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Molecular determinants involved during *Staphylococcus aureus* colonization and/or infection

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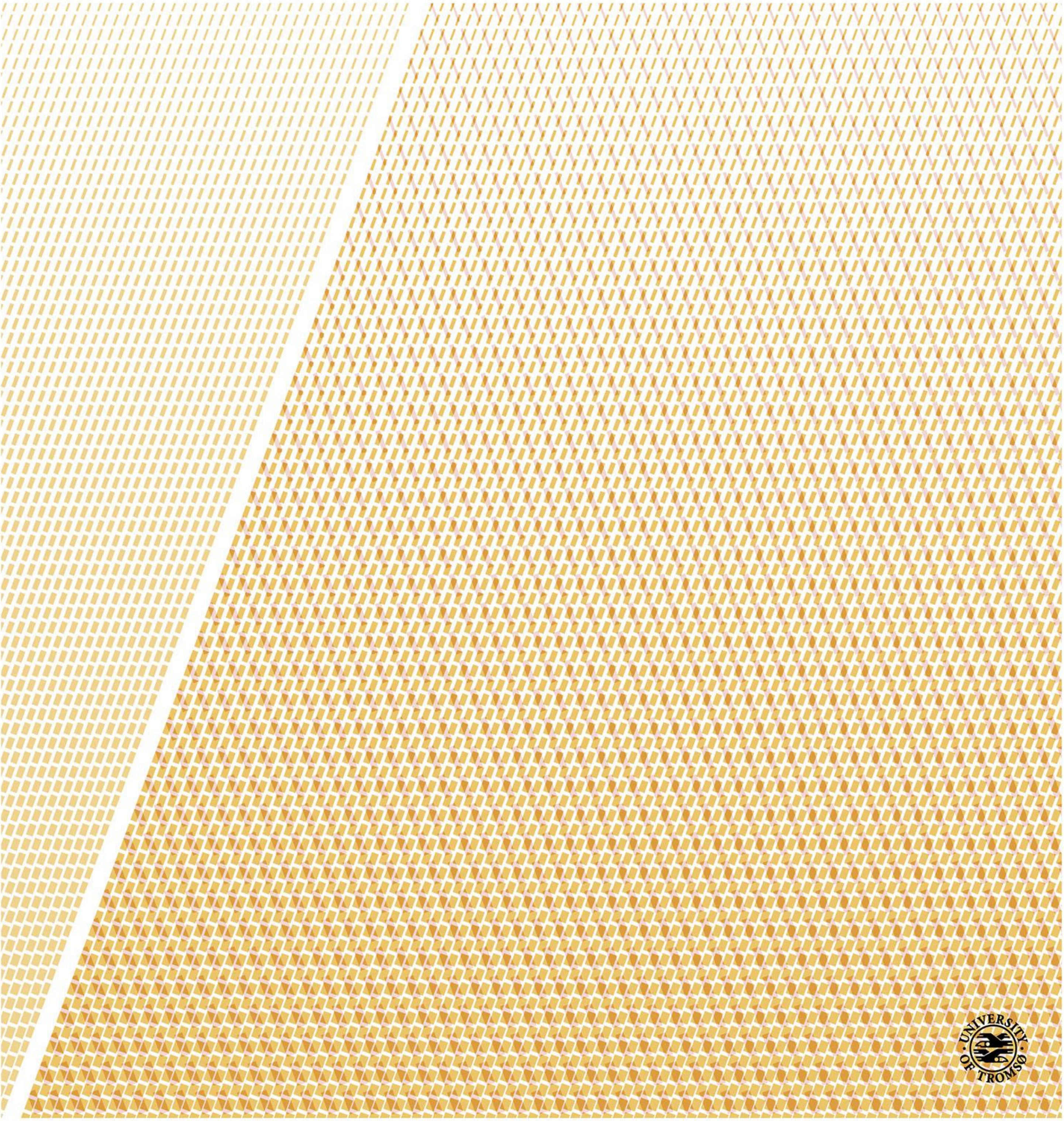
Fatemeh Askarian
A dissertation for the degree of Philosophiae Doctor – April 2014



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Tromsø 2014

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CONTENT

LIST OF PAPERS

Paper I

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Paper III

Fatemeh Askarian, Nina M. van Sorge, Anne-Merethe Hanssen, Diep Bao Dzung, Jos A. G. van Strijp, Johanna U. E. Sollid, **Mona Johannessen**. *Staphylococcus aureus* SdrD promotes bacterial adherence to keratinocytes and whole blood survival. Manuscript.

PREFACE

Given the link between the compelling impact of *Staphylococcus aureus* colonization on the risk and outcome of infection, strategies to prevent nasal colonization could be an appealing method of fighting infection. The ability of *S. aureus* to colonize and infect a host is a balance between its multitudes of virulence factors and the host immune defence mechanisms. Despite the multiple bacterial factors known to be associated with colonization, little is known about the relative contribution of the host determinants. This study explores selected molecular determinants associated with colonization and/or infection, to present a new insight on *S. aureus* interactions with the human host. In theory, the breakage of the interaction between host and *S. aureus* may open new avenues for developing novel therapeutic strategies. However, pursuit of such golden goal merits further investigations.

INTRODUCTION

Staphylococcus aureus persistently colonizes the anterior nares of 10%-35% of healthy individuals ([146, 216, 217] and references within), and can also be found in a number of other anatomical sites [57]. *S. aureus* numerous adhesive and invasive factors, recruitment of resistance to multiple antibiotics, as well as host susceptibility are the main determinants associated with colonization and/or infection [291]. Despite developments in medical care, mortality due to *S. aureus* bacteremia in the developed world is 20-30% [294]. Hence, there is an urgent need for novel strategies to successfully treat staphylococcal infections. To achieve this, increased knowledge and understanding of the molecular determinants involved in the complex of host immune system and *S. aureus* interactions are highly prioritized.

The host innate immune response, as the first line of defense against *S. aureus*, is an imperative factor, highly associated with the outcome of staphylococcal infections. The human innate immunity recognizes a wide range of “pathogen associated molecular pattern” (PAMP), which are highly conserved among pathogens, through “pattern-recognition receptors” (PRRs) such as “toll-like receptor” (TLRs) [139]. In addition, the complement system is a crucial and efficient part of the innate immune system which quickly recognizes *S. aureus* and facilitates its handling by phagocytes [253]. Protective immunity against *S. aureus* is not reported and recurrent staphylococcal infections frequently appear [141]. Thus, the host innate immune response interference by *S. aureus* is pivotal for avoidance of prompt elimination by the defense system and consequently, establishing a critical population size. Therefore, understanding *S. aureus* immune evasion mechanisms has been an area of intense research.

In this project, we investigated selected molecular determinants involved during host-microbe interactions, which may be associated with colonization and/or infection.

STAPHYLOCOCCUS AUREUS AND COLONIZATION/INFECTION OF THE HUMAN HOST

Description of species

Scientific classification of *S. aureus* is as following [188]:
Kingdom: Bacteria
Phylum: Firmicutes
Class: Bacilli
Order: Bacillales
Familly: Staphylococcaceae
Genus: *Staphylococcus*
Species: *Staphylococcus aureus*

S. aureus is a highly adaptive Gram-positive, non-motile, non-spore forming and facultative anaerobe coccus. It is distinguished from other staphylococcal species based on positive catalase and coagulase as well as negative oxidase results. The species was named *aureus* due to the golden color of colonies on solid media [110].

Nowadays, the genus *Staphylococcus* consists of 49 species and 26 subspecies (http://www.bacterio.net/staphylococcus.html, accessed 06. Feb. 2014). *S. aureus* has the advantage of growing under high-salt conditions promoting *S. aureus* colonization on the human skin [92].

Genome and molecular typing

Genome sequencing of *S. aureus* has enabled researchers to investigate questions regarding resistance, virulence, as well as outbreaks. The genome size of *S. aureus* varies between 2.5 to 2.9 megabases (Mb) and possesses approximately 2,400 to 2,800 open reading frames (ORF). The *S. aureus* genome is composed of approximately 80% core and 20% accessory genes where the latter mostly consist of mobile genetic elements (MGEs) [86]. The core genome is conserved among different lineages and comprised of genes associated with metabolic, regulatory, housekeeping, and adhesive functions [170]. A “core variable” (CV) contains genes encoding surface proteins and their regulators, as well as virulence genes. It is localized within the core genome and makes up to 10 to 12% of the *S. aureus* genomes [171, 173].

The presence of accessory genes in *S. aureus*, apart from the core genome, may promote or suppress the pathogenic potential of a given isolate [152, 195]. The MGEs in *S. aureus* consist of e.g. bacteriophages, *S. aureus* pathogenicity islands (SaPIs), plasmids, transposons, and staphylococcal cassette chromosomes (SCC). The MGEs approximately encode 50% of the known virulence factors and actively contributes to horizontal transfer of DNA among *S. aureus* isolates [86, 173]. Horizontal transfer of genes can induce disparate combinations of virulence factors, which may promote host-specific adaptations of clones [115].

Different typing approaches offer the possibility of investigating distribution of clones in the community and hospital, particularly during outbreak circumstances. In other words, typing of the bacteria is crucial for resolving transmissions routes and infection surveillance [283]. Nowadays, typing techniques used for studies of *S. aureus* population structure include; staphylococcal protein A typing (*spa* typing), Multilocus Sequence Typing (MLST), DNA microarrays, and Pulsed Field Gel Electrophoresis (PFGE) [154].

The *spa* gene is localized in the core variable genome [154]. *Spa* typing is a sequence-based method, where the variable number tandem repeat (VNTR) region of the *spa* gene is analyzed. The VNTR region consists of a variable number of short tandem repeats (24-27 bp), and the *spa* type is determined based on the number and order of these short repeats. The recognized *spa* type is grouped into clusters, *spa* clonal complex (CC) groups, using the “Based Upon Repeat BURST” algorithm. This method has high a discriminatory power for outbreak, as well as population investigations [108, 197].

MLST is based on the sequence of internal fragments of seven housekeeping genes including; *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *ygiL* [68]. Comparison of sequence variation within these housekeeping genes against known alleles provides an allelic profile, which identifies the sequence type (ST). Moreover, by using eBURST analysis (WWW.MLST:net), related sequence types, can be clustered into CCs [63, 64, 69]. The MLST is frequently used in population investigations and evolutionary epidemiology. However, the discriminatory power of this method is not sufficient for studies of *S. aureus* outbreaks [196].

The DNA microarray system is based on the whole genome of *S. aureus* and can be used for population investigations. Multiple smaller DNA microarrays have also been developed that detects genes associated with, e.g. virulence, adhesion, or antimicrobial resistance in *S. aureus* [59, 205, 259]. However, the present typing methods are not adequately discriminatory, but this challenge is set to convert with the introduction of whole-genome sequencing (WGS) technique. The WGS enables researchers to compare the genetic differences between organisms with the single base pair resolution. It provides the sufficient discriminatory power for studying *S. aureus* outbreaks, as well as population structure and is becoming faster and cheaper (reviewed in [237]).

Based on typing, the population of *S. aureus* strains can be grouped into different clusters. The *S. aureus* populations, associated with humans, consist of 10 dominant and numerous minor lineages. The dominant lineages are often specified by their CC number [173]. The ten dominating human *S. aureus* lineages, CC1, CC5, CC8, CC12, CC15, CC22, CC25, CC30, CC45, and CC51 consist of colonizing as well as invasive isolates of methicillin-resistant *S. aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA) [68]. *S. aureus* population structure based on isolates colonizing people in different parts of the world, displays huge geographical divergence in the most commonly found CCs [68, 194, 256].

Carriage site and host range

S. aureus colonization in humans can occur at multiple body sites including; anterior nares [299, 322], skin [322], perineum [249], vagina [97], axillae [49, 249, 322], different part of the digestive system including pharynx [10, 249, 322], gastrointestinal tract [250, 322], urinary tract [208] and throat [322]. Although, the nares have been known as the main niche and reservoir of *S. aureus* in humans [299, 322], several studies have indicated higher prevalence of *S. aureus* in the throat [104, 164, 186, 215].

S. aureus can also colonize animals e.g. dogs, cats, rabbits, pigs, cattle, horses, parrots, bats and chinchillas [207]. Various genetic analyses have shown that animal-associated *S. aureus* is not commonly found in human-associated lineages. This reflects the presence of host specific barriers between *S. aureus* animal and human lineages. Notably, both lineages are closely related to each other and only a few particular genes or gene combinations may contribute to host specificity and adaptation [286].

