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***STREPTOCOCCUS PYOGENES*:**
life within the macrophage

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“Väntat så länge på just den här dan, och det är skönt att den äntligen kommer”

Kenta Gustafsson

ABSTRACT

Streptococcus pyogenes is a versatile human pathogen causing a wide array of diseases ranging from uncomplicated throat and skin infections to invasive life-threatening diseases with a mortality rate of up to 60%. It is ranked number nine on the list of worst infectious diseases world wide, causing 500 000 deaths yearly. The work presented in this thesis was initiated upon finding viable bacteria in tissue macrophages of patients with severe *S. pyogenes* infections. This intracellular bacterial reservoir was linked to prolonged bacterial persistence at the tissue site. It was therefore of interest to investigate how the bacteria could survive within professional phagocytes that have evolved for the specific purpose of degrading invading microbes. Hence, the overall aim of the thesis was to decipher streptococcal survival strategies in macrophages.

Certain bacterial serotypes are more efficient in causing invasive infections than others, as they are equipped with special tools to facilitate invasion and survival. We have identified the bacterial M1-protein as an important factor for intracellular survival in macrophages. Studies on entry and intracellular trafficking revealed that the bacteria are residing inside membrane-bound compartments, which do not fuse with the lysosomal compartments; thus enabling persistence and replication. Thereto, M1-protein was shown to suppress the host-inflammatory response upon *S. pyogenes* infection. Regulation of bacterial intracellular signaling is vital for bacterial survival and it also affects host responses. Data are supporting a role for the two-component gene regulatory system, Ihk/Irr, during adaption to the intracellular environment while another regulatory system, CovR/S, possibly facilitates infectivity of disseminating bacteria. In addition, we show that the arachidonic acid metabolite, prostaglandin E_2, which is produced by host cells upon infection, has a negative impact on macrophage bactericidal responses, thus enabling bacterial survival inside the host cell.

The studies comprised in this thesis contribute to a deeper understanding of the host-pathogen interplay during severe *S. pyogenes* infections, in particular with regards to mechanisms contributing to intracellular survival in host cells. The results demonstrate that *S. pyogenes* ability to persist within macrophages is enabled through distinctly regulated mechanisms involving both host and bacterial factors.

S. pyogenes used to be considered as an extracellular pathogen, however, it is becoming increasingly apparent that it is important to consider also an intracellular source; thus affecting choice of antimicrobial agents. In addition, work presented in this thesis has identified several novel targets, both on the bacterial side and on the host side, which may be suitable candidates for intervention.

LIST OF PUBLICATIONS

This thesis is based on two publications and one manuscript. The individual papers are referred to by Roman numerals.

I. **Hertzén E**, Johansson L, Wallin R, Schmidt H, Kroll M, Rehn A, Kotb M, Mörgelin M, Norrby-Teglund A. M1 Protein-dependent Intracellular Trafficking Promotes Persistence and Replication of *Streptococcus pyogenes* in Macrophages.
Journal of Innate Immunity. 2010 Aug;27(2): 234-245.

II. **Hertzén E**, Johansson L, Kansal R, Hecht A, Dahesh S, Nizet V, Kotb M, Norrby-Teglund A. *Streptococcus pyogenes* Utilize the *ihk/irr* Two-Component Gene Regulatory System During Intracellular Survival in Human Macrophages. Manuscript.

III. Goldmann O, **Hertzén E**, Hecht A, Schmidt H, Lehne S, Norrby-Teglund A, Medina E. Inducible Cyclooxygenase Released Prostaglandin E_2 Modulates the Severity of Infection Caused by *Streptococcus pyogenes.*
Journal of Immunology. 2010 Jul;19(185); 2372-2381

ADDITIONAL PAPER

Shannon O, **Hertzén E**, Norrby-Teglund A, Mörgelin M, Sjöbring U, Björck L. Severe Streptococcal Infection is Associated with M protein-Induced Platelet Activation and Thrombus Formation.
Molecular Microbiology. 2007 Jul;27,**65**(5), 1147–1157.

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
Atg	Autophagy-related
APC	Antigen-presenting cell
ARF	Acute rheumatic fever
ATP	Adenosine triphosphate
BM	Bone marrow
cAMP	Cyclic adenosine monophosphate
COP1	Coat protein 1
COX	Cyclooxygenase
CR	Complement receptor
DC	Dendritic cell
EEA-1	Early endosomal antigen 1
ER	Endoplasmic reticulum
EP	E prostanoid
Fc-R	Fc-receptor
FnBP	Fibronectin-binding protein
GFP	Green fluorescent protein
GRAB	G-related α-$_2$-macroglobulin-binding protein
GTPase	Guanosine triphosphatase
IdeS	Immunoglobulin G-degrading enzyme of *S. pyogenes*
IL	Interleukin
iNOS	Inducible nitric oxide synthase
LAMP	Lysosomal associated membrane protein
LC3	Light chain 3
LDH	Lactodehydrogenase
LLO	Listeriolysin O
LPS	Lipopolysaccharide
LTA	Lipoteichoic acids
MBL	Mannose binding lectin
mga	Multiple gene regulator
MHC	Major histocompatibility complex
MR	Mannose receptor
MyD88	Myeloid differentiation primary response gene 88

NF-κB	Nuclear factor kappa B
NK cell	Natural killer cell
NLR	Nod-like receptor
NO	Nitric oxide
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PGE2	Prostaglandin E2
PKA	Protein kinase A
PMN	Polymorphonuclear cell
PRR	Pattern recognition receptors
PTS	phosphate-specific transport system
RALP	RofA-like protein family
RHD	Rheumatic heart disease
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RR	Response regulator
SIC	Streptococcal inhibitor of complement
SKA	Streptokinase
SLO	Streptolysin O
SLS	Streptolysin S
SNARE	Soluble NSF-attachment protein receptor protein
SpeB	Streptococcal exotoxin B
STSS	Streptococcal toxic shock syndrome
T3SS	Type three secretion system
TCR	T-cell receptor
TCS	Two-component system
TEM	Transmission electron microscope
TNF	Tumor necrosis factor
TLR	Toll-like receptor
T_H	T-helper cell
T_c	T-cytotoxic cell

1 INTRODUCTION

1.1 HOST DEFENCE MECHANISMS AGAINST PATHOGENS

The function of the immune system is to sense and protect the host from foreign substances. It is a delicate machinery with a variety of adaptations depending on the site of invasion and the type of invader. The primary defense is called the innate immune system and consists of several branches. Skin and mucosal surfaces lining the cavities in the body represent a major barrier for invading pathogens. However, sometimes, these barriers are compromised, allowing the intruder to become invasive, whereafter it comes in contact with the cellular immune system.

1.1.1 Cellular components of the immune system

Cellular immunity is divided into a primary and a secondary response upon challenge. The primary immune response is the first line of defense that recognizes conserved microbial patterns and cannot differentiate between small variations in foreign particles and it does not have the capacity to memorize a pathogen exposure. It is activated within hours of infection and comprises phagocytes, such as macrophages, dendritic cells (DCs), and polymorphonuclear cells (PMNs), and natural killer (NK) cells. Antimicrobial peptides, components of the complement system and cytokines are also part of the innate immunity (1).

The second part of the immune response, which kicks in at a later stage, up to days after infection, is characterized by immunological memory and is called the adaptive immunity. It can be divided into two parts, the humoral and the cell-mediated response. The main effector cells are the lymphocytes, which are divided into two groups; B lymphocytes, the antibody-producing cells that mediate the humoral immunity and T lymphocytes that provide the cell-mediated immunity (2). Antibodies secreted by B cells recognize microbes and neutralize for example secreted toxins and target the microbes for destruction by effector cells (3). Adaptive immunity is initiated by lymphocyte recognition of a foreign antigen. Antigen-presenting cells (APCs), namely macrophages, DCs and B cells are cells that upon encounter with a foreign particle, phagocytose and degrade it. During the process of degradation, peptides are derived which can be presented on MHC class I or class II molecules. MHC molecules with the processed peptide on the APCs bind to the T cell receptor on T cells, which then become activated and gain effector functions, such as secretion of cytokines and cytotoxicity. There are two types of T cells, $CD4^+$ T cells that are activated by APCs presenting antigen on MHC class II molecules, mainly derived from exogenous antigens, and $CD8^+$ T cells that are activated by antigens on MHC class I molecules, derived from both exogenous and endogenous antigens such as bacteria, viruses or tumor cells (2, 4). Antigens

derived from intracellular bacteria that are residing inside phagosomes of host cells are presented on MHC class II, while antigens from bacteria escaping from the phagosome and consequently residing in the cytosol, are presented on MHC class I molecules (4). $CD4^+$ T cells generally functions as T helper (T_H) cells secreting cytokines, while $CD8^+$ T cells develop into T cytotoxic (T_c) cells (1, 2).

Repeated exposure to a microbe results in a faster and more prominent adaptive immune response since it is provided with memory lymphocytes that recognize the antigen (5). This is in contrast to the innate immune response that will always react with the same power. The ability of T and B cells to express receptors that can discriminate between small differences in structure between antigens forms the basis of the concept of diversity. This ability differentiates receptors of cells of the innate immunity, which can only recognize a limited number of patterns shared by several microbes. It is estimated that the innate immune system can recognize 10^3 different patterns while the adaptive immune system can recognize 10^7 or more specific antigens (2). Table 1 presents general characteristics of the innate and the adaptive immune system.

Characteristics	**Innate immunity**	**Adaptive immunity**
Specificity	For structures shared by groups of related microbes	For antigens of microbes
Diversity	Limited	Very large
Memory	None	Yes
Time to action	Within hours	Within days
Components		
Physical and chemical barriers	Skin; mucosal epithelia; antimicrobial compounds	Lymphocytes in epithelia; antibodies secreted at epithelial surfaces
Blood proteins	Complement	Antibodies
Cells	Macrophages, DCs PMNs, NK cells	T and B lymphocytes

Table 1. Selected basal features of innate and adaptive immunity.
Adapted from Abbas et al., Cellular and Molecular Immunology, Fifth edition, 2005 (2).

1.1.1.1 Pattern-associated molecular patterns and their receptors

Upon encounter with a pathogen, innate immune cells recognize microbial structures on the intruder known as pathogen-associated molecular patterns (PAMPs). The receptors on immune cells recognizing the PAMPs are called pattern recognition receptors (PRRs). They recognize a variety of PAMPs including double-stranded RNA from replicating viruses, DNA from bacteria, lipopolysaccharide (LPS) expressed by Gram-negative bacteria and lipoteichoic acids (LTA) from Gram-positive bacteria (6). Moreover, each type of PAMP is recognized by different receptors on host cells in the tissue or in the circulation (2, 7). The most common receptor type is Toll-like receptor (TLR) family (8), which comprises 10 known receptors in humans and 12 in mice. About half of the TLRs are expressed on the cell surface enabling recognition of PAMPs from extracellular bacteria, fungi and protozoa. These include TLR 1,

2, 4, 5 and 6, while the remaining TLRs (3, 7, 8, 9) are located inside the cells in endosomes/lysosomes where they recognize nucleic acid PAMPs from various viruses and bacteria (9). TLR10 is expressed on the surface; however, its activating ligand remains unknown. TLR activation generally induces transcription of inflammatory cytokines. Other membrane-bound receptor types are mannose receptor (MR) and mannose-binding lectin (MBL) receptor. Moreover PPRs called NOD (nucleotide-binding oligomerization domain)-like receptors (NLRs) are present in the cytoplasm where they recognize various PAMPs from bacteria, viruses and fungi. Recognition of a PAMP by an NLR can lead to activation of the NF-κB (Nuclear factor kappa B) or MAP-kinase pathway inducing transcription of pro-inflammatory cytokines (6). Recently, numerous studies have reported that also bacterial RNA in the cytoplasm, identified by RIG-I or MDA-5 receptors, can induce type I interferon responses, something that previously has been thought of as a strictly anti-viral response (10). Triggering of TLRs induces a wide range of responses from secretion of type I interferons and induction of an antiviral state to macrophage activation (2).

1.1.1.2 The complement system

Another important system involved in both innate and adaptive immunity is the complement system. It consists of several plasma proteins that upon activation by pathogens carry out several central functions in the immune response, including:

- causing lysis of cells, bacteria and viruses
- targeting antigens for subsequent phagocytosis by opsonization
- binding to specific complement receptors on host cells to induce cellular functions, such as secretion of inflammatory molecules
- performing clearance of immune complexes in the circulation

The proteins comprising the complement system are circulating in an inactive state in the serum. Upon activation, several factors assemble into a functional pore forming membrane attack complex (MAC), which causes lysis of the target. Activation occurs via three different mechanisms: 1) binding of C1 complex to the FcRs of antibodies attached to pathogenic surfaces. 2) recognition of PAMPs, or 3) binding of MBL to the pathogen which acts as an opsonin (11, 12).

1.1.1.3 Cytokine responses during infection

Activation of the immune system results in production of a variety of cytokines inducing different effector functions. T_H cells are secreting cytokines that can direct the type of immune response that is mediated by effector cells participating in the host response. A T_H1 response leads to a pro-inflammatory cytokine profile that supports inflammation and activates macrophages and T_c while a T_H2 response mainly activates B cells and immune responses that depend on antibodies (13).

TNF-α and IL-1β are hallmark mediators of immune activation and are secreted by a variety of cells upon infection. They are both potent pleiotropic cytokines that stimulate activation of cells and TNF-α is responsible for many of the systemic complications seen in severe infections such as sepsis and toxic shock (14, 15). IL-12 is inducing IFN-γ secretion and subsequent macrophage activation to kill intracellular pathogens. In addition, IL-12 enhances the cytolytic function of NK cells and $CD8^+$ T cells (16, 17). Secretion of type I interferons such as IFN-α and IFN-β are important tools to keep control of viral infections (2, 18). IL-6 is involved in promoting adaptive immunity by inducing proliferation and antibody production of B cells (12). In summary, upon encounter of a foreign particle, the immune system is activated and many factors are secreted to promote specific responses depending on the invader. However, when an immune reaction has been initiated, several cytokines are secreted to regulate and confine the host cell responses through different mechanisms, such as inhibition of IL-12 production, promoting down-regulation of MHC class II molecule expression and limiting proliferation and activation of effector molecules. This is maintained by for example IL-10 and TGF-β (1, 2).

1.1.2 The macrophage and its role during infection

Macrophages are important cells of the immune system and they have an essential role in this thesis. They have the capacity to engulf and degrade pathogens as well as to present antigens to T cells and thereby initiate a powerful activation of the adaptive immune response. This section comprises a detailed description of macrophage effector functions during infection.

