Bacteria – host interplay in Staphylococcus aureus infections

Jakub Kwiecinski

Department of Rheumatology and Inflammation Research
Institute of Medicine
Sahlgrenska Academy at University of Gothenburg

UNIVERSITY OF GOTHENBURG

Gothenburg 2013

Cover illustration by Katarzyna Babis

Bacteria – host interplay in Staphylococcus aureus infections

jakub.kwiecinski@rheuma.gu.se

ISBN 978-91-628-8713-1

Printed in Gothenburg, Sweden 2013
Ale Tryckteam AB, Bohus

To my Parents

Bacteria – host interplay in Staphylococcus aureus infections

Jakub Kwiecinski

Department of Rheumatology and Inflammation Research, Institute of Medicine
Sahlgrenska Academy at University of Gothenburg
Göteborg, Sweden

ABSTRACT

Staphylococcus aureus infections are a major healthcare challenge and new treatment alternatives are needed. The key to new therapies is understanding the interplay between bacterial virulence factors and host immune response, which decides on disease outcome. *S. aureus* produces numerous virulence factors. Among them are the surface proteins and soluble factors, like staphylokinase (Sak) – a protein activating host plasminogen. Recently characterized subset of leukocytes, the natural killer T-cells (NKT) respond rapidly to bacterial challenge and link innate and adaptive immunity. Activation of NKT cells might possibly affect the outcome of *S. aureus* infections.

In this thesis, I explored the role of certain bacteria components (surface proteins, Sak) and host factors (NKT cells, plasminogen) during infectious process. Various mouse infection models (*S. aureus* skin infections, septic arthritis, and sepsis), as well as in vitro models and collections of clinical bacterial isolates were used.

Staphylococcal surface proteins were crucial for establishment of abscess-like skin infection in mice. Activation of host plasminogen by Sak was an important element for staphylococcal invasion into the skin and establishment of new infectious sites. However, once infection was established, Sak diminished the infection severity and reduced the damage. Benifical effect of plasminogen activated by Sak was also observed in *S. aureus* systemic infection. On the host side, the NKT cells were involved in experimental *S. aureus* sepsis, but they didn't appear to have a significant impact on the disease outcome. However, sulfatide treatment activating the type II NKT cells significantly reduced mortality in experimental *S. aureus* sepsis.

Staphylococcal infection is a complex process, regulated by various staphylococcal factors interacting with host: both by surface proteins and by secreted proteins like Sak. Those bacterial factors might be potential future treatment targets for limiting disease severity. Another potential treatment strategy is to activate type II NKT cells, which downregulates exaggerated immune response in *S. aureus* sepsis, leading to less tissue damage and better survival.

Keywords: Staphylococcus aureus, staphylokinase, NKT cells, surface proteins

ISBN: 978-91-628-8713-1

SAMMANFATTNING PÅ SVENSKA

Staphylococcus aureus är en farlig bakterie. Den orsakar många slags infektioner, till exempel hudinfektioner, ledinfektioner och livshotande blodinfektioner. För att utveckla bättre sätt att behandla och förhindra sådana infektioner, behöver vi förstå hur *S. aureus* kan orsaka dem, och hur våra kroppar försvarar sig mot denna bakterie. Målet för denna avhandling var att upptäcka hur *S. aureus* interagerar med värden (det vill säga, med oss) under infektion, och hur olika faktorer som produceras av bakterien interagerar med faktorer som produceras av våra kroppar. Denna avhandlings fynd kretsar kring tre ämnen:

1. Ytproteiner. På ytan av bakteriecellen finns många proteiner. *S. aureus* använder dem för att interagera med omgivningen, för att binda till ämnen i vår kropp och försvara sig mot vårt immunsystem. I denna avhandling visar jag att dessa ytproteiner bidrar till framkallandet av hudinfektion.

2. Stafylokinas. *S. aureus* kan aktivera det humana fibrinolytiska systemet (systemet som är ansvarigt för att lösa upp blodkoagel, men det kan också lösa upp många andra strukturer i kroppen). Stafylokocken aktiverar det fibrinolytiska systemet genom att sekreera en speciell molekyl som kallas stafylokinas. I denna avhandling upptäckte jag att detta fibrinolytiska system används av bakterien för att penetrera in i huden och orsaka infektion. Tack vare stafylokinas kan *S. aureus* helt enkelt lösa upp barriärer och ta sig in i kroppen. Däremot, när bakterien väl tagit sig in, börjar stafylokinas agera till dess nackdel, och gör infektionen (både hudinfektion och blodinfektion) mindre allvarlig.

3. NKT-celler. En särskild grupp celler i vårt immunsystem, NKT-cellerna, ansvarar för att koordinera vårt försvar mot bakterier. I denna avhandling fann jag att om dessa celler tas bort gör det ingen skillnad för hur allvarlig en blodinfektion blir – trots deras förmodade roll i antibakteriellt försvar. Däremot, jag fann också att om vi använder särskilda läkemedel som stimulerar NKT-celler, för att göra dem mer aktiva, så ökar dessa överlevnaden vid blodinfektion orsakad av *S. aureus*.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Kwiecinski J, Josefsson E, Mitchell J, Higgins J, Magnusson M, Foster T, Jin T, Bokarewa M. Activation of plasminogen by staphylokinase reduces the severity of *Staphylococcus aureus* systemic infection.
J Infect Dis 2010; 202: 1041-1049.

II. Kwiecinski J, Jacobsson G, Karlsson M, Zhu X, Wang W, Bremell T, Josefsson E, Jin T. Staphylokinase promotes the establishment of Staphylococcus aureus skin infections while decreasing disease severity.
Accepted for publication in J Infect Dis, 2013.

III. Kwiecinski J, Jin T, Josefsson E. Surface proteins of Staphylococcus aureus play an important role in experimental skin infection in mice.
Manuscript

IV. Kwiecinski J*, Rhost S*, Löfbom L, Månsson JE, Cardell SL#, Jin T#. Sulfatide attenuates experimental *Staphylococcus aureus* sepsis through a CD1d dependent pathway.
Infect Immun 2013; 81: 1114-1120.

*, # - contributed equally

CONTENT

ABBREVIATIONS

ClfA, ClfB	clumping factor A, clumping factor B
CRP	C-reactive protein
DIC	disseminated intravascular coagulation
Eap	extracellular adherence protein
EDIN	epidermal cell differentiation inhibitor
ET	exfoliative toxin
FnbA, FnbB	fibronectin binding protein A, fibronectin binding protein B
ICAM	intercellular adhesion molecule
IL	interleukin
LPS	lipopolysaccharide
MeSH	medical subject heading index
MHC	major histocompability complex
NK cell	natural killer cell
NKT cell	natural killer T cell
PAI	plasminogen activator inhibitor
plg	plasminogen
Sak	staphylokinase
SasF	*Staphylococcus aureus* surface protein F
SE	staphylococcal enterotoxin
SEl	staphylococcal enterotoxin-like toxin

Spa	staphylococcal protein A
TNF-α	tumor necrosis factor α
tPA	tissue-type plasminogen activator
TSST	toxic shock syndrome toxin
uPA	urokinase-type plasminogen activator
vWbp	von Willebrand factor-binding protein

1 INTRODUCTION

1.1 Infections and *Staphylococcus aureus*

Infectious diseases plagued humankind all through the history [1]. Only during the last decades and in the western countries, infectious diseases ceased to be the main cause of death, replaced by cancer and cardiovascular diseases [2]. However, infections still remain amongst the leading causes of death, with microbial sepsis alone responsible for nearly 10% of deaths in USA [3]. In the future, threats caused by microorganisms might unfortunately again become even more serious due to increasing bacterial resistance to chemotherapy and to growing numbers of elderly and immunocompromised individuals, who are especially susceptible to infections. Ironically, physicians sometimes win the battles with cancer or save seemingly hopeless cases in intensive care units, but later loose the fight with common microbes.

One of the leading pathogens responsible for infections nowadays is *Staphylococus aureus*. In developed countries, it is the most prevalent species isolated from infections of hospital inpatients, and one of the most frequently isolated from outpatients [4-5]. It is also a major, though frequently ignored, source of diseases in developing countries [6]. It can cause a wide range of infections: both minor and life-threatening, local and systemic, acute and chronic. Among them are the subjects of this thesis, including sepsis/bacteraemia, septic arthritis, and skin and soft tissue infections.

1.1.1 Hallmarks of *S. aureus* infections

Certain characteristic features are shared by all *S. aureus* infections. It is usual for this pathogen to cause metastatic infections: spreading from one infectious foci to the neighbouring tissues or to distant organs, through bloodstream [7]. Staphylococcal infections also frequently become chronic, and tend to recur at later time [8-11]. Finally, severe *S. aureus* infections often cause disease sequelae, leaving patients with permanent or long-lasting disabilities and organ damages [10, 12].

1.1.2 S. aureus sepsis

Sepsis is nowadays a leading cause of mortality in hospital intensive care units [3], and *S. aureus* is the most common cause of bloodstream infections [13]. Even with proper treatment, staphylococcal bacteraemia has 10-29% mortality rate, reaching 56% mortality if septic shock develops[14-15].

Bacteremia (presence of bacteria in blood) can lead to development of sepsis, that is the systemic inflammatory response occurring during infection [16]. The host mounts a disproportionate immune response to bacteria, leading to an excessive systemic inflammation that damages many organs. Due to the loss of immune regulation and to subsequent anti-inflammatory response induced by excessive inflammation, a severe immunosupression develops in later stages, allowing bacteria to multiply freely. Staphylococci from blood may spread to numerous organs leading to metastatic infections [17]. Inflammation in sepsis is associated with severe hemostatic abnormalities in form of excessive coagulation, leading to a disseminated intravascular coagulation (DIC) in peripheral tissues [18]. This is followed by a shortage of coagulation factors and platelets, consumed by the uncontrolled coagulation [18], what can lead to severe bleeding. The combination of damage caused by inflammation, coagulation, and bacterial growth leads to multiple organ dysfunction, shock and eventually death.

1.1.3 *S. aureus* arthritis

Infectious arthritis (joint infection) is a potentially devastating condition [12, 19]. The most common microorganisms causing it is *S. aureus* [12, 19]. Bacteria could reach the joint by spreading from a neighboring bone or soft tissue infection, or can be directly introduced by a foreign body trauma, but the most common way is the hematogenous route. *S. aureus* present in the blood (from bacteraemia or from other infectious foci) reaches synovial capillaries, and from them, in a manner not yet understood, it moves inside the joint cavity and into synovium [19]. Once inside the joint, the pathogen will multiply, leading to recruitment of immune cells and outburst of inflammation. The activity of bacterial products and host factors induced by inflammation together lead to destruction of cartilage, and – if not stopped – will eventually cause bone remodeling, destruction of joint surface, bone ankyloses and joint contracture [19]. Joint inflammation will persist even after the infection is cleared, and activity of immune cells will perpetuate the joint destruction process [12]. During septic arthritis, there is a significant risk of further bacterial spread from infected joints to blood and other tissues, what sometimes leads to sepsis and death [19].

1.1.4 *S. aureus* skin and soft tissue infections

S. aureus is the leading pathogen responsible for skin and soft tissue infections [20-21]. Staphylococcal skin infections are a big and varied group [21-22]. The minor ones are impetigo (infection of epidermis), ecthyma (severe impetigo, with involvement of dermis) and folliculitis (infection of hair follicles) [21]. Deep follicullitis can transform into a more severe

infection: furuncles. Skin abscesses, probably developing from minor skin infections, are known as carbuncles and are associated with a marked pus accumulation. Systemic spread of bacteria from those abscesses is not uncommon [21]. Infection of subcutaneous fat – cellulitis – can be limited, but it can also develop into a severe case with significant mortality [14, 21]. Infection of muscles, pyomyositis, occurs mainly in tropical countries and is associated with enormous pus accumulation in infected tissue [14, 21]. *S. aureus* has also recently become a common cause of a necrotizing fasciitis [23-24]. This rare infection of deep skin and subcutaneous tissues is a quickly progressing necrosis, spreading along the fascial plane, frequently leading to sepsis and death, or leaving survivors with an extensive body damage [23-24]. *S. aureus* is also one of the leading causes of skin wound infections, both in cases of chronic wounds [25] and wounds due to surgery or trauma [26]. Although bacteremia is more common in the severe cases of skin infections, even the mild superficial cases carry a risk of systemic spread. Therefore skin and soft tissue infections are the most commonly reported sources of systemic bacteraemia [27].

1.1.5 *S. aureus* colonisation

Despite its dangerous potential, *S. aureus* in most people cause only mild infections or asymptomatic colonization. About 20% of the population is persistently colonized, and further 30% are intermittently colonized [28]. The most common site for *S. aureus* carriage are the anterior nares of the nose [28]. Colonization could be also found in certain areas of skin, in pharynx and perineum, on hands, or even less frequently – in vagina and axillae [28]. There is also an increasing prevalence of gastrointestinal carriage of *S. aureus*, especially in infants, probably related to changing lifestyles [29].

1.1.6 Treatment of *S. aureus* infections

There are three main approaches to treatment of staphylococcal infections [7]. The primary goal is the removal of infecting bacteria as well as damaged tissues and inflammatory infiltrates – therefore abscesses are drained, infected joints undergo lavage, and if necessary a larger scale debridement is performed in soft tissue infections. In minor cases, like superficial abscesses, this might be sufficient and no other treatment is needed. Usually an additional approach – antibiotic therapy – is necessary, though increasing resistance of *S. aureus* to common chemotherapeutics makes this challenging. Finally, a supportive treatment is needed to maintain homeostasis if organ dysfunctions develop during infection.

Possibilities of disease prevention are limited to controlling the spread of multi-resistant strains, isolation of patients spreading bacteria in hospital environments and elimination of staphylococcal colonization in high-risk groups by an aggressive chemotherapy [7].

Perspectives for future treatment and of *S. aureus* diseases are bad. The vaccine development is a history of repeated failures [30]. Since introduction of antibiotics, no new concepts in treatment of staphylococcal diseases have appeared. Even in case of antibiotics, the future is not bright. As the pharmaceutical industry paid little attention to development of new antibacterial compounds in recent decades (preferring to concentrate on more profitable activities [31]), the new drugs are being developed too slow to catch up with the increasing bacterial resistances. There has been a tremendous increase in understanding of mechanisms involved in pathogenesis of staphylococcal infections, but all this knowledge about biology and virulence of *S. aureus* didn't lead to development of new drugs or treatments. There has been a significant increase in survival of severe staphylococcal infections over the last decades of the 20th century [32], probably reflecting faster diagnostics and improved life-support techniques. There is, however, no further decrease in mortality in the 21st century [33], so one might wonder, if we have already reached the limit of what can be done to fight *S. aureus*.

1.2 Virulence factors of *Staphylococcus aureus*

S. aureus is an interesting pathogen, possessing numerous virulence factors and showing extensive adaptation to the host's attacks [7].

1.2.1 Cell wall

S. aureus cells are cocci, with a diameter of approximately 1 μm. The cell is surrounded by a typical gram-positive cell wall, of 20 - 40 nm thickness. Peptidoglycan, the main component of the staphylococcal cell wall, is a polymer of alternating β-1,4- linked N-acetylglucosamine and N-acetylmuramic acid, with attached tetrapeptides composed of L-alanine, D-glutamine, L-lysine and D-alanine. Tetrapeptides attached to neighboring polymers are cross-linked by 5-glycine bridges, turning the entire structure into one big scaffold surrounding the cell [2]. In addition to peptidoglycan, the other important components of the cell wall are teichoic acids: polymers

of ribitol phosphate. They are either attached to peptidoglycan, or to the lipids of the cell membrane (then they are known as lipoteichoic acids) [2]. Specific modifications of the staphylococcal cell wall (including O-acetylation of N-acetylmuramic acid and modifications of teichoic and lipoteichoic acids reducing the surface's negative charge) makes it resistant to antibacterial host protein lysozyme and less susceptible to defensins, lactoferrins and myeloperoxidase [34]. When sensed by immune receptors, the staphyloccal cell wall induces a strong inflammatory response [7]. The surface of the cell wall is additionally covered with polysaccharide capsule, which provides defense from phagocytosis [7].

1.2.2 Toxins

S. aureus secrets a wide array of toxins, which can be divided into three main groups: membrane-active agents, superantigens and Rho-inactivating toxins.

The first group are "lysing toxins": α-toxin, β-toxin, leukotoxins like γ-toxin or Panton-Valentine leukocidin, and phenol-soluble modulins, like δ-toxin [35]. All of them interact with membranes of host's cells and – under some conditions – can cause lysis of those cells. Some, like α-toxin, can target various cell types and lead to massive damage in infected sites. Other, like the leukotoxins, are more specific and target mainly leukocytes, blocking the immune response. In addition to damaging host cell's membranes, those toxins have also other properties: for an example α-toxin and leukocidins when used at sub-lytic concentrations can directly stimulate inflammatory responses and lead to cytotoxicity without the membrane damage [35]. Phenol-soluble modulins also induce inflammation and stimulate chemotaxis of leukocytes [35]. Surprisingly, some of the toxins also act as adhesion molecules or play a role in biofilm formation [36-39].

Superantigens are molecule causing a massive, non-specific, polyclonal activation of T-cells and subsequent massive cytokine release and deregulation of immune response. This is achieved by staphylococcal superantigens binding to Major Histocompability Complex (MHC) molecules on the surface of antigen presenting cells and to the T-cell receptor. As a result, there is a crosslinking of MHC and the T-cell receptor, sending an activation signal to T cell irrespective of its antigen specificity. Exact mechanism of this activation is still not clear, and probably additional interaction of superantigens with CD28 co-receptor on T-cells's surface is also involved [40]. *S. aureus* secretes various toxins with extremely high superantigenic potential: toxic shock syndrome toxin 1 (TSST-1), staphylococcal enterotoxins (SE) A-E, G-J and staphylococcal enterotoxin-

like toxins (SEl) K-R, U, U2 and V [35, 41]. In addition to superantigenicity, SEs (but not SEls) cause typical food poisoning after ingestion. Whether or not this is independent of their superantigenic activity is debatable [35, 41]. Also exfoliative toxins (ET) A, B and D have some superantigenic properties, but they seem to be weak. The main action of ETs is that of specific proteinases, damaging the epidermis by cleaving desmosomes in the basal epidermis layer [35].

Rho GTPases are important regulators of cytoskeleton activity in eukaryotic cells. Their inactivation leads to numerous abnormalities, including changes in cell shape and movements. Some *S. aureus* strains secrete toxins with such Rho-inactivating capacity. Those are known as epidermal cell differentiation inhibitors (EDIN) A, B and C [42]. They block keratinocyte differentiation *in vitro*, but probably *in vivo* their activity is directed against various types of cells and not limited to epidermis.

1.2.3 Enzymes and other secreted molecules

S. aureus secretes numerous extracellular enzymes, which digest or modify host tissues and host proteins: proteases, lipases, fatty-acid modifying enzyme, catalase, hyaluronidase and nucleases. Those enzymes help bacteria in tissue penetration, digest complex molecules to provide nutrients and inhibit activities of the immune system [43].

In addition to enzymes, staphylococci secrete also other proteins, which are supposed to interact with host tissues and immune system. Examples are staphylococcal complement inhibitor [34, 44], chemotaxis inhibitory protein of *Staphylococcus aureus* [34] and many other molecules.

S. aureus secretes also coagulases and staphylokinase – molecules interacting with host's coagulation and fibrinolysis.

1.2.4 *S. aureus* effects on coagulation and fibrinolysis

Control of blood clot formation ("coagulation") and its subsequent dissolution ("fibrinolysis") is essential for keeping hemostatic balance in human body [45-46]. During coagulation, a cascade of coagulation factors leads to activation of prothrombin (inactive zymogen) into thrombin. Thrombin, then, turns soluble fibrinogen into insoluble fibrin, which is additionally stabilized by crosslinking by activated factor XIII. In this way, a fibrin mesh (the clot) is formed. During fibrinolysis, an inactive zymogen, plasminogen (plg), is turned into an active plasmin by tissue-type

plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA). Also some products of coagulation cascade (eg. kallikrein) can activate plg. Plasmin cleaves fibrin into soluble fibrin degradation products (e.g. D-dimer) and so removes the clot. Activity of tPA and uPA is inhibited by plasminogen activator inhibitors 1 and 2 (PAI-1, PAI-2). Plasmin is directly inhibited by α-2-antiplasmin, α -2-macroglobulin and thrombin activatable fibrinolysis inhibitor. A careful balance of coagulation and fibrinolysis ensures that neither hemorrhages, nor unnecessary coagulation (like DIC) occurs [45-46].