There is an exchange of genes encoding virulence factors between animal- and human-associated *S. aureus* lineages. Clearly, acquisition of virulence genes by specific host-adapted isolates, that enable them to colonize and infect new hosts, can be dangerous. Previously, it has been demonstrated that a dominant livestock-associated methicillin-resistant *S. aureus* (LA-MRSA), ST 398, was adapted to humans by acquisition of additional genetic material including, Panton-Valentine Leukocidin (PVL) and phages encoding human specific innate-immune evasion factors [72]. Several invasive human infections, e.g. endocarditis, osteomyelitis, and ventilator-associated pneumonia are caused by ST398 [58, 185]. Interestingly, recent whole-genome analysis of ST 398 suggested that this strain originated from methicillin susceptible *S. aureus* (MSSA), crossed species barriers from humans to livestock, and is adapting back to humans through acquisition of virulence genes [238]. Nowadays, methicillin-resistant *S. aureus* (MRSA) is increasingly recognized within the animal kingdom, and a huge concern has been raised due to its presence in the animal world, particularly in pigs, as these may serve as a reservoir for human infection and colonization [207, 312].

Transmission

The transmission potential of a pathogen is obviously influenced by “transmissibility” and “duration of infectivity” [187]. If an individual is a carrier of *S. aureus,* this can enhance the risk of additional individual or surrounding contamination [153]. *S. aureus* nasal carriers with rhinitis, also called “cloud” individuals, are able to disperse a high load of this bacterium to the environment [269]. *S. aureus* can survive for several months on any kind of surface [317]. Typical transmission of *S. aureus* occurs mainly due to direct skin to skin contact, or contact with recently contaminated surfaces [50, 202]. Hands play an important role in the transmission of *S. aureus* from surfaces to the nasal niche/other body site and vice versa [318]. A strong correlation between hand carriage and nasal carriage has been reported previously [274]. Moreover, host determinants, e.g. colonization status [153] and immune impairment, as well as capability of *S. aureus* in colonization on the corneal layer of the skin with different properties, e.g. low temperature, low pH, high osmolarity, nutrient restrictions, antimicrobial peptides and interference of commensal microorganisms [203], contribute to successful transmission and acquisition of *S. aureus*.

Pattern of nasal colonization

S. aureus frequently colonizes the human skin and mucus, either for long or short periods throughout life. “Vestibulum nasi” has been known as one of the major sites for *S. aureus* colonization in humans. Nasal carriage of *S. aureus* is identified by a subclinical inflammatory response, which is inadequate for elimination of *S. aureus* [44, 242]. The presence of *S. aureus* has been confirmed in the associated keratin and mucous debris, cornified layer of squamous epithelium as well as hair follicles in the vestibulum nasi [289]. The prevalence of *S. aureus* nasal carriers varies among various groups as well as different age groups [10, 231]. For instance, a high prevalence has been reported in infants [231], white people [44, 322], males [65, 322] and among patients with several diseases (reviewed in [317]) such as HIV [214], diabetes mellitus [174], atopic dermatitis [321], end stage liver disease [29, 31] and in dialysis patients [145]

S. aureus nasal carriers can be classified into persistent carriers and non-persistent carriers/non-carriers [297]. This is based on the “culture rule” where at least two nasal swabs are required for accurate prediction of carriage status [216]. Within a healthy population, approximately 10-35 % are reported to be persistently colonized with a high load of *S. aureus* in the anterior nares, while non-carriers or intermittent carries have low or no detectable bacterial load ([146, 216, 217] and references within). The detection of persistent carriers is pivotal in determining the risk of subsequent infections. In many cases, persistent carriers are colonized by a single isolate of *S. aureus* over a long time. In contrast, intermittent carriers may carry various strains over time [65, 119, 300].

Clinical significance, colonization versus infection

S. aureus is one of the most medically important pathogens, can be the cause of human superficial and systemic infections. The pathogen can be detected both in the community (“Community-Acquired” (CA)) and in the hospital setting (“Hospital-Acquired” (HA) or nosocomial) [272]. The prevalence of antimicrobial resistance among HA isolates are significantly greater than CA isolates, demonstrating that HA isolates are epidemiologically distinct from CA isolates. This can imply the presence of a resident microflora in the hospitals [239].

MRSA was identified in the 1960s as a nosocomial pathogen [14]. In the 1990s CA-MRSA rapidly emerged globally [295], and the MRSA rates have increased worldwide during the last decades [282]. MRSA is a significant global public health concern and associated with higher morbidity, mortality and financial costs [91]. Although, data from the European Antimicrobial Resistance Surveillance Network (EARS-Net) from 2002-2009 show a notable decrease in the proportion of MRSA among *S. aureus* strains in the participating countries, the proportion is still over 25 % in more than one fourth of them [81]. Clinical studies describe a high risk of bacteremia among MRSA nasal carriers with multiple hospitalizations or central venous catheter [107].

Globally, 39% of skin and soft tissue infections, 22% of bloodstream infections and 20-30% bacteremia mortality are caused by *S. aureus* [54, 294]. *S. aureus* infections generally involve a carrier of the bacteria either through autoinfection or cross-infection [54]. The persistent carrier may result in high dispersal of the *S. aureus* to the surroundings [146, 216, 217]. Clinical studies suggest a high risk of bacteremia among nasal carriers [45, 107, 127, 146, 240] and non-bacteremic *S. aureus* healthcare associated infections[147, 148, 182, 267].The relation between *S. aureus* carriage and infection is verified by the fact that in more than 80% of *S. aureus* nosocomial bacteremia, carrier strains and infecting isolates have the same genotype [304, 316]. However, despite the high risk of infection in *S. aureus* carriers, only a minority of them suffers any detrimental effects of their co-existence. The incidence of carriage has been estimated 1,000 times higher than infections [163].

Access to the host's internal tissues or vasculature is crucial for initiating of *S. aureus* infection. Once inside the host, the bacterium goes through an alteration in gene expression leading to the controlled production of virulence determinants that promote infection [292]. *S. aureus* infection principally can be classified into several types: **(1)** superficial infection, e.g. boils, furuncles and lesions, which are localized in the skin or other sites of the body; **(2)** deep-seated infections, e.g. systemic or life threatening infections such as endocarditis, osteomyelitis, brain abscesses, meningitis, pneumonia, and bacteremia; **(3)** hospital-acquired infection, e.g. surgical site infection and infections associated with indwelling medical devices such as joint prostheses, cardiovascular devices and artificial heart valves; **(4)** toxinoses, e.g. scaled skin syndrome, food poisoning (release of enterotoxin), and toxic shock syndrome (release of superantigens into the blood) [2, 74].

THE HOST IMMUNE RESPONSES

The skin

The skin is a vital physical barrier, with constitutive innate immune responses, providing the first line of defence against pathogens encountered in the environment [156, 211]. The human skin structure is composed of two main compartments including epidermis, the outer compartment, and dermis, the inner compartment [211].

Epidermis is composed of four different layers including corneal, granular, spinous and basal layers, from top to bottom (Fig. 1) and is continuously being renewed. The basal layer consists of one row of undifferentiated keratinocytes, which divides frequently. Keratinocytes migrate from the basal layer to the corneal layer and simultaneously go through a maturation process. The corneal layer consists of dead mature keratinocytes, corneocytes, which are devoid of organelles and is highly responsible for the barrier function of the skin. This layer does not exist in other epithelium cells that are exposed to the environment such as gut and lung [156, 211]. Dermis consists of connective tissue, e.g. collagen and elastin fibers, sweat glands, sebaceous glands, hair follicles, and vasculatures [156, 211].

Both dermis and epidermis participate in cutaneous immune responses. The surface of the skin has some basic properties such as low pH and temperature [96], corneal barrier [156, 211], production of antimicrobial peptides by the corneal layer [223, 262], as well as normal skin microflora or commensals [95], which protect the host against pathogens. In addition, there are numerous immune cells residing both in epidermis, e.g. Langerhans cells, as well as dermis, e.g. macrophages, dendritic cells, natural killer cells (NK), plasma cells, fibroblasts, B-cells and T-cells, which also contribute in cutaneous immune responses [156, 211].

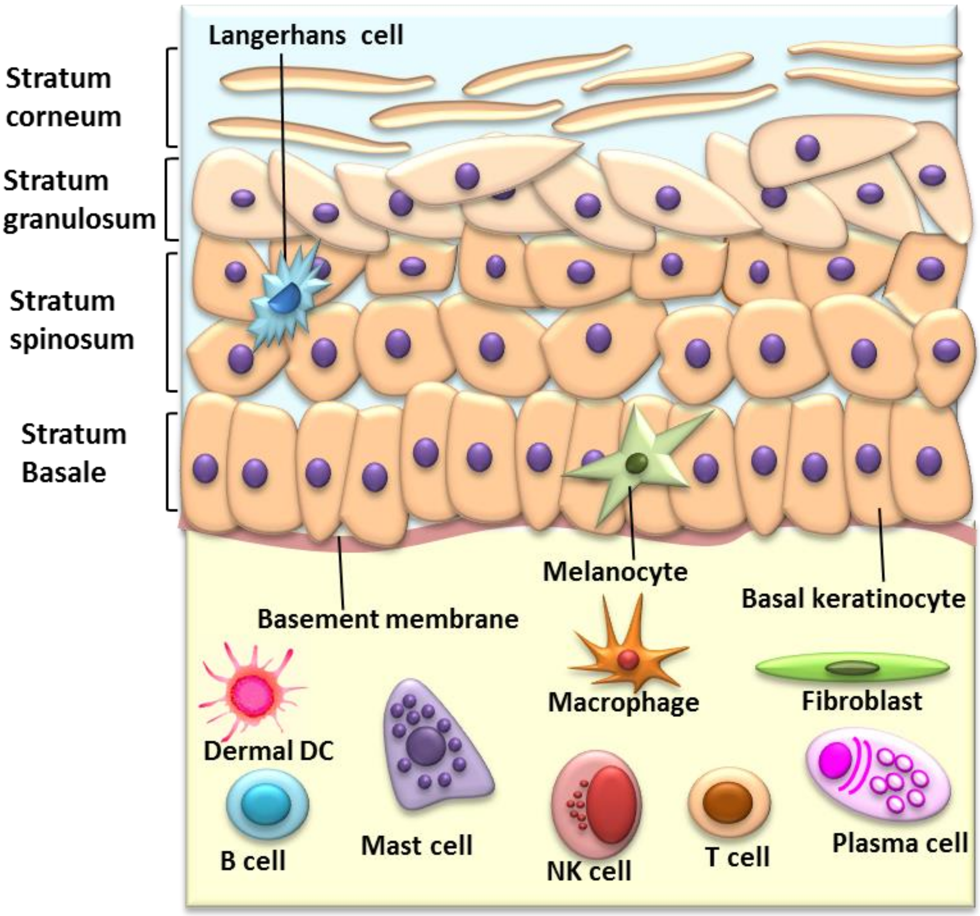

Figure 1. Skin anatomy. Epidermis consists of several layers of keratinocyte cells at various differentiation levels. Langerhans cells are found in epidermis whereas, natural killer (NK) cells, dendritic cells (DC), plasma cells, mast cells, macrophages, fibroblasts, B-cells and T cells are found in dermis. Based on [211].

Innate immunity

The immune system has traditionally been classified into the innate, which we are born with, and adaptive components, which we acquire. The major distinction between these two systems lies in the mechanisms and receptors used for molecular recognition. In simple words, the innate immunity comprises the first line of host defense during infection and plays an important role in the early recognition of pathogen and subsequent induction of proinflammatory responses against invading pathogens [193]. The adaptive immunity is in charge of eradication of pathogens in the late phase of infection, as well as production of immunological memory [124].

The innate immune responses are dependent on recognition of microbial conserved structures such as the pathogen-associated molecular patterns (PAMPs), mediated through germ-line encoded pattern recognition receptors (PRRs), e.g. Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors [204]. In addition, the complement system can be considered as a humoral “master alarm system” of the innate immunity [151].

In this thesis, the innate immunity is of focus, and TLR signaling and complement system will be briefly described.

Toll-like receptor signaling

The human Toll-like receptors (TLRs) consist of 10 members (TLR1- TLR10) and play crucial roles in induction of human innate immune responses [3, 287]. They can recognize various conserved microbial structures [3, 221], collectively called PAMPs [287]. Each TLR can recognize a distinct set of PAMPs, derived from a diverse range of microbial pathogens such as bacteria, fungi, protozoa, and viruses [4, 287]. Recognition can be either through a direct interaction, e.g. TLR1/TLR2, TLR3 and TLR9 [128, 162, 177], or indirectly through an accessory PAMP-binding molecule, e.g. interaction between LPS and MD2-TLR4 complex [142].

Cellular distribution of Toll-like receptors is diverse. Some of the TLRs, e.g. TLR1, -2, -4, -5, -6 and -10, are expressed on the cell surface and are specialized in recognition of PAMPs as well as endogenous misplaced proteins. Others, e.g. TLR3, -7, -8 and -9, are mainly localized in intracellular compartments such as lysosomes, endosomes and endolysosomes and mainly recognize nucleic acids [124, 138, 204] (Fig. 2). TLRs are expressed in most cell types either in an inducible or constitutive manner. However, antigen presenting cells (APCs) including macrophages, dendritic cells (DCs) and B lymphocytes (B-cells) are constitutively expressing the TLR proteins [204]. All members of TLRs, except TLR2, are functionally activated as homodimers. TLR2 is able to form heterodimers with either TLR1 or TLR6 to achieve specificity for the various bacterial lipoproteins repertoire (reviewed in [67]).

