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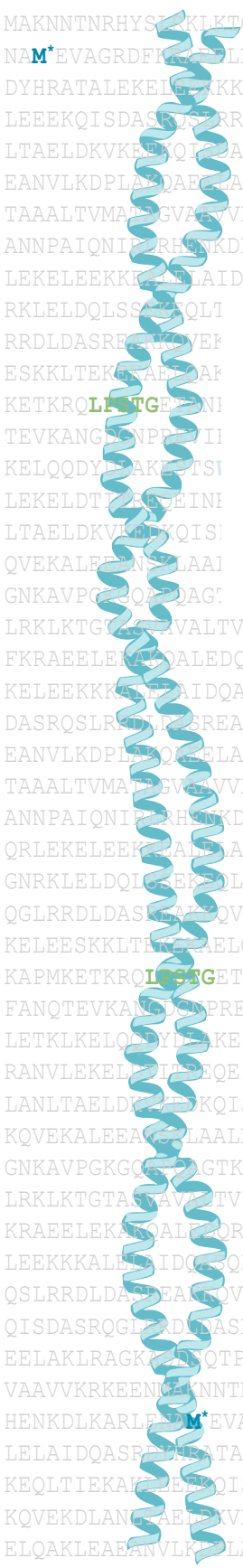

Bacterial virulence or exorbitant host response?

On innate immunity against the streptococcal M1 protein

SANDRA PERSSON
DEPARTMENT OF CLINICAL SCIENCES | LUND UNIVERSITY

Bacterial virulence
or exorbitant host response?

Bacterial virulence or exorbitant host response?

On innate immunity against the streptococcal M1 protein

Sandra Persson

LUND
UNIVERSITY

DOCTORAL DISSERTATION

by due permission of the Faculty of Medicine, Lund University, Sweden.
To be defended on October 18th 2019 at 9 AM in Belfragesalen, Biomedical Center, Lund, Sweden.

Faculty opponent
Professor Dr. Shiranee Sriskandan
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Abstract

Bacterial infection is inevitable throughout a lifetime. What differs between mild and serious infections is the pathogen responsible and how the host immune system responds to it. A fundamental role to prevent infection is a proper physical barrier such as the skin and mucosa. However, sometimes abrasions or injuries are still introduced, being potential ports of entry for pathogenic bacteria. An appropriate activation of innate immunity is necessary for bacterial elimination and wound healing. Special precautions are needed to prevent excessive host response that can result in severe tissue injury and aggravate the state of infection. This thesis has focused on studying the initial reactivity towards the streptococcal virulence factor M1 protein, and the consequent innate host response *in vitro*, modelling a streptococcal skin infection. The first part of the thesis describes the vigilant activity of keratinocytes triggering PAMP-signaling when encountering the streptococcal virulence factor. This signaling pathway resulted in cytokine release, especially IL-8, enabling recruitment and activation of leukocytes. We continued by studying the delicate balance between bacterial virulence and host response to avoid excessive inflammation. The bacterial protein was not cytotoxic, however, it completely abolished any wound-healing abilities of keratinocytes *in vitro*. We concluded that M1 protein can aggravate the state of streptococcal skin infection, and that it is rather a bacterial virulence mechanism than due to an exorbitant host response. Moreover, we decided to investigate if CAP, a partly ionized gas with antiseptic abilities, can also modulate the inflammatory activities triggered by the streptococcal M1 protein. For the first time, we have shown that CAP is able to modulate a specific bacterial virulence factor and abolish its detrimental activities in favor of the host. Our results also show that CAP exert its antivirulence and antimicrobial functions without disturbing fundamental innate immune responses. The final part of this thesis identifies new virulence factors of Leptospira responsible for triggering massive HBP release from neutrophils. Elevated HBP levels were found in serum samples from patients suffering from Leptospirosis and could potentially be a diagnostic biomarker for the disease. In conclusion, this thesis explores new mechanisms and host responses to combat bacterial virulence important for the pathology of infectious diseases.

Key words: Innate immunity, infection, bacterial virulence factors, cold atmospheric plasma, kINPen MED, streptococcal M1 protein, HBP

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Lagom,

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Table of Content

List of papers

Paper I — Vigilant keratinocytes trigger pathogen-associated molecular pattern signaling in response to streptococcal M1 protein.
Sandra T. Persson, Laura Wilk, Matthias Mörgelin, Heiko Herwald. *Infection and Immunity*. 2015 Dec;83(12):4673-81. Doi:10.1128/IAI.00887-15.

Paper II — Leucocyte recruitment and molecular fortification of keratinocytes triggered by streptococcal M1 protein.
Sandra T. Persson, Simon Hauri, Johan Malmström, Heiko Herwald. *Cellular Microbiology*. 2018 Jan;20(1). Doi:10.1111/cmi.12792.

Paper III — Cold atmospheric plasma disarms M1 protein, an important streptococcal virulence factor.
Sandra T. Persson, Simon Ekström, Praveen Papareddy, Heiko Herwald. *Journal of Innate Immunity,* 2019 Sep. Doi:10.1159/000502959.

Paper IV — Heparin-binding protein release is strongly induced by leptospira species and is a candidate for an early diagnostic marker of human leptospirosis.
Mônica L. Vieira, **Sandra Persson**, Mônica Lopes-Ferreira, Eliete C. Romero, Karin Kirchgatter, Ana Lucia T. O. Nascimento, Heiko Herwald. *The Journal of Infectious Diseases*. 2019 Feb 23;219(6):996-1006. Doi:10.1093/infdis/jiy589.

Abbreviations

AMPs	antimicrobial peptides
ATP	adenosine triphosphate
CAP	cold atmospheric plasma
DNA	deoxyribonucleic acid
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
FGF	fibroblast growth factor
HDX	hydrogen deuterium exchange
HBP	heparin-binding protein
HMGB1	high mobility group box-1
HSPs	heat shock proteins
IGF	insulin growth factor
IgG	immunoglobulin G
IL	interleukin
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
LTA	lipotechoic acid
MAPK	mitogen-activated protein kinase
NETs	neutrophil extracellular traps
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
PAMP	pathogen associated molecular pattern
PMNs	polymorphonuclear neutrophils
PRRs	pattern recognition receptors
SSIs	surgical site infections
TLR	Toll-like receptor
TGF	Transforming growth factor
TNF	tumor necrosis factor

Abstract

Bacterial infection is inevitable throughout a lifetime. What differs between mild and serious infections is the pathogen responsible and how the host immune system responds to it. A fundamental role to prevent infection is a proper physical barrier such as the skin and mucosa. However, sometimes abrasions or injuries are still introduced, being potential ports of entry for pathogenic bacteria. An appropriate activation of innate immunity is necessary for bacterial elimination and wound healing. Special precautions are needed to prevent excessive host response that can result in severe tissue injury and aggravate the state of infection. This thesis has focused on studying the initial reactivity towards the streptococcal virulence factor M1 protein, and the consequent innate host response *in vitro*, modelling a streptococcal skin infection. The first part of the thesis describes the vigilant activity of keratinocytes triggering PAMP-signaling when encountering the streptococcal virulence factor. This signaling pathway resulted in cytokine release, especially IL-8, enabling recruitment and activation of leukocytes. We continued by studying the delicate balance between bacterial virulence and host response to avoid excessive inflammation. The bacterial protein was not cytotoxic, however, it completely abolished any wound-healing abilities of keratinocytes *in vitro*. We concluded that M1 protein can aggravate the state of streptococcal skin infection, and that it is rather a bacterial virulence mechanism than due to an exorbitant host response. Moreover, we decided to investigate if CAP, a partly ionized gas with antiseptic abilities, can also modulate the inflammatory activities triggered by the streptococcal M1 protein. For the first time, we have shown that CAP is able to modulate a specific bacterial virulence factor and abolish its detrimental activities in favor of the host. Our results also show that CAP exert its antivirulence and antimicrobial functions without disturbing fundamental innate immune responses. The final part of this thesis identifies new virulence factors of Leptospira responsible for triggering massive HBP release from neutrophils. Elevated HBP levels were found in serum samples from patients suffering from Leptospirosis and could potentially be a diagnostic biomarker for the disease. In conclusion, this thesis explores new mechanisms and host responses to combat bacterial virulence important for the pathology of infectious diseases.

Introduction

The skin – a protective barrier

The three layers of the skin and their resident cells

The skin is the primary interface between the body and the environment. It is an incredibly complex organ with the main functions of protection, thermoregulation and sensation (1). It provides a first line of defense against microbial pathogens and physical and chemical insults (2). The skin is made up of three layers, the epidermis, dermis, and the hypodermis, with significant differences in anatomy and function (3, 4).

The epidermis

The epidermis is the most outer layer of the skin, being in contact with the environment. It is responsible for skin color, texture, and moisture (5). Keratinocytes are the predominant cell type of this layer and are accountable for the formation of the epidermal water barrier by making and secreting lipids (6). Keratinocytes also participate in the formation of vitamin D by ultraviolet (UV) light-dependent activation of cholesterol precursors (7). However, their main function is to maintain the physical barrier by developing into the stratum corneum (8).

The epidermis is divided into several layers including the stratum corneum (the most superficial portion of the epidermis), stratum lucidum (present in hairless skin of palms and soles of the feet), stratum granulosum, stratum spinosum, and stratum basale (the deepest portion of the epidermis) (5, 9). *Stratum corneum*, 20-30 cell layers, the uppermost layer, is made up of keratin and dead keratinocytes (corneocytes). *Stratum granulosum*, 3-5 cell layers, contains cells with keratohyalin granules and lamellar granules that function as a glue, keeping cells stuck together. *Stratum spinosum*, 8-10 cell layers, contains cells with cytoplasmic processes, that extend outward and contact neighboring cells by desmosomes. Keratinization begins in this layer and dendritic cells (Langerhans cells), as well as some T cells, can also be found. *Stratum basale*, 1-3 cell layers, is the deepest layer separated from the dermis by the basement membrane (basal lamina) and attached to it by hemidesmosomes. The cells found in this layer are mitotically active stem cells producing keratinocytes, melanin producing melanocytes and Merkel cells, tactile cells with mechanoreceptors for light touch sensations (5, 9).

The dermis and hypodermis

The dermis is located under the basement membrane, a thin layer of extracellular matrix (ECM) proteins, and consists of two layers with distinct fibroblast cells and provides most of the mechanical and elastic strength to the skin (3). The papillary layer is the upper layer composed of loose connective tissue and contacts epidermis. The reticular layer is the deeper layer, which is thicker, less cellular, and consists of dense connective tissue and bundles of collagen fibers (3, 5). The sweat glands, hair, hair follicles, muscles, sensory neurons, and blood vessels are also located in the dermis (10). Below the dermis comes the subcutaneous fat called hypodermis, sometimes also referred to as subcutaneous fascia. It is the deepest layer of skin and mostly contain adipose lobules along with some hair follicles, sensory neurons, and blood vessels. The major cell types of this layer are fibroblasts, adipocytes and macrophages (3).

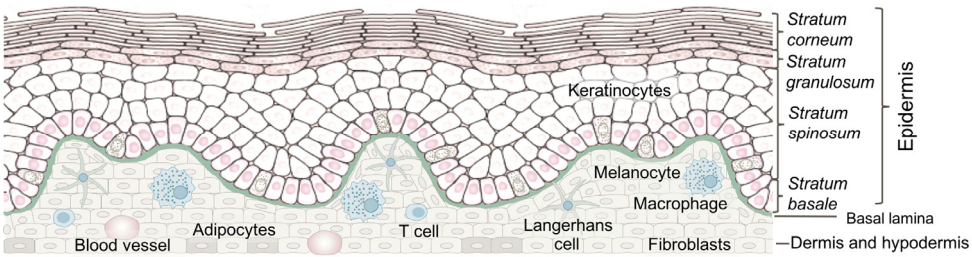

Figure 1. The different layers and resident cells of the human skin.

