Identification of phagosomal escape relevant factors

in *Staphylococcus aureus* infection

DISSERTATION

zur Erlangung des Akademischen Grades

doctor rerum naturalium

(Dr. rer. nat.)

im Fach Biologie

eingereicht an der Fakultät für Biologie

der Julius-Maximilians-Universität Würzburg

vorgelegt von

Magdalena Urszula Grosz, M. Sc.

geboren am 25.06.1985 in Kattowitz

Würzburg, August 2015

Eingereicht am:

Mitglieder der Prüfungskommission:

Vorsitzender:

Erstgutachter: Prof. Dr. Thomas Rudel

Zweitgutachter: PD Dr. Knut Ohlsen

Tag des Promotionskolloquiums:

Doktorurkunde ausgehändigt am:

Table of contents

Summary

Staphylococcus aureus is a facultative Gram-positive human pathogen which can cause different severe infections. Staphylococci are phagocytosed by professional and non-professional phagocytes; they are strongly cytotoxic against eukaryotic cells and have been proposed to play a role in immune evasion by spreading within migrating phagocytes. This study investigated the post invasive events upon *S. aureus* infection. Strains which are able to escape the phagosome were identified and the responsible toxins were determined. Thereby innovative insights into host pathogen interaction were obtained.

A novel class of small amphipathic peptides with strong surfactant-like properties, the phenol soluble modulins, particularly PSMα as well as the leukocidin LukAB, are involved in phagosomal escape of the clinical *S. aureus* strains LAC, MW2 and 6850 in non-professional and professional phagocytes. Whereas, PSMβ, δ-toxin, α-toxin, β-toxin or phosphatidyl inositol-dependent phospholipase C did not affect phagosomal escape. By blocking the bacterial DNA-dependent RNA polymerase with rifampicin phagosomal escape is determined to start approximately 2.5 hours post infection. Phagosomal escape further was required for intracellular replication of *S. aureus*. Strains which are not able to escape cannot replicate in the acidic vacuole, whereas, the host cytoplasm offers a rich milieu for bacterial replication. Additionally, phagosomal escape, with intracellular bacterial replication induces the subsequent host cell death. This could be confirmed by an infection assay including *S. aureus* knockout mutants in *psmα* or *lukAB* which were significantly less cytotoxic, compared with those infected with escape-positive wild type strains.

Further, this study showed that phagosomal escape is not only mediated by bacterial toxins. Since, the phagocyte-specific cognate receptors for both escape relevant toxins, FPR2 (PSMα receptor) and CD11b (LukAB receptor) are produced in epithelial and endothelial cells only after infection with *S. aureus* in a calcium dependent fashion. The knockdown of both receptors using siRNA prevents *S. aureus* to escape the phagosome. Furthermore, blocking intracellular calcium release with the inositol trisphosphate receptor (IP3R) inhibitor 2-APB prohibits upregulation of *fpr2* and *cd11b* and subsequently phagosomal escape of *S. aureus*.

To conclude, the current study clarifies that phagosomal escape and host cell death are interplay of both, bacterial toxins and host cell factors.

Zusammenfassung

Staphylococcus aureus ist ein fakultativ Gram-positives Humanpathogen, dass verschiedene schwerwiegende Infektionen verursachen kann. Staphylokokken werden von professionellen und nicht-professionellen Phagozyten (Fresszellen) zu gleich aufgenommen. Desweitern sind sie stark zytotoxisch für eukaryotische Zellen. Außerdem wird vermutet, dass sie sich mittels migrierender Phagozyten dem angeborenen Immunsystem entziehen können. In dieser Studie werden die post-invasiven Ereignisse während einer Staphylokokken Infektion untersucht. Im Detail wurden Stämme identifiziert die aus den Phagosomen entkommen können und die dafür verantwortlichen Toxine. Im Zuge dessen wurden neue Erkenntnisse der Interaktion zwischen Bakterien und Wirtszellen gewonnen.

Eine neue Klasse von kleinen amphiphatischen Peptiden mit starken grenzflächenaktiven Eigenschaften (Surfactant), die sogenannten Phenol soluble modulins (PSMs) im Besonderen PSMα sowie das Leukozidin LukAB, sind am phagosomalen Ausbruch der klinisch relevanten *S. aureus* Stämmen LAC, MW2 und 6850 in nicht professionellen und professionellen Phagozyten involviert. Hingegen, sind PSMβ, δ-toxin, α-toxin, β-toxin oder Phosphatidylinositol abhängige Phospholipase C nicht am phagosomalen Ausbruch beteiligt. Durch die Hemmung der bakteriellen DNA-abhängigen RNA Polymerase mit Rifampicin wurde der Zeitpunkt für den Ausbruch auf etwa 2,5 Stunden nach der Infektion eingegrenzt. Der phagosomale Ausbruch ist weiterhin für die intrazelluläre Replikation von *S. aureus* notwendig. Während Stämme, die nicht ausbrechen können in der angesäuerten Vakuole nicht replizieren können, bietet das Zytoplasma ein reichhaltiges Milieu für die Vermehrung. Zudem wird der Pathogen induzierte Zelltod erst nach dem phagosomalen Ausbruch und mit anschließender Vermehrung ermöglicht. Nachgewiesen wurde dies mittels *psmα* und *lukAB* defizienten Mutanten welche signifikant weniger zytotoxisch waren als der Wildtyp Stamm. Diese Studie zeigt darüber hinaus, dass der phagosomale Ausbruch nicht nur durch bakterielle Toxine vermittelt wird. Sondern, dass die Phagozyten-spezifischen Rezeptoren für beide relevanten Toxine, FPR2 (PSMα Rezeptor) und CD11b (LukAB Rezeptor), in Epithel- und Endothelzellen nach Infektion mit *S. aureus* calciumabhängig produziert werden und für den Ausbruch notwendig sind. Der knockdown beider Rezeptoren mittels siRNA verhindert den Ausbruch. Wird der intrazelluläre Calciumstrom mittels des Inositoltrisphosphat Rezeptor (IP3R) Inhibitor 2-APB blockiert können die Gene *fpr2* und *cd11b* nicht hochreguliert werden und der Ausbruch wird ebenfalls verhindert.

Folglich zeigt diese Studie, dass der phagosomale Ausbruch und Pathogen induzierte Zelltod sowohl durch bakterielle Toxine als auch Wirtsfaktoren vermittelt wird.

1 Introduction

1.1 *Staphylococcus aureus*

Staphylococcus aureus (*S. aureus*) is a gram-positive, facultative anaerobic, nonmotile coccal bacterium which appears on microscopic image in clusters with a diameter of 0.8-1.2 µm (Lowy, 1998). The surgeon Sir Alexander Ogston identified staphylococci for the first time 1880 (Aberdeen, United Kingdom) in pus from an abscess in a knee joint (Ogston, 1984). Later, Rosenbach established the name *Staphylococcus aureus,* by the official system of nomenclature at that time, according to its coloured pigmentation when growing on agar plates. The designation consists of two ancient Greek parts *σταφυλόκοκκος*, "grape-cluster berry" as well Latin *aureus*, “golden” (Rosenbach, 1884). Staphylococci belong to the family of staphylococcaceae with 44 different species. Cocci do not sporulate and have a circular genome composed of ~2.8 million nucleotides which correspond to a thousandth part of the human genome with about three billion nucleotides (Kuroda et al., 2001). The core genome is composed of genes existing in all strains, including genes for essential functions like cellular metabolism, growth and replication. The variable part of the genome is about 10 % and differs between different linages. Up to 20 % of the genome consists of mobile genetic elements which often encode virulence factors and resistance genes (Lindsay et al., 2006).

S. aureus is a commensal as well as human pathogen that colonizes mucosal surfaces and is responsible for a wide variety of serious acute and chronic infections. Approximately 20 % of the healthy human population is asymptomatic permanently colonized and 60 % are transiently colonized whereas 20 % seem to be seldom or never concerned (Williams, 1963, Eriksen et al., 1995). Isolates of infected patients were analysed and exhibited that at least nosocomial infections are mostly endogenous (Wertheim et al., 2004) whereas nasal carriage is a major risk factor for several types of infections. Locations for infection sites include the skin, axillae, rectum, vagina, pharynx and gastrointestinal tract (Wertheim et al., 2005).

S. aureus developed some strategies to evade its elimination by the host´s immune system. It expresses several factors to interfere with the efficiency of professional phagocytes including neutrophils and macrophages which represent the first line of defence against infection. Secreted proteins by staphylococci inhibit complement activation and neutrophil chemotaxis or induce their lysis, neutralize antimicrobial defensin peptides and their cell surface is modified not to offer a target for the host. *S. aureus* is able to survive in phagosomes; the opsonisation by antibodies is inhibited through polysaccharides and protein expression. Superantigens change the normal humoral immune response which results in anergy and immunosuppression [reviewed in (Foster, 2005)].

1.2 Virulence factors

1.2.1 *S. aureus* toxins including exotoxins, enterotoxins and leukocidins

1.2.1.1 α-haemolysin (α-toxin)

S. aureus α-haemolysin, known as well as α-toxin, is considered as a prototype for the class of small β-barrel pore-forming cytotoxic toxins (Bhakdi and Tranum-Jensen, 1991, Parker and Feil, 2005). It is secreted as a water soluble monomer of 33.2 kDa (Bhakdi and Tranum-Jensen, 1991) and binds the host cell membrane by oligomerization into a heptamer (Figure 1) (Song et al., 1996, Gouaux, 1998). Electron microscopy led to the discovery of ring-like structures 10 nm in diameter with 6-7 subunits and a central pore of 2-3 nm (Fussle et al., 1981). The heptametrical structure was confirmed by resolving the crystal structure (Song et al., 1996). Pore formation allows passage of Ca^{2+} and K^{+}, ATP, and low molecular weight molecules up to 4 kDa (Bhakdi and Tranum-Jensen, 1991).

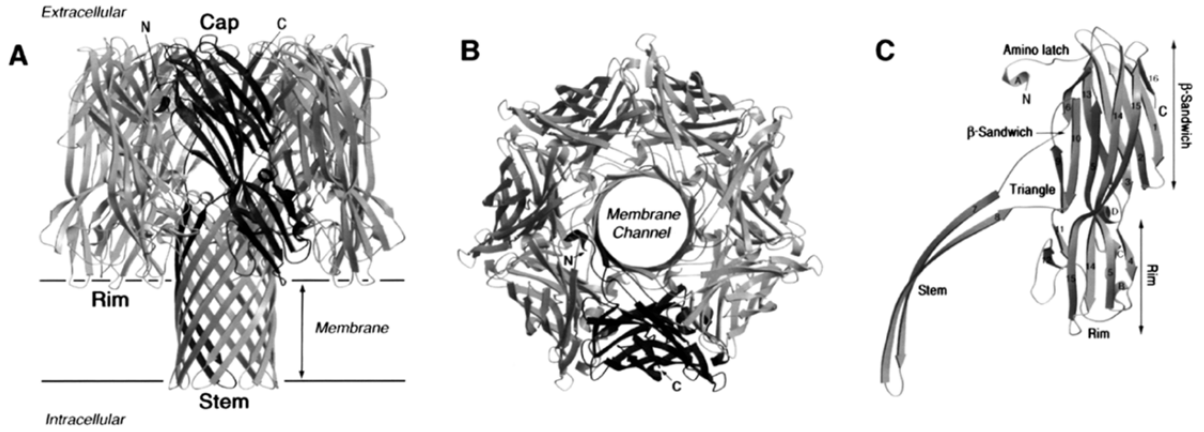

Figure 1: Structure of α-toxin. A) Division of a pore in different regions: Cap region to enter the pore, the membrane interfacing region (Rim) and membrane perforating stem. B) Top view of the heptamer. C) Ribbon representation of a monomer with the amino latch, β-sandwich domain, triangle region, rim, and stem. Figure published in (Gouaux, 1998).

In the early 1980s the gene encoding α-toxin was discovered utilizing a recombinant phage-based strategy that allows *E. coli* to lyse red blood cells (Kehoe et al., 1983). The *hla* locus is present in a single copy on the chromosome with conserved primary amino acid sequence in all sequenced *S. aureus* strains (Gray and Kehoe, 1984). Alpha toxin production is controlled by several global regulatory systems. Its main expression is controlled by the accessory gene regulator (*agr*) locus via the regulatory RNA molecule RNAIII (Novick et al., 1993). Further, the *in vitro* expression is modulated by the regulatory systems *sarA* and *saeR/S*, respectively (Xiong et al., 2006). Investigations with *S. aureus* wild-type and *hla* knockout mutant in strain LAC yielded that α-toxin is important for programmed cell death of human monocytes, T cells and B cells (Nygaard et al., 2012). Interestingly, *hla* contributes to eosinophil necrosis whereas no cell death was observed in α-toxin

treated neutrophils (Valeva et al., 1997, Prince et al., 2012). Significant differences in α-toxin sensitivity between human and rabbit erythrocytes, saturable toxin binding as well as temporal dynamics suggested a proteinaceous eukaryotic toxin receptor (Hildebrand et al., 1991). Furthermore, membrane lipids seem to be important for α-toxin binding as cholesterol or sphingomyelin depletion inhibits toxin binding and cytotoxicity. Vice versa, the addition of exogenous phosphocholine disrupts toxin binding and impairs haemolysis of rabbit erythrocytes (Valeva et al., 2006). Wilke and Wardenburg identified a member of a disintegrin and zinc-dependent metalloprotease (ADAM10) by mass spectroscopic analysis with rabbit and human erythrocyte ghosts as high-affinity α-toxin host binding receptor at low toxin concentrations (Wilke and Bubeck Wardenburg, 2010), whereas, pore formation at higher concentrations might be receptor-independent. The interaction of α-toxin with ADAM10 is required for pore formation and toxin-mediated cytotoxicity and toxin binding is followed by disruption of focal adhesions (Wilke and Bubeck Wardenburg, 2010). In turn cleavage of the adherence junction protein E-cadherin in alveolar epithelial cells results in disruption of the epithelial barrier function, followed by lethal acute lung injury (Inoshima et al., 2011). ADAM10 is a member of a protein family expressed as a type I transmembrane protein in diverse tissues and cells. It is composed of an N-terminal signal sequence followed by a prodomain (metalloprotease domain), a disintegrin domain, a cysteine-rich domains which facilitates protein-protein interactions at the cell surface, an epidermal growth factor (EGF)-like repeat, a transmembrane helix and a cytoplasmic C-terminus (Edwards et al., 2008). The proteolytic ectodomain release by ADAM protease is also known as “shedding” and constitutes a key mechanism for regulating the function of cell surface proteins. The absence or expression level of ADAM10 could be an explanation for cell type and species specificity (Berube and Bubeck Wardenburg, 2013).

1.2.1.2 β-toxin

S. aureus β-toxin (*hlb*) is a sphingomyelinase of type C by degrading cell surface sphingomyelins of eukaryotic cells. Further, β-toxin is a “hot-cold” haemolysin, after switching the temperature from 37 °C to 4 °C haemolysis will be completed on sheep blood agar plates. This is a result of changes in the physical properties of the cell membrane. More, it has been shown to induce apoptosis by generation of the ceramide that is produced after sphingomyelin hydrolysis (Bayles et al., 1998). Beta-toxin belongs to the structural-superfamily of DNase I (Pearl et al., 2003) and is able to form covalent cross-links to itselfs in the presence of DNA thereby producing insoluble nucleoprotein *in vitro*. In addition, β-toxin stimulates biofilm formation *in vivo* in rabbit endocarditis model (Huseby et al., 2010). However, most virulent *S. aureus* strains are phenotypically *hlb* negative due to prophage

insertion into the structural gene (Coleman et al., 1991). Moreover, up to 97 % of *S. aureus* isolated from bovine mastistis were producing β-toxin whereas only 10 -15 % of isolates from humans were β-toxigenic (Aarestrup et al., 1999) suggesting that it only plays a minor role in human pathogenesis. Interestingly, a lack of both, catalase and β-toxin, has been demonstrated to result in attenuated virulence in murine and bovine models but to increased intracellular survival of bacteria in macrophages and epithelial cells (Martinez-Pulgarin et al., 2009).

1.2.1.3 Bi-component toxins: Leukotoxins

Bi-component toxins are composed of two polypeptides, an S-subunit ("slow eluting") and F-subunit ("fast eluting") based on their electrophoretic mobility. Both act synergistically to form cation selective pores in the plasma membrane of target cells. As a result cell death is induced by osmotic imbalance in connection with the cation flux (Menestrina et al., 2003). There are different leukotoxins produced by *S. aureus*: γ-hemolysin (HlgAB and HlgCB), Panton-Valentine leukocidin (PVL; LukS-PV and LukF-PV) (Panton and Valentine, 1932), LukED and LukEDv (Gravet et al., 1998, Morinaga et al., 2003) and LukGH (Ventura et al., 2010) also termed LukAB (Dumont et al., 2011). Studies on toxin structure (Olson et al., 1999) and the underlying mechanism of pore formation were mainly performed with γ-hemolysin or PVL, but are thought to be similar for all these toxins. Each polypeptide binds to the cell surface first as a monomer before oligomerizing into a prepore formed by four S components alternatively arranged with four F components. The complete hetero-octamer permits as a β-barrel pore the passage through the plasma membrane leading to host cell lysis (Menestrina et al., 2001).

PVL is highly cytotoxic against human neutrophils, to a lesser degree against monocytes and macrophages, but is not cytotoxic for lymphocytes (Gauduchon et al., 2001, Panton and Valentine, 1932). Moreover, PVL is species specific since human and rabbit neutrophils are highly susceptible, whereas murine or macaque neutrophils are resistant to the leukocidin. The molecular basis for differences in PVL responsiveness is not understood, although a myeloid-specific receptor has been proposed (Gauduchon et al., 2001). PVL is encoded by a prophage which is found in a variety of CA-MRSA isolates (Vandenesch et al., 2003). Its expression has been linked to soft tissue infections and necrotizing pneumonia (Gillet et al., 2002). However the role of PVL in necrotizing pneumonia is controversial, since a suitable animal model of the disease do not exist (Bubeck Wardenburg et al., 2007, Voyich et al., 2006, Labandeira-Rey et al., 2007). Spaan et al. resolved the present contradictory results by identifying the human complement receptors C5aR and C5L2 as host targets of PVL (Spaan et al., 2013). LukS-PV subunit binds C5aR and C5L2 thereby inducing pore formation. In addition, the core region of C5aR determines the species specificity of PVL and is critical to induce

pore formation. PVL-induced pore formation by C5aR is blocked by the chemotaxis inhibitory protein of *S. aureus* (CHIPS) resulting in protection of human neutrophils (Spaan et al., 2013).

The most recently identified leukocidin, LukAB (also termed as LukGH), was discovered by exoproteome analysis (Dumont et al., 2011). The sequence similarity among these leukocidins ranges from 60 % to 80 % with the exception of LukAB, which is only 30–40 % similar to the other leukocidins. LukAB is required and sufficient to kill human neutrophils, macrophages and dendritic cells. Furthermore, contributes *lukAB* expression *in vivo* within abscess in a mouse model to the pathogenesis of MRSA (Dumont et al., 2011). Exposure of *S. aureus* to polymorphonuclear leukocytes (PMNs) activates the *lukAB* promotor. LukAB cytotoxicity proceeds not only through extracellular bacteria but also facilitates the escape of *S. aureus* phagocytosed by PMNs (DuMont et al., 2013b). The α-subunit of the αM/β1 integrin (CD11b/CD18), also known as macrophage-1 antigen (MAC-1), is a complement receptor and were identified as cellular receptor for LukAB (DuMont et al., 2013a). LukAB directly interacts with CD11b, specifically it I-domain, which is responsible for the species specificity of LukAB. CD11b thus renders human neutrophils sensitive for LukAB. Extracellular *S. aureus* use the LukAB receptor for membrane destruction while phagocytosed staphylococci profit LukAB-mediated targeting of CD11b to cause cell damage and promotes escape from within (DuMont et al., 2013a). An additional difference between LukAB and other leukotoxins (e.g. PVL) is a markedly attenuated and slowed calcium ion flux in PMNs (Yanai et al., 2014).

1.2.1.4 Phenol soluble modulins

Phenol soluble modulins (PSMs) are a family of small amphipathic peptides. Initially, PSMs were isolated from the phenol-soluble fraction of *S. epidermidis* culture filtrate as a proinflammatory complex (Mehlin et al., 1999). PSMs are secreted into the growth media and they can be identified using high-performance liquid chromatography, electrospray ionization-mass spectrometry and Edman degradation (Gonzalez et al., 2012, Wang et al., 2007).

PSMs were categorized into six types: PSMα, PSMβ; PSMγ (also kwon as S. aureus δ-toxin; *hld)*; PSMδ; PSMε and PSM-*mec*. PSM operons are encoded by the core genome (Wang et al., 2007, Mehlin et al., 1999). The PSM-*mec* peptide is located in staphylococcal chromosome cassette methicillin-resistance island (SCC*mec*) which is an important mobile genetic element (MGE) of MRSA strains that is involved in resistance to antibiotics and metal (Queck et al., 2009). There are currently 11 SCC-*mec* types (SCC*mec* I-X), and for *S. aureus* SCC*mec* types II, III and VIII were identified (Chatterjee et al., 2011). PSMs can be classified into two groups based on molecular weight and length of peptide chain. The shorter α-type PSMs with a length of 20-26 amino acids (aa) include

PSMα1-4, PSMγ and PSM-*mec* (Figure 2 A) and the longer β-type PSMs (PSMβ1-2) with 44 aa. These peptides are usually below 5 kDa (Wang et al., 2007). All four PSMα peptides are encoded by *psm*α operon. There is only little or no homology between other known protein-coding genes from database and for *psm*α genes, with one exception for δ-toxin with approximately 40 % sequence similarity to *S. epidermis* δ-toxin (Mehlin et al., 1999). *S. aureus* δ-toxin is encoded by *hld* gene, with in the RNAIII domain of the accessory gene regulator (*agr*) quorum-sensing operon. PSMβ peptides share 35-73 % identity with gonococcal growth inhibitor 1, 2 and 3 of *Staphylococcus haemolyticus* and to SLUSH polypeptides (Frenette et al., 1984). Because gonococcal growth inhibitors and SLUSH peptides have a haemolytic activity (Wade et al., 2012, Donvito et al., 1997), similar molecular weights and aa composition as *S. aureus* PSMβ (Mehlin et al., 1999), it is possibly that the staphylococcal peptides exhibits similar biological functions.

The structure of PSMs were analysed with circular dichroism and revealed that they are organized in a highly conserved amphipathic α-helices (Figure 2 C) with hydrophilic and hydrophobic sides (Queck et al., 2008, Wang et al., 2007). Their haemolytic activity is characterized by pore formation with membrane perforation and subsequently cell death (Figure 2 D). Thereby, PSM peptides insert into hydrophobic membranes to form cation selective ion channels (Mehlin et al., 1999). The hydrophobic domains interact with the cell membrane whereas the hydrophilic domains form the inner aqueous pore, hence PSM activity can be receptor independent (Donvito et al., 1997).

Most sequenced *S. aureus* strains encode PSMs, but the comparison between CA-MRSA and HA-MRSA strains of *in vitro* cultures demonstrate that CA-MRSA strains produce PSMs in high concentrations which contribute to their virulence (Wang et al., 2007). The expression of PSMs is controlled by the agr quorum-sensing system. It is composed of two transcriptional units RNAII and RNAIII which originate from the bidirectional P2 and P3 promoters, respectively (Yarwood and Schlievert, 2003). The P2 promoter encodes four proteins that generate the sensing mechanism, namely AgrA, AgrC, AgrD and AgrB (Novick, 2003). Transcription of P3 operon induces RNAIII, a regulatory RNA that induces many virulence factors, including toxins (δ-toxin; TSS toxin-1), surface adhesins, the capsule and proteases (Arvidson and Tegmark, 2001). Mutation in the RNAII region of *agr* leads to blocked PSM peptide expression, including PSMα, PSMβ and SCC*mec*-encoded PSM-*mec* (Vuong et al., 2004, Queck et al., 2009). Further, it was shown that *agrA* binds directly to the promotor of the *psm* operon (Vuong et al., 2004, Wang et al., 2007) and exhibits its direct regulatory function on PSM expression (Queck et al., 2008).

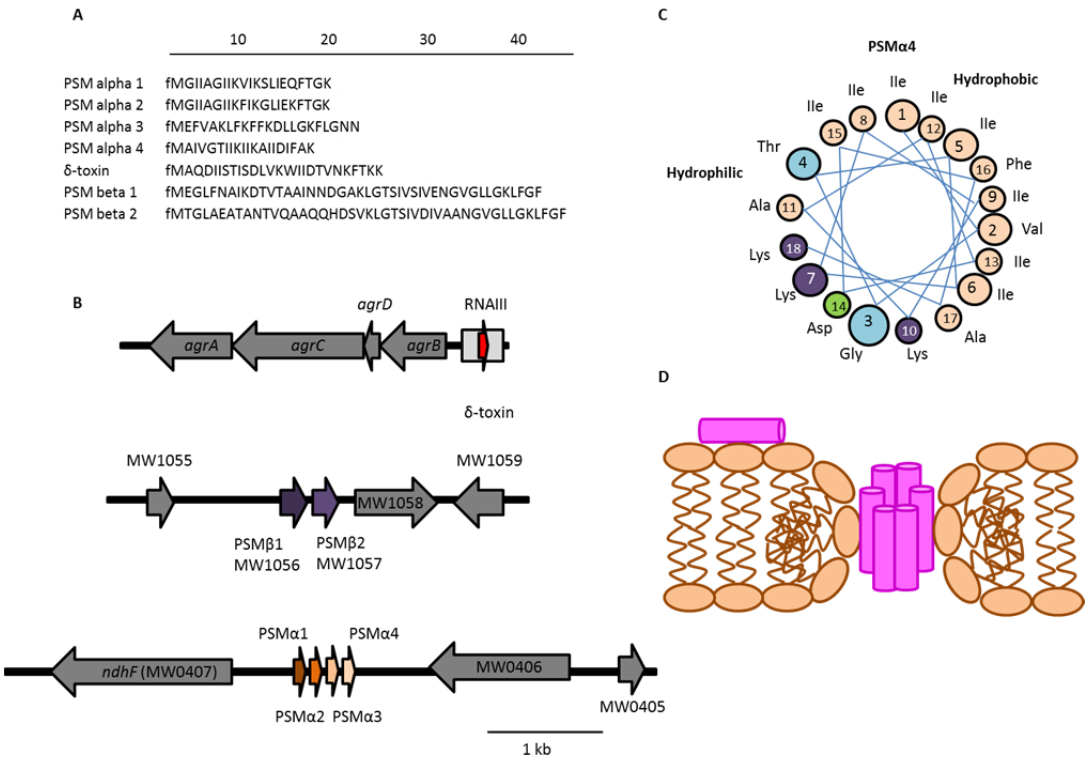

Figure 2: Phenol-soluble modulins (PSMs) in *S. aureus*. A) Amino acid sequence of the PSMs which are formylated (f) at the N-terminal methionine residue. Scale bar indicate amino acid length. B) Location of PSM genes in the genome of USA400 MW2. C) Example of the α-helical wheel arrangement for PSMα4. D) Receptor independent pore formation by PSMs. Figure modified from (Wang et al., 2007, Otto, 2014).

PSMs are secreted by a specific phenol-soluble modulin transporter (Pmt). The Pmt is made up of four genes (*pmtA*, *pmtB*, *pmtC* and *pmtD*) encoding an ATP-binding cassette (ABC) transporter with two separate membrane parts (PmtB and PmtD) and two ATPases (PmtA and PmtC) (Chatterjee et al., 2013). Furthermore, the authors of the study showed that the pmt system is regulated by agr system and that the transporter was essential for bacterial growth, immunity towards secreted PSMs and defence against PSM-mediated bacterial interference (Chatterjee et al., 2013).

PSMs have multiple functions in staphylococcal pathogenesis. Among other things they efficiently lyse erythrocytes (Cheung et al., 2012) and neutrophils (Wang et al., 2007, Cheung et al., 2012), regulates biofilm development (Periasamy et al., 2012) and trigger receptor-mediated inflammatory responses. Especially PSMα showed the most pronounced proinflammatory activity, as neutrophil activation was indicated by $gp91^{phox}$ and CD11b expression, further chemotaxis and Ca^{2+} release was measured as well as release of cytokine interleukin (IL)-8 but not tumor necrosis factor (TNF)-α or IL1-β. From all tested PSM peptides, PSMα3 caused the most substantial lysis of neutrophils. Further, it was shown that clarified culture media of PSMα deletion strains possess a greatly reduced capacity

to lyse human neutrophils. By contrast PSMβ and δ-toxin have only weak leukocidal properties and are less chemotactic for neutrophils (Wang et al., 2007).

While, the cytolytic activity of PSMα is receptor independent, is the inflammatory response triggered by interaction of formyl peptide receptor 2 (FPR2) with all know PSM peptide classes (Kretschmer et al., 2010). FPR2 is a G-coupled receptor with 7 transmembrane domains and is present on the surface of neutrophils and leukocytes (Fu et al., 2006). FPR2 previously has been implicated in control of endogenous inflammatory processes (Kretschmer et al., 2010). FPR2 senses PSMs in nanomolar concentrations and initiates proinflammatory neutrophil responses to CA-MRSA but not HA-MRSA. Inhibition of FPR2 or deletion of PSMs led to reduced detection of abilities of CA-MRSA by neutrophils (Kretschmer et al., 2010).

Experiments with human serum demonstrate that cytolytic activity of PSMs was inhibited. Further, serum treatment fully blocked cell lysis and FPR2 activation of neutrophils, suggesting that PSMs can only exert their function as intracellular toxins in intracellular or lipoprotein-free niches (Surewaard et al., 2012). Surewaard and colleagues showed that PSM-dependent cytolysis of neutrophils happened after phagocytosis and deletion of *psm*α significantly decreased bacterial survival. Hence, PSMα is necessary and sufficient for increased neutrophil cell death and increased survival of *S. aureus* (Surewaard et al., 2012). Knockout mutants in *psm*α showed significantly decreased ability to cause skin lesions and markedly reduced mortality bacteraemia mice model (Wang et al., 2007).

Biofilms are designated as agglomerations of bacteria surrounded in an extra cellular matrix (ECM) (Costerton et al., 1995). Bacteria form biofilms as protection against host defence during colonization and infection, by decreasing the efficiency of antimicrobial peptides and by preventing phagocytosis (Otto, 2006). Biofilm generation includes three stages: attachment, maturation and detachment (Otto, 2008, O'Toole et al., 2000). PSMs were identified as the key players in biofilm maturation processes, more in precisely in formation of channel-containing biofilm structure, detachment, control of expansion and dissemination from biofilms *in vivo*. The absence of already one class of PSMs or δ-toxin shown influence on biofilm volume, thickness, roughness and channel formation. Further, *agr* deficient mutants produce extremely thick biofilms as a result of abnormal biofilm development caused by the lack of PSM-dependent biofilm structuring and expansion (Periasamy et al., 2012).

1.2.2 Staphylococcal enzymes, superantigens and protein A

Extracellular proteases produced by *S. aureus* have important roles in virulence. They degrade host molecules or interfere with host metabolic or signalling cascades. Currently there are 11 major

proteolytic enzymes which consist of aureolysin (Aur) a metalloprotease, staphopain A (ScpA) and staphopain B (SspB) which are two cysteine proteases, a serine glutamyl endopeptidase (serine protease SspA or V8), and six serine-like proteases that are SspA homologues (SplABCDEF) (Reed et al., 2001, Shaw et al., 2004). These serine protease-like proteins are maximally expressed during the transition from exponential into stationary growth phase and are positively controlled by the *agr* system. A *spl* operon deletion mutant revealed no difference in virulence compared to the parental strain (Reed et al., 2001). Aureolysin cleaves after hydrophobic residues, including the protein insulin B. In addition, PSMs are degraded by aureolysin thereby influencing bone remodelling during osteomyelitis (Cassat et al., 2013). Thus, proteases appear to be important for pathogen-mediated evasion of the human complement system (Jusko et al., 2014). The exfoliative toxin serine protease cleaves desmosomal cadherin's of the superficial skin layer causing a severe damage of the skin known as staphylococcal scaled skin syndrome (SSSS) (Bukowski et al., 2010). Further, invasion into tissue is facilitated by secreted proteases by cleaving factors, such as human a1-proteinase inhibitor (Potempa et al., 1986), the heavy chains of human immunoglobulin (Prokesova et al., 1992) and elastin (Potempa et al., 1988). *S. aureus* is able to disseminate by degrading human fibronectin, fibrinogen and kininogen by staphopain B, which thereby mimics the specificity of plasma serine proteases (Massimi et al., 2002). Also, Kolar et al. revealed that extracellular proteases are important for resisting phagocytosis by human leukocytes, and proteomic analysis demonstrated exo-proteases as key mediators of virulence-determinant stability (Kolar et al., 2013).

S. aureus superantigens (SAgs) include a class of exotoxins (enterotoxins) and the toxic shock syndrome toxin-1 (TSST-1). They are able to activate T cells which exclusively express the major histocompatibility complex (MHC) class II molecules (Marrack and Kappler, 1990, Dellabona et al., 1990). SAgs share the ability to bind with low affinity to the variable region (V) of the beta chain of the T cell receptor via their canonical structure, composed of smaller N-terminal domain with two β-sheets and a larger C-terminal domain with a central helix and a five-strand β-sheet (Li et al., 1999). Principal dual binding is required for T cell activation and subsequent cytokine release. The first disease linked to SAgs was staphylococcal food poisoning which appeared in the 1930s (Dack et al., 1930). Further, TSS caused by TSST-1 was described first in 1927 by Franklin Stevens (Stevens, 1927) and termed later "toxic shock syndrome" by Todd and colleagues in 1978 (Todd et al., 1978). The pathogenesis is due to SAgs-induced massive cytokine release by activated T cells, but at the same time lacking neutralizing antibodies to the particular SAgs with symptoms including fever, rash, hypotension, multiorgan involvement and convalescent desquamation [reviewed in (McCormick et al., 2001)].

Additional studies showed possible role for SAgs in disease including Kawasaki disease, atopic dermatitis, and chronic rhinosinusitis [reviewed in (Xu and McCormick, 2012)]. However, it has to be resolved which role or benefit SAgs play during *S. aureus* life-cycle.

Staphylococcal protein A (SpA) is a covalently cell wall anchored protein of 42 kDa present in most *S. aureus* strains. It is composed of repeated units (A-E), each unit is able to bind the Fc part of different IgG subclasses in a non-antigen specific manner (Uhlen et al., 1984). Expression of SpA makes staphylococci less susceptible to neutrophils as a result of the IgG Fc-binding property of SpA (Peterson et al., 1977). Actually, studies including strains with a mutation in the gene encoding protein A were slightly less virulent and caused less septic arthritis in a mouse model (Palmqvist et al., 2002).

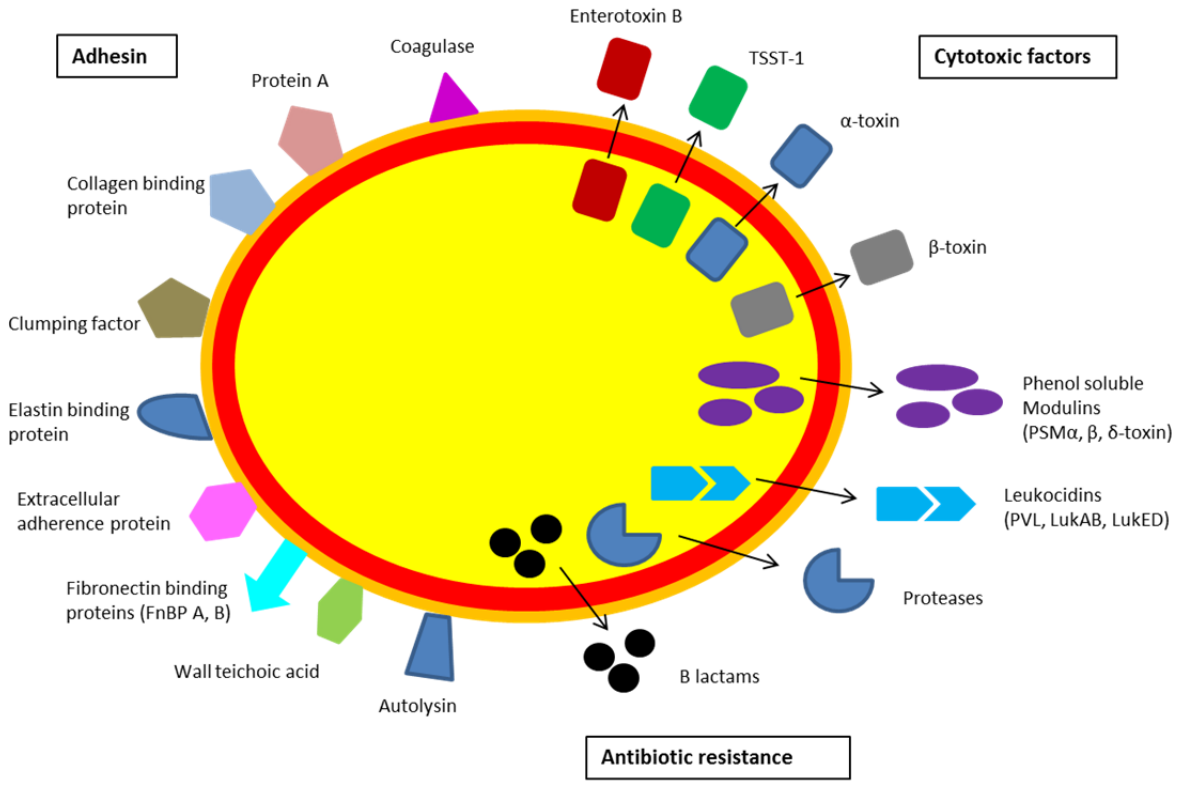

Figure 3: Overview of *S. aureus* pathogenic factors. *S. aureus* express surface proteins which contribute to adherence, and secreted proteins such as toxins, leukocidins and proteases which mediated cytotoxicity and β-lactams which contribute to antibiotic resistance.

1.3 Pathogenesis and medical relevance of MRSA and *S. aureus*

In the 1940s, *S. aureus* infections were routinely treated with penicillin but staphylococci quickly developed resistance by acquiring the gene penicillinase (Barber and Rozwadowska-Dowzenko, 1948). Subsequently, methicillin was introduced but only about one year later the first methicillin-resistance *S. aureus* (MRSA) (Barber, 1961) strains appeared and spread throughout the world

(Barrett et al., 1968). Methicillin resistance is caused by the *mecA* gene which is encoded on a mobile genetic element found in all MRSA strains, a genomic island designated staphylococcal cassette chromosome *mec* (SCC-*mec*) (Katayama et al., 2000). To date, *S. aureus* developed resistance to almost all available antibiotics. Multidrug-resistant MRSA strains that are unaffected to methicillin and several other antibiotics become frequent in the hospital settings (Lowy, 2003).

MRSA is the leading cause for hospital-associated (HA) infections (Lowy, 1998) whereby nasal colonisation with staphylococci increases the risk of infection for patients during treatment especially, for postoperative wound infections or bacteraemia (von Eiff et al., 2001, Wenzel and Perl, 1995). Furthermore, carrier of MRSA showed a 13-fold increased risk to develop an MRSA-infection during inpatients stay compared with those colonised with MSSA strains (Cosgrove et al., 2005). In the United States, about half a million people acquire staphylococcal infections each year thus raising health-care costs by 14 billion dollars per year (Noskin et al., 2007). In European countries HA-MRSA infections are estimated to affect more than 150,000 patients annually resulting in approximately EUR 380 million to EU healthcare system (Kock et al., 2010). Since the 1990s, community-associated methicillin-resistant *S. aureus* (CA-MRSA) increased throughout the world with first cases described from Australia (O'Brien et al., 2004) and the United States (Herold et al., 1998). About 90 % of CA-MRSA infections are present as skin and soft tissue infections. However, CA-MRSA may cause different types of infection, ranging from mild skin infections to severe abscesses, sepsis, and necrotizing pneumonia (Kaplan et al., 2005, Seybold et al., 2006). Infection with CA-MRSA is not focused to a specific group but the combination of close body contact and low personal hygiene may cause an outbreak, demonstrating that everyone can be infected by CA-MRSA (Otto, 2010).

Virulence factors play an important role in developing diseases however it is not completely understood for all factors. Surface proteins are called “microbial surface components recognizing adhesive matrix molecules” (MSCRAMMs) that facilitates adherence to host tissue. They bind polysaccharides such as collagen, fibronectin, and fibrinogen. MSCRAMMs appear to play a role in initiation of endovascular infections, bone and joint infections and prosthetic-device infections (Patti et al., 1994, Menzies, 2003). Staphylococci can form biofilms on host and prosthetic surfaces, which are used for persistence and allows evasion of host defences and antimicrobials (Donlan and Costerton, 2002). *In vitro*, *S. aureus* invades and survives inside epithelial and endothelial cells which permit to escape host defences, mainly in endocarditis (Moreillon et al., 2002). Small-colony variants (SCVs) contribute to persistence as well as to recurrent infections (Proctor et al., 1995). Invasive skin infections and necrotizing pneumonia are caused by CA-MRSA, expression leukocidins (PVL) and abscess formation are associated with capsular polysaccharides (Foster, 2005, Tzianabos et al., 2001). To name only a few examples how *S. aureus* virulence factors contribute to disease development.

1.4 Invasion mechanisms of *S. aureus* in non-professional phagocytes

S. aureus possesses different ways to adhere to the host cell surfaces (Figure 4). Internalization occurs on the one hand via a zipper-like mechanism which involves integrins, fibronectin (Fn) and fibronectin-binding proteins (FnBPs) in non-professional phagocytes. Integrins belongs to a superfamily of cell adhesion receptors. They are cation-dependent glycoprotein transmembrane receptors containing noncovalently associated α- and β-subunits; both subunits contribute to the ligand-binding side of the heterodimer. Important ligands are intercellular adhesions molecules (ICAMs) which are present on inflamed endothelial cells or antigen-presenting cells. The extracellular binding domain of integrins recognizes RGD or LVD sequences in ligands including Fn, fibrinogen, vitronectin, or laminin (Takada et al., 2007). Fibronectin is a glycoprotein located in the extracellular matrix and it is well-known to be involved in adhesion of many bacteria, including *S. aureus*. Staphylococci express two FnBPs encoded by the genes *fnbA* and *fnbB* (Jonsson et al., 1991). Fibronectin forms a bridge between FnBPs on bacterial side and integrin α5β1 on host cell side. However, it is not an active bacterial process because formaldehyde-fixed and live staphylococci were equally invasive. FnBP deficient mutants lost invasiveness in multiple cell types including epithelial cells, endothelial cells and fibroblast (Sinha et al., 1999, Peacock et al., 1999). FnBP mediated *S. aureus* invasion was shown as well for osteoblast (Ahmed et al., 2001) and keratinocytes (Edwards et al., 2011). The contribution of integrin α5β1 during *S. aureus* internalization in non-professional phagocytes was demonstrated by blockage experiments with antibodies which reduced bacterial uptake in 293T cells (Sinha et al., 1999), HUVECs (Massey et al., 2001) and keratinocytes (Kintarak et al., 2004). Furthermore, Dziewanowska and colleagues identified that the host heat-shock protein Hsp60 interacts with FnBPs. The pretreatment of epithelial cells with a specific antibody for Hsp60 significantly reduced *S. aureus* internalization (Dziewanowska et al., 2000). Another heat-shock protein which is linked to *S. aureus* internalization is Hsc70. It was shown that Hsc70 interacts with *S. aureus* autolysin (Atl). *Atl*-deficient strains were to a lesser extent endocytosed by eukaryotic cells. Two mechanisms have been proposed for Atl-dependent internalization i) a direct interaction with Hsc70 or ii) an indirect interaction via Fn connected to integrin α5β1 (Hirschhausen et al., 2010). The extracellular adherence protein (Eap) stimulates the adherence of staphylococci to epithelial cells (Palma et al., 1999) and to fibroblasts (Hussain et al., 2002). Eap is a secreted protein which can bind back to the bacterial surface (SERAM) and binds to fibronectin, fibrinogen and prothrombin, forms oligomers and agglutinates *S. aureus* (Haggar et al., 2003). However, the host cell receptor of Eap-dependent invasion is hitherto unknown.