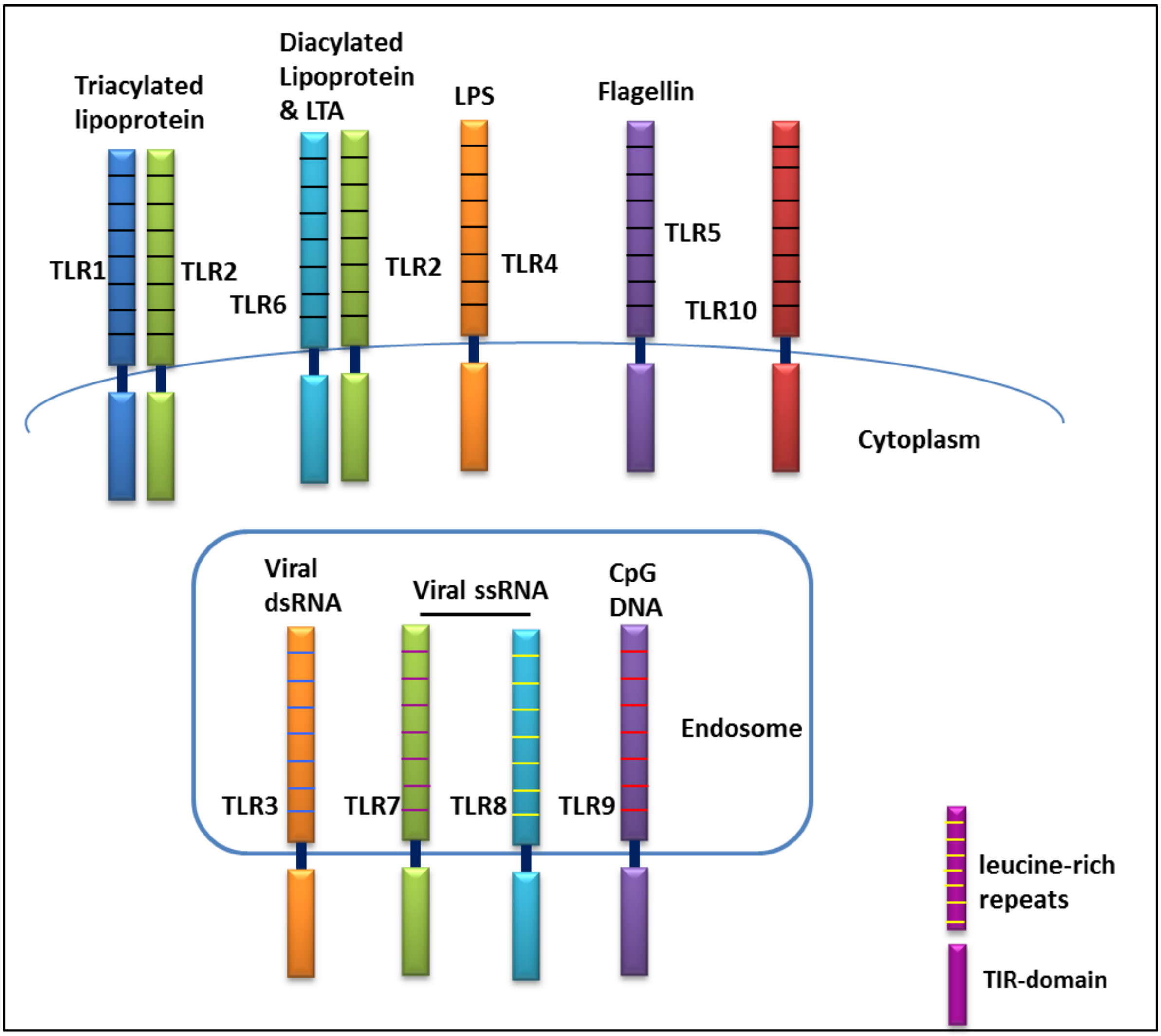

Figure 2. TLRs localization and their ligands. Plasma membrane and endosomal localized TLRs recognize the indicated ligands. Natural ligand for TLR10 has not been identified yet. Based on **[204, 287]**

The TLRs are transmembrane glycoproteins composed of an extracellular or luminal ligand binding domain, a transmembrane domain and a cytoplasmic region of around 200 amino acids, recognized as the Toll/IL-1 receptor (TIR) domain (Fig. 2). The extracellular domain contains leucine-rich repeats (LRRs) [221, 275, 287] and the intracellular TIR domain recruits appropriate TIR-containing adaptor protein(s) such as myeloid differentiation factor 88 (MyD88), MyD88 adaptor-like protein (Mal, also known as TIR-associated protein or TIRAP), TIR-domain-containing adaptor protein including interferon-β (TRIF), TRIF-related adaptor molecule (TRAM), or sterile adaptor α- and armadillo-motif-containing protein

(SARM). TIRAP is distinctly known as a bridging molecule for MyD88 in the context of TLR2 and TLR4 signal transduction [3, 287].

The ligand binding to the receptor induces TLR dimerization and subsequently recruitment of the cytoplasmic TIR-containing adaptor proteins, such as MyD88 or TIRAP. MyD88 consists of an N-terminal death domain (DD), an intermediate domain (ID), and a C-terminal TIR domain (TIR), which is associated with the TIR domain of TLRs [287]. The ID and DD of MyD88 are associated with the IL-1R-associated kinase 4 (IRAK4) and IRAK1 respectively. Binding of IRAK4 leads to phosphorylation of IRAK1 [126, 287]. Activation of IRAKs induces recruitment of tumor necrosis factor receptor-associated factor 6 (TRAF6) to the receptor complex. Phosphorylated IRAK1 and TRAF6 are dissociated from the receptor complex and associates with another complex consisting of transforming growth factor-β-activated kinase-1 (TAK1), TAK1 binding protein 1 (TAB1) and TAB2. This subsequently leads to activation of two different signaling pathways, such as nuclear factor kappa B (NF-κB) through the I kappa B kinase (IKK) complex and the mitogen-activated protein kinases (MAPKs) [4, 137, 138] (Fig. 3).

The IKK complex plays a key role in production of proinflammatory responses through TLR-induced NF-κB activation. IKK complex is composed of the kinases IKKα and IKKβ as well as the regulatory subunit IKKγ/NEMO. TAK1 phosphorylates and subsequently activates the IKK complex, which phosphorylates IκB, targeting it for proteasomal degradation. NF-κB is then released and translocates into the nucleus where the transcription factor binds to κB sites. NF-κB regulates a broad range of genes associated with the host immune responses [100, 138] (Fig. 3).

Several members of MAPK kinases (MKKs), e.g. MKK3, -4, -6 and -7, are involved in induction of the MAPK signaling pathway. Upon TAK1-mediated phosphorylation of MKKs, MKK3/6 and MKK4/7 phosphorylate and subsequently activate p38 and c-Jun N-terminal kinase respectively. Ultimately, transcription factor activator protein 1 (AP-1) is activated due to induction of MAPK signaling [30, 143]. The TLR induced- NF-κB and -MAPKs play a crucial role in induction of pro-inflammatory host responses through secretion of cytokines and chemokines [138] (Fig. 3).

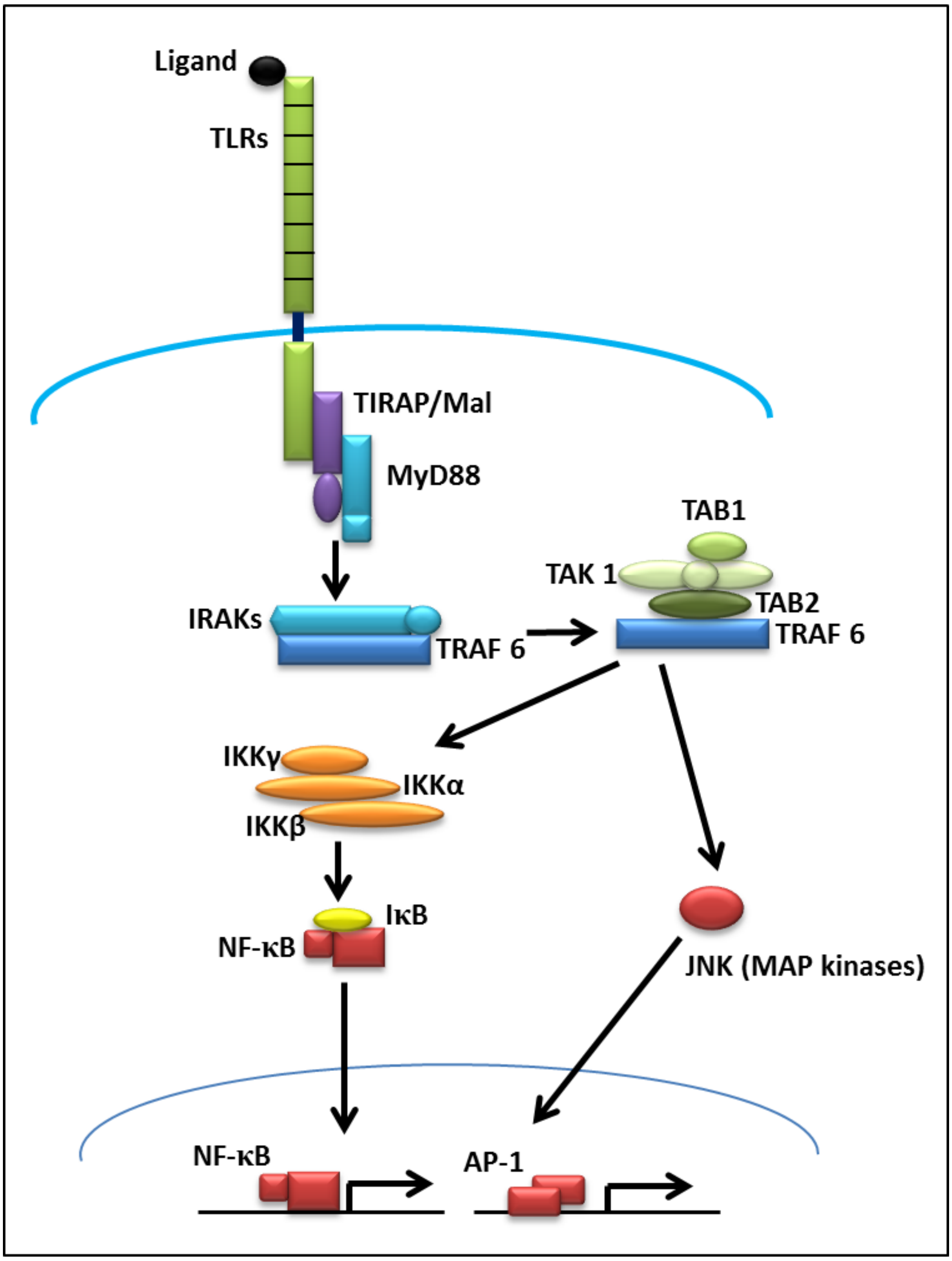

Figure 3. Schematic view of stimuli- induced TLR2-mediated signaling. TLRs, e.g. TLR2, signaling pathway is mainly mediated through MyD88-dependent pathway. TIRAP are sorting adaptors used by TLRs such as TLR2 and TLR4. MyD88 recruits and IRAKs and TRAF6 and ultimately induces pro- inflammatory responses through activation of MAPK and NF-κB. TIRAP: TIR associated protein, MyD88: myeloid differentiation factor-88, IRAK: IL-1 receptor associated kinase, TRAF: tumor necrosis factor receptor-associated factor, TAK1: growth factor-β-activated kinase-1, TAB: TAK1 binding protein 1, IKK: IkappaB kinase, MAPK: mitogen activated protein kinase, NF-κB: nuclear factor kappa B, Ap-1: activator protein 1. Based on [138, 287].

Complement system

The complement system is an “upstream arm” of innate immunity [13] and forms a strong immune barrier. Upon entrance of pathogens, this system initiates its function immediately and produces a regulated and efficient antimicrobial response [332]. The main activities of the complement system in innate immunity are (1) labeling of pathogens or immunogenic particles with C3b or iC3b molecules to facilitate phagocytosis, (2) attraction of phagocytes through production of anaphylactic peptides or chemoattractants such as C3a and C5a, and (3) direct lysing of Gram-negative bacteria through the membrane attack complex (MAC) [158, 310].

