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PHAGOCYTIC CELLS AND *STREPTOCOCCUS PYOGENES* IN INVASIVE INFECTIONS: AN INFLAMMATORY RELATIONSHIP

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my family

POPULAR SCIENTIFIC SUMMARY

Streptococcus pyogenes is a bacterium capable of causing a wide range of diseases, from mild infections like tonsillitis (strep-throat) to life threatening conditions like necrotizing fasciitis (NF).

Also known as flesh-eating disease, NF is a rapidly progressing disease characterized by substantial destruction of the body's soft tissues. In severe streptococcal infections, the immune defense mechanisms are activated in an attempt to eradicate the bacteria. However, an excessive immune response can also be harmful and cause damage to the patient's own cells and tissue.

This thesis aimed to study interactions between the immune system and the bacteria, and how these may contribute to the disease.

Focusing on neutrophils and macrophages, the foot soldiers of the immune system, and the inflammatory molecule HMGB1 we could see that there are high amounts of HMGB1 in infected tissue, and that HMGB1 can attract neutrophils. We also found that streptococci are potent activators of neutrophils, leading to release of inflammatory molecules. In contrast, we found that macrophages become more anti-inflammatory during streptococcal infections. We have previously seen that streptococci can infect and survive inside macrophages, and it is tempting to speculate that the anti-inflammatory phenotype may promote bacterial survival.

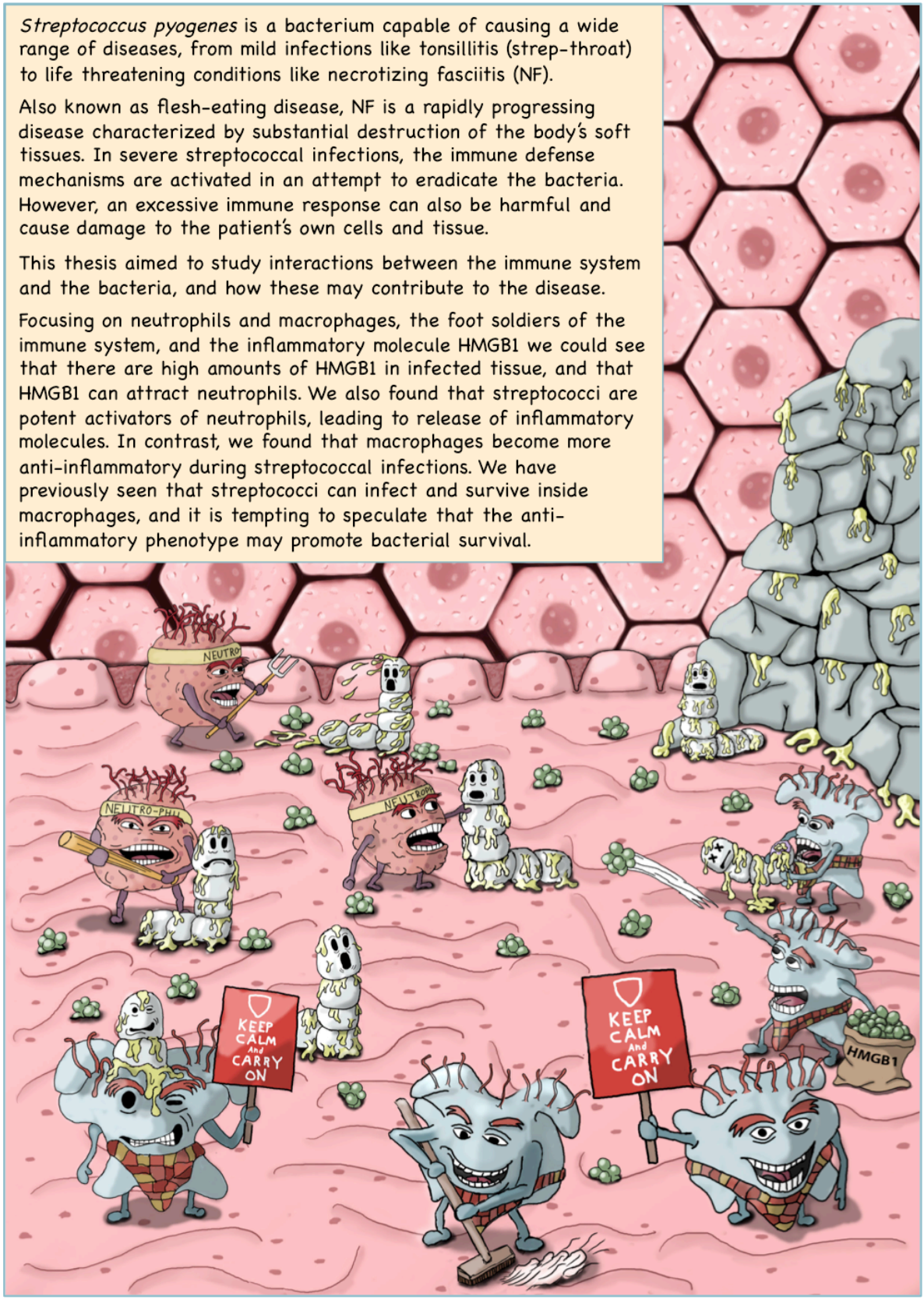


Illustration by Andreas Mielonen

ABSTRACT

Severe *Streptococcus pyogenes* infections, such as streptococcal toxic shock syndrome and necrotizing soft tissue infections, are rare but life threatening conditions. They are characterized by high bacterial load and a hyper-inflammatory state. The aim of this thesis was to investigate interactions between *S. pyogenes* and the phagocytic cells neutrophils and macrophages and how this correlates to cell activation and inflammation.

First, we investigated high mobility group box 1 (HMGB1), a marker of inflammation and necrosis, as a potential biomarker and mediator of tissue pathology in *S. pyogenes* tissue infections. Analysis of tissue biopsies collected from patients with streptococcal soft tissue infections of varying severity showed that HMGB1 was present in the tissue, and that the amount correlates with severity. Further investigations showed that HMGB1 co-localized with IL-1β suggesting the potential for immunostimulatory complexes to form at the site of infection. HMGB1 was also demonstrated to act as a chemoattractant for neutrophils.

Next, we assessed neutrophil activation and degranulation in response to different bacterial species, focusing on the release of the sepsis-associated factors heparin-binding protein (HBP) and resistin. Stimulations of neutrophils showed that streptococcal strains were potent inducers of neutrophil activation and degranulation. The results also showed a difference in signaling requirements for the release of HBP and resistin, respectively. While HBP release was mainly dependent on a previously described mechanism involving dual ligation of integrins and Fc-receptors, the release of resistin appeared to be multifactorial and involve multiple bacterial structures and host signaling pathways.

Finally, we set out to define the macrophage phenotype present at the site of infection. Using a multi-parameter imaging workflow, we were able to assess the phenotype of macrophages present at the site of infection, in tissue from patients with severe *S. pyogenes* soft tissue infections as well as infected organotypic skin tissue models. These investigations showed that macrophages in *S. pyogenes* infected tissue displayed a shift towards a more anti-inflammatory M2-like phenotype, in spite of the hyper-inflammatory environment in the tissue. Gene expression analysis of infected patient tissue, skin tissue models as well as a murine model of severe streptococcal soft tissue infection showed an overrepresentation of signaling pathways associated with anti-inflammatory macrophage polarization.

Taken together these findings highlight the complex pathophysiology of severe *S. pyogenes* infections, where on the one hand the bacteria and mediators present in the infected tissue potently activates neutrophils. While macrophages on the other hand, display a more anti-inflammatory phenotype upon infection, potentially promoting intracellular survival and persistence of *S. pyogenes*. Emphasizing the importance of careful patient characterization with regards to immune status, to ensure optimal treatment.

LIST OF SCIENTIFIC PAPERS

I. Johansson L, **Snäll J**, Sendi P, Linnér A, Thulin P, Linder A, Treutiger CJ, Norrby-Teglund A. HMGB1 in severe soft tissue infections caused by *Streptococcus pyogenes*. *Frontiers in Cellular and Infection Microbiology (2014) Jan 30;4:4.*

II. **Snäll J**, Linnér A, Uhlmann J, Siemens N, Ibold H, Janos M, Linder A, Kreikemeyer B, Herwald H, Johansson L, Norrby-Teglund A. Differential neutrophil responses to bacterial stimuli: Streptococcal strains are potent inducers of heparin-binding protein and resistin-release. *Scientific Reports (2016) Feb 18;6:21288.*

III. **Snäll J**, Itzek A, Nookala, Benis N, Thänert R, Mukundan S, Saccenti E, Hyldegaard O, Nekludov N, Karlsson Y, Oppegaard O, Arnell O, Pieper D, Martins dos Santos V, Kotb M, INFECT Study group, Svensson M and Norrby-Teglund A. Macrophage phenotype in group A *Streptococcus* infected soft tissue: an anti-inflammatory shift in a hyper-inflammatory milieu. *Manuscript.*

LIST OF ADDITIONAL PAPERS, NOT INCLUDED IN THE THESIS

- Mairpady Shambat S, Siemens N, Monk IR, Mohan DB, Mukundan S, Krishnan KC, Prabhakara S, **Snäll J**, Kearns A, Vandenesch F, Svensson M, Kotb M, Gopal B, Arakere G, Norrby-Teglund A. A point mutation in AgrC determines cytotoxic or colonizing properties associated with phenotypic variants of ST22 MRSA strains. *Scientific Reports (2016) Aug 11;6;31360*

- Neumann A, Papareddy P, Westman J, Hyldegaard O, **Snäll J**, Norrby-Teglund A, Herwald H. Immunoregulation of Neutrophil Extracellular Trap Formation by Endothelial-Derived p33 (gC1q Receptor). *Journal of Innate Immunity (2017) Oct 17.*

CONTENTS

LIST OF ABBREVIATIONS

ACIA	Aquired computerized image analysis
APC	Antigen presenting cell
CARS	Compensatory anti-inflammatory response syndrome
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
ERK	Extracellular signal-regulated kinase
fMLP	N-formyl-methionyl-leucyl-phenylalanine
GM-CSF	Granulocyte-macrophage colony stimulating factor
GPCR	G-protein-coupled receptors
HA	Hyaluronic acid
HBP	Heparin-binding protein
ICAM	Intercellular adhesion molecule
IHC	Immunohistochemistry
IFN	Interferon
IL	Interleukin
IRAK	Interleukin-associated kinase
ITAM	Immunoreceptor tyrosine-based activation motif
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
LPS	Lipopolysaccharide
LTB_4	Leukotriene B_4
MAP	Mitogen-activated protein
M-CSF	macrophage colony stimulating factor
Mga	Multiple gene regulator
MHC	Major histocompatibility complex
MPO	Myeloperoxidase
MyD88	Myeloid differentiation primary response 88
NADase	NAD^+-glycohydrolase
NADPH	Nicotinamide adenine dinucleotide phosphate
NETs	Neutrophil extracellular traps
NF	Necrotizing fasciitis
NHDF	Normal human dermal fibroblast
NLRs	NOD-like receptors
NSTI	Necrotizing soft tissue infection
PAMP	Patogen-associated molecular pattern

PI3K	Phosphatidylinositol 3-kinases
PRR	Pattern recognizing receptors
PSGL	P-selectin glycoprotein ligand
RAGE	Receptor for advanced glycosylated end products
Ralp	RofA-like protein regulators
RLR	RIG-I like receptors
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SIC	Streptococcal inhibitor of complement
SIRS	Systemic inflammatory response syndrome
Ska	Streptokinase
SLO	Streptolysin O
SLS	Streptolysin S
SMEZ	Streptococcal mitogen exotoxin Z
Spe	Streptococcal pyrogenic exotoxin
SpyCEP	*S. pyogenes* cell-envelope protease
SSA	Streptococcal superantigen
STAT	Signal transducer and activator of transcription
STSS	Streptococcal toxic shock syndrome
Syk	Spleen tyrosine kinase
TCS	Two component systems
TGF	Transforming growth factor
Th	T-helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
T-regs	Regulatory T-cells

1 BACKGROUND

This thesis work has been focused around the immune mediated pathophysiology of invasive infections caused by *Streptococcus pyogenes*. These are complex multifactorial infections and the pathogenesis is dependent on a wide range of bacterial virulence factors that interact with numerous host cells, immune effector molecules and extracellular matrix components.

In particular, this thesis has investigated the role of, high mobility group box 1 (HMGB1) (a marker linked to necrosis and inflammation), neutrophil activation and degranulation as well as polarization of macrophages, in severe streptococcal soft tissue infections. While these topics at first glance may appear quite distinct, they all represent central components of the immune defense against infections as well as the immune mediated pathology in severe streptococcal infections.

1.1 *STREPTOCOCCUS PYOGENES* INFECTIONS

S. pyogenes is a Gram-positive, facultative anaerobic bacterium that grows in chains and forms beta-hemolysis on blood agar plates.

Listed as number 9 on the list of global killer pathogens, causing an estimated 500.000 deaths yearly *S. pyogenes* is an important human pathogen. It can cause a wide range of diseases in immunocompetent individuals, ranging from non-invasive superficial infections of the skin and throat such as impetigo and pharyngitis, to severe life threatening conditions like necrotizing fasciitis (NF) and streptococcal toxic shock syndrome (STSS) (1). Severe invasive streptococcal infections like NF and STSS are rare, affecting approximately three out of every 100.000 individuals (2, 3). However, they still represent a substantial health problem as they cause significant morbidity and mortality, with an overall case fatality of up to 50% despite intensive care and antibiotic treatment (4).

NF is a form of necrotizing soft tissue infection (NSTI). This is a rapidly progressing infection of the subcutaneous soft tissue, characterized by inflammation and tissue necrosis, often accompanied by extreme pain. NSTIs can be subdivided based on the microbiological aetiology into type I (poly-microbial infections), type II (mono-microbial infections) or type III (infections by marine organisms). While they can be caused by a variety of microbes, *S. pyogenes* represents the dominant pathogen in mono-microbial (type II) NSTIs, which tend to occur in otherwise healthy immunocompetent individuals (5, 6). NF is often associated with systemic toxicity, and 30-50% of patients with NF caused by *S. pyogenes* will also develop STSS (4), in which case the fatality rates increase further and may reach over 60% (7). STSS is a systemic condition characterized by excessive immune activation, mediated by bacterial toxins so-called superantigens, resulting in hypotensive shock and multiple organ failure (8). While STSS often occurs in association with a localized infection, such as NF, the port of entry is unknown in about 50% of cases (8, 9). In these cases a transient bacteremia originating from the oropharynx has been proposed as the bacterial source (8).

In addition, *S. pyogenes* infections may be followed by autoimmune post-streptococcal sequelae, like acute rheumatic fever, rheumatic heart disease and glomerulonephritis (10). These conditions are more commonly found in developing countries where repeated superficial infections of the throat or skin, left untreated, are believed to underlay these sequelae (1).

1.1.1 Epidemiology

S. pyogenes isolates can be identified and characterized using different serological or genotyping methods. One of the traditionally most commonly used methods is M-typing. This is a serological method, established by Rebecca Lancefield in the 1920s, based on the surface expressed M-protein and type-specific sera targeting the hyper-variable region (11). Serological typing can also be performed targeting another surface protein called the T-protein (12) or serum opacity factor, a lipoproteinase expressed by approximately half of *S. pyogenes* strains, and that causes increased opacity in serum (13). Today, typing of *S. pyogenes* strains is most commonly done by sequencing of the hyper-variable part of the *emm* gene (which encodes the M-protein) (14), and there is currently more than 200 different *emm*-types identified (15). Antibodies to the hyper-variable part of the M-protein are type-specific, and as a consequence, individuals that have been infected by a certain *emm*-type, typically develop protective antibodies towards that *emm*-type but remain susceptible to other types (16).