The functional barrier

Maintaining skin integrity is of paramount importance for host survival. The epidermis provides most of the protection from the environment, as the dermis is very permeable once the epidermis is removed (10). The mechanisms by which it is able to do this include the production of antimicrobial peptides (AMPs), resident epidermal Langerhans cells, and transient epidermal T-cells (8). Adherence between neighboring keratinocytes is maintained by tight junction complexes, especially in stratum granulosum, which also form an important intracellular and protective barrier (11). In addition, the dryness of the outer layer of the epidermis and the continuous shedding of keratinocytes assist in preventing any sustained growth of organisms on the skin (2).

Resting keratinocytes produce baseline levels of mediators and immune-competent surface receptors which are necessary to maintain endogenous signaling during skin homeostasis. Whenever needed, keratinocytes are activated and expression levels as well as signaling pathways are switched from maintenance to a regulatory state, recruiting immune cells and releasing AMPs (12, 13). Activation occurs either through a receptor-mediated signaling to various stimuli such as microbial proteins, endogenous molecules

or cytokines from other cells, or as a reaction to direct keratinocyte damage by UV light, mechanical or chemical irritation or other events impairing skin barrier function (14).

Healing a wound

The immune system

If the physical barrier is disrupted by abrasions or injuries it makes a potential port of entry for pathogenic bacteria. With an invading pathogen, the immune system is activated to eliminate any infection (15). The immune system can be subdivided into two complex branches, the innate and the adaptive immune system. The innate immune system gives an immediate and broad range host response, while the adaptive branch takes time, several days to weeks, to produce and gives highly specific immunity. Together they form the human defense against infectious diseases and preserve body homeostasis (16). Primitive organisms such as invertebrates and plants do not have an adaptive system and are therefore completely dependent on the defense mechanisms of the innate immunity, highlighting the effectiveness of this primary system (17, 18).

The innate immunity

The innate immune system is characterized by its instant and direct response to any invading pathogen. This system is evolutionary ancient and exists in all living organisms. It has evolved a multitude of strategies to combat microbial pathogens and their toxins including the production of AMPs, the complement- and coagulation systems, acute-phase proteins and professional phagocytes (19, 20). This immunological branch involves the activation of monocytes, macrophages, mast cells, eosinophils, natural killer (NK) cells and especially neutrophils (21). Neutrophils are key mediators of innate immunity as they are the first immune cell recruited to an infection site where they capture, engulf and destroy pathogens through processes like degranulation, NETosis and phagocytosis (22). Macrophages can also engulf microbes and damaged tissue but more importantly, together with dendritic cells, they also initiate the adaptive immune system, such as B- and T cell responses, by antigen presentation via MHC class II antigen (21).

The adaptive immunity

The adaptive immunity is highly specific and facilitates long-term protection by developing a memory against certain pathogens. It involves activation of B- and T cells towards antigens from a pathogen presented on the surface of macrophages and dendritic cells or exposed by antibodies (16). In order to activate native T cells, the antigen presenting cell needs to be transported to the lymph node where T cells are located. T cells are then directed to produce cytokines to activate other effector cells, or they are primed for immediate attack of infected host cells (23). B cells are essential for the humoral immune response by producing highly specific antibodies. Antibodies recognize and bind to the surface of pathogens and their toxins to either directly neutralize its activity or to facilitate the recognition of the pathogen by phagocytes (24). Besides antibody production, B cells also generate immunological memory, present antigens to T cells and regulate different cytokine productions (25). Due to the high specificity and immunological memory of the adaptive immune system, these processes are the fundamental principles behind vaccination programs in use today.

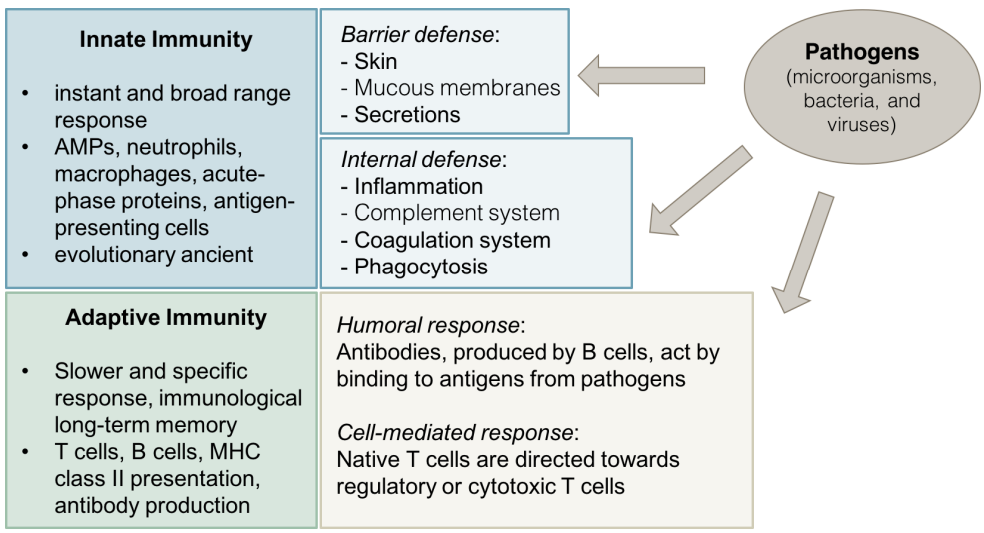

Figure 2. Overview of the innate and adaptive immune system.

Microbial recognition, protection and elimination

Inflammation

The detection and identification of foreign substances are necessary to prevent and combat potential danger. Components and microbial structures from pathogens, referred to pathogen-associated molecular patterns (PAMPs), can be recognized by cells of the innate immune system via pattern recognition receptors (PRRs) (15). The most

characterized PRRs in mammalians are Toll-like receptors (TLRs), which can be found on many cell types including macrophages, neutrophils and epithelial cells (26, 27). Furthermore, the innate immune system is not only active against pathogens, but also recognizes endogenous molecules released by stressed cells or damaged and necrotic tissues. These molecules are referred to as damage-associated molecular patterns (DAMPs) (28). The recognition of PAMPs and DAMPs triggers intracellular signaling resulting in the release of inflammatory mediators such as cytokines, chemokines and vasoactive peptides to recruit immune cells (29). These mediators induce the classical signs of inflammation including redness, swelling, heat, pain and itching (30).

TLR signaling and their specificities

The TLRs are composed of leucine-rich repeats in the extracellular ligand-binding domain and a cytoplasmic Toll/IL-1 receptor (TIR) domain interacting with adaptor molecules such as MyD88, IL-1 receptor-associated kinases (IRAKs) and tumor necrosis factor receptor-associated factor-1 (TRAF6) (20, 31). The intracellular signal is then transduced by several different kinases activating down-stream mitogen-activated protein kinase kinases (MKKs), the inhibitor of NF-κB (IκBα) and mitogen-activated protein kinases (MAPKs) such as ERK1/2, JNK and p38. These kinases activate the transcription factors AP-1 and NF-κB, which finally leads to the expression of pro-inflammatory cytokines (32). TLRs can be found in either the plasma membrane (TLR1, 2, 4, 5, 6) or intracellularly in endosomes (TLR3, 7, 8, 9). Membrane bound TLRs recognize mainly microbial membrane components such as lipids, lipoproteins and proteins, whereas intracellular TLRs distinguish microbial nucleic acids (31, 33). For example, TLR4 is the major receptor for LPS from the surface of Gram-negative bacteria and TLR2 binds to peptidoglycan, lipotechoic acid and M protein from Gram-positive *Streptococcus pyogenes* (34, 35). Further, TLR4 is also the receptor for various DAMPs (HMGB1, HSPs, human DNA and ATP molecules) released following tissue injury caused by mechanical force, excessive heat or cold, chemical insult, radiation or hypoxia (36).

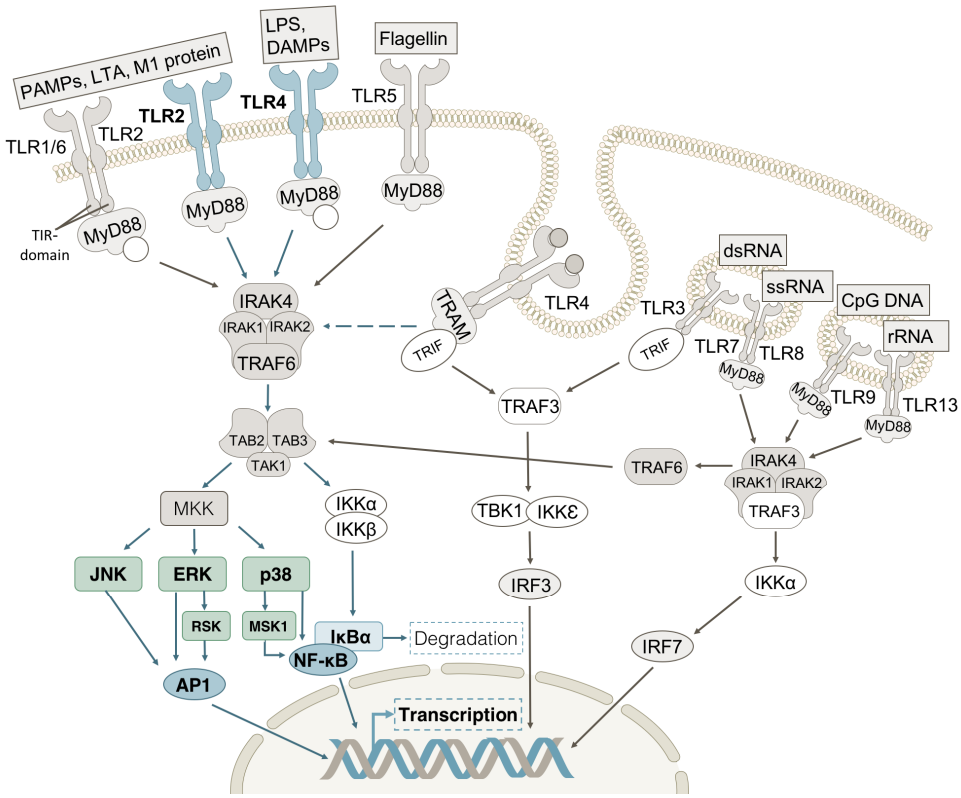

Figure 3. Schematic representation of intracellular TLR signaling.

Keratinocytes as active sentinels

As keratinocytes represent the first barrier against exogenous pathogens in human skin, it is no surprise that they express several vigilant TLRs on their surfaces (37, 38). By their expression, keratinocytes are able to sense microbes and discriminate between harmless commensal organisms and harmful pathogens. They can also recognize DAMPs and irritants, toxins, haptens and UV-dependent danger through the activation of the inflammasome, a large multiprotein complex, to process and secrete key pro-inflammatory cytokines. This in turn results in the activation of tissue-resident immune cells that continue an operative inflammatory strategy (14).

Over the past decade, it has become increasingly apparent that keratinocytes and other resident skin cells produce a number of antimicrobial molecules important for maintaining skin homeostasis (39). Studies of AMPs in many organ systems have shown that they are involved in a wide range of activities including direct microbial killing, chemotaxis, modification of inflammatory responses, angiogenesis, and wound healing (40). Cathelicidins and defensins are two classes of AMPs that have been well

characterized and studied in the skin. The cathelicidin protein hCAP18, as well as human β-defensins (hBDs-1, -2, and -3) are produced in keratinocytes of the superficial epidermis and packaged in lamellar bodies prior to extrusion to the stratum corneum (41, 42). The essential role of AMPs in protecting the skin against bacterial infection has been demonstrated in mice deficient in the murine cathelicidin gene, which have increased susceptibility to severe skin infection by group A streptococci (43). Together these observations illustrate the immune defense potential of keratinocytes acting directly through storage and processing of antimicrobial peptides (44).

In addition to AMP production, keratinocytes constitutively secrete, or are induced to release, numerous cytokines, including IL-1, IL-6, IL-10, IL-18 and tumor necrosis factor (TNF). Keratinocytes are also an important source of chemokines and express chemokine receptors, and can thus modulate immune responses by attracting different cell types into the skin. As an example, by expressing CC-chemokine ligand 20 (CCL20), activated keratinocytes can selectively attract effector T cells. Activated keratinocytes can also recruit monocytes and neutrophils to the epidermis by producing GROα and IL-8 (45).