The complement system is composed of different (>30) plasma, and cell-bound proteins [248]. The system is activated through three distinct pathways including the classical pathway (CP), the lectin pathway (LP) and the alternative pathway (AP) [273, 332]. Initiation of the CP depends on the presence of a distinct antibody-antigen interaction on the bacterial surface [332] and initiates through activation of the C1 complex. Immunoglobulin M and G (IgM and IgG) are the only group of antibodies that are able to activate the CP [15]. The C1 complex consists of the recognition protein, C1q, and the serine proteases, C1r and C1s. The complex binds to the Fc region of immunoglobulins via C1q molecule. Subsequently, this activates the associated serine protease C1r that later triggers activation and cleavage of the C1s molecule. Activated C1s cleaves C4 and C2 molecules and generates C3 convertase (C4b2a) [62, 332] (Fig. 4).

The LP is activated through attachment of mannose-binding lectin (MBL) or ficolin to an array of carbohydrate structures, polysaccharides, on the microbial surface. MBL and ficolin form a complex with multiple MBL-associated serine proteases (MASPs) including MASP1, MASP2, MASP3 as well as small MBL-associated protein (sMAP), which are the major effectors of the LP. Activated MASP2 cleaves C4 and C2 molecules, thereby generating the C3 convertase, C4b2a. In addition, MASP1 can cleave central component of complement, C3 molecule, directly [62] (Fig. 4).

The AP acts as an amplification pathway for both the CP and LP through increasing the C3 convertase formation and amplification of C3 cleavage. Spontaneous hydrolysis of C3, C3(H_2O), generates small traces of C3b molecule. Activated C3b molecule binds to activated factor B (Bb), which in turn is cleaved by factor D (D). Consequently, the AP C3 convertase, C3bBb is generated [13, 62] (Fig. 4).

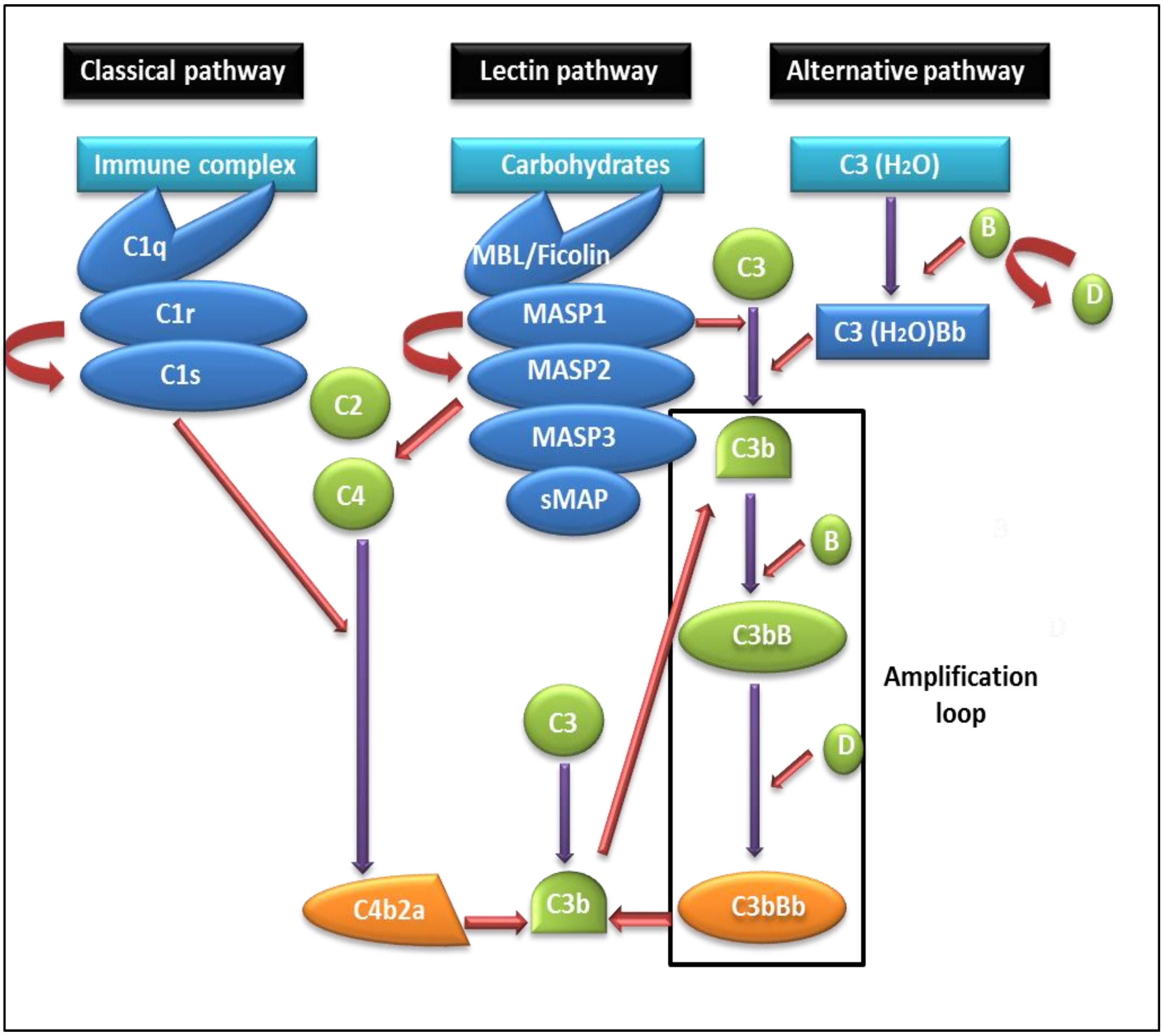

Figure 4. Schematic representation of the three pathways for complement activation including, the classical (CP), alternative (AP) and lectin pathway (LP). In the CP, IgG-/IgM-bound bacteria are recognized by C1-complex. In the LP, carbohydrate structures on pathogens are recognized by ficolin/MBL, while the AP is initiated on the surface of targets without the involvement of recognition molecules. The CP and LP trigger the formation of C3 convertase (C4b2a) and further promote C3b accumulation. The AP works as the CP and LP amplifier. MBL: mannose-binding lectin, MASP: MBL-associated serine protease 1, sMAP: small MBL-associated protein. Based on: [62].

The C3 convertases cleave C3 molecules to C3b molecules and a small peptide chemoattractant, C3a [62]. The deposited C3b molecules can form new convertases and consequently amplify opsonization. However, a major part of C3b molecules is further processed by factor I using factor H as co-factor, into its inactive derivative, iC3b molecules. Either the generated C3b or iC3b facilitate eradication of pathogens through being recognized by complement receptors on the phagocytes [255, 333] (Fig. 4).

If the activation cascade progresses further, additional C3b molecules bind to the CP; LP and AP C3 convertases and consequently generate C5 convertases (C4b2a3b and C3bBbC3b). The C5 convertase cleaves C5 molecules to C5a and C5b peptides. The C5a is a strong anaphylactic peptide and a potent chemoattractant, whereas C5b fragment is a part of terminal complement complex (TCC), which plays an important role in formation of the membrane-attack complex (MAC). The TCC is important in defence against Gram-negative pathogens [332].

Phagocytes

Macrophages, dendritic cells, and neutrophils are professional phagocytes [71, 260]. Approximately, 60% of the leukocyte population in the blood is composed of neutrophils, which are the main phagocytes [6]. Neutrophil-mediated killing is the key host defense mechanism, which protects the host against acute bacterial infections, e.g. staphylococcal infection [277]. Recruitment of neutrophils to the site of infection is a multistep procedure. This procedure is initiated through activation of the endothelial cells, which is followed by rolling of neutrophils along the vessel wall. Thereafter, neutrophils firmly attach to the endothelial cells and finally transmigrate into the tissue, a process called “extravasation” [302]. Upon arrival of neutrophils into the tissue, a chemotactic gradient directs them toward the invading source [21]. This gradient is caused by production of chemoattractants either through activated host cells, e.g. chemokines or cytokines, or complement derived activation products, e.g. anaphylatoxins C3a and C5a, as well as bacterial fragments, e.g. formyl peptides and phenol-soluble modulins (PSMs) in the case of *S. aureus* [277].

Neutrophil mediated phagocytosis depends on opsonization of the target microbe by complement or other innate immune components, and/or immunoglobulins. Opsonin-coated microorganisms attach to the specific receptors on the surface of phagocytes, e.g. complement receptors (CRs) or Fc_{Υ} receptors ($\mathrm{Fc}_{\Upsilon}\mathrm{Rs}$), resulting in endocytosis of the pathogen and formation of a phagosome [6, 277]. Thereafter, the phagosome fuses with lysozyme to form a phagolysosome (Fig. 5). The phagolysosome contains different antimicrobial agents, such as reactive oxygen species, nitrogen intermediates, proteolytic and degradative enzymes, which contributes in destruction of the pathogen [53, 71].

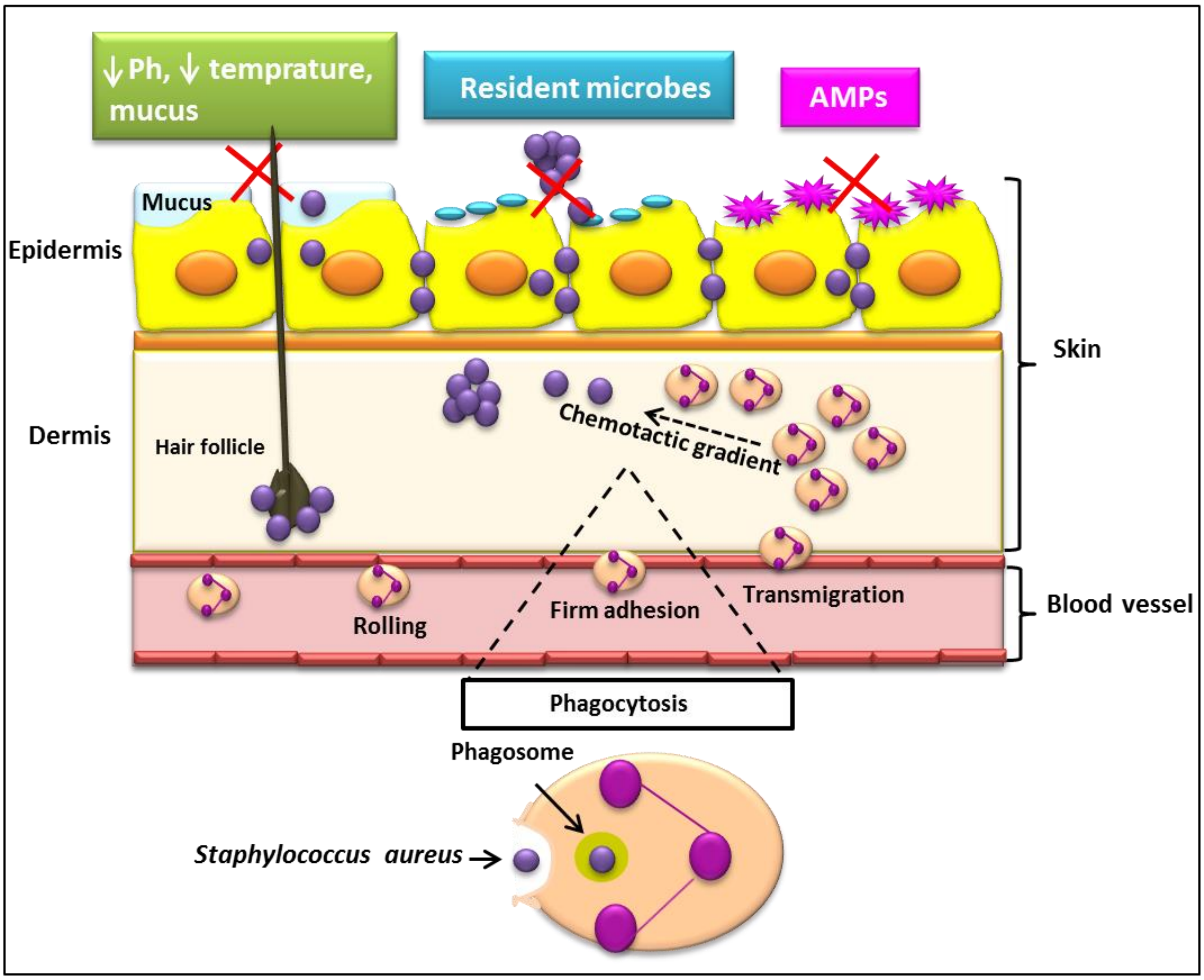

Figure 5. Schematic presentation of normal skin innate immune mechanisms and neutrophil mediated phagocytosis of *S. aureus*. For simplicity, the epidermis structure is presented as one layer of cells instead of several layers. The breakage of the skin barriers increases the risk of staphylococcal infection. Neutrophil mediated phagocytosis of *S. aureus* results in endocytosis of the pathogen and formation of a phagosome. AMP: antimicrobial peptide. Based on [203] with modifications.

S. AUREUS AND HUMAN HOST INTERACTIONS

The interaction between *S. aureus* and its human host is crucial during staphylococcal colonization in a challenging environment, such as nasal epithelium or epidermal keratinocytes, as well as infection [76]. Understanding the mechanisms by which *S. aureus* can colonize, evade the host's immunity, and survive within the host is essential for further development and intervention of novel staphylococcal therapies.

