Dzmitry Sinitski

Strain-specific interactions of *Staphylococcus aureus* with human endothelium

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Dzmitry Sinitski

aus Riga

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Dekanin/Dekan: Prof. Dr. Wolf-Michael Weber

Erster Gutachter: Prof. Dr. Hans-Joachim Schnittler

Zweite Gutachterin: Prof. Dr. Susanne Fetzner

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Abbreviations

AD	arginine-deiminase
Agr	accessory gene regulator
AIDS	acquired immuno-dificency syndrome
ANOVA	analysis of variance
AP-1	activator protein 1
ATP	adenosine triphosphate
AVBB	annexin V binding buffer
BSA	bovine serum albumin
CA-MRSA	community acquired methicillin-resistant *Staphylococcus aureus*
cDNA	complementary desoxyribonucleic acid
ddH_2O	double-distilled water
DMSO	dimethyl sulfoxide
dNTP	desoxynucleotides triphosphate
Eap	extracellular adhesion protein
ECM	extracellular matrix
EDIN	epidermal cell differentiation inhibitor
EDTA	ethylene diamine tetraacetic acid
Emp	envelope-associated protein
FAK	focal adhesion kinase
FC	fold change
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FnBPA	fibronectin binding protein A
FnBPB	fibronectin binding protein B
GlcNAc	N-Acetylglucosamine
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hsp60	heat-shock protein 60
HUVEC	human umbilical vein endothelial cells
IA	inhibition assay
IF	immunofluorescence

LA	lipoteichoic acid
LS	lysostaphin
MAPK	mitogen-activated protein kinases
MDP	muramyldipeptide
MF	mean flourescence
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
MRSA	methicillin-resistant *Staphylococcus aureus*
MSCRAMMs	microbial surface component recognizing adhesive matrix molecules
NF-kB	nuclear factor kappa B
NK cell	natural killer cell
NLRP3	NLR family, pyrin domain containing 3
NOD2	nod-like receptor 2
OD	optical density
PAMP	pathogen associated molecular pattern
PBP	penicillin-binding protein
PBS	phosphate-buffered saline
PCA	principal component analysis
PFA	paraformaldehyde
PG	peptidodoglycan
PI	propidium iodide
PTK	protein tyrosine kinase
PTSAg	pyrogenic toxin superantigens
RIG-I	retinoic inducible gene-I-like receptor
RNI	reactive nitrogen intermediates
ROI	reactive oxygen intermediates
RT-qPCR	real time quantitative polymerase chain reaction
SCCmec	staphylococcal cassette chromosome mec
SD	standard deviation
SEB	staphylococcal exotoxin B
SERAMs	secretable expanded repertoire adhesive molecules

SP	signaling pathway
spA	staphylococcal protein A
STAT1	signal transducer and activator of transcription 1
TER	trans-endothelial resistance
TLR	toll-like receptor
TNFR1	tumor necrosis factor receptor 1
TNF-α	tumor necrosis factor alpha
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
TSB	tryptic soy broth
TSST-1	toxic shock syndrome toxin

1.Introduction

1.1. Principles of bacteria-host interactions and microbial virulence

Every mammalian organism interacts with a broad variety of microorganisms such as fungi and bacteria (Zasloff, 2002). Most of these interactions are symbiotic or commensal in nature and in these relationships, the host microorganism population is usually referred to as microflora (Tlaskalova-Hogenova *et al.*, 2004). Host microflora plays an important role in maintaining body homeostasis and contributes to the natural resistance of the host to other types of infection (Berg, 1996).

In other cases, some microorganisms can be pathogenic to the host organism. Their pathogenic properties can compromise host immunity and result in the development of disease. The specific clinical outcomes of many infectious diseases are determined by the broad spectrum of host-pathogen interactions.

Host-pathogen interactions describe infectious processes that include the interplay between microorganism virulence factors and the response mechanisms of host defense (Casadevall and Pirofski, 2000).

To understand the concept of host-pathogen interaction the terms bacterial virulence and pathogenicity must also be understood. Virulence is defined as the relative capacity of a pathogen to overcome the host immune response and is determined by a particular set of bacteria virulence factors (Sparling, 1983). Common bacterial virulence factors include toxins, enzymes, antigens, invasins and adhesins. Virulence factors modulate different steps of infection such as attachment, proliferation, tissue damage, invasion and intracellular persistence of the pathogen. The summarized effects of virulence factors result in the development of disease and are categorized by bacteria pathogenicity (Casadevall and Pirofski, 1999).

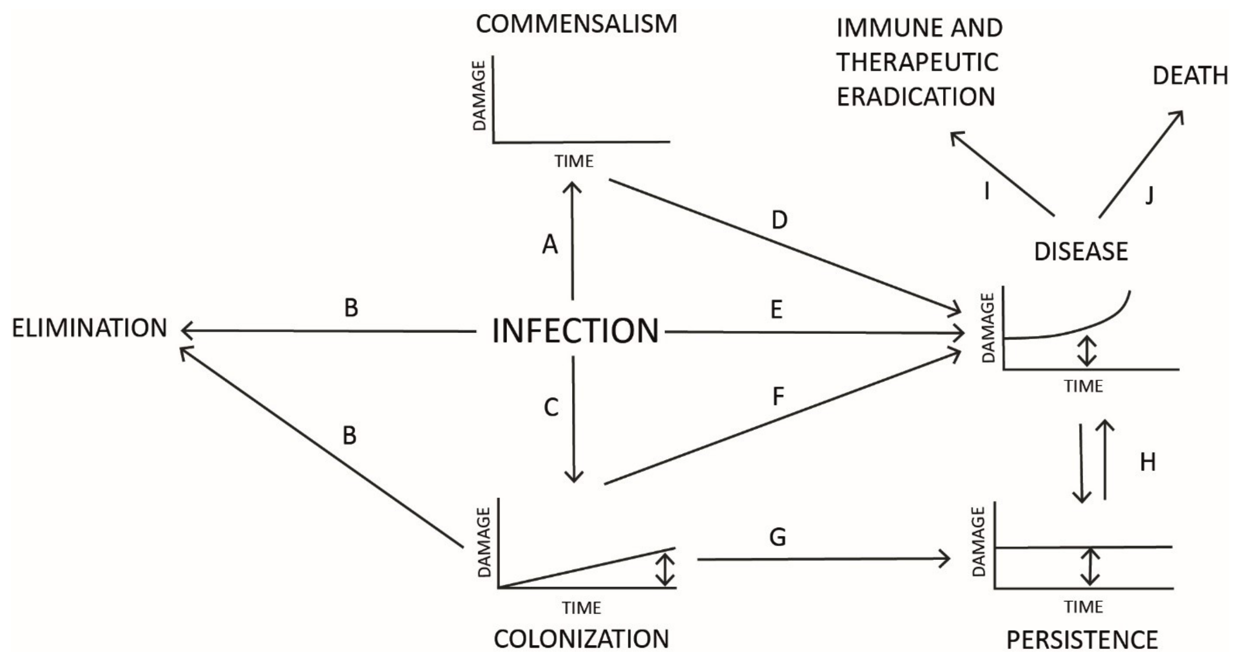


Figure 1. The model of host-pathogen interaction (Casadevall and Pirofski, 2000).
After initial invasion microorganisms have developed several strategies corresponding to disease development and level of the damage to the host. Single-headed arrows indicate: A, commensal state causing no damage to the host; B and C, initial colonization with gradually growing damage with subsequent elimination of infection; D, E and F, disease development as result of impaired immune system, state of colonization or initial pathogen virulence; G, result of colonization and inability of immune the system to eradicate pathogen is chronicity and latency of pathogen persistence; H, persistent infection reactivation leads to disease or disease silencing to persistence development; I and J, major outcomes of infection disease – death as result of irreversible damage to the host or therapeutic eradication of pathogen with reversible or irreversible host organism damage. The various states are shown in the figures of damage/time dependency. Double-headed arrows stand for conditions of variable amounts of damage.

Bacteria pathogenicity can be described based on the parameters of pathogen-induced host damage. Theoretically, overall damage to the host can occur directly via the pathogen or indirectly via pathogen-induced hyperactivation of the host defense mechanism. Infections with weak host immune response and serious infection outcomes usually refer to pathogen-mediated damage, while strong host immune response refers to host-mediated damage. Therefore, the outcome of a particular disease largely depends on host reactivity and/or direct capability of the pathogen to destroy cells or tissue. As a result, many parameters including the structure and functions of the host might be altered (Casadevall and Pirofski, 1999) (Fig. 1).

Taking into account the concepts mentioned above, this study will focus on endothelium-*S. aureus* infections as a particular example of host-pathogen interaction.

1.2. Staphylococcus *aureus*

More than 20 species of *Staphylococcus* are known (Garrity, 2004), but the most prominent human pathogen among them is *Staphylococcus aureus*. First recorded by Rosenbach in 1884, *S. aureus* was described as type of bacteria forming yellow colonies. The taxonomic classification of *S. aureus* places it as part of the genus *Staphylococcus* in the bacterial family *Staphylococcaceae (Micrococcaceae)* (Todar, 2008-2012).

S. aureus are gram-positive spherical cocci approximately 1 µm in diameter that form grape-like clusters. This characteristic growth can be explained by cell division occurring in three perpendicular planes without sister cell detachment (Todar, 2008-2012). The majority of bacterial strains show hemolytic properties on blood agar. In metabolic terms, *S. aureus* are facultative anaerobes, catalase-positive, oxidase-negative bacteria and the majority of documented strains are coagulase-positive.

1.2.1. *S. aureus* is a human pathogen

S. aureus mainly colonizes the nasal-pharyngeal passages of healthy carriers, but it can also be regularly found at other locations including the skin, oral cavity and gastrointestinal tract (Todar, 2008-2012). About 30% of healthy individuals carry *S. aureus* persistently and up to 70% carry it transiently. This high level of transport can be partly explained by *S. aureus'* adherent properties to human mucosa surfaces through a number of surface adhesins that include *can*, *fnbpa*, *fnbpb*, *eap* etc. Variability and redundancy of *S. aureus* adhesins allow attachment to multiple cell types, such as platelets, epithelial and endothelial cells, phagocytes as well as different extracellular matrix proteins (Jefferson, 2009).

Due to broad range of virulence factors including adhesins, *S. aureus* has been reported to cause a spectrum of varying infections ranging from minor skin boils to severe life-threatening diseases such as septic shock or endocarditis (Lowy, 1998). Several host factors have been reported to predispose staphylococcal infection: diabetes mellitus, use of intravenous catheters, drug abuse, AIDS, etc. (Archer, 1998).

1.2.2. Antibiotic resistance of *S. aureus*

Current treatment strategies of *S. aureus* born infections have become less effective as the pathogen has developed antibiotic resistance to broad spectrum of anti-bacterial drugs.

The first report of methicillin-resistant *Staphylococcus aureus* (MRSA) was documented in the early 1960´s in regard to nosocomial infections (Colley *et al.*, 1965). Today, antibiotic tolerant strains are becoming increasingly prevalent and the high speed of methicillin resistance acquisition has become a major problem in many clinical facilities (Witte *et al.*, 1997). Global survey suggested that, among all *S. aureus*–infected patients, MRSA isolates are involved in 46% of cases of lung infection, 38% of urinary tract infections, 30% of bloodstream infections, and 30% of skin/soft tissue infections, respectively (Diekema *et al.*, 2001). Although MRSA was initially described as a minor nosocomial pathogen, community acquired methicillin-resistant *S. aureus* (CA-MRSA) infections are predominant and widespread in modern communities (DeLeo *et al.*, 2010).

MRSA strain multi-drug resistance is acquired by a large transmissible element called staphylococcal cassette chromosome *mec* (SCC*mec*) (Sjostrom *et al.*, 1975; International Working Group on the Classification of Staphylococcal Cassette Chromosome, 2009). The mecA gene complex determines methicillin resistance and is found on the MRSA chromosome. One of these genes functions by encoding a penicillin-binding protein (PBP), PBP2a, whose purpose is to synthesize a cell wall by linking peptidoglycan chains (de Jonge *et al.*, 1992; Goffin and Ghuysen, 1998). Due to acquired PBP2a low affinity to all β-lactams, the protein performs cell wall biosynthesis even in the presence of antibiotics, which are not capable of inactivating cell wall biosynthesis (Brown and Reynolds, 1980; Hartman and Tomasz, 1984).

Another strategy used by *S. aureus* to withstand antibiotic treatment is its ability to form small colony variant (SCV) subpopulations and biofilm (Chuard *et al.*, 1997). The background of SCV physiological changes are mutations in genes involved in the electron transport chain. This results in a reduction of adenosine triphosphate (ATP) levels and a slowing down of bacteria metabolism. It is believed that such alterations influence bacterial intracellular fitness and are responsible for an increased tolerance to antibiotics (Proctor *et al.*, 1998).

Biofilm formation is yet another mechanism used by *S. aureus* to tolerate antibiotic therapy and promote infection (Mah and O'Toole, 2001). The ability of bacteria to attach to

a solid surface of biotic or abiotic origin leads to the formation of biofilm, which represent a microbial community covered with an exopolysaccharide matrix and related proteins. Antibiotic treatments often fails as drugs are not able to diffuse past the polymeric capsule of biofilm to reach the targeted bacteria (Stewart and Costerton, 2001).

Considering the ever increasing number of life-threatening *S. aureus* infectious diseases and the rapid development of multi-drug resistant strains, it is clear that this pathogen is a priority in infectious disease research (Plata *et al.*, 2009).

1.3. *S. aureus* virulence factors and their role in infection process

Each step of the infection process depends on the interplay between *S. aureus* virulence factors and the host immune system (Archer, 1998), which determines whether the infection remains localized or enters the bloodstream to cause a systemic response (Jefferson, 2009). For this reason, it is important to determine which particular virulence factors are expressed by a bacterium. This knowledge would allow for a more accurate prediction of pathogen behavior during the different steps of *S. aureus* infection including host colonization and the formation of a local infection/abscess or subsequent dissemination and/or the development of sepsis with the formation of septic metastases/toxinosis (Archer, 1998).

Different groups of *S. aureus* virulence factors have already been classified based on their respective functions (Table 1). These virulence factors have been shown to contribute to the infection progress and include adhesins anchoring bacteria to the host cell surface, invasins responsible for successful tissue invasion, inhibitors of phagocytosis, exo- and endotoxins and other factors modulating host immune response as well as antibiotic resistance determinants (Zecconi and Scali, 2013).

Table 1. Virulence determinants of *S. aureus*

Virulence factor name	Abbreviation	Main function	Proven effects	References
Microbial surface components recognizing adhesive matrix molecules (MSCRAMMs)				
Fibronectin-binding protein A and B	FnBPA and FnBPB	Binds to fibronectin and fibrinogen	Staphylococcal uptake promotion by non-professional phagocytes. Activation of endothelial cells	Heying, 2007
Clumping factor A	ClfA	Binds to fibrinogen	Inhibition of phagocytosis	Clarke, 2006
Clumping factor B	ClfB	Binds to fibrinogen	Interaction with epithelium, tissue colonization properties	O´Brien, 2002
Collagen-binding adhesin	Cna	Binds to collagen I and IV	Role in septic arthritis development	Patti, 1994
Serin-aspartic acid-rich proteins	Sdr proteins	Binds to ECM	Nasal colonization	Sabat, 2006
S. aureus surface protein	Sas proteins	Binds to ECM	Nasal epithelim binding	Roche 2003
S. aureus protein A	Spa	Binds to Fc chain of immunoglobulins, von Willebrand factor, TNFR1, mammalian gC1q-R	Prevents opsonization and phagocytosis, modulates TNF signaling, role in pneumonia progression	Cedergen, 1993; Gomez, 2004; Hartleib, 2000; Nguyen, 2000
Secreted expanded repertoire adhesive molecules (SERAMs)				
Extracellular adherence protein	Eap	Binds to ECM, ICAM-1, interfare ICAM-1-LFA-1 interaction	Impaired neutrophil and T cell involvement, prevents T cell division, inhibits MAPK signaling	Chavakis, 2002; Haggar, 2004; Athanasopoulos, 2006, Sobke 2006, Lee 2002
Coagulase	Coa	Activates prothrombin	interaction with platelets	Panizzi, 2006; Sawai, 1997
ECM binding protein	Emp	Interacts with ECM	Unknown	Hussain, 2001
Extracellular fibrinogen binding protein	Efb	Binds to fibrinogen, binds to complement factors C3b and C3d	Complement inhibition, inhibition of opsonophagocytosis, blocks platelets aggregation	Lee, 2004; Lee 2004; Hammel, 2007; Shannon, 2005
IsdA		Binds to various receptor including ECM	Adherence to desquamated epithelium	Clarke, 2004

Sbi		Binds to IgG	Binds to IgG and β2 glycoprotein	Zhang, 1998
Enzymes				
Catalase	CatA	Hydrogen peroxide neutralization	Crucial enzyme for survival, persistence and colonization	Cosgrove 2007
Alkylhydroxide reductase	AhpC	Catalase activity	Together with CatA is crucial enzyme for survival, persistence and colonization	Cosgrove 2007
Proteases (V8 protease, aureolysin, staphopain)	Sasp, Aur, ScpA	Tissue invasion and other effects on pathogenesis	Activation-inactivation and modification of various pathogen and host molecules	Sieprawska-Lupa, 2004; Shaw, 2004
Fatty acid-modifying enzyme	FAME	Fatty acid modification	Inactivation of bactericidal fatty acids	Kapral, 1992
Staphylokinase	Sak	Plasminogen activation	Anti-defensin, inhibition of complement	Jin 2004
Toxins				
α-toxin	Hla	Pore formation activity	Induction of inflammatory response	Fournier, 2005
β-Hemolysin	Hlb	Lysis of cytokine-producing cells	Cytotoxicity	Dinges, 2000
δ-hemolysin	Hld	Neutrophil/monocyte binding	TNF-alpha induction, chemoattraction	Somerville, 2003
γ-hemolysin; Panton-Valentine leukocidin; leukocidins D, E, M	Hlg, PVl, LukD, LukE, LukM	γ-hemolysin causes hemolysis, PVL activates and lyses neutrophil/monocyte	Role in necrotizing pneumonia due to influence on expression and representation of staphylococcal proteins	Labandeira-Rey,2007
Superantigen toxins (staphylococcal enterotoxin, TSS)	SE	Food poison, toxic shock syndrome	T cell activation, facilitation of MHC-II-TCR interaction	Thomas, 2007
Formylpeptides	fMLPs	binds to formyl peptide receptor (FPR)	Chemoattractants expression	Le, 2002
Anti-inflammatory ligands				
Chemotaxis inhibitory protein of *S.aureus*	CHIPS	Binds to C5aR and FPR	Inhibition of chemotaxis	de Haas 2004, Postma 2004, Haas, 2004
FPR-like 1 inhibitory protein	FLIPr	Binds to FPR-like 1 receptor	Inhibition of chemotaxis	Prat, 2006
Staphylococcal complement inhibitor	SCIN	Stabilizes C2a-C4b and Bb-C3b enzymes	Inhibition of complement	Roojakkers, 2005

Cell wall factors				
Teichoic acids	LTA, WTA	Binds to Toll-like receptor 2	Activation of cytokines and chemokines expression	Fedtke, 2004

Table 1. Selected major virulence factors of *S. aureus* their functions and role in pathogenicity (Chavakis *et al.*, 2007).

S. aureus adhesins facilitate adherence of the pathogen to the host cell membrane at the initial stage of infection. They are usually divided into two groups: a microbial surface component recognizing adhesive matrix molecules (MSCRAMMs) (Foster *et al.*, 2014) and secretable expanded repertoire adhesive molecules (SERAMs) (Sinha and Herrmann, 2005). The first group includes fibronectin binding proteins A and B (*fnbpa*, *fnbpb*) as well as clump factors A and B (*clfa*, *clfb*) which are anchored into the bacterial cell wall and mediate pathogen attachment through binding extracellular matrix proteins such as fibrinogen, fibronectin and collagen (Heying *et al.*, 2007; Clarke and Foster, 2006). The second group of adhesins including *eap* and *emp* proteins belong to a group of secreted adhesion molecules. These facilitate pathogen adhesion only after the initial binding to the respective ligand of the host origin such as ICAM-1 or extracellular matrix proteins. Additionally, this group of adhesins has been shown to be involved in the inhibition of leukocyte adhesion and recruitment to the endothelial surface (Haggar *et al.*, 2004).

Another group of virulence factors is the invasins. This group consists of heterogeneous virulence factors that include different proteolytic enzymes such as lipases (Cadieux *et al.*, 2014), proteases (Kolar *et al.*, 2013) and nucleases (Berends *et al.*, 2010) or hyaluronidase with the function of invading surrounding tissues and protecting the pathogen from the host's immune defense (Lowy, 1998).

S. aureus also expresses a broad spectrum of toxins such as alpha-toxin, sigma-toxin and Panton-Valentine leucocidin, which influence phagocytic activity, the migration of leukocytes and cause affected cells to be induced to commit cell death (Chavakis *et al.*, 2007). It is postulated that *S. aureus* virulence factors such as *spa*, *clfa*, *clfb* and staphylokinase (*sak*) are also involved in evading the immune system by protecting bacteria from host immunoglobulin opsonization and compliment system (Rooijakkers *et al.*, 2005).

Various effects and functions of bacterial virulence factors have been studied using wild-type and mutants forms of pathogen in *in vitro* models [typically different cell cultures] and

in vivo models [murine models of septic arthritis] (Palmqvist *et al.*, 2002) or systemic infection models (Kropec *et al.*, 2005) etc.

In clinical study of *S. aureus,* isolating virulence factor expression, revealed doubts that a single virulence factor predominantly defines the success and severity of infection (Peacock *et al.*, 2002). Moreover, the deletion of single or multiple virulence factors could not guarantee a decrease in the severity of infection or eliminate the pathogen's ability to cause infection (Jonsson *et al.*, 1985; Patti *et al.*, 1994). Such observations can be partly explained by the broad spectrum of *S. aureus* virulence determinants, which often have similar functions and are capable of substitution during infection (Arvidson and Tegmark, 2001).

It is difficult to trace the function of a specific staphylococcal virulence factor during disease development (Fournier and Philpott, 2005; Foster *et al.*, 2014), although correlations between isolates´ virulence and certain diseases have been documented (Nilsson *et al.*, 1999). For example, the importance of collagen binding factor (*cna*) was demonstrated in a study using *S. aureus* mutants in a septic arthritis murine model. The *S. aureus cna* mutants had lower adhesion rates to cartilage and induced lower immune response (Patti *et al.*, 1994). In the murine animal model, Protein A (*spa*) virulence factor was shown to propagate arthritis development accompanied by an increased mortality rate when compared with the bacteria deletion *spa* mutants (Palmqvist *et al.*, 2002).

Although the pathogen has developed a wide array of virulence factors, the host also has a set of defense counter measures, which are discussed in the next section.

1.4. The innate immune system response to *S. aureus* infections

For a host organism without a functional immune system, any infectious disease has the potential to be life-threatening. Innate and acquired immune system responses are two different mechanisms of host defense (Luster, 2002). An innate immune response is the non-specific "quick" answer by an organism to any kind of invading pathogen or antigen (Tosi, 2005).

Three important defense components of the innate immune system against *S. aureus* infection are antimicrobial peptides, compliment system and phagocytes (Rooijakkers *et al.*, 2005). The phagocytes include diverse populations of mast cells, dendritic cells,

macrophages, granulocytes, NK cells, T cells and non-professional phagocytes usually presenting as epithelial and endothelial cells (Basset *et al.*, 2003).
The cellular defense response starts with the initial recognition of antigen signals by specific receptors (Fig. 2) of immune competent cells of first line of defense – the skin and mucosal epithelium or endothelium, which respond by starting the production of chemoattractants to recruit immune competent cells to the site of infection (Philpott *et al.*, 2001).

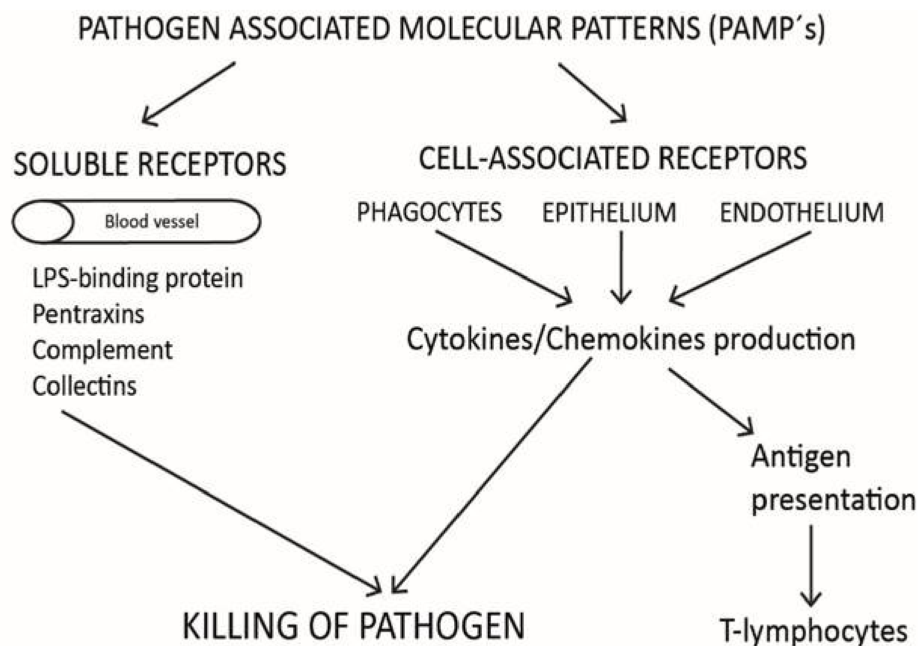


Figure 2. Pattern recognition receptors and their role in innate and adaptive immunity (Basset *et al.*, 2003).

Recent studies suggested the important role of the endothelium in primary immune response to *S. aureus*. Thus, the role of endothelium can be viewed as initial detection of pathogen invasion, activation of pro-inflammatory phenotype and providing signals to professional phagocytes such as macrophages and/or neutrophils, which, in turn, responsible in ensnaring and killing the pathogen (Harding and Kubes, 2012; Rooijakkers *et al.*, 2005).

1.4.1. Endothelium in the innate immunity

With regard to *S. aureus*-induced immune response, it is necessary to underline the important role of the endothelium. This type of tissue is an important component of the host immune system and plays a crucial role in innate and acquired immunity. The endothelium forms the inner surface of blood and lymphatic vessels. Some calculatory approaches suggest a total surface area in the range of four to seven thousand square meters including about 10^{13} endothelial cells in the average human adult (Lemichez *et al.*, 2010).

The barrier function of endothelium plays a central role in regulating vascular homeostasis, angiogenesis, permeability, solute exchange, vascular tone, coagulation/anticoagulation cascade and inflammation (Michiels, 2003). Endothelial cells react to environment changes by switching off their anti-thrombotic, anti-inflammatory phenotype to allow for a vasodilated state with the initiation of inflammation and coagulation events (Dandona, 2002).

Endothelial cells have been shown to detect various pathogen associated molecular patterns. This ability classifies endothelium as part of the defense mechanism by which innate immunity detect and eradicate potential invaders (Harding and Kubes, 2012).

Upon sensing the pathogen via pattern recognition receptors, endothelial cells transition into an activated state. The resulting endothelium pro-inflammatory activation involves the expression of surface adhesion molecules and the production of chemoattractants and cytokines to initiate inflammation (Szmitko *et al.*, 2003).

Inflammatory response of endothelium is a combination of modifications including vasodilation, endothelial cells pro-inflammatory response and barrier function dysregulation. These changes make endothelium inflammatory response one of the key regulators of the interactions between blood leukocytes and the site of infection, autoimmune process or tumor development (Valbuena and Walker, 2006).

Inflamed endothelium attracts leukocytes by secreting cytokines and chemokines and interacts with immune cells via overexpressed adhesion molecules such as E-selectin, P-selectin and ICAM-1. After adherence, leukocytes cross the endothelium barrier and extravasate to the locus of inflammation (Carlos and Harlan, 1994).

In this way, it would be correct to consider endothelium as an important immunological organ whose response is vital during the different stages of infection: from pro-inflammatory activation to eradication of intracellular pathogens (Wood, 2006).

1.4.2. Endothelium – *S. aureus* infection

In most cases, *S. aureus* can enter the blood through damaged skin or by translocation through endothelium from the initial site of infection (Edwards and Massey, 2011). Once a pathogen has access to the bloodstream it can cause the formation of multiple abscesses in distant organs as well as over-activating the immune response, partially due to endothelium inflammation. Blood infections can result in septic shock and are known to lead to multi-organ failure with potentially fatal outcomes (Archer, 1998).

The interactions of *S. aureus* with human endothelial cells has a big impact on the infection process in a number of human diseases (Table 2) including endocarditis, the formation of metastases, sepsis development and other clinical complications (Lowy, 1998).

Furuncle or carbuncle
Surgical wound infection
Botryomycosis
Hospital-asquired bacteremia
Acute or right-sided endocarditis
Hematogenous osteomyelitis
Septic arthritis
Epidural abscess
Brain abscess
Hospital-asquired pneumonia
Empyema
Septic shock
Toxic shock syndrome
Scalded skin syndrome
Food-borne gastroenteritis

Table 2. Infection diseases and syndromes where *S. aureus* is the primary pathogen. Infections where *S. aureus* and endothelium interactions are playing a crucial role in pathogenesis are marked in bold (Archer, 1998).

A typical response of infected endothelium, at tissue level, includes increased permeability, pro-inflammatory response (Schouten *et al.*, 2008) and, is in some cases, accompanied endothelium-mediated bacteria translocation through the endothelial monolayer (Aird, 2003).

Loss of barrier function by endothelium is one of the hallmarks of inflammation development, leading to serious tissue damage and increasing the severity of infection (Aird, 2003).

It has been shown that abundant *S. aureus* alpha-toxin, due to its pore-forming activity, is able to lead to the loss of cell to cell contacts and dramatically increases endothelial permeability. The mechanism of junction disintegration during this process can be partly explained by actin cytoskeleton contraction due to Ca^2+ influx through cellular alpha-toxin-built-pores (Hocke *et al.*, 2006). Additionally, it was shown that alpha-toxin interacts with its receptor A-disintegrin leading to increased metalloprotease activity and the disruption of cadherin adherent junctions (Powers *et al.*, 2012). Staphylococcal exotoxin B (SEB) also acts in intracellular gap formation and barrier failure via protein tyrosine kinase (PTK) phosphorylation (Campbell *et al.*, 1997).

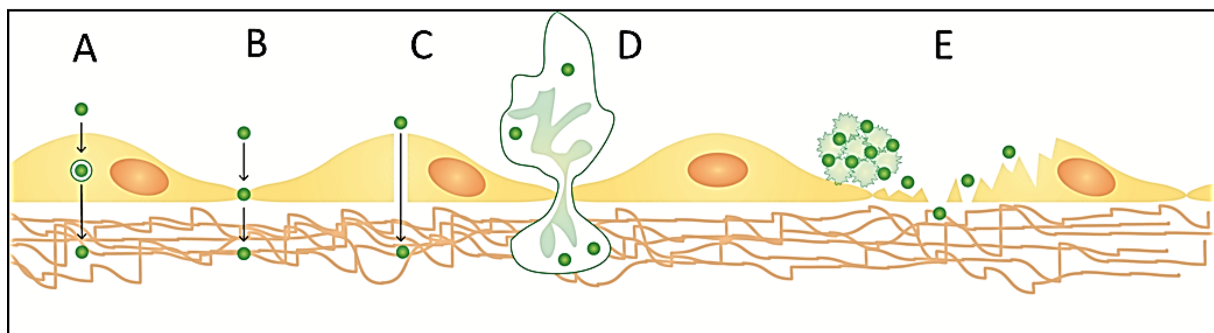
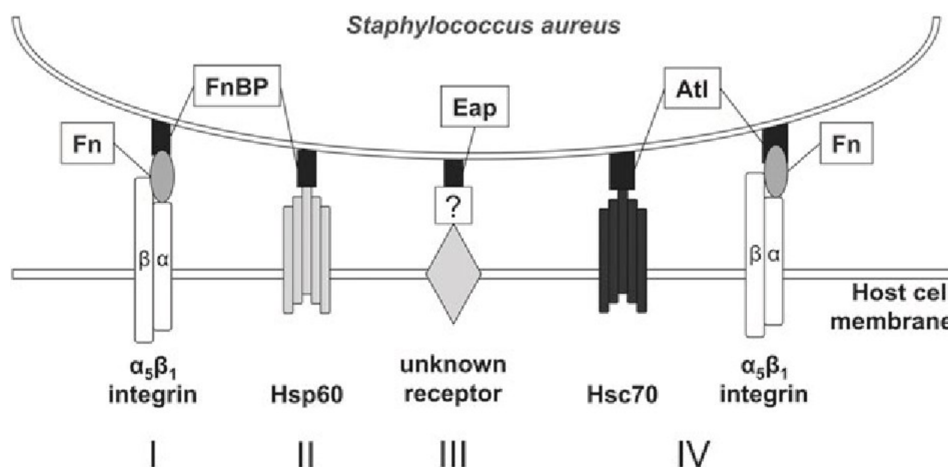


Figure 3. Possible mechanisms of *S. aureus* translocation through endothelium barrier. A. Transcytosis, B. Paracytosis, C. Macroaperture formation mediated by EDIN toxin, D. Phagocytotic transport, E. Endothelium damage and thrombus formation (Modified according to (Edwards and Massey, 2011)).

In order to reach the bloodstream, a bacterium has to cross the endothelial cell barrier (Fig. 3). *S. aureus'* ability to colonize and translocate through endothelium is perfectly suited to facilitate bypassing this obstacle and the mechanism used also allows less well adapted intracellular pathogens to similarly disseminate and escape from local immune response (Lemichez *et al.*, 2010)

It is reported that bacteria can exploit several different mechanisms to cross the endothelial barrier. One such tactic is transcytosis; a process by which pathogens are able to pass the endothelial barrier through inner cellular compartments that allow traffic in both directions. It is currently unknown whether *S. aureus* can translocate through the endothelial monolayer by transcytosis (Edwards and Massey, 2011).

Paracytotic pass and cross along junction openings may occur during *S. aureus* infection as toxins produced and endothelial oxidative stress as well as cytokine release can cause junctional gaps to form (Scriba *et al.*, 2008; Jonkam *et al.*, 2009). In the case of septic endocarditis, *S. aureus* causes endothelial damage and thrombus formation which is used as a gateway for pathogen translocation and subsequent dissemination (Mylonakis and Calderwood, 2001). There is evidence that staphylococcus can cross the endothelium by concealing itself inside infected professional phagocytes where it can survive for significant periods of time (Kubica *et al.*, 2008). Another possible mechanism used to reach the bloodstream involves the formation of macroapertures, large channels in the cellular membrane that allow bacteria to cross the endothelial barrier. Macroaperture formation has been reported to be mediated by *S. aureus* toxins named epidermal cell differentiation inhibitors (EDINs), which modulate the actin cytoskeleton by interacting with host cell Rho GTPases (Boyer *et al.*, 2006; Edwards and Massey, 2011).



Fugure 4. Mechanisms involved in *S. aureus* internalization by non-professional phagocytes including endothelium. (I) The major mechanism for *S. aureus* internalization by non-professional phagocytes is determined by FnBPs interaction with α5β1 integrins via Fn. (II) FnBPs also bind to human Hsp60 thereby contributing to *S. aureus* internalization. Hsp60 may function as a co-receptor for a5b1 integrins. (III) The *S. aureus* extracellular adherence protein Eap has been shown to play a role in *S. aureus* internalization. A host cell receptor for Eap is not known. (IV) Autolysin/adhesins Atl from *S. aureus* also promotes staphylococcal internalization by host cells. As the host cellular receptor, the heat shock cognate protein Hsc70 was identified (Hirschhausen *et al.*, 2010).

Endothelial tissue shows specific reactions during *S. aureus* infection at the cellular level. The first step of host-pathogen interactions begin with bacteria adhering to the cell surface (Fig. 4). The mechanism of adhesion includes bacteria interacting with adhesins and their counterparts at the endothelial surface. Bacterial fibronectin binding proteins (FnBPs) attach to the cell surface by direct adhesion of FnBP to heat shock protein 60 (Hsp 60) (Dziewanowska *et al.*, 2000) or through a fibronectin molecular bridge that connects FnBP with integrin α5β1 (Sinha *et al.*, 1999). One more potential player is autolysin/adhesion factor Atl. It has been shown, on endothelial cell line Ea.hy 926, that Atl is responsible for adhering the heat shock cognate protein Hsc70 on the cell surface and promotes the internalization of pathogens (Hirschhausen *et al.*, 2010). Other bacterial adhesins have also been shown to play a role in the adhesion to endothelial cells such as *can* (collagen adhesion), *clfa* and *clfb* (fibrinogen adhesion) and *eap* (plasma proteins adhesion). However, the specific receptors on the endothelial cell surface exploited by these factors are largely unknown (Sinha and Herrmann, 2005).

Following adhesion, integrins function as endocytotic receptors and promote complex signaling to stimulate bacteria uptake. Integrin-mediated uptake of *S. aureus* has been demonstrated to be co-localized by a number of integrin-associated proteins: vinculin, paxillin, zyxin, tensin, focal adhesion kinase (FAK) and Src kinase (Hoffmann *et al.*, 2011). These proteins´ interactions result in the reorganization of actin and the internalization of the pathogen (Agerer *et al.*, 2005).

