like protein (SclA)⁹, and Protein SIC¹⁰. A multiple gene regulator called Mga¹¹ regulates the expression of these proteins in response to environmental factors, such as temperature and the CO₂ level¹². Streptococcal strains producing OF, express three different M-like proteins, whereas OF-negative strains express one to three different M-like proteins¹³.

Proteins of the M protein family can be divided into two different classes based on the type of repeat region they express in their C-terminal part; class A proteins express A repeats and

class C proteins express C repeats. Many strains express proteins from both class A and C, whereas some strains only express class C proteins¹⁴. Mrp proteins belong to class A, whereas the Emm proteins express C repeats. Emm proteins can further be divided into subclasses according to their cross-reactivity with antibodies (Abs) directed against the C repeats of the M6 protein. Streptococci expressing Emm proteins belonging to class I share antigenic epitopes with the M6 protein and do not express OF, whereas strains bearing class II Emm proteins produce OF, but do not have epitopes cross-reacting with the M6 protein¹⁵.

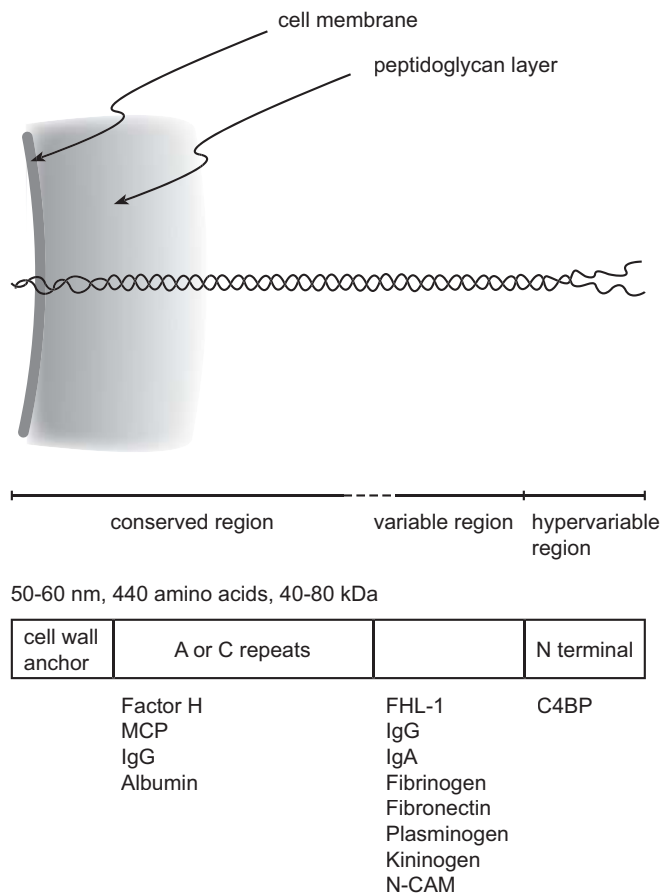


Figure 1.1 A schematic drawing of the M protein and its different regions. The approximate binding sites for different ligands are shown.

The M protein is thought to be important in different stages of streptococcal infection. It has been proposed to mediate adhesion to keratinocytes through the binding to CD46 (MCP)¹⁶ and to epithelial cells by interactions with fibronectin¹⁷ and mucins¹⁸. In addition to adhesion, the interaction between M protein and fibronectin has been shown to mediate

internalization of the bacteria¹⁷. The binding of kininogens has been predicted to contribute to increased vascular permeability through the release of bradykinin^{19, 20}. In addition to the proteins mentioned above, the M protein also binds fibrinogen²¹ (Paper IV), proteins regulating complement activation²²⁻²⁴, immunoglobulins (Igs)²⁵⁻²⁷, N-CAM²⁸, plasminogen²⁹, and albumin²⁷. The binding of plasma proteins may act as sterical hindrance for Abs and complement deposition.

Fibrinogen (Fg) is a 340 kDa glycoprotein present in plasma at 3 mg/ml. It consists of six polypeptide chains linked together by disulphide bridges. Upon damage of host tissue, due to injury or infection, the coagulation cascade is activated. The coagulation system is composed of several plasma proteins present as inactive forms. The activation of the coagulation cascade results in the cleavage of Fg to fibrin, and to the formation of a fibrin network that covers the damaged tissue. In addition, the interaction between fibrin(ogen) and platelets, will lead to the accumulation of these cells and stabilization of the fibrin clot. All virulent strains of *S. pyogenes* bind Fg and the binding of Fg to M proteins has been reported to reduce the deposition of complement on the bacterial surface, leading to inefficient killing of the bacteria^{30, 31}.

The binding of proteins regulating the complement system (see Chapter 2) is an important mechanism to inhibit complement deposition (opsonization) on the bacterial surface, thereby inhibiting the subsequent binding to phagocytic cells, ingestion, and killing. In particular OF⁺ strains bind C4b-binding protein (C4BP), a negative regulator of the classical pathway, and this interaction has been demonstrated to reduce complement deposition and subsequent killing of the bacteria^{32, 33}. In contrast, the binding of inhibitors of the alternative pathway, Factor H (FH) and FH-like protein 1 (FHL-1), does not influence the deposition of C3b on the bacterium or the ability of *S. pyogenes* to survive in human blood³⁴.

1.2.2 Fibronectin-Binding Proteins

Fibronectin (Fn) consists of two similar approximately 250 kDa subunits covalently linked to each other by two disulfide bonds. Fn is an ubiquitous extracellular matrix (ECM) protein and is expressed by many different cells. It is important for adhesion, cell migration, differentiation, growth, and fetal development³⁵. There are two forms of Fn; soluble Fn found in plasma (300 µg/ml) expressed by hepatocytes, and insoluble Fn found in the ECM as multimeric fibrils and secreted as a soluble dimer by different cells. The Fn molecule has binding sites for integrins, heparin, fibrin, collagen, and four binding sites for Fn itself³⁵. The classic Fn receptor is considered to be $\alpha_3\beta_1$, but Fn binds to several other integrins, including $\alpha_4\beta_1$ and $\alpha_4\beta_7$. The integrins bind to an RGD (arginine, glycine, and aspartic acid) motif in Fn, but an RGD-independent binding to $\alpha_4\beta_1$ has also been found³⁶. In solution, Fn has a compact structure, where the binding sites for Fn are hidden inside the molecule. Upon binding to integrins on cell surfaces, the Fn molecule undergoes a conformational change and is expanded, exposing the Fn binding sites (active Fn). The Fn molecule can now bind to other cell-associated Fn molecules, creating Fn fibrils. In addition, other ECM proteins are also attached to the Fn fibrils³⁷.

S. pyogenes expresses several proteins that bind Fn. So far, more than ten different Fn-binding proteins have been found in *S. pyogenes*, including the M protein, lipoteichoic acid (LTA) protein F1 (sfbI), protein F2, FBP54, and OF³⁸. These proteins play an important role in the colonization step; they bind to fibronectin in the ECM of epithelial cells, thus participating in adhesion. In the internalization process, bacteria-bound Fn binds to $\alpha_5\beta_1$ on the surface of epithelial cells, thereby activating the cells cytoskeleton and leading to the efficient internalization of the bacterium by these normally non-phagocytic cells^{17,39}. By mediating their own uptake into epithelial cells, the bacteria evade the host immune system and inside the cells, the bacteria are also well protected from those antibiotics that do not penetrate into cells. However, Fn-binding is not only beneficial to the bacterium. In a mouse model of *S. pyogenes* infection, it was found that bacteria expressing a Fn-binding protein were less virulent and spread less efficiently to the spleen as compared to a strain not expressing Fn-binding proteins⁴⁰.

1.2.3 Capsule

The surface of *S. pyogenes* is covered by a hyaluronic acid capsule. The expression of capsule varies greatly between different strains and, in addition, capsule production is stimulated upon contact with the host⁴¹. Highly encapsulated strains show resistance against killing in human blood⁴² and have been found to be more virulent and to cause invasive disease⁵. In addition, the hyaluronate capsule binds to CD44 on keratinocytes, thereby stimulating the opening of intercellular junctions and facilitating tissue penetration⁴³.

1.2.4 Other Virulence Factors

In addition to the M proteins and Fn-binding proteins, *S. pyogenes* expresses several other virulence factors (surface-bound or secreted). Many of them are involved in the establishment of infection (adhesion and internalization). Others are important for the spreading of the bacteria in tissues or for inhibiting the effective opsonization of the bacterium by complement components or Abs.

The surface-bound C5a peptidase cleaves the chemoattractant C5a⁴⁴ (see also Chapters 2 and 3), thereby inhibiting the recruitment of phagocytic cells to the site of infection. OF is expressed by approximately half of the *S. pyogenes* strains⁴⁵. It is present both as a surface-associated and as a secreted protein. OF mediates opalescence in mammalian serum by cleaving apolipoprotein A1 present in high-density lipoprotein⁴⁶. Furthermore, OF binds Fg⁴⁷ and Fn⁴⁸. The importance of OF as virulence factor has been demonstrated in a mouse model where an OF-expressing strain was more virulent than a strain deficient in OF-expression⁴⁸. The surface-bound protein GRAB (protein G-related α_2 M-binding) inhibits the degradation of other surface-bound streptococcal proteins by binding the proteinase inhibitor α_2 -macroglobulin⁴⁹. Collagen-like proteins, Scls, have been found to be important in a mouse model of soft tissue infection⁵⁰.

The secreted protein streptococcal inhibitor of complement (SIC) inhibits insertion of the membrane attack complex by binding complement components C5b-7^{51,52} (see Chapter 2). In addition to modulating the effect of complement deposition, *S. pyogenes* can also interfere

with the interactions with IgG. EndoS hydrolyzes the N-linked carbohydrate of IgG⁵³, thereby interfering with its ability to trigger phagocytosis⁵⁴. The IgG-degrading enzyme of *S. pyogenes* (IdeS), also called Mac, cleaves IgG in the hinge region⁵⁵ and binds to FcγRIIIb⁵⁶ and has been demonstrated to protect *S. pyogenes* from phagocytosis. *S. pyogenes* secretes two hemolysins – SLS, an oxygen-stable streptolysin, and SLO, which is oxygen-sensitive. These lysins have been shown to damage keratinocytes⁵⁷ and, in addition, SLO impairs the ability of neutrophils to kill *S. pyogenes*⁵⁸.

A number of pyrogenic exotoxins are expressed by *S. pyogenes*; streptococcal pyrogenic exotoxins A-J (SpeA-J), the streptococcal superantigen (SSA), and the streptococcal mitogenic exotoxin Z. These proteins act as superantigens and bind to T cells, thereby stimulating the massive release of inflammatory cytokines leading to tissue damage and shock⁵. In addition to acting as a superantigen, SpeB can also function as a cysteine proteinase. A number of host proteins are cleaved by SpeB; e.g. IgG⁵⁴, H-kininogen⁵⁹, fibronectin, and vitronectin⁶⁰. SpeB also modulates the surface of the bacterium by cleaving surface-bound proteins, e.g. M protein and C5a peptidase⁶¹. In addition, SpeB also cleaves the fibronectin-binding protein F1, thereby reducing the adhesion to and entry into host cells⁶².

Streptokinase, a protein secreted by all strains of *S. pyogenes*, converts plasminogen to plasmin⁶³. This protease degrades fibrin clots and several ECM proteins including fibronectin and laminin. The generation of plasmin activity has been shown to facilitate the spreading of *S. pyogenes* in transgenic mice expressing human plasminogen⁶⁴.

The Complement System

2

The immune system consists of the adaptive, also called the acquired, and the innate immune system. After an infection, the adaptive immune system has developed a “memory” that will recognize the pathogen if the host is reinfected. Antibodies (Abs) are the central part of this memory and are present in secretions, plasma, and all tissue fluids. They recognize an invading pathogen and bind to its surface, thereby labelling it for binding to Fc receptors expressed on phagocytic cells. In addition to mediating Fc-mediated phagocytosis, Abs can also activate the complement system. It should also be mentioned that Abs are involved in mediating allergic reactions and auto-immune diseases.