Toll like receptors (TLRs), especially TLR2, are also relevant in *S. aureus* infection. Their cytoplasmic domain is required for signalling to activated NF-κB (Uematsu and Akira, 2006) and the extracellular

motif (leucine rich repeat, LRR) is responsible for the recognition of pathogen-associated molecular patterns (PAMPs) (Akira and Takeda, 2004). TLR2 recognize different PAMPs including lipopeptides, LTA, PGN or PSMs of *S. epidermidis* (Akira et al., 2006). The receptor is involved in internalization of *S. aureus* in human umbilical cord blood-derived mast cells (Rocha-de-Souza et al., 2008). CD36, a membrane glycoprotein and member of the class of B scavenger receptor family, serves as a coreceptor for TLR2. CD36 acts as sensor for diacylglyceride (DAG) recognition through the TLR2/6 heterodimer resulting in bacterial invasion in phagocytic cells (Hoebe et al., 2005). The overexpression on CD36 in 293T cells lead to the uptake of *S. aureus* (Stuart et al., 2005) and in HeLa even a colocalization of CD36 with staphylococci was observed (Leelahavanichkul et al., 2012).

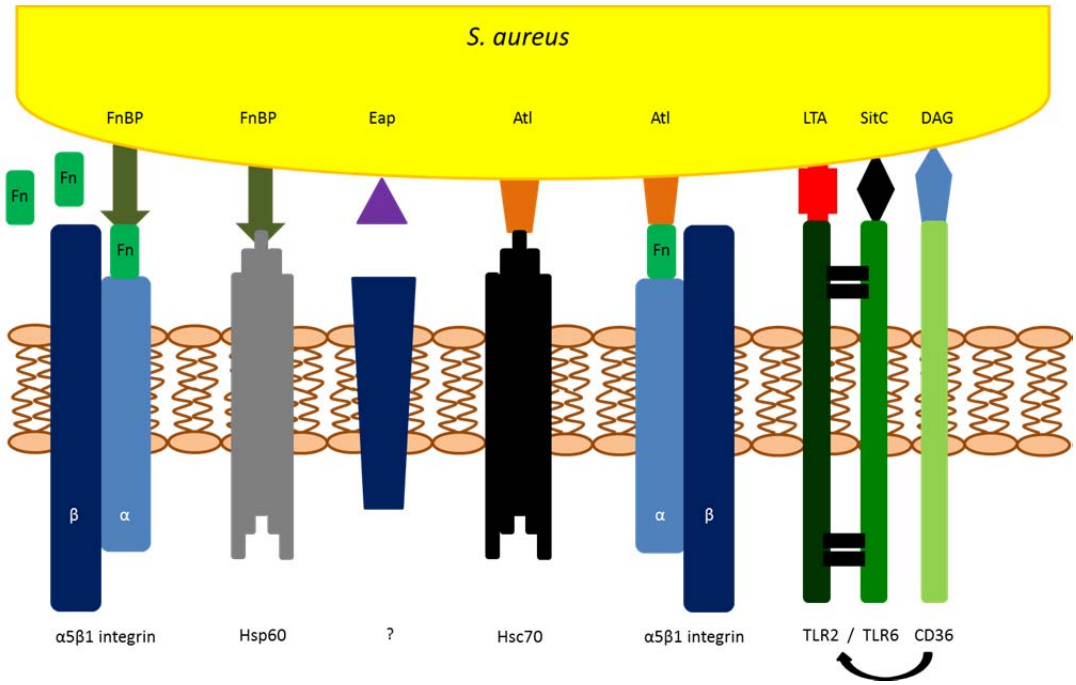

Figure 4: Different mechanisms and receptors involved in *S. aureus* internalization in non-professional phagocytes. The first mechanism for internalization involved α5β1 integrin receptor and is mediated by Fibronectin (Fn) and Fibronectin-binding protein (FnBP). Heat-shock protein (Hsp) 60 interacts with FnBPs as well mediating internalization. Extracellular adherence protein (Eap) contributes to staphylococcal adherence and internalization with currently unknown host cell receptor. *S. aureus* autolysin (Atl) can bind the host chaperon Hsc70 or via Fn to α5β1 integrin resulting in internalization. Further, TLR2/TLR6 dimer recognizes PAMPs including lipoteichoic acid (LTA) and lipoprotein (SitC) thereby facilitating *S. aureus* invasion. CD36 acts as coreceptor of TLR2/TLR6 and recognises diacylglycerides (DAG) of *S. aureus*. Figure modified from (Alva-Murillo et al., 2014, Hirschhausen et al., 2010).

1.5 Phagosomal escape

Phagocytosis is a property of professional phagocytes including polymorphonuclear leukocytes (neutrophils), dendritic cells and macrophages, but it is not unique to these cells. So-called non-professional phagocytes include epithelial cell, endothelial cells, keratinocytes and osteoblasts, which readily take up adhesin/invasin producing bacteria by a zipper-mechanism (ligand-receptor interaction) [reviewed in (Haas, 2007)]. Phagosomal maturation includes the acidification of the

phagosomal lumen to pH 4.5 by a membrane-located proton-pumping adenosine trisphosphate (ATPase) complex and bacterial clearance [(Figure 5)reviewed in (Haas, 2007)].

Bacterial pathogens developed several strategies to avoid phagolysosomal killing. Maturation of the endocytic vesicles is delayed by pathogens such *Chlamydia* spp. (Eissenberg et al., 1983), *Mycobacterium tuberculosis* (Armstrong and Hart, 1975) or *Legionella pneumophila* (Tilney et al., 2001), whereas destruction of the endocytic membrane has been described for *Listeria monocytogenes* through pore-formation by listeriolysin O (LLO) (Gaillard et al., 1987). Further bacteria which destroy the endocytic membrane are *Shigella flexneri*, *Rickettsia* spp. (Goetz et al., 2001) or *Francisella tularensis* (Clemens et al., 2004).

In 1998 Bayles and colleagues showed that bovine mastitis isolates of *S. aureus* escaped from endosomes of bovine mammary epithelial cell line (MAC-T) and subsequently induced programmed cell death in the host cell (Bayles et al., 1998). Patients with cystic fibrosis (CF) are frequently colonized with *S. aureus* and suffer to persistent and relapsing infection which may be related to staphylococci actively residing inside epithelial cells. Kahl and colleagues investigated that induction of apoptosis requires bacterial internalization and replication (Kahl et al., 2000). How *S. aureus* escapes form epithelial cells is not known in much detail. A functional agr system is required for phagosomal escape as *agr*-mutants were unable to escape from MAC-T cells (Shompole et al., 2003). Whereas in CF cells escape seem to be mediated by agr-dependent α-toxin (Jarry et al., 2008) there is no evidence for α-toxin dependent escape in CFTR proficient cells (Giese et al., 2009, Lam et al., 2010).

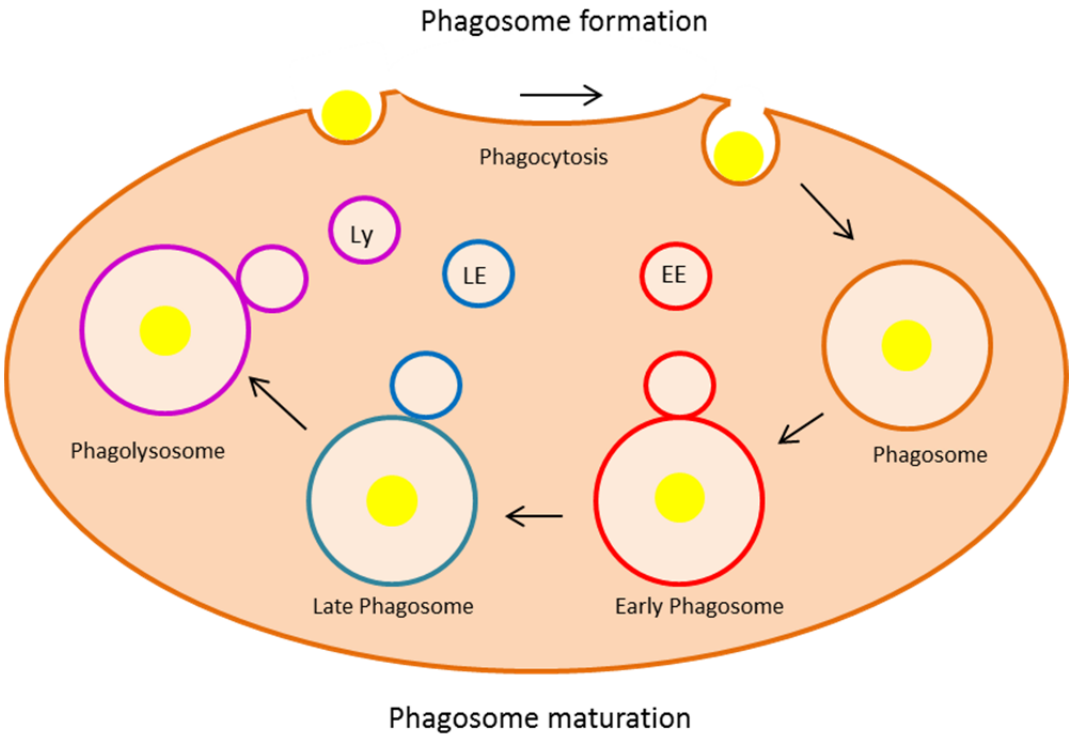

Figure 5: Schematic overview of phagosome maturation. Phagocytosis of bacteria via phagocytic receptors is followed by cytoskeleton rearrangements and pseudopod extensions to surround and fuse around the bacteria creating a phagosome. The early endosome loses plasma membrane associated actin and phosphatidylinositol 4,5-bissphotphate (PtdIns(4,5)P2. Early phagosome interacts with early phagosomes and is positive for ras-related protein 5 (Rab5), early endosome antigen 1 (EEA1), syntaxin 13, endobrevin and phosphatidylinositol 3-phosphate (PI(3)P). The late phagosome becomes more acidic inside (pH 5-6) lost some of the early phagosome markers and acquires late markers. Late Phagosome is positive for Rab7, mannose-6-phosphate receptor (M6PR), vesicle-associated membrane protein 7 (VAMP7), syntaxin7, vATPase, lysosomal associated membrane protein 1/2 (LAMP1-/-2). The phagolysosome is formed when late phagosomes fuse with lysosomes which contain hydrolases (nucleases, lipases, glycosidases, proteases and cathepsins) and a highly acidic content (pH 4.5). Phagolysosomes are decorated with LAMP-1, MSPR and vATPase H^+ ion pump.

1.6 *S. aureus* induced host cell death

Induction of programmed cell death (apoptosis) in mammalian cells involves different signalling pathways. The caspase-dependent signalling is receptor-mediated (extrinsic) and initiated by ligand binding to a death receptor. In type I cells (pro-) caspase 8 and caspase 3 are activated, resulting in apoptosis (Figure 6).

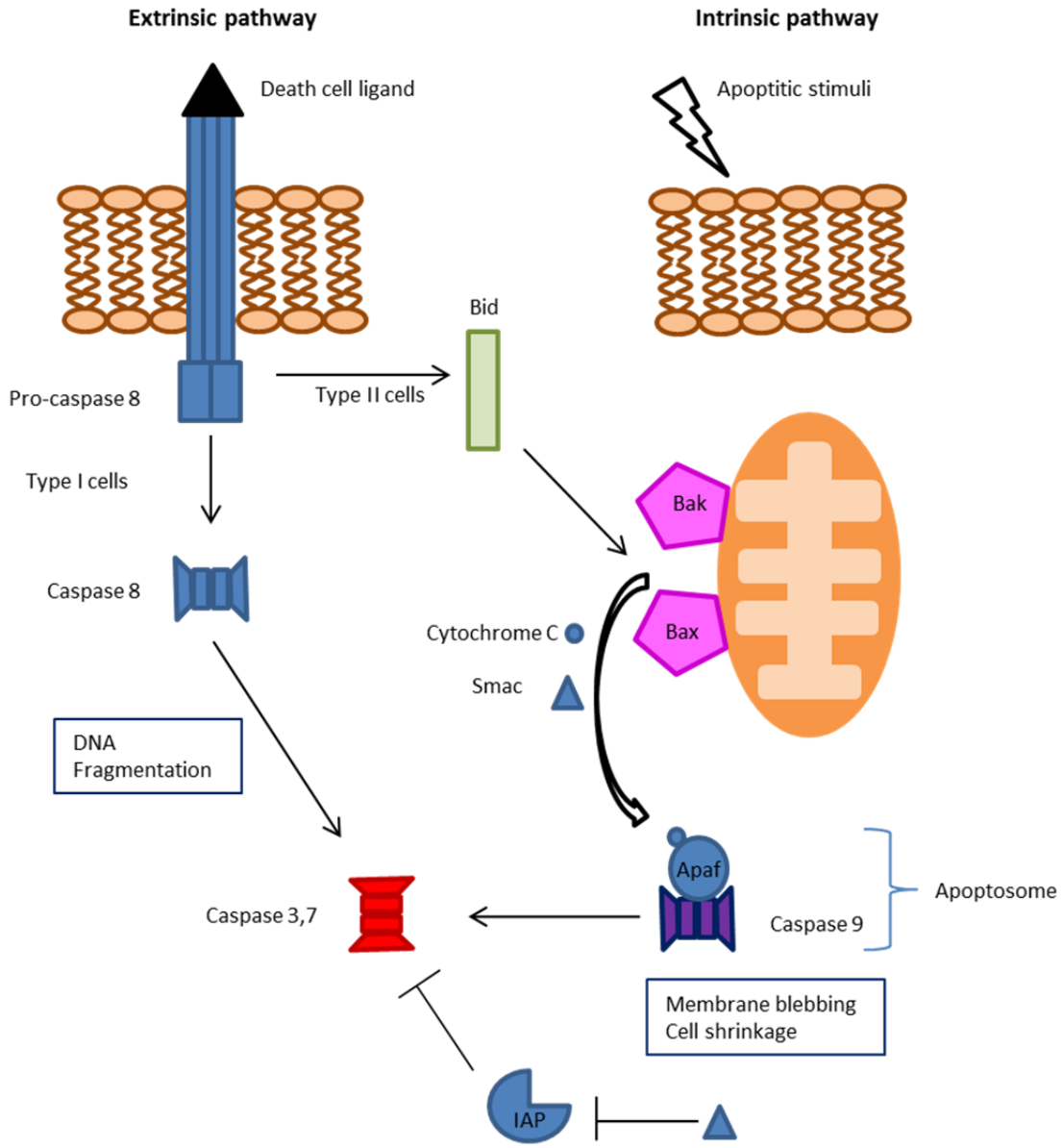

Figure 6: Intrinsic and extrinsic pathway of apoptosis. Ligation of the cell death receptor results in the activation of pro-caspase 8. In type I cells, activates caspase 8 pro-caspase 3, which cleaves target proteins, leading to apoptosis. In type II cells, caspase 8 cleaves Bid, which induces the translocation, oligomerization and insertion of Bax and Bak into the mitochondrial outer membrane. This is followed by the release of cytochrome c which forms with apoptosis activation protein 1 (Apaf1) and caspase 9 the apoptosome. This results in activation of pro-caspase 9, which leads to activation of pro-caspase 3. The intrinsic, mitochondria-mediated pathway is sensed by apoptotic stimuli and is controlled by Bcl-2 family proteins (regulation of cytochrome c release), inhibitor of apoptosis proteins (IAPs) (inhibition of caspase), and second mitochondria activator of caspases (Smac) and Omi (negative regulation of IAPs).

In type II cells Bid is cleaved which leads to cytochrome c release out of mitochondria. The intrinsic, mitochondria-mediated pathway requires apoptotic stimuli and is controlled by Bcl-2 family proteins which regulate cytochrome c release. Formation of the apoptosome complex (Apaf-1, cytochrome c, pro-caspase 9) activates caspase 9 and induces cell death. Lastly, the caspase-independent pathway

is mediated by perforin and granzyme and is characterized by formation of single-stranded DNA nicks and the appearance of apoptotic morphology (Orrenius et al., 2003)].

In the past most publications dealing with *S. aureus* induced host cell death were focusing on α-toxin effects by staphylococcal α-toxin (Nygaard et al., 2012, Bantel et al., 2001, Haslinger et al., 2003, Thay et al., 2013, Guan et al., 2013, Yu et al., 2013). Nygaard and colleagues demonstrate that α-toxin has an impact on plasma membrane permeability in monocytes, T cells and B cells but not PMNs. Further, the membrane permeability due to α-toxin resulted in induction of programmed cell death during *S. aureus* LAC infection (Nygaard et al., 2012). α-toxin of intact bacteria and the supernatant, both induced cell death in Jurkat T cells and activates caspase 3, 8 and 9 via the intrinsic death pathway independently of a death receptor (Bantel et al., 2001). While α-toxin induces apoptosis in Jurkat T cells only by the mitochondrial pathway, in peripheral blood mononuclear cells (MNC) endogenous TNF-α and the death receptor pathway are involved (Haslinger et al., 2003). In HUVECs apoptosis is induced by α-toxin supernatant in dose and time dependent manner and caused enhanced expression of TNF-α, caspase 3 and caspase 8. By contrast, the inhibition of TNF-α with neutralizing antibodies and caspase 3 and 8 inhibiting peptides decrease α-toxin induced apoptosis (Guan et al., 2013). α-toxin and staphylococcal enterotoxin B (SEB) induced apoptosis in the epithelial-like ECV304 cells via similar mechanisms by activation of caspase 3 and 8 in time and dose dependent manner (Yu et al., 2013). Other publications either connected *S. aureus* PSMα to lysis and killing of neutrophils (Wang et al., 2007) or LukAB killing of human neutrophils, macrophages and dendritic cells (Dumont et al., 2011).

1.7 Aim of the study

Staphylococcus aureus as a facultative intracellular pathogen causes severe infections and becomes more and more resistant to available antibiotics. Therefor it is important to understand what occurs during infection with phagocytosed *S. aureus* as well as with the host to develop new drug targets and strategies against infection.

The aim of this study was to investigate the post-invasive events during *S. aureus* infection. First, *S. aureus* strains which are able to escape the phagosome should be identified. Therefore a flow-cytometric based method with FITC-labelled staphylococci were used which allows the distinction between phagocytosed and escape bacteria. After finding some escape positive strains, next the escape relevant factors should be investigated using knockout mutants in different toxins such as the well-known α-toxin, β-toxin, δ-toxin and a new class of toxins the phenol-soluble modulins (PSMs) or leukocidins. The timing of phagosomal escape should be resolved as well. To date, most published

studies which were interested in host-pathogen interaction focused on professional phagocytes such as neutrophils. Therefore this study is exercising non-professional phagocytes including epithelial and endothelial cell lines since they are affected by infection as well. Further, should be investigated if a connection between phagosomal escape and pathogen-induced cell death exists. By a genome-wide RNA interference (RNAi) screen host cell factors were identified to be crucial for *S. aureus* mediated cell death. Particularly intracellular calcium signalling was influenced by *S. aureus*. In this work the host cell contribution to phagosomal escape of *S. aureus* should be revealed as well.

2 Material and Methods

2.1 Material

2.1.1 Bacterial strains

Table 1*:* Bacterial strains

Strain	Description	Reference
6850	Clinical osteomyelitis isolate, MSSA, producer of *hlb*, PSMs, *hld* and *plc*; t185	(Balwit et al., 1994)
6850 pJL74	6850 containing plasmid pJL74, 10 erm^R	This study
6850 Δ*hla*	*hla* deletion in 6850	Sudip Das, University Würzburg
6850 Δ*psm*α	*psm*α deletion in 6850, 10 cm^R	(Grosz et al., 2014)
6850 Δ*psm*α pJL74	6850 Δ*psmα* containing plasmid pJL74, 10 erm^R	This study
6850 Δ*psm*α pTXΔ	*psm*α deletion in 6850 containing empty plasmid (Δ*xylR* repressor)	This study
6850 Δ*psm*α pTXΔα1-4	6850 Δ*psm*α complemented for PSMα expression	(Grosz et al., 2014)
6850 pCG33	*hlb* (β-toxin) deletion in 6850, 10 erm^R	(Grosz et al., 2014)
6850 Δ*plc*	*plc* knockout in 6850	(Grosz et al., 2014)
Cowan I	Phenotypically without *hla* and *hlb*; t076	ATCC 12598
LAC	CA-MRSA USA300 LA county jail outbreak; t008	(Maree et al., 2007)
LAC pJL74	LAC containing plasmid pJL74, 10 erm^R	This study
LAC Δ*psm*α	*psm*α deletion in LAC, 150 $spec^R$	(Wang et al., 2007)
LAC Δ*psm*α pMV158	LAC Δ*psm*α containing MV158 GFP, 150 $spec^R$ 20 tet^R	This study
LAC pTXΔ16	LAC with empty plasmid pTXΔ16 (Δ*xylR* repressor), 12.5 tet^R	(Wang et al., 2007)
LAC Δ*psm*α pTXΔ16	LAC Δ*psm*α containing plasmid (Δ*xylR* repressor), 150 $spec^R$ 12.5 tet^R	(Wang et al., 2007)
LAC Δ*psm*α pTXΔα1-4	LAC Δ*psm*α complemented for PSMα expression, 150 $spec^R$ 12.5 tet^R	(Wang et al., 2007)
LAC Δ*psm*β	*psm*β kockout in LAC, 150 $spec^R$	(Wang et al., 2007)
LAC Δ*hld*	*hld* kockout in LAC	(Wang et al., 2007)
LAC Δ*hla*	*hla* kockout in LAC	(Bose et al., 2013)
LAC Δ*pvl*	PVL kockout in LAC; Δ*luk*SF, 150 $spec^R$	(Voyich et al., 2006)
LAC Δ*luk*AB	*luk*AB knockout in LAC	C. Wolz, University Tübingen
LAC Δ*luk*AB pJL74	LAC Δ*luk*AB containing plasmid pJL74, 10 erm^R	This study
LAC Δ*luk*AB Δpsmα	*luk*AB and *psm*α knockout in LAC, 10 tet^R	C. Wolz, University

		Tübingen
LAC *agr*$^-$	*agr* deficient LAC, 3 tet^R	C. Wolz, University Tübingen
LAC *agr*$^-$ pJL74	LAC *agr*$^-$ containing plasmid pJL74, 3 tet^R 10 erm^R	This study
LAC *agr*$^-$ pPSMα	LAC*agr*$^-$ deficient LAC complemented for PSMα expression, 3 tet^R 10 cm^R	C. Wolz, University Tübingen
LAC *agr*$^-$ pPSMβ	LAC*agr*$^-$ deficient LAC complemented for PSMβ expression, 3 tet^R 10 cm^R	C. Wolz, University Tübingen
MW2	MRSA, USA400 PFGE type, isolated 1998 in North Dakota, USA; t128	(Baba et al., 2002)
MW2 Δ*psm*α	*psm*α kockout in MW2, 150 spec^R	(Wang et al., 2007)
MW2 pTXΔ16	MW2 containing empty plasmid pTXΔ16 (Δ*xylR* repressor), 12.5 tet^R	(Wang et al., 2007)
MW2 Δ*psm*α pTXΔ16	MW2 Δ*psm*α containing empty plasmid pTXΔ16 (Δ*xylR* repressor), 150 spec^R 12.5 tet^R	(Wang et al., 2007)
MW2 Δ*psm*α pTXΔα1-4	MW2 Δ*psm*α complemented in PSMα expression, 150 spec^R 12.5 tet^R	(Wang et al., 2007)
MW2 Δ*psm*β	*psm*β kockout in MW2, 150 spec^R	(Wang et al., 2007)
MW2 Δ*hld*	*hld* kockout in MW2	(Wang et al., 2007)
MW2 Δ*pvl*	PVL kockout in MW2; Δ*luk*SF, 150 spec^R	(Voyich et al., 2006)
RN4220		(Kreiswirth et al., 1983)
RN4220 pJL74	RN4220 containing plasmid pJL74,10 erm^R	This study
RN4220 pMV158	RN4220 containing plasmid pMV158, 20 tet^R	This study
SA113	Restriction-deficient mutant derived from strain NCTC 8325; *rsbU*$^-$, *agr*$^-$, *tcaR*$^-$	(Iordanescu and Surdeanu, 1976)
SA113 pPSMα	SA113 complemented for PSMα expression, 10 cm^R	Julia Kolter (Masterthesis)
Newman	Clinical isolat	(Duthie, 1952)
RN1HGtcaR (HG003)	NTCT8325 (RN1) *tcaR* and *rsbU* repaired	(Herbert et al., 2010);
ST239	MRSA	(Feil et al., 2008)

2.1.2 Cell lines

Table 2: Cell lines

Cell line	Description	Reference
293T	Human embryonic kidney cells, carrying a plasmid containing the temperature sensitive mutant of SV40 T-antigen	DSMZ; ACC 635
293T-Lamp-1-YFP	293T cells expressing the lysosomal-associated membrane protein 1 fused to YFP (Lamp-1-YFP) (Giese et al., 2009)	(Grosz et al., 2014)
EA.hy926	Human umbilical vein endothelial cells fused with thioguanine-resistant clone of A549	(Edgell *et al.,* 1983)
HeLa	Human cervix carcinoma cells	DSMZ; ACC 57
HeLa229 YFP-Fc	HeLa cells expressing the escape marker YFP-Fc	(Giese et al., 2011)
HeLa229 YFP-CWT	HeLa cells expressing the escape marker YFP-CWT	(Giese et al., 2011)
HUVEC	Human umbilical vein endothelial cells	ATCC® CRL-1730™
THP-1	Human monocytic cell line derived from an acute monocytic leukaemia patient	(Tsuchiya *et al.*, 1980)
hFob 1.19	Human osteoblast cells; containing SV40 large T antigen	ATCC® CRL-11372™
MG-63	Human osteosarcoma fibroblast cells	ATCC® CRL-1427™

2.1.3 Plasmids

Table 3: Plasmids

Name	Description/Resisence	Source
pJL74	Green fluorescent protein; erm^R	J. Liese
pMV158 GFP	Green fluorescent protein; tet^R	J. Liese

2.1.4 Antibodies

Table 4: Antibodies

Antibody	Origin and Dilution	Company
Actin (8432)	Mouse, 1:5000	Sigma Aldrich
FPR2 (PAB14680)	Rabbit, 1:6000	Abnova
Anti-mouse IgG HRP linked sc2005	Goat, 1:3000	Santa Cruz
Anti-rabbit IgG HRP linked sc 2004	Goat, 1:3000	Santa Cruz

2.1.5 Oligonucleotides

Table 5: Oligonucleotides for sequencing

Name	Sequence (5´-3´)
TrueSeq Sencse	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
TrueSeq Antisense	CAAGCAGAAGACGGCATACGAGAT-NNNNNN-GTGACTGGAGTTCAGACGTGTGCTCTTCCG ATC

NNNNNN = 6 n barcode for muliplexing

Table 6: Oligonucleotides for qRT-PCR

Name	Sequence (5´-3´)	Target
PSM alpha fwd	GGCCATTCACATGGAATTCGT	*S. aureus psmα*
PSM alpha rev	GCCATCGTTTTGTCCTCCTG	*S. aureus psmα*
PSM beta fwd	GGCACAAGCATTGTGGCAT	*S. aureus psmβ*
PSM beta rev	ATTGCTTCTGCTAGTCCAGTCATTT	*S. aureus psmβ*
gyrB fwd	TACAGGAATCGGTGGCGACT	*S. aureus gyrB*
gyrB rev	ACAACGGTGGCTGTGCAATA	*S. aureus gyrB*
agrA fwd	ATGGAAATTGCCCTCGCAA	*S. aureus agrA*
agrA rev	CCAACTGGGTCATGCTTACGA	*S. aureus agrA*
rpoD fwd	ACGTAAGTTCGCTTCTGCAAGTCT	*S. aureus rpoD*
rpoD rev	TCTTAGTGCCCCTCCAGGTGT	*S. aureus rpoD*
CD11b fwd	ACTGGTGAAGCCAATAACGCA	Human *cd11b*
CD11b rev	TCCGTGATGACAACTAGGATCTT	Human *cd11b*
FPR2 fwd	TCTTGCTCTAGTCCTTACCTTGC	Human *fpr2*
FPR2 rev	AATGACAAACCGGATAATCCCTC	Human *fpr2*
GAPDH fwd	GAAATCCCATCACCATCTTCCAGG	Human *gapdh*
GAPDH rev	GACCCCCAGCCTTCCATG	Human *gapdh*

Table 7: siRNA oligonucleotides (stock concentration 10 µM; mixture of four siRNAs against same target)

Name	Function	Company
siALLStar	Negative siRNA control	Qiagen
siITGAM	Integrin alpha so known as cluster of differentiation molecule 11b (CD11b)	Qiagen
siFPR2	Formyl peptide receptor 2	Qiagen
siCell death control	Transfection control	Qiagen

2.1.6 Kits

Table 8: Kits

Name	Comments	Company
Agencourt RNAClean XP kit	Fragmentation	Beckman Coulter Genomics
DNA Mini Kit	DNA isolation	Qiagen
*mir*Vana™ miRNA Isolation Kit, with Phenol	RNA isolation	Ambion® Life Technologies
RNease Mini Kit	RNA isolation	Qiagen
QIAPrep Spin Miniprep Kit	Plasmid isolation	Qiagen
Quantitec Transcription	Reverse Transcrition	Qiagen

2.1.7 Antibiotics

Table 9: Antibiotics for genetically manipulated organisms

Abbrevation	Name	Stock solution	Final concentration
erm	Erythromycin	10 mg/ml EtOH	10 µg/ml
tet	Tetracycline	10 mg/ml EtOH	3, 12.5, 20 µg/ml
atet	Anhydrotetracycline	200 µg/ml EtOH	100 ng/ml
amp	Ampicillin	100 mg/ ml in H_2O	100 µg/ml
cm	Chloramphenicol	10 mg/ml in H_2O	10 µg/ml
spec	Spectinomycin	150 mg/ml in DMSO	150 µg/ml
blasti	Blasticidin	1 mg/ml in DMSO	3 µg/ml
G418	Geneticin	100 mg/ml in DMSO	300 µg/ml
rif	Rifampicin	10 µg/ml in EtOH	10 ng/ml

2.1.8 Buffers, solutions and media

Table 10: Media and solutions for cell culture

Medium/Chemical	Company
DMEM/F-12, GlutaMAX™ with / and no phenol red	Gibco
RPMI1640	Gibco
M200	Gibco
DPBS	Gibco
Fetal calf serum	PAA
Penicillin/Streptomycin	Sigma Aldrich
Sodium pyruvate	PAA

Table 11: Buffers and Solutions for infection assay

Buffer/Medium	Ingredients
Completed medium	10 % FCS, 1 % Penicillin/Streptomycin, (1 % Sodium pyruvate only for RPMI1640)
Invasion medium	1 % human serum albumin, 10 mM Hepes
HSA/PBS	1 % human serum albumin in PBS
FITC solution	Buffer A: 0.42 g $NaHCO_3$ resuspended in 10 ml H_2O Buffer B: 0.53 g Na_2CO_3 resuspended in 10 ml H_2O Add 5.8 ml of Buffer A into Buffer B FITC-DMSO: 1 mg FITC resuspended in 1 ml DMSO FITC solution: FITC-DMSO was diluted 1:10 in Buffer A/B mix
Crystal violet solution	3.75 g Crystal violet, 250 ml EtOH (100 %), 1.25 g NaCl, 24 ml, Formaldehyd (36 %), 226 ml water
Elution buffer	25 ml SDS (20 %), 475 ml PBS
Annexin binding buffer	10 mM HEPES/NaOH pH7.4, 10 mM NaCl, 5 mM $CaCl_2$

Table 12: Buffers and media for bacteria cultivation

Buffers and media	Ingredients and Company
Mueller Hinton	Sigma Aldrich
Tryptic soy broth (TSB)	Sigma Aldrich
Sheep blood agar	BioMérieux
Electroporation buffer	0.5 M Saccarose, Glycerol

Table 13: Buffer, solutions for SDS-PAGE and Western blot

Buffer and solutions	Ingredients
10x SDS buffer 1L	30.275 g Tris, 144 g glycine, 10 g SDS
10x Semi dry transfer buffer 1L	24 g Tris, 113 g glycine, 2 g SDS
1x Semi dry transfer buffer	10x semi dry buffer diluted to 1x with 20% (v/v) methanol
Blocking solution	1xTBS with 5% (w/v) dry milk powder
10x TSB	60.5 g Tris, 87.6 g NaCl, adjust to pH 7.5 with HCl
2x Laemmli buffer	100 mM Tris/HCl (pH 6.8), 20% (v/v) glycerol, 4% (w/v) SDS, 1.5% (v/v) 2-mercaptoethanol, 0.004% bromophenol blue
SDS upper gel solution	For 5 ml: 3.4 ml H_2O, 0.83 ml 30% (v/v) acrylamid-bisacrylamid mix (5:1), 0.63 ml 1.0 M Tris/HCL (pH 6.8), 0.1 ml 10% (w/v) SDS, 0.05 ml 10% APS, 5 µl TEMED
SDS lower gel solution	For 5ml: 2.3 ml H_2O, 1.3 ml 30% (v/v) acrylamid-bisacrylamid mix (5:1), 0.05 ml 10% (w/v) SDS, 1.3 ml 1.5 M Tris/HCL (pH 8.8), 0.05 ml 10% APS, 0.003 µl TEMED
ECL solution 1	2.5 mM lumiol, 0.4 mM *p*-coumaric acid
ECL solution 2	100 mM Tri/HCL pH 8.5, 0.02 % H_2O_2

2.1.9 Chemicals

Table 14: Chemicals

Name	Company
2-APB	Sigma Aldrich
7AAD (559925)	BD Bioscience
APC AnnexinV (550475)	BD Bioscience
DMSO (Dimethysulfoxid)	Sigma Aldrich
FITC (Fluorescein isothiocyanate)	Invitrogen
HiPerFect® HTS Reagent	Qiagen
Lysostaphin	AMBI Products
Moviol	Invitrogen
Monensin	Flucka BioChemika
PMA (Phorbol-12-myristat-13-acetat)	Cell Signalling
Propidium iodide	Flucka BioChemika
RNA*later*	Qiagen
SYBR® Green I Dye	Life Technologies
Staurosporin	Sigma Aldrich
TritonX-100	Panreac AppliChem

2.1.10 Equipment

Following technical devices were used in this study: 2100 Bioanalyzer (Agilent Technology), Accuri C6 Flow Cytometer (BD), Chemiluminescence camera system (Intas), cold centrifuge CT14RE (Himac), Electrophoresis Chambers (BioRad), FACScalibur (BD), FACSaria III (BD), FastPrep (MP Biomedicals) Hera Cell 240i incubator (Thermo), Hera Safe sterile bench (Thermo), HiSeq2500 (Illumina), Leica DMIR RB (Leica), Megafuge 1.0R (Haraeus), Micropulser (BioRad), NanoDrop 1000 spectrophotometer (Peqlab Biotechnology), Neubauer counting chamber (Laboroptic), Plate Reader infinite 200 (Tecan), Step One Plus RT PCR system (Applied Biosystems), Semi dry Blotter (Peqlab), Thermal cycler GS1 (G-STORM), TCS SP5 confocal microscope (Leica), UltraspecTM 3100pro (Amersham Biosciences Corp.), Vortexer shaker Reax 2000 (Heidolph),

2.1.11 Software programs and webpages

Table 15: Software programs and webpages

Software/Tools	Description	Source (URL/Company)
Word 2010	Text processing tool	Microsoft
Excel 2010	Calculation and graphing tool	Microsoft
PowerPoint 2010	Graphing tool	Microsoft
ImageJ	Image editing	http://imagej.nih.gov/ij/
StepOne™ Software	qRT-PCR evaluation	Applied Biosystems
BD Accuri C6 Sofware	Flow cytometric evaluation	BD
CellQuest Pro	Flow cytometric evaluation	BD
EndNote X7	Reference management software	Thomsom Reuters
PyMOL	Crystal structer of proteins	http://www.pymol.org/
GeneCard®	Information about gene function	http://www.genecards.org/

2.2 Methods

2.2.1 Bacterial culture techniques

2.2.1.1 *S. aureus* cultivation

S. aureus were grown on TSB agar plates or sheep blood agar plates if necessary supplemented with the appropriate antibiotics over night at 37 °C. Liquid cultivation was performed in Mueller Hinton bouillon (MHB) at 37 °C without agitation or TSB media at 37 °C and 190 rpm.

S. aureus stocking samples were performed by resuspending *S. aureus (*grown on agar plates) in MHB media containing 10 % glycerine and were stored at -80 °C.

2.2.1.2 Growing curves

With an overnight culture of *S. aureus*, the main culture was inoculated with an optical density (OD) of 0.01. OD was measured at 540_{nm} every 30 min until stationary phase was reached.

2.2.1.3 Generation electrocompetent *S. aureus*

For generation of electrocompetent *S. aureus* 5 ml of an overnight culture was diluted in 100 ml TSB medium. Next, the OD_{540} were measured and adjusted to an initial concentration of 0.5, and then the culture was incubated for at least 40 min at 37 °C and 150 rpm. After reaching an OD_{540} of 0.6-0.8 the culture was transferred into two 50-ml tubes and incubated for 15 min on ice. Thereafter culture was spun down at 3000 rpm for 10 min at 4 °C and the supernatant was discarded. Pellets were resuspended in 25 ml ice cold sterile water by pipetting up and down, further 25 ml water was added and spun down again. Pellets were resuspended in 25 ml ice cold water and spun down, and then dissolved in 10 ml water and spun down. Finally pellets were resuspended in 10 ml ice cold sterile 10 % glycerine and spun down for 15 min at 4000 rpm after discarding the supernatant pellets were resuspended in 5 ml glycerine. After a final centrifugation step pellets were dissolved in 0.35 ml glycerine and pooled. Aliquots of 70 µl were pipetted into pre-cooled tubes and stored at -80 °C.

2.2.1.4 Electroporation of *S. aureus*

For electroporation of *S. aureus*, electrocompetent bacteria were thawed on ice, 140 µl Electroporation Buffer was added to 70 µl-aliquots (2.2.1.3) and incubated at room temperature for 15 min. For every preparation 1 µl plasmid was used, the mixture was incubated for further 20 min. The samples were pipetted into electroporation cuvettes and placed in the appliance MicroPulser (BioRad, München, Germany). After electroporation, samples was transferred with 1 ml pre-warmed TSB medium into 1.5 ml tubes and incubated for 1 h at 37 °C and 150 rpm. Then, electroporated samples were spun down, 800 µl of the supernatants were discarded and 50 µl and 100 µl suspension were plated on appropriate antibiotic agar plates. Plates were in incubated at 37 °C for 24 – 48 h.

2.2.1.5 Determination of the minimal inhibitory concentration of *S. aureus*

To detect the minimal inhibitory concentration (MIC) of antimicrobial agents, like rifampicin, against *S. aureus* a dilution series and an ETEST® were performed. For the dilution series, rifampicin and DMSO (control) were diluted in 1:2 steps from an initial concentration of 512 µg ml^{-1} - 0,001 µg ml^{-1} in a 96-well plate. An overnight culture of *S. aureus* 6850 was adjusted to an OD of 0.1 and 0.001 and added to the rifampicin dilution series. Plate was incubated for 1 day at 37 °C and 5 % CO_2. MIC was defined by survey of each well for bacterial growth.

Another technique to define the MIC is a ETEST®(Biomérieux), a MIC Test Strip is applied onto an *S. aureus* 6850 inoculated agar surface, the performed exponential gradient of rifampicin is immediately transferred to the agar matrix. Plates were incubated for 18-24 hrs at 37 °C. A symmetrical inhibition ellipse centred along the strip was formed. The MIC is read directly from the scale in terms of µg ml-1 at the point where the edge of the inhibition ellipse intersects the strip MIC Test Strip.

2.2.2 Cell culture methodes and infection

2.2.2.1 Cell passage

The culture of the different cell lines was performed in T25 or T75 tissue flasks (Sarstedt, Nümbrecht, Germany) at 37 °C and 5 % CO_2 in a humidified atmosphere unless specified otherwise. Depending on size and doubling time of the different cell lines but at least twice per week, cells were passaged by dilution in media at rates between 1:10 and 1:3. All cell lines were tested routinely negative for contamination with *Mycoplasma* by PCR. The epithelial 293T cells (human embryonic kidney), endothelial EA.hy926 and osteosarcoma MG-63 cells were grown in DMEM/F-12 (1:1) GlutaMax™-1 supplemented with 10 % fetal calf serum (FCS) and penicillin/streptomycin (50 U ml^{-1} and 50 µg ml^{-1}, respectively). For the osteoblastic hFob 1.19 medium was complemented with 0.3 mg ml^{-1} G418 and the cells were cultivated at 34°C and 5 % CO_2. HeLa (from human cervix), endothelial EA.hy926 and the monocytes THP-1 were grown in RPMI 1640 GlutaMax™ medium with 10 % FCS, penicillin/streptomycin and 1 % sodium pyruvate. The human umbilical vein endothelial cell line HUVEC was grown in M200 with supplements 200s.

2.2.2.2 siRNA Transfection

Small interfering RNA (siRNA) are double-stranded RNA nucleotides, with 3’ overhangs at each end that can be used to interfere with the translation of proteins by binding to and promoting the

degradation of messenger RNA at specific sequences. Therefore, they prevent the production of specific proteins based on the nucleotide sequence of their corresponding mRNA (Tsai, 2007).We used this method to knowdown specific proteins. As control for a successful knowdown, a cell death siRNA was included in each experiment. Further controls were siRNA ALLStar or GFP. HeLa YFP-CWT was transfected two days prior infection with HiPerFect® HTS Reagent (Qiagen). In a 12-well plate, 1.5 µl HiPerFect® HTS Reagent, 1 µl siRNA (final 16.6 nM) and $5x10^4$ cells per well were used. Transfection was stopped 24 hrs later, cells were washed with PBS and incubated for further 24 h in full medium.

2.2.2.3 *S. aureus* infection

Cells were seeded in 12-well-plates or 24-well-plates one day before infection with one expectation. THP-1 monocytes have to be treated with PMA (0.025 µg ml final concentration) for at least 48 h to differentiate into macrophage. The bacterial overnight (o/n) culture was prepared in 20 ml Mueller-Hinton broth in 50-ml-tubes (Greiner) without shacking for 16 h at 37 °C. Antibiotics were added when necessary. Next day the o/n culture was spun down, washed with PBS and resuspended in PBS containing 1 % human serum albumin (HSA). The optical density was measured at 540_{nm} and adjusted to OD of 1. The cells were washed with invasion medium (without FCS and antibiotics) supplemented with 1 % HSA and 10 mM Hepes. Infection was performed in 500 µl invasion medium, 50 µl of the bacterial suspension was added and spun down for 5 min, 800 rpm. After 50 min cells were treated with 20 µg ml^{-1} lysostaphin (AMBI, Lawrence, NY) for 10 min to eliminate the extracellular bacteria. Next cells were washed and incubated in FCS containing medium until the end of the experimentation.

