Human humoral and cellular immune responses to *Staphylococcus aureus* lipoproteins and Panton-Valentine Leukocidin (PVL)

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Table of contents

Abbreviations

2D	Two-dimensional
APC	Antigen presenting cell
BCR	B cell receptor
E. coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
g	Standard gravity
Ig	Immunoglobulin
IL	Interleukin
INF	Interferon
LB	Luria-Bertani medium
Lgt	Prolipoprotein diacylglyceryl transferase
LPS	Lipopolysaccharides
MRSA	Methicillin-resistant *S. aureus*
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PVL	Panton-Valentine leukocidin
S. aureus	*Staphylococcus aureus*
Sag	Superantigen
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SpA	Staphylococcus protein A
TCR	T cell receptor
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TSB	Tryptic soy broth

Summary

Staphylococcus aureus is present in around a third of the human population as a constant commensal in the anterior nares, in a third as an intermittent commensal, and a third are non-carriers. However, *S. aureus* is also a dangerous pathogen, responsible for many types of infections. The severity of these infections ranges from moderate symptoms - such as folliculitis and furunculosis - to grievous conditions, including sepsis, deep abscesses, pneumonia, osteomyelitis, and infective endocarditis. Recently, the emerging of methicillin-resistant *S. aureus* strains has aggravated the health problem. Treating infections caused by the invasive strains has become ineffective with conventional antibiotics. Noticeably, transmission of *S. aureus* has occurred not only in healthcare settings but also in the community; furthermore, transmission between humans and domestic animals has been reported. Although studies about host-pathogen interactions of *S. aureus* have advanced our knowledge in the last decades, we still have not fully understood mechanisms of the immune system in responses to *S. aureus*. The aim of this study is to unravel interactions of the human adaptive immune system to selected *S. aureus* virulence factors. In particular, the study focuses on two aspects: the reaction of human antibodies to the bacterial extracellular proteins in *S. aureus*-induced furunculosis with an emphasis on Panton-Valentine Leukocidin and responses of the adaptive immune system to membrane-bound lipoproteins of *S. aureus*.

Furunculosis is a variety of hair follicle infection in which *S. aureus* is one of the chief causal pathogens involved. The corresponding bacterial strains are generally capable of producing of a pore-forming toxin, known as Panton-Valentine Leukocidin (PVL). Recently, the emerging of *pvl*-positive methicillin-resistant *S. aureus* has become a problem for treating the bacterially caused furuncles. Colonization with the bacteria is a risk factor for development of chronic or recurrent boils. It is not yet known why furunculosis patients are

largely infants or young adults. In this context, we untangled the responses of antibody IgG antibodies to *S. aureus* extra-cellular factors, notably the PVL toxin, in families in which the patients were children. Multiplex PCR demonstrated that *S. aureus* clones, isolated from the patients' wounds but also from the nares of family members, harbored genes coding for PVL toxin. *Spa*-typing highlighted that bacterial genotypes were very similar in each family. This suggests that transmission of *pvl*-positive *S. aureus* took place between family members. The finding also raises the question why only the young patients but not family members who were colonized by the same *S. aureus* clones suffered from furunculosis. 2D immune proteomics procedures showed a tendency of higher IgG titers against bacterial virulence factors in family healthy members than in patients. PVL-specific antibodies were measured using ELISA, in which patients' PVL-specific IgG titers were low. This supports the idea that antibodies, probably in conjunction with T cells, might contribute to clinical protection in furunculosis. This research will serve as a foundation for future studies, in which our results should be validated in a larger cohort.

Among *S. aureus*' virulence factors are lipoproteins, which are anchored in the bacterial cell membrane. Lipoproteins perform various functions in colonization, immune evasion, and immunomodulation. These proteins are potent activators of the complex of innate immune receptors termed Toll-like receptors (TLR) 2 and 6. This study addressed the specific B-cell and T-cell responses to lipoproteins in human *S. aureus* carriers and non-carriers. 2D immune proteomics and ELISA approaches revealed that titers of serum antibody (IgG) binding to the *S. aureus* lipoproteins were very low or even unmeasurable in healthy individuals except for the lipoprotein SaeP. Only patients with cystic fibrosis or epidermolysis bullosa who were heavily exposed to the bacteria, generated an antibody response also to lipoproteins. Proliferation assays and cytokine profiling data showed only subtle responses of T cells in healthy individuals; three out of eight tested

lipoproteins did not elicit proliferation. Hence, the robust activation of the innate immune system by *S. aureus* lipoproteins does not translate into a strong adaptive immune response. Reasons for this may be inaccessibility of lipoproteins for B cells as well as ineffective processing and presentation of the antigens to T cells.

The main findings implicate that family members can serve as *S. aureus* reservoirs causing recurrent furunculosis in young patients and that antibodies may provide partial protection from such infections by *S. aureus*. We have found that, different from proteins that are secreted by *S. aureus*, lipoproteins which anchored in the bacterial cell membrane, do not trigger strong responses from the human adaptive immune system. This suggests that these proteins remain mostly hidden in the bacterial cell-wall.

Chapter 1. Introduction

1. Back ground information on *Staphylococcus aureus*

1.1. Two faces of *Staphylococcus aureus*

Staphylococcus aureus was first discovered in 1880 in Aberdeen, Scotland, by the surgeon Alexander Ogston, from a patient with postoperative wound suppuration and abscesses. *S. aureus* is a Gram-positive coccus, and appears in grape-like clusters with a golden-yellow color. Some *S. aureus* strains produce capsules. In the genus *Staphylococcus*, it is known as one of the most pathogenic species for human.

Being a commensal bacterium, *S. aureus* can be found on the skin and mucous membranes, as well as in various other tissues. The anterior nares are the most frequent niche of these bacteria. A great number of studies showed that about 20% (range 12–30%) of individuals are persistent *S. aureus* nasal carriers, approximately 30 % are intermittent carriers (range 16–70%), and about 50% (range 16–69%) are non-carriers (Broker et al., 2014; Holtfreter and Broker, 2005; Wertheim et al., 2005).

S. aureus is known to be a major human pathogen causing a wide range of infections, from relatively mild skin infections such as folliculitis and furunculosis to life-threatening conditions, including sepsis, deep abscesses, pneumonia, osteomyelitis, and infective endocarditis (Rooijakkers et al., 2005; Schaffer and Lee, 2008; Tong et al., 2015; van Belkum et al., 2009).

Nosocomial infections

In many cases, after hospital treatment, patients are accidentally infected by *S. aureus*, so-called nosocomial infections. Nosocomial infections, also called hospital-acquired or healthcare-associated infections, are side results of treatment at a hospital or a healthcare service unit (Plata et al., 2009). *S. aureus* is one of

the most frequent causes of nosocomial infections and has been accepted as one of the most common causes of bloodstream infections (Holtfreter et al., 2009; van Belkum et al., 2009). Additionally, nasal *S. aureus* carriage has been specified as a risk factor for development of the infections not only among patients but also in hospital staffs. In the hospital environment, doctors, nurses and workers can be sources for transmission of *S. aureus* (Uhlemann et al., 2014; Wertheim et al., 2005).

Antibiotic resistance

In recent years, the resistance of *S. aureus* strains to more antibiotics has become a worldwide health problem. Nosocomial infections with methicillin-resistant strains (MRSAs) are spreading quickly throughout the world (Boyle-Vavra and Daum, 2007). Noticeably, the infections have spread not only in healthcare centers, but also in the community, and may be responsible for more complicated syndromes in patients (Boyle-Vavra and Daum, 2007; Byers and Decker, 2008). Moreover, transmission of MRSA strains between domestic animals and humans has been reported (Becker et al., 2015; Bosch et al., 2015). The methicillin-resistant strains harbor the gene *mecA*, which encode the resistance to β-lactam antibiotics. Very often, these strains also harbor the *pvl* gene, coding for a virulence factor, known as Panton-Valentine Leukocidin (PVL). Lately, the emergence of community-associated methicillin-resistant *S. aureus* strains in several countries has been linked to presence of the *pvl* gene, but studies to demonstrate the role of PVL in the pathogenesis of such strains have given conflicting results (Miller and Kaplan, 2009; Otto, 2012; van Belkum et al., 2009; Watkins et al., 2012).

Nasal carriage

In spite of its aptitude for living in many sites of skin and mucosal surfaces of carriers, the primary niche of *S. aureus* is the anterior nares. Carriage of this

bacterium in the nose appears to be important in the epidemiology and pathogenesis of the infection (Kluytmans et al., 1997). Persistent carriers usually harbor one *S. aureus* clone, and carriage is more common among children than adults. This pattern of carriage changes between the ages of 10 and 20 years in many people, but details on this change are still limited. In addition, persistent carriage tends to protect from the invasion of foreign strains, but the effect size decreases when antibiotics are applied (Kluytmans et al., 1997). Theoretically, the elimination of nasal carriage, e.g. by mupirocin treatment, would reduce the rate of infection (Wertheim et al., 2005).

To date, there is an under discussion issue whether the nasal carriage of *S. aureus* is contributing to acquiring of MRSA or not. Krebes and co-authors found no evidence that carriers are more sensitive to receive MRSA (Krebes et al., 2011), while Ghasemzadeh-Moghaddam and co-workers identified nasal colonization with *S. aureus* as an important factor for acquiring new MRSA strains (Ghasemzadeh-Moghaddam et al., 2015).

1.2. Virulence factors of *Staphylococcus aureus*

S. aureus strains are responsible for a wide range of infectious diseases, and many of the bacteria's virulence factors are involved in the pathogenesis (see figure 1). First of all, many proteins enhance infectious processes of *S. aureus*. One group are surface bound proteins, termed adherence molecules, which enable colonization and invasion of host cells (Plata et al., 2009). The most common adherence proteins are: fibronectin binding protein A and B (FnbpA and FnbpB), collagen binding protein (Cna), clumping factors A and B (ClfA and ClfB), plasma-sensitive surface protein (Pls) and a remarkable protein, called protein A (SpA). Besides the attachment-improving properties common to all these proteins, protein A also has the ability to bind antibody IgG via their Fc parts, resulting in improved bacterial escape from opsonization and phagocytosis (Plata

et al., 2009). Additionally, many secreted proteins, e.g., degradative enzymes such as proteases, lipase, etc., support the invasion of *S. aureus*.

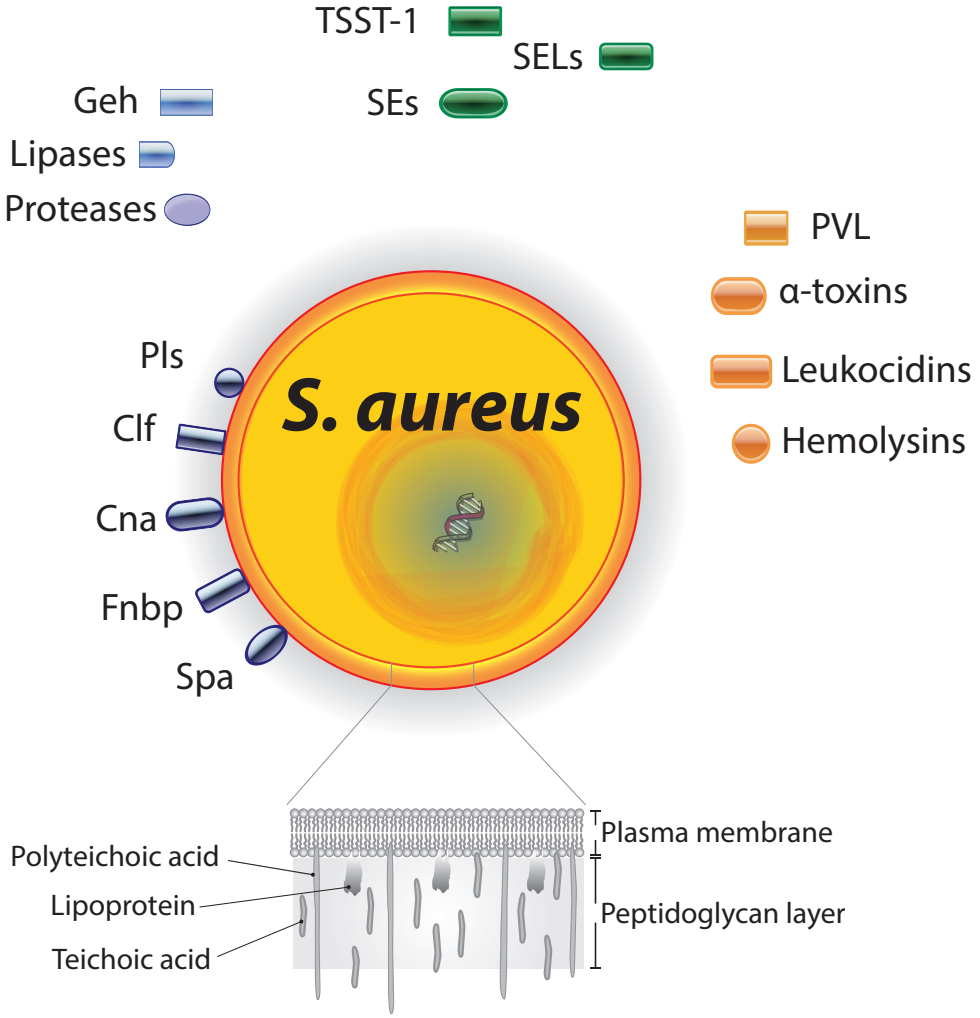

Figure 1. Virulence factor of S. aureus.

Virulence factors of *S. aureus* usually are secreated proteins—superantigens (TSST-1, staphylococcal enterotoxins [SEs], staphylococal enterotoxin-like proteins [SELs]), proteases, glycerol ester hydrolase [Geh], lipases, pore-forming toxins (Panton-Valentine Leukocidin [PVL], α-toxin, leukocidins, hemolysins). Another protein group, surface proteins, also contribute in invasion of the pathogen *S. aureus*. The figure is adapted from Gordon and Lowy, 2008.

Toxins are also among these secreted proteins. It is believed that they play a key role in *S. aureus* infection and are associated with serious syndromes. A number

of the toxins belong to the known group of staphylococcal superantigens (SAgs), including: toxic shock syndrome toxin (TSST-1), staphylococcal enterotoxins (SEA–SEE, SEG–SEJ, SES and SET), the staphylococcal enterotoxin-like toxins (SElK–SELlR and SElU) (Grumann et al., 2008). Proteins in this group greatly stimulate both the innate and the adaptive immune system and are involved in gastroenteritis as well as toxic shock syndrome (Holtfreter and Broker, 2005). Another group comprises exfoliative toxins, causing staphylococcal scalded skin syndrome, occurring most commonly in children (van Belkum et al., 2009). Another group of virulence factors includes many substances acting on the cell membrane and resulting in disruption of host cells, such as α-toxin, also called α-hemolysin (Hla); β-hemolysin, γ-hemolysin, leukocidin and Panton-Valentine Leukocidin. Most of them are at least bicomponent toxins. Although numerous *S. aureus* virulence factors are known, many questions remain concerning the exact role of each, be it in colonization, persistence or infection (Plata et al., 2009; van Belkum et al., 2009).

Staphyloccocus aureus Panton-Valentine Leukocidin

PVL is a virulence factor consisting of two components: LukS-PV and LukF-PV (see figure 2). These protein parts act as a pore-forming toxin, resulting in target cell lysis. Initially, LukS-PV binds to the cell surface that consequently initiates binding of LukF-PV. Then, these components form a hetero-octamer pore on the surface of host cells (Pedelacq et al., 1999). The targets of PVL toxin are neutrophils, monocytes and macrophage but not lymphocytes (Spaan et al., 2013). Genes encoding for LukS-PV and LukF-PV are located on a prophage which is integrated in the core genome of *S. aureus*. Two–3% of *S. aureus* wild-type clones carry PVL encoding genes; mostly *pvl*-positive isolates are associated with community-acquired methicillin-resistant infections caused by *S. aureus* (Boyle-Vavra and Daum, 2007; Loffler et al., 2010; Melles et al., 2006).

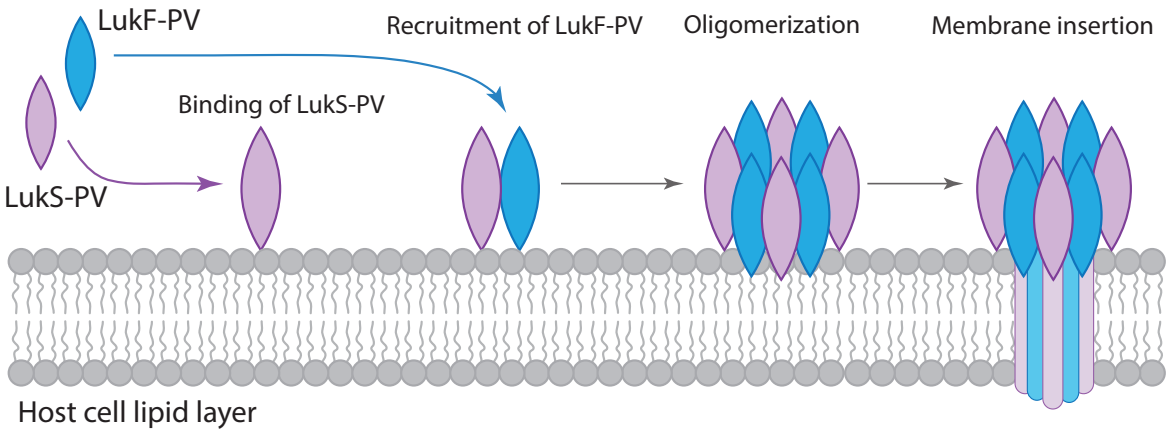

Figure 2. Pore-forming Panton-Valentine Leukocidin model.

Panton-Valentine Leukocidin subunits, LukF and LukS, involve in pore-forming in host cell membrane. Initially, LukS recognizes and binds to the envelope of leukocytes. Consequently, LukF is recruited to the binding site. Following, an octameric structure is assembled which consists of alternating 8 subunits of LukS and LukF. In last stage, the formation of a β-barrel pore leads to insertion through the host cell lipid layer. The illustration is adapted from Alonzo and Torres, 2014.

Pvl-positive *S. aureus* strains are strongly linked to skin and soft-tissue infections (SSTIs). As opposed to *pvl*-negative strains, these strains are more likely acquired from the community (Grumann et al., 2014; Shallcross et al., 2013). The severity of *S. aureus*-caused SSTIs is in a range from surface skin infections to deep tissue abscesses. In 2006, Moran et al. reported that 98% of MRSA *S. aureus* isolated from SSTI patients harbored the *pvl* gene (Moran et al., 2006). The majority of skin and soft-tissue cases reported in the UK military are caused by *pvl*-positive *S. aureus* strains (Lamb and Morgan, 2013). *Pvl*-positive *S. aureus* clones play a role in the development of furuncles (boils), particularly in young adults (Yamasaki et al., 2005). Family transmission of *pvl*-positive *S. aureus* isolates associated with skin infections has been described Perez-Roth et al., 2010.

Staphylococcus aureus lipoproteins

Lipoproteins, which are attached to the *S. aureus* membrane via a diacylglycerol linkage, are considered as promising potential vaccine targets (see figure 3). As virulence factors, these proteins are involved in various functions, including transport, signal transduction, and as membrane transporters they also contribute to antibiotic resistance (Kovacs-Simon et al., 2011; Sheldon and Heinrichs, 2012). Heterodimers of the innate immune receptors TLR2 and TLR6 recognize diacylated lipopeptides of Gram-positive bacteria, while complexes of TLR2 and TLR1 sense triacylated lipopeptides from Gram-negative bacteria (Kang et al., 2009). However, adaptive immune responses to these lipoproteins are largely unexplored.

Only few studies based on animal models have focused on the adaptive immune response. Lipoprotein FhuD2 vaccination resulted in specific anti lipoprotein antibodies and conferred protective immunity in murine infection models (Mishra et al., 2012). Another two lipoproteins, NWMN_0601 (MntC) and NWMN_0364, elicited IgG and T cell responses in cattle immunized with heat-killed intact *S. aureus* Newman cells (Lawrence et al., 2012). As reviewed, a phase II *S. aureus* vaccine trial has been conducted with three antigens were applied, among them the MntC. Trial was not successful (Jansen et al., 2013). Lipoproteins from *S. aureus* Newman led to enhanced activation of dendritic cells and induced Th1 and Th17 differentiation in mice (Schmaler et al., 2011). There are, however, few publications regarding the human response to *S. aureus*. Diep and colleagues showed human IgG binding to lipoproteins, although low titers of the antibodies were observed (Diep et al., 2014). Zielinski et al. have shown populations of specific Th1 and Th17 cells specific for *S. aureus* in humans. The triggering antigens, however, were not determined (Zielinski et al., 2011). To date, no systematic analysis of the natural human adaptive immune response to *S. aureus* lipoproteins has been published.

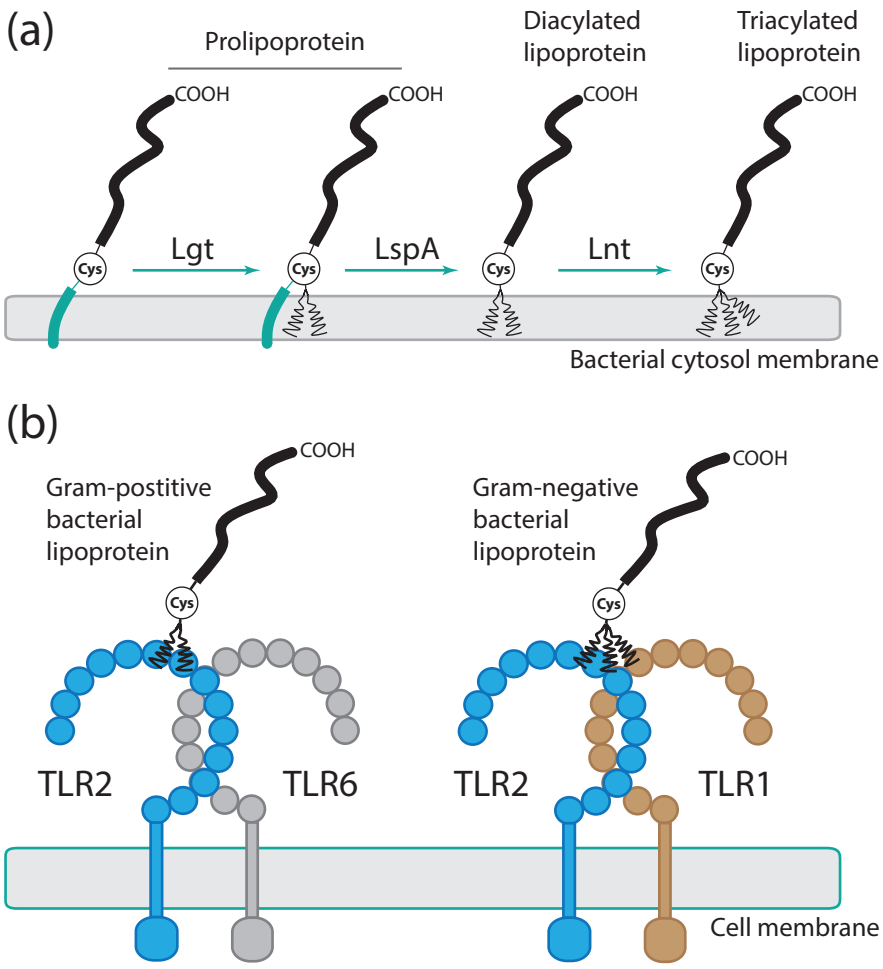

Figure 3. Bacterial lipoproteins: synthesis and recognition by Toll-like receptors.

(a) The maturation of lipoproteins. Prolipoprotein diacylglyceryl transferase (Lgt) enzyme transfers a diacylglycerol group to the cysteine position. LspA cleaves off the signal peptide. In Gram-negative bacteria, a third acyl group will be bound to cysteine by catalyzing of enzyme Lnt. (b) TLR2 together with TLR6 or TLR1 can recognize lipoproteins from Gram-positive and -negative bacteria, respectively. This figure is adapted from Wells, 2011.

2. Recognition of *S. aureus* by immune system

In general, the mammalian immune system uses two strategies to prevent infections. The innate immune system, together with physical, chemical and cellular barriers, protects the hosts from invasion at a very early stage.

Collaboratively, the adaptive immune system, in a specific response, then contributes to eliminating the pathogen in re-encounter scenarios.

2.1. The innate immune system and *S. aureus*

Recognizing bacteria

The innate immune system can sense *S. aureus* using pattern recognition receptors (PRRs). Expressed on the cells located in skin—such as Langerhans cells, macrophages, dendritic cells, mast cells—these receptors can sense microbe-associated molecular patterns (MAMPs) which are conserved in micro-organisms. Host cells use Toll-like receptors (TLR1/6 with TLR2) to recognize lipid tails of bacterial lipoproteins (Kang et al., 2009); these receptors are localized on membranes of the cells or endosomes. Another type of PRR is nucleotide-binding oligomerization domain proteins (NODs), which are free-floating in the host cell's cytosol. The NOD1 and NOD2 bind to muramyl dipeptide, degraded from the bacterial peptidoglycan layers (Fournier and Philpott, 2005). These bacterial outer cell layers can also be detected via peptidoglycan recognition proteins (PGRPs). These PGRPs exist as extracellular proteins. Tumor necrosis factor-α receptor 1 may also play a role as PRR by binding to *S. aureus* protein A (Krishna and Miller, 2012). After recognizing MAMPs, the PRRs trigger signaling cascades, involving, for example, the mitogen-activated protein kinase (MAPK) or the nuclear factor-κB (NF-κB). Subsequently, host cells secrete cytokines, chemokines, adhesion molecules, and also antimicrobial peptides. The antimicrobial peptides (e.g., defensins), which are about 50 amino acids in length, are able to recognize and eliminate bacteria. In general, anionic and hydrophobic properties of the bacterial cell surface make antimicrobial attack on these cells possible. Small peptides can interfere in the protein synthesis and folding of proteins as well as in DNA synthesis and the cell wall structure (Gottlieb et al., 2008).

Important innate immune cells

Neutrophils have been shown to play a hallmark role in *S. aureus* clearance. Neutrophils are the first phagocytic cells to migrate to sites of infection, and subsequently they activate many mechanisms to eliminate *S. aureus*. First, neutrophils envelop *S. aureus*, which are already opsonized, using Fc and complement receptors. Then bacteria can be killed in different ways. The most common way is using reactive oxygen species, such as O_2-, H_2O_2 and HOCl. Oxidative bursts can either destroy bacterial cells directly or damage the bacterial membrane, which then supports enzymatic damage, resulting in cell death. Antimicrobial peptides inside the phagosome such as cathelicidins and defensins, can also kill the bacteria. Other proteinases such as neutrophil elastase, neutrophil collagenase proteinase 3, as well as acid hydrolases, digest bacterial cell components. In addition, neutrophils possess proteins, which function as nutrient sequesters, preventing the uptake of iron or vitamins by *S. aureus*. In addition, these leukocytes can use neutrophil extracellular traps (NETs) to facilitate the killing of *S. aureus*. These extracellular structures are released through a cell death program. Composed of decondensed chromatins and proteases, the NETs trap the bacteria, then other phagocytic cells eliminate them (Branzk et al., 2014; Miller and Cho, 2011; van Kessel et al., 2014).

Dendritic cells are known as a link between the innate and adaptive immune systems. Dendritic cells do not kill *S. aureus* as efficiently as neutrophils or macrophages, but they contribute to bacterial eradication by coordinating host responses to *S. aureus* infection. They orchestrate protective inflammation responses during *S. aureus* infection, mainly through releasing IL-12 (Novak et al., 2010). In a dendritic cell-depleted murine model, it was observed that more neutrophils were recruited to sites of the infection than in non-depleted mice, but the ability of neutrophils to kill *S. aureus* was impaired (Schindler et al., 2012).

2.2. Adaptive immune responses to *S. aureus*

In any discussing about host-protective responses against *S. aureus*, the adaptive immune system must be mentioned. Adaptive immunity enables highly specific responses with a broad range of antigen receptor repertoires and a more rapid reaction upon the second encounter with the same antigen or pathogen. The fast secondary response of the adaptive immune system enhances and focusses the innate immune reaction and can reduce tissue damage associated with it.

Humoral immunity and B cells

Antibodies, which comprise the humoral immune response, and are secreted by B cells, include 4 main types: IgM, IgG, IgA and IgE. IgMs exist very early in life. Acting as a penta-or hexameric structure, IgM has a high avidity, even if each binding site has only moderate affinity for the antigenic epitope. Therefore, IgM is able to bind to many different antigens and provide protection. In Kawasaki disease (a rare, childhood form of casculitis), serum IgMs can bind to 4 superantigens of *S. aureus* (SEA, SEB, SEC and TSST-1). The patient's IgM titers increased through the first to the fourth weeks in the clinical course (Matsubara et al., 2006).

In a study about IgAs against lysate of *S. aureus* 8520 Δ*spa*, a low level of this antibody was observed for 2- to 23-month-old infants, which then increased, maintaining stable levels between the ages of 4 and 18 years (Dryla et al., 2005). In another study conducted on a cohort including 15 persistent carriers and 19 non-carriers, IgA binding to 19 *S. aureus* antigens was analyzed using Luminex technology. Data showed a diverse binding; significant differences between persistent and non-carriers were observed on TSST-1, SEA, ClfA and ClfB (Verkaik et al., 2009).

