Improving diagnosis and treatment of *Staphylococcus aureus* infections

Experimental studies

Sanne van den Berg

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Improving Diagnosis and Treatment of *Staphylococcus aureus* Infections

Experimental Studies

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Staphylococcus aureus infecties

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Overige leden:	Prof.dr. J.D. Laman
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Copromotor:	Dr. I.A.J.M. Bakker-Woudenberg

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1

General introduction, aim and outline of the thesis

Introduction

Staphylococcus aureus is an opportunistic pathogen that causes a variety of infections, ranging from mild skin infections like furuncles and impetigo, to severe, life-threatening infections including endocarditis, osteomyelitis and pneumonia.[1] Invasive infections are frequently associated with *S. aureus* bacteremia.[2] Despite antibiotic treatment, the infection-related mortality rate in patients with *S. aureus* bacteremia is still 17%.[3]

In addition to being a pathogen, *S. aureus* can also asymptomatically colonize the skin and mucosa of humans.[1] The nose is the primary reservoir for *S. aureus*,[4] which is persistently colonized in 20-30% of the healthy population.[5-7] Also extra-nasal sites may be colonized by *S. aureus*, including hands (27%), perineum (22%), forearm (20%), pharynx (10-20%), skin of the chest (15%), skin of the abdomen (15%), neck (10%), ankle (10%), axilla (8%), and vagina (5%).[4] Persistent nasal carriage of *S. aureus* is generally assumed to be a risk factor for the development of *S. aureus* infection,[5,8,9] including a three-fold higher risk of acquiring *S. aureus* bacteremia. However, *S. aureus* bacteremia-related mortality appeared significantly lower in nasal *S. aureus* carriers than in non-carriers.[8] **Further studies on nasal carriage of *S. aureus* in relation to mortality from invasive *S. aureus* infections are needed.**

The human nasal inoculation model[10-14] is an ideal way to study nasal colonization of *S. aureus* in humans. While studies involving this artificial inoculation model are very informative, animal models may provide a more detailed investigation by allowing for more risky interventions under highly controlled conditions in a more homogenous host population. Mice are often used as experimental animals in biomedical research, but these are not natural nasal *S. aureus* carriers.[15] To establish nasal colonization in mice without using very high *S. aureus* inocula, antibiotic pre-treatment is required which reduces the resistance to colonization exerted by the local resident microbiota.[16] In addition, the evolutionary relatedness between mice and humans is rather weak. **A well-defined human-like *S. aureus* model in experimental animals more closely related to humans is essential, but currently lacking.**

Several studies have shown that in adult people, irrespective of their nasal *S. aureus* carriage state at the time of sampling, a broad range of anti-staphylococcal antibodies is present in the circulation.[17-19] A role for certain anti-staphylococcal antibodies in

the protection against *S. aureus* bacteremia-related death is suggested by a number of studies. In carriers, *S. aureus* bacteremia usually has an endogenous origin.[4,20] Because of exposure to *S. aureus*, carriers may have developed anti-staphylococcal antibodies that are protective against bacteremia-related mortality. In a prospective study in patients, nasal *S. aureus* carriage state was assessed on admission to the hospital, and a serum sample was collected. When patients developed *S. aureus* bacteremia, the invasive *S. aureus* strain was stored. Nasal *S. aureus* carriers who developed endogenous *S. aureus* bacteremia had a stronger and broader pre-bacteremia immunoglobulin G (IgG) response to their own invasive, endogenous *S. aureus* strain compared to bacteremic non-carriers, who are confronted with an exogenous *S. aureus* strain.[21] Other clinical studies have shown that, compared to non-carriers, nasal *S. aureus* carriers have significantly elevated IgG levels against toxic shock syndrome toxin 1 (TSST-1),[19] staphylococcal enterotoxin A (SEA)[19] and factor effecting methicillin resistance (FmtB).[17] These higher levels of neutralizing anti-staphylococcal antibodies in carriers suggest a role of specified anti-staphylococcal antibodies in lowering the risk of bacteremia-related mortality.

In spite of these observations, both carriers and non-carriers of *S. aureus*, harboring a broad range of anti-staphylococcal antibodies in their circulation,[17-19] can become infected by *S. aureus*, which argues against a role for anti-staphylococcal antibodies in the protection against *S. aureus* infection. In addition, clinical studies concerning active or passive immunization to prevent, to reduce or to cure *S. aureus* infection have, so far, not shown protection.[22-24] The role of anti-staphylococcal antibodies in protection against *S. aureus* bacteremia-related death is, thus, not yet fully understood. Our knowledge of what components of the immune system are important in prevention, reduction or cure of *S. aureus* infection is deficient. **Regarding antigen-specific anti-staphylococcal antibodies, it remains to be further investigated which at the onset of *S. aureus* infection may contribute to a favorable outcome of *S. aureus* infection.** These studies are difficult to perform in humans, as both carriers and non-carriers harbor complex mixtures of anti-staphylococcal antibodies.[17-19] In an experimental model, non-human primates may be very suitable for these types of studies because of their relatedness to humans. However, the use of non-human primates in experimental research encounters ethical dilemmas. Mostly, rodents are used in biomedical research. In addition, mice that are free of *S. aureus* and anti-staphylococcal antibodies are available and appropriate to study the role of specific anti-*S. aureus* immunoglobulins in protection against *S. aureus* bacteremia-related

death. **To enable these studies in mice, techniques that are able to simultaneously assess a broad range of anti-staphylococcal antibodies in small-volume serum samples[25] need to be optimized for mouse serum.**

In general, rapid diagnosis of infection is essential, particularly in severe infections, as delays in treatment may lead to poor outcomes. Diagnosis of *S. aureus* infection is based on the medical history, clinical signs and symptoms, cultures from the site of suspected infection, and the measurement of host response factors to infection including C-reactive protein (CRP) and other markers of inflammation. In addition to rapid diagnosis, insight into the prognosis of *S. aureus* infection, i.e. predicting whether a complicated or a non-complicated course of disease will develop, is important to further support clinical decisions on antibiotic and additional treatments. **To this aim, there is a need for reliable biomarkers predicting the outcome of *S. aureus* infection.** While CRP is useful as a marker for the presence of infection, by itself it is not a predictor of infection outcome.[26,27]

Treatment of infections caused by methicillin-susceptible *S. aureus* (MSSA) or methicillin-resistant *S. aureus* (MRSA) strains needs improvement as treatment failure of severe *S. aureus* infection is illustrated by the high mortality rates.[3,28-30] In the pre-antibiotic era, mortality rates of *S. aureus* bacteremia were shockingly high, reaching 83%.[31] The introduction of antibiotics in the 1940s and 1950s resulted in much better outcomes.[32] Traditionally, *S. aureus* infections were treated with penicillins, including methicillin. After the introduction of the antibiotic methicillin in 1959, within two years the first methicillin-resistant *S. aureus* (MRSA) strains were described.[33] Unfortunately, MRSA infections are now prevalent within many hospitals worldwide, and are associated with increased mortality and length-of-stay for patients.[34-36] Recent years have also witnessed a spread of antibiotic-resistant staphylococci outside the health care setting and, alarmingly, today community-acquired MRSA is a well-known entity.[37] Vancomycin treatment is one of the few therapeutic options available for patients with MRSA infection. Unfortunately, in 2002 the first vancomycin-resistant *S. aureus* (VRSA) isolates were identified in the United States,[38] and in 2013 the first patient with a VRSA infection manifested in Portugal.[39] These are worrying developments in view of the lean pipeline for novel therapeutics[40] to treat infections with antibiotic-resistant *S. aureus* strains. **Therefore, there is an urgent need to develop novel, non-**

antibiotic-based anti-staphylococcal therapies to enhance, or even replace, current antibiotic treatment.

Active or passive immunization as prophylactic treatment of patients at risk for *S. aureus* infections may be such a non-antibiotic-based treatment strategy, as a role of anti-staphylococcal antibodies in protection against *S. aureus* bacteremia-related death is suggested by a number of clinical studies. In this respect, studies have been performed in animal models of *S. aureus* infection, in patients at risk for *S. aureus* infection, and in *S. aureus*-infected patients.[22-24] While this novel therapeutic approach appeared to be successful in most animal models, this was never shown to be successful in protection against or cure of *S. aureus* infections in patients.

The lack of efficacy of active and passive immunization in patients at risk for *S. aureus* infection and in *S. aureus*-infected patients may be related to the heterogeneity of *S. aureus* strains causing infections.[41,42] **Focus on conserved *S. aureus* antigens that are expressed by the vast majority of *S. aureus* strains,[42] preferably in an immunization strategy targeting multiple *S. aureus* antigens simultaneously, may be important to increase the therapeutic effectiveness of antimicrobial interventions.**

Aim and outline of the thesis

The general aim of the research described in this thesis is to increase the knowledge on the improvement of diagnosis and treatment of *S. aureus* infections. This research included experimental studies *in vitro*, *ex vivo*, and *in vivo*, which may serve as a base for clinical studies.

To gain insight into the role of nasal *S. aureus* carriage in the acquisition and the course of *S. aureus* infections, a human-like model in experimental animals is essential, but currently lacking. In **chapter 2**, we investigate whether rhesus macaques are nasal carriers of *S. aureus*, and as such could provide a model of human nasal *S. aureus* carriage.

The role of nasal *S. aureus* carriage and certain anti-staphylococcal antibodies in protection against *S. aureus* bacteremia-related death, as suggested by a number of studies, is not yet fully understood. Mice that are free of *S. aureus* and anti-staphylococcal antibodies are pre-eminently appropriate to further study the role of antibodies

in this respect. In **chapter 3**, we optimize the bead-based flow cytometry technique already available for the assessment of a broad anti-staphylococcal antibody profile in small-volume human serum samples for use in mice. Using this mouse-adapted multiplex assay, we investigate the anti-staphylococcal antibody profiles for various *S. aureus* infections in mice.

In **chapter 4**, we study the role of mild *S. aureus* skin infection on the course of subsequent endogenous or exogenous *S. aureus* bacteremia in mice, and evaluate levels of protection in relation to anti-staphylococcal antibody levels following skin infection.

To further support clinical decisions on antibiotic treatment, there is a need for reliable biomarkers supporting diagnosis of *S. aureus* infection and providing insight into the prognosis of the infection. In **chapter 5**, we assess the role of selected cytokines as biomarkers for the presence and the prognosis of severe *S. aureus* bacteremia in mice.

Treatment of MSSA or MRSA infections needs improvement, which is illustrated by the high mortality rates.

In **chapter 6**, we review the studies on active and passive immunization in animal models of *S. aureus* infection, in patients at risk for *S. aureus* infection, and in *S. aureus*-infected patients.

In **chapter 7**, we investigate the efficacy of a human monoclonal antibody against the immunodominant staphylococcal antigen A (IsaA), a conserved *S. aureus* antigen, in mice with bacteremia caused by either a MSSA or a MRSA strain.

In **chapter 8**, we investigate the protective effect of active immunization with an octa-valent *S. aureus* antigen mixture on the course and outcome of *S. aureus* bacteremia and *S. aureus* skin infection in mice.

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2

Rhesus macaques (*Macaca mulatta*) are natural hosts of specific *Staphylococcus aureus* lineages

Sanne van den Berg, Willem J.B. van Wamel, Susan V. Snijders, Boudewijn Ouwerling, Corné P. de Vogel, Hélène A. Boelens, Rob J.L. Willems, Xander W. Huijsdens, Frank A.W. Verreck, Ivanela Kondova, Peter J. Heidt, Henri A. Verbrugh, Alex van Belkum

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Abstract

Currently, there is no animal model known that mimics natural nasal colonization by *Staphylococcus aureus* in humans. We investigated whether rhesus macaques are natural nasal carriers of *S. aureus*. Nasal swabs were taken from 731 macaques. *S. aureus* isolates were typed by pulsed-field gel electrophoresis (PFGE), *spa* repeat sequencing and multi-locus sequence typing (MLST), and compared with human strains. Furthermore, the isolates were characterized by several PCRs. Thirty-nine percent of 731 macaques were positive for *S. aureus*. In general, the macaque *S. aureus* isolates differed from human strains as they formed separate PFGE clusters, 50% of the isolates were untypeable by *agr* genotyping, 17 new *spa* types were identified, which all belonged to new sequence types (STs). Furthermore, 66% of macaque isolates were negative for all superantigen genes. To determine *S. aureus* nasal colonization, three nasal swabs from 48 duo-housed macaques were taken during a 5 month period. In addition, sera were analyzed for immunoglobulin G and A levels directed against 40 staphylococcal proteins using a bead-based flow cytometry technique. Nineteen percent of the animals were negative for *S. aureus*, and 17% were three times positive. *S. aureus* strains were easily exchanged between macaques. The antibody response was less pronounced in macaques compared to humans, and nasal carrier status was not associated with differences in serum anti-staphylococcal antibody levels. In conclusion, rhesus macaques are natural hosts of *S. aureus*, carrying host-specific lineages. Our data indicate that rhesus macaques are useful as an autologous model for studying *S. aureus* nasal colonization and infection prevention.

Introduction

In the light of the rapid, worldwide emergence of antibiotic resistance in and the lack of an effective long-term elimination strategy against *Staphylococcus aureus* nasal carriage, new approaches are needed to prevent staphylococcal carriage and its consequent diseases. Many different *S. aureus* animal models have been described for studying the pathogenesis of *S. aureus* colonization and infection. These models have provided insight into the role of bacterial virulence genes and have assisted in the estimation of vaccine efficacy. Models have been set up in various species, such as insects, worms, mice, rats, guinea pigs, hamsters, chickens, rabbits, sheep, dogs, pigs, and cows.[1-4] Notably, most of these animals, unlike humans, are not natural nasal carriers of *S. aureus*, only pigs, sheep, and cows may be naturally colonized by *S. aureus*. For instance, *S. aureus* sequence type (ST) 398 strains belong to a biotype associated with pigs and other species of livestock.[5,6] *S. aureus* strain RF122 is a member of a bovine mastitis-associated clone that is genetically different from human clones of *S. aureus*.[7,8] Humans can acquire these *S. aureus* strains during intensive short-term exposure to livestock, but in most cases the strain is lost again within 24 hours.[9] However, lack of a natural, human-like animal model of nasal *S. aureus* carriage is still a problem. Therefore, we investigated whether a non-human primate could provide a natural model for human nasal carriage of *S. aureus*.

The rhesus macaque (*Macaca mulatta*) belongs to the old world monkeys and has been used in several studies involving *S. aureus*. Kuklin et al*.* used rhesus macaques to study the immunogenicity of IsdB.[10] Protection against lethal SEB aerosol exposure by passive transfer of SEB-specific antibodies was also studied in macaques.[11] In addition to these protection studies, rhesus macaques were also used for safety evaluations. For example, the tolerability and potential toxicity of the thrombolytic agent staphylokinase was investigated in healthy rhesus macaques.[12] To our knowledge, natural nasal *S. aureus* carriage and the consequences on natural immunity in rhesus macaques has never been studied before.

Using a cross sectional set-up, we isolated 287 *S. aureus* strains from 731 rhesus macaques after nasal sampling. We compared *S. aureus* strains isolated from rhesus macaques and humans. Furthermore, we followed a group of 48 rhesus macaques in time for studying persistence of nasal carriage of *S. aureus*. In addition, serum samples from these macaques were analyzed for anti-staphylococcal immunoglobulin G and A (IgG and IgA) levels.

Materials and methods

Study population

For comparison of *S. aureus* strains isolated from rhesus macaques with those from humans, 731 rhesus macaques from the breeding colony of the Biomedical Primate Research Center (Rijswijk, the Netherlands) were studied. These animals were of Indian, Burmese and Chinese origin. These macaques were housed in groups of 2-44 individuals. Furthermore, 48 young rhesus macaques that were recently imported from China were followed in time for studying the persistence of *S. aureus* nasal carriage as well as their serum anti-staphylococcal antibody levels. These animals were duo-housed in 4 different animal rooms. Physical contact with the macaques in the neighboring cage was possible. In each room 2 groups of cages were located opposite to each other.

Human *S. aureus* strains

For reasons of comparison, 56 human isolates of *S. aureus* were included. These carriage (n=30) and bacteremia derived (n=20) MSSA isolates have been described before.[13,14] Three MSSA isolates from animal care-takers and 3 *S. aureus* strains for which the genome sequence is known were included as well (N315, Mu50, MRSA252).

Ethics statement

Sampling of the longitudinally screened macaques was approved by the Animal Experiments committee of the Biomedical Primate Research Center (Dierexperimentencommissie (DEC), which is the ethical committee installed and officially recognized as required by the Dutch Law on Experimental Animals and which is the Dutch analogue for the IACUC). The approval number is: DEC#579, dated October 28, 2008. The study was conducted in compliance with all relevant Dutch laws and in agreement with international and scientific standards and guidelines.

Sampling for bacteriology of the animals in the breeding groups was performed as part of their yearly routine health screening, which is by Dutch law not considered to be an animal experiment. Therefore no permission of the ethical committee was necessary for this part of the study. All samplings were performed under ketamine anesthesia (which is routine for all health checks of non-human primates) and all efforts were made to minimize stress and suffering of the animals. The housing, care and handling of all animals were according to the Dutch Law on Experimental Animals

and the European Directive 86/609/EEC. The research described is in accordance to the Weatherall Report recommendations for good welfare.

Human samples were described before.[13,14] For carriage isolates and serum samples, volunteers provided written informed consent and the local Medical Ethics Committee of the Erasmus Medical Center Rotterdam approved the study (MEC-2007-106). For bacteremia derived isolates, *S. aureus* strains were collected for routine culture. The Medical Ethics Committee of the Erasmus Medical Center Rotterdam approved the study (MEC-2007-106, addendum 2).

Sampling procedures

A total of 731 macaques were sampled once for nasal carriage of *S. aureus*, while another 48 macaques were screened three times during a 5 month period. Nasal cultures were taken by streaking both anterior nares using a sterile cotton swab (Swab Transystem, Greiner Bio One) during regular animal medical check-up. All swabs were processed within 24 hours. Nasal swabs were plated on a Columbia sheep blood agar plate-medium (bioTRADING). Plates were read after one and two days of incubation at 35°C. Identification of *S. aureus* was based on colony morphology and coagulase plasma test (Becton Dickinson) and confirmed by API Staph (bioMérieux).

Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) of *Sma*I digested chromosomal DNA from all *S. aureus* strains from rhesus macaques and 56 strains from humans was performed as described previously.[15] Relatedness among the PFGE profiles was evaluated with Bionumerics software (version 3.0; Applied Maths). A dendrogram was produced using the Dice coefficient and an unweighted-pair group method using arithmetic averages (UPGMA). Band tolerance was set at 2.0%.

Anti-staphylococcal antibodies

IgG and IgA antibody levels in serum directed against the following antigens were semi-quantified: *S. aureus* proteins clumping factor A and B (ClfA and ClfB); surface protein G (SasG); iron-responsive surface determinants A and H (IsdA and IsdH); fibronectin-binding proteins A and B (FnbpA and FnbpB); serine-aspartate dipeptide repeat protein D and E (SdrD and SdrE); staphylococcal enterotoxins A-E, G-J, M-O, Q, and R (SEA - SEE, SEG - SEJ, SEM - SEO, SEQ, SER); toxic shock syndrome toxin 1 (TSST-1); chemotaxis inhibitory protein of *S. aureus* (CHIPS); staphylococcal comple-

ment inhibitor (SCIN); extracellular fibrinogen-binding protein (Efb); exfoliative toxin A and B (ETA and ETB); alpha toxin; γ hemolysin B (HlgB); leukocidin D, E, F, S (LukD, LukE, LukF, LukS); and staphylococcal superantigen-like proteins 1, 3, 5, 9, and 11 (SSL1, SSL3, SSL5, SSL9, and SSL11). Antibodies were semi-quantified simultaneously in a single multiplex assay using a bead-based flow cytometry technique (xMap; Luminex Corporation). Methods have been described elsewhere,[14,16,17] with the exception that a 1:50 dilution of R-phycoerythrin (RPE)-conjugated AffiniPure goat anti-human secondary IgA was used. Tests were performed in independent duplicates, and the median fluorescence intensity (MFI) values, reflecting semiquantitative antibody levels, were averaged. In each experiment, control beads (no protein coupled) were included to determine nonspecific binding. In the event of nonspecific binding, the nonspecific MFI values were subtracted from the antigen-specific results.

Serum samples from 47 out of the 48 rhesus macaques from China were analyzed. Anti-staphylococcal antibody levels in these sera were compared to those in sera from 20 Dutch healthy human volunteers.[14] Pooled serum from all rhesus macaques included in the present study or from humans was used as a standard.

DNA isolation

DNA isolation of *S. aureus* was performed for representative isolates from each PFGE cluster. Total DNA of *S. aureus* was isolated with the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions, or with MagNA Pure LC DNA isolation kit III (bacteria, fungi) using the MagNA Pure LC instrument (Roche Diagnostics).[18]

spa genotyping

PCR for amplification of the *S. aureus* protein A (*spa*) repeat region was performed according to the published protocol.[19] PCR products were purified with the QIAquick PCR Purification Kit (Qiagen) and sequenced using two amplification primers from a commercial supplier (SeqLab). The forward and reverse sequence chromatograms were analyzed with the Ridom StaphType software (Ridom GmbH).

Multi-locus sequence typing

For one representative *S. aureus* isolate of each *spa* type, the sequence type (ST) was determined using multi-locus sequence typing (MLST).[20] goeBURST,[21] as implement in Phyloviz software (http://www.phyloviz.net/wiki/), and which used the same priority rules for linking STs as eBURST but with a global optimization, was used to infer

relatedness between STs. goeBURST was run on the whole *S. aureus* MLST database (http://saureus.mlst.net/; interrogated July 2011) supplemented with STs of rhesus macaque isolates not present in the *S. aureus* MLST database at the time of interrogation. Clonal complexes (CCs) are defined as STs that are linked through single locus variants (SLVs) and are named on the basis of the predicted founder ST, which is the ST having the most SLVs.

Staphylococcal antigen PCRs

Methicillin resistance was detected with *mecA*-specific primers.[22] Six sets of multiplex PCRs were established to amplify 19 superantigen genes (*sea* to *see*, *seg* to *ser*, *seu*, *tst*), *eta*, *etd* and *agr-1* to *-4*, as described previously.[23] The presence of the *scn*, *chp*, *clfA*, *clfB*, *efb*, *fnbA*, *fnbB* and *tst* genes was determined by PCR as well.[13,24-26]

Statistical analysis

Statistical analyses were performed with SPSS software, version 15.0 (SPSS). The Mann-Whitney U test was used to compare median differences in anti-staphylococcal antibody levels. Differences were considered statistically significant when 2-sided *P*-values were < 0.05.

Results

Characterization of *S. aureus* isolates in rhesus macaques

A total of 731 macaques was screened once for presence of *S. aureus* in their nasal cavity. Thirty-nine percent (287/731) of these cultures were positive for *S. aureus*, clearly indicating that rhesus macaques can be *S. aureus* nasal carriers.

S. aureus isolates from the 287 culture positive macaques as well as all collected isolates from the 48 macaques that were sampled three times (a total of 355 isolates) were typed using molecular methods including PFGE. This revealed 11 major clusters (Figure 1). Five of these consisted of only rhesus macaque isolates, one comprised only human isolates, while five clusters included isolates from both macaques and humans.

For further characterization of the *S. aureus* isolates from rhesus macaques, *spa* genotyping was performed on representative isolates from each PFGE cluster (n=108) (Table 1). These isolates were all *spa*-positive, re-confirming that these were indeed *S.*

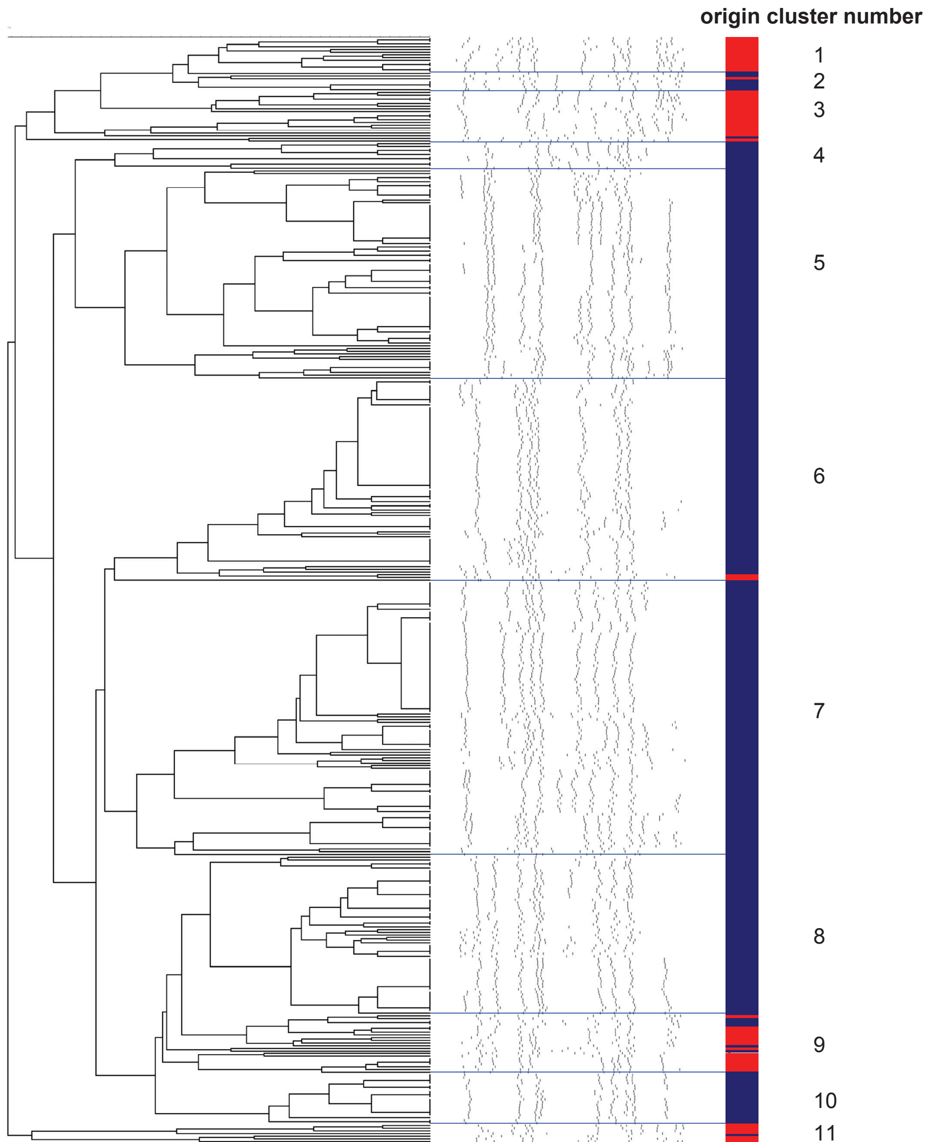

Figure 1. Dendrogram of the PFGE data of *S. aureus* strains isolated from rhesus macaques and humans. Blue color indicates *S. aureus* strains isolated from rhesus macaques, red color indicates *S. aureus* strains isolated from humans. Five clusters consisted of only rhesus macaque isolates, one comprised only human isolates, and five clusters included isolates from both macaques and humans.

Table 1. Different *spa* types found in 108 representative *S. aureus* strains isolated from rhesus macaques.

spa type		No. of isolates	Percentage of isolates
t189		1	0.9
t516		21	19.4
t729		15	13.9
t786		4	3.7
t1814		3	2.8
t4167	new	11	10.2
t4168	new	18	16.7
t4980	new	2	1.9
t4981	new	1	0.9
t5010	new	2	1.9
t5011	new	1	0.9
t5012	new	1	0.9
t5013	new	6	5.6
t5014	new	1	0.9
t5015	new	4	3.7
t5016	new	1	0.9
t5017	new	5	4.6
t5018	new	5	4.6
t5135	new	3	2.8
t5136	new	1	0.9
t5137	new	1	0.9
t5458	new	1	0.9

aureus strains. This revealed 22 different *spa* types, of which five have already been described before. Consequently, we identified 17 new *spa* types not included in the Ridom SpaServer database at the time of analysis. These new *spa* types comprised 59% of the isolates. The most prominent clone was t516, which comprised 21 isolates (19%). *Spa* types t4168, t729 and t4167 were represented by 18 (17%), 15 (14%) and 11 (10%) isolates, respectively. Nine *spa* types were present as single isolates. Each major PFGE cluster comprised 1 to 8 different *spa* types, though the repeat succession of different *spa* types within a cluster were clearly related in some cases (Table 2).

For one *S. aureus* isolate of each *spa* type, we performed MLST and compared STs found in macaque strains with all STs available in the *S. aureus* MLST database at the moment of interrogation (Figure 2). Seventeen different STs were found, which were all not present in the *S. aureus* MLST database at the time of interrogation. ST2099, ST2100 and ST2134 were part of CC45, which mostly contained STs found in human *S.*

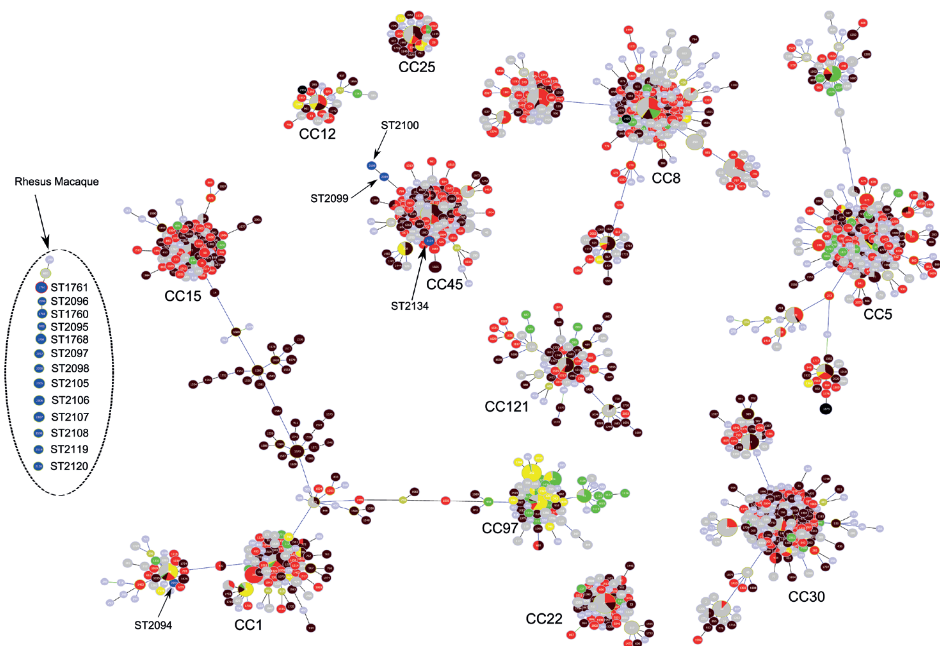

Figure 2. Partial population snapshot of *S. aureus*. This snapshot was created by goeBURST v1.2 software using the dataset downloaded from http://saureus.mlst.net/ that included 2010 STs representing 3887 isolates. A subset of this dataset (1304 STs, representing 2875 isolates) is presented in this figure including all major Clonal Complexes (CCs) supplemented with all rhesus macaque ST as far as they were not part of the major CCs. The area of each circle in the goeBURST diagram corresponds to the relative abundance of the STs in the input data. The names of major CCs have been indicated. ST colors refer to the source *S. aureus* was isolated from. STs representing macaque isolates are indicated in blue and by their ST number; brown colors correspond with community-acquired human isolates; red colors correspond with hospital-acquired human isolates; green colors correspond with animal isolates; yellow colors correspond with *S. aureus* isolates from food; grey, light green, and light blue colors correspond with isolates with unknown source.

aureus isolates. ST2099 and ST2100 were SLVs. Isolates with these 3 STs were found in different PFGE clusters. ST2094 had relations with STs in CC1, which contained a lot of STs found in human isolates as well. The PFGE pattern of this *S. aureus* isolate differed from all other macaque isolates, as this isolate was found in PFGE cluster 11, in which this isolate was the only one from macaque origin. All other STs found in the macaque isolates (ST1760, ST1761, ST1768, ST2095, ST2096, ST2097, ST2098, ST2105, ST2106, ST2107, ST2108, ST2119, ST2120) were not part of the larger CCs, but were present as singletons. ST1760 and ST2096 as well as ST1768 and ST2095 were SLVs, and were both found in PFGE clusters 7 and 6, respectively, clusters with mainly isolates from macaques. Isolates with STs present as a singleton

Table 2. Relatedness of *spa* types within each PFGE cluster.

PFGE cluster	*spa* types in PFGE cluster	Repeat succession										
1	-											
2	t4980	4	- 16	- 21	- 12	- 12	- 17	- 13	- 39	- 13		
	t5135	210	- 23	- 34	- 34	- 16	- 17	- 34	- 17	- 17	- 23	- 17
3	-											
4	t516	8	- 16	- 2	- 25	- 51	- 68	- 2	- 24	- 2	- 24	
	t5014	15	- 12	- 16	- 16	- 2	- 25	- 34	- 24	- 17	- 24	- 17
	t5015	15	- 12	- 16	- 16	- 25	- 25	- 34	- 24	- 17	- 17	- 17
	t5017	8	- 34	- 2	- 43	- 34	- 43	- 43	- 16	- 2	- 17	- 83
5	t516		8	- 16	- 2	- 25	- 51	- 68	- 2	- 24	- 2	- 24
	t5011		8	- 16	- 16	- 25	- 25	- 34	- 24	- 17	- 17	- 17
	t5015	15	- 12	- 16	- 16	- 25	- 25	- 34	- 24	- 17	- 17	- 17
6	t516	8	- 16	- 2	- 25	- 51	- 68	- 2	- 24	- 2	- 24	
	t729	7	- 12	- 21	- 17	- 13	- 34	- 34	- 34	- 33	- 34	
	t786	7	- 12	- 21	- 17	- 13	- 34	- 34	-	33	- 34	
	t1814	7	- 12	- 21	- 17	- 34	- 34	- 34	-	33	- 34	
	t5012	8	- 34	- 2	- 43	- 34	- 43	- 43	- 16	- 2	- 17	- 16
	t5136	4	- 2	- 17	- 17	- 34	- 24	- 17	- 17	- 17		
	t5458	210	- 23	- 34	- 17	- 34	- 22	- 34	- 17	- 23	- 34	
7	t4167	7	- 23	- 17	- 22	- 249	- 12	- 117	- 24	- 25	- 17	
	t4168	8	- 23	- 21	- 17	- 13	- 13	- 13	- 24	- 23		
	t4980	4	- 16	- 21	- 12	- 12	- 17	- 13	- 39	- 13		
	t5013	7	- 23	- 23	- 17	- 34	- 21	- 12	- 20	- 24		
	t5016	8	- 34	- 2	- 43	- 34	- 43	-			17	- 83
	t5017	8	- 34	- 2	- 43	- 34	- 43	- 43	- 16	- 2	- 17	- 83
	t4981	8	- 16	- 2	- 43	- 17	-			34		
	t5018	8	- 16	- 2	- 43	- 17	- 173	- 34	- 17	- 34		

Table 2. Relatedness of *spa* types within each PFGE cluster. (continued)

PFGE cluster	*spa* types in PFGE cluster	Repeat succession
8	t4168	8 - 23 - 21 - 17 - 13 - 13 - 13 - 24 - 23
	t5010	8 - 23 - 12 - 17 - 13 - 13 - 13 - 24 - 23
9	t4167	7 - 23 - 17 - 22 - 249 - 12 - 117 - 24 - 25 - 17
	t4168	8 - 23 - 21 - 17 - 13 - 13 - 13 - 24 - 23
	t5015	15 - 12 - 16 - 16 - 25 - 25 - 34 - 24 - 17 - 17 - 17
	t5137	4 - 16 - 13
10	t4168	8 - 23 - 21 - 17 - 13 - 13 - 13 - 24 - 23
11	t189	7 - 23 - 12 - 21 - 17 - 34

Table 3. Presence of genes encoding superantigens, exfoliative toxins, and *agr* types in 32 representative *S. aureus* strains isolated from rhesus macaques.

	Gene[a]												
Isolate no.	***seg***	***sei***	***sem***	***sen***	***seo***	***etd***	***agr-1***	***agr-2***	***agr-3***	***agr-4***	**Gene profile**	**PFGE cluster**	**Origin isolates in PFGE cluster**[b]
08-1178	-	-	-	-	-	-	+	-	-	-	1	5	RM
08-1192	-	-	-	-	-	-	+	-	-	-	1	4	RM
08-1276	-	-	-	-	-	-	+	-	-	-	1	7	RM
08-1494	-	-	-	-	-	-	+	-	-	-	1	7	RM
08-1495	-	-	-	-	-	-	+	-	-	-	1	7	RM
08-1185	+	+	+	+	+	-	+	-	-	-	2	8	RM
08-1415	+	+	+	+	+	-	+	-	-	-	2	8	RM
08-1197	+	-	-	-	-	-	-	-	-	+	3	6	RM+HV
08-1255	+	-	-	-	-	-	-	-	-	-	4	11	RM+HV
08-1299	-	-	-	-	-	-	-	-	-	+	5	5	RM
08-1514	-	-	-	-	-	-	-	-	-	+	5	5	RM

Table 3. Presence of genes encoding superantigens, exfoliative toxins, and *agr* types in 32 representative *S. aureus* strains isolated from rhesus macaques. (continued)

Isolate no.	Gene[a]										Gene profile	PFGE cluster	Origin isolates in PFGE cluster[b]
	seg	***sei***	***sem***	***sen***	***seo***	***etd***	***agr-1***	***agr-2***	***agr-3***	***agr-4***			
08-1332	-	-	-	-	-	-	-	+	-	-	6	4	RM
08-1342	-	-	-	-	-	-	-	-	+	-	7	6	RM+HV
08-1404	-	-	-	-	-	-	-	-	-	-	8	5	RM
08-1412	-	-	-	-	-	-	-	-	-	-	8	6	RM+HV
08-1423	-	-	-	-	-	-	-	-	-	-	8	7	RM
08-1430	-	-	-	-	-	-	-	-	-	-	8	7	RM
08-1432	-	-	-	-	-	-	-	-	-	-	8	7	RM
08-1487	-	-	-	-	-	-	-	-	-	-	8	6	RM+HV
08-1504	-	-	-	-	-	-	-	-	-	-	8	7	RM
08-1507	-	-	-	-	-	-	-	-	-	-	8	7	RM
08-1528	-	-	-	-	-	-	-	-	-	-	8	7	RM
08-1737	-	-	-	-	-	-	-	-	-	-	8	2	RM+HV
08-1749	-	-	-	-	-	-	-	-	-	-	8	7	RM
08-1756	-	-	-	-	-	-	-	-	-	-	8	7	RM
08-1419	-	-	-	-	-	+	-	-	+	-	9	7	RM
08-1420	-	-	-	-	-	+	-	-	+	-	9	7	RM
08-1486	+	-	+	+	+	-	-	-	-	-	10	7	RM
08-1583	+	+	+	+	+	-	-	-	-	-	11	6	RM+HV
08-1602	+	+	+	+	+	-	-	-	+	-	12	9	RM+HV
08-1765	+	+	+	+	+	-	-	-	+	-	12	7	RM
08-1630	-	-	-	-	-	+	-	-	-	-	13	2	RM+HV
No. of positive isolates (%)	8 (25)	5 (16)	6 (19)	6 (19)	6 (19)	3 (9)	7 (22)	1 (3)	5 (16)	3 (9)			

[a] All isolates were negative for *sea*, *seb*, *sec*, *sed*, *see*, *seh*, *sej*, *sek*, *sel*, *sep*, *seq*, *ser*, *seu*, *tst*, and *eta*.

[b] Isolates belong to typically rhesus macaque (RM) PFGE clusters or to mixed clusters including both macaque and healthy human volunteer (HV) strains.

were distributed among PFGE clusters 4 to 9, which were clusters with mainly isolates from rhesus macaques.

Next, we determined the genomic presence of *mecA*, 19 superantigen genes, and *eta* and *etd* by PCR. None of the 108 selected isolates harbored the *mecA* gene, which indicates that none of these isolates were MRSA. Thirty-two isolates that were representative for each *spa* type were selected for testing presence of superantigen genes and *eta* and *etd* (Table 3). These genes were absent in 66% (21/32) of these isolates. The *etd* gene was detected in 9% of the isolates (3/32), 16% of the isolates were *sei*-positive (5/32), 19% were positive for *sem*, *sen*, and *seo* (6/32), and 25% were positive for *seg* (8/32). Genes encoding SEA to SEE, SEH, SEJ to SEL, SEP to SER, SEU, TSST-1 and ETA were absent in all 32 isolates. Only 6 isolates harbored a complete or incomplete *egc* locus. Four of these were strains from PFGE clusters comprising only macaque isolates, while 2 isolates belong to clusters including both macaque and human isolates.

These 32 isolates were also *agr* genotyped (Table 3). The most common *agr* type was type I (22%), followed by *agr* type III (16%), type IV (9%), and type II (3%). Sixteen isolates (50%) did not belong to any of the 4 known *agr* types, which is intriguing. Isolates with *agr* type I or type II belonged to PFGE clusters comprising only macaque isolates, while isolates with other *agr* types belonged to clusters including both macaque and human isolates. Overall, 13 combinations of *agr* type and toxin genes were found. Two of the *agr* type I isolates, two of the *agr* type III isolates and two of the unknown *agr* type isolates harbored a complete or incomplete *egc* locus. Five *agr* type I isolates, the single *agr* type II isolate, one *agr* type III isolate, two *agr* type IV isolates, and 12 isolates with an unknown *agr* type harbored none of the superantigen and exfoliative toxin genes. *S. aureus* isolates with the same *agr* type and toxin genes were distributed among the different PFGE clusters.

S. aureus nasal carriage in rhesus macaques

Forty-eight (4 groups of 12) macaques were screened three times for nasal carriage of *S. aureus* over a 5 month period. The four groups of twelve macaques were housed in separate rooms. For 9 macaques (18.8%), all nasal culture results were negative. Eighteen macaques (37.5%) had one positive culture, 13 (27.1%) had two positive cultures, and for 8 macaques (16.7%) *S. aureus* was found in all three cultures.

Isolates from these 48 duo-housed macaques were also typed by PFGE, and *spa* types were determined for representative isolates from each PFGE cluster (Figure 3).

room no.	cage no.	screen 1 PFGE type	screen 1 *spa* type	screen 2 PFGE type	screen 2 *spa* type	screen 3 PFGE type	screen 3 *spa* type
1	1	A	t5013	A'	t5013	B	t5013*
	2	B	t5013	B	t5013*	-	-
	3	-	-	-	-	-	-
	4	-	-	-	-	-	-
	5	-	-	B	t5013*	-	-
	6	-	-	-	-	B	t5013*
	7	-	-	-	-	B	t5013*
	8	-	-	B'	t5013*	-	-
	9	C	t5018	-	-	B	t5013*
	10	B	t5013	-	-	-	-
	11	-	-	B	t5013*	B	t5013*
	12	-	-	C	t5018*	B	t5013*
2	1	D	t5017	D	t5016*	D	t5016*
	2	-	-	D	t5016*	D	t5016*
	3	-	-	D	t5016*	-	-
	4	-	-	-	-	-	-
	5	-	-	D	t5016*	D	t5016*
	6	-	-	C'	t5018	C'	t5018*
	7	-	-	D	t5016*	-	-
	8	-	-	-	-	D	t5016*
	9	-	-	D	t5016*	D	t5016*
	10	D	t5016	D	t5016*	H	§
	11	D	t5017	G	t5135	-	-
	12	D	t5017	-	-	-	-
3	1	-	-	-	-	G	t5135*
	2	-	-	-	-	-	-
	3	-	-	G	t5135	-	-
	4	-	-	G	t5135	-	-
	5	-	-	-	-	-	-
	6	-	-	C	t5018*	-	-
	7	-	-	C'	t5018*	-	-
	8	C'	t5018	C'	t5018*	C'	t5018*
	9	C	t5018	C	t5018*	A	t5013*
	10	B	t5013	B	t5013*	-	-
	11	C'	t5018	-	-	-	-
	12	-	-	-	-	C'	t5018*
4	1	E	t516	E'	§	E	t5458
	2	A	t5013	F	t4167	A	t5013*
	3	-	-	-	-	-	-
	4	-	-	A	t5013*	-	-
	5	-	-	-	-	-	-
	6	-	-	-	-	-	-
	7	F	t4167	F	t4167*	A	t5013*
	8	F'	t4167	-	-	F'	t4167*
	9	-	-	-	-	-	-
	10	-	-	F	t4167*	A	t5013*
	11	-	-	-	-	A	t5013*
	12	-	-	A	t5013*	A	t5013*

Figure 3. Persistence of *S. aureus* nasal carriage in rhesus macaques. Macaques were duo-housed. Physical contact to the macaques in the neighboring cage was possible. Each block indicates an animal room, horizontal lines in blocks indicate which macaques were not able to have physical contact. Red color indicates the "epidemic" strain in each room. – indicates a *S. aureus* negative nasal culture. * indicates that the isolate was not *spa* genotyped itself, *spa* type was derived from typed isolates with identical PFGE pattern. § indicates that the isolate was not *spa* genotyped, and this could not be derived from typed isolates with identical PFGE pattern. *S. aureus* strains were frequently exchanged between macaques in the same room.

