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HOST-PATHOGEN INTERACTIONS IN INVASIVE STAPHYLOCOCCUS AUREUS INFECTIONS

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Stockholm 2016

Front Cover: The mage on the cover page shows 3D volume rendering of lung tissue model expressing ADAM10 (green), E-cadherin (red) and epithelial cells (blue; DAPI) stimulated with a staphylococcal supernatant from a necrotizing pneumonia strain.

Published by Karolinska Institutet.
Printed by E-Print AB 2016

ISBN 978-91-7676-290-5

Host-pathogen interactions in invasive *Staphylococcus aureus* infections

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To My Beloved Family

ABSTRACT

Staphylococcus aureus is a versatile human pathogen causing a wide range of diseases from uncomplicated skin and soft tissue infections to life-threatening invasive diseases like endocarditis, bacteremia, necrotizing pneumonia, and fasciitis. The pathogen has become increasingly resistant to β-lactam antibiotics, and of special concern is the rise in community-acquired (CA)-MRSA strains, as specific CA-MRSA clones have been associated with highly aggressive infections. The ability of *S. aureus* to cause such a multitude of infections is linked to the production of a wide array of virulence factors. Several virulence factors have been implicated in disease pathogenesis, including the exotoxins Panton-Valentine Leukocidin (PVL), alpha-toxin (α-toxin), superantigens and phenol soluble modulins. This thesis project aimed to characterize *S. aureus* strains in the community as well as through use of clinical invasive isolates and human lung/skin organotypic tissue models explore the role of specific staphylococcal toxins and virulence regulation in the pathologic events leading to the destructive infections in lung and skin.

In **paper I**, molecular characterization of Indian community *S. aureus* isolates to determine their lineage and to analyze their virulence and immune-evasion factors was conducted. The percentage of methicillin resistance was 26% in carrier isolates while 60% among disease isolates. 69% of the isolates were positive for PVL genes along with combinations of many other toxins. The patterns of presence and absence of virulence and immune evasion factors strictly followed the sequence type (ST). We are reporting several new STs, which have not been reported earlier, along with factors influencing virulence and host-pathogen interactions. Next, we demonstrate in **paper II** that community *S. aureus* strains displayed stable phenotypic response profiles, defined by either proliferative or cytotoxic responses. The cytotoxic supernatants contained significantly higher levels of α-toxin as compared to proliferative supernatants. Furthermore, a significant association between *agr* type and phenotypic response profile was found, with *agr* I and *agr* IV strains being predominantly cytotoxic whereas *agr* II and III strains were proliferative. This differential response profiles associated with certain *S. aureus* strains with varying toxin production abilities could have an impact on disease outcome and may reflect upon the existence of specific pathotypes.

In **paper III** we focused on the pathogenesis of CA *S. aureus* severe pneumonia, in particular, the impact of exotoxins produced by strains isolated from varying severity of lung infections on human host cells and in human 3D organotypic lung tissue. α-toxin had a direct damaging effect on the epithelium whereas PVL contributed indirectly to the tissue pathology by triggering lysis of neutrophils. We demonstrated that severe tissue pathology is associated with a combination and high levels of both α-toxin and PVL, and fatal outcome correlated with higher toxin production in pneumonia. Notably, both α-toxin and PVL mediated cytotoxic effect and epithelial disruption was significantly abrogated by addition of polyclonal intravenous immunoglobulins.

In **paper IV** we focused on skin and soft tissue infections caused by ST22 strains, one of the most critically expanding MRSA clones world-wide. Here we identified a mechanism for which new variants, cytotoxic vs. persistent phenotype, can emerge. We link this phenotype switch to a specific mutation of receptor histidine kinase AgrC. The phenotypic switch to a persistence phenotype is associated with upregulation of bacterial surface proteins, less severe skin tissue damage, resistance to antimicrobials, and induction of autophagy. In contrast, cytotoxic phenotype strains showed upregulated exotoxin expression and caused infections characterized by inflammasome activation and severe skin tissue pathology. This study shows a strong effect of a single amino acid substitution in AgrC as a critical factor contributing to virulence properties and infection outcome.

Together, the studies in this thesis demonstrate that several different toxins will contribute to tissue pathology, but they target different cells and their impact may be tissue-specific. Also, distinct functional differences between the isolates were identified that are likely to contribute to disease outcome. Such insight should promote the development of novel diagnostics or therapeutic strategies.

LIST OF SCIENTIFIC PAPERS

This thesis is based on three publications and one manuscript. The individual papers are referred to by roman numerals

I. **Shambat S**, Nadig S, Prabhakara S, Bes M, Etienne J and Arakere G. Clonal Complexes and virulence factors of Indian *Staphylococcus aureus* from the Community. *BMC Microbiology, (2012) 12:64.* doi: 10.1186/1471-2180-12-64.

II. **Mairpady Shambat S**, Haggar A, Vandenesch F, Lina G, van Wamel WJB, Arakere G, Svensson M, Norrby-Teglund A. Levels of Alpha-Toxin Correlate with Distinct Phenotypic Response Profiles of Blood Mononuclear Cells and with agr Background of Community-Associated *Staphylococcus aureus* Isolates. *PLoS One, (2014) 9(8): e106107.* doi: 10.1371/journal.pone.0106107

III. **Mairpady Shambat S**, Chen P, Nguyen Hoang A.T, Bergsten H, Vandenesch F, Siemens N, Lina G, Monk I.R, Foster T.J, Arakere G, Svensson M*, and Norrby-Teglund A.* Modelling staphylococcal pneumonia in a human 3D lung tissue model system delineates toxin-mediated pathology. *Disease Models and Mechanisms, (2015) 8: 1413-1425.* doi: 10.1242/dmm.021923.

IV. **Mairpady Shambat S***, Siemens N *, Monk I.R, Mohan B. D, Mukundan S, Krishnan K C, Prabhakara S, Snäll J , Kearns A, Vandenesch F, Svensson M, Kotb M, Gopal B, Arakere G, and Norrby-Teglund A. A phenotype switch in the successful MRSA ST22 clone dictates infection outcome.
* Equal contribution, *Submitted Manuscript*

LIST OF ADDITIONAL PAPERS

- Prabhakara S, Khedkar S, **Mairpady Shambat S**, Srinivasan R, Basu A, Norrby-Teglund A, Narain Seshasayee A S, Arakere G. Genome Sequencing Unveils a Novel Sea Enterotoxin-Carrying PVL Phage in *Staphylococcus aureus* ST772 from India. *PLoS One, (2013) 8(3):e60013.* doi: 10.1371/journal.pone.0060013.

- Siemens N, Chakrakodi B, **Mairpady Shambat S**, Morgan M, Bergsten H, Hyldegaard O, Skrede S, Arnell P, Johansson L, INFECT Study Group, Juarez J, Bosnjak L, Mörgelin M, Svensson M*, and Norrby-Teglund A*. Biofilm in group A streptococcal necrotizing soft tissue infections (Submitted; Under review).

CONTENTS

LIST OF ABBREVIATIONS

MRSA	methicillin-resistant *S. aureus*
MSSA	methicillin-susceptible *S. aureus*
CA-SA	community-acquired *S. aureus*
MLST	multilocus sequence typing
PFGE	pulsed-field gel electrophoresis
ST	sequence type
CC	clonal complexes
SPA	Staphylococcal protein A
SCC*mec*	staphylococcal cassette chromosome mec
PBP2a	penicillin binding protein 2a
MIC	minimum inhibitory concentration
UTI	urinary tract infections
SSTIs	skin and soft tissue infections
MGEs	mobile genetic elements
Agr	accessory gene regulator
ADAM10	A Disintegrin and Metalloprotease 10
PVL	Panton-Valentine leucocidin
GPCRs	G-protein-coupled receptors
TLR	Toll-like receptors
HlgAB/HlgCB	Gamma-hemolysin
PSMs	Phenol soluble modulins
Hld	δ-hemolysin
FPR2	N-formyl-peptide receptor 2
DCs	Dendritic cells
SAgs	Staphylococcal superantigens
SEIs	staphylococcal enterotoxin-like toxins
TSST-1	toxic shock-syndrome toxin 1
APCs	antigen presenting cells
AIP	auto inducing peptide
PAMPs	pathogen-associated molecular patterns
NLRs	nod-like receptors
SCIN	staphylococcal complement inhibitor

CHIPS	chemotaxis inhibitory protein of staphylococci
LTA	Lipoteichoic acid
MSCRAMMs	microbial surface components recognizing adhesive matrix molecule
LFA-1	lymphocyte function associated antigen-1
ICAM	intracellular adhesion molecules
Efb	extracellular fibrinogen-binding protein
Ecb	extracellular complement-binding protein
Sbi	second binding protein of immunoglobulin
PBMC	peripheral blood mononuclear cells

1 INTRODUCTION

1.1 STAPHYLOCOCCUS AUREUS

Staphylococcus aureus is a gram-positive, non-motile, and a common commensal of humans. It stably colonizes the nares, axillae, the skin, and can be present persistently among approximately one-third of the human population, whereas another one-third of the population are colonized intermittently (1-3). *S. aureus* was first isolated from the surgical wounds in 1882 by the Scottish surgeon Sir Alexander Ogston (4). Based on its appearance it was classified as *Staphylococcus* (from the Greek staphylos ["grape"] and kokkos ["berry" or "seed"]) (5). Later, after a couple of year's German physician; Friedrich J. Rosenbach described pigmented colonies of staphylococci isolated from humans and proposed the nomenclature *Staphylococcus aureus* (from the Latin aurum ["gold"]). The golden yellow pigmentation of the colonies is due to a membrane-bound carotenoid called staphyloxanthin, which protects *S. aureus* from reactive oxygen species and phagocytic killing (6). *S. aureus* is a significant cause of human infections globally. It can cause a wide variety of infections ranging from minor skin infections to fatal necrotizing pneumonia or necrotizing fasciitis as well as bloodstream infections leading to severe disease manifestations such as sepsis, infective endocarditis, and deep-seated abscesses. Apart from that it is also estimated that 30–35% of healthy human individuals carry *S. aureus* on the skin or nasal nares (7, 8). The pathogen has become increasingly resistant to β-lactam antibiotics and methicillin-resistant *S. aureus* (MRSA), which are resistant to all available penicillin's and other β-lactam antimicrobial drugs, is now a leading cause of hospital-acquired infections (9-11) MRSA infections are associated with greater lengths of hospital stay, higher mortality and increased costs than infections caused by methicillin-susceptible *S. aureus* (MSSA) (12). In the US, *S. aureus* infection is among the leading causes of death by any single infectious agent (13). *S. aureus* is the most frequently occurring pathogen in hospitals and the second most common pathogen in outpatient (14). Despite substantial advances in health care as well as medical treatment, the morbidity and mortality caused by *S. aureus* is still continuously increasing. In addition and of special concern is the rise in community-acquired *S. aureus* (CA-SA) strains, as specific CA-MRSA clones are associated with highly aggressive infections, including severe skin and soft tissue infections, necrotizing fasciitis and necrotizing pneumonia, in otherwise healthy individuals.

1.1.1 Epidemiology

S. aureus is one of the most commonly occurring pathogen in hospitals and long-term hospital stay is related to an increasing morbidity and mortality due to infection. Approximately 400,000 cases of *S. aureus* infections are reported per year between 2003-2005 in the USA alone (15) and MRSA infections kill approximately 20,000 hospitalized

American patients each year (13). Taken together with the fact that (a) antibiotics is as a strong driver for resistance, (b) there is a rampant antibiotic consumption in many developing countries where MRSA is hyper endemic, and (c) the striking intercontinental spread of virulent MRSA clones, MRSA strains being pandemic, with dissemination of specific HA-MRSA clones from the 1960s, CA-MRSA clones from the 1990s, it is obvious that MRSA represent a significant health threat for both developing and developed countries. Till few years ago, the distinction between hospital associated methicillin-resistant *S. aureus* (HA-MRSA) and CA-MRSA was clear. CA-MRSA strains had distinctly different antibiotic sensitivities with low MIC values for oxacillin or imipenem as compared to HA-MRSA infected patients (16). But in the last 5 to 7 years, CA-MRSA has infiltrated the hospitals and is replacing HA-MRSA, mainly in countries where CA-MRSA is highly prevalent (17, 18). The morbidity and high mortality of these infections, together with a rapid rise in the incidence of CA-MRSA world-wide, suggest that some CA-MRSA strains are more virulent and transmissible than are traditional HA-MRSA strains. Although the increase in CA-MRSA infection has been well recognized, few studies have focused on the current epidemiological status of CA-MSSA strains. Most of the MSSA clones are identified as being Panton-Valentine Leukocidin (PVL) positive and belong to more diverse genetic backgrounds as compared to MRSA strains (14). A study conducted by McCaskill et. al. between 2001 and 2006 in Texas children hospital demonstrated an increase in the proportion of CA-MSSA infections (19). Similarly, a recent prospective study comparing the characteristics and outcomes of PVL positive MRSA and PVL positive MSSA in pneumonia cases demonstrated that methicillin resistance is not associated with severity of *S. aureus* pneumonia (20). Since CA-MSSA strains are most plausible reservoirs of CA-MRSA further epidemiological surveillance studies and infection control efforts focusing MSSA strains as well must be reinforced (14).

1.1.2 Molecular Typing of *Staphylococcus aureus*

An important tool for understanding the nomenclature and epidemiology of these globally distributed strains is molecular typing to determine clonality of strains. The current nomenclature of *S. aureus* is mainly based on various methods of molecular genotyping techniques that are being used. Presently there are four primary methods that are widely recognized for typing *S. aureus* strains i.e. multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), spa typing, and SCC*mec* typing (21, 22). MLST is based on sequencing of fragments of seven specific housekeeping genes (*arc, aroE, glpF, gmk, pta, tpi, yqiL).* Sequence variations among each individual genes are given different allele numbers, and all the seven alleles are linked together to form a unique allelic profile called sequence type (ST). Related ST types sharing at least five out of the seven alleles are grouped into single clonal complexes (CC) (22).

PFGE is a kind of fingerprinting method wherein whole genomic DNA of *S. aureus* is digested using a restriction enzyme called *SmaI*. This creates a banding pattern based on the size of each digested fragment in a matrix gel. This method is highly used in investigations of outbreaks involving closely related *S. aureus* strains (22).

Spa typing is also a sequencing based method wherein the highly polymorphic variable region of Staphylococcal protein A (*spA*) gene consisting of number of tandem repeats, typically 24 bp in length are sequenced. These duplicating repeats contribute towards a unique pattern called spa types.

Almost all MRSA strains carry SCC*mec* elements which include *mecA* gene encoding for penicillin-binding protein 2a (PBP2a) contributing towards the methicillin resistance (23). The *mecA* gene is found integrated into a specific chromosomal region called the *orfx* by horizontal gene transfer and hence is called staphylococcal cassette chromosome mec (SCC*mec)* (24). Based on their structural organization and genetic content SCC*mec* element type I-XI are identified, which are mainly distinguished by the type of *ccr* gene complex that mediates the site-specific excision and insertion and the class of *mec* complex that they bear (25). Conventionally HA-MRSA strains are found to usually carry large SCC*mec* types I, II, or III, while CA-MRSA strains are usually characterized by the possession of smaller SCC*mec* types IV and V (Figure 1) (26).

