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Analysis of the molecular interplay between Streptococcus pyogenes and its human host

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Analysis of the molecular interplay between *Streptococcus pyogenes* and its human host

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During S. pyogenes infections, human plasma leaks into the site of infection as a consequence of inflammation. This thesis shows that S. pyogenes rapidly alters its expression of extracellular and intracellular proteins in response to human plasma. In addition, the pathogen also expresses multiple variants of its important virulence factors, M1 protein and C5a peptidase, when exposed to plasma. The function of modified M1 protein and C5a peptidase is not yet elucidated but is suggested to have important implications for the pathogenicity of S. pyogenes.

Opsonizing IgG recognizes and mediates the elimination of bacteria during infection. Here, the identification and characterization of a novel IgG cleaving cysteine proteinase of S. pyogenes, denoted IdeS, is described. IdeS facilitates S. pyogenes evasion of Fc-mediated phagocytosis by specifically cleaving the hinge region of IgG1, IgG2, IgG3, and IgG4. Moreover, data suggesting that neutrophil proteinases release immunogenic epitopes from IdeS are presented. This is a novel mechanism by which S. pyogenes exploits the human immune system and prevents its virulence factors from being eliminated by opsonizing immunoglobulins.

Key words: Streptococcus pyogenes, M protein, C5a peptidase, IdeS, Immunoglobulin, Cysteine proteinase, Neutrophil, Elastase, Cathepsin G

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Cover image
A model of amino acids 49-339 from IdeS (Protein Data Bank 1Y08) generated by Mattias Collin using VMD 1.8.4b111 (Humphrey, W., Dalke, A. and Schulten, K., "VMD - Visual Molecular Dynamics", J. Molec. Graphics, 1996, vol. 14, pp. 33-38.) and Tachyon ray tracer (http://jedi.ks.uiuc.edu/~johns/raytracer/).

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List of Papers

This thesis is based on the following publications referred to in the text by their roman numerals (I-IV):

I. **Johansson, B. P**., Levander, F., von Pawel-Rammingen, U., Berggård, T., Björck, L., James, P. The protein expression of *Streptococcus pyogenes* is significantly influenced by human plasma. *J Proteome Res*. (2005) **4**:2302-11.

II. von Pawel-Rammingen, U., **Johansson, B. P**., Björck. L. IdeS, a novel streptococcal cysteine proteinase with unique specificity for immunoglobulin G. *EMBO J*. (2002) **21**:1607-15.

III. von Pawel-Rammingen, U., **Johansson, B. P**., Tapper, H., Björck, L. *Streptococcus pyogenes* and phagocytic killing. *Nat Med*. (2002) **8**:1044-5.

IV. **Johansson, B. P**., Moritz, L., Nilsson, R., von Pawel-Rammingen, U. Neutrophil serine proteinases remove immunogenic epitopes from the streptococcal IgG cleaving enzyme IdeS, without affecting the biological activity of the enzyme. *Manuscript*.

List of Abbreviations

aa	Amino acids
ARF	Acute rheumatic fever
CG	Cathepsin G
CRP	C-reactive protein
DSF-9	NH_2-terminal peptide of IdeS (DSFSANQEI)
EndoS	Endoglycosidase of Streptococci
GAS	Group A Streptococci
IdeS	Immunoglobulin degrading enzyme of *S. pyogenes*
$IdeS^{C94G}$	Enzymatically inactive IdeS
IL-8	Interleukin-8
Ig (A, D, E, G, M)	Immunoglobulin (A, D, E, G, M)
Igs	Immunoglobulins
kDa	Kilodalton
LC-MS	Liquid chromatography- mass spectrometry
LPS	Lipopolysaccharide
MBL	Mannan-binding lectin
MAC	Membrane attack complex
NE	Neutrophil elastase
PMN	Polymorphonuclear leukocytes or neutrophils
Pr3	Proteinase 3
PSGN	Post-streptococcal glomerulonephritis
RRs	“Stand alone” regulatory networks
Scl (A, B)	Streptococcal collagen-like protein (A or B)
Scp (A, B)	C5a peptidase of group A/B streptococci
SIC	Streptococcal inhibitor of complement
Sof	Serum opacity factor
SPA	Serum amyloid P component
Spe (A, B, C)	Streptococcal pyrogenic exotoxin (A, B, C)
TCSs	Two-component signal transduction systems

Abstract

The common human pathogen *Streptococcus pyogenes* is the causative agent of numerous mild and severe clinical conditions. It expresses a number of secreted or cell wall-anchored proteins that modulate the human immune system and facilitate colonization and spread of the pathogen in the human host.

During *S. pyogenes* infections, human plasma leaks into the site of infection as a consequence of inflammation. This thesis shows that *S. pyogenes* rapidly alters its expression of extracellular and intracellular proteins in response to human plasma. In addition, the pathogen also expresses multiple variants of its important virulence factors, M1 protein and C5a peptidase, when exposed to plasma. The function of modified M1 protein and C5a peptidase is not yet elucidated but is suggested to have important implications for the pathogenicity of *S. pyogenes*.

Opsonizing IgG recognizes and mediates the elimination of bacteria during infection. Here, the identification and characterization of a novel IgG cleaving cysteine proteinase of *S. pyogenes*, denoted IdeS, is described. IdeS facilitates *S. pyogenes* evasion of Fc-mediated phagocytosis by specifically cleaving the hinge region of IgG1, IgG2, IgG3, and IgG4. Moreover, data suggesting that neutrophil proteinases release immunogenic epitopes from IdeS are presented. This is a novel mechanism by which *S. pyogenes* exploits the human immune system and prevents its virulence factors from being eliminated by opsonizing immunoglobulins.

Introduction

Annually, more than 25% of all deaths world wide are caused by bacterial, viral, or parasitic infections (*143*). The Gram-positive bacterium *Streptococcus pyogenes* (Group A streptococci, GAS) is one of the major human pathogens that causes substantial morbidity and mortality on a global scale (*29*). The molecular interplay between *S. pyogenes* and its human host has been studied in detail during the last decades, and numerous streptococcal virulence factors have been identified and characterized. This thesis aims at describing some of the mechanisms by which *S. pyogenes* avoids detection and elimination by the human immune system, with special emphasis on streptococcal proteins that modulate immunoglobulin mediated phagocytosis. The present investigations section at the end of the thesis summarizes the original findings on which this thesis is based.

Host-bacteria relations

The human body is built up by at least 200 highly specialized eukaryotic cell types that cooperate and influence each other's activities (*7, 176, 200*). In contrast, bacteria are unicellular prokaryotic cells without any distinct organelles. They replicate mainly by binary fission, and can divide every 20 minutes under optimal conditions. The rapid replication rate in combination with their susceptibility to spontaneous mutations enables bacteria to quickly adapt to environmental changes.

Mucosal surfaces and epithelial membranes of the human body are constantly colonized by bacterial species, referred to as commensals or the normal flora (*7, 176, 200*). Commensal organisms normally do not cause infection in healthy individuals and can sometimes even be beneficial for the host by producing vitamins or lactic acid, as well as competitively preventing pathogens from colonizing the body surface. In contrast, the interior body is sterile and invading organisms are rapidly attacked and eradicated by the human immune system. However, bacteria that are not part of the normal flora are often equipped with an array of molecules (mainly proteins) that enable them to circumvent the antimicrobial activities of the immune system, and thus colonize epithelial membranes or disseminate into sterile tissue where they produce symptoms of disease. Such microbes are called pathogens and their strategies for evading the host immune system are numerous.

The definitions of virulence and pathogenicity have been debated to some extent (*32, 33*). This thesis defines pathogens as microorganisms that cause disease in healthy

human hosts. Therefore, commensals that normally do not infect humans, but cause disease under optimal conditions (e. g. in immuno-suppressed individuals) are not included by this definition. Moreover, virulence factors are here defined as extracellular molecules of a pathogen that directly interact with host molecules, and thereby facilitate survival and spread of the pathogen in the human host.

Streptococcus pyogenes

S. pyogenes is a major human pathogen characterized by growing in chain-like structures (See Fig. 1), and lysis of red blood cells when grown on blood agar plates (ß-hemolysis) (*54*). It is a Gram-positive bacterium with a thick peptidoglycan cell wall enveloping a single cell membrane. The pathogen is a common colonizer of skin and mucous membranes in the upper respiratory tract, where it causes relatively mild clinical conditions such as impetigo or pharyngitis respectively (*53*). However, invasive strains can penetrate into deeper tissues and cause severe and potentially life-threatening conditions such as necrotizing fasciitis (soft tissue destruction), streptococcal toxic shock syndrome (hypotension and multi-organ failure), sepsis, pneumonia and meningitis. These infections have rapid progressions, and can cause death within a couple of days if left untreated with penicillin.

In addition, post-infectious sequelae such as acute rheumatic fever (ARF) and post-streptococcal glomerulonephritis (PSGN) sometimes follow the acute clinical conditions caused by *S. pyogenes* (*54*). ARF is often developed after untreated pharyngitis and affects organs such as the heart valves, joints, brain and skin. An important streptococcal virulence factor, denoted M protein, has immunogenic epitopes resembling human myosin and tropomyosin, and is therefore believed to contribute to the development of ARF as a consequence of cross-reacting antibodies (*96, 203*).

In contrast to ARF, which exclusively follows streptococcal throat infections, PSGN can develop after both untreated skin infections as well as throat infections, and is characterized by an intense inflammation of the kidney (*54*). The etiology of PSGN is not clear and immune complex deposition and cross-reacting antibodies are examples of proposed mechanisms for PSGN development (*186, 190*). A recent study funded by the WHO demonstrated an annual prevalence of over 600 million cases of pharyngitis and 111 million cases of severe streptococcal skin infections world-wide (*29*). Perhaps even more concerning is that at least 517,000 deaths occur each year due to severe GAS diseases (e. g. acute rheumatic fever, post-streptococcal glomerulonephritis, and invasive

infections). These numbers emphasize that *S. pyogenes* is an important global cause of morbidity and mortality.