1.1.2.1 Pattern recognition receptors

Recognition of a pathogen occurs via an array of receptors expressed on the cellular membrane. Interaction with a pathogen can occur directly via PAMPs and PRRs or indirectly via opsonins on the pathogenic surface. Opsonins include for example antibodies, which are recognized by the Fc-receptors (FcRs) and complement components that are recognized by the complement receptors (CRs). Most microbial structures are recognized by more than one receptor, and some receptors may also interact with each other. Each receptor has a different role during the process, some mediate adhesion while others are part of the internalization process and certain receptors are involved in both. An example is ligation of the fibronectin receptor at the surface of the macrophage, which induces signaling and activation of the otherwise inactive complement receptor CR3 to mediate phagocytosis (19, 20). Macrophages express a family of receptors called scavenger receptors, which upon ligation can induce phagocytic uptake. They differ from FcRs and CRs since they can recognize and bind their target without opsonins. The most well known receptors are scavenger receptor A and macrophage receptor with collagenous structure, which recognize many bacterial ligands including LTA on Gram-positive bacteria and the lipid A component of LPS on Gram-negatives (21). The c-type lectin domain receptors identify carbohydrates on pathogens. One example is the MR, which has been shown to bind the bacterial cell wall

glycolipid mannosylated lipoarabinomannan (ManLam) of *Mycobacteria* and thereby induce bacterial uptake (22).

1.1.2.2 Maturation of the phagosome

Upon recognition and uptake of pathogens, the macrophage induces a variety of effector functions. At first the intruder is surrounded by a membrane structure, which forms the vesicle, called the phagosome. Initially it contains extracellular fluid and is not harmful to the pathogen. However, phagolysosomal maturation is instantly initiated, as this is the default endosomal-lysosomal degradation pathway. Consequently, many intracellular pathogens have developed mechanisms to avoid this pathway, which is further described in section 1.3.1.2.

Maturation of the phagosome includes recruitment and fusion of vesicles containing hydrolytic enzymes and protons, and the pH of the phagosome is constantly decreasing during progression to the phagolysosome (Figure 1).

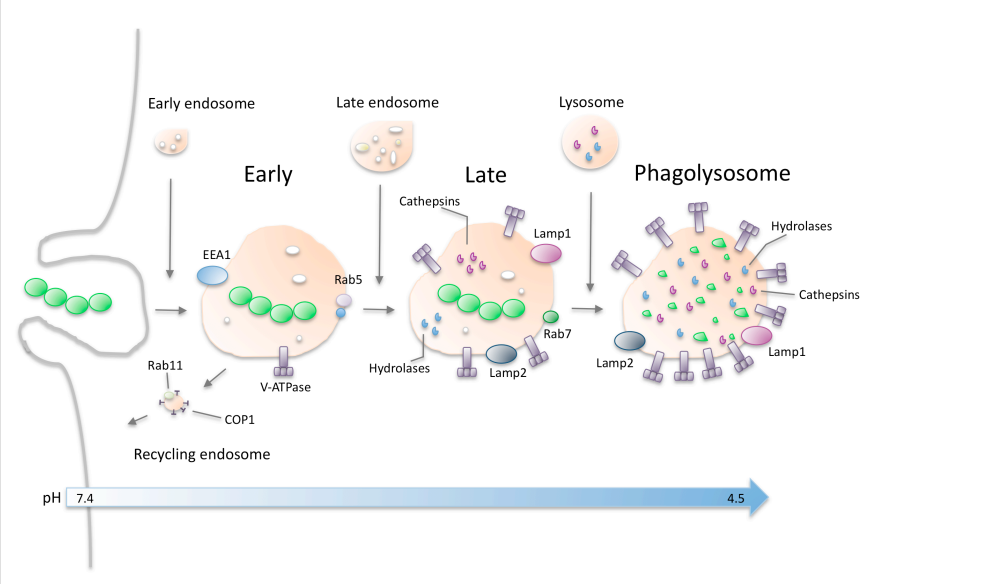

Figure 1. General overview of phagosomal maturation.

Recruitment of vesicles is maintained by small guanosine triphosphatases (GTPases). The phagosome is quickly becoming an early endosome through fusion events organized by the GTPase Rab5 (23) using several effectors such as early endosomal antigen 1 (EEA-1) and soluble NSF-attachment protein receptor proteins (SNARE), which mediate docking and fusion of cellular membranes. EEA-1 is thought to act as a bridge that brings early endosomes to incoming endocytic vesicles (24). The pH of the early endosome is mildly acidic (pH 6.1-6.5) and has a weak hydrolytic activity (25). In addition, membrane parts of the phagosome are recycled to the plasma membrane via fission events governed by coat protein 1 (COP1), Arp and GTPases such as Rab11 (26, 27). When these recycling proteins are removed, the phagosome progress to a later stage called the late endosome. It is characterized by an

increase in hydrolytic enzymes and a lower pH ranging from 5.5-6.0, through acquisition of a proton-pumping vacuolar (V)-ATPases (28) and fusion with lysosome associated membrane proteins (LAMPs) (29). Formation of the late endosome is regulated by Rab7 GTPases that coordinate the traffic between endosomes and lysosomes (30). The final maturation stage is the phagolysosome, where pathogens are degraded in a lumen that has been reported to be as low as pH 4.5 (31). The acidity is created by accumulation of V-ATPases and in addition the acidity activates maturation of hydrolytic enzymes such as endopeptidases and hydrolases. These enzymes are produced in a distinct order, where cathepsin H is predominant in early phagosomes while cathepsin S is more common in late phagosomes (32).

1.1.2.3 Reactive oxygen species and antimicrobial proteins

Bacterial killing is also achieved by reactive oxygen species (ROS), produced by the NADPH oxidase 2 (NOX2). When activated, NOX2 transfers electrons from cytosolic NADPH to molecular oxygen, releasing O_2^- into the phagosomal lumen. Inside the phagosome O_2^- forms H_2O_2, which again reacts with O_2^-, generating hydroxyl radicals and singlet oxygen (33). These highly reactive ROS efficiently kill the pathogens inside the phagosome. In addition to ROS, macrophages produce nitric oxide (NO) and reactive nitrogen species (RNS). NO is produced upon activation of the inducible nitric oxide synthase (iNOS) by pro-inflammatory cytokines. In contrast to ROS, NO is produced in the cytosol but can cross the phagosomal membrane where it reacts with ROS resulting in highly toxic compounds (34).

In addition, macrophages have a variety of antimicrobial effector molecules, including antimicrobial peptides, scavenger molecules and ion transporters. They can be divided into two functional groups; one that limits bacterial growth, bacteriostatic, and one that induces bacterial lysis, bacteriocidal. To affect the bacterial growth, scavenger molecules can be transferred into the phagosome, such as lactoferrin that bind available Fe^{2+}, which is important for some bacterial species (35). Another mechanism is to insert transporters that export divalent cations such as Fe^{2+}, Zn^{2+} and Mn^{2+} important for house keeping genes and virulence (36). Antimicrobial peptides are effector molecules that target the microbial membrane to induce bacterial lysis. They also possess a variety of immunomodulatory functions stimulating for example cytokine release and chemotaxis (37, 38).

1.1.2.4 Autophagy

The autophagosome is a machinery used to degrade cellular rest products by collecting them in a double membrane organelle that is transported to the lysosome forming an autolysosome. This system can be utilized to eliminate cytosolic pathogens since a number of invaders have evolved ways to escape from the endosomal-lysosomal pathway in to the cytosol (39, 40). The autophagosome is formed by rough endoplasmic reticulum (ER) via (Autophagy-related) Atg1 and Atg14 regulatory genes and is marked by light chain 3 (LC3) (41). Apart from degrading the pathogen, the autophagy system

also mediates additional immune effector functions such as increasing endogenous cytoplasmic presentation of antigens via major histocompatibility complex (MHC) class II (42). Intracellular pathogens that are captured by autophagy include Mycobacterium (43), Francisella (44) and Listeria (45). Interestingly, polymorphisms in an autophagy regulatory gene have been linked to predisposition to tuberculosis and to the chronic inflammatory bowel disease, Crohn's disease, in humans (46, 47). In addition, *in vivo* animal models confirm the importance of functional autophagic regulators during bacterial infections (48).

1.1.2.5 Macrophage polarization

Similarly to the T_H1 and T_H2 responses of T cell polarization, macrophages can be divided into M1 and M2 phenotype, with specific effector functions, depending on the signaling molecules in the surrounding environment (Figure 2)(49, 50). The classical M1 response is triggered by microbial products or IFN-γ, inducing an anti-microbial response with secretion of for example IL-12, IL-23, an increased capacity to present antigens and to produce ROS (51). The alternative activation of macrophages is triggered by mediators such as IL-4, IL-13, IL-10, TLR-ligands and prostaglandins inducing a M2 type of macrophage (52, 53). The M2 macrophages are poorly bactericidal and several pathogens induce this phenotype to control the host cell response (54, 55). It was recently reported that polymorphism in a transcription factor regulating macrophage polarization leads to higher susceptibility of autoimmune diseases resulting from increased inflammation (56).

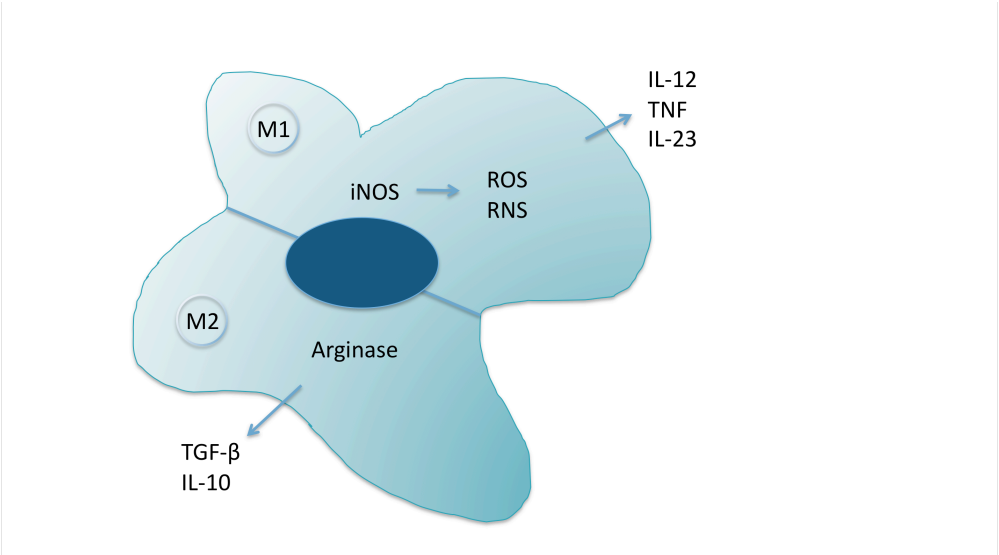

Figure 2. General characteristics of M1 and M2 polarization.

1.1.2.6 NF-κB activation

Recognition of pathogens by the different PRRs involves, in the majority of cases, signaling through the adaptor molecule myeloid differentiation primary-response gene (MyD88) (57). Signaling via MyD88 eventually leads to activation of the cytosolic NF-κB, which regulates effector functions of both innate and adaptive immunity. There are five different mammalian NF-κB proteins, p65/relA, RelB, c-Rel, p50/p105 and p52/p100. They form hetero- and homodimers that depending on the constellation result in differential signaling. NF-κB is also equipped with negative regulators, the p50 and p52 which act as repressors and thereby inhibit inflammatory responses (58). NF-κB proteins are present in the cytoplasm in an inactive state, which is maintained through association with inhibitory IκB proteins, thereby limiting nuclear access. Upon stimulation of the cell, the IκB protein is phosphorylated and subsequently degraded, thus releasing NF-κB, which translocates to the nucleus and induces gene transcription of inflammatory effector molecules (59, 60). Interestingly, accumulation of p50 homodimers have been reported in LPS-tolerent monocytes in septic patients and in M2 polarized macrophages (50, 61, 62).

1.1.2.7 Prostaglandin E_2 during immune responses

Prostaglandin E_2 (PGE_2) is produced by a wide array of cells, including macrophages, and has been shown to affect immune responses during infection and inflammation (63). It is dependent on the inducible enzyme cyclooxygenase (COX). PGE_2 acts via G-protein-coupled receptors, called E prostanoid (EP) receptors. Engagement of these receptors results in different effector functions depending on the cell type, receptor subtype, and signal of activation. Release of PGE_2 is known to induce a T_H2 – type of response with suppression of T cell proliferation as well as inhibition of IFN-γ secretion (63, 64). In addition, it stimulates cytokine production by DCs, macrophages and B cells towards a T_H2 response (65). Recent reports underscore the exploitation of PGE_2 during *Francisella* infection of macrophages (66) and during *S. pyogenes* infection *in vivo (65, 67)*. It appears that these bacteria can utilize PGE_2 to facilitate infection by promoting an anti-inflammatory host response.

1.1.3 Phagocytosis and other uptake mechanisms

There are multiple ways of pathogen uptake by phagocytes. In most cases, the primary interaction induces a signaling cascade, which results in rearrangement of the actin cytoskeleton and subsequent reformation of the plasma membrane to assist the procedure, Figure 3. Often, host cell integrins are involved in activation and modulation of the actin filaments via phosphorylation of cytoplasmic domains. Conventional phagocytosis is probably the most well-known uptake mechanism by cells. The discovery of the capacity of cells to engulf particles awarded Elie Metchnikoff the Nobel prize in 1908. Since then this process has been investigated in detail and much progress has been made in understanding the molecular events underlying this procedure, which activates a variety of signaling cascades with their effector

functions (68). Several receptor-interactions induce phagocytosis such as binding of immunoglobulin to FcRs or complement proteins to CRs and binding of PAMPs to TLRs or scavenger receptors to mention a few. Often the same pathogen is recognized by several receptors inducing multiple signaling cascades with the final goal to eliminate the invader (69). Different mechanisms of phagocytosis with characteristic morphology have been described such as zippering, triggering and coiling phagocytosis. It is evident that the uptake mechanism differs depending on the prey, the host cell and which receptors are involved in recognition. The zippering pathway involves formation of a tight vacuole that surrounds the pathogen and transfers it into the cell. *Listeria* and *Yersinia* are both taken up via this pathway (70). The triggering/micropinocytic pathway is used for example by *Salmonella* and *Shigella* where the pathogens induce membrane ruffling by secreting factors into the host cells stimulating uptake in a spacious vacuole (71). *Legionella* is taken up by coiling phagocytosis, during which the host cell membrane forms long pseudopodia that tightly encircles the bacterium prior to entry (72).

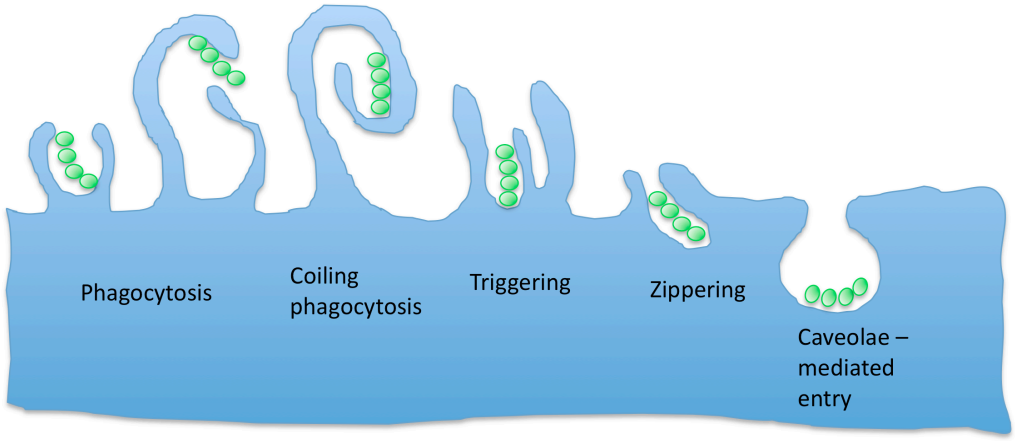

Figure 3. Simplified schematic illustration of various entry mechanisms.