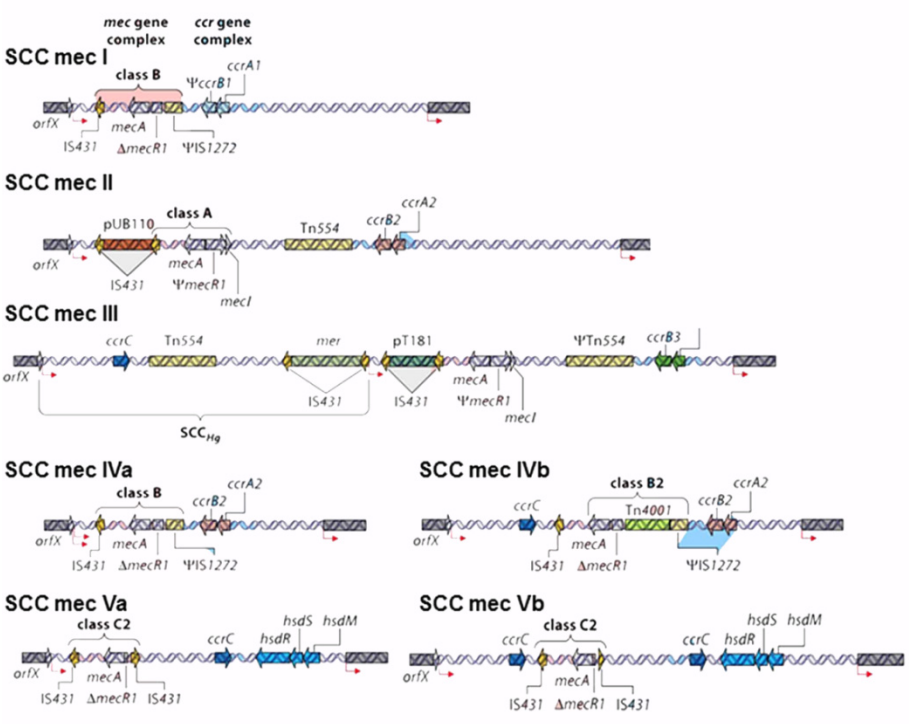


Figure 1: Classification of SCC*mec* type based on *ccr* complex and *mec* complex type. Adapted and modified from (12)

1.1.3 Hospital-Associated Methicillin-Resistant *Staphylococcus aureus*

MRSA strains that are circulating in the hospital settings are classified as HA-MRSA and are mostly responsible for invasive MRSA infections. HA-MRSA is highly prevalent worldwide with North and South America demonstrating high rates (>50%), while in other parts such as Asia, Australia, Africa and some European countries intermediate rates of around 25-50% are reported. The lowest prevalence of HA-MRSA strains are generally seen in Netherlands and Scandinavian countries (27, 28). The most common and frequently reported genotypes of HA-MRSA strains are CC5, CC8, CC22, CC30, and CC45 (7, 29, 30). Strains belonging to CC22 and CC30 type are widespread globally, whereas CC45 is commonly found in USA and Europe (7, 17) and strains from CC8 and CC5 are frequently isolated in Asia (Figure 2) (31).

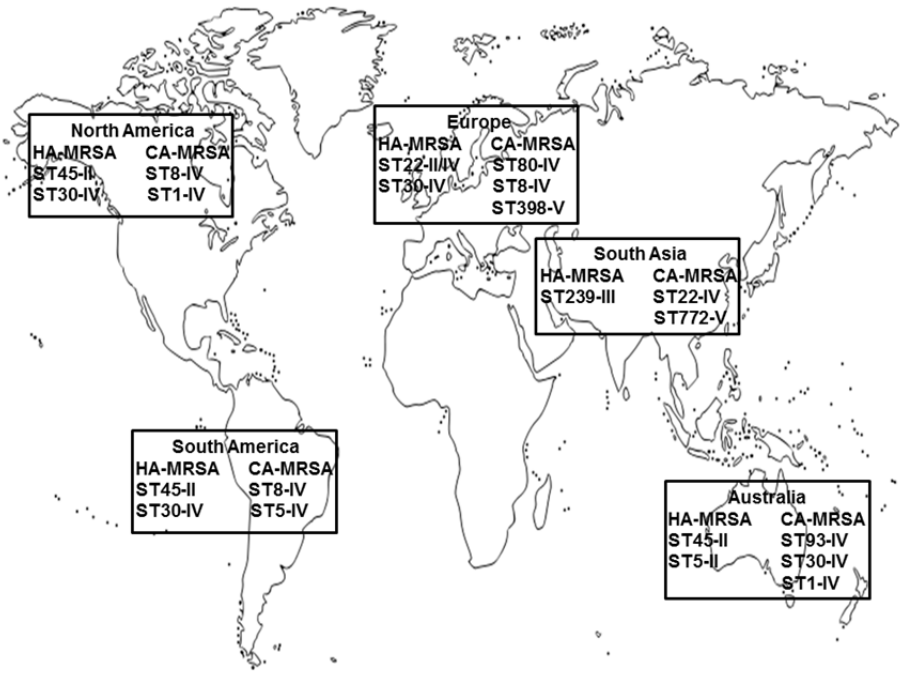


Figure 2: Global distribution of major MRSA lineages by sequence type

1.1.4 Community-Associated Methicillin-Resistant *Staphylococcus aureus*

CA-MRSA has become a widespread problem in many developed as well as developing countries around the world during the last decades (8, 12). The definition for CA-MRSA infection is based on several clinical and bacteriological criteria i.e. (i) MRSA infection diagnosed for an outpatient or within 48 h of hospitalization, (ii) patients lacking the following HA-MRSA risk factors including hemodialysis, surgery, residence in a long-term care facility or hospitalization during the previous year, the presence of an indwelling catheter or a percutaneous device at the time of culture, or previous isolation of MRSA from the patients (32), and (iii) CA-MRSA strains differ from HA-MRSA strains in their molecular characteristics (8, 12) carrying specific antibiotic cassette such as SCC*mec* IV or SCC*mec*

V. Furthermore CA-MRSA strains commonly lack multiple antibiotic resistance genes, except to β-lactams antibiotics, and frequently have different exotoxin gene profiles, e.g. PVL, pathogenicity islands, ACME elements genes (Figure 3) (12, 33, 34). Various MRSA clones have spread between the community and hospitals, particularly CA-MRSA strains are being transmitted in the hospital settings and the circulation of HA-MRSA strains that occurs in the community makes the distinction between CA-MRSA and HA-MRSA very difficult (18). Studies of global transmission of CA-MRSA identified 5 major intercontinental pandemic MRSA clones, which were found to have evolved from 2 distinct evolutionary lineages (35). However, there is a striking geographic variation in predominant CA-MRSA clones with most cases in the US belonging to CC8 (USA300 and USA400), whereas in Europe ST80 is predominate and in Australia ST93 (Figure 2) (8). In Sweden, which is a low prevalence country, imported MRSA strains through travel and immigrations represent a large proportion of the cases and importantly, regions with the highest risk for MRSA in travelers showed a correlation with community-acquisition (36). CA-MRSA infections mainly occur in healthy young individuals, through skin-to-skin contact. The major clinical manifestation associated with CA-MRSA is skin and soft tissue infections (SSTIs) (about 70-80%), but severe life-threatening infections such as necrotizing fasciitis, necrotizing pneumonia, and severe sepsis have also been reported (12, 37, 38). CA-MRSA strains also cause wound infection, surgical site infections, urinary tract infections (UTI), meningitis, sinusitis and eye infections (Figure 4) (39-41).

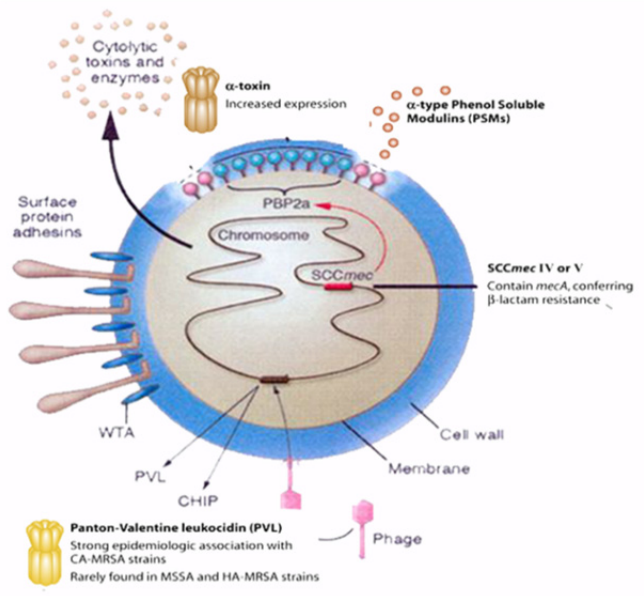


Figure 3: Main characteristic features of CA-MRSA strains (12).

1.1.5 Pulmonary Infections

S. aureus is one of the major pathogens implicated in the development of pneumonia in both CA and HA infections including MSSA and MRSA (42-44). Traditionally, *S. aureus* accounts

for approximately one-third of pleural empyema cases (45) and these infections usually occur as a result of hematogenous spread or via local extension from other infected source (46). These respiratory infections lead to a wide range of outcomes, from asymptomatic colonization to fulminant invasive clinical outcome, and the host immune response plays a significant role in determining the consequence of these infections. Severe, invasive CA-MRSA infections especially necrotizing pneumonia have a high mortality rate, even when optimal therapeutic treatments are used. It is a distinct syndrome characterized by a massive influx of neutrophils into the lung parenchyma, formation of abscesses, hemoptysis, high fever, and lung lesions, often requiring mechanical ventilation. Cases of necrotizing MRSA pneumonia are often associated with a prior respiratory viral infection, predominantly influenza (47) and occur most often in children, young adults and immunocompromised patients (48). Although the incidences of CA-pneumonia is fairly low (around 2 to 3% in the USA, around 10% in the United Kingdom and around 3% in Australia (Figure 4)) the rapid expansion of these community-acquired virulent and highly transmissible *S. aureus* strains is a major cause of concern. The emergence of severe necrotizing pneumonia is usually epidemiologically linked to infection with distinct CA-MRSA strains carrying genes for PVL. This association between *S. aureus* strains carrying genes for PVL and necrotizing pneumonia was first identified in USA (37) and then in France (49) followed by subsequent studies of community-associated pneumonia in otherwise healthy individuals (50, 51). Molecular epidemiological studies have identified a dominance of a few major clones of *S. aureus* strains wide spread throughout USA and Europe, and the mechanism for their success is attributed to their abilities to acquire genes of both antimicrobial resistance and virulence genes through horizontal gene transfer.

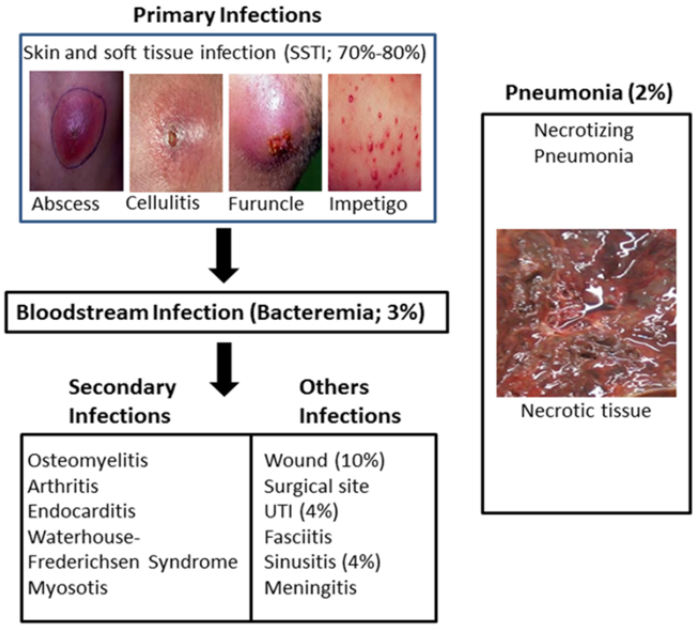


Figure 4: Burden and infections caused by CA-*S. aureus* strains

1.1.6 Skin and Soft Tissue Infections

CA-*S. aureus* strains are most commonly associated with SSTIs. Any breach in the skin barrier function that occurs due to a trauma, and/or surgical procedures can cause entry of *S. aureus* into the subcutaneous tissues. Apart from that *S. aureus* may also cause infection without any barrier breach including at hair follicles causing folliculitis, bullous or superficial lesions, confluent abscess like furuncles and carbuncles all such infections are classified as SSTIs (Figure 4) (12, 52). Over the past fifteen years, the emergence of CA-MRSA strains associated with SSTI infections has increased and it has become apparent that CA-MRSA epidemic is not only replacing the endemic SSTI strains but also increasing the burden of SSTIs (8, 53). The earliest cases reported of CA-MRSA strains causing SSTIs in USA was in the late 1990s and was mainly caused by USA400 strain type (54). Since the 2000s, it has been replaced predominantly by USA300 strain type contributing to more than 50% of the SSTI cases in the USA alone (55). Apart from that, a 3 fold increase in the admission rates for abscess and cellulitis is found in the United Kingdom, similarly in Australia, it was identified about 48% increase in cutaneous abscess as well as an increase in outpatients CA-MRSA strains attributed to SSTI (56, 57). Differential gene expression of virulence factors such as alpha-toxin (α-toxin), PVL, phenol soluble modulins (PSM), Staphylococcal Protein A (SPA), cell surface associated factors and ACME elements have contributed towards these enhanced virulence properties of CA-MRSA strains (Figure 3) (58). Similarly, PVL-positive CA-MRSA strains are isolated usually from deep-seated skin infections such as furuncle, carbuncle, and cellulitis (59), whereas superficial skin infections and impetigo are related to PVL-negative CA-MRSA strains (60). A study on nasal carriage of MRSA identified a correlation between nasal carriage and development of SSTI, suggesting that nasal carriage of MRSA strains can contribute towards the risk factor for SSTI (60).

1.2 VIRULENCE FACTORS

S. aureus express a wide array of virulence factors that enables its survival during infection. The ability to secrete a diverse repertoire of immune evasion factors, including cytotoxins (hemolysins, cytolytic peptides, leucocidins), immunomodulatory proteins (superantigens, complement-inhibitory proteins), and factors that prevent immune cell recognition (SPA, among others) contribute in various ways to disease pathogenesis (61, 62). The majority of these virulence factors is encoded on mobile genetic elements, such as plasmids or prophages, and is transferred between strains by horizontal gene transfer (53, 61). Each of these molecules destabilizes the host immune system in many different ways and mediates resistance mainly towards innate immune defenses. The expression of these virulence factors adds to the multi-faceted action triggering pathological immune response, support

bacterial proliferation and evade elimination in the host. Several of these toxins are specific towards human cells, indicating the longstanding adaptation of *S. aureus* as a human pathogen.

1.2.1 α-toxin

α-toxin is one of the major and by far the most carefully well examined secreted virulence factor of *S. aureus.* The majority of the strains produce α-toxin. The toxin is cytotoxic to a wide range of cell types particularly was initially described as exhibiting dermonecrotic and neurotoxic factor and has since been shown to be cytotoxic. The mature protein contains 293 amino acid residues and has a molecular weight of 33 kDa, composed of beta-sheets (65%) and alpha-helical structures (10%). The production of α-toxin is under the control of the global accessory gene regulator (*agr*) (63) and it is produced during the late exponential growth phase of bacterial culture. The expression of α-toxin is found to be increased upon interaction with epithelial cells (64-66) and correlates with the virulence of *S. aureus* strains (67, 68). It is produced as monomeric component and these secreted monomers integrate into the membrane of target cells and form cylindrical heptamers (Figure 5) (69). This binding usually occurs in two different ways. At higher concentrations, α-toxin nonspecifically adheres to the cell membrane (70) and this oligomeric form induces lyses of eukaryotic cells. However at low concentrations, A Disintegrin and Metalloprotease 10 (ADAM10) is involved as a specific receptor for α-toxin (71). The species and cellular specificity exhibited by α-toxin correlate with ADAM10 expression (71). Interaction of α-toxin with both membrane lipids and its cell surface receptor indicates the cooperative nature of these interfaces in the modulation of toxin binding, assembly, and cytotoxicity (Figure 5). The α-toxin binding to ADAM10 at the cell surface induces the metalloprotease catalytic activity resulting in cleavage of E-cadherin (71, 72). This, in turn, causes loss of interaction between adjacent cells at the adherens junction, thereby disrupting the epithelial barrier function. Pore formation by the toxin triggers rapid release of ATP, K+ ions and influx of extracellular calcium into the cell, results in leaky gaps between cells leading to apoptotic cell death (73). In addition, α-toxin generate a chemokine gradient that facilitates neutrophil and other immune cell recruitment in the lung (74), and it induces inflammatory responses in multiple cell types resulting in the release of cytokines and pro-inflammatory mediators (75, 76). Intoxication with α-toxin triggers (NLRP3) inflammasome, caspase-1 activation and induces IL-1β secretion in macrophages and monocytes. (Figure 6) (77, 78). These inflammatory stimuli, associated cell death via pyroptosis, exert a deleterious effect on the local tissue microenvironment, increasing the reactivity of the vasculature, promoting tissue edema, and modulating host immunity. Due to its important role in pathogenesis, α-toxin has been an active focus of vaccine development. Toxin antibodies have shown protective role in mice

(79) and rabbits (80, 81) models of pneumonia. Furthermore, structural analogues of α-toxin have been shown to reduce epithelial damage in *S. aureus* pneumonia (82, 83).

