Molecular effects of *Staphylococcus aureus* toxins and their contribution to pathogenesis

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Contents

Abbreviations

Abbreviation	Meaning
ACME	Arginine catabolic element
ADAM10	Disintegrin and metalloprotease domain-containing protein 10
agr	Accessory gene regulator
APC	Professional antigen presenting cell
CA	Community associated
CD	Cluster of differentiation
CDR2	Complementary determining region 2
DC	Dendritic cell
Dsg1	Desmoglein 1
EC	Extracellular cadherin domain
ET	Exfoliative toxin
ETL	Enterotoxin-like toxin
FoxP3	Forkhead box P3
FR3	Framework region 3
HA	Hosptial associated
HL	Hemolysin
HLA	Human leukocyte antigen
HMGB1	High-mobility group protein B1
IgA	Immunoglobulin A
IL	Interleukin
Isd	Iron-regulated surface determinant system
MHC	Major histocompatibility complex
MRSA	Methicillin resistant Staphylococcus aureus
MΦ	Macrophage
NK	Natural killer cell
NLRP3	Nucleotide-binding domain and leucine-rich repeat containing gene family, pyrin domain containing 3 protein
OB	Oligonucleotide-oligosaccharide binding domain
PMN	Polymorphonuclear cell
Poly(I:C)	Polyinosinic:polycytidylic acid
PSM	Phenol soluble modulin
PVL	Panton-Valentine leukocidin
S.aureus	*Staphylococcus aureus*
SCC	Staphylococcal chromosome casette
SE	Staphylococcal enterotoxin
SEl	Staphylococcal enterotoxin-like toxin
SSL	Superantigen-like proteins
SSSS	Staphylococcal scaled-skin syndrome
TCR	T cell receptor
TGFβ	Tumor growth factor β
TNF	Tumor necrosis factor
T_{reg}	Regulatory T cell
TSST-1	Toxic shock syndrome toxin 1

Abstract

The gram-positive bacterium *Staphylococcus aureus* (*S.aureus*) is carried by up to 30% of the population and can cause disease, including skin and soft tissue infections. But also more severe symptoms like abscesses, sepsis and pneumonia were described. In the past, pathogenic *S.aureus* strains were mainly present in hospitals. However, since the beginning of the 1990s *S.aureus* strains emerged which can also be found outside of hospitals. The success of such community-associated strains is based on a high expression of a broad variety of virulence factors including genes for antibiotic resistance and exotoxins. Such exotoxins can be subdivided into three groups: The first group consists of hemolysins which have the ability to lyze blood cells, namely erythrocytes, phagocytes and lymphocytes. This effect is based on the formation of pores in the membrane of the affected cell, which disturbs the ion metabolism and results in depletion of small molecules like ATP. Lysis of erythrocytes could hereby help *S.aureus* to attain iron out of the erythrocyte-derived proteins hemoglobin and myoglobin. Lysis of immune cells could provide a mechanism of immune evasion, since phagocytes and lymphocytes are required for an efficient clearance of *S.aureus* infections. The second group comprises the superantigens. Staphylococcal superantigens cross-link Vβ chains of distinct T-cell receptor variants present on $CD4^+$ T cells with major histocompatibility complex (MHC) class II molecules of professional antigen presenting cells (APC). This cross-linking does not require antigen specifity of the MHC class II molecules and can result in activation in up to 20% of the overall T cell population. T cell activation is followed subsequently by anergy and depletion of the respective T cell clones, which could facilitate *S.aureus* infections because T cells play an important role in clearance of bacterial infections. The third group is the group of exfoliative toxins, which can cause proteolytic cleavage of the cadherin desmoglein-1 (Dsg1). Dsg1 is responsible for cell-cell interactions within the skin and its cleavage results in disruption of the skin barrier. The disruption of the skin barrier was described to be an important factor for a successful establishment of an infection by *S.aureus*.

Taken together, the described staphylococcal exotoxins are important virulence factors enhancing the ability of *S.aureus* to establish and maintain an infection. Although hemolysins, superantigens and exfoliative toxins require further investigations to determine the impact of the toxins more in detail, this knowledge could be used in the future to develop drugs and vaccines for the treatment or prevention of *S.aureus* infections.

Introduction

Development of community associated *S.aureus* strains

Since the discovery of penicillin in 1928 by Alexander Fleming many diseases caused by bacteria became treatable [1]. However, the control of infections by *Staphylococcus aureus* (*S.aureus*), a gram-positive coccal bacterium, was only partially achieved: Already in 1942 the first penicillin-resistant strains of *S.aureus* were described and the number of penicillin resistant strains permanently increased in the following decades [2]. Also the introduction of methicillin in 1959 was not very fruitful considering that methicillin resistant strains were already described two years after the introduction of the antibiotic [2]. Methicillin resistant *S.aureus* (MRSA) strains subsequently spread in hospitals worldwide [2] establishing a pandemic which is still present today. Besides these emerging health care-associated MRSA (HA-MRSA) strains, in the beginning of the 1990s MRSA strains were described which were not restricted to hospitals but were able to infect healthy individuals in the community [3]. Unlike the HA-MRSA strains, community-associated MRSA strains (CA-MRSA) could infect individuals without any need for prior risk predisposition factors or illnesses [4]. To be able to establish successful infection in healthy individuals, such CA-MRSA strains required additional virulence factors and the ability to spread efficiently [4]. And indeed, CA-MRSA strains like USA300, the most prevalent strain in the USA have been shown to express virulence factors like PVL, α-hemolysin (α-HL) or phenol soluble modulins (PSM) enhancing the strains capacities of colonization, infection and transmission [5, 6,7,8].

The success of *S.aureus* is also reflected in the present staphylococcal colonization rates: A study done between 2001 and 2004 revealed that 30% of the US population was positive for *S.aureus* while 1.5% carried a MRSA strain with increasing prevalence [9]. In certain risk groups MRSA is even more prevalent: Besides of hospital patients (64%) [10] in the US for example also prison inmates (2.5-5.9%) [11], military personnel (3%) [12], athletes (1-25%) [13], children in day care (3-24%) [14] and men who have sex with men (2.2-25,7%)[15] are positive for one or more MRSA strains.

Symptoms and impact of CA-MRSA infections

A colonization rate of 30% of the population indicates that *S.aureus* is mostly commensal and hence, causing usually no pathophysiologic symptoms. However, especially MRSA strains can potentially cause disease including a broad range of clinical symptoms: The most abundant symptoms of MRSA infections are skin and soft tissue infections, which can be found in approximately 90% of the diseased patients depending on the immune status of the patient and the virulence of the MRSA strain [16]. MRSA related skin and soft tissue infections can cause mild symptoms like cellulitis, furuncles or rashes, but also severe complications can occur [17]. Especially the abundant CA-MRSA strains have also been shown to cause increasingly more severe and even life-threatening symptoms including abscesses, sepsis, myositis, necrotizing pneumonia and fasciitis [18, 19, 20]. Also a pathophysiological state described as toxic shock syndrome has been described after MRSA infection, including symptoms like edema, desquamination, sore throat, myalgias, fever, scarlatiniform rashes and multiple organ failure [21,22], resulting in a 3-5% mortality rate in children [23]. Altogether, this causes an annually mortality rate of 6.3 per 100.000 persons caused by MRSA [24].

The increased prevalence of CA-MRSA associated disease is also causing a growing financial burden for the national health systems: Alone in the USA the costs for treatment and control of *S.aureus* infections are approximately 14.5 billion $ per year [25].

Staphylococcal toxins

The symptoms associated with *S.aureus* infections are caused by a broad range of exotoxins, all potentially helping to establish colonization and survival or to make nutrients accessible [8]. Hereby the toxins can be divided in three different groups, depending on their structure and targets. The first group consists of hemolysins, of which some are produced by almost all *S.aureus* strains. Hemolysins target cells of the immune system or erythrocytes and are causing lysis of these cells [8]. The second group includes the superantigens, toxins which have been described to cause T cell anergy and clonal depletion of different subsets of T cells as a long-term effect [26]. The third group comprises the exfoliative toxins which can cause disruption of epidermal cell layers [27].

These toxins are also key molecules of the success of the emerging CA-MRSA strains: Comparison between HA-MRSA and CA-MRSA revealed that production levels of many toxins like the hemolysins are significantly higher in CA-MRSA strains [19]. An extreme example for this is the expression of PVL, which is produced in high levels by the CA-MRSA strains USA300 and USA1000, but not at all by the most common HA-MRSA strains USA100, USA200 and USA500 [6].

Besides of the toxins mentioned above, also various other molecules are secreted by *S.aureus* including different proteases, complement binding proteins or molecules involved in biofilm formation, all potentially increasing the fitness of *S.aureus* by interactions with various human proteins [28,29,30]. However, these molecules do not directly result in cell death and thus, are not considered as toxins within this work.

Prevalent strains and their characterization

Nowadays, CA-MRSA is pandemic all over the world [19]. Hereby the predominance of certain CA-MRSA strains varies depending on the geographical area [5] (**Figure 1**). The classification of these strains is dependent on the presence of virulence factors like PVL, α-HL or PSM [5, 7]. In the USA, the CA-MRSA strain USA300 is the most abundant strain, causing more than 80% of the MRSA infections [31]. Although originating from the low virulence and methicillin-sensitive *S.aureus* (MSSA) subtype CC8 [32], USA300 combines a high virulence with an efficient transmissibility and colonization [6]. First designated USA500, it acquired staphylococcal chromosome cassettes (SSC), genomic islands including the genes *ccrA* and *ccrB* [33]. The integrases encoded by *ccrA* and *ccrB* allow a fast acquisition of genes coding for virulence factors from other strains [33]. An important example for this is SCCmec type IV presumably acquired by MSSA subtype CC8 by horizontal gene transfer originating from *S.epidermidis* [34]. SCCmec type IV includes several genes for antibiotic resistance like *mecA*, an enzyme encoding for the “penicillin binding protein 2a” which inhibits several β-lactam antibiotics like methicillin or penicillin [33]. This multiresistant CA-MRSA strain developed furthermore an increased cytolytic potential by a higher expression of α-HL and PSM compared to the original CC8 strain. After subsequent acquisition of virulence factors like PVL and arginin catabolic elements (ACME) by USA500 the virulence increased even more, forming the strain USA300 [33, 35].