Epidemiological studies of *S. pyogenes* strains have shown that the prevalence of different M-types in the community vary over time and geographic area. Studies have, however, also shown an association between certain M-types and specific clinical presentations. For instance, M1- and M3-type isolates are over-represented among severe invasive infections like NF and STSS (2, 17).

1.1.2 Pathogenesis

S. pyogenes is an exclusively human pathogen, and has adapted its expression of virulence factors accordingly. The pathogenesis of invasive *S. pyogenes* infections is complex and involves several different bacterial factors as well as host mediators (18, 19).

The bacterium expresses a wide array of virulence factors, which may be divided into surface associated or secreted factors (Fig. 1). Importantly, there is a considerable heterogeneity among streptococcal strains, and the virulence factors expressed by these strains. Also, several factors have multiple functions, and their role in pathogenesis may vary during different stages of infection. Described below are some virulence factors that have been found to be important in the pathogenesis of severe streptococcal infections.

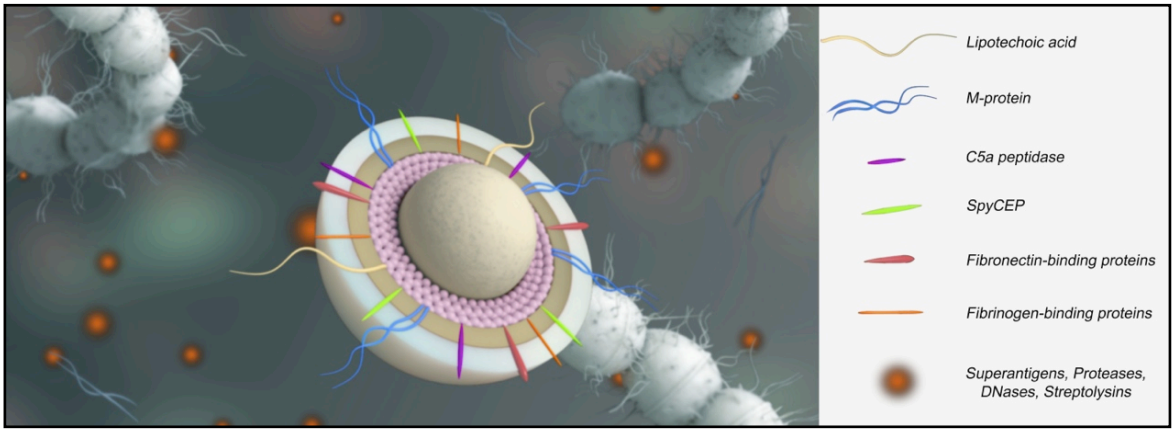


Figure 1. Schematic overview of selected surface-associated or secreted streptococcal virulence factors. *Illustration by Andreas Mielonen*

1.1.2.1 Surface-associated factors

In general, surface-associated factors are important for adherence to host cells and evasion of phagocytosis, through various mechanisms including host mimicry and inactivation of host effector molecules.

S. pyogenes express an anti-phagocytic hyaluronic acid (HA) capsule, with HA similar to that of the human connective tissue. In addition to allowing the bacteria to disguise itself as an immunological "self", HA also enables the bacteria to adhere to epithelial cells by interacting with CD44 (19, 20).

The bacteria also express surface associated fibronectin-binding proteins. To date, there are at least 11 fibronectin-binding proteins described in *S. pyogenes*. They mediate adhesion and invasion of human cells through binding of the human extracellular matrix protein fibronectin. In addition, some fibronectin-binding proteins have also been shown to be anti-phagocytic, inhibiting complement opsonization of the bacteria (21).

C5a peptidase is an enzyme that is expressed on the streptococcal surface, where it is capable of cleaving and inactivating human C5a, an important chemoattractant for human immune cells (22). *S. pyogenes* cell-envelope protease (SpyCEP) is another enzyme expressed on the streptococcal surface, capable of cleaving and inactivating chemoattractants for human immune cells (22). It cleaves interleukin (IL)-8, which is an important chemoattractant for human neutrophils, and has hence been suggested to impair neutrophil recruitment to the site of infection. In murine models, SpyCEP activity has been observed to decrease IL-8 dependent neutrophil recruitment and bacterial killing, as well as play an important role in bacterial dissemination (23, 24). Furthermore, an increased SpyCEP activity was reported in clinical isolates from patients with invasive as compared to non-invasive *S. pyogenes* infection, and as the four isolates recovered from fatal cases had the highest SpyCEP activity, the data suggested a potential link to disease severity (25).

One of the most well studied virulence factors of *S. pyogenes* is the M-protein, the same protein as used for serological M-typing or genetic *emm*-typing. The protein itself is a fibrillar protein that protrudes from the streptococcal surface like a strand of hair. One of the best-known properties of the M-protein is its ability to inhibit phagocytosis, which is achieved by interference with opsonization and deposition of complement. For instance, the M-protein can bind host plasma proteins like albumin and fibrinogen, thereby coating the bacterial surface with these proteins and hindering complement deposition (26). It can also bind the human complement inhibitor C4b-binding protein, which retains its inhibitory function after being bound by the M-protein and can prevent complement deposition on the bacterial surface (27, 28).

More recent studies have identified additional functions of the M-protein, including binding and adherence to host cells by interacting with extracellular matrix proteins like collagen and fibronectin (26). Also, the M-protein has been shown to mediate survival of intracellular *S. pyogenes* in neutrophils (29, 30) and macrophages (31).

The M-protein can be cleaved of the bacterial surface by either bacterial or human proteases. An important finding is that shedded M-protein has pro-inflammatory properties, and may interact with monocytes through toll-like receptor (TLR)2 and induce the expression of cytokines like IL-6, IL-1β and tumor necrosis factor (TNF) (32). Observations have also been made, suggesting that soluble M-protein may act as a superantigen and induce T-cell activation and subsequent release of large amounts of cytokines like lymphotoxin α and interferon (IFN)γ (33).

Importantly for this thesis, soluble M-protein can form complexes with the human protein fibrinogen, which in turn are capable of activating neutrophils. In a seminal study by Herwald et al. it was shown that M1-protein-fibrinogen complexes are able to bind β2-integrins on the neutrophil surface, thereby activating the cell and inducing the release of heparin-binding protein (HBP) (34). HBP is a potent inducer of vascular leakage and has been proposed to play an important role in the pathophysiology of circulatory failure associated with severe sepsis and septic shock (35, 36).

1.1.2.2 Secreted factors

While many of the factors expressed on the streptococcal surface are important for adherence and evasion of phagocytosis, secreted factors mainly contribute to immune evasion mechanisms as well as dissemination and growth of the bacteria.

Two well-characterized secreted factors of *S. pyogenes* are streptolysin O (SLO) and streptolysin S (SLS), which are pore-forming hemolysins capable of lysing erythrocytes, platelets and neutrophils. While most *S. pyogenes* strains produce both SLO and SLS there are some strains that do not produce these factors, or only produce one or the other (37). SLO and SLS have also been show to aid in the dissemination of *S. pyogenes,* by inducing death of infected phagocytes and degradation of intercellular epithelial junctions (38-40). In addition,

a streptolysin-dependent escape of intracellular streptococci into the cytosol has been reported (41-43).

NAD^+-glycohydrolase (NADase) is a toxin secreted from the bacteria that promotes cytotoxicity by depletion of energy stores, through cleavage of nicotinamide and adenosine diphosphoribose in host cells. It has also been reported to interfere with innate immune response by decreasing inflammasome dependent IL-1β secretion from activated macrophages (44). Furthermore, it was recently shown that SLO and NADase may form complexes, stabilizing these two factors and augmenting the cytotoxic effect of SLO (45).

In addition, *S. pyogenes* may also secrete several factors capable of degrading various extracellular matrix components, thereby aiding in the spread of bacteria through tissue planes, a characteristic feature of invasive streptococcal tissue infections. These factors include amongst others; DNA degrading enzymes (DNases) like Sda1, enzymes that degrade HA (hyaluronidases) and streptokinase (Ska) that can activate the human enzyme plasminogen to plasmin, which in turn can degrade blood clots and fibrin barriers (37).

Streptococcal pyrogenic exotoxin (Spe)B is a broad-spectrum cysteine protease secreted by the bacteria. SpeB has many substrates and can degrade both bacterial as well as human factors. Some of the bacterial substrates include factors like superantigens, Ska, protein F1 and Sda1. Human substrates include various human extracellular matrix components, as well as several other factors including, immunoglobulin, chemokines, fibrinogen and plasminogen and antimicrobial peptide LL-37 (46). Due to this broad proteolytic activity targeting also bacterial virulence factors, SpeB expression is tightly regulated. Among others *S. pyogenes* has a sophisticated protection at the bacterial surface through expression of a bacterial surface protein that bind the human protein α2-macroglobulin (47). α2-macroglobulin is an abundant protease inhibitor and was shown to entrap and inhibit SpeB, thereby, rendering the bacteria protected against SpeB-degradation of surface associated virulence factors.

S. pyogenes also secretes toxins with superantigenic activity, and recent studies have revealed that they are important for *S. pyogenes* adherence and colonization (48). To date, 11 distinct streptococcal superantigens have been identified; SpeA, SpeC, SpeG-M, streptococcal superantigen (SSA) and streptococcal mitogen exotoxin Z (SMEZ) (49). Superantigens are characterized by their ability to bind the major histocompatibility complex (MHC) class II molecule on antigen presenting cells (APCs) and the Vβ chain of the T-cell receptor, without prior processing (49). This unconventional interaction bypassing the normal rules for antigen processing and presentation leads to a T-cell activation involving up to 20% of the resting T-cell population, as compared to a conventional antigen that activates an antigen-specific T-cell clone, constituting less than 0.01% of the resting T-cell population. This massive T-cell activation leads to release of excessive levels of pro-inflammatory cytokines like IL-1β, IL-2, TNF and IFNγ, often referred to as a "cytokine storm" (50). Importantly, this excessive cytokine release has been shown to be an important contributor to hypotensive shock and organ failure observed in patients with severe streptococcal infections (51, 52).

1.1.2.3 Regulation of virulence factors

The regulation of virulence factors is complex as the bacteria needs to be appropriately equipped during the different stages of infections, involving factors for adherence and colonization of the host, as well as factors for invasion, immune modulation and dissemination. The expression of these factors is typically regulated through two component systems (TCS) and stand-alone regulators. TCSs are often used by the bacteria to sense and respond to signals from the extracellular environment. They generally consist of two parts; a transmembrane sensory histidine kinase (that recognizes the extracellular signal) and an intracellular cytoplasmic response regulator (that transduces the signal from the kinase, and either activates or represses gene expression) (53). The CovR/S system is one of the best-characterized TCS in *S. pyogenes*, and controls many of the virulence factors important for infection.

In a murine model of *S. pyogenes* skin and soft tissue infection, a switch from a non-invasive to an invasive phenotype was observed in response to a spontaneous mutation within the CovR/S system. This mutation was associated with reduced expression of SpeB and increased expression of the DNase Sda1 (54). Moreover, the down-regulation of SpeB seen in the invasive CovR/S mutant has been proposed to protect several surface-associated and secreted virulence factors (55), thereby promoting invasive infection and bacterial dissemination. In support of this notion, Kansal et al. reported an inverse correlation between disease severity and expression of SpeB among M1T1 isolates from invasive streptococcal infections (56). However, analysis of tissue biopsies from patients with *S. pyogenes* NSTI has shown high levels of SpeB at the site of infection (57), and bacterial cultures directly from patient tissue sections revealed the majority of patient biopsies showed a presence of both SpeB-positive and SpeB-negative clones (58). Also, a study investigating a large collection of approximately 10.000 clinical isolates found that the majority of isolates expressed SpeB (59).

Stand-alone regulators activate or repress transcription without input from an equivalent sensory histidine kinase, as the TCSs. However, that is not to say that these regulators do not respond to input from other signaling components, just that the sensory elements have not been identified. For the most part these regulators contain DNA-binding domains and can interact directly with the promoters of the genes that they control. The best characterized stand-alone regulators are multiple gene regulator (Mga) and RofA-like protein regulators (Ralps) that regulate genes involved in adherence and colonization and genes involved in internalization and persistence, respectively (53).

1.2 IMMUNE RESPONSES

The primary function of the immune system is to sense and protect the host from infectious agents and foreign elements. Constituting a number of different cell types, tissues and organs, the immune system is typically divided into innate and adaptive immunity. Traditionally, innate immunity is characterized by a rapid response to conserved microbial patterns, recognized by a large number of cells. Adaptive immunity, however, is composed of a small number of cells with the capacity of recognizing specific pathogens. Because of the small number, the responding cells have to proliferate and expand to attain sufficient numbers to mount an effective response, a process that takes several days. Importantly, the adaptive immune response is also able to produce long-lived cells that exist in a dormant state and rapidly get activated upon another encounter with the specific pathogen, so called immunologic memory, permitting a more effective response against that pathogen (60). Innate and adaptive immunity are often referred to as separate arms of the immune response, however, they typically act in concert, and synergy between them is essential for an effective immune response.

1.2.1 Innate immunity

The innate immune response is often referred to as the "first line defense" against pathogens, it is phylogenetically well conserved and can be found in essentially the same template from primitive life-forms to humans (61). However, before encountering any of the innate effector cells, the invading pathogen first has to cross the anatomic and physical barriers surrounding the body, like skin, respiratory epithelium and the epithelium of the gastro-intestinal tract. These are not just passive borders, but have several anti-microbial mechanisms of their own, like mucus secretion, cilia, peristalsis, resident microbial flora and antimicrobial peptides (62). If the outer barriers are breached the next line of defense are the innate effector cells, including professional phagocytes like macrophages, DCs and neutrophils, as well as lymphoid cells such as innate lymphoid cells, natural killer cells, gamma delta T cells and mucosal-associated invariant T-cells (62).

The cells of the innate immune system are capable of recognizing a wide spectrum of pathogens through evolutionary conserved patterns that are crucial for the pathogen to survive, so called pathogen-associated molecular patterns (PAMPs). The receptors responsible for recognizing PAMPs are usually grouped together as pattern recognizing receptors (PRRs). However, there are several different types of PRRs, capable of recognizing different types of structures like RNA and DNA from replicating intracellular pathogens, and factors like lipopolysaccharide (LPS) of Gram-negative bacteria and lipoteichoic acid of Gram-positive bacteria (63)

The TLR family is one of the best-characterized classes of PRRs. To date, 10 distinct TLRs have been identified in humans, of these approximately half are expressed on the cell surface while the others are located inside the cell in intracellular vesicles (64). Two other important forms of PRRs are NOD-like receptors (NLRs) and RIG-I like receptors (RLRs). These

receptors can be found in the cytoplasm of the cell and while RLRs mainly recognize viral RNA, the NLRs recognize a wide range of ligands including bacterial RNA (63).

Fc- and complement receptors represent two other groups of receptors that are important in the host defense against pathogens. They interact indirectly with the invading pathogen via antibodies or complement deposited on the bacterial surface and promotes phagocytosis by innate immune cells.

1.2.2 Adaptive immunity

Lymphocytes are the main effector cells of the adaptive immunity; they are produced by the bone marrow and can be divided into B- and T-lymphocytes. The adaptive responses are based primarily on the antigen-specific receptors expressed by these cells. B-cells have B-cell receptors and can upon activation produce antigen-specific antibodies capable of recognizing and neutralizing pathogens or toxins. Antibodies are also important for opsonization of bacteria for phagocytosis and destruction by effector cells like neutrophils and macrophages (65). T-cells, on the other hand, express T-cell receptors and can be divided into CD4+ T-helper (Th) cells and CD8+ cytotoxic T-cells, based on expression of surface markers and functions. CD8+ cytotoxic cells are important for the destruction of infected cells and tumor cells while CD4+ Th cells are more important for activation and regulation of the immune response (65). Furthermore, upon activation CD4+ Th cells can differentiate into different subsets depending on the signals and cytokines present at the site of activation. These subsets include among others Th1, Th2, Th17, as well as regulatory T-cells (T-regs) and have different roles in control of the immune response and homeostasis, either by activation or suppression of inflammatory pathways (60).