In macaques with more than one *S. aureus* positive nasal culture, a strain replacement occurred in half of the animals.

It seemed that there was one most prevalent "epidemic" strain in each animal room. This strain spread from one or a couple of macaques to most others. This phenomenon was clear for rooms 1 (PFGE type B, *spa* type t5013), 2 (PFGE type D, *spa* type t5016) and 3 (PFGE type C', *spa* type t5018), while for room 4, the epidemic strain was less clear (PFGE type A, *spa* type t5013, or PFGE type F, *spa* type t4167). In some cases, a macaque did not acquire the epidemic strain, e.g. the macaque in animal room 2, cage 6 had two nasal cultures positive with a *S. aureus* isolate with PFGE type C', while the epidemic isolate had PFGE type D.

Anti-staphylococcal antibodies in rhesus macaques

Serum samples from 47 of the 48 macaques that were screened three times for nasal carriage were analyzed for anti-staphylococcal antibodies directed against 40 different *S. aureus* proteins. The MFI values reflecting the serum IgG and IgA levels for each macaque and each antibody isotype are shown in Figure 4. An extensive diversity in individual antibody responses was observed, similar to the findings in humans. Individual macaques had high antibody levels against a number of antigens, while anti-staphylococcal antibody levels against other antigens were low.

For most antigens, there was no apparent quantitative difference in antibody level between macaques with 0, 1, 2, or 3 positive cultures. However, the median serum levels of IgG directed against ClfA were significantly higher in rhesus macaques with one positive culture than in those without positive culture (MFI value, 657 vs. 191; $P < 0.05$). Additionally, median IgA levels were higher in macaques without a positive culture than in those with three positive cultures for LukF (532 vs. 167; $P < 0.05$). In macaques with three positive cultures, median IgA levels were higher than in macaques without positive culture for SdrE (881 vs. 200; $P < 0.05$). In macaques with one positive culture, median IgA levels were higher than in macaques with three positive cultures for LukD (1580 vs. 599; $P < 0.005$), SEC (658 vs. 23; $P < 0.05$), and SSL11 (204 vs 22; $P < 0.05$). In general, a larger number of positive cultures was not related to elevated or decreased anti-staphylococcal antibody levels. Other comparisons were also made (non-carriers *vs.* intermittent *vs.* persistent carriers; carriers of the most prominent isolate in the animal room *vs.* carriers of another isolate). Again, no association was found between nasal carrier status and anti-staphylococcal antibody levels in serum (data not shown).

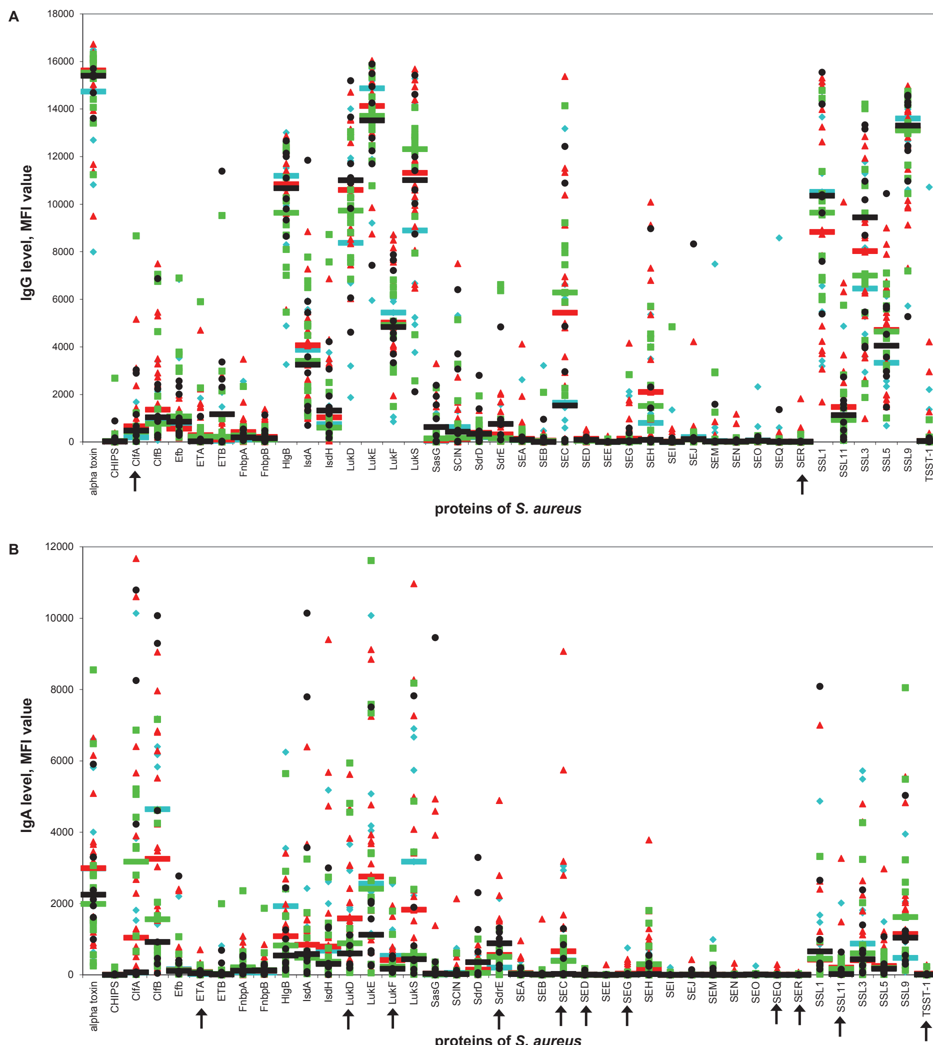

Figure 4. Relation between *S. aureus* nasal colonization and level of anti-staphylococcal IgG (A) and IgA (B). Antibody levels are reflected by median fluorescence intensity (MFI) value. Each symbol represents a single rhesus macaque. Blue diamonds represent macaques without *S. aureus* positive culture, red triangles represent macaques with one positive culture, green squares represent macaques with two positive cultures, and black circles represent macaques with three positive cultures. Median values are indicated by horizontal lines. Arrows indicate statistically significant differences in median values (Mann-Whitney U test). More *S. aureus* positive cultures were not related to high – or low – anti-staphylococcal antibody levels. CHIPS, chemotaxis inhibitory protein of *S. aureus*; Clf, clumping factor; Efb, extracellular fibrinogen-binding protein; ET, exfoliative toxin; Fnbp, fibronectin-binding protein; HlgB, γ hemolysin B; Isd, iron-responsive surface determinant; Luk, leukocidin; SasG, *S. aureus* surface protein G; SCIN, staphylococcal complement inhibitor; Sdr, serine-aspartate dipeptide repeat protein; SE, staphylococcal enterotoxin; SSL, staphylococcal superantigen-like protein; TSST-1, toxic shock syndrome toxin 1.

Table 4. Median fluorescence intensity (MFI) values reflecting antigen-specific IgG and IgA levels in rhesus macaques (RM) and healthy human volunteers (HV).

	MFI value, median (range)				MFI value, median (range)		
***S. aureus* protein (antibody isotype)**	**RM**	**HV**	***P*[a]**	***S. aureus* protein (antibody isotype)**	**RM**	**HV**	***P*[a]**
alpha toxin (IgG)	14337 (7438-15574)	14587 (6355-17465)	0.285	SEA (IgG)	88 (17-3565)	3839 (29-11365)	< 0.001
alpha toxin (IgA)	1540 (139-4729	1775 (215-9549)	0.154	SEA (IgA)	16 (1-449)	247 (5-9345)	< 0.001
CHIPS (IgG)	20 (0-2580)	13310 (2933-16281)	< 0.001	SEB (IgG)	34 (0-2836)	4223 (190-13622)	< 0.001
CHIPS (IgA)	1 (0-130)	7494 (957-11238)	< 0.001	SEB (IgA)	2 (0-673)	90 (2-398)	< 0.001
ClfA (IgG)	454 (16-8250)	4029 (670-13449)	< 0.001	SEC (IgG)	4551 (1-14006)	12720 (4713-15884)	< 0.001
ClfA (IgA)	580 (4-6237)	673 (145-4730)	0.373	SEC (IgA)	280 (0-4896)	600 (63-3943)	0.004
ClfB (IgG)	1066 (65-7518)	7272 (2499-14127)	< 0.001	SED (IgG)	84 (3-533)	800 (33-7924)	< 0.001
ClfB (IgA)	1232 (0-6962)	2357 (255-10038)	0.125	SED (IgA)	1 (0-85)	19 (0-736)	< 0.001
Efb (IgG)	622 (162-6325)	4238 (770-12855)	< 0.001	SEE (IgG)	3 (0-229)	258 (7-3017)	< 0.001
Efb (IgA)	69 (0-1575)	4775 (257-8125)	< 0.001	SEE (IgA)	0 (0-151)	12 (0-1856)	< 0.001
ETA (IgG)	156 (10-5670)	234 (16-14891)	0.217	SEG (IgG)	37 (0-3861)	517 (5-4385)	0.002
ETA (IgA)	30 (1-366)	19 (6-1127)	0.742	SEG (IgA)	5 (0-353)	22 (0-432)	0.035
ETB (IgG)	72 (0-10405)	55 (1-2855)	0.515	SEH (IgG)	1282 (0-10804)	1400 (9-15602)	0.468
ETB (IgA)	3 (0-1023)	20 (1-1529)	0.026	SEH (IgA)	88 (0-2083)	44 (0-2158)	0.821
FnbpA (IgG)	208 (20-3374)	1269 (222-6132)	< 0.001	SEI (IgG)	4 (0-5154)	257 (0-3544)	< 0.001
FnbpA (IgA)	73 (1-1047)	744 (26-5261)	< 0.001	SEI (IgA)	0 (0-111)	8 (0-840)	< 0.001
FnbpB (IgG)	111 (9-1089)	478 (54-2758)	< 0.001	SEJ (IgG)	105 (5-7274)	2538 (95-16246)	< 0.001
FnbpB (IgA)	15 (2-253)	253 (19-2603)	< 0.001	SEJ (IgA)	1 (0-217)	25 (0-2236)	< 0.001
HlgB (IgG)	8519 (2595-10365)	10761 (2979-13212)	0.001	SEM (IgG)	12 (0-7325)	307 (8-2738)	< 0.001
HlgB (IgA)	474 (10-2799)	556 (44-6394)	0.233	SEM (IgA)	1 (0-354)	56 (0-1128)	< 0.001
IsdA (IgG)	2811 (508-8602)	2665 (576-5183)	0.732	SEN (IgG)	7 (0-1166)	631 (0-2672)	< 0.001
IsdA (IgA)	248 (13-3997)	357 (9-8058)	0.940	SEN (IgA)	0 (0-155)	7 (0-2743)	< 0.001
IsdH (IgG)	929 (44-8170)	3921 (605-9420)	< 0.001	SEO (IgG)	40 (7-2009)	306 (147-653)	< 0.001
IsdH (IgA)	311 (3-4328)	294 (43-2117)	0.661	SEO (IgA)	3 (0-117)	20 (4-544)	< 0.001

Table 4. Median fluorescence intensity (MFI) values reflecting antigen-specific IgG and IgA levels in rhesus macaques (RM) and healthy human volunteers (HV). (continued)

S. aureus protein (antibody isotype)	MFI value, median (range)			*S. aureus* protein (antibody isotype)	MFI value, median (range)		
	RM	**HV**	***P*[a]**		**RM**	**HV**	***P*[a]**
LukD (IgG)	1082 (1852-15031)	12226 (3781-16731)	0.011	SEQ (IgG)	5 (0-7125)	341 (0-3763)	< 0.001
LukD (IgA)	641 (0-3418)	694 (106-4088)	0.753	SEQ (IgA)	0 (0-132)	19 (0-2219)	< 0.001
LukE (IgG)	12386 (5270-14191)	12541 (5955-15590)	0.732	SER (IgG)	3 (0-1797)	63 (0-2654)	< 0.001
LukE (IgA)	1181 (28-5431)	664 (38-7766)	0.524	SER (IgA)	0 (0-40)	9 (0-357)	0.001
LukF (IgG)	7321 (1240-12631)	10579 (2984-14996)	0.007	SSL1 (IgG)	8783 (1520-14022)	4683 (518-11914)	0.005
LukF (IgA)	321 (0-2554)	433 (38-3916)	0.041	SSL1 (IgA)	198 (0-4066)	195 (6-2947)	0.956
LukS (IgG)	10470 (1939-14385)	8734 (1662-16976)	0.135	SSL11 (IgG)	867 (43-8824)	1107 (122-8955)	0.311
LukS (IgA)	602 (0-6321)	1015 (13-10014)	0.524	SSL11 (IgA)	46 (3-1495)	96 (8-2162)	0.215
SasG (IgG)	89 (3-3376)	197 (3-2791)	0.093	SSL3 (IgG)	7280 (898-12949)	7813 (1618-15432)	0.403
SasG (IgA)	8 (0-5408)	33 (4-3950)	0.009	SSL3 (IgA)	321 (5-3062)	519 (24-5234)	0.244
SCIN (IgG)	187 (5-7259)	11333 (7572-15438)	< 0.001	SSL5 (IgG)	4138 (621-9547)	3267 (511-5928)	0.021
SCIN (IgA)	14 (0-1220)	2945 (211-10036)	< 0.001	SSL5 (IgA)	97 (6-1293)	115 (27-933)	0.264
SdrD (IgG)	233 (17-2844)	1683 (400-6924)	< 0.001	SSL9 (IgG)	11968 (4788-13605)	9255 (1005-15161)	0.150
SdrD (IgA)	64 (3-2205)	634 (48-4758)	< 0.001	SSL9 (IgA)	444 (35-3523)	138 (20-1158)	0.001
SdrE (IgG)	250 (28-6655)	2045 (251-8626)	< 0.001	TSST-1 (IgG)	32 (4-9350)	12051 (0-16927)	< 0.001
SdrE (IgA)	218 (0-3017)	604 (28-3650)	0.015	TSST-1 (IgA)	4 (0-125)	932 (10-3504)	< 0.001

[a] Differences in antigen-specific MFI values between groups were considered to be statistically significant at $P < 0.05$ (Mann-Whitney U test).

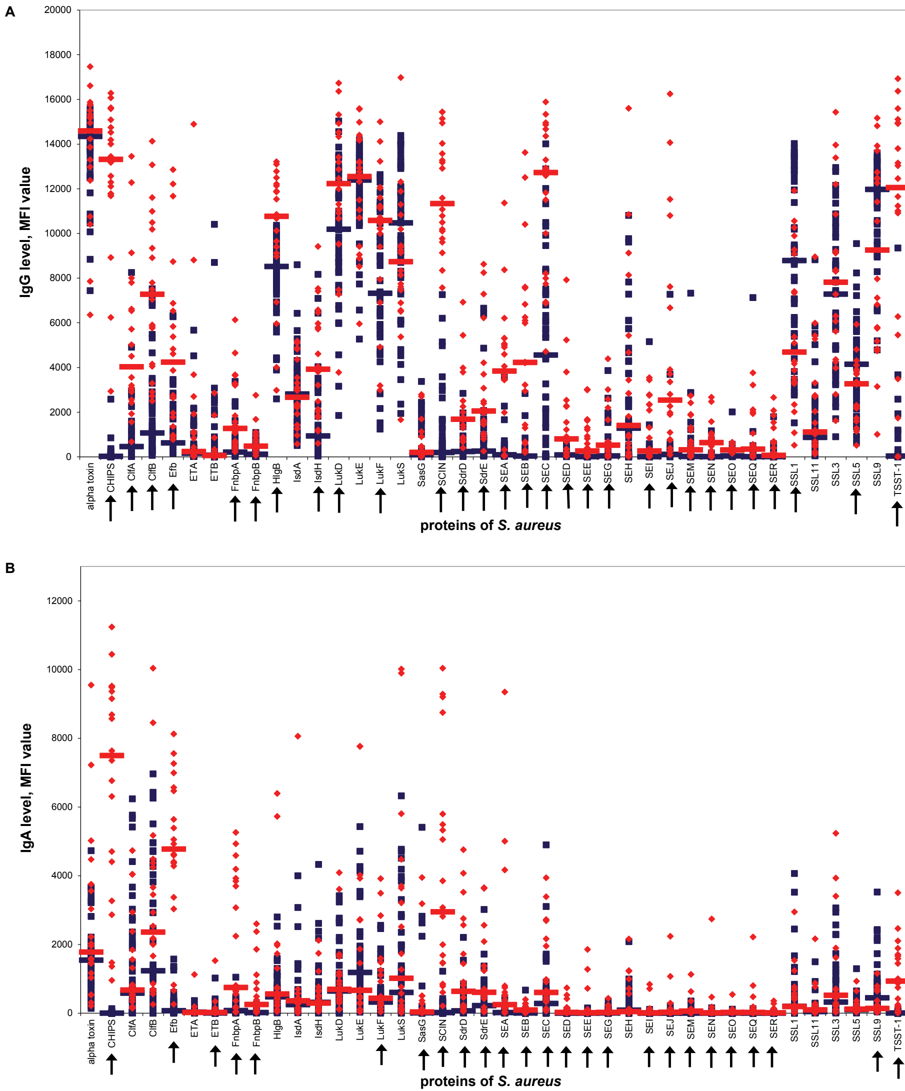

Figure 5. Level of anti-staphylococcal IgG (A) and IgA (B) in rhesus macaques and human volunteers. Antibody levels are reflected by median fluorescence intensity (MFI) value. Each symbol represents a single rhesus macaque or human. Blue squares represent healthy rhesus macaques and red diamonds represent healthy human volunteers. Median values are indicated by horizontal lines. Arrows indicate statistically significant differences in median values (Mann-Whitney U test). Anti-staphylcoccal IgG and IgA levels were significantly different between rhesus macaques and humans for 29 and 25 out of 40 antigens, respectively. CHIPS, chemotaxis inhibitory protein of *S. aureus*; Clf, clumping factor; Efb, extracellular fibrinogen-binding protein; ET, exfoliative toxin; Fnbp, fibronectin-binding protein; HlgB, γ hemolysin B; Isd, iron-responsive surface determinant; Luk, leukocidin; SasG, *S. aureus* surface protein G; SCIN, staphylococcal complement inhibitor; Sdr, serine-aspartate dipeptide repeat protein; SE, staphylococcal enterotoxin; SSL, staphylococcal superantigen-like protein; TSST-1, toxic shock syndrome toxin 1.

Isolates from rhesus macaques differ from those from humans in PFGE type, *spa* type, *agr* type, and prevalence of superantigen genes. To determine whether these different *S. aureus* isolates induce different humoral antibody responses, anti-staphylococcal antibody levels in sera of the 47 rhesus macaques were compared to those in sera of 20 healthy human volunteers (Figure 5). Significant differences in anti-staphylococcal IgG and IgA levels between rhesus macaques and healthy volunteers were observed for 29 and 25 out of 40 antigens, respectively (Table 4). In general, the median antibody serum levels in humans were higher than those in macaques. However, the median serum IgG level directed against SSL1 and SSL5 was significantly higher in macaques than in humans. The median serum IgA level directed against SSL9 was significantly higher in macaques than in humans.

Association of anti-staphylococcal antibody levels with presence of genes

To study whether the low antibody levels in rhesus macaques were due to absence of the genes encoding the antigens, 10 isolates from the macaques that were screened three times were selected for determination of the presence of genes encoding antigens against which a low MFI value was found in macaques, while MFI values in humans were high (SCIN, CHIPS, ClfA, ClfB, Efb, TSST-1, FnbpA, and FnbpB). Results are shown in Table 5. None of the isolates from rhesus macaques were *scn*-, *tst*-, or *fnbB*-positive, which explains why antibody levels were at background level. On the other hand, all 10 isolates were *clfB*- and *efb*-positive, and the macaques that carry these isolates had high antibody levels against these antigens. Nine out of 10 *S. aureus* isolates did not possess the gene encoding for CHIPS, which explains why the MFI values were at background level. The macaque from which the *chp*-positive strain was isolated also had MFI values at background level. Eight isolates were *clfA*-positive, with corresponding high MFI values for 6 macaques. Interestingly, the two macaques from which the *clfA*-negative strains were isolated, still had high MFI values. Nine isolates possessed the gene encoding FnbpA, which corresponds with MFI values just above background level (range 22-616). However, the macaque with the *fnbA*-negative *S. aureus* strain had MFI values above background level as well. These differences are probably due to a previous exposure to another *S. aureus* strain.

Table 5. Association of anti-staphylococcal antibody levels with presence of genes in 10 *S. aureus* strains isolated from rhesus macaques.

	SCIN			CHIPS			ClfA			ClfB		
Isolate no.	**Gene**	**MFI value, IgG**	**MFI value, IgA**	**Gene**	**MFI value, IgG**	**MFI value, IgA**	**Gene**	**MFI value, IgG**	**MFI value, IgA**	**Gene**	**MFI value, IgG**	**MFI value, IgA**
1	-	356	2	-	6	0	-	664	82	+	291	45
2	-	6404	77	-	23	1	+	2893	8252	+	6871	10067
3	-	5	3	-	15	1	+	3033	10785	+	997	4604
4	-	305	2	-	8	0	+	142	660	+	816	238
5	-	759	44	-	65	0	+	275	5213	+	1382	805
6	-	3702	26	+	173	3	-	282	4223	+	2423	9292
7	-	3067	127	-	30	2	+	26	7	+	866	462
8	-	492	11	-	886	0	+	27	37	+	2232	310
9	-	155	9	-	338	11	+	664	3549	+	462	549
10	-	27	76	-	8	27	+	17	1574	+	192	1574

	Efb			TSST-1			FnbpA			FnbpB		
Isolate no.	**Gene**	**MFI value, IgG**	**MFI value, IgA**	**Gene**	**MFI value, IgG**	**MFI value, IgA**	**Gene**	**MFI value, IgG**	**MFI value, IgA**	**Gene**	**MFI value, IgG**	**MFI value, IgA**
1	+	1011	107	-	14	2	+	60	36	-	12	23
2	+	2332	2773	-	58	27	+	545	254	-	477	169
3	+	2564	402	-	180	9	+	181	100	-	1132	255
4	+	1156	101	-	10	0	+	157	56	-	78	86
5	+	373	48	-	43	16	+	165	616	-	63	223
6	+	279	321	-	37	0	+	192	544	-	161	300
7	+	416	25	-	30	22	+	131	22	-	324	13
8	+	679	61	-	37	1	+	508	134	-	149	74
9	+	3777	1069	-	197	5	+	74	51	-	24	35
10	+	2003	1061	-	8	4	-	356	502	-	26	263

Discussion

Nasal carriage of *S. aureus* plays a key role in the epidemiology and pathogenesis of staphylococcal infections.[27,28] Eradication of *S. aureus* from the nose has proven to be effective in the reduction of staphylococcal infections.[29-32] This indicates that the anterior nasal region is a primary ecological reservoir of *S. aureus*,[33,34] although throat and perineum are important reservoirs as well.[35-37] However, nasal re-colonization may occur within weeks to months in those who have successfully been decolonized.[38,39] In order to develop new strategies in prevention of staphylococcal disease, acquiring additional knowledge about the underlying mechanisms of *S. aureus* nasal carriage is important. Artificial inoculation of human volunteers offers opportunities to study these mechanisms.[40] While such semi-clinical studies in humans remain the most informative, animal models of *S. aureus* colonization sometimes enable a more detailed investigation of the processes involved in pathogenesis by allowing for more risky interventions.

In the present study, we describe for the first time natural nasal *S. aureus* carriage in rhesus macaques. The nasal cavity of these macaques appears to be an important reservoir of *S. aureus*, as is also observed in humans. In a single screening of their noses, 39% of the 731 rhesus macaques had a positive culture, which is comparable to the human situation with ~20% persistent carriers and ~30% intermittent carriers.[28,41,42] Most of these *S. aureus* isolates were different from human *S. aureus* isolates: rhesus macaque isolates formed separate PFGE clusters, 59% had one of the 17 *spa* types that were not yet described, the previously described *spa* types t189, t516, t729, t786, and t1814 were rare as well (Ridom SpaServer), in most macaque isolates genes encoding superantigens were not present, and half of the isolates could not be *agr* typed. Moreover, in the majority of macaque isolates new STs were found which were not related to described STs. Three isolates were part of CC45, and one was part of CC1. These macaque isolates were therefore more comparable to human isolates than the other ones. Differences between macaque and human *S. aureus* isolates are underlined by major differences in both anti-staphylococcal antibody levels and gene content of the *S. aureus* isolates between these two species. This indicates that new host-specific lineages of *S. aureus* have now been found in rhesus macaques.

In contrast to the macaque *S. aureus* isolates, most human strains harbor at least one gene encoding superantigens, on average 5 or 6 genes, among which those encoding the *egc* superantigens SEG, SEI, SEM, SEN, and SEO are most prevalent.[23,43,44]

These genes were also most prevalent in the macaque isolates. The relatively low number of isolates containing superantigen genes could also be due to the fact that the primers used in this study were designed for *S. aureus* strains isolated from humans. Therefore, it cannot be concluded that most macaque *S. aureus* isolates do not carry superantigen genes at all, but that, at least, if they are present, these genes differ from those found in human *S. aureus* isolates.

In addition, half of the macaque isolates could not be typed with the current *agr* multiplex PCR system. Of those that could be typed, most belonged to *agr* types I or III, which is in contrast to human *S. aureus* isolates. These *agr* types are also the largest groups (*agr* type I 35% and type III 38%), but 25% of the isolates contains *agr* type II.[45] In contrast, *agr* type IV is hardly found among human isolates (2%), while 19% of the typeable macaque isolates harbored this type. The *agr* untypeable *S. aureus* isolates probably have a deletion of the *agr* locus or extensive sequence variation at the primer sites and could therefore not be typed. This further emphasizes that *S. aureus* isolates in rhesus macaques differ from those in humans.

Next, we studied the persistence of nasal *S. aureus* carriage in 48 rhesus macaques. PFGE and *spa* typing of these isolates, as well as the absence of certain antigen genes while serum anti-staphylococcal antibody levels against these antigens were high, suggest that persistent nasal *S. aureus* carriage as observed in humans is not present among these rhesus macaques. Human persistent carriers are usually colonized by the same *S. aureus* strain over a long time period,[46] while half of the studied rhesus macaques carried different *S. aureus* isolates, even over a 5 month period. Frequent transmission of *S. aureus* isolates rather than persistent nasal carriage was suggested by the presence of one or two epidemic strains in each animal room.

The absence of persistent nasal carriage was underlined by the absence of an association between nasal carrier status and anti-staphylococcal antibody levels in serum of rhesus macaques, as was observed in humans. Differences in IgG levels between human persistent carriers and non-carriers were reported for alpha hemolysin, major autolysin, IsdA and IsdH, immunodominant secretory antigen A (IsaA), major histocompatibility complex class II analogue protein w (Map-w), ClfB, TSST-1, and SEA. IgA levels were different between persistent carriers and non-carriers for TSST-1, SEA, ClfA, and ClfB.[14,47,48]

In addition, the differences in anti-staphylococcal antibody levels in rhesus macaques and humans, independent of nasal carrier status, again underlined the differences between *S. aureus* isolates in humans and those in macaques. The major

immunogenic proteins in human *S. aureus* strains do not seem to elicit a humoral immune response in rhesus macaques.

In conclusion, the present study demonstrates that rhesus macaques are natural hosts of *S. aureus*, and that *S. aureus* isolates from rhesus macaques versus those from humans differ in many aspects. Therefore, rhesus macaques are not suitable for studying *S. aureus* persistent carriage as is seen in humans. However, the nasal cavity is an important reservoir in both species, which implies that the rhesus macaque provides an interesting model for studying short term nasal colonization, and in particular bacterial factors involved in adherence. Furthermore, rhesus macaques provide an autologous system in which transmission of *S. aureus* strains between individuals can be studied, and are therefore a useful model for studying infection prevention. Whether data from these studies can be directly extrapolated to humans is unclear. However, possibilities for extrapolation from rhesus macaque to human appear reasonable because of their close evolutionary relatedness.

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3

A multiplex assay for the quantification of antibody responses in *Staphylococcus aureus* infections in mice

Sanne van den Berg, M. Gabriela Bowden, Tjibbe Bosma, Girbe Buist,
Jan Maarten van Dijl, Willem J.B. van Wamel, Corné P. de Vogel, Alex van Belkum,
Irma A.J.M. Bakker-Woudenberg

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Abstract

Staphylococcus aureus causes a variety of infections. Knowledge about the physiological role of most *S. aureus* antigens in colonization and infection is only limited. This can be studied by measuring antigen-specific antibody responses. In this study, we optimized the multiplex microsphere bead-based flow cytometry technique for mouse serum samples. We analyzed immunoglobulin G (IgG) levels directed against 26 *S. aureus* proteins in a single small-volume mouse serum sample. We assessed possible cross reactivity. Furthermore, we analyzed serum samples from mice with different types of *S. aureus* infections caused by different *S. aureus* strains. The results show that cross reactivity between proteins on microspheres and serum antibodies towards other proteins was limited. We found that lung-infected mice had a higher and broader IgG response than skin-infected mice. Clearly, the site of infection influences the IgG profile. Next, we compared sera from mice with intravenously-induced bacteremia caused by different *S. aureus* strains. We showed different IgG responses depending on the causing *S. aureus* strain. It is concluded that the bead-based multiplex *S. aureus* antibody assay can be successfully applied to determine the immunogenicity of different *S. aureus* proteins in relation to the site of infection and the *S. aureus* strain causing the infection.

Introduction

Staphylococcus aureus causes a diverse arsenal of infections, ranging from superficial skin infections (furuncles and impetigo) to invasive infections such as abscesses, pneumonia, endocarditis, and bacteremia.[1] Little is known about the precise physiological role of many if not most *S. aureus* antigens that are important in colonization and infection. For some infections, some or at least one of the *S. aureus* antigens important during infection are known. For example, superantigens such as toxic shock syndrome toxin 1 (TSST-1) are predominant in Toxic Shock Syndrome;[2] staphylococcal enterotoxins are known to cause food poisoning;[3] and Panton Valentine Leukocidin (PVL) is involved in necrotizing pneumonia.[4]

Immunogenicity of antigens in these processes can be studied by assessing the antigen-specific antibody responses elicited. It is known that levels of antibodies to TSST-1, staphylococcal enterotoxin A (SEA), and clumping factors A and B (ClfA and ClfB) are significantly higher in persistent carriers of *S. aureus* than in non-carriers.[5] Colonized children have higher immunoglobulin G (IgG) levels against chemotaxis inhibitory protein of *S. aureus* (CHIPS), extracellular fibrinogen-binding protein (Efb), and iron-responsive surface determinant H (IsdH), and higher immunoglobulin A (IgA) levels against CHIPS, iron-responsive surface determinant A (IsdA), and IsdH than non-colonized children in both the first and second years of life.[6] Although for instance the course of the humoral immune response in *S. aureus* bacteremia patients as well as in pediatric patients infected with community-associated *S. aureus* has been investigated, the importance and therapeutic effect of antibody induction in many other diseases remain enigmatic.[7,8] While clinical studies remain the most informative in this respect, animal models of *S. aureus* infection enable investigation of antibody responses to specific *S. aureus* antigens under similar conditions of *S. aureus* colonization and infection as are encountered by humans. In this way animal studies may provide insight into the potential role of *S. aureus* antigens in the important processes of colonization and infection. Experimental animal models of different *S. aureus* infections have been developed, and mice are frequently used as models.

For quantification of circulating antibody levels, conventional immunological techniques such as the Enzyme-Linked ImmunoSorbent Assay (ELISA) can be applied. This technique is time- and serum-consuming, and antibodies against only one antigen can be measured in one separate ELISA. To assess levels of antibodies directed against a broad range of antigens, multiple mice need to be bled to yield enough serum and

this may confound observations due to inter-experiment variations. The microsphere bead-based flow cytometry technique (xMap, Luminex Corporation) permits the simultaneous analysis of antibodies for up to 100 different antigens from a single, small-volume serum sample.[9] To our knowledge, this technique has as yet only been used for measuring antibodies against *S. aureus* proteins in human serum samples.[5,10,11] In the present study, we optimized the Luminex technology to quantify IgG antibodies directed against a broad panel of *S. aureus* proteins in mouse serum, and we assessed cross reactivity. In addition, this technique was applied to analyze serum samples from mice with different types of *S. aureus* infections caused by different *S. aureus* strains.

Materials and methods

Serum samples from mice immunized with monovalent staphylococcal vaccine candidates

Female BALB/cOlaHsd mice (6-8 weeks old, specified pathogen free) were immunized intranasally (5 mice per group) with monovalent Gram-positive Enhancer Matrix (GEM)-based vaccines containing ClfA, Efb, or TSST-1. One dose of vaccine consisted of 2.5 x 10^9 GEM-particles containing 8.0, 2.0, or 2.1 µg ClfA, Efb, or TSST-1, respectively, in a volume of 10 µL. Another group of mice was immunized subcutaneously (4 mice per group) with monovalent GEM-based vaccines containing endonuclease (Nuc), peptidoglycan hydrolase (LytM), or immunodominant staphylococcal antigen A (IsaA). One dose of vaccine consisted of 2.5 x 10^9 GEM-particles containing 25.0, 10.0, or 17.5 µg Nuc, LytM, or IsaA, respectively, in a volume of 100 µL. The immunization schedule consisted of three doses given at 10-day intervals. Animal experiments were performed with approval of the Animal Experimentation Committee of the University of Groningen, the Netherlands. Sera were collected before immunization and 2 weeks after the last immunization.

Serum samples from mice with lung infection or skin infection caused by *S. aureus* strain LAC

Sera from mice with lung infection or skin infection caused by *S. aureus* strain LAC (USA300) were obtained from Dr. M.G. Bowden and prepared as described.[4] In short, female BALB/c mice (6 weeks old, specified pathogen free) were inoculated intranasally (5 x 10^7 cfu in 20 µL, 9 mice) for lung infection or intradermally (1 x 10^7 cfu in 50

μL, 10 mice) for skin infection with *S. aureus* strain LAC. Sera were collected 5 weeks after infection.

Serum samples from mice with intravenously-induced bacteremia caused by different *S. aureus* strains

Female BALB/cBYJ mice (11-13 weeks old, specified opportunistic pathogen free) were inoculated intravenously via the tail vein with *S. aureus* clinical isolate P or *S. aureus* clinical isolate S (7 x 10^4 cfu in 100 μL, 5 mice per group). Animal experiments were performed with approval of the Institutional Animal Care and Use Committee of the Erasmus University Medical Center Rotterdam, the Netherlands. *S. aureus* clinical MSSA isolates P and S were kindly provided by Dr. G. Buist (University Medical Center Groningen, Groningen, the Netherlands). The characterization of these *S. aureus* isolates based on proteomic analysis has been described by Ziebandt et al.[12] Sera were collected before and 2, 3, and 5 weeks after infection.

Proteins

The following purified proteins of *S. aureus* were coupled to Sero-MAP beads: Nuc; LytM; IsaA; ClfA and ClfB; IsdA and IsdH; fibronectin-binding proteins A and B (FnbpA and FnbpB); Efb; staphylococcal complement inhibitor (SCIN); alpha toxin; γ hemolysin B (HlgB); leukocidin D, E, F, and S (LukD, LukE, LukF, and LukS); staphylococcal enterotoxins A-C (SEA-SEC); TSST-1; and staphylococcal superantigen-like proteins 1, 3, 5, 9, and 11 (SSL1, SSL3, SSL5, SSL9, and SSL11). G. Buist (University Medical Center Groningen, Groningen, the Netherlands) supplied Nuc, LytM, and IsaA.[12] ClfA was kindly provided by T. Bosma (BiOMaDe Technology, Groningen, the Netherlands). ClfB, IsdA, IsdH, FnbpA, and FnbpB were expressed and purified as described previously.[5] The constructs were provided by T. Foster (Trinity College, Dublin, Ireland). J.I. Flock (Karolinska Institutet, Stockholm, Sweden) supplied Efb.[13] S. Rooijakkers (University Medical Center Utrecht, Utrecht, the Netherlands) provided SCIN.[14] Alpha toxin, HlgB, LukD, LukE, LukF, LukS, SEA, and SEC were prepared as described previously.[11] SEB and TSST-1 were provided by S. Holtfreter and D. Grumann (University of Greifswald, Greifswald, Germany).[15] SSL1, SSL3, SSL5, SSL9, and SSL11 were a gift from J.D. Fraser (University of Auckland, Auckland, New Zealand).[16]

The coupling procedure was performed as described elsewhere.[5,10,17] In short, 25 μg of protein was added to 5.0 x 10^6 microspheres. This amount of protein was found to be optimal. As an activation buffer, we used 100 mmol/L monobasic sodium phosphate

(pH 6.2). To activate the carboxyl groups on the surface of the beads, 10 μL of 50 mg/mL *N*-hydroxysulfosuccinimide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide was used (Pierce Biotechnology). The coupling buffer consisted of 50 mmol/L 2-(*N*-morpholino)-ethanesulfonic acid (pH 5.0; Sigma-Aldrich). The final concentration of microspheres was adjusted to 3000 beads/μL with blocking-storage buffer (PBS-BN) consisting of phosphate-buffered saline (PBS), 1% bovine serum albumin, and 0.05% sodium azide (pH 7.4). The microspheres were protected from light and stored at 4°C until use. For control beads, the coupling procedure was performed in the absence of *S. aureus* protein. In each experiment, control beads were included to determine nonspecific binding. In case of nonspecific binding, the median fluorescence intensity (MFI) values were subtracted from the protein-specific results. As a negative control, PBS-BN was included.

Multiplex *S. aureus* antibody assay

IgG levels in serum directed against the above mentioned proteins were quantified simultaneously using a bead-based flow cytometry technique (xMap; Luminex Corporation). Methods have been described elsewhere.[5,10,17] In brief, 50 μL of serum, diluted 1:100 in PBS-BN was incubated with the microspheres in a 96-well 1.2-μm polyvinylidene fluoride filter microtiter plate (Millipore) for 35 minutes at room temperature on a Thermomixer plate shaker (Eppendorf). The plate was washed twice with PBS-BN that was aspirated by vacuum manifold. The microspheres (3000 beads per color per well) were resuspended in 50 μL of PBS-BN. In separate wells, 50 μL of a 1:100 dilution of R-phycoerythrin (RPE)-conjugated AffiniPure goat anti-mouse IgG (Abcam) was added. The plate was incubated for 35 minutes at room temperature on the plate shaker at 800 rpm and washed. The microspheres were resuspended in 100 μL of PBS-BN. Measurements were performed on the Luminex 100 instrument (BMD) using Luminex IS software (version 2.3). Tests were performed in independent duplicates, and MFI values, reflecting semi-quantitative antibody levels, were averaged. The coefficient of variation (CV) was calculated for each serum sample and averaged per protein.

The multiplex *S. aureus* antibody assays (serum incubated with the different fluorescence-colored protein-coupled beads mixed in one well) were developed. Two multiplex assays were used, one including Nuc, LytM, ClfA, and IsaA (multiplex 1), the other including ClfB, IsdA, IsdH, FnbpA, FnbpB, Efb, SCIN, alpha toxin, HlgB, LukD, LukE, LukF, LukS, SEA, SEB, SEC, TSST-1, SSL1, SSL3, SSL5, SSL9, and SSL11 (multiplex 2). Multiplex 2 was verified in a previous study[7] using human pooled serum (HPS). Multiplex

1 was verified in the present study using HPS. HPS was obtained from 36 healthy human donors of unknown *S. aureus* nasal carriage state.[5] MFI values for HPS obtained with the multiplex assay 1 were compared with the results for HPS obtained with singleplex assays (serum incubated with each different color of protein-coupled beads in separate wells). Cross reactivity between proteins and antibodies was assessed using serum samples from mice immunized with monovalent staphylococcal vaccines. Serum samples from mice with lung infection or skin infection caused by *S. aureus* strain LAC and from mice with intravenously-induced bacteremia caused by *S. aureus* isolate P or isolate S were analyzed. Mouse pooled serum (MPS) was used as a positive control. For MPS, mice inoculated intravenously with 5 x 10^5 cfu of *S. aureus* isolate P were bled 5 weeks after infection. Serum from non-infected mice was used as a negative control.

Statistical analysis

Statistical analyses were performed with SPSS software, version 15.0 (SPSS). The Mann-Whitney U test was used to compare median differences in anti-staphylococcal IgG levels. Differences were considered statistically significant when 2-sided *P*-values were < 0.05.

Results

Optimization and verification of the multiplex *S. aureus* antibody assay

In multiplex 1 and multiplex 2, a 1:100 dilution of mouse serum and a 1:100 dilution of RPE-conjugated AffiniPure goat anti-mouse IgG were found to be optimal. Next, multiplex 1 was verified using HPS. MFI values obtained for HPS with multiplex 1 were 76%, 80%, 94%, and 95% for Nuc, LytM, ClfA, and IsaA, respectively, of the MFI values obtained with the singleplex assays, indicating that multiplex 1 was approved for use. In multiplex 1 and multiplex 2, serum incubated with control beads (beads without protein coupled on their surface) resulted in median MFI values for IgG of 8 (range, 5 - 85), indicating that nonspecific binding was low. The negative control (PBS-BN) incubated with protein-coupled beads also resulted in low MFI values (≤12).

Reproducibility of the multiplex *S. aureus* antibody assay

For multiplex 1 and multiplex 2, inter-assay variation was investigated and calculated from MFI values obtained for MPS, which was included on each 96-wells plate. MFI values were averaged per protein. The median CV was 16%, and the range was 7% (IsaA) to 39% (LukF). The relatively high CV for LukF was due to the low MFI values, being close to 0.

Antibody profile in sera from mice immunized with monovalent staphylococcal vaccine candidates

To assess whether proteins on the microspheres cross reacted with serum antibodies directed against other proteins, the antibody profile in serum samples from mice immunized with GEM-based monovalent staphylococcal vaccines was determined. The

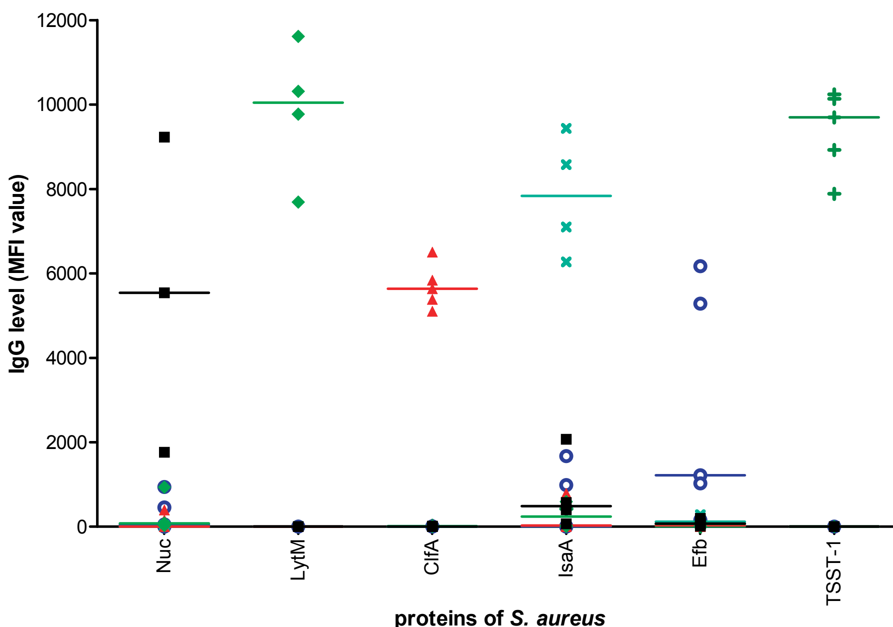

Figure 1. Median fluorescence intensity (MFI) values reflecting levels of protein-specific IgG for six *Staphylococcus aureus* proteins in sera from mice immunized with GEM-based monovalent staphylococcal vaccines. Different symbols represent mice immunized with different vaccines: black squares for Nuc containing vaccine, light green diamonds for LytM containing vaccine, red triangles for ClfA containing vaccine, turquoise crosses for IsaA containing vaccine, blue circles for Efb containing vaccine, and dark green plus signs for TSST-1 containing vaccine. Median levels of anti-staphylococcal IgG are indicated by horizontal lines. Nuc, endonuclease; LytM, peptidoglycan hydrolase; Clf, clumping factor; Isa, immunodominant staphylococcal antigen; Efb, extracellular fibrinogen-binding protein; TSST, toxic shock syndrome toxin.

MFI values reflecting serum IgG levels for individual mice are shown in Figure 1. In serum from protein-vaccinated mice, median serum IgG levels directed against the vaccine protein were high, while IgG levels against the other proteins were low.