In a non-immune host, the innate immune system plays an important role in the defence against invading microorganisms. The complement system is part of the innate immune system, consisting of several proteins acting in cascades, aiming at destroying foreign particles – “non-self”, either directly or by marking them for subsequent destruction by specific cells. The system also encompasses its own regulators, protecting “self” from complement attack. In addition, it links the adaptive to the innate immune system. The activation of the complement system is tightly regulated at different levels, protecting the host cells from its otherwise devastating effects. Small complement components released during the activation are potent mediators of inflammation, leading to increased vascular permeability and recruitment and activation of inflammatory cells. Deficiencies either in complement components or in the proteins regulating their activation may lead to difficulties clearing certain infections or to development of different immune complex diseases. The focus of this text will be the role of the complement system in the clearance of invading microorganisms.

2.1 Activation of the Complement System

The complement system can be activated by three different pathways (Figure 2.1), converging in a common step leading to the lysis of foreign particles or in the deposition of complement components on the surface of the particle, making it recognizable to phagocytic cells expressing complement receptors. The three pathways are initialized by different stimuli; immune complexes (The Classical Pathway), carbohydrates (Lectin Pathway), or foreign surfaces (The Alternative Pathway). The classical and the lectin pathways converge after the

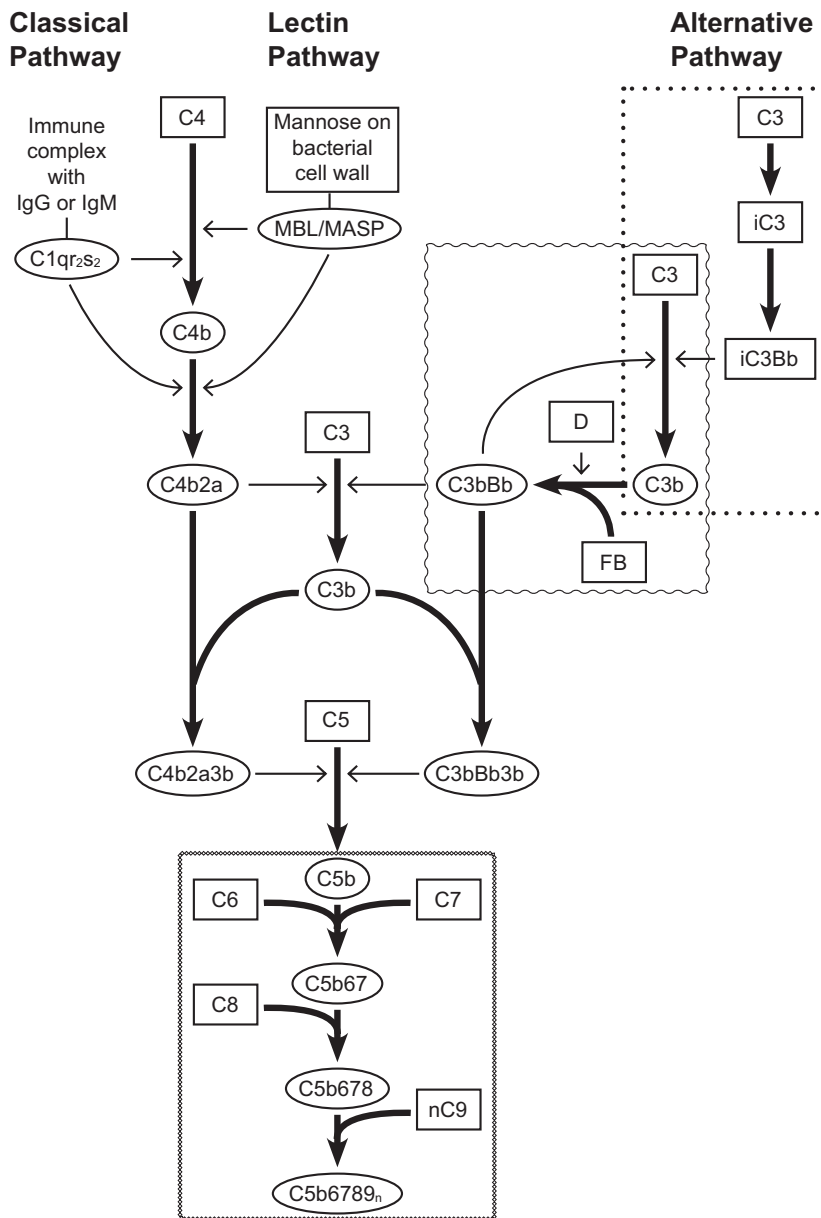


Figure 2.1 An overview of the complement system. Components in rectangles are soluble, whereas components inside ovals are surface bound. The dotted rectangle denotes the C3 tick-over reaction, the wavy rectangle the amplification loop of C3 activation, and the grey rectangle the Terminal Pathway.

initial steps. The classical pathway is a link to adaptive immunity. The complement system is capable of massive amplification; even the smallest initiating stimulus leads to efficient activation.

The Classical Pathway is initiated when an immune complex containing IgM or IgG is bound by C1 (consisting of 1 C1q, 2 C1r, and 2 C1s). Upon binding, one C1r first activates itself before it activates the other C1r and the two C1s. C1s can then bind and activate C4, yielding C4a and C4b*, an unstable product that is rapidly covalently bound to either amine or hydroxyl groups on proteins or carbohydrates on cell surfaces, yielding surface-bound C4b. Cell-bound C4b, in turn, binds C2, which is cleaved by C1s, resulting in C2b and the surface-bound complex C4b2a, the classical pathway C3 convertase.

The Lectin Pathway is initiated in an Ab-independent fashion: mannose binding protein (MBP) can bind to carbohydrates such as mannose and N-acetyl-glycosamine on bacterial surfaces. MBP then forms a complex with mannan-binding protein associated serine protease (MASP). This complex has the same function as C1 in the classical pathway, i.e. cleaving C4 to C4b and C2 to C2a, resulting in the same C3 convertase, C4bC2a.

The Alternative Pathway is another possibility to activate the complement cascade. In plasma, C3 is spontaneously and continuously hydrolyzed to an active form, iC3 (C3(H₂O)), an event termed *C3 tick-over*. iC3 binds factor B, yielding iC3B. Factor B is cleaved by factor D resulting in Ba and the complex iC3Bb, a fluid phase C3 convertase cleaving C3 into C3a and C3b*, an unstable product most of which is hydrolyzed and inactivated by water. However, if it comes in contact with a foreign surface, such as bacteria, it will bind covalently to the surface and initiate an *amplification loop of C3 activation* resulting in more C3b being deposited on the surface. The surface-bound C3b interacts with factor B, which is cleaved by factor D to give Ba and the surface-bound complex C3bBb, the alternative pathway C3 convertase.

The two C3 convertases then bind and cleave C3, yielding the C5 convertases C4b2a3b and C3bBb3b, respectively. These convertases bind and cleave C5 into C5a and C5b, initiating **the Terminal Pathway**. C5b, still bound to its convertase, binds C6 and C7, forming a hydrophobic complex, which is released from the convertase and inserts itself into lipid bilayers. C8 then interacts with the complex, making the membrane slightly permeable. Step-wise addition of up to 14 C9 monomers is followed, resulting in a pore (the membrane attack complex, MAC) that effectively lyses the target cell.

2.2 Regulation of the Complement System

If not regulated, activation of the complement system can cause massive inflammation and severe damage to unprotected cells and tissue. An unregulated activation of complement would not only cause local damage, it would also rapidly lead to depletion of complement factors, making the host complement deficient and thus susceptible to disease. The activation cascade is partly regulated by the instability of the convertases and the transient ability of C4b*, C3b*, and the precursors of the MAC to bind to target surfaces. There are also

proteins that regulate the complement cascade at different points. Some of the regulators are membrane-bound, whereas others are present circulating freely in plasma. Regulators of the complement system are summarized in Table 2.1. The modes of action of the different regulators are either by enzymatic cleavage to inactivate a complement component or by destabilizing the convertase complexes, thus leading to their dissociation. Only one positive regulator is known, properdin, which stabilizes C3bBb by binding to C3b.

Table 2.1. *Regulators of the complement system. P, plasma; M, membrane; CP, Classical Pathway; AP, Alternative Pathway; TP, Terminal Pathway.*

Regulator (localization)	Function	Pathway(s)
C1inh (P)	- binds activated C1, dissociating the complex	CP, LP
C4BP (P)	- promotes dissociation of convertases - cofactor for FI mediated cleaving of C4b	CP
FH and FHL-1 (P)	- promotes dissociation of convertases - cofactor for FI mediated cleaving of C3b	AP
FI (P)	- cleaves C3b and C4b in the presence of a cofactor	CP, AP
MCP (M)	- cofactor for FI mediated cleaving of C3b and C4b	CP, AP
DAF (M)	- promotes dissociation of convertases	CP, AP
CR1(P and M)	- promotes dissociation of convertases - cofactor for FI mediated cleaving of C3b and C4b	CP, AP
S protein (P)	- binds C5b67, preventing its binding to membranes	TP
Clusterin (P)	- binds C5b67, preventing its binding to membranes	TP
HRF (M)	- binds C8, blocking the assembly of the MAC	TP
CD59 (M)	- blocks the insertion of more than one C9 in C5b-8	TP
Properdin (P)	- stabilizes C3bBb	AP

The classical pathway is inhibited by C1 inhibitor (C1inh) which binds to the activated C1 complex, leading to the dissociation of C1s and C1r from C1q. Further down in the cascade, C4b-binding protein (C4BP) destabilizes the two convertases C4b2a and C4b2a3b, leading to their dissociation. Factor H (FH) and factor H-like protein-1 (FHL-1) have the same effect on two convertases of the alternative pathway by binding to C3b. Factor I (FI) is a key regulator of the complement system, degrading both C4b and C3b, using different cofactors. In addition to promoting the dissociation of the convertases, C4BP, FH, and FHL-1 can act as cofactors in the FI-mediated degradation of C4b and C3b, C4BP being specific for C4b and FH and FHL-1 for C3b. Membrane cofactor protein (MCP, CD46) is a membrane-bound protein, binding to C4b and C3b in the convertases and acting as a cofactor for FI-mediated degradation of these

molecules, thus inactivating the convertases. Decay accelerating factor (DAF, CD55) is also membrane-bound and binds both C4b and C3b in the convertases, but instead of acting as a cofactor for cleavage, it destabilizes the complexes, leading to their dissociation. CR1 is one of the complement receptors expressed on different cells. It is, however, also an important regulator of complement. First, it can (like MCP) act as a cofactor for FI in the degradation of both C3b and C4b. Second, it destabilizes the convertases, thus leading to their inactivation by dissociation (like DAF).⁶⁵

The terminal pathway is also regulated by different proteins, both soluble and membrane-bound. S protein and clusterin are plasma proteins that bind C5b67, thereby preventing its binding to membranes. Membrane-bound homologous restriction factor (HRF) binds C8, thereby blocking the assembly of the MAC. Another membrane-bound regulator of the terminal pathway is CD59 which inhibits the formation of MAC by binding to C8 in the C5b678 complex and blocking the insertion of more than one C9.

In addition to surface-bound and circulating regulators, there are other modes of regulating complement activation. On a bacterial surface, deposited C3b has a high affinity for factor B, resulting in the formation of a stable C3bBb complex, whereas, on “self” surfaces, bound C3b binds FH, and C3b is subsequently inactivated by FI. The precise mechanism behind this difference is not fully understood, but the presence of acidic carbohydrates, e.g. sialic acid, seem to favour the degradation of deposited C3b, thus protecting “self” surfaces from amplified C3b deposition. Nucleated cells can also either internalize and degrade MAC or shed MAC on membrane vesicles and in this way protect themselves from the effects of MAC.

The released fragments of C3, C4, and C5 (C3a, C4a, and C5a) are potent mediators of inflammation, C5a being the most potent, followed by C3a. They are also called anaphylatoxins because, when released in large amounts, they lead to the same reactions as in anaphylactic shock: histamine release and smooth muscle contraction leading to vascular leakage. The anaphylactic peptides are regulated by inhibitors in plasma, restricting their activity to the site of complement activation. Their principal inactivator is serum carboxypeptidase-N (anaphylatoxin inactivator), which cleaves the anaphylatoxins in their carboxy-terminal end, making them much less potent.