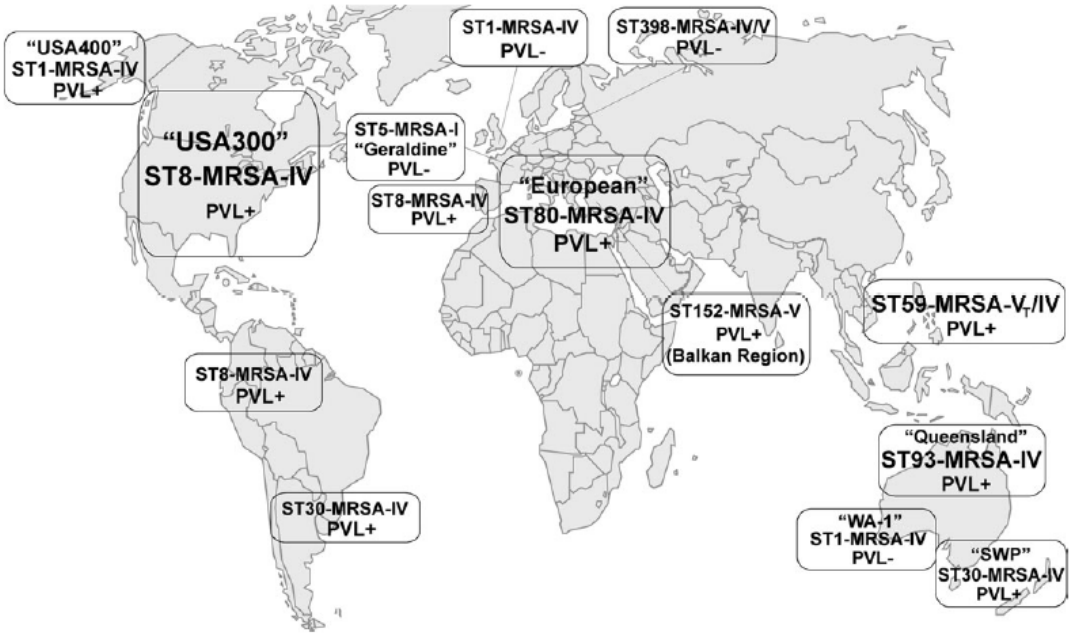

Figure 1: Worldwide distribution of CA-MRSA strains, showing the most dominant CA-MRSA strains. Since the presence of PVL in a CA-MRSA strain indicates high virulence and is important for the characterization of a strain, PVL presence is indicated for every strain [adapted from 5].

Aim of this work

CA-MRSA strains are becoming an emerging health care problem worldwide resulting in an increased prevalence of *S.aureus* infections and subsequent complications. Such complications can be in case of necrotizing fasciitis, pneumonia or the development of a toxic shock syndrome even fatal. The increasing success of CA-MRSA strains like the highly virulent USA300 strain is hereby based on the rapid formation of resistance against antibiotics and expression of other virulence factors including a variety of toxins contributing to immune evasion of the strain [16]. Since predominant CA-MRSA strains like USA300 or ST80 are resistant to the most of the common antibiotics [2], new therapies against CA-MRSA are required to reduce the disease and financial burden and help to control the emerging CA-MRSA pandemic. Promising targets for therapeutic interventions and the development of vaccines are hereby the toxins themselves, since these have been shown to be crucial factors for the high virulence of CA-MRSA strains [19].

This work aims to review the current literature about molecular mechanisms of staphylococcal toxins and their contribution to pathogenesis. The gained knowledge could be potentially used in the future for the development of vaccines or new therapeutic interventions interfering with staphylococcal toxin effects.

Molecular effects of *S.aureus* toxins and their contribution to pathogenesis

S.aureus is expressing a broad range of different virulence factors enhancing its ability to establish and maintain infection in humans. Such virulence factors include also several exotoxins which can be divided into three subgroups: As first group, *S.aureus* expresses hemolysins which act on cell membranes forming lytic pores [19, 37]. The second class is described as superantigens, which cause a long-term dysfunction of specific T cells [38]. The third group consists of exfoliative toxins involved in the development of the staphylococcal scaled-skin syndrome (SSSS) and includes several proteases responsible for the disruption on skin integrity [27]. Importantly, the expression of nearly all virulence factors secreted by *S.aureus* are depended on the activity of the accessory gene regulator *agr*, a regulon responsible for the alteration of gene expression in response to immune reactions and dependent on quorum sensing [39]. Especially CA-MRSA strains like USA300 have been shown to have strong activities of *agr* activity, resulting in high levels of produced exotoxins [39].

The following part aims to list the known staphylococcal exotoxins and to explain their molecular modes of action. At the end of each toxin subgroup a short tabular summary about the toxins belonging to the respective group, their prevalence within clinical *S.aureus* isolates and their mechanism of action is given.

Pore forming hemolysins

Pore forming hemolysins are produced by almost all human pathogenic *S.aureus* strains. However, a large variation of expression levels between *S.aureus* strains was detected, whereas especially CA-MRSA strains like USA 300 produce high levels of PVL, α-HL and PSM **(table 1)** [6, 19, 40]. As the name indicates, hemolysins are causing lysis and subsequent death of blood cells [8, 40]. Hereby primarily erythrocytes and leukocytes are affected, whereas the selectivity for a cell type is dependent on the lipid composition of the membrane and on other partially unknown receptors [8, 19, 41]. Lysis of cells by hemolysins is always based on the formation of a pore in the cell membrane of the affected cell, which can be based on a transmembrane β-barrel structure or on enzymatic cleavage of membrane lipids [37, 42]. Disruption of the membrane integrity and pore formation subsequently results in leakage of ions like K^+, Na^+ and Ca^{2+} and small molecules like ATP. [8, 37, 43]. Hereby especially the influx of Ca^{2+} into the cell can have serious side effects since Ca^{2+} is involved in a variety of signal cascade mechanisms [8]. An example for this is the α-HL activity on epithelial cells: Due to influx of Ca^{2+} into the cell and the associated osmotic stress, epithelial cell start swelling which results in the loss of the epithelial integrity and increased vascular permeability. An increased vascular permeability can subsequently result in the formation of an edema [8].

The following part should explain the different hemolysins more in detail with regard to their structure and mechanism of action.

α-hemolysin

α-HL is produced by >99% of the described *S.aureus* strains, including human pathogenic CA-MRSA strains like USA300 or USA400 [7, 44]. Although α-HL does not cause lysis of human neutrophils, α-HL cytotoxicity was proven on human cells including lymphocytes, monocytes, erythrocytes and epithelial cells [45, 46].

The α-HL monomer is a polypeptide with a length of 293 amino acids consists of approximately 65% β strand and 10% α-helical structures. These structures fold into a tertiary structure containing a cap domain important for oligomerization and pore formation, a rim domain which is involved in membrane binding and a prestem domain which is covered by the cap domain [8, 37, 47]. The toxicity of α-HL is based on the formation of a heptameric pore with an internal diameter of about 1-2nm [8, 47]. The process of pore formation includes overall three steps, namely binding of the α-HL monomers to the target membrane, heptamerization of the monomers into a prepore complex and transition of the prepore into a functional transmembrane pore [48]. Important for the binding of water-soluble α-HL monomers to the membrane bilayer is the rim region, which serves as a membrane anchor: The high presence of aromatic and cationic amino acid residues in the rim domain interact with the phospholipid head groups of the membrane lipids sphingomyelin, phosphatidylcholine and cholesterol and form hydrogen bonds [49]. Afterwards, heptamerization of the α-HL monomers into a non-lytic prepore occurs, generating a cylindrical structure with a diameter of approximately 100Å [37, 47] **(Figure 2)**. The molecular basis of the interactions responsible for heptamerization are up to date not completely understood, but it was supposed that amino acid residues in the twelve antiparallel β strands of the cap region play a role since mutation of certain residues inhibits oligomerization [37, 47, 50].

The final transition of the non-functional heptameric prepore into the functional lytic pore is mediated by interactions of the N-terminal latch regions and domains within the cap region with other integrated α-hemolysin monomers in close proximity, since mutations in these regions inhibited formation of lytic heptamers, but not of non-functional prepores [47, 51]. Interactions of the N-terminal latch and cap regions subsequently induce conformational changes of the prestem regions, which are built up of a β-hairpin structure including 37 amino acid residues. The seven prestem domains present in the prepore assemble into an amphiphilic 52Å long β barrel structure with a diameter of 26Å which penetrates the membrane bilayer [37, 52]. The β barrel is hereby stabilized by the interaction of the β-strands with each other via hydrogen bonds [52]. Furthermore, the uncharged residues of the β-barrel are responsible for integration of α-HL into the cell membrane bilayer by formation of numerous van der Waals interactions [49]. On the other side, the negatively charged residues of the β-hairpins point into the middle of the pore allowing influx of Ca^{2+} and Na^{+} ions and outflow of K^{+} ions or bigger molecules like hemoglobin and ATP [8, 37, 43].

Interestingly, also non-lytic activities of α-HL were described: *Inoshima et al*. discovered recently a mechanism of α-HL dependent cell injury which is not based on the formation of transmembrane pores: The group showed that α-HL can also bind to the surface protein “Disintegrin and metalloprotease domain-containing protein 10” (ADAM10) [53]. ADAM10 is a metalloprotease responsible for ectodomain cleavage of several integrins and of E-cadherin, a protein crucially required for adherens junctions within the vascular epithelium [53, 54]. Binding of α-HL to ADAM10 on alveolar epithelial cells causes an upregulation of ADAM10 activity which results in an increased cleavage of the targets of ADAM10. The subsequent loss of the integrity of the vascular epithelium caused by an increased cleavage of E-cadherin can subsequently promote inflammation and as a

result of this pneumonia, a symptom associated with *S.aureus* infections [53]. Moreover, an activation of the "Nucleotide-binding domain and leucine-rich repeat containing gene family, pyrin domain containing 3 protein" (NLRP3)-inflammasome by α-HL was shown by *Craven et al.*[55]. The NLRP3-inflammasome is a signaling complex mediating responses to endogenous danger signals including *S.aureus* derived molecules [55]. The activation of NLRP3-dependent signaling pathways subsequently promotes apoptosis by the activation of the protease caspase-1 and the release of pro-inflammatory molecules including "high-mobility group protein B1 (HMGB1) [55].