Antibody profile in sera from mice with lung infection or skin infection caused by *S. aureus* strain LAC

The MFI values reflecting serum IgG levels for individual mice at 5 weeks after infection are shown in Figure 2. The protein-specific antibody levels showed substantial inter-individual variability. Median IgG levels in sera from non-infected mice were low and comparable to the negative control (PBS-BN). In both lung-infected mice and skin-infected mice, median serum IgG levels directed against Nuc, IsaA, Efb, alpha toxin, LukE, LukS, and SSL1 were significantly increased compared to non-infected

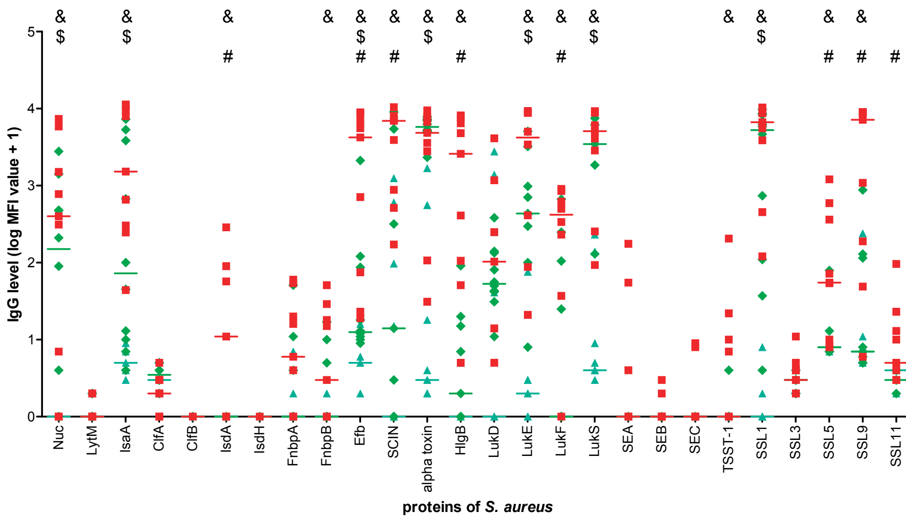

Figure 2. Median fluorescence intensity (MFI) values reflecting levels of protein-specific IgG for 26 *Staphylococcus aureus* proteins in sera from mice 5 weeks after induction of lung infection or skin infection caused by *S. aureus* strain LAC. Different symbols represent mice with different infections: red squares for mice with lung infection, green diamonds for mice with skin infection, and turquoise triangles for non-infected mice. Median levels of anti-staphylococcal IgG are indicated by horizontal lines. Statistically significant differences in median values are indicated between mice with lung infection and non-infected mice (&), between mice with skin infection and non-infected mice ($), and between mice with lung infection and mice with skin infection (#). Differences in protein-specific MFI values between groups were considered to be statistically significant at $P < 0.05$ (Mann-Whitney U test). Nuc, endonuclease; LytM, peptidoglycan hydrolase; Isa, immunodominant staphylococcal antigen; Clf, clumping factor; Isd, iron-responsive surface determinant; Fnbp, fibronectin-binding protein; Efb, extracellular fibrinogen-binding protein; SCIN, staphylococcal complement inhibitor; Hlg, γ hemolysin; Luk, leukocidin; SE, staphylococcal enterotoxin; TSST, toxic shock syndrome toxin; SSL, staphylococcal superantigen-like protein.

mice. Interestingly, differences between mice with lung infection or with skin infection caused by the same strain were also observed. Median IgG levels directed against IsdA, FnbpB, SCIN, HlgB, LukF, TSST-1, SSL5, and SSL9 were significantly increased only in lung-infected mice and not in skin-infected mice.

Antibody profile in sera from mice with intravenously-induced bacteremia caused by different *S. aureus* strains

The MFI values reflecting median IgG levels in mice before and at various intervals after infection are shown in Figure 3. Differences between *S. aureus* isolate P-infected mice and *S. aureus* isolate S-infected mice were only calculated for median IgG levels found at 5 weeks after infection. In both groups, isolate P-infected mice and isolate S-infected mice, one out of five mice died. Although protein-specific median IgG levels for SEA and TSST-1 were low, the median IgG levels were significantly increased in isolate S-infected mice compared to isolate P-infected mice. For Nuc, IsdA, Efb, SSL1, and SSL5 median IgG levels were significantly increased in isolate S-infected mice compared to isolate P-infected mice. Median IgG levels directed against most *S. aureus* proteins (for example Efb, HlgB, LukD, and LukF) increased with progression

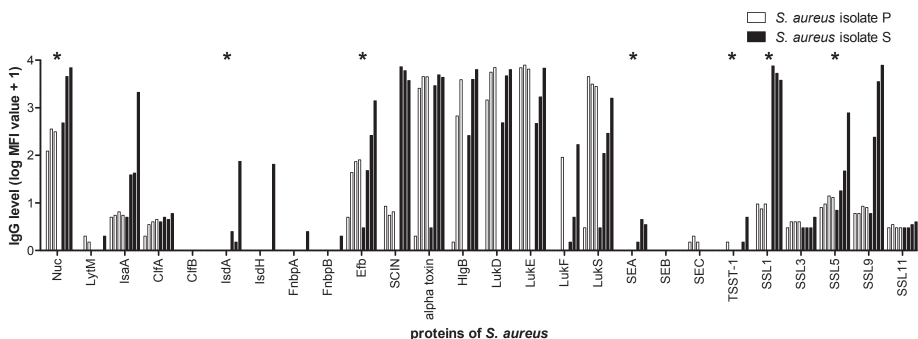

Figure 3. Median fluorescence intensity (MFI) values reflecting levels of protein-specific IgG for 26 *Staphylococcus aureus* proteins in sera from mice at various intervals after induction of intravenously-induced bacteremia caused by different *S. aureus* strains. Bars from left to right reflect IgG levels before infection, and at 2 weeks, 3 weeks, and 5 weeks after infection, respectively. Statistically significant differences in median IgG values are indicated between mice with bacteremia caused by *S. aureus* isolate P and mice with bacteremia caused by *S. aureus* isolate S at 5 weeks after bacterial inoculation (*). Differences in protein-specific MFI values between groups were considered to be statistically significant at $P < 0.05$ (Mann-Whitney U test). Nuc, endonuclease; LytM, peptidoglycan hydrolase; Isa, immunodominant staphylococcal antigen; Clf, clumping factor; Isd, iron-responsive surface determinant; Fnbp, fibronectin-binding protein; Efb, extracellular fibrinogen-binding protein; SCIN, staphylococcal complement inhibitor; Hlg, γ hemolysin; Luk, leukocidin; SE, staphylococcal enterotoxin; TSST, toxic shock syndrome toxin; SSL, staphylococcal superantigen-like protein.

of bacteremia up to a maximum at 5 weeks after infection, whereas towards some *S. aureus* proteins the maximum IgG levels were found at 2 or 3 weeks after infection (for example SCIN, alpha toxin, and SSL1).

Discussion

The multiplex *S. aureus* antibody assay is a suitable tool for investigating the humoral immune response against *S. aureus* proteins and may provide further insight into the role of these antigens in nasal colonization and infections with *S. aureus* in humans.[5,7,11] With this assay antibodies directed to at least 26 proteins can be measured in small volumes of serum, which in this respect is an advantage over the conventional ELISA technique. In the present study, we adapted this multiplex *S. aureus* antibody assay for use in experimental *S. aureus* infections in mice. The assay was optimized and verified for measuring IgG levels in mouse serum. For this purpose, sera from mice immunized with GEM-based monovalent staphylococcal vaccines were used. The use of this type of vaccines was described before by Audouy et al*.* as an efficient delivery system for mucosal vaccination.[18,19] We showed that cross reactivity between proteins on microspheres and serum antibodies towards other proteins was limited. It was concluded that the multiplex *S. aureus* antibody assay can successfully be applied for measuring serum antibody levels specific for *S. aureus* proteins.

In the present study, the multiplex *S. aureus* antibody assay was used to characterize the IgG profile in sera from mice with lung infection or skin infection caused by the same *S. aureus* strain LAC. Our data showed that the site of infection influences the IgG profile. Mice with severe lung infection had a higher and broader IgG response compared to mice with skin infection. Differences were most striking for the sortase-anchored surface protein IsdA, the immune modulators Efb and SCIN, the members of the leukotoxin family HlgB and LukF, and superantigen-like proteins SSL5, 9, and 11. Brown et al*.* already described the characteristics of these mice infected with severe lung infection or skin infection caused by *S. aureus* strain LAC, in terms of the course of infection, histopathology and quantitative cultures from the infected tissue.[4] Mice in both infection groups survived the infection. In their study, the antibody reactivity to a panel of *S. aureus* proteins was measured 4 weeks after skin infection with *S. aureus* strain LAC. These mice developed a significant response to LukF, LukS, alpha

toxin, and Efb. We also observed increased IgG levels against LukS and alpha toxin at 5 weeks after skin infection. However IgG levels for LukF and Efb were low.

Next, the multiplex *S. aureus* antibody assay was applied to characterize the IgG profile in sera from mice with similar infections, intravenously-induced bacteremia, caused by different *S. aureus* strains, isolate P or isolate S. These studies revealed different IgG responses against both *S. aureus* isolates. This observation in mice correlates well with data obtained in patients with *S. aureus* bacteremia, in whom antibody responses during the course of infection were specific for each patient.[7] In mice with bacteremia caused by *S. aureus* isolate S we observed a broader IgG response compared to mice with bacteremia caused by *S. aureus* isolate P, indicating that each *S. aureus* strain, exhibiting its own specific protein expression during infection, generates a characteristic IgG antibody profile over time. Most striking were the IgG levels for the sortase-anchored surface protein IsdA, the immune modulator Efb, superantigen-like proteins SSL1 and 5, and the nuclease Nuc, being significantly increased in isolate S-infected mice compared to isolate P-infected mice.

Summarizing, the data from the present study show that a bead-based multiplex *S. aureus* antibody assay can be successfully applied for investigating IgG responses related to *S. aureus* infections in mice. Only a small serum volume in the order of one to a few microliters is required. With this technique the immunogenicity of different proteins during the course of different *S. aureus* infections can be determined in mice. When measuring antibody levels in sera from patients, it is hard to assess the humoral immune response towards the causative *S. aureus* strain in infection, as patients probably had some or more previous encounters with different *S. aureus* strains. The use of *S. aureus*-free mice, which never have had contact with *S. aureus* before induction of the experimental infection, enables to assess and quantify the primary antibody responses to specific *S. aureus* proteins, and to investigate whether the immunogenicity of *S. aureus* proteins depended on the site of infection and/or the *S. aureus* isolate causing the infection. Whereas our study was focused exclusively on IgG directed against *S. aureus* proteins, it should be noted that cell-wall components, such as capsular polysaccharides 5 and 8,[20] peptidoglycan[21] and lipoteichoic acid,[22] are also known to be immunogenic. In future studies we will include the analysis of the host response against these cell-wall components as well. Moreover, next to IgG levels, other immunoglobulins and their subclasses will be investigated.

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4

Mild *Staphylococcus aureus* skin infection improves the course of subsequent endogenous *S. aureus* bacteremia in mice

Sanne van den Berg, Corné P. de Vogel, Alex van Belkum,
Irma A.J.M. Bakker-Woudenberg

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Abstract

Staphylococcus aureus carriers with *S. aureus* bacteremia may have a reduced mortality risk compared to non-carriers. A role for the immune system is suggested. Here, we study in mice the effect of *S. aureus* skin infection prior to an endogenous or exogenous *S. aureus* bacteremia, and evaluate levels of protection in relation to anti-staphylococcal antibody levels. Skin infections once or twice (interval 3 weeks) by a clinical *S. aureus* isolate (isolate P) or *S. aureus* strain 8325-4 were induced in mice free of *S. aureus* and anti-staphylococcal antibodies. Five weeks later, immunoglobulin G (IgG) levels in blood against 25 *S. aureus* antigens were determined, and moderate or severe bacteremia caused by *S. aureus* isolate P was induced. *S. aureus* skin infections led to elevated levels of anti-staphylococcal IgG in blood. One skin infection improved the course of subsequent severe endogenous bacteremia only. A second skin infection further improved animal survival, which was associated with further increased pre-bacteremia serum IgG levels against Efb, IsaA, LukD, LukE, Nuc, PrsA and WTA. In conclusion, in mice *S. aureus* skin infection reduces the severity of subsequent endogenous *S. aureus* bacteremia only. Anti-staphylococcal IgG against specified antigens may contribute to this effect.

Introduction

About 20% of the healthy human population persistently carries *Staphylococcus aureus* in their nose.[1-3] Although carriage of *S. aureus* is usually asymptomatic, this bacterial species can also cause infections. These include skin and soft tissue infections such as furunculosis, and also life-threatening invasive diseases such as pneumonia and bacteremia.[4] Nasal carriage of *S. aureus* is a major risk factor for the development of surgical site infections caused by *S. aureus*.[5-9] Moreover, Wertheim et al.[10] suggested that carriers have a three-fold higher risk than non-carriers of acquiring hospital-associated *S. aureus* bacteremia, while the mortality risk in carriers with bacteremia might be lower. Intranasal application of mupirocin effectively decolonizes and prevents invasive *S. aureus* infections in patients receiving long-term dialysis treatment[11-14] as well as surgical site infections.[15] Moreover, Bode et al. showed that rapid detection of *S. aureus* nasal carriage followed by immediate decolonization of nasal and extranasal sites with mupirocin nasal ointment and chlorhexidine gluconate soap reduced the risk of hospital-associated *S. aureus* infections by nearly 60%. As a result, hospital stay was reduced by almost two days on an average length of stay of 14 days.[16]

In spite of the higher risk of acquiring nosocomial *S. aureus* bacteremia in *S. aureus* carriers, the risk of death due to bacteremia might be lower once carriers have acquired bacteremia. An explanation for this has not yet been provided, although a role for the immune system has been proposed. More than 80% of health care-associated *S. aureus* infections are caused by an endogenous strain.[10,17] This suggests that because of long-term exposure to the colonizing *S. aureus* strain, carriers may have developed antibodies or cellular immune responses that protect against bacteremia-related death. Non-carriers may have developed similar responses that protect against colonization, which prevent them from being a carrier more than being protected against invasive disease. Several studies have been conducted comparing anti-staphylococcal antibody levels in carriers and non-carriers. Carriers show higher immunoglobulin G (IgG) levels than non-carriers against toxic shock syndrome toxin 1 (TSST-1), staphylococcal enterotoxin A (SEA)[18] and the factor effecting methicillin resistance (FmtB).[19] In contrast, compared to carriers, IgG levels in non-carriers are significantly higher against alpha toxin, major autolysin (Atl), iron-responsive surface determinant A and H (IsdA and IsdH), immunodominant staphylococcal antigen A (IsaA),[19] extracellular adherence protein (Eap), haptoglobin-hemoglobin binding protein A (HarA), and clumping factor

B (ClfB).[20] In addition to these descriptive studies, the prospective clinical study of Kolata et al.[21] also suggested a contribution of antibodies against the colonizing *S. aureus* strain in the improvement of the course and outcome of *S. aureus* bacteremia. In this study, *S. aureus* carriers who developed endogenous *S. aureus* bacteremia showed a stronger and broader pre-bacteremia IgG response to their own invasive, endogenous *S. aureus* strain compared to non-carriers, who develop an exogenous *S. aureus* bacteremia. Recently, Montgomery et al.[22] showed in an experimental study in mice that *S. aureus* skin and soft tissue infection (SSTI) protects against secondary SSTI. This protection was mediated by antibody and interleukin (IL) 17A and inhibited by interferon (IFN) γ. Conclusions regarding the antigen-specificity of these antibodies were not drawn. Their observation suggests, in addition to a role of humoral immunity, a protective role of cellular immunity.

In humans, conclusive studies on the exact influence of *S. aureus* carriage and the role of humoral and/or cellular immunity on the course and outcome of subsequent *S. aureus* infection are difficult as both carriers and non-carriers harbor anti-staphylococcal antibodies. In non-carriers, these antibodies may be induced by *S. aureus* carriage or (sub-)clinical infection in the past. Studies in mice initially free of *S. aureus* and anti-staphylococcal antibodies may provide further insight.

In the present study in mice, we investigated whether the course of *S. aureus* bacteremia is influenced by one or two prior *S. aureus* skin infections and whether this is dependent on the *S. aureus* strain (endogenous or exogenous) causing the skin infection. We focused in this study on the humoral immunity only, by analyzing the pre-bacteremia IgG levels against a broad panel of 25 *S. aureus* antigens following skin infection, and we assessed whether improvement in the course of *S. aureus* bacteremia was associated with pre-bacteremia IgG levels.

Materials and methods

Bacteria

Bacterial strains used were a clinical *S. aureus* isolate and *S. aureus* strain 8325-4, a well-characterized laboratory strain (MSSA, ST8).[23] *S. aureus* isolate P (gift from G. Buist, University of Groningen, University Medical Center Groningen, Groningen, the Netherlands) is a clinical isolate recovered from blood of a septic patient and was previously described and analyzed by proteomics by Ziebandt et al. (community-acquired

MSSA, ST7, *agr*-type 1).[24] Staphylococci were grown overnight at 35°C on Colombia III blood agar (Becton Dickinson). Cultures of *S. aureus*, grown in Brain Heart Infusion broth (Becton Dickinson) until OD_{560} ~ 1.0, were stored with 5% glycerol at -80°C. For infection, a suspension of staphylococci was defrosted and centrifuged for 10 minutes at 14,000 x *g*. The *S. aureus* pellet was resuspended in saline, and diluted to obtain the desired inoculum.

Animals

Specified opportunistic pathogen-free (SOPF) female BALB/cBYJ mice were obtained from Charles River. These *S. aureus*-free animals were 11-13 weeks old on the day of infection, and were given food and water *ad libitum*. Before each experiment, one mouse per group was sacrificed to confirm the *S. aureus*-free status in terms of cultures of fresh fecal and nasal microbiota and the absence of anti-staphylococcal IgG levels in blood (see below). The animal experimental protocols adhered to the rules laid down in the Dutch Animal Experimentation Act and the EU Animal Directive 2010/63/EU.

Model of *S. aureus* skin infection

The method of induction of *S. aureus* skin infection in mice was adapted from Brown et al.[25] In short, the lower back of the mice was shaved and cleaned with 70% ethanol under general anesthesia after using a mixture of medetomidine (Sedator®, 0.5 mg/kg; Eurovet Animal Health), midazolam (Midazolam, 5 mg/kg; Actavis) and fentanyl (Fentanyl, 0.05 mg/kg; Hameln Pharmaceuticals). *S. aureus* isolate P (3-6 x 10^7 cfu) or *S. aureus* 8325-4 (5-10 x 10^7 cfu) was injected intradermally (50 µL). The mice were antagonized using a mixture of atipamezole (Antisedan®, 2.5 mg/kg; Orion Corporation), flumazenil (Flumazenil, 0.5 mg/kg; Pharmachemie) and naloxon (Naloxon, 1.2 mg/kg; Orpha-Devel Handels und Vertriebs). Anesthetic and antagonistic agents were administered intraperitoneally, in a total volume of 175 and 250 µL, respectively. Mouse body weight was assessed three times a week. Two and five weeks after intradermal *S. aureus* inoculation, blood was withdrawn from the tail artery to determine anti-staphylococcal IgG levels in serum. Blood was collected in a Microvette® CB300 tube (Sarstedt) and sera were prepared and stored at -80°C. Presence of *S. aureus* in intestines and nasopharynx five weeks after intradermal *S. aureus* inoculation was determined by culturing fresh feces and nasopharyngeal lavage in phenol-red mannitol salt broth (PHMB; Becton Dickinson) at 35°C for 7 days. Nasopharyngeal lavage was performed by flushing the nares with 5 mL sterile phosphate buffered saline +

0.4% Tween 20 (Sigma-Aldrich). When PHMB demonstrated a yellow color, this was subcultured overnight at 35°C on Colombia III blood agar. *S. aureus* was identified based on colony morphology and Slidex Staph Plus agglutination testing (bioMérieux).

Model of *S. aureus* bacteremia

Bacteremia was induced by inoculation of 100 μL of *S. aureus* isolate P into the tail vein. A *S. aureus* inoculum at the 50% lethal dose (1-2 x 10^5 cfu) or at the 100% lethal dose (5-8 x 10^5 cfu) was used for establishment of moderate or severe bacteremia, respectively. Clinical signs of illness in each mouse were evaluated twice daily during the experiment, and mice that displayed severe signs of illness were euthanized by CO_2 exposure. Euthanized mice were considered as deaths, as pilot experiments showed that mice with severe signs of illness die before the next time point. Animal survival rate over 14 days after infection was monitored. At day 28, *S. aureus* load in blood and organs of surviving animals was determined after sacrificing the mice by CO_2 exposure. A blood sample was taken via a transcutaneous cardiac puncture and collected in a vial containing Lithium Heparin (Sarstedt). The lungs, spleen, liver, and kidneys were removed aseptically and homogenized (Polytron, Kinematica) in 2 mL of saline for 10 seconds at 30,000 rpm at room temperature. Undiluted homogenate suspensions and blood as well as 10-fold serial dilutions in saline were plated onto Colombia III blood agar. After incubation overnight at 35°C, colonies were counted.

Experimental set-up to study the course of *S. aureus* bacteremia in relation to prior *S. aureus* skin infection

Experimental set-up is shown in Figure 1. Skin infection with *S. aureus* isolate P or with *S. aureus* 8325-4 (n=15 per group) was induced 35 days before induction of bacteremia. Controls received intradermal inoculation of saline. In case of skin infection twice, three weeks after the first skin infection, a second skin infection using the same *S. aureus* strain was applied, near the inoculation site of the first skin infection. Bacteremia caused by *S. aureus* isolate P was always established at five weeks after the first skin infection.

Quantification of serum anti-staphylococcal IgG levels

Serum IgG levels against the following antigens were semi-quantified: alpha toxin; clumping factors A and B (ClfA and ClfB); extracellular fibrinogen-binding protein (Efb); ESAT-6-like protein (EsxA) and CFP-10-like protein (EsxB); formyl peptide receptor-

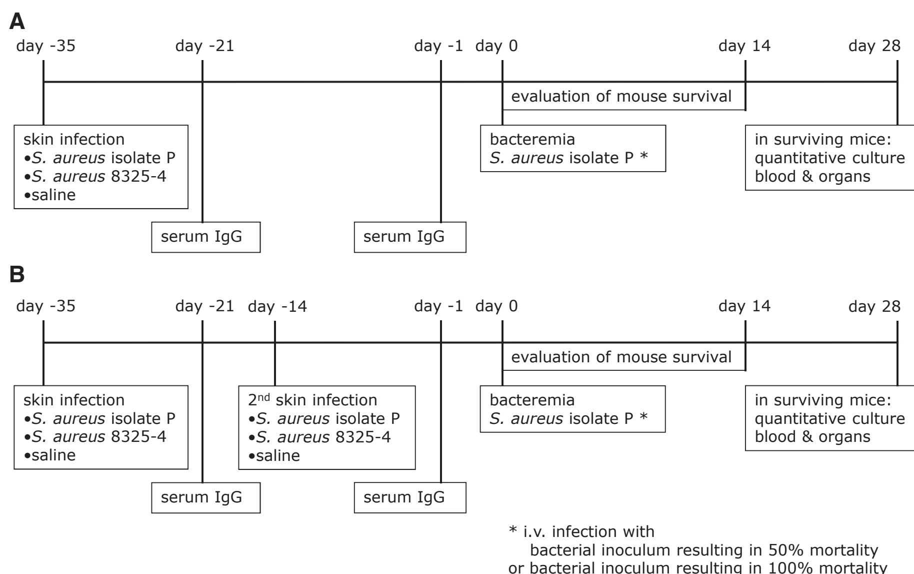


Figure 1. Experimental set-up to study the course of *S. aureus* bacteremia in relation to prior *S. aureus* skin infection. (A) Skin infection once at 35 days before bacteremia. (B) Skin infection twice at 35 days and 14 days before bacteremia.

like1 inhibitory protein (FLIPr); fibronectin-binding protein A (FnbpA); immunodominant staphylococcal antigen A (IsaA); iron-responsive surface determinants A and H (IsdA and IsdH); lipase; leukocidins D and E (LukD and LukE); peptidoglycan hydrolase (LytM); endonuclease (Nuc); peptidoglycan (PG); a parvulin-type peptidyl-prolyl *cis/trans* isomerase (PrsA); hypothetical protein SA0104; serine-aspartate dipeptide repeat protein D (SdrD); staphylococcal superantigen-like proteins 1, 3, 5, and 10 (SSL1, SSL3, SSL5, and SSL10); and wall teichoic acid (WTA). Genes encoding these *S. aureus* antigens were all present in both *S. aureus* isolate P and *S. aureus* 8325-4.

Alpha toxin, LukD, and LukE were prepared as described previously.[26] ClfB, FnbpA, IsdA, IsdH, and SdrD were expressed and purified as described previously.[18] The constructs were kindly provided by T. Foster (Trinity College, Dublin, Ireland). All other antigens were kindly provided by other research groups, as indicated in the Acknowledgments.

IgG levels were semi-quantified simultaneously in multiplex assays using a bead-based flow cytometry technique (xMap; Luminex Corporation). Methods have been described elsewhere.[27-29] Tests were performed in independent duplicates, and the median fluorescence intensity (MFI) values, reflecting semi-quantitative antibody levels,

were averaged. In each experiment, control beads (no antigen coupled) were included to determine nonspecific binding. In case of nonspecific binding, the nonspecific MFI values were subtracted from the antigen-specific values.

Serum samples from mice with skin infection caused by *S. aureus* isolate P or *S. aureus* 8325-4 (n=10 per group), and from mice with placebo skin infection were analyzed. Sera from three non-infected mice were used as negative controls.

Statistical analysis

The Mann-Whitney U test was used to compare median differences in anti-staphylococcal IgG levels in different groups. The Wilcoxon Signed Rank test was used to compare anti-staphylococcal IgG levels in paired samples. The Bonferroni correction was applied to correct for multiple testing. As a result, *P*-values < 0.002 were considered to be statistically significant. These statistical analyses were performed using the Statistical Package of Social Sciences version 17.0 for Windows (SPSS Inc.).

The Fisher's exact test was used to compare differences in *S. aureus* colonization status. The log rank test was used to determine statistical differences in animal survival rate between groups. Differences were considered statistically significant when

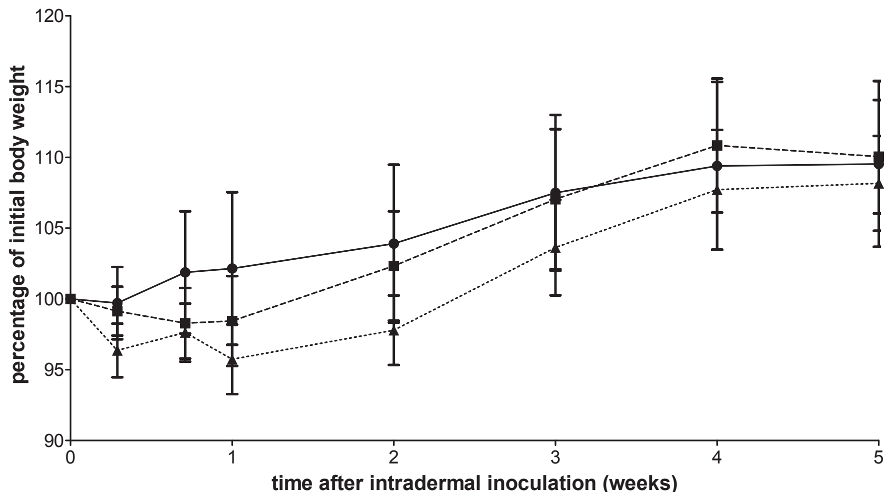


Figure 2. Body weight loss of mice with skin infection caused by *S. aureus* isolate P (■), *S. aureus* 8325-4 (▲), or placebo skin infection, saline inoculation (●) (n=10 per group). Data are expressed as mean ± SD (error bars).

2-sided *P*-values were < 0.05. GraphPad Prism 5 for Windows (GraphPad Software Inc) was used for these statistical analyses.

Results

S. aureus skin infection

Mice infected with *S. aureus* isolate P or *S. aureus* 8325-4 developed a scab (area of ~1.5 cm^2) at the inoculation site within 1 week. Body weight loss was only minor (Figure 2) and mice experienced little discomfort. Five weeks after infection, when *S. aureus* bacteremia was induced, the skin had healed and body weight was restored. *S. aureus* cultures from the inoculation site were always negative at week 5 after skin infection. However, the intestines of mice with skin infection once or twice, caused by *S. aureus* isolate P or *S. aureus* 8325-4, were always culture positive for the infecting *S. aureus* strain. Nasopharynges were not always culture positive: *S. aureus* was cultured from mice after *S. aureus* isolate P skin infection once (3 of 10 mice) or twice (5 of 10 mice), and after *S. aureus* 8325-4 skin infection once (1 of 10 mice) or twice (4 of 10 mice). No significant differences in number of nasopharynx culture positive mice were observed.

Anti-staphylococcal IgG levels following skin infection

Serum anti-staphylococcal IgG levels were assessed two and five weeks after *S. aureus* isolate P skin infection once or twice (Table 1) or after *S. aureus* 8325-4 skin infection once or twice (Table 2). Before infection (week 0), IgG levels were at background values. In mice with skin infection once caused by *S. aureus* isolate P or *S. aureus* 8325-4, IgG levels against a number of *S. aureus* antigens were observed at week 2 and 5 after infection, but these did not change significantly over time. However, following the second skin infection with *S. aureus* isolate P, the IgG levels against IsaA, Nuc, PrsA, and WTA were significantly elevated at week 5 ($P < 0.002$), whereas the second skin infection with *S. aureus* 8325-4 resulted in a significant rise in IgG levels at week 5 against Efb, IsaA, and IsdA ($P < 0.002$).

Table 1. *S. aureus* isolate P skin infection. Median fluorescence intensity (MFI) values reflecting levels of antigen-specific IgG against 25 *S. aureus* antigens in sera from mice (n=10 per group) before skin infection (week 0), at week 2 or week 5 after skin infection once, and at week 5 after skin infection twice.

Antigen	Median MFI week 0 (range)	Skin infection once		Skin infection twice	*P*-value (skin infection once) week 2 vs week 0[a]	*P*-value (skin infection once) week 5 vs week 0[a]	*P*-value (skin infection once) week 5 vs week 2[b]	*P*-value (week 5) skin infection twice vs once[a]
		Median MFI week 2 (range)	Median MFI week 5 (range)	Median MFI week 5 (range)				
alpha toxin	0 (0-1)	644 (203-1712)	5693 (4402-6704)	3994 (3381-5922)	0.011	0.011	0.005	0.007
ClfA	0 (0-0)	0 (0-0)	0 (0-4)	0 (0-5)	1.000	0.584	0.317	0.304
ClfB	0 (0-0)	0 (0-0)	0 (0-14)	0 (0-4)	1.000	0.584	0.317	0.358
Efb	3 (1-6)	4 (2-8)	11 (7-1536)	143 (33-1590)	0.611	0.011	0.005	0.070
EsxA	5 (5-6)	5 (5-6)	6 (4-8)	5 (3-8)	0.661	0.495	0.313	0.569
EsxB	3 (3-4)	4 (3-24)	5 (3-46)	4 (2-177)	0.551	0.062	0.028	0.119
FLIPr	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-44)	1.000	1.000	1.000	0.068
FnbpA	0 (0-0)	0 (0-3)	0 (0-14)	0 (0-0)	0.584	0.584	0.317	0.317
IsaA	5 (4-6)	12 (6-1504)	8 (5-1509)	3885 (247-8090)	0.014	0.034	0.386	0.001 *
IsdA	103 (3-106)	77 (0-108)	30 (0-94)	70 (0-432)	0.498	0.236	0.012	0.072
IsdH	63 (27-68)	38 (21-61)	36 (11-50)	41 (20-1065)	0.236	0.236	0.475	0.257
lipase	6 (6-6)	9 (8-14)	8 (7-20)	80 (9-8188)	0.011	0.011	0.959	0.002
LukD	0 (0-0)	0 (0-0)	103 (1-1986)	1595 (34-5177)	0.584	0.011	0.005	0.019
LukE	2 (0-2)	235 (13-777)	4470 (2110-6930)	6487 (4921-7596)	0.011	0.011	0.005	0.023
LytM	1 (1-3)	2 (0-5)	2 (1-3)	4 (2-66)	0.865	0.441	0.918	0.015
Nuc	2 (1-3)	6 (0-270)	405 (2-3999)	4554 (930-5686)	0.128	0.028	0.013	0.001 *
PG	6 (4-6)	6 (5-6)	6 (5-7)	6 (5-7)	0.302	0.395	0.777	0.223
PrsA	32 (28-33)	405 (194-900)	3282 (1728-4490)	4568 (3592-5838)	0.011	0.011	0.005	0.0019 *
SA0104	5 (4-6)	6 (4-19)	6 (4-34)	6 (5-9)	0.125	0.106	0.574	0.939
SdrD	0 (0-0)	0 (0-10)	0 (0-22)	0 (0-5)	0.584	0.584	0.317	0.942
SSL1	0 (0-0)	0 (0-50)	1 (0-63)	21 (0-34)	0.215	0.148	0.500	0.292
SSL3	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	1.000	1.000	1.000	1.000
SSL5	0 (0-0)	0 (0-2)	3 (0-274)	0 (0-20)	0.584	0.098	0.028	0.033
SSL10	0 (0-0)	0 (0-2)	1 (0-12)	0 (0-2)	0.147	0.098	0.324	0.606
WTA	1 (0-1)	1 (0-2)	4 (2-10)	22 (4-78)	0.283	0.011	0.008	0.0019 *

[a] Mann-Whitney U test

[b] Wilcoxon signed ranks test

* $P < 0.002$

Table 2. *S. aureus* 8325-4 skin infection. Median fluorescence intensity (MFI) values reflecting levels of antigen-specific IgG against 25 *S. aureus* antigens in sera from mice (n=10 per group) before skin infection (week 0), at week 2 or week 5 after skin infection once, and at week 5 after skin infection twice.

Antigen	Median MFI week 0 (range)	Skin infection once		Skin infection twice	*P*-value (skin infection once) week 2 vs week 0[a]	*P*-value (skin infection once) week 5 vs week 0[a]	*P*-value (skin infection once) week 5 vs week 2[b]	*P*-value (week 5) skin infection twice vs once[a]
		Median MFI week 2 (range)	Median MFI week 5 (range)	Median MFI week 5 (range)				
alpha toxin	0 (0-1)	1129 (354-2341)	5545 (2110-6932)	4401 (2878-6605)	0.011	0.011	0.005	0.258
ClfA	0 (0-0)	0 (0-0)	0 (0-1)	0 (0-0)	1.000	0.584	0.317	0.317
ClfB	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-2)	1.000	1.000	1.000	0.317
Efb	3 (1-6)	4 (2-8)	8 (6-12)	24 (15-80)	0.445	0.017	0.009	0.0002 *
EsxA	5 (5-6)	5 (3-6)	6 (4-6)	6 (5-6)	0.865	0.670	0.366	0.335
EsxB	3 (3-4)	3 (3-12)	4 (3-6)	4 (2-39)	0.733	0.351	0.483	0.731
FLIPr	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	1.000	1.000	1.000	1.000
FnbpA	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-2)	1.000	1.000	1.000	0.147
IsaA	5 (4-6)	7 (4-61)	6 (5-166)	186 (12-6838)	0.235	0.089	0.918	0.001 *
IsdA	103 (3-106)	54 (0-129)	82 (0-221)	525 (120-1145)	0.397	0.612	0.161	0.001 *
IsdH	63 (27-68)	34 (28-60)	36 (14-53)	37 (19-61)	0.398	0.237	0.359	0.650
lipase	6 (6-6)	12 (5-166)	141 (8-1992)	405 (8-6865)	0.011	0.011	0.008	0.364
LukD	0 (0-0)	0 (0-61)	7 (0-112)	11 (2-57)	0.304	0.037	0.012	0.325
LukE	2 (0-2)	5 (0-345)	57 (5-1008)	118 (7-1743)	0.034	0.011	0.022	0.364
LytM	1 (1-3)	2 (1-3)	2 (1-3)	3 (1-22)	0.492	0.293	0.429	0.333
Nuc	2 (1-3)	1 (0-13)	2 (0-15)	11 (1-1646)	0.497	0.670	0.130	0.031
PG	6 (4-6)	6 (5-6)	6 (4-7)	6 (5-7)	0.664	0.389	0.589	0.373
PrsA	32 (28-33)	190 (43-303)	1074 (153-2502)	3167 (264-4967)	0.011	0.011	0.005	0.034
SA0104	5 (4-6)	6 (5-8)	5 (4-8)	7 (4-23)	0.034	0.391	0.087	0.030
SdrD	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-3)	1.000	1.000	1.000	0.317
SSL1	0 (0-0)	1 (0-93)	45 (0-3834)	41 (1-4112)	0.148	0.062	0.043	0.545
SSL3	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	1.000	1.000	1.000	1.000
SSL5	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-5)	1.000	1.000	1.000	0.317
SSL10	0 (0-0)	0 (0-5)	0 (0-6)	1 (0-5)	0.215	0.148	0.564	0.209
WTA	1 (0-1)	1 (0-2)	2 (0-7)	3 (1-12)	0.122	0.383	0.443	0.068

[a] Mann-Whitney U test

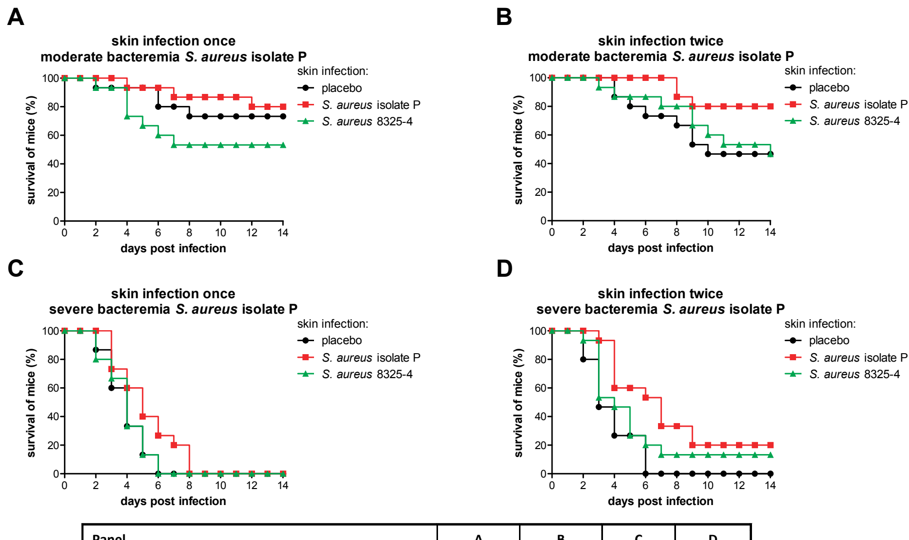
[b] Wilcoxon signed ranks test

* $P < 0.002$

Course of *S. aureus* bacteremia in mice with prior skin infection

Bacteremia was always induced by *S. aureus* isolate P irrespective of the strain used for skin infection. Animal survival rate after bacteremia was monitored over a 14-day evaluation period. After this time point, no further changes in animal survival were observed (data not shown).

The course of moderate *S. aureus* bacteremia in mice with prior skin infection is shown in Figures 3A and B. In mice with placebo skin infection, survival of bacteremic mice declined gradually, until 47-73% at day 14. Survival rates of mice with placebo skin infection once or twice were comparable. After skin infection once or twice with *S. aureus* isolate P or *S. aureus* 8325-4, animal survival rate of bacteremic mice was not significantly improved compared to placebo skin infection.



Panel	A	B	C	D
Skin infection *S. aureus* isolate P or *S. aureus* 8325-4	once	twice	once	twice
Bacteremia *S. aureus* isolate P	moderate	moderate	severe	severe
Statistical evaluation	***P*-values**			
S. aureus isolate P *vs* placebo	0.639	0.052	0.033 ★	0.001 ★
S. aureus 8325-4 *vs* placebo	0.208	0.828	0.954	0.240
S. aureus isolate P *vs S. aureus* 8325-4	0.094	0.070	0.038 ★	0.119

Figure 3. Animal survival rate in mice with moderate or severe *S. aureus* bacteremia caused by *S. aureus* isolate P and prior skin infection (once or twice). Skin infection was induced by *S. aureus* isolate P (■) or *S. aureus* 8325-4 (▲); placebo skin infection (●). Animal survival rate in different groups (n=15 per group) was compared using the log-rank test, and *P*-values are indicated in the table. * statistically significant ($P < 0.05$).

The course of severe *S. aureus* bacteremia in mice with prior skin infection is shown in Figures 3C and D. In mice with placebo skin infection, survival of bacteremic mice declined gradually, and at day 6 all mice had died. Survival rates of mice with placebo skin infection once or twice were comparable. An increased animal survival rate of bacteremic mice was observed only after prior skin infection with *S. aureus* isolate P. The time to death of bacteremic mice was increased after skin infection once (P = 0.033), and was further prolonged after the second skin infection (P = 0.001). Skin infection once or twice with *S. aureus* 8325-4 had no effect on the course of bacteremia.

In mice that survived moderate bacteremia (Figures 3A and B) or severe bacteremia (Figure 3D), *S. aureus* was never cultured from blood, lungs, spleen, and liver at day 28. In 17-60% of surviving mice, cultures from kidneys were *S. aureus*

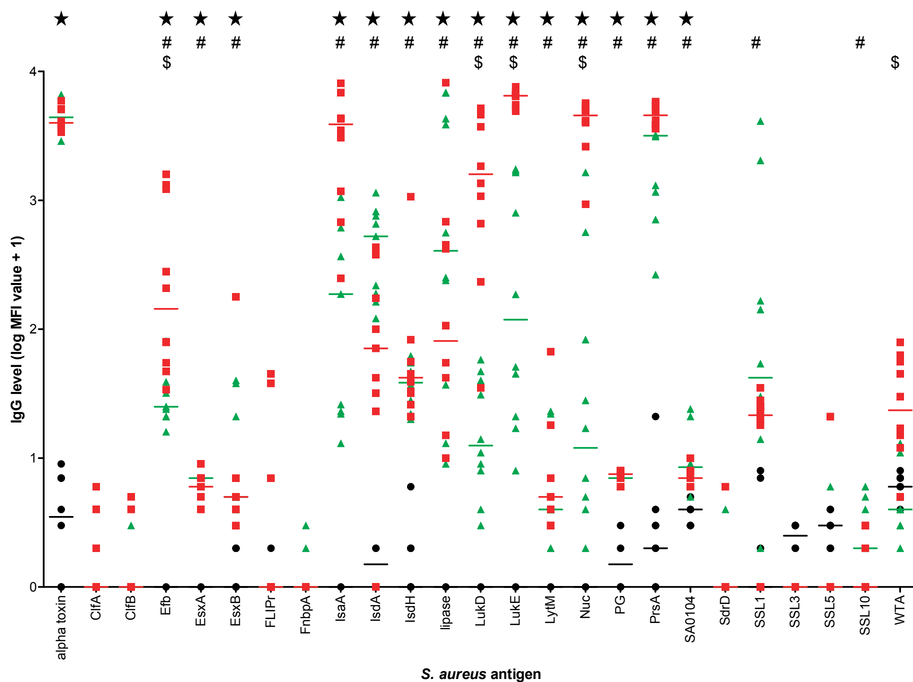


Figure 4. Median fluorescence intensity (MFI) values reflecting levels of antigen-specific IgG against 25 *S. aureus* antigens in sera from mice (n=10 per group) at week 5 after *S. aureus* skin infection twice. Skin infections were induced by *S. aureus* isolate P (■) or *S. aureus* 8325-4 (▲); placebo skin infection (●). ∗ indicates significant differences ($P < 0.002$) between mice with *S. aureus* isolate P skin infection and mice with placebo skin infection. # indicates significant differences ($P < 0.002$) between mice with *S. aureus* 8325-4 skin infection and mice with placebo skin infection. $ indicates significant differences ($P < 0.002$) between mice with *S. aureus* isolate P skin infection and mice with *S. aureus* 8325-4 skin infection.

positive, but differences in number of *S. aureus* positive kidneys between the mice that survived moderate bacteremia were not observed.

We assessed whether protection against *S. aureus* bacteremia was associated with increased levels of anti-staphylococcal IgG induced by *S. aureus* skin infection. To this aim, in mice receiving skin infection twice caused by *S. aureus* isolate P or *S. aureus* 8325-4, serum IgG levels at week 5 after skin infection, the day of induction of *S. aureus* isolate P bacteremia were compared. IgG levels are shown in Figure 4. In *S. aureus* isolate P skin infected mice, IgG levels against alpha toxin, Efb, EsxA, EsxB, IsaA, IsdA, IsdH, lipase, LukD, LukE, LytM, Nuc, PG, PrsA, and SA0104 were significantly higher than in mice with placebo skin infection. In *S. aureus* 8325-4 skin infected mice, IgG levels against Efb, EsxA, EsxB, IsaA, IsdA, IsdH, lipase, LukD, LukE, LytM, Nuc, PG, PrsA, SA0104, SSL1 and SSL10 were significantly higher than in mice with placebo skin infection. Comparing both IgG profiles, IgG levels against Efb, LukD, LukE, Nuc, and WTA were significantly higher in *S. aureus* isolate P skin infected mice than in *S. aureus* 8325-4 skin infected mice.

Discussion

In the present study, we showed for the first time that mild *S. aureus* skin infection improved the course of subsequent endogenous *S. aureus* bacteremia. The use of *S. aureus*-free mice enabled us to draw conclusions in this respect. In humans, conclusive studies on the exact influence of *S. aureus* exposure on the outcome of *S. aureus* bacteremia remain difficult, as both carriers and non-carriers have been exposed to *S. aureus*.[18-21] Wertheim et al.[10] already suggested that carriers have a decreased risk of death due to *S. aureus* bacteremia compared to non-carriers, hypothesizing that anti-staphylococcal antibody levels or components of the cellular immune system that are increased in carriers may play a role in protection against death due to *S. aureus* bacteremia. Our observations in the present study in mice are in line with the suggestions made by Wertheim et al.