Furthermore, *Francisella* and *S. pyogenes* are taken up by yet another mechanism inducing formation of large asymmetric spacious pseudopod loops (73, 74). In addition, there is a mechanism called caveolae-mediated entry, which involves induction of invaginations in the plasma membrane, which morphologically differs from the other pathways, and has been shown to mediate uptake into host cells for several pathogens (75, 76).

1.2 *STREPTOCOCCUS PYOGENES*

1.2.1 Disease spectrum

The Gram-positive *Streptococcus pyogenes*, also called β-hemolytic group A Streptococcus, is estimated to cause 500 000 deaths yearly, (Table 2) (77), rendering it number nine on a scale of worst infectious diseases world wide. It has a wide disease spectrum ranging from uncomplicated throat and skin infections to invasive life-threatening diseases with a mortality rate of up to 60% (78-80).

Disease	Number of existing cases	No of new cases each year	No of deaths per year
Severe S. pyogenes diseases			
Rheumatic heart disease	16,24 million	460 000	349 000
Acute post streptococcal glomerulonephritis	N/A	472 000	5000
Invasive S. pyogenes diseases	-	663 000	163 000
Total severe cases	**18,1 million**	**1,78 million**	**517 000**
Superficial S. pyogenes diseases			
Pyoderma	111 million	-	-
Pharyngitis	-	616 million	-

Table 2. Number of *S.pyogens* cases world wide. Adapted from (77).

1.2.1.1 Uncomplicated throat and skin infections

The majority of *S. pyogenes* infections are throat infections, which world wide account for an estimated 616 million cases each year (Table 1) (77). Most people come in contact with *S. pyogenes* in young age, resulting in tonsillitis/pharyngitis or impetigo that are easily treatable with penicillin. However, recurrent tonsillitis is relatively common, and it has been suggested to be associated with bacteria persisting inside epithelial cells of the tonsils, thus avoiding killing by penicillin (81). There are also high numbers of asymptomatic carriers in the population, especially among children. Pharyngitis is seasonal and more prevalent during fall and winter. *S. pyogenes* also cause skin infections such as erysipelas and impetigo/pyoderma, which are commonly more frequent during the warmer periods of the year. Erysipelas is characterized by a localized inflammation of the superficial layer of the skin whereas cellulitis affects the subcutaneous part of the skin and the soft tissue (82). Another infection caused by *S. pyogenes* is scarlet fever, which is usually associated with throat infections; but it can also arise from infections at other locations (83).

1.2.1.2 Post -streptococcal sequelae

Pharyngitis can sometimes result in acute rheumatic fever (ARF), an autoimmune disease that is most common in the developing world where repeated throat infections, left untreated, can lead to this sequelae. In some cases ARF can progress into rheumatic heart disease (RHD), causing permanent heart valve damages and risk of death due to heart failure (84). Acute glomerulonephritis is a kidney inflammatory disease that can arise following streptococcal infections, most commonly after skin infections. Association between certain M types and pharyngeal or skin infections that coincided with outbreaks of ARF respectively glomerulonephritis led to the terminology rheumatogenic and nephritogenic types (85).

1.2.1.3 Severe invasive infections

The infections described in the section above are non-severe and responding well to antibiotic therapy, if not progressing into the different sequeale described. Nonetheless, *S. pyogenes* may also give rise to severe invasive manifestations such as necrotizing fasciitis and streptococcal toxic shock syndrome (STSS). Although these infections are relatively rare, they represent a substantial health problem due to their high mortality and morbidity, which has given them the nickname “flesh-eating bacteria” commonly used by the lay press. Puerperal sepsis involving infection of the uterus that rapidly becomes systemic is also a severe manifestation that may be caused by *S. pyogenes*. This was a frequent cause of maternal deaths during the 1700 and 1800 centuries (84). Another severe state is streptococcal sepsis, which develop upon entry of *S. pyogenes* into the blood stream. This can occur through a wound, an insect bite or by transition through the tissue from an infection of the mucosa or epidermis. However, in 50% of the cases, the portal of entry is not known (86). *S. pyogenes* sepsis can rapidly progress into STSS, which is characterized by an enormous activation of the immune system mediated by bacterial toxins, so called superantigens, which are described in more detail in the pathogenesis section. This condition is associated with hypotensive shock and multiple organ failure early in the course of infection (83). The mortality rate is high, often exceeding 40%, despite prompt antibiotics in most cases. Necrotizing fasciitis is a rapidly advancing soft tissue infection characterized by fascial necrosis. Approximately 50% of the necrotizing fasciitis cases also develop STSS, which is associated with even higher mortality rates of up to 60% (78-80). An important problem with these rapidly progressing infections is the difficulty to make the right diagnosis at admission to the hospital. This is due to often unclear general initial symptoms such as malaise, fever, flu-like symptoms and signs of soft-tissue infections like localized swelling, pain and erythema. A hallmark symptom is severe pain. Known risk factors include skin trauma, drug abuse, surgery, diabetes, varicella, and burns in addition to genetic predisposition and lack of type-specific antibodies (83, 87-89).

During these severe invasive infections, patients are given intravenous antibiotics, commonly a β-lactam and clindamycin, and due to the heavy vascular leakage, massive amounts of intravenous fluids (86). Intravenous polyspecific immunoglobulin G (IVIG) has been suggested as an effective

additional therapy in STSS by virtue of its anti-inflammatory properties, as well as ability to neutralize superantigens and increasing the opsonizing effect in these patients with often very low antibody titers of streptococcal-specific antibodies (90-92).

1.2.2 Epidemiology

S. pyogenes are most commonly characterized based on M-typing. The first method for serotyping was established in the 1920's by Rebecca Lancefield (93). The M-protein is expressed on the bacterial surface and the typing method relies on variations in the aminoterminal part of the protein in different *S. pyogenes* strains. The M-proteins are extracted and then studied in a precipitin-based test with standardized M-type specific sera. However, there were several problems with this typing method, including difficulties in producing high titers of type-specific antisera, and the high numbers of non-typeable strains. An alternative method is the opacity test. Approximately half of the *S. pyogenes* strains express the opacity factor, a lipoproteinase causing serum to increase in opacity. Antibodies against the opacity factor are type-specific and correlate with the M-type (94, 95). Another test used to distinguish strains and to correlate specific strains to outbreaks is T-typing, which is based on an agglutination assay. The T-protein is also expressed on the bacterial membrane and T-antigens are often M-type specific, rendering them a good alternative when the M-protein is not identifiable (96). Today, typing is commonly done by *emm*-sequencing (97) and currently, there are more than 150 different *emm*-types identified (98). These are all methods used in the research laboratory, however upon clinical diagnosis of patients, immune responses towards extracellular products such as streptolysin O (SLO), DNase B, hyaluronidase, NADase, and streptokinase may be included.

A recent review of the distribution of the streptococcal M-types world wide revealed that there are large differences between the high-income countries, USA, Canada and Europe as compared to low-income countries in Africa and the Pacific (99). The data further showed variations in disease spectrum, where pharyngitis and invasive disease were the most prevalent causes of infection in the high-income countries in contrast to low-income countries where the post-streptococcal sequelae, ARF, RHD and acute post-streptococcal glomerulonephritis are dominating. However, it should be noted that a major drawback is that there is a lack of data from most of the low-income countries making it difficult to get a conclusive analysis (99).

1.2.3 Vaccine development

Vaccine development of *S. pyogenes* has been discussed for decades, and C5a peptidase and especially the M-protein have been proposed as interesting targets (100, 101). Only one vaccine has so far been processed for clinical trials. That is a 26-valent vaccine, intended to target the most predominantly occurring M-strains in the US (102). One concern with this vaccine, as highlighted in the review by Steer et al (103) is that the M-types included in the vaccine are not the same ones causing infections in low-income countries

where most of the deaths occur. Another major concern with serotype-based vaccines is the risk of serotype replacement in the society. This was seen after the introduction of a 7-valent pneumococcal vaccine towards invasive strains in the US, where the number of pneumococcal infections with the 7 serotypes included in the vaccine was reduced (104), whereas strains of other serotypes increased with 45% (105, 106). One way to overcome the serotype dependency is to create a vaccine with specificity towards the conserved part of the M-protein, which is identical in all strains. A difficulty with this vaccine design is cross-reactivity of epitopes in the conserved region with human myosin, which is expressed in heart-, brain- and joint tissues (85), see Figure 4. However, identification of these specific epitopes on the M-protein opened up possibilities to create peptides that avoid the cross-reactive parts of the protein (107). A new vaccine candidate of the conserved region was recently reported to induce a wide humoral and cellular response in proliferation assays of RF/RHD patients and healthy controls (108). Its efficiency during *in vivo* conditions remains to be shown.

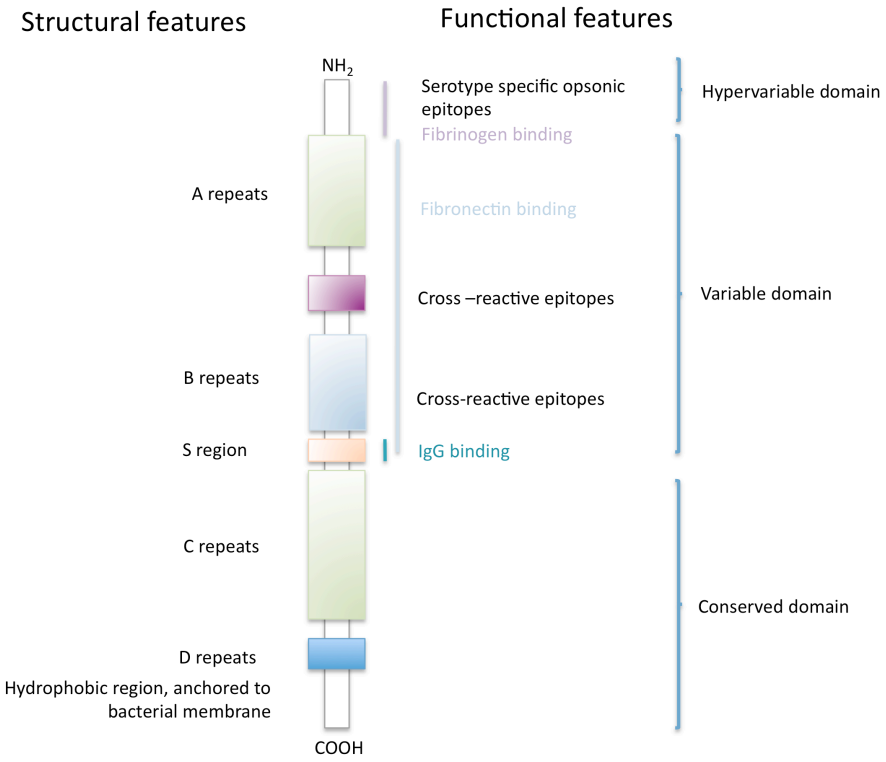

Figure 4. Schematic picture of the M1-protein with the hyper-specific and cross-reactive regions indicated.

1.2.4 Pathogenesis/virulence factors

S. pyogenes has a wide disease spectrum, causing infections in many different locations and with varying severity. It has a large array of bacterial factors enabling this versatile behavior and rendering the bacteria easily adaptable to the current surroundings. Several strains have become associated with different diseases. This is evident by the association of specific types with particular disease manifestations, such as the association of M1 and M3 strains with outbreaks of STSS and necrotizing fasciitis and M28 with puerperal sepsis.

In general, around 10% of the genome of sequenced strains consists of genetic mobile elements such as prophages and prophage-like elements, which accounts for most of the heterogeneity among the strains (109). The clinical significance of differential genome sequence has been underscored in several studies. Sequencing of 114 isolates of M28 serotype revealed a new genetic element, not previously found in other sequenced *S. pyogenes* strains but is related to regions in *Streptococcus agalactiae* (Group B streptococcus), a pathogen important in neonatal infections (110). One of the proteins encoded by this genetic element, R28, was subsequently shown to bind to vaginal epithelial cells *in vitro* and suggested to facilitate the colonization of the female genital tract (110, 111). Another example is the invasive M1 clone that has been studied in the papers of this thesis (112) and the M3 serotype, both of which have been associated with severe invasive diseases (113-116). Comparisons of their genome sequences and that of other serotypes reveled that the M1 and M3 have a specific set of phage-encoded genes. For example, new phage-encoded variants of SpeA were found upon sequencing of contemporary strains of M1 and M3 serotype, and it has been speculated that these phage-encoded genes contribute to the increase in severity in these strains (117, 118).

1.2.4.1 Adhesion

S. pyogenes express various cell-surface proteins, which enable binding to host cells. Recently a pili-like structure was identified and shown to be involved in bacterial epithelial cell adherence and biofilm formation (119-121). Also lipoteichoic actid (LTA) and C5a peptidase have been reported to have adhesive roles. In addition to its adhesive properties, C5a peptidase cleaves the complement component C5a (122, 123). However the most well described adhesins are the fibronectin binding proteins (FnBPs). Protein F1 (PrtF1/Sfbl) is essential for Fn-mediated adherence (124, 125) and it can also interfere with the complement system through inhibition of C3 deposition on the bacterial surface, thereby avoiding phagocytosis (126). It has also been shown to mediate invasion into host cells (127). Nevertheless, it is not expressed by all strains, some strains lacking PrtF1 express PrtF2 instead, a protein critical for adherence of M49 to epithelial cells. Interestingly, the M1 strains lack both of the proteins but encode FbaA, another protein involved in adherence to epithelial cells (128). The M3 and M18 serotypes encode FbaB, which also has specificity for fibronectin and mediates adhesion to epithelial cells (129). In addition, the M3 serotype have a bacteriophage encoding a phospholipase A_2 (SlaA) that has been reported to contribute to bacterial adhesion of pharyngeal

epithelium (130). In summary, there is a complex interplay and many factors are involved in the process of bacterial adhesion to host cells. It has been proposed that the hydrophobic interactions of the LTA initiates contact between the bacteria and the host cell allowing other adhesins such as the PrtF1 to form high-affinity interactions (131).

1.2.4.2 M-protein

The M protein is a classical virulence determinant of *S. pyogenes*. It is composed of two polypeptide chains in an alpha-helical coiled-coil composition that forms fibrils on the bacterial surface. In addition to the surface location, it can be shedded by enzymatical activity, excerted by bacterial or host cell proteases (132, 133). M-protein possesses anti-phagocytic activity, which is achieved via interference with the complement system and inhibition of complement opsonization (134-136). Studies of the adhesive effect of M-protein have reported some opposing results. For example, M protein can bind to Hep-2 epithelial cells and keratinocytes but not to buccal or tonsillar epithelial cells (137, 138). Both M-protein and PrfF1 have been shown to be differentially regulated by changes in the atmosphere with PrtF1 being expressed when the O_2-level is increased, whereas M-protein at higher CO_2 (139). It has been proposed that during infections of the epidermis (higher O_2), PrtF1 is expressed and mediates binding to cutaneous surfaces, while in the deeper tissue M protein is expressed and mediates binding to keratinocytes (140). This is also the case during infection of respiratory epithelial cells, where the oxygen level regulates expression of virulence factors important for adherence and invasion into the tissue (141).