S. aureus tissue infection induces migration of phagocytes to the infection site in order to eradicate the bacterium and neutrophils are the most crucial phagocytic cells. Patients with congenital neutrophil deficiencies or abnormality of neutrophil functions, such as chronic granulomatosis, suffer from cutaneous, respiratory, periodontal or soft tissue, as well as sever fatal infections often caused by *S. aureus* [6, 203, 277]. *S. aureus* targets and interferes with the neutrophil-mediated host defence, targeting extravasation, chemotaxis, opsonization and phagocytosis [53, 277]. Interestingly, once *S. aureus* enters into the professional phagocytes, it uses different strategies to reduce the efficiency of the antimicrobial mechanisms [53]. It takes the benefit of these cells for transport through the bloodstream and distribution throughout the human body [290]. In addition, *S. aureus* produces cytolytic toxins and proteins, which mediate lysis of the host cells and manipulate death of phagocytic cells [53, 306]. This causes the release of surviving bacteria and their toxins into the infected tissue, which consequently leads to local inflammation an infection [290].

In this section of thesis, the *S. aureus* virulence factors promoting colonization, as well as infection of the human host, will be discussed.

S. aureus adhesion during colonization/infection

S. aureus expresses a variety of surface-associated as well as secreted proteins, which mediates attachment to mucus, plasma proteins, epithelial cells, endothelial cells, and extracellular matrix (ECM), as well as evasion of the host immune responses [279]. Staphylococcal adhesins can be structurally classified into "secreted expanded repertoire adhesive molecules" (SERAMs) (reviewed in [33]) and cell wall-anchored (CWA) proteins, which are covalently attached to peptidoglycan (reviewed in [77]), as well as non-protein materials such as wall teichoic acid (WTA) [114].

The SERAMs of *S. aureus* are structurally unrelated secreted adhesins, containing ECM binding properties. Some of its proposed members are coagulase (Coa), fibrinogen binding protein A (FbpA), von Willebrand factor binding protein, extracellular fibrinogen-binding protein (Efb), extracellular adhesive protein (Eap), and extracellular matrix binding protein (Emp). SERAMs can either bind to various host ECM components, such as fibronectin and fibrinogen or facilitate bacterial adhesion to host cells (Table 1). In addition, some of the SERAMs, e.g. Efb, contribute in immune evasion and promote *S. aureus* pathogenicity [33].

The CWA proteins promote adhesion of *S. aureus* to the ECM and other molecules on the host cell, and may facilitate immune evasion. These proteins have been recently classified into four distinct classes based on structural and functional properties including, **(1)** the microbial surface component recognizing adhesive matrix molecules (MSCRAMMs) family, e.g. clumping factor A and B (ClfA and ClfB), serine-aspartate repeat (Sdr) C, D and E, collagen adhesin (Cna) and fibronectin-binding proteins A and B (FnBPA and FnBpB); **(2)** the near iron transporter (NEAT) family, e.g. Iron-regulated surface determinant A and B (IsdA and IsdB); **(3)** the three-helical bundle, e.g. Protein A and **(4)** the G5-E repeat family, e.g. *S. aureus* surface protein G (SasG). These proteins contain an N-terminal secretory signal sequence and a C-terminal sorting signal, where the latter is involved in covalent anchoring of the protein to the staphylococcal cell wall peptidoglycan due to cleavage of the conserved LPXTG motif by sortase (reviewed in [77]).

Twenty-four different CWA proteins may be expressed by *S. aureus* (reviewed in [77]) and the combinations and expression levels of the proteins may vary among *S. aureus* strains [189]. Some of the CWA genes such as *isdA*, *fnbpA*, are present in all 11 lineages of *S. aureus*, whereas others are absent in the majority, e.g. *sdrE, cna*, or few of the lineages, e.g. *clfA*, *clfB*, *sasG, sdrC*, *sdrD* [189]. Moreover, occurrence of allelic variation in the functional domain of the same type of CWA proteins, e.g. FnBP, can influence on the protein-ligand binding strengths and subsequently their contribution in colonization and pathogenesis [25, 179, 181]. Thus, the success of *S. aureus* adherence to the host interaction partners may depend on the correct combination, allelic variation, and the expression level of CWA proteins. In parallel, the proper expression and allelic variation of the human host interaction partners are also determinants in colonization [77, 129].

Certain CWA proteins may influence or alter the adhesive properties of other adhesins. SasG, for instance, masks the ability of exponentially grown *S. aureus* cells expressing ClfB and FnBPs to bind to cytokeratin 10, fibronectin and/or fibrinogen [46]. An individual adhesin can usually interact with several host molecules [279], e.g. ClfB binds to the soluble plasma protein fibrinogen [311], cytokeratin K10 [220, 319] and K8 [101], the major components of squamous cells, as well as to loricrin [209], which is the main component of the cornified cell envelope that is found in terminally differentiated epidermal cells. Another example is IsdA that binds to involucrin, loricrin, and cytokeratin K10 [41]. Moreover, one host molecule can interact with multiple CWA proteins, e.g. fibrinogen binds to ClfA [191] and ClfB [311].

Several CWA proteins are expressed during colonization [23, 24]. Microarray analysis of *S. aureus* nasal isolates reveals high expression level CWA proteins, which are essential in *S. aureus* adhesion to the squamous cells [23]. For instance, transcriptional analysis of the *clfB* gene has shown elevated expression after several days of colonization [23]. Additionally, increased expression levels of SasD and SdrH have been reported among carrier compared to non-carrier isolates [210].

Different CWA proteins, such as ClfB, IsdA, SdrC, SdrD, SasG and SasX, promote *S. aureus* adhesion to squamous cells [39, 46, 47, 166, 209, 251]. Using an *in vivo* model investigating rodent nasal colonization, ClfB [209, 261] and IsdA [39] were demonstrated to promote *S. aureus* colonization. ClfB also contributed in human colonization [319]. Notably, *clfB* and *isdA*–deficient *S. aureus* can still adhere to human desquamated epithelial cells [39], which indicates the role of other components of CWA proteins in *S. aureus* adhesion.

Interestingly, some of the CWA proteins display other functions in addition to adhesion, which magnify their role in *S. aureus* colonization. For instance, IsdA decreases *S. aureus* cellular hydrophobicity, which provides resistance to the innate host bactericidal human skin fatty acids [40]. Other bacterial factors, such as WTA [313], transglycolase SceD [280], as well as several other virulence factors, contribute in adherence of *S. aureus* to host cells (Table 1).

Table 1. A selection of *S. aureus* virulence factors involved in adhesion to the host

Protein group	**Bacterial determinant**	**Abbreviation**	**Adhesion to**	**References**
CWA-MSCRAMM	Fibronectin binding protein A, B	FnBPA, FnBPB	Fibrinogen (* FnBPA), fibronectin & elastin	[26, 140, 228] & reviewed in [77]
CWA-MSCRAMM	Clumping factor A, B	ClfA, ClfB	Desquamated epithelial cells and cytokeratin (*ClfB), immobilized fibrinogen (*ClfA)	Reviewed in [77]
CWA-MSCRAMM	Collagen adhesin	Cna	Collagen-rich tissue	[335]
CWA-MSCRAMM	Serine-aspartate repeat proteins C, D	SdrC, SdrD	Desquamated epithelial cells	[47]
CWA-MSCRAMM	Bone sialoprotein-binding protein	Bbp	Fibrinogen, bone sialoprotein	[301]
Cell-surface protein	Elastin-binding protein	EbpS	Elastin	[225]
CWA-NEAT motif family	Iron-regulated surface determinant A, B, H	IsdA, IsdB, IsdH	Haem, haemoglobin (IsdA, IsdB, IsdH) & desquamated epithelial cells, fibrinogen fibronectin, cytokeratin 10, loricrin (*IsdA)	Reviewed in [77]
CWA- G5-E repeat family	*S. aureus* surface protein G	SasG	Desquamated epithelial cells	[251]
CWA-structurally uncharacterized	*S. aureus* surface protein X	SasX	Desquamated epithelial cells	[166]
SERAM	Extracellular matrix binding protein	Emp	ECM	[192]
SERAM	Extracellular adhesive protein	Eap	ECM	Reviewed in [109]
SERAM	von Willebrand factor binding protein	vWbP	Prothrombin, fibrinogen & vW factor	Reviewed in [33]
Cell- Surface protein	ECM-binding protein homologue	Ebh	ECM	[38]
Cell- Surface protein	Plasmin sensitive protein	Pls	Lipid of the host cells	[120]
Cell-wall component	Wall teichoic acid	WTA	Primary nasal epithelial cells	[313]

* The interaction to host target is only reported for this protein. CWA: Cell wall anchored protein, ECM: Extracellular Matrix, MSCRAMM: microbial surface component recognizing adhesive matrix molecule, NEAT: near iron transporter family, SERAM: secreted expanded repertoire adhesive molecules.

Molecular determinants of nasal colonization

There is a consistent mechanical removal of *S. aureus* in the anterior nares through shedding of squamous epithelial cells and mucus. In order to remain in the anterior nares, the bacteria must proliferate and evade the host immune responses [314]. *S. aureus* nasal carriage is a multifactorial process which involves **bacterial factors**, e.g. bacterial interference with commensal organisms [95, 96], absence or presence of adhesins (see previous section), **host factors**, e.g. host genetic factor, variation in number and nature of host nares receptors for bacterial adherence (reviewed in [229, 314]), constitutive properties of the skin, e.g. low pH and temperature and corneal layer, (reviewed in [211]), immune responses, presence of anti-staphylococcal component in nasal secretions and serious underlying diseases (reviewed in [229, 314]), as well as **environmental factors**, e.g. hospitalization [89] (Fig. 6). The relative importance of these factors in nasal colonization needs further elucidation. However, it has been proposed that host factors play a crucial role while bacterial factors may determine which strain is carried rather than carriage status [231].

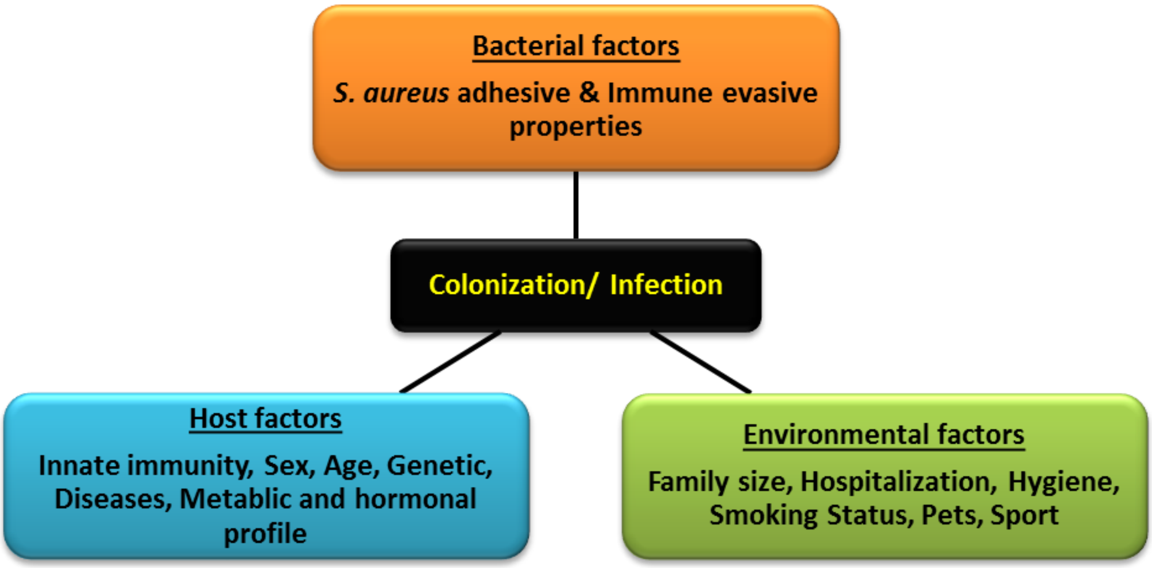

Figure 6. Host-microbe-environment interplay. Proposed interaction between bacterial, host and environmental risk factors associated in *S. aureus* colonization/infection. Based on [222].

A recent publication based on Danish middle-aged and elderly twins demonstrated that host genetic factors had a minor effect on the *S. aureus* carrier state [7]. However, several single nucleotide polymorphisms (SNPs) have been suggested to be crucial determinant for the *S. aureus* persistent nasal carriage [219, 314]. These include polymorphisms in the genes encoding TLR2, Mannose-binding lectin (MBL), C-reactive protein (CRP), glucocorticoid-receptor, vitamin D receptor, defensin, complement factor H, complement C1 inhibitor, as well as in the promoter of interleukin 4 (IL-4) [60, 61, 93, 219, 257, 296, 298, 307]. The expression of host interaction partners may also contribute in *S. aureus* skin colonization and/or infection. Fibronectin, for instance, is not present in the stratum corneum of healthy skin, but is expressed in the skin of atopic dermatitis (AD) patients, which may partly explain the high susceptibility of these patients to the *S. aureus* colonization [35]. Additionally, the skin lesions from AD patients show a high level of Th2 cytokines, e.g. IL-4, which increase *S. aureus* mediated fibrinogen and fibronectin attachment [35, 36].