Neutrophil responses

It is not sufficient to sense microbes, the host must also be able to eliminate the intruder and clear off any danger. Here, neutrophils exert a significant contribution serving several different functional responses, including phagocytosis, degranulation, and the release of nuclear material in the form of neutrophil extracellular traps (NETs). Neutrophils are produced in large numbers in the bone marrow from hematopoietic stem cells. Mature neutrophils contain granules and secretory vesicles that store specific proteins relevant to kill microbes. The granules are divided into primary (azurophil) granules, secondary (specific) granules and tertiary (gelatinase) granules. Together they store an arsenal of antimicrobial enzymes, including elastase, myeloperoxidase, cathelicidins, defensins, and matrix metalloproteinases, which are used to kill invading pathogens (22, 46). Heparin-binding protein (HBP) is a neutrophilic-derived inactive serine proteinase involved in endothelial permeability and vascular leakage (47, 48). It is contained in the secretory vesicles and azurophilic granules from neutrophils and is an established marker of neutrophil degranulation (49, 50). Granules are normally prevented from being released until receptors of the neutrophil surface are activated. Plasma membrane- or phagosomal membrane receptors are directing cytoplasmic proteins, via phosphorylation by kinases, to the mobilization and secretion of granular contents by degranulation. The release of each type of granule appears to be regulated by different intracellular signaling pathways, usually involving increased intracellular Ca^{2+} levels as a crucial second messenger in the activation of exocytosis. Degranulation is a very complex process and requires important control mechanisms as the granules are highly enriched in tissue-destructive proteases (51).

Upon activation by a range of mediators, including IL-8 and LPS, neutrophils can generate a web of extracellular fibers known as NETs, composed of DNA, histones, and antimicrobial proteins, which are highly effective at trapping and killing bacteria. Intracellular reactive oxygen species, processed by myeloperoxidase, can trigger NET formation (52) which amplify the effectiveness of antimicrobial components by concentrating them in this fibrous network and reducing their exposure to host tissues (53, 54). Phagocytosis involves the ingestion of a microorganism into a phagocytic vacuole that upon maturation becomes a phagolysosome. Within the phagolysosome, the microorganism is destroyed by the action of low pH, reactive oxygen species and degrading enzymes. The reactive oxygen intermediates are initiated in a series of reactions by NADPH oxidase from superoxide and hydrogen peroxide (55). All reactive oxygen intermediates kill microbes by reacting with different molecular targets including microbial lipids, proteins, and nucleic acids. The host itself is not resistant to these reactive oxygens and they may cause substantial injury to healthy tissues. This is one disadvantage due to the lack of specificity of the otherwise fast and efficient innate immune system (21).

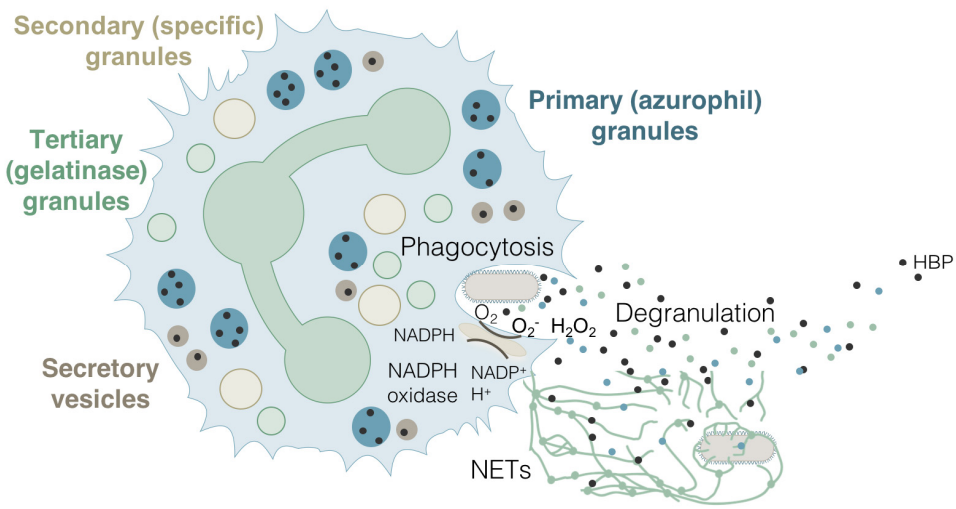

Figure 4. Neutrophil attack against pathogen including phagocytosis, degranulation and NETosis.

The healing orchestra

Immune cell infiltration

The process of wound healing from skin infections is complex and highly organized to control homeostasis, inflammation, proliferation, and remodeling. As tissue injury causes disruption of blood vessels and extravasation of blood constituents, the blood clot re-establishes homeostasis temporarily (56). Platelets are responsible for creating the clot and also act chemotactic to macrophages and fibroblasts by the secretion of several mediators, such as platelet-derived growth factor (57). Infiltrating neutrophils cleanse the wounded area from microbes and their secreted products and are then phagocytosed by macrophages (58). Monocytes can also be recruited to the site of injury by different cytokines that also triggers their formation into inflammatory and reparative macrophages. These macrophages produce other important cytokines and growth factors (TGF-a, TGF-b, IL-1, IGF-1) for the initiation and propagation of new tissue formation (59). Eventually, T cells and other members of the adaptive immune system may become involved at later stages if specific pathogens are not combatted by the initial innate immune responses. There is no need of infiltrating immune cells for wound healing, as demonstrated by excellent wound healing capacity of fetal skin lacking immune cell responses (60) and by studies using immune cell knock-out mouse models (61-64), as long as no infection is involved. Such studies have only revealed a pivotal role for the dendritic epidermal T cells, important for keratinocyte proliferation and wound closure by their release of keratinocyte growth factors, fibroblast growth factor 7 (FGF-7) and FGF-10 (65).

Proliferation and tissue remodeling

There is no clear transition from inflammation to repair as these phases overlap in time and function. Reepithelialisation of wounds begins within hours after an injury at the wound margins and involves keratinocytes proliferating and migrating to repair the epidermal abrasion. The stimuli for the migration and proliferation of epidermal cells during reepithelialisation have not yet been determined (66). Endothelial cells in the dermis respond to injury by proliferating, migrating and forming new blood vessels during angiogenesis (60). The epithelial cells undergo phenotypic alterations, dissolve the intercellular desmosomes and form peripheral cytoplasmic actin filaments, which allow cell movement. Furthermore, the epidermal and dermal cells no longer adhere to each other, because of the dissolution of hemidesmosomal links between the epidermis and the basement membrane, which allows the lateral movement of epidermal cells. The expression of integrin receptors on the epidermal cells allows them to interact with the ECM proteins at the margins of the wound, also enabling their migration and scrutiny of desiccated eschar from viable tissue. Degradation of the ECM is required for the epithelial migration between the collagenous dermis and the fibrin eschar and depends on the activation of the plasminogen activator and collagenase (matrix

metalloproteinase 1), produced by epidermal cells (56). As this process progresses, fibroblastic cells are attracted to the wound to secrete collagenous ECM known as granulation tissue that provide several growth factors, structural integrity and scaffolding characteristics. The provisional ECM is gradually replaced with a collagenous matrix, also produced by fibroblasts which then undergo apoptosis (67). Transition from granulation tissue to a proper scar is dependent on continued synthesis and catabolism of collagen at a low rate. This collagen remodeling is controlled by several proteolytic enzymes termed matrix metalloproteinases and their tissue inhibitors, which are secreted by macrophages, epidermal cells, and endothelial cells, as well as fibroblasts (68). In superficial wounds, the skin heals to form a tissue that is indistinguishable from the intact skin, but if a wound is sufficiently deep or large, the wound will heal with scar formation. Scars are characterized by the absence of skin appendages, such as hair follicles and sweat glands, and by a collagenous matrix that differs in structure from that of intact tissue. In unfortunate circumstances, the normal scar is replaced by pathological fibrotic tissue, which can result in hypertrophic or keloid scars (69).

Infectious diseases – a global health issue

Since the discovery of conventional antibiotics, in the late 1920s, and the initiation of vaccination programs, the morbidity and mortality of many infectious diseases has been significantly reduced (70, 71). Despite these advances, infectious diseases still bring significant contribution to morbidity and mortality across the world (72). One of the major challenges of infectious diseases is the increasing development of antibiotic resistant bacteria (73, 74). Although efforts are being made to limit the spread of resistance, by restraining the use of conventional antibiotics, there is still substantial work left to be done. Hospitals need faster and better tools for clinical diagnostics of infections and their severities, as well as completely new innovations for their treatments. Without antibiotic treatments, there would be no possibility to perform any type of surgeries or combat serious infections. Therefore, it is crucial to come up with alternative therapies and new antibiotic approaches to be able to continue and to improve this era of global health and development (75).

Bacterial infections of the skin

Complicated skin and surgical site infections (SSIs) are among the most common infections treated in the hospital setting (76, 77). SSIs are explicitly increasing the duration of hospital stay for patients and the direct costs of their hospitalization (78). Skin and soft tissue infections collectively refer to microbial invasions of the different skin layers and of the underlying soft tissues, inducing a host response. The majority of

skin infections tend to resolve within 7–10 days, and the estimated prevalence among hospitalized patients was 7–10 % in 2005 (76). The increased number of patients with immunosuppressed conditions (due to immunosuppressive drugs, cancer treatment or transplant surgery), of invasive medical techniques and surgical wound infections, may contribute to the increasing incidence of severe skin and soft tissue infections over the last decades (79, 80). Physical or chemical assaults of the skin can induce disruption of the cutaneous barrier and predisposing it to bacterial penetration, growth and multiplication. Such loss of skin integrity may be caused by scratches, burns or ulcers, bites or surgical wounds, along with inflammatory dermatoses and viral or fungal infections (81). Also, variations of skin pH and temperature, dryness and maceration can be predisposing factors involved in the development of severe skin infections (82, 83).

Accountable pathogens

The most commonly implicated pathogens in skin and soft tissue infections include Gram-positive bacteria, such as *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Enterococcus* spp. Gram-negative and mixed organisms can be additionally encountered in complicated cases of skin and soft tissue infections, typically *Pseudomonas aeruginosa*, *Escherichia coli*, *Enterobacte*r spp., and *Klebsiella* spp. (84). Bacterial skin infections can be related to the bacterial etiology, the environmental source, and patient risk factors. For example, the most common pathogen causing cellulitis in the community are *S. pyogenes* (85, 86). Patients with diabetes mellitus, chronic renal failure, or patients who are intravenous drug abusers predominantly encounter staphylococcal infections. Nosocomial acquired skin infections tend to be more associated with *S. aureus* and *P. aeruginosa* (87, 88). Further, *S. aureus*, *Enterococcus* spp., *E. coli*, *P. aeruginosa*, and *Enterobacter* spp. are most frequently isolated in SSIs (89). Meanwhile, diabetic foot ulcers are usually polymicrobial, with three to five organisms isolated per infection involving both gram-positive and gram-negative bacterial strains (87, 88).

Far down the skin

The depth of the skin that is reached by invading microbes is crucial for the clinician to establish the correct diagnosis, and is in direct correlation with disease severity (83). Epidermal infections caused by *S. pyogenes* and *S. aureus* include impetigo, ecthyma, and erysipelas. Impetigo involves the outer keratin layer of the skin, resulting in crusty lesions. Ecthyma is a deeper form of impetigo usually starting as a vesicle, small blister or a pustule. Erysipelas affects the epidermis and the upper dermis, recognized by an intense red skin color and a raised, demarcated border of infection. This cutaneous inflammation is accompanied by chills, fever, and toxicity (83, 90). Dermal infections consist of advanced erysipelas, cellulitis and necrotizing fasciitis. Cellulitis is a spreading inflammation occurring in the deeper dermis and subcutaneous tissues. It has a pinkish tone with less defined edges compared to erysipelas, indicative of a more severe

infection and associated with bacteremia. Clinical findings also include distinct local pain, tenderness, and swelling, as well as fever, chills, and malaise (82, 90). Necrotizing fasciitis is described to involve the subcutaneous tissues and deep fascia, associated with massive tissue destruction. Later stages of the disease involve all layers of the skin and also muscles as well. At this stage, the blood supply becomes compromised with hemorrhage and hypoxia, resulting in a purple or violaceous appearance and rapid progression of tissue destruction (90, 91). Purulent skin and soft tissue infections are mostly caused by staphylococcal spp., whereas streptococcal infections are commonly less or non-purulent (90).