The role of mild *S. aureus* skin infection once or twice on the course and outcome of subsequent endogenous or exogenous invasive *S. aureus* disease was investigated in mice. We established local *S. aureus* skin infection caused by either *S. aureus* isolate P, a clinical sepsis isolate,[24] or *S. aureus* 8325-4, a frequently used sequenced strain.[23] Subsequent bacteremia was always induced by *S. aureus* isolate P. Skin infec-

tion led to *S. aureus* colonization in intestines and nasopharynges, and with respect to the humoral immune response, anti-staphylococcal IgG in blood was found. The skin infection described above was mild and transient, simulating a mild *S. aureus* infection in humans. We showed that prior *S. aureus* skin infection improved the course of severe *S. aureus* isolate P bacteremia provided that bacteremia was caused by the endogenous *S. aureus* strain. The protective effect was not observed in exogenous bacteremia caused by *S. aureus* isolate P when skin infection was induced by *S. aureus* 8325-4. While a single prior skin infection resulted in delayed time to death in severe endogenous *S. aureus* bacteremia, in moderate endogenous *S. aureus* bacteremia, prior skin infection did not. This lack of a protective effect in moderate *S. aureus* bacteremia may be related to the relatively small window to detect significant differences in this respect. Whereas in the present study bacteremia was always caused by *S. aureus* isolate P, in future experiments it would be informative to study the effects of skin infection on the course and outcome of subsequent *S. aureus* 8325-4 bacteremia.

We also demonstrated that a second skin infection following the first skin infection further enhanced the protective effect in severe endogenous *S. aureus* bacteremia. Also in the model of moderate endogenous *S. aureus* bacteremia, mortality was reduced, although this difference was only borderline significant ($P = 0.052$). Interestingly, following the second skin infection, pre-bacteremia serum IgG levels were elevated as well. As protection against *S. aureus* bacteremia was associated with strong increases in pre-bacteremia anti-staphylococcal IgG levels, this suggests that IgG resulting from prior mild skin infection may potentially act protective against antigens determining the outcome of *S. aureus* bacteremia. Future studies including successful adoptive transfer of antibody purified from serum of skin infected mice to naive mice prior to *S. aureus* bacteremia may confirm the protective role of pre-bacteremia IgG.

Regarding the pre-bacteremia IgG levels, most striking were the IgG levels against IsaA, Nuc, PrsA, and WTA, which were further elevated following the second skin infection. These IgG levels may contribute to the delayed time to death and improved animal survival in mice with *S. aureus* bacteremia. Next to IgG against these *S. aureus* antigens, pre-bacteremia IgG against Efb, LukD, and LukE may also contribute in this respect. These IgG levels were elevated in mice with skin infection twice and endogenous bacteremia compared to mice with skin infection twice and exogenous bacteremia.

Our observation that *S. aureus* skin infection was protective in the course of subsequent endogenous *S. aureus* bacteremia in mice are in line with those obtained in

a recently published experimental study in mice by Montgomery et al.[22] They studied the effect of *S. aureus* skin infection (SSTI) on a secondary SSTI caused by the same *S. aureus* strain. It was shown that skin infection protected against the subsequent skin infection. In addition, they demonstrated that this protection was mediated by antibody and IL-17A and inhibited by IFN-γ as shown by antibody transfer to naive mice and neutralization of IL-17A or IFN-γ prior to infection, respectively. While in their study IgG levels against Hla and IsdB were assessed, the present study in mice included IgG levels against a broad panel of 25 *S. aureus* antigens. Future studies in mice on the effect of immunization targeting IsaA, Nuc, PrsA, WTA, Efb, LukD and LukE prior to the induction of *S. aureus* bacteremia may shed further light on the protective role of these anti-staphylococcal IgGs in bacteremia. In addition to these antigens, antibodies against *S. aureus* antigens not included in the present study may also be associated with protection against *S. aureus* bacteremia. Further insight may be obtained from adoptive antibody transfer studies.

Next to the potential role of pre-bacteremia anti-staphylococcal antibodies, components of cellular immunity are expected to contribute as well to the improvement of the animal survival rate in mice with endogenous *S. aureus* bacteremia, as the cellular immune response has a role in *S. aureus* infections.[22,30,31] Regarding the role of cellular immunity in reduction of *S. aureus* bacteremia-related mortality, conclusions cannot be drawn from the present study which was focused on the humoral immune response prior to *S. aureus* bacteremia. However Montgomery et al.[22] showed that in their model of SSTI protection against the second skin infection in BALB/c mice was mediated by both antibody and IL-17A. It would be very interesting to investigate the role of IL-17A and other components of cellular immunity in the *S. aureus* bacteremia model used in the present study.

In addition to pre-bacteremia immune responses, the consequences of exposure to *S. aureus* in the intestines and nasopharynx following *S. aureus* skin infection may also contribute to the improvement of the animal survival rate. As quantitative cultures from the intestinal and nasopharyngeal flora were not performed, definite conclusions on the role of intestinal and nasopharyngeal colonization cannot be drawn.

In conclusion, we show that *S. aureus* skin infection prior to *S. aureus* bacteremia improved the outcome of endogenous invasive infection. Increased levels of protection may be associated with the elevated levels of anti-staphylococcal IgGs against specified antigens. The observation that anti-*S. aureus* IgG may contribute to improvement of the course and outcome of endogenous *S. aureus* bacteremia opens new

perspectives to investigate the protective capacity of active and passive immunization as a non-antibiotic-based treatment regimen in patients with *S. aureus* bacteremia. Further studies using other *S. aureus* strains are needed to generalize and support our conclusions. In this respect, also the role of cellular immunity should be further investigated.

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5

Distinctive cytokines as biomarkers predicting fatal outcome of severe *Staphylococcus aureus* bacteremia in mice

Sanne van den Berg, Jon D. Laman, Louis Boon, Marian T. ten Kate, Gerjo J. de Knegt, Rob M. Verdijk, Henri A. Verbrugh, Jan L. Nouwen, Irma A.J.M. Bakker-Woudenberg

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Abstract

Invasive *Staphylococcus aureus* infections are frequently associated with bacteremia. To support clinical decisions on antibiotic therapy, there is an urgent need for reliable markers as predictors of infection outcome. In the present study in mice, bacteremia was established by intravenous inoculation of a clinical *S. aureus* isolate at the LD_{50} inoculum. As potential biomarkers for fatal outcome, blood culture (qualitative and quantitative), serum levels of C-reactive protein (CRP), as well as 31 selected cytokines and chemokines were assessed during the first three days of infection. A positive *S. aureus* blood culture, the quantitative blood culture, CRP levels, and levels of eight cytokines were indicative for the presence of *S. aureus* bacteremia. However, only tumor necrosis factor (TNF) α, interleukin (IL) 1α, and keratinocyte chemoattractant (KC; a functional homologue of human IL-8) were each significantly elevated in eventually non-surviving infected mice versus eventually surviving infected mice. In severe *S. aureus* bacteremia in mice, TNF-α, IL-1α, and KC are biomarkers predicting fatal outcome of infection. KC was a biomarker elevated irrespective the progression of infection, which is very interesting regarding clinical application in view of the heterogeneity of patients experiencing bacteremia in this respect.

Introduction

Staphylococcus aureus is an important opportunistic pathogen that causes a variety of infections, from relatively mild infections such as skin infections and food poisoning, to life-threatening conditions such as necrotizing pneumonia and osteomyelitis.[1] The invasive infections are frequently associated with *S. aureus* bacteremia.[2] Shorr et al. showed that *S. aureus* was the most common bacterial isolate in 6,697 bloodstream infections in the USA, and *S. aureus* was more strongly associated with mortality than any other bacterial pathogen.[3]

A clinically significant bacteremia is generally defined as the isolation of bacteria from one or more peripheral venous blood culture samples collected from a patient with associated relevant clinical symptoms of systemic infection as defined on the 2001 International Sepsis Definitions Conference.[4] When sepsis is strongly suspected, an appropriate treatment regimen for each patient is designed.

Blood culture confirms the presence of bacteremia, and allows identification of the causative infectious agent. Rapid diagnosis and (antibiotic) treatment of bacteremia is essential, as any delay in treatment may lead to worse outcome. On the other hand, antibiotic use should be limited, and proper decision on initiating antibiotic therapy or adjustment of antibiotic choice in therapy already started in severe bacteremia is highly important. Knowledge of how to predict fatal outcome in patients with *S. aureus* bacteremia is currently lacking. To support clinical decisions on these issues, there is an urgent need for reliable markers that can be used as predictors of outcome of infection. An obvious candidate biomarker may be C-reactive protein (CRP), which is the classic acute phase protein. CRP is released by the liver to the blood, and activates the complement system. This marker is currently used to determine inflammation and tissue damage.[5] Systemic levels of CRP are elevated in septic traumatized patients compared to non-septic traumatized patients.[6] Synthesis of CRP by hepatocytes starts very rapidly, and CRP can be detected in plasma after 6-12 hours, and a plateau is reached after 20-72 hours. Plasma half-life of CRP is about 19 hours.[7-9]

Bacteremia can lead to sepsis and multiple organ failure, and eventually to death when therapy fails. For many years, it was assumed that sepsis is a consequence of an overwhelming inflammatory reaction of the patient to microorganisms. Neutralization of single pro-inflammatory cytokines like tumor necrosis factor (TNF) α or interleukin (IL) 1 in animal models of sepsis resulted in protection against lethal sepsis.[10-14] In contrast to these murine studies, inhibition of these cytokines has not provided clini-

cal benefit to patients with severe sepsis.[15-20] Therefore, it is clear that not only the pro-inflammatory reaction forms the basis for adverse outcome in sepsis, immune suppression by anti-inflammatory reactions is involved as well. As cytokines are important in sepsis, they are candidate biomarkers for presence and fatal outcome of *S. aureus* bacteremia. Clinical studies determined cytokine levels in sepsis or sepsis-like situations. Two studies assessed cytokine levels in healthy volunteers after lipopolysaccharide (LPS) injection. Dandona et al.[21] found elevated serum levels of TNF-α, and Van der Poll et al.[22] described a rise in IL-10 plasma levels. Cytokine levels in septic patients were measured as well. These studies did not focus on a single causative infectious agent. Levels of IL-1β,[23] IL-2,[24] IL-6,[23,25-28] IL-8,[24] IL-10,[22,23,26-28] TNF-α,[23,28,29] and interferon (IFN) γ[28] were elevated in septic patients compared to normal levels. Levels of IL-6,[23,26] IL-10[22,30] and TNF-α[30] were predictive for fatal outcome in patients with sepsis, while IL-10[23,26] levels were predictive for survival. It is difficult to evaluate the value of changes in cytokine profile during bacteremia in patients due to differences in the causative infectious agents in these studies. In addition, only a limited number of cytokines were included in these studies, which hinders the comparison of results obtained from the various studies.

Hence, we hypothesized that cytokines are useful as biomarkers for fatal outcome of *S. aureus* bacteremia. The present study in mice was performed to identify biomarkers predicting fatal outcome of severe *S. aureus* bacteremia. We focused on blood culture, CRP, and selected cytokines. To this aim, we established an *in vivo* model of severe *S. aureus* bacteremia in mice, using a clinical *S. aureus* isolate. The use of a *S. aureus* inoculum resulting in 50% mortality allowed comparing eventually surviving infected mice and eventually non-surviving infected mice in the same model.

Materials and methods

Bacteria

A clinical *S. aureus* isolate (isolate P), recovered from a septic patient, was used. This isolate was kindly supplied by G. Buist (University Medical Center Groningen, the Netherlands) and described by Ziebandt et al. (CA-MSSA, MLST type 7, *agr*-type 1, *pvl*-negative).[31] Staphylococci were grown overnight at 35°C on Colombia III blood agar (Becton Dickinson). Cultures of *S. aureus*, grown in Brain Heart Infusion broth (Becton Dickinson) until OD_{560} ~ 1.0, were stored at -80°C.

Animals

Specified opportunistic pathogen free (SOPF) female BALB/cBYJ *S. aureus*-free mice (11-13 weeks at day of infection) were obtained from Charles River, and were given food and water *ad libitum*. Before each experiment, *S. aureus*-free status was checked by culture of fresh fecal and nasal microbiota as well as by confirming the absence of anti-staphylococcal IgG levels against 54 antigens using Luminex technology.[32]

Ethics statement

The experimental protocols adhered to the rules specified in the Dutch Animal Experimentation Act (1977) and the published Guidelines on the Protection of Experimental Animals by the Council of the EC (1986). The Institutional Animal Care and Use Committee of the Erasmus University Medical Center Rotterdam approved the present protocols.

S. aureus bacteremia

A suspension of *S. aureus* was defrosted and centrifuged for 10 minutes at 14,000 x *g*. The *S. aureus* pellet was resuspended in saline, and diluted to obtain the desired inoculum. To establish bacteremia, 100 μL of *S. aureus* isolate P was injected into the tail vein.

Various *S. aureus* inocula, resulting in 0-100% mortality, were injected intravenously (n=5 per inoculum) to determine the inoculum-dependent cumulative mouse mortality. Clinical signs of illness in each mouse were evaluated twice daily. Mice with bad fur were scored -2. Mice with bad fur and hunched back were scored -3. Mice with bad fur and hunched back and that were instable, were scored -4. These mice showed severe signs of illness and were euthanized by CO_2 exposure. Euthanized mice were considered as death, as pilot experiments showed that mice with severe signs of illness died before the end of the experiment. Animal survival 14 days after inoculation was monitored and cumulative mortality was calculated. For experiments, groups of mice (n=25) were infected with a *S. aureus* inoculum at the 50% lethal dose (LD_{50}). Animal survival rate, body weight, and discomfort were monitored over 14 days. From mice that were euthanized due to severe signs of illness, blood, lung, spleen, liver, and kidneys were cultured to confirm that they died because of *S. aureus* infection only.

At various intervals after infection, the bacterial load in blood and infected organs was determined. Mice (n=4 per time point) were sacrificed at 1, 17, or 48 hours by CO_2 exposure. A blood sample was taken via (transcutaneous) cardiac puncture and collected in a vial containing Lithium Heparin (Sarstedt). The lungs, spleen, liver, and kidneys were removed aseptically and homogenized (Polytron, Kinematica) in 2 mL

of saline for 10 seconds at 30,000 rpm at room temperature. Undiluted homogenate suspensions and blood and 10-fold serial dilutions of homogenates and blood in saline were plated onto Colombia III blood agar. After overnight incubation at 35°C colonies were counted.

The histopathological changes in infected tissues of non-surviving, euthanized mice (n=4) were determined in animals sacrificed using an overdose of pentobarbital (Ceva Sante Animale). The *in situ* re-expanded lungs and the other organs were processed as described before.[33] Tissue sections (4 μm) were stained with haematoxylin and eosin. A pathologist examined the sections microscopically.

Parameters to monitor infection progression and outcome

Blood cultures

Blood was withdrawn from the tail artery of infected mice at 17, 48, and 72 hours after infection (n=25), collected in a Microvette® CB300 capillary tube containing Lithium Heparin (Sarstedt) and cultured as described above.

C-reactive protein

For quantification of CRP, blood was withdrawn from the tail artery of infected mice at 1, 17, or 48 hours after infection. Blood was collected in a Microvette® CB300 capillary tube (Sarstedt) and sera were prepared and stored at -80°C. To exclude effects of animal handling, a control group receiving saline only was included (n=10). CRP was measured via solid phase ELISA kit (LifeDiagnostics) according to the manufacturer's instructions, as described by Reichelt et al.[34] All samples were run in duplicate and reported as an average of two determinations.

Cytokines

Cytokine concentrations in serum were assessed at 1, 17, or 48 hours after infection. To exclude effects of animal handling on cytokine levels, a control group receiving saline only was included (n=10). Cytokine levels were determined using the Milliplex® MAP kit (Millipore) in duplicate, following the manufacturer's user manual using the Luminex 100 instrument (Biomedical Diagnostics). Selected cytokines were: granulocyte colony stimulating factor (G-CSF), granulocyte monocyte colony stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF), interferon (IFN) γ, leukemia inhibitory factor (LIF), tumor necrosis factor (TNF) α, interleukin (IL) 1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, and

IL-17A. Selected chemokines were: eotaxin (CCL1), IFN-γ inducible protein of 10 kDa (IP-10; CXCL10), keratinocyte chemoattractant (KC; CXCL1; functional homologue of human IL-8[35]), lipopolysaccharide induced CXC chemokine (LIX; CXCL5-6), monocyte chemoattractant protein 1 (MCP-1; CCL2), monokine induced by IFN-γ (MIG; CXCL9), macrophage inflammatory protein 1α (MIP-1α; CCL3), MIP-1β (CCL4), MIP-2 (CXCL2), the chemokine regulated-upon-activation normal T-cell expressed and secreted (RANTES; CCL5), and vascular endothelial growth factor (VEGF). Selected cytokines and chemokines are representative for both pro- and anti-inflammatory cytokines.

Statistical analysis

Quade's rank analysis of covariance was used to compare body weight of placebo-inoculated mice, surviving infected mice and non-surviving infected mice. Fisher exact test was used to compare numbers of mice with positive blood cultures in groups of surviving infected mice versus non-surviving infected mice. Also, *S. aureus* counts in blood and organs, and serum levels of CRP and cytokines in groups of placebo-inoculated mice, surviving infected mice and non-surviving infected mice were compared using the Mann-Whitney U test. Binary logistic regression analysis using all variables associated with presence or fatal outcome of *S. aureus* bacteremia in the Mann-Whitney U test (*P*-values < 0.01) was performed to determine the biomarkers for presence and fatal outcome of *S. aureus* bacteremia. Receiver operating characteristic (ROC) curves were constructed and markers with area under ROC curve > 0.8 are considered indicative as biomarker.

Results

Course of *S. aureus* bacteremia

To establish the LD_{50} inoculum in the *S. aureus* bacteremia model, groups of mice were intravenously infected with different inocula (data not shown). *S. aureus* at 1×10^4 cfu did not result in mortality, while 2×10^6 cfu *S. aureus* resulted in 100% mortality. The LD_{50} inoculum was calculated to be 3×10^5 cfu. The survival of mice infected with the LD_{50} inoculum declined gradually over 14 days (Figure 1A). After this time point, no changes in animal survival were observed. Infected mice decreased in body weight compared to placebo-inoculated mice. Body weight of eventually non-surviving infected mice was significantly lower compared to eventually surviving infected mice and to placebo-inoculated mice ($P < 0.01$; Quade's rank analysis of covariance; Figure 1B). Discomfort

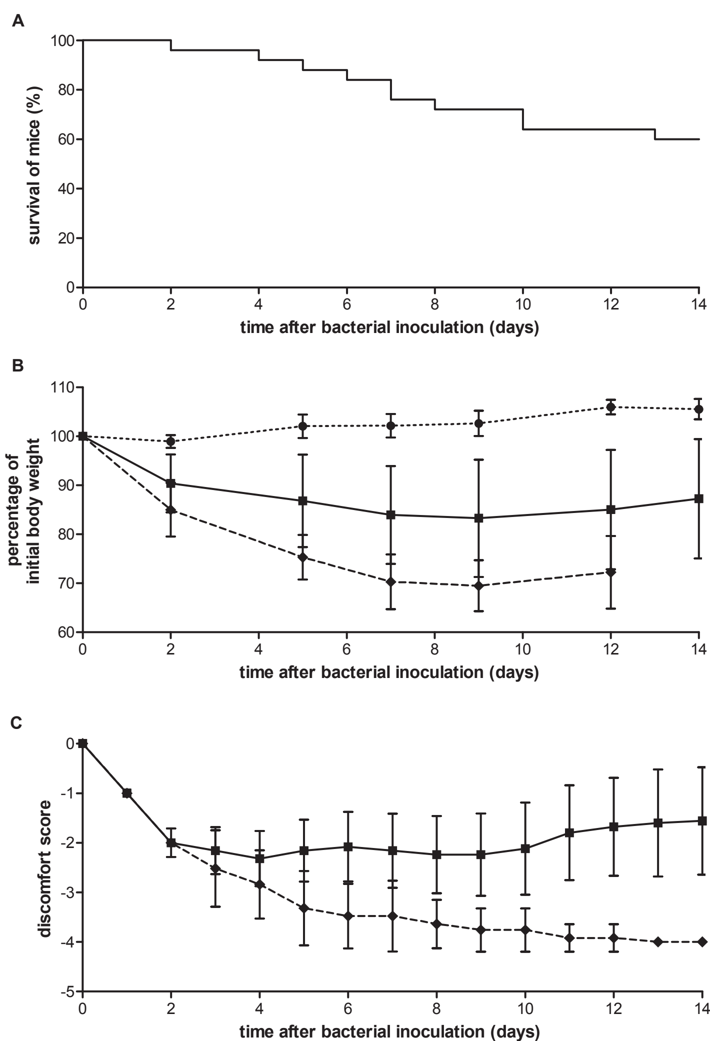

Figure 1. Time course of bacteremia in mice infected with *S. aureus* at the LD_{50} inoculum. (A) Survival of infected mice (n=25). (B) Mouse body weight, shown as mean ± SD (error bars). Body weight of non-surviving infected mice was significantly lower compared to surviving infected mice and to placebo-inoculated mice ($P < 0.01$; Quade's rank analysis of covariance). (C) Discomfort score, shown as mean ± SD (error bars). Discomfort score -2: bad fur. Discomfort score -3: bad fur, hunched back. Discomfort score -4: bad fur, hunched back, instability, euthanasia needed. Placebo-inoculated mice (● n=12), surviving infected mice (■ n=25), non-surviving infected mice (◆ n=25).

increased in all infected mice from two days after infection. In surviving infected mice, this remained stable until day 10 after infection and then decreased again, whereas the discomfort score in non-surviving infected mice further increased. Animals that showed severe signs of illness were euthanized and were scored -4 (Figure 1C). During the course of bacteremia, *S. aureus* load in blood and organs did not significantly change over time (Figure 2). Only in kidneys, *S. aureus* load tended to increase over time.

The course of *S. aureus* bacteremia was further characterized by histopathology of lungs, spleen, liver, and kidneys. Organs of infected mice that had to be euthanized early (day 4-5) and mice that had to be euthanized later (day 10-11) were compared (Figure 3). Most striking were renal bacterial abscesses in all euthanized mice. Bronchopneumonia was observed in both early euthanized mice and in one of the late euthanized mice. Bronchioles were filled with neutrophils. The hepatic sinusoids were dilated with a subtle increase of sinusoidal neutrophils in mice that had to be euthanized early. Mice that had to be euthanized late also developed hepatic microabscesses. The spleen showed lymphodepletion, which was more profound in late euthanized mice compared to early euthanized mice. *S. aureus* was primarily found intra- and perivascularly.

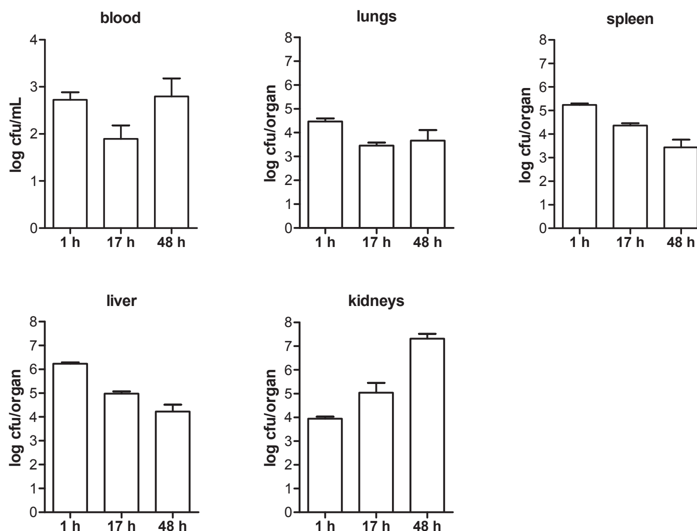

Figure 2. *S. aureus* counts in blood and organs from mice with bacteremia. At indicated time points after intravenous inoculation of *S. aureus* at the LD_{50} inoculum, mice were sacrificed and quantitative cultures of blood and organs were performed (n=4 per time point). Results are expressed as mean ± SD (error bars). Statistically significant differences (Mann-Whitney U test) in *S. aureus* load were not found. h, hours.

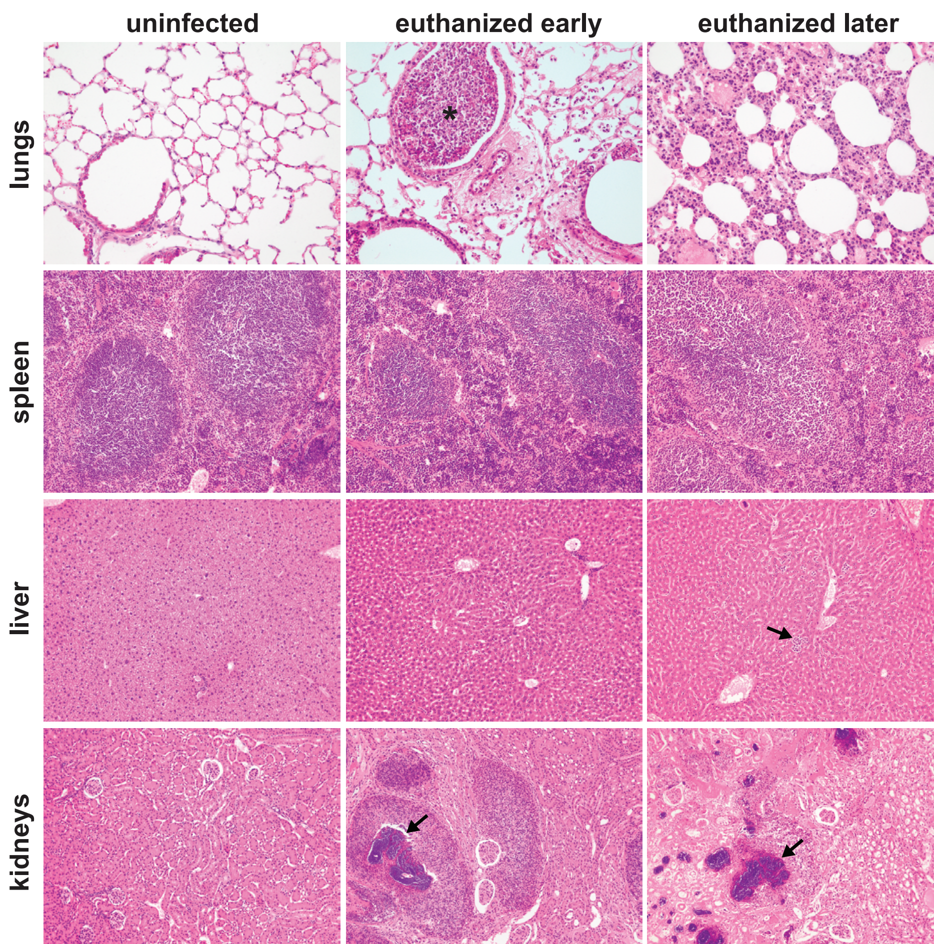

Figure 3. Representative histopathological features in lungs, spleen, liver and kidneys of mice with *S. aureus* bacteremia. Haematoxylin and eosin stained tissue sections are shown. Lungs (200x), spleen (100x), liver (100x), and kidneys (100x). Uninfected mice; mice with *S. aureus* bacteremia that were euthanized early (day 4-5); mice with *S. aureus* bacteremia that were euthanized later (day 10-11). In lungs, asterisk indicates bronchopneumonia with neutrophils. In liver, arrow indicates hepatic microabscesses with neutrophils. In kidneys, arrows indicate abscesses in kidneys.

S. aureus load in blood in surviving infected mice versus non-surviving infected mice

We determined whether conventional blood culture is indicative for fatal infection outcome (Figure 4). The number of animals with positive blood cultures was not significantly different between groups of surviving infected mice and non-surviving infected mice at all time points. Quantitative blood cultures showed substantial inter-

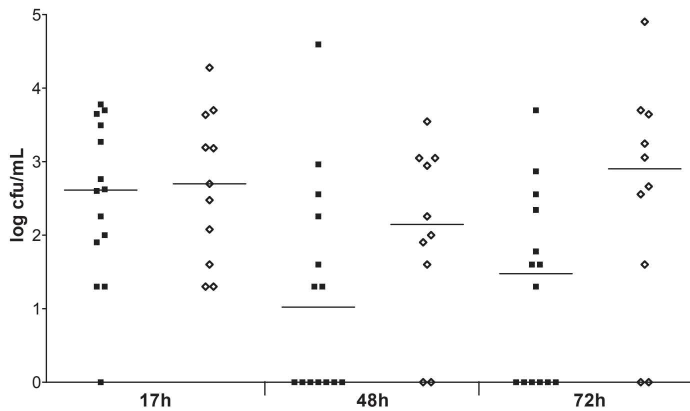

Figure 4. *S. aureus* counts in blood from mice with bacteremia at indicated time points. Mice were inoculated intravenously with *S. aureus* at the LD_{50} inoculum (n=25). Each symbol represents a single mouse. Median values are indicated by horizontal lines. Surviving infected mice (■), non-surviving infected mice (◇). Statistically significant differences in numbers of animals with positive blood cultures (Fisher exact test) or *S. aureus* counts (Mann-Whitney U test) between groups of surviving infected mice versus non-surviving infected mice were never observed. h, hours.

individual variability. At 72 hours, cfu counts tended to be higher in non-surviving infected mice compared to surviving infected mice, although this was not statistically significant. Blood cultures were negative in all surviving infected animals at 2 weeks after inoculation (data not shown).

C-reactive protein levels in surviving infected mice versus non-surviving infected mice

We determined whether serum CRP levels are indicative for presence and fatal outcome of *S. aureus* bacteremia in mice (Figure 5). CRP levels were significantly elevated in infected mice compared to placebo-inoculated mice at both 17 and 48 hours after infection ($P < 0.01$). No significant differences were found between surviving infected mice and non-surviving infected mice.

Cytokines in surviving infected mice versus non-surviving infected mice

Serum levels of 31 selected cytokines were assessed. Significant differences between *S. aureus*-infected mice versus placebo-inoculated mice as well as between non-surviving infected mice versus surviving infected mice are shown in Table 1.

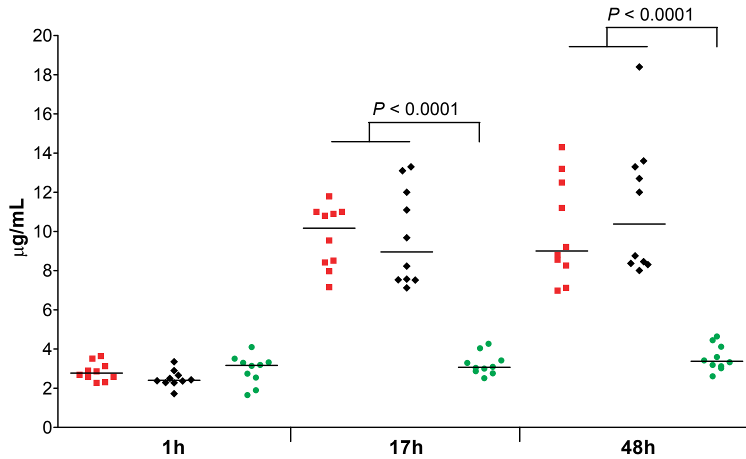

Figure 5. Serum C-reactive protein (CRP) levels in mice with *S. aureus* bacteremia at indicated time points. Mice were inoculated intravenously with *S. aureus* at the LD_{50} inoculum. Each symbol represents a single mouse. Red squares represent surviving infected mice (n=10); black diamonds represent non-surviving infected mice (n=10); green circles represent placebo-inoculated mice (n=10). Median values are presented by horizontal lines. Statistically significant differences between infected mice and placebo-inoculated mice are indicated. Statistically significant differences were never found between surviving infected mice and non-surviving infected mice. *P*-values < 0.01 (Mann-Whitney U test) are shown. h, hours.

Comparing infected mice to placebo-inoculated mice, 21 cytokines were elevated in infected mice ($P < 0.01$; Mann-Whitney U test). From these, 3 cytokines (TNF-α, IL-6, and KC) were already elevated early at 1 hour after infection and remained elevated at 17 and 48 hours after infection. IL-10 was elevated at 1 and 17 hours as well, but was not elevated anymore at 48 hours. At both 17 and 48 hours after infection, 12 cytokines were elevated (G-CSF, M-CSF, IFN-γ, IL-1α, IL-1β, IL-12p70, IL-15, IL-17A, IP-10, MCP-1, MIG, and MIP-1α) in infected mice compared to placebo-inoculated mice. GM-CSF, MIP-1α, MIP-2, and RANTES were only elevated at 17 hours, while eo-taxin was only elevated at 48 hours after infection. Logistic regression analysis showed that G-CSF, TNF-α, IL-1α, IL-6, IL-12p70, IP-10, KC, and MIP-1α were discriminative between infected mice and placebo-inoculated mice. Levels of these 8 cytokines in individual mice are shown in Figure 6. Levels of G-CSF showed an impressive increase between 1 and 17 hours after infection, and remained high at 48 hours. Levels of IL-12p70, IL-1α, IL-6, KC, and MIP-1α showed an increase between 1 and 17 hours as well, although this increase was less pronounced than for G-CSF. The levels of these 5 cytokines remained elevated at 48 hours. IP-10 levels showed an initial increase between 1 and 17 hours as well, but decreased again between 17 and 48 hours.

Table 1. Cytokines in serum from mice with *S. aureus* bacteremia at various time points after infection.

	Elevated due to infection[a]			Elevated in non-surviving infected mice versus surviving infected mice[a]		
Cytokine/chemokine	**1h**	**17h**	**48h**	**1h**	**17h**	**48h**
G-CSF		**<0.0001**	**<0.0001**			
GM-CSF		0.0044				
M-CSF		0.0039	0.0099			
IFN-γ		<0.0001	0.0017			
TNF-α	**<0.0001**	**<0.0001**	**<0.0001**			**0.0004**
IL-1α		**0.0073**	**0.0001**			**0.0065**
IL-1β		0.0037	0.0031			0.0028
IL-6	**<0.0001**	**<0.0001**	**<0.0001**			
IL-10	0.0001	0.0003				
IL-12p70		**<0.0001**	**0.0001**			
IL-15		0.0001	0.0003			
IL-17A		0.0002	0.0007			
eotaxin (CCL1)			0.0012			0.0025
IP-10 (CXCL10)		**<0.0001**	**0.0008**			
KC (CXCL1)	**0.0089**	**<0.0001**	**<0.0001**	**0.0043**	**0.0025**	**0.0090**
MCP-1 (CCL2)		<0.0001	<0.0001			
MIG (CXCL9)		0.0001	0.0008			
MIP-1α (CCL3)		**0.0001**	**0.0019**			
MIP-1β (CCL4)		0.0015				
MIP-2 (CXCL2)		0.0003				
RANTES (CCL5)		0.0024				

[a] Only *P*-values for the 21 cytokines that were significantly elevated (Mann-Whitney U test, $P < 0.01$) in infected mice (n=20) compared to placebo-inoculated mice (n=10) are shown. Cytokines for which area under ROC curve was > 0.8 (logistic regression analysis) are indicated in bold.

Levels of TNF-α were elevated at 1, 17, and 48 hours. The elevated cytokine levels in infected mice were not the result of animal handling during drawing of blood, as cytokine levels in placebo-inoculated mice were consistently low. Cytokine levels in surviving infected mice decreased over time, and were near or comparable to those in placebo-inoculated mice 2 weeks after inoculation (data not shown).

Comparing non-surviving infected mice to surviving infected mice, we also found differences in the cytokine levels. Serum levels of 5 cytokines were elevated in non-surviving infected mice compared to surviving infected mice ($P < 0.01$; Mann-Whitney U test). From these, KC was already elevated early at 1 hour after infection in non-surviving infected mice, and remained elevated at 17 and 48 hours after infection. The other 4 cytokines (TNF-α, IL-1α, IL-1β, and eotaxin) were elevated in non-surviving

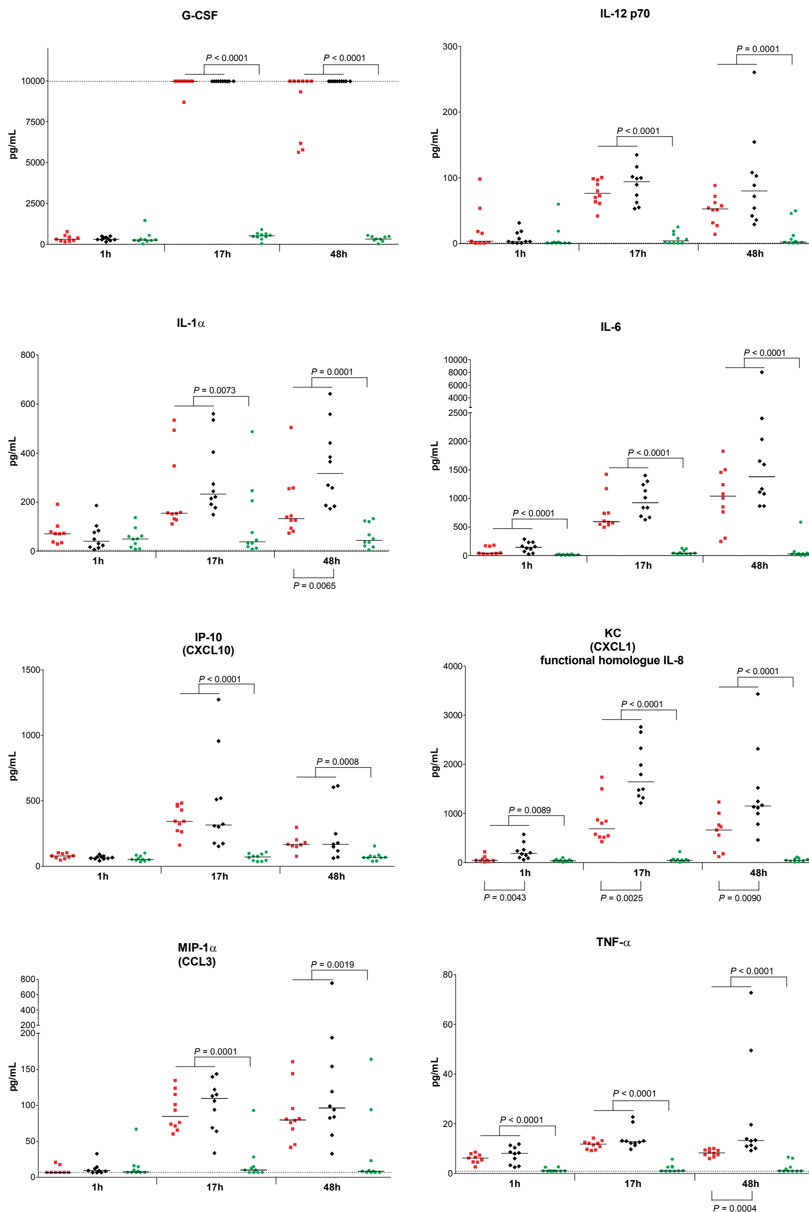

Figure 6. Serum cytokine levels elevated due to *S. aureus* bacteremia in mice at indicated time points. Mice were inoculated intravenously with *S. aureus* at the LD_{50} inoculum. Each symbol represents a single mouse. Red squares represent surviving infected mice (n=10); black diamonds represent non-surviving infected mice (n=10); green circles represent placebo-inoculated mice (n=10). Median values are presented by horizontal lines. Statistically significant differences in cytokine levels between infected mice versus placebo-inoculated mice are indicated above the x-axis. Statistically significant differences in cytokine levels between surviving infected mice versus non-surviving infected mice are indicated below the x-axis. *P*-values < 0.01 (Mann-Whitney U test) are shown. h, hours.

infected mice only at 48 hours after infection. Logistic regression analysis showed that TNF-α, IL-1α, and KC were discriminative between non-surviving infected mice and surviving infected mice. Each of the three cytokines showed an area under ROC curve > 0.8. Combining these cytokines in the analysis did not further improve the area under ROC curve.

Discussion

Early diagnosis of *S. aureus* bacteremia is crucial to reduce mortality rates. In addition, optimization of antibiotic use is of high importance. To this aim, novel and reliable markers as predictors of infection outcome are urgently needed. In this study in mice, we determined whether blood culture, CRP, and cytokines are potential biomarkers for presence and outcome of *S. aureus* bacteremia. We have established a model of *S. aureus* bacteremia in mice that were *S. aureus*-free before infection. In this way, all (immune) responses in these animals were due to the inoculated *S. aureus* strain only. A clinical *S. aureus* isolate recovered from a septic patient was used at an inoculum resulting in 50% mortality of mice. This experimental design allowed comparison of surviving and non-surviving mice infected with the same *S. aureus* inoculum. Staphylococci mainly migrated to the kidneys, resulting in an increase of bacterial load and bacterial microabscesses in the kidneys were observed. Similar observations were described by Cheng et al.[36] The model proved to be very reproducible, and provides a robust experimental system to investigate *S. aureus* bacteremia.

When a patient is suspected of bacteremia, blood is drawn for identification of the causative infectious agent, and cultured in broth to make sure that low bacterial blood counts will also be detected. Using this method, a quantitative blood culture cannot be assessed. In the current study in mice, we investigated whether presence of *S. aureus* bacteremia or *S. aureus* quantitative blood counts are biomarkers predicting fatal outcome of *S. aureus* bacteremia. A positive *S. aureus* blood culture did not discriminate between eventually non-surviving infected mice and eventually surviving infected mice. The *S. aureus* blood counts tended to be higher in non-surviving infected mice compared to surviving infected mice, but this was not statistically significant. We concluded that neither the positive *S. aureus* blood culture nor the *S. aureus* counts in blood could be used as biomarkers indicative for fatal infection outcome.

We included CRP as a potential biomarker for presence and fatal outcome of *S. aureus* bacteremia as this marker is frequently used in clinical practice to determine and monitor inflammation and infection in general. Circulating CRP levels directly reflect the intensity of the pathological processes that stimulate the CRP production.[7] The present study showed that CRP can be used as a biomarker for presence of severe *S. aureus* bacteremia in mice. However, serum CRP levels were not discriminative between surviving infected mice and non-surviving infected mice, and therefore CRP is not a biomarker for fatal outcome of infection. These data in mice are in concordance with findings in bacteremic patients, as CRP is a biomarker for presence of *S. aureus* bacteremia, but does not predict outcome of infection.[6] As CRP is not specific for *S. aureus* infection, elevated CRP levels will not indicate bacteremia caused by *S. aureus* only.

In addition, we assessed whether cytokines can be used as biomarker in *S. aureus* bacteremia. According to the binary logistic regression analysis, TNF-α, IL-1α, KC, G-CSF, IL-6, IL-12p70, IP-10, and MIP-1α are indicative for presence of *S. aureus* bacteremia in mice, while TNF-α, IL-1α, and KC were also potential biomarkers for fatal outcome of this infection. As the last three cytokines each had a very high area under ROC curve, and combination of these cytokines did not improve this, each of these can be used individually as biomarkers for fatal outcome. Diagnosis of *S. aureus* bacteremia cannot be based solely on elevated levels of these cytokines, as cytokine levels will rise in other infections as well. Until now, cytokine levels in mice with *S. aureus* bacteremia are not well studied. Ashare et al*.* assessed cytokine levels in a murine model simulating polymicrobial sepsis and showed that levels of TNF-α and IL-1β in liver were elevated in septic mice compared to controls.[37] Osuchowski et al*.*, using the same murine model, showed that plasma levels of MCP-1, MIP-2, and TNF-α had a robust correlation with outcome of infection.[38] Discrepancies between these studies and the present study could be explained by differences in the biomaterial in which cytokines were measured, differences in the causative infectious organism(s), and differences in cytokines included for assessment in these studies. In the present study, we selected a broad panel containing 31 cytokines, covering the main cytokines found in related studies, and including both pro- and anti-inflammatory cytokines. Most other studies measured only a limited number of cytokines, up to 16.

In the present study, 3 cytokines (TNF-α, IL-1α, and KC) emerged as biomarkers for fatal outcome of *S. aureus* bacteremia. TNF-α is primarily produced by activated macrophages and stimulates the acute phase reaction. It induces apoptotic cell death, attracts neutrophils and stimulates phagocytosis. IL-1α is also mainly produced by

activated macrophages early after onset of infection, and plays one of the central roles in the regulation of immune responses. It activates lymphocyte proliferation and increases number of blood neutrophils. KC is the murine functional homologue of human IL-8[35] and is an important neutrophil chemoattractant. Neutrophils appear to be important in *S. aureus* sepsis, as TNF-α, IL-1α and KC/IL-8 all attract these cells. In patients with *S. aureus* bacteremia, neutrophils are activated as well.[39] At both 1 and 17 and 48 hours after infection, KC was elevated in non-surviving infected mice compared to surviving infected mice. A biomarker elevated irrespective the progression of infection, such as KC, is very interesting for clinical application in view of the heterogeneity of patients experiencing bacteremia in this respect.