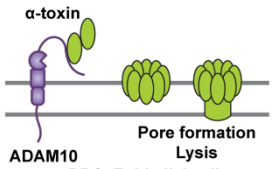
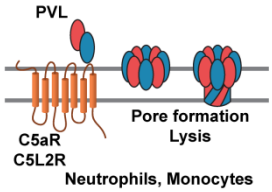
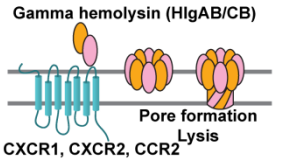
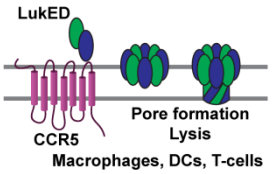
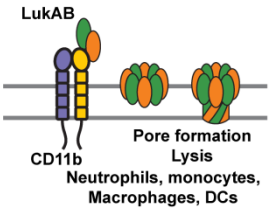
Toxin/Receptor/cell type specificity	Genome location	Strain distribution	Species specificity	Cytotoxic dose	Sublytic activity
	Core genome	99% of all isolates	Yes, Rabbit > Human	80ng-100ng/ml	Yes, Proinflammatory effects
	φSa2	2-3% of all isolates	Yes, Rabbit and human only	60ng-100ng/ml	Yes, Neutrophil priming Inflammasome activation
	Core genome	99% of all isolates	Unclear	1ug-10ug/ml	HlgAB: not known HlgCB: Ca2+ release
	Pathogenicity Island	70% of all isolates	No, toxic across species	2ug-5ug/ml	Unclear
	Core genome	Unknown	Yes, Human and rabbit	2ug-5ug/ml	Unclear

Figure 5: Cell specificities and receptor mediated targeting of *S. aureus* cytotoxins (84-86)

1.2.2 Bicomponent Pore-Forming Leucocidins

The bicomponent leucocidins produced by *S. aureus* are capable of exerting a potent lytic activity on a variety of host immune cells of the myeloid lineage, namely monocytes, macrophages, and neutrophils. Van de Velde in his studies first demonstrated leukocidal activity of *S. aureus* and over the past 100 years research has advanced from that of a single lytic toxin molecule to the identification of six different leucocidins (HlgAB, HlgCB, LukAB, PVL, LukED and LukMF) (87). The first pore-forming leucocidin to be purified from *S. aureus* was PVL. It consists of two subunits designated as S (slow; LukS-PV), and the other as F (fast; LukF-PV), based on their elution profiles (88-90). These bicomponent toxins are cytotoxic only when both the subunits are combined and cytolytic activity requires; (i) each of

the monomeric subunits (S and F) are secreted; (ii) initially S subunit recognizes proteins and/or lipids on the host surface in a species- and cell type-specific manner, followed by F subunit recruitment and recognizing each other; (iii) the subunits accumulate into an octameric structure with alternating F and S components forming a prepore. Furthermore, the stem domains unfold causing structural shifts and insert into the cell membrane to form pore formation (Figure 5) (91, 92). PVL is encoded within a lysogenic temperate phage φSLT (93, 94). The *lukS*-PV gene encodes a 312-amino-acid protein with a molecular weight of 32kDa similarly LukF-PV protein is a 325 amino-acid protein with a molecular weight of 34kDa (95). The leucocidin gene is regulated by a number of master regulators and external signals provide major inputs into their altered gene expression. Under favorable environmental conditions, Agr-mediated RNAIII negatively regulates the leucocidin repressor Rot inducing increased production of leucocidins especially PVL (85). Similarly, SarA indirectly facilitates leucocidin expression by positively regulating the expression of RNAIII. Upon external stimuli, SaeRS two-component system induces direct binding of SaeR to leucocidin promoters and inducing its production (85). PVL is present in only approximately 2 to 5% of all *S. aureus* isolates it received a lot of attention as specific PVL-positive CA-MRSA types (such as USA300) were epidemiologically linked to the emergence of severe necrotizing infections in particular necrotizing pneumonia (96, 97). Although compelling epidemiological data, direct evidence for a role of PVL as a virulence determinant was sought through experimental murine models of acute pneumonia, soft tissue or sepsis. Isogenic PVL-positive and PVL-negative *S. aureus* strains were used in these animal studies and provided conflicting results (67, 98). The role of PVL in staphylococcal virulence and disease has since been greatly debated. Recent studies have emphasized the importance of host susceptibility to PVL with a strong and rapid cytotoxic activity against neutrophils isolated from human, but not from murine or non-human primates (99). A role for PVL was then further substantiated in a rabbit model of necrotizing pneumonia in which PVL significantly enhanced the tissue injury, inflammation, and death of the animals (100). The transmembrane G-protein-coupled receptors (GPCRs) C5aR and C5L2 were recently identified as the cellular receptors of PVL (101). The recognition by PVL is mediated by both the core membrane-spanning portions as well as their extracellular N-terminal region (101). PVL “S” subunit is highly specific against human cells expressing C5aR especially neutrophils, while less specific against murine and macaque cells (Figure 6). The binding of LukS-PV to C5aR not only exerts cytotoxic effect but also induces priming and activation of neutrophils promoting proinflammatory responses (Figure 6) (102, 103). The cytotoxic effect of PVL leads to the release of proinflammatory mediators such as IL-8 and also induces substantial cellular damage by the release of tissue-damaging enzymes. At sublytic concentrations PVL (i) induces granule exocytosis and increased bactericidal property of neutrophils (Figure 6) (85), (ii) triggers (NLRP3) inflammasome, (iii) induces IL-1β secretion

mediated by potassium efflux, (iv) causes NF-κB activation as a result of calcium influx and by engaging Toll-like receptors (TLR2 and TLR4) and (v) apoptosis due to mitochondrial disruption (Figure 6) (85).

Gamma-hemolysin (HlgAB/HlgCB) is encoded in the core genome; both share the same F subunit (HlgB), but differ in their S subunit (HlgA and HlgC) and are expressed by 99% of *S. aureus* strains (Figure 5) (104, 105). These leucocidins are cytotoxic towards red blood cells and are usually found upregulated during blood stream infection (106). HlgCB also targets the same C5aR as that of PVL to exert cytotoxic effect against neutrophils and macrophages (Figure 6) (107). Similarly CXCR1, CXCR2, and CCR2 are identified as the cellular receptors of HlgAB (Figure 6) (107).

LukED is the one and only leucocidin which exhibits broad activity on different cell types as well as from different species (85). LukED is found to be lineage specific in its gene expression and is present in approximately around 70% of *S. aureus* strains (Figure 5) (108, 109). LukED targets CCR5 as its cellular receptor to lyse macrophages, T cells and dendritic cells (Figure 6) (110). Similarly chemokine receptors CXCR1 and 2 are utilized to exert its cytotoxic effect towards neutrophils, monocytes and NK cells (Figure 6) (111).

LukAB is the most recently identified *S. aureus* leukotoxin (112, 113) and is the only leukotoxin that is found to be secreted as well as cell surface associated (113). Human CD11b a component of the integrin Mac-1 is required by LukAB to exert cytotoxicity towards neutrophils, macrophages, and monocytes and determines its cell and species specificity (114). This leukotoxin also enhances *S. aureus* intracellular survival and facilitate escape upon phagocytosis by human neutrophils.

1.2.3 Phenol-Soluble Modulins

PSMs are a family of small amphipathic α-helical peptides broadly divided into two subfamilies, (i) PSMα including δ-hemolysin (Hld) being short around 20-26 amino acids long and (ii) PSMβ which are long 40-44 amino acids in length (65). PSMs are core genome-encoded genes at three different locations, PSMα1- PSMα4 within the *psmα* operon PSMβ1 - PSMβ2 within the *psmβ* operon and δ-hemolysin encoded within the RNAIII of the *agr* system. The PSMs are regulated by the *agr* system independent of the RNAIII mediated regulation (115) and also influence the expression α-toxin (116). The production of PSMs correlates with the capacity of *S. aureus* strains to cause invasive infections (117) and CA-MRSA strains usually show an increased production of PSMs as compared to HA-MRSA (118). PSMα peptides especially PSMα3 effectively lyse human leukocytes and erythrocytes (118) and also they facilitate neutrophil killing upon phagocytosis (119, 120), mainly mediated by strong expression of *agr* regulating the PSMs production within neutrophil

phagosome (121). PSMs are sensed by leukocytes via N-formyl-peptide receptor 2 (FPR2) which leads to pro-inflammatory responses, including the release of IL-8, recruitment of neutrophils, activation, and lysis of neutrophils and DCs (118, 122) contributing to inflammation and severity of tissue injury.

1.2.4 Superantigens

Staphylococcal superantigens (SAgs) are secreted toxins which act as a potent T cell-mitogen and can activate or stimulate T cells at very low concentrations. The staphylococcal SAgs are basically divided into three different subclasses (i) staphylococcal enterotoxins (SEs); (ii) staphylococcal enterotoxin-like toxins (SEls); and (iii) toxic-shock-syndrome toxin 1(TSST-1) (123). Till now 24 different staphylococcal SAgs have been identified of which 12 are enterotoxins; 11 enterotoxin-like proteins and finally TSST-1 (124). *S. aureus* isolates exhibit a high heterogeneity in terms of carrying SAg genes, as they are encoded on mobile genetic elements, such as bacteriophages, plasmids, and pathogenic genomic islands. 80% of the strains express genes for around five to six different SAgs (125) and their expression is regulated by four different global regulators such as Agr, SarA, SarB and SaeRS (125). The hallmark of SAgs is that they activate and stimulate a large fraction of T cells by directly cross-linking without prior processing certain TCR Vβ domains with conserved structures on MHC class II molecules expressed on professional APCs resulting in massive proliferation of T cells, cytokine production and apoptosis (Figure 6) (126). TSST-1 causes toxic shock syndrome (TSS) defined by high fever, rash, desquamation, vomiting, diarrhea, and hypotension, frequently resulting in multiple organ failures. Similarly, enterotoxins (SEA and SEB) are shown to play a major role in skin (atopic dermatitis) and airway (asthma) allergies by modulating the levels of IgE antibodies (95, 127, 128).

1.2.5 Staphylococcal Protein A

SPA is one of the major virulence factors which is present abundantly on the cell surface and expressed by the majority of *S. aureus* strains. It is also one of the most complex components of *S. aureus.* It exhibits multiple interactions against host immune machinery contributing towards immune evasion and pathogenesis. SPA has multiple domains. The N-terminal region consists of a signal peptide with five repeated domains (E, D, A, B, C) which interacts with IgG and promotes resistance towards phagocytosis (129). The carboxyl terminal contains a variable region of 24bp repeat sequence called Xr region, which is widely used in epidemiological typing of *S. aureus* strains (130). SPA is found to directly interact with TNFR1 and EGFR via its IgG binding domains studied extensively in epithelial cells and macrophages (131). SPA engagement at TNFR1 and EGFR leads to increased levels of TNFR1 at the cell surface causing subsequent shedding of a soluble form of TNFR1 due to the action of ADAM17 (Figure 8) (132). Apart from that TNFR1 and SPA interaction induces

a pro-inflammatory signaling response by induction of IL8 release in airway epithelial cells via MAP kinase and NF-κB pathway (Figure 8) (131, 132). Activation of a cascade of signaling pathway by SPA facilitates invasion of *S. aureus* across epithelial barriers by causing cleavage of tight junction proteins such as occludin and E-cadherin (133).

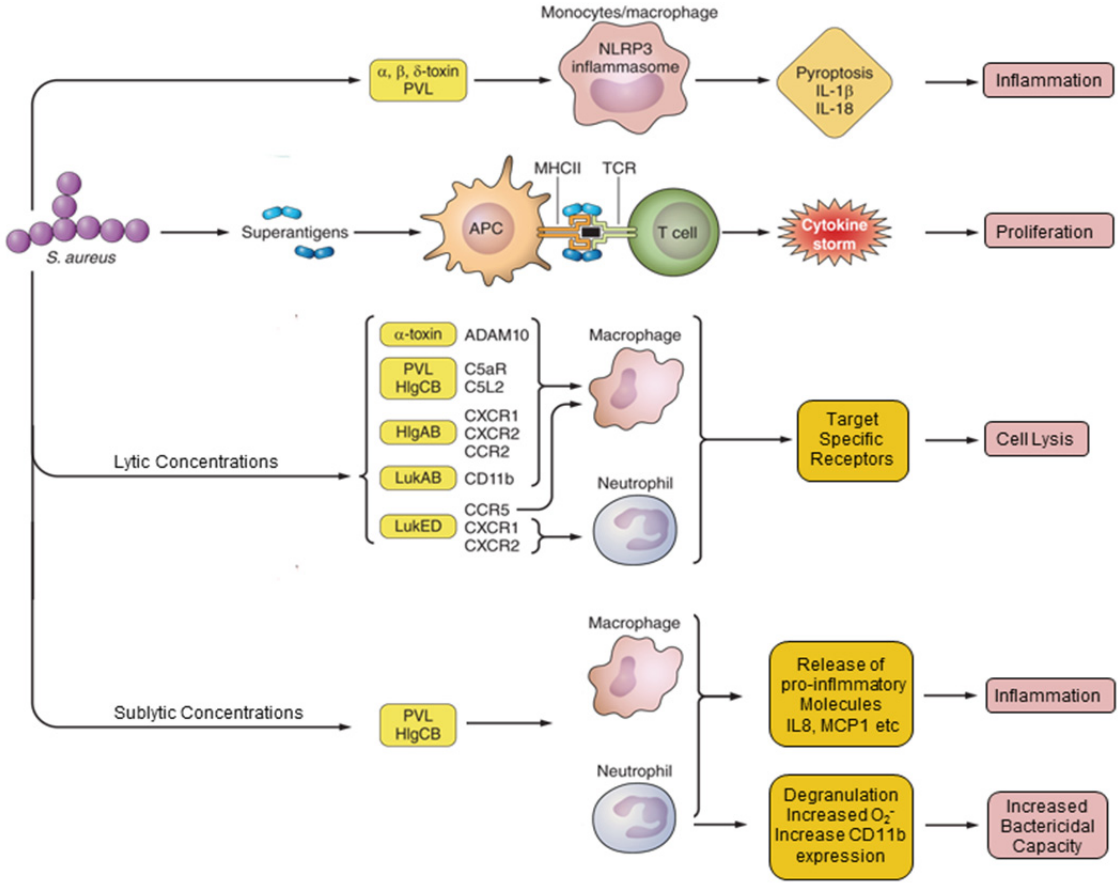


Figure 6: Overview of *S. aureus* toxins action on host immune cells. Adapted and modified from (134).

1.3 THE AGR SYSTEM

The *agr* operon is around 3.5kb in size and consists of two transcriptional units RNAII and RNAIII, which expression is controlled by the P2 and P3 promoters respectively (135). The RNAII encodes for four domains, AgrB, AgrD, AgrC and AgrA (135), where the AgrD encodes for peptide precursor which is processed to form a 7-9 amino acids long pheromone with a thiolactone ring structure called the auto inducing peptide (AIP) (136). AgrB is a transmembrane endopeptidase responsible for processing pro peptide (AgrD) into its functionally active final octapeptide form (137). The AgrC and AgrA act as the bacterial two-component signal transduction system with AgrC being a receptor histidine kinase which gets phosphorylated upon binding of AIP and AgrA acting as a response regulator (Figure 7) (138, 139). AIPs are produced during the exponential growth phase of the bacteria and upon reaching a threshold cell density, the *agr* locus gets autoactivated (135). Upon activation by

AgrC-mediated phosphorylation, AgrA binds to the P2 and P3 promoters of RNAII and RNAIII respectively (140). AgrA can also directly bind to the promoters regulating the expression of PSM α and β peptides (Figure 7) (115). Four classes of *S. aureus* AIPs, known as (AGR I, II, III, and IV) are present, which differ from each other based on the variations in the amino acid sequence (138). This diversity among the AIPs causes inter strain cross-inhibition i.e. peptide of one class activates the *agr* operon of its homologous group and in turn suppresses the heterologous groups (137, 138). Specific *agr* classes are also associated with specific *S. aureus* genetic background and several studies have reported a strong link between the *agr* class and particular clonal complexes (141). For example clonal lineages CC8, CC22, CC45 and CC395 usually harbor *agr*-I, while CC5, CC12, and CC15 isolates are characterized by *agr*-II, CC30 isolates often carry *agr*-III, and CC121 harbor *agr*-IV (142). The limited literature suggests a possible link between different *agr* classes and certain staphylococcal syndromes (143) with strains belonging to *agr* type IV are more commonly linked with exfoliative syndromes while strains belonging to *agr*-I and II are usually overrepresented with cases of endocarditis (143).