Adaptive immunity is initiated by lymphocyte recognition of foreign antigens by the respective antigen-specific receptors. While B-cells are capable of recognizing antigens on their own, T-cells need to get antigens presented by MHC class I or II on antigen presenting cells (66).

1.2.3 Cytokines and alarmins

Activation of the immune system results in production and release of a variety of cytokines and chemokines that in turn induce effector functions like growth, differentiation, migration and activation of immune cells.

Initial activation of the innate immune cells initiates a signaling cascade resulting in the activation of nuclear factor–κB (NF-κB) and, among other functions, the transcription and translation of cytokines and chemokines like TNF, IL-1β, IL-6 and IL-8. The primary purpose of the cytokines and chemokines released during the initial stages of infection is to enhance leukocyte recruitment to the site of infection. TNF and IL-1β have both been shown to have effects on the vasculature, promoting vasodilatation and increased capillary

permeability. IL-6 is an important mediator of the acute phase response, IL-12 has been shown to be important for the activation of T-cells and development of adaptive immunity, and IL-8 is an important chemoattractant for neutrophils (67). In parallel with the pro-inflammatory cytokines and chemokines there is also a production of anti-inflammatory mediators like IL-10, transforming growth factor (TGF)β and numerous soluble cytokine receptors, aiming to counterbalance the inflammation and ensure that the inflammation does not get out of control (67).

IL-1β is a quintessential pro-inflammatory cytokine that broadly affects inflammatory processes, and tight control of its production is required. IL-1β is synthesized as an inactive zymogen, which needs to be activated by a cysteine protease, typically caspase-1 (68). Caspase-1 is also synthesized as an inactive zymogen, and is activated only after incorporation into and activation of the inflammasome. The inflammasome is a large, multi-protein complex present in the cytosol of innate immune cells that is assembled in response to cytosolic recognition of microbial products or endogenous molecules released from damaged or dying cells. In addition to cytokine processing inflammasomes also regulate pyroptosis, which is a form of cell death (69).

Several recent studies have highlighted inflammasome activation and IL-1β as important contributor to the pathophysiology of severe soft tissue infections caused by *S. pyogenes* (70, 71). Notably LaRock et al. recently showed that also streptococcal SpeB can cleave the IL-1β zymogen (72), and they propose that IL-1β might be a sensor of bacterial proteolysis and hence of infection.

Alarmins constitute a group of constitutively expressed, endogenous molecules with chemotactic and immune activating properties. Normally located inside the cells, it is only when they are released into the extracellular space by damaged or dying cells that they become immune activating. Notably, these alarmins are recognized by PRRs, and therefore they have also been termed danger-associated molecular patterns (DAMPs) (73).

1.2.3.1 High mobility group box 1

HMGB1 is an alarmin that has been implicated in a variety of clinical conditions, including arthritis, sepsis, and chronic kidney disease (74). Originally identified as a nuclear protein involved in chromatin remodeling, HMGB1 was discovered as a late mediator of sepsis lethality in a seminal study by Wang and colleagues in 1999 (75). Compared to classical sepsis-associated cytokines like TNF and IL-1β, which reach their maximum concentrations within hours after challenge and then decline to normal levels quite rapidly, HMGB1 displays delayed kinetics. It starts to appear in circulation after 8h, and peak levels are seen after 16-32h (75). The delayed kinetics, in combination with an observed protective effect of injections with HMBG1-neutralizing antibodies in murine models, has raised an interest in HMGB1 as a potential target for treatment in sepsis (75). Furthermore, a study by Sundén-

Cullberg et al. found elevated levels of HMGB1 in circulation in patients with severe sepsis or septic shock (76).

Being a nuclear protein, there are two main ways for HMGB1 to reach the extracellular environment, either through passive release by injured or necrotic cells, or by being secreted by activated immune cells like macrophages, DCs and natural killer cells (77-79). Inside the cell, HMGB1 shuttles between the nucleus and the cytoplasm, an energy driven process involving posttranslational modifications like acetylation and phosphorylation (80). Upon active secretion, HMGB1 is retained in the cytoplasm by hyper-acetylation and stored in secretory lysosomes (80, 81). While the mechanism for loading hyper-acetylated HMGB1 into the secretory lysosomes is not fully elucidated, studies have shown that HMGB1 release from activated immune cells is mediated by inflammasome activation (82, 83).

Once released, HMGB1 has many receptors, including amongst others TLR4, TLR2, receptor for advanced glycosylated end products (RAGE) and CD24-Siglec-10 (84-87). Depending on the receptor, engagement of HMGB1 may lead to induction of processes like cell proliferation, cell migration, production and release of pro-inflammatory cytokines. In addition to direct receptor engagement, HMGB1 can also form potent immunostimulatory complexes with molecules like LPS, IL-1β and CXCL12, and subsequently signal, indirectly, through the receptor of the partner-molecule (88-90).

It has become increasingly evident that posttranslational modifications of HMGB1 effect not only its intracellular localization, but also its properties outside the cell. While acetylation does not appear to affect the binding and activities of extracellular HMGB1, the protein harbors three cysteine residues and the redox state of these appears to play essential roles. For instance, it has been shown that it is only when all three cysteines are fully reduced that HMGB1 is able to bind CXCL12 and signal through CXCR4, and thereby promote cell migration (91). However, when HMGB1 has a disulfide bond between two of the cysteines (C23 and C45) it can bind to TLR4 and promote activation of NF-κB and transcription of multiple pro-inflammatory cytokines (92, 93). Fully oxidized HMGB1 (terminal oxidation), which can be found in apoptotic cells and at inflammatory sites, loses its immunostimulatory effects and instead induces tolerance (94) (Fig. 2).

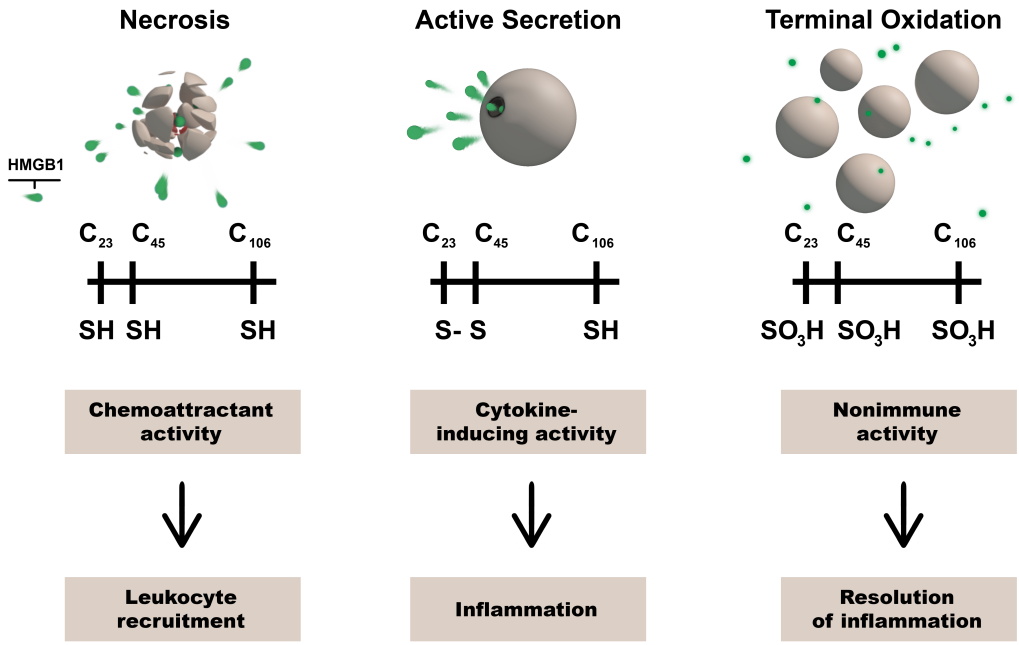


Figure 2. Overview of different forms of HMGB1 and their influence on inflammation.
Illustration by Andreas Mielonen

Furthermore, HMGB1 can bind TLR9 and TLR2 when in complex with CpG-oligodeoxynucleotides and nucleosomes, respectively, thereby activating cytokine production from macrophages and DCs (95-97). HMGB1 can also signal directly through RAGE, resulting in NF-κB activation as well as activation of mitogen-activated protein (MAP) signaling kinases (98). However, the redox state in these interactions is unknown (99).

During tissue damage HMGB1 orchestrates two key events, recruitment of leukocytes and activation of recruited leukocytes. In light of the findings that the different redox states of HMGB1 are present in the tissue sequentially (98), it has been proposed that; when released by necrotic cells in an injured tissue HMGB1 forms chemotactic complexes with CXCL12, which is present at low levels in extracellular fluids. HMGB1 may also promote production of additional CXCL12 by binding and signaling through the RAGE receptor. In the extracellular space, the oxidizing conditions will affect the fully reduced form of HMGB1 and convert it into the disulfide form. The leukocytes recruited by the HMGB1-CXCL12 complexes will encounter the disulfide form and start producing cytokines, chemokines and secrete more HMGB1. Finally, the fully oxidized form of HMGB1 will lose its pro-inflammatory effects and instead promote resolution of inflammation and tolerance (99).

Despite its association with sepsis, inflammation and necrosis, little is known regarding the role of HMGB1 in severe streptococcal soft tissue infections, which are often associated with systemic toxicity and sepsis. Furthermore, considering the pronounced tissue damage present at the site of infection HMGB1 should be released and may contribute to the observed immunopathology. This is explored in **Paper I**.

1.3 PHAGOCYTIC CELLS

Neutrophils, monocytes and macrophages represent the major groups of innate phagocytic cells. These cells engulf large particles like dying cells and pathogenic bacteria, through a process called phagocytosis. This process was first described by Elie Metchnikoff, for which he was awarded the Nobel prize in 1908 (100).

Neutrophils are efficient phagocytes capable of engulfing particles in less than 20 seconds (101), while macrophages require several minutes for a similar target (102). The initial step in phagocytosis is recognition of the bacteria or dying cell to be taken up, a process mediated by PRRs and other receptors. After recognition, the cellular membrane extends around the pathogen and encloses it in a vesicle (the phagosome). Initially the phagosome mainly contains extracellular fluids and is not especially harmful to the pathogen (103). However during phagosomal maturation, intracellular vesicles and granules are recruited and fused with the phagosome, thereby delivering bactericidal molecules (104). As the phagosome matures the pH decreases and becomes acidic. Neutrophil and macrophage phagosomal pH regulation differs, and while the macrophage phagosome gradually acidifies the neutrophil phagosome is initially alkaline and remains neutral for a prolonged period (103). The acidification of the phagosome leads to activation of hydrolytic enzymes like endopeptidases and hydrolases that kill the bacteria. Bacterial killing is also mediated by reactive oxygen species (ROS) and reactive nitrogen species (RNS) that are delivered to the phagosome by membrane bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (105).

1.3.1 Neutrophils

Neutrophils, also known as polymorphonuclear leukocytes, are often referred to as the foot soldiers of innate immunity. Constituting about 40-70% of circulating leukocytes in the blood of healthy individuals, the main job of these cells is to phagocytose and kill invading pathogens.

Neutrophils are derived from hematopoietic stem cells in the bone marrow, a process regulated by granulocyte colony stimulating factor (106). During their maturation, the cells pass through several steps where they develop additional features, like expression of surface receptors, phagocytic ability, intracellular granules and the ability to produce ROS (107). Traditionally neutrophils have been considered to be very short lived, only surviving a mere 5-10 hours if not activated and recruited to an infection site, which increases their life span (108). Recent findings have however indicated that neutrophils may remain for as long as 5 days in circulation under homeostatic conditions (109).

Neutrophils circulate in the blood stream, and are recruited to the site of infection through a process initiated by activation of the endothelium. Inflammatory mediators released from cells in the infected tissue activate the endothelial cells of the local post-capillary venules, resulting in expression of various adhesion molecules and integrins. The adhesion molecules and integrins bind their respective counterpart expressed on the neutrophil surface, allowing

the neutrophil to roll along, adhere and finally migrate through the vessel wall. To guide the neutrophils to the site of infection both the endothelial cells as well as the activated cells in the infected tissue secrete chemotactic molecules like leukotriene B_4 (LTB_4), C5a and IL-8 (110-113).

As previously mentioned, the main function of neutrophils is to engulf and kill invading pathogens. For this purpose they are equipped with several mechanisms for killing, such as the ability to release neutrophil extracellular traps (NETs), phagocytosis (described above) and degranulation (113). NETs are structures made up of long DNA strands, which are ejected from the neutrophil during a special kind of controlled cell death called NETosis. The DNA strands are decorated with antimicrobial granule proteins like histones, myeloperoxidase (MPO) and LL-37, forming a meshwork where bacteria may be trapped and killed outside the cell (110, 114, 115). Neutrophils are densely packed with intracellular granules, which contain antimicrobial and inflammatory effector molecules. Degranulation is the release of these molecules into the phagosome or the surrounding tissue. It is a strictly controlled process that involves granule recruitment, docking, priming and fusion with either the phagosomal or cellular membrane. Receptor recognition and cell activation induces remodeling of the cytoskeleton and granule mobilization. As the granules reach the target membrane, they dock and fuse with the membrane and release the granular content (116).

1.3.1.1 Neutrophil surface receptors and their signaling

Extracellular signals trigger an array of functional responses in neutrophils, leading to the infiltration and accumulation of neutrophils in the infected tissue, and mounting of an effective anti-microbial response. There are several different classes of receptors expressed on the surface of neutrophils, like G-protein-coupled receptors (GPCRs), Fc-receptors, adhesion receptors and cytokine receptors, as well as PRRs. Receptor engagement leads to activation of multiple complex intracellular signaling pathways, many of which are not completely understood, resulting in processes like chemotactic migration, phagocytosis, degranulation and NET release (117) (Fig. 3).

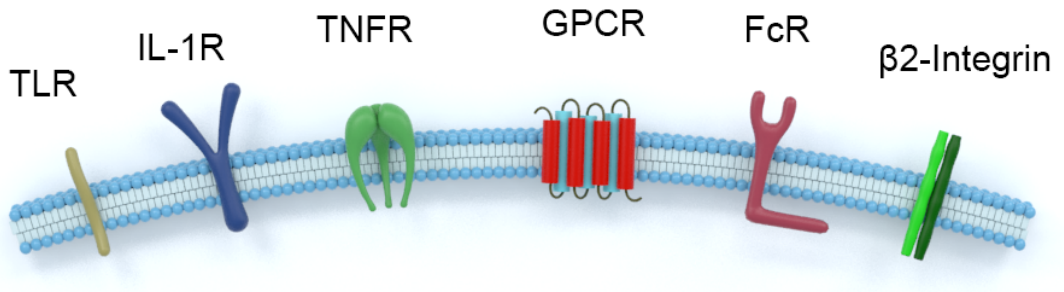


Figure 3. Overview of neutrophil surface receptors. There are several different classes of receptors expressed on the surface of neutrophils, like G-protein-coupled receptors (GPCRs), Fc-receptors (FcR), adhesion receptors (β_2-integrin) and cytokine receptors (TNFR and IL-1R), as well as PRRs (TLR). Receptor engagement leads to activation of multiple complex intracellular signaling pathways, resulting in processes like chemotactic migration, phagocytosis and degranulation. *Illustration by Andreas Mielonen*

During inflammation, bacterial and host derived immune stimulatory factors are abundant, and these factors stimulate endothelial cells nearby to produce and display adhesion molecules at the luminal side. As neutrophils circulate in the blood stream they continuously and randomly probe the vessel wall, and upon encounter with activated endothelial cells, neutrophils slow down and migrate out of the vessel through a multistep process of cell rolling, adhesion and transmigration (111).

