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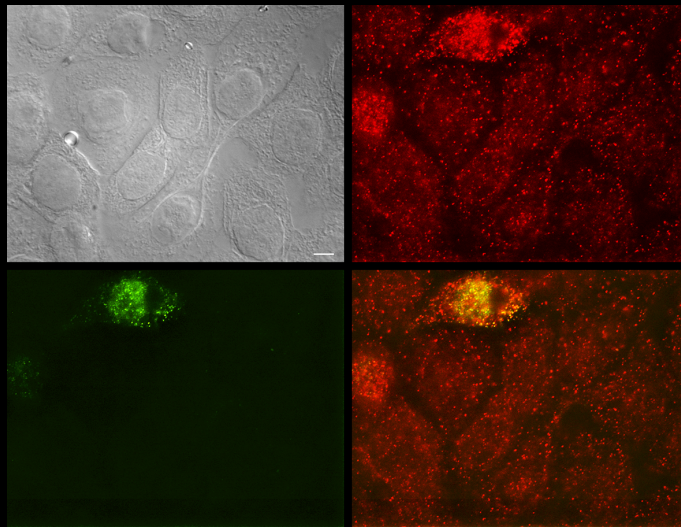
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Molecular and microscopical analysis of pathogenic streptococci

- studies on surface proteins interacting with human cells and extracellular matrix



Marta Bober

Department of Clinical Sciences, Lund
Division of Infection Medicine
Faculty of Medicine, Lund University
2011



Academic Dissertation

Molecular and microscopical analysis of pathogenic streptococci

- studies on surface proteins interacting with human cells and extracellular matrix

Marta Bober

With the permission from the Faculty of Medicine at Lund University,
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Belfragesalen, Biomedical Center, Lund, Sweden

Faculty opponent

Associate Professor **Melody N. Neely**
Department of Immunology and Microbiology
Wayne State University
Detroit, Michigan, USA



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Molecular and microscopical analysis of pathogenic streptococci

- studies on surface proteins interacting with human cells and extracellular matrix

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Cover image:

Fluorescence microscopy picture of human keratinocytes incubated with the streptococcal Slr protein. Green color shows surface associated Slr and red color shows intracellular Slr.

Experiment performed by Marta Bober
Photo by Dr. Pontus Nordenfelt

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To my family

Science is always wrong.
It never solves a problem
without creating ten more.

George Bernard Shaw

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List of papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals (I-III):

Paper I

Bober M, Enochsson C, Collin M, Mörgelin M. Collagen VI is a subepithelial adhesive target for human respiratory tract pathogens. *J Innate Immun.* 2010 Feb;2(2):160-6. Epub 2009 Aug 1.

Paper II

Bober M, Mörgelin M, Olin AI, von Pawel-Rammingen U, Collin M. The membrane bound LRR lipoprotein Slr, and the cell wall-anchored M1 protein from *Streptococcus pyogenes* both interact with type I collagen. *PLoS One.* 2011;6(5):e20345. Epub 2011 May 31.

Paper III

Bober M, Lood R, Nordenfelt P, Allhorn M, Collin M. Slr from *Streptococcus pyogenes* binds to non-muscle myosin IIA and internalizes into human keratinocytes. Manuscript.

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Abstract

Association to specific host tissue structures allows pathogenic bacteria to establish an infection and facilitates the spread within its host. Interactions between bacterial surface structures and human proteins might determine the outcome of the infection. *Streptococcus pyogenes* (*S. pyogenes*), is a human pathogen mostly causing localized infections of the skin and respiratory tract, but it is also capable of causing severe invasive disease such as necrotizing fasciitis, sepsis and toxic shock.

Until recently, *S. pyogenes* has been considered as a strictly extracellular pathogen, but several studies has shown that it is capable of invading and surviving intracellularly in several human cell types. In this thesis I have investigated the interaction of *S. pyogenes* and *Streptococcus pneumoniae* (*S. pneumoniae*) with collagen type VI, the binding of *S. pyogenes* M1 protein and leucine rich (Slr) protein to collagen type I, and Slr's interaction with human keratinocytes. The adherence of *S. pyogenes* and *S. pneumoniae* to collagen type VI in murine upper and lower airways was restricted to the proximity of the NH₂ - and COOH-terminal globular domains of collagen type VI, and for *S. pyogenes* this interaction was mediated by the M1 protein.

The M1 protein and Slr are co-expressed on the bacterial surface and both bind to collagen type I with high affinity. Slr exhibited multiple binding sites to collagen I, both to the monomeric and the fibrillar structure, with the most binding concentrated to the overlap region of the collagen I fibril. Slr is able to adhere to and internalize into human keratinocytes (HaCaT) cells in a time dependent manner and we were able to identify non-muscle myosin IIA as a potential cellular ligand for Slr. We could further confirm the presence of non-muscle myosin IIA in HaCat cell lysate and that Slr binds to non-muscle myosin IIA.

In summary, we have characterized a novel adhesin of *S. pyogenes*, Slr, as a LRR containing lipoprotein that, in concert with the M1 protein, might utilize collagens as adhesive targets during the infection process. Slr and M1 bind to collagen type I, and M1 also binds to collagen type VI, a process that might play a role in the primary step of infection in the skin and respiratory tract. Furthermore, Slr binds to non-muscle myosin IIA possibly resulting in the bacterial adherence to and internalization into human keratinocytes. This could contribute to *S. pyogenes* avoidance of the immune system and/or bacterial dissemination into deeper tissues.

Populärvetenskaplig sammanfattning

Vi är omringade av olika mikroorganismer såsom bakterier och svampar. Bakterier kan leva från de mest ogästvänliga miljöer som arktisk is och varma källor på havsbotten till varje människans hud och mag- tarmkanal. Bakterier som bor på och inuti oss spelar en stor roll i vårt välbefinnande. De hjälper oss att smälta maten och hindrar andra mer farliga bakterier från att bosätta sig på vår hud. Vi använder oss av olika bakterier för att tillverka vacciner som skyddar oss från sjukdom och de används i tillverkning av diverse matprodukter såsom ostar och öl.

En av skyddsmekanismerna mot bakterier är de fysiska barriärerna såsom hud och slemhinnor. En del av bakterierna kan under vissa omständigheter orsaka lokal skada på hud och slemhinnor, men kan även ibland sprida sig djupare in i vävnaden eller ta sig till blodbanan. För att kunna ta sig in och kolonisera sin värd måste bakterierna kunna binda till ytstrukturer på celler eller till proteiner som finns i det extracellulära matrixet (ECM, som är ett lager av olika proteiner som finns under celler och som bland annat ger stadga till cellerna) som finns under cellerna.

Detta gör bakterierna med hjälp av proteiner som finns på deras cellyta. Det extracellulära matrixet finns i alla typer av vävnader i kroppen, men speciellt mycket i bindväv och brosk. I ECM finns det olika proteiner, ett av dessa är kollagen som bildar fiberstrukturer. Dessa fiberstrukturer ger stadga och är mycket motståndskraftiga mot slitande krafter. Kollagen står för ca 25 % av det totala mängden protein i våra kroppar. Kollagen består av tre peptidkedjor (korta proteiner) snurrade runt om varandra och bildar en trippelhelixform. Trippelhelixen bildas inne i cellen och kallas då prokollagen. Prokollagen molekylerna utsöndras av cellerna och går ihop för att bilda tjockare kollagenfiber. Det finns olika grupper av kollagener beroende på uppbyggnad, funktion och var i kroppen de finns. Bakterier har ett flertal olika proteiner som kan binda till kollagen.

Streptococcus pyogenes (*S.pyogenes*) som mitt avhandlingsarbete har handlat om har ett ytprotein Cpa som kan binda till kollagen typ I som är väldigt vanlig i hud. *S.pyogenes* är en gram-positiv bakterie som oftast växer i par eller kedjor. Den kan orsaka relativt ofarliga infektioner såsom halsfluss och hudinfektioner (sk. impetigo som förekommer oftast hos barn), men kan även orsaka livshotande tillstånd som blodförgiftning och nekrotiserande fasciit (tillstånd där bakterien angriper djupare vävnad, muskler och fett). Jag har undersökt om streptokocker kan binda till olika kollagener, som i sin tur kan under-

lätta för bakterien att hålla sig kvar i det infekterade området eller möjliggöra bakteriens spridning i kroppen.

I delarbete I har vi undersökt om *Streptococcus pneumoniae* (*S. pneumoniae*) och *S. pyogenes* kan binda till kollagen typ IV. Detta kollagen finns i övre och nedre luftvägarna och kan vid mekanisk skada eller som följd av en virusinfektion bli blottat och då tillgängligt för bakterier. Vi kunde visa att kollagen typ VI hos möss finns i luftstrupen och bronkerna (som är förgreningar från luftstrupen) genom att använda oss av antikroppar som är riktade mot kollagen typ VI. Vi kunde visa att hela *S. pneumoniae* och *S. pyogenes* bakterier och proteiner som klipptes från bakteriens yta kunde binda till kollagen typ VI. Med hjälp av elektronmikroskopi (en metod där man använder sig av elektroner istället för ljus för att erhålla en bild) kunde vi visa att kollagen typ VI kan binda till bakteriens yta, att de klippta proteinerna från bakterierna binder till en specifik del på kollagen typ VI fibern (den globulära domänen) och att det för *S. pyogenes* är M1-proteinet som står för bindningen till kollagenet.

I delarbete II undersökte vi om M1-proteinet och Slr proteinet från *S. pyogenes* kan binda till kollagen typ I (som är väldigt vanligt i hud) och kan använda det som hjälp att bibehålla sitt fäste vid infektion. Vi producerade Slr proteinet i *E. coli* (som är en lätt manipulerad bakterie som ofta används för att producera proteiner) och kunde visa att det är ett lipoprotein som innehåller leucinrika (LRR) områden med likhet till InlA som är ett protein hos *Listeria monocytogenes* (en annan typ av bakterie som kan orsaka sjukdom hos människan). Vi kunde visa att både Slr och M1-proteinet kan binda till kollagen typ I och att dessa proteiner under en period finns samtidigt på bakteriens yta. Vi gick sedan vidare med att genetiskt manipulera *S. pyogenes* så att den inte kan uttrycka hela Slr proteinet på sin yta. Därefter jämförde vi ursprungsstammen (AP1, som har både M1-proteinet och Slr på sin yta) och två mutanters (MB1 som saknar Slr och MC25 som saknar M1-proteinet) kapacitet att binda till kollagen typ I. Vi kunde, med olika metoder, visa att AP1 band starkast medan MB1 och MC25 hade minskad bindningsförmåga.