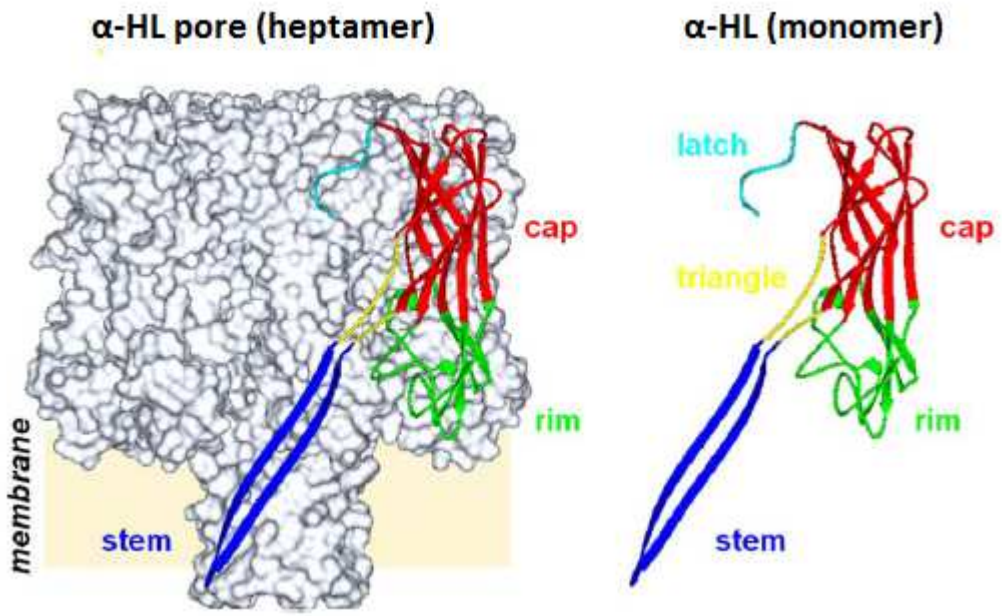

Figure 2: Assembled α-hemolysin pore (left), consisting of seven α-HL monomers (right). The pore consists of a stem, rim and cap region, furthermore of a flexible triangle and a latch region. Hereby the hydrophobic stem region represents the transmembrane domain, the rim region serves as a membrane anchor and the external cap region as stabilization and oligomerization domain [adapted from 37].

β-hemolysin

β-hemolysin[1] (β-HL) production was reported in 11% of the healthy carriers of *S.aureus* and 13% of the carriers with S.aureus septicemia [56]. β-HL is produced as a precursor protein with a molecular weight of approximately 39kDa and is encoded by the *hlb* gene [8]. After cleavage of the signal sequence, the mature β toxin with a molecular mass of approximately 29-30kDa is generated [57]. Mature β-HL acts on various cells including red blood cells [57], immune cells [42] or epithelial cells [8] by its activity as a sphingomyelinase [42][2]. Sphingomyelin, the target of β-HL is broadly abundant in the outer layer of the lipid bilayer of cell membranes and can make up to 15% of the total phospholipids of a cell, depending on the cell type and species [58]. Furthermore, lyso-phosphatidylcholine (LPC) was identified as a target for β-HL. LPC can make up to 3% of the lipids in cell membranes [58, 59].

[1] B hemolysin is in the literature also known as sphingomyelinase C or hot-cold hemolysin. To simplify matters in this work only the name β hemolysin is used.
[2] Hereby the sphingomyelinase activity is highest at temperatures below 37°C [8].

Crystallographic studies revealed that mature β-HL folds into a sandwich structure with four layers consisting of four α-helices and 14 β-strands forming two central sheets [42] (**Figure 3**). The catalytic centre of β-HL is in a hollow build by the central β-sheets and surrounded by several hydrophobic residues. These hydrophobic residues are important for hydrophobic interactions with the sphingosine part of sphingomyelin and β-HL [42]. Moreover, binding is supported by hydrogen bonds of three residues to the polar phosphorylcholine of the head group of sphingomyelin [42]. Cleavage of sphingomyelin by β-toxin is mediated by the active site of the enzyme (two His, one Asn and one Asp residue) which hydrolyzes the phosphodiester bond connecting the phosphorylcholine and the ceramide [42]. This reaction is stabilized by two Mg^{2+} molecules within the active centre of β-HL [42]. Degradation of sphingomyelin results in a release of phosphorylcholine and ceramide (N-acylsphingosine) [58]. The pores formed in that way cause loss of essential ions and depletion of ATP from cells, which can result in death of the respective cells [42, 60].

Furthermore, as a consequence of β-hemolysin activity ceramide is released as cleavage product. The release of ceramide serves as activation signal for neutrophils and macrophages by activation of the NFκb pathway and causes subsequently inflammation [61]. This was found to be associated with further sequelae like injury of lung or corneal epithelia [58, 62].

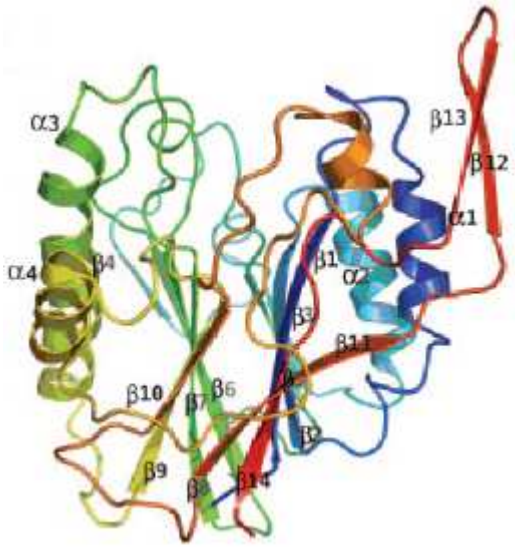

Figure 3: Ribbon model of β-HL, showing its structure consisting of four α-helices and 14 β-strands. The colors used in this model range from blue (N-terminus) to red (C-terminus). The active centre of β-HL is in the hollow in the middle of the depicted model [Adapted from 42].

γ- hemolysin, Panton-Valentine leukocidin and related toxins

Besides of α-HL, also eleven other β-barrel forming toxin components were described with a weight of 31-38kDa which have the potential to lyze leukocytes and erythrocytes [37, 63]. Several combinations of such toxins were described, whereas γ-hemolysin (γ -HL) consisting of LukF and Hlg2 and PVL consisting of LukF-PV and LukS-PV are the best described ones.

The toxicity of the closely related γ-HL and Panton-Valentine leukocidin (PVL) is based on the formation of a bicomponent transmembrane pore with β-barrel structure similar to α-HL [37, 64].

The proteins involved in this process are can be divided into 2 classes, depending on their amino acid sequence: The class of F proteins which consists of five proteins including LukF-PV and LukF on one hand, the class of S proteins with six known proteins comprising Hlg2 and LukS-PV. Interestingly, these proteins have some conserved regions in common with α-HL, whereas the homology of the F proteins with α-HL is approximately 30% and the homology of S proteins about 20% [64, 65]. Comparing the classes of S and F proteins with each other gives a homology of 30%, proteins within one class share about 70% of the amino acid sequence [65].

The assembly of a lytic octameric pore consisting of S and F proteins includes in general five steps[3]: First, the F protein binds to the membrane bilayer (1), then the S protein binds to the F protein forming a heterodimer (2). After reorientation of the dimer (3), four S/F-protein dimers form an octameric prepore (4). This prepore is transformed into the functional lytic pore after insertion of the amphipatic β-hairpins of the prestem domain into the membrane (5) (See as an example for this process **Figure 4**) [37, 64]. The following part gives a more detailed description of the exact mechanisms and proteins involved in the formation process of the functional γ-HL and PVL pores[4].

γ -HL

The basis of pore formation by γ-HL is binding of LukF to the membrane lipid bilayer. The LukF monomer consists homologous to α-HL of an extracellular cap domain, an attached rim domain and a prestem domain, which is covered by the cap domain [66]. Attachment of LukF is dependent on the rim domain recognizing and binding the hydrophilic head groups of the membrane lipids by hydrogen bonds [66, 37]. Binding of the LukF monomer results in exposition of a further binding site which is located within the cap domain of LukF on the cytosolic site. This interface 1 consists of amino acids of a flexible loop of LukF which connects two β-strands (β2 and β3) [64]. After binding of LukF to the membrane and subsequent structural changes, interface 1 site can then be recognized by several amino acid residues of the β 1 strand in the cap domain of Hlg2, forming electrostatic interactions [64]. Binding of residues of the Hlg2 cap domain to LukF induces several structural changes in LukF and Hlg2 which are required for the further assembly of the pore: First, the N-terminal latch domain of LukF is released from the β-sheets by which it was previously covered, because of steric restrain caused by the altered positions of interface 1 after Hlg2 binding [64]. Moreover, interface 2 of the LukF domain becomes exposed which can be bound afterwards by Hlg2 of another dimer, forming electrostatic interactions [64]. Establishment of electrostatic interactions between both interfaces of LukF with Hlg2 induces release of the prestem structures of the LukF and Hlg2 components from their accordant cap regions and subsequent assembly of a prepore [64]. This causes a reorganization of the β-hairpins involved in the prestem, which subsequently penetrate the membrane bilayer [52]. This forms the functional transmembrane β-barrel, stabilized by hydrogen bonds between the β-strands [52, 64]. The functionally assembled pore has a diameter of 114Å and a height of 93Å [64].

[3] This was proven for γ-HL, leukocidin and PVL. Although not proven crystallographically, it is likely that the other known heterooctameric β-barrel hemolysins form a pore in a similar way since all of them are consisting of S and F proteins and cause similar symptoms [37, 63].

[4] γ-HL and PVL were chosen as examples, since these proteins are the best described bicomponent hemolysins.

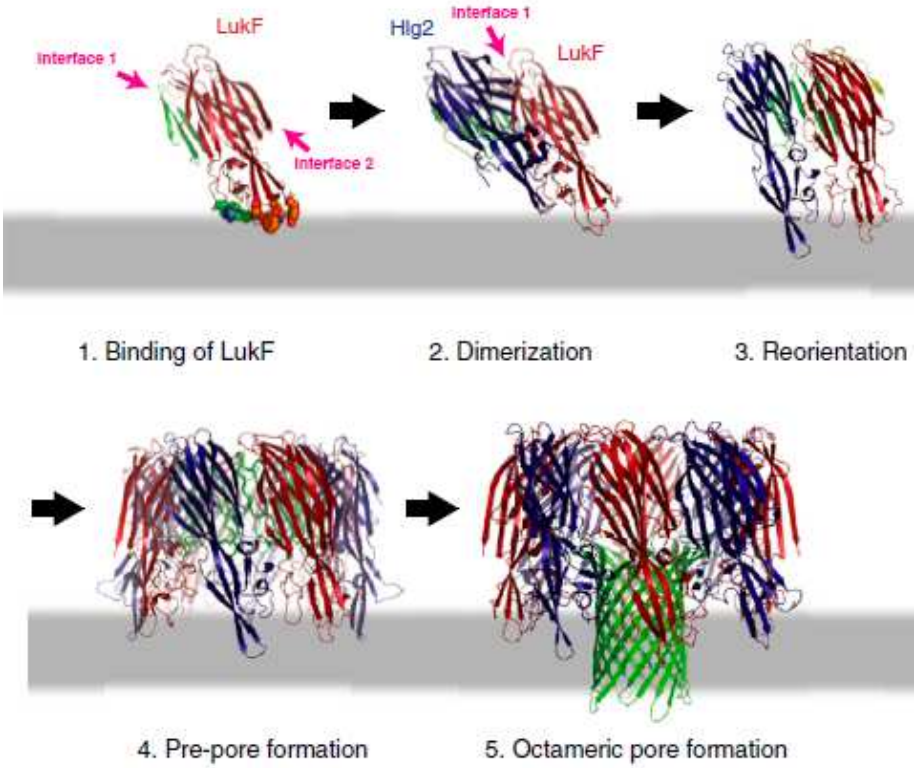

Figure 4: Mechanism of γ-HL formation. After attachment of the rim region of the LukF monomer to the membrane bilayer (1), LukF dimerizes with Hlg2 (2). The dimerization induces reorientation of the molecule (3), exposing a second binding interface for Hlg2. This allows binding of further LukF/Hlg2 heterodimers and prepore formation (4). After octamerization, structural changes occur resulting in penetration of the prestem region into the membrane bilayer and assembly of the functional pore [picture adapted from 64].