In the present study, we exhaustively examined blood culture, CRP and cytokines as biomarkers for presence of severe *S. aureus* bacteremia and fatal outcome of infection in mice. This study provides evidence that in severe *S. aureus* bacteremia in mice, TNF-α, IL-1α, and KC each can be useful as biomarkers predicting fatal outcome of infection. Blood cultures (qualitative or quantitative) and CRP are not discriminative in this respect. As elevated levels of these cytokines are indicative for *S. aureus* bacteremia, these cytokines cannot be used to diagnose *S. aureus* bacteremia; instead these cytokines might be useful as biomarkers for outcome of infection in patients already diagnosed with *S. aureus* bacteremia. Several clinical studies have shown that it is difficult to evaluate changes in cytokine profile during bacteremia in patients, and this is mainly due to differences in causative infectious agents. Studies focused on a single, known causative infectious organism might be more informative. In the clinical setting, infecting *S. aureus* strains will vary between patients. In the present study in mice, only one *S. aureus* clinical isolate was used. So, we should be careful in extrapolating the results obtained in mice to the clinical setting. As the host as well as the *S. aureus* strain are both important determinants in the infectious process, and only one mouse strain and one *S. aureus* strain were included in the present study, it is currently unknown whether the observations obtained can be generalized. Therefore, we conclude that the current results in mice suggest that cytokines might be reliable biomarkers for fatal outcome of *S. aureus* bacteremia in patients. Currently, data on appropriate biomarkers are lacking and only when bacteremia is strongly suspected in patients, antibiotic treatment is started. Based on the present study in mice, a clinical study on the role of selected cytokines in predicting the infection outcome is warranted. These data will improve decisions on the start and choice of antibiotic therapy.

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6

Strengthening the immune system as an antimicrobial strategy against *Staphylococcus aureus* infections

Sanne van den Berg, Irma A.J.M. Bakker-Woudenberg

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Abstract

Staphylococcus aureus asymptomatically colonizes the nose of about 20% of the healthy population, but can also cause mild to severe infections. Antibiotic treatment of these infections is not always successful, resulting in substantial therapy failure. Therefore, more effective treatment is urgently required. Among alternative antimicrobial intervention strategies, both active and passive immunization in the prevention and cure of *S. aureus* infection is investigated in experimental animals and patients. The translational value of animal studies is determined by a proper selection of bacterial target, infection model, treatment modalities, and outcome parameters. In experimental animals, various infection models are described for studying the efficacy of immunization. Most of these studies focusing on a broad range of bacterial targets were successful in prevention, reduction or cure of infection. The efficacy of immunization focused on a limited number of bacterial targets was also investigated in *S. aureus* infected patients or individuals that are at high risk for *S. aureus* infection. In these studies, final conclusions on the efficacy of immunization cannot be drawn.

Introduction

About 20% of the healthy population persistently carries *Staphylococcus aureus* in their nose.[1] Carriage of *S. aureus* is asymptomatic, although it can also cause infection. *S. aureus* infection represents a serious burden to global public health, particularly with respect to infections in healthcare-related settings. In this respect, *S. aureus* is not only associated with serious infections such as surgical wound infections and pneumonia, but also with serious invasive disease, including sepsis, which may result in endocarditis, osteomyelitis and meningitis.[2] Further, methicillin-resistant *S. aureus* (MRSA) has now become prevalent within hospitals, leading to a significantly increased risk of mortality and increased length of stay for hospitalized patients.[3] Alarmingly, recent years have also witnessed the spread of resistant staphylococci outside of the healthcare setting, with the prevalence of community-acquired MRSA now increasing.[4] Even more alarming is the emergence of vancomycin- and mupirocin-resistant *S. aureus* strains, which represent a major threat to the control of *S. aureus* infections within both the hospital and community setting.[5,6] These worrying developments indicate that there is an urgent need to develop novel long-lasting anti-staphylococcal therapies to enhance, or even replace, current antibiotic-related therapies. Among alternative, non-antibiotic-related, antimicrobial intervention strategies, both active and passive immunization in the prevention and cure of *S. aureus* infection is investigated in experimental animals and patients. These novel treatment strategies need to be thoroughly evaluated before they can be applied within the clinical setting. Although *in vitro* assays provide a hint of the efficacy of both active and passive immunization strategies against *S. aureus* infection, they may be inadequate or even misleading for the prediction of actual *in vivo* efficacy. Therefore, studies *in vivo* are of high importance. When utilizing *in vivo* animal models, a well-planned experimental study design is essential in order to generate data with a high translational value for patients using a minimum input of animals. In this respect, a proper selection of the bacterial target, infection model, treatment modalities, and outcome parameters is crucial. In this chapter, an overview of the study design as well as the results obtained with immunization in experimental animals and clinical studies is provided.

Bacterial target

Selection of the *S. aureus* target is the first and probably most essential step in designing a study on immunization as antimicrobial strategy. The criteria to which the ideal target should meet need to be defined.

Immunogenicity of the bacterial target is required to be successful in immunization studies in *S. aureus* infection. This means that antibodies against the target are expected to be produced. An indication which *S. aureus* antigen should be targeted with immunization is provided by studies comparing anti-staphylococcal antibody levels in *S. aureus* carriers or *S. aureus* infected patients with non-carriers or non-infected patients, respectively. Antibodies against these antigens may play important roles in *S. aureus* colonization and infection. For example, immunoglobulin G (IgG) against staphylococcal enterotoxin A (SEA) and toxic shock syndrome toxin 1 (TSST-1) is elevated in carriers compared to non-carriers,[7] while IgG against glucosaminidase, γ hemolysin B (HlgB), leukocidin F (LukF), SA0688, staphylococcal complement inhibitor (SCIN), staphylococcal superantigen-like proteins 1 and 5 (SSL1 and SSL5) are higher in patients with *S. aureus* bacteremia than in non-infected patients.[8]

Although a broad panel of *S. aureus* antigens appears to be immunogenic, these antibodies are not always sufficient to eliminate *S. aureus* from the nose of *S. aureus* carriers.[7] Moreover, the anti-staphylococcal antibodies present in healthy individuals do not prevent them from getting infected with *S. aureus* as carriers of *S. aureus* have higher anti-staphylococcal antibody levels[7] and also a higher chance on developing *S. aureus* infections than non-carriers.[9] This might support the exclusion of these antigens as target for immunization as antimicrobial strategy.

Immunization based on more than one *S. aureus* antigen as target is expected to be more valuable than immunization focusing on a single antigen, as each *S. aureus* carrier or *S. aureus* infected patient develops a unique immune response against different *S. aureus* antigens. Another reason for the selection of multiple targets for immunization strategies is the presence of functional redundancy. *S. aureus* produces many antigens, of which some have similar or comparable functions. When one of these antigens is absent in a *S. aureus* strain, another antigen will take over this function.

S. aureus targets without sequence variation in the immunoglobulin binding domain should be preferred above targets with genetic variation. This increases the chance that the variable region of the antibodies, either induced by active immunization or administered as passive immunization, is able to bind to the epitope on the *S. aureus* target, and will enhance a successful treatment of the *S. aureus* infection. As McCarthy and Lindsay[10] showed sequence variation in 25 surface bound *S. aureus* proteins as well as in 13 secreted proteins amongst the 58 *S. aureus* genomes studied, they emphasize that immunization as antimicrobial strategy should contain (antibodies against) cocktails of antigens representing all variants of the *S. aureus* targets.

A polyvalent strategy may not be necessary when the *S. aureus* target is conserved on all *S. aureus* strains. Choosing a conserved *S. aureus* target will enhance the success of immunization as antimicrobial strategy in infections which are caused by a wide variety of *S. aureus* strains. This is of main importance as the *S. aureus* strains causing infections in patients are very heterogenous.[8,11] Ziebandt et al.[11] observed that 7 extracellular proteins were produced by all 17 clonally different *S. aureus* strains (immunodominant staphylococcal antigen A (IsaA), lipase (Lip), peptidoglycan hydrolase (LytM), nuclease (Nuc), SA0620, SA2097, and SA2437). A further nine proteins (aureolysin (Aur), glycerol ester hydrolase (Geh), glycerophosphoryl diester phosphodiesterase (GlpQ), alpha toxin, HlgB, SA0570, SA1812, staphylococcus serine protease A and B (SspA and SspB)) were identified in at least 80% of these strains. Especially the 7 proteins are interesting targets for monovalent immunization strategies towards all *S. aureus* strains.

Another consideration that has to be taken into account when selecting the bacterial target for immunization as antimicrobial strategy is that the selected target needs to be accessible for antibodies enabling binding of antibodies to the target. To this aim, the target needs to be exposed on the bacterial surface, or has to be excreted. In addition, target expression during *S. aureus* infection is needed.

Treatment modalities

Important choices concerning treatment modalities are active or passive immunization as well as dosing.

Active or passive immunization

In active immunization, also called vaccination, antigenic material (vaccine) is administered before infection, and aims for stimulation of the immune system to develop adaptive immunity. This vaccine may consist of intact, but inactivated or attenuated *S. aureus* cells, or purified components of the bacterium. In case the target is immunogenic, active immunization will result in an antibody response directed against the target. Moreover, the antigen will elicit an effector T cell response.

The use of an adjuvant in active immunization is highly preferred in order to induce inflammation at the injection site, which will enhance the immune response. It is known that immunization with purified proteins without adjuvant leads to a poor immune response. In addition, adjuvants cause soluble protein antigens to aggre-

gate and precipitate forming particles, which will facilitate their efficient uptake by antigen-presenting cells and reduce the rate at which the antigen is cleared from the system. Various adjuvants are used in active immunization studies concerning *S. aureus*. Complete Freund's adjuvant (CFA) is commonly used. This is an emulsion of killed mycobacteria and mineral oil into which antigens are vigorously mixed. In experimental animals, the use of CFA should be limited because of its painful reaction and potential for tissue damage. Incomplete Freund's adjuvant (IFA) only contains the mineral oil, and is often used as adjuvant in booster immunizations after CFA as adjuvant in the first immunization. Next to CFA/IFA, Ribi adjuvant, amorphous aluminium hydroxyphosphate sulphate adjuvant (AAHSA), cholera toxin, toxoids, saponin, Abisco-100 and alum are also described as adjuvants in active immunization against *S. aureus* infections in experimental animals.

In passive immunization, antibodies directed against the bacterial target are injected into the body. Passive immunization can be administered before infection (prophylactic treatment) or after infection (therapeutic treatment). Although passive immunization will provide a quick response, the benefit is short-lasting as the antibodies once injected will naturally be broken down. In passive immunization, the type of antibodies used is of importance. Antibodies can be monoclonal or polyclonal, directed against one or more epitopes of the target, respectively. Next, antibodies may be derived from human origin in order to be directly applicable in the clinical setting, or they may have an animal origin, with better chances for protective efficacy in an animal model.

Dosing

All kinds of dosing schedules and routes of administration are used in the various studies on active and passive immunization as antimicrobial strategy.

In studies concerning active immunization in experimental models, animals are immunized at least two times, and in one study even ten times. Booster immunizations after the primary exposure may lead to long-lasting immunity through activation of immunological memory cells. Time intervals between two consecutive injections vary as well. In most studies, this interval is 1-2 weeks. Immunization injections are mostly given via the subcutaneous (s.c.) or intramuscular (i.m.) route, while intranasal (i.n.), intraperitoneal (i.p.), intravenous (i.v.), and intratracheal (i.t.) injections are also described. In some studies, two routes of administration are combined. In general, dose-finding is necessary for each target and route of administration. In contrast to the animal studies, in clinical studies active immunization is performed only once, via the i.m. route.

In passive immunization studies in most experimental models, animals are immunized with a single prophylactic gift of antibodies. This gift is administered 2 days to several hours before infection. In only a few passive immunization studies, therapeutic treatment is applied. The antibodies are mainly administered via the i.p. route, while i.v. administration is also described. Again, dose-finding is necessary. In contrast to the animal studies, in clinical studies passive immunization is performed via the i.v. route, either as prophylactic or as therapeutic treatment. Number of immunizations range from a single gift to immunization twice daily.

Outcome parameters

To monitor the course of infection and to assess the efficacy of immunization, relevant outcome parameters are of high importance.

Overall, in experimental animal studies, clinical signs of illness, including body temperature, behavior, and appearance are monitored over time. In models of skin infection, also the lesion size over time is monitored. In lethal infection models, disease progression score over time is assessed. In all models, after dissection of the animals, bacterial load in blood and relevant organs may be determined. It always needs to be confirmed that the *S. aureus* isolate recovered from the infected animals is identical to the *S. aureus* isolate inoculated.

In clinical studies, next to clinical signs of illness, prevention of (relapse of) *S. aureus* infection is an important outcome parameter to assess the efficacy of immunization as antimicrobial strategy.

Experimental animal studies

Regarding the design of *in vivo* studies in experimental animals and the interpretation of the results obtained, one has to realize that animal models of infection only mimic the infection in humans, mainly because the infective dose used in artificially induced infections in animals is different from the naturally acquired infection in patients. At the same time, animal models – provided well characterized – are needed and accepted as well for studying the potential efficacy of novel treatment strategies, as they provide the unique opportunity to study treatment efficacy under similar conditions of

intensity and duration of infection. Uniform groups of patients are difficult to obtain because of differences in underlying clinical conditions.

A proper experimental design when performing animal studies is always required to generate maximum results with a minimum input of animals. Next to choice of bacterial targets, treatment modalities and outcome parameters, proper selection of the infection model is of main importance.

Infection model

The experimental infection model is characterized by the animal species, type of infection, and the infecting *S. aureus* strain used. Relevant choices have to be made in light of the research question that needs to be answered, and will be discussed.

Animal species

In experimental models of *S. aureus* infection, a variety of animal species are used. A prerequisite of the selected animal species is that it has to be susceptible to *S. aureus*.

Mice are often used as experimental animals, being relatively inexpensive compared to other animal species, easy to maintain and easy to handle. Moreover, much is known about mouse genetics and immunology, and knock-out mouse strains are available. In addition, immunologic reagents applicable in mouse tissues are widely available.

In experimental *S. aureus* studies, next to different strains of mice, also rats, cotton rats, rabbits, sheep, cattle and rhesus macaques are used for evaluation of the efficacy of active or passive immunization. The cotton rat has been described as a good model for *S. aureus* nasal colonization as nasal histology of cotton rats is comparable to that of humans. Moreover, pretreatment with antibiotics, being required to establish nasal colonization in mice, is not needed in cotton rats.[12] In contrast to rodents and rabbits, sheep and cattle may be naturally colonized and infected by *S. aureus*. This is interesting for studying the potential efficacy of treatment. However, these *S. aureus* strains are genetically different from those found in humans.[13] Rhesus macaques, being naturally colonized by *S. aureus* in their noses are the most suitable experimental animals.[14] However, while rhesus macaques are genetically more related to humans than cotton rats, sheep and cattle are, ethical issues limit the use of large groups of macaques.

In addition to the selection of the animal species, the microbial status of the experimental animal needs attention. Microbiological standardization is an important tool to

achieve reproducible animal experiments. Most commercial suppliers of experimental animals have a health monitoring program based on the FELASA Recommendations.[15] The supplier lists the organisms that are not present in the experimental animal. In this respect, animals with a specified pathogen free (SPF) status are not tested for the presence of *S. aureus*, while those with a specified opportunistic pathogen free (SOPF) status are free of *S. aureus*. In order to maintain the microbial health status in the facility where the animal experiments are performed, it is important that animals are housed in individually ventilated cages. Disinfected gloves should be worn when handling these animals, to prevent transmission of human colonizing *S. aureus* strains to the animals.

Infection type

S. aureus can cause a variety of infections,[9] ranging from mild skin infections to more severe infections like arthritis, endocarditis and sepsis, as shown in Figure 1. Many types of infection models are described, some more extensively characterized than others. For each research question, the most suitable experimental infection should be selected, and clinically relevant infections that urgently need alternative treatment should be preferred. The following models have been published for studying immunization as antimicrobial strategy in *S. aureus* infections:

- nasal carriage
- skin infection, wound infection
- keratitis
- mastitis
- catheter-related infection
- endocarditis
- renal abscess
- pneumonia
- septic arthritis
- bacteremia
- sepsis
- shock

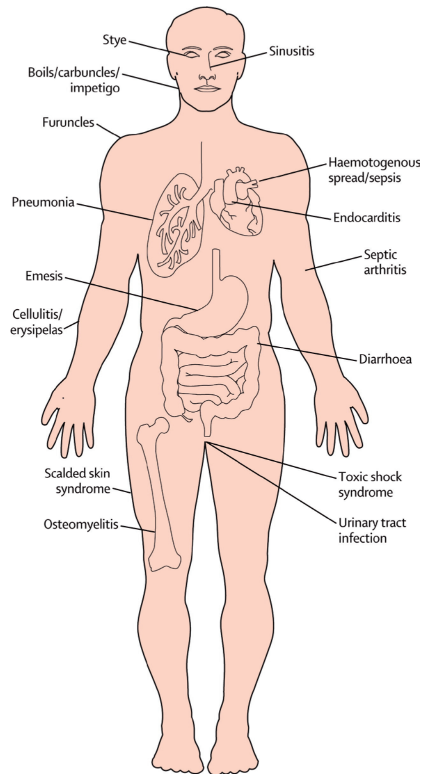

Figure 1. *S. aureus* can cause a wide range of infections. Figure adapted from Wertheim et al.[9]

Infecting strain

Many different *S. aureus* strains were used among which clinical isolates as well as sequenced strains. Originally, sequenced strains were clinical isolates as well, but they are passaged *in vitro* for many times. The choice of *S. aureus* strain depends on the research question.

As bacterial strains may lose virulence by *in vitro* passage, animal passage of the infecting strains is needed to maintain virulence of the *S. aureus* strain.

Results of immunization in experimental infection models

Numerous studies in animal models are published concerning immunization against *S. aureus* infections, including a wide range of bacterial targets and both active and passive immunization. Most studies concentrate on a specific infection model, and the experimental approach in these studies is summarized in Table 1.

Nasal carriage

In this infection model, mice or cotton rats are i.n. challenged with *S. aureus*. To assess the efficacy of immunization, noses are surgically removed and homogenized to determine numbers of viable *S. aureus*.

Active immunization with iron-responsive determinant A or H (IsdA or IsdH), factor effecting methicillin resistance (FmtB), SA2666 or the glucosaminidase domain of major autolysin (Atl) was studied by Clarke et al.[16] Only immunization with IsdA or IsdH protected against nasal carriage.

Schaffer et al.[17] investigated whether active immunization with clumping factor B (ClfB) resulted in lower *S. aureus* load in the nose of mice. While s.c. immunization with ClfB resulted in lower *S. aureus* colonization levels, i.n. immunization with ClfB did not protect mice against nasal colonization. Passive immunization targeting both capsular polysaccharide 5 (CP5) and ClfB reduced the bacterial load in the nose of mice as well, as observed by Pozzi et al.[18]

Skin infection, wound infection

For the model of skin infection, mice are challenged with *S. aureus* via the intradermal (i.d.) or s.c. route. In some cases, bacteria are mixed with dextran beads to promote abscess formation. Efficacy parameters include body weight changes, size of the abscess, hyperemia (increased blood flow), skin injury, and viable *S. aureus* load in the abscess.

Kennedy et al.[19] studied both active and passive immunization focused on alpha toxin in a murine model of skin infection. Both immunization strategies resulted in reduction of abscess size. Passive immunization targeting alpha toxin was also studied by Foletti et al.[20] They also observed a reduced abscess size and a reduction of the bacterial burden in the abscess.

Table 1. Experimental approach in animal studies on immunization as antimicrobial strategy in *S. aureus* infection.

Experimental model	Reference	Experimental animal	Bacterial target	Active immunization			Passive immunization			
				Number of immunizations	Route of immunization	Adjuvant	Number of immunizations	Route of immunization	P/T[1]	Type of antibodies[2]
Nasal colonization	[16]	Cotton rat	IsdA, IsdH, FmtB, SA2666, Atl	3	s.c.	Ribi				
	[17]	Mouse	ClfB	1 or 2	s.c.	CFA, IFA				
				3	i.n.	Cholera toxin				
	[18]	Mouse	CP5, ClfB				1	i.p.	P	Rabbit pAb
Skin infection	[19,20]	Mouse	alpha toxin	3	i.m.	CFA, IFA	1 or 2	i.p.	P	Rabbit, human pAb
	[21]	Mouse	LukF, LukS	2	s.c.	CFA, IFA				
				5	i.n.	Cholera toxin				
	[22]	Mouse	AIP-4				1	i.p.	P	Mouse mAb
	[23]	Mouse	RAP	1	?	CFA				
	[18]	Mouse	PNAG, alpha toxin, CP5, ClfB, CP8, IsdB				2	i.p.	P	Rabbit pAb
Wound infection	[24]	Mouse	Eap, ClfA, Efb	3	s.c.	Abisco-100				
Keratitis	[25]	Rabbit	alpha toxin	3	s.c.	CFA	1	in cornea	P	Rabbit pAb
Mastitis	[26]	Rabbit	alpha toxin	8	i.m., i.v.	CFA, -				
	[27]	Cattle	SEC	3	i.m.	-				
	[28,29,33]	Cattle, sheep	Whole cell	2 or 3	s.c.	α- and β-toxoids, IFA	1	i.p.	P	Rabbit pAb
	[30,31]	Mouse	FnbpA	2	s.c.	CFA, IFA	1	intra-mammary	P	Rabbit pAb
	[32]	Mouse	ClfA				1	i.p.	P	Human mAb
Catheter-related infection	[34,35]	Mouse, rat	IsdB				1	i.p.	P	Mouse, human mAb
	[36]	Mouse	IsaA				2	i.v.	P	Mouse mAb

Table 1. Experimental approach in animal studies on immunization as antimicrobial strategy in *S. aureus* infection. (continued)

Experimental model	Reference	Experimental animal	Bacterial target	Active immunization			Passive immunization			
				Number of immunizations	Route of immunization	Adjuvant	Number of immunizations	Route of immunization	P/T[1]	Type of antibodies[2]
Endocarditis	[37]	Rat	Fnbp	3	s.c.	CFA, IFA				
			Collagen binding protein	3	s.c.	CFA, IFA				
	[38,39]	Rabbit	ClfA				1	i.v.	P/T	IGIV with elevated anti-ClfA levels
	[40,41]	Rat	CP5, teichoic acid	3 or 9	s.c.	-	1	i.p.	P	Rabbit pAb
	[42]	Rabbit	Whole cell	10	i.v., i.p.	-, -				
	[43]	Rat	FnbpB	3	s.c.	Saponin				
Renal abscess	[44,45]	Mouse	IsdA, IsdB, SdrD, SdrE	2	i.m.	CFA, IFA	1	i.p.	P	Rabbit pAb
	[46]	Mouse	Protein A	2	i.m.	CFA, IFA				
	[47]	Mouse	PNAG	3	i.p.	-	2	i.p.	P	Rabbit pAb
			CP5+CP8				2	i.p.	P	Human pAb
Pneumonia	[21,48]	Mouse	alpha toxin	2	i.m.	CFA, IFA	1	i.p.	P	Rabbit pAb
			LukF, LukS	2	s.c.	CFA, IFA	1	i.p.	P	Rabbit pAb
				5	i.n.	Cholera toxin				
	[18,20,49]	Mouse	alpha toxin, PNAG	2	i.m.	CFA, IFA	1 or 2	i.p.	P/T	Mouse, rabbit, human mAb
Septic arthritis	[50]	Mouse	ClfA	2	s.c.	CFA, IFA	1	i.p.	P	Rat, rabbit, human pAb
	[51]	Mouse	Cna	3	s.c.	CFA, IFA				
Bacteremia	[43,52]	Mouse	FbnpA, FnbpB				1 or 2	i.v.	P	Rat, rabbit pAb
	[53]	Mouse	ABC transporter				1	i.v.	T	Rabbit pAb
	[54]	Mouse	CP5, CP8				1	s.c.	P	Human pAb

Table 1. Experimental approach in animal studies on immunization as antimicrobial strategy in *S. aureus* infection. (continued)

Experimental model	Reference	Experimental animal	Bacterial target	Active immunization			Passive immunization			
				Number of immunizations	Route of immunization	Adjuvant	Number of immunizations	Route of immunization	P/T[1]	Type of antibodies[2]
Sepsis	[44]	Mouse	IsdA, IsdB, SdrD, SdrE	2	i.m.	CFA, IFA				
	[34,35,45, 55-57]	Mouse	IsdA, IsdB	3	i.m.	AAHSA	1	i.p.	P	Mouse, human mAb, rabbit pAb
	[51,58]	Mouse	Cna, Fnbp	3	s.c.	CFA, IFA, -	1	i.p.	P	Rat pAb
	[59]	Mouse	SEA	1 or 3	s.c.	CFA, IFA	1	i.p.	P	Rat pAb
	[60]	Mouse	SEC	3	i.n.	Cholera toxin				
	[54]	Mouse	CP5	3	s.c.	Exoprotein A of *P. aeruginosa*				
	[61]	Mouse	Hypothetical protein 2160	4	i.p., s.c.	CFA, IFA				
	[20,22,62]	Mouse	ClfA, AIP-4, alpha toxin				1	i.p.	P	Mouse, human mAb
	[47,63,64]	Mouse	PNAG				1 or 2	i.p.	P	Rabbit pAb, human mAb
	[36]	Mouse	IsaA				2	i.v.	P	Mouse mAb
	[65]	Mouse	Whole cell				2	i.v., i.p.	P	IGIV
Shock	[66,67]	Rhesus macaque	SEB	3	i.m., i.t.	Alum	?	?	P/T	Chicken pAb

[1] Prophylactic (P) or therapeutic (T) treatment.

[2] Polyclonal antibodies (pAb), monoclonal antibodies (mAb).

Active immunization with leukocidin F or S (LukF or LukS) was studied by Brown et al.[21] While s.c. immunization protected mice against body weight loss, i.n. immunization was not effective.

Three studies used *S. aureus* mixed with dextran beads for infection. Park et al.[22] infected mice with *S. aureus*, and at the same time, mice were passively immunized targeting autoinducing peptide 4 (AIP-4). This passive immunization reduced hyperemia and skin injury. In the study of Balaban et al.,[23] mice were actively immunized with RNAIII activating protein (RAP). This immunization reduced the incidence of cutaneous lesions, as well as the lesion size. Pozzi et al.[18] passively immunized mice targeting poly-N-acetylglucosamine (PNAG) and alpha toxin; CP5 and ClfB; or capsular polysaccharide 8 (CP8) and iron-responsive determinant B (IsdB). These immunizations resulted in a reduction in bacterial load in the abscesses.

A model of wound infection in mice was described by Schennings et al.[24] A full thickness wound was punched out through the skin, which was subsequently infected with *S. aureus*. Mice were actively immunized with extracellular adhesion protein (Eap), clumping factor A (ClfA), and extracellular fibrinogen binding protein (Efb). This reduced the number of infected wounds.

Keratitis

One study, by Hume et al.,[25] investigated both active and passive immunization targeting alpha toxin in a keratitis model. In this infection model, rabbits are infected with *S. aureus* in the cornea. Both immunization strategies targeting alpha toxin protected against cornea erosion.

Mastitis

Mice, rabbits, sheep or cattle are challenged with *S. aureus* in their mammary glands. Prevention of mastitis is the efficacy parameter to assess efficacy of immunization.

In the study of Adlam et al.,[26] rabbits were actively immunized with alpha toxin, resulting in prevention of mastitis.

Active immunization with staphylococcal enterotoxin C (SEC) was studied by Chang et al.[27] in a mastitis model in cattle. Immunization prevented mastitis in lactating cattle.

Active immunization of cattle with whole, inactivated *S. aureus* was investigated by Nordhaug et al.,[28] resulting in a reduced incidence of spontaneous *S. aureus* mastitis.

Leitner et al.[29] actively immunized cows with killed *S. aureus* strains, and showed that reduction of the number of infected cows was obtained.

Fibronectin-binding protein A (FnbpA) was the target for both active and passive immunization of mice in the studies of Mamo et al.[30,31] Both ways of immunization prevented mastitis.

Tuchscherr et al.[32] passively immunized mice targeting ClfA, CP5, or CP8, resulting in reduction of the *S. aureus* load in the infected mammary glands.

Watson[33] used a mastitis model in sheep. In this study, animals were actively immunized with killed cells of *S. aureus*, which resulted in a decline of mastitis.

Catheter-related infection

For the catheter-related infection model, mice or rats are used. A central venous catheter is surgically implanted. After this surgery, *S. aureus* is inoculated i.v. or via the catheter. Bacterial load on the catheter and in blood is determined to assess the efficacy of immunization. In one study also the *S. aureus* load in liver, lung, spleen and kidneys are used as outcome parameters.

Passive immunization targeting IsdB was studied by both Brown et al.[34] and Ebert et al.,[35] in models in mice and rats. Brown et al. observed a higher number of culture-negative catheters in mice. In the study of Ebert et al., passive immunization targeting IsdB was successful as well: a reduction of bacterial load in blood and on the catheter was observed in rats.

In a study of Lorenz et al.,[36] IsaA was the target for passive immunization in their model in mice. This resulted in a lower bacterial load in the kidneys, while the *S. aureus* load on the catheter was not reduced.

Endocarditis

In the endocarditis model, rats or rabbits are used. A catheter is introduced via the right carotid artery into the left ventricle, touching the aortic valve. *S. aureus* is inoculated via the i.v. or i.p. route. Efficacy parameters include the staphylococcal load on the heart valves and the attached vegetations, in blood and in kidneys.

Schennings et al.[37] studied active immunization of rats with Fnbp or collagen binding protein. While active immunization with Fnbp reduced the bacterial load on heart valves and attached vegetations, immunization with collagen binding protein did not.

Vernachio et al. studied the efficacy of immunoglobulins for intravenous use (IGIV) from plasma donors with elevated levels of anti-ClfA antibody. In their first study,[38]

rabbits were treated with this IGIV in addition to vancomycin treatment. This resulted in a reduction of bacteremia. When in a second study,[39] this IGIV was tested alone in the same infection model, a lower bacterial load on cardiac valve vegetations and in blood was obtained.

CP5 was the target selected for immunization in studies by Lee et al.[40] and Nemeth and Lee[41] in rats. Lee et al. studied passive immunization, while in the study of Nemeth and Lee, rats were either actively immunized with killed *S. aureus* cells expressing CP5, or passively immunized. Whereas Lee et al. observed a lower prevalence of endocarditis in immunized rats,[40] this was not observed in the study by Nemeth and Lee.[41] Next to passive immunization targeting CP5, Nemeth and Lee also studied passive immunization targeting teichoid acid. This strategy did not protect against staphylococcal endocarditis.

Active immunization of rabbits with killed whole cell *S. aureus* was studied by Greenberg et al.[42] This did not result in reduction of the incidence of endocarditis or renal abscesses.

Rennermalm et al.[43] published a variation on the catheter-related infection model: a combined arthritis/endocarditis model. In this model, rats are i.v. challenged with *S. aureus*, and a corticosteroid is injected into the mandibular joint resulting in septic arthritis in the joint. After a week, animals are catheterized to induce endocarditis. Animals were actively immunized with fibronectin-binding protein B (FnbpB), which did not protect rats against the development of arthritis, but reduced the bacterial load on aortic valves as well as in the kidneys.

Renal abscess

Mice are used for the renal abscess model. *S. aureus* is inoculated i.v., and to assess the efficacy of immunization, the kidneys are removed to determine the bacterial load and to study histopathology.

Stranger-Jones et al.[44] actively immunized mice with IsdA, IsdB, serine-aspartate dipeptide repeat protein D (SdrD) and/or serine-aspartate dipeptide repeat protein E (SdrE). While immunization with individual antigens did not protect against *S. aureus* challenge, the combined vaccine reduced the bacterial load in kidneys below detection level, and prevented the formation of abscesses in the kidneys.

Kim et al.[45] studied IsdA and IsdB as targets for active immunization in mice. Passive immunization reduced the bacterial load in the kidneys as well as the number of abscesses. The size of abscesses was not reduced.

The group of Kim also performed a study on active immunization with protein A in mice.[46] They observed a reduction in bacterial load in the kidneys, as well as smaller and less abscesses in this organ.

PNAG was the selected target in a study of McKenney et al.[47] Both active and passive immunization strategies in mice resulted in a reduction of staphylococcal load in the kidneys. In the same study, passive immunization targeting CP5 and CP8 was examined, which did not result in a reduced bacterial load in the kidneys.

Pneumonia

In this infection model, mice are challenged i.n. by *S. aureus*. Disease progression and animal survival, staphylococcal load in lungs and pathology of the lungs are parameters to assess the efficacy of immunization strategies.

In a number of studies, immunization focused on alpha toxin was investigated. Bubeck Wardenburg et al.[48] observed in mice that active immunization resulted in a reduced mortality, as well as a reduced number of *S. aureus* in lungs and less pathology. Passive immunization of mice showed similar results. Ragle et al.[49] performed an almost identical study in mice. Results obtained were identical to those of Bubeck Wardenburg et al. Foletti et al.[20] included only passive immunization targeting alpha toxin in their study, which completely protected mice against death. In a study of Pozzi et al.,[18] passive immunization targeting both PNAG and α-toxin resulted in 100% survival of mice as well.

LukF and LukS were targets selected for active immunization by Brown et al.[21] While i.n. immunization protected mice against death, s.c. immunization did not. Remarkably, passive immunization targeting LukF and LukS, used by Bubeck Wardenburg et al.,[48] did not protect mice against pneumonia.

Septic arthritis

In the model of septic arthritis, mice are challenged i.v. with *S. aureus* and develop septic arthritis when untreated. Bacterial load in joints, degree of arthritis (assessed by clinical evaluation and histopathology) and animal survival are evaluated to assess the efficacy of immunization.

A study by Josefsson et al.[50] examined whether active and passive immunization targeting ClfA protected mice against septic arthritis. Active immunization with ClfA resulted in less severe arthritis. Passive immunization targeting ClfA suppressed the development of arthritis and protected mice against septic death.

Nilsson et al.[51] evaluated active immunization with collagen adhesin (Cna), which resulted in less severe arthritis and improved the survival of mice.

Bacteremia

Mice are infected with *S. aureus* via the i.v. or i.p. route, resulting in bacteremia. The parameters used to assess the efficacy of immunization include body weight and the *S. aureus* load in blood, spleen, liver, kidneys and peritoneal cavity.

Passive immunization targeting FnbpB was studied by Rennermalm et al.[43] Immunization resulted in less decrease of body weight. Rozalska and Wadström[52] passively immunized mice targeting FnbpA or FnbpB, which resulted in a reduction of the bacterial load in liver and peritoneal cavity, and a more rapid clearance of bacteria from blood.

An ABC transporter was target for passive immunization in the study of Burnie et al.[53] This resulted in a lower bacterial load in spleen, liver and kidneys.

Passive immunization targeting CP5 and CP8 was studied by Fattom et al.[54] This resulted in less bacteremic mice.

Sepsis

In this model, mice are challenged with *S. aureus* via the i.p. or i.v. route, resulting in sepsis. Disease progression is scored, and parameter for effective immunization is improved survival of animals.

Many targets for immunization were studied in the sepsis model in mice. Stranger-Jones et al.[44] assessed the efficacy of active immunization with IsdA, IsdB, SdrD and/ or SdrE. Remarkably, only the combination vaccine protected mice against death, while immunization with individual antigens did not.

In contrast to these observations, Kuklin et al.[55] and Joshi et al.[56] showed that active immunization with IsdB alone resulted in improved animal survival. Passive immunization targeting IsdB improved survival of mice as well.[34,35,56,57] Kim et al.[45] passively immunized mice targeting IsdA or IsdB, which also protected against septic death.

Zhou et al.[58] studied active immunization with both Cna and Fnbp. This resulted in improved survival of mice. Nilsson et al.[51] studied passive immunization targeting Cna. This also protected mice against death.

SEA was selected by Nilsson et al.[59] as target for active and passive immunization. Both immunization strategies improved survival of mice. The target selected for active

immunization in the study of Hu et al.[60] was SEC. Immunization improved animal survival.

Active immunization with CP5 was studied by Fattom et al.[54] This resulted in improved mouse survival.

Glowalla et al.[61] used hypothetical protein 2160 for active immunization. This strategy improved the survival of mice.

A number of different research groups showed that passive immunization targeting ClfA, PNAG, AIP-4, IsaA, or alpha toxin protected mice against septic death.[20,22,36,47,62-64]

Commercially available human IGIV was used by Farag et al.[65] IGIV administered via i.v. route did not protect mice against septic death, while i.p. administration of IGIV improved animal survival.

Shock

In contrast to the infection models mentioned above, in this model, rhesus macaques are used. These animals are not challenged with viable *S. aureus*, but with an aerosol of a lethal dose of staphylococcal enterotoxin B (SEB). Disease progression (anorexia, progressive depression, shock) and animal survival are outcome parameters to assess the efficacy of immunization.

Lowell et al.[66] actively immunized macaques with SEB. This resulted in protection against severe illness and death. Passive immunization targeting SEB was studied by LeClaire et al.[67] These antibodies protected macaques against death.

Clinical studies

While most immunization strategies in experimental animals have been proven to be effective in prevention, reduction or cure of *S. aureus* infection, clinical trials in humans are performed to investigate this treatment strategy. In contrast to studies in animals, it is always difficult to obtain uniform groups of patients in clinical trials due to variation in underlying diseases and in history of exposure to *S. aureus*. Proper definition of the patient population is of major importance.

Regarding the immunization studies against *S. aureus* infection, in all patients groups *S. aureus* infections are highly prevalent.

Results of immunization in clinical Phase I and Phase II/III studies

A limited number of bacterial targets for immunization were studied in humans. In Phase I studies, safety, tolerability and immunogenicity are tested in healthy adults. In Phase II/III studies, safety, tolerability, immunogenicity and sometimes efficacy are studied in *S. aureus* infected patients or individuals that are at high risk for *S. aureus* infection. The results obtained are summarized in this way.

Phase I studies

For SA75®, a vaccine composed of whole killed *S. aureus*, a Phase I study is finished. SA75® was shown to be safe and immunogenic.[68] Currently, no further studies with this vaccine are running.

PentaStaph® is a vaccine comprising CP5, CP8, polysaccharide type 336, Panton Valentine Leukocidin (PVL), and alpha toxin, studied in Phase I.[69] However, this product is currently abandoned.

Three other Phase I studies are ongoing. One study concerns the safety and immunogenicity of GSK Biologicals Staphylococcal 4-component Investigational Vaccine (GSK2392102A). Two studies assess the safety of a 4-antigen *S. aureus* vaccine (SA4Ag) or a 3-antigen *S. aureus* vaccine (SA3Ag). In these two studies, the effect on the presence of *S. aureus* on the skin and within the nose, throat and perineum of healthy adults is determined as well.[70]

Phase II/III studies

A number of Phase II/III clinical studies in *S. aureus* patients or individuals at high risk for *S. aureus* infection are summarized below.

Patients receiving hemodialysis

As *S. aureus* infection is a prominent cause of complications and death among patients receiving hemodialysis,[71] adequate treatment to prevent these infections is needed.

StaphVAX® is a vaccine containing CP5 and CP8. These polysaccharides are conjugated to nontoxic recombinant *Pseudomonas aeruginosa* exotoxin A. The vaccine was administered by a single i.m. injection. This active immunization did not provide protection against *S. aureus* bacteremia. In a post-hoc analysis, evaluating the per-

formance of the vaccine during various shorter time periods, StaphVAX® was shown to reduce *S. aureus* bacteremia through 40 weeks follow-up.[72,73]

Another vaccine, containing whole killed *S. aureus* combined with α-toxoid, was also investigated for prevention of staphylococcal infection in patients receiving hemodialysis. However, no effects of active immunization were found on the incidence of peritonitis, catheter-related infections, or *S. aureus* nasal carriage.[74]

Patients undergoing cardiothoracic surgery

Patients undergoing cardiothoracic surgery are also a group of patients that needs adequate treatment, as infections due to *S. aureus* are serious complications, resulting in substantial morbidity and mortality.[75]

V710® is a vaccine containing IsdB. This vaccine was administered once via the i.m. route to patients undergoing cardiothoracic surgery. The rate of all surgical site or invasive *S. aureus* infections was not reduced. This active immunization strategy was associated with even higher mortality among patients who developed *S. aureus* infection.[76]

Currently, studies with the vaccine StaphVAX® (described in 'Patients receiving hemodialysis') concerning safety and immunogenicity in patients undergoing cardiothoracic surgery are running.[70]

Patients undergoing orthopedic joint surgery

In orthopedic patients, *S. aureus* accounts for the majority of surgical site infections.[77] A treatment strategy that provides protection during the post-operative period would address an important unmet medical need.

Safety and immunogenicity of the vaccine StaphVAX® (described 'Patients receiving hemodialysis') are currently assessed in patients undergoing orthopedic joint surgery.[70]

Patients with bacteremia

S. aureus is a leading cause of both hospital- and community-acquired *S. aureus* bacteremia, and the current therapy is often not successful.[78] More powerful treatment is needed.

Aurexis® (tefibazumab) is a humanized monoclonal antibody that binds to ClfA. In patients with positive *S. aureus* blood cultures, who were treated with standard antibiotic therapy, this passive immunization was administered once via the i.v. route,

which was well tolerated. However, the number of patients developing *S. aureus* bacteremia-related complications that were not present at baseline or developing relapse of bacteremia was equal in patients treated with Aurexis® compared to those who received placebo-treatment.[79]

AltaStaph® contains polyclonal antibodies from humans immunized with the vaccine StaphVAX® (described in 'Patients receiving hemodialysis'), and targets CP5 and CP8. Bacteremic patients were passively immunized with two i.v. injections, next to standard antibiotic treatment. The median time to resolution of *S. aureus* bacteremia was not significantly different between the AltaStaph® group and the placebo group. The time to hospital discharge was reduced in the AltaStaph® group. However, due to the small sample size, this study was not powered to show efficacy, and therefore, this passive immunization strategy warrants further investigation.[80]

Patients with deep-seated infection

Treatment failure of deep-seated MRSA infections often occurs. Higher vancomycin concentrations are thought to be necessary. However, this results in a higher risk of nephrotoxicity.[81] Therefore, alternatives for vancomycin treatment are urgently needed.

Aurograb® is a single-chain monoclonal antibody variable fragment binding to an ABC transporter. Patients with deep-seated MRSA infection were passively immunized twice daily via the i.v. route, next to vancomycin. No additional efficacy was observed in patients treated with both antibiotics and Aurograb®, and therefore Aurograb® was not further developed.[69,82]

Very low birth weight infants

S. aureus is one of the most common organisms causing late onset sepsis, the leading cause of death in infants with very low birth weight.[83] More adequate treatment of this infection is needed to lower mortality.

Veronate® (INH-A21) is an intravenous immunoglobulin from donors with high levels of antibodies directed against ClfA. Premature infants were passively immunized with four i.v. infusions of this IGIV. Bloom et al.[84] showed that this IGIV had potential to reduce *S. aureus* sepsis and mortality in this group, but statistical significance was not reached. In a study of DeJonge et al.,[85] Veronate® failed to reduce the incidence of late onset sepsis in premature infants.

AltaStaph® (described in 'Patients with bacteremia'), polyclonal antibodies targeting CP5 and CP8, was applied in very low birth weight neonates. Passive immunization with two i.v. infusions of AltaStaph® induced high levels of CP5 and CP8 specific antibodies, but rates of *S. aureus* bacteremia were similar in the treatment and the placebo group. However, due to the small sample size, this study was not sufficiently powered to detect small differences in outcome, and therefore a study including more patients is needed.[86]

Pagibaximab® is a humanized monoclonal antibody targeting lipoteichoic acid (LTA). Patients were immunized twice via the i.v. route. This antibody was shown to be safe and tolerable in very low birth weight infants.[87] Its efficacy has not been evaluated until now.

Concluding remarks

Antibiotic treatment of *S. aureus* infections is not always successful. Due to the emergence of antibiotic resistance, alternatives for antibiotic treatment are urgently needed. Active and passive immunization could be alternative, non-antibiotic-related, antimicrobial intervention strategies. Various studies in animal models and humans have been conducted in this field. In animal models, a study design focused on a clinically-relevant infection, a proper bacterial target and well-considered dosing will enhance the translational value of the results obtained.

A range of experimental models of *S. aureus* infections are described to study the efficacy of active or passive immunization as antimicrobial strategy. The animal models are well selected, as they represent the most common *S. aureus* infections in the clinical setting. A broad panel of *S. aureus* targets has been studied in these experimental models. Active and passive immunization targeting these *S. aureus* antigens have mostly shown to be successful in prevention, reduction or cure of infection.

In contrast to the numerous studies in experimental animals, only a limited number of clinical studies in *S. aureus* infected patients or patients at high risk for *S. aureus* infection has been performed. In most of these clinical studies, *S. aureus* bacteremia/sepsis is a common infectious complication, and is therefore used as outcome parameter for efficacy in immunization studies.

Clinical Phase II/III studies have been performed focused on the bacterial targets CP5, CP8, IsdB, ClfA, ABC transporter, or whole cell *S. aureus*. Successful results were obtained in patients receiving hemodialysis, who are at high risk for *S. aureus*

infection. Active immunization with CP5 and CP8 (StaphVAX®) resulted in a reduction of *S. aureus* bacteremia. Similar observations were obtained in the experimental *S. aureus* sepsis model in mice, showing that active immunization with CP5 improved the survival of mice. This indicates the translational value of the results obtained in this experimental mouse sepsis model to the clinical setting.

Other studies in patients with *S. aureus* bacteremia and in very low birth weight infants who are at high risk for *S. aureus* sepsis, investigating the efficacy of passive immunization with antibodies targeting CP5 and CP8 (AltaStaph®), were less successful in providing protection against *S. aureus* bacteremia. Lack of power because of the low sample size might contribute to the lack of success of immunization. In contrast, in the experimental *S. aureus* bacteremia model in mice, passive immunization with antibodies targeting CP5 and CP8 has been shown to protect mice against bacteremia. Based on these encouraging results in this animal model, more extended studies on the efficacy of AltaStaph® in patients with *S. aureus* bacteremia are warranted.