Nasal fluids contain complement proteins [28], defensins [43], lysozyme as well as immunoglobulins (IgA and IgG) and are a part of host defence against *S. aureus* [134]. *S. aureus* is resistant to lysozyme because of the cell wall modifying enzyme O-acetyltransferase (OatA) and WTA [16]. Also, the nasal secretion from carriers was defective in killing nasal carrier isolates of *S. aureus in vitro* [44], even though it contains elevated levels of α-defensins and human β-defensin 2 [296]. The human β-defensin 3 [201] and cathelicidin LL-37 [324] kill *S. aureus* effectively *in vitro* and the generation of human β-defensin 3 from skin and nasal secretion can be induced by the presence of *S. aureus* [199]. However, its level is significantly lower in persistent carriers than non-carriers [328]. Another trait found among carriers is presence of hemoglobin in nasal fluids. This may contribute in *S. aureus* colonization via inhibition of *agr* system [241].

All these finding demonstrate that several host determinants are associated with nasal colonization.

***S. aureus* innate immune evasion**

Upon entry of *S. aureus* into subepidermal tissues in the body or blood, several cellular and proteinaceous components of host innate immunity collaborates. Innate immune responses against *S. aureus* thoroughly depend on both complement system activation, as well as polymorphonuclear leukocyte (neutrophil) mediated phagocytosis [75, 253]. Neutrophils can recognize *S. aureus* using different receptors such as TLR2 [264] or formylated peptide receptor (FPR) that recognizes formylated peptide releasing by growing bacteria [52]. In the absence of antibodies, the LP of the complement system is activated by recognizing *S. aureus* through MBL and ficolins, which detect LTA and peptidoglycan [183]. Additionally, the AP can be directly activates by peptidoglycan [253]. Accordingly, deposition of the C3 molecule on the *S. aureus* surface, as well as activation of TLR2, FPR and C5a-R signaling pathways lead to an efficient neutrophil mediated phagocytosis [253, 277].

However, *S. aureus* avoid the success of the innate immune components through a hide and seek strategy by interfering with TLR recognition, restraining complement deposition or activation, as well as the chemotaxis of neutrophils (reviewed in [75, 77, 141, 253]). Additionally, several members of CWA proteins such as ClfA [102, 103], Cna [135], SdrE [268] and protein A [73, 169, 232], also interfere with innate and adaptive immune responses. Moreover, *S. aureus* produces several secreted proteins with lytic properties towards neutrophils (reviewed in [75, 277]).

In the following, *S. aureus* virulence factors associated with TLR recognition and complement evasion will be described.

Evasion of TLRs signaling

TLR2 is one of the receptors, recognizing *S. aureus* derived products [78] and its role in defense against *S. aureus* is pivotal. TLR2- [117, 284, 288] and MyD88-deficient [288] mice are hypersusceptible to *S. aureus* infection. In TLR2-deficient mice clearance of *S. aureus* is slower than in wild-type mice, peritoneal macrophages are insensitive to lipoteichoic acid (LTA) [117] and neutrophils are incapable to eliminate *S. aureus* due to a failure in the oxidative burst in response to this bacterium, other than phagocytosis [125]. Patients with genetic defects in TLR signaling pathways, such as MyD88-, or IRAK4-deficiencies, are highly susceptible to infections by Gram-positive bacteria [233, 303]. Deficiency of TLR2, but not TLR4, enhances colonization of MRSA strains in a mouse model of nasal carriage [87]. Additionally, particular polymorphisms in human genes, which encode TLRs or related signaling components, can influence host susceptibility to bacterial infections. For instance, humans with TLR2 Arg753Gln polymorphisms display high susceptibility to infection with *Mycobacterium tuberculosis* and other Gram-positive bacteria such as *S. aureus* [27]. All these findings suggest that the TLR2-MyD88/IRAK-4 pathway is crucial for defense against *S. aureus*.

Understanding bacterial evasion mechanisms through interference with TLR recognition has been an area of intense research. Several studies have been conducted in order to interrupt TLR function in bacteria. Most studies have been carried out on Gram-negative bacteria, confirming the presence of several bacterial molecules targeting most steps in the TLR-NF-κB signaling pathway (Reviewed in [130]). However, our related knowledge on Gram-positive pathogens is limited.

Staphylococcal superantigen-like proteins (SSLs), previously called staphylococcal enterotoxin-like toxins (SETs) [161], consist of 14 different exoproteins displaying low amino acid sequence homologies. The molecular masses of these proteins are approximately 25-35 kDa. There are some structural similarities and sequence homology between SSL proteins, toxic shock syndrome toxin 1 (TSST-1) and enterotoxins. However, they do not display superantigenic properties, and their role was long unknown [20, 168, 323]. The SSL1-SSL11 proteins are encoded by genes located on staphylococcal pathogenicity island 2 (SaPI2), while SSL12-SSL14 are encoded by genes located on immune evasion cluster 2 (IEC2) [70, 131]. The structure of SSL protein as well as TSST-1 and enterotoxins, is composed of a C-terminal β-grasp fold (β-GF), involved in binding to various soluble ligands, and an N-terminal oligonucleotide/oligosaccharide-binding domain (OB) associated with nucleic acid recognition [1, 8, 9, 224, 323]. The SSLs family is involved in the pathogenesis of *S. aureus* and some of them interfere with the host immune proteins [11, 18-20, 51, 122, 123, 227, 308, 309]. Recently, SSL3 and to a lower extent SSL4, was found to inhibit activation and consequently proinflammatory cytokine production via direct binding to the extracellular domain of TRL2. SSL3 significantly suppressed IL-8 production by HEK cells expressing TLR1/2 and TLR2/6 dimers [12]. Additionally, SSL3 inhibited tumor necrosis factor alpha

(TNF-α) production from murine macrophages in response to heat-killed *S. aureus* and related TLR2 ligands such as peptidoglycan and lipopeptide [326].

Evasion of Complement system

S. aureus has developed various mechanisms to modulate the host complement responses at different stages of the cascade (Fig. 7). *S. aureus* modulates complement activation and recognition through different strategies including direct targeting of human immunoglobulins, MBL, complement components e.g. C1q complex and the C3 molecule [158, 334]. Several human immunoglobulin binding proteins are expressed that prevent CP activation, including staphylococcal protein A (SpA), second binder of immunoglobulin (Sbi), SSL7, and SSL10 [20, 111, 123, 330]. The CWA protein Cna, interferes with the interaction of the C1q and C1r components through binding to the collagen domain of the C1q molecule, which consequently prevents the CP activation [135]. Additionally, staphylokinase (Sak)-mediated plasmin deposition prevents *S. aureus* recognition through inactivating of IgG indirectly [254]. WTA binds MBL and blocks the LP activation [133]. *S. aureus* uses two different mechanisms to inactive the complement C3 through enzymatic cleavage [334]. One strategy is to cleave C3 by use of staphylococcal metalloprotease aureolysin [159]. Alternatively, human plasminogen binds to extracellular fibrinogen-binding protein (Efb), Sbi, or triosephosphate isomerase (TPI) and is converted to plasmin either by *S. aureus* itself through Sak, or the human activator, upa. The active plasmin may then cleave C3 [17, 22, 80, 150, 157].

Formation of C3 convertases is crucial for activation of complement amplification [332], which consequently can influence *S. aureus* opsonization. Five different proteins that directly target C3 convertases, known as C3 convertase inhibitors, are secreted. These includes staphylococcal complement inhibitor A (SCIN-A) and its homologues (SCIN-B and SCIN-C) [252], extracellular fibrinogen-binding protein (Efb), and extracellular complement-binding protein (Ecb) [34, 105, 106, 131, 247]. SCIN-A, -B and -C blocks C3 processing by “freezing” the C3 convertase [252]. Efb and Ecb bind to C3 and C3b proteins, resulting in a conformational change in the C3b, thereby preventing its binding to Factor B. The net result is inhibition of the formation of the C3 convertase [34, 105, 106, 247].

The complement regulators down-regulate convertase activity and play an important role in protection of host tissues against excessive activities of the complement system [332]. *S. aureus* contributes to complement evasion by recruiting and attracting host regulators to its surface. For example, Sbi binds to the human complement regulator factor H and factor H-related proteins, as well as the C3b protein, resulting in formation of a tripartite complex, which consequently blocks the AP activation [113]. Moreover, ClfA and SdrE binds to the human C3 protease factor I and factor H respectively, thereby promoting the destruction of C3b molecules [102, 103, 268].

S. aureus also produces several proteins inhibiting C5 activation and consequently neutrophil migration. For example, SSL7 binds to C5 and inhibits C5a and C5b generation [131, 160], while Efb and Ecb are putative inhibitors of C5a-mediated immune responses [131]. Additionally, chemotaxis inhibitory protein of *S. aureus* (CHIPS) also modulates the C5a-mediated immune responses via high affinity binding to the C5aR and thereby preventing recruitment of neutrophils [121, 235, 236]. Panton-Valentine Leukocidin (PVL) also binds to the C5a receptor (C5aR) and C5L2, modulating the C5a-mediated immune responses [276]. The C5b molecule is involved in formation of the membrane attack complex (MAC) [332], which is not assumed to induce lytic action in Gram-positive bacteria due to their thick cell wall. Interestingly, SCIN, Efb, Ecb and SSL7 block MAC-mediated erythrocyte hemolysis. However, the relevance of MAC inhibition by *S. aureus* is unclear [158]. Moreover, the three human terminal complement regulators vitronectin, β2GP1 and CFHR1 and can be recruited to the surface of *S. aureus* through their binding to extracellular matrix binding protein (Emp), Sbi and an unknown protein respectively (reviewed in [334]). The various levels of the complement system and *S. aureus* interference are summarized in figure 7.

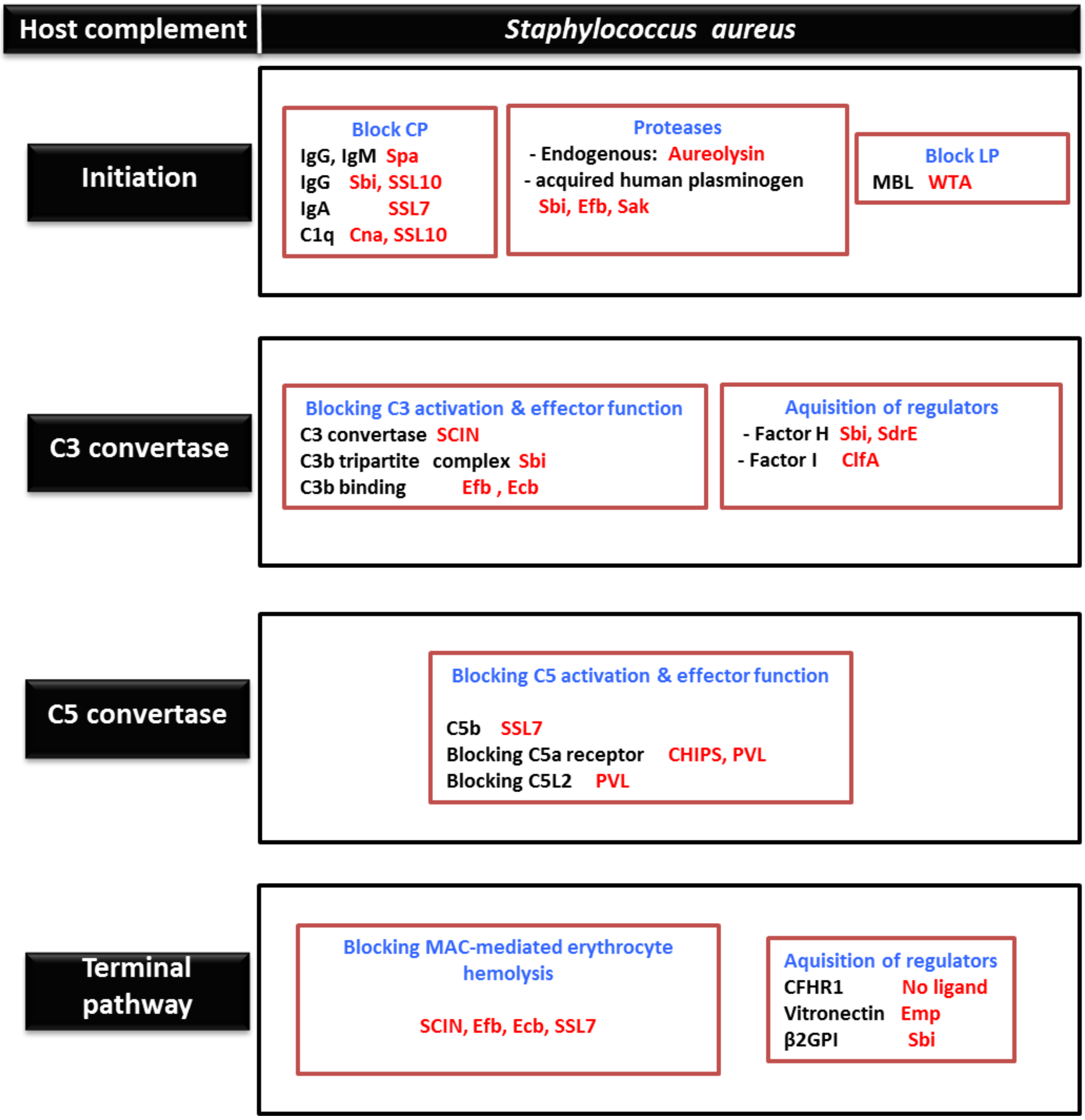

Figure 7. *S. aureus* interferes with human complement response on different levels. The host complement system can be divided into various levels such as initiation, C3 convertase, C5 convertase and terminal pathway. The levels and proteins involved are visualized in black. Strategies of evasion are marked in blue, and staphylococcal proteins involved in evasion at the particular levels are visualized in red. Spa: staphylococcal protein A, Sbi: Second binder of immunoglobulin, SSLs: Superantigen-like proteins , Sak: Staphylokinase, Cna: Collagen adhesin, WTA: Wall teichoiic acid, SCIN: Staphylococcal complement inhibitor, Efb: extracellular fibrinogen-binding protein, Ecb: extracellular complement-binding protein, SdrE: Serine-aspartate repeat protein E, ClfA: Clumping factor A, CHIPS: Chemotaxis inhibitory protein of *S. aureus*, PVL: Panton-Valentine Leukocidin, Emp: extracellular matrix binding protein. Based on [334].