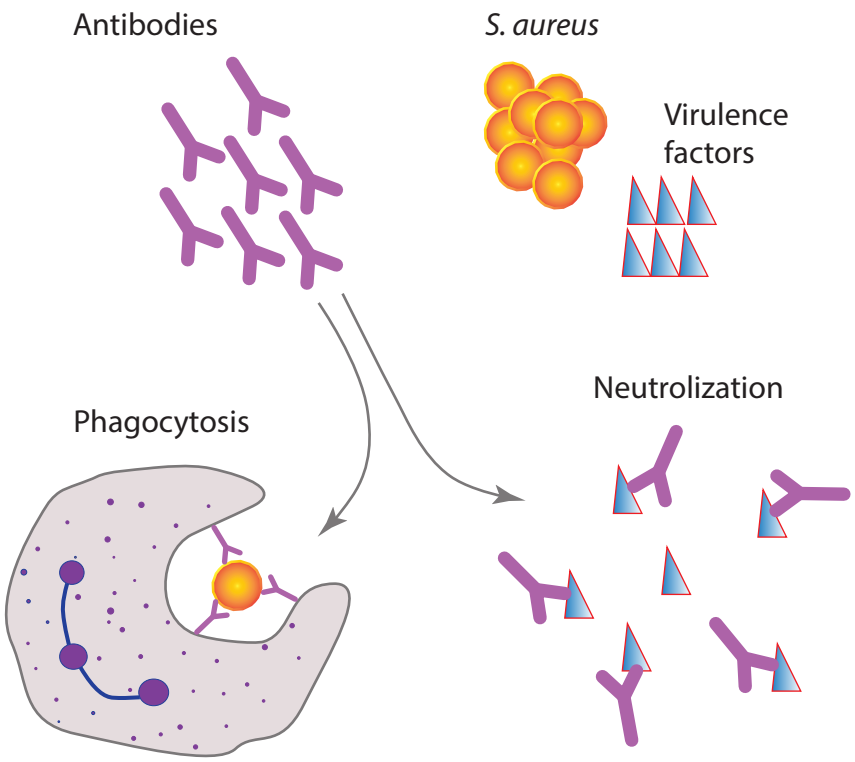

Figure 4. Functions of antibodies in S. aureus clearance.

S. aureus-binding antibodies contribute in opsonizing the bacteria, then support clearance of *S. aureus* by phagocytosis. In addition, antibodies can neutralize *S. aureus* virulence factors. The figure is adapted from Pozzi et al., 2015.

IgGs, acting as monomers, are produced in the specific immune response (see figure 4). They have important roles in neutralizing many virulence factors of *S. aureus* (Brown et al., 2012; Cheng et al., 2010; Park et al., 2014; Stentzel et al., 2015; Thomsen et al., 2014). It was found that a broad range of *S. aureus* proteins, especially extracellular proteins, can be bound by IgG. With high specific binding of IgG to pathogen, phagocytosis takes place more efficiently on opsonized bacteria. This contributes to host protection from *S. aureus*. For instance, *S. aureus* carriers usually have higher titers of *S. aureus*-specific IgG than do *S. aureus* non-carriers, and the carriers have a lower risk of severe manifestations caused by *S. aureus* such as sepsis (van der Kooi-Pol et al., 2013a).

Antibodies do not always have a positive impact. For instance, IgEs are not mentioned any study about protection against *S. aureus*, rather, they are linked to allergy. In asthma patients, IgEs against *S. aureus* enterotoxins are correlated with the severity of this disease (Bachert et al., 2012).

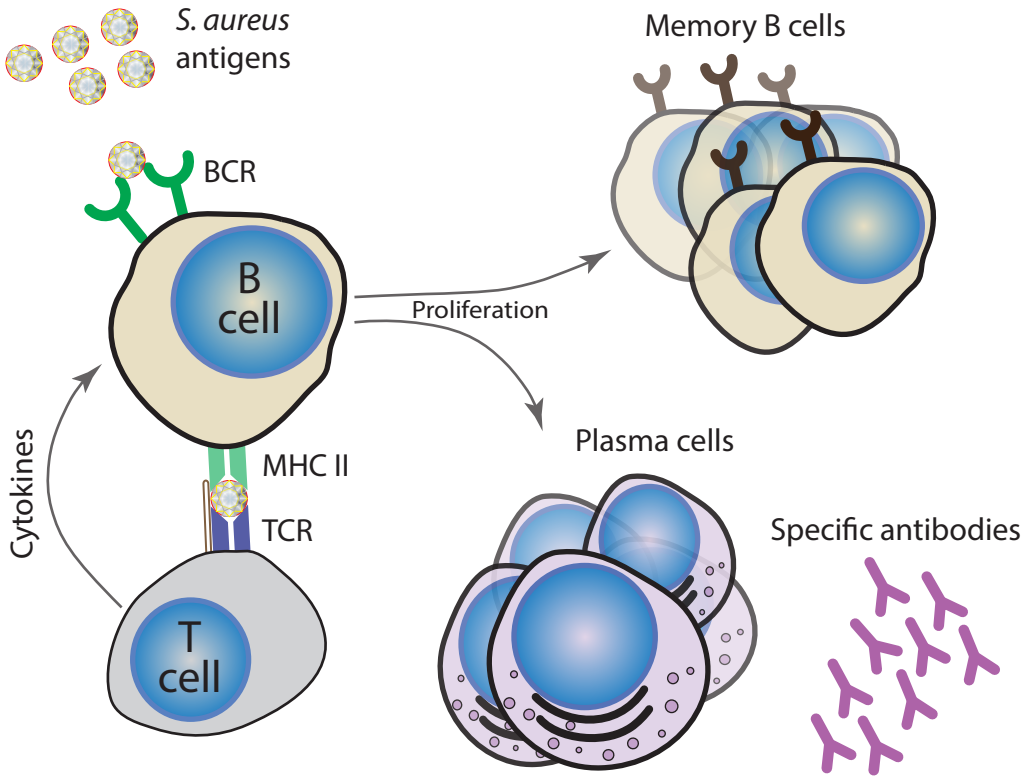

Figure 5. B cell activation and antibody class switch.

B cells are activated when they contact antigens with their BCRs. With support from T cells, B cells proliferate and undergo Ig class switch, resulting in generation of memory B cells and long-lived plasma cells. These cells release high affinity antibodies of classes IgG, IgA and IgE. This illustration is adapted from Pozzi et al., 2015.

The existence of IgG, IgA, and IgE, which bind to *S. aureus* proteins, implies that a memory immune response is raised to this pathogen. Adaptive immune cells, B cells and T cells, contribute to this process. The class switch of antibodies from IgM requires B cells and helper T cells, as well as antigen presenting cells

(APCs). Fully activated T cells release cytokines to induce B cell proliferation and then differentiation into antibody-secreted plasma cells (see figure 5) (Broker et al., 2014).

In a different scenario, B cell receptors (BCRs) are strongly cross-linked the same antigen and B cells can be activated without T-cell help. Antibodies produced lack class switch recombination and somatic hyper-mutation. Only low affinity IgMs are generated.

T cell response

In general, helper T cells can only recognize bacterial antigens which have already been processed by antigen-presenting cells (APCs). When invasive pathogens are taken up during phagocytosis, they are lysed by phagosomes or endosomes in host cells. Digested bacterial peptides are then bound to proteins encoded in the major histocompatibility complex (MHC) and expressed on the surface. T cell receptors (TCRs) recognize these short peptides in the context of MHC molecules. When other activation signals are received, T cells proliferate and rapidly differentiate into different subsets.

With support from helper T cells, the adaptive immune system attacks the same pathogen faster and with greater specificity in re-encounter scenarios. At the first contact with an antigen, the number of T cells specific to an antigen in the total pool is very low with frequencies lower than 0.01% (Kirsch et al., 2012; Kolata et al., 2015). In re-encounters with the same antigen, there are obvious differences in the way T cells respond compared to that of the primary response. First, a great quantity of T cells exists which react with the antigens. Second, specialized T cells subsets direct the quality of the immune response to the antigens via the secretion of cytokines (see figure 6).

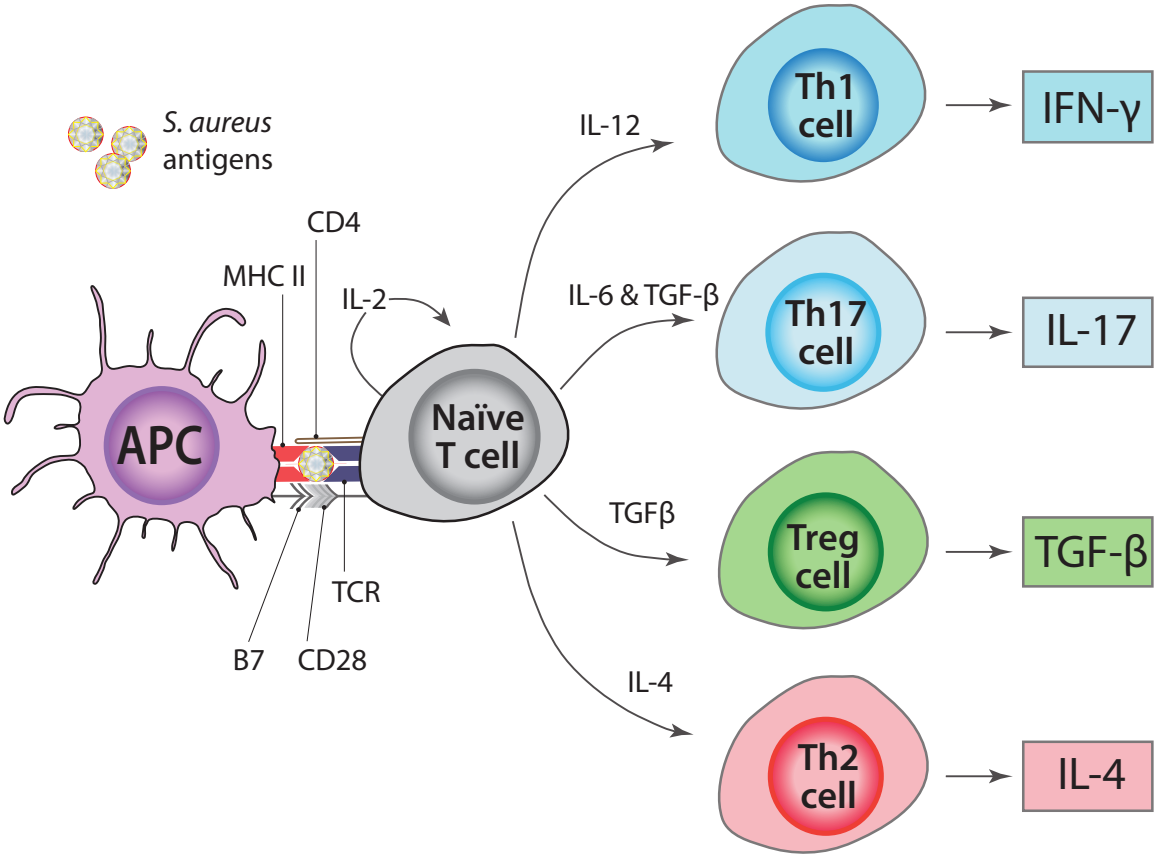

Figure 6. T cell differentiation and its subsets.

T cells are activated by antigen-presenting cells (APCs) such as dendritic cells. Afterwards, naïve $CD4^+$ T cells proliferate and differentiate into major different effector T cell subsets—T helper 1 (Th1), Th17, Th2 and regulatory T cells (Tregs). Each subset is characterized by its unique cytokine profile. The illustration is adapted from (Zou and Restifo, 2010).

T helper cells of type 1 (Th1), have ability to support B cells producing high-affinity IgG. In addition, Th1 cells release IFN-γ which contributes to enhancing phagocytosis by generating a greater oxidative burst (Lin et al., 2009).

Another T cell subset, called Th17, is known as a controller of phagocytes. They can indirectly induce the maturation of neutrophils and the recruitment of these leukocytes to the infection sites. It is well known that neutrophils are the most important innate cells involved in the eradication of *S. aureus*. Cytokine IL-17, released by Th17 cells, promotes the killing of the engulfed bacterial cells. These T cells also release cytokine IL-22, and together with IL-17, these cytokines

induce the generation of anti-microbial peptides in keratinocytes (McGeachy, 2013; Zielinski et al., 2012).

Type 2 T helper cells (Th2) drive the secretion of IgEs, which generally trigger allergic reactions. Furthermore, Th2 cells release IL-4 and IL-13, which promote the colonization of *S. aureus* on damaged skin of atopic dermatitis patients (Travers, 2014).

To balance reactions, driven by two distinct T cell groups: Th2 and Th1-Th17, regulatory T cells (Tregs) contribute to maintaining immune tolerance and immune homeostasis in general. Among their effector mechanisms are anti-inflammatory cytokines. Moreover, these T cells can stimulate the antibody class switch to IgA that is present in high concentrations on mucosal membranes (Broker et al., 2014).

3. *S. aureus*-related diseases studied in this work

3.1. Furunculosis

Furunculosis is a type of hair follicle infection in which boils (furuncles) usually develop on the skin of patients. Many pathogens are involved in furunculosis, with *S. aureus* being one of the most common causal pathogens. Colonization with *S. aureus* is a risk factor for developing of chronic or recurrent furunculosis. The corresponding bacterial strains are generally either methicillin-sensitive or methicillin-resistant *S. aureus* acquired from the community. They are usually PVL-positive. Currently, the spread of *pvl*-positive MRSA *S. aureus* is a problem for treating of furunculosis. In 7 out of 10 severe furunculosis cases, PVL toxin was identified in *S. aureus* strains. The infected patients are often under 18 years old. Healthy people may carry *pvl*-positive *S. aureus* strains without symptoms, but frequency is low. (Demos et al., 2012; Hay and Morris-Jones, 2012; Ibler and Kromann, 2014).

Many studies showed that *S. aureus* strains from the lineage with sequence types ST30, ST80, ST121, ST159 are overrepresented in recurrent skin infections compared with symptom-free colonization (DeLeo et al., 2010; Demos et al., 2012; Larsen et al., 2008; Masiuk et al., 2010).

3.2. Epidermolysis bullosa

Epidermolysis bullosa (EB) is a rare disease with an incidence of 1:50,000. Patients have a mutation in the integrin α6β4 cell adhesion molecule that leads to loss of skin integrity. *S. aureus* may cause severe infection in EB patients, because the breach of the skin paves the way for invasion. *S. aureus* nasal carriage is an important risk factor for these infections, and the existence of these bacteria can contribute to the development of chronic wounds (van der Kooi-Pol et al., 2013b). Certain salient points of *S. aureus* colonization in relation to epidermolysis bullosa deserve mention: colonization of patients with EB is not limited to specific genetic lineages of *S. aureus*; EB patients may carry up to 4 different *S. aureus* types (MLVA classification, which is used for MRSA strains), and auto-inoculation (self-transmission) of EB patients occurs frequently between the higher respiratory tract and wounds. However, severe systemic *S. aureus* infection is rare in EB (van der Kooi-Pol et al., 2014).

3.3. Cystic fibrosis

Cystic fibrosis (CF) is caused by a mutation in both alleles of the gene encoding cystic fibrosis transmembrane conductance regulator (CFTR). When this protein CFTR does not function properly, mucous membranes become thin; this mostly affects the lungs, leading to their dysfunction. The upper and lower respiratory tracts of CF patients are usually infected by different types of bacteria. Notably, *S. aureus* is one of the earliest bacteria colonizing infants and children who suffer from CF. The rise of MRSA is a risk factor for CF patients. In the case of chronic lung infection, *S. aureus* exists as small colony variants (SCVs). It is known that

SCVs are promoted by products of *P. aeruginosa*. SCVs are associated with higher rates of antimicrobial resistance. These small *S. aureus* colonies can be found in biofilms, which show even greater antibiotic resistance. CF patients bear more hypermutation strains of *S. aureus* than does nasal colonization in healthy subjects (Goss and Muhlebach, 2011; Parkins and Floto, 2015; Yonker et al., 2015).

4. Aims of this study

Antibodies against *pvl*-positive *S. aureus*

Furunculosis patients are frequently children and young adults whose family members are also colonized by *S. aureus* but free of symptoms. Based on the hypothesis that symptom-free colonized family members have developed a protective immune response, the present study seeks to answer some related questions.

- Do family members act as a source of infection?
- Do symptom-free family members have anti-*S. aureus* antibodies which the patients lack?
- How do antibodies react to the *S. aureus* PVL toxin?

Adaptive immune system response to *S. aureus* lipoproteins

As virulence factors, lipoproteins are important for colonization and invasion of *S. aureus*. However, little is known about the adaptive immune system response to this protein class. To elucidate this, the present study was guided by the following questions:

- Do humans generate antibodies (IgG) binding to *S. aureus* lipoproteins?
- Can lipoproteins trigger proliferation of human T cells?

Chapter 2. Patients, materials and methods

1. Patients

Human serum

In a furunculosis cohort, sera were collected from patients and healthy family members belonging to three different families (see table 1). Serum samples were stored at −80 °C.

Table 1. Family member information in the furunculosis cohort.

Family	Serum ID	Member	Status	Age
1	P1	Child	Patient	5
	M1	Mother	Carrier	29
	F1	Father	Non-carrier	31
2	P2	Child	Patient	12
	M2	Mother	Non-carrier	NA
	F2	Father	Non-carrier	NA
3	P3	Child	Patient	2
	M3	Mother	Non-carrier	38
	F3	Father	Carrier	39
	B3a	Brother	Carrier	13
	B3b	Brother	Non-carrier	9

NA: not available.

In an epidermolysis bullosa cohort, sera were collected from nine patients, who carried from 1 to 4 different *S. aureus* isolates. Serum samples were stored at −80 °C.

In a lipoprotein trial, besides using serum samples from furunculosis and epidermolysis bullosa cohorts, we used other serum cohorts: cystic fibrosis (CF) and a set of sera of healthy individual (SH). In the cystic fibrosis cohort, serum samples were collected from eighteen patients. The SH cohort consists in a collection of sixteen sera from *S. aureus* carriers and sixteen sera from *S. aureus* non-carriers, all of them free of symptoms.

Peripheral blood mononuclear cells

Blood cells and sera were freshly collected from ten volunteers (Lgt cohort). Afterward, peripheral blood mononuclear cells (PBMCs) were isolated using ficoll gradient centrifugation. Isolated mononuclear cells were then directly used in T cell proliferation assays.

2. Materials

Staphylococcus aureus strains

In the furunculosis cohort, *S. aureus* strains were collect from families in which children had recurrent furunculosis symptoms (see table 2). Bacterial strains were not only collected from the pediatric patients but also from their relatives (father, mother, siblings).

Table 2. S. aureus strains collected in the furunculosis cohort.

Family	Strain ID	Source	Site	Location
1	P1 NOSE	Child (Patient)	Nose	Göttingen, Germany
	P1 WOUND		Wound	
	P1 TREATED		Treated wound	
	M1 NOSE	Mother (Carrier)	Nose	
2	P2 V412	Child (Patient)	Nose	Rostock, Germany
3	P3 V08364	Child (Patient)	Nose	Rostock, Germany
	P3 V10504		Wound	
	F3 V12159	Father (Carrier)	Nose	
	F3 V12160		Perineum	
	F3 V03453		Nose	
	F3 V04383		Nose	
	F3 V08370		Nose	
	B3a V12155	Brother (Carrier)	Nose	
	B3a V08366		Perineum	
	B3a V02228		Nose	
	B3a V04373		Nose	

In the lipoprotein study, we used four laboratory *S. aureus* strains and their isogenic *lgt* mutants (see table 3 for details).

Table 3. S. aureus laboratory strains used in the lipoprotein study.

No	Strain	Genome type	*Spa* type	Selection marker
1	COL WT	Wild type	t008	
2	COL Δ*lgt*	Lgt deficient	t009	Erythromycin
3	Newman WT	Wild type	t008	
4	Newman Δ*lgt*	Lgt deficient	t008	Erythromycin
5	RN4220 WT	Wild type	t211	
6	RN4220 Δ*lgt*	Lgt deficient	t211	
7	SA113 WT	Wild type	t211	
8	SA113 Δ*lgt*	Lgt deficient	t211	Erythromycin

Kits

The Kits used for all experiments are listed below in table 4.

Table 4. Kits used in this study.

Purpose	Company	Type
Cytokine measurement	DB Biosciences, Germany	Cytometric Bead Array (CBA) Flex Sets (Human)
DNA isolation	QIAGEN, Germany	DNeasy Blood & Tissue Kits
ELISA substrate	DB Biosciences, USA	TMB Substrate Reagent Set
PCR product purification	Macherey-Nagel, Germany	NucleoSpin Gel and PCR Clean-up
Western blot substrate	Thermo Fisher Scientific, USA	SuperSignal West Femto Maximum Sensitivity Substrate

Equipment

The devices used in this study are listed below in table 5.

Table 5. Equipment used in this study.

Equipment	Company	Type
1D blotter	ProteinSimple, USA	Simon
Autoclave	Tuttnauer, USA	2540 EL
Balance	Sartorius, Germany	BP 1200
Balance	Sartorius, Germany	WP 120 S

Equipment	Company	Type
Blotter	Millipore, USA	Milliblot Graphite Electroblotter II
Cabinet	Thermo Fisher Scientific, Germany	Heraeus Hera Safe
Casting Chamber	Bio-Rad, Germany	Mini-PROTEAN Tetra Handcast Systems
Casting Chamber	Bio-Rad, Germany	PROTEAN Plus Multi-Casting Chamber
Casting Chamber	Bio-Rad, Germany	Mini-PROTEAN 3 Multi-Casting Chamber
Casting Stand	Bio-Rad, Germany	Mini-PROTEAN® Tetra Cell Casting Stand with Clamp Kit
Centrifuge	Thermo Fisher Scientific, Germany	Heraeus Megafuge 16R
Centrifuge	Heraeus, Germany	Biofuge fresco
CO_2 Incubator	Binder, Germany	CB160
Electrophoresis chamber	Bio-Rad, Germany	PROTEAN Plus Dodeca Cell
Electrophoresis chamber	Bio-Rad, Germany	Mini-PROTEAN 3 Dodeca Cell
First-dimension IEF	GE Healthcare, Sweden	Multiphor II Electrophoresis System
Gel scanner	Epson, Japan	Epson Expression 1680 Professional
Gel spot cutter	Bio-Rad, Germany	ExQuest Spot Cutter
Heater	Eppendorf, Germany	Thermomixer comfort
Magnetic stirrer	Heidolph, Germany	MR3001
Microscope	Olmpus, Japan	CX21LED
PCR	Biometra, Germany	Tprofessional Themocycler
pH meter	Mettler Toledo, Germany	FiveEasy PE20
Photometer	Tecan, Autrisa	Infinite M100 Pro
Power supply	Bio-Rad, Germany	PowerPac Basic 300 V Power Supply
Power supply	Bio-Rad, Germany	PowerPac 300
Scanner	GE Healthcare, Germany	Typhoon 9400
Scanner	Intas, German	ChemoCam HR3200 chemiluninescent scanner

Equipment	Company	Type
Shaker	Edmund Bühler, Germany	KL2
Spectrophotometer	Thermo Scientific, Germany	Nanodrop 2000
Spectrophotometers	GE Healthcare, Germany	Ultrospec 2100 pro
Thermo cycle	Biometra, Germany	Tgradient
Tumbling table	Biometra, Germany	WT12
Vortex	Heidolph, Germany	REAX Top

Buffers

The buffers used are listed below in table 6.

Table 6. Buffers used in this study.

Name	Final concentration
Blocking buffer	PBS/T Nonfat milk powder 5% (w/v)
Fix solution	Ethanol 40% (v/v) Acetic acid 10% (v/v)
Loading buffer (3x)	Tris-Cl pH 6.8 0.24 M Glycerol 30% (v/v) SDS 0.06% (v/v) Bromophenolblue 0.06% (w/v) B-mercaptoethanol 0.16% (v/v)
PBS	NaCl 137 mM KCl 2.7 mmM Na_2HPO_4 10 mM KH_2PO_4 1.8 mM
PBS/T	PBS Tween 20 0.05% (v/v)
RHB	Urea 8 M Thiourea 2 M CHAPS 2% (w/v)
RHD	RHB 83% (v/v) Isopropanol 10% (v/v) Glycerol 5% (v/v) DTT 2.5% (w/v) Pharmalyte pH 3–10 1% (v/v) Pharmalyte pH 8.5–10.5 1% (v/v)

Name	Final concentration
Running buffer	Tris 25 mM Glycine 190 mM SDS 0.1% (w/v)
TBE	Tri-Borate 98 mM EDTA 2 mM
TBS	Tris 50 mM NaCl 150 mM Adjust pH to 7.6 by HCl
TBS/T	PBS 99.9% (v/v) Tween 20 0.1% (v/v)
Transfer buffer	Tris 25 mM Glycine 190 mM Methanol 20% (v/v)
Tris 1.5 M, pH 6.8	Tris 1.5 M Adjust pH to 6.8 by HCl
Tris 1.5, pH 8.8	Tris 1.5 M Adjust pH to 8.8 by HCl
Washing solution	Ethanol 0.5% (v/v) Acetic acid 0.5 (v/v)

Primers and recombinant proteins

Primers used for multiplex PCR and *spa* typing are listed below as described in (Holtfreter et al., 2007) (see table 7).

Table 7. Primers for multiplex PCR and spa typing.

Class	Gene		Primer (5'-3')
Accessory gene regulator (agr)	*agr-1*	agr1	gtcacaagtactataagctgcgat
		pan agr	atgcacatggtgcacatgc
	agr-2	Agr2	tattactaattgaaaagtgccatagc
		pan agr	atgcacatggtgcacatgc
	agr-3	Agr3	gtaatgtaatagcttgtataataatacccag
		pan agr	atgcacatggtgcacatgc
	agr-4	agr1	cgataatgccgtaatacccg
		pan agr	atgcacatggtgcacatgc
Super antigens	*sea*	Nsea-1	gaaaaaagtctgaattgcagggaaca
		Nsea-2	caaataaatcgtaattaaccgaaggttc
	seb	Nseb-1	attctattaaggacactaagttaggga
		Nseb-2	atcccgtttcataaggcgagt
	sec	Nsec-1	cttgtatgtatggaggaataacaaaacatg

Class	Gene		Primer (5’-3’)
		Nsec-2	catatcataccaaaaagtattgccgt
	sed	Nsed-1	gaattaagtagtaccgcgctaaataatatg
		Nsed-2	gctgtatttttcctccgagagt
	see	Nsee-1	caaagaaatgctttaagcaatcttaggc
		Nsee-2	caccttaccgccaaagctg
	seg	Nseg-1	tctccacctgttgaagg
		Nseg-2	aagtgattgtctattgtcg
	seh	Sseh-1	caactgctgatttagctcag
		Sseh-2	gtcgaatgagtaatctctagg
	sei	Gsei-1	ctygaattttcaacmggtac
		Gsei-2	aggcagtccatctcctg
	sej	Gsej-1	tcagaactgttgttccgctag
		Gsej-2	gaattttaccaycaaaggtac
	sek	Nsek-1	atgccagcgctcaaggc
		Nsek-2	agattcatttgaaaattgtagttgattagct
	sel	Nsel-1	gcgatgtaggtccaggaaac
		Nsel-2	catatatagtacgagagttagaaccata
	sem	Nsem-1	ctattaatctttgggttaatggagaac
		Nsem-2	ttcagtttcgacagttttgttgtcat
	sen	Gsen-1	cgtggcaattagacgagtc
		Gsen-2	gattgatyttgatgattatkag
	seo	Nseo-1	agtttgtgtaagaagtcaagtgtaga
		Nseo-2	atctttaaattcagcagatattccatctaac
	sep	Gsep-1	gaattgcagggaactgct
		Gsep-2	ggcggtgtcttttgaac
	seq	Gseq-1	acctgaaaagcttcaagga
		Gseq-2	cgccaacgtaattccac
	ser	Nser-1	agcggtaatagcagaaaatg
		Nser-2	tcttgtaccgtaaccgtttt
	ses	Ses3	aaaaactggatgaattagagtggaa
		Ses5	ttccttcaaccgctttgttc
	set	Set3	aatgacttatttttgtgtaatcaagtg
		Set5	ccaaatgctaagtctgattctcg
	seu	Nseu-1	aatggctctaaaattgatgg
		Nseu-2	atttgatttccatcatgctc
Virulence factors	*arcA*	SarcA-1	gcagcagaatctattactgagcc
		SarcA-2	tgctaacttttctattgcttgagc
	eta	Neta-1	actgtaggagctagtgcatttgt
		Neta-2	tggatacttttgtctatctttttcatcaac
	edt	Setd-1	cccgttgattagtcatgcag

Class	Gene		Primer (5’-3’)
		Setd-2	tccagaatttcccgactcag
	mecA	SmecA-1	gtgaagatataccaagtgatt
		SmecA-2	atgcgctatagattgaaaggat
	pvl	Spvl-1	atcattaggtaaaatgtctggacatgatcca
		Spvl-2	gcatcaa(g/c=s)tgtattggatagcaaaagc
	tst	Ntst-1	ttcactatttgtaaaagtgtcagacccact
		Ntst-2	tactaatgaatttttttatcgtaagccctt
	mw765	Smw756-1	tggttagctatgaatgtagttgc
		Smw756-2	gtccatcctctgtaaattttgc
	mw1409	Smw1409-1	caaattttgaaaactttacgc
		Smw1409-2	tccaggattaaaagaagcg
Housekeeping gene	*16SrRNA*	16SrRNA-1	aactctgttattagggaagaaca
		16SrRNA-1	ccaccttcctccggtttgtcacc
	gyrase	gyr-1	agtacatcgtcgtatactatatgg
		gyr-2	atcacgtaacagttcaagtgtg
	nuc	Snuc-1	gcgattgatggtgatacggtt
		Snuc-2	agccaagccttgacgaactaaagc
Spa type	*spa*	*Spa*-5’	taaagacgatccttcggtgagc
		Spa-3’	cagcagtagtgccgtttgctt

Primers and gene loci information for recombinant lipoproteins are listed below in table 8.

Table 8. Primers for cloning lipoproteins.