PVL

PVL consists of an octamer generated out of the two proteins LukF-PV (34kDa) and LukS-PV (32kDa) [67]. Like α-HL or Hlg2 and LukF of γ-HL, LukF-PV and LukS-PV monomers consist of a cap domain, a rim domain for membrane binding and a prestem domain, covered by the cap domain [68] (**Figure 5**). Furthermore a flexible triangle domain links the cap with the prestem domain [67]. However, in contrast to Hlg2 of γ-HL, also LukS-PV can also bind directly to the cell membrane. Binding of the rim domains of LukS-PV and LukF-PV to the membrane is hereby mediated by the rim domain containing several hydrophobic amino acid residues, which integrate into the membrane bilayer serving as molecular anchor [67].

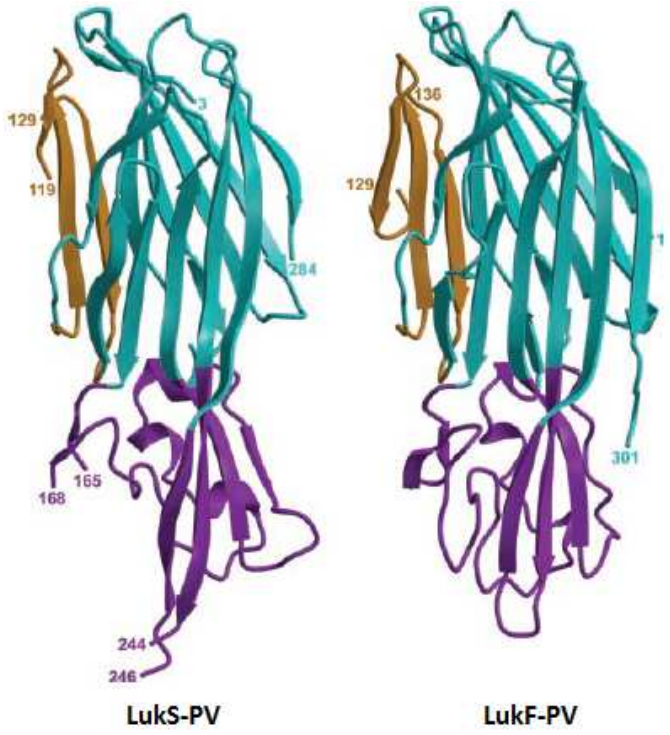

Figure 5: Ribbon model of the LukF-PV and LukS-PV monomers. The purple part represents the rim domain, the cyan part the cap domain and the orange part the prestem domain. The numbers indicate the position of the amino acid residues [adapted from 68].

Although the octameric pore structure of PVL is not determined crystallographically up to date, a description of the binding sites involved in octamerization is available from *in silico* predictions. As first step for octamerization, dimerization of the membrane-bound monomers is required. Monomers in close proximity can hereby potentially interact with two distinct binding interfaces, similar to γ-HL. Within the area of the first interface site (interface 1), the formation of 22 hydrogen bonds including one salt bridge between LukF-PV and LukS-PV is predicted [67]. This would allow tight binding of the monomers. Remarkably, on the other interface site (interface 2) of the monomers, only five hydrogen bonds including two salt bridges can be potentially formed, which is due to the presence of more hydrophobic residues. Stabilization of this binding is therefore more dependent on hydrophobic interactions, which potentially involves three residues on LukS-PV and four residues on LuKF-PV. The presence of the three salt bridge interactions is presumably important since it was shown that salt bridges within pore structures are often required for the recognition of molecules and interface stability [67]. A role of the salt bridges in the recognition of molecules is also suggested by the prediction that the salt bridges are formed on the top of the PVL molecules, which represents the boundary of PVL to the extracellular environment [67]. Based on these predictions and the high homology to the monomers LukF and Hlg2, it is likely that octamerization occurs similar to γ-HL: After assembly of LukF-PV/LukS-PV heterodimers, tetramerization of two of such heterodimers occurs, followed by the formation of an octamer by interactions of two of these tetramers [67]. This results in the assembly of a nonfunctional prepore with a predicted diameter of 115Å, a height of 70Å and a structure parallel to α-Hl and γ-HL. In respect to the structure of the LukS-PV and LukF-PV monomers and the pore formation mechanism of α-HL and γ-HL, the functional octameric pore is likely to be generated after conformational changes and insertion of the stem domain into the membrane induced by the octamerization. A key player in these conformational

changes could be the N-terminal domains of LukS-PV and LukF-PV: It is predicted that these domains are located on top of the prestem domains [67]. Oligomerization presumably shifts these domains in such a manner that they come in closer proximity to the prestem domains. This could initiate the translocation of the prestem domains into the membrane, which finally forms the lytic pore [67]. Hereby the lytic effect was shown especially on PMNs, which could give an explanation for the necrotizing effect of high PVL concentrations: Lysis of PMNs could result in the release of the cytotoxic granules of PMNs, including reactive oxygen metabolites and different proteases, which result in further injury of tissue [36] (**Figure 6**).

Besides of this, also other roles of LukS-PV in *S.aureus* virulence were postulated: After cleavage of the N-terminal signal peptide of LukS-PV, it was shown that the released peptide binds with its highly positively charged N-terminus to extracellular heparan sulfates [41]. This binding is based on the negative charge of heparan sulfates, which allows the formation of strong ion bonds [41]. Moreover, the newly generated C-terminus presumably binds to the cell wall of S.aureus, since the signal peptide can be found on cell wall derived material [41]. Taken together, this binding could provide support for the attachment of *S.aureus* to epithelial cells [41]. *Zivkovic et al.* found also a role of PVL in necrotizing pneumonia in humans and in mice during CA-MRSA infections which are not based on the formation of lytic pores [69]: In fact, this effect is caused by binding of PVL to toll-like receptor 2 (TLR2) molecules in combination with the coreceptor CD14 on alveolar macrophages [69]. The subsequent downstream effects of TLR2 signaling include the activation of the MAPK and NFκb pathways, which results in an activation of alveolar macrophages accompanied with a massive production and release of proinflammatory cytokines like interleukin-1β (IL-1β),monocyte chemotactic protein-1 (MCP-1) interleukin-8 (IL-8) and tumor necrosis factor-α (TNF-α) [36, 69] (Figure 5). High levels of IL-1β and TNF-α can cause strong inflammation with subsequent cell death which could exacerbate the necrotizing pneumonia caused by *S.aureus* [36, 69].

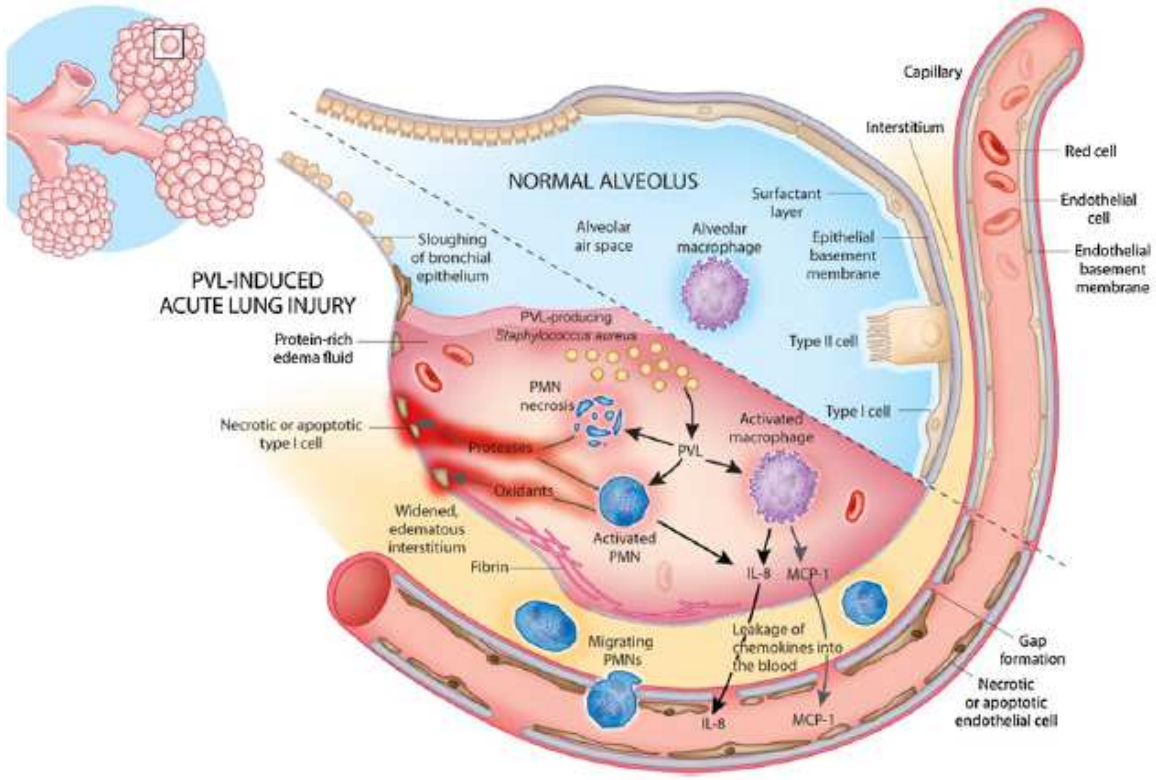

Figure 6: Overview showing mechanisms of alveolus toxicity by PVL produced by *S.aureus*. The toxic effect is hereby mediated by direct lysis of PMN and subsequent release of cytotoxic granules. Furthermore, also high levels of proinflammatory chemokines released into the blood stream and subsequent inflammation could promote necrotizing pneumonia [Adapted from 36].

Other leukocidins

Several other groups reported the formation of lytic transmembrane pores based on S and F proteins in a similar way like PVL and γ-HL [63, 70] (**table 1**). It is assumed that hereby the combination of any of the described six S and five F proteins could potentially generate an active lytic pore permeable for Ca^{2+} and other ions or small molecules [63, 71, 72]. The mechanism of some of these pore forming leukocidins like the initially described leukocidin (LUK) is partially solved [73, 74], however the crystal structures and contribution of such leukocidins to *S.aureus* virulence remain to be elusive.