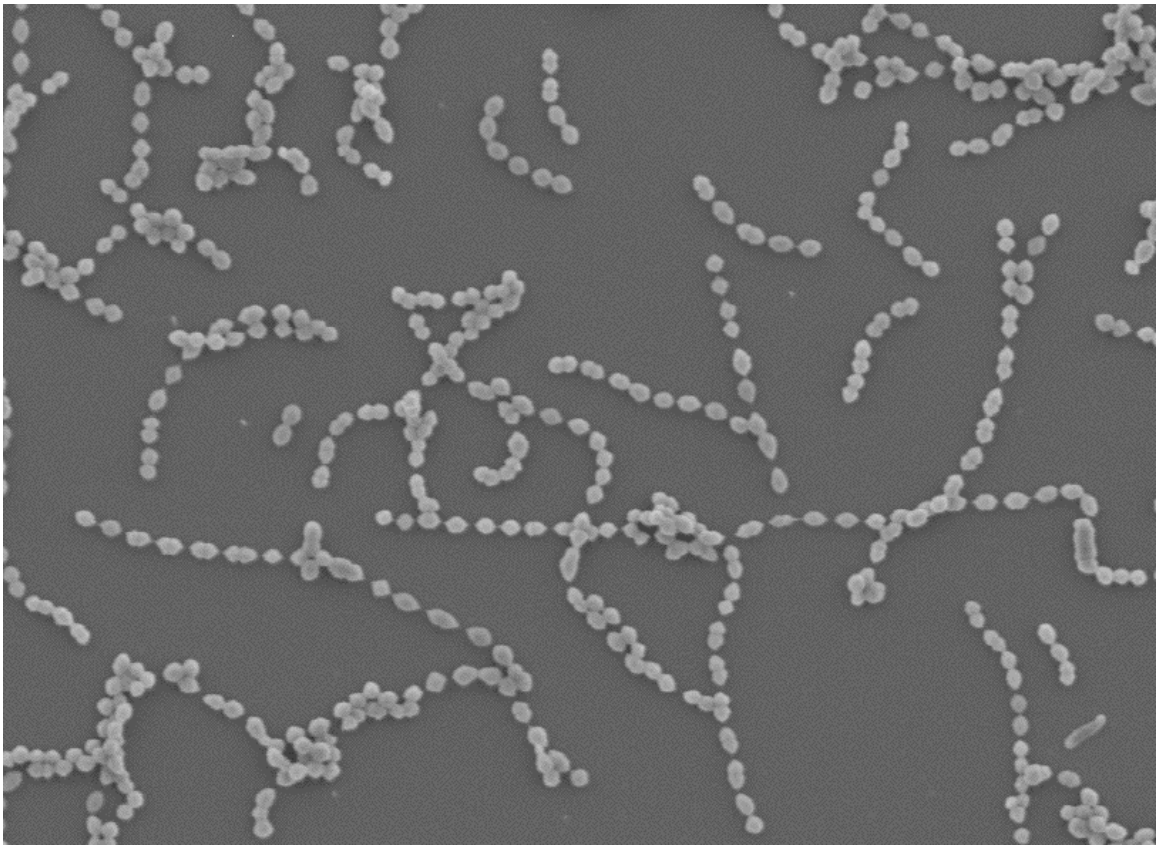

Figure 1. Electron micrograph of S. pyogenes growing in chains.

Classification of *S. pyogenes*

Streptococci are classified into groups based on a major carbohydrate in their cell-wall. *S. pyogenes* differs from other streptococcal species by possessing the group A carbohydrate in their cell wall, hence the name (Group A streptococci) (*119*). The expression of M- or T proteins on the bacterial surface further differentiates *S. pyogenes* strains into serotypes (*120*). Serotyping has been invaluable during the past 60 years for determining the relationship between different *S. pyogenes* strains and the varied clinical manifestations inflicted by these organisms. In brief, serotyping is based on a procedure where antisera raised against specific clinical isolates are absorbed with heterologous strains to remove antibodies that are not specific for the chosen strain. This procedure has allowed the production of a large number of M-type specific sera, and over 100 different serotypes have been described (*65*). In contrast to M proteins, T proteins are resistant against trypsin. By treating individual strains to trypsin prior to antibody absorption, approximately 25 distinct T-antigens have been described. Since production of M-type

precipitating antisera is very expensive and labor-intensive, serotyping by sequencing the hypervariable 5´ end of the M protein gene (*emm*) is becoming increasingly common (*64, 65*).

S. pyogenes genome

The sequencing of *S. pyogenes* genome has provided researchers with new insight into the evolution, metabolic activities, and virulent properties of the pathogen (*71*). To date, seven genomes from five different M-types have been successfully sequenced (two M1 strains (*70, 196*), two M3 strains (*14, 146*), one M6 strain (*9*), one M18 strain (*187*), and one M28 strain (*82*)). Moreover, an additional seven genomes are in progress (http://www.genomesonline.org). Comparative analyses have shown that the chromosomal sequences are well preserved between different *S. pyogenes* strains, and that the genomic variability observed is mainly due to single nucleotide polymorphisms and prophage DNA elements (*82*).

The human immune system

To prevent being colonized by pathogenic microorganisms, the human body is equipped with an armament of cells, proteins, peptides, and other molecules that recognize and kill everything that is identified as non-self (*173*). These antimicrobial components are referred to as the human immune system and can be divided into the innate and adaptive immune system. While the innate immune system relies on non-specific distinction of self and non-self, the adaptive immune system is highly specific for a particular pathogen and improves by prior exposure to the pathogen. However, it is important to realize that the separation of the immune system into two classes is not absolute, since components of each class often cooperate in eradicating microorganisms. For instance, phagocytosis of bacteria by neutrophils (innate) is greatly enhanced by opsonizing antibodies (adaptive).

Opsonization

There are two major phases of any immune response; recognition of the antigen and a reaction to eradicate it. Opsonization is a process where opsonins (e.g. C3b, C3bi and immunoglobulins (Igs)) label the antigen and make it more susceptible to phagocytosis by immune cells (*176*). Although phagocytes have some intrinsic ability to recognize and bind bacteria directly, the phagocytic process is greatly improved when opsonins have bound to the bacterial surface.

Immunoglobulins

The acquired immune system relies heavily on Igs to identify and mediate killing of infecting microbes (*173*). Igs are found in all body fluids and are synthesized in billions of forms, each with a different amino acid (aa) sequence that recognizes and binds to a unique antigen. The primary function of Igs is to bind antigens, and in a few cases this has a direct effect such as the neutralization of bacterial toxins (*6, 183*). However, most of the time Igs act as opsonins that are recognized by secondary "effector" constituents that eradicate the bacteria (*162*). Igs covering a surface are able to activate the classical pathway of complement, which makes the pathogen vulnerable to the membrane attack complex (MAC) or phagocytosis via complement receptors. In addition, opsonizing Igs also enhance phagocytosis as their Fc domain is recognized by Fc receptors located on the surface of phagocytic cells.

Upon antigen recognition, human B-cells generate an immunological response by producing five immunoglobulin isotypes (IgA, IgD, IgE, IgG, and IgM) (*173*). The different classes of Igs have slightly different functions and are found at different locations in the human body. IgA represents 15-20% of the human serum Ig pool but is also common on cutaneous surfaces and in mucous secretions such as saliva and milk. It defends the host against skin infections and recognizes respiratory pathogens. IgD and IgE are scarce in blood but are found at the surface of immune cells (*98*). IgE plays a role in parasite immunity and is commonly associated with allergic diseases such as asthma (*99*). IgM accounts for approximately 10% of the Ig pool and is often seen in the early immune response against bacteria and other microorganisms. IgG is the major Ig in normal human blood and accounts for 70-75% of the total Ig pool. It is also the major Ig of secondary immune responses and the exclusive antitoxin class.

IgG

IgG is the most abundant immunoglobulin isotype in blood and is produced in large quantities during the secondary immune response (*173*). The heavy chain is often referred to as γ and differs from the heavy chains of other immunoglobulin isotypes. For instance, the μ chain of IgM differ from γ chains in amino acid sequence and has an extra constant region domain in place of the IgG hinge. Moreover, within human IgG four subclasses (IgG1, IgG2, IgG3, and IgG4) have been identified with slightly different amino acid compositions in the hinge and Fc-region. The subclasses differ in antigen recognition and in their ability to activate the classical pathway of complement. While protein epitopes

are primarily recognized by IgG1, IgG3 and IgG4, carbohydrates are recognized by IgG1 and IgG2. With the exception of IgG4, IgGs also activate the classical pathway of complement (*8, 112*).