Invasive infection by *Streptococcus pyogenes*

S. pyogenes is a human pathogen that mainly causes throat- and skin infections, like pharyngitis, scarlet fever, impetigo, erysipelas and cellulitis. The vast majority of streptococcal infections are uncomplicated, but some cases develop into sepsis, necrotizing fasciitis and streptococcal toxic shock syndrome (STSS), which all display an extremely rapid disease progression and high mortality rates (92). STSS is characterized by a systemic toxicity and fever, resulting in hypotension and multiple organ failure. About 70 % of the STSS cases are accompanied by necrotizing fasciitis or myositis (93). Necrotizing fasciitis, also known as the flesh-eating disease, is an uncontrolled infection of the hypodermis that leads to massive tissue destruction, often requiring surgical intervention in addition to intravenous antibiotic therapy (94, 95). Still, STSS with necrotizing fasciitis causes mortality rates about 30-60 %, and can kill a patient within 72-96 h (96). Necrotizing fasciitis can be monomicrobial, most commonly caused by group A streptococci but also by *S. aureus*, or polymicrobial infections. Polymicrobial cases typically involve either group A streptococci or *S. aureus*, plus a multitude of other less virulent Gram-positive bacteria such as α-hemolytic *Streptococcus* spp. or *Staphylococcus epidermidis* (97). It remains to be clarified if these polymicrobial cases represent a primary monomicrobial invasive infection that subsequently has been colonized by environmental microbes or if the polymicrobial organisms participate in a symbiotic infectious process (98). The progression of a streptococcal infection into necrotizing fasciitis has not yet been clarified. The combined action of many potent proteases and other degradative virulence factors expressed by the invading streptococci together with tissue-damaging enzymes released by innate host leukocytes is believed to be involved. Also, ischemia from microvascular thromboses, a common histological feature of necrotizing fasciitis by group A streptococci, may significantly contribute to the progression of tissue damage (99).

Group A streptococci is a Gram-positive and non-motile coccus with round to ovoid shape ranging from 0.6–1.0 μm in diameter. They divide in a single plane and therefore appears in pairs or short chains. Longer chains of streptococci can be recovered from pure cultures grown in enriched liquid media. It is a β-hemolytic

facultative anaerobe inducing complete lysis of erythrocytes (100). Strains of *S. pyogenes* are serologically categorized based on the antigenic differences in the N-terminal region of the M protein expressed on the streptococcal surface, originally devised by Rebecca Lancefield and colleagues during the early 1900s (92, 101, 102). The streptococcal serotype M1 is a highly prevalent strain, and together with serotype M3, among the most common serotypes causing invasive disease (103-105). Genetic and proteomic analyses of invasive strains have exhibited unusual epidemiologic features and increased virulence, unlike other less virulent streptococcal strains (106-108). Increased virulence of the notorious M1 serotype can be attributed to its diversification through phage mobilization and its ability to sense and adapt to different host environments, selected to survive and invade host tissue (109). These abilities are recognized by specific virulence factors. *S. pyogenes* has developed an extensive repertoire of virulence factors to mediate adhesion to host tissues, enable dissemination of bacteria, immune evasion or that modulate host immune responses, which are comprehensively reviewed by Cunningham (92). However, despite decades of study, many fundamental questions bearing on streptococcal pathogenesis from superficial to invasive infections remain unanswered (110). Still, today, this knowledge deficit drives an intense research effort to improve our understanding of the epidemiology, molecular mechanisms, and host-pathogen interactions underlying streptococcal necrotizing fasciitis and STSS in human patients.

Bacterial Virulence

Streptococcal virulence factors

Approximately 90 % of the total gene content is shared among all group A streptococci. The other 10 % of variable genetic material is attributed to the presence or absence of prophage-like elements that encode specific virulence factors, including secreted toxins, adhesins, degradative enzymes, superantigens, and drug-resistance genes (111). The expression of different virulence factors has also been found to be growth-phase-dependent and regulated by transcriptional sensing mechanisms (112). Several reports have documented that particular clones of M1 and M3 strains, most commonly associated with STSS, share particular virulence factor expression patterns not present in non-invasive strains (113, 114). Genetic recombination of bacteriophage-harboring genes among different *S. pyogenes* serotypes, such as the streptococcal pyrogenic exotoxin A and C, have been demonstrated important for invasive infections (115). Further analysis has revealed several virulence factors necessary for the establishment of streptococcal infections, as well as associated with invasive disease.

Capsule and cell wall

S. pyogenes is surrounded by a thick cell wall composed of peptidoglycans and lipoteichoic acid (LTA). LTA mediates adhesion of streptococcus to fibronectin on oral epithelial cells (116, 117), and together with peptidoglycan innate immune responses are triggered via the TLR2 (118). Outside the cell wall, streptococci form a hyaluronic acid capsule, with important anti-phagocytic functions (119), and enables adhesion to keratinocytes via CD44-binding (120). The capsule can also facilitate throat colonization *in vivo* (121). As the ability to encapsulate varies, it is considered more common in virulent strains causing severe streptococcal infections than in strains causing uncomplicated pharyngitis (122).

Secreted virulence factors

Among the most potent virulence factors known are the superantigens. They have an ability to by-pass normal T cell activation by crosslinking the MHC class II of the antigen-presenting cells and the T cell receptor, resulting in massive and uncontrolled release of T cell associated cytokines. So far, 11 superantigens have been described in *S. pyogenes*, namely the streptococcal pyrogenic exotoxins (Spe) A, C and G-M, the streptococcal superantigen (SSA), and the streptococcal mitogenic exotoxin (SmeZ) (123, 124). Streptococcal pyrogenic exotoxin B (SpeB), is a cysteine proteinase with broad substrate specificity. It can release the potent inflammatory mediator bradykinin from H-kininogen (125), activate metalloproteases, including MMP-2 and MMP-9, involved in coagulation (126, 127), degrade the antibacterial peptide LL-37 and release dermatan sulfate that inhibits AMPs (128, 129). SpeB can also cleave ECM proteins such as fibronectin and vitronectin (130, 131) and release endogenous proteins, such as the M protein, from the bacterial surface (132). SpeB and two other secreted streptococcal proteinases, IdeS and EndoS, are able to degrade structures of immunoglobulins, hindering their immune active functions (133, 134). These proteolytic activities have obvious implications for understanding bacterial dissemination and tissue destruction in invasive infections. Streptococcal inhibitor of complement (SIC), another important virulence factor, inhibits multiple host derived molecules, including the complement membrane attack complex, lysozyme, α- and β-defensins, secretory leukocyte proteinase inhibitor, IFN- , MIG, and LL-37. These combined effects result in increased GAS-resistance to phagocytosis and bactericidal activity (135-137). Further, SpeA and SpeB induce human mononuclear cells to synthesize TNFα, IL-1β and IL-6, suggesting that they could mediate the fever, shock, and organ failure observed in patients with STSS (138-140). Streptolysin O and S (SLO and SLS) are two streptococcal proteins responsible for lysis of host cells. SLO forms large pores in eukaryotic membranes by the binding to cholesterol (141), and can, together with the hyaluronic acid capsule, induce keratinocyte apoptosis accompanied by cell detachment and loss of epithelial integrity (142). SLS compose the β-hemolytic (breakdown of erythrocytes) properties of S. pyogenes (143). Both have

been demonstrated to contribute to virulence and the induction of necrotic lesions in vivo (144). Streptococci also secrete streptokinase, a plasminogen activator, and DNases required for pharyngitis and is believed to significantly contribute to necrotizing fasciitis pathogenesis (145, 146). DNase mutant streptococcal strains have been demonstrated less virulent in mouse models of skin infections and bacteremia (146). DNase inactivation also increased the susceptibility of group A streptococci to phagocytosis and elimination by host phagocytes. Subsequent studies have confirmed that DNases from group A streptococcus function in pathogenesis by degrading host-protective neutrophil extracellular traps (147). Finally, the *S. pyogenes* cell envelope protease (SpyCEP), a proteolytic enzyme secreted from streptococci with specific activity against the human leukocyte–recruiting chemokine IL-8 (148, 149). SpyCEP contributes to host-pathogen interactions during invasive infections, as demonstrated in a mouse skin infection model, where the SpyCEP-deficient strain caused greater neutrophil influx, leading to more leukocyte degranulation and degradative enzyme release, resulting in significantly larger skin lesions. SpyCEP was also demonstrated to inhibit leukocyte activity through cleavage of granulocyte chemotactic protein 2 (GCP-2) and growth-related oncogene alpha (GROα) (150). The cumulative data confirm that group A streptococci has developed several specific virulence mechanisms to protect and avoid the human innate immune system. It also provides additional evidence that host-derived factors such as neutrophil proteases and degradative enzymes contribute to tissue damage and other characteristics of invasive streptococcal infections.

Surface bound virulence factors

M and M-like proteins are the most abundant streptococcal surface protein and are expressed by all streptococcal strains. The protein family is organized into an α-helical coiled-coil dimer (151) with a conserved carboxy-terminal domain, a variable central part, and a hyper-variable amino-terminal end (152). The carboxy-terminal is anchored to the cell wall via an LPxTG motif, shared among many surface bound proteins of Gram-positive cocci (153). The gene encoding M protein is called *emm* and is controlled by the Mga regulon (multiple gene regulator of group A streptococcus) (154). The Mga regulon determines the expression of several virulence factors including M protein and the structurally related M-like proteins (*mrp*, *enn*, *protH* and others), which shares similar binding properties and rendering the bacteria resistant to phagocytic killing (92, 155-157). These virulence factors interact with a large number of host proteins, such as fibrinogen (158), fibronectin and albumin (159, 160), IgG (160), kininogens (161), factor H (162), factor H-like protein (FHL-1) (163), and C4b-binding protein (C4BP) (164). The binding of host molecules to M protein contributes to bacterial infection mainly through its ability to impede phagocytosis of streptococci by human leukocytes. Conversely, type-specific antibodies against the M protein enhances phagocytosis and confer resistance to challenge with group A streptococcus of the same M serotype (165). Further, group A streptococci produce

the protease SpeB that cleaves the terminal portion of the M-protein, rendering the organism more susceptible to phagocytosis by human phagocytes but still more resistant to phagocytosis as in the presence of type-specific antibodies (166). Streptococci are also able to survive inside phagocytes by M protein-dependent mechanisms, as M protein-negative mutants are efficiently killed off (167-169). The α-helical coiled-coil dimer is also responsible for adhesion to keratinocytes (170), promotes intracellular invasion into epithelial cells (171) and is enabling throat colonization in a baboon model of streptococcal pharyngitis (121). Moreover, M protein have been implicated in the generation of cross-reactive autoantibodies against human myosin and collagen, classical characteristics found in rheumatic heart fever (172, 173).

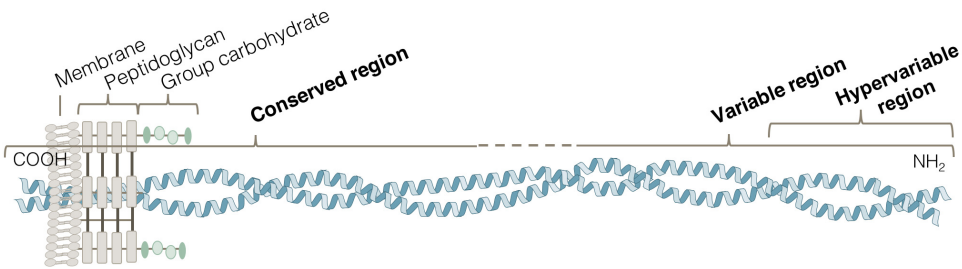

Figure 5. Structure representation of the streptococcal M protein protruding the bacterial cell membrane and cell wall.