Agr-mediated up-regulation of virulence factors plays a crucial role in *S. aureus* infection properties and determines the disease progression (137, 141). Inversely down-regulation of PSMs and up-regulation of cell surface components have been implicated in bacterial biofilm formation and colonization phenotype of *S. aureus* (137, 141). Any mutation in Agr causing dysfunction of Agr system is correlated with a persistent phenotype, especially identified in *S. aureus* bacteremia (144). In general, Agr-dependent up-regulation of virulence factors, proteases, degenerative exoenzymes and down-regulation of cell surface components reflect the sequential requirements for establishing specific virulence properties during bacterial infection. The major toxins that are under the regulation of Agr include α-toxin, a family of bi-component leukocidins, peptide toxins PSMs, many secreted proteases and cell surface protein SPA (137).

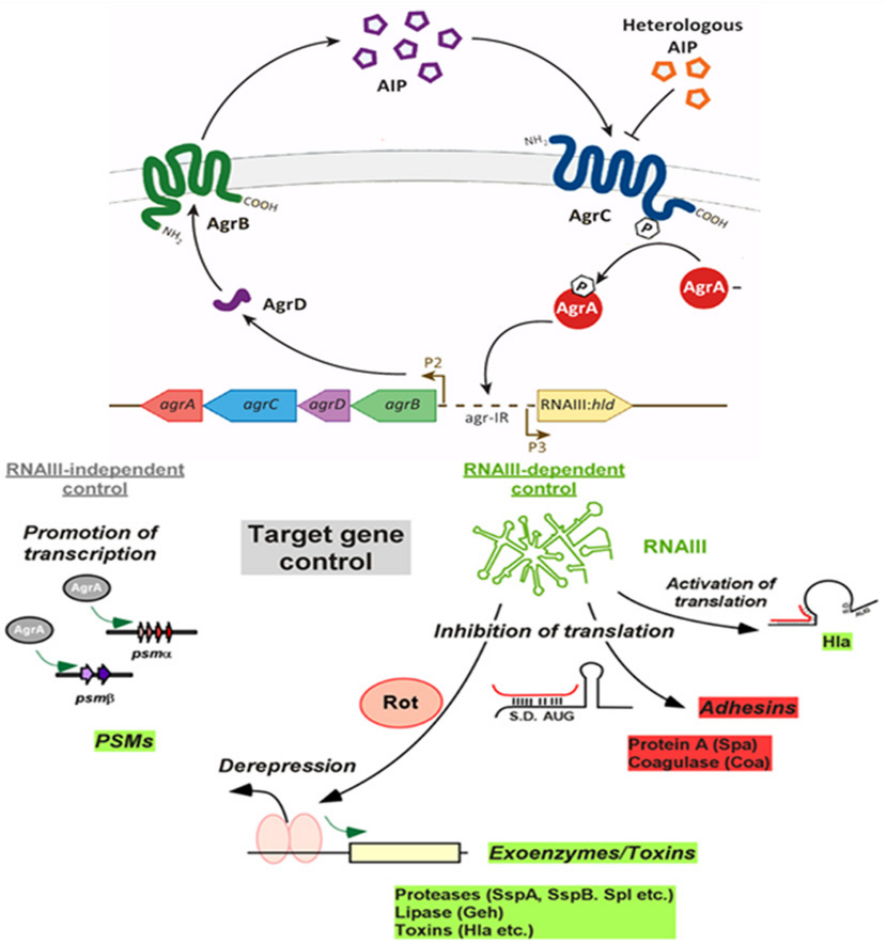


Figure 7: Agr control of quorum-sensing and virulence regulation in *S. aureus*. Modified and adapted from (137, 145).

1.4 HOST-PATHOGEN INTERACTIONS

Interactions between *S. aureus* and the host immune system and the nature of the host immune response evoked are key factors in determining the outcome during the infection. The major function of the immune system is to recognize and protect the host from various infectious agents and foreign substances. The primary defense machinery is called the innate immune system and it represents the first line of defense and consists of several branches (146). The major effector mechanisms include (i) epithelial barrier, (ii) antimicrobial peptide production, (iii) complement system and (iii) phagocytosis. The initial recognition and response is triggered by epithelial cell signaling to recruit immune cells like macrophages, dendritic cells, monocytes, neutrophils, and T-cells. During the initial hours innate immune cells like macrophages and neutrophils responds by directly killing through phagocytosis. Initiation of production of proinflammatory mediators like antimicrobial components, cytokines, and chemokines, further helps in recruiting immune cells for more efficient response and aid in the elimination of the pathogen. The recognition of pathogen-associated molecular patterns (PAMPs) by TLRs and NLRs are considered essential for the first crucial step of innate immune response (147, 148). Furthermore, the innate immune system guides

the development of pathogen-specific adaptive immune response by presenting processed antigens to T cells (149). These harmonized actions between innate and adaptive arms of the immune system are fundamental for eliminating the pathogen.

S. aureus is a potent human pathogen by virtue of its many immune evasion strategies including avoiding of TLR recognition (150, 151). *S. aureus* expresses surface- associated adhesins which confer the ability to adhere cells and tissues, helps in escaping phagocytosis and provides resistance against host defense peptides (152, 153). Secreted proteins like SPA and second immunoglobulin-binding protein (Sbi) prevent IgG mediated opsonization (68, 131, 154). *S. aureus* produces complement evasion proteins like staphylococcal complement inhibitor (SCIN) and chemotaxis inhibitory protein of staphylococci (CHIPS). SCIN aids in protection against phagocytosis and neutrophil-mediated killing (155) while CHIPS inhibit neutrophil and monocyte migration (156). Finally, exotoxins such as cytotoxins cause cell and tissue damage; activate inflammasome mediators and induce excessive proinflammatory mediator production that impedes the efficient clearing of *S. aureus*.

1.4.1 *S. aureus* and Airway Epithelial Response

Polarized epithelial cells play a crucial role in mucosal defenses by sensing the pathogen that gains access to the airway and in response stimulating a cascade of downstream signaling pathway. The airway epithelial cells regulate the interface between “self” and the environment exposure of “non-self” antigens especially pathogens. Epithelial barrier induces the production of several cytokines, which plays an important role in antibacterial response. It also regulates the efficient recruitment and activation of neutrophils and interaction between the epithelium and local immune cells shapes the host response to *S. aureus* (157).

1.4.1.1 TLR signaling

The airway epithelium expresses a complete array of Toll-like receptors (TLR). Cell surface components of *S. aureus* is the primary factor recognized by the epithelial cell surface exposed pattern recognition receptors (PRR). Lipoteichoic acid (LTA) in the cell wall of *S. aureus* is recognized by the TLR2, which causes significant inflammation and neutrophils recruitment as response to epithelial infection (158, 159). Similarly a pore-forming bi component toxin such as PVL is also recognized by TLR2 by the epithelial cells resulting in NFκB signaling (Figure 8) (160). *S. aureus* and TLR interaction induce the production of several cytokines such as IL-8, GM-CSF, TNF and TGF-α and β, and it also induces the secretion of antimicrobial peptides by the epithelial cells (132, 161, 162). These cytokines plays a crucial role in recruiting and maintaining the survival of neutrophils in the airway (162).

1.4.1.2 The Inflammasome

Inflammasomes are large cytosolic multiprotein complexes that control the activation of proteolytic enzymes caspases. Assembly of these complexes is dependent upon cytosolic sensing of pathogen-associated molecular patterns (PAMPs) such as cell surface molecules, toxins, and other harmful agents by the host PRRs during infection (163). Activation of caspase-1 downstream regulates the maturation of pro IL1β and IL18 onto active form as well as induction of pyroptosis (164). Several of the *S. aureus* exotoxins like α-toxin; bi component leucocidins etc. activate the NLRP3 inflammasome via TLR and pore-formation. Inflammasome activation downstream signals Caspase-1 mediated activation of proinflammatory IL1 family cytokines such as IL1β and IL18, which in turn induces cell death via pyroptosis (134, 165). *S. aureus* peptidoglycan and LTA produce the primary stimulus followed by pore-forming toxins (α, β, γ-toxins and PVL) providing with the secondary stimulus for inflammasome activation (102, 166-168). IL1β also regulates the production of IL17 by γδ T cells and blocking the IL1 signaling reduces toxin-induced tissue pathology (169).

1.4.1.3 Type I IFN signaling

Type I IFN activation is commonly associated with *S. aureus* lung infection (134, 170). *S. aureus* PAMPs gaining access to the endosomal or cytosolic compartments induces type I IFN responses in turn resulting in the induction of STAT1, 2 and 3 transcription factors (Figure 8) (134, 171). Type I IFN influences the cell functions of a variety of cell types in the lung as well as induces anti-microbial responses of these cell types (171). The ability of different *S. aureus* strains to cause lung tissue damage has been linked to their ability to activate IFNβ signaling (172).

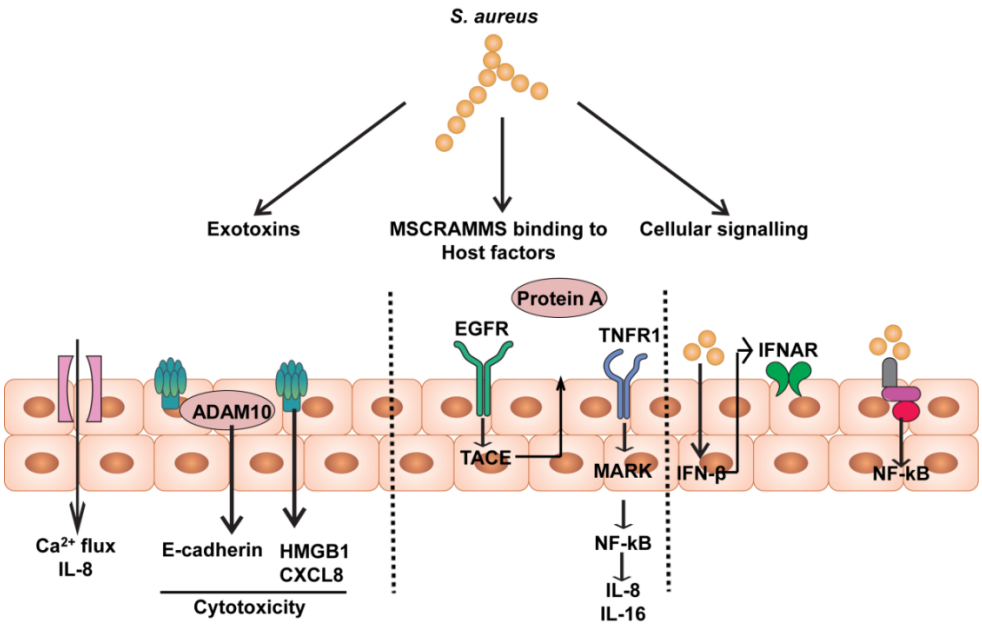


Figure 8: *S. aureus* virulence factors and lung epithelial function and signaling (134, 173)

1.4.2 *S. aureus* and Skin

Skin acts as a most important barrier that protects the body from pathogens. It is divided into three basic layers: the corneal, epidermis and dermis. The corneal layer acts as a major physical barrier, consisting of terminally differentiated keratinocytes (174, 175). While the epidermis is continuously reformed due to the migration of keratinocytes passively to the corneal layer, the dermis is composed of collagen and elastin fibers (174, 175). Apart from being a physical barrier skin also acts as a major immunological barrier especially against pathogens. PAMPs from the microorganisms are recognized by the TLRs and NLRs expressed on the surface of the keratinocytes (174, 176). This recognition induces the production of inflammatory mediators like cytokines, chemokines and antimicrobial peptides against the invading pathogens. This cutaneous immune response also triggers recruitment of immune cells like neutrophils as well as activates resident immune cells like Langerhans cells, macrophages, NK cells, plasma cells, T and B cells, which together can coordinate an efficient cutaneous immune response to clear the pathogens (174-176).

1.4.2.1 Response during skin colonization

The surface of the skin has developed certain attributes to prevent the colonization of *S. aureus*, such as low temperature and low pH. The skin effects the expression of bacterial cell surface factors such as ClfA, CLfB and FnbpA and thereby reducing the growth of *S. aureus* (177, 178). Keratinocytes constitutively express antimicrobial peptides which directly affect the bacterial growth (bacteriostatic or bactericidal) as well as regulate the resident immune cell response against *S. aureus* infection (179, 180). However, *S. aureus* colonizing in the skin is found to produce aureolysin a metalloproteinase which inhibits the antimicrobial peptides activity (181). At the same time, *S. aureus* has evolved to colonize the skin by expressing a wide array of microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) proteins on its cell surface to promote binding and adherence (182, 183).The superantigens produced by *S. aureus* skew the T cell immune response more towards Th2 phenotype and thus contributing towards enhanced skin colonization (184).

1.4.2.2 Response during skin infection

The PRRs on the surface of the keratinocytes recognize the *S. aureus* cell surface factors such as LTA, peptidoglycan etc. and activates the production of proinflammatory molecules such as cytokines, chemokines and tissue-resident immune cells (185, 186). Pore-forming toxins of *S. aureus* such as α-toxin, bicomponent leucocidins such as PVL, LukED etc. causes keratinocyte lysis and activates inflammasome signaling. Activation of caspase-1 by inflammasome is crucial for cleavage of pro- IL1β onto its active form (187). IL1β mediated neutrophil recruitment is required for *S. aureus* clearance and plays a crucial role against

deep-seated *S. aureus* skin infections like cellulitis and folliculitis (188, 189). Both IL1α and IL1β contribute equally against superficial skin infection such as impetigo (188). In addition secretion of IL17 by T cell subsets such as Th17 cells, NKT cells, and γδ T cells also plays a critical role in neutrophil recruitment as a host defense against cutaneous *S. aureus* infections (190, 191).

1.4.3 *S. aureus* and Neutrophils

Neutrophils are critical components of the first line of host defense against infectious agents as they are capable of producing non-specific antimicrobial effector responses. The majority of healthy individuals are usually protected against severe *S. aureus* infections, largely due to the protective role played by neutrophils in host immune responses. To facilitate rapid recruitment to the site of infection large numbers of neutrophils are maintained in the circulation. Circulating neutrophils are recruited to the site of infection mainly by host-derived chemotactic factors, among others IL-8, GCP-2, complement factor C5a, HMGB1 etc. (192-194). The whole process of neutrophil recruitment to the tissue site is broadly divided into four steps including rolling adhesion, integrin activation, firm adhesion and transmigration. Initially, neutrophils adhere to the blood vessel by repetitive ligand-receptor binding (192, 193). Further, neutrophil-endothelial cell interaction takes place by the activation of lymphocyte function associated antigen-1 (LFA-1); followed by LFA-1 binding onto intracellular adhesion molecules (ICAM-1 and ICAM-2) on endothelial cells causing neutrophil capture (192, 193, 195). This induces cytoskeletal rearrangements initiating morphological changes from spherical to flat in neutrophils, causing transmigration from the endothelium into infected tissue (195). The primary role of neutrophils in the host-defense mechanism is phagocytosis of pathogens recognized by the PRRs (196, 197). This process is usually enhanced when the microbes are opsonized and recognized by IgG and complement receptors on neutrophils (198, 199). Upon phagocytosis NADPH oxidase complex generates high levels of reactive oxygen species (ROS); this superoxide anion is readily converted into hydrogen peroxide, other secondary oxygen derivative, and hypochlorous acid which contribute towards microbicidal activity (200, 201). Apart from this neutrophils also produces several degradative enzymes and cationic peptides like cathepsins, elastase, defensins, proteinases etc. contained in its granules which facilitate the efficient killing of bacterial pathogens (202, 203).

1.4.3.1 S. aureus neutrophil evasion

S. aureus is capable of overcoming neutrophil-mediated killing by producing several secreted and surface bound factors that alter the functional capacity of neutrophils. The chemotaxis of both neutrophils and monocytes are inhibited by CHIPS through directly binding to the C5a and FPR, and also thereby inhibiting phagocytosis (156, 204).

Complement-mediated opsonization of *S. aureus* is inhibited by SCIN, extracellular fibrinogen-binding protein (Efb) and extracellular complement-binding protein (Ecb). Similarly SPA and Sbi interacts with the Fc region of the immunoglobulins to inhibit phagocytosis (155, 205, 206). To protect and counter the cytotoxic effect of ROS and antimicrobial peptides inside the phagosome *S. aureus* is known to produce superoxide dismutases, catalase, hydroperoxide reductase and staphyloxanthin (207-209). Furthermore, *S. aureus* secretes several of the cytotoxins including PSMs, PVL, and LukAB which specifically target neutrophils by causing pore formation and inducing osmotic lysis (90, 106, 112, 113, 118).