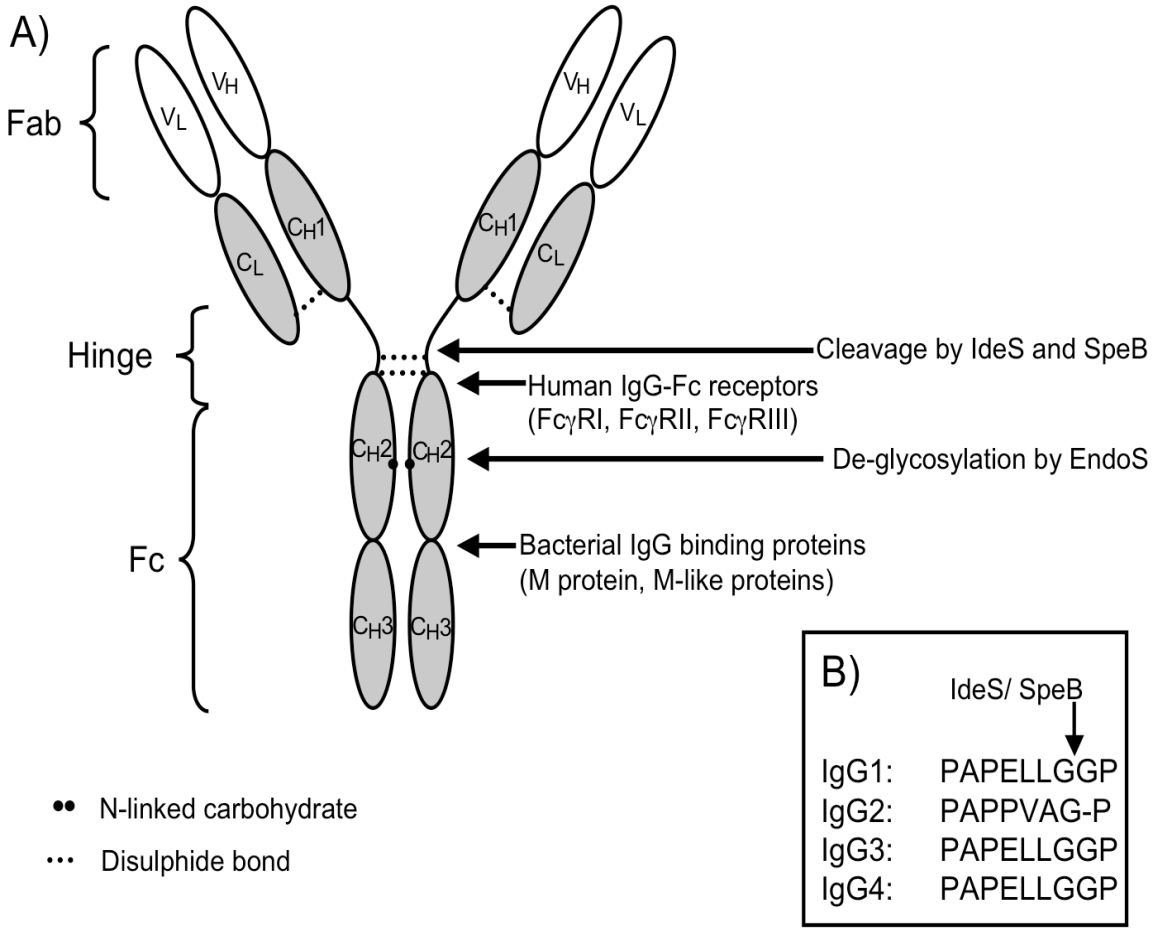

Figure 2. Structure of human IgG

A) *Sites that interact with human or bacterial proteins are indicated by arrows.* ***B)*** *The amino acid sequences of human IgG1, IgG2, IgG3 and IgG4 hinge regions. IgG2 has a slightly different amino acid sequence at the cleavage site of IdeS and SpeB, possibly explaining the reduced activity of IdeS against IgG2.*

Structure of IgG

The basic structure of an immunoglobulin molecule consists of two light chains, of about 220 amino acids, and two heavy chains of approximately 440 amino acids (See Fig. 2)(*177*). Both the heavy and light chains are organized into repeating globular segments called Ig domains. A light chain consists of one variable (V_L) and one constant domain (C_L), whereas a heavy chain contains one variable (V_H) and three constant domains (C_H).

It is the variable regions of the NH_2-terminal part of the light and heavy chains that come together to form the antigen-binding site (Fab). The remaining constant part of the IgG molecule (Fc) binds to Fcγ-receptors on phagocytic cells (which initiates phagocytosis), and activates complement (*162*). The Fab and Fc regions are connected through a flexible hinge region. Interestingly, the flexible structure of the hinge region makes it vulnerable to proteinase activities, which several pathogens take advantage of during infection. For instance, the two secreted cysteine proteinases of *S. pyogenes*, SpeB and IdeS, enhance streptococcal survival in human immune blood by cleaving the hinge region of opsonizing IgG (*48, 206*).

Complement

The complement system, which consists of approximately 30 serum or membrane-bound proteins, acts as an important member of the immune system through 1) opsonization and phagocytosis of invading organisms, 2) chemotaxis and activation of leukocytes, 3) direct lysis of microorganisms and cells, 4) clearance of immune complexes, and 5) induction of antibody responses (*121*). There are three separate routes by which the complement system can be activated, called the classical pathway, the mannan-binding lectin pathway, and the alternative pathway (see Fig. 3). The classical pathway is activated by the complement complex C1q that binds the Fc-domain of IgG or IgM that have bound to antigens (*112, 131*). The mannan-binding lectin pathway is activated by the serum protein mannan-binding lectin (MBL) that recognizes mannose-rich carbohydrate structures on the surface of bacterial species (*150*). The alternative pathway is activated by molecular structures on invading organisms or other foreign structures, independent of antibody deposition. Initiation of the alternative pathway is dependent on continuous deposition of C3b on surfaces, which in part may be achieved by low-level activation of the classical pathway.

Common for all three pathways is that after complement activation, different C3-convertases are formed (denoted C4b2a, C3bBb and iC3Bb). The C3-convertases catalyze the key reaction in the complement system, the conversion of C3 to C3b, which is deposited on microbial surfaces and is recognized as an opsonin by complement receptors 1 and 3 (CR1 and CR3). Complement also protects its host from microbial infections by forming membrane attack complexes (MAC), which are inserted into the membrane of Gram-negative bacteria and cause subsequent lysis of the microbes. In contrary, Gram-positive bacteria are resistant to MAC because of their thick

peptidoglycan cell-wall (*103*). A third mechanism by which complement eradicates microbes is by the generation of C3a and C5a that function as anaphylatoxins, neutrophil attractants, as well as activators for neutrophil opsonophagocytosis.

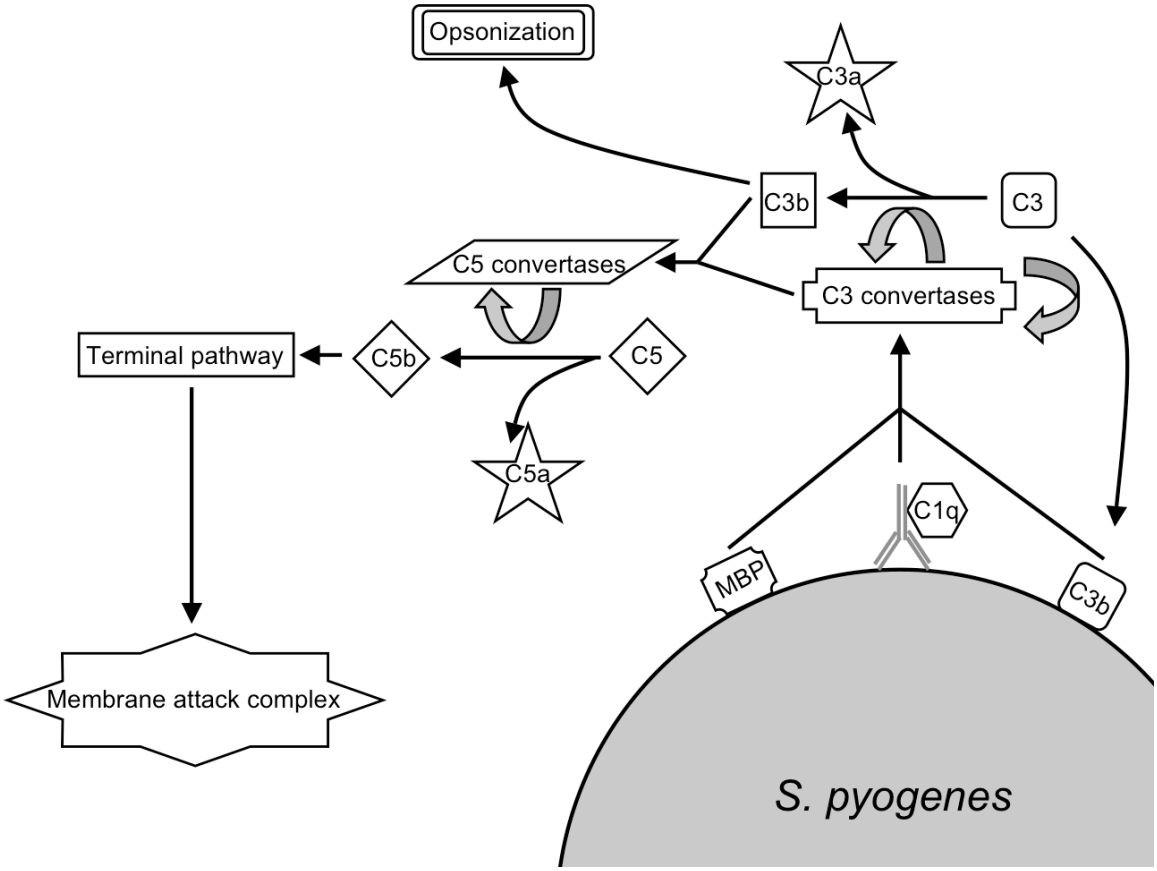

Figure 3. Schematic diagram of the complement cascades

MBP (lectin pathway), immune complexes (classical pathway), and C3b (alternative pathway) mediate the formation of C3 convertases, that in turn cleaves C3 into C3b. C3b acts as an opsonin but can also transform C3 convertases into C5 convertases that initiate the formation membrane attack complexes. The star-shaped boxes indicate anaphylatoxins.

The complement system is an obvious target for pathogenic microorganisms trying to survive and multiply in the human body. There are several ways by which *S. pyogenes* avoids complement attack. For instance, M proteins bind complement regulators factor H (*95*), factor H-related protein 1 (*115*), and C4BP (*199*) to the bacterial surface. Moreover, Fba binds factor H and factor H-related protein 1 (*154*), C5a peptidase cleaves the anaphylatoxin C5a (*44, 92*), SIC inhibits the formation of MAC (*4*), and Ig

binding proteins (*17*) and Ig modulating enzymes inhibit antibody-mediated complement activation (*50, 206*).

Neutrophils and phagocytosis

Neutrophils (Polymorphonuclear leukocytes, PMNs) are essential effector cells of the human innate immune system and provide the primary defense against pathogenic microorganisms (*122, 181*). The cells are widely distributed throughout the body, but when an infection occurs they are recruited to the site of infection as a consequence of inflammatory responses. Upon inflammation, capillary permeability is stimulated and blood supply into the inflamed area is increased. Moreover, leukocytes are stimulated and migrate out of the venules and into the surrounding tissues upon chemotactic responses. In the earliest stages of infection, neutrophils are particularly prevalent, but in later stages other phagocytic cells such as monocytes and lymphocytes also migrate towards the infection. Neutrophils eradicate invading microorganisms such as bacteria through phagocytosis or by releasing antimicrobial components into the site of infection. Although neutrophils bind, engulf and kill non-opsonized particles, the process is greatly enhanced by opsonins such as Igs or complement. After engulfment, neutrophils utilize at least two mechanisms in order to kill bacteria, the oxidative or non-oxidative pathway (*211*). While the oxidative pathway, or "respiratory burst", involves production of highly reactive oxidizing radicals that are harmful to bacteria (*83*), the non-oxidative pathway is composed of proteinases and antimicrobial agents that are stored in the specific or azurophil granules of neutrophils (*10*). However, activated neutrophils do not only kill bacteria through phagocytosis, but also release fibers composed of granule proteins and chromatin into the extracellular environment (*22*). These fibers form extracellular traps that bind and kill both Gram-positive and Gram-negative bacteria.