I delarbete III har vi undersökt om Slr kan binda till och ta sig in i mänskliga hudceller. Förmågan att ta sig in i mänskliga celler kan hjälpa bakterien att sprida sig i kroppen samt att kunna undkomma vårt immunförsvar som ska döda bakterierna vid en infektion. Vi fann att Slr kan binda till hudceller samt kan ta sig in i cellerna. Vi identifierade

myosin 9 som det protein i hudcellerna som Slr kan använda sig av för att binda till och ta sig in i hudcellerna. Myosiner är en familj av proteiner som är involverade i celltillväxt och rörlighet, men studier har visat att de kan vara inblandade i bakteriers inträde i mänskliga celler.

Sammanfattningsvis så har jag i mitt avhandlingsarbete karakteriserat ett nytt protein hos *S. pyogenes*, Slr, ett lipoprotein med LRR-segment som tillsammans med M1-proteinet kan binda till kollagener i början av en infektion. Slr och M1 binder till kollagen typ I, och M1 binder även till kollagen typ VI, en mekanism som kan vara viktig för att orsaka infektion i hud och andningsorgan. Slr kan även binda till myosin 9 och på detta sätt binda till och ta sig in i mänskliga hudceller. Denna mekanism kan resultera i att bakterien möjligtvis kan komma undan immunsystemet och/eller sprida sig till djupare vävnad. Dessa arbeten ger oss en större förståelse för hur infektionsprocessen går till.

Abbreviations

AMP	Antimicrobial peptide
AP	Antibacterial peptide
C (number)	Complement factor (number)
CD46	membrane co-factor 46 protein
C4BP	C4b-binding protein
DC	Dendritic cell
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EndoS	Endoglycosidase of streptococci
FHL-1	Factor H-like protein 1
FN	Fibronectin
GAG	Glycosaminoglycan
GAS	Group A Streptococci
HaCaT	Human keratinocytes
HDL	High-density lipoprotein
IdeS	Immunoglobulin G-degrading enzyme of streptococci
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILK	Integrin-linked kinase
InlA	Internalin A
Lpp	Lipoprotein
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
Lsp	Lipoprotein signal peptidase II
LTA	Lipotechoic acid
MAC	Membrane attack complex
MBL	Mannose binding lectin
MHC	Major histocompatibility complex
MIG	Monokine induced by interferon gamma
MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules
NK	Natural killer

NOD	Nucleotide-binding oligomerization domain
OF	Opacity factor
PAMP	Pathogen-associated molecular pattern
PG	Proteoglycan
PI3K	Phosphatidylinositol 3-kinase
PrtF1	Protein F1
PRR	Pattern-recognition receptor
PTK	Protein-tyrosine kinase
SBP	Substrate-binding protein
Scl (A, B)	Streptococcal collagen-like protein (A, B)
SfbI	Streptococcal fibronectin-binding protein I
SIC	Streptococcal inhibitor of complement
SLO	Streptolysin O
Slr	Streptococcal leucine rich protein
SLRRP	Small leucine-rich repeat proteoglycan
Spe (A, B, C)	Streptococcal pyrogenic exotoxin (A, B, C)
TCR	T cell receptor
Th	T helper
TNF	Tumor necrosis factor
TLR	Toll-like receptor

1 Introduction

Stephen Jay Gould, the evolutionary biologist and prolific science writer, said that we live in the age of bacteria [76] and I can only agree. Microorganisms can be found virtually everywhere: from arctic ice and geothermal vents in the ocean depths to every person's skin and intestines. Microbes that inhabit humans play a major part in the cycle of life, from helping us to digest food to being the source of vitamins, vaccines as well as being involved in the production of certain foods such as cheese and beer.

Although most of microorganisms have a beneficial role, some are capable of causing great harm to humans and have had a big impact on humanity throughout our history. One of the most known outbreaks is the black death, that was caused by *Yersinia pestis*. This bacterium struck Europe for the first time in 1347 and over the coming 80 years it wiped out approximately 75 % of the European population.

My work however has been performed on a somewhat nicer bacterium, the Gram-positive *Streptococcus pyogenes* (*S. pyogenes*). This bacterium causes localized and mild diseases such as pharyngitis and impetigo (more commonly known as strep throat and skin infection usually occurring in childhood), but also has the ability to cause life threatening conditions such as necrotizing fasciitis and toxic shock. The main subject of my thesis was to study one of *S. pyogenes* surface proteins, the streptococcal leucine rich repeat (LRR) protein (Slr), and try to elucidate its role as a potential virulence factor. This thesis will describe the pathogenesis of *S. pyogenes*, virulence factors involved in colonization and invasion as well as the immunological response of the human host.

2 Bacterial proteins

Surface molecules in bacteria range from very large protein structures like flagella to smaller proteins and non-protein molecules such as polysaccharides. For human pathogens, these surface molecules have been perfectly tuned to mediate attachment and invasion of specific cells and to be able to persist in infected tissues. The cell envelope of Gram-positive bacteria is composed of a cytoplasmic membrane, a 15-80 nm thick cell wall layer of peptidoglycan, and polysaccharide structures that are linked to the surface of the peptidoglycan. Some Gram-positive bacteria also have a carbohydrate capsule loosely associated with the cell wall. The cytoplasmic membrane is composed of a phospholipid bilayer and is involved in (i) active transport of molecules into the cell, (ii) generation of energy by oxidative phosphorylation, (iii) secretion of enzymes and toxins, and (iv) synthesis of precursors of the cell wall. The peptidoglycan layer of most bacteria is composed of β -(1,4) linked *N*-acetylglucosamine and *N*-acetylmuramic acid (MurNAc-GlcNAc). Attached to the MurNAc are short peptides that can be cross-linked to each other and form a mesh-like layer providing rigid support for the cell. There are three general categories of protein anchoring in the Gram-positive bacterial cell wall: proteins that are anchored to the cell wall via their COOH-terminal ends, proteins that bind by charge and hydrophobic interactions, and lipoproteins (Lpp) that are anchored to the membrane via their NH₂-terminal region (see Figure 1). This chapter will give a brief introduction to the NH₂- and COOH-terminally anchored proteins.

2.1 The NH₂-terminally anchored lipoproteins

The Gram-positive Lpp was first discovered in 1981, when three research groups could demonstrate that the extracellular penicillinase of *Bacillus licheniformis* existed in a post-translationally lipidated, membrane associated form which was comparable to Braun's lipoprotein from *Escherichia coli* [117, 200, 154]. Structural studies that followed revealed that a diacylglycerol moiety is thioether linked to an NH₂-terminal cysteine of this Lpp and that this lipid group orientates the protein by anchoring it to the inner leaflet of the outer membrane [210]. Processing of bacterial Lpps is dependent on both signal peptide features (directing protein export) and an lipobox motif. The features of typical Lpp signal peptides are well established and consist of an n-domain (that contains the charged amino acids lysine and/or arginine), a central hydrophobic h-domain and a cleavage c-region [106, 84, 217, 28].

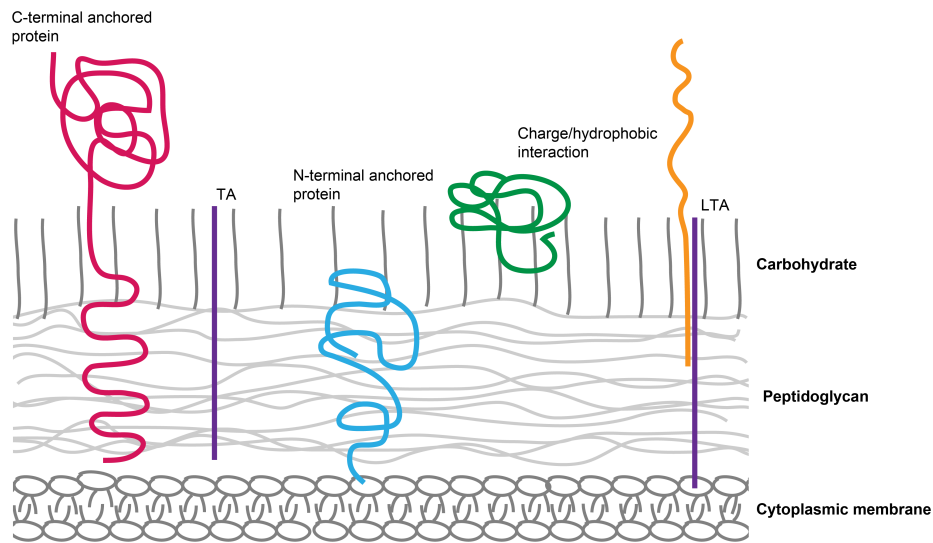


Figure 1: Surface structure of the Gram-positive bacteria cell wall. Lipoproteins have a lipid linked through a cysteine at the NH_2 -terminus. COOH-terminal anchored proteins are attached in the peptidoglycan through a LPXTG motif. Certain proteins are attached via charge and/or hydrophobic interactions with the cell surface (some proteins can also be bound ionically to the lipoteichoic acid). The lipoteichoic acid (LTA) is a carbohydrate-phosphate polymer linked to the cytoplasmic membrane via a fatty acid, while teichoic acid (TA, composed of a similar polymer) is linked through a phosphodiester to the peptidoglycan. This figure is adapted from the textbook *Gram-Positive Pathogens* by Fishetti *et al.* [67].