For this, neutrophils express adhesion receptors like selectins, selectin ligands and integrins. Selectins are single-chain transmembrane glycoproteins that recognize a large number of carbohydrate containing cell surface molecules. L-selectin and P-selectin glycoprotein ligand-1 (PSGL-1) are expressed by neutrophils, and interact with P- and E-selectins on endothelial cells. They mediate transient interactions between leucocytes and the vessel wall during the initial phases of leukocyte extravasation, and slow down the neutrophil (111, 117).

Selectin-mediated interactions trigger an intracellular signaling cascade, where activated immunoreceptor tyrosine-based activation motif (ITAM)-bearing domains signal through tyrosine kinases like spleen tyrosine kinase (Syk) and phosphatidylinositol 3-kinases (PI3K), resulting in activation of integrins and slow rolling of the neutrophil (111, 118).

Integrins are heterodimeric transmembrane glycoproteins, made up by an α-chain and a β-chain. To date at least 18 different α chains and 8 different β chains have been identified, which may be paired in specific combinations depending on cell type (119). One important integrin family expressed by neutrophils is the β_2-integrin family that consists of the β_2-integrin (CD18) and an exclusive α-chain. Two important members of the β_2-integrin family are $\alpha L\beta_2$ (CD11a/CD18 or LFA-1), expressed by all circulating leukocytes, and $\alpha M\beta_2$ (CD11b/CD18 or Mac-1), which is primarily expressed by myeloid cells like neutrophils,

macrophages and monocytes (117). LFA-1 and Mac-1 interactions with adhesion molecules like intercellular adhesion molecule (ICAM)1, expressed by endothelial cells, are important for adhesion and transmigration. In activated neutrophils, LFA-1 adopts a high-affinity conformation leading to increased binding to the endothelium. This results in slow rolling and complete arrest of the neutrophil. Arrested neutrophils start spreading and crawling along the endothelium, primarily using Mac-1. After transmigration, the neutrophil migrate through the tissue using β_2-integrins and other not fully characterized adhesion receptors (111, 117).

After the neutrophil has left the blood vessel, and moved out into the tissue, it follows a chemotactic gradient toward the site of infection. During this process the chemoattractants bind to their respective neutrophil receptors, which are often GPCRs. Neutrophils express several different GPCRs including among others formyl-peptide receptors and receptors for classical chemoattractants like C5a, LTB_4 and IL-8 (113). In addition to chemotaxis, activation of these receptors also induces neutrophil responses like ROS production and degranulation. Activation of GPCRs leads to dissociation of the intracellular G-protein complex, and induction of intracellular signaling cascades leading to increase in intracellular calcium as well as activation of molecules like extracellular signal-regulated kinase (ERK) and p38 MAP kinases (117).

Neutrophils also express a number of cytokine receptors, these include conventional cytokine receptors that recognize cytokines like IL-4, IL-6 and IL-12, members of the IL-1/TLR-receptor super family, and members of the TNF-receptor super family. Engagement of these receptors triggers diverse biological functions like differentiation, activation and coordination of inflammatory responses, but also apoptosis (117).

Conventional cytokine receptors are thought to signal primarily through janus kinase (JAK)-signal transducer and activator of transcription (STAT) dependent pathways. IL-1/TLR-receptor super family, however, have been shown to signal trough an myeloid differentiation primary response 88 (MyD88) and interleukin-associated kinase (IRAK) dependent pathway, resulting in the activation of NF-κB, as well as MAP kinases like ERK, c-Jun N-terminal kinase (JNK) and p38. The TNF receptor family signals through recruitment of complexes with intracellular adapters, leading to the activation of NF-κB and MAP kinases, or activation of pro-apoptotic pathways and caspases depending on the complex (117, 120)

At the site of infection, phagocytosis represent the major mechanism of removing pathogens. It is an active, receptor-mediated, process during which particles are internalized by the cell (described above). The interaction between the cell and the pathogen can either be direct through PRRs or mediated by opsonins like antibodies.

Recognition of antibody-opsonized pathogens is mediated by Fc-receptors, capable of binding the Fc-domain of antibodies bound to antigen. Two Fc-receptors expressed by neutrophils are the low-affinity receptors FcγRIIa (CD32a) and FcγRIIIb (CD16b), they are thought to play important roles in neutrophil activation by immune complexes. It has been proposed that activation requires engagement of both receptors, where CD16b makes the initial contact and binding of the immune complexes after which synergistic ligation of both

receptors leads to full activation of the cell (117, 121). Activated neutrophils may also express the high-affinity Fc-receptor FcγRI (CD64), which is of diagnostic value (122, 123) but otherwise relatively poorly understood (117). Fc-receptor signaling is initiated through intracellular ITAM-bearing domains, via tyrosine kinases like Syk and results in NF-κB mediated responses like cytokine and chemokine release as well as NF-κB independent responses like oxidative burst and degranulation (124).

As previously mentioned, neutrophils express PRRs to recognize both PAMPS and alarmins. TLRs are members of the IL-1/TLR-receptor super family, and signal via MyD88 and NF-κB to induce cytokine production and other pro-inflammatory processes (117, 120).

Given the role of neutrophil dysfunction in the pathogenesis of sepsis (125) and the heavy neutrophilic infiltration seen at the local site of infection in severe streptococcal soft tissue infections (57), neutrophil receptors and receptor activation may play an important role in the pathophysiology of these conditions. This is further explored in **Paper II**.

1.3.1.2 Neutrophil granules

An important hallmark of mature neutrophils is that they are densely packed with intracellular granules containing about 300 different proteins. These granules can be classified as primary, secondary, tertiary or secretory vesicles (126). They are formed at different stages during neutrophil differentiation in the bone marrow, with the primary granules appearing first, followed by the secondary, and the tertiary towards the final stages of maturation before the cell migrate into the blood stream. The secretory vesicles are believed to be formed by endocytosis, occurring after the neutrophil has entered the blood stream (127). The content of each respective granule is believed to be controlled at gene expression level during cell development, and the proteins expressed will be packed into the granules being formed at that time (128).

There is a hierarchical order in which the granules are released (Fig. 4). Secretory vesicles are the first to be released; this generally takes place as soon as the neutrophil makes contact with the activated endothelium. The vesicles are packed with adhesion molecules that will cover the membrane and allow the neutrophil to adhere to the endothelium (129). In addition, the secretory vesicles also contain HBP which loosen the tight junctions between the endothelial cells, thereby aiding the neutrophil in transmigration from the blood to the tissue (130).

The tertiary granules are the next to be released, they contain matrix degenerating enzymes and membrane receptors that are important for the neutrophil to transmigrate across the vessel wall and move through the tissue. As the neutrophil is moving through the tissue, secondary and primary granules undergo partial exocytosis. This mobilizes receptors for extracellular matrix components as well as matrix-degrading enzymes like collagenases and proteases that brake down the extracellular matrix and facilitate migration. Upon encounter with bacteria, the neutrophil activates its antimicrobial functions by releasing primary and secondary granules into the phagosome or out into the tissue (129, 131).

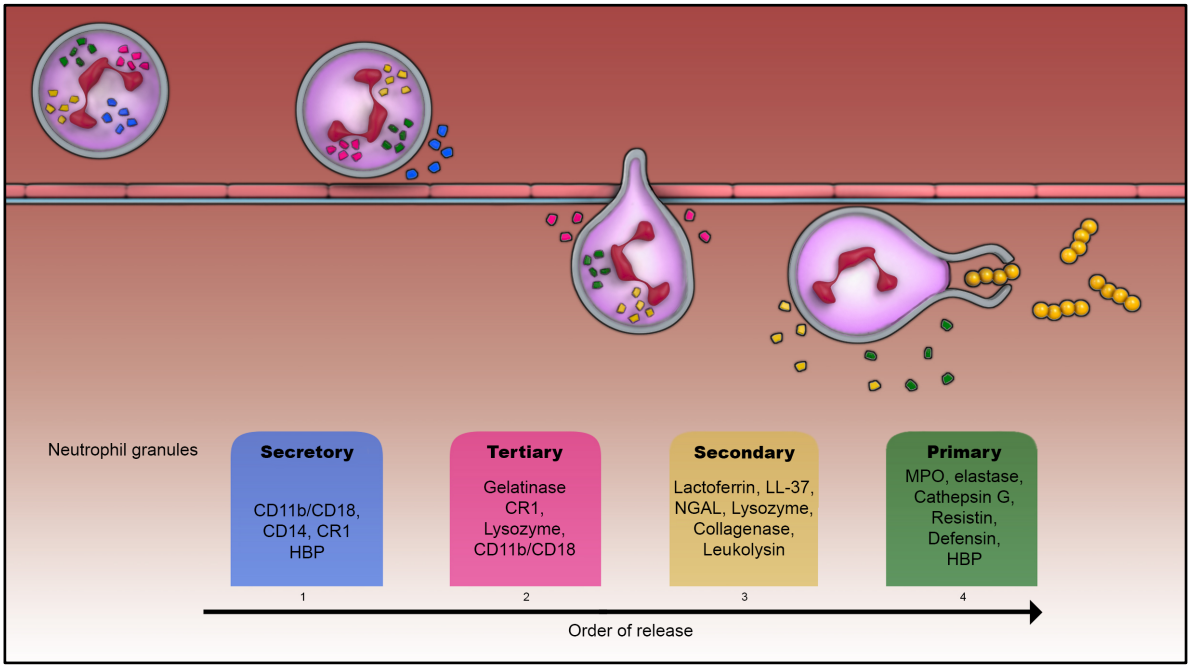


Figure 4. Order of granule release during neutrophil recruitment. Activated endothelial cells express adhesion molecules and integrins, allowing the neutrophil to start rolling along, adhere to and finally migrate through the vessel wall. When the neutrophil makes contact with the endothelium it releases the secretory vesicles (blue). The tertiary granules (pink) are the next to be released; they contain matrix degenerating enzymes and are important for transmigration and for the neutrophil to move through the tissue. The secondary (yellow) and primary granules (green) are the last to be released; they contain antimicrobial factors, hydrolases and proteases and are important for pathogen killing.

Illustration by Andreas Mielonen

1.3.1.3 Heparin-Binding Protein

HBP (also known as azurocidin or cationic antimicrobial protein of 37kD) was initially recognized for its broad antimicrobial effects (132). It has however become evident that HBP also has potent immunomodulatory effects, including recruitment and activation of macrophages, monocytes and neutrophils (133) as well as being a chemoattractant for T-cells (134).

HBP is stored in two granule compartments, both in the secretory vesicles and the primary granules (135). While the primary granules are mobilized at a late stage during neutrophil recruitment, the secretory vesicles are released into the extracellular environment upon contact with the activated endothelium. At the endothelium HBP can increase the expression of adhesion molecules and further boost leukocyte recruitment (136).

Another important feature of HBP is its ability to impair the endothelial barrier function. By increasing paracellular permeability, HBP may induce plasma leakage and edema formation, which are hallmark features of inflammation and shock (35, 137). Several studies have investigated HBP as a potential biomarker in severe infections, and found elevated HBP levels in plasma of patients with severe sepsis or septic shock (36, 138). In some patients elevated plasma levels of HBP could be detected up to 12 hours prior to signs of circulatory failure (36). Furthermore, a study assessing the potential of various biomarkers to discriminate between viral- and bacterial infections, serum levels of HBP was only exceeded by procalcitonin and white blood cell counts (139). However, in a different study involving patients admitted to the emergency care unit, the authors were unable to detect a difference in HBP levels between patients with septic- or non-septic shock (140).

Importantly, HBP has been detected in the tissue of patients with streptococcal soft tissue infections, ranging from erysipelas to NF (32, 141). Also, as previously mentioned, HBP is readily released by neutrophils in response to complexes formed by the streptococcal M1-protein and human fibrinogen, which in turn induces vascular leakage (34).

1.3.1.4 Resistin

Another protein present in the intracellular granules of neutrophils is resistin (126). There is however some controversy regarding which granule compartment resistin primarily resides in. While Johansson et al. found the primary granules to be the principle source of resistin (142), Boström et al. observed that the majority of resistin was stored in the secondary granules (143).

Although initially discovered as a peptide hormone mediating insulin resistance in mice (144), several studies in humans have since associated resistin with both acute and chronic inflammatory conditions (145, 146). While adipocytes represent the main source of resistin in mice (147), neutrophils and monocytes represent the main source in humans (143, 148, 149). However, resistin has also been found in human adipocytes (150), pancreatic islands (151)

and placental tissue (152). Murine and human resistin share only 55% homology at the protein level, which may explain the different findings regarding source and function (153).

The lack of a known receptor hampered the understanding of the biological functions mediated by resistin for a long time. Studies showed that resistin contributed to inflammation by inducing TNF and IL-6 release in macrophages and adipocytes through activation of NF-κB and JNK signaling pathways (150, 154). However, in 2010 observations were made that resistin competes with LPS for binding to TLR4, and that signals evoked by resistin are mediated through NF-κB and MAP-kinase signaling mechanisms (155). Also, in 2014 another receptor capable of binding resistin was found. Adenylyl cyclase-associated protein 1 was identified as capable of binding resistin and eliciting pro-inflammatory signals in human monocytes (156). Furthermore, resistin has also been shown to stimulate expression of adhesion molecules on endothelial cells (157), platelets (158) and monocytes (159).

In 2007, Sundén-Cullberg et al. investigated the plasma levels of resistin in patients with severe sepsis and found it to be a marker of disease severity (160), something that has also been confirmed by later studies (161-163). Furthermore, studies investigating resistin in relation to inflammation and neutrophil activation have found that serum levels of resistin were positively correlated with levels of pro-inflammatory cytokines in individuals with helminth infection, and that high levels may impede parasite clearance (164). Resistin has also been found to promote neutrophil activation and NET formation (165).

Importantly for this thesis, Johansson et al. found high levels of resistin in both circulation and at the site of infection in severe streptococcal infections, and that neutrophils represent the dominant source (142). The same study also showed that resistin was readily released from neutrophils in response to *S. pyogenes*.

1.3.2 Macrophages

Macrophages are, like neutrophils, phagocytic cells of the innate immune system. Together with monocytes and dendritic cells they make up the mononuclear phagocyte system. Strategically placed throughout the body, macrophages are specialized at ingesting and processing debris, dead cells and foreign materials. They are also important for the recruitment of other immune cells during infection and inflammation (166).

Tissue-resident macrophages are present in several tissues throughout the body, and play important roles in organ development and tissue homeostasis. Originating from embryonic progenitors, these cells self-renew locally without the need for replenishment by infiltrating monocytes (167-172). However, in some tissues, like the gut and dermis, the macrophage population is dependent on a continuous supply of infiltrating monocytes. During inflammation the affected tissues are also enriched with monocyte-derived macrophage like cells, though influx of monocytes from the blood stream (173-175).

Monocytes are innate effector cells, equipped with inflammatory and bactericidal capabilities (176) that are derived from hematopoietic progenitors in the bone marrow where development, homeostasis and proliferation is regulated by macrophage colony stimulating factor (M-CSF) (177). Monocytes are released from the bone marrow to the blood stream, where they constitute about 10% of total leukocytes (178). In circulation, monocytes can remain for up to 1-2 days after which they are removed, unless recruited into tissue in response to danger (179). Monocytes are recruited to the site of infection by chemokines like CCL2 (180). Upon entering the tissue, the monocytes differentiate into monocyte-derived effector cells (i.e. monocyte-derived macrophage like cells) with different functions depending on the local microenvironment in the tissue. Despite their different origin and ontogeny, tissue-resident macrophages and monocyte-derived macrophage like cells share many functions and markers, making it difficult to distinguish these cells (181).