Recent reports have highlighted the inflammatory properties of the M-protein as summarized by Oehmcke et al. (142). A major finding was that shedded M-protein was a powerful inducer of vascular leakage (133), which is one of the most prominent complications associated with severe streptococcal infection. In addition, M-protein has proven to act as a superantigen activating a massive T cell response (143), and also to interact with monocytes via TLR2 resulting in their production of interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α) and IL-1β (144).

1.2.4.3 Extracellular products

A hallmark of the invasive diseases is major tissue destruction and systemic toxicity (82), which is to a large extent due to secreted factors, including different bacterial enzymes and exotoxins. Two well-characterized haemolysins are SLO and Streptolysin S (SLS). SLO is a pore forming lysin, which is toxic to PMNs and platelets in addition to erythrocytes. Moreover, it has been shown to prevent degradation of *S. pyogenes* in pharyngeal epithelial cells by disrupting lysosomes and thereby avoiding a pH-dependent destruction (145). Recently it has also been proven to have a role in dissemination of bacteria from infected phagocytic cells by inducing cell death (146, 147). SLS is produced upon contact with serum and it is one of the most potent cytotoxins known, affecting PMNs, platelets and sub-cellular organelles. Recently, SLS was shown to

facilitate invasion into deeper tissues by degradation of epithelial intercellular junctions (148). Other factors enabling spread from one tissue layer to another include secreted DNAses (DNase A, B, C, D and Sda1), hyaluronidase, which degrades hyaluronic acid present in the connective tissue and streptokinase (SKA), which acts to promote dissolving of clots (137). Streptococcal exotoxin B (SpeB) is a potent cysteine protease, with many substrates such as human extracellular matrix, immunoglobulins, complement components, IL-1β and the antimicrobial peptide LL-37 (149). However, SpeB can also cleave endogenous substrates, which demands a tight regulation of the gene expression (149). Streptococcal inhibitor of complement (SIC), a secreted protein produced by only a few serotypes including M1 strains, can bind to the insertion site of complement proteins inhibiting phagocytosis of the bacteria as well as inactivating antimicrobial peptides (150, 151). GRAB (G-related α-$_2$-macroglobulin-binding protein), is a membrane bound protein that binds α-$_2$-macroglobulin, a highly abundant proteinase inhibitor found in human plasma. Binding of α-$_2$-macroglobulin to the bacterial surface via GRAB lead to entrapment of SpeB at the bacterial surface, and consequently renders the bacteria protected from SpeB-mediated degradation of virulence factors (152). Furthermore, this complex protects against the antimicrobial peptide LL-37, as these small peptides enter the complex and are degraded by SpeB (153). A recently described virulence factor is the *S. pyogenes* cell envelope proteinase (*Spy*CEP), which has also been shown to impede the host response by inactivating chemotactic signals, in particular IL-8 (154, 155).

1.2.4.4 Pyrogenic exotoxins

The streptococcal pyrogenic (fever producing) exotoxins are central mediators of STSS and necrotizing fasciitis. They consist of the phage-encoded SpeA and SpeC, as well as chromosomally encoded SpeG, SpeH, SpeJ, SpeK, SpeL, SSA, SMEZ and SMEZ-2. They all have superantigen activity inducing a massive immune activation, which results in liberation of large amounts of inflammatory cytokines including among others TNF and IFN-γ (156, 157). Bacterial superantigens are characterized by their ability to bind intact, without prior cellular processing, to the outer part of the MHC class II-molecule and the T cell receptor (TCR) (Figure 5). Binding occurs to a subset of T cells bearing specific Vβ sequences in their TCR, thereby activating 20-30% of the entire resting T cell population (158, 159) which leads to a massive cytokine storm. This excessive immune activation results in the systemic toxicity associated with STSS (86).

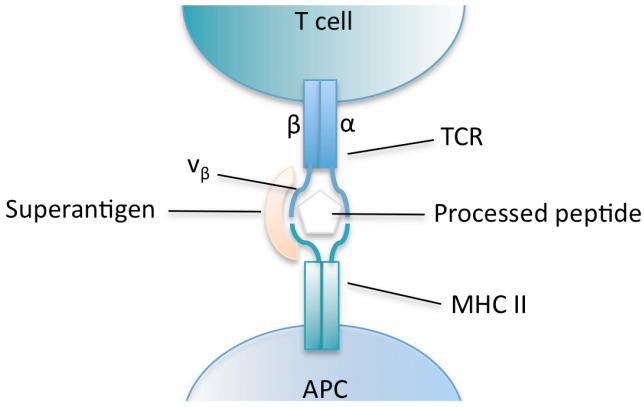

Figure 5. Schematic figure of the MHC II-TCR interaction with a superantigen bound to the outer part of the complex.

1.2.5 Regulation of virulence factors

The pathogenesis of severe invasive diseases is complex and involves an array of bacterial components that contribute to initial adherence/colonization, invasion and modulation of immune responses. It is a complex scenario with many players involved. In the following section bacterial regulation of virulence factors is discussed.

1.2.5.1 Two component systems

S. pyogenes regulate its own gene expression in a sophisticated way in order to adapt to the environment during infection. The bacteria express a number of virulence factors during the process and different factors are expressed depending on the stage of infection. These proteins are regulated through distinct mechanisms where stand alone regulators (160) and two component systems (TCS) (161) are the principal types. Through genome sequencing it has been estimated that *S. pyogenes* have approximately 100 response regulator homologs. Out of these, 13 belong to the TCS, which normally consist of two parts, a sensory and a regulatory component (161). The sensor component is typically a transmembrane protein with an extracellular sensor histidine kinase that recognizes one or more specific environmental signal(s). Examples of such signals include changes in ion concentration, osmotic pressure or pH. Upon sensing the activation signal, the histidine kinase is activated and a phosphoryl group is transferred to the response regulator (RR). The RR is cytoplasmic and the majority of them are transcription factors with DNA binding domains that act by repressing or activating transcription of specific genes upon phosphorylation (161, 162). In *S. pyogenes* 4 TCSs have been studied in more detail, i. e. CovR/S, FasBCAX, SptR/S and Ihk/irr. The CovR/S is in control of many of the virulence factors and is described below. FasBCAX has homology to quorum sensing systems in *Staphylococcus aureus* and *Streptococcus pneumoniae,* and it has been shown to regulate adhesins, such as fibronectin/fibrinogen binding proteins as well as hemolytic activity and streptokinase transcription in a growth phase dependent manner (163). SptR/S was recently shown to be important for *S. pyogenes* persistence in human

saliva (164). Ihk/Irr activation has been shown to be induced upon encounter of blood cells, and it is involved in resistance to phagocytic degradation in neutrophils (165, 166). Recently, a new TCS, TrxSR was identified and found to be repressed by CovR. It is the first TCS shown to regulate Mga expression, described later in this section (167).

1.2.5.2 CovR/S

The best characterized TCS in *S. pyogenes* is the CovR/S (168), control of virulence, formerly named CsrR/S for capsule synthesis regulator. The name was changed when the system was found to regulate also other genes than those involved in capsule synthesis (169), including the hyaluronic capsule, SKA, SLS, SpeB, and Mac-1–like protein (Mac)/Immunoglobulin G-degrading enzyme of *S. pyogenes* (IdeS). The CovR/S system acts primarily by repressing transcription of the genes that it regulates (169, 170). However, when *S. pyogenes* encounters stress, such as during infection, CovS acts by de-phosphorylating CovR enabling transcription of the repressed genes that are needed for invasion (171). Colonies of CovR mutants are for example mucoid due to the expression of the capsule gene leading to an increased production of hyaluronic acid (171). CovR/S regulation affects approximately 15% of the bacterial genome of which many encoded genes are virulence factors (172).

In a murine model of skin and soft tissue infections; a transition from non-invasive to invasive phenotype was noted during disease progression after infection with a M1T1 strain (173). Sequencing of isolates from these infections revealed a mutation in the *covS* gene, resulting in decreased expression of SpeB, which is positively regulated by CovS. This renders the bacteria more virulent by higher expression of virulence factors that would otherwise be degraded by SpeB. Also microarray comparisons of bacteria from pharyngeal and invasive murine infections revealed mutations in *covR/S* (174). However, *covS* seems to have a very distinct way of fine-tuning gene expression of CovR repressed genes as reported by Treviño et al. (175). Some early studies on clinical isolates from severe and non-severe infections indicated an inverse relationship between SpeB expression and disease severity (176). Recently there have been reports of human isolates of invasive infections with this mutation in the CovR/S regulatory system indicating that this also happens during infection in the natural host (177). Interestingly, in a recent case-report, two isolates were taken from the same patient, one from the blood and one from the pharynges. When sequencing the strains, the sole difference was a mutation in the *covS* gene of the blood isolate, rendering it invasive and enabling the progression into the blood stream (178). However, analyses from tissue biopsies of patients with necrotizing fasciitis revealed high levels of SpeB at the tissue site of *S. pyogenes* infection (179); thus, underscoring the fine tuned expression of virulence factors at different sites and stages of infection.

1.2.5.3 Ihk/Irr

The Ihk/Irr TCS was identified by Federle et al, who were studying regulation of virulence factors and especially systems with homology to the PhoP-PhoS TCS in *Bacillus subtilis (169)*. The PhoPS system is widely distributed among bacterial species and is involved in regulating virulence factors through sensing of the phosphate concentration in the surrounding environment (180). The Pho sensing subunit is located in the inner membrane and is interacting with an ATP-binding cassette (ABC) type phosphate-specific transport system that during low phosphate conditions activated the regulatory unit leading to up- or down regulation of the affected gene (180). ABC transporters are important components for bacterial metabolism as they import and export for example nutrients and ions for different purposes, such as virulence and general nutrition. Iron acquisition through iron ABC uptake systems is an example of a process designated to bacterial virulence. Specific iron-binding proteins, such as transferrins and lactoferrins, are transported through the system, and iron is extracted from these by the bacteria (181).

It has been shown that *ihk/irr* is highly up-regulated during infection of human PMNs (182), macrophages (**Paper II**), in whole blood and in saliva (164, 166). Importantly, studies in human PMNs as well as in mice infected with wild type or an *ihk/irr* mutant revealed an important role of this TCS in resistance to ROS during infection (165). Another regulatory system that has been involved in resistance to ROS is PerR, peroxide stress response regulator (183). PerR was recently shown to be important for resistance to oxidative burst of *S. pyogenes* in human whole blood as well as for bacterial survival in a baboon model of pharyngitis (184). PerR is also found in *Bacillus subtilis* and *Staphylococcus aureus*, where it is known to regulate peroxidases, such as catalase and alkylhydroperoxide reductase, in addition to coordination of the oxidative stress responses and iron metabolism in these species (185, 186). *S. pyogenes* do not express catalase, which other Gram-positive bacteria use to degrade H_2O_2 to H_2O and O_2, but instead it has several alternative peroxidases such as alkyl hydroperoxidase, glutathione peroxidase and NADH peroxidase. These have been shown to be important for ROS detoxification both *in vitro* during different O_2 conditions and in *in vivo* infections of murine model systems (183, 187-189).

1.2.5.4 Stand-alone regulators

Stand-alone regulators are transcriptional regulators that are primarily involved in adaptation to the host environment during infection and regulate for example carbohydrate utilization and metabolism (190). Their activation signals and sensory elements remain unknown (162). The three most defined regulators are the multiple gene regulator (mga), the RofA-like protein family (RALPs) and the Rgg/RopB. They have basically been divided into different functional expression periods during infection, where the mga regulates genes involved during adherence and colonization, the RALPs co-ordinate gene expression during the internalization and persistent phase and the RopB/Rgg system regulate genes involved during dissemination, as reviewed in (160). The TCSs

act to fine-tune the events described above by their ability to sense the surroundings (162). Gene regulators also regulate each other during the infection process. One example is the Rgg that repress both mga and CovR/S, while *ihk/irr* on the other hand is down-regulated following Rgg inactivation (191). At the same time expression of SpeB can repress Rgg (192).

In general it is believed that during the initial phase of streptococcal infection, Mga is upregulated and also the Ihk/Irr system, resulting in increased amounts of adhesins, such as fibronecting-binding proteins, M and M-like proteins, that are important for adherence and invasion into host cells and tissues as reviewed in (160). During the second stage, both CovR/S and RALPs are active; there is a bacterial replication ongoing, repair of oxidative damage and high protein turnover. During dissemination, it has been suggested that Rgg is expressed whereas the mga regulon remains suppressed and the *speB, slo, speG, grab* genes are transcribed resulting in spread of infection by increased enzymatic activity (160), see Figure 6.

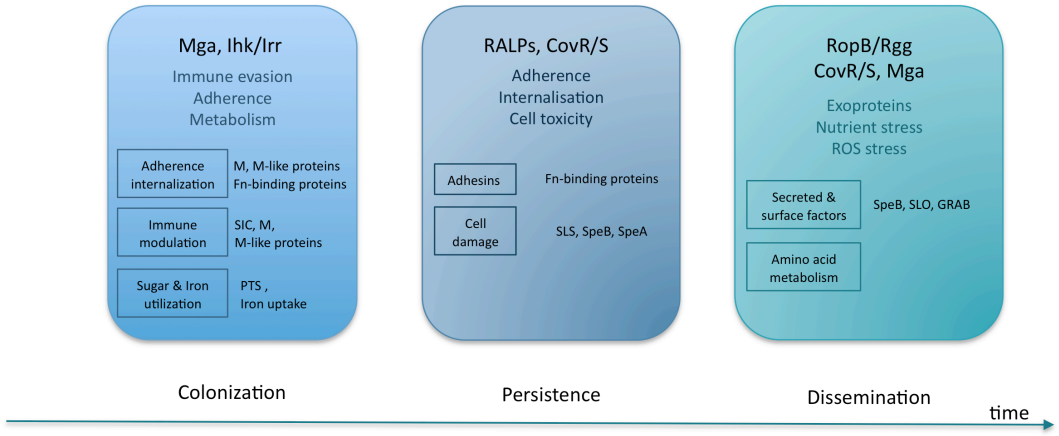

Figure 6. General characteristics of gene regulation during *S. pyogenes* infection. Adapted from (160).