Many other *S. aureus* targets have been selected for immunization studies in animal infection models. In the experimental bacteremia and sepsis models in mice, successful results were obtained with active or passive immunization focusing on IsdA, IsdB, SdrD, SdrE, Cna, FnbpA, FnbpB, SEA, SEC, hypothetical protein 2160, ClfA, PNAG, AIP-4, IsaA, alpha toxin, and/or ABC transporter, or whole cell *S. aureus*. From these bacterial targets, only IsdB, ClfA, ABC transporter or whole cell *S. aureus* were investigated in clinical Phase II/III immunization studies. Efficacy was never observed in these studies.

Until now, in most clinical studies immunization as antimicrobial strategy was investigated in relation to *S. aureus* bacteremia/sepsis. Based on the successful immunization studies in experimental animal models of other *S. aureus* infection, such as skin/wound infection, keratitis, mastitis, catheter-related infection, endocarditis, renal abscess, pneumonia and arthritis, studies in patients with *S. aureus* infections other than bacteremia/sepsis investigating the efficacy of immunization as antimicrobial strategy seem warranted.

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7

A human monoclonal antibody against the conserved staphylococcal antigen IsaA protects mice against *Staphylococcus aureus* bacteremia

Sanne van den Berg,* Hendrik P.J. Bonarius,* Kok P.M. van Kessel, Goffe S. Elsinga, Neeltje Kooi, Hans Westra, Tjibbe Bosma, Magdalena M. van der Kooi-Pol, Danny G.A.M. Koedijk, Herman Groen, Jan Maarten van Dijl, Girbe Buist, Irma A. J. M. Bakker-Woudenberg

* These authors contributed equally to this study.

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Abstract

Due to substantial therapy failure and the emergence of antibiotic-resistant *Staphylococcus aureus* strains, alternatives for antibiotic treatment of *S. aureus* infections are urgently needed. Passive immunization using *S. aureus*-specific monoclonal antibodies (mAb) could be such an alternative to prevent and treat severe *S. aureus* infections. The invariantly expressed immunodominant staphylococcal antigen A (IsaA) is a promising target for passive immunization. Here we report the development of the human anti-IsaA IgG1 mAb 1D9, which was shown to bind to all 26 *S. aureus* isolates tested. These included both methicillin-susceptible and methicillin-resistant *S. aureus* (MSSA and MRSA, respectively). Immune complexes consisting of IsaA and 1D9 stimulated human as well as murine neutrophils to generate an oxidative burst. In a murine bacteremia model, the prophylactic treatment with a single dose of 5 mg/kg 1D9 improved the survival of mice challenged with *S. aureus* isolate P (MSSA) significantly, while therapeutic treatment with the same dose did not influence animal survival. Neither prophylactic nor therapeutic treatment with 5 mg/kg 1D9 resulted in improved survival of mice with *S. aureus* USA300 (MRSA) bacteremia. Importantly, our studies show that healthy *S. aureus* carriers elicit an immune response which is sufficient to generate protective mAbs against invariant staphylococcal surface antigens. Human mAb 1D9, possibly conjugated to for example another antibody, antibiotics, cytokines or chemokines, may be valuable to fight *S. aureus* infections in patients.

Introduction

Staphylococcus aureus represents a major burden for public health, particularly in health care settings. This pathogen produces multiple virulence factors, which facilitate the colonization of susceptible hosts and can lead to serious infections, such as surgical wound infection and pneumonia.[1,2] Life-threatening invasive diseases, such as sepsis, endocarditis, osteomyelitis and meningitis, can arise when *S. aureus* enters the blood stream. The risk of intravascular and systemic infection by *S. aureus* increases when the first line barrier in host defense is disrupted by surgery, intravascular catheters, implants, mucosal damage or trauma. Antibiotic-resistant variants of *S. aureus*, such as methicillin-resistant *S. aureus* (MRSA), are frequently encountered in hospitals, and lead to significantly increased mortality and length of stay for patients.[3,4] In recent years, a spread of resistant staphylococci has also been observed outside health care settings, due to community-acquired MRSA.[5] Therefore, there is a major need to develop novel long-lasting anti-staphylococcal therapies to enhance, or even replace the currently applied therapies. Given the fact that antibiotic-resistant *S. aureus* strains typically emerge within the first years after the introduction of novel antibiotics,[5] there is a critical need for alternative antimicrobial intervention strategies.

Passive immunization with human(ized) monoclonal antibodies (mAbs) seems a highly attractive alternative for treatment with antibiotics. Several anti-staphylococcal mAbs have shown efficacy in animal models as well as safety in phase I clinical trials.[6] Nevertheless, efficacy of passive immunization in phase II or III clinical trials is not yet observed. The bacterial targets of these antibodies were selected based on accessibility for antibody therapy and, in some cases, a role in bacterial virulence. However, none of these mAbs was designed to neutralize targets present on all *S. aureus* strains, the so-called conserved antigens, and at the same time targeting bacterial factors essential for bacterial growth and virulence.

Using a combination of proteomics, genomics, bioinformatics and immunological approaches, we identified conserved immunogenic determinants of relevant *S. aureus* isolates, of which the immunodominant staphylococcal antigen A (IsaA) is one example. IsaA is a conserved protein that is invariably produced by all *S. aureus* isolates tested so far.[7] Notably, IsaA is a non-covalently cell wall-bound lytic transglycosylase,[8-10] which is co-regulated with the glycylglycine endopeptidase LytM.[11] Based on its role in cell wall growth and division and the invariant expression in a wide range of *S. aureus* isolates, IsaA can be regarded as a standard cellular housekeeping protein.

Surface-exposed protein domains of IsaA of clinically relevant *S. aureus* isolates have been identified by gel-free proteomics.[12] In humans, IsaA is highly immunogenic, and *S. aureus*-infected patients have increased anti-IsaA IgG levels.[8,13-15] Whether the high anti-staphylococcal antibody titers protect against severe invasive *S. aureus* infections remains to be shown. However, this is well conceivable since a murine mAb targeting IsaA described by Lorenz et al.[16] was shown to be protective in mouse models of catheter-related *S. aureus* infection and *S. aureus* sepsis. Altogether, these findings suggested that IsaA is an interesting target for passive immunization.

In the present study, we have generated a fully human mAb with specificity against IsaA by single-cell PCR cloning of the IgG genes from IsaA-specific B cells donated by *S. aureus* carriers. This mAb was characterized *ex vivo* and its potential efficacy to protect mice against *S. aureus* infection was assessed in a bacteremia model. The results show that our approach to generate human mAbs targeting conserved staphylococcal surface proteins works and that it has the potential to develop a novel-class therapy to treat *S. aureus* infections.

Materials and methods

Bacteria

The studies included the sequenced *S. aureus* isolates Mu50, MW2, N315, COL, 8325-4, MRSA252, MSSA476, USA300 and Newman, and 16 clinical isolates (A, C, E-F, M-X) that were previously analyzed by proteomics.[7] Recombinant strains lacking IgG-binding proteins included *S. aureus* NewmanΔ*spa*Δ*sbi*[17] and SH1000Δ*spa*.[18] The *S. aureus* isolates P (MSSA) and USA300 (MRSA) were used in animal experiments. The *S. aureus* USA300 strain used in the present study is a clinical isolate of the USA300 pulsed-field gel electrophoresis type (data not shown), and is referred to as "*S. aureus* USA300".

Selection of blood donors

Healthy nasal *S. aureus* carriers were selected as potential donors for protective human antibodies via assessment of left and right anterior nares samples using transswabs (MWE) as described earlier.[15] *S. aureus* identification was based on colony morphology after growth on blood agar with 5% sheep blood, Gram staining, catalase test and Pastorex Staph Plus test (Biorad).

Isolation and immunodetection of IsaA

The *isaA* gene was PCR-amplified from chromosomal DNA of *S. aureus* NCTC8325 using the primers IsaAHis.fw (AGGCACTCACCATGGGAGCTGAAGTAAACGTTGATCAAG) and IsaAHis.rev (GTGATGTCGAATTCCGAATCCCCAAGCACCTAAACCTTG). The resulting PCR product specified IsaA with a C-terminal hexa-histidine tag (IsaA-His_6). This fragment was cleaved with the restriction enzymes *Nco*I and *Hin*dIII (restriction sites are underlined in the primer sequences) and ligated into these sites of plasmid pET24d (Novagen), resulting in plasmid pET24dIsaA. For IsaA production, an overnight culture of *E. coli* BL21DE3 (pET24dIsaA) was diluted 1:100 in fresh Lysogeny Broth (BD). Four hours after induction of IsaA production with 1 mM isopropyl-β-D-thiogalactopyranoside, cells were harvested, resuspended in binding buffer (20 mM sodium phosphate buffer, 0.5 M NaCl, 30 mM imidazole, pH 7.3) with 6 M urea, and disrupted in a French Pressure cell (Thermo Scientific) chilled on ice. Cleared lysate was mixed with His Mag Sepharose™ Ni magnetic beads (GE Healthcare) and the IsaA-His_6 protein was isolated and purified according to the instructions of the supplier. Pooled fractions containing the purified IsaA-His_6 were dialyzed against PBS.

Cell and supernatant fractions from overnight cultures of *S. aureus* SH1000Δ*spa* were prepared as described before.[17] Protein samples were separated using NuPAGE gels (Life Technologies) according to the manufacturer's instructions and proteins were transferred to a Protran nitrocellulose membrane (Whatman) by semi-dry blotting (75 minutes at 1 mA/cm^2). Membranes were incubated with specific rabbit antibodies against IsaA (kindly provided by N. Sakata[9]) or with the human mAb. After incubation with IRDye 800 goat anti-rabbit (IsaA) or IRDye 800 goat anti-human IgG antibodies respectively, the signals were detected using the Odyssey system (LI-COR Biosciences).

Production and selection of human monoclonal antibody

Blood was donated by nasal *S. aureus* carriers. The Independent Ethics Committee of the Foundation 'Evaluation of Ethics in Biomedical Research' approved the protocol for blood donation. The protocol is registered by QPS Groningen (code 04132-CS011). All volunteers provided their written informed consent.

Human monoclonal IgG1 antibodies were generated using the mCHR protocol (molecular Cloning of the Human Response) according to US 7807415 B2[19] with some modifications, being the electronic sorting of IsaA-specific memory B cells using biotinylated IsaA-His_6, CD27 and CD19, and the cloning of mAbs as described[20,21] from single cell sorted IsaA ELISPOT-positive wells. The mAb named "1D9" is used in the

present study. The human mAb directed against Protective Antigen (PA) of *Bacillus anthracis* (IQNPA) was described before[22] and used as an isotype control mAb.

Antigen ELISA

ELISA plates were coated with IsaA-His_6 (100 or 250 ng/well) or PA (100 ng/well), diluted in carbonate coating buffer (71 mM $NaHCO_3$, 29 mM Na_2CO_3, pH 9.6). Plates were blocked for 1 hour at 37°C with PBS containing 1% BSA. Purified plasma or antibodies were used in a titration range starting at 20 or 1000 ng/mL. Binding of antibodies was monitored by addition of goat or rabbit anti-human-HRP, diluted in PBS with 0.05% Tween-20 (PBST) and 5% FCS and incubated for 1 hour at 37°C. Between each step, the wells were washed 3 times with PBST. Reaction was visualized with TMB substrate (Kem-en-Tec Diagnostics) and stopped by H_2SO_4. The plates were read with the Magellan 2 program of an ELISA reader, which was set on 450 nm absorbance and a reference filter of 620 nm. Samples were determined positive if the OD_{450} was > 3 times the OD_{450} of PBS 1x (plasma of donors) or supernatant from B cells cultivated in Linolea complete medium (i.e. IMDM containing BSA, insulin, ethanolamine and β-mercaptoethanol). To check for aspecific antibody binding, the samples were also tested on a plate coated with 4% BSA.

S. aureus whole cell ELISA

Bacteria were grown overnight in HEPES-buffered IMDM without phenol red (Life Technologies), diluted in fresh medium, and subsequently cultured until the mid-exponential growth phase (OD_{660} ~ 0.5). Bacteria were washed with PBS and stored at -20°C. ELISA plates were coated with 5 x 10^6 cfu per well in PBS for 18 hours at 4°C. Plates were blocked first with 4% BSA in PBST and subsequently with 100 μg/mL normal guinea pig IgG (Jackson ImmunoResearch Laboratories) in PBST containing 1% BSA to block surface expressed IgG Fc-binding proteins of *S. aureus*. Binding of biotinylated human mAbs was detected with HRP-labeled streptavidin (Dako) and visualized with TMB substrate as above.

Animals

Specified pathogen-free female BALB/cBYJ mice were obtained from Charles. Animals were 11-13 weeks old at the day of infection, and were given food and water *ad libitum*.

The animal experimental protocols adhered to the rules laid down in the Dutch Animal Experimentation Act and the EU Animal Directive 2010/63/EU.

Effect of 1D9 on *S. aureus* growth

Bacteria were grown overnight in IMDM or MHB (Oxoid), diluted 1:50 in fresh medium and mixed with an equal volume of buffer or human mAbs with or without additional 10% mouse serum. Wells were loaded with 200 µL of this mixture and incubated with shaking for 18 hours at 37°C in a BioScreen C growth analyzer (Oy Growth Curves Ab Ltd). OD_{600} was measured every 15 minutes.

Isolation of neutrophils

Human neutrophils were isolated from heparinized blood of healthy volunteers as described.[23] Murine neutrophils were isolated from bone marrow of uninfected mice. Therefore, the femur and tibia of both hind legs were prepared, flushed with HBSS (Life Technologies) containing 0.1% HSA, 15 mM EDTA, and 25 mM HEPES, and residual erythrocytes were lysed with ice cold water. To enrich for neutrophils, cells were loaded onto a discontinuous Percoll (GE Healthcare) gradient of 81% and 62.5% and centrifuged for 30 minutes at 1,500 x *g* without brake. Cells were recovered from the band formed between the 62.5% and 81% layer, washed and resuspended in RPMI/HSA. Cell purity was determined by specific staining with PE-labeled rat anti-Ly-6G (Gr-1) mAb (Life Technologies) and flow cytometry (FACSCalibur; BD) (purity 75-85%).

Quantitative determination of neutrophil activation and oxidative burst

The immune complex (IC) induced oxidative burst of neutrophils was determined by luminol enhanced chemiluminescence. White microplates (Cliniplate; Thermo Scientific) were coated with 5 µg/mL HSA or IsaA-His_6 in 50 µL of 0.1 M carbonate buffer (pH 9.6) for 1 hour at 37°C. After washing with PBS, wells were blocked with 10% FCS and subsequently incubated with antibodies in PBS/1% FCS, for HSA with 1 µg/mL rabbit anti-HSA (Sigma Aldrich) or normal rabbit IgG (Jackson ImmunoResearch Laboratories), for IsaA-His_6 with 3 µg/mL 1D9 or IQNPA. After a final wash with PBS, 100 µL HBSS/0.1% HSA containing 150 µM luminol was added and the plate was loaded into a CentroLB 960 microplate luminometer (Berthold Technologies). The reaction was initiated by the addition of 50 µL neutrophils in HBSS/HSA at 1.25 x 10^6 cells/mL for human and 5 x 10^6 cells/mL for murine neutrophils, respectively. Oxidative burst was continuously recorded for 30 minutes at 37°C and expressed as relative light units.

Phagocytosis assay

S. aureus isolate P and USA300 were labeled with 100 μg/mL FITC for 1 hour at 4°C, washed with RPMI/HSA and stored at -20°C. Phagocytosis with human or mouse neutrophils was performed as described,[24] but extended to 30 minutes for murine neutrophils and including normal mouse serum. Bacteria to cell ratio was 10:1 (MOI 10).

IsaA expression by *S. aureus* isolates P and USA300

For detection of IsaA expression by *S. aureus* isolates P and USA300, strains were grown in BHI (BD) and stored as described in '*S. aureus* whole cell ELISA'. Around 10^5 cfu of bacteria were inoculated in BHI, IMDM, or serum of BALB/cBYJ mice (Charles River). Culture samples were collected 6 and 24 hours after inoculation. Cfu's of (un) diluted suspensions were determined after overnight growth at 35°C on blood agar with 5% sheep blood. Cell fractions were prepared and analyzed by Western blotting and immunodetection as described in 'Isolation and immunodetection of IsaA'.

Infection model of *S. aureus* bacteremia

For assessment of the efficacy of 1D9 in protection against death due to *S. aureus* bacteremia, mice were treated intravenously (i.v.) with either 1D9 (5 mg/kg in a volume of 100 μL) or saline (n=12 per group). *S. aureus* bacteremia was induced as described before,[25] using inocula of 2-4 x 10^5 cfu for *S. aureus* isolate P or 4-7 x 10^5 cfu for *S. aureus* USA300. Three hours before or after induction of bacteremia, 1D9 was administered. Discomfort and animal survival rate over 14 days after infection was monitored as described before.[25]

For characterization of the early course of bacteremia, the bacterial load in blood, lungs, spleen, liver, and kidneys was assessed at 1, 6, or 24 hours after infection (n=4 per time point) as described.[25]

For determination of 1D9 serum levels over time, mice were treated i.v. with 1D9 (5 mg/kg in a volume of 100 μL). Three hours after treatment, bacteremia was induced by either *S. aureus* isolate P or USA300 (n=3 per group). Uninfected mice were included as well. Blood was withdrawn from the tail artery of infected mice at 1, 6, and 24 hours after infection and collected in a Microvette® CB300 capillary tube (Sarstedt). Sera were prepared and stored at -80°C. Serum levels of 1D9 were determined by antigen ELISA as described above.

Statistical analysis

For comparison of cfu counts in blood and organs of infected mice, cfu counts were log_{10} transformed before analysis. The *t* test was used for comparison of cfu counts in mice with *S. aureus* isolate P bacteremia and mice with USA300 bacteremia (IBM SPSS Statistics version 20; IBM Corporation). As multiple comparisons were made, a Bonferroni correction was applied. As a result, *P*-values < 0.003 were considered statistically significant.

Mean cfu counts at 1, 6, and 24 hours were compared using one-way ANOVA (IBM SPSS Statistics version 20). The log rank test was used to determine statistical differences in animal survival rates (GraphPad Prism 5 for Windows; GraphPad Software Inc.). Differences were considered statistically significant when two-sided *P*-values were <0.05.

Results

Donor selection and generation of antibodies

Six volunteers were identified as nasal *S. aureus* carriers, of which five had significant IgG levels for IsaA (Figure 1A). Of the latter group, donor T7-1 also tested positive for PA, which was used as a control (Figure 1B). Material from this donor was not used to prevent collection of high-binding non-specific antibodies. Blood from the remaining four donors was tested for the presence of monoclonal IsaA-specific B cells. In this case, only donor T7-5 tested positive as judged from an ELISPOT analysis after selection of IsaA-binding B lymphocytes. Human mAbs were generated, and six of these were found to bind purified IsaA-His_6. Only one monoclonal, named "1D9", was positive for IsaA-His_6 and negative for the control antigens (Figure 1C and D). As shown by Western blotting, the human mAb 1D9 was equally effective in binding the cell-associated and secreted forms of native IsaA as an IsaA-specific polyclonal control antibody from rabbit (Figure 1E).

Binding of 1D9 to *S. aureus*

After the generation of the human anti-IsaA mAb 1D9, we assessed whether this mAb was able to bind to *S. aureus* as well. To check for interference of binding of 1D9 via the Fc-region to protein A and Sbi, *S. aureus* NewmanΔ*spa*Δ*sbi* was included. 1D9 bound concentration-dependent to *S. aureus* isolate P, USA300, Newman wild-type and NewmanΔ*spa*Δ*sbi* (Figure 2A). Binding of control mAb IQNPA to *S. aureus*

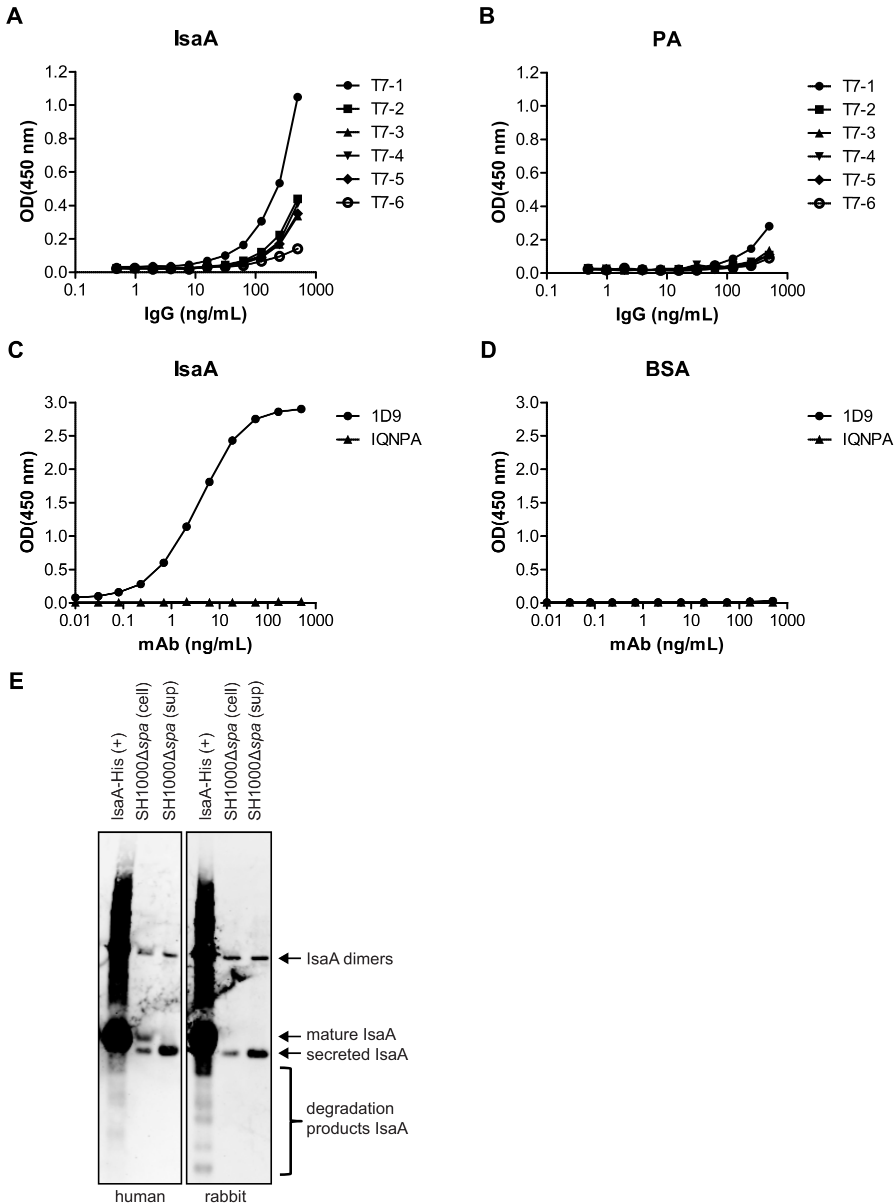

Figure 1. Donor selection and generation of human monoclonal antibody 1D9 specific for IsaA and *S. aureus*. (A, B) Serum IgG levels against IsaA-His$_6$ or PA in 6 nasal *S. aureus* carriers. (C, D) Concentration-dependent binding of 1D9 and isotype control antibody (IQNPA) to IsaA-His$_6$ or BSA. (E) Binding of 1D9 to native and IsaA-His$_6$. Western blot analysis of native IsaA in cell (cell) and supernatant (sup) fractions of *S. aureus* SH1000Δ*spa* and purified IsaA-His$_6$ using human mAb 1D9 (left panel) or rabbit polyclonal anti-IsaA (right panel). For data in panels A-D, 3 independent experiments were performed. Representative curves are shown.

isolate P and USA300 was low, but considerable to Newman wild-type despite blocking surface expressed IgG Fc-binding proteins with non-related guinea pig IgG (Figure 2B). In addition, 1D9 bound to 9 sequenced and 16 defined clinical *S. aureus* isolates, including both MSSA and MRSA isolates (Figure 2C). Binding of IQNPA (100 ng/mL) was relatively low for most strains (Figure 2D).

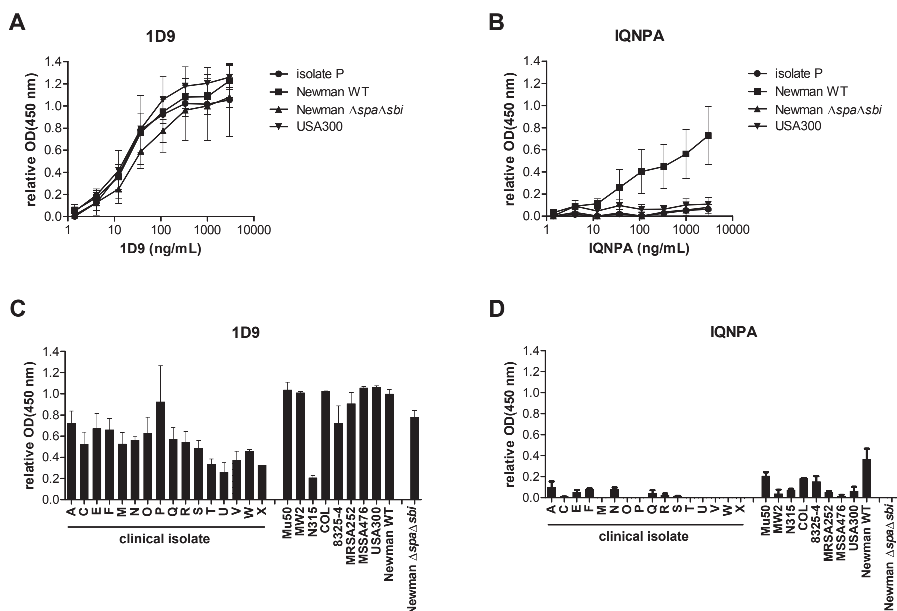

Figure 2. Binding of 1D9 to *S. aureus* in whole cell ELISA assay. Plates were coated with cells of various *S. aureus* strains harvested at mid-exponential growth. Biotinylated 1D9 or the isotype control antibody IQNPA was added. Absorption values (at 450 nm) are plotted relative to binding of 1000 ng/mL 1D9 to *S. aureus* Newman*Δspa*Δ*sbi*. Experiments were performed in triplicate. Mean ± SD is shown. (A) Concentration-dependent binding of 1D9 to *S. aureus* NewmanΔ*spa*Δ*sbi*, Newman wild-type (WT), isolate P, or MRSA USA300. (B) Concentration-dependent binding of isotype control antibody IQNPA to *S. aureus* NewmanΔ*spa*Δ*sbi*, Newman wild-type (WT), isolate P, or USA300. (C) Binding of 100 ng/mL 1D9 to *S. aureus* NewmanΔ*spa*Δ*sbi*, various clinical *S. aureus* isolates including *S. aureus* isolate P, or the sequenced *S. aureus* strains Mu50, MW2, N315, COL, 8325-4, MRSA252, MSSA476, USA300 and Newman wild-type (WT). (D) Binding of 100 ng/mL isotype control antibody IQNPA to *S. aureus* NewmanΔ*spa*Δ*sbi*, various clinical *S. aureus* isolates including *S. aureus* isolate P, or various sequenced *S. aureus* strains as specified for panel C.

Lack of effect of 1D9 on multiplication of *S. aureus*

Although binding of 1D9 to *S. aureus* was clearly detected, the addition of up to 30 µg/mL of 1D9 to *S. aureus* isolate P neither affected its multiplication in MHB nor in IMDM (data not shown). Serum supplementation up to 10% did not change the normal growth of *S. aureus* isolate P, neither with nor without the addition of 1D9.

Activation of human and murine neutrophils by 1D9

To explore the potential Fcγ receptor (FcγR) stimulation of human mAb 1D9 for both human and murine cells, isolated neutrophils were challenged with IsaA/1D9 or HSA/rabbit-anti-HSA immune complexes (IC) to generate an oxidative burst. The luminol-enhanced chemiluminescence was properly initiated with both control IC and IsaA/1D9 IC (Figure 3). Omission of either antigen or specific IgG or exchange with isotype control IgG did not activate the oxidative burst. This shows that IC containing the human mAb 1D9 can specifically interact with the FcγRs that are expressed on the surface of human and murine neutrophils.

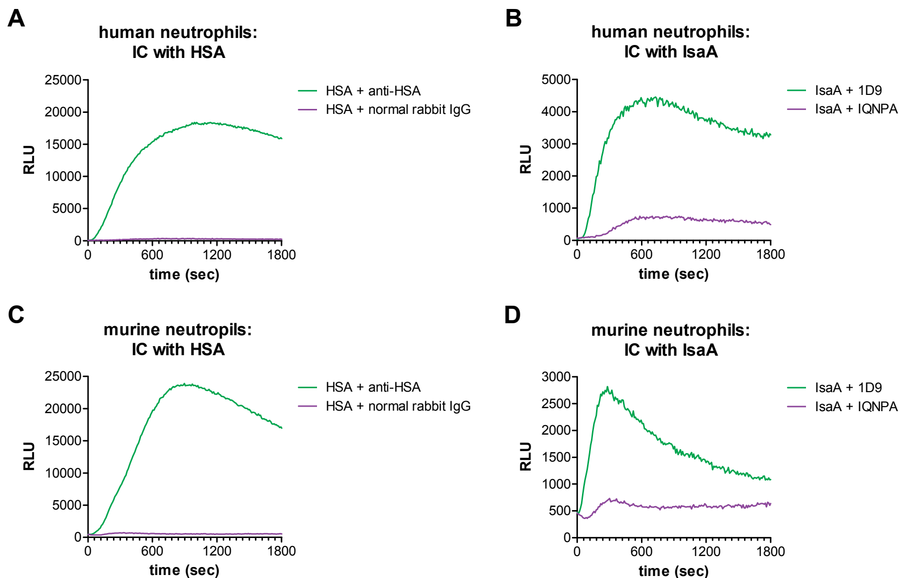

Figure 3. Activation of human or murine neutrophils by 1D9. Plates were coated with 5 µg/mL HSA (A, C) or IsaA (B, D), blocked with 10% fetal calf serum and incubated with 1 or 3 µg/mL rabbit anti-HSA or 1D9, respectively. Control antibodies were normal rabbit IgG or human mAb IQNPA. Neutrophils (human neutrophils (A, B) 1.25 x 10^6 cells/mL, murine neutrophils (C, D) 5 x 10^6 cells/mL) were added to initiate the reaction. Oxidative burst was measured for 30 minutes at 37°C. RLU, relative light units. Three independent experiments were performed. Representative curves are shown.

Lack of effect of 1D9 on the phagocytosis of *S. aureus* by human and murine neutrophils

As expected, normal human serum was found to be an efficient opsonin source for phagocytosis of *S. aureus* isolate P (Figure 4) and USA300 (data not shown) by human neutrophils. Inactivation of the complement system by heating diminished the potency, but phagocytosis was still promoted. Normal mouse serum promoted phagocytosis of *S. aureus* isolate P and USA300 as well, but required an intact complement system. The autologous combination of murine neutrophils and mouse serum resulted in the most efficient phagocytosis observed in the present experiments. However, in all combinations tested, 1D9 (10 μg/mL) did not initiate or improve phagocytosis with either human or murine neutrophils.

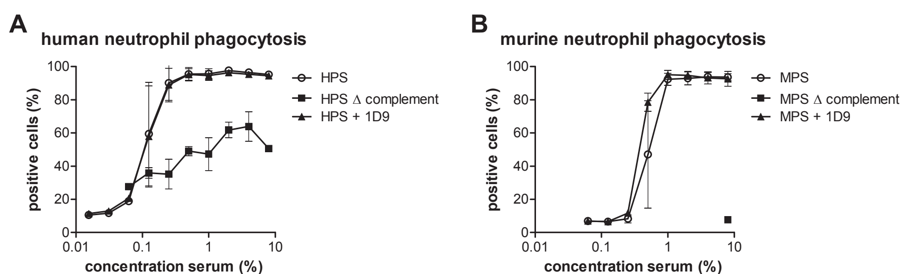

Figure 4. Phagocytosis of *S. aureus* isolate P by human (A) and mouse (B) neutrophils. Serial dilutions of normal human pooled serum (HPS) or normal mouse pooled serum (MPS), with or without (Δ) complement (inactivated by heating), were mixed with 1D9 (10 μg/mL). *S. aureus* isolate P, labeled with FITC, was added and preincubated with sera. Subsequently, human or murine neutrophils (bacteria to cell ratio 10:1) were added and incubated for 15 (human) or 30 (mouse) minutes on a shaking platform at 37°C. The number of cells containing fluorescent bacteria (positive cells) was determined using flow cytometry. Experiments were performed in triplicate. Mean ± SD is shown.

IsaA expression by *S. aureus* isolates P and USA300

To estimate the production of IsaA by the *S. aureus* isolates P and USA300 in different media and at different stages of growth, both were grown in BHI, IMDM, or pooled serum from BALB/c mice. The expression of IsaA was more or less identical during growth in IMDM, mouse serum (Supplementary Figure S1) or BHI (data not shown). In addition to the IsaA-specific band, a protein A band was observed due to the aspecific binding of IgG. After 24 hours of growth in mouse serum, a much higher expression of protein A was observed in *S. aureus* USA300 than in isolate P.

Early course of *S. aureus* bacteremia in mice

To study the course of *S. aureus* isolate P or USA300 bacteremia, the bacterial load in blood and organs of infected mice sacrificed at various time points was determined (Figure 5). In both infections, ~99% of the staphylococci had disappeared from blood already after 1 hour. The bacterial load in spleen and liver decreased within the first

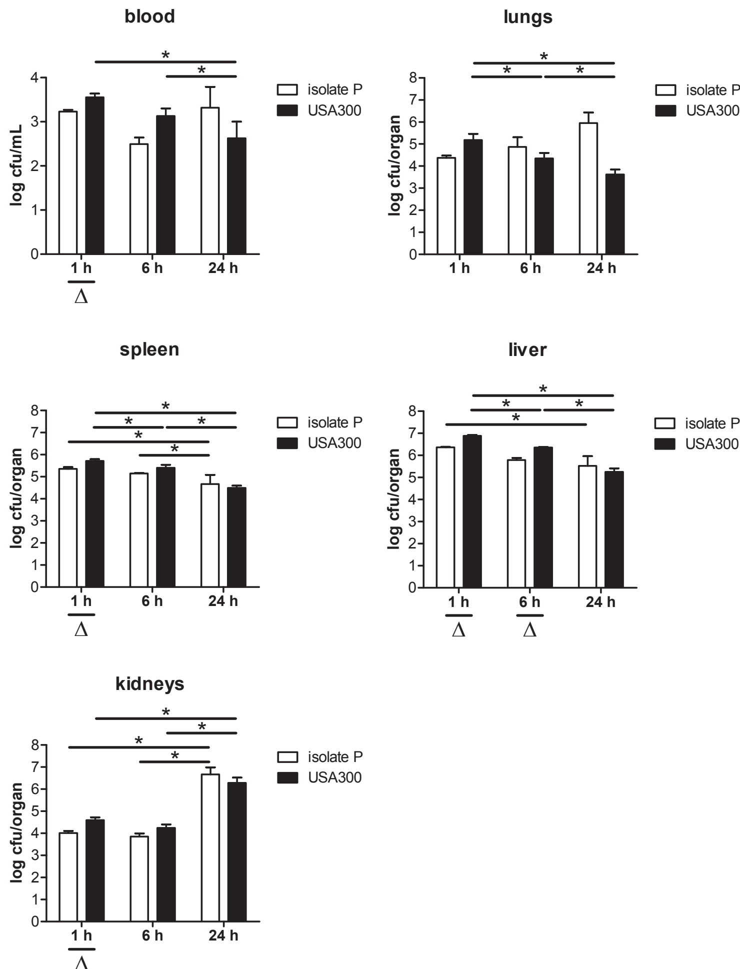

Figure 5. *S. aureus* counts in blood and organs from mice with *S. aureus* bacteremia. Mice (4 per group) were infected with 2-4 x 10^5 cfu of *S. aureus* isolate P (open bars) or with 4-7 x 10^5 cfu of *S. aureus* USA300 (black bars), by intravenous inoculation, and were sacrificed at indicated time points. Quantitative cultures of blood and organs were performed. Mean and SD are shown. Asterisks indicate statistically significant differences in cfu counts between mice with similar infection (one-way ANOVA, $P < 0.05$). Triangles indicate statistically significant differences in cfu counts between mice with *S. aureus* isolate P bacteremia and mice with *S. aureus* USA300 bacteremia (*t* test, $P < 0.003$).

24 hours after infection, while the *S. aureus* numbers in the kidneys increased. Only in mice with *S. aureus* USA300 bacteremia, staphylococcal load in blood and lungs decreased. At 1 hour after infection, the bacterial load in blood, spleen, liver, and kidneys was higher in mice with *S. aureus* USA300 bacteremia than in mice with isolate P bacteremia.

Course of serum 1D9 levels over time

Mice were treated i.v. with 5 mg/kg 1D9. Three hours later, bacteremia was induced by either *S. aureus* isolate P or USA300. Antibody levels at 1, 6, and 24 hours were assessed (Figure 6). In uninfected mice, serum 1D9 levels remained stable during 24 hours. In infected mice, the mean 1D9 titers showed a log reduction compared to uninfected mice. Although variability in 1D9 titers between individual mice was observed, these levels were stable during 24 hours. No differences in 1D9 titers were observed between mice with either *S. aureus* isolate P or USA300 bacteremia.

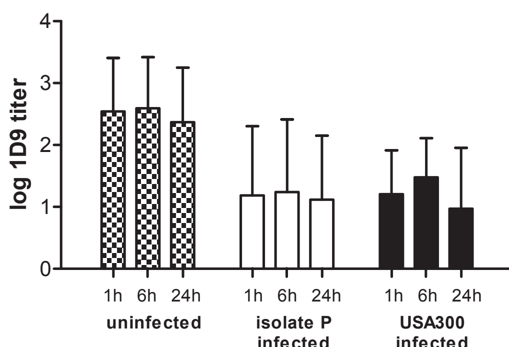

Figure 6. Course of serum 1D9 levels over time. Mice (3 per group) were treated intravenously with 5 mg/kg 1D9. Three hours later, bacteremia was induced by either *S. aureus* isolate P or *S. aureus* USA300. Uninfected mice were included as well. At indicated time points after infection, serum levels of human mAb 1D9 were assessed using ELISA plates coated with 250 ng of purified IsaA per well. Mean and SD are shown.

Protective effect of 1D9 in mice with *S. aureus* bacteremia

The *in vivo* efficacy of 1D9 was assessed in the murine model of *S. aureus* bacteremia caused by either *S. aureus* isolate P or USA300. Mice were treated i.v. with 1D9 or a placebo. Saline was used for the placebo treatment, as pilot experiments showed that survival of saline-treated mice was comparable to that of mice treated with IQNPA (5 mg/kg, data not shown). Survival of placebo-treated mice declined gradually over 14

days. Of the mice with *S. aureus* isolate P bacteremia, 25-42% survived the study period, while 17-42% of the mice with *S. aureus* USA300 bacteremia survived. After this time period, no changes in animal survival were observed. Survival rate of placebo-treated mice was comparable in all groups. Prophylactic treatment with 5 mg/kg 1D9 resulted in increased time to death and a significantly improved survival rate of mice infected with *S. aureus* isolate P (P = 0.0057; Figure 7A). In contrast, prophylactic treatment with 5 mg/kg 1D9 did not result in an improvement in the survival rate of mice infected with *S. aureus* USA300 (P > 0.05; Figure 7C). Therapeutic treatment with 5 mg/kg 1D9 did not improve the survival rate of mice with *S. aureus* isolate P or USA300 bacteremia (Figure 7B and D).

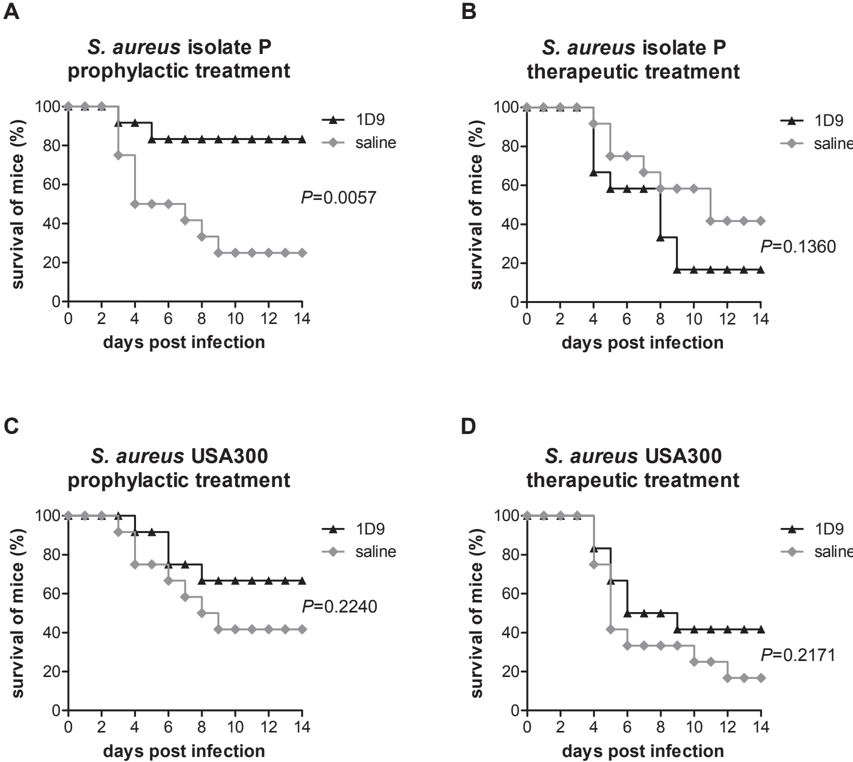

Figure 7. Efficacy of 1D9 in mice with *S. aureus* bacteremia. Mice (12 per group) were infected with 2-4 x 10^5 cfu of *S. aureus* isolate P or with 4-7 x 10^5 cfu of *S. aureus* USA300, by intravenous inoculation. Animals were treated intravenously with 1D9 (5 mg/kg) or placebo (saline) 3 hours before (A, C) or 3 hours after (B, D) infection. Animal survival was monitored for 14 days. The significance of protection compared to that for animals receiving placebo was measured with the log rank test.

Discussion

The present study describes the generation of a fully human monoclonal IgG1 antibody directed against the conserved *S. aureus* antigen IsaA. In the used mCHR protocol,[20,21] B lymphocytes were enriched for IsaA-binding B cells and subsequently cloned. This resulted in one mAb, 1D9, that did bind specifically to both purified and native IsaA-His_6 as well as to a broad panel of 26 clinical *S. aureus* isolates, including MSSA and MRSA strains among which *S. aureus* isolate P (MSSA) and USA300 (MRSA), the strains used for further experiments *ex vivo* and *in vivo*.

Although 1D9 bound to all 26 MSSA and MRSA strains studied, the strains differed in binding capacity. This likely reflect variations in the expression of IsaA as previously shown,[7] which may impact on the bacterial surface display of IsaA. Binding of the isotype control human mAb IQNPA to the *S. aureus* strains also varied, probably reflecting variations in expression of protein A and Sbi upon growth on IMDM. 'Aspecific' binding of 1D9 by protein A and Sbi can be considered as low, as binding of 1D9 to *S. aureus* NewmanΔ*spa*Δ*sbi* was only slightly lower compared to its binding to Newman wild-type. Notably, the expression of protein A by *S. aureus* USA300 turned out to be much higher compared to isolate P upon 24 hours of growth in mouse serum. This suggests that the protein A-mediated IgG binding of *S. aureus* may be different under different growth conditions, which underscores the importance of selecting the most appropriate *in vitro* assay and culture conditions for predicting the *in vivo* efficacy.

Despite binding of 1D9 to IsaA and a broad panel of *S. aureus* strains grown in IMDM, 1D9 at a concentration of 10 µg/mL did not result in enhanced opsonophagocytosis of *S. aureus* isolate P or USA300 by human or murine neutrophils *ex vivo*. In a study of Kelly-Quintos et al., opsonophagocytic activity of human mAbs targeting PNAG was assessed using human neutrophils.[26] They used mAb concentrations ranging from 25-1.5 µg/mL, and a concentration of 12 µg/mL resulted in maximum opsonophagocytic activity in most cases. Nevertheless, IsaA-1D9 IC did stimulate the FcγR on human and murine neutrophils as measured by the generation of an oxidative burst, indicating that the human mAb 1D9 binds to the FcγR on murine neutrophils. However, it is important to bear in mind that the absence of enhanced opsonophagocytosis of *S. aureus* by neutrophils *ex vivo* does not exclude the possibility that enhanced opsonophagocytosis occurs *in vivo*.

Our results on the *ex vivo* characterization of the human anti-IsaA mAb 1D9 are in line with data obtained with UK-66P[16] and hUK-66,[27] murine and humanized anti-IsaA

mAbs, respectively. These mAbs also bound to different *S. aureus* isolates, and, in the presence of *S. aureus*, activated murine neutrophils. Moreover, incubation of *S. aureus* with UK-66P or hUK-66 resulted in enhanced bacterial killing in neutrophils. In the presence of UK-66P, uptake of *S. aureus* by neutrophils was not enhanced.

