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Karolinska Institutet, Stockholm, Sweden

HOST-PATHOGEN INTERACTIONS IN SEVERE GROUP A STREPTOCOCCAL INFECTIONS

Pontus Thulin

Karolinska Institutet

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To my Grandparents

ABSTRACT

Streptococcus pyogenes, also called group A *Streptococcus* (GAS), is an important human pathogen ranked number 9 on the list of global killer pathogens. It causes a wide spectrum of disease, including uncomplicated superficial infections of the skin and throat, to life-threatening invasive infections and post-streptococcal sequelae.

This thesis aims to increase the understanding of pathogenic mechanisms contributing to severe soft tissue infections caused by GAS. To investigate host-pathogen interactions at the local site of infection, a tissue biopsy material collected from patients with severe soft tissue infections was used. The studies revealed that tissue biopsies from the site of infection contained high bacterial load, high amounts of the streptococcal exotoxins SpeB and SpeF, and heavy infiltration of inflammatory cells. A potent pro-inflammatory cytokine response was evident in the biopsies, and the amount of Th1 cytokines was significantly associated with severity of infection. The results indicated a critical role for superantigens, the cysteine protease SpeB and pro-inflammatory cytokines in severe GAS tissue infections.

Viable bacteria could be detected in the tissue even late after onset of infection and prolonged intravenous antibiotic therapy. In the tissue biopsies GAS, which is normally considered an extracellular pathogen, could be found intracellularly in host cells, predominantly macrophages. The patient studies further implied a potential role of SpeB for intracellular survival. An *in vitro* infection model was used to confirm these data. The studies provided evidence that GAS could infect and survive, for a prolonged time, intracellularly in human monocytes and macrophages. Furthermore, infection with a *spe*B-deficient mutant verified a role for SpeB in promoting intracellular survival. This intracellular persistence of GAS at the tissue site may have evolved as an immune escape mechanism as well as a strategy to avoid antibiotic eradication, which may explain the high bacterial load present even long after onset of infection.

Important components of the host immune response are the antimicrobial peptides. The antimicrobial peptide LL-37 has been reported to be important in the control of GAS infections. Analyses of LL-37 in our patient biopsy material revealed high amounts of the mature peptide in all biopsies, which correlated positively to bacterial load. Studies of interaction between the peptide and SpeB, which has previously been reported to inactive LL-37, revealed a strong co-localization of SpeB and LL-37 around the bacteria. Hence, the study provided *in vivo* support that SpeB-mediated inactivation of LL-37 may represent a bacterial resistance mechanism at the infected tissue site.

These studies have identified pathogenic mechanisms that likely contribute to the severity of these infections, and consequently, are targets for intervention. Improved therapeutic strategies are required to limit the morbidity and mortality associated with severe GAS infections.

LIST OF PUBLICATIONS

I. Norrby-Teglund A, **Thulin P**, Gan BS, Kotb M, McGeer A, Andersson J and Low DE. *Evidence for Superantigen Involvement in Severe Group A Streptococcal Tissue Infections.* J Infect Dis. 2001 184;853-860.

II. **Thulin P**, Johansson L, Low DE, Gan BS, Kotb M, McGeer A and Norrby-Teglund A. *Viable Group A Streptococci in Macrophages during Acute Soft Tissue Infection.* PLoS Medicine 2006 3(e53);0371-0379.

III. **Thulin P***, Johansson L*, Sendi P, Hertzén E, Linder A, Åkesson P, Low DE, Agerberth B, and Norrby-Teglund A. *LL-37 in severe Streptococcus pyogenes soft tissue infection.* Manuscript submitted.
* Equal contribution of authors

CONTENTS

LIST OF ABBREVIATIONS

GAS	Group A *Streptococcus*
GRAB	G-related $\alpha_2$macroglobulin-binding protein
hCAP18	Human cationic antimicrobial protein of 18kDa
IFN	Interferon
IL	Interleukin
IVIG	Intravenous polyspecific immunoglobulin
MF	Mitogenic factor
NET	Neutrophil extracellular traps
NF	Necrotizing fasciitis
Spe	Streptococcal pyrogenic exotoxin
STSS	Streptococcal toxic shock syndrome
TNF	Tumor necrosis factor

INTRODUCTION

"Killer bug" and "Flesh-eating bacteria" – The headlines in the lay press about severe invasive *Streptococcus pyogenes* infections were many and frightening during the 1990ies. It was almost as if a new pathogen had arisen. *Streptococcus pyogenes*, also commonly referred to as Group A *Streptococcus* (GAS), has however, been with us for a long time. Already Hippocrates described a devastating "flesh eating disease", which in its clinical description is highly similar to what we today call necrotizing fasciitis (NF)[32].

During the last 20 years we have witnessed an increase in severe GAS infections world wide, and a new severe form of GAS disease with high mortality rates was described and named streptococcal toxic shock syndrome (STSS) [23]. This resulted in intense research in the field that taught us a lot about the epidemiology of invasive GAS diseases, the strains that cause them and the molecular interactions with the human host. Despite these advances invasive GAS infections remain a global health problem with an estimated 163 000 deaths yearly [20]. We obviously need more knowledge about the mechanisms by which GAS cause disease in order to improve prevention and treatment of these infections. This thesis aimed to increase understanding of the pathogenesis of severe invasive GAS infections with particular emphasis on host-pathogen interactions at the local tissue site of infection.

Streptococcus pyogenes

Streptococcus pyogenes is a Gram-positive, non-motile, spherical bacterium that grows in chains. It was described and named by Rosenbach in 1884, and is distinguished from other streptococci by the presence of the Lancefield group A carbohydrade in its cell wall. When grown on blood agar, it results in β-hemolysis, and is consequently referred to as β-hemolytic GAS. GAS can be divided into serotypes based on a long, fibrillar protein called the M-protein. This is a highly variable protein with at least 124 types described [41], and since the introduction of nucleotide sequencing of the gene encoding M-protein, i.e. *emm*-sequencing, new types are added regularly. Historically M-typing was introduced by Rebecca Lancefield in 1928 with the serology method using specific reference sera [74]. Serotyping was further developed in 1946 by the addition of T-typing (trypsin resistant antigen), which is based on identification of different variants of the T-antigen [75]. The two typing systems are generally used in combination.

Epidemiology

A large proportion of people carrying GAS are asymptomatic, and this asymptomatic carriage range between 1-12% [10, 55, 138]. Asymptomatic carriage is consistently higher in children and decreases with age. GAS infections are generally more common during the winter months [28, 29, 108], whereas asymptomatic carriage rates do not change with season [10, 55, 138].