1.3.2.1 Macrophage polarization

Both monocyte-derived and tissue resident macrophages are highly plastic cells, and are capable of changing their phenotype in response to changes in their local microenvironment, a process known as polarization (173). Traditionally macrophages and macrophage like cells have been classified into two distinct functional subsets; M1 (classically activated) and M2 (alternatively activated) macrophages, a terminology adopted from the T-cell field corresponding to Th1 and Th2 cells (182, 183) (Fig. 5). However, this classification is based on *in vitro* studies where cells are polarized with distinct cytokines. More recent studies assessing a wider repertoire of polarizing agents have shown that the M1 and M2 dichotomy only represents two extremes of a continuum of functional sates (184-188).

The complexity of macrophage polarization is reflected by some of the issues frequently raised in the field; the inability of *in vitro* studies to mimic the complex relationships and interactions seen *in vivo* (189), and the general lack of specific markers to study macrophage populations, in combination with the fact that many show overlapping expression profiles (181).

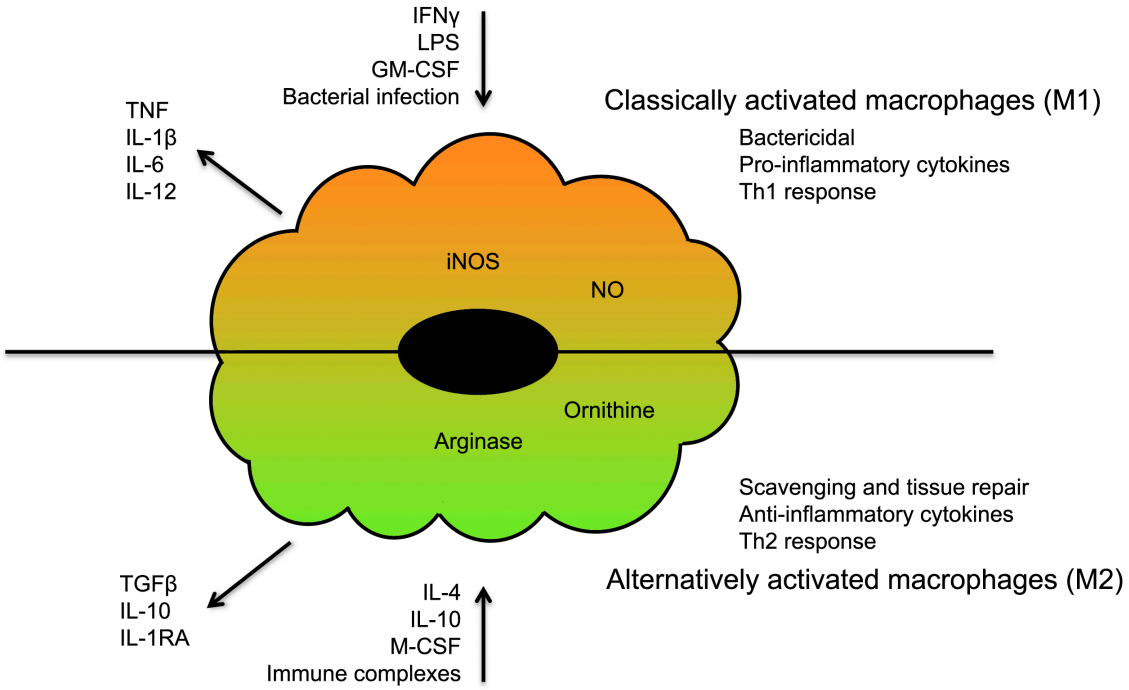


Figure 5. General concepts and properties of M1 and M2 macrophages
IL-1RA; IL-1 receptor antagonist

1.3.2.2 M1 macrophages

Microbial components, alone or in combination with cytokines such as IFNγ or granulocyte-macrophage colony stimulating factor (GM-CSF), can induce M1 macrophages. These cells promote Th1 responses, and possess strong microbicidal and tumoricidal activity (166). They are characterized by high antigen presentation capacities and high production of antimicrobial molecules like ROS and RNS. They have been associated with expression of surface markers such as CD80, CD64 and CD40 (190-193) and production of several pro-inflammatory cytokines including IL-12, IL-1, TNF, IL-6, type I IFNs and IL-23 (166, 182, 194). Furthermore, they recruit Th1 lymphocytes by production of chemokines like CXCL9 and CXCL10 (195). Key transcription factors associated with induction of M1 activation include NF-κB, STAT1 and activator protein-1, which regulate the expression of a large number of inflammatory genes (189, 196). High numbers of M1 macrophages can be found in inflamed tissues (197, 198), and typically thought of as being involved in initiating and sustaining the inflammatory process (181).

1.3.2.3 M2 macrophages

Prototypical Th2 cytokines like IL-4 and IL-13, and anti-inflammatory cytokines like IL-10 and TGFβ are classical M2 polarizing agents (197, 199, 200). Glucocorticoids, as well as simultaneous stimulation with immune complexes and anti-inflammatory molecules, can also induce M2 polarizing gene programs (178). M2 macrophages can be divided in to sub-groups like M2a, M2b and M2c, based on the polarizing stimuli and secreted cytokine profile (181, 182, 201). However, questions have been raised whether these exist *in vivo* (181). In general, M2 macrophages are considered to have anti-inflammatory functions, promote tissue repair and remodeling, and to be involved in the defense against parasite infections and other polarized Th2 responses (181, 184). These cells generally show little or no secretion of pro-inflammatory cytokines, while increased secretion of anti-inflammatory mediators like IL-10 and IL-1 receptor antagonist (189, 202, 203). *In vitro* studies have associated M2 macrophages with increased expression of certain markers, including CD163, CD206 and CD200R (190-193). Moreover, M2 macrophages direct the immune response by secreting chemokines like CCL17, CCL22, CCL24 that recruit cells like Th2 cells, T-regs, eosinophils and basophils (195). Studies have implicated factors like STAT6 and STAT3 as important for M2 polarization, and activation of these has been shown to reduce expression of pro-inflammatory cytokines and genes involved in inflammation (196). While M1 macrophages and their products can induce inflammation, the presence of M2 macrophages is usually associated with resolution of inflammation and tissue healing (204).

1.3.2.4 Macrophage polarization during inflammation

Macrophage polarization is a dynamic process that is both transient and plastic (205-207). One of the major roles of macrophages during the initial stages of inflammation is to secrete inflammatory cytokines like TNF, IL-1 and IL-6 that triggers the immune response and defends the host from invading pathogens. During later stages of inflammation, however, macrophages down-regulate production and secretion of inflammatory mediators while the production of anti-inflammatory cytokines like IL-10 and TGFβ increases and they actively remove damaged or dead cells (208, 209). During resolution and healing, macrophages support maturation of the regenerating tissues by remodeling and reorganizing extracellular matrix, vasculature and scar tissue (173).

Studies have shown that *in vitro* stimulation of M2 macrophages with M1 signals, or vice versa, may re-polarize already differentiated cells (173). However, while M2 macrophages can change to M1 macrophages, the reversed is considered to occur only in particular circumstances like mild inflammation. It is generally considered that the M1 macrophages most likely represent an end-stage killer cell that dies during the inflammatory response, possibly succumbing to its own nitric oxide production (173, 210).

Often, acute inflammation, resolution and regeneration are overlapping processes during which M1 macrophages are important to initiate the inflammatory response while M2 macrophages are fundamental for resolution and regeneration (204, 211, 212). Hence, it has

been proposed that rather than distinct populations, M1 and M2 signatures do not necessarily exclude each other and may often in fact coexist (189). In support of this, mixed macrophage phenotypes have been observed during the course of a variety of disease settings, like atherosclerotic plaques (213) and some murine tumors (214).

Macrophage polarization has also been studied in sepsis, and it has been suggested that NF-κB activation in M1 macrophages drives the initial hyper-inflammatory phase, while during the later phase macrophages display a more anti-inflammatory phenotype and impaired NF-κB activation (196). The phenomenon of reduced NF-κB activation in response to a secondary stimulation with LPS is known as endotoxin tolerance and has been shown to be induced by chronic signaling through TLR4, which leads to induction of various negative regulators of NF-κB signaling (215-217). In support of this, increased numbers of monocytes express phenotypic markers associated with M2 macrophages have been observed in patients with sepsis. The increased number of monocytes expressing M2 macrophage markers was associated with a lower proportion of IFNγ producing T-cells and higher production of T-regs (218).

1.3.2.5 Bacterial manipulation of macrophage polarization

A multi-study review of transcriptional responses in mononuclear phagocytes to bacteria or bacterial components identified a common response program, which mainly included an up-regulation of M1 associated genes like TNF, IL-6, IL-12, IL-1β, IL-7R, IL-15 AR, CCL2, CCL5, and CXCL8, CCR7 (219). M1 responses have typically been associated with protection against bacterial infections, and have been seen to aid the host in control of bacteria like *Listeria monocytogenes*, *Salmonella typhimurium*, *Mycobacterium tuberculosis*, *Mycobacterium ulcerans* and chlamydia infections (219-224). Several lines of evidence show that pathogenic bacteria, especially intracellular species, have developed mechanisms to interfere with macrophage polarization to enhance their own survival (225). Some bacteria accomplish this by interfering with M1 polarization, to reduce inflammation and bactericidal functions (173, 225).

Studies on the Gram-positive bacterium *Staphylococcus aureus* have showed that it is capable of inhibiting NF-κB activity in murine macrophages and thereby shift the phenotype of the cell from anti-microbial to functionally inert (226). It has also been observed that in a murine model of catheter-associated *S. aureus* biofilms, the infection was associated with limited macrophage infiltration (227). It was also observed that; *in vitro* generated bone marrow derived macrophages, which managed to infiltrate the biofilm, displayed a skewed response characterized by decreased expression of pro-inflammatory mediators and increased expression of the M2 associated factor arginase-1 (227).

Studies on murine macrophages in *S. pyogenes* infection have demonstrated a mixed activation program, including markers characteristic for both M1- and M2- activation (228). Importantly for this study, Thulin et al. identified an intracellular niche for *S. pyogenes* in

macrophages at the site of infection in streptococcal soft tissue infections (57). Moreover, Hertzén et al. showed that *S. pyogenes* is able to survive and replicate inside monocyte-derived macrophage like cells by an M1-protein dependent inhibition of fusion between the phagocytic vacuole harboring the bacteria, and the lysosome (31). In the same study infected macrophage like cells harboring live bacteria showed a skewed NF-κB activation, as compared to cells where the bacteria were efficiently killed (31), suggesting a dampened inflammatory response.

Taken together these findings make it tempting to speculate that *S. pyogenes* may skew the macrophage phenotype towards a more anti-inflammatory M2-like phenotype thereby impairing the bactericidal effects of the macrophage, and promoting its own intracellular persistence and survival. This is investigated in **Paper III**.

2 AIM

The overall aim of this thesis project was to advance our understanding regarding host-pathogen interactions in severe invasive infections caused by *S. pyogenes*.

Large numbers of phagocytic cells, such as neutrophils and macrophages, can be found in the infected tissue, and excessive inflammation has been associated with disease severity. Hence it was of interest to investigate potential harmful effects of interactions between neutrophils, macrophages and bacteria, leading to host cell activation and inflammation.

Specific aims included:

- To explore HMGB1 responses in severe soft tissue infections caused by *S. pyogenes*.
- To investigate whether the degree of neutrophil activation and degranulation vary depending on the bacterial stimuli.
- To define the macrophage phenotype present at the site of infection and specifically to explore whether *S. pyogenes* infection has an impact on macrophage polarization.

3 RESEARCH APPROACH

Detailed descriptions of the various experimental procedures used in this thesis can be found in the respective articles and manuscript. Common for all projects is the use of human cells and tissue systems, patient tissue and visualization by *in situ* imaging to study the local infection in a setting as clinically relevant as possible. Presented below are some methodological considerations for selected methods.

3.1 BACTERIAL ISOLATES

The bacterial isolates used throughout the various studies are clinical isolates, handled with minimum passaging. A benefit of using clinical isolates with minimum passaging as opposed to laboratory strains is that clinical isolates in addition to being clinically relevant are more likely to retain their virulence properties. In contrast, laboratory strains have often adapted to growth in rich media and lack of threat, making them less virulent.

However, it should be noted that throughout the studies the bacterial isolates were grown in rich media, which may have affected their respective virulence properties as compared to growth in infected tissue. The various media were chosen to supply optimal growth conditions and support exotoxin production for the respective bacteria. As the growth phase of the bacteria is known to affect the virulence profile, we chose to use bacteria from stationary phase (16-18h) during which the exotoxins are abundantly produced. This resembles previous *in vivo* findings with a high bacteria load and high expression of virulence factors at the site of infection (34, 51, 57).

3.2 HUMAN PRIMARY CELLS

3.2.1 Neutrophils

We used primary human neutrophils, isolated from whole blood of healthy donors. Using cells from different donors, rather than cell lines, may give larger variation between experiments, but it reflects real life, as different individuals will respond differently to pathogens and stimuli.

Neutrophils get activated easily and are short-lived, so experiments were carefully planned and executed. Studies have shown that neutrophils follow the circadian rhythm and may exhibit stronger response in the beginning of the active phase (morning) as compared to later in the day (229, 230). In order to minimize variation introduced by the circadian rhythm, we tried to isolate the cells and perform the experiments around the same time. Working with neutrophils requires careful handling, as they are readily activated during the isolation process. Isolation using density gradient centrifugation may trigger a mild activation of neutrophils, resulting in release of secretory vesicles and altered migratory behavior (231). In fact, inconsistent migratory patterns of neutrophils after density gradient isolation posed a

considerable problem during the studies performed for **Paper I**, when we tried to assess potential chemotactic effects of HMGB1. As isolated neutrophils failed to give consistent results, we adapted an assay based on whole blood and thereby we were able to generate reproducible data.

A potential way to avoid some of the issues associated with primary human neutrophils, like donor variation, would be to use a cell line. The HL-60 cell line is a human leukemia cell line that can be transformed into neutrophil-like cells (232, 233) and is frequently used to study neutrophil responses. The neutrophil-like cells are however considerably different from primary neutrophils, and for example, only contain one of the granule types normally found in these cells (234).

3.2.2 Monocytes

Primary human monocytes were isolated from buffy coats, and allowed to differentiate into macrophage like cells either in cell cultures or organotypic skin tissue models. As with primary neutrophils, the use of cells from different donors may give larger variation between experiments, however, as different individuals will respond differently to pathogens and stimuli it may reflect real life to a larger extent.

As for neutrophils, a potential way around this variability would be the use of cell lines. However, observations of human monocytic cell lines, that can be transformed into macrophages, such as THP-1 cells and U937 showed that they differ considerably with regards to adherence, morphology, intracellular granularity as well as infection efficiency (235).

3.3 MICROSCOPY

3.3.1 Light microscopy

A key method used throughout the studies is immunohistochemical (IHC) staining of tissue sections, in combination with acquired computerized image analysis (ACIA). IHC is well established and widely used to study distribution and localization of proteins in tissue, and the ACIA protocol used allows for a semi-quantitative analysis of the immunolabeled tissue. In short, the ACIA value yielded is presented as the percentage of positively stained area, multiplied by the mean intensity of the stained area (51).

3.3.2 Fluorescence microscopy

An advantage of immunofluorescence (IF) compared to IHC is that IF is superior when it comes to investigate more than one marker at the same time. Hence, we often use this method for investigating two or more markers at the same time. Throughout the project, we have used

IF to investigate cell subsets present at the site of infection, co-localization and phenotypic shift. In addition to optimization of the staining and imaging procedures, considerable effort has also been put towards image analysis and quantification. Presented below are two of the analysis methods developed.