2.3 Biological Roles of the Complement System

The important roles of the complement system are easily understood when studying patients suffering from deficiencies in this system. Deficiencies of almost all components of the complement system, including the regulators, have been reported. Deficiencies of components of the classical pathway lead to the inability to clear immune complexes, thus leading to immune complex disease. Patients suffering from C3 deficiency will not be able to opsonize bacteria, since C3 is a pivotal component in all three pathways. C3 deficiency will therefore lead to an increased susceptibility to bacterial infections. Deficiencies of terminal pathway components cause increased susceptibility to particularly *Neisseria* infections⁶⁶.

The end point of complement activation on the surface of an invading organism is the formation of the MAC complex leading to complete lysis of the cell. However, e.g. Gram-positive bacteria often express a thick cell wall protecting the membrane from a direct attack by MAC. However, the deposition of complement components on the surface of such organisms, opsonization, is not without effect, since surface bound C4b and C3b and its “break down products” (iC3b and C3dg) are readily recognized by specific complement receptors on phagocytes leading to the effective uptake and killing of these organisms.

C3a and, in particular, C5a are potent chemoattractants leading to the recruitment of inflammatory cells and activation of phagocytes at the site of infection.

The complement system is also involved in the clearance of apoptotic cells via complement-mediated phagocytosis⁶⁷.

2.4 Complement Receptors

The different components of C3 act as powerful opsonins, marking “non-self” to be recognized by the host’s immune cells. There are four different complement receptors (CRs), designated CR1-4. Their alternative names, cellular distribution, and ligands are summarized in Table 2.2. In addition to acting as a regulator of complement activation, CR1 is mainly involved in the elimination of immune complexes⁶⁵ and binding of complement-opsonized particles, but has also been shown to be involved in phagocytosis⁶⁸. CR2 is an important link between innate and adaptive immunity and has been implicated in maintenance of B cell tolerance against “self”-antigens⁶⁹ and in the regulation of the IgE production⁷⁰. CR3 is involved in adhesion and phagocytosis. CR4 is has been implicated in phagocytosis^{68, 71}. For a more detailed description of CR3 (and CR4) see Chapters 3 and 4.

Table 2.2 Complement receptors, their cellular distribution, ligands, and functions.^a

Receptor	Alternative name	Cellular distribution	Ligands
CR1	CD35	Neutrophils Monocytes Macrophages Erythrocytes B cells Follicular dendritic cells Glomerular epithelial cells	C3b > iC3b C4b
CR2	CD21	B cells Follicular dendritic cells Epithelial cells of cervix Epithelial cells of nasopharynx	iC3b C3dg Epstein-Barr virus Interferon- α
CR3	CD11b/CD18 $\alpha_M\beta_2$ Mac-1 Mo-1	Neutrophils Monocytes Macrophages NK cells Follicular dendritic cells	iC3b fibrinogen ICAM-1 factor X certain bacteria zymosan
CR4	CD11c/CD18 $\alpha_X\beta_2$ p150.95	Neutrophils Monocytes Tissue macrophages Dendritic cells	iC3b fibrinogen

^a In part adopted from Immunology, 4th edition, Roitt, Brostoff, and Male

The Neutrophil

3

The neutrophils are part of the body's first line of defence against invading organisms and their main purpose is to recognize, ingest and kill intruders. Patients suffering from defects in neutrophil production or function are susceptible to bacterial infections.

The neutrophils are 10-20 μm in diameter and make up 50-60% of the total leukocyte population. These cells are also called polymorphonuclear neutrophils (PMNs) or granulocytes, referring to their lobular-shaped nucleus and their large amount of granules. They mature from myelocytes in the bone marrow at an amazing rate of 100×10^9 per day, adding up to as much as 1.5 tons in a lifetime. The process of maturation takes 10-12 days, but can be accelerated in the case of infection or stress⁷². The neutrophils circulate with the blood, and in the case of the appropriate signals from a site of infection or inflammation they extravasate into the tissue, where they participate in the killing of invading microbes and mediation of acute inflammation. Once they have left the blood stream, they stay in the tissue. The neutrophils life in the blood is very short, with a half-life of 4.5 h⁷³, whereas in tissue, they can live for a few days. The blood pool of neutrophils is renewed within 2-3 days. Neutrophils in the blood die by apoptosis and are cleared by macrophages in the liver, spleen, or lungs⁷⁴.

3.1 Neutrophil Granules and Secretory Vesicles

During their maturation in the bone marrow, the neutrophils are equipped with different granules, containing various proteins, enzymes, and surface receptors important for extravasation and recognition and killing of microorganisms (Table 3.1). The granules are formed during different stages of maturation, and proteins synthesized simultaneously are packaged together^{75, 76}. The first granules to be produced are the azurophil granules (primary granules), followed by specific granules (secondary granules), gelatinase granules (tertiary granules), and secretory vesicles.

The azurophil granules are not easily exocytosed in response to stimulation⁷⁷. These granules are rich in antimicrobial proteins and acidic hydrolases and their primary contribution to neutrophil function is to participate in the killing and degradation of phagocytosed microorganisms in the phagosome. Myeloperoxidase (MPO) amplifies the toxic potential of H_2O_2 formed by the NADPH oxidase⁷⁸. The major constituents of the azurophil granules are

the small, cationic α -defensins. These cytotoxic peptides show antimicrobial activity against bacteria, fungi, enveloped viruses and protozoa⁷⁹. Bactericidal/permeability-increasing protein (BPI) mediates killing of Gram-negative bacteria⁸⁰. Proteinase-3, elastase, and cathepsin G are serine proteases with proteolytic activity against many ECM proteins⁷⁹. In addition, these enzymes are also antimicrobial and induce the activation of e.g. endothelial and epithelial cells, and macrophages⁸¹.

The specific granules participate in the antimicrobial activities of the neutrophil by emptying their content into the phagosome or the exterior of the cell. These granules are rich in antimicrobial substances such as hCAP-18, lactoferrin, and lysozyme. The C-terminal part of hCAP-18, LL-37, has antimicrobial activity against both Gram-positive and Gram-negative bacteria⁸². Lactoferrin impairs bacterial growth by sequestering iron⁸³. Lysozyme cleaves peptidoglycan polymers of bacterial cell walls and is present in all of the granules, but its highest concentrations are found in specific granules. Upon neutrophil activation, cytochrome b₅₅₈ is translocated to the phagosomal membrane or to the surface of the cell, where it functions as part of the NADPH oxidase. Two metalloproteases (MMPs) are found in specific granules; collagenase (MMP-8) and gelatinase (MMP-9).

The gelatinase granules contain gelatinase and leukolysin (MMP-25). The MMPs are stored as inactive proforms and are activated upon exocytosis. These proteases degrade many ECM components such as collagens, laminin, and fibronectin, and are thought to play a role in migration⁸¹. The gelatinase granules also contain membrane receptors and antimicrobial peptides.

Table 3.1 Selected contents of neutrophil granules and secretory vesicles, for a review see reference [79].

high	Density			low
Azurophil granules	Specific granules	Gelatinase granules	Secretory vesicles	
MPO	CD11b/CD18	CD11b/CD18	CD11b/CD18	
BPI	Cytochrome b ₅₅₈	Cytochrome b ₅₅₈	Cytochrome b ₅₅₈	
HBP	hCAP-18	fMLP-R	CD14	
Defensins	Lactoferrin	β_2 -Microglobulin	CR1	
Proteinase-3	Lysozyme	Gelatinase	C1q-R	
Cathepsins	Collagenase	Lysozyme	DAF	
Elastase	Gelatinase	NRAMP-1	HBP	
Lysozyme			Plasma proteins	

Secretory vesicles contain plasma proteins, which suggests that these vesicles are formed by endocytosis⁸⁴. These vesicles contain cell surface receptors needed at the early stages of neutrophil activation and they are easily exocytosed upon stimulation⁷⁷. Their mobilization leads to the upregulation of CD11b/CD18, the complement receptor 1 (CR1), CD14 (the LPS/ lipoteichoic receptor), fMLP-receptors, and CD16 (the Fc γ RIIIb receptor) on the neutrophil surface⁷⁹. In addition, L-selectin is shed from the neutrophil surface⁸⁵. Due to these changes on the cell surface, the neutrophil can now be firmly attached to the vascular endothelium. The

secretory vesicles are the most easily mobilized, and fuse with the plasma membrane when the neutrophil is activated in the blood stream. This is a means of the neutrophil to regulate its surface expression of adhesion molecules. In addition to surface receptors, the secretory vesicles also contain heparin-binding protein (HBP)⁸⁶, also known as azurocidin or CAP37, which increases vascular permeability⁸⁷, attracts monocytes⁸⁸, and shows antimicrobial activity⁸⁹.

Degranulation of neutrophil granules and secretory vesicles occurs in response to increased intracellular Ca^{2+} concentration. Secretory vesicles are released at the lowest concentration, followed by the gelatinase and specific granules. The azurophilic granules are released at the highest concentration^{77, 90}. In addition to elevations of intracellular Ca^{2+} concentrations, degranulation has also been found to be dependent on the small RhoGTPase Rac2⁹¹.

3.2 Oxidative burst – the NADPH Oxidase

In addition to the microbicidal components in the different granules, the neutrophil also utilizes an oxygen-dependent mechanism for the killing of microorganisms. The NADPH oxidase is composed of one membrane-bound and four cytosolic components. The membrane-bound component is cytochrome b_{558} , mainly present in the membranes of specific and gelatinase granules (85%), but also in the the membrane of secretory vesicles and in the plasma membrane⁹². The cytosolic components are p40^{phox} , p47^{phox} , p67^{phox} , and Rac2⁹³. Upon activation and exocytosis or fusion of granules, the cytosolic components translocate to the cytochrome b_{558} located in the plasma membrane or in the membrane of the newly formed phagosome, and the active NADPH oxidase is formed. Cytochrome b_{558} mediates the translocation of one electron from NADPH to oxygen, generating superoxide anion radical, $\text{O}_2^{\cdot-}$, which is dismutated to hydrogen peroxide, H_2O_2 . Neither $\text{O}_2^{\cdot-}$ nor H_2O_2 has the potential to kill microorganisms, but react with one another and/or other species, generating different toxic compounds. In addition, MPO delivered to the phagosome through the fusion of azurophilic granules, potentiates the microbial effect of H_2O_2 by inducing the formation of reactive intermediates. The MPO- H_2O_2 system mediates the oxidation of e.g. halide compounds, tyrosine, and nitrite into the microbicidal compounds hypochlorous acid (HOCl), tyrosine radicals, and reactive nitrogen intermediates⁹⁴. Activation of the NADPH oxidase can occur in the plasma membrane of the cell, resulting in a localized extracellular production of reactive oxygen species; in the membrane of phagosomes; and in granule membranes⁹². The role of the NADPH oxidase in the killing of invading pathogens is further discussed in section 3.5. The NADPH oxidase has also been shown to be activated independently of phagocytosis⁹⁵. Besides being involved in killing of microorganisms, the products of the respiratory burst are thought to play a part in apoptosis⁹⁶ and intracellular signalling⁹⁷.

The importance of a functioning NADPH oxidase in the killing of invading pathogens is demonstrated in patients suffering from chronic granulomatous disease (CGD), caused by defects in one or more of the components of the NADPH oxidase. Although neutrophils from these patients are able to phagocytose microorganisms, the phagocytosed microbes are not always killed, and CGD patients suffer from severe recurrent fungal and bacterial infections.

These infections are mainly caused by catalase-producing pathogens and/or microorganisms that are resistant to non-oxidative killing mechanisms, such as various *Aspergillus* species, *Staphylococcus aureus*, and enteric Gram-negative bacteria.⁹⁸ By degrading the small amounts of H₂O₂ produced by the bacteria themselves, catalase prevents MPO from utilizing H₂O₂ for the production of reactive oxygen species, hence inhibiting the subsequent killing of the pathogen.

3.3 Recruitment of Neutrophils to the Site of Infection

To be able to ingest and kill an invading microorganism, the neutrophils must be recruited to the site of infection. In response to inflammatory mediators released, circulating neutrophils are activated. In addition, the endothelial cells lining blood vessels adjacent to the infection site upregulate the expression of receptors that interact with neutrophils. The recruitment of neutrophils is summarized in Figure 3.1.