The intracellular fate of *S. aureus* in the inner compartment of endothelial cells depends on bacteria virulence determinants. After phagocytosis bacteria normally stay in phagosome vesicles that are acidified after fusing with lysosomes. However, internalized bacteria have developed different strategies to avoid cellular defense mechanisms and resist low pH conditions (Sinha and Fraunholz, 2010). The first such strategy is to form small colony variants and persist inside infected cells without provoking a defense reaction (Garzoni and Kelley, 2009). A second strategy involves preventing phago-lysosomal fusion and/or disrupting the surrounding phagosomal membrane to enter into the cytoplasm. It has been shown that phagosomal escape of *S. aureus* in endothelial cells depends on several staphylococcal virulence factors including δ-toxin and sphingomyelinase β-toxin (Giese *et al.*, 2011). There is currently no data surrounding intracellular replication of *S. aureus* in primary endothelium, although bacterium has been shown to proliferate in cytoplasmic

milieu of professional and non-professional phagocyte cell lines upon phagosomal escape (Grosz *et al.*, 2014). In addition, *S. aureus* has been shown to invade and persist in the intracellular environment, but rapid intracellular growth was not observed in human osteoblasts (Ellington *et al.*, 2001) or epithelium (Kahl *et al.*, 2000).

Endothelial cells are able to 'sense' the presence of *S. aureus* once bacteria ligands have bound to a certain set of the receptors of the innate immune system (Opitz *et al.*, 2009). Bacterial cell wall components or genome degradation products have been demonstrated to induce endothelial expression of Toll-like receptors 2 and 4 (Faure *et al.*, 2001), intracellular receptor NOD2 (Davey *et al.*, 2006; Oh *et al.*, 2005), RIG-I receptor and TNFR1 (Imaizumi *et al.*, 2002).

Pro-inflammatory endothelial response is a fast reaction in response to sensing a staphylococcus infection. Inflammation of endothelial cells can be activated by a number of *S. aureus* determinants such as alpha-toxin (Pruefer *et al.*, 2002), fibronectin binding protein A (*fnbpa*) and components of polysaccharide capsule and cell wall including muramyldipeptide (MDP) and lipoteichoic acid (LA) (Heying *et al.*, 2007). As a result of activation, endothelial cells release a number of chemokines and cytokines and upregulate adhesion molecules VCAM-1, ICAM-1 and E-selectin (Tessier *et al.*, 1998). The cytokines attract and the adhesion molecules mediate the binding of leukocytes to the endothelium (Beekhuizen *et al.*, 1997).

Initial receptor activation leads to phosphorylation events in the MAPK signaling cascade, a complex process of interplay between kinases which subsequently results in the activation of nuclear factor NF-kB and other transcription factors such as STAT1, AP-1 etc. (Aird, 2003). Respective upregulation of genes contributing to endothelial immune response upon *S. aureus* invasion has demonstrated an emphasized expression of molecules from all six cytokine families such as interleukins, cytotoxic cytokines, colony-stimulating factors, interferons, growth factors and chemokines (Fournier and Philpott, 2005).

During infection, host cells can be seriously damaged and subsequently be induced into apoptosis. This process has been shown to involve death receptor signaling, stress stimuli and damage by intracellular bacterial replication (Kahl *et al.*, 2000) or bacterial toxins (Dinges *et al.*, 2000). The process of endothelium cell death plays an important role in amplifying the immune response and eliminating damaged/mutated cells from affected tissue (Thompson, 1995).

Currently, little is known about the mechanism of inducing cell death in endothelium upon *S. aureus* infection. It is suggested that for host cell death to occur in response to infection, *S. aureus* has to be taken up into the cell and must remain viable once inside, as demonstrated by using UV-inactivated bacteria and phagocytosis inhibitors (Menzies and Kourteva, 1998). Other studies have reported that pathogen-induced endothelial cell death requires invasiveness and haemolytic features by which agr and sigB regulators need to be functional (Haslinger-Loffler *et al.*, 2005a). Staphylococcal pyrogenic toxin superantigens (PTSAgs) such as TSST-1 have also been reported to be cytotoxic for human pulmonary artery endothelium (Lee *et al.*, 1991) as well as staphylococcal enterotoxin B (SEB) for porcine endothelial cells (Campbell *et al.*, 1997).

Esen and colleagues showed the importance of JNK phosphorylation, releasing cytochrome-C, activating caspases 3 and 8 and the role of acidic sphingomyelinase during *S. aureus*-mediated endothelium cell death (Esen *et al.*, 2001). Furthermore, inflammasome NLRP3 seems to be one of the factors that contribute significantly to *S. aureus*-induced cell death in endothelium (Yin *et al.*, 2009) and endothelium activation (Xiang *et al.*, 2011; Xiao *et al.*, 2013). However, in vice versa, *S. aureus* infection can lead to NLRP3 activation (Sinha and Fraunholz, 2010), which likely occurs through mitochondrion toxin-mediated release of cathepsin (Munoz-Planillo *et al.*, 2009).

While the endothelium quickly responds to staphylococcus infection in a variety of ways including helping activate an immune response to combat the pathogen, the condition of the host immune system prior to infection is of crucial importance. Nevertheless, bacteria also have variety of mechanisms allowing them to evade the immune system and induce the development disease regardless of the physical condition of the host.

1.5. Different strains of *S. aureus* cause diverse responses in endothelium

The infectious capacity of a particular *S. aureus* strain is mostly related to the genetic modulation of the bacterial genome, a process which can result in a type of adaptation for sufficient survival, colonization and growth within a host.

Strain-specific host responses are determined by differences in the expression profile of virulence factors by particular *S. aureus* strain. A single mutation has the potential to alter any stage of pathogenesis even going so far as to change the entire scenario of infection.

One of the factors crucial for S. aureus virulence is functional *agr* global regulator. Specifically, it was shown that, during infection, isolated *S. aureus* strains undergo mutations in the agr regulatory system (Shopsin *et al.*, 2008). This agr system is one of the determinants that contribute to pathogen virulence (Traber *et al.*, 2008) and an agr deficiency has been shown to support intracellular bacterial persistence and escape from host cell inflammatory response (Matussek *et al.*, 2005). Similar effects are shown by small-colony variants (SCV) of *S. aureus*. SCV upregulate Crp/Fnr genes involved in arginine-deiminase pathway (AD) due to a mutation of the hemB gene. This pathway has also been shown to play a role in bacteria intracellular persistence. The ATP produced in the AD pathway protects SCV strains from acidification. Additionally, small-colony variants have lower virulence factor production and a subsequent weaker immune response from the host which results in a very effective strategy for long-term persistence in host cells (Seggewiss *et al.*, 2006).

Using a murine renal abscess model, it was demonstrated that *S. aureus* virulence factors have distinctive functions during different stages of infection. In the experiment, mice were infected with several *S. aureus* mutants. Effects of virulence factors in dissemination, abscess formation, abscess maturation and secondary metastase development were studied. It was shown that following intravasal injection, *S. aureus* was cleared from the blood within 6 hours. Since bacteria were found in peripheral tissues/organs, a trans-endothelial/trans-epithelial transport mechanism is assumed to take place, although this was not directly addressed in the study. Following 5 days post-infection, *S. aureus* clumping factor A and B (ClfA and ClfB) mutants demonstrated decreased survival with murine blood showing impaired bacterial loads and abscess formation. Mutants of iron uptake genes sdrD, isdA and B showed a decrease in bacterial loads and reduced abscess formation. Extracellular adhesion protein (Eap), envelope-associated protein (Emp) and staphylococcal protein A (spA) virulence factors were demonstrated to be involved in abscess maturation, bacterial dissemination after abscess rupture and *de novo* abscesses formation (Cheng *et al.*, 2009). Differences in infectious dynamics discovered in the mice model could be partly the consequence of the different endothelial responses to the different *S. aureus* mutants.

Even at very early points in time strain-specific *S. aureus* infection can have diverse effects on the inflammatory reactions in endothelium. These includes changing the paracellular endothelial barrier function and the expression of cell adhesion molecule-1s including ICAM-1. Furthermore, some strains are known to have the ability to translocate through the endothelium (Kramko *et al.*, 2013).

These findings were uncovered in an experiment where endothelial cells were infected with 22 different clinical isolates of *S. aureus* as well as two well-characterized lab strains causing diverse and strain-specific changes in para- and transcellular endothelial barriers (Kramko *et al.*, 2013).

Endothelial cells respond to *S. aureus* infection by upregulating pro-inflammatory molecules at the gene expression level. Heterogeneous endothelial expression response to *S. aureus* was demonstrated using 18 clinical isolates. In particular, pro-inflammatory cytokines such as IL-6, IL-8, GM-CSF, GRO-α, RANTES (Strindhall et al., 2002) and the adhesion molecules E-selectin and ICAM-1 (Strindhall et al., 2005) were diversely expressed upon strain-specific infection. Furthermore, genes involved in cell growth, apoptosis and cellular interactions (Matussek *et al.*, 2005) were also differentially expressed. In another study where endothelial cells (HUVEC) were infected by septic or carriage isolates, similar responses for both groups of isolates were observed. Interestingly, gene expression variations were not higher between the groups of different origin when compared to the variations of gene expression within the same group (Stark *et al.*, 2009). This result is further supported with the fact that there is a certain common gene pool in endothelial cells containing genes influenced by all *S. aureus* strains, likely by non-specific virulence determinants. Naturally, specific genes also exist and are only activated in the endothelium in the presence of a specific strain (Grundmeier *et al.*, 2010).

In conclusion, it is clear that the interaction between endothelium and *S. aureus* is a multifactorial process of a great importance in facilitating normal innate and acquired immune response to staphylococcal infection. The role of endothelial cells as a natural barrier between different inner environmental conditions further supports its function and importance in homeostasis and mediating infection outcomes.

1.6. Aim of the study

Although it is clear that different strains of *S. aureus* cause different endothelial responses, the specific molecular mechanisms involved in these diverse reactions is not clearly understood. The main goal of this study is to enrich the existing knowledge concerning distinct endothelial responses to strain-specific *S. aureus* infections using an endothelial cell culture model and bacterial clinical isolates.

Our goals are:

- To understand the background of the strain-specific inflammatory response of infected HUVEC
- To study strain-specific HUVEC gene expression upon infection with *S. aureus* strains with distinctive virulence expression profiles
- To study strain-specific HUVEC cell death induction
- To determine the central mechanism involved in HUVEC cell death upon infection with *S. aureus*

2. Materials and methods

2.1. Materials

2.1.1. Reagents and materials

Name of the reagent	***Manufacturer***
Accutase	Merck (Darmstadt, Germany)
Agar-B	Difco Laboratories (Becton Dickinson, Heidelberg, Germany)
Bovine serum albumine (BSA)	GE healthcare (Buckinghamshire, UK)
Calcium chloride	Merck (Darmstadt, Germany)
Endothelial growth factor (Cattle eyes)	German slaughterhouses
DMSO	Sigma (Deisenhofen, Germany)
Ethanol (absolute)	J.T.Baker (Deventer, Netherlands)
Gentamicin sulfate salt	PAA (Pasching, Austria)
Glass coverslips, D=15 mm	Gerhard Menzel GmbH (Braunschweig, Germany)
Glass slides (76x26 mm)	Gerhard Menzel GmbH (Braunschweig, Germany)
Glass counting chamber	Sigma (Deisenhofen, Germany)
Glutaraldehyde solution 25%	Sigma (Deisenhofen, Germany)
Glycerol	Calbiochem (Bad Soden, Germany)
Glycine	Applichem (Darmstadt, Germany)
HCl (37%)	Applichem (Darmstadt, Germany)
HEPES	PAA Laboratories (Cölbe, Germany)
Lysostaphin	Sigma (Deisenhofen, Germany)
ß- Mercapthoethanol	Sigma (Deisenhofen, Germany)
Mounting medium	Agilent Technologies (Santa Clara, United States)
Paraformaldehyd (PFA)	Merck (Darmstadt, Germany)
PCR-film	Eppendorf (Homberg, Germany)
PCR plates (96-well, colorless)	Eppendorf (Homberg, Germany)

PBS Ca^{2+}-, Mg^{2+}-	Gibico (Carlsbad, USA)
Sodium chloride	Merck (Darmstadt, Germany)
KAPA2G Fast Readymix with Dye	PeQlab (Erlangen, Germany)
Tryptic Soy Broth (TSB)	Sigma (Deisenhofen, Germany)
Tumor necrosis factor α (TNF-α)	Life Technologies (Darmstadt, Germany)
Yeast extract	Difco Laboratories (Heidelberg, Germany)

2.1.2. Equipment

Equipment	***Manufacturer***
Autoclave (Systec DX-65)	Systec (Linden, Germany)
Shaking Incubator (GFL 3031)	Heraeus instrument (Hanau, Germany)
Safety Cabinet (safe 2020)	Thermo Fisher Scientific (Waltham, USA)
Automated cell counter (TC20)	BioRad Laboratories (München, Germany)
Centrifuges (5804R, 5415R, 5415D, 5424R, 5430R and 5810R)	Eppendorf (Homberg, Germany)
CO_2-incubator (Heracell ™240i)	Thermo Fisher Scientific (Waltham, USA)
Inverted confocal microscope (LSM780 ELYRA PS.1)	Carl Zeiss (Jena, Germany)
Electronic scales 1474 (max. 1600.0g)	Sartorius (Göttingen , Germany)
Freezer (-80°C)	Sanyo (Moriguchi, Germany)
Freezer (-20°C)	Sanyo (Moriguchi, Germany)
Fridges with freezers (-20°C)	Liebherr (Bulle, Switzerland)
Heating/drying oven (Model 100-800)	Memmert (Schwabach, Germany)
HiScan-SQ array scanner	Illumina (San Diego, California, USA)
Ice Flaker AF103	Scotsman (Illinois, USA)
Inverted Microscope (Axio observer Z1)	Carl Zeiss (Jena, Germany)
Inverted Microscope (Axio Vert.A1)	Carl Zeiss (Jena, Germany)

Mastercycler™ ep realplex Real-time PCR System	Eppendorf (Homberg, Germany)
Mastercycler™ flexlid nexus gradient	Eppendorf (Homberg, Germany)
Cube 6, flow cytometer	Sysmex Partec (Görlitz, Germany)
pH-meter (PB-11)	Sartorius (Goettingen, Germany)
UV/visible nanophotometer	IMPLEN (München, Germany)
Vortex mixer –MS3	IKA (Staufen, Germany)
Micro-centrifuge, Sprout™	Kisker Biotech (Steinfurt, Germany)
Water bath	GFL (Burgwedelf, Germany)

2.1.3. Solutions, buffers, medium.

All buffers, solutions and medium were prepared using sterile filtered ddH_2O unless otherwise indicated.

Standard TSB medium for bacteria cultivation (1L):

Tryptic Soy Broth	30 g

30 g of tryptic soy broth was dissolved in ddH_2O water and autoclaved.

TSB agar plates (1L):

Tryptic Soy Broth	30 g
Agar	15 g

30 g of tryptic soy broth and 15 g of agar-b was dissolved in water and autoclaved. After autoclaving, solution was distributed into plastic Petri dishes (Sarstedt, Nümbrecht, Germany)

Binding buffer for anti-annexin V antibodies staining (10x):

Water solution of:

HEPES	0.1 M
Sodium Chloride	1.4 M
$CaCl_2$	25 mM

When using 10x solution was diluted to 1x and pH was adjusted (recommended pH is 7.2).
2% Paraformaldehyde (PFA) for cells fixation:

2% PFA was prepared in PBS and dissolved at 60^oC. Add 1M of NaOH until the solution clears. The pH was adjusted to 7.2. Prepared solution was aliquoted and frozen at -20^oC.

2 mM glycine:

Glycine was prepared in PBS in concentration 2 mM.

1% BSA:

1% BSA was prepared in PBS and stored at +4^oC.

Gentamycin solution:

10 mg/ml gentamycin sulfate salt in ddH_2O.

2.1.4. Commercial reagent kits

Name	***Manufacturer***
RNeasy Mini Kit (50)	Qiagen (Venlo, The Netherlands)
Illumina TotalPrep RNA Amplification Kit	Life Technologies (Darmstadt, Germany)
HumanHT-12 v4 Expression BeadChip Kit	Illumina (San Diego, California, USA)
HumanHT-12 v4 Expression BeadChip	Illumina (San Diego, California, USA)
Reverse Transcription Core Kit	Eurogentec (Liege, Belgium)
Nuc-View 488 Caspase-3 assay kit for live cells	Biotium (Hayward, CA, USA)

2.1.5. Antibodies/reagents

Name of antibody/reagent	***Conjugate***	***Manufacturer***	***Application****
Mouse anti-human CD54 (clone 1H4)	FITC	Immunotools (Friesoythe, Germany)	FC
Mouse IgG2b isotype control	FITC	Immunotools (Friesoythe, Germany)	FC
Recombinant chicken Annexin V	FITC	Immunotools (Friesoythe, Germany)	FC
Propidium Iodide	-	Sigma (Deisenhofen, Germany)	FC
Phalloidin	TRITC	Sigma (Deisenhofen, Germany)	IF
NQDI-1	-	Axon Medchem (Groningen, The Netherlands)	IA
SB203580	-	Life Technologies (Darmstadt, Germany)	IA
PD98059	-	Life Technologies (Darmstadt, Germany)	IA
Wortmannin	-	Merck (Darmstadt, Germany)	IA
Bisindolylmaleimide I	-	SantaCruz (Dallas, Texas, USA)	IA
SP600125	-	SantaCruz (Dallas, Texas, USA)	IA

*FC – flow cytometry, IF – immunofluorescence, IA – inhibition assay

2.1.6. Bacteria strains

Strain	***Characteristic***	***Reference***
S. carnosus TM300	Non-pathogenic *Staphylococcus*	(Schleifer and Fischer, 1982)
S. aureus K9976	Sepsis isolate (blood), MSSA, hemolysis 4	(Kramko *et al.*, 2013)
S. aureus K3010	Sepsis isolate (blood), MRSA, hemolysis 3	(Kramko *et al.*, 2013)
S. aureus 6850	Derivative from sepsis with metastatic bone infection, MSSA, hemolysis 3	(Balwit *et al.*, 1994)
S. aureus K9657/04	Sepsis isolate (blood), MSSA, hemolysis 0-1	(Grundmeier *et al.*, 2010)
S. aureus K70058396	Sepsis isolate (blood), MRSA, hemolysis 3-4	(Kramko *et al.*, 2013)
S. aureus 8325-4	Derivative of conjunctivitis isolate NCTC 8325, MSSA, hemolysis 2	(Kramko *et al.*, 2013)
S. aureus K10485	Sepsis isolate (blood), MRSA, hemolysis 0-1	(Kramko *et al.*, 2013)
S. aureus K528	Sepsis isolate (blood), MSSA, hemolysis 3	(Kramko *et al.*, 2013)
S. aureus K1801/10	Sepsis isolate (blood), MRSA, hemolysis 2	(Kramko *et al.*, 2013)
S. aureus K2900/10	Sepsis isolate (blood), MRSA, hemolysis 0-1	(Kramko *et al.*, 2013)

2.1.7. Cell culture reagents

Chemicals and materials used during cell culture manipulation.

Name	***Manufacturer***
M199 medium	Sigma (Deisenhofen, Germany)
0.2% collagenase A solution	Roche (Mannheim, Germany)
0.05% trypsin-ethylene diamine tetraacetic acid (EDTA)	Merck Millipore, Billerica, USA
0.5% gelatine	Sigma (Deisenhofen, Germany)
Complete Promocell medium for endothelium	Promocell (Heidelberg, Germany)
Penicillin/Streptomycin (P/S)	PAA Laboratories (Cölbe, Germany)
Fetal calf serum (FCS)	Pan Biotech (Seoul, South Korea)
6-well and 12-well plates (for cell culture)	Greiner bio-one (Frickenhausen, Germany)
Cell culture flask, 25 cm^2	Greiner bio-one (Frickenhausen, Germany)

2.2. Methods

2.2.1. Isolation and maintenance of human umbilical vein endothelial cell culture (HUVEC)

Human umbilical cords were obtained from Münster University Hospital (UKM). The Jaffe isolation method was used in accordance with the permission of the ethics commission (2009-537-f-S) (Jaffe *et al.*, 1973).

The umbilical cords pieces of 20-30 cm were briefly prepared with a sterile scalpel. Both ends were closed with clamps and the cord was soaked for 30-60 seconds in 70% ethanol. Afterwards, the umbilical vein was cannulated with a blunt needle and injected by the pre-warmed PBS until the efflux was transparent. After cleaning, the vein was perfused with 0.2% collagenase A solution, closed and incubated in warm PBS for 10 minutes in a suitable volume. Following this, the cord was agitated gently to enable the cell detachment. The collagenase solution containing endothelial cells was collected into a falcon tube with pre-warmed M199 medium containing 10% FCS, 1% P/S, 1% sodium pyruvate and L-Glutamine. After centrifuging at 1200 rpm for 5 min, supernatant was discarded and the cell pellet was re-suspended in complete M199. The ECs were cultured in a cell culture flask coated with 0.5% gelatin T25 and incubated at 95% air humidity, 5% CO_2, and 37°C. Prior to the M199 medium being changed the next day, the cells were washed twice with pre-warmed PBS to clear the culture from red blood cells and debris. The medium was exchanged every two days.

Upon cell splitting, the culture medium was discarded and the cells were washed once with pre-warmed PBS. For approximately 2-3 minutes, 1 ml 0.05% trypsin-ethylene diamine tetraacetic acid (EDTA) was added to detach the cells at room temperature (RT). After the trypsin was removed, the flask was shaken to enable a complete detachment of the cells. The endothelial cells were collected by adding 1 ml of complete Promocell medium with 2% FCS and 1% P/S. Seeding procedure was dependent on required volume, cell density and desired coating.

2.2.2. Coating cell culture plates with or without glass coverslips

The HUVEC were seeded in 6 or 12-well cell culture plates with coated plastic bottoms with or without glass coverslips. For the immunostaining of endothelial cells, 12-well cell culture plates with glass coverslips were used.

To begin, the sterile glass coverslips were briefly put into each well of the 12-well culture plate. The glass coverslips were then coated with cross-linked gelatin using the following procedure: washing once with PBS, adding pre-warmed 0.5% gelatin upon 30 min incubation at 37°C. After removing the gelatin, 2% glutaraldehyde water solution was applied for 15 min at RT. The glutaraldehyde was then discarded before adding 70% ethanol. Treatment with 70% ethanol lasted up to 1 hour to fully neutralize remaining glutaraldehyde. After this application, the wells were washed with PBS for 5 intervals of 5 minutes. The wells were then incubated overnight with PBS containing 2 mM glycine at RT. Prior to use, plates were washed again with PBS for 5 intervals of 5 minutes.

The plastic plates without glass coverslips were coated with 0.5% pre-warmed gelatin solution for 30 minutes and incubated at 37°C.

2.2.3. Preparation of bacteria inoculum

The culture of *Staphylococcus aureus* was seeded on TSB agar plate. After overnight incubation at 37°C, a single colony of each respective strain was picked and re-suspended in 10 ml of TSB medium for 16 hours in a shaking incubator at 37°C (until stationary phase).

Following incubation, the bacteria suspension was transferred into 15 ml falcon tube and centrifuged for 5 min at 5000 rpm (RT). The medium was discarded and bacteria were re-suspended in 3 ml of PBS. The optical density of bacteria suspension was estimated by OD_{600} measurement using IMPLEN nanophotometer. Afterwards, the bacteria suspension was added to TSB/20% glycerol medium to OD_{600}=1. The number of the bacteria was calculated using glass counting chamber using the following formula:

Bacteria number/1 ml= cells/square x dilution factor x 1.25×10^{6}

The aliquots were frozen and stored at -20°C prior to usage. Before infection, aliquots were defrosted at RT and centrifuged for 5 minutes at 5000 rpm. The medium was discarded and the cell pellet was re-suspended in 1 ml of complete Promocell medium without antibiotics.

2.2.4. Maintaining a HUVEC culture

Before seeding, the amount of HUVEC in the sample was calculated using an automated cell counter. HUVEC were seeded on 15 mm glass coverslips or plastic culture plates with a cell density of 30.000 cells/cm². The cells were incubated in complete Promocell medium containing antibiotics until confluence.

Prior to infection, cells were washed once with pre-warmed PBS and the medium was exchanged with complete Promocell medium lacking antibiotics.

2.2.5. Infection of HUVEC with *Staphylococcus aureus*

The HUVEC were infected with respective *S. aureus* strain in MOI = 50 and incubated for one, two or three hours depending on the experimental design (see results section). Following initial infection, cell cultures were treated with 20 µg/ml of the lysostaphin (LS) for 30 min. Subsequently, the cells were rinsed a single time with pre-warmed PBS.

After LS application, infected cells were incubated in complete Promocell medium with or without antibiotics respective to the experimental design (see results section). HUVEC were incubated until the respective post-infection (p.i.) time point had passed.

2.2.6. Immunostaining and fluorescence microscopy during caspase-3 assay

30 minutes before the experiment was terminated, 5 µM of caspase-3 substrate was added to living cells by being dissolved in complete Promocell medium containing 15 µg/ml of gentamycin for 30 minutes at RT. At 7 hours p.i., the cells were fixed with 2% PFA for ten minutes at RT.

After washing the cells twice with PBS, cells were labeled with diluted (1:500) palloidin-TRITC (1 mg/ml, Deisenhofen, Germany) in PBS for 30 minutes, before being subsequently washed three times in PBS and mounted in Dako fluorescent mounting medium.

Afterwards, coverslips with stained cells were mounted onto microscopy glass slides. Fluorescent microscopy was performed using 40x oil objective with 488 and 561 nm laser channels.

2.2.7. Annexin V/PI apoptotic assay

After initial infection, cells were detached using 0,5 mM of pre-warmed accutase. In turn, the cells were re-suspended in annexin V binding buffer (AVBB) and centrifuged for 5 min at 1600 rpm at RT. The supernatant was discarded and the cell pellet was re-suspended in 100 µl of the new AVBB.

Each aliquot was divided into four respective samples and each of the following was transferred: 5 µl of the Annexin V, 10 µl of the PI, 5 µl of the Annexin V and 10 µl of the PI or nothing. After 15 minutes of incubation at RT in darkness, samples were washed with PBS/1 % BSA one time. After centrifugation, the supernatant was discarded and the cell pellet was re-suspended in AVBB and analyzed by flow cytometry.

2.2.8. *Staphylococcus aureus* intracellular survival

The infected cells were detached using 0,5 mM of pre-warmed accutase. Each sample was divided in two aliquots. The first aliquot was used for counting the number of HUVEC using an automated cell counter. The second was used to estimate the number of intracellular bacteria for three post-infection points in time.

At the respective time point based on experimental design, the HUVEC aliquot was lysed by application of 1% Triton X-100. After a series of dilutions in PBS, the 100 µl of bacteria suspension was seeded on TSB agar plates. This was followed by overnight incubation and counting the colonies the next day.

2.2.9. Infected HUVEC phase contrast microscopy

The time lapse recording of infected HUVEC by phase contrast microscopy was performed for a 24 hour period post-infection. In the experiment, 6 or 12 independent movies were simultaneously acquired. Each frame corresponds to five minutes of infection.

2.2.10. Protein kinases inhibition assay

The pharmacological inhibitors of ASK1, p38, PKC, PI3K, ERK 1/2 and JNK were applied. All of the inhibitors were dissolved in DMSO, aliquoted and stored at -20°C. HUVEC were treated with mitogen-activated protein kinase inhibitors prior and during infection (see results section). To observe the effect of a respective kinase inhibitor, time lapse recordings of infected HUVEC by phase contrast microscopy were performed for a 24 hour period post-infection.

2.2.11. Gene expression study

2.2.11.1. The mRNA isolation and reverse transcription

Total mRNA was isolated from HUVEC lysate using a RNeasy mini kit in accordance with the protocol laid out by the manufacturer. The quality and concentration of the mRNA was measured using the IMPLEN nanophotometer. Prepared mRNA was frozen at -80°C or processed by reverse transcription. Subsequently, the mRNA was used to synthesize the cDNA using the Reverse Transcriptase Core Kit and following the following scheme:

Reagent	***Volume***
500 ng of mRNA+ddH_2O	4.05 µl
10x RT Buffer	1 µl
25 mM $MgCl_2$	2 µl
2.5 mM dNTP	2 µl
Oligo dT	0.5 µl
RNase Inhibitor	0.2 µl
Euroscript RTase	0.25 µl

The thermocycler was programmed with the following parameters:

Step	***Temperature***	***Duration***
Primer annealing	25°C	10 min
Extension	48°C	30 min
Denaturation	95°C	5 min
Cooling	4°C	as needed

The samples were twice diluted with ddH_2O and frozen at -20^oC or immediately used for RT-qPCR.

2.2.11.2. Real-time qPCR

During all procedures, the samples were cooled to 4^oC. cDNA samples were mixed with oligonucleotides and SYBR Green qPCR Master Mix in the following order:

Reagent	***Volume***
cDNA	0.8 µl
2x SYBR Green Master mix	9.0 µl
Primer forward	1.7 µl
Primer reverse	1.7 µl
ddH_2O	6.8 µl

The real-time qPCR was processed using the Eppendorf realplex thermocycler with the respective program:

Step	***Temperature***	***Duration***
1 cycle:		
Denaturation	95^oC	5:00
40 cycles:		
Denaturation	95^oC	10 sec
Annealing/elongation	55^oC	15 sec
Annealing/elongation	60^oC	15 sec
1 cycle (Melting curve):		
Denaturation	95^oC	15 sec
Annealing	60^oC	15 sec
Gradient temperature increase		20 min
Denaturation complete	95^oC	15 sec

The two-step qPCR with melting curve was performed for all gene expression experiments. Specific human primers were designed by using Universal Roche Probe Library (Roche, 1996-2015) (Table 4). The oligonucleotides were ordered and synthesized by Eurogentec.

Genes name		*Primers 5´→3´*
	Reference genes	
E-selectin	Forward	GAGTGCACATCTCAGGGACA
	Reverse	ACTGCCAGGCTTGAACATTT
ICAM-1	Forward	CCTTCCTCACCGTGTACTGG
	Reverse	AGCGTAGGGTAAGGTTCTTGC
VCAM-1	Forward	TGCACAGTGACTTGTGGACAT
	Reverse	CCACTCATCTCGATTTCTGGA
GAPDH	Forward	GAGGGTCTCTCTCTTCCTCTTGT
	Reverse	CTCCTCTGACTTCAACAGCGACA
	Genes for microarray verification	
CLDN11	Forward	CCCGGTGTGGCTAAGTACAG
	Reverse	CAACAAGGGCGCAGAGAG
CLDN23	Forward	GGACCAGTGGGGCTACTTC
	Reverse	AGCGAGGTGACCATGAGTG
CEACAM1	Forward	CCCATCATGCTGAACGTAAA
	Reverse	AGGGCCACTACTCCAATCAC
ACTA2	Forward	CCTATCCCCGGGACTAAGAC
	Reverse	AGGCAGTGCTGTCCTCTTCT
ARHGAP1	Forward	TTGTGTTTAGTGCCTGTCGAA
	Reverse	TACTGGTCCAGGGTGTGCTT
FAM107A	Forward	CTGCAGTGCCCCTTTGAG
	Reverse	TCCTTCTCTGGTGGTTTTTCC
DCP1A	Forward	AGCCAAGGATGAGTATGAGAGG
	Reverse	AGCTGAGTGCTTGGCTGTAAC
FLNC	Forward	GCCTCCCTCTCGGATGAC

	Reverse	GGCTGGTTCACCTTGAGC
CCL5	Forward	TGCCCACATCAAGGAGTATTT
	Reverse	CTTTCGGGTGACAAAGACG
CCL20	Forward	GCTGCTTTGATGTCAGTGCT
	Reverse	GCAGTCAAAGTTGCTTGCTG
CXCL10	Forward	GAAAGCAGTTAGCAAGGAAAGGT
	Reverse	GACATATACTCCATGTAGGGAAGTGA
CXCL11	Forward	AGTGTGAAGGGCATGGCTA
	Reverse	TCTTTTGAACATGGGGAAGC
ATF3	Forward	CGTGAGTCCTCGGTGCTC
	Reverse	GCCTGGGTGTTGAAGCAT
EGR1	Forward	AGCCCTACGAGCACCTGAC
	Reverse	GGTTTGGCTGGGGTAACTG
EGR2	Forward	TTGACCAGATGAACGGAGTG
	Reverse	TGGTTTCTAGGTGCAGAGACG
CD47	Forward	TGCTGCTCCAGACACCTG
	Reverse	CTACCAGGGGCCACATCTC
CD68	Forward	GTCCACCTCGACCTGCTCT
	Reverse	CACTGGGGCAGGAGAAACT
CD83	Forward	CGGTCTCCTGGGTCAAGTTA
	Reverse	TGTCCCCTGAGGTGGTCTT
GBP1	Forward	CCAGTGCTCGTGAACTAAGGA
	Reverse	TGTCATGTGGATCTCTGATGC
GBP2	Forward	CCCTAGTTCTGCTCGACACTG
	Reverse	AGGCAAAGATCCAGGAGTCA
STX11	Forward	AGATGTTCCACCAAAAACACG
	Reverse	AAGAACACCTGCCAAGCTGA
RND1	Forward	GCGAAGGATTGCTATCCAGA
	Reverse	GGTATCCCAGAGACTAAGCTCCA

RND3	Forward	GCGCTGCTCCATGTCTTC
	Reverse	GCCGTGTAATTCTCAAACACTG
TAP1	Forward	TCTCGCTGTTCCTGGTCCT
	Reverse	TCTTGTAGAATCCAGTCAGTGAGG
BCL3	Forward	GCCTCAGCTCCAATGGTC
	Reverse	GAGGAGCCATGGGGAATC
BIRC3	Forward	GATGAAAATGCAGAGTCATCAATTA
	Reverse	CATGATTGCATCTTCTGAATGG
CASP1	Forward	CCTTAATATGCAAGACTCTCAAGGA
	Reverse	TAGCTGGGTTGTCCTGCACT
CASP7	Forward	CCGAGACTTTTAGTTTCGCTTT
	Reverse	CCTGATCATCTGCCATCGT
CFLAR	Forward	TCCTGAACAGTACTATTTCGTGTGA
	Reverse	AGCATGAGATATAAAATGAAAAGAAGG
TNFRSF12A	Forward	GACCGCACAGCGACTTCT
	Reverse	CACGAAGGTCAGGCTCAGA
CXCR4	Forward	GGATATAATGAAGTCACTATGGGAAAA
	Reverse	GGGCACAAGAGAATTAATGTAGAAT

Table 4. The oligonucleotides were used in the study.

2.2.11.3. Quantification of RT-qPCR results

The results of RT-qPCR were obtained as Ct (cycle threshold) values. The fold expression change was calculated by $2^{-\Delta\Delta Ct}$ method of relative gene expression (Schmittgen and Livak, 2008).

Where, fold change = $2^{-\Delta\Delta Ct}$

, and –ΔΔCt = [(Ct gene of interest – Ct internal control) sample 1 – (Ct gene of interest – Ct internal control) calibrator)]. Ct of the internal control refers to the Ct value of housekeeping gene GAPDH.

2.2.12. Microarray performance

Total mRNA was isolated using the RNeasy Mini kit in accordance with the supplier's instructions. cDNA was synthesized using the Reverse Transcriptase Core Kit (Eurogentec). Biotinylated and amplified RNA for direct hybridization was transcribed using the Illumina TotalPrep RNA Amplification Kit (Life Technologies) in accordance with the manufacturer´s instructions (Fig. 5).

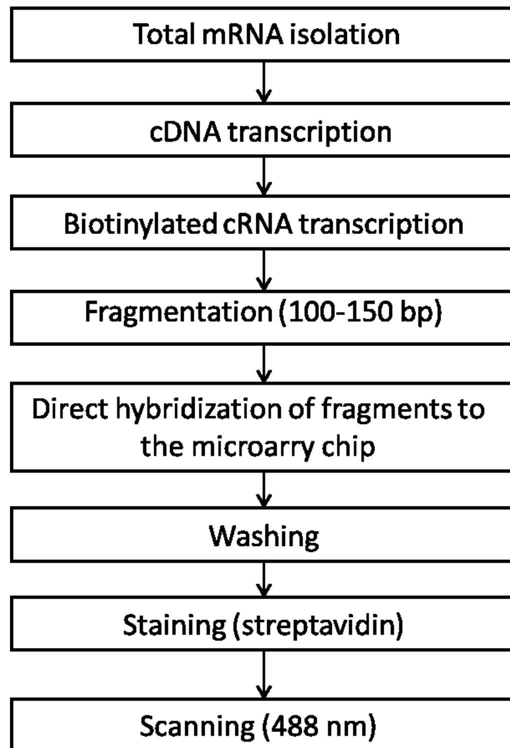


Figure 5. The schematic procedure of microarray preparation.

For microarray, Illumina BeadChip Technology (Human HT-12 v4 Expression BeadChip) was used and processed using an Illumina HiScan-SQ array scanner. Quality controls and differential expression analyses were performed according to the Illumina Custom Model installed in the Genome Studio software (Illumina).

2.2.13. Statistics

The microarray data for the group comparisons was summarized as mean ± SD and expression differences between strains were analyzed by use of a student's t-test and ANOVA followed by post-hoc Bonferroni adjustments. For the other tests, statistical analyses between two groups were performed by double-sided Student's *t*-test. The data was presented as mean ± SD. P-values equal or less than 0.05 were considered as significant.

3. Results

3.1. *Staphylococcus aureus* strains that actively translocate prevent TNF-α-induced ICAM-1 upregulation in human endothelial cells

A recent study (Kramko *et al.*, 2013) demonstrated a diverse endothelial response upon infection with different strains of *S. aureus* (Kramko et al., 2013). This includes the capacity of the particular strain to upregulate ICAM-1 expression (Fig. 6).