Another important group A streptococcus surface molecule is the C5a peptidase (ScpA), a serine protease that specifically cleaves and inactivates complement protein C5a (174). As a result, ScpA can decrease leukocyte recruitment to the infection site and allows enhanced group A streptococcus survival (174, 175). *S. pyogenes* also expresses a number of plasmin-, plasminogen- and fibronectin-binding proteins, such as α-enolase (176), glycerhaldehyde-3-phosphate dehydrogenase (177), protein F1 (Sfb1) (178) and streptococcal opacity factor (179). Binding of plasmin and plasminogen seems to be important for streptococcal virulence (180, 181) while fibronectin-binding promotes adhesion and internalization into host epithelial cells (182-184).

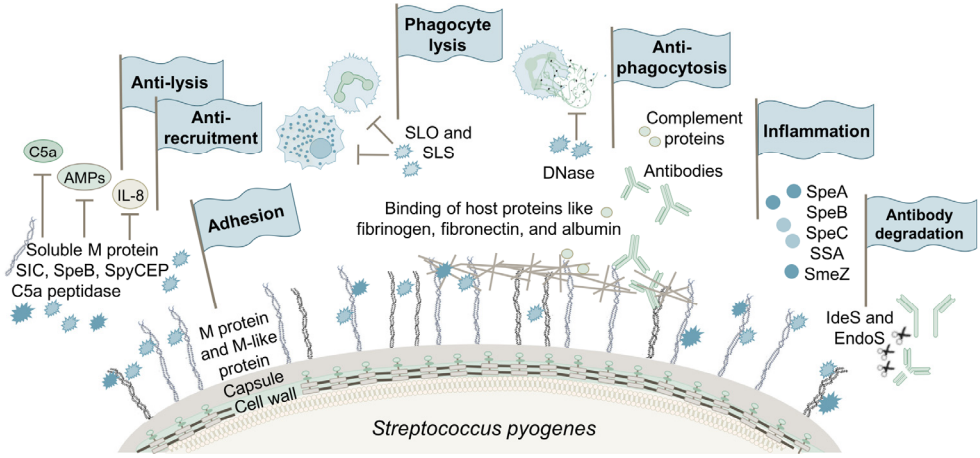

Figure 6. Summary of the arsenal of virulence factors from *Streptococcus pyogenes*.

Host and microbe interactions

The commensal community

During a lifetime, we encounter numerous of microorganisms which can be beneficial or threatening to our survival. These microorganisms are referred to as commensals or pathogens, respectively (185). However, the vast majority of microbes we encounter are commensals, sharing a symbiotic relationship important for our wellbeing. Commensals can provide several essential functions including the production of certain vitamins, energy harvesting and maintaining epithelial integrity (186, 187). The skin is a primary target for infections, and in some cases, caused by normal constituents of the normal microbiota. In the context of genetic predisposition associated with barrier or regulatory network defects, commensal microorganisms can induce inflammatory exacerbations that can contribute to the initiation and amplification of several skin disorders (187). One important mechanism of commensals is their capacity to prevent pathogens from adhering and establishing infection of certain niches, referred to as colonization resistance (188). In the skin, commensals are able to actively prevent colonization of pathogenic microbes such as *Streptococcus pyogenes*, *Staphylococcus aureus*, *Pseudomonas* spp. and *Candida albicans*. As an example, sebaceous glands are relatively anoxic and support the growth of facultative anaerobes such as *Propionibacterium acnes*, a common skin commensal bacterium. *P. acnes* produces several lipases that can hydrolyze triglycerides present in sebum, releasing free fatty acids that contribute to the low pH (~ 5) of the skin surface. Many pathogenic bacteria are inhibited of acidic pH, preventing the growth of for instance *S. aureus* and *S. pyogenes* and instead favoring the growth of coagulase-negative staphylococci and *Corynebacteria* (189, 190). *Staphylococcus epidermidis*, a commensal bacterium, has been

demonstrated to selectively inhibit the growth of *S. aureus* and group A streptococcus by their own AMP production and by modulating the innate host response (191, 192). Also, LTA from *S. epidermidis*, can reduce skin inflammation and trigger AMP production from keratinocytes by binding to TLR2 and TLR3 receptors (193, 194). However, *S. epidermidis* is also the most frequent cause of hospital-acquired infection from medical devices such as catheters or heart valves (195). Virulent strains of this organisms can form biofilms, which protects them from the host immune system and antibiotics. Increasing levels antibiotic resistance has also been detected for *S. epidermidis*, particularly to oxacillin or methicillin, and therefore the microbe is assumed to be a reservoir for spreading antibiotic-resistance genes to the closely related but more virulent organism, *S. aureus* (196).

Bacterial host-adaptation

The immune system and host environment induce a number of antimicrobial pathways to prevent an infection when encountering a pathogen. The invading pathogen responds by adapting to that inflammatory environment using several gene regulatory systems. These gene regulatory systems, such as Mga regulon, CovR/S system and the mtsR-prsA-SpeB axis, is crucial components to the rapid environmental adaptation needed for the pathogenesis of invasive infections. The Mga is a DNA-binding protein that activates the expression of several important virulence genes in *S. pyogenes* in response to changing environmental conditions (197). The Mga regulon controls the *mrp*, *emm*, *enn* and *scpA* gene expression which translates to the M- and M-like proteins (92, 155-157), but also regulates the expression of SpeB (198), SIC (199), C5a peptidase (200) and collagen-like protein (SclA) (201). Mga regulon is influenced by conditions that signify favorable growth conditions and subsequently regulates genes and operons involved in metabolism and sugar utilization as well, supporting the establishment of infection (202). The other large regulator of virulence in *S. pyogenes* is the CovR/S two-component system. Function-altering mutations in either the sensor kinase (CovS) component or the transcriptional repressor (CovR) component of the CovR/S regulatory system underlie massive transcriptome changes, involved in the transition from uncomplicated to invasive infection profile of *S. pyogenes* (203). Gene expression studies performed on group A streptococcal strains with inactivating mutations in CovR/S revealed a marked dysregulation of many virulence factors, including SpeB, SpyCEP, streptokinase, and SIC, as well as the Mga regulon (204). Thus, dysregulation of the CovR/S two-component regulatory system is associated with invasive transitioning and plays a central role in directing infection phenotype. The CovR/S system normally responds to different stimuli like elevated temperature, high osmolarity, low pH, low iron levels, elevated magnesium and presence of LL-37 to maintain bacterial survival in different environmental conditions (205, 206). Single nucleotide polymorphism (SNP) analysis has yielded novel insight into the molecular pathogenesis of group A streptococcal necrotizing fasciitis by identifying the mtsR-prsA-SpeB axis (207). *In vivo* experiments, using knock-out mouse and primate

models, showed that inactivation of mtsR, a transcriptional repressor, upregulated the expression of prsA, a peptidyl-prolyl isomerase required for posttranslational maturation of the cysteine protease virulence factor SpeB (208) resulting in smaller lesions with less tissue destruction, and reduced systemic bacterial dissemination (207). Genomic data generated from different M protein serotype strains can improve our understanding of the associations between strain serotype and disease phenotype, and pinpoint important virulence factors and their mechanisms. Likewise, genomic data derived from strains within M protein serotypes could give clues on molecular pathogenesis and subclone emergence. Such investigations are crucial for the formation of new hypotheses supporting novel concepts to elude antibiotic resistance and proceed vaccine development.

Critical immune activation or exorbitant host response

Due to the colonization of commensals, the skin has evolved both active defense mechanisms and tolerogenic pathways to reserve skin- and immune homeostasis. The skin needs to ensure proper activation of immune responses adjusted to various challenges. Many of the systemic effects of cytokine mediators are aimed at attracting cells, diverting blood flow to the affected tissue and favor the influx of cells capable of killing invading microbes (14). Thus, the presence of a virulent bacterial strain is necessary but not sufficient to cause invasive infections. Host susceptibility to group A streptococcus infection is inversely related to the quantity of specific antibodies against streptococcal virulence factors such as SpeA and M protein (105). For example, patients with antibodies to pyrogenic exotoxins but not to M protein may develop pharyngitis or bacteremia but will not develop scarlet fever or STSS. Patients with antibodies against both M protein and pyrogenic exotoxins are immune to the establishment of such group A streptococcus infections. Patients with no antibody response to pyrogenic exotoxins are more susceptible in developing pharyngitis or scarlet fever and, if they also lack antibody response towards M protein, more susceptible to invasive infection (209). Further, several recent investigations studying the role of neutrophils in skin repair have shown that the presence of neutrophils may actually be unfavorable for healing (21). Results from using antibody-based methods to reduce neutrophils in mice suggested that neutrophils can suppress healing, as depletion of neutrophils led to significantly faster reepithelialization of wounds. The redundancy of neutrophils when recovering from an injury is also exemplified by the superior wound healing capacity of fetal skin, which virtually lack immune cells (60). Furthermore, neutrophils can actually aggravate tissue injury when amplifying the inflammatory response and direct the release of toxic effectors (210). Examples of effectors that can cause tissue damage are the ROS-molecules, such as superoxide and hydrogen peroxide, the myeloperoxidase (MPO), as well as non-oxidative mechanisms such as proteolytic enzymes (MMPs, elastase and collagenase) and antimicrobial proteins (defensins and cathelicidins) contained inside the neutrophil granules. Noteworthy, those effectors do not always cause damage to the tissue, although damage may occur through overstimulation of

neutrophils and massive release of these mediators (211, 212). Also, it is important to note that chronic, non-healing wounds in humans display an exaggerated inflammatory response which results in delayed or impaired healing mechanisms (213). The character of excessive inflammatory responses to infections has been the subject of many investigations, and falls in the category of sepsis research. Sepsis and septic shock is the systemic consequences of an inadequate immune activation towards an infection, resulting in severe morbidity including fever, increased heart- and breathing rate, hypotension and multiorgan failure (214-216). This life-threatening condition is caused by the same mechanisms that are essential for our survival towards infections and evolved as our protection against invading pathogens. Exploring the factors regulating host–microbial interactions in the skin is a particularly compelling line of research because of the rising incidence of inflammatory disorders affecting this compartment. The next challenge is to develop novel approaches to address skin homeostasis, response to infection and disease states in the context of its complexity.

Cold Atmospheric Plasma – an antiseptic innovation

Plasma – the fourth state of matter

Plasma is a partially ionized gas and is described as the fourth state of matter following solids, liquids and gases (217). It is generated by energizing gas to a critical point at which electrons dissociate from atoms, freeing charge carriers, ions, active radicals and excited molecules. The resulting ionized gas contains charged particles, but the overall charge remains electrically neutral (218). Natural plasmas exist in the universe including the solar corona surrounding the sun and stars, in solar winds, and in the earth's ionosphere (219-221). Natural plasmas are estimated to account for more than 99 % of the visible universe and can be seen on earth as lightning and aurora borealis (northern lights) (222, 223). Plasmas can also be artificially produced for surface treatments, like decontamination of surgical equipment, or used in displays and fluorescent lamps (224-226). Previous plasma applications utilized the thermal energy for the desired effects (thermal plasmas). In thermal plasmas, the temperature of electrons, ions and neutrals are the same, resulting in a very high gas temperature. Non-thermal plasmas utilize the difference in mass and energy consumption ensuing that only smaller electrons are heated, whereas the bigger atoms and neutrons, and the plasma collectively, remains at room- or body temperatures (217). Current research, particularly in the biomedical sector, focuses on non-thermal plasmas where the generation of reactive species and charged particles can induce chemical modifications and drive and catalyze complex biochemical reactions. Non-thermal plasmas that are operating at an atmospheric pressure is referred to cold atmospheric plasma (CAP). Important factors for the plasma desired effects is the flux of active charged particles (Ar^+ and N_2^+) and uncharged reactive species of atoms and molecules (O_3, OH, H_2O_2,

NO etc.). Two types of CAP, direct and indirect, are differentiated depending on the plasma generation source. In direct plasma, the tissue target acts as one of the plasma electrodes causing a current to pass through the body. The plasma device and tissue surface need to be at close and constant distance (~ 1 mm). Indirect plasmas are produced between two electrodes and transported to desired target or tissue through a gas flow, delivering the active species directly and via diffusion. In general, CAP deliver up to 10^9 to 10^{10} active agents per cm^2 and second, and a typical application duration is 10 to 100 s (10^{10} to 10^{12} active molecules/ cm^2). The active agent delivery is comparable to a lotion with 0.1 % to 1 % concentration of active agents (2.5 x 10^{10} to 2.5 x 10^{11}) but without the need of immersing the active components in a carrier medium (217).