The first interactions of neutrophils with endothelial cells are weak and occur via different selectins expressed both on neutrophils and endothelial cells. L-selectin (CD62L) is exclusively found on leukocytes and is constitutively expressed. P-selectin (CD62P) is stored inside endothelial cells and is translocated to the surface soon after stimulation with inflammatory mediators⁹⁹ and binds to the neutrophil surface via P-selectin glycoprotein ligand 1 (PSGL-1)¹⁰⁰. Further stimulation of endothelium by cytokines leads to the upregulation of E-selectin (CD62E) after a few hours¹⁰¹, and the expression of ligands for neutrophil L-selectin is initiated. The ligands for the three selectins are mostly glycoproteins carrying e.g. Sialyl Lewis^x¹⁰². The weak interactions and the shear stress exerted by the blood flow cause the neutrophils to roll on the endothelium. There is also evidence that $\alpha_4\beta_1$ is involved in the early events of adhesion to endothelium¹⁰³.

During the process of rolling, L-selectin is shed from the surface of the neutrophil¹⁰⁴ and the secretory vesicles fuse with the plasma membrane, thereby upregulating the expression of β_2 integrins, primarily CD11b/CD18, on the surface. These integrins are further activated through “inside-out signalling” (see Chapter 4) induced by the action of chemoattractants, cytokines, and growth factors¹⁰⁵. In addition, endothelial cells in areas of inflammation release reactive oxygen species which also trigger the activation of the β_2 integrins¹⁰⁶. CD11a/CD18 (LFA-1) binds to intercellular adhesion molecules 1 and 2 (ICAM-1 and ICAM-2) on the endothelial cells, and CD11b/CD18 binds ICAM-1¹⁰⁷. The expression of ICAM-1 is upregulated on inflamed endothelium, whereas ICAM-2 is constitutively expressed^{108, 109}. These interactions lead to the adhesion of the neutrophil to the endothelium. Furthermore, engagements of the β_2 integrins induce the surface expression of β_1 integrins such as $\alpha_2\beta_1$ and $\alpha_4\beta_1$ ¹¹⁰, which both have been found to be implicated in neutrophil adhesion and migration in tissue.

The adhesion of the neutrophil to the endothelium and the upregulation of the expression of β_2 integrins cause the neutrophil to spread on the endothelium. The neutrophil crawls on the endothelium following the chemotactic gradient of surface-bound chemoattractants, a process called haptotaxis. β_2 integrin engagement induces the release of HBP which mediates

an increase in endothelial permeability^{86,87}. In addition, once the neutrophil is attached to the endothelium, gelatinase granules release their contents of ECM-degrading enzymes, which degrade the basement membrane, thereby facilitating the migration of neutrophils into the tissue^{81, 111, 112}.

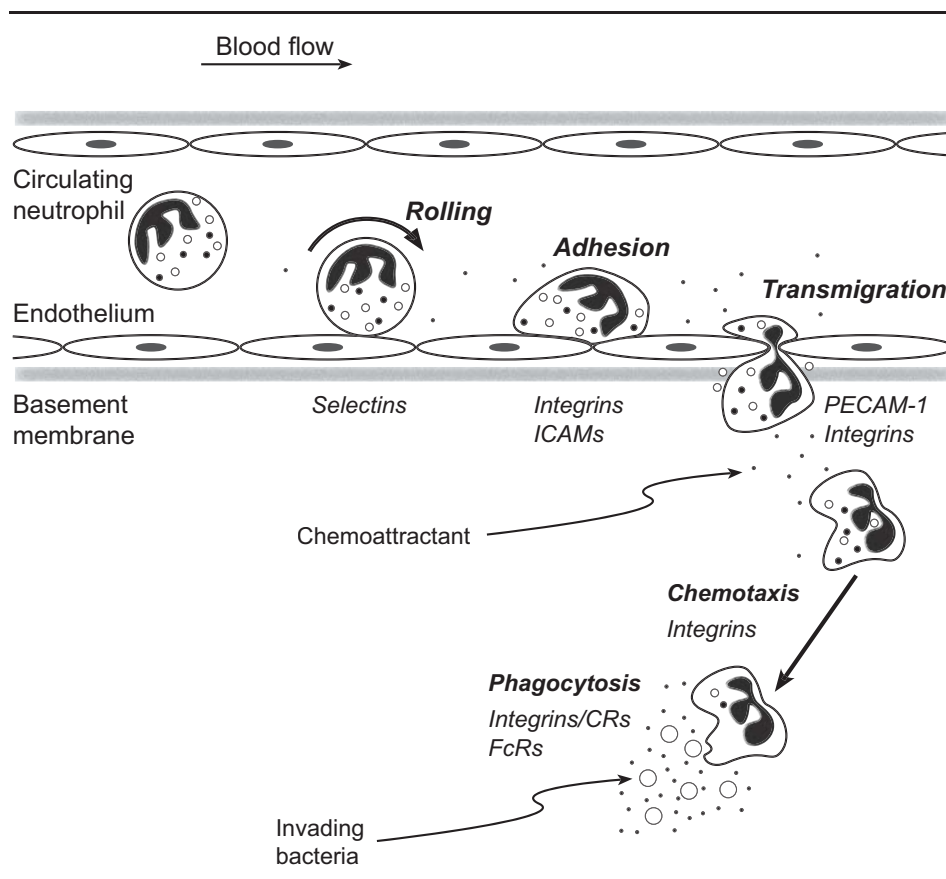


Figure 3.1 The process of neutrophil recruitment to a site of infection. The different steps and the receptors involved are indicated.

The final step in extravasation is transendothelial migration which is a rapid event, occurring mainly at tricellular corners¹¹³, although neutrophils have also been shown to use a transcellular route^{114, 115}. Both endothelial cells and neutrophils express the platelet endothelial adhesion molecule-1 (PECAM-1). Transendothelial migration is mediated by PECAM-1 homophilic interactions¹¹⁶. Extravasation and the concomitant release of granules containing surface receptors have also been demonstrated to alter the responsiveness of the neutrophil, e.g. to substances activating the NADPH oxidase¹¹⁷.

In the tissue, the neutrophil moves along the chemotactic gradient of soluble chemoattractants, a process known as chemotaxis, until it reaches the site with the highest concentration. The movement through tissue is mainly mediated by the matrix-binding integrins. The neutrophils possess an intricate mechanism for recognizing the direction of the chemotactic gradient; with receptors working in sequence, desensitizing each other, resulting in the favouring of signals from the end target (C5a and formylated peptides) over “earlier” chemotactic signals (e.g. IL-8 and LTB₄)^{118, 119}.

3.4 Phagocytosis

When the neutrophil has reached the site of infection, it must be able to recognize, bind, and eliminate the invading microorganism. Using different surface receptors recognizing various ligands, the neutrophil binds invading microorganisms. The neutrophil has receptors for antibodies (Fc receptors, FcRs), complement components (complement receptors, CRs), and for conserved bacterial structures (pattern recognition receptors). FcRs and CRs can mediate phagocytosis. Although in the end, the effect of these two events is internalization of the target, the two processes occur by somewhat different mechanisms. FcR-mediated phagocytosis has been found to be accompanied by the activation of the small GTPases Rac and Cdc42, membrane ruffling and the formation of pseudopods (Type I or zipper phagocytosis), whereas in complement mediated phagocytosis the small GTPase RhoA is activated and the target sinks into the cell (Type II or trigger phagocytosis)^{120, 121}. However, these experiments were performed using sheep red blood cells that are significantly larger than live bacteria. In human neutrophils, complement-mediated phagocytosis of pansorbins has been demonstrated to activate Cdc42 and Rac¹²².

3.4.1 Fc Receptors (FcRs)

In order to be able to bind foreign targets coated with Abs, phagocytosing cells express FcRs. Neutrophils express one Fc α R (Fc α RIa) and three different Fc γ Rs on their surface (Fc γ RIa, Fc γ RIIa, and Fc γ RIIIb)¹²⁰. The Fc γ Rs belong to the immunoglobulin gene superfamily and have a common homologous extracellular IgG-binding domain¹²³.

Fc γ RIa (CD64) is expressed on neutrophils stimulated with interferon γ (IFN γ) and exists as a multimeric complex where one α chain is associated with a dimer of disulphide-linked γ chains. The α chain contains three Ig-like extracellular domains, which are responsible for IgG binding, and it binds monomeric IgG with high affinity. Each γ chain contains one immunoreceptor tyrosine activation motif (ITAM) which is responsible for signal transduction.¹²³

Fc γ RIIa (CD32) is constitutively expressed on neutrophils and consists of one single α chain with two extracellular Ig-like domains and one intracellular ITAM¹²³. It binds IgG with low affinity and does only bind IgG in immune complexes or aggregates. Fc γ RIIa is considered to be the most important FcR on neutrophils¹²⁴ with 20 000 - 40 000 copies per cell¹²⁵.

Fc γ RIIIb (CD16) is present only on neutrophils¹²³ where it is expressed constitutively. It is the most abundant of the Fc γ Rs with 100 000 – 300 000 copies per cell¹²⁵, and, in addition, large amounts of Fc γ RIIIb are stored in secretory vesicles^{126, 127}. It consists of only one α chain with two Ig-like extracellular domains, and has no transmembrane or cytoplasmic domains. Instead, it is linked to the cell membrane by a glycosyl phosphatidyl inositol (GPI) anchor.¹²³ Fc γ RIIIb is not alone able to trigger phagocytosis, but instead it works in cooperation with either CR3 or Fc γ RIIa¹²⁸⁻¹³².

Upon crosslinking of the Fc γ R, Src kinases associate to the receptor, are activated, and phosphorylate tyrosine residues within the ITAM. These phosphotyrosines are binding sites for other signalling molecules, e.g. the tyrosine kinase Syk.¹³³ Syk phosphorylates and activates an array of signalling molecules, including PI3-K and PLC γ ¹²³. Later downstream the small GTPases Rac and Cdc42 are activated¹²¹. Fc γ R signalling results in increase of intracellular free Ca²⁺, rearrangement of the actin cytoskeleton and finally phagocytosis¹²⁰.

3.4.2 Complement Receptors (CRs)

Neutrophils express three different receptors for complement components: CR1 (CD35), CR3 (CD11b/CD18, $\alpha_M\beta_2$), and CR4 (CD11c/CD18, $\alpha_X\beta_2$). Of these, CR3 is the most abundant, followed by CR1 and CR4. CR1 is a single-chain transmembrane protein recognizing particles covered with C3b, C4b, and iC3b, having highest affinity for C3b. CR1 is considered to mainly participate in the binding of phagocytic prey, and not mediating phagocytosis by itself. In addition, CR1 plays a role in the regulation of complement activation (see Chapter 2). The β_2 integrin CR3 is the main receptor mediating phagocytosis of complement-opsonized particles, binding iC3b (and C3b). In addition, it also binds different matrix proteins. CR4 is also a β_2 integrin and binds iC3b, and has also been implicated in phagocytosis^{68, 71}. However, its role in phagocytosis is not fully understood. For a more detailed description of the integrins, see Chapter 4.

3.4.3 Pattern Recognition Receptors

In addition to CRs and FcRs, which recognize molecules deposited on bacterial surfaces, neutrophils also express receptors that directly bind to bacterial components. One of the most studied groups of such receptors is the group of toll-like receptors, TLRs. So far, ten different TLRs have been identified, out of which neutrophils express all but one (TLR3)¹³⁴. Neutrophil TLRs recognize LPS (TLR4), bacterial lipoproteins and LTA (TLR2 as a heterodimer with TLR1 or TLR6), flagellin (TLR5), unmethylated DNA (TLR9), and single-stranded viral RNA (TLR7)¹³⁵. Engagement of TLRs result in IL-8 production, shedding of L-selectin, priming for fMLP-mediated superoxide generation, decreased chemotaxis, and increased phagocytosis¹³⁴.

3.5 Killing of Microorganisms

The mechanisms by which ingested microbes are killed have been extensively studied. Previously, it was generally believed that the NADPH oxidase in combination with the actions of MPO and the antimicrobial proteins and proteases stored in the granules constituted two different killing mechanisms. However, the relative importance of direct killing by reactive oxygen species generated by the NADPH oxidase and halogenation accomplished by the MPO-H₂O₂ system has been questioned, since the experiments supporting this idea were not carried out under conditions resembling the phagosome regarding e.g. pH and protein concentrations¹³⁶. Instead, the granule enzymes released into the phagosome are thought to be activated by the conditions produced by the influx of ions that compensate for the charge differences induced by the activity of the NADPH oxidase. In this model, the primary role for the NADPH oxidase would thus be to generate an environment in the vacuole facilitating the activation of the different antimicrobial enzymes and not to mediate killing by the production of reactive oxygen species. Hence, in the phagosome, the cationic antimicrobial proteins are activated and released from the negatively charged proteoglycans (to which they are bound when stored in granules) by elevations in pH and the influx of K⁺¹³⁷. In support of this theory are the observations that individuals deficient in MPO are capable of killing microorganisms⁹⁸, and the finding that double knock-out mice for elastase and cathepsin G show defects in bacterial killing similar to mice lacking a functioning NADPH oxidase¹³⁷.