In some cases GAS colonization results in infections that, in the vast majority of cases, are uncomplicated and either self limiting or easily treated with antibiotics. These include pharyngotonsillitis and otitis of mucous membranes, as well as superficial skin infections such as impetigo and erysipelas (**Figure 1**). Of these, pharyngotonsillitis is the most common with an estimated 616 million cases world wide each year [20]. Impetigo is mainly seen in children, while erysipelas is preferably seen in the elderly. Although regarded as non-severe and self-limiting, antibiotic treatment is recommended for these infections since it is thought to prevent the development of severe complications and post-streptococcal sequelae.

Two types of post-streptococcal sequelae are distinguished clinically. After pharyngeal GAS infections, some patients develop acute rheumatic fever which is caused by cross reactivity between GAS proteins and host tissues including cardiac tissue [50]. Acute glomerulonephritis is another sequelae to GAS skin and throat infections, which is likely caused by immune complexes trapped in the kidney glomeruli with concomitant inflammation (Reviewed in [154]). These sequelae are only sporadically seen in Western countries today, but represent a major health problem in developing countries. Especially rheumatic fever causes morbidity and mortality in developing countries due to its potential complication of rheumatic heart disease which develops in 60 % of patients with rheumatic fever. World wide, more than 200 000 people is estimated to succumb to this streptococcal complication [20]. The global number of cases of acute post streptococcal glomerulonephritis has been estimated to equal cases of rheumatic fever, but the mortality is substantially lower (**Table 1**).

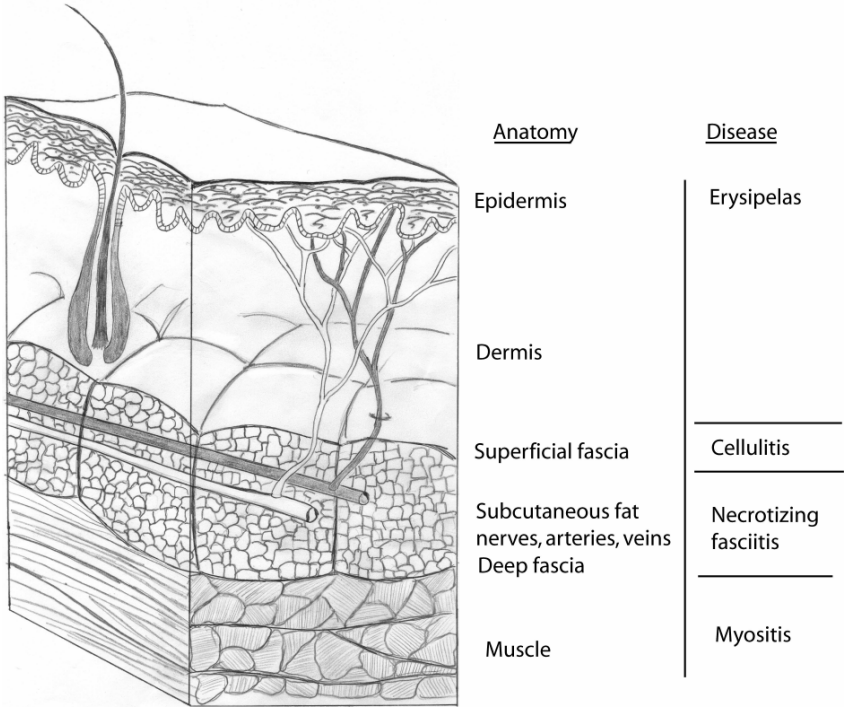

Figure 1: Schematic illustration and clinical classification of soft tissue infections. Modified based on [49]

Table 1: Global burden of group A streptococcal infections*

Disease Manifestation	Incidence	No. of deaths
Rheumatic heart disease	282 000	233 000
Rheumatic heart disease complications (endocarditis and stroke)	178 000	116 000
Acute post streptococcal glomerulonephritis	472 000	5 000
Invasive infections	663 000	163 000

*Adopted from [20]. The table shows estimated numbers.

The reason for the differences in incidence of rheumatic fever seen in developed and developing countries are debated. It is generally believed that early antibiotic treatment in developed countries is responsible for the decreased incidence of rheumatic fever, but as the morbidity and mortality of GAS disease decreased already before the introduction of antibiotic treatment [120], and since outbreaks of have been reported in for example USA [24] it seems likely that also other factors, such as hygiene, crowding, endemicity of streptococcal infection and changing virulence of GAS are involved.

The incidence of invasive infections is generally reported to be 1-4/100 000 population [29, 77, 109, 110, 151], although higher frequencies are seen in for example African Americans [108] and indigenous Australians [105]. Invasive GAS disease manifestations involve among others cellulitis, septicaemia, puerperal sepsis, NF and myositis. The most severe form of streptococcal disease is STSS. Untreated STSS rapidly leads to death, and despite adequate medical intervention mortality rates can reach 60% [29, 37]. Altogether invasive GAS disease world wide has been estimated to lead to more than 160 000 deaths every year.

Invasive GAS infections are treated with intravenous antibiotics, generally penicillin and clindamycin. If skin or soft tissue is involved, debridement is usually performed. A beneficial effect of intravenous polyspecific immunoglobulins (IVIG) as adjunctive therapy for STSS has also been reported [27, 66], and this may decrease the need for debridement. IVIG is recommended in the treatment of STSS in Sweden since 2004. The mechanistic actions of IVIG in severe invasive GAS infections probably include opsonisation of bacteria, neutralization of virulence factors and modulation of cytokine responses [7, 9, 92, 101, 129]. Whether also patients with NF would benefit from IVIG has not yet been proven, but a case series showed a potential beneficial effect in particular with respect to a more conservative surgical approach [100].

Epidemiological studies have identified a number of predisposing factors for NF and STSS. These include HIV-infection, injecting drug usage, diabetes mellitus, alcohol abuse, cardiac disease, lung disease, cancer, number of persons at home and contact with children with sore throat [29, 42]. Furthermore, a lack of antibodies against surface and secreted GAS proteins has been reported to predispose for invasive disease [3, 8, 56, 103]. The clinical course of invasive disease is linked to the HLA haplotype of the patient, and the association has been linked to superantigen-mediated inflammatory responses [71].

The mortality of severe invasive GAS infections is high despite prompt antibiotic therapy. In NF-patients, the mortality was recently reported to be 20% [47], but in STSS cases mortality commonly exceeds 50%. The highest mortality rate among patients with GAS soft tissue infections was seen in patients above 65 years of age [128], and in studies of NF extremes of age, <1 or >60 years, were associated with increased mortality [21].