Phenol soluble modulins

Phenol soluble modulins (PSM) were originally described in *S.epidermidis* strains as virulence factors [75]. In 2007 *Wang et al.* described that also particular *S.aureus* strains are secreting altogether seven distinct proteins similar to the PSMs of *S.epidermidis*. These proteins were subdivided into the groups of α-phenol soluble modulins (α -PSM), which are mostly encoded by the *psmα* operon, and β-phenol soluble modulins (β -PSM) encoded by the *psmβ* operon [40]. The group of α-PSM includes four polypeptides consisting of 20-22 amino acids which are named PSMα1-4 [40]. Moreover, δ-toxin a peptide with 26 amino acid residues produced by 97% of the characterized *S.aureus* strains belongs to this group [8]. δ-toxin as well as PSMα1-4 possess an amphipatic α helix domain with conserved key residues [77].

The toxic effect of α-PSMs is hereby based on the ability of PSM-α to recruit, activate and lyze human neutrophil granulocytes, whereas PSMα3 showed additional capacities to lyze erythrocytes and monocytes [40]. Although the molecular interactions between PSMs and neutrophil granulocytes are so far not crystallographically solved, studies of *Kerr et al.* could give a potential explanation: δ-toxin is organized in an amphipatic helix and incubation of δ-toxin with bilayer membranes results in an integration of the toxin into the membranes. Hereby on average six δ-toxin monomers form a transmembrane pore with a weak selectivity and rapid transitions between closed and open conformations [78]. The integrity of the pore is ensured by the formation of H-bonds among side chains of at least nine amino acid residues per δ-toxin monomer. These H-bonds occur inter- and intrahelical and are therefore responsible for the stability of the helices themselves and for the stability of the pore [78]. Since δ-toxin and all αPSMs 1-4 share an amphipatic helical domain with several key residues, this mechanism could also cause the lytical effect of the other α-PSMs on neutrophil granulocytes, monocytes and erythrocytes [77].

Besides of α-PSMs, some of the human pathogenic *S.aureus* strains like USA300 do also produce β-PSMs [77]. In contrast to α-PSMs, the two known β-PSMs 1 and 2 (44 amino acids each) are not lytic in humans and have only a weak association with upregulation of proinflammatory cytokines like TNFα [40, 77]. Considering that both β-PSMs possess an amphipatic helix domain with conserved key residues like α-PSMs, also other molecular determinants seem to play a role in α-PSM function [77].

Summary of the known human pathogenic hemolysins:

Name	Gene frequencies in clinical *S.aureus* isolates (CA-MRSA isolates)	Mechanism of action
α -HL [37]	<99%	Oligomerization of seven membrane-bound alpha-HL results in the formation of a prepore. Conformational changes induce penetration of α -HL monomer prestem domains into the membrane.
β -HL [42, 56]	11%	Proteolytic cleavage of the membrane components sphingomyelin and LPC.
γ -HL (lukF/Hlg2) [37, 64]	<99%	Oligomerization of four S- and four F-protein monomers on the target membrane results in the formation of an octameric non-lytic prepore. Structural changes induce penetration of the prestem domains of the S-/P-proteins into the membrane and formation of the lytic pore.
PVL (lukF-PV/lukS-PV) [37, 64]	2% (<99%)	
LUK (lukF/lukS) [37, 64]	<99%	The exact mechanism of action was not yet determined. However, the structure and high homology of the S-/ F-proteins indicate a mechanism analogous to PVL and γ -HL.
LukE/D (lukD/lukE) [63]	30%	
LukG/H (LukG/lukH) [76]	<99%	
Luk-R (LukF-R/LukS-R) [72]	Not determined	

Table 1: Overview about the known hemolysins derived from human clinical *S.aureus* isolates[5]. Round brackets behind the toxin name indicate the names of the involved F-/S-proteins.

[5] *Gravet et al.* detected also other combinations of S-/F-proteins resulting in the formation of a lytic pore [63]. Such pore complexes are not listed in this work due to the lack of further data.

Pyrogenic toxin superantigens

Generally, exogenous molecules possess antigenic capacity if they can be efficiently endocytosed by professional antigen presenting cells (APC), degraded in their phagolysosomes and subsequently presented on major histocompatibility (MHC) class II molecules. These loaded MHC class II molecules can be recognized afterwards by the T cell receptor (TCR) of peptide specific $CD4^{+}$T cells resulting in a clonal expansion of such T cells. Usually these T cells with specificity for a distinct antigen are only present in low numbers and are accounting for 0.001% to 0.0001% of the overall T cell population [79]. The following immune response based on the recognition of specific peptides is directed and creates a cytokine milieu favorable for further specific T and B effector cell activation. This activation is strongly dependent on the specificity of the TCR recognizing certain epitopes presented on MHC class II by APC. By that mechanism the immune system ensures an appropriate immune response, leaving unspecific T cells unstimulated [38, 79].

However, the by approximately 63% of the *S.aureus* strains produced pyrogenic superantigens (PTSAgs) have been shown to crosslink the MHC class II molecules with the Vβ chain of TCRs directly (**figure 7, table 2**) [38, 80, 81]. Hereby superantigens don't have to be processed and loaded to MHC class II molecules priorily. As a result of this, PTSAgs activate all T cell clones with a TCR bearing the recognized Vβ chain which can be up to 20% of the overall T cell population [38]. The activation of such a high partition of T cells causes a massive release of proinflammatory cytokines like interleukin-2 (IL-2), interferon-γ (IFNg) and tumor necrosis factor α (TNFa). Such enormous levels of these cytokines can result in an immune response potentially causing a toxic shock syndrome, which can include various symptoms: Among fever, malaise, diarrhea, conjunctivitis, myalgias, headache and rashes also life-threatening symptoms including shock, hypotension, renal failure, intravenous coagulation and thrombocytopenia were described as symptoms [26, 82].

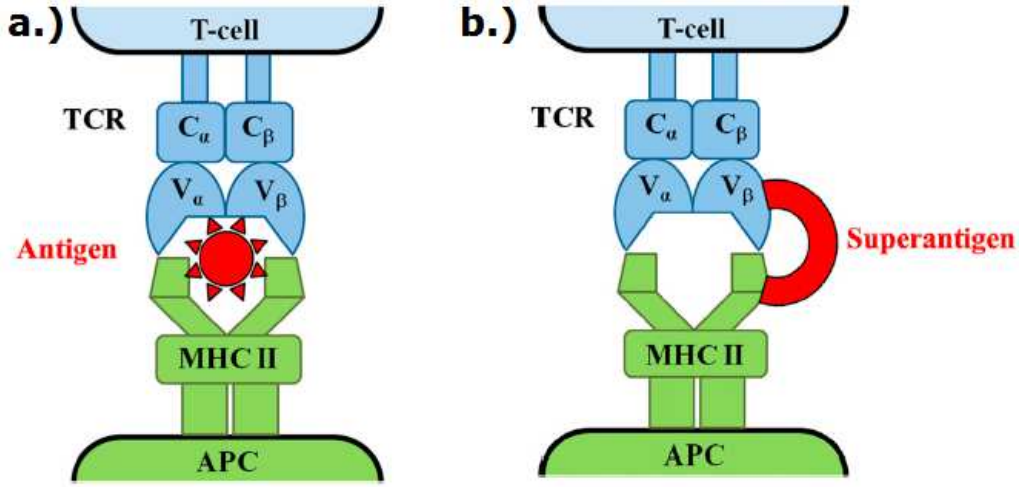

Figure 7: Schematic overview showing T cell activation in a physiological situation and by superantigens. a.) Antigen presentation on MHC class II molecules on APC. Only T cells with an antigen-specific TCR can bind to loaded MHC class II molecules and become activated. b.) Superantigens crosslink MHC class II molecules on APC with the Vβ chain of T cells, resulting in activation of these T cells. Antigen specificity is hereby not required [picture adapted from 38].

A broad range of different forms of such superantigens were described, each belonging to one of the following three classes[6]: The first group includes toxic shock syndrome toxin 1 (TSST-1), the second group comprises the staphylococcal enterotoxins and the third group consists of the enterotoxin-like toxins [26]. All of these superantigen groups have a distinct two domain protein structure in common. The domain A consists of an α-helix and a C-terminal β-grasp motif, the domain B includes an N-terminal oligonucleotide-oligosaccharide binding (OB) domain [83]. Besides of such superantigens also superantigen-like proteins exist: Superantigen-like proteins were primarily considered as superantigens since they have structural similarities with superantigens [84]. Unlike superantigens those proteins do not bind T cells but bind sialyl lewis^X residues of P-selectin glycoprotein type-1 (PSGL1) on leukocytes [85].This inhibits extravasation of leukocytes and as a consequence of this local inflammation [86]. However, because superantigen-like proteins do not possess cytolytic potential this group is not included in detail in this work. The following part should explain the original superantigens including the underlying mechanisms of toxin action.

Toxic shock syndrome toxin 1

Toxic shock syndrome toxin 1 (TSST-1) (also known as enterotoxin F) is a 194 amino acid long peptide with a molecular weight of 22kDa and was first described by *Bergdoll et al*. in 1981 [87]. A recent study revealed that up to 75% of the clinical MRSA isolates produced TSST-1, whereas the concentrations of produced TSST-1 varied up to 170-fold [88]. The mechanism of TSST-1 action is based on binding of the toxin to the human Vβ chain 2.1 (hVb2.1) of the TCR by intermolecular contacts to the complementary determining region 2 (CDR2). Although this region is highly homologous to other Vβ chain subtypes like hVb4, TSST-1 is specific for hVb2.1. This specificity is due the requirement of the co-recognition of another region on the TCR, called framework region 3 (FR3) [89] (**Figure 8**). Unlike any other TCR, the FR3 region of hVb2.1 includes a glutamate residue on position 61 and a lysine residue on position 62, which are recognized by TSST-1[89].