Protein	Loci*	Primer
IsdE	SAUSA300_1032	atggtaggtctcaaatgcaatcttccagttctcaagaatcaa
		atggtaggtctcagcgctttttttatccttataaaataaatcatataat
MntC	SAUSA300_0618	atggtaggtctcaaatgggtactggtggtaaacaaagcag
		atggtaggtctcagcgcttttcatgcttccgtgtacagtttc
Opp1A	SAUSA300_2411	atggtaggtctcaaatgggcggtaataaaggtttagaggag
		atggtaggtctcagcgctttatactgcatttcattgaatggtaa
Opp3A	SAUSA300_0891	atggtaggtctcaaatggctaatgacgatggtatttattcag
		atggtaggtctcagcgctttttttcttcttacctgtttctttatc
Opp4A	SAUSA300_0892	tagtgtggtctccaatgggaaaaagcagtaataaag
		gtcgagggtctctgcgctagcttctttagttaaatt
PstS	SAUSA300_1283	atggtaggtctcaaatgggtggcggtaatggtggcagt
		atggtaggtctcagcgctttttttgtcttcagacttttttatcatc
SirA	SAUSA300_0117	atggtaggtctcaaatgagtgggaattcaaataaacaatcatc
		atggtaggtctcagcgcttttgattgtttttcaatatttaacttttc

Protein	Loci*	Primer
SstD	SAUSA300_0721	atggtaggtctcaaatgggtaacaattctgataaagaacaatc
		atggtaggtctcagcgctttttacaactttatcaagttcctcaat

*database www.uniprot.com

Software

All software used in this study is listed below in table 9.

Table 9. Software used for analysis data.

Name	Company	Description
Adobe Illustrator CS6	Adobe, USA	Image production
Adobe Photoshop CS5	Adobe, USA	Image editing
Delta2D v4.4	DECODON, Germany	2D gel analysis
FCAP Array v3.0	Soft Flow, Hungary	Cytokine evaluation
GraphPad Prism v6.02	GraphPad software, USA	Statistics and Graph
Microsoft Excel 2007 and 2013	Microsoft, USA	Statistics and data organization
Primer D'Signer v1.1	IBA, Germany	Primer designer
R v3.1.1	The R Foundation	Statistics and Graph
Ridom StaphType v2.2.1	Ridom, Germany	*Spa* gene typing
Rstudio v0.98.1062	Rstudio, USA	Statistics and Graph
Seqscape v2.6	Applied Biosystems, USA	DNA sequence analysis

3. Methods

Molecular biology

3.1. *S. aureus* cultivation

Wild types *S. aureus* and their isogenic mutant strains used in this study are described in table 3. The *lgt* mutation (*Δlgt*) confers on *S. aureus* the ability to release lipoproteins into the culture medium. A protein-A-deletion mutant, *S. aureus* RN4220 Δ *lgt*Δ*spa*, was generated as described elsewhere (Stentzel et al., 2014) to enable investigation of the IgG responses at pIs in the acid range, avoiding non-specific IgG binding to protein A. To isolate extracellular proteins, bacteria were cultivated in tryptic soy broth (TSB) at 37 °C and shaken at 100

linear rpm under iron-limiting conditions of 600 µM 2,2'-bipyridyl (Stentzel et al., 2014). After letting settle for 3.5 hours, the bacterial culture supernatant was filtered through a 0.22-µm filter and precipitated with trichloroacetic acid. Proteins were diluted in RHB buffer and stored at −80 °C (Holtfreter et al., 2009).

3.2. DNA isolation

The preparation of genomic DNA from *S. aureus* was performed with the DNeasy Blood and Tissue Kit with minor modifications. The bacteria were cultivated overnight in tryptic soy broth (TSB) at 37 °C, and 200 rpm. The cell pellet was collected from 1 mL medium using centrifugation at 7,500 rpm in 10 minutes and suspended in 124 µL lysis buffer (20 mM Tris-Cl pH 8; 0,2 mM EDTA, and 1.2% v/v Triton X-100). Then 36 µL lysozyme (100 mg/mL) and 20 µL lysostaphin (1 mg/mL) were added. Bacterial cells were lysed at 37 °C for 30 minutes. Afterwards, 25 µL of protease K and 100 µL of AL buffer were added and vortex mixed. The solution was incubated for 30 minutes at 65 °C. Then 200 µL of ethanol was added to the sample, and vortex mixed. The mixture was then transferred to a DNeasy Mini spin column and centrifuged at 8,000 rpm for 1 minute, after which the flow-through solution was discarded. In next step, 500 µL of AW1 buffer was added to the column, and the column was centrifuged for 1 minute at 8,000 rpm, discardng the flow-through solution afterwards. 500 µL of AW2 was added to the column and centrifuged for 3 minute at 13,000 rpm, discarding flow-through solution. The column was air dried and put in a 1.5-mL tube. 100 µL of DNase-free water was added to the center of the column's membrane. An incubation step was carried out for 1 minute at room temperature. The DNA solution was eluted using centrifugation at 8,000 rpm for 1 minute.The DNA concentration was measured using Nanodrop 2000 (Themor Scientific, Germany) or Infinity M200 Pro (Tecan, Austria). The samples were then stored at −20 °C.

3.3. Multiplex PCR

Multiplex PCR was conducted using GoTaq® Flexi DNA Polymerase (PROMEGA, USA) according to the manufacturer's instruction. PCR components were mixed as described in table 10. For each reaction, 40 ng of DNA was used. The positive and negative controls used in the multiplex PCR are listed in table 11. The thermal cycles for multiplex PCR are given in table 12.

Table 10. Components of multiplex PCR.

Multiplex	**Component**					**Control**		
	dNTP (mM)	**$MgCl_2$(mM)**	**GoTaq (U/µl)**	**Primer (µM)**		**positive**	**negative**	**Program**
Multiplex agr (*agr-1, agr-2, agr-3, agr-4*)	0.1	5	0.04	agr1	0.3	+agr	*E. coli*	SAg-/ agr-mpx
				agr2	0.3			
				agr3	0.3			
				agr4	0.3			
				pan agr	0.3			
Multiplex I (*16SrRNA, sea, sec, seh, tst*)	0.1	5	0.04	16SrRNA-1/2	0.15	+I	8325	SAg-/ agr-mpx
				Nsea-1/2	0.3			
				Nsec-1/2	0.2			
				Nseh-1/2	0.2			
				Ntst-1/2	0.2			
Multiplex II (*eta, etd, nuc, sed, sek*)	0.1	5	0.04	Neta-1/2	0.2	+II	8325	SAg-/ agr-mpx
				Netd-1/2	0.15			
				Snuc-1/2	0.15			
				Nsed-1/2	0.1			
				Nsek-1/2	0.34			
Multiplex III (*seb, see, sel, sem, seo*)	0.1	5	0.04	Nseb-1/2	0.15	+III	8325	SAg-/ agr-mpx
				Nsee-1/2	0.15			
				Nsel-1/2	0.2			
				Nsem-1/2	0.2			
				Nseo-1/2	0.286			
Multiplex IV (*sej, seg, sen, seq, ses*)	0.1	5	0.04	Gsej-1/2	0.3	+IV	8325	SAg-/ agr-mpx
				Gseg-1/2	0.15			
				Gsen-1/2	0.3			

Multiplex	Component					Control		Program
	dNTP (mM)	$MgCl_2$(mM)	GoTaq (U/µl)	Primer (µM)		positive	negative	
				Gseq-1/2	0.2			
				Ses-3/5	0.15			
Multiplex V (*sei, sep, ser, set, seu*)	0.1	5	0.04	Gsei-1/2	0.2	+V	8325	SAg-/ agr-mpx
				Gsep-1/2	0.15			
				Nser-1/2	0.15			
				Set-3/5	0.15			
				Nseu-1/2	0.15			
CA-MRSA mpx I (*16SrRNA, pvl, MW756, gyr, mecA*)	0.1	5	0.04	16SrRNA-1/2	0.1	+SHIP	8325	CMRSA-mpx
				gyr-1/2	0.08			
				SmecA-1/2	0.16			
				Smw756-1/2	0.15			
				Spvl-1/2	0.15			
CA-MRSA mpx II (*etd, arcA, seh, nuc, MW1409*)	0.1	5	0.04	SarcA-1/2	0.1	+SHIP	8325	CMRSA-mpx
				Setd-1/2	0.3			
				Smw1409-1/2	0.3			
				Snuc-1/2	0.1			
				Sseh-1/2	0.4			

Table 11. Positive and negative controls used in multiplex PCR.

Multiplex PCR	Positive control (gene)	Negative control
+ agr	FRI913 (*agr1*)	*E. coli*
	FRI137 (*agr2*)	
	TY114 (*agr3*)	
	A920210 (*agr4*)	
+ I	FRI913 (*sea*, *sec*, *tst*)	NCTC8325-4
	FRI137 (*sec*, *seh*)	
+ II	FRI1151m (*sed*)	NCTC8325-4
	TY114 (*etd*)	
	A920210 (*eta*)	
	FRI913 (*sek*)	
+ III	FRI913 (*see*)	NCTC8325-4
	CCM5757 (*seb*)	
	FRI137 (*sel*, *sem*, *seo*)	

Multiplex PCR	Positive control (gene)	Negative control
+ IV	FRI137 (*sen*, *seg*)	NCTC8325-4
	COL (*seq*)	
	FRI1151m (*sej*)	
	Baltimore 1060 (*ses*)	
+ V	FRI137 (*sei*, *ser*, *seu*)	NCTC8325-4
	N315 (*sep*)	
	Baltimore 1060 (*set*)	
+ SHIP	CMRSA80 (06-00300; *lukPV*, *etd*, *mecA*)	NCTC8325-4
	CMRSA1 (07-00814; *MW756*)	

Table 12. Thermal cycles for multiplex PCR.

SAg-/ agr-mpx PCR			
Steps	**Temperature**	**Time**	**Number of cycles**
Initial denaturation	95 °C	5 minutes	1
Denaturation	95 °C	30 seconds	25
Annealing	55 °C	30 seconds	
Extension	72 °C	1 minute	
Final extension	72 °C	7 minutes	
Storage	4 °C	∞	1
CMRSA-mpx PCR			
Steps	**Temperature**	**Time**	**Number of cycles**
Initial denaturation	95 °C	5 minutes	1
Denaturation	95 °C	30 seconds	30
Annealing	60 °C	30 seconds	
Extension	72 °C	1 minute	
Final extension	72 °C	7 minutes	
Storage	4 °C	∞	1

3.4. *Spa* typing

A *spa* gene fragment was amplified using a pair of primer Spa-5’ and Spa-3’ (table 7) using Platinum® Taq DNA Polymerase High Fidelity (Invitrogen, Germany) in accordance with the manufacturer’s instructions. The thermal cycle conditions used for PCR are: initial denaturation at 94 °C in 5 minutes; 30 cycles

of denaturation at 94 °C for 45 seconds, annealing at 60 °C for 45 seconds, extension at 72 °C for 90 seconds; final extension at 72 °C for 10 minutes.

The PCR product was visually checked by agarose electrophoresis (see 3.3.5). Images of gels were captured using GenoPlex2 (Vwr, England). The DNA fragments were purified using the QIAquick PCR purification kit (QIAGEN, Germany). Primers *Spa*-5' and *Spa*-3' were directly used to sequence *spa* repeats. The DNA sequence was analyzed using Ridom StaphType v2.2.1 (Ridom, Germany). Elucidated *Spa* types have been submitted to the Ridom *spa* server.

3.5. Agarose gel electrophoresis

DNA was separated in 1% arose gel in TBE buffer. A volume of 10 µL DNA solution was mixed with 5× loading buffer (Life technologies, US) and pipetted into each well. Under a constant voltage of 200 V from Powerpac (Bio-Rad, Germany), DNA was separated in the gel within the electrophoresis chamber (Bio-Rad, Germany). Redsafe (Intron Biotechnology, North Korea) staining was used for detecting nucleic acids by the fluorescence emitted. Images of gels were captured by GenoPlex2 (Vwr, England).

3.6. Gene cloning

Total DNA of *S. aureus* strain USA300 (NC_007793) was used for gene cloning. DNA fragments, encoding for lipoproteins, were amplified in a volume of 200 µL using Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen, Germany) according to the manufacturer's instructions with a PCR program: an initial denaturation at 95 °C for 5 minutes; 30 cycles of denaturation at 95 °C for 5 minutes, annealing at 56 °C for 1 minutes, extension at 72 °C for 1.5 minutes; and a final extension at 72 °C for 5 minutes.

PCR products and the vector pPR-IBA1 (IBA, Germany) were digested using the restriction enzyme *Bsa*I (NEB, USA), then purified using the QIAquick PCR purification kit (QIAGEN, Germany). To prevent self-ligation, the 5' phosphate

group of the linear vector pPR-IBA1 was removed using Shrimp Alkaline Phosphatase (Life Technologies, USA). The vector and PCR products were ligated using the Fast-Link DNA ligation kit (BioZym, Germany).

The ligation product then was directly transformed in *E. coli* using One Shot Max Efficiency Dh5α-T1 (Invitrogen, US) following the manufacturer's instructions. Plasmids were purified according High Pure Plasmid isolate kit (Roche, Switzerland). Using two restriction enzymes, *Hin*dIII and *Xba*I (Life Technologies, US), we identified recombinant plasmids carrying genes of interest. The resulting DNA plasmids were sequenced using universal vector primers. Software Seqscape v.2.6 was used to analyze and confirm correct open reading frames prior to overexpression recombinant proteins.

Recombinant PVL components, LukF-PV and LukS-PV, were generated in *E. coli* and purified using a His-tag system (Loffler et al., 2010). Recombinant HlgC was overexpressed in *E. coli* and purified using a Strep-tag system (Kloppot et al., 2015).

3.7. *Spa* mutant *S. aureus* strain

The *spa* gene encoding for protein A was depleted using the phage transduction method. The phage ϕ11 functioned as a gene transporter from donor *S. aureus*-strain SA113 *ery*Δ*spa*. The phage was introduced into the *S. aureus* strain SA113 *ery*Δ*spa* cells in TSB medium (supplemented with erythromycin 20 µg/mL, $MgSO_4$ 10 mM and $CaCl_2$ 10 mM) at 200 rpm and 37 °C until bacteria were lysis completely (about 4 hours). The culture was then centrifuged at 4,500 rpm for 10 minutes and filtered using a 0.22-µm diameter filter. This phage lysate was diluted to a concentration of 10^{10} phages/mL and stored at 4 °C. To knockout target *spa* gene in a recipient *S. aureus* strain, the phage lysate was mixed with the recipient bacteria (10^9 cells) at serial dilutions: 1:5, 1:50 and 1:500 (cells: phages) in 0.2 mL Luria-Bertani (LB) medium supplemented with $CaCl_2$ 10 mM. These mixtures were kept at 37 °C for 25 minutes in a water bath. Then, 0.5 mL

of ice-cold sodium citrate 2 mM was added to each mixture. Bacterial cells were collected using centrifugation at 10,000 ×*g* for 10 minutes at 4 °C, then suspended in 0.5 mL of sodium citrate 2 mM and placed in ice for 3 hours. 100 µL aliquots were spread onto LB agar plates (supplemented with erythromycin 20 µg/mL and sodium citrate 2 mM) and incubated for 12–72 hours at 37 °C. Single clones were transferred 2 more times to the LB agar plates. The genome types of mutants were checked using PCR with primers specific for genes encoding for protein A and erythromycin-resistant protein (table 7). ELISAs were used to confirm whether protein A was depleted completely or not; this method can detected the protein A from the whole bacterial cell (4×10^8 cells) using naïve mouse serum at a 1:200 dilution as primary antibody (Stentzel et al., 2014).

3.7. Overexpression of recombinant proteins

To optimize protein overexpression, signal peptides and lipid tail binding sites (Lipobox) were removed as defined by conserved sequence motifs (Sheldon and Heinrichs, 2012). The lipoprotein- encoding DNA fragments were amplified and cloned in the pPR-IBA1 vector (IBA, Götingen, Germany) to add an N-terminal strep-tag for affinity purification of the resulting fusion protein. Lipoproteins were overexpressed in *E. coli* BL21(DE3)pLysS (Invitrogen, USA) grown in LB medium (supplemented with 100 µg/mL ampicillin). Expression was induced by treating cultures shaken at 100 rpm with 0.5 mM of IPTG at 37 °C. Sonication-disrupted cell supernatants were applied to strep-Tactin columns (IBA, Germany) following the manufacturer's instructions.

Afterwards, recombinant lipoproteins were desalted using Spectra/Por 1 (6-8 kDa) membranes (Spectrum, USA) at a ratio of 1:200 in PBS buffer without Ca^{2+} and Mg^{2+} (Biochrom, Germany) (1 mL of protein solution is desalted with 200 mL of PBS buffer) at 4 °C twice within 48 hours. Contaminating lipopolysaccharides (LPS) were removed by the use of EndoTrap red columns (Hyglos, Germany) following manufacturer's instructions. In the last step, we

used the Endosafe-PTS (Charles River, USA) to detect the amount of LPS remaining in protein samples.

3.8. Extracting extracellular proteins

Extracellular proteins were precipitated using trichloroacetic acid (TCA). The cultivated medium was centrifuged at 9,000 ×*g* for 10 minutes at 4 °C. The supernatant was filtered through a 0.22-µm filter. Cool TCA (125 g of TCA dissolved in 125 mL of water) was slowly added to the filtered supernatant to obtain a ratio 1:10 (1 mL of TCA added to in 10 mL of supernatant). Protein precipitation took place overnight at 4 °C; this solution was then centrifuged at 11,000 ×*g* for 60 minutes. The supernatant was removed gently using a pipette. Protein pellets were washed and suspended in cool ethanol 70% five times, then centrifuged at 11,000 ×*g* for 10 minutes at 20 °C. The pellets were then dried at room temperature overnight and dissolved in RHB buffer. Samples were centrifuged at 11,000 ×*g* for 20 minutes at 20 °C, after which the supernatant was collected and stored at −20 °C. Protein concentration was measured with the Bradford method using a photometer Ultrospec pro 2100 (GE Healthcare Bio-Sciences, US).

3.9. SDS-PAGE

For SDS-PAGE analysis, gel was prepared in two parts: the stacking gel and the separating gel, with components of which are shown in table 13. A mini gel (8.3 × 7.3 × 1.0 cm) was casted using casting equipment for a mini size following manufacturer's instructions (Bulletin 6201, Bio-Rad, US).

Table 13. Recipe for casting stacking and separating gels.

Component	**Stacking gel 4% Volume (5 mL)**	**Separating gel 12% Volume (5 mL)**
1. Acrylamide/Bis 30%	0.66 mL	2 mL
2. Tris 1.5 M, pH 8.8	-	1.25 mL
3. Tris 0.5 M, pH 6.8	1.26 mL	-
4. SDS 10%	50 µL	50 µL

Component	Stacking gel 4% Volume (5 mL)	Separating gel 12% Volume (5 mL)
5. H_2O	3 mL	1.68 mL
6. APS 10%	25 µL	25 µL
7. TEMED	5 µL	2.5 µL

Protein samples were mixed with loading buffer (3×) and loaded into each well. Under a voltage of 120 V, proteins were separated by molecular weight in running buffer (see table 6).

For visualization, we used the coomassie blue staining method (Coomassie Brilliant Blue R250 0.1% [m/v], methanol 50% [v/v], glacial acetic acid 10% [v/v], H_2O 40% [v/v]). Gels were incubated in staining solution for about 60 minutes, then exposed to destaining solution (methanol 50% [v/v], glacial acetic acid 10% [v/v], H_2O 40% [v/v]) for 30 minutes. This step was repeated at least 3 times until gel background was as transparent as possible. Shaking was performed at 100 rpm at room temperature for all staining and washing steps. Finally, the gel image was generated using Canon scanner (Canon, Japan).

3.10. 2D gel electrophoresis

For separating proteins with an isoelectric point (pI) range of 4–7

Proteins were loaded on a 7-cm Immobiline Dry strips (GE Healthcare, Germany) using rehydration loading technique following manufacturer's instructions. 30 µg of extracellular proteins was prepared in rehydration solution for the strip as shown in table 14.

Table 14. Rehydration components for pI 4–7.

Component	Volume (µL)
1. RHB	106.75
2. Protein (10 µg/µL)	3
3. DTT in RHB[1]	12.5
4. Pharmalyte pH 3–10	2.5
5. Bromophenol 1% (m/v)	0.25

[1] freshly add 0.0175 g of DTT to 400 µL of RHB buffer

The rehydration solution was then shaken for 30 minutes at 8,000 rpm and 20 °C to dissolve the proteins completely. An additional centrifuging of 10 minutes at 13,000 rpm eliminated precipitates. A 7-cm strip was rehydrated with 125 µL of the rehydration solution overnight at 20 °C (under a layer of mineral oil).

The sample-loaded strip was washed 2 times with H_2O to remove the oil layer, then soaked in DTT 15 mM. The strip was then mounted in the IEF chamber and covered with a layer of mineral oil as per manufacturer's instructions. The running program was set as follows: step 1: 200 V with a power of 1 Vh was applied for 1 second; step 2: 3,500 V with a power of 2,800 Vh was applied for 90 minutes; step 3: 3,500 V with a power of 5,200 Vh was applied for 90 minutes. Protein separation in the strip took place at 20 °C for about 5 hours.

For separating proteins in pI range of 6–11

Proteins were loaded on a 7-cm Immobiline Dry strips (GE Healthcare, Germany) using the cup loading technique as manufacturer's instructions. The strip was first rehydrated in the solution described in table 15.

Table 15. Rehydration components for pI 6–11.

Component	**Volume (µL)**
1. RHB	84.75
2. Isopropanol	12.5
3. Glycerol 50% in RHB	12.5
4. DTT 25% in RHB[1]	12.5
5. Pharmalyte pH 3–10	1.25
6. Pharmalyte pH 8.5–10.5	1.25
7. Bromophenol 1%	0.25

[1]freshly add 0.025 g DTT to 100 µL of RHB buffer

A volume of 125 µL rehydrating solution was used for each strip. Hydration was carried out overnight at 20 °C under a layer of mineral oil. After removing the mineral oil using water, the rehydrated strip was mounted in the IEF chamber. 30

μg of proteins in solution was prepared as shown in table 16 and then added to every cup according to manufacturer's instructions.

Table 16. Loading buffer components for pI 6–11.

Component	Volume (μL)
1. RHB	64.8
2. Proteins (10 μg/μL)	3
3. Isopropanol	10
4. Glycerol 50% in RHB	10
5. DTT 25% in RHB[1]	10
6. Pharmalyte pH 3–10	1
7. Pharmalyte pH 8.5–10.5	1
8. Bromophenol 1%	0.2

[1]freshly add 0.025 g DTT to 100 μL RHB buffer

The strip was then covered by mineral oil. Proteins were separated at 20 °C as follows: step 1: 200 V with a power of 1 Vh was applied for 1 second; step 2: 3,500 V with a power of 2,800 Vh was applied for 90 minutes; step 3: 3,500 V with a power of 3,700 Vh was applied for 65 minutes.

Equilibration

The protein-loaded strip was then equilibrated. The strip was first shaken in 2.5 mL of solution A (equilibration buffer with DTT 1% [m/v]) for 15 minutes, then secondly soaked in 2.5 mL of solution B (equilibration buffer with iodoacetamide 2.5% [m/v], bromophenol 0.02‰ [m/v]).

In a next step, proteins were separated by molecular weight using SDS-PAGE (section 3.9, chapter 2). Gels were cast using a PROTEAN Plus Multi-Casting Chamber (Bio-Rad, USA).

3.11. Protein identification

For protein identification, the extracellular bacterial proteins were first separated on 11-cm Immobiline Dry strips (GE Healthcare, Germany) both in pI ranges of 4–7 and 6–11, and, then on 12.5% polyacrylamide gels. Gels were stained with

Flamingo (Bio-Rad, Germany) and scanned using a Typhoon 9400 scanner (GE Healthcare, England) at 532 nm with a resolution of 100 µm. Protein identification by mass spectrometry was carried out on a Proteome-Analyser 4800 (AB Sciex, USA). GPS explorer v3.6 (AB Sciex, USA) and the MASCOT search engine v2.2.02 (Matrix Science, England) were used to search the obtained peak lists against a database of protein sequences derived from the genome sequences of the *S. aureus* strains, depending on the propose of the experiments. The mass spectrometry was carried out in Department of Functional Genomics, University Medicine Greifswald.

Immunological methods

3.12. Human sera collection and preparation

Sera were collected using DB Vacutainer SST tubes (DB, Germany). 4 mL of blood was added to each tube. Inverting 5 times is required to mix clot activator with blood. The tubes were then let stand vertically for 30 minutes. Serum was separated using swing-head centrifugation at 1,300 ×*g* for 15 minutes at 20 °C. The upper serum layer was aliquoted and stored at −80 °C.

3.13. 2D immunoblot

For immunoblotting, the extracellular bacterial proteins were separated by isoelectric focusing on strips 7-cm Immobiline Dry Strips (GE Healthcare, England) over a pI range of 6–10 and, in the case of strain RN4220 Δ*spa*Δ*lgt* over a pI range of 4–7. In the second dimension, proteins were subsequently fractionated by molecular mass on 12.5% SDS polyacrylamide gels. Proteins were then blotted onto a PVDF membrane Immobilon-P (Millipore, USA). The human serum pool was used as a source of primary antibodies and incubated with the membrane at a dilution of 1:10,000 in blocking buffer (NaCl 140 mM, Tris 50 mM, Tween 20 0.1% [v/v], non-fat milk powder 5% [w/v], pH 7.6). IgG binding was then detected with peroxidase-conjugated goat anti-human IgG

(Dianova, Germany) with a chemiluminescent substrate (ECL substrate, SuperSignal West Femto Maximum Sensitivity Substrate, Pierce, USA) using a ChemoCam HR3200 chemiluninescent scanner (INTAS, Germany). All experiments were performed in three technical replicates, which were superimposed using the Delta2D software v4.4 (Decodon, Germany).

3.14. ELISA

100 ng of lipoprotein was diluted in the carbonate buffer pH 9.0 (CANDOR Bioscience, Germany), used to coat wells of a F96 Cert MaxiSorp Nunc-Immuno plate (NUNC, Denmark), and let stand overnight at 4 °C. The coating solution was discarded, and after washing 3 times with PBS-T (NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 1.8 mM, Tween 20 0.05% [v/v]), the plate was blocked in PBS-T with 2% non-fat milk powder for 1 hour at room temperature (RT) on a shaker (50 rpm). Serum probes were used as the source of primary antibody (IgG) at different dilutions for 1 hour at RT on a shaker (50 rpm). Goat-anti-human-IgG 0.8 mg/mL (Jackson ImmunoResearch, USA) was used as the secondary antibody at a dilution of 1:50,000. Following washing, the plates were incubated with 3,3',5,5'-tetramethylbenzidine as the substrate (TMB Substrate Reagent Set, BD Biosciences, USA) for 15 minutes and optical density was measured at 450 nm. The human serum pool described above and PBS (Biochrom, Germany) were used as positive and negative controls, respectively. All measurements were performed in duplicate, and the means were used for analysis.

3.15. PBMCs isolation

Human peripheral blood mononuclear cells (PBMCs) were isolated using the Pancoll (PAN-Biotech, Germany) density gradient centrifugation at the room temperature. 15 mL of fresh blood was diluted by PBS 1× (Biochrom, Germany) at a ratio of 1:1, then gently transferred to the top of the Pancoll layer. Cells were

centrifuged at 1,580 ×g for 15 minutes without brakes. The PBMC layer was transferred to a new tube, washed with 50 mL of PBS buffer, and centrifuged at 610 ×g for 10 minutes. Supernatant was discarded and erythrocytes were disrupted by adding 700 µL of water, then gently mixed for 60 seconds. Another washing step was carried out, and the cell pellet was collected using a centrifugation at 150 ×g for 10 minutes. An additional disrupting platelet step was required if the cell pellet was still red. In the last step, PBMCs were diluted in R10F medium (RPMI1640 supplemented with penicillin 200 U/mL, streptomycin 200 µg/mL, L-glutamine 4 mM and fetal calf serum 10% [v/v]) and counted using a hemocytometer.

3.16. T cell proliferation assay

10^5 human peripheral blood mononuclear cells per well (96-well plate) were cultured in 200 µL R10F medium. Proteins were serially diluted to final concentrations of from 25 µg/mL to 0.006 µg/mL and used to stimulate PBMCs. Incubation in flat- bottom 96- well plates was performed for 7 days at 37 °C, in an atmosphere of 5% CO_2. Each experiment was carried out in triplicate. Afterwards, 0.5 µCi/well of tritiated thymidine (3H-TdR) (PerkinElmer, USA) was added to the cell culture and incubation was continued for a further 17 hours. Proliferation of PBMCs was measured via 3H-TdR incorporation (Kolata et al., 2015).

3.17. Cytokine profiling

Cell culture supernatants of PBMCs incubated with *S. aureus* antigens or controls as described above (see 3.3.16) were collected on day 7. Secreted cytokines (GM-CSF, IFN-γ, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-17A, TGF-β1, TNF) were measured using Cytometric Bead Array (CBA) Flex Sets (DB, Germany) following the manufacturer's instructions.

Concentrations were determined using FCAP Array software v3.0 (Soft Flow Inc., USA). All measurements were performed in triplicate.

4. Software and statistical analysis

4.1. DNA analysis

DNA sequences were analyzed using Seqscape v2.6. Alignment settings were: gap penalty (22.5), extension penalty (8.5) and library matches (20). For *spa* typing, duplicate DNA sequencing results were used. For lipoprotein gene cloning, triplicate DNA sequence results were analyzed, and the clones with correct (without gap) open reading frames were selected for protein overexpression.

4.2. Protein analysis

The MASCOT search engine v2.2.02 (Matrix Science, England) and GPS explorer v3.6 (AB Sciex, USA) were used to search the obtained peak lists against a database of protein sequences derived from the genome sequences of the *S. aureus* strains USA300 (NC_007793) as described (section 3.11, chapter 2). Protein identifications with the highest scores were selected as identified proteins.

To check the homology between *S. aureus* lipoproteins and all human proteins, we used the NCBI blast-algorithm: blastp (protein-protein BLAST) with the following conditions: [Database:] All non-redundant GenBank CDS translations+PDB+SwissProt+PIR+PRF excluding environmental samples from WGS projects (Number of sequences: 67615144) and [Organism:] Homo (taxid: 9605) (Database date: 10 June 2015 at www.ncbi.com/blast).