Biological effects of plasma treatments

Recent progress in understanding plasma, together with the development of plasma sources within tissue tolerable temperatures has put an increasing focus on its application in health care. In 1996, the first paper using atmospheric pressure plasma to destroy microorganisms was published, and paved the way for the plasma medicine research field (227). Since then, CAP has been shown to exert antibacterial efficacy against Gram-positive and Gram-negative bacteria (228-234), biofilm-producing bacteria (235, 236), virus, fungi and spores (237-242). The exact mechanisms of bacterial killing by CAP remains unclear. However, the charged particles in plasma has been concluded to play the essential role for bacterial inactivation by rupturing the outer layer of the cell membrane (243-246). It is generally accepted that low dose of plasma treatment can stimulate eukaryotic cell viability and enhance proliferation, differentiation, and migration, while high dose induces cell apoptosis and necrosis (217, 247, 248). Further, the resistance against plasma treatment is different between normal and cancerous cells, enabling CAP to selectively kill cancer cells while being less damaging to normal cells (249-252). Subsequent risk assessment studies have demonstrated that CAP is non-mutagenic and well tolerated by healthy tissues (253, 254). Plasma treatment interacts with keratinocytes on a molecular level by increasing β_2-integrin expression and reducing E-cadherin and EGFR expression in HaCaT-keratinocytes (255). CAP treatment was further shown to induce expression of IL-8, TGF-β1, and TGF-β2 as well as the antimicrobial β-defensins in keratinocytes (256). CAP has also been reported to have beneficial wound healing abilities using animal models (257-261) and it has been demonstrated successful in treating infected and chronic wounds in clinical trials (262-267). The CAP-created reactive oxygen- and nitrogen species (O, O_3, OH, H_2O_2, NO, NO_2 etc.) play the most important role in all plasma treatment processes (246, 268), and a detailed discussion of their biological contributions can be found in (269).

The kINPen MED plasma tool

The kINPen MED is a commercially available atmospheric pressure argon plasma jet, approved as a medical device for the use in patients, since 2013 (218). It has a hand-held pen-like design developed for biomedical applications to allow precise and arbitrary movements (270). It is operated by using a power supply unit and argon gas to expulse the plasma. The electrical safety of the kINPen is certified and complies with EU standards (certificate number 609.003.1). The plasma is generated by applying a sinusoidal voltage (2-6 kVpp) with a frequency of 0.1-1.1 MHz to the central electrode, resulting in a typical plasma effluent length of 9-12 mm and 1 mm diameter. Its use is intended for the treatment of non-healing wounds and ulcers of the human skin where the plasma can operate effectively regardless of the type of infection or any bacterial multidrug resistance (218).

Figure 7. Visualization of cold atmospheric plasma using the kINPen MED.

The dramatic increase in prevalence of multidrug-resistant pathogens requests for therapeutics with novel mechanisms as treatment alternatives for the future. An immediate immune response is critical as soon as the skin barrier is disrupted to prevent microorganisms from establishing infection. However, because cytokines of such immune responses are important mediators of shock and STSS, strategies to inhibit or neutralize their effects may provide useful actions (271, 272). Further, neutralization of circulating bacterial toxins was requested already back in 2000 in a review by Stevens DL., suggesting commercialization of intravenous gamma-globulins for treating STSS (103). Deactivation of potent bacterial virulence factors together with microbial killing, as reported for plasma application, would be desirable for future antiseptics of infected wounds in the health care system.

Aims of thesis

General aims with this thesis were to investigate innate host responses towards bacterial virulence factors that can explain symptoms from serious infections. Superficial infections can under very unfortunate circumstances develop into devastating and life-threatening diseases. Such transformation is dependent on bacterial virulence factors. Exploring the mechanisms and identifying important virulence factors is crucial to prevent severe complications, find better treatment options and enabling to save more patients in the future.

Specific aims for each paper were;

Paper I

To investigate if keratinocytes participate immunologically towards the streptococcal virulence factor M1 protein, which is the most prominent surface protein and can be released from *Streptococcus pyogenes*.

Paper II

To clarify if the streptococcal virulence factor M1 protein can aggravate the severity of streptococcal skin infections, as M1 protein-related strains is overrepresented in invasive streptococcal diseases.

Paper III

To investigate if cold atmospheric plasma, a partially ionized gas, can modulate the streptococcal virulence factor M1 protein and what impact such treatment would have on the host response.

Paper IV

To study the pathophysiological mechanisms responsible for leptospirosis symptomatology involving hypotension and vascular leakage, as these symptoms can initiate multiorgan failure and aggravate the disease outcome.

Present investigations

Paper I

Vigilant keratinocytes trigger pathogen-associated molecular pattern signaling in response to streptococcal M1 protein.

The streptococcal M1 protein is the most abundant protein on the surface of *S. pyogenes* and is responsible for bacterial adherence and evasion of phagocytosis. The streptococcal virulence factor has been extensively studied as the M1 serotype is among the most prevalent strain found in invasive streptococcal infections. The M1 protein interacts and binds to several host proteins such as fibronectin, kininogen, fibrinogen, IgG3 and albumin. The virulence factor can cause a number of inflammatory reactions from immune cells such as neutrophils, monocytes and T cells mediated by the crosslinking of β_2-integrins and by the binding of TLR2 and T-cell receptors, respectively. However, these reactions are triggered following an already established infection. The bacteria are usually initiated in the skin or throat, still the interactions with cells from these niches have not been as extensively studied. We wanted to investigate if keratinocytes, the major constituent of the skin, respond to the streptococcal M1 protein and if so, how would that response develop.

Gene expression profiling of keratinocytes treated with streptococcal M1 protein were analyzed by microarray analysis resulting in 498 differently expressed genes. The vast majority of upregulated genes were involved with Toll-like receptor (TLR) signaling pathway and cytokine production, specially interleukin 8 (IL-8). Our initial experiment provided several clues about keratinocytes reaction towards the bacterial protein and the forthcoming experiments focused on confirming the proposed hypothesis. We confirmed the activation of TLR-signaling pathway, via phosphorylated MAP-kinases and degradation of IκBα and subsequent translocation of the transcription factors AP-1 and NF-κB into the nucleus. We also studied the cytokine release from M1 protein stimulated keratinocytes. Out of 102 different cytokines, chemokines and growth factors analyzed, we found increased levels of 10 inflammatory proteins in response to the streptococcal virulence factor. Further analysis, including inhibition of the TLR2, TLR4, and the MAP-kinases ERK and p38, revealed an exclusive TLR2-dependent signaling pathway for the release of the chemotactic factor IL-8. The other inflammatory mediators released is probably under other receptor signaling pathways,

as the pro-inflammatory IL-1 receptor could be a suggestion. In microarray analysis, gene expression for the chemokine IL-8 gave the highest fold change score between M1 protein treated and control samples, being upregulated almost 33 times the baseline levels. M1 protein induced IL-8 release was compared to other streptococcal M proteins and SIC (streptococcal inhibitor of complement), another virulence factor from *S. pyogenes*, and revealed M1- and M5 protein exclusively potent, and both are associated with invasive serotypes. However, the M1 protein was evidently the strongest inducer of IL-8. We concluded the keratinocytes as vigilant arbitrators, triggering pathogen-associated molecular pattern signaling in response to streptococcal M1 protein and that neutrophil attraction through IL-8 secretion could be an important protection that keratinocytes exert in response to streptococcal skin infection.

Paper II

Leucocyte recruitment and molecular fortification of keratinocytes triggered by streptococcal M1 protein.

Group A streptococci is a major human pathogen associated with millions of skin and throat infections each year worldwide. The bacteria can evoke a variety of skin diseases ranging from simple pyoderma and impetigo to severe cellulitis and necrotizing fasciitis. *S. pyogenes* of the M1 serotype is commonly associated with invasive streptococcal infections and development of streptococcal toxic shock syndrome. Despite modern intensive care, these infections are still correlated with high mortality rates and responsible for more than 150,000 deaths annually. The M1 protein is a powerful inducer of inflammatory responses for several human cell types, but the reason why M1 protein-related strains is over-represented in invasive streptococcal diseases is still not understood. This study was undertaken to investigate if soluble M1 protein can aggravate the severity of streptococcal skin infections in respect to inflammation, leucocyte recruitment, and tissue remodeling as seen in patients with cellulitis and necrotizing fasciitis.

As M1 protein stimulated keratinocytes responded primarily with the release of IL-8, we wanted to investigate the chemotactic properties of leukocytes from such cell supernatants. Because there is a lot of interactive signaling between immune cells we decided to use a mixed population of leukocytes in order to resemble the inflammatory course as much as possible. Since many diseases are driven by an exorbitant host immune system, we wanted to clarify whether the streptococcal virulence factor is involved in aggravating invasive skin infections or trigger an overreaction of the host response. We found that the streptococcal M1 protein did not induce chemotaxis, however supernatants from M1 protein stimulated keratinocytes were able to recruit twice the number of leukocytes compared to control following a chemotactic assay.

Increased levels of heparin-binding protein (HBP) demonstrated activation and degranulation by recruited neutrophils, as HBP is a specific neutrophil degranulation marker, when encountering M1 protein. To our surprise, a lactate dehydrogenase (LDH) assay demonstrated that neither M1 protein nor activated leukocytes were cytotoxic to keratinocytes. Instead, mass spectrometry analysis revealed a molecular fortification of keratinocytes when encountering the bacterial virulence factor. Noteworthy, mass spectrometry analysis also demonstrated the incredible purity of the M1 protein purification procedure used in our studies. Furthermore, we found increased levels of proteins involved in cell stress, immunomodulation, survival and proliferation, as well as structure and cell secretion. However, the M1 protein completely abolish wound healing properties of keratinocytes, as demonstrated using scratch assays. The impaired wound healing was in part explained by inhibited cell proliferation. We also found impaired wound healing and IL-8 release from M1 protein stimulated keratinocytes to be dependent on the structural confirmation of the bacterial protein as the denatured (heat-inactivated) M1 protein had lost this ability. Our study suggests an important role for the bacterial protein in the pathology of severe streptococcal skin infection. Still, we concluded these findings to rather be a streptococcal virulence mechanism than an overwhelming host response. Based on our findings, we believe that M1 protein is an interesting target for drug development.

Paper III

Cold atmospheric plasma disarms M1 protein, an important streptococcal virulence factor.

Cold atmospheric plasma (CAP) has been demonstrated to be a successful antiseptic for chronic and infected wounds. CAP is a partially ionized gas, producing reactive oxygen and nitrogen species, ions, and charged particles as well as thermal and ultraviolet radiation. It is an interesting clinical treatment option because it has antimicrobial activity towards many bacterial species, without any resistance reported, as well as demonstrated well-tolerated by eukaryotic cells. Though CAP-treatment is antiseptic towards all types of bacterial species tested and current research is deciphering its pro-wound healing mechanisms, little is known whether the effect of CAP treatment can abolish the detrimental activity of bacterial virulence factors. We therefore decided to investigate if CAP can modulate the inflammatory activities triggered by the streptococcal virulence factor M1 protein.