In addition to intracellular killing of invading microorganisms, neutrophils can mediate extracellular killing by releasing the content of its granules into the extracellular space. Neutrophils have also been reported to form so called neutrophil extracellular traps (NETs) consisting of released chromatin and granule proteins, that bind both Gram-positive and Gram-negative bacteria, degrade virulence factors, and kill the trapped bacteria¹³⁸.

The Integrins

4

Integrins are important surface receptors linking the ECM to the cytoskeleton. They are present on most cell types and found in many animal species, ranging from sponges to mammals¹³⁹. These glycosylated surface proteins play important roles in intercellular communication and interactions with the ECM. Integrins are composed of two non-covalently linked chains, one α and one β chain. So far, 18 different α chains and 8 different β chains have been identified. One specific α chain can form dimers with different β chains, thus allowing the formation of at least 24 different integrins (Figure 4.1). Each integrin can bind several different ECM proteins, and many matrix proteins are recognized by several integrins. The ligand recognition site is located in the I domain of the α chain^{140, 141}. In addition, the β chain has an I-like domain which is also important for ligand binding^{140, 142}. The I domain contains a “metal ion-dependent adhesion site” – MIDAS, which coordinates Mg^{2+} or Mn^{2+} and binds to negatively charged residues in the ligand^{140, 143}. Furthermore, the ligand often contains an RGD (arginine, glycine, and aspartic acid) motif important for binding. However, this motif is not a prerequisite for binding to β_2 integrins¹⁴⁴. In addition to ECM molecules, integrins also bind soluble ligands and counter-receptors on other cells.

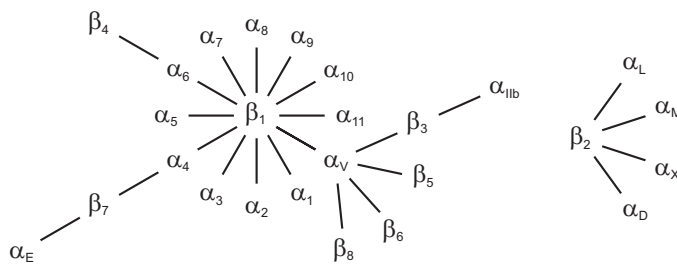


Figure 4.1 The integrin family. The 24 identified heterodimers are indicated.

On a resting cell, the integrins have a conformation with low affinity for their ligand, with the I domain and its MIDAS motif covered by the β chain¹⁴⁴ – the “closed” or inactive state. To be able to bind a ligand, the integrin has to be activated. In addition to affinity changes, the mobility of the integrin in the plasma membrane can be altered, facilitating the local

clustering of integrins. Activation of integrins is referred to as “inside-out” signalling. Upon activation, the integrin changes its conformation, exposing the ligand-binding domains¹⁴⁴ (“open” or active state) and/or can move in the plasma membrane. Different integrins are regulated and activated by different stimuli inducing different signalling pathways. Ligands for G protein-coupled receptors (GPCRs) (e.g. the chemoattractants IL-8, fMLP, and C5a) are known to activate integrins, but the signalling mechanisms responsible for integrin activation are not fully understood. In addition to GPCRs, other receptors such as Fc γ Rs can induce the activation of integrins. The activated integrin binds its ligand with high affinity and transmits signals into the cell – “outside-in” signalling.

Leukocyte integrins are involved in many cellular functions; such as adhesion to the vessel wall prior to extravasation, migration, and recognition and phagocytosis of invading bacteria. Cell-cell contacts mediated by integrins are involved in antigen presentation and cytotoxicity.

4.1 Neutrophil Integrins

Neutrophils express integrins of the β_1 , β_2 , and β_3 family. Their alternative names, selected ligands, and effector functions are indicated in Table 4.1.

Neutrophils express approximately 10^5 integrins of the β_1 (CD29) family (also called very late antigens (VLAs))¹⁴⁵. These integrins bind different components of the ECM and play important roles in adhesion and migration. The major β_1 integrins expressed on human neutrophils are $\alpha_5\beta_1$ and $\alpha_6\beta_1$ ¹⁴⁵. VLA-5 ($\alpha_5\beta_1$) binds fibronectin with high affinity and has been found to be involved in the migration of neutrophils. VLA-4 ($\alpha_4\beta_1$) binds fibronectin and is expressed at very low concentrations on human neutrophils, but is upregulated upon neutrophil transmigration¹⁴⁶. Directional migration in fMLP-mediated chemotaxis has been shown to require $\alpha_4\beta_1$ and $\alpha_L\beta_2$ (LFA-1)¹⁴⁷.

Several studies have demonstrated crosstalk between the β_1 and the β_2 integrins. For example, engagement of activated β_1 integrins has been found to regulate β_2 integrin-mediated adhesion¹⁴⁸⁻¹⁵⁰ and ligation of β_2 integrins induce the surface expression of β_1 integrins¹¹⁰.

The β_2 integrins are exclusively found on leukocytes (lymphocytes, monocytes, and neutrophils). The β_2 integrins are involved in recruitment, adhesion, migration, and phagocytosis. Unstimulated neutrophils express approximately 10^5 β_2 integrins, and upon stimulation the expression can increase up to fivefold¹⁵¹. On neutrophils, the β_2 chain (designated CD18) forms dimers with three different α chains; α_L (CD11a), α_M (CD11b), and α_X (CD11c). Each integrin has one or more names; hence CD11a/CD18 is also called LFA-1 (lymphocyte function-associated antigen 1) or $\alpha_L\beta_2$; CD11b/CD18 has four alternative names (CR3, Mac-1, Mo-1, and $\alpha_M\beta_2$); and CD11c/CD18 is termed CR4, gp150/95, or $\alpha_X\beta_2$. The most abundant β_2 integrin on neutrophils is CD11b/CD18, followed by CD11a/CD18.

Leukocyte adherence deficiency (LAD) syndrome is a condition where the number of β_2 integrins is absent or greatly reduced on the leukocytes due to a mutation in the β_2 gene. LAD patients suffer from recurrent life-threatening bacterial infections, impaired wound healing,

and tissue remodelling¹⁵². Furthermore, the accumulation of neutrophils and monocytes at sites of infection is impaired, and leukocytes from these patients show defects in adhesion and signalling¹⁴⁴.

Table 4.1 Neutrophil integrins, selected ligands and roles in neutrophil function.

Integrin	Alternative names	Selected Ligands	Effector functions
$\alpha_2\beta_1$	CD49b/CD29 VLA-2	collagen laminin	adhesion migration
$\alpha_3\beta_1$	CD49c/CD29 VLA-3	collagen laminin fibronectin	adhesion (?)
$\alpha_4\beta_1$	CD49d/CD29 VLA-4	fibronectin VCAM-1	rolling adhesion migration
$\alpha_5\beta_1$	CD49e/CD29 VLA-5	fibronectin	adhesion migration chemotaxis
$\alpha_6\beta_1$	CD49f/CD29 VLA-6	laminin	adhesion chemotaxis
$\alpha_9\beta_1$	CD49i/CD29 VLA-9	fibronectin VCAM-1	adhesion chemotaxis
$\alpha_T\beta_2$	CD11a/CD18 LFA-1	ICAM-1 ICAM-2 ICAM-3	adhesion diapedesis
$\alpha_M\beta_2$	CD11b/CD18 CR3 Mac-1 Mo-1	ICAM-1 ICAM-2 fibrinogen iC3b	adhesion chemotaxis phagocytosis degranulation respiratory burst
$\alpha_X\beta_2$	CD11c/CD18 CR4 gp150/95	fibrinogen iC3b	adhesion
$\alpha_V\beta_3$	CD51/CD61	PECAM-1 fibronectin vitronectin	adhesion migration

Fc γ R-mediated activation of $\alpha_M\beta_2$ avidity has been demonstrated to involve PI3-K and cytoskeletal rearrangement. In contrast, GPCR-mediated avidity activation of the same integrin was found to be independent of PI3-K and rearrangement of the actin cytoskeleton. However, both of these receptors were found to activate PAK1, an effector of the small GTPases¹⁵³. IL-8-mediated activation of β_2 integrins has been shown to require the activation of the small GTPase RhoA¹⁰⁵. The chemokine-mediated activation of PI3-K has also been demonstrated to activate cytohesin-1, a GEF for the GTPase ARF, which induces LFA-1 activation by direct interaction with the β_2 chain, leading to increased mobility and clustering of the integrin. However, cytohesin-1 was not found to be involved in affinity triggering¹⁵⁴.

CD11b/CD18 is the dominant complement-binding receptor on neutrophils¹⁵⁵, binding iC3b deposited on invading microorganisms. CD11b/CD18 must first be activated in order to be able to phagocytose the microorganism. In addition to the pathways discussed above, it has also been proposed that co-ligation of the lectin site in CD11b/CD18 by microbial surface carbohydrates activates the receptor, making the binding of iC3b deposited on the bacterial or fungal surface sufficient to trigger phagocytosis¹⁵⁵. In addition to mediating complement-dependent phagocytosis, CD11b/CD18 has been shown to interact with the GPI-anchored Fc γ RIIIb to facilitate Ab-mediated phagocytosis and respiratory burst. Hence, neutrophils from LAD patients demonstrate impaired Ab-dependent phagocytosis¹³². For a detailed discussion on the signalling events elicited upon β_2 integrin engagement, see section 4.2.

The $\alpha_v\beta_3$ integrin binds PECAM-1, vitronectin and fibronectin. Interactions between $\alpha_v\beta_3$ expressed on monocytes and endothelial PECAM-1 have been implicated in transendothelial migration of these cells¹⁵⁶ and this integrin has also been demonstrated to be involved in neutrophil migration¹⁵⁷.

4.2 β_2 Integrin Signalling in Human Neutrophils

The signalling events elicited upon engagement of β_2 integrins in human neutrophils have been extensively studied during the last decade. Most studies have been focused on the signals triggered by β_2 integrin-mediated adhesion to different ECM proteins or in response to Ab-mediated clustering of the integrins. The signalling events elicited by the activation of β_2 integrins are summarized in Figure 4.2.

β_2 integrins have no intrinsic enzymatic activity, but rely on the recruitment and activation of other proteins. One of the first events in the β_2 integrin signalling cascade is the activation of **non-receptor tyrosine kinases**. So far, three families of non-receptor tyrosine kinases have been implicated in β_2 integrin signalling; the Src family tyrosine kinases, Syk, and possibly also the FAK family kinases. Three Src kinases have been identified in neutrophils; Fgr, Hck, and Lyn. Mice lacking one of the Src kinases have normal neutrophil functions, whereas leukocytes from the double (*hck^{-/-}fgr^{-/-}*) and triple (*hck^{-/-}fgr^{-/-}lyn^{-/-}*) knock-out mice display impaired migration and reduced inflammatory response¹⁵⁸⁻¹⁶², indicating the importance of these kinases in neutrophil function and “overlapping” functions between the kinases. Another tyrosine kinase, Syk, is normally activated by Src kinase-mediated phosphorylation and has

been shown to form multiprotein complexes with the integrin itself and Src kinases¹⁶³. In neutrophils, spreading, respiratory burst, and degranulation have been shown to be dependent on Syk^{164, 165}. The activation of the FAK family kinases in neutrophils is dependent on Src and Syk, but the importance of these tyrosine kinases in neutrophil function remain largely unknown. One possibility is that FAK acts as a docking protein recruiting other proteins to a signalling complex¹⁶⁶.