4.3. 2D immunoblot analysis

Images from flamingo, coomassie staining and 2D immunoblot were normalized before using in Delta2D software v4.4. Pictures were cropped and converted into 8-bit gray images using Photoshop CS5.

To analyze gel images, first, each fused image was made from three images of experimental results. The merging parameters were set as follows: process images before fusion (do not filter); fusion type (union); adjust image sizes (common region) and process fused image (don’t filter). Second, spots were detected automatically (settings: 8.15, 5%) and edited manually to eliminate artefacts. The spots were then labeled for further use as described elsewhere (Kolata et al., 2011).

4.4. Online data

The *spa* ridom server was used for identifying *spa* genes and submitting *spa* types for the *S. aureus* research community. Uniprot, NCBI, Kregg and Aureowiki were used as databases to get acquire information for lipoproteins.

4.5. Statistical analysis

GraphPad Prism version 6.02 software was used for statistical analysis. Suitable tests were selected depending on the types of experiments. Significance was set at p-value < 0.05.

Chapter 3. Results

1. Furunculosis

1.1. Genetic diversity of *pvl*-positive *S. aureus* strain

The furunculosis cohort consisted of 3 families (see table 1), in which one child suffered from furunculosis. Bacterial swabs were collected from all family members and all *S. aureus* isolates were tested (see table 2).

To study the diversity of the virulence genes of the *S. aureus* isolates in each family, we used multiplex PCR (section 3.3, chapter 2). With this method, a set of virulence genes can be analyzed. The set of investigated genes was selected based on their functions and conservation. The 16S RNA gene is a positive control for *S. aureus*. This gene encodes 16S ribosome RNA, which is conserved in the species *S. aureus* (Krimmer et al., 1999). 16S RNA gene sequencing is the widely used conventional technique for classifying bacteria (Gurtler and Barrie, 1995). The *nuc* gene is a housekeeping gene of *S. aureus*. Encoding for thermostable nuclease, this gene is a specific marker for *S. aureus* (Brakstad et al., 1992). *Gyr*, another *S. aureus* specific gene, is used as an indicator for this bacterium. *Gyr* encodes an enzyme called gyrase, which is essential for the bacterial DNA replication (Alt et al., 2011; Margerrison et al., 1992). *Agr* is a gene encoding for a global regulator of virulence gene expression (Abdelnour et al., 1993). There are 4 subclasses of *agr*: *agr 1*, *agr 2*, *agr 3* and *agr 4*, which are linked to different *S. aureus* clonal complexes (Holtfreter et al., 2007). A methicillin resistance gene, *mecA*, was also used as a marker in multiplex PCR. This gene codes for a protein known as penicillin-binding protein 2a (Ubukata et al., 1989). *MW1409* is a marker for phage (Zhang et al., 2008a). *MW756* is a gene from a genomic island, and is usually linked to strain USA400 (Zhang et al., 2008a). USA300 (CC8) isolates harbor the arginine catabolic mobile element (ACME). The *arcA* gene is associated with the ACME and may function as a

strain-specific marker (Ellington et al., 2008). The two exfoliative toxins *eta* and *etd*, are related to skin diseases (Yamaguchi et al., 2002). Moreover, *etd* is also used as a marker for ST80-CA-MRSA clones (Holmes et al., 2005; Holtfreter et al., 2007).The *pvl* gene encodes for Panton Valentine leukocidin, which is believed to facilitate in invasion of host cells. This toxin promotes host cell lysis by forming pores in the cell membrane (Boyle-Vavra and Daum, 2007). An array of genes encoding for superantigens including *tst* and a set of enterotoxins was tested by multiplex PCR. Some of them are encoded on a gene cluster, known as enterotoxin gene clusters (*egc*) comprising the superantigen genes (*seg*, *sei*, *sem*, *sen*, *seo*, *seu*) (Holtfreter et al., 2004). These superantigens can bind to the conserved of T cell receptors, causing a dysfunctional T cell response (Dinges et al., 2000).

Multiplex PCR results show that all disease-causing isolates in in the affected children as well as most colonizing strains in family members harbored the *pvl* gene (see table 17). In family 1, it seems that in patient P1 *S. aureus* strains, isolated from different sites, had the same virulence factor patterns. In family 3, two different clones isolated from patient P3: The clone from the nose did not carry the *pvl* gene, while one from a wound showed the existence of this gene. *Eta* and *etd* genes may also be involved in skin infections, but were not found in any members of the three families. *Agr* subclass 4 was found in families 1 and 3, which was linked to lineage CC121 carrying the *pvl* gene as shown a study in Vorpommern, Germany (Masiuk et al., 2010). In contrast, the *pvl*-positive strain P2 V412 in family 2 had *agr* subclass 3. The strain also harbored superantigen SEA, which belongs to the non-*egc* group. Notably, isolate P2 V412 possessed gene *MW1409* which is a specific marker prophage φSa2mw. It has been known that this prophage is associated to *pvl*-positive *S. aureus* (Zhang et al., 2008b).

Spa typing is an additional classification method, which is based on DNA sequences of a gene encoding for protein A. In this gene, there is a repeat region

consisting of many tandems, 17–20 base pairs in length each. The combination of the tandems confers diversity on the *spa* genes. The *spa* type results are shown in table 18. In family 1, three *S. aureus* isolates belonged to 2 lineages, T2392 and T5007, differing only in one repeat between the isolates of the nose and wound of the affected child. This result is well compatible with the multiplex PCR data, which gave the same results in all strains suggesting a clonal origin. In family 2, the clone from the patient had *spa* type T017. In family 3, we identified two *spa* types T1458 and T284. Clone T1458 was unique and only isolated from the patient's nose, while clone T284 was found both in the patient's wound and other family members. The nasal *S. aureus* T1458 isolate carried superantigen SEA (a non-*egc* Sag) but—uniquely in the furunculosis cohort—no PVL, which is in agreement with its commensal behavior.

Table 17. Multiplex PCR results.

Family	**Strain ID**	**Source**	**Location**	***Pvl***	***Agr***	**Non-*egc* Sag**	***Egc* Sag**	**Others**
Family 1	P1 NOSE	Nose	Göttingen	+	4		gimnou	*gyr, nuc*
	P1 WOUND	Wound	Göttingen	+	4		gimnou	*gyr, nuc*
	P1 TREATED	Treated wound	Göttingen	+	4		gimnou	*gyr, nuc*
	M1 NOSE	Nose	Göttingen	+	4		gimnou	*gyr,nuc*
Family 2	P2 V412	Nose	Rostock	+	3	a	gimnou	*gyr,nuc,mw1409*
Family 3	P3 V08364	Nose	Rostock	-	3	atq		*gyr,nuc*
	P3 V10504	Wound	Rostock	+	4		gimnou	*gyr,nuc*
	F3 V12159	Nose	Rostock	+	4		gimnou	*gyr,nuc*
	F3 V12160	Perineum	Rostock	+	4		gimnou	*gyr,nuc*
	F3 V03453	Nose	Rostock	+	4		gimnou	*gyr,nuc*
	F3 V04383	Nose	Rostock	+	4		gimnou	*gyr,nuc*
	F3 V08370	Nose	Rostock	+	4		gimnou	*gyr,nuc*
	B3a V12155	Nose	Rostock	+	4		gimnou	*gyr,nuc*
	B3a V08366	Perineum	Rostock	+	4		gimnou	*gyr,nuc*
	B3a V02228	Nose	Rostock	+	4		gimnou	*gyr,nuc*
	B3a V04373	Nose	Rostock	+	4		gimnou	*gyr,nuc*

P: patient; M: mother; F: father; B: brother.

g, i, m, n, o, u: enterotoxin genes *seg, sei, sem, sen, seo, seu* of the egc cluster respectively.

a, t, q: enterotoxin genes *sea, tst, seq* of the non-egc cluster respectively.

+: positive, -: negative.

Table 18. Spa typing results.

Family	Strain ID	*Spa* type	*Spa*-type repeats succession	MLST
Family 1	P1 NOSE	t2392	14-44-13-17-17-17-17-17-23-18-17	NA
	P1 WOUND	t5007	14-44-13-17-17-17-17-17-17-23-18-17	NA
	P1 TREATED	t5007	14-44-13-17-17-17-17-17-17-23-18-17	NA
	M1 NOSE	t5007	14-44-13-17-17-17-17-17-17-23-18-17	NA
Family 2	P2 V412	t017	15-12-16-16-02-16-02-25-17-24-24	ST30
Family 3	P3 V08364	t1458	121-21-17-17-23-24	NA
	P3 V10504	t284	14-44-13-17-17-23-18-17	ST121
	F3 V12159	t284	14-44-13-17-17-23-18-17	ST121
	F3 V12160	t284	14-44-13-17-17-23-18-17	ST121
	F3 V03453	t284	14-44-13-17-17-23-18-17	ST121
	F3 V04383	t284	14-44-13-17-17-23-18-17	ST121
	F3 V08370	t284	14-44-13-17-17-23-18-17	ST121
	B3a V12155	t284	14-44-13-17-17-23-18-17	ST121
	B3a V08366	t284	14-44-13-17-17-23-18-17	ST121
	B3a V02228	t284	14-44-13-17-17-23-18-17	ST121
	B3a V04373	t284	14-44-13-17-17-23-18-17	ST121

P: patient; M: mother; F: father; B: brother.
NA: not available.

Table 19. Selected S. aureus clones for 2D immunoblot.

Family	Strain ID	Member	Source	*Spa* type	*Pvl*	*Agr*	Non-*egc* Sag	*Egc* Sag	Others
Family 1	P1 NOSE	P1	Nose	t2392	+	4		gimnou	*gyr, nuc*
	P1 WOUND		Wound	t5007	+	4		gimnou	*gyr, nuc*
Family 2	P2 V412	P2	Nose	t017	+	3	a	gimnou	*gyr,nuc,mw1409*
Family 3	P3 V08364	P3	Nose	t1458	-	3	atq		*gyr,nuc*
	B3a V08366	B3a	Perineum	t284	+	4		gimnou	*gyr,nuc*

P: patient; F: father.
g, i, m, n, o, u: enterotoxin genes *seg, sei, sem, sen, seo, seu* of the egc cluster respectively.
a, t, q: enterotoxin genes *sea, tst, seq* of the non-egc cluster respectively.
+: positive, -: negative.

1.2. Protein identification on 2D gels

Based on data from multiplex PCR and *spa* typing, we selected 5 strains from 3 families (see table 19). These strains had different genotypes and four of them harbored the gene encoding for the PVL toxin.

To identify extracellular proteins from the selected *S. aureus* isolates for further experiments, we used a mass spectrometry method. Gel spots were cut, digested in-gel and identified. We found that PVL was expressed *in vitro* in TSB medium. In addition, all strains expressed a typically broad range of extracellular proteins. They included enterotoxins, pore-forming toxins, house-keeping proteins, and enzymes (for details see tables 20 and 21, figures 16, 17, 18, 19 and 20).

1.3. IgG binding to extra-cellular *S. aureus* proteins

First the extracellular proteomes of the five *S. aureus* strains were determined by 2D SDS-PAGE followed by mass spectrometric identification of the protein spots. Following this, the anti-*S. aureus* antibody response was characterized in patients and family members using 2D immunoblots. Spots of IgG binding were identified by matching them with the corresponding spot in the 2D gel. Table 20 shows a list of the identified proteins with or without antibody binding. In general, we found IgG binding to known immunodominant proteins of *S. aureus*. It was observed that IgG bound to PVL (see table 20 and figures 21, 22 and 23).

Table 20. Identified proteins and their antibody IgG.

Family	**Family 1**						**Family 2**			**Family 3**										
***S. aureus*/ member**	**P1 NOSE**			**P1 WOUND**			**P2 V412**			**P3 V08364**					**B3a V08366**				
Protein	**P1**	**M1**	**F1**	**P1**	**M1**	**F1**	**P2**	**M2**	**F2**	**P3**	**M3**	**F3**	**B3a**	**B3b**	**P3**	**M3**	**F3**	**B3a**	**B3b**
50Sribo	Y																		
A5IQG7				Y		Y	Y												
A7WXV0	Y		Y																
Aerolysin							Y	Y	Y										
AmiD6															Y	Y	Y	Y	Y
Amidase																			
Atl	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
B-channform											Y	Y	Y	Y					
B-fo-CYTO																			
B-lacta	Y		Y	Y	Y	Y									Y	Y	Y	Y	Y
Blar1			Y								Y	Y	Y	Y	Y	Y	Y	Y	Y
Cell-SURF		Y	Y	Y		Y				Y	Y		Y	Y	Y	Y	Y	Y	Y
ChaP																			
Chips													Y						
Cys-PRO				Y	Y	Y													
E5qwp1							Y	Y	Y										
Emp										Y	Y	Y	Y						
EntG																			
Fib											Y								

Family	Family 1						Family 2			Family 3										
***S. aureus*/ member**	**P1 NOSE**			**P1 WOUND**			**P2 V412**			**P3 V08364**					**B3a V08366**				
Protein	**P1**	**M1**	**F1**	**P1**	**M1**	**F1**	**P2**	**M2**	**F2**	**P3**	**M3**	**F3**	**B3a**	**B3b**	**P3**	**M3**	**F3**	**B3a**	**B3b**
FtsI											Y								
Geh	Y	Y	Y		Y	Y	Y	Y	Y						Y	Y	Y	Y	Y
GlpQ	Y	Y	Y				Y		Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Hla		Y		Y	Y	Y				Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
HlgB	Y	Y	Y	Y	Y	Y	Y	Y	Y		Y	Y	Y	Y	Y	Y	Y	Y	Y
HlgC		Y		Y	Y	Y	Y	Y		Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
HlgC2		Y	Y	Y	Y	Y									Y	Y	Y	Y	Y
Hyaluronate	Y	Y																	
IsaB																			
IsdA							Y				Y		Y	Y					
Leu-ASH4											Y	Y	Y	Y					
Leukoci										Y	Y	Y	Y	Y					
Lip	Y	Y	Y		Y	Y	Y		Y						Y	Y	Y	Y	Y
LtaS	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
LukE										Y	Y	Y	Y						
LukF-PV	Y	Y	Y													Y	Y	Y	Y
LukL1	Y	Y		Y		Y		Y	Y		Y	Y	Y	Y					
LukS-PV							Y	Y	Y										
LysM	Y	Y	Y							Y	Y	Y	Y	Y		Y		Y	
MapW2																			Y
Mco									Y										

Family	Family 1						Family 2			Family 3										
***S. aureus*/ member**	**P1 NOSE**			**P1 WOUND**			**P2 V412**			**P3 V08364**					**B3a V08366**				
Protein	**P1**	**M1**	**F1**	**P1**	**M1**	**F1**	**P2**	**M2**	**F2**	**P3**	**M3**	**F3**	**B3a**	**B3b**	**P3**	**M3**	**F3**	**B3a**	**B3b**
MreC																			
Nuc		Y	Y					Y		Y	Y		Y		Y	Y	Y	Y	Y
Nucleotidase							Y	Y	Y										
Peni-BIND	Y	Y	Y						Y									Y	
PurF	Y	Y	Y													Y			
Pvl-F				Y	Y	Y	Y	Y	Y		Y	Y	Y	Y					
Q2ywb3																			
Rnj1		Y	Y													Y			Y
RplA											Y	Y	Y	Y					
RplC																			
RplF																			
RplM											Y	Y							
RpoC			Y																
Saa																			
SasH															Y	Y	Y	Y	Y
Sbi							Y	Y	Y										
Scp	Y	Y	Y							Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Sek																			
Sem											Y	Y	Y	Y					
Serine-PRO																			
Set11																			

Family	Family 1						Family 2			Family 3									
***S. aureus*/ member**	**P1 NOSE**			**P1 WOUND**			**P2 V412**			**P3 V08364**					**B3a V08366**				
Protein	**P1**	**M1**	**F1**	**P1**	**M1**	**F1**	**P2**	**M2**	**F2**	**P3**	**M3**	**F3**	**B3a**	**B3b**	**P3**	**M3**	**F3**	**B3a**	**B3b**
Sle1	Y		Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y		Y		Y	Y
SplB	Y	Y	Y												Y	Y	Y	Y	Y
SplC																			
SplF																			
Ssa2																			
SsaA2																			
SspB																			
SspP			Y	Y	Y	Y									Y	Y		Y	Y

P: patient; F: father; M: mother; B: brother

Y	Protein with IgG binding
	Protein without IgG binding
	No protein identified

To compare differences of IgG antibody binding to bacterial proteins between each family member, we determined the intensities of spots on the 2D immunoblots. The intensity of a spot reflects quantity of IgG which can bind to proteins represented in the spot (figure 7). In family 1, spot intensities of two selected *S. aureus* strains for each family member were compared. Figure 7a shows antibody binding to the non-invasive nasal isolate (P1 NOSE) and figure 7b shows IgG binding to the invasive wound isolate (P1 WOUND). Titers of antibodies bound to extracellular proteins from the invasive isolate were not higher than those of the non-invasive isolate, which was expected because both are closely related and can be assumed to be derivatives of the same *S. aureus* strain. With both invasive and non-invasive strains, antibodies from healthy family members showed a higher binding signal compared to those of the patient (see figures 7(a) and 8(a)). In family 2, two healthy family members did not did not demonstrate a stronger IgG response to exo-proteins of invasive strain P2 V412 than did their affected child (see figures 7(c) and 8(c)). In the family 3, healthy family members showed a stronger response that did the patient both to P3 V08364 (non-invasive) and B3a V08366 (invasive) strains.

In families 1 and 3, it was observed that the numbers of spots detected on 2D immunoblots from healthy members were higher than those of patients (see figure 7(a)(b)(d)(e)). Furthermore, spot numbers may reflect whether IgG antibodies can bind to a broader range of *S. aureus* proteins. Comparing invasive and non-invasive cases, results showed more spots of IgG binding in invasive strains (see figure 7).

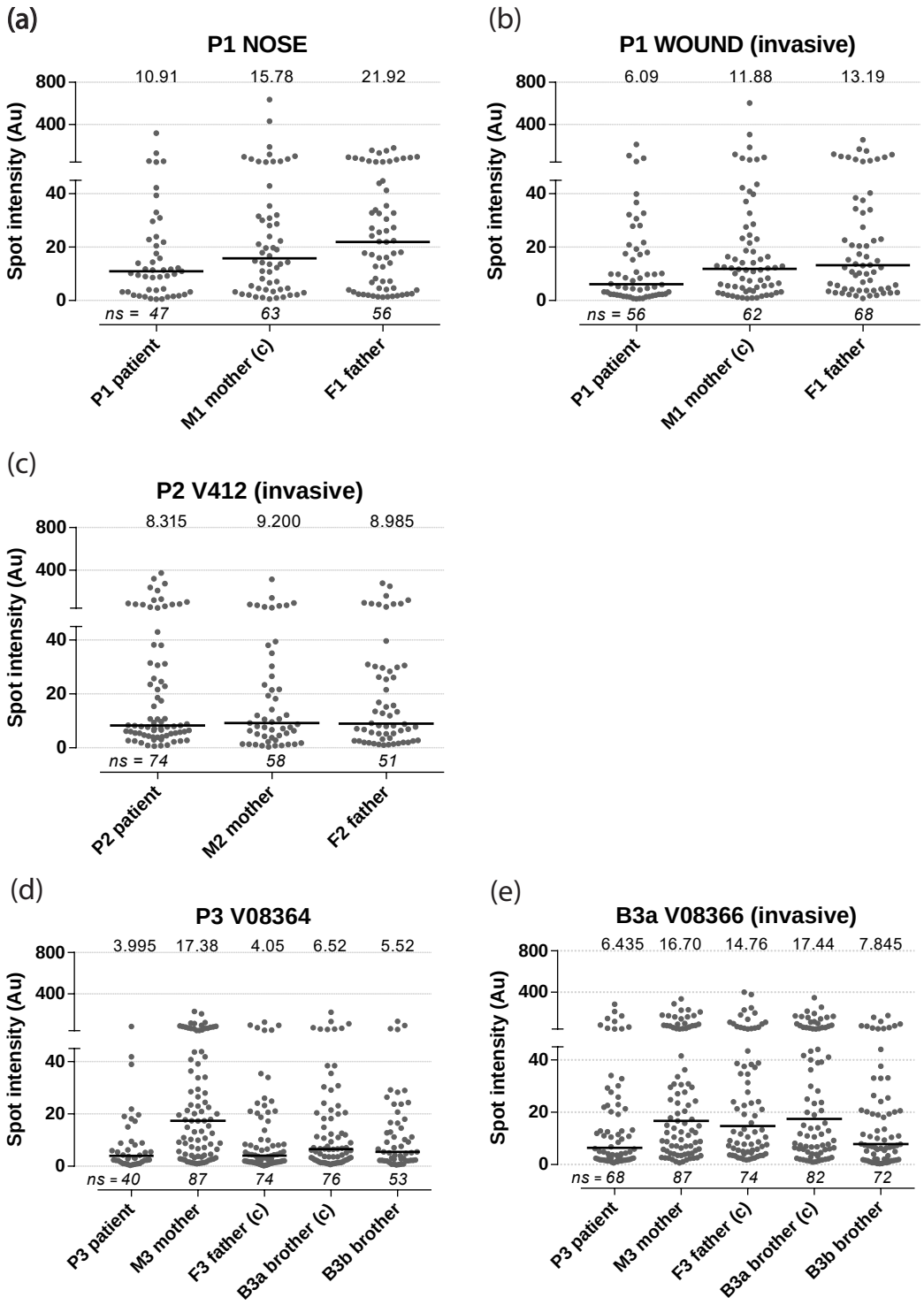

Figure 7. Spot intensities from each family.

Spot intensities were calculated using software Delta2D v4.4. Medians of three data sets are showed with numbers above the columns (a)(b) Spot intensities from strains P1 NOSE and P1 WOUND of family 1, in which the mother is a *S. aureus* carrier. (c) In family 2, the patient was infected by clone P2 V412, while the *S. aureus* strain was not isolated from the parents.

(d)(e) In family 3, isolate P3 V08364 was unique and isolated from the patient. The invasive clone was not only found in patient but also in the carriers, the father and the brother B3a. Numbers of spots (ns) are depicted below graphs.

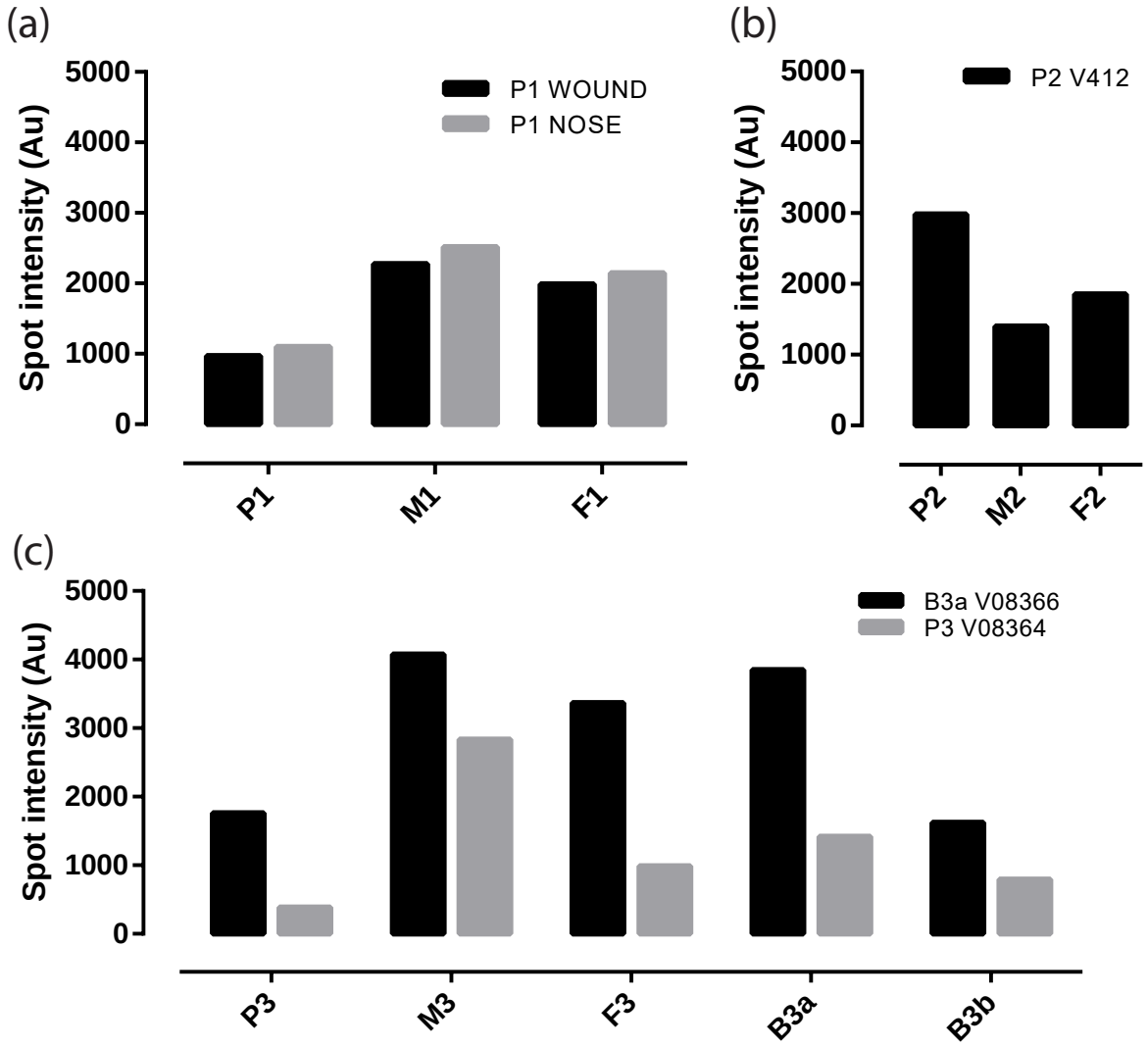

Figure 8. Summing up spot intensities.

Spot intensities were summed for each individual. The figure depicts IgG binding signal (spot intensity) for families 1 (a), 2 (b), 3 (c).

Next, the total signal of spot intensities was calculated (see figure 8). From every family member, the data reflect a total binding signal to extracellular proteins of a *S. aureus* clone. A stronger total signal implies a higher IgG antibody response. In two of the three families, where healthy family members were also colonized by *S. aureus*, antibody binding was weaker in the patient than in the healthy

individuals. This was not the case, however, in family 2 where the healthy father and mother were not colonized by *S. aureus*.

1.4. IgG binding to recombinant PVL proteins

ELISAs were conducted to investigate the binding of IgG to PVL. The toxin PVL is a two-component toxin, involved in forming pores on targeted host cells. The results (see figure 9) shows that a trend in which antibody binding to LukF-PV and LukS-PV of healthy individuals is higher than that of patients. This was due to the results of family, where the symptom-free members had more anti-PVL antibodies than the patient. In the other two families there was no such difference. There was concordance between anti-LukF-PV and anti-LukS-PV binding.

Because protein sequences of LukS-PV and γ-hemolysin C (HglC) are around 70–80% identical (Croze et al., 2009; Prevost et al., 1995), the question arose as to whether any cross reactivity exists between antibodies against LukS-PV and antibodies against γ-hemolysin C. ELISA was used to answer this question. IgG binding was stronger in healthy individuals than in the three patients. A correlation test showed that there was a weak correlation between IgG binding to LukS-PV and HlgC (see figure 10) implying that the antibody response to LukS-PV as well as to γ-hemolysin was specific. Thus in healthy family members and patients alike, there was a specific IgG response—against PVL components, which was higher in individuals who were exposed to PVL-positive *S. aureus* strains but remained free of furunculosis.

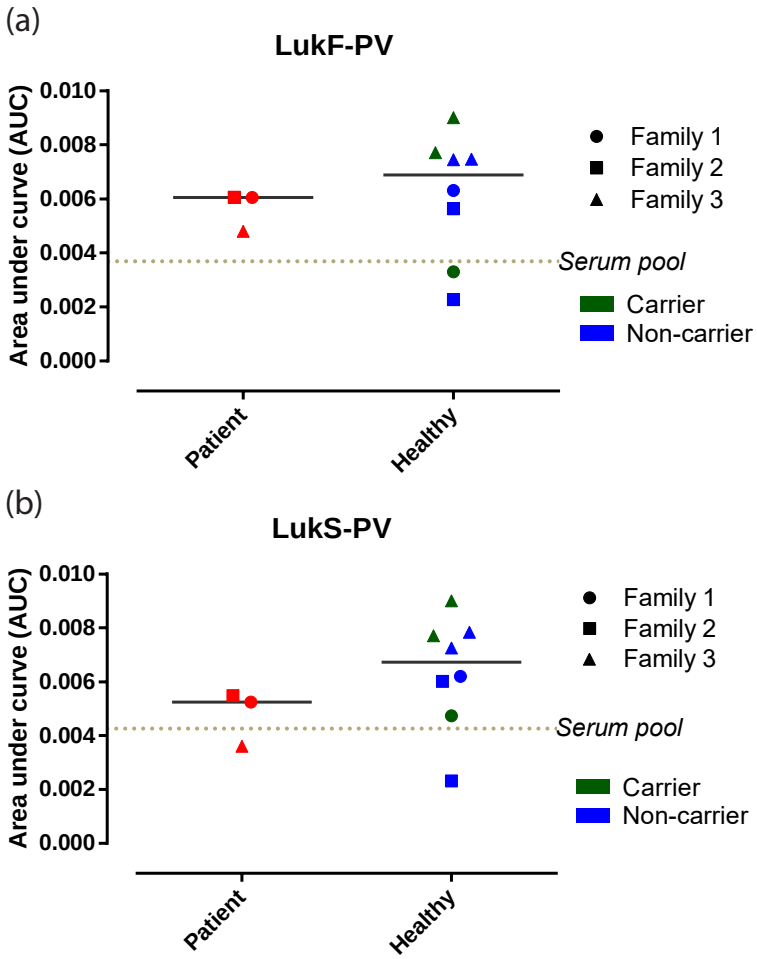

Figure 9. ELISA results of LukF- and LukS-PV.