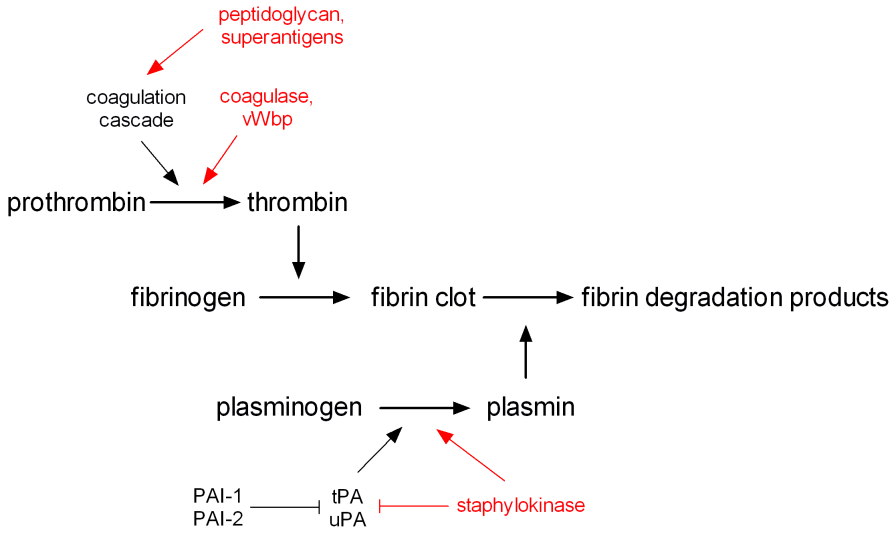


Figure 1. Effects of S. aureus on coagulation and fibrinolysis. Selected components of human coagulation and fibrinolytic system and their interactions are colored black. Staphylococcal components and interactions with human system are colored red. Pointed arrows indicate transition or stimulation. Blunt-ended arrows indicate inhibition. Explanation of abbreviations in the text.

S. aureus has numerous strategies to interact with the coagulation and fibrinolytic pathways and to "hijack" them (Fig. 1). Cell wall peptidoglycan and superantigenic toxins can induce the coagulation cascade indirectly, by initiating inflammation and stimulating blood mononuclear cells, what in turn triggers the cascade [47-48]. *S. aureus* can also exert much stronger effect: its secreted proteins, coagulase and von Willebrand factor-binding protein (vWbp) activate prothrombin into thrombin and directly initiate coagulation and fibrin deposition [49-50]. Also in respect to fibrinolysis, *S. aureus* can control the host's mechanisms. Staphylokinase (Sak) secreted by bacteria

activates plg to plasmin and induces clot lysis [51]. At the same time, Sak competes out the activity of host activators tPA and uPA, giving bacteria complete control over the fibrinolytic system [52]. Notably Sak has also a puzzling activity not related to fibrinolysis: it protects bacteria from antimirobial peptides, α-defensins [53], by binding with them. Intriguingly, this binding results in Sak losing its capacity to activate plg [54].

The staphylococcal capacity to coagulate blood and deposit fibrin is very important for the virulence [49, 55]. However, the virulent effects of Sak and fibrinolysis induced by bacteria are not investigated yet.

1.2.5 Surface proteins

A characteristic group of staphylococcal surface proteins are the "surface-anchored" proteins. A common trait of most of them is the presence of a conserved C-terminal sorting signal, containing an LPXTG sequence. After the protein is secreted through the cytoplasmic membrane, this sequence is recognized by sortase enzymes, which cleaves the sequence and subsequently covalently attaches the protein to a 5-glycine bridge in the cell wall peptidoglycan. *S. aureus* has two such sortases: sortase A and B, with sortase B attaching solely IsdC protein, while sortase A is responsible for all other proteins [56-57]. Many of the surface-anchored proteins had been identified and studied up till 2001. After that, search of sequenced staphylococcal genomes identified even more putative surface-anchored proteins carrying LPXTG sequence, named *Staphylococcus aureus* surface (Sas) proteins: SasA-SasK. Several of those were later studied in detail and given more specific names.

Most of the surface-anchored proteins act as adhesins or bind host proteins and structures. Many of them also interact with the host's immune system. List of known binding ligands and activities of surface-anchored proteins is presented in Table 1.

Table 1. Surface-anchored proteins of S. aureus

Protein	Binds to ligand	Known activities
Protein A Spa	IgG, IgM, TNF-α receptor [58] IgE [59] platelet receptor gC1qR/p33 [60] von Willebrand factor [61]	prevention of antibody-mediated phagocytosis, activation of B-lymphocytes, induction of inflammation [58] activation of mast cells [59] inhibition of osteoblast activity [62-63]
Fibronectin binding proteins A and B FnbA, FnbB	fibronectin, elastin, fibrinogen [58] Hsp60 [64]	adhesion and invasion into host's cells [58]
Clumping factor A ClfA	Fibrinogen [58] platelet membrane 118 kDa protein [65]	inactivation of complement C3b opsonin [66] prevention of phagocytosis [58, 66] adhesion to host's cells [58] platelet aggregation [67-68]
Clumping factor B ClfB	fibrinogen, cytokeratin [58] loricrin [69]	adhesion to host's cells [58, 70] nasal colonization [69, 71] platelet aggregation [68]
Collagen adhesin Cna	collagen [58]	adhesion to cartilage [58]
Serine-aspartate repeat-containing protein C, D and E SdrC, SdrD, SdrE	complement factor H [72]	adhesion to host's cells [70] evasion of complement [72] platelet aggregation [68]
Bone sialoprotein binding protein Bbp	bone sialoprotein [73]	-
Plasmin-sensitive protein Pls	lipids and glycolipids [58, 74]	-
Serine-rich adhesin for platelets SraP	?	adhesion to platelets [75]
Iron-regulated surface determinant A, B, C and H IsdA, IsdB, IsdC, IsdH	fibrinogen, fibronectin, fetuin, haemin, haptoglobin, transferring, hemoglobin [58] platelet integrin GPIIb/IIIa [76]	heme acquisition [77] adhesion and invasion into host's cells [70, 76, 78] prevention of phagocytosis [79]

S. aureus surface protein F SasF	?	resistance to fatty-acids [80]
S. aureus surface protein G SasG	?	adhesion to host cells [81-82] biofilm formation [81]
S. aureus surface protein X SasX	?	adhesion to host cells, biofilm formation [83]
S. aureus surface proteins SasB, SasC, SasD, SasH, SasK	?	-
Biofilm-associated protein Bap	Hsp90 [84]	biofilm formation [85] decrease internalization into host's cells [84]

In addition to surface-anchored LPXTG proteins, on the staphylococcal cell surface there are also many other noncovalently attached proteins [58]. Those molecules are not as well characterised and identified as LPXTG-containing proteins. Examples of them are extracellular adherence protein (Eap) and proteins involved in plg binding.

Eap is a multifunctional protein, it acts both as an adhesin and as an immunomodulatory molecule [86]. Eap was shown to bind various plasma proteins, extracellular matrix structures and cell surfaces [86]. It also interacts with the immune system in several ways: 1) it blocks binding of leukocytes to ICAM-1 and therefore prevents their extravasation from circulation to infection area; 2) it interacts with antigen-presenting cells, changing the pattern of acquired immune response; and 3) it induces T-cell death [86]. The overall effect of those interaction is unclear, and perhaps dependent on timing and concentrations of Eap. It is assumed that the main outcome is suppression of inflammation and immune response. In addition to those activities, Eap can also activate platelets [87].

S. aureus can bind host plg on its surface. This plg can later be activated into plasmin by either host plasminogen activators, or by bacteria's own Sak. This gives staphylococci a strong, surface-associated proteolytic activity. It was speculated that such activity helps bacteria in tissue destruction and spreading to other sites to cause metastatic infection [88-91]. Surface-bound plasmin can also cleave immunoglobulins and complement attached to bacteria, therefore protecting them from phagocytosis [92]. Notably, surface-bound plasmin is not susceptible to host's plasmin inhibitors like alpha-2-

antiplasmin [93]. It is not entirely clear which staphylococcal surface proteins are responsible for plg binding, but it seems to be mediated by several distinct molecules: α-enolase [93], inosine 5'-monophosphate dehydrogenase [93], ribonucleotide reductase subunit 2 [93] and triosephosphate isomerase [94]. Why and how those molecules (known for their intracellular functions) are displayed on the staphylococcal cell surface remains unclear.

1.2.6 Functions of virulence factors

S. aureus possess an enormous arsenal of potential virulence factors, and only some of them were described above, divided into several categories. In addition to classifying staphylococcal proteins and structures according to their location or biochemistry, as above, one can also divide them according to their functions. Various classification schemes were created, for an example into factors involved in tissue invasion, evasion of immunity, biofilm formation and secretion of toxins [95]. Another possible division is into factors that mediate adherence, facilitate tissue destruction, promote iron uptake, binds to plasma proteins to evade complement, antibodies or phagocytosis, lyse host cells and manipulate immune responses [35]. The most practical division of staphylococcal proteins and structures according to their function is probably the following:

1. Mediating adherence to host cells and tissues. This is a crucial factor necessary to establish a colonisation, otherwise microbes would be easily "washed away" [35].
2. Providing *S. aureus* with nutrition. This includes both provision of iron (probably the most crucial and most limited factor for bacterial growth in human body [77]) and of other nutrients coming from damaged host tissues.
3. Promoting bacterial spreading and invasion into tissues.
4. Evading host's immune response. This includes both straightforward inhibition of complement and phagocytosis [44], as well as modulation of the entire inflammatory response [34].

It is obvious, that those are not completely distinct categories and they frequently overlap. More importantly, the same molecule frequently plays different functions. That is the case of surface proteins, which are usually involved in adhesion, cell invasion, interaction with immune system and even acquisition of nutrients – all functions in one protein! Nevertheless, those categories are useful when trying to organize thinking about functions of particular proteins during an infection.

1.3 Immune response to *Staphylococcus aureus*

When *S. aureus* (carrying all the virulence factors) comes in contact with the host, the host does not remain inert. To the contrary: host senses the bacteria, interacts with it, and when needed – attempts to fight it. Some components of immune system involved in this fight are described below.

1.3.1 Complement system

Complement system is composed of a number of plasma proteins, helping ("complementing") phagocytes in the struggle against pathogens [96]. Presence of microbes can activate complement through different pathways, but they all end with an assembly of the C3-convertase complex. It cleaves C3 protein into C3a, which has proinflammatory activity, and C3b, which attaches to the staphylococcal surface and acts as an opsonin, increasing phagocytosis of the microbe. Activity of complement was shown to be crucial for defence against systemic *S. aureus* infections [97-98]. Not surprisingly, staphylococcus developed numerous strategies to inhibit the complement [44].

1.3.2 Phagocytes: neutrophils and macrophages

Neuthrophils, the most abundant phagocytes of the immune system, play a central role in protection against *S. aureus*. In the infectious site, they kill microbes with phagocytosis, oxidative burst, antimicrobial peptides, enzymes degrading bacterial components and with proteins sequestering essential nutrients needed for bacterial growth [22]. Both in local skin infections and in systemic infections, depletion of neutrophils greatly aggravates the disease [99-100]. Patients with defects in neutrophil recruitment or function are at greatly increased risk of *S. aureus* infections [22].

Monocytes and macrophages have a more ambiguous role in staphylococcal infection. They ingest invading *S. aureus*, therefore they help to clear the infection and prevent mortality caused by bacterial overgrowth and spread [101]. On the other hand, macrophages promote tissue damage and increase inflammation [101]. This is probably linked to the capacity of macrophages to secrete high amounts of TNF-α, as this cytokine was also showed to have the same double-edged effect on staphylococcal infection [102], and blocking TNF-α decreases tissue damage and reduces overactive inflammation [103].

1.3.3 T-cells

Early studies showed that CD8+ T-cells play no role in *S. aureus* infections, while CD4+ T-cells aggravate the disease [104-105]. Stimulation with superantigen-secreting *S. aureus* leads to a massive clonal expansion of certain CD4+ T-cells. This massive activation leads to increased inflammation and increased tissue pathologies [105]. However, recently a subset of CD4+ T-cells was identified with completely different activity during *S. aureus* infection: the Th17 cells. This subset appears to play a strong role in protecting body against microbial infection, as they coordinate and promote neutrophil recruitment to infected sites [22]. Another subset of T cells, γδ T cells, was also shown to protect mice against *S. aureus* skin infection [106]. Humans, unlike mice, don't have γδ T cells residing in epidermis, so it is not clear if those cells play same role in human infections. There is also no data on their role in systemic infections.

1.3.4 Natural Killer T-cells

Another unusual subset of T-cells are the Natural Killer T (NKT) cells. NKT cells, unlike most T cells, don't recognize protein antigens. Instead, NKT cells recognize lipid and glycolipid antigens presented on the CD1d receptor (an MHC class I – like molecule) [107]. NKT cells are capable of secreting vast array of cytokines, and therefore are thought to regulate immune responses [107]. This, together with their capability to detect non-protein antigens makes them a potential bridge between innate and adaptive immune systems.

NKT cells are divided into two types [107]. Type I NKT cells (also known as invariant NKT cells) always express an invariant Vα14-Jα281 (in mice) or Vα24-Jα18 (in humans) α-chain of a T-cell receptor, whereas type II NKT cells use a diverse T-cell receptor repertoire. Additionally, most of the type II cells recognize sulfatide (a self-glycolypid derived from myelin) presented on CD1d [108], and therefore could be pharmacologically activated by injection of sulfatide. Those two types of NKT cells probably have different, or even opposite activities in immune responses [107].

NKT cells are implicated in mechanisms of various infections [109]. By fast reaction to bacterial lipids (or even to activated antigen-presenting cells alone) they can potentially prime immune response and accelerate pathogen clearance. On the other hand, intensive cytokine secretion by NKT cells can contribute to unnecessary inflammation and tissue damage. There are therefore conflicting data on the positive or negative role of NKT cells in different infections [109]. Their place in *S. aureus* infections is only partially

studied. Some NKT cells can be activated by staphylococcal superantigens [110-111] and whole heat-killed bacteria [112]. Type I NKT cells are probably involved in protection of intestine against colonization by pathogens including *S. aureus* [113] and application of compounds activating type I NKT cells decreased severity of experimental staphylococcal muscle infection and urinary tract infection [114-115]. The role of NKT cells in systemic *S. aureus* infections remains unknown.

1.3.5 NK cells

Natural killer (NK) cells possibly play a protective role during staphylococcal infections [116-117]. However, this was concluded from studies of mice with depletion of NK1.1+ cells. It is known now, that this kind of depletion would potentially remove not only NK cells, but also NKT cells, so one should be careful with interpretation of those findings.

1.3.6 B-cells

Staphylococcal infection results in a marked activation of B-cells [118]. Contact with *S. aureus* leads to antibody production and nearly all adults produce antibodies against *S. aureus* and its components [119]. In general, B-cells does not seem to play any important role in determining outcome of staphylococcal infections [120], though slight protective effect of antibodies is possible [119].

1.4 Events in *Staphylococcus aureus* infections

Considering numerous types of infections caused by *S. aureus*, there can be no single "typical mechanism of staphylococcal infection". Detailed events and mechanisms in each kind of infection might differ from the other kinds. Perhaps overlooking this complexity is partly responsible for failed attempts to find a "universal cure" for staphylococcal diseases. However, certain key events probably take place in many kinds of infections, and certain general schemes, valid for many cases, can be imagined (Fig. 2).

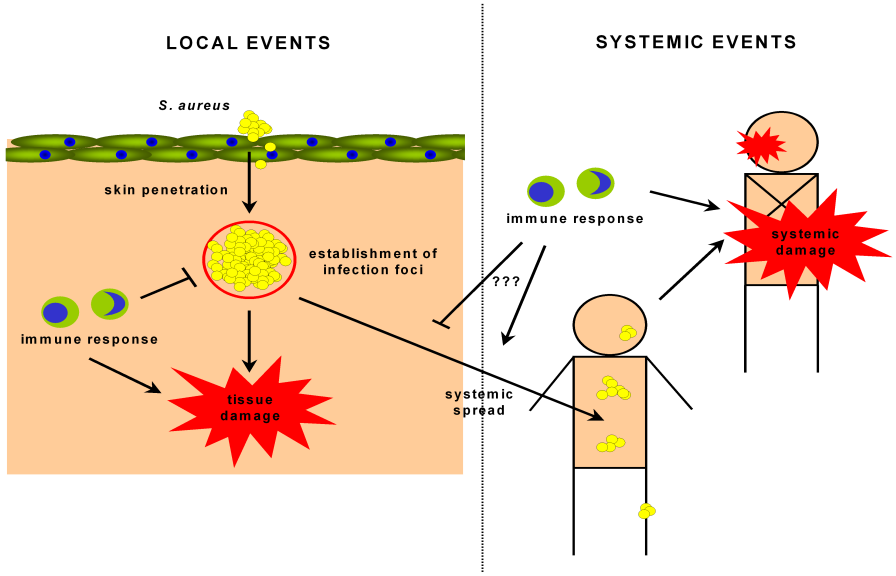


Figure 2. Simplified sequence of events during S. aureus infection – from breach of skin barrier and establishment of primary infection, to metastatic systemic infection.

1.4.1 Entry into the body

The human body is separated from the environment by epithelium: layers of tightly adhering cells with underlying basal membrane. This includes skin and linings of respiratory, gastrointestinal and urinary tracts. For bacteria to enter the body and cause infection, they need to get through those barriers. In case of *S. aureus* this happens frequently after a local trauma, when the epithelial layer is destroyed. But there are also other hypothetical possibilities for staphylococci to cross those barriers, even in absence of trauma or in presence of only minimal damage (microtrauma). ETs, Panton-Valentine leukocidin and V8 proteinase were suggested to help in penetration from the skin surface [95, 121]. ETs and V8 proteinase could increase permeability of the skin by damaging tight contacts between epidermal cells [95, 121], while leukocidin could help bacteria to bind hair surfaces and enter hair follicles, a common site of skin infection.

1.4.2 Local immune response and establishment of infectious foci

Upon contact with bacteria (or upon ingesting them), epithelial cells initiate an immune reaction: they secrete cytokines to attract immune cells and secrete antibacterial peptides like β-defensins and LL-37 to kill the intruders [122]. Apart from keratinocytes, which are the main sentinel detecting skin infection, dendritic cells, mast cells, macrophages and T cells (including NKT cells) resident in the skin are likely involved in the early stages of detection of microbial invasion and induction of inflammation [123].

Inflammatory mediators released by cells detecting staphylococcal invasion attract more leukocytes to the infection site. Phagocytes (neutrophils and macrophages) appear already after a couple of hours, while T-cells are recruited to infection sites after 48 h. This order of appearance and timing is probably universal irrespective of the tissue involved, as it was observed both in skin and in joint infections [104, 124].

Over a couple of days an abscess is usually formed in the infected site. "Mature" staphylococcal abscess has a characteristic structure. Multiplying bacteria are localized in the centre, surrounded by a fibrin capsule, in turn surrounded by numerous neutrophils (necrotic or viable, depending on exact localisation in the abscess), and all this separated from the tissue by another capsule, perhaps also composed of fibrin [125]. The fibrin layer protects *S. aureus* from neutrophil attacks [126], therefore elimination of bacteria from an abscess is a big challenge to the immune system. Formation of this elaborate abscess structure requires active participation of bacteria. Surface proteins and proteins inducing coagulation are suggested to play the main role in this process [125]. Interestingly, abscess formation requires also the presence of neutrophils [127], though there is no doubt that their main role in the local infection is fighting off the bacteria and preventing the establishment of infectious foci.

1.4.3 Systemic spread of infection

In some cases infection does not remain limited to the original infection site. The abscess might rupture and leak, or bacteria might escape from it and make their way into the bloodstream. It is not known what factors allows for this penetration through tissue and entry to the circulation. However, various authors suggested that activation of host plg by Sak might play a role [88-90]. Plasmin, giving staphylococci a strong proteolytic activity, would break through the fibrin layer and later digest tissues (both directly and indirectly, by activating latent tissue metaloproteinases [89]) to pave bacteria a way for

spreading. Similar mechanisms were observed in *Yersinia pestis* and streptococci [128-129], which also secrete some kinds of bacterial plasminogen activators. This hypothesis was however not yet investigated in the case of staphylococci.

After reaching the bloodstream, staphylococci needs to escape it and enter tissues again to establish metastatic infections. To do this, they need to pass through the lining of blood vessels (endothelium), what poses similar challenges as passing through epithelium to enter the body. FnbA, FnbB and many other surface proteins, which mediate invasion into host cells, were suggested to help breaking through a barrier between blood and organs [58, 130]. Also teichoic and lipoteichoic acids, as well as EDIN toxins are hypothesised to play a role in this process [130].

A completely different vision of *S. aureus* dissemination was also proposed. A certain proportion of staphylococci can survive phagocytosis by macrophages and neutrophils, and they can remain viable inside phagocytes for a prolonged time [131-133]. This means, that bacteria can potentially be carried inside those cells away from the original infectious site into other parts of the body, and initiate new infection foci. If this hypothesis turns out to be true, it means that leukocytes are "Trojan horses" spreading the disease inside the body [130, 134].