2.2.2.4 Flow cytometry analysis

2.2.2.4.1 pH dependent escape assay

To investigate the behaviour of intracellular staphylococci, infected cells were analysed in a flow cytometric escape assay. By use of fluorescein isothiocyanate (FITC) labelled *S. aureus* it is possible to measure the acidity of the bacterial microenvironment and hence if the bacteria are located in phagosomes or within the cytoplasm of the host cell. FITC has maximum fluorescence intensity at neutral pH but fluorescence is reversibly quenched at acidic pH (Figure 7). Quenching of the FITC fluorescence signal and recovery of fluorescence upon neutralization thus can be used to indicate phagosomal escape.

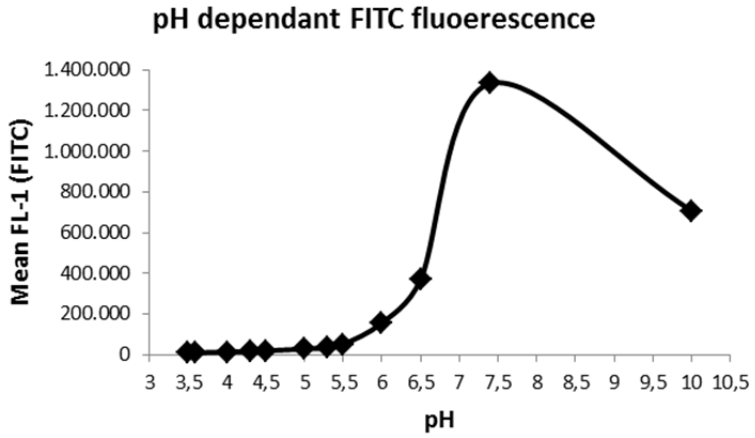

Figure 7: FITC fluorescence has a maximum at neutral pH. FITC labelled *S. aureus* was incubated in medium with different pH and the fluorescence was measured by flow cytometry.

Phagosomal escape was investigated in non-professional phagocytes (like 293T, HeLa, EA.hy926, HUVEC, MG-63 and hFob 1.19) and professional phagocytes (differentiated THP-1 cells). For the assay host cells were seeded one day before infection in 24-well-plates to a concentration that yielded a confluent cell layer at the time of infection. Bacterial o/n cultures were washed with PBS and stained with 3 ml FITC solution for 30 min at 37 °C. Stained bacteria were washed with PBS and the OD_{540nm} was adjusted to 1.

Infected cells were harvested 2 h p.i. as well as 6 h p.i. For that cells were washed with PBS, trypsinized and the reaction was stopped by adding 1 ml full medium. Cells were pelleted by centrifugation, resuspended in 1 ml PBS/HSA and each sample was split into two fractions. Fraction one was treated with 1 µl 50 µM monensin (solved in ethanol) , fraction two with 1 µl 100 % ethanol as solvent control. The samples were incubated for 10 min at 37 °C. The ionophore monensin forms complexes with monovalent cations and transport them across lipid membranes of cells (Mollenhauer et al., 1990). Based on the neutralized pH in the infected host cells upon monensin treatment, the previously acid-quenched FITC fluorescence is recovered. The resulting difference in FL-1 fluorescence, difference in arbitrary fluorescence units (ΔAFU), of monensin treated and untreated samples at 2 h p.i. and 6 h p.i. was used as reading for intracellular pH (Figure 8).

Measurements were performed with a FACScalibur (Beckton Dickinson) or Accuri C6 (Beckton Dickinson) flow cytometer using a fixed forward scatter (FSC) and side scatter (SSC) gating. PI-positive cells or cell debris in the FL-3 channel were excluded for the analysis after addition of 0.5 µg ml^{-1} propidium iodide. For the calculation of the AFU (displayed in negative values) the invasion rate was determined for the monensin or ethanol-treated samples according to:

$$AFU = \frac{(\text{FL-1 mean cells} \times \%\ \text{gated cells}) \times 100}{\text{FL-1 mean bacteria}}$$

And in a following step the ΔAFU were calculated by:

$$\Delta AFU = \frac{(AFU_{Ethanol}) - (AFU_{Monensin}) \times 10000}{AFU_{Monensin}}$$

Larger negative values correspond to more-acidic pH values and less negative values relate neutral pH values. Each biological experiment was performed with not infected cells, infected with Cowan I (control for escape negative strains) and 6850 (control for escape positive strains) in duplicates.

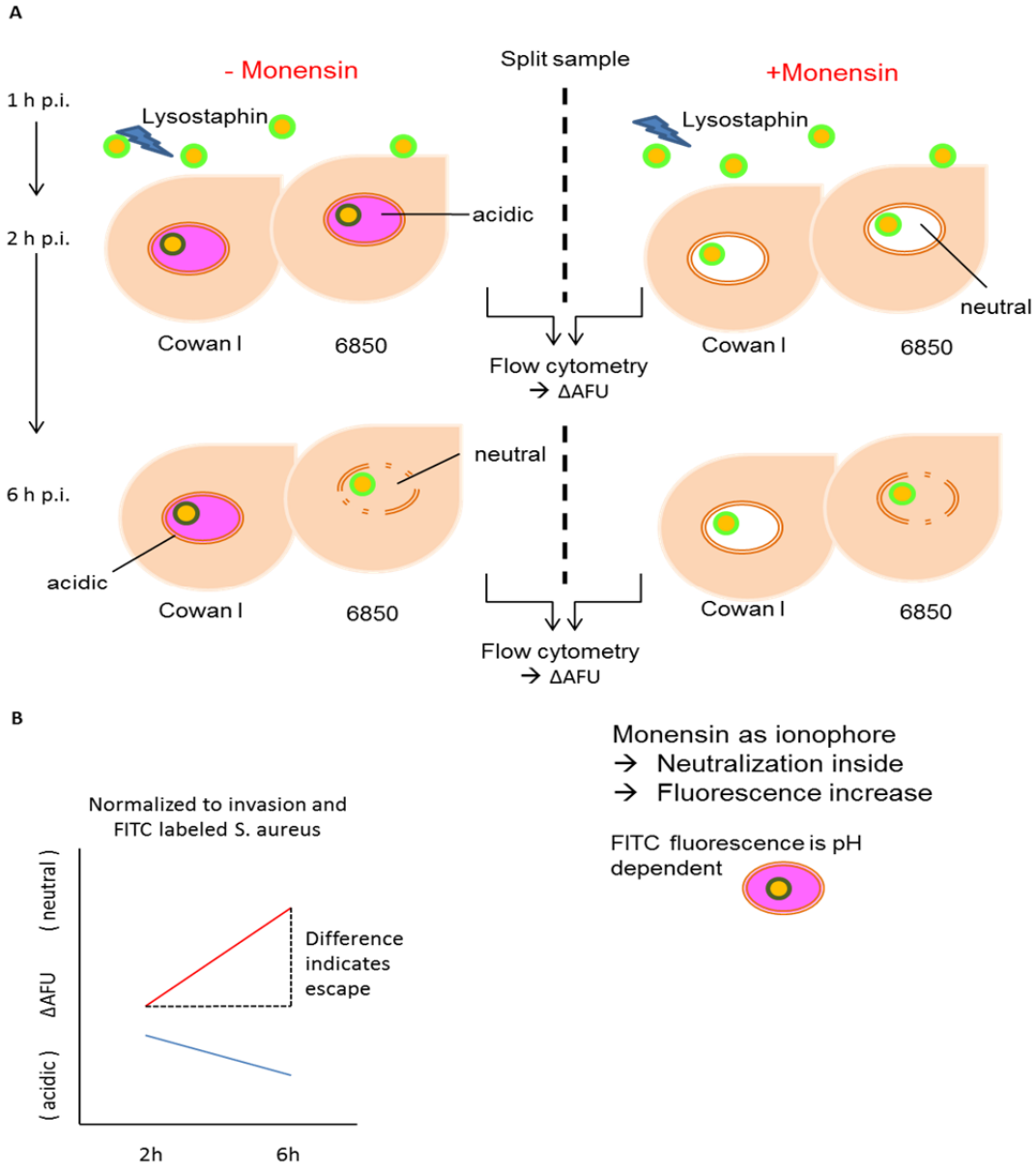

Figure 8: Schematic representation of a flow cytometric escape assay. Cells were infected with FITC labelled *S. aureus*, extracellular bacteria were removed by lysostaphin treatment and each sample was split into a monensin-treated or ethanol-treated (solvent control) fraction. Quenching of the FITC fluorescence by low pH was used as a marker of acidification (A). Changes of ΔAFU towards more neutral values (less negative) at 6 h p.i. when compared to 2 h p.i. indicate phagosomal escape (B).

2.2.2.4.2 Intracellular replication with GFP-expression *S. aureus*

Intracellular replication of *S. aureus* can be monitored via flow-cytometry using GFP-expressing strains. Cells were infected as described in chapter 2.2.2.3, trypsinized and transferred in FACS-tubes. Uninfected cells (control) were measured as well. A shift in green fluorescence (Mean in FITC-A) indicated intracellular replication. The measurements were performed with a laser line at 488 nm (excitation) and an emission filter with 530/30 (green) nm.

2.2.2.4.3 Annexin V/7-AAD

Infected and uninfected cells (control) were trypsinized and centrifuged for 5 min at 800xg. Pellets were resuspended in 50 µl of APC Annexin V/7-AAD (BD bioscience) solution and incubated in the dark at room temperature for 10 min. The final concentration of APC annexin V and 7AAD was 18 µl ml^{-1} dissolved in annexin V binding puffer. For FACS analysis samples were diluted with 250 µl annexin V binding buffer and measured with AriaIII. APC is excited by the Red (637 nm) laser, FITC is excited by the Blue (488 nm) laser and PI is excited by the Green (552 nm) laser hence the compensation values are minimal.

2.2.2.5 Microscopic based escape marker recruitment assay

Alternatively, phagosomal escape was visualized by escape marker recruitment (Giese et al., 2011). For this assay reporter cells were used that constitutively expressed yellow fluorescent protein either fused to the Fc part of human immunoglobulin G (IgG; YFP-Fc) or to the cell wall-targeting domain (CWT) of lysostaphin (CWT, YFP-CWT) in the host cytoplasm. The escape signal is generated by the recruitment of cytoplasmic YFP-Fc to protein A or YFP-CWT to *S. aureus* peptidoglycan (Grundling and Schneewind, 2006) upon disruption of the phagosomal membrane barrier (Giese et al., 2011). The co-localization of fluorescent bacteria with yellow fluorescent escape marker indicated translocation of the pathogen to the host cell cytosol (Giese et al., 2011) Most *S. aureus* strains express Protein A on their cell surface (DeDent et al., 2007) that binds the Fc part of the fusion protein or pentaglycine cross bridges in the cell wall (Grundling and Schneewind, 2006) which interact with the CWT part of the protein. Sample preparation and microscopy was performed basically as published in (Giese et al., 2011). For the microscopy, samples were washed with PBS twice and fixed with 4 % (w/v) formaldehyde (PFA) in PBS^{2+} for 30 min at room temperature. After a washing step, cells were quenched by PBS with 50 mM NH_4Cl for further 5 min. To visualize the actin cytoskeleton and DNA were counterstained with fluorophore-conjugated phalloidin (Invitrogen, Karlsruhe, Germany) and 5 µg ml^{-1} Hoechst 34580 or 4',6-diamidino-2-phenylindole (DAPI; Sigma, Taufkirchen, Germany). The

samples were incubated for 30 min in staining solution, were rinsed with PBS and mounted on glass slides with Mowiol reagent (Invitrogen, Karlsruhe, Germany). For semi-automatic escape quantitation image series were obtained from three biological replicates and two technical duplicates each. Image acquisition was performed with either a 40x/1.3 objective on a LSM510 Meta confocal laser scanning microscope (Zeiss, Jena Germany) or 63x/1.4 objective on a Leica TCS SP5 confocal laser scanning microscope (Leica, Wetzlar, Germany). Image analysis was performed with ImageJ (Abramoff et al., 2004). Localization to LAMP1-YFP-positive vesicles and immunofluorescence analyses were performed as described elsewhere (Giese et al., 2009).

2.2.2.6 Lysostaphin protection assay

Internalized and recovered colony forming units (CFUs) were determined by lysostaphin protection assay. Cells were infected as described in chapter 2.2.2.3. Then, 1-2 h and 6 h post infection cells were washed twice with PBS and trypsinized. Reaction was stopped by addition of FCS containing full medium and cells were transferred into 1.5-ml tubes and spun down. Pellets were resuspended in water, mixed and incubated for 10 min at 37 °C. The cell lysates were serially diluted in PBS/HSA, selected dilution were plated on TSB agar plates and incubated at 37 °C for one day.

2.2.2.7 Crystal violet assay

Host cell viability was measured with a crystal violet assay. Cells were seed two days before infection in a 96-well plate to reach confluence on day of infection. An overnight culture of *S. aureus* was prepared (chapter 2.2.2.3) and cells were infected with an OD of 0.1. In each experiment cells were incubated with staurosporin (final concentration 1 µM and 3 µM) and TritonX-100 (final concentration 1 %) as positive controls. Bacterial internalization was stopped 1 h after infection and extracellular staphylococci were removed with lysostaphin treatment. Cells were washed with PBS and incubated with Medium containing FCS for further 3 h and 21 h at 37 °C and 5 % CO_2. Next, cells were washed three times with PBS and stained with 50 µl crystal violet solution (Table 11) at room temperature for 30 min. Staining solution was removed, cells were washed four times with PBS and then dried overnight. Crystal violet was eluted with 200 µl elution buffer (Table 11) per well for 4 and 24 h. Ten samples per well were measured at 620 nm with the Tecan InfinitePro 200 plate reader.

2.2.2.8 Monitoring of the viable cell concentration with Trypan Blue

For monitoring the percentage of viable cells were washed with PBS and trypsinized. The reaction was stopped by adding FCS containing medium. An aliquot of this cell suspension was diluted 1:1 with 0.4 % Trypan Blue (Gibco®) and pipetted into a Neubauer hemacytometer (Labor Optik, United Kingdom). Dead and viable cells were discriminated microscopically by colour.

2.2.3 RNA methods

2.2.3.1 RNA isolation

Cells were infected as described in chapter 2.2.2.3. Next, trypsinized cells were spun down and the pellets were resuspended in 350 µl RLT buffer, to lyse the cells, supplemented with ß-Mercapotethanol (1 µl/ml). RNA isolation was performed with the RNease Kit (Qiagen, Hilden, Germany) according to attached manufacture protocol. Briefly, lysed cells were diluted with 1 volume of 70 % ethanol, the sample was transferred to an RNeasy Mini spin column and centrifuged for 15 sec at ≥8000 rpm. The flow-through was discarded and the column was wash first with 700 µl Buffer RW1 and twice with 500 µl Buffer RPE. RNA was eluted with 30 µl RNase free water. RNA concentration and quality was measured with a Nanodrop (PeqLab).

For RNA isolation of *S. aureus* overnight or shacking cultures, bacterial pellets were resuspended in 600 µl RLT buffer with ß-Mercaptoethanol and transferred into lysin matrix B tubes (MP Biomedicals, Eschwege, Germany). Bacteria were homogenized in a FastPrep with following settings: 45 sec and 6.5 m/sec speed. The samples were put immediately on ice, spun quickly down and transferred into RNase-free tubes. Following steps was done according manufacture protocol of the RNease Mini Kit (Qiagen, Hilden, Germany). RNA was elated with 50 µl RNase free water. RNA concentration and quality was measured with a Nanodrop (PeqLab).

2.2.3.2 Reverse transcription

Transcription of 500 ng – 1000 ng RNA into cDNA was performed with the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). RNA was mixed with RNase-free water and 2 µl 7x gDNA Wipeout Buffer to genomic DNA. Samples were incubated at 42 °C for 5 min and placed on ice again. Further, 1 µl RT Primer Mix, 4 µl 5x Quantiscript RT Buffer and 1 µl Quantiscript Reverse Transcriptase was added to each sample and incubated for 30 min at 42 °C. Finally samples were heated up to 95 °C for 3 min. Transcribed cDNA was stored at -20 °C.

2.2.3.3 Polymerase Chain Reaction (PCR)

The conventional polymerase chain reaction (PCR) was performed to pre-test the primer pairs and templets (cDNA) for quantitative Real-time PCR (qRT-PCR). A 50 µl reaction was made up of 32,5 µl water; 10 µl 5x Buffer; 1 µl dNTP-Mix (25 mM); 2,5 µl forward and reverse primer (100 µM) and 1 µl cDNA (500 µg ml^{-1}). PCR program include initial denaturation at 98 °C for 1 min, followed by 30 cycles at 98 °C for 10 sec, primer binding at 60 °C for 15 sec and elongation by 72 °C for 45 sec. Followed by 5 min at 72 °C.

2.2.3.4 Gel electrophoresis

Amplification products from a conventional PCR, to pre-test the quality of the primer pairs for the qRT-PCR or qRT-PCR products to examine the size or exclude double bands were examined by gel electrophoresis. Products were load on a 1.5-2 % agarose gel with HD Green DNA stain (INTAS). Products were displaced with 8 µl 6x loading dye and electrophoresis was performed at 100 Volt for 30 - 45 min.

2.2.3.5 Quantitative *real-time* PCR (qRT-PCR)

Quantitative Real-time PCR (qRT-PCR) was performed using StepOne Plus PCR system (Applied Biosystems) with a standard PCR program, the initial denaturation was performed at 95 °C, followed by 40 cycles at 95 °C for 15 sec and 65 °C for 1 min. At the end of each PCR run a melting curve was performed. The 20 µl reaction solution was composed of nuclease-free water, 2x SYBR® Green Mix contains SYBR® Green I Dye, AmpliTaq Gold® DNA polymerase, dNTPs wit dUTP, Passive Reference and optimized buffer components. Further, 0.3 mM forward and reverse primer and 5-20 ng cDNA as template. To exclude contaminations a non-template control of each primer pair was include in each PCR run.

Analysis of relative gene expression for eukaryotic genes was done according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). For normalization the geometrical mean (Vandesompele et al., 2002) of the expression levels of the gene encoding Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used. The not infected (n.i.) cells served as a control. For calculation of the fold changes the following formulas were used: First ΔCT has to be calculated by: $\Delta CT = CT_{\text{target gene}} - CT_{\text{GAPDH}}$; next $\Delta\Delta CT = \Delta CT_{inf} - \Delta CT_{n.i.}$ and finally fold change results as $2^{-\Delta\Delta CT}$.

For quantification of relative gene expression of bacterial genes, the relative amount of the target gene in comparison to an internal control gene was determined. The housekeeping gene gyrase

subunit B (*gyrB*) was used for the normalization step. For calculation of the relative gene expression [RQ] of a gene, the cycle threshold (CT) of the target gene and housekeeping gene were subtracted using following formula: $2^{-\Delta CT}$ (Livak and Schmittgen, 2001).

2.2.3.6 Dual-RNA Seq

A recently developed RNA sequencing technique offers a new approach to study and analyse host and pathogen transcriptomes in parallel. RNA-seq is parallel sequencing of RNA or rather the corresponding cDNA and is based on next-generation sequencing (NGS) platforms. After the sequencing reaction, the generated sequence stretches (reads) are mapped onto a reference genome to deduce the expression state of any given transcript in the sample (reviewed in (Westermann et al., 2012)). The big advantage of this method is that there is no need to separate physically eukaryotic and bacterial parts after an infection. It is time saving and there no loss of biological material.

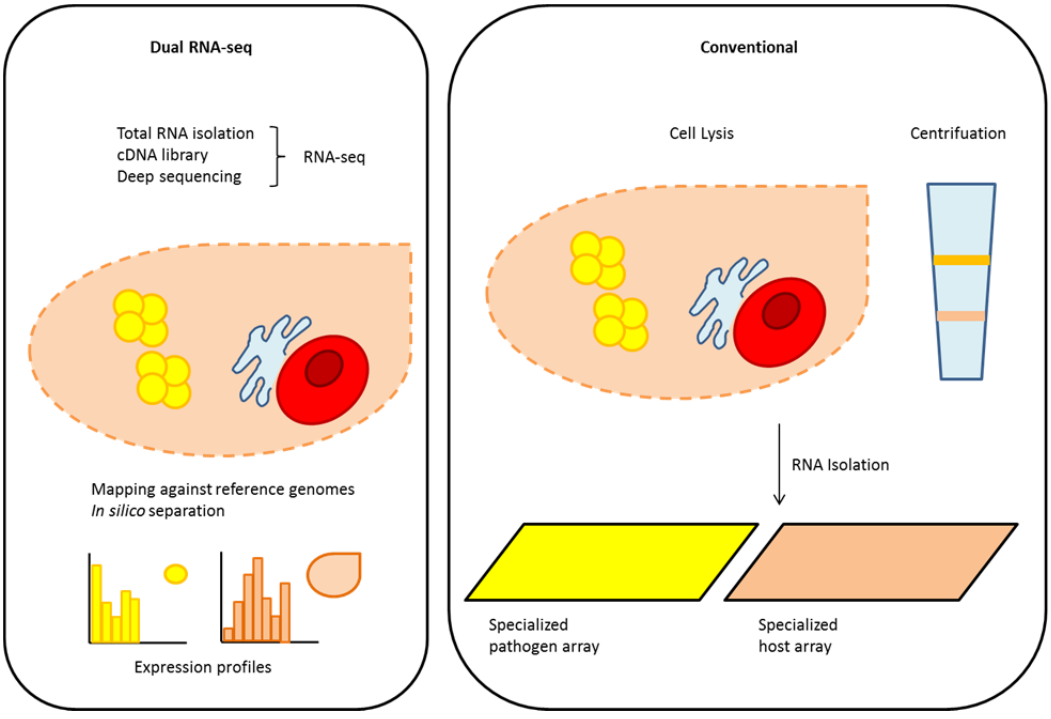

Figure 9: Difference between conventional and Dual RNA-seq transcriptomes. To date a separation of host and pathogen was necessary by using probe-based methods. Meanwhile Dual RNA-seq allows isolating total RNA without separation. The classification between host and pathogen take place at the bioinformatics level. Figure modified from (Westermann et al., 2012).

In order to determine the transcription of host and pathogen simultaneously dual RNA-seq was performed. 293T cells were inoculated with *S. aureus* 6850 or the escape-deficient mutant 6850

Δ*psm*α in 6-well-plates so that between 70-90 % of the whole cell population was infected. Samples were collected 2 h and 6 h post infection and two wells per sample were used to prepare RNA. As control RNA was isolated from uninfected cells which were spiked with S. aureus overnight cultures at the same proportion as infected cells with. RNA isolation was performed with the *mir*Vana™ miRNA Isolation Kit with Phenol (Ambion®) according to the manufacture's protocol. RNA quality was analysed with the Agilent 2100 Bioanalyzer (Agilent Technologies). Further, preparation steps were performed in the research group of Prof. Dr. Jörg Vogel by Alexander Westermann (Institute for Molecular and Infection Biology (IMIB), Würzburg, Germany). Fragmentation was done using ultrasound per sample four times for 30 sec. Fragments below 20 nt were removed using Agencourt RNAClean XP kit (Beckman Coulter Genomics) according to the manufacture's protocol f. Next RNA samples were transcribed into cDNA. Libraries for Illumina sequencing (Hiseq) were made by Vertis Biotechnology AG (Freising, Germany) as described previously (Berezikov et al., 2006) without size-fragmentation step prior to cDNA synthesis. RNA samples were poly(A)-tailed using poly(A) polymerase. The 5´-trisphosphates were removed by applying tobacco acid pyrophosphatase (TAP) resulting in 5´-monophosphat. Then, a RNA adapter was ligated to the 5´-monophosphat. First-strand cDNA was synthesized by an oligo(dT)-adapter primer and the MLV transcriptase. The cDNA concentration was increased to 20-30 ng/µl using a high fidelity DNA polymerase by PCR-based amplification. 3´-sequencing adapter include a library-specific barcode for multiplex sequencing. cDNA insert was flanked by the following adapter sequences: 5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT-3' (True Seq Sense primer) and 5'-CAAGCAGAAGACGGCATACGAGAT-NNNNNN-GTGACTGGAGTTCAGACGTGTGCTCTTC CGATC(dT25)-3' (True Seq Antisence NNNNNN primer). The resulting cDNA libraries were sequenced using HiSeq 2500 system (Illumina) in single read mode and running 100 cycles.

Analysis of the Dual RNA-seq data was performed by Dr. Konrad Förstner (research group of Prof. Dr. Jörg Vogel, IMIB, Würzburg, Germany). Data are supported in FASTQ format; the quality trimming was applied with a Phred quality score (Ewing et al., 1998) of 20. This means, there is a probability of one incorrect base out of 100 bases with a base accuracy of 99 %.

2.2.4 Protein biochemical methods

2.2.4.1 SDS-PAGE

Protein samples were prepared in 2x Laemmli buffer and immediately incubated at 95 °C for 20 min for protein denaturation. The protein separation was performed under denaturing conditions in presence of SDS in 7.5% - 12% polyacrylamide gels by gel electrophoresis (SDS-PAGE) with 120 V.

2.2.4.2 Western blot

Proteins separated by SDS-PAGE were transferred to a PVDF membrane for protein blotting. PVDF membrane were activated in methanol for 1 min and transferred into semi-dry buffer. Protein blotting were performed in a semi dry apparatus with a sandwich consistent of Whatman paper, PVDF membrane, acrylamide gel and Whatman paper for 2 hours at 1 mA per cm^2. For immunoblotting membranes were incubated in blocking solution (5% milk powder in 1x TBS) for 1 h at RT, then incubated in appropriate first antibody (diluted in 1x TBS with 5% milk powder) over night at 4 °C. Membranes were washed three times for 10 min with 1x TBS and incubated with horseradish-peroxidase (HRP)-coupled secondary antibody (1:3000 in 1x TBS with 5% milk powder) for 1 h, RT. Membranes were washed again with TBS and ECL-mix was add on the blots. Chemiluminescence was detected by the photo-sensitive Intas imager system.

2.2.5 Statistical analysis

The significance (p-value; *P*) was determined with the two tailed student´s t-test by Excel 2010 (Microsoft). $P > 0.05$ = n.s.; $P \leq 0.05$ = *; $P \leq 0.01$ = **; $P \leq 0.001$ = ***.

3 Results

3.1 Phagosomal escape of MSSA 6850 and CA-MRSA strains LAC and MW2

3.1.1 Phagosomal escape in epithelial and endothelial cell lines

Only a minority of *S. aureus* strains is able to escape the phagosomal compartment in non-professional phagocytes such as epithelial and endothelial cells (Lam et al., 2010), however this constituted clinically relevant strains which prompted this study to investigate the involved virulence factors and post escaped events.

Flow cytometry and microscopy were used to identify escape positive strains in epithelial and endothelial cells. The flow cytometry escape assay has the advantage to detect the intracellular pH of *S. aureus* infected cells with the help of the pH dependent fluorescein isothiocyanate (FITC) dye. Staphylococci were labelled with FITC prior to infection. Consequently, it was possible to discriminate between escaped staphylococci, which had a brighter fluorescence signal in the FL1 channel than not escaped staphylococci (Figure 10).

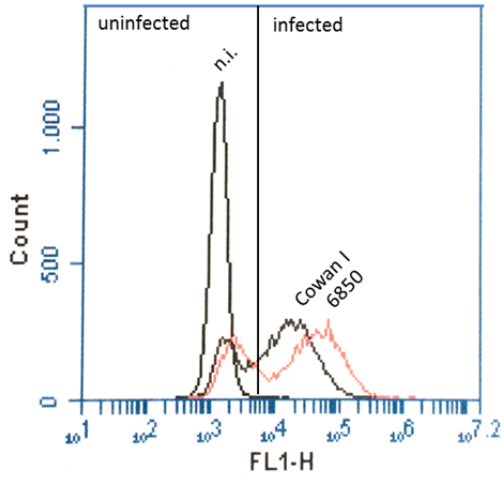

Figure 10: Increase in FITC fluorescence intensity in FL1-H channel of *S. aureus* infected cells due to phagosomale escape. 293T cells infected with *S. aureus* 6850 had a stronger FITC fluorescence than infected with Cowan I at 6 h post infection. Uninfected cells (n.i.) appeared on the left side of the histogram.

The community-associated methicillin-resistant *S. aureus* (CA-MRSA) strains USA300 LAC (CDC, 2003), USA400 MW2 (Baba et al., 2002), together with the strong cytotoxic strain 6850 (Balwit et al., 1994) translocate to the host cell cytoplasm 6 h post infection (p.i), while non-cytotoxic *S. aureus* strain Cowan I (ATCC 12598) continue in an acidified vacuole (Figure 11 A; (Grosz et al., 2014)). Translocation of *S. aureus* strains LAC, MW2 and 6850 from the acidic compartment to cytoplasm was showed by significant changes of ΔAFU between 2 h and 6 h p.i. (LAC: $P = 0.003$; MW2: $P = 0.012$;

6850: *P* = 0.002). The comparison of ΔAFU values 6 h p.i. between the escape positive strains and Cowan I was significant as well (*P* < 0001). Other tested *S. aureus* strains such as Newman, RN1HGtcaR and ST239 did not translocate to the cytoplasm (Figure 11 B).

Additionally, phagosomal escape was verified using microscopy upon escape marker recruitment (Giese et al., 2011) with strain LAC (Figure 11 C, arrowhead) in 293T YFP-Fc cells and strain 6850 in 293T LAMP1 YFP-Fc cells (Grosz et al., 2014). For semi-automatic escape quantitation image series were obtained from three biological replicates with two technical duplicates each using a confocal microscope. The escape signal is generated by the recruitment of cytoplasmic YFP-Fc to protein A to *S. aureus* peptidoglycan (Grundling and Schneewind, 2006) upon disruption of the phagosomal membrane barrier. The co-localization of fluorescent bacteria with yellow fluorescent escape marker indicated translocation of the pathogen to the host cell cytosol (Giese et al., 2011) and was analysed with ImageJ (Abramoff et al., 2004).

Vice versa, the absences of a LAMP1-YFP-positive membrane around fluorescent *S. aureus* strain 6850 (red, SNARF1) 6 h p.i. indicated phagosomal escape (Figure 11D), since LAMP1 is known as a lysosomal/endosomal marker (Jarry and Cheung, 2006). Escape deficient strain Cowan I was surrounded by a LAMP1-YFP positive membrane during the complete infection time.

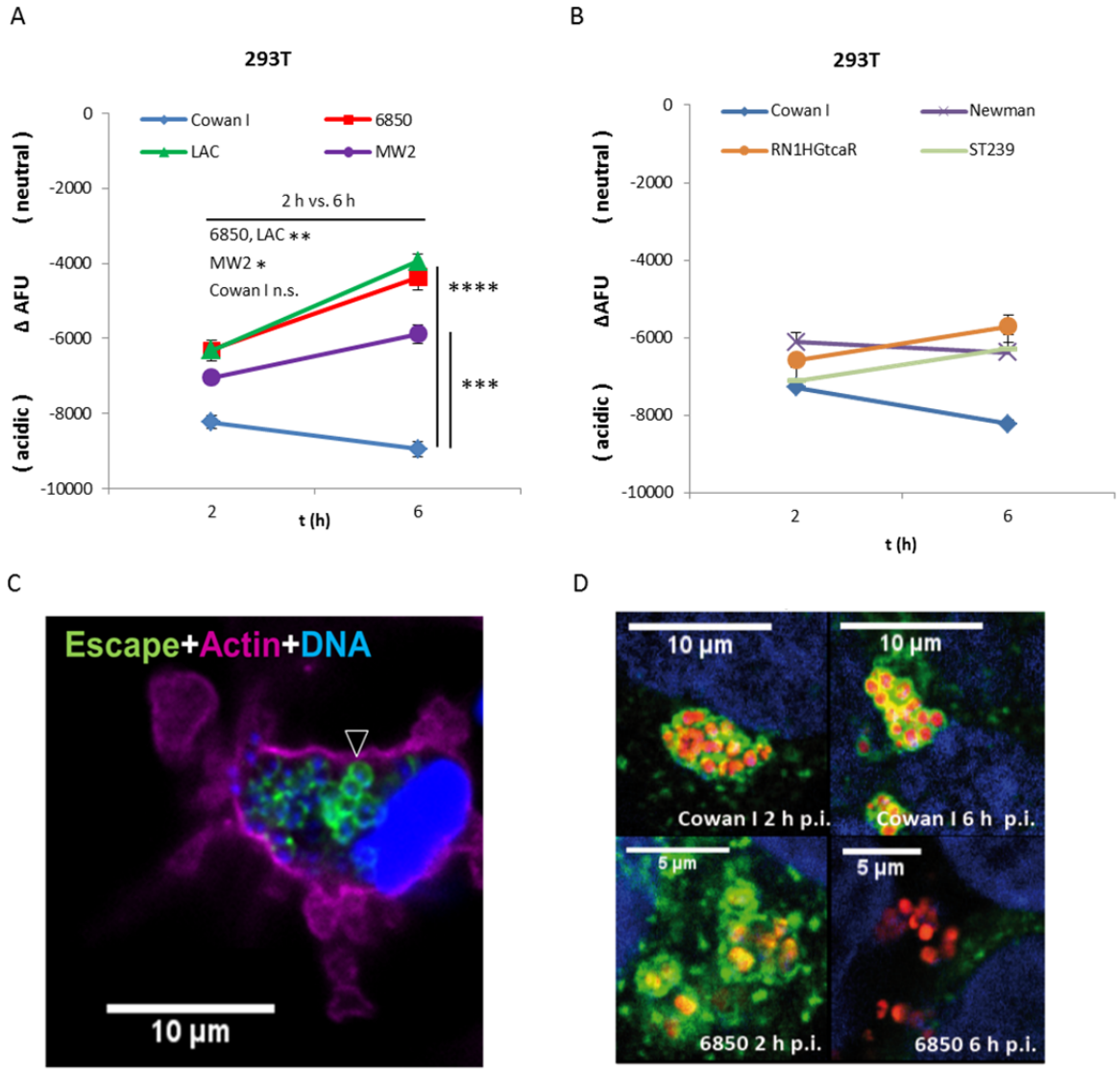

Figure 11: Phagosomal escape of CA-MRSA strains LAC and MW2, as well as MSSA 6850. A) Phagosomal escape of strains LAC, MW2 and 6850 in 293T cells were identified by flow cytometric escape assay. Intracellular pH was determined by the fluorescence intensity of FITC-labelled endocytosed staphylococci. Strongly negative ΔAFU values correlate with acidification of *S. aureus* containing phagosomes. The translocation of bacteria to neutral cytoplasm is mirrored in less negative values. Data shown are mean values from at least 3 independent experiments performed in duplicates ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < .0001. B) Infection of 293T cells expressing the fluorescent escape marker YFP-Fc with *S. aureus* LAC resulted in YFP-Fc recruitment (green) to bacterial cell wall (arrowhead) and thereby indicating phagosomal escape. C) S. aureus strains Newman,RN1HGtcaR and ST239 show no significant difference in AFUs 2 h and 6 h p.i. D) Infection of 293T cells, expressing the fluorescent phagosome marker LAMP1-YFP-Fc, with *S. aureus* strains 6850 and Cowan I resulted in LAMP-1-YFP-Fc recruitment (green) to bacterial cell wall 2 h p.i. and 6 h p.i. only for Cowan I. Absence of LAMP1-YFP indicated phagosomal escape of 6850 6 h p.i.

3.1.2 *S. aureus* escapes 2.5 h post infection

To determine when phagosomal escape starts, the bacterial protein biosynthesis was blocked indirectly by treatment of infected cells with rifampicin. First, minimal inhibitory concentration (MIC) for rifampicin was tested by an ETEST® and revealed a MIC of 16 ng ml^{-1} for strains 6850 and LAC. Infected cells were treated with 10 ng ml^{-1} rifampicin at different time points (1-6 h post infection in

1 hour intervals) and revealed a time window for *de novo* synthesis of required virulence factors for phagosomal escape in 6850 (Figure 12 A) and LAC (Figure 12 B) at 2.5 h post infection. Whereas rifampicin treatment at 1 or 2 h p.i. blocked phagosomal escape efficiently, treatment at later time points indicated no influence on bacterial translocation to the host cell cytoplasm. Protein aqueous factors are transcribed in the first hours of infection and mediate phagosomal escape of strains 6850 and LAC (Grosz et al., 2014).

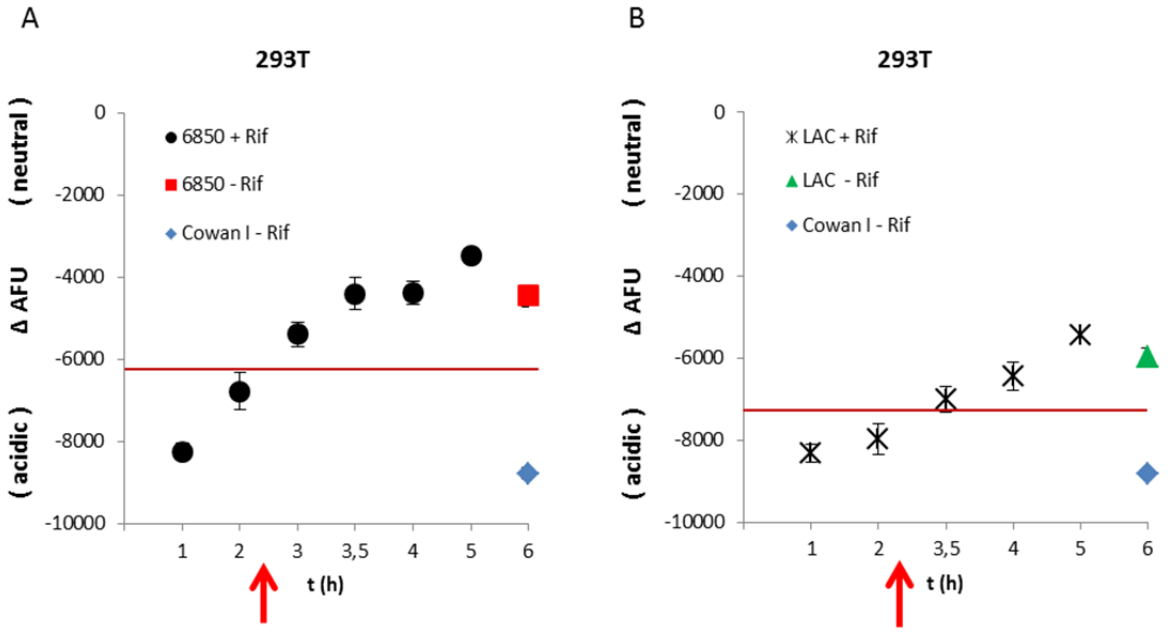

Figure 12: Monitoring the phagosomal escape of *S. aureus* strains 6850 (A) and LAC (B) in 293T cells. Translocation to the host cell cytoplasm commenced about 2.5 hours post infection (arrow) in both strains. By applying rifampicin to intracellular staphylococci at several time points (1 h p.i. - 6 h p.i.) and measuring the pH microenvironment at 2 h and 6 h p.i. revealed that only early treatment (1 h and 2 h p.i.) with rifampicin could block phagosomal escape. Treatment after 3 h p.i. resulted in less negative ΔAFU values by means of 6850 without rifampicin treatment (6850 –Rif). Data shown are mean values of at least 3 independent experiments performed in duplicates ± SEM.

3.2 *S. aureus* phagosomal escape is a synergistic process involving multiple toxins

3.2.1 α-toxin, β-toxin, PIPLC and PVL not contribute to phagosomal escape in non-professional phagocytes

The identification of the escape positive strains 6850, LAC and MW2 (chapter 3.1) supported investigation in toxin mediated escape. α-toxin (*hla*) a β-barrel, channel-forming toxin (Gouaux, 1998) is highly expressed in *S. aureus* strains LAC, MW2 and (Figure 13) was reported to be required for phagosomal escape of *S. aureus* strain RN6390 in cystic fibrosis epithelial cell line (Jarry et al., 2008). In addition α-toxin induces programmed cell death of human T-cells, B-cells, and monocytes during LAC infection (Nygaard et al., 2012) by membrane permeabilization (Nygaard et al., 2013).

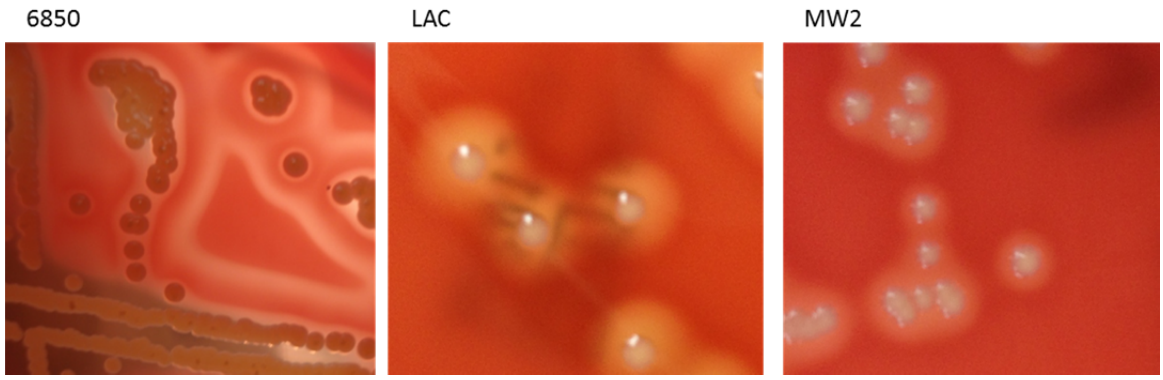

Figure 13: Haemolysis pattern of *S. aureus* strains 6850, LAC and MW2 on sheep blood agar plates. 6850 is an α-toxin and β-toxin producer. MRSA LAC and MW2, both express α-toxin, but lack β-toxigenicity due to an insertion of a prophage genome in their structural *hlb* genes.