Sequence analysis of both Gram-negative and Gram-positive Lpp signal peptides suggests that they are usually shorter than the secretory signal peptides, with c-regions that are shorter and typically contain apolar amino acids. Thus, the c-region is a continuation of the h-domain that is distinguished primarily by the conserved sequence preceding the invariant lipid-modified cysteine [84, 28].

During the signal peptide-directed export of the prolipoprotein (proLpp), the enzyme prolipoprotein diacylglycerol transferase (Lgt) uses phospholipid substrates and catalyses the addition of a diacylglycerol lipid unit onto the thiol of a conserved cysteine that is located within the lipobox motif in the cleavage domain of the proLpp signal peptide [172, 185]. The signal peptide is removed by a lipoprotein signal peptidase II (Lsp) enzyme which cleaves within the lipobox to release the lipidated cysteine as the NH_2 -terminus of the mature Lpp [184, 28]. These two steps have been confirmed to be necessary for protein lipidation in Gram-positive bacteria [246, 241, 10, 166, 121].

A third step, where the Lpp NH₂-terminus is further modified by addition of an amide-linked fatty acid, is apparently not conserved since homologues of the enzyme lipoprotein aminoacyl transferase are not found in the genomes of low G/C Gram-positive bacteria [218].

Lpps in Gram-positive bacteria perform as substrate-binding proteins (SBPs) in ABC transportersystems ; they are involved in antibiotic resistance ; in cell signalling ; in protein export and folding ; in conjugation and exhibit other functions [95, 247, 201, 234, 211]. Several Lpps have also shown promise as vaccine candidates [111]. Many of the Lpps, particularly the SBPs, perform functions equivalent to those of periplasmic proteins of Gram-negative bacteria. Molecular genetic studies and genome sequencing, have identified a large number of putative Lpp genes on the basis of the presence of a possible lipobox sequences at the NH₂-termini of protein sequences. Putative Lpps may represent at least 0.5-8 % of the bacterial proteome [35, 218, 78]. However, it remains possible that a large number of these putative Lpps might be false-positives, with the presence of a cysteine within their signal sequences resulting in misidentification.

My work focuses on the lipoprotein Slr from *Streptococcus pyogenes* (*S. pyogenes*) that contains leucine rich repeats (LRR) with similarity to Internalin A (InlA), a LRR lipoprotein from *Listeria monocytogenes* (*L. monocytogenes*). Proteins containing LRRs vary widely in functions and can be found in both prokaryotes and eukaryotes, but the bacterial LRR proteins, especially from *Listeria* species, are important virulence factors that mediate adhesion and cellular invasion through protein-protein interactions [19]. The LRRs are approximately 20 to 30 amino acids long with the defining feature of a 11-amino acid residue sequence LxxLxLxxNxL (x being any amino acid). The amount of LRRs might consist of 2 to 45 repeats, divided into a conserved and variable segments [9, 59]. The conserved amino-terminal stretch forms the β -strand and the variable segment is a COOH-terminal stretch varying in length, sequence and structure.

The arrangement of the repeats results in a horseshoe-shaped structure with the β -sheet on the concave side and the variable stretches on the convex side [53, 108]. Slr contains a NH₂-terminal signal sequence containing 21 amino acid ending with TLIA amino acid sequence (see Figure 2). There are 13 LRRs in Slr spanning over amino acid numbers 421 to 705 forming the β -sheets compared to 15 LRRs in InlA. The COOH-terminal end of Slr contains histidine rich repeat sequences, but lacks a cell wall-anchoring motif, while

InlA carries a classical LPtTG cell wall-anchoring motif [23, 194]. This protein's actions will be further discussed in the section "**Virulence factors of *S. pyogenes***".

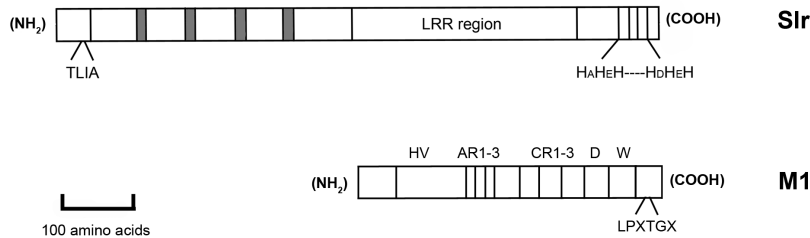


Figure 2: Schematic picture of Slr and M1 protein from *S. pyogenes*. The histidine triad motifs in Slr are shown in gray and a TLIA containing lipobox at the NH₂-terminus of the protein. Stated are the LPXTGX anchoring motif located at the COOH-terminus and the blocks A, B, C and D of the M1 protein. The abbreviations used are AR= A repeat, CR= C repeat, D= D block, HV= hypervariable, W= wall-spanning LRR= leucine rich repeat

2.2 The COOH-terminally anchored proteins

In the 1980s came the first publications describing the genes coding for protein A from *Staphylococcus aureus* (*S. aureus*) and M protein from *S. pyogenes* [224, 88]. The immunoglobulin G (IgG)-binding protein A has served as a model protein for elucidating the molecular details of protein sorting to the cell wall, mainly done by Schneewind and co-workers [69]. An examination of the COOH-terminus revealed a conserved part that contains a hexapeptide sequence known as the LPXTGX motif [64]. Following the LPXTGX motif is a hydrophobic stretch of amino acids and a short charged tail, all three which are necessary for the anchorage of protein A to the cell wall [190]. Upon correct insertion of protein A in the membrane, the protein is cleaved between the threonine and glycine residue in the LPXTGX motif [149]. The free threonine residue is then linked to the pentaglycine crossbridge of the staphylococcal peptidoglycan via an amide bond [151, 189, 219, 220]. Internalin A, a LPXTGX containing protein of *L. monocytogenes* is linked to the listerial peptidoglycan in the same manner [49] indicating that this mechanism might be universal in Gram-positive bacteria. The transpeptidation of the threonine residue and the pentaglycine crossbridge is catalysed by a small transmembrane protein denoted sortase [136, 221]. Lipid II, a precursor of cell wall synthesis, might act as the substrate for the transpeptidation followed by a transglycosylation with the protein becoming a part of the cell wall.

The M protein may be considered as an archetypical molecule when it comes to studies of the COOH-terminally anchored proteins of *S. pyogenes*. A characteristic of this protein is the presence of regions with sequence repeats and a conserved anchor. The M protein is composed of four repeat blocks, differing in size and sequence [88] (see Figure 2). The hypervariable NH₂-terminal (A-repeats, each composed of 14 amino acids), a less variable region rich in glutamate and glutamine (B-repeats, each composed of 25 amino acids), followed by conserved C-repeats (2.5 blocks of 42 amino acids) and four short D-repeats [16, 143, 238, 88]. These repeats make up the central helical rod region of the M protein due to high helical potential of the amino acids found in this segment [65, 167]. Further actions and structural differences of the M and M-like proteins will be discussed in the section "**Virulence factors of *S. pyogenes***".

3 Pathogenic streptococci

3.1 *Streptococcus pyogenes*

S. pyogenes that are also called group A streptococci are Gram-positive bacteria that grow in chains (see Figure 3). This bacterium was discovered by Louis Pasteur in 1879, but the generic name *Streptococcus* was proposed in 1884 by Alan Rosenbach. One of the most important characteristics in identification of streptococci is the type of hemolysis that can be observed when the bacterium is grown on blood-agar plates. α -hemolytic streptococci form a green zone around the colonies due to incomplete lysis of blood cells in the agar, while

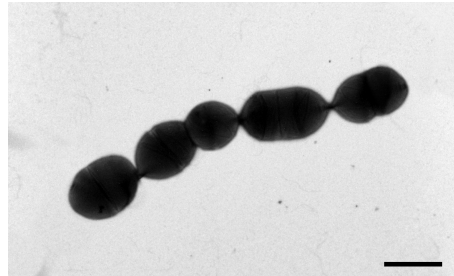


Figure 3: *Streptococcus pyogenes* growing in chains. Scale bar = 1 μm

β -hemolytic streptococci form a clear zone as a result of complete lysis of blood cells. Some streptococci are not able to lyse blood cells and are referred to as γ -hemolytic. *S. pyogenes* belongs to the class of β -hemolytic streptococci and there are several classification systems of the β -hemolytic streptococci. One classification system is based on the immunological properties of carbohydrate structures in the cell wall. The group A streptococcal carbohydrate is composed of *N*-acetyl- β -D-glucosamine linked to a polymeric rhamnose backbone [137], distinguishing it from the other streptococci, such as group B, C, F and G streptococci. Another defining characteristic is the composition of surface proteins, with the most common serotyping being based on the differences in the M protein expressed by the bacterium [118, 119]. To this day over 180 types of M protein have been classified, with the serotype M1 and M3 being the most common clinical isolates [48]. *S. pyogenes* primarily infects mucous tissues and the skin and this chapter will give an overview of the virulence factors and diseases caused by this pathogen.

3.2 Virulence factors of *S. pyogenes*

The term virulence is used to define the microbes' capacity to cause disease, a feature that distinguishes pathogens from non-pathogens. Virulence factors, individual microbial components, have been defined as factors that when deleted impairs virulence, but not necessarily the viability of the microbe [32]. Several components of *S. pyogenes* have been described to be virulence factors, and these include surface structures and secreted

molecules. The pathogenicity of a microbe, the ability to cause disease, is a term that is debated and has differing definitions [33]. A pathogen in a generally used definition, is a microbe that can cause disease in the host. However, this definition does not include "pathogens" that do not cause disease in every host they colonize. *S. pyogenes* is able to colonize the upper respiratory tract of some individuals without causing disease and is not able to cause disease in individuals that have developed specific antibodies towards a certain serotype from previous exposure [47, 159]. The proteins that are considered to be virulence factors exhibit structural and functional diversity, thus making it possible for the pathogen to use different strategies in order to colonize and infect the host.