1.4.4 Response to systemic infection

Spread of staphylococci in the body, or severe local infection, lead to systemic inflammation. Activated immune cells secrete vast amounts of proinflammatory cytokines, like IL-6 and TNF-α, which is called a "cytokine storm". Those further increase the activity of the immune system what in extreme cases can lead to organ damage, and DIC. At the same time, inflammation induces expression of anti-inflammatory cytokines, such as IL-10, which are responsible for regulating the immune response. Elevated levels of both pro- and anti- inflammatory cytokines in circulation reflects severity of infection and inflammation. Elevated levels of proinflammatory cytokines in circulation will signal liver to produce and release "acute phase proteins". This is a broad name for various proteins which increase in circulation during inflammation. Acute phase proteins includes C-reactive protein (CRP), complement components, serum amyloid A and many proteins involved in coagulation and fibrinolysis: PAI-1, fibrinogen, prothrombin, von Willebrand factor, plg, α -2-macroglobulin and others.

Elevated levels of PAI-1, induced by inflammation, efficiently inhibits circulating plg activators. This decreases plasmin activity, inhibits fibrinolysis and moves the balance towards increased coagulation [88]. Such effect is observed both in infected humans and in experimentally infected mice [135]. Inflammatory cells additionally enhance coagulation by expressing tissue factor, which initiates the coagulation cascade[18]. This leads to formation of fibrin clots and thrombi in the inflamed area, which is probably meant to cut-off the infected tissue from the rest of the body and limit the infection [136]. In case of *S. aureus*, the host's coagulation is potentially additionally strengthened by staphylococcal own coagulases. Fibrinogen (produced in excess by liver) and fibrin further stimulate immune cells to secrete cytokines, perpetuating the inflammation [88]. When massive intravascular coagulation occurs, as it is in severe sepsis, the platelets are consumed due to clot formation and their numbers drop down. Plg and plasmin, though their activity is greatly reduced during severe inflammation, appear to play an important role not only in decreasing disseminated coagulation, but also in regulation of cytokine production. Data on the exact role of plasmin(ogen) in severe inflammation during infection and on its mechanism of action are partly contradictory, but most point to plg activation as a positive factor preventing organ damage and mortality [135, 137-138].

2 GOALS

A lot is known about pathology and mechanisms of staphylococcal infections. There are, however, perhaps even more unknowns. Here are some of them:

1. Coagulation induced by *S. aureus* has been shown to play an important role in various infections, but the interactions of staphylococci with the fibrinolytic system remain unstudied. Activation of fibrinolysis by Sak has been suggested to help in bacterial spreading, but this hypothesis has not been tested yet.
2. Another area, where the knowledge is lacking, is the role of NKT cells in staphylococcal systemic infections. The field of NKT research is now rapidly evolving, and NKT cells emerge as important regulators of the entire immune system. Therefore questioning the role of NKT cells in staphylococcal sepsis should be a logical continuation of previous research on immune responses to staphylococci.
3. Great importance of surface proteins for staphylococcal infections has been convincingly shown in many systemic infection models. Surprisingly, their role in events during local infection did not attract equal attention.

Those issues will be addressed in this thesis, and the contribution of Sak, surface proteins and NKT cells to virulence in various infections will be explored.

In case of Sak, the questions asked will be: Does Sak help *S. aureus* in spreading through physiological barriers and tissues? Can it help staphylococci to penetrate into the skin? Can it promote their systemic spread from an infected skin? What is the impact of Sak on virulence in both systemic infections and localized skin infections?

In case of NKT cells, the questions asked will be: How do the type I and type II NKT cells affect the outcome of *S. aureus* sepsis? And can activation of type II NKT cells by the sulfatide change the course of the disease?

In case of surface proteins, the questions asked will be: Do the surface proteins play a role in localized skin infection? If yes, which of the surface proteins is most crucial for the virulence?

3 METHODOLOGICAL CONSIDERATIONS

3.1 Defining "virulence" and "virulence factors"

Efforts to understand microbial virulence and to control it are in the center of nowadays research in medical microbiology. Each year several hundreds of new articles on virulence of *S. aureus* are indexed in PubMed database. There are numerous established techniques to study those issues, and most scientists probably have an intuitive feeling in this subject, but the concepts of "virulence" and "virulence factors" are somewhat imprecise and are defined in various ways [139-141].

According to MeSH medical subject heading index, the virulence is "the degree of pathogenicity within a group or species of microorganisms or viruses as indicated by case fatality rates and/or the ability of the organism to invade the tissues of the host [142]." From a more ecological perspective, virulence has been described as the capacity of the pathogen to decrease the fitness (that is, the ability to both survive and reproduce) of the host. Those definitions concentrate on the damage associated with the infecting microorganism, but it should be noted that in many cases it is not the pathogen itself, but the host's response that causes the damage. Virulence therefore can be seen as a phenomenon arising from a specific interaction of the pathogen and the host [141]. If we accept this viewpoint, speaking of virulence of particular bacteria without making references to the condition of the host makes little sense. One should also notice, that increasing virulence is not necessarily increasing the microbe's fitness. It rather seems that many pathogens have some evolutionary "optimal virulence" levels [143-144]. This is especially significant in case of microbes that depend on their host for the spreading, like in pathogens spread by person-to-person contact or from parents to their offsprings.

Another troublesome idea is the one of "virulence factors". MeSH defines them as "those components of an organism that determine its capacity to cause disease but are not required for its viability per se [142]", but this definition has been extensively criticized [139, 141]. If virulence is interpreted as resulting from a bacterium-host relation, then the capacity to cause a disease rises from an interplay of the environmental, host and bacterial factors, and concentration solely on components of the

microorganism makes little sense [141]. This is especially striking in case of opportunistic pathogens, when the main factor causing the disease is the immunosuppressed state of the host. Therefore a broader definition of virulence factors could be needed, which would take into the account not only the bacteria factors, but also the context of the host's condition and the particular infection setting. However, it is questionable if such a broad definition would be useful for the typical virulence-oriented microbiological research.

An additional issue arises with factors necessary for viability of the pathogen. Many factors known to induce damage during infection (for an example bacterial DNA and cell walls) are at the same time needed for bacterial survival. The MeSH definition excludes such factors, but some alternative definitions prefers to include them [139, 141]. The virulence research is considered with finding potential ways to prevent damage done to the host by microbial factors. Therefore it seems unpractical to artificially divide those factors into ones needed and not needed for bacterial viability. Perhaps, from research point of view, the most useful definition of virulence factor is "a microbial component that can be potentially removed, blocked or modified to decrease the pathogen's virulence".

3.2 Practical approach to virulence measurement

Despite all the theoretical difficulties, the methods commonly used to measure virulence are very straightforward. In this thesis numerous methods assessing damage to the host were used [Papers I-IV]. The most obvious readout was the mortality, but also others were employed: decreased weight (indication of systemic deleterious effect of the disease), damage to the tissues seen histopathologically, swelling and clinical signs of local inflammation (inflammation indicates body's attempt to fight invading bacteria, it also inevitably damages the tissues) and systemic markers of inflammation (cytokines and PAI-1). Another readout used was the number of surviving/proliferating bacteria in the infected sites. This is not directly a measurement of virulence, but measuring bacterial survival provides important information about capacity of *S. aureus* to resist immune attacks and capacity of the host to fight the infection. Intensive proliferation in tissues is also presumably harmful for the host and quantification of viable bacteria provides an estimate of potential damage.

3.3 Identifying virulence factors: Koch's postulates

Considering the doubts with definition of virulence factors, it comes as no surprise that there is no one, universally accepted method to identify them [139, 145]. There have been, however, an attempt to systematize the search for bacterial virulence factors using certain criteria. A list named "molecular Koch's postulates" (after the original Koch's postulates, used to identify if a particular pathogen is responsible for the disease) was created to guide virulence factor research [139, 146]. They have never been strictly followed and it is easier to find this list in a first-year textbook than in everyday research practice. Since they were formulated 25 years ago, the understanding of "pathogen" and "virulence" has significantly changed, and some kinds of important host-pathogen interactions turned out not to fit into the frames of the postulates [146]. Nevertheless, the postulates provides a valuable inspiration for intellectual scrutiny of the scientific data – after all, even their author stressed that they are meant to be a basis of dialogue, not a set of rules. The postulates are [146]:

1. The phenotype or property under investigation should be associated with pathogenic members of a genus or pathogenic strains of a species.
2. Specific inactivation of the gene associated with the suspected virulence trait should lead to a measurable loss in pathogenicity or virulence.
3. Reversion or allelic replacement of the mutated gene should lead to restoration of pathogenicity.

One could imagine a similar list for studying the host factors responsible for virulence and identifying components of immune system involved in disease:

1. An immune component must be present at the infection site or must show some other distinct reaction to the infectious process.
2. Specific inactivation of the immune component should lead to an increase (or decrease) in virulence.
3. Increasing the number, activity etc. of the immune component should lead to an opposite effect on virulence than the inactivation.

It seems hard (if not impossible) to apply all of the mentioned criteria to every gene or immune subset of interest, but the more the postulates are

fulfilled, the more certain is the identification of a virulence factor. I will address here in more detail three postulates, with their potential application to *S. aureus* research: the association of a factor with virulent strains, testing changes in virulence and alternatives to specific inactivation of virulence genes.

3.4 Studying virulence factors in clinical *S. aureus* strains

3.4.1 What to compare?

Identification of factors associated with pathogenic strains is challenging in case of bacterial species that are commensal and/or opportunistic bacteria. In case of opportunists, there are no "typical" pathogenic strains, and the factor which causes the virulence is the host's immunosuppression or introduction of bacteria into an unusual location in the host's body. On the other hand, in case of commensal species, sometimes it is possible to identify specific strains responsible for causing disease. This is for an example the case of *Neisseria meningitidis*: meningococcal colonization could progress to meningitis almost exclusively in case of certain virulent strains, while non-virulent strains nearly always remain harmless colonizers [147].

How is the situation in case of *S. aureus*, usually described as a commensal and an opportunistic pathogen? Attempts to identify specific virulent strains of *S. aureus* has until now provided equivocal data [148-150]. Some analyses point to differences in prevalence of specific genes or genotypes between isolates from carriers and infected subjects [151-152], but other claim that the strains responsible for colonization and infection are essentially the same [153-154].

Considering the difficulties with identification of "pathogenic" strains of *S. aureus*, in this thesis I attempted to approach this problem from a different direction [Paper II]. Instead of asking "what factors distinguish commensal from pathogenic strains?", I searched for virulence factor by asking "can we identify factors associated with a particular type or severity of the infection?".

3.4.2 Primary vs secondary infection

First comparison was between isolates from primary and secondary skin and soft tissue infections [Paper II]. The division into "primary" and "secondary" skin infection is not a commonly used one [21], but it was previously

successfully employed to identify factors involved in pathogenesis of skin infections [155-156]. “Secondary” infections were defined as those occurring on the already damaged skin: after trauma, surgery or other skin diseases. Such wounded skin does not act anymore as a barrier to infection. The situation is different in “primary” skin infections, defined as infections occurring on previously healthy skin (like staphylococcal impetigo, folliculitis or skin abscesses). To induce such infection, *S. aureus* probably takes advantage of micro-damages of epidermis and hair follicles, but most of the barrier-function of the skin is still present in those cases. Virulence factors needed for breaking skin barrier and establishing the infection will probably be more useful and common in the isolates from primary infections than in isolates from secondary cases, where no barrier-breaking is needed.

3.4.3 Uncomplicated vs invasive infection

Another comparison was between isolates from invasive and from uncomplicated infections [Paper II]. This is a commonly used classification of staphylococcal infections [20]. Uncomplicated skin infections were defined as cases of skin wounds in conjunction with clinical signs of local infection, but without involvement of other organs, no positive cultures from otherwise sterile organs, no clinical signs of systemic infection (fever >38.5°C, tachycardia, hypotension) and no need for in-hospital treatment. Invasive infections were defined as cases with positive cultures from otherwise sterile internal sites (e.g. blood, joint fluid, cerebrospinal fluid, bone biopsy, samples from deep tissue abscesses), with clinical signs of systemic infection and with a need for in-hospital treatment. Progression from local infectious foci to a disseminated invasive infection is (as has been discussed in introductory chapters) probably a multifactorial process. Most likely it involves both the staphylococcal factors, the host-related factors and their interplay. As there are so many factors involved, there is a risk that searching for a single factor will be inefficient and a great care has to be taken when interpreting such findings.

3.4.4 How to compare?

The final issue to consider is the method of assaying for the presence of the putative virulence factor. This is usually done with genotypic methods [152, 154-159]. However, the presence of the gene of interest in the genome does not mean that it is being transcribed during the infection, and that it has an opportunity to affect the virulence. An interesting example is the α-haemolysin: many S. aureus strains harbor a conserved variant of α-haemolysin’s gene, coding for an inactive protein, but differing from the wild-type variant just by a single point mutation [160]. Most routine

genotyping methods will not discriminate those two, leading to erroneous data. On the other hand, phenotypic assays for the putative virulence factors also suffer from several shortcomings. They measure if the factor is really expressed (or even how much is it expressed), but this is done in the in vitro setting. While it is possible to choose in vitro conditions which hopefully resemble conditions during a real infection, the expression in the infected tissue might still differ from the one observed in the laboratory setting. Therefore, no method can be considered truly optimal – however, in this thesis I decided to use a phenotypic method, as it provides data not only about a prevalence of the studied factor (in this case, the Sak), but also about its amount secreted by the bacteria in vitro [Paper II].

3.5 Animal models

Laboratory models are essential for virulence research, as they allow to directly measure the outcomes in controlled conditions. The Koch's postulates (both original and the "molecular" ones) specifically ask for use of models to identify pathogens and virulence mechanisms. Infection models are only simplified, abstract approximations of the real infection. No model can be truly and completely equivalent to a real-life infection, and each model carries a set of assumptions. Usually models reflect only certain aspects of real infection, concentrating on a particular stage, symptom, or specific part of host-pathogen interaction. Many models are especially invalid in simulating the initiation of the infection, as they are not spontaneous, but are initiated by researchers. Finally, some models might employ non-physiological conditions, to better concentrate on a particular aspect of the studied disease. But quite often even the models which attempt to be "real-like", are quite distant from infections occurring in patients. The very idea of modeling natural events in a lab setting, with purified substances and standardized objects, is heavily loaded with theoretical assumptions (though the researchers are frequently unaware of them) [161]. All this has to be considered when analyzing and interpreting the results. However, despite all the shortcomings of the laboratory infection models, they contributed enormously to our understanding of human diseases. It is nearly certain, that they will continue to provide us new knowledge on virulence in near future as well.

Numerous model organisms have been used for testing virulence of *S. aureus* and exploring the mechanisms of staphylococcal infection. Those range from unicellular amoebas [162-163], through plants [164-166], nematode worms [167-170], insects [171-174], fish [132, 175-176], birds [177-179], finishing

with rodents [98, 124], other mammals and even humans [180-181]. Of all this variety, the most common and versatile model organism – used also in this thesis – is the laboratory mouse.

3.5.1 Laboratory mouse in studies of staphylococcal virulence

The laboratory mouse has numerous advantages, which made it the most popular model species. Mice are easy to handle and small, decreasing the space and cost needed to keep and breed them, and they have accelerated lifespan, allowing studying effects spanning the entire lifetime of an organism. Numerous research tools (such as well-defined inbred, outbred or transgenic strains and established experimental protocols) are available speeding up the research, and rich literature assists in design and interpretation of new experiments. However, there are also many shortcomings of the mouse models in infection research. Most importantly, the genes involved in response to inflammation in mice probably correlate poorly with the genomic response in humans [182]. Mice have also evolved in a radically different environment than humans and their immune system is adapted to deal with different set of infectious agents [183]. *S. aureus* is known to colonize skin of laboratory mice and to cause some infections, what means that murine staphylococcal infection models are not completely artificial. However, one should note that the frequency of colonization in laboratory mice (6-11%) is much lower than in humans (approx. 20%, or up to 50%, if transient colonization is included), and frequency of colonization in wild mice remains unknown [184]. Spontaneous *S. aureus* infections in mice are rare, and the most typical kind of disease – a suppurative inflammation of the preputial glands – does not resemble any human infections (though *S. aureus* also sometimes causes wound infections in mice, and those parallel the clinical situation in humans) [185]. One should also not forget that staphylococcal strains derived from animals frequently differ from human strains [186-187]. This further contributes to artificiality of the murine models if *S. aureus* strains isolated from humans are used to infect rodents.

The potential pitfalls of mouse models have been realized by many biomedical researchers, and some even suggest that paradoxically more knowledge is available now about the mouse biology than about the human one [183]! Therefore, over-reliance on murine models receives increasingly more critique. The best solution to this "mouse trap of biomedical research" is increasing number of translational studies, where results obtained in mouse models are immediately compared with human data and "translation" of findings into facts meaningful for human health is performed [183]. In this

work, such approach was attempted in one paper [Paper II], where the research program moves back and forth between human clinical data, *in vitro* experiments and results of mouse studies, building one unifying theory.

To perform a translational research, scientists commonly attempt to narrow the gap between murine models and real situations [183]. This include "humanization" of mice – use of transgenic animals expressing human proteins or carrying human cell subsets. Conversely, models can also be more "mouse-real-life-like" when bacteria and infection types occurring naturally in mice are used. Similar attempts were taken in this some parts of this thesis. Sak, a staphylococcal protein that was investigated, can activate human, but not mouse plg. Therefore results obtained in a normal mouse model would most likely be misleading. In an attempt to circumvent this problem, "humanized" mice, expressing human plg (h-plg), were used [Papers I-II]. Expression was driven under a control of the mouse albumin gene regulatory sequences and it resulted in levels of h-plg in mouse plasma corresponding to approximately 17% of levels in normal human plasma [129]. Noteworthy, also the *S. aureus* strain LS-1, used in many infection models in this thesis [Papers I-IV], is probably not a human strain. It was isolated from a spontaneous outbreak of staphyloccal infection in mouse, when bacteria infected wounds and then spread hematogenously to cause bacteraemia, osteitis and septic arthritis [188]. Use of the bacterial strain originating from mouse in mouse model increases the resemblance to a real-life situation.

The final key to minimizing pitfalls of mouse models is the careful design and interpretation, taking into the account the shortcomings of the mouse as an imperfect substitute of human, and of laboratory models as mere approximations of reality. Of various models available, in this thesis, the models of systemic infection (sepsis and septic arthritis) and skin and soft tissue infection were chosen.

3.5.2 Murine *S. aureus* sepsis models

A basic prerequisite for development of human sepsis is bacteraemia, the presence of microbes in the bloodstream [98]. This in turn activates the cascade of events potentially leading to sepsis and septic shock. Therefore mouse models of sepsis are usually created in a straightforward way – by an intravenous injection of *S. aureus* (except certain models concentrating on the later events in the septic shock, which are induced by injection of staphylococcal toxins without viable bacteria [103, 135, 189]). The crucial factor is the injected microbial inoculum: too low will fail to elicit the systemic inflammatory response leading to sepsis and subsequent shock, too

high will lead to a rapid death of all animals. This dose needs to be further adjusted to the particular *S. aureus* strain used, as they differ in ability to induce sepsis [98]. Indeed, various models used by researchers differ mainly in respect to used inocula and time-course of infection. There is no comparison of different models available, but it is reasonable to suspect, that different mechanisms might be of different importance in models with fast and slow onset of mortality. In certain models, animals begin to die in less than 24 h after inoculation [190], or show rapid mortality during first 24 – 48 h [191]. In the model used in this thesis [Paper I, Paper IV], animals showed first signs of infection (changed appearance and behavior, body weight loss) already 24 h after injection of *S. aureus*, but they began to die from the day 3 onwards. Even though the mortality began quite late, the important events occur in the host's organisms already during the first days of infection and they could determine the final outcome [192]. In this model, the mortality is the most obvious readout of virulence, but additional information is provided by differences in weight loss and by number of bacteria present in tissues. Analysis of inflammation markers in blood can complement this with information about host's immune reaction.

3.5.3 Murine *S. aureus* arthritis models

The easiest method of inducing staphylococcal arthritis is to directly inoculate bacteria into the joint cavity. This approach has been used and is especially useful to answer the specific question about interaction of bacterium and immune system with joint's components [53, 137]. However, septic arthritis in humans is usually hematogenously spread from other infectious foci [19]. Therefore, to induce arthritis, *S. aureus* needs to adapt to host's environment, survive bactericidal components of blood, reach the joint's synovium and penetrate into joint cavity [98]. To include those crucial steps of septic arthritis, a hematogenous-spread *S. aureus* arthritis model is used [98]. It resembles a sepsis model, but the bacterial inoculum is reduced, to avoid systemic shock response. After injection into the bloodstream, detectable *S. aureus* colonization appears in numerous tissues and organs within several hours [193-194]. In case of most of the organs, *S. aureus* normally fails to establish permanent infectious foci and is soon cleared by the immune system, but it can persist in at least joints and kidneys [193-194] (and perhaps, in some instances, bones [195]), what leads to typical symptoms of arthritis complicated by renal abscesses and sometimes chronic osteomyelitis. Infected joints become red and swollen (first symptoms appearing within a few days from inoculation, though sometimes they might be visible already 24 h after infection). In humans, septic arthritis can lead to sepsis. Also in this mouse model, some of infected animals develop severe

disease and die. The higher the infectious dose, the higher the mortality and the higher resemblance of the model to the sepsis model. In fact those two models are not really separate, but form certain continuum, with "obvious sepsis" and "obvious septic arthritis" on two ends, and various degrees of "mixed situation" depending on the doses of inocula. Therefore the entire condition: mixture of sepsis and septic arthritis, accompanied by renal abscesses, could be termed a "systemic infection" [Paper I]. To assess severity of the disease, clinical symptoms in the infected joints can be scored during the disease and the damage to joint structures can be scored by histopathology. Numbers of *S. aureus* persisting in kidneys can be counted. Weight loss and mortality rates provide important data on disease severity. Analysis of inflammation markers in blood can complement this with information about the host's immune reaction [98].