Severity of GAS infections had been observed to decline for several decades, and it was thought that GAS virulence was decreasing [120]. It was that sort of belief that made it possible to conduct experiments with human subjects as described below:

> "Experimental inoculation of 7 strains of Group A streptococci failed to result in either colonization or infection of normal intact skin of human volunteers. Inoculation on skin damaged by superficial scarification resulted in localized infections when 1 x 10(4) or more organisms were inoculated into the wound by rubbing and covered with an impermeable plastic film. Intradermal inoculation resulted in localized cellulitis, regional lymphadenopathy, and fever."[80]

Just a few years later, the first report was published describing a case of an aggressive invasive episode of GAS disease [23], and it was soon to be followed by other, similar reports [40, 136]. During the 1990ies, the world witnessed an increase in invasive GAS disease, including the newly described STSS. It was recognized that these outbreaks were mainly due to M1 and M3 strains of GAS [81], and further studies implicated one strain specifically, referred to as the invasive M1T1 strain. This strain had disseminated globally and was found to be involved in a large number of invasive disease episodes. Although M1 and M3 GAS strains have been associated with large outbreaks of severe invasive disease, also infection with other serotypes may result in severe invasive diseases.

In Sweden, the incidence of invasive GAS infections is relatively high, as compared to other developed countries, with an incidence of 3/100 000 population [28]. Larger outbreaks of invasive GAS infections were reported in the winter of 88/89 and 94/95. M1T1 strains were found to dominate during these peaks [137, 142]. Since July 2004, invasive GAS infections have to be reported to the Swedish Institute of Infectious Disease Control, and according to available data at (www.smittskyddsinstitutet.se),

2007 will likely show the highest incidence ever reported in Sweden with 292 cases identified during the first six months of 2007.

Importantly, it has been recognized that herd immunity affects the degree of dissemination of a particular strain in the population [56], and the frequency of a strain in the population is usually linked to its prevalence in invasive infections [39, 124]. In conclusion, the incidence of invasive infection is likely a combination of the invasiveness of the strain, the prevalence of the strain in the population, the strain-specific herd immunity, and the prevalence of predisposing factors within the population.

The human immune system

The human body is constantly in contact with potentially harmful bacteria, viruses and parasites, and to avoid infection we need protective mechanisms that prevent these organisms to cause infections. These mechanisms can be divided into physical barriers, innate and adaptive immunity. Innate immunity is immediate and non-adaptive, and consequently important in the initial response to infection. Adaptive immunity takes several days to become fully evolved, is antigen-specific, has memory, and is primed by the innate response.

Innate immunity has a soluble and a cellular arm. The soluble arm is composed of complement and antimicrobial peptides, while the cellular arm is composed of among others macrophages, neutrophils, and mast cells. These cells have several classes of pattern-recognition receptors present at the surface of the cell or in the cytoplasm, which recognize evolutionary pathogen-specific products, such as lipopolysaccharide and peptidoglycan of bacterial cell walls.

Important components include the *antimicrobial peptides* that are expressed by many different cell types. Their functions are diverse, ranging from antimicrobial action, modulation of inflammation and phagocyte recruitment to wound healing, angiogenesis and anti-cancer effects Reviewed in [69, 85, 155]. The mode of antimicrobial action varies and although destabilization of microbial cell membranes is the most common, other antimicrobial actions have been described (reviewed in [17]. Antimicrobial peptides are generally divided into two main groups, defensins and cathelicidins [156]. In this thesis, the focus is on the cathelicidins, which are linear peptides found in all vertebrates. In humans and mice only one cathelicidin is found, LL-37 and CRAMP, respectively. LL-37 is synthesized by many celltypes and tissues, including skin and intestinal epithelial cells [45, 59], sweat glands [94], neutrophils, monocytes, natural killer (NK) cells and mast cells [2, 26, 33]. The peptide is expressed as a proform, human cationic antimicrobial peptide of 18 kDa (hCAP18), which is trimmed into the mature antimicrobial peptide LL-37 via proteinase 3 in neutrophils [130] and kallikrein 5 and 7 in the skin [153]. LL-37 can also be further processed yielding smaller peptides with antimicrobial activity [93, 153].

Other soluble components of major importance for the innate immune response are the group of blood plasma proteins known as *complement*. The name refers to its ability to "complement the antimicrobial activity of antibodies". Some functions are to opsonize

bacteria, induce inflammation/chemotaxis, and bacterial lysis. These activities need cooperation of several proteins, many of which are proteases. The complement cascade can be initiated in three different ways dependent on either antigen-antibody complexes (classical pathway), mannose on the pathogen surface (lectin pathway), or spontaneous activation of complement factor C3 (alternative pathway).

Phagocytosis is a main defence mechanism against infections and the main phagocytic cells are neutrophils and macrophages, both of which are specialized in uptake and degradation of bacteria. Phagocytes have different ways to recognize pathogens, including pattern recognition receptors that recognize different pathogen-derived molecules (lipopolysaccharide, mannose, glucan, and flagellin), or receptors for pathogen-bound complement and antibodies which engagement lead to phagocytosis of pathogens. Upon phagocytosis, the phagosome is acidified and usually fuses with lysosomes. Lysosomes contain several toxic substances such as nitric oxide, reactive oxygen species and antimicrobial peptides that can mediate killing and degradation of phagocytosed pathogens (reviewed in [126]).

Neutrophils are highly efficient phagocytes with a short half-life. They also have numerous granules with a large array of antimicrobial peptides as discussed earlier. Neutrophils are also capable of producing "neutrophil extracellular traps" (NETs) composed of histones, DNA and LL-37 [16]. These nets are thought to mediate killing of entrapped bacteria through the high concentration of LL-37 in these nets.

The precursors of *macrophages* are found in the blood as monocytes. Differentiation occurs as they migrate out into the tissue. Macrophages reside in the tissues and are therefore part of the early recognition of invading pathogens.

Adaptive immunity is composed of a humoral (soluble) and a cellular arm represented by B and T cells, respectively. The activation of B- and T-cells is dependent on antigen presenting cells, such as macrophages and dendritic cells, which present processed peptides in complex with Major Histocompatibility Complexes (MHC). There are two classes of MHC molecules, class I is present on all nucleated cells in the body and present antigens from intracellular sources, whereas MHC class II is mainly expressed on antigen presenting cells and present antigens derived from extracellular sources.