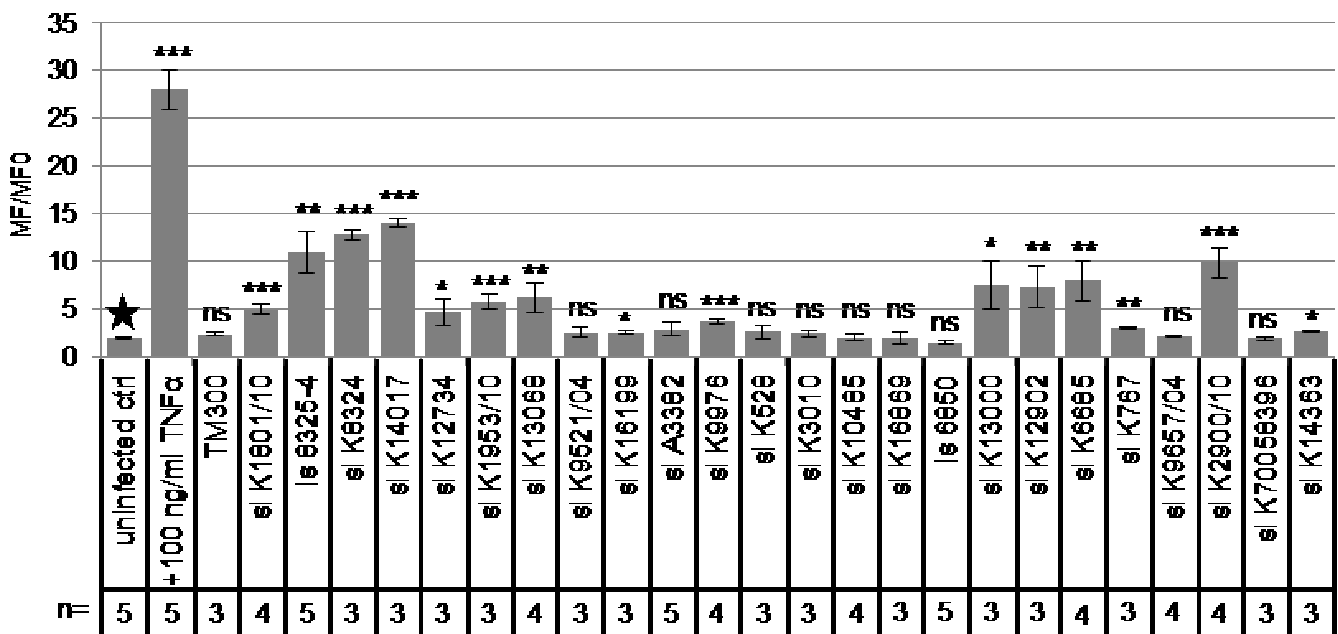
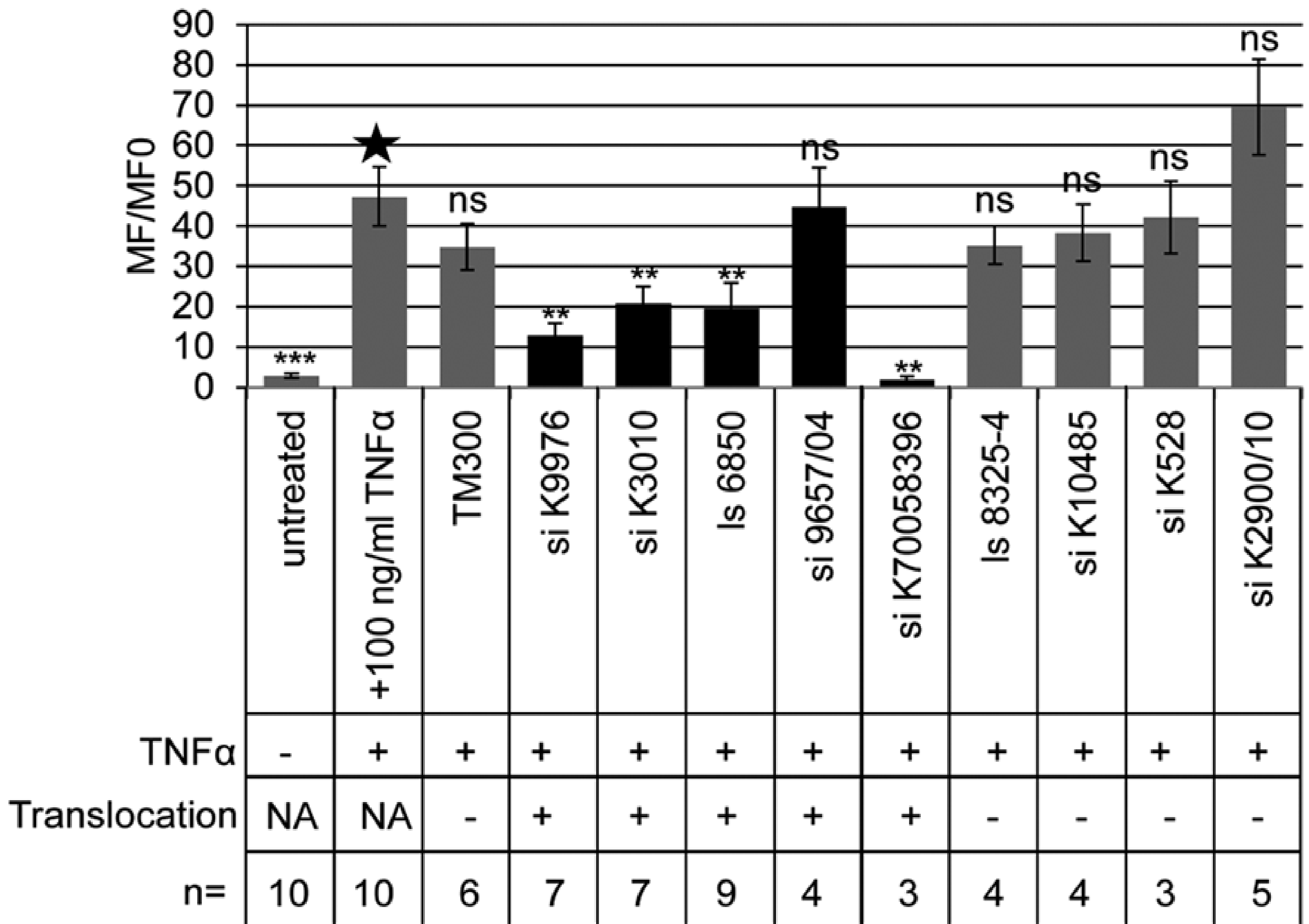


Figure 6. ICAM-1 surface expression was determined in HUVEC after infection with *S. aureus* strains (MOI of 50) as indicated by calculation of MF/MF0 (mean fluorescence ratio) values obtained from flow cytometry. Data shown is the mean from n independent experiments, as indicated, ± SEM. The p-values were determined related to mock-treated control (bold star). *=p≤0.05, **=p≤0.005, ***=p≤0.0005, ns=not significant (Kramko *et al.*, 2013).

Nine *S. aureus* strains did not demonstrate significant upregulation of ICAM-1 (Fig. 6) while 14 others did. Two strains (6850 or K70058396) out of 24 did not upregulate ICAM-1 expression (Kramko *et al.*, 2013) but in contrast did transmigrate through the endothelium monolayer. Next, we tested whether strains that do not induce ICAM-1 expression have the ability to influence immune response suppression in endothelium. To evaluate this, endothelial cells were infected and subsequently challenged by TNF-α, a cytokine that is known to upregulate ICAM-1, and increases permeability (Goldblum *et al.*, 1993; Yang *et al.*,

2005). TNF-α alone was used as a positive control while a mock treatment served as the negative control. The data shows that 6850, K70058396, K3010 and K9976 strains inhibited the TNF-α-induced upregulation of ICAM-1 and translocated trough the cell layer while all other tested strains showed no inhibitory effect (Fig. 7).



TNFα	-	+	+	+	+	+	+	+	+	+	+	+
Translocation	NA	NA	-	+	+	+	+	+	-	-	-	-
n=	10	10	6	7	7	9	4	3	4	4	3	5

Figure 7. Efficiently translocating *S. aureus* strains diminish the TNF-α-induced ICAM-1 expression in endothelial cells. HUVEC were infected with *S. aureus* with a MOI of 50 followed by a depletion of not internalized bacteria. Strains were subsequently treated with 100 ng/ml of recombinant TNFα for 6 hours and then ICAM-1 surface expression was determined by calculation of MF/MF0 values obtained from flow cytometry. Data shown represents the mean from n independent experiments, as indicated, ± SEM. The p-values were determined respectively to the TNF-α treated control (bold star). **=p≤0.005, ***=p≤0.0005, ns=not significant, NA=not applicable (Kramko *et al.*, 2013).

The data demonstrates that the activation of endothelium can be blocked by particular strains of *S. aureus* (Kramko *et al.*, 2013).

3.2. *S. aureus* display strain-related pathophysiological features in EC

To further study strain-specific endothelial response, the following strains displaying select characteristics were chosen (Fig. 10). The selection was based on diverse pathophysiological effects of *S. aureus* previously studied (Kramko *et al.*, 2013). ICAM-1 expression was analyzed by flow cytometry, barrier function was examined by a determination of the transendothelial electrical resistance (TER) from impedance spectroscopic measurements and bacterial transmigration was evaluated by use of a transwell filter assay (Kramko *et al.*, 2013) (Fig. 8).

Distinctive *S. aureus* strains were deliberately used in all following experimentations to study a maximal broad range of endothelial response upon infection.

strain	K1801/10	8325-4	K70058396	6850	K9657/04	K2900/10
MF(ICAM-1)/MF0(isotype)	5	11	NS	NS		9.9
ΔTER	0.75	0.6	NS	0.23		
MF/MF0(bacteria+TNF)/MF/MF0(TNF)	ND	NS	0.04	0.4		
(Translocated bacteria)*10^5	NS	NS	22	9.4	10	

= pro-inflammatory parameter
= anti-inflammatory parameter
= pro-inflammatory strain
= anti-inflammatory strain
= incomplete anti-inflammatory strain
= areactive strain

Figure 8. Effects of selected *S. aureus* strains on early endothelial response compared to controls.

Strains were assigned as pro-inflammatory when they upregulated pro-inflammatory cell adhesion molecules (e.g. ICAM-1), decreased paraendothelial barrier function and did not transmigrate. Strains were assigned anti-inflammatory when they did not provoke upregulation of cell adhesion molecules, did not decrease the TER, decreased the pro-inflammatory response in endothelium after TNF-α challenge and were able to

transmigrate. One strain that features mostly anti-inflammatory properties, however, moderately down-regulated the TER. To classify it, we termed this strain incomplete anti-inflammatory. Strains K9657/04 and K2900/10 that affected only one of the parameters we labelled as areactive.

3.2.1. Expression of virulence factors is strain-related

To better characterize the selected *S. aureus* strains, virulence factor expressions were determined (Silke Niemann, Institute of Medical Microbiology). This data represents virulence factor expression levels of planktonic bacteria in reach medium after three hours of incubation for *fnbpA* and six hours of incubation for all other virulence factors.

The strain-specific expression of global virulence regulators including toxins and adhesins was determine for each selected *S. aureus* strain (Table 3). Strains with a lesser pro-inflammatory effect in the endothelium demonstrated a comparatively higher expression of *psm* (phenol-soluble modulin), *sae*, *agr* and toxins *hla* (α-toxin) while *sarA* had low levels of expression in all strains being investigated. Strains that influenced a profound pro-inflammatory response expressed much less of the global regulator and toxin, particularly *psm* and *sae*.

Gene expression investigated by RT-PCR

strain / virulence factor	agr	sarA	sae	hla	psm	emp	eap	fnba
K1801/10	4,1	0,2	9,5	2,5	7,7	0,0075	0,04	0,2
8325-4	0,06	0,01	2,0	0,7	0,006	0,014	0,3	0,03
6850	5,9	0,2	17,9	7,6	88,3	1,8E-05	1,0	0,03
K7005839	24,1	0,5	33,3	26,5	424	2,6E-05	0,0001	0,008
K9657/04	8,6	0,3	1,6	0,002	7,4	9,2E-06	3,3E-05	0,1
K2900/10	0,9	0,09	1,1	0,0002	0,004	7,5E-07	2,6E-05	0,13

Table 3. Virulence factors expression of selected *S. aureus* isolates. In red is labeled the strain´ highest gene expression among the isolates. Data is the mean from 3 independent experiments (The experiment was done by Silke Niemann, institute of Medical Microbiology).

The expression levels of global regulators in the strains K70058396 and 6850 were very high. Interestingly, these strains belong to the anti-inflammatory and incomplete anti-inflammatory strain categories, easily transmigrating through the endothelium. In contrast, however, the strains 8325-4 and K1801/10 showed comparatively weak expressions of global regulators and toxins (Table 5) and belong to pro-inflammatory or incomplete pro-inflammatory strains. Strain K9657 and strain K2900/10 displayed mixed behaviors and displayed a low expression of global regulators (Table 3).

3.3. Morphodynamics of cytotoxic effect of *S. aureus* strains in infected endothelial cell cultures

To further characterize the strain-specificity of *S. aureus*-endothelial interactions we investigated cytotoxic effect of strain-specific infection. In particular, we looked for apoptosis and necrosis, but we also tested the capability for invasion, intracellular bacterial persistence and growth.

In order to investigate the particular behaviors of *S. aureus* in endothelial cells, samples were infected and subsequent time lapse recordings of confluent endothelial cell cultures were performed for 24 hours (illustrated in the scheme (Fig. 9). TNF-α is a pro-inflammatory drug but also exhibits pro-apoptotic activity and served as positive control.

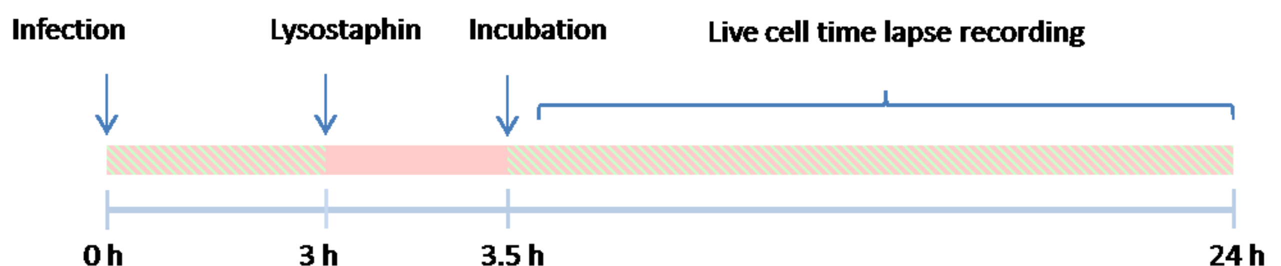


Figure 9. General scheme of live cell time lapse recording experiment.

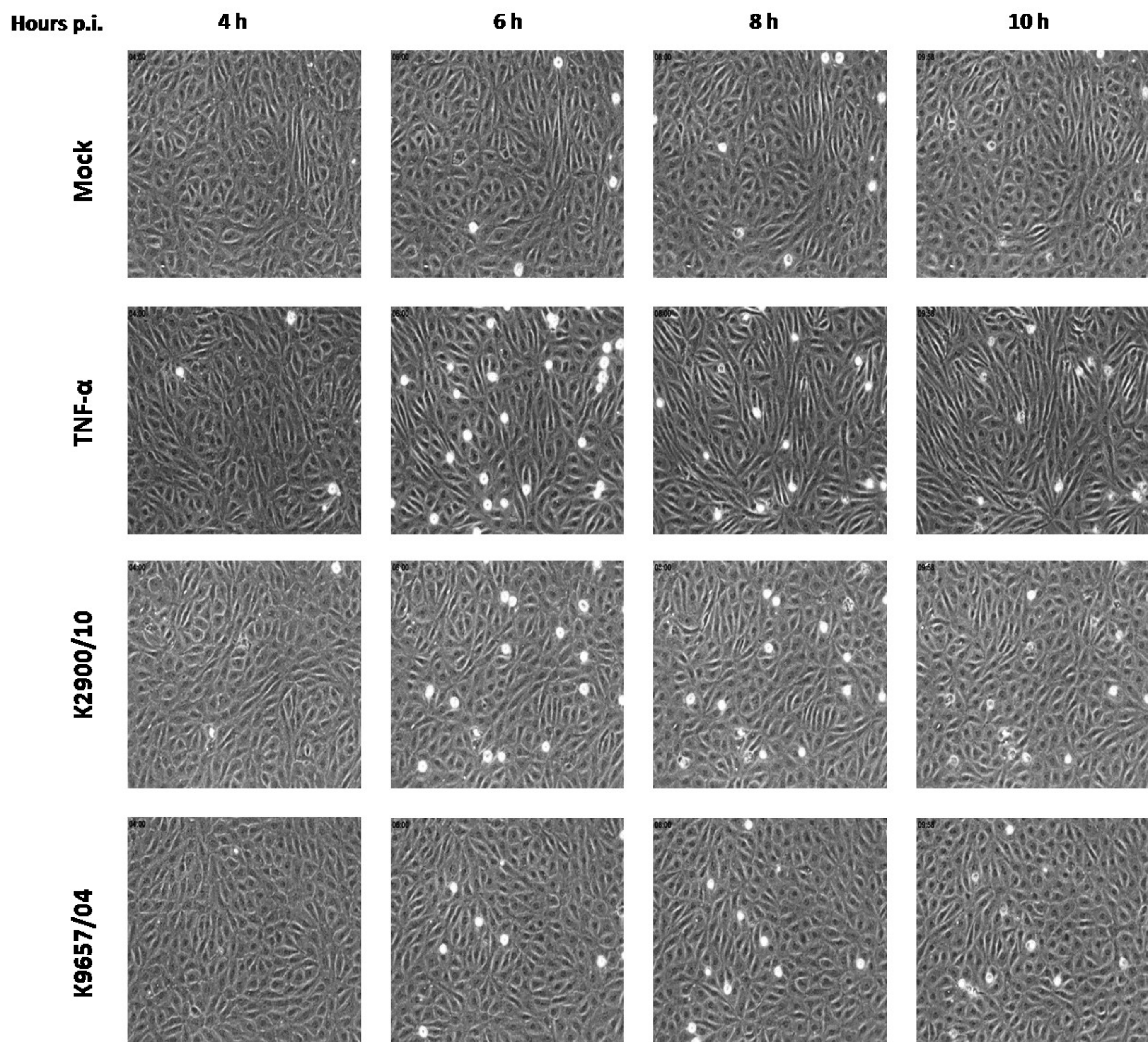


Figure 10. Live cell time lapse recording of *S. aureus* infected endothelium (20x). Images were taken after 4, 6, 8 and 10 hour post-infection from an image sequence of 24 hours infection.

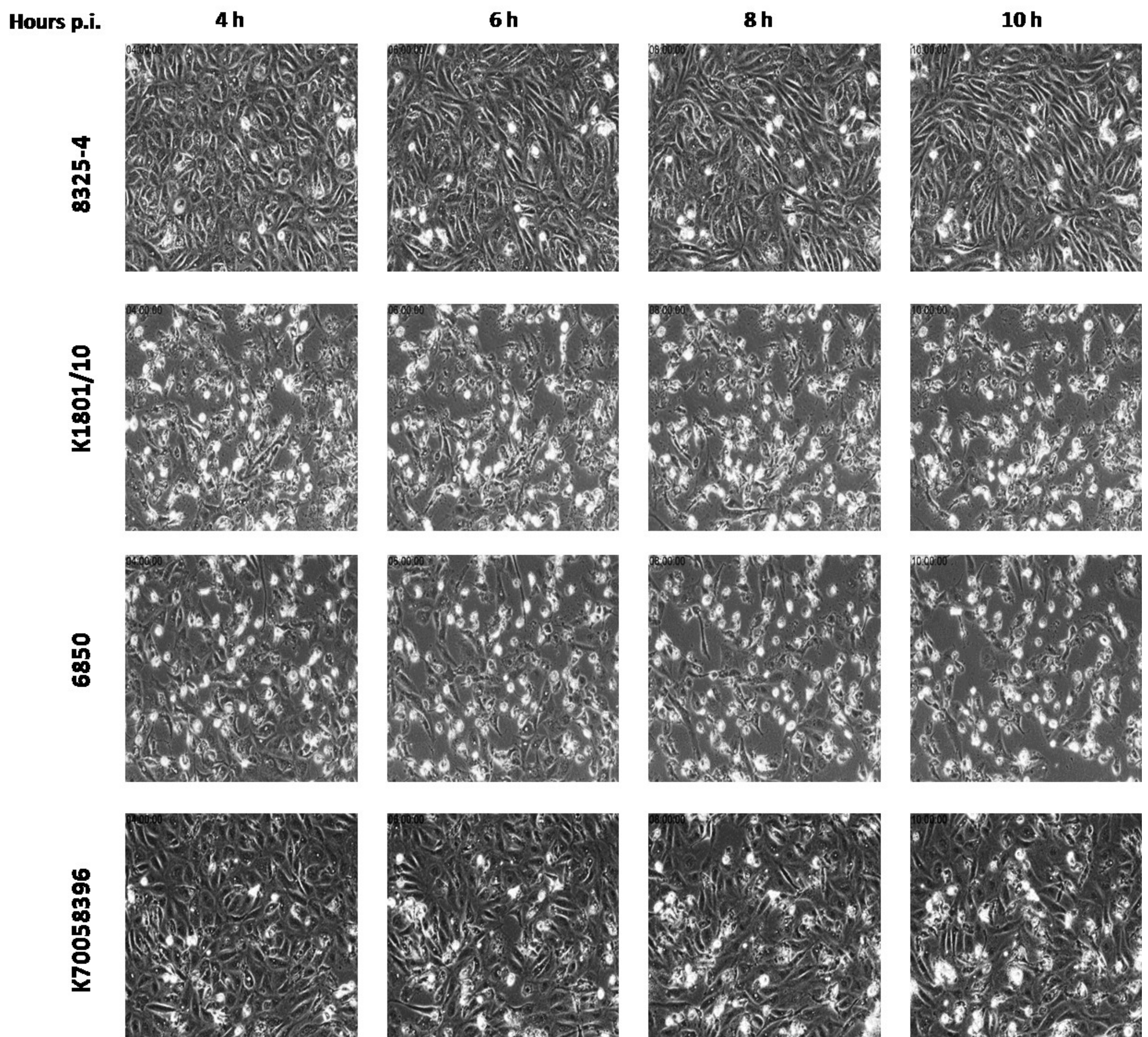


Figure 10 (Continued). Live cell time lapse recording of *S. aureus* infected endothelium (20x). Images represent 4, 6, 8 and 10 hour post-infection. The images were exported from respective 24 hours live cell imaging movies for each treatment.

Infection of the endothelial cells with strains 8325-4, K2900/10 and K9657/04 did not visually compromise endothelial monolayer integrity and induced only minor endothelial cell death, a behavior which corresponds to rounded-up cells being pushed out from the cell layer, within 10 hours following infection (Fig. 10) and were designated as non-cytotoxic. In contrast, infection with the *S. aureus* strains K1801/10, 6850 and K70058396 caused massive endothelial cell damage, likely by apoptosis or necrosis, with cell gap junction

formation starting at 4 hours post-infection (Fig. 10). Treatment with TNF-α led to endothelial cells elongating and inducing visible apoptotic changes after 6 hours of incubation (Fig. 10).

The damaged endothelial cells showed similar apoptotic morphologies when compared with TNF-α-induced cell death (Fig. 11, A). Cells lost cell-to-cell adhesion, were rounded-up, and displayed characteristic blebbing (Fig. 11, A). The process of endothelial cell death for some infected cells includes the formation of a large membrane bubble and a shrinking of the nuclei (Fig. 11, B).

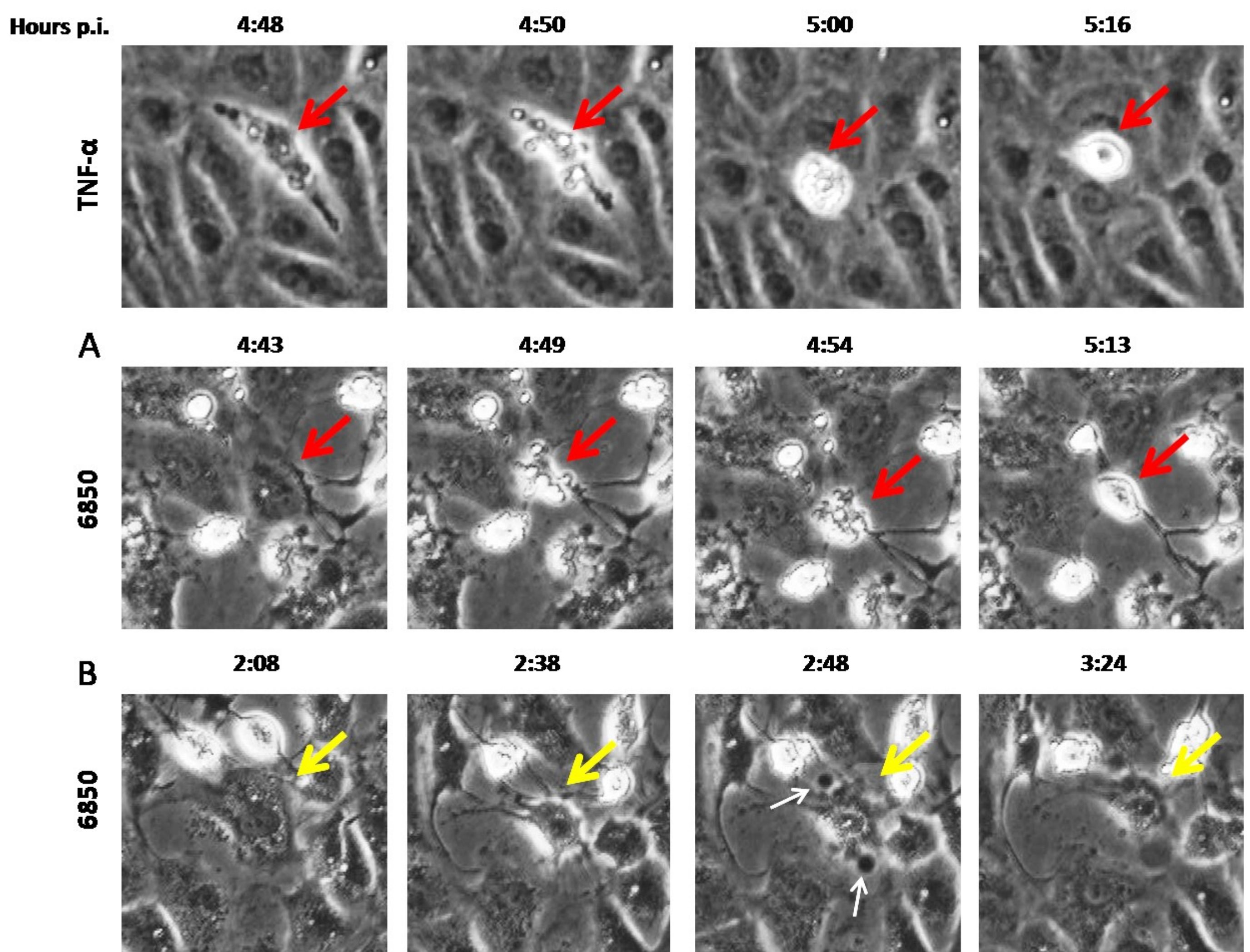


Figure 11. Cytotoxic *S. aureus* strain 6850 induces profound endothelial cell death. TNF-α-induced endothelium cell death is presented for comparison. Red arrows indicate endothelial cell death with membrane blebbing. Yellow arrows demonstrate endothelial cell death with big membrane bubbles (white arrows) and shrunken nuclei.

3.3.1. Strain-specific *S. aureus* release from infected endothelial cells by "outbursts"

During morphodynamic study, we discovered a 'burst-like' repeated release of bacteria from endothelial cells infected by the K70058396 strain. Particularly at the beginning, cells appeared to be rounded, possibly due to cell swelling, followed by a sudden rupturing process taking approximately 15 minutes and causing a massive release of intracellular bacteria into the medium (Fig. 12, 13). The entire process was termed "outburst". Bacteria "outbursts" were observed to start between 7-10 hours after infection with the K70058396 strain. Based on evidence from the time lapse movies it appears that these "outbursts" are repeated several times by the very same cell (Fig. 13).

Among all isolates we used during this experiment, "outburst" activity was observed only with strain K70058396.

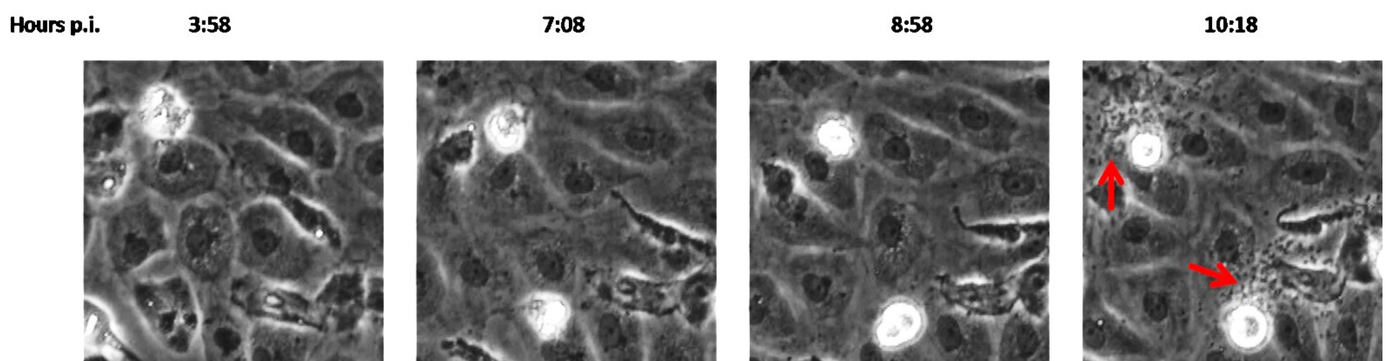


Figure 12. HUVEC infected with *S. aureus* strain K70058396. Red arrows indicate bacteria "outbursts" from endothelial cells.

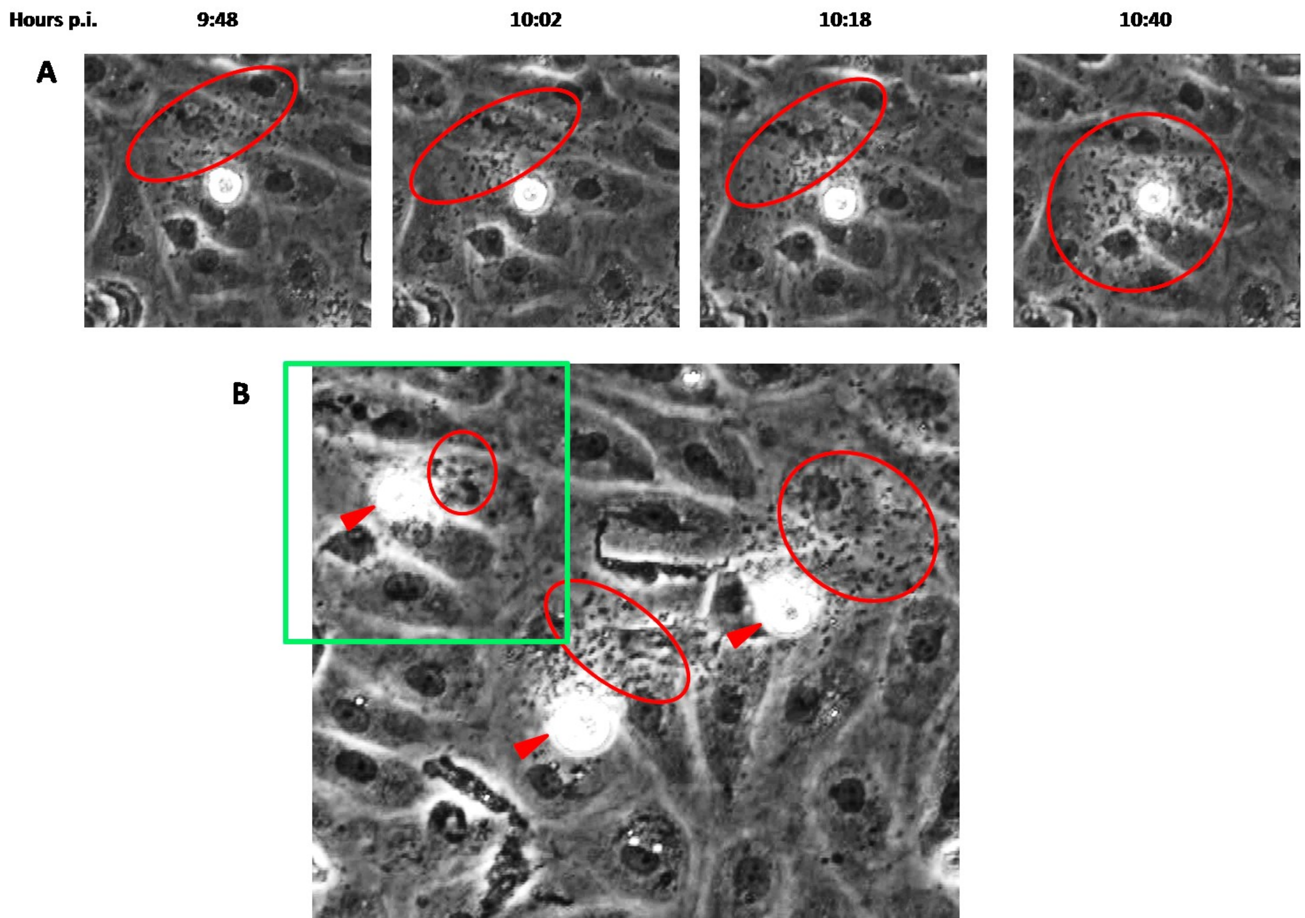


Figure 13. (A) HUVEC infected with *S. aureus* strain K70058396. Red circles indicate repeated bacteria "outbursts" from the same endothelial cell. (B) HUVEC infected with *S. aureus* strain K70058396 – 10 hours post-infection (enlarged). Red arrows indicate bacteria "outbursts" from several endothelial cells. In red circles are indicated released bacteria. Green box corresponds to area illustrated above (Fig. 13; A).

The data indicates that intracellular survival and bacterial growth may occur in several separated intracellular compartments. Since *S. aureus* strain K70058396 was the only one that displayed this behavior, we theorize that such strains benefit from specialized mechanisms that enable bacterial survival and in human endothelium.

3.4. Cytotoxic *staphylococcus aureus* strains are able to survive and grow in endothelium

As demonstrated above, the apoptotic *S. aureus* strains K70058396 showed the capacity to cause "outbursts" from infected endothelial cells. We hypothesize that *S. aureus* intracellular survival and multiplication can be a characteristic sign identifying cytotoxic strains. To confirm intracellular survival and quantify growth rate of chosen *S. aureus* isolates, an intracellular survival assay was designed. Specifically, HUVEC cultures were

grown to confluency and infected with three cytotoxic *S. aureus* strains K1801/10, 6850 and K70058396 for one hour with MOI=50. As a negative control we infected endothelium with non-cytotoxic strain 8325-4. Cells were infected for 1 hour closely followed by a depletion of extracellular bacteria using lysostaphin for 30 minutes. Subsequently, infected cells were divided and further cultured with and without antibiotics until the experiment was terminated after 2,5; 4,5 and 7,5 hours post-infection (Fig. 14). HUVEC were then washed , lysed and a plaque assay was performed.

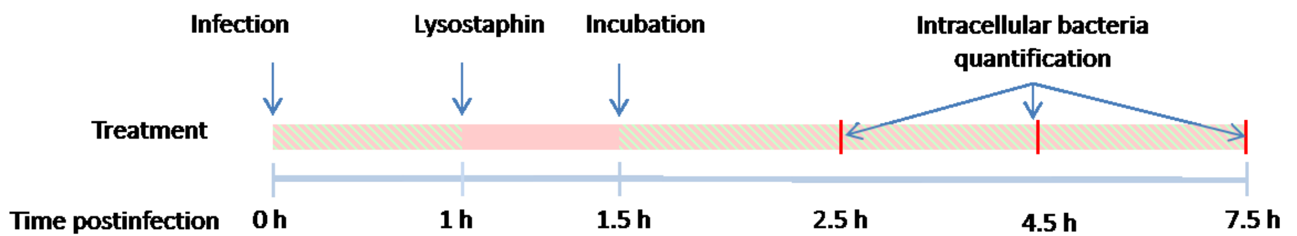


Figure 14. The scheme of bacteria intracellular survival experiment

S. aureus isolates showed different traits to survive and multiply intracellularly. The three cytotoxic strains showed significant intracellular growth by which strain K70058396 demonstrated a growth rate three times higher than 6850 and six times higher than strain K1801/10 (Fig. 15; A). In contrast, the non-cytotoxic strain 8325-4 did not show significant intracellular growth, but rather continued to reside inside the cells.

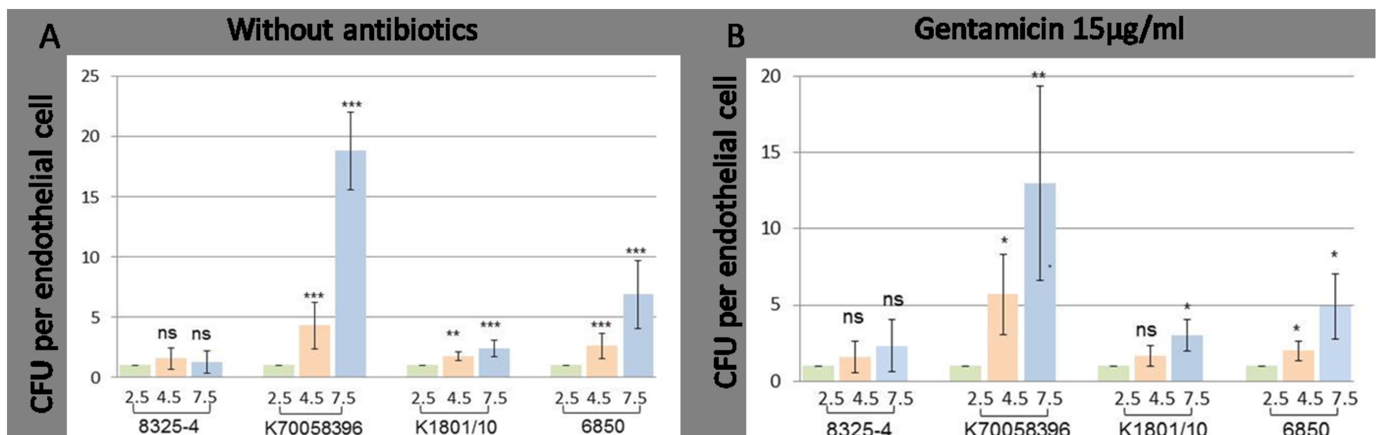


Figure 15. Intracellular *S. aureus* survival in HUVEC (A) without antibiotics or (B) with 15 µg/ml Gentamicin. The data shows the number of colony forming units (CFU) per one endothelial cell. The colored columns indicate different points in time. The data shown is the mean from at least three independent experiments, indicated as ± SEM. The p-values related to 2.5 hours incubation time point. *=p≤0.05, **=p≤0.01, ***=p≤0.005, ns= not significant. The number of colonies was normalized to the number of endothelial cells in the sample, and recalculated as number of colony forming unit (CFU) per one endothelial cell.