3.5.4 Murine *S. aureus* skin infection models

Various models of staphylococcal skin infections have been developed over the years. One approach was to apply bacteria directly on the surface of the previously damaged skin. In those models skin was scraped with scalpel blade [196], abraded with sandpaper [197], tape-stripped [198-199], cut [200] or inflamed [201-202]. Those methods suffered from poor reproducibility and standardization, and our attempts to replicate them failed. Another developed approach is a direct subcutaneous injection of bacteria – either mixed with a foreign carrier material to reduce the required infectious dose [203-204], or just *S. aureus* alone [124, 196]. This approach is more reproducible and easier to standardize. Following bacterial inoculation a subcutaneous abscess and/or skin necrosis develops, depending on the inoculum size, bacterial strain and mice used [203]. The development of skin abscesses is closer to a typical course of skin and soft tissue infection. This subcutaneous injection model was used in this thesis [Papers II-III]. Readouts included measuring the diameter of formed lesions (or scoring them using clinical scoring scale), inflammatory markers in blood, histological damage to infected area, as well as counting viable bacteria present in skin, or disseminating to other organs.

There are, however, certain difficulties with the model. It seems to have a smaller discriminative power than the sepsis or arthritis models, so depending on the intended use of the model, the timing and injected inocula might need adjusting to fit the conditions where the model has maximum sensitivity [Paper III]. Also, in immunocompetent mice, the host's response is very efficient – a large inoculum is needed to induce the disease, and the immune system anyway manages to limit the infection at the injection site, quickly clears it and prevents systemic spread. Only after neutrophils are removed,

the susceptibility of mice to infection rises, and a systemic spread from infected skin to other organs appears [99]. Due to the high resistance of mice to infection, numerous models used immunosuppressed mice [196-197, 200-201]. This was also done in this thesis: to study the systemic spread from infected skin, I immunosuppressed mice with an alkylating cytostatic drug – cyclophosphamide [Paper II].

3.5.5 Infectious dose in mouse models and human reality

One striking difference between human situation and all of the mouse models is the inoculum size. Due to obvious reasons, the initiation of real spontaneous disease in humans was never investigated. However, one could imagine that usually the number of bacteria responsible for the initiation of the infection must have been rather low. At the same time, to induce disease in mice, a very large inocula, of millions of *S. aureus* cells are used. No obvious explanation of this discrepancy is available. It might point either to artificiality of our models, or to our wrong imaginations about "real" human infections. It is however unclear, how to investigate this issue in an ethical and efficient way.

3.6 Identifying factors involved in virulence: knocking-out, knocking-in and more

Classical approach to identifying virulence factors, mentioned in "molecular Koch's postulates" is a specific inactivation of a putative factor. This is commonly done by a creating mutant strains with removed ("knocked-out") gene encoding the factor if interest. This is also the method used in this thesis [Paper III]. There are, however other possible approaches – and they were explored in my research as well.

3.6.1 Case of clumping factor A

Instead of removing the entire protein under investigation, it might be possible to just render it inactive. This approach was attempted in the past with ClfA [205]. At that time, the known activity of ClfA was binding the host's fibrinogen. A mutant version of the protein was constructed, with two amino acids from the active site exchanged, so that the mutated protein lost its capacity to bind fibrinogen, but retained its size and three-dimensional conformation [205]. The gene coding for normal ClfA in *S. aureus* strain Newman was exchanged for the gene encoding mutated protein, creating a strain that can be used as a tool in identifying the role of fibrinogen binding

by ClfA in virulence. This strain was created in Tim Foster's lab and was also used in this thesis [Paper III]. However, this strain also reveals potential difficulties inherent in this approach: after some time, it appeared that in addition to fibrinogen binding, ClfA also plays an important role in inhibition of complement-mediated phagocytosis [66]. The mutated version of the protein turned out to have en exactly opposite property: instead of inhibiting, it promotes complement-mediated phagocytosis [66]. This finding prompted re-evaluation of results obtained with the mutant strain and lead to a serendipitous discovery of the complement's role in the skin infections, described in this thesis [Paper III]. That illustrates the great care which needs to be taken when designing and analyzing results obtained with "inactivated" putative virulence factors – but it also shows great potential of this approach.

3.6.2 Case of staphylokinase

S. aureus has a vast array of virulence factors, and usually each strain carries just some of them. This might pose certain difficulties when attempting to "knock-out" a particular factor for testing in a model: it requires that the bacterial strain optimal for the model has the factor of interest. This was the case of Sak and strain LS-1. *S. aureus* LS-1 was originally isolated from murine spontaneous infection [188] and therefore was used to establish and optimize the staphylococcal arthritis and sepsis models [98, 194]. However, like many other strains of animal origin [206], it lacks Sak. Therefore, instead of inactivating the putative factor, the opposite was done: the gene encoding Sak was inserted into the genome of strain LS-1 ("knocked-in"). This gave the additional opportunity to insert the gene either fused to its original promoter (resulting in 'normal' levels of Sak expression), or to the strong promoter of protein A (resulting in a very pronounced expression during exponential phase). Those strains was created by Tao Jin in Tim Foster's lab and were used in this thesis [Papers I-II]. Therefore, instead of comparing just strains secreting or not secreting the factor, it was possible to compare the non-secreting strain with two strains of varying level and timing of secretion.

3.6.3 Case of NKT cells

The specific inactivation of cell subsets or immune proteins is useful for the study of immune components and their role in virulence (see previous discussion of Koch's postulates). But again, this is not always possible – and this was the case of NKT cells studies in this thesis [Paper IV]. NKT don't interact with MHC, like usual T cells, but are instead restricted by CD1d, an MHC-like molecule. Mice with CD1d-deficiency have therefore a markedly decreased number of NKT cells and they can't perform their physiological

functions [207]. Those “NKT knock-out” mice are valuable to study the role of NKT cells in infection, and were also used in this thesis. However, NKT cells are not a homogenous population, and they consist of at least two subsets: type I and type II. As each subset have probably different or even opposite activity [107], removing them both at the same time might yield misleading results. It is possible to use mice lacking only type I NKT cells (mice with “knock-out” of Jα18, a part of invariant T-cell receptor specific for type I NKT [208]) – and so I did in the thesis. Unfortunately, there are no specific genes that could be inactivated to produce a type II NKT cell deficient mouse – so an alternative approach is needed to study the role of type II NKT. Luckily, type II NKT recognize sulfatide (self-lipid derived from myelin), and treatment of mice with sulfatide results in activation of type II NKT. In this way, instead of analyzing the effect of removing a cell subset during infection, I investigated the opposite: the effect of activation of this cell subset. Combination of all those approaches, though forced by necessity and lack of alternative research tools, proved to be effective and provided valuable information on NKT cells in staphylococcal infection [Paper IV].

3.7 Other methods used in the thesis

All methods used in the thesis are described in details in the original publications [Paper I-IV]

4 RESULTS

Key findings of the papers included in this thesis are presented below. More detailed descriptions are contained in the papers I-IV attached to the thesis.

4.1 Paper I

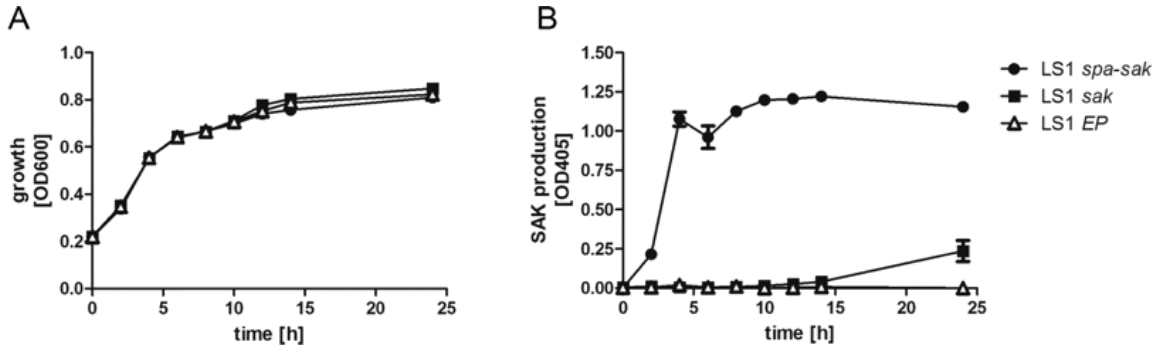
4.1.1 Interaction of Sak and host plg was studied using congenic *S. aureus* strains and human plg transgenic mice.

A mouse model system for studying the role of Sak in pathogenesis was developed. It consisted of "Sak knock-in" *S. aureus* strains and transgenic mice expressing human plg (h-plg) in addition to normally expressed mouse plg.

Three congenic strains based on *S. aureus* LS-1 and differing in Sak secretion were developed by Tao Jin. In one of those strains (LS-1*sak*), the Sak gene was expressed from its own original promoter. In the other strain (LS-1*spa-sak*), the Sak gene was fused to the promoter of the protein A. Third strain (LS-1*EP*) had no gene encoding Sak (instead carried an empty vector) and served as control.

All three strains had the same growth rates *in vitro* (Fig. 3A). Strain LS-1*spa-sak* began to secrete Sak early during the exponential growth phase and the overall secretion was very high. Strain LS-1*sak* began to secrete Sak only at the end of exponential growth phase, and the overall secretion was moderate. No Sak secretion could be detected in the control LS-1*EP* strain (Fig. 3B).

Figure 3. In vitro growth of (A) and Sak secretion by (B) three congenic S. aureus strains differing in Sak secretion.



Plasma from transgenic h-plg mice was found to react to Sak *in vitro*. After addition of recombinant Sak, plasmin activity increased in plasma from h-plg animals. Addition of supernatant from a Sak-secreting *S. aureus* strain caused lysis of clot formed in plasma from h-plg mice. Neither plasmin activity nor clot lysis was observed in plasma from wild-type animals. This confirmed that Sak has biological activity in h-plg, but not in wild-type mice.

4.1.2 Activation of plasminogen by staphylokinase decreases severity of systemic S. aureus infections

To observe effects of Sak on septic arthritis and mild systemic infection, mice were injected intravenously with an arthritic dose of *S. aureus* congenic strains. This lead to development of arthritis, weight loss and a small degree of mortality. The mortality was decreased when the Sak-secreting strains LS-1*spa-sak* and LS-1*sak* were injected into h-plg mice. No reduction of mortality was observed when strain LS-1*EP* was injected in h-plg mice (Fig 4). Interaction of Sak with host plg had, however, no effect on weight loss, bacterial counts in kidneys or frequency and severity of the arthritis.

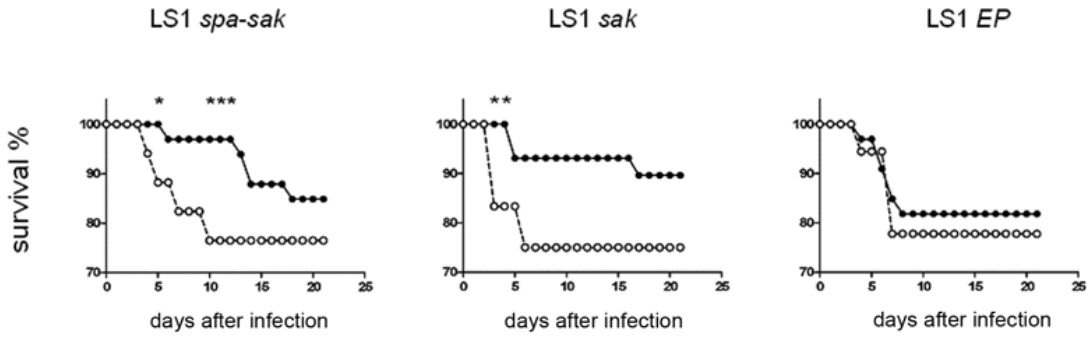


Figure 4. Survival of h-plg transgenic mice (black circles) and wild-type mice (white circles) after intravenous inoculation with an arthritogenic dose of congenic S. aureus strains.

When a higher, septic dose of *S. aureus*, was injected into mice, they developed severe sepsis and many of them died. Interaction of Sak with host plg did not affect the mortality in this case. However, activation of host plg by Sak reduced the weight loss: h-plg mice injected with LS-1*spa-sak* strain lost less weight than wild-type mice injected with the same strain (Fig 5).

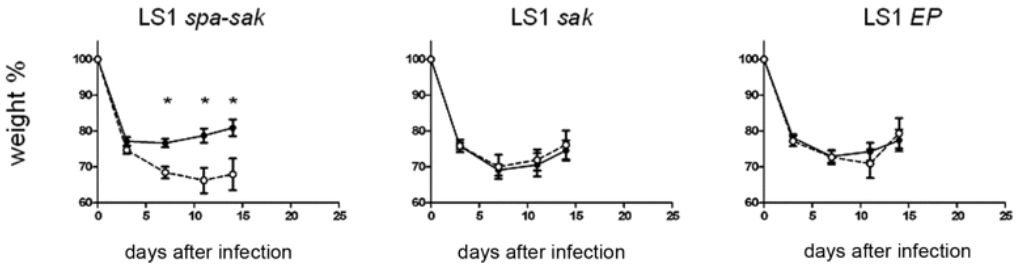


Figure 5. Weight loss of h-plg transgenic mice (black circles) and wild-type mice (white circles) after intravenous inoculation with septic dose of congenic S. aureus strains.

In addition to better weight development, h-plg animals injected with LS-1*spa-sak* fought the invading staphylococci more efficiently, as shown by reduced bacterial counts in kidneys (Fig. 6).

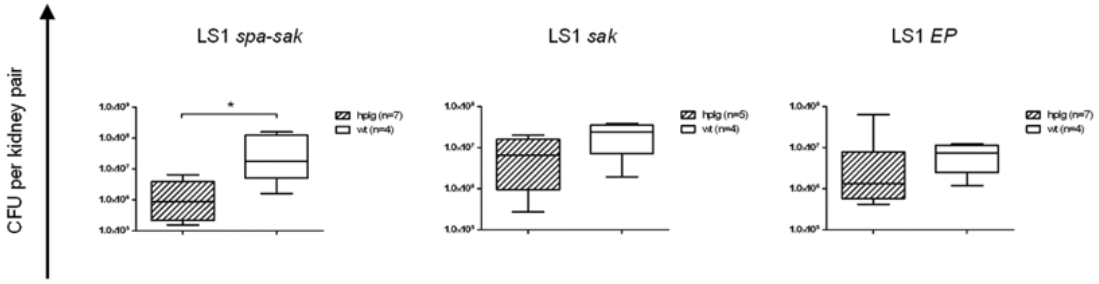


Figure 6. Bacterial loads in kidneys of h-plg transgenic mice and wild-type mice 14 days after intravenous inoculation with septic dose of congenic S. aureus strains.

4.2 Paper II

4.2.1 Activation of plasminogen by staphylokinase increases S. aureus penetration through skin physiological barriers and promotes establishment of primary skin infections

When three *S. aureus* congenic strains, differing in their secretion of Sak, were tested for their capability to penetrate through physiological barriers in a transwell system, a clear pattern was observed. In case of all tested barriers including fibrin clots, complete plasma clots, reconstituted basal membranes

and keratinocyte monolayers, the strain LS-1*spa-sak* secreting high amounts of Sak was the first one to penetrate through the barrier. It was followed by the strain LS-1*sak*, which secretes moderate amounts of Sak. The Sak non-secreting strain LS-1*EP* had difficulties with penetrating through the barrier in all the cases (Fig. 7). This effect was dependent on presence of plg in the tested system, and on ability of Sak to activate plg into plasmin. Addition of plasmin inhibitor or removal of plg from the system greatly diminished the capability of bacteria to penetrate through the barriers.

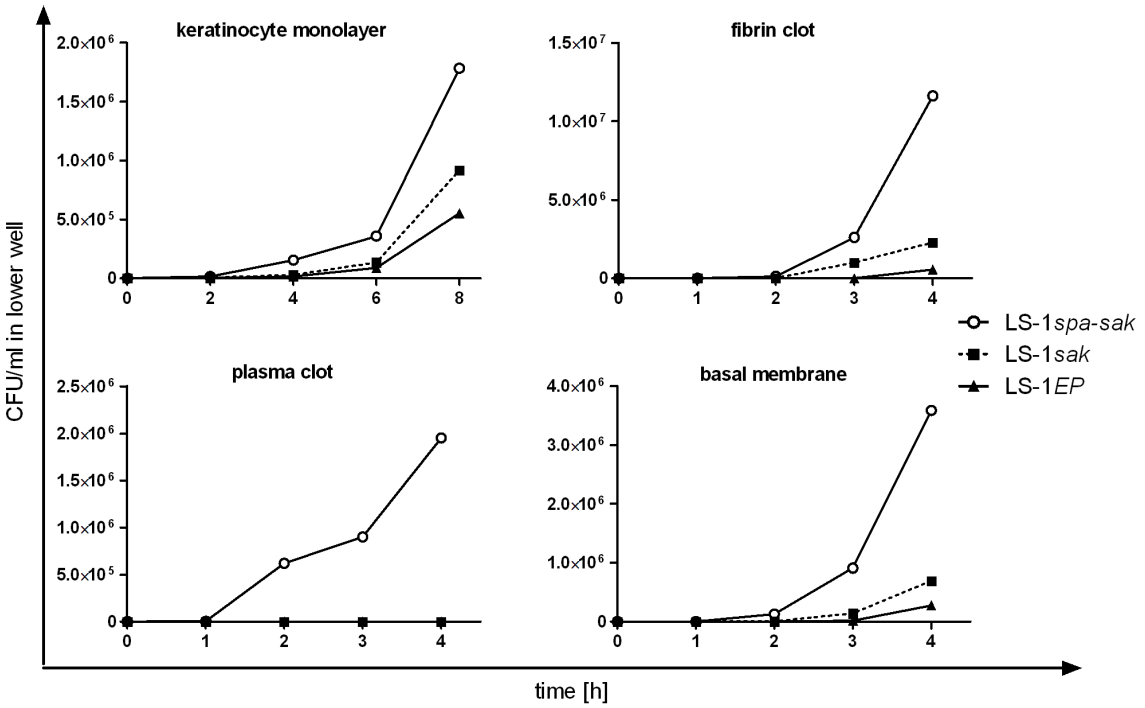


Figure 7. Penetration of congenic S. aureus strains through physiological barriers in a transwell in vitro test system.

This observation was further confirmed in a more real-life setting of an *ex vivo* skin biopsy from a h-plg mice. When cultures of LS-1*spa-sak* or LS-1*sak* were placed on the surface of shaved skin, they were able to penetrate through epidermis and invade the dermis. In contrast, the Sak negative strain, LS-1*EP*, remained on the skin surface (Fig. 8).

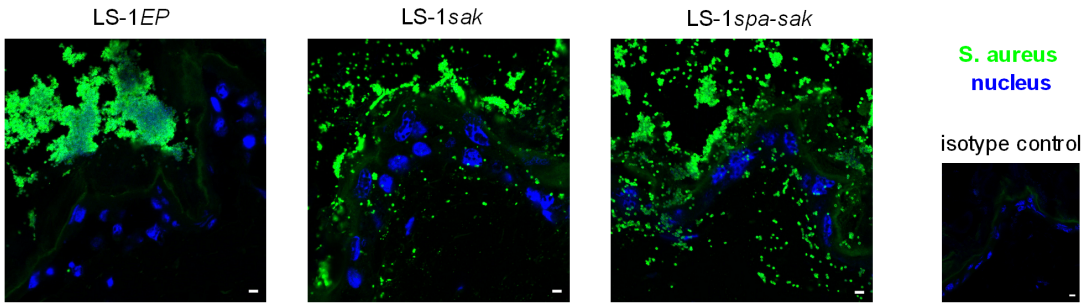


Figure 8. Penetration of congenic S. aureus strains into the ex vivo skin biopsy, seen in fluorescence microscope. Dermis is located to the bottom of the photos and surface of the biopsy to the top. Slight unspecific staining of corneocytes marks border of the skin.

The findings from the *in vitro* and *ex vivo* systems were further elaborated by analysis of Sak secretion by clinical *S. aureus* strains isolated from skin and soft tissue infections in humans. Isolates from primary skin lesions secreted Sak not only significantly more frequently, but also in higher amounts than isolates from cases of secondary skin infections (Fig. 9). This underlines an association of Sak with primary skin infections, where bacteria need to penetrate into healthy skin to establish infection.