A temperature study designed to mimic the different conditions during infection in skin, throat and deep tissue revealed that 9% of the genome was altered between 29ºC to 37ºC (193). Especially genes encoding secreted proteins, such as hemolysins and proteins, involved in iron homeostasis, were shown to be temperature-dependent. Many metabolic enzymes involved in glycolysis and fatty acid metabolism were down-regulated at 29ºC as well as virulence regulator RofA. Instead genes encoding proteins involved in iron homeostasis were up-regulated at 29ºC. At 40ºC, the ferric uptake repressor, Fur, was down-regulated, indicating the need for iron during fever. During infection of the throat, the bacteria come in contact with saliva. Microarray studies of *in vitro* growth of *S. pyogenes* in human saliva revealed a new TCS, the SptR/S, which is essential for bacterial persistence in this type of environment. SptR/S is positively regulating a number of virulence factors such as DNases, SpeB, the hyalyronic acid capsule and genes encoding carbohydrate transporters. During early infection, genes encoding proteins involved in adherence and resistance to the host such as M-protein and SIC as well as oxidative stress responses,

ATP generation and pH-balance, were up-regulated. However as the infection proceeds metabolic genes and virulence factors such as DNases and pyorgenic enzymes as well as stress-response regulators were most active (164). Infection of human blood almost alters a third of the bacterial genome. Here, also M-protein and SIC are up-regulated, as well as the regulatory systems; mga, rofA and Ihk/Irr and as infection progresses, transcription of covR/S increases (166). A study of the carbohydrate regulator, catabolite control protein A, showed that growth of a mutant strain was dependent of the media. Reproduction in human saliva was heavily affected while growth in rich cell culture media did not affect reproduction significantly, indicating the importance of carbohydrate regulation for replication and virulence (194).

1.2.6 *S. pyogenes* as an intracellular pathogen

Traditionally *S. pyogenes* has been considered as an extracellular pathogen. The first evidence of intracellular *S. pyogenes* was published in the mid 1990's. Following pharyngitis infection, intracellular bacteria have been found in the epithelial cells lining the mucosa (81). In *in vitro* models, invasion and survival of bacteria has been shown in epithelial (195, 196) and endothelial cells (197). In patients, formation of an intracellular reservoir, protecting from penicillin treatment, has been proposed to contribute to treatment failure and recurrent tonsillitis (81). In addition, investigations of biopsies from patients with severe streptococcal tissue infections revealed viable bacteria inside macrophages despite prolonged antibiotic therapy (179). This was the first evidence of *S. pyogenes* inside professional phagocytes during human infection. In addition, persistence inside macrophages has been confirmed *in vitro* (179). *S. pyogenes* has also been shown to circumvent degradation in PMNs through avoidance of azurophilic fusion with the phagosome (198-200). Various internalization mechanisms have been reported for different host cells as shown in table 3. These mechanisms were discussed in section 1. 1. 3, delineating uptake mechanisms by host cells.

Cell type	Mechanism	M-type	Reference
Epithelial cells	Caveolae Zipper-like,actin-dependent	M12 M1	Rohde et al. (75) Dombek et al. (201)
Endothelial cells	Zipper-like, actin-dependent Caveolae	M3 M12	Nerlich et al. (202) Rohde et al. (75)
Macrophages	Phagocytosis, actin-dependent	M1	**Paper I**
PMNs	Phagocytosis, actin-dependent	M1	Staali et al. (199)

Table 3. Entry pathways of *S. pyogenes* into various human host cells.

It has been shown that M1 *S. pyogenes* are able to induce up-regulation of integrin expression on epithelial cells and thereby promote uptake (203). Upon invasion of M12 *S. pyogenes* into endothelial and epithelial cells, intracellular bacteria can reside in caveosomes and avoid the endosomal-lysosomal

degradation pathway as there is no co-localisation of the different markers of the forming phagolysosome and the pathogens (75). In keratinocytes, *S. pyogens* has been reported to circumvent phagolysosomal destruction through secretion of SLO, which has been shown to act in the same manner as the pore-forming toxin, listeriolysin O of *Listeria* species, and thereby enable bacterial escape from the phagosome (145, 204). In PMNs the M1-protein inhibits azurophilic fusion with the phagosome (198). In macrophages, *S. pyogenes* associate with the early endosomes but not with the late endosomes or lysosomes and as in PMNs, the avoidance of phagosomal maturation is dependent on expression of M1-protein (**Paper I**). Studies of intracellular *S. pyogenes* in human epithelial cells have reported a caspase 9-dependent induction of apoptosis upon infection (205, 206). In another study using epithelial cells, *S. pyogenes* were shown to ultimately be degraded via autophagy after SLO-induced phagosomal escape into the cytosol (204). The autophagy compartment was characterized by expression of LC3 and Rab7 (207). Cell death has also been reported during infection in macrophages, however the mechanism is still debated since SLO was suggested to induce caspase-1 associated pyroptosis in one study (147) and apoptosis in another (146). In summary, there are many different mechanisms described and despite intense research during the past two decades there are still many aspects that remain unknown.

Next section comprises an introduction to the interactions between the host and bacteria, focusing on traditional intracellular pathogens and their survival strategies.

1.3 HOST-PATHOGEN INTERACTIONS

1.3.1 Survival mechanisms of intracellular bacteria

As cells and pathogens have lived side by side through millions of years, several microbes have evolved systems to circumvent their destruction by the immune system. A number of pathogens have created systems to avoid intracellular degradation (208-210) and some have even generated ways to form a safe environment within the host, aimed for intracellular replication (211). These are so-called professional intracellular bacteria. Entry into host cells is gained via different mechanisms. Many involve activation of a signaling cascade that induces rearrangement of the actin cytoskeleton and formation of membrane protrusions enabling bacterial uptake. Below is a table containing uptake mechanisms of selected intracellular bacteria into macrophages or epithelial cells. Next section comprises an introduction to bacterial survival strategies.

Pathogen	Mechanism	Cell type	Reference
Listeria	Zipper – actin dependent – via CR	Epithelial	(70)
Shigella	Trigger – actin dependent	-"-	(70)
Salmonella	-"-	-"-	(70)
Legionella	Phagocytosis – via CR	Macrophage	(212), (213)
Francisella	-"-	-"-	(73)
Mycobacterium	Phagocytosis- various receptors	-"-	(213)
Coxiella	Phagocytosis - αvβ3 integrin	-"-	(214), (213)
Yersinia	Zipper – actin dependent	Epithelial	(70)

Table 4. Entry mechanisms of various professional intracellular bacteria into host cells.

1.3.1.1 Manipulation of cellular signaling and effector functions

One of the most well known intracellular pathogens is *Mycobacterium tuberculosis*. It can avoid phagosomal degradation and stay dormant in lung granulomas for long periods of time. It has a large array of mechanisms to prevent destruction through manipulation of the host cell response. One important immune response towards *M. tuberculosis* is activation of CD4+ T cells and their subsequent release of TNF-α and IFN-γ, which in turn induce a pro-inflammatory response and activation of macrophages and DCs. However, *M. tuberculosis* interfere with IFN-γ and NF-κB signaling through secretion of ESAT-6, which inhibits activation downstream of TLR-2 and subsequent induction of pro-inflammatory responses (215). Upon recognition of microbial products, the inflammasome is activated through caspase-1 activation with subsequent IL-1β secretion in the host cell. Inhibition of caspase-1 activation has been demonstrated to be mediated by factors expressed by *Mycobacterium* and *Yersinia* (216, 217). In addition, *Mycobacterium* can induce IL-10 secretion, which promote bacterial replication (218). IL-10 levels are also

high during chronic infection of *C. burnetii* promoting the M2 macrophage phenotype, which is poorly bactericidal (219). Recently, Shigella was shown to prevent phosphorylation of a specific subset of NF-κB-regulated genes encoding chemokines, including the important IL-8, thereby reducing infiltration of PMNs to the infection site. Other species that interfere with NF-κB signaling are *Salmonella* and *Yersinia* (220). Some species have developed mechanisms to circumvent oxygen radicals. *Salmonella* express superoxide dismutases that detoxify ROS secreted by the host cell (221) and like *Mycobacterium* and *Legionella* it detoxifies peroxynitrite to nitrite to inhibit oxidation of bacterial DNA (222). Yet another way to limit host response is utilized by *Francisella* that is able to alter its LPS structure, resulting in a compound that is a less potent activator of iNOS (223). This mechanism of antigenic variation to reduce immune recognition is also utilized by *Salmonella* and *Mycobacterium (224)*.

1.3.1.2 Manipulation of intracellular trafficking

Many intracellular bacteria have evolved mechanisms to interfere with the endosomal-lysosomal degradation pathway. There are basically three different mechanisms. The first is to inhibit maturation of the phagosome at some stage before fusion to the lysosome, second to escape from the phagosome before it starts to mature or finally to adapt to the phagosomal compartment (222), see Table 5. *M. tuberculosis*-containing phagosomes associate with the early endosomal markers, but it prevents further fusion and acidification with the late endosomal marker Rab 7 (225). In contrast, *C. burnetti* is contained in a vacuole that is acidified. It associates initially with EEA-1 and later with LAMP-1, CD63 and M6PR and interestingly, bacterial viability is not affected by the low pH. The mechanism behind the avoidance of lysosomal fusion and degradation is suggested to be inhibition of Rab7-recruitment to the phagosome (219).

Pathogen	**Mechanism to avoid degradtaion**	**Vacuole markers**	**Replicate in vacuole**	**Reference**
Listeria	Escape phagosome	-, LAMP-1	-	(213)
Shigella	-"-	-	-	(70)
Rickettsia	-"-	-	-	(70)
Salmonella	Avoid maturation to lysosome	Phalloidin, ER	+	(222)
Legionella	-"-	Rab1	+	(213)
Francisella	-"-	EEA-1, LAMP-1/2, Rab7	-	(40)
Mycobacterium	-"-	Rab5	+	(222)
Coxiella	Adapt to lysosome	EEA-1, LAMP-1, CD63, M6RP, LC3	+	(219)

Table 5. Mechanisms to manipulate endosomal-lysosomal degradation and markers associated with the phagosomal compartment of selected professional intracellular species.

Salmonella has yet another way of forming a compartment that accumulates markers of the endoplasmic reticulum as well as proteins from both early and late endosomes and finally from the trans-golgi network (226). Intracellular *Francisella* are contained within a vesicle that recruits EEA-1, followed by Lamp-1, Lamp-2 and Rab 7 (40). This vesicle bursts upon acquisition of the vATPase pump, which induces the escape mechanism into the cytoplasm where replication occurs (227, 228). Similarly, acidification of the phagosome is a mechanism that also has been shown to induce burst of the *Listeria*-containing phagosome (229). In addition, increase of Ca^{2+}, which is required for fusion of endosomes and/or lysosomes, is inhibited by *Mycobacterium* and *Listeria* through interference in signaling of vesicle trafficking and perforation of the phagosome respectively (229, 230).

Other bacterial species, such as *Listeria*, *Shigella* and *Rickettsia*, escape from the phagosomal compartment to the cytoplasm in order to avoid degradation. *Listeria* expresses listeriolysin O (LLO), by which it forms pores in the vesicle membrane and escape into the cytosol. This can occur as early as five minutes after infection and is followed by replication (39). However, it has recently been shown that during circumstances of low LLO levels, *Listeria* stay in the phagosome where it replicates slowly. The *Listeria*-containing phagosome acquires LAMP-1 positivity but it does not degrade the bacteria and this persistence has been suggested to contribute to chronic infection (231). *Shigella* escape the phagosome through secretion of lytic virulence factors (232) via its type three secretion system (T3SS), described below. Replication is initiated upon entry to the cytosol and autophagy is circumvented by factors processed by the T3SS (71). Interestingly, all three species posses the capacity to use host cell actin as a means of motility to promote intracellular and intercellular dissemination (233). Table 5 presents an overview of the proteins associated with the bacteria-containing vacuoles.

Many Gram-negative bacteria have T3SSs, which are machineries forming a cylindrical needle-like organelle enabling transport of bacterial proteins into the host cell, facilitating pathogenesis (234). Studies of Salmonella pathogenicity island 2 (SPI-2) have revealed a T3SS encoding machinery that delivers factors into the vacuole inhibiting lysosomal fusion and oxidative stress as well as inhibition of NADPH oxidase fusion with the phagosome (235-237). These are all functions which are important for intracellular survival (210). *Legionella* encode a T4SS with which it can modulate the maturation process of the phagosome avoiding the endosomal-lysosmal pathway. Rab1 is recruited and it directs trafficking of ER-vesicles to the *Legionella*-containing phagosome (238). Ultimately, *Legionella* replicates inside a large vacuole with some properties of a phagolysosome before the host cell is lysed to release bacteria to the surroundings (239). Replication within a vacuole requires material to maintain the integrity of the membrane and to produce progeny. In *Legionella*, a machinery has been located to transfer substrate across the target host cell membrane to the replicating vacuole (240). In *Salmonella* SPI-2, *SifA* has been implicated in maintaining the replication vacuole, as mutants fail to keep the integrity of the vesicle resulting in bacteria in the cytosol (233).

2 AIMS OF THE THESIS

This project was initiated upon finding viable bacteria in tissue macrophages of patients with severe *S. pyogenes* infections. The intracellular bacterial reservoir was linked to prolonged bacterial persistence at the tissue site. It was therefore of interest to investigate how the bacteria could survive within these professional phagocytes that has evolved only for the specific purpose of degrading invading microbes. Hence, the overall aim of the thesis was to decipher streptococcal survival strategies in macrophages. The specific aims were the following:

1) To study bacterial entry and intracellular trafficking of *S. pyogenes* in human macrophages (Paper I).

2) To investigate survival, replication and dissemination strategies of *S. pyogenes* in human macrophages. (Paper I).

3) To analyze bacterial gene expression of intracellular *S. pyogenes* in macrophages and relate this to extracellular bacteria in order to identify factors supporting intracellular survival (Paper II).

4) To investigate the functional aspects of gene regulation during intracellular survival and dissemination of *S. pyogenes* from human macrophages. (Paper II).

5) To study host-response mechanisms to intracellular *S. pyogenes* with focus on Prostaglandin E_2 (Paper III).

3 COMMENTS ON METHODOLOGY

Detailed descriptions of the experimental procedures are found in the papers on which the thesis is based. In this section I will discuss advantages and disadvantages with methods and explain the rationale for choice of methods.

In vitro infection assays

As *S. pyogenes* is a human-specific pathogen, the studies are focused mainly on human cells. Investigation of soft tissue from patients with severe *S. pyogenes* infections revealed macrophages as the main host cell of bacteria. For that reason, we have developed an *in vitro* assay with primary human monocyte-derived macrophages (hMDM) using cells isolated from buffy coats from healthy donors. Naturally, there are limitations working with primary cells such as to number of cells per donor, inter-donor variations, availability of cells, time to prepare and maintain the cells. However, we have found that the positive aspects of working with primary cells overcome the drawbacks, including the fact that they mimic our findings in patients. During the years, we have also assessed different human monocytic cell lines, such as THP-1 and U937, in order to complement work with primary cells. However, these cells differed in several important aspects as compared to primary cells. Both THP-1 and U937 have to be transformed in order to differentiate into macrophages, but even in the differentiated state, they did not adhere in the same manner as the primary cells. In addition, the morphology and the intracellular granularity of the cells were different as well as the infection efficiency. In conclusion, our experience with these cell lines is that they are not comparable to primary cells in our experimental set-up.

One limit with the primary hMDM is its questionable oxidative stress responses. However, this problem has also been reported for human monocytic cell lines such as THP-1 (241) and may be a general aspect when working with human macrophages.

In vivo infection assays

Despite *S. pyogenes* being a human pathogen, there are animal models that are useful when studying specific aspects of infection *in vivo*. In this thesis, a murine model was used in collaboration with another research group working with murine *S. pyogenes* infection.