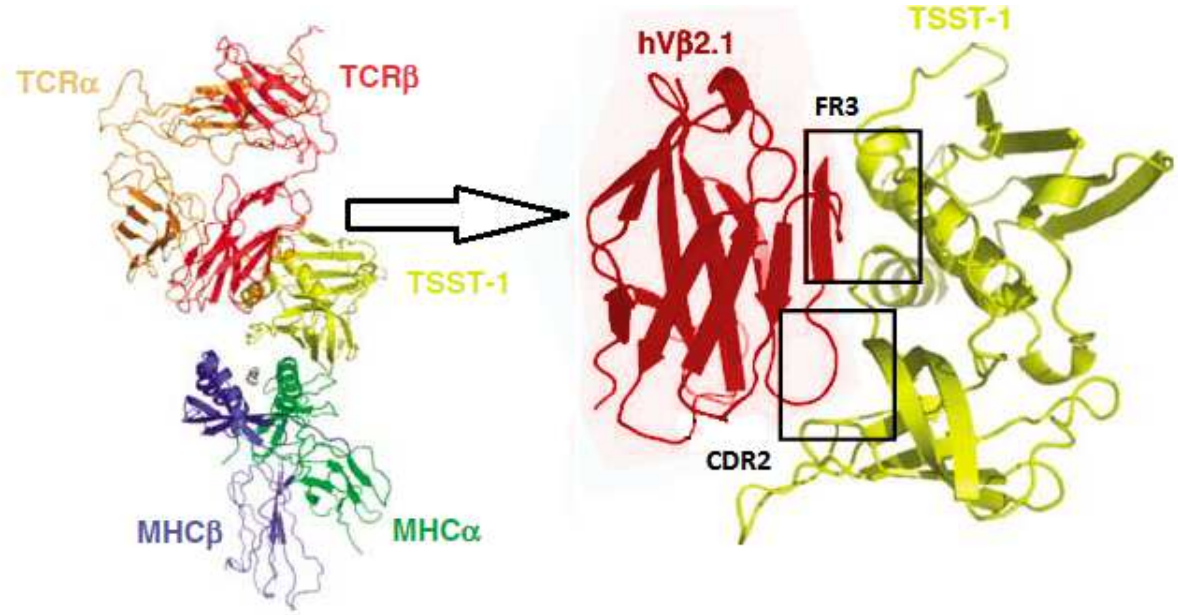

Figure 8: Ribbon model of TSST-1 mediated interaction between MHC class II molecule on APC and the TCR of a T cell. The black boxes indicate the binding regions CDR2 and FR3 on hVβ2.1 [adapted from 89].

[6] The subdivision in the distinct classes is dependent on the binding mechanism of the PTSAg to the MHC class II molecules and the TCR.

On the other site TSST-1 binds to the α-subunit of the MHC class II molecules, whereas binding properties depend on the allelic variant of the MHC class II molecule. Hereby the most studies were done with the MHC class II subtype HLA-DR1, a well studied MHC class II subtype [57, 90]. The binding between the MHC α-subunit of HLA-DR1 and TSST-1 is based on the interaction of altogether three contact regions including 24 residues of TSST-1 and 20 residues of DR1. The extensive binding of TSST-1 to HLA-DR1 is due to hydrophobic interactions, hydrogen bonds and salt bridges [57]. Binding of TSST-1 to MHC class II molecules does not essentially require binding of a specific antigen into the binding groove of the MHC class II molecule, but the presence of certain antigens increased TSST-1 binding up to 5000-fold [6, 22].

Enterotoxins

Altogether, 9 staphylococcal enterotoxins named staphylococcal enterotoxin A –J (SEA-SEJ) with several isoforms were found in human *S.aureus* isolates[7]. The name enterotoxin comes from the fact that enterotoxins can play a causative role in Staphylococcal food poisoning because they can induce emesis by induction of serotonin release into the intestine [91]. Although the amino acid sequence homology of such enterotoxins is only between 22% (SEG and SED) or 67% (SEB and SEC), all enterotoxins possess like the other groups of superantigens a conserved two-domain topology consisting of a β-barrel and a β-grasp motif linked by a conserved junction[90]. The junction between the β-barrel and the β-grasp motif in enterotoxins forms a shallow depression which is important for TCR binding and mutations in this domain drastically inhibit binding of the enterotoxin to the TCR [90].

However, the binding domain specific for interactions with MHC class II molecules differs between the different enterotoxins: The enterotoxins SEA, SEC1-3[8], SED[9], SEE, SEH and SEJ possess a zinc binding domain and interaction of these toxins with MHC class II molecules is dependent on the presence of Zn^{2+} ions [22, 94, 95]. Such enterotoxins possess next to a low-affinity binding domain shared with SEB and SEG also a high affinity binding domain specific for the β-chain of MHC class II molecules [22][10]. This high affinity binding site binds MHC class II molecules on the polymorphic β-chain by the C-terminal domain of the superantigens [22]. The enterotoxins SEB and SEG link MHC class II molecules and TCR only on the basis of the low-affinity binding site: Mutational analysis indicates that SEB exclusively use a low affinity binding site on the α-chain of MHC class II. *Kappler et al.* postulated that binding to MHC class II by SEB is primarily mediated by the N-terminal residues of an α-helix and hydrophobic residues in a β-strand of SEB [96]. For SEG the exact binding mechanism is not determined so far, but since SEG possesses the same binding residues like SEB and a sequence homology of 44.1%, the SEG binding mechanism is presumably similar to the binding mechanism of SEB [94].

[7] Staphylococcal enterotoxin F was renamed to TSST-1. In this work only the name TSST-1 was used and any information referring exclusively to enterotoxins does not refer to TSST-1.
[8] 3 isoforms of SEC were described which differ in 9 (SEC1/SEC3) or 4 (SEC2/SEC3) amino acids [92].
[9] SED binds MHC class II molecules as a homodimer [93].
[10] SEH binds MHC class II molecules exclusively by the C-terminal domain [22].

Enterotoxin-like toxins

Staphylococcal enterotoxin-like toxins (SEls) share the characteristic three dimensional structures of enterotoxins and TSST-1 containing the two conserved polypeptide domains. However in contrast to enterotoxins, SEls do not necessarily induce emesis and are not associated with staphylococcal food poisoning [20]. Up to now the SEls K-R, U, U2 and X have been described, also occurring in highly virulent CA-MRSA strain like USA 300 [26, 83]. Due to their similar structure SEls presumably cross-link the TCR of T cells and MHC class II molecules of APC similar to enterotoxins, although interactions were not solved crystallographically so far [26, 97].

Furthermore, *Thomas et al.* showed that *S.aureus* can quickly generate new superantigens of the SEl class by recombination of genes coding for toxins within the enterotoxin gene cluster [97]. Thus, it is likely that more SEls will arise in the future.

Summary of the known human pathogenic PTSAgs:

Name	Gene frequencies in clinical MSSA (CA-MRSA) isolates[11]	Primary mechanism of action
TSST-1 [88, 89, 98]	25-33% (75%)	TSST-1 cross-links the Vβ-chain of TCRs with MHC class II molecules of APC. This results in an unspecific primary activation and proliferation of T cells and subsequent anergy and clonal depletion. Binding of peptides into the MHC class II binding groove is hereby not essential but increases TSST-1 binding up to 5000-fold.
SEA [97-100]	17-54% (58-95%)	All SEs cross-link the Vβ-chain of TCRs with MHC class II molecules on APC, independent of their antigen specificity. This results in an unspecific primary activation and proliferation of T cells and subsequent anergy and clonal depletion. Binding of peptides into the MHC class II binding groove hereby does not alter the affinity of the SE to the MHC class II molecule.
SEB [97-100]	4-30% (3-23%)	
SEC [97-100]	5-25% (95-97%)	
SED [97-100]	5-13% (0-19%)	
SEG [97-100]	55-89% (0-19%)	
SEH [97-100]	10-17% (65-100%)	
SEI [97-100]	63-91% (1%)	
SEJ [97-100]	9% (1%)	
SEl-K [97-100]	8% (96%)	The mechanism of action of SEls is so far only paritally determined. However, the structure and high homology of SEls to SEs indicate a mechanism analogous to the mechanism of action of ETs.
SEl-L [97-100]	9-25% (96-97%)	
SEl-M [97-100]	44-63% (0%)	
SEl-N [97-100]	63-81% (0%)	
SEl-O [97-100]	63-82% (0%)	
SEl-P, Q, R, U, U2, V [97-100]	Not determined	

Table 2: Overview about the known superantigens derived from clinical *S.aureus* isolates. Moreover, *Thomas et al.* postulated that the appearance of further SEl in the future is likely since *S.aureus* can generate new SEls by recombination of existing gene loci [97].

[11] Toxin frequencies can vary significantly, depending on the study set-up and the region from which *S.aureus* isolates were obtained. If different frequencies were found, a range of the postulated toxin frequencies is given.

Exfoliative toxins

Exfoliative toxins (ETs) are detectable in approximately 5%-10% of all clinical *S.aureus* isolates including MRSA-strains [27] (**table 3**). In human isolates three different isoforms (ETA, ETB and ETD) were described which occurrence is strongly dependent on the geographic region [27]. Besides of ETA, ETB and ETD also several other ETs were described affecting a broad range of animals like pigs, chickens, mice and guinea-pigs [101]. ETs can cause several symptoms in humans with different severity, primarily affecting neonates and children. The symptoms are generally described as bullous impetigo if the symptoms are locally bordered or Staphylococcal scalded skin syndrome (SSSS) if symptoms are generalized. In both cases the formation of blisters and exfoliation occurs which can cover to up to 90% of the body surface in case of SSSS [27]. Bullous impetigo and SSSS are usually accompanied with rashes and fragile, thin-roofed and flaccid bullae containing liquid or pus. Areas affected by bullous impetigo or SSSS are positive for the Nickolsky sign, which means that the affected skin peels off easily after putting slight pressure on it [27]. Besides, the formed bullae rupture easily releasing the contained liquid or pus [102]. In case of SSSS, the symptoms in newborns usually start localized affecting the umbilical stump, the inner ear, conjunctiva or the upper respiratory tract. In adults the origin of SSSS is often difficult to determine although catheterization, infection of artheriovenous shunts, septic arthritis or parenteral infection have been described as potential sources [27]. Besides of the formation of blisters and rashes like in bullous impetigo, SSSS can be accompanied with systemic symptoms including fever, malaise and conjunctivitis [103]. The formation of bullae in vast areas of the body can furthermore impair the patient's skin function in several ways: Besides of extensive loss of fluid also a poor body temperature control and superinfections have occurred as secondary complications [104]. Furthermore it was described that epidermal loss in neonates can be associated with staphylococcal sepsis including tachycardia, hypotension, neutropenia or respiratory distress [27].

The molecular reason for the formation of bullae is the ability of exfoliative toxins to act as serine proteases: All three exfoliative toxins affecting humans have significant sequence homologies with other trypsin-like serine proteases including a catalytic triad as active centre [105]. And indeed, *Hanakawa et al*. showed that the three exfoliative toxins ETA, ETB and ETD found in human isolates are specific for a serine residue on amino acid position 381 followed by a glutamate in the human Desmoglein 1 (Dsg1) protein [106] (**Figure 9**). As desmosomal protein of the cadherin family, Dsg1 is required for cell-cell-adhesions of keratinocytes and responsible for the integrity of the epidermis [107]. The serine residue recognized by ETs is in a putative Ca^{2+} binding site of Dsg1 and abundance of Ca^{2+} is essential for ET function [106]. Subsequent proteolytic cleavage of Dsg1 between the subunits EC3 and EC4 results in loss of cell-cell adhesion and epidermal detachment [105] (**Figure 10**).