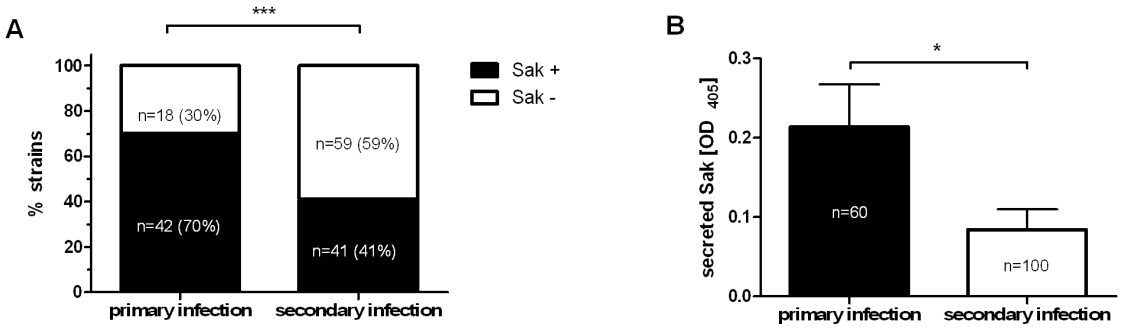


Figure 9. Frequency (A) and amount (B) of Sak secreted by clinical S. aureus isolates from human primary and secondary skin infections.

4.2.2 Activation of plasminogen by Sak does not promote systemic spread from the skin infection site

To measure the capacity of Sak to promote systemic spread, *S. aureus* was injected subcutaneously into neutropenic mice and bacteria spreading to kidneys, livers and spleens were counted at various times. No matter if the injected strain was secreting Sak (LS-*1spa-sak*) or not (LS-1*EP*), or if it was injected into mice in which Sak can activate plg (h-plg mice) or not (wild-type mice), no differences in bacterial spread to internal organs were observed.

4.2.3 Activation of plasminogen by Sak reduces severity of skin infection

When strain LS-1*EP*, not secreting Sak, was injected subcutaneously, the size of skin lesions was similar irrespective of mouse strain used (Fig. 10). In contrast, when one of the Sak-secreting strains (LS1*spa-sak* or LS1*sak*) was injected, there were clear differences in disease severity: lesions were smaller in h-plg mice than in wild-type animals (Fig. 10).

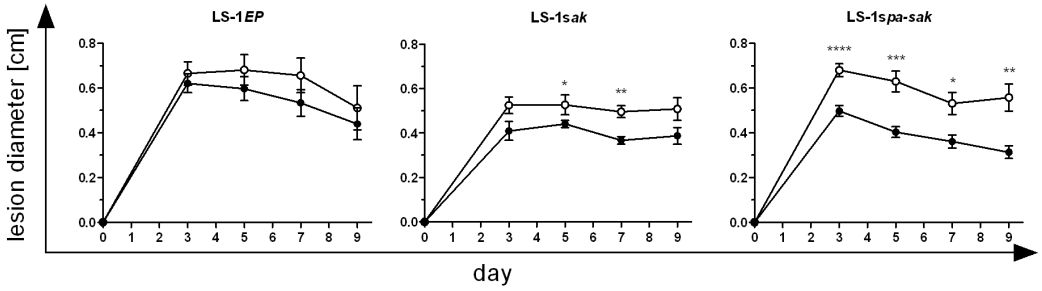


Figure 10. Sizes of skin lesions induced by subcutaneous injection of congenic S. aureus strains into immunosupressed h-plg (black circles) and wild-type (white circles) mice.

This difference in infection severity was not only limited to macroscopically smaller lesions. Also histopathological analysis of changes and analysis of inflammation markers (cytokines IL-6 and IL-10, and acute phase protein PAI-1) pointed to reduced tissue damage and milder inflammation in mice where Sak secreted by bacteria activated host plg. This effect of reducing infection severity was, however, not apparent when immunocompetent mice were used (Fig. 11).

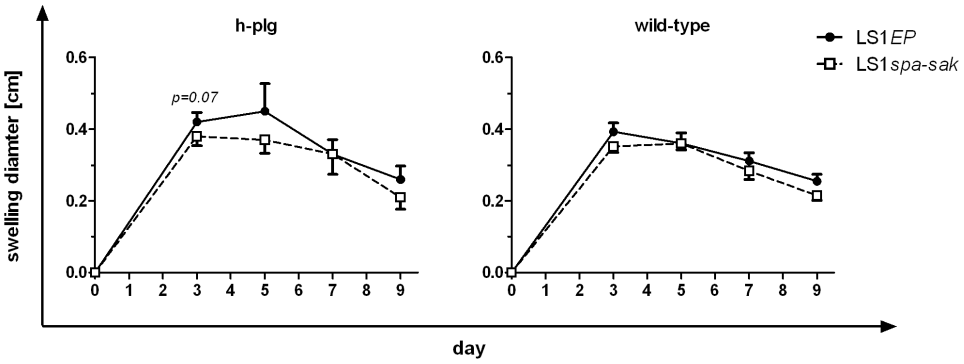


Figure 11. Sizes of skin lesions induced by subcutaneous injection of congenic S. aureus strains into immunocompetent h-plg and wild-type mice.

When secretions of Sak by clinical *S. aureus* isolates from humans was tested, again a virulence-reducing capability of Sak was observed. When comparing isolates from uncomplicated skin infections and from invasive infections, the strains from complicated invasive cases secreted significantly lower amounts of Sak. There was, however, no difference in the frequency of strains secreting Sak between those two groups (Fig. 12).

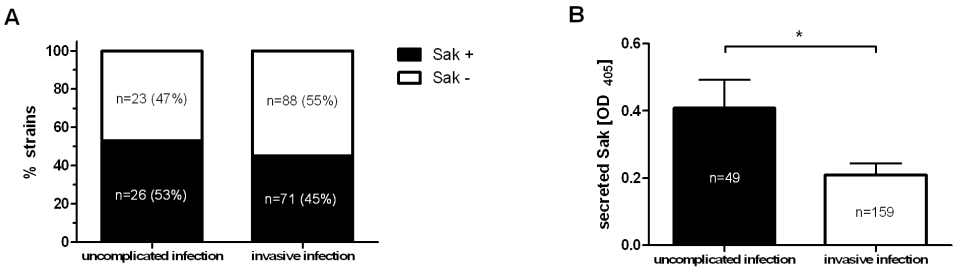


Figure 12. Frequency (A) and amount (B) of Sak secreted by clinical S. aureus isolates from human uncomplicated skin infections and invasive infections.

4.2.4 Activation of plasminogen by Sak promotes the drainage of skin lesions.

Some of the skin lesions caused by *S. aureus* injections spontaneously opened and drained. This was rare in wild-type mice, and happened equally frequently irrespectively of the injected staphylococcal strain. However, in h-plg animals there was significantly more opening if LS-1*spa-sak* strain was injected, than when LS-1*EP* was used.

4.3 Paper III

4.3.1 Sortases are essential for virulence in an abscess model of skin infection

Knock-out mutations of the genes for srtA and srtB, enzymes responsible for covalently attaching proteins to staphylococcal surface, had a pronounced effect on virulence in mouse skin abscess infection model. After subcutaneous injection, the mutant strain lacking sortases caused significantly less swelling (Fig. 13B,D) and had significantly lower viable counts in the infected spot than the wild-type strain (Fig. 13A,C). This effect was apparent already on the first day of infection and persisted throughout whole course of the disease (Fig. 13E).

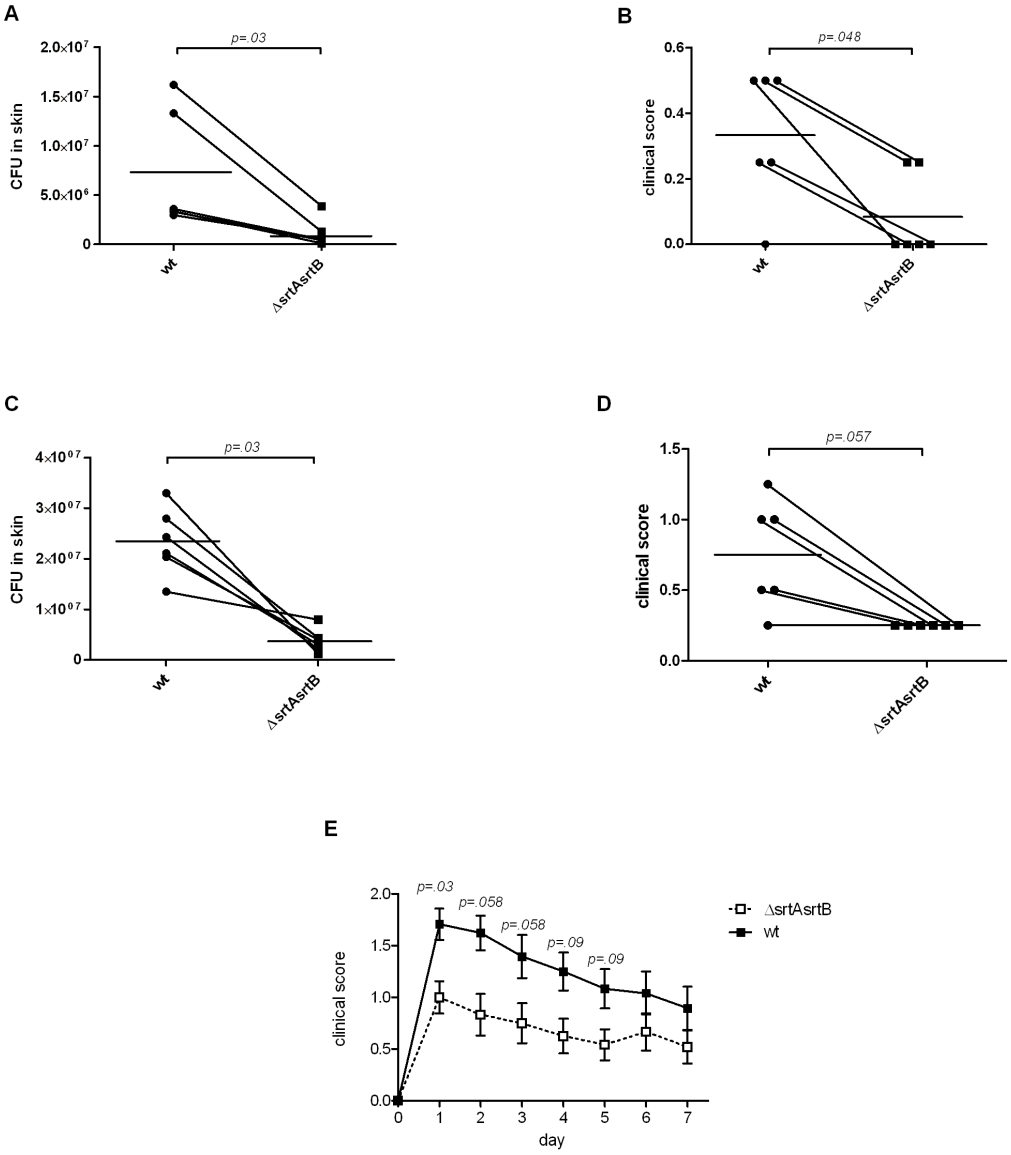


Figure 13. Effects of sortase on skin virulence in an abscess model. Wild-type (wt) and congenic srtAsrtB mutant S. aureus strains were injected subsutaneously at lower (A, B) or higher (C, D) concentrations of bacteria. Skin swelling (B, D) and viable counts in skin (A, C) were measured on day 2 of infection. In another experiment, mice were injected with a medium number of bacteria and skin swelling was followed daily (E).

4.3.2 Several surface proteins play a role in skin infection

To identify which surface proteins of *S. aureus* might be responsible for the virulence in skin abscess infection model, mice were injected subcutaneously with strains lacking selected surface proteins and their wild-type strain counterparts. Protein A, FnbA and FnbB, and Eap were found to be associated with virulence in this model. No such association was observed for SasF protein and for ClfA.

The mutant form of ClfA, ClfAPYI, caused strongly diminished virulence of the strain carrying it. As removing the entire ClfA protein had no impact on the virulence, this property of ClfAPYI was a surprising and unexpected finding.

4.4 Paper IV

4.4.1 NKT type I cells don't affect survival in systemic S. aureus infection

Increased numbers and activation of NKT type I cells was observed in spleen during staphylococcal sepsis (Fig. 14).

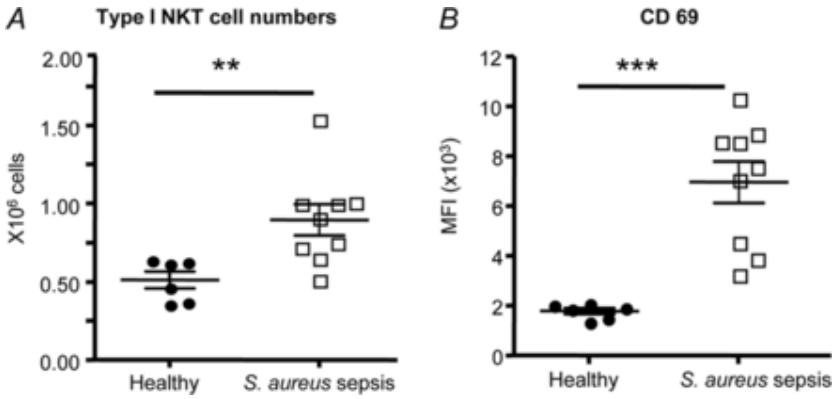


Figure 14. Flow cytometry analysis of splenocytes from mice on day 3 of infection and from healthy controls, showing the absolute number of type I NKT cells (A) and mean fluorescence intensity of activation marker CD69 on type I NKT cells (B).

However, they didn't seem to affect the development of systemic infection, as the mortality in Jα18-deficient mice (which lack NKT I cells) did not differ from the mortality in wild-type animals (Fig. 15A).

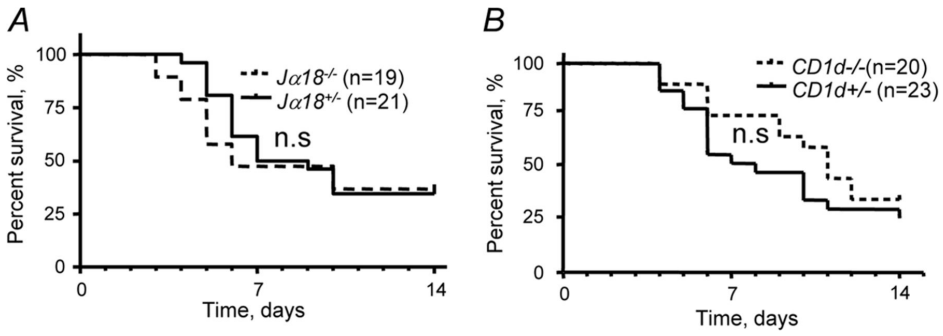


Figure 15. Survival of mice lacking type I NKT cells (Jα18-/- mice, A) and mice lacking all NKT cells (CD1d-/- mice, B) after inoculation with septic dose of S. aureus.

4.4.2 NKT type II probably don't affect the systemic S. aureus infection

There are no good methods available to identify NKT type II cells and there are no available mutant mouse strains lacking them. It is therefore hard to determine their role during staphylococcal sepsis. There were indirect suggestions from flow cytometry data that they are activated by *S. aureus* infection, but no final conclusions could be drawn. *S. aureus* sepsis induced same mortality in wild-type animals and in CD1d-deficient mice (which lacks both type I and type II NKT cells), suggesting that both NKT I and NKT II play no role in virulence in this model (Fig. 15B).

4.4.3 Sulfatide treatment decreases severity of staphylococcal sepsis through activation of NKT type II cells

Injection of sulfatide on day 0 and 3 of infection protected mice from mortality in staphylococcal sepsis (Fig. 16). The same effect was not observed when sulfatide was injected only on day 3.

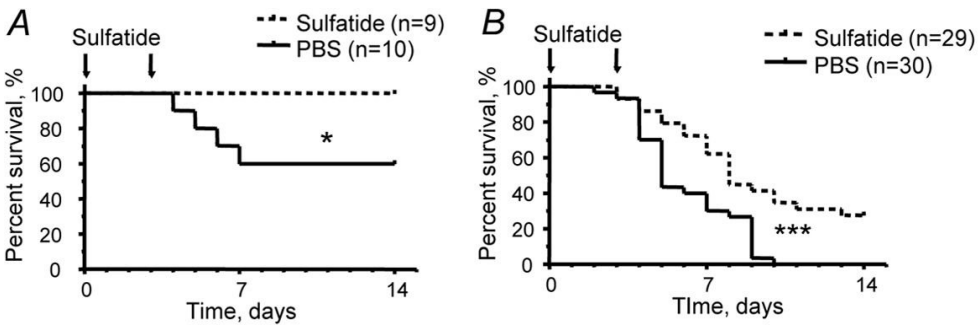


Figure 16. Survival of mice treated with sulfatide or PBS (control) after inoculation with low (A) or high (B) septic dose of S. aureus.

The protective effect was associated with significantly decreased levels of proinflammatory cytokines TNF-α and IL-6. Also the markers for DIC were improved in mice receiving sulfatide: platelet counts were significantly increased, and there was a trend towards increased plasmin activity and lower fibrinogen levels. Despite the diminished systemic inflammation and coagulation, the counts of bacteria in kidneys remained unchanged.

The protective effect of sulfatide disappeared when CD1d-deficient mice were used, but remained when infection was induced in Jα18-deficient mice. Therefore, the effect was dependent on presence of some NKT cells, but not on the NKT type I cells. This points to NKT type II as responsible immune cells for protecting mice against mortality in systemic *S. aureus* infection after sulfatide treatment.

5 DISCUSSION

5.1 Paper I and II – Sak and virulence

Those papers aimed at identifying the impact of Sak on virulence in systemic (paper I) and local skin infections (paper II).

5.1.1 Can a mouse model describe the interaction of Sak with plg during infection?

Experimental studies of Sak in animal models has been hampered by the specificity of Sak for human plg (h-plg). Development of mice expressing h-plg gave an opportunity to conduct such studies. Sak was able to exert its effects in plasma from those h-plg transgenic mice, making them a suitable tool for studying Sak.plasminogen interactions during infection.

5.1.2 Does Sak secretion change virulence in systemic infections?

When animals were infected with an *S. aureus* strain that secretes no Sak, the disease severity was same irrespective if transgenic h-plg or wild-type mice were used. This suggests that the presence of h-plg alone does not affect the disease. However, when mice were infected with Sak-secreting *S. aureus* strains, the disease was more severe in wild-type than in h-plg animals. Therefore, activation of the host's plasminogen by staphylococcal Sak reduces damage during systemic infection. This finding is perhaps not surprising, when compared with the data about other bacterial plg activators. Neither the plg activator from streptococci, nor from *Yersinia pestis* increase virulence in systemic bloodstream infections [128-129].

5.1.3 Does Sak secretion promote establishment of skin infections?

Data from *in vitro* transwell experiments, showed that activation of plg by Sak promotes staphylococcal spreading through different components of skin barriers; data from *ex vivo* biopsies showed ability of Sak to promote bacterial entry into the skin; and data from clinical isolates, showed close association of Sak-secreting isolates with primary skin infections. All results point in one direction. Sak seems to play an important role in *S. aureus*

penetration from skin surface through epidermis and therefore helps in establishing a skin infection.

In the skin, plg is present mainly in the basal layer of the epidermis and in the walls of hair follicles [209]. Therefore one can imagine that Sak secreted by skin colonizing *S. aureus* can activate plg, especially when microdamages are present, which allow Sak to easily diffuse into the basal layer. Plasmin activated by Sak would then cleave the components of basal membranes and cell-to-cell adhesions, paving a way for bacteria to penetrate into dermis and establish infection foci. Production of antibacterial LL-37 by skin cells upon contact with staphylococci will make the penetration even easier, as LL-37 greatly increases the activity of Sak [210]. One can also speculate, that an intensive local proteolysis caused by plasmin will provide large amounts of nutrients for staphylococci forming new infectious foci in the skin. This hypothesis, however, would require further experimental confirmation.

5.1.4 What is the effect of Sak on an already existing skin infection?

It has been hypothesized, that activation of plg by bacteria is an universal mechanism for spreading from the original infection site and causing metastatic infections [88-90]. This, however, didn't appear to be true for *S. aureus*. Activation of host plg by Sak did not increase systemic spread from skin infection in mouse model. Data from clinical staphylococcal isolates, showing increased Sak secretion by non-invasive isolates, might even suggest that high Sak secretion limits systemic spread. This puts Sak in clear opposition to other bacterial plg activators, which are known to promote bacterial metastasis [128-129]. However, data from clinical isolates need to be treated carefully. Staphylococcal surface proteins appear to be of great importance for severity of skin infections [Paper III], therefore differences in surface proteins expressed by clinical isolates can easily overshadow weak effects of Sak and confuse the outcome. Plasmin, resulting from Sak's activity, can cleave some surface proteins (e.g. Pls) and can damage many of the ligands for other surface proteins. Therefore the skin virulence of each strain might be a result of the interplay between Sak and the specific set of surface proteins carried by this strain. Such a complicated setting probably can't be understood just by analyzing frequencies of individual virulence factors in various isolate collections. The future of virulence research might therefore lie in the approach of systems biology, where numerous factors and their interactions are analyzed simultaneously [149].