Bacterial strains

All *in vitro* infections have been carried out with a clinical M1T1 STSS isolate, 5448, of *S. pyogenes* (89). This strain is well characterized in the group and is widely used in the field. An advantage using clinical strains is the preserved virulence in contrast to lab strains, which tend to loose virulence when adapting to rich media and lack of threat.

Generation of GFP-expressing bacteria

To facilitate visualization of *S. pyogenes* during intracellular infection and trafficking, we created GFP-expressing bacteria using the clinical isolate. Incorporation of GFP enabled monitoring of both viability and location in relation to other fluorescent markers during infection, both with fixed samples and during live cell imaging. One aspect of GFP-expression is that it can be quenched by low pH. However, being aware of that risk, we have throughout the project kept monitoring bacterial DNA in addition to GFP-expression, to ensure visualization of GFP-negative cocci.

Fluorescence and light microscopy

Microscopy-based methods have been very useful tools during the course of these thesis projects. It has enabled visualization of the bacterial behavior during infection and its association/lack of association with different compartments as the infection progress. Antibodies specific for proteins expressed during the different stages of phagosomal maturation were used to visualize the endosomal-lysosomal degradation pathway. Although microscopy can be used to quantify expression in combination with appropriate software, this is often more easily achieved by other methods such as flow cytometry described below.

Electron microscopy

Electron microscopy was something that we decided to use to study the uptake mechanism as well as the interaction of intracellular bacteria with vacuoles. This method enables visualization of structural details of the plasma membrane as well as the organelles within the host cell at very high magnification.

Flow cytometry

Flow cytometry is a useful quantitative method. It enables measurements of various cellular characteristics through sorting on size, granularity and binding to various fluorescent antibodies. We used it to measure uptake of bacteria in different settings as well as to study differentiation markers on macrophages.

Western Blot

Western blot was used to investigate phosphorylation of activation molecules. It is a classic method to study proteins. Nowadays phosphoflow is emerging as a very useful tool to look at phosphorylation.

PCR and quantitative real-time PCR

PCR was used to reverse-transcribe RNA to cDNA. Quantitative real-time PCR was used to verify the gene expression seen in the microarray analysis. The advantage with the real-time compared to the conventional PCR is that it allows for quantification of the gene, which enables comparison of gene expression between different genes.

Microarray

Whole-genome microarray was used to get an overview of the bacterial gene expression of intracellular bacteria. This method is an easy way to collect much information at the same time, which allows for further dissection and deeper analysis of specific genes of interest. We had the opportunity to perform these studies in collaboration with Prof. Malak Kotb, who has access to customized serotype-specific arrays for the clinical isolate that we are working with.

4 RESULTS AND DISCUSSION

This part comprises a discussion of the results achieved in the papers on which this thesis is based. The first section focuses on intracellular *S. pyogenes* in human macrophages and our finding that they can avoid lysosomal degradation. The second part describes intracellular replication and how intracellular bacteria later egress out of the cells leading to continued infection. The third section highlights gene expression of intracellular *S. pyogenes* and regulation of virulence factors important for intracellular survival. Finally, the influence of host-derived PGE_2 to intracellular survival is discussed in the last section. **Paper I-III** can be read in full at the end of this thesis.

4.1 INTRACELLULAR TRAFFICKING AND SURVIVAL STRATEGIES

S. pyogenes is classically considered as an extracellular pathogen. However, during the last decades it has become evident that it is capable of surviving inside a number of cell types in different tissues. Highly invasive clones have emerged with the ability to cause severe infections raising the number of critical *S. pyogenes* infections, especially in the western communities. In particular, the M1 and the M3 strains have been isolated from patients with severe invasive infections (78, 112, 115, 242).

The first evidence of invasion and intracellular persistence was shown in respiratory epithelial cells in the mid 1990's (195, 196). Since then, a variety of cells have been studied in relation to invasion and intracellular survival. During *in vitro* studies of PMNs it has been shown that *S. pyogenes* can resist fusion of azurophilic granules (74, 179). Thulin et al. (179), investigated a large collection of biopsies from patients suffering from severe tissue infections, including necrotizing fasciitis and severe cellulitis, caused by *S. pyogenes*. Results from this study revealed that the bacteria could survive in the tissue despite prolonged antibiotic treatment. Detailed analysis of the tissue demonstrated viable bacteria harboring inside cells, and furthermore, characterization of the cells revealed that the main host cell for *S. pyogenes* at the tissue site are macrophages (179). The intracellular bacterial survival could also be verified in an in vitro infection model (179). This was the first evidence that *S. pyogenes* can persist inside macrophages during severe acute infections; thus, highlighting a problem with antibiotic eradication of *S. pyogenes* at the tissue site of infection and need for improved future treatment strategies for these severe invasive infections.

The aim with this project was to identify streptococcal survival strategies and bacterial factors contributing to the persistence seen in the biopsy material. The *in vitro* model previously established was further developed and used in detailed kinetic assays to monitor uptake and intracellular trafficking of *S. pyogenes* over time. Cells were infected with a clinical isolate of M1 serotype from a patient suffering from STSS (89). Transmission electron microscopy (TEM) was employed to get a detailed insight to the bacterial entry process.

The analyses revealed that the bacteria are taken up via formation of long spacious pseudopod loops that are encircling the pathogen, translocating it to the cellular membrane (Figure 1A and 1B, **paper I**). This mechanism has previously been reported to describe the uptake of *Francisella tularensis* into human macrophages (73). Many uptake mechanisms involve reformation of the cellular membrane including rearrangement of the actin cytoskeleton. Hence, we assessed actin-dependency and found that the bacteria associate with the filaments and that blocking of actin polymerization inhibited uptake significantly (Figure 1D and 1E, **paper I**), indicating the participation of the host cell machinery for efficient *S. pyogenes* intake. Involvement of actin filaments has previously been reported for *S. pyogenes* uptake into epithelial and endothelial cells (197, 201). However, a study comparing fibronectin-binding protein Sfb1+/- strains reported an actin-independent uptake mechanism of the Sfb1+ strain in contrast to an actin-dependent uptake of the Sfb1- strain into epithelial cells (243). In light of this, it is important to consider differences among streptococcal strains in their expression of surface molecules, which may affect entry processes. The M1 strains, which is the serotype used in this study, do not express Sfb1, which could explain their actin-dependency. Similarly, actin-dependency was recently reported for an M3 serotype, also lacking SfbI, during invasion of endothelial cells (202). Other bacterial strains relying on an actin-dependent uptake include *Salmonella* and *Brucella* (244). *Legionella* and *Francisella* utilize actin in addition to complement receptor binding and the presence of complement factor C3 (73, 212).

Furthermore, assessment of the intracellular location revealed that *S. pyogenes* are contained in membrane-surrounded compartments where they remain during the entire infection until dissemination (Figure 2A, **paper I**). At no time point was *S. pyogenes* found in the cytosol (**paper I**), in contrast to results reported for *S. pyogenes* in human pharyngeal epithelial cells, where secretion of SLO enable bacterial escape from the phagosome to the cytosol (145). Upon encounter of a pathogen, macrophages normally engulf and transfer the foreign particle through the endosomal-lysosomal degradation pathway for final destruction in the phagolysosome, and as described earlier, many bacterial species have evolved mechanisms to circumvent their destruction (71). To assess *S. pyogenes* intracellular trafficking, detailed analysis of kinetic experiments using antibodies specific for different markers of the endosomal-lysosomal pathway was conducted. Following uptake *S. pyogenes* associate with the early endosomes up to 15 minutes post infection but at later time points post-infection, when pathogens are normally located in the phagolysosome, *S. pyogenes* are to be found in vacuoles distinct from the phagolysosomes, Figure 7 (Figure 2B, 2C and 2D, **paper I**).

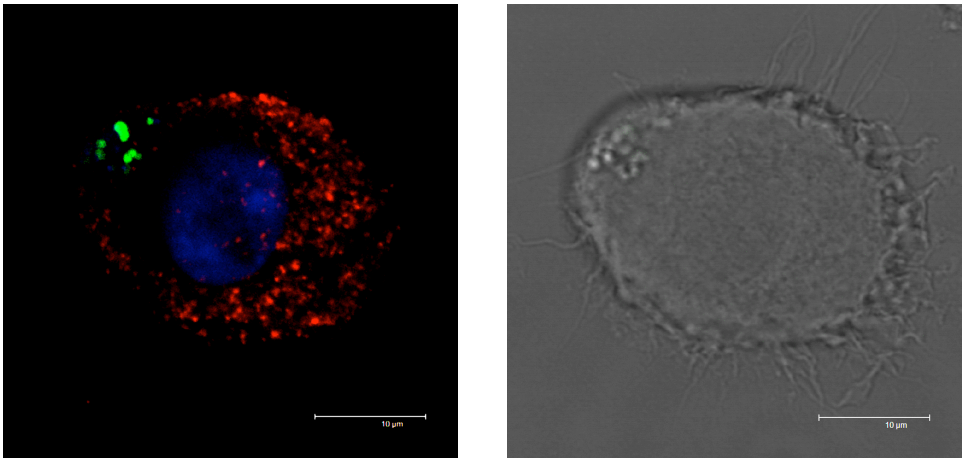

Figure 7. Confocal pictures of GFP-expressing *S. pyogenes* inside human macrophages 1h post infection. *S. pyogenes* are located separated from phagolysosomal compartments in red.

In fact, they were residing inside actin-surrounded compartments at late time points post infection (unpublished data) which is in line with a strategy previously reported to be essential for *Salmonella typhimurium* (245). That study showed that *S. typhimurium*, via a pathogenicity island distinct from the one inducing actin-dependent uptake, induces formation of actin filaments surrounding the bacteria. This actin-containing vacuole offer the bacteria a safe niche as experiments with an actin-depolymerising drug reduced the number of bacteria inside the vacuole and negatively affected replication (245).

Avoidance of phagosomal maturation by inhibiting fusion of lysosomal vesicles with the phagosome at various stages of the maturation process is a method employed by intracellular *Salmonella*, *Brucella* and *Legionella*, visualized in Figure 1 in the introduction *(211, 246, 247)*. *F. tularensis* traffics the endosomal pathway similarly to *S. pyogenes* as they also transiently associate with the early endosomal compartment. However, in contrast to *S. pyogenes*, the *Francisella*-containing vesicle progress to associate with late endosomal markers and upon acidification it rapidly burst to release the bacteria in the cytosol at approximately 3h of infection. The released particles can then replicate in the cytosol (228).

Several intracellular bacterial species have developed sophisticated machineries, which secrete effector molecules in order to facilitate invasion and intracellular survival, such as T3SS found in *Salmonella* and *Shigella*, T4SS in *Legionella* and type six – like secretion system found in *Francisella (248-251)*. These effector molecules can, in addition to enhancing bacterial entry, also affect signal transduction and modulate host cell responses to the intruders favor. *S. pyogenes* has no known secretion system like the ones found in Gram-negative bacteria. However, based on our results demonstrating impaired lysosomal fusion, an interesting candidate molecule to explore was the M-protein, which has been implicated in resistance to phagosomal maturation in human PMNs (198, 199). To this end we employed a M1-protein deficient isogenic mutant (ΔM1) in our infection system. M1-protein was shown to be an essential component for intracellular survival as significantly ($P < 0.03$)

lower counts were recovered following infection with the mutant as compared to the wild type (wt) (Figure 3A and 3B, **paper I**). Recent data using a complemented ΔM1-strain restored that function, resulting in high numbers of surviving bacteria (Hertzén et al., unpublished).

In order to get a more detailed view of the vesicle containing the mutant, we used transmission electron microscopy. The electron micrographs revealed fusion events of vacuoles with ΔM1 *S. pyogenes*-containing phagosomes, something that was not seen in phagosomes with wt bacteria (Figure 3C and 3D, **paper I**). Similarly, in confocal microscopy analysis, the M1-mutant, but not the wt co-localized with phagolysosomal compartments (Figure 3E and 3F, **paper I**). Thus, our data demonstrates that M1-protein is required for prevention of phagosomal maturation in macrophages. This is in line with what has been previously shown during *S. pyogenes* infection in PMNs (198-200). However, the bacterial strain used in the PMN studies is deficient in the whole mga operon, resulting in defective expression of protein H (an M-like protein), SIC and C5a peptidase in addition to M1-protein. The data in **paper I** directly implicates M1-protein in this process. In the study by Nordenfelt et al., the aim was to identify the mechanism behind M1-protein mediated impaired lysosomal fusion with a special focus on azurophilic granules. To this end, they used the human promyelocytic cell line, HL-60, which has functional azurophilic granules but lacks specific granules normally involved in maturation of the phagosome (200). In conclusion, much remains to be elucidated regarding the exact molecular mechanisms involved in steering the phagosomal maturation.

The inflammatory response towards foreign particles is classically regulated by NF-κB. Microbial products or IFN-γ induce activation of intracellular signaling, which results in phosphorylation of NF-kB (59). Activation of the inflammatory pathway can thereby be studied by investigating the phosphorylation status of NF-kB components. The p65 subunit is an activating unit while the p50 and p52 homodimers are repressors lacking a transcription activation domain. Many intracellular bacteria are able to modulate the host cell response to infection. Normally, a pro-inflammatory reaction is induced with a release of a variety of cytokines and chemokines to activate and recruit more immune cells to the site of infection. However, this response can be affected in different ways. A recent study showed that p50 regulation induces an M2 macrophage phenotype, thereby creating an anti-inflammatory response (62). Studying wt and ΔM1 *S. pyogenes* infection of macrophages revealed that wt bacteria suppress activation of the NF-kB induced host response as compared to ΔM1 bacteria, which showed significantly higher phosphorylation of both IκBα and p65 (Figure 4, **paper I**). Additional gene expression experiments supported this finding, as TNF-α levels were 8-fold higher in the ΔM1 as compared to the wt bacteria (**paper I**). Thus, M1-protein can be considered as a virulence factor modulating the host-cell inflammatory signaling during infection, which is important in the defense against intracellular pathogens. Other bacterial species that interfere with macrophage polarization include among others, Salmonella species that suppress M1 polarized cytokine production of IL-12 (252), *Brucella* inhibiting TNF production (253) and *Mycobacteria* which inhibit induction of NF-kB activation (215). A microarray study of *S. pyogenes* infected murine macrophages showed a mixed response with up-regulation in both M1

and M2 associated genes (254). It is tempting to speculate that this may reflect differences in infected versus uninfected cells, so that the infected cells are those displaying the M2 phenotype whereas uninfected the M1 phenotype. It would be of interest to further investigate this by isolation of a pure population of infected cells by sorting on fluorescently labelled intracellular bacteria.

In **Paper I** we demonstrate mechanisms underlying *S. pyogenes* entry and intracellular survival in human macrophages. It is interesting that M1-expressing *S. pyogenes* are taken up by an actin-dependent mechanism, as has also been shown for M3 *S. pyogenes (202)*, which both lack the SfbI protein that normally induce fibronectin-mediated uptake. Future studies should aim to decipher the receptors involved in this process. M1-expressing *S. pyogenes* encode the FbaA protein whereas M3-expressing bacteria have the FbaB protein. Uptake of M1-expressing *S. pyogenes* to epithelial cells have been reported to bridge fibronectin or laminin with M1-protein (255). However, bacterial entry into macrophages is not dependent on M1-protein expression (**paper I**). Upon entry, *S. pyogenes* is transiently associated with the endosomal compartments but can via expression of the M1-protein direct the phagosomal trafficking to avoid fusion with lysosomal vesicles. The importance of M1-protein further emphasized by its ability to modulate the host cell signaling and thereby reduce the pro-inflammatory response. The first part of **Paper I** underscores the importance of M1-protein for intracellular survival in human macrophages. In the next section I will describe findings that enable further understanding of the intracellular stage of infection and dissemination of *S. pyogenes*.