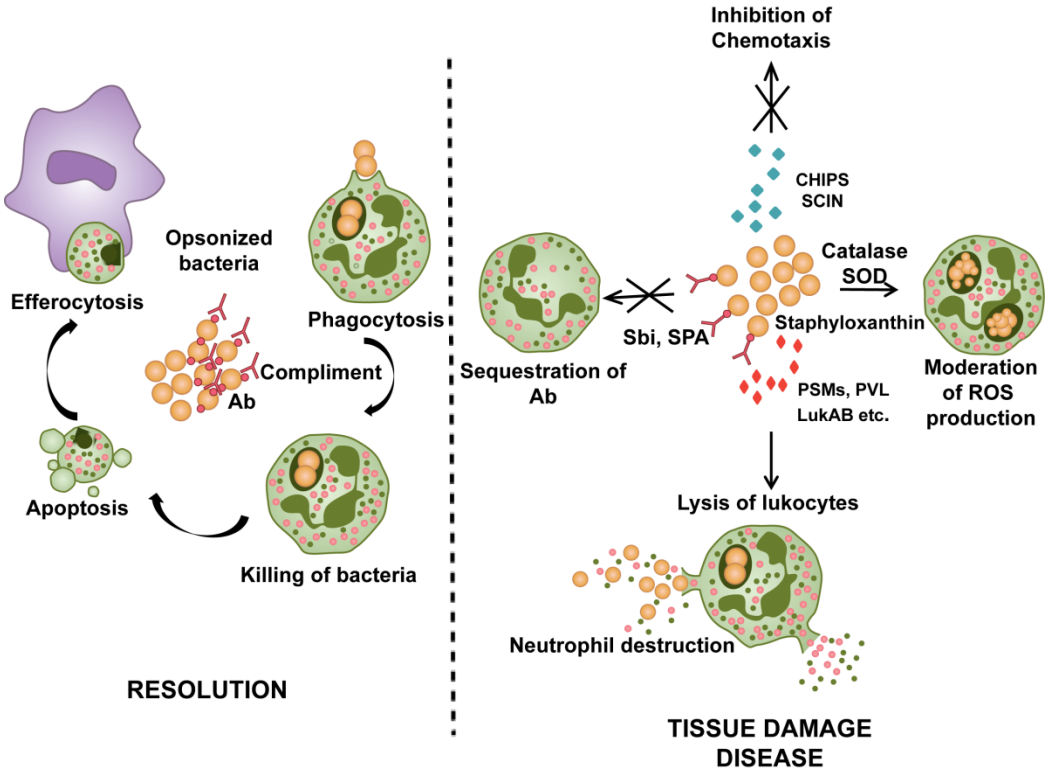


Figure 9: *S. aureus* neutrophil evasion mechanism (197, 210)

2 AIMS

This project focuses on severe invasive infections caused by *S. aureus*. The incidences of these infections are rising, and unusually severe pathological signs are associated with certain Community Acquired (CA) *S. aureus* types with superior transmissibility and virulence. Here we seek to obtain insight into CA *S. aureus* infections, including molecular characterization of strains in the community, mechanistic studies to identify mechanisms contributing to lethal necrotizing *S. aureus* infections, as well as understand the role of specific staphylococcal toxins and virulence regulation in the pathologic events leading to the destructive infections in lung and skin.

Specific aims are:

- To establish the antibiotic resistance profile, genetic lineage and to determine toxins and virulence traits of *S. aureus* strains isolated from both colonizers and patients with invasive infection.

- To determine how diversity in exotoxin profiles among CA *S. aureus* strains translates into virulence-associated functional responses.

- To delineate the role of specific staphylococcal exotoxins in mediating tissue injury and to define correlates of severe lung pathology associated with pneumonia.

- To identify the underlying mechanism of the genetically related clinical ST22 MRSA strains displaying starkly different phenotypic response profile and to explore how this influences infection outcome.

3 METHODOLOGY

A detailed description of the experimental procedures used in this thesis is found in the respective articles and manuscripts. In this section, a brief summary and overview of some of the experiments are presented here.

Bacterial strains

A total of 68 *S. aureus* isolates were included in **paper I**, of which 38 were from healthy nasal carriers and 30 from various infection sites. *S. aureus* isolates were selected based on the growth in chromogenic agar and further characterized using various biochemical and molecular confirmatory experiments. In **paper II,** 38 strains from the above heterogeneous cohort of CA *S. aureus* strains were selected randomly to represent different ST types and toxin profiles from both colonized as well as from patients with varying infections. Similarly, this study also included a confirmatory homogenous cohort of 31 isolates collected from patients with CA *S. aureus* pneumonia. In the next study in **paper III,** we focused specifically on lung infections of varying severity. Clinical *S. aureus* isolates collected from pleural fluid of patients with varying severity of pneumonia, including two cases of necrotizing pneumonia (NP753 and NP796) and one milder case of lung empyema (LE2332) were selected from the characterized strain collection in **paper I**. The results obtained from these strains were then confirmed using a larger cohort of strains causing pneumonia. In **paper IV** we specifically focused on ST22 MRSA isolates causing skin infection. This thesis overall focuses on well-characterized clinical *S. aureus* strains causing various infections. An advantage of using clinical strains in contrast to certain lab strains is due to the fact that virulence properties are well preserved as well as ensures results of high clinical relevance.

Proliferation assay

This assay is commonly used to functionally asses the superantigen-mediated proliferation of immune cells, i.e. T-cells. The superantigen-mediated proliferative responses will be influenced by the presence of cytotoxins eliciting cytotoxicity and targeting specific immune cells. Phytohemagglutinin-L (PHA) was used as a positive control for polyclonal T cell activation and expansion.

Organotypic tissue models

We speculate that tissue-specific pathogen-elicited host responses may explain tissue tropism and disease manifestations of acute severe bacterial infections. Thus, it is likely that pathogen-specific toxins and host derived inflammatory mediators at the local site of infection both contribute significantly to disease progression and outcome. In this context,

human tissue models can capture important aspects of host associated responses to infections, since many important human pathogens (e.g. *group A streptococcus and S. aureus*) induce species-specific responses. To approach this we have used an innovative human 3D organotypic human lung/skin tissue model system in combination with bacterial stimulation assays as means to predict virulence and pathogen-elicited tissue responses.

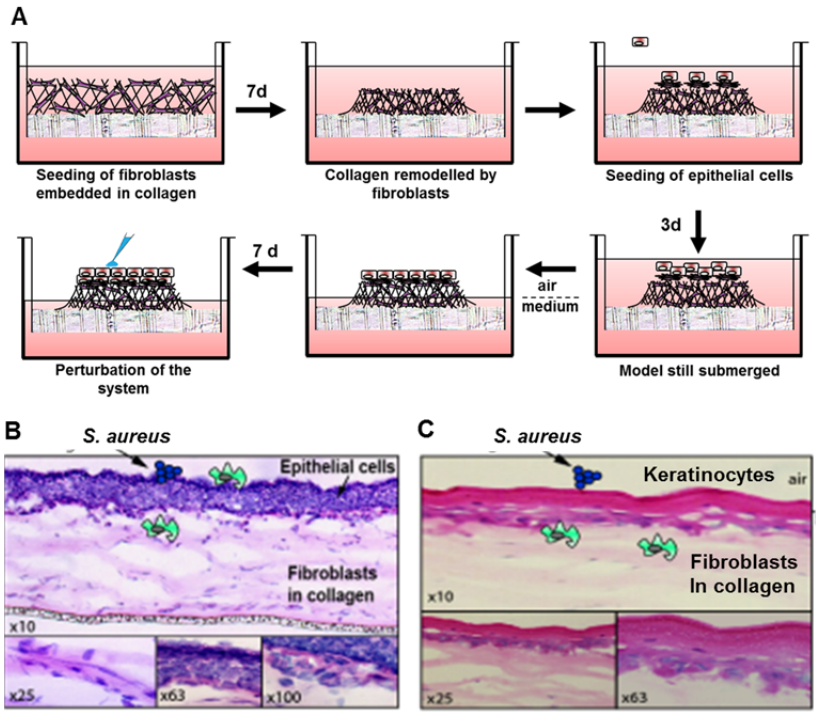


Figure 10: A) Schematic drawing depicting setting up of organotypic tissue models. Representative hematoxylin/eosin staining image of (B) Lung tissue model, and (C) Skin tissue model. Modified and adapted from (211, 212).

To investigate the impact of *S. aureus* on human tissue-specific cells, we used a human 3D organotypic lung/skin tissue model in which cells retain their differentiated cellular phenotypes in an *in vivo* like architecture; thus allowing for experiments under more physiologic conditions. Epithelial cells are stratified on a layer of fibroblasts as shown in (Figure 10). In our model, epithelial and fibroblast cell lines are used as they allow for reproducibility as compared to using genetically non-uniform and potentially impure primary cells. In these tissue models, stromal cells acquire a polarized phenotype and a large number of cell-cell contacts between stromal cells occur. Also, production and deposition of important tissue components are seen. Thus, the model is well suited for studies on tissue-specific responses to bacterial provocation in a physiological relevant setting. These systems, therefore, provide unique models in which mechanisms of disease manifestations and the events directing acute severe infections can be monitored in real-time.

Confocal microscopy and live imaging

Microscopy based methods have been very useful and a widely used technique during the course of this thesis projects. Confocal microscopy was used to characterize and quantify the structural and cellular proteins in the tissue models stimulated with bacterial toxins or live infection. It was also used to visualize and determine the localization/association of *S. aureus* with host cellular compartments during infection.

Live imaging method was employed mainly due to the fact that this technique enables studies on visualization and quantification of tissue integrity determination, cellular process and localization of molecules in real time within the live tissue. Lung tissue models were constructed using GFP expressing epithelial cells and were stimulated with bacterial supernatants or pure toxins. Live imaging experiments was performed over a time period of 16 hours at 20 min intervals between each image and were captured by acquiring 3D z-stacks at 3 µm z-dimension resolution, the volume of 512 x 512 µm in x and y-direction and 120-150 µm in the z direction. Finally, tissue integrity was determined by quantifying the total intensity sum of GFP expression over the period of time after stimulation. Due to the more transparent nature of the tissue models as compared to the real tissues, deep penetration of the laser into the models was possible, with a reduced scattering of the light.

4 RESULTS AND DISCUSSION

4.1 MOLECULAR CHARACTERIZATION OF *STAPHYLOCOCCUS AUREUS* STRAINS FROM THE COMMUNITY

S. aureus is a significant cause of human infections and an emerging health problem globally. The prevalence of *S. aureus* infections varies worldwide among countries. The increasing use of antibiotics has resulted in multi-drug resistant *S. aureus* due to selective pressure. Consequently, the emergence of MRSA infections is a major cause of concern in most countries, with high antibiotic resistance including Vancomycin resistance which in some cases has left physicians with limited options for treatment (7, 213). In India, antibiotics consumption is rampant and the rate of MRSA in clinical samples, as well as carriage in the community, is high (30-70%) (214). The advent of CA-MRSA into the hospitals replacing the HA-MRSA strains in the past decade has resulted in increased drug resistance, mainly in countries where the prevalence is high (215). Studies on Indian CA-MRSA especially their molecular characteristics and clonal complexes present have not been studied in detail. Therefore, in the first study of this thesis we characterized Indian CA-MRSA and MSSA strains from rural and urban healthy carriers and disease isolates from pus, blood etc. from patients visiting hospitals. The aim was to study the distribution of SCC*mec* elements, sequence types (STs) and their toxins and virulence factors profile in the community and hospital environment. Carriers were chosen to include only those with no identified risk factors for MRSA acquisition including, prior hospitalization, use of antibiotics and surgeries in the past year. Carrier isolates (n=38) were obtained from nasopharynx swabs. Disease isolates (n=30) were recovered from wounds, pleural fluid and blood cultures in hospitals from 4 different cities within India. The isolates were characterized using MLST, *spa* typing, PFGE, SCC*mec* typing, *agr* typing, virulence gene content and antibiogram (CLSI 2005). Molecular characterization was complemented by virulence gene microarray of selected isolates, the analysis is presented in Table 1 **(Paper-I)**.

4.1.1 Antibiotic Resistance

Among the isolates, the overall MRSA rate was 41% with 26% of carrier isolates and 69% of disease isolates containing *mecA* gene. Antibiotic sensitivity to five antibiotics was tested and the following resistance percentages were observed: oxacillin (n =16; 24%), cefoxitin (n =18; 27%), erythromycin (n =12; 18%), gentamycin (n =20; 29%) and tetracycline (n = 0). The antibiotic sensitivity pattern among carrier and disease MRSA isolates were similar to each other (Figure 11). A major difference in antibiotic sensitivity pattern was identified between carrier and disease MSSA isolates where the majority of the carrier MSSA isolates (76%) were sensitive to all antibiotics tested in contrast to only 33% of the disease MSSA isolates being sensitive to all antibiotics. Among disease, MSSA isolates 25% of them had resistance determinants for gentamicin and/or erythromycin (Figure 11). This is in

accordance with a follow-up study on a larger cohort showing MSSA strains exhibiting increased resistance towards certain antibiotics (216). Similarly, few isolates were neither completely sensitive nor resistant to the antibiotics tested in terms of their MIC values; such strains were categorized as intermediate resistant strains.

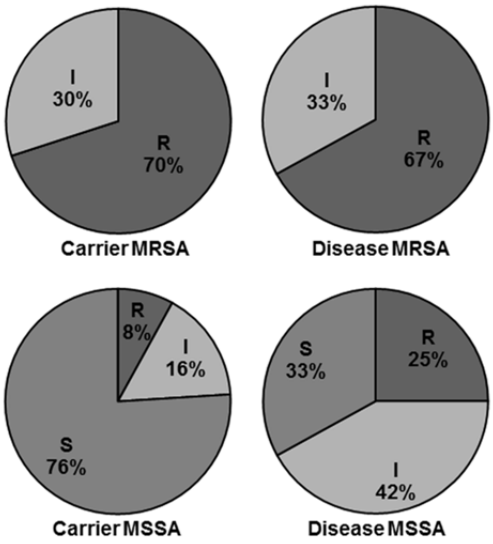


Figure 11: Antibiotic resistance pattern among carrier and disease MSSA and MRSA isolate. R: Resistant, I: Intermediate resistant, S: Sensitive

4.1.2 Major MLST types and their virulence gene profile

In **paper I**, even from a small number of strains collections of 68 isolates, 15 different STs including isolates resembling *S. aureus* from animal origin were identified. MSSA isolates were more diverse and heterogeneous in their genetic background as compared to MRSA isolates. The major ST type (ST22, ST772, and ST121) represented more than 10% of the isolates and ST1208 and ST672 were identified as emerging clones.

ST22 formed the dominant clone both among carriers and disease isolates making up 28% of the total strain collection. Sixty eight percent of the ST22 isolates were *mecA* positive and had SCC*mec* IV element, and a PFGE pattern resembling that of EMRSA-15 but exhibiting some variation (217). These strains are found to replace the traditional HA-MRSA strains in many countries like Germany, Portugal and Singapore (218-220). Similarly, ST239 HA-MRSA strains with SCC*mec* III or IIIA that previously circulated in the hospital environment are being replaced by ST22 MRSA strains (221). All ST22 isolates were belonging to *agr* type I, capsular type 5 and positive for PVL and *egc.*

ST772 was the second major clone with 69% of the strains being methicillin-resistant, belonging to SCCmec type V and *agr* type II. All the isolates were capsular type 5, PVL, *egc* positive, and contained combination of several enterotoxin genes. The type V SCC*mec* cassette in these strains contained the second *ccrC* region similar to that of SCC*mec* ZH47 mosaic cassette as previously reported. The above noted low-level resistance towards oxacillin might be attributed towards the composite nature of the SCC*mec* type V cassette in these strains (222). This was further confirmed by whole genome sequencing studies on

these Indian ST772 strains, identifying a novel type V (5C2) SCC*mec* element in many while the others have a composite SCC*mec* type V element (5C2&5) formed by the integration of type V SCC*mec* into an MSSA carrying SCC element (223).

ST121 clones were detected in 10% of the isolates and all the strains were methicillin sensitive and negative for *mecA* gene. All ST121 strains identified in this study belong to *agr* type IV, capsular type 8, and are PVL and *egc* positive.