To examine the effect of α-toxin in epithelial and endothelial cells, HeLa cells, 293T cells and EAhy.926 were infected with wild-type strains 6850 and LAC as well as the corresponding *hla* knockout mutant strains. Flow cytometric escape analysis showed that the *hla* mutants were able to escape just like the corresponding wild type strains (Figure 14 A, C). Panton-Valentine leukocidin (PVL) is a staphylococcal leukotoxin, belongs to a family of pore-forming toxins and is encode by two genes *luk*S and *luk*F as a bicomponent toxin (Diep et al., 2006). Since, PVL is cytotoxic against neutrophils (Gauduchon et al., 2001) knockout mutants (Δ*luk*SF) in strains LAC and MW2 were tested for phagosomal escape. Recently the human complement receptors C5aR and C5L2 were identified for LukSF which are expressed predominantly on neutrophils and determined consequently a cell and species specificity for PVL (Spaan et al., 2013). Thus, the *luk*SF deficient strains LAC and MW2 escaped as wild-type strains in epithelial cell lines 293T and HeLa (Figure 14 A, B). Strain 6850 produce the sphingomyelinase β-toxin in high amounts (Figure 13) which possesses a phospholipase C activity. It was shown that overexpression of β-toxin together with δ-toxin mediates phagosomal escape in non-professional phagocytes (Giese et al., 2011). Most clinical strains as well as the here investigated MRSA strains LAC and MW2 do not express a functional β-toxin due to insertion of a lysogenic prophage in the gene of β-toxin. Anyway the involvement of β-toxin for phagosomal escape was checked in strain 6850 and its corresponding mutant (6850 pCG33 *hlb*-). Mutant and wild-type strain escaped with similar efficiencies indicating that β-toxin was not involved in escape (Figure 14). *S. aureus* 6850 additional displays a strong phosphatidyl inositol-dependent phospholipase C (PIPLC; *plc*) activity (data not shown). As homologues of PIPLC are involved in phagosomal escape in *L. monocytogenes* (Goldfine et al., 1995), a *plc* knockout in strain 6850 was generated and measured for phagosomal escape in HeLa cells. There are no significant differences between wild-type and mutant, both shows same efficiencies in recruitment of the YFP-Fc escape marker (Figure 14 D). To confirm these results an avirulent *S. aureus* SA113 (Iordanescu and Surdeanu, 1976) overexpressing

PIPLC was also tested. The transgenic strain displayed very low escape rates when only Plc (0.08 ± 0.12 %) was overexpressed. Thus, these results demonstrate that α-toxin, β-toxin and Plc were not required for phagosomal escape of clinically relevant *S. aureus* (Grosz et al., 2014). This results indicate that other toxins are required for phagosomal escape.

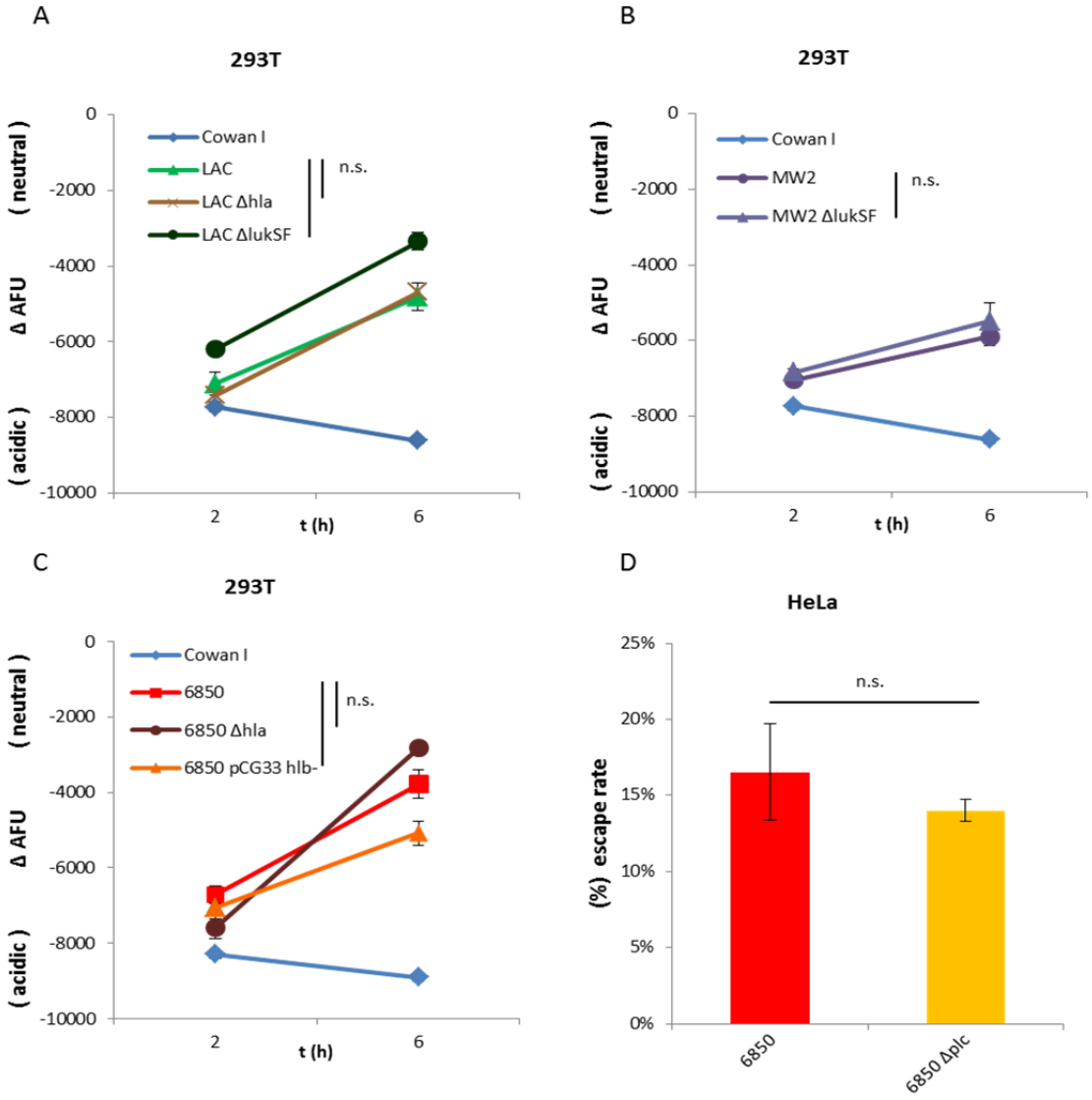

Figure 14: α-toxin, β-toxin and Plc do not contribute to phagosomal escape of *S. aureus*. Mutant strains of LAC (A) or 6850 (C) which were deficient for α-toxin production still escaped from the phagosomes with similar efficiencies as the wild-type or Δ*luk*SF (PVL) negative strains (A, B), whereas Cowan I remained in an acidic compartment. C) A knockout of β-toxin in strain 6850 escaped just as the wild-type strain. Data shown are mean values of at least 3 independent experiments performed in duplicates ± SEM. D) A knockout of *plc* in strain 6850 does not affected efficiency of phagosomal escape in HeLa YFP-Fc cells.

3.2.2 PSMα but not PSMβ or δ-toxin (PSMγ) is required for phagosomal escape

The last identified *S. aureus* toxins are phenol-soluble modulins (PSMs), PSMα expressed by LAC, was shown to be the most virulent gene cluster in a mouse model of infection (Wang et al., 2007). There

are two types of PSMs, PSMα 1-4 belong to the α-type and PSMβ1-2 belongs to the β type, further δ-toxin (PSMγ) is similar to α-type and is encoded by *agr* within RNAIII (Janzon et al., 1989). Giese et al. demonstrate that overexpression of both, δ-toxin (*hld*) and β-toxin, but not either factor alone, was able to mediate phagosomal escape of a non-cytotoxic laboratory strain (Giese et al., 2011). Hence, PSMs appear to be promising factors for phagosomal escape, in this study their contribution was investigated in non-professional and professional phagocytes as well.

Determination of the pH microenvironment by flow cytometry escape assay revealed that there was no difference in ΔAFU values between wild-type strains LAC, MW2 and the corresponding mutants in *hld* (Figure 15 C, D). But PSMα mutants of MRSA LAC and MW2, as well as 6850 were unable to reduce the acidification signal in 293T cells (Figure 15 A, B, D), HeLa cells and EA.hy 926 cells (Figure 16). The complementation of PSMα1-4 in 6850 Δ*psmα* (6850 Δ*psmα* pTXΔpsmα1-4) restored the wild-type phenotype (Figure 15 A). Knockout mutants in PSMβ escaped in similar efficiencies as corresponding wild-type strains.

Results from the microscopic recruitment assay displayed similar results as the flow cytometry escape assay. *S. aureus* LAC Δ*psmα* showed a significantly reduced escape rate in HeLa YFP-Fc cells (2.35±0.92 %) when compared with wild-type strain (20.85±0.63 %; Figure 17 A). PSMβ and δ-toxin deficient mutants escaped with similar efficiencies as wild-type strain, nevertheless all mutants showed no changes for invasiveness (Figure 17 B).

Testing the different PSM types with different approaches revealed that only PSMα is required for phagosomal escape in non-professional phagocytes such as HeLa, 293T or EA.hy926 cells (Grosz et al., 2014).

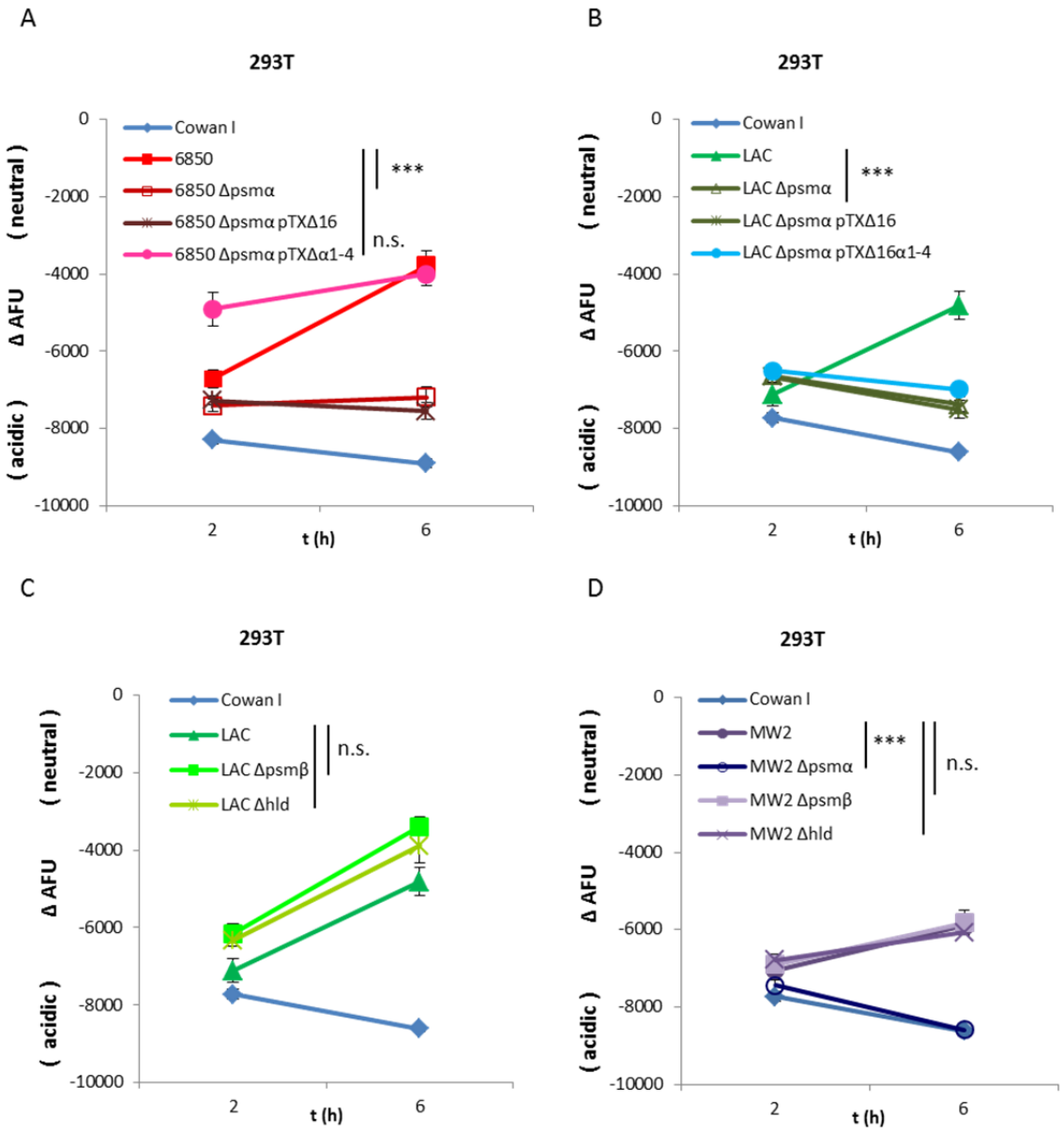

Figure 15: PSMα is required for phagosomal escape of *S. aureus*. Only PSMα knockout failed to de-acidify the microenvironment, with a significant different ΔAFU values compared to wild-type 6850 (A), LAC (B) and MW2 (D). Complementation of PSMα restored the phenotype in 6850 (A) and partially in LAC. By contrast, PSMβ and δ-toxin were not required for phagosomal escape (B-D) and the vector control (pTXΔ16) had no effect on escape as well. Data represent flow-cytometric analysis 2 and 6 h p.i. in 293T cells. Data are displayed as mean values from at least 3 independent experiments performed in duplicates ± SEM. ****P* < 0.001

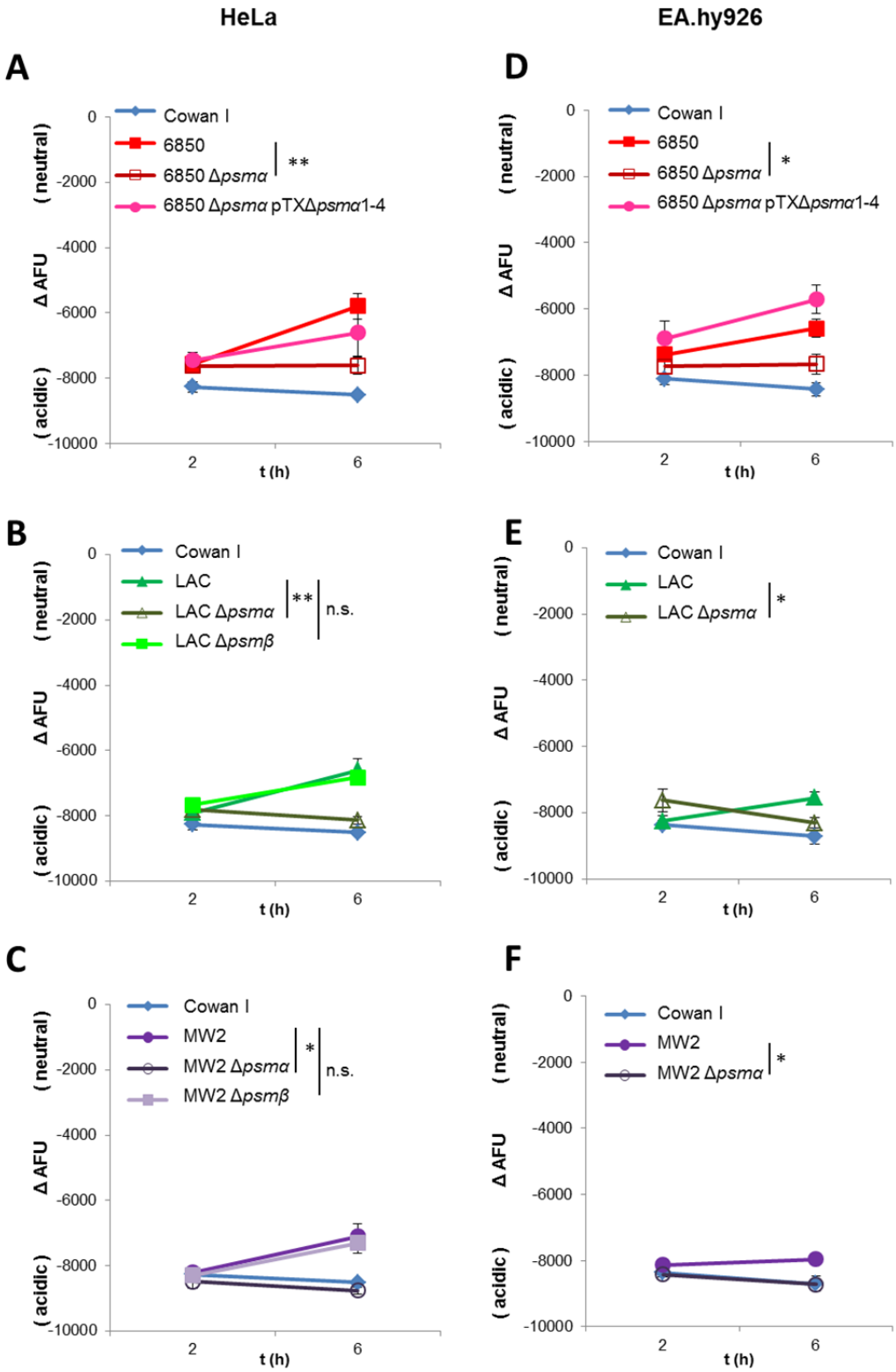

Figure 16: PSMα mediates phagosomal escape of *S. aureus* strains LAC and MW2 in epithelial cells (HeLa) and endothelial cells (EA.hy926). A) HeLa cells and B) EA.hy926 cells were infected with the indicated strains. Only psmα deficient strains displayed AFUs which correspond to acidic pH thereby indicating that there is no phagosomal escape. Data are displayed as means form at least 3 independent experiments performed in duplicates ± SEM. * P <0.05, * P < 0.01.

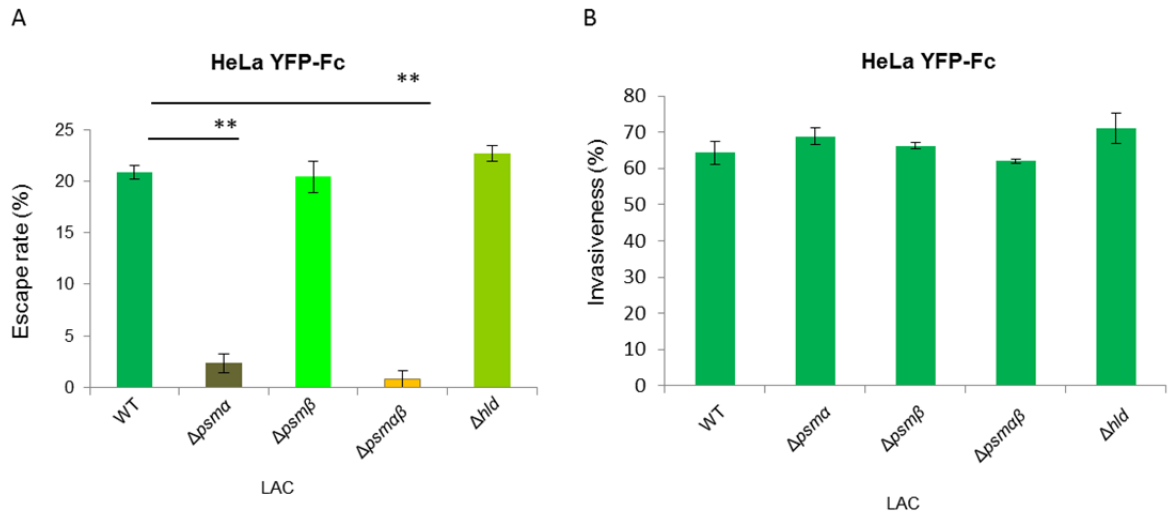

Figure 17: Recruitment of the escape marker YFP-Fc in *S. aureus* LAC, LAC Δ*psm*β and LAC Δ*hld* infected HeLa cells. A) There is a significantly lower escape rate for LAC Δ*psm*α and the double knock-out mutant (Δ*psm*αβ). B) The invasiveness in HeLa YFP-Fc cells between wild-type and mutants were the same.

3.2.3 Expression changes of *psm*α, *psm*β and *agr*A during infection

Based on the results from flow-cytometric escape assay which indicated PSMα as required factor for phagosomal escape, changes in gene expression of *psm*α, *psm*β and *agr*A from phagocytosed *S. aureus* were investigated by qRT-PCR. In wild-type *S. aureus* LAC intracellular *psm*α expression levels (RQ) first increased from inoculum (0.57±0.20) to 2 h p.i. (3.01±1.13) and then were found at low levels at 6 h p.i. (0.29±0.29). Similar dynamic of RQ values were found for mutant strain LAC Δ*psm*β (inoculum: 0.69±0.19; 2 h p.i.: 3.65±0.18; 6 h p.i.: 0.53±0.13). In both strains the *agr*A activity was moderate at 2 h p.i. (wt: 1.12±0.20; Δ*psm*β 1.75±0.40) and 6 h p.i. (wt: 0.49±0.09; Δ*psm*β 0.6±0.14) and in LAC wild-type *psm*β levels significantly decreased during infection time course (2 h p.i.: 1.02±0.55; 6 h p.i.: 0.08±0.05) (Figure 18 A). In the context of LAC Δ*psm*α, *psm*β as well as *agr*A gene expression rose at 2 h p.i. (*psm*β: 2.8±0.7; *agr*A: 4.52±2.1) and stay high at 6 h p.i. (*psm*β: 1.31±0.83; *agr*A: 2.3 ±1.3) when compared with LAC or LAC Δ*psm*β (Figure 18 A). Gene expression profile demonstrate differences between Δ*psm*α mutant and the escape positive wild-type and Δ*psm*β mutant, the expression of *agr*A and *psm*β remained at higher levels in non-escapers compared to escape positive strains.

A quite similar expression outline was observed for 6850 wild-type and 6850 Δ*psm*α mutant (Figure 18 B). Once more *psm*α and *psm*β expression increased in the wild-type from inoculum (*psm*α: 0.16±0.20; *psm*β: 0.34±0.19) to 2 h p.i. (*psm*α: 0.78±0.36; *psm*β: 2.1±0.83) and dropped again to low levels at 6 h p.i. (*psm*α: 0.14±0.07; *psm*β: 0.27±0.11). On the contrary, in the escape-deficient *psm*α

mutant the relative expression of *psm*β started with 2.04±0.72, increase at 2h p.i. to 9.2±2.56 and stay high at 6 h p.i. with 4.96±2.35 (Figure 18 B).

Gene expression analysis thus benefits the necessity of PSMα for phagosomal escape of clinically relevant MRSA LAC and highly cytotoxic 6850 within non-professional phagocytes.

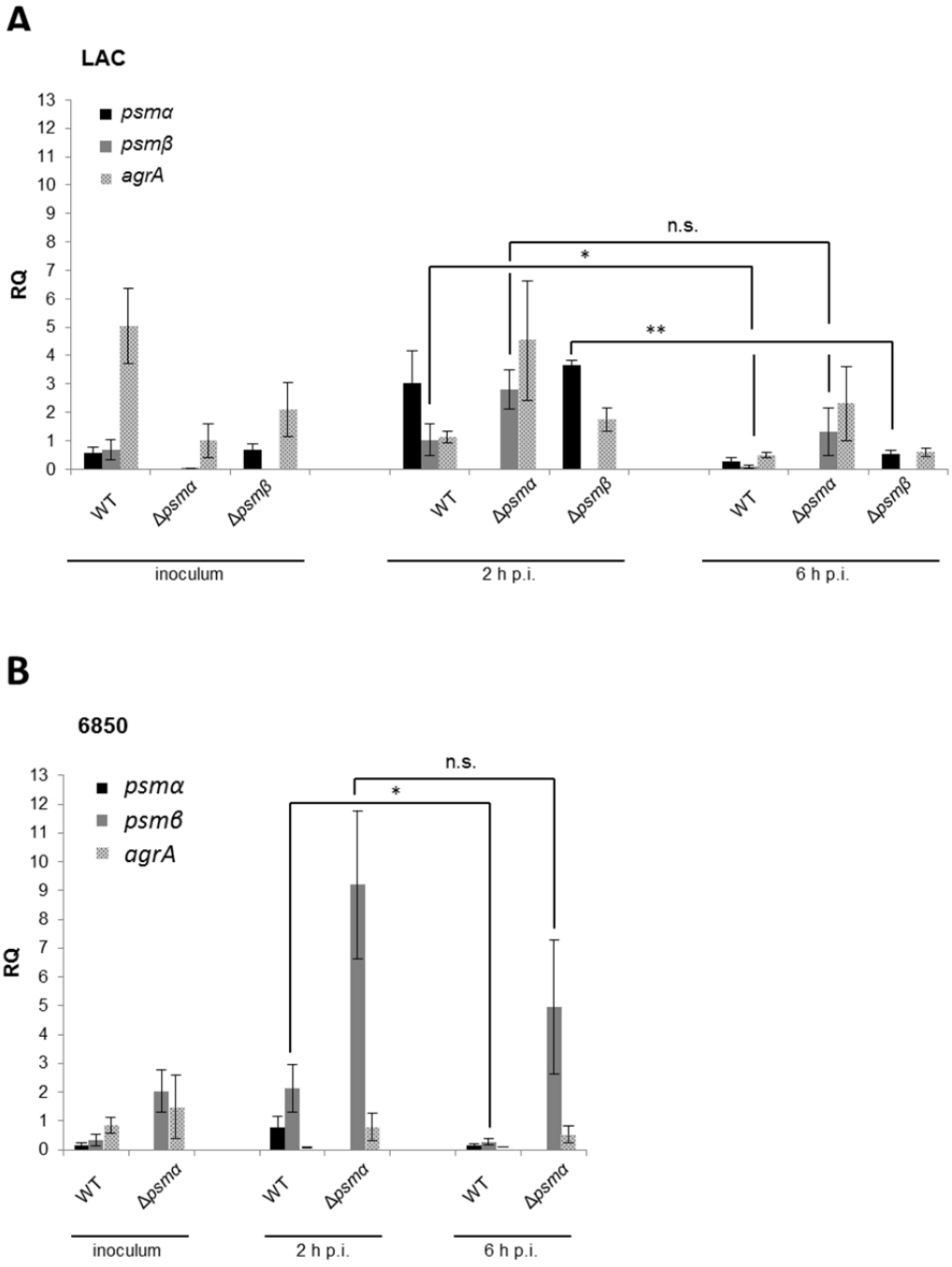

Figure 18: Expression profile of *psm*α, *psm*β ang *agr*A during *S. aureus* infection. In escape-deficient *S. aureus* (Δ*psm*α) *psm*β and *agr*A remain transcriptionally active. Relative expression levels (RQ; $2^{-\Delta CT}$) of the genes *psm*α, *psm*β and *agr*A at 2 and 6 h p.i and of the inoculum as measured by qRT-PCR in 293T cell infected with (A) *S. aureus* strain LAC, LAC Δ*psm*α and LAC Δ*psm*β or (B) 6850 and 6850 Δ*psm*α. Expression was normalized to the housekeeping gene gyrase subunit B (*gry*B). Data shown are results of 3 independent experiment performed in triplicates ± SEM. **P* < 0.05, ***P* < 0.01

3.2.4 Phagosomal escape in osteoblasts

S. aureus infection causes severe infections, including haematogenous osteomyelitis, postsurgical and implant-related infections (Lew and Waldvogel, 2004) therefore phagosomal escape in osteoplastic cells were investigated.

For cell culture infection model, the human fetal osteoblastic cells (hFOB1.19) were used; these cells have the ability to differentiate into mature osteoblasts expressing a normal osteoblastic phenotype. hFob1.19 cells become highly infected by *S. aureus* strain LAC pJL74 (GFP expressing) as evidenced by microscopic image 1 h p.i. (Figure 19 A). The flow cytometry escape assay revealed that *S. aureus* strain 6850 and LAC were able to translocate to the neutral host cell cytoplasm 6 h post infection. Whereas, Δ*psmα* mutants in both strains remained in acidic vacuoles as wells as strain Cowan I. The knockout of PSMβ, LukSF and β-toxin in *S. aureus* 6850 (Figure 19 B, C) had no effect on escape equal to results shown for epithelial and endothelial cells (chapter 3.2.2).

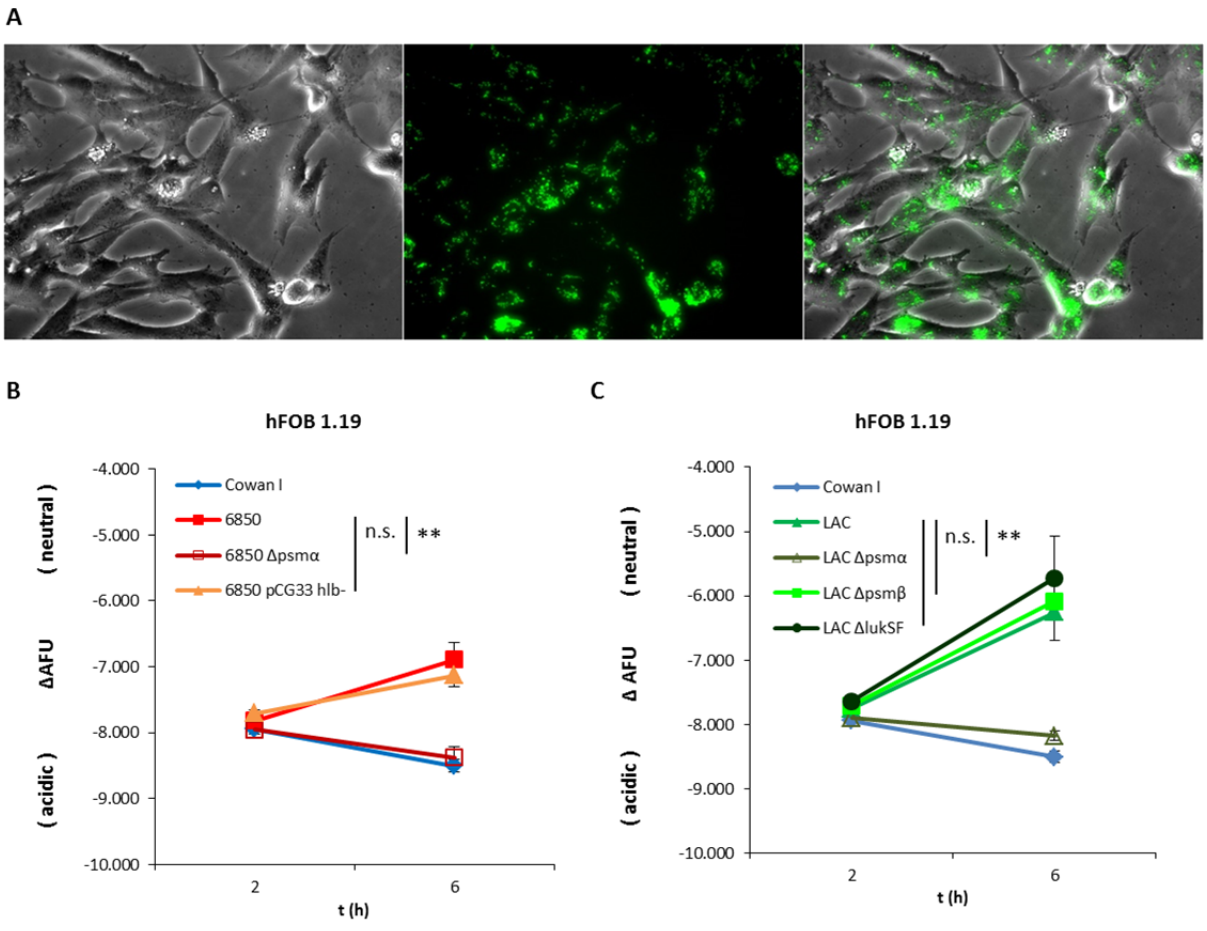

Figure 19: *S. aureus* infects highly the osteoblastic cell line hFob1.19 and escape from the phagosome 6 h post infection. A) hFOB1.19 cells becomes highly infected with LAC pJL74 expressing a GFP plasmid constitutively. There was phagosomal escape for *S. aureus* 6850 (A) and LAC by measuring pH microenvironment of infected hFOB1.19 cells. PSMα deficient mutants (B, C) showed significant less ΔAFU values as corresponding wild-type strains. Sphingomyelinase β-toxin, PSMβ and LukSF knockout mutants escaped with the same efficiency as wild-type strains. Data are displayed as mean values from at least 3 independent experiments performed in duplicates ± SEM. $^{**}P < 0.01$.

3.2.5 PSMα is not sufficient for phagosomal escape

Knockout of *psmα* in strains LAC, MW2 and 6850 prevented phagosomal escape in epithelial, endothelial cells (chapter 3.2.2) and osteoblasts (chapter 3.2.4) thereby indicating crucial role for PSMα. To investigate if PSMα alone is sufficient for phagosomal escape an accessory gene regulator (*agr*) mutant in MRSA LAC was complemented with a plasmid encoding PSMα. As *agr* of *S. aureus* is a global regulator of virulence associated protein genes (Novick et al., 1993), strains deficient or altered in *agr* produce no functional toxin, including PSMs. MRSA LAC deficient in *agr* (LAC *agr*-) was not able to translocate to the host cell cytoplasm as compared with wild-type strain that showed ΔAFU values representing neutral pH. However, the expression of PSMα in LAC *agr*- was not sufficient to restore wild-type phenotype since there is no significant difference between agr deficient and PSMα complemented strain (Figure 20 A). Similarly PSMα expression in the agr negative S. aureus SA113 (Iordanescu and Surdeanu, 1976) does not lead to translocation of the bacteria to the cytosol of 293T cells (Figure 20 B). These results illustrate that PSMα expression is not sufficient for phagosomal escape.

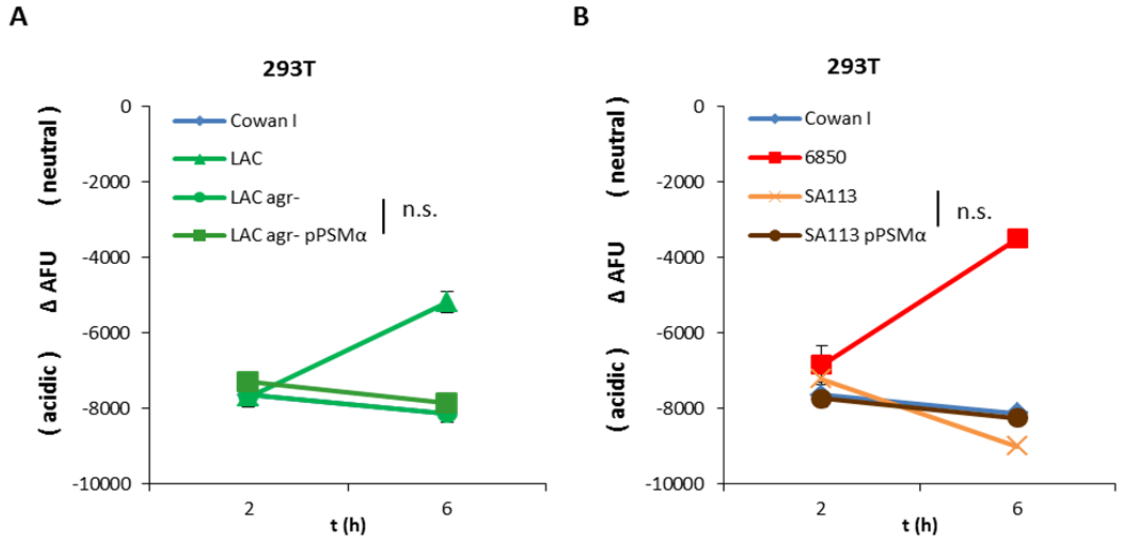

Figure 20: Overexpression of PSMα is not sufficient for phagosomal escape in 293T cells. A) MRSA LAC deficient in *agr* is not able to translocate to the host cell cytoplasm as the parental strain, due to altered toxin production. The complementation with PSMα is not sufficient for phagosomal escape. B) The *agr* deficient *S. aureus* wild-type strain SA113 remains in the phagosomal compartment as the control strain Cowan I. Transformation of SA113 with pPSMα did not lead to phagosomal escape while the escape positive control *S. aureus* 6850 escaped successfully. Data are displayed as mean values from at least 3 independent experiments performed in duplicates ± SEM.

3.2.6 LukAB and PSMα are required for phagosomal escape

As escape assays showed that PSMα expression is not sufficient for phagosomal escape a synergistic cofactor was hypothesized. From current literature leukocidin LukAB seems to be a promising

candidate although staphylococcal escape (DuMont et al., 2013b) or host killing mediated by LukAB were only reported for human neutrophils (DuMont et al., 2013a).

Thus, *S. aureus* LAC Δ*luk*AB was tested for phagosomal escape in different non-professional phagocytes. *LukAB* deficient LAC was not able to escape the phagosome from HeLa and 293T cells, primary human umbilical vein endothelial (HUVEC) cells and osteoblasts hFOB1.19 whereas the wild-type S. aureus strain escaped efficiently (Figure 21). Similarly, mutants in *psm*α, *agr* and a double knockout (Δ*psm*αΔ*luk*AB) were not able to translocate to the cytoplasm of HUVEC cells. Thus, both LukAB and PSMα are required for phagosomal escape in epithelial, endothelial and osteoblastic cell lines.

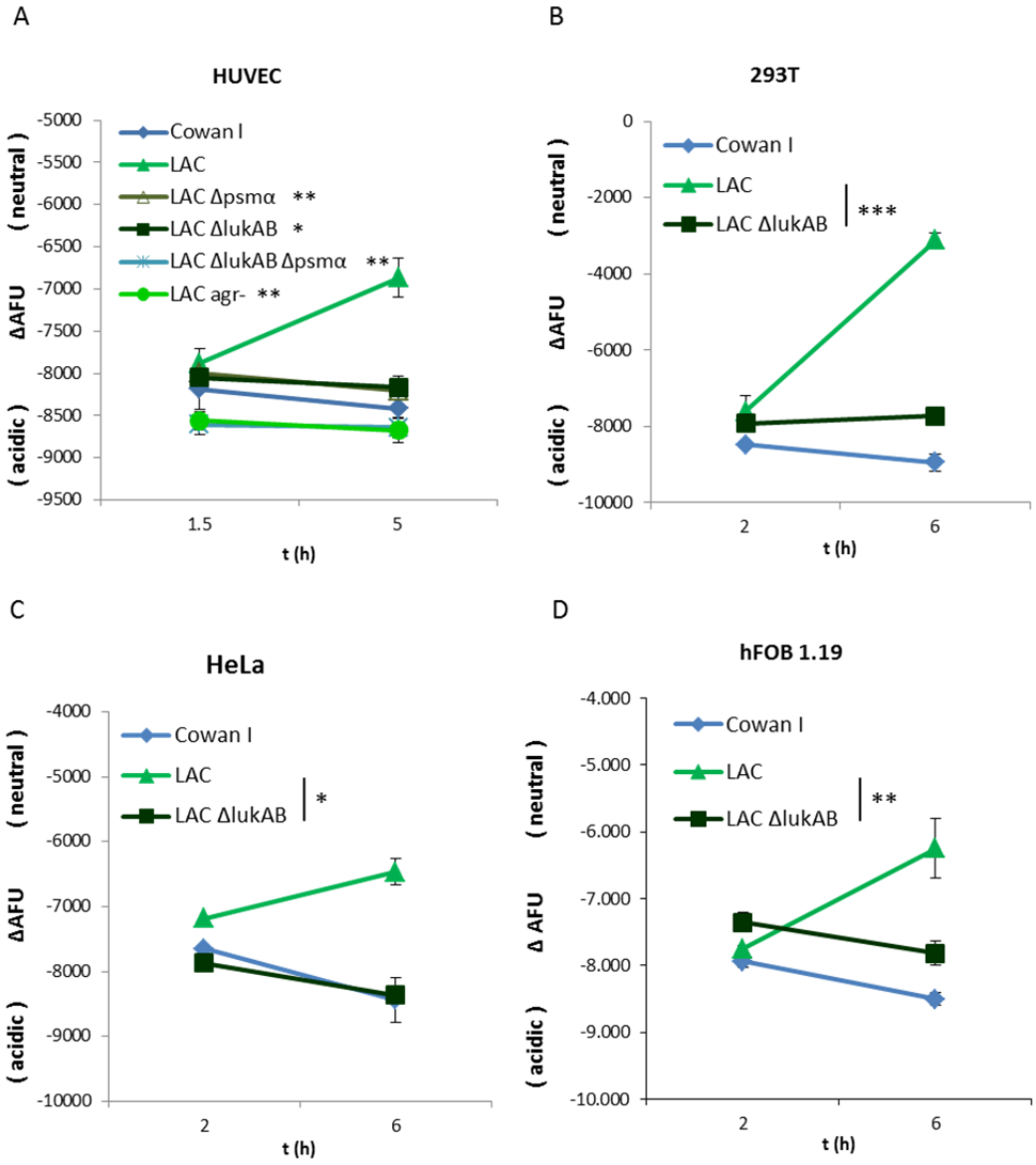

Figure 21: LukAB mediates phagosomal escape in endothelial, epithelial and osteoblastic cells. There is no phagosomal escape with LAC Δ*luk*AB in HUVEC (A), 293T (B), HeLa (C) and hFOB1.19 cells (D). Further, Δ*psm*α, double knock-out Δpsmα Δ*luk*AB and *agr* deficient mutants display ΔAFU values as Cowan I (control) in HUVECs (A). Data are displayed as means from at least 3 independent experiments performed in duplicates ± SEM. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

3.2.6.1 *cd11b* expression during *S. aureus* infection

Knockout of *lukAB* in MRSA LAC showed an escape deficiency in epithelial cells (293T, HeLa), endothelial cells (HUVEC) and osteoblasts (chapter 3.2.6). LukAB has been previously shown to bind integrin alpha M (ITGAM) also known as cluster of differentiation molecule 11B (cd11b) in neutrophils (DuMont et al., 2013a) since *lukAB* showed an escape phenotype also in non-professional phagocytes the relative expression of *cd11b* in infected HUVEC and 293T cells were measured. RNA was isolated 1 h p.i. as well as 5 h p.i. qRT-PCR showed a significant increase in *cb11b* expression in HUVECs during later stages of infection when compared with 1 h p.i. time points (*S. aureus* 6850 5 h p.i. 42.54±9.83 fold increase; *S. aureus* LAC 5 h p.i. 3.91±1.60 fold increase of cd11b transcription) (Figure 22 A). In infected 293T cells with *S. aureus* 6850 is the rel. *cd11b* expression significantly increased 5 h p.i. compared with 1 h p.i. by a factor of 5.39±1.72 (Figure 22 B).

The relative *cd11b* expression in infected HUVEC was found to be increased to similar levels when compared to those of PMA-treated THP-1 cells infected with *S. aureus*: 1h p.i. resulted in 23.01 fold change increase in *cd11b* transcripts whereas 3 h p.i. demonstrated a 42.89 fold change. Without PMA there is no *cd11b* expression in THP-1 monocytes (Figure 22 C).