To exclude the secondary internalization by extracellular bacteria released by destroyed endothelial cells, the same experiment was performed in the presence of 15 µg/ml gentamicin. Gentamicin is an antibiotic that is hardly taken up in the cells and requires over 10 hours until a concentration equilibrium can be reached (Seral *et al.*, 2003; Easmon, 1979). Thus, Gentamycin has weak activity against intracellular *S. aureus* (Mohamed *et al.*, 2014).

The presence of Gentamicin did not change the results (Fig. 15; B), which further demonstrates the efficient intracellular survival and intracellular growth tactics of cytotoxic *S. aureus* strains. Intracellular growth and survival seems to be a characteristic for cytotoxic strains and is most likely related to *S. aureus*-induced endothelial cell death in a strain-specific manner.

3.5. *S. aureus*-induced endothelial cell death

3.5.1. Cytotoxic strains of *S. aureus* induce early apoptosis in endothelial cells.

In the next series of experiments our goal was to better understand cell death induced by *S. aureus*. To differentiate between *S. aureus* early/late apoptosis and/or necrosis in HUVEC, a Annexin V/propidium iodide (PI) assay was performed. The Annexin V binds phosphatidylserine on the cell surface which is then used as the marker for early apoptosis while PI and double stained positive cells indicate necrosis or late apoptosis. After infection, extracellular bacteria were depleted by treatment with lysostaphin for 30 minutes, washing and further incubation until 18 hours past the initial time of infection. Subsequently, the percentage of Annexin V positive, PI positive or double-positive cells was determined by flow cytometry. 1 mM hydrogen peroxide was applied to cell cultures for one hour. The experimental time scale is illustrated in figure 16 (Fig. 16).

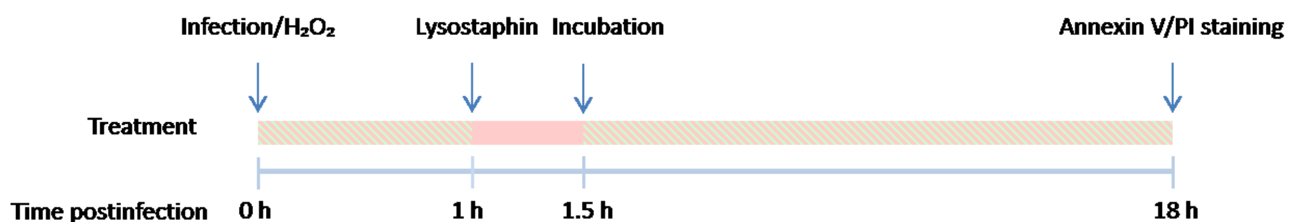


Figure 16. The scheme of Annexin V/PI apoptotic assay.

Indeed, the *S. aureus* strains K1801/10, 6850 and K70058396 showed 70% of Annexin V positive cells for all three cytotoxic isolates. The number of PI and double stained positive cells did not show significant changes with any of the strains compared to the negative control. Data indicates that apoptosis is the main method of cell death. In contrast, the non-cytotoxic strain 8325-4 lacked the ability to induce any apoptotic changes when compared to the mock treatment. Hydrogen peroxide-induced a significant increase in late apoptosis as seen by an increase of double positive cells (Fig. 17).

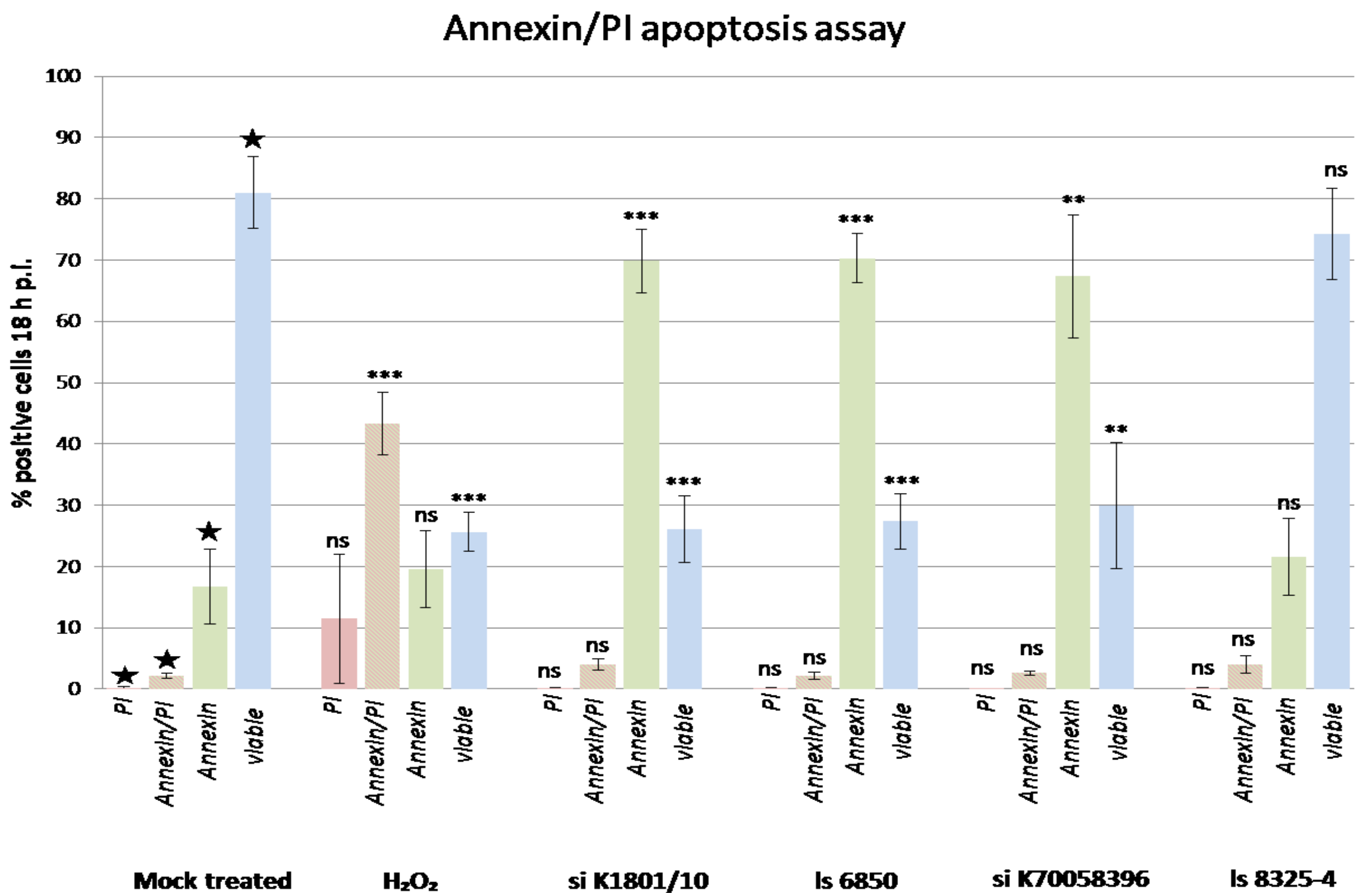


Figure 17. Annexin V/PI apoptosis assay by flow cytometry. The percentage of Annexin V, PI and double positive endothelial cells was determined after infection with *S. aureus* strains (MOI of 50) at 18 hours post-infection. The data shown is the mean from four independent experiments, indicated as ± SEM. The p-values related to mock-treated control (bold star). *=p≤0.05, **=p≤0.005, ***=p≤0.005, ns= not significant.

To further confirm the induction to apoptosis by cytotoxic *S. aureus* strains, we performed a caspase-3 apoptosis assay in living cells by adding a membrane permeable caspase-3 substrate. Endothelial cells were infected with the test strains and the caspase-3 substrate was added for 30 minutes at room temperature before formaldehyde fixation and microscopic analyses (Fig. 18). Unfortunately, the caspase substrate needs to be added at room temperature in accordance with the manufacturer's instructions. It is well known that down-regulation of temperature from 37°C to room temperature (about 21°C) can cause a partial loss of cell-cell adhesion that was observed in all of our cell cultures (Fig. 19). In the case of the positive control sample, cells were treated with 1 mM hydrogen peroxide for one hour before the culture medium and lysostaphin were applied to every test sample (Fig. 18).

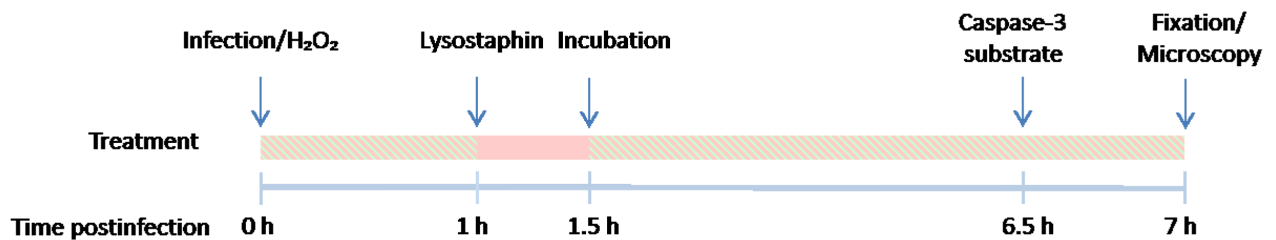


Figure 18. The scheme of caspase-3 apoptotic assay.

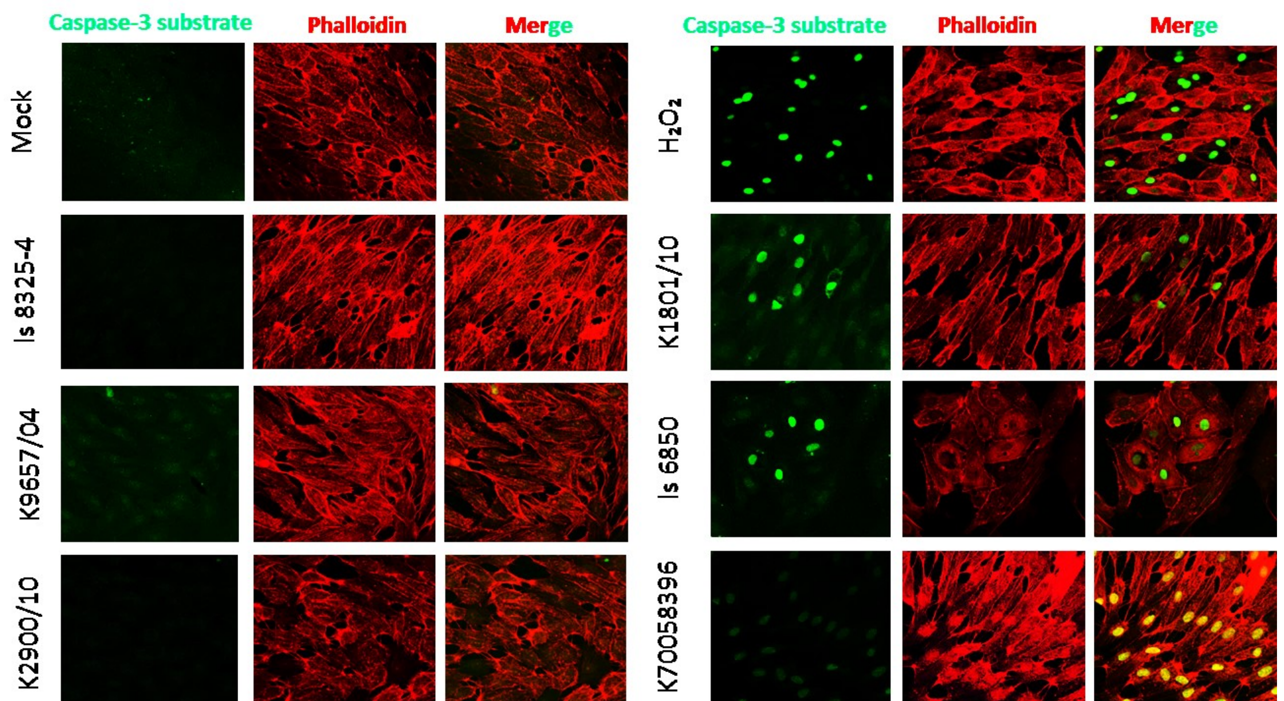


Figure 19. SIM microscopy of infected HUVEC treated with caspase-3 substrate.

The cytotoxic *S. aureus* strains K1801/10, 6850, K70058396 and hydrogen peroxide treatments induced caspase-3 activation, which led to cytosolic caspase-3 substrate cleavage and translocation of free nuclear dye into the nuclei of the endothelium. Therefore, the fluorescent signal in the nucleus corresponds to caspase 3 active cleavage of the caspase-3 substrate. To quantify the intensity of the nuclei fluorescence signal, we used ImageJ Software. For quantification, we used three randomly acquired, 40x magnification, fields of view of each sample for the four independent experiments (Fig. 20) giving a total of 12 fields in total each containing 30-50 cells.

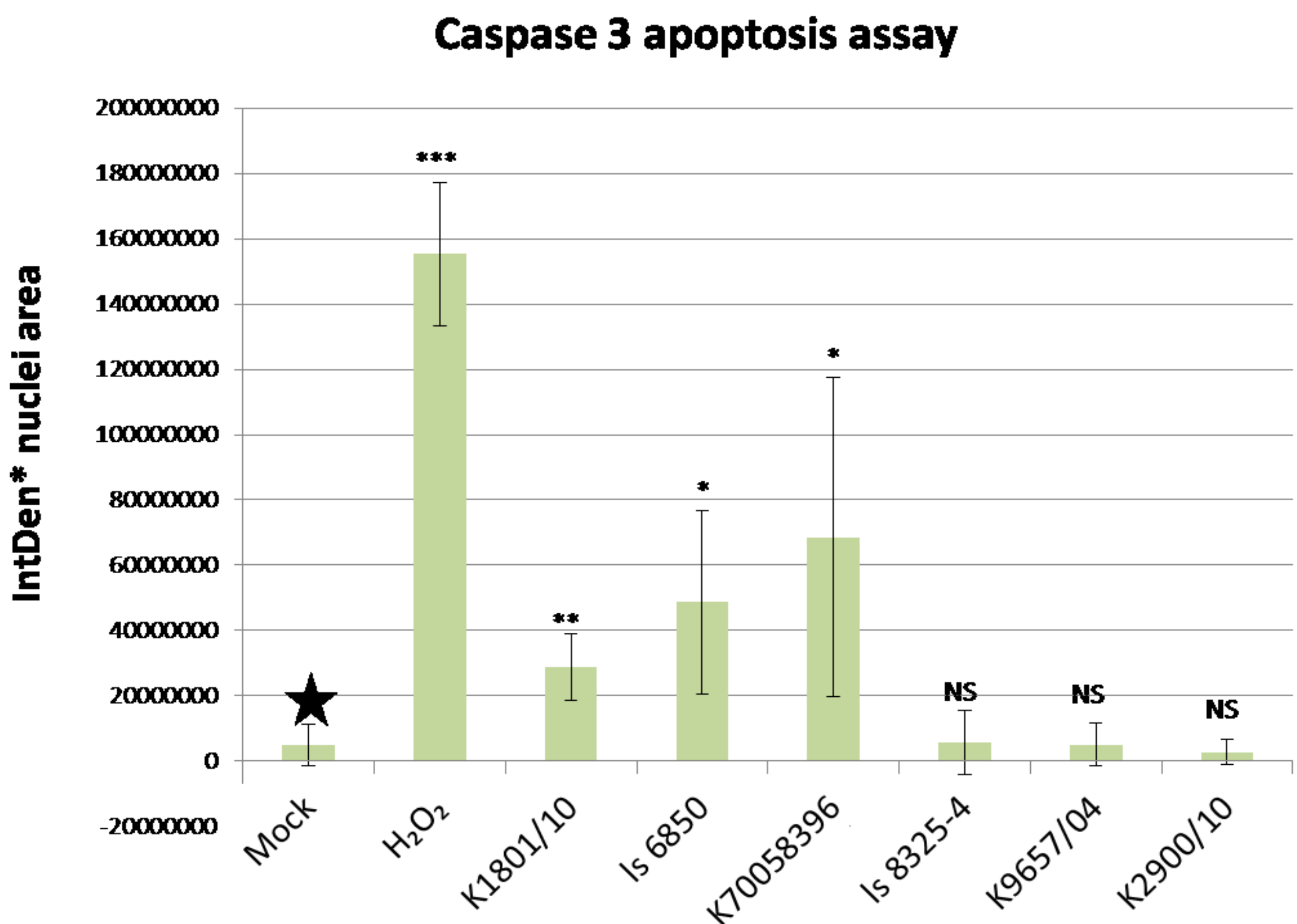


Figure 20. Caspase-3 apoptosis assay. The integrated signal density of caspase-3 cleaved substrate was multiplied by number of fluorescent events. The data shown is the mean from four independent experiments, indicated as ± SEM. The p-values related to mock-treated control (bold star). *=p≤0.05, **=p≤0.01, ***=p≤0.005, ns= not significant.

Data demonstrates that cytotoxic *S. aureus* strains K1801/10, 6850 and K70058396 prefer to induce early apoptosis in endothelial cells while strains Is 8325-4, K9657/04 and K2900/10 appear to lack this ability.

3.6. Global transcriptome analyses of HUVECs infected with different isolates of *S. aureus*

S. aureus strains demonstrated strain-specific differences in their interactions with the endothelium. Strains varied greatly in their inflammatory result, influence of apoptotic properties and translocation behavior. To identify mechanisms and signaling pathways that might be involved in these processes and result in these variations, we performed a whole genome transcriptome analysis using Illumina DNA-microarray technology.

3.6.1 The study of gene expression and experimental design

S. aureus strain selection was based on the pathophysiological behaviors exemplified in each as described in chapter 3.2. For the transcriptome analyses, we infected HUVEC and isolated mRNA at 7 hours post-infection, respectively (Fig. 21). The treatment with 100 ng/ml of TNF-α was used as a positive control. The isolated mRNA was used for reference gene study prior to the microarray experiment and it's quality was checked by an optical density ratio test.

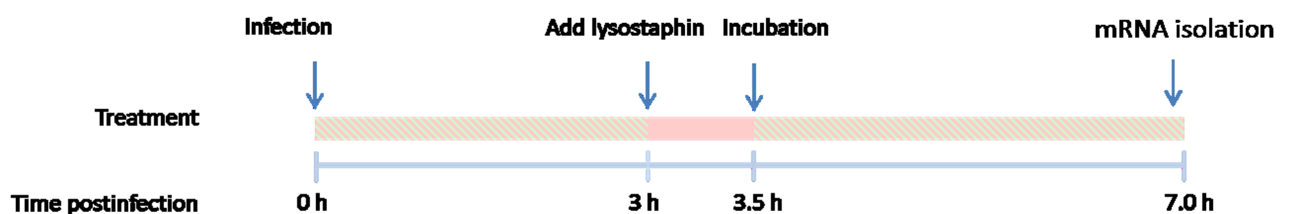


Figure 21. The scheme of HUVEC infection for expression studies.

Prior the microarray assay, we tested expression response of reference genes ICAM-1, VCAM-1 and E-selectin by RT-qPCR. These genes are already known to be differentially upregulated in endothelium upon *S. aureus* infection as well as after TNF-α stimulation.

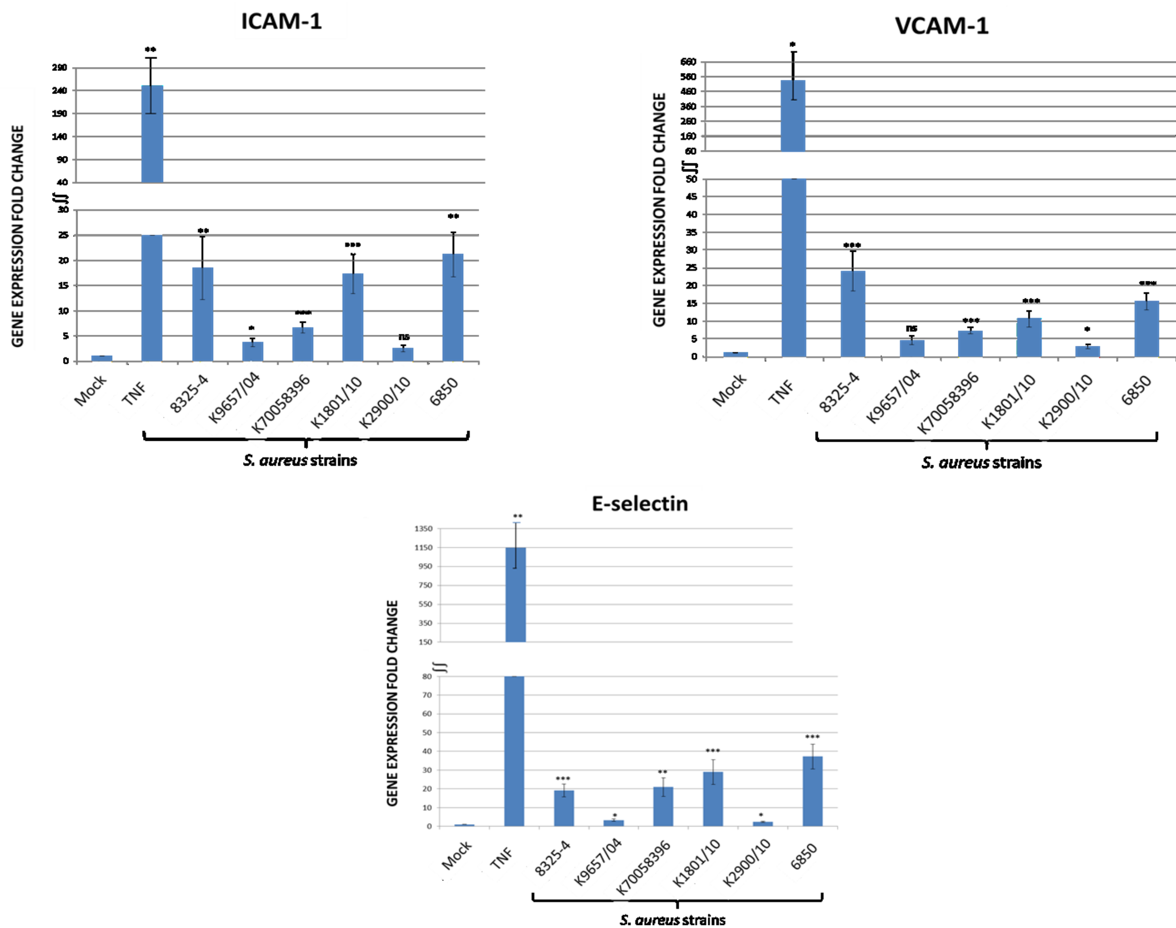


Figure 22. Differential expression of endothelial adhesion molecules upon *S. aureus* infection or TNF-α treatment. Data shown is the mean from 5 independent experiments, as indicated, ± SEM. The p-values were determined related to a mock-infected control, respectively. *=p≤0.05, **=p≤0.005, ***=p≤0.0005, ns=not significant

S. aureus infection and TNF-α treatment caused high upregulation of endothelial pro-inflammatory response genes in reference to ICAM-1, VCAM-1 and E-selectin expression (Fig. 22). The result of the expression of pro-inflammatory response genes was considered during experimental design and was used to validate the reliability of the experiments.

The microarray experiment (Fig. 23) was performed using isolated HUVEC mRNA after infection with six selected *S. aureus* strains and TNF-α treatment. HUVEC were infected and mRNA was isolated in accordance to protocol illustrated in figure 24 (Fig. 24).

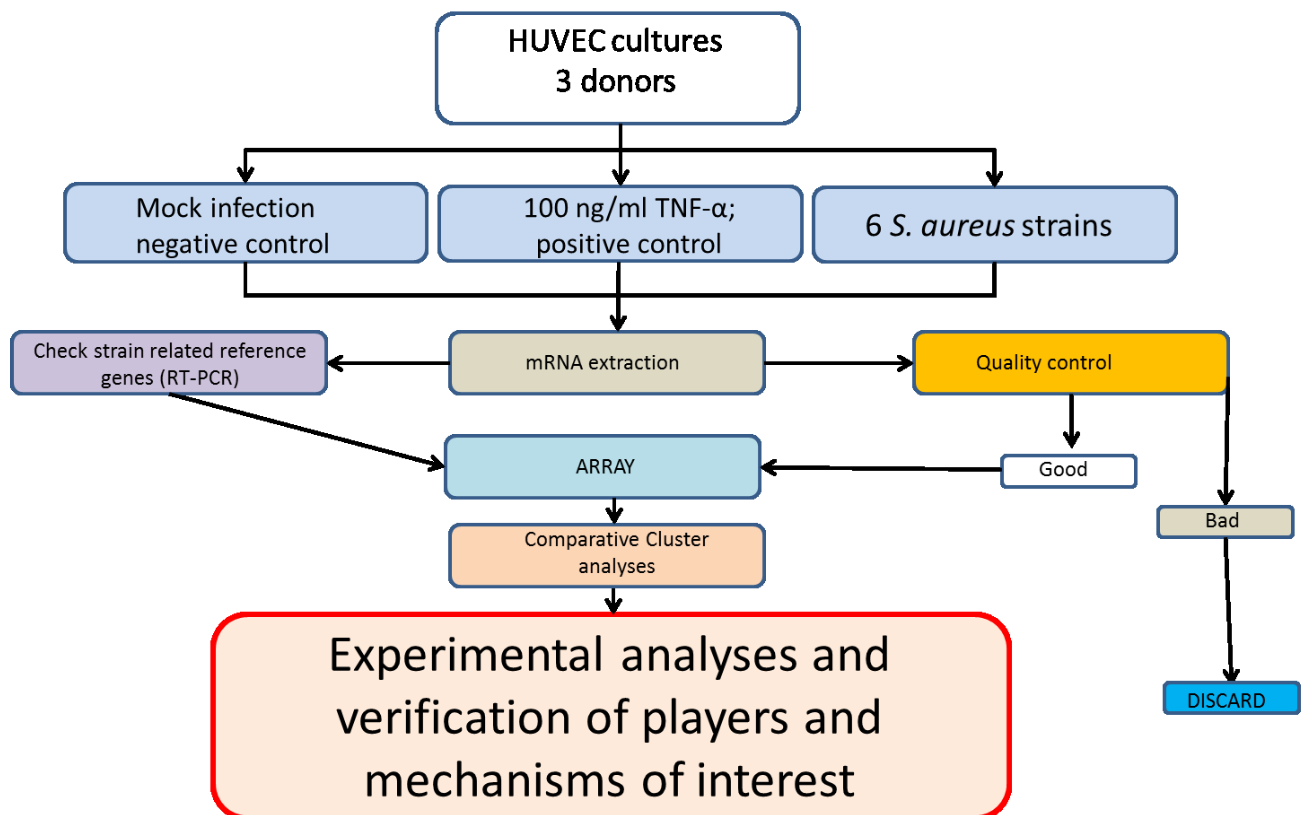


Figure 23. Microarray experiment

3.7. Microarray controls

3.7.1 Microarray internal controls

Transcriptome analysis was performed using DNA-microarray technology (Illumina) in cooperation with the Institute of Human Genetics in Münster, headed by Prof. Dr. Monika Stoll. Experimental procedures were performed according to the scheme (Fig. 23). For the transcriptome analysis, a HumanHT-12 v4 Expression BeadChip microarray detection chip was used.

Prior to expression, data analyses' internal quality controls governing microarray performance were considered. The microarray internal controls represent a technical note, which describes the concept; metrics and techniques ensure the quality of collected data. Based on the expected quality standards, a decision was made concerning further analyses (Illumina, 2010) (Table 6).

Control Metric	Expected value	Notes**
Hybridization Controls*	High > Medium > Low	There are three spikes in concentrations of Cy3-labeled oligos with perfect matches to control oligos on the BeadChip. The data will be considered acceptable if the signal intensities from these hybridization controls are present at low, medium, and high signal levels, respectively.
Low Stringency*	PM (perfect match) > MM2 (mismatch)	MM2 (oilgo with two mismatches to a control oligo on the beadchip) and PM (oligo with a perfect match to a control oligo on the beadchip). These controls provide a way of judging if the stringency is too low. Ideally, the ratio of PM to MM2 should be higher. If this ratio is low then the stringency is not high enough.
Biotin and High Stringency*	High	The biotin control is biotin labeled oligo that binds to an oligo on the BeadChip and stains with Streptavidin-Cy3. The low intensity for the biotin control indicate complications with the staining procedure.
Negative Controls (Background and Noise)	Low	The negative controls provide a measure for the stringency of the hybridization which depends on various conditions such as temperature, formamide concentration, high temperature wash and other factors. The data will be considered acceptable if the signal for the negative signal is very low or absent.
Gene Intensity (Housekeeping and All Genes)	Higher than Background (Housekeeping > All Genes)	Gene intensity is used to evaluate the quality of the samples. The signal level of housekeeping genes should be a very high when compared to the signal of all genes.
Labeling and Background	If used, Labeling > Background; Otherwise, Labeling ≈ Background	Labelled controls come from external nucleic acids (RNA from Bacillus sp.) that are spiked into the reverse transcription reaction. These controls track the efficiency of reverse transcription reaction and staining. The labeling and background controls should have approximately equal and low signal intensities. The background value provides a measures of the signal intensity resulting from the auto florescence of the surface array and nonspecific binding of target or stain molecules. High levels of background cause an overall loss of sensitivity in the experiment.

Table 4. Microarray quality controls. * Sample-independent controls. ** Description of internal controls ((YCGA), 2015)

Internal controls are distinguished as sample-dependent and sample-independent controls (Table 4). Sample-independent controls evaluate BeadChip and reagent

performance, efficiency of hybridization and the staining process. The sample-dependent controls are used to evaluate sample quality and performance. They include negative controls, gene intensity and labeling as well as the background controls (Illumina, 2010). The specific report on microarray internal controls is illustrated in figure 24 (Fig. 24). The reliability of each internal control parameter corresponded to the manufacturer´s requirements (Table 6).

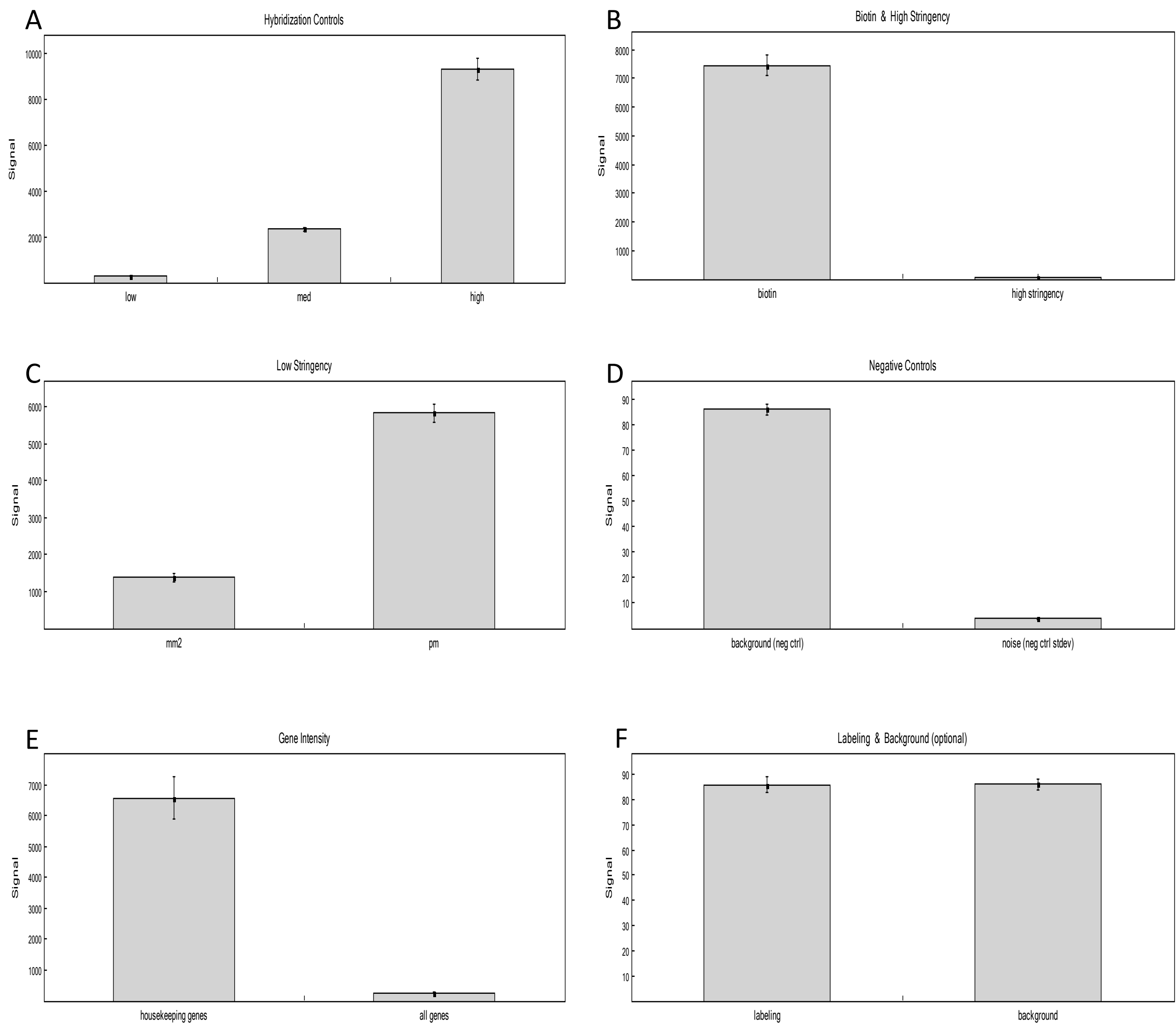
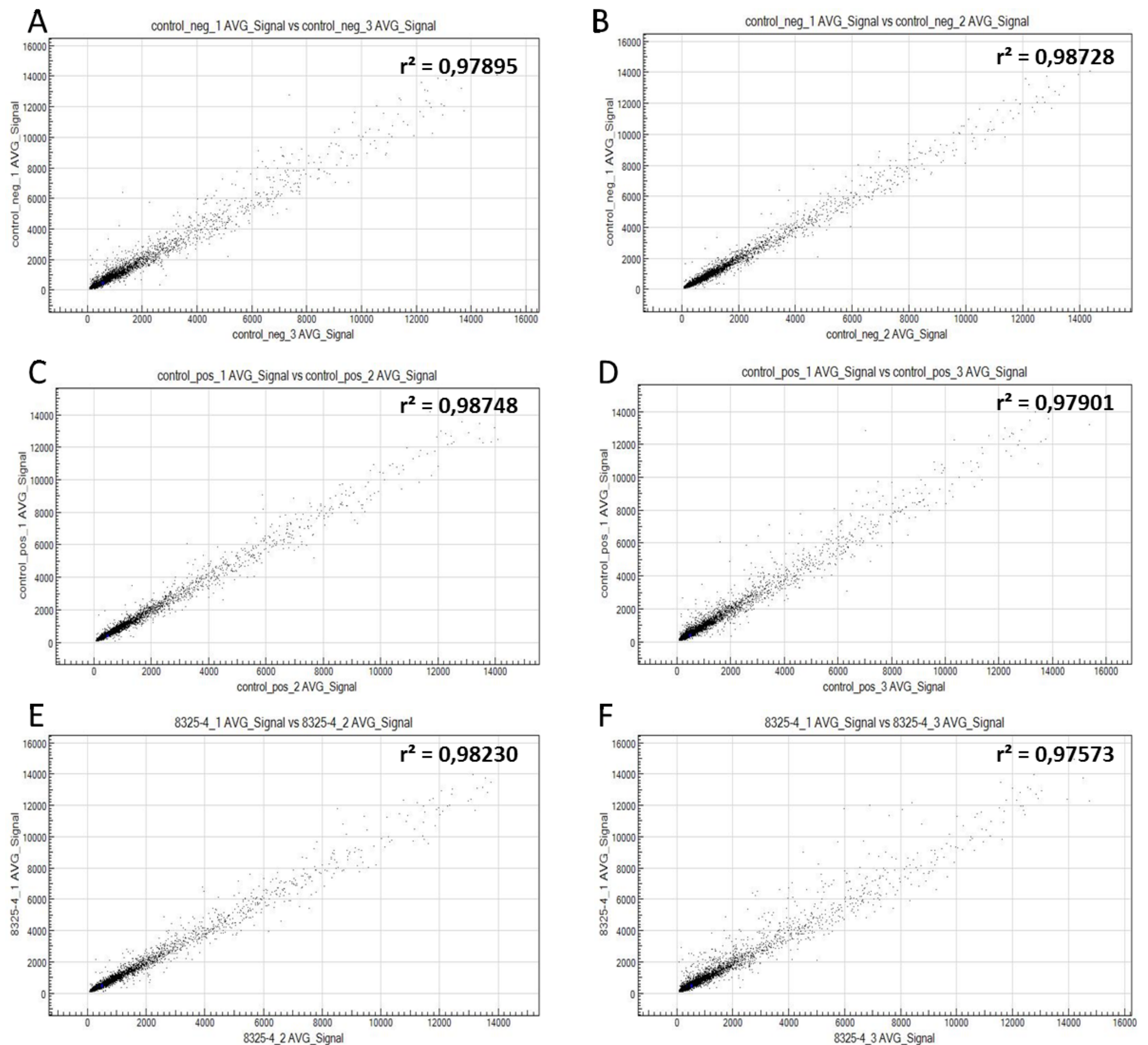
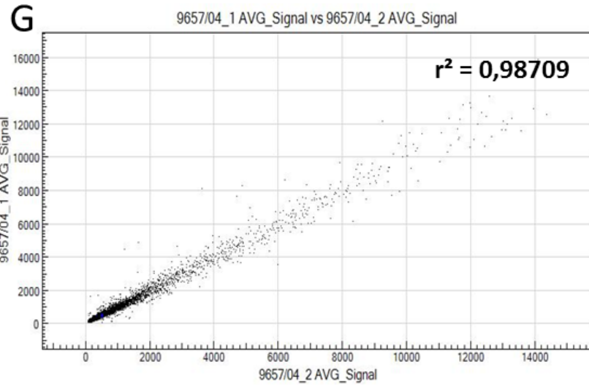


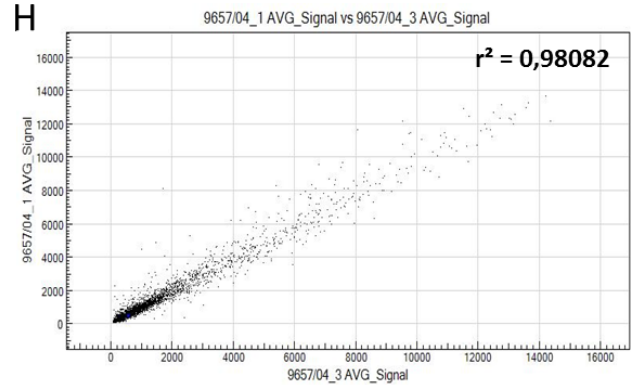
Figure 24. Control summary plot. A. Hybridization controls; B. Biotin and high stringency; C. Low stringency; D. Negative controls ; E. Gene intensity (Housekeeping genes and All genes); F. Labeling and background.