Azurophilic serine proteinases

The azurophil granules (also called primary granules) of neutrophils contain three serine proteinases denoted neutrophil elastase (NE), cathepsin G (CG) and proteinase 3 (Pr3). The content of NE has been estimated at approximately 4 μg per 10^6 cells, making it a major component of neutrophil granules (*128*). Neutrophils that have been activated by pro-inflammatory mediators rapidly translocate NE, CG and Pr3 to the external surface of the plasma membrane. Stronger and more potent activation signals (e. g. when the cells bind to immune complexes) mediate release of the proteinases into the environment. Several studies have demonstrated that neutrophil serine proteinases play an

important role in the immune response against pathogenic bacteria. For instance, NE degrades outer membrane proteins in *E. coli*, which leads to membrane damage and killing of the bacteria (*12*). In addition to killing bacteria directly, NE and CG have indirect antimicrobial activities. CG contains an internal antimicrobial peptide sequence that is released by clostripain *in vitro* (*182*), and NE activates the antimicrobial peptide cathelicidine that is present in human airway secretions, thus killing both Gram-positive and Gram-negative bacteria (*47*).

NE has also been demonstrated to degrade and inactivate virulence factors of enterobacteria (*210*). Similar studies with *S. pyogenes* show that the streptococcal M protein is released from the bacterial surface by neutrophil serine proteinases, but retains its affinity to fibrinogen. The pathogenic M1/fibrinogen complexes that are formed activate neutrophils and induce a potentially lethal inflammatory response in the host (*90*). Furthermore, we suggest in Paper IV that *S. pyogenes* utilizes NE and CG to release immunogenic epitopes from its cysteine proteinase IdeS, and thus prevents IdeS from being opsonized and eliminated during infection.

Neutrophil receptors

Phagocytes express a broad spectrum of receptors on their cell surfaces that recognize and mediate internalization of bacteria and other microbes. (See Table I for more information).

Fcγ-receptors

IgG-opsonized particles are recognized by surface receptors that bind to the Fc-region of IgG (FcγRs) (*55, 169*). Activated receptors bind IgG-opsonized microbes and trigger internalization through actin polymerization beneath the particle (*1*). To date, three classes of receptors have been identified in mice, the activating FcγRI and FcγRIII, and the inhibitory FcγRIIb. Activating FcγRs contain ITAM motifs in their intracellular domain that recruit kinases and activates phosphorylation cascades, whereas inhibitory FcγRs contain an ITIM motif that recruits phosphatases and thus inhibits signaling (*55, 169*).

Complement receptors

To date, four complement receptors have been identified (CR1, CR2, CR3 and CR4), although CR2 has not been described as a phagocytic receptor. CR1 binds a number of microbial opsonins including complement components C1q, C3b, and C4b, as well as

mannan-binding lectin (*79, 113*). Although CR1 alone is unable to mediate phagocytosis, colligation with Fc receptors on the neutrophil surface triggers engulfment of opsonized particles (*79*). CR3 and CR4 recognize iC3b that has bound to bacterial surfaces. However, internalization signaled by these receptors requires a second activation step, e.g. TNFα or LPS, that increases the number of receptors at the cell surface (*18*), enhances receptor affinity (*104*), and allows the receptors to trigger phagocytosis (*216*). In addition to mediating phagocytosis, CR3 is also involved in other biological processes such as neutrophil adhesion and migration (*52, 57*).

Table I. Receptors that participate in phagocytosis of microbes

Receptors	**Ligands**
Complement receptors	
CR1 (CD35)	MBL-, C1q-, C3b-, C4b-opsonized particles (*79*)
CR3 ($\alpha_M\beta_2$, CD11b/CD18, Mac 1)	iC3b-opsonized particles (*61*)
CR4 ($\alpha_X\beta_2$, CD11c/CD18)	iC3b-opsonized particles (*175*)
Fc-receptors	
FcγRI (CD64)	IgG-, CRP-opsonized particles (*19, 20, 169*)
FcγRII (CD32)	IgG-, CRP-opsonized particles (*19, 20, 169*)
FcγRIII (CD16)	IgG-, CRP-opsonized particles (*19, 20, 169*)
FcεRI	IgE-opsonized particles (*166*)
FcεRII (CD23)	IgE-opsonized particles (*171*)
FcαRI (CD89)	IgA-opsonized particles (*202*)
Scavenger receptors	
Mannose receptor (CD206)	Mannan (*63*)
CD14	LPS, peptidoglycan (*59, 178*)
C1qR(P)	C1q, MBL, SPA (*149*)
Dectin-1	B 1,3-glucan (*25*)
SRA	Bacteria, LPS, Lipoteichoic acid (*158*)
MARCO	Bacteria (*201*)
Other receptors	
$\alpha_5\beta_1$ (CD49e/CD29)	Fibronectin/Vitronectin-opsonized particles (*21*)

CRP; C-reactive protein
LPS; Lipopolysaccharide
MBL; Mannan-binding lectin
SPA; Serum amyloid P component

Immuno-modulating proteins of *S. pyogenes*

S. pyogenes evasion of opsonizing IgG

As previously described, opsonizing IgG eliminates pathogenic bacteria by activating the classical pathway of complement or by enhancing phagocytic killing. Although *S. pyogenes* survives and multiplies in human blood, opsonizing antibodies from immune donors usually eliminate the bacteria. To protect itself from the detrimental effects of opsonizing IgG, *S. pyogenes* expresses proteins that either bind, cleave, or de-glycosylate IgG. Strikingly, most IgG modulating proteins of *S. pyogenes* are expressed during logarithmic growth *in vitro*, thus possibly suggesting that they are important for establishing streptococcal infections *in vivo*.

IgG binding surface proteins of *S. pyogenes*

The M protein family consists of M proteins and M-like proteins (e. g. Arp, Protein H, Protein Sir, and Mrp) (*76, 80, 193, 194*). They protrude from the surface of *S. pyogenes* in a hair-like structure and bind several host proteins to the bacterial surface, including IgG (*88*), IgA (*127*), albumin (*179*), fibrinogen (*106*), plasminogen (*15*), fibronectin (*74*), kininogens (*13*), and others (*148*). Most *S. pyogenes* strains express one *emm* gene and up to two genes encoding M-like proteins located in the Mga regulon.

While the COOH-terminal part of M proteins is highly conserved, the variability between different M types increases as the structure reaches the NH_2-terminus and the absolute NH_2-terminus is unique for each M-protein. The hypervariable NH_2-terminus of M proteins is the major epitope for opsonizing IgG, which means that patients infected with *S. pyogenes* mainly develop antibodies toward the specific strain that caused the infection. By subjecting M proteins to post-translational modifications, *S. pyogenes* can alter its non-immune binding to IgG and modify the immunogenic NH_2-terminus (*40, 163-165, 174*). Another mechanism by which *S. pyogenes* prevents antibody recognition is by generating genetically distinct subpopulations during infection (*145, 157*). Such events result in M proteins of different sizes and partly changed amino acid sequences in the hyper-variable region, thus allowing daughter cells to avoid antibody recognition during infection.

Antiphagocytic properties of M proteins

M protein expressing *S. pyogenes* strains survive in human blood lacking type-specific antibodies, whereas M protein lacking strains are rapidly killed (*54*). Therefore, M

proteins have been assigned anti-phagocytic properties. However, this definition has proven less accurate as wild type *S. pyogenes* strains are as rapidly engulfed by neutrophils as mutant strains lacking M or M-like proteins on the bacterial surface (*191*). However, wild type strains survive intracellularly whereas strains deficient in M proteins are rapidly killed after phagocytosis (*191, 192*). The exact mechanism of the antiphagocytic properties of M proteins is not known but several studies link this feature of M proteins to its binding of fibrinogen (*30, 172, 214*). Moreover, some M proteins bind the Fc-region of IgG and block the interaction between IgG and C1q of complement on the bacterial surface. This non-immune binding of IgG leads to a reduced surface deposition of the opsonin C3b on the bacterial surface (*17*).

IgG modulating enzymes of *S. pyogenes*

Although Igs are common targets for bacterial pathogens, few specific IgG modulating proteinases have been identified in bacterial pathogens. However, *S. pyogenes*, *Prevotella intermedia* and *Prevotella nigrescens* all express IgG cleaving cysteine proteinases (*49, 100, 206*). In *S. pyogenes*, three IgG modulating enzymes have been identified. These include one endoglycosidase (EndoS) and two cysteine proteinases (SpeB and IdeS) that are discussed more extensively below.

In contrast to the relatively small number of bacterial IgG cleaving proteinases, IgA-proteinases have been described for a number of bacterial species that colonize or infect the mucosal membranes of humans, such as oral streptococci (*111*), *Haemophilus influenzae* (*110, 135*), *Streptococcus pneumoniae* (*110, 135*), and *Neisseria meningitidis* (*144*). Interestingly, no specific IgA-protease has been described in *S. pyogenes*, although the streptococcal cysteine proteinase SpeB degrades the COOH-terminus of IgA (*48*).

EndoS

The endoglycosidase of streptococci (EndoS) is secreted into the environment during *S. pyogenes* infections (*49*). It hydrolyzes the conserved aspargine-linked glycan on the heavy chain of IgG, and thus alters the structural stability of the antibody. Interestingly, EndoS treatment of immune blood contributes to increased survival of *S. pyogenes in vitro* (*50*). This is due to reduced binding of IgG to Fc receptors and impaired classical pathway-mediated activation of complement (*50, 151, 162*). Both the glycan structure of IgG and the tertiary structure of EndoS are important for the endoglycosidase activity, as an increase of IgG denaturation leads to a gradual decrease of EndoS activity (*48*). To date, EndoS and EndoE (*51*) from *Enterococcus faecalis* are the only examples of

bacterial endoglycosidases that hydrolyze the glycan of native IgG, and little is known about their regulation *in vivo*. However, EndoS expression is increased when *S. pyogenes* interacts with human PMNs *in vitro* (*208*), and patients suffering from streptococcal infections develop anti-EndoS antibodies (*5*).