3.3.2.1 Co-localization of granule proteins in neutrophils

In **Paper II**, we were interested in co-localization between HBP, resistin and MPO in primary human neutrophils. Co-localization can be divided into two main components; co-occurrence (spatial overlap of two probes) and correlation (relationship between two probes) (236). In this case, we did not believe that there would be a relationship between these molecules (co-regulation or complex formation), and were in fact only interested in whether they could be found in the same granule compartment. Hence, we chose to look at co-occurrence rather than correlation.

To study this, we stimulated, fixed and immunolabeled neutrophils. Confocal microscopy was used to capture high-resolution image-stacks of the cells. For co-localization analysis the 3D-image analysis software Imaris was used (Fig. 6). In short, the cells were identified and segmented using the "surface"-function, which yielded a mask for the body of the cell (Fig. 6 "Masking of cell body"). For identification of the intracellular vesicles, the "cell" function was used. This function allows for the semi-automated identification and segmentation of vesicle-like structures within a defined area, based on size and intensity thresholds set by the user (Fig. 6 "Identification of vesicles"). Intracellular vesicles were identified and segmented for each channel/marker (HBP, resistin and MPO, respectively) (Fig. 6 "Masked vesicles"), and assessed for co-localization using the "coloc"-function. Total number of co-localized pixels was determined using orthogonal regression analysis of the image scatterplot in combination with Pearson coefficient.

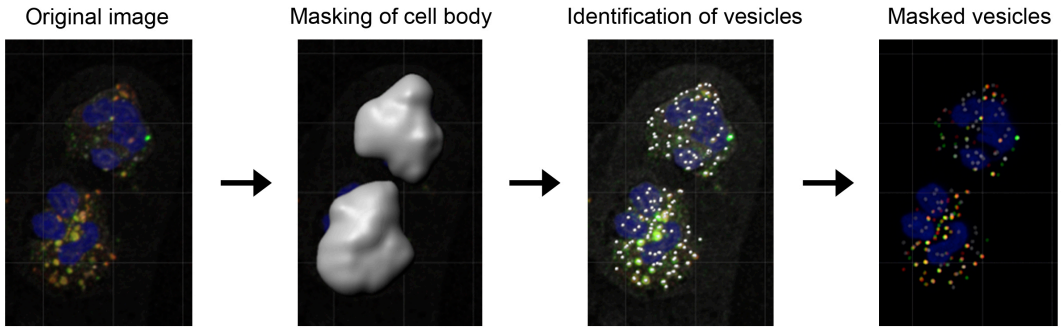
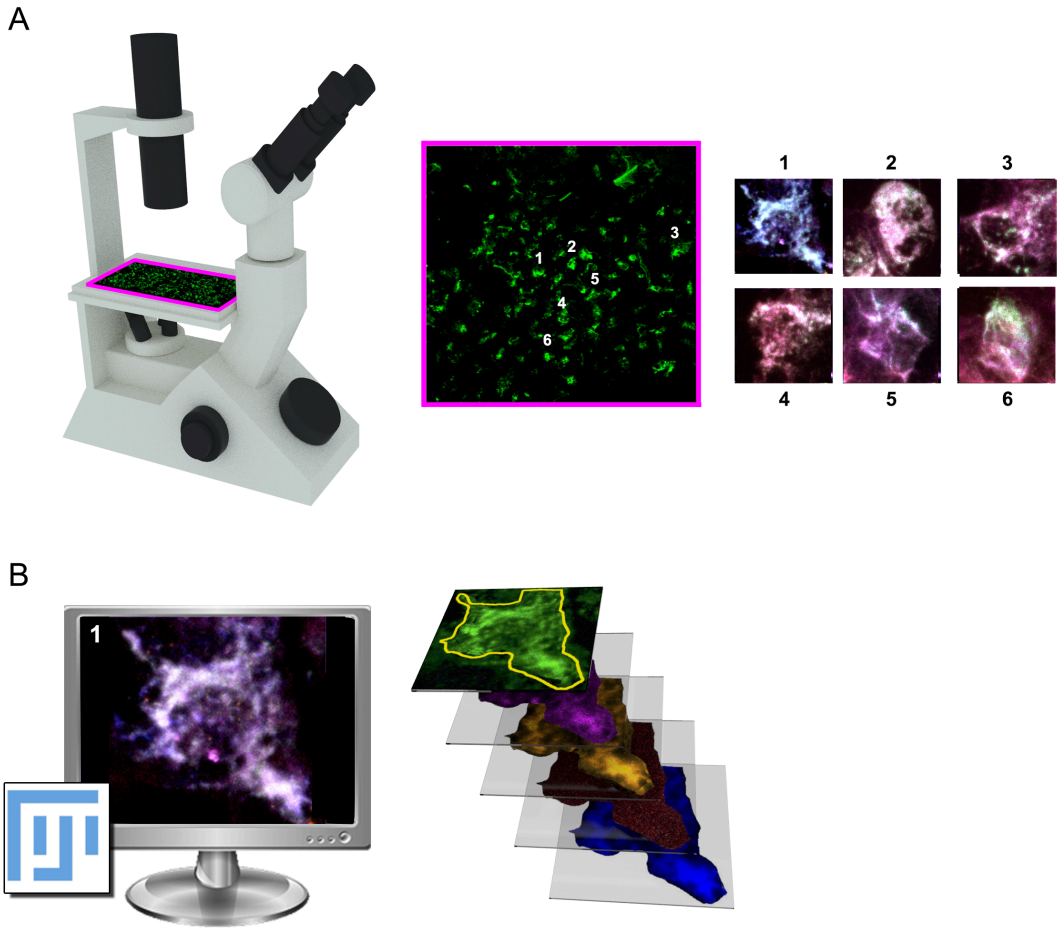


Figure 6. Strategy for identification and segmentation of intracellular granules. Image-stacks were imported into the image analysis software Imaris. In order to segment intracellular vesicles for co-localization analysis, the body of the cell was defined (Masking of cell body), after which intracellular vesicles containing the respective markers were identified and segmented (Identification of vesicles). Finally, the masked vesicles were assessed for percent co-localized pixels.

3.3.2.2 Phenotyping of macrophages in snap-frozen tissue

In **Paper III** we were interested in assessing macrophage phenotype at the site of infection in NSTI. Flow cytometry is a well established and widely used method for phenotyping of immune cells and allows for the simultaneous analysis of multiple markers. Flow cytometry requires single-cell suspensions, ideally prepared from fresh tissue, although there are studies showing that snap-frozen tissue blocks may be thawed and disrupted into single cell suspensions with comparable results (237, 238). However, it has been noted that this procedure does not work equally well for all tissues, and that necrosis has a negative impact on the results (239). Considering that our patient tissue consists of snap-frozen tissue removed during debridement surgery, with a high degree of inflammation and necrosis, it was deemed unlikely that thawed and disrupted biopsies would yield reliable results. Hence, we set out to investigate microscope-based approaches for phenotyping of macrophages in tissue. In short; the IF stained tissue was imaged using a confocal microscope with a spectral detector, to allow for the discrimination of more than four colors (Fig. 7). A reference spectrum was obtained for each fluorophore by imaging single stained samples for each respective probe using a single excitation source. All reference spectra were stored in a spectral library, and later used for unmixing of the imaged samples using the unmixing algorithm of the NIS Elements software. For imaging of the stained samples, we started by acquiring an overview image over the entire biopsy/model, at relatively low resolution (20x), using only the CD68-channel. The CD68-positive cells were then identified and selected for high-resolution (100x) spectral imaging (Fig. 7A). The unmixed images were then analyzed using the image analysis software ImageJ. For the analysis, the unmixed image was imported into the program and split into single channel images. The CD68-image was used to define the area of the cell. A threshold was applied to the image in order to generate a binary image of the CD68-positive area. The binary image was further processed to fill any holes left by the threshold within the cell area, and then transformed into a mask. The mask was applied to the other channels, and the intensity of the respective channel measured within the mask (Fig. 7B).



Marker	Mean	Maximum
CD68	410.3	2797
CD80	357	3038
CD64	101.56	1301
CD163	99.81	1609
CD206	499.11	3940

Figure 7. Multi-parameter microscopy for phenotyping of macrophages in tissue. Sections of snap-frozen tissue were immunolabeled, using antibodies against CD68 (macrophage marker) and phenotypic markers. An overview image of the sample was obtained using the CD68-channel at low magnification (20x). The CD68-positive cells were then identified and selected for high-resolution (100x) spectral imaging (A). After spectral unmixing, the images were imported and analyzed using the image analysis software ImageJ. Cells were first segmented based on the CD68-signal, a mask of the cell area was created and the intensity of the respective phenotypic markers measured inside the mask (B).

3.4 3D ORGANOTYPIC SKIN TISSUE MODEL

A majority of our knowledge regarding biological- and cellular processes is derived from studies of 2D cell cultures, where one type of cell is cultivated on a plastic surface. While these studies have provided understanding on individual cellular responses they may fail to capture important physiological responses in a more complex setting such as the tissue milieu. Hence, there is an interest in culturing cells in 3D cultures that mimics the morphological and functional features of human tissue, containing several cell types, extracellular matrix and other important tissue components. In **Paper III** we were interested in studying polarization of macrophages in response to *S. pyogenes* infection. Considering that it is likely that pathogen-derived virulence factors and host-derived inflammatory mediators both contribute to disease progression at the site of infections, as well as the importance of microenvironmental signals in macrophage polarization, we chose to use an organotypic skin tissue model. The skin model has previously been shown to be a robust tool to model *S. pyogenes* tissue infections (58, 240). Also, the cells in these models retain their differentiated cellular phenotypes in an *in vivo* like architecture.

In this model, normal human dermal fibroblasts (NHDFs) are grown in a collagen matrix for a period of 7 day, during which the cells acquire a polarized phenotype, remodel the matrix and develop a large number of cell-cell contacts. After 7 days keratinocytes are seeded on top of the fibroblast-collagen matrix and form a confluent layer, after which the model is air-exposed for a period of up to 7 days. During the air-exposure the keratinocytes stratify and form an epithelium similar to that of normal skin. In order to study macrophage polarization, monocytes from healthy donors were isolated and implanted into the model between the fibroblast-collagen matrix and the keratinocyte layer, and allowed to differentiate into monocyte-derived macrophages like cells in the tissue like environment for a period of up to 11 days.

In this skin model we utilized long term cultured NHDFs and an immortalized keratinocyte cell line in order to maximize the reproducibility, as compared to genetically non-uniform and potentially impure freshly isolated primary cells. However, for the reasons stated above we chose to utilize primary human monocytes as a source of macrophage-like cells.

3.5 ETHICAL CONSIDERATIONS

All studies were conducted in accordance with the Helsinki declaration, and were approved by the local ethics committees of Karolinska University Hospital, the University of Toronto and Lund University Hospital, as well as the regional ethics committee of Stockholm, the regional ethics committee in Gothenburg, the Ethical Review Board at the National Committee on Health Research Ethics in Copenhagen, and the regional ethics committee Vest, Norway. Written informed consent was obtained from all patients or their legal guardians, as well as healthy controls; all samples were coded or provided anonymously.

4 RESULTS AND DISCUSSION

This part comprises a discussion around some of the main findings presented in **Paper I-III**. The first section focuses on HMGB1 as a potential biomarker and mediator of tissue pathology. The second part assesses neutrophil activation and degranulation in response to different bacterial species, focusing on the release of the sepsis-associated factors HBP and resistin. Finally, the third section highlights macrophage phenotype in *S. pyogenes* infections.

4.1 HMGB1 AT THE SITE OF INFECTION

4.1.1 HMGB1 as a potential biomarker for tissue pathology

Due to the association of HMGB1 with various inflammatory conditions and its release from necrotic cells, it was of interest to explore HMGB1 responses in *S. pyogenes* infections. We hypothesized that HMGB1 would be increased in the most severe soft tissue infections. To test our hypothesis, we utilized two different collections of tissue biopsies from *S. pyogenes* infected patients. The first collection consisted of biopsies collected from patients with severe streptococcal soft tissue infection, i.e. severe cellulitis and NF, while the other collection came from patients with erysipelas (previously described by Linder et al. (141)). Erysipelas is typically an uncomplicated localized infection of the superficial layer of the skin. Importantly, the erythema can be clearly distinguished from the surrounding tissue and there is no necrosis (241).

Using IHC we were able to detect HMGB1 in all biopsies, where it demonstrated both a distinct intracellular- as well as a diffuse extracellular staining pattern (**Fig. 1 and 2, Paper I**). Notably, all cells were not positive for HMGB1, despite it being a constitutively expressed nuclear factor. This has been observed by others as well (242), and is likely due to technical limitations, such as insufficient permeabilization of the nuclear envelope or epitope availability inside the nucleus.

From patients with severe soft tissue infections, biopsies were collected from both central and distal, not visually inflamed, areas. Using *in situ* image analysis we were able to obtain a semi-quantitative measurement of the amount of HMGB1 in the tissue. This showed higher amounts in tissue from central areas of the lesion as compared to samples taken in distal areas. Also, higher levels were noted in tissue from patients with severe infections as compared to patients with erysipelas. From one of the erysipelas patients, biopsies had been collected from the center of infection, a distal area (5 cm outside the lesion) and a healthy area (other non-infected leg). In these biopsies, HMGB1 levels increased with more involved and inflamed tissue (**Fig. 2, Paper I**). Taken together these results show that levels of HMGB1 correlate with severity of infection and tissue involvement (distal versus center), suggesting that HMGB1 might be a potential biomarker for severe soft tissue infections.

The presence of HMGB1 in the tissue from patients with erysipelas, where no necrosis is present, suggested that in addition to passive release through necrosis there is also an active

secretion of HMGB1 from activated cells. An active secretion was also implied by confocal microscopy, showing a vesicular staining pattern for HMGB1 in infiltrating immune cells. Further analysis identified macrophages as the dominant source of intracellular HMGB1 at the site of infection, and infection of monocyte-derived macrophages like cells with clinical *S. pyogenes* isolates resulted in release of HMGB1 (**Fig. 4, Paper I**). These results are in line with a previous report showing release of HMGB1 by activated monocytes, through a non-classical vesicle mediated pathway (81).

4.1.2 HMGB1 and other inflammatory mediators at the site of infection

Given the hyper-inflammatory state at the site of infection, and that HMGB1 has been shown to form immunostimulatory complexes with cytokines like IL-1β (243) and CXCL12 (90) *in vitro,* we sought to investigate the presence of these markers in the tissue biopsies.

IHC stainings of consecutive biopsy sections showed HMGB1 in the same areas as both IL-1β and CXCL12. Using confocal microscopy, we could however only see an overlap between HMGB1 and IL-1β (**Fig. 5, Paper I**). The noted co-localization of HMGB1 and IL-1β shows the potential for complex formation *in vivo*, but to prove that such complex formation occur at the infected tissue site other techniques, such as proximity ligation assay or co-immunoprecipitation, are required.

In addition to CXCL12 and IL-1β, HMGB1 is also capable of forming immunostimulatory complexes with LPS (243). In light of this, as well as reports on other immunostimulatory complexes formed between streptococcal proteins and host factors like; neutrophil activating streptococcal M1-protein-fibrinogen complexes (34) and streptococcal inhibitor of complement (SIC)-histone 4 complexes that boost cytokine production (*Westman et al., Manuscript*). It would be of interest to see whether HMGB1 forms complexes also with streptococcal factors. If so, it would be tempting to speculate that the hyper-inflammatory state seen in these infections may in part be explained by the presence of such complexes.