3.2.1 Surface bound virulence factors

When it comes to surface structures, the most studied and described is the cell wall-anchored M protein. This protein appears as hair-like projections on the bacterial surface and is composed of two polypeptide chains in a α -helical coiled coil configuration that transverse the cell wall [167]. The M protein is encoded by the *emm* gene while the M-like proteins are encoded by *enn* or *mrp* genes. Most *S. pyogenes* strains possess one *emm* gene and zero to two *enn/mrp* genes, located between the gene encoding the Mga regulator and the gene encoding the C5a peptidase [89, 169]. The *S. pyogenes* strains expressing opacity factor (OF+), a apolipoproteinase that cleaves the apoprotein AI from high-density lipoprotein (HDL) causing opalescence in serum [169], encode class II Emm proteins, while OF- strains encode class I Emm proteins [169, 89, 15]. The M proteins have a hypervariable NH₂-terminal region, while the Mrp and Enn proteins display a less variable NH₂-terminal region [16, 143, 237, 238]. The Emm I proteins bind to fibrinogen, factor H, factor H-like protein 1 (FHL-1), IgG, kininogens and plasminogen. In contrast, the Emm II proteins are not able to bind fibrinogen, Factor H or FHL-1, but often bind to C4b-binding protein (C4BP) or IgA [150].

The M protein has been shown to contribute to *S.pyogenes*' survival in human blood [63], and due to this effect it has been attributed anti-phagocytic properties. Another virulence factor with anti-phagocytic properties is the hyaluronic acid capsule [233, 68]. The mechanisms of these effects have been suggested to include evasion of detection by coating the bacterial surface with fibrinogen [239], as well as inactivation of the complement system through binding of factor H [90]. However, recent studies have shown that the M protein mediates intracellular survival in neutrophils [206, 205, 144] pointing out that survival in blood does not necessarily mean evasion from internalization into

phagocytic cells. This has been confirmed by several other studies, in macrophages [215] and in *in vivo* models in mice [140, 139]. The M protein and M-like proteins have also been shown to interact with a number of human plasma proteins such as fibrinogen, plasminogen and H-kininogen [148, 12, 99, 158] as well as factors involved in regulating the complement system such as C4BP, factor H and FHL-1 [90, 109, 214]. A large number of M and M-like proteins are also able to interact with immunoglobulins (Igs). A study performed by Åkesson *et al.* showed that the interaction between the M protein, protein H and IgG blocked the interaction between IgG and complement factor C1q, thus inhibiting the activation of the classical pathway of the complement system [2, 16]. It has also been shown that the interaction between the M protein and IgA blocks the binding of IgA to the neutrophil receptor CD89, thus inhibiting the effector functions of IgA [168].

Other surface molecules of *S. pyogenes* involved in virulence are the complement inhibiting C5a peptidase [235], protein F that promotes cellular adhesion and invasion via binding to fibronectin [81, 213], collagen-like surface proteins such as SclA and SclB [128, 129, 176, 177, 236], serum opacity factor (SOF) that binds fibronectin [45, 175, 187], and the lipoteichoic acid that contributes to adherence and has cytotoxic effects on cells [196, 46].

The protein G-related α_2 -macroglobulin (α_2 -M) binding protein (GRAB) has been shown to contribute to virulence in a mouse model [178], and the collagen-like proteins SclA and SclB contribute to virulence in skin infection in a mouse model [177, 128, 129]. The LRR containing lipoprotein, Slr, a putative virulence factor in *S. pyogenes* has been shown to in concert with the M1 protein bind to collagen type I (Paper II) [23] and might utilize non-muscle myosin IIA as a receptor to gain entry into human keratinocytes (Paper III). During a *S. pyogenes* infection in humans, antibodies against Slr are developed, and a study showed that a Slr negative strain was less resilient against neutrophil phagocytosis *in vitro* and less virulent in a mouse infection model [179, 227], further confirming that Slr might be a virulence factor.

The streptococcal protease SpyCEP, a cell wall-anchored serine protease, inactivates IL-8 [54, 248], reduces neutrophil extracellular trap formation [249]. This results in diminished bacterial killing and has been shown to be involved in bacterial dissemination within soft tissues [116].

3.2.2 Secreted virulence factors

Other types of virulence factors are secreted products. These include several types of enzymes, cytolysins, superantigens and exotoxins. The pyrogenic exotoxins A (SpeA) and C (SpeC) have superantigenic properties that include T-cell activation without antigen presentation. They have been implicated in the development of toxic shock due to their ability to induce an inappropriate immune response [110]. Streptococcal pyrogenic exotoxin B (SpeB), has been shown to cleave a number of host proteins such as H-kininogen leading to the release of kinins causing vascular permeability [86], the cytokine precursor IL-1 β resulting in an active IL-1 β [100], vitronectin and fibronectin [101]. SpeB is able to degrade antibacterial peptides (APs) and Igs [21, 41, 188, 56, 102], thus interfering with phagocytosis processes and APs induced bacterial lysis. SpeB can also degrade proteoglycans, resulting in dermatan sulphate release that inactivates APs [188]. It also cleaves the surface bound C5a peptidase and M proteins [11]. Cleavage of the M proteins results in altered binding of IgG to the bacterium [174]. The released fragment of C5a peptidase retains its C5a degrading activity and the M protein fragment is still able to bind plasma proteins [11]. Cleavage of M protein can be inhibited by α_2 -M, suggesting that it might act as a physiological inhibitor of SpeB [178]. The role of SpeB as a virulence factor has been confirmed using *in vivo* infection models [222, 130]. The streptococcal inhibitor of complement (SIC) is a secreted protein that inhibits the formation of the membrane attack complex [4, 61] by interacting with the C5b67 complex before insertion into the cell membrane [4, 61]. SIC also binds to the APs, thus blocking their antibacterial effects [55, 70, 62].

Several secreted products from *S. pyogenes* are able to interact and degrade human Igs. A endoglycosidase from *Streptococcus pyogenes* (EndoS) is able to remove the glycan moiety from the γ -chains of human IgG resulting in impairment of the effector functions of IgG [42]. A second enzyme, the immunoglobulin G-degrading enzyme of *Streptococcus pyogenes* (IdeS) is a cysteine proteinase that cleaves IgG in the hinge region [164, 232]. Recent studies showed that IdeS blocks the development of IgG induced arthritis in a mouse model of disease [147], is able to remove IgG from the blood circulation of rabbits without any side effects, and cures mice from a IgG induced thrombocytopenia [97]. Streptolysin O (SLO) is a cytolysin, that together with other virulence factors, enhances tissue damage and the inflammatory response [183, 197]. A mouse infection model showed decreased mortality in subjects infected with the SLO- strain as compared to the wild type strain [124].

Table 1: Some virulence factors from *S. pyogenes*

Virulence factor	Location	Function	Ref
Capsule		Anti-phagocytic	[233, 68]
C5a peptidase	LPXTG	Complement inhibition	[235]
EndoS and IdeS	Secreted	IgG degradation	[42, 164, 232]
GRAB	LPXTG	α_2 M-binding	[178]
M-protein	LPXTG	Survival in blood, invasion, adherence	[87, 205, 206]
Opacity factor	LPXTG	Cleavage of apoproteins in HDL	[45, 186, 187]
Protein H	LPXTG	Anti-phagocytic, IgG-binding	[2, 105]
Protein F	LPXTG	Adhesion, invasion	[160, 94]
SclA and SclB	LPXTG	Survival in blood, invasion, adherence	[205, 206, 87]
Sda1	Secreted	DNA degrading	[30, 208, 40]
SIC	Secreted	MAC-inhibition	[61, 4]
Slr	TLIA-lipobox	Collagen-binding	[23]
SpeB	LPXTG	Tissue degradation	[127]
SpyCEP	LPXTG	IL-8 degradation, survival	[54, 249]
Streptolysin O	Secreted	Tissue degradation	[183, 197]

Sda1, a bacteriophage-encoded DNase [209], promotes DNA degradation in the extracellular traps of neutrophils [30, 208], has been shown to promote virulence in a mouse model of infection [30], and is involved in hypervirulence resulting in a higher likelihood of bacterial dissemination within soft tissues [40, 228].

3.3 Diseases caused by *S. pyogenes*

The Strep-EURO program, responsible for analyzing data gathered from 11 participating countries, reported the epidemiology of severe *S. pyogenes* diseases in Europe during the 2000s [96]. An approximate number of 2.46 cases per 100,000 was reported in Finland, 2.58 in Denmark, 3.1 in Sweden, and 3.31 in the United Kingdom [96]. Carapetis *et al.*, looking globally, could conclude that *S. pyogenes* might be regarded as one of the most important human pathogens both in terms of morbidity and mortality [31]. A CDC report from 2008, stated that approximately 9,000-11,500 cases of invasive GAS disease (3.2-3.9 per 100,000) occur each year in the US with a mortality rate of 10-15 % [34].

3.3.1 Acute infection

Acute diseases occur most often in the respiratory tract, bloodstream, or the skin. Some strains of streptococci show a predisposition for the respiratory tract; others, for the skin.

Generally, isolates from the pharynx and respiratory tract do not cause skin infections. *S. pyogenes* is the leading cause of uncomplicated bacterial pharyngitis and tonsillitis commonly referred to as strep throat. Other respiratory infections include sinusitis, otitis, and pneumonia. Infections of the skin can be superficial (impetigo, involving the epidermal layer of the skin) or deep (cellulitis, when the infection spreads into subcutaneous tissue) [22]. The most susceptible for skin infections are pre-pubertal children. In some cases the infection can spread into deeper tissue leading to necrotizing fasciitis (the destruction of fascia and fat), endocarditis, and toxic shock syndrome (TSS) [47].

3.3.2 Post streptococcal sequelae

There are a number of streptococcal post-infection conditions including: acute rheumatic fever and glomerulonephritis. Acute rheumatic fever is a consequence originating from pharyngeal infections resulting in carditis and joint inflammation. Persistence of the bacterium on pharyngeal tissues such as the tonsils has been associated with an increased risk of rheumatic fever. Acute glomerulonephritis can follow infections of the pharynx or the skin. The pathogenic mechanisms in acute glomerulonephritis include immune-complex formation, alteration of glomerular tissue, cross-reacting antibodies, and complement activation caused by streptococcal components deposited in the kidney [48].