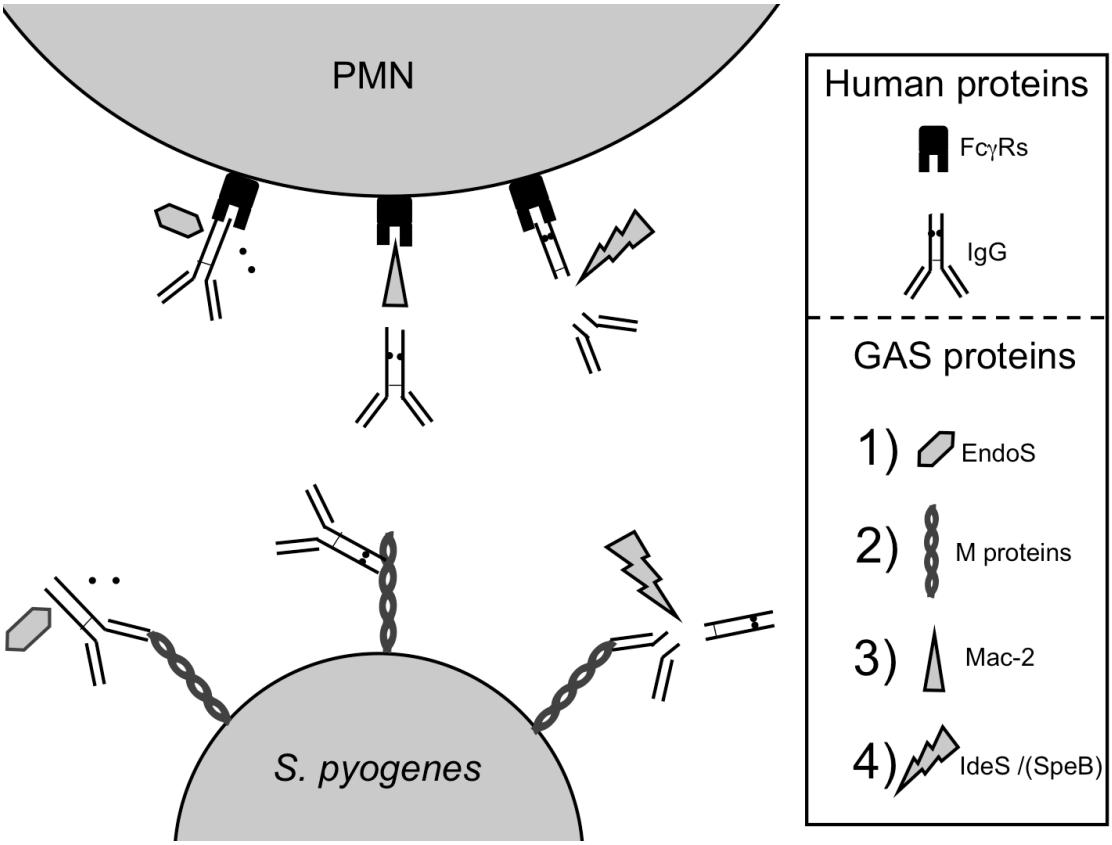

Figure 4. S. pyogenes evasion of opsonizing IgG

S. pyogenes evades IgG mediated phagocytosis by at least four distinct mechanisms. ***1)*** *EndoS alters the structural stability of IgG by hydrolyzing the conserved NH_2-linked glycan on IgG Fc-domains.* ***2)*** *Fc-binding to M proteins inhibits complement deposition and Fc-mediated phagocytosis.* ***3)*** *Mac-2 hinders neutrophil recognition of opsonized particles by binding to FcγRII and FcγRIII on the neutrophil surface.* ***4)*** *IdeS and SpeB inactivates opsonizing IgG by specific cleavage of the hinge region.*

SpeB

The streptococcal pyrogenic exotoxin B (SpeB) is the classical cysteine proteinase of *S. pyogenes*. It is secreted as a 40 kDa zymogen during stationary growth and its expression is repressed by glucose and other nutrients *in vitro* (*62*). Despite limited sequence homology, the SpeB zymogen has structural homologies to the papain super-family of

proteinases (*105*). Upon secretion, the propeptide is released and an active 28kDa proteinase is generated. The molecular events leading to propeptide removal and generation of active SpeB are not fully understood. However, it is known that the zymogen form has some proteolytic activity under reducing conditions, and that the propeptide can be removed by autocatalysis (*58*). In addition, the intracellular protein RopA has also been demonstrated to be important for the generation of active SpeB (*58, 105, 133*).

The catalytic site of SpeB is composed of a catalytic dyad (Cys192-His340), instead of the catalytic triad (Cys-His-Asn), commonly associated with cysteine proteinases (*129, 130*). Active SpeB degrades or activates several human proteins (summarized in Table II) and releases streptococcal proteins from the bacterial surface, including IgG-binding M and M-like proteins (*16, 168*). The released IgG/M protein-complexes activate and consume complement at a distance from the pathogen and thereby prevent complement activation at the bacterial surface (*17*).

SpeB has proteolytic activities against human immunoglobulins (*48*). It cleaves human IgG between glycine residues 236 and 237 in the hinge region, generating two stable Fab fragments and one Fc fragment (*49*). SpeB cleavage of opsonizing IgG facilitates streptococcal survival in whole blood, suggesting that IgG cleavage by SpeB is a mechanism for *S. pyogenes* to escape Fc mediated phagocytosis and complement deposition (*50*). Moreover, SpeB also cleaves the COOH-terminal region of the heavy chains of human IgA, IgD, and IgM into small fragments, whereas the heavy chain of IgE is completely degraded (*48*).

IdeS/Mac-1

IdeS (also called Mac-1) was recently independently discovered by Lei *et al* and von Pawel-Rammingen *et al* (*123, 206*). It is a 35kDa cysteine proteinase that in contrast to SpeB cleaves IgG in the hinge region with a unique specificity. The expression of IdeS is maximal during logarithmic growth *in vitro* and it is negatively regulated by the streptococcal *covR-covS* two-component gene regulatory system (*123*). IdeS cleaves all subclasses of human IgG in the hinge region between glycine residues 236 and 237 (of IgG1), thus generating two stable Fab fragments and one Fc fragment (Fig. 2a, Fig. 4). However, IgG2 is slightly more resistant towards the endopeptidase activity of IdeS, possibly as a result of its altered amino acid composition at the cleavage site (Fig 2b). Several studies have confirmed that the proteolytic activity of IdeS is extremely specific,

and no other substrates have been identified (*2, 205, 206*). It has also been proposed that IdeS has an exosite that binds to the C_H2 domain of human IgG prior to cleavage, explaining why synthetic and naturally occurring substrates (e.g. IgA, IgD, IgE, and IgM), with high sequence similarities to the hinge region of IgG, are not cleaved by IdeS (*205*). The presence of an exosite is not a novel concept and has previously been suggested for other types of bacterial Ig cleaving proteinases (*41*).

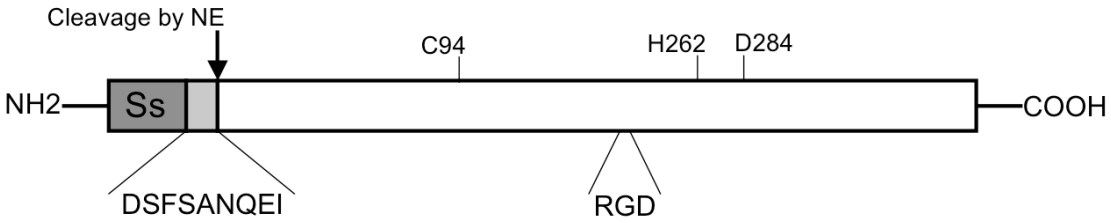

Figure 5. Primary structure of IdeS

The signal sequence (Ss), the aa residues that form IdeS catalytic site (C94, H262, D284) and the DSF-9 peptide (DSFSANQEI) released by NE are indicated.

IdeS is believed to act as a major virulence factor during infections with *S. pyogenes* by removing the Fc part of opsonizing IgG that has bound to the streptococcal surface. As previously discussed, removal of Fc regions from opsonizing IgG inhibits complement deposition and Fc mediated phagocytosis, and several assays have confirmed that IdeS prevents killing of *S. pyogenes in vitro* (*123, 206, 207*). Although an isogenic IdeS mutant strain has been generated, IdeS importance for *S. pyogenes* colonization and spread during infection has never been investigated *in vivo* (*184*). However, antibodies against IdeS are found in both acute phase- and convalescent phase serum from patients suffering from mild and invasive *S. pyogenes* infections (*5, 6*) and the enzyme is proteolytically active in pH and salt concentrations found in skin (pH 5.5-6.5), human plasma (pH 7.4) and saliva (pH 7.4) (*205*). A recent study suggested IdeS as a suitable vaccine candidate against *S. pyogenes* infections, as it is nontoxic to mice, is present in all *S. pyogenes* serotypes, and induces protective immune responses after immunization of mice (*109, 153*).

Structure of IdeS

The *ideS* gene translates into a protein of 339 amino acids of which the 29 most NH_2-terminal residues encode a putative signal sequence (*206*) (Fig. 5). Apart from the putative signal sequence, an RGD motif, commonly found in bacterial and viral

pathogens, is located at residues 214–216 (*97, 123, 206*). This motif is involved in the interaction of IdeS with vitronectin and platelet receptors (*2*). Site-directed mutagenesis and crystal structure analysis have identified Cys94, His262 and Asp284 as active site residues, and Asp286 as important for the structure of IdeS catalytic site (*125, 212*). Interestingly, IdeS does not contain a propeptide and is thus already proteolytically active upon translation (*206*).

The three dimensional structure of IdeS reveals that the enzyme belongs to the papain super-family of proteinases despite weak sequence homologies (*3, 212*). By forming symmetric dimers IdeS can increase IgG specificity and enzyme cooperativity (*3*). Similarity searches against the human genome reveal that the middle one third of the IdeS sequence has a significant homology to the human CR3 subunit CD11b (26% identity and 45% similarity over IdeS residues 139-322). It was first suggested that IdeS prevents opsonophagocytosis of *S. pyogenes* by binding and sterically blocking the Fc receptor FcγRIIIb, located on the neutrophil surface (*123*). However, it was recently demonstrated that IdeS does not have affinity to human Fc-receptors and that the anti-phagocytic properties of IdeS are exclusively due to its endopeptidase activity (*2, 206*).