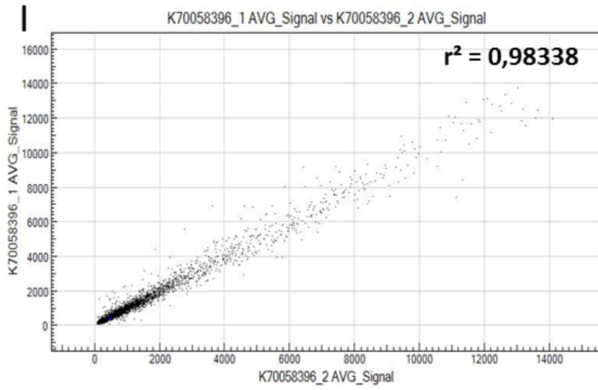
3.7.2. Determining sample similarity by scatter plotting.

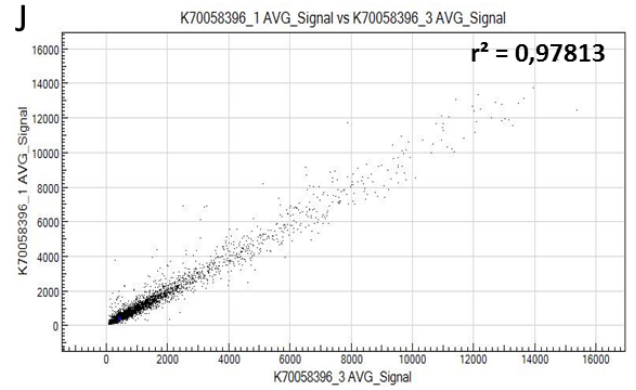
The scatter plots compare the data on transcription expression levels between two samples and represents the homogeneity of replicate expression. Biological replicates should exhibit similar transcript levels. The linear correlation values (r^2) was > 0.95 in all samples for hybridization replicates (Illumina, 2010) (Fig. 25).

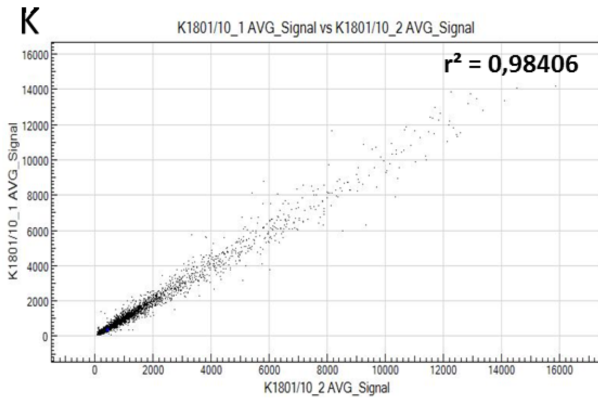


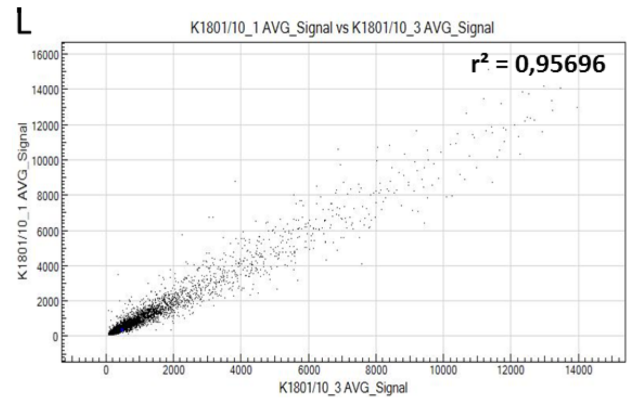

G
9657/04_1 AVG_Signal vs 9657/04_2 AVG_Signal
r² = 0,98709
9657/04_1 AVG_Signal
9657/04_2 AVG_Signal


H
9657/04_1 AVG_Signal vs 9657/04_3 AVG_Signal
r² = 0,98082
9657/04_1 AVG_Signal
9657/04_3 AVG_Signal


I
K70058396_1 AVG_Signal vs K70058396_2 AVG_Signal
r² = 0,98338
K70058396_1 AVG_Signal
K70058396_2 AVG_Signal


J
K70058396_1 AVG_Signal vs K70058396_3 AVG_Signal
r² = 0,97813
K70058396_1 AVG_Signal
K70058396_3 AVG_Signal


K
K1801/10_1 AVG_Signal vs K1801/10_2 AVG_Signal
r² = 0,98406
K1801/10_1 AVG_Signal
K1801/10_2 AVG_Signal


L
K1801/10_1 AVG_Signal vs K1801/10_3 AVG_Signal
r² = 0,95696
K1801/10_1 AVG_Signal
K1801/10_3 AVG_Signal

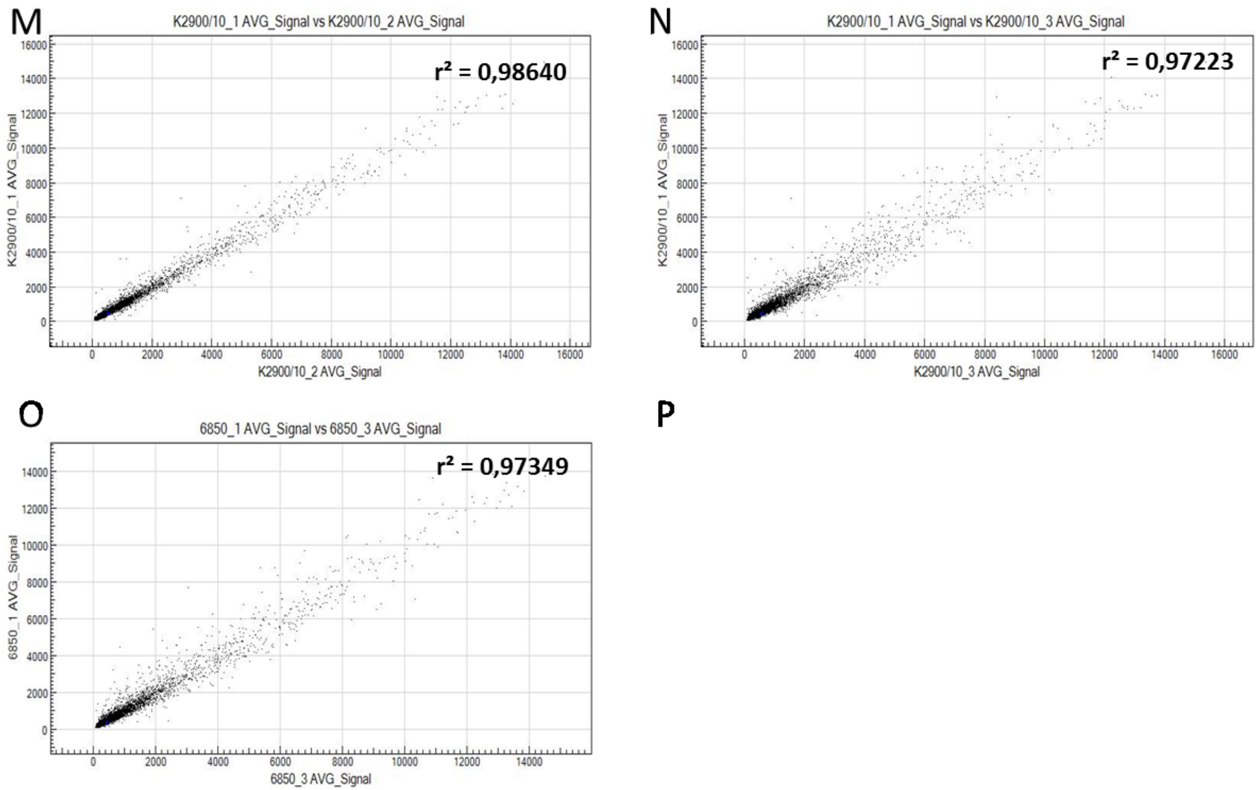


Figure 25. The data quality diagnosis by scatter plot. A.- P. Plotted detection values for sample replicates.

The control parameters' assessment of the performed microarray confirmed the high validity and reliability of the obtained data.

3.7.3. Validation of microarray data by RT-qPCR

One of the most important steps to validate microarray results is to confirm expression data with RT-qPCR. This tactic is most frequently used as an independent gene expression profiling method.

The mRNA used for microarray analysis data-validation was isolated through an identical treatment protocol as previously described for microarray and reference gene analyses (Fig 11). Based on the microarray analysis data, we selected 30 genes from each of the three functional groups for validation. The selected genes are characteristic of the following functional groups: a) apoptosis (6 genes, Fig. 26); b) immune response (13 genes, Fig. 27), c) cytoskeleton/development (9 genes, Fig. 28).

RT-PCR validates gene array data sets

Apoptotic genes

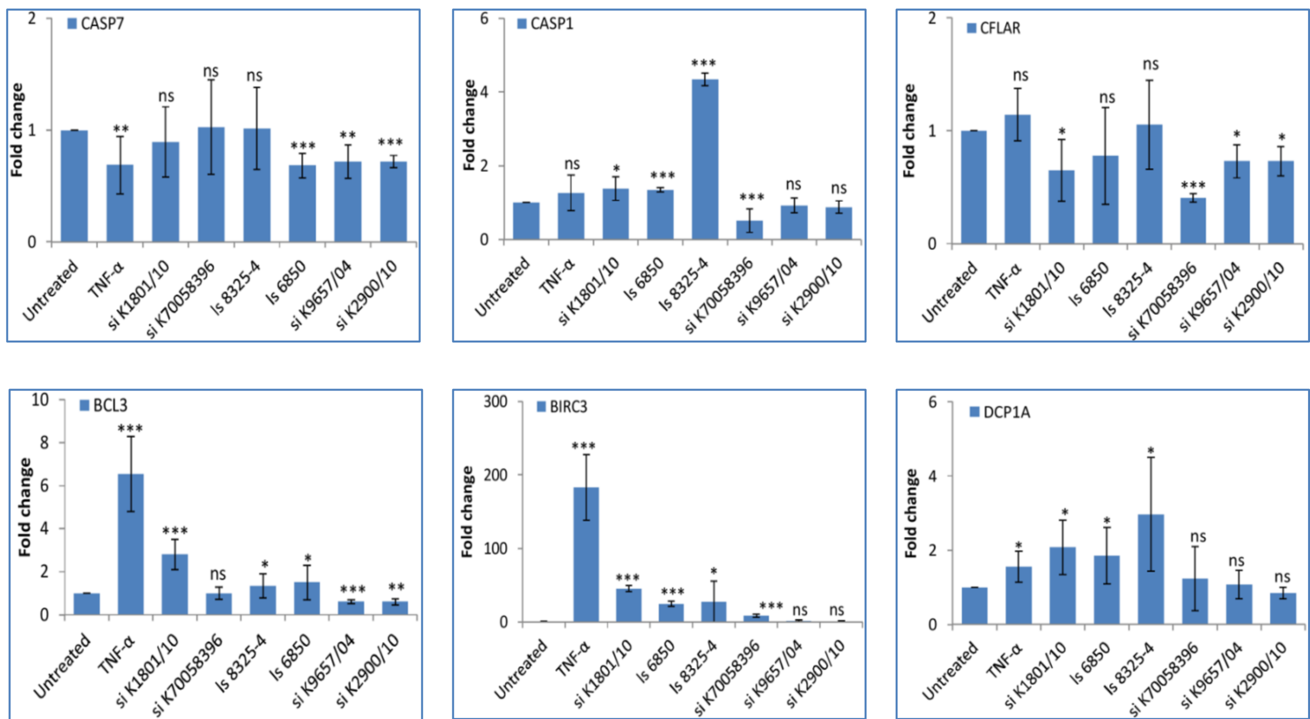


Figure 26. Expression of the selected endothelial genes involved in endothelial cell death by RT-qPCR. The data shown is the mean from minimum three independent experiments, indicated as ± SEM. The p-values were determined related to mock-infected control. *=p≤0.05,**=p≤0.005, ***=p≤0.0005, ns=not significant.

Immune response gene

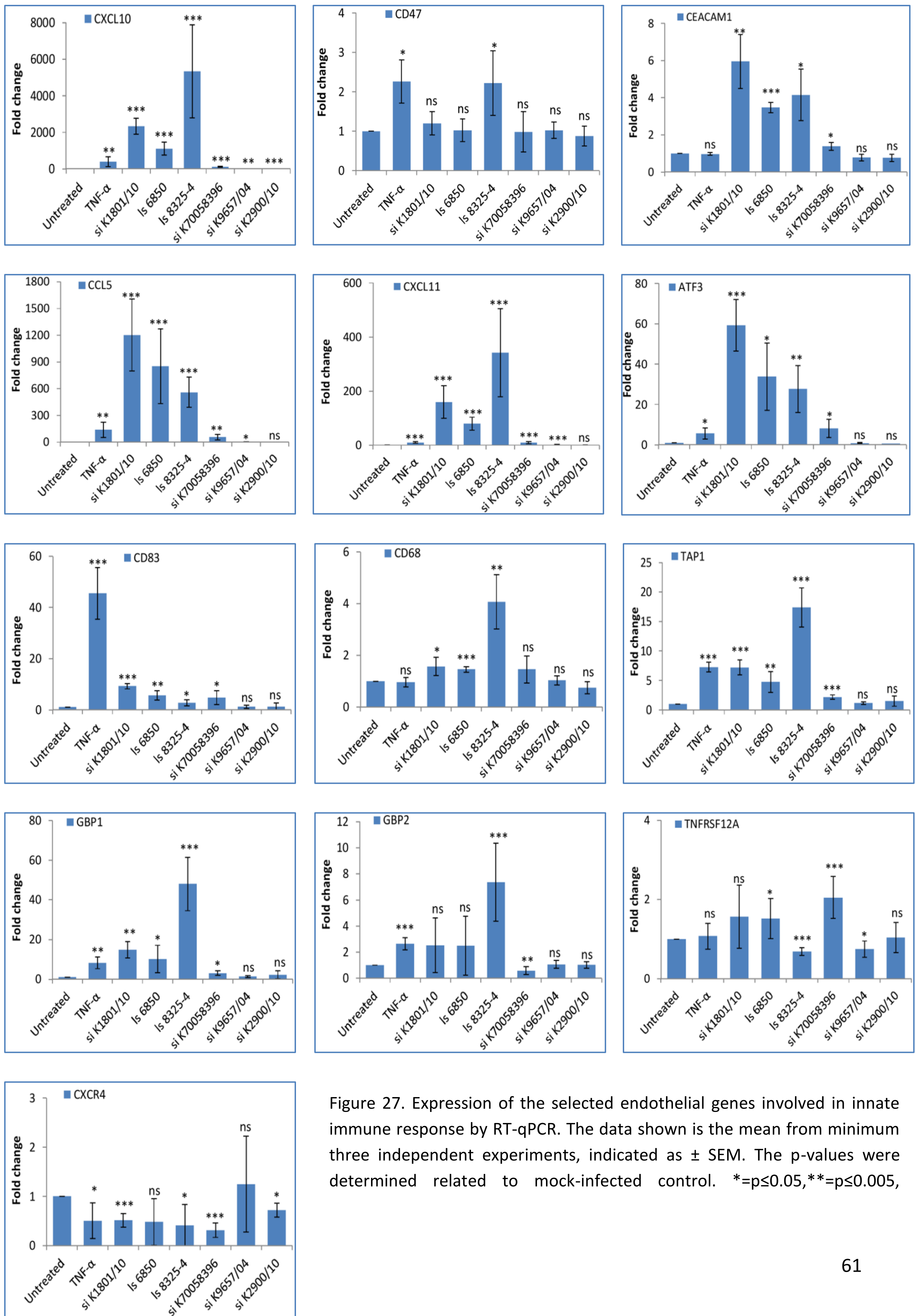


Figure 27. Expression of the selected endothelial genes involved in innate immune response by RT-qPCR. The data shown is the mean from minimum three independent experiments, indicated as ± SEM. The p-values were determined related to mock-infected control. *=p≤0.05,**=p≤0.005,

Cytoskeleton/Development

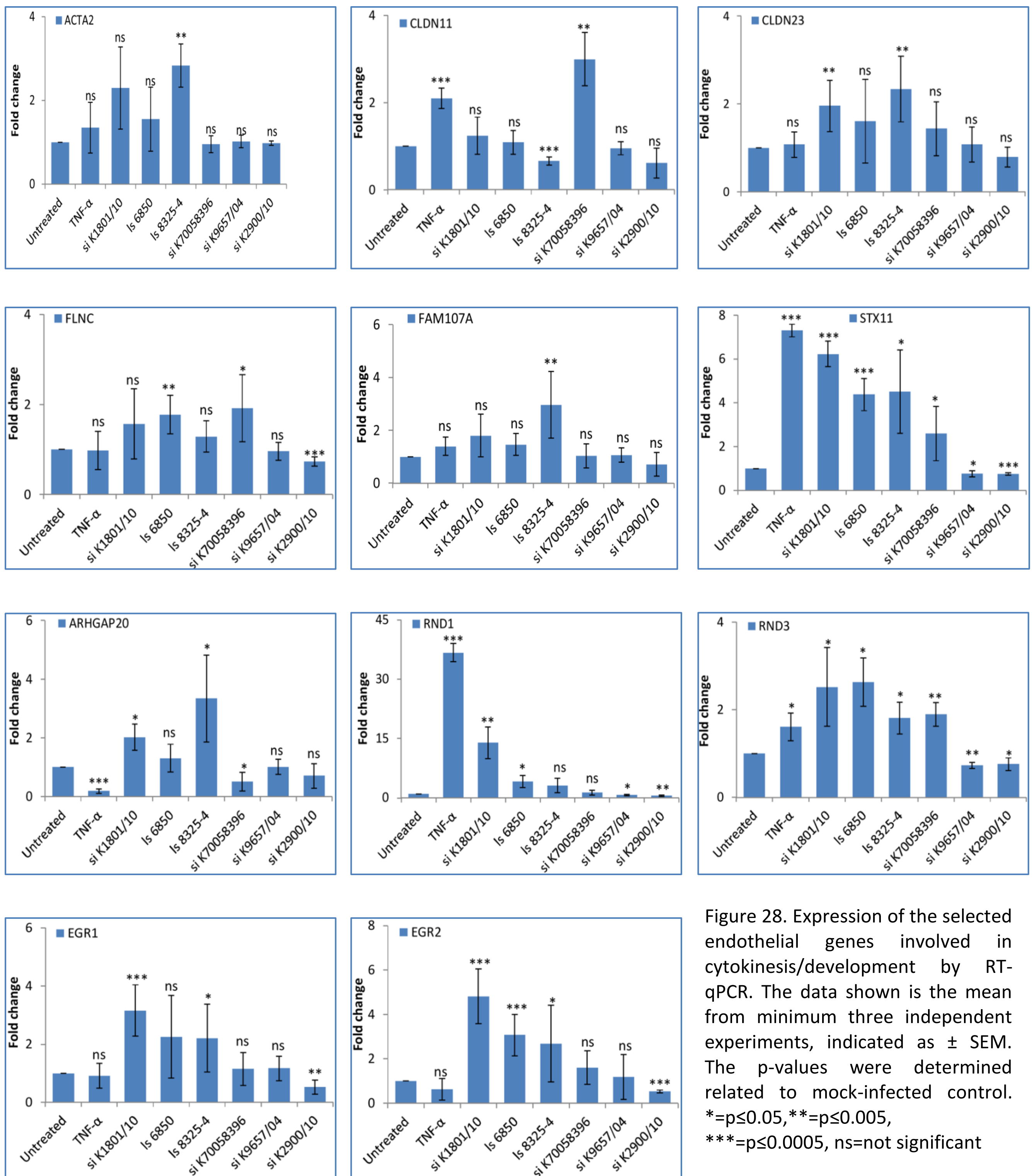


Figure 28. Expression of the selected endothelial genes involved in cytokinesis/development by RT-qPCR. The data shown is the mean from minimum three independent experiments, indicated as ± SEM. The p-values were determined related to mock-infected control. *=p≤0.05, **=p≤0.005, ***=p≤0.0005, ns=not significant

Validation by RT-qPCR expression confirmed that 95% of the genes display the same expression pattern as seen in the microarray analysis. Due to the higher sensitivity of RT-qPCR, we also saw changes in differential gene expression for the nine transcripts with

strains K9657/04 and K2900/10 demonstrating a much weaker response when compared to the other four isolates (Fig. 26-28).

The high correlation of the microarray data and the values obtained from RT-qPCR designate that this experiment has yielded reliable data which can be used for further bioinformatical data analyses.

3.7.4. Principal component analysis

Principal component analysis (PCA) is a statistical method that allows for the visualization and investigation of variability in a given dataset. This is achieved by assigning so-called principal components, representing the principal directions along which data variation is highest (Powell V., 2014). When applied to microarray data, the PCA identifies the set of genes with the greatest variability, which is invaluable in these experiments as differential expression between few treatments should be investigated.

We used PCA as a first approach to analyze gene expression data before applying threshold or any other sorting approach (Fig. 28). The gene set assigned to PC1 seems best to reflect differences between the strains, grouping them in the separate clusters with only few intersecting points. Interestingly, areactive strains are grouped together with the negative control. The pro-inflammatory strain 8325-4, on the other hand, is grouped close to the positive control, pro-inflammatory mediator TNF-α. Data analysis assigns PC1 gene ontology (GO)-terms including response to type I interferon and response to a virus, associated with STAT- and IRF-dependent transcription.

PC2 appears to reflect the differences between endothelial cell cultures isolated from different donors, thereby pulling data from separate replications of the same treatment along the y-axis. PC2 is assigned GO-terms including translation, cell adhesion and wound healing, which might be associated with transcription factors such as SPII, GABPA, Egr1 (Fig. 33).

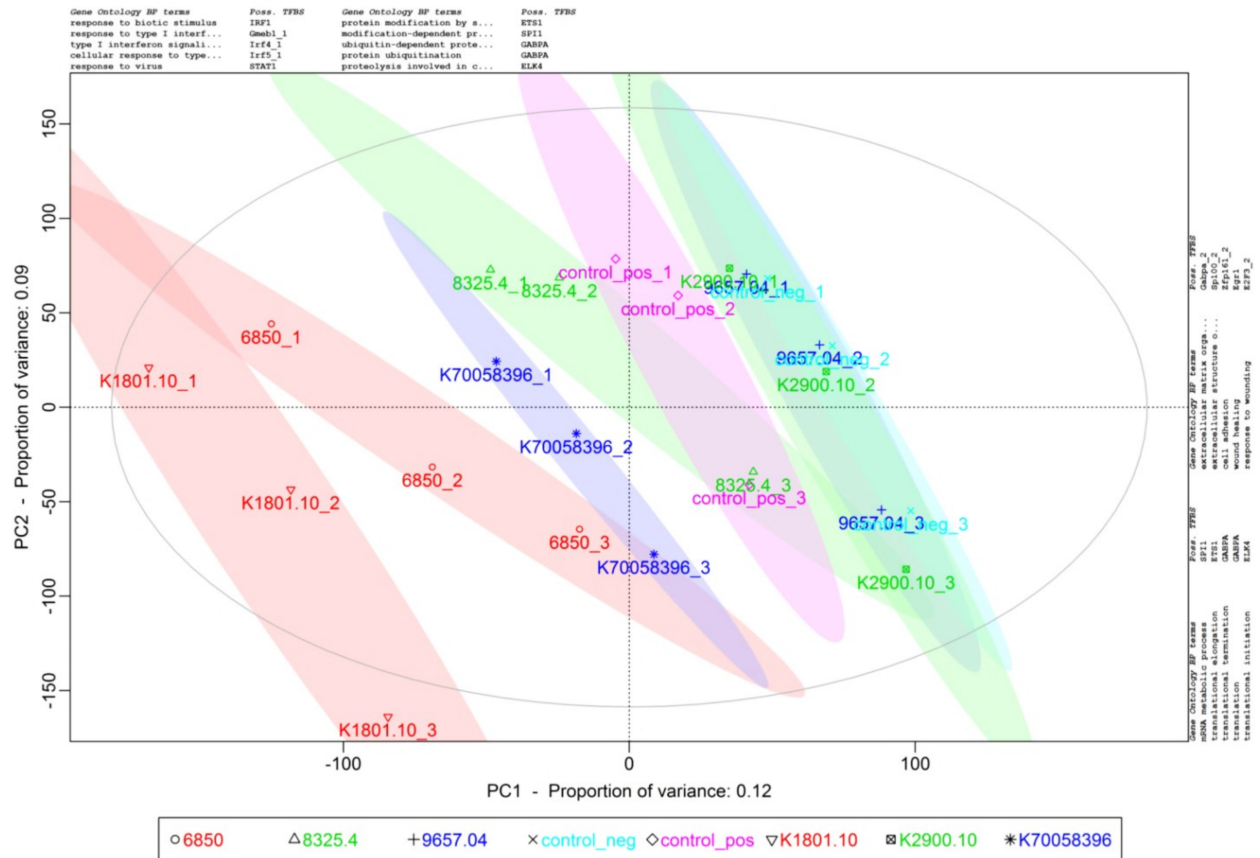


Figure 28. Principal component analysis of microarray data. Components ranged from the largest to the smallest variations; the first two components are plotted. Three replications of the same treatment are encircled.

The PCA results confirmed strain-specific differences in the transcriptional responses of the infected endothelium.

3.8. Microarray results

3.8.1. Differential expression of HUVECs infected with different *S. aureus* strains

The overall result of the microarray with all treatments showed significant differences in expression of 4968 of the 34695 HUVEC genes screened. In the following analysis, we included the expression data of 875 genes. 505 of these were significantly up-regulated to twice their original level of expression and 370 were significantly down-regulated to less than 60% of their original level of expression by at least one of the treatments.

The statistics for obtained data were analyzed by the statistical test package of GenomeStudio Software (Illumina). Gene expression was considered statistically significant when P-values were lower than 0.05. The statistically significant data showed an upregulation to more than twice the original level of expression and a downregulation of less than 60% of their original level of expression; values that were used in further analyses.

	TNF	K1801/10	6850	8325-4	K70058396	K9657/04	K2900/10
↑	176	255	160	173	60	2	0
↓	214	355	118	51	38	0	0
Unique genes:							
↑	114	88	10	73	3	0	0
↓	126	248	28	23	7	0	0

Figure 29. Number of HUVEC´ differentially expressed genes infected with *S. aureus* isolates and TNF-α treated in comparison with mock infection. In the red box is the number of differentially expressed genes for the strains that did not affect endothelial gene expression response. The data represents three independent experiments. The p-values were determined in relation to differential expression of mock-treated control and detection p-values respectively. The differential p-value: $p \leq 0.05$. The detection p-value: $p \leq 0.05$.

The strain K1801/10 induced the differential expression of 610 transcripts. Strains 8325-4 and 6850 caused relatively high levels of gene expression changes to occur, including a respective 278 and 224 differentially expressed genes. Strain K70058396 changed the expression levels of 98 genes.

A similar gene expression trait is also true for the differential expression of unique transcripts. Unique genes are defined as genes that change expression only during a single treatment such as infection or TNF-α. The highest number of unique gene expression changes was observed for strain K1801/10 – 336 transcripts and the lowest for strain K70058396 with only 10 genes. Strains K2900/10 and K9657/04 did not show expression changes for almost any of the transcripts, and were therefore excluded from further group analyses (Fig. 29).

TNF-α treatment induced a differential expression of 390 gene transcripts with high levels of unique gene expression, namely 240 gene transcripts.

3.8.2. Differential expression of HUVEC innate immunity genes

In the next stage, the transcriptome of all differentially expressed genes was grouped using functional clustering approach. Particularly, "The Database for Annotation, Visualization and Integrated Discovery" (DAVID) v6.7 software was used in which clusters were selected in accordance to Fisher exact p-value≤0.05 based on their enrichment score (Huang da *et al.*, 2009b; Huang da *et al.*, 2009a). The Fisher exact p-value determines whether a particular user gene list is associated (enriched) for a particular signaling pathway when compared to the sets of genes taken-up by random chance. Additionally, the online database "GeneCards" (for functional annotation of genes) was used to verify particular gene functions (GeneCards, 1996-2015). Gene lists, analyzed by these two approaches, were divided into four main groups: genes related to innate immunity (Table 5), apoptotic genes (Table 6), genes related to cytokinesis and development (Table 7) and genes encoding transcription factors and signaling molecules (Table 8).

Gene symbol	***Gene bank accession number***	***Full gene name***	***Fold change TNF-α treated***	***Fold change K1801/10***	***Fold change 6850***	***Fold change K70058396***	***Fold change 8325-4***
			Pos. Control	*S. aureus* strains			
Genes related to innate immunity							
Cytokines and chemokines							
CCL2	NM_002982.3	chemokine (C-C motif) ligand 2	21,33	9,87	7,97	6,79	7,33
CCL20	NM_004591.1	chemokine (C-C motif) ligand 20	22,86	3,88	2,45	NS	NS
CCL5	NM_002985.2	chemokine (C-C motif) ligand 5	2,1	17,77	10,9	1,95	NS
CCL8	NM_005623.2	chemokine (C-C motif) ligand 8	NS	11,43	8,84	NS	19,61
CX3CL1	NM_002996.3	chemokine (C-X3-C motif) ligand 1	71,43	25,29	23,4	5,62	21,97
CXCL1	NM_001511.1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	10,17	9,11	3,55	5,45	NS
CXCL10	NM_001565.1	chemokine (C-X-C motif) ligand 10	16,7	84,8	64,62	12,32	107,3
CXCL2	NM_002089.1	chemokine (C-X-C motif) ligand 2	9,25	11,12	4,79	NS	NS

CXCL9	NM_002416.1	chemokine (C-X-C motif) ligand 9	NS	4,5	2,26	NS	8,49
IL10	NM_000572.2	interleukin 10	NS	3,85	2,69	NS	NS
IL6	NM_000600.1	interleukin 6 (interferon, beta 2)	NS	13,3	NS	NS	NS
IL8	NM_000584.2	interleukin 8	25,97	10,11	7,42	NS	NS
Adhesion molecules							
CD47	NM_001025080.1	CD47 molecule	1,78	NS	1,5	NS	2,22
CD93	NM_012072.2	CD93 molecule	0,79	0,59	0,83	0,85	NS
CEACAM1	NM_001024912.1	carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein)	NS	3,47	2,96	NS	2,7
CLEC14A	NM_175060.1	C-type lectin domain family 14, member A	0,61	0,59	0,68	0,75	NS
ICAM1	NM_000201.1	intercellular adhesion molecule 1	37,43	5,03	3,49	NS	3,53
LGALS8	NM_201544.1	lectin, galactoside-binding, soluble, 8	NS	NS	NS	NS	2,07
SDC4	NM_002999.2	syndecan 4	2,39	2,37	1,75	1,25	NS
SELE	NM_000450.1	selectin E	50,35	8,1	NS	NS	NS
Antigen presentation/reseptors							
CD274	NM_014143.2	CD274 molecule	NS	2,42	1,77	NS	1,98
CD68	NM_001251.2	CD68 molecule	NS	1,81	1,69	NS	2,41
CD83	NM_004233.3	CD83 molecule	11,13	3,7	3,35	NS	NS
COLEC12	NM_130386.1	collectin sub-family member 12	0,79	0,58	NS	0,68	0,75
IFI30	NM_006332.3	interferon, gamma-inducible protein 30	NS	1,48	1,27	NS	2,44
KLRG1	NM_005810.3	killer cell lectin-like receptor subfamily G, member 1	0,53	0,57	NS	NS	NS
PSMB10	NM_002801.2	proteasome (prosome, macropain) subunit, beta type, 10	1,72	NS	NS	NS	2,19

PSMB8	NM_148919.3	proteasome (prosome, macropain) subunit, beta type, 8 (large multifunctional peptidase 7)	1,22	1,85	1,67	NS	3,02
PSMB9	NM_002800.4	proteasome (prosome, macropain) subunit, beta type, 9 (large multifunctional peptidase 2)	1,35	1,68	1,62	NS	3,13
PTX3	NM_002852.2	Pentraxin 3, Long	3,76	2,34	2,06	2,49	NS
TAP1	NM_000593.5	transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	5,9	6,97	6,2	2,93	11,59
TAP2	NM_000544.3	transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)	1,46	1,36	1,23	NS	2,63
TLR3	NM_003265.2	toll-like receptor 3	NS	1,7	1,67	NS	3,38
Complement							
CFB	NM_001710.4	complement factor B	NS	2,3	NS	NS	NS
F3	NM_001993.2	coagulation factor III (thromboplastin, tissue factor)	4,79	2,73	NS	NS	NS
PLAUR	NM_002659.2	plasminogen activator, urokinase receptor	NS	2,73	2,25	1,86	NS
SERPINE1	NM_000602.1	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	3,98	3,19	2,62	NS	NS
Interferon response							
EPSTI1	NM_033255.2	epithelial Stromal Interaction Protein 1	NS	12,62	12,44	5,32	27,21

GBP1	NM_002053.1	guanylate binding protein 1, interferon-inducible, 67kDa	4,37	8,81	7,6	2,35	16,99
GBP2	NM_004120.3	guanylate binding protein 2, interferon-inducible	1,73	NS	1,77	NS	3,55
GBP4	NM_052941.2	guanylate binding protein 4	1,8	3,31	2,49	NS	5,09
GBP5	NM_052942.2	guanylate binding protein 5	NS	3,17	1,84	NS	3,55
FAM111A	NM_022074.2	family with sequence similarity 111, member A	1,32	1,5	NS	NS	2,04
FAM46C	NM_017709.2	family with sequence similarity 46, member C	1,26	1,36	NS	NS	2,13
HERC5	NM_016323.1	hect domain and RLD 5	NS	13,53	11,11	3,12	13,69
HERC6	NM_001013005.1	hect domain and RLD 6	NS	4,41	3,74	1,61	7,13
IFI16	NM_005531.1	interferon, gamma-inducible protein 16	NS	1,68	1,93	NS	2,69
IFI35	NM_005533.2	interferon-induced protein 35	1,42	4,29	3,33	NS	10,56
IFI44	NM_006417.2	interferon-induced protein 44	NS	6,13	5,93	2,94	8,36
IFI44L	NM_006820.1	interferon-induced protein 44-like	NS	6,88	5	2,58	16,66
IFI6	NM_002038.2	interferon, alpha-inducible protein 6	NS	NS	NS	NS	3,28
IFIH1	NM_022168.2	interferon induced with helicase C domain 1	4,64	9,86	8,91	2,85	17,38
IFIT1	NM_001548.2	interferon-induced protein with tetratricopeptide repeats 1	NS	44,67	40,92	18,71	45,26
IFIT2	NM_001547.3	interferon-induced protein with tetratricopeptide repeats 2	2,77	41,91	41,21	16,29	41,57
IFIT3	NM_001031683.1	interferon-induced protein with tetratricopeptide repeats 3	2,11	26,83	20,86	7,7	29,92

IFIT5	NM_012420.1	interferon-induced protein with tetratricopeptide repeats 5	NS	2,48	2,91	2,17	4
IFITM1	NM_003641.2	interferon-induced transmembrane protein 1 (9-27)	NS	5,71	5,42	2,65	9,92
IFNB1	NM_002176.2	interferon, beta 1, fibroblast	NS	44,8	18,6	NS	NS
ISG15	NM_005101.1	ISG15 ubiquitin-like modifier	NS	16,48	12,27	6,1	18,43
ISG20	NM_002201.4	interferon stimulated exonuclease gene 20kDa	5,26	10,88	9,65	NS	15
MX1	NM_002462.2	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	NS	7,85	7,08	4,05	10,92
MX2	NM_002463.1	myxovirus (influenza virus) resistance 2 (mouse)	NS	5,31	NS	NS	14,52
TRIM21	NM_003141.3	tripartite motif-containing 21	2,12	2,9	3,11	1,92	5,57
TRIM22	NM_006074.2	tripartite motif-containing 22	1,44	1,84	1,7	NS	3,63
TRIM25	NM_005082.4	tripartite motif-containing 25	NS	NS	1,59	NS	3,03
TRIM38	NM_006355.2	tripartite motif-containing 38	0,87	1,65	1,29	NS	2,13
TRIM56	NM_030961.1	tripartite motif-containing 56	1,76	1,86	1,53	1,28	2,17
USP18	NM_017414.3	ubiquitin specific peptidase 18	NS	6,49	5,51	2,33	10,63
Other genes involved in immune response							
ANGPT2	NM_00111888 8.1	angiopoietin 2	NS	0,59	NS	NS	NS
APOL3	NM_030644.1	apolipoprotein L, 3	4,68	NS	2,04	NS	5,01
CH25H	NM_003956.2	cholesterol 25-Hydroxylase	0,68	4,9	2,27	NS	NS
GCH1	NM_000161.2	GTP cyclohydrolase 1	2,42	2,94	2,85	1,44	4,1
IDO1	NM_002164.4	indoleamine 2,3-dioxygenase 1	1,92	7,83	3,62	1,25	20,65
LOC10012	XM_001721430	hypothetical	NS	2,53	NS	NS	NS

9362	.1	LOC100129362					
LRRC33	NM_198565.1	leucine rich repeat containing 33	0,35	0,56	0,65	NS	0,63
OAS1	NM_002534.2	2',5'-oligoadenylate synthetase 1, 40/46kDa	NS	6,43	4,3	2,13	9,89
OAS2	NM_002535.2	2'-5'-oligoadenylate synthetase 2, 69/71kDa	NS	7,94	6,78	3,05	13,28
OAS3	NM_006187.2	2'-5'-oligoadenylate synthetase 3, 100kDa	NS	3,48	2,85	1,6	6,84
OASL	NM_003733.2	2'-5'-oligoadenylate synthetase-like	NS	49,95	29,84	6,08	39,17
PTGS2	NM_000963.1	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	NS	9,25	6,39	NS	3,9
RFTN1	NM_015150.1	raftlin, lipid raft linker 1	NS	NS	0,58	NS	NS
RFTN2	NM_144629.1	raftlin family member 2	0,58	NS	NS	0,56	NS
SP100	NM_001080391.1	SP100 nuclear antigen	NS	1,96	1,46	NS	2,78
SP110	NM_080424.1	SP110 nuclear body protein	NS	3,69	3,49	2,02	7,8
SPAG9	NM_172345.1	sperm associated antigen 9	1,51	2,19	2,22	NS	NS

Table 5. The HUVEC expression of the immune response genes upon infection with *S. aureus* blood isolates. The genes were divided in several subgroups in accordance to their specific function. Each gene symbol corresponds with the full gene name; “Gene Bank” accession number; and fold of transcriptional change for every *S. aureus* isolates and TNF-alpha treatment. The significantly upregulated genes are shown in red; the significantly downregulated genes in blue; and the not significantly expressed genes in grey. The data shown is the mean from three independent experiments. The p-values were determined respectively to differential expression of mock-treated control and detection p-values. The differential p-value: p≤0.05; ns= not significant. The detection p-value: p≤0.05; ns= not significant.