B cells express and secrete immunoglobulins, also called *antibodies*, which are the effector molecules of humoral immunity. Antibodies are diverse molecules in that each B cell expresses only one type of antibody that will recognize only one specific part of an antigen. Upon binding its antigen, B-cells can become either anergic, that is, non-responsive, or activated in which case the B-cell starts to proliferate. The progeny turns into plasma cells that produce and secrete large amounts of its surface B-cell receptor, antibodies, or it becomes a memory B-cells that will be available if the body is invaded by the same pathogen once again. Activation of B-cells is generally dependent on helper T-cells (see below). Antibodies function in neutralizing toxins and viral particles, and mark pathogens for phagocytosis. Antibodies also induce complement activation thereby increasing phagocytosis and inflammation at the site of infection.

Two main types of *T cells* are recognized, $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells. $CD4^+$ T cells recognize antigens presented on MHC II molecules, while $CD8^+$ T-cells recognize antigens presented on MHC I molecules. $CD4^+$ T cells are called helper T cells (Th) by virtue of their importance in the initiation of immune responses to particular pathogens. Upon antigenic stimulation, $CD4^+$ T cells become activated, expand and differentiate into effector cells, i.e. T-helper 1 (Th1) cells, Th2 cells or Th17 cells. These subsets are characterized by their differential production of cytokines, which direct specific immune responses (see "Cytokines" below, and **Table 2**). Th1 cells promote activation of cellular immune responses important in intracellular infections, whereas Th2 cells stimulate an antibody response against extracellular bacteria and toxins. The Th17 response is also important in protection from extracellular pathogens.

Table 2: T helper subsets, and their hallmark cytokine reponses

Th1	Th2	Th17
IL-2	IL4	IL17
TNF-β	IL5	
IFN-γ	IL10	

IFN, Interferon; IL, Interleukin; TNF, Tumor necrosis factor;

Cells of the immune system interact not only through cell-cell interactions, but also via soluble messengers called *cytokines*. They can exert their effect on the same cell that released it (autocrine effect), on neighbouring cells (paracrine effect) or on distant cells (endocrine effect). The effects are generally pleiotropic, and several cytokines may interact to mediate a certain response. Cytokines can be divided into groups based on receptor-specificity, producer cells, or function. The classification based on function commonly includes pro- and anti-inflammatory cytokines as well as chemokines. The latter of which are cytokines that mediate chemotaxis of immune cells. Some of the classical pro-inflammatory cytokines include IL-1, IL-12, TNF-α and IFN-γ, whereas IL-4, IL-10, IL-11, IL-13 and IL-1ra are commonly regarded as anti-inflammatory.

Pathogenic mechanisms of GAS infections

GAS has numerous virulence factors that contribute to its ability to efficiently colonize humans and cause disease (**Table 3**). A few major virulence factors, and the once that are of particular interest for this thesis will be highlighted in this section.

The *M-protein* is a multifunctional protein, classically known for its anti-phagocytic properties [14]. Recently, soluble M-protein has been reported to have potent pro-inflammatory activities both by activation of neutrophils [53], platelets [127] as well as T cells through its action as a superantigen [116].

Table 3: Selected virulence factors of group A *Streptococcus*

Immune function	GAS virulence factor	Mechanism
Antimicrobial peptides (AMP)	SpeB	Degradation of AMPs
	SIC	Inactivation of AMPs
	DNases	Degradation of NETs
Complement	C5a peptidase	Degrades C5a
Phagocytosis	Hyaluronic acid capsule	Antiphagocytic
	SpeB	Induction of apoptosis, intracellular survival, adhesion
	Streptolysins O & S	Cytotoxic; SLO – secretion system
	Streptococcal chemokine protease (ScpC)	Degrades IL-8
Antibodies	SpeB	Ig degradation (all classes)
	IdeS	IgG degradation
	EndoS	IgG modification
Adaptive immunity	Protein H	Cytostatic intracellularly
	Superantigens	Excessive cell activation/proliferation

Spe, Streptococcal pyrogenic exotoxin; SIC, Streptococcal inhibitor of complement, IdeS, Immunoglobuli G-degrading enzyme of *S. pyogenes*

GAS produces several proteases, which are important for virulence. IdeS and SpeB have been shown to degrade immunoglobulins (Reviewed in [152]) thereby inhibiting opsonisation. Another recently described protease, streptococcal chemokine protease C, has been shown to degrade the chemokine IL-8 and consequently impair recruitment of neutrophils into the infected tissue [54].

The streptococcal *cysteine protease SpeB* has been shown to contribute to both tissue tropism and pathogenesis [73, 82-84, 141, 144, 147], although contradictory data have been published [4, 6, 63]. SpeB is found in all GAS strains and is produced as a proenzyme that is autocatalytically cleaved to form a mature cysteine protease [34]. It has a wide variety of targets, many of which are physiologically important host proteins. These include among others extracellular matrix proteins, antimicrobial peptides such as LL-37, IL-1β-precursor, tissue metalloproteases etc. [22, 64, 65, 106, 143]. SpeB has also been found to release or degrade streptococcal proteins including

M- and M-like proteins, fibronectin binding proteins, streptokinase and superantigens [13, 107, 121, 123]. This has been suggested to potentiate GAS dissemination due to loss of binding to host cells [13]. Expression of SpeB could however also be a potential threat to the cocci, since, for example the M-protein is involved in protection from phagocytosis [14], and streptokinase have been shown to be of importance for invasive infections [58, 140]. Expression of SpeB therefore needs to be tightly regulated. The finding that GAS produce a surface bound protein called GRAB (G-related α2-macroglobulin binding protein) that bind the major protease inhibitor in human blood, α_2-macroglobulin thus explained how GAS could protect itself from proteases of both host and GAS origin [122]. It was also found that GRAB bound α_2-macroglobulin created a cage around active SpeB, creating a sheet of SpeB close to the bacterial surface that protected GAS against the antimicrobial peptide LL-37[106] (**Figure 2**).