Mac-2

Studies of *ides* gene variations between 31 different *S. pyogenes* isolates identified a novel IdeS variant denoted Mac-2 (*124*). IdeS and Mac-2 are closely related and share approximately 80% sequence identity. Most of the amino acid differences are located in the middle one third of the protein sequences. Interestingly, all tested strains encode either the gene for IdeS or Mac-2, but both genes have never been identified in the same strain.

In contrast to IdeS that efficiently binds and cleaves IgG in the hinge region, Mac-2 only has weak affinity and endopeptidase activity against human immunoglobulins (*2, 124*). Instead, Mac-2 binds to FcγRII and FcγRIII, located on the neutrophil surface, and thus prevents binding of IgG to these receptors. Although not yet demonstrated in a bactericidal assay, it has been proposed that Mac-2 also interferes with Fc-mediated phagocytosis by blocking IgGFc/ Fc-receptor interactions (*2*).

Table II. Comparison of the two cysteine proteinases of S. pyogenes

	SpeB	**IdeS**
Size	40kDa zymogen/ 28kDa active form	35 kDa
3D-structure	*Papain super-family*	*Papain super-family*
Catalytic site	Catalytic dyad (Cys192-His340)	Catalytic triad
Propeptide	*Yes*	*No*
RGD motif	Yes	Yes
Expression in vitro	*Stationary growth phase*	*Logarithmic growth phase*
Regulators	CovR/CovS (down), Rgg/RopB (Up), RALP (down), Ihk/Irr (Up)	CovR/CovS (down), Rgg/RopB (Down), Ihk/Irr (Up)
Location	*Secreted*	*Secreted*
Degradation by PMNs	Zymogen No/ active Yes	No
Substrates	*1) IgA, IgD, IgE, IgG, IgM (48, 49)* *2) MMP-2 (26)* *3) Dermatan sulphate (180)* *4) Fibrinogen (136)* *5) Fibronectin (108)* *6) Vitronectin (108)* *7) H-kininogen (89)* *8) Interleukin 1β (107)* *9) Streptococcal surface proteins (16, 167)*	*IgG*
Biological activity	1) Facilitates bacterial spread (*108*) 2) Inhibits antimicrobial peptides (*180*) 3) Degrades fibrinogen (*136*) 4) Activates cytokines (*107*) 5) Releases proinflammatory peptides (*89*) 6) Releases streptococcal surface proteins (*16, 167*) 7) Prevents Fc-mediated phagocytosis (*50*)	Prevents Fc-mediated phagocytosis

Other immuno-modulating proteins of *S. pyogenes*

C5a peptidase

C5a peptidase (ScpA) is a 130 kDa subtilisin-like serine proteinase located on the bacterial surface. ScpA inactivates and depletes the anaphylatoxin C5a from the site of infection by cleaving the substrate between His67 and Lys68 (*38, 45*). The substrate of ScpA, C5a, is important for the host defense against bacterial infections. It increases vascular permeability, attracts neutrophils to the site of infection, and stimulates neutrophil opsonophagocytosis. All human isolates of β-hemolytic streptococci, including *S. pyogenes*, express ScpA or ScpA-homologues on their bacterial surfaces (*38, 42*). Like many other virulence factors of *S. pyogenes*, ScpA expression is regulated by the mga-regulon and the protein is therefore assumed to be present on the bacterial surface early during infection. Recently, the crystal structure of ScpA from group B streptococci (ScpB) was determined (*23*). It revealed that the active site of ScpB is flanked by two RGD sequences, and it was suggested that binding of integrins to ScpB greatly enhanced the enzymes proteolytic activity. Apart from depleting C5a from the bacterial surface, ScpA has also been demonstrated to promote non-opsonizing entry of *S. pyogenes* into epithelial cells (*39, 161*). This is an interesting observation since it is becoming increasingly clear that *S. pyogenes* survives intracellularly during infection (*191, 192*). Immunogenicity studies have identified ScpA as an attractive target in the development of vaccines against *S. pyogenes* since it is highly immunogenic in children infected with *S. pyogenes* pharyngitis, and intranasal immunizations of ScpA in mice prevent streptococcal infections (*155, 185*).

SpyCEP

Lethal necrotizing soft tissue infections caused by *S. pyogenes* are characterized by an absence of neutrophils at the site of infection (*46, 91, 197*). Even though this feature can in part be explained by the C5a cleaving actions of ScpA, Hidalgo-Grass *et al* recently demonstrated that supernatants of invasive *S. pyogenes* strains specifically cleave and inactivate interleukin-8 (IL-8) (*91*). IL-8 is a major chemokine that is essential for neutrophil transmigration through the endothelial surfaces of venules and attracts neutrophils to sites of infection (*140*). To date, the IL-8 cleaving enzyme of *S. pyogenes* has only been crudely purified from growth medium by ammonium sulfate precipitation (*60*). However, supernatant fractions with IL-8 degrading activity contained a protein encoded by the open reading frame *Spy0416* . Spy0416 (also called SpyCEP) belongs to

the subtilisin family of proteinases and contains a cell wall-anchoring motif (LPXTGX) suggesting that it is present on the streptococcal surface (*101*). Although subtilisin homologues have been characterized in *S. pyogenes* (ScpA), *Bacillus* species (*43, 141*), and other Gram-positive bacteria (*67, 84*), IL-8 degradation by a bacterial proteinase is a novel concept among pathogenic bacteria.

SIC

Some strains of *S. pyogenes* secrete the streptococcal inhibitor of complement (SIC), which was originally identified as an inhibitor of the membrane attack complex (MAC) (*4*). The distribution of the *sic* gene was first reported to be restricted to M1 and M57 strains (*4, 85*), but a recent study reported occurrences of the gene in several other M types (*134*). Apart from inhibiting complement, SIC also inhibits other components of the innate immune system, such as lysozyme, α- and ß- defensins, LL-37, and the secretory leukocyte proteinase inhibitor SLPI (*68, 69, 75*). SIC is highly immunogenic in humans and its structure is highly variable between and within different *S. pyogenes* strains, possibly as a response to the need for the molecule to escape immune pressure (*189*). The high diversity of the *sic* gene (*94*), the recovery of new SIC variants within an epidemic wave (*93*), and the fact that the *sic* gene is present in all highly virulent M1 isolates, suggests that SIC has an important role during *S. pyogenes* infections. This view is emphasized by a recent study, which reported that SIC expressing M1 strains are more persistent in mice than SIC-negative strains after nasal inoculations (*132*).

Regulation of S. pyogenes protein expression

S. pyogenes regulates its protein expression in response to extracellular stimuli such as temperature, oxygen-levels and iron concentrations (See Table III for more information). Environmental signals from the host or other bacteria trigger positive-acting or negative-acting gene regulatory systems that in turn stimulate the expression of extracellular proteins. The regulatory networks in *S. pyogenes* are divided into two-component signal transduction systems (TCSs) and “stand alone” response regulators (RRs). While TCSs recognize extracellular signals through transmembrane kinases, the exact mechanism of RRs sensing abilities is still unclear (*118*). The genome sequences of *S. pyogenes* serotypes M1, M3 and M18 revealed an average of 13 characterized or putative TCSs per genome (*14, 70, 118, 187*), suggesting that gene regulation is important for the pathogenicity of *S. pyogenes*. To date, only three RRs (mga, RALP, and Rgg/RopB) and three TCSs (CovR/CovS, FasBCAX and Ihk/Irr) have been studied in some detail with

respect to their influence on host-bacteria interactions. In table III, environmental signals that influence RR and TCS expression, and their impact on virulence gene expression in *S. pyogenes* are summarized.

In addition to responding to different host environments, bacteria also use their sensing abilities to communicate with each other through quorum sensing (*209*). Quorum sensing provides a mechanism for bacteria to modulate their protein expression in response to population density, and to synchronize their expression of virulence factors (*27*).

Mga (Multiple gene regulator of group A streptococci)

Mga is the most characterized transcriptional regulator in *S. pyogenes*. It is found in all GAS serotypes, and homologs have been identified in several Gram-positive pathogens, including *Streptococcus dysgalactiae* and *Streptococcus pneumoniae* (*78, 198, 204*). Virulence factors that are regulated by Mga include M and M-like proteins, streptococcal collagen-like protein (SclA), serum opacity factor (Sof), C5a peptidase (ScpA) and streptococcal inhibitor of complement (SIC) (*54, 170*). Several studies have demonstrated that the Mga expression is maximal during logarithmic growth and that elevated CO_2 levels, increased temperature and iron-limiting growth conditions activate the Mga regulon (*28, 137, 159*). Beside external stimuli, Mga also binds within its own promoter and thus elevate Mga expression by auto-regulation (*139*), whereas RALP and Rgg/RopB both decrease Mga expression *in vitro* (*78, 138*).

Interestingly, since a down-regulation of genes encoding surface-attached proteins will not immediately lead to a smaller number of surface-attached proteins on the bacterial surface, it has become evident that *S. pyogenes* utilizes proteolytic cleavage to down-regulate cell-wall attached proteins. Thus, SpeB releases M1 protein (*16*), Protein H (*16*), ScpA (*16*) and Protein F1 (*152*) from the bacterial surface.

Table III. Important regulatory networks in S. pyogenes and their impact on protein expression.

Regulon family	Regulon	Signals	Up-regulated genes	Down regulated genes	References
RRs	Mga	Temperature, CO_2, iron, plasma, logarithmic growth	*emm, scpA, sof, mrp, arp, sclA, sic, mga*	*SclB*	(*28, 54, 102, 137, 159, 167, 170*)
	Rgg/RopB	Temperature, stationary growth	*speB, covR/covS, ihk/irr*	*ideS, sagA, slo, mga*	(*34-37, 133, 188*)
	RALP	Temperature, super oxide, anaerobic conditions, stationary growth	*prtF, rofA*	*prtF2, speA, speB, mga, nra*	(*11, 73, 117, 142, 156, 160, 188*)
TCS	CovR/CovS	Blood, late logarithmic growth, stationary growth	----------	Capsule genes, *sagA, speB, ideS, grab, ska, mspA*	(*81, 86, 87, 123, 126, 213*)
	FasBCAX	Temperature, late logarithmic growth	*sagA*	*fbp54, mrp*	(*116, 188*)
	Ihk/Irr	ROS, PMN contact	*sic, grab, ideS, endoS, speB*	*emm1*	(*66, 208*)

Present investigations

As previously described, *Streptococcus pyogenes* produces a number of extra cellular proteins that modulate the immunological response directed against the bacteria during infection. A goal of our research is to identify these immuno-modulating proteins and understand their role during infection. In this section, the papers on which this thesis is based are summarized and discussed in a broader context.