Despite no effect of Sak on systemic spread seen in our experimental setting, another effect was observed: activation of host plg by Sak reduced the severity of skin lesion, promoted drainage of infected sites and therefore accelerated healing. This effect was clearly seen in immunosuppressed mice. A similar trend occurred in immunocompetent mice, but it was too weak to draw definite conclusions.

5.1.5 How does Sak reduce virulence?

The finding of a bacterial component that reduces virulence, rather than increases it, is somehow surprising. Nevertheless, it was observed in case of both systemic and local infection. Such effect is supported by previously published data gathered from clinical isolates, where isolates secreting Sak were associated with milder bacteraemia cases [211]. How is this effect of Sak mediated? Coagulation induced by bacteria plays an important role in staphylococcal virulence and is needed for proper formation of infectious foci [49, 55]. Counteracting this coagulation with fibrinolysis induced by Sak would therefore reduce the virulence. Also binding to various host ligands in extracellular matrix plays an important role in staphylococcal infection: it allows bacteria to attach and establish infectious foci and it can protect from phagocytosis (for example by binding fibrinogen on the cell's surface [205, 212-213]). Sak may counteract this by inducing extensive proteolysis, destroying potential attachment sites in tissues and removing fibrinogen from bacterial surface. Finally, plasminogen and active fibrinolytic system have been shown to play some protective role during staphylococcal infections [135, 137-138]. This protective effect would then be strengthened by Sak, which promotes fibrinolysis.

5.1.6 Why does Sak reduce virulence? A summary

The presence of proteins that decrease the virulence of a pathogen, so called the "virulence suppressors", has been described in various bacterial species [214]. It is therefore possible that Sak acts as a virulence suppressor in staphylococcal infections, especially in the systemic ones. The question remains – why does *S. aureus* secrete such a "self-sabotaging" protein? The answer perhaps lies in the natural life cycle of this pathogen. It normally persists as a colonizer of the nasal cavity, and causes minor, localized, suppurative skin infections. It spreads from person to person from the colonized noses and suppurating wounds and abscesses. The activity of Sak reveals adaptation to this lifestyle, as it plays an important role in initiating skin infections. Invasive infections are rare and they do not provide bacteria with opportunity for transfer to new hosts. Dissemination to internal organs is rather a "dead end" for staphylococci and systemic spread or damage to

internal organs is not beneficial to *S. aureus*. Therefore there is no evolutionary pressure to eliminate factors which, like Sak, reduce virulence in systemic infections. Sak remains a widespread protein in *S. aureus* strains, because it plays an important role in the staphylococcal lifestyle: it promotes skin infections (the role of Sak in nasal carriage has not been investigated, but it might play some role as well). Compared to that, the virulence-limiting effect of Sak in systemic infections, though important for humans, remains irrelevant from the point of view of the staphylococci.

5.2 Paper III – staphylococcal surface proteins in skin infections

A multitude of *S. aureus* skin infection models testifies about the difficulties in development of a reproducible, reliable and realistic animal model. In this thesis, I used a model previously developed in our department [124], and adapted it to study the effects of surface proteins on virulence. Optimal infectious dose and time point for evaluation were determined. This allowed to find some answers about the staphylococcal surface proteins in skin infections.

5.2.1 Do cell-wall anchored surface proteins play a role in skin infections?

Without sortase enzymes, staphylococci can't covalently attach surface proteins to staphylococcal cell walls, and are therefore greatly limited in their interactions with the surrounding environment. In the abscess model of skin infection, a mutant lacking sortases A and B induced less clinical symptoms and had reduced viability in the skin. This clearly shows the importance of sortases and surface proteins covalently attached by sortases to cell surface for the local skin infection. A similar finding was observed previously for the systemic infections [56-57], what stresses the common role of surface proteins for staphylococcal virulence in different conditions.

5.2.2 Which surface proteins are responsible for virulence in skin infection?

Several mutant strains of *S. aureus*, lacking some of the surface proteins, were tested in the skin abscess model to identify which specific proteins might be responsible for the virulence in skin infection. Tested proteins included both covalently attached (that is, sortase-dependent) protein A,

FnbA and FnbB, SasF and ClfA, as well as the Eap protein, attached non-covalently to bacterial surface. The reduced virulence of the mutant lacking sortases probably results form a combined effect of several proteins missing, therefore effects of removing only one protein at the time are expected to be smaller than the effect observed in the sortase mutant. This of course makes identifying individual proteins involved in virulence a challenge, as even after the optimization the skin model lacks high sensitivity and discriminative power, and the observed effects were small. Nevertheless, certain proteins were successfully identified as involved in skin virulence. Those were protein A, FnbA and FnbB, and Eap. No impact on skin virulence (at least in this model) was observed after removal of SasF or ClfA.

The identified skin virulence factors (protein A, FnbA and FnbB, Eap) function as adhesins, attaching bacteria to surrounding structures. Protein A and Eap additionally play an immune modulatory role: protein A induces inflammation, while Eap has mainly antiinflammatory activity. Perhaps one could imagine, that a successful establishment of an abscess in skin first needs the inflammation and the influx of neutrophils, to help in formation of the characteristic abscess structure, and then needs anti-inflammatory activity of Eap to prevent clearance of the abscess? Such role of Eap as protector of abscess from phagocytes was previously suggested in kidney abscesses [125]. FnbA and FnbB are known to mediate internalization of staphylococci into host's cells. Whether this intracellular strategy of *S. aureus* play any role in skin infection is not known - but it can be reasonably suspected.

5.2.3 Why ClfAPYI causes reduced virulence?

The mutant *S. aureus* strain lacking ClfA had virulence in skin comparable to the wild-type strain. It was therefore reasonable to suspect, that also the strain carrying its mutated variant ClfAPYI will retain unchanged virulence. Surprisingly, strains carrying ClfAPYI had strongly reduced virulence. This is probably not due to the first two known differences between ClfAPYI and wild-type ClfA, that is lack of fibrinogen binding and lack of complement inactivation in the mutated protein. If this was the case, the same effect would have been observed after removal of the ClfA protein. Therefore at the current stage the difference has to be ascribed to the third know difference between the two proteins: ClfAPYI increases complement deposition on bacterial surface [66]. If this is the case, it would point to a central role of complement-mediated phagocytosis in host's defense against skin infections.

5.2.4 Are SasF and ClfA involved in skin virulence?

SasF and ClfA were not found to be involved in virulence in the abscess model. But this doesn't mean that they play no role in real human infections. As all models are merely approximations of the real disease, those two proteins might play a role at some pathogenic event not properly reflected in this model. Moreover, staphylococci have an over-abundance of virulence factors, many of them with overlapping activities. It is therefore possible, that in the strains used in the experiments, the functions of removed proteins were compensated by other surface proteins – for example by other fibrinogen-binding and anti-complement proteins compensating for loss of ClfA. Perhaps on a different genetic background, SasF and ClfA would appear to be important for virulence. Therefore definitive conclusions should not be drawn too hastily.

5.2.5 Surface proteins in staphylococcal skin infection – summary

Obtained data strongly support the theory, that surface proteins (both attached by sortases, and some of the ones attached by other means) play an important role during staphylococcal skin infection and abscess formation. Similar results were observed previously for abscess formation in kidneys [215]. This means that the general mechanisms of staphylococcal abscess formation in local infections are the same, irrespective of infection site, with small differences caused by specific locations. That makes surface proteins an attractive drug target, possibly efficient over a wide range of infection types. However, as this study concentrated just on one point of the "molecular Koch's postulates" – namely, the identification of proteins which can be removed to decrease virulence – more studies are needed to address other parts of the postulates.

5.3 Paper IV – NKT cells in staphylococcal sepsis

In this paper I attempted to answer if NKT cells play any role during *S. aureus* sepsis, and if they can be used as a therapeutic target. Therefore, three questions were posed: Do NKT cells respond to infection? Does inactivation or removal of NKT cells changes virulence? Does stimulation of NKT cells affect virulence?

5.3.1 Do NKT cells react to infection?

A clear increase in number and activation of NKT type I cells was observed after injection of staphylococci. As there is no technical possibility to identify NKT type II cells in the flow cytometry, a definite conclusion about their response to infection couldn't be reached. However, the observed activation of NKT cells which don't show the type I surface marker (that is, presumed type II cells) suggests that also type II NKT cells respond to the staphylococcal infection.

5.3.2 Do NKT cells affect virulence in sepsis?

Previous data from models of gram-negative sepsis (LPS-induced shock) showed that NKT type I cells contribute to mortality by secreting proinflammatory cytokines [216-217]. Also in polymicrobial sepsis model, NKT type I cells contribute to mortality [218]. However, in case of *S. aureus* sepsis studied in this thesis, mice lacking NKT type I cells did not differ in the mortality from wild-type animals. It suggests, that despite becoming activated, NKT type I does not decide the outcome of systemic staphylococcal infection. One may speculate, if this is due to the cells reacting differently to a gram-positive stimulus than to a gram-negative one, or to unique features of staphylococcal infections.

There are no available mutant mouse strains that lack NKT type II cells, so it was not possible to precisely test their role in a same way as with type I cells. CD1d-deficient mice lacking both types of NKT cells have improved survival of LPS-induced shock [216] but data on polymicrobial sepsis are contradictory. In one study CD1d-deficient mice have an unchanged survival [219], but in another study blocking CD1d decreased the mortality [218] (though it is hard to draw firm conclusions from studies based on blocking of the CD1d receptor, as NKT cells in some cases might be activated even in absence of CD1d [217]). In case of *S. aureus* sepsis studied in this thesis, mice lacking CD1d did not differ in the mortality from wild-type animals. It is, however, not completely clear how to interpret this result. Unchanged infection severity in CD1d deficient mice might be due to 1) none of the NKT subsets affecting the outcome; or 2) the type II cells affecting the disease outcome only in presence of type I NKT cells. Such a crosstalk between NKT cells, with type II cells inhibiting host-damaging effect mediated by type I cells has already been reported [220-221]. This, however, is probably not the case in *S. aureus* sepsis, as NKT type I cells don't play any detrimental role here (as discussed above). Data from CD1d-deficient mice therefore probably points to both kinds of NKT cells having no impact on disease outcome, despite NKT cells being activated by the infection.

5.3.3 What is the effect of activation of NKT type II cells?

To clarify the role of type II NKT cells in the *S. aureus* sepsis, I used sulfatide injections. This treatment is known to activate NKT type II cells. Activation of NKT type II cells showed a strong effect on the sepsis: sulfatide treatment prevented mortality. Apparently, sulfatide injections lead to decreased levels of proinflammatory cytokines and, subsequently, prevented the development of disseminated intravascular coagulation. This effect was observed even in the absence of type I cells, but was dependent on the presence of CD1d. This suggests sulfatide treatment exerted its effect through the type II NKT cells.

Activation of type II NKT cells appear to have a potential as a preventive measure against staphylococcal sepsis. Sulfatide treatment, when applied after the disease already had developed (on day 3 after *S. aureus* inoculation) couldn't prevent the mortality. However, one cannot conclude whether this means that active type II NKT cells play their role only very early in the course of infection process, or is it just because the treatment protocol has not been optimized.

5.3.4 NKT cells and staphylococcal sepsis – summary.

Total deficiency of all NKT or deficiency of type I NKT cells appeared to have no effect on the outcome of *S. aureus* sepsis. Intriguingly, activation of NKT type II cells by sulfatide reduced the mortality rates associated with staphylococcal sepsis. It remains unclear why type II NKT didn't show this potential in physiological conditions. One may hypothesize that this is because their activation by *S. aureus* was too low, or their activation was too slow to fit into the "time-window of opportunity". While activation of NKT type I cells was not investigated in this thesis, it is possible that they also posses such "hidden potential". This is suggested by previous reports of muscle and urinary tract infections alleviated by pharmaceutically activating type I cells [114-115, 220-221]. However, systemic infections might differ greatly in their pathophysiology from the local infections, so those data can't be extrapolated without experimental confirmation.

6 CONCLUSION

The findings presented in this thesis contributes to our understanding of the mechanisms involved in *S. aureus* infections, which can be simplified and summarized as follows (Fig. 17). Sak helps *S. aureus* to break the skin barrier. Then, once bacteria are present in the skin, surface proteins participate in establishment of infectious foci. Local damage, associated with this stage of infection is decreased by the interaction of Sak with plasminogen in immunosuppressed individuals. When the systemic infection occurs, Sak expression alleviates the *S. aureus* sepsis through plg activation. During systemic infection, activation of NKT type II cells by sulfatide significantly improves the survival of the disease. Type I NKT cells, on the other hand, appear not to have any effect during systemic infection.

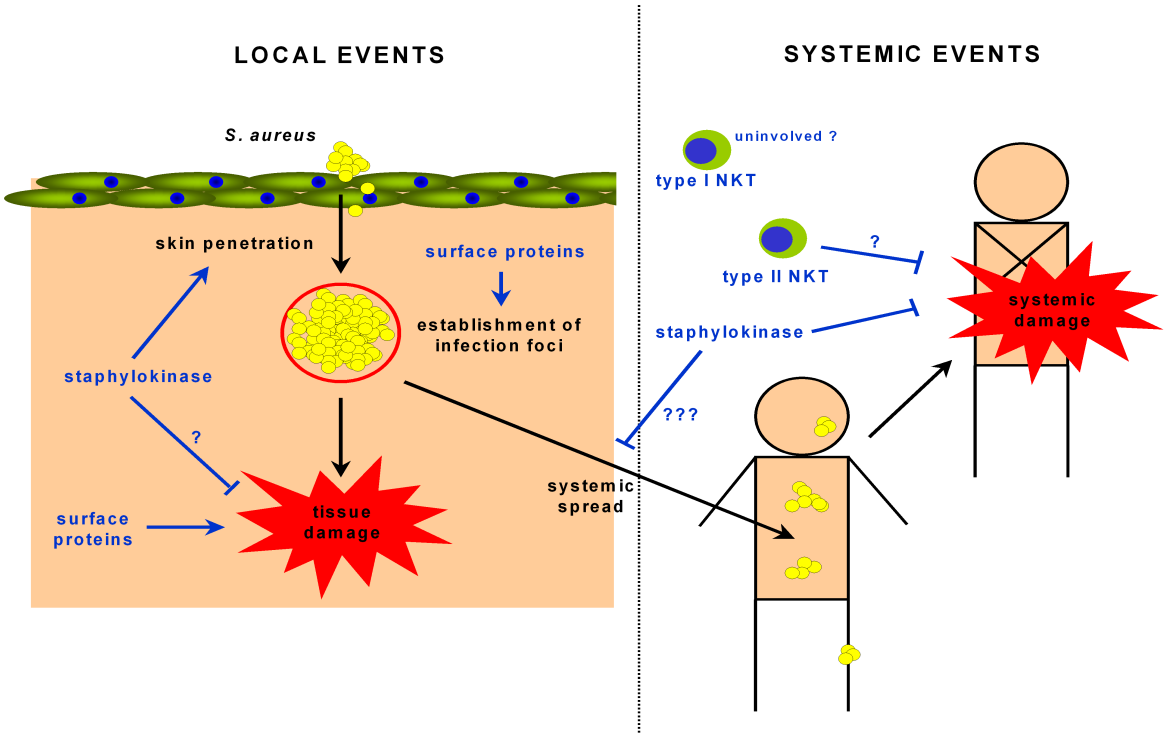


Figure 17. The involvement of Sak, surface proteins and NKT cells in different phases of S. aureus infection. Pointed arrows indicate progression or stimulation. Blunt-ended arrows indicate inhibition.

The complex picture becomes even more complicated, when one tries to think simultaneously about host and bacterial factors. Activity of Sak requires availability of the host's plg. As plg availability and activity of the entire fibrinolytic system are greatly affected by the grade of inflammation, also the effect of Sak in the particular patients might vary. The beneficial outcome of NKT type II cell activation clearly shows that the outcome of infection depends on the activity of the immune system at the time point when the infection begins. Therefore the host's response to staphylococci might differ depending on whether there is already some inflammation present in the body or not. Surface proteins, with their immunomodulatory actions, will affect the inflammation, therefore leading indirectly to changed activity of Sak and NKT cells. In real life situation, all interactions between bacterial and hosts factors form an intricate network, which might be unique to each particular infection case. Studying it will be a challenge.

ACKNOWLEDGEMENTS

It has been a great joy and a great intellectual adventure to work at the Department of Rheumatology and Inflammation Research. I am indebted to all of you, and I thank you all!

First of all, **Tao**, who has been the best supervisor that one could imagine! You managed to handle my troublesome character and guided me on the way to becoming a real scientist. You always took great care of me, believed in me, encouraged me and listened to me. I feel how much I have developed over those years thanks to your support, guidance and inspiration. Thank you for making me your PhD student!

I was lucky to have excellent co-supervisors: great researchers and wise people. **Elisabet**, with whom we spent a great deal of fun time chatting about science, culture, travels and all the things in between. **Maria**, who told me some important things about life and career. And **Tomas**, who was the first one to work with staphylococci in the department.

I began working in our department under the supervision of **Inger**. Despite my disorganized and chaotic nature, she managed to successfully guide me through my first Swedish research project.

I can't imagine completing this PhD without **Malin** and **Ing-Marie**. When I came here, they taught me all the skills and methods for working in our labs and animal house. And now, they keep on providing advice and help whenever I need it.

Without our great technicians, past and present – **Christina**, **Margareta** and **Berit** – my work would be much more troublesome, if not impossible.

I was lucky to have great collaborators: **Linda**, **Sara** and **Susanna**, who introduced me to the world of NKT cells; **Xuefeng**, who is our molecular biology expert; **Gunnar**, who helped me with his clinician's view on staphyloccal infections; and all my other co-authors.

Making this thesis would be impossible without all the **mice**. I'm grateful to those little creatures.

I would have never been here if not for **Andrej Tarkowski**, who gave me the chance to work in our Department and discover a world of rheumatology.

I did my first steps in a real research world with **Sigrun Eick** and **Kinga Wójcik**. Thank you for believing in me!

Our department is such a great place to work thanks to **all of its members**, and especially all other **PhD students** (past and present), who made this place so vivid and full of emotions.

Thanks to **Abukar**, I had a chance to see how it is to supervise a tallented and friendly student. That was a pleasant experience!

When I began my PhD I didn't have to live under the bridge thanks to **Dmitriy**, who shared his flat with me.

Marcin B and **Marcin K**, with whom I arrived to our Department and shared a flat: living and working with you was a lot of weird and geeky fun!

I was lucky to have met here fantastic people. **Luisa** and **Lucija**, who traveled with me far to the north. **Jessica** and **Leo**, who traveled with me far to the south. **Beata**, who provided the needed break at work with our nice chats. **Johan**, the most British gentleman of all the Swedes.

Bogusia and **Ryszard** in Stockholm and **Jolanta** in Göteborg took great care of me and made me feel as if I had my family on this side of the sea.

My amazing friends in Poland, **Emilia** and **Ewa**, have constantly supported me and helped me pass through the darkest moments.

Last but not least, **my beloved family**. My parents, **Roma** and **Andrzej**. My grandmas, **Lila** and **Marysia**. Thank you for the never ending love, support and care. I love you!

REFERENCES

1. Hare, R., The Antiquity of Diseases Caused by Bacteria and Viruses, A Review of the Problem from a Bacteriologist's Point of View, in Diseases in Antiquity, D. Brothwell and A.T. Sandison, Editors. 1967, Charles C Thomas Publisher: Springfield, IL, USA.

2. Brock, T.D. and M.T. Madigan, Biology of microorganisms. 6th ed. 1991, Englewood Cliffs, N.J.: Prentice Hall. xix, 874 p.