In **paper I**, we have characterized Indian *S. aureus* isolates which belong to a wide variety of ST types. Even in a small collection of isolates (n=68), strains belonging to 15 different STs were identified, with MSSA isolates exhibiting the greatest heterogeneity. Similar diversity among MSSA strains has been recently reported from a study conducted in China (224). The most obvious bias in the study is the limited number of isolates collected, but our results are in part concordant with the existing literature: the two major MRSA STs (ST22 and ST772) being reported earlier, as well as a greater heterogeneity among MSSA, isolates as compared to MRSA strains (221, 224, 225). Many of the STs are being reported for the first time in this study. Sixty nine percent of the isolates carried PVL genes and the superantigen genes *egc* were found in 84% along with many other toxins. This high prevalence of PVL and *egc* genes among Indian MSSA and MRSA isolates is unlike the situation in other South Asian countries, where only MSSA strains contain PVL (226, 227). In addition, detailed molecular analysis of Indian *S. aureus* isolates along with their virulence repertoire is reported here for the first time which might lead to a better understanding of the characteristics of circulating clones in the community and the disease caused by these strains.

4.2 COMMUNITY *S. AUREUS* ELICITS STABLE CYTOTOXIC OR PROLIFERATIVE RESPONSES IN HUMAN PBMC: LINK TO *AGR* TYPE AND ALPHA-TOXIN LEVELS

The ability of *S. aureus* to cause multitude of infections is linked to the production of a wide array of virulence factors. Several virulence factors have been implicated in disease pathogenesis, including Panton-Valentine Leukocidin (PVL), alpha-toxin (α-toxin), superantigens and phenol soluble modulins (67, 98, 118, 228). These virulence factors are regulated and controlled by a global regulator system called the *agr* system. A recent study demonstrated that allelic variation on *agr* translates into significant differences in expression of several virulence factors (229).

In **paper II** we wanted to assess the virulence properties and functional responses of community *S. aureus* strains with distinct toxin profiles as characterized in **paper I**. In particular, we sought to analyze the responses elicited by bacterial supernatants prepared

from overnight cultures of the isolates. Such supernatants contain a mixture of secreted virulence factors including both superantigens and cytotoxins. Based on humoral responses against superantigens and cytotoxins that develop in patients, it is clear that these toxins are expressed *in vivo*. Although we cannot account for potential differences *in vitro* and *in vivo* expression of virulence factors, we believe that the use of supernatants more closely reflects what the patients are exposed to as compared to purified toxins. Exactly how these different combinations of toxins affect virulence and host responses is not yet fully elucidated.

A heterogeneous cohort of community *S. aureus* strains (n= 38), both MRSA and MSSA, collected from colonized individuals and patients with varying *S. aureus* infections were used in this study (**paper II**). This isolate collection had been characterized with respect to antibiotic resistance profile, molecular typing including Sequence Type (ST), Agr-types, and toxin profile as determined by microarray in the previous study **(paper-I)**.

4.2.1 Functional properties of clinical *S. aureus* isolates: proliferative or cytotoxic profiles

Bacterial supernatants were initially tested in a proliferation assay, in which peripheral blood mononuclear cells (PBMC) were stimulated with different dilutions (1:50, 1:100 and 1:1000) of the bacterial supernatants (Figure 1A and 1B **paper II**). This is commonly used to functionally assess superantigenic responses elicited by *S. aureus* isolates but the response will be influenced by the presence of cytotoxins. The supernatants demonstrated striking differences in their ability to trigger proliferative responses, and they displayed robust response profiles, either a proliferative or cytotoxic. A proliferative profile was denoted when strains induced proliferative responses at all dilutions. A cytotoxic profile was assigned when the strains did not elicit a proliferative response in the more concentrated supernatants (1:50 and 1:100). This was not mainly due to lack of superantigenic activity, as strong proliferative responses were noted in the most diluted samples; thus, indicating a cytotoxic effect rather than lack of superantigen-mediated proliferation. To assess this further, flow cytometry analysis was performed using stimulated cells stained for dead cell marker plus cell surface CD3, HLA-DR, and CD45. Proliferative supernatants induced expansion of T cells, while cytotoxic supernatants induced significant cell death (Table 1 and Figure 1E; **paper II**). Thus, demonstrating that in some strains the superantigens trigger proliferative responses, but this activity is masked by toxin-mediated cytotoxic responses. The two functional profiles could be found in both colonizing isolates as well as from invasive cases, and there was no significant association between functional profile and ST-type/clonal complex or MRSA/MSSA (Table 1; **paper II**).

4.2.2 Significant association between agr type and proliferative or cytotoxic profile

Expression of many secreted *S. aureus* virulence factors, including superantigens and cytotoxins, is controlled by a global regulator called the *agr* (accessory gene regulator) system. The *agr* is an autoinducing, quorum-sensing two-component system for which four different allelic variants were identified (*agr* I-IV). Comparison of functional profile to *agr* type revealed a significant correlation with *agr* II and *agr* III strains predominantly showing a proliferative profile whereas *agr* I and IV strains were cytotoxic (Figure 1F; **paper II**). As this analysis was conducted on a highly heterogeneous strain collection, including both colonizing and invasive CA *S. aureus* strains from India, we wanted to also test a more homogenous isolate collection. To this end, we tested isolates collected from patients with CA *S. aureus* pneumonia in France. Also in this isolate collection, a similar association with *agr* I and *agr* IV being significantly more cytotoxic than *agr* II or III was noted (Figure 1G; **paper II**). By virtue of *agr* being a global regulator of virulence factors including both cytotoxins and superantigens, it seems likely that the noted association is linked to varying toxin production.

4.2.3 α-toxin expression correlates with cytotoxicity against PBMC

Next, we sought to delineate the factors responsible for the cytotoxic response profile. *S. aureus* express a wide array of virulence factors, which contribute in various ways to disease pathogenesis. Among several putative determinants of community *S. aureus* virulence, the cytotoxins α-toxin and PVL have been implicated in severe invasive infections. To assess their potential contribution to the functional profile, the amounts of α-toxin and PVL were determined in all bacterial supernatants. The results demonstrated a striking correlation between the amount of α-toxin and cytotoxic response; the cytotoxic supernatants had significantly higher levels of α-toxin than did the proliferative, regardless of whether the strains were collected from the patients, colonized or CA pneumonia cohort (Figure 2A; **paper II**). In contrast, there was no significant correlation between the proliferative or cytotoxic profile of strains and PVL expression (Figure 2B; **paper II**). Thus, the data strongly implied that α-toxin contributed to the noted cell death in PBMCs. This was further confirmed by stimulating PBMC with PHA and pure α-toxin, which revealed a clear dose response where higher amounts of α-toxin resulted in higher cytotoxicity (Figure 3A; **paper II**) as well as stimulation experiments using isogenic α-toxin and PVL mutants (Figure 3C and 3D; **paper II**). Of note, the majority of the cytotoxic supernatants contained ≥ 225 ng/ml α-toxin, whereas proliferative supernatants always had low levels of α-toxin productions i.e. < 221ng/ml. Thus, it is tempting to speculate that there exists a critical threshold of α-toxin concentration that determines the cytotoxic response and underlines the importance of quantifying the toxin levels rather than the traditional epidemiological method of just determining their presence at the genetic level. The amount of toxins secreted by the

individual strains seems to influence their virulence property and may play a crucial role in disease outcome. Furthermore, in accordance with the above results significantly, higher levels of α-toxin were found in strains belonging to cytotoxic *agr* type I and IV as compared to the proliferative *agr* type II and III (Figure 2C; **paper II**). A study assessing variation in kinetics and degree of *agr* signaling showed that strains belonging to *agr* type I and IV had the earliest and strongest *agr* signaling induction as compared to *agr* type II and III strains (229). The molecular basis for this hierarchy in signaling strength and link between *agr* type and response profile due to varying levels of secreted AIPs is not yet fully elucidated. Furthermore, the clinical relevance of these distinct functional profiles remains to be shown.

In conclusion, the study in **paper II**, reveals robust functional response profiles, either proliferative or cytotoxic, in community *S. aureus* isolates. The response profiles were associated with *agr* type and low or high α-toxin production, and it seems likely that distinct functional response profiles could influence the clinical manifestation and that it might reflect specific pathotypes. To explore such a linkage would require large epidemiologic materials consisting of strains from different genetic lineages and, *agr* types combined with well-defined clinical data. Similarly, whether the differential phenotypic response profiles elicited by *S. aureus* strains can be utilized as a diagnostic or as an epidemiological surveillance tool to differentiate between strains capable of causing cytotoxic response versus strains eliciting a superantigen-mediated systemic response in patients can at present only be speculated upon.

4.3 TOXIN-MEDIATED PATHOLOGY IN A HUMANIZED LUNG TISSUE MODEL EXPOSED TO *S. AUREUS* TOXINS

Necrotizing pneumonia is one of the most severe manifestations of invasive CA *S. aureus* infections. PVL-positive strains and CA *S. aureus* have been epidemiologically linked to these life-threatening infections (49, 98). However, studies of pathogenesis have implicated different toxins, including PVL, α-toxin and phenol-soluble modulins (67, 98, 118). These contradicting results have partly been attributed to use of animal models significantly less sensitive to the toxins as compared to humans (99). Although necrotizing pneumonia is recognized as a toxin-mediated disease, the mechanisms underlying the clinically important tissue destruction remains unclear. Progress in the field has been hampered by the lack of experimental models that allows for studies of toxin-mediated effects in human lung tissue. This underscores the importance of using clinically relevant susceptible host for the study of toxin-mediated pathology.

In **paper III**, we focused on the pathogenesis of CA *S. aureus* necrotizing pneumonia, in particular elucidation of the tissue destructive events. To investigate the impact of specific *S.*

aureus exotoxins on human tissue-specific cells, we used a human 3D organotypic lung tissue model. Our working hypothesis is that several different toxins will contribute to tissue pathology, but they may target different cells and their impact may be tissue-specific.

4.3.1 Necrotizing pneumonia isolates mediated strong cytotoxicity and increased tissue disruption

To study toxin-mediated tissue pathology, contributing to *S. aureus* pneumonia three CA-MSSA *S. aureus* strains isolated from patients with a lung focus of infection, including two severe cases of necrotizing pneumonia (strains NP796 and NP753) and one milder with lung empyema (strain LE2332), were selected from the strain collection described in **paper I**. These strains were characterized with respect to molecular types and virulence profiling using microarray analyses (**paper I** and **paper III** Table S1). The two necrotizing pneumonia isolates were both ST121 type, *agr* type IV and shared an identical exotoxin gene profile, whereas the lung empyema strain was an ST30 type and *agr* type III (**paper III**; Table 1). As a reference strain, the epidemic CA-MRSA USA300 strains 11358 and LUG2012 was used. Initially, we tested the bacterial supernatants from overnight cultures of these strains in a proliferation assay as explained in **paper II**. The supernatants elicited starkly different responses i.e. both necrotizing pneumonia strains displayed cytotoxic response profiles whereas the lung empyema strain a proliferative response profile (Figure 12A). Cytotoxicity was further confirmed by adding bacterial supernatants together with the PHA, which resulted in a reduction of PHA-response in the presence of the necrotizing pneumonia supernatants, but not the lung empyema supernatant (Figure 12B). Flow cytometry analysis in PBMC cultures exposed to supernatants from the necrotizing pneumonia strains showed that 97% of the cells were positive for dead cell marker (Figure 12C).

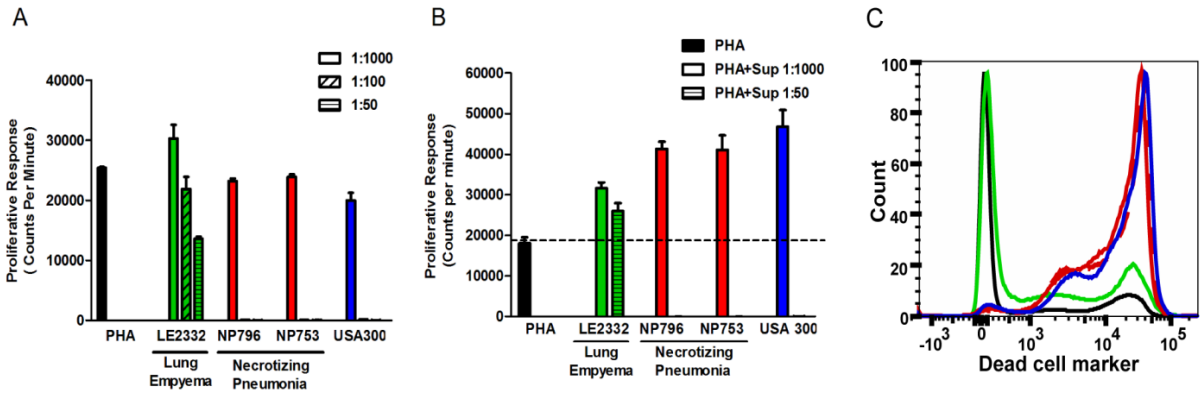


Figure 12: Representative proliferative or cytotoxic responses by human PBMCs stimulated with indicated dilution of bacterial supernatants. A) Stimulation with bacterial supernatants alone. B) Stimulation with the addition of PHA. C) Representative histogram of flow cytometry data as defined by dead cell marker positivity among PBMC stimulated with LE2332 (green), NP753 (red full), NP796 (red dashed), USA300 (11358) (blue), unstimulated (black).

Also, other relevant cell types such as human primary neutrophils and lung epithelial cells were exposed to the supernatants. The necrotizing pneumonia isolates were highly cytotoxic to both lung epithelial cells and neutrophils whereas the lung empyema isolate lacked cytotoxic activity towards lung epithelial cells and was moderately cytotoxic towards neutrophils (Figure 1A and 1B; **paper III**). This corresponded to higher levels of PVL and α-toxin being produced by the two necrotizing pneumonia isolates, as compared to the lung empyema isolate that produced low levels of PVL but no α-toxin (Table1; **paper III**). Taken together, the data revealed cell-specific responses, demonstrated by α-toxin targeting PBMC (**paper II**) and lung epithelial cells but not neutrophils (**paper III**), whereas PVL only targeting neutrophils. Using the lung tissue model, histological analysis of sections of the tissue model revealed that in unstimulated models, the epithelial cells remained intact on top of the fibroblast matrix layer while tissue injury including breakage of the epithelial mucosa barrier as well as dissociation of epithelial cells from the fibroblast matrix layer was accentuated in lung tissue models exposed to necrotizing pneumonia isolates as compared to the lung empyema isolate (Figure 1C and 1D; **paper III**). Exposure of lung tissue models to toxins secreted by strains causing necrotizing pneumonia (NP753, NP796) vs lung empyema (LE2332) exhibited varying response and the epithelial damage elicited by these strains correlated with the observed clinical severity.

4.3.2 α-toxin and PVL mediated cell-specific cytotoxicity contributes towards epithelial damage

In **paper II,** we report that α-toxin contributes to cytotoxicity against PBMC, and it is well recognized that the main target of PVL is neutrophils. We, therefore, quantified the levels of α-toxin and PVL in the bacterial supernatants and the results showed that the two necrotizing pneumonia isolates produced high levels of both PVL and α-toxin, whereas the lung empyema isolate produced moderate levels of PVL but low levels of α-toxin (Table1; **paper III**). This furthermore suggests that α-toxin might be a key factor mediating epithelial damage. To test this assumption and to determine the kinetics of cytotoxic events, live imaging experiments of lung tissue models exposed to supernatants or pure toxin was performed. Epithelial disruption was evident within 2 to 3 hours and maximum cytotoxicity was attained by 6 hours in NP753 supernatant and pure α-toxin stimulated lung tissue models (Figure 2C and 2D; **paper III**). Further experiments from toxin-deficient mutants verified that α-toxin was the major mediator of epithelial disruption which is in line with previous findings (230, 231).

A recent study identified ADAM10, a metalloprotease, as a receptor for α-toxin (71). In line with this finding, we found that lung epithelial cells expressed high levels of ADAM10; PBMCs expressed intermediate levels whereas neutrophils expressed low levels of ADAM10. Thus, the noted cell-specificity in α-toxin mediated cytotoxicity was coupled to a differential receptor expression. Previous studies have described that interaction between α-

toxin and ADAM10 may result in an activation of the protease activity of ADAM10 and consequently lead to cleavage of the tight junction protein E-cadherin (72). We found a distinct loss of E-cadherin in lung model stimulated with necrotizing pneumonia strain as compared to the lung empyema strain (Figure 2H and 2I; **paper III**). This further supports that α-toxin has a key role in tissue damage, not only through its direct cytolytic activity but also through its interaction with the metalloprotease ADAM10 resulting in loss of important adherence junction proteins such as E-cadherin.