4.1.3 HMGB1 and neutrophil chemotaxis

IHC stainings made during this study, as well as in **Paper II** and by Johansson et al. (244), show a large neutrophil infiltration in the infected tissue. Also, co-staining of HMGB1 and neutrophils showed that, while not co-localizing, neutrophils were present in areas with HMGB1 and in some regions the neutrophils seemed to surround HMGB1-positive areas **(Fig. 4, Paper I)**. These findings, in combination with the fact that HMGB1 has been described to have chemotactic effects (74), made us investigate whether the observed staining pattern was due to a chemotactic effect of HMGB1 on neutrophils. Using a transmigration assay, we could demonstrate that HMGB1 had a chemotactic effect on primary human neutrophils, almost to the same extent as the known neutrophil chemotactic factor IL-8 **(Fig. 4, Paper I)**. While others have reported a concentration dependency for the chemotactic

effect of HMGB1 on neutrophils (245), we did not see any such effects in our system despite a similar experimental set-up.

Together these data show that HMGB1 is a potential biomarker for tissue pathology in severe streptococcal soft tissue infections, and as a substantial co-localization with IL-1β was noted, it seems likely that immunostimulatory IL-1β-HMGB1 complexes may be formed at the site of infection. The data also shows that HMGB1 has chemotactic effect on neutrophils. Whether HMGB1 is a mediator of neutrophil induced tissue pathology cannot be concluded by this study, as more mechanistic data is required. The role of HMGB1 as a promoter of inflammation has been difficult to study due to a postnatal lethality of knock out mice. However, recently Huebener et al., using a conditional ablation model, showed that HMGB1 exerts an essential role in neutrophil recruitment to necrotic sites during sterile inflammation, resulting in amplification of tissue injury and decreased survival (246).

4.2 NEUTROPHIL ACTIVATION IN RESONSE TO BACTERIA

In light of the large neutrophil recruitment during sepsis and tissue infections, and that aberrant neutrophil activation may cause tissue pathology (247). As well as the fact that several studies have reported on aberrant neutrophil responses in sepsis with respect to survival, migration and functionality (125, 248-250), it was of interest to investigate neutrophil activation in response to bacterial stimuli. For this purpose, we focused on sepsis and used bacterial isolates from septic patients in combination with primary human neutrophils from healthy donors. To assess neutrophil activation, we measured the release of granule proteins HBP and resistin, as they have both been associated with severity of sepsis (138, 160).

We investigated neutrophil activation in response to different bacterial stimuli by stimulating primary human neutrophils with fixed clinical bacterial isolates, collected from patients with septic shock, or filtered bacterial culture supernatants. From a clinical perspective it would have been more relevant to use live bacteria. However, for this study it was deemed necessary to use fixed bacteria as live infection would have been associated with confounders like varying degree of phagocytosis, intracellular replication and toxin-mediated cytotoxicity, complicating interpretation of results with regards to bacterial factors triggering neutrophil activation. Moreover, previous studies by our group, comparing the stimulatory effect of live and fixed bacteria showed a similar degree of activation and degranulation, at least with regards to resistin release (244).

4.2.1 Neutrophil activation in response to different bacterial stimuli

Stimulation of neutrophils with filtered bacterial culture supernatants, from overnight cultures of *S. pyogenes* isolates failed to induce release of either HBP or resistin. This was true for all tested isolates, irrespective of serotype.

In a recent report by Uhlmann et al. varying neutrophil responses to culture supernatants from *S. pyogenes* isolates were seen (251). A stimulatory effect was only seen in supernatants from bacterial strains where the protease SpeB was not present, i.e. SpeB-negative strains. The lack of response in SpeB-positive supernatants could be linked to a SpeB mediated degradation and inactivation of the neutrophil stimulatory factor phosphoglycerate kinase (PGK).

The expression of SpeB in invasive clinical strains is controversial. It has been proposed that SpeB-negative strains are hype-virulent, and selected for during invasive infection (252, 253). In line with this, it has also been reported that SpeB expression by clinical isolates is inversely correlated with severity of invasive disease (253). A selection for SpeB-negative strains during invasive infection would argue against SpeB being present in the clinical isolates used in **Paper II**. However a recent report, assessing SpeB expression in a large strain collection, of approximately 10.000 clinical isolates, showed that the majority of strains expressed SpeB (59). Furthermore, analyses of tissue biopsies from patients with NSTI have revealed high levels of SpeB at the site of infection (57, 141, 254). In addition Siemens et al. isolated a mixed population of SpeB-positive and SpeB-negative clones from infected tissue in *S. pyogenes* NSTI (58). SpeB is an important virulence factor, capable of degrading both human and bacterial factors. As such it is likely that its role differs during different stages of infection, and that expression might be beneficial at certain stages while unfavorable at others. Thus, it is plausible that the clinical isolates used in **Paper II** are all SpeB-positive and therefore, the bacterial supernatants failed to elicit neutrophil activation. Another possibility for the lack of stimulatory effect of the bacterial culture supernatants may be low levels of SLO, as another report observed HBP-release in response to bacterial culture supernatants containing SLO (255). However, SpeB may inactivate also this factor.

Visualization of neutrophils exposed to fixed bacteria for 2h showed considerable differences, with an almost complete aggregation of the cells in response to *S. pyogenes*. However, only minor aggregation could be seen in response to *S. aureus*, and even less in response to *Escherichia coli* (**Fig. 3A, Paper II**). Aggregation of neutrophils into clusters, so called neutrophil swarming, is an emerging concept within infection and inflammation, and has been proposed to be a way for neutrophils to isolate and contain microbial invaders (256). Swarming behavior of neutrophils has also been proposed to contribute to establishment and progression of infection, where transiently swarming neutrophils take up microbial pathogens and then act as motile reservoirs carrying the microbes away from the neutrophil swarm into nearby tissues (257). Which role neutrophil swarming plays in the pathogenesis of *S. pyogenes* infections remains to be determined. However, in the light of the fast spread of the bacteria during soft tissue infections it tempting to speculate that *S. pyogenes* may use the swarming behavior of neutrophils to promote bacterial spread and dissemination.

Measurement of HBP and resistin release from neutrophils stimulated with fixed bacteria revealed an increased degranulation and factor release in response to *S. pyogenes* and other streptococcal strains, as compared to *S. aureus* and *E. coli* (**Fig. 3 and 5, Paper II**). This was also evident when stimulating neutrophils with purified bacterial proteins, and streptococcal M1-protein triggered an increased release of both HBP and resistin as compared to LPS.

Notably, the neutrophil stimulations showed a considerable inter-donor variation in *S. pyogenes* triggered HBP release, with some donors responding by releasing high amounts of HBP, while others released low amounts. This was only noted for HPB and not for resistin release. Donor variation in streptococcal-triggered HBP release has previously been reported in a study assessing neutrophil responses to streptococcal M1-protein (258). The donor variation was linked to the presence of high titers of specific antibodies against the M1-protein in the high-responder, and proposed to be induced by complexes containing M1-protein, fibrinogen and IgG, triggering HBP release through dual engagement of both β2-integrin and Fc-receptors (258). In line with this report, dot-blot analysis (assessing antibodies against streptococcal M1-protein) of plasma from a high and a low responder indicated higher antibody titers in plasma from the high responder (**Fig. 5, Paper II**).

In this study, we observed high HBP release not only to streptococcal M1-protein and *S. pyogenes*, but also to other streptococcal species like group B, C and G *Streptococcus* as well as *Streptococcus viridans.* While the precise mechanism for this is not known it is tempting to speculate that it may be mediated by cross-reactive antibodies against M- and M-like proteins, as these proteins share homologies in central regions.

As HBP is an inducer of vascular leakage, and as vascular leakage and circulatory failure are prominent features of severe sepsis and septic shock, it is tempting to speculate that so called high responders have an elevated risk of developing severe disease. It would be of interest for future studies to assess if there is an increased risk for individuals with high titers of activating antibodies to develop severe disease. However, as these studies will require serum samples taken before infection to screen for the presence of antibodies, murine studies may be required.

4.2.2 Signaling requirements for release of HBP and resistin

The presence of plasma proteins, particularly fibrinogen, has been shown to be important for neutrophil activation and HBP release triggered by the streptococcal M1-protein (34, 258). This was evident also in this study, as neutrophils stimulated in the presence of plasma responded by releasing HBP while neutrophils stimulated in the absence of plasma did not. Notably this was only seen for release of HBP, and not resistin (**Fig. 5, Paper II**). Something, which led us to further investigate the signaling involved in resistin release. We started by attempting to identify the bacterial factors involved in streptococcal-triggered neutrophil activation.

We first assessed the role of surface attached M1-protein by stimulating neutrophils with an M1-deficient *S. pyogenes* mutant. These results showed a marginal reduction in resistin release induced by the M1-deficient mutant suggesting that also other factors are involved in activation. Therefore we utilized a panel of *S. pyogenes* strains deficient in gene regulatory systems, affecting more than one factor. Data from these experiments confirmed a limited effect of M- and M-like proteins on neutrophil activation and resistin release, as a mutant lacking Mga (a positive regulator of genes coding for M- and M-like proteins) triggered release of similar amounts of resistin as the wild type strain. In contrast, bacterial mutants deficient in regulators affecting the FCT-region triggered altered resistin release profiles, implicating factors like fibronectin- and collagen binding protein as well as the T-pilus, in *S. pyogenes* triggered resistin release from neutrophils (**Fig. 5, Paper II**).

Revisiting the findings by Uhlmann et al., PGK is also a potential candidate for neutrophil activation and resistin release. PGK is an anchorless adhesin, a so-called moonlighting protein with important functions in both bacterial metabolism as well as virulence (259, 260). While PGK is released by the bacteria, it has been proposed to re-associate with the membrane by a yet unknown mechanism (261). Considering its stimulatory properties in supernatants, it is tempting to speculate that secreted PGK re-associates with the bacterial surface and is thus able to trigger neutrophil activation and resistin release also in our systems. However, to test this we would need to generate a bacterial mutant lacking PGK. Uhlmann et al. attempted to generate a PGK deficient mutant but were not successful; suggesting that deletion of PGK might be lethal due to its essential role in glycolysis.

Next we decided to explore host signals and receptors involved in neutrophil activation and resistin release. We started by investigating the intracellular signaling pathways involved in *S. pyogenes* triggered resistin release by treating neutrophils with pharmacological inhibitors of selected molecules. These results showed that inhibition of Src-family or p38 MAP kinases resulted in a small, but consistent reduction in resistin release. However, these molecules are used by multiple receptors and signaling pathways, so to further dissect the signals needed for resistin release we used blocking antibodies against β2-integrins and TLR2. In accordance with previous publications (34) blocking of the β2-integrin resulted in a reduction in streptococcal M1-protein triggered activation, and release of resistin. This reduction was however not seen when neutrophils were stimulated with whole bacteria (*S. pyogenes*). In contrast, when TLR2 was blocked there was a reduction in resistin release triggered by *S. pyogenes* but not M1-protein (**Fig. 5, Paper II**). These findings are in line with M1-protein triggered activation being mediated at least in part by β2-integrins, while other receptors (like TLR2) are more important for resistin release triggered by whole bacteria. It should, however, be noted that the observed reductions in resistin release were far from complete, suggesting a multifactorial activation involving multiple signals and receptors.

4.2.3 Synchronized release of HBP and resistin

The differences in signaling requirements for HBP and resistin release prompted us to investigate the subcellular localization of these two proteins. For this purpose, we stimulated neutrophils with streptococcal M1-protein, LPS or N-formyl-methionyl-leucyl-phenylalanine (fMLP), and stained them for HBP, resistin and MPO (a marker of primary granules) using IF. Confocal microscopy revealed numerous granules positive for each marker. However, while HBP and resistin could be found to co-localize with MPO, i.e. primary granules, there was also a substantial amount of the proteins that did not (**Fig. 4, Paper II**). Both HBP and resistin have been reported to localize to other granule subsets, secretory vesicles and secondary granules (135, 143), respectively, and it seems likely that these would be the granules harboring the HBP and resistin not co-localized with MPO. Furthermore, co-localization analysis showed that there was little overlap between HBP and resistin in unstimulated cells. The degree of co-localization did however increase in response to stimulation, especially in response to stimulation with M1-protein (**Fig. 4, Paper II**), suggesting a potential synchronized release of these two factors. The notion of a synchronized release was further supported by neutrophil stimulations, revealing similar release kinetics for HBP and resistin in response to both fixed *S. pyogenes* as well as M1-protein.

We also compared levels of HBP and resistin in acute phase plasma of patients with severe sepsis or septic shock. Consistent with previous reports (36, 138, 160-163, 244), both HBP and resistin levels were elevated in patients as compared to non-infected critically ill patients. While there was no significant differences in the levels of HBP or resistin in samples from patients infected with Gram-positive or Gram-negative bacteria. There was a stronger correlation between the two in patients with Gram-positive bacterial infection, and in plasma from patients with STSS the correlation was even stronger (**Fig. 1, Paper II**). Thus further supporting the idea of a synchronized release of HBP and resistin, also in a clinical setting.

Taken together the results from this study indicate that streptococcal species are potent inducers of neutrophil activation and degranulation. Given their greater stimulatory capacity, neutrophil activation and degranulation may play a central role in the pathology of invasive streptococcal infections. The importance of neutrophils and neutrophil activation is also highlighted by the findings from **Paper I** where we saw a chemotactic effect of HMGB1 on neutrophils and found large amounts of HMGB1 as well as a positive correlation with HMGB1 levels and presence of neutrophils in the infected tissue. Furthermore, gene expression analysis performed in **Paper III** on infected tissue from patients with *S. pyogenes* NSTI, highlighted neutrophil degranulation as an important pathway.

While the current study was focused on sepsis, it also included analyses of HBP and resistin in tissue from patients with severe streptococcal soft tissue infections, and showed that both were be readily detectable. However, it would be of interest to expand the study and analyze tissue from patients with severe soft tissue infections caused by other bacterial species as well, and test the hypothesis that there is less HBP and resistin released at the site of infection in these infections.

4.3 MACROPHAGE POLARIZATION IN STREPTOCOCCAL SOFT TISSUE INFECTIONS

As previously mentioned, macrophages and monocyte-derived macrophage like cells constitute a heterogeneous population of cells. However, they do share many functions and markers (181), and are important in our defense against bacterial infections. Reports from our group have shown that the affected tissue from patients with severe *S. pyogenes* infections demonstrate high inflammation, including infiltration of large numbers of both macrophages and neutrophils (51, 57). These reports also show that *S. pyogenes* may infect and persist inside macrophages at the site of infection (57), and that monocyte-derived macrophage like cells harboring live *S. pyogenes, in vitro,* display a suppressed NF-κB activation (31). As several bacterial species have been shown to skew macrophage polarization towards an anti-inflammatory phenotype, thereby promoting their own survival (219), we wanted to investigate the macrophage phenotype at the site of infection in NSTI caused by *S. pyogenes*.

4.3.1 Multi-parameter imaging to assess macrophage phenotype

In order to further investigate macrophage phenotype at the site of infection, we used a multi-parameter imaging workflow, which allowed for assessment the expression of phenotypic markers associated with macrophage polarization at the single cell level. Based on a review of the literature and availability of compatible antibodies, we chose to test a panel of phenotypic markers associated with either M1- (CD64 and CD80) or M2 (CD163 and CD206) macrophages.

First we tested this panel on monocyte-derived macrophage like cells stimulated with prototypical M1 (IFNγ+LPS) or M2 (IL-4+IL-10) polarizing agents, which showed a mixed expression pattern of the markers, i.e. all markers were expressed by all cells but at different levels. While we could readily detect CD64, CD80 and CD206 using microscopy, CD163 was barely detectable. Furthermore, flow cytometry analysis of polarized cells showed an increased expression of CD80 on M1- as compared to M2-polarized or unstimulated cells. Reversely, CD206 expression was increased on M2- as compared to M1-polarized and unstimulated cells, which is in line with the literature (190-193). Based on these results, we assessed an inflammatory index based on the ratio between CD80 and CD206 (CD80/CD206). Both with respect to confocal microscopy and flow cytometry, this index was able to distinguish between M1- and M2 polarized cells (**Fig. 2, Paper III**).