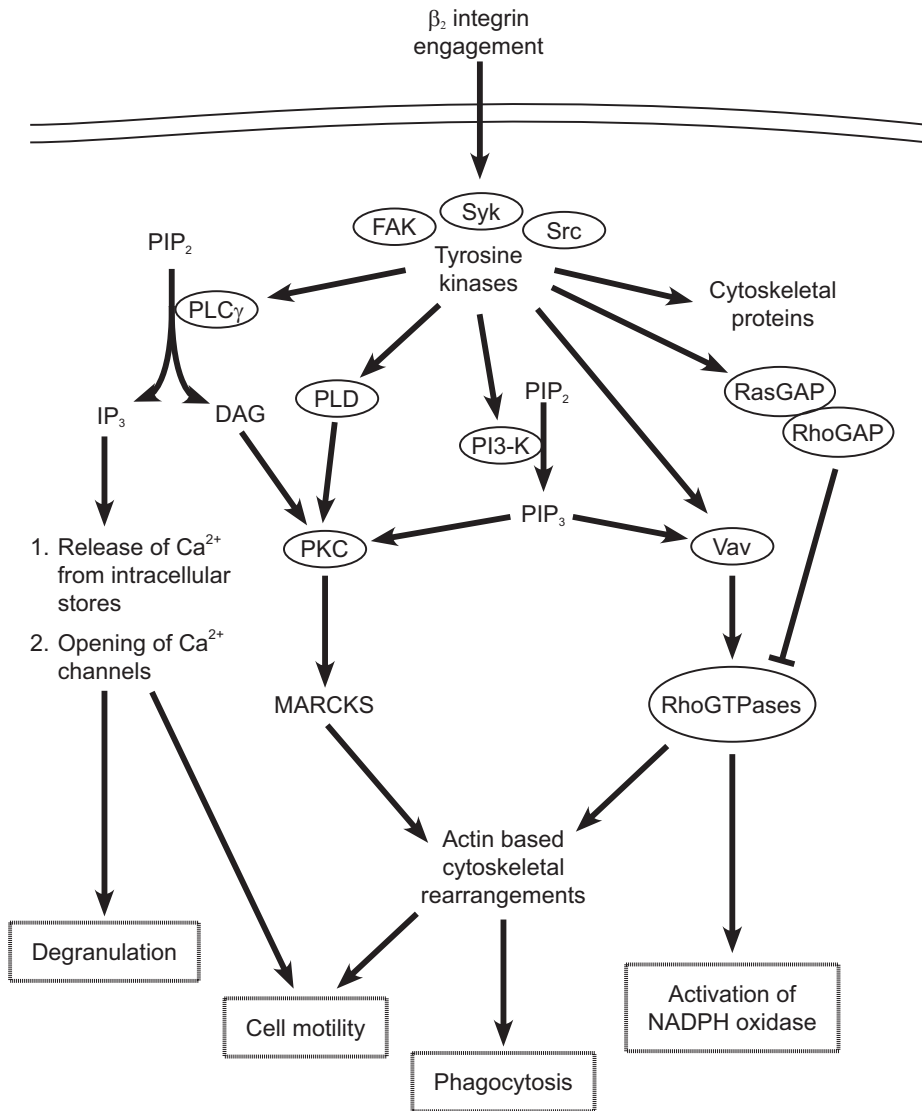


Figure 4.2 A model for β_2 integrin signalling. For a review see references [166] and [167].

A number of proteins are tyrosine phosphorylated upon β_2 integrin engagement, e.g. tyrosine kinases, cytoskeletal proteins, regulators of the small RhoGTPases, and the integrin subunits themselves. Functions such as cytoskeletal rearrangement, motility, and survival, and the production of reactive oxygen intermediates have been demonstrated to depend on the integrin-dependent activation of tyrosine kinases¹⁶⁷.

Phosphoinositide 3-kinase (PI3-K) is one of the proteins activated downstream of tyrosine kinases. PI3-K is a lipid kinase phosphorylating phosphoinositides at the 3' position of the inositol ring. Upon β_2 integrin engagement in neutrophils, PI3-K activation has been demonstrated to occur possibly through binding to Src tyrosine kinases¹⁶⁸. In addition, PI3-K has also been shown to be activated, by the engagement of β_2 integrins or other receptors, either through association with the docking protein FAK (mouse fibroblasts)¹⁶⁹, or through interactions with the small GTPase Ras^{168, 170, 171}. Activation of PI3-K is associated with its translocation to the plasma membrane where it mainly catalyzes the phosphorylation of phosphatidylinositol-(4,5)-bisphosphate (PIP₂) resulting in phosphatidylinositol-(3,4,5)-trisphosphate (PIP₃). PIP₃ recruits and activates signalling proteins containing PH (pleckstrin homology) domains. Signalling molecules activated by PI3-K and their downstream targets are involved in different cellular responses, including cytoskeletal rearrangements¹⁷².

Phospholipase Cy2 (PLC γ 2) can also be activated by tyrosine phosphorylation in β_2 integrin signalling¹⁷³. This protein catalyzes the breakdown of PIP₂ to diacylglycerol (DAG) and inositol-(1,4,5)-triphosphate (IP₃). IP₃ stimulates the release of Ca²⁺ from intracellular stores¹⁷⁴, which, in turn, leads to actin cytoskeleton rearrangements and the opening of Ca²⁺ channels in the plasma membrane with the subsequent influx of Ca²⁺ from the extracellular environment. The early localized increase of Ca²⁺ is considered to be important for tight adhesion and spreading, while the second wave of Ca²⁺ may be involved in cell movement¹⁶⁷.

Protein kinase C (PKC) is a large family of serine/threonine kinases which can be divided into three classes based on their structure and the way they are activated; the classical PKCs are Ca²⁺- and DAG-dependent; the novel PKCs require DAG for activation, but not Ca²⁺; and the atypical PKCs are independent of both Ca²⁺ and DAG¹⁷⁵. PKCs from all three subclasses are found in neutrophils and PKC and its downstream targets are involved in several cell functions, e.g. activation of the NADPH oxidase⁹², phagocytosis¹⁷⁶, and the regulation of the cytoskeleton^{176, 177} and its association to the plasma membrane. Some studies put phospholipase D (PLD) downstream of PKC activation¹⁷⁸, whereas the signalling events following clustering of β_2 integrins involve first the activation of PLD and thereafter the activation of PKC¹⁷⁶. This signalling sequence has also been shown to be important for complement-mediated phagocytosis¹⁷⁹. Activated PLD mediates the generation of diglyceride¹⁷⁶, and PLD-activity has been shown to coincide with the generation of reactive oxygen species and lactoferrin secretion¹⁸⁰.

4.2.1 The Rho Family of small GTPases

The Rho family of small GTPases (RhoGTPases) comprises more than 20 different proteins. They are approximately 25 kDa and can associate with the membrane through interaction with their C-terminal end. In neutrophils, three different members have been found to play

important roles; Rho, Rac (Rac 1 and Rac2, of which Rac2 comprises >95% of the total Rac¹⁸¹), and Cdc42. Rho is involved in the formation of stress fibres¹⁸², Rac induces the formation of membrane ruffles¹⁸³, and Cdc42 is implicated in the formation of filopodia¹⁸⁴. In addition, Rac2 is a component of the NADPH oxidase⁹³ and has been found to be important for degranulation⁹¹. The RhoGTPases have also been shown to be involved in different cellular processes, such as transcription, cell-cycle progression, and cell adhesion¹⁸⁵.

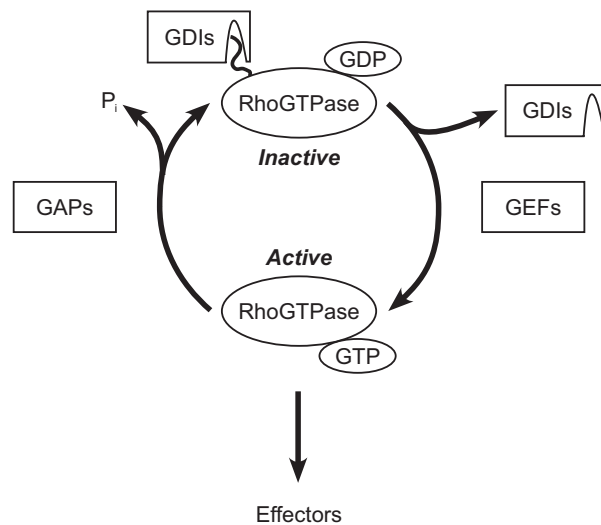


Figure 4.3 Regulation of the activity of the small Rho GTPases.

The Rho GTPases cycle between a GTP-bound (active) state and a GDP-bound (inactive) state. In their GTP-bound state, they can bind to downstream effector proteins. The activation of RhoGTPases is controlled by guanine nucleotide exchange factors (GEFs) and the deactivation is regulated by GTPase activating proteins (GAPs) (Figure 4.3). GEFs facilitate the exchange of GDP to GTP. Vav, a GEF for the RhoGTPases¹⁸⁶, is activated during β_2 integrin signalling, either through direct tyrosine phosphorylation¹⁸⁷ or downstream PI3-K¹⁸⁸. In addition, Rho GTPases have been shown to be activated by the direct interaction with PIP₂¹⁸⁹ or PIP₃¹⁹⁰. Since RhoGTPases possess a low intrinsic GTPase activity (hydrolysis of GTP to GDP), GAPs are needed to accelerate the hydrolysis of GTP. The best characterized GAP for Rho is p190RhoGAP and this protein is activated in a Src kinase-dependent manner¹⁹¹, which leads to the deactivation of RhoA after β_2 integrin ligation in human neutrophils. Alternatively, the GAP activity of p190RhoGAP may be regulated through β_2 integrin-dependent tyrosine phosphorylation of p190RhoGAP-interacting proteins, e.g. p120RasGAP¹⁶⁶. In addition to GEFs and GAPs, the RhoGTPases are regulated by guanine nucleotide dissociation inhibitors (GDIs). These proteins interact with the lipid-modified C-terminal of the GDP-bound form of the GTPase¹⁹², thereby controlling the cellular location of the GTPase. When the GTPase dissociates from the GDI, it is able to translocate from the cytosol to the membrane.

Studies of phagocytosis by macrophages/cell lines have lead to the general conclusion that phagocytosis mediated by FcRs is dependent on the activation of Cdc42 and Rac2, whereas complement-mediated phagocytosis induces the activation of RhoA¹²¹. However, β_2 integrin engagement in human neutrophils has been shown to activate both RhoA¹⁹¹ and Cdc42, but not Rac¹⁹³. In addition, complement-mediated phagocytosis of pansorbins by human neutrophils has been shown to activate both Rac and Cdc42¹²². Possible reasons for the discrepancies between these studies could be the cell type studied (mouse macrophage cell line or human neutrophils) and/or the size differences of the phagocytosed particles (red blood cells or pansorbins).

Downstream targets of the RhoGTPases include different proteins regulating the cytoskeleton. Rho kinase (ROK) is activated by Rho and has been demonstrated to be involved in the formation of focal adhesions and stress fibres in fibroblasts^{194,195}. ROK phosphorylates myosin light chain (MLC)¹⁹⁶ and inhibits MLC phosphatase¹⁹⁷. Together, these two events lead to an increase in MLC phosphorylation which promotes the assembly of actomyosin filaments and cell contractility. LIM kinase (LIMK) is another protein that is phosphorylated by ROK¹⁹⁸. Activation of LIMK leads to the stabilization of actin filaments. Together with increased actin polymerization, MLC phosphorylation and LIMK activation lead to the formation of stress-fibres¹⁸⁵.

GTP-bound Cdc42 activates Wiscott-Aldrich syndrome protein (WASP)¹⁹⁹ and related proteins. These proteins interact with the ARP2/3 complex, which stimulates actin polymerization by increasing the number of nucleation sites, leading to increased cellular content of actin filaments²⁰⁰⁻²⁰². Both Rac and Cdc42 regulate the activity of p21-activated kinases (PAKs)¹⁸⁵. The GTPases bind to the Cdc42/Rac interacting binding (CRIB) domain in PAK²⁰³. PAK activity has been shown to be involved in cytoskeleton dynamics²⁰⁴ and to be required for directed cell motility²⁰⁵. Furthermore, in human neutrophils stimulated with chemoattractants or opsonized particles, PAK has been localized to regions of actin polymerization in membrane ruffles, lamellipodia, pseudopodia, and phagocytic cups²⁰⁶. PAK phosphorylates and inactivates MLC kinase²⁰⁷ which leads to decreased actomyosin contraction. In addition, PAK also phosphorylates and activates LIMK²⁰⁸ which induces actin filament stabilization. Together with increased actin polymerization, these two events lead to the formation of lamellipodia and membrane ruffles¹⁸⁵.