The efficacy of 1D9 was assessed in our murine bacteremia model caused by *S. aureus* isolate P (MSSA) or USA300 (MRSA). *S. aureus* isolate P is a community-acquired strain recovered from the blood of a septic patient,[7] and *S. aureus* USA300 is one of the most frequent causes of community-acquired infections in the United States.[28] A prophylactic 1D9 dose of 5 mg/kg resulted in a significantly improved animal survival rate in *S. aureus* isolate P bacteremia, but was not protective in USA300 bacteremia. Therapeutic treatment with 1D9 at the same dose was not effective in both infection models. As we observed that at 3 hours after i.v. inoculation *S. aureus* had multiplied and disseminated to the lungs, spleen, liver and kidneys, while mice were still bacteremic, a possible explanation for therapeutic failure of this single 1D9 dose might be an insufficient bioavailability of 1D9 to reach *S. aureus* which had already multiplied and disseminated to the organs to induce protection.

Although 1D9 at a dose of 5 mg/kg protected mice from death due to *S. aureus* isolate P bacteremia, no protective efficacy was observed in USA300 bacteremia. This difference in efficacy cannot be explained by differences in the volume of distribution for 1D9, as 1D9 titers in mice with *S. aureus* isolate P and mice with USA300 bacteremia were comparable. As IsaA protein sequence is conserved among *S. aureus* strains, differences in epitope structure are unlikely, and cannot explain the difference of efficacy in the two infection models. Uneven IsaA expression levels can also not clarify this difference in efficacy, as both in chemically defined culture media and in mouse serum IsaA expression by these *S. aureus* strains was comparable. Regarding *S. aureus* USA300, surface exposure of IsaA had also been shown before by surface shaving using trypsin and subsequent proteomic analysis.[12] However, substantial differences in protein A expression were observed when these *S. aureus* strains were cultured in mouse serum. The presence of higher amounts of protein A on *S. aureus* USA300 may lead to higher levels of 'aspecific' 1D9 binding and, consequently, to an increased survival of this strain due to more effective evasion of the host immune response.[29] Other 1D9 doses, or treatment combining 1D9 and a control antibody blocking protein A and Sbi may overcome this problem. It cannot be excluded that another dosing schedule of 1D9 could positively influence the outcome of infection. The addition of control antibody was not included in this study, and further experiments in this respect

are needed. In addition, studies including *S. aureus* isogenic mutant strains lacking *spa* and/or *sbi* may further elucidate the differences in protective capacity of 1D9.

Lorenz et al.[16] also demonstrated the prophylactic activity of their murine anti-IsaA mAb UK-66P in a murine *S. aureus* sepsis model caused by *S. aureus* MA12 (MSSA) or USA300 (MRSA). Explanations for the discrepancy between their observations and our data may be the different dosing schedules of mAbs, being 2 doses at start of infection and 24 hours later (Lorenz et al.) and 1 dose at 3 hours before infection (present study). Also, the evaluation periods to assess protective capacity were different, being 8 days (Lorenz et al.) and 14 days (present study). An evaluation period of 8 days in the present study would not have changed our conclusions.

As the *in vivo* prophylactic efficacy of 1D9 in *S. aureus* isolate P bacteremia cannot be correlated to enhanced *ex vivo* phagocytosis of *S. aureus* by neutrophils or inhibition of *S. aureus* multiplication, alternative explanations for the protective effect of 1D9 have to be considered. For example, other phagocytes such as macrophages and monocytes could play a role in the 1D9-mediated protection. Furthermore, a protective effect through a non-classical mechanism as proposed by Pancari et al.[30] may be relevant. They showed that protection conferred by their human anti-IsdB mAb was dependent on complement, phagocytes and lymphocytes rather than on Fc functionality, classical complement activation or direct inhibition of growth. In addition, although 1D9 did not improve phagocytosis *ex vivo*, this does not exclude the possibility that enhanced phagocytosis occurs *in vivo*.

In contrast to animal studies showing the prophylactic efficacy of passive immunization in *S. aureus* infections, studies performed in humans were not yet promising.[31-36] The lack of efficacy of passive immunization in humans with *S. aureus* infections might partly be explained by the choice of the antigen targets that were selected for the production of antibodies in these studies, not being invariably conserved antigens present on the surface of all *S. aureus* strains.

The results of the present study show that our approach to clone IgG targeting conserved staphylococcal surface proteins from *S. aureus* carriers is feasible. In addition to cloning protective mAbs from infected or vaccinated donors,[37,38] using the mCHR protocol it is possible to clone protective mAbs from healthy *S. aureus* carriers without history of infection or vaccination.

In conclusion, the mCHR protocol used in the present study allows the generation of fully human mAbs against invariably expressed targets on the surface of *S. aureus*. Importantly, such mAbs can be cloned directly from selected B cells donated by healthy

nasal *S. aureus* carriers. The human anti-IsaA mAb 1D9 thus generated was shown to be effective in prophylactic treatment of mice with *S. aureus* isolate P (MSSA) bacteremia. These data are consistent with the results obtained by Lorenz et al.[16] and Oesterreich et al.[27] using murine and humanized anti-IsaA mAbs, respectively. The protective capacity of human mAb 1D9 as shown in the present study, opens new ways to investigate the efficacy of 1D9, possibly conjugated to for example another antibody, antibiotics, cytokines or chemokines, in a clinical study to fight *S. aureus* infections.

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HPJB, GSE, NK, HW and HG have been employees of IQ Therapeutics when they were involved in the present studies. TB is an employee of LanthioParma. The University Medical Center Groningen owns Intellectual Property related to the use of monoclonal antibodies against *S. aureus*. These potential conflicts of interest have had no influence on the content of this manuscript. All other authors declare no competing financial interests.

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Supplementary data

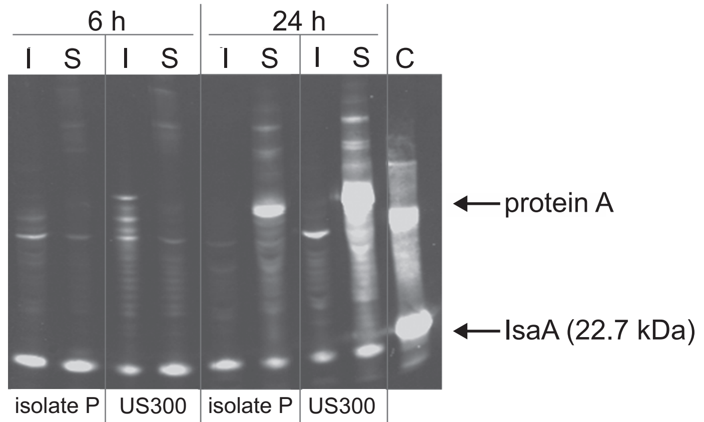

Figure S1. IsaA expression by *S. aureus* isolate P and *S. aureus* USA300. Both strains were grown in IMDM (I) or mouse serum (S) and after 6 or 24 hours culture samples were taken. Equal amounts of cellular proteins collected from each culture at the indicated time points were loaded based on the determined number of cfu as described in the Materials and methods. As a positive control for immunodetection, isolated IsaA-His_6 was loaded in lane C. Bands specific for IsaA or the staphylococcal protein A are marked with arrows.

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Active immunization with an octa-valent *S. aureus* antigen mixture in models of *S. aureus* bacteremia and skin infection in mice

Sanne van den Berg,* Dennis G.A.M. Koedijk,* Jaap Willem Back, Jolanda Neef, Annette Dreisbach, Jan Maarten van Dijl, Irma A.J.M. Bakker-Woudenberg, Girbe Buist

* These authors contributed equally to this study.

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Abstract

Proteomic studies with different *Staphylococcus aureus* isolates have shown that the cell surface-exposed and secreted proteins IsaA, LytM, Nuc, the propeptide of Atl (pro-Atl) and four phenol-soluble modulins α (PSMα) are invariantly produced by this pathogen. Therefore the present study was aimed at investigating whether these proteins can be used for active immunization against *S. aureus* infection in mouse models of bacteremia and skin infection. To this end, recombinant His-tagged fusions of IsaA, LytM, Nuc and pro-Atl were isolated from *Lactococcus lactis* or *Escherichia coli*, while the PSMα1-4 peptides were chemically synthesized. Importantly, patients colonized by *S. aureus* showed significant immunoglobulin G (IgG) responses against all eight antigens. BALB/cBYJ mice were immunized subcutaneously with a mixture of the antigens at day one (5 µg each), and boosted twice (25 µg of each antigen) with 28 days interval. This resulted in high IgG responses against all antigens although the response against pro-Atl was around one log lower compared to the other antigens. Compared to placebo-immunized mice, immunization with the octa-valent antigen mixture did not reduce the *S. aureus* isolate P load in blood, lungs, spleen, liver, and kidneys in a bacteremia model in which the animals were challenged for 14 days with a primary load of 3 x 10^5 cfu. Discomfort scores and animal survival rates over 14 days did not differ between immunized mice and placebo-immunized mice upon bacteremia with *S. aureus* USA300 (6 x 10^5 cfu). In addition, this immunization did not reduce the *S. aureus* isolate P load in mice with skin infection. These results show that the target antigens are immunogenic in both humans and mice, but in the used animal models do not result in protection against *S. aureus* infection.

Introduction

Staphylococcus aureus is a widespread Gram-positive bacterium that colonizes the skin and anterior nares of about 20-30% of the healthy human population.[1] Although mainly a harmless colonizer, *S. aureus* can cause invasive diseases like skin and soft tissue infections, and can be responsible for severe infections in humans like pneumonia, endocarditis and osteomyelitis,[1] which are frequently associated with *S. aureus* bacteremia.[2] *S. aureus* in its methicillin-resistant form (MRSA) is the most important cause of antibiotic-resistant health care-associated infections worldwide.[3,4] In the case of MRSA, a single genetic element makes *S. aureus* resistant to the most frequently prescribed class of antimicrobials - the β-lactam antibiotics, including penicillins, cephalosporins, and carbapenems.[5] A high incidence of MRSA is encountered in hospitals, resulting in prolonged hospital stays and in higher mortality rates,[3,4] and limited effectiveness of alternative treatment regimens. Glycopeptides, especially vancomycin, are currently used as first-line treatment of MRSA infections. Unfortunately, this has led to the emergence of vancomycin-intermediate and vancomycin-resistant MRSA.[6] In addition, there is raising concern that the current first-line treatment for MRSA infection will become increasingly ineffective. Since in the past 25 years no novel small-molecule antibacterial drugs have been discovered,[7] and the development pipeline of new antimicrobials remains lean,[8] new ways of treatment of *S. aureus* infections such as immunization need to be explored. Several strategies of passive and active immunization in *S. aureus* infections have been studied in experimental infection models, but until now none of these have been proved to be effective in clinical studies.[9-11]

Novel target identification strategies have been applied to screen for new antigenic targets for immunization. Invariant immunogenic determinants of relevant *S. aureus* isolates have been successfully identified in previous studies using a combination of proteomics, genomics, bioinformatics and immunological approaches.[12-15] A complete inventory of predicted secreted proteins of sequenced *S. aureus* strains has been made.[12] Proteomic analysis of the exoproteomes of 25 clinical *S. aureus* isolates showed that only seven of these secreted proteins (IsaA, Lip, LytM, Nuc, SA0620, SA2097, and SA2437) were produced by all clinical isolates studied.[13] In a proteolytic shaving approach of *S. aureus* cells, multiple surface-exposed proteins were identified among which IsaA, Nuc, Atl and the PSMα1 peptide.[14]

In the present study, we used IsaA, LytM, Nuc, pro-Atl, and PSMα1-4 as targets for active immunization in *S. aureus*-infected mice. These *S. aureus* antigens were all selected by the previous target identification strategies, and are all potential virulence factors of *S. aureus*. The phenol-soluble modulins (PSMs) are highly potent surfactants that facilitate the movement of *S. aureus* over moist surfaces by colony spreading, and they are involved in the metastatic escape of staphylococcal cells from biofilms thereby representing an enormous risk factor for serious invasive disease.[16] High levels of PSM production are supposed to contribute to the high virulence and epidemic behavior of community-acquired MRSA lineages.[17]

Staphylococcal major autolysin (Atl) is a bifunctional autolysin composed of a signal peptide for protein secretion, a propeptide, and domains with amidase and glucosaminidase activity. After proteolytic cleavage, the active domains are involved in cell separation and they have been shown to be cell surface-exposed.[18] It is believed that the propeptide of Atl (pro-Atl) has a role in the folding and/or activation of Atl and that it would be degraded rapidly upon proteolytic processing of the Atl protein.

Immunodominant staphylococcal antigen A (IsaA) and autolysin M (LytM) have been shown to be peptidoglycan hydrolases with transglycosylase and glycyl-glycine endopeptidase specific activity, respectively.[15] The secreted nuclease (Nuc) has been shown to limit the formation of biofilms due to the degradation of extracellular DNA.[19]

In previous studies, IsaA, LytM, Nuc, pro-Atl and PSMα1-4 have all been shown to be immunogenic. Using a multiplex assay to quantify antibody responses against 26 staphylococcal proteins, we found that mice with a *S. aureus* USA300 pneumonia or skin infection showed good IgG responses against IsaA and Nuc, while anti-LytM levels were low.[20] In contrast, in mice immunized with monovalent staphylococcal vaccines containing IsaA, Nuc or LytM, the highest IgG responses were obtained against the latter antigen. Using the same multiplex assay, it has been shown that 75% of the patients with the blistering disease Epidermolysis bullosa (EB) that were colonized with *S. aureus*, have increased IgG responses against some secreted, cell wall and membrane-bound staphylococcal proteins.[21] Within the EB patient group, the highest response was detected against IsaA, while for Nuc and LytM moderate responses were detected. These data show that both in mice and in human individuals a good IgG response is generated against IsaA, LytM and Nuc. Moreover, passive immunization of mice with murine anti-IsaA results in a 30% reduction of several *S. aureus* strains, among which *S. aureus* USA300 strain, likely because phagocytosis is triggered, resulting in killing of *S. aureus*.[22] Holtfreter et al. showed in their review that Atl has

been identified in different studies as a well-recognized target of the human immune system.[23] Recently, we showed that pro-Atl is exposed on the outside of *S. aureus* cells and is recognized by antibodies from four out of six patients that were colonized with *S. aureus*.[24] Within a patient group of hospitalized adults with an invasive *S. aureus* infection, in those patients who did not develop sepsis, higher levels of immunoglobulin G (IgG) responses were detected against exotoxins among which PSMα3.[25]

In the present study in mice, we examined the protective efficacy of active immunization with an octa-valent antigen mixture containing IsaA-His_6, LytM-His_6, Nuc-His_6, His_6-pro-Atl and the PMSα1-4 peptides. In mice immunized with a mixture of these antigens, generated IgG response was determined by ELISA. Subsequently, the protective capacity of this immunization strategy was studied in mouse models of MSSA or MRSA bacteremia and in a mouse model of MSSA skin infection, which are clinically highly relevant *S. aureus* infections.[26]

Materials and methods

Bacterial strains and growth conditions

Strains and plasmids used in this study are listed in Table 1. *Lactococcus lactis* strains were grown at 30ºC in M17 broth (Oxoid) supplemented with 0.5% w/v glucose

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant phenotype(s) or genotype(s)	Source or reference
Strains		
E. coli BL21DE3	Allows IPTG-inducible expression of P_{T7}	Novagen
L. lactis NZ9700	Nisin producer	55
L. lactis PA1001	MG1363 *pepN*::*nisRK*, allows nisin inducible expression, Δ*acmA* Δ*htrA*	56
S. aureus isolate P	Community-acquired MSSA patient isolate	13
S. aureus USA300	Community-acquired MRSA isolate	27
S. aureus N315	Hospital-acquired MRSA	57
Plasmids		
pET24d::*isaA*::his_6	Kan^R, pET24d containing *isaA* with C-terminal his_6	35
pPA180::*lytM*::his_6	Cm^R, pPA180 containing *lytM* with C-terminal his_6	20
pNG400::*nuc*::his_6	Cm^R, nisin inducible expression via P_{nisA} of Nuc with C-terminal his_6	35
pNG4110::*proAtl*	Cm^R, nisin inducible expression via P_{nisA} of the pro-Atl peptide fused to SS_{usp45}, and a N-terminal his_6	This study

Kan^R, kanamycin resistance gene; Cm^R, chloramphenicol resistance gene; P_{T7}, IPTG inducible T7-promoter; P_{nisA}, nisin inducible promoter; his_6, 6 histidine-tag; SS_{usp45}, signal sequence of *usp45*.

(GM17). When necessary the medium was supplemented with chloramphenicol (5 µg/mL) for plasmid selection. For *in vivo* studies, *S. aureus* isolate P and USA300 were used and grown in Brain Heart Infusion broth (Becton Dickinson). *S. aureus* isolate P is a community-acquired MSSA strain recovered from the blood of a septic patient and was previously analyzed by proteomics.[13] *S. aureus* USA300 is one of the most frequent causes of community-acquired infections in the United States.[27]

Construction of His_6-pro-Atl expression plasmids

For cloning of the DNA fragment coding for the propeptide of Atl (pro-Atl) in the plasmid pNG4110, the primers atlpro.F1 (ATAT<u>GGATCC</u>GCTGAGACGACACAAGATCAAACTACTA ATAAAAACG) and atlpro.R2 (ATATG<u>CGGCCG</u>CTTAAGCGCTAAAAGTAGTTACTTTAGGT GTCGCTTCAGTTTTAGC) were used with chromosomal DNA of *S. aureus* strain N315 as a template. Using this vector, a N-terminally His-tagged fusion protein can be secreted from *L. lactis*.[28] PCR product and vector were digested using *Bam*HI and *Not*I (cleavage sites are underlined in the primers sequences). After ligation the resulting plasmids were transferred into *L. lactis* PA1001 by electro transformation with selection on chloramphenicol. A nucleotide sequence analysis of the cloned inserts was performed by Eurofins DNA.

Protein isolation, purification and quantification

For the production and isolation of IsaA-His_6, an overnight culture of *E. coli* BL21DE3 (pET24d::*isaA*::*his_6*) was diluted 1:100 in fresh lysogenic broth with 50 µg/mL kanamycin. Induction was performed at OD_{600} ~ 0.5 for 4 hours by adding 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Duchefa). The culture was centrifuged and the pellet was resolved in binding buffer (20 mM Na-Phosphate, pH 7.4, 0.5 M $NaCl_2$, 60 mM imidazole) containing 6 M urea. After sonification (Sonicator S-4000, Misonix) the supernatant was mixed with binding buffer (1:1) and HisLink beads (Promega) for 1 hour at 4°C under shaking conditions. Protein elution was performed with binding buffer containing 500 mM imidazole.

For the production and isolation of LytM-His_6, Nuc-His_6 and His_6-pro-Atl, overnight cultures of *L. lactis* PA1001 were diluted 1:2 in GM17 medium. Nisin induction for protein expression was performed at OD_{600} ~ 0.5 for 16 hours by adding the culture supernatant (1:1000) from an overnight culture of *L. lactis* NZ97000. For analysis of the extracellular production of His_6-pro-Atl by *L. lactis* PA1001 (pNG4110::*proAtl*), proteins from the culture supernatant were precipitated using trichloric acid (TCA;

10% w/v). The culture was centrifuged and the pellet was washed with acetone and dried. For the isolation of Nuc-His_6, from the supernatant fraction of a culture of *L. lactis* PA1001 (pNG400::*nuc*::*his_6*), HisLink beads were added for 1 hour at 4°C under shaking conditions after which HisLink beads were collected. For isolation of LytM-His_6 cells of *L. lactis* PA1001 (pPA180::*lytM*::*his_6*) were harvested and disrupted in binding buffer using a Sonicator S-4000. Purification was performed with Mag beads (GE Healthcare) for 1.5 hours under shaking conditions at 4°C. Elution of Nuc-His_6 and LytM-His_6 was done with binding buffer containing 500 mM imidazole. The flow-through, wash and elution fractions were analyzed by LDS-PAGE. Protein samples were mixed with LDS buffer and incubated at 95°C for 10 minutes, separated by LDS-PAGE using precast 10% NuPage gels (Life Technologies) and stained with Simply Blue™ Safe Stain (Life Technologies).

The fractions containing the purified His-tagged proteins of IsaA-His_6, LytM-His_6, Nuc-His_6 or His_6-pro-Atl were pooled and dialyzed (G2-Float-A-Lyzer, Spectrum Europe) against PBS and concentrated with the Speedvac Concentrator Plus (Eppendorf Nederland).

Protein concentrations were determined with the DC Protein Assay (Bio-Rad) according to the instructions of the supplier, using Bovine Serum Albumin (Sigma-Aldrich) as a standard, or with the Nanodrop ND-1000 (Thermo Fisher Scientific) using the extinction coefficient calculated for each of the proteins.

PSMα1-4 were synthesized as described previously,[16] with the addition of a GGG-Lys(ε-biotin). The peptides were mixed in a 1:1:1:1 molar ratio, and incubated in PBS in a stoichiometric ratio to avidin (Thermo Fisher Scientific Inc.).

Human plasma

Whole blood donations from EB patients were collected under the approval of the medical ethics committee of the University Medical Center Groningen (approval no. NL27471,042,09) upon written informed patient consent, and with adherence to the Helsinki Guidelines.[21] The Independent Ethics Committee of the Foundation 'Evaluation of Ethics in Biomedical Research' (Assen, the Netherlands), approved the protocol for blood donations from healthy volunteers. This protocol is registered by QPS Groningen (code 04132-CS011). The required informed consent was obtained from all EB patients and healthy volunteers included in the present studies.

Protein detection and activity assays

For Western blot analyses, proteins separated by LDS-PAGE were blotted onto a nitrocellulose membrane (Protran®, Schleicher & Schuell BioScience). Immunodetection was performed using anti-His-tag antibodies (Invitrogen™, Life Technologies) and rabbit polyclonal antibodies raised against the amidase or glucosaminidase domains of Atl (gift from Motoyuki Sugai, Hiroshima University, Japan[29]). Dilutions (1:1000) of the collected human plasma[21] were used to detect the IgG responses against IsaA-His_6, LytM-His_6 and Nuc-His_6. Equal amounts of the three proteins were loaded on the gel and after blotting strips of the blot were incubated with the different plasma samples. Bound primary antibodies were visualized using specific fluorescently labeled secondary antibodies (IRDye 800 CW, Li-Cor Biosciences). Membranes were scanned for fluorescence at 800 nm using the Odyssey Infrared Imaging System (Li-Cor Biosciences).

To show expression of the PSMα1-4 peptides by *S. aureus* isolates P and USA300, both strains were tested for the ability to spread on tryptic soy soft agar plates (0.24% agar). The spreading assay was performed as described.[16]

Peptidoglycan hydrolase activity of IsaA-His_6 and LytM-His_6 was detected by a zymogram staining technique using SDS-polyacrylamide (12.5%) gels containing 0.1% (w/v) autoclaved cell wall fragments of *S. aureus* RN4220 isolated as described previously.[30] After electrophoresis, the gels were gently shaken at room temperature for 24 hours in three to five changes of 100 mL of 25 mM Tris-HCl (pH 6.0) containing 1% (v/v) Triton X-100 for protein renaturation.

Pepscan analysis

To determine whether regions of the *S. aureus* PSMα1-4 peptides were recognized by human IgGs, libraries of linear 15-mer peptides were synthesized with an overlap on solid support (Pepscan), as previously described.[31] The peptide libraries were probed with heat-inactivated human sera, in a dilution of 1:1000, with goat-anti-human-HRP conjugate (SouthernBiotech) as a secondary antibody, and developed with 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (Sigma-Aldrich). A charge-coupled device camera was used to register absorbance at 405 nm. For every single Pepscan dataset, the data were normalized to the average signal intensity of the analysis. Furthermore, the signals for every single protein were normalized to the median of the corresponding protein. In addition the standard deviations of the normalized data sets were calculated for each protein. Peptides with a signal exceeding the median plus twice the

standard deviation and normalized signal intensity higher than three were regarded as being immunogenic domains.

Animals

Specified pathogen free female BALB/cBYJ mice were obtained from Charles River. Mice were housed in individually ventilated cages, 3-4 mice per cage. Animals were 11-13 weeks old at the day of infection, and were given food and water *ad libitum*. All animal experiments were performed in accordance with the rules laid down in the Dutch Animal Experimentation Act and the EU Animal Directive 2010/63/EU (permit number: EMC2694).

Immunization procedure

Purified Nuc-His$_6$, LytM-His$_6$, IsaA-His$_6$, His$_6$-pro-Atl, and PSMα1-4 were emulsified 1:1 with TiterMax® Gold adjuvant (Sigma-Aldrich). Mice were immunized subcutaneously in the flank with 100 µL formulated vaccine on days -70 (5 µg of each antigen), days -42, and -14 (25 µg of each antigen). PSMα1-4 was considered as a single antigen, and therefore a total of 5 or 25 µg of this 1:1:1:1 mixture was used per immunization. Control mice received 100 µL PBS emulsified with adjuvant. Mice were randomly allocated to either the vaccine or the placebo group. At days -71 and -1, blood was withdrawn from the tail artery. Sera were examined by ELISA for IgG titers with specific antigen-binding activity.

ELISA

ELISA plates (Greiner Bio-One) were coated with 250 ng of the antigens in coating buffer (0.05 M carbonate-bicarbonate, pH 9.6-9.8) and incubated for three days at 4°C. The plates were blocked for 45 minutes at 37°C in coating buffer with 2.5% milk powder (Oxoid). After washing, serial twofold dilutions of the sera were made in PBS containing 0.05% Tween-20 (PBST) and incubated for 2 hours at 37°C. After washing with PBST, the plates were incubated with GaM/IgG-HRP (SouthernBiotech) (1:5000 in PBST) for 90 minutes at 37°C. Finally, the peroxidase reaction was visualized using o-phenylene-diamine (Sigma-Aldrich) for 30 minutes at room temperature. The reaction was stopped by adding 2 M H_2SO_4. The plates were measured at 492 nm in a plate reader (Biotek Powerwave XS2, Beun de Ronde).

Infection model of *S. aureus* bacteremia

Immunized mice (n=8 per group) were challenged on day 0 by intravenous inoculation of 100 µL of *S. aureus* isolate P (3 x 10^5 cfu) or *S. aureus* USA300 (6 x 10^5 cfu) as described previously.[32] Discomfort and animal survival rate over 14 days after infection were monitored. For discomfort score, clinical signs of illness in each mouse were evaluated twice daily as described before.[32] Mice with bad fur were scored -2. Mice with bad fur and hunched back were scored -3. Mice with bad fur and hunched back and that were instable were scored -4. These mice showed severe signs of illness and were euthanized by CO_2 exposure. At day 14 after infection in surviving mice, *S. aureus* load in blood, lungs, spleen, liver and kidneys was determined. Mice were sacrificed by CO_2 exposure and exsanguinated by cardiac puncture. Blood was collected in a vial containing Lithium Heparin (Sarstedt). Organs were removed aseptically and homogenized using a gentleMACS™ Dissociator (Miltenyi Biotec) in 2 mL of saline. Cfu's of (un-)diluted blood and organ homogenates were determined after overnight growth on trypticase soy agar with 5% sheep blood (Becton Dickinson).

Infection model of *S. aureus* skin infection

Immunized mice (n=4 per group) were challenged on day 0 by intradermal inoculation of 50 µL of *S. aureus* isolate P (3 x 10^7 cfu).[33] Animal body weight and lesion size over 7 days after infection were monitored. Lesions were measured with a caliper. Lesion size was calculated by using the formula $A = \pi(L \times W)/2$, where L is length and W is width of the lesion.[34] At day 7 after infection, mice were sacrificed by CO_2 exposure. A circular area (diameter 14 mm) of the skin lesion was removed aseptically and homogenized using a gentleMACT™ Dissociator in 2 mL saline. Serial dilutions of skin homogenates were cultured on phenol-red mannitol salt agar (Becton Dickinson). Culture plates were incubated at 35°C for 48 hours and at room temperature for 5 days. Identification of *S. aureus* was based upon colony morphology on the PHMA. Suspected colonies were cultured overnight on trypticase soy agar with 5% sheep blood (Becton Dickinson). A latex agglutination test (Staph Plus Latex; DiaMondial) was then performed.

Statistical analysis

The Mann-Whitney U test was used to compare median differences in bacterial load in different groups. The log rank test was used to determine statistical differences in animal survival rate between groups. Correlations between IgG titers and time to

death were assessed using Pearson's correlation coefficient. GraphPad Prism 5 for Windows (GraphPad Software Inc.) was used for these statistical analyses. Quade's rank analysis of covariance was used to compare discomfort score, skin lesion size and body weight in different groups over time. These statistical analyses were performed using the Statistical Package of Social Sciences version 17.0 for Windows (SPSS Inc.). P-values < 0.05 were considered to be statistically significant.

Results

Isolation and purification of *S. aureus* antigens

In an earlier study on epitope mapping of surface proteins of *S. aureus*, the N-terminal propeptide of Atl (pro-Atl) was shown to be cell surface exposed.[24] Therefore, this protein domain was chosen as a candidate target for the screening of human antibodies against *S. aureus*. After cloning the His_6-pro-Atl fusion protein was produced by *L. lactis* and expression and secretion of the peptide was proven (data not shown). Western detection showed that all fusion proteins used in this study contained the His-tag needed for protein isolation (data not shown).

The *S. aureus* antigens LytM-His_6, Nuc-His_6, and His_6-pro-Atl were isolated from *L. lactis* whereas IsaA-His_6 was isolated from *E. coli*. The isolated and purified His-tagged proteins were verified using LDS-PAGE and subsequent protein staining (Figure 1). Using zymographic analysis it was shown that purified LytM-His_6 and IsaA-His_6 had

Figure 1. LDS-PAGE detection of the purified and dialyzed *S. aureus* antigens. Fifteen µg of LytM-His_6 (1), Nuc-His_6 (2), IsaA-His_6 (3) or His_6-pro-Atl (4) was loaded. The molecular weight of marker proteins is indicated (in kDa).

retained their enzymatic peptidoglycan-degrading activity while Nuc-His_6 was still able to hydrolyze DNA.[35] The synthetic PSMα1-4 peptides have been shown to be able to generate spreading indicating they are equally active as the naturally produced peptides.[16] These data show that the antigens were successfully isolated and the IsaA-His_6, LytM-His_6, and Nuc-His_6 fusions had retained their biological activity.

Production of antigens by *S. aureus* isolate P and USA300

To show the production of the selected antigens by the *S. aureus* isolates P (MSSA) and USA300 (MRSA) that were used in the *in vivo* studies, different protein detection methods were used. For *S. aureus* USA300 all antigens used in this study had been identified in earlier (proteomics) studies (Table 2). Also for the *S. aureus* isolate P, most of the antigens were identified by proteomics in earlier studies (Table 2). Expression of the Atl protein by *S. aureus* isolate P was detected using specific polyclonal antibodies as mentioned in the Materials and methods (data not shown). As pro-Atl is the propeptide of the autolysin Atl, which was detected by proteomic studies, pro-Atl was concluded to be expressed by both *S. aureus* strains.[24] The expression of the PSMα1-4 peptides has been detected indirectly by using a plate assay in which cells of *S. aureus* isolates P or USA300 showed a spreading phenotype on a soft agar plate, indicating that both strains produce the PSM peptides (Table 2, data not shown). These data show that all antigens used in this study are produced and secreted by *S. aureus* isolates P and USA300.

Table 2. Proteins and peptides used in this study.

Name	NCBI number / sequence*	Function	Identification antigen in		Production	Mw (dD)	PI
			isolate P	USA300			
IsaA-His_6	SA2356 (N315)	Transglycosylase	(1)	(2)	*E. coli*	22.7	6.3
Nuc-His_6	SA0746 (N315)	Thermonuclease	(1)	(3)	*L. lactis*	20.1	9.7
LytM-His_6	SA0265 (N315)	Glycyl-glycine endopeptidase	(1)	(3)	*L. lactis*	33.1	6.4
His_6-pro-Atl	USA300HOU_0997 (USA300)	Propeptide autolysin Atl	Western (5)	(1)	*L. lactis*	19.4	7.9
PSMα1	MGIIAGIIKVIKSLIEQFTGKGGGGK$#	Spreading/Toxin	Spreading (6)	(2)/(4)	Synthetic	3.07	10.5
PSMα2	MGIIAGIIKFIKGLIEKFTGKGGGGK$#	Spreading/Toxin	Spreading (6)	(2)/(4)	Synthetic	3.09	10.7
PSMα3	MEFVAKLFKFFKDLLGKFLGNNGGGGK$#	Spreading/Toxin	Spreading (6)	(2)/(4)	Synthetic	3.41	10.4
PSMα4	MAIVGTIIKIIKAIIDIFAKGGGGK$#	Spreading/Toxin	Spreading (6)	(2)/(4)	Synthetic	2.98	10.6

* the sequences GGK$# stand for the addition of the GGG-Lys(ε-biotin) to each of the peptides

(1) Identified by proteomics[13]

(2) Identified by proteomics[14]

(3) Identified by proteomics[24]

(4) Identified using spreading assay[16]

(5) Detection of Atl using specific antibodies (see Materials and methods)

(6) Spreading as determined by plate assay (results not shown)

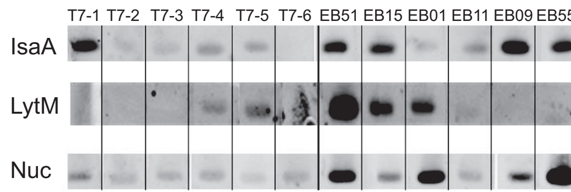

Figure 2. Western detection of immune responses against the antigens IsaA-His$_6$, LytM-His$_6$ and Nuc-His$_6$. Sera of healthy volunteers (T7-1 till T7-6) and EB patients (EB51, 15, 01, 11, 09, 55) were used.

Detection of IgG responses against the selected antigens in EB patients

To assess the IgG responses against IsaA-His$_6$, LytM-His$_6$ and Nuc-His$_6$, immunodetection was performed using sera of healthy donors and EB patients (Figure 2). From this analysis it is clear that EB patients have a higher although variable IgG response against all three proteins in comparison to the healthy donors. The Atl propeptide has been shown to be recognized by antibodies present in sera of EB patients.[24]

As the PSMα1 has previously been shown to be exposed on the cell surface of *S. aureus*, we included the PSMα1, α2, α3, and α4 in an epitope mapping approach using the PepScan technology.[31] This analysis showed that 5 out of 7 EB patients have an above average response against the peptides tested (Figure 3). Three out of 7 plasma samples from EB patients show a very high reactivity against the N-terminal regions of the PSMα1, α2, and α3 peptides. These results show that besides the IsaA-His$_6$, LytM-His$_6$ and Nuc-His$_6$ proteins, the PSM peptides are also well recognized by the humoral immune system.

PSM	AA	Peptide	T7-1	T7-2	T7-3	T7-4	EB01	EB09	EB51	EB11	EB15	EB53	EB02
PSMα1	1 to 15	MGIIAGIIKVIKSLI	63	144	77	143	**860**	162	**1099**	189	240	73	**324**
PSMα1	7 to 21	IIKVIKSLIEQFTGK	59	119	79	148	251	119	185	162	214	72	249
PSMα2	1 to 15	MGIIAGIIKFIKGLI	61	153	96	165	**1018**	192	**1079**	250	**311**	89	**507**
PSMα2	7 to 21	IIKFIKGLIEKFTGK	58	137	83	167	226	164	307	227	284	81	**331**
PSMα3	1 to 15	MEFVAKLFKFFKDLL	52	77	70	86	**1069**	79	**1025**	78	182	58	**278**
PSMα3	7 to 21	FKFFKDLLGKFLGNN	46	142	91	135	108	103	121	161	199	73	**459**
PSMα4	1 to 15	MAIVGTIIKIIKAII	54	125	76	148	121	101	104	120	216	66	263
PSMα4	7 to 21	TIIKIIKAIIDIFAK	58	134	92	165	**387**	149	152	147	292	82	**466**

Figure 3. Heatmap of anti-PSMα1-4 reactivity. Plot of IgG responses from sera of volunteers (T7-1 till T7-4) and EB patients (EB01, 09, 51, 11, 15, 53, 02) against the N- (1 to 15) and C- (7 to 21) terminal parts of the PSMα1-4 peptides. Colors represent a gradient of reactivity against the various peptides (green is low and red is high). Peptides with a signal exceeding the median plus twice the standard deviation are boxed.

Immunization with an octa-valent mixture of *S. aureus* antigens does not reduce bacterial load in mice with *S. aureus* isolate P bacteremia

Groups of mice were immunized by subcutaneous injection with 5 µg of purified IsaA-His_6, LytM-His_6, Nuc-His_6, His_6-pro-Atl, and PSMα1-4 each, emulsified in TiterMax® Gold adjuvant on day -70. On days -42 and -14, they were boosted with this mixture containing 25 µg of each purified antigen. Blood samples were collected before and after immunization, and specific serum IgG levels were determined by ELISA, demonstrating that these antigens generated humoral immune responses to immunization (Figure 4). The response against His_6-pro-Atl was on average 10-fold lower compared to the other 4 antigens.

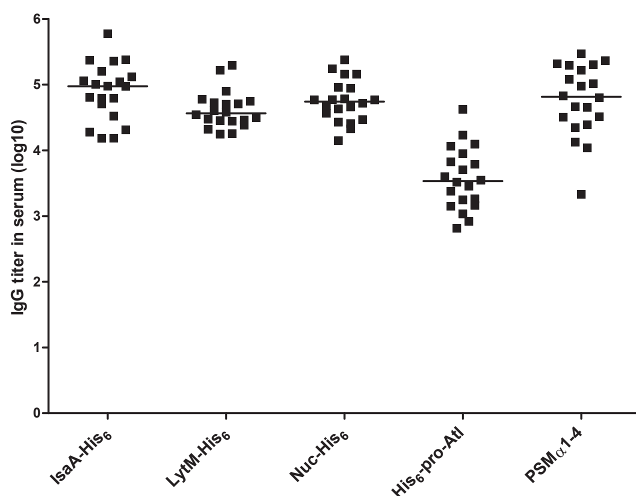

Figure 4. IgG titers in serum of mice after immunization with the octa-valent mixture. Mice (n=20) were immunized subcutaneously with the mixture containing IsaA-His_6, LytM-His_6, Nuc-His_6, His_6-pro-Atl, and PSMα1-4 at days -70 (5 µg of each antigen), days -42, and -14 (25 µg of each antigen). IgG titers on day -1 were assessed. Each symbol represents a single mouse. Median values are indicated by horizontal lines.

Mice were challenged at day 0 with mild *S. aureus* isolate P bacteremia (n=8 immunized mice, n=8 placebo-immunized mice), and animal discomfort and survival rate over 14 days after infection were monitored. At day 14, surviving mice were sacrificed, and blood and organs were removed for assessment of the bacterial load. In placebo-immunized mice, discomfort increased, while animal survival declined over time. At day 14, 6 out of 8 placebo-immunized mice were still alive. *S. aureus* load in

kidneys of these mice was high, while bacterial load in lungs and liver was low, and blood and spleen were culture negative (Figure 5).

Compared to placebo-immunized animals, immunization with the octa-valent antigen mixture did not reduce the *S. aureus* load in blood, lungs, spleen, liver, and kidneys (Figure 5). Furthermore, discomfort score and animal survival rate over 14 days did not differ between immunized and placebo-immunized mice (data not shown).

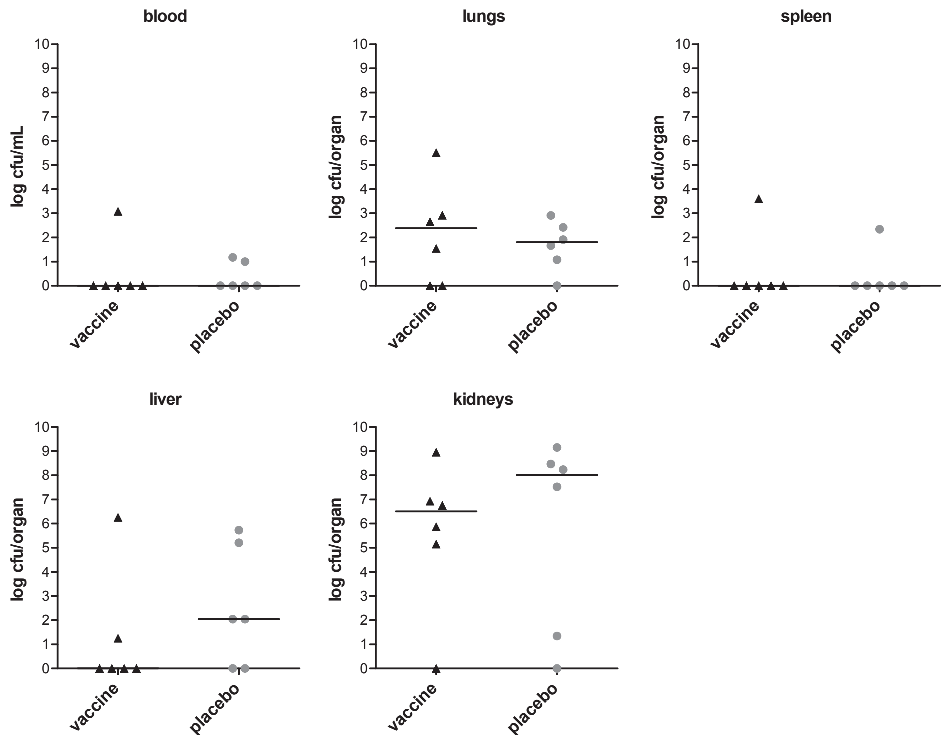

Figure 5. Bacterial load in immunized mice with *S. aureus* isolate P bacteremia. Mice (n=8) were immunized with the octa-valent vaccine containing IsaA-His_6, LytM-His_6, Nuc-His_6, His_6-pro-Atl, and PSMα1-4, or with placebo. Animals were infected by intravenous inoculation of *S. aureus* isolate P (3×10^5 cfu). At day 14, surviving mice were sacrificed and quantitative cultures of blood, lungs, spleen, liver, and kidneys were performed. Each symbol represents a single mouse. Median values are indicated by horizontal lines. Statistically significant differences (Mann-Whitney U test) in *S. aureus* load were not observed.

Immunization with an octa-valent *S. aureus* antigen mixture is not protective against mortality due to *S. aureus* USA300 bacteremia in mice

To assess whether the lack of protective effect of immunization with the octa-valent mixture was *S. aureus* strain-dependent, we included a lethal mouse model of MRSA bacteremia as well. As immunized mice showed excellent IgG titers, the immunization schedule was not adapted. In this model, mice were challenged with severe *S. aureus* USA300 bacteremia (n=8 immunized mice, n=8 placebo-immunized mice), and animal discomfort and survival rate over 14 days after infection were monitored. In placebo-immunized mice, discomfort increased, while animal survival declined gradually over time, and at day 9, all placebo-immunized mice had died (Figure 6).

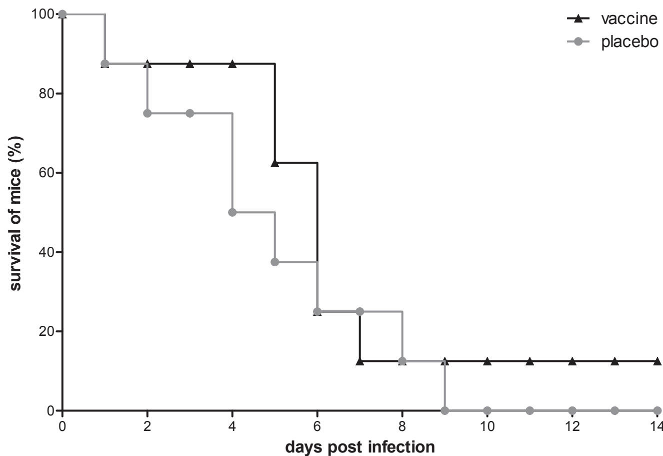

Figure 6. Survival of immunized mice with *S. aureus* USA300 bacteremia. Mice (n=8) were immunized with the octa-valent vaccine containing IsaA-His$_6$, LytM-His$_6$, Nuc-His$_6$, His$_6$-pro-Atl, and PSMα1-4, or with placebo. Animals were infected by intravenous inoculation of *S. aureus* USA300 (6 x 10^5 cfu), and were monitored for 14 days. A statistically significant difference (log rank test) in animal survival rate was not observed.

In this model of severe MRSA bacteremia, immunization with the octa-valent mixture containing IsaA-His$_6$, LytM-His$_6$, Nuc-His$_6$, His$_6$-pro-Atl, and PSMα1-4 did not protect against mortality due *S. aureus* USA300 bacteremia (Figure 6). Furthermore, the discomfort score over 14 days did not differ between immunized and placebo-immunized mice, and IgG levels and time to death were not correlated (data not shown).

Immunization with an octa-valent *S. aureus* antigen mixture does not protect against *S. aureus* isolate P skin infection

Absence of protection of the octa-valent mixture in *S. aureus* bacteremia does not exclude a possible (lack of) protection in other types of *S. aureus* infections. To assess whether this antigen mixture elicits a protective immune response against *S. aureus* skin infection, immunized mice were challenged with *S. aureus* isolate P via the intradermal route (n=4 immunized mice, n=4 placebo-immunized mice). Animal body weight and lesion size over 7 days after infection were monitored. At day 7, mice were sacrificed and the *S. aureus* load in skin lesions was assessed. In placebo-immunized mice, body weight loss over 7 days was minor, with a maximum of 6.5%. Size of the skin lesion increased over time until 2.8-5.2 cm^2 at day 7. At this time point, median *S. aureus* load in the skin lesion was 2 x 10^8 cfu/cm^2 (Figure 7).