Components of PVL toxin, LukF-PV and LukS-PV, were recombinantly expressed and used as antigens in an ELISA. ELISAs were conducted to determine whether antibody IgG bind to the protein units LukF-PV (a) and LukS-PV (b). A serum pool (from 20 healthy donors) represents as the base-line of the healthy population. Significant difference was not found between patient and healthy groups using Mann-Whitney test.

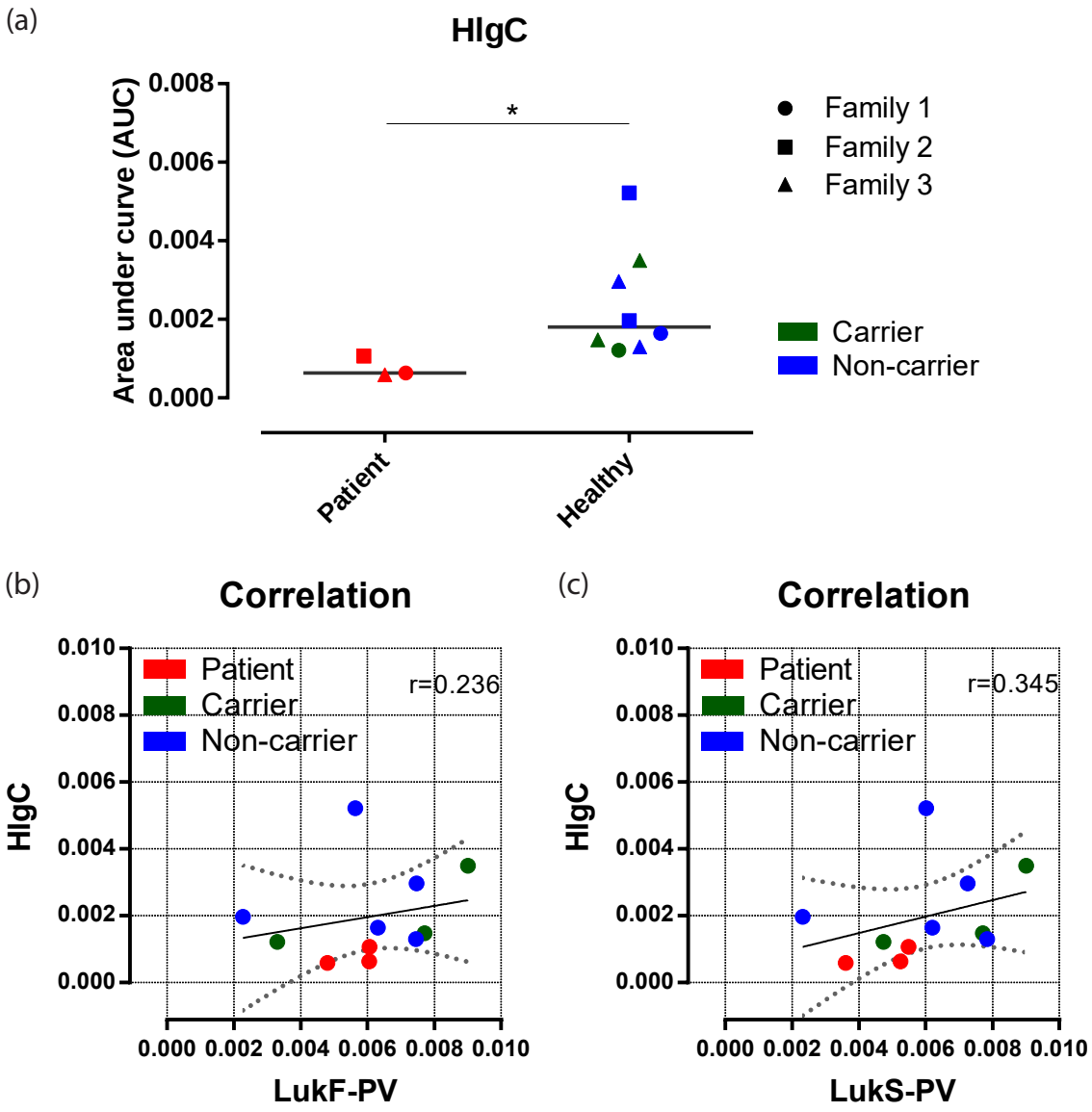

Figure 10. Correlation between antibody binding to HlgC and PVL subunits.

(a) Sera from the furunculosis cohort were used to conduct ELISAs, in which recombinant HlgC functions as an antigen. Mann-Whitney test showed a significant difference between patient and healthy groups (*, $p < 0.05$) (b) (c) No correlation was found between antibodies binding to HlgC and those binding LukF-PV or LukS-PV components. Correlation coefficients for the data were calculated using Pearson test.

2. Lipoproteins of *S. aureus*

2.1. The release of lipoproteins by *lgt* defective *S. aureus* mutants

To investigate the human natural immune response to lipoproteins, we used *S. aureus lgt* mutant strains. In the absence of the enzyme prolipoprotein diacylglyceryl transferase (Lgt), lipoproteins have no lipid tails, and, consequently cannot be anchored to the bacterial cell membrane but instead are released into the medium.

Four *S. aureus* wild type (WT) strains and their isogenic *lgt* mutations (Δ*lgt*) were examined (table 3). The bacteria were grown in cell culture under iron-limited conditions using 600 µM 2,2'-bipyridyl. Protein extracts derived from the culture supernatants of WT and isogenic *lgt* mutants were compared after separation on 2D gels. In the pI range 6–11, numerous novel spots were observed in the lgt mutant of strain RN4220 (figure 11). The novel spots were excised and the proteins identified by mass spectrometry (supporting information figure 24, table 22). Among them were isoforms of four lipoproteins: MntC, Opp1A, SirA and SstD (figure 11). Similar results were obtained with the *S. aureus* strains Colombia (COL), Newman, and SA113 (supporting information figure 25). In the pI range of 4–7, protein-A deletion mutants were used (Δ*spa*) to avoid non-specific IgG binding in subsequent tests. RN4220 Δ*lgt* and Δ*lgt*Δ*spa* strains shared very similar spot patterns on the 2D gels, although some spots in the protein extracts of the mutant strain were of higher intensity. The corresponding proteins were identified by mass spectrometry and shown not to be lipoproteins (supporting information figure 26).

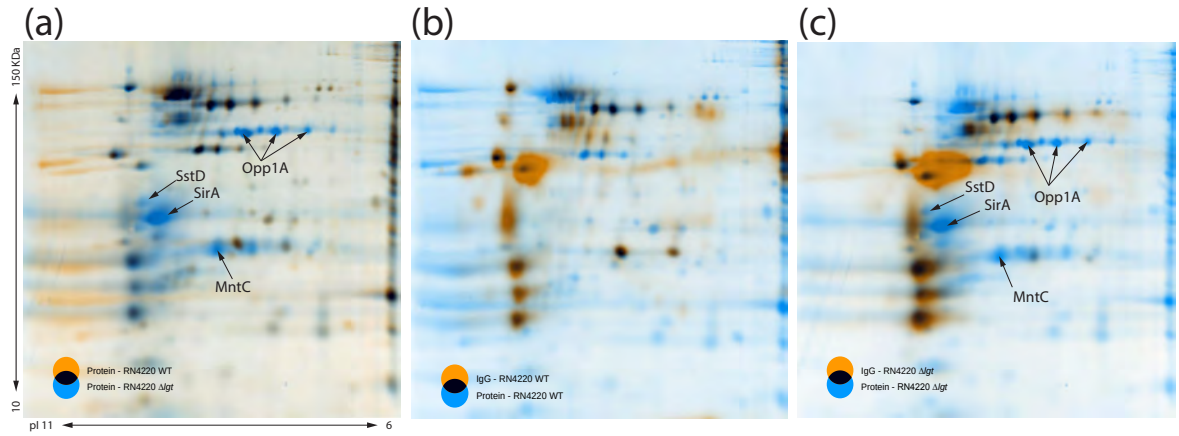

Figure 11. No antibody binding to lipoproteins in 2D immunoblot.

S. aureus extracellular proteins were separated on 2D gels. The superimposed image was created by software Delta2D version 4.4. Proteins were identified by mass spectrometry (MALDI-TOF). A human serum pool was used as a source of IgG for staining immunoblots. (a) Superimposition of 2D gel images of extracellular proteins derived from an *lgt*-competent *S. aureus* strain RN4220 (orange) and its isogenic *lgt* mutant (blue). The blue spots represent presumptive lipoproteins, which cannot be membrane bound in the absence of Lgt function. Mass spectrometry analysis identified these proteins as lipoproteins. (b)(c) Superimposition of the 2D immunoblot and the gel shows the human serum IgG binding to *S. aureus* extracellular proteins; blue: proteins from *S. aureus*, orange: IgG-bound proteins. IgG binding to *S. aureus* lipoproteins was not observed (blue spots in panel A). Similar testing in the pH range 4–7 revealed no secreted lipoproteins (see supporting information figure 26). Experiments were carried out in duplicate with similar results.

2.2. Proteomics and immune proteomics of *S. aureus* lipoproteins

The proteins separated by 2D-SDS PAGE were transferred onto membranes and incubated with a human serum pool from 20 healthy adults. IgG binding to numerous *S. aureus* proteins present in both WT and lgt mutant of strains RN4220 was seen in the pI range of 6–11. In contrast, none of the lipoproteins selectively released by the RN4220 Δ*lgt* mutant were as targeted by human serum IgG (figure 11, supporting information figure 24). Since no lipoproteins with pI between 4 and 7 were identified, IgG binding was not investigated in this pI range (supporting information figure 26).

2.3. Low antibody binding to recombinant *S. aureus* lipoproteins

To be able to study the human antibody response directed at *S. aureus* lipoproteins on a broader basis, the four lipoproteins identified on the immunoblots were expressed in recombinant form. Four additional lipoproteins were selected which perform different functions: IsdE is part of the heme transporter system IsdDEF (Pluym et al., 2007), Opp3A and Opp4A are oligopeptide ABC transporters (Bohn et al., 2010), and PstS is a periplasmic phosphate-binding protein functioning as a phosphate ABC transporter (Brautigam et al., 2014). All eight lipoproteins were cloned from the DNA of the *S. aureus* strain USA300. Based on the known lipoprotein sequence motifs, the signal sequences were removed, including the cysteine residue required for Lgt-mediated lipid tail attachment. A C-terminal strep tag sequence was attached to permit affinity purification on strep-tactin columns. The two extra-cellular *S. aureus* antigens, IsdB and Plc, were generated in a similar way and used as controls. IsdB transports heme to bacterial cells (Zorman et al., 2013) and Plc degrades membrane lipids and membrane-bound proteins (White et al., 2014). Final LPS concentrations were lower than 0.07 ng/mL.

The recombinant lipoproteins as well as IsdB and Plc (non-lipoprotein controls) were used as ligands in an ELISA to study serum IgG binding in a cohort of healthy adults comprising 16 *S. aureus* carriers and 16 *S. aureus* non-carriers. Corroborating the results of the immunoblots, there was very little if any IgG binding to the eight lipoproteins. Carriers and non-carriers did not differ in this respect. In contrast, strong IgG binding to IsdB as well as Plc was observed, in both carriers and non-carriers (figure 12).

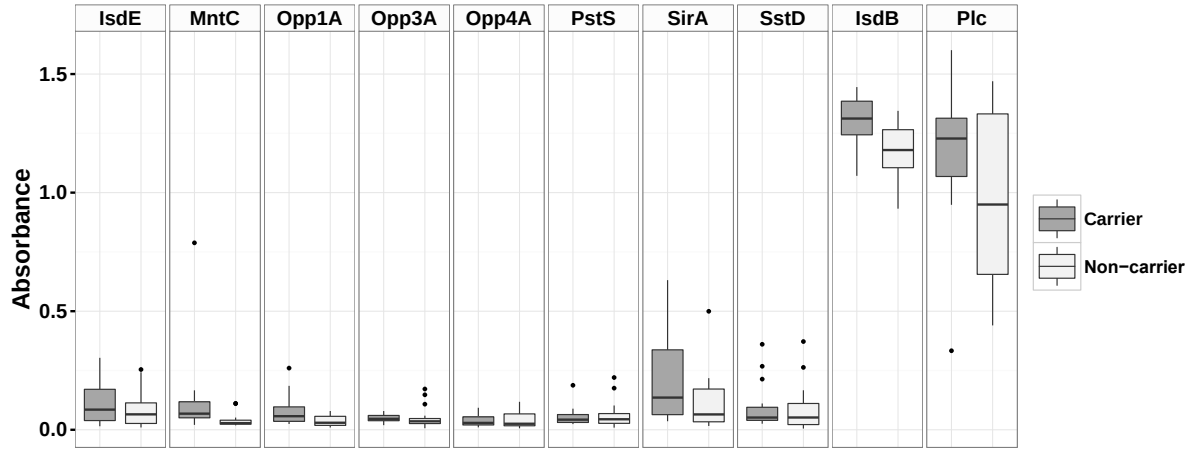

Figure 12. Flat levels for IgG against recombinant lipoproteins.

Recombinant lipoproteins (100 ng per cavity) were coated on ELISA plates and incubated with human serum samples—16 samples from *S. aureus* non-carriers and 16 samples from *S. aureus* carriers—each at a dilution of 1:100. After incubation with the goat anti-human-IgG-POD secondary antibody, IgG binding was measured using TMB substrate. The absorbance at OD450 nm showed at best weak IgG binding. IsdB and Plc, known immunodominant proteins, were included as controls.

2.4. T cell proliferation and cytokine secretion in response to recombinant lipoproteins

We then studied the possible function of lipoproteins as canonical T cell antigens. The eight recombinant lipoproteins, devoid of lipid tails, as well as the control antigens Plc and tetanus toxoid (TT), were used to test the human T cell response in ten healthy volunteers (figure 13). As expected, the control antigens induced a robust proliferation response already at very low concentrations (0.006 µg/mL). Most individuals have T cell memory of TT, a typical recall antigen, and also of Plc, as has been shown previously (Kolata et al., 2015). The proliferation elicited by the lipoproteins was variable and much lower than the response to TT or Plc (see supporting information figure 27). Opp1A, Opp4A and SirA did not trigger cells division at all, even at the maximum concentration of 25 µg/mL. MntC and SstD triggered proliferation only at the high concentration of 25 µg/mL, whereas

IsdE, Opp3A and PstS induced the cells to respond at concentrations from 6.25 µg/mL. Thus, these lipoproteins only stimulated weak to moderate proliferation of PBMCs, if at all.

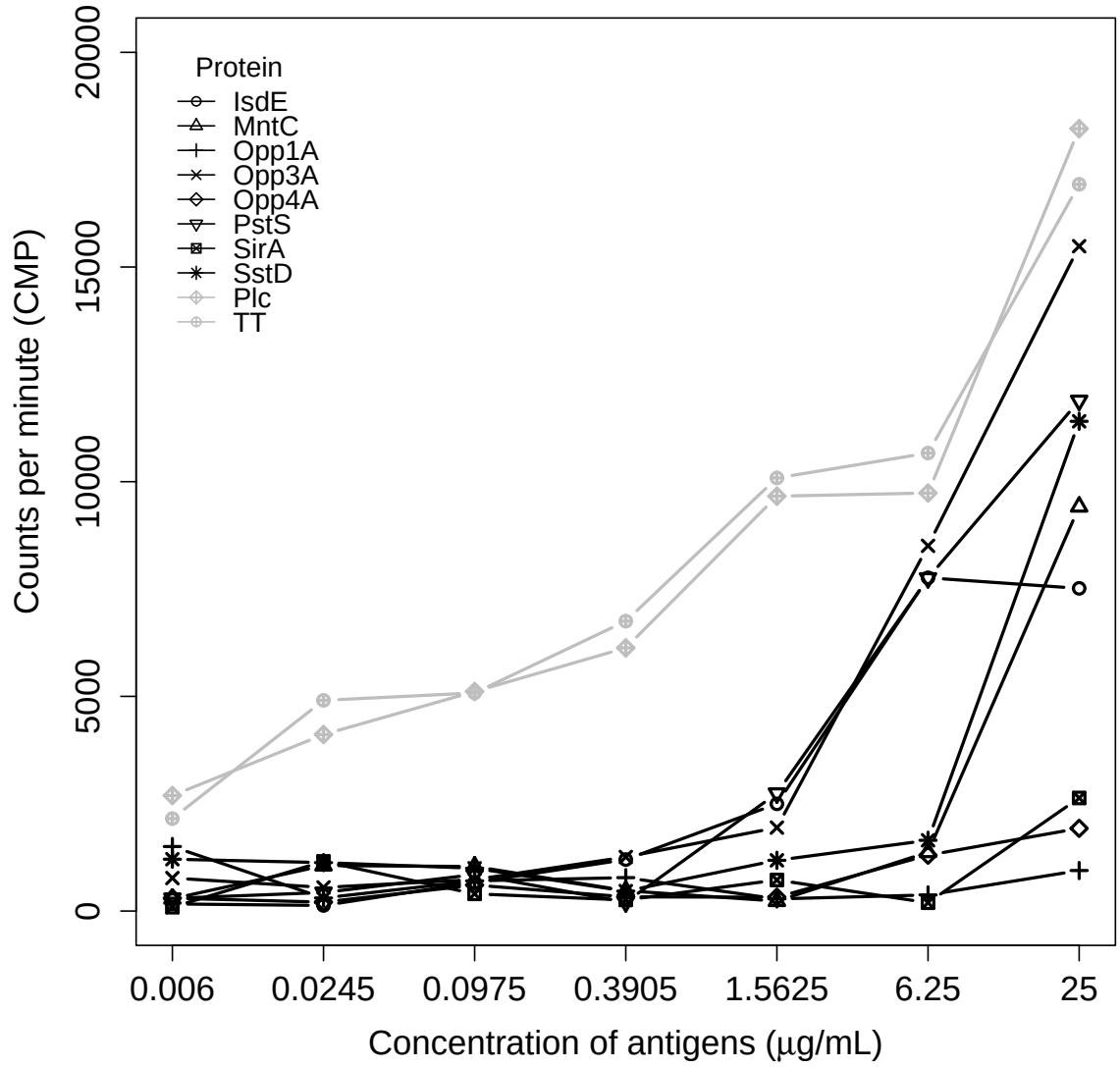

Figure 13. A weak response from T cells to lipoproteins.

Proliferation assays were conducted to investigate how PBMCs respond to *S. aureus* lipoproteins. Fresh human blood samples from 10 individuals were used to isolate PBMCs. Antigens were applied in serial dilution from 0.006 to 25 ng/µL. The recall antigen tetanus toxoid (TT) and the immune dominant protein *S. aureus* phospholipase C (Plc) were included as controls. Proliferation was measured by 3H-thymidine incorporation. Experiments were performed in triplicate. Means of the average T cell responses of the 10 individuals are depicted.

Lipoproteins stimulated PBMCs only at high antigen concentrations (≥ 6.25 ng/µL). Three of the five tested lipoproteins (Opp1A, Opp4A and SirA) did not trigger proliferation at all.

To elucidate the quality of the cellular immune response to lipoproteins without lipid tails, cytokine measurements were conducted. Supernatants from cultures with moderate or high proliferation (≥ 5,000 cmp) in response to maximal antigen concentration (25 µg/mL) were selected, and the following cytokines were measured: GM-CSF, IFN-γ, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-17A, TGF-β1, TNF. The lipoproteins Opp1A, Opp4A and SirA were excluded from this analysis, since they did not induce any proliferation in PBMCs. The results are shown in (figure 14) where the three lipoproteins IsdE, Opp3A and PstS (but not MntC and SstD) triggered the strongest proliferation, and induced the release of significant concentrations of IFN-γ, IL-17A, IL-1, IL-12 and IL-10, whereas tetanus toxoid elicited relatively higher amounts of IL-3, IL-4, IL-5, IL-9 and IL-13 (supporting information figure 28). The Th2 bias in the response to the recall antigen TT has been noted before (Kolata et al., 2015; Rowe et al., 2000).

Thus, cytokine release and the proliferative response to these lipoproteins were positively correlated. The three proteins which moderately stimulated the lymphocytes to divide, IsdE, Opp3A and PstS, also induced the secretion of Th1/Th17 cytokines (see supporting information figure 28).

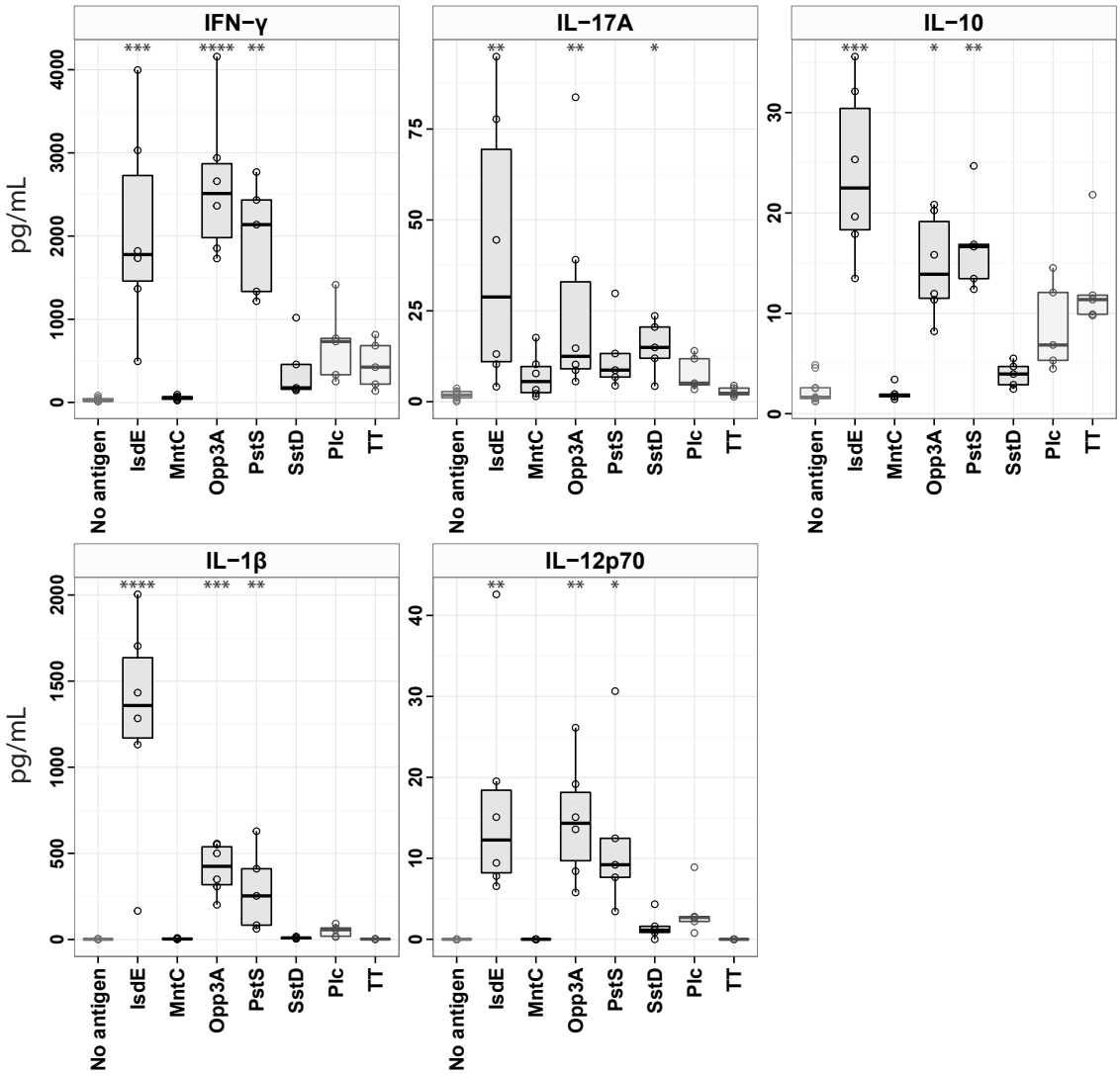

Figure 14. Lipoproteins triggering the release of inflammatory cytokines.

Cytokine profiling was conducted in five proliferation-stimulated lipoprotein assays. Cytokine secretion during the proliferative response was analyzed by cytokine bead assay (BD Biosciences). A box-and-whisker plot visualized median, upper quartile, lower quartile, maximum, minimum and outliers for each dataset. The three lipoproteins IsdE, Opp1A, and PstS induced the secretion of the Th1/Th17 cytokines IL-17A and INF-γ. Other cytokines, including IL-1β, IL-10, and IL-12p70, were also induced by these lipoproteins. In contrast, MntC and SstD weakly stimulated the secretion of cytokines belonging to the Th1/Th17 group. Kruskal-Wallis tests were conducted using GraphPad Prism v6.02 software to calculate p-values. Asterisks at the top of each panel correspond to significant differences versus to non-antigen (No Ag) control as following *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$;

p>0.05 data not shown (for more details about other cytokines see supporting information figure 28).

2.5. Antibody binding to recombinant lipoproteins in patients extensively exposed to *S. aureus*

Sera from the EB, CF and furunculosis cohorts were used in ELISAs to investigate the response of IgG to lipoproteins when individuals had been heavily exposed to *S. aureus* for a long period. Results (see figure 15) show that titers of antibodies from EB and CF patients binding to MntC, Opp1A and SirA were higher than those of family members in the furunculosis cohort and healthy individuals (SH and Lgt). In contrast, antibody binding to the bacterial non-lipoprotein antigens was similar in all tested cohorts.

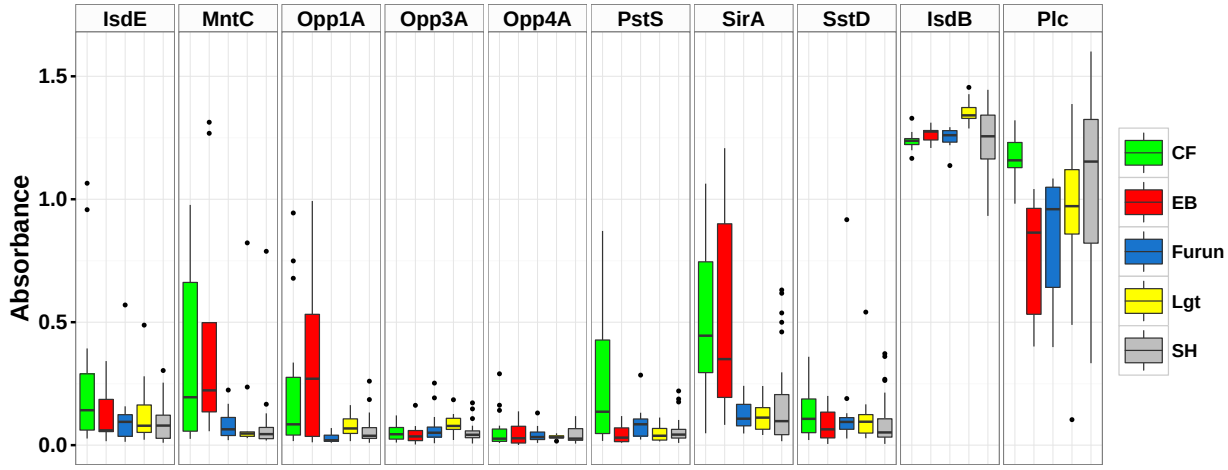

Figure 15. Antibodies from CF and EB cohorts binding to lipoproteins.

Sera from cohorts CF (n=18), EB (n=8), furunculosis—Furun (n=10), and Lgt (n=10) were also used to conduct ELISAs. Results shows that titers IgG from CF and EB cohorts binding to MntC, Opp1A and SirA are higher than in the furunculosis, Lgt and SH cohorts.

Chapter 4. Discussion

1. Diversity of *pvl*-positive *S. aureus* strains

In the furunculosis cohort, the invasive *S. aureus* clones from 3 families carried a gene coding for LukS-PV, a component of the pore-forming toxin PVL. This toxin was frequently associated with furunculosis. Other toxins encoded by *eta* and *etd* genes, such as those involved in staphylococcal scalded skin syndrome (Bukowski et al., 2010; Plano, 2004), were not identified in furunculosis clones. *Pvl*-positive *S. aureus* strains have been known as causes of recurrent skin and soft tissue infections in the community, especially in healthy young individuals (Boan et al., 2015; Issartel et al., 2005; Yamasaki et al., 2005). In this cohort, the three patients were children at age from 2 to 12. Thus the possibility that PVL contributed directly to furunculosis is high. *Pvl*-positive strains have also been linked to methicillin resistance (Miller and Kaplan, 2009; Otto, 2012; Tong et al., 2015; Watkins et al., 2012). However, multiplex PCR results did not show any markers for methicillin resistance in this cohort. This emphasizes that not only MRSA but also MSSA can cause recurrent skin and soft tissue infections.

In families 1 and 3, *S. aureus* strains were isolated from patients and healthy members. *Spa*-typing and multiplex PCR (see table 17 and 18) showed that bacterial genotypes were very similar in each family indicative of inter-transmission. In a study in The Netherlands, the authors demonstrated that family members act as reservoirs of *S. aureus* and may contribute to spreading these bacteria (Huijsdens et al., 2006). Close physical contact is the main reason for the bacterial spreading. A number of studies have found that invasive strains originate from the parents and circulate family members. In a case report, Cocchi et al suggested *S. aureus* may have been transmitted from father (recurrent soft tissue infection) causing necrotizing pneumonia in his child (Cocchi et al., 2013). Furthermore, transmission from mothers to infants has been reported (Chatzakis

et al., 2011), and intra-household transmission has also been documented (Knox et al., 2012). The household is a major community-based reservoir for methicillin resistant *S. aureus* (Knox et al., 2015). This means that in order to prevent reinfection of susceptible individuals all household members should be de-colonized of *S. aureus*.