3.8.3. Differential expression of cell death-related genes in endothelium

Gene symbol	*Gene bank accession number*	*Full gene name*	*Fold change TNF-α treated*	*Fold change K1801/10*	*Fold change 6850*	*Fold change K70058396*	*Fold change 8325-4*
			Pos. Control	*S. aureus* strains			
Apoptosis							
Pro-apoptotic genes							
AXUD1	NM_033027.2	Cysteine-Serine-Rich Nuclear Protein 1	2,78	7,48	3,4	2,45	2,52
ARHGEF6	NM_004840.2	Rac/Cdc42 guanine nucleotide exchange factor (GEF) 6	0,67	0,52	0,59	0,62	NS
BCL3	NM_005178.2	B-cell CLL/lymphoma 3	3,26	2,39	1,67	1,5	NS
BCL6	NM_001706.2	B-cell CLL/lymphoma 6	NS	2,96	1,82	NS	NS
CASP1	NM_033292.2	caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)	NS	NS	NS	NS	3
CASP7	NM_033338.4	caspase 7, apoptosis-related cysteine peptidase	1,44	1,62	1,69	1,22	2,19
DUSP1	NM_004417.2	dual specificity phosphatase 1	NS	3,43	2,75	NS	NS
PMAIP1	NM_021127.1	phorbol-12-myristate-13-acetate-induced protein 1	1,98	11,61	8,8	2,17	5,03
PLEKHF1	NM_024310.2	pleckstrin homology domain containing, family F (with FYVE domain) member 1	0,75	2,18	2,57	1,39	2,83
PHLDA1	NM_007350.3	pleckstrin homology-like domain, family A, member 1	2,63	4,23	2,63	2,09	NS
RIPK1	NM_003804.3	receptor (TNFRSF)-interacting serine-threonine kinase 1	NS	2,46	1,66	NS	2,42

TXNIP	NM_006472.1	thioredoxin interacting protein	0,36	NS	1,94	NS	2,85
TNFSF10	NM_003810.2	tumor necrosis factor (ligand) superfamily, member 10	NS	5,45	3,98	NS	9,69
TP53INP1	NM_033285.2	tumor protein p53 inducible nuclear protein 1	NS	0,45	0,55	0,69	0,77
Anti-apoptotic genes							
ACVR1	NM_001105.2	activin A receptor, type I	NS	0,59	0,71	0,71	NS
ANGPTL4	NM_001039667.1	angiopoietin-like 4	NS	10,07	NS	5,45	NS
BIRC3	NM_182962.1	baculoviral IAP repeat-containing 3	10,4	6	4,33	NS	NS
CBX4	NM_003655.2	chromobox homolog 4 (Pc class homolog, Drosophila)	NS	2,34	1,82	1,3	2,31
MCL1	NM_021960.3	myeloid cell leukemia sequence 1 (BCL2-related)	1,6	2,83	2,43	1,7	2,21
NR4A2	NM_006186.2	nuclear receptor subfamily 4, group A, member 2	NS	3,46	3,14	NS	NS
NUAK1	NM_014840.2	NUAK family, SNF1-like kinase, 1	0,22	0,4	0,42	0,64	0,56
NUAK2	NM_030952.1	NUAK family, SNF1-like kinase, 2	3,62	2,34	1,52	NS	NS
PARP10	NM_032789.1	poly (ADP-ribose) polymerase family, member 10	NS	1,79	1,56	NS	3,2
PARP12	NM_022750.2	poly (ADP-ribose) polymerase family, member 12	1,36	2,62	2,3	NS	4,27
PARP14	NM_017554.1	poly (ADP-ribose) polymerase family, member 14	3,13	2,9	1,96	NS	5,24
PARP9	NM_031458.1	poly (ADP-ribose) polymerase family, member 9	1,45	3,66	3,44	2,03	7,13
TIPARP	NM_015508.2	TCDD-inducible poly(ADP-ribose) polymerase	0,6	4,08	3,81	1,55	3,02

TNFAIP3	NM_006290.2	tumor necrosis factor, alpha-induced protein 3	16,46	11,49	5,15	NS	NS
Apoptosis regulatory genes							
C8orf4	NM_020130.2	chromosome 8 open reading frame 4	10,55	8,38	4,05	NS	NS
C21orf63	NM_058187.3	chromosome 21 open reading frame 63	2,28	2,44	1,83	1,8	NS
CCNA1	NM_003914.2	cyclin A1	NS	2,97	2,64	NS	NS
DCP1A	NM_018403.2	DCP1 decapping enzyme homolog A (S. cerevisiae)	1,34	2,28	1,93	1,26	2,18
DDIT4	NM_019058.2	DNA-damage-inducible transcript 4	0,48	1,79	NS	NS	0,51
DHX58	NM_024119.1	DEXH (Asp-Glu-X-His) box polypeptide 58	1,6	2,29	1,98	NS	3,93
DUSP19	NM_080876.2	dual specificity phosphatase 19	NS	7,4	5,04	1,77	7,22
DUSP5	NM_004419.3	dual specificity phosphatase 5	NS	2,27	2,59	1,81	2,43
EIF2AK2	NM_002759.1	eukaryotic translation initiation factor 2-alpha kinase 2	NS	2,87	2,78	NS	4,37
FAM188A	NM_024948.2	chromosome 10 open reading frame 97	NS	0,6	0,77	NS	0,82
GADD45B	NM_015675.1	growth arrest and DNA-damage-inducible, beta	NS	4,3	2,31	NS	NS
GDF15	NM_004864.1	growth differentiation factor 15	NS	2,7	NS	1,84	NS
HES1	NM_005524.2	hairy and enhancer of split 1, (Drosophila)	0,46	2,29	NS	NS	NS
HK2	NM_000189.4	hexokinase 2 pseudogene; hexokinase 2	NS	1,56	2,28	1,79	1,53
INDO	NM_002164.3	indoleamine 2,3-dioxygenase 1	1,93	7,94	NS	1,24	19,16
KIAA0196	NM_014846.2	KIAA0196	0,7	0,58	0,62	0,76	NS
MLKL	NM_152649.1	mixed lineage kinase domain-like	NS	2,43	2,26	1,48	3,99

NLRP8	NM_176811.2	NLR family, pyrin domain containing 8	NS	2,12	NS	NS	NS
PLSCR1	NM_021105.1	phospholipid scramblase 1	NS	3	2,28	NS	4,1
PPP1R15A	NM_014330.2	protein phosphatase 1, regulatory (inhibitor) subunit 15A	1,52	4,15	2,27	1,68	1,62
PRMT6	NM_018137.1	protein arginine methyltransferase 6	NS	0,55	0,76	NS	NS
REEP1	NM_022912.1	receptor accessory protein 1	0,63	0,55	0,66	0,6	0,72
SLC40A1	NM_014585.3	solute carrier family 40 (iron-regulated transporter), member 1	0,52	0,29	0,45	0,42	NS
TRIM69	NM_080745.3	tripartite motif-containing 69	NS	2,21	1,5	NS	NS
VIP	NM_003381.2	vasoactive intestinal peptide	NS	2,3	3,07	1,64	NS
XAF1	NM_199139.1	XIAP associated factor 1	1,26	2,75	2,45	1,61	5,72

Table 6. The HUVEC expression of genes related to cell death upon infection with *S. aureus* blood isolates. Genes are divided in several subgroups: pro-, anti-apoptotic and regulatory cell death genes. Each gene symbol corresponds with the full gene name; "Gene Bank" accession number; and fold of transcriptional change for every *S. aureus* isolate and TNF-alpha treatment. The significantly upregulated genes are shown in red; the significantly downregulated genes in blue; and the not significantly expressed genes in grey. The data shown is the mean from three independent experiments. The p-values were determined respectively to differential expression of mock-treated control and detection p-values. The differential p-value: $p \leq 0.05$; ns= not significant. The detection p-value: $p \leq 0.05$; ns= not significant.

The data indicates that the strain-specific differential expression of HUVEC genes related to cell death showed the strongest induction with cytotoxic *S. aureus* K1801/10 isolate (55 genes) and 6850 isolate (34 genes). However, the cytotoxic strain K70058396 only affected the expression of nine gene transcripts. The non-cytotoxic strain 8325-4 caused a change in expression of only 29 genes.

This outcome raised the question as to which factors and what mechanism could cause such a difference and change the expression levels of cell death-related genes so dramatically during strain-specific *S. aureus*-induced infection. To answer this question we conducted a comprehensive analysis of *S. aureus*-induced endothelial cell death regarding HUVEC gene expression, which is described in the following chapters.

3.8.4. Differential expression of HUVEC cytoskeleton and developmental genes

The genes of cytokinesis & development (Table 7) and transcription factors & signaling groups (Table 8) are listed below. These groups might hold important information concerning the mechanisms and signaling pathways involved in cytoskeleton regulation and infection signaling during pathophysiological infection changes in endothelium.

Gene symbol	*Gene bank accession number*	*Full gene name*	*Fold change TNF-α treated*	*Fold change K1801/10*	*Fold change 6850*	*Fold change K70058396*	*Fold change 8325-4*
			Pos. Control	*S. aureus* strains			
Cytokinesis and development							
Cytoskeleton/adhesion/migration							
ACTA2	NM_001613.1	actin, alpha 2, smooth muscle, aorta	NS	2,28	1,81	1,45	NS
AGGF1	NM_018046.3	angiogenic factor with G patch and FHA domains 1	0,75	0,57	0,73	0,75	NS
C16orf30	NM_024600.1	TMEM204 (Transmembrane Protein 204)	0,63	0,54	0,62	0,62	NS
CALM1	NM_006888.2	calmodulin 3 (phosphorylase kinase, delta); calmodulin 2 (phosphorylase kinase, delta); calmodulin 1 (phosphorylase kinase, delta)	NS	0,56	NS	NS	NS
CLDN11	NM_005602.4	claudin 11	NS	NS	NS	2,28	NS
CLDN23	NM_194284.1	claudin 23	NS	NS	NS	NS	3,13
CNKSR3	NM_173515.2	membrane associated guanylate kinase, WW and PDZ domain containing 1; CNKSR family member 3	6,11	2,78	3,33	1,53	NS

COL3A1	NM_000090.2	collagen, type III, alpha 1	NS	0,46	NS	NS	NS
DIXDC1	NM_001037954.2	DIX domain containing 1	0,6	0,56	0,62	0,68	0,75
FAM107A	NM_007177.1	family with sequence similarity 107, member A	NS	NS	NS	NS	2,16
FLNC	NM_001458.2	filamin C, gamma (actin binding protein 280)	NS	1,53	1,9	2,08	NS
FLRT2	NM_013231.4	fibronectin leucine rich transmembrane protein 2	NS	0,4	0,45	0,6	0,58
LIMCH1	NM_014988.1	LIM and calponin homology domains 1	0,51	0,49	0,47	0,48	NS
LRCH2	NM_020871.2	leucine-rich repeats and calponin homology (CH) domain containing 2	NS	1,3	NS	NS	2,27
MARVELD2	NM_144724.1	MARVEL domain containing 2	0,64	0,57	0,61	0,67	0,66
MYH10	NM_005964.1	myosin, heavy chain 10, non-muscle	NS	0,51	0,55	0,68	0,63
NDN	NM_002487.2	necdin homolog (mouse)	0,75	0,57	NS	NS	NS
PAFAH1B1	NM_000430.2	platelet-activating factor acetylhydrolase, isoform Ib, subunit 1 (45kDa)	NS	0,47	0,71	0,74	0,79
PPAP2B	NM_177414.1	phosphatidic acid phosphatase type 2B	1,92	1,86	2,66	1,56	NS
SPAG9	NM_172345.1	sperm associated antigen 9	1,51	2,19	2,22	NS	NS
TEK	NM_000459.2	TEK tyrosine kinase, endothelial	NS	0,53	NS	NS	NS
GTPase activity							
ARHGAP20	NM_020809.2	Rho GTPase activating protein 20	0,56	1,77	1,43	NS	2,01
ARHGAP28	NM_030672.2	Rho GTPase activating protein 28	NS	0,47	NS	NS	NS
ARL1	NM_001177.3	ADP-ribosylation factor-like 1	NS	0,56	0,72	0,74	NS
ARL4A	NM_0010371	ADP-ribosylation	0,6	0,43	0,45	0,54	0,61

	64.1	factor-like 4A					
FAM13B	NM_001101800.1	family with sequence similarity 13, member B	NS	0,51	0,62	0,67	NS
RAB24	NM_001031677.1	RAB24, member RAS oncogene family	NS	1,6	NS	NS	2,06
RASIP1	NM_017805.2	Ras interacting protein 1	NS	0,71	NS	NS	0,59
RND1	NM_014470.2	Rho family GTPase 1	5,62	6,24	NS	NS	NS
RND3	NM_005168.3	Rho family GTPase 3	NS	2,98	3,35	2,5	1,9
RGL1	NM_015149.2	ral guanine nucleotide dissociation stimulator-like 1	NS	NS	NS	NS	2,05
TBC1D4	NM_014832.1	TBC1 domain family, member 4	NS	0,63	0,66	NS	0,53
Intracellualr transport/endocytosis							
AP3M2	NM_006803.2	adaptor-related protein complex 3, mu 2 subunit	0,47	0,56	0,75	NS	NS
ANKFY1	NM_020740.1	ankyrin repeat and FYVE domain containing 1	NS	1,21	1,23	NS	2,16
FNBP1L	NM_017737.3	formin binding protein 1-like	NS	0,57	NS	NS	NS
MALL	NM_005434.3	mal, T-cell differentiation protein-like	NS	NS	2,11	NS	NS
PEX11B	NM_003846.1	peroxisomal biogenesis factor 11 beta	NS	0,48	0,6	0,67	NS
SH3D19	NM_001009555.2	SH3 domain containing 19	0,68	0,54	0,57	0,68	0,69
SIRPA	NM_080792.1	Signal-Regulatory Protein Alpha	0,65	0,55	0,63	0,63	0,73
STX11	NM_003764.2	syntaxin 11	3,81	4,86	4,13	2,22	2,51
VPS36	NM_016075.2	vacuolar protein sorting 36 homolog (S. cerevisiae)	0,68	0,5	0,66	0,72	NS
VPS4B	NM_004869.2	vacuolar protein sorting 4 homolog B (S. cerevisiae)	NS	0,57	NS	0,81	NS

Vasculature development/tissue remodeling							
ADAMTS1	NM_006988.3	ADAM Metallopeptidase With Thrombospondin Type 1 Motif, 1	NS	5,21	9,92	3,2	NS
ADAMTS9	NM_182920.1	ADAM metallopeptidase with thrombospondin type 1 motif, 9	NS	NS	2,24	NS	NS
BAMBI	NM_012342.2	hypothetical LOC729590; BMP and activin membrane-bound inhibitor homolog (Xenopus laevis)	NS	4,52	2,62	NS	NS
BMP6	NM_001718.2	bone morphogenetic protein 6	NS	0,53	0,67	NS	NS
EFNB2	NM_004093.2	ephrin-B2	NS	0,49	NS	NS	NS
FAP	NM_004460.2	fibroblast activation protein, alpha	NS	2,4	1,58	NS	NS
GPR180	NM_180989.3	G protein-coupled receptor 180	NS	1,41	NS	1,18	2,02
ID1	NM_181353.1	inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	0,4	2,33	NS	NS	NS
ID2	NM_002166.4	inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	NS	2,37	1,67	NS	NS
KLHL12	NM_021633.2	kelch-like 12 (Drosophila)	NS	0,49	0,69	0,73	NS
MAPK6	NM_002748.2	mitogen-activated protein kinase 6	1,26	0,54	0,62	0,76	0,74
NRP1	NM_003873.3	neuropilin 1	0,59	0,59	NS	NS	NS
TGFBR3	NM_003243.2	transforming growth factor, beta receptor III	0,56	0,57	0,59	0,68	NS

Table 7. The HUVEC expression of genes related to adhesion, cell motion and development upon infection with *S. aureus* blood isolates. The genes are divided in several subgroups in accordance to their specific function. Each gene symbol corresponds with the full gene name; "Gene Bank" accession number; and fold of transcriptional change for every *S. aureus* isolates and TNF-alpha treatment. The significantly upregulated genes are shown in red; the significantly downregulated genes in blue; and the not significantly expressed genes in grey. The data shown is the mean from three independent experiments. The p-values were determined respectively to differential expression of mock-treated control and detection p-values. The differential p-value: p≤0.05; ns= not significant. The detection p-value: p≤0.05; ns= not significant.

3.8.4.1. Strain-specific expression of structural and developmental genes

Within the group of genes related to cytoskeletal/intracellular trafficking/vasculature development, the most differentially expressed transcripts observed are the strains K1801/10, 8325-4, and 6850. These strains have the strongest effects on endothelial barrier function, as indicated by TER decrease performed in previous studies (Kramko *et al.*, 2013). However, the amount of upregulated genes in the group of strains affected TER was similar. For this reason, we assume that these genes might be involved in the structural organization and control of cell junctions, the cytoskeleton and small GTPase´s proteins relating to junctional integrity and endothelial barrier function regulation. Strain K70058396, which does not induce a TER decrease, showed the lowest changes in expression of the group by upregulating six and downregulating three transcripts.

The data related to differentially expressed cytoskeletal genes can be of great help to better understand the regulation of endothelial permeability and bacteria translocation during staphylococcal infection. For example, RND1 is the gene encoding a protein that belongs to the Rho GTPase family. The RND1 is known to play a role in cortical actin inhibition (Nobes *et al.*, 1998). This gene is upregulated by TNF-α and strain K1801/10 treatments, making it a possible candidate participating in endothelial permeability regulation during *S. aureus* infection.

In contrast to the upregulated genes, the downregulated transcripts display greater variability. In particular, 29 genes were down-regulated in HUVEC following infection with K1801/10, while only 3 of them corresponded to 8325-4 infected cells. However, the majority of these genes are also involved in various cell functions including tissue remodeling, vascular development and intracellular trafficking. For example, predominantly downregulated by strain 6850 and K1801/10, genes COL3A1 and MYH10 are induced by developmental stimuli or the BMP6 gene, which participates in tissue remodeling.

3.8.5. Differential expression of HUVEC transcription factors and signaling molecules

Differentially expressed genes encoding transcription factors and signaling molecules are listed in the table 8 (Table 8).

Gene symbol	***Gene bank accession number***	***Full gene name***	***Fold change TNF-α treated***	***Fold change K1801/10***	***Fold change 6850***	***Fold change K70058396***	***Fold change 8325-4***
			Pos. Control	*S. aureus* strains			
Transcription factors and signalling							
AIRE	NM_000659.1	autoimmune regulator	NS	2,33	NS	NS	NS
ATF3	NM_001030287.1	activating transcription factor 3	NS	14,46	7,62	NS	NS
BATF2	NM_138456.3	basic leucine zipper transcription factor, ATF-like 2	NS	5,26	3,87	1,78	8,26
CEBPB	NM_005194.2	CCAAT/enhancer binding protein (C/EBP), beta	NS	2,28	1,76	NS	NS
CEBPD	NM_005195.2	CCAAT/enhancer binding protein (C/EBP), delta	5,29	5,89	5,08	1,8	5,14
CXCR7	NM_020311.1	chemokine (C-X-C motif) receptor 7	9,1	3,19	2,84	2,2	NS
DDX58	NM_014314.2	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	2,17	7,58	7,31	2,78	15,23
DDX60	NM_017631.3	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60	1,48	2,97	2,33	1,41	6,17
DACH1	NM_080759.3	dachshund homolog 1 (Drosophila)	0,41	0,55	NS	NS	NS
DKK1	NM_012242.2	dickkopf homolog 1 (Xenopus laevis)	NS	NS	3,38	3,3	NS
DLL1	NM_005618.2	delta-like 1 (Drosophila)	NS	1,78	NS	NS	2,79
EGR1	NM_001964.2	early growth response 1	NS	4,02	2,39	NS	NS
EGR2	NM_000399.2	early growth	NS	3,71	2,89	1,59	1,59

		response 2					
ELF1	NM_172373.2	E74-like factor 1 (ets domain transcription factor)	NS	1,86	2,27	NS	2,38
ELF4	NM_001421.1	E74-like factor 4 (ets domain transcription factor)	NS	2,11	NS	1,3	NS
FOS	NM_005252.2	v-fos FBJ murine osteosarcoma viral oncogene homolog	NS	17,22	7,07	NS	NS
FOSB	NM_006732.1	FBJ murine osteosarcoma viral oncogene homolog B	NS	30,41	25,84	11,2	NS
FOXO1	NM_002015.2	forkhead box O1	NS	0,59	NS	NS	NS
HOXA9	NM_152739.2	homeobox A9	0,49	0,47	NS	NS	NS
HNRNPA0	NM_006805.3	heterogeneous nuclear ribonucleoprotein A0	NS	0,49	NS	NS	NS
JUN	NM_002228.3	jun oncogene	NS	5,82	3	2,76	NS
JUNB	NM_002229.2	Jun B Proto-Oncogene	1,66	3,67	1,79	NS	1,48
IER3	NM_052815.1	immediate early response 3	NS	NS	0,53	NS	0,6
IRAK2	NM_001570.3	interleukin-1 receptor-associated kinase 2	5,71	3,47	2,64	1,9	NS
IRF1	NM_002198.1	interferon regulatory factor 1	7,82	8,17	7,02	2,99	9,49
IRF7	NM_004029.2	interferon regulatory factor 7	1,37	6,95	4,17	2,42	7,81
IRF9	NM_006084.3	interferon regulatory factor 9	1,28	2,04	2,25	1,71	3,41
JAK2	NM_004972.2	Janus kinase 2	1,17	1,64	1,61	NS	2,86
KLF2	NM_016270.2	Kruppel-like factor 2 (lung)	NS	2,39	2,07	NS	NS
KLF4	NM_004235.3	Kruppel-like factor 4 (gut)	NS	15,98	8,7	NS	NS
MAP3K8	NM_005204.2	mitogen-activated protein kinase kinase kinase 8	1,82	3,1	2	1,14	NS
MYD88	NM_002468.2	myeloid differentiation primary response gene (88)	NS	1,96	1,88	1,33	3,27

NFKBIA	NM_020529.1	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	8,59	5,2	3,56	2,48	NS
NFKBIZ	NM_0010054 74.1	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	2,48	6,61	5,03	1,82	NS
RIPK2	NM_003821.4	receptor-interacting serine-threonine kinase 2	13,7	7,89	6,22	2,18	NS
SOCS1	NM_003745.1	suppressor of cytokine signaling 1	NS	2,19	1,78	1,34	3,02
SOCS3	NM_003955.3	suppressor of cytokine signaling 3	NS	2,41	1,68	NS	1,55
STAT1	NM_139266.1	signal transducer and activator of transcription 1, 91kDa	0,56	1,53	1,4	NS	3,31
STAT2	NM_005419.2	signal transducer and activator of transcription 2, 113kDa	NS	1,82	1,81	NS	4,61
TNFRSF12A	NM_016639.1	tumor necrosis factor receptor superfamily, member 12A	NS	1,82	NS	2,17	NS
TNFAIP3	NM_006290.2	tumor necrosis factor, alpha-induced protein 3	16,46	11,49	5,15	NS	NS
TNFSF13B	NM_006573.3	tumor necrosis factor (ligand) superfamily, member 13b	NS	2,38	2,26	NS	8,02
TRAFD1	NM_006700.1	TRAF-type zinc finger domain containing 1	1,42	1,71	1,52	NS	3,34
TRIB1	NM_025195.2	tribbles homolog 1 (Drosophila)	2,21	4,45	3,69	2,37	2,11
TNFRSF1B	NM_001066.2	tumor necrosis factor receptor superfamily, member 1B	0,42	0,47	0,51	NS	NS
TSC22D2	NM_014779.2	TSC22 Domain Family, Member 2	NS	2,44	1,89	1,56	NS

UNC93B1	NM_030930.2	unc-93 homolog B1 (C. elegans); unc-93 homolog B6 (C. elegans); unc-93 homolog B3 pseudogene (C. elegans); similar to unc-93 homolog B1 (C. elegans)	NS	1,35	1,25	NS	2,05
ZC3HAV1	NM_024625.3	zinc finger CCCH-type, antiviral 1	1,27	5,49	5,48	2,22	5,37
ZFP36	NM_003407.1	zinc finger protein 36, C3H type, homolog (mouse)	3,01	11,91	4,61	NS	NS
ZFYVE26	NM_015346.2	zinc finger, FYVE domain containing 26	NS	NS	NS	NS	2,1

Table 8. The HUVEC expression of transcription factors and signaling regulators upon infection with *S. aureus* blood isolates. The genes are divided in several subgroups in accordance to their specific functions. Each gene symbol corresponds with the full gene name; "Gene Bank" accession number; and fold of transcriptional change for every *S. aureus* isolates and TNF-alpha treatment. The significantly upregulated genes are shown in red; the significantly downregulated genes in blue; and the not significantly expressed genes in grey. The data shown is the mean from three independent experiments. The p-values were determined respectively to differential expression of mock-treated control and detection p-values. The differential p-value: p≤0.05; ns= not significant. The detection p-value: p≤0.05; ns= not significant.

The specific mechanical background of strain-specific induction with differential expression as well as the role of particular transcripts still requires further investigation. For example, transcriptional factors JUN, JUNB and FOS, which are predominantly differentially expressed upon infection with cytotoxic strains, where shown to play a role in a variety of cellular mechanisms including cell survival (Kriehuber *et al.*, 2005). Therefore, a functional study is required to elucidate the exact mechanisms playing role during *S. aureus* infection.

3.9. *S. aureus* strain-related induction of genes involved in endothelial cell death

3.9.1. Cytotoxic strains of *S. aureus* induces cell death-related gene expression in endothelial cells

Based on the microarray results, we chose to focus on a group of cell death-related genes to study *S. aureus*-induced endothelial apoptosis in greater detail. To that end, with respect to *S. aureus*-induced cytotoxicity, we analyzed genes that were differentially regulated upon endothelial infection with the four selected strains: 6850, K1801/10, K70058396 and 8325-4. Considering many different cell death mechanisms such as necrosis, apoptosis, necroptosis etc. to avoid bias (Yipp and Kubes, 2013), we divided the cell death-related genes into two groups. One group contained genes involved in cell death induction (Table 9) while the second group contained cell death prevention (Table 10). Treatment of endothelium with TNF-alpha served as the positive control for inflammation just as before as well as an extrinsic inductor of the apoptotic pathway through the TNF-R1 receptor (Beg and Baltimore, 1996).

Cytotoxic strain relative gene expression

Expression of the genes related to cell death induction						
			S. aureus strains			
Gene symbol	Acsess number	Pos. Control	Cytotoxic			Non-cytotoxic
		TNF treated	K1801/10	6850	K70058396	8325-4
ATF3	NM_001030287	NS	14,46	7,62	NS	NS
RIPK2	NM_003821	13,7	7,89	6,22	2,18	NS
JUN	NM_002228	NS	5,82	3	2,76	NS
PHLDA1	NM_007350	2,63	4,23	2,63	2,09	NS
DUSP1	NM_004417	NS	3,43	2,75	NS	NS
AXUD1	NM_033027	2,78	7,48	3,4	2,45	2,52
BCL6	NM_001706	NS	2,96	1,82	NS	NS
PPP1R15A	NM_014330	1,52	4,15	2,27	1,68	1,62
JUNB	NM_002229	1,66	3,67	1,79	NS	1,48
BCL3	NM_005178	3,26	2,39	1,67	1,5	NS
PTGS2	NM_000963	NS	9,25	6,39	NS	3,9
PMAIP1	NM_021127	1,98	11,61	8,8	2,17	5,03
RIPK1	NM_003804	NS	2,46	1,66	NS	2,42
FOXO1	NM_002015	NS	0,59	NS	NS	NS
TP53INP1	NM_033285	NS	0,45	0,55	0,69	0,77
ARHGEF6	NM_004840	0,67	0,52	0,59	0,62	NS
TNFRSF1B	NM_001066	0,42	0,47	0,51	NS	NS
DUSP5	NM_004419	NS	2,27	2,59	1,81	2,43
PLEKHF1	NM_024310	0,75	2,18	2,57	1,39	2,83
CASP7	NM_033338	1,44	1,62	1,69	1,22	2,19
BATF2	NM_138456	NS	5,26	3,87	1,78	8,26
MLKL	NM_152649	NS	2,43	2,26	1,48	3,99
TNFSF10	NM_003810	NS	5,45	3,98	NS	9,69
TXNIP	NM_006472	0,36	NS	1,94	NS	2,85
CASP1	NM_033292	NS	NS	NS	NS	3
TNFSF13B	NM_006573	NS	2,38	2,26	NS	8,02
Fold change	NS	≥10	≥5<10	≥2<5	≥1.2<2	<0.8

Non-cytotoxic strain relative gene expression

Table 9. HUVEC´ transcripts related to cell death by four *S. aureus* isolates and TNF-alpha. The data shows the mean from three independent experiments. The p-values related to respectively differential expressions of mock-treated control and detection p-values. The differential p-value: p≤0.05, ns= not significant. The detection p-value: p≤0.05, ns=not significant. Cytotoxic strain relative gene expression = gene FC (cytotoxic strain)/gene FC (non-cytotoxic strain). Non-cytotoxic strain relative gene expression = gene FC (non-cytotoxic strain)/gene FC (cytotoxic strain).

Cytotoxic strain relative gene expression

Expression of the genes related to cell death prevention						
			S. aureus strains			
Gene symbol	Acsess number	Pos. Control	Cytotoxic			Non-cytotoxic
		TNF treated	K1801/10	6850	K70058396	8325-4
TNFAIP3	NM_006290	16,46	11,49	5,15	NS	NS
ANGPTL4	NM_00103966	NS	10,07	NS	5,45	NS
C8orf4	NM_020130	10,55	8,38	4,05	NS	NS
BIRC3	NM_182962	10,4	6	4,33	NS	NS
GADD45B	NM_015675	NS	4,3	2,31	NS	NS
DDIT4	NM_019058	0,48	1,79	NS	NS	0,51
NR4A2	NM_006186	NS	3,46	3,14	NS	NS
CCNA1	NM_003914	NS	2,97	2,64	NS	NS
GDF15	NM_004864	NS	2,7	NS	1,84	NS
C21orf63	NM_058187	2,28	2,44	1,83	1,8	NS
NUAK2	NM_030952	3,62	2,34	1,52	NS	NS
HES1	NM_005524	0,46	2,29	NS	NS	NS
CEBPB	NM_005194	NS	2,28	1,76	NS	NS
TRIM69	NM_080745	NS	2,21	1,5	NS	NS
MCL1	NM_021960	1,6	2,83	2,43	1,7	2,21
CBX4	NM_003655	NS	2,34	1,82	1,3	2,31
IER3	NM_052815	NS	NS	0,53	NS	0,6
NUAK1	NM_014840	0,22	0,4	0,42	0,64	0,56
ACVR1	NM_001105	NS	0,59	0,71	0,71	NS
PARP12	NM_022750	1,36	2,62	2,3	NS	4,27
DHX58	NM_024119	1,6	2,29	1,98	NS	3,93
PARP10	NM_032789	NS	1,79	1,56	NS	3,2
PARP14	NM_017554	3,13	2,9	1,96	NS	5,24
PARP9	NM_031458	1,45	3,66	3,44	2,03	7,13
XAF1	NM_199139	1,26	2,75	2,45	1,61	5,72
INDO	NM_002164	1,93	7,94	NS	1,24	19,16
Fold change	NS	≥10	≥5<10	≥2<5	≥1.2<2	<0.8

Non-cytotoxic strain relative gene expression

Table 10. HUVEC´ transcripts related to cell death prevention by four *S. aureus* isolates and TNF-alpha. The data shows the mean from three independent experiments. The p-values related to respectively differential expressions of mock-treated control and detection p-values. The differential p-value: p≤0.05, ns= not significant. The detection p-value: p≤0.05, ns=not significant. Cytotoxic strain relative gene expression = gene FC (cytotoxic strain)/gene FC (non-cytotoxic strain). Non-cytotoxic strain relative gene expression = gene FC (non-cytotoxic strain)/gene FC (cytotoxic strain).

The gene order was defined by the ratio between the FC of the cytotoxic strain K1801/10 to the FC of the non-cytotoxic strain 8325-4. This ratio was named, “cytotoxic strain relative gene expression”. The highest relative gene expression of cytotoxic strain K1801/10 corresponds to the highest FC of this strain while the lowest relative gene expression of K1801/10 corresponds to the highest FC of the non-cytotoxic strain 8325-4. The cytotoxic strain relative gene expression decreases from the top to the bottom of the table (see legend Table 9, 10).

For a better visualization of the strain-specific transcriptions of cell death-related genes (Table 9, 10), we generated a “filled radar chart” (Fig. 34, 35). The cytotoxic *S. aureus* strains K1801/10, 6850 and K70058396 showed distinctive expression patterns of genes related to cell death induction (Fig. 30) and prevention (Fig. 31). The expression patterns of the non-cytotoxic strain 8325-4, for both gene groups, differed from the ones induced by cytotoxic strains (Fig. 30, 31).

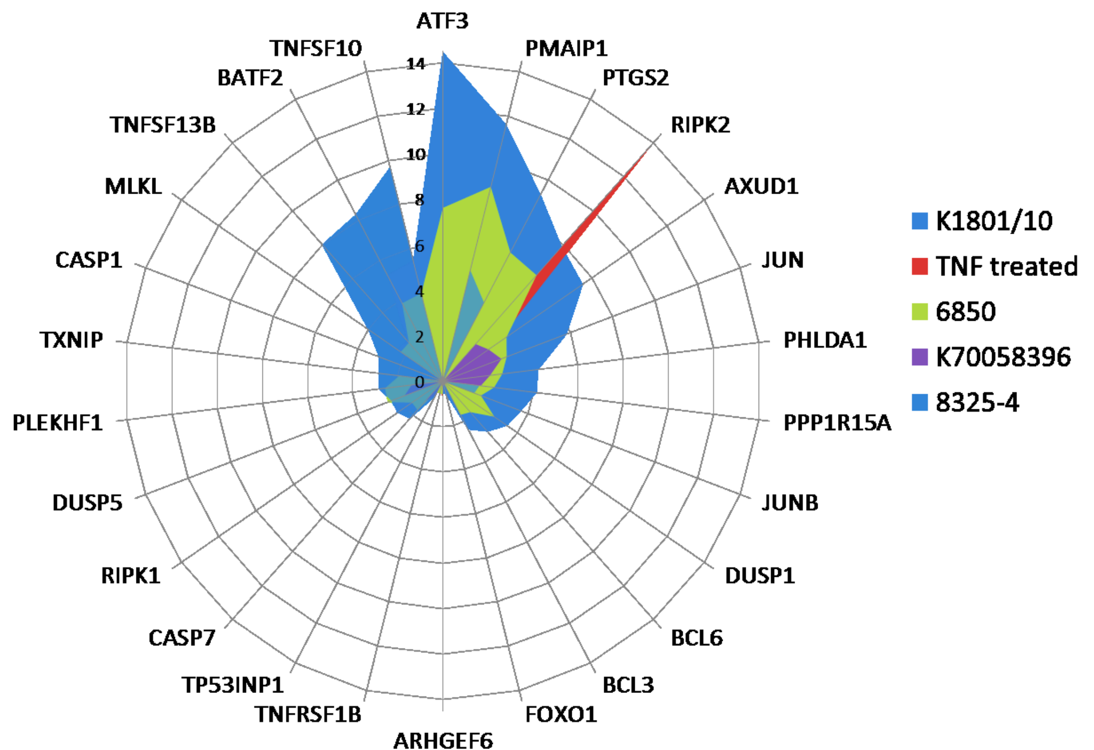


Figure 30. HUVEC´ gene expression of transcripts related to cell death prevention by four *S. aureus* isolates and TNF-alpha represented in filled radar chart. Numbers represent gene fold change.