Present Investigation

It is well established that the expression of M protein confers *S. pyogenes* with the ability to resist killing when incubated in human blood²⁰⁹ and it has been proposed that the binding of different plasma proteins is important. However, the precise molecular mechanisms are unknown. In blood, the neutrophils are the main cells responsible for the killing of invading microorganisms. These cells possess an array of receptors important for different stages of elimination of invading microorganisms, e.g. binding, ingestion, and killing. One such family of receptors is the integrin family, which is involved in binding to ECM proteins, chemotaxis, binding of complement-opsonized microorganisms, and phagocytosis. The aim of this study was to further investigate the mechanisms explaining the ability of the M protein to mediate resistance to killing by human blood, to study the molecular interactions between human neutrophils and *S. pyogenes*, and to investigate the signals evoked in human neutrophils upon contact with *S. pyogenes*.

Streptococcal M5 protein prevents neutrophil phagocytosis by interfering with CD11b/CD18 receptor-mediated association and signalling (Paper I)

To investigate the mechanisms by which *S. pyogenes* avoids being killed in whole blood, we incubated a wild type strain expressing the M5 protein (M5 bacteria) or a deletion mutant not expressing the M5 protein (Δ M5 bacteria) with human neutrophils in the presence of plasma from non-immune individuals. After different periods of time, we counted the bacteria in the mixtures and compared with the amount of bacteria present at the beginning of the experiment. As expected, the wild-type strain avoided being killed, whereas the number of the deletion mutant had started to decrease already within the first hour of incubation.

To investigate why more Δ M5 than M5 bacteria were killed by the neutrophils, we incubated human neutrophils with bacteria in the presence of plasma from non-immune individuals. After 3 h, the samples were centrifuged onto glass slides, fixed, and to be able to distinguish between extra- and intra-cellular bacteria a double-staining technique was used. The samples were examined by fluorescence microscopy. We found that of neutrophils incubated with Δ M5 bacteria, more than 20% were associated with bacteria, whereas only 7% of the neutrophils that had been incubated with the wild type strain were associated with bacteria, despite the presence of at least 300 times more bacteria in the M5 samples. Moreover, four times more

Δ M5 than M5 bacteria had become associated with the neutrophils, demonstrating that M5 protein expression limits the adhesion to human neutrophils. However, the proportion of ingested bacteria relative to the number of bacteria associated with the neutrophils did not differ between the two strains. Thus, of the bacteria that are bound to the neutrophils, the same proportion is ingested, regardless of M protein expression.

Neutrophil adhesion is largely mediated by complement receptors (CRs). To investigate the relationship between CR activation and the killing of Δ M5 bacteria, we studied the effect of two different complement inhibitors on the killing of Δ M5 bacteria. By impairing complement activation with either of the two complement inhibitors, the killing of Δ M5 was inhibited, indicating a role for complement in the elimination of Δ M5 bacteria. The adhesion of Δ M5 to neutrophils was inhibited by the addition of anti-CD11b Abs. Furthermore, the killing of Δ M5 was also blocked by the addition of blocking Abs directed against CD11b. Together, these results clearly indicate a role for complement activation and CD11b/CD18 (CR3) in the adhesion and the subsequent killing of *S. pyogenes*.

Engagement of CD11b/CD18 on human neutrophils is known to trigger the activation of tyrosine kinases¹⁶⁶ and the subsequent tyrosine phosphorylation of various proteins, including proteins that might be involved in phagocytosis. We found an increase in tyrosine phosphorylation in whole cell lysates of neutrophils that had been incubated with Δ M5 compared to neutrophils incubated with wild type bacteria. The increase in tyrosine phosphorylation of neutrophil proteins could be inhibited by the addition of genistein, a broad tyrosine kinase inhibitor, or by complement impairment. In addition, the killing of Δ M5 by neutrophils was inhibited by the addition of genistein. Together, these findings support the role of CR-mediated tyrosine kinase activation in the killing of Δ M5 bacteria by human neutrophils.

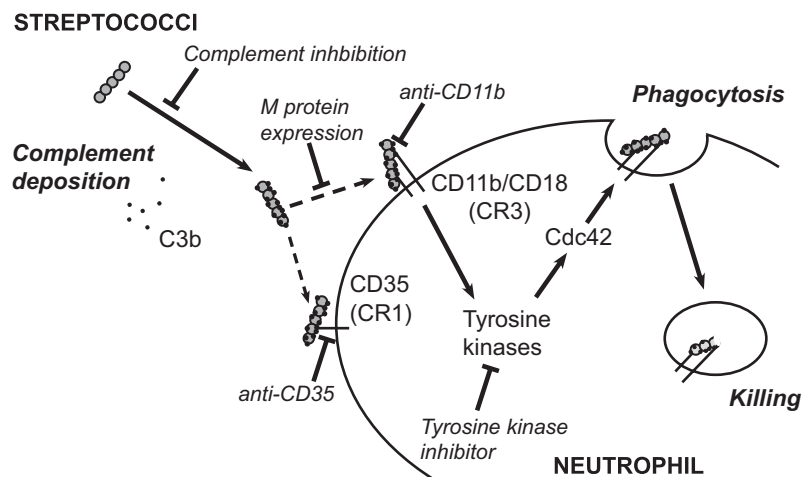


Figure 5.1 A schematic drawing illustrating the key events in the complement-mediated adhesion and phagocytosis of Δ M5 bacteria by human neutrophils. (Modified from Paper I, *J Immunol* (2004) 172: 3798-807)

Phagocytosis is accompanied by the activation of Rho GTPases¹²¹ and the activation of these GTPases has been shown to be dependent on tyrosine kinases²¹⁰. We found that CR3-mediated killing of Δ M5 bacteria triggers the activation of Cdc42, but not Rac2, and that this activation was dependent on tyrosine phosphorylation. In addition, the activation of Cdc42 could be inhibited by impaired complement activation or by the addition of anti-CD11b Abs. The results presented in Paper I are summarized in the model depicted in Figure 5.1.

Critical role for CD11b/CD18 but not for FcRs, in the killing of S. pyogenes by neutrophils in human immune serum (Paper II)

S. pyogenes is killed by human neutrophils in the presence of opsonizing serotype-specific Abs. To elucidate the mechanisms by which *S. pyogenes* is killed in the presence of immune serum, we identified three donors in whose blood specific *S. pyogenes* strains were unable to grow. We found that the component in serum responsible for the killing of the bacteria was IgG directed against the variable N-terminal part of the M protein.

Neutrophils express several receptors known to mediate the killing of opsonized microorganisms. To determine the receptor(s) expressed on neutrophils mediating the killing of *S. pyogenes* in the presence of type-specific Abs, we added function-blocking Abs directed against different FcRs and CRs to a mixture of neutrophils, serum from immune individuals, and bacteria. Only Abs directed against CD11b, but not Abs directed against FcRs or other CRs, blocked the killing of the bacteria. Killing of the bacteria in the presence of immune serum was also inhibited by the addition of either of two complement inhibitors. Furthermore, more C3 and C4 fragments were deposited on bacteria incubated in immune serum as compared to bacteria that had been incubated with serum from non-immune individuals. Together, these findings emphasize the role of complement in the killing of *S. pyogenes* in the presence of immune serum and suggest that the primary role of the M protein-specific Abs is to activate complement.

To further investigate the role of complement activation, sera from immune individuals were depleted from C1q and Factor D. When depleted serum was used in the survival experiments, the bacteria grew. The killing of the bacteria was restored when adding Factor D, but not C1q, to the mixture, strongly indicating a role for the alternative pathway of complement activation.

Once ingested, the phagocytosed microorganisms are killed by the combined action of oxidative and non-oxidative mechanisms¹³⁶. It is generally believed that phagocytosis mediated by FcRs, but not by CR3, leads to oxidative burst. However, we show that, in serum from immune individuals, CR3-mediated killing of *S. pyogenes* is accompanied by induction of the respiratory burst. Inhibition of complement activation and Abs directed against CD11b, but not against CD32 (Fc γ RIIa), reduced the activation of the respiratory burst.

In accordance with the results in Paper I, CR3-mediated killing of *S. pyogenes* in the presence of serum from immune individuals was accompanied by increased levels of activated Cdc42 and this activation was inhibited by impaired complement activation.

In summary, M protein-specific Abs activate the complement cascade resulting in complement-deposition on the surface of *S. pyogenes* and the subsequent CR3-mediated binding and killing. The activation of CR3 is accompanied by activation of the small GTPase Cdc42 and induction of the respiratory burst.

A role for the fibrinogen-binding regions of streptococcal M proteins in phagocytosis resistance (Paper III)

All virulent strains of *S. pyogenes* bind fibrinogen (Fg), but the role of Fg-binding in the pathogenesis of this bacterium is not known. To study the molecular requirements and biological consequences of Fg-binding, we studied a *S. pyogenes* strain expressing the M1 protein and the strain M5 Manfredo (also used in Paper I). Both of these M proteins bind Fg. However, we found that these two M proteins bind to different regions of Fg, since one protein failed to block the Fg-binding of the other. To determine which part of the M protein is responsible for Fg-binding, different deletion mutants of the M5 protein lacking different regions of the protein were constructed. The proteins were expressed in *Escherichia coli* and tested for Fg-binding. The protein lacking all four B repeats (M5 Δ B) failed to bind Fg in several test systems. In addition, the Δ M5 strain expressing M5 Δ B on its surface did not bind Fg, demonstrating that the B repeats of M5 are required for Fg-binding.

The M1 protein has two B repeats which show little similarity to the B repeats in the M5 protein. However, the B repeats in M1 are located in the same area of the protein as the B repeats in M5. Based on their size, repetitive structure, and location in the protein we hypothesized that the B repeats of M1 are responsible for Fg-binding. To further investigate the Fg-binding properties of the B repeats of the M1 and M5 proteins, the IgA-binding region of Emm4, a streptococcal protein that does not bind Fg, was replaced with the B repeats from the two M proteins, resulting in the two chimeric proteins Emm4/M5B1-4 and Emm4/M1B1-2. The two chimeric proteins both bound Fg and Δ M5 streptococci expressing either of the two proteins also bound Fg.

Finally, M5, M5 Δ B, Emm4, Emm4/M1B1-2, and Emm4/M5B1-4 were expressed on the surface of the Δ M5 strain. The resulting strains were incubated for 3 h in human whole blood and tested for survival. The strain expressing the M5 Δ B protein showed reduced survival as compared to the strain expressing the entire M5 protein. Bacteria expressing the Emm4 protein were readily killed. In contrast, strains expressing proteins harbouring Fg-binding B repeats, either originating from the M1 (Emm4/M1B1-2) or the M5 (Emm4/M5B1-4) proteins, resisted killing. Taken together, these results clearly demonstrate the importance of Fg-binding for the ability of the M protein to confer *S. pyogenes* with resistance to killing in whole blood.

To summarize, we localized the Fg-binding of the M1 and M5 proteins to the B repeats and demonstrated that the binding of this plasma protein is important for the resistance to killing in human blood.

Adhesion of Streptococcus pyogenes to phagocytic cells is achieved by two distinct mechanisms that are both blocked by M protein expression (Paper IV)

Most studies of *S. pyogenes* – neutrophil interactions have been done under conditions reflecting bacteremia, i.e. at a low bacteria to neutrophil ratio (multiplicity of infection, MOI). Under these conditions, as demonstrated in Papers I and II, the deposition of complement components and binding to CD11b/CD18 on the neutrophils mediate adhesion and subsequent ingestion and killing. However, at the initial stages of infection, occurring at skin or mucosal surfaces, the bacteria to neutrophil ratio is much higher. In Paper IV, we investigated the molecular interactions between *S. pyogenes* and phagocytes in different model systems reflecting a low and high MOI, respectively, and the role of M protein expression in these situations.

In the model reflecting the conditions in bacteremia (low MOI) the adhesion of $\Delta M5$ to neutrophils was inhibited by the addition of anti-CD11b Abs, but not by Abs directed against β_1 and β_3 integrins, clearly establishing the role of complement deposition and CD11b/CD18 (CR3) in the adhesion of *S. pyogenes* to human neutrophils.