3.4 *Streptococcus pneumoniae*

Although *S. pneumoniae* only plays a minor role in my thesis work, it is still worth mentioning. This bacterium is a common commensal and a frequent colonizer of the human nasopharyngeal cavity. However, during appropriate conditions it can cause infections such as community-acquired pneumonia, otitis media, and meningitis. All invasive clinical isolates are encased in a polysaccharide capsule, that protects the bacterium from phagocytosis [133]. To this day over 90 capsule serotypes have been identified and most of them originate from patients with disease, implying that the capsule is one of the most important virulence factors of *S. pneumoniae* [85, 161]. In addition to the capsule, *S. pneumoniae* expresses a variety of virulence factors such as adhesins or enzymes, that contributes to the pathogenicity of *S. pneumoniae* [13, 79]. For a detailed description of pneumococcal virulence factors see references stated in this section.

4 Bacterial-Host interactions

The attachment to host surfaces is an important event in host colonization and the development of diseases caused by microbial pathogens [240]. After entering the host, pathogens adhere to the cell surfaces or to the extracellular matrix (ECM) utilizing different surface structures. The bacterial adhesins that recognize ECM components are commonly referred to as MSCRAMMs: microbial surface components recognizing adhesive matrix molecules [162, 163]. This chapter will give an overview of some ECM components, skin cells, and their interaction with streptococci.

4.1 The extracellular matrix

Connective tissue is defined as any supporting tissue that lies in between other tissues and consists of cells embedded in a large amount of extracellular matrix (ECM). In most connective tissues the macromolecules making up the ECM are secreted by fibroblast and form an intricate network filling the extracellular space, forming a supportive framework holding cells and tissues together. Two main classes of macromolecules constitute the ECM: glycosaminoglycans (GAGs), that are polysaccharide chains usually covalently linked to proteins in the form of proteoglycans, and fibrous proteins such as collagen, elastin, laminin and fibronectin that have both structural and adhesive functions.

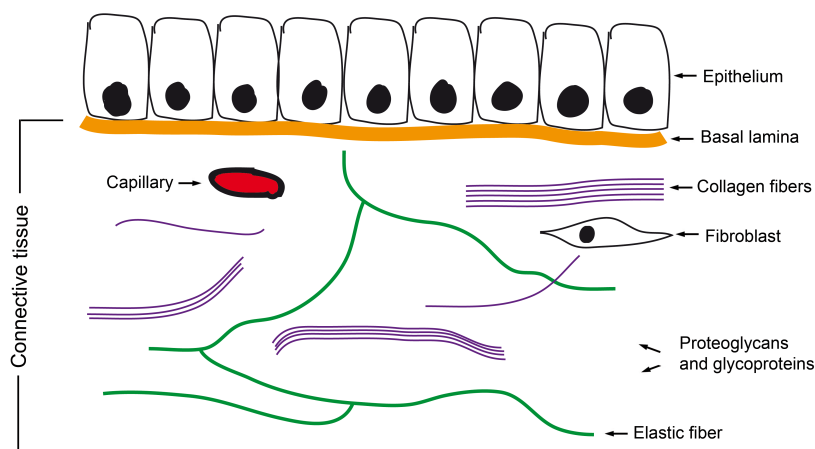


Figure 4: Connective tissue components

GAGs are polysaccharide chains composed of repeating disaccharide units. One of the two sugars is always an amino sugar (*N*-acetylglucosamine or *N*-acetylgalactosamine) and the second sugar is usually an uronic acid. Due to the sulfate or carboxyl groups on most of the sugars, GAGs are highly negatively charged. There are four main groups of GAGs classified according to their sugars, the linkage between sugars, and the number and location of sulfate groups: (i) hyaluronan, (ii) chondroitin and dermatan sulfate, (iii) heparan sulfate, and (iv) keratan sulfate [195]. All GAGs (with the exception for hyaluronan) are covalently attached to a polypeptide chain (core protein) in the form of proteoglycans. The core protein is made on the ribosomes and the GAGs chains are assembled on the core protein in the Golgi apparatus. The complete proteoglycan is then secreted to the ECM. Proteoglycans have a role in chemical signaling between cells, they can also bind and regulate the activity of secreted proteins such as proteases and protease inhibitors [14, 92]. GAGs and proteoglycans can associate with each other forming huge polymeric complexes, and they also associate with fibrous matrix proteins and basal lamina thus forming extremely complex structures [107, 195]. The dermatan sulfate proteoglycans, such as decorin, belong to the small leucine-rich repeat proteoglycan family (SLRRPs) [113, 138, 91]. Other examples are the proline arginine-rich end leucine-rich repeat protein (PRELP), chondroadherin (CHAD), and biglycan [195, 108, 18]. These molecules interact with fibrillar collagens and are thought to be involved in stabilizing the fibril interactions [226, 83, 192, 173].

Table 2: Some common proteoglycans.

Proteoglycan	Type	Location	Function	Ref
Aggregan	chondroitin and keratan sulfate	cartilage	mechanical support	[52]
Biglycan	chondroitin/dermatan sulfate	cell surface, matrix	binds TGF- β	[66, 152]
Decorin	chondroitin/dermatan sulfate	connective tissues	binds type I collagen and TGF- β	[114]
Perlecan	heparan sulfate	basal lamina	structural function	[156]

Collagens are the most abundant proteins in mammals, constituting approximately 25% of the total protein mass. The typical feature of collagen is its triple helical structure, with three collagen polypeptide chains (α -chains) wound around each other forming a superhelix [29, 57]. Collagens are rich in proline and glycine, both important for the

formation of the superhelix. Proline stabilizes the helical conformation in each α -chain, while glycine spaced at every third residue allows the three α -chains to be packed tightly together [73, 58]. The main types of collagens found in the connective tissues are types I, II, III, V and XI. These are called fibrillar collagens, upon secretion they assemble into collagen fibrils that often aggregate into larger cable-like structures known as collagen fibers [212, 73]. Collagen type IX and XII are fibril-associated and are thought to link collagen fibrils to each other and to other components of the ECM. Collagen types IV and VII are network-forming collagen, where type IV constitutes a major part in basal lamina, while type VII forms anchoring fibrils that helps attach the basal lamina to the underlying connective tissue [181].

Collagen synthesis starts with the production of precursor chains, called pro- α chains on membrane bound ribosomes, the pro- α are then relocated to the endoplasmic reticulum (ER) where each pro- α is combined with two others forming a hydrogen-bonded, triple-stranded, helical molecule called pro-collagen. The precursor also contains additional amino acids, pro-peptides, located at both their NH₂- and COOH-terminal end. The pro-collagen molecule is secreted from the cell, the pro-peptides are removed converting the pro-collagen into collagen that can then assemble into collagen fibrils. The pro-peptides are necessary to guide the intracellular formation of the triple-stranded molecule and to prevent self-assembly of collagen fibrils within the cell.

Table 3: Summary of some collagens and their distribution. References originate from Gelse *et al.* and Ricard-Blum *et al.* [73, 181]

	Type	Form	Distribution
Fibrillar	I	fibril	bone, skin, tendons, internal organs
	II	fibril	cartilage
	III	fibril	internal organs, skin, blood vessels
	V	fibril (with type I)	bone, skin, tendons, internal organs
	XI	fibril (with type II)	cartilage
Fibril-associated	IX	with type II fibrils	cartilage
	XII	with some type I fibrils	ligaments, tendons
Network-forming	IV	sheetlike network	basal lamina
	VII	anchoring fibrils	under stratified squamous epithelia
Basement membrane	VI	sheetlike network	lung, blood vessels

Bacterial adhesins that interact with ECM components such as collagen, fibronectin, fibrinogen and laminin-related polysaccharides have been identified in both Gram-negative

and Gram-positive bacteria [125, 193, 162]. For Gram-positive cocci, collagen binding proteins might be important factors in tissue tropism and organ-specific infection. For instance, protein Cpa of *S.pyogenes* has been shown to bind to collagen I and thus can have an important role in the bacterium's primary adhesion to its host [112]. Recently the M1 protein has been shown to bind collagen type VI (Paper I) and collagen type I (Paper II). M1 protein is also able to utilize GAGs as receptors to promote bacterial adhesion [72]. Binding of collagen type I by group G streptococci M-like protein FOG [155], as well as adhesion to basement membrane collagen type IV by group A streptococci M3 protein [51] has been shown. However, some strains of *S. pyogenes* cannot bind directly to collagen but they can gain access to these components via prebound fibronectin (FN) [50]. SfbI acts as a FN binding adhesin and thus represents a virulence factor for the bacteria's pathogenesis.

4.2 Host cells

All living organisms are composed of cells, some of a single cell while others are multicellular systems with groups of cells with specialized functions. During my thesis I worked with keratinocytes, human skin cells, and this chapter will give an overview of the human skin and how bacteria interacts with its cells. The largest skin is organ in the human body and its integrity is essential to maintain normal homeostasis [170]. The skin is made up of two distinct layers, the dermis and the epidermis, both attached to and separated by a sheet of ECM constituting the basement membrane [107, 216]. The dermis is located beneath the epidermis and mainly consists of connective tissue. It is mostly populated by ECM-producing fibroblasts, but also contains blood vessels, hair follicles and sweat glands [202]. The epidermis functions as a semi-permeable barrier that allows perspiration but hinders microorganisms from gaining entry into the body. It is tightly anchored via integrins to the basement membrane to withstand wear and tear from external environment and it consists of several layers of squamous epithelium (keratinocytes).

Bacterial proteins of *S. pyogenes*, such as the M protein, M-like proteins and streptococcal fibronectin-binding protein I (SfbI) are all capable of binding to human skin cells [3]. The M1 protein binds to the membrane cofactor protein (CD46) on keratinocytes via factor H-like repeats present in CD46 [47, 126, 60]. *S. pyogenes* is usually considered to be an extracellular pathogen, but its ability to invade epithelial cells has been known for some time [120]. The mechanism of hiding within cells has several benefits, it might help the bacterium to spread to deeper tissues, evade the immune response and treatment

with non-permeating antibiotics such as penicillin. There are two main mechanisms that bacteria utilize in order to allow cellular invasion. One is the use of secretion systems that trigger internalization by directly injected proteins, which in turn will induce membrane and cytoskeleton rearrangements. The second mechanism is the expression of surface proteins that functionally mimic natural ligands of host receptors.