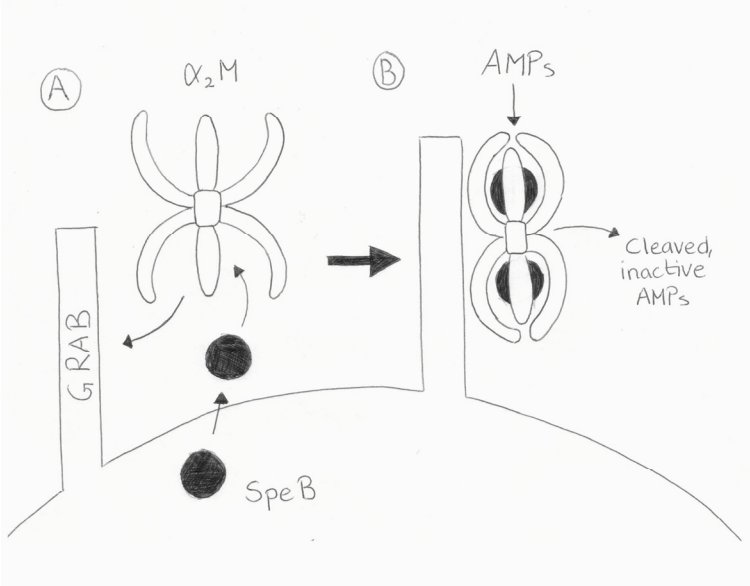

Figure 2: Schematic model of the formation of a protective α2-macroglobulin-proteinase complex on the surface of GAS. A) GAS express GRAB on its surface to which α2-macroglobulin binds. Secreted SpeB is trapped in the bound α2-macroglobulin molecule in a proteolytically active form. B) Antimicrobial peptides (AMPs) such as LL-37 can penetrate the complexes which result in degradation of the AMPs. Modified based on [106].

Initially four *DNases* were described and named DNaseA-D [148-150] but lately two new DNases have been characterized – Streptococcal DNase 1 (Sda1) [5]and Streptococcal DNase α (Sdα) [51](**Table 4**). DNases have been suggested to liquefy pus, to degrade DNA from lysed bacteria, and to degrade NETs [18, 139, 145] [19]. The newly described GAS DNase Sda1 is both necessary and sufficient for NET degradation and neutrophil resistance [19]. DNaseB is of particular interest for this thesis by virtue of its superantigenic activity [102]. It has been found to represent a major part of the DNase activity [89, 132, 150], and to cause permeabilization of blood vessels in the lung [87]. DNaseB was renamed to mitogenic factor in 1993 due to its mitogenic activity [60], but was shortly thereafter named Streptococcal pyrogenic exotoxin F (*SpeF*) due to its induction of pyrogenic cytokines [102].

Table 4: DNases produced by group A *Streptococcus*

Name	Alternative name	Reference
MF	SpeF, DNaseB, SdaB, Spd	[38, 60, 61, 148]
MF2	Spd1, DNaseC*, Sdγ/MF2-v*	[18, 148]
MF3	Spd3, Sdβ, DNaseA*	[52, 148]
MF4	Spd4	[12, 95]
DNaseD	SdaD	[119, 149]
Sda1		[5]
Sdα	Sdn*	[51]

MF, Mitogenic factor; Spe, Streptococcal pyrogenic exotoxin; Spd, Streptococcal phage encoded DNase; Sd/Sdn, Streptodornase and Sda, Streptococcal DNase.
*Suggested, but not proven identity.

Superantigens are a family of microbial proteins with ability to induce massive inflammatory responses. This is achieved by circumventing the normal rules for antigen processing and presentation. Superantigens interact without prior cellular processing with the MHC class II molecules on antigen presenting cells binding outside of the antigen-binding cleft. It also interacts with the Vβ-regions of the αβ heterodimeric T cell receptor and each superantigen activate T cells in a Vβ-specific manner, i.e. only T cells bearing certain Vβs will be activated (**Figure 3**) [86]. This activates the interacting cells to proliferate and to produce large amounts of cytokines. There are about 20-50 different Vβ regions recognized in humans and in mice, hence, one superantigen can activate 2-20% of the resting T-cell population, as compared to approximately 0.1% for a conventional antigen. The activation phase may be followed by apoptosis of most of the expanded T-cell clone, leaving a few surviving anergized (non-responsive) cells [78]. GAS produces several potent superantigens, including SpeA-M, the streptococcal superantigen (SSA) and the streptococcal mitogenic exotoxin (Sme) Z (**Table 5**). The number of superantigens that have been described in GAS points to an important role in the pathogenesis, and it has been suggested that a dysregulated immune response is beneficial for the bacteria.

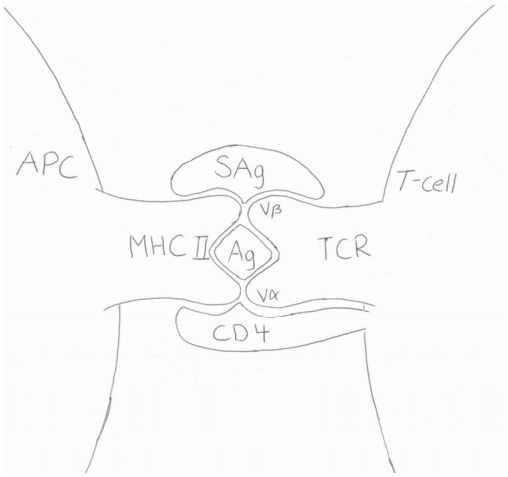

Figure 3: Schematic model of a superantigen (SAg) bidning to the MHC class II on the antigen presenting cell (APC) and the T cell receptor (TcR) on a $CD4^+$ T cell. Modified based on [70].

The first step in establishment of infection is attachment to host cells. GAS express a large number of *adhesion proteins*, generally called adhesins, that can bind to host cells and extracellular matrix (Reviewed in [25]). Many of the adhesins are M or M-like proteins. GAS also express pili, which mediates attachment to tonsils and skin [1, 43, 135]. Studies of colonization of skin revealed that the M-protein mediates binding to the membrane cofactor CD46 on keratinocytes [111], while protein F1 mediates binding to Langerhans cells in epithelia [112].

Table 5: Streptococcal superantigens and their Vβ specificity in humans.