4.2 INTRACELLULAR REPLICATION AND SPREAD OF THE INFECTION

A previous report from the host lab showed frequencies of intracellular streptococci in macrophages that implied a potential intracellular multiplication (179). This was further addressed in **paper I**, where a six log increase, compared to initial inoculum, was noticed when quantifying the number of bacteria over time (Figure 3B). TEM was used to clarify this phenomenon and electron micrographs revealed the characteristic septum of dividing bacteria inside macrophages (Figure 5D, **paper I**). *S. pyogenes* are still contained in membrane-bound compartments upon replication, in contrast to *F. tularensis* that is taken up by a similar mechanism. Currently, we don't know how acquisition of substrates for replication is achieved within the vacuole. Further studies are needed to address this. It is clearly so that *S. pyogenes* express a unique gene profile during the early stages of infection, which likely promote survival and replication, **paper II** discussed below. Other bacterial species utilize proteins from the ER to form the replicative compartment, such as *Legionella* (256) and *Brucella* (257) that can be visualized for example by immunoflourescent stainings of infected cells or by fractionation and western blot staining. As mentioned previously, *S. typhimurium* requires an actin-containing replicative niche in order to multiply (245), this type of actin-dependency would be interesting to investigate during *S. pyogenes* infection, as we have seen that they are contained within such a compartment at late time points post infection (unpublished data).

However, at 6-8 hours after infection, a shift in morphology of the bacterial compartments can be noticed. The membranes of compartments with wt bacteria appear less dense and not equally tight fitted around the bacteria, as compared to ΔM1 or paraformaldehyde (PFA)-fixed wt bacteria (Figure 3C, 3D, 5A, 5B and 5C, **paper I**), thereby indicating that the bacterial dissemination is an active process. Accordingly, membranes of phagosomes containing wt-bacteria are dissolving and bacteria are egressing out of the cells (Figure 5A, **paper I**). At late time points post-infection, host cells are completely dissolved (Figure 5A, **paper I**). However, cells infected with the ΔM1 mutant or PFA-fixed wt bacteria did not show any morphology changes at later time points post-infection (Figure 5B and 5C, **paper I**). To further analyze the functional outcome of *S. pyogenes* infection, discharge of the cytosolic enzyme lactodehydrogenase (LDH) from host cells was quantified. Consequently, wt *S. pyogenes* infection induce a time-dependent release of LDH, indicating a loss of integrity of the cellular membrane, which was not seen in uninfected cells over time (Figure 5E and 5F, **paper I**). This has previously been reported in human monocytes and PMNs upon *S. pyogenes* infection (147). Recent studies have pointed out SLO as a major factor inducing host cell death of macrophages via apoptosis or oncosis (146, 147). Another lysine that has been shown to be important for bacterial intracellular life is the Listeriolysin O from Gram-positive *Listeria*, which results in escape from the phagosomes in to the cytosol (39).

In the studies describing the death-inducing effect of SLO mentioned above, killing was seen more rapidly, at three hours post infection as opposed to our results of cell death from 8 to 12 hours post infection. This difference in time may be explained by utilization of differential infection systems. In the studies described, murine macrophages, a human monocytic cell line and human blood-derived monocytes were used whereas we have been using human monocyte-derived macrophages throughout our studies. The rationale for using human macrophages is that it is the predominant host cell for *S. pyogenes* during severe invasive infections which was identified in human biopsies and was then hypothesized as being an intracellular source of bacteria in the tissue (179).

A clinically important question that was obvious to answer was; are the bacteria able to re-infect surrounding cells after dissemination from the host cells? The previous reports of viable intracellular *S. pyogenes* in patient biopsies and *in vitro* (179) pointed to an intracellular source that might contribute to dissemination and continued infection. Accordingly, an *in vitro* model system of dissemination was established, and the results showed that intracellular *S. pyogenes* are able to migrate out of the primary infected cells and re-infect neighboring cells, thereby spreading the infection (Figure 5G, **paper I**).

S. pyogenes used to be considered as an extracellular pathogen. The findings presented in **paper I** add on to the recent appreciation that they can survive inside phagocytic cells. Avoidance of phagosomal maturation is a pre-requisite for survival and is dependent on expression of M1-protein. The bacteria replicate in membrane-surrounded compartments as visualized by TEM and the increase in bacterial numbers can be quantified by plating of supernatants. Finally an increase of LDH in the supernatant as well as morphological

changes of the host cells reveled that they are being lysed. Importantly, the bacterial survival enable dissemination of the infection since it was shown that primary infected macrophages can release bacteria to infect surrounding cells, thereby closing the infection circle as visualized below in Figure 8.

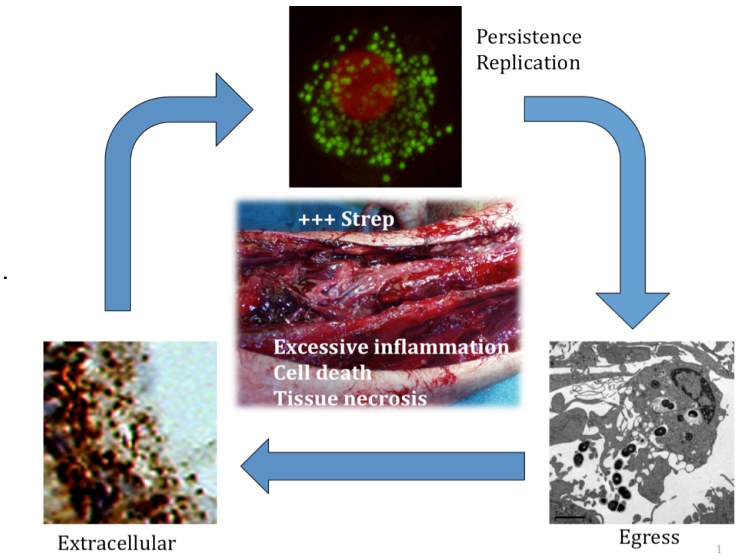

Figure 8. Infection cycle of invasive *S. pyogenes*

4.3 GENE REGULATION IN INTRACELLULAR *S. PYOGENES*

During infection *S. pyogenes* regulate expression of its virulence factors in a very distinct manner. The different sensing components and mechanisms of regulation enable bacterial adaptation to the changing environment throughout infection (162). The two most common mechanisms of controlling gene expression utilized by *S. pyogenes* are the stand-alone regulators and the TCSs that together fine-tune the bacterial response and survival within the host cell (162). As described above, we could demonstrate the importance of M1-protein for survival in macrophages. Gene expression of *S. pyogenes* grown in human whole blood (166), serum (164) and several murine (258, 259) and monkey (260, 261) *in vivo* models have revealed some insight into streptococcal pathogenesis. However, there are no studies, to our knowledge, on bacterial gene expression during intracellular survival in human macrophages.

In **paper II** we used customized microarrays with printed oligomers of the M1T1 strain 5448 and quantitative real-time PCR to study the gene expression of intracellular *S. pyogenes* in human macrophages. The microarray technique is powerful as it enables analysis of the whole bacterial genome at once, resulting in an overview of the gene expression.

As we aimed to target the early time point of the infection process when bacteria are adapting to the intracellular compartment as well as preparing for

replication, gene expression was determined at 2h post infection of human macrophages. At this time point, 145 genes were differentially expressed as compared to extracellular bacteria (Figure 1A, **paper II**). In another study, investigating the gene expression of intracellular *S. pyogenes* in human PMNs, 276 genes were differentially transcribed at three hours of infection (182). As in our study, the largest group, when dividing these genes into functional categories, contained hypothetical genes (Figure 1B, **paper II**) (182). However, of the ones with a known function, the major categories of up-regulated genes were those involved in cell wall synthesis and energy production (Figure 1B, **paper II**). In addition, also a group of phage-encoded genes were up-regulated (Figure 1B, **paper II**). A common way to transfer virulence factors between bacteria is via transposable genetic elements, such as bacterial phages (262). Comparison of the genomes of the clinical isolate 5448, the strain used in this thesis, and the lab strain SF370, both of the M1T1 serotype, revealed a 95% homology (263). Interestingly, the majority of the 5% remaining genes were phage-related, and more prominent, in the clinical 5448 isolate it is noteworthy that 78% of the 5448-specific sequences are shared by two M3 strains, MGAS315 and SSI-1, which are also associated with invasive diseases (263). Two important phage-encoded virulence factors found in invasive M1 serotype are the superantigen SpeA and the DNase Sda1, to which homologs can be found also in the invasive M3 subtypes. It has previously been suggested that invasive subclones of the same serotype share genetic elements that render them more successful infectants (264, 265).

Bacterial metabolism and regulation of import/export of nutrients and virulence factors is dependent on several transport systems. An ABC transporter, *spy2032/2033*, was shown to have increased expression at 2h post-infection (Figure 2B, **paper II**), which confirms previous studies of *S. pyogenes* growth in human blood (166). In the former study, expression of *spy2032/2033* was associated with increased virulence. Up-regulation during infection in macrophages was also verified using q-RT-PCR (Figure 2A, **paper II**). In addition, another ABC transporter, specific for iron transport, *spy0385*, was up-regulated both in the microarray and in the q-RT-PCR assay (Figure 2B, **paper II**). In a mouse soft-tissue infection model, the PerR iron transport regulator was highly up-regulated, underscoring the importance of iron metabolism during infection (258). Bacterial growth within the host cell is dependent on efficient acquisition of iron (266), why several studies report an increased expression of regulators during infection. Also, bacteria deficient in iron regulators are more susceptible to oxidative stress (267, 268).

Intracellular bacteria in macrophages showed a down-regulation of genes associated with oxidative stress (Figure 1B, **paper II**), as compared to results reported during infection in PMNs, which show both up-regulated genes and expression of oxidative species (182, 198). The down-regulation of genes involved in oxidative stress during macrophage infection might be explained by *S. pyogenes* inhibiting maturation of the phagosome (**paper I**), which renders activation of oxidative stress-regulated genes unnecessary.

Interestingly, the TCS Ihk/Irr, recently identified to support resistance to oxidative stress in PMNs (165, 182), was up-regulated at 2h post-infection (Figure 2B, **paper II**). To verify this up-regulation, RNA from intracellular

bacteria was analyzed using q-RT-PCR, which confirmed the increase (Figure 2A, **paper II**). Increased expression of *ihk/irr* has also been reported during *in vivo* infections of murine models and during infection of human blood and serum (164, 166, 258). These studies of *S. pyogenes,* and the fact that Ihk/Irr belongs to a conserved family of gene regulators that are found in several bacterial species (269, 270), where it has a role in mediating intracellular survival, indicate that it is possibly involved in adaptation and in resistance to host cell responses during *S. pyogenes* infections. To further investigate the role of Ihk/Irr in the pathogenesis of *S. pyogenes* infection, we created an Ihk/Irr-deficient mutant (ΔIhk/Irr). Interestingly, plating of infection media from cells infected with wt or ΔIhk/Irr revealed a significantly reduced number of colony forming units from cells infected with the mutant as compared with the wt (Figure 3A and 3B **paper II**), thus implying that Ihk/Irr has a role in survival, alternatively in bacterial dissemination. This matter is currently under investigation.

During the infection process, bacteria regulate virulence factors in a sophisticated manner, often specific systems are active at different phases. CovR/S is a negative regulator of expression of several virulence factors, such as SpeB, SIC, SpyCEP, Ska and Sda1 (271). To further look into the dynamics of gene regulation during infection, expression of *ihk/irr* and the *covR/S* systems were compared by q-RT-PCR, at 2h and 6h post-infection. Here we included a later time-point at 6h post-infection, as we hypothesized that a different gene expression profile would be required during early adaptation phase compared to later phases of infection. Early during infection, *ihk/irr* was up-regulated whereas the expression decreased as the infection proceeded (Figure 4B, **paper II**). The opposite was true for *cov*R/S, which had higher activity at 6h of infection as compared to 2h post-infection (Figure 4A, **paper II**), thus demonstrating the temporal expression of different TCSs during various stages of infection.

CovR/S is a negative regulator through repression of gene expression (169), and it has recently been appreciated that invasive *S. pyogenes* infections often results from strains with a mutation in the *covR/S* TCS (272). Mutation in *covR/S* results in an invasive phenotype, as the normally repressed virulence factors then can be secreted (173, 273). CovS positively regulate SpeB, but upon mutation, *speB* is down-regulated and cannot inhibit the action of the secreted virulence factors. This phenotype has recently been reported for human clinical isolates (177), importantly, and in contrast to this, analysis of SpeB expression in tissue biopsies from human subjects reveal high amounts of the protease (179). This reflects the fine-tuned gene regulation during the infection process, which is depending on several factors including the location and the stage of infection. It may seem contradictory that *covR/S* is up-regulated at time of dissemination in this study, since it acts by repressing virulence factors. However, one of the known genes to be suppressed by CovR/S is *hasA*, which encodes the hyaluronic capsule. The capsule is a potent anti-phagocytic factor, why low encapsulation would increase the probability of being phagocytosed by a host cell after dissemination from the primary infected cell. This would then be a way of spreading the infection.

The M1-protein was naturally an interesting target to analyze, as previous studies have shown its importance during the intracellular stage in macrophages (**paper I**) and in PMNs (198, 199). We hypothesized that as the bacteria replicate and start to adapt for host cell escape and re-infection of surrounding cells, expression of the M1-protein would increase. mRNA expression of M1-protein at 2h and 6h of infection revealed a 2-fold increase over time (data not shown). It is tempting to speculate that this expression is increased at later time points post-infection when the majority of cells are lysed and the bacteria demonstrate high capacity to infect new cells. Actually, a recent study investigating clinical isolates identified higher expression of M-protein in M1, M3 and M6 serotypes, which are known to be associated with invasive diseases, as compared other serotypes (274). To further analyze the functional outcome of differential gene expression, macrophages were infected for 2h and 6h, after which they were lysed and new cells were re-infected with bacteria from the lysates (see experimental out-line in Figure 9). After 2h of infection, lysates from newly infected cells were plated and colony-forming units were counted. Preliminary data on infectivity supported the hypothesis that bacteria later in the infection stage have better capacity to infect and adapt in new host cells, and thereby indicate the importance of differential gene expression during the infection process (Figure 4C, **paper II**).

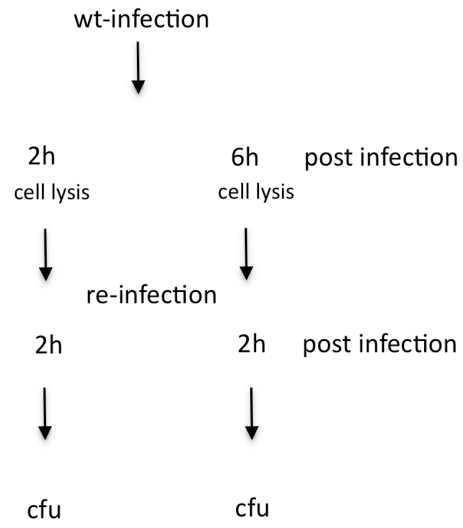

Figure 9. Overview of the experimental set-up.