To study the interactions at a higher MOI, we measured aggregation of phagocytes induced by *S. pyogenes* in the presence of human plasma. In contrast to bacteria expressing M protein, streptococci not expressing M protein induced aggregation of the phagocytes. Inhibition of complement activation did not affect the aggregation of the phagocytes, excluding a role for complement in the interactions between *S. pyogenes* and phagocytes at high MOI. By analysing plasma proteins that had bound to $\Delta M5$ bacteria, we identified fibronectin (Fn) as a major protein deposited on streptococci not expressing M protein. A fragment of Fn known to account for the binding of Fn to *S. pyogenes* inhibited the aggregation induced by streptococci not expressing M protein. In addition, these bacteria were able to induce aggregation of the phagocytes in the absence of plasma when Fn was added to the mixture. To further confirm the role of Fn for aggregation, we used plasma from mice carrying an inducible tissue-specific deletion of the Fn gene. In the presence of Fn-deficient plasma, streptococci not expressing M protein failed to induce aggregation of the phagocytes.

Phagocytes express different integrins known to bind Fn. To determine the integrins that are involved in the Fn-mediated adhesion to streptococci, Abs directed against different integrins and different peptides mimicking integrin-binding sites in Fn were added to the mixtures of phagocytes, streptococci not expressing M protein, and plasma. Aggregation of the phagocytes was inhibited by peptides mimicking sequences in Fn that account for binding to $\alpha_5\beta_1$ and $\alpha_v\beta_3$. In contrast, a control peptide or a peptide mimicking the $\alpha_4\beta_1$ integrin binding site in Fn was not able to inhibit aggregation. In addition, neither Abs directed against α_M (CD11b) or α_4 inhibited the aggregation. However, Abs directed against α_5 , β_1 , and β_3 inhibited the aggregation, further supporting a role for these integrins in the interaction with *S. pyogenes* at high MOI.

In another model mimicking conditions relevant for the initial recognition of bacteria by phagocytes at mucosal or skin surfaces, streptococci were immobilized on a cover slip and subjected to a continuous flow of phagocytes. The cells adhered more efficiently to streptococci not expressing M protein than to wild type bacteria. Further, the adhesion was inhibited by a peptide mimicking the sequences in Fn that account for binding to $\alpha_5\beta_1$ and $\alpha_v\beta_3$ and by Abs directed against β_1 , and β_3 , but not by anti- α_M (CD11b) Abs.

In conclusion, at low MOI, resembling the situation in bacteremia, the interaction between *S. pyogenes* and phagocytes depends on the binding of complement components deposited on the bacterial surface and CD11b/CD18 (CR3) on the neutrophil surface. In contrast, at high MOI, resembling the situation on skin and mucosal surfaces, Fn bound to the surface of *S. pyogenes* interacts with $\alpha_5\beta_1$ and $\alpha_v\beta_3$ expressed by the phagocytes.

Conclusions

- In the presence of plasma from non-immune individuals, *S. pyogenes* avoids killing by human neutrophils by M protein-mediated inhibition of adhesion to the neutrophils
- Phagocytosis of *S. pyogenes* in the presence of plasma from non-immune individuals is mediated by CD11b/CD18 and is accompanied by activation of tyrosine kinases and the small GTPase Cdc42
- In the presence of serum from immune individuals, the killing of *S. pyogenes* is mediated through activation of the complement system by M protein-specific Abs and activation of CD11b/CD18 and Cdc42 followed by phagocytosis
- The B repeats of the M1 and M5 proteins are responsible for the Fg-binding to M1 and M5 streptococci and the Fg-binding B repeats of M1 and M5 are important for *S. pyogenes* in mediating resistance to killing in human blood
- At low MOI, resembling the situation in bacteremia, the interaction between *S. pyogenes* and phagocytes depends on the binding of complement components deposited on the bacterial surface and CD11b/CD18 (CR3) on the neutrophil surface
- At high MOI, resembling the situation on skin and mucosal surfaces, Fn bound to the surface of *S. pyogenes* interacts with $\alpha_3\beta_1$ and $\alpha_v\beta_3$ expressed by the phagocytes

Populärvetenskaplig Sammanfattning

Streptococcus pyogenes (Grupp A streptokocker) är en bakterie som oftast orsakar vanliga okomplicerade infektioner hos människan, t ex halsfluss, scharlakansfeber och svinkoppor. Denna bakterie kan dock även ge upphov till allvarliga och livshotande tillstånd där patienten kan vara död inom loppet av några få dygn – *S. pyogenes* kallas därför ofta för ”mördarbakterie” i kvällspressen. Varje år drabbas ungefär 600 000 människor världen över av sådana allvarliga infektioner och av dessa dör ca 30%. Efter obehandlade infektioner orsakade av *S. pyogenes* drabbas vissa patienter av akut reumatisk feber (ARF), som angriper bl a hjärtat och lederna. ARF är en viktig orsak till hjärt-kärlsjukdomar i utvecklingsländerna och orsakar 230 000 dödsfall årligen. Sammanlagt är runt 15 miljoner människor drabbade av ARF. WHO uppskattar att omkring 500 000 människor dör varje år till följd av allvarliga infektioner orsakade av *S. pyogenes*.

För att kunna infektera människan är *S. pyogenes* utrustade med diverse s k *virulensfaktorer*. Dessa virulensfaktorer är olika proteiner som ansvarar för vidhäftning till mänskliga celler, underlättar för bakterien att tränga sig djupare in i vävnaden eller förhindrar människans immunsystem från att döda bakterien. En sådan viktig virulensfaktor är det s k M-proteinet, som *S. pyogenes* har på sin yta. M-proteinet bildar en spiral och två spiraler snurrar sig runt varandra (se Figur 1.1). Det finns fler än 100 olika typer av M-protein. En bakterie har bara en typ av M-proteiner på sin yta. När vi fått en infektion bildar kroppen *antikroppar* mot den infekterande bakteriens M-protein. Dessa antikroppar skyddar oss mot att bli sjuka av samma bakterie en gång till. Detta förklarar varför vi kan få halsfluss flera gånger under livet – det finns ju fler än 100 olika M-proteiner! M-proteinet har visat sig vara viktigt för flera olika steg i infektionsprocessen; ansvara för vidhäftning till mänskliga celler, möjliggöra för bakterien att ta sig djupare in i vävnaden och skydda mot människans försvar. En viktig egenskap hos M-proteinet är dess förmåga att skydda *S. pyogenes* mot effektiv avdödning genom att hindra vita blodkroppar från att äta upp (*fagocytera*) bakterien det sitter på. M-proteinet binder olika proteiner som finns i blodplasma, t ex proteiner som reglerar komplementsystemet (se nedan) och fibrinogen.

Komplementsystemet tillhör vårt medfödda försvar mot inkräktande mikroorganismer. Detta system består av en kaskad av flera olika proteiner (se Figur 2.1) och kan aktiveras direkt på en främmande yta eller av antikroppar som fastnat på t ex en bakterie. Komplementsystemet har i princip två viktiga funktioner; punktera främmande celler så att de dör och att märka

ut främmande celler så att de känns igen av strukturer (*komplementreceptorer*) på vita blodkroppar så att dessa celler kan fagocytera och döda inkräktarna.

De vita blodkropparna (*neutrofilerna*) finns i blodet och är viktiga celler när det gäller att försvara oss mot infekterande mikroorganismer. Dessa celler tillverkas i benmärgen med en förunderlig hastighet av 100×10^9 per dag (totalt så mycket som 1.5 ton neutrofiler under ett helt liv!). De tar ungefär 10 dagar för att mogna i benmärgen och lever i blodet endast några timmar. Efter 2-3 dagar är hela gruppen neutrofiler utbytt. Vid en infektion kallas neutrofilerna dit (se Figur 3.1) och vandrar från blodet ut i vävnaden för att känna igen, fagocytera och döda de invaderande bakterierna. I vävnaden kan neutrofilerna leva ett par dagar. För att känna igen, binda och fagocytera bakterierna har neutrofilen olika strukturer, *receptorer*, på sin yta. Dessa receptorer fäster till antikroppar eller komplementkomponenter som fastnat på bakterieytan. Neutrofilerna är packade med små blåsor som innehåller ämnen som är giftiga för bakterier och när de har fagocyterat bakterierna tömmer de ut dessa ämnen i blåsan med bakterier. Tillsammans med mycket giftiga syreradikaler som också bildas vid denna process bidrar dessa ämnen till att bakterierna oskadliggörs och dör.

En viktig grupp av receptorer på neutrofiler är *integrinerna*. Dessa består av två olika kedjor, en α -kedja och en β -kedja. Integriner har visat sig vara viktiga för neutrofilen då den tar sig från blodet ut i vävnaden, för rörelse genom vävnaden och för igenkänning och fagocytos av invaderande mikroorganismer. Integriner binder till olika strukturer i kärlväggen och i vävnaden och till komplementkomponenter på t ex bakterier. En viktig grupp integriner på neutrofiler är de sk β_2 -integrinerna. Dessa integriner är viktiga för att neutrofilen ska kunna ta sig ut ur blodet och fram till bakterierna i vävnaden. Dessutom är en av β_2 -integrinerna, $\alpha_M\beta_2$ (även kallad komplementreceptor 3, *CR3*) en av de viktigaste receptorena för komplementkomponenter och kan även "säga till" (*signalera*) till neutrofilen att den ska fagocytera bakterierna.

Målet med den här avhandlingen var att vidare studera de mekanismer som ligger till grund för M-proteinets förmåga att förse *S. pyogenes* med egenskapen att motstå avdödning i mänskligt blod, att studera de molekylära interaktionerna mellan neutrofiler och *S. pyogenes*, samt att studera signalering i neutrofiler som kommit i kontakt med denna bakterie.

I delarbete I studerade vi vilka mekanismer som ligger till grund för förmågan hos *S. pyogenes* att motstå avdödning av neutrofiler i närvaro av plasma från icke-immuna individer. Vi använde två olika stammar; en vildtyp-stam som uttrycker ett M-protein som kallas M5 och en mutant stam som inte uttrycker något M5-protein ($\Delta M5$). Vi fann att anledningen till att M5 stammen inte dödas var att M5-proteinet hindrade neutrofilerna att känna igen bakterien och fästa vid den. Genom att använda $\Delta M5$ som modell visar vi att neutrofilen huvudsakligen känner igen streptokocker och dödar dessa via CR3-medierad fagocytos. Vidare fann vi att aktivering av CR3 via interaktion med komplementkomponenter på ytan av *S. pyogenes* leder till aktivering av sk *tyrosinkinaser* och aktivering av det lilla RhoGTPaset Cdc42.

I delarbete II studerade vi hur antikroppar från immuna individer leder till avdödning av *S. pyogenes* genom neutrofiler. Vi fann att de antikroppar som ledde till avdödning var riktade mot den yttersta delen av M-proteinet och att dessa antikroppar ledde till aktivering av

komplementsystemet. Avdödning i närvaro av M-protein-specifika antikroppar skedde även i detta fall via aktivering av CR3 och Cdc42.

Alla sjukdomsframkallande stammar av *S. pyogenes* binder fibrinogen som finns i plasma, men vilken roll denna bindning spelar för infektionsprocessen är inte helt klarlagd. I delarbete III visar vi att M-proteiner binder fibrinogen till de delar av M-proteinet som kallas för B-domänerna. Vi fann dessutom att fibrinogen-bindningen till M-proteinet var viktig för bakteriens förmåga att motstå avdödning i mänskligt blod, eftersom bakterier med M-proteiner utan B-domäner dödades medan vildtypen överlevde.

De modellsystem som används i delarbete I - III liknar förhållanden som råder vid blodförgiftning, dvs i lösning och med få bakterier och många neutrofiler (ett lågt "multiplicity of infection", *MOI*). I de första stadierna av en infektion (som sker på hud och slemhinnor) är förhållandena dock de omvända, dvs många bakterier i förhållande till neutrofiler (ett högt *MOI*). I delarbete IV använde vi olika modellsystem för att studera interaktionerna mellan *S. pyogenes* och neutrofiler vid olika *MOI*. Vi fann att vid ett lågt *MOI* beror interaktionen mellan *S. pyogenes* och neutrofiler på bindningen av komplementkomponenter på bakterieytan till CR3 på neutrofilen. Vid ett högt *MOI* däremot, interagerar fibronektin som bundit till bakterieytan med två andra integriner, $\alpha_5\beta_1$ och $\alpha_v\beta_3$, på neutrofilerna.

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