Figure 9: Schematic overview of Dsg1, including a transmembrane region (TM), a signal peptide (S), a propeptide sequence (P) and 4 extracellular cadherin domains (EC1-4). Vertical black lines indicate calcium binding sites, the black arrow between EC3 and EC4 indicates amino acid position 381, which is the ET-cleaving site [adapted from 108].

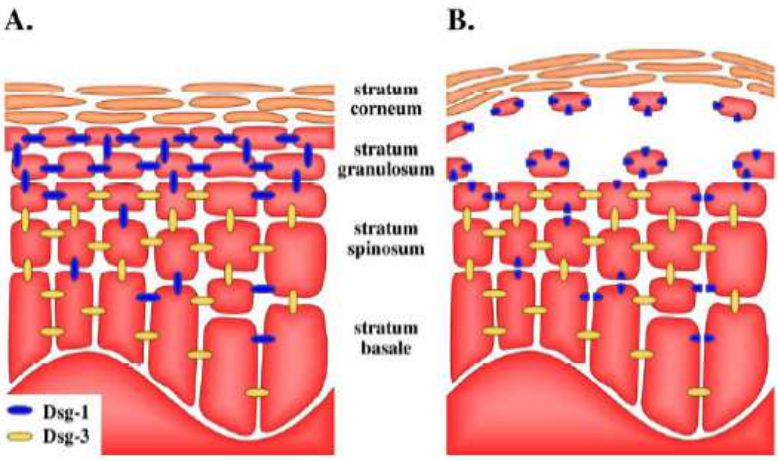

Figure 10: Schematic overview about the exfoliative toxin effect: A. represents the epidermal structure in a healthy individual; B. shows the situation after ET exposure. ETs specifically cleave Dsg1, resulting in separation of keratinocytes in the stratum granulosum. That causes detachment of the stratum corneum, the outermost layer of the epidermis [adapted from 105].

Furthermore, several groups postulated that ETs possess also mitogenic capacity for T cells, resulting in the non-specific T cell proliferation similar to PTSAgs [109, 110]. On the other hand, several other groups which tried to reproduce these findings found no superantigenic activity of exfoliative toxins [111, 112]. Also no molecular mechanism of action for the superantigenic effect has been described so far.

Exfoliative toxin A

Exfoliative toxin A (ETA) has a size of 27kDa and its production was reported from a broad range of S.aureus strains [113]. Furthermore, ETA is the most produced ET in European countries and the USA: For example studies in France revealed that up to 96% of the ET-positive isolates produced ETA [27]. ETA possesses a catalytic triad responsible for the proteolytic attack on the serine residue position 381 (E381) homologous to ETB and ETD [102] (**Figure 11**).

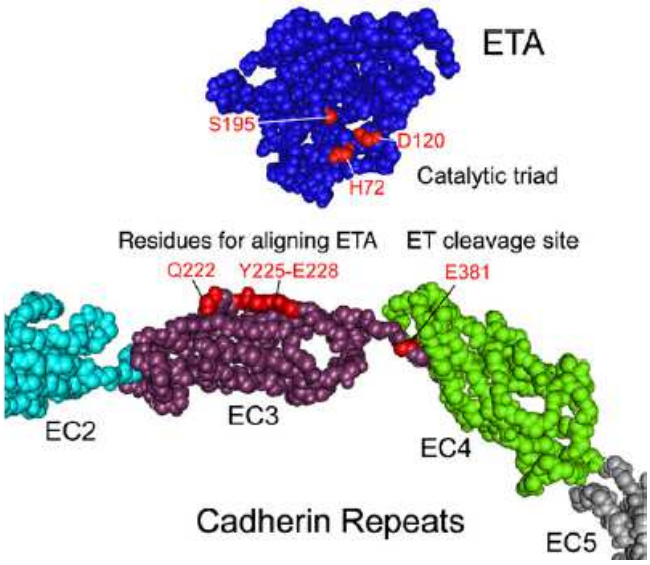

Figure 11: Mechanism of action of ETA: After aligning of ETA on the residues Q222 and Y225 to E228 on Dsg1, ETA specifically cleaves Dsg1 on the residue E381 mediated by the catalytic triad in the active centre of ETA [adapted from 99].

Exfoliative toxin B

Exfoliative toxin B (ETB) has a size of 27.3kDa was found in up to 52% of human ET-producing S.aureus isolates in the USA [27]. ETB is unlike ETA not encoded by the genome but on a plasmid and is approximately 40% homologous to ETA [114]. The three dimensional structure of ETB is similar to the structure of ETA including the conserved key catalytic site residues [114]. Although the exact mechanism of action of ETB was less precisely investigated than of ETA, the high homology and similar structure suggests a common catalytic mechanism [114].

Exfoliative toxin D

Yamaguchi et al. described in 2002 the presence of a novel chromosomally encoded exfoliative toxin with a size of 27.2kDA found in human S.aureus isolates [115]. This toxin was named exfoliative toxin D (ETD) and is predominantly found in furuncles and abscesses [115, 116]. A study done in France revealed that ETD was found in 10.5% of S.aureus isolates, mostly derived from European CA-MRSA strains [116]. ETD sequence is 40% identical to ETA and 59% identical to ETB and includes the same conserved catalytic key residues like ETA and ETB [115]. Therefore it is likely that the mechanism is similar to the mechanism of ETA and ETB.

Summary of the known human pathogenic ETs:

Name	Gene frequency in clinical *S.aureus* isolates[12]	Mechanism of action
ETA [114, 117]	5%	Proteolytic cleavage of the desmosomal cadherin Dsg1; Some data indicates that staphylococcal ETs possess superantigenic potential comparable to PTSAgs.
ETB [114, 117]	0.6%-2.5%	
ETD [116, 117]	4.6%-10.5%	

Table 3: Overview about the ETs found in human clinical *S.aureus* isolates.

[12] Toxin frequencies can vary significantly, depending on the study set-up and the region from which *S.aureus* isolates were obtained. If different frequencies were found, a range of the postulated toxin frequencies is given.

Discussion

Summary

S.aureus is a gram-positive bacterium which is carried by up to 30% of the population and can cause complications especially in combination with other predisposing factors like immune deficiency [9]. These complications include various symptoms like soft tissue and skin infections, but also more severe symptoms like abscesses, edema, sepsis and pneumonia [19, 21, 22]. The worldwide emerge of *S.aureus* is hereby based on the expression of genes for antibiotic resistance and a variety of exotoxins [7, 8, 19]. Moreover, especially MRSA strains quickly develop resistance against new antibiotics which gave such strains the ability to spread in hospitals worldwide [2]. From the beginning of the 1990s also MRSA strains were described which could also cause disease without any prior infections or immune deficiency and which were not restricted to hospitals [3]. Analysis of clinical *S.aureus* isolates revealed that especially these CA-MRSA strains like the highly pathogenic strain USA300 produce high levels of exotoxins. An important role of the staphylococcal exotoxins is also provided by knockout experiments of toxins like PVL or α-HL in animal models: This was associated with significantly decreased virulence or even inhibited a staphylococcal infection completely [7]. To understand the toxin actions and their contribution to *S.aureus* pathogenicity and immune evasion more in detail, this work aims to summarize the current literature on molecular mechanisms of the staphylococcal toxins.

This knowledge could be used in the future for the development of therapeutical interventions interfering with toxin binding and action. Besides, a detailed understanding of the toxin structures and their antigenicity could also be used for the development and improvement of vaccines based on toxin components as antigens.

The role of hemolysins

The group of hemolysins comprises toxins assembled out of one type of monomer like α-HL or the PSMs, bicomponent hemolysins like PVL and γ-HL and the sphingomyelinase β-HL. Although these toxins differ in their structure, all of them have the ability to lyze blood cells by the formation of transmembrane pores. The lysis of blood cells has mainly two benefits for *S.aureus*: First, lysis of erythrocytes results in release of hemoglobin and myoglobin, two molecules possessing heme groups with a central iron atom [42]. After release of hemoglobin and myoglobin into the blood stream, *S.aureus* can subsequently take up the hemoglobin and myoglobin molecules to extract the iron utilizing the “iron-regulated surface determinant system” (Isd), a multiprotein iron scavenging system [118]. That is important for *S.aureus* since iron is a limiting nutrient and required for a broad range of metabolical processes [42, 118]. Second, lysis of cells of the immune system could provide an immune evasion mechanism of *S.aureus*: Especially neutrophil granulocytes, macrophages and monocytes are essential for the primary immune response of the host against *S.aureus* [19, 40]. *Diep et al.* postulated that lysis of leukocytes could especially be an important factor at an early stage of the infection to establish a successful infection in the lung to prevent rapid clearance of *S.aureus* [36].

However, whereas the mechanism of toxin action and the benefit of it on a molecular basis is widely undisputed, the literature provides different opinions about the impact of hemolysins in human infections: On one hand, animal experiments with rabbits and mice indicate an important role especially for PVL, α-HL and α-PSM, since knockout or artificial mutations of the genes of these toxins

severely reduce the pathogenicity of highly virulent strains like USA300 [7, 40]. It is also remarkable that only about 2% of all analyzed *S.aureus* strains possess the *luk*-PV genes, whereas almost 100% of the clinical CA-MRSA strains are positive for these genes [119]. Considering the high virulence of the CA-MRSA strains, this underlines a potential role for PVL. Studies done by *Lina et al.* indicate furthermore a role of PVL in the development of pneumonia in humans, because the presence of PVL was found to be significantly associated with community-associated pneumonia and invasive skin infections [20]. Further indication that hemolysins are important virulence factors are also provided by studies which investigated the expression levels of hemolysins in different strains: High levels of α-PSMs are mainly produced by common CA-MRSA strains like USA300 and USA400 and are associated with enhanced virulence of these strains compared to HA-MRSA strains [40, 120]. The CA-MRSA strain USA300 for example produces 5.93-fold more PSMα3 compared to the HA-MRSA strains USA100 and USA200 [35]. The role of α-PSMs as major determinants of CA-MRSA virulence is also underlined by mouse experiments in which *psmα* knockout strains caused a significantly reduced mortality of the mice compared to the respective wild type strain [40]. Also knockout experiments of α-HL indicate an important role in staphylococcal virulence: For example the *S.aureus* strain LAC, derived from the for humans virulent CA-MRSA strain USA300, is expressing high levels of α-HL [7]. Experimental inoculation of mice with LAC resulted in a mortality of approximately 80% within 72h after inoculation of the mice [7]. Knockout of the *hla* gene coding for α-HL abrogated mortality completely. Similar data was also found for other human pathogenic strains like USA400 and Newman, a well described strain which is also commonly used in animal models [7, 44].