Superantigen	**Vβ specificity**
SpeA	2.1, 12.2, 14.1, 15.1
SpeB	8
SpeC	2.1, 3.2, 12.5, 15.1
SpeF	2, 4, 8, 15, 19
SpeG	2.1, 4.1, 6.9, 9.1, 12.3
SpeH	2.1, 7.3, 9.1, 23.1
SpeI	6.9, 9.1, 18.1, 22
SpeJ	2.1
SpeK/L	1.1, 5.1, 23.1
SpeL/M	1.1 5.1, 23.1
SpeM	1.1, 5.1, 23.1
SSA	1.1, 3, 15
SmeZ-1	2.1, 4.1, 7.3, 8.1

Spe, Streptococcal pyrogenic exotoxin; SSA, Streptococcal superantigen; Sme, Streptococcal mitogenic exotoxin

(Partly adopted from Superantigens Ed: Kotb and Frasier 2007)

GAS was for a long time considered an exclusively extracellular pathogen, but evidence for active internalization, survival and possibly also multiplication in human cells have recently been presented. Initially internalization was observed into epithelial cells [76] [113] and endothelial cells [48]. In tonsils, intracellular bacteria were suggested to explain recurrent tonsillitis [114, 118]. Lately intracellular survival has been found to encompass also neutrophils [88, 134], and macrophages (Paper II). In neutrophils, GAS were found to be able to escape the phagocytic vacuole [134], but whether this also takes place in macrophages is not known. Proteins involved in cellular internalization are called *invasins* and in GAS serum opacity factor [146], and Protein F1 [62, 91] have been implicated as invasins of epithelial cells. Internalization has been found to be mediated via at least two distinct pathways, one actin dependent and one actin independent [76, 90]. Different internalization mechanisms have been reported for different M-types and cell types [72, 115]. Bacterial intracellular survival strategies have been suggested to include escape from the phagocytic vacuole in epithelial cells [76], and in neutrophils, inhibition of fusion of the phagosome with azurophilic granules [133]. Studies of gene transcription in phagocytes in response to intracellular GAS have shown that in neutrophils proapoptotic pathways are induced which lead to accelerated rate of apoptosis and survival of GAS [68]. Gene transcription in macrophages was found to be "atypical" in that both pro-inflammatory markers, notably TNF-α, IL-1, IL-6, and anti-inflammatory markers, notably IL-10 and IL1ra were upregulated [46].

To summarize, GAS is equipped with an array of virulence factors that promote bacterial colonization, invasiveness and contribute to the pathogen's ability to cause disease in humans.

AIMS OF THIS THESIS

The overall objective of this thesis was to increase the understanding of pathogenic mechanisms contributing to severe soft tissue infections caused by GAS. For this purpose, host-pathogen interactions at the local site of infection were investigated.

Specific aims were to study host and bacterial factors in relation to severity of soft tissue infection, in particular the role of:

1) Specific streptococcal factors, i.e. superantigens and the cysteine protease,
2) Host inflammatory and antimicrobial responses, and
3) Bacterial localization and killing.

MATERIAL AND METHODS

The material and methods are described in detail in respective paper. Common for all papers is the use of a tissue biopsy material and visualization/quantification of responses by *in situ* imaging, described briefly below.

Patient tissue biopsy material

The articles presented in this thesis are based in large on a patient material consisting of tissue biopsies from severe soft tissue infections (Paper I - III). The patients were identified through active surveillance in Ontario, Canada, during 1995–1997. All patients received intravenous clindamycin in combination with a β-lactam antibiotic at admission. The biopsy material was collected and stored as described in paper I. Biopsies, including skin, subcutaneous tissue, muscle, or fascia, were collected at surgical procedures performed on different days, ranging from 1–20 d after diagnosis. The tissue from which biopsies were taken was graded, based on a clinical assessment at the time of sampling, according to the following definition. Clinical grade 1 = normal tissue, no evidence of inflammation. Grade 2 = inflamed tissue (erythema and edema), including 2a = cellulitis (inflammation of skin or soft tissue, but excluding fascia and muscle); 2b = fasciitis (inflammation of skin or soft tissue and fascia); and 2c = NF (necrotic fascia). In several patients, biopsies of different clinical grades were collected at the same time point. Clinical evaluation, classification of patients, and cell marker/bacterial antigen analyses were performed independently and blinded by different investigators and at different hospitals.

In paper III, a second patient material consisting of punch biopsies from the epicenter of infection of patients with erysipelas was included as a material representing a localized, superficial streptococcal infection characterized by low bacterial load. This material was collected at Lund University Hospital during 2006.

The studies were approved by the Human Subjects Review Committee of the University of Toronto and of Lund University, and informed consent was obtained from all patients.

Immunostaining and *in situ* imaging

The methods are described in detail in (Paper I – III). Briefly, the biopsies were sectioned and immunohistochemically stained for GAS, streptococcal factors, and host factors by use of specific antibodies. The stainings were evaluated by microscopy and quantified by acquired computerized image analysis (ACIA). The positive signal was related to the total cell area (based on hematoxylin counterstain). The results are presented as ACIA values: percentage of positively stained area x mean intensity of positive staining.

RESULTS AND DISCUSSION

Severe invasive GAS infections have received a lot of attention for the last two decades, due to rise in incidence noted world wide. Intense research implicated superantigens as the main mediators of the systemic toxicity associated with these infections. The studies also revealed that the magnitude of superantigen-induced cytokine responses was determined by the individuals' HLA class II type and consequently severity of infection [71]. Importantly, this study demonstrated unique HLA class II association for STSS and NF patients, respectively. Hence, the pathogenic mechanisms for these two clinical entities are likely at least in part different. The majority of studies focused on the systemic toxicity, i.e. STSS, whereas much less was done at the local site of infection. This thesis aimed at investigating pathogenic mechanisms contributing to severe soft tissue infections, and the results are described below.

Role of streptococcal exotoxins at the local tissue site of infection (Papers I and II)

To investigate host-pathogen interactions at the local site of infection, a tissue biopsy material collected from patients with severe soft tissue infection was used. This allowed for analyses of host and bacterial factors at the local site of infection. GAS has numerous virulence factors that contribute to its ability to cause disease. In paper I and II, the potential influence of superantigens and the cysteine protease SpeB was investigated, as they have been ascribed pro-inflammatory and proteolytic activities likely to affect the tissue injury.

Eleven distinct superantigens have been identified so far, and GAS isolates commonly harbours the genes encoding 4 - 6 of those. The patient material includes patients infected with GAS isolates of varying serotypes and superantigen gene profiles. Although the superantigen profile varies between isolates, the genomically encoded SpeB and SpeF are present in all and hence, represented suitable candidates for analyses. SpeB was of interest not only by virtue of its superantigenic activity but also for its potent proteolytic and pro-inflammatory activities.