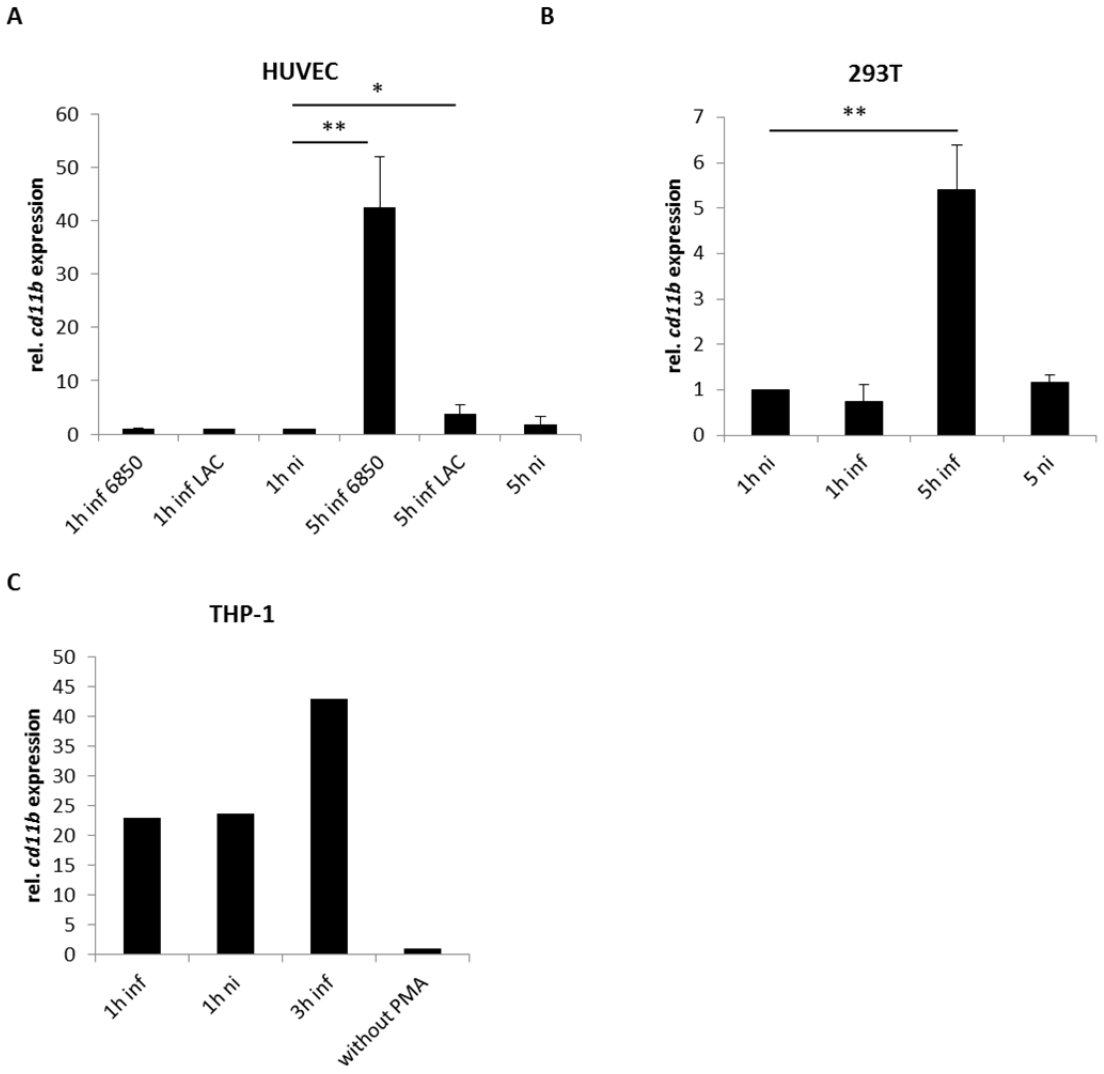

Figure 22: Complement receptor CD11b is upregulated during *S. aureus* infection in endothelial and epithelial cell lines. qRT-PCR revealed that after infection with *S. aureus* 6850 (A, B, C) or LAC (A) *cd11b* which encodes the receptor for LukAB is upregulated. Expression was normalized to the human housekeeping gene Glycerinaldehyd-3-phosphat-Dehydrogenase (*gapdh*) and to the not infected (ni) cells according following formula: $2^{-\Delta\Delta CT}$. Data shown (A) are results of 3 independent experiment performed in triplicates ± SEM. **P* < 0.05, ***P* < 0.01. As a control *cd11b* expression was measured in professional phagocytes (THP-1) untreated and PMA-treated. Results from (C) represent 1 experiment performed in triplicates.

3.2.6.2 *fpr2* expression during *S. aureus* infection

The formyl-peptide receptor FPR2 (Fu et al., 2006) is the receptor for PSMα in neutrophils (Kretschmer et al., 2010). Similar to *cd11b* (12 fold change at 4.5 h p.i. compared to 1 h p.i.) *fpr2* transcripts increased (5.56 fold change, Figure 24 A) at later time points of infection in 293T cells. Furthermore, there is no *fpr2* expression when 293T cells are infected with LAC Δ*psmα* (Figure 24 B).

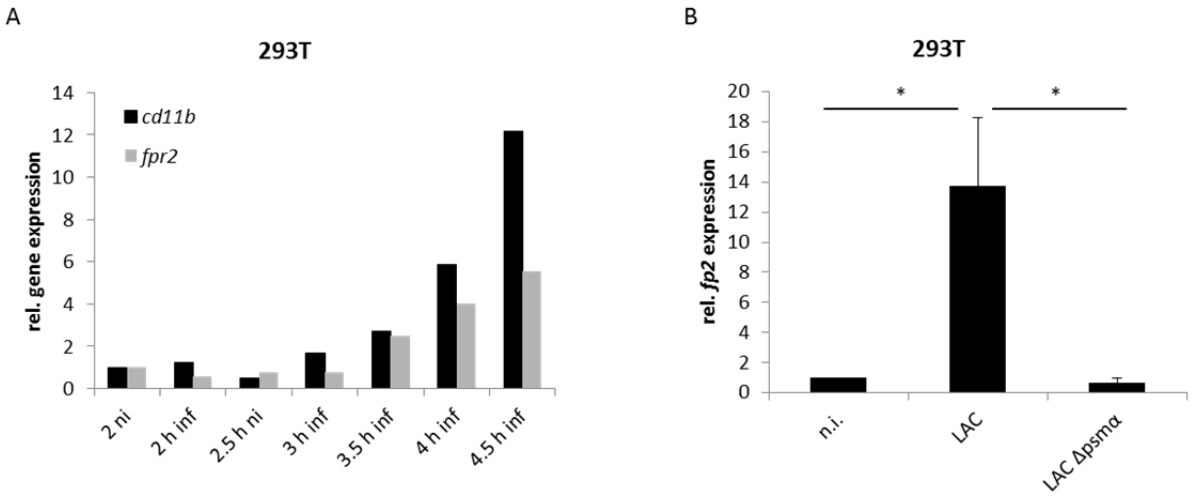

Figure 23: *cd11b* is the gene encoding the receptor for LukAB and *fpr2* is the gene encoding the receptor for PSMα, both receptors are upregulated during infection of 293T cells. Expression was normalized to the human housekeeping gene Glycerinaldehyd-3-phosphat-Dehydrogenase (*gapdh*) and to the not infected (ni) cells according following formula: $2^{-\Delta\Delta CT}$. Data represent 1 experiment performed in triplicates (A). *fpr2* is expressed only after infection with *S. aureus* expressing PSMα peptides (B).

To test wheater the upregulation of fpr2 results in a functional protein, SDS Page and Western blot analysis were performed. Only in cells infected with *S. aureus* 6850 a protein band of 38 kDa was detected, which corresponds to the size of FPR2. Further, there is no signal when cells are infected with 6850 Δ*psmα* or Cowan I (Figure 24 A). The FPR2 receptor thus is expressed only after infection with *S. aureus* expressing PSMα peptides. In order to investigate if PSMα-dependent phagosomal escpae of S. aureus required the presence of FPR2, HeLa YFP-CWT escape marker cells were transfected with a small interfering RNA (siRNA) against FPR2 and a negative controle (siALL Star) and were subsequently infected with *S. aureus* 6850. Interestingly, escape signal in the negative controle is more prominent as in cells with silenced FPR2 (Figure 24 B) illustrating that PSMα might use FPR2 as cellular receptor to initate phagosomal escape even in non-professional phagocytes that are usually devoid of the receptor.

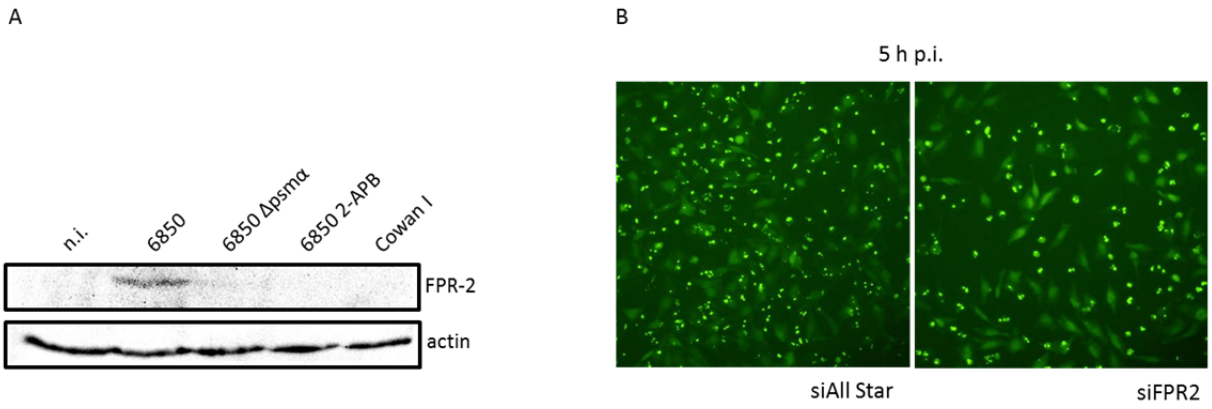

Figure 24: Protein expression of FPR2 receptor in *S. aureus* infected 293T cells. A) The functional peptide for FPR2 receptor with 38 kDa is prominent after infection with *S. aureus* 6850. Actin is the protein loading control. B) Silencing of FPR2 in HeLa YFP-CWT reduced the escape signal for *S. aureus* 6850 5 h post infection.

3.2.7 Phagosomal escape in macrophages

To investigate the relevance of phagosomal escape in professional phagocytes THP-1 cells were activated with phorbol-12-myristate-13-acetate-differentiated (PMA) for differentiation into macrophages and were infected with *S. aureus* 6850. Flow-cytometric escape analysis demonstrated that 6850 is able to escape the phagosome of macrophages, whereas Cowan I remained in an acidic environment (Figure 25 A). Similar, THP-1 YFP-CWT reporter cells infected with 6850 (Figure 25 C) and LAC (Figure 25 B, D) at an MOI of 10 indicated that 6850 shows an escape rate of 25.6±0.3 % in the microscopic recruitment assay whereas Δ*psmα* mutant displayed only 5.5±0 % of translocating bacteria. The complemented strain (Δ*psmα* pTXpsmα1-4) displayed an escape rate (12.2±2.8 %) similar to the wild-type strain (Figure 25 C). Also, wild-type strain LAC (5.4±1.6 %) and complemented mutant (3.9±1.6 %) escaped significantly better when compared with the isogenic Δ*psmα* mutant (0.8±0.3 %) (Figure 25 D).

Thus, 6850 and LAC are able to escape form professional phagocytes and PSMα is required for a phagosomal escape (Grosz et al., 2014).

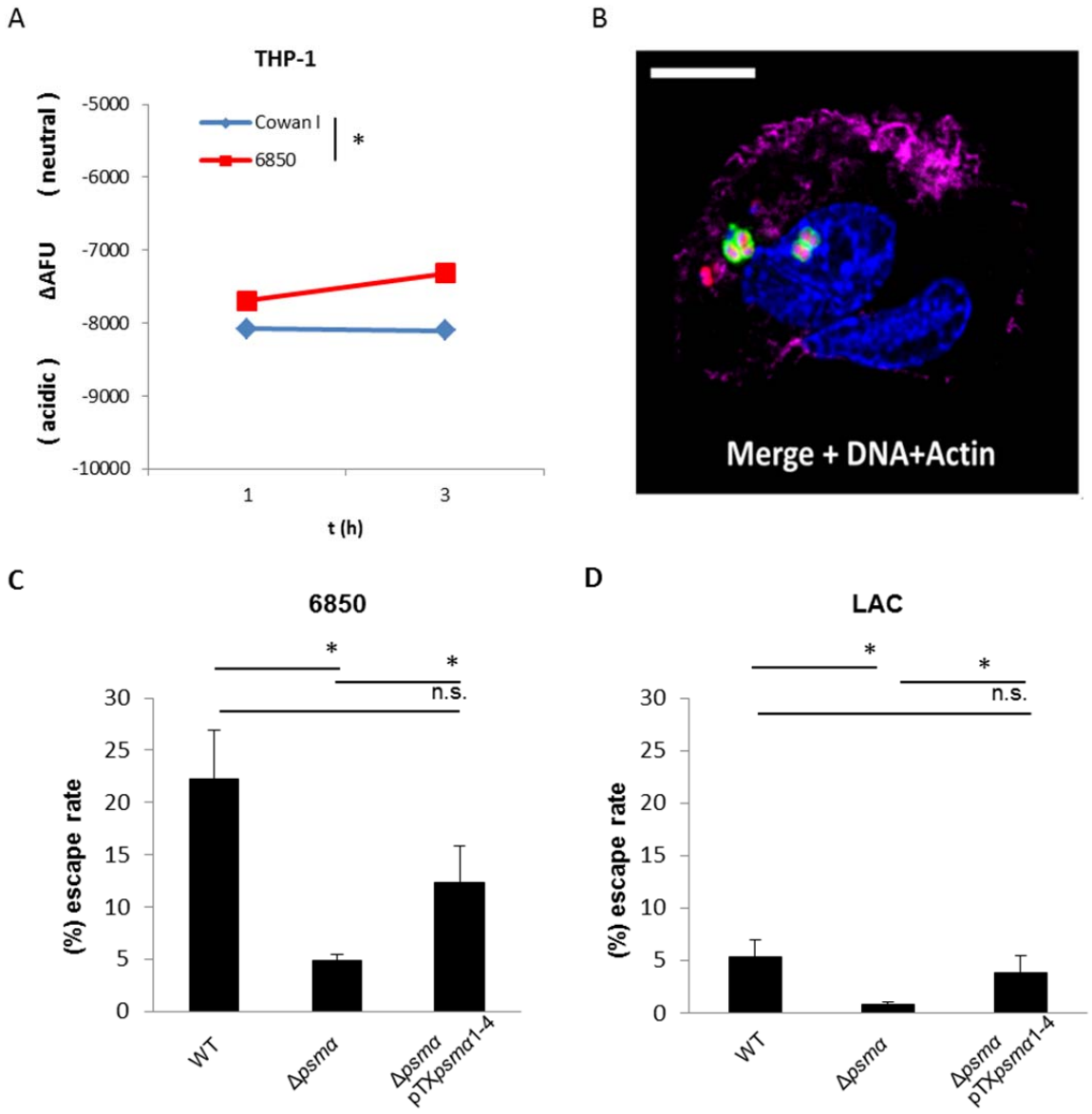

Figure 25: MRSA LAC and MSSA 6850 escape for phagosomes of professional phagocytes THP-1 cells. A) Phagosomal escape of *S. aureus* 6850 for THP-1 phagosomes was determined by measuring pH microenvironment by flow-cytometry. B) Escape of wild-type *S. aureus* LAC (green) from THP-1 phagosomes as shown by YFP-Fc escape marker recruitment (green). While, psmα deficient mutants in strains 6850 (C) and LAC (D) were not able to escape. A complementation by expression of psmα *in trans* restored the wild-type phenotype. Data are displayed as mean values from at least 3 independent experiments performed in duplicates ± SEM. *$P < 0.05$.

3.3 Cytoplasmic replication of *S. aureus* after phagosomal escape

Since *S. aureus* was shown to escape from phagosomes of professional and non-professional phagocytes, the fate of the intracellular bacteria was determined by lysostaphin protection assays.

The epithelial cell line 293T as well as the phagocytic cell line THP-1 was infected with *S. aureus* strains. Cells were osmotically lysed (293T cells 2 h and 6 h p.i. and THP-1 cells 1 h and 3 h p.i.) and released bacteria were plated on agar plates. Whereas, the escape-competent *S. aureus* 6850

replicates intracellularly in 293T cells (from 20.0±7.8 CFUs at 2 h p.i. to 59.0±12.84 CFUs at 6 h p.i.) no replication was observed for *S. aureus* 6850 Δ*psmα* (from 16 ± 4.94 CFUs at 2 h p.i. to 25 ± 5.58 CFUs at 6 h p.i.) or Cowan I (from 6 ± 1.01 CFUs at 2 h p.i. to 6 ± 0.88 CFUs at 6 h p.i.). *S. aureus* 6850 had a 5 fold increased intracellular replication compared with 6850 Δ*psmα* and Cowan I (Figure 26 A). Illustrating that numbers of escape-deficient bacteria did not increase intracellularly for the latter time of infection.

Similar results were demonstrated for *S. aureus* LAC and its escape-negative mutant strains: wild-type LAC replicated (from 9.0±2.37 CFUs at 2 h p.i. to 45.0±10.18 CFUs at 6 h p.i.) whereas the isogenic mutant did not (LAC Δ*psmα* from 8.0±0.87 CFUs at 2 h p.i. to 14±3.27 CFUs at 6 h p.i.; LAC Δ lukAB from 15 CFUs at 2 h p.i. to 5 CFUs at 6 h p.i.; LAC Δ*psm*αΔ*luk*AB from 6.3 CFUs at 2 h p.i. to 11.6 CFUs at 6 h p.i. and LAC agr- from 12.5 CFUs at 2 h to 16 CFUs at 6 h p.i.). The relative fold changes (2 h p.i. versus 6 h p.i.) were significantly lower for all tested mutant strains compared with wild-type LAC (Δ*psm*α: 1.08±0.39; ΔlukAB: 1.65±0.92; Δ*psm*αΔ*luk*AB: 1.89±0.54; *agr*-: 1.40±0.24; Figure 26 B).

Microscopy was used to visualize this statement, 293T cells were infected with GFP expressing *S. aureus* 6850, 6850 Δ*psm*α and LAC Δ*lukAB* (Figure 26 C). There are no invasion differences between wild-type and mutant strains, 293T cells became highly infected with green fluorescent bacteria 1 h post infection. After continuing infection with wild-type 6850 cells became a rounding shape, started to detach and were completely full with bacteria. Most of the cells which were infected with either 6850 Δ*psmα* or LAC Δ*lukAB* appeared with single bacteria per cell 5 h p.i. (Figure 26 C). These results were corroborated by flow cytometry using GFP-expressing bacteria.

Interestingly, addition of a supernatant of a bacterial overnight culture constitutively expressing PSMα1-4 was able to restore intracellular replication of green-fluorescent 6850 Δ*psmα* (Figure 26 D) in HeLa cells. The supernatant of this PSMα complemented mutant was able to induce *fpr2* in not infected HeLa cells, whereas the supernatant of the PSMα deficient mutant was not able to induce *fpr2* comparable to not treated cells (without SN; Figure 26 E).

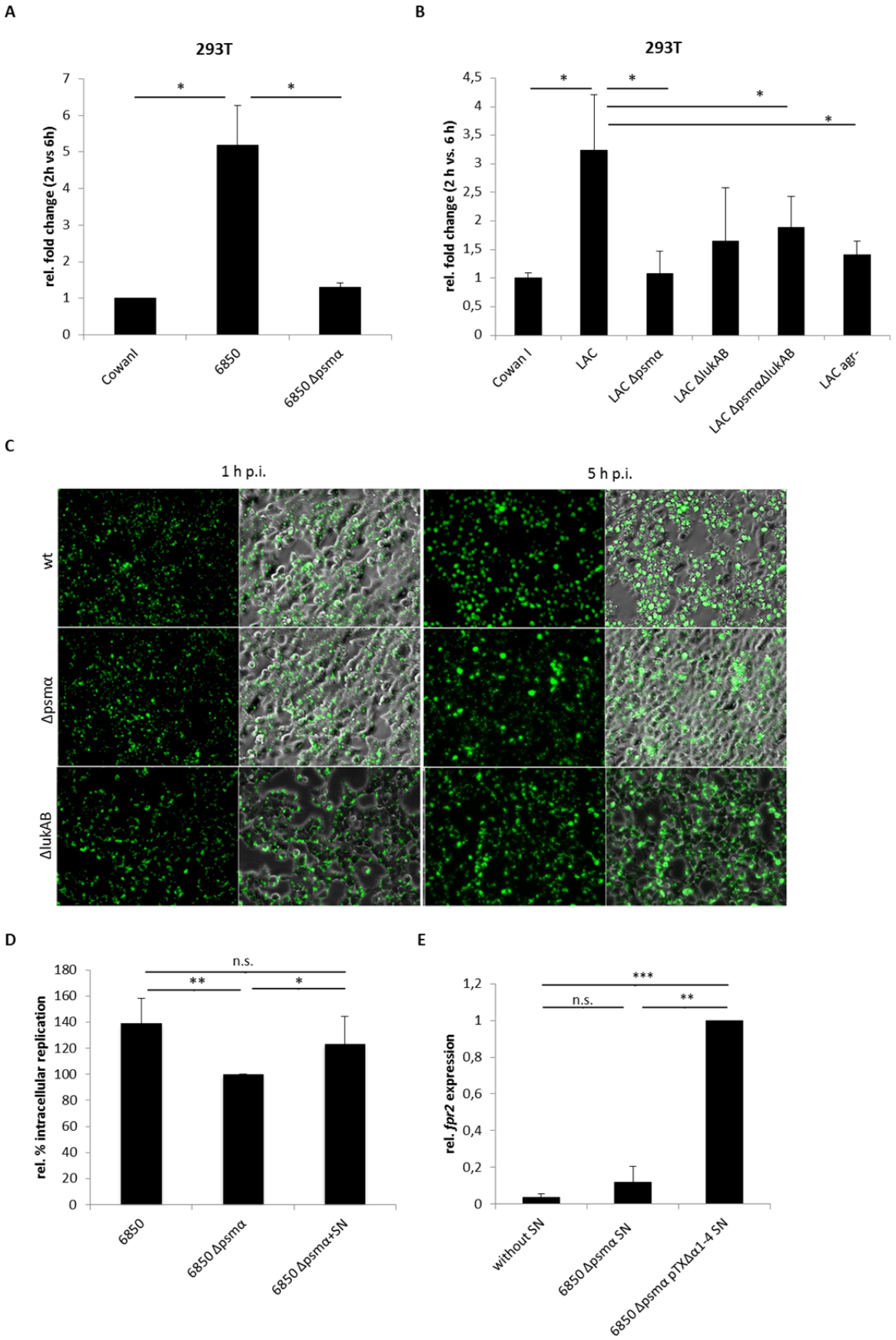

Figure 26: *S. aureus* replicates in the host cell cytoplasm. The escape positive strains 6850 (A) and LAC (B) replicate intracellularly as evidenced by the increased number of recovered CFU per host cell, expressed here as fold change (2 h vs.

6 h p.i.) values. The escape negative strains Cowan I, 6850 Δ*psm*α (A), LAC Δ*psm*α, LAC Δ*luk*AB and LAC *agr*- (B) displayed significantly less recovered CFUs. C) 293T cells were infected with GFP expressing S. aureus strains 6850, 6850 Δpsmα and LAC ΔlukAB. At 1 hp.i. there are no differences between wild-type and mutant strains, but at 5 h p.i. there are more round and detaching cells, which are filled with 6850 compared with those infected with the mutant strains. Cells infected with *psm*α or *lukAB* deficient mutants display less replication, only a few cells are completely filled with staphylococci. D) Supernatant of 6850 Δ*psmα*pTXΔα1-4 with consitutive expression of PSMα allows intracellular replication of 6850 Δ*psmα* in HeLa cells. Replication correspond to increased GFP signal of replicating staphylococci and measured by flow-cytometry. E) Hela cells were incubated with 50 % of the culture supernatant of bacterial overnight for 1 h, thereafter RNA was isolated. Supernatant was steril filtered twice and treated with lysostaphin. Expression was normalized to *gapdh* and to 6850 Δ*psm*α pTxα1-4 SN treated cells according following formula: $2^{-\Delta\Delta CT}$. Data shown are mean values of at least 3 independent experiments performed in duplicates ± SEM. **P*< 0.05, ***P*< 0.01.

S. aureus 6850 also replicated within THP-1 cells (11.5±6.3 CFUs at 2 h p.i. to 28±15.5 CFUs at 6 h p.i.), whereas Cowan I and the PSMα mutant were recovered with lower CFUs (Cowan I: 19.5±6.3 CFUs at 2 h p.i. to 18±5.6 CFUs at 6 h p.i. and 6850 Δ*psmα*: 19±1.9 CFUs at 2 h p.i. to 15±9.9 CFUs at 6 h p.i.). Thereby strain 6850 replicated with a significantly greater fold change compared to Cowan I and 6850 Δ*psmα* (Figure 27 A). In these experiments the host cell numbers were monitored. Initially, $1.25x10^5$ cells/ml and well were seeded for an infection experiment. At 3 h p.i. Cowan I and 6850 Δ*psmα* infected cells displayed counts of about $1.2x10^5$ cells/ml which correspond the initial cell numbers. However, host cells infected with the wild-type 6850 decreased in number (3 h p.i.: $7x10^4$ cells/ml).

Together these results indicated that intracellular *S. aureus* replication within the host cell cytoplasm upon phagosomal escape in a relatively short time span and that phagosomal escape of the bacteria might influence host cell viability.

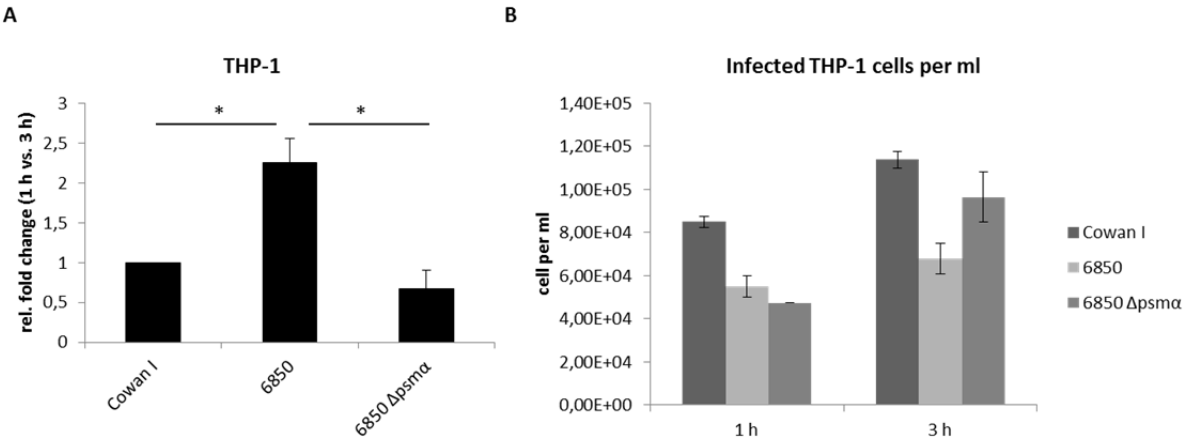

Figure 27: *S. aureus* replicates in the host cell cytoplasm of macrophages. A) The escape positive *S. aureus* 6850 replicated in THP-1 cells 3 h p.i. while Cowan I and 6850 Δ*psm*α displays constant recovered CFUs. B) There are less THP-1 cells infected with 6850 compared to Cowan I or 6850 Δ*psm*α. Data shown are means of at least 3 independent experiments performed in duplicates ± SEM. **P*< 0.05.

3.4 *S. aureus* induced host cell death is linked to phagosomal escape

Based on phagosomal escape of MRSA LAC and MW2 as well as MSSA 6850 which is followed by cytoplasmic replication and beyond mutants deficient in PSMα and LukAB remain in phagosomes without replication, were the linkage between phagosomal escape and *S. aureus* induced host cell death investigated.

Host cell death was monitored with Annexin V/7AAD staining using flow-cytometry (in cooperation with A.-C. Winkler). After a cell has entered apoptosis, the negatively charged phospholipids are transported from inner side of cellular membrane to the outer cell surface by the protein scramblase. Annexin V, which is labelled to a fluorescent protein, binds only phospholipids oriented at the outer cell surface and indicates apoptotic cells. Necrotic cells with a destroyed cell membrane are marked by 7AAD. Investigations with LAC and 6850 and their corresponding escape-negative mutants were performed in HUVEC and HeLa cells. Flow-cytometric assays showed that there is a signal for Annexin V but not for 7AAD, demonstrating no cell lysis upon *S. aureus* infection. HUVECs infected with *S. aureus* LAC showed four times as much annexin positive cells as uninfected cells (18±11 % pos. cells, in cooperation with A.-C. Winkler). Cell death rate is significantly reduced down to 60-70 % when cells were infected with either LAC *psm*α (72±5 % pos. cells) or LAC *luk*AB (60±3 % pos. cells) knockout mutants (Figure 28 A). Cell counts of infected HUVECs showed supporting results, infected cells with LAC or 6850 had less residual cells per millilitre as for Cowan I, *psm*α or *luk*AB knockout mutants (Figure 28 B).

Similar results were obtained for *S. aureus* infected HeLa cells. Cell death rate for uninfected (28±8 % pos. cells), infected with LAC *psm*α (84±6 % pos. cells) or LAC *luk*AB (77±10 % pos. cells) as well as the *agr* deficient (60±7 % pos. cells) mutant were significantly reduced up to 60-84 % annexin positive cells (Figure 28 C) compared with wild-type infected HeLa cells. Similarly, HeLa cells infected with Cowan I (43±3.2 % pos. cells) or 6850 Δ*psm*α (65±5.2 % pos. cells) had a significantly reduced cell death rate compared with 6850 infected cells (Figure 28 D). Shown results support that there was no strain or cell line specific.

Survival of infected HeLa cells was tested by a colorimetric approach in a crystal violet assay to validate results obtained in Annexin V/7AAD assays. HeLa cells were infected for 24 h and stained with crystal violet, the amount of dye correlates with survived host cells. There are significantly more survived cells infected with Cowan I (80±5 % survived cells) or LAC Δ*psm*α (67±2 % survived cells) as with wild-type LAC (41±6 % survived cells). As controls were staurosporin (4±2 % survived cells) and TritonX-100 (11±1 % survived cells) treated cells included (Figure 28 E).

These results indicate that phagosomal escape of *S. aureus* and pathogen induced host cell death are linked.

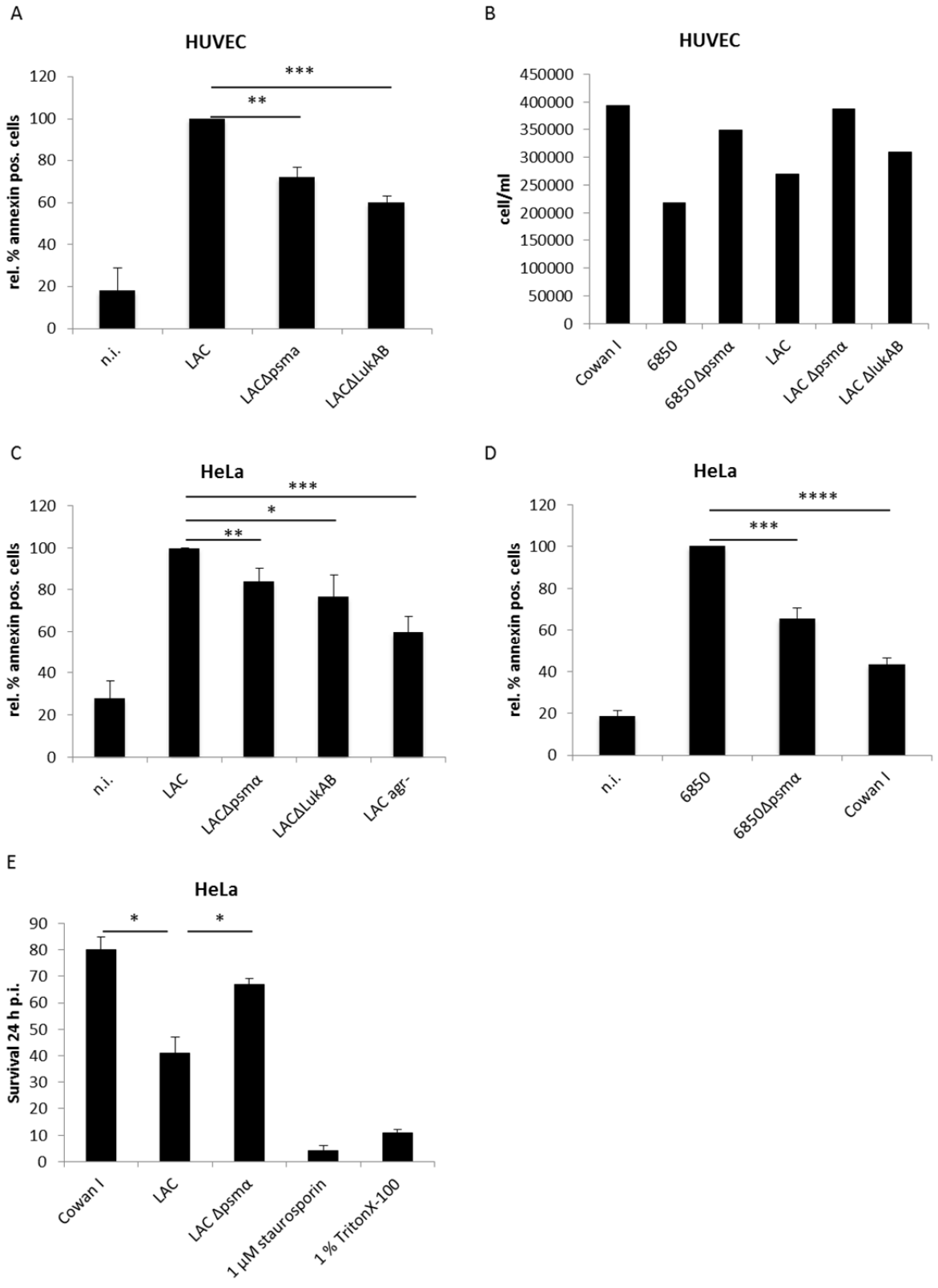

Figure 28: Escape deficient *S. aureus* strains cause less host cell death. A) The *psmα* and *lukAB* knockout mutants showed less cell death (annexin positive pos. cells) than wild-type infected in HUVEC and (C) HeLa cells. B) There are less residual HUVEC cells per mililiter infected with 6850 and LAC. Data represent 1 experiment. C) HeLa cells infected with Cowan I or

6850 Δ*psmα* showed less annexin positive cells. D) HeLa cells infected with Cowan I, LAC or LAC Δ*psmα* for 24 hours were stained with crystal violet. Staining intensity correlates with survived cells. There aere significantly more survived cells infected with Cowan I and LAC Δ*psmα* compared to wild-type infected. A, C, and D in cooperation with A.-C. Winkler. Data shown are mean values of at least 3 independent experiments performed in duplicates ± SEM. *P< 0.05, **P< 0.01, ***P< 0.001, ****P< 0.0001

3.5 Host cell contribution to phagosomal escape

3.5.1 Role of intracellular calcium for phagosomal escape, replication and host cell death

3.5.1.1 Phagosomal escape is blocked by IP3R inhibitor 2-APB

As a whole genome RNA interference (RNAi) screen (Dissertation Winkler, 2015) of HeLa cells infected with *S. aureus* 6850 revealed that intracellular calcium plays a role during infection, a main receptor for Ca^{2+} release the inositol 1,4,5-trisphosphat receptor (IP3R) was blocked by its inhibitor called 2-APB. Involvement of intracellular calcium in phagosomal escape was measured.

HUVECs infected with LAC or 6850 were treated with 2-APB (30 µM final concentration) and phagosomal escape was measured by flow cytometry escape assay. 2-ABP treatment prevents phagosomal escape of *S. aureus* LAC and 6850 in HUVEC cells (Figure 29 A, B). Further, was shown that 2-APB treatment had an impact on bacterial growh in cells, there was no intracellular replication of *S. aureus* after inhibitor treatment (Dissertation Winkler, 2015).

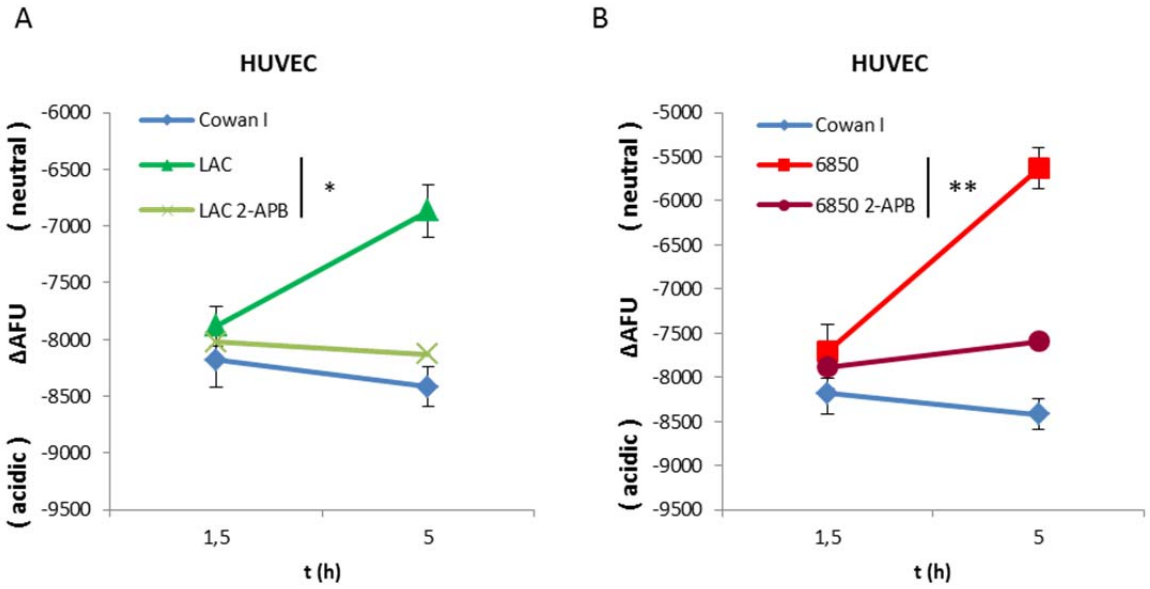

Figure 29: Intracellular calcium is necessary for phagosomal escape of *S. aureus*. Blocking the intracellular Ca^{2+} release with IP3R inhibitor 2-APB prevented phagosomal escape of LAC (A) and 6850 (B) in HUVEC cells. Data shown are mean values of at least 3 independent experiments performed in duplicates ± SEM. *P< 0.05, *P< 0.01.

3.5.1.2 Changes in *fpr2* and *cd11b* expression after 2-APB treatment

Since, the IP3R inhibitor 2-APB blocked phagosomal escape of *S. aureus* (chapter 3.5.1.1) and the receptors CD11b for LukAB and FPR2 for PSMα were upregulated during infection (chapters 3.2.6.1 and 3.2.6.2), qRT-PCR was performed to investigate changes in *cd11b* and *fpr2* expression of *S. aureus* infected 293T cells treated with 2-APB.

Initially, there was no expression of *fpr2* and *cd11b* one hour p.i. but at later time point of infection gene expression was approximately five fold enriched. In samples treated with 2-APB remained the rel. expression of *fpr2* and *cd11b* significantly low similar as in samples 1 h p.i. or in uninfected cells (Figure 30). Thus, IP3R inhibitor 2-APB had an effect on the regulation of both receptors CD11b and FPR2, which plays a role in phagosomal escape.

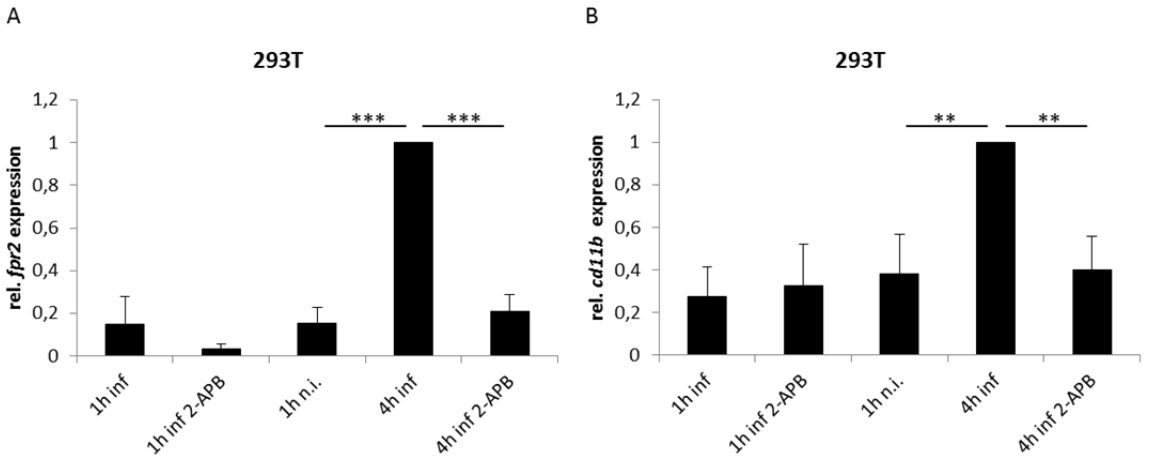

Figure 30: The IP3R inhibitor 2-APB repress the *fpr2* and *cd11b* expression during infection. Expression was normalized to the human housekeeping gene Glycerinaldehyd-3-phosphat-Dehydrogenase (*gapdh*) and to the infected cells according following formula: $2^{-\Delta\Delta CT}$. Data shown are results of 3 independent experiment performed in triplicates ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$.

3.5.1.3 *S. aureus* induced host cell death is blocked by 2-APB

Phagosomal escape and intracellular replication of *S. aureus* was mandatory for host cell death (chapters 3.3 and 3.4) since IP3R inhibitor 2-APB prevented phagosomal escape of *S. aureus* (chapter 3.5.1.1), Annexin V/7ADD staining was investigated of *S. aureus* infected and 2-APB treated HUVEC cells.

HUVECs were infected with *S. aureus* LAC and treated with 2-APB, as a control non-cytotoxic *S. aureus* Cowan I was included in the cell death assays. The host cell death rate was significantly reduced after 2-APB treatment down to 38±22 % with no significant difference to Cowan I infected cells (Figure 31).

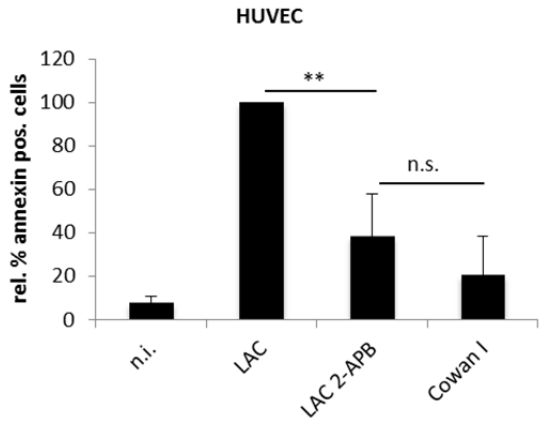

Figure 31: *S. aureus* induced host cell death was reduced after 2-APB treatment. Infection of HUVECs with *S. aureus* LAC prevented cell death after 2-APB treatment by Annexin/7AAD assay. HUVECs were infected with Cowan I (neg. control). Data shown are results of 3 independent experiment performed in triplicates ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$.

3.6 Host cell factors affected in *S. aureus* infection identified with dual RNA-seq

To investigate changes in gene expression during infection of the host and pathogen in parallel, samples were analysed by Dual RNA-seq sequencing. Therefore RNA was isolated from infected 293T cells with *S. aureus* 6850 and escape-deficient 6850 Δ*psm*α at 2 h p.i and 6 h p.i. Sequencing was performed with HiSeq 2500 system (Illumina) in single read mode with 100 running cycles.[1] The quality trimming was applied with a Phred quality score of 20.

The numbers of reads per lane of *S. aureus* genes (purple; Figure 32) were too low to perform further bioinformatical analysis.[2] Human genes reached read numbers over $1.5x10^7$ (green; Figure 32). Most of the aligned reads are classified as rRNA and tRNA (Figure 33 A, B). The human genes which were involved in *S. aureus* infection had been aligned and the top ten hits with a fold-change ≥ 2 and p-value ≤ 0.05 are listed in the chapter appendix (Table 16, Table 17, Table 18, Table 19, Table 20).