*3. Angus, D.C., et al., Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. Crit Care Med, 2001. **29**(7): p. 1303-10.*

*4. Styers, D., et al., Laboratory-based surveillance of current antimicrobial resistance patterns and trends among Staphylococcus aureus: 2005 status in the United States. Ann Clin Microbiol Antimicrob, 2006. **5**: p. 2.*

*5. Diekema, D.J., et al., Survey of infections due to Staphylococcus species: frequency of occurrence and antimicrobial susceptibility of isolates collected in the United States, Canada, Latin America, Europe, and the Western Pacific region for the SENTRY Antimicrobial Surveillance Program, 1997-1999. Clin Infect Dis, 2001. **32 Suppl 2**: p. S114-32.*

*6. Nickerson, E.K., et al., Staphylococcus aureus disease and drug resistance in resource-limited countries in south and east Asia. Lancet Infect Dis, 2009. **9**(2): p. 130-5.*

*7. Lowy, F.D., Staphylococcus aureus infections. N Engl J Med, 1998. **339**(8): p. 520-32.*

*8. Chang, F.Y., et al., Staphylococcus aureus bacteremia: recurrence and the impact of antibiotic treatment in a prospective multicenter study. Medicine (Baltimore), 2003. **82**(5): p. 333-9.*

*9. Sreeramoju, P., et al., Recurrent skin and soft tissue infections due to methicillin-resistant Staphylococcus aureus requiring operative debridement. Am J Surg, 2011. **201**(2): p. 216-20.*

*10. Jacobsson, G., E. Gustafsson, and R. Andersson, Outcome for invasive Staphylococcus aureus infections. Eur J Clin Microbiol Infect Dis, 2008. **27**(9): p. 839-48.*

*11. Jacobsson, G. and S. Nasic, Long-term outcome of invasive Staphylococcus aureus infections. Scand J Infect Dis, 2012. **44**(5): p. 350-4.*

*12. Tarkowski, A., Infection and musculoskeletal conditions: Infectious arthritis. Best Pract Res Clin Rheumatol, 2006. **20**(6): p. 1029-44.*

13. Pfaller, M.A., et al., Survey of blood stream infections attributable to gram-positive cocci: frequency of occurrence and antimicrobial susceptibility of isolates collected in 1997 in the United States, Canada, and

Latin America from the SENTRY Antimicrobial Surveillance Program. SENTRY Participants Group. Diagn Microbiol Infect Dis, 1999. ***33****(4): p. 283-97.*

14. Klevens, R.M., et al., Invasive methicillin-resistant Staphylococcus aureus infections in the United States. JAMA, 2007. ***298****(15): p. 1763-71.*

15. Wyllie, D.H., D.W. Crook, and T.E. Peto, Mortality after Staphylococcus aureus bacteraemia in two hospitals in Oxfordshire, 1997-2003: cohort study. BMJ, 2006. ***333****(7562): p. 281.*

16. Levy, M.M., et al., 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference. Crit Care Med, 2003. ***31****(4): p. 1250-6.*

17. Huttunen, R. and J. Aittoniemi, New concepts in the pathogenesis, diagnosis and treatment of bacteremia and sepsis. J Infect, 2011. ***63****(6): p. 407-19.*

18. Levi, M. and H. Ten Cate, Disseminated intravascular coagulation. N Engl J Med, 1999. ***341****(8): p. 586-92.*

19. Nade, S., Septic arthritis. Best Pract Res Clin Rheumatol, 2003. ***17****(2): p. 183-200.*

20. Dryden, M.S., Skin and soft tissue infection: microbiology and epidemiology. Int J Antimicrob Agents, 2009. ***34 Suppl 1****: p. S2-7.*

21. Hedström, S.Å., Skin and Soft Tissue Infections, in The Ever Present Pathogens. An update on staphylococci and staphylococcal infections., F. Espersen, S.Å. Hedström, and C.O. Solberg, Editors. 1999, Pharmacia & Upjohn: Göteborg. p. 185-207.

22. Miller, L.S. and J.S. Cho, Immunity against Staphylococcus aureus cutaneous infections. Nat Rev Immunol, 2011. ***11****(8): p. 505-18.*

23. Das, D.K., M.G. Baker, and K. Venugopal, Risk factors, microbiological findings and outcomes of necrotizing fasciitis in New Zealand: a retrospective chart review. BMC Infect Dis, 2012. ***12****: p. 348.*

24. Tsitsilonis, S., et al., Necrotizing fasciitis: is the bacterial spectrum changing? Langenbecks Arch Surg, 2013. ***398****(1): p. 153-9.*

25. Siddiqui, A.R. and J.M. Bernstein, Chronic wound infection: facts and controversies. Clin Dermatol, 2010. ***28****(5): p. 519-26.*

26. Fry, D.E. and P.S. Barie, The changing face of Staphylococcus aureus: a continuing surgical challenge. Surg Infect (Larchmt), 2011. ***12****(3): p. 191-203.*

27. Wilson, J., et al., Trends in sources of meticillin-resistant Staphylococcus aureus (MRSA) bacteraemia: data from the national mandatory surveillance of MRSA bacteraemia in England, 2006-2009. J Hosp Infect, 2011. ***79****(3): p. 211-7.*

*28. Wertheim, H.F., et al., The role of nasal carriage in Staphylococcus aureus infections. Lancet Infect Dis, 2005. **5**(12): p. 751-62.*

*29. Lindberg, E., et al., Effect of lifestyle factors on Staphylococcus aureus gut colonization in Swedish and Italian infants. Clin Microbiol Infect, 2011. **17**(8): p. 1209-15.*

*30. Daum, R.S. and B. Spellberg, Progress toward a Staphylococcus aureus vaccine. Clin Infect Dis, 2012. **54**(4): p. 560-7.*

31. Angell, M., The truth about the drug companies : how they deceive us and what to do about it. Rev. and updated. ed. 2005, New York: Random House Trade Paperbacks. xxviii, 319 p.

*32. Benfield, T., et al., Increasing incidence but decreasing in-hospital mortality of adult Staphylococcus aureus bacteraemia between 1981 and 2000. Clin Microbiol Infect, 2007. **13**(3): p. 257-63.*

*33. Laupland, K.B., T. Ross, and D.B. Gregson, Staphylococcus aureus bloodstream infections: risk factors, outcomes, and the influence of methicillin resistance in Calgary, Canada, 2000-2006. J Infect Dis, 2008. **198**(3): p. 336-43.*

*34. Chavakis, T., K.T. Preissner, and M. Herrmann, The anti-inflammatory activities of Staphylococcus aureus. Trends Immunol, 2007. **28**(9): p. 408-18.*

35. Grumann, D., U. Nubel, and B.M. Broker, Staphylococcus aureus toxins - Their functions and genetics. Infect Genet Evol, 2013.

*36. Anderson, M.J., et al., Alpha-Toxin Promotes Staphylococcus aureus Mucosal Biofilm Formation. Front Cell Infect Microbiol, 2012. **2**: p. 64.*

*37. Caiazza, N.C. and G.A. O'Toole, Alpha-toxin is required for biofilm formation by Staphylococcus aureus. J Bacteriol, 2003. **185**(10): p. 3214-7.*

*38. Periasamy, S., et al., Phenol-soluble modulins in staphylococci: What are they originally for? Commun Integr Biol, 2012. **5**(3): p. 275-7.*

*39. Schwartz, K., et al., Functional amyloids composed of phenol soluble modulins stabilize Staphylococcus aureus biofilms. PLoS Pathog, 2012. **8**(6): p. e1002744.*

*40. Fraser, J.D., Clarifying the mechanism of superantigen toxicity. PLoS Biol, 2011. **9**(9): p. e1001145.*

*41. Fraser, J.D. and T. Proft, The bacterial superantigen and superantigen-like proteins. Immunol Rev, 2008. **225**: p. 226-43.*

*42. Czech, A., et al., Prevalence of Rho-inactivating epidermal cell differentiation inhibitor toxins in clinical Staphylococcus aureus isolates. J Infect Dis, 2001. **184**(6): p. 785-8.*

43. Arvidson, S., Extracellular Enzymes, in Gram-Positive Pathogens, V.A. Fischetti, et al., Editors. 2000, ASM Press: Washington, D.C.

44. *Laarman, A., et al., Complement inhibition by gram-positive pathogens: molecular mechanisms and therapeutic implications. J Mol Med (Berl), 2010.* ***88****(2): p. 115-20.*

45. *Furie, B. and B.C. Furie, Thrombus formation in vivo. J Clin Invest, 2005.* ***115****(12): p. 3355-62.*

46. *Cesarman-Maus, G. and K.A. Hajjar, Molecular mechanisms of fibrinolysis. Br J Haematol, 2005.* ***129****(3): p. 307-21.*

47. *Mattsson, E., et al., Peptidoglycan from Staphylococcus aureus induces tissue factor expression and procoagulant activity in human monocytes. Infect Immun, 2002.* ***70****(6): p. 3033-9.*

48. *Mattsson, E., H. Herwald, and A. Egesten, Superantigens from Staphylococcus aureus induce procoagulant activity and monocyte tissue factor expression in whole blood and mononuclear cells via IL-1 beta. J Thromb Haemost, 2003.* ***1****(12): p. 2569-76.*

49. *Cheng, A.G., et al., Contribution of coagulases towards Staphylococcus aureus disease and protective immunity. PLoS Pathog, 2010.* ***6****(8): p. e1001036.*

50. *McAdow, M., D.M. Missiakas, and O. Schneewind, Staphylococcus aureus secretes coagulase and von Willebrand factor binding protein to modify the coagulation cascade and establish host infections. J Innate Immun, 2012.* ***4****(2): p. 141-8.*

51. *Bokarewa, M.I., T. Jin, and A. Tarkowski, Staphylococcus aureus: Staphylokinase. Int J Biochem Cell Biol, 2006.* ***38****(4): p. 504-9.*

52. *Jin, T., et al., Staphylokinase reduces plasmin formation by endogenous plasminogen activators. Eur J Haematol, 2008.* ***81****(1): p. 8-17.*

53. *Jin, T., et al., Staphylococcus aureus resists human defensins by production of staphylokinase, a novel bacterial evasion mechanism. J Immunol, 2004.* ***172****(2): p. 1169-76.*

54. *Bokarewa, M. and A. Tarkowski, Human alpha -defensins neutralize fibrinolytic activity exerted by staphylokinase. Thromb Haemost, 2004.* ***91****(5): p. 991-9.*

55. *Vanassche, T., et al., Inhibition of staphylothrombin by dabigatran reduces Staphylococcus aureus virulence. J Thromb Haemost, 2011.* ***9****(12): p. 2436-46.*

56. *Jonsson, I.M., et al., The role of Staphylococcus aureus sortase A and sortase B in murine arthritis. Microbes Infect, 2003.* ***5****(9): p. 775-80.*

57. *Mazmanian, S.K., et al., Staphylococcus aureus sortase mutants defective in the display of surface proteins and in the pathogenesis of animal infections. Proc Natl Acad Sci U S A, 2000.* ***97****(10): p. 5510-5.*

58. *Clarke, S.R. and S.J. Foster, Surface adhesins of Staphylococcus aureus. Adv Microb Physiol, 2006.* ***51****: p. 187-224.*

59. Genovese, A., et al., Bacterial immunoglobulin superantigen proteins A and L activate human heart mast cells by interacting with immunoglobulin E. Infect Immun, 2000. ***68****(10): p. 5517-24.*

60. Nguyen, T., B. Ghebrehiwet, and E.I. Peerschke, Staphylococcus aureus protein A recognizes platelet gC1qR/p33: a novel mechanism for staphylococcal interactions with platelets. Infect Immun, 2000. ***68****(4): p. 2061-8.*

61. Hartleib, J., et al., Protein A is the von Willebrand factor binding protein on Staphylococcus aureus. Blood, 2000. ***96****(6): p. 2149-56.*

62. Claro, T., et al., Staphylococcus aureus protein A binding to osteoblast tumour necrosis factor receptor 1 results in activation of nuclear factor kappa B and release of interleukin-6 in bone infection. Microbiology, 2013. ***159****(Pt 1): p. 147-54.*

63. Widaa, A., et al., Staphylococcus aureus protein A plays a critical role in mediating bone destruction and bone loss in osteomyelitis. PLoS One, 2012. 7(7): p. e40586.

64. Dziewanowska, K., et al., Staphylococcal fibronectin binding protein interacts with heat shock protein 60 and integrins: role in internalization by epithelial cells. Infect Immun, 2000. ***68****(11): p. 6321-8.*

65. Siboo, I.R., et al., Clumping factor A mediates binding of Staphylococcus aureus to human platelets. Infect Immun, 2001. ***69****(5): p. 3120-7.*

66. Hair, P.S., et al., Clumping factor A interaction with complement factor I increases C3b cleavage on the bacterial surface of Staphylococcus aureus and decreases complement-mediated phagocytosis. Infect Immun, 2010. ***78****(4): p. 1717-27.*

67. Loughman, A., et al., Roles for fibrinogen, immunoglobulin and complement in platelet activation promoted by Staphylococcus aureus clumping factor A. Mol Microbiol, 2005. ***57****(3): p. 804-18.*

68. O'Brien, L., et al., Multiple mechanisms for the activation of human platelet aggregation by Staphylococcus aureus: roles for the clumping factors ClfA and ClfB, the serine-aspartate repeat protein SdrE and protein A. Mol Microbiol, 2002. ***44****(4): p. 1033-44.*

69. Mulcahy, M.E., et al., Nasal colonisation by Staphylococcus aureus depends upon clumping factor B binding to the squamous epithelial cell envelope protein loricrin. PLoS Pathog, 2012. ***8****(12): p. e1003092.*

70. Corrigan, R.M., H. Miajlovic, and T.J. Foster, Surface proteins that promote adherence of Staphylococcus aureus to human desquamated nasal epithelial cells. BMC Microbiol, 2009. ***9****: p. 22.*

71. Wertheim, H.F., et al., Key role for clumping factor B in Staphylococcus aureus nasal colonization of humans. PLoS Med, 2008. ***5****(1): p. e17.*

*72. Sharp, J.A., et al., Staphylococcus aureus surface protein SdrE binds complement regulator factor H as an immune evasion tactic. PLoS One, 2012. **7**(5): p. e38407.*

*73. Tung, H., et al., A bone sialoprotein-binding protein from Staphylococcus aureus: a member of the staphylococcal Sdr family. Biochem J, 2000. **345 Pt 3**: p. 611-9.*

*74. Josefsson, E., et al., The surface protein Pls of methicillin-resistant Staphylococcus aureus is a virulence factor in septic arthritis. Infect Immun, 2005. **73**(5): p. 2812-7.*

*75. Siboo, I.R., H.F. Chambers, and P.M. Sullam, Role of SraP, a Serine-Rich Surface Protein of Staphylococcus aureus, in binding to human platelets. Infect Immun, 2005. **73**(4): p. 2273-80.*

*76. Miajlovic, H., et al., Direct interaction of iron-regulated surface determinant IsdB of Staphylococcus aureus with the GPIIb/IIIa receptor on platelets. Microbiology, 2010. **156**(Pt 3): p. 920-8.*

*77. Tiedemann, M.T., D.E. Heinrichs, and M.J. Stillman, Multiprotein heme shuttle pathway in Staphylococcus aureus: iron-regulated surface determinant cog-wheel kinetics. J Am Chem Soc, 2012. **134**(40): p. 16578-85.*

78. Zapotoczna, M., et al., Iron-regulated surface determinant B (IsdB) promotes Staphylococcus aureus adherence to and internalization by non-phagocytic human cells. Cell Microbiol, 2012.

*79. Visai, L., et al., Immune evasion by Staphylococcus aureus conferred by iron-regulated surface determinant protein IsdH. Microbiology, 2009. **155**(Pt 3): p. 667-79.*

*80. Kenny, J.G., et al., The Staphylococcus aureus response to unsaturated long chain free fatty acids: survival mechanisms and virulence implications. PLoS One, 2009. **4**(2): p. e4344.*

*81. Corrigan, R.M., et al., The role of Staphylococcus aureus surface protein SasG in adherence and biofilm formation. Microbiology, 2007. **153**(Pt 8): p. 2435-46.*

*82. Roche, F.M., M. Meehan, and T.J. Foster, The Staphylococcus aureus surface protein SasG and its homologues promote bacterial adherence to human desquamated nasal epithelial cells. Microbiology, 2003. **149**(Pt 10): p. 2759-67.*

*83. Li, M., et al., MRSA epidemic linked to a quickly spreading colonization and virulence determinant. Nat Med, 2012. **18**(5): p. 816-9.*

*84. Valle, J., et al., Bap, a biofilm matrix protein of Staphylococcus aureus prevents cellular internalization through binding to GP96 host receptor. PLoS Pathog, 2012. **8**(8): p. e1002843.*

*85. Cucarella, C., et al., Bap, a Staphylococcus aureus surface protein involved in biofilm formation. J Bacteriol, 2001. **183**(9): p. 2888-96.*

86. Harraghy, N., et al., The adhesive and immunomodulating properties of the multifunctional Staphylococcus aureus protein Eap. Microbiology, 2003. ***149****(Pt 10): p. 2701-7.*

87. Bertling, A., et al., Staphylococcal extracellular adherence protein induces platelet activation by stimulation of thiol isomerases. Arterioscler Thromb Vasc Biol, 2012. ***32****(8): p. 1979-90.*

88. Bergmann, S. and S. Hammerschmidt, Fibrinolysis and host response in bacterial infections. Thromb Haemost, 2007. ***98****(3): p. 512-20.*

89. Lahteenmaki, K., S. Edelman, and T.K. Korhonen, Bacterial metastasis: the host plasminogen system in bacterial invasion. Trends Microbiol, 2005. ***13****(2): p. 79-85.*

90. Nitsche-Schmitz, D.P., M. Rohde, and G.S. Chhatwal, Invasion mechanisms of Gram-positive pathogenic cocci. Thromb Haemost, 2007. ***98****(3): p. 488-96.*

91. Bhattacharya, S., V.A. Ploplis, and F.J. Castellino, Bacterial plasminogen receptors utilize host plasminogen system for effective invasion and dissemination. J Biomed Biotechnol, 2012. ***2012****: p. 482096.*

92. Rooijakkers, S.H., et al., Anti-opsonic properties of staphylokinase. Microbes Infect, 2005. 7(3): p. 476-84.

93. Molkanen, T., et al., Enhanced activation of bound plasminogen on Staphylococcus aureus by staphylokinase. FEBS Lett, 2002. ***517****(1-3): p. 72-8.*

94. Furuya, H. and R. Ikeda, Interaction of triosephosphate isomerase from Staphylococcus aureus with plasminogen. Microbiol Immunol, 2011. ***55****(12): p. 855-62.*

95. Iwatsuki, K., et al., Staphylococcal cutaneous infections: invasion, evasion and aggression. J Dermatol Sci, 2006. ***42****(3): p. 203-14.*

96. Molne, J. and A.E. Wold, Inflammation. 2007, Stockholm: Liber.

97. Sakiniene, E., T. Bremell, and A. Tarkowski, Complement depletion aggravates Staphylococcus aureus septicaemia and septic arthritis. Clin Exp Immunol, 1999. ***115****(1): p. 95-102.*

98. Tarkowski, A., et al., Model systems: modeling human staphylococcal arthritis and sepsis in the mouse. Trends Microbiol, 2001. ***9****(7): p. 321-6.*

99. Molne, L., M. Verdrengh, and A. Tarkowski, Role of neutrophil leukocytes in cutaneous infection caused by Staphylococcus aureus. Infect Immun, 2000. ***68****(11): p. 6162-7.*

100. Verdrengh, M. and A. Tarkowski, Role of neutrophils in experimental septicemia and septic arthritis induced by Staphylococcus aureus. Infect Immun, 1997. ***65****(7): p. 2517-21.*

101. Verdrengh, M. and A. Tarkowski, Role of macrophages in Staphylococcus aureus-induced arthritis and sepsis. Arthritis Rheum, 2000. ***43****(10): p. 2276-82.*

102. Hultgren, O., et al., TNF/lymphotoxin-alpha double-mutant mice resist septic arthritis but display increased mortality in response to Staphylococcus aureus. J Immunol, 1998. ***161****(11): p. 5937-42.*

103. Fei, Y., et al., The combination of a tumor necrosis factor inhibitor and antibiotic alleviates staphylococcal arthritis and sepsis in mice. J Infect Dis, 2011. ***204****(3): p. 348-57.*

104. Abdelnour, A., et al., Role of T lymphocytes in experimental Staphylococcus aureus arthritis. Scand J Immunol, 1994. ***39****(4): p. 403-8.*

105. Abdelnour, A., et al., Clonal expansion of T lymphocytes causes arthritis and mortality in mice infected with toxic shock syndrome toxin-1-producing staphylococci. Eur J Immunol, 1994. ***24****(5): p. 1161-6.*

106. Molne, L., et al., Role of gamma/delta T cell receptor-expressing lymphocytes in cutaneous infection caused by Staphylococcus aureus. Clin Exp Immunol, 2003. ***132****(2): p. 209-15.*

107. Rhost, S., et al., Immunomodulatory type II natural killer T lymphocytes in health and disease. Scand J Immunol, 2012. ***76****(3): p. 246-55.*

108. Patel, O., et al., Recognition of CD1d-sulfatide mediated by a type II natural killer T cell antigen receptor. Nat Immunol, 2012. ***13****(9): p. 857-63.*

109. Skold, M. and S.M. Behar, Role of CD1d-restricted NKT cells in microbial immunity. Infect Immun, 2003. ***71****(10): p. 5447-55.*

110. Hayworth, J.L., et al., CD1d-independent activation of mouse and human iNKT cells by bacterial superantigens. Immunol Cell Biol, 2012. ***90****(7): p. 699-709.*

111. Ragin, M.J., N. Sahu, and A. August, Differential regulation of cytokine production by CD1d-restricted NKT cells in response to superantigen staphylococcal enterotoxin B exposure. Infect Immun, 2006. ***74****(1): p. 282-8.*