Establishment of an *S. pyogenes* infection is a complex process that requires distinct mechanisms regulating every step. *S. pyogenes* is a versatile pathogen that is able to cause a variety of infections in different tissues, which is possible through bacterial adaptation as a reaction on the surrounding environment. Gene expression during the intracellular stage of infection of human macrophages was investigated in **paper II**. Overall, the major changes are associated with adaptation to the intracellular environment. Two key groups of up-regulated genes were those involved in cell wall synthesis and energy production. This is also the case during infection in human PMNs (182). Additionally, phage-encoded genes were up-regulated, which is likely linked to

virulence promotion as well as some transporters important for acquisition of metals and nutrients. Studies of specific genes revealed a temporal expression of two TCSs, *ihk/irr* and *covR/S*, indicating the fine-tuned gene regulation and confirming their previously reported importance during infection. Furthermore, the functional importance of gene regulation was shown in a survival assay.

In conclusion, intracellular *S. pyogenes* actively change their expression profile as to enable an intracellular persistence. The recent understanding that *S. pyogenes* have the ability to survive intracellularly has opened up a new research field with many aspects to explore in regards to gene regulation and adaptation. Future experiments aiming to answer these questions will be important to improve our understanding of host-pathogen interactions during severe *S. pyogenes* infections.

4.4 COX-2 EFFECT ON INTRACELLULAR SURVIVAL

The two previous studies have focused on uptake, trafficking and intracellular survival of *S. pyogenes* from the bacterial point of view. In **Paper III**, focus is shifted to study how the host cell response can affect *S. pyogenes* infection. Upon infection, the immune system is activated and cells in the surroundings of the bacteria start to secrete signaling molecules. One such molecule is PGE_2, which genes has been shown to be strongly induced upon *S. pyogenes* infection in a susceptible mouse model (67). PGE_2 is an arachidonic acid metabolite that is produced by various cells in response to inflammation (63) and it has previously been showed to facilitate intracellular survival of several bacterial species (275, 276). Thus, our aim with **paper III** was to study the effect of PGE_2 in *S. pyogenes* infection.

Firstly, we analyzed biopsies from patients with varying severity of streptococcal tissue infection for COX-2, the rate-limiting enzyme converting arachidonic acid into PGE_2. The results revealed a significant correlation with disease severity and amount of COX-2 in the tissue (Figure 1, **paper III**). Similarly, *S. pyogenes* infection of mice resulted in induction of *cox-2* mRNA as compared to uninfected controls. Investigation of COX-2 protein in the liver, revealed clear staining in infected mice as compared to uninfected (Figure 2B, **paper III**). To ascertain an effect of the COX-2 expressed, the plasma concentrations of PGE_2 was determined and found to be significantly elevated in infected mice. In addition, an inhibitor of COX-2 reduced the levels of PGE_2 to those seen in the plasma of uninfected control mice (Figure 2C, **paper III**). In summary, these experiments show that COX-2 is up-regulated upon *S. pyogenes* infection in an *in vivo* model and promote PGE_2 production.

Activated macrophages have long been known to be potent producers of prostaglandin with COX-2 being responsible for processing of the active component, PGE_2 (277, 278). Indeed, bone marrow (BM) murine macrophages were shown to be strong producers of COX-2, shown both at the RNA and protein level (Figure 3A and 3B, **paper III**) and COX-2 could be identified in BM murine macrophages and primary human monocytes (Figure 3C and supplementary figure 1, **paper III**).

To further decipher the functional role of COX-2 we used an experimental model utilizing mice deficient in the COX-2 gene. Infection of the COX-2 $^{-/-}$ mice reveled a significant improved survival (Figure 4A, **paper III**) and no detectable PGE_2 was present in the serum of infected as compared to wt mice (data not shown). Pro-inflammatory cytokines has previously been reported to affect disease progression during *S. pyogenes* infection (279), and in fact, investigation of serum levels revealed that COX-2 $^{-/-}$ mice produced significantly lower amounts of IL-6 and IFN-γ (Figure 4C and 4D, **paper III**). Based on these results we hypothesized that a COX-2 inhibitor could be a potential therapeutic strategy. Consequently, mice were given a COX-2 inhibitor 2h prior and 4h after *S. pyogenes* infections, which resulted in prolonged survival, lower amount of bacteria in the liver and lower IL-6, IFN-γ and TNF-α levels as compared to vehicle-treated mice (Figure 4E, 4F, 4G, supplementary figure 3A and 3B, **paper III**).

In light of our data of intracellular *S. pyogenes* in macrophages, as well as previous reports that PGE_2 can inhibit intracellular killing of *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumoniae* in macrophages (275, 276), we wanted to investigate this aspect of PGE_2 function in *S. pyogenes* infection of BM macrophages. Indeed, Figure 5A, **paper III**, shows a clear dose-dependent inhibition of bacterial killing in BM macrophages as well as in human monocytes (Supplementary figure 4, **paper III**). These data suggest a suppressive effect of PGE_2 on intracellular killing.

To study the mechanism by which PGE_2 mediated its effect on bacterial survival, we investigated the interactions in more detail. The effect of PGE_2 can be exerted through ligation of four types of membrane bound E prostanoid (EP) receptors (EP1-EP4) (280). Identification of the receptor involved in the suppressive effect on intracellular survival of *S. pyogenes* in macrophages was achieved through agonist/antagonist assays using compounds blocking the PGE_2-receptor interaction or replacing PGE_2 in the receptor interaction. Those studies revealed that EP2 is the receptor mediating the effector function of PGE_2 in macrophages (Figure 5, **paper III**). The effect of EP2 inhibition could be shown both *in vivo* and *in vitro*.

Intracellular signaling via EP2 occurs through cyclic AMP (cAMP) expression (64), and investigation of the cAMP levels during *S. pyogenes* infection in macrophages revealed a dose-dependent increase in the intracellular cAMP levels (Figure 6A, **paper III**). Similar amounts of cAMP were found when the EP2 agonist, butaprost was used, as compared to the EP3 agonist, soprostone, (Figure 6B, **paper III**). Functional evidence was gained as addition of forskolin or rolipram, known to increase cAMP levels in macrophages, resulted in higher numbers of viable intracellular *S. pyogenes* (Figure 6C and 6D, **paper III**). To identify the molecular mechanism in detail we went further down in the signaling cascade. cAMP acts through activation of two downstream effector molecules, the protein kinase A (PKA) and the exchange protein directly activated by cAMP (Epac) (281). Activation of PKA resulted in suppression of antimicrobial effects and consequently higher numbers of intracellular bacteria as compared to the levels in non-treated cells. In contrast, inhibition of PKA prevented the immune suppressive effect and as a result, less bacteria were to be found (Figure 7A, **paper III**).

It has previously been reported that cAMP-activated PKA can inhibit ROS release from macrophages (275, 282). Production of ROS by NADPH oxidase is one of the major host cell responses to *S. pyogenes* by macrophages (254). We hypothesized that PGE_2 bound to EP2 resulting in cAMP-elevation leads to PKA activation that finally inhibited formation of ROS. This hypothesis was tested through investigation of ROS production in PGE_2-treated and non-treated macrophages. Figure 7B, **paper III**, clearly shows that PGE_2 treated cells do not produce ROS while non-treated cells do, thus supporting the hypothesis.

In conclusion, prostaglandin secreted from various cells is converted to PGE_2 by the inducible enzyme COX-2. PGE_2 binds to the EP2 receptor on the macrophage membrane and increase cAMP levels in the cell. cAMP in turn activates PKA which have an inhibitory effect on ROS production, resulting in a deficient bacterial killing.

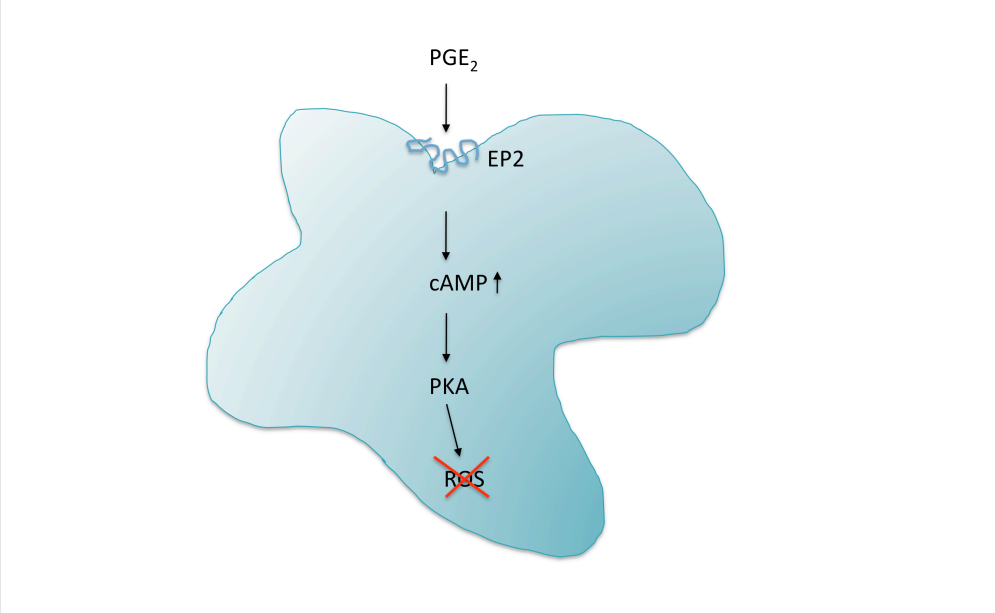

Figure 10. Schematic presentation of the results in **paper III**.

The results from the present study reveal a way to interfere with the suppression of ROS-production. Non-steroidal anti-inflammatory drugs (NSAID) are commonly used for patients with fever and inflammatory symptoms. However, these are non-selective COX inhibitors, which may mask initial symptoms of severe *S. pyogenes* infections and thereby delay correct diagnosis resulting in worsening the outcome (283). Therefore it is important to use selective inhibitors to target this interaction, which may provide a new treatment strategy. Further studies are needed to clarify these mechanisms and their clinical outcome.

5 CONCLUDING REMARKS

The studies comprised in this thesis contribute to a deeper understanding of the host-pathogen interplay during severe *S. pyogenes* infections, in particular with regards to mechanisms contributing to intracellular survival in host cells. The results demonstrate that *S. pyogenes* ability to persist within macrophages is enabled through distinctly regulated mechanisms involving both host and bacterial factors.

Certain bacterial serotypes are more efficient in causing invasive infections than others, as they are equipped with special tools to facilitate invasion and survival. We have identified M1-protein as an important factor for intracellular survival in macrophages. The possibilities of *S. pyogenes* to affect phagosomal maturation, thereby creating a safe-haven and replicative niche crucial for continued disease progression. The molecular events underlying this mechanism and the potential of M1-protein as a target for intervention remain to be defined in future studies. Another important question is whether the noted phagosomal maturation arrest mediated by the M1-protein is a property shared among different M proteins or unique to the M1-protein. Regulation of bacterial intracellular signaling is vital for bacterial survival and it also affects host responses. Data are supporting a role for the TCS Ihk/Irr during adaption to the intracellular environment while CovR/S possibly facilitates increased infectivity of disseminating bacteria. In addition, we show that PGE_2, which is produced upon infection, has a negative impact on macrophage bactericidal responses. What can be done to inhibit bacterial persistence and modulation of the host cell response? Work presented in this thesis has identified different host and bacterial factors that may serve as potential targets for novel therapeutic strategies in severe *S. pyogenes* infections. In addition, host pre-disposition to disease progression has to be taken into account and at present, there are large knowledge gaps in this area. Future research in this new field is thus strongly needed. *S. pyogenes* used to be considered as an extracellular pathogen, however, it is now important to evaluate ways to interfere with its intracellular capacity.

6 POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Det uppskattas att *Streptococcus pyogenes* orsakar 500 000 dödsfall varje år, vilket ger en världsrankning som nummer nio i listan på de värsta infektionssjukdomarna. *S. pyogenes* är en bakterie som de flesta kommer i kontakt med under barndomen då den är en vanlig orsak till halsfluss och svinkoppor (impetigo). Man uppskattar att det förekommer 616 miljoner halsinfektioner orsakade av *S. pyogenes* i världen varje år. Dessa infektioner är lätta att bota med antibiotika. Emellertid kan *S. pyogenes* även orsaka aggressiva system- och mjukdelsinfektioner, vilka är svårare att behandla och som snabbt kan utvecklas till livshotande infektioner. Dessa infektioner har hög dödlighet och morbiditet trots adekvat antibiotikabehandling. Studier av vävnadsprover från patienter med dessa allvarliga infektioner påvisade levande bakterier inuti immunceller, så kallade makrofager. *S. pyogenes* kan alltså överleva inuti celler och därmed undvika antibiotika samt kroppens eget immunförsvar. I denna avhandling har vi kartlagt hur bakterierna kan överleva inuti cellerna samt om de använder cellerna som ett slags skyddsrum för att kunna tillväxa och sedan sprida infektionen vidare till andra delar av kroppen.

Vi har kunnat se att bakterierna tas upp av cellerna på ett nytt och annorlunda sätt jämfört med andra bakterier och när de väl är inne i cellerna uttrycker de ett speciellt protein (dvs. M-proteinet) som ger dem möjlighet att manipulera cellens sätt att ta död på dem. Vi har också kunnat se att de kan föröka sig inuti cellen. *S. pyogenes* kan även sprida sig till närliggade celler genom att spränga cellväggen. För att öka förståelsen för hur *S. pyogenes* överlever i cellerna så tillämpade vi en metod för att undersöka genuttrycket hos bakterierna när de är inuti cellerna, så kallad microarray. Vi kunde då konstatera att ämnes-omsättningen påverkas när bakterien måste anpassa sig till miljön i cellerna samt att bakterien uttrycker andra faktorer än utanför vilka bidrar till en ökad överlevnadschans. En av de här faktorerna är ett regulatoriskt så kallat två-komponentssystem, Ihk/Irr, vilket tidigare visats vara viktigt vid överlevnad inuti en annan immuncell, neutrofilen. Det verkar alltså ha en central roll för streptokockens liv inuti cellen.

Genom att undersöka hur cellen reagerar på infektion har vi även kunnat konstatera att bakterien kan dämpa aktivering av cellens immunsvar. Även i en musmodell kunde vi även se att bakterien inducerar en celltyp (typ 2 makrofag) som inte är kapabel till att bryta ned bakterien och därmed kan den överleva inuti cellen.

Sammanfattningsvis kan man säga att vi med de arbeten som ingår i den här avhandlingen, har kartlagt specifika faktorer som har betydelse för *S. pyogenes* överlevnad och fortsatta infektion i humana makrofager. Dessa faktorer är intressanta som målprotein för utveckling av nya behandlingsmetoder av svåra streptockockinfektioner orsakade av *S. pyogenes*.

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