The protein expression of *Streptococcus pyogenes* is significantly influenced by human plasma (Paper I)

Virulent *S. pyogenes* strains temporally control their transcription of virulent and metabolic genes in response to environmental changes. Even though we are just beginning to understand the regulatory networks during infections with *S. pyogenes*, several factors including temperature, O_2-pressure, and ion-concentration have been shown to influence the streptococcal protein expression (*28, 147, 159*). During infection, *S. pyogenes* will at some point encounter human plasma. Superficial infectious sites with inflammation contain plasma as a consequence of vascular leakage, and invasive strains penetrate into the blood stream. Even though plasma is a rich growth medium, it is also a reservoir for opsonizing antibodies, complement, and other components of the human immune system. By expressing an array of extracellular proteins that interfere with various host defense mechanisms, the bacteria prevent recognition and subsequent killing during infection (*54*).

In Paper I, we show that *S. pyogenes* rapidly remodels its cellular metabolism and virulence pathways in response to human plasma. A proteome map was generated and *S. pyogenes* protein expression was analyzed when grown in human plasma and compared to the protein expression of bacteria grown in standard laboratory growth medium. In general, *S. pyogenes* increases its protein expression when grown in human plasma. A majority of the up-regulated proteins were either important for cell maintenance or involved in various metabolic pathways. However, the expression of two major streptococcal virulence factors, M1 protein and C5a peptidase, were not only up-regulated in bacteria grown in plasma but also expressed in multiple forms. This suggests that *S. pyogenes* subjects virulent proteins to post-translational modifications when exposed to human plasma. MS/MS analysis of the different M1 protein spots revealed two distinct M1 forms, an intact native form, and a processed form lacking 13 amino acid residues in the NH_2-terminus.

The precise mechanism responsible for the modification of the M1 protein and C5a peptidase, and their relevance for *S. pyogenes* pathogenicity is currently unknown. However, previous studies have suggested that the M1 protein can be subjected to phosphorylation and/or proteolytic processing on the bacterial surface, which alters the virulent phenotype *S. pyogenes* (*40, 163-165, 174*). It has also been reported that M proteins of different sizes and with partly changed amino acid composition in the hyper-variable NH_2-terminus can occur, as genetically distinct subpopulations develop during bacterial growth (*145, 157*) The subtilisin-like cell wall-anchored proteinase C5a peptidase was also expressed in multiple forms in bacteria grown in plasma. Although heterogeneity has been described for the C5a peptidase gene between different *S. pyogenes* strains, post translational modifications or gene heterogeneity of the C5a peptidase gene within the same strain have never been reported before (*114*).

IdeS, a novel streptococcal cysteine proteinase with unique specificity for immunoglobulin G (Paper II).

S. pyogenes has evolved several mechanisms to circumvent the antimicrobial effects of Igs in order to colonize and spread in the human host (*49, 54, 215*). In Paper II we describe the identification and characterization of a novel IgG cleaving enzyme of *S. pyogenes* denoted IdeS (Immunoglobulin G-degrading enzyme of *S. pyogenes*). The project started with the observation that a mutant strain, lacking the *SpeB* gene, was able to cleave human IgG when incubated with 10% human plasma. In addition, IgG was cleaved in growth medium from the virulent M1 strain AP1, grown under conditions repressing SpeB activity. These findings indicated that *S. pyogenes* expresses a novel IgG cleaving enzyme, distinct from SpeB.

By fractionating the growth medium and following the enzymatic activity, a major band of approximately 34 kDa was associated with the IgG-cleaving activity. NH_2-terminal sequencing of the 34 kDa band gave a perfect match to a reading frame of 339 amino acids designated Spy0861. Analyzing the amino acid sequence of the Spy0861 reading frame revealed that IdeS contains a signal sequence, but no cell wall-attachment signal (LPXTGX), confirming that IdeS is secreted rather than cell wall-attached (*72, 101*). Furthermore, like many other bacterial virulence factors, IdeS contains an RGD motif, suggesting that it might have affinity to host cell integrins (*97*). Interestingly, a cysteine and a histidine were also found in the amino acid sequence, indicating that IdeS belongs to the proteinase class of cysteine proteinases. This was confirmed through the incubation of recombinantly expressed IdeS with different proteinase inhibitors.

A number of experiments demonstrated the unique specificity of IdeS and NH_2-terminal analysis of hydrolyzed IgG showed that IdeS cleaves the hinge region of human IgG between glycine residues 236 and 237, generating two stable monomeric Fab fragments and one Fc fragment (Fig. 2 and 4). All subclasses of IgG are cleaved by IdeS but IgG2 is slightly more resistant to the endopeptidase activity. Interestingly, the IgG antibody response directed against M proteins on the bacterial surface is mainly distributed between IgG1 and IgG3 (*31*), suggesting that IdeS cleaves surface bound IgG with high efficiency. The other human immunoglobulin classes (IgA, IgD, IgE and IgM) are not cleaved or degraded by IdeS, and no additional protein bands appeared on SDS-PAGE gels when human plasma was incubated with IdeS.

Most extracellular proteinases expressed by Gram-positive bacteria have a propeptide located between the signal sequence and the mature protein. Interestingly, IdeS does not contain a propeptide and is thus already proteolytically active upon translation. Propeptides provide stability and prevent enzymes from degrading intracellular bacterial molecules. It is plausible that the extreme specificity of IdeS enables streptococci to express active IdeS without exposing intracellular proteins to degradation.

Even though a PCR screening for the *ideS* gene revealed that IdeS is widespread among different *S. pyogenes* strains, only five out of 11 tested strains expressed IgG-cleaving activity. Although puzzling at first it is now evident that the endopeptidase negative strains express the proteolytically inactive Mac-2 rather than IdeS (*124*).

By following the growth of AP1, and continuously measuring the secretion of IdeS into the growth medium, it was determined that IdeS is secreted during logarithmic growth *in vitro*. This finding, in combination with previously published data demonstrating that SpeB is not expressed until late stationary growth phase under laboratory conditions (*34*), suggests that the two IgG-cleaving enzymes of *S. pyogenes* are expressed at different stages of infection and have complementary, rather than additive roles during infection.

Although SpeB has proteolytic activities against human Igs *in vitro*, it is questionable whether SpeB has a role in cleaving opsonizing IgG during streptococcal infections *in vivo*. Elimination of IgGs is probably especially important for *S. pyogenes* during an early stage of infection, since it postpones the immune systems detection of the pathogen. In contrast to other IgG-modulating proteins of *S. pyogenes* that are expressed during logarithmic growth, SpeB is repressed until nutrition is scarce or the bacteria

reaches stationary growth phase *in vitro* (*34*). Moreover, the large number of substrates interacting with SpeB during an infection also suggests that Igs are not the primary target of SpeB, since the enzyme will be occupied with substrates other than IgG.

As a final set of experiments, phagocytosis assays demonstrated that IdeS prevents Fc-mediated killing of the bacteria by human neutrophils. We concluded that the anti-phagocytic property of IdeS was a direct consequence of its ability to release the Fc fragment from opsonizing IgG that has bound to the surface of *S. pyogenes*.

Streptococcus pyogenes and phagocytic killing (Paper III)

Human neutrophils express an array of receptors that recognize Igs or complement that have bound to bacterial surfaces. Two important leukocyte receptors are the Complement receptor 3 (CR3, CD18/CD11b, or Mac 1), and the Fc-receptor FcγRIIIb (also called CD16). Previous studies have demonstrated that CR3 and FcγRIIIb are physically and functionally linked at the surface of human neutrophils and cooperate in binding and ingesting bacteria (*24, 77, 195*).

After the submission of Paper II to *EMBO J*, Lei *et al* described a streptococcal protein designated Mac (*123*). Interestingly, Mac and IdeS are identical and Lei *et al* confirmed our results demonstrating that IdeS interferes with neutrophil opsonophagocytosis. However, instead of preventing opsonophagocytosis by cleaving opsonizing IgG, Lei *et al* suggested an alternative mechanism based on molecular mimicry. They postulated that the sequence homology shared between IdeS and CR3, enables IdeS to bind to CR3, and block the closely linked FcγRIIIb from interacting with opsonizing IgG.

In Paper III we examined whether the enzymatic activity is important for IdeS interference with phagocyte functions. By replacing the catalytic cysteine residue with a glycine residue by site directed mutagenesis, a catalytically inactive IdeS mutant protein was generated ($IdeS^{C94G}$). Although native IdeS and enzymatically inactive $IdeS^{C94G}$ interacted with the surface of human neutrophils in a similar manner, only native IdeS significantly increased the survival of *S. pyogenes* in phagocytosis assays and increased bacterial survival in immune blood. Hence, the results in Paper III show that the enzymatic activity of IdeS is essential for its ability to interfere with phagocytosis and subsequent killing of *S. pyogenes*, and that steric hindrance of FcγRIIIb *per se* is not sufficient. Later, it has been demonstrated that Mac-2 blocks the interaction between IgG

and Fc receptors by competitively bind to FcγRII and FcγRIII, whereas IdeS does not have any affinity towards neutrophil Fc receptors (*2*).

Neutrophil serine proteinases remove immunogenic epitopes from the streptococcal IgG cleaving enzyme IdeS, without affecting the biological activity of the enzyme (Paper IV)

Neutrophils store the serine proteinases NE, CG and Pr3 in their primary granules (*56*). Upon stimulation, activated neutrophils express all three proteinases on the cell surface or release them into the environment. NE has been assigned antimicrobial activities and degrades virulence factors of Gram-negative bacteria with pronounced sensitivity (*210*).