To include also neutrophils in this setting and to decipher contribution of PVL-mediated neutrophil lysis on epithelial damage, culture supernatants from neutrophils stimulated with the bacterial supernatants were added to the lung models, alone or in combination with bacterial supernatants/pure α-toxin (Figure 3A-C; **paper III**). The results revealed that addition of stimulated neutrophil supernatants further augmented the tissue pathology. In lung models, addition of neutrophil supernatant stimulated with lung empyema strain enhanced the level of tissue destruction to the same degree as in models stimulated with necrotizing pneumonia strains (Figure 3A; **paper III**). The data thus showed that PVL contributed to epithelial damage indirectly by triggering neutrophil lysis. Neutrophil-released contents like granule proteases and other factors have previously been shown to cause epithelial damage (100, 232). Taken together, this implies that both α-toxin and PVL will impact tissue destruction by targeting different cells and that most severe tissue damage is elicited by a combined action of α-toxin and PVL.

We also analyzed bacterial supernatants containing known amounts of α-toxin and PVL in a larger collection of CA-pneumonia strains (Table S2; **paper III**). The results revealed that α-toxin levels correlated with cytotoxicity against lung epithelial cells (Spearman r=0.68; p=<0.0001) but not with neutrophils. In contrast PVL levels correlated with cytotoxic response towards neutrophils (Spearman r=0.56; p=0.001) but not with lung epithelial cells. Similar results were obtained also in lung tissue model experiments where α-toxin levels correlated with direct epithelial damage while high levels of PVL correlated with neutrophil-mediated epithelial damage (Figure 5B and 5C; **paper III**). Notably dividing the strains according to their clinical outcome demonstrated that strains expressing high levels of α-toxin and PVL were largely found in the non-survivor cohort and that higher cytotoxicity towards both epithelial cells and neutrophils was more prevalent among strains associated with fatal outcome as compared to survivors. These data on clinical isolates is concordant with our previous observation of a combined role for both high α-toxin and PVL levels in severe disease manifestations. These data underlines the importance of quantifying the levels of toxins rather than solely measuring their presence at gene level as well as determining the cytotoxic activity of these strains in relevant cell types in clinical and epidemiological studies.

4.3.3 Augmented inflammation, tissue necrosis and chemotactic responses induced by *S. aureus* toxins in lung tissue model.

Furthermore to characterize the level of tissue pathology caused by the bacterial supernatants, the tissue was analyzed for HMGB1 and CXCL8. The results revealed that HMGB1 and CXCL8 expression were accentuated in epithelial layer exposed to necrotizing pneumonia strains as compared to lung empyema (Figure 4; **paper III**). Thus, these inflammatory responses are in complete agreement with the differential tissue damage elicited by the strains. As necrotizing pneumonia is characterized by a massive influx of neutrophils to the site of infection, we explore whether stimulated epithelium influenced neutrophil migration. Using a transwell migration assay, conditioned media from *S. aureus* toxins exposed lung tissue models induced a strong chemotactic response and caused significant migration of neutrophils over a broad concentration range (Figure 4G and 4H; **paper III**). Notably, these effects were seen even at sublytic concentrations of the toxins and it can be noted that this induction of neutrophil migration, infiltrating the tissue environment can further exacerbate tissue pathology.

In our previous studies in **paper II** we showed that IVIG efficiently inhibited α-toxin-mediated cytotoxicity and superantigen-mediated proliferation (Figure 4A and 4B; **paper II**). Here we tested whether this toxin-mediated tissue pathology can be blocked by addition of IVIG. Live imaging experiments revealed that epithelial damage mediated by both bacterial supernatant and supernatants from stimulated neutrophil was completely abrogated by IVIG (Figure 13, Figure 6D and 6E; **paper III**). In line with previous reports (233-235), our data support a beneficial role of IVIG in pneumonia through its broad spectrum of antibodies against toxins can prevent toxin-mediated tissue damage.

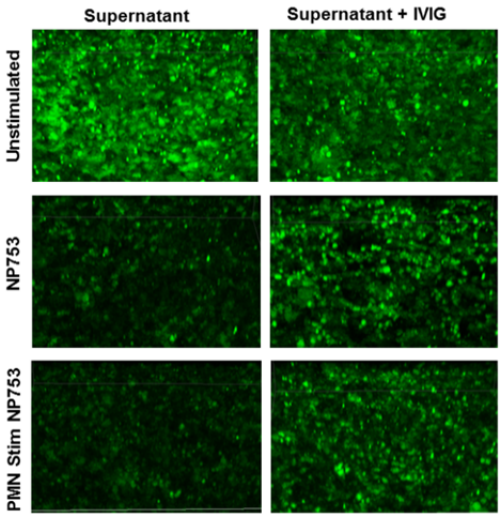


Figure 13: Live imaging analyses of GFP-expressing lung tissue stimulated with supernatant from NP753 or NP753 stimulated neutrophils without IVIG (left panel) and with IVIG (right panel).

In this study in **paper III** we demonstrate that severe lung tissue pathology is associated with a combination of high levels of both α-toxin and PVL, and fatal outcome correlated with higher toxin production and cytotoxic activity in pneumonia isolates. Both α-toxin and PVL were found to elicit strong upregulation of chemokines in the lung epithelium which resulted

in increased neutrophil chemotaxis. This demonstrates that toxin-mediated pathology is not limited to cytolytic events. Taken together, the data demonstrate a dual role for the toxins involving both cytolytic and chemotactic responses, and underscore the importance of targeting multiple toxins and inflammatory pathways in the treatment of severe *S. aureus* pneumonia (Figure 14). The study in **paper III** mainly focused on understanding toxin-mediated pathology under well-defined conditions using a human 3D lung tissue model, and which may not necessarily reflect the full course of infection as live bacterial infection may trigger many different pathways in terms of adaptation, spatial distribution, and interactions with the host. Hence, further experiment's focusing on long-term live infections on tissue models made with innate immune cells can open up new avenues for detailed studies of host-pathogen interactions, testing of novel antibiotics treatment and intervention strategies in a human tissue-like setting.

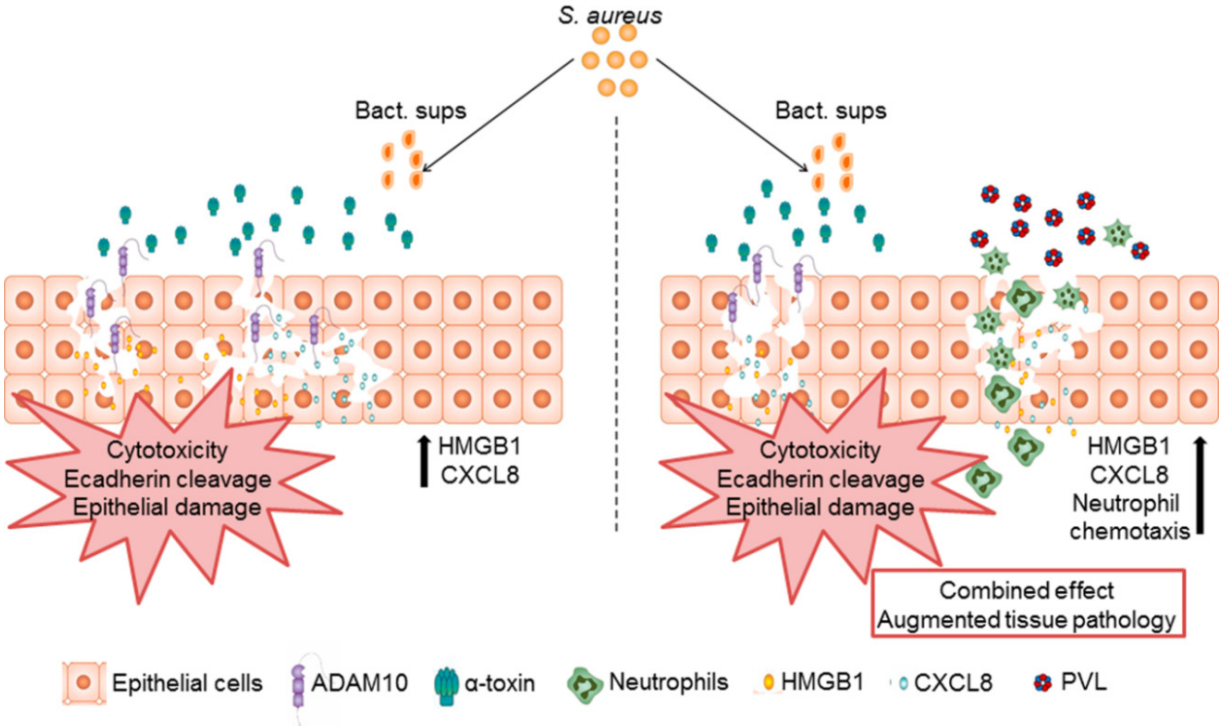


Figure 14: Schematic presentations of the results in **paper III.**

4.4 PHENOTYPE SWITCH IN *S. AUREUS* ST22 STRAINS CAUSING SKIN INFECTIONS IS REGULATED BY A POINT MUTATION OF RECEPTOR HISTIDINE KINASE AGRC

Staphylococcus aureus is one of the major causes of skin and soft tissue infections. Epidemic MRSA-15 (EMRSA-15), belonging to sequence type ST22 is one of the most critically expanding MRSA clone world-wide. Recent studies have identified ST22 as most markedly expanding MRSA clone in Europe (236). Apart from that in countries like India where the prevalence of MRSA is high, ST22 MRSA strains are replacing the previously

circulating HA-MRSA strains in a hospital environment (221). Several studies have reported PVL positive ST22 strains being associated with skin infections (237-239) as well as outbreaks of neonatal infections, which are particularly concerning (240). *S. aureus* expresses a wide array of virulence factors, including a plethora of secreted toxins and a broad range of cell surface virulence factors implicated in pathogenesis during infections. The expression of these virulence factors is under the control of the accessory gene regulator composed of Agr A, B, C and D domains (138). RNA III, the effector RNA molecule of the Agr system governs the expression of multiple genes, typically high RNAIII expression results in reduced expression of cell surface proteins (115).

Previous studies, in particular, **paper II** and **paper III** have focused on *S. aureus* exotoxin-mediated virulence properties and its contribution towards tissue pathology. In this study (**paper IV**) the focus shifted to understand the role of Agr system a global virulence regulator of *S. aureus* in determining the virulence response profile. The reason for this is that we identified a single amino acid substitution in *agrC* was responsible for starkly different phenotypes, e.g. a cytotoxic vs. persistent phenotype in genetically highly related ST22 clones causing skin infections.

4.4.1 Distinct phenotypical profiles of ST22 strains due to a single point mutation in agrC

In **paper I** we found that strains belonging to ST22 type formed the dominant clone both among carriers and disease isolates making up 28% of the total strain collection. Also, our study in which we defined robust functional responses of community *S. aureus* strains with distinct toxin profiles (**paper II)** identified different phenotypic virulence profiles in PBMC exposed to strains from a different genetic background as well as within the same ST type, including ST22 strains. Here we focused on two ST22 MRSA skin infection isolates, M37 isolated from a recurrent skin infection and PUNE08 from an acute skin and soft tissue infection which displayed identical virulence gene profile as determined by microarray analysis in **paper I,** and yet revealed starkly different phenotypic response profile in PBMC. M37 induced proliferation, whereas PUNE08 was cytotoxic towards PBMC (Figure 1A; **paper IV**). In **paper II** the difference in phenotypic response profile was linked to the Agr type, and considering the key regulatory effect of Agr system on virulence gene expression, sequence analysis of the whole Agr region was performed. Sequence analyses identified a single point mutation in *agrC* at nucleotide position 667 (amino acid position: 223) in strain M37, leading to an amino acid substitution from tyrosine (Y) to cysteine (C). Molecular modelling and biophysical studies on these point mutation identified that amino acid substitution in the AgrC (Y223C) affects the oligomeric assembly of AgrC due to aggregation in solution and leads to destabilization of AgrC-AgrA interaction (Figure 1F and 1G; **paper IV**). Furthermore to confirm that the observed phenotypic difference is linked to the point

mutation, allelic replacement mutants (Y223C and C223Y) were generated in M37 and PUNE08 strains (Figure 2A; **paper IV**). A distinct phenotypic switch was observed when the wild-type strains and their respective mutants were tested for their proliferative activity and cytotoxicity in different cell types (Figure 2C and 2D; **paper IV**). PUNE08 wt strain switched from cytotoxic to a proliferative phenotype in the mutant PUNE08 agrC-Y223C, whereas opposite responses were noted for M37 and its mutant.

To test the impact of such a phenotypic switch due to the *agrC* mutation, for infection outcome a series of experiments including virulence gene expression measurement, infection of keratinocytes, infection of human organotypic skin tissue models as well as *in vivo* infection using a murine skin infection model, were performed. The results demonstrated that Tyrosine at $AgrC_{223}$ induces a higher exotoxin expression profile resulting in an increased cytotoxicity and severe epithelial disruption and tissue damage in both *in-vitro* and *in vivo* infection experiments. In contrast, strains harboring Cysteine at $AgrC_{223}$ demonstrated upregulation of genes encoding cell surface virulence factors, resulting in an increased adherence to and internalization into keratinocytes, and caused negligible epithelial damage *in vitro* as well as *in vivo* (Figure 3, 4 and 6; **paper IV**). Taken together the results demonstrated that strains harboring Cysteine at $AgrC_{223}$ showed characteristic features of a colonizing/persistent phenotype (241, 242), while the cytotoxic activity is dependent on Tyrosine at $AgrC_{223}$.

4.4.2 Cytotoxic vs persistence phenotype in a human 3D skin model

During the infection process of keratinocytes or human 3D skin model cytotoxic strains induced upregulation of Caspase 1 and IL1β indicating inflammasome activation. In contrast, reduced caspase 1 and IL1β levels were observed in cells and tissue models infected with colonizing/persistent strains. The persistent strains showed greater co-localization with LAMP1 as well as LC3-positive autophagic compartments as compared to cytotoxic strains, thus differing from the cytotoxic strains in terms of its intracellular locality (Figure 5A-5J; **paper IV**). Persistent strains also induced significant induction of autophagy as compared to cytotoxic strains (Figure 5F and 5I; **paper IV**). Inflammasome activation is considered as one of the major mechanisms for *S. aureus* clearance in skin (166, 243) and recent studies have shown that autophagy induction has the capacity to consume the inflammasome components (244, 245). Along the same line, our data indicate that inflammasome-mediated protective host responses are less pronounced in infections from persistent strains as compared to cytotoxic strains. Previous studies have shown that a common feature of persistent *S. aureus* strains is resistance against antibiotics and antimicrobial peptides, such as LL37 which is abundantly expressed in the skin where it contributes towards the first line of defense against bacterial infections (246, 247). LL-37 was readily detectable in organotypic skin tissues infected with persistent M37 wt and PUNE08 agrC-Y223C strains,

but not in tissue infected with the cytotoxic strains PUNE08 and M37 agrC-C223Y, which is likely a consequence of the greater disruption of the tissue (Figure 15). Determination of minimum inhibitory concentrations (MIC) of LL-37 revealed that persistent M37 wt and PUNE08 agrC-Y223C strains were highly resistant against LL37 mediated killing as compared to the cytotoxic PUNE08 wt and M37 agrC-C223Y strains (Figure 15 and Figure 6K; **paper IV**). The mechanism underlying this difference in susceptibility towards LL-37 has not yet been explored and warrants further studies.

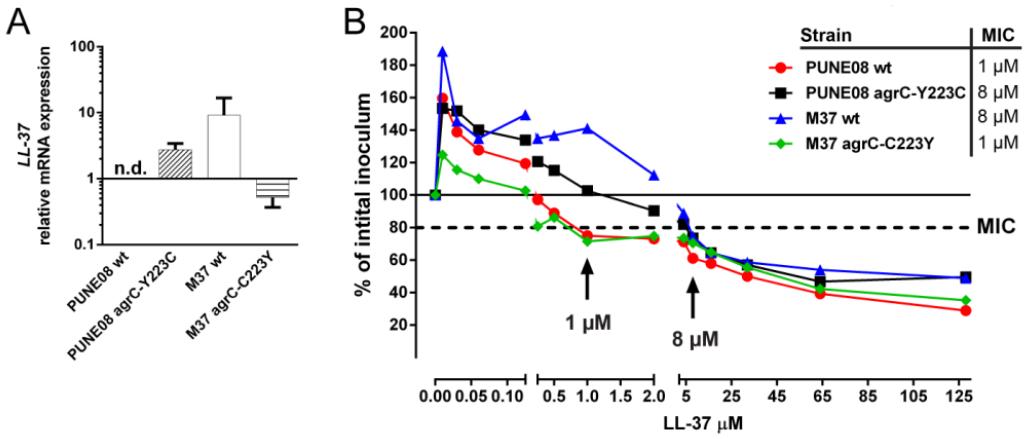


Figure 15: A) Relative mRNA expression of *LL-37* during the 24 h skin tissue model infection. The data represent the mean values ± SD from three independent infections (n=3). (B) Representative graph showing the effect of LL-37 on *S. aureus* strains incubated with different concentrations as indicated. The data shows the percentage of initial inoculum determined by CFU analysis in relation to peptide-free control cultures.