[1] Performed by Alexander Westermann (IMIB, University Wuerzburg)
[2] Performed by Dr. Konrad Förstner (Core Unit Systemmedizin, University Wuerzburg)

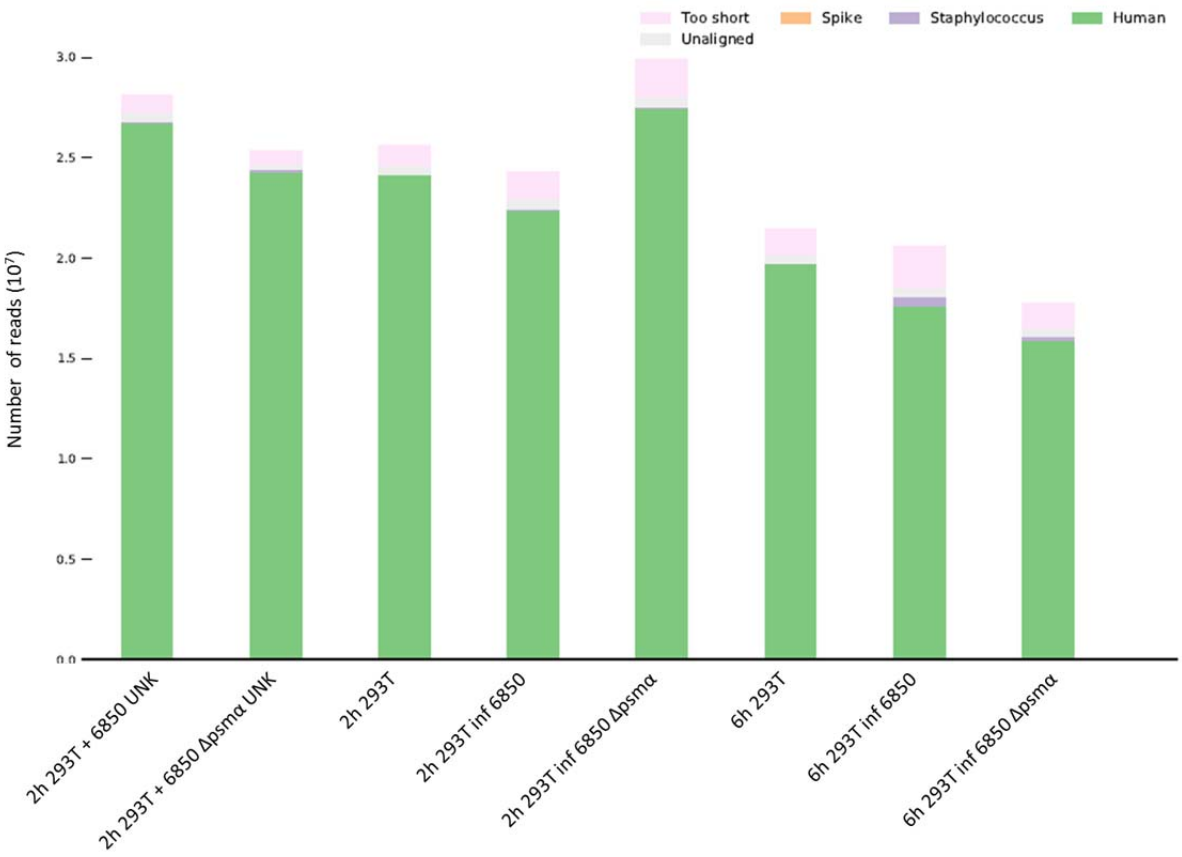

Figure 32: Number of reads from infected 293T cells dual RNA-seq data including distribution in human, staphylococcus too short, spice and unaligned parts reads. [3]

[3] Provided by Dr. Konrad Förstner (Core Unit Systemmedizin, University Wuerzburg)

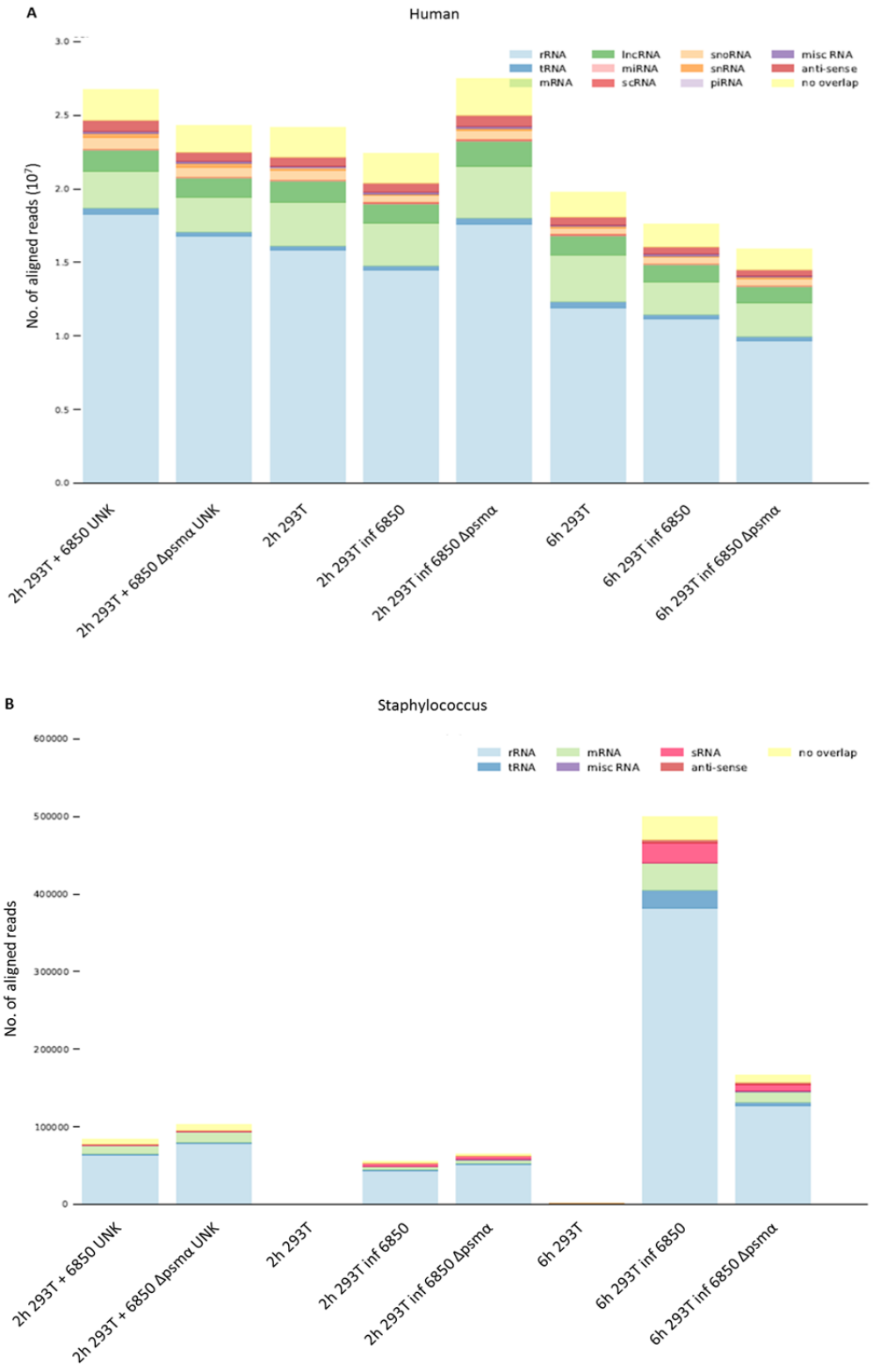

Figure 33: Numbers of aligned reads from human (A) and staphylococcus (B) transcriptomes. Distribution in regions like: rRNA, tRNA, mRNA, micsRNA, sRNA, anit-sense and no overlap. [4]

[4] Provided by Dr. Konrad Förstner (Core Unit Systemmedizin, University Wuerzburg)

4 Discussion

S. aureus is a successful human pathogen which not only exists as an extracellular pathogen but is also able to invade different cell types. Staphylococci are actively taken up by professional phagocytes including polymorphonuclear neutrophils (PMNs) and macrophages. Recently, many publications showed that they are efficiently invading non-professional phagocytes as well, including epithelial and endothelial cells, fibroblasts, keratinocytes, and osteoblasts (Dziewanowska et al., 1999, Jevon et al., 1999, Peacock et al., 1999, Kintarak et al., 2004). Professional phagocytes, as the first line of defence, should recognize and eliminate staphylococci to embank the infection. But are there any advantages for invading bacteria e.g. in epithelial or endothelial cells? Once they are inside host cells they are no more visible for the immune system. Further, cytosolic bacteria developed different strategies to survive inside the host, including modulation of phagolysosomal maturation or disruption of the phagosomal membrane. *L. monocytogenes* escapes the phagosome by phospholipase C-dependent pore formation with listeriolysin O (LLO) (Goldfine et al., 1995). A further example is *Rickettsia* spp. which disrupts the membrane using hemolysin C and phospholipases (Whitworth et al., 2005). In this work escape-positive *S. aureus* strains and the escape mechanism should be revealed and described.

4.1 Phagosomal escape of specific *S. aureus* strains in different types of non-professional phagocytes

For *S. aureus* it was shown that they are able to translocate into the host cell cytoplasm (Bayles et al., 1998, Jarry and Cheung, 2006). However, it seems that only few strains are able to escape the phagosome (Lam et al., 2010) and there is less known about involved virulence factors. For instance, phagosomal escape in cystic fibrosis (CF) cells is mediated by the pore-forming α-toxin (Jarry and Cheung, 2006, Jarry et al., 2008) but this is not sufficient within non-CF cells (Giese et al., 2009, Lam et al., 2010).

Different *S. aureus* strains were investigated for phagosomal escape, initially in human embryonic kidney cells (HEK 293T). Studying epithelial cells is important as *S. aureus* colonizes preferentially epithelial structures (Foster, 2004, Schwab et al., 1993) and is involved in renal abscess formation (Baradkar et al., 2009, Dougherty et al., 1991). Further, 293T cells are an established cell culture model for *S. aureus* infection (Sinha et al., 1999, Maya et al., 2012). A flow-cytometric assay was used to detect phagosomal escape. This assay is based on the intracellular acidification after phagocytosis and de-acidification upon phagosomal escape. Fluorescence intensity of ingested FITC-labelled staphylococci correlates with intracellular pH, with decreased arbitrary fluorescence units (AFUs) in

acidic phagosomes due to FITC quenching or increased AFUs after translocation to the host cell cytoplasm (Figure 8). 293T cells infected with several virulent or previously studied *S. aureus* strains including 6850, USA300 LAC, USA400 MW2 (Figure 11 A), Newman, RN1HGtcaR (HG003), ST239 (Figure 11 B), and SA113 (Figure 20 B) show different results. Phagosomal escape was only detected for LAC and MW2 as well as for 6850 (Figure 11 A). These results are not surprisingly as LAC and MW2 are well-known community-associated MRSA strains which cause severe infection, in worst cases even death, in otherwise healthy adults outside of the healthcare settings (Chambers, 2005, Nair et al., 2014). In addition, strain 6850 is a highly cytotoxic methicillin-sensitive strain isolated from a patient with bacteraemia associated with osteomyelitis and septic arthritis (Vann and Proctor, 1987, Balwit et al., 1994). These results were confirmed in further cell lines: HeLa (Figure 16 A, B, C), endothelial cell lines including EA.hy926 (Figure 16 D, E, F) and primary human umbilical vein endothelial cells (HUVEC; Figure 21 A) or osteoblasts hFob1.19 (Figure 19 B, C).

Another model to monitor phagosomal escape of *S. aureus* depends on the cytoplasmic expression of the escape markers YFP-Fc or YFP-CWT in reporter cell lines. Thereby, HeLa cells and 293T cells displayed phagosomal escape for strain LAC (Figure 11 B). The escape signal was displayed in terms of green ring-structures after binding of *S. aureus* protein A to cytoplasmic YFP-Fc which was quantifiable with ImageJ. For strain 6850 escape was visible as well, unfortunately the automatic quantification was problematic. There were no complete ring-structures but rather dots arranged in a “ring-like” formation (not shown). Additionally, lysosomal-associated membrane protein 1 (LAMP1) positive vesicles were visible only for escape negative strain Cowan I but not for 6850 in 293T Lamp1-YFP cells at late-infection time points (Figure 11 D).

In line with Lâm and colleagues, phagosomal escape were measured for some virulent strains (Lam et al., 2010) but was not a common event for all *S. aureus* strains probably due to different toxin production or escape relevant factors. Additionally, escape was not limited to a single cell type.

Usually, phagosomal escape was determined by measuring intracellular pH at 2 h and 6 h p.i. in terms of less negative ΔAFUs. In order to detect a defined time frame for phagosomal escape, rifampicin treatment was included into the infection assay. Rifampicin blocks the bacterial DNA-dependent RNA-polymerase and as a result escape-relevant proteins are not synthetized. Rifampicin was added in intervals of one hour and measured at 2 h and 6 h p.i. Treatment after 3 h p.i. did not prevent phagosomal escape of strains 6850 or LAC. Generally, measurement at 2 h p.i. reveals strong negative ΔAFUs similar to an acidic environment. In the given situation, phagosomale escape of strain 6850 must happen after 2 h but before 2.5 h p.i. (Figure 12 A) and 30 min later in case of strain LAC (Figure 12 B). This is late in comparison to other pathogens, since *S. flexneri* translocated to cytoplasm after lysis of phagosomal membrane already between 15 – 30 min after infection

(Sansonetti et al., 1986). *L. monocytogenes* escapes from vacuoles of non-activated macrophages within 30 min of infection (Myers et al., 2003, Henry et al., 2006) and *Rickettsia conorii* belongs to the quickest escapers with 12 min post infection (Teysseire et al., 1995). Although, *S. aureus* escaped little later it seem not to have a negative effect on their viability. Late escape maybe correlates with expression time of escape relevant factors.

4.2 Alpha-toxin, beta-toxin, Pantone-Valentine leukocidin and Phosphoinositide phospholipase C are not escape relevant factors

S. aureus strains 6850, LAC and MW2 escaped from phagosomes of epithelial and endothelial cells (Figure 11) although factors which are involved in mediating escape had to be investigated. α-toxin was in focus of research to induce cell death in T-cells, B-cells and monocytes (Nygaard et al., 2012) or to facilitate phagosomal escape in CF cells (Jarry and Cheung, 2006). Hence, α-toxin KO mutants in strain 6850 and LAC were tested. But there was no difference in ΔAFU between mutants and appropriate wild-type strains (Figure 14 A, C). Subsequently, these results support previous findings that α-toxin plays exclusively a role in phagosomal escape in CF cells (Jarry et al., 2008, Giese et al., 2009, Lam et al., 2010). However, it should be mentioned that in year 2010 the metalloprotease ADAM10 was published as receptor for α-toxin, low toxin concentration leads to receptor interaction and pore formation (Wilke and Bubeck Wardenburg, 2010) and toxin binding is followed by disruption of focal adhesions. The absence or expression level of ADAM10 could be an explanation for the cell type and species specificity (Berube and Bubeck Wardenburg, 2013) and could be the reason why α-toxin mutants were without phenotype in epithelial cells in this work.

Further, Giese and colleagues showed that δ-toxin acts with β-toxin in a synergistic manner when both toxins were recombinantly overexpressed in an otherwise avirulent laboratory *S. aureus* strains (Giese et al., 2011). Besides this strain 6850 is an extreme β-toxin producer and for that reason it could be that 6850 translocates to the cytoplasm with the help of β-toxin. On these grounds mutants deficient in β-toxin or δ-toxin were tested. Here KO mutant's showed a different result as the overexpressing strains, β-toxin and δ-toxin were not necessary for phagosomal escape in HeLa and 293T cells (Figure 14 C; Figure 15 C, D). Knock-out of a gene might be a more precise application as an overexpression, in rare cases the overexpression of a gene corresponds to its natural gene expression. Additionally phosphoinositide phospholipase C (PI-PLC) was checked because a homologue in *L. monocytogenes* was required for phagosomal escape (Goldfine et al., 1995), Δ*plc* did not reveal an escape phenotype, either (Figure 14 D). Not surprisingly, Δ*lukSF* (PVL) mutants showed same phenotype as wild-type strains due to missing necessary host cell receptors C5aR and C5L2

which are apparently expressed on neutrophils (Spaan et al., 2013). The Δ*lukSF* mutants have to be distinguished as a control similar to Cowan I in epithelial and endothelial cell lines. However, escape relevant factors are still missing.

4.3 Escape relevant toxins

4.3.1 PSMα is necessary for phagosomal escape in non-professional phagocytes

Recently a new class of toxins was identified, the phenol-soluble modulins (PSMs) which are claimed to play a role in staphylococcal virulence because of their ability to activate and lyse neutrophils (Wang et al., 2007). Staphylococci express beside δ-toxin, two types of PSMs: α-type (PSMα1-4) and β-type (PSMβ1-2). Examination *in vivo* revealed that infection of mice with LAC Δ*psmα* caused less skin lesions in comparison to wild-type and in a bacteraemia model with MW2 Δ*psmα* survived significantly more mice compared to wild-type infected. Concerning α-helical and amphipathic structure of PSMs, they possess most likely a membrane-damaging activity (Wang et al., 2007) similar to non-ionic detergents as has been published for δ-toxin (Kreger and Bernheimer, 1971). For this reason knock-out mutants in both types of PSMs were checked if they are still able to escape phagosomes of non-professional phagocytes. In line with previous publications, only PSMα-deficient mutants were unable to translocate to the cytoplasm of 293T, HeLa and EA-hy926 cells (Figure 15 A, B, D; Figure 16). By contrast, PSMβ mutants had no effect on escape efficiency in epithelial cells (Figure 15 C, D; Figure 16). These impressive results showed that phagosomal escape of clinically relevant *S. aureus* strains such as the epidemic CA-MRSA strains LAC, MW2 and the highly cytotoxic MSSA 6850 was mediated by a common PSMα-dependent mechanism despite distinct genetically backgrounds.

PSMs are acting rather from within a cell than as extracellular peptides. Same assumed Surewaard and colleagues by neutralization of PSMs with serum lipoproteins. Human serum totally inhibits PSM-mediated activation, attraction and lysis of neutrophils (Surewaard et al., 2012). Laabei and colleagues performed a study with individual PSM peptides against phospholipid vesicles and T cells to get insight into which specific PSM type mediates cell membrane disruption (Laabei et al., 2014). Lysis of vesicles containing 10 and 30 mol% cholesterol was obtained mostly by the PSMα peptides (PSMα1-3). Whereas, PSMβ type peptides and PSMα4 have little effect on vesicles and cytotoxicity at concentration comparable to PSMα1-3. They presume that the degree of alpha-helicity which differs between all PSMs correlates with their lytic activity. Furthermore, they showed cholesterol dependent lysis. For this reason differences in cholesterol concentration in phagosomes from

different cell types and species may influence PSM-mediated phagosome escape as well (Laabei et al., 2014).

Examination of *psm*α, *psm*β and *agr* mRNA expression during infection revealed that in wild-type *S. aureus* LAC those genes were initially up regulated (inoculum vs. 2 h p.i.; before escape) and afterwards down regulated (6 h p.i.; after escape). But, there was a different expression pattern when compared with the non-escaping LAC Δ*psm*α mutant. While escape-positive LAC Δ*psm*β mutant had a similar gene expression profile as wild-type displayed LAC Δ*psm*α still increased *psm*β and *agr* mRNA expression at 6 h p.i. (Figure 18 A). Since, *S. aureus* remained in the phagosome, an adversarial environment for bacteria *agr* as global regulator for virulence seems to be still active. Changes in gene expression were similar for 6850 and Δ*psm*α mutant (Figure 18 B). Consequently, phagosomal uptake or escape had an influence on PSM and Agr expression.

Osteomyelitis is a common manifestation of invasive *S. aureus* infection (Lew and Waldvogel, 2004), in this regard the highly cytotoxic strain 6850 was isolated from a patient with severe osteomyelitis (Balwit et al., 1994, Vann and Proctor, 1987). In children, staphylococcal osteomyelitis accounts for approximately 2.5 out of every 1,000 hospital admissions (Gerber et al., 2009) and among adults, osteomyelitis frequently complicates open fractures, soft tissue infections and diabetes (Lew and Waldvogel, 2004). Treatment is complex and involves one or more surgical debridement followed by prolonged antimicrobial therapy. Despite appropriate therapy patients develop complications such as septicaemia, deep venous thrombosis, and pathologic fractures (Belthur et al., 2012) which are related to the ability of *S. aureus* to tempt bone destruction. This renders therapy by confining antimicrobial penetration to the infectious centre. An enhanced insight into the mechanism that promotes bone destruction could facilitate development of new and improved strategies that limit bone destruction and reduce prolonged antimicrobial therapy. However, little is known regarding factors that promote bone destruction. Recently, *sae* was found to be critical for osteomyelitis pathogenesis, the sae-regulated protease aureolysin as a significant determinant and PSMs as aureolysin-degraded peptides that trigger osteoblast cell death and bone destruction (Cassat et al., 2013). Hence, it was worth to investigate phagosomal escape in human fetal osteoblastic cells (hFOB1.19) infected with *S. aureus*.

Osteoblasts become highly infected by *S. aureus* and strains 6850 and LAC were able to escape from phagosomes (Figure 19). Similar as for epithelial or endothelial cells only the *psm*α knock-out mutant failed to escape the phagosome of hFOB1.19 cells (Figure 19 B, C). Thus, *psm*α-mediated phagosomal escape might be an initial step in evolving severe osteomyelitis with bone destruction.

4.3.2 PSMα is not sufficient for phagosomal escape

To proof if PSMα is sufficient for phagosomal escape in an otherwise avirulent non-escaping strain SA113 or *agr* deficient LAC, strains were complemented with a plasmid coding *psm*α operon. Surprisingly, complementation of PSMα did not restore the wild-type phenotype of LAC and SA113 pPSMα did not escape. Both complemented strains show AFUs which corresponds to an acidic pH, consequently no phagosomal escape occurred (Figure 20). Consequently, PSMα is not sufficient and there is more than one factor which is involved in mediating phagosomal escape. Since earlier publications in this field showed that δ-toxin together with β-toxin mediated phagosomal escape in HeLa cells (Giese et al., 2011) same could be possible for PSMα peptides.

4.3.3 Influence of *S. aureus* on relative *fpr2* and *cd11b* expression

Although, for leukocidin LukAB was reported to mediate escape and host cell death (DuMont et al., 2013b, DuMont et al., 2013a) solely in neutrophils, a knock-out mutant was tested in diverse non-professional phagocytes. Surprisingly, there was a strong phenotype without LukAB in endothelial cells (HUVEC), epithelial cell lines (293T and HeLa), and osteoblasts (hFOB1.19). Flow-cytometric assays showed that without LukAB there was a significant difference in ΔAFU between wild-type and LukAB KO mutant for all tested cell lines (Figure 21).

In neutrophils or macrophages LukAB acts via the human complement receptor CD11b (DuMont et al., 2013a), for this reason expression of *cd11b* was investigated during infection. As it was expected, there was no messenger RNA in non-infected cells and in cells 1 h post infection. But the gene expression was significantly upregulated 5 h p.i. for strain 6850 and LAC in HUVECs and 293T cells, which correlates with timing of phagosomal escape *S. aureus*. The expression level differs between host cell types, but HUVECs and THP-1 cells which serve as control for relative *cd11b* expression show almost the same expression results (Figure 22).

The receptor expression in non-professional phagocytes might be not that strong as in neutrophils but was sufficient for a visible phenotype. It is likely that LukAB and PSMα act somehow together to mediate escape from phagosomes. However, it cannot be ruled out that there are even more factors which contribute to this event.

S. aureus infection induced *cd11b* expression (Figure 22) therefor the expression of the human formyl peptide receptor 2 (FPR2/ALX) was investigated as well. PSMs sensed FPR2 receptor at nanomolar concentrations and initiated proinflammatory neutrophil response in CA-MRSA. At the same time leukocyte lysis remained receptor independent (Kretschmer et al., 2010). To date is known that FPR2 is expressed on neutrophils, monocytes, macrophages, dendritic cells and microglial

cells. FPR2 activation induces chemotaxis, exocytosis and superoxide generation in neutrophils (Fu et al., 2006). Whereas the FPR1 receptor recognize N-formylated methionine followed by hydrophobic amino acids (Fu et al., 2006). The structural property of a determine FPR2 ligand is still unknown. The α-helical amphipathic structure may play a role in receptor binding but more demanding techniques will be necessary to clarify common features of FPR2 ligands (Kretschmer et al., 2010).

A kinetic study was performed to investigate *cd11b* and *fpr2* expression. With ongoing *S. aureus* 6850 infection *cd11b* and *fpr2* were time-dependently upregulated (Figure 23 A). A comparison of the relative *fpr2* expression between uninfected cells, cells infected with LAC or LAC Δ*psmα* showed that there was only mRNA expression in wild-type infected cells. For further verification, FPR2 were tested on protein level by SDS-PAGE and Western blot. A functional protein was only obtained after infection with 6850. There is no signal in not infected cells, or in cells infected with Cowan I or 6850 Δ*psmα* (Figure 24 A). To confirm that FPR2 plays a role in phagosomal escape, the receptor was silenced with a siRNA transfection in HeLa cells expressing the escape marker. Without FPR2 receptor the escape signal was markedly reduced compared to the control cells (siAll Star; Figure 24 B).

There must be a kind of signalling between staphylococci and host cells whereby FPR2 is upregulated during infection, more precisely only during phagosomal escape because not escaping strains like Cowan I or 6850 Δ*psmα* did not induce FPR2 during infection. Maybe due to different surface decoration of staphylococci there are different host responses. *S. aureus* is possibly able to modulate the gene expression of the host cell in that way to escape more efficiently. But how this is possible is so far not known.

Although *cd11b* was highly upregulated in *S. aureus* 6850 and LAC infected non-professional phagocytes it was not possible to visualize the CD11b protein by SDS-PAGE and Western blot. The protein level might be too low in HeLa cells for protein detection.

4.3.4 Phagosomal escape in professional phagocytes

After showing that *S. aureus* escaped efficiently from non-professional phagocytes with the help of PSMα, a great interest exists what happens in professional phagocytes. Moreover, neutrophils were attracted and lysed by PSMα (Wang et al., 2007). In this work, PMA activated THP-1 cells were infected with *S. aureus* 6850, PSMα-deficient mutant, and the complemented mutant and analysed by flow-cytometry or microscopy.

Measuring the intracellular pH as well as monitoring the escape-marker, showed that *S. aureus* 6850 and LAC were able to escape the phagosomes of macrophages within 3 h p.i. However, PSMα-

deficient mutants remained inside acidic vacuoles (Figure 25). There was no significant difference between wild-type and the complemented mutant. It seems that strain 6850 was even more potent to escape phagosomes of macrophages with a fivefold increased efficiency compared to strain LAC. However, in epithelial cells were the measured escape rates greater than in macrophages. THP-1 cells possess certainly a stronger bactericidal activity and possibly eliminate some staphylococci before they can escape.

4.4 Cytoplasmic replication of *S. aureus* only after phagosomal escape

S. aureus phagosomal escape was mediated by PSMα and LukAB, subsequently it was interesting if there are differences between escaping and non-escaping bacteria in host cells. For this reason CFU assays and flow-cytometric measurements, respectively, of internalized bacteria were performed in 293T cells.

Not escaping strains like Cowan I or RN4220 did not show increasing CFUs counts at 6 h p.i. whereas 6850 was fivefold enriched in infected cells. A non-escaping *psmα* KO mutant replicated neither. Same results were achieved for LAC and LAC Δ*psmα*. This is in line with all not escaping mutants: Δ*lukAB*, Δ*psmα*Δ*lukAB* and *agr*$^-$ had significantly lower recovered CFUs compared to wild-type (Figure 26 A, B, C). These results demonstrated that staphylococci inside phagosomes cannot replicate though survive at least up to 6 h p.i. In addition, it showed impressively how resistant they are to adverse conditions. This was not surprisingly as it has been published before, that small colony variants (SCVs) of *S. aureus* were able to survive in phagoendosomes of non-professional phagocytes up to five days (Schröder et al., 2006). SCVs are metabolically inactive, without toxin production and have been described to persist even in cells for prolonged periods (Schröder et al., 2006, Sendi and Proctor, 2009). Nevertheless, the compartment of replication exhibits some differences to previous published data, beneath prior was shown that the induction of autophagy is required for *S. aureus* replication inside a vacuole and only than staphylococci escape to the cytoplasm (Schnaith et al., 2007). Here presented results contradict, as strains and mutants which were not able to escape exhibits no changes in recovered CFUs at all.

Experiments with conditioned media showed that the overnight culture supernatant (SN, sterile filtrate of bacterial overnight culture) of 6850 Δ*psmα*pTXΔα1-4, a mutant which express PSMα constitutively, induced replication of otherwise non-replicating 6850 Δ*psmα* (Figure 26 D). PSMα peptides are small in size and due to their amphipathic character they can likely cross cellular membranes. As LukAB was not deleted in strain 6850 Δ*psmα*pTXΔα1-4 it is possible that LukAB contribute to escape and replication, as well.

Furthermore, the supernatant of the bacterial overnight culture of 6850 Δ*psmα*pTXΔα1-4 was enough to induce *fpr2* in not infected HeLa cells. However, not treated cells or the supernatant of the bacterial overnight culture of 6850 Δ*psmα* were not sufficient to induce *fpr2*. Thus PSMα peptides somehow are able to induce their receptor on the cell surface or intracellularly if they are able to cross the cell membrane.

Consequently, intracellular replication is only possible after phagosomal escape, takes place in the host cell cytoplasm and is PSMα and LukAB dependent in non-professional phagocytes.

If *S. aureus* can replicate in epithelial cells a comparison to professional phagocytes was suggestive. Interestingly, in THP-1 macrophages *S. aureus* 6850 were able to double whereas Cowan I and 6850 Δ*psmα* showed no significant changes in recovered CFUs. Additionally, cell counts of infected THP-1 cells did not decrease 3 h p.i. (Figure 27).

In non-professional and professional phagocytes *S. aureus* replicated in the host cell cytoplasm after phagosomal escape. Replication in epithelial cells was more efficiently than in THP-1 cells which seem reasonable.

4.5 *S. aureus* induced host cell death mediated by PSMα and LukAB

Human cells infected with *S. aureus* become sooner or later apoptotic depending on cell type and infection load. Because, PSMα and LukAB play an important role in phagosomal escape and intracellular replication, their contribution to host cell death was investigated.

There are different approaches for monitoring cell death, this work focused mainly on measuring the fluorescence increase of APC Annexin V by flow-cytometry (in cooperation with A.-C. Winkler). Cells that undergo programmed cell death are distinguished by outwardly oriented phosphatidylserines (PS) which only then bind Annexin V (Koopman et al., 1994). To date PS are considered to be the best characterized “eat me” signals on the apoptotic cell surface (Fadok et al., 2001, Fadok et al., 1998). In parallel cells should be treated with propidium iodide (PI; 7AAD) which is a DNA staining dye (Krishan, 1975) to detect perforated necrotic cells.

S. aureus infected cells were never PI positive, consequently staphylococcal infection induced no necrosis in HeLa and HUVEC cells. However, changes in annexin V positivity were indicated, whereas uninfected cells had a basal level of about 20 % annexin positive cells, showed wild-type *S. aureus* (LAC and 6850) infected cells fivefold enriched values (Figure 28). Cell death was determined in different cell types such as HUVEC (Figure 28 A) and HeLa cells (Figure 28 C, D). The deletion of *psmα*

or *lukAB* in *S. aureus* had a strong impact on pathogen induced cell death; the annexin signal was significantly reduced, between 16-40 %, for both mutants in HUVEC and HeLa cells. Additionally, HeLa cells infected with 6850 Δ*psmα* had 40 % less annexin positive cells compared to wild-type infected cells. *S. aureus* strains such as Cowan I or LAC *agr*$^-$ showed even more reduced cytotoxicity due to failed toxin expression. Accordingly, pathogen induced cell death needs two upstream events: phagosomal escape and bacterial replication. Hence, blocking of *S. aureus* escape or replication should prevent host cell death.

A look into the literature showed that α-toxin is implemented in *S. aureus* induced host cell death in leucocytes (Bantel et al., 2001, Haslinger et al., 2003). Further, α-toxin induced caspase activation and apoptosis through mitochondrial cytochrome c release independently of death receptor signalling in lymphocytes and monocytes (Bantel et al., 2001). Additionally was published, that α-toxin was required for pathogen induced cell death in peripheral blood mononuclear cells in a dose and time dependent manner with induced breakdown of the mitochondrial membrane potential and the intrinsic activation of caspase-3, -8 and -9 (Haslinger et al., 2003).

This work did not investigate the involvement of caspase activation during apoptosis but the effect of α-toxin on phagosomal escape. There was no evidence that α-toxin plays a role in phagosomal escape in epithelial and endothelial cells (Figure 14). As phagosomal escape is necessary for host cell death it can be concluded that α-toxin does not mediate host cell death in epithelial and endothelial cells however in other cell types including T cells, B cells or monocytes (Haslinger et al., 2003, Nygaard et al., 2012). Staphylococci express numerous toxins dependent on growth phase and strain; further it was published that some of these toxins bind specific host cell receptors which are not expressed on each cell type. Thus, *S. aureus* toxin production might be seen as an adaption to its environment. While Panton-Valentin leukocidin (PVL) induces predominantly rapid activation and cell death in human and rabbit neutrophils, there is no influence on murine or simian cells (Löffler et al., 2010) neither on human epithelial and endothelial cells (Grosz et al., 2014) lacking necessary C5aR receptor (Spaan et al., 2013). The phenol-soluble modulins seems to have a more “universal” ability. Apparently PSMs have a cytotoxic effect on many eukaryotic cell types, such as erythrocytes (Cheung et al., 2012), osteoblasts (Cassat et al., 2013, Rasigade et al., 2013), epithelial and endothelial cells (Giese et al., 2011, Grosz et al., 2014), monocytes (Wang et al., 2007) and PMNs (Wang et al., 2007, Surewaard et al., 2013). They act more from within rather than as extracellular toxins based on their inactivation by serum lipoproteins (Surewaard et al., 2012). This work demonstrated that PSMα acts from within, as well, facilitating *S. aureus* to escape from phagosomes. So far cytotoxicity of the bi-component leukotoxin LukAB was selectively shown for leukocytes such as monocytes, macrophages, dendritic cells, and PMNs (Dumont et al., 2011) which express CD11b the host cell receptor for

LukAB. Here, new cell types were identified as the deletion of LukAB showed no phagosomal escape in epithelial cells (293T, HeLa), HUVEC cells and osteoblasts. Further the LukAB deficient mutant failed to replicate intracellularly and to induce host cell death. The verification of *cd11b* in infected epithelial and endothelial cells explained failed phagosomal escape of LAC Δ*lukAB* in these cell types.

Summarizing, if there is no phagosomal escape due to missing PSMα and LukAB expression, then there is no intracellular replication and significantly less cell death (Figure 34).

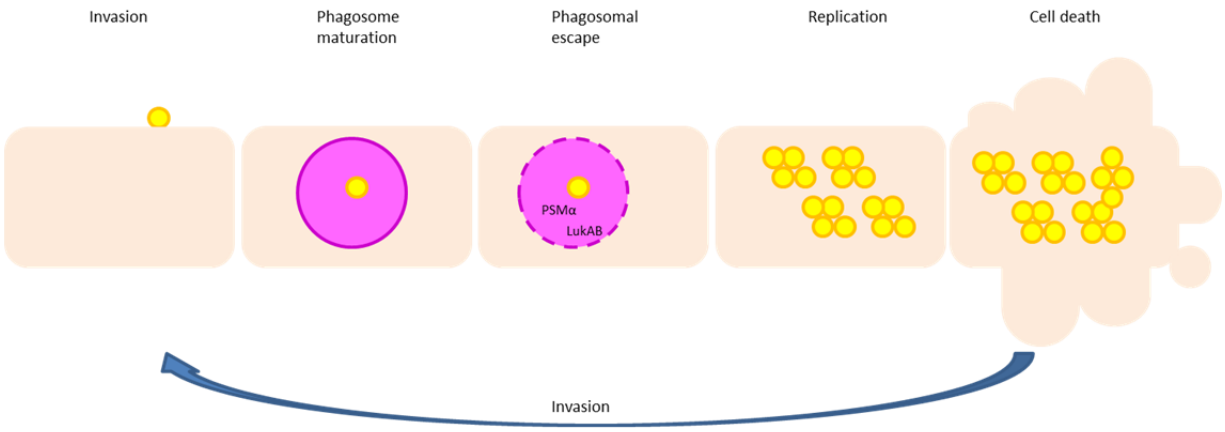

Figure 34: Lifecycle of intracellular *S. aureus*. Staphylococci are facultative intracellular pathogens; bacteria are phagocytosed by non-professional phagocytes. Strains expressing PSMα and LukAB are able to escape the phagosome. In the host cell cytoplasm they start to replicate. The fast bacterial replication contributes to pathogen induced cell death.

4.6 Host cell contribution to phagosomal escape, bacterial replication and cell death

A whole genome RNA interference (RNAi) screen (Dissertation Winkler, 2015) revealed that a large cluster of cytotoxicity-associated factors are connected to calcium signalling. Intracellular calcium plays a crucial role in the control of many cellular processes including cell motility, secretion, cell proliferation, cell differentiation, cancer and apoptosis (Berridge et al., 2000). The release of intracellular calcium, from store sites that are found primarily in the ER, is regulated by the second messenger inositol 1,4,5-trisphosphate (IP3). The glycoprotein IP3 receptor is located in the membrane, binding of IP3 to the receptor triggers the release of Ca^{2+} through this channel (Yoshida and Imai, 1997). 2-APB is an inhibitor for the IP3R thereby blocking the Ca^{2+} release. When HUVEC cells were treated with 2-APB and infected with *S. aureus* strains 6850 or LAC no phagosomal escape was measured by flow-cytometric pH-based escape assay (Figure 29). Thus, intracellular calcium might play a role in phagosomal escape. Noteworthy, 2-APB repressed *S. aureus* induced *fpr2* and *cd11b* expression, as well (Figure 30). This was validated by SDS-PAGE and Western blot as well, after the 2-APB treatment and 6850 infection there was no FPR2 signal quantifiable. And in consequence,

as 2-APB was shown to block phagosomal escape less cell death was obtained in *S. aureus* infected HUVEC cells (Figure 31).

Summarizing, phagosomal escape of *S. aureus* is not only mediated by bacterial toxins as previously assumed but rather is influenced by host cell factors including intracellular calcium signalling. How 2-APB interfere into this process remains to be investigated in future.

4.7 Host cell relevant genes which are involved in *S. aureus* infection identified by screening with Dual RNA-seq sequencing

To investigate the host-pathogen interaction on gene level, cells were infected with escape positive 6850 and 6850 Δ*psmα* mutant. Samples were analysed by dual RNA-seq 2 h and 6 h post infection. Advantage of this method is that there is no need to separate bacteria and eukaryotic RNA for sequencing.

Unfortunately, the coverage of staphylococcal genes was too low to produce reliable bioinformatical analysis[5] (Figure 32). Sample contains usually 95 % eukaryotic RNA and 5 % bacterial RNA (Westermann et al., 2012) but in this work was the bacterial proportion even less (Figure 33). To improve the result it would be appropriate to infected with a higher multiplicity of infection (moi). However, the eukaryotic genes which were identified[2] (two-fold enriched; $P \geq 0.05$) didn´t yield a suggestive pattern so far (Table 16, Table 17, Table 18, Table 19, Table 20, Table 21). Loading the enriched genes in the web free available analysis tools Kyoto Encyclopaedia of Genes and Genomes (KEGG) or String9.1 to generate pathways failed. This experiment has to be repeated with a higher coverage to get significant results and to generate signalling pathways. But already gained results can be compared with other sequenced data. Nevertheless, by means of this screening date the expression of *cd11b* after infection was identified and verified by qRT-PCR. An improved experimental design as well as the repetition of at least two times could improve existing data.

4.8 Conclusion and outlook

S. aureus invades professional and non-professional phagocytes via zipper-like mechanism, whereas fibronectin-binding proteins (FnBPs), fibronectin (Fn), and integrin α5β1 forms a bridge between staphylococci and eukaryotic cells. Internalized staphylococci get into endosomes which fuse with lysosomes and becomes acidic phagolysosomes which are designed to eliminate pathogens.

[5] Performed by Dr. Konrad Förstner (Core Unit Systemmedizin, University Wuerzburg)

Nevertheless, a small community of *S. aureus* strains were able to escape from phagosomes. This work revealed that the expression of PSMα and LukAB was necessary for phagosomal escape (Figure 35). Further, it is possible that PSMα needs LukAB as a co-factor in mediating escape. Single expression of PSMα in an avirulent background did not suffice to induce phagosomal escape. Once staphylococci were in the host cell cytoplasm they started to replicate which in turn leads to pathogen induced cell death. Strains deficient in PSMα and LukAB expression remained in vacuoles, they cannot replicate but neither becomes degraded 6 h post infection. Further, *S. aureus* infection caused the upregulation of *cd11b* and *fpr2*, both receptors were not expressed in uninfected cells. The complement receptor CD11b binds the bi-component leukotoxin LukAB and the formyl peptide receptor 2 binds PSMα. How this receptors become upregulated is yet not known, but strains deficient in corresponding toxins were not able to induce mRNA expression. Further, this study showed that the bacterial culture supernatant with PSMα1-4 enables 6850 Δ*psmα* to escape and replicate in the cytoplasm. Phagosomal escape, replication and induced host cell death are linked to each other. Moreover, the host cell had also a contribution to these events. Silencing of FPR2 with siRNA transfection blocked phagosomal escape and replication. These means bacterial toxins and host receptors are required for pathogen induced host cell death. Additionally, treatment with 2-APB which is an inhibitor for the inositol trisphosphate receptor (IP3R) blocked phagosomal escape and host cell death as well. Usually IP3R release calcium from ER why intracellular calcium is involved in phagosomal escape. Additionally, 2-APB treatment reverse *S. aureus* induced *fpr2* and *cd11b* expression. Thus, *S. aureus* induced host cell death is a multifactorial process including bacterial toxins and host cell factors (Figure 35).