The tissue was first assessed for presence of GAS by use of antiserum against the Lancefield group A carbohydrate. The staining identified cocci of the correct size and *in situ* imaging revealed a positive correlation between amount of bacteria and severity of disease (defined by the clinical grade of the biopsy)(Paper I). SpeB and SpeF could be detected in the vast majority of tissue biopsies, and the amount of each exotoxin correlated to bacterial load ($p<0.0006$ and 0.02 for SpeB and SpeF, respectively)(Paper I and II). The tissue was further analyzed for SpeA, as this is one of the classical streptococcal superantigens. The gene for SpeA, which is phage-encoded, was found in isolates from 5 of the patients from whom 17 biopsies were available. However, SpeA could not be detected in any of the biopsies (Thulin et al. unpublished data). This is in agreement with the report by Kazmi et al. that demonstrated a differential expression of SpeA and SpeB [67]

Further analyses aimed to characterize the inflammatory response in the biopsies. Massive infiltration of inflammatory cells including macrophages and T cells, in particular $CD4^+$ T cells was found (Paper I). Determination of the cytokine expression profile revealed high levels of pro-inflammatory cytokines including IL1, IL8, and TNFα, and in particular the Th1 cytokines TNFβ and IFNγ. The latter of which were significantly associated with severity of tissue infection ($p<0.03$). The Th1 specificity of this response was further strengthened by the finding that an upregulated expression of the Th1-associated chemokine receptor CCR5 together with a lack of the Th2-associated CXCR4. Taken together, the cytokine profile at the tissue site has the characteristics of a superantigen response.

Previous reports [99, 104] have demonstrated a lack of TNFβ and IFNγ in the circulation of patients with severe invasive GAS disease, despite detectable amounts of superantigens in plasma of patients with STSS [98, 131]. Interestingly, this is exclusive for the Th1 cytokines, as a potent pro-inflammatory response with significantly upregulated IL-2, IL-6 and TNF-α is found in the circulation of these patients [99]. To assess whether this could be due to preferential homing of superantigen-induced Th1 responses, expression of homing receptors specific for extravasation of T cells to the inflammatory site was assessed. Both CD44 and cutaneous leukocyte antigen (CLA) were selected, as they are receptors directing activated T cells to the inflamed skin and furthermore both have been shown to be activated respectively upregulated by bacterial superantigens [31, 79]. Both of these receptors were highly expressed in the tissue and correlated significantly with the magnitude of Th1 cytokine response ($p<0.02$).

To conclude this part, studies of papers I and II demonstrate the presence of the superantigens and the cysteine protease SpeB. Furthermore, the data suggest a critical role for superantigens through their induction of excessive Th1 cytokines at the local site of infections in patients with severe soft tissue infections.

Bacterial localization in the tissue (Paper II)

One important observation in paper I was the finding of high amounts of bacteria even late after onset of infection. This was further elaborated on in paper II using an expanded patient material. Similarly to results in paper I, a correlation between bacterial load and severity of infection was seen. Importantly, biopsies collected more than 3 days after onset of infection still contained bacteria and in 50% high bacterial load was detected. A highly relevant question in this respect is whether the bacteria in the tissue are viable. Detection of GAS in the tissue by staining for the group A carbohydrate obviously does not allow for conclusions regarding viability. However, the staining revealed cocci with intact morphology, which together with the prolonged expression of GAS exotoxins, would imply that they are indeed viable. One biopsy collected at day 7 after diagnosis had, at the time of sampling, been cultured on a blood agar plate. This revealed a zone of inhibition around the biopsy, but growth of GAS further away (**Figure 4**). Hence, this biopsy contained viable bacteria. For the snap-frozen biopsies, bacterial viability was evaluated by use of a bac light viability stain, which discriminates between dead and viable bacteria. This revealed that large amounts of viable bacteria were detected in the majority of biopsies (74%) and most were

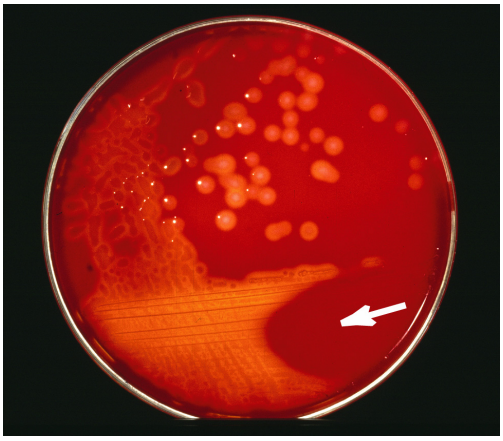

Figure 4: Blood agar culture of a tissue biopsy obtained day 7 after onset of GAS NF. The arrow indicate an inhibitory zone where the biopsy was placed

associated with cells in the tissue. This is a major concern as these patients have been on intravenous antibiotics, many for a prolonged time. The bacteria were tested and found to be sensitive to the antibiotics used, i.e. penicillin and clindamycin. It has been questioned whether the antibiotic concentration at the tissue site is adequate as the tissue infection may lead to impaired penetrance. As shown in figure 4, the biopsy contained substances that inhibited bacterial growth, such as antibiotic or the host antimicrobial peptides.

As studies have reported that infiltration of neutrophils does not occur into infected tissues in NF patients, nor in murine and zebra fish models [36, 54, 117], we assessed infiltration of phagocytic cells to see whether an impaired response could partly explain the bacterial persistence. Contrary to previous studies, heavy infiltration of neutrophils was evident in all biopsies (Paper II), and the amount correlated positively to bacterial load ($p<0.02$). Similar results were seen for macrophages with an even higher significant correlation to bacterial load ($p<0.0016$). Hence, the data show that there is no impairment in influx of phagocytic cells to the inflamed tissue site in these patients.

At this point, we started to investigate a potential intracellular source of GAS, which in the literature had been proposed to promote bacterial persistence in pharyngotonsillitis [114]. The immunohistochemical staining pattern for GAS in the tissue demonstrated three main patterns of mainly extracellular bacteria, mainly intracellular bacteria or a combination of intra- and extra-cellular bacteria (Paper II, Fig 2).
Confocal microscopy confirmed the presence of bacteria intracellularly in host cells, predominantly macrophages. GAS could also be seen in other cell types, including neutrophils, but macrophages were the main cells containing GAS in the tissue.

In the studies of SpeB, the highest expression was seen in severely involved tissue biopsies where the bacteria showed a combination of intra- and extracellular localization. In these biopsies, SpeB showed a cocci-staining pattern indicating an accumulation at the bacterial surface, which was confirmed by confocal microscopy. Whether SpeB in this form contributes to adhesion or internalization is as of yet unknown, but is supported by the report of Hytonen et al [57] where strepadhesive activity of SpeB was demonstrated.