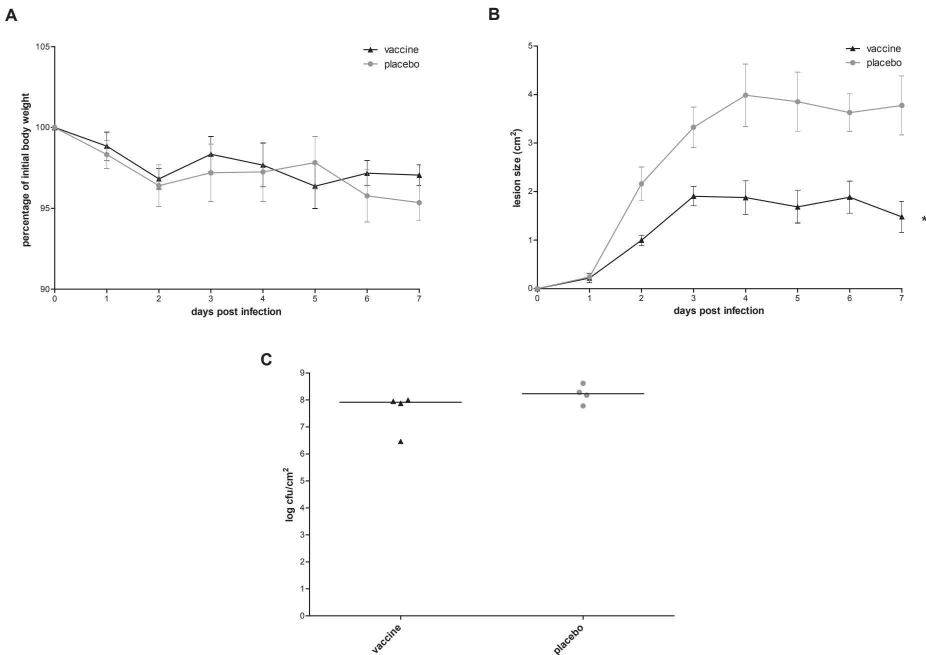

Figure 7. Course of infection in immunized mice with *S. aureus* isolate P skin infection. Mice (n=4) were immunized with the octa-valent vaccine containing IsaA-His_6, LytM-His_6, Nuc-His_6, His_6-pro-Atl, and PSMα1-4, or with placebo. Animals were infected by intradermal inoculation of *S. aureus* isolate P (3 x 10^7 cfu), and were monitored for 7 days. (A) Animal body weigh after *S. aureus* skin infection was not affected by vaccination (Quade's rank analysis of covariance). (B) Lesion size was significantly reduced by vaccination, as indicated by the asterisk (Quade's rank analysis of covariance). (C) *S. aureus* load in the skin lesion at day 7 was not reduced by vaccination (Mann-Whitney U test).

Compared to placebo-immunized mice, immunization with the octa-valent antigen mixture did not limit the body weight loss over time (Figure 7A), while size of the skin lesion was reduced in immunized mice (Figure 7B). *S. aureus* load in skin lesions at day 7 was not reduced in immunized mice (Figure 7C).

Discussion

In view of the high mortality rates of *S. aureus* infections,[36-39] the emergence of antibiotic-resistant *S. aureus* strains[6] and the lack of new antimicrobials in the development pipeline,[8] alternative treatment strategies for *S. aureus* infections are urgently needed. One approach is treatment through immunization targeting *S. aureus* antigens. This may be an interesting substitute for or additive to the currently used antibiotics. A role of certain anti-staphylococcal antibodies in protection against *S. aureus* infection-related death is suggested by a number of studies in humans.[21,40-42] Next to these suggestions from clinical studies, a number of studies in *S. aureus*-infected experimental animals showed protective effects of active or passive immunization. Notwithstanding these promising results obtained in experimental animals, no efficacy of immunization is observed in clinical studies.[9-11]

The lack of protective capacity of immunization targeting for example CP5 and CP8,[43,44] IsdB,[45] or ClfA[46] in *S. aureus*-infected patients may be related to the choice of targets for immunization. Therefore, in the present study, we selected *S. aureus* antigens that are accessible for antibodies based on proteomic analysis of exoproteomes of *S. aureus*[13] and a proteolytic shaving approach of *S. aureus*.[14] This strategy resulted in the selection of IsaA, LytM, Nuc, pro-Atl, and PSMα1-4 as targets for active immunization. Previous studies showed that these eight *S. aureus* antigens are immunogenic in humans,[21,23-25,41] and may therefore be applicable in the clinical setting. Moreover, monoclonal antibodies against IsaA enhance the killing of *S. aureus* in whole blood samples from healthy subjects and patients prone to staphylococcal infections,[47] and passive immunization with these monoclonal antibodies can lead to protection against *S. aureus* infections in mice.[22,48]

Using the hosts *L. lactis* and *E. coli*, all selected *S. aureus* antigens (IsaA, LytM, Nuc, and pro-Atl) were successfully isolated. No major degradation products were observed after isolation, and IsaA-His_6, LytM-His_6, and Nuc-His_6 fusion proteins were all biologically active after purification, indicating they had retained their natural con-

formation. In addition, these three fusion proteins as well as the PSMα1-4 peptides were well recognized by IgG present in plasma of healthy volunteers and EB patients. These results indicate that His-tagged fusions did not affect binding of IgG to these antigens. In addition, in a previous study, it has been shown that the Atl propeptide is recognized by antibodies present in sera of EB patients.[24]

The potentially protective capacity of immunization was evaluated in mice immunized with a mixture of IsaA, LytM, Nuc, pro-Atl and PSMα1-4. In the use of an octa-valent antigen cocktail we followed the approach of Stranger-Jones et al.,[49] who immunized mice with IsdA, IsdB, SdrD, and SdrE, either in a combination or individually. They observed that single antigen immunization elicited no or only very modest protection against *S. aureus* abscess formation or *S. aureus* lethal challenge, whereas immunization with the cocktail completely protected in both *S. aureus* infections. In the present study immunization of mice with the octa-valent antigen mixture of IsaA, LytM, Nuc, pro-Atl and PSMα1-4 resulted in detectable IgG responses against all eight antigens. Although anti-pro-Atl levels were slightly lower these were still in the expected range.[49,50] This observation indicated that all antigens were recognized well by the immune system of the mice.

After the final booster immunization, mice were infected with *S. aureus*. Clinically relevant models were used: mild bacteremia by *S. aureus* isolate P (MSSA), lethal bacteremia by *S. aureus* USA300 (MRSA), and transient *S. aureus* isolate P skin infection. It was shown that active immunization with the octa-valent mixture of IsaA-His_6, LytM-His_6, Nuc-His_6, His_6-pro-Atl, and PSMα1-4, although resulting in high anti-staphylococcal IgG levels, did not protect mice against mild *S. aureus* isolate P bacteremia, severe *S. aureus* USA300 bacteremia, or *S. aureus* isolate P skin infection.

The lack of protection by active immunization with the octa-valent *S. aureus* antigen mixture in our mouse infection experiments may be related to the study design or the presumed role of anti-staphylococcal antibodies in protection against these *S. aureus* infections. Regarding the study design, the *S. aureus* isolates used and the type of *S. aureus* infections studied are clinically relevant.[13,26,27] Other investigators used these infection models as well, and demonstrated that active or passive immunization targeting *S. aureus* antigens can lead to protection in mice.[9-11] In addition, both *S. aureus* strains produced all eight *S. aureus* antigens. In conclusion, failure to show protective activity upon active immunization cannot be explained by the choice of *S. aureus* strains or the choice of infection models.

A more plausible explanation for the lack of protection of the octa-valent antigen mixture in the present study may be related to the presumed role of anti-staphylococcal antibodies in protection against *S. aureus* infection. Although previous studies suggested a role of anti-staphylococcal antibodies in protection against death in *S. aureus* carriers,[21,40-42,50,51] their role may be overestimated. Possibly, IgG against IsaA-His_6, LytM-His_6, Nuc-His_6, His_6-pro-Atl, and PSMα1-4, or anti-staphylococcal IgG in general, has no or only a limited role in protection against *S. aureus* infections. It may be conceivable that antibodies against *S. aureus* antigens in humans are just a result of prior exposure in carriers and non-carriers, via *S. aureus* colonization or previous (sub-)clinical *S. aureus* infection, while their protective capacity is limited. Previous exposure to *S. aureus* in carriers may also result in improved cellular immunity, which could also protect against *S. aureus* infection-related death, as was already suggested by Joshi et al.[52] Beside this, the production of virulence factors such as toxins and immune evasion proteins by *S. aureus*[53,54] might overwhelm the generated humoral immune response.

Although passive immunization with monoclonal antibodies against IsaA protected mice against *S. aureus* infection,[22,48] in the present study no protection was obtained after active immunization with a mixture including IsaA. A possible explanation for this discrepancy may be related to insufficient binding of polyclonal antibodies induced by active immunization to relevant epitopes of IsaA in order to provide protection against *S. aureus* infection, in contrast to the monoclonal antibodies administered by passive immunization, clearly binding to relevant epitopes of IsaA.

In conclusion, active immunization with an octa-valent mixture containing IsaA-His_6, LytM-His_6, Nuc-His_6, His_6-pro-Atl and the PMSα1-4 peptides does not protect mice against *S. aureus* bacteremia and *S. aureus* skin infection. The observations suggest that these polyclonal anti-staphylococcal antibodies do not provide protection against *S. aureus* infection. Consequence of the present study should not be abandoning of research focusing on immunization in *S. aureus* infections, as other investigators obtained promising results in this respect. Instead, future research should focus on novel treatment strategies combining immunization with antibiotic treatment and/or cytokine administration.

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9

Summarizing discussion

S. aureus can cause a wide range of infections, varying from relatively mild infections like furuncles and impetigo, to severe, life-threatening infections including endocarditis, osteomyelitis and pneumonia.[1] The invasive infections are frequently associated with *S. aureus* bacteremia.[2] Antibiotic treatment of severe infection caused by methicillin-susceptible *S. aureus* (MSSA) or methicillin-resistant *S. aureus* (MRSA) strains is not always successful, which is illustrated by the high morbidity and mortality rate of invasive *S. aureus* infections.[3-6]

As *S. aureus* is an opportunistic pathogen, it as such may also colonize the nose in humans. About 20% of the healthy population is a persistent nasal *S. aureus* carrier, while 30% is an intermittent carrier of this bacterium.[7-10] The question arises whether *S. aureus* carriage is predisposing to *S. aureus* infection. On the other hand, it is questioned whether *S. aureus* carriage improves the outcome of *S. aureus* infection thanks to the presence of anti-staphylococcal antibodies in carriers resulting from continuous exposure to *S. aureus*. In this respect, previous clinical studies suggest that nasal *S. aureus* carriers have on the one hand an increased risk of acquiring *S. aureus* bacteremia, but on the other hand, a decreased risk of *S. aureus* bacteremia-related death.[11] A number of studies propose a role of certain anti-staphylococcal antibodies in protection against *S. aureus* bacteremia-related death.[12-15]

The observation that anti-staphylococcal antibodies might be involved in the acquisition and outcome of *S. aureus* infection opens new ways for active or passive immunization as a novel potential treatment approach to enhance or even replace the currently applied therapies. In view of the emergence and spread of antibiotic-resistant *S. aureus* strains, among which MRSA,[16-20] and the lean pipeline for novel therapeutics,[21] there may be a role for non-antibiotic-based antimicrobial intervention strategies, including immunization, to prevent, to reduce, or to cure *S. aureus* infection. In **chapter 6**, we have provided an overview on studies concerning active and passive immunization in animal models of *S. aureus* infection, in patients at risk for *S. aureus* infection, and in *S. aureus*-infected patients. Most of the studies in experimental animals, focusing on a broad range of various bacterial targets, showed success of active or passive immunization in prevention, reduction or cure of *S. aureus* infection. The efficacy of immunization focused on a limited number of *S. aureus* targets has also been investigated in humans at high risk of *S. aureus* infection and in *S. aureus*-infected patients. Until now, active or passive immunization against *S. aureus* did not prevent, reduce or cure *S. aureus* infection. Insufficient power because of a low sample size in some

clinical studies, as well as the heterogeneity of *S. aureus* strains causing infections in humans may contribute to the failure of treatment through immunization in patients.

The animal studies concerning immunization as a non-antibiotic-based antimicrobial intervention strategy were performed in many different models of *S. aureus* infections, in various animal species ranging from mice to macaques. It is obvious that for these kind of studies, an experimental model closely related to humans seems to be most appropriate. In **chapter 2**, we have investigated whether rhesus macaques could serve as a non-human primate model for this purpose. Our observations showed that rhesus macaques are natural hosts of *S. aureus*. The nasal cavity of these macaques is an important reservoir of *S. aureus*, as is also observed in humans. The isolated *S. aureus* strains appeared to be host-specific, as they differ from human *S. aureus* isolates in many aspects (PFGE clustering, *spa*-types, content of superantigen-encoding genes, *agr*-types, STs). These differences were underlined by major differences between humans and macaques in serum anti-staphylococcal antibody levels and profile. In contrast to human *S. aureus* carriers, persistent nasal *S. aureus* carriage of the same strain over a long period was not observed in rhesus macaques. **We conclude that rhesus macaques are natural hosts of specific *S. aureus* lineages, and provide an interesting model for studies concerning the protective capacity of immunization as a novel potential treatment strategy.**

It is known that the use of macaques in experimental research encounters ethical dilemmas. Beside this, rhesus macaques are not an appropriate model for studies on the role of anti-staphylococcal antibodies in the protection against *S. aureus* bacteremia, as they are carriers of *S. aureus*. For such studies, experimental animals that are initially free of *S. aureus* and anti-staphylococcal antibodies are needed. Mice that fulfill these criteria are available and we used these mice for studies concerning this research question.

To investigate the association between anti-staphylococcal antibody levels and the course and outcome of *S. aureus* infections in mice, quantification and characterization of antibody profiles in small-volume serum samples is needed. For this purpose, in **chapter 3** we have optimized the microsphere bead-based flow cytometry technique which was already described for application in human serum samples,[22] for use in mouse serum samples. A major advantage of this technique is that only 1 μL of serum is required. Next, we have investigated IgG profiles in sera from mice with different

types of *S. aureus* infection caused by different *S. aureus* strains. We observed that the site of infection as well as the causative *S. aureus* strain clearly influenced the IgG profile. **Through these studies, we have validated this multiplex assay for the quantification and characterization of antibody responses in small-volume serum samples of *S. aureus*-infected mice. This assay can be applied to determine the immunogenicity of *S. aureus* antigens in relation to the site of infection and the *S. aureus* strain causing the infection.**

From clinical studies, the role of nasal *S. aureus* carriage and specific anti-staphylococcal antibodies in protection against *S. aureus* bacteremia-related death is not fully understood. In **chapter 4**, we have described studies in this respect using two different MSSA strains: isolate P, a clinical sepsis isolate,[23] and 8325-4, a well-characterized laboratory strain.[24] We induced skin infection caused by *S. aureus* isolate P or *S. aureus* 8325-4 in mice. Subsequent bacteremia was always induced by *S. aureus* isolate P. We assessed the association between anti-staphylococcal antibodies resulting from the skin infections and the course and outcome of subsequent endogenous or exogenous *S. aureus* bacteremia. This study demonstrated that mild skin infection did improve the course and outcome of subsequent endogenous *S. aureus* bacteremia in mice, but not the course and outcome of exogenous *S. aureus* bacteremia. These observations in mice are in line with the suggestions derived from these studies in humans, suggesting that carriers may have a reduced risk of *S. aureus* bacteremia-related mortality.[11]

Pre-bacteremia IgG levels against Efb, LukD, LukE, Nuc, and WTA were higher in skin infected mice with endogenous *S. aureus* bacteremia than in skin infected mice with exogenous *S. aureus* bacteremia. We also found that, compared to mice with skin infection once, in mice with skin infection twice IgG levels against IsaA, Nuc, PrsA, and WTA were elevated at the onset of endogenous *S. aureus* bacteremia. Interestingly, compared to skin infection once, a second skin infection further reduced the severity of subsequent endogenous *S. aureus* bacteremia. As an increased level of protection against death due to endogenous *S. aureus* bacteremia was associated with increased pre-bacteremia IgG levels against these *S. aureus* antigens, these antibodies might contribute to the improved course and outcome of endogenous *S. aureus* bacteremia.

Our results are in line with those obtained in a recently published experimental study in mice by Montgomery et al.[25] They studied the effect of *S. aureus* skin infection on a secondary skin infection caused by the same *S. aureus* strain. It was shown

that skin infection protected against the subsequent skin infection. In addition, they demonstrated that this protection was mediated by antibody and IL-17A and inhibited by IFN-γ as shown by antibody transfer to naive mice and neutralization of IL-17A or IFN-γ prior to infection, respectively. Conclusions regarding the antigen-specificity of these antibodies were not drawn.

We conclude that in our infection model in mice pre-bacteremia anti-staphylococcal IgG levels resulting from mild *S. aureus* skin infection are associated with an improved course and outcome of subsequent endogenous *S. aureus* bacteremia. Studies including adoptive transfer of antibodies are needed to confirm a causal relationship between elevated anti-staphylococcal antibody levels and improved levels of protection against *S. aureus* bacteremia-related mortality.

Correlates of immune protection in human *S. aureus* colonization and disease are still not sufficiently understood, and it is unknown whether a protective immune response can be produced in humans. However, next to the presumptive evidence that anti-staphylococcal antibodies might have a role in the outcome of *S. aureus* infection, a role of components of the cellular immune system is suggested. Several studies in patients with impaired Th17 cell function have proposed a role for IL-17A in protection against *S. aureus* infection.[26-28] In addition, experimental studies in mice also show a protective role of IL-17A.[25,29,30] *S. aureus*-primed γδ T cells which display enhanced IL-17A production are shown to have a protective role in *S. aureus* peritonitis in mice.[31] As a role of components of cellular immunity was not investigated in our study in chapter 4, conclusions in this respect cannot be drawn.

The observations in chapter 4 warranted further studies on the potential role of passive and active immunization in the prevention, reduction, or cure of severe *S. aureus* infections. Chapter 6 provides a review of studies in humans at high risk of *S. aureus* infection and in *S. aureus*-infected patients, showing failure of treatment through immunization. This may be partially explained by the heterogeneity of *S. aureus* strains causing infection in humans.[23,32] The use of experimental animals provides a unique opportunity to explore the efficacy of active or passive immunization. In **chapter 7**, we have used mouse models of severe *S. aureus* bacteremia caused by isolate P (a clinical MSSA sepsis isolate[23]) or USA300 (a frequent cause of community-acquired MRSA infections in the United States[33]), which models are clinically relevant because of the major need for effective treatment. In view of the heterogeneity of *S. aureus*

strains causing infections in humans, for immunization, the selection of a conserved *S. aureus* antigen that is expressed by all *S. aureus* strains as bacterial target is important. As a study by Ziebandt et al.[23] shows that IsaA is such a conserved *S. aureus* antigen, IsaA was selected as a target for passive immunization in our study. We produced a human mAb ("1D9") targeting IsaA. As a first step, we assessed the usability of this human mAb in our mouse model by performing various *in vitro* and *ex vivo* experiments. Our mAb 1D9 was shown to bind to purified IsaA as well as to a broad range of *S. aureus* isolates, including both MSSA and MRSA strains. This binding did not influence the multiplication of *S. aureus* strains in medium containing mouse serum. It was shown that immune complexes consisting of IsaA and 1D9 stimulated both human and mouse neutrophils to generate an oxidative burst, which indicated a specific interaction of the immune complex with the Fcγ receptor expressed on the surface of both human and mouse neutrophils. In our *ex vivo* phagocytosis assay, despite binding of the mAb to *S. aureus*, 1D9 did not initiate any phagocytosis of *S. aureus* isolate P or *S. aureus* USA300 by human or mouse neutrophils.

After these *in vitro* and *ex vivo* characterizations, the human mAb 1D9 as an alternative non-antibiotic-based treatment was further investigated *in vivo* in mice, initially using the model of *S. aureus* isolate P bacteremia. In a previous study (not published) in this model, flucloxacillin treatment resulted in 100% survival of mice compared to 17% survival in the group of placebo-treated mice. By confirming the therapeutic efficacy of flucloxacillin, in this respect this MSSA infection model was validated. Before investigating the efficacy of 1D9, the blood circulation time of the human mAb 1D9 in mice was determined. In uninfected mice during the first 24 hours after injection, the 1D9 levels in blood remained stable. In mice with *S. aureus* isolate P bacteremia, mean 1D9 levels were also stable in the first 24 hours after injection, but levels were 10-fold lower compared to uninfected mice. This loss of detectable mAb 1D9 may be the result of binding to *S. aureus* or leakage from the blood to the infected organs. After characterization of the blood circulation time, we investigated the efficacy of a single dose of 5 mg 1D9/kg in the *S. aureus* isolate P bacteremia model. While prophylactic treatment with 1D9 improved the course and outcome of *S. aureus* isolate P bacteremia, therapeutic treatment did not. The lack of therapeutic effect may be explained by insufficient availability of 1D9 to reach *S. aureus*, which had already multiplied and disseminated to lungs, spleen, liver, and kidneys. Possibly, the therapeutic dosing schedule is not yet optimal in this mouse model.

Next to the isolate P (MSSA) infection, we also investigated the efficacy of 1D9 in our mouse model of *S. aureus* USA300 (MRSA) bacteremia. Neither prophylactic nor therapeutic administration of our anti-IsaA mAb (5 mg/kg) improved the course and outcome of USA300 bacteremia. The failure of 1D9 in this infection could not be explained by lower 1D9 blood concentrations, as the 1D9 levels were similar in both MSSA and MRSA bacteremia models. A possible explanation of the lack of success of passive immunization with 1D9 in *S. aureus* USA300 bacteremia might be a higher expression of protein A by *S. aureus* USA300 compared to *S. aureus* isolate P, and as a result a higher aspecific and nonfunctional binding of 1D9 to *S. aureus* USA300. Our data from Western blot analyses did confirm a substantially higher protein A expression by *S. aureus* USA300 in mouse serum. In view of these observations, the dosing schedule of the human anti-IsaA mAb 1D9 in mice with *S. aureus* USA300 infection needs to be optimized. **We conclude that in this MSSA infection model passive immunization with a human monoclonal antibody targeting IsaA, a conserved *S. aureus* antigen, protects mice against death due to *S. aureus* bacteremia. This conclusion cannot yet be extrapolated to *S. aureus* strains in general. Possibly, further optimization of the dosing schedule of mAb 1D9, either alone or in combination with other mAbs or antibiotics, may expand the usability of this immunization strategy.**

Next to passive immunization targeting a conserved *S. aureus* antigen, we also investigated the efficacy of active immunization. In **chapter 8**, we have examined the protective capacity of active immunization with an octa-valent *S. aureus* antigen mixture in two *S. aureus* infection models. The results in this chapter have shown that IsaA, LytM, Nuc, pro-Atl, and PSMα1-4 are immunogenic in humans, as patients colonized with *S. aureus* had significant IgG levels against these antigens. Active immunization of mice with this cocktail of *S. aureus* antigens resulted in significant IgG levels, indicating the immunogenicity of these antigens in mice. However, these high pre-infection IgG levels against IsaA, LytM, Nuc, pro-Atl, and PSMα1-4 in mice did not result in protection against *S. aureus* isolate P (MSSA) or USA300 (MRSA) bacteremia or *S. aureus* isolate P skin infection. Although prophylactic passive immunization with the human anti-IsaA mAb showed protective capacity against *S. aureus* isolate P bacteremia in mice (chapter 7), active immunization with the octa-valent antigen mixture including IsaA did not (chapter 8). This latter lack of efficacy may be explained by insufficient binding of polyclonal antibodies to relevant epitopes of IsaA in

order to provide protection against *S. aureus* infection, in contrast to the monoclonal antibodies administered by passive immunization, clearly binding to relevant epitopes of IsaA. **We conclude that polyclonal anti-staphylococcal antibodies against IsaA, LytM, Nuc, pro-Atl, and PSMα1-4 resulting from active immunization do not provide protection against *S. aureus* infection.**

Beside the need for improvement of therapeutic outcome in *S. aureus* infection, further optimization of the diagnosis of *S. aureus* infection may also contribute to the improvement of infection management. A more comprehensive diagnosis is important in decisions on the start of antibiotic therapy. Currently, diagnosis of *S. aureus* infection is based on medical history, clinical signs and symptoms, bacterial cultures from the site of suspected infection, and the measurement of host response factors including CRP levels. In addition, there are developments in molecular diagnostics aiming for a reduced time to identification of the infectious agent. Identification of specific biomarkers to gain insight into the prognosis of *S. aureus* infection may further reduce the time to start directed antibiotic therapy, aiming for a lower risk of morbidity and mortality. In **chapter 5**, we have assessed the role of cytokines as biomarkers for the presence of and outcome in severe *S. aureus* bacteremia in mice. In this study, TNF-α, IL-1α, KC (CXCL1), G-CSF, IL-6, IL-12p70, IP-10 (CXCL10), and MIP-1α (CCL3) appeared to be biomarkers for severe *S. aureus* bacteremia. Among these cytokines, TNF-α, IL-1α, and KC were also shown to be predictors of fatal outcome of *S. aureus* bacteremia. From these, KC, a functional homologue of human IL-8,[34] was elevated irrespective of the progression of infection in mice, which is very interesting regarding its potential role as biomarker in *S. aureus* infected patients, in which the progression of infection is mostly unknown. **We conclude that in this infection model in mice, distinctive cytokines are biomarkers predicting fatal outcome of severe *S. aureus* bacteremia.**

Future perspectives

The experimental studies in animals described in this thesis may serve as a basis for studies in patients with *S. aureus* infections.

Regarding diagnosis of *S. aureus* infection, based on our studies in mice identifying individual cytokines to be biomarkers for fatal outcome of *S. aureus* bacteremia, clinical studies are warranted to identify certain cytokines predicting the outcome of *S. aureus* infection (bacteremia or other severe infections). Detection of specific biomarkers, together with rapid identification of the causative infectious agent, may further reduce the time to start directed antibiotic therapy, and in this way lower the risk of morbidity and mortality.

Regarding the involvement of immunization in the prevention, reduction or cure of *S. aureus* infection, our studies in mice support the concept that this alternative non-antibiotic-based treatment approach might be valuable to enhance the current antibiotic treatment. Based on our studies in mice, passive immunization seems to be more promising than active immunization. Selection of a conserved *S. aureus* target, expressed by all *S. aureus* strains, for immunization is important to provide protection against a broad range of *S. aureus* strains causing the infection. Up to now, most studies are performed in rodents (studies described in present thesis and studies summarized in chapter 6). To increase the translational value of these results to the clinical setting, studies in small numbers of rhesus macaques should be encouraged to confirm the results obtained with immunization in rodent models.

Since the role of active immunization in the treatment of *S. aureus* infection is not very encouraging as shown in experimental and clinical studies performed so far, stimulation of the cellular immune system, e.g. by targeting the induction of specific subsets of γδ T cells that secrete IL-17A, as another approach in the non-antibiotic-based treatment strategy in *S. aureus* infections should be further explored. Currently, studies on this subject are ongoing.

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10

Nederlandse samenvatting

Staphylococcus aureus is een bacterie die op de huid en slijmvliezen van veel gezonde personen aanwezig is. Zo'n 20-30% van de gezonde bevolking draagt deze bacterie altijd bij zich (persisterend dragers), waarbij *S. aureus* vooral in de neus wordt gevonden en er geen ziektesymptomen zijn. Naast asymptomatisch dragerschap kan *S. aureus* ook veel verschillende infecties veroorzaken. Deze variëren van relatief milde huidinfecties, zoals steenpuisten en krentenbaard, tot ernstige, levensbedreigende infecties als longontsteking, hersenvliesontsteking en bloedbaaninfectie (bacteriëmie). *S. aureus* dragers lijken een grotere kans te hebben op het ontwikkelen van een ernstige *S. aureus* infectie dan niet-dragers. Daarentegen zouden dragers een kleiner risico dan niet-dragers hebben om te overlijden aan deze infectie.

Totdat de antibiotica werden geïntroduceerd in de jaren 40 en 50 van de vorige eeuw was het aantal patiënten dat overleed aan een *S. aureus* bacteriëmie schrikbarend hoog: zo'n 80%. In 1959 werden patiënten voor het eerst behandeld met het antibioticum methicilline, waardoor de sterfte door *S. aureus* infecties daalde. Echter, al binnen 2 jaar na de introductie van dit antibioticum werden de eerste methicilline-resistente *S. aureus* (MRSA) stammen gevonden. Momenteel is het antibioticum vancomycine een van de laatste redmiddelen in de behandeling van MRSA infecties, maar helaas zijn er inmiddels ook vancomycine-resistente *S. aureus* (VRSA) stammen beschreven, aanvankelijk in de Verenigde Staten en in 2013 ook in Europa. Omdat de behandeling van *S. aureus* infecties met antibiotica lang niet altijd succesvol is, mede als gevolg van resistente *S. aureus* stammen, en omdat de farmaceutische industrie nauwelijks nieuwe antibiotica ontwikkelt, zijn er zeer dringend alternatieve behandelingen nodig die niet op antibiotica gebaseerd zijn.

In het eerste deel van dit proefschrift is een studie beschreven die beoogt meer duidelijkheid te verkrijgen in de rol van *S. aureus* dragerschap in het beloop van *S. aureus* infecties. Het daarna volgend onderzoek is gericht op verbetering van de diagnose van *S. aureus* bacteriëmie en het vergroten van inzicht in de prognose van de infectie. Ten slotte zijn een tweetal studies verricht naar alternatieve behandelingen (anders dan antibiotica) van *S. aureus* infecties. De verschillende vraagstellingen zijn onderzocht binnen diermodellen van *S. aureus* infectie die wij voor dit doel hebben opgezet.

Al eerder verrichte studies van andere onderzoekers aangaande de rol van *S. aureus* dragerschap in het beloop van *S. aureus* infecties suggereren dat antistoffen hierbij

een rol zouden kunnen spelen. Niet-dragers zouden antistoffen hebben die beschermen tegen dragerschap en infectie, terwijl dragers antistoffen zouden hebben die een beschermende rol spelen in het beloop van de infectie. Welke antistoffen in deze situaties nu echt van belang zijn is in mensen moeilijk te onderzoeken omdat zowel dragers als niet-dragers een grote variëteit aan antistoffen tegen *S. aureus* in het bloed hebben. Om deze reden worden vaak proefdiermodellen gebruikt. Uiteraard gaat bij de keuze van een proefdier de voorkeur uit naar een dier dat evolutionair gezien dicht bij de mens staat. Apen zouden daarom een goed model kunnen zijn voor dit doel. Maatschappelijk gezien zijn hier echter wel dilemma's.

In **hoofdstuk 2** hebben we resusapen onderzocht op *S. aureus* dragerschap door kweken af te nemen vanuit de neus. Onze studie heeft laten zien dat deze apen inderdaad *S. aureus* in de neus bij zich dragen, maar dat de *S. aureus* stammen bij de apen verschillen van de *S. aureus* stammen die bij mensen gevonden worden. Bovendien zijn de antistofprofielen in het bloed van resusapen en van mensen heel verschillend. Om deze redenen zien wij resusapen niet als ideaal proefdiermodel voor het bestuderen van de rol van antistoffen in het beloop van *S. aureus* infecties. Voor studies op dit gebied zouden proefdieren die geen drager van *S. aureus* zijn en geen antistoffen hebben tegen *S. aureus* het meest geschikt zijn. Muizen vrij van *S. aureus* zijn verkrijgbaar, en wij hebben ervoor gekozen het onderzoek naar de rol van antistoffen in het beloop van *S. aureus* infecties in deze muizen te verrichten. Voor deze experimenten in muizen dienen de concentraties van antistoffen in kleine hoeveelheden bloed gemeten te worden. Een bestaande techniek voor het meten van antistoffen tegen veel verschillende *S. aureus* antigenen tegelijk in kleine hoeveelheden bloed van mensen hebben we in **hoofdstuk 3** geoptimaliseerd voor toepassing bij de muis. Met de voor de muis aangepaste techniek hebben we antistofprofielen gemeten in bloed van muizen met verschillende infecties veroorzaakt door verschillende *S. aureus* stammen. We hebben aangetoond dat het antistofprofiel verschillend is afhankelijk van de soort *S. aureus* infectie en de *S. aureus* stam die de infectie veroorzaakt in de muis.

In **hoofdstuk 4** wordt de associatie tussen bescherming tegen overlijden aan *S. aureus* bacteriëmie en antistofniveaus aan het begin van die bacteriëmie onderzocht. Om dit te bestuderen werd bij muizen een *S. aureus* huidinfectie aangebracht, als gevolg waarvan antistoffen tegen verschillende *S. aureus* antigenen werden gevormd en aangetoond in het bloed. Vijf weken na de huidinfectie werd een *S. aureus* bacteriëmie geïnduceerd en het beloop van de *S. aureus* infectie werd gevolgd. Voor de huidinfectie en de bacteriëmie werden dezelfde *S. aureus* stammen (endogene

bacteriëmie) of verschillende *S. aureus* stammen (exogene bacteriëmie) gebruikt. Dankzij de huidinfectie bleken de muizen beschermd te zijn tegen overlijden aan de later volgende endogene *S. aureus* bacteriëmie. Een voorafgaande huidinfectie beschermde niet tegen overlijden door exogene *S. aureus* bacteriëmie. In het geval van de endogene bacteriëmie bleken muizen die twee keer een huidinfectie hadden ondergaan beter beschermd tegen overlijden dan muizen die één huidinfectie hadden doorgemaakt. Door de antistofprofielen nader te onderzoeken, kwantitatief (hoogte van de titer) en kwalitatief (tegen welke antigenen van de bacterie gericht), werd ook aangetoond dat antistofniveaus tegen bepaalde antigenen vlak voor de bacteriëmie verhoogd waren in muizen met een endogene bacteriëmie vergeleken met muizen met een exogene bacteriëmie. Dit suggereert dat in dit muisinfectiemodel antistoffen een rol zouden kunnen spelen in de bescherming tegen overlijden aan *S. aureus* bacteriëmie. Verdere studies zijn nodig om een causaal verband aan te tonen tussen hoge antistofniveaus en bescherming tegen overlijden aan endogene *S. aureus* bacteriëmie. In zo'n studie zouden antistoffen uit muizen met een *S. aureus* huidinfectie moeten worden overgebracht naar muizen zonder huidinfectie. De bescherming tegen *S. aureus* bacteriëmie daarna zou dan ook zichtbaar moeten zijn.

Naast de rol van dragerschap in het beloop van *S. aureus* bacteriëmie hebben we in dit proefschrift onderzocht hoe de diagnose van *S. aureus* bacteriëmie verder te verbeteren en hoe meer inzicht te verkrijgen in de prognose van de infectie. Deze kennis zou kunnen bijdragen aan het verlagen van de ziektelast en sterfte door *S. aureus* infecties.

In **hoofdstuk 5** hebben we in muizen onderzocht of cytokines (signaalstoffen die door cellen van ons afweersysteem worden uitgescheiden) biomarkers kunnen zijn voor de aanwezigheid en het beloop van *S. aureus* bacteriëmie. Aangetoond werd dat bepaalde cytokines inderdaad biomarkers zijn die de aanwezigheid en het beloop van de *S. aureus* bacteriëmie voorspellen. Sommige cytokines in een vroege fase van de infectie, andere in een latere fase van de infectie. Een van die cytokines, KC (functioneel homoloog aan humaan IL-8) bleek een biomarker te zijn onafhankelijk van het stadium van de infectie. Dit is een belangrijke bevinding in termen van klinisch belang gezien het feit dat bij patiënten met *S. aureus* bacteriëmie meestal weinig inzicht is in welke fase van de infectie de patiënten zich bevinden. Een studie in patiënten met

S. aureus infecties zal moeten uitwijzen of en zo ja welke cytokines ook in mensen biomarkers zijn voor de aanwezigheid en het beloop van de *S. aureus* infectie.

Zoals al eerder in dit hoofdstuk genoemd is er een dringende behoefte aan alternatieven (anders dan antibiotica) in de behandeling van *S. aureus* infecties vanwege de verspreiding van resistente *S. aureus* stammen en het gebrek aan nieuwe antibiotica. Daarom hebben we in dit proefschrift ook onderzoek gedaan naar mogelijke alternatieve behandelingen. De bevinding dat antistoffen een rol zouden kunnen spelen in het beloop van *S. aureus* infecties zoals aangetoond in hoofdstuk 4 biedt perspectieven voor immunisatie als een mogelijke alternatieve behandeling. Bij actieve immunisatie (vaccinatie) wordt preventief een antigeen toegediend, waarop het lichaam antistoffen tegen dit antigeen zal vormen. Bij passieve immunisatie worden de antistoffen tegen een antigeen op het laboratorium bereid en daarna aan de mens toegediend, hetzij preventief, hetzij in de situatie van een reeds bestaande *S. aureus* infectie. Een groot aantal studies op het gebied van actieve en passieve immunisatie bij *S. aureus* infecties zijn gedaan, en daarvan is in **hoofdstuk 6** een overzicht gegeven. Veel studies op dit gebied zijn uitgevoerd in proefdieren, en in de meeste gevallen zijn de resultaten veelbelovend. Daarentegen laten de studies in mensen met een verhoogd risico op *S. aureus* infectie of in patiënten met *S. aureus* infectie geen positieve resultaten zien.

In **hoofdstuk 7 en 8** hebben we de beschermende werking van immunisatie als alternatief voor behandeling met antibiotica bestudeerd in muismodellen van *S. aureus* infectie.

De studies in **hoofdstuk 7** zijn gericht op passieve immunisatie met monoklonale antistoffen (genaamd 1D9) tegen het *S. aureus* antigeen IsaA. Dit antigeen komt tot expressie op de overgrote meerderheid van *S. aureus* stammen, wat belangrijk is gezien de heterogeniteit van *S. aureus* stammen die infecties veroorzaken bij de mens. Ons *in vitro* onderzoek toonde aan dat 1D9 daadwerkelijk bindt aan het antigeen IsaA en aan de *S. aureus* bacterie, en dat de Fc-staart van 1D9 een interactie aangaat met de Fcγ receptor op neutrofiele granulocyten afkomstig van muis of mens. Het monoklonaal 1D9 remt niet de vermenigvuldiging van *S. aureus* in medium met muisserum en initieert niet de fagocytose van *S. aureus*.

Na deze *in vitro* experimenten hebben we de beschermende werking van 1D9 onderzocht in onze muismodellen van bacteriëmie veroorzaakt door een methicilline-gevoelige *S. aureus* (MSSA) of bacteriëmie door een methicilline-resistente *S. aureus* (MRSA). Preventieve 1D9 behandeling bleek te beschermen tegen overlijden aan de

MSSA bacteriëmie, maar niet tegen overlijden aan de MRSA bacteriëmie. In beide modellen bood therapeutische 1D9 behandeling geen bescherming. Verder onderzoek in deze studie heeft geresulteerd in mogelijke verklaringen voor de afwezigheid van bescherming door 1D9 in genoemde gevallen.

De studies in **hoofdstuk 8** zijn gericht op actieve immunisatie met een combinatie van acht antigenen, IsaA, LytM, Nuc, pro-Atl en vier PSMα's. Nadat we hadden aangetoond dat deze antigenen immunogeen zijn in de mens, hebben we onderzocht of actieve immunisatie met een combinatie van deze antigenen beschermt in onze muismodellen van MSSA of MRSA bacteriëmie en in ons MSSA huidinfectiemodel. Hoewel muizen na immunisatie hoge antistofspiegels ontwikkelden tegen deze antigenen, bleek deze actieve immunisatie niet te beschermen tegen de MSSA bacteriëmie en de MRSA bacteriëmie en niet tegen de MSSA huidinfectie. Ondanks deze weinig hoopvolle bevindingen dient naar onze overtuiging nog niet de deur te worden gesloten voor immunisatie als mogelijke alternatieve behandeling van *S. aureus* infectie, omdat onze eigen studies in muizen (hoofdstuk 4 en 7) en studies van andere onderzoekers hebben aangetoond dat antistoffen wel degelijk een rol spelen in de bescherming tegen *S. aureus* infectie.

Met de in dit proefschrift beschreven experimentele studies hopen we een basis te hebben gelegd voor vervolgonderzoek dat kan bijdragen aan de opzet van klinische studies bij patiënten met een hoog risico op *S. aureus* infectie en patiënten met *S. aureus* infecties.

Appendixes

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Dankwoord

♫ Zing, vecht, huil, bid, lach, werk en bewonder! ♫ Dat is hoe mijn proefschrift tot stand is gekomen. Maar ook: ♫ Niet zonder ons! ♫ Ik wil daarom ook iedereen ontzettend bedanken die op de een of andere manier heeft bijgedragen aan dit proefschrift. Natuurlijk wil ik ook een aantal mensen in het bijzonder bedanken voor hun bijdragen. Hier komt-ie:

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Prof.dr. Van Dijl, beste Jan Maarten. Al voordat mijn aanstelling op het Erasmus MC officieel van start ging, heb ik je in Groningen leren kennen als de projectleider van het AntiStaph project. We hebben veel samengewerkt in dit project, en later ook in het AntiBact project. Dank dat je nu plaats hebt willen nemen in de leescommissie.

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Curriculum Vitae

Sanne van den Berg was born on April 10th, 1984 in Oeffelt, the Netherlands. After finishing her secondary school (gymnasium) at Merletcollege in Cuijk in 2002, she started studying Biomedical Sciences at the Radboud University in Nijmegen. In her last year, she followed her final internship at the Laboratory of Pediatrics & Infectious Diseases of Radboudumc in Nijmegen. She was involved in the development of an otitis media mouse model to study the pathogenesis of this disease and the efficacy of vaccines in this respect; this is where her interest was created in vaccine research in experimental animals. After graduating in 2007, she continued the work on otitis media for a couple of months in the same department. In February 2008, she started with her PhD research in Rotterdam at the department of Medical Microbiology & Infectious Diseases of Erasmus MC under supervision of dr. Irma Bakker-Woudenberg. Her research focused on improving diagnosis and treatment of *Staphylococcus aureus* infections, using experimental animal models. The majority of this research was performed as part of two TI Pharma projects (AntiStaph T4-213 and AntiBact T4-502). From 2013, Sanne is also involved in Tuberculosis research at the same department.

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Sanne van den Berg,* Dennis G.A.M. Koedijk,* Jaap Willem Back, Jolanda Neef, Annette Dreisbach, Jan Maarten van Dijl, Irma A.J.M. Bakker-Woudenberg, Girbe Buist. Active immunization with an octa-valent *S. aureus* antigen mixture in models of *S. aureus* bacteremia and skin infection in mice. Submitted for publication.

* These authors contributed equally to the work.

PhD portfolio

Name:	Sanne van den Berg
Erasmus MC department:	Medical Microbiology & Infectious Diseases
Promotor:	Prof. dr. Henri A. Verbrugh
Supervisor:	Dr. Irma A.J.M. Bakker-Woudenberg

PHD TRAINING	**Year**
In depth courses Postgraduate School Molecular Medicine	
Animal Imaging Workshop by AMIE 'From mouse to man'	2009
Seminars and workshops	
Departmental Journal Clubs (oral presentations)	2008-2014
Departmental Research Meetings (oral presentations)	2008-2014
PhD Day Erasmus MC	2008, 2009, 2011
Symposium and workshops 'Molecular Microbiology of Infectious Diseases'	2008
Molecular Medicine Get Out of your Lab days	2009
Course 'Drug Development Cycle'	2011
Course 'Business and Entrepreneurial Skills'	2011
Workshop 'Onderhandelen'	2013
Workshop 'Determinants of Response & Variability in Early Clinical Drug Development', Tres Cantos, Spain	2013
Workshop 'Photoshop & Illustrator CS6'	2013
Symposium 'Ethische toetsing in de keten van de gezondheidszorg: van dierproeven naar onderzoek met mensen'	2013
(Inter)national conferences	
Scientific Spring Meeting TI Pharma	2008
Luminex Planet xMAP	2008
Molecular Medicine Day	2009
Scientific Spring Meeting NVMM	2009
Scientific Spring Meeting TI Pharma (oral presentation)	2009
Scientific Spring Meeting NVMM (oral presentation)	2010
Scientific Spring Meeting TI Pharma	2010
ASM General Meeting, San Diego (poster presentation)	2010
Scientific Spring Meeting NVMM (poster presentation)	2011
Scientific Spring Meeting NVMM	2012
ICAAC, San Francisco (poster presentation)	2012
Scientific meetings	
Departmental Research Day (oral presentation)	2008
Departmental Research Day (oral presentation)	2009
Departmental Research Day	2010
Departmental Research Day (poster presentation)	2012
Grants	
Travel grant Erasmus Trustfonds	2012
TEACHING	
Lecturing	
Lecture Research Master Infection & Immunity, 'Animal models'	2012, 2013
Supervision of students	
Supervision of 2nd year medical students laboratory practicals 'Vaardigheidsonderwijs Infectieziekten'	2008-2014