About the importance of β-HL and other hemolysins like γ-HL or other bicomponent hemolysins less is known, since less data is available quantifying the impact of these toxins of *S.aureus* infections. However, the ability of β-HL has the potential to lyse *in vitro* human lymphocytes, monocytes and PMN, indicating that β-HL could play a role in enhancing the virulence of *S.aureus* [42, 60]. Also a role of γ-HL as virulence factor is likely, since it is found in up to 99% of the *S.aureus* strains [71]. This is also suggested by an epidemiological study by *Peacock et al.*, who found a significant association between the presence of the *hlg* gene coding for γ-HL and an increased invasiveness of the respective *S.aureus* strains [121].

On the other hand, several groups postulated that the role of hemolysins as staphylococcal virulence factors could be less important than supposed: For example the experimental setups to determine the lytic capacities of hemolysins could cause bias, since the capability of some hemolysins to lyze rabbit or mouse blood cells is higher than the capability to lyze the respective human cells [8]. This was already shown for *in vivo* experiments to determine the toxicity of α-HL, which has the ability to lyze rabbit erythrocytes approximately 1000-fold more efficient than human erythrocytes [8]. Thus, extrapolation of the results to the *in vivo* situation in humans could be invalid. Moreover, not all animal experiments suggested a link between enhanced virulence and the investigated hemolytic toxin: For example *Voyich et al.* found no significant differences comparing the virulence of PVL-producing and non-producing CA-MRSA strains in mouse experiments [122][13]. Also another explanation why especially CA-MRSA strains produce high levels of PVL does exist: It was postulated that PVL is encoded by an integrated bacteriophage genome within the genome of *S.aureus* [119]. Since integrated bacteriophages upregulate transcription of viral genes under stress conditions and

[13] The significance of these findings was doubted by several groups stating that mouse leukocytes are not lyzed by PVL as in rabbits or humans. This would mean that the chosen animal model is not suitable to represent the *in vivo* situation in humans [123, 124].

treatment of CA-MRSA with antibiotics induces stress, a high level of PVL expression could be in fact the result of treatment of clinical CA-MRSA cases and not the cause of the disease [119].

The significance of β-HL in virulence contribution of *S.aureus* is discussed and some groups described β-HL only as minor virulence factor since it is mainly active on temperatures colder than 37°C [125, 126]. Moreover, a hemolytic effect in low concentrations against erythrocytes was so far mainly demonstrated with sheep erythrocytes and not in rabbit or human erythrocytes [8].

Taken together, epidemiological and experimental data suggest an important role for hemolysins like PVL, α-HL, γ-HL and PSMs in *S.aureus* infections, based on the formation of lytic transmembrane pores. This makes such hemolysins promising targets for therapeutic interventions or as antigens for vaccine development. However, the exact impact of some hemolysins remains to be elusive since the relevance of the existing data for the in *vivo* situation in humans is conflicting.

The role of PTSAgs

Intuitively, cross-linking of APC and T cells and subsequent activation of cross-linked T cells by superantigens seems to be adverse for *S.aureus*, since T cells play an important role in the clearance of pathogens and proinflammatory signals generally generate a hostile environment for pathogens [38, 127]. However, the expression of superantigens is beneficial for *S.aureus* after primary proliferation and expansion of the activated T cells, because superantigens induce T cell anergy and clonal depletion as long-term effect [22, 79]. This is caused by the induction and generation of $CD4^+CD25^+FoxP3^+$ regulatory T cells (T_{reg}), which suppress further expansion of T effector cells mediated by the release of interleukin-10 and tumor growth factor β (TGFβ) [128, 129, 130]. A long-term stimulus of superantigens during chronic infection could also be advantageous for *S.aureus*, because APC present superantigens and other staphylococcal derived antigens at the same time to T cells. Hereby a linked suppression was observed, also attenuating T cells reactive to other antigens than superantigens [128, 129]. This could hamper an effective T cell response even further.

Moreover, findings of *Kim et al* and *Tanriver et al.* could give another explanation: *Kim et al.* postulated, that RAG knockout mice lacking functional T cells show strong hypersensitivity against poly(I:C), a broadly used immunostimulant simulating viral infections. Such mice died 12-24h after treatment with a poly(I:C) dose sublethal for wild type mice by an abnormal secretion of cytokines by cells of the innate immune system and subsequent shock. This effect was abolished by an adoptive transfer of T lymphocytes proving a suppressive role of T cells in cytokine release by cells of the innate immune system [57]. Based on those findings, *Tanriver et al.* investigated in mouse studies in which way increased levels of suppressive T cells after PTSAg stimulation have an effect on cells of the innate immune system. The group found that PTSAg-specific T_{reg} cells were not only able to inhibit activation, but also migration and entry of natural killer cells (NK) and granulocytes to the local lymph nodes, which is important for the establishment of a local inflammation [129]. Due to this mechanism *S.aureus* could be able to avoid effective clearance of local infections by the immune system of the host.

An important role of PTSAgs is also suggested by epidemiological data: For example, epidemiological studies revealed that in clinical isolates of CA-MRSA strains significantly more often enterotoxins are produced than in less virulent MSSA strains [100, 121, 131] **(table 2)**. For instance *Fey et al.* found

that 31 out of 32 analyzed CA-MRSA strains produced either SEB or SEC. In contrast, none of the 32 analyzed HA-MRSA strains produced SEB or SEC [131].

Taken together, superantigens provide an immune evasion mechanism for *S.aureus* by preventing the generation of functional T effector cells, specific for staphylococcal antigens [22, 79]. Furthermore, the inhibition of local inflammation could be beneficial in the early establishment of an infection, since local inflammation is required to attract and activate leukocytes [129]. An important role for staphylococcal survival is also suggested by epidemiological data, since especially the highly virulent CA-MRSA strains produce PTSAgs [100, 131].

The role of exfoliative toxins

The ETs found in human *S.aureus* isolates cleave the epidermal cadherin Dsg1. Dsg1 is hereby responsible for the cell-cell junctions within the epithelial layers, generating the barrier function of the skin. Hence, proteolytic cleavage of Dsg1 destroys the cell-cell adhesions resulting in the rupture of epidermal integrity and epidermal detachment, which is described as SSSS [99, 105]. The subsequent loss of the barrier function of the skin is beneficial for *S.aureus*, because it facilitates percutaneous invasion [102]. Besides of the proteolytic capacity for the cleavage of Dsg1, also controversial data exists that also ETs might have superantigenic capacities to induce T lymphocyte proliferation: *Monday et al.* showed that purified ETA and ETB have besides of its proteolytic activity also the potential to induce proliferation of specific T cell subsets [109]. This is also in line with the results of *Rago et al.*, postulating that specific mutations of ETA decrease its mitogenic effects towards T cells [110]. In contrast to such findings, for example *Plano et al.* state that neither purified ETA nor ETB has the ability to promote proliferation of lymphocytes [111]. Furthermore, it was also not yet successful to prove the mitogenic effect of ETs structurally or mechanistically, for example by crystallography. This increases the chance that the observed T cell proliferation by ETs was caused by unknown confounding factors like for example contaminations [112].

Whether ETs possess a mitogenic activity or not, the overall contribution of ETs to *S.aureus* virulence seems to be lower than of hemolysins or the PTSAgs: Only approximately 5-11% of the characterized *S.aureus* strains express ETs and most studies don't describe an association of ETs and an enhanced virulence of the producing strain [116, 117, 132]. For example *Yokota et al.* found that only one out of 82 MRSA strains isolated from outbreaks in neonatal intensive care units in Japan produced ETA, whereas none of them produced ETB [132]. But on the other hand, there is also evidence that expression of ETs could increase the fitness of *S.aureus*: *Yamaguchi et al.* described in 2002 the emergence of three clonally different ET-producing MRSA groups in Japan [133]. Also *Peacock et al.* indepently found a significant association of ETA and enhanced invasiveness of *S.aureus* [121].

Taken together, the present literature suggests a role of ETs especially in the establishment of a staphylococcal infection and the development of SSSS, based on the proteolytic activity of ETs [27, 99]. This could facilitate the percutaneous invasion of *S.aureus* [27]. The mitogenic activity however remains to be investigated more in detail due the high controversity of the available publications and the lack of data explaining the mechanism of this mitogenicity. Since the most epidemiological studies do not find ETs significantly associated with highly pathogenic strains, the contribution of ETs to *S.aureus* virulence can be considered as of minor importance compared to hemolysins and PTSAgs.

Concluding remarks and outlook

This work describes that staphylococcal toxins are important virulence factors for *S.aureus* and explains the underlying mechanisms of their toxicity. Because of their important role in staphylococcal virulence, the development of therapeutic interventions or vaccines interfering with toxin binding and action could be a promising approach to reduce the disease and financial burden of *S.aureus* infections [134]. However, this work also shows that the high virulence of *S.aureus* cannot be attributed to a single or a few toxins, but is in fact the result of a broad range of different toxins and other virulence factors. The precise impact of the particular toxins in human infections remains mostly elusive, since the conclusions of the available epidemiological data are strongly dependent on the study-setup and the regions from which the analyzed isolates were obtained. Thus, a better epidemiological description of the genotypes of pathogenic *S.aureus* strains and their levels of toxin expression could provide a better picture of the importance of the toxins. Another problem is that *in vivo* mechanisms of toxin action were mainly described in animal models including sheep, mice and rabbits [7, 8]. The results describing the impact of the analyzed toxins varied significantly or were even contradictory, depending on the used experimental setup or animal model [7, 36, 110, 111]. Therefore further investigation to determine the underlying molecular reasons resulting in such conflicting outcomes could be fruitful in order to develop a suitable animal model which allows valid extrapolation to the *in vivo* situation in humans. Moreover, it could be fruitful to analyze the less described staphylococcal toxins more in detail, since for example other bicomponent hemolysins assembled of other S and F proteins than γ-HL or PVL are produced by up to 99% of human clinical *S.aureus* isolates, depending on the respective proteins [63, 64]. Although such toxins were only associated with weak hemolytic and leukotoxic activity, for example a role in dermonecrosis was described [8, 63]. Last but not least, further efforts to verify the hypothetical models of toxin binding and action of some toxins could improve the certainty of the postulated mechanisms: For example the proposed mechanisms of pore formation by PVL and PSMs require further investigation, since the postulated mechanisms of pore formation are only based on *in silico* predictions or the crystallographic structures of the non-functional monomers [67, 68, 78]. Solving the crystal structures of the functional pores could verify the presumed mechanisms of action and thus, giving more reliable information about potential drug targets to interfere with.

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Declaration in lieu of an oath

Herewith I affirm that I wrote this report by myself. I furthermore affirm that I labeled all information derived from the work of others.

Utrecht, …….. ……

(Urs Mörbe)