Since IdeS and neutrophils are closely associated during streptococcal infections, we were interested in investigating whether IdeS and NE influence each other through proteolytic cleavage. By incubating recombinant IdeS with purified neutrophils, and analyzing the integrity of IdeS by SDS-PAGE, we observed an extra protein band of approximately 33 kDa. NH_2-terminal sequencing revealed that the additional protein band represents an NH_2-terminally processed IdeS protein (ΔN-IdeS), lacking the 9 outmost amino acids (DSFSANQEI, DSF-9, see Fig. 5). By repeating the experiment with class specific inhibitors, and analyzing recombinant IdeS incubated with purified neutrophil serine proteinases, we concluded that NE and CG were responsible for the release of DSF-9. In contrast, purified Pr3 did not generate the 33 kDa band, but instead efficiently degraded IdeS in a dose dependent manner. Liquid chromatography-mass spectrometry (LC-MS) identified DSF-9, together with intrinsic peptides and other peptides corresponding to IdeS NH_2-terminus. Since processed IdeS remains stable in the presence of neutrophil proteinases, we suggest that the internal peptides identified result from the degradation of miss folded proteins. However, the three-dimensional structure of IdeS reveals that the NH_2-terminus consists of at least 12 aa that are accessible to proteinase cleavage, thus explaining the presence of NH_2-terminally cleaved peptides.

Interestingly, IdeS retains its enzymatic activity and specificity after proteolytic cleavage by NE and CG, and ΔN-IdeS protects the bacteria against Fc-mediated killing as efficiently as native IdeS. We therefore suggest that evolutionary pressure has driven *S. pyogenes* to express an IdeS protein that is proteolytically active under conditions of inflammation. In contrast to IdeS, active SpeB is degraded by neutrophils within 60 minutes. Although SpeB also degrades Igs, its main function during infection is probably not directly associated with activated neutrophils, which might explain its rapid degradation.

In addition to the intact endopeptidase activity of ΔN-IdeS, we observed that IgG and patient serum preparations against IdeS were preferentially directed against IdeS NH_2-terminus rather than the intrinsic peptides identified by LC-MS. Although more patient serums are needed to confirm this observation, these results suggests that IdeS releases immunogenic peptides that otherwise would opsonize and lead to the elimination of the protein.

Conclusions

- *S. pyogenes* significantly remodels its cellular metabolism and virulence pathways in response to human plasma.
- M1 protein and C5a peptidase, two cell wall-attached virulence factors of *S. pyogenes*, are increasingly expressed and modified in response to human plasma.
- IdeS is a novel IgG cleaving cysteine proteinase secreted by *S. pyogenes* during logarithmic growth phase.
- IdeS cleaves the hinge region of human IgG1, IgG2, IgG3, and IgG4 with a unique specificity.
- IdeS prevents Fc-mediated killing of *S. pyogenes* by releasing the Fc fragment from opsonizing IgG that has bound to streptococcal surfaces.
- In contrast to virulence factors of Gram-negative bacteria, IdeS retains its endopeptidase activity after exposure to NE and CG.
- Immunogenic peptides are released from IdeS after exposure to NE and CG.

Populärvetenskaplig sammanfattning på svenska

Streptococcus pyogenes (också kallade grupp A streptokocker) är en vanlig sjukdomsframkallande bakterie som nästan alla någon gång blir infekterad av. Oftast infekterar *S. pyogenes* halsen och ger då upphov till halsfluss, eller huden, vilket orsakar svinkoppor eller scharlakansfeber. Det otäcka med infektioner som orsakas av *S. pyogenes* är att de i vissa fall kan utvecklas till allvarliga och direkt livshotande tillstånd om man inte behandlas med antibiotika. Exempel på akuta och livshotande tillstånd som orsakas av *S. pyogenes* är blodförgiftning, nekrotiserande fasciit (när bakterierna bryter ner muskulaturen) och ett chock-tillstånd som leder till att patientens organ kollapsar och slutar fungera.

Utöver de kliniska tillstånd som exemplifierats ovan, kan obehandlade halsflussar och hudinfektioner även följas av minst lika allvarliga följdsjukdomar såsom reumatisk feber och glumerolunefrit. Nyligen publicerades en vetenskaplig artikel som uppskattade att mer än 600 miljoner människor årligen drabbas av halsfluss och att ungefär 500 000 människor dör av akuta streptokockinfektioner eller av dess följdsjukdomar. Även om antalet dödsfall i Sverige till följd av streptokockinfektioner är relativt lågt, kostar dessa samhället stora summor pengar i form av uteblivna arbetsdagar och läkarbesök.

Alla djur har någon form av immunförsvar som skyddar deras kroppar från att infekteras av mikroorganismer. Människans immunförsvar är avancerat och består av en mängd specialiserade celler, proteiner och andra molekyler, som samarbetar för att känna igen och avdöda sjukdomsframkallande organismer. Ytterst förenklat kan man likna celler vid en levande organism, medan proteiner är cellernas verktyg.

På samma sätt som immunförsvarets celler använder proteiner för att känna igen och döda mikroorganismer, uttrycker *S. pyogenes* en mängd egna proteiner som förstör eller förvillar kroppens immunförsvar. Många års forskning har lett till att vi nu har en ganska god förståelse om de molekylära interaktioner som utspelar sig mellan streptokocken och immunförsvaret under en infektion med *S. pyogenes*. Denna avhandling bygger på fyra vetenskapliga artiklar som alla berör det molekylära samspelet mellan *S. pyogenes* och den mänskliga värden.

Delarbete I

När den mänskliga kroppen registrerar att den blivit infekterad av en sjukdomsframkallande organism, svarar den genom att försätta det infekterade området i ett tillstånd av inflammation. Inflammationen gör att olika bakterieavdödande celler och

proteiner kan ta sig ut till det infekterade området och förhindrar på så sätt att infektionen sprider sig. Vid inflammation finns det också alltid plasma närvarande. Plasma består av blodvätska samt olika proteiner. I delarbete I undersöker vi hur streptokocker reagerar när de hamnar i en miljö som innehåller plasma. Förekomst av plasma signalerar till *S. pyogenes* att de är inne i en mänsklig kropp, samt är ett tecken på att en inflammatorisk process har dragits igång. Det är intressant att veta vilka försvarsmekanismer bakterierna då uttrycker för att förhindra upptäckt av immunförsvaret. Intressant nog visade det sig att *S. pyogenes* snabbt arrangerar om sitt uttryck av proteiner på bakterieytan när de kommer i kontakt med plasma. Genom att förhöja mängden C5a-peptidas och M-proteiner (två viktiga streptokockproteiner som lurar människans immunförsvar) på bakterieytan gör sig bakterien redo att möta det ankommande immunförsvaret. Kanske ännu mer intressant är upptäckten att *S. pyogenes* uttrycker olika varianter av C5a-peptidaset och M-proteinet när de kommer i kontakt med plasma. Den upptäckten är intressant med tanke på att en förändring av proteiners tredimensionella struktur kan medföra att de uppträder på nya sätt. Än så länge kan man inte dra några säkra slutsatser kring varför bakterierna uttrycker olika former av C5a-peptidaset och M-proteinet, men det är troligt att de har en betydande roll för *S. pyogenes* sjukdomsframkallande egenskaper.

Delarbeten II, III och IV

När plasma läcker ut i infektionshärden följer stora mängder antikroppar med. Antikroppar är proteiner som snabbt och effektivt binder upp till bakterieytor och andra kroppsfrämmande element. En antikropp som har bundit upp till en bakterieyta känns lätt igen av andra komponenter av immunförsvaret som snabbt avdödar bakterien. Eftersom antikroppar har en så viktig roll i kroppens immunförsvar är de en given angreppspunkt för bakterier som vill hålla sig gömda i kroppen. Man vet sedan tidigare att *S. pyogenes* skyddar sig mot antikroppsattacker på tre sätt: 1) Genom att själv binda upp antikroppar till sin yta på ett sätt som förhindrar celler och andra proteiner från att känna igen antikropp-bakteriekomplexet, 2) genom att klippa av en sockermolekyl från antikroppen vilket leder till att antikroppen förlorar mycket av sin stabilitet, samt 3) genom att uttrycka ett enzym som klyver och förstör antikroppar.

I delarbete II identifierar och karaktäriserar vi ett tidigare okänt streptokockenzym (IdeS) som klyver den mänskliga antikroppen IgG i två delar. Denna klyvning gör att *S. pyogenes* överlever när de inkuberas med celler som i vanliga fall snabbt hade avdödat

bakterierna. Detta försök i kombination med att IdeS förhindrar avdödning av *S. pyogenes* i immunt blod visar vilken stor betydelse IdeS tycks ha för bakteriernas förmåga att undgå upptäckt av kroppens immunförsvar. I motsats till det tidigare kända streptokockenzymet (SpeB), klyver IdeS IgG med en oerhörd specificitet. Vi visar också att IdeS uttrycks under förhållanden som tros likna den tidiga fasen av en streptokockinfektion, tillika den fas då antikroppsklyvning borde vara av störst betydelse.

I delarbete III visar vi att det är den enzymatiska aktiviteten som är avgörande för IdeS förmåga att rädda *S. pyogenes* från immunförsmedierad avdödning. Genom att ändra om genen som kodar för IdeS, framställde vi en IdeS-variant som är identisk med vanligt IdeS förutom att den inte kan klyva IgG. När *S. pyogenes* utsätts för specifika förhållanden som normalt sett dödar bakterien, förhindrar IdeS till skillnad från IdeS-varianten, att *S. pyogenes* avdödas.

I delarbete IV undersöker vi vad som händer med IdeS när immunförsvaret väl har känt igen *S. pyogenes* och slagit på alla sina bakteriedödande mekanismer. En försvarsmekanism som immunförsvaret använder sig av är att skicka ut två enzymer som kallas för elastas och cathepsin G. Elastas och cathepsin G kan förstöra bakteriella proteiner och ibland även döda bakterier. Intressant nog visar vi att IdeS behåller sin förmåga att klyva IgG även efter att det har kommit i kontakt med elastas och cathepsin G. Vi tror inte att detta är en slump utan att *S. pyogenes* under evolutionens gång har utformat IdeS så att de ska tåla angrepp från mänskliga enzymer. En annan observation är att små fragment av IdeS klyvs loss av elastas och cathepsin G. Dessa fragment tycks vara särskilt lätta för kroppens immunförsvar att känna igen och patienter som tidigare varit infekterade med *S. pyogenes* har antikroppar riktade mot dessa fragment i större utsträckning än mot det resterande proteinet. Att medvetet designa IdeS så att lättigenkänliga delar klyvs av efter att IdeS har upptäckts är en intressant tanke som ytterligare förstärker intrycket av att *S. pyogenes* är exceptionellt välanpassad för att hålla sig undan människans immunförsvar.

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