In family 1, two *S. aureus* isolates from the patient may be of clonal origin. They shared identical virulence gene patterns, and two *spa* types were only different in one tandem, number 17 (see table 18). This means there might be an evolution of these *S. aureus* strains.

The *S. aureus* strain from family 1 was a hitherto unknown *spa* type and sequence type (see table 18). These strains may belong to a new *pvl*-positive *S. aureus* lineage, prevalent in Göttingen, Germany. The invasive strains from families 2 and 3 respectively showed sequence types ST30—a lineage commonly found in Europe—and ST121, which has been linked to *pvl*-positive *S. aureus* clones in Szczecin, Poland, respectively (Masiuk et al., 2010). The other well-known *S. aureus* sequence types ST1, ST5, ST8, ST22, ST59, ST80, ST93, ST152 and ST377, which have already been linked to *pvl*-positive *S. aureus,* were not found in this study (Boakes et al., 2012; DeLeo et al., 2010; Masiuk et al., 2010; Monecke et al., 2011; Shore et al., 2014).

2. Antibody binding to the *S. aureus* extra-cellular proteome including PVL

A combination of 2D-gel and 2D-immunoblot data provided a panoramic view of the antibody response to the *S. aureus* extra-cellular proteome in patients and their healthy family members. The number of spots on 2D immunoblots underlines the broad range of *S. aureus* antigens that can be bound by antibodies, while intensities of spots may reflect the amount of antibody binding.

In family 1, the number of spots and spot intensities of the mother M1 (carrier) were higher than those of the child—P1 (patient). The father, F1, also had high IgG binding titers (see figure 7) implying exposure to *S. aureus*. The 2D-gel and 2D-immunoblot datasets revealed that the two *S. aureus* strains from the patient's nose and wound had very similar protein patterns, and, not surprisingly, IgG response patterns. This corroborates the genotype data pointing at a close clonal relationship between the strains.

In family 2, at the time point of sample collection, it seems that father (F2) and mother (M2), both non-carriers, had not been challenged by with the invasive strain P2 V412, whereas the child—P2 (patient)—showed high titers of anti-*S. aureus* IgG (see figure 7).

In family 3, the father (F3) and the oldest brother (B3a) were carriers and had high IgG titers. The mother (M3) might be an intermittent carrier; her IgG titers are even higher than those of the carriers F3 and B3a The patient (P3) in this family had the lowest spot intensities and spot numbers (see figure 7).

In summary, in two of three families the non-symptomatic members, many of them carriers, had more anti-*S. aureus* antibodies in the serum than the affected child. In the third family the unaffected parents were non-carriers, and had lower antibody binding to *S. aureus* antigens than the young furunculosis patient. It is known that carriers have higher IgG titers to extracellular *S. aureus* proteins, which may provide protection for them. The more antibodies a person has at the time of infection, the better the outcome (Montgomery et al., 2014; Stentzel et al., 2015).

Because of natural contact with *S. aureus*, antibodies, which bind bacterially secreted proteins and cell-wall components, are common in humans (Glasner et al., 2015; Kumar et al., 2005). To date, antibodies directed against many *S. aureus* proteins have been identified (Holtfreter et al., 2010; Kolata et al., 2011). For instance, the basic bacterial proteins have corresponding antibodies, including

Atl, Aaa, IsaB and IsdA (Holtfreter et al., 2009). Additionally, there are highly conserved proteins, most of which are known as virulence factors of *S. aureus* such as α-hemolysin (Hla), cysteine protease A and B (SspA, SspB), phospholipase C (Plc), serine-protease-like protein B and E (SplB, SplE), staphylococcal protease (V8 protease, SspA), immunodominant staphylococcal antigen A (IsaA) and TSST-1. Further, it has been reported that some proteins exist which are abundant in *S. aureus* but have no corresponding antibodies, for instance, lipase (Lip) and some unknown proteins, and even SAgs encoded by the *egc* gene on phage SaPI3 (Holtfreter and Broker, 2005; Holtfreter et al., 2009; Kolata et al., 2011).

Antibodies perform many important functions; for example, they can inhibit superantigen-induced T-cell activation by neutralizing of the toxins, and especially antibodies against TSST-1 could prevent patients from toxic shock syndrome (Holtfreter et al., 2004).

Of particular interest in furunculosis is PVL and the antibody response to this toxin, which is strongly associated with skin and soft tissue infections. Both 2D immunoblots and ELISAs with the recombinant units LukF- and LukS-PV demonstrated that PVL-specific antibodies were similarly low in furunculosis patients and healthy unrelated controls but higher in the patients' symptom-free family members (in family 3). In the case of γ-hemolysin C the difference between patients and healthy family members was even more pronounced. Because there is no correlation between ELISA data of LukS-PV and HlgC, we can conclude that the human immune system developed specific antibodies against PVL. PVL is expressed in a minority of *S. aureus* isolates and one can assume that in many adults the immune system has never encountered the toxin, which explains the low baseline healthy unrelated control individuals. The symptom-free family members of patients with *pvl*-positive *S. aureus*, however, had evidently mounted an antibody response to the toxin, while all patients had

antibody binding in the range of healthy controls most of whom may be assumed to have never encountered PVL. This means *S. aureus*-expressed PVL triggered the immune system *in vivo*. In addition, Thomsen et al discovered high titers of IgG against both LukF-PV and LukS-PV in children who were in convalescing compared to children with acute *S. aureus* infections (Thomsen et al., 2014). Assumably, *S. aureus* used this leucocidin as a virulence factor in invasion (Croze et al., 2009). Another study showed that antibodies can neutralize PVL toxin (Fritz et al., 2013). Hence, high titers of PVL-specific IgG probably confer advantages on healthy people and contribute to protection during exposure to *pvl*-expressing *S. aureus* strains.

In view of the wealth of *S. aureus* virulence factors, many of which are redundant, it is, however, unlikely that antibody binding to PVL is sufficient to prevent patients from developing furunculosis. Other studies have shown the importance of antibodies neutralizing numerous *S. aureus* virulence factors in host protection from infection and its complications (Adhikari et al., 2012; Fritz et al., 2013; Glasner et al., 2015; Stentzel et al., 2015; Thomsen et al., 2014; Yeaman et al., 2014). Moreover, in recent years the importance of T cells has been stressed by many authors (Joshi et al., 2012; Kolata et al., 2015; Lawrence et al., 2012; Zielinski et al., 2012).

3. Marginal levels of antibody (IgG) against *S. aureus* lipoproteins in healthy adults

The results of 2D proteomics showed that *S. aureus* lipoproteins were released into the culture medium in Lgt-deficient *S. aureus* strains. The absence of the enzyme Lgt means that there is no lipid tail attached to the nascent lipoproteins. The resulting immature proteins thus cannot anchor to the cytoplasmic membrane and are then released from the bacterial cells (Stoll et al., 2005). All four lipoproteins identified in the bacterial supernatants of Lgt-deficient *S. aureus*

participate in ion transport, presumably they were strongly induced by the iron limitation stress (Stentzel et al., 2014). MntC is a magnesium-binding protein (Gribenko et al., 2013); Opp1A, also known as a NikA-like protein, belongs to an ABC transporter system for nickel and cobalt (Hiron et al., 2010) and SirA is required for the acquisition of ferrite Fe(III) (Grigg et al., 2010). SstD has been shown to interact with ferric catecholamine and catechol siderophores (Beasley et al., 2011).

To investigate the humoral immune response to lipoproteins, we used the 2D immunoblotting approach, with a serum pool as the source of human IgG. Briefly, results on blots (figure 11) indicated that there is no antibody binding to the four identified lipoproteins. In contrast, IgG binding was observed on other spots, which are known to be either cell-surface or secretion proteins (for instance Atl, Geh, Nuc or IsdB) (supporting information figure 24). In a second approach, serum IgG binding to eight function-known and twenty function-unknown recombinant lipoproteins was tested in individual *S. aureus* carriers and non-carriers. This confirmed that the natural human antibody response to *S. aureus* lipoproteins was very low in *S. aureus* carriers and non-carriers alike, irrespective of the function of the tested lipoprotein. This confirms and extends the findings of Diep and co-workers, who found low antibody binding to lipoproteins even in patients with *S. aureus* infection (Diep et al., 2014).

Many studies have indicated that lipoproteins are cytoplasmic membrane-bound (Hutchings et al., 2009; Okuda and Tokuda, 2011; Sheldon and Heinrichs, 2012). Therefore, they extend into the bacterial cell wall and are covered by the peptidoglycan layers. Thus, we hypothesized that hiding in the cell wall may be a suitable explanation for the lack of the antibody response. The crystal structure information available for IsdE and MntC (2Q8P and 4K3V, respectively; protein data base www.pdb.org) provides support for the hypothesis. The diameters in the three dimensions of the crystalized structure of IsdE are 63.53, 63.53, and

144.25 Å (Grigg et al., 2007), and those of MntC are 67.48, 68.36, and 107.91 Å (Gribenko et al., 2013). Hence, the maximum diameter of these lipoproteins is less than the 300–1000 Å thickness of the Gram-positive peptidoglycan layer (Silhavy et al., 2010) by a factor of at least two, suggesting that these proteins may be largely inaccessible for B cells in intact bacteria. This idea is further supported by a study which shows that *S. aureus* surface wall teichoic acids prevent bacterial recognition and opsonization by antibodies (Gautam et al., 2015).

However, lipoproteins are not immunologically "silent" per se, since immunization of mice with recombinant FhuD2 resulted in a robust antibody response, which then conferred protection by significantly inhibiting growth of *S. aureus in vivo* (Mishra et al., 2012). In addition, vaccination of cattle with heat-killed bacteria elicited antibodies against two lipoproteins: NWMN_0601 (MntC) and NWMN_0364. Possibly, heat treatment disrupts the bacterial cell wall, thus facilitating lipoprotein release or exposure, which might explain the difference in the MntC-directed antibody response between naturally *S. aureus*-exposed humans and vaccinated cattle (Lawrence et al., 2012).

4. Lipoproteins triggered a low level of T cell response

Turning to cellular immunity, lipoproteins could have two mutually non-exclusive effects: First, they are potent stimulators of innate immune cells, which recognize the lipid tails as danger signals via TLR2/6 pattern recognition receptors. Such danger signals are a prerequisite for the initiation of an inflammatory T cell response, irrespective of the antigen-specificity of the T cells. Studying infection of mice with *lgt* mutant *S. aureus*, Schmaler and co-workers showed that lipoproteins are potent stimulators of T cells, whose antigen-specificity was not examined. The resulting release of IFN-γ, IL-17 and IL-10 was dependent on the presence of TLR2 and MyD88 signaling (Schmaler et al., 2011).

In addition to the danger signals conveyed by the lipid tail, the protein fragment of lipoproteins could prime an antigen-specific T cell response. This requires that lipoproteins be taken up by antigen-presenting cells, proteolytically cleaved into peptides, and presented to T cells in complex with MHC class II molecules. Such MHC/peptide complexes are recognized by antigen-specific T cells with the T cell receptor (TCR). The TCR is the master switch for activating naïve T cells for proliferation and differentiation into effector T cells, of which numerous subtypes with specialized functions have been described (Broker et al., 2014).

To discriminate the antigen-specific T cell response to lipoproteins from non-specific stimulation effects of their lipid tails, recombinant proteins were generated representing the protein parts of eight lipoproteins without lipid tails. Five of the eight lipoproteins tested triggered cell division to a low or moderate degree in PBMCs of human *S. aureus* carriers and non-carriers (figure 13), whereas the classical recall antigen tetanus toxoid (TT) and the immune dominant protein Plc induced proliferative responses at a higher rate, as already indicated in other studies (Kolata et al., 2015). Opp1A, Opp4A and SirA did not react at all. Mntc and SstD only triggered lymphocytes at the highest concentration. Since it is possible to vaccinate with recombinant lipoproteins (Anderson et al., 2012; Mishra et al., 2012), ineffective processing and presentation of natural lipoproteins imbedded in the *S. aureus* cell wall could explain the lack of a strong cellular immune response to this group of antigens.

The low grade T cell reaction to lipoproteins in healthy adults could be a second factor contributing to the weak human antibody response to *S. aureus* lipoproteins. For differentiation into antibody-producing plasma cells, most B cells need the assistance of T cells in addition to antigen contact (Broker et al., 2014).

Cytokine profiling showed that PBMCs responded to lipoproteins in different ways (figure 14 and supporting information figure 28). The three most mitogenic

lipoproteins IsdE, Opp3A and PstS induced production of IFN-γ, IL-17A, IL-10, IL-12 and IL-1β. This agrees well with the dominant T cell reaction profile to *S. aureus* described by Zielinski and co-workers as well as in a previous study by our group (Kolata et al., 2015; Zielinski et al., 2012). In this respect, lipoproteins appear as typical *S. aureus* antigens.

The production of IFN-γ and IL-17A confers a protective immune response by supporting B lymphocyte differentiation to specific IgG producing cells and recruiting neutrophils for phagocytosis. In fact, Lawrence and colleagues, showed that an immune response of this quality could be induced by vaccinating cattle with heat-inactivated *S. aureus* particles. When challenged with recombinant MntC, the vaccinated animals developed an immune response characterized by both IFN-γ and MntC-specific IgG (Lawrence et al., 2012). In contrast, only a slight MntC-specific humoral or cellular immune response was detectable in humans naturally exposed to *S. aureus* in this study as well as in that by Diep and co-workers (Diep et al., 2014).

In summary, the present study shows that human IgG binds marginally at best to *S. aureus* lipoproteins. We propose that lipoproteins are buried inside bacterial cell membranes, and are thus not easily accessible for B cells. Likewise, lymphocyte proliferation and cytokine profiling results indicated that lipoproteins weakly triggered the T cell response. This may be due to the ineffective presentation of antigens to T lymphocytes. This study briefly provides an overview of the *in vivo* reaction of the human adaptive immune system to lipoproteins of *S. aureus*.

5. *S. aureus*-exposed patients' antibody binding to lipoproteins

Results of ELISA conducted using sera of CF and EB cohorts showed that patients, who were heavily exposed to *S. aureus* for a long time had mounted an antibody response to some lipoproteins. This was not the case, however, in

furunculosis patients, which is in agreement with the findings described above, showing an overall moderate anti-*S. aureus* antibody response in such patients. In the sera of CF and EB patients, antibody binding to MntC, Opp1A and SirA was also seen on 2D immunoblots using *lgt*-mutant *S. aureus* increases the chance of lipoproteins being released from cell, e.g., upon bacterial cell death. The amount of a lipoprotein may be a main factor in triggering the adaptive immune response. However, during long-term extensive exposure to large amounts of *S. aureus*, such as in CF and EF, some lipoproteins are evidently released in sufficient amounts to trigger an antibody response in the patients.

In summary, this study lends support to the notion that robust immune memory of *S. aureus*, as it is reflected by high titer specific serum IgG, provides clinical protection from *S. aureus*-mediated disease. In case of skin and soft tissue infection antibodies directed against PVL and γ-hemolysin may be of special importance. To elucidate the target molecules of the human anti-*S. aureus* antibody response, this work focused on lipoproteins, which hitherto had not been extensively studied because they are retained in the bacterial membrane and not regularly released by the bacteria in large amounts. The study shows that this class of bacterial fitness and virulence factors is not immunodominant; both antibody and T cell responses were low in healthy adults. Only under special circumstances, in patients with very high bacterial loads, some lipoproteins can trigger an antibody response.

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Supplemental data

Table 21. Protein identification for the furunculosis study.

Protein	Description	Mass (Da)	Isoelectric point	Uniprot ID
50SriboL5	50S ribosomal protein L5	18318	9.99	D8HDX8
A5IQG7	Putative uncharacterized protein	18594	9.6	A5IQG7
A7WXV0	Putative uncharacterized protein	33094	6.28	A7WXV0
Aerolysin	Aerolysin/Leukocidin family protein	38502	9.7	E5QRN2
AmiD6	N-acetylmuramoyl-L-alanine amidase	138280	10.1	E5QQT8
Amidase	N-acetylmuramoyl-L-alanine amidase	69171	6.47	D2NAM6
Atl	Bifunctional autolysin	136682	10.07	D2N627
B-chann form	B-channel forming cytolysin	38662	8.98	A5IUH2
B-lacta	B-lactamase	31395	10.33	D2N8R0
BlaR1	Regulatory protein	69468	10.01	D2N8Q9
Cell-surf	Cell surface hydrolase	32594	10.05	D2N9D0
ChaP	CHAP domain containing protein	16870	10.61	A5IVX1
ChiPs	Chemotaxis inhibitory protein	17058	10.11	A6QIG7
Cys-pro	Staphopain cysteine proteinase	44170	10.11	D8HCT9
E5QWP1	Putative uncharacterized protein	33543	9.81	E5QWP1
Emp	Extracellular matrix protein-binding protein emp	38193	10.35	Q2YWL4
EntG	Enterotoxin type G	29940	6.94	P0A0L6
Fib	Fibrinogen-binding protein	18765	10.45	A6QG59
FtsI	Cell division protein	77224	9.73	D3EWU8
Geh	Glycerol ester hydrolase	72344	9.51	Q2YVD0
GlpQ	Glycerophosphoryl diester phosphodiesterase	34051	8.58	D9RNE7
Hla	A-hemolysin	35973	9.19	Q2G1X0
HlgB	γ-hemolysin component B	36711	9.81	P0A075
HlgC	γ-hemolysin component C	35584	9.75	Q7A019

Protein	Description	Mass (Da)	Isoelectric point	Uniprot ID
Hyaluronate	Hyaluronate lyase	92534	8	D8HDV0
Iron-reg	Iron-regulated heme-iron binding protein	71456	9.45	D2N6A2
IsaB	Immunodominant staphylococcal antigen B	19370	10.23	Q2FDM1
IsdA	Iron (Fe2+)-regulated surface determinant protein	39133	10.34	E5QY13
Leu-ASH4	Combined Leukocidin/ASH4 hemolysin	40462	9.95	A5IUH3
Leukoci	Leukocidin/hemolysin toxin family protein	38664	9.37	D9RBI7
Lip	Triacylglycerol lipase	76479	9.2	D9REL4
Lip1	Lipase 1	76601	8.45	Q6GDD3
Lip2	Lipase 2	76522	9.47	Q6GCF1
LtaS	Glycerol phosphate lipoteichoic acid synthase	77160	9.56	E5QSC4
LukE	Leukotoxin E subunit	34747	10.05	Q2YTQ3
LukF	Leukocidin/hemolysin toxin family F subunit	38686	9.06	A6QIL7
LukF-PV	Panton-Valentine leukocidin subunit F	36962	9.57	A8Z264
Lukl1	Uncharacterized leukocidin-like protein 1	38662	8.98	Q7A4L0
Lukl2	Uncharacterized leukocidin-like protein 2	40434	9.95	Q2FFA2
LukS-PV	Panton-Valentine leukocidin subunit S	35313	9.62	A8Z265
LysM	LysM domain protein	35923	9.99	D8HFU6
MapW2	Cell surface protein	65574	10.53	A8Z2V4
Mco	Multicopper oxidase	50854	9.96	Q6GIX3
MreC	Rod shape-determining protein	31010	9.6	A5ITH3
Nuc	Staphylococcal thermonuclease	25121	9.93	Q2YWL2
Peni-bind	Penicillin-binding protein, transpeptidase	77224	9.73	A5IT80
PurF	Amidophosphoribosyltransferase	54397	6.56	Q5HH14
Pvl-F	Probable leukocidin F subunit	38686	8.72	D8HCW8
Q2YWB3	Putative uncharacterized protein	25690	8.58	Q2YWB3
Rnj1	Ribonuclease J 1	62669	7.33	Q2FHZ1
RplA	50S ribosomal protein L1	24546	9.43	Q6GJD0
RplC	50S ribosomal protein L3	23718	10.5	A6QJ92

Protein	Description	Mass (Da)	Isoelectric point	Uniprot ID
RplF	50S ribosomal protein L6	19787	10.12	A5IV19
RplM	50S ribosomal protein L13	16333	9.75	A5IV02
RpoC	DNA-directed RNA polymerase subunit β	135409	6.94	P60284
Saa	Staphopain A	44204	10.11	P65825
SasH	5'-nucleotidase	84289	9.86	E5QV61
Sbi	Immunoglobulin-binding protein	50192	9.94	Q6GE15
Scp	SCP-like extracellular	33605	9.81	A5ISM0
Sek	Staphylococcal enterotoxin	27727	8.32	Q5HHK0
Sem	Staphylococcal enterotoxin type I	28154	8.95	D9RN09
Serine-pro	Serine proteinase	26054	9.61	D8HCB7
Set11	Staphylococcal exotoxin 11	25670	9.41	Q2YVM7
Sle1	N-acetylmuramoyl-L-alanine amidase	36024	10	Q6GJK9
SplB	Serine protease	26141	9.58	A5ITX7
SplC	Serine proteinase	25971	7.72	D8HCB6
SplF	Serine proteinase	25647	9.83	D8HCB5
Ssa2	Staphylococcal secretory antigen	29651	9.09	D9RCG1
SsaA2	Staphylococcal secretory antigen	29327	9.1	Q2G2J2
SspB	Cysteine protease	44048	10.01	E5QS33
SspP	Staphopain A	44142	10.07	Q6G824
TraP	Signal transduction protein	19615	6.89	D8HCE0

Table 22. Protein identification of the lipoprotein study.

Label	Description	Mass (Da)	pI	Uniprot ID
1	Oligopeptide permease, peptide-binding protein	60019	9.23	Q2FE32
2	Oligopeptide permease, peptide-binding protein	60019	9.23	Q2FE32
3	Oligopeptide permease, peptide-binding protein	60019	9.23	Q2FE32
4	Oligopeptide permease, peptide-binding protein	60019	9.23	Q2FE32
5	Oligopeptide permease, peptide-binding protein	60019	9.23	Q2FE32
6	Oligopeptide permease, peptide-binding protein	60019	9.23	Q2FE32
7	Oligopeptide permease, peptide-binding protein	60019	9.23	Q2FE32
8	Oligopeptide permease, peptide-binding protein	60019	9.23	Q2FE32
9	Iron-regulated surface determinant protein B	72192	9.6	Q2FHV2
11	ABC transporter, substrate-binding protein	34741	9.16	Q2FJ07
12	ABC transporter, substrate-binding protein	34741	9.16	Q2FJ07
13	Iron compound-binding protein SirA	36744	9.76	Q2FKE4
14	Transferrin receptor	37854	9.93	Q2FIQ4
14	Iron compound-binding protein SirA	36744	9.76	Q2FKE4
16	A-hemolysin	35973	9.19	Q2FHS2
18	Iron-regulated surface determinant protein B	72192	9.6	Q2FHV2
19	Iron-regulated surface determinant protein B	72192	9.6	Q2FHV2
20	Iron-regulated surface determinant protein B	72192	9.6	Q2FHV2
22	A-hemolysin	35973	9.19	Q2FHS2
23	Iron-regulated surface determinant protein A	38746	10.26	Q2FHV1
24	N-acetylmuramoyl-L-alanine amidase sle1	35836	9.99	Q2FJH7
24	Iron-regulated surface determinant protein A	38746	10.26	Q2FHV1
25	Autolysin	137423	10.09	Q2FI25
26	Autolysin	137423	10.09	Q2FI25
27	Iron-regulated surface determinant protein B	72192	9.6	Q2FHV2
28	Triacylglycerol lipase	76676	7.66	Q2FDJ1

29	Iron-regulated surface determinant protein B	72192	9.6	Q2FHV2
29	Triacylglycerol lipase	76418	9.55	Q2FJU4
29	Iron-regulated surface determinant protein B	72192	9.6	Q2FHV2
30	Iron compound-binding protein SirA	36744	9.76	Q2FKE4
31	Amino acid ABC transporter, amino acid-binding protein	28904	9.88	Q2FE84
32	Iron-regulated surface determinant protein A	38746	10.26	Q2FHV1
33	Thermonuclease	25120	9.72	Q2FIK2
35	Iron-regulated surface determinant protein C	24855	9.42	Q2FHV0
38	Iron compound-binding protein SirA	36744	9.76	Q2FKE4

Table 23. Recombinant lipoproteins information

Name	Uniprot locus	NCBI locus	Protein sequence	Identical*
IsdB	SAUSA300_1028	WP_001041586.1	MNKQQKEFKSFYSIRKSSLGVASVAISTLLLLMSNGEAQ AAAEETGGTNTEAQPKTEAVASPTTTSEKAPETKPVANA VSVSNKEVEAPTSETKEAKEVKEVKAPKETKEVKPAAK ATNNTYPILNQELREAIKNPAIKDKDHSAPNSRPIDFEMK KKDGTQQFYHYASSVKPARVIFTDSKPEIELGLQSGQFW RKFEVYEGDKKLPIKLVSYDTVKDYAYIRFSVSNGTKAV KIVSSTHFNNKEEKYDYTLMEFAQPIYNSADKFKTEEDY KAEKLLAPYKKAKTLERQVYELNKIQDKLPEKLKAEYK KKLEDTKKALDEQVKSAITEFQNVQPTNEKMTDLQDTK YVVYESVENNESMMDTFVKHPIKTGMLNGKKYMVMET TNDDYWKDFMVEGQRVRTISKDAKNNTRTIIFPYVEGK TLYDAIVKVHVKTIDYDGQYHVRIVDKEAFTKANTDKS NKKEQQDNSAKKEATPATPSKPTPSPVEKESQKQDSQK DDNKQLPSVEKENDASSESGKDKTPATKPTKGEVESSST TPTKVVSTTQNVAKPTTASSKTTKDVVQTSAGSSEAKDS APLQKANIKNTNDGHTQSQNNKNTQENKAKSLPQTGEE SNKDMTLPLMALLALSSIVAFVLPRKRKN	0
IsdE	SAUSA300_1032	WP_011443629.1	MISVVILTSCQSSSSQESTKSGEFRIVPTTVALTMTLDKLD LPIVGKPTSYKTLPNRYKDVPEIGQPMEPNVEAVKKLKP THVLSVSTIKDEMQPFYKQLNMKGYFYDFDSLKGMQKS ITQLGDQFNRKAQAKELNDHLNSVKQKIENKAAKQKKH PKVLILMGVPGSYLVATDKSYIGDLVKIAGGENVIKVKD RQYISSNTENLLNINPDIILRLPHGMPEEVKKMFQKEFKQ NDIWKHFKAVKNNHVYDLEEVPFGITANVDADKAMTQ LYDLFYKDKK	30
MntC	SAUSA300_0618	WP_000737654.1	MKKLVPLLLALLLLVAACGTGGKQSSDKSNGKLKVVTT NSILYDMAKNVGGDNVDIHSIVPVGQDPHEYEVKPKDIK KLTDADVILYNGLNLETGNGWFEKALEQAGKSLKDKKV IAVSKDVKPIYLNGEEGNKDKQDPHAWLSLDNGIKYVK	0

Name	Uniprot locus	NCBI locus	Protein sequence	Identical*
			TIQQTFIDNDKKHKADYEKQGNKYIAQLEKLNNDSKDK FNDIPKEQRAMITSEGAFKYFSKQYGITPGYIWEINTEKQ GTPEQMRQAIEFVKKHKLKHLLVETSVDKKAMESLSEE TKKDIFGEVYTDSIGKEGTKGDSYYKMMKSNIETVHGS MK	
Opp1A	SAUSA300_2411	WP_001229077.1	MRKLTKMSAMLLASGLILTGCGGNKGLEEKKENKQLTY TTVKDIGDMNPHVYGGSMSAESMIYEPLVRNTKDGIKPL LAKKWDVSEDGKTYTFHLRDDVKFHDGTPFDADAVKK NIDAVQENKKLHSWLKISTLIDNVKVKDKYTVELNLKE AYQPALAELAMPRPYVFVSPKDFKNGTTKDGVKKFDGT GPFKLGEHKKDESADFNKNDQYWGEKSKLNKVQAKVM PAGETAFLSMKKGETNFAFTDDRGTDSLDKDSLKQLKD TGDYQVKRSQPMNTKMLVVNSGKKDNAVSDKTVRQAI GHMVNRDKIAKEILDGQEKPATQLFAKNVTDINFDMPT RKYDLKKAESLLDEAGWKKGKDSDVRQKDGKNLEMA MYYDKGSSSQKEQAEYLQAEFKKMGIKLNINGETSDKIA ERRTSGDYDLMFNQTWGLLYDPQSTIAAFKAKNGYESA TSGIENKDKIYNSIDDAFKIQNGKERSDAYKNILKQIDDE GIFIPISHGSMTVVAPKDLEKVSFTQSQYELPFNEMQYK	36
Opp3A	SAUSA300_0891	WP_000197019.1	MTRKFRTLILILIATIALSGCANDDGIYSDKGQVFRKILSS DLTSLDTSLITDEISSEVTAQTFEGLYTLGKGDKPVLGVA KAFPEKSKDGKTLKVKLRSDAKWSNGDKVTAQDFVYA WRKTVDPKTGSEFAYIMGDIKNASDISTGKKPVEQLGIK ALNDETLQIELEKPVPYINQLLALNTFAPQNEKVAKKYG KNYGTAADRAVYNGPFKVDDWKQEDKTLLSKNQYYW DKKNVKLDKVNYKVIKDLQAGASLYDTESVDDAVITAD QVNKYKDNKGLNFVLTTGTFFVKMNEKQYPDFKNKNL RLAIAQAIDKKGYVDSVKNNGSIPSDTLTAKGIAKAPNG KDYASTMNSPLKYNPKEARAHWDKAKKELGKNEVTFS MNTEDTPDAKISAEYIKSQVEKNLPGVTLKIKQLPFKQR VSLELSNNFEASLSGWSADYPDPMAYLETMTTGSAQNN	47