The gene expression of transcripts in treatment with the cytotoxic strain K70058396 was generally lower than for the other isolates. However, relative gene expression of K70058396 had similar traits when compared with other cytotoxic isolates. Relative gene expression for transcription factors and apoptosis regulators such as JUN, RIPK2, PHLDA1 (Table 11, 12) was higher for all cytotoxic strains compared to the non-cytotoxic strain 8325-4 (Fig. 30).

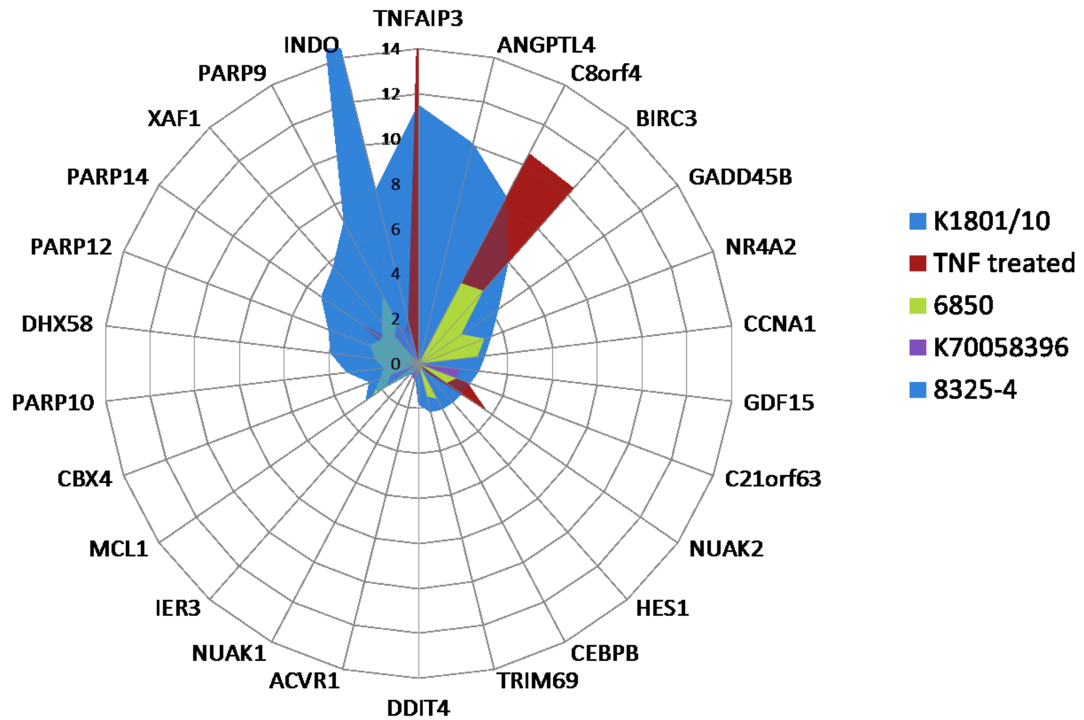


Figure 31. HUVEC´ gene expression of transcripts related to cell death prevention by four *S. aureus* isolates and TNF-alpha represented in filled radar chart. Numbers represent gene fold change.

Considering the strain-specific differences in differentially regulated HUVEC cell death genes, we assume that different transcription factors might be involved in their transcription.

3.9.2. Different transcription factors involved in *S. aureus*-induced cell death gene expression

We further investigated transcription factors that might control cell death-related gene expression upon *S. aureus* infection. The results are listed below and correspond to the enrichment score of the genes´ overrepresented binding sites for particular transcription factors (TFs) (by Pscan Software (Zambelli *et al.*, 2009)). Promoter region analysis of signaling pathways indicated a high enrichment score for TFs involved in interferon signaling, such as NF-κB and PI-3K signaling (Table 11).

Transcription factors involved in apoptotic gene regulation									
TNF	**p-value**	**K1801/10**	**p-value**	**6850**	**p-value**	**K70058396**	**p-value**	**8325**	**p-value**
REL	5,16E-06	CREB1	2,01E-04	CREB1	1,54E-05	E2F3	4,11E-04	STAT1	2,76E-06
NFKB1	2,59E-05	RELA	5,92E-04	ESR2	3,77E-04	CREB1	1,85E-03	STAT2::STAT1	1,66E-05
RELA	5,73E-05	NFKB1	6,03E-04	TAL1::TCF3	7,89E-04	E2F4	2,39E-03	IRF1	7,60E-05
ESR2	4,80E-04	REL	1,14E-03	NR4A2	8,38E-04	E2F1	2,75E-03	Nr5a2	4,51E-04
Nr1h3::Rxra	5,21E-03	Nr5a2	1,53E-03	ESR1	1,45E-03	Mafb	3,06E-03	IRF2	9,71E-04
Zfx	5,94E-03	ESR2	1,92E-03	Esrrb	1,53E-03	E2F6	3,32E-03	SRF	2,13E-03
TFAP2A	7,16E-03	ESRRA	2,14E-03	SRF	1,71E-03	EBF1	5,78E-03	FOXF2	2,38E-03
ESR1	1,61E-02	FOXD1	2,24E-03	Nr5a2	2,25E-03	NFKB1	7,03E-03	FOXP2	2,71E-03
CREB1	1,89E-02	HINFP	2,60E-03	REL	2,45E-03	FOXO3	1,96E-02	EWSR1-FLI1	5,06E-03
Pax5	1,93E-02	ESR1	2,82E-03	NFKB1	2,77E-03	NFIL3	2,01E-02	Foxo1	8,09E-03

Table 11. Promoter region analysis of HUVEC´ genes related to cell death by Pscan. The different colors correspond to the analysis of different gene sets of the HUVEC treatments. For each treatment, a list of enriched transcription factors and their respective p-value is presented. The p-values of the enrichment scores ranking relate to the compared probability of matrix set used in the analysis to set of genes taken by chance.

To begin, the promoter region analysis demonstrated a high significance for TF CREB1 for all cytotoxic *S. aureus* strains. Furthermore, TFs NFKB1, REL and RELA involved in NF-kB pathway were also involved in the regulation of cell death-related gene expression by cytotoxic strains, but were much less significant. Finally, the promoter region analysis for the non-cytotoxic strain 8325-4 showed a high significance for interferon signaling TFs. Particularly, these included STAT1, STAT1::STAT2 and IRF1 while TFs CREB1 and NFKB1 corresponding to NF-kB and PI-3K pathways were completely absent. The promoter region analysis for the TNF-alpha treatment showed the highest significance for the TFs of the NF-kB pathway. TF CREB1 was also significantly enriched, but less so than for cytotoxic *S. aureus* isolates (Table 11).

Based on this data, we hypothesize that cytotoxic *S. aureus* strains modulate cell death-related gene expression in HUVEC via TF of PI-3K cascade, namely CREB1. Apoptotic gene expression induced by cytotoxic isolates partly overlaps with TNF-alpha-induced cell death transcript expression, likely through TFs of the NF-kB signaling pathway. However, it seems that the non-cytotoxic strain of *S. aureus* affects cell death gene expression through the interferon signaling pathway, which may not participate in *S. aureus*-induced endothelial cell death.

3.10. Influence of kinase signaling on the survival of endothelium upon *S. aureus* infection

The downstream transcription factors NF-kB and CREB1 require additional activation by MAPKs (Wen *et al.*, 2010). In turn, the signaling kinases p38, ASK1 (Yang *et al.*, 2008), JNK and ERK ½ (Yang *et al.*, 2000) were also shown to contribute to cell death regulation in *S. aureus* infected endothelium (Esen *et al.*, 2001). The function of PKC and PI3K kinases was reported to regulate the activation of respective mitogen-activated protein kinases (MAPKs) which thereby implies a possible role in cell death regulation.

The differential expressions of JUN, JUNB, BCL3 and FOS genes induced by cytotoxic isolates partly proves the involvement of MAPKs during the infection process. Transcription of these genes was shown to depend on AP-1 and/or c-JUN transcription factors activated by MAPKs (Kappelmann *et al.*, 2014).

3.10.1. Inhibition of protein kinases affects HUVEC survival upon *S. aureus* infection

Based on bioinformatic analyses of cell death gene expressions and the data concerning their transcription regulators, we attempted to verify the role of HUVEC protein kinases in promoting endothelial cell death under apoptotic stimuli.

To investigate the role of a particular kinase in *S. aureus*-induced endothelial cell death in greater detail, we applied specific inhibitors of six protein kinases prior to HUVEC infection or H_2O_2 application. The name of the respective inhibitor and particular time of pre-incubation are listed below (Table 12).

Inhibitor	**Working concentration**	**Time of pre-incubation in hours**	**Mode of action**	**References**
NQDI-1	25 µM in DMSO	3	Selective inhibitor of apoptosis signal-regulating kinase 1 (ASK1, MAP3K5). Prevents apoptosis via inhibition of caspase-3 activation	(Volynets *et al.*, 2011; Song *et al.*, 2014)
SB203580	10 µM in DMSO	1	Pyridinyl imidazole inhibitor of p38 mitogen-	(Cuenda *et al.*, 1995)

			activated protein (MAP) kinase	
PD98059	10 µM in DMSO	1	PD98059 is a potent and selective inhibitor of ERK1/2 (p44/p42 MAPK) by MEK1/2.	(Alessi *et al.*, 1995)
Wortmannin	1 µM in DMSO	0,5	Cell-permeable, selective and irreversible inhibitor of phosphatidylinositol 3-kinase (PI3K)	(Fukao and Koyasu, 2003)
Bisindolylmaleimide I	5 µM in DMSO	1	Highly selective, cell-permeable, and reversible protein kinase C (PKC) inhibitor.	(Toullec *et al.*, 1991)
SP600125	25 µM in DMSO	0,5	Cell-permeable, selective and reversible inhibitor of c-Jun N-terminal kinase	(Bennett *et al.*, 2001)

Table 12. MAPK kinases inhibitors.

After pre-treatment with respective kinase inhibitors (Table 14), HUVEC were infected with the selected *S. aureus* strains according the standard protocol (Fig. 32) with the notable addition of inhibitors.

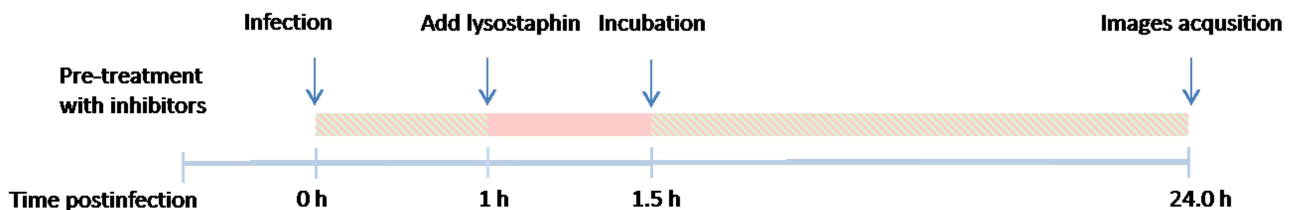


Figure 32. The scheme of MAPK kinases inhibition assay.

The application of ASK-1 and p38 MAPK kinases inhibitors (NQDI-1 and SB203580) were able to rescue endothelial cells from the apoptotic stimuli of hydrogen peroxide as well as the three pro-apoptotic strains of *S. aureus* K1801/10, 6850 and K70058396. In all cytotoxic strains and H_2O_2 treated cells, we observed fewer apoptotic events, decreased gap formation and the maintenance of a partially intact HUVEC monolayer. Endothelial cell death was not observed in mock treatments with or without inhibitors and there were no discernible differences for strain 8325-4 (Fig. 33, 34). The treatments with either ASK-1 or p38 MAPK kinase inhibitors demonstrated similar effects. The similar results of ASK1 and

p38 inhibition on endothelial survival can be explained by the same mechanism, since ASK1 is an upstream activator of p38 MAPK. The observed effect implies that these kinases play a role in cell death signaling under various apoptotic stimuli including cytotoxic *S. aureus* infection.

ASK 1 inhibitor

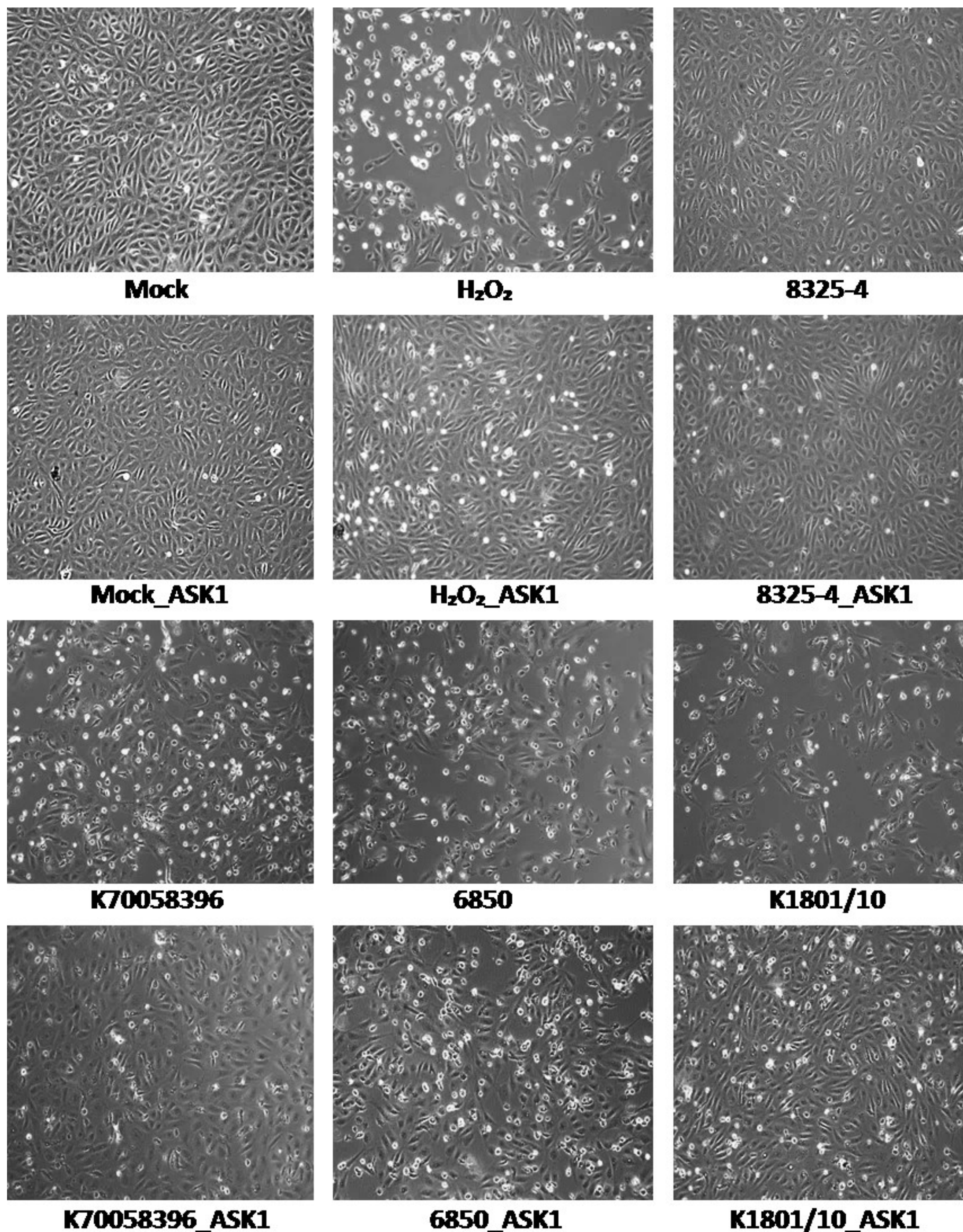


Figure 33. HUVEC infected with K70058396 and K1801/10 *S. aureus* strains with and without ASK1 inhibitor compared to the mock control and H_2O_2 treatments at 24 hours post-infection (20x).

p38 inhibitor

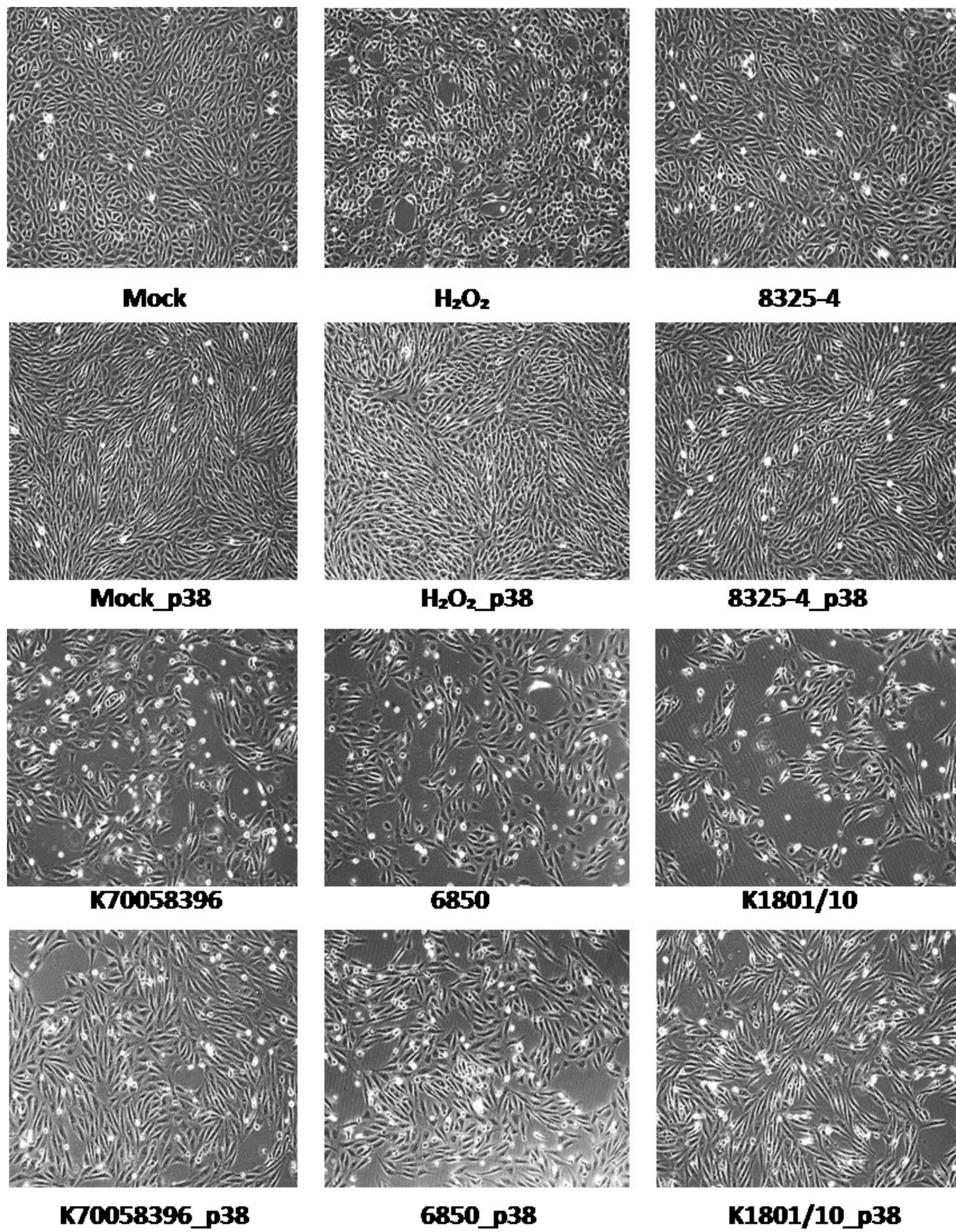


Figure 34. HUVEC infected with K70058396 and K1801/10 *S. aureus* strains with and without p38 inhibitor compared to the mock control and H_2O_2 treatments at 24 hours post-infection (20x).

ERK ½ inhibitor

During inhibition of ERK ½ MAPK kinase (PD 98059), we saw a decrease in endothelial cell death and diminished gap formation with H_2O_2 and cytotoxic strains K1801/10 and 6850. The strain K70058396 induced the same level of endothelial cell death with or without ERK ½ inhibitor. Controls receiving mock treatments and strain 8325-4 did not demonstrate any changes (Fig. 35).

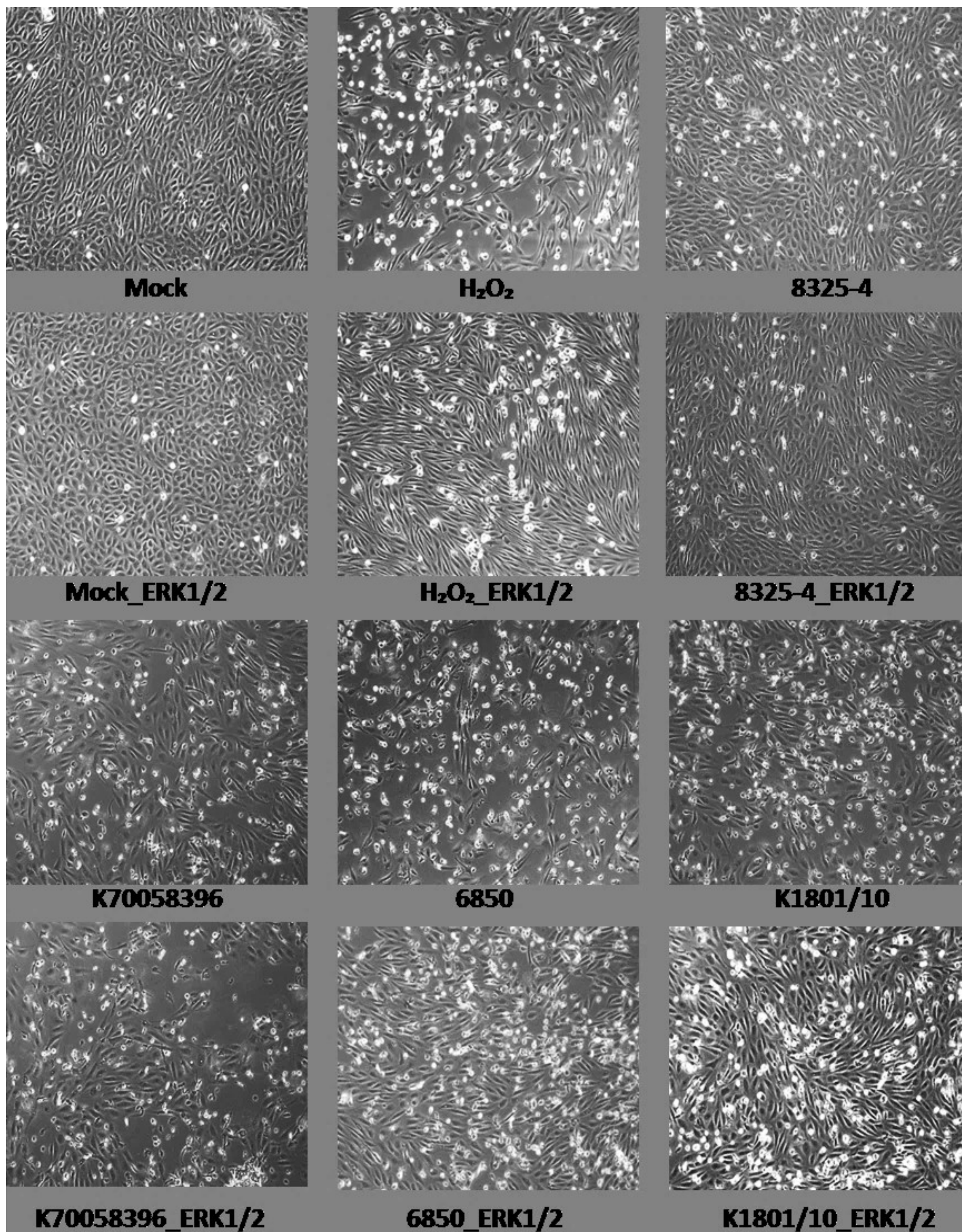


Figure 35. HUVEC infected with K70058396 and K1801/10 *S. aureus* strains with and without ERK1/2 inhibitor compared to the mock and H_2O_2 treatments at 24 hours post-infection (20x).

PKC inhibitor

In comparison to the ERK ½ inhibitor, it seems that PKC kinase has a pro-survival effect on endothelial cells as its inhibition lead to an increase of HUVEC cell death upon apoptotic stimuli. When PKC inhibitor (Bisindolylmaleimide I) was applied, exposure to hydrogen peroxide and cytotoxic *S. aureus* strains caused greater endothelial cell death (Fig. 36). Controls receiving mock treatments and strain 8325-4 showed no differences in induced cell death.

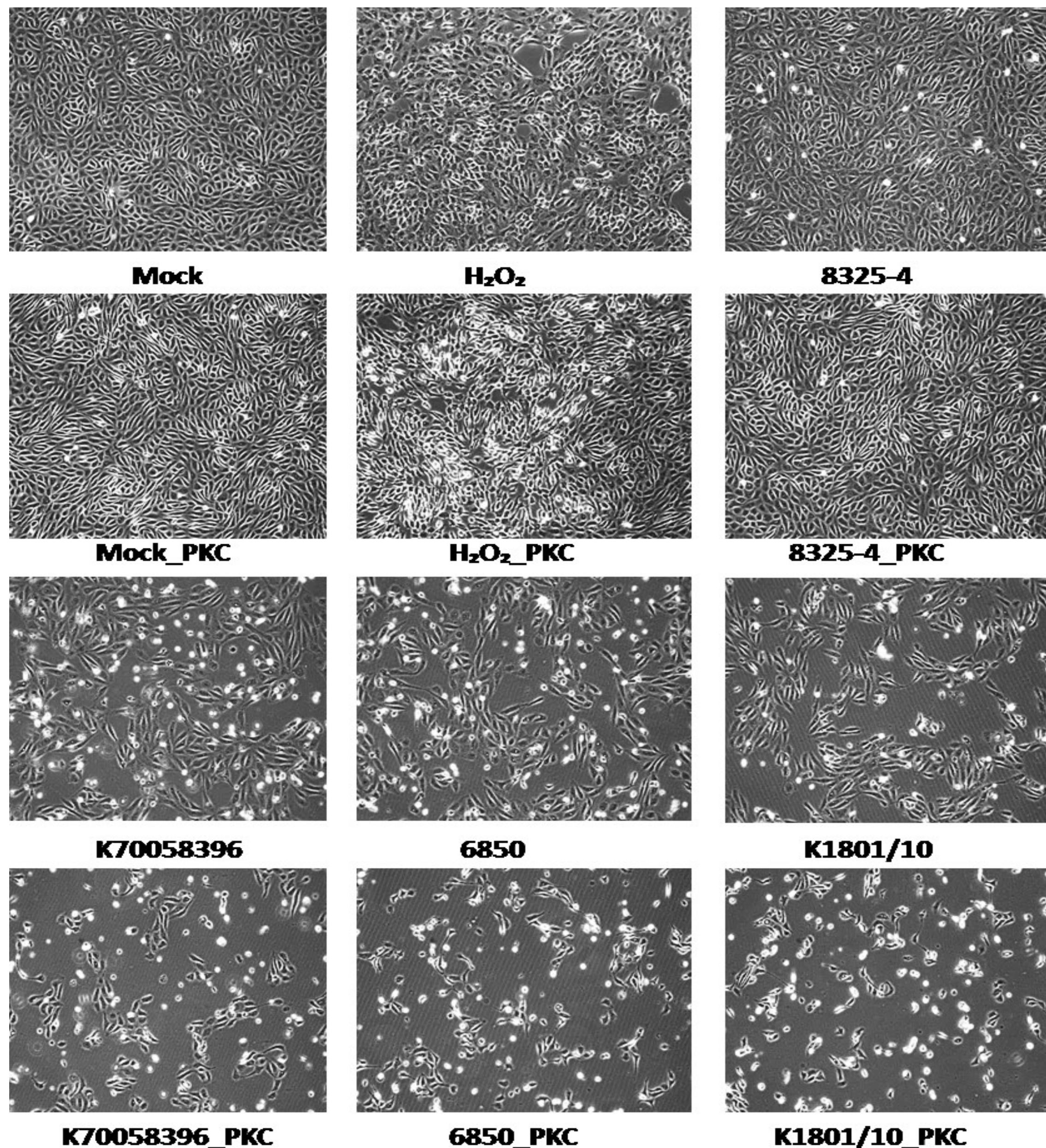


Figure 36. HUVEC infected with K70058396 and K1801/10 *S. aureus* strains with and without PKC inhibitor compared to the mock control and H_2O_2 treatments at 24 hours post-infection (20x).

PI3K inhibitor

The application of PI3K inhibitor (Wortmannin) resulted in a loss of cells from the substrate (Fig. 37) with any level of treatment. Furthermore, applications of both H_2O_2 and PI3K inhibitor further decreased the number of surviving cells when compared with H_2O_2 alone. The infection of the cells with *S. aureus* strains had no visible effect on cell survival and did not induce cell death. This finding demonstrates that PI3K is required for viability and maintenance of an intact cell monolayer (Fig. 37).

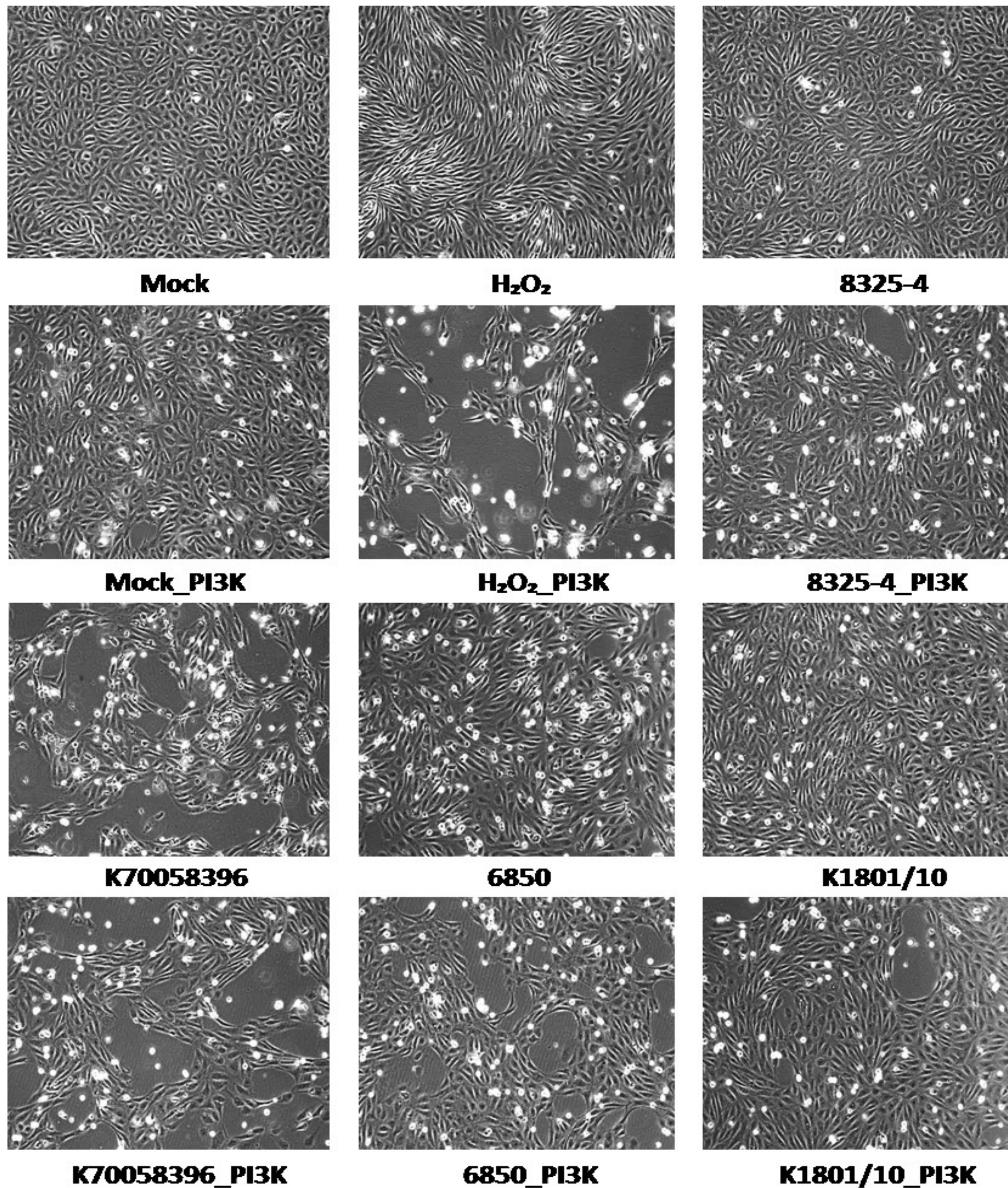


Figure 37. HUVEC infected with K70058396 and K1801/10 *S. aureus* strains with and without PI3K inhibitor compared to the mock control and H_2O_2 treatments at 24 hours post-infection (20x).

JNK inhibitor

When exposed to JNK kinase inhibition, endothelial cells started to die shortly after pretreatment with the SP600125 inhibitor. The JNK kinase inhibition seems to have an absolutely lethal effect on endothelium and it's effects did not differ for any of the treatments after 24 hours following infection (Fig. 38).

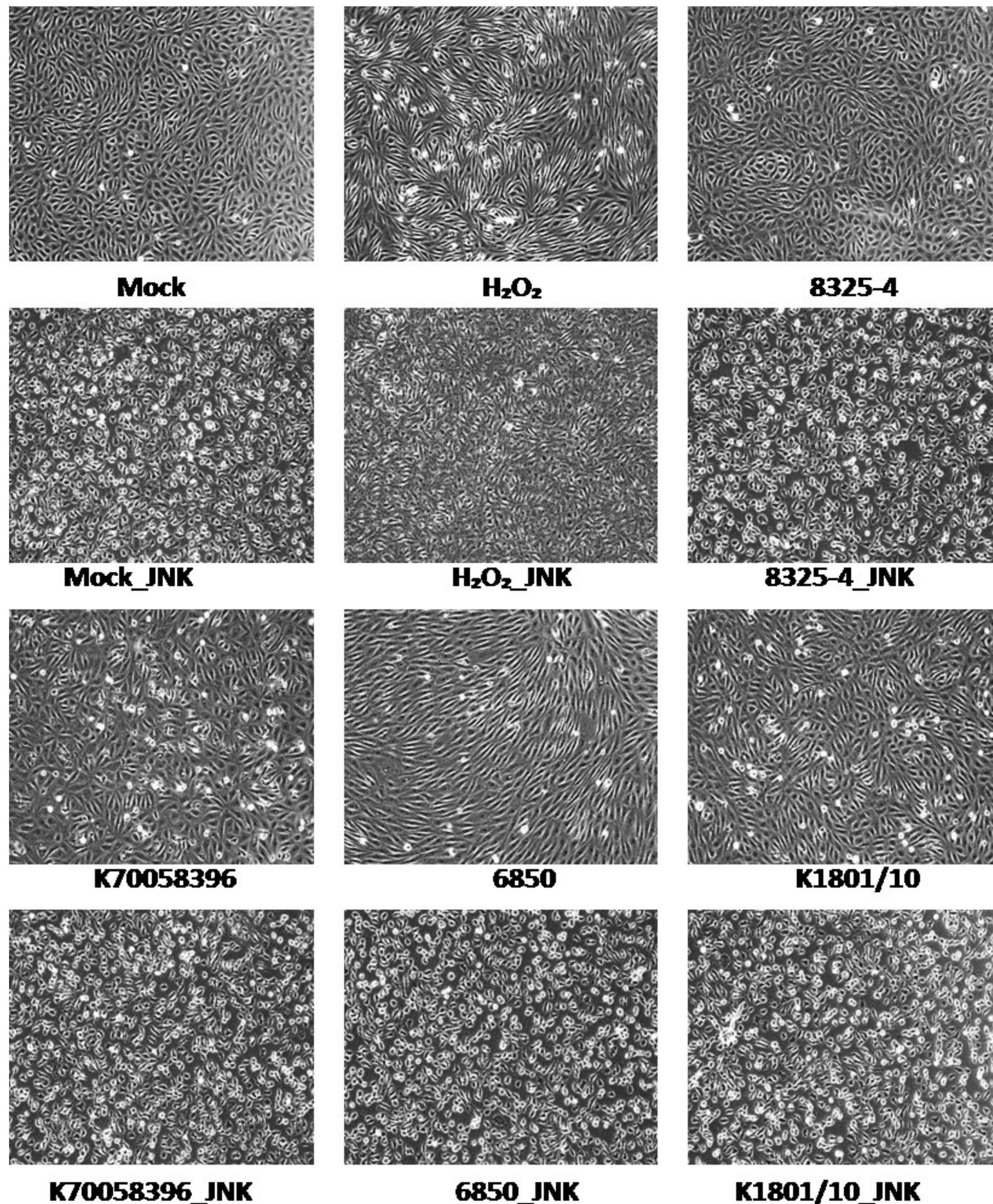


Figure 38. HUVEC infected with K70058396 and K1801/10 *S. aureus* strains with and without JNK inhibitor compared to the mock control and H_2O_2 treatments at 24 hours post-infection (20x).

The study suggests a possible involvement of regulatory kinases in *S. aureus*-induced endothelial cell death. Our results suggest that phosphorylated ASK-1, p38, and possibly ERK ½ kinases can transduce apoptotic signals causing *S. aureus*-induced endothelium cell death after the endothelium has been infected with cytotoxic strains. On the other hand, we assume that phosphorylated PKC kinase is important for endothelium survival upon induction with a pro-apoptotic stimuli. Kinases PI3K and especially JNK appear to be absolutely necessary under normal homeostatic endothelial conditions as their inhibition was shown to lead to cell death induction without any cytotoxic agents. The possible mechanisms involved in these observed effects will be addressed in further detail in the discussion.