The internalization of GAS into epithelial cells has been shown to be mediated by binding of fibronectin to the M1 protein or SfbI/PrtF1, as well as binding of plasminogen for serotypes other than the M1, resulting in integrin mediated uptake via $\alpha1\beta1$ - and $\alpha5\beta1$ -integrin [230, 198, 12]. The complex of M1 or SfbI/PrtF1 and fibrinogen induces clustering of integrins located on the cell surfaces, that in turn activates different signaling pathways. Activation of pathways such as phosphatidylinositol 3-kinases (PI3K), integrin-linked kinase (ILK), focal adhesion kinase and paxillin have been identified during M1-mediated internalization leading to an actin polymerization-based zipper like uptake [230, 171, 229]. *S. pyogenes* strains expressing SfbI can use caveolae as an entry point, a mechanism that differs from the zipper-like [182]. SfbI/PrtF1 are able to form complexes consisting of focal adhesion kinase, Src kinase, paxillin and Rho GTPases that results in the uptake of the bacterium [160]. PI3K activation resulting in induction of paxillin phosphorylation has recently been shown for both M1- and SfbI-mediated invasion [229], while Nerlich *et al.* showed that internalization of SfbI/PrtF1/M1 negative GAS strain of serotype M3 involves the Src family of protein-tyrosine kinase (PTK) [153].

5 The immune system

We are constantly exposed to a variety of microorganisms and as mentioned earlier, most of them do not cause any harm, they can even be beneficial to us. These different bacterial species are referred to as the normal flora. They can prevent colonization of pathogens by competing for space and nutrients, they produce substances that inhibit growth and even kill nonindigenous species as well as synthesize and excrete vitamins that the host can use as nutrients. However, pathogenic species have evolved mechanisms that allow them to colonize and invade the human host. In order to defend ourselves against invading pathogens we have evolved several protection systems. The human immune defense is composed of the innate immunity, that depends on physical barriers and pattern recognition, and the adaptive immunity, that recognizes specific antigens. This chapter will give a brief introduction to our immune system.

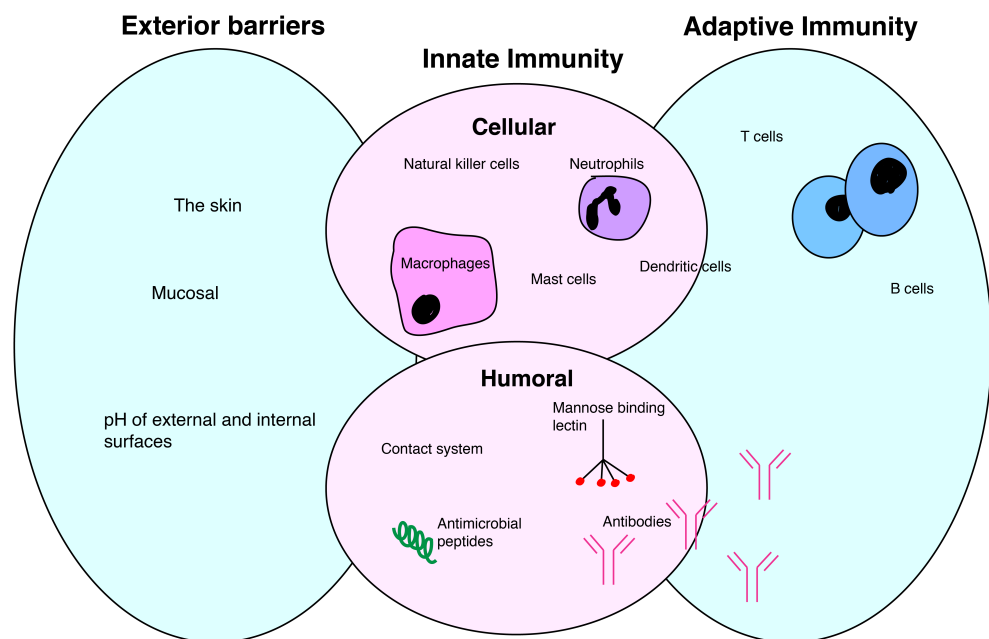


Figure 5: Simplified summary of the human immune system

5.1 Innate immunity

Innate immunity is the first line of defense when we encounter a microbe and is represented by different blood cells, antimicrobial peptides (AMPs), as well as contact and complement system. Skin and the mucosal linings of bodily cavities are composed of a number of different cell types, keratinocytes, dendritic cells and macrophages, that aid the defense by producing AMPs and cytokines. The ionicity and pH of body fluids, such as sweat and saliva, exhibit bacteriostatic properties. At the moment there are two theories explaining the activation of the innate immunity. One is the danger theory, where host cells that are injured release signals that activate antigen-presenting cells [135] and the other is the pattern-recognition theory where microbial particles induce the response. Pattern-recognition receptors (PRRs) are present on a variety of cells including epithelial cells, dendritic cells (DCs), neutrophils, monocytes/macrophages and natural killer (NK) cells [93]. PRRs recognize microbial conserved structures called pathogen-associated molecular patterns (PAMPs). PAMPs are essential for microbial survival and pathogenicity and some common PAMPs for different classes of pathogens are LPS, PG and LTA. The best characterized PRRs in mammalian species are the Toll-like receptors (TLRs), they are evolutionary conserved and have affinity for several types of PAMPs [141]. Pathogens that are not recognized by TLRs can be recognized by the members of the cytosolic recognition family NOD (nucleotide-binding oligomerization domain), such as NOD1 or NOD2 [104]. Once a pathogen has been identified, the humoral and cellular innate defenses target the pathogen and tries to eliminate it. The cellular immunity involves various cells of the innate immune system, while the humoral immunity involves secreted products such as AMPs, secreted proteins (lactoferrin and lysozyme) and the complement system.

5.1.1 The cellular innate immunity

Leukocytes (white blood cells) patrol our bodies looking for injured cells and invading organisms. Leukocytes of the innate immunity include: phagocytes such as dendritic cells, macrophages and neutrophils, and non-phagocytic cells such as mast cells, basophils, eosinophils and NK cells. These cells are specialized to deal with different pathogens at different stages of infection. This section will describe some of the cells involved in innate immunity.

Macrophages - Mature macrophages differentiate from circulating monocytes and they are present in peripheral tissues where they can encounter pathogens during the early

stages of infections. They can employ a wide array of antimicrobial mechanism when encountering a pathogen. These mechanisms include phagocytosis, induction of reactive oxygen and nitrogen species as well as AMPs. In addition they will also release several inflammatory mediators, such as TNF- α , IL-1 and IL-6, when they interact with pathogens [223, 36, 37].

Mast cells - Mast cells reside in connective and mucosal tissues and upon encountering pathogens they will release inflammatory mediators, such as TNF- α and histamine, that are responsible for recruiting neutrophils to the infection site [223, 36, 37].

Neutrophils - Neutrophils are recruited from circulation upon infection by cytokines and chemokines produced by cells resident in tissues. They phagocytose and kill pathogens using a variety of mechanisms. Neutrophils contain several types of granules, including azurophilic (also known as primary granules) granules filled with antimicrobial proteins, peptides and proteolytic enzymes [98, 242], the specific (or secondary) granules that also contain antimicrobial components, but are also involved in activation of oxidative burst [26] and the gelatinase (or tertiary) granules.

Dendritic cells - The immature DCs reside in peripheral tissues and express a number of PRRs, such as TLRs and phagocytic receptors [223, 36, 37]. Upon encountering a pathogen, several antimicrobial mechanisms will be induced, such as the production of nitric oxide. They also have a role in initiation of the adaptive immunity [93].

5.1.2 The humoral innate immunity

PRRs mediated recognition of pathogens result in a production of cytokines and chemokines. The production of chemokines is regulated by cytokines in an antagonistic or synergistic way, that directs the type of immune response. The recruited cells of the innate immunity amplify the production of cytokines and chemokines, resulting in sustained immune response that ultimately results in recruitment of cells belonging to the adaptive immunity [38, 77, 132].

Interferons (IFNs) - IFNs act as major regulators of the innate immune system that are produced due to a variety of stimuli. The type I IFNs (IFN- α , IFN- β) are expressed by most cell types after exposure to viruses, double-stranded viral RNA and PAMPs [103, 122, 199]. The only type II IFN is IFN- γ , which is produced mainly by NK cells

as a response to cytokines [122]. IFN- γ induces macrophages to produce proinflammatory cytokines such as TNF- α and IL-2, it induces production of APs as well as reactive oxygen and nitrogen species in phagocytes [20, 191].

IL-1 β and TNF- α - They are mainly produced by T helper (Th) cells and monocytes/macrophages [77, 207, 225], but other cells such as keratinocytes and DCs are able to produce IL-1 β and TNF- α [74, 115]. The production of IL-1 β and TNF- α result in activation of NF- κ B and an increased transcription of genes that mediate the innate immune response [123, 207].

Chemokines - They are a family of cytokines with chemotactic properties that regulate trafficking of leukocytes [8, 20, 131], but can also have direct antimicrobial activity [245]. They are divided into four families, due to the arrangement of their conserved N-terminal cysteine motifs, C, CC, CXC and CX3C (X is a non-conserved amino acid) [7, 6, 8, 131, 39]. Binding of a chemokine to its receptor leads to cellular activation, an increase in cellular calcium that causes a cellular response [131, 146]. The chemokine MIG (CXCL9) recruits NK cells and T cells and has been shown to exhibit antibacterial activity in a GAS infection pharyngitis model [55]. IL-8 (CXCL8) exhibits chemotactic and activating effect on neutrophils and has a positive effect during wound healing [145, 180].