Name	Uniprot locus	NCBI locus	Protein sequence	Identical*
			TDWGNKEYDQLLKVARTKLALQPNERYENLKKAEEMF LGDAPVAPIYQKGVAHLTNPQVKGLIYHKFGPNNSLKH VYIDKSIDKETGKKKK	
Opp4A	SAUSA300_0892	WP_000517187.1	MGKLIKYISILLIVVLVLSACGKSSNKDEGVKDATKTETS KHKGGTLNVALTAPPSGVYSSLLNSTHADSVVEGYFNES LLATDKKIRPKAYIASWKDIEPAKKIEFKIKKGIKWHDGN ELKIDDWIYSIEVLANKDYEGAYYPSVENIQGAKDYHEG KTDHISGLKKIDDYTMQVTFDKKQENYLTGFITGPLLSK KYLSDVPIKDLAKSDKIRKYPIGIGPYKVKKIVPGEAVQL VKFDDYWQGKPALDKINLKVIDQAQIIKAMEKGDIDVA NDATGAMAKDAKSSNAGLKVLSAPSLDYGLIGFVSHDY DKKANKTGKVRPKYEDKELRKAMLYAIDREKWIKAFFN GYASEINSFVPSMHWIAANPKDLNDYKYDPEKAKKILD KLGYKDRDGDGFREDPKGNKFEINFKHYSGSNPTFEPRT AAIKDFWEKVGLKTNVKLVEFGKYNEDLANASKDMEV YFRSWAGGTDPDPSDLYHTDRPQNEMRTVLPKSDQYLD DALDFEKVGIDEKKRKDIYVKWQKYMNDELPGLPMFQ GKSITIVNDKVRNLDIEIGTDQSLYNLTKEA	20
Plc	SAUSA300_0099	WP_000710576.1	MKKCIKTLFLSIILVVMSGWYHSAHASDSLSKSPENWMS KLDDGKHLTEINIPGSHDSGSFTLKDPVKSVWAKTQDKD YLTQMKSGVRFFDIRGRASADNMISVHHGMVYLHHELG KFLDDAKYYLSAYPNETIVMSMKKDYDSDSKVTKTFEEI FREYYYNNPQYQNLFYTGSNANPTLKETKGKIVLFNRM GGTYIKSGYGADTSGIQWADNATFETKINNGSLNLKVQ DEYKDYYDKKVEAVKNLLAKAKTDSNKDNVYVNFLSV ASGGSAFNSTYNYASHINPEIAKTIKANGKARTGWLIVD YAGYTWPGYDDIVSEIIDSNK	27
PstS	SAUSA300_1283	WP_000759232.1	MKKWQFVGTTALGATLLLGACGGGNGGSGNSDLKGEA KGDGSSTVAPIVEKLNEKWAQDHSDAKISAGQAGTGAG FQKFIAGDIDFADASRPIKDEEKQKLQDKNIKYKEFKIAQ DGVTVAVNKENDFVDELDKQQLKAIYSGKAKTWKDVN	0

Name	Uniprot locus	NCBI locus	Protein sequence	Identical*
			SKWPDKKINAVSPNSSHGTYDFFENEVMNKEDIKAEKN ADTNAIVSSVTKNKEGIGYFGYNFYVQNKDKLKEVKIK DENGKATEPTKKTIQDNSYALSRPLFIYVNEKALKDNKV MSEFIKFVLEDKGKAAEEAGYVAAPEKTYKSQLDDLKA FIDKNQKSDDKKSDDKKSEDKK	
SirA	SAUSA300_0117	WP_001045111.1	MNKVIKMLVVTLAFLLVLAGCSGNSNKQSSDNKDKETT SIKHAMGTTEIKGKPKRVVTLYQGATDVAVSLGVKPVG AVESWTQKPKFEYIKNDLKDTKIVGQEPAPNLEEISKLKP DLIVASKVRNEKVYDQLSKIAPTVSTDTVFKFKDTTKLM GKALGKEKEAEDLLKKYDDKVAAFQKDAKAKYKDAW PLKASVVNFRADHTRIYAGGYAGEILNDLGFKRNKDLQ KQVDNGKDIIQLTSKESIPLMNADHIFVVKSDPNAKDAA LVKKTESEWTSSKEWKNLDAVKNNQVSDDLDEITWNL AGGYKSSLKLIDDLYEKLNIEKQSK	0
SstD	SAUSA300_0721	WP_000754443.1	MKKTVLYLVLAVMFLLAACGNNSDKEQSKSETKGSKD TVKIENNYKMRGEKKDGSDAKKVKETVEVPKNPKNAV VLDYGALDVMKEMGLSDKVKALPKGEGGKSLPNFLESF KDDKYTNVGNLKEVNFDKIAATKPEVIFISGRTANQKNL DEFKKAAPKAKIVYVGADEKNLIGSMKQNTENIGKIYDK EDKAKELNKDLDNKIASMKDKTKNFNKTVMYLLVNEG ELSTFGPKGRFGGLVYDTLGFNAVDKKVSNSNHGQNVS NEYVNKENPDVILAMDRGQAISGKSTAKQALNNPVLKN VKAIKEDKVYNLDPKLWYFAAGSTTTTIKQIEELDKVVK	0

*maximum percent identical to human proteins (NCBI database)

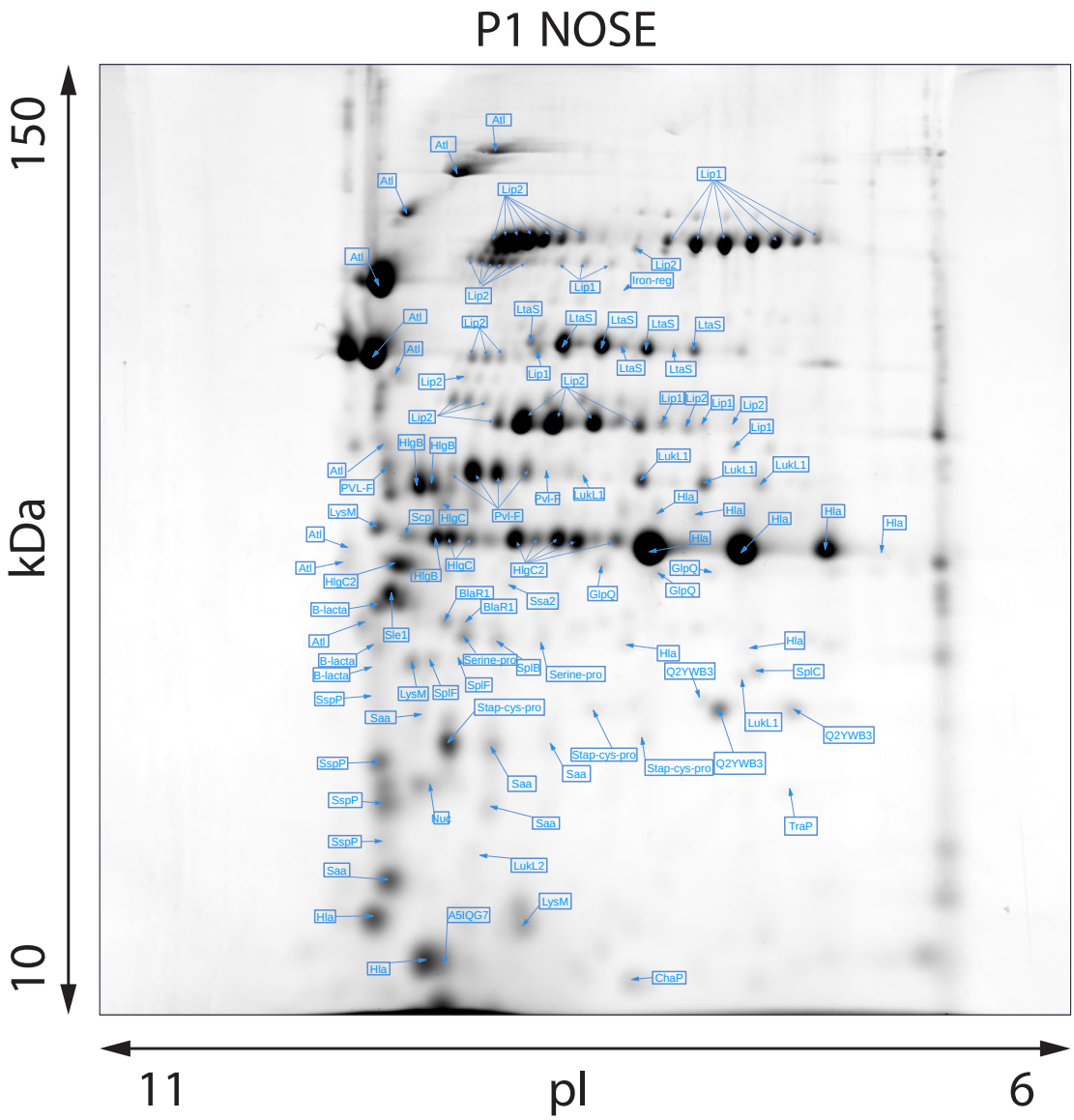

Figure 16. Protein identification of the S. aureus P1 NOSE strain.

The extracellular proteins of *S. aureus* P1 NOSE were extracted at the stationary growth phase, then separated on 11-cm 2D SDS PAGE with pI in a range of 6–11. The gels were stained using Flamingo (Bio-Rad, Germany). In-gel proteins were cut using ExQuest Spot Cutter (Bio-Rad, Germany) and identified by the Proteome-Analyser 4800 (AB Sciex, USA). GPS explorer v3.6 (AB Sciex) and the MASCOT search engine v2.2.02 (Matrix Science, London, England) were used to search the obtained peak lists against a database of protein sequences derived from the genome sequences of the all *S. aureus* strains available in the Uniprot database.

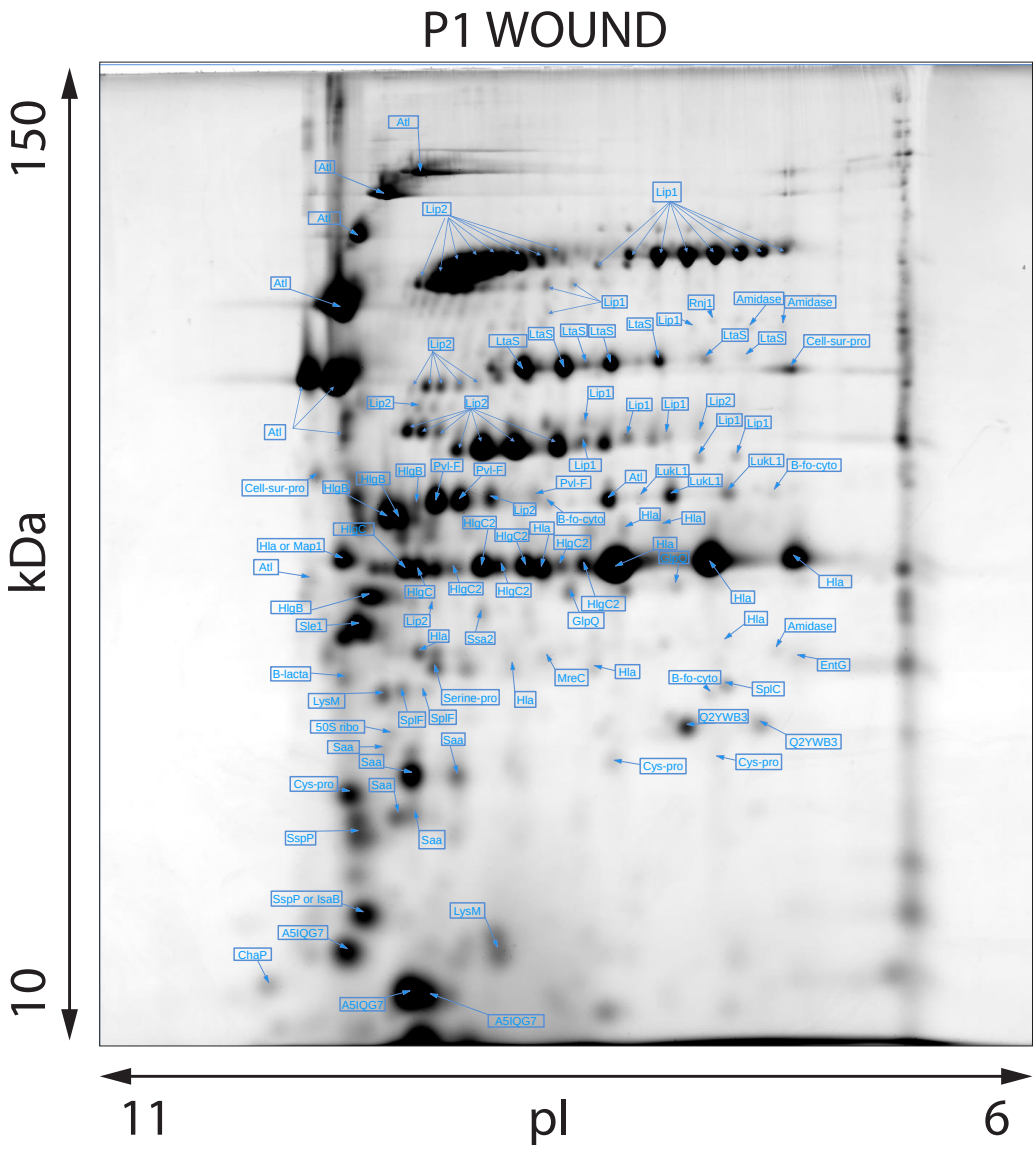

Figure 17. Protein identification of S. aureus P1 WOUND strain.

The extracellular proteins of *S. aureus* P1 WOUND were extracted at the stationary growth phase, then separated on 11-cm 2D SDS PAGE with pI in a range of 6–11. The gels were stained using Flamingo (Bio-Rad, Germany). In-gel proteins were cut using ExQuest Spot Cutter (Bio-Rad, Germany) and identified by the Proteome-Analyser 4800 (AB Sciex, USA). GPS explorer v3.6 (AB Sciex) and the MASCOT search engine v2.2.02 (Matrix Science, London, England) were used to search the obtained peak lists against a database of protein sequences derived from the genome sequences of the all *S. aureus* strains available in the Uniprot database.

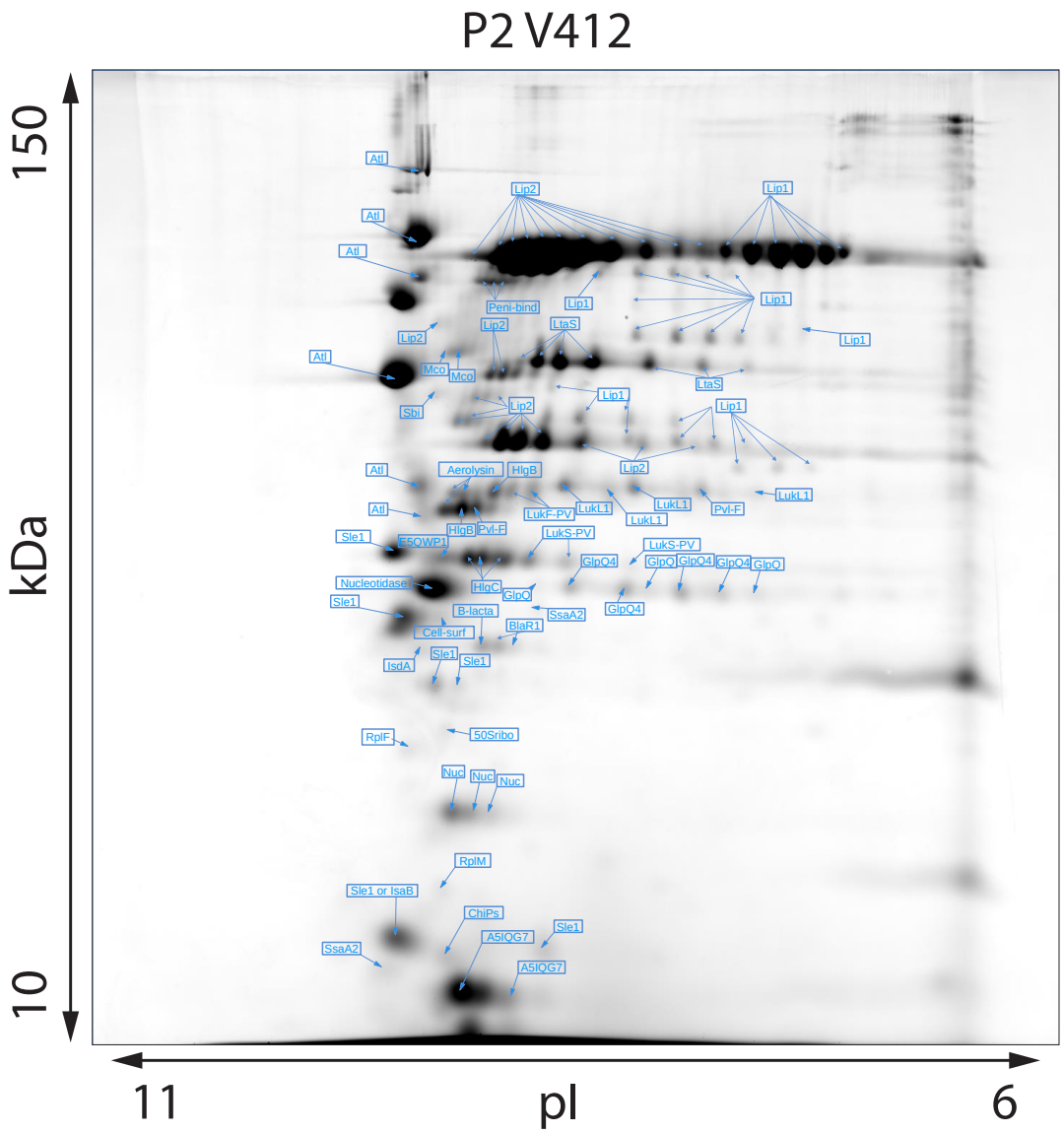

Figure 18. Protein identification of S. aureus P2 V412 strain.

The extracellular proteins of *S. aureus* P2 V412 were extracted at the stationary growth phase, then separated on 11-cm 2D SDS PAGE with pI in a range of 6–11. The gels were stained using Flamingo (Bio-Rad, Germany). In-gel proteins were cut using ExQuest Spot Cutter (Bio-Rad, Germany) and identified by the Proteome-Analyser 4800 (AB Sciex, USA). GPS explorer v3.6 (AB Sciex) and the MASCOT search engine v2.2.02 (Matrix Science, London, England) were used to search the obtained peak lists against a database of protein sequences derived from the genome sequences of the all *S. aureus* strains available in the Uniprot database.

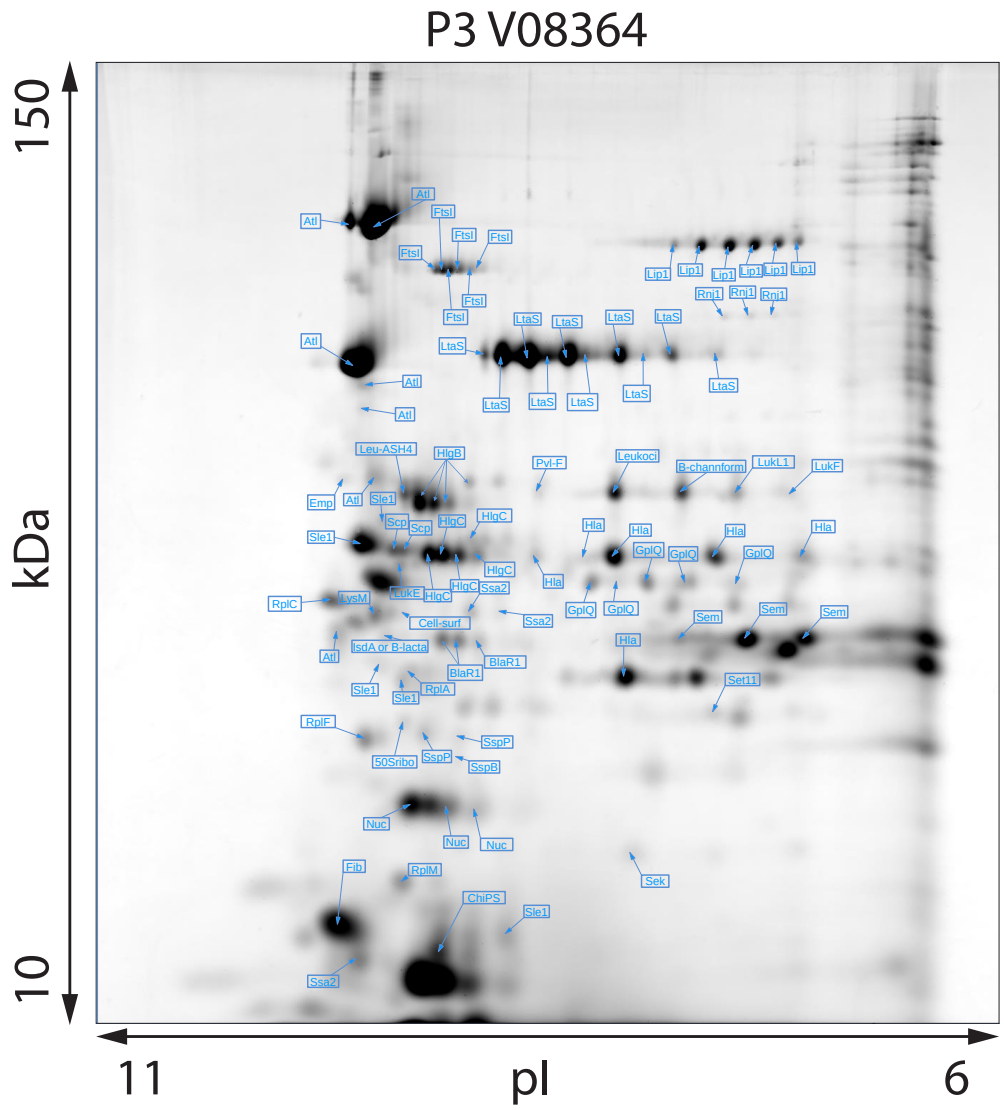

Figure 19. Protein identification of S. aureus P3 V08364 strain.

The extracellular proteins of *S. aureus* P3 V08364 were extracted at the stationary growth phase, then separated on 11-cm 2D SDS PAGE with pI in a range of 6–11. The gels were stained using Flamingo (Bio-Rad, Germany). In-gel proteins were cut using ExQuest Spot Cutter (Bio-Rad, Germany) and identified by the Proteome-Analyser 4800 (AB Sciex, USA). GPS explorer v3.6 (AB Sciex) and the MASCOT search engine v2.2.02 (Matrix Science, London, England) were used to search the obtained peak lists against a database of protein sequences derived from the genome sequences of the all *S. aureus* strains available in the Uniprot database.

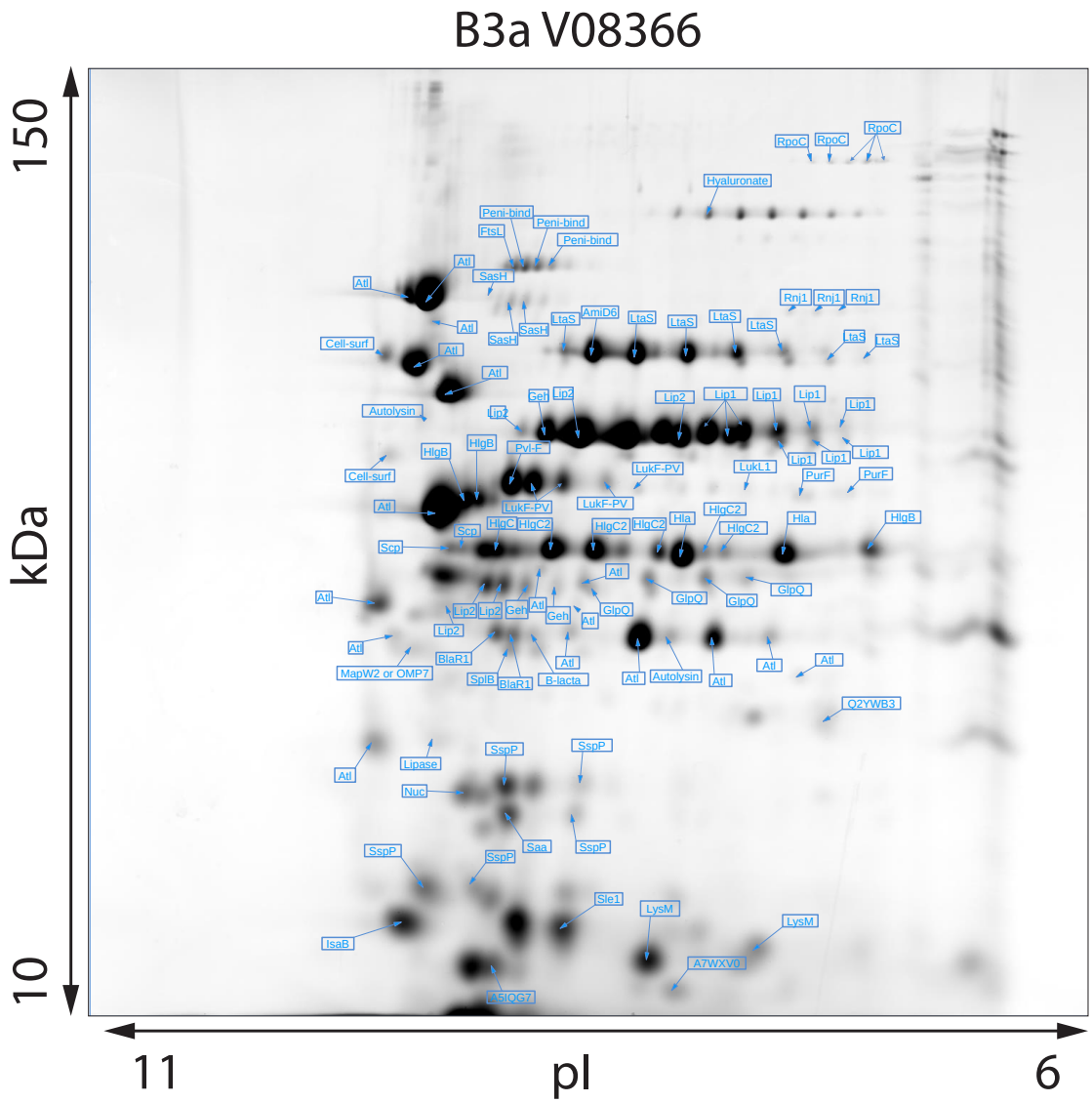

Figure 20. Protein identification of S. aureus B3a V08366 strain.

The extracellular proteins of *S. aureus* B3a V08366 were extracted at the stationary growth phase, then separated on 11-cm 2D SDS PAGE with pI in a range of 6–11. The gels were stained using Flamingo (Bio-Rad, Germany). In-gel proteins were cut using ExQuest Spot Cutter (Bio-Rad, Germany) and identified by the Proteome-Analyser 4800 (AB Sciex, USA). GPS explorer v3.6 (AB Sciex) and the MASCOT search engine v2.2.02 (Matrix Science, London, England) were used to search the obtained peak lists against a database of protein sequences derived from the genome sequences of the all *S. aureus* strains available in the Uniprot database.

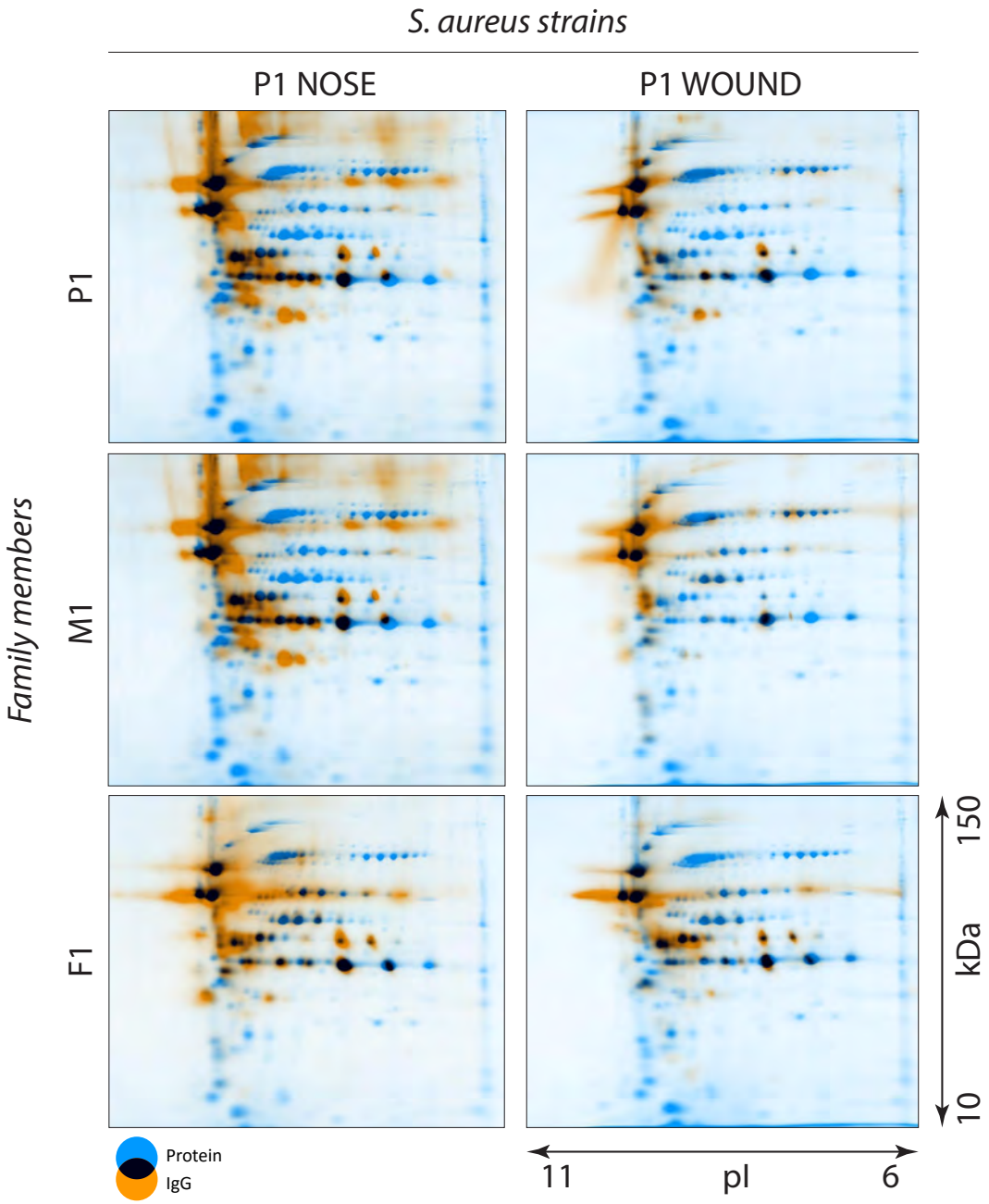

Figure 21. Superimposed protein and IgG binding patterns in family 1.