We used the kINPen plasma jet, operated with pure argon as gas-supply, to generate CAP. We found that CAP-treatment of the bacterial virulence factor withdraw its capacity to impair wound healing mechanisms, as studied using the *in vitro* scratch assay. CAP-treatment of keratinocytes without addition of bacterial protein was no different in wound healing activities compared to untreated control cells. Also, the IL-

8 secretion and apoptotic turnover was reduced when M1 protein was treated with CAP. Moreover, we found improved cell morphology and viability of keratinocytes added with CAP-treated M1 protein as visualized by scanning electron microscopy. In order to elucidate the mechanisms behind CAP-dependent inactivation of the bacterial virulence factor we employed experiments such as SDS-PAGE and fibrinogen-binding assay, thus resulting in no obvious difference between native and CAP-treated M1 protein. Instead, a collaboration with the Swedish National Infrastructure for Biological Mass Spectrometry (BioMS) made it possible to analyze posttranslational modifications by hydrogen deuterium exchange mass spectrometry (HDX-MS). As CAP is demonstrated to create reactive oxygen or nitrogen species, HDX-MS provides a suitable technique for investigation of oxidative modifications possibly introduced to the M1 protein following CAP-treatment. The main observations were an almost complete oxidation of Met81 in the CAP-treated sample, probably provoking a conformational change of the N-terminal region. Finally, we studied the effect of CAP on fundamental innate immune responses like sustaining a keratinocytic barrier, leukocyte recruitment and phagocytosis, and pro-inflammatory HBP- and IL-6 secretion. Results show an insignificant effect on the host immune system, suggesting that the host response to an infection is not impaired as a consequence of the treatment. To conclude, short CAP-treatments (2-15 s), sufficient for bacterial killing and tissue-tolerability, can also modify and disarm the detrimental activities of the streptococcal M1 protein, reduce M1 protein-triggered inflammation, and improve host recovery during wound healing. Our study presents the first investigation of CAP-modulation of a bacterial virulence factor. Our data support the usefulness of CAP-application to combat skin- or surgery-related bacterial infections, and as an alternative to regular antibiotics. Further, we believe that CAP can be used to destroy the pernicious activity of other virulence factors across different bacterial species. Thus, the data presented in this study may lead to novel concepts for the development of new antimicrobial therapies.

Paper IV

Heparin-binding protein release is strongly induced by Leptospira species and is a candidate for an early diagnostic marker of human leptospirosis.

Leptospirosis is caused by a pathogenic spirochete *Leptospira*, and is one of the most prevalent zoonotic diseases. Leptospires can enter their host via skin or mucosa and rapidly disseminate to surrounding tissues through the bloodstream. Infections vary from non-symptomatic to severe conditions characterized by jaundice, hypotension, acute lung injury, hemorrhages, and multiorgan failure. The pathophysiological mechanisms responsible for leptospirosis symptomatology are poorly understood. The host immune responses and inflammation seem to be main determinants, still there is

a substantial lack of specific host response and bacterial virulence factor interactions. Heparin-binding protein (HBP) is a neutrophilic protein stored in the secretory and azurophilic granules of neutrophils. As HBP is released upon neutrophil activation, it acts as an important multifunctional inflammatory mediator and induce cytoskeletal rearrangements of endothelial cells resulting in increased vascular permeability. Clinical studies have revealed the release of HBP in response to many bacterial diseases and is considered as a biomarker for severe infections like sepsis. Because of the pathological signs, hypotension and multiorgan failure, of severe leptospirosis we hypothesized that HBP release could be involved in leptospirosis disease progression.

By studying HBP levels after addition of leptospira cells or their culture supernatants to human blood, we found that different species of leptospira and their secreted products induce massive HBP release from human polymorphonuclear neutrophils (PMNs). These data also suggest a surface associated and/or secreted Leptospiral virulence factor responsible for the HBP induction. We confirmed the HBP release was triggered through controlled degranulation, excluding cell lysis, by analyzing O_2^- production and LDH leakage from leptospira-treated PMNs. By employing different signal transduction inhibitors and Ca^{2+} chelators, we established that HBP release in human blood induced by leptospira and their secreted products is dependent on the interaction of a receptor like structure at the neutrophil surface involving several downstream intracellular signaling, involving PI3K, p38 MAPK and Ca^{2+} influx. Aiming to identify specific Leptospiral virulence factors responsible of triggering HBP mobilization, we purified membrane proteins from the *L. interrogans* L1-130 by TX-114 solubilization method, following anion exchange chromatography fractionation. Fractions positive in triggering HBP release were analyzed by SDS-PAGE and identified to the proteins Lsa63 and LipL45 by mass spectrometry. These proteins were recombinantly expressed and tested for their ability to stimulate HBP release from PMNs. Our data show that Lsa63 and LipL45 both significantly induced HBP degranulation, with Lsa63 being the most potent, also transduced by controlled signaling pathways as demonstrated previously using whole bacteria stimulation of PMNs. As HBP induces vascular leakage, we tested the endothelial permeability following incubation with PMN exudates triggered by the leptospiral recombinant proteins. All recombinant proteins were able to increase the endothelial permeability indirectly, however, the increased permeability did not correlate with the induced HBP levels, as in this case LipL45-induced exudates was the most aggravating. Finally, we measured HBP levels in sera from patients with leptospirosis during the early and the convalescent phase of the disease. Patients with leptospirosis exhibited high levels of HBP, especially in sera from the early phase. When comparing the HBP levels in sera with other tropical diseases with similar initial symptomatology and also bacterial infections, the mean HBP levels in leptospirosis samples were considerably higher. Therefore, we suggest the use of HBP as an early biomarker of human leptospirosis, complementing the established and definitive specific leptospirosis diagnostic methods

such as polymerase chain reaction analysis of blood samples. This study contributes with specific host response and bacterial virulence factor interactions, previously not reported, to explain some of the pathophysiological mechanisms behind leptospirosis. As suggested, screening HBP levels would be a simple diagnostic test that could greatly enhance the early leptospirosis recognition and improve the rate of successful treatments of leptospirosis in the future.

Concluding remarks

Infection by a bacterial strain can be overcome by the actions of an appropriate host immune response. The very same bacterial strain can trigger a fatal condition in another patient. Such disease progression can be caused by increased bacterial virulence or triggered by an overwhelming host response. The fascinating battle between the two is crucial for the survival of the patient and the bacteria as well. In the end, how do we know what to treat?

As the M1-serotype is overrepresented in invasive streptococcal infections, the initial interactions of the virulence factor M1 protein and human keratinocytes were studied. *Paper I* demonstrate the immunological response of keratinocytes encountering the streptococcal M1 protein. A TLR2-dependent pro-inflammatory signaling pathway was activated, resulting in the release of several cytokines and chemokines, especially IL-8. Comparing the IL-8 induction between different M proteins, M1 and M5 were the only potent triggers, both associated with invasive streptococcal serotypes.

In order to clarify if the M1 protein is involved in aggravating the severity of streptococcal skin infections, *paper II* approached the subtle balance between bacterial virulence and overwhelming host response. Keratinocytes were fortified towards recruited and activated leukocytes, still, the M1 protein completely abolished their wound healing abilities. Taken together, M1 protein can aggravate streptococcal skin infection, however, this would be an act of bacterial virulence alone and not because of a deteriorate immune system.

By studying the host response towards a bacterial virulence factor, *paper I-II* suggest that the streptococcal M1 protein is an interesting target for drug development. *Paper III* establishes a novel benefit of using cold atmospheric plasma (CAP) to combat bacterial virulence in skin infections. In addition to being antiseptic, CAP abolished all detrimental effects caused by the M1 protein, without disturbing fundamental innate immune responses. This novelty might be transferable to other virulence factors across different bacterial species.

The streptococcal M1 protein is a well-known and characterized virulence factor, making it easier to study what host responses it triggers. In *paper IV*, the study was conducted from an opposite direction. By reviewing the symptomatology of leptospirosis, including hypotension and vascular leakage, clues from the

pathophysiological mechanisms was noted and could be confirmed experimentally. Massive HBP release was found when human blood encountered leptospira or their secreted products. Specific leptospiral virulence factors, Lsa63 and LipL45, was identified as responsible for triggering neutrophil degranulation containing HBP. Consequently, endothelial permeability was increased by the actions of these virulence factors. Further, the massive HBP secretion triggered by leptospira and their virulence factors could be used to better diagnose the infection and distinguish it from other tropical diseases.

In order to cause infection, bacterial strains have evolved several mechanisms involving a number of different virulence factors. Host responses are needed to combat any infection, still, overwhelming host responses can lead to an uncontrolled intensification of inflammatory mediators, vascular permeability and subsequent multiorgan failure. These are classic characteristics of sepsis and septic shock. When an infection has progressed into a septic state, symptoms caused by host responses need to be treated. Remaining infections should preferably be treated by controlling bacterial virulence, proliferation and survival without disturbing the host response. Progress in the knowledge of these different aspects of infection biology are essential for future therapeutic advances, including design of new pharmaceutical compounds, antibacterial treatments, regenerative strategies, and correction of inherited disorders.

Future perspectives

Times have passed where most serious infections had a fatal outcome. Times have also passed where serious infections would always be controlled and cured. Since the discovery of antibiotics in late 1920s, conventional antibiotic treatments have been used and misused for several decades. Today, as a result, the bacterial resistance towards conventional antibiotics is increasing, creating an uncertainty on how we will approach infectious diseases in the future.

As already in progress, by limiting the use of conventional antibiotics, we can prolong their antibiotic power. A significant contribution of this work would be better and faster diagnostics, with the ability to identify and discriminate between controlled and severe infections using supervised machine learning. Prospective observational cohort studies can give important clinical patient information to preprogram differences of milder and life-threatening conditions to enable future severity predictions. However, we might need to start measuring completely different parameters to be able to perform such predictions in the future. Identification of causative bacterial strains would allow specific antibiotic treatments, reducing the use of broad spectrum antibiotics and limiting the development of antibiotic resistance. One approach to develop such diagnostics is by searching for biomarkers within the host response that is specific or distinguishable for particular microbes or infections, as we showed in *paper IV*. I also want to emphasize the interesting future of mass spectrometry analysis. This technique is very sensitive and specific in identifying bacterial species and it is much faster than conventional diagnostics. Several mass spectrometric-based techniques are already established, but they come with a significant cost and requires highly educated personnel to be run. Still I believe that every hospital or microbiological laboratory will have a mass spectrometer running in the future.

Further, I consider that studying bacterial virulence and host responses allows the recognition of interesting targets for drug development to either reduce symptomatology triggered by the host or to limit bacterial dissemination. Still, most interestingly would be to find completely new approaches to eliminate bacteria and disarm their virulence factors. The results from deactivation of bacterial virulence factors by intravenous administration of immunoglobulins to patients suffering from STSS has been controversial and unfortunately not yet verified additional clinical benefits over conventional antibiotics. As outlaid in this thesis (*paper I-III*), cold atmospheric plasma (CAP) could be a very interesting antiseptic tool to prevent the

development of serious skin infections or surgery-related infections. Surgical site infections (SSIs) alone cost the society about 0,5 - 1 billion SEK every year and consumes lots of antibiotic prescriptions (Socialstyrelsen, 2006, Art.nr 2006-123-12). I also believe that CAP is an interesting tool to use within the hospital care in the future to limit contact-dependent bacterial spread, like we use ethanol today. As we want to continue our abilities to perform surgeries, cure serious infections and save patients, we are in great need of new innovations. To this end, there is only research and creativity to rescue us.

Populärvetenskaplig sammanfattning

Människan har utvecklats i symbios med bakterier sedan begynnelsen. Att kunna skilja mellan opportunistiska och patogena bakterier har varit avgörande för vår överlevnad och har bidragit till hur vårt immunsystem är uppbyggd idag. Under evolutionens gång har vårt immunsystem lärt sig att ständigt hålla sig alert och övervakande efter invaderande mikroorganismer och oskadliggöra dem för att hålla oss friska. För att upprätthålla denna funktion har immunförsvaret utvecklat olika typer av försvarsmekanismer. För att kunna studera samspelet mellan människan och bakterier har forskningen och läkarsamfundet delat in våra försvarsmekanismer i de fysiska barriärerna, såsom huden och våra slemhinnor, det medfödda och det adaptiva immunförsvaret.