Antibacterial peptides (APs) - The characteristics of APs are the combined cationic and hydrophobic properties [165] and their main target is the negatively charged bacterial membrane. The interaction of APs with the bacterial membrane result in a pore formation within the membrane, thus disrupting the osmotic balance and increased pressure that results in the lysis of the bacterium [24, 165]. The best characterized APs are the cathelicidins, such as LL-37 (the only human cathelicidin) and defensins (α , β). The production and secretion of LL-37 and tissue specific β -defensin is performed by epithelial cells [24], but other cell types such as platelets, monocytes and NK cells can produce APs.

5.1.3 The complement system

The complement system includes more than 30 plasma proteins and membrane-associated proteins, it is self amplifying, highly regulated and its activation leads to immune complex formation and bacterial opsonisation. The complement system can be activated in

three ways: (i) the classical, (ii) the alternative and (iii) the lectin pathway.

The classical pathway - This pathway is initiated by the interaction between the C1 complex (the complex is composed of C1q, C1r and C1s) and IgM or IgG [82]. Binding of C1q to Igs leads to conformational changes, the cleavage of C1s by C1r results in an enzymatically active C1 complex. This complex proceeds to cleave C4 and C2 resulting in the formation of C3 convertase (C4b2a) that cleaves C3 into C3a and C3b. C3b receptors on phagocytes render the phagocytes to clear immune complexes or to phagocytose bacteria that are coated with C3b. C3b can also associate with C3 convertase forming the C5 convertase (C4b2a3b), that after processing and associating with C5-9 will form the membrane attack complex (MAC). MAC causes lysis of primarily Gram-negative bacteria.

The alternative pathway - This pathway is antibody independent and starts via spontaneous activation of plasma C3 to C3(H₂O), factor B will bind C3(H₂O) and this complex will be cleaved by factor D. Cleavage by factor D results in the release of C3Ba, but a part of factor B (Bb) will remain in the complex. The Bb part of the complex will cleave C3 to C3a and C3b, C3b might associate with Bp forming C3bBb, the C3 convertase of the alternative pathway.

The lectin pathway - This pathway is initiated when the mannose binding lectin (MBL), that recognizes bacterial carbohydrates, binds to bacterial surfaces. Following association with the bacterial carbohydrates, the MBL and MBL-associated serine protease complex is formed.

This complex activates C4 and the downstream effects that follow are identical to the ones in the classical pathway.

5.1.4 The contact system

The human contact system involves three serine proteinases: (i) coagulation factor XI and XII, (ii) plasma kallikrein and (iii) high molecular weight kininogen (HK) [17, 43]. Activation of the contact system is a consequence of these molecule's interaction with negatively charged surfaces and results in the activation of the intrinsic pathway of coagulation, the release of bradykinin that is a potent pro-inflammatory peptide, and the production of APs.

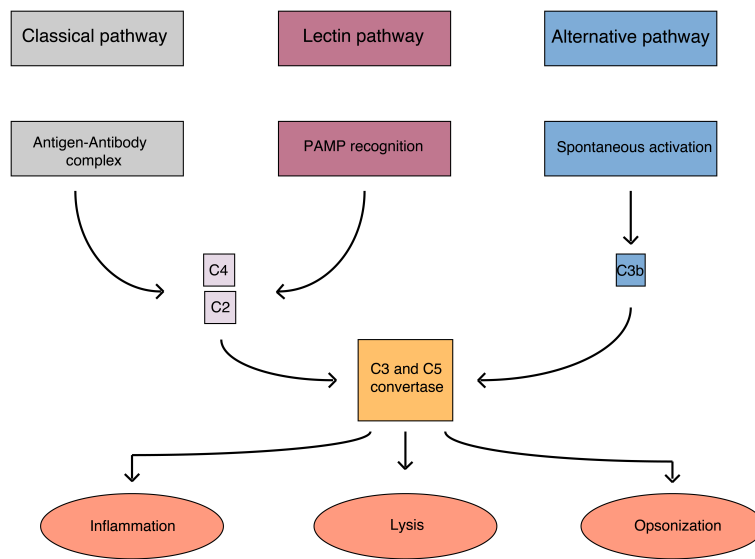


Figure 6: Simplified overview of the complement system

Cleavage of HK by activated plasma kallikrein generates bradykinin that is involved in fever induction, increased vascular permeability and capillary leakage [157]. In *S. pyogenes* infection HK is bound to the M protein on the bacterial surface, it can be cleaved by SpeB resulting in the release of bradykinin as well as APs [86, 71]. In spite of the risk for the bacterium to cleave HK, it might also be a source to gain nutrients from plasma.

5.2 Adaptive immunity

An adequate response to invading pathogens requires action from both the innate and the adaptive immunity. The switch from innate to adaptive immunity is to a certain extent dependent on cytokines and chemokines derived from epithelial cells. DCs activate naïve $CD4^+$ T cells that can differentiate into various effector cells, such as Th1, Th2 and regulatory T cells. The cell's development is directed by specific cytokines, resulting in mature Th cells that in turn produce cytokines that direct the immune responses mediated by effector cells, such as B cells and cytotoxic T cells.

5.2.1 The cellular adaptive immunity

The cellular adaptive immunity is mediated by T cells. The T cell activation is driven by antigen processing, and the peptide fragment presentation by major histocompatibility complex (MHC) class I or II. MHC class I is expressed in all nucleated cells, while MHC class II is mainly expressed by antigen presenting cells [5]. The T cell receptor (TCR) binds to the antigen-MHC complex, with co-stimulatory signals such as CD80, CD86 and CD28 that are present on the T cell, resulting in T cell activation [75]. The T cells are subgrouped into CD4⁺ and CD8⁺ T cells. CD4⁺ cells recognize peptides that are presented by MHC class II, and upon activation they give either a Th1 or a Th2 response. The Th1 response is characterized by IL-2 and IFN- γ secretion, leading to enhanced phagocytosis, while Th2 response releases cytokines such as IL-4, IL-5, IL-10 and IL-13 that promote B cell activation resulting in antibody production [204]. CD8⁺ cells, also referred to as cytotoxic T cells, recognize peptides presented on MHC class I. Cells infected with viruses or bacteria, as well as tumor cells, will present foreign peptide to the cytotoxic T cell that will induce apoptosis of the infected cell [243].

5.2.2 The humoral adaptive immunity

The humoral adaptive immunity is mediated by immunoglobulins (Igs) that are produced as a response to antigens (microbial components) present on the surface of phagocytes that have engulfed and destroyed the invading microbe. Igs are produced by cells of the adaptive immunity, the circulating B lymphocytes. The interaction between the antigen and B lymphocyte results in B cell maturation into a plasma cell. The plasma cells will then produce and secrete the Igs that will target the antigen the B cell was introduced to. B cell receptor heterogeneity is increased after activation through somatic hypermutation and class switch recombination. Somatic hypermutations, point mutations in the antigen-binding part of the Ig molecule, give rise to high-affinity antibodies. Class switch recombination, achieved by DNA deletions, refer to the change of the antibody classes from IgM or IgD on naïve B cells, to IgG, IgE or IgA on activated cells without altering the antigen specificity [134]. The role of Igs is to recognize and aid the destruction of microbes via interaction with Fc-receptors on phagocytic cells or by initiating the classical pathway of the complement system. There are five classes of Igs: IgA, IgE, IgD, IgM and IgG [5], that have slightly different functions and locations in the human body.

6 Present investigation

6.1 Paper I

As previously mentioned, collagens are one of the most abundant proteins in the human body and has been reported as adhesive substrates for a number of pathogenic bacteria and clinical isolates [244]. Pathogenic bacteria can overcome the subepithelial barriers (i.e. the basement membrane) and cause deep tissue infections. In this paper we investigated whether collagen type VI in upper and lower airways might be a target for bacterial adhesion. Immunohistochemistry on paraffin sections using a FITC-labeled collagen VI antibody visualized the location of collagen type VI in murine pharynx and bronchus in the subepithelial lamina propria. The same observation was made using electron microscopy (EM). Collagen type VI was distributed close to collagen type I fibrils distant to the epithelial basement membrane. In order to investigate the adhesion capacity of bacterial proteins to murine pharynx and bronchus, cyanogen bromide (CNBr) extracted surface proteins from *S. pyogenes* and *S. pneumoniae* were conjugated with colloidal gold, incubated with paraffin sections and visualized using EM. The bacterial proteins were frequently observed to be co-localized with collagen type VI. In order to identify airway matrix components serving as substrates for *S. pyogenes* and *S. pneumoniae*, we performed ligand blot experiments. Pharyngeal and bronchial samples were extracted with SDS, separated by SDS-PAGE, transferred to a PVDF membrane and incubated with CNBr radio-labeled protein extracts from *S. pyogenes* and *S. pneumoniae*. The extract bound to a few distinct bands with the molecular mass of approximately 130 and 140 kDa. Comparison with the Coomassie stained collagen VI and I standards revealed that these bands exhibited a similar migration pattern as the $\alpha 2$ -chain of collagen type VI and the $\alpha 1$ - and $\alpha 2$ -chains of collagen type I.

To assess a relative value of collagen binding to streptococci we performed binding assays. Collagen type VI fibrils were purified from bovine cornea and radio-labeled with 125 Iodine. A fixed amount of collagen was added to bacterial suspensions, incubated, centrifuged and the relative amount of radioactivity bound to the bacterial pellet was determined. Both pathogens strongly adsorbed collagen type VI and turned out to be rather similar to the classical fibrillar collagen type I. The basement membrane collagen type IV showed much weaker binding. These data establishes collagen type VI as a relevant target for streptococcal adhesion in the extracellular matrix in the upper and lower airways. We further investigated the *in vitro* binding of collagen type VI to the bacterial surface

using negative staining and transmission EM. This procedure has the advantage that no protein modification is required. We incubated collagen type VI fibrils with bacterial suspensions and could observe that the bacterial surfaces were covered with collagen type VI. Co-localization experiments using gold-conjugated antibodies against collagen VI and the M1 protein revealed the M1 protein as a novel collagen type VI adhesin, this was confirmed using the M-like protein H where no binding was observed. In order to map the binding site in the collagen type VI fibril, CNBr protein extracts were labeled with colloidal gold particles and added to collagen type VI. Visualization using EM revealed that gold conjugates of both bacterial extracts bound close to the globular domains on collagen type VI fibrils. Similar interactions were observed for gold-labeled M1 protein, but not for protein H, thus confirming the novel role of M1 as a specific adhesin for collagen type VI.