112. Doisne, J.M., et al., Cutting edge: crucial role of IL-1 and IL-23 in the innate IL-17 response of peripheral lymph node NK1.1- invariant NKT cells to bacteria. J Immunol, 2011. ***186****(2): p. 662-6.*

113. Nieuwenhuis, E.E., et al., Cd1d-dependent regulation of bacterial colonization in the intestine of mice. J Clin Invest, 2009. ***119****(5): p. 1241-50.*

114. Lin, K.H., et al., In vivo protection provided by a synthetic new alpha-galactosyl ceramide analog against bacterial and viral infections in murine models. Antimicrob Agents Chemother, 2010. ***54****(10): p. 4129-36.*

115. Minagawa, S., et al., Activation of natural killer T cells by alpha-galactosylceramide mediates clearance of bacteria in murine urinary tract infection. J Urol, 2005. ***173****(6): p. 2171-4.*

116. Nilsson, N., et al., Protective role of NK1.1+ cells in experimental Staphylococcus aureus arthritis. Clin Exp Immunol, 1999. ***117****(1): p. 63-9.*

117. Small, C.L., et al., NK cells play a critical protective role in host defense against acute extracellular Staphylococcus aureus bacterial infection in the lung. J Immunol, 2008. ***180****(8): p. 5558-68.*

118. Palmqvist, N., et al., Bacterial cell wall-expressed protein A triggers supraclonal B-cell responses upon in vivo infection with Staphylococcus aureus. Microbes Infect, 2005. ***7****(15): p. 1501-11.*

119. Jacobsson, G., et al., Antibody responses in patients with invasive Staphylococcus aureus infections. Eur J Clin Microbiol Infect Dis, 2010. ***29****(6): p. 715-25.*

120. Gjertsson, I., et al., Are B lymphocytes of importance in severe Staphylococcus aureus infections? Infect Immun, 2000. ***68****(5): p. 2431-4.*

121. Hirasawa, Y., et al., Staphylococcus aureus extracellular protease causes epidermal barrier dysfunction. J Invest Dermatol, 2010. ***130****(2): p. 614-7.*

122. Schroder, J.M., The role of keratinocytes in defense against infection. Curr Opin Infect Dis, 2010. ***23****(2): p. 106-10.*

123. Nestle, F.O., et al., Skin immune sentinels in health and disease. Nat Rev Immunol, 2009. ***9****(10): p. 679-91.*

124. Molne, L. and A. Tarkowski, An experimental model of cutaneous infection induced by superantigen-producing Staphylococcus aureus. J Invest Dermatol, 2000. ***114****(6): p. 1120-5.*

125. Cheng, A.G., et al., A play in four acts: Staphylococcus aureus abscess formation. Trends Microbiol, 2011. ***19****(5): p. 225-32.*

126. Guggenberger, C., et al., Two distinct coagulase-dependent barriers protect Staphylococcus aureus from neutrophils in a three dimensional in vitro infection model. PLoS Pathog, 2012. ***8****(1): p. e1002434.*

127. Cho, J.S., et al., Neutrophil-derived IL-1beta is sufficient for abscess formation in immunity against Staphylococcus aureus in mice. PLoS Pathog, 2012. ***8****(11): p. e1003047.*

128. Sodeinde, O.A., et al., A surface protease and the invasive character of plague. Science, 1992. ***258****(5084): p. 1004-7.*

129. Sun, H., et al., Plasminogen is a critical host pathogenicity factor for group A streptococcal infection. Science, 2004. ***305****(5688): p. 1283-6.*

130. Edwards, A.M. and R.C. Massey, How does Staphylococcus aureus escape the bloodstream? Trends Microbiol, 2011. ***19****(4): p. 184-90.*

131. Kubica, M., et al., A potential new pathway for Staphylococcus aureus dissemination: the silent survival of S. aureus phagocytosed by human monocyte-derived macrophages. PLoS One, 2008. ***3****(1): p. e1409.*

132. Prajsnar, T.K., et al., A privileged intraphagocyte niche is responsible for disseminated infection of Staphylococcus aureus in a zebrafish model. Cell Microbiol, 2012. ***14****(10): p. 1600-19.*

133. Gresham, H.D., et al., Survival of Staphylococcus aureus inside neutrophils contributes to infection. J Immunol, 2000. ***164****(7): p. 3713-22.*

134. Thwaites, G.E. and V. Gant, Are bloodstream leukocytes Trojan Horses for the metastasis of Staphylococcus aureus? Nat Rev Microbiol, 2011. ***9****(3): p. 215-22.*

135. Klak, M., et al., Tranexamic acid, an inhibitor of plasminogen activation, aggravates staphylococcal septic arthritis and sepsis. Scand J Infect Dis, 2010. ***42****(5): p. 351-8.*

136. Fourrier, F., Severe sepsis, coagulation, and fibrinolysis: dead end or one way? Crit Care Med, 2012. ***40****(9): p. 2704-8.*

137. Guo, Y., et al., Protective effects of plasmin(ogen) in a mouse model of Staphylococcus aureus-induced arthritis. Arthritis Rheum, 2008. ***58****(3): p. 764-72.*

138. Guo, Y., et al., Beneficial and detrimental effects of plasmin(ogen) during infection and sepsis in mice. PLoS One, 2011. ***6****(9): p. e24774.*

139. Wassenaar, T.M. and W. Gaastra, Bacterial virulence: can we draw the line? FEMS Microbiol Lett, 2001. ***201****(1): p. 1-7.*

140. Casadevall, A. and L.A. Pirofski, Host-pathogen interactions: redefining the basic concepts of virulence and pathogenicity. Infect Immun, 1999. ***67****(8): p. 3703-13.*

141. Casadevall, A. and L. Pirofski, Host-pathogen interactions: the attributes of virulence. J Infect Dis, 2001. ***184****(3): p. 337-44.*

142. Medical Subject Headings. 2013, U.S. National Library of Medicine.

143. Gilchrist, M.A. and D. Coombs, Evolution of virulence: interdependence, constraints, and selection using nested models. Theor Popul Biol, 2006. ***69****(2): p. 145-53.*

144. Kirchner, J.W. and B.A. Roy, Evolutionary implications of host-pathogen specificity: fitness consequences of pathogen virulence traits. Evolutionary Ecology Research, 2002. ***4****(1): p. 27-48.*

145. Alksne, L.E., Virulence as a target for antimicrobial chemotherapy. Expert Opin Investig Drugs, 2002. ***11****(8): p. 1149-59.*

146. Falkow, S., Molecular Koch's postulates applied to bacterial pathogenicity--a personal recollection 15 years later. Nat Rev Microbiol, 2004. ***2****(1): p. 67-72.*

147. Katz, L.S., et al., Using single-nucleotide polymorphisms to discriminate disease-associated from carried genomes of Neisseria meningitidis. J Bacteriol, 2011. ***193****(14): p. 3633-41.*

148. van Belkum, A. and D.C. Melles, Not all Staphylococcus aureus strains are equally pathogenic. Discov Med, 2005. ***5****(26): p. 148-52.*

149. Priest, N.K., et al., From genotype to phenotype: can systems biology be used to predict Staphylococcus aureus virulence? Nat Rev Microbiol, 2012. ***10****(11): p. 791-7.*

150. van Belkum, A., et al., Co-evolutionary aspects of human colonisation and infection by Staphylococcus aureus. Infect Genet Evol, 2009. ***9****(1): p. 32-47.*

151. Melles, D.C., et al., Natural population dynamics and expansion of pathogenic clones of Staphylococcus aureus. J Clin Invest, 2004. ***114****(12): p. 1732-40.*

152. Peacock, S.J., et al., Virulent combinations of adhesin and toxin genes in natural populations of Staphylococcus aureus. Infect Immun, 2002. ***70****(9): p. 4987-96.*

153. Feil, E.J., et al., How clonal is Staphylococcus aureus? J Bacteriol, 2003. ***185****(11): p. 3307-16.*

154. Lindsay, J.A., et al., Microarrays reveal that each of the ten dominant lineages of Staphylococcus aureus has a unique combination of surface-associated and regulatory genes. J Bacteriol, 2006. ***188****(2): p. 669-76.*

155. del Giudice, P., et al., Primary skin abscesses are mainly caused by Panton-Valentine leukocidin-positive Staphylococcus aureus strains. Dermatology, 2009. ***219****(4): p. 299-302.*

156. Sjolund, M. and G. Kahlmeter, Staphylococci in primary skin and soft tissue infections in a Swedish county. Scand J Infect Dis, 2008. ***40****(11-12): p. 894-8.*

157. Luedicke, C., et al., Molecular fingerprinting of Staphylococcus aureus from bone and joint infections. Eur J Clin Microbiol Infect Dis, 2010. ***29****(4): p. 457-63.*

158. Monecke, S., et al., Comparative genomics and DNA array-based genotyping of pandemic Staphylococcus aureus strains encoding Panton-Valentine leukocidin. Clin Microbiol Infect, 2007. ***13****(3): p. 236-49.*

159. Monecke, S., et al., DNA microarray-based genotyping of methicillin-resistant Staphylococcus aureus strains from Eastern Saxony. Clin Microbiol Infect, 2008. ***14****(6): p. 534-45.*

160. O'Reilly, M., B. Kreiswirth, and T.J. Foster, Cryptic alpha-toxin gene in toxic shock syndrome and septicaemia strains of Staphylococcus aureus. Mol Microbiol, 1990. ***4****(11): p. 1947-55.*

161. Sismondo, S., An introduction to science and technology studies. 2nd ed. 2010, Chichester, West Sussex, U.K. ; Malden, MA: Wiley-Blackwell. ix, 244 p.

*162. Huws, S.A., et al., Interactions of some common pathogenic bacteria with Acanthamoeba polyphaga. FEMS Microbiol Lett, 2008. **282**(2): p. 258-65.*

*163. Yang, L., et al., Pattern differentiation in co-culture biofilms formed by Staphylococcus aureus and Pseudomonas aeruginosa. FEMS Immunol Med Microbiol, 2011. **62**(3): p. 339-47.*

*164. Zhang, Y., et al., Duckweed (Lemna minor) as a model plant system for the study of human microbial pathogenesis. PLoS One, 2010. **5**(10): p. e13527.*

*165. Prithiviraj, B., et al., Staphylococcus aureus pathogenicity on Arabidopsis thaliana is mediated either by a direct effect of salicylic acid on the pathogen or by SA-dependent, NPR1-independent host responses. Plant J, 2005. **42**(3): p. 417-32.*

*166. Prithiviraj, B., et al., Plant models for animal pathogenesis. Cell Microbiol, 2005. **7**(3): p. 315-24.*

*167. Sifri, C.D., et al., Caenorhabditis elegans as a model host for Staphylococcus aureus pathogenesis. Infect Immun, 2003. **71**(4): p. 2208-17.*

*168. Begun, J., et al., Staphylococcus aureus virulence factors identified by using a high-throughput Caenorhabditis elegans-killing model. Infect Immun, 2005. **73**(2): p. 872-7.*

*169. Wu, K., et al., Caenorhabditis elegans as a host model for community-associated methicillin-resistant Staphylococcus aureus. Clin Microbiol Infect, 2010. **16**(3): p. 245-54.*

*170. Garsin, D.A., et al., A simple model host for identifying Gram-positive virulence factors. Proc Natl Acad Sci U S A, 2001. **98**(19): p. 10892-7.*

*171. Jensen, R.L., et al., Limitations in the use of Drosophila melanogaster as a model host for gram-positive bacterial infection. Lett Appl Microbiol, 2007. **44**(2): p. 218-23.*

*172. Miyazaki, S., et al., Evaluation of Staphylococcus aureus virulence factors using a silkworm model. FEMS Microbiol Lett, 2012. **326**(2): p. 116-24.*

*173. Needham, A.J., et al., Drosophila melanogaster as a model host for Staphylococcus aureus infection. Microbiology, 2004. **150**(Pt 7): p. 2347-55.*

*174. Wu, K., et al., Assessment of virulence diversity of methicillin-resistant Staphylococcus aureus strains with a Drosophila melanogaster infection model. BMC Microbiol, 2012. **12**: p. 274.*

*175. Li, Y.J. and B. Hu, Establishment of multi-site infection model in zebrafish larvae for studying Staphylococcus aureus infectious disease. J Genet Genomics, 2012. **39**(9): p. 521-34.*

176. Prajsnar, T.K., et al., A novel vertebrate model of Staphylococcus aureus infection reveals phagocyte-dependent resistance of zebrafish to non-host specialized pathogens. Cell Microbiol, 2008. ***10****(11): p. 2312-25.*

177. Daum, R.S., et al., A model of Staphylococcus aureus bacteremia, septic arthritis, and osteomyelitis in chickens. J Orthop Res, 1990. ***8****(6): p. 804-13.*

178. Wilder, T.D., et al., Differences in the pathogenicity of various bacterial isolates used in an induction model for gangrenous dermatitis in broiler chickens. Avian Dis, 2001. ***45****(3): p. 659-62.*

179. Zhou, Q., et al., Role of interleukin-6 in the pathogenesis of an avian model of Staphylococcus aureus arthritis. Poult Sci, 2007. ***86****(6): p. 1245-50.*

180. Savin, J.A., Experimental infections of man with Staphylococcus aureus. Br J Dermatol, 1974. ***90****(4): p. 475-9.*

181. Singh, G., R.R. Marples, and A.M. Kligman, Experimental Staphylococcus aureus infections in humans. J Invest Dermatol, 1971. ***57****(3): p. 149-62.*

182. Seok, J., et al., Genomic responses in mouse models poorly mimic human inflammatory diseases. Proc Natl Acad Sci U S A, 2013. ***110****(9): p. 3507-12.*

183. Alam, M.-u. and W. Muller, Studying Immunology in Mice, in The Laboratory Mouse, H.J. Hedrich, Editor. 2012, Academic Press: London.

184. Pritchett-Corning, K.R., J. Cosentino, and C.B. Clifford, Contemporary prevalence of infectious agents in laboratory mice and rats. Lab Anim, 2009. ***43****(2): p. 165-73.*

185. Clifford, C.B. and K.R. Pritchett-Corning, Bacterial Infections of Laboratory Mice, in The Laboratory Mouse, H.J. Hedrich, Editor. 2012, Academic Press: London.

186. Sung, J.M., D.H. Lloyd, and J.A. Lindsay, Staphylococcus aureus host specificity: comparative genomics of human versus animal isolates by multi-strain microarray. Microbiology, 2008. ***154****(Pt 7): p. 1949-59.*

187. Lowy, F.D., How Staphylococcus aureus adapts to its host. N Engl J Med, 2011. ***364****(21): p. 1987-90.*

188. Bremell, T., et al., Outbreak of spontaneous staphylococcal arthritis and osteitis in mice. Arthritis Rheum, 1990. ***33****(11): p. 1739-44.*

189. Stiles, B.G., et al., Correlation of temperature and toxicity in murine studies of staphylococcal enterotoxins and toxic shock syndrome toxin 1. Infect Immun, 1999. ***67****(3): p. 1521-5.*

190. Adhikari, R.P., et al., Novel structurally designed vaccine for S. aureus alpha-hemolysin: protection against bacteremia and pneumonia. PLoS One, 2012. 7(6): p. e38567.

191. McAdow, M., et al., Coagulases as determinants of protective immune responses against Staphylococcus aureus. Infect Immun, 2012. ***80****(10): p. 3389-98.*

192. Edwards, A.M., et al., Staphylococcus aureus host cell invasion and virulence in sepsis is facilitated by the multiple repeats within FnBPA. PLoS Pathog, 2010. ***6****(6): p. e1000964.*

193. Nilsson, I.M., et al., Role of the staphylococcal accessory gene regulator (sar) in septic arthritis. Infect Immun, 1996. ***64****(11): p. 4438-43.*

194. Bremell, T., et al., Experimental Staphylococcus aureus arthritis in mice. Infect Immun, 1991. ***59****(8): p. 2615-23.*

195. Horst, S.A., et al., A novel mouse model of Staphylococcus aureus chronic osteomyelitis that closely mimics the human infection: an integrated view of disease pathogenesis. Am J Pathol, 2012. ***181****(4): p. 1206-14.*

196. Kraft, W.G., et al., Cutaneous infection in normal and immunocompromised mice. Infect Immun, 1986. ***52****(3): p. 707-13.*

197. Abe, Y., H. Akiyama, and J. Arata, Production of experimental staphylococcal impetigo in mice. J Dermatol Sci, 1992. ***4****(1): p. 42-8.*

198. Hahn, B.L., et al., Systemic dissemination and cutaneous damage in a mouse model of staphylococcal skin infections. Microb Pathog, 2009. ***47****(1): p. 16-23.*

199. Kugelberg, E., et al., Establishment of a superficial skin infection model in mice by using Staphylococcus aureus and Streptococcus pyogenes. Antimicrob Agents Chemother, 2005. ***49****(8): p. 3435-41.*

200. Akiyama, H., et al., Staphylococcus aureus infection on cut wounds in the mouse skin: experimental staphylococcal botryomycosis. J Dermatol Sci, 1996. ***11****(3): p. 234-8.*

201. Akiyama, H., et al., Staphylococcus aureus infection on experimental croton oil-inflamed skin in mice. J Dermatol Sci, 1994. ***8****(1): p. 1-10.*

202. Sabala, I., et al., Anti-staphylococcal activities of lysostaphin and LytM catalytic domain. BMC Microbiol, 2012. ***12****: p. 97.*

203. Bunce, C., et al., Murine model of cutaneous infection with gram-positive cocci. Infect Immun, 1992. ***60****(7): p. 2636-40.*

204. Noble, W.C., The production of subcutaneous staphylococcal skin lesions in mice. Br J Exp Pathol, 1965. ***46****(3): p. 254-62.*

205. Josefsson, E., et al., Fibrinogen binding sites P336 and Y338 of clumping factor A are crucial for Staphylococcus aureus virulence. PLoS One, 2008. ***3****(5): p. e2206.*

206. Devriese, L.A., A simplified system for biotyping Staphylococcus aureus strains isolated from animal species. J Appl Bacteriol, 1984. ***56****(2): p. 215-20.*

207. Chen, Y.H., et al., Impaired NK1+ T cell development and early IL-4 production in CD1-deficient mice. Immunity, 1997. ***6****(4): p. 459-67.*

208. Cui, J., et al., Requirement for Valpha14 NKT cells in IL-12-mediated rejection of tumors. Science, 1997. ***278****(5343): p. 1623-6.*

209. Burge, S.M., J.M. Marshall, and S.A. Cederholm-Williams, Plasminogen binding sites in normal human skin. Br J Dermatol, 1992. ***126****(1): p. 35-41.*

210. Braff, M.H., et al., Staphylococcus aureus exploits cathelicidin antimicrobial peptides produced during early pneumonia to promote staphylokinase-dependent fibrinolysis. J Infect Dis, 2007. ***195****(9): p. 1365-72.*

211. Jin, T., et al., Fatal outcome of bacteraemic patients caused by infection with staphylokinase-deficient Staphylococcus aureus strains. J Med Microbiol, 2003. ***52****(Pt 10): p. 919-23.*

212. Higgins, J., et al., Clumping factor A of Staphylococcus aureus inhibits phagocytosis by human polymorphonuclear leucocytes. FEMS Microbiol Lett, 2006. ***258****(2): p. 290-6.*

213. Josefsson, E., et al., Protection against experimental Staphylococcus aureus arthritis by vaccination with clumping factor A, a novel virulence determinant. J Infect Dis, 2001. ***184****(12): p. 1572-80.*

214. Bliven, K.A. and A.T. Maurelli, Antivirulence genes: insights into pathogen evolution through gene loss. Infect Immun, 2012. ***80****(12): p. 4061-70.*

215. Cheng, A.G., et al., Genetic requirements for Staphylococcus aureus abscess formation and persistence in host tissues. FASEB J, 2009. ***23****(10): p. 3393-404.*

216. Dieli, F., et al., Resistance of natural killer T cell-deficient mice to systemic Shwartzman reaction. J Exp Med, 2000. ***192****(11): p. 1645-52.*

217. Nagarajan, N.A. and M. Kronenberg, Invariant NKT cells amplify the innate immune response to lipopolysaccharide. J Immunol, 2007. ***178****(5): p. 2706-13.*

218. Hu, C.K., et al., The role of hepatic invariant NKT cells in systemic/local inflammation and mortality during polymicrobial septic shock. J Immunol, 2009. ***182****(4): p. 2467-75.*

219. Etogo, A.O., et al., NK but not CD1-restricted NKT cells facilitate systemic inflammation during polymicrobial intra-abdominal sepsis. J Immunol, 2008. ***180****(9): p. 6334-45.*

220. Arrenberg, P., I. Maricic, and V. Kumar, Sulfatide-mediated activation of type II natural killer T cells prevents hepatic ischemic reperfusion injury in mice. Gastroenterology, 2011. ***140****(2): p. 646-55.*

221. Halder, R.C., et al., Type II NKT cell-mediated anergy induction in type I NKT cells prevents inflammatory liver disease. J Clin Invest, 2007. ***117****(8): p. 2302-12.*