In conclusion, our study on ST22 *S. aureus* strains shows that natural AgrC variants with starkly different phenotypes exists. Similarly, clinical isolates exhibiting *agr*-defective phenotypes due to mutation or dysfunction of the Agr system have been reported to be beneficial for survival and persistence within the host (244, 248, 249) . Both AgrC and AgrA regions are identified as hot spots for mutations leading to dysfunction of the Agr system (250). Notably, G55R in AgrC and L181I amino acid substitutions are observed in CC30 and ST80 lineages respectively, which are found to contribute towards decreased virulence and increased colonization abilities in these strains (249, 251). However, the point mutation identified in this study has not been reported previously. We link this phenotype switch to a naturally occurring specific mutation of receptor histidine kinase AgrC. Our data show that cytotoxic strains elicited severe tissue pathology in both *in vitro* human skin tissue model as well as *in vivo* murine model. Similarly, persistent intercellular strains can exploit the phagolysosome and autophagy compartments for their intercellular survival (Figure 16).The phenotypic switch to a persistent clone is inherently associated with resistance to antimicrobials, which will starkly reduce the antibiotic treatment efficacy. Whether this phenotypic switch represents a mechanism that provide a reservoir for dissemination and

become difficult to eradicate seems likely but remains to be proven. Similarly, the clinical relevance of strains with persistent phenotype causing recurrent skin infection, while cytotoxic strains causing acute skin infections needs to be addressed using a larger cohort of well-defined strains with appropriate clinical data on patients.

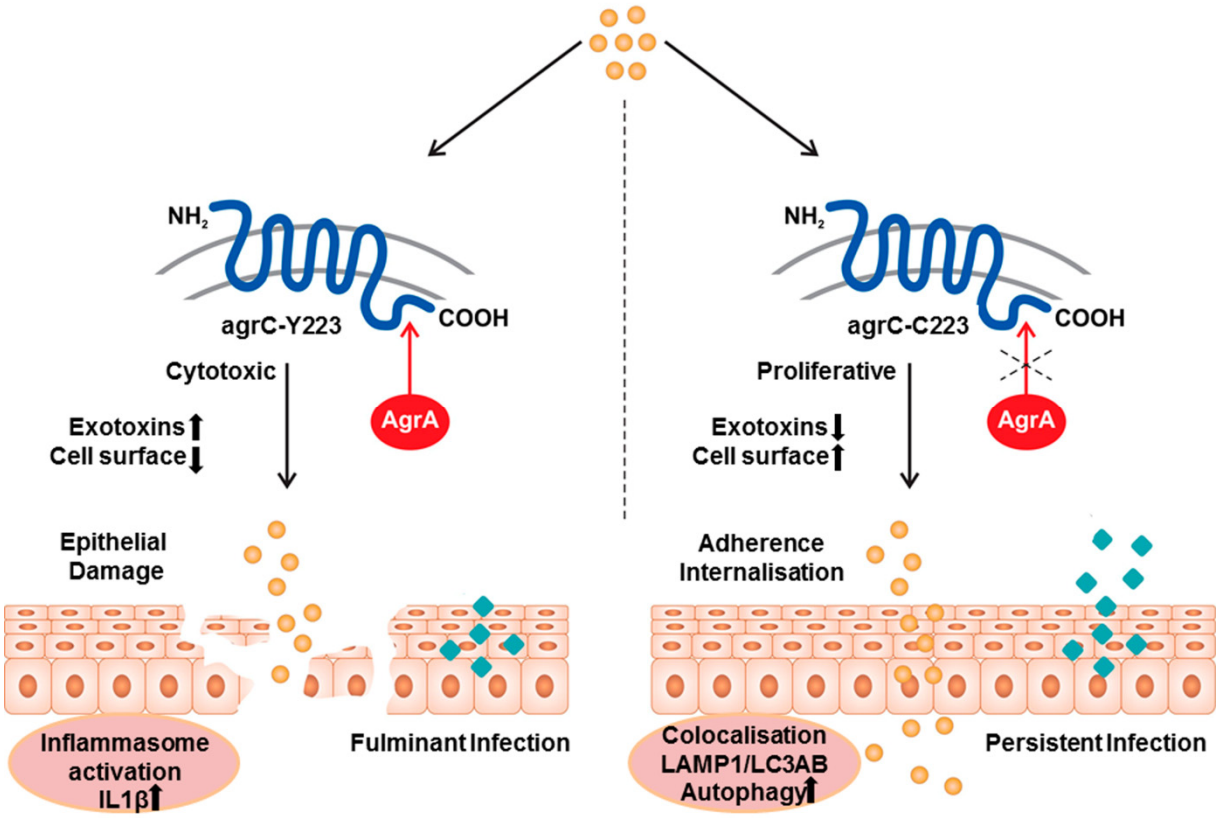


Figure 16: Schematic presentations of the results in **paper IV.**

5 CONCLUDING REMARKS AND FUTURE ASPECTS

S. aureus represents a significant health threat in both developing and developed countries. The incidences of these infections are rising, and unusually severe pathological signs are associated with certain *S. aureus* strains. Here we use clinical isolates with identical or related genetic background but with varying toxin profiles in clinically relevant human cell and tissue model systems to increase our understanding of the role of specific staphylococcal toxins in the pathologic events leading to the destructive infections in lung and skin.

Paper I

- Indian *S. aureus* strains were highly diverse and heterogeneous with all major clonal complexes circulating in the population.
- The majority of the isolates were positive for *PVL* and *egc* genes along with combination of many other virulence factors

The probability of MSSA conversion to MRSA is high in India due to rampant consumption of antibiotics. There lies a possibility of PVL positive MSSA strains acquiring SCC*mec* element to become PVL positive MRSA resulting in emergence of new variants. Hence prospective active surveillance studies including detailed molecular characterization of larger strain collections need to be conducted.

Paper II

- Community *S. aureus* isolates reveal robust functional response profiles, either proliferative or cytotoxic, in PBMC.
- High α-toxin levels correlate with the cytotoxicity against PBMC demonstrating the importance of assessing concentrations and not solely presence of genes.
- Significant association between proliferative or cytotoxic profiles and *agr* type

The clinical relevance of these distinct functional profiles remains to be shown, as well as whether these phenotypic response profiles reflect specific pathotypes contributing to specific disease manifestation needs to be addressed. Another unexplored question is to understand the significance of expression levels and combined effects of superantigens and cytotoxins systemically as well as locally.

Paper III

- Severe lung tissue pathology is associated with high levels of both α-toxin and PVL
- Both α-toxin and PVL were found to elicit strong upregulation of chemokines in the lung epithelium which resulted in increased neutrophil chemotaxis

- Data demonstrate a dual role for the toxins involving both cytolytic and chemotactic responses and underscore the importance of targeting multiple toxins and inflammatory pathways in the treatment of severe *S. aureus* pneumonia.

The human 3D lung tissue model represents a powerful tool allowing for detailed studies of host-pathogen interactions and testing of novel intervention studies in a human tissue like setting. Further optimization of the tissue model to include innate immune cells during relatively long-term infection studies to understand the adaptation, spatial distribution of the bacteria and the host factors during pathological events needs to be explored.

Paper IV

- Naturally occurring single amino acid variant at $AgrC_{223}$ of ST22 MRSA strains determines cytotoxic or persistence virulence phenotypes
- Y223C substitution causes aggregation and destabilization of AgrC-AgrA interaction
- High cytotoxicity, induced inflammasome activation, and severe skin pathology is linked to Tyrosine in $AgrC_{223}$
- Cysteine in $AgrC_{223}$ determines a persistence phenotype with limited tissue pathology, but enhanced exploitation of autophagy and increased resistance to LL-37

The phenotypic switch may represent a mechanism for which new variants emerge and may contribute to the ongoing persistence of particularly successful clones, such as the ST22; however, this remains to be proven. Although we provide molecular and biochemical data on specific mutation leading to a diverse signaling capacity, differential virulence gene profiles and different infection outcome, the triggers driving these events are unclear. Further studies to identify if this mechanism is evolutionary occurring and actively driven by *S. aureus* or caused by the human host immune compartments warrant further investigations.

6 ACKNOWLEDGEMENTS

My sincere thanks to **Karolinska Institutet (Center for Infectious Medicine),** where the projects included in this thesis were performed and all the **funding agencies** that supported my Ph.D. studies. I am grateful for providing me with this opportunity to work in an scientifically inspiring and a multi-cultural work environment.

Firstly I wish to thank my main supervisor, **Anna Norrby-Teglund** for giving me an opportunity to pursue Ph.D. studies under her guidance, welcoming me to Stockholm and into her research group. You have been wonderful and enthusiastic all throughout these years. I am grateful to your entire positive attitude towards research and for all the scientific inputs. I am amazed by your multitasking abilities and getting things done in a timely manner. It has been a great privilege to be your student over these years.

Mattias Svensson, my co-supervisor, thanks for all the scientific insights, support, and guidance. You have been very generous and kind enough in offering assistance during shifting apartments in my initial days in Stockholm. Thank you for making me feel comfortable during my initial years and always being there to listen to all my relevant/irrelevant ideas with great patience and enthusiasm.

Gayathri Arakere, I am greatful to you for introducing me to *S. aureus* world by giving a good strong foundation, inspiring me each day into research through your charming personality and sheer dedication towards science. Thank you for being a wonderful mentor, well-wisher, and great family friend.

To all my present group members: It has been a great joy to know and work with you all. **Nikolai**, you are a great colleague and a wonderful friend to have. Thank you for all the great science/non-science discussions, inputs we had, it was always such a pleasure to talk to you. I am grateful to you for enduring my all work related frustrations and giving some great timely suggestions. **Julia**, thanks for “Uidiiiii-Ohrschläpprli” and, for keeping the lab environment lively, for organizing our Friday food and dance parties. **Johanna**, thanks for being the best office neighbor and for your great one liners!!!. **Helena**, thanks for tolerating me as your master project supervisor.

To my past group members: **Linda**, I still miss you for all your laughter and being an energy bundle, for bringing great joy and spreading happiness around. **Erika**, thanks for introducing me to entire CIM and giving me a detailed tour of the green lab, helping me with the initial days of apartment hunting. **Anna Linner,** thanks for being a sweet and nice colleague. **Axana**, thanks for starting Staph experiments and giving me a good start into my Ph.D. studies. **Janos**, thanks for all the nice discussion we had in the green lab.

Mattias Svensson group: **Puran**, thank you for your interest in the Staph project, for all live the imaging adventures, all the wonderful discussions we had through these years. **Anh Thu**, thank you for introducing me to the world of tissue modelling and for being such a warm person and a kind friend. **Julius**, for being a wonderful green lab colleague. **Sofia** and **Magda**, for sharing some FACS antibodies.

Gayathri Arakere's lab members: **Sushma** and **Savitha**, thank you for being a good support system during all good and bad times in SDTC. Many thanks to **Gen. Raghunath**, **Gangadhar**, **Dakshayini**.

For all my collaborators: **Prof. Tim Foster**, thank you for your keen interest in my project and hosting me in your lab for two months. It was a great learning experience. You were very generous and kind to show me around and making me feel so comfortable during my stay in Dublin. Thank you **Ian Monk** for your patience and for teaching me the techniques of generating *S. aureus* mutants. It has been a wonderful experience working along with you. **Simon**, thank you for all insightful discussions in the lab and dinner outings during ISSSI 2014, Chicago.

Prof. Jerome Etienne, thank you for your overall interest in my research career, for your contribution, and teaching me some tricks/tips of writing epidemiological paper and being such wonderful host during ISSSI 2012. **Michele Bes**, thank you for providing the microarray data on Indian *S. auerus* strains

Prof. Francois Vandenesch, thank you for the fruitful collaborations, providing us with the toxin quantification data, nice collection of Pneumonia strains and sharing crucial, *S. aureus* mutant strains which have made a big impact on our study.

Prof. Gerard Lena, thank you for a wonderful collaboration, scientific and technical inputs.

I am grateful to **Malak Kotb** for being a great collaborator and her group members **Santosh** and **Karthickeyan** for providing us with *in-vivo* data.

Thank you, **Prof. B. Gopal**, for allowing to me to use your lab and facilities during my research stays in India, for being a wonderful collaborator and helping us out with structural and biophysical studies, all his group members especially **Disha Mohan B** for performing all the experiments.

Thank you **Willem J. B. van Wamel** for performing and providing Luminex studies and data on *S. aureus* toxins. **Prof. Angela Kearns**, thank you for providing us ST22 reference strain and its sequence data.

Many thanks to **Margit** for being a friendly and a cheerful administrator for CIM. Thank you, **Lena**, **Anette**, **Elizabeth**, and **Hernan** for taking care of all laboratory stuff and helping CIM run in an organized manner.

I would like to thank all past and present members of CIM- **Emma**, **Su**, **Pär**, **Edwin H**, **Ginny**, **Marton, David, Joana, Benedict, Monika, Nicole, Christine, Egle, Luca, Venkat, Sushil, Anette, Heinrich, Martha, Vivian, Ebba, Matthias, Martin, Senait, Marianne, Vikki, Erna, Lisa, Steph, Jakob, Renata, Hongya, Dominiq, Sam, Lakshmikanth, Steve, Margit, Edwin L, Sebastian, Avinash, Salah, Sayma, Jubyer, Anna Rao, Aline, Moni**, all other PhD students and Post Docs in the Department for making CIM a nice working place. A special thanks to all the **Green Lab Members**.

Thank you **Sush-Johan** for organizing nice dinners, parties and all the mid-summer celebrations, songs, Swedish games, friendly talks.

Jagadeesh-Rekha, thank you for being great friends, apartment neighbors, thank you for all late night chats, foods, and helping me out in all aspects whenever required.

Ramana-Sandhya, thank you for being kind friends, taking good care of me during my initial years in Stockholm, a great source of information and for all your honest suggestions. **Sandhya** for all wonderful food, especially the best Pappu ever.

Kedar-Shruthi, thank you for all the cherishing memories of childhood and native, all great outings, making us feel at home while being away from home. You are my extended Mangalorean family in Stockholm.

Sougat-Beauty, Srinivas-Sindhu, Srinivas Reddy, Sridharan Ganesan, Lalit-Deepti, thank you for being wonderful KI Huddinge (Flemingsberg) gang. **Jyothi Prakash, Mahesh, Shridhar-Smitha**, our Stockholm Kannada-Mangalore friends, for all the movies and awesome fun times we had together.

Thank you all my Indian friends: **Harsha-Shreya, Sajit-Shawon, Nilesh, Shahul, Suhas-Ashwini, Pradeep, Adithya, Shreemanta, Martin, Daya, Usman, Kishan** thank you for all the parties, food, Indian festival celebrations.

Huge thanks to 'Dushtas'- **Pradeep, Karthik-Nandini, Viveka-Vathsala, Vikram-Rachna, Shaithilya-Swathi,** for being an integral part of my life. You all are my wonderful lifelong friends and family.

My beloved family: Thank you **Somayaji Uncle-Sumedha Aunty** for being a wonderful support system for our family and for all your kindness. **Srinivas Mama** for inspiring and motivating me to take up basic life sciences/research.

Thank you **Ajja, Akka-Bhava, Sarvesh Mama-Varalaxmi Atte, Murthy Mama-Padmaja Atte, Atte-Mava** for being a great caring and loving family, for being there for me whenever needed.

Thank you **Amma** you are my great source of inspiration, strength and courage and also for just being there for me always. **Bhavya**, my loving wife thank you for your patience, understanding, and all the support. Thank you for pitching in and contributing towards making the art work of the thesis. I am so lucky and happy to share my life with you.

This thesis is dedicated to my family, friends, colleagues and everyone who have contributed and supported me directly or indirectly all these years in this journey. I would like to take this chance to thank everyone who has helped me all these years during my stay here in Stockholm, Sweden.

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