S. aureus extracellular proteins were separated on 2D gels. The superimposed images were created by software Delta2D version 4.4. Serum from each family 1 members were used as total IgG for staining immunoblots. Superimpositions of the 2D immunoblot and the gel are shown; blue: proteins from *S. aureus*, orange: IgG-bound proteins. IgG binding to *S. aureus* PVL toxin subunits was observed (see table 20).

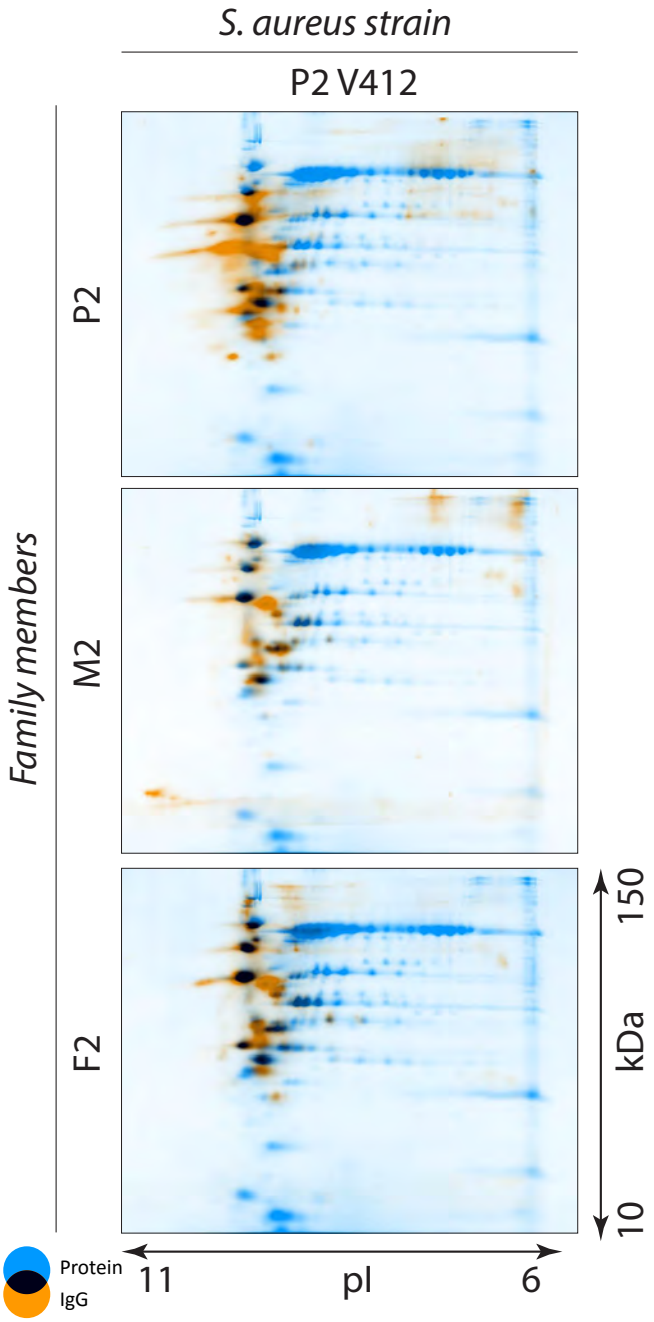

Figure 22. Superimposed protein and IgG binding patterns from family 2.

S. aureus extracellular proteins were separated on 2D gels. The superimposed images were created by software Delta2D version 4.4. Serum from all family 2 members were used as total IgG for staining immunoblots. Superimpositions of the 2D immunoblot and the gel are shown; blue: proteins from *S. aureus*, orange: IgG-bound proteins. IgG binding to *S. aureus* PVL toxin subunits was observed (see table 20).

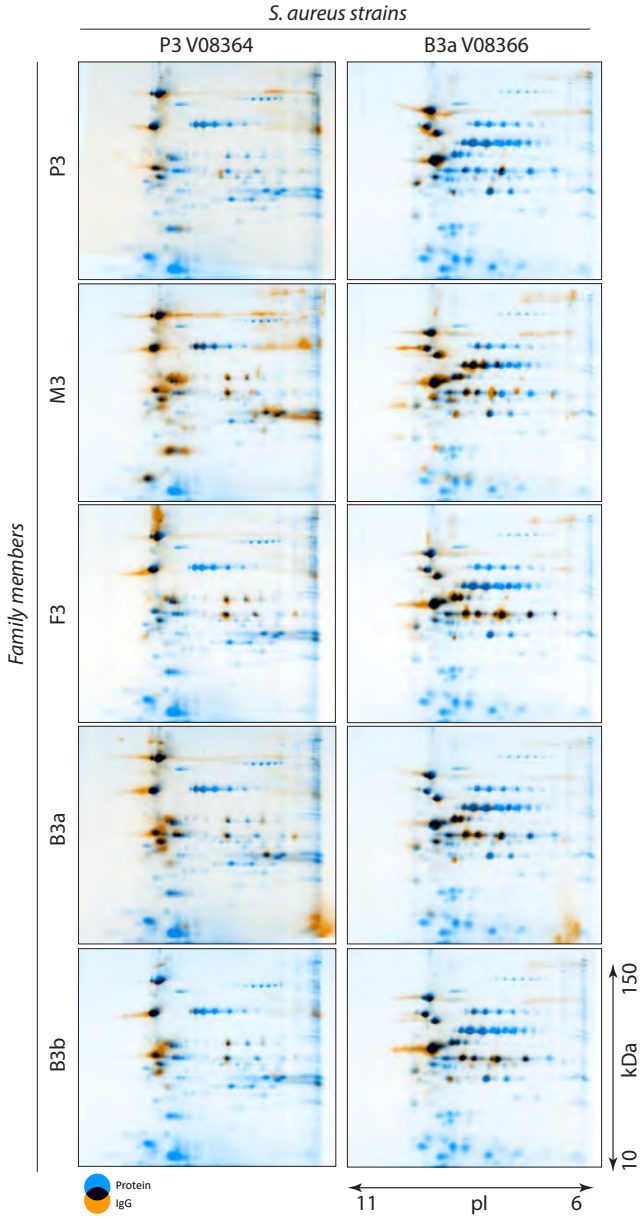

Figure 23. Superimposed protein and IgG binding patterns from family 3.

S. aureus extracellular proteins were separated on 2D gels. The superimposed images were created by software Delta2D version 4.4. Sera from each family 3 members were used as total IgG for staining immunoblots. Superimpositions of the 2D immunoblot and the gel are shown; blue: proteins from *S. aureus*, orange: IgG-bound proteins. IgG binding to *S. aureus* PVL toxin subunits was observed (see table 20).

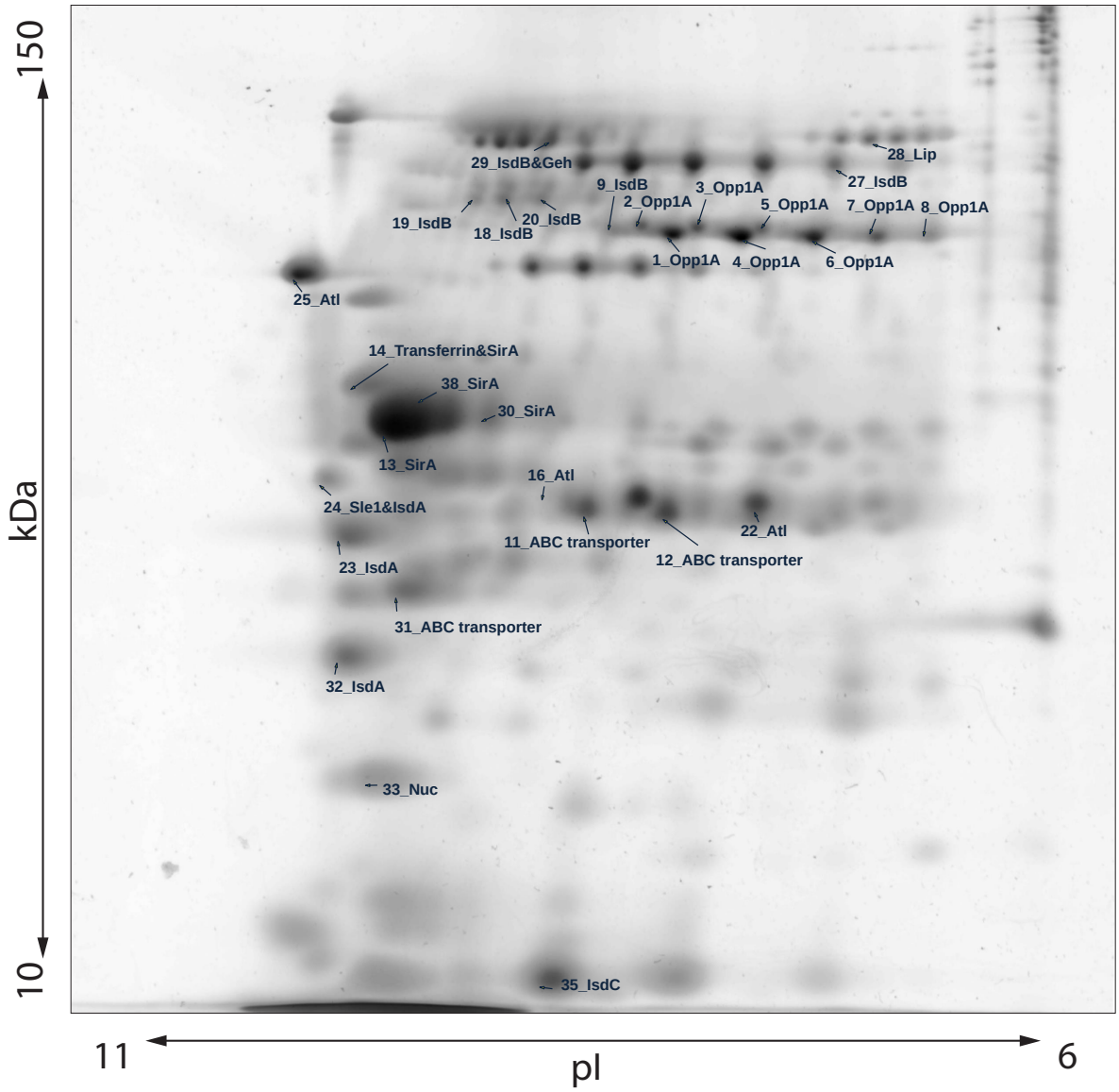

Figure 24. Protein identification of S. aureus RN4220 strain.

Extracellular proteins (100ng) of RN4220 Δ*lgt* strain were separated on Immobiline Dry Strips (GE Healthcare, Germany) in the pI range of 6–11. The second dimension separation was run on SDS-PAGE. Flamingo fluorescent gel stain (Bio-Rad, Germany) was used as described in the manufacturer's instructions. Spots were cut out and prepared for in-gel trypsin digestion. Mass spectrometry analysis of the gel-digested solutions was conducted on a Proteome-Analyser 4800 (AB Sciex, USA) (see table 22).

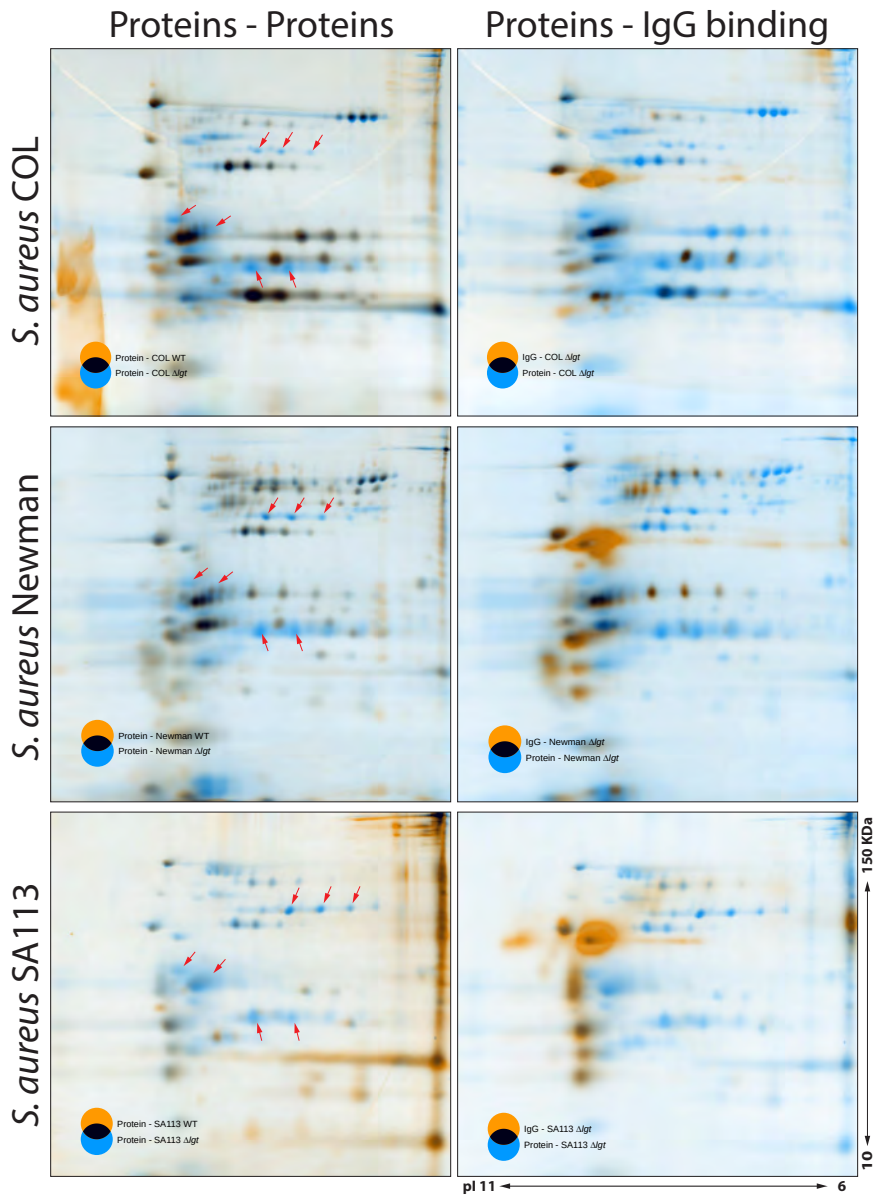

Figure 25. Expression of lipoproteins and no IgG binding to them.

Extracellular proteins from the three *S. aureus* strains COL, Newman, and SA113 as well as their isogenic *lgt* mutations were extracted and then separated in the pI range of 6–11. After a second dimension run on SDS-PAGE, the gels were analyzed using the software Delta2D v4.4. Blue spots on superimposed images indicate presumptive lipoproteins. A human serum pool was used for immunoblot analysis. No IgG binding to the presumptive lipoproteins was observed on superimposed created from 2D gels and immunoblots of the *S. aureus* Δ*lgt* strains. Each experiment was carried out in duplicate.

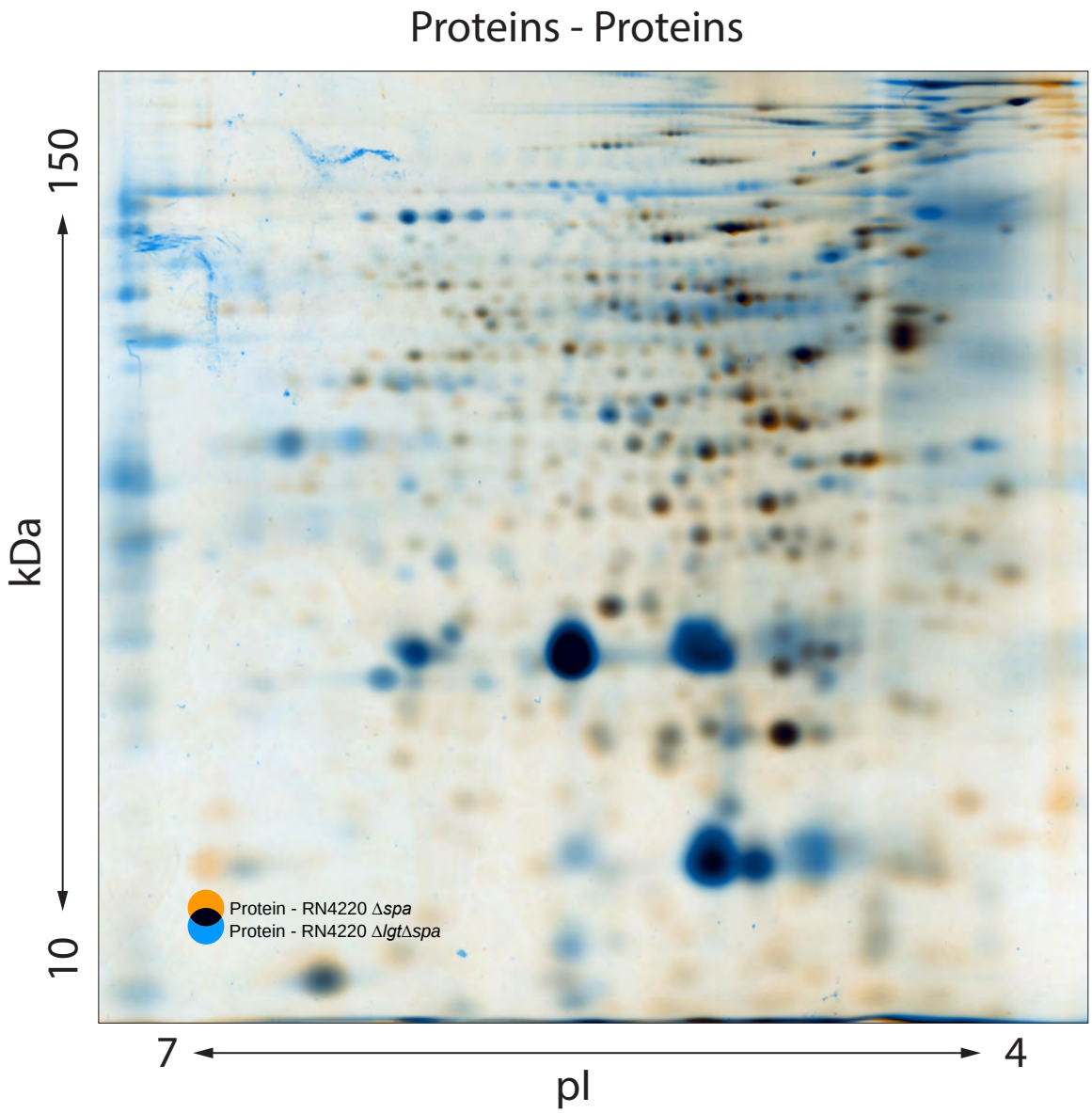

Figure 26. No lipoprotein found in an acidic range.

To conduct the experiment at pI 4–7, a *S. aureus* protein A mutant strain (Δ*spa*) was generated from the RN4220 Δ*lgt* strain by phage transduction. Then extracellular proteins of the RN4220 Δ*lgt* and RN4220 Δ*spa*Δ*lgt* strains were extracted and separated by IEF at pI 4–7. Subsequently, the second dimension superimposed images of gels were created using the software Delta2D v4.4. No lipoprotein was identified. Experiments were repeated twice.

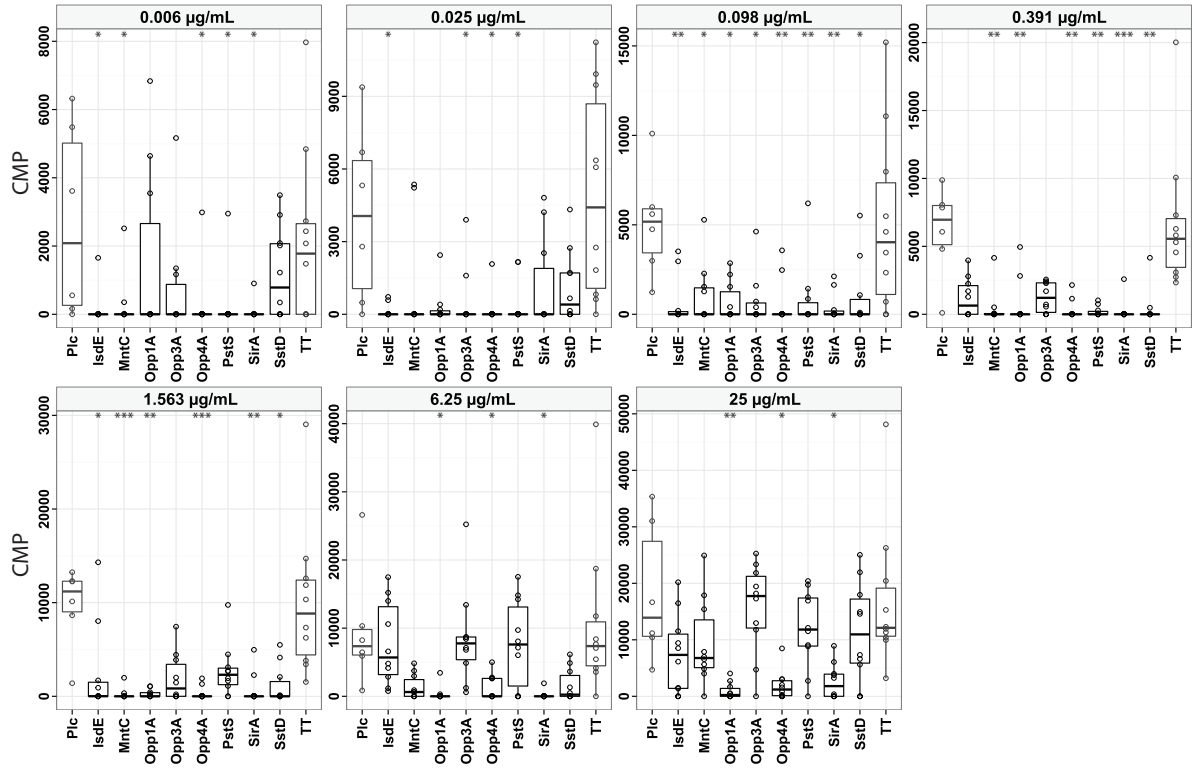

Figure 27. T cell proliferation at concentrations of antigens.

Plc was used as positive control in the proliferation assay, and provoked BPMCs even at low concentrations. At a concentration of 6.25 µg/mL, the three proteins IsdE, Opp3A and PstS stimulated PBMCs early on. Kruskal-Wallis tests were conducted to calculate p-values. Asterisks at the top of the panels depict significant differences to Plc responses as follows *, $p<0.05$; **, $p<0.01$; $p>0.05$ data not shown.

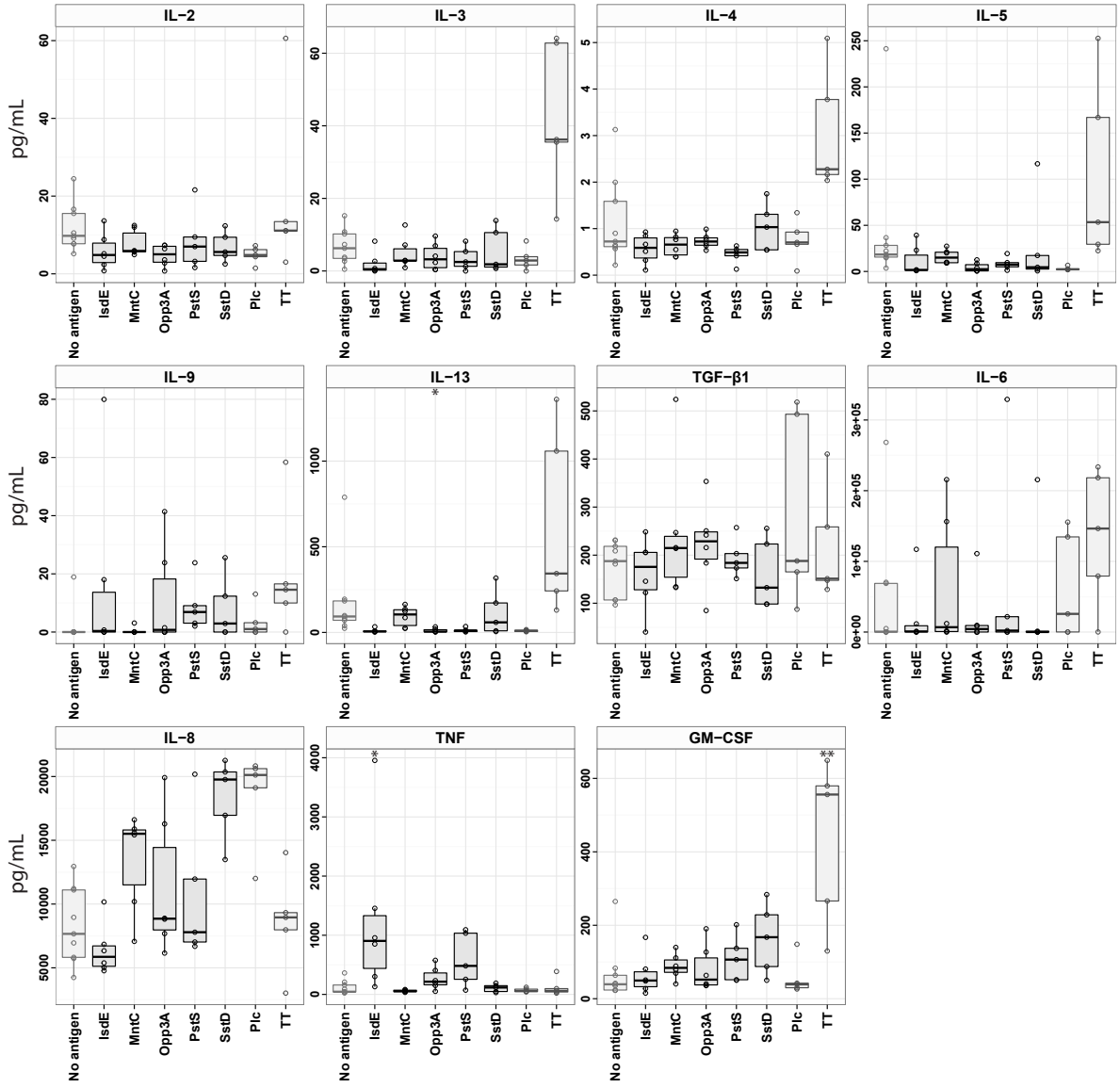

Figure 28. The secretion of other cytokines.

PBMCs isolated from fresh blood were used for proliferation assays. Supernatants were collected at day 7 of experiments and used for cytokine measurements. CBA flex kits were used to measure the concentrations of cytokines. Each experiment was repeated in triplicate. A box-and-whisker plot visualized median, upper quartile, lower quartile, maximum, minimum and outliers for each dataset. Kruskal-Wallis tests were conducted to calculate p-values. Asterisks at the top of each panel correspond to significant differences to non-antigen (No Ag) control as follows *, $p<0.05$; **, $p<0.01$; $p>0.05$ data not shown.

List of tables

List of figures

Eigenständigkeitserklärung

Hiermit erkläre ich, dass diese Arbeit bisher von mir weder an der Mathematisch-Naturwissenschaftlichen Fakultät der Ernst-Moritz-Arndt-Universität Greifswald noch an einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion eingereicht wurde.

Ferner erkläre ich, dass ich diese Arbeit selbstständig verfasst und keine anderen als die darin angegebenen Hilfsmittel und Hilfen benutzt und keine Textabschnitte eines Dritten ohne Kennzeichnung übernommen habe.

Greifswald, den 17/12/2015

Vu Hai Chi

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Curriculum vitae

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Education

2011–Present	University of Greifswald, Germany	PhD candidate, Host-Pathogen Interaction and Immunology
2008–2011	Institute of Biotechnology, Vietnam & University of Greifswald, Germany	Diploma Equivalent, Molecular Biology and Genomics
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Awards

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Research experience

2011–Present	Department of Immunology, Institute of Immunology and Transfusion Medicine, University Medicine Greifswald, Germany Project: Investigate human adaptive immune response on virulence factors of *Staphylococcus aureus* using an immunoproteomic approach. Head: Prof. Dr. Barbara M. BRÖKER
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Research interest

Host-pathogen interaction—infection immunology
Evolution of host-pathogen relationship

Publications

Chi Hai Vu, Julia Kolata, Sebastian Stentzel, Manuela G. Salazar, Leif Steil, Susanne Engelmann, Michael Hecker, Uwe Völker, Barbara M. Bröker (2015) *Human adaptive immune response to membrane-bound lipoproteins of S. aureus* (in preparation).

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Conference

August 2015	"*Human adaptive immune response to S. aureus lipoproteins*". Meeting of PhD students in SFB 34, Wuerzburg, Germany (Oral presentation).
September 2014	"*Adaptive immune response to membrane-bound lipoproteins of S. aureus*". 44rd Annual Meeting of the German Society for Immunology, Bonn, Germany (Poster).
October 2012	"Protective anbibodies in furunculosis?". 4th Autumn School of German Society for Immunology, Bad Schandau, Germany (Oral presentaion).
April 2012	"*Human IgG response to Staphylococcus aureus-induced furunculosis*". Joint meeting of GRK 840 and TRR 34, Vilm, Germany (Poster).