4.3.2 Macrophage phenotypes in *S. pyogenes* infected tissue

Next, we decided to use our imaging workflow to investigate the macrophage phenotype at the site of infection in *S. pyogenes* NSTI. For this purpose, we chose to use a tissue biopsy material collected from NSTI patients, with a particular focus on patients infected by *S. pyogenes*. In addition to assessing macrophage phenotype, we also characterized these

biopsies with regards to the degree of inflammation, tissue destruction, bacterial load as well as the bacterial load in the tissue.

We could see a reduced inflammatory index (CD80/CD206) in macrophages from *S. pyogenes* infected tissue, as compared to *S. aureus* infected tissue, suggesting a more anti-inflammatory M2-like phenotype in *S. pyogenes* infection. However, there was no apparent relation between macrophage inflammatory index and *S. pyogenes* serotype, bacterial load or the amount of viable bacteria. In order to compare the inflammatory index of macrophages from patients with *S. pyogenes* infections, to that of macrophages present in healthy skin, we collected and analyzed tissue specimens from healthy individuals undergoing plastic surgery. The macrophage inflammatory index was higher in healthy controls compared to *S. pyogenes* infected tissue. (**Fig. 3, Paper III**). From two *S. pyogenes* infected patients, samples were collected from the same tissue at different time points after hospitalization. Analyses of these samples showed that in the patient where the inflammation and tissue destruction worsened over time, the inflammatory index remained equally low at both time points. However, in the patient where the inflammation and tissue destruction was reduced over time, the inflammatory index increased (**Fig. 3, Paper III**). Taken together, the data suggest that there is a shift towards an anti-inflammatory M2-like phenotype in macrophages from *S. pyogenes* infected tissue, in spite of the infections generally being associated with a high degree of inflammation.

To validate the findings from the patient tissue, we utilized an organotypic skin tissue model, which has been shown to be a robust tool to study host-pathogen interactions in a tissue like environment (58). We implanted the model with primary human monocytes, which were allowed to differentiate for 10 days before bacterial challenge. At this time point, CD68+ cells could be detected in the model by both flow cytometry and confocal microscopy. Microscopic analysis of models infected by the clinical *S. pyogenes* isolate 2006 revealed a reduced inflammatory index in monocyte-derived macrophage like cells from infected as compared to uninfected models. Furthermore, flow cytometry analysis of enzymatically-digested models revealed that the monocyte-derived macrophage like cells from *S. pyogenes* infected models had a phenotype more similar to that of models treated with M2-polarizing agents (IL-4+IL-10), as compared to models treated with M1- polarizing agents (IFNγ+LPS) **(Fig. 4, Paper III**), further supporting the notion of a shift towards an anti-inflammatory phenotype in macrophages, in response to *S. pyogenes* infection.

4.3.3 Over-represented pathways in *S. pyogenes* infected tissue

To get a broader view of the host response to *S. pyogenes* infection, we analyzed gene expression in *S. pyogenes* infected tissue by RNA sequencing and differential gene expression analysis. To reduce the level of complexity, we started by analyzing the skin tissue model, which only contains three different types of cells: keratinocytes, fibroblasts and monocyte-derived macrophage like cells.

Comparison of *S. pyogenes* infected and uninfected models revealed an up-regulation and overrepresentation of mediators involved in IL-37-, IL-10-, IL-20- and IL-4/IL-13-signaling pathways, as well as terminal pathway of complement. Interestingly, several of the implicated pathways have also been implicated in M2-polarization (219, 262, 263). To further investigate this, we identified the differentially expressed genes linked to the implicated pathways, focusing on factors previously reported to be associated with either M1 or M2 macrophages. Normalized gene expression values showed a mixed response among the selected markers in infected compared to uninfected models (**Fig. 5, Paper III**).

These results are in line with a previous report assessing macrophage polarization in response to *S. pyogenes* infection, in mice, which showed a mixed expression profile with regards to M1 and M2 markers (228). However, an additional report by Veckman et al. reported an increased production of M1-associated chemokines by *S. pyogenes* infected monocyte-derived macrophage like cells, as well as chemotactic effect of culture supernatants from macrophage like cells on Th1 cells (264). While the observed differences may be due to different experimental set-ups including the fact that our cells were in a tissue-like environment and the experiments by Veckman et al. were performed in *in vitro* monocultures. Veckman et al. also appeared to focus on M1-associated chemokines; hence, it is possible that the cells were also producing M2-associated chemokines, and we did in fact see an up-regulation of M1-associated genes as well.

To assess if the implicated pathways could also be detected in patients, gene expression data from *S. pyogenes* infected patient tissue were compared to healthy control tissue. Analysis of differentially expressed genes implicated several pathways within the immune system, more than identified in the infected organotypic skin models. Notably, both IL-4/IL-13- and IL-10-signaling were implicated also in the patient tissue. The larger number of implicated pathways identified in the real tissue likely reflects the greater complexity of the patient tissue. Several of the pathways, i.e. neutrophil degranulation, TLR-regulation, cross-presentation and ROS/RNS production by phagocytes, are of interest considering our previous findings of hyper-inflammatory responses and neutrophil activation and degranulation **(Paper I and II)**. We also analyzed gene expression data from a murine NSTI-model, utilizing different HLA class II transgenic mice with varying susceptibility to *S. pyogenes* infection. Also these results verified IL-4/IL-13- and IL-10 signaling, as well as neutrophil degranulation, as over-represented pathways in infected versus uninfected mice (**Fig. 6, Paper III**).

Taken together these data indicate that *S. pyogenes* infection is associated with a shift in macrophage phenotype, towards an anti-inflammatory M2-like state, despite the fact that the tissue generally has a high degree of inflammation.

In the sepsis-field, the terms systemic inflammatory response syndrome (SIRS) and compensatory anti-inflammatory response syndrome (CARS) are used as a conceptual framework to describe the immunologic responses observed in the patients, with an initial hyper-inflammatory state, followed by a state of immunosuppression when the patient is prone to acquire nosocomial infections (265). Though traditionally being viewed as

sequential events, SIRS and CARS have been proposed to take place at the same time (266, 267). This is very much in line with the findings presented here where we can see an anti-inflammatory macrophage phenotype in a hyper-inflammatory tissue, highlighting the complexity of these conditions.

In further support of this mixed inflammatory concept, a report by Hansen et al. showed increased levels of classical pro-inflammatory mediators like IL1β, IL-6 and TNF as well as elevated levels of IL-10 in plasma from NSTI patients, collected within the same project as the tissue biopsies used here (71).

Several recent studies have reported on inflammasome activation and IL-1b release from macrophages in response to S. pyogenes infections (268), and IL-1b was recently recognized as a key regulator contributing to susceptibility to S. pyogenes NSTI (70, 71). Hence, it will also be of interest to study IL-1b and inflammasome activation in the M2-like macrophages identified in the infected tissue.

One limitation of this study is that we were unable to assess the effect of macrophage phenotype on *S. pyogenes* survival. This would require the ability to simultaneously identify live bacteria and macrophage phenotype at the single cell level, which is currently not possible. Furthermore, future studies are warranted to delineate the function of the observed macrophage phenotypes in the infected tissue, by assessing the presence of cytokines, antimicrobial mediators as well as bacterial killing.

5 CONCLUDING REMARKS

In this thesis host-pathogen interactions at the site of infection in severe streptococcal infections have been explored. In particular, interactions of the bacteria with neutrophils and macrophages have been in focus, as previous reports have shown a high infiltration of these cells at the site of infection and a positive correlation to severity of the infection (57). We investigated this by utilizing a combination of analyses of patient tissue biopsies with *in vitro* infection or stimulation experiments using primary human cells and a 3D skin tissue model. Microscopy analyses have been used to visualize the interactions, including also establishment of multi-parameter microscopic method for single-cell phenotyping in tissue biopsy sections.

Paper I

- HMGB1 can be detected in tissue biopsies collected from patients with severe streptococcal soft tissue infections, and its levels correlates with severity of disease.
- HMGB1 is present in the same areas as IL-1β and co-localization of the two factors suggests that immunostimulatory complexes may form.
- HMGB1 has chemotactic effects on neutrophils.

Our results indicate the potential value of HMGB1 as a biomarker for tissue pathology in severe soft tissue infections. A biomarker that can detect necrosis in the deeper tissue would be of great clinical value as these infections are often difficult to diagnose and prompt diagnosis is critical to save lives and limbs. In addition, our results show that HMGB1 has the potential to form immunostimulatory complexes and recruit neutrophils, which could be harmful in the severe infections as they are characterized by an excessive inflammatory response. However, further investigations are needed to determine whether HMGB1 is a mediator of the tissue pathology observed in severe streptococcal soft tissue infections. Given its ability to form immunostimulatory complexes with e.g. LPS, it is also of interest to explore whether HMGB1 can also form immunostimulatory complexes with streptococcal factors. Also, the co-presence of IL-1β and HMGB1 warrants further studies on how HMGB1 relates to inflammasome activation during infection. This is of particular relevance as IL-1β has been identified as a key network in to *S. pyogenes* NSTI (70).

Paper II

- Streptococcal strains are potent inducers of neutrophil activation and degranulation resulting in release of the sepsis-associated factors HBP and resistin.
- There are differences in signaling requirements involved for the release of HBP and resistin, respectively. While HBP release is mainly dependent on a previously described mechanism involving M-protein dependent dual ligation of integrins and Fc-receptors, the release of resistin is multifactorial and involves multiple bacterial structures and host signaling pathways.
- HBP and resistin are mainly localized within different intracellular granules, but display a synchronized release upon streptococcal activation.

Our results show that neutrophil responses differ considerably depending on the bacterial stimuli with streptococcal strains being potent inducers of neutrophil activation and degranulation, resulting in a synchronized release of HBP and resistin. We could also detect both HBP and resistin in circulation of septic patients as well as in the infected tissue from patients with severe streptococcal soft tissue infections. Future studies are needed to pinpoint whether the neutrophil response profile is different in patients with invasive infections caused by different pathogens. Notably, the gene expression profiling presented in **Paper III** demonstrate an overrepresentation of the neutrophil degranulation pathways in patients with *S. pyogenes* NSTI. Future studies will explore whether this is a unique feature for streptococcal elicited infections or also evident in NSTI of other aetiology.

Paper III

- Macrophages in *S. pyogenes* infected tissue display an anti-inflammatory phenotype, in spite of the hyper-inflammatory environment.
- Gene expression analysis of severe streptococcal soft tissue infection showed an overrepresentation of signaling pathways associated with anti-inflammatory macrophage polarization.

Our results show that macrophages in severe streptococcal soft tissue infections display a more anti-inflammatory M2-like phenotype in both patient tissue biopsies as well as in *S. pyogenes* infected skin tissue models. As M2 macrophages are typically associated with impaired bacterial killing, the results imply that this phenotype switch promotes bacterial intracellular survival. How this relates to functional responses elicited by the macrophages has yet to be investigated. Future studies should also investigate whether the observed shift in phenotype promotes intracellular survival of the bacteria, and thereby bacterial persistence. We also intend to follow-up on recent studies implicating inflammasome activation in macrophages (268), and to explore how this relates to macrophage phenotype and intracellular bacterial survival at the tissue site of infection.

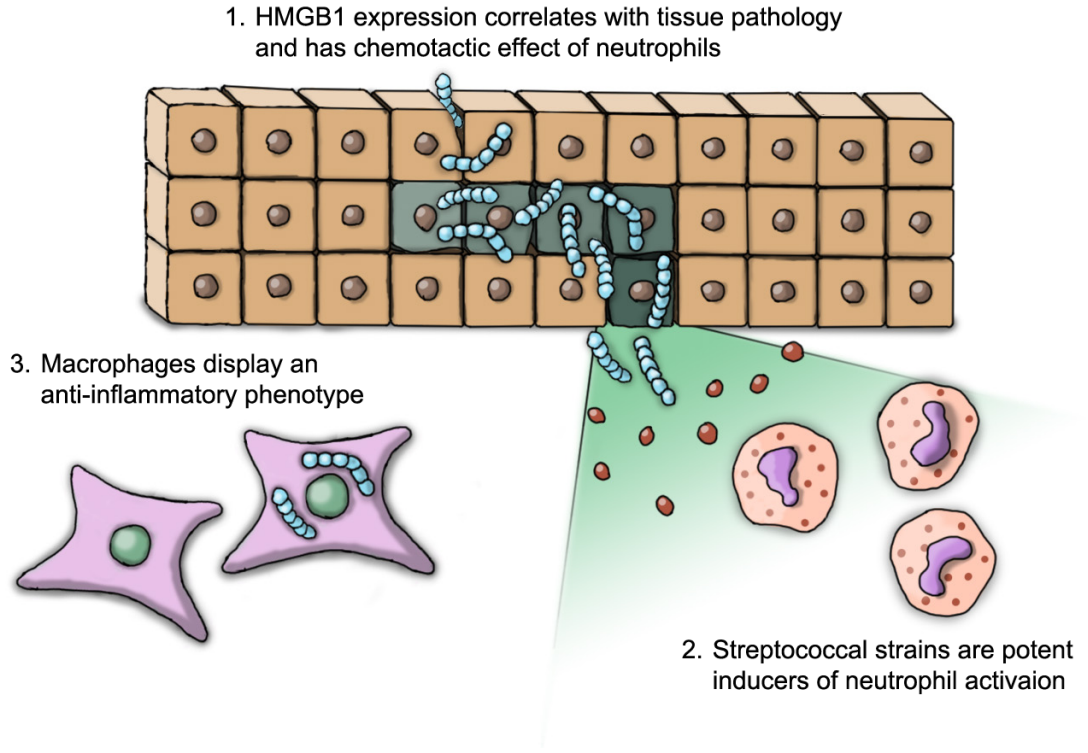


Figure 8. Summary of findings in paper I-III.

Illustration by Andreas Mielonen

Taken together, the findings from this thesis project highlight the complex pathophysiology of severe *S. pyogenes* infections, where on the one hand the bacteria and mediators present in the tissue potently activates neutrophils. While macrophages on the other hand, display a more anti-inflammatory phenotype, potentially promoting intracellular survival and persistence of *S. pyogenes* (Fig. 8).

It also underlines the importance of careful patient characterization in order to pinpoint the immune status of the patient to identify what that patient needs in terms of immunomodulatory treatment; thus, allowing for personalized medicine in severe infections. With respect to neutrophil activation, streptococcal patients might have an excessive neutrophil degranulation as compared to other aetiologies. Also among patients with *S. pyogenes* infections, there may be variation in neutrophil responses determined by the patients' antibody titers to virulence factors where high titers are linked to increased HBP release. Such difference should likely be taken into account to optimize the therapeutic strategies. To obtain such a personalized therapy, there is a great need for improved understanding of the pathogenesis as well as diagnostic tools to determine the response profile in individual patients. That is what this thesis work has been about, trying to characterize and further our understanding regarding the host-pathogen interactions at the site of infection, and how they contribute to the pathology of these diseases.

Also, patients may display different macrophage phenotypes. How this is functionally related to the outcome of an infection still remains to be elucidated. However, if an anti-inflammatory macrophage phenotype supports intracellular survival of the bacteria and thereby persistence it would potentially be beneficial to try and polarize the macrophages towards a phenotype with more efficient bacterial killing. Having that said, recent studies have shown that ageing mice have increased number of inflammatory monocytes, but that these monocytes are dysfunctional with regards to bacterial killing (269).

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