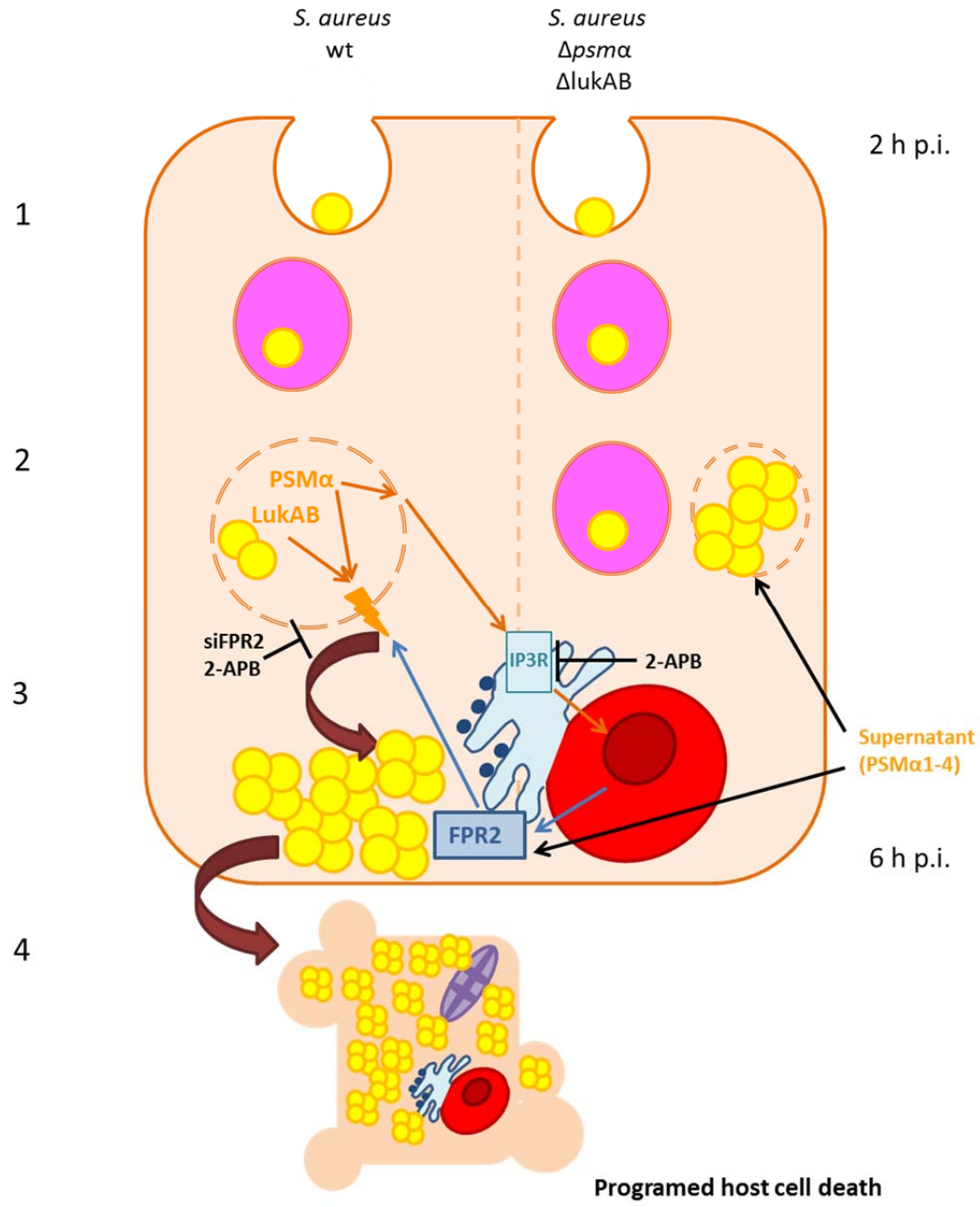

Figure 35: Model of post-invasive event in *S. aureus* infection *in vitro*. *S. aureus* enter the host cell via a zipper-like mechanism and ends up in an endosomal compartment. Endosomes becomes acidic phagosomes after fusion with lysosomes. *S. aureus* escapes with the help of two toxins: PSMα and LukAB. The genes *fpr2* and *cd11b* which encodes the receptors for the toxins are upregulated during infection. Upon phagosomal escape *S. aureus* replicates in the cytoplasm inducing host cell death. Supernatant containing PSMα1-4 enables 6850 Δ*psmα* to escape the phagosome and to replicate. Silencing of FPR2 by siRNA transfection blocks phagosomal escape. Intracellular calcium plays possibly a critical role in phagosomale escape as blocking the release of Ca^{2+} by IP3R from ER with inhibitor 2-APB prevents *S. aureus* to escape.

In future, the interaction of PSMα and LukAB as well as, their interaction with host cells has to be further examined. It is still not known how these toxins are able to induce their own host cell receptors, especially in cells which usually don't express CD11b or FPR2. Further, it would be advantageous to get information about the localization of both receptors. Fluorescence microscopy

proved somehow problematic due to strong unspecific antibody reaction with surface expression of staphylococcal protein A. Methods which are antibody independent would be promising for example FPR2 or CD11b coupled to a SNAP-tag® or Halo Tag®.

The induction of *fpr2* could be tested with synthetized PSMα peptides instead with supernatant of a bacterial overnight culture. The culture supernatant contains other toxins which might change the host cell response, too. Additional, PSMα peptides allow the measurement of calcium flux which was not possible to date with *S. aureus* infected cells.

The host-pathogen interaction should be resolved with Dual RNA-seq sequencing which provide information on gene level of both, *S. aureus* and eukaryotic cells. However, one experiment was not enough for a sufficient coverage, and especially the staphylococcal genes were completely underrepresented. A repetition of the Dual RNA-seq experiment with a higher coverage as performed before would perhaps provide significant results for cytotoxicity relevant genes on both sides. Maybe further escape relevant factors might be found.

Microinjection of staphylococci into eukaryotic cells might deliver new insights if progress is the same as after conventional infection. Is bacterial replication possible or is the invasion with the related signalling necessary for the so far known progress of phagosomal escape, replication and host cell death?

As host cell death was induced only after phagosomal escape of *S. aureus* and the treatment with 2-APB blocked the escape and subsequently host cell death, 2-APB should be tested for its application *in vivo.* Furthermore, it would be conceivable to use 2-APB as a new approach for therapy in human beings; it could serve as an alternative for “traditional” antibiotic treatment as *S. aureus* develops continuously resistance against antibiotics.

5 Literature

AARESTRUP, F. M., LARSEN, H. D., ERIKSEN, N. H., ELSBERG, C. S. & JENSEN, N. E. 1999. Frequency of alpha- and beta-haemolysin in Staphylococcus aureus of bovine and human origin. A comparison between pheno- and genotype and variation in phenotypic expression. *Apmis,* 107**,** 425-30.

ABRAMOFF, M. D., MAGALHÃES, P. J. & RAM, S. J. 2004. Image Processing with ImageJ. *Biophotonics International,* 11**,** 36-42.

AHMED, S., MEGHJI, S., WILLIAMS, R. J., HENDERSON, B., BROCK, J. H. & NAIR, S. P. 2001. Staphylococcus aureus fibronectin binding proteins are essential for internalization by osteoblasts but do not account for differences in intracellular levels of bacteria. *Infect Immun,* 69**,** 2872-7.

AKIRA, S. & TAKEDA, K. 2004. Toll-like receptor signalling. *Nat Rev Immunol,* 4**,** 499-511.

AKIRA, S., UEMATSU, S. & TAKEUCHI, O. 2006. Pathogen recognition and innate immunity. *Cell,* 124**,** 783-801.

ALVA-MURILLO, N., LOPEZ-MEZA, J. E. & OCHOA-ZARZOSA, A. 2014. Nonprofessional phagocytic cell receptors involved in Staphylococcus aureus internalization. *Biomed Res Int,* 2014**,** 538546.

ARMSTRONG, J. A. & HART, P. D. 1975. Phagosome-lysosome interactions in cultured macrophages infected with virulent tubercle bacilli. Reversal of the usual nonfusion pattern and observations on bacterial survival. *J Exp Med,* 142**,** 1-16.

ARVIDSON, S. & TEGMARK, K. 2001. Regulation of virulence determinants in Staphylococcus aureus. *Int J Med Microbiol,* 291**,** 159-70.

BABA, T., TAKEUCHI, F., KURODA, M., YUZAWA, H., AOKI, K., OGUCHI, A., NAGAI, Y., IWAMA, N., ASANO, K., NAIMI, T., KURODA, H., CUI, L., YAMAMOTO, K. & HIRAMATSU, K. 2002. Genome and virulence determinants of high virulence community-acquired MRSA. *Lancet,* 359**,** 1819-27.

BALWIT, J. M., VAN LANGEVELDE, P., VANN, J. M. & PROCTOR, R. A. 1994. Gentamicin-resistant menadione and hemin auxotrophic Staphylococcus aureus persist within cultured endothelial cells. *J Infect Dis,* 170**,** 1033-7.

BANTEL, H., SINHA, B., DOMSCHKE, W., PETERS, G., SCHULZE-OSTHOFF, K. & JANICKE, R. U. 2001. alpha-Toxin is a mediator of Staphylococcus aureus-induced cell death and activates caspases via the intrinsic death pathway independently of death receptor signaling. *J Cell Biol,* 155**,** 637-48.

BARADKAR, V. P., MATHUR, M. & KUMAR, S. 2009. Renal and perinephric abscess due to Staphylococcus aureus. *Indian J Pathol Microbiol,* 52**,** 440-1.

BARBER, M. 1961. Methicillin-resistant staphylococci. *J Clin Pathol,* 14**,** 385-93.

BARBER, M. & ROZWADOWSKA-DOWZENKO, M. 1948. Infection by penicillin-resistant staphylococci. *The Lancet***,** 641-644.

BARRETT, F. F., MCGEHEE, R. F., JR. & FINLAND, M. 1968. Methicillin-resistant Staphylococcus aureus at Boston City Hospital. Bacteriologic and epidemiologic observations. *N Engl J Med,* 279**,** 441-8.

BAYLES, K. W., WESSON, C. A., LIOU, L. E., FOX, L. K., BOHACH, G. A. & TRUMBLE, W. R. 1998. Intracellular Staphylococcus aureus escapes the endosome and induces apoptosis in epithelial cells. *Infect Immun,* 66**,** 336-42.

BELTHUR, M. V., BIRCHANSKY, S. B., VERDUGO, A. A., MASON, E. O., JR., HULTEN, K. G., KAPLAN, S. L., SMITH, E. O., PHILLIPS, W. A. & WEINBERG, J. 2012. Pathologic fractures in children with acute Staphylococcus aureus osteomyelitis. *J Bone Joint Surg Am,* 94**,** 34-42.

BEREZIKOV, E., VAN TETERING, G., VERHEUL, M., VAN DE BELT, J., VAN LAAKE, L., VOS, J., VERLOOP, R., VAN DE WETERING, M., GURYEV, V., TAKADA, S., VAN ZONNEVELD, A. J., MANO, H., PLASTERK, R. & CUPPEN, E. 2006. Many novel mammalian microRNA candidates identified by extensive cloning and RAKE analysis. *Genome Res,* 16**,** 1289-98.

BERRIDGE, M. J., LIPP, P. & BOOTMAN, M. D. 2000. The versatility and universality of calcium signalling. *Nat Rev Mol Cell Biol,* 1**,** 11-21.

BERUBE, B. J. & BUBECK WARDENBURG, J. 2013. Staphylococcus aureus alpha-toxin: nearly a century of intrigue. *Toxins (Basel),* 5**,** 1140-66.

BHAKDI, S. & TRANUM-JENSEN, J. 1991. Alpha-toxin of Staphylococcus aureus. *Microbiol Rev,* 55**,** 733-51.

BOSE, J. L., FEY, P. D. & BAYLES, K. W. 2013. Genetic tools to enhance the study of gene function and regulation in Staphylococcus aureus. *Appl Environ Microbiol,* 79**,** 2218-24.

BUBECK WARDENBURG, J., BAE, T., OTTO, M., DELEO, F. R. & SCHNEEWIND, O. 2007. Poring over pores: alpha-hemolysin and Panton-Valentine leukocidin in Staphylococcus aureus pneumonia. *Nat Med,* 13**,** 1405-6.

BUKOWSKI, M., WLADYKA, B. & DUBIN, G. 2010. Exfoliative toxins of Staphylococcus aureus. *Toxins (Basel),* 2**,** 1148-65.

CASSAT, J. E., HAMMER, N. D., CAMPBELL, J. P., BENSON, M. A., PERRIEN, D. S., MRAK, L. N., SMELTZER, M. S., TORRES, V. J. & SKAAR, E. P. 2013. A secreted bacterial protease tailors the Staphylococcus aureus virulence repertoire to modulate bone remodeling during osteomyelitis. *Cell Host Microbe,* 13**,** 759-72.

CDC 2003. Methicillin-resistant Staphylococcus aureus infections among competitive sports participants - Colorado, Indiana, Pennsylvania, and Los Angeles County, 2000-2003. *Conn Med,* 67**,** 549-51.

CHAMBERS, H. F. 2005. Community-associated MRSA--resistance and virulence converge. *N Engl J Med,* 352**,** 1485-7.

CHATTERJEE, S. S., CHEN, L., JOO, H. S., CHEUNG, G. Y., KREISWIRTH, B. N. & OTTO, M. 2011. Distribution and regulation of the mobile genetic element-encoded phenol-soluble modulin PSM-mec in methicillin-resistant Staphylococcus aureus. *PLoS One,* 6**,** e28781.

CHATTERJEE, S. S., JOO, H. S., DUONG, A. C., DIERINGER, T. D., TAN, V. Y., SONG, Y., FISCHER, E. R., CHEUNG, G. Y., LI, M. & OTTO, M. 2013. Essential Staphylococcus aureus toxin export system. *Nat Med,* 19**,** 364-7.

CHEUNG, G. Y., DUONG, A. C. & OTTO, M. 2012. Direct and synergistic hemolysis caused by Staphylococcus phenol-soluble modulins: implications for diagnosis and pathogenesis. *Microbes Infect,* 14**,** 380-6.

CLEMENS, D. L., LEE, B. Y. & HORWITZ, M. A. 2004. Virulent and avirulent strains of Francisella tularensis prevent acidification and maturation of their phagosomes and escape into the cytoplasm in human macrophages. *Infect Immun,* 72**,** 3204-17.

COLEMAN, D., KNIGHTS, J., RUSSELL, R., SHANLEY, D., BIRKBECK, T. H., DOUGAN, G. & CHARLES, I. 1991. Insertional inactivation of the Staphylococcus aureus beta-toxin by bacteriophage phi 13 occurs by site- and orientation-specific integration of the phi 13 genome. *Mol Microbiol,* 5**,** 933-9.

COSGROVE, S. E., QI, Y., KAYE, K. S., HARBARTH, S., KARCHMER, A. W. & CARMELI, Y. 2005. The impact of methicillin resistance in Staphylococcus aureus bacteremia on patient outcomes: mortality, length of stay, and hospital charges. *Infect Control Hosp Epidemiol,* 26**,** 166-74.

COSTERTON, J. W., LEWANDOWSKI, Z., CALDWELL, D. E., KORBER, D. R. & LAPPIN-SCOTT, H. M. 1995. Microbial biofilms. *Annu Rev Microbiol,* 49**,** 711-45.

DACK, G. M., GARY, W. E., WOOLPERT, O. & WIGGERS, H. 1930. An Outbreak of Food Poisoning proved to be due to a Yellow Hemolytic Staphylococcus. *Journal of Preventive Medicine,* 4**,** 167-175.

DEDENT, A. C., MCADOW, M. & SCHNEEWIND, O. 2007. Distribution of protein A on the surface of Staphylococcus aureus. *J Bacteriol,* 189**,** 4473-84.

DELLABONA, P., PECCOUD, J., KAPPLER, J., MARRACK, P., BENOIST, C. & MATHIS, D. 1990. Superantigens interact with MHC class II molecules outside of the antigen groove. *Cell,* 62**,** 1115-21.

DIEP, B. A., GILL, S. R., CHANG, R. F., PHAN, T. H., CHEN, J. H., DAVIDSON, M. G., LIN, F., LIN, J., CARLETON, H. A., MONGODIN, E. F., SENSABAUGH, G. F. & PERDREAU-REMINGTON, F. 2006. Complete genome sequence of USA300, an epidemic clone of community-acquired meticillin-resistant Staphylococcus aureus. *Lancet,* 367**,** 731-9.

DONLAN, R. M. & COSTERTON, J. W. 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev,* 15**,** 167-93.

DONVITO, B., ETIENNE, J., DENOROY, L., GREENLAND, T., BENITO, Y. & VANDENESCH, F. 1997. Synergistic hemolytic activity of Staphylococcus lugdunensis is mediated by three peptides encoded by a non-agr genetic locus. *Infect Immun,* 65**,** 95-100.

DOUGHERTY, F. E., GOTTLIEB, R. P., GROSS, G. W. & DENISON, M. R. 1991. Neonatal renal abscess caused by Staphylococcus aureus. *Pediatr Infect Dis J,* 10**,** 463-6.

DUMONT, A. L., NYGAARD, T. K., WATKINS, R. L., SMITH, A., KOZHAYA, L., KREISWIRTH, B. N., SHOPSIN, B., UNUTMAZ, D., VOYICH, J. M. & TORRES, V. J. 2011. Characterization of a new cytotoxin that contributes to Staphylococcus aureus pathogenesis. *Mol Microbiol,* 79**,** 814-25.

DUMONT, A. L., YOONG, P., DAY, C. J., ALONZO, F., 3RD, MCDONALD, W. H., JENNINGS, M. P. & TORRES, V. J. 2013a. Staphylococcus aureus LukAB cytotoxin kills human neutrophils by targeting the CD11b subunit of the integrin Mac-1. *Proc Natl Acad Sci U S A,* 110**,** 10794-9.

DUMONT, A. L., YOONG, P., SUREWAARD, B. G., BENSON, M. A., NIJLAND, R., VAN STRIJP, J. A. & TORRES, V. J. 2013b. Staphylococcus aureus elaborates leukocidin AB to mediate escape from within human neutrophils. *Infect Immun,* 81**,** 1830-41.

DUTHIE, E. S. 1952. Variation in the antigenic composition of staphylococcal coagulase. *J Gen Microbiol,* 7**,** 320-6.

DZIEWANOWSKA, K., CARSON, A. R., PATTI, J. M., DEOBALD, C. F., BAYLES, K. W. & BOHACH, G. A. 2000. Staphylococcal fibronectin binding protein interacts with heat shock protein 60 and integrins: role in internalization by epithelial cells. *Infect Immun,* 68**,** 6321-8.

DZIEWANOWSKA, K., PATTI, J. M., DEOBALD, C. F., BAYLES, K. W., TRUMBLE, W. R. & BOHACH, G. A. 1999. Fibronectin binding protein and host cell tyrosine kinase are required for internalization of Staphylococcus aureus by epithelial cells. *Infect Immun,* 67**,** 4673-8.

EDWARDS, A. M., POTTER, U., MEENAN, N. A., POTTS, J. R. & MASSEY, R. C. 2011. Staphylococcus aureus keratinocyte invasion is dependent upon multiple high-affinity fibronectin-binding repeats within FnBPA. *PLoS One,* 6**,** e18899.

EDWARDS, D. R., HANDSLEY, M. M. & PENNINGTON, C. J. 2008. The ADAM metalloproteinases. *Mol Aspects Med,* 29**,** 258-89.

EISSENBERG, L. G., WYRICK, P. B., DAVIS, C. H. & RUMPP, J. W. 1983. Chlamydia psittaci elementary body envelopes: ingestion and inhibition of phagolysosome fusion. *Infect Immun,* 40**,** 741-51.

ERIKSEN, N. H., ESPERSEN, F., ROSDAHL, V. T. & JENSEN, K. 1995. Carriage of Staphylococcus aureus among 104 healthy persons during a 19-month period. *Epidemiol Infect,* 115**,** 51-60.

EWING, B., HILLIER, L., WENDL, M. C. & GREEN, P. 1998. Base-calling of automated sequencer traces using phred. I. Accuracy assessment. *Genome Res,* 8**,** 175-85.

FADOK, V. A., BRATTON, D. L., FRASCH, S. C., WARNER, M. L. & HENSON, P. M. 1998. The role of phosphatidylserine in recognition of apoptotic cells by phagocytes. *Cell Death Differ,* 5**,** 551-62.

FADOK, V. A., DE CATHELINEAU, A., DALEKE, D. L., HENSON, P. M. & BRATTON, D. L. 2001. Loss of phospholipid asymmetry and surface exposure of phosphatidylserine is required for phagocytosis of apoptotic cells by macrophages and fibroblasts. *J Biol Chem,* 276**,** 1071-7.

FEIL, E. J., NICKERSON, E. K., CHANTRATITA, N., WUTHIEKANUN, V., SRISOMANG, P., COUSINS, R., PAN, W., ZHANG, G., XU, B., DAY, N. P. & PEACOCK, S. J. 2008. Rapid detection of the pandemic methicillin-resistant Staphylococcus aureus clone ST 239, a dominant strain in Asian hospitals. *J Clin Microbiol,* 46**,** 1520-2.

FOSTER, T. J. 2004. Nasal colonization by Staphylococcus aureus. *Nat Med,* 10**,** 447; author reply 447.

FOSTER, T. J. 2005. Immune evasion by staphylococci. *Nat Rev Microbiol,* 3**,** 948-58.

FRENETTE, M., BEAUDET, R., BISAILLON, J. G., SYLVESTRE, M. & PORTELANCE, V. 1984. Chemical and biological characterization of a gonococcal growth inhibitor produced by Staphylococcus haemolyticus isolated from urogenital flora. *Infect Immun,* 46**,** 340-5.

FU, H., KARLSSON, J., BYLUND, J., MOVITZ, C., KARLSSON, A. & DAHLGREN, C. 2006. Ligand recognition and activation of formyl peptide receptors in neutrophils. *J Leukoc Biol,* 79**,** 247-56.

FUSSLE, R., BHAKDI, S., SZIEGOLEIT, A., TRANUM-JENSEN, J., KRANZ, T. & WELLENSIEK, H. J. 1981. On the mechanism of membrane damage by Staphylococcus aureus alpha-toxin. *J Cell Biol,* 91**,** 83-94.

GAILLARD, J. L., BERCHE, P., MOUNIER, J., RICHARD, S. & SANSONETTI, P. 1987. In vitro model of penetration and intracellular growth of Listeria monocytogenes in the human enterocyte-like cell line Caco-2. *Infect Immun,* 55**,** 2822-9.

GAUDUCHON, V., WERNER, S., PREVOST, G., MONTEIL, H. & COLIN, D. A. 2001. Flow cytometric determination of Panton-Valentine leucocidin S component binding. *Infect Immun,* 69**,** 2390-5.

GERBER, J. S., COFFIN, S. E., SMATHERS, S. A. & ZAOUTIS, T. E. 2009. Trends in the incidence of methicillin-resistant Staphylococcus aureus infection in children's hospitals in the United States. *Clin Infect Dis,* 49**,** 65-71.

GIESE, B., DITTMANN, S., PAPROTKA, K., LEVIN, K., WELTROWSKI, A., BIEHLER, D., LAM, T. T., SINHA, B. & FRAUNHOLZ, M. J. 2009. Staphylococcal alpha-toxin is not sufficient to mediate escape from phagolysosomes in upper-airway epithelial cells. *Infect Immun,* 77**,** 3611-25.

GIESE, B., GLOWINSKI, F., PAPROTKA, K., DITTMANN, S., STEINER, T., SINHA, B. & FRAUNHOLZ, M. J. 2011. Expression of delta-toxin by Staphylococcus aureus mediates escape from phago-endosomes of human epithelial and endothelial cells in the presence of beta-toxin. *Cell Microbiol,* 13**,** 316-29.

GILLET, Y., ISSARTEL, B., VANHEMS, P., FOURNET, J. C., LINA, G., BES, M., VANDENESCH, F., PIEMONT, Y., BROUSSE, N., FLORET, D. & ETIENNE, J. 2002. Association between Staphylococcus aureus strains carrying gene for Panton-Valentine leukocidin and highly lethal necrotising pneumonia in young immunocompetent patients. *Lancet,* 359**,** 753-9.

GOETZ, M., BUBERT, A., WANG, G., CHICO-CALERO, I., VAZQUEZ-BOLAND, J. A., BECK, M., SLAGHUIS, J., SZALAY, A. A. & GOEBEL, W. 2001. Microinjection and growth of bacteria in the cytosol of mammalian host cells. *Proc Natl Acad Sci U S A,* 98**,** 12221-6.

GOLDFINE, H., KNOB, C., ALFORD, D. & BENTZ, J. 1995. Membrane permeabilization by Listeria monocytogenes phosphatidylinositol-specific phospholipase C is independent of phospholipid hydrolysis and cooperative with listeriolysin O. *Proc Natl Acad Sci U S A,* 92**,** 2979-83.

GONZALEZ, D. J., OKUMURA, C. Y., HOLLANDS, A., KERSTEN, R., AKONG-MOORE, K., PENCE, M. A., MALONE, C. L., DERIEUX, J., MOORE, B. S., HORSWILL, A. R., DIXON, J. E., DORRESTEIN, P. C. & NIZET, V. 2012. Novel phenol-soluble modulin derivatives in community-associated methicillin-resistant Staphylococcus aureus identified through imaging mass spectrometry. *J Biol Chem,* 287**,** 13889-98.

GOUAUX, E. 1998. alpha-Hemolysin from Staphylococcus aureus: an archetype of beta-barrel, channel-forming toxins. *J Struct Biol,* 121**,** 110-22.

GRAVET, A., COLIN, D. A., KELLER, D., GIRARDOT, R., MONTEIL, H. & PREVOST, G. 1998. Characterization of a novel structural member, LukE-LukD, of the bi-component staphylococcal leucotoxins family. *FEBS Lett,* 436**,** 202-8.

GRAY, G. S. & KEHOE, M. 1984. Primary sequence of the alpha-toxin gene from Staphylococcus aureus wood 46. *Infect Immun,* 46**,** 615-8.

GROSZ, M., KOLTER, J., PAPROTKA, K., WINKLER, A. C., SCHAFER, D., CHATTERJEE, S. S., GEIGER, T., WOLZ, C., OHLSEN, K., OTTO, M., RUDEL, T., SINHA, B. & FRAUNHOLZ, M. 2014. Cytoplasmic replication of Staphylococcus aureus upon phagosomal escape triggered by phenol-soluble modulin alpha. *Cell Microbiol,* 16**,** 451-65.

GRUNDLING, A. & SCHNEEWIND, O. 2006. Cross-linked peptidoglycan mediates lysostaphin binding to the cell wall envelope of Staphylococcus aureus. *J Bacteriol,* 188**,** 2463-72.

GUAN, J., ZHU, X., YU, F., YANG, W., LIU, T., ZHANG, T., LIN, N., LIU, Y. & LIU, C. 2013. Role of alpha-toxin-induced apoptosis of umbilical vein endothelial cells in vertical infection of Staphylococcus aureus L-form. *Nan Fang Yi Ke Da Xue Xue Bao,* 33**,** 619-24.

HAAS, A. 2007. The phagosome: compartment with a license to kill. *Traffic,* 8**,** 311-30.

HAGGAR, A., HUSSAIN, M., LONNIES, H., HERRMANN, M., NORRBY-TEGLUND, A. & FLOCK, J. I. 2003. Extracellular adherence protein from Staphylococcus aureus enhances internalization into eukaryotic cells. *Infect Immun,* 71**,** 2310-7.

HASLINGER, B., STRANGFELD, K., PETERS, G., SCHULZE-OSTHOFF, K. & SINHA, B. 2003. Staphylococcus aureus alpha-toxin induces apoptosis in peripheral blood mononuclear cells: role of endogenous tumour necrosis factor-alpha and the mitochondrial death pathway. *Cell Microbiol,* 5**,** 729-41.

HENRY, R., SHAUGHNESSY, L., LOESSNER, M. J., ALBERTI-SEGUI, C., HIGGINS, D. E. & SWANSON, J. A. 2006. Cytolysin-dependent delay of vacuole maturation in macrophages infected with Listeria monocytogenes. *Cell Microbiol,* 8**,** 107-19.

HERBERT, S., ZIEBANDT, A. K., OHLSEN, K., SCHAFER, T., HECKER, M., ALBRECHT, D., NOVICK, R. & GOTZ, F. 2010. Repair of global regulators in Staphylococcus aureus 8325 and comparative analysis with other clinical isolates. *Infect Immun,* 78**,** 2877-89.

HEROLD, B. C., IMMERGLUCK, L. C., MARANAN, M. C., LAUDERDALE, D. S., GASKIN, R. E., BOYLE-VAVRA, S., LEITCH, C. D. & DAUM, R. S. 1998. Community-acquired methicillin-resistant Staphylococcus aureus in children with no identified predisposing risk. *Jama,* 279**,** 593-8.

HILDEBRAND, A., POHL, M. & BHAKDI, S. 1991. Staphylococcus aureus alpha-toxin. Dual mechanism of binding to target cells. *J Biol Chem,* 266**,** 17195-200.

HIRSCHHAUSEN, N., SCHLESIER, T., SCHMIDT, M. A., GOTZ, F., PETERS, G. & HEILMANN, C. 2010. A novel staphylococcal internalization mechanism involves the major autolysin Atl and heat shock cognate protein Hsc70 as host cell receptor. *Cell Microbiol,* 12**,** 1746-64.

HOEBE, K., GEORGEL, P., RUTSCHMANN, S., DU, X., MUDD, S., CROZAT, K., SOVATH, S., SHAMEL, L., HARTUNG, T., ZAHRINGER, U. & BEUTLER, B. 2005. CD36 is a sensor of diacylglycerides. *Nature,* 433**,** 523-7.

HUSEBY, M. J., KRUSE, A. C., DIGRE, J., KOHLER, P. L., VOCKE, J. A., MANN, E. E., BAYLES, K. W., BOHACH, G. A., SCHLIEVERT, P. M., OHLENDORF, D. H. & EARHART, C. A. 2010. Beta toxin catalyzes formation of nucleoprotein matrix in staphylococcal biofilms. *Proc Natl Acad Sci U S A,* 107**,** 14407-12.

HUSSAIN, M., HAGGAR, A., HEILMANN, C., PETERS, G., FLOCK, J. I. & HERRMANN, M. 2002. Insertional inactivation of Eap in Staphylococcus aureus strain Newman confers reduced staphylococcal binding to fibroblasts. *Infect Immun,* 70**,** 2933-40.

INOSHIMA, I., INOSHIMA, N., WILKE, G. A., POWERS, M. E., FRANK, K. M., WANG, Y. & BUBECK WARDENBURG, J. 2011. A Staphylococcus aureus pore-forming toxin subverts the activity of ADAM10 to cause lethal infection in mice. *Nat Med,* 17**,** 1310-4.

IORDANESCU, S. & SURDEANU, M. 1976. Two restriction and modification systems in Staphylococcus aureus NCTC8325. *J Gen Microbiol,* 96**,** 277-81.

JANZON, L., LOFDAHL, S. & ARVIDSON, S. 1989. Identification and nucleotide sequence of the delta-lysin gene, hld, adjacent to the accessory gene regulator (agr) of Staphylococcus aureus. *Mol Gen Genet,* 219**,** 480-5.

JARRY, T. M. & CHEUNG, A. L. 2006. Staphylococcus aureus escapes more efficiently from the phagosome of a cystic fibrosis bronchial epithelial cell line than from its normal counterpart. *Infect Immun,* 74**,** 2568-77.

JARRY, T. M., MEMMI, G. & CHEUNG, A. L. 2008. The expression of alpha-haemolysin is required for Staphylococcus aureus phagosomal escape after internalization in CFT-1 cells. *Cell Microbiol,* 10**,** 1801-14.

JEVON, M., GUO, C., MA, B., MORDAN, N., NAIR, S. P., HARRIS, M., HENDERSON, B., BENTLEY, G. & MEGHJI, S. 1999. Mechanisms of internalization of Staphylococcus aureus by cultured human osteoblasts. *Infect Immun,* 67**,** 2677-81.

JONSSON, K., SIGNAS, C., MULLER, H. P. & LINDBERG, M. 1991. Two different genes encode fibronectin binding proteins in Staphylococcus aureus. The complete nucleotide sequence and characterization of the second gene. *Eur J Biochem,* 202**,** 1041-8.

JUSKO, M., POTEMPA, J., KANTYKA, T., BIELECKA, E., MILLER, H. K., KALINSKA, M., DUBIN, G., GARRED, P., SHAW, L. N. & BLOM, A. M. 2014. Staphylococcal proteases aid in evasion of the human complement system. *J Innate Immun,* 6**,** 31-46.

KAHL, B. C., GOULIAN, M., VAN WAMEL, W., HERRMANN, M., SIMON, S. M., KAPLAN, G., PETERS, G. & CHEUNG, A. L. 2000. Staphylococcus aureus RN6390 replicates and induces apoptosis in a pulmonary epithelial cell line. *Infect Immun,* 68**,** 5385-92.

KAPLAN, S. L., HULTEN, K. G., GONZALEZ, B. E., HAMMERMAN, W. A., LAMBERTH, L., VERSALOVIC, J. & MASON, E. O., JR. 2005. Three-year surveillance of community-acquired Staphylococcus aureus infections in children. *Clin Infect Dis,* 40**,** 1785-91.

KATAYAMA, Y., ITO, T. & HIRAMATSU, K. 2000. A New Class of Genetic Element, Staphylococcus Cassette Chromosome mec, Encodes Methicillin Resistance in Staphylococcus aureus. *Antimicrob Agents Chemother,* 44**,** 1549-55.

KEHOE, M., DUNCAN, J., FOSTER, T., FAIRWEATHER, N. & DOUGAN, G. 1983. Cloning, expression, and mapping of the Staphylococcus aureus alpha-hemolysin determinant in Escherichia coli K-12. *Infect Immun,* 41**,** 1105-11.

KINTARAK, S., WHAWELL, S. A., SPEIGHT, P. M., PACKER, S. & NAIR, S. P. 2004. Internalization of Staphylococcus aureus by human keratinocytes. *Infect Immun,* 72**,** 5668-75.

KOCK, R., BECKER, K., COOKSON, B., VAN GEMERT-PIJNEN, J. E., HARBARTH, S., KLUYTMANS, J., MIELKE, M., PETERS, G., SKOV, R. L., STRUELENS, M. J., TACCONELLI, E., NAVARRO TORNE, A., WITTE, W. & FRIEDRICH, A. W. 2010. Methicillin-resistant Staphylococcus aureus (MRSA): burden of disease and control challenges in Europe. *Euro Surveill,* 15**,** 19688.

KOLAR, S. L., IBARRA, J. A., RIVERA, F. E., MOOTZ, J. M., DAVENPORT, J. E., STEVENS, S. M., HORSWILL, A. R. & SHAW, L. N. 2013. Extracellular proteases are key mediators of Staphylococcus aureus virulence via the global modulation of virulence-determinant stability. *Microbiologyopen,* 2**,** 18-34.

KOOPMAN, G., REUTELINGSPERGER, C. P., KUIJTEN, G. A., KEEHNEN, R. M., PALS, S. T. & VAN OERS, M. H. 1994. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood,* 84**,** 1415-20.

KREGER, A. S. & BERNHEIMER, A. W. 1971. Disruption of bacterial protoplasts and spheroplasts by staphylococcal delta hemolysin. *Infect Immun,* 3**,** 603-5.

KREISWIRTH, B. N., LOFDAHL, S., BETLEY, M. J., O'REILLY, M., SCHLIEVERT, P. M., BERGDOLL, M. S. & NOVICK, R. P. 1983. The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. *Nature,* 305**,** 709-12.

KRETSCHMER, D., GLESKE, A. K., RAUTENBERG, M., WANG, R., KOBERLE, M., BOHN, E., SCHONEBERG, T., RABIET, M. J., BOULAY, F., KLEBANOFF, S. J., VAN KESSEL, K. A., VAN STRIJP, J. A., OTTO, M. & PESCHEL, A. 2010. Human formyl peptide receptor 2 senses highly pathogenic Staphylococcus aureus. *Cell Host Microbe,* 7**,** 463-73.

KRISHAN, A. 1975. Rapid flow cytofluorometric analysis of mammalian cell cycle by propidium iodide staining. *J Cell Biol,* 66**,** 188-93.

KURODA, M., OHTA, T., UCHIYAMA, I., BABA, T., YUZAWA, H., KOBAYASHI, I., CUI, L., OGUCHI, A., AOKI, K., NAGAI, Y., LIAN, J., ITO, T., KANAMORI, M., MATSUMARU, H., MARUYAMA, A., MURAKAMI, H., HOSOYAMA, A., MIZUTANI-UI, Y., TAKAHASHI, N. K., SAWANO, T., INOUE, R., KAITO, C., SEKIMIZU, K., HIRAKAWA, H., KUHARA, S., GOTO, S., YABUZAKI, J., KANEHISA, M., YAMASHITA, A., OSHIMA, K., FURUYA, K., YOSHINO, C., SHIBA, T., HATTORI, M., OGASAWARA, N., HAYASHI, H. & HIRAMATSU, K. 2001. Whole genome sequencing of meticillin-resistant Staphylococcus aureus. *Lancet,* 357**,** 1225-40.

LAABEI, M., JAMIESON, W. D., YANG, Y., VAN DEN ELSEN, J. & JENKINS, A. T. 2014. Investigating the lytic activity and structural properties of Staphylococcus aureus phenol soluble modulin (PSM) peptide toxins. *Biochim Biophys Acta,* 1838**,** 3153-61.

LABANDEIRA-REY, M., COUZON, F., BOISSET, S., BROWN, E. L., BES, M., BENITO, Y., BARBU, E. M., VAZQUEZ, V., HOOK, M., ETIENNE, J., VANDENESCH, F. & BOWDEN, M. G. 2007. Staphylococcus aureus Panton-Valentine leukocidin causes necrotizing pneumonia. *Science,* 315**,** 1130-3.

LAM, T. T., GIESE, B., CHIKKABALLI, D., KUHN, A., WOLBER, W., PANE-FARRE, J., SCHAFER, D., ENGELMANN, S., FRAUNHOLZ, M. & SINHA, B. 2010. Phagolysosomal integrity is generally maintained after Staphylococcus aureus invasion of nonprofessional phagocytes but is modulated by strain 6850. *Infect Immun,* 78**,** 3392-403.

LEELAHAVANICHKUL, A., BOCHAROV, A. V., KURLANDER, R., BARANOVA, I. N., VISHNYAKOVA, T. G., SOUZA, A. C., HU, X., DOI, K., VAISMAN, B., AMAR, M., SVIRIDOV, D., CHEN, Z., REMALEY, A. T., CSAKO, G., PATTERSON, A. P., YUEN, P. S., STAR, R. A. & EGGERMAN, T. L. 2012. Class B scavenger receptor types I and II and CD36 targeting improves sepsis survival and acute outcomes in mice. *J Immunol,* 188**,** 2749-58.

LEW, D. P. & WALDVOGEL, F. A. 2004. Osteomyelitis. *Lancet,* 364**,** 369-79.

LI, H., LLERA, A., MALCHIODI, E. L. & MARIUZZA, R. A. 1999. The structural basis of T cell activation by superantigens. *Annu Rev Immunol,* 17**,** 435-66.

LINDSAY, J. A., MOORE, C. E., DAY, N. P., PEACOCK, S. J., WITNEY, A. A., STABLER, R. A., HUSAIN, S. E., BUTCHER, P. D. & HINDS, J. 2006. Microarrays reveal that each of the ten dominant lineages of Staphylococcus aureus has a unique combination of surface-associated and regulatory genes. *J Bacteriol,* 188**,** 669-76.

LIVAK, K. J. & SCHMITTGEN, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods,* 25**,** 402-8.

LÖFFLER, B., HUSSAIN, M., GRUNDMEIER, M., BRÜCK, M., HOLZINGER, D., VARGA, G., ROTH, J., KAHL, B. C., PROCTOR, R. A. & PETERS, G. 2010. Staphylococcus aureus Panton-Valentine Leukocidin Is a Very Potent Cytotoxic Factor for Human Neutrophils. *PLoS Pathog,* 6.

LOWY, F. D. 1998. Staphylococcus aureus infections. *N Engl J Med,* 339**,** 520-32.

LOWY, F. D. 2003. Antimicrobial resistance: the example of Staphylococcus aureus. *J Clin Invest,* 111**,** 1265-73.

MAREE, C. L., DAUM, R. S., BOYLE-VAVRA, S., MATAYOSHI, K. & MILLER, L. G. 2007. Community-associated methicillin-resistant Staphylococcus aureus isolates causing healthcare-associated infections. *Emerg Infect Dis,* 13**,** 236-42.

MARRACK, P. & KAPPLER, J. 1990. The staphylococcal enterotoxins and their relatives. *Science,* 248**,** 705-11.

MARTINEZ-PULGARIN, S., DOMINGUEZ-BERNAL, G., ORDEN, J. A. & DE LA FUENTE, R. 2009. Simultaneous lack of catalase and beta-toxin in Staphylococcus aureus leads to increased intracellular survival in macrophages and epithelial cells and to attenuated virulence in murine and ovine models. *Microbiology,* 155**,** 1505-15.

MASSEY, R. C., KANTZANOU, M. N., FOWLER, T., DAY, N. P., SCHOFIELD, K., WANN, E. R., BERENDT, A. R., HOOK, M. & PEACOCK, S. J. 2001. Fibronectin-binding protein A of Staphylococcus aureus has multiple, substituting, binding regions that mediate adherence to fibronectin and invasion of endothelial cells. *Cell Microbiol,* 3**,** 839-51.

MASSIMI, I., PARK, E., RICE, K., MULLER-ESTERL, W., SAUDER, D. & MCGAVIN, M. J. 2002. Identification of a novel maturation mechanism and restricted substrate specificity for the SspB cysteine protease of Staphylococcus aureus. *J Biol Chem,* 277**,** 41770-7.

MAYA, S., INDULEKHA, S., SUKHITHASRI, V., SMITHA, K. T., NAIR, S. V., JAYAKUMAR, R. & BISWAS, R. 2012. Efficacy of tetracycline encapsulated O-carboxymethyl chitosan nanoparticles against intracellular infections of Staphylococcus aureus. *International Journal of Biological Macromolecules,* 51**,** 392-399.

MCCORMICK, J. K., YARWOOD, J. M. & SCHLIEVERT, P. M. 2001. Toxic shock syndrome and bacterial superantigens: an update. *Annu Rev Microbiol,* 55**,** 77-104.

MEHLIN, C., HEADLEY, C. M. & KLEBANOFF, S. J. 1999. An inflammatory polypeptide complex from Staphylococcus epidermidis: isolation and characterization. *J Exp Med,* 189**,** 907-18.

MENESTRINA, G., DALLA SERRA, M., COMAI, M., CORAIOLA, M., VIERO, G., WERNER, S., COLIN, D. A., MONTEIL, H. & PREVOST, G. 2003. Ion channels and bacterial infection: the case of beta-barrel pore-forming protein toxins of Staphylococcus aureus. *FEBS Lett,* 552**,** 54-60.

MENESTRINA, G., SERRA, M. D. & PREVOST, G. 2001. Mode of action of beta-barrel pore-forming toxins of the staphylococcal alpha-hemolysin family. *Toxicon,* 39**,** 1661-72.

MENZIES, B. E. 2003. The role of fibronectin binding proteins in the pathogenesis of Staphylococcus aureus infections. *Curr Opin Infect Dis,* 16**,** 225-9.

MOLLENHAUER, H. H., JAMES MORRÉ, D. & ROWE, L. D. 1990. Alteration of intracellular traffic by monensin; mechanism, specificity and relationship to toxicity. *Biochimica et Biophysica Acta (BBA) - Reviews on Biomembranes,* 1031**,** 225-246.

MOREILLON, P., QUE, Y. A. & BAYER, A. S. 2002. Pathogenesis of streptococcal and staphylococcal endocarditis. *Infect Dis Clin North Am,* 16**,** 297-318.

MORINAGA, N., KAIHOU, Y. & NODA, M. 2003. Purification, cloning and characterization of variant LukE-LukD with strong leukocidal activity of staphylococcal bi-component leukotoxin family. *Microbiol Immunol,* 47**,** 81-90.