6.2 Paper II

Proteoglycans of the SLRRP family associate with collagens forming complex structures that constitute the ECM. The Slr protein of *S. pyogenes* has been previously reported to be a putative LRR containing lipoprotein by Reid *et al.* and Waldemarsson *et al.* [179, 227]. One might hypothesize that due to the LRR domain, Slr might be involved in the binding to collagens. We have previously shown that the M1 protein is able to bind to collagen type VI (Paper I) and in this paper we investigated whether Slr and the M1 protein are able to bind collagen type I. The lipoproteins from Gram-positive bacteria are not as well studied as cell wall-anchored proteins, but several recent studies have suggested that lipoproteins also are important for immune evasion and adherence during colonization and infection. The lipoproteins of *S. pyogenes* have been linked to metal acquisition, most of them belong to ABC transport systems and some have been shown to be virulence factors in animal models of infection [95, 247, 201, 234]. LRR proteins from *Listeria* species are virulence factors that mediate adhesion and cellular invasion through protein-protein interactions [19]. It is of interest to investigate if the streptococcal LRR lipoprotein Slr might be involved in host-pathogen interactions.

The *slr* gene encoding Slr could be found in all 32 strains of different M serotypes. We were able to show that Slr and the M1 protein expression on the bacterial surface reached their peaks at different time points during the logarithmic growth phase, but during a substantial amount of time in late logarithmic growth phase, both proteins were present at the bacterial surface. We investigated the characteristics of Slr and were

able to confirm that Slr is a LRR containing lipoprotein with similarity to InlA from *L. monocytogenes*. Binding experiments showed that Slr binds to collagen type I in the native and denatured form *in vitro* and that the interaction takes place in the vicinity of the COOH- and NH₂-terminal ends as well as approximately 70 and 100 nm further in on the monomeric collagen type I. We constructed a Slr mutant strain using a single crossover mutagenesis approach and performed a series of binding experiments using the wild type strain AP1, our Slr mutant MB1, and a M1 protein mutant strain MC25. The experiments showed that elimination of Slr or the M1 protein from the bacterial surface did not have a major effect on the strains' total capacity to bind collagen type I. A similar binding pattern of Slr and M1 towards collagen could also be observed in direct binding assays, leading to the conclusion that both proteins can use collagen type I as a ligand. Protein localization experiments using gold-conjugated antibodies revealed no inhibition in binding of Slr antibodies to Slr by the M1 protein on the bacterial surface as previously described for the M6 protein [227]. Under the conditions studied here, the gold-conjugated anti-M1 and anti-Slr antibodies were able to bind simultaneously to the bacterial surface, leading us to the conclusion that the M1 protein and Slr are simultaneously accessible for protein-protein interactions on the bacterial surface. The elimination of either the M1 protein or Slr might reveal otherwise covered binding sites for collagen type I on the remaining protein in accordance with the camouflaging described by Waldemarsson et al. [227]. This might explain why there was not a major reduction in total collagen I binding capacity of the Slr and M1 mutants compared to the wild type strain.

The presence of and possible implications for the host-pathogen interactions of the four histidine triad motifs in the N-terminal part of Slr were not approached during this study, but such motifs have been identified in four proteins of *S. pneumoniae* and might be involved in metal or nucleoside binding [1] and have been shown to alter complement deposition of the bacterial surface [142]. Therefore, enzymatic or other functions than collagen binding of Slr, cannot be excluded.

6.3 Paper III

In paper II we were able to show that Slr is similar to InlA from *L. monocytogenes* and can utilize collagen type I as adhesin that might be of importance during colonization and infection [23]. InlA is a LRR protein that aids in the uptake of *L. monocytogenes* into non-phagocytic cells via E-cadherin and Met [25]. Due to the similarities between

Slr and InlA it can be hypothesized that Slr might exhibit properties involved in the adhesion and uptake into non-phagocytic cells. Previous studies have shown that adhesion and uptake of streptococci is mediated via CD46 for the M1 serotype [60, 126] or through an integrin associated mechanism in several GAS serotypes [230]. In order to investigate the binding and internalization capacity of Slr we used human keratinocytes (HaCaT). These cells originate from normal adult skin, but are immortalized and highly differentiated due to spontaneous transformation *in vitro* [27]. Using flow cytometry and fluorescence microscopy techniques we were able to show that Slr binds to and is internalized into HaCaT cells. MASCOT MS/MS analysis identified the Slr cellular ligand as non-muscle myosin IIA. The presence of, and interaction with Slr, non-muscle myosin IIA in HaCaT cells was further confirmed using Western blot.

Myosins belong to the family of molecular motors proteins involved in processes such as cell motility and cell repair. However, non-conventional myosin VIIa has been shown to be involved in InlA mediated internalization. Myosin VIIa together with transmembrane protein vezatin are recruited at the bacterial site of entry and inhibition of either protein severely impaired the internalization of *L. monocytogenes* [203]. A myosin II mediated mechanism that contributes to invasion of *Salmonella typhimurium* into non-phagocytic cells has been recently described by Hänich et al. [80]. This mechanism seems to be dependent on RhoA activation, that acts as a myosin II contractility activator [44] and the regulation of myosin II via Rho kinase has been proposed to control the position of *Salmonella* containing vacuole within macrophages [231]. The downstream effects of Slr's entry into keratinocytes have to be clarified in order to fully understand the implications of this mechanism. It can be hypothesized that Slr is involved in the activation of myosin IIA, perhaps facilitating the uptake of *S. pyogenes* into keratinocytes.

6.4 Conclusions

- *S. pyogenes* and *S. pneumoniae* can bind to collagen type VI in murine pharynx and bronchi.
- For *S. pyogenes* the binding of collagen type VI is mediated by the M1 protein.
- The Slr protein is a LRR containing lipoprotein.
- The Slr and M1 protein of *S. pyogenes* are able to utilize collagen type I as an extracellular matrix ligand.
- The Slr protein is able to adhere to and internalize into human keratinocytes, and this interaction is mediated by binding to non-muscle myosin IIA.

6.5 Future perspectives

During my thesis work I have characterized a novel adhesin of *S. pyogenes*, Slr, as a LRR containing lipoprotein. Slr along with the M1 protein can utilize collagens as adhesive targets during the infection process. Slr and M1 are able to bind to collagen type I, and M1 also binds to collagen type VI, a process that might play a role in the primary step of infection in the skin and respiratory tract. Furthermore, Slr binds to non-muscle myosin IIA, which could result in the bacterial adherence to and internalization into human keratinocytes. This could contribute to *S. pyogenes* avoidance of the immune system and/or bacterial dissemination into deeper tissues.

So, where do we go from here? There are many directions future projects can take, but here are some that I would address first:

- With recombinantly expressed fragments of Slr and the M1 protein and collagen fragments, we would be able to determine where the binding sites are located.

- A double mutant strain (with no Slr and M1 expression) in binding experiments would exclude or reveal interactions between collagen type I/VI with other surface proteins of *S. pyogenes*.
- Elucidate the down stream effects, such as stress fiber formation or other effector mechanisms, of Slr's binding to non-muscle myosin IIA in keratinocytes.
- A mouse model of infection with wild type and mutant strains might share more light on Slr as a putative virulence factor and give a an *in vivo* perspective.

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Appendix

Paper I

Paper II

Paper III

Molecular and microscopical analysis of pathogenic streptococci

Association to specific host tissue structures allows pathogenic bacteria to establish an infection and facilitates the spread within its host. Interactions between bacterial surface structures and human proteins might determine the outcome of the infection. *Streptococcus pyogenes* (*S. pyogenes*), is a human pathogen mostly causing localized infections of the skin and respiratory tract, but it is also capable of causing severe invasive disease such as necrotizing fasciitis, sepsis and toxic shock. Until recently, *S. pyogenes* has been considered as a strictly extracellular pathogen, but several studies has shown that it is capable of invading and surviving intracellularly in several human cell types. In this thesis I have investigated the interaction of *S. pyogenes* and *Streptococcus pneumoniae* (*S. pneumoniae*) with collagen type VI, the binding of *S. pyogenes* M1 protein and leucine rich (Slr) protein to collagen type I, and Slr's interaction with human keratinocytes. The adherence of *S. pyogenes* and *S. pneumoniae* to collagen type VI in murine upper and lower airways was restricted to the proximity of the NH₂ - and COOH-terminal globular domains of collagen type VI, and for *S. pyogenes* this interaction was mediated by the M1 protein. The M1 protein and Slr are co-expressed on the bacterial surface and both bind to collagen type I with high affinity. Slr exhibited multiple binding sites to collagen I, both to the monomeric and the fibrillar structure, with the most binding concentrated to the overlap region of the collagen I fibril. Slr is able to adhere to and internalize into human keratinocytes (HaCaT) cells in a time dependent manner and we were able to identify non-muscle myosin IIA as a potential cellular ligand for Slr. We could further confirm the presence of non-muscle myosin IIA in HaCaT cell lysate and that Slr binds to non-muscle myosin IIA. In summary, we have characterized a novel adhesin of *S. pyogenes*, Slr, as a LRR containing lipoprotein that, in concert with the M1 protein, might utilize collagens as adhesive targets during the infection process. Slr and M1 bind to collagen type I, and M1 also binds to collagen type VI, a process that might play a role in the primary step of infection in the skin and respiratory tract. Furthermore, Slr binds to non-muscle myosin IIA possibly resulting in the bacterial adherence to and internalization into human keratinocytes. This could contribute to *S. pyogenes* avoidance of the immune system and/or bacterial dissemination into deeper tissues.



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