Den fysiska barriären ansågs tidigare inte vara en del av vårt immunförsvar. Men med tiden har vi förstått dess betydelse och aktiviteter för att hålla borta inkräktare. En intakt och frisk fysisk barriär är avgörande för att förhindra uppkomsten av infektioner och för vårt allmäntillstånd. Det medfödda immunförsvaret är ett snabbt och reaktivt skydd som ingriper likartat oavsett infektion och agerar genom vad vi associerar till inflammation. Cellulära medlare och deras kemiska signaler kan aktivera rodnad, svullnad, klåda och feberkänsla vid bekämpning av mikroorganismer. Men mikroorganismer har också utvecklat en arsenal av motverkande försvarsmekanismer för sin överlevnad, så kallat virulensfaktorer, och då dessa kan vara mycket listiga och specifika, så räcker inte alltid det medfödda immunförsvaret till. Därför har människan och andra däggdjur också utvecklat det adaptiva immunförsvaret. Detta system är mer återhållsamt och tidskrävande vid första påträffandet av en specifik patogen. Men det vidhåller ett utmärkt minne och reagerar väldigt effektivt vid framtida infektioner utav samma patogen, och kan då till och med föregå oss obemärkta.

Vi blir medvetna om vårt immunförsvars brister vid till exempel uppkomsten av eksem, utslag, allergi, autoimmuna sjukdomar eller återkommande infektioner. Men för majoriteten av människor fungerar vårt skydd i högsta grad. För vissa infektioner kan immunförsvaret själv utlösa livsfarliga tillstånd så som vid sepsis, även kallat blodförgiftning. Sepsis är när kroppens immunförsvar överreagerar på en infektion som nått blodomloppet och skapar massiv aktivering av immunsystemet och okontrollerad utsöndring av dess kemiska signaler, exempelvis heparin-bindande protein (HBP). Detta kan orsaka blodtrycksfall och omfattande syrebrist i kroppens organ som slutligen kan leda till att kroppen upphör att fungera. Denna typ av infektion tillskrivs ofta grupp

A-streptokocker, *Streptococcus pyogenes*. Denna bakterieart är en allmän sjukdomsalstrare och orsakar vanligtvis halsfluss och vissa hudsjukdomar såsom svinkoppor och rosfeber. Men vid oförutsedda omständigheter orsakar den även allvarliga tillstånd som cellulit, nekrotiserande fasciit, sepsis och barnsängsfeber.

Denna avhandling fokuserar på samspelet mellan den fysiska barriären i form av vår hud och det medfödda immunförsvaret vid påträffande av *S. pyogenes*. Invasiva och livshotande infektioner har ofta associerats med *S. pyogenes* serotyp M1. Denna skadliga bakterie innehar ofantligt många virulensfaktorer för att överleva i människan. Men en av de mest potenta och mest studerade är virulensfaktorn M1 protein. Streptokockers cellyta är till största del täckt av M protein som tränger igenom cellmembranet och cellväggen och kan även frisättas från bakterieytan. Man har delat in streptokocker i serotyper beroende på uppbyggnaden av deras M proteiner och idag finns över 250 olika beskrivna.

Det första delarbetet i denna avhandling (***Paper I***) har studerat hur keratinocyter, den mest förekommande celltypen i huden, reagerar vid påträffandet av M1 protein och demonstrerar hudcellernas immunologiska deltagande för att varna och aktivera det medfödda immunsystemet för streptokockinfektion. Arbetet visar hur M1 protein binder till en immunologisk receptor på hudcellernas yta som sätter igång en signalkaskad för att slutligen frisätta kemiska signaler vilka är viktiga medlare för det medfödda immunförsvaret. Denna aktivitet var uteslutande för M-serotyper associerade med invasiva streptokockprofiler, M1 och M5, och aktiverades inte av andra M proteiner som testades. I det andra delarbetet (***Paper II***) studerades balansen mellan bakteriell virulens och hudskada vållat av det egna immunförsvaret. Då hudcellernas immunologiska aktivitet var specifikt riktat mot farligare M-serotyper, kännetecknades aktiviteten som en eventuell förklaring till varför dessa M-serotyper kan orsaka allvarligare och djupare hudinfektioner. Konsekvenserna av det medfödda immunförsvarets aktivitet gentemot M1 protein skulle därför utredas vidare. Hudcellernas integritet och vitalitet studerades efter att dem stimulerats med M1 protein och aktiverade leukocyter, cellerna som ingår i det medfödda immunsystemet, men ingen skada kunde relateras till oproportionerlig immunaktivering. Istället identifierades många frisatta faktorer från M1-stimulerade hudceller som verkar skyddande och oskadliggörande av immunförsvarets antibakteriella attacker. M1 proteinet hade heller ingen demolerande effekt på hudcellerna, men däremot så förstörde virulensfaktorn de sårläkande egenskaperna hos hudceller. Konklusionen av detta blev att M1 protein kan medverka till att förvärra hudinfektioner, men att det helt och hållet beror på dess bakteriella virulens och inte av ett oreglerat immunförsvar.

Kall atmosfärisk plasma är en joniserad gas och antiseptisk teknik som utvärderas för tillämpning inom sårläkning och behandling av ytliga infektioner. Tekniken har varit framgångsrik för att läka kroniska infektioner och ingen resistensutveckling hos bakterier har förekommit. Däremot har ingen tidigare studerat om eller hur kall

atmosfärisk plasma interagerar med specifika virulensfaktorer från bakterier. Det tredje avhandlingsarbetet (***Paper III***) utreder om kall atmosfärisk plasma kan modulera M1 proteinet och hur det modifieras av behandlingen. Arbetet visar att kall atmosfärisk plasma kan avvärja alla skadliga och inflammatoriska effekter som M1 proteinet framkallar utan att påverka det egna immunförsvarets aktiviteter. Verkningsmekanismen identifierades till att plasmabehandlingen inducerar en oxidation av aminosyran metionin81 som troligen orsakar en konfirmations-förändring av proteinets N-terminala sida. Metoden är ett intressant alternativ till konventionella antibiotika och mycket potent då en behandling på 2–15 sekunder är tillräckligt för både antivirulent och antibakteriell aktivitet. Dessutom är den antivirulenta aktiviteten troligen tillämpbar på andra bakteriella virulensfaktorer. Denna studie kartlägger en av plasmabehandlingens fördelaktigheter och stödjer kall atmosfärisk plasma som behandlingsalternativ och prevention av hud- och operationsrelaterade infektioner.

Det sista avhandlingsarbetet (**Paper IV**) behandlar sjukdomen Leptospiros, en infektion som orsakas av bakterier från släktet Leptospira och sprids från djur till människa. Leptospira smittar genom kontakt med hud och slemhinnor, och sprids sedan vidare inuti kroppen. Symptomen kan variera från milda med huvudvärk, muskelvärk och feber, till väldigt allvarliga och livshotande såsom gulsot, meningit lungblödning, blodtrycksfall och organsvikt. Patofysiologin bakom Leptospiros är ännu inte väldokumenterad och virulensmekanismerna för Leptospira är näst intill okända. Därmed har detta delarbetet fokuserat på att studera hur Leptospira interagerar med det medfödda immunsystemet och identifiera de ansvariga virulensfaktorerna. Arbetet redovisar en massiv frisättning av HBP från neutrofiler i mänskligt blod när det kommer i kontakt med Leptospira bakterier. HBP är en inflammatorisk molekyl som även ökar blodkärlens genomsläpplighet. Under kontrollerade omständigheter underlättar HBP för leukocyter att vandra igenom kärlväggen från blodomloppet ut i vävnaden där infektion har uppstått. Vid en okontrollerad situation och massiv HBP frisättning kan det istället leda till blodtrycksfall och organsvikt, karaktäristiskt för både sepsis och allvarlig Leptospiros. HBP frisättningen i respons mot Leptospira kartlagdes till en aktiv utsöndring från neutrofilerna och kunde härledas till två stycken membranbundna virulensfaktorer, Lsa63 och LipL45. Höga HBP nivåer uppmättes även i serum från patienter med Leptospiros. Dessa nivåer jämfördes med andra tropiska infektioner, vilka var anmärkningsvärt lägre, och föreslår att HBP-mätning kan användas som biomarkör för att urskilja Leptospiros från andra tropiska infektioner och därmed förbättra den terapeutiska behandlingen.

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It is the mark of an educated mind to be able to entertain a thought without accepting it.

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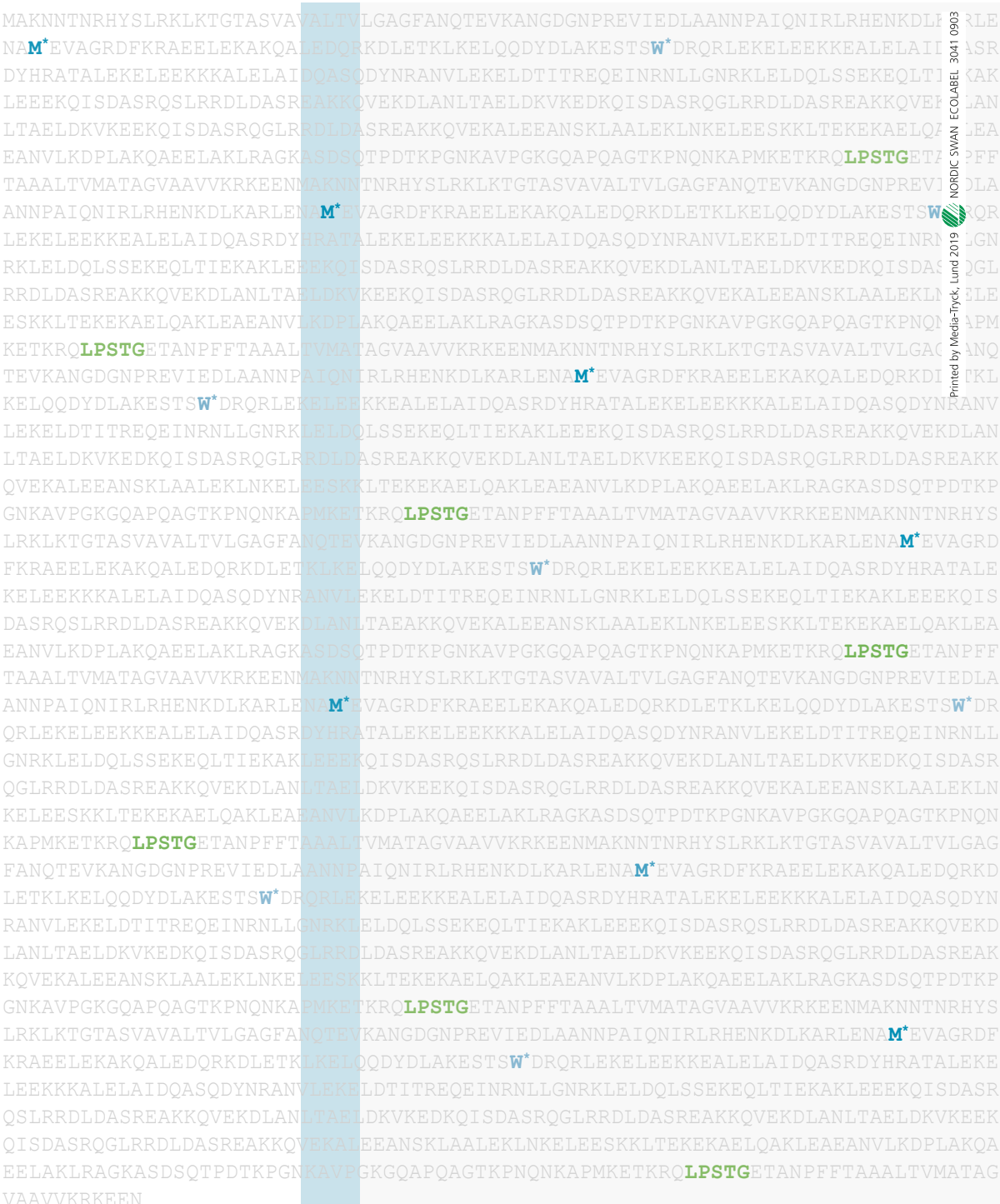

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