MYERS, J. T., TSANG, A. W. & SWANSON, J. A. 2003. Localized reactive oxygen and nitrogen intermediates inhibit escape of Listeria monocytogenes from vacuoles in activated macrophages. *J Immunol,* 171**,** 5447-53.

NAIR, R., AMMANN, E., RYSAVY, M. & SCHWEIZER, M. L. 2014. Mortality among patients with methicillin-resistant Staphylococcus aureus USA300 versus non-USA300 invasive infections: a meta-analysis. *Infect Control Hosp Epidemiol,* 35**,** 31-41.

NOSKIN, G. A., RUBIN, R. J., SCHENTAG, J. J., KLUYTMANS, J., HEDBLOM, E. C., JACOBSON, C., SMULDERS, M., GEMMEN, E. & BHARMAL, M. 2007. National trends in Staphylococcus aureus infection rates: impact on economic burden and mortality over a 6-year period (1998-2003). *Clin Infect Dis,* 45**,** 1132-40.

NOVICK, R. P. 2003. Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Mol Microbiol,* 48**,** 1429-49.

NOVICK, R. P., ROSS, H. F., PROJAN, S. J., KORNBLUM, J., KREISWIRTH, B. & MOGHAZEH, S. 1993. Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. *Embo j,* 12**,** 3967-75.

NYGAARD, T. K., PALLISTER, K. B., DUMONT, A. L., DEWALD, M., WATKINS, R. L., PALLISTER, E. Q., MALONE, C., GRIFFITH, S., HORSWILL, A. R., TORRES, V. J. & VOYICH, J. M. 2012. Alpha-toxin induces programmed cell death of human T cells, B cells, and monocytes during USA300 infection. *PLoS One,* 7**,** e36532.

NYGAARD, T. K., PALLISTER, K. B., ZUREK, O. W. & VOYICH, J. M. 2013. The impact of alpha-toxin on host cell plasma membrane permeability and cytokine expression during human blood infection by CA-MRSA USA300. *J Leukoc Biol,* 94**,** 971-9.

O'BRIEN, F. G., LIM, T. T., CHONG, F. N., COOMBS, G. W., ENRIGHT, M. C., ROBINSON, D. A., MONK, A., SAID-SALIM, B., KREISWIRTH, B. N. & GRUBB, W. B. 2004. Diversity among community isolates of methicillin-resistant Staphylococcus aureus in Australia. *J Clin Microbiol,* 42**,** 3185-90.

O'TOOLE, G., KAPLAN, H. B. & KOLTER, R. 2000. Biofilm formation as microbial development. *Annu Rev Microbiol,* 54**,** 49-79.

OGSTON 1984. Classics in infectious diseases. "On abscesses". Alexander Ogston (1844-1929). *Rev Infect Dis,* 6**,** 122-8.

OLSON, R., NARIYA, H., YOKOTA, K., KAMIO, Y. & GOUAUX, E. 1999. Crystal structure of staphylococcal LukF delineates conformational changes accompanying formation of a transmembrane channel. *Nat Struct Biol,* 6**,** 134-40.

ORRENIUS, S., ZHIVOTOVSKY, B. & NICOTERA, P. 2003. Regulation of cell death: the calcium-apoptosis link. *Nat Rev Mol Cell Biol,* 4**,** 552-65.

OTTO, M. 2006. Bacterial evasion of antimicrobial peptides by biofilm formation. *Curr Top Microbiol Immunol,* 306**,** 251-8.

OTTO, M. 2008. Staphylococcal biofilms. *Curr Top Microbiol Immunol,* 322**,** 207-28.

OTTO, M. 2010. Basis of virulence in community-associated methicillin-resistant Staphylococcus aureus. *Annu Rev Microbiol,* 64**,** 143-62.

OTTO, M. 2014. Staphylococcus aureus toxins. *Curr Opin Microbiol,* 17**,** 32-7.

PALMA, M., HAGGAR, A. & FLOCK, J. I. 1999. Adherence of Staphylococcus aureus is enhanced by an endogenous secreted protein with broad binding activity. *J Bacteriol,* 181**,** 2840-5.

PALMQVIST, N., FOSTER, T., TARKOWSKI, A. & JOSEFSSON, E. 2002. Protein A is a virulence factor in Staphylococcus aureus arthritis and septic death. *Microb Pathog,* 33**,** 239-49.

PANTON, P. N. & VALENTINE, F. C. O. 1932. Staphylococcal toxin. *Lancet* 219**,** 506-508.

PARKER, M. W. & FEIL, S. C. 2005. Pore-forming protein toxins: from structure to function. *Prog Biophys Mol Biol,* 88**,** 91-142.

PATTI, J. M., ALLEN, B. L., MCGAVIN, M. J. & HOOK, M. 1994. MSCRAMM-mediated adherence of microorganisms to host tissues. *Annu Rev Microbiol,* 48**,** 585-617.

PEACOCK, S. J., FOSTER, T. J., CAMERON, B. J. & BERENDT, A. R. 1999. Bacterial fibronectin-binding proteins and endothelial cell surface fibronectin mediate adherence of Staphylococcus aureus to resting human endothelial cells. *Microbiology,* 145 (Pt 12)**,** 3477-86.

PEARL, F. M., BENNETT, C. F., BRAY, J. E., HARRISON, A. P., MARTIN, N., SHEPHERD, A., SILLITOE, I., THORNTON, J. & ORENGO, C. A. 2003. The CATH database: an extended protein family resource for structural and functional genomics. *Nucleic Acids Res,* 31**,** 452-5.

PERIASAMY, S., JOO, H. S., DUONG, A. C., BACH, T. H., TAN, V. Y., CHATTERJEE, S. S., CHEUNG, G. Y. & OTTO, M. 2012. How Staphylococcus aureus biofilms develop their characteristic structure. *Proc Natl Acad Sci U S A,* 109**,** 1281-6.

PETERSON, P. K., VERHOEF, J., SABATH, L. D. & QUIE, P. G. 1977. Effect of protein A on staphylococcal opsonization. *Infect Immun,* 15**,** 760-4.

POTEMPA, J., DUBIN, A., KORZUS, G. & TRAVIS, J. 1988. Degradation of elastin by a cysteine proteinase from Staphylococcus aureus. *J Biol Chem,* 263**,** 2664-7.

POTEMPA, J., WATOREK, W. & TRAVIS, J. 1986. The inactivation of human plasma alpha 1-proteinase inhibitor by proteinases from Staphylococcus aureus. *J Biol Chem,* 261**,** 14330-4.

PRINCE, L. R., GRAHAM, K. J., CONNOLLY, J., ANWAR, S., RIDLEY, R., SABROE, I., FOSTER, S. J. & WHYTE, M. K. 2012. Staphylococcus aureus induces eosinophil cell death mediated by alpha-hemolysin. *PLoS One,* 7**,** e31506.

PROCTOR, R. A., VAN LANGEVELDE, P., KRISTJANSSON, M., MASLOW, J. N. & ARBEIT, R. D. 1995. Persistent and relapsing infections associated with small-colony variants of Staphylococcus aureus. *Clin Infect Dis,* 20**,** 95-102.

PROKESOVA, L., POTUZNIKOVA, B., POTEMPA, J., ZIKAN, J., RADL, J., HACHOVA, L., BARAN, K., PORWIT-BOBR, Z. & JOHN, C. 1992. Cleavage of human immunoglobulins by serine proteinase from Staphylococcus aureus. *Immunol Lett,* 31**,** 259-65.

QUECK, S. Y., JAMESON-LEE, M., VILLARUZ, A. E., BACH, T. H., KHAN, B. A., STURDEVANT, D. E., RICKLEFS, S. M., LI, M. & OTTO, M. 2008. RNAIII-independent target gene control by the agr quorum-sensing system: insight into the evolution of virulence regulation in Staphylococcus aureus. *Mol Cell,* 32**,** 150-8.

QUECK, S. Y., KHAN, B. A., WANG, R., BACH, T. H., KRETSCHMER, D., CHEN, L., KREISWIRTH, B. N., PESCHEL, A., DELEO, F. R. & OTTO, M. 2009. Mobile genetic element-encoded cytolysin connects virulence to methicillin resistance in MRSA. *PLoS Pathog,* 5**,** e1000533.

RASIGADE, J. P., TROUILLET-ASSANT, S., FERRY, T., DIEP, B. A., SAPIN, A., LHOSTE, Y., RANFAING, J., BADIOU, C., BENITO, Y., BES, M., COUZON, F., TIGAUD, S., LINA, G., ETIENNE, J., VANDENESCH, F. & LAURENT, F. 2013. PSMs of hypervirulent Staphylococcus aureus act as intracellular toxins that kill infected osteoblasts. *PLoS One,* 8**,** e63176.

REED, S. B., WESSON, C. A., LIOU, L. E., TRUMBLE, W. R., SCHLIEVERT, P. M., BOHACH, G. A. & BAYLES, K. W. 2001. Molecular characterization of a novel Staphylococcus aureus serine protease operon. *Infect Immun,* 69**,** 1521-7.

ROCHA-DE-SOUZA, C. M., BERENT-MAOZ, B., MANKUTA, D., MOSES, A. E. & LEVI-SCHAFFER, F. 2008. Human mast cell activation by Staphylococcus aureus: interleukin-8 and tumor necrosis factor alpha release and the role of Toll-like receptor 2 and CD48 molecules. *Infect Immun,* 76**,** 4489-97.

ROSENBACH, F. J. 1884. Mikroorganismen bei den Wundinfektionskrankheiten des Menschen.

SANSONETTI, P. J., RYTER, A., CLERC, P., MAURELLI, A. T. & MOUNIER, J. 1986. Multiplication of Shigella flexneri within HeLa cells: lysis of the phagocytic vacuole and plasmid-mediated contact hemolysis. *Infect Immun,* 51**,** 461-9.

SCHNAITH, A., KASHKAR, H., LEGGIO, S. A., ADDICKS, K., KRONKE, M. & KRUT, O. 2007. Staphylococcus aureus subvert autophagy for induction of caspase-independent host cell death. *J Biol Chem,* 282**,** 2695-706.

SCHRÖDER, A., KLAND, R., PESCHEL, A., VON EIFF, C. & AEPFELBACHER, M. 2006. Live cell imaging of phagosome maturation in Staphylococcus aureus infected human endothelial

cells: small colony variants are able to survive in lysosomes. *Med Microbiol Immunol,* 195**,** 185-94.

SCHWAB, U. E., WOLD, A. E., CARSON, J. L., LEIGH, M. W., CHENG, P. W., GILLIGAN, P. H. & BOAT, T. F. 1993. Increased adherence of Staphylococcus aureus from cystic fibrosis lungs to airway epithelial cells. *Am Rev Respir Dis,* 148**,** 365-9.

SENDI, P. & PROCTOR, R. A. 2009. Staphylococcus aureus as an intracellular pathogen: the role of small colony variants. *Trends Microbiol,* 17**,** 54-8.

SEYBOLD, U., KOURBATOVA, E. V., JOHNSON, J. G., HALVOSA, S. J., WANG, Y. F., KING, M. D., RAY, S. M. & BLUMBERG, H. M. 2006. Emergence of community-associated methicillin-resistant Staphylococcus aureus USA300 genotype as a major cause of health care-associated blood stream infections. *Clin Infect Dis,* 42**,** 647-56.

SHAW, L., GOLONKA, E., POTEMPA, J. & FOSTER, S. J. 2004. The role and regulation of the extracellular proteases of Staphylococcus aureus. *Microbiology,* 150**,** 217-28.

SHOMPOLE, S., HENON, K. T., LIOU, L. E., DZIEWANOWSKA, K., BOHACH, G. A. & BAYLES, K. W. 2003. Biphasic intracellular expression of Staphylococcus aureus virulence factors and evidence for Agr-mediated diffusion sensing. *Mol Microbiol,* 49**,** 919-27.

SINHA, B., FRANCOIS, P. P., NUSSE, O., FOTI, M., HARTFORD, O. M., VAUDAUX, P., FOSTER, T. J., LEW, D. P., HERRMANN, M. & KRAUSE, K. H. 1999. Fibronectin-binding protein acts as Staphylococcus aureus invasin via fibronectin bridging to integrin alpha5beta1. *Cell Microbiol,* 1**,** 101-17.

SONG, L., HOBAUGH, M. R., SHUSTAK, C., CHELEY, S., BAYLEY, H. & GOUAUX, J. E. 1996. Structure of staphylococcal alpha-hemolysin, a heptameric transmembrane pore. *Science,* 274**,** 1859-66.

SPAAN, A. N., HENRY, T., VAN ROOIJEN, W. J., PERRET, M., BADIOU, C., AERTS, P. C., KEMMINK, J., DE HAAS, C. J., VAN KESSEL, K. P., VANDENESCH, F., LINA, G. & VAN STRIJP, J. A. 2013. The staphylococcal toxin Panton-Valentine Leukocidin targets human C5a receptors. *Cell Host Microbe,* 13**,** 584-94.

STEVENS, F. A. 1927. The occurence of Staphylococcus aureus infection with a scarlitiniform rash. *Jama,* 88**,** 1957-1958.

STUART, L. M., DENG, J., SILVER, J. M., TAKAHASHI, K., TSENG, A. A., HENNESSY, E. J., EZEKOWITZ, R. A. & MOORE, K. J. 2005. Response to Staphylococcus aureus requires CD36-mediated phagocytosis triggered by the COOH-terminal cytoplasmic domain. *J Cell Biol,* 170**,** 477-85.

SUREWAARD, B. G., DE HAAS, C. J., VERVOORT, F., RIGBY, K. M., DELEO, F. R., OTTO, M., VAN STRIJP, J. A. & NIJLAND, R. 2013. Staphylococcal alpha-phenol soluble modulins contribute to neutrophil lysis after phagocytosis. *Cell Microbiol,* 15**,** 1427-37.

SUREWAARD, B. G., NIJLAND, R., SPAAN, A. N., KRUIJTZER, J. A., DE HAAS, C. J. & VAN STRIJP, J. A. 2012. Inactivation of staphylococcal phenol soluble modulins by serum lipoprotein particles. *PLoS Pathog,* 8**,** e1002606.

TAKADA, Y., YE, X. & SIMON, S. 2007. The integrins. *Genome Biol,* 8**,** 215.

TEYSSEIRE, N., BOUDIER, J. A. & RAOULT, D. 1995. Rickettsia conorii entry into Vero cells. *Infect Immun,* 63**,** 366-74.

THAY, B., WAI, S. N. & OSCARSSON, J. 2013. Staphylococcus aureus alpha-toxin-dependent induction of host cell death by membrane-derived vesicles. *PLoS One,* 8**,** e54661.

TILNEY, L. G., HARB, O. S., CONNELLY, P. S., ROBINSON, C. G. & ROY, C. R. 2001. How the parasitic bacterium Legionella pneumophila modifies its phagosome and transforms it into rough ER: implications for conversion of plasma membrane to the ER membrane. *J Cell Sci,* 114**,** 4637-50.

TODD, J., FISHAUT, M., KAPRAL, F. & WELCH, T. 1978. Toxic-shock syndrome associated with phage-group-I Staphylococci. *Lancet,* 2**,** 1116-8.

TSAI, C. S. 2007. Biomacromolecules: Introduction to structure, function and informatics. *Wiley-Liss.*

TZIANABOS, A. O., WANG, J. Y. & LEE, J. C. 2001. Structural rationale for the modulation of abscess formation by Staphylococcus aureus capsular polysaccharides. *Proc Natl Acad Sci U S A,* 98**,** 9365-70.

UEMATSU, S. & AKIRA, S. 2006. Toll-like receptors and innate immunity. *J Mol Med (Berl),* 84**,** 712-25.

UHLEN, M., GUSS, B., NILSSON, B., GATENBECK, S., PHILIPSON, L. & LINDBERG, M. 1984. Complete sequence of the staphylococcal gene encoding protein A. A gene evolved through multiple duplications. *J Biol Chem,* 259**,** 1695-702.

VALEVA, A., HELLMANN, N., WALEV, I., STRAND, D., PLATE, M., BOUKHALLOUK, F., BRACK, A., HANADA, K., DECKER, H. & BHAKDI, S. 2006. Evidence that clustered phosphocholine head groups serve as sites for binding and assembly of an oligomeric protein pore. *J Biol Chem,* 281**,** 26014-21.

VALEVA, A., WALEV, I., PINKERNELL, M., WALKER, B., BAYLEY, H., PALMER, M. & BHAKDI, S. 1997. Transmembrane beta-barrel of staphylococcal alpha-toxin forms in sensitive but not in resistant cells. *Proc Natl Acad Sci U S A,* 94**,** 11607-11.

VANDENESCH, F., NAIMI, T., ENRIGHT, M. C., LINA, G., NIMMO, G. R., HEFFERNAN, H., LIASSINE, N., BES, M., GREENLAND, T., REVERDY, M. E. & ETIENNE, J. 2003. Community-acquired methicillin-resistant Staphylococcus aureus carrying Panton-Valentine leukocidin genes: worldwide emergence. *Emerg Infect Dis,* 9**,** 978-84.

VANDESOMPELE, J., DE PRETER, K., PATTYN, F., POPPE, B., VAN ROY, N., DE PAEPE, A. & SPELEMAN, F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol,* 3**,** Research0034.

VANN, J. M. & PROCTOR, R. A. 1987. Ingestion of Staphylococcus aureus by bovine endothelial cells results in time- and inoculum-dependent damage to endothelial cell monolayers. *Infect Immun,* 55**,** 2155-63.

VENTURA, C. L., MALACHOWA, N., HAMMER, C. H., NARDONE, G. A., ROBINSON, M. A., KOBAYASHI, S. D. & DELEO, F. R. 2010. Identification of a novel Staphylococcus aureus two-component leukotoxin using cell surface proteomics. *PLoS One,* 5**,** e11634.

VON EIFF, C., BECKER, K., MACHKA, K., STAMMER, H. & PETERS, G. 2001. Nasal carriage as a source of Staphylococcus aureus bacteremia. Study Group. *N Engl J Med,* 344**,** 11-6.

VOYICH, J. M., OTTO, M., MATHEMA, B., BRAUGHTON, K. R., WHITNEY, A. R., WELTY, D., LONG, R. D., DORWARD, D. W., GARDNER, D. J., LINA, G., KREISWIRTH, B. N. & DELEO, F. R. 2006. Is Panton-Valentine leukocidin the major virulence determinant in community-associated methicillin-resistant Staphylococcus aureus disease? *J Infect Dis,* 194**,** 1761-70.

VUONG, C., DURR, M., CARMODY, A. B., PESCHEL, A., KLEBANOFF, S. J. & OTTO, M. 2004. Regulated expression of pathogen-associated molecular pattern molecules in Staphylococcus epidermidis: quorum-sensing determines pro-inflammatory capacity and production of phenol-soluble modulins. *Cell Microbiol,* 6**,** 753-9.

WADE, J. D., LIN, F., HOSSAIN, M. A. & DAWSON, R. M. 2012. Chemical synthesis and biological evaluation of an antimicrobial peptide gonococcal growth inhibitor. *Amino Acids,* 43**,** 2279-83.

WANG, R., BRAUGHTON, K. R., KRETSCHMER, D., BACH, T. H., QUECK, S. Y., LI, M., KENNEDY, A. D., DORWARD, D. W., KLEBANOFF, S. J., PESCHEL, A., DELEO, F. R. & OTTO, M. 2007. Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. *Nat Med,* 13**,** 1510-4.

WENZEL, R. P. & PERL, T. M. 1995. The significance of nasal carriage of Staphylococcus aureus and the incidence of postoperative wound infection. *J Hosp Infect,* 31**,** 13-24.

WERTHEIM, H. F., MELLES, D. C., VOS, M. C., VAN LEEUWEN, W., VAN BELKUM, A., VERBRUGH, H. A. & NOUWEN, J. L. 2005. The role of nasal carriage in Staphylococcus aureus infections. *Lancet Infect Dis,* 5**,** 751-62.

WERTHEIM, H. F., VOS, M. C., OTT, A., VAN BELKUM, A., VOSS, A., KLUYTMANS, J. A., VAN KEULEN, P. H., VANDENBROUCKE-GRAULS, C. M., MEESTER, M. H. & VERBRUGH, H. A. 2004. Risk and outcome of nosocomial Staphylococcus aureus bacteraemia in nasal carriers versus non-carriers. *Lancet,* 364**,** 703-5.

WESTERMANN, A. J., GORSKI, S. A. & VOGEL, J. 2012. Dual RNA-seq of pathogen and host. *Nat Rev Microbiol,* 10**,** 618-30.

WHITWORTH, T., POPOV, V. L., YU, X. J., WALKER, D. H. & BOUYER, D. H. 2005. Expression of the Rickettsia prowazekii pld or tlyC gene in Salmonella enterica serovar Typhimurium mediates phagosomal escape. *Infect Immun,* 73**,** 6668-73.

WILKE, G. A. & BUBECK WARDENBURG, J. 2010. Role of a disintegrin and metalloprotease 10 in Staphylococcus aureus alpha-hemolysin-mediated cellular injury. *Proc Natl Acad Sci U S A,* 107**,** 13473-8.

WILLIAMS, R. E. 1963. Healthy carriage of Staphylococcus aureus: its prevalence and importance. *Bacteriol Rev,* 27**,** 56-71.

WINKLER, A.-C. 2015. *Dissertation: Identification of human host factors involved in Stapyhlococcus aureus 6850 infection.* Wuerzburg.

XIONG, Y. Q., WILLARD, J., YEAMAN, M. R., CHEUNG, A. L. & BAYER, A. S. 2006. Regulation of Staphylococcus aureus alpha-toxin gene (hla) expression by agr, sarA, and sae in vitro and in experimental infective endocarditis. *J Infect Dis,* 194**,** 1267-75.

XU, S. X. & MCCORMICK, J. K. 2012. Staphylococcal superantigens in colonization and disease. *Front Cell Infect Microbiol,* 2**,** 52.

YANAI, M., ROCHA, M. A., MATOLEK, A. Z., CHINTALACHARUVU, A., TAIRA, Y., CHINTALACHARUVU, K. & BEENHOUWER, D. O. 2014. Separately or combined, LukG/LukH is functionally unique compared to other staphylococcal bicomponent leukotoxins. *PLoS One,* 9**,** e89308.

YARWOOD, J. M. & SCHLIEVERT, P. M. 2003. Quorum sensing in Staphylococcus infections. *J Clin Invest,* 112**,** 1620-5.

YOSHIDA, Y. & IMAI, S. 1997. Structure and function of inositol 1,4,5-trisphosphate receptor. *Jpn J Pharmacol,* 74**,** 125-37.

YU, F. L., LIU, T. T., ZHU, X., YANG, W. X., ZHANG, T., LIN, N., LIU, Y., LIU, C. S., JIANG, J. & GUAN, J. C. 2013. Staphylococcal enterotoxin B and alphatoxin induce the apoptosis of ECV304 cells via similar mechanisms. *Mol Med Rep,* 8**,** 591-6.

6 Appendix

6.1 Abbreviations

µ	Micro
7-AAD	7-Aminoactinmycin D
α	Alpha
AFU	Arbitrary fluorescence units
2-APB	2-Aminoethoxydiphenyl borate
AA	amino acid
ABC	ATP binding cassette
ADAM10	Disintegrin and metalloproteinase domain containing protein 10
Agr	accessory gene regulator
amp	Ampicillin
atet	Anhydrotetracycline
Atl	autolysin
ATP	adenosine triphosphate
Aur	aureolysin
Bcl-2	B cell lymphoma 2
β	beta
blasti	Blasticidin
CA-MRSA	community-acquired MRSA
cDNA	complementary DNA
CD11b	cluster of differentiation molecule 11B (=ITGAM)
CF	cystic fibrosis
CFU	colony forming units
CHIPS	chemotaxis inhibitory protein of *S. aureus*
cm	Chloramphenicol
c/ml	cells per millilitre
CT	cycle threshold
C-terminal	carboxyl-terminal
CWT	C-terminal cell wall-targeting domain
δ/Δ	Delta
DAG	diacylglycerin
DMEM	Dulbecco´s modified Eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleosid trisphosphate
E. coli	*Escherichia coli*
EE	early endosomal
EGF(R)	epidermal growth factor (receptor)
e.g.	*exempli gratia*, for example
erm	erythromycin
EtOH	Ethanol
GP91	glycoprotein 91
Eap	extracellular adherent protein
EDTA	ethylenediaminetetetraacetic acid
ER	endoplasmatic reticulum
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanate

Fig.	Figure
Fn	fibronectin
FnBP	fibronectin binding protein
FPR2	formyl peptide receptor 22
fwd	forward
LRR	leucine rich repeat
gapdh	Glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
G418	Geneticin
gyrB	gyrase subunit B gene name
h	hours
HA-MRSA	hospital-acquired MRSA
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hla	alpha toxin gene name
Hlb	beta toxin gene name
HSA	human serum albumin
Hsp60	heat shock protein 60
inf	infected
IL-1β	interleukin 1 beta
IP3R	Inositol trisphosphate 3 receptor
ITGAM	Integrin Alpha M (=CD11b)
k	kilo
kb	kilo base
kDa	Kilo Dalton
KO	knock-out
L	litre
LAMP 1/2	lysosomal associated membrane protein 1/2
LE	late endosomal
LLO	Listeriolysin O
LPS	Lipopolysaccharide
LY	lysosomal
LukAB	Leukocidin A/B
M	Molar
m	milli
MEFs	mouse embryonic fibroblasts
MHB	Mueller Hinton bouillon
min	Minute
MOI	multiplicity of infection
MRSA	methicillin resistant *S. aureus*
mRNA	messenger RNA
nM	nanomolar
n.i.	not infected
N-terminal	amino-terminal
OD_{540nm}	optical density measured at a wavelength of 540nm
P	p-value
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	Paraformaldehyde
p.i.	post infection
PI-PLC	Phosphoinoside phospholipase C
PMN	polymorphonuclear leukocytes

Pmt	phenol-soluble modulin transporter
PSM	phenol-soluble modulin
PVL	Panton-Valentine-Leukocidin
qRT-PCR	quantitative real-time PCR
rev	reverse
rif	Rifampicin
RNA	ribonucleic acid
RNAi	RNA interference
rpm	revolutions per minute
RT	room temperature
RQ	relative expression levels
s	seconds
SSSS	Staphylococcal scaled skin syndrome
S. aureus	*Staphylococcus aureus*
SAgs	*S. aureus* superantigens
SCV	small colony varients
saeR/S	gene regulatory system
sarA	staphylococcal accessory regulator
SEM	standard error of the mean
S. epidermidis	*Staphylococcus epidermidis*
SD	standard deviation
siRNA	small interfering RNA
Ssp	Staphopain
spec	Spectinomycin
tc	Tetracycline
TSB	Tris buffered saline
TSST-1	Toxic shock syndrome 1
TLR	toll like receptor
U	enzyme unit
wt	wild-type
WTA	wall teichoic acid
YFP	yellow fluorescent protein

6.2 Additional results

Following tables contain the results from the dual RNA-seq screen with genes which were at least two fold enriched.[6] The information including name, acronym and function which are listed in the following tables are collected by the webpage called GeneCards®.

[6] Provided by Dr. Konrad Förstner (Core Unit Systemmedizin, University Wuerzburg)

Table 16: Enriched genes in 293T cells 2 h post infection, with 6850 wild-type vs. 6850 Δ*psm*α. Genes with a fold change > 2 and P-value ≤ 0.05

Acronym	Name	Function	Fold change
COLGALT2	Collagen beta(1-O)galactosyltransferase 2	Beta-galactosyltransferase activity; transfers beta-galactose to hydroxylysine residues on	2.37
AC010894.4	No name	AC010894.4 processed pseudogene	4.10
PROS1	Protein S (Alpha)	Anticoagulant plasma protein	2.04
CDV3P1	CDV3 Homolog (Mouse) Pseudogene	CDV3P1 is a pseudogene	6.97
GSTO3P	Glutathione S-Transferase Omega 3, Pseudogene	GSTO3P is a pseudogene	2.58
TIFA	TRAF-Interacting Protein With Forkhead-Associated Domain	Adapter protein which mediates the IRAK1 and TRAF6 interaction following IL-1 stimulation, resulting in the downstream activation of NF-kappa-B and AP-1 pathways	2.60
SAP30	Sin3A-Associated Protein	Involved in the functional recruitment of the Sin3-histone deacetylase complex (HDAC) to a specific subset of N-CoR corepressor complexes	2.76
SGCE	Sarcoglycan, Epsilon	Component of the sarcoglycan complex, a subcomplex of the dystrophin-glycoprotein complex which forms a link between the F-actin cytoskeleton and the extracellular	2.87
UAP1L1	UDP-N-Acteylglucosamine Pyrophosphorylase 1-Like	Converts UDP and GlcNAc-1-P into UDP-GlcNAc, and UDP and GalNAc-1-P into UDP-GalNAc	2.22
THNSL1	Threonine synthase-like 1 (*S. cerevisiae*)	Potent inducer of osteoblastic production of IL6. May act to exacerbate inflammation and/or bone turnover under inflammatory conditions	2.40

Table 17: Enriched genes in 293T cells 2 h post infection, with uninfected vs. 6850 wild-type. Genes with a fold change > 2 and P-value ≤ 0.05

Acronym	Name	Function	Fold change
PEX10	Peroxisomal biogenesis factor 10	Involved in import of peroxisomal matrix proteins.	2.41
THAP3	THAP domain containing, apoptosis associated protein 3	Component of a THAP1/THAP3-HCFC1-OGT complex that is required for the regulation of the transcriptional activity of RRM1	2.63
TMEM201	Transmembrane protein 201	Isoform SAMP1 may define a distinct membrane domain in the vicinity of the mitotic spindle	2.01
MAD2L2	MAD2 mitotic arrest deficient-like 2 (yeast)	A component of the mitotic spindle assembly checkpoint that prevents the onset of anaphase until all chromosomes are properly aligned at the metaphase plate.	2.09
RP11570P14.1	No name	Pseudogene expressed in kidney	2.10
DMAP1	DNA methyltransferase 1 associated protein 1	Involved in transcription repression and activation.	2.11
MUTYH	MutY homolog	Involved in oxidative DNA damage repair	2.43
NSRP1P1	nuclear speckle splicing regulatory protein 1 pseudogene 1	NSRP1P1 is a pseudogene	2.45
C1orf54	chromosome 1 open reading frame 54	C1orf54 is a open reading frame	3.18
DPM3	dolichyl-phosphate mannosyltransferase polypeptide 3	Stabilizer subunit of the dolichol-phosphate mannose (DPM) synthase complex; tethers catalytic subunit DPM1 to the ER	2.18

Table 18: Enriched genes in 293T cells 2 h post infection, with uninfected vs. 6850 Δ*psm*α. Genes with a fold change > 2 and P-value ≤ 0.05

Acronym	Name	Function	Fold change
RP11480I12.5	No name	RP11480I12.5 is a pseudogene	3.47
C1orf101	Chromosome 1 open reading frame 101	An important paralog of this gene is CATSPERD.	4.16
FAHD2B	Fumarylacetoacetate hydrolase domain containing 2B	Probable mitochondrial acylpyruvase which is able to hydrolyze acetylpyruvate and fumarylpyruvate in vitro	2.3
RNF25	Ring finger protein 25	E3 ubiquitin-protein ligase that mediates ubiquitination and subsequent proteasomal degradation of NKD2	2.23
SLC4A3	Solute carrier family 4 (anion exchanger), member 3	Plasma membrane anion exchange protein of wide distribution.	5.09
RGS14	Regulator of G-protein signalling 14	The protein attenuates the signalling activity of G-proteins by binding, through its GoLoco domain, to specific types of activated, GTP-bound G alpha subunits	3.66
PRRT1	Proline-rich transmembrane protein 1	PRRT1 is a protein-coding gene	5.09
HABP4	Hyaluronan binding protein 4	May be involved in nuclear functions such as the remodeling of chromatin and the regulation of transcription	2.12
ANAPC2	Anaphase promoting complex subunit 2	Component of the anaphase promoting complex/cyclosome (APC/C), a cell cycle-regulated E3 ubiquitin ligase that controls progression through mitosis and the G1 phase of the cell cycle	2.08
ZNF485	Zinc finger protein 485	May be involved in transcriptional regulation	2.86

Table 19: Enriched genes in 293T cells 6 h post infection, with 6850 wild-type vs. 6850 Δ*psm*α. Genes with a fold change > 2 and P-value ≤ 0.05

Acronym	Name	Function	Fold change
RNVU1-6	RNA, variant U1 small nuclear 6	U1 spliceosomal RNA - U1 is a small nuclear RNA (snRNA) component of the spliceosome (involved in pre-mRNA splicing)	3.18
NBPF13P	Neuroblastoma breakpoint family, member 13, pseudogene	This gene is a member of the neuroblastoma breakpoint family (NBPF)	2.10
RNVU1-14	RNA, variant U1 small nuclear 14	U1 spliceosomal RNA - U1 is a small nuclear RNA (snRNA) component of the spliceosome (involved in pre-mRNA splicing)	2.07
FCGR2B	Fc fragment of IgG, low affinity IIb, receptor (CD32)	Low affinity receptor for the Fc region of immunoglobulin gamma complexes. Involved in the phagocytosis of immune complexes and in the regulation of antibody production by B-cells	2.29
RNA5SP67	RNA, 5S ribosomal pseudogene 67	RNA5SP67 is a pseudogene	2.28
PLA2G4A	Phospholipase A2, group IVA	The enzyme is activated by increased intracellular Ca^{2+} levels and phosphorylation, resulting in its translocation from the cytosol and nucleus to perinuclear membrane vesicles	2.40
LINC01036	Long intergenic non-protein coding RNA 1036	LINC01036 is a RNA gene and is affiliated with the non-coding RNA class	2.15
MARC2	mitochondrial amidoxime reducing component 2	As a component of the benzamidoxime prodrug-converting complex required to reduce N-hydroxylated prodrugs, such as	2.10

		benzamidoxime	
ZNF513	Zinc finger protein 513	Transcriptional regulator that plays a role in retinal development and maintenance	2.49
KIAA1841	No name	Uncharacterized protein KIAA1841	2.13

Table 20: Enriched genes in 293T cells 6 h post infection, with uninfected vs. 6850 wild-type. Genes with a fold change > 2 and P-value ≤ 0.05

Acronym	Name	Function	Fold change
ISG15	ubiquitin-like modifier	Ubiquitin-like protein which plays a key role in the innate immune response to viral infection	3.00
SCNN1D	Sodium channel, non-voltage-gated 1, delta subunit	Mediates the electrodiffusion of the luminal sodium through the apical membrane of epithelial cells.	24.66
U1	U1 small nuclear	U1 is a RNA gene	2.11
CNR2	Cannabinoid receptor 2 (macrophage)	Heterotrimeric G protein-coupled receptor for endocannabinoid 2-arachidonoylglycerol mediating inhibition of adenylate cyclase. May function in inflammatory response, nociceptive transmission and bone homeostasis	4.41
MAN1C1	Mannosidase, alpha, class 1C, member 1	Involved in the maturation of Asn-linked oligosaccharides. Trim alpha-1,2-linked mannose residues from Man(9)GlcNAc(2) to produce first Man(8)GlcNAc(2) then Man(6)GlcNAc and a small amount of Man(5)GlcNAc	3.34
PTAFR	Platelet-activating factor receptor	Seven-transmembrane G-protein-coupled receptor for platelet-activating factor (PAF) that localizes to lipid rafts and/or caveolae in the cell membrane	3.89
SNORD85	Small nucleolar RNA, C/D box 85	SNOR85 is a RNA gene, and is affiliated with the snoRNA class	3.79
ERMAP	Erythroblast membrane-associated protein	Possible role as a cell-adhesion or receptor molecule of erythroid cells	9.52
ST3GAL3	ST3 beta-galactoside alpha-2,3-sialyltransferase 3	Type II membrane protein that catalyzes the transfer of sialic acid from CMP-sialic acid to galactose-containing substrates.	2.77
AGBL4	ATP/GTP binding protein-like 4	Metallocarboxypeptidase that mediates deglutamylation of target proteins. Catalyzes the deglutamylation of polyglutamate side chains generated by post-translational polyglutamylation in proteins such as tubulins	3.25

Table 21: Enriched genes in 293T cells 6 h post infection, with not infected vs. 6850 Δ*psm*α. Genes with a fold change > 2 and P-value ≤ 0.05

Acronym	Name	Function	Fold change
SCNN1D	Sodium Channel, Non-Voltage-Gated 1, Delta Subunit	These channels transport Na+ across the epithelium using energy from the Na+, K+-ATPase, and constitute one of the essential mechanisms in regulation of sodium balance, blood volume and blood pressure.	12.46
MAN1C1	Mannosidase, alpha, class 1C, member 1	Involved in the maturation of Asn-linked oligosaccharides	3.56
SNORA73B	Small nucleolar RNA, H/ACA box 73B	RNA gene, and is affiliated with the snoRNA class.	2.02
RP4-604A21.1	No name	RP4-604A21.1 known processed pseudogene	2.48
MATN1	Matrilin 1, cartilage matrix protein	Cartilage matrix protein is a major component of the extracellular matrix of non-articular cartilage. It binds to collagen	3.47
ADC	Arginine decarboxylase	Include ornithine decarboxylase activator activity and arginine decarboxylase activity. An important paralog of this gene is ODC1.	4.83
ERMAP	Erythroblast membrane-associated protein	Possible role as a cell-adhesion or receptor molecule of erythroid cells	7.06
RPS15AP11	Ribosomal protein S15a pseudogene 11	PRS15AP11 is a pseudogene	2.51
MSH4	MutS homolog 4	Involved in meiotic recombination. Required for reciprocal recombination and proper segregation of homologous chromosomes at meiosis	3.17
RP11-422P24.9	No name	RP11-422P24.9 known processed pseudogene	2.13

6.3 List of publications and poster presentations

Publications

Grosz M., Kolter J., Paprotka K., Winkler A.-C., Schäfer D., Chatterjee S. S., Geiger T., Wolz C., Ohlsen K., Otto M., Rudel T., Sinha B. and Fraunholz M. Cytoplasmic replication of *Staphyloccous aureus* upon phagosomal escape triggered by phenol-soluble modulin α. Cell Microiol, 16, 451-65.

Grosz M.[+] and Winkler A.-C.[+], Oesterreich B., Fraunholz M., Ohlsen K., Otto M., Wolz C., Rudel T. *S. aureus* induced host cell death requires phagosomal escape and calcium signalling. (in preparation)

Winkler A.-C.[+] and **Grosz M**.[+], Ade C., Onyango M., Rudel T. A shRNA screen revealed that the AKT1 survival pathway is crucial for enhanced invasion and intracellular replication of *S. aureus*. (in preparation)

[+] These authors contribute equally to this work.

Poster Presentations

Grosz M., Schäfer D., Chatterjee S. S., Goerke C., Wolz C., Otto M., Fraunholz M., Sinha B. Small membrane-active peptides (PSMα) are required for phagosomal escape by *S. aueus* in non-professional phagocytes. Gordon Research Conference Staphylococcal Disease (2011, Lucca, Italy).

Grosz M., Schäfer D., Chatterjee S. S., Goerke C., Wolz C., Otto M., Fraunholz M., Sinha B. Small membrane-active peptides (PSMα) are required for phagosomal escape by *S. aueus* in non-professional phagocytes.63 . Jahrestagung der Deutschen Gesellschaft für Hygiene und Mikrobiologie (DGHM) e.V. (2011, Dresden, Germany).

Grosz. M., Kolter J., Paprotka K., Winkler A.-C., Geiger T., Chatterjee S. S., Wolz C., Otto M., Rudel T., Sinha B. and Fraunholz M. Phenol-soulbe modulin α triggers phagosomal escape o *Staphylococcus aureus* and implicates cytoplasmic replication. 65 . Jahrestagung der Deutschen Gesellschaft für Hygiene und Mikrobiologie (DGHM) e.V. (2013, Rostock, Germay). Posterpreis.

Grosz. M. and Winkler A.-C., Oesterreich B., Geiger T., Otto M., Fraunholz M., Rudel T. Host cell death is initiated by phagosomal escape of *S. aureus* and is a multifactorial interaction between the host and bacterial toxins. 16th International Symposium on Staphylococci and Staphylococcal Infections (2014, Chicago, USA)

6.4 Danksagung

An dieser Stelle möchte ich mich bei allen bedanken, die mich bei der Erstellung dieser Arbeit unterstützt haben.

Ich möchte Herrn Prof. Dr. Thomas Rudel für die Möglichkeit meine Doktorarbeit am Lehrstuhl für Mikrobiologie zu verfassen danken, vor allem aber für seine Unterstützung beim Arbeitsplatzwechsel und für die Übernahme meines Erstgutachters. Vielen Dank für die sehr gute Betreuung, für die zahlreichen Gespräche aber auch für die konstruktive Kritik.

Bei PD Dr. Knut Ohlsen bedanke ich mich herzlich für die Übernahme des Zweitgutachtens. Außerdem möchte ich mich für die gute Zusammenarbeit und die netten Gespräche bedanken.

Bei Dr. Martin Fraunholz möchte ich mich herzlich für seine stets geöffnete Türe bedanken, für die vielen Diskussionen, Tipps und für die große Hilfsbereitschaft in den vergangenen Jahren.

Ein riesiges Dankeschön an Dr. Ann-Cathrin Winkler, für eine hervorragende Kooperation, die mir viel Freude bereitet hat. Ich danke Dir auch für die netten Kaffeepausen in denen wir Energie sammeln konnten, um den Laboralltag gemeinsam zu meistern und nicht zu verzweifeln.

Vielen Dank auch an Alexander Westermann und Dr. Konrad Förstner für die große Unterstützung (Planung und Auswertung) beim Dual RNA-seq Versuch.

Ein ganz besonderer Dank an alle jetzigen und ehemaligen Arbeitskollegen der Mikrobiologie, die mich während meiner Promotion begleitet haben. Danke Annette, Christine, Lisa, Christine und Michaela, dass ihr mich in eure Mädelsrunde so lieb aufgenommen habt!

Ich bedanke mich auch herzlich bei Heidi, Nora und Babsi für die super Zeit in der alten AG Sinha. Vielen Dank für eure Unterstützung bei der Laborarbeit und dafür dass aus Arbeitskollegen auch Freunde werden können.

Auch meiner ganzen Familie, vor allem Mama, Papa, Johanna und Martin und meinen vielen lieben Freunden, hier möchte ich meine älteste und beste Freundin Katharina nennen, gebührt ein ganz besonderes Dankeschön! Vielen Dank für eure Unterstützung, Motivation, euer offenes Ohr und für eure Liebe.

Zum Schluss möchte ich mich bei einem ganz besonderen Menschen in meinem Leben bedanken, bei meinem Mann Jordan. Ohne dich wäre ich jetzt nicht da wo ich jetzt bin. Vielen Dank, dass du in jeder Lebenslage für mich da bist, dass du mich immer motivierst das Beste zu geben, das ich immer auf dich und deine Hilfe zählen kann und dass du nicht aufhörst an mich zu glauben.

6.5 Selbstständigkeitserklärung

Ich erkläre ehrenwörtlich, dass die vorliegende Arbeit von mir selbständig und nur unter Verwendung der angegebenen Quellen und Hilfsmittel angefertigt wurde.

Diese Dissertation hat weder in gleicher noch in ähnlicher Form in einem anderen Prüfungsverfahren vorgelegen.

Ich habe früher, außer den mit dem Zulassungsgesuch urkundlich vorgelegten Graden, keine weiteren akademischen Grade erworben oder zu erwerben versucht.

Würzburg den,