To confirm our data of an intracellular persistence of GAS in macrophages, an *in vitro* model system with human monocyte-derived macrophages was used. Infection with two clinical M1T1 STSS isolates revealed that GAS could survive for at least 20 hours within human macrophages as determined by bacterial viability staining. Removal of extracellular antibiotics in the *in vitro* infection system lead to a rapid resurgence of GAS into the growth medium, and the rate with which GAS multiplied in these experiments were not possible to obtain in pure GAS cultures grown in either Todd Hewitt or cell culture medium. This indicates that intracellular bacteria were released from intracellular sources to the growth medium, and the actual numbers even point to a potential intracellular replication of bacteria.

The *in vitro* infection system was also used to further study a potential role of SpeB for intracellular bacterial survival. For this purpose, an isogenic *spe*B-deficient mutant was compared to the wildtype strain. The two strains showed a similar infection frequency and no difference between the 2 strains at the 4 hours time point. In contrast, at 12 hours the *spe*B-mutant demonstrates significant decreased intracellular survival as compared to the wildtype strain ($p<0.008$). Hence, these data provided support for a role of SpeB in promoting intracellular survival, but the mechanisms remains to be defined.

In conclusion, this paper demonstrates for the first time that GAS resides intracellularly in macrophages during acute soft tissue infections. We believe that this promotes bacterial persistence at the tissue site and possibly that it may also contribute to dissemination of infection. Future studies aim to elucidate the entry pathways and survival strategies of GAS intracellularly. Another interesting aspect of this finding is that as an intracellular pathogen it becomes a potential target for NK cells and CD8+ cells. The increasing insight of GAS as an intracellular pathogen, opens up new avenues of research regarding pathogenic mechanisms, protective immunity and therapeutic strategies.

Role of LL-37 in GAS soft tissue infections (Paper III)

As noted above, cultivation of GAS from a tissue biopsy indicated the presence of bacterial growth inhibitory substances in the tissue. These could be antibiotics and/or host antimicrobial peptides. Animal models have indicated that the mouse cathelicidin CRAMP is of importance for control of GAS infection [15, 33, 97]. GAS has also been shown to be highly sensitive to the human cathelicidin LL-37 [35]. In paper III, the presence of LL-37 at the inflamed tissue site was evaluated by immunohistochemical staining and its amount related to bacterial amount. The results revealed a strong expression in all biopsies which correlated positively to the bacterial load ($p=0.042$, $r=0.46$). Although an up-regulation of LL-37 was expected as a normal response to infection, the fact that the tissue contains viable bacteria during prolonged time would imply that LL-37 does not contribute efficiently to bacterial killing in the tissue.

To seek further insight into the lack of antimicrobial effect of LL-37 in the tissue, a number of potential explanations were explored. The first was to determine whether the mature peptide was present. LL-37 is generated from proteolytic processing of the

proform hCAP18, upon secretion. The staining pattern suggested the presence of both proform and mature peptide in the tissue, which could be confirmed by western blot analysis. Since many cell types are known producers of LL-37, it was of interest to investigate the major source. Double staining for LL-37 and various cell markers revealed that neutrophils were the main producers of LL-37, which was supported by the finding that neutrophil infiltration significantly correlated with LL-37 levels (p=0.0001, r=0.8). In contrast, monocytes only rarely contained LL-37, and LL-37 producing mast cells were found but only in low numbers.

In order to relate the LL-37 response seen in the severe soft tissue infections, to that of a superficial streptococcal infection characterized by low bacterial load, a biopsy material from erysipelas patients was included. Also in these biopsies LL-37 expression was found, and expression pattern was similar to that of deep tissue infections. It is worth noting that the amount of both GAS and LL-37 was similar in erysipelas and deep tissue infections.

Potent GAS counter-strategies against antimicrobial peptides have been described, including degradation of LL-37 by SpeB [125]or streptococcal inhibitor of complement (SIC) [44]. SIC is only expressed by a few GAS serotypes, and our material includes patients infected with strains known to lack this protein. Hence, it was reasonable to focus on SpeB as a potential inactivator of LL-37 in these patients. In our previous study (Paper II) SpeB was identified on the GAS surface. Notably, Nyberg et al had shown that such surface localization was mediated via the streptococcal protein GRAB which bound the major protease inhibitor in human plasma, α_2-makroglobulin[106](**Figure 2**). This protease inhibitor formed a cage-like structure enclosing SpeB in an active form, which was found to protect GAS from LL-37 mediated killing. In the tissue biopsies, we had observed surface-associated SpeB in severely involved biopsies with high inflammatory parameters such as high expression of LL-37, large amounts of GAS as well as of inflammatory cells. To obtain support for SpeB-mediated inactivation of LL-37 in severe soft tissue infections, we first confirmed the presence of SpeB with a polyclonal antibody which yielded a staining pattern of distinct cocci. Importantly, the cocci remained unstained when using an antibody recognizing the inactive SpeB precursor specifically, indicating that surface-attached SpeB is the proteolytically active SpeB. Triple-staining for GAS, LL-37 and SpeB confirmed a co-localization of these factors in the patient biopsies. These data collectively support the model proposed by Nyberg et al, and shows that the association occurs *in vivo.* This likely contributes to the lack of significant bacterial killing of LL-37 despite its presence in high amounts in the tissue.

It is becoming increasingly evident that antimicrobial peptides act not only as antimicrobial agents but also as immmunomodulatory and chemotactic factors [11, 96]. LL-37 has been recognized to induce chemotaxis of neutrophils as well as $CD4^+$ T cells [2, 30]. It is noteworthy that both these cell types are present in high amounts in the inflamed tissue (Paper I and II), and it is tempting to speculate that this could in part be due to the presence of LL-37. In this scenario, the effect of LL-37 would be detrimental rather than beneficial for the host, as it could exacerbate the already hyperinflammatory state without the desired antimicrobial effect.

CONCLUSIONS

This thesis is based on studies of host-pathogen interactions at the local tissue site in patients with severe soft tissue infections. The studies revealed that the pathogenesis at the tissue site involves:

- High expression of superantigens and the cysteine protease SpeB
- Excessive cytokine production, in particular pro-inflammatory and Th1 cytokines
- Heavy infiltration of inflammatory cells including macrophages, neutrophils, and CD4+ T-cells
- Viable bacteria for a prolonged period after intravenous antibiotic therapy
- Intracellular survival of GAS in macrophages
- SpeB as a factor promoting intracellular survival
- Failure of LL-37 to mediate significant bacterial killing despite presence in high amounts
- SpeB-mediated inactivation of LL-37 is likely contributing to the impaired function of LL-37

These studies have identified pathogenic mechanisms that likely contribute to the severity of these infections, and consequently, targets for intervention. Improved therapeutic strategies are required to limit the morbidity and mortality associated with severe GAS infections.

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