4. Discussion

Staphylococcus aureus is member of the family *Staphylococcaceae* and a facultative anaerobic, gram-positive bacterium that can be found in about 50% of the human population on the skin and within the upper parts of the respiratory tract (Wertheim *et al.*, 2005). However, *S. aureus* can cause diverse infections ranging from simple skin boils via abscesses up to sepsis and the development of septic shock (Archer, 1998). The specific development of these diverse infections depends both on the immune status of the host and virulence of the given strain. *S. aureus* virulence is a subject of high variability and genetic plasticity that are also thought to be the main contributor to disease variability. However, an evident relationship between particular strains, their virulence factors and observed pathophysiological behavior is not always clear (Foster *et al.*, 2014). Treatment of *S. aureus* infections is complicated with the occurrence of antibiotic resistance, often to multiple antibiotics. This problem is of particular significance in combination with severe diseases such as endocarditis, osteomyelitis, sepsis and septic shock, as the lethality with these complicating circumstances, irrespective to full scale intensive care treatment, still ranges between 32% to 58% (Romero-Vivas *et al.*, 1995; Cosgrove *et al.*, 2003).

The development of severe diseases which mostly arise from local infections is usually associated with bacteremia, where host pathogen encounter occurs in the vasculature (Hickey and Kubes, 2009). Once in the blood, the majority of the bacteria are cleared by professional phagocytes (Gresham *et al.*, 2000), but the vascular endothelium has also been shown to be infected (Weidenmaier *et al.*, 2005). This phenomenon might be of significant relevance in the development of sepsis and septic shock with the formation of septic metastases since the bacteria need to overcome the endothelial cell layer. In this way, infection by *S. aureus* and transmigration of the pathogen through the endothelial cell layer might be one of the key steps in sepsis and septic shock development.

However, despite the it’s clear importance, the wide variability of *S. aureus* strains, its virulence factors and varying pathophysiological behavior mean that current research has yet to see the development of a clear concept describing the interrelationship between disease development and strain specificity.

In this study, we tested 24 strains and identified which showed extreme pathophysiological behavior in endothelial cells with respect to pro-inflammatory and anti-

inflammatory reactivity. The majority of the tested strains caused a mixture of pro- and anti-inflammatory responses in endothelium, which is consistent with the diversity of related disease development (Fig. 39). However, several of the tested strains displayed clear anti-inflammatory behavior along with the allowance of bacterial transmigration as well as the blockage of a TNF-α induced activation. Other strains caused a pro-inflammatory phenotype that, in contrast, did not allow for bacterial transmigration. With this knowledge, we used isolated strains as tools in order to uncover the fundamental mechanisms and signaling pathways in endothelium and better understand the diversity of *S. aureus* infections. Here, we performed a genome wide RNA array analyses following the infection of endothelial cells with the chosen strains. We quickly identified different mechanisms and signaling pathways controlling *S. aureus*-induced apoptosis, intracellular bacterial survival and inflammatory versus anti-inflammatory reactivity.

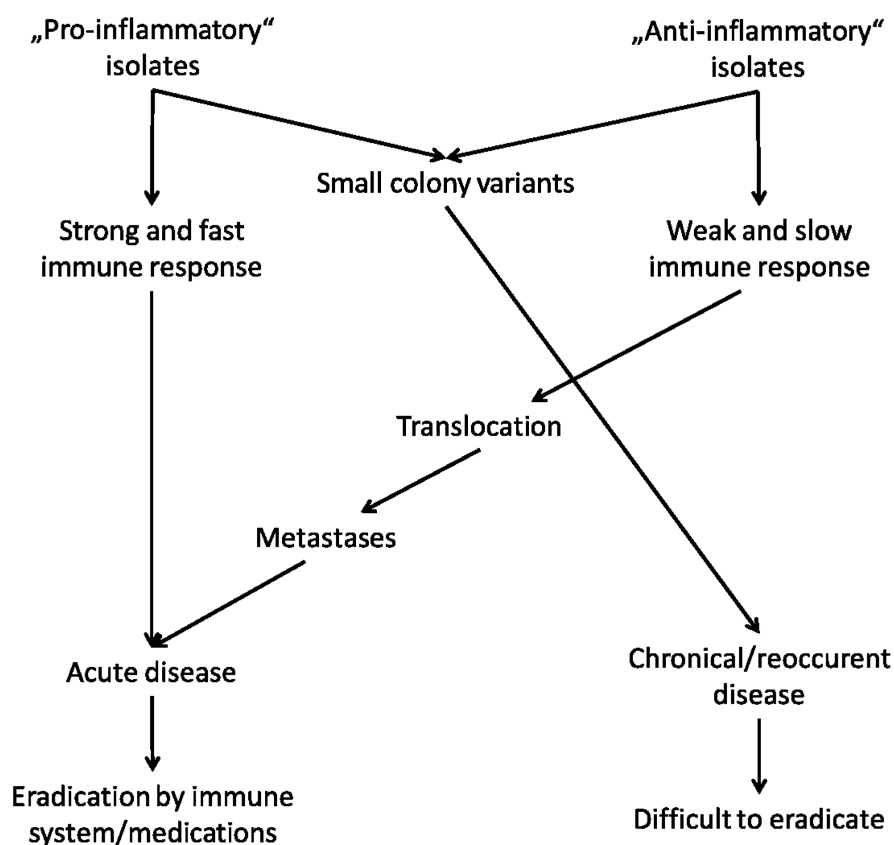


Figure 39. The proposed scheme of the host response to different *S. aureus* isolates.

Clarifying the mechanism behind the ability of some *S. aureus* strains to down-regulate immune response can be of a significant clinical importance. It is more than likely that, in some cases, complications during *S. aureus* infection could be related to modulation of the immune system reactivity. This criterion needs to be confirmed in animal models or in vivo and can be used for evaluating alternative medical interventions.

4.1. HUVEC expression study and microarray data analysis

To study the strain-specific endothelial response to *S. aureus* infection at a the level of gene expression, we performed a microarray assay due to its reputation as a broadly used method to investigate changes in cellular gene transcription caused by vast number of stimuli. In our research, we aimed to investigate early transcriptional changes of endothelium infected by six different *S. aureus* strains differing in their infectious properties as discussed in the results. To accomplish this, we extracted total endothelial mRNA and reverse transcribed as well as labelled target mRNA through several washing steps. The final step of the experiment included the loading of nucleic acid on a beadchip, hybridization of the transcripts with oligonucleotides on the beadchip surface and detection of fluorescent signal. This technique has the advantage of being high speed, highly specific and allows screening biological replicates. The disadvantages of this experimental design include affordability and rather low flexibility. The oligonucleotide array requires expensive specialized equipment and beadchips are costly. Furthermore, the complexity of beadchip production causes it to be a product restricted to centralized manufacturing facilities thereby limiting its flexible usage (Facility, 2015).

The raw data of the microarray was analyzed using specialized software (Genome Studio) which provided the normalized data of differential expression fold change and significance for each gene of each sample when compared to the untreated control. Further bioinformatical analyses offer additional features to perform functional group clustering, gene ontology analysis, promoter region analysis etc. These methods are widely used to work with large transcriptional datasets obtained during microarray. The basic principle of any of these bioinformatical analyses is to decrease the rate of data variability and group it in accordance to its functional similarity, by attribution to a same process or regulatory

pathway. During the microarray analyses, we used DAVID and PScan online software to predict possible induction mechanisms of endothelial response upon strain-specific infection.

The DAVID software analyzes sets of transcriptome data, grouping the genes and finally predicting their possible involvement in shared pathways or functions. PScan software provides predictions of transcription factors that may be involved in the transcriptional regulation of genes of a particular uploaded set. DAVID and PScan prediction is based on the same principle of calculating enrichment scores for the data of interest. These scores are generated using data from a number of sources and databases and the result reflects whether a particular user gene list is associated (enriched) with a particular signaling pathway compared to the sets of genes taken by random chance.

It is important to understand that outcomes of bioinformatics analyses give a hint as to which processes may be involved in an observed phenomenon, but this does not qualify as a legitimate discovery. For this reason, each conclusion based solely on bioinformatic analyses should be confirmed on a functional level.

Before beginning with sophisticated analytical approaches it is important to do an initial data grouping of a transcriptional dataset. For example, the expression activation of “basic” immune response includes a set of endothelium genes that are differentially expressed during infection with any of the tested *S. aureus* strains. The differential expression of these genes is induced by non-specific *S. aureus* pathogen associated molecular patterns (PAMPs). The strain-specific *S. aureus* virulence factors, determinants and metabolites can cause specific differential gene expression corresponding to infection with a single, particular strain or a group of strains (Table 13).

S. aureus strains

Gene symbol	K1801/10	6850	K70058396	8325-4
Non-specific response (fold change)				
CCL2	9,87	7,97	6,79	7,33
CX3CL1	25,29	23,4	5,62	21,97
CXCL10	84,8	64,62	12,32	107,3
TAP1	6,97	6,2	2,93	11,59
AXUD1	7,48	3,4	2,45	2,52
Group-specific response (fold change)				
ATF3	14,46	7,62	NS	NS
IL-10	3,85	2,69	NS	NS
CD83	3,7	3,35	NS	NS
SERPINE1	3,19	2,62	NS	NS
BIRC3	6	4,33	NS	NS
Strain-specifically expressed genes (fold change)				
HES1	2,29	NS	NS	NS
MALL	NS	2,11	NS	NS
CLDN11	NS	NS	2,28	NS
CLDN23	NS	NS	NS	3,13
CASP1	NS	NS	NS	3

Table 13. The example of gene expression group comparison.

The qualitative analysis indicates the principle differences and possible mechanisms of gene expression induction. The quantitative analysis reflects overall differences in level of gene expression changes during the host-pathogen interaction, which can reflect stimuli intensity or possible active immunomodulation during infection.

The analysis of differentially expressed genes during all treatments brought us to the conclusion that gene expression of non-specific transcripts induced by all *S. aureus* strains is likely induced by the same type of stimulus. This stimulus leads to a non-specific response that can be triggered by characteristic antigen determinants presenting in all *S. aureus* isolates. For example, cell wall peptidoglycan and lipoteichoic acid, muramyl di-peptide and bacterial RNA/DNA are well-known activators of transcription factors NF-kB and STAT1 via

cellular pattern recognition receptors (PRRs) (Loos *et al.*, 2006). Indeed, all *S. aureus* isolates that were used in our study were able to induce upregulation of chemokines CX3CL1, CXCL10 and CCL2, whose expression is known to be regulated by activated transcription factors NF-kB and STAT1 (Isozaki *et al.*, 2008) (Table 13).

It seems that the fold of expression change in the group of non-specifically expressed transcripts correlates with the level of endothelial receptor induction. It was shown that *S. aureus* strain-specific expression of bacterial capsules can mask surface peptidoglycan and, therefore, prevent phagocytosis (Wilkinson *et al.*, 1979) and likely lower NF-kB signaling via TLR 2 (Luong and Lee, 2002). Similarly, prevention of pathogen-induced phagolysosome fusion (Lam *et al.*, 2010) or bacteria resistance to acidification in lysosomes (Lam *et al.*, 2010) can be prerequisite for a low activation of NOD 2 or RIG-I intracellular receptors.

The strain-specific expression of strain-specific genes or group-specific genes demonstrated qualitative differences. We assume that differentially expressed endothelial genes that respond only to infection with particular isolate can point out principal differences in infection strategies between *S. aureus* strains. We hypothesize that the expression of strain-specific *S. aureus* virulence determinants is the reason for distinctive endothelial expression profiles and partly explains the broad variations in *S. aureus* infection scenarios.

4.2. HUVEC infected with the *Staphylococcus aureus* isolates demonstrate strain-specific gene expression response

During infection, pathogens are able to modulate host gene expression. The endothelium attempts to detect potential invaders and “switch on” defense mechanisms, which allow for a prevention of pathogen survival and dissemination. To remove dead or infected cells, host cells are release a number of cytokines and chemokines which attract professional immune cells to the site of inflammation. The pathogen can modulate host gene expression response by masking its location or inducing apoptosis to eliminate immune cells responsible for combatting infection.

Endothelial cells actively participate in the host immune response. Aside from their important role in inflammation and signaling to specialized immune cells, they also regulate barrier function and thus provide an obstacle to spreading pathogens. All these functions

are closely connected to endothelium gene expression response upon infection with *S. aureus*.

In this study, we identified early endothelial strain-specific expressions of HUVEC infected with six *S. aureus* strains and the pro-inflammatory response of HUVEC treated with TNF-α. The microarray results largely confirmed previous reports, which demonstrated a highly diverse endothelial expression response to different *S. aureus* strains (Grundmeier *et al.*, 2010; Stark *et al.*, 2009; Matussek *et al.*, 2005). Based on strain-related endothelium expression, we attempted to elucidate particular differences in endothelium gene expression and the possible mechanisms involved in their regulation. The first important observation confirmed a highly heterogeneous differential expression of HUVEC upon strain-specific infection. The number of differentially regulated genes varied from 610 transcripts affected by pro-inflammatory K1801/10 to only 98 transcripts by affected by anti-inflammatory K70058396.

The second significant observation was that two pro-inflammatory strains appeared to induce the highest differential expression of genes involved in innate immune response, as in the number of transcripts and level of gene expression fold change.

We believe that large variations in strain-related endothelial expression can be explained by a selection of *S. aureus* strains with different infectious properties, which simultaneously reflect the differences in bacterial virulence factor expression. Moreover, previous studies have suggested that important parameters influencing cellular response to *S. aureus* infection are determined by: *S. aureus* ability for successful adhesion and invasion and aggressiveness, including cytotoxicity and intracellular survival (Haslinger-Loffler *et al.*, 2005). Additionally, it has been reported that cellular response is linked to inflammatory and cytotoxic properties of *S. aureus* antigens such as LTA, PNG and TSST-1 (Mattsson *et al.*, 2008).

Interestingly, we found that strains expressing high quantities of virulence regulators, toxins and adhesins, induced fewer changes in gene expression in endothelial cells while strains expressing low quantities of virulence factors, in comparison, provoked many more changes in gene expression in the endothelium. This intriguing data indicates that the virulence factors actively control gene expression in infected endothelium. These findings also support an earlier observation describing how *S. aureus* strains expressing many virulence factors are either highly invasive or aggressive (Iwatsuki *et al.*, 2006).

For example, anti-inflammatory strain K70058396 with highly expressed virulence factors appeared to induce a weak endothelial expression response in compare with the other three isolates. It seems that a high expression of virulence factors by particular *S. aureus* strain can actively silence endothelial response and benefit bacteria by avoiding immune defense, thereby improving survival. However, we observed other strategies in avoiding endothelial response such as expressing a low amount of virulence factors, as demonstrated by strains K2900/10 and K9657/04.

As such, we speculate that the isolates expressing the highest amounts of toxins are more prominent in intracellular survival and are likely able to multiply intracellularly. This hypothesis was supported and accepted as three *S. aureus* strains showing the highest production of virulence factors appeared to survive and grow intracellularly. Remarkably, the anti-inflammatory strain K70058396, with the highest production of virulence factors, had the highest rate of intracellular growth. It seems, however, that endothelial pro-inflammatory activation can diminish this ability as one pro-inflammatory strain did not demonstrate intracellular growth and the second had a low rate of intracellular multiplication when compared with anti-inflammatory strain K70058396.

Due to the logical consequences of high bacteria virulence of the multiplying strains, one could suggest stronger cytotoxic properties of such isolates. Indeed, three *S. aureus* strains that were able to survive and grow intracellularly induced apoptosis, which was demonstrated by Annexin/PI and caspase-3 assay.

To study strain-specific mechanisms of cell death regulation in infected endothelium, we first analyzed a subset of endothelial differentially-regulated genes related to cell death. The transcriptional data gave us a hint as to which transcriptional factors and pathways could play a role in the differential expression of endothelial genes related to cell death.

We found that infected endothelium showed a high variation of apoptotic gene expression upon strain-specific *S. aureus* infection. However, the main outcome of apoptotic gene expression analysis showed differences in subsets of up/down pro- and anti-apoptotic genes induced by cytotoxic and non-cytotoxic *S. aureus* isolates.

We could thereby conclude that the differential expression of cell death genes related to strain-specific activation of transcription is mediated by specific virulence factors. Furthermore, we proposed that particular virulence factors could induce gene expression via defined endothelial transcription factors. This was suggested by the fact that cytotoxic

strains likely up/down-regulate gene expression via CREB1 and NF-kB transcription factors. In contrast, the non-cytotoxic strain 8325-4 induces cell death-related genes via transcription factors IRF1 and STAT1 belonging to the interferon signaling pathway (Fig. 40). Interestingly, the separate activation of NF-kB and interferon signaling pathways requires the involvement of different receptors which implies that pro-apoptotic stimuli have a connection with the NF-kB signaling pathway.

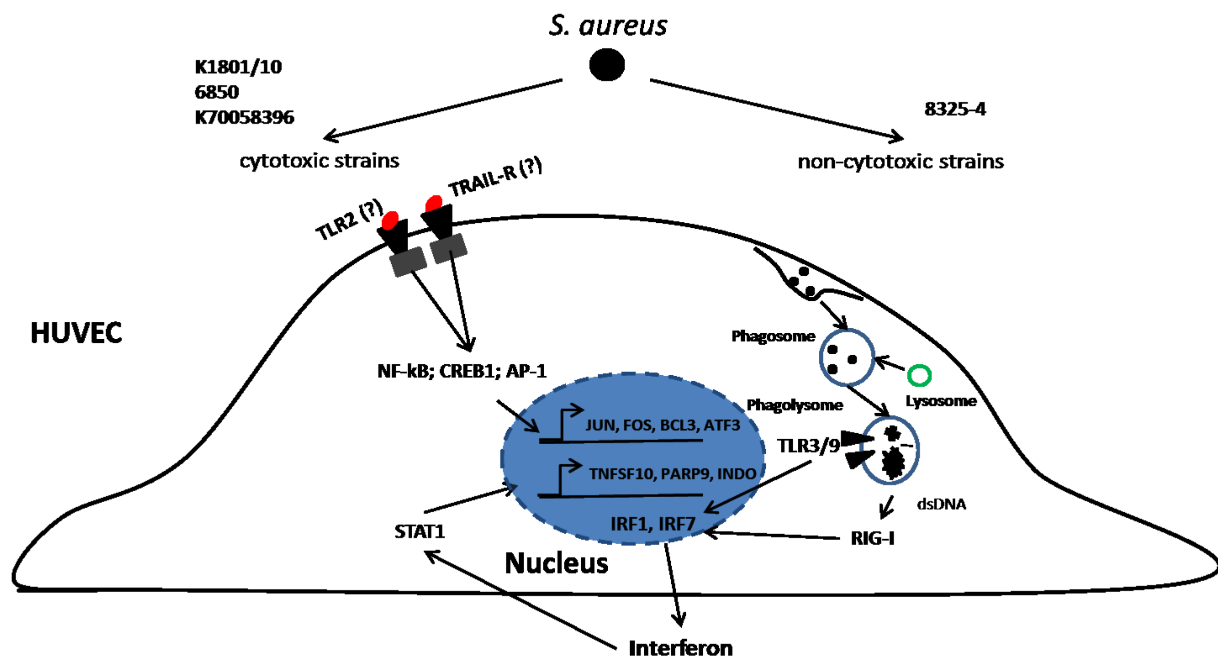


Figure 40. Proposed model based on microarray data analyses. Depending on the cytotoxicity, *S. aureus* strains induce gene expression via the activation of different transcription factors.

4.3. Strain-specific mechanisms of HUVEC cell death induction

Based on transcriptional data analyses, we hypothesize that there are key differences in the ability to induce cell death between the different *S. aureus* isolates. It has been previously described that apoptosis is mediated through extrinsic or intrinsic pathways (Dhanasekaran and Reddy, 2008). Based on our research data, we propose that cytotoxic strains kill infected endothelial cells by induction of both extrinsic and intrinsic pathways. We propose that the extrinsic death receptor pathway is most likely activated by TLR 2 and/or tumor necrosis factor receptor 1 accompanied by the activation of apoptosis-inducing ligand receptor (TRAIL-R). The extrinsic death receptor pathway was shown to induce gene expression through NF-kB and CREB1 transcription factors (Hadad *et al.*, 2011).

Additionally, this conclusion is supported by the differential expression of TNFAIP3, RIPK2, and BIRC3 genes that are upregulated by cytotoxic strains and by TNF-α treatment. The expression of these genes is a hallmark of NF-kB-mediated transcription. Interestingly, the activation of this TRAIL receptor leads to the upregulation of c-Fos pro-oncogenic gene (Siegmund *et al.*, 2001), which is highly expressed by endothelium infected exclusively by cytotoxic strains. Part of the extrinsic apoptotic pathway involves mitogen-activated protein kinases (MAPK) and the activation by reactive oxygen species (ROS). It seems that cytotoxic strains induce MAPK phosphorylation which, in turn, activate ATF3, JUN (Kappelmann *et al.*, 2014) and other transcription factors responsible for pro-apoptotic gene expression such as BCL3 (Amundson *et al.*, 1999).

The second possibility to induce endothelial apoptosis that could be exploited by cytotoxic *S. aureus* strains is the activation of the intrinsic apoptotic pathway. The intrinsic apoptotic pathway is tightly bound with the extrinsic pathway and has been reported to play a role in *S. aureus*-mediated HUVEC apoptosis. The intrinsic apoptotic pathway includes activation of BCL-protein family members, which compromise mitochondrion integrity upon apoptotic stimuli (Esen *et al.*, 2001). The intrinsic pathway is also interconnected with the extrinsic through activated death receptors, ROS or MAPK kinases which can also activate BCL proteins (Cross *et al.*, 2000). Activated BCL proteins activate caspase 3 through cytochrome-C release and induction of apoptosis-activating factor 1 (Apaf-1) (Dhanasekaran and Reddy, 2008).

One of the central questions we looked to investigate was why infection with pro-inflammatory isolate 8325-4 does not cause apoptosis in endothelium. We assume that non-cytotoxic strain 8325-4 mainly activates cell death-related gene expression via IRF1 and STAT1 transcription due to intracellular bacteria degradation and presentation of DNA/RNA to intracellular receptors of the interferon signaling pathway such as TLR 3 and 9 or RIG-I.

To begin, this assumption can be partially proven by the observed overexpression of TRAIL, INDO, BATF2 and Poly (ADP-Ribose) Polymerase (PARPs) genes by 8325-4 strain (Fig. 45). These genes belong to the group of interferon-inducible genes and are regulated by IRF1 and STAT1 transcription factors (Gong and Almasan, 2000; Ma *et al.*, 2011; Juszczynski *et al.*, 2006). In addition, the strain 8325-4 demonstrated intracellular persistence more so than intracellular growth. This may implicate impaired growth due to the possible destruction by activated endothelium.

As such, we speculate that the expression of cell death-related genes by 8325-4 strains through the interferon signaling pathway cannot lead to endothelium apoptosis, most likely due to the need for additional pro-apoptotic stimuli. For instance, TRAIL ligand, whose expression is highly upregulated by the 8325-4 strain, was shown to play a role in the extrinsic apoptotic pathway. However, its primary induction does not lead to apoptosis in every cell type. For certain cell types, it was shown that the additional activation of the intrinsic apoptotic pathway is needed to induce apoptosis (Almasan and Ashkenazi, 2003). Moreover, it was demonstrated that TRAIL-induced HUVEC apoptosis is regulated by surface expression of TRAIL-R3 receptor (Zhang *et al.*, 2000). This means that a significant increase in apoptosis could occur, but only upon removal of TRAIL-R3 from the HUVEC surface TRAIL ligand (Zhang *et al.*, 2000). Therefore, TRAIL stimulation requires additional stimuli in order to induce apoptosis in endothelium.

Other receptors such as TLR4, TLR3, NOD2 and RIG-I can also recognize staphylococcal determinants and are likely able to modulate NF-kB, CREB1 or STAT1 activation as well as play a role in endothelium defense response (Takeuchi *et al.*, 1999; Kato *et al.*, 2011).

4.4. Verification of the role of protein kinases phosphorylation in *S. aureus*-induced endothelial apoptosis

To verify the role of the extrinsic apoptotic pathway in endothelial cell death induction we performed a kinase inhibition assay. The selection of kinases was based on previous studies where *S. aureus'* ability to modulate phosphorylation/de-phosphorylation events in MAPK pathway was demonstrated, which had a very significant impact on the cellular response to infection. In particular, staphylococcal peptidoglycan-induced activation of TLR2 was shown to activate such regulatory kinases as p38 and JNK MAPK (Into and Shibata, 2005; Esen *et al.*, 2001). JNK, p38 and ERK ½ MAPK signaling is a complex process which has also been shown to involve a number of other upstream regulatory kinases such as PKC, PI3K and ASK1 (Fukao and Koyasu, 2003; Kappelmann *et al.*, 2014). Currently, the exact mechanism of how kinase interplay affects apoptosis is not known, although it is hypothesized to occur through the regulation of certain transcription factors and cell death effectors (Cross *et al.*, 2000).

During the experiment, JNK, PI3K, ERK 1/2, PKC, p38 and ASK1 were inhibited with commercially available selective inhibitors. It was found that the inhibition of stress-responsive kinase ASK1 and its downstream partner p38 allowed for the rescue of infected and hydrogen peroxide treated cells. This data confirms the previously described involvement of ASK1 and p38 in *S. aureus*-mediated cellular death, though the direct anti-apoptotic effect of ASK1 and p38 inhibition for infected endothelium had not been previously described prior to this experiment.

We hypothesize that cytotoxic strains of *S. aureus* induce endothelium apoptosis through a stronger activation of TLR 2 signaling and/or higher ROS production to activate caspase-3-mediated apoptosis via ASK1 and p38 MAPK signaling. This assumption is supported by the fact that ASK1 kinase plays an important role in cellular stress response and has been shown to be activated in the TLR 2 signaling cascade or by ROS. The downstream kinase p38 have been shown to be activated by and play a role in caspase-3-mediated apoptosis (Into and Shibata, 2005).

By contrast, the role of PKC appeared to have an anti-apoptotic function. The effect of increased cell death upon PKC inhibition indicated an anti-apoptotic role of PKC signaling in infected endothelium and a possible involvement in the inhibition of ASK1-p38 signaling. Furthermore, PKC was shown to inhibit p38 phosphorylation in bovine leukocytes (Yamamori *et al.*, 2000).

Another member of MAPK ERK ½ can be activated by staphylococcal surface antigens challenging the PRRs (Ratner *et al.*, 2001); however, this was not shown for infected endothelium. This MAPK has anti-apoptotic properties and can become activated through a similar pathway with p38 or JNK (Takeda *et al.*, 2007). In our experiment, the inhibition of ERK ½ did not influence endothelium survival upon *S. aureus* infection which suggests it has a limited role in endothelium cell death regulation during staphylococcal infection. It seems that activated endothelial ERK ½ has a pro-apoptotic effect upon hydrogen peroxide treatment which indicates its "selectivity" in cell death regulation.

The other regulatory kinase PI3K was shown to be involved in VEGF signaling in endothelium with strong anti-apoptotic functions. This has been demonstrated as, upon growth factor activation, PI3K mediates endothelial proliferation (Bullard *et al.*, 2003). Additionally, PI3K has been shown to inhibit p38 and other MAPK phosphorylation (Fukao and Koyasu, 2003), which are features that might contribute to cell death prevention. As

expected, the endothelium inhibition of PI3K had a strong apoptotic effect irrespective to the applied treatment. This finding indicates the possible role of one more regulatory kinases in endothelial cell death prevention upon infection. Interestingly, co-regulation between PKC and PI3K kinases has already been shown (Cross *et al.*, 2000), whose anti-apoptotic effect during infection could be regulated synergically.

Considering that JNK is activated by ASK1 in the same pathway as p38 and based on previous research suggesting its role in endothelium apoptosis (Esen *et al.*, 2001), we expected that JNK inhibition would have an anti-apoptotic effect. Surprisingly, sustained endothelial JNK inhibition led to a state of 100% cellular death irrespective to the treatment applied. Therefore, the role of JNK kinase in cell death has to be elucidated by a different experimental approach.

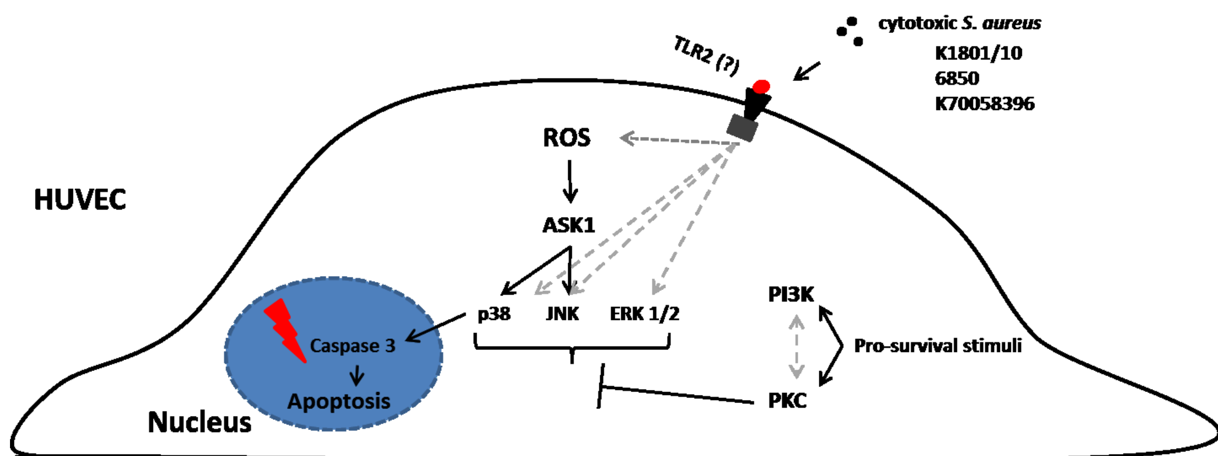


Figure 41. The model of endothelium apoptosis regulation by kinase signaling upon infection with cytotoxic *S. aureus* strains. Grey arrows represent literature data that was not tested in our study.

In conclusion, we propose a model of kinase interaction in endothelium apoptotic regulation. The cytotoxic *S. aureus* strain can trigger surface receptor TLR 2 and induce ROS production. These changes lead to ASK1 and p38 MAPK activation and cell death induction through caspase-3 dependent mechanisms. PI3K and PKC regulatory kinases play a role in inhibiting ASK1-p38 phosphorylation and promoting cell survival. The role of JNK and ERK ½ kinases in this process is not clear and should still be elucidated. Non-apoptotic strains cannot induce apoptotic changes, most likely due to the hypo-stimulation of TLR 2 and/or low ROS production with low or no ASK1/p38 activation (Fig. 41).

To summarize, we suspect that both the extrinsic and intrinsic apoptotic pathways are playing a role in endothelial cell death induced by cytotoxic *S. aureus* strains. However, the particular role of each pathway requires further clarification. In this study, we attempted to explain the background of strain-specific *S. aureus*-induced apoptosis based on transcriptional data, apoptotic and kinase inhibition assays along with the data of strain-specific *S. aureus* intracellular survival (Fig. 42).

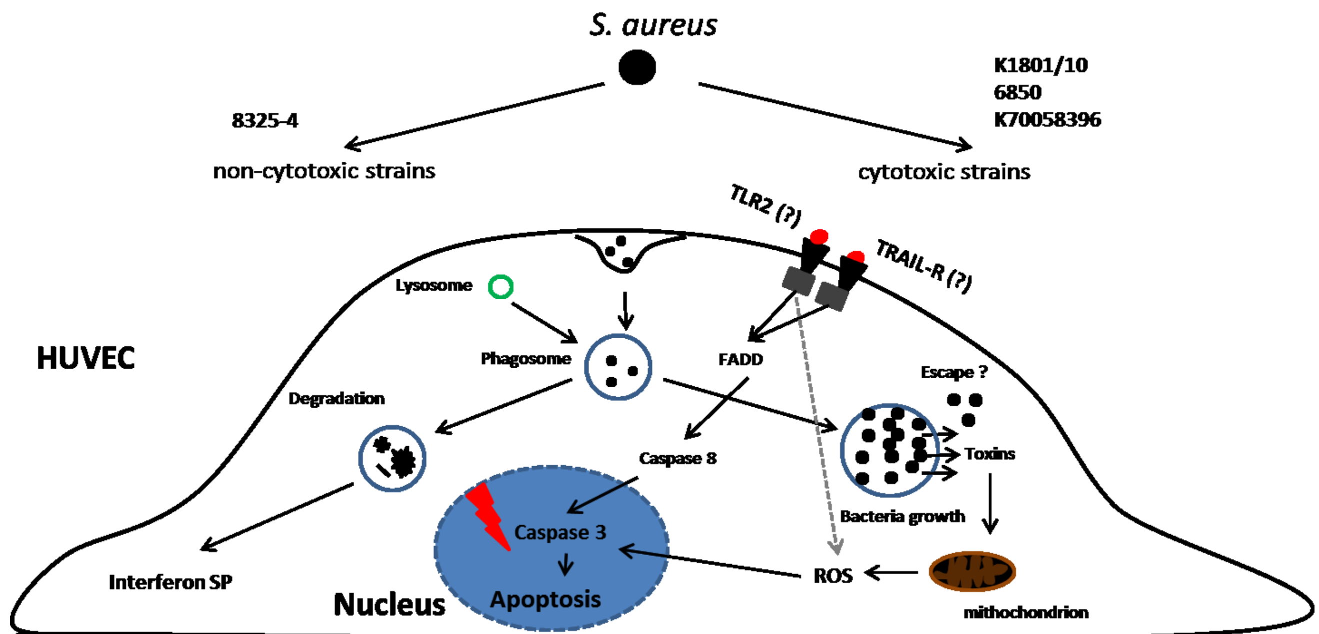


Figure 42. Hypothetical scheme of endothelium cell death induction by cytotoxic *S. aureus* strains.

Summary

Staphylococcus aureus is a prominent, wide-spread human pathogen causing various clinical complications. *S. aureus* antibiotic resistance is currently a prevalent clinical problem that leads to an increased cost of treatment, duration of hospitalization and severe diseases. One of the complications during *S. aureus* treatment is the broad variation of strains each representing different virulence and pathogenic potentials which complicates predicting infection outcomes and choosing the right course of treatment to eradicate the pathogen. During many *S. aureus*-born life-threatening infections, interactions of the pathogen with endothelium is of critical importance. For this reason, this study focused on understanding strain-specific *S. aureus*-endothelium interactions as they are the key to better understanding *S. aureus* infection strategies and clarifying the specific endothelium regulation mechanisms at work during infection. We were especially interested in examining early endothelial response to strain-specific *S. aureus* infection. The early endothelial response is of great importance at the initial stage and overall success of the infection as well as for the situation of systemic endothelial response to a massive release of pathogen into the bloodstream.

A number of studies have been performed to better understand how particular bacterial virulence factors influence systemic responses in endothelium. In particular, *S. aureus* adhesins facilitate adhesion of the pathogen to endothelium as well as, after uptake, provoking endothelium immune response accompanied by cell death induction. The immune response of endothelium leads to the activation of pro-inflammatory phenotypes and the gene expression of genes involved in immunity, cell death, cytokinesis and other cell functions.

In this study we focused to uncover fundamental *S. aureus*-induced pathophysiological relevant mechanisms in endothelial cells in the early stages of infection. After screening 24 septic isolates with respect to pro- and anti-inflammatory early responses in endothelium, we observed a wide heterogeneity. However, several strains were identified as exhibiting extreme behaviors just after infection. In particular, we found two strains with clear anti-inflammatory properties and two strains with pro-inflammatory properties. Furthermore, three of these four strains induced cytotoxicity while one strain had no such effect. Some of the *S. aureus* strains showed strong anti-inflammatory effects, even upon TNF-α stimulation

while others did not alter TNF-α-induced endothelial response. We used these "extreme" strains for microarray analyses and found a tremendous differences in the gene expression patterns in endothelium early after infection (7 hours). Each isolate changed the expression of a distinctive number of genes with specific levels of expression change. We propose that a number of differentially regulated genes and their level of differential expression are related to the specific virulence of a particular *S. aureus* isolate. The strain-specific endothelial gene expression analysis showed a probability to be promoted by a number of activated transcription factors likely activated through PRRs and interferon signaling pathways.

The expression data was used to predict possible mechanisms and pathways involved in endothelium defense response, with the focus on cell death induction. We reported that *S. aureus* strains vary in their ability to cause endothelium cell death. We divided them into two groups of pro-apoptotic and non-apoptotic isolates. The pro-apoptotic isolates showed early apoptotic changes in Annexin V/PI assay and were shown to induce apoptosis by activation of caspase 3. We assumed that cytotoxic isolates of *S. aureus* are able to activate caspase 3 through TLR 2, TLR 4 signaling, and via reactive oxygen species production (ROS), due to intracellular multiplication and toxin-mediated mitochondria challenge.

We suggested that strain-specific differences in apoptosis induction depend on variations in invasiveness, toxin production and intracellular growth between cytotoxic isolates. These differences partially determine strain-specific differential phosphorylation of apoptosis-regulated kinase 1 (ASK1), p38, PI3K and PKC kinases, which regulate the process of endothelium cell death.

There are still many questions left unanswered regarding the precise mechanisms of anti-inflammatory properties and cytotoxic effects of *S. aureus* isolates. The particular *S. aureus*-induced pathways responsible for apoptosis and gene expression induction need to be confirmed through molecular biology testing.

The results of this study will help deepen our understanding of the pathophysiological processes at work during *S. aureus*-endothelium interactions. Information concerning the mechanisms of interplay between pathogen virulence factors and the host defense response can be used to enhance targeted clinical diagnostics and development new non-antibiotics therapies to improve treatment and slow the development of new resistant strains.

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