Internalization and survival of S. aureus in host cells

S. aureus has traditionally been known as an extracellular pathogen. Now, we know that *S. aureus* can be internalized, and survive in professional phagocytic cells, as well as non-professional phagocytic cells, e.g. fibroblasts, endothelial cells, osteoblasts, keratinocytes (reviewed in [82, 271]).

The intracellular life style enhances *S. aureus* persistence in host tissue as bacteria are protected against host antimicrobial components and immune responses [266]. Indeed, intracellular *S. aureus* localization has been found in turbinate- and tonsil-biopsies from patients with recurrent rhinosinusitis or tonsillitis, respectively [42, 329] and demonstrated in mice experimental models of mastitis and a rat model of endocarditis [112]. Additionally, it has been demonstrated that *S. aureus* use host cells for its conveyance and dissemination from the site of infection [290].The intracellular localization of *S. aureus* in various cells may increase the risk of relapsing infection and/or contribute to the establishment of chronic infections [82, 234, 293]. During intracellular infection, *S. aureus* may alter the phenotype into small colony variants (SCVs), which increases resistance to intracellular immune responses as well as possibility of therapeutic failure [266, 293, 305]. SCVs can rapidly return to their wild-type form after leaving intracellular milieu [293].

Internalization of *S. aureus* into the non-professional phagocytes is mediated through actin-rearrangement of the host cell (reviewed in [79, 82]). FnBPs on the bacterial surface binds to fibronectin that connects bacterial proteins to $\alpha_5\beta_1$ integrins at the host cell surface (Fig. 8), which induces a zipper-type uptake of *S. aureus* [270]. However, internalization into non-professional phagocytes can also be achieved by several other bacterial-host cell interactions. First, FnBP can bind directly to heat shock protein 60 (Hsp60), present on the membranes of human and bovine epithelial cells [56]. Second, Eap can contribute to internalization of *S. aureus* Newman into epithelial cells and fibroblasts by an FnBPs-independent mechanism [109]. Further, the interaction of staphylococcal autolysin (Atl) with heat shock cognate protein (Hsc70) has been shown to be involved in internalization into an endothelial cell line [116]. The difference in the bacterial uptake among various cell lines may also depend upon the expression and availability of the host cellular receptors (reviewed in [178]). However, some of the staphylococcal proteins such as α-toxin interfere with integrin-mediated adhesion and internalization of *S. aureus* by the human host [167, 325].

Intracellular degradation of unwanted material, e.g. pathogens, can be carried out via a process called autophagy. Thus, pathogens are taken up by host cells in autophagosomes and degraded after autophagosomal fusion with the lysosome [165]. *S. aureus* strains, expressing the accessory gene regulator- (*agr*-) related virulence factors or α-hemolysin (Hla), display a clear resistance against autophagic removal by preventing autophagosome maturation. These strains escape from the autophagosome into the cytoplasm, leading to death of the host cell and bacterial release [263]. The involvement of single virulence factors, such as pore-forming toxins in *S. aureus* intracellular survival, depends on bacterial strains and type of host cell. For example, the main target of PVL and phenol-soluble modulins (PSMs) is the neutrophils (review in [277]). The α-, β-, δ-toxin and β-PSM target a much broader spectrum of cells such as epithelial and endothelial cells in staphylococcal escape from the phagoendosomes [85, 90, 198, 320]. Interestingly, the role of PSMs in lysing of osteoblasts has recently been demonstrated [246]. CA-MRSA displays efficient lysis of polymorphonuclear leukocyte (PMN) after phagocytosis in comparison to other strains of *S. aureus* [149]. Additionally, in *S. aureus* LAC (USA300), a prominent CA-MRSA strain, leukocidin AB (LukAB), also known as leukocidin G/H (LukGH), may also have a role in intracellular lysis [55].

S. aureus protects itself against phagocytic killing and can survive inside PMNs with the help of various factors that are dependent on the global regulator, *sarA*, which controls the synthesis and secretion of several virulence factors [94]. Several staphylococcal enzymes such as staphyloxanthin [175], super oxide dismutase [136], surface factor promoting resistance to oxidative killing (SOK) [184], catalase (KatA) and alkyl hydroperoxide Reductase (AhpC) [48] contribute to resistance against neutrophil killing. *S. aureus* survival within PMNs depends on the multiplicity of infection (MOI), as well as bacterial growth phase. Notably, the number of intracellular viable *S. aureus* increases when bacteria from the stationary phase of growth and high MOI is presented to the PMNs [265].

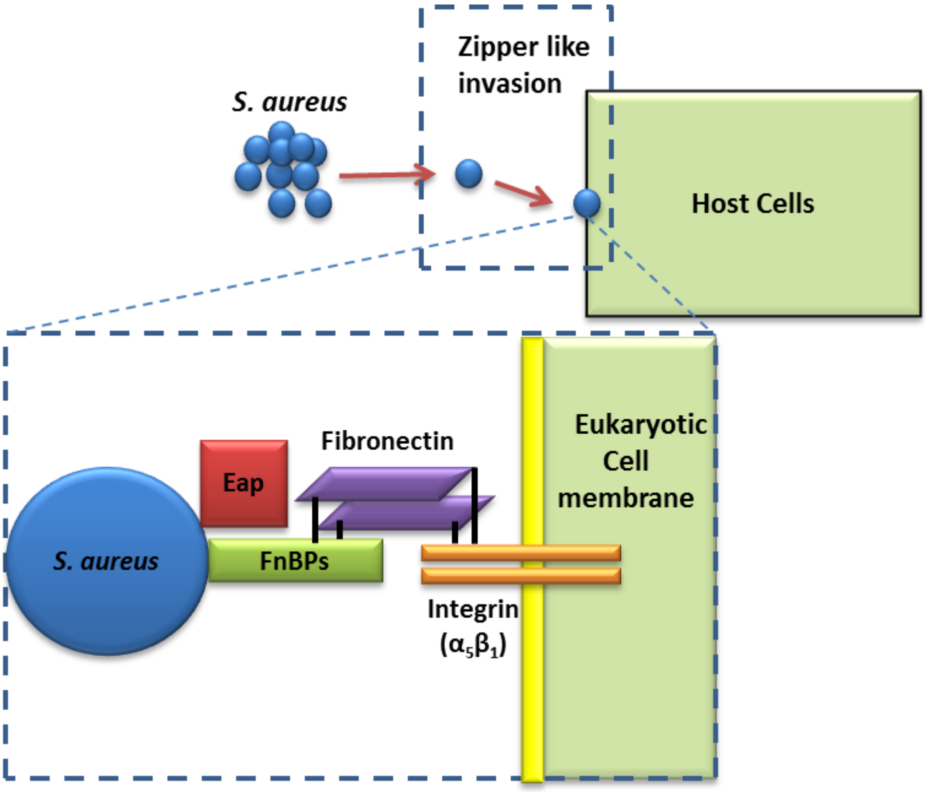

Figure 8. Schematic diagram illustrating proteins involved in initial phase of *S. aureus* internalization into the non-professional phagocytes. FnBPs: Fibronectin binding protein A, B, Eap: Extracellular adherence protein. Based on [82] with minor modification.

OBJECTIVES

Molecular studies of *S. aureus*-host interactions are of importance for better understanding of determinants associated with colonization and/or infection. Being a successful colonizer and/or invader requires the ability to adhere to the host cells/tissues, and ability to play hide and seek with the defense system. *S. aureus* MSCRAMMs are multifunctional proteins, which display a crucial role both in colonization and infection as adhesive and evasive molecules. Another immune evasion mechanism has been identified in Gram-negative bacteria, where TIR containing proteins negatively interfere with intracellular component of the TLR signaling pathway as well as induction of pro-inflammatory responses. However, whether Gram-positive bacteria express such mechanisms remain elusive.

The aims of this study were to explore selected molecular determinants associated with S. *aureus* colonization and/or infection and contribute to the knowledge about *S. aureus* interactions with the human host. Thus, the following questions were specifically addressed:

- Can a small subset of *S. aureus* nasal isolates from healthy individuals, belonging to different *spa* types, induce different responses in the presence of keratinocytes? Are certain bacterial traits beneficial for colonization?

- Is the *S. aureus* TIR domain protein (TirS) expressed? Can this protein interfere with host signaling and immune evasion?

- Can SdrD of *S. aureus* NCTC8325 contribute to host cells adhesion, invasion, and immune evasion?

GENERAL DISCUSSION

Colonization by *S. aureus* occurs when the bacteria exist as a commensal on the surface of the skin or mucus without any signs or symptoms of infection. Importantly, breakage of the skin barriers promotes transformation of *S. aureus* from a commensal colonizer to an invading pathogen. Studies on *S. aureus* gene regulation suggest downregulation of virulence genes during colonization and upregulation during infection [218]. *S. aureus* avoids host recognition or diminishes the subsequent immune activation for survival in a human host (reviewed in [88, 155, 202]). Additionally, the residential flora of the host organ, e.g. anterior nares, is a formidable challenge for *S. aureus,* since the presence of certain bacterial competitors can preclude carriage [176].

The presence of an optimal fit and highly specific interactions between *S. aureus* and the human host has been suggested. The huge variations in the combination of virulence factors, as well as allelic variations among *S. aureus* isolates, may determine the bacterial fitness. It has been suggested that, the gene combinations crucial for severe infections may be the same as those associated with *S. aureus* colonization [172]. The host factors including the various polymorphisms, the expression level of the ligands/receptors for bacterial attachment, as well as host immune responses are additional determinants for tolerance or eradication of *S. aureus* (reviewed in [129, 231, 314]). Thus, the host genotype and bacterial factors may be determinants of the carriage status and the carrying strain respectively.

In this study, we have investigated the variation of host cell responses to a small series of *S. aureus* nasal isolates **(paper I)**, as well as two determinants involved in *S. aureus* immune evasion including staphylococcal TIR containing protein (TirS) (**paper II**) and SdrD protein (**paper III**). However, the latter turned out to be multifunctional (**paper III**).

Adhesion/invasion and role in host-microbe interactions

S. aureus expresses several MSCRAMMs that are associated with adhesion and/or invasion of non-phagocytic cells through binding to the ECMs (reviewed in [77]). *S. aureus* internalization and survival within the host cells may protect the bacterium from immune responses, antibiotic treatments as well as promoting the establishment of chronic or frequent relapse of staphylococcal infection [5, 178, 266].

Microarrays confirmed the presence of several genes encoding CWA proteins such as *clfB*, *isdA*, *sdrD* and *sdrC* in all selected *S. aureus* isolates **(paper I)**. Additionally, our study **(paper III)** revealed that disruption of *sdrD* in *S. aureus* NCTC8325 suppressed bacterial attachment, internalization, and survival within human keratinocytes. The promoting function of CWA proteins such as ClfB, IsdA, SdrC, SdrD, SasG, SasX, in *S. aureus* adhesion to squamous cells has been demonstrated previously [39, 46, 47, 166, 209, 251]. However, there is a considerable functional redundancy between surface proteins in *S. aureus*. Thus, it is challenging to demonstrate the significant role of a single protein in adhesion or evasion. To circumvent the problem of redundancy, a single CWA protein can be expressed individually in a substitutive host such as *L. lactis* or *S. carnosus* (reviewed in [77]). Therefore, we included *L. lactis* expressing SdrD in some of our experiments, which confirmed the role of SdrD in adhesion to keratinocytes **(paper III).**

Our findings in the **paper I** demonstrated that *S. aureus* nasal isolates, belonging to different *spa* types, display variability in several traits as well as host cell responses *in vitro*. The genetic background of a given *S. aureus* isolate, which can be determined by the *spa* type, can predict the magnitude of invasiveness at the cellular level *in vitro* [315]. Indeed, our results in **paper I** suggested a huge variability among the studied *S. aureus* isolates in their attachment to and internalization into human keratinocytes. The host cell invasion is relying on the expression of staphylococcal surface protein, e.g. FnBPs, which engage host cell fibronectin and $\alpha_5\beta_1$ integrin [270].

Our results demonstrated that seven out of the eight studied *S. aureus* nasal isolates were positive for either FnBPA/FnBPB or both **(paper I)**. The oligos' binding sites of the probes on the microarray are highly specific. Thus, absence of the *fnb* or *fnbB* genes in one of the selected isolate may be due to the allelic variation of this gene. This limitation may also be the reason for the absence of *fnb* or *fnbB* in some of the tested lineages in the previous study [189]. However, FnBP-independent invasion of human keratinocytes have been previously demonstrated for some of the *S. aureus* isolates [144]. Presence of SdrD resulted in an increased level of internalized bacteria **(paper III)**, but whether the internalization is FnBP-dependent or -independent remains elusive. The cytotoxic outcome and sub-cellular localization of ingested *S. aureus* are greatly strain-dependent [83], however, whether the eight studied isolates in **paper I** vary in these properties remain to be investigated.

Immune evasion strategies and role in colonization/infection

Structural mimicry of host proteins is an effective strategy for pathogens to manipulate host immune responses [281]. TLR-mediated signaling plays a pivotal role in the upregulation of host innate immune responses through PAMPs recognition and subsequently induction of pro-inflammatory responses such as secretion of cytokines and chemokines [4, 124, 137, 287]. Bacterial TIR containing proteins have been identified in a wide range of bacteria that contribute to evasion of host immune system (reviewed in [245]). Genes encoding the bacterial TIR-containing proteins are generally localized within mobile genetic elements. Thus, the high possibility of lateral transmission of these genes has been suggested [331]. A TIR containing protein was identified in *S. aureus* MSSA476 through a database search analysis, named TirS and investigated further in **paper II**.

We demonstrated a TirS specific inhibitory effect against stimuli-induced TLR2-mediated NF-κB activation, JNK phosphorylation, and cytokine production upon its ectopic expression in eukaryotic cells **(paper II)**. One of the major limitations of the ectopic expression study is high cytosolic concentrations of the target protein. This is due to constitutive expression of the gene of interest by a strong promoter. Thus, the results of ectopic expression of TirS were confirmed by an infection experiment using MSSA476 wild type, MSSA476Δ*tirS* and complemented strain MSSA476Δ*tirS* +pTirS in a transwell system **(paper II)**.

The negative interference of the bacterial TIR protein with the TLR signaling pathway and consequently inhibition of NF-κB activation has been reported for several Gram-negative bacteria such as *Salmonella enterica* [212], *Escherichia coli* [37], *Brucella sp.* [37, 243], *Yersinia pestis* [244] and *Paracoccus dentrificans* [180]. Previously, *S. aureus* interference with recognition by TLR2 through SSL3 has been demonstrated [12, 326]. Improving our knowledge on bacterial immune evasion strategies triggering TLR-NF-κB signaling pathway may be of high medical interest and provide an alternative option for treatment of inflammatory diseases in the future [130].

Genetic disorders in TLR signaling pathways can affect the susceptibility of the infection with Gram-positive pathogens such as *S. aureus* [117, 233, 285, 288]. In order to study whether TirS increases *S. aureus* virulence, pathogenicity of MSSA476 wild type versus an isogenic mutant MSSA476 Δ*tirS* was compared in an intravenous and a skin abscess mouse infection models. The results revealed that the presence of TirS increased the bacterial load in multiple organs upon systemic infection **(paper II)**. Our results are consistent with studies using *E. coli, Brucella sp.* and *S. enterica* wild type containing the TIR domain protein [37, 212, 243]. However, *Y. pestis* containing the TIR domain protein did not influence on the bacterial virulence in a mouse model of bubonic plague [278]. Interference of TirS with TLR and JNK signaling pathways was confirmed in the **paper II**. Recently, the role of TLR2 in phagocytosis and autophagy induction via JNK signaling was demonstrated in *S. aureus* (NCTC8325)-stimulated murine macrophages [66]. Although different strains were used, but the high bacterial survival in infected mice by MSSA476 wild type may be due to TirS-mediated reduction in production of proinflammatory cytokines/chemokines and/or reduction in autophagy. However, this remains to be investigated.

TirS influences the production of proinflammatory cytokines *in vitro* (**paper II**). Obviously, further comparison of cytokine levels in the serum of mice intravenously infected with MSSA476 wild type versus isogenic mutant MSSA476Δ*tirS* could add value to our results. Based on literature, both TNF-α [98] and TLR receptor signaling pathways [4, 124, 137, 287] can induce the activation of the NF-κB-reporter. TirS inhibited PAMP-induced TLR2-mediated NF-κB activation, but not TNF-α (**paper II**). Thus, induction of TNF-α receptor-mediated NF-κB activation by bacteria in the host cell may mask the effect of TirS on PAMP-TLRs. Therefore, the choice of time points is of high importance in order to pinpoint the TirS effect. Additionally, the serum volume that can be extracted from an individual mouse was only 0.1 to 0.15 ml in our initial studies and numerous animals would be needed to optimize these experiments. Thus, regarding the three R's of animal ethics (replacement, refinement and reduction), we chose not to include cytokine analysis in our mouse systemic infection modeling. Although much can be learned from murine modeling of infection, one of the main problems with the approach of using mouse models is the presence of a host difference. Thus, the interpretation of results in animal models should be carried out cautiously [77, 200].

Immune evasion mechanisms are important for bacteria during infection and perhaps also colonization. Survival of *S. aureus* in an extracellular environment depends on overcoming opsonophagocytosis, mediated through complement components and antibodies (reviewed in [334]). Our finding in paper **III** demonstrated the role of SdrD in immune evasion, which yielded a reduction of C3b deposition, increasing blood survival, and reduction of PMN-mediated phagocytosis. Several other MSCRAMMs such as ClfA, Cna and SdrE, interfere with complement-mediated activities of immunity [102, 103, 135, 268].

The meeting between selected virulence factors and host

TirS and SdrD were both found in culture media (**paper II & III**). A common assumption about secreted proteins is that they contain a signal peptide. However, TirS is lacking such a peptide and was still found outside host cells (**paper II**). Proteins without the signal peptide or cell wall bound proteins may be released into culture media by different mechanisms. One possibility is secretion of membrane vesicles (MVs) into the extracellular milieu, which is a common feature of *S. aureus* [99]. Another option is release due to bacterial expression of autolysin, which is termed “nonclassical protein secretion.” Indeed, the two cell wall anchored proteins such as SdrD and protein A were both found in the secretome, together with several cytosolic proteins without signal peptide [226]. Recently, an ATP binding cassette transporter with previously unknown function, was found to be involved in the release of phenol-soluble modulins [32]. Therefore, a third option is release through un-identified receptors. SdrD contains an LPXTG motif and was still found in the culture supernatant. This could be either due to proteolytic cleavage or bacterial death. However, this remains to be investigated.

The mechanism of transfer of TirS into the host cells requires further investigation. Gram-negative bacteria can directly inject their effectors into the host cells using the Type III or Type IV secretion systems (T3SS or T4SS) [84, 213], or the effectors can be secreted into the medium and afterwards be taken up by the host cells. An example of the latter is TcpC from *E. coli* that enters into the host cell through cholesterol-rich lipid rafts [37]. Another possibility is MVs, which play an important role in transportation of several virulence-associated components into the host cell [99]. However, if any of these or other mechanisms is used by TirS to enter cells remains to be elucidated.

Spread of virulence factors

S. aureus virulence genes are generally localized on mobile genetic elements (MGE), such as pathogenicity islands (SaPIs), phages, or staphylococcal cassette chromosomes (SCC). This may enhance the possibility of their acquisition by other *S. aureus* stains through horizontal gene transfer and provide *S. aureus* an advantageous pathogenic strategy to adapt to the human host [190]. It has been demonstrated that mobile accessory virulence genes are not disseminated consistently among *S. aureus* strains. Additionally, some virulence genes can be carried on more than one element ([206] and references within).

The *tirS* gene integrated into the staphylococcal cassette chromosome SCC_{476} element [118] **(paper II)**. It seems that the existence of the *tirS* gene is an advantage for MSSA476 enhancing its virulence through subversion of the fast-acting innate immune response **(paper II)**. However, so far the prevalence of *tirS* has been reported in a limited number of other sequenced *S. aureus* isolates **(paper II)**. The time will show whether the prevalence of TirS is increasing or decreasing. The origin of TirS is not known yet. However, it has been suggested that coagulase-negative staphylococci (CoNS) serve as reservoirs for *S. aureus* SCCmec elements (references within [327]). Whether the acquisition of *tirS* by MSSA476 is adapted from the (CoNS), merits further investigations.

The *sdr* locus consists of *sdr*C, *sdr*D and/or *sdr*E, but all three genes are not necessarily present in the same strain [132, 258]. At least two *sdr* genes exist in all studied *S. aureus* isolates [132] and *sdrC* is always reported in the *sdr* locus [230]. The sdr locus of NCTC8325 contains *sdrC* and *sdrD* (**paper III**). The genes encoding surface protein complexes are mostly localized on the “core variable” [173], and some of these genes, e.g. *sdrD*, are not present in all studied *S. aureus* isolates ([132, 189, 258] and **paper III**). Interestingly, more than 50% of tested MSSA and approximately all tested MRSA isolates contain *sdrD* in their genome ([258] and references within). Our results demonstrated that the prevalence of *sdrD* was significantly higher among invasive isolates (37.1%) than nasal isolates (28.5%) (**Paper III**). This may suggest that although the presence of *sdrD* is not crucial for *S. aureus* survival and growth, it confers benefits for invasive isolates as they often contain SdrD.

CONCLUDING REMARKS AND OUTLOOK

Much has been learned over the past years about *S. aureus* colonization and infection of the human host. However, many questions remain unsolved. Our results are consistent with those of other studies and suggest that *S. aureus* and host interactions are strongly influenced by various parameters such as strains, MOI and host genetic background. The success of colonization and infection is, therefore, a complex interplay between a specific isolate and human individual. An effective adherence to the host cell, as well as ability to evade the host immune response is critical steps in *S. aureus* colonization and infection.

Multiple bacterial determinants are known to be involved in colonization and infection. SdrD was found to facilitate adherence of *S. aureus* NCTC8325 to skin as well as enhancing survival in human blood. The virulence factor TirS was found to interfere with PAMP-induced TLR2 signaling and increase bacterial accumulation in mice. These results suggest that SdrD may be a determinant involved in both infection and colonization. On the other hand, TirS may be more important during infection than colonization. Several questions should be addressed in future studies. The prevalence of *sdrD* was higher among invasive isolates than nasal isolates and has been associated with bone infections. Thus, it is important to focus on the role of SdrD in bone infections. In addition, the exact mechanisms behind the SdrD contribution in C3b deposition and high blood survival need to be evaluated further. Other questions that merit further investigations are whether and/how *tirS* is spread among *S. aureus* strains as well as a mechanism(s) by which TirS enters into the host cell.

In summary, *S. aureus* has several adhesive and immune evasive factors, where the number and combination depend on the strain. Here, we found that the combination of factors not only influence adhesive and invasive properties, but also host responses. We also studied two determinants in more detail, TirS and SdrD. Both were found to have immune evasive properties, while the latter turned out to be multifunctional. Hopefully, our results may improve our understanding on some determinants associate with *S. aureus* colonization and infection and provide us with more knowledge on the complex interaction between this pathogen and the human host. However, further studies on the molecular aspects of interactions between *S. aureus* and host cells are needed in order to obtain future targets for infection prevention and/or therapy.

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PAPER I

Staphylococcus aureus nasal isolates from healthy individuals cause highly variable host cell responses *in vitro*, The Tromsø Staph and Skin Study

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PAPER II

A *Staphylococcus aureus* TIR Domain Protein Virulence Factor Blocks TLR2-Mediated NF-κB Signaling

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PAPER III

Staphylococcus aureus SdrD promotes bacterial adherence to keratinocytes and whole blood survival

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APPENDIX

Review paper

Bacterial interference with